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BIO-ORGANIC CHEMISTRY QUARTERLY REPORT

June through August 1963

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BIO-ORGANIC CHEMISTRY QUARTERLY REPORT

June through August 1963

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October 2, 1963

1. THE EFFECTS OF 8-METHYL LIPOIC ACID ON THE EVOLUTION OF OXYGEN AND REDUCTION OF CARBON DIOXIDE DURING PHOTOSYNTHESIS

J. A. Bassham, Horst Egeter, Frances Edmonston, and Martha Kirk

During the past quarter we have studied transient changes in concentrations of intermediate compounds of the photosynthetic carbon-reduction pathways in Chlorella pyrenoidosa; these changes accompany the addition of 8-methyl lipoic acid to a suspension of the algae that have been photosynthesizing with $^{14}CO_2$ under steady-state conditions. The results of these experiments are in press.[1] They will only be summarized in this report.

Methods of achieving steady-state photosynthesis, of measuring rates of CO_2 and $^{14}CO_2$ uptake and O_2 evolution, of analyzing the radioactive products of photosynthesis, and of determining the ^{14}C labeling and active-pool concentrations of these compounds were the same as those published previously.[2, 3, 4] In most of the experiments reported here, 10 mg of 8-methyl lipoic acid in 200 μliters of ethanol was added to 80 ml of 2% (wet-packed vol/vol suspension) Chlorella pyrenoidosa, following 10 min photosynthesis with $^{14}CO_2$ under steady-state conditions.

The results may be summarized as follows:

1. Complete inhibition of O_2 evolution and of CO_2 uptake occurred within 2 min (the time of response of the CO_2 and O_2 analyzers). This inhibition partly disappeared after 20 to 25 min (recovery period).

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1. J. A. Bassham, Horst Egeter, Frances Edmonston, and Martha Kirk, Biochem. Biophys. Res. Commun. 13, 144 (1963).

2. J. A. Bassham and M. Kirk, Biochim. Biophys. Acta 43, 447 (1960).

3. D. C. Smith, J. A. Bassham, and M. Kirk, Biochim. Biophys. Acta 48, 299 (1961).

4. J. A. Bassham and M. Kirk, Synthesis of Compounds from $^{14}CO_2$ by Chlorella in the Dark Following Preillumination, in Microalgae and Photosynthetic Bacteria, Ed. Japanese Society of Plant Physiologists, (University of Tokyo Press, 1963) p. 493.

2. The level of phosphoglyceric acid dropped with 15 sec to less than 25% of its steady-state level and remained at a low level until the recovery period.

3. The level of ribulose-1, 5-diphosphate rose slightly for about 1 min and then declined to about one-half its steady-state value, where it remained.

4. Levels of fructose-1, 6-diphosphate and of sedoheptulose-1, 7-diphosphate rose rapidly, while levels of fructose-6-phosphate and of sedoheptulose-7-phosphate declined. All these changes occurred within 1 to 4 min after addition of the inhibitor.

5. Glycolic acid labeling increased continuously until the recovery period.

6. Sucrose labeling, after leveling off during the first 6 to 8 min, increased very markedly until 20 min, apparently at the expense of glucose-6-phosphate, which declined by about the same amount during the same period.

We also found that photosynthesis could be inhibited by the addition of similar concentrations of lipoic acid alone. Kinetic studies of ^{14}C labeling of compounds accompanying such inhibition are in progress.

Although various interpretations of these results are possible, further studies with this and other related inhibitors will be required before some choice of interpretations can be made. The magnitude and immediacy of some of these effects suggest important roles of disulfide compounds, or compounds that react with disulfides, in the mecahnism of the primary carbon-reduction cycle of photosynthesis.

2. FURTHER ^{14}C AND ^{15}N TRACER STUDIES OF AMINO ACID SYNTHESIS DURING PHOTOSYNTHESIS BY CHLORELLA PYRENOIDOSA

J. A. Bassham and Martha Kirk

In previous Quarterly Reports we described results of dual-tracer ($^{15}NH_4^+$ and $^{14}CO_2$) studies of amino acid synthesis by photosynthesizing Chlorella pyrenoidosa.[1,2] We concluded that there are at least two pools of the primary amino acids (perhaps three in the case of glutamic acid) and that the most important route of incorporation of NH_4^+ ion occurs via glutamic acid. No evidence was found for a previously suspected[3] direct amination to give alanine, since the ^{15}N labeling of alanine was consistent with its formation by transamination with previously formed glutamic acid-^{15}N. In order to obtain a comparison on ^{14}C and ^{15}N labeling of glutamic acid (in $\mu M/min/cm^3$ of algae), we divided by 5 the μM of ^{14}C found in glutamic acid. This gives an average labeling of glutamic acid, and is most meaningful if the rates of labeling of all the carbon-atom positions of glutamic acid are about equal. We have now carried out a partial degradation of the labeled glutamic acid in order to measure the ^{14}C labeling of the C-1 carbon atom (the carboxyl carbon adjacent to the carbon bonded to nitrogen).

We have also determined the ^{15}N labeling of the amide nitrogen of glutamine.

Glutamic Acid Carboxyl Labeling

The published method[4] for labeling the C-1 carboxyl carbon was modified to give the following procedure. The ^{14}C-labeled glutamic acid from one or more paper chromatograms was eluted with water and diluted (in radioactivity) by adding 1 or 2 mg of unlabeled glutamic acid. Ten mg of sodium citrate buffer (pH 2.5) and 5 mg of ninhydrin were added, and 0.5 ml of the solution was placed in a 30-ml conical flask. This was attached, via a vacuum stopcock, to one arm of a Y-tube connected to a vacuum line. A flask containing NaOH solution was attached through a vacuum stopcock to the other arm of the Y-tube. The solutions in both flasks were frozen and the entire system was evacuated.

The stopcock to the amino acid-ninhydrin solution was closed, and that solution was boiled for about 30 min (until the initial purple color had disappeared).

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1. J. A. Bassham and Martha Kirk, in Bio-Organic Chemistry Quarterly Report, UCRL-10934, June 1963.

2. J. A. Bassham, Martha Kirk, and G. J. Crowley, in Bio-Organic Chemistry Quarterly Report, UCRL-10634, Jan. 1963, p. 1.

3. B. C. Smith, J. A. Bassham, and M. Kirk, Biochim. Biophys. Acta 48, 299 (1961).

4. David M. Greenberg and Morton Rothstein in Methods in Enzymology Eds. S. P. Colowick and N. W. Kaplan (Academic Press Inc., New York 1957), Vol. IV, p. 711.

Table 2-II. The labeling of the amide nitrogen of glutamine from Chlorella following photosynthesis with $^{15}NH_4^+$. Steady-state experiment 52.

Sample	Time (min)	^{15}N (%)	Comparable glutamic acid (%)
1 + 2	7.5 (av)	3.7	24.8 (av)
3	15	4.0	49.4
5 + 6	38 (av)	3.3	50.3 (av)
7 + 8	75 (av)	2.3	53.3 (av)

The percentage labeling of the amide nitrogen of glutamine is much lower than the labeling of the amino nitrogen of glutamic acid, or, for that matter, the amino group of alanine (see previous Quarterly Report[1]). The absolute rate of incorporation of ^{15}N into the amide group depends also on the pool size. Although we have not determined the absolute pool size of glutamine so far, it is clear from the color developed by spraying chromatograms of Chlorella extracts that the glutamine pool is much smaller than that of glutamic acid. Thus, in this experiment, the rate of incorporation of ^{15}N into the amide group of glutamine appears to be much lower than the rate into glutamic acid.

However, a confusing aspect of the results is that the amount of ^{15}N in the glutamine amide nitrogen does not increase after 15 min. This result may suggest that there is a very small pool of glutamine, the amide nitrogen of which is rapidly saturated. If this is true, the rate of turnover through this small pool conceivably could be very large and the amount of ^{15}N incorporated via this pathway could also be large.

3. TWO-DIMENSIONAL HIGH-VOLTAGE LOW-TEMPERATURE PAPER ELECTROPHORESIS OF ^{14}C-LABELED PRODUCTS OF PHOTOSYNTHESIS WITH $^{14}CO_2$

Wolfgang Rohr and J. A. Bassham

Introduction

Paper chromatography in combination with autoradiography has proved to be a highly useful analytical tool in elucidating the carbon-reduction cycle of photosynthesis as it is now known.[1,2] However, in spite of many efforts,[3,4,5] all attempts have failed to produce unequivocal evidence for the existence of the six-carbon beta-keto-acid diphosphate (2-carboxy-3-keto-pentitol-1, 5-diphosphate), which has been proposed[1] as the first real reaction product of $^{14}CO_2$ fixation. Such a compound, as well as the unidentified early $^{14}CO_2$-fixation product reported by Shkolnik and Doman,[6] would be highly labile. It might well be that the analysis by ordinary two-dimensional paper chromatography, mostly employed in past studies, is not sufficiently mild because of time required, temperature at which performed, and reactivity of solvents during chromatography. It seemed worth while to attempt to find conditions for the separation of early photosynthesis products by high-voltage electrophoresis at low temperatures because this method should be faster and milder than paper chromatography. Also, since it depends on different molecular properties, it might supplement chromatographic procedures.

It seemed improbable that all the products could be separated at any one pH, but two-dimensional electrophoresis at two pH values appeared more promising.

In a recent paper, J. M. Galmiche reported the separation of photosynthesis products for short times with $^{14}CO_2$ by one-dimensional paper electrophoresis at 0°C.[7] He studied mainly the influence of light intensity,

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1. J. A. Bassham and M. Calvin, The Path of Carbon in Photosynthesis (Prentice-Hall, Englewood Cliffs, N. J., 1957).

2. M. Calvin and J. A. Bassham, The Photosynthesis of Carbon Compounds (W. A. Benjamin, Inc., New York, 1962).

3. J. A. Bassham, M. Kirk, and M. Calvin, Proc. Natl. Acad. Sci. U.S. 44, 491 (1958).

4. V. Moses and M. Calvin, Proc. Natl. Acad. Sci. U.S. 44, 260 (1958).

5. V. Moses, R. J. Ferrier, and M. Calvin, Proc. Natl. Acad. Sci. U.S. 48, 1644 (1962).

6. M. Ya Shkolnik and N. G. Doman, Biokhimiya 25, 276 (1960).

7. J. M. Galmiche, Compt. Rend. Acad. Sci. Paris 254, 1169 (1962).

and its spectral composition, on $^{14}CO_2$ incorporation. No new compound resembling the expected first stable products of $^{14}CO_2$ fixation were reported.

This paper describes a simple apparatus designed for two-dimensional high-voltage electrophoresis at low temperature. With this apparatus we were able to reproduce to some extent the one-dimensional chromatography obtained by J. M. Galmiche. Furthermore, we report here the first two-dimensional separation of early products of photosynthesis.

Materials and Methods

The Apparatus

In addition to other factors encountered with electrophoresis in general, high-voltage electrophoresis is accompanied by marked heating of the paper sheets. Therefore, adequate and constant cooling is the most important technical problem with this method. We have suitably dissipated the generated heat by immersing the paper (wet with buffer) in a bath of decahydronaphthalene (Decalin) between two cooling coils, which were supplied independently with a circulating cooling mixture (equal volumes of water and ethylene glycol) from an insulated tank by means of two pumps. The contents of the tank were kept at -8° to -10°C by a refrigeration unit.

The actual apparatus (Fig. 3-1) consists of a square box of Lucite (66.4×66.4×11.4 cm, inside measurements), having two buffer vessels placed at opposite sides. Each buffer-vessel capacity is approximately 1800 ml when filled to the edge (buffer-vessel measurements: 46.8×7.7×5.7 cm). Below the paper sheet is a square plate of Lucite (48.2×48.2×0.65 cm) that contains 13 rows of 13 holes (2.9 cm in diameter). The paper is actually supported by 12 rows of 12 glass beads (5 mm) fixed in the space between the holes, thus giving point support to the paper as well as free circulation of the cooling Decalin.

Above and below the paper and its support are cooling coils, the upper of which is mounted to the cover of the box. The cover also bears the two platinum electrodes. The cover itself can be raised and lowered by a pulley. The lower cooling coil rests on square blocks of Lucite that occupy most of the space between the buffer vessels, thereby decreasing the volume of Decalin necessary to fill the system. Nitrogen pressure is used to push the Decalin from a glass container below the apparatus up into the system. The pressure is released to drain the box. The temperature at various points near the paper is measured by nine thermometers inserted through the cover and placed over the actual separation area—almost touching the paper sheet.

Buffers

Out of a number of tested buffer systems, the following ones proved suitable for our purposes:

pH 4.4 (for the first dimension)
11760 ml distilled water
150 ml glacial acetic acid
90 ml pyridine

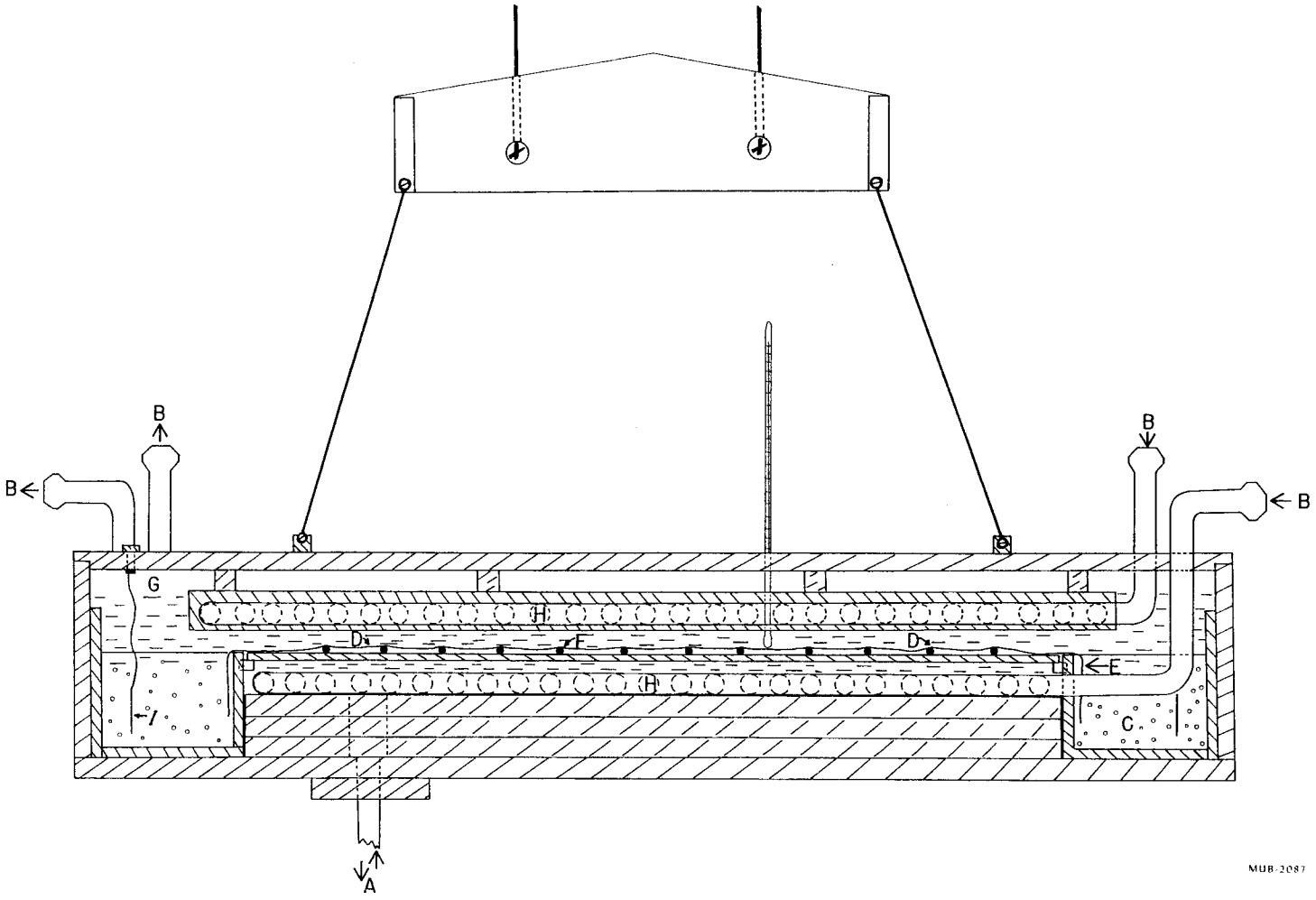

Fig. 3-1. Cross section of apparatus for two-dimensional high-voltage low-temperature electrophoresis.
(a) Decalin; (b) ethylene-glycol mixture; (c) buffer vessel; (d) sheet of paper; (e) paper wicks; (f) plastic support with glass beads; (g) Decalin; (h) cooling coils; (i) electrode.

8 g ethylenediaminetetraacetic acid (EDTA)
pH 2.0 (for the second dimension)
4500 ml distilled water
290 ml glacial acetic acid
130 ml 25% formic acid

Papers

Whatman No. 3MM paper, generally used in electrophoresis, has sufficient wet strength to be handled for chromatography in two dimensions with one drying operation in between. Whatman No. 1 is satisfactory for one-dimensional chromatography but is often torn by the supporting beads during the subsequent drying operation. Ederol No. 202 is extremely difficult to handle, because of its low wet strength.

Preparation of Suspensions of Plant Material

Bean leaves and algae were allowed to fix $^{14}CO_2$ for a short period (1 to 30 sec). Subsequent killing and extraction of the material resulted in a suspension that contained the whole material.

Bean Leaves

A single leaf or an excised di- or trifoliate leaf (depending on the size) from a bean plant was placed in an illumination chamber of 80 ml volume and exposed to $^{14}CO_2$ as described elsewhere.[7,8] Subsequently, the leaf was plunged into isopentane at -160°C and crushed into smaller pieces. These were ground to a fine powder in a homogenizer cooled with dry ice–acetone. The final suspension was obtained by resuspending the lyophylized isopentane suspension in various solvents (e.g., 80% methanol, ice water, formamide), at temperatures below zero, to a volume of 200 µl per 100 mg fresh weight of the starting material.

Algae

Chlorella pyrenoidosa, grown in continuous culture, was harvested and resuspended in 10^{-3} M KH_2PO_4/K_2HPO_4 buffer, giving a 1% suspension (wet-packed volume/suspension volume), 20 ml of which was placed in a simplified "lollipop" immersed in a water bath. Air containing 2% CO_2 was passed through the vessel, which was illuminated from either side by a photospot lamp. After 10 to 15 min of photosynthesis, the air was replaced by nitrogen for 60 sec. Then, 0.5 ml of $NaH^{14}CO_3$ solution (0.06 M, 1.7 mC/ml) was added with shaking. After a short period of photosynthesis, the algae suspension was run into 80 ml of cold methanol (below 0°C). This suspension was then vacuum evaporated to 1.5 ml at a temperature below 0°.

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8. J. A. Bassham, Photosynthesis, in Radioactive Isotopes in Physiology, Diagnostics, and Therapy, Eds. H. Schweigk and F. Turba (Springer-Verlag, Berlin, 1961) 2nd ed., Volume 2, pages 449-478.

Electrophoretic Technique

An almost-square sheet of paper (48.4×46.4 cm) was dipped into buffer solution, blotted between filter paper, and placed in position under the Decalin, which filled the box to a level of 9.5 cm. The paper was connected by paper wicks to the buffer vessels, which were equally filled with 1600 ml of buffer. These wicks were strips 48.4 cm long and 9 cm wide, made from the same paper; they overlapped the edge of the paper sheet by 1 to 1.5 cm.

After 45 to 60 min, when the paper had reached hydraulic equilibrium, the system was cooled to 0°C and the sample to be separated was applied at the origin as a straight line by means of calibrated micropipettes. An aliquot protion (75 to 200 µl) of suspension was used for a one-dimensional run, giving a starting line of 28 to 40 cm; the values for a two-dimensional run are 10 to 25 µl and 2 to 4 cm. After a run was completed, the Decalin was drained from the system, the wicks were removed, and the paper (clamped to its support) was placed in a drying box. The drying operation required about 90 min, after which the electrophoretogram was put on film for radioautography.

For two-dimensional separation, the dry paper was sprayed with the more acidic buffer after the first drying, with care taken not to wet a strip 5 to 6 cm wide in order to keep in position the components that were separated in the first dimension. The wet sections of the paper were blotted again, and the paper was put in position with the direction of the separation at right angles to the first one. After application of the paper wicks the two buffer fronts started moving together, thus bringing about a concentration of the single zones to single spots. When the whole strip was completely wet with the buffer, the system was cooled down and the second run was started.

The radioactive zones and spots of the electrophoretograms were analyzed by use of paper-chromatographic separation and radioautography with known compounds as markers.

Results

A radioautograph of a one-dimensional electrophoretogram from a 12-sec photosynthesis experiment with algae shows eight zones. These migrate to the anode as shown in Fig. 3-2 (3.7 kV, 70 mA, 30 min).

Zone 1: phosphoenolpyruvic acid and two other very weakly labeled compounds
Zone 2: 3-phosphoglyceric acid, 2-phosphoglyceric acid
Zone 3: ribulose diphosphate and one other weakly labeled compound
Zone 4: malic acid
Zone 5: still not known
Zone 6: still not known
Zone 7: fructose-6-phosphate and glucose monophosphate
Zone 8: sedoheptulose monophosphate [and uridine diphosphate glucose ? (UDPG)]

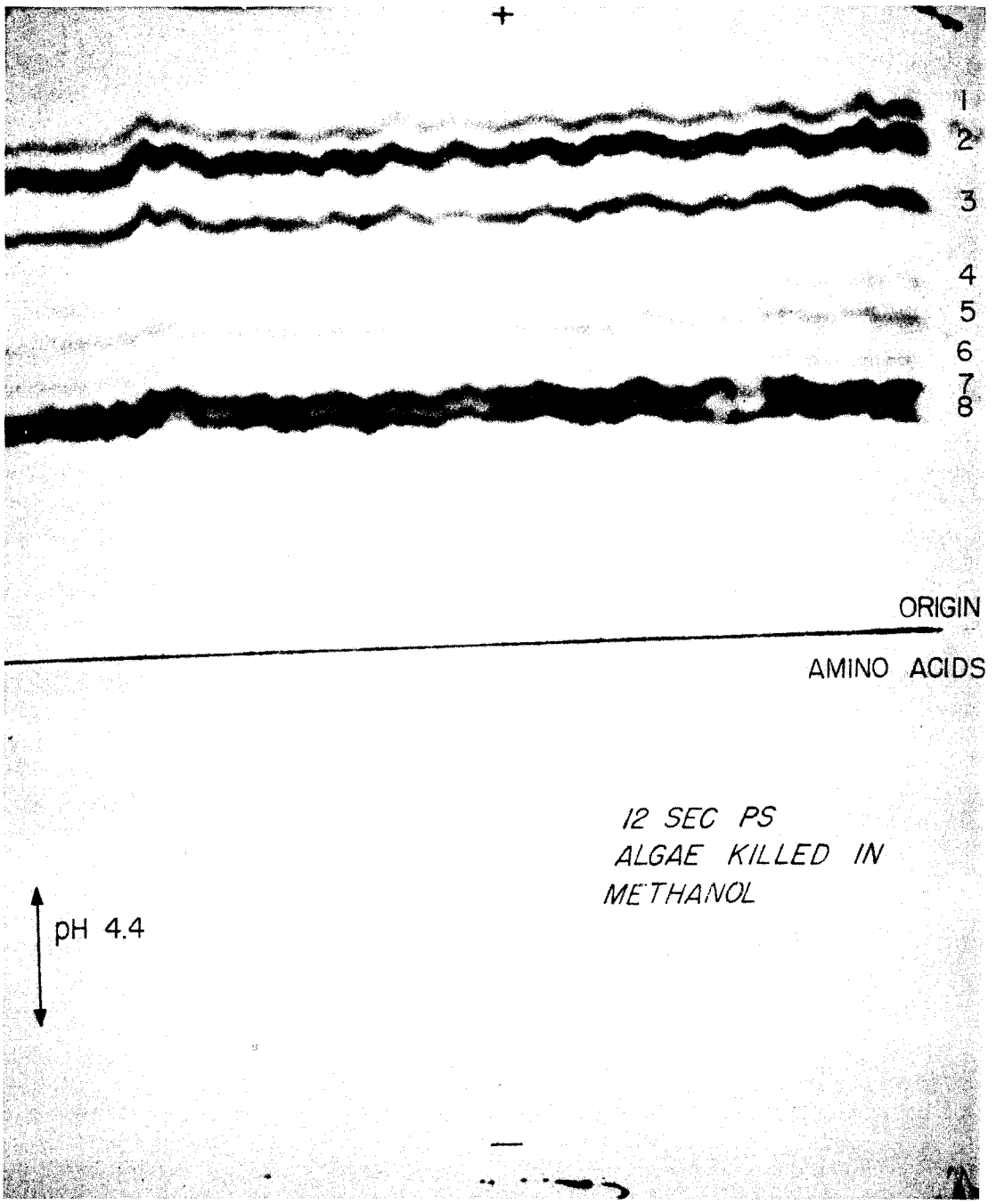

Fig. 3-2. Radioautograph of a one-dimensional electrophoretogram from a 12-sec photosynthesis experiment with algae. Conditions: pH 4.4, 3.7 kV, 70 mA, 30 min.

Origin: the radioactivity found at the starting line could not be eluted by water, acetone, or methanol. Part of it came off on treatment with 1 N NaOH.

The two weakly labeled zones beyond the origin contain substances such as free sugars and neutral amino acids.

Figure 3-3 shows a radioautograph of a two-dimensional electrophoretogram. On the basis of the known electrophoretic behavior of the single groups of compounds encountered in early photosynthesis, such as phosphorylated sugars, amino acids, etc., one can predict, to some extent, the area in which they should be located on a two-dimensional electrophoretogram.

Under the conditions employed, all phosphorylated compounds should be located on the anodic side of the second starting line, where the moving buffer fronts come together. According to their low mobility at pH 2.0, the carboxylic acids should be found in close proximity to this line.

Only amino acids (except cysteic acid) should migrate into the cathodic section, with aspartic and glutamic acid well separated from the rest of the primary amino acids. As can be seen from Fig. 3-3, all compounds analyzed so far behave as expected except for one spot (labeled ×), which is found close to aspartic acid. By determinations with authentic material it was shown that this compound is neither γ-hydroxyglutamic acid nor β-methylaspartic acid. There remains the possibility that a phosphorylated neutral amino acid migrates as an anion in the first run at pH 4.4, but is then hydrolyzed by the second buffer at pH 2.0. That would explain the appearance of a neutral amino acid in this region, but this possibility has not been checked so far.

The general pattern of separation could be reproduced many times and is always characterized by the symmetry and compactness of the spots.

Thus far, no new ^{14}C-labeled products of photosynthesis have been identified, but a number of as yet unidentified spots have been seen on the radioautographs. Even if new compounds are not discovered, it should be of interest to compare the kinetics of labeling of photosynthetic products analyzed by this method with previous kinetic studies in which paper-chromatographic analysis was used.

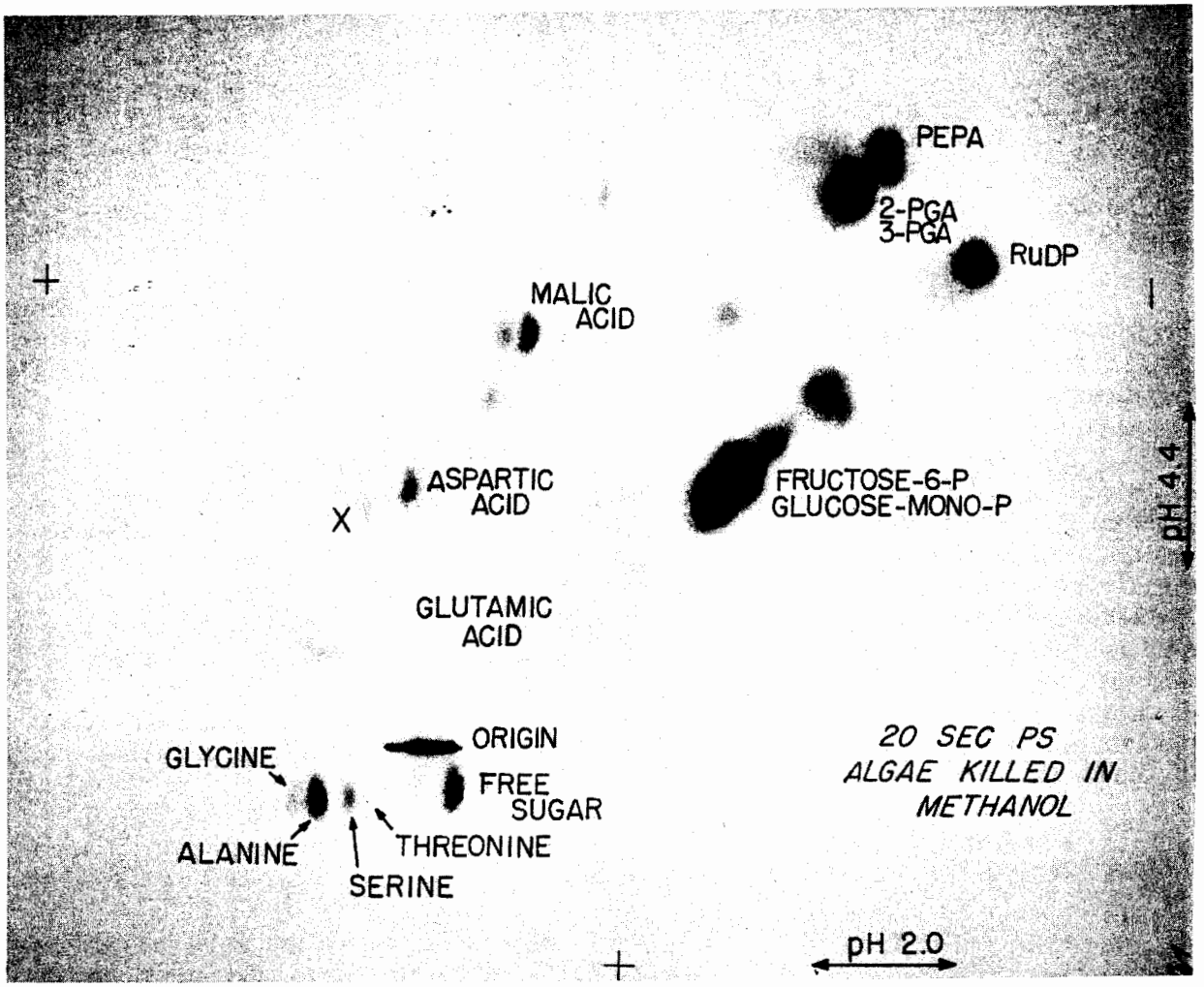

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Fig. 3-3. Radioautograph of a two-dimensional electrophoretogram from a 20-sec photosynthesis experiment with algae. Conditions: pH 4.4, 2.8 kV, 135 mA, 42 min
pH 2.0, 3.7 kV, 100 mA, 40 min.

4. A SEARCH FOR ENZYMIC AND NONENZYMIC REACTIONS BETWEEN THIAMINE DERIVATIVES AND SUGAR PHOSPHATES

Horst Egeter

Introduction

It has been suggested that photosynthetic carbon dioxide reduction might be mediated by a multifunctional organized enzyme system containing thiamine pyrophosphate chloride (TPP)* as one cofactor.[1]

The "active" intermediates of some enzymic reactions where TPP is involved as a coenzyme (such as the decarboxylation of pyruvate to acetaldehyde and CO_2) have been isolated by Holzer and coworkers.[2] One such compound is "active glycolaldehyde" (see Fig. 4-1), which is formed during a transketolase reaction in which fructose-6-phosphate reacts with glyceraldehyde-3-phosphate to give a tetrose phosphate and xylulose-5-phosphate.[2] In all such reactions, adducts are formed between a functional carbonyl group of the substrate and the active site of the TPP molecule, which is the carbon atom 2 of the thiazole moiety, as has been shown by the model experiments of Breslow[3] and later by Ingraham.[4] In view of the findings of Krampitz[5] and associates, who succeeded in carrying out nonenzymic reactions with thiamine and a variety of aldehydes, it appeared that it might be possible to perform some reactions between thiamine derivatives and a two-carbon fragment with a phosphate group adjacent to the carbonyl function. Glycoaldehyde phosphate was chosen for some attempts because the supposed adduct in the hypothetical mechanism of carboxylation of ribulose diphosphate[1] might be:

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*Abbreviations used in this report: Th, thiaminechloride; TPP, thiamine pyrophosphate chloride; RuDP, ribulose diphosphate; G-1,6-P, glucose 1,6-diphosphate; F-6-P, fructose-6-phosphate; GA1P, glycolaldehyde phosphate; F-1,6-P, fructose 1,6-diphosphate; PGA, 3-phosphoglyceric acid.

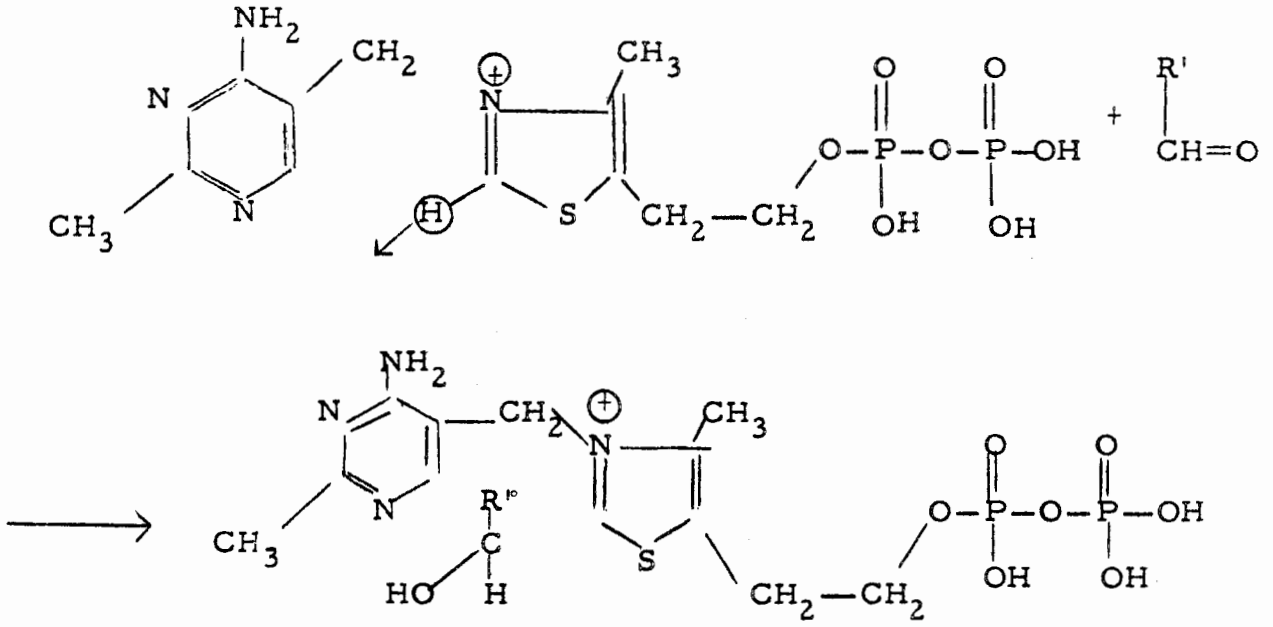
1. J. A. Bassham, The Mechanisms of the Reactions of the Carbon Reduction Cycle of Photosynthesis, UCRL-10521, Aug. 1962.

2. H. Holzer, F. da Fonseca-Wollheim, G. Kohlhaw, and Ch. W. Woenckhaus, Ann. N. Y. Acad. Sci. 98, 453 (1962).

3. R. Breslow, J. Am. Chem. Soc. 80, 3719 (1958); 81, 3080 (1959).

4. L. L. Ingraham, J. Am. Chem. Soc. 84, 3109 (1962).

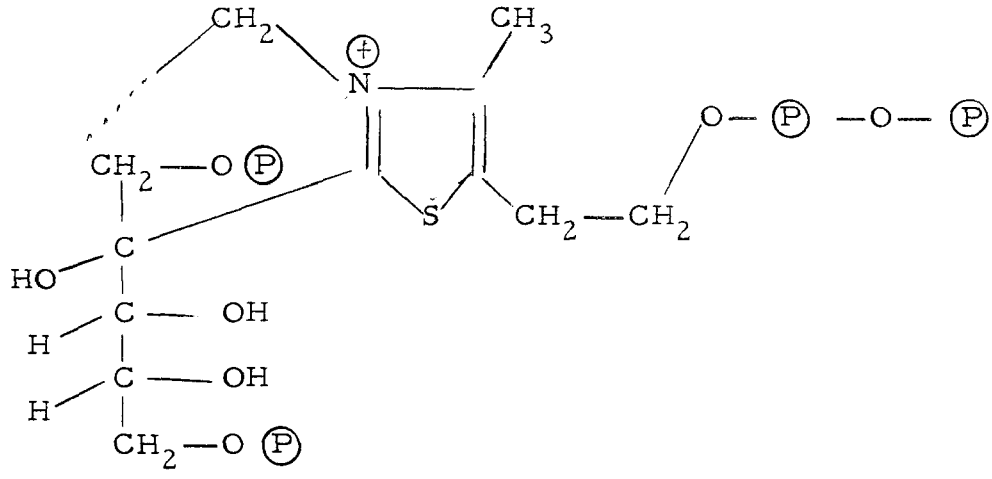
5. L. O. Krampitz, Ann. N. Y. Acad. Sci. 98, 401 (1962).



For "active acetaldehyde," R' = CH_3

For "active glycolaldehyde," R' = CH_2OH

Fig. 4-1. Formation of "active aldehydes" with thiamine pyrophosphate.



Such a compound could be split to give TPP-CHOH-$CH_2OPO_3H_2$ plus triose phosphate. Besides, nonenzymic reactions were attempted with unlabeled F-6-P, F-1,6-P, RuDP, and labeled GA1P-^{14}C and RuDP-^{14}C. The ^{14}C-labeled compounds have also been incubated with TPP in the presence of carboxydismutase enzyme under complete deprivation of air in an argon atmosphere. In the case of RuDP, carbonic anhydrase was also present to facilitate removal of CO_2 from the system.

Experimental Procedure

General

The molar ratio of Th or TPP to sugar phosphate that we used was in the range of 1:3 to 10:1. The reactions were incubated at a temperature of 45°C in aqueous solution for 2 to 24 hr in a reaction mixture, the pH of which was adjusted to 8.3 to 8.9 with either tris buffer or NaOH. The final volume of 1.2 to 2.0 ml was reduced, after an incubation period, by evaporating to a small volume (about 150 µl) and subjected to one- or two-dimensional paper chromatography. The single constituents were treated simultaneously in separate vessels under the same conditions in order to have reference chromatograms for comparison.

Sample Reaction Mixtures

(a) GA1P: Glycolaldehyde phosphate (42.0 mg, 0.3 mmole) was liberated from the glycolaldehyde phosphate diethyl acetal dicyclohexylamine salt[6] by shaking with ion-exchange resin [Dowex AG 50 W - X 8 (H^+)] in 2.0 ml water, then heating for 5 hr at 40°C. After this, 0.1 mM Th or TPP was added, the pH was adjusted to 8.8, and the solution was incubated at 45°C for 12 hr; during this time the pH was checked and readjusted every 2 hr.

6. Kindly supplied by Dr. C. E. Ballou, Department of Biochemistry, University of California, Berkeley.

(b) F-6-P: Fructose-6-phosphate (78.0 mg, 0.3 mmole), obtained from the corresponding Ba salt, and 0.1 mmole Th or TPP in 1.5 ml of water were incubated for 16 hr at 42°C after adjusting the pH to 8.2 and checking occasionally.

(c) F-1,6-P: Fructose diphosphate (170.0 mg, 0.5 mmole), prepared from the trisodium salt, and 0.1 mmole Th or TPP in a final volume of 1.1 ml were incubated at 40 to 45°C for about 5 hr. The pH was adjusted to 8.4.

Paper-Chromatographic Method

Reaction mixtures were applied to various chromatographic papers (Whatman No. 1 or 4, Ederol No. 202). These were developed in one or two dimensions. Four different solvent systems were used. In the first direction papers were developed with either "semistench"[7] or freshly prepared n-butanol–acetic acid–water (5:2:3) for approximately 19 to 28 hr. In the second direction either freshly prepared n-butanol–propionic acid–water (14:10:9) or n-butanol–ethanol–water (4:2:3) was used for about the same time. For locating the spots, a phosphate spray (according to Hanes[8]) or the thiochrome spray for specific detection of thiamine derivatives[9] (showing fluorescence in uv light) was used.

Carboxydismutase Enzyme

This enzyme was isolated in the usual way from spinach chloroplasts by using isotonic sucrose tris buffer (pH 8.0) and fractionating the extract obtained by osmotically leaching the washed chloroplasts in ammonium sulfate.[10]

Preparation of Radioactive RuDP

This substance was prepared with a 1% suspension of Chlorella pyrenoidosa by 90-sec photosynthesis with $H^{14}CO_3^-$, as reported by Pon.[10] The combined 80% methanol–20% ethanol extracts were evaporated to a smaller volume, then the lipids were removed by extraction with petroleum ether. After further evaporation to a volume less than 1 ml, the extract was applied as a band on Whatman No. 4 (oxalic acid-washed paper) and allowed to develop with "semistench" for 48 hr. The RuDP, located by radioautography, was eluted from the paper and dephosphorylated with Polidase-S. Subsequently, it was chromatographed, identified with authentic marker compounds, cut out, re-eluted, and several times rechromatographed

7. Gerald J. Crowley, V. Moses, and Johannes Ullrich, in Bio-Organic Chemistry Quarterly Report, UCRL-10743, March 1963, p. 1.

8. C. S. Hanes, Nature 164, 1102 (1949).

9. D. Siliprandi, Biochim. Biophys. Acta 14, 52 (1954).

10. Ning G. Pon, Studies on the Carboxydismutase System and Related Materials, (Ph. D. Thesis), UCRL-9373, Aug. 1960.

in one dimension with n-butanol–propionic acid–water (on Whatman No. 4) until it showed a single band in the radioautography. This band contained also G-1, 6-P and F-1, 6-P, as shown by chromatography of the free sugars obtained by phosphatase action. It was not possible to achieve complete purification of ribulose diphosphate.

Preparation of Radioactive GA1P

The sugar monophosphates and PGA from paper chromatograms from the above photosynthesis experiment were eluted and submitted to periodate cleavage. To 1.2 ml aqueous eluate were added 64 mg of $NaIO_4$ in 0.5 ml water; the pH of the solution was 4.9. After 4 hr at 42°C, the solution was reduced in volume and was applied to paper and developed by one-dimensional chromatography on Ederol No. 202 paper with n-butanol–acetic acid–water. Labeled compounds, located by radioautograph, were eluted and chromatographed in two dimensions using "semistench" and n-butanol–propionic acid–water. The compounds on the paper were washed with diethyl ether to remove traces of the solvents, and subsequently eluted with water.

Positive Reaction with 1, 2-Acetaldehyde-C^{14}

We incubated 0.01 mmole Th or TPP and 0.1 mmole 1, 2-acetaldehyde-C^{14} (total activity 100 μC) at 44°C in about 0.8 ml aqueous solution, the pH of which was adjusted to 8.7 with 2 N NaOH. After 5 hr and after 26 hr, samples were taken and run on two-dimensional chromatograms. A radioactive area on the radioautograph that coincided with a sprayed spot of a thiamine derivative on the paper was found and was cut out and run once more. After that the coincidence did not change. There was no difference in the patterns of the 5-hr and 26-hr samples.

Enzymic Reactions

(a) RuDP-^{14}C-Th, or -TPP, respectively: Usually 300 μl carboxydismutase (protein content approximately 4.0 mg) in 0.05 mM tris buffer (pH 7.6) and 50 μl (200 units) carbonic anhydrase were placed in one arm of a two-sidearm vessel. The substrates placed in the other part consisted of 200 μl RuDP-^{14}C eluate, 500 μl Th or TPP (3 to 10 $\times 10^{-3}$ mmole in 0.05 mM tris (pH 7.9)), 50 μl $MgCl_2$ solution (10^{-2} mmole), and 50 μl carbonic anhydrase (200 units). Prior to mixing, the vessel was cautiously evacuated to a low pressure and then argon was admitted. The incubation was carried out at 38°C for 3 hr or at room temperature for 6 hr, and finally stopped by addition of hot methanol. After centrifugation and concentration of the supernatant solution to a volume of about 150 μl, one part of the sample was placed on the origin of a two-dimensional chromatogram. At the same time as the enzyme experiment, the same reaction mixture without any carboxydismutase was incubated in the same way.

(b) GA1P-^{14}C-Th or TPP: The thiamine derivative (3.5×10^{-3} mmole) in 500 μl aqueous solution, and 200 μl of the purified GA1P-^{14}C eluate were adjusted to a pH of about 8.7, either by adding solid tris buffer, 1 N NaOH, or $NaHCO_3$. For the enzymic reactions, 25 μl of $MgCl_2$ (5×10^{-3} mmole) was also added. Carboxydismutase (100 μl, protein content 3.5 mg) in tris buffer (pH 7.1) was placed in the second bulb of the reaction vessel. The

treatment that follows before and after the incubation interval was the same as that described in the preceding section.

Proteolytic Cleavage of the Enzyme

Attempts were made to liberate the suspected prosthetic TPP from carboxydismutase by utilization of the highly active proteolytic enzyme, "Pronase" (Streptomyces griseus protease, California Corporation for Biochemical Research, Los Angeles), which is reported[11] to have a low substrate specificity and is capable of hydrolyzing as much as 90% of the peptide bonds in protein. The enzyme (500 µl, approximately 6.5 mg of protein) in 0.1 mM tris buffer (pH 7.9), Pronase (65 µg), and 50 µl of ethanol were mixed and incubated at 39°C for 48 hr or 96 hr. The pH was checked every few hours and, if necessary, adjusted by addition of 0.1 N NaOH. The control experiments were done with added TPP in a ratio 1 mole protein: 1 mole TPP (assuming the molecular weight of the enzyme as 3×10^5). At the end of the incubation the volume was reduced under low pressure in a rotating evaporator to about 100 µl and submitted to two-dimensional chromatography. Sometimes, prior to this, the enzymes were removed by precipitation with hot methanol.

Results

In all these experiments, whether with or without the addition of carboxydismutase, a weakly alkaline medium was used because the maintenance of a pH between 7.8 and 8.9 was assumed to be important for the necessary protonation at the active position 2 of the thiazole moiety. Although the reactions were carried out in the same way as reported for the formation of adducts with free aldehydes, no reactions have been detected for carbonyl phosphates in which a phosphate group is adjacent to a carbonyl group. Perhaps steric hindrance of this bulky phosphate group prevents a nucleophilic attack at the carbonyl. Actually, there was at no time any difference between the chromatographic pattern of the singly treated constituents and that of the reaction mixture. Even by use of isolated, labeled RuDP-^{14}C and GA1P-^{14}C, a coincidence between the radioautographs and the position of the thiamine derivative on the paper was not observable. Because no thiamine derivative could be detected with the very sensitive thiochrome test after proteolytic splitting of different carboxydismutase preparations, apparently no TPP is implicated in the mode of action of the carboxydismutase system. Moreover, it appears that formation of "active aldehyde" addition compounds between TPP and sugar phosphates does not occur with keto sugars phosphorylated in the C-1 position.

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11. M. Nomoto and Y. Narahshi, J. Biochem. (Tokyo) 48, 593 and 906 (1960).

5. THE CYTOCHROME CONTENT OF PURIFIED SPINACH CHLOROPLAST LAMELLAE

John Biggins and R. B. Park

Highly purified lamellae from spinach chloroplasts were prepared[1] and anlyzed for cytochrome by difference spectrophotometry as follows:

1. A solution with an optical density of 2 at 677 mμ was prepared. It was buffered with 0.05 M tris at pH 8.

2. A base line was determined (soln vs soln) with 1-cm cuvettes and full-scale expansion, on a Cary automatic recording spectrophotometer (Model 14) fitted with a light-scattering attachment. The base line was from 600 mμ to 500 mμ. This spectral region was chosen because it includes the region of the ferrocytochrome α and β bands, and because optical absorption by lipid pigments is minimal.

3. The reference cuvette was oxidized with 10^{-3} M potassium ferricyanide. No change was noted in absorbency, which indicated that any cytochrome present was ferricytochrome.

4. The solution in the sample cuvette was reduced with a small amount of solid sodium dithionite. Here, an increase in absorbency was noted at 560 mμ and 530 mμ. This indicated the presence of ferrocytochrome b_6.

5. The experiment was repeated at various concentrations of chlorophyll in the cuvettes. Below 0.01 mg chlorophyll per ml, the 560 mμ deflection on reduction was negligible. Above 0.15 mg chlorophyll per ml, erratic base lines were encountered.

The results of these experiments are summarized below:

Chlorophyll concentration, (mg/ml)	Δ absorbence, at 560 mμ after reduction
0.0263	0.006
0.0583	0.0123
0.075	0.014
0.095	0.0193
0.16	0.025
0.304	0.038

1. R. B. Park and N. G. Pon, J. Mol. Biol. 6, 105 (1963).

The ratio of chlorophyll concentration to the change in absorbence (optical density) at 560 mμ is constant. For a chlorophyll concentration of 0.1 g/liter, the Δ absorbence (upon reduction) at 560 mμ is 1.94×10^{-2} optical density units. Assuming a molar extinction coefficient at this wave length of 2.27×10^4 for the b-type ferrocytochrome,[2] the molar ratio chlorophyll/ferrocytochrome b_6 = 128.5. It therefore appears that we have about one cytochrome b_6 molecule for every 130 chlorophyll molecules. It is likely, then, that the minimum photosynthetic unit (or Mn unit) described by Park and Pon,[1] of 115 chlorophyll molecules, contains one cytochrome b_6. Cytochrome f, the c-type cytochrome described by Davenport and Hill,[3] appears to have been removed by the washings and sonication in these preparations of lamellae.

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2. H. Schichi and D. P. Hackett, J. Biol. Chem. 237, 2955 (1962).

3. H. E. Davenport and R. Hill, Proc. Roy. Soc. London B, 139, 327 (1952).

6. THE OSMIUM TETROXIDE FIXATION OF CHLOROPLAST LAMELLAE

R. B. Park

A common method of preparing leaf tissue for electron microscopic examination is by fixation of the leaves with neutral 2% OsO_4, followed by drying and embedding in plastic. Thin sections are sliced from the plastic block and viewed in the electron microscope. The electron-opaque areas correspond to regions of selective osmium deposition within the tissue.

In chloroplasts, OsO_4 reacts primarily with the membrane and with the chlorophyll-containing lamellae. These lamellae perform the light reactions and associated electron-transport reactions of photosynthesis. [1] A lamella consists of two layers, each of which is about 100 Å thick. The single layers are still active when separated. An OsO_4-stained cross section of a two-layered lamella is seen as two dark lines separated by about 100 Å (see Fig. 6-1).

We wish to learn exactly which chemical compounds within the lamellae react with the OsO_4, since such information will allow further localization of chemical compounds within chloroplast lamellae. Some preliminary experiments are described here that were designed to find the amount of osmium taken up by a known weight of lamellae. One object of this work is to see whether this amount of osmium corresponded to the abundance of some given chemical structure (such as olefinic double bonds) within the lamellae. This experiment was performed as follows.

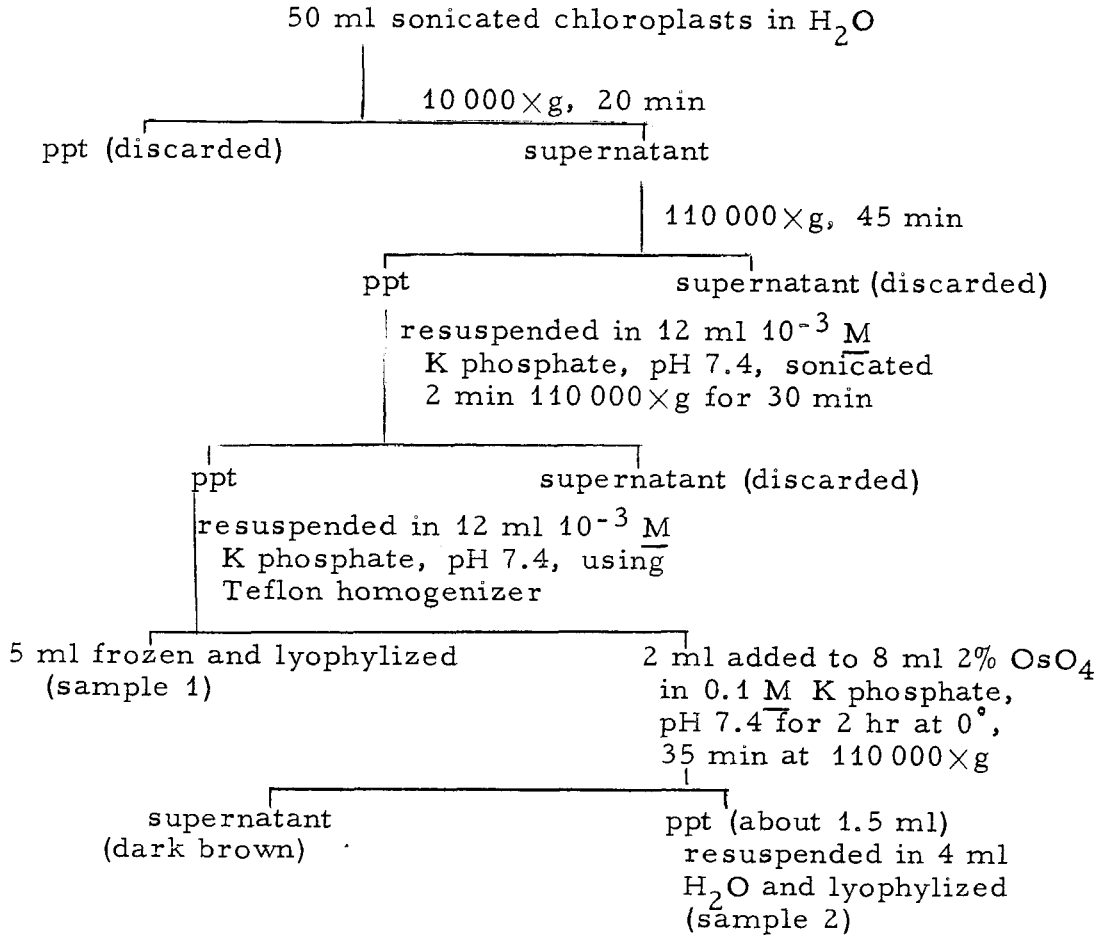
Sonicated chloroplasts were prepared following the method of Park and Pon. [1] The sonicated chloroplasts were then treated as shown on next page. Samples 1 and 2 were weighed, and these weights were corrected to 1 ml of original suspension from which the samples were prepared (see Table 6-I).

Table 6-I. Dry weights of equivalent amounts of OsO_4-treated and control lamellae.

Sample	Observed weight (mg)	Original suspension (mg/ml)
1	185.3	37.05
2	108.7	54.35

The weight of sample 2 was increased 47% by osmium treatment. If one assumes that the majority of the osmium has formed osmic esters (see Fig. 6-2) with unsaturated fatty acids of the lamellae, [2] there are 68 μmoles of osmium per ml of lamellae or 0.92 μmoles of osmium per mg of lamellae.

1. R. B. Park and N. G. Pon, J. Mol. Biol. 3, 1 (1961).
2. W. Stoeckenius, J. Biophys. Biochem. Cytol. 5, 491 (1959).


50 ml sonicated chloroplasts in H_2O
10 000×g, 20 min
ppt (discarded)
supernatant
110 000×g, 45 min
ppt
supernatant (discarded)
resuspended in 12 ml 10^{-3} M K phosphate, pH 7.4, sonicated 2 min 110 000×g for 30 min
ppt
supernatant (discarded)
resuspended in 12 ml 10^{-3} M K phosphate, pH 7.4, using Teflon homogenizer
5 ml frozen and lyophylized (sample 1)
2 ml added to 8 ml 2% OsO_4 in 0.1 M K phosphate, pH 7.4 for 2 hr at 0°, 35 min at 110 000×g
supernatant (dark brown)
ppt (about 1.5 ml) resuspended in 4 ml H_2O and lyophylized (sample 2)

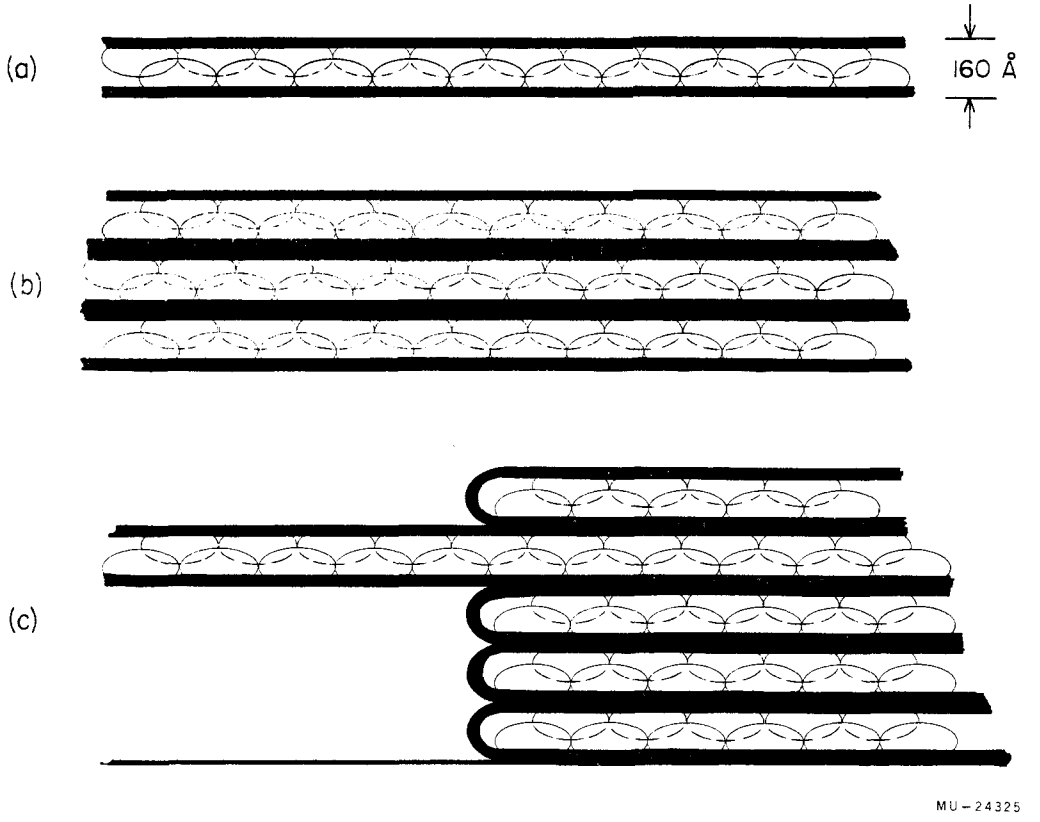


Fig. 6-1. OsO_4-stained chloroplast lamellae as viewed in thin section in the electron microscope. (a) blue-green algae; (b) Euglena; (c) higher plants.

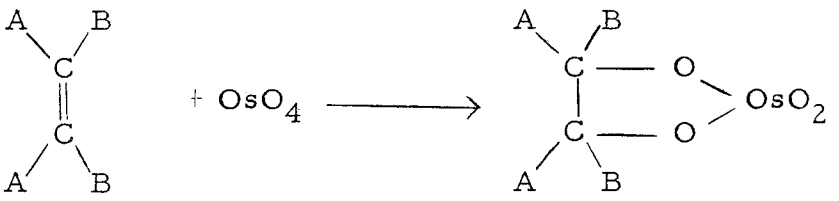


Fig. 6-2. Formation of osmic esters from olefinic double bonds and OsO_4.

One can calculate from the data of Wolf et al.[3] that each chloroplast fatty acid contains an average of 2.2 double bonds. Thus, in the minimum molecular-weight unit (980 000 per mol Mn) described by Park and Pon,[4] there are about 1 440 double bonds in fatty acids. Since a 47% increase in weight of the unit occurs on OsO_4 treatment, one can calculate that the amount of osmium esterified is 442 000/254 = 1 740 atoms per Mn unit. Since the unit contains about 1 440 double bonds in fatty acids, the dark lines in Fig. 6-1 may represent, mainly, osmic esters of fatty acids.

Methanol extraction of samples 1 and 2 removed 51% and 17% respectively, of the sample mass. As determined spectrophotometrically, about 10% of the chlorophyll was removed from the osmium-treated sample; in contrast, essentially all of the chlorophyll may be removed from the control on methanol extraction.

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3. P. T. Wolf, J. G. Coniglio, and J. T. Davis, Plant Physiol. 37, 33 (1962).

4. R. B. Park and N. G. Pon, J. Mol. Biol. 6, 105 (1963).

7. KINETICS OF EXOENZYMES AND APPLICATIONS TO THE DETERMINATION OF THE SEQUENCE OF NUCLEIC ACIDS

Charles Cantor and Ignacio Tinoco, Jr.

Introduction

Three of the methods proposed for determining the sequence of bases in nucleic acids are the overlap method,[1] selective labeling,[2] and the use of specific endonucleases.[3] Each of these would require a subsequent analysis of oligomers containing many nucleotides. We wish to study the use of exonucleases to obtain the base sequence of oligomers and to find the sequence of bases near the ends of polynucleotides. We discuss first the general kinetics of exoenzymes and then the applications to determination of base sequences.

We have used two different approximations to calculate the kinetics of exoenzymes. In one case, the free-enzyme concentration is assumed to be constant. This is applicable to systems with high enzyme concentration and to all systems after the reaction has proceeded through the first two or three intermediates. In addition, a steady-state treatment, useful in the limit of low enzyme concentration, is also derived.

Theory

For all the calculations we use a standard simple model for the mechanism of enzyme action:

$$S_1 + E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES_1 \xrightarrow{k_3} E + S_2 + B_1 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES_2 \xrightarrow{k_3} E + S_3 + B_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{etc.} \qquad (1)$$

Here S_1 symbolizes the polymer $B_1B_2\cdots B_N$; S_n is the polymer $B_nB_{n+1}\cdots B_N$; ES_n is the enzyme-substrate complex of the exoenzyme and S_n. Note that the rate constants (K_1, K_2, and K_3) are taken to be independent of the substrate. This reaction scheme can be described by the following set of 3N linear differential equations, where the dot above a concentration variable means the derivative with respect to time:

$$\begin{aligned}
(\dot{S}_1) &= -k_1(E)(S_1) + k_2(ES_1), \\
(\dot{S}_n) &= -k_1(E)(S_n) + k_2(ES_n) + k_3(ES_{n-1}), && \text{for all } n \neq 1, \\
(\dot{ES}_n) &= k_1(E)(S_n) - (k_2 + k_3)(ES_n), && \text{for all n's}, \\
(\dot{B}_n) &= k_3(ES_n), && \text{for all n's}
\end{aligned}$$

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1. F. Sanger, in Currents in Biochemical Research, D. E. Green, Ed., (Interscience, New York, 1956).
2. S. Mandeles and I. Tinoco, Jr., Biopolymers 1, 183 (1963).
3. W. E. Rice and R. M. Bock, J. Theoret. Biol. 4, 260 (1963).

with boundary conditions

$$[S_n(0)] = [B_n(0)] = [ES_n(0)] = 0 \qquad \text{for all } n \neq 1,$$

$$[B_1(0)] = 0; \quad [S_1(0)] = S_0; \quad [ES_1(0)] = ES_0.$$

Units are adjusted so that $S_0 + ES_0 = 1$, and therefore each $B_n(\infty) = 1$. Since the total amount of enzyme bound in complexes,

$$\sum_{n=1}^{N} ES_n(t) = \frac{1}{k_3} \sum_{n=1}^{N} \dot{B}_n,$$

is very close to constant [see Eqs. (15) and (16)], the concentration of free enzyme,

$$E = E_0 - \sum_{n=1}^{N} ES_n(t),$$

is a constant. Let $k_1(E) = k$. When using very high enzyme concentrations one must realize that significant amounts of ES_1 may form during the time that it takes to mix the enzyme with the initial undegraded substrate. This can be accommodated by allowing $ES_1(0)$ to be greater than zero.

The Laplace transform, $L[f(t)]$, is defined by $\int_0^\infty f(t)e^{-st}dt$, where s is an arbitrary variable; by partial integration it can be shown that

$$L[f'(t)] = sL[f(t)] - f(0). \tag{2}$$

Since we know all the boundary conditions at time t = 0, it is convenient to take the Laplace transform of each of the above differential equations.

Define $L(ES_n) = w_n$,
$L(S_n) = u_n$,
$L(B_n) = y_n$.

By taking the Laplace transform of each of the differential equations and using (2) and the above boundary conditions, we have

$$su_1 - S_1(0) = -ku_1 + k_2w_1, \tag{3}$$

$$su_n = -ku_n + k_2w_n + k_3w_{n-1}, \qquad \text{for all } n \neq 1, \tag{4}$$

$$sw_1 - ES_1(0) = ku_1 - (k_2 + k_3)w_1, \tag{5}$$

$$sw_n = ku_n - (k_2 + k_3)w_n, \qquad \text{for all } n \neq 1, \tag{6}$$

$$sy_n = k_3w_n, \qquad \text{for all n's.} \tag{7}$$

Now, by simultaneous algebraic solution of these equations, we can find a recursion relationship for w_n and then substitute this in (7) to find the Laplace transform of B_n in terms of the rate constants and the nonzero boundary conditions:

$$w_n = \frac{kk_3 w_{n-1}}{s^2 + s(k + k_2 + k_3) + kk_3} ,$$

$$w_1 = \frac{(s + k)ES_0 + kS_0}{s^2 + s(k + k_2 + k_3) + kk_3} ,$$

$$w_n = \frac{(kk_3)^{n-1}[(s + k)ES_0 + kS_0]}{[s^2 + s(k + k_2 + k_3) + kk_3]^n} ,$$

and

$$y_n = k_3 w_n / s = \frac{k_3(kk_3)^{n-1}ES_0}{[s^2 + s(k+k_2+k_3) + kk_3]^n} + \frac{(kk_3)^n[ES_0 + S_0]}{s[s^2 + s(k+k_2+k_3) + kk_3]^n} . \tag{8}$$

The denominator can be factored as $(s-a_1)(s-a_2)$, where

$$a_{1,2} = \frac{-(k+k_2+k_3) \pm \sqrt{(k+k_2+k_3)^2 - 4kk_3}}{2} ,$$

and (8) can be rewritten as (9), since $kk_3 = a_1 a_2$.

$$y_n = \frac{k_3(a_1a_2)^{n-1}ES_0}{(s-a_1)^n(s-a_2)^n} + \frac{(a_1a_2)^n(ES_0 + S_0)}{s(s-a_1)^n(s-a_2)^n} . \tag{9}$$

Taking the inverse Laplace transform of (9), we find

$$B_n(t) = k_3(a_1a_2)^{n-1}ES_0 L^{-1}[(s-a_1)^{-n}(s-a_2)^{-n}]$$

$$+ (a_1a_2)^n(ES_0 + S_0)L^{-1}[s^{-1}(s-a_1)^{-n}(s-a_2)^{-n}]. \tag{10}$$

It is known that $L^{-1}[(s-a_1)(s-a_2)]^{-1} = (a_1-a_2)^{-1}[\exp(a_1t) - \exp(a_2t)]$. By successive applications of $\frac{\partial^2}{\partial a_1 \partial a_2}$ to both sides of this equation we find

$$L^{-1}[(s-a_1)^{-n}(s-a_2)^{-n}] = \frac{1}{(n-1)!}\sum_{i=0}^{n-1}\frac{t^i(2n-2-i)!(-1)^{n-1+i}[\exp(a_1t)+(-1)^{i+1}\exp(a_2t)]}{(n-1-i)!(i)!(a_1-a_2)^{2n-1-i}}$$

$$\equiv I_n.$$

By similar methods we can show

$$II_n \equiv L^{-1}[s^{-1}(s-a_1)^{-n}(s-a_2)^{-n}]$$

$$= \frac{1}{(a_1a_2)^n} + \frac{(-1)^{n-1}}{(n-1)!}\sum_{i=0}^{n-1}\frac{(2n-2-i)!}{(n-1-i)!(a_1-a_2)^{2n-1-i}}\sum_{p=0}^{i}\frac{(-1)^p t^p[\exp(a_1t)]}{p!\,a_1^{i+1-p}}$$

$$+ \frac{(-1)^{n-1}}{(n-1)!}\sum_{i=0}^{n-1}\frac{(2n-2-i)!}{(n-1-i)!(a_2-a_1)^{2n-1-i}}\sum_{p=0}^{i}\frac{(-1)^p t^p[\exp(a_2t)]}{p!\,a_2^{i+1-p}}\,.$$

Using the above set of sums, we can rewrite (10) as

$$B_n(t) = ES_0(a_1a_2)^n(k)^{-1}I_n + (ES_0 + s_0)(a_1a_2)^n\,II_n\,. \qquad (11)$$

In the steady-state approximation, Eqs. (5) and (6) are equal to zero. By algebraic manipulation as before, we find

$$w_1 = \frac{kS_0}{s(k_2+k_3)+kk_3}$$

$$w_n = \frac{kk_3w_{n-1}}{s(k_2+k_3)+kk_3} = \frac{(kk_3)^{n-1}kS_0}{[s(k_2+k_3)+kk_3]^n}$$

and $$y_n = \frac{(kk_3)^nS_0}{s[s(k_2+k_3)+kk_3]^n} = \frac{b^nS_0}{s(s+b)^n}\,,$$

where

$$b = \frac{kk_3}{(k_2+k_3)}\,.$$

Taking the inverse Laplace transform, we have

$$B_n(t) = b^n S_0 L^{-1}[s^{-1}(s+b)^{-n}] = b^n S_0 L^{-1}[s^{-1}(s-C)^{-n}],$$

where $C = -b$. It is known that $L^{-1}[s^{-1}(s + C)^{-1}] = -C^{-1} + C^{-1}e^{Ct}$. By successive application of $\partial/\partial C$ to both sides of this equation we find

$$L^{-1}[s^{-1}(s - C)^{-n}] + (-C)^{-n} + \sum_{i=0}^{n-1} \frac{t^i[\exp(Ct)(-1)^{n-1-i}]}{C^{n-i}\, i!} \qquad (12)$$

and

$$B_n(t) = S_0\left(1 - \sum_{i=0}^{n-1} \frac{bt^i[\exp(-bt)]}{i!}\right), \text{ the steady-state case.} \qquad (13)$$

In the special case in which $a_1 = a_2 = A$, Eq. (11) does not hold, since $(a_1 - a_2)$ is zero. Instead, using (12) and assuming $ES_1(0) = 0$, we have

$$II_n = L^{-1}[s^{-1}(s - A^{2n})] = A^{-2n} + \sum_{i=0}^{2n-1} \frac{t^i[\exp(At)(-1)^{2n-1-i}]}{A^{2n-i}\, i!}.$$

Now, $A = a_1 = a_2$ implies $(k-k_3)^2 + k_2^2 + 2k_2(k_3+k) = 0$, which for $k, k_2, k_3 > 0$ has only the solution $k = k_3 = A = a$; $k_2 = 0$.

Therefore

$$B_n(t) = S_0\left(1 - \sum_{i=0}^{2n-1} \frac{(at)^i[\exp(-at)]}{i!}\right). \qquad (14)$$

By use of the original differential equations, expressions for $S_n(t)$ and $ES_n(t)$ can be derived. In the steady-state approximation and the case $a_1 = a_2$, these are particularly simple.

Steady State	$a_1 = a_2$; $ES_0 = 0$
$ES_n(t) = \dfrac{bS_0(bt)^{n-1}[\exp(-bt)]}{k_3(n-1)!}$	$ES_n(t) = S_0 \dfrac{(at)^{2n-1}[\exp(-at)]}{(2n-1)!}$
$S_n(t) = \dfrac{S_0(bt)^{n-1}[\exp(-bt)]}{(n-1)!}$	$S_n(t) = \dfrac{S_0(at)^{2n-2}[\exp(-at)]}{(2n-2)!}$

Another quantity of interest is the total rate at which a monomer is evolved from an infinite polymer. This is the slope of a plot of monomer concentration versus time.

In the steady-state case

$$\sum_{n=1}^{\infty} \dot{B}_n(t) = S_0 b \sum_{n=1}^{\infty} \frac{(bt)^{n-1}[\exp(-bt)]}{(n-1)!} = bS_0. \qquad (15)$$

In the case $a_1 = a_2$; $ES_1(0) = 0$,

$$\sum_{n=1}^{\infty} \dot{B}_n(t) = S_0 a \sum_{n=1}^{\infty} \frac{(at)^{2n-1} e^{-at}}{(2n-1)!} = a(1/2 - 1/2e^{-2at})S_0. \qquad (16)$$

Thus, by measuring the total rate of monomer evolution it may be possible to determine the kinetic parameters a and b and perhaps to decide whether or not an exoenzyme is acting under "steady-state" conditions.

We can also find the time at which the rate of evolution of any given monomer is a maximum.

In the steady state $\ddot{B}_n = 0$ implies $t = (n-1)/b$. (17)

In the case $a_1 = a_2$, $\ddot{B}_n = 0$ implies $t = (2n-1)/a$. (18)

Equations (11), (13), and (14) were evaluated for several sets of rate constants on the IBM 7090 digital computer.

Discussion

In order to use an exoenzyme to determine the sequence of monomers in a polymer, it is desirable that each monomer be evolved in as short and as separate a time interval as possible so that its presence may be determined before the picture is clouded by the presence of other monomer units. By changing the kinetic parameters k, k_2, and k_3 it is possible to change the time interval in which a specific monomer is evolved. Figure 7-1 shows the concentration of free monomer units 1, 3, 5, and 25 as a function of time, for two different cases. Since the ideal case would be a step function, one can see that the case $a_1 = a_2$ has monomers coming off much more sharply and distinctly than in the steady state. The first steady-state monomer is given off more rapidly at first because the steady-state treatment implicitly assumes that there is some ES_1 present at zero time. This can be compensated for by assuming that $ES_1(0)$ is not equal to zero for the non-steady-state solutions; however, our calculations indicate that making this assumption does not change the shapes of the curves, but only displaces them towards positive time (see Table 7-I).

To compare the effect of different choices of parameters we have defined a sharpness parameter,

$$P = \frac{\text{time that } B_n = 0.9 - \text{time that } B_n = 0.1}{\text{time spacing between two successive curves at } B_n = 0.5}. \qquad (19)$$

This dimensionless parameter is plotted in Fig. 7-2 and also shown in Table 7-I. Note that for $a_1 = a_2$ the 24th monomer comes off as sharply as the 13th monomer of the steady state. From Table 7-I one can see that the steady state is an accurate approximation when either k_1E or K_3 is much less than or much greater than k_2, but the approximation breaks down badly when $k_1E = k_2 = k_3$ or when k_2 is much less than both k_3 and k_1E.

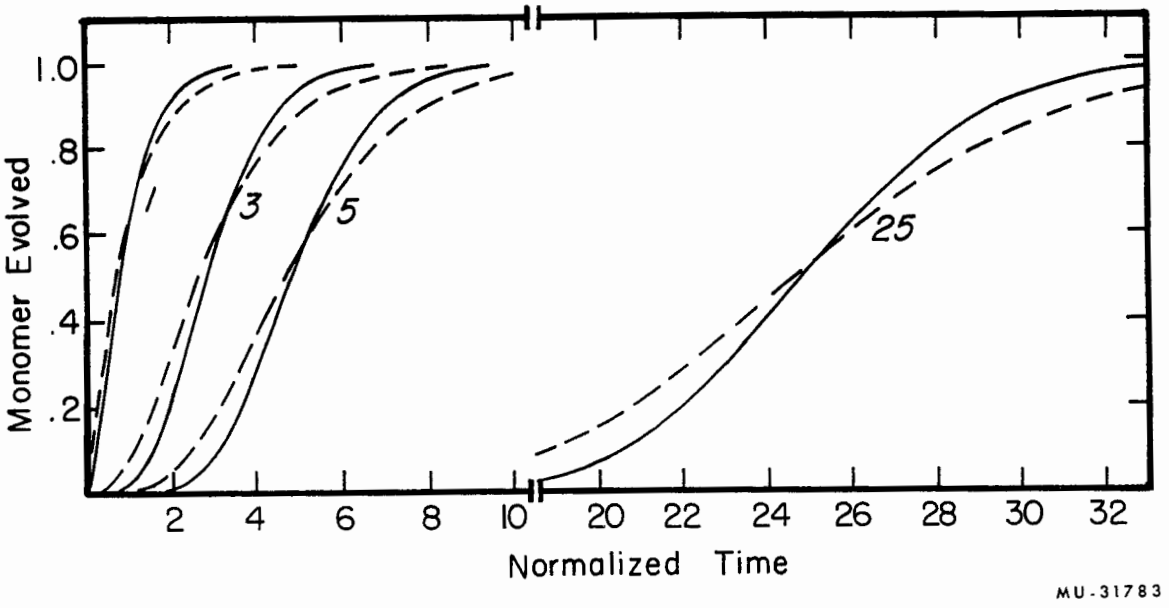

Fig. 7-1. The amount of monomer number n evolved as a function of time for n = 1, 3, 5, and 25.
--------- steady state; b = 1, S_0 = 1
———— $a_1 = a_2 = 1$; $S_0 = 1$, $ES_0 = 0$

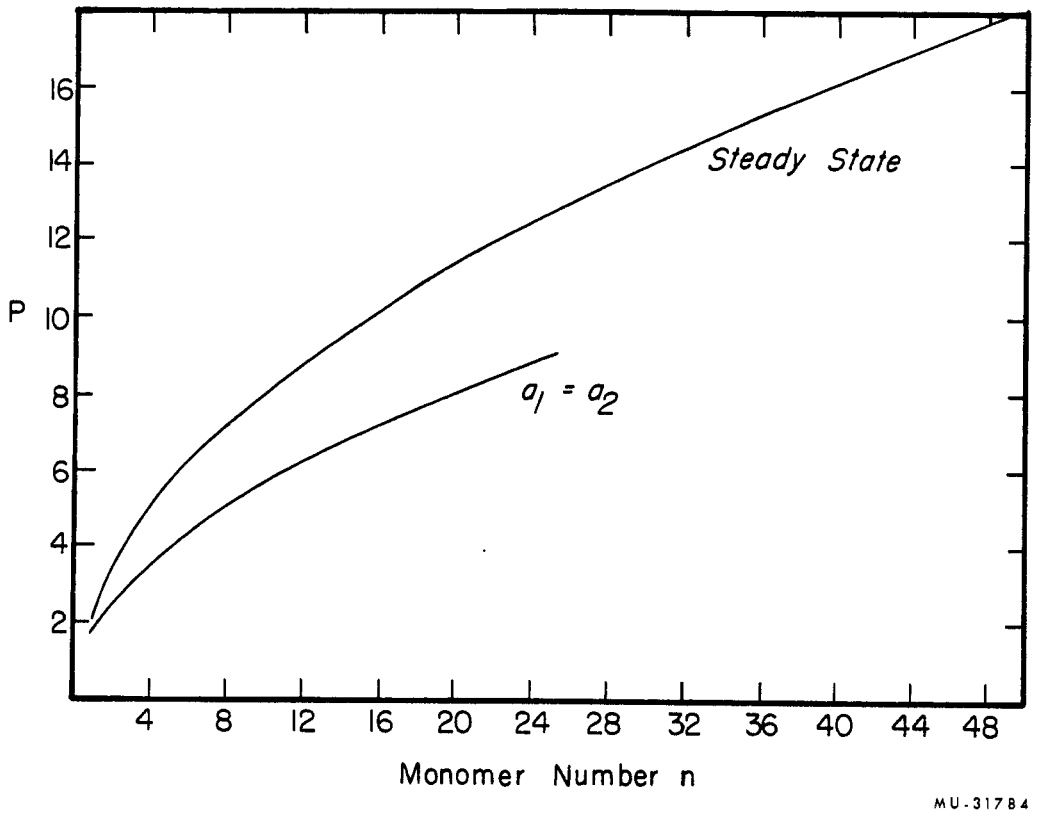

Fig. 7-2. The sharpness parameter P as a function of monomer number n for the steady-state case and the case $a_1 = a_2$.

Table 7-I. Sharpness parameter P for several sets of kinetic parameters.

			Monomer number (n)				
k_1E	k_2	k_3	1	2	3	4	5
Steady state			2.2	3.4	4.2	4.9	5.6
10^{-3}	1	1	2.2	3.4	4.2	5.0	5.6
10^{-4}	1	1[a]	2.2	3.4	4.2	5.0	5.6
10^{3}	1	1	2.2	3.4	4.2	5.0	5.6
10^{3}	1	10^{-4}	2.2	3.4	4.2	5.0	5.6
1	1	1[a]	2.2	3.3	3.9	4.5	4.9
10^{3}	10^{-3}	10^{2}	2.2	3.3	4.0	4.6	5.2
1.5	0.16	1.3	1.8	2.7	3.3	3.7	4.3
1100	10^{-3}	10^{3}	1.8	2.6	3.1	-	-
$a_1 = a_2$			1.7	2.5	3.1	3.6	4.0

[a]The calculated P's were identical whether $ES_0 = 1$ or $ES_0 = 0$.

When k_1E and k_3 are approximately equal and both are much greater than k_2, the approximation $a_1 = a_2$ is not a bad one. All of the cases calculated have P values that fall between the steady-state case and the $a_1 = a_2$ case for all monomers. On this basis we shall assume that these two cases represent the extremes of curve sharpness available in this system. In the $a_1 = a_2$ case each monomer is evolved in the minimum time interval and with the best separation between two successive monomers. Thus, this case represents the easiest way to analyze the reaction products in order to determine the sequence of monomers in the polymer. It is reasonable that this case offers the best possible separation of monomer units, since if $k_2 \neq 0$, each time an enzyme-substrate complex decomposes without cleaving a monomer unit the enzyme is freed to attack any of the various polymer species in the reaction mixture.

The number of monomer units being evolved simultaneously at an appreciable rate is another criterion that would determine the feasibility of using an exoenzyme to determine the sequence of units in a polymer. This is illustrated in Fig. 7-3 for the two extreme cases. If it is possible to adjust the experimental conditions, so that k_1E and k_3 are both much greater than k_2, much clearer evidence for the sequence of monomers will be obtained.

From our calculated curves we have observed that the time interval between the rise of one monomer to 0.5 concentration and the rise of the next monomer to 0.5 concentration is approximately constant. In the light of Eqs. (17) and (18), and the shapes of the curves (see Fig. 7-1), this is quite reasonable.

Consider a specific experiment using RNA and a pure exonuclease. The presence of an appreciable endonuclease activity will greatly obscure all the following results, and obtaining an exonuclease free of endonuclease may be one of the major stumbling blocks in doing these hypothetical experiments in the laboratory. The enzyme and the RNA are rapidly mixed in solution and allowed to react, and aliquots are taken from the reaction mixture at specific intervals. The mononucleotides are removed from the aliquot by dialysis, and the base composition of the dialysate is determined by spectroscopic and chromatographic analysis. The amount of each of the four nucleotides in the solution at each time is plotted, producing curves like those in Figs. 7-4a, 7-4c, 7-5, and 7-6. By comparing Fig. 7-4a with 7-4c, one can see that it should be fairly easy to distinguish a sequence like GAAG from GAGA by this method. In practice, the determination of sequence would be done by mathematical analysis of the experimental curves, but many sub-sequences like the two in Figs. 7-4a and 7-4c can be determined by inspection.

Figures 7-4b and 7-4d show data that would be obtained from a different type of experiment. At specific time intervals the reaction mixture would be swept clean of all nonpolymeric material and the eluate would be analyzed for mononucleotides. The curves measure the amount of nucleotide given off during a specific time interval. By inspection, the sequence of small fragments may be obtainable in this manner.

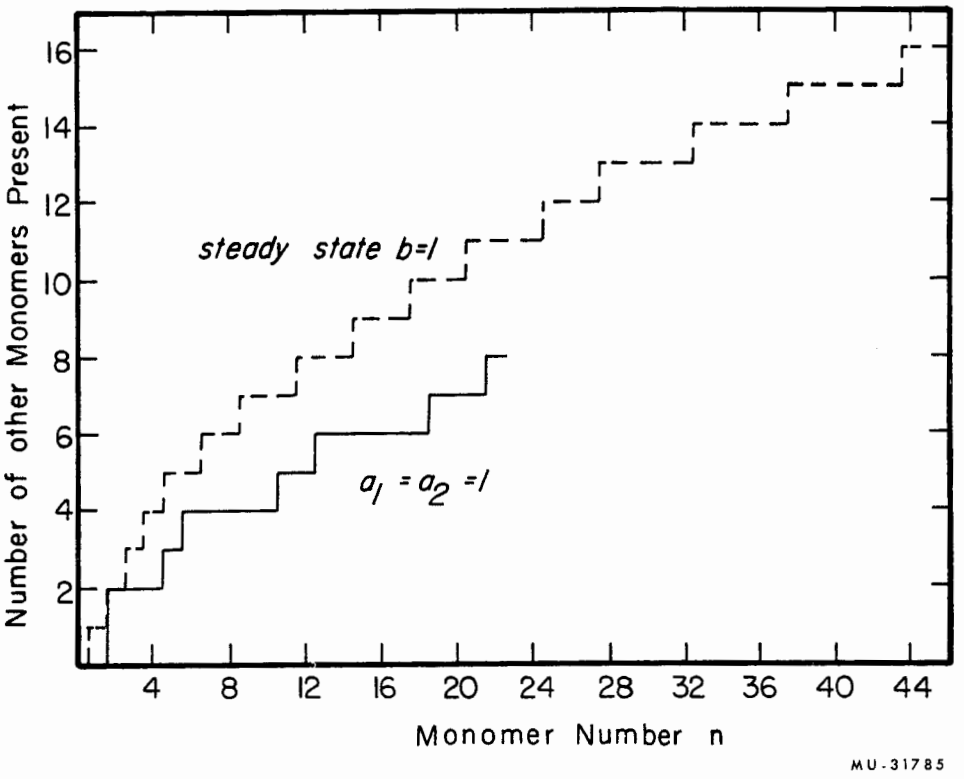

Fig. 7-3. The number of other monomer units present between 0.1 and 0.9 concentration when monomer number n reaches 0.5 concentration.

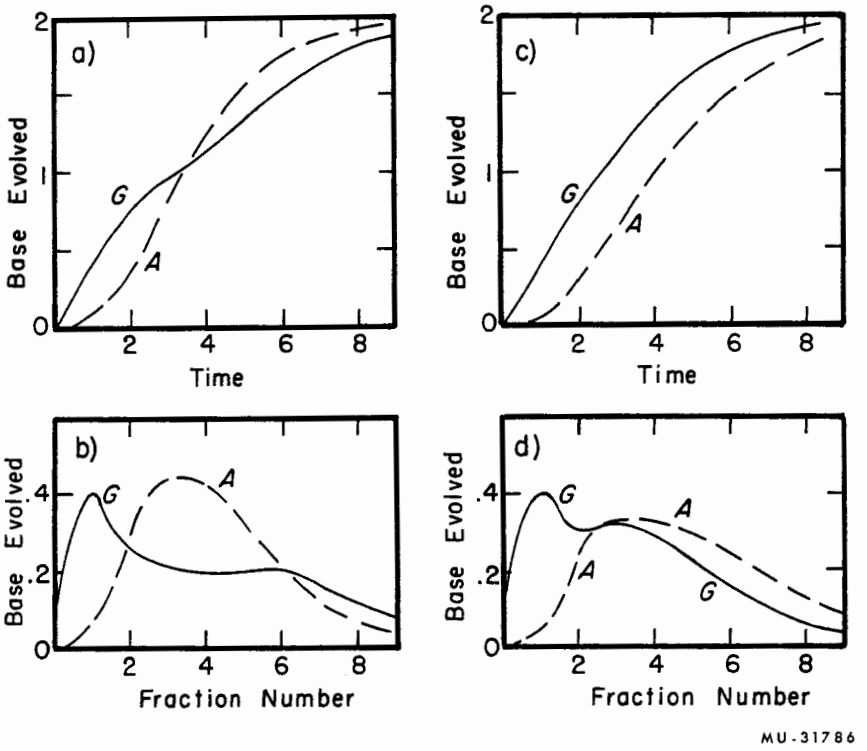

Fig. 7-4. Two methods of distinguishing GAAG from GAGA. See text for explanation. All data calculated for $k_1E = 1.5$, $k_2 = 0.16$, $k_3 = 1.3$.
(a) GAAG: Data from aliquots
(b) GAAG: Data from fractions
(c) GAGA: Data from aliquots
(d) GAGA: Data from fractions

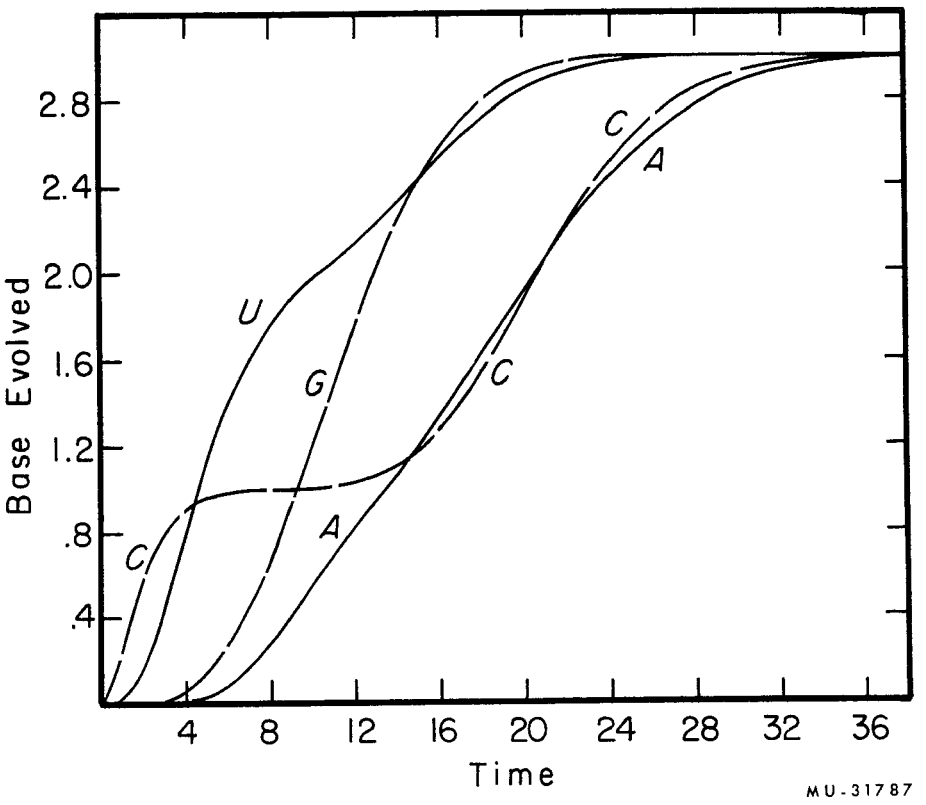

Fig. 7-5. Predicted kinetic data from degradation of CUUGAGGUACCA by an exonuclease; $a_1 = a_2 = k$.

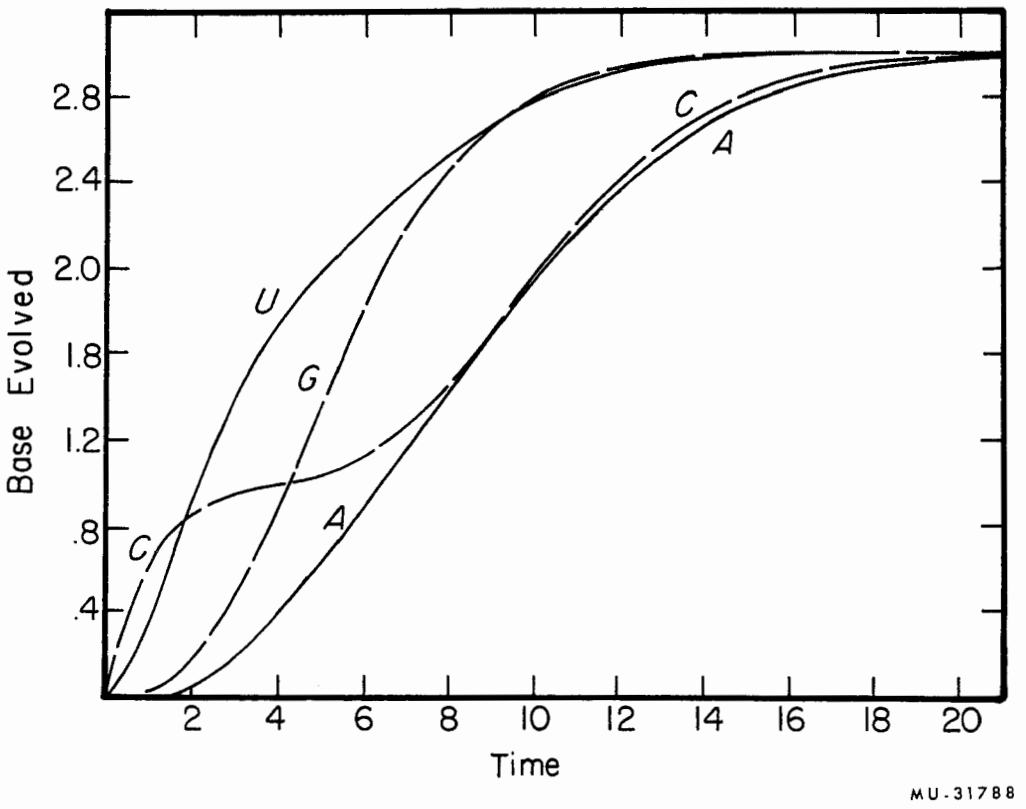

Fig. 7-6. Predicted kinetic data from degradation of CUUGAGGUACCA by an exonuclease; steady-state conditions, b = 1.

We can illustrate how the sequence of a random twelve-mer can be obtained by qualitative study of kinetic data. Figure 7-5 shows data that might be obtained from exoenzyme degradation of a twelve-mer. From the asymptotes the base composition is determined as 3A, 3G, 3U, and 3C. Now, from the total amount of base given off we can learn a from Eq. (16), assuming that the reaction took place under conditions for which this approximation is valid. If we use this a to plot out the curves for twelve distinguishable bases we find that curves 1, 2, 4, and 5 coincide with the C, U, G, and A curves respectively up to a concentration of about 0.8. Therefore, these positions are the left-most locations of each of these bases. The second base is U, the fourth is G, and the fifth is A. We now know five of the bases of the sequence:

C U _ G A _ _ _ _ _ _ _ A.

Now, the only possibility for the third base is U, since this is the only base whose concentration has greatly surpassed 1 before the fourth base starts coming off at an appreciable rate. The curve of U vs time has a shoulder and then reaches a maximum slope again at time 15. Since a = 1, Eq. (18) predicts that the eighth base is U.

The known sequence is now

C U U G A _ _ U _ _ _ A.

Notice that all of the G has been given off before the third U, base number 8, has reached a concentration of 3. Therefore, the second and third G's must come at positions 6 and 7. This leaves three bases to go, in positions 9, 10, and 11. Two of these are C and one is A. Thus, there are three possibilities for the sequence of the last four bases:

CCAA, CACA, or ACCA.

From Fig. 7-5 one can see that from time 15 to 21 the amount of base A that has been evolved is greater than base C. From Fig. 7-4a and 7-4c we can rule out both CCAA and CACA, since if the terminal bases were in these sequences there would always be more C than A given off. The only possibility remaining is the correct sequence,

C U U G A G G U A C C A.

In practice, after this qualitative construction of the sequence, the kinetics of the proposed sequence would be calculated from (11), (13), or (14) and compared with the experimental curves.

The steady-state kinetic data do not give a very clear indication of the sequence. Figure 7-6 shows the data that would be expected for the same twelve-mer discussed above if the enzyme were operating under steady-state conditions. The shoulder of the U curve, the intersection of the A and C curves, and the relative values of the curves near the asymptotes are obscured or missing entirely. From this and other data given previously we can conclude that to maximize the amount of base sequence information obtainable, one should employ exonucleases under conditions for which the steady-state approximation is not valid.

With an exoenzyme it will be easier to determine the sequence of bases of an oligomer than the sequence of the terminal bases of a longer polymer, since the presence of later bases will eventually confuse the evidence of the earlier ones. For nucleic acids, however, the availability of exonucleases that attack specifically from either the 5' end or the 3' end will be of great help. The sequence of longer oligomers may be determined by using two different enzymes, since each enzyme will provide clear information about the end bases, and the overlap of the two sets of kinetic data should help to clarify the sequence of bases in the middle.

Throughout the kinetic treatment we have assumed that the rate constants are independent of the substrate. If this is not true, the problem becomes much more complex, but it is still soluble. The inverse Laplace transforms of Eq. (10) would have to be expanded by partial fractions and then inverted term by term, and the resulting expression for the base concentrations would be much more complicated. The analysis of experimental data would also be much more difficult. If the constants were not all the same, the spacing between successive monomer concentrations of 0.5 would not be a constant, and Eqs. (15), (16), (17), and (18) would not hold. It is currently believed, however, that for venom phosphodiesterase there is no kinetic specificity towards substrates of different purines or pyrimidines.

8. BRAIN BIOCHEMISTRY AND BEHAVIOR IN RATS

G. T. Pryor, Hiromi Morimoto, and Barbara Olton

As part of a continuing effort to relate brain biochemistry to behavioral and genetic differences in rats, we have determined the concentration of serotonin (5-hydroxytryptamine or 5-HT) and acetylcholine (ACh) and the activities of 3, 4-dihydroxyphenylalanine decarboxylase (DOPAD), L-glutamic acid decarboxylase (GAD), monamine oxidase (MAO), acetylcholinesterase (AChE), and cholinesterase (ChE) in six strains of rats.

Although the functions of serotonin and γ-aminobutyric acid (GABA) have not yet been clearly defined, both compounds have been implicated in neural functions. The enzymes, DOPAD and MAO, respectively, synthesize and degrade serotonin. GAD converts L-glutamic acid by decarboxylation into GABA. AChE degrades acetylcholine, a substance well established as a neurotransmitter. The function of ChE in brain is still obscure, although a role in glial cell function has been suggested.

Serotonin has been estimated by a fluorometric procedure described previously.[1] Sensitive assay methods have been developed for the estimation of DOPAD, GAD, and MAO, utilizing ^{14}C-containing substrates. These methods, which are described in this report, have enabled us to determine the activity of each of these enzymes separately in areas of the cortex and subcortex of individual rats. In addition, these assay methods have been made compatible with the spectrophotometric determinations of AChE and ChE in aliquots of the same homogenates.[2]

The three paired strains of rats in which these enzyme activities were determined were: S_1 and S_3; Roderick Dempster High (RDH) and Roderick Dempster Low (RDL);[3] and Olson High (OH) and Olson Low (OL). The S_1 and S_3 rats are descendants of strains developed by Tryon for maze-bright and maze-dull behavior many years ago in the Psychology Department.[4] Without further selection, the S_1 decendants are superior to the S_3 decendants in numerous maze tests. These strains have previously been shown to differ in AChE and ChE activity, as well as in acetylcholine content of the brain.[3]

The RDH and RDL strains were selectively bred for high and low AChE activity to test the simple hypothesis that AChE would provide a measure of maze ability. This did not prove to be the case, although a revised hypothesis incorporating the ratio of AChE to acetylcholine concentration in brain can explain the results.[5] More recently, Olson has selected animals

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1. H. Morimoto and G. Pryor, in Bio-Organic Chemistry Quarterly Report, UCRL-9652, April 1961, p. 216.

2. E. L. Bennett, D. Krech, and M. R. Rosenzweig, Fed. Proc. 22, 334 (1963).

3. T. H. Roderick, Genetics 45, 1123 (1960).

4. R. C. Tryon, Yearbook Natl. Soc. Stud. Educ. 39, Part I, 111 (1940).

5. M. R. Rosenzweig, D. Krech, and E. L. Bennett, Psychol. Bull. 57, 476 (1960).

again on the basis of maze behavior. The OH' s were selected to have superior maze performance to the OL' s on the Lashley III maze. However, the studies of the maze and biochemical characteristics of this pair of strains have not been completed. Preliminary data indicate no differences in either AChE or ACh for these two strains.

Methods

Ten littermate pairs were selected from each strain. One of each pair was used for serotonin assay; its littermate was sacrificed for enzymatic assay. The rats were about 100 days old.

Tissue Sections and Preparation

For serotonin. Whole brains (with cerebellum removed) were placed directly into 60-ml round-bottom centrifuge tubes (32 mm by 128 mm) containing 2 ml of NaCl-saturated 0.1 N HCl. Sections were immediately homogenized and extracted with butanol.

For enzymatic studies. Cortical sections were as follows: 40-to-50-mg samples were removed from the visual area (V) and somesthetic area (S); after removal of V and S sections, the remaining dorsal cortex (DC) was stripped off; the ventral cortex (VC) comprised the remaining cortical areas. Subcortical sections were as follows: medulla and pons (M + P) as a single sample; cerebellum (Ce); hypothalamus (H); and remaining subcortical brain (RSB). After removal, these sections were placed on dry ice until assay. Sections were homogenized in 0.03 M potassium phosphate buffer (pH 7.0) at concentrations of 20 mg/ml for DC, VC, and M + P; 10 mg/ml for V, S, and RSB; and 5 mg/ml for H sections.

Serotonin

Serotonin was determined with an Aminco-Bowman spectrophotofluorometer, using the method of Bogdanski, et al.[6] with some modification, as described previously.[1] This procedure involves extraction of serotonin into salt-saturated butanol, and its return to an aqueous phase by addition of 2 volumes of heptane. Fluorescence at 550 mμ in 3 N HCl, when activated at 295 mμ, allows assay without interference from most other normally occurring materials in brain.

Acetylcholinesterase and Cholinesterase

These analyses are based on a spectrophotometric method described by Ellman et al.[7] and modified by Bennett et al.[2] For acetylcholinesterase (AChE), acetylthiocholine is used as substrate in the presence of 5, 5' -dithio-bis-(2-nitrobenzoic acid) or DTNB. Thiocholine, the enzymatic product, reacts with DTNB, forming the yellow anion, 2-nitrothiobenzoate, which absorbs maximally at 412 mμ. These assays were done in 0.45 M potassium phosphate buffer (pH 7.5) and required 2 to 10 mg of tissue.

6. D. F. Bogdanski, A. Pletscher, B. B. Brodie, and S. Udenfriend, J. Pharmacol. 117, 82 (1956).

7. G. L. Ellman, K. D. Courtney, V. N. Androes, Jr., and R. M. Featherstone, Biochem. Pharmacol. 7, 88 (1961).

For cholinesterase (ChE), butyrylthiocholine was used as substrate, with DTNB, and with a selective inhibitor of AChE — 1:5-bis-(4 tri-methyl-ammoniumphenyl) pentane-3-one diiodide. These assays were done in 0.03 M potassium phosphate buffer (pH 7.0) and required 5 to 20 mg of tissue.

All assays were done on a Beckman DU spectrophotometer equipped with a Gilford Automatic Cuvette positioner, optical-density converter, and recorder. Reaction rates were automatically recorded for 10 min and were linear during this interval.

Monoamine Oxidase

Brain homogenates (from 5 to 10 mg) were made up to a total volume of 1.0 cc with 0.03 M potassium phosphate buffer (pH 7.0) in 25-ml glass-stoppered Erlenmeyer flasks. After 2 min of prewarming in a metabolic shaker, 20 μg of 5-hydroxytryptamine-2 ^{14}C oxalate (specific activity 0.94 mC/mg; supplier: New England Nuclear Corp.) was added. Incubation was at 37°, 35 strokes/min. After a 1-hr incubation, reaction mixtures were transferred to preheated 12-ml centrifuge tubes in a boiling water bath. The reaction mixtures were held at 100° for 5 min, and the tubes were then centrifuged for 20 min at 3000 rpm. A portion (0.1 ml) of supernatant was spotted at 2-in. intervals along the long axis of an 18-1/4 by 22-1/2-in. sheet of Whatman No. 1 paper (8 spots/sheet). Sheets were developed in butanol: acetic acid:water (5:1:4) and autoradiographs prepared by exposure of x-ray film to the paper for 1 week to 10 days. In this solvent system, serotonin has a Rf value of 0.51, and its oxidative deaminated product, 5-hydroxy-indoleacetic acid (5-HIAA) has a Rf of 0.82. Internal standards were used with each experiment. Radioactivity was counted with an automatic spot counter.[8]

Decarboxylase Activity

Decarboxylations were run in special one-arm flasks (see Fig. 8-1). The total volume of each cell was approximately 6 ml. For assays, 0.1 ml of substrate was added to the side arm, 0.1 ml pyridoxal phosphate to the main compartment of the cell, and homogenate plus buffer added until the total volume was 1.2 ml. The side arm was capped with a rubber serum cap (No. F-1) and the cell attached to an evacuating apparatus (Fig. 8-2) by a 2-in. length of rubber tubing. Cells were evacuated to 2 to 3 cm Hg pressure, and the gaseous phase replaced by N_2. This evacuation was repeated twice. Each cell was then placed under a slight vacuum and sealed by a screw clamp on the rubber tubing. After incubation at 37°, the enzymatic reaction was terminated by addition of 0.3 ml 5 N H_2SO_4.

To collect evolved CO_2, 2 ml of phenethylamine reagent[9] was added to scintillation bottles and the bottles evacuated with a vacuum apparatus.

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8. V. Moses and K. K. Lonberg-Holm, Anal. Biochem. 5, 11 (1963).

9. F. H. Woeller, Anal. Biochem. 2, 508 (1961).

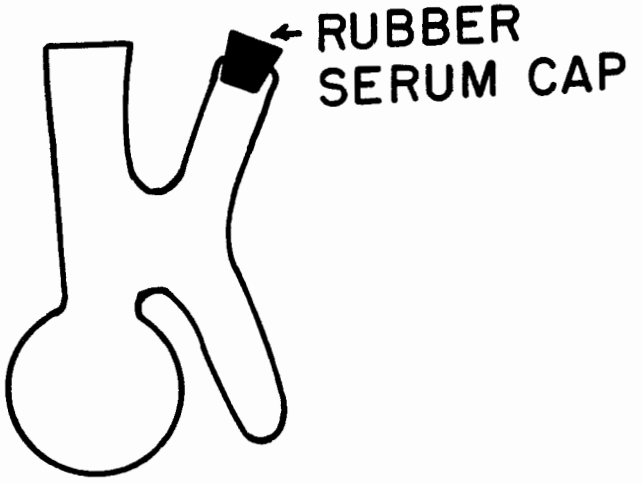

Fig. 8-1. Incubation cell for decarboxylation.

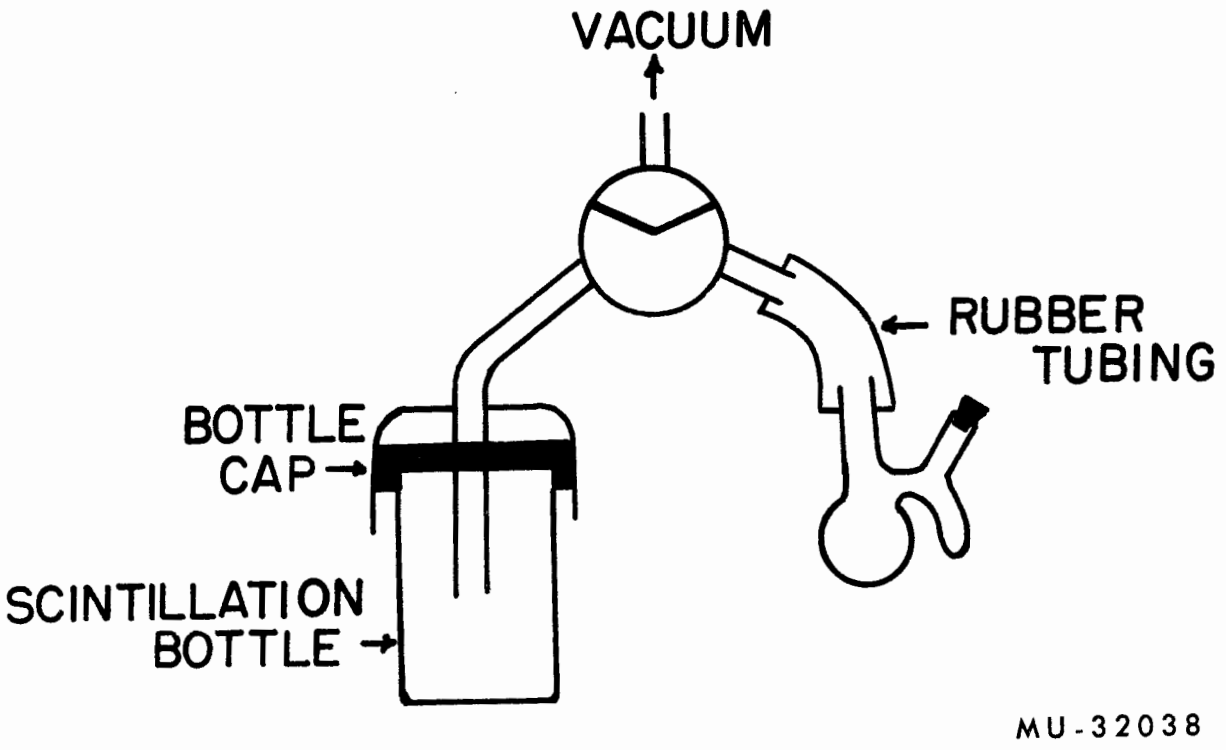

Fig. 8-2. Evacuation apparatus for the decarboxylation.

The incubation cells and scintillator bottles were then interconnected by means of a three-way stopcock. After 5 min, 0.5 mmole of $NaHCO_3$ was added to the acid-killed homogenates with a hypodermic syringe through the serum cap. Nitrogen gas was then passed through the cell to ensure complete recovery of CO_2. The entire collection period for 10 cells requires 25 min. Scintillation fluid #2 (toluene, dioxane, ethanol, naphthalene, PPO, and POPOP) was added, and samples were counted in a Packard liquid scintillation counter.

3, 4-dihydroxyphenylalanine decarboxylase (DOPAD)

Lovenberg[10] has shown the identity of 5-hydroxytryptophan decarboxylase (5-HTP decarboxylase) and DOPAD.[8] DOPAD decarboxylates DOPA 6.4 times as rapidly as it decarboxylates 5-HT. Optional conditions for this enzyme were pH 7.0 and DOPA concentration 5×10^{-4} M. For assay, 0.2 μC of 3, 4-dihydroxyphenylalanine-1-^{14}C was used per cell. Pyridoxal phosphate was required as a cofactor; 0.1 mg gave maximal activity. Under these conditions, decarboxylating activity after 2 hr decreases to approximately 75% of the initial rate. No effect was observed under an atmosphere of air, but reactions were run routinely in a nitrogen atmosphere at 37° in a metabolic shaker (117 strokes/min). All cells were given a 2-min preincubation, and the reaction was initiated by mixing DOPA with the homogenates. The reaction was stopped after 2 hr by addition of 0.3 ml of 5 N H_2SO_4.

L-Glutamic Acid Decarboxylase (GAD)

Optimal conditions were 1×10^{-3} M L-glutamic acid, pH 8.0. We used 0.2 μC of DL-glutamic acid-1-^{14}C (New England Nuclear Corp.) per cell. Fifteen μg of pyridoxal phosphate gave maximal activity. Under an atmosphere of air, initial activity was 40% of the activity in a N_2 atmosphere. GAD activity was not stable when the material was stored for 1 week at -22°. However, activity was retained if tissues were kept on dry ice overnight. Assays were run routinely for 1 hr at 37° at pH 7.0 under an atmosphere of nitrogen.

Results

Preliminary experiments on serotonin levels of 100-day old S_1 and S_3 rats showed large strain differences (Table 8-I) in brain serotonin.

In further experiments the same six strains of rats were used: S_1 and S_3; RDH and RDL; and OH and OL. Littermates of these animals were analyzed for five enzymes. Weight data are presented in Table 8-II, relative regional activities of five enzymes in Table 8-III, and enzyme activity and 5-HT concentration in different brain sections of the six strains of rats in Table 8-IV. These values for DOPAD, GAD, and MAO correspond to comparable sections in other species.[11, 12, 13] Although ChE, GAD, and

10. W. Lovenberg, H. Weissbach, and S. Udenfriend, J. Biol. Chem. 237, 89 (1962).

11. R. Kuntzman, P. A. Shore, and D. Bogdanski, J. Neurochem. 6,226 (1961).

12. I. P. Lowe, E. Robins, and G. S. Eyerman, J. Neurochem. 3, 8 (1958).

13. D. F. Bogdanski, H. Weissbach, S. Udenfriend, J. Neurochem. 1, 272 (1957).

MAO are fairly constant throughout the brain, AChE and DOPAD vary widely. DOPAD activity is localized predominantly in gray matter and is low in white matter and the cerebellum. Its distribution throughout the brain roughly follows the serotonin distribution.[8] The proportionally high value of ChE for subcortical sections (compared with cortical ChE) is probably related to higher glial cell counts in these sections.

The data from individual sections were combined to give total cortex (V + S + DC + VC), subcortex (RSB + H + M + P), and total brain (cortex + subcortex). The data are summarized in Table 8-IV for the six strains. In all measures, S_1 have higher levels than S_3; the other two pairs of strains do not show a consistent pattern of differences in brain biochemistry. Differences in DOPAD and GAD activity are in the same direction as maze performance for the three pairs.

Table 8-V summarizes qualitative differences of seven chemical measures for the six strains. Maze performance on the Lashley III maze is also indicated (performance on the Lashley III maze gives comparable directional results to performance on the Dashiell maze and the Hebb-Williams maze for S_1 and S_3 and for RDH and RDL;[3] no other behavioral data are available for OH and OL).

Discussion

Although there is little doubt that acetylcholine has a neurotransmitter function, it is probably not the only transmitter substance.

Both serotonin and GABA (γ-aminobutyric acid) have, because of their biological properties and neural distribution, been suggested as neurohumors (compounds affecting nerve activity).

Rosenzweig, Krech, and Bennett have reported numerous studies relating maze behavior with the acetylcholine-acetylcholinesterase system.[5] In Table 8-V, relative levels of acetylcholine (ACh), cholinesterase (ChE), and acetylcholinesterase (AChE) for the six strains are indicated.

There does not seem to be any simple relation between maze performance on the Lashley III maze and the acetylcholine-acetylcholinesterase system. However, GAD and DOPAD levels both follow the behavior differences between these three pairs of strains. Results of cholineacetylase levels may also prove rewarding on these strains.

A more comprehensive analysis of data is in progress. In addition, data on other species are being analyzed. A replication is planned on strain differences, and effects of behavioral conditions will be investigated.

Table 8-I. Mean brain serotonin levels in S_1 and S_3 rats.

	S_1				S_3			
Age	N	5 HT (mμg)	Wet weight of tissue (mg)	5 HT (mμg/g)	N	5 HT (mμg)	Wet weight of tissue (mg)	5 HT (mμg/g)
100 days	10	851	1308	651	13	789	1435	551

Table 8-II. Weights of tissue samples from six rat strains.

	S_1	S_3	RDH	RDL	OH	OL
Body wt. (g)	300.2	306.9	224.0	324.4	371.3	315.0
Brain: V (mg)	65.63	73.99	64.09	73.99	75.24	73.46
S	52.80	56.18	49.80	55.05	55.85	56.73
DC	260.8	297.0	256.6	295.2	316.8	302.6
VC	318.1	344.5	304.9	341.5	365.0	352.5
H	60.85	60.95	54.50	61.89	61.98	60.08
M+P	168.0	197.6	159.0	194.0	192.4	188.4
RSB	433.6	482.0	417.7	461.0	524.9	488.1
Ce	234.7	277.0	228.5	270.2	277.9	258.0
Total cortex	697.3	771.6	675.4	765.7	812.9	785.4
Total sub-cortical brain	657.8	728.5	610.9	710.9	758.8	723.2
Total brain	1600	1777	1515	1747	1850	1766

Table 8-III. Relative regional activities (expressed as percent of remaining subcortical brain) (RSB).

Section	AChE	ChE	DOPAD	GAD	MAO
V	21	62	*	60	72
S	25	61	*	67	70
DC	25	60	25	79	74
VC	45	59	34	54	83
Ce	16	83	*	64	67
M+P	47	101	60	48	58
H	43	120	100	83	118
RSB	100	100	100	100	100

*V, S, and Ce sections were insufficient in size and activity for assay.

Table 8-IV. Enzyme activity and 5-HT concentration in different brain sections from six rat strains

Enzyme	Section	S_1	S_3	RDH	RDL	OH	OL
AChE[a]	Cortex	85.4	72.9	71.8	62.6	70.1	70.0
	Subcortex	186.2	181.6	185.8	164.1	180.9	178.2
	Total brain	134.5	125.9	126.2	111.5	123.5	121.9
ChE[a]	Cortex	2.95	2.76	2.93	2.62	2.75	2.90
	Subcortex	5.39	5.13	5.68	5.28	5.06	5.37
	Total brain	4.14	3.91	4.23	3.89	3.86	4.08
DOPAD[b]	Cortex	1.72	1.47	1.28	1.59	1.36	1.21
	Subcortex	5.05	5.00	4.71	4.75	5.05	4.31
	Total brain	3.47	3.35	3.06	3.26	3.30	2.83
MAO[c]	Cortex	4.85	4.77	5.16	4.91	4.84	4.79
	Subcortex	6.21	6.12	6.16	6.25	6.25	6.38
	Total brain	5.52	5.43	5.63	5.55	5.52	5.56
5-HT[d]	Total brain	812.6	742.5	810.8	790.3	709.4	730.7
GAD[e]	Cortex	6.49	5.85	5.98	6.05	6.39	5.93
	Subcortex	7.48	7.42	7.49	7.79	8.76	7.93
	Total brain	7.10	6.55	6.62	6.79	7.33	6.82

a. AChE and ChE expressed as mM acetylthiocholine hydrolyzed per min per mg $\times 10^{10}$

b. DOPAD expressed as μM CO_2 decarboxylated per g per 2 hr

c. MAO expressed as percent serotonin metabolized per mg per hr

d. 5-HT expressed as mμg per g brain

e. GAD expressed as μM CO_2 decarboxylated per g per hr

Table 8-V. Comparison of maze performance and brain biochemistry of three pairs of rat strains.

	S_1 S_3	RDH RDL	OH OL
Maze performance Lashley III (fewer errors)	>	<	>
AChE activity	>	>	=
ACh conc	>	=	=
ChE activity	>	>	<
DOPAD activity	>	<	>
MAO activity	⩾	=	=
S-HT conc	>	=	⩽
GAD activity	>	<	>

9. EXPERIMENTS ON CLASSICAL CONDITIONING AND LIGHT HABITUATION IN PLANARIANS

Allan Jacobson and Reeva Jacobson

Part I

Several studies in recent years have demonstrated that planarians (common, fresh-water flatworms) are capable of acquiring a conditioned response.[1] The training pattern in these studies consists of the following. An animal is placed in a water-filled channel and is permitted to move about freely. Sudden illumination of the channel typically elicits few visible reactions from the animal. An appropriate electric current passed through the water, however, invariably evokes at once a longitudinal body contraction, which is maintained for the duration of the current.

If paired presentations of light and shock (in that order) are repeatedly administered to the animals, then a progressive increase in the animal's reactivity to the light (prior to the shock) generally occurs,[2] whereas presentation of unpaired light and shock does not enhance the animal's reactivity to light.[3] Trained animals that are sectioned transversely show, upon regeneration, considerable retention of the conditioned response.[4]

The present research represents attempts to replicate these findings.

Methods

Several experiments were carried out, with some changes in procedure. This section describes the overall training operation, and particulars associated with a given experiment are presented in the section on results.

Subjects

Several varieties of planarians were used: Specimens of Dugesia dorotocephala obtained from the H. A. Dahl Co., Berkeley, California; Dugesia tigrina from Carolina Biological Supply Co., Elon College, N. C.; D. tigrina from Carolina Biological Supply Co., Powell Laboratory Gladstone, Oregon, and D. tigrina from Ann Arbor, Michigan (generously provided by Dr. Margaret Clay). Results from these different groups will be presented separately.

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1. A. L. Jacobson, Psychol. Bull. 60, 74 (1963).

2. R. Thompson and J. V. McConnell, J. Comp. Physiol. Psychol. 48, 65 (1955).

3. R. Baxter, Classical Conditioning in the Planarian, (Master's Thesis), University of Florida, 1961, (unpublished).

4. J. V. McConnell, A. Jacobson, and D. P. Kimble, J. Comp. Physiol. Psychol. 52, 1 (1959).

All animals were maintained in the dark in bowls containing local spring water and were fed freshly hatched brine shrimp three times a week.

Apparatus

Animals were trained in a semicircular channel grooved into a white Lucite block. The channel was 10 in. long, 1/2 in. wide, and 3/16 in. deep. Brass electrodes were set into the channel at either end. Background illumination was provided by a 7-1/2-watt bulb (in some cases diminished by means of a Variac). Overhead illumination (hereafter the conditioned stimulus or CS) was provided by two adjacent student lamps, located 11 in. above the trough. Each lamp contained a 100-watt frosted bulb. Current (hereafter designated the unconditioned stimulus or UCS) was delivered either by means of a step-down transformer or by a 6-volt dry cell connected to an inductorium (#308, Harvard Apparatus Co.) The light-shock sequence was preset and was activated by a single button. An accessory switch served to transfer power from one complete apparatus unit to another, so that several animals could be run in a given session.

Procedure

On each day of training the subject worm S was transferred from its home bowl to the training trough by means of a No. 3 sable brush. S was then given 5 min of undistrubed "habitation" time, following which the day's training was begun. A single trial consisted of a 3-sec CS presentation, during the last sec of which the UCS was also administered. Twenty to twenty-five such trials (referred to as a "trial set") were given to each S, with a minimum intertrial interval of 1 min. After a day's trials, S was returned to its home bowl and kept in the dark until the next training session.

Trials were given only when S was gliding smoothly and was oriented longitudinally between the two electrodes. The experimenter observed and recorded responses occurring during the first 2 sec of the CS. S was judged to have made a response when it deviated noticeably from its straight-line locomotion, either by contracting longitudinally, by turning its head to left or right, or by raising its head from the grooved channel.

Occasionally S would come to rest in the trough. At these times S was stimulated gently with the brush and the trial was delayed until S had resumed smooth locomotion.

Results

Experiment 1

Subjects: D. dorotocephala from the H. A. Dahl Co.
CS: Two 100-watt bulbs, delivering 3-sec flashes
UCS: Output of 6-volt dry cell passed through a Harvard inductorium
Backlight: 7-1/2-watt bulb
No. of trials per day: 25

As Table 9-I illustrates, the group averages varied irregularly; a high score on one day did not necessarily foretell a high score on the next day.

Table 9-I. Number of responses to light by individual subjects (D. dorotocephala) per set of 25 training trials during Experiment 1.

Worm No.	Trial sets																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	12	11	1	8	9	3	11	7	9	15	1	14	6	5	12	11	10	5	3	12
2	11	2	10	19	15	13	2	14	2	16	7	14	2	5	6	7	13	10	5	5
3	12	4	9	13	2	11	20	13	13	7	10	13	10	11	8	14	19	16	8	2
4	13	7	4	10	11	8	15	6	15	12	10	5	8	6	3	13	8	11	8	6
5	3	2	6	6	7	14	18	12	5	15	3	10	5	3	6	15	18	7	7	1
6	3	0	4	5	6	11	15	2	8	15	7	4	3	7	12	5	2	5	2	7
7	13	1	7	17	13	11	3	5	14	13	2	8	3	2	6	8	10	2	2	0
8	4	5	16	15	12	16	19	20	19	21	8	19	11	18	8	4	4	3	12	7
9	3	14	17	13	10	18	19	11	16	19	12	9	3	11	4	7	18	10	2	16
10	9	4	17	14	13	10	20	7	5	7	4	9	1	4	7	7	8	10	7	5
11	7	0	1	14	2	10	12	3	13	12	8	10	0	8	6	3	16	13	9	17
12	4	7	15	16	7	14	11	15	16	14	11	8	2	9	6	11	20	17	6	13
13	8	1	4	4	18	11	13	8	8	9	8	4	3	6	7	3	16	3	6	1
14	6	5	9	24	21	17	8	9	9	18	6	4	2	5	9	3	23	4	8	2
15	0	13	13	22	14	13	19	5	20	16	5	9	1	8	5	8	12	2	5	5
16	3	4	18	7	15	23	7	12	13	8	12	0	11	12	9	21	4	17	1	17
Average	6.9	5.0	9.4	12.9	10.9	12.1	15.1	9.3	11.6	13.6	7.4	8.1	4.4	7.5	7.1	8.8	12.7	8.4	5.7	7.3

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Experiment 2

Subjects: D. tigrina from Gladstone, Oregon.
CS, UCS, backlight, and trials/day were the same as in Experiment 1.
Factual data are presented in Table 9-II.

Table 9-II. Number of responses to light by individual subjects (D. tigrina) per set of 25 training trials during Experiment 2.

Worm No.	Trial sets													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	11	7	5	9	10	6	14	7	13	5	12	3	4	3
2	4	4	4	10	7	13	10	4	5	6	14	10	7	5
3	6	8	3	3	1	3	5	11	4	4	17	14	17	15
4	8	11	2	7	7	4	2	6	2	3	12	5	4	4
Average	7.3	7.5	3.5	7.3	6.3	6.5	7.8	7.0	6.0	4.5	13.8	8.0	8.0	6.3

No clear trend is evident in the group data. Although one animal (No. 3) did reach and maintain a response level considerably higher than its initial level, the other S' s showed no stable increase in response to the CS.

Experiment 3

Subjects: D. tigrina from Carolina Biological Supply Co., Elon College, N. C.
CS, UCS, backlight, and trials/day were the same as in Experiment 1.
Factual data are presented in Table 9-III.

Table 9-III. Number of responses to light by individual subjects (D. tigrina from N. C.) per set of 25 training trials during Experiment 3.

Worm No.	Trial Sets									
	1	2	3	4	5	6	7	8	9	10
1	0	5	5	2	4	3	4	7	6	9
2	3	8	8	4	2	4	10	5	6	6
3	2	3	3	0	1	2	4	3	6	4
4	3	2	4	2	2	5	9	1	4	2
Average	2.0	4.5	5.0	2.0	2.3	3.5	6.8	4.0	5.5	5.3

Experiment 4

In Experiment 4 we used D. tigrina from a source that had yielded good "conditioners" in the past.

Subjects: D. tigrina from Ann Arbor, Michigan.
CS, UCS, backlight, and trials/day were the same as in Experiment 1.
Factual data are presented in Table 9-IV.

The data obtained from this group were markedly different from those of the previous groups (see Table 9-IV and Fig. 9-1). The average response rate is doubled in 75 trials and tripled in 250 trials. It increases little thereafter but is maintained for an additional 250 trials at a level considerably higher than the initial level. The responses of individual animals were often maintained at relatively high levels, but no animal responded to more than 84% of the stimuli on any given day. Especially noteworthy is the record of animal No. 4, who responded at least 19 times out of 25 for 7 days in a row, but on no day during this period exceeded 21.

Although this "ceiling" does not negate the conspicuous increases that did occur, it did necessitate an adjustment of the criterion level previously employed (23 responses out of any 25 consecutive trials).[1]

Experiment 5

Subjects: D. tigrina from Ann Arbor, Michigan.
CS, UCS, and backlight were the same as Experiment 1; number of trials per day was 20.

In Experiment 5 a criterion level of 18 responses during any 20 consecutive trials was adopted, and the training of any S was terminated at that point. In addition, each day's session was reduced from 25 to 20 trials. Table 9-V shows factual data obtained during this experiment.

As in Experiment 4, a marked increase in mean response level is evidenced in these data (Table 9-V and Fig. 9-2). Furthermore, of the 13 S's, 10 reached criterion (18 out of 20) in 300 trials or fewer. Of the remaining five S's, two (Nos. 10 and 11) were below 50% response rate at the termination of training, while the other three (Nos. 6, 7, and 8) were at about a 70% response rate. S's that reached criterion required from 175 to 300 trials to do so.

Upon attaining criterion, each S was bisected transversely, and simultaneously an untrained control S was bisected. Approximately 3 weeks after transection, training was initiated for regenerated experimental and control S's. This training was conducted in "blind" fashion. As a result of sickness among the regenerating animals, data for this experiment were meager.

Experiment 6

Conditions were the same as in Experiment 5.

Inspection of Table 9-VI reveals no significant difference between experimental and control animals. This result may be viewed as a failure to replicate previous findings.[4]

Table 9-IV. Number of responses to light by individual subjects (D. tigrina) per set of 25 training trials during Experiment 4.

Worm No.	Trial sets																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	6	8	7	7	9	8	6	11	15	10	12	6	14	10	15	19	15	20	12	18
2	3	4	12	9	7	10	8	10	11	14	10	14	15	9	8	10	18	19	11	12
3	6	7	6	6	10	12	7	8	10	12	15	12	16	14	15					
4	2	7	6	4	5	3	10	8	19	19	20	19	20	21	19	11	11	17	13	15
5	3	5	6	3	14	7	12	11	5	17	5	3	7	16	10	12	15	15	17	13
6	6	10	12	16	15	11	15	10	8	16	4	12	12	14	15	8	11	13	20	15
7	3	9	13	8	14	11	11	11	13	9	14	13	17							
8	7	6	9	10	9	16	12	13	16	15	11	17	20							
9	5	8	13	7	8	6	16	15	17	8	12	9	15							
10	5	11	6	3	3	8	7	14	17	15	8	6	14							
Average	4.6	7.5	9.0	7.3	9.4	9.2	10.4	11.1	13.1	13.5	11.1	11.1	15.0	14.0	13.7	12.0	14.0	16.8	14.6	14.6

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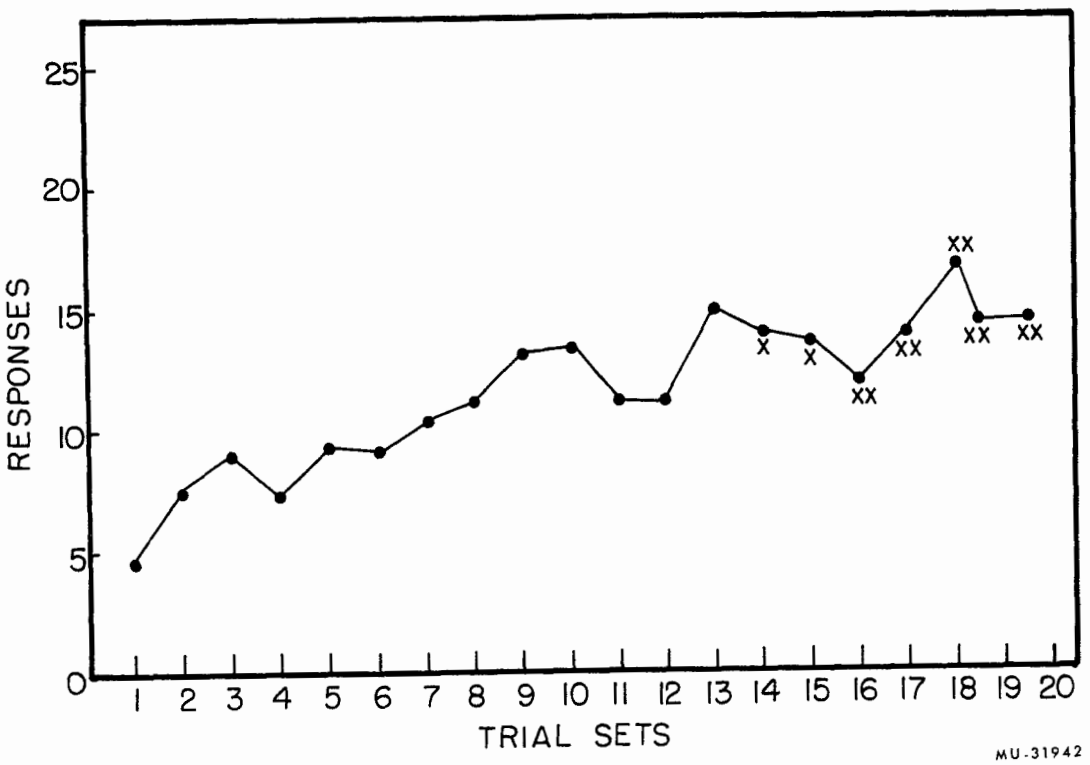

Fig. 9-1. Average number of responses per set of 25 trials by 10 D. tigrina (X, N = 6; XX, N = 5; ●, N = 10).

Table 9-V. Number of responses to light by individual subjects (D. tigrina) per set of 20 training trials during Experiment 5.

Worm No.	Trial sets														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	1	5	6	7	11	7	16	11	6	17[a]					
2	3	7	5	10	9	10	18[a]								
3	6	7	8	12	7	14	9[a]								
4	3	2	6	7	7	13	15	9	15	17[a]					
5	0	7	5	4	10	7	8	14	14	11	15	6	9	11	18[a]
6	0	5	4	3	11	11	14	10	15	15	13	11	8	16	14
7	2	7	5	6	11	9	12	13	15	15	16	16	10	13	15
8	1	7	7	13	6	10	14	13	9	14	14	11	13	10	14
9	3	8	11	12	8	17	14	18[a]							
10	1	1	1	2	5	13	7	4	6	7	9	11	7	8	8
11	4	4	6	3	5	13	9	14	5	11					
12	0	4	8	8	6	10	11	8	20[a]						
13	1	2	13	9	13	16	15	11[a]							
14	5	8	15	14	11	11	17	15	10[a]						
Average	1.9	4.9	6.5	7.4	8.1	11.5	12.7								

[a]Training was terminated when a subject responded at least 18 times during any 20 consecutive trials, which may have been held on two consecutive days.

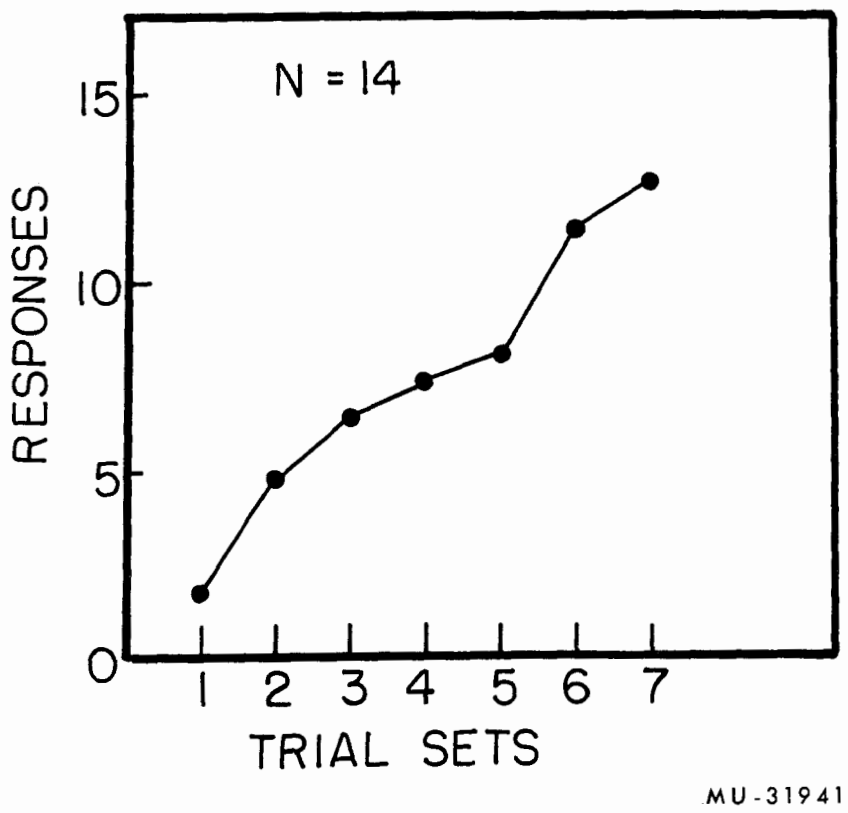

Fig. 9-2. Average number of responses per set of 20 trials by 14 D. tigrina.

Table 9-VI. Number of responses to light by individual subjects per set of 20 training trials during Experiment 6. Eight subjects had been regenerated from bisected planarians (Nos. 2, 3, 7, and 13) of Experiment 4.

	Worm No.	Trial sets 1	2	3	4	5	6	7	8	9	10	11	12
Regenerated head of	3	4	10	14	14	12	14	10	9	9	14	18	15
Regenerated head of	2	10	11	10	3	5	9	7	2	2	4	1	
Regenerated head of	7	11	9	9	10	11	13	18	14	3	7		
Regenerated head of	13	9	13	7	10	17	8	9	9	5			
	Average	8.5	10.8	10.0	9.3	11.3	11.0	11.0	8.5	4.8			
Regenerated tail of	3	8	14	8	12	13	7	5	4	13	16	10	8
Regenerated tail of	2	11	12	11	8	5	9	3	4	10	9	7	
Regenerated tail of	7	7	8	11	8	8	8	9	9	9	6		
Regenerated tail of	13	0	8	9	7	4	6	7	8	11			
	Average	6.5	10.5	9.8	8.8	7.5	7.5	6.0	6.3	10.8			
Regenerated head of control		8	9	5	12	11	9	1	7	9	13	11	11
Regenerated head of control		7	11	12	11	6	10	4	6	11	5	6	
Regenerated head of control		7	4	8	6	6	11	12	12	10	12		
Regenerated head of control		13	10	7	11	6	4	10	9	6			
	Average	8.8	8.5	8.0	10	7.3	8.5	6.8	8.5	9.0			
Regenerated tail of control		died											
Regenerated tail of control		died											
Regenerated tail of control		6	10	6	2	12	6	8	2	died			
Regenerated tail of control		14	6	10	10	8	5	4	9	5			
	Average	10.0	8.0	8.0	6.0	10.0	5.5	6.0	5.5				

Two additional points deserve comment:
(1) no increase in response rate was found as training progressed, and
(2) as has been noted, many animals either died or became sickly during regeneration (the cause of this sickness has not been ascertained).

Experiment 7

In Experiment 7, three animals were trained under the conditions of Experiment 4, except that the background illumination impinging on the trough was diminished (by means of a Variac).

Subjects: D. tigrina from Ann Arbor, Michigan.
CS: Two 100-watt bulbs
UCS: Output of 6-volt dry cell passed through a Harvard inductorium
Backlight: 7-1/2-watt bulb diminished by Variac
No. of trials per day: 25

The results of this experiment, shown in Table 9-VII, approximate those of Experiments 4 and 5, despite the small N. One S (No. 2) never exceeded a 50% response level, while the other 2 S's did so repeatedly. No appreciable improvement in level of performance appears to result from diminishing the background illumination.

Table 9-VII. Number of responses to light by individual D. tigrina per set of 25 training trials during Experiment 7. The backlight in this experiment was reduced.

Worm No.	Trial sets													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	2	8	6	5	10	11	13	2	18	16	21	18	15	14
2	2	5	2	3	5	7	10	6	10	5	6	6	2	10
3	4	8	9	5	15	6	22	15	18	20	9	10	17	22
Average	2.7	7	5.7	4.3	10	8	15	7.7	15.3	13.7	12.	12.7	12.7	15.3

Experiment 8

In Experiment 8, alternating current was used as UCS instead of the dry cell plus inductorium. Two voltage levels were employed—10-volt and 20-volt, measured at the electrodes. (After 250 trials, subjects 1 to 4 were switched from 10 V to 20 V.)

Subjects: D. tigrina from Ann Arbor, Michigan.
CS: 10-volt or 20-volt ac
Backlight: 7-1/2-watt diminished by Variac
No. of trials per day: 20

Table 9-VIII. Number of responses by D. tigrina per set of 20 training trials during Experiment 8. The unconditioned stimulus was 10- or 20-volt ac.

Worm No.	Trial sets												
	10-volt										20-volt		
	1	2	3	4	5	6	7	8	9	10	11	12	13
1	1	2	2	3	7	10	9	10	3	1	5	4	7
2	2	1	5	2	8	4	5	4	0	1	4	4	0
3	6	6	5	1	8	6	7	5	7	3	2	0	2
4	2	2	3	4	5	3	9	3	6	2	1	7	9
5	0	0	3	6	8	8	6						
6	9	12	7	2	8	1	4						
7	8	8	7	4	11	3	7						
8	8	4	7	5	4	5	8						
Average	4.5	4.9	4.9	3.4	7.4	5.0	6.9	5.5	4.0	1.8	3.0	3.8	4.5

Worm No.	All 20-volt												
1	6	9	14	9	7	6	13	9	15	10	13	6	9
2	4	4	8	3	4	6	6	5	9	9	11	16	8
3	1	3	12	4	1	4	5	9	17	13	4	5	7
4	2	3	5	1	4	4	6	10	12	7	3	2	1
Average	3.3	4.8	9.8	4.3	4.0	5.0	7.5	8.3	13.3	9.8	7.8	7.3	6.3

Any difference between 10-volt and 20-volt UCS would appear to favor the latter. In Table 9-VIII, the 10-volt group showed no clear increase, whereas the 20-volt group improved somewhat up to 180 trials. Even this gain, however, was lost with continued training.

Conclusions

From the foregoing data, several general conclusions appear warranted, within the prevailing conditions:

(1) Of the several varieties of planarians tested, specimens of D. tigrina from Ann Arbor yielded the best training results.

(2) The Harvard inductorium was a more effective UCS than the ac current.

(3) Although appreciable gains can be produced, the establishment of a high, stable response level in individuals S's remains an outstanding problem for future research in this Laboratory.

Part II

Westerman has reported habituation to light in planarians.[5] When 25 exposures per day to a 3-sec burst of light were given, S's reached a zero level of responding in from 250 to 500 exposures. Westerman also found that automatically presented "pretraining" trials (200 to 600 trials) reduced the initial level of responding in subsequent individual testing.[6] We attempted to replicate these findings.

Methods

Subjects

Specimens of Dugesia dorotocephala, obtained from Turtox Biological Supply Co., Chicago, were used. They were maintained on an artificial light-dark cycle and were fed freshly hatched brine shrimp twice a week.

Apparatus

The apparatus used in these habituation studies was the same as that used in the previously reported conditioning experiments except, of course, that the shock was disconnected. The bursts of light (CS) were provided by two 40-watt bulbs. Backlighting was provided by undimmed 7-1/2-, 10-, or 25-watt bulbs. As measured at the trough, the CS produced 400 foot-candles, and the 25-watt backlight produced 22-foot-candles.

Procedure

Twenty-five trials per day were administered, with a minimum intertrial interval of 30 sec. As in the conditioning studies, a response consisted of any noticeable deviation in locomotion.

Some animals were "pretrained" prior to individual training. In these cases, S was placed in the trough and was exposed to one CS every 40 sec, as administered by a preset timing device. In subsequent individual testing, control animals were run in blind fashion along with the pretraining S's. Control animals were either "totally" naive, or were matched to experiments in terms of handling and apparatus exposure (habituation controls).

Results

Experiment 1

Backlight: 25-watt bulb
Pretraining: None
N = 10

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5. R. A. Westerman, Science 140, 676 (1963).

6. R. A. Westerman, (California Institute of Technology, Pasadena, California), personal communication.

The results (shown in Table 9-IX) are in marked contrast to those of Westerman. Although several animals reached a zero level of responding, no trend is evident in the group means, despite the fact that training was prolonged.

Table 9-IX. Number of responses per subject set of 25 trials (no pretraining) measured during Experiment 1.

Worm No.	Trial sets									
	1	2	3	4	5	6	7	8	9	10
1	3	2	10	0	7	5	4	6	9	2
2	3	3	4	9	10	9	5	4	9	6
3	6	6	2	2	7	3	7	1	2	0
4	6	19	9	5	4	3	10	5	1	3
5	7	10	12	2	5	3	1	7	1	
6	3	4	7	6	2	3	0	0[a]	1	
7	3	4	3	17	9	4	12	5	9	7
8	6	3	13	11	13	7	5	3	1	1
9	3	7	5	1	2	5	6	0	4	7
10	1	6	4	2	0	6	6	4	8	3
Av.	4.1	6.4	6.9	5.5	5.9	4.8	5.6	3.5	4.5	3.6
	11	12	13	14	15	16	17	18	19	20
1	2	7	4	7	8	1	5	3	6	0
2	6	5	1	5	8	6	3	3	3	2
3	0[a]	3	10	5	1	3	1	7	1	2
4	3	10	2	7	6	8	11	22	6	19
5										
6										
7	15	10	3	0	0[a]	0	3	3	5	1
8	5	7	4	3	1	0	0[a]	1	4	3
9	8	1	5	3	7	4	2	8	2	4
10	9	10	11	10	2	7	5	9	9	3
Av.	6.0	6.6	5.0	5.0	4.1	3.6	3.7	7.0	4.5	4.2
	21	22	23	24	25					
1	1	2	0	5	4					
2	3	2	6	13	3					
3	3									
4										
5										
6										
7	7	0	0[a]							
8	7	7	1	2	1					
9	0	3	5							
10	4	1	6							

[a]Indicates that the criterion of 50 consecutive trials without a response was obtained.

Experiment 2

Backlight: 25-watt bulb
Pretraining: 367 trials in one session
Total N = 52

Although some differences between the groups appear -- e.g., all the 0's and 1's occur in the experimental group (see Table 9-X) — the difference in means is negligible.

Table 9-X. Number of responses to light per subject in 25 trials during Experiment 2.[a]

Experimentals	Controls
0	8
1	4
2	3
3	5
2	5
2	4
2	6
6	5
1	3
2	3
2	2
5	2
1	4
0	3
2	7^{+}
4	6^{+}
1	3
4	3
2	
2	6^{+}
8	4^{+}
6	2^{+}
4	3^{+}
12	7^{+}
9	7^{+}
4	5^{+}
5	
Average 3.4	4.4

[a]The experienced planarians had been preconditioned to 367 flashes of light in one session prior to testing. Control planarians indicated by $^{+}$ were matched to experimental planarians in terms of handling and apparatus exposure.

Experiment 3

Backlight: 10-watt bulb
Pretraining: 367 trials in one session
Total N = 10
Results are shown in Table 9-XI.

Table 9-XI. Number of responses per subject per set of 25 trials during Experiment 3.

	Experimentals	Controls
	1	3
	8	7
	10	3
	4	14
	6	10
Average	5.8	7.4

Experiment 4

Backlight: 10-watt bulb
Pretraining: 1800 trials in one session
Total N = 8
Results are shown in Table 9-XII.

Table 9-XII. Number of responses per subject per set of 25 trials during Experiment 4.

	Experimentals	Controls
	13	10
	3	11
	5	3
	7	13
Average	7.0	9.3

Experiment 5

Backlight: 7-1/2-watt bulb
Pretraining: None
N = 4
Results are shown in Table 9-XIII.

Table 9-XIII. Number of responses per subject per set of 25 trials during Experiment 5.

Worm	Trial sets					
No.	1	2	3	4	5	6
1	11	5	6	16	12	6
2	13	13	0	8	6	8
3	4	12	12	11	4	3
4	1	2	13	12	0	6
Av.	7.3	8.0	7.8	11.8	5.5	5.8

Experiment 6

Backlight: 10-watt bulb
Pretraining: None
N = 4
Results are shown in Table 9-XIV.

Table 9-XIV. Number of responses per subject per set of 25 trials during Experiment 6.

Worm No.	Trial sets							
	1	2	3	4	5	6	7	8
1	3	9	7	8	9	10	5	8
2	4	15	12	18	10	5	5	5
3	12	16	5	11	12	8	3	4
4	8	11	5	7	7	4	0	4
Average	6.8	12.8	7.3	11.0	9.5	6.3	3.3	5.3

Experiment 7

Backlight: 25-watt bulb
CS: None
Pretraining: None
Purpose: To test "spontaneous" response level
N = 2
Results: During 25 trials, one animal responded once and one didn't respond at all

Experiment 8

Backlight: 25-watt bulb
Pretraining: 367 trials on each of 3 consecutive days
Total N = 16

	Experimentals	Habituated Controls
	3	5
	10	9
	1	4
	3	7
	2	4
	3	0
	5	3
	5	2
Average	4.0	4.3

Experiment 9

Backlight: 25-watt bulb
Pretraining: 200 trials per day for 1, 2, or 3 days
Total N = 18

	Experimentals	Controls
After 200 trials	3	8
	5	3
	5	6
After 400 trials	1	4
	9	1
	1	2
After 600 trials	4	2
	3	1
	2	6
Average	3.7	3.7

Discussion of Experiments 3 through 9

Neither experiments on group pretraining (3, 4, 8, 9) nor those on individual training (5, 6) yielded the desired trends. In habituation experiments, various procedures failed to demonstrate a phenomenon normally considered very simple and demonstrated reliably elsewhere. The causes of this discrepancy remain undetermined.

Part III

For conditioning work described in Parts I and II, we used an inductorium or alternating current as UCS. It was recently reported that a polarized dc current served as a most effective UCS.[7] Conditioning was achieved, however, only when the direction of the current was such as to produce a contraction of the animal's posterior end—i.e., when the animal was oriented towards the cathode. When the current produced anterior contraction (anodal orientation), little improvement in performance was observed. Further, conditioning in the "tail-shock" subjects was rapid (clear increases in responding from day 1 to day 2, day 2 to day 3, etc.), orderly, and consistent from subject to subject.

For the remaining conditioning work in this Laboratory we used polarized direct current.

Method

Subjects

Two varieties of planarians were tested: specimens of D. tigrina from Gladstone, Oregon, and D. dorotocephala, collected locally in Golden Gate Park, San Francisco. They were maintained in the dark between

7. C. D. Barnes and B. G. Katzung, Science 141, 728 (1953).

training sessions, and were fed brine shrimp twice a week. The D. dorotocephala were kept and run in water from their home lake.

Apparatus

The apparatus employed was that of the previous conditioning experiments, except for the shock mechanism. The shock was delivered by a dc stimulator, which was set to produce 100 pulses of 5-min duration, alternating with an equal number of 5-min interruptions. The experimenter could, by operating a switch, change the direction of the current in accordance with the subject's orientation at the moment. Backlighting was provided by a 7.5-watt bulb, dimmed by a Variac.

Procedure

The procedure was identical to that in the earlier experiments: 25 trials were given per day (except where otherwise indicated), with a 1-min intertrial interval. Experimental animals received the usual overlapping, light-shock pattern. Control groups ("pseudoconditioning") received the same number of light and shock stimuli, but each given pair of stimuli was separated by an interval of 15 sec. Any difference between groups could thus be attributed to temporal association. In addition, either experimental or control animals could receive either of two types of shock: cathodal orientation ("tail shock") or anodal orientation ("head shock"). In practice, only three of these combinations were employed: experimental head shock, experimental tail shock, and control (pseudoconditioning) tail shock.

Results

Experiment 1

Species: D. tigrina from Oregon
CS: Two 100-watt bulbs
UCS: 25 volts

It is apparent from Tables 9-XV and 9-XVI that no appreciable enhancement of response level occurred in either group as training progressed. There was a consistent but small difference between the groups. In Experiment 2, in an effort to approximate Barnes' s conditions more closely, several subjects were run, beginning at 5 a. m.

Table 9-XV. Number of responses per set of 25 trials by D. tigrina receiving "head shock" by a polarized current (25 volts) during Experiment 1.

Worm No.	Trial sets										
	1	2	3	4	5	6	7	8	9	10	11
2	3	3	7	4	9	7	6	13	14		
4	3	8	1	3	1	0	7	4	3	5	6
5	0	0	0	2	1	0	2	4	3		
6	4	2	13	10	14	4	3	8	8	13	
7	0	10	9	7	5	8	8	1	6	3	4
9	5	14	3	3	0						
10	1	0	1	3	0						
11	1	2	1	2	2	0					
12	2	2	2	1	2						
13	4	8	4	2	0						
14	1	1	0	9	0						
15	0	1	1	1							
Average	2.0	4.2	3.5	3.9	3.1						

Table 9-XVI. Number of responses to light per set of 25 trials by D. tigrina receiving "tail shock" by a polarized current (25 volts) during Experiment 1.

Worm No.	Trial sets										
	1	2	3	4	5	6	7	8	9	10	11
2	0	2	3	4	6	4	7	10	13		
4	7	16	11	11	12	13	6	6	13	14	9
5	8	11	3	13	9	6	12	11	7	10	
6	11	8	11	8	9	3	7	7			
7	4	8	11	8	7	8	10	10	11	15	
8	2	6	13	4							
9	8	5	6	4	7						
10	3	11	4	4	9						
11	1	2	3	0	4	2					
12	6	4	4	3	3						
13	3	11	3	2							
14	5	4	1	0	3						
15	1	4	1	1							
Average	4.6	7.1	5.7	4.8	6.9						

Experiment 2

Subjects: D. tigrina from Oregon
CS: Two 100-watt bulbs
UCS: 25 volts

Despite the small N, it is clear that time of training made little difference in the results, as shown in Table 9-XVII.

Table 9-XVII. Number of responses to light per set of 25 trials by D. tigrina receiving "tail" or "head shock" (25 volts) from a polarized current during Experiment 2. Tests were made at 5 a.m.

	Tail Shock					Head Shock			
Worm	Trial sets				Worm	Trial sets			
No.	1	2	3	4	No.	1	2	3	4
1	0	5	3	2	3	6	4	3	4
2	5	9	8	5	4	2	0	0	-

Experiment 3

Subjects: D. dorotocephala
CS: Two 100-watt bulbs
UCS: 25 volts

Factual data are presented in Table 9-XVIII.

Table 9-XVIII. Number of responses to light by D. dorotocephala receiving shock from a polarized current (25 volts) during Experiment 3. The two overhead lights were 100 W.

	Tail Shock							Head Shock					
Worm	Trial sets						Worm	Trial sets					
No.	1	2	3	4	5	6	No.	1	2	3	4	5	6
1	10	20	16	10	19	25	3	1	6	1	11	9	2
2	14	9	13	14	13		4	8	6	4	4	15	

Experiment 4

These results appeared somewhat promising and were extended further in Experiment 4. Two conditions were changed: CS was reduced from 100-watt bulbs to 40-watt bulbs so as to decrease variability and the initial level of responding, and the UCS was reduced to 20 volts.

Subjects: D. dorotocephala
CS: Two 40-watt bulbs
UCS: 20 volts

Table 9-XIX. Number of responses by D. dorotocephala to "head shock" (20 volts) per set of 25 trials during Experiment 4. Two 40-watt lights were used overhead.

Worm No.	Trial sets							
	1	2	3	4	5	6	7	8
3	2	4	8	9	10	0	14	14
7	2	7	7	5	13	15	4	10

Table 9-XX. Number of responses per set of 25 trials by D. dorotocephala receiving "tail shock" (20 volts) during Experiment 4. Animals were retrained 1 week later. Two 40-watt overhead lights were used.

Worm No.	Initial training								Re-test							
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
1	1	15	16	20	22	a			20	20						
2	1	6	6	10	4	a			13	8	8	12	5	10	14	7
4	3	8	7	16	11	8	21	17	11	14	13	8	15			
5	1	9	13	9	15	16	16	16	15	a						
6	6	15	15	19	a				18	17	a					
8	6	16	19	22	a				13	17	21	a				
9	5	12	15	13	15	18	18	a	20	17						
10	8	19	17	17	a				22	a						
11	2	9	8	10	16	17	20	13	10	7	13	12	5	10		
12	3	9	15	14	18	a			22	a						
13	8	20	19	21	a				20	18	a					
14	2	13	18	17	17	20	18	19	19	16	a					
15	3	15	14	a					15	14	21	a				
16	5	13	16	14	13	11	a		5	6	8	13				
Average	4	13	15	16.5	17	18	19	17	16	14	14	11	7	10	14	7

a indicates the planarians reached the preset criterion of 23 responses out of 25 consecutive trials.

Table 9-XXI. Number of responses per set of 25 trials by D. dorotocephala receiving unpaired light and "tail shock" (pseudoconditioning) during Experiment 4. Three animals were retrained 1 week later.

Worm	Worm training								Re-test	
No.	1	2	3	4	5	6	7	8	1	2
17	2	13	7	12	10	12	16	17	10	
18	5	14	13	13	15	14	2	15	18	19
23	5	9	17	11	17	a			19	15
25	4	8	10	16	7	8	5	13		
26	14	11	8	12	9	13	11	10		
27	0	4	3	2	4	4	4	6		
28	10	12	8	9	7	4	8	5		
Average	5	12	8	12	9	12	6.5	11.5		

a indicates the planarians reached the preset criterion of 23 responses out of 25 consecutive trials.

The data of Table 9-XIX suggest no clear pattern of improvement, although the N is too small to draw final conclusions. Table 9-XX, describing the performance of the cathodally oriented S's, has several interesting features. Of 14 animals so trained, 10 reached the preset criterion (23 responses out of any 25 consecutive trials) within 200 trials. The overall group medians show a regular increase, even though S's were dropped from the calculations upon reaching criterion. These data contrast somewhat with those of Table 9-XXI, which presents the results of the pseudoconditioning (light and shock unpaired) group. Of the latter group, one S out of seven reached criterion, and successive medians exhibit erratic variation.

The second part of Tables 9-XX and 9-XXI show retraining data after a lapse of 1 week from the termination of original training. These results are incomplete, especially in the case of the control group. These incomplete data indicate that all but S no. 2 retained some of the original training.

One aspect of these results, which does not appear in the tables, led us to terminate this experiment prematurely: namely, that as training proceeded, some S's of both groups exhibited an increasing lethargy in the experimental apparatus and had to be stimulated to move. This effect was most conspicuous from day 1 to day 2, and although it diminished thereafter, it was nonetheless undesirable. This lethargy occurred equally in both experimental and control groups. It was conspicuous again on retraining (in some animals), and it was at this point that we decided to attempt to eliminate it.

Experiment 5

In Experiment 5, then, the intensity of the shock was decreased from 20 to 15 volts.

Subjects: D. dorotocephala
CS: Two 40-watt bulbs
UCS: 15 volts

No marked differences between groups, nor any clear trends, are apparent in Table 9-XXII. Moreover, the same lethargy was observed as occurred in Experiment 4. In the final two experiments, a UCS of 20 volts was used, but only 15 trials were given per day. Experiment 6 tested animals that had been in the laboratory for several weeks, and Experiment 7 employed the same procedures on freshly collected animals.

Table 9-XXII. Number of responses by D. dorotocephala subjected to sets of 25 trials of "tail shock" under conditioning and pseudoconditioning during Experiment 5. A 15-volt polarized potential was applied.

Worm No.	Tail-shock conditioning Trial sets					Worm No.	Tail-shock pseudoconditioning Trial sets				
	1	2	3	4	5		1	2	3	4	5
19	7	12	13	15	14	21	3	9	5	3	15
20	0	9	11	10	10	22	6	7	11	10	10

Experiment 6

Subjects: D. dorotocephala
CS: Two 40-watt bulbs
UCS: 20 volts
No. of trials/day: 15
Note: "Old" animals

Experiment 7

Subjects: D. dorotocephala
CS: Two 40-watt bulbs
UCS: 20 volts
No. of trials/day: 15
Note: Freshly collected animals

Only 60 trials were given in these experiments, but in light of the rapid improvement found by Barnes, the failure of the S's to show conditioning may be viewed as significant. Table 9-XXIII summarizes the results of Experiment 6 and 7.

Table 9-XXIII. Number of responses by D. dorotocephala during each set of 15 daily trials during Experiments 6 and 7. A polarized potential of 20 volts was applied. The planarians had been in the laboratory for several weeks (Part A) or were freshly collected (Part B).

Worm No.	Conditioning Trial sets 1	2	3	4	Worm No.	Pseudoconditioning Trial sets 1	2	3	4
				Part A					
1	2	0	2	9	5	2	1	4	10
2	0	2	3	3	6	4	1	0	2
3	1	0	3	4	7	2	1	0	1
4	1	1	2	2	8	4	1	3	1
Average	1.0	0.8	2.5	4.5		3.0	1.0	1.8	3.5
				Part B					
1	4	4	2		5	3	8	6	5
2	1	2	6	3	6	2	5	1	4
3	4	3	1	1	7	1	4	1	0
4	0	1	6	9	8	3	7	5	5
Average	2.3	2.5	3.8	4.3		2.3	6.0	3.3	3.5

Summary and Conclusions

A painstaking series of investigations on classical conditioning and habituation in planarians has not yielded a training procedure adequate for proposed biochemical manipulations. Although it is not accurate to say that training could not be achieved (see, for example, Figs. 9-1 and 9-2 of Part I), but the training process was not brought under the desired degree of control. Until the crucial discrepancies between these and prior studies are isolated, we can only report that there are conditions under which clear-cut training does not occur.

That statement, however, does not dismiss the problem. We were particularly puzzled because we failed to obtain reliable habituation in planarians. Habituation to a repetitive stimulus is generally considered to be one of the simplest cases of behavioral modification, is practically universal throughout the animal kingdom, and was demonstrated in planarians by Westerman.[5] We are led to conclude that some uncontrolled (and unknown) variable influenced the animals' behavior sufficiently to mask the presumably more subtle habituation. Such a conclusion is further suggested by the conditioning results, and also by the results to be considered briefly now.

Recently Lee reported successful operant conditioning in planarians.[8] When turning off an aversive bright light was contingent on breaking a

8. R. M. Lee, Science 139, 1048 (1963).

photobeam, experimental animals showed markedly more efficient behavior than controls. Preliminary attempts (in which the present writers are not involved) to replicate Lee's findings in this Laboratory have been unsuccessful (see Sec. 10 of this quarterly report). It is too early, however, to judge the ultimate success of this project, and it certainly merits a strong effort. If an unknown common variable is disrupting all the training operations, it can be as easily discovered in one device as another, and the Lee apparatus has the advantages of automation. It should be noted, though, that the phenomena (regeneration, cannibalism) shown with classical conditioning in planarians have yet to be tested in the Lee apparatus.

Finally, we believe that relative failure and success in training planarians reflect differences in conditions of training rather than in the capacities of the animals. This view is supported by the fact that concurrently with research in this Laboratory, positive results have been obtained in other laboratories (such as by Barnes, Lee, McConnell, and Westerman).

10. OPERANT CONDITIONING IN PLANARIANS

Janet L. Alvarez, Henry Schott, and Frank T. Upham

Numerous reports have appeared recently suggesting that planarians can be trained to give simple conditioned responses and that these worms, so trained, provide a promising medium for investigating the possible molecular basis of learning and memory.[1,2,3] In order to pursue similar experiments, we wished to develop a technique for training large numbers of animals in some relatively automatic apparatus. We desired an apparatus in which stimuli could be presented to the subjects, their responses observed, and appropriate data recorded—all by some suitable electromechanical means. Such a device would assure maximum objectivity in the data-collecting process, and at the same time would require a minimum of attendance by scientific personnel.

With these goals in mind, we decided to build an apparatus to duplicate that described by Lee.[4] He reported that in his experiments the "response measured was the animal's passage through a narrow beam of light directed at a photocell, and the reinforcement was the termination of an intense light."

Experimental

Apparatus

The training apparatus was housed in an open-fronted wooden box, 36 in. long, 9 in. deep, and 15 in. high, divided into two units. In the center of the top of each unit there was a 40-W electric bulb. Eight in. beneath each bulb was placed a pair of adjacent cells for the subjects. These were made of clear Lucite, cylindrically shaped, 1 in. deep and 3/4 in. in diameter. Protruding into each cell from the side was a rectangular block, 1/8 in. thick and extending 1/2 in. across the cell. A space of 2 mm between the bottom of these blocks and the bottom of the cells permitted the worms to swim freely underneath. A minute photocell (IN 2175) was inserted in each of the blocks, 1/8 in. from the edge of the cell. Each pair of cells rested on a metal plate in which 0.5-W light bulbs were located. Small holes, about 1 mm in diameter, located in the top surface of the plates immediately under the photocells, permitted fine beams of light to pass through the bottoms of the cells and up to the detectors in the rectangular blocks. By swimming under the block, the worm could pass through the beam of light directed at the photocell.

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1. J. B. Best, Sci. Am. 208 (2), 55 (1963).

2. J. V. McConnell, A. L. Jacobson, and D. P. Kimble, J. Comp. Physiol. Psychol. 52, 1 (1959).

3. W. C. Corning and E. R. John, Science 134, 1363 (1961).

4. R. M. Lee, Science 139, 1048 (1963).

Each unit contained one cell for the experimental subject and one for the control.

Black curtains across the front of the units shielded the cells from extraneous light. Cooling was provided by water running through the metal plates under the cells.

The circuitry was designed (for each unit) so that when the experimental subject passed through the small beam of light, the overhead light went off. The worm's passage and the turning off of the light were recorded in separate channels of a multiple-channel Esterline-Angus recorder. The light that illuminated both the experimental and control cells remained off for 15 min and then automatically went on again; the recorder indicated this event. The light then stayed on until the experimental worm again passed through the narrow light beam. The photocell in the adjacent control cell was connected to a third recording pen. Every time the control worm passed under the block and through the narrow beam, the event was recorded but no change occurred in the overhead light.

The pen records allowed us to compare the pattern of responses made by the experimental worms, which controlled the lights, with those of the controls. The controls received identical light-and-dark stimulation, but their responses did not influence that stimulation. Since the bright light is an aversive stimulus,[4] it was expected that the experimental subjects would learn to swim under the block and through the narrow beam each time the overhead light went on. The controls, on the other hand, should show no particular correspondence between the onset of the light and their passage through the photocell beam.

Lee reports that, in his very similar apparatus, "only the reinforced subjects produced steady, spaced behavior".[4] In his work (after 20 to 30 hr), the experimental animals responded rather consistently by passing through the photocell beam shortly after the light went on. On the other hand, the controls showed very low rates of responding, and often stopped swimming altogether.

Worms

Several species of worms were used in the apparatus. In particular, we tried to use Cura foremani specimens, since this was the species with which Lee obtained such promising results. Unfortunately, the specimens available to us were either too small or too inactive to break the light beam more than 3 or 4 times in periods as long as 60 hr, and therefore we abandoned their use. Finally, we used exclusively planarians from ponds in Golden Gate Park. These are a large, dark, egg-laying variety—probably Dugesia dorotocephala.

Subjects were collected on three occasions: at the end of June, on July 23, and on August 21, 1963.

Environmental Conditions

Initially the training apparatus was located, and the worms were housed, in a large upstairs laboratory with daylight coming in the windows.

At the end of July, the apparatus and housing cupboard were moved to a small basement room where all the light was artificial and where it was almost totally dark a great deal of the time. (The data obtained here are referred to as "modified conditions".)

At the time of this move, we installed several other environmental changes. We had been disturbed that the response rate of our subjects was substantially lower than that reported by Lee, and we hoped that the modifications would bring our results into agreement with his. These changes are discussed below, and all the environmental conditions are summarized at the end of this section.

All subjects were maintained in shallow bowls housed in a dark cupboard, except when they were in the training cells. No particular effort was made to shield them from occasional exposure to light, as when the cupboard door was opened to remove subjects or for feeding. The temperature of the water in the bowls was 26 °C in the laboratory upstairs; it dropped to 23 °C in the basement room.

The animals were fed on bits of liver, twice weekly, for 3 to 6 hr. After the worms were fed, the bowls and water were changed. Bottled spring water was used in the bowls and training cells while we were upstairs. We tried using filtered pond water (Table 10-II subjects—see "Results" and "Discussion"), but when it showed no obviously advantageous effect on either the health or the performance of the worms, we reverted to the more convenient bottled water (Table 10-III subjects).

In the training cells the temperature of the water, while we were upstairs, was 26 °C. Hypothesizing that this might be too warm (Lee ran his worms at 16 °—18 °C and maintained them at 18 °C), an improved cooling mechanism was installed when we moved. This brought the temperature of unit 1 down to 22 °C and of unit 2 down to 17 °C.

In a further effort to increase the activity rate of the subjects we installed a device for bubbling air through the training cells. The device never worked properly in unit 2, but it was operative for all subjects in unit 1 throughout the period covered in Tables 10-II and 10-III.

Summary of environmental conditions

Table 10-I[a] Subjects	Table 10-II[a] Subjects	Table 10-III[a] Subjects
Collected end of June	Collected 7/23/63	Collected 8/21/63
Trained 1 to 4 weeks after capture	Trained 1 to 4 weeks after capture	Trained 5 days after capture
Housed at 26 °C	Housed at 23 °C	Same as Group 2
Trained at 26 °C	Trained at 22 °C (unit 1)	Same as Group 2
	17 °C (unit 2)	Same as Group 2
Lab had windows	Lab had no windows	Same as Group 2
Cells not aerated	Unit 1, aerated water	Same as Group 2
Bottled water	Pond water	Bottled water

[a] The tables refer to the data presented under "Results" and "Discussion".

Results

The data obtained from our apparatus are summarized in Tables 10-I, 10-II, and 10-III. The tables cover three different chronological periods, during each of which the environmental conditions were altered slightly. Each subject has been designated by numbers and letters. The first number indicates the unit in which the worm was tested, the second designates the chronological order of the experiment, and the letter refers to experimental (E) or control (C) worms. All the data of these tables were obtained exclusively with the Golden Gate Park worms.

The data that we have obtained do not show any pattern, such as Lee reports, which could be interpreted as evidence of a training process. We did obtain evidence of a diurnal activity cycle under certain conditions. The data of Tables 10-I, 10-II, and 10-III are organized to illustrate this cycle.

Table 10-I contains the data from the experiments conducted in an upstairs laboratory in diffuse daylight. The data of Tables 10-II and 10-III are those obtained in the experiments conducted in a small, almost totally dark, basement laboratory.

Table 10-I. Responses of planarians in operant conditioner in upstairs laboratory in diffuse daylight.

Dates of time in apparatus[a]	Worm No.	Total time in apparatus (hr)	Total counts	Counts 8a.m.-8p.m. No.	Counts 8a.m.-8p.m. %	Counts 8a.m.-8p.m. No.	Counts 8a.m.-8p.m. %	Comments
3 p.m. 7/5 to	1-1-E	73	15	11	73	4	27	
4 p.m. 7/8	1-1-C	22	27	17	63	10	37	(b)
11 a.m. 7/9 to	1-2-E	140	101	67	66	34	34	
9 a.m. 7/15	1-2-C	140	57	51	90	6	10	
3 p.m. 7/5 to	2-2-E	213	150	101	68	49	32	(c)
10 a.m. 7/15	2-2-C	213	340	216	64	124	36	(c)
2 p.m. 7/18 to	1-3-E	23	0	-	-	-	-	
1 p.m. 7/19	1-3-C	23	22	21	95	1	5	
4 p.m. 7/15 to	2-3-E	87	88	54	62	34	38	
7 a.m. 7/19	2-3-C	87	149	112	75	37	25	
2 p.m. 7/19 to	1-4-E	97	2	0	0	2	100	
3 p.m. 7/23	1-4-C	97	41	29	71	12	29	
	Totals	1215	992	679	69	313	31	

[a] The periods of time are not multiples of 24 hr, and there is a total of 32 more hr between 8 p.m. and 8 a.m. than vice-versa (i.e., more nighttime hr). It is unlikely that the percentage of daylight counts would have increased materially had the worms remained in the apparatus to finish out these 24-hr periods. This is because all but three worms had ceased to be very active when it was decided to discontinue their respective runs.

[b] Died after 22 hr in the apparatus.

[c] Removed once for 22 hr for apparatus repair.

Table 10-II. Responses of planarians in operant conditioner in almost dark basement laboratory.

Dates of time in apparatus	Worm No.	Total time in apparatus (hr)	Total counts	Counts 8 a.m. - 8 p.m.		Counts 8 p.m. - 8 a.m.		Comments
				No.	%	No.	%	
4 p.m. 7/31 to	1-6-E	288	267	161	60	106	40	
4 p.m. 8/12	1-6-C	-	-	-		-	-	(a)
4 p.m. 8/5 to	2-6-E	96	64	35	55	29	45	
4 p.m. 8/9	2-6-C	-	-	-	-	-	-	(a)
4 p.m. 8/12 to	1-7-E	144	124	30	24	94	76	
8 p.m. 8/15 and 8 p.m. 8/16 to 4 p.m. 8/19	1-7-C	144	113	53	47	60	53	(b)
4 p.m. 8/12 to	2-7-E	96	59	34	58	25	42	
4 p.m. 8/16	2-7-C	96	49	25	51	24	49	
8 p.m. 8/16 to	2-8-E	-	-	-	-	-	-	(a)
8 p.m. 8/18	2-8-C	48	61	16	26	45	74	
1 p.m. 8/19 to	2-9-E	120	35	30	86	5	14	
1 p.m. 8/24	2-9-C	-	-	-	-	-	-	(a)
9 a.m. 8/20 to	1-8-E	96	3	3	100	0	0	
9 a.m. 8/24	1-8-C	96	16	15	94	1	6	
	Totals	1244	791	402	51	389	49	

[a] Prolonged electronic defect made it impossible to include data.

[b] Brief electronic defect, 8 p.m. 8/15. Run resumed 8 p.m. 8/16.

Table 10-III. Responses of planarians in operant conditioner in almost dark basement laboratory a few days after experiments in Table 10-II.

Dates of time in apparatus	Worm No.	Total time in apparatus (hr)	Total counts	Counts 8 a.m. - 8 p.m.		Counts 8 p.m. 8 a.m.	
				No.	%	No.	%
8 a.m. 8/26 to	1-10-E	96	59	22	37	37	63
8 a.m. 8/30	1-10-C	96	7	6	83	1	17
8 a.m. 8/26 to	2-10-E	96	50	31	62	19	38
8 a.m. 8/30	2-10-C	96	68	44	65	24	35
	Totals	384	184	103	56	81	44

Discussion

Learning

Examination of all the individual records reveals no patterning that can be construed as evidence for "learning." There were no consistent changes in the behavior of any of the experimental animals during the course of "training." Neither were there any consistent differences between the activity patterns of the experimental subjects and the controls.

We cannot suggest an adequate hypothesis for the discrepancy between our results and those of Lee. The experiments will be continued and some minor modifications will be made in the training cells in hope of obtaining more encouraging response patterns.

Total Activity under Various Environmental Conditions

Numbers are given in Tables 10-I, 10-II, and 10-III for "Total counts" and "Total hr in apparatus." A calculation of the average number of counts per hr in the apparatus, for each group of worms, suggests we have not been successful in our efforts to advance the response rate of the subjects. The individual totals for subjects in unit 1, compared with those in unit 2, do not give a good indication whether or not the aerated water is helpful; nor is there sufficient information to judge what is an optimum temperature. We badly need further study of these issues, since the summary in Table 10-IV indicates that the activity rate has probably declined during the course of the experiments.

Table 10-IV. Comparison of responses per hour by planarians in this series of experiments in operant conditioner.

		Total time (hr)	Total counts	Counts per hr
Table 10-I	Initial conditions	1215	992	0.82 ± 0.03[a]
Table 10-II	Modified conditions	1244	791	0.63 ± 0.04
Table 10-III	Modified conditions and freshly captured worms	384	184	0.48 ± 0.05

[a]Errors quoted in this table and in Table 10-V, are statistical errors based on the total number of events.

Diurnal Activity under Various Environmental Conditions

Data are given in Tables 10-I, 10-II, and 10-III indicating the percent of total responses that occurred between 8 a.m. and 8 p.m. for each worm and each group of worms. Table 10-V summarizes some of these data.

Table 10-V. Summary of diurnal variation in response of planarians in operant conditioner.

		Total counts	Counts 8 a.m. -8 p.m.	% Counts 8 a.m. -8 p.m.
Table 10-I	Natural day-night cycle	992	679	69 ± 3
Table 10-II	Artificial light, extensive dark	791	402	51 ± 4
Table 10-III	Artificial light, extensive dark, freshly captured worms	184	103	56 ± 7

There is a statistically significant difference between the worms in Table 10-I and those in Table 10-II. The fresh worms shown in Table 10-III were selected to begin a study of possible causes for the shift in diurnal activity pattern that occurred coincidentally with the relocation of the apparatus and other changes in the environment.

11. MANGANESE PORPHYRIN COMPLEXES

Linda K. Phillips

Several transition-metal porphyrin chelates have been studied in this Laboratory, in the hope of finding models for similar biologically important compounds. Loach has investigated the redox and photochemistry of manganese hematoporphyrin IX, which is water-soluble.[1] On the other hand, the phthalocyanine and etioporphyrin complexes, which have been studied recently by Yamamoto and Phillips, are in general nearly insoluble.[2,3] For instance, manganese(II) phthalocyanine is more soluble in pyridine than in any other solvent investigated, but even in pyridine reaches a concentration of only about 5×10^{-4} M.

This low solubility leads to serious experimental difficulties. The organic solvents which must be used (principally pyridine) are likely to contain trace impurities of oxidants or reductants. For the usual sample concentration of 10^{-5} M, an impurity of, say, 1 ppm would be of about the same molarity as the sample. The redox chemistry of iron etioporphyrin in pyridine solution has not been reproducible, nor has the behavior of manganese etioporphyrin in the presence and "absence" of water, again in pyridine as solvent. We are now trying to find conditions for the preparation, by gas chromatography, of more nearly pure pyridine. In addition, since it would be advantageous to work with more soluble compounds, some preliminary experiments have been done with the methyl ester of manganese pheophorbide-a.

Preparation

About 25 mg of pheophorbide-a methyl ester was mixed with a tenfold excess of $MnAc_2$ in chloroform. The electronic spectrum agreed with that[4] of methyl pheophorbide-a, and did not change even after long standing and heating. The $CHCl_3$ was evaporated, and the residue dissolved in dimethylformamide to which was added some solid $NaHCO_3$. Again, little reaction was observed until the mixture was heated on the steam bath.

Water and benzene were now added, the plan being to extract the chelate and the unreacted chlorin into the benzene layer. Surprisingly, only the methyl pheophorbide-a appeared in the benzene, whereas the water layer exhibited an unfamiliar spectrum (almost identical to that in ethanol; see Fig. 11-1). To the eye, both layers were the same dark green.

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1. Paul A. Loach and Melvin Calvin, Biochemistry 2, 361 (1963).

2. G. Engelsma, Akio Yamamoto, E. Markham, and Melvin Calvin, J. Phys. Chem. 66, 2517 (1962).

3. Akio Yamamoto, Linda K. Phillips, and Melvin Calvin, "Phthalocyanine Manganese and Etioporphyrin I Manganese Complexes," manuscript in preparation.

4. G. Zieger and H. T. Witt, Z. Phys. Chem. (Frankfurt) (N. F.) 28, 286 (1961).

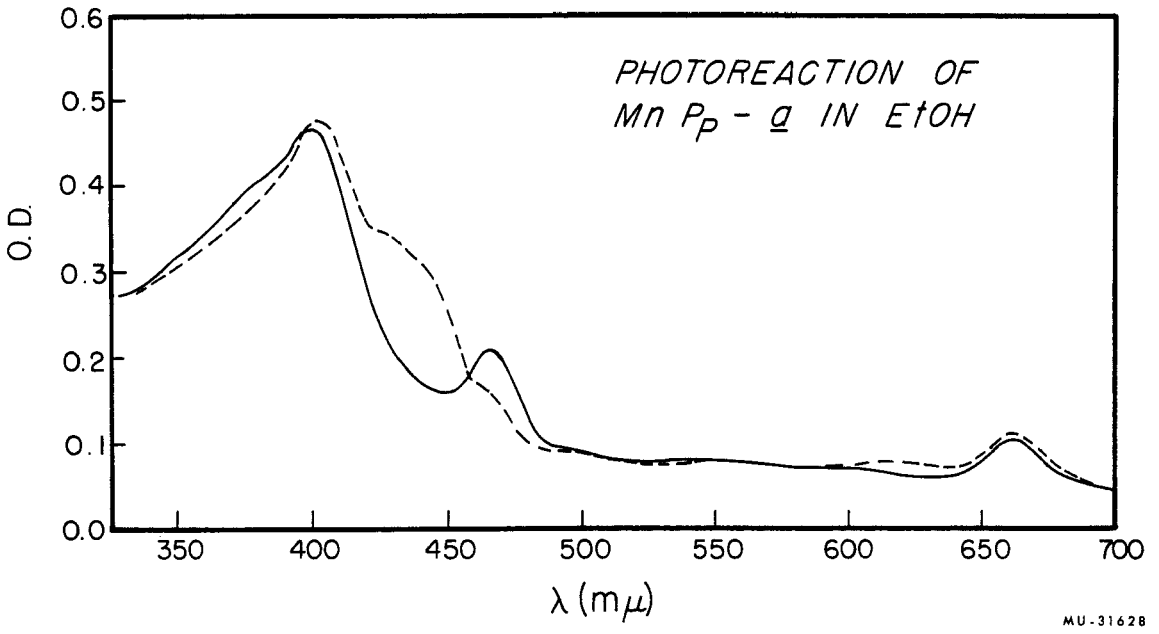


Fig. 11-1. Photoreaction of manganese pheophorbide-a ester in ethanol. Solid line, Mn(III) complex; dashed line, after 1.5 hr illumination (Mn(II) complex).

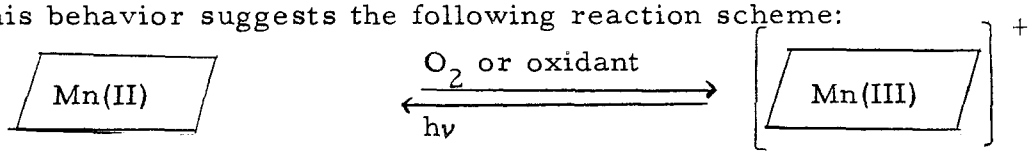
The inorganic salts and most of the water were removed from what is believed to be the Mn(III) chelate, by repeated evaporation and dissolution in EtOH. The final alcoholic solution was used without further purification. The sample was probably allomerized (although resolution was not good enough to unambiguously detect accompanying spectral shifts), and the methyl group may have been replaced by ethyl.

Reactions

To compare this complex with those previously studied, a solution of optical density about 0.5 (i. e., on the order of 10^{-5} to 10^{-6} M) was placed in a 1-cm Pyrex cuvette in which all reactions were carried out. No rigorous attempt was made to exclude water.

In the absence of oxygen a photoreaction occurred that was apparently nearly complete after 1.5 hr illumination. The original spectrum reappeared after the sample had stood overnight in the dark; there was little or no loss of pigment. Exposure to air speeded up the back reaction. There was no noticeable spectral change (except possibly a slight decrease in over-all intensity, suggesting ring destruction) upon illumination in air for 18 minutes. Light from a G. E. photospot lamp (RS-2), filtered through glass and water, was used for all illuminations.

This behavior suggests the following reaction scheme:



where the parrellelogram represents the pheophorbide ring.

The unidentified substance oxidized during the photoreaction would provide the oxidant for the spontaneous dark back reaction. The Mn(III) complex is the stable form in air.

Thus, the behavior of manganese pheophorbide-a methyl (or ethyl) ester in EtOH is similar to that of manganese etioporphyrin in pyridine.[2] More careful experiments are clearly indicated. We need to determine redox potentials, and the effects of solvents, added ligands, and changes in concentration of the complex, as well as possible mechanisms for the reactions.

12 EPR STUDIES OF SOME COMPLEX ORGANIC SOLUTIONS

David F. Ilten, Maurits Kronenberg, and R. H. Ruby

Photoinduced electron paramagnetic resonance (EPR) signals have been observed in solutions of electron acceptors (e.g., chloranil) in various organic solvents.[1,2] An important step in determining the nature of the paramagnetic species involved is the study of the growth and the decay of electron-spin signals in carefully purified materials. The present work deals with purification procedures and with a method for measuring EPR rise and decay times of the order of 20 milliseconds.

Purification Procedures

p-Chloranil was recrystallized repeatedly from benzene after the hot solution had been treated with Norit. No impurities were detectable with thin-layer chromatography. o-Chloranil was recrystallized from 1,2-dichloroethane. p-Dioxane was prepared by vapor-phase chromatography (VPC) using an Aerograph "Autoprep" (Wilkens Instrument and Research, Inc.,) and a "Ucon polar" column. The purified dioxane showed no impurities in the VPC trace. Tetrahydrofuran (THF) was repeatedly distilled until only a trace of impurity could be found by VPC. Again a "Ucon polar" column was used.

Physical Measurements

The solutions to be studied were placed in quartz tubes of 4 mm i.d. and degassed by freezing, evacuating, and melting. This procedure was repeated ten times until a constant pressure of 10^{-5} mm of Hg was attained. The solutions showed no EPR signal in the dark. The samples were irradiated through slots of a Varian #4531 rectangular cavity. The light source was a G.E. AH-6 high-pressure Hg lamp located 70 cm from the cavity and focused by means of two glass lenses. The time of irradiation was controlled with a camera shutter.

The samples studied were those that had exhibited "fast" rise and decay times during preliminary investigations.[2] In order to obtain a better estimate of these times it was necessary to reduce the time constant of the EPR spectrometer to 30 msec. This, however, resulted in a decrease of the signal-to-noise ratio to approximately 1. To circumvent this difficulty the signal was averaged over a number of passes using an integrator.[3,4] That is, the sample was irradiated for 200 msec until a signal had built up.

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1. G. Lagercrantz and M. Yhland, Acta Chem. Scand. 16, 1043 (1962).

2. D. Ilten and M. Kronenberg, in Bio-Organic Chemistry Quarterly Report, UCRL-10743 March 29, 1963, p. 58.

3. M. P. Klein and G. W. Barton, Jr., Rev. Sci. Instr. 34, 754 (1963).

4. The integrator used in these experiments was a Computer of Average Transients (CAT, Mnemotron Corp., Pearl River, New York).

The light was then shut off until the spin had disappeared. At this time the sample was again irradiated. The signal increased with the number of irradiations, whereas the noise increased only as the square root of this number. Thus, when 100 irradiations were made the signal-to-noise ratio was increased from 1 to 10. The time constants for the shutter and the EPR spectrometer limited experimental measurement to nothing faster than approximately 20 msec. The rise times for the samples appeared to approach this limiting value, so it will be necessary to reduce these instrumental time constants still further.

Results

The following systems were studied and showed a photoinduced signal; their colors and relative rise times are indicated parenthetically:

p-chloranil p-dioxane	(yellow)	(fast)
p-chloranil THF	(yellow)	(fast)
o-chloranil thiophene	(red)	(fast)
o-chloranil carbon tetrachloride	(red)	(slow)
crystalline solid o-chloranil	(red)	(slow)

The signals had a g value of approximately 2. They were all about 15 gauss wide, showed no hyperfine structure, and were asymmetric. Figures 12-1 through 12-3 show the experimental curves for the rise and decay times of some of the systems. It should be noted that one of the difficulties in analyzing the data is determining when the shutter is actually opened and closed. The 10 to 90% rise time for the shutter was approximately 10 msec. For the first three systems listed above, the maximum spin concentration was reached within 100 msec. After the shutter was closed the spin concentrations decayed to zero in 50 to 150 msec. The o-chloranil–thiophene system showed a photoinduced signal, whereas the p-chloranil–thiophene system did not. For this reason studies were made on o-chloranil in CCl_4 and on solid o-chloranil. In both cases a slowly growing photoinduced EPR signal was observed, but in neither case was a fast signal seen. No signal was found in any of the solvents alone. This evidence seems to indicate that the photoinduced EPR signal is attributable to an interaction between o-chloranil and thiophene, and that such an interaction does not occur between p-chloranil and thiophene.

In Fig. 12-4 the log of maximum spin concentration, minus the spin concentration at a time t after the light has been turned on, is plotted as a function of t. This gives an approximately straight line, indicating that the rise is roughly exponential. In Figures 12-5 to 12-7 are plotted the decay curves for various samples. These data were obtained as before by adjusting the magnetic field to the point where the derivative of the absorption was at a maximum, and then shutting off the light and observing the decrease in the maximum value. The decay curves are complicated and may indicate the presence of two or more paramagnetic species.

Figure 12-8 compares the growth curves of p-chloranil and dioxane with and without a neutral density filter (Bausch and Lomb, o.d. 0.9) to reduce the intensity of irradiation to about 12.5% of its maximum value. It appears to require a longer time for the spin concentration to reach its maximum value when the filter is used.

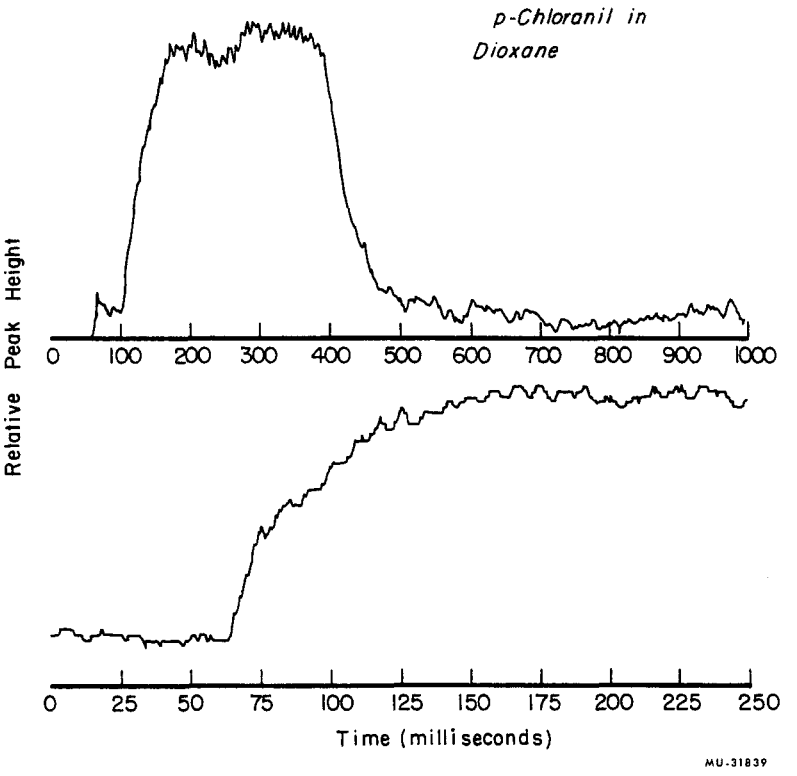

Fig. 12-1(a).Rise and decay of EPR signal for p-chloranil in dioxane (top). (b). Rise time (expanded scale) (bottom).

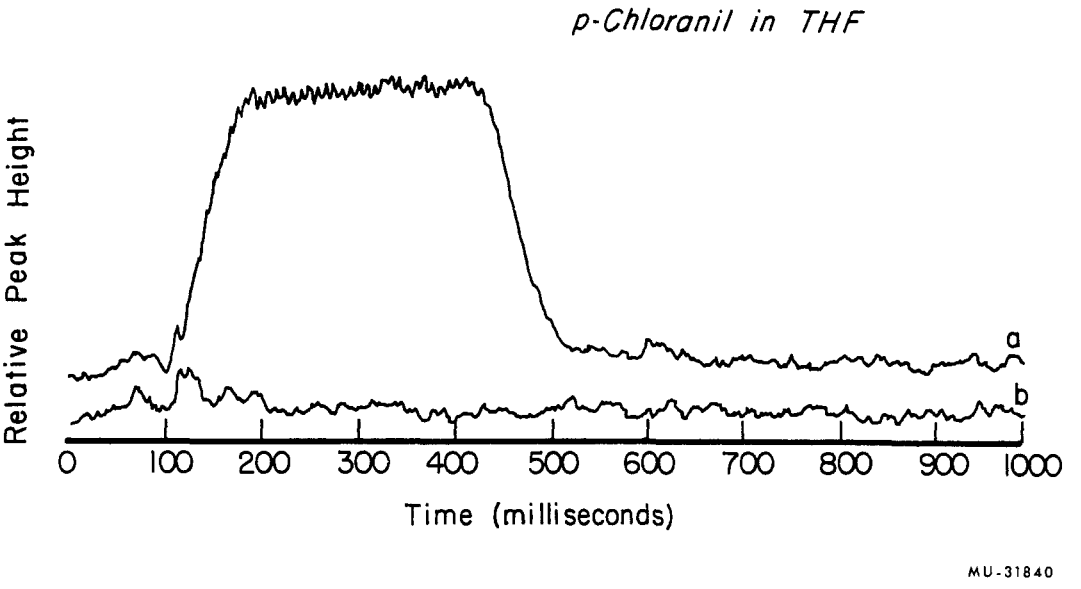

Fig. 12-2. Rise and decay of EPR signal for p-chloranil in tetrahydrofuran (THF) (a) and signal off magnetic resonance to show noise (b).

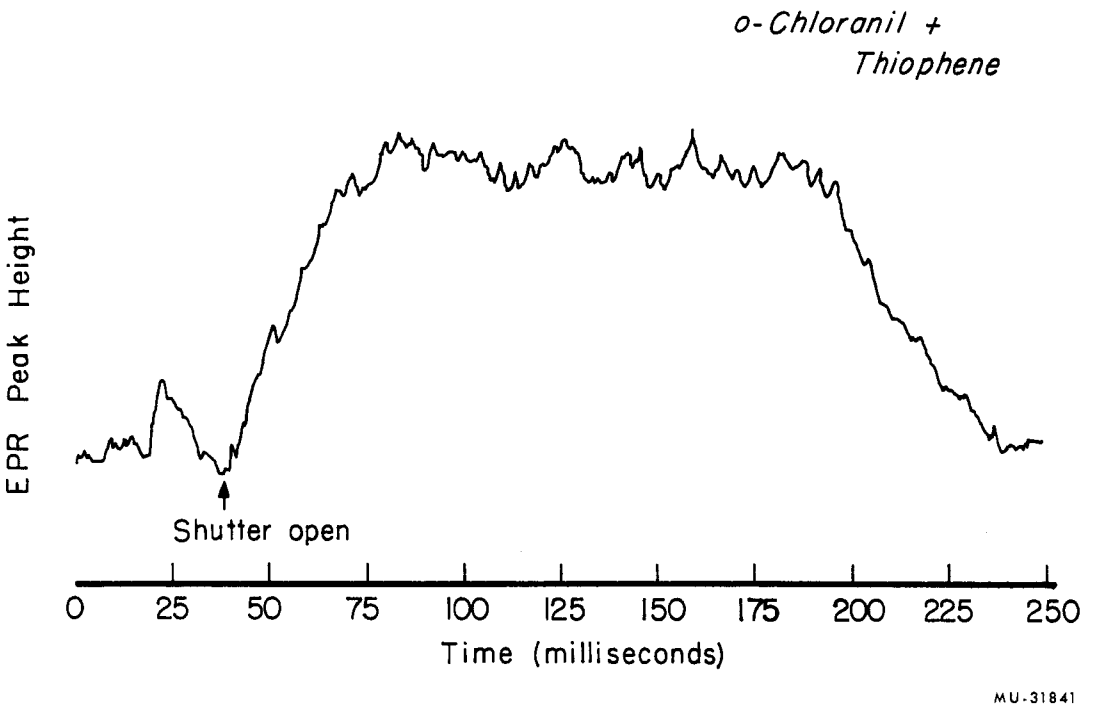

Fig. 12-3. Rise and decay of EPR signal for o-chloranil in thiophene.

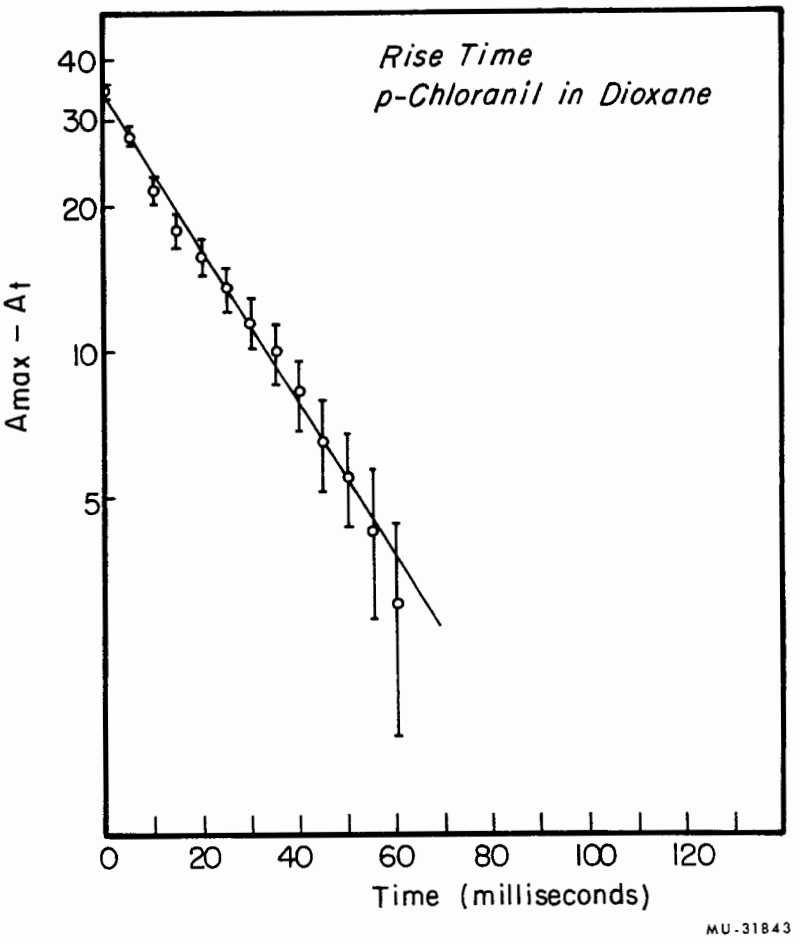

Fig. 12-4. Semilog plot of rise of EPR signal for p-chloranil in dioxane. A_{max} denotes maximum amplitude of signal in Fig. 12-1a. Again the units are arbitrary. A_t denotes signal amplitude at time t after light has been turned off.

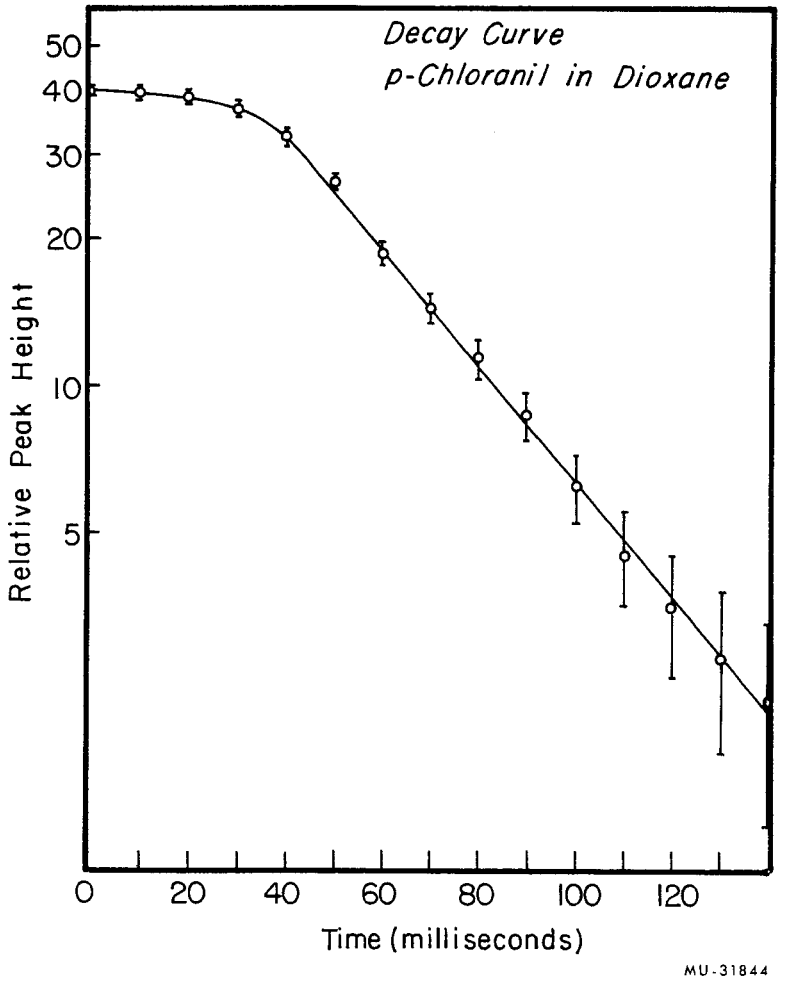

Fig. 12-5. Decay of EPR signal for p-chloranil in dioxane. The light was turned off at t = 0. Plot of log relative peak height vs time.

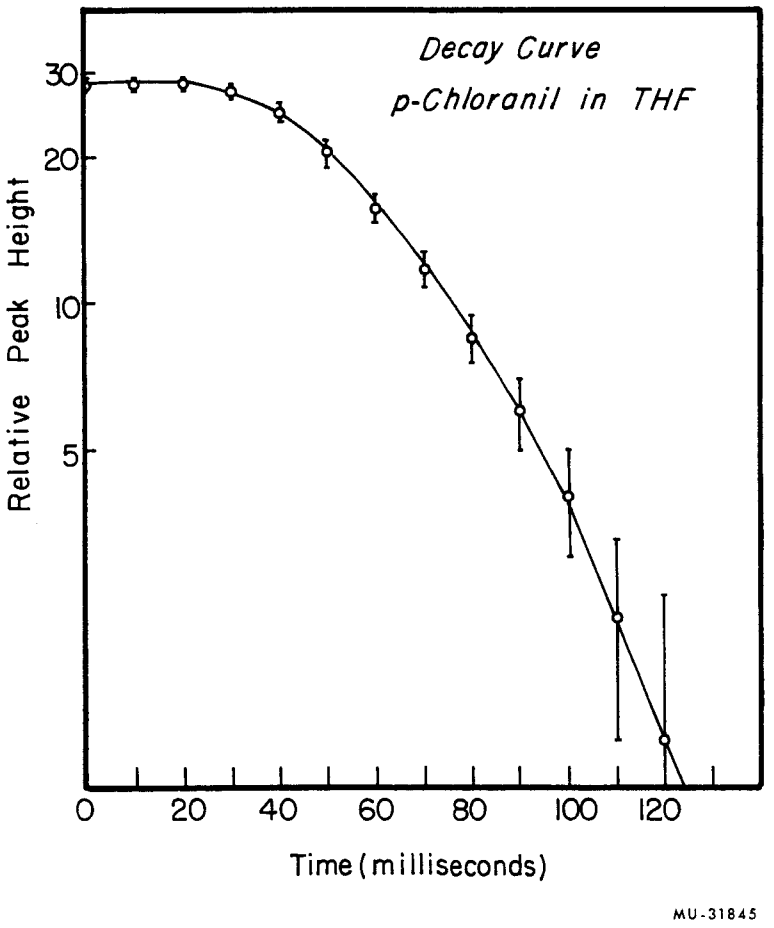

Fig. 12-6. Decay of EPR signal for p-chloranil in THF. The light was turned off at t = 0. Plot of log relative peak height vs time.

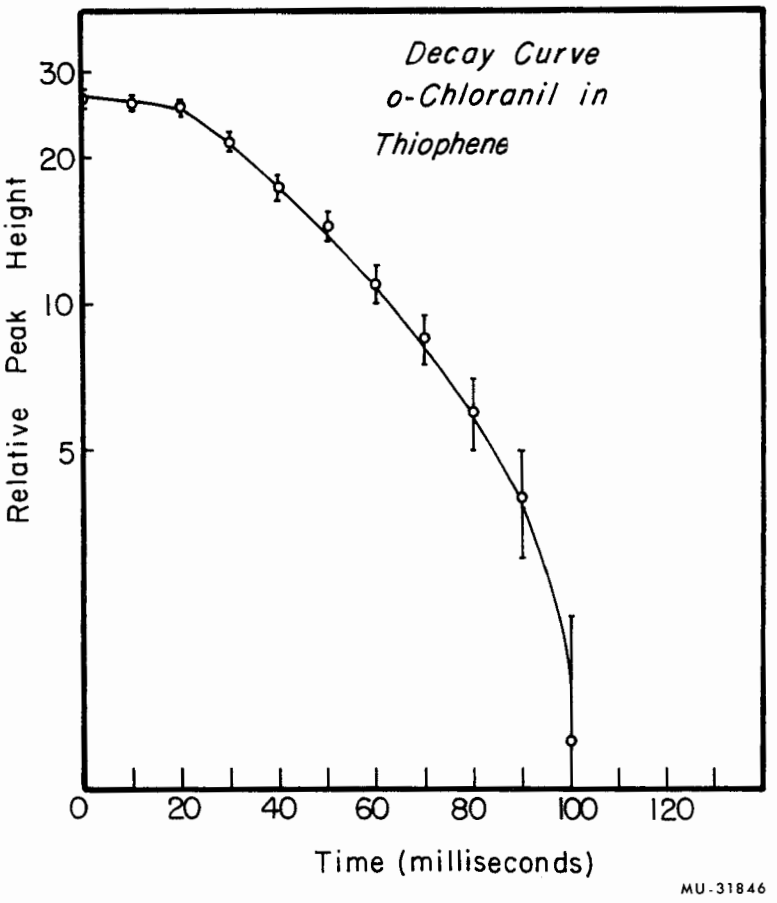

Fig. 12-7. Decay of EPR signal for o-chloranil in thiophene. The light was turned off at t = 0. Plot of log relative peak height vs time.

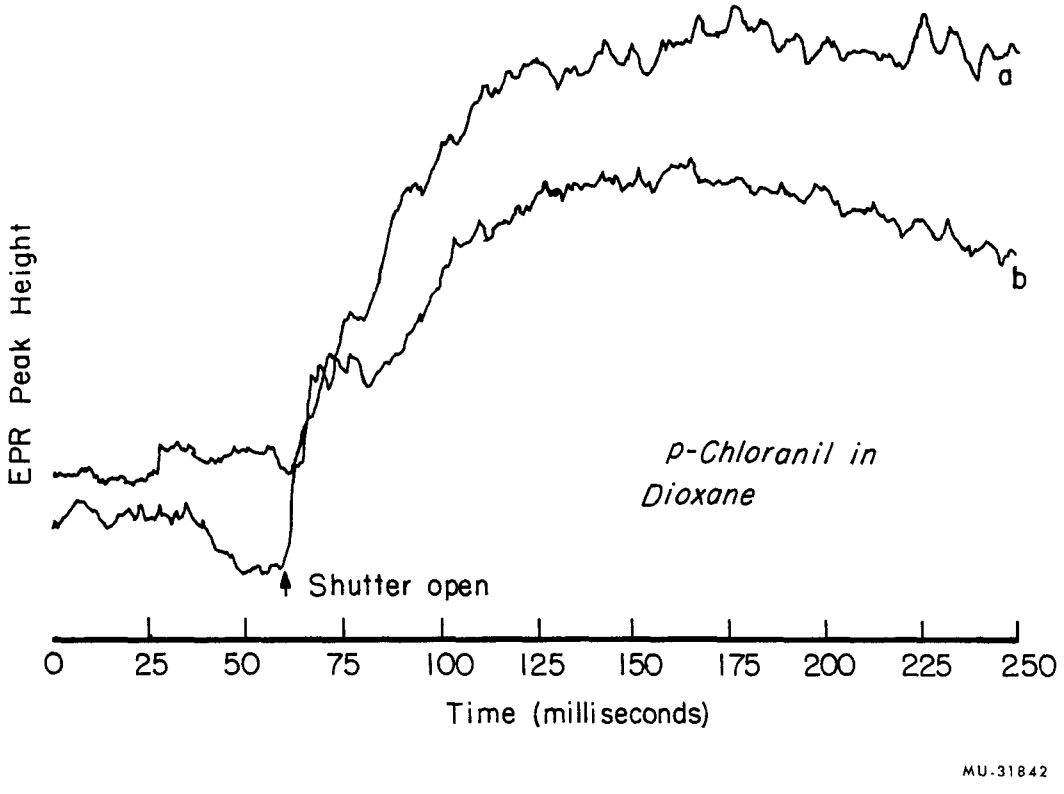

Fig. 12-8. Rise of EPR signal for p-chloranil in dioxane (a); same, except light is attenuated to 12.5% of its intensity in (a) by means of a neutral density filter (b).

A preliminary study of the temperature dependence of the spin concentration shows that at 77° there is no fast signal, but that a long-lived signal slowly builds up as irradiation is continued.

13. TRANSIENT RESPONSE OF LIGHT-INDUCED PHOTOSYNTHETIC ELECTRON PARAMAGNETIC RESONANCE SIGNALS: *RHODOSPIRILLUM RUBRUM* CHROMATOPHORES

R. H. Ruby

Introduction

Use of electron paramagnetic resonance (EPR) in the examination of the physical mechanism of primary quantum conversion in photosynthesis has two requirements;(1) that the signals observed are in fact produced in the primary energy-conversion process, as opposed to chemical reactions at a subsequent time; (2) that the features characterizing the EPR signal may be explained in terms of the variables that describe the physical and chemical system in which the energy conversion takes place.

We here report preliminary results of experiments designed to correlate the observation of EPR in a photosynthetic system with the observation of optical-absorption changes in the same system. This information supplements positive evidence previously obtained in this Laboratory, [1] and provides further information about variables that may be used to characterize the system.

The system chosen for our experiments was *R. rubrum* chromatophores, chosen primarily for the reproducible EPR signals it yields. The samples were prepared as previously described. [2]

Action spectrum.

The observed unpaired electrons appear when light is absorbed by chlorophyll molecules. [1] Therefore, one might expect that the wavelength dependence of a light-induced signal resulting from the primary quantum-conversion process would closely resemble the shape of the optical-absorption spectrum of the chlorophyll. This wavelength dependence of the action spectrum appears to have much broader peaks than the optical-absorption spectrum. We believe that this was caused by the use of saturating light intensities when action spectra were taken.

Preliminary to obtaining an action spectrum, we studied the dependence of the signal size upon light intensity (using white light). We hoped to estimate the energy input at which saturation would occur. These data are presented in Fig. 13-1. The expected saturation phenomena did not occur, but rather the signal appears to be proportional to the logarithm of the relative intensity of the light.

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1. Melvin Calvin and G. M. Androes, Science 138, 867 (1963).

2. G. M. Androes, M. F. Singleton, and M. Calvin, Proc. Natl. Acad. Sci. U. S. 48, 1022 (1963).

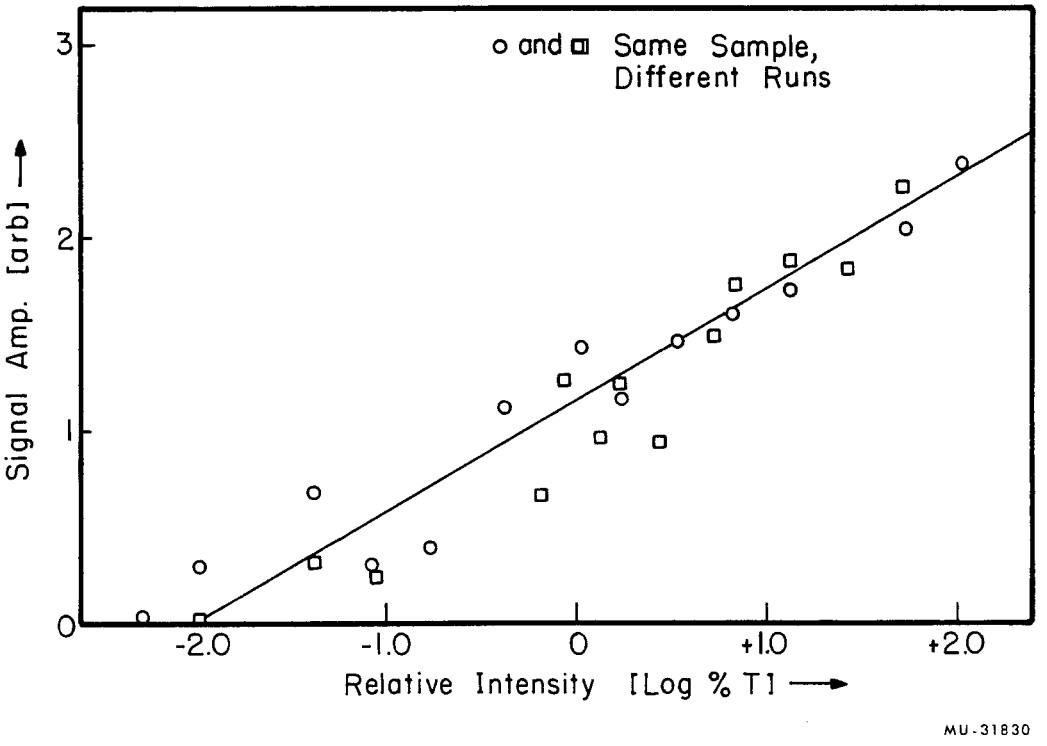

Fig. 13-1. The EPR signal, the maximum in the derivative of the absorption curve, versus the logarithm of the relative intensity of white light irradiating the sample at 300 °K. The light, produced by a 1000-W projection lamp operating at 50 V, was filtered by water and an ir filter, and focused on the sample. Reduced intensities, obtained by inserting neutral density filters, were plotted as the log of % transmission of the filter [log % T]. Oxygen was removed by bubbling argon gas through the sample for 45 min (O. D. = 0.79).

Initially, in taking data, a large scatter occurred. This was reduced by removing O_2 from the chromatophores. In addition, it was observed that the sample would produce a signal in response to light intensities falling on the sample from the room lights, and a lighttight box was constructed to enclose the apparatus. At the low light intensities expected to produce signals with a linear response to the light, the scatter became high owing to a small signal-to-noise ratio (S/N).

An action spectrum was taken, using the lowest light intensity consistent with a workable S/N, shown in Fig. 13-2. The light-induced signal with rapid kinetics, of the order of seconds or better, is plotted. To facilitate comparison, the optical absorption spectrum of R. rubrum chromatophores is presented as the ratio of the light absorbed to the light incident on the sample. Experimental detail is described in the figure caption.

The increased resolution of the peak indicates that light saturation was indeed part of the cause of a broadened peak in the action spectrum. Further effort was postponed until the S/N of the apparatus could be increased.

Transient response of light.

The characteristic times of growth and decay of the EPR signal are being determined in response to a square-shaped pulse of light. These times are being studied in terms of some variables that we may control. These variables are the light intensity (I), the optical density (O. D.), the reduction-oxidation potential (E_h) determined by an external redox couple, and the temperature of the sample (T).

Apparatus

The time resolution required by this experiment means that any resistance-times-capacitance (RC) time constant in the system must be $\leq$ 10 milliseconds. This produces a subsequent loss of S/N of the EPR spectrometer that is normally operated with a time constant $\approx$ 1 sec. A digital integrator ("CAT", Mnemotron Corp.), described by Klein and Barton,[3] was used to regain the required S/N.

A block diagram of the apparatus is shown in Fig. 13-3. The light source is a pulsed, neon-gas-filled lamp as described by Kuntz et al.[4] The light passes through a filter, which removes $\mu \leq 6200$ Å, and is focused on the cavity. Thus, the light falling on the cavity contains wavelengths primarily in the region from 6200 Å to 7000 Å, with an estimated maximum intensity $\approx 10^{-3}$ mW/cm^2. The light may be turned on and off in approximately 1 (μsec). The limiting factor in the time resolution of the apparatus

3. Melvin P. Klein and George W. Barton, Jr., Rev. Sci. Instr. 34, 754 (1962).

4. Irwin D. Kuntz, Jr., Paul A. Loach, and Melvin Calvin, Absorption Changes in Bacterial Chromatophores, submitted to Biophys. J.

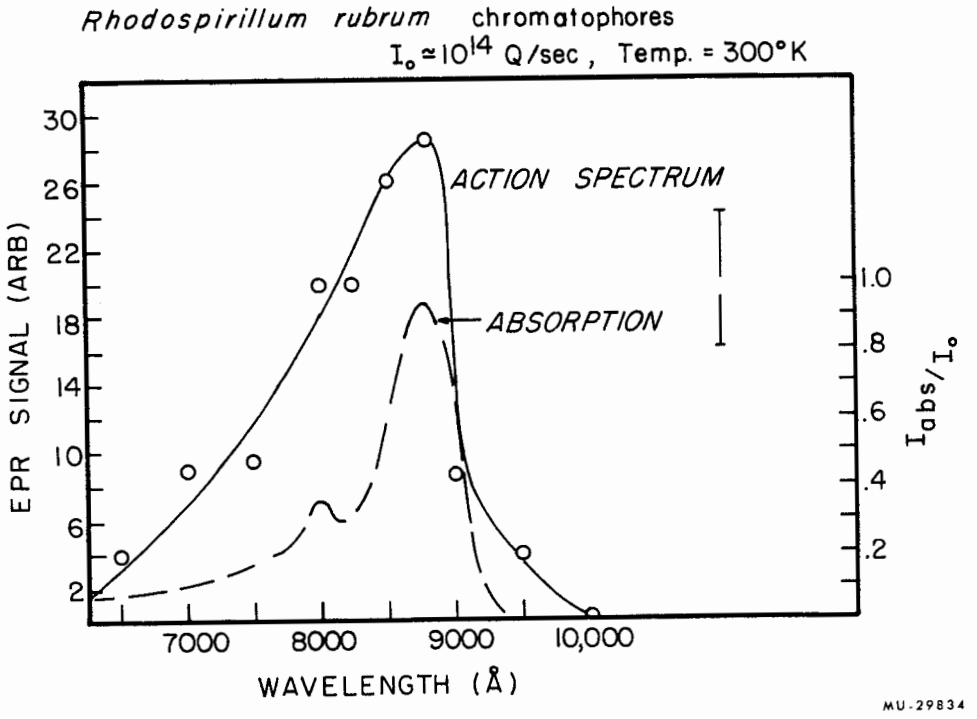

Fig. 13-2. The action spectrum of R. rubrum chromatophores in aqueous suspension. The slit width of the monochrometer corresponds to $\Delta\lambda$ = 52 Å. A cutoff filter was used passing $\lambda > 6200$ Å; T = 300 °K; $I_0 \approx 10^{14}$ Q/sec·cm^2. Oxygen removed by passage of argon. Q ≈ quanta. (O. D. = 0.6)

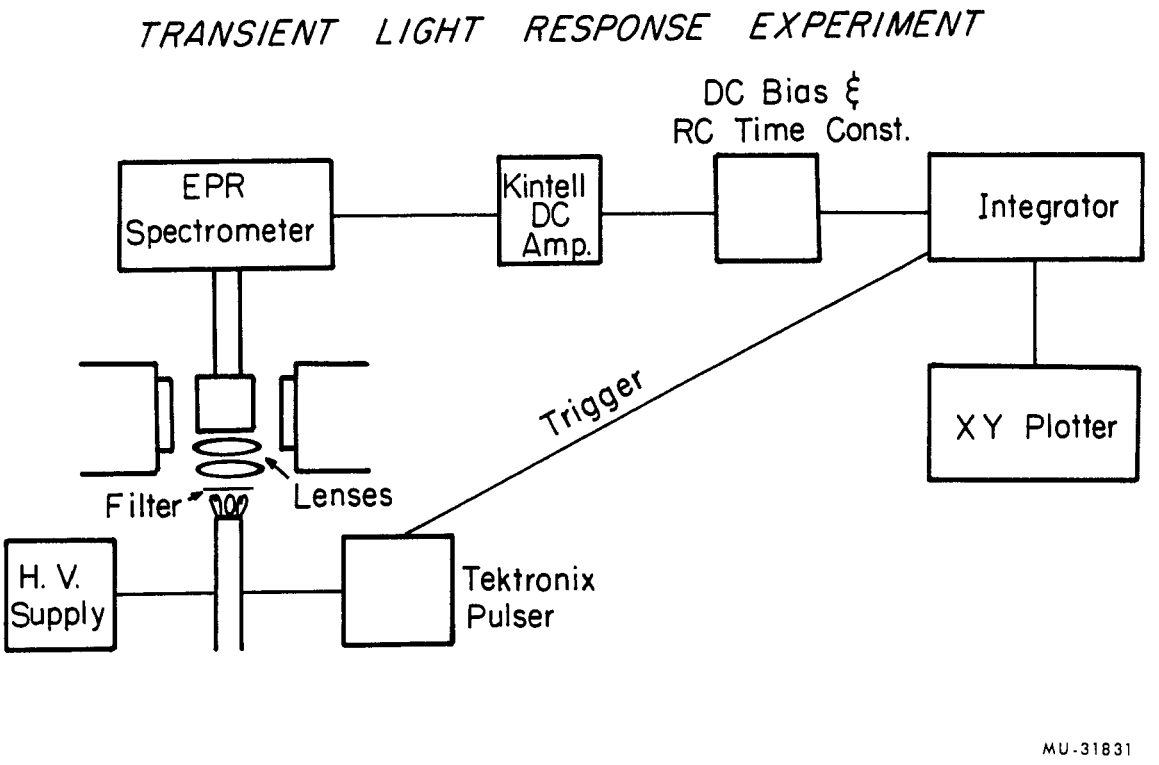

Fig. 13-3. Block diagram of the apparatus used in obtaining measurements of the transient response of the EPR signal to a square-shaped pulse of light.

was an RC time constant of ≈ 10 msec at the input to the integrator. This reduced the noise input to the integrator, thus reducing the experimental time required to obtain a given S/N. The integrated signal was then recorded on an XY plotter.

Response to a light pulse

The signal observed, $S_{(t)}$, is the maximum in the derivative of the EPR absorption curve. The response of this signal to the light when it is turned on and off is shown in Fig. 13-4. Figure 13-5 shows an example of the growth of the signal when the light is turned on (when an expanded time scale is used). Both the growth and the decay may be described in terms of exponential curves. Figures 13-6 and 13-7 show the plots of the signal growth and decay on semilogarithm paper. Thus we see that the growth may be described by an expression

$$S_{(t)} = A[1 - \exp(-t/\tau_r)]$$

characterized by the rise time τ_r. The growth rate is then $k_r = 1/\tau_r$. The decay is similarly of the form

$$S_{(t)} = B[\exp(-t/\tau_d)] + C[\exp(-t/\tau'_d)].$$

There are two decay times, τ_d and τ'_d. These characteristic rise and decay times will be used to describe the signal response. A, B, and C are the amplitudes of each component in the signal, where A = B + C.

Optical density

The rise time was measured at several values of the O. D. No major change in the rise time was observed. The values were as follows:

O. D.	τ_r ● 20%
4.6	420 msec
2.3	325 msec
1.2	360 msec

The redox potential was +0.25 volt, and the bacteria were a 7-day growth.

Intensity

Figure 13-8 shows the variation of the growth rate (k_r), the steady state signal (S), and the initial slope ($dS/dt\,|_{t=0}$) with changes in the intensity of the light. The initial slope and the growth rate appear to depend linearly on the light intensity, although neither of them extrapolates through zero. The steady-state value of the signal changes only by approx 20% when the light intensity is changed roughly 1 order of magnitude. Thus, the intensity of the light may still be partially saturating. Any attempt at interpretation should await the taking of more experimental points, but these plots show the qualitative behavior.

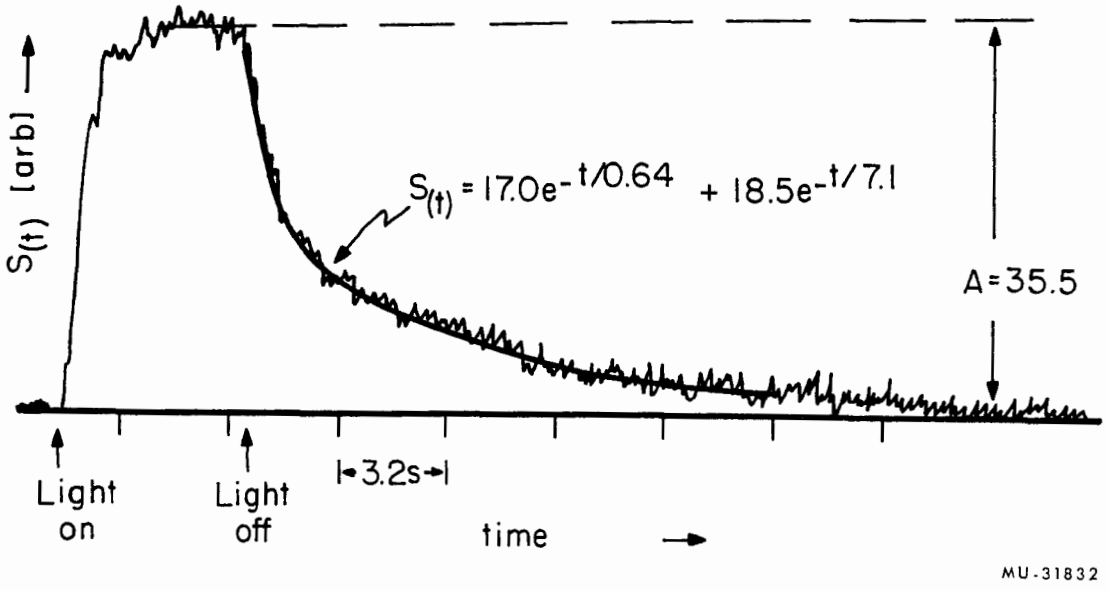

Fig. 13-4. The transient response of the EPR signal to a square pulse of light intensity. O. D. = 3.9; E_h = +0.40 volt. Growth of bacteria = 6 days. The quantity A is the steady-state value of the signal. Time is shown in seconds. Also shown is the exponential fit to the decay curve obtained in Fig. 13-7.

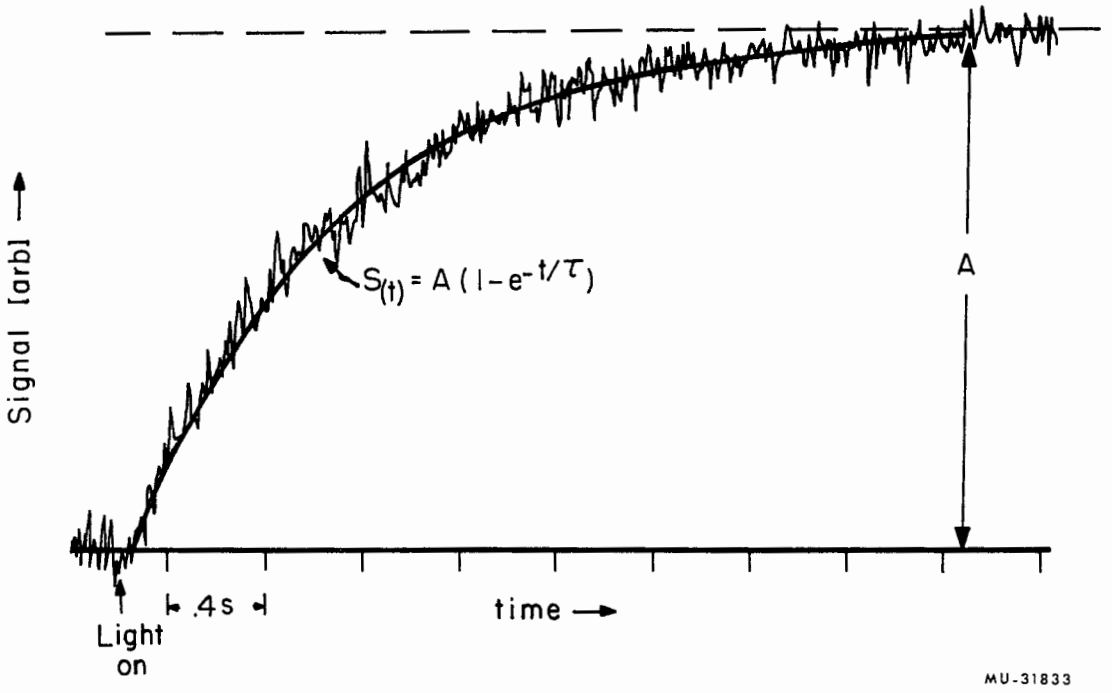

Fig. 13-5. The growth of the EPR signal $S_{(t)}$ when the light is turned on. O. D. = 3. Bacteria growth = 7 days. The solid curve is a fit of the expression $S = A[1 - \exp(-t/0.90)]$.

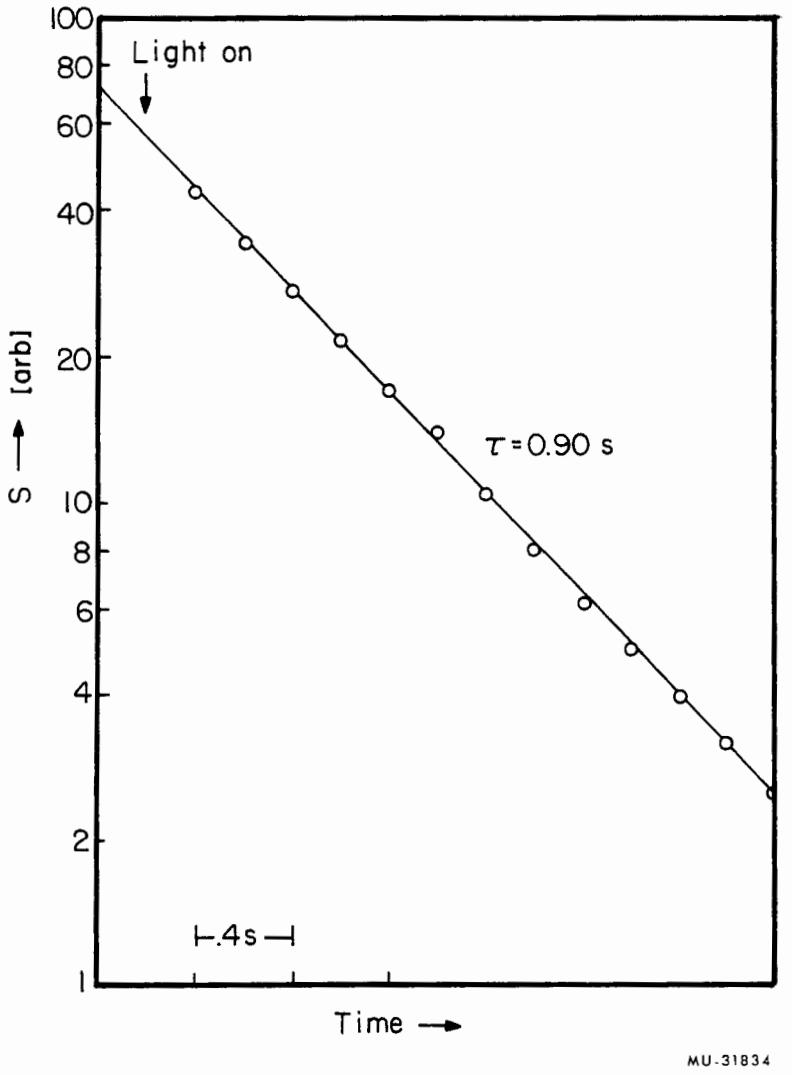

Fig. 13-6. A semilog plot of the growth of the EPR signal when the light is turned on, as shown in Fig. 13-5.

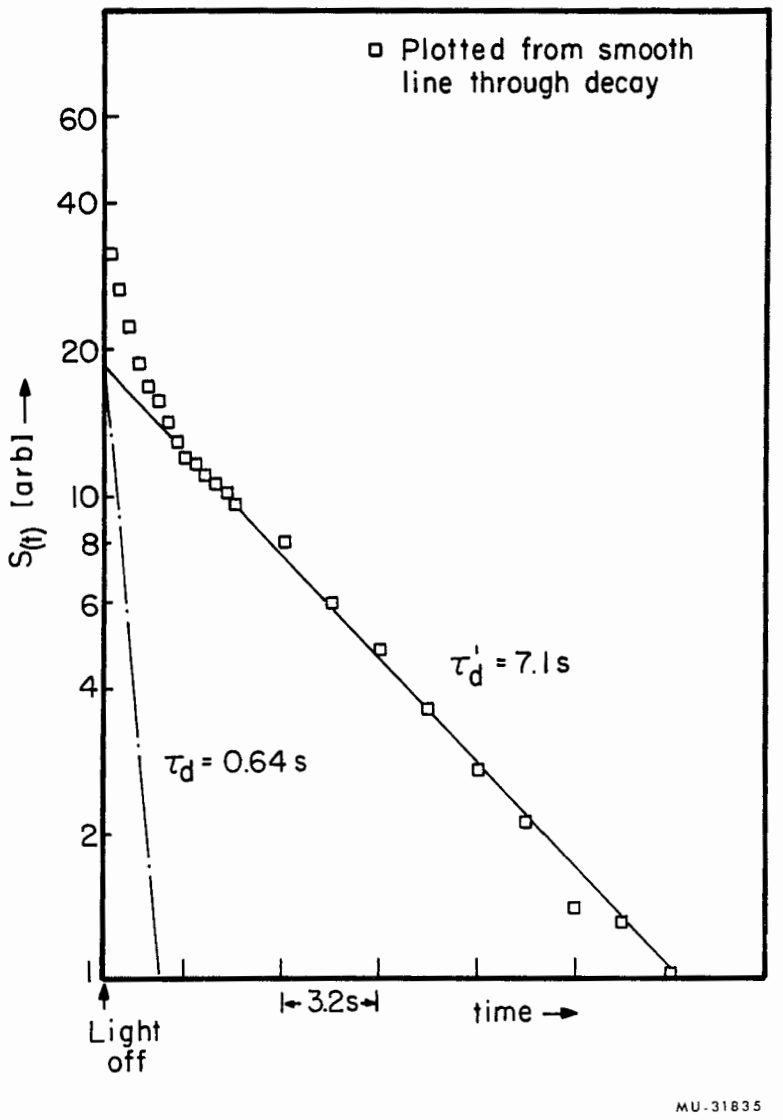

Fig. 13-7. A semilog plot of the decay of the EPR signal after the light is turned off, as shown in Fig. 13-4. The slower decay is extrapolated back to the origin and the difference between the data points and this line is plotted as the lower set of points. The straight line through these points then determines the time constant of the faster component of the decay curve.

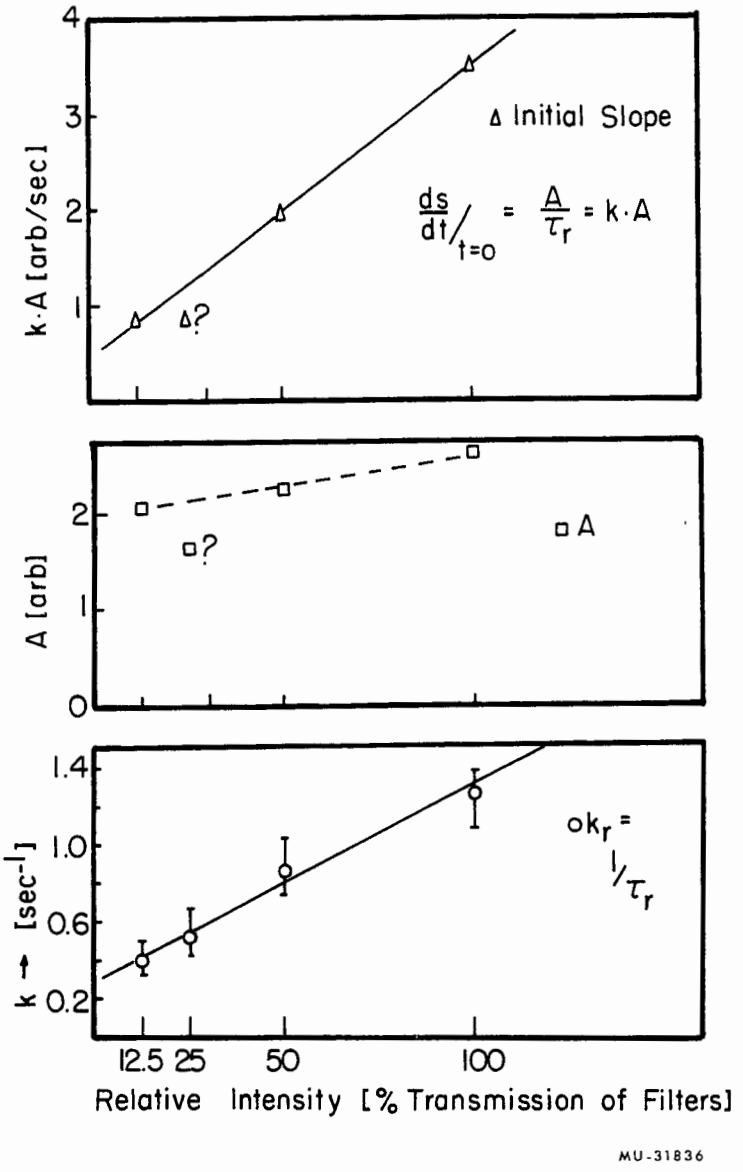

Fig. 13-8. Graphs of the growth rate k_r, the steady-state value A, and the initial slope $dS/dt\big/_{t=0} = kA$ of the EPR signal as a function of the relative intensity of the light pulse. The intensity was varied by insertion of neutral density filters, and is plotted in terms of % transmission of the filters.

Redox potential

The effect of varying the redox potential by a ferri-ferrocyanide couple is shown in Fig. 13-9. All three rates appear to be changing near the midpoint of the titration, which occurs at E_h = +0.45 volt. [1]

Temperature

A comparison was made of the transient response to light of a dried film of chromatophores at 300 °K and at 77 °K. When the sample was cooled to 77 °K (using liquid nitrogen) the steady-state value of the signal when irradiated with the neon lamp decreased to a value that made the taking of transient data difficult. A white-light source was substituted, and the pulse was shaped by a mechanical camera shutter with an opening time of approx 10 msec. The signal response had the following characteristics:

T	τ_r	τ_d	τ'_d
300 °K	100 msec	(100 msec)	10 sec
77 °K	50 msec	100 msec	(1 sec)

At each temperature there appeared to be two decays. At 300 °K the primary component, in magnitude, was the longer decay; while at 77 °K the primary component was the shorter decay. In each case the lesser component is uncertain.

Apparatus revision

The preliminary experiments described indicated a need for an increased S/N.

The spectrometer has been completely overhauled and slightly modified (Fig. 13-10). A new automatic frequency control (AFC) was installed, which operates at 60 kc modulation frequency. A preamplifier was constructed with a narrow bandpass centered at 100 kc to reduce interaction between the paramagnetic resonance detection and the AFC circuits.

It was found that the best S/N was obtained when the preamplifier is operated at full gain, and when the gain of the Varian 100-kc box is reduced to keep the signal level on scale. The cavity is uncoupled to produce a microwave-detector crystal current reading midscale on its meter. In this condition, the crystal detector is being operated with a linear voltage response to the microwave power. Also, the S/N is maximum under this condition when the klystron is being regulated on the sample cavity.

The result has been an increase in the S/N. The apparent sensitivity is now $< 10^{13}$ spins of Cr^{3+} in MgO with 1 G modulation.

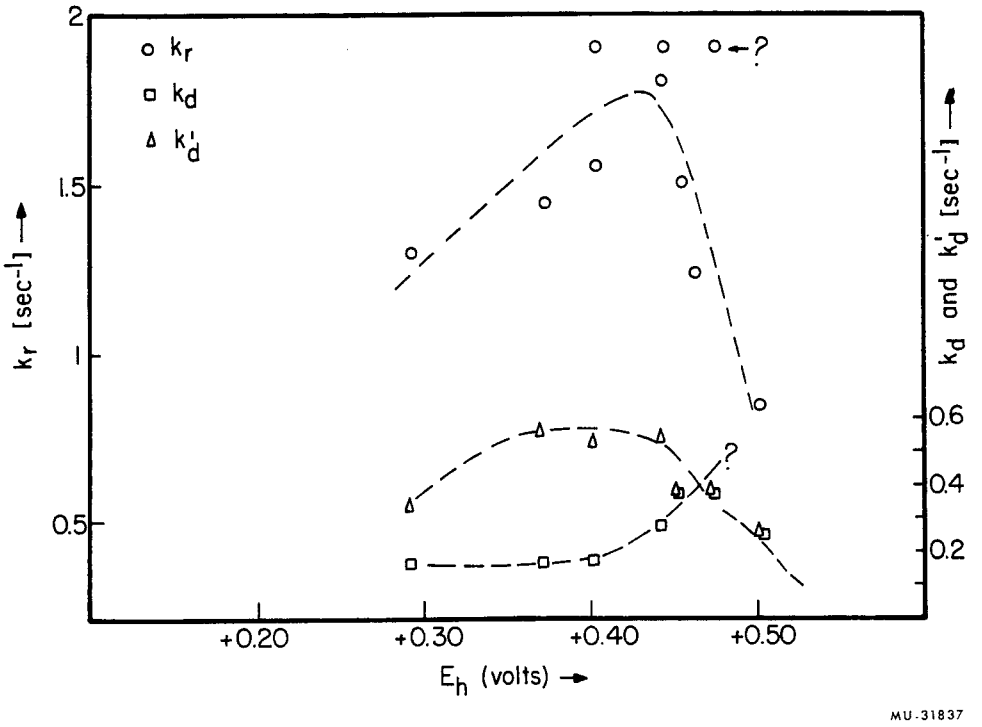

Fig. 13-9. Growth and decay rates versus redox potential.

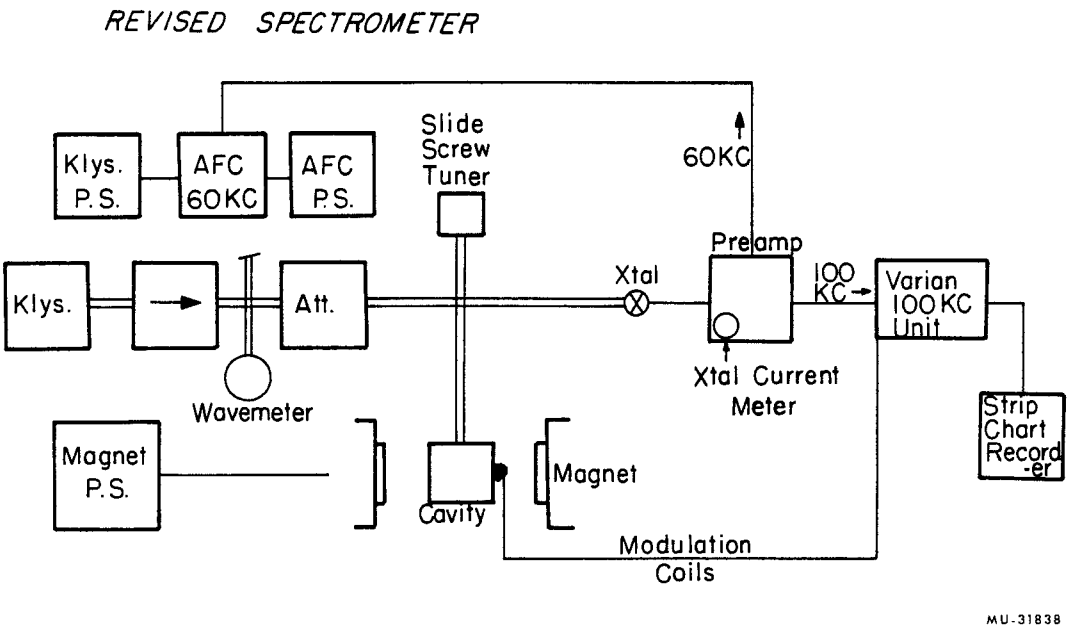

Fig. 13-10. Block diagram of the modified EPR spectrometer.

Discussion

We have observed a rapid reversible light-induced signal in a dried film of R. rubrum at 77 °K, and have measured rise and decay times of the order of 100 msec. This observation is similar to the earlier one recorded by Sogo et al. [5]

The dependence upon redox, and the independence from O. D., of the rise times indicate that the rise time is not related to the number of photosynthetic units in the sample, but is related to the density, inside the unit, of chlorophyll molecules that may be oxidized in the primary quantum-conversion process.

In conversation with I. Kuntz, who is performing parallel experiments in the optical absorption of R. rubrum, the following correlations between the EPR and optical-absorption measurements were made.

The over-all qualitative behavior of the EPR signal is consistent with that observed in optical-absorption changes, with respect to changes in the temperature, redox potential, and intensity. A comparison of the crudely quantitative decay rates of the EPR signal at room temperature and at a redox potential of +0.4 volt indicates that the kinetics of the EPR signal are most closely allied to the optical-density changes at 4330 Å. Our tentative identification of the EPR signal on the basis of these transient response studies indicates that the continuation of such studies will be productive, but that a positive identification will require work with the use of more experimental control.

A series of parallel experiments with EPR and optical absorption of chromatophores of R. rubrum, in which the sample geometry and other operating conditions are being made identical, has been initiated.

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5. P. Sogo, M. Jost, and M. Calvin, Radiation Res. Suppl. 1, 511 (1959).

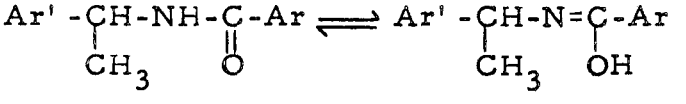
14. STUDIES ON THE TAUTOMERISM OF AMIDES

Lech Skulski and G. C. Palmer

Introduction

Despite the large amount of research concerned with the structure of amides, some doubt remains about their structure. Several contradictory opinions appear in the literature.[1-7]

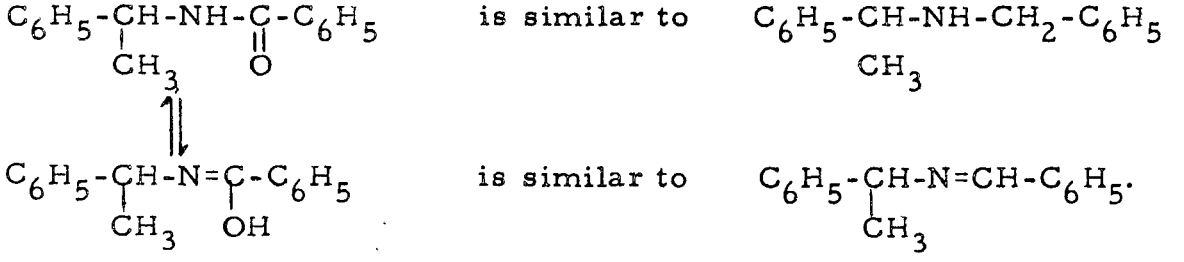
The solvent effect on the direction of the optical rotation of benzoyl derivatives of α-methylbenzyl-and α-p-tolylethyl-amines has been interpreted in terms of an amide-iminol equilibrium:



(A) (B)

The position of the equilibrium is solvent-dependent.[1]

The authors have shown that the optical rotary dispersion (ORD) curve of N-(α-methylbenzyl)-benzamide in benzene is similar to that of N-benzyl-α-methylbenzylamine (in both benzene and methanol), whereas in methanol it is similar to the ORD curve of N-benzal-α-methylbenzylamine.[1] The aurhors give the comparison:



1. V. M. Potapov, A. P. Terent'ev, et al., Zh. Obshch. Khim. 32, 1187 (1962), and previous papers from this series.

2. A. Hantzsch, Chem. Ber. 64, 661 (1931).

3. Z. Piasek and T. Urbanski, Tetrahedron Letters, 723 (1962); Z. Piasek and T. Urbanski, Bull. Acad. Polon. Sci., Ser. Sci. Chim. 10, 113 (1962).

4. C. A. Grob and B. Fischer, Helv. Chim. Acta 38, 1794 (1955).

5. F. Nerdel and H. Liebig, Ann. Chem. 621, 42 (1959); F. Nerdel, et al., Ann. Chem. 627, 106 (1959).

6. P. G. Puranik and K. V. Ramiah, Proc. Indian Acad. Sci., Ser. A, 54, 69 (1961).

7. R. A. Nyquist, Spectrochim. Acta 19, 509 (1963).

They also show that there is no change in the sign of rotation for the benzoyl derivatives of N-benzal-α-methylbenzylamine from benzene to methanolic solutions.

The discovery of the existence of two crystal forms of the optically active N-benzoyl- and N-(3, 5-dinitro-benzoyl)-α-methylbenzylamines was adduced as supporting evidence for the comparison.

Hantzsch reasoned that the iminol form was the predominant one, basing his opinion on the uv spectra of amides of trichloroacetic acid and aromatic acids.[2] He also gave the boiling- and melting-point relationships of some amides and imino ethers as supporting evidence.

Piasek and Urbanski[3] have made studies of the infrared spectra of urea in the solid state and in aqueous and alcoholic solutions. They conclude that as much as a few percent of the imidol form is present in these solutions, and that urea freshly crystallized from these solutions also contains some of the imidol form in the solid state.

Grob and Fischer compared the spectra of the amides, N,N-dimethyl amides, and methyl imino ethers of four α,β-acetylenic acids, and concluded that no significant amount of iminol is present in solutions of the primary amides.[4]

Nerdel says that the benzamides of α-methylbenzylamine are associated in solution via >N-H· · · O=C< bridges and this association affects the optical rotation.[5]

Puranik and Ramiah have also studied the association of primary and secondary amides in various solvents using ir and Raman spectra.[6] Nyquist reports that ir spectra show that secondary amides are in trans configuration in dilute solution.[7]

Results and Discussion

To check the correctness of the assumption that N-(α-methylbenzyl)-benzamide exists predominantly in the iminol form in methanolic solutions, a series of acyl (mostly acetyl and benzoyl) derivatives of optically active and racemic α-methylbenzylamines, 1, 1-diphenylmethylamine, benzylamine, and N-methylbenzylamine were synthesized. N-deuterated analogs of these compounds were also prepared.

Spectropolarimetric measurements of the ORD of N-benzoyl-, N-acetyl-, N-formyl-, and N-trifluoroacetyl-α-methylbenzylamines were made in benzene and methanol solutions at 25° (see Figs. 14-1 through 14-4). [The ORD curve of N,N-dibenzyl-α-methylbenzylamine in methanol (Fig. 14-5) is included for comparison.]

The experimental observations reporting that the rotation of optically active N-(α-methylbenzyl)-benzamides is of opposite sign in methanol from what it is in benzene and other nonhydroxylic solvents, have been confirmed,[1] as well as that of the existence of two different solid forms of these compounds.[8]

8. We could not, however, obtain two forms of the racemic compound.

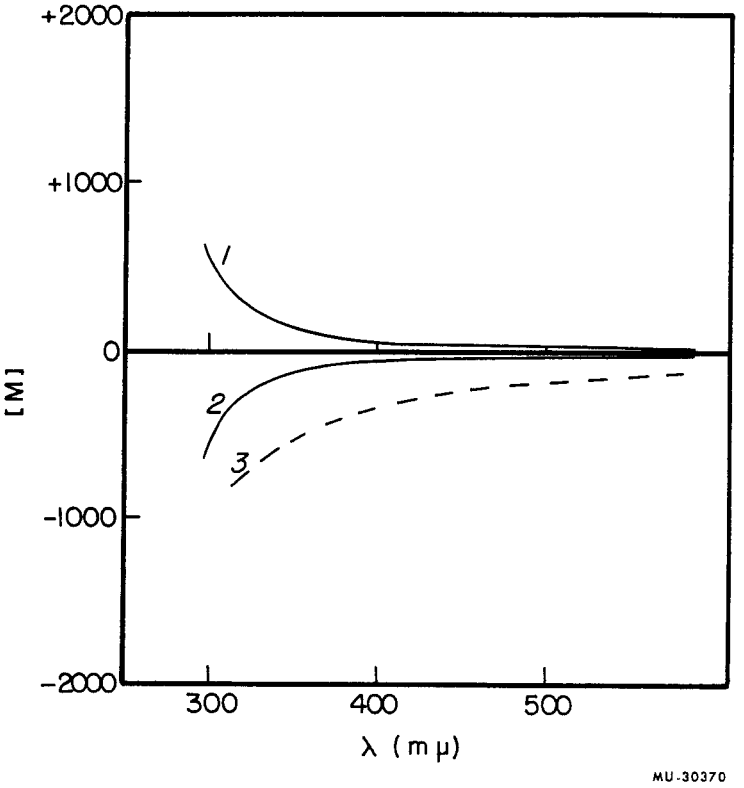


Fig. 14-1. ORD curves of N-(α-methylbenzyl)-benzamides at 25°: 1. l-form in methanol; 2. d-form in methanol; 3. l-form in benzene.

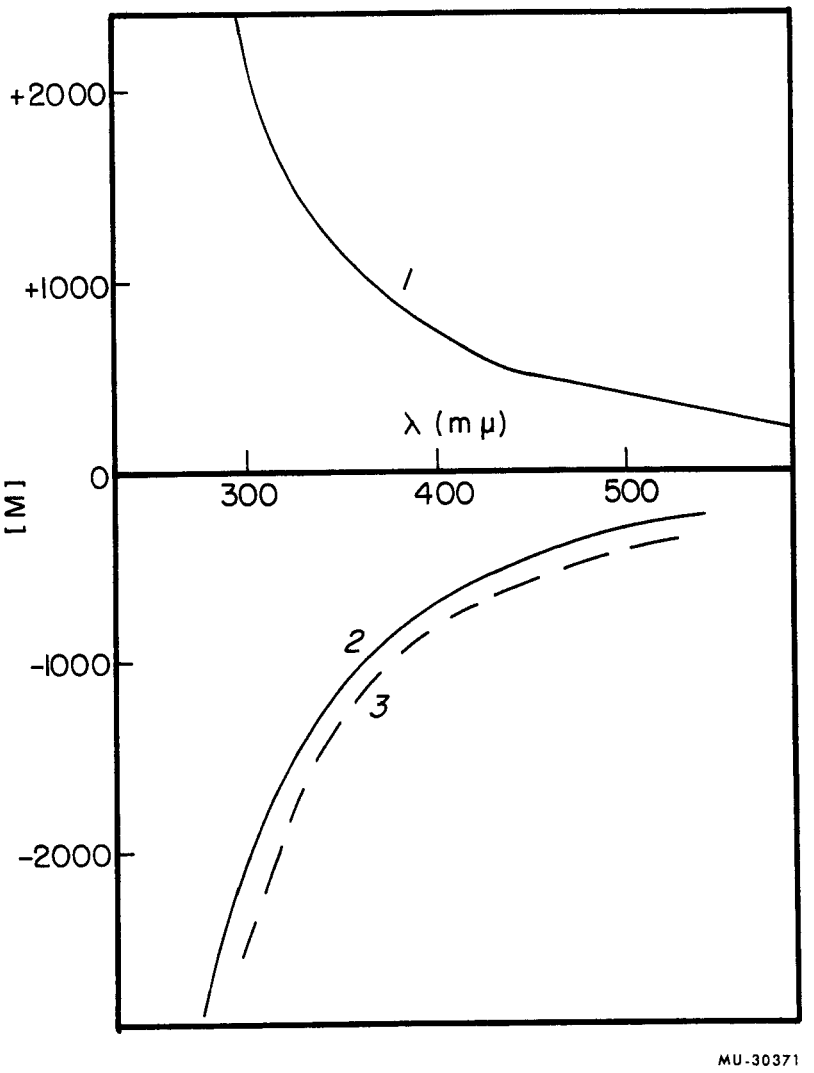


Fig. 14-2. ORD curves of N-(α-methylbenzyl)-acetamides at 25°: 1. d-form in methanol; 2. l-form in methanol; 3. l-form in benzene.

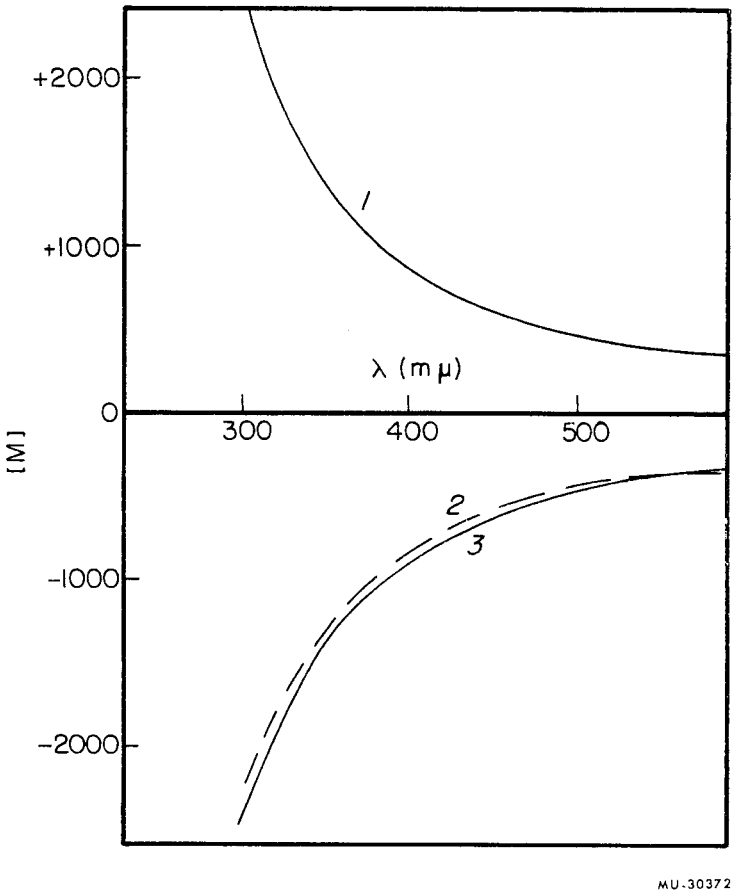


Fig. 14-3. ORD curves of N-(α-methylbenzyl)-formamides at 25°: 1. d-form in methanol; 2. l-form in benzene; 3. l-form in methanol.

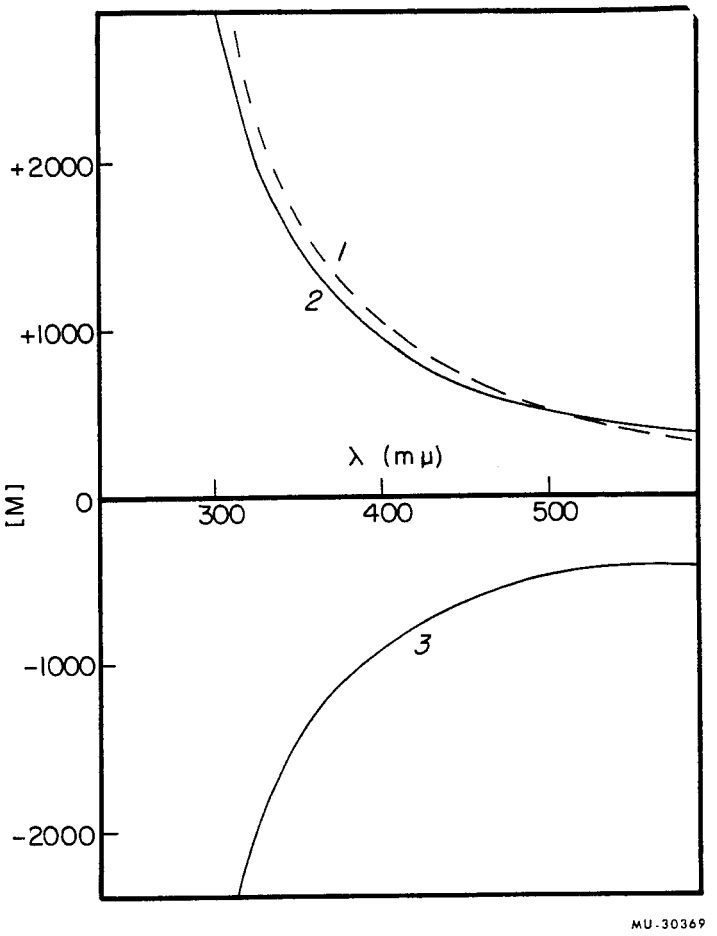


Fig. 14-4. ORD curves of N-(α-methylbenzyl)-trifluoroacetamides at 25°: 1. d-form in benzene; 2. d-form in methanol; 3. l-form in methanol.

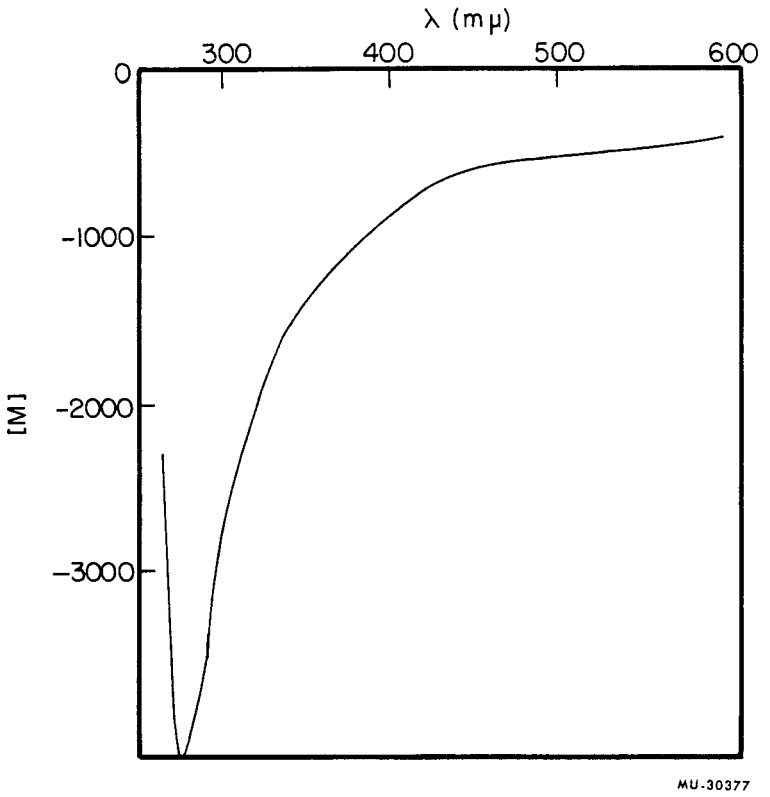
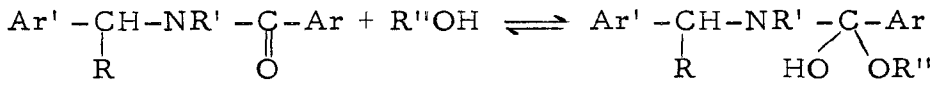


Fig. 14-5. ORD curve of N, N-dibenzyl-1-α-methylbenzylamine in methanol.

No difference in the sign of rotation between methanolic and benzene solutions has been found for the other optically active acyl derivatives. See Figs. 14-2 through 14-4 and Refs. 5 and 9, where $[\alpha]_D$ or [M] values in different solvents are given.

Proton NMR studies carried out on the solutions of the optically active compounds and related amides in CCl_4, $CDCl_3$, CS_2, anhydrous dioxane, CH_3OH, and CH_3OD (predominantly at room temperature) confirm the assumption that all the amides studied exist predominantly in the amide form (A) in methanol as well as in the other solvents used. This viewpoint is supported by the fact that the same splitting of the CH_2 and CH hydrogen resonances (due to spin-spin coupling) by the hydrogen of the adjacent NH group is seen in all these solvents, including methanol (Figs. 14-6 and 14-7). This splitting disappears when the hydrogen of the NH group is replaced by deuterium or a methyl group. See also Refs. 10 and 11, where the proton NMR spectra of several secondary and tertiary amides have been discussed. The iminol form (B) thus cannot be present in significant amount if it exists at all in solutions. Preliminary NMR studies at different temperatures reveal that in some of these molecules the apparent hindrance to free rotation is observed.[10]

The ultraviolet (uv) spectra of these compounds in various solvents give no indication of an equilibrium of the type C $\rightleftharpoons$ D,



(C) (D)

(where R = H, alkyl, or aryl;
R' = H or alkyl;
R'' = H or alkyl),

with appreciable amounts of the hemiacetal or hydrate form (D) existing in alcoholic or aqueous[12] solutions (see Figs. 14-8 through 14-14). The formation of hemiacetal or hydrate of the type (D) in alcoholic or aqueous solutions would be expected to shift the fairly intense band at 220 to 230 mμ to shorter wavelengths and lower intensities.

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9. R. Huisgen and C. Ruchardt, Ann. Chem. 601, 21 (1956).

10. C. Franconi, Z. Elektrochem. 65, 645 (1961).

11. V. J. Kowalewski and D. G. Kowalewski, J. Chem. Phys. 32, 1272 (1960).

12. L. Skulski, Bull. Acad. Polon. Sci., Ser. Sci. Chim. 10, 201 (1962), where only the locations of the K bands are given.

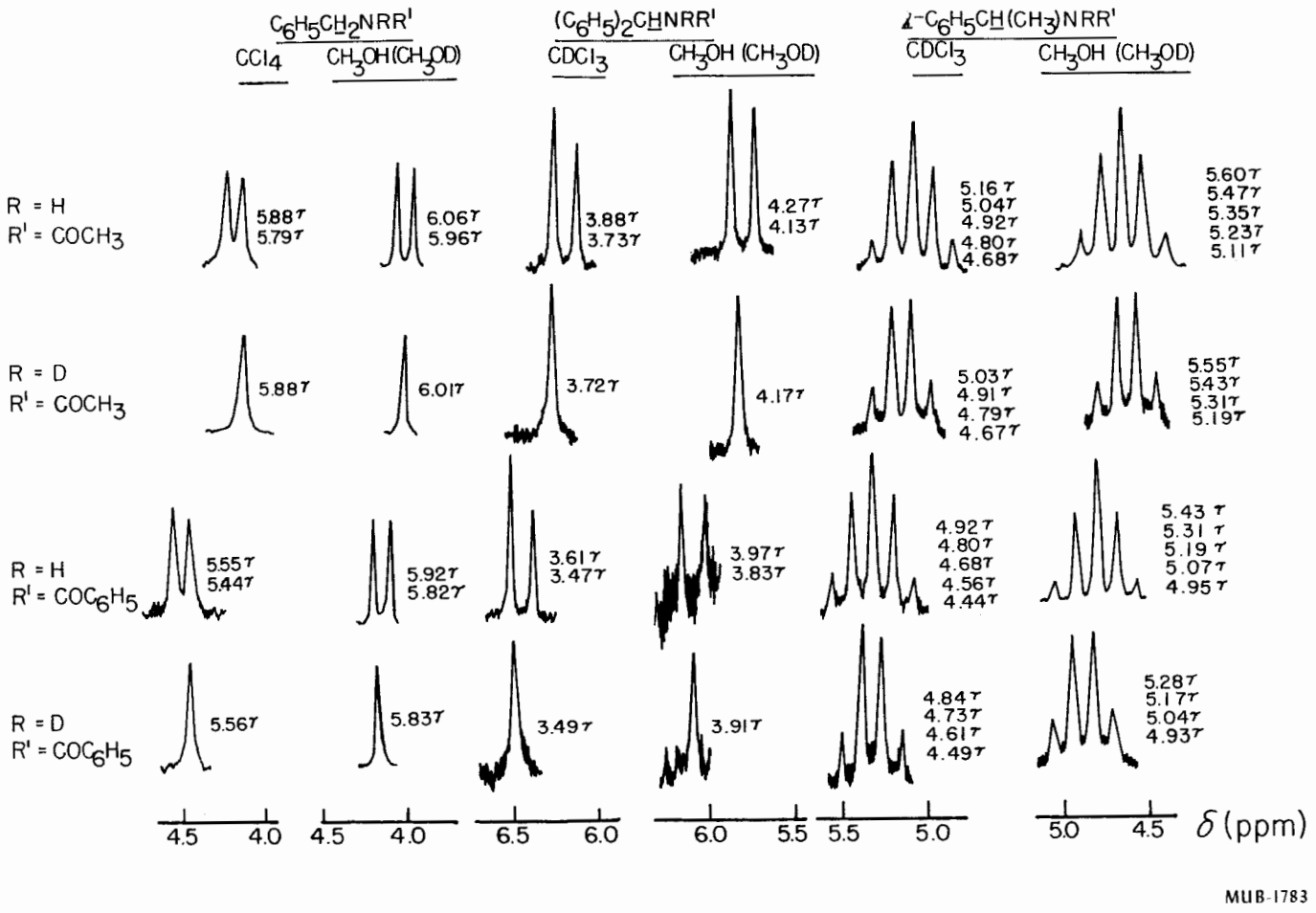


Fig. 14-6. Proton NMR spectra of the benzylic hydrogen atoms of substituted amides. All NMR spectra were taken with a Varian A-60 spectrometer. The abscissa is given in δ units.

$$\delta_{ppm} = \frac{\Delta(Me_4Si) \times 10^6}{\text{oscillator freq. (cps)}}$$

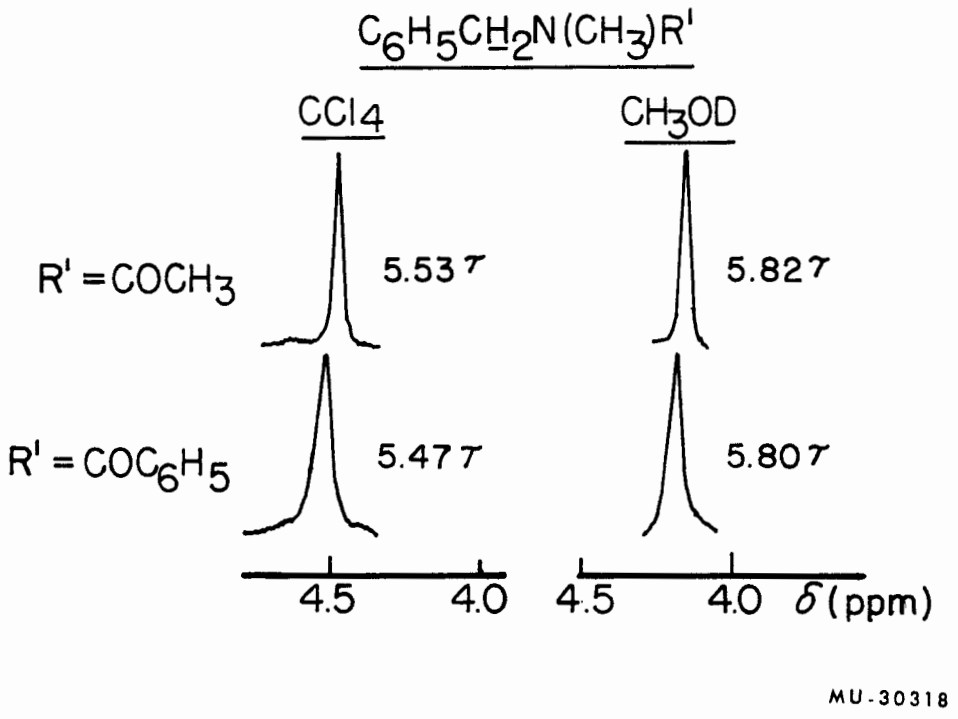


Fig. 14-7. Proton NMR spectra of the benzylic hydrogen atoms of the acetyl and benzoyl derivatives of N-methylbenzylamine. All NMR spectra were taken with a Varian A-60 spectrometer. The abscissa is given in δ units. Spectra in CH_3OD run at approx 60 °C, due to broadening of the peaks at room temperature.

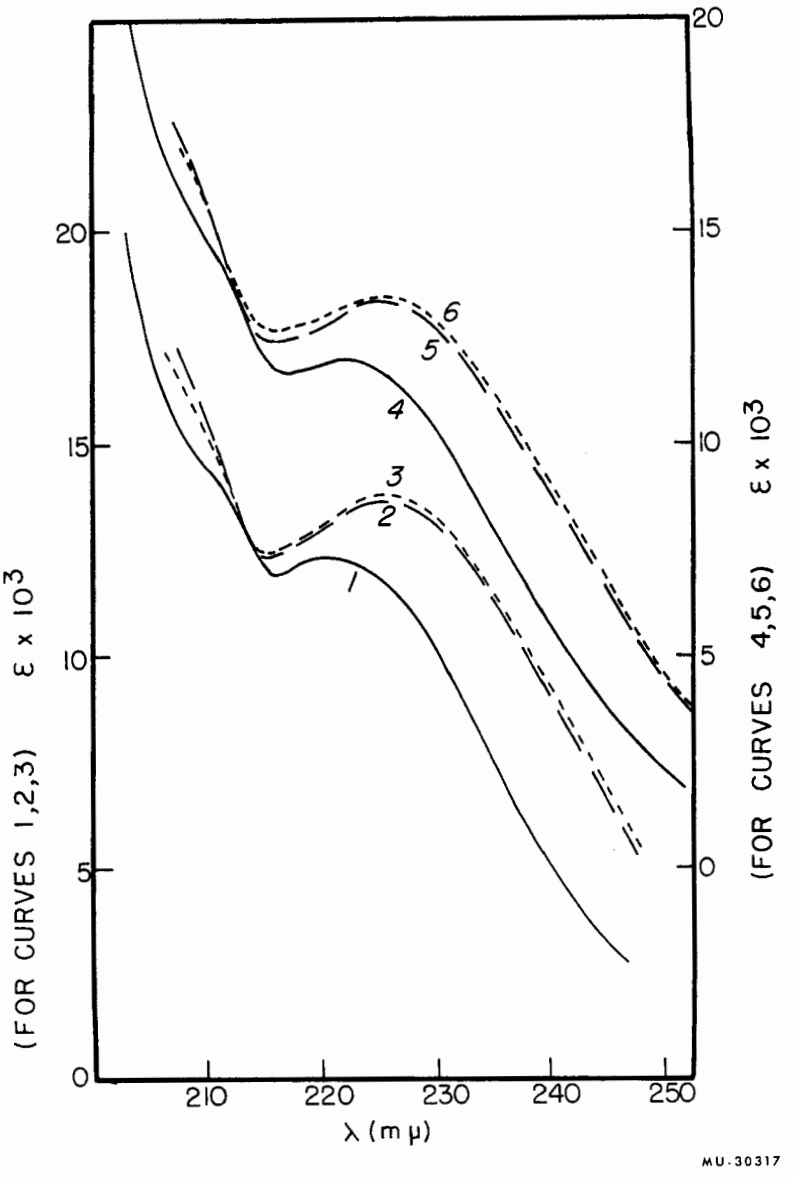


Fig. 14-8. Ultraviolet absorption spectra of N-benzyl-benzamide (curves 1-3) and N-(1-α-methylbenzyl)-benzamide (curves 4-6) in three different solvents(c = 0.2 mM); 1, 4 in cyclohexane; 2, 5 in mixtures of 80% ethanol and 20% cyclohexane; 3, 6 in mixtures of 80% methanol and 20% cyclohexane. ε is the molar extinction coefficient.

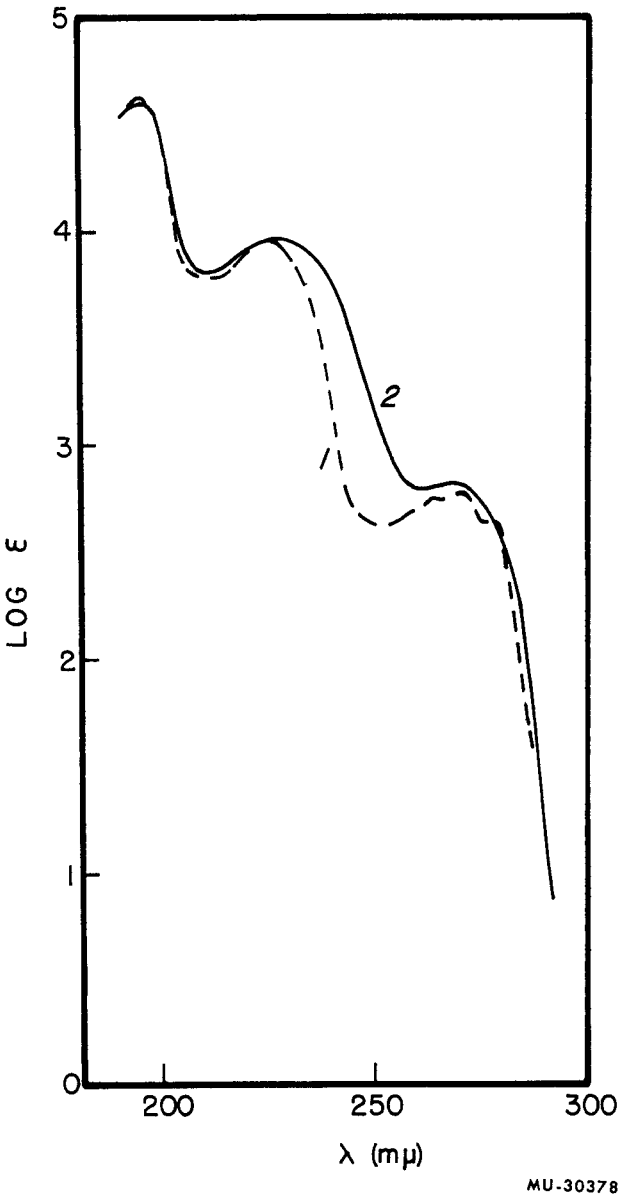


Fig. 14-9. Ultraviolet absorption spectra of benzamide (reference 12): 1, in cyclohexane (c = 0.090 mM); 2, in water (c = 0.479 mM).

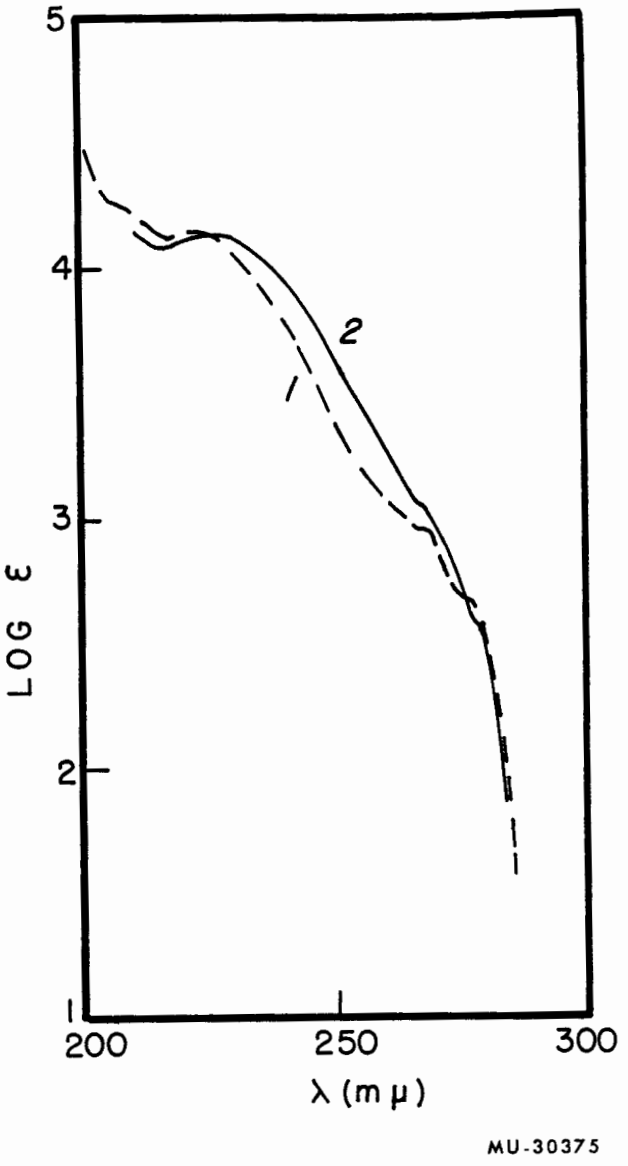


Fig. 14-10. Ultraviolet absorption spectra of N-benzylbenzamide (c = 0.2 mM): 1, in cyclohexane; 2, in methanol.

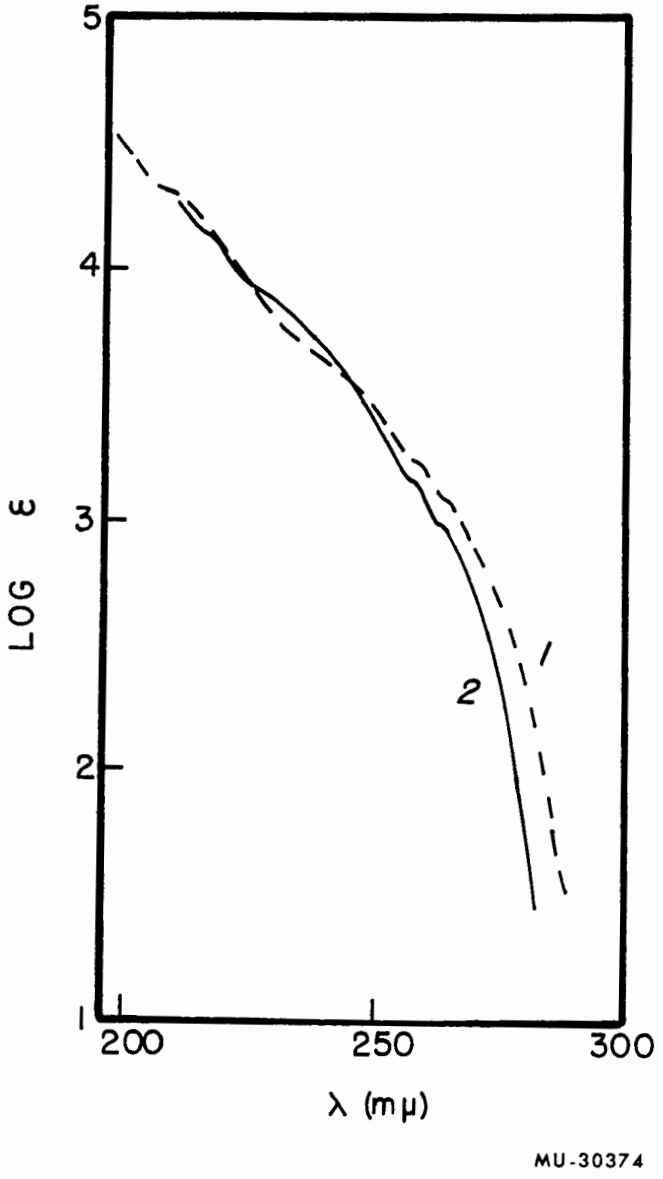


Fig. 14-11. Ultraviolet absorption spectra of N-methyl-N-benzyl-benzamide (c = 0.2 mM): 1, in cyclohexane; 2, in methanol.

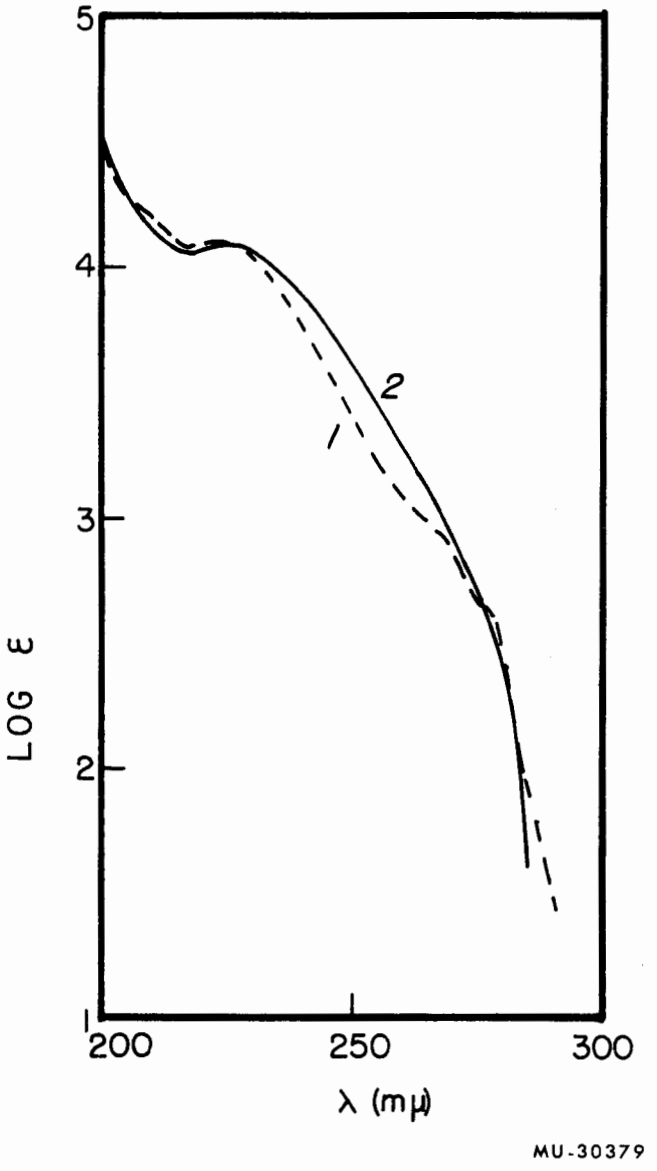


Fig. 14-12. Ultraviolet absorption spectra of N-(1-α-methylbenzyl)-benzamide (c = 0.2 mM): 1, in cyclohexane; 2, in methanol.

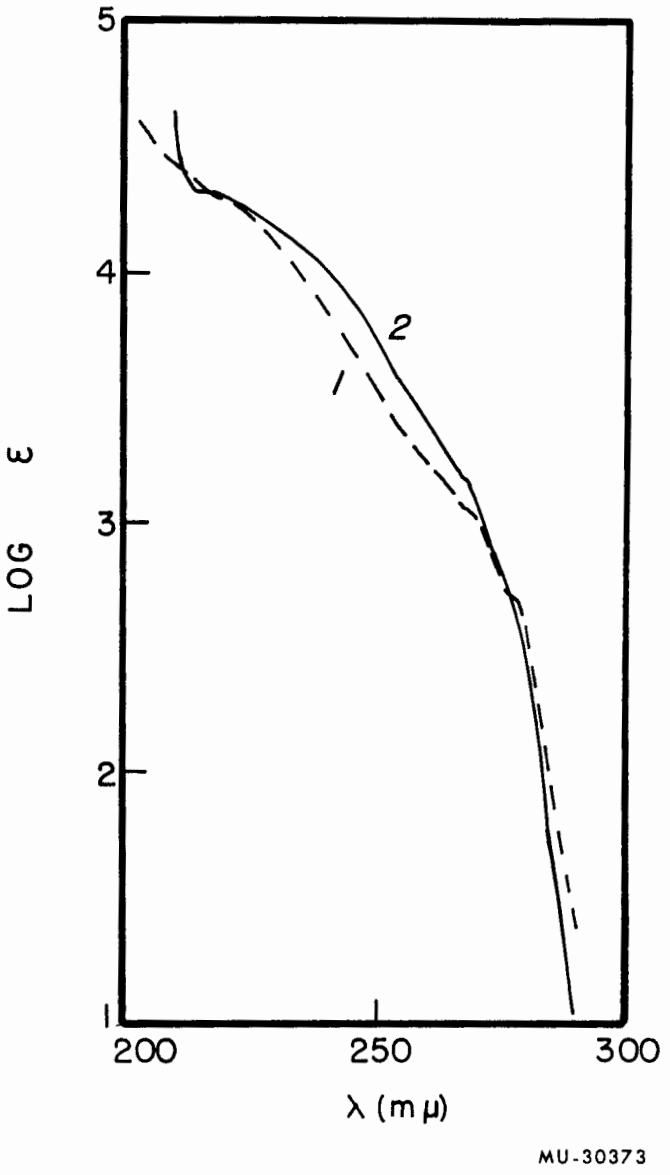


Fig. 14-13. Ultraviolet absorption spectra of N-diphenylmethylbenzamide (c = 0.2 mM): 1, in cyclohexane; 2, in methanol.

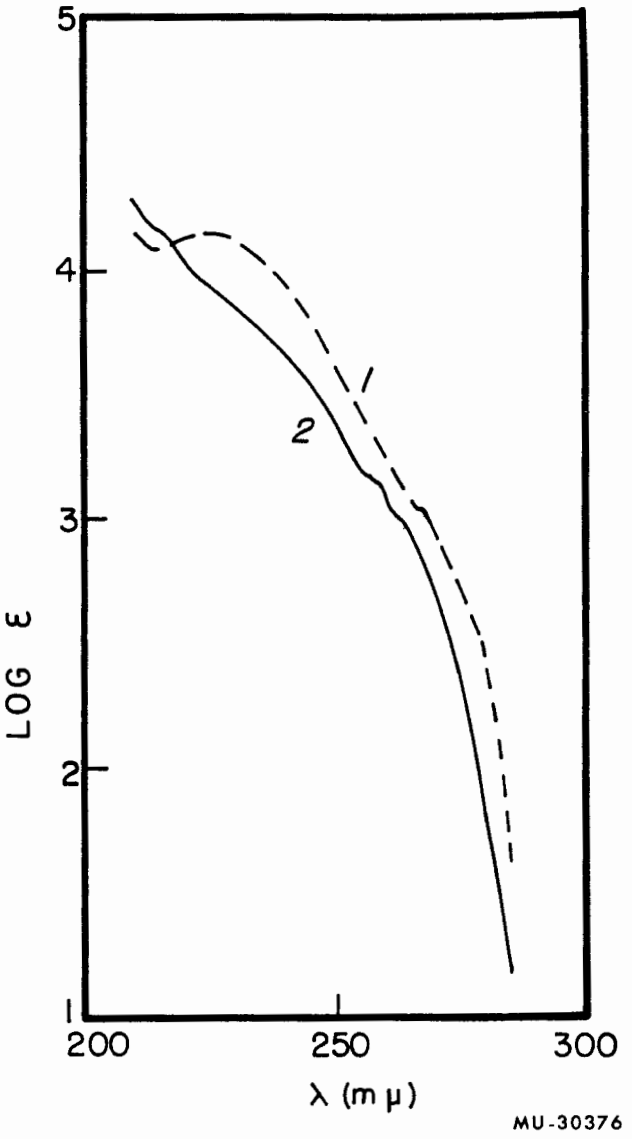
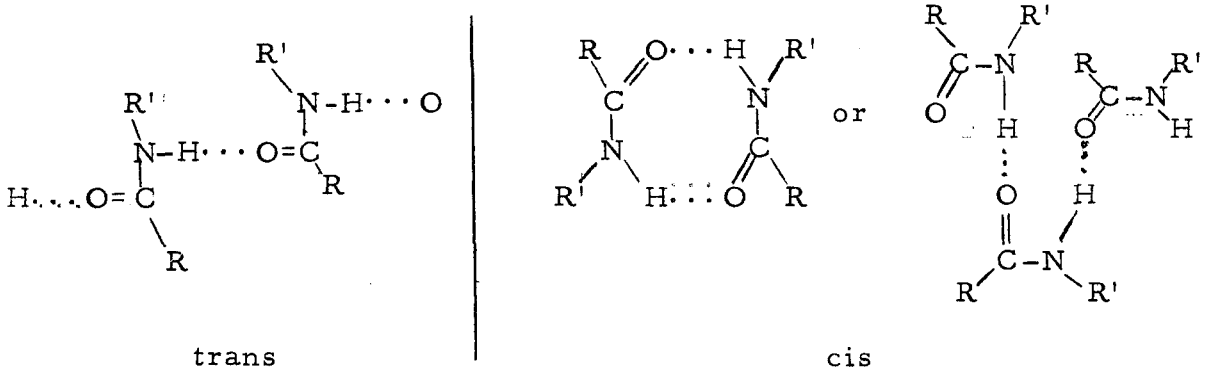


Fig. 14-14. Ultraviolet absorption spectra in methanol (c = 2 mM): 1, N-benzyl-benzamide; 2, N-methyl-N-benzyl-benzamide.

The infrared spectra of these amides give no proof of the presence of the iminol form in the solid state. Table 14-I lists the three most reliable amide-absorption bands. These bands are well known and their origins have been established. [13, 14]

The carbonyl absorption bands for the optically active benzamides and for N-(1, 1-diphenylmethyl)-benzamide show only a negligible shift (approximately 1 cm^{-1}) from one crystal form to the other (see Table 14-I and Figs. 14-15 through 14-22). If one of the two crystal forms were the iminol form (R-N=COHR'), the carbonyl absorption at about 6.1 μ (1640 cm^{-1}) would be expected to shift to about 6.2 μ (1610 cm^{-1}) for the N=C absorption. [13, 14]

From the N-H stretching and amide II absorption bands of the optically active compounds, it appears that the different crystal forms of this compound are hydrogen-bonded "cis-trans" isomers with respect to the amide bond: [14]



trans cis

Bellamy gives the range 3320 to 3270 cm^{-1} for the trans-bonded N-H stretching mode and 3180 to 3140 cm^{-1} for the cis mode. [14] For the optically active N-(α-methylbenzyl)-benzamides, the band for the lower melting isomer (3329 cm^{-1}) is close to the region for the trans form. The band of the higher melting form (3247 cm^{-1}) may be due to a "cis form" which has its band shifted from the normal cis N-H region due to steric crowding of the α-methylbenzyl group.

The shift of the amide II band in the optically active compounds (from 1523 cm^{-1} to 1537 cm^{-1}) may also be a trans-cis shift. See Ref. 14, page 217, where a shift of this type is predicted for trans-cis isomerization.

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13. H. M. Randal, et al., Infrared Determination of Organic Structures (Van Nostrand Co., New York, 1949), 10-13.

14. L. J. Bellamy, The Infrared Spectra of Complex Molecules, 2nd ed., (Methuen, London, 1958), Chapter 12.

Table 14-I. Selected absorption bands in solid-state ir spectra of amides (Values given in frequency units — cm^{-1}).

Type of absorption	l-$C_6H_5CH(CH_3)NHCOC_6H_5$ [a]		$(C_6H_5)_2CHNHCOC_6H_5$		$C_6H_5CH_2NHCOC_6H_5$ [b]
	mp 122 to 123°	mp 127 to 128°	mp 171 to 172°	mp 176 to 177°	mp 107 to 109°
N-H stretch	3329 m	3247 ms	3316-3309 m,br	3299 m	3323 m, br
Amide I (C=O) stretch	1632 vs	1633 vs	1639 vs	1640 vs	1639 vs
Amide II	1523 s	1537 s	1519 s	1538 s	1542 s
	l-$C_6H_5CH(CH_3)NDCOC_6H_5$ [a]		$(C_6H_5)_2CHNDCOC_6H_5$ [c]		$C_6H_5CH_2NDCOC_6H_5$ [b]
	mp 122.5 to 123.5°	mp 127 to 129°	mp 171 to 172°	mp 175 to 177°	mp 107 to 108°
N-H stretch	2480 m 2391 w	2463 m (2429) mw 2400 m	2465 m (2448) w 2403 w	2464 m (2450) w 2407 m	(2494) m 2483 m 2476 m (2454) m 2442 m 2403 w 2390 w
Amide I (C=O) stretch	1629 vs	1627 vs	1631 vs	1631 vs	1631 vs
Amide II	1428 s	1430 s	1423 s	1423 s	1421 s

a. Spectra were taken using the KBr disk technique; all others were taken using Nujol mulls.

b. The sample appeared to be partially dissolved in Nujol.

c. Results not very reproducible; both forms are obviously mixtures.

s - strong; m - medium; w - weak; br - broad, v - very.
( ) indicates a shoulder.

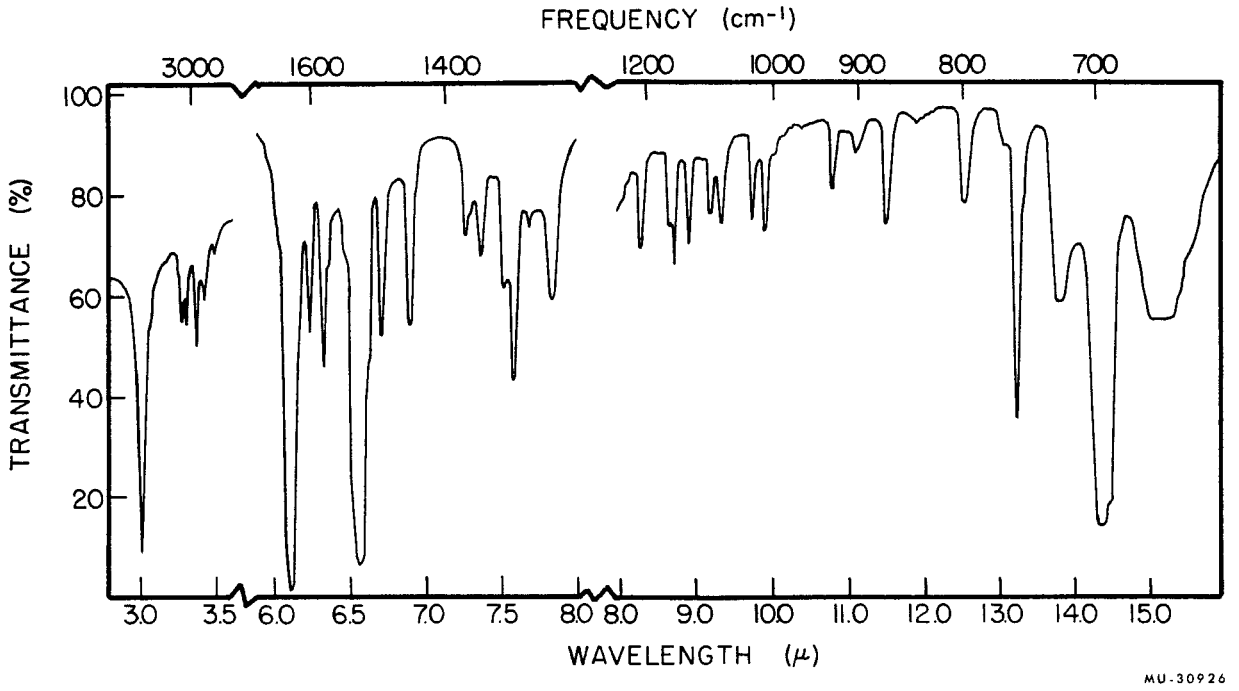

Fig. 14-15. Solid-state ir absorption spectrum of N-(1-α-methylbenzyl)-benzamide, mp 123 to 124°, from methanol in KBr disk.

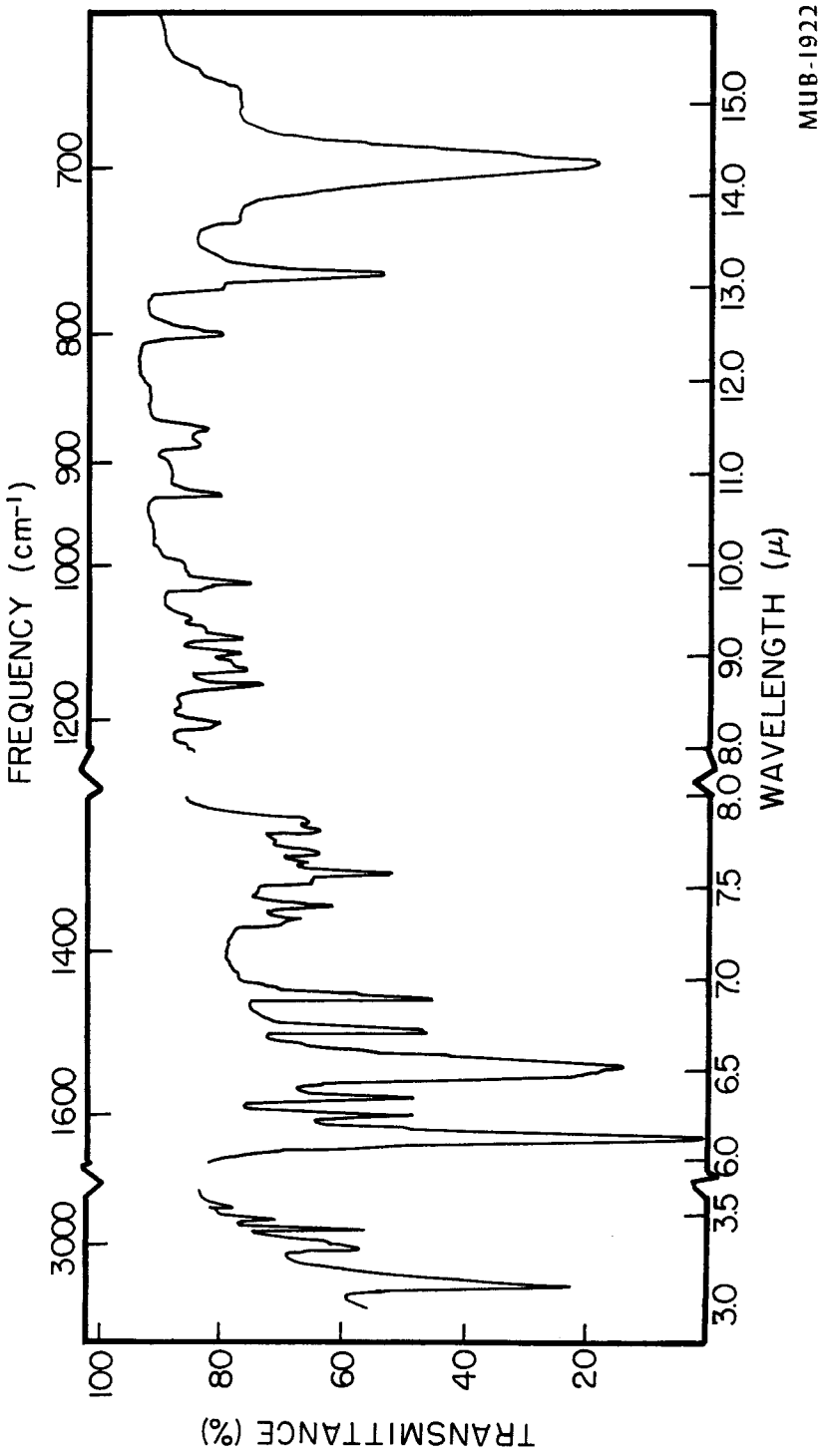

Fig. 14-16. Solid-state ir absorption spectrum of N-(1-a-methylbenzyl)-benzamide, mp 127 to 129°, from benzene-heptane, in KBr disk.

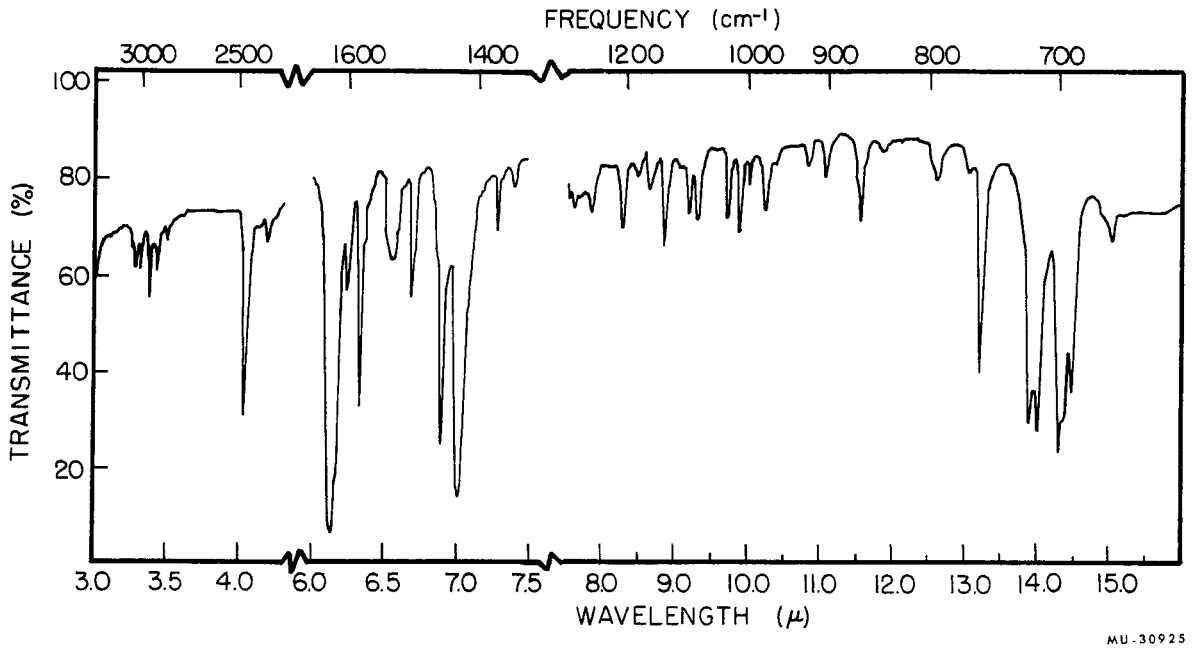

Fig. 14-17. Solid-state ir absorption spectrum of N-deutero-N-(1-α-methylbenzyl)-benzamide, mp 122.5 to 123.5°; from EtOD + D_2O, in KBr disk.

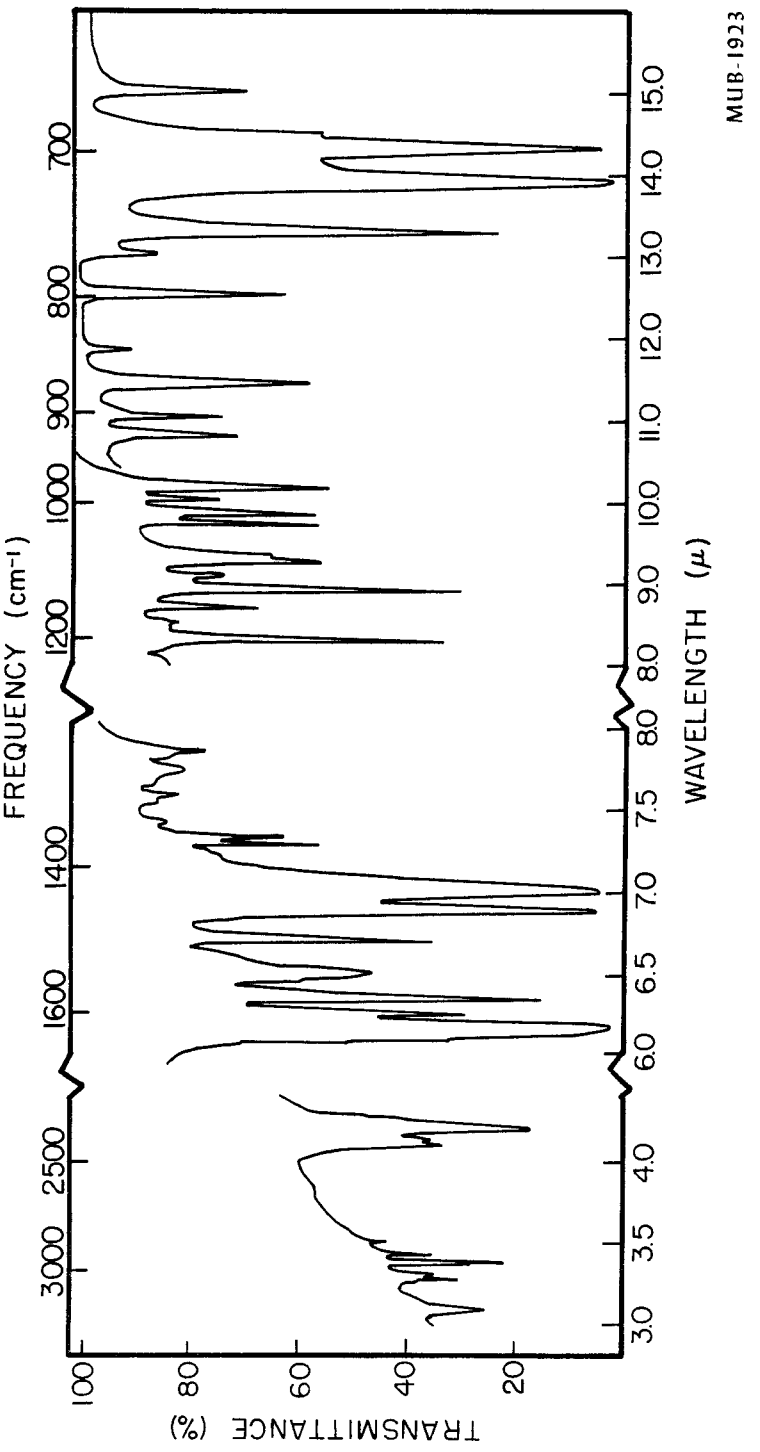

Fig. 14-18. Solid-state ir absorption spectrum of N-deutero-N-(1-α-methylbenzyl)-benzamide, mp 128 to 129°, melted and resolidified, in KBr disk.

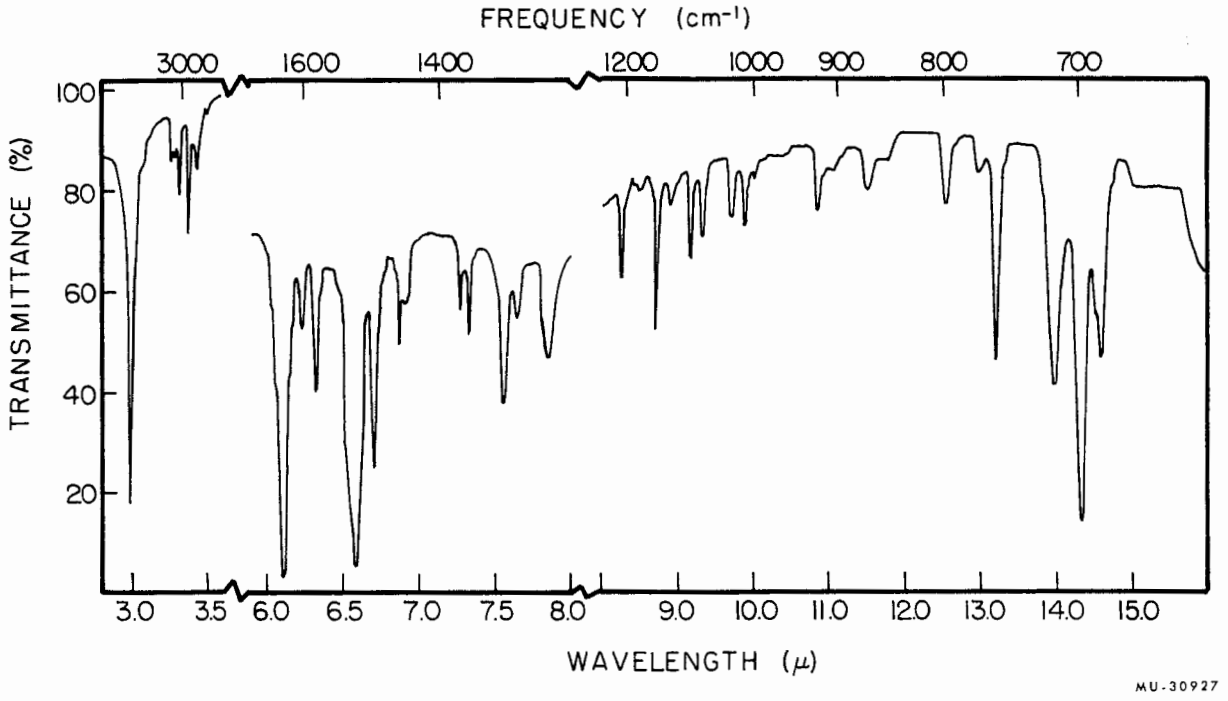

Fig. 14-19. Solid-state ir absorption spectrum of N-(1-α-methylbenzyl)-benzamide, mp 124 to 125.5°, in KBr disk.

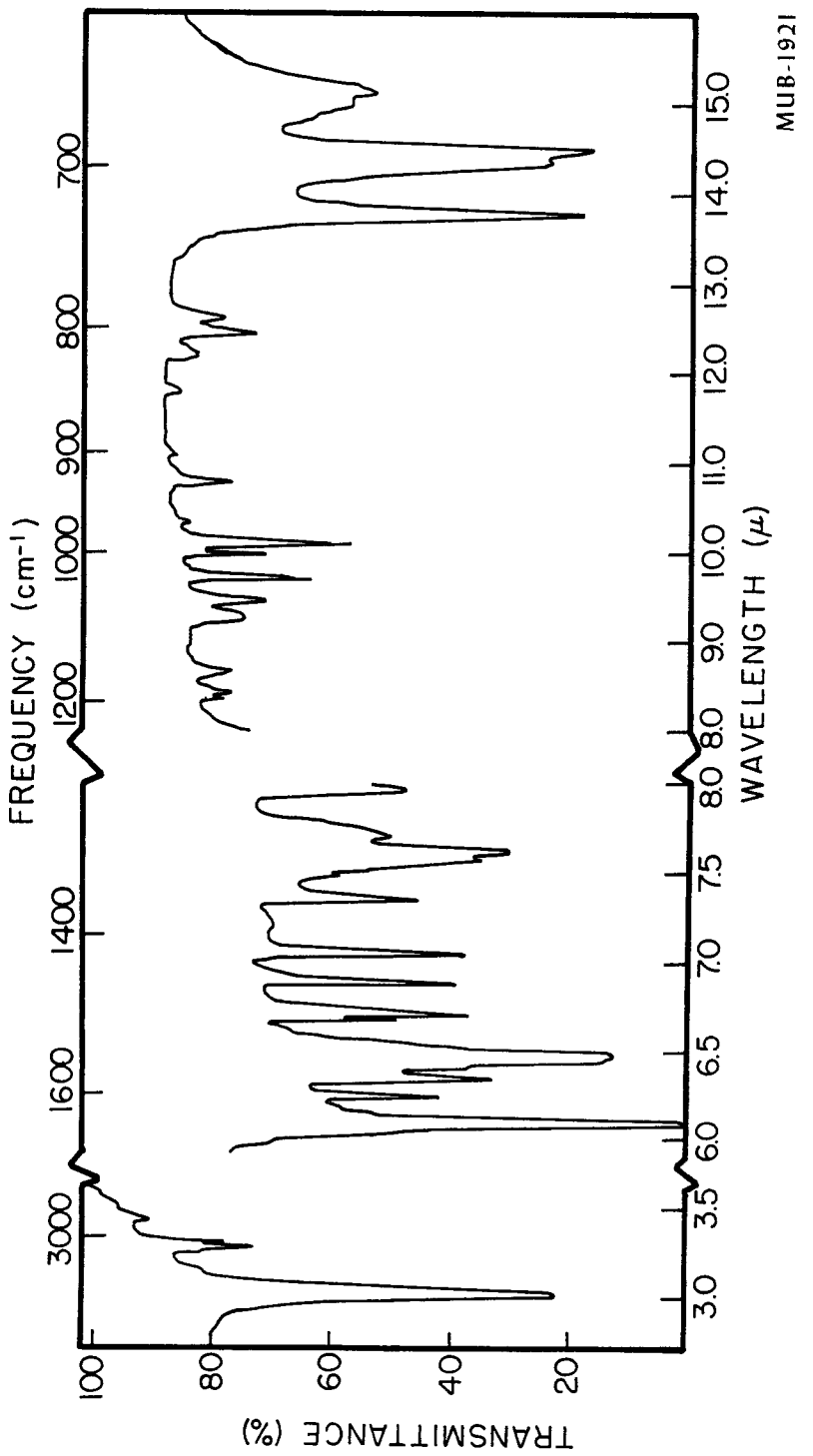

Fig. 14-20. Solid-state ir absorption spectrum of N-benzylbenzamide, mp 107 to 109°, in KBr disk.

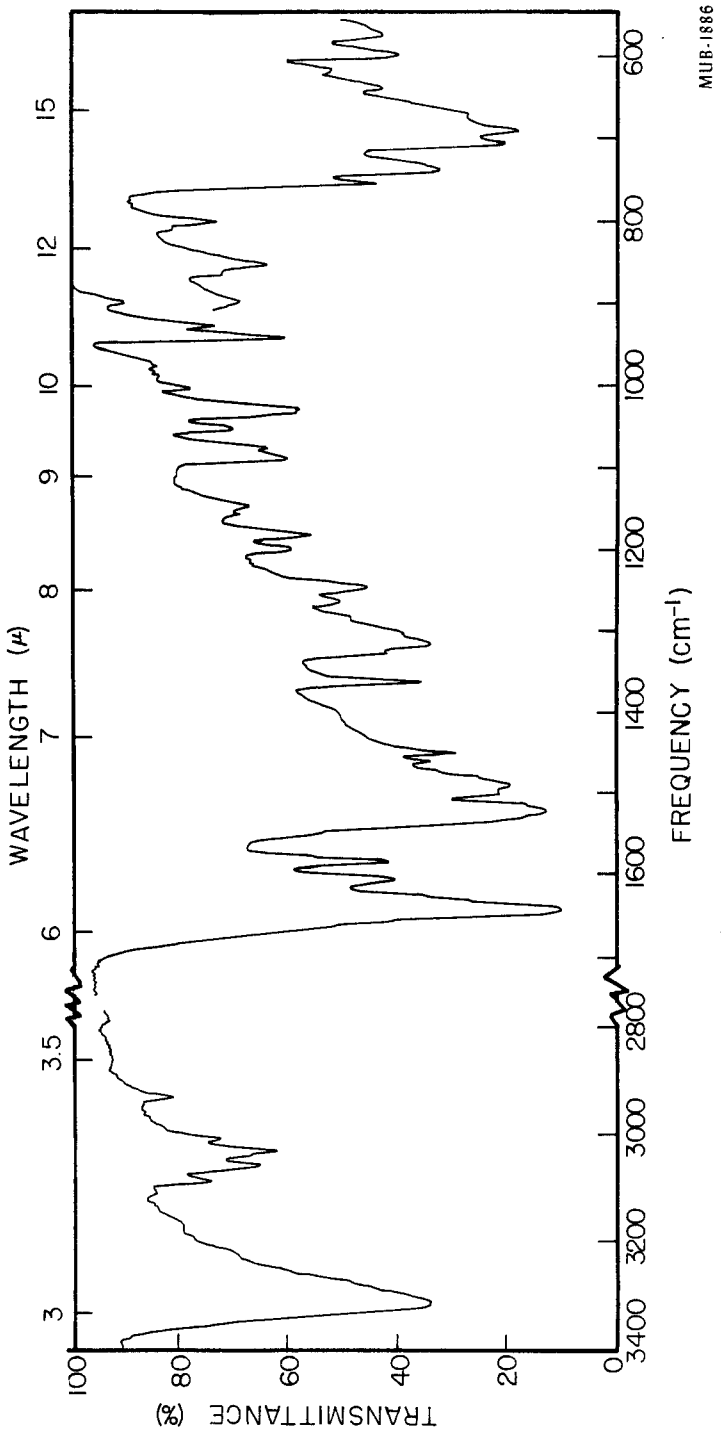

Fig. 14-21. Solid-state ir spectrum of N-(1, 1-diphenylmethyl)-benzamide crystallized from methanol (mp 171 to 172°) in KBr disk.

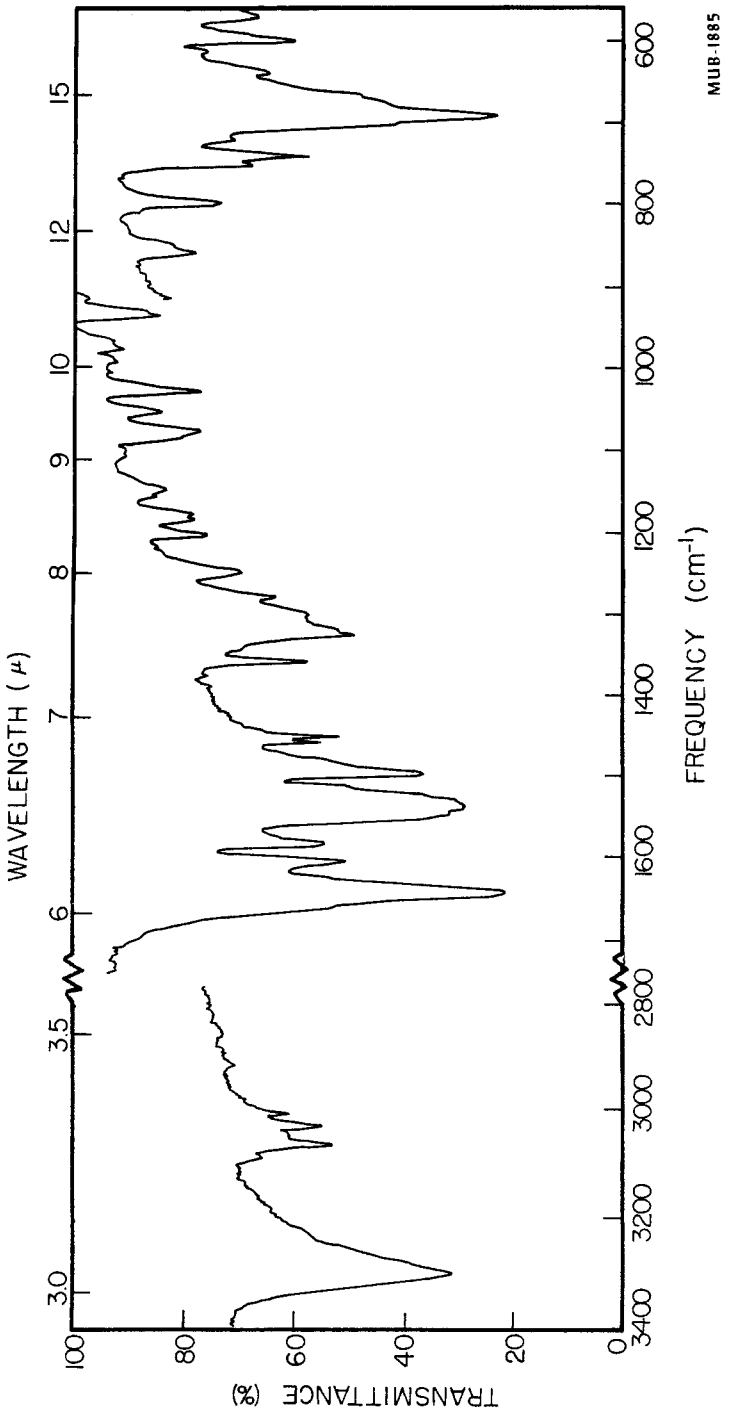

Fig. 14-22. Solid-state ir spectrum of N-(1, 1-diphenylmethyl)-benzamide crystallized from xylene (mp 176 to 177°), in KBr disk.

Table 14-I and Figs. 14-21 and 14-22 show that the N-H stretching band for N-(1, 1-diphenylmethyl)-benzamide varies only slightly (about 10 to 15 cm^{-1}) from one form to the other. The significance of this shift is questionable. It may also result from a cis-trans type of isomerization, with the cis form actually only a slightly modified trans form due to steric crowding of the bulky diphenylmethyl group. The shift of the amide II band (see Table 14-II) would seem to confirm this cis-trans isomerization. However, the small change in the N-H stretching frequency may indicate only a change of the hydrogen bonding in the crystal (e. g., formation of cyclic instead of linear polymers).

A cis-trans relationship seems highly likely from the physical "manipulations" of the two compounds. In each case, the stable lower-melting isomer is obtained by crystallization at lower temperatures (from cooled methanol), whereas the less stable higher-melting isomer is obtained by either solidification of the melted compound or crystallization from warm to hot solutions (benzene or heptane or both in one case and xylene in the other); the best results are obtained by seeding the hot solutions with a melted and solidified sample. The lower-melting isomer is converted to the higher-melting one by prolonged mixing with KBr (as shown by systematic ir studies). This also is probably a heating effect, since the sample is noticeably much warmer after prolonged mixing. The higher-melting isomer is entirely transformed into the lower melting one after standing a few days at room temperature.

These physical "manipulations" suggest that the higher melting isomer is the cis-form and the lower melting one the trans-form, in agreement with ir studies.

Experimental

Reagents

The following were used without purification: dl-α-methylbenzylamine, dl-α-aminophenylacetic acid, benzylamine (all from Eastman Kodak Co.), and N-methylbenzylamine (The Matheson Co., Inc.). All compounds described below were purified by recrystallization from appropriate solvents or distilled until no further changes in mp or bp (or both) and ir spectra were seen. The physical properties and analytical data for the compounds prepared are compiled in Tables 14-II and 14-III.

Optically active α-methylbenzylamines were prepared by resolution of the racemic amine with l-malic and d-tartaric acids.[15] Measurements of optical activity were carried out with a tube 0.1 decimeter (dm) long (for amines without solvent), and with a 2.0-dm tube for l-α-methylbenzylamine-d-tartrate (in 8% aqueous solution):

15. A. W. Ingersoll, Org. Syn., Coll. Vol. II, 506.

Table 14-II. Physical constants of compounds prepared during this investigation.

Amine	Derivative	mp (°C) (uncorrected)	ANALYSES Carbon (%) Calc.	Carbon (%) Found	Hydrogen (%) Calc.	Hydrogen (%) Found	Nitrogen (%) Calc.	Nitrogen (%) Found	Optical rotation
dl-α-phenylethylamine, $C_6H_5CH(CH_3)NH_2$	N-benzoyl	124-127 (from C_6H_6+Hp)	80.1	80.0	6.7	6.7	6.2	6.2	
		124-125.5 (from dilute MeOH)	80.1	80.1	6.7	6.7	6.2	6.3	
l-α-phenylethylamine, $C_6H_5CH(CH_3)NH_2$	N-formyl	46.5-47.5	72.5	72.3	7.4	7.4	9.4	9.4	
	N-acetyl	104-105	73.5	73.2	8.0	7.8	8.6	8.4	$[\alpha]_D^{28°} = -147.3°$ in MeOH(c=0.01)
	N-trifluoro-acetyl	96-98	55.4	55.5	4.6	4.45	6.5	6.2	$[\alpha]_D^{28°} = -145.4°$ in MeOH(c=0.01)
	N-benzoyl	122-123 (from dilute EtOH)	80.1	80.3	6.7	6.6	6.2	6.1	
		127-128 (from Hp+ $CHCl_3$)	80.1	80.0	6.7	6.8	6.2	6.0	
d-α-phenylethylamine, $C_6H_5CH(CH_3)NH_2$	N-formyl	47.5-49	72.5	72.7	7.4	7.2	9.4	9.3	
	N-acetyl	104-106	73.5	73.5	8.0	8.05	8.6	8.4	$[\alpha]_D^{26°} = +154.1°$ in MeOH(c=0.01)
	N-trifluoro-acetyl	97-98	55.4	55.5	4.6	4.8	6.5	6.3	$[\alpha]_D^{28°} = +148.4°$ in MeOH(c=0.01)
	N-benzoyl	127-128*	80.1	80.22	6.7	6.6	6.2	6.1	$[\alpha]_D^{25°} = +20.8°$ in $CHCl_3$(c=0.025)
dl-α-phenylglycine, $C_6H_5CH(COOH)NH_2$	N-acetyl	about 199-203 (subl.)	62.2	62.0	5.7	5.8	7.2	7.1	
	N-benzoyl	180-182	70.6	70.7	5.1	5.2	5.5	5.5	
1,1-diphenylmethylamine (benzhydrylamine) $(C_6H_5)_2CHNH_2$	N-acetyl	149.5-150	80.0	80.1	6.7	6.7	6.2	6.4	
	N-benzoyl	171-172 (from MeOH)	83.6	83.5	6.0	5.95	4.9	4.9	
		176-177(from xylene)	83.6	83.5	6.0	5.9	4.9	5.1	
benzylamine, $C_6H_5CH_2NH_2$	N-acetyl	58-62 (from Et_2O)	72.45	72.8	7.4	7.75	9.4	9.2	
		62-64 (melted)	72.45	72.6	7.4	7.2	9.4	9.4	
	N-benzoyl	107-109 (from dilute EtOH)	79.6	79.5	6.2	6.2	6.6	6.8	
		107-109 (from Hp+ C_6H_6)	79.6	79.7	6.2	6.1	6.6	6.5	
N-methylbenzylamine, $C_6H_5CH_2NHCH_3$	N-acetyl	39-41	73.6	73.6	8.0	8.1	8.6	8.5	
	N-benzoyl	40.5-43	80.1	79.9	6.7	6.6	6.2	6.4	
methylamine, CH_3NH_2	N-benzoyl	81.82	71.1	70.9	6.7	6.6	10.4	10.3	
l-α-phenylethylamine, $C_6H_5CH(CH_3)NH_2$	N-benzyl	bp 143-145°/4 mm	85.4	85.6	8.0	8.15	6.6	6.3	
	N,N-dibenzyl	75-76	87.7	87.8	7.6	7.7	4.65	4.5	
d-α-phenylethylamine, $C_6H_5CH(CH_3)NH_2$	N-benzyl	bp 133-137°/2 mm	85.4	85.1	8.0	7.9	6.6	6.5	

*From a mixture of heptane and benzene; compound crystallized from dilute EtOH had mp 122 to 123°.

Table 14-III. Molecular rotation of derivatives of optically active α-phenylethylamines in methanol and in benzene. Measurements were carried out with a cell 5.0 cm (and 1.0 cm--data in parentheses). Concentrations (g per 100 cc of solution) are given in parentheses marked with an asterisk [( )*].

	N-formyl-α-phenylethylamines			N-acetyl-α-phenylethylamines		
	L-form		D-form	L-form		D-form
λ in mμ	C_6H_6 (0.2)*	CH_3OH (0.1)*	CH_3OH (0.1)*	C_6H_6 (0.25)*	CH_3OH (0.1)*	CH_3OH (0.1)*
589	-355	-337	+352	-311	-228	+225
500	-443	-468	+460	-415	-290	+405
436	-637		+656	-629	-535	-529
405	-788	-865	+821	-789	-676	+718
381	-904		+913	-867	-732	+845
365	-1120	-1180	+1160	-1090	-963	+963
334	-1530	-1650	+1600	-1525	-1345	+1338
313	-2050	-2110(-1920)	+2080(+2270)	-2020	-1740(-1850)	+1818(+2089)
302	-2200	(-2390)	+2410(+2600)	-2305	-2030(-2064)	+2060(+2366)
297				-2500	(-2211)	+2402(+2154)
292		-2880	+2780(+3010)		-2210(-2505)	+2464(+2774)
289			+2950		(-2310)	+2572(+3411)
285		-3120(-3070)	+3030(+3390)		-2480(-2513)	+2673(+2872)
280					(-2864)	(+3297)
275			+3620(+3850)		-2790(-3010)	+3215(+3395)
270		(-3740)	(+4460)		(-3003)	+2295(+3819)
265			(+5220)		-2480(-4075)	+1208(+4488)
260					(-3395)	(+3933)
254		(-6070)	(+6240)		(-4847)	+1152(+5810)
248		(-7450)	(+7520)		(-7377)	(+6675)
242						(+8111)
237.5						(+9955)
234.5		(-10860)	(+11700)			(+11000)

	N-trifluoroacetyl-α-phenylethylamines			N-benzoyl-α-phenylethylamines			N,N-dibenzyl-α-phenylethylamines
	D-form		L-form	L-form		D-form	L-form
	C_6H_6 (0.21)*	CH_3OH (0.1)*	CH_3OH (0.1)*	C_6H_6 (1.0)*	CH_3OH (1.0)*	CH_3OH (1.0)*	CH_3OH (0.1)*
589	+321	+373	-421	-124	+15	-19	-415
546					+22		
500	+510	+517	-469	-181		-27	-536
461					+32		
436	+788	+703	-700	-258		-38	-668
405	+1010	+938		-322	+63	-50	-843
381	+1140	+1030	-995	-356		-62	-969
365	+1430	+1320	-1220	-445	+102	-99	-1200
334	+2040	+1780	-1760	-617	+188	-185	-1625
313	+2780	+2440(+2300)	-2410(-2350)	-816	+344(+365)	-311	-2310
302	+3250	+2900(+2740)			+510(+554)	-500	-2620(-1960)
297	+3600	(+3150)	(-3020)		+630(+670)	-642	(-2950)
292	+3600	+3250(+3450)					-3300(-3550)
289		+3250			+620(+993)		-3320
285		+3510(+4233)	-3570(-3170)				-3380(-3760)
275		(+4860)	(-3840)				(-4090)
270		(+5470)					(-3430)
265		(+6690)	(-6060)				(-2300)
254		(+8340)	(-8000)				

l-α-methylbenzylamine-d-tartrate: $[\alpha]_D^{28^\circ} = +13.2^\circ$,

l-α-methylbenzylamine, bp 185 to 187°: $[\alpha]_D^{25.5^\circ} = -38.07^\circ, -38.20^\circ$; $[\alpha]_D^{23^\circ} = -38.33^\circ$,

d-α-methylbenzylamine, bp 185 to 187°: $[\alpha]_D^{25.5^\circ} = +37.10^\circ, +37.22^\circ, +37.14^\circ$.

The literature[15] gives bp 184 to 185° and $[\alpha]_D^{25^\circ} = +39.2^\circ$ to 39.7° (without solvent) for the d-amine, and $[\alpha]_D = +13.0^\circ$ to 13.2° for the l-amine-d-tartrate (in 8% aqueous solution).

N-(α-Methylbenzyl)-Formamides

The optically active amines were refluxed for several hours with an excess of 98% formic acid containing a few drops of concentrated H_2SO_4. The formic acid was removed under vacuum, the residues neutralized with a saturated solution of $NaHCO_3$, and extracted with benzene or ether. The extracts were dried over Na_2SO_4 and solvents removed under vacuum. The oily residues were distilled (bp about 175/15 mm), then crystallized from a mixture of petroleum ether and ether or CCl_4. The white needles were washed with petroleum ether and dried. The literature[9] reports mp 46 to 47° and bp 110°/0.001 mm.

N-(α-Methylbenzyl)-Trifluoroacetamides

The optically active amines were dissolved in dry benzene, an excess of trifluoroacetic anhydride was added, and the mixtures were left overnight at room temperature. The next day the volatile materials were removed under vacuum and the residues were recrystallized consecutively from aqueous alcohol, CCl_4, and a mixture of benzene and heptane. The long white needles were washed with petroleum ether and dried.

N-(α-Methylbenzyl)-Acetamides

The optically active amines were dissolved in dry benzene and acetylated with an excess of acetic anhydride at room temperature. After a few hours the volatile compounds were removed under vacuum, and the residues were crystallized consecutively from ligroine, CCl_4, and a mixture of heptane and CCl_4. The white rectangular crystals were washed with petroleum ether and dried.

According to the literature,[5, 9] the mp of the optically active compounds crystallized from benzene-petroleum ether were 101 to 102°, and 102.5 to 103.5°.

N-benzyl-α-methylbenzylamines and N,N-dibenzyl-l-α-methylbenzylamine

Optically active amine (4.4 g) was heated on the water bath for 7 hr with 5.0 g of freshly distilled benzyl chloride and then left overnight at room temperature. Most of the solidified reaction mixtures were dissolved

in about 100 cc of boiling water. After cooling, the undissolved oil was extracted with ether. The ethereal solutions were evaporated under vacuum and the residual oil dissolved in methanol. The methanolic solutions were filtered and slowly evaporated under vacuum at room temperature. The pale yellow crystals of N,N-dibenzyl derivative of the l-form had a mp of 74 to 75° (the d-form was not as pure). Recrystallization from methanol yielded 1.2 g of pure N,N-dibenzyl-l-α-methylbenzylamine in the form of white needles.

The aqueous solutions of the optically active N-benzyl-α-methylbenzylamines were evaporated under vacuum, and small white crystals (about 4 g) were filtered, crystallized from hot water (mp 181 to 183°), and dissolved in hot water, and the hydrochlorides were decomposed with dilute NaOH solution. The free amines were extracted with ether and the extracts dried over solid NaOH. The ether was evaporated and the residue distilled under vacuum. The above procedure is similar to that used by Parck, who reported a bp of 171°/15 mm for N-benzyl-l-α-methylbenzylamine, a mp of 177° for its hydrochloride, and a mp of 74° for the optically active dibenzyl derivatives.[16]

N-(α-Methylbenzyl)-Benzamides

The racemic and optically active amines were benzoylated by the Schotten-Baumann method, with an excess of benzoyl chloride and 10% aqueous NaOH solution. The crystalline products were crystallized several times from different solvents.

The observations reporting the existence of two different forms of optically active N-(α-methylbenzyl)-benzamide have been essentially confirmed.[17,18] It is sometimes possible to obtain, by crystallization from dilute methanol or ethanol, the crystalline form (small colorless needles) that melts completely at 122 to 123°, solidifies at temperatures above 123°, and melts again at 127 to 128°. Crystallization from heptane or a mixture of heptane and benzene usually yields the higher-melting form (large colorless needles), which melts at 128 to 129°. Both forms, when melted and resolidified, melt at 128 to 129°. The conditions of crystallization and the rate of cooling the solutions are, however, important factors. It is sometimes possible to obtain the lower-melting form even from nonhydroxylic solvents. To obtain the higher-melting form from a hot benzene-heptane mixture by slow crystallization, it is advisable to seed the warm solution with the melted and resolidified sample.

Systematic ir studies showed that the form with a higher mp, on standing at room temperature, is slowly transformed into the one with a lower mp. It was also established that when using the KBr-pressed-pellet technique more time is required to mix the sample with KBr—the lower-melting form is gradually transformed into the higher-melting one when the mixing with KBr is prolonged.

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16. K. Parck, J. Prakt. Chem. (2), 86, 284 (1912).
17. V. M. Potapov, A. P. Terent'ev, Dokl. Akad. Nauk. SSSR 132, 626 (1960).
18. V. M. Potapov, A. P. Terent'ev, Zh. Obshch. Khim. 31, 1720 (1961).

The double-melting phenomenon and the differences in the ir spectra are observed only for the optically active benzoyl derivatives of α-methylbenzylamine. The racemic compound crystallized from dilute methanol (short colorless needlelike crystals) or from benzene-heptane (long needles) reveals some small differences in its mp (Table 14-II), but the ir spectra are apparently the same (as well as for melted and resolidified samples). The mp given in the literature for the benzoyl derivative of the racemic amine are 120° [19] and 124°. [5]

1, 1-Diphenylmethylamine (benzhydrylamine)

This compound was obtained by reduction of benzophenone oxime with sodium in ethanolic solution. [20, 21] The crude amine was distilled under vacuum, bp 137°/3.6 mm.

N-(1, 1-Diphenylmethyl)-Acetamide

The amine (5.5 g) was refluxed for 12 hr with 50 cc of glacial acetic acid and 10 cc of acetic anhydride. The solvents were then removed under vacuum. The yellow precipitate was dissolved in dilute methanol, refluxed for 1 hr, and the solution filtered and cooled. The white crystals of N-(1, 1-diphenylmethyl)-acetamide were recrystallized from benzene-cyclohexane (long colorless needles) and have the same mp as the substance that has been resolidified. Melting points from 146 to 147° (needles from dilute ethanol) and 148° (colorless needles from benzene) are given in the literature. [22, 23]

N-(1, 1-Diphenylmethyl)-Benzamide

This compound was obtained by the Schotten-Baumann method. The crude product was recrystallized from different solvents, and some differences in the melting points and ir spectra were found when dilute alcohol or hydrocarbon solvents were used for crystallization. From dilute aqueous methanol the compound had mp 171 to 172° (needles), but solidified again, with a mp of 175.5 to 177°. From hot xylene solution seeded with the resolidified sample, the compound crystallized in the form of long needles with a mp of 176 to 177°. But in this case results were even less reproducible than with the optically active N-(α-methylbenzyl)-benzamides, and very often samples were obtained that softened or partially melted at 171°, and melted completely at 177 to 179°.

The melting points recorded in the literature are 166 to 167° (needles from ethanol) and 172°. [22, 24]

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19. M. Kann, J. Tafel, Chem. Ber. 27, 2306 (1894).
20. A. Lachman, Org. Syn. 10, 10 (1930).
21. H. H. Fox and W. Wenner, J. Org. Chem. 16, 225 (1951).
22. H. L. Wheeler, Am. Chem. J. 26, 345 (1901).
23. G. W. H. Cheeseman, J. Chem. Soc. 1957, 115.
24. M. Busch and L. Leefhelm, J. Prakt. Chem. (2), 77, 1 (1907).

Racemic α-Acetamidophenylacetic Acid

This[25] was obtained by slow addition of twice the theoretical amount of acetic anhydride to the solution of dl-α-phenylglycine in aqueous NaOH solution at 5°. Crystallization from water yielded white needles. A melting point of 199° (from water) is given in the literature.[26]

Racemic α-Benzamidophenylactiv acid

This was prepared by the method of Minovici, using sodium bicarbonate as alkali.[25] The crude product was extracted several times with hot benzene, washed well with not benzene, and crystallized from dilute acetone and ethanol, which furnished needlelike crystals. The literature reports mp of 174° and 178° from alcohol, and 178.0 to 178.5° (from dilute acetone).[26, 27, 28]

N-Benzylacetamide

This was obtained by refluxing benzylamine and a mixture of acetic acid and acetic anhydride. After a few hours the solvent was removed under vacuum. The residue was dissolved in dilute ethanol and refluxed for 1 hour. The solution was filtered and the solvent evaporated under vacuum. The residue was crystallized from benzene-ligroine and from ether. White needles and needlelike plates were obtained. The literature reports mp 59°, and 60 to 61° (plates from ether or petroleum ether).[29]

N-Benzylbenzamide

This compound was prepared by the Schotten-Baumann method. The products obtained by crystallization from different solvents — dilute methanol or benzene — and the resolidified ample had identical mp and ir spectra. Reported mp: 105 to 106° (plates from benzene), 105 to 107° (needles from dilute ethanol).[30, 31]

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25. The amides of dl-α-phenylglycine were prepared, but not reported in the NMR studies, since they were sufficiently soluble (for NMR studies) only in dioxane and methanol, and these solvents obscured some of the essential parts of the spectra.

26. S. Searles and G. J. Cvejanovich, J. Am. Chem. Soc. 72, 3200 (1950).

27. S. Minovici, Bull. Soc. Chim. Romania 2, 8 (1920); Chem. Abs. 14, 3228 (1920).

28. R. E. Steiger, J. Org. Chem. 9, 396 (1944).

29. H. Amsel and A. W. Hofmann, Ber. 19, 1284 (1886).

30. E. Beckmann, Ber. 23, 3331 (1890).

31. C. Blacher, Ber. 28, 432 (1895).

N-Methyl-N-Benzylacetamide

This compound was prepared in the same way as N-benzylacetamide. The residue remaining when the solvent was removed was dissolved in ether. The ethereal solution was extracted with dilute hydrochloric acid, neutralized with aqueous $NaHCO_3$ solution, dried over Na_2SO_4, filtered and evaporated. The residue was distilled, bp 101°/0.7 mm, and finally the compound was crystallized from ether-ligroine. The white plates were washed with petroleum ether. The literature reports a mp of 43 to 44° and a bp of 97 to 99°/0.1 mm.[32]

N-Methyl-N-Benzylbenzamide

This was prepared by the Schotten-Baumann method. The reaction mixture was extracted with ether, the ethereal solution was extracted a few times with a 10% solution of NaOH, dried over Na_2SO_4, and the ether was removed under vacuum. The compound was crystallized from mixtures of ether-ligroine and chloroform-ligroine yielding colorless lustrous plates. Reported mp was 44°.[33]

N-Methylbenzamide

This compound was prepared and was crystallized twice from methanol and ethanol, yielding white plates.[34] Reported mp were 78° and 80°.[33, 34]

Deuterated Compounds

The exchange with D_2O or C_2H_5OD was effected by dissolving the acetyl and benzoyl derivatives of 1-α-methylbenzylamine, 1, 1-diphenylmethylamine, and benzylamine in C_2H_5OD and precipitating with D_2O by crystallization from D_2O or C_2H_5OD, or from a mixture of C_2H_5OH and D_2O. This procedure was repeated a few times until the ir spectra revealed that the substances were deuterated in not less than about 95%.

Physico-Chemical Measurements and Analyses

All microanalyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley. The melting points were determined with a Fisher-Johns melting-point apparatus and are uncorrected, as are the boiling points given. The ultraviolet (uv) absorption spectra were determined in absolute methanol, cyclohexane, and isooctane, by means of a Cary 14 recording spectrophotometer and 0.25, 1.0, and 2.0 cm silica cells. The ir studies of the solid compounds

32. J. A. King and F. H. McMillan, J. Am. Chem. Soc. 77, 2814 (1955).

33. J. V. Braun and J. Weismantel, Ber. 55, 3165 (1922).

34. P. van Romburgh, Rec. Trav. Chem. 4, 384 (1885).

were carried out on Perkin-Elmer 237 and 421 instruments. For systematic ir studies on the influence of the time of mixing the samples with KBr, a WIG-L-BUG Amalgamator (Crescent Dental Mfg. Co., Chicago, Ill.; model 5A) was used, and the mixing time was changed within the limits of 30 to 300 seconds.

The ORD studies were performed with a nonrecording device essentially similar to that used by Potapov and Terent'ev;[35] anhydrous methanol and benzene were used as solvents. Solutions were placed in 1.0- and 5.0-cm calibrated silica cells. The data obtained are listed in tables or presented in the form of graphs in the text.

Conclusions

The solvent effect on optical rotation must be accounted for in terms other than amide-iminol tautomerism. The best explanations would probably lie in the different intermolecular complexes (monomers vs dimers or polymers) formed in different solutions or in the different barriers to rotation about the amide bond, possibly resulting in different positions of the cis-trans equilibrium of the amides in different solvents.

The two crystal forms can most likely be explained in terms of cis-trans isomerization about the amide bond or by different intermolecular complexes in the solid state. The amide-iminol tautomerism, although extremely unlikely, cannot be entirely ruled out as the cause of these two crystal forms.

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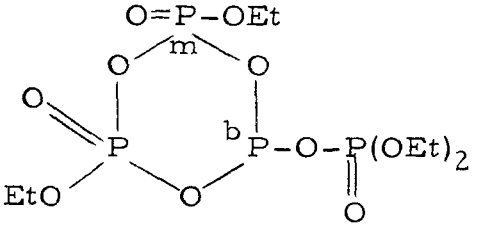
35. V. J. Potapov and A. P. Terent'ev, Zh. Obshch. Khim. 31, 1003 (1961).

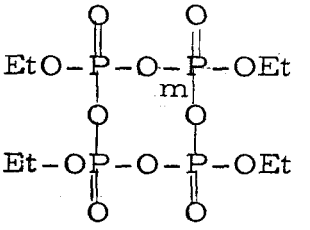
15. STRUCTURE AND MECHANISM OF HYDROLYSIS OF THE PRODUCT OF REACTION OF P_2O_5 AND ETHYL ETHER

Gilbert Weill, Melvin P. Klein, and Melvin Calvin

The product of the reaction of P_2O_5 and ethyl ether, which has the composition of a metaphosphate ester $[PO_3(C_2H_5)]_n$, has been the subject of recent discussions. Rätz and Thilö[1] have shown that the product of hydrolysis of this ester could result from the splitting of a mixture of the two compounds



and



I II

However, the NMR spectrum obtained by Van Wazer[2] on the crude compound shows three peaks, the chemical shifts of which are characteristic of terminal (t), middle (m), and branched (b) phosphorus atoms but in the proportions $P_t = 25\%$, $P_m = 72\%$, $P_b = 3\%$. The inequality of P_b and P_t seemed to rule out the proposed structure.

The use of this compound as an activating agent in a number of condensation reactions[3] has indicated its extraordinarily high reactivity towards OH groups, and has raised the question of the mechanism of the condensation reaction. The titration of a solution of the polyphosphate ester in water immediately after dissolving shows two primary acid groups for each initial P_4O_{10} molecule (Fig. 15-1, curve 1). Pollmann has studied the changes in ir spectra of a solution in chloroform upon addition of small quantities of water, and has suggested an opening of a ring based upon the disappearance of a broad peak attributed to cyclic metaphosphate.[4]

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1. R. Rätz and E. Thilö, Ann. Ber. Chem. 272, 173 (1951).

2. J. Van Wazer, C. F. Callis, J. N. Shoolery, and R. C. Jones, J. Am. Chem. Soc. 74, 5715 (1956).

3. G. Schramm and H. Wissmann, Chem. Ber. 91, 1073 (1955); G. Schramm, H. Grötsch, and W. Pollmann, Angew. Chem. Intern. Ed. Engl. 1, 1 (1962).

4. W. Pollmann, (Max Planck Institute für Virusforschung) personal communication.

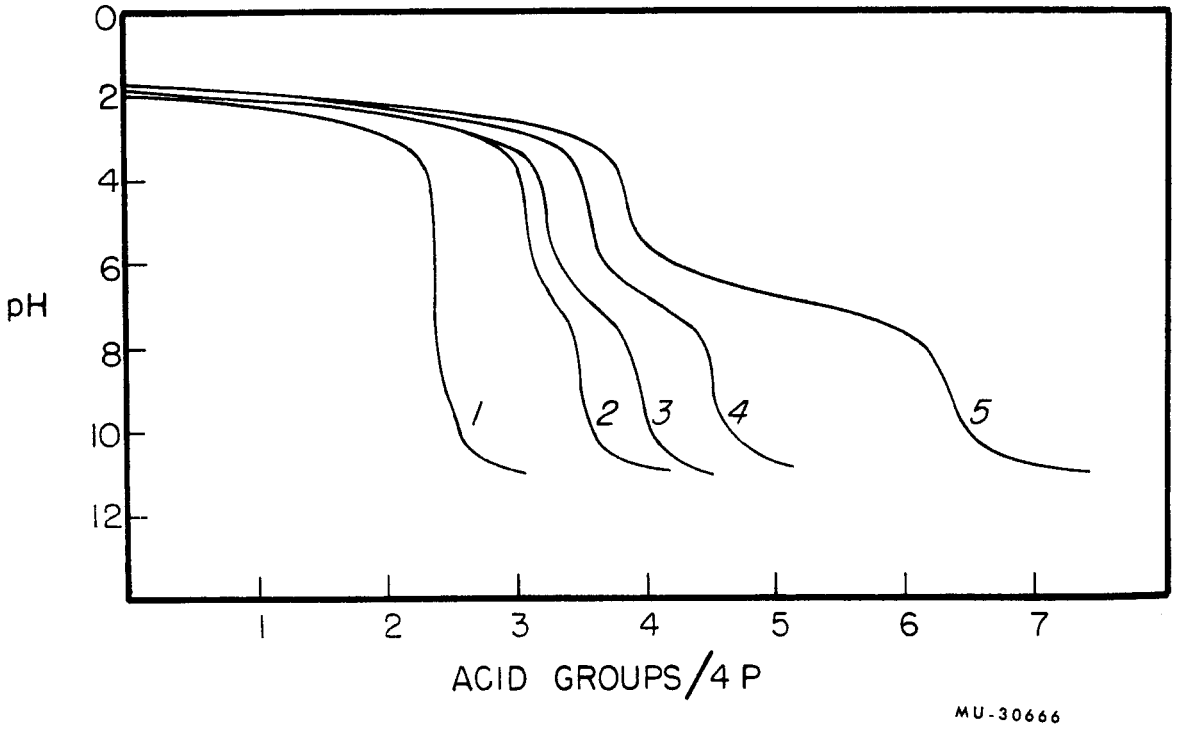


Fig. 15-1. Titration of the polymetaphosphate ester in water as a function of time (a) immediately after dissolving, (b) after 6 hr, (c) after 24 hr, (d) after 52 hr, (e) after boiling 1 hr.

The NMR technique seemed suitable for a fine analysis of the structure and mechanism of hydrolysis of this compound. We have used a Varian spectrometer operating at 24.6 Mc. Phosphoric acid (85%) was used as an external standard and no attempt was made to measure the chemical shift with an accuracy greater than ± 2 ppm.

A first attempt to reproduce Van Wazer's experiment on an undiluted sample prepared a few days before the observation produced the results shown in Fig. 15-2. The chemical shifts are identical to those published by Van Wazer–$\delta P_t = 16$, $\delta P_m = 29$, $\delta P_b = 42$–and the proportion is only slightly different from his, with an unequal proportion of P_b (6%) and P_t (32%).

To prevent partial hydrolysis during storage or transfer of the viscous material into the narrow tube, we have introduced directly into the tube a freshly prepared chloroform solution of the compound in the absence of humidity, and evaporated part of the solvent under vacuum. The spectrum so obtained (Fig. 15-3a) and its integral (Fig. 15-3f) show approximately equal proportions of P_b and P_t and therefore permits structure I. (Fig. 15-4 shows a similar series of spectra in ethanol rather than water.) The relative amounts of phosphorus in structures I and II can then be calculated:

$$C_I \approx 2 \times (15\% + 13\%) = 56\%$$

$$C_{II} \approx 44\%.$$

The changes of the spectrum upon addition of small quantities of water (Fig. 15-3b, c, d, e) reveal the following features.

In a first step (b) the P_b peak disappears and the P_t peak increases while P_m remains practically constant. No resonance corresponding to orthophosphate derivatives appears.

In a second step (c) the concentration of P_m decreases and the concentration of P_t increases until P_t and P_m are roughly equal.

The concentration of P_t becomes higher than P_m and a small quantity of orthophosphoric derivative P_s is found (c), followed by disappearance of P_m with increasing amounts of P_s (d).

The same experiment performed with added ethanol gives the same pattern of results (Fig. 15-4). From the relative amounts of the different species of phosphorus found during these three steps, the following conclusions are drawn:

1. The structure I is more sensitive to hydrolysis than structure II. The first bond to be split is the bond connecting the "branched" phosphorus to another phosphorus of the ring.

2. The ring opening of structure II follows until transformation of the two compounds into two linear tetrapolyphosphates ($P_t/P_n = 1$) is complete.

3. The hydrolysis of the tetrapolyphosphate proceeds preferentially through formation of two pyrophosphate structures. This observation fits with the rate of appearance of secondary acidities evident in Fig. 15-1.

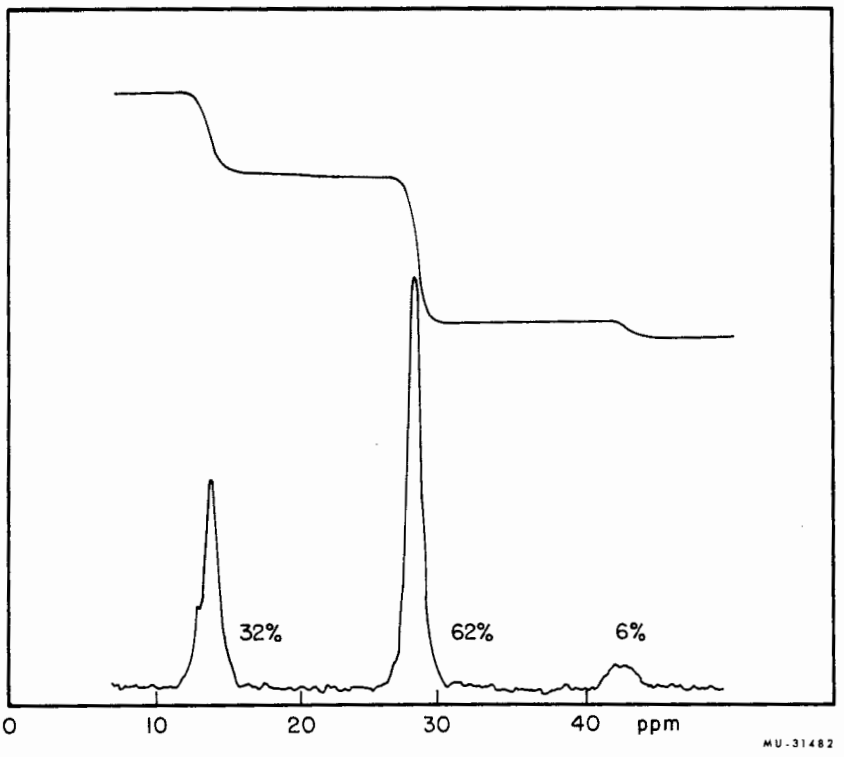


Fig. 15-2. NMR spectrum of a crude sample of polymetaphosphate ester and integration curve (no solvent).

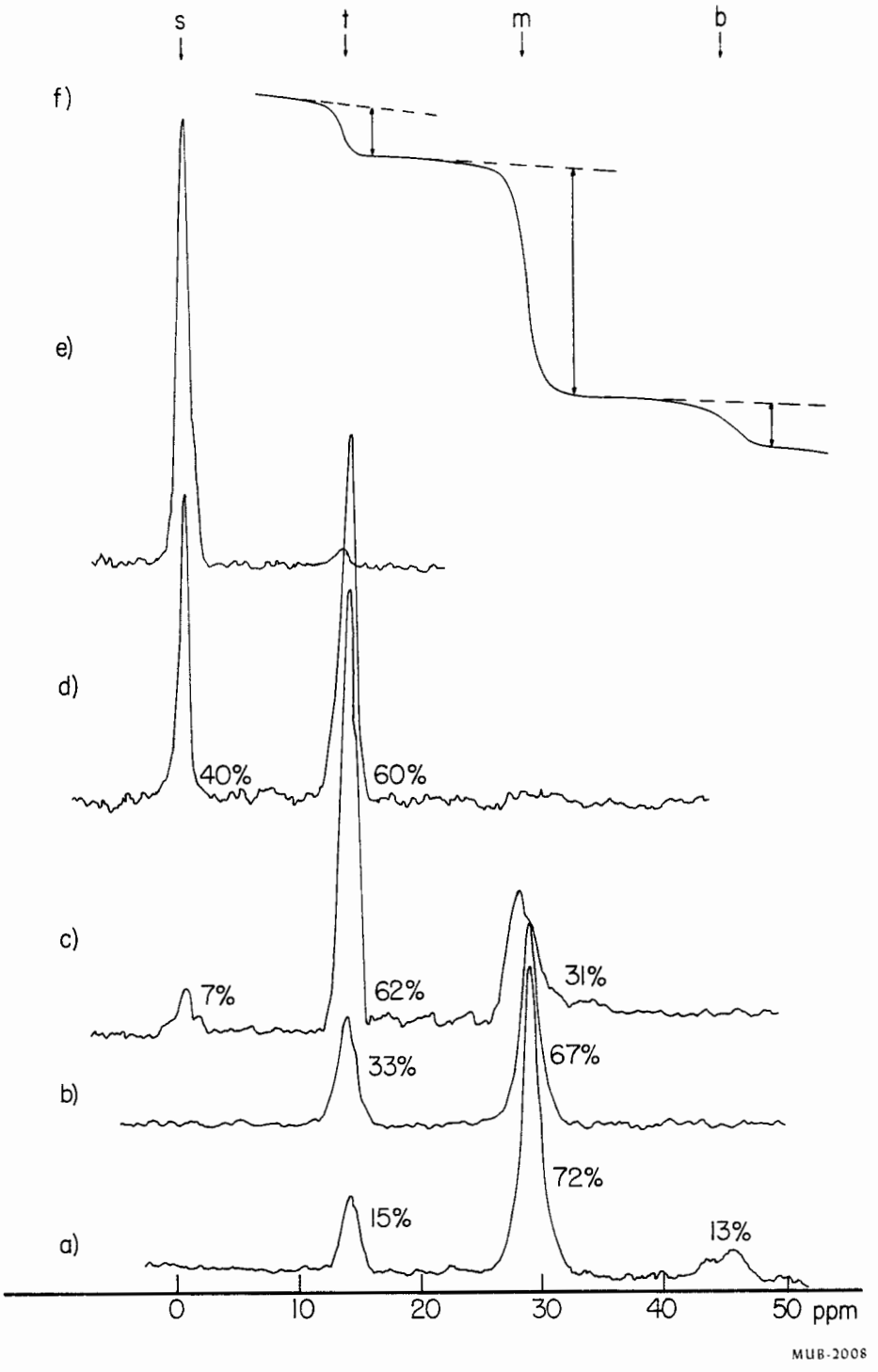


Fig. 15-3. NMR spectrum of a concentrated solution of freshly prepared polymetaphosphate ester in chloroform: (a) and (f) sample in absence of water and its integral, respectively, (b) with less than one equivalent of H_2O per 4 P, (c) with a large excess of water after 5 hr, (d) with an excess of water after 20 hr, (e) with an excess of water after boiling (each trace may be at a different amplification).

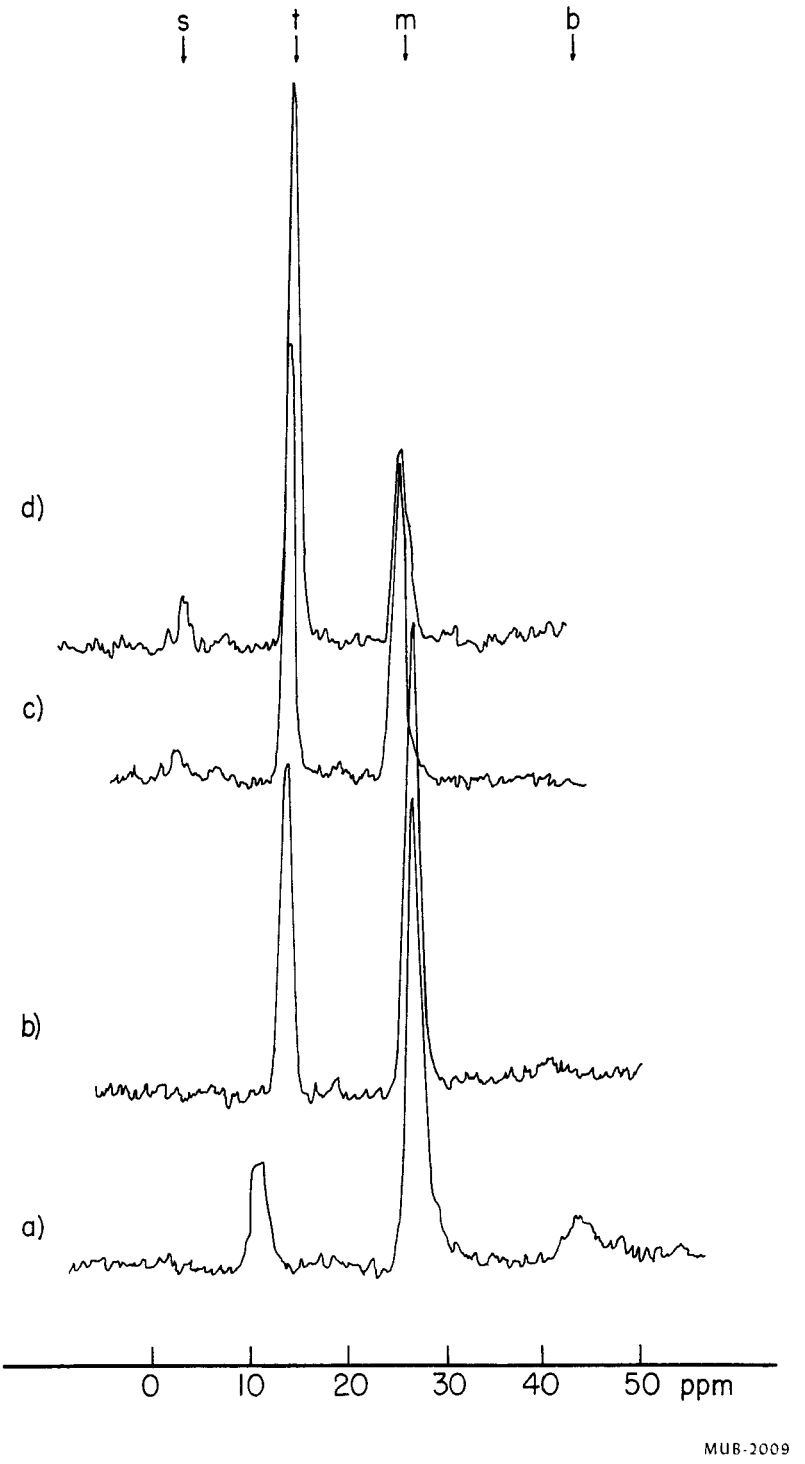


Fig. 15-4. Series similar to that of Fig. 15-3, but using ethanol as additive in place of water.

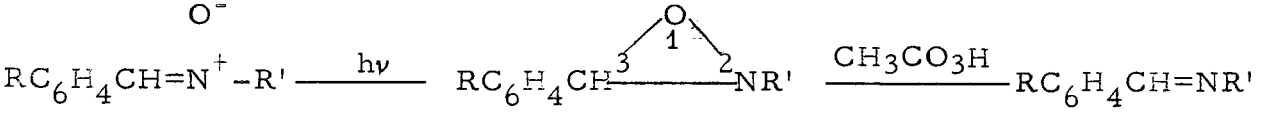
It is probable that the relative amounts of structures I and II depend on the preparation and could account for some variation in the behavior of this material in polycondensation reactions. However, a ratio of the order of 1:1 can be expected from the original structure of P_4O_{10} and the observed higher stability of structure II towards hydrolysis.

16. A STUDY OF THE IRRADIATION PRODUCTS OF SEVERAL NITRONES

Janet S. Splitter

Certain nitrones have been known for many years to be sensitive to light.[1-3] For example, it has been reported that N-α-diphenylnitrone, when irradiated, yields a mixture of benzanilide, benzaldehyde, nitrosobenzene, azoxybenzene, and o-hydroxyazobenzene.[1] In recent years, several nitrones were prepared in the course of a study of the irradiation products of o-nitrostilbenes[4] and all were found to be photosensitive in solution. Following the report by Emmons[5a] of the synthesis of oxaziridines by the peracetic acid oxidation of certain imines, the suggestion arose that oxaziridines might be early irradiation products of nitrones. At this time, others also considered this same possibility.[6,7]

In a prior communication three oxaziridines (II, II, and III) synthesized by the peracetic acid method were shown to be identical to the irradiation product of the corresponding nitrone:[8]



A B C

1. L. Alessandri, Atti accad. Lincei, 19, II, 122; Chem. Zentr. 1910, II, 1043; Chem. Abstr. 5, 276 (1911).
2. O. Brady and A. McHugh, J. Chem. Soc. 125, 547 (1924).
3. L. Chardonnens and P. Heinrich, Helv. Chim. Acta 32, 656 (1949).
4. J. Splitter and M. Calvin, J. Org. Chem. 20, 1086 (1955).
5a. W. Emmons, J. Am. Chem. Soc. 78, 6208 (1956); 79, 5739 (1957). Others (5b, c) also reported this same reaction.
5b. L. Horner and E. Jürgens, Chem. Ber. 90, 2184 (1957).
5c. H. Krimm, Chem. Ber. 91, 1057 (1958).
6. M. Kamlet and L. Kaplan, J. Org. Chem. 22, 576 (1957).
7. F. Kröhnke, Ann. Chem. 604, 203 (1957).
8. J. Splitter and M. Calvin, J. Org. Chem. 23, 651 (1958).

In this report, the types of compounds are designated by capital letters and the specific compounds by roman numerals, as follows.

A = nitrone
B = oxaziridine
C = imine
D = benzamide
E = formanilide
F = aldehyde and hydroxylamine
G = benzylamine
H = cation (from nitrone)
J = cation (from oxaziridine)

I $R = NO_2$, $R' = C_2H_5$
II $R = NO_2$, $R' = t\text{-}C(CH_3)_3$
III $R = H$, $R' = t\text{-}C(CH_3)_3$
IV $R = NO_2$, $R' = C_6H_5$
V $R = H$, $R' = C_6H_5$
VI $R = (CH_3)_2N$, $R' = C_6H_5$
VII $R = (CH_3)_2N$, $R' = m\text{-}NO_2C_6H_4$

Because the peracetic acid oxidation method could be used only on acid-stable imines, and only for n-alkyl oxaziridines relatively stable at room temperature, the method was useful only for a limited number of oxaziridines.

Since the time of the previous report other reports have described the isolation of oxaziridines formed by the irradiation of the corresponding nitrones.[9,10] Similar oxaziridines have been synthesized by another method.[11]

In this study, several N,α-diarylnitrones (IV, V, VI, VII) were investigated, and in every case the first irradiation product was quite unstable, the stability varying according to substituent and solvent. The ultraviolet (uv) absorption spectra of the first irradiation products of nitrones IV and V in ethanol were very similar to those of oxaziridines II and III, respectively. By a modified method, the first irradiation products from nitrones IV and V in ethanol contained a high percentage of active oxygen (measured by release of I_2 from KI). All the N,α-diarylnitrones, IV, V, VI, and VII, produced first irradiation products that were rearranged or hydrolyzed or both, or reacted further in the dark to yield end products that varied in amount with different solvents. In Fig. 16-1 are shown the spectra of nitrone VI, oxaziridine VI, and all the products resulting from oxaziridine VI:

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9. R. Bonnett, V. M. Clark, and Sir A. Todd, J. Chem. Soc. 1959, 2102.
10. L. H. Sternbach, B. A. Keochlin, and E. Reeder, J. Org. Chem. 27, 4671 (1962).
11. E. Schmitz, R. Ohne, and D. Murawaki, Angew. Chem. 73, 708 (1961).

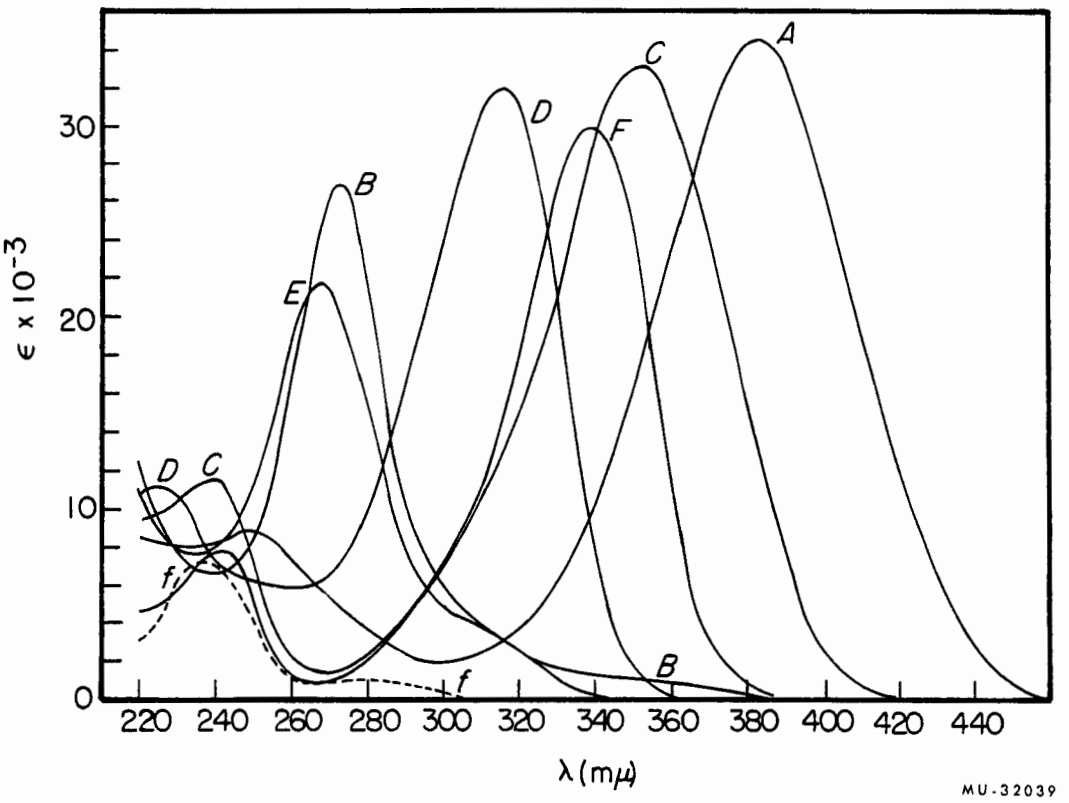
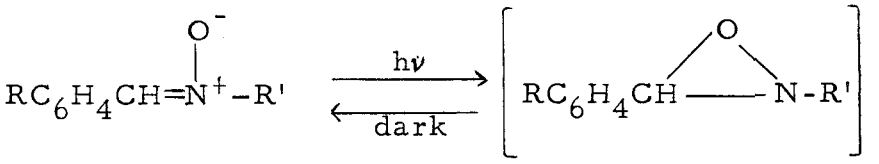
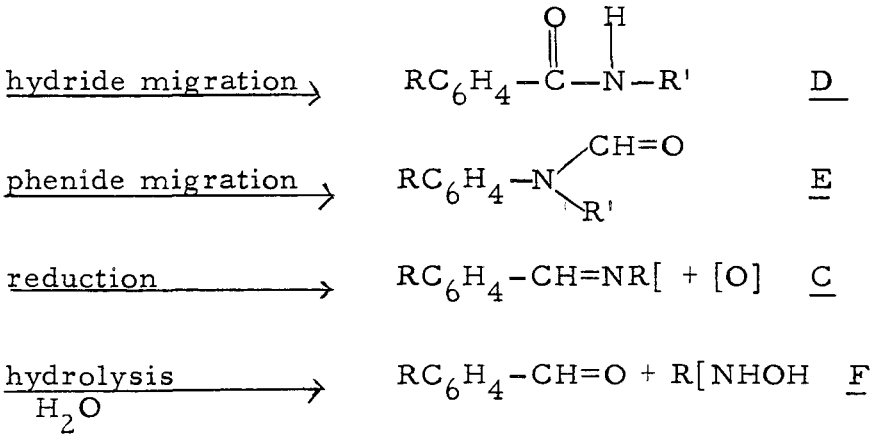


Fig. 16-1. Absorption spectra in absolute ethanol except where noted: (A) α-(p-dimethylaminophenyl)-N-phenyl-nitrone; (B) 3-(p-dimethylaminophenyl)-2-phenyloxaziridine (in ether); (C) N-(p-dimethylaminobenzylidene) aniline (D) 4-dimethylaminobenzanilide; (E) N-(p-dimethylaminophenyl) formanilide; (F) p-dimethylaminobenzaldehyde; (f) n-phenylhydroxylamine.



A B



D E C F

These dark reactions were very similar to those that Emmons found for the 2-alkyl-3-aryloxaziridines.[5a] Thus, it was concluded that N, α-diaryl-nitrones also form oxaziridines as the initial products of irradiation.

In Table 16-I are given the yields of the various products, both those isolated and those determined spectrophotometrically from the irradiation of seven nitrones in several kinds of solvents. It is apparent from this table that substituent differences result in great variation in the stability and reactions of the oxaziridines. In addition, there is a certain amount of solvent dependence in the course of rearrangement of oxaziridines IV, V, and VI.

Because of the reactivity of the 2, 3-diaryloxaziridines IV, V, VI, and VII, the spectra of these oxaziridines (Fig. 16-2) were recorded immediately after irradiation of the corresponding nitrones (Fig. 16-3). The spectra of oxaziridines VI and VII could not be obtained in ethanol because of extensive rearrangement. That of VI was obtained in ether and isooctane and that of VII in ether. Kamlet and Kaplan's spectral data in methanol after irradiation of three N, α-diarylnitrones were not of oxaziridines alone, but of mixtures of oxaziridines with rearranged products.[6] This was due to the 1-hour irradiation time, whereas only a few minutes were sufficient at 10^{-5} M concentrations.

Table 16-II gives the λ_{max} and extincition coefficient (ϵ) of oxaziridines obtained immediately after the minimum irradiation required for complete disappearance of the nitrone. The ϵ was determined by assuming 100% conversion of the nitrone to the oxaziridine. For comparison, the

Table 16-I. Percent yields of products from the irradiation of seven nitrones in various solvents[a]

Nitrone A	Solvent	Oxaziridine B		Benzamide D		Formanilide E		Nitrone A	Imine C	Hydrolysis products F	
		iso-lated	active O_2%[a]	iso-lated	spect. deter.	iso-lated	spect. deter.	spect. deter.	spect. deter.	isolated as resin	spect. deter. as aldehyde
I	CH_3CN	35	63								
II	C_2H_5OH	40	91								
III	CH_3CN	b	90								
IV	C_2H_5OH	c	(72)	30	31	18	19				39
	CH_3COCH_3	c		75	78		5		4	12	
	C_6H_6	c		72	72	trace	4				23
	iso-C_8H_{18}	c			60						35
V	C_2H_5OH	c	(75)	5	11	53	69	trace	trace		19
	CH_3COCH_3	c		81	85	6	7	trace		8	
	C_6H_6	c			53		15	trace			
	iso-C_8H_{18}	c		70	72		3	trace		24	20
VI	C_2H_5OH	c				73	86	4	5		5
	CH_3COCH_3	c		40	47	25[d]	21	22	trace		trace
	C_6H_6	c			trace		80	4	trace		trace
	iso-C_8H_{18}	c			6		75	9	trace		trace
VII	C_2H_5OH	c					6	94[e]			
	CH_3COCH_3	c						74			
	C_6H_6							88			

a. Active oxygen contents were determined on irradiated ethanol solutions of nitrones, assuming 100% conversion to the oxaziridine. The figures in parentheses were obtained by analysis immediately after irradiation. There was no active oxygen content immediately after irradiation of nitrones VI and VII in ethanol, because in this solvent there was rearrangement of the oxaziridines when the measurement was made.

b. 97% nitrone re-formed with heat.

c. Not isolated because these oxaziridines were unstable at room temperature.

d. The yield is larger than that determined spectrophotometrically because the isomerized nitrone was reirradiated.

e. The cis nitrone was formed first.

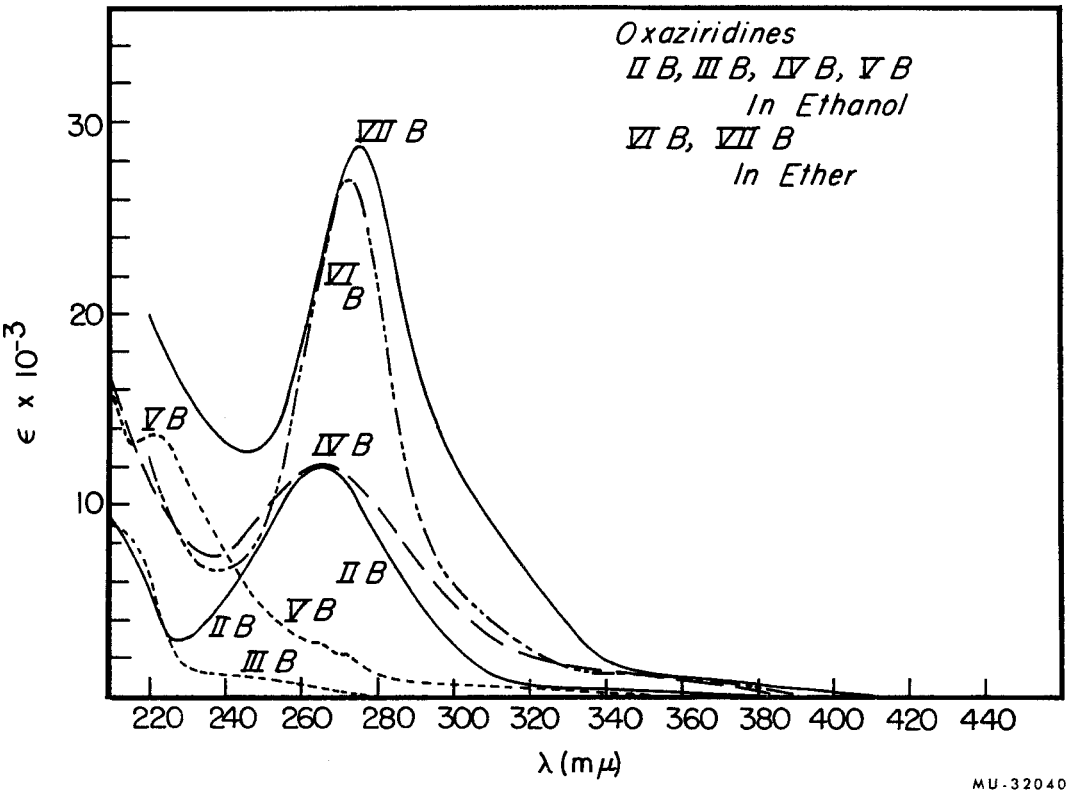


Fig. 16-2. Absorption spectra of the substituted oxaziridines.

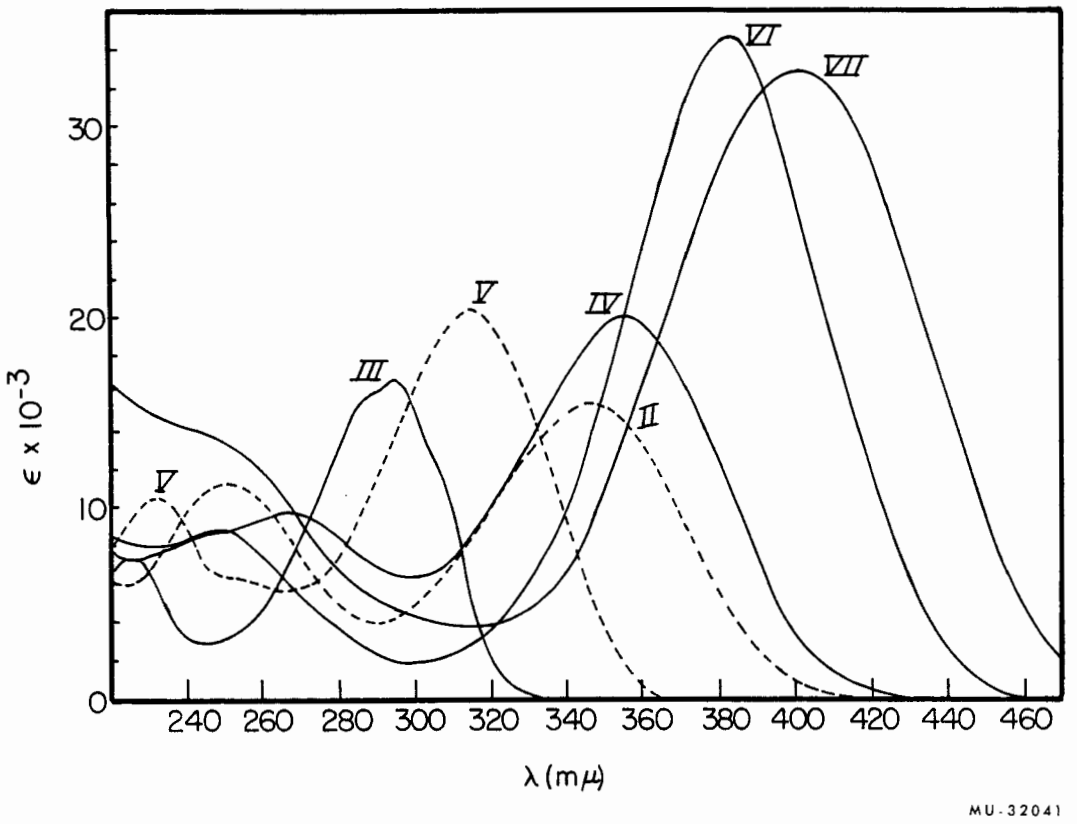


Fig. 16-3. Absorption spectra of the substituted nitrones in absolute ethanol.

Table 16-II. Comparison of uv absorption maxima of the oxaziridines with the corresponding nitrones, imines, benzamides, and benzylamines in ethanol.[a]

	Oxaziridine (B) mμ and (ε)	Nitrone (A) mμ and (ε)	Imine (C) mμ and (ε)	Benzamide (D) mμ and (ε)	Benzylamine (G) mμ and (ε)
III	210(9000) 249(930)[c]	225(7400) 295(16,700)[c]	247(17,000)[d]	225(10,000)[b] 267(800)[b]	208(8000) 258, 264(160)[e]
V	222(13,600) 265(2800) 272(2200)	232(10,800)[f] 314(20,300)[f]	264(16,000)g 317(10,000)g	270(12,600)[h] 306s(2000)	250(12,600)[i] 294(2000)
II	268(11,900)[j]	250(11,200) 346(15,400)	280(17,000)[k]	265(12,600)[l]	272(10,000)[m]
IV	266(12,100)	265(9500)[f] 355(20,000)[f]	290(12,500)g 340(9300)g	242(16,500) 292(9200)	246(16,000)[n] 270(12,600)[n] 332s(630)[n]
VI	272(27,000)[q]	248(8800) 383(34,600)	358(34,000)[p]	305(22,000)[o] 316(32,000)	
VII	275(28,700)[q]	250s(13,300) 401(32,800)	358(34,000)[r]		

a. Absorption maxima (λ) in mμ; molar absorptivities (ε) in parentheses; s after wavelength figures indicates a shoulder.
b. K. Miescher, A. Marxer, and E. Urech, Helv. Chim. Acta 34, 1 (1951); R' = H.
c. W. Emmons, J. Am. Chem. Soc. 79, 5739 (1957).
d. G. E. McCasland and E. C. Horswill, ibid. 73, 3923 (1951); R' = methyl.
e. P. Grammaticakis, Bull. Soc. Chim. France, 1947, 664; R' = H.
f. M. Kamlet and L. Kaplan, J. Org. Chem. 22, 576 (1957), in methanol.
g. P. Grammaticakis, Bull. Soc. Chim. France, 1951, 965.
h. A. Kotera, S. Shibata, and K. Sone, J. Am. Chem. Soc. 77, 6183 (1955).
i. E. A. Smirnov, Sbornik Statei Obschei Khim. 2, 1394 (1953).
j. W. Emmons, (in acetonitrile) J. Am. Chem. Soc. 79, 5739 (1957).
k. A. Burawoy and J. Critchley, Tetrahedron 5, 340 (1959); R' = methyl.
l. P. Grammaticakis, Bull. Soc. Chim. France, 1953, 207; R' = H.
m. A. Burawoy and E. Spinner, J. Chem. Soc. 1955, 2557; R' = H.
n. V. Ismailskii and E. Smirnov, Zhur Obsch. Khim. 25, 1400 (1955).
o. Ref. 1 for R' = H, R = $p(CH_3)_2NC_6H_4$.
p. R. L. Reeves and W. F. Smith, J. Am. Chem. Soc. 85, 724 (1963), in 50% ethanol.
q. In ether.
r. Estimated ε.

maxima and intensities of the corresponding nitrones, imines, benzamides, and benzylamines are included. As previously reported by Emmons, the oxaziridine ring does not appear to have any characteristic absorption.[5a] However, as seen from Table 16-II, the spectral characteristics of the oxaziridines appear to be somewhere between those of the corresponding benzamide and benzylamine--that is, the 3-aryl electronic system doesn't interact with the oxygen electronic system as fully as it does in the benzamide. This is analogous to the absorption data found for α-phenylpropylene oxide,[12] in contrast to those for acetophenone and benzyl alcohol. The authors concluded that there was considerable conjugation between the aromatic ring and the oxide grouping.[12] Also, the oxaziridine spectra (Fig. 16-1) are somewhat similar in shape to that of α-phenylpropylene oxide.[12] The interaction of the 2-aryl electronic system with the oxaziridine nitrogen is also somewhat restricted from what it is in the aryl amine. This may be due to a possible distortion of the nonbonding "p" orbitals of the nitrogen atom as a result of the strained-bond angles in the oxaziridine ring.

The 2,3-diaryloxaziridines IV, V, VI, and VII from cations with dilute sulfuric acid in ethanol or acetone solution in sufficient concentrations to be measured spectrophotometrically. Only nitrones VI and VII and imines VI and VII form appreciable amounts of cations under the same conditions. In a recent report the conjugate acid of imines V and VI were reported to have the λ_{max} at a longer wavelength and with a higher ϵ than the parent imines.[13] With their conditions, the pseudo first-order decay half-time of the conjugate acid of imine VI (9.4 sec) was far greater than that of imine V (0.03 sec). A higher concentration of acid was used in their work than in this study.

In Fig. 16-4 are given the absorption spectra of oxaziridine cations IV and V in ethanol and of VI and VII in acetone. In Fig. 16-5 are given the absorption spectra of nitrone and oxaziridine VI and their respective cations in acetone. In Table 16-III are tabulated the absorption maxima and intensities of the cations from these oxaziridines, nitrones, and imines that exhibited cation formation with dilute sulfuric acid. The pseudo first-order decay half times of the cations are also given. It is evident that the cations absorb at longer wavelengths and with higher intensities than their parent compounds. Reeves and Smith concluded that the cation from imine VI was the conjugate acid.[13] Similarly, the cations from nitrones VI and VII would be the conjugate acids.

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12. T. W. Campbell, S. Linden, S. Godshalk, and W. A. Young, J. Am. Chem. Soc. 69, 880 (1947).

13. R. L. Reeves and W. F. Smith, J. Am. Chem. Soc. 85, 724 (1963).

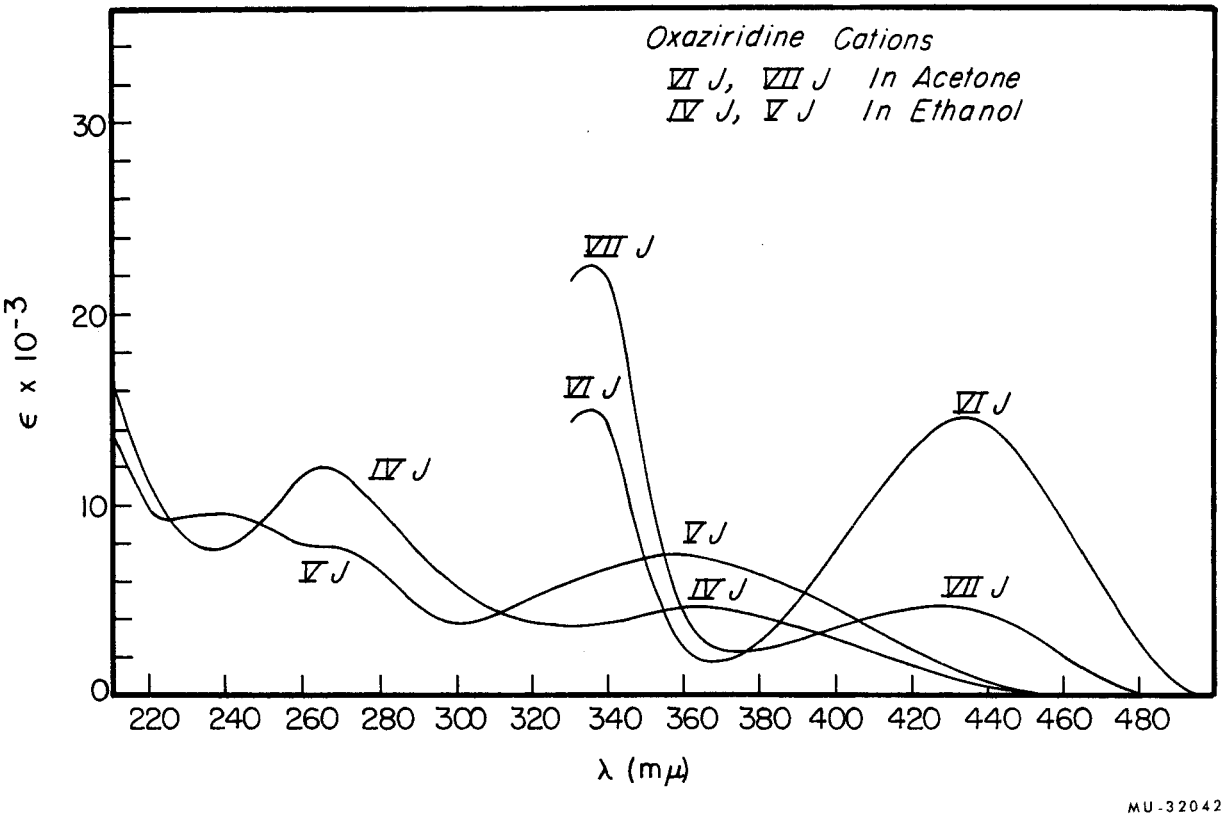


Fig. 16-4. Absorption spectra of the substituted oxaziridine cations. The cations were formed by adding to 3.5 ml of oxaziridine solution 1 drop of 0.1 N H_2SO_4 for VI J and VII J and 1 drop of 2.5% H_2SO_4 (by vol) for IV J and V J.

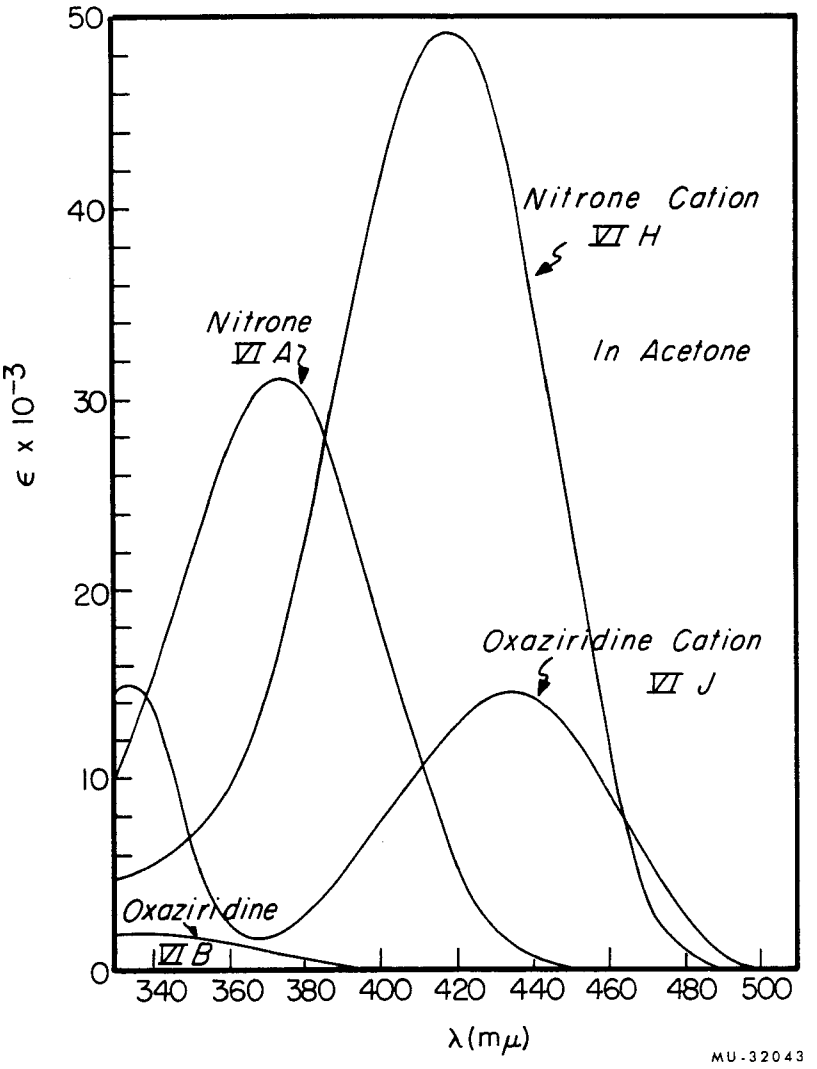


Fig. 16-5. Absorption spectra of nitrone VI, oxaziridine VI, and their respective cations, which were formed by adding 1 drop of 0.1 N H_2SO_4 to 3.5 ml of solution.

Table 16-III. Ultraviolet absorption maxima and decay half-times of the cations formed from oxaziridines, nitrones, and imines with dilute sulfuric acid[a] at 26°.

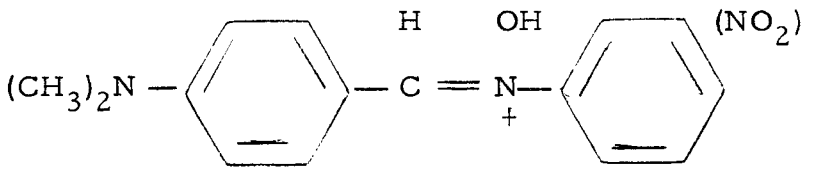
Cation from	In EtOH λ_{max}, ϵ	In CH_3COCH_3 λ_{max}, ϵ	In EtOH $t_{1/2}$, sec	In acetone $t_{1/2}$, sec
Oxaziridine				
IV	365(4600)[b] 365(4300)[c]	370(700)[b] 370(1500)[c]	270	10
V	358(8500)[b] 358(4500)[c]	360[d](2400)[b] 360[d](2000)[c]	70	10
VI	Rearr.	434(14,500)[b, c]	rearr.	1150
VII	Isom.	430(5000)[b] 430(4600)[c]	isom.	250
Nitrone				
VI	420(49,000)[b] 410(35,000)[c]	419(49,000)[b, c]	340	2400
VII	430(37,000)[b] 415(32,000)[c]	427(45,000)[b] 427(42,000)[c]	35	300
Imine				
VI	435(47,000)[b, c]	435(43,000)[b, c]	45	840
VII	440	445	2	45

a. ϵ based on nitrone or imine used. The pseudo first-order decay half-time was determined with the 0.1 N H_2SO_4 as in footnote c; not extrapolated to 0 time. In ethanol, the time to reach maximum concentration of cation was 60 sec for oxaziridine IV, and 20 sec for oxaziridine V; in acetone, the time was 15 sec for oxaziridine VII; the time for all others was only a few seconds.

b. For 3.5 ml of approx 10^{-5} M solution, 1 drop of 10% H_2SO_4 (by vol) was used.

c. For 3.5 ml of approx 10^{-5} M solution, 1 drop of 0.1 N H_2SO_4 was used.

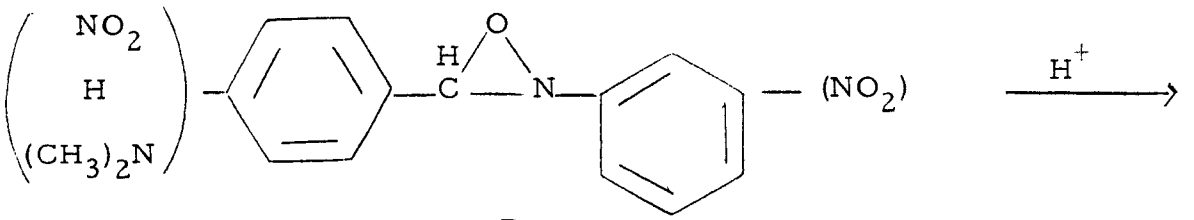
d. The λ_{max} is approximate.



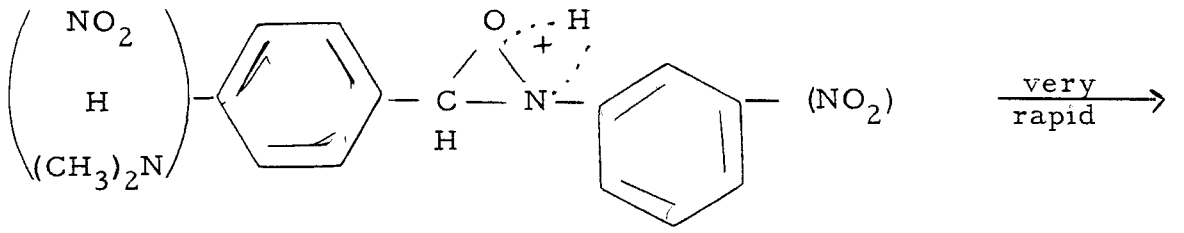
VI H, VII H

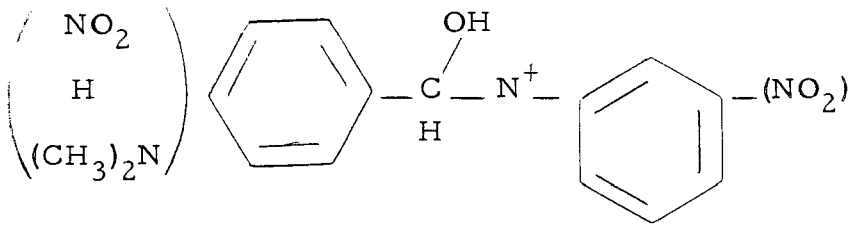
The absorption spectra of the nitrone conjugate acids were very similar to those of the respective imine conjugate acids, but the decay half times were far longer. The conjugate acids of the nitrones could be neutralized with reversion back to the nitrones. Irradiation of nitrone cation VI resulted in the formation of oxaziridine cation VI.

The absorption spectra of the 2, 3-diaryloxaziridine cations were similar in shape but exhibited different characteristics from those of their respective nitrone cations. On neutralization of the oxaziridine cations, hydrolysis occurred. For oxaziridine cation VII there was no evidence of C–O bond breakage, as was found for oxaziridine VII. Neutralization of oxaziridine cation VII did not yield the nitrone. The 2-alkyl-3-aryl oxaziridines did not form measurable amounts of cations under the same conditions used for the 2, 3-diaryloxaziridines. From these observations, the following structures are proposed for the intermediate and the oxaziridine cation:



B





J

In the series of oxaziridine cations IV, V, and VI, the oxaziridine cation VI was formed more easily than the others by the lower concentration of acid. Oxaziridine cation VII, with the m-nitro group, had a lower ϵ of the long-wavelength band and shorter decay half time than oxaziridine cation VI. The decay half times of the oxaziridine cations were longer in ethanol than in acetone, but those of the nitrone and imine cations were shorter in ethanol.

Kinetic Data

The kinetic data for the disappearance of the oxaziridines were obtained by several different methods. One method was to follow the rate of disappearance of oxaziridines IV and V in ethanol, and IV, V, and VI in acetone. This was done by adding dilute sulfuric acid to portions of the irradiated solution of the corresponding nitrones and measuring by spectrophotometric means the cation formed.

The major reaction of oxaziridine VII in all the solvents was isomerization to the nitrone, so nitrone formation was followed spectrophotometrically as a means of determining the rate of oxaziridine disappearance. In ethanol only, the first product formed was unstable and had characteristics expected of the cis-nitrone VII. The rate of nitrone formation from oxaziridine VI in all solvents was followed in the same way as for oxaziridine VII. In ethanol there was some evidence that cis-nitrone VI was formed before the trans-nitrone VI.

In the nonpolar solvents (benzene and isooctane), the formation of benzamides IV and V from oxaziridines IV and V, respectively, was followed spectrophotometrically through their own absorption spectra. The disappearance of oxaziridine VI in the same solvents was followed spectrophotometrically, since its spectrum and that of the products formed did not completely overlap. Figures 16-6, 16-7, 16-8, and 16-9 show the data obtained on the rates of disappearance of oxaziridines IV, V, and VI in ethanol, acetone, benzene, and isooctane. Figure 16-10 shows the data for oxaziridine VII in ethanol, acetone, and benzene.

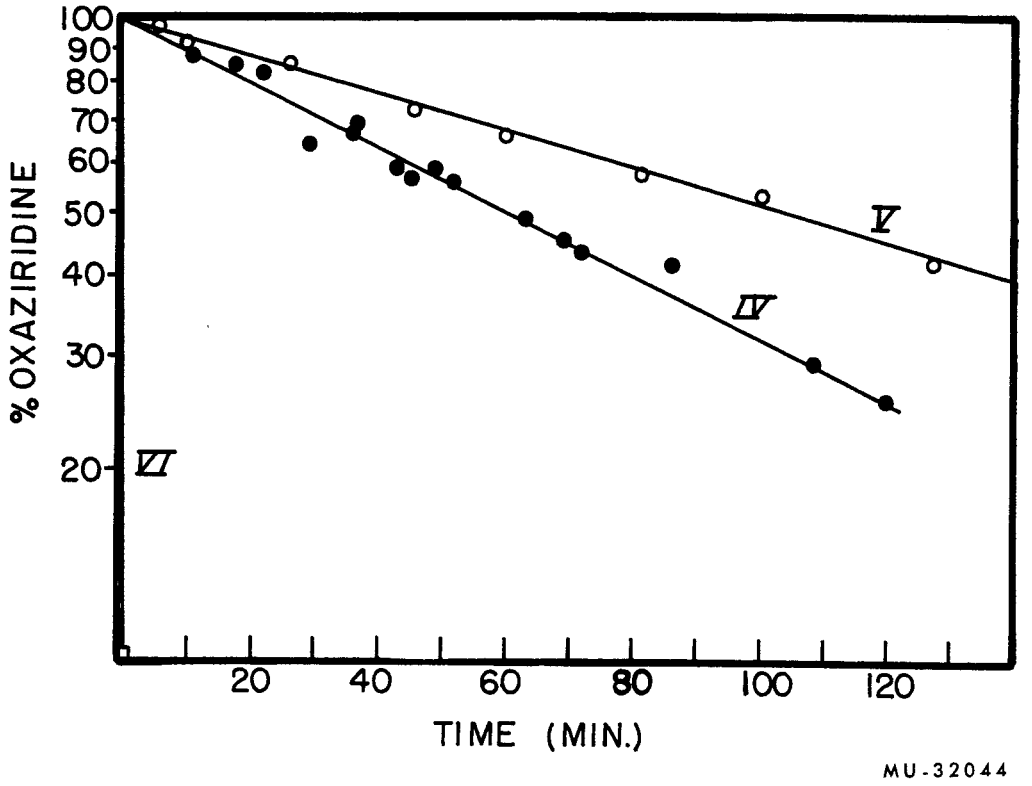


Fig. 16-6. Rates of disappearance of oxaziridines in absolute ethanol.

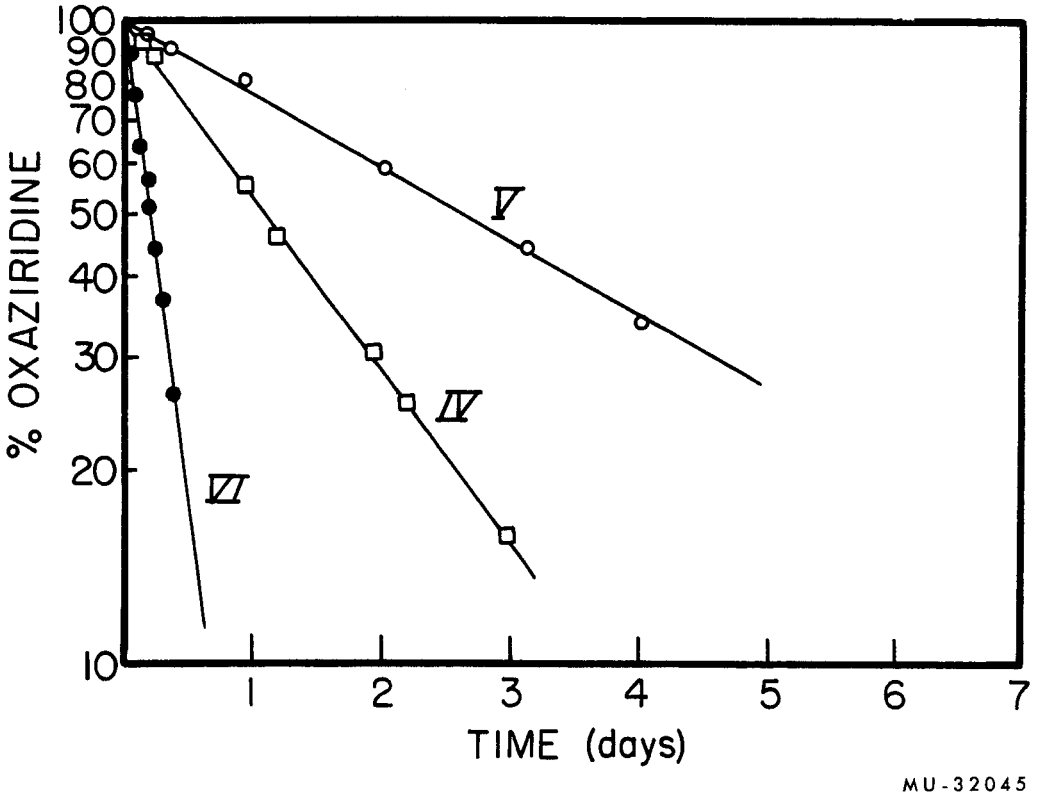


Fig. 16-7. Rates of disappearance of oxaziridines in acetone.

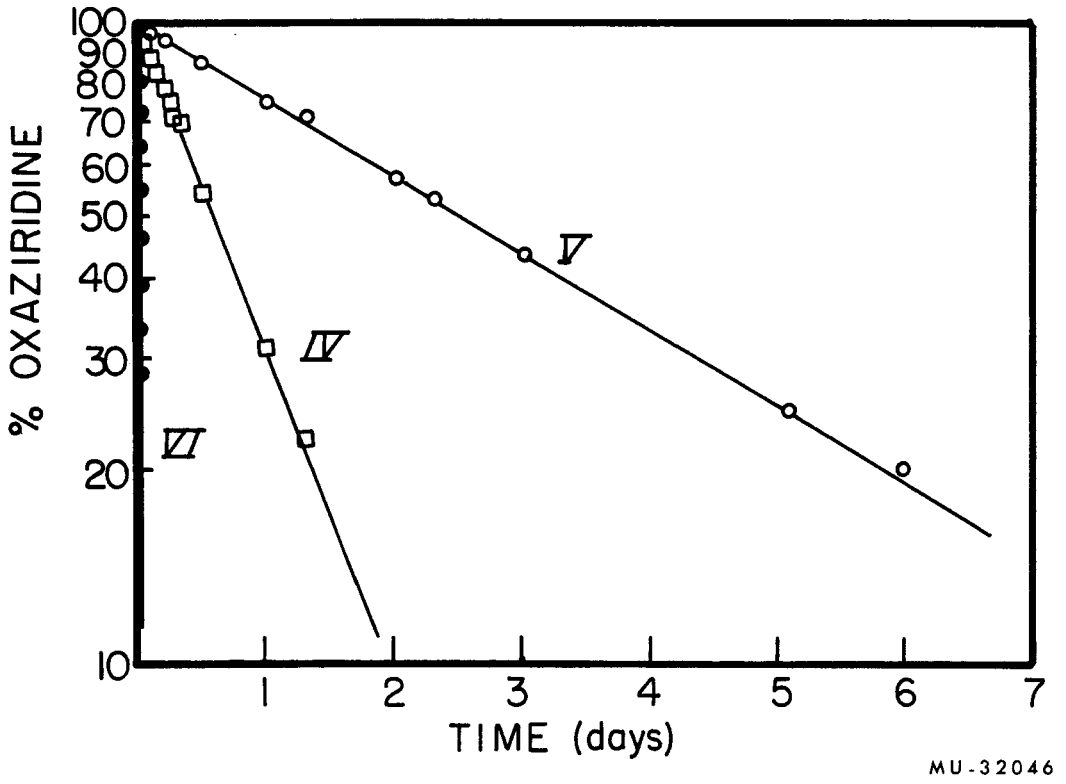


Fig. 16-8. Rates of disappearance of oxaziridines in benzene.

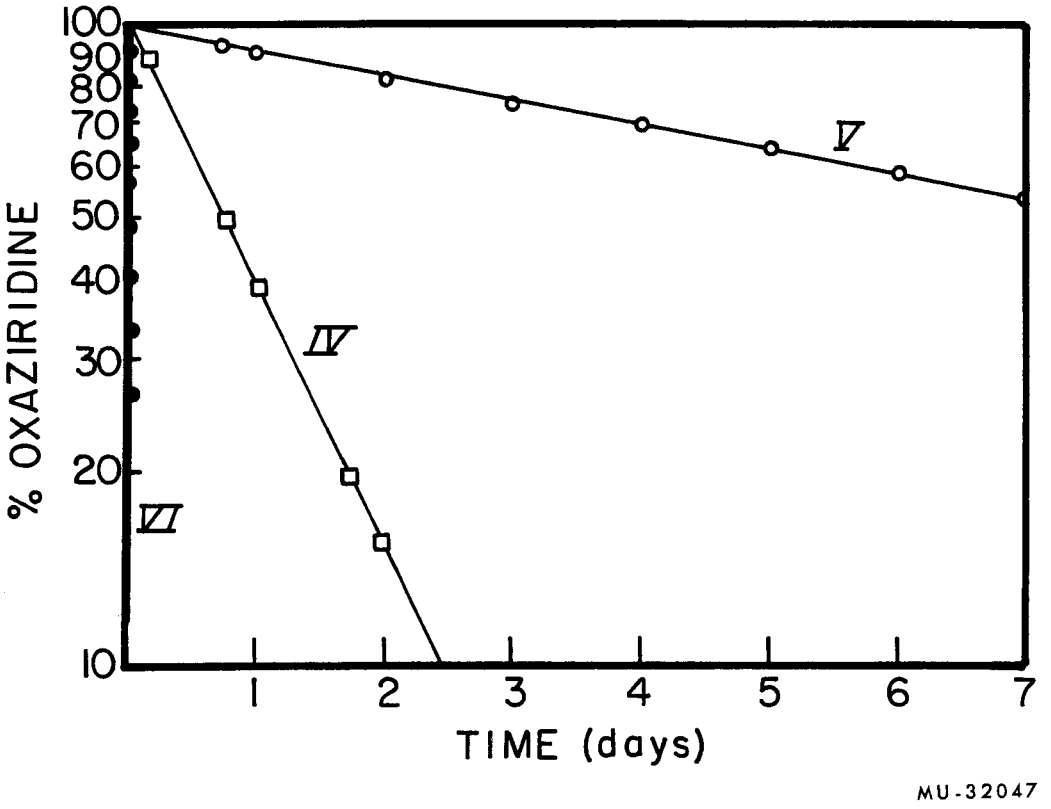


Fig. 16-9. Rates of disappearance of oxaziridines in isooctane.

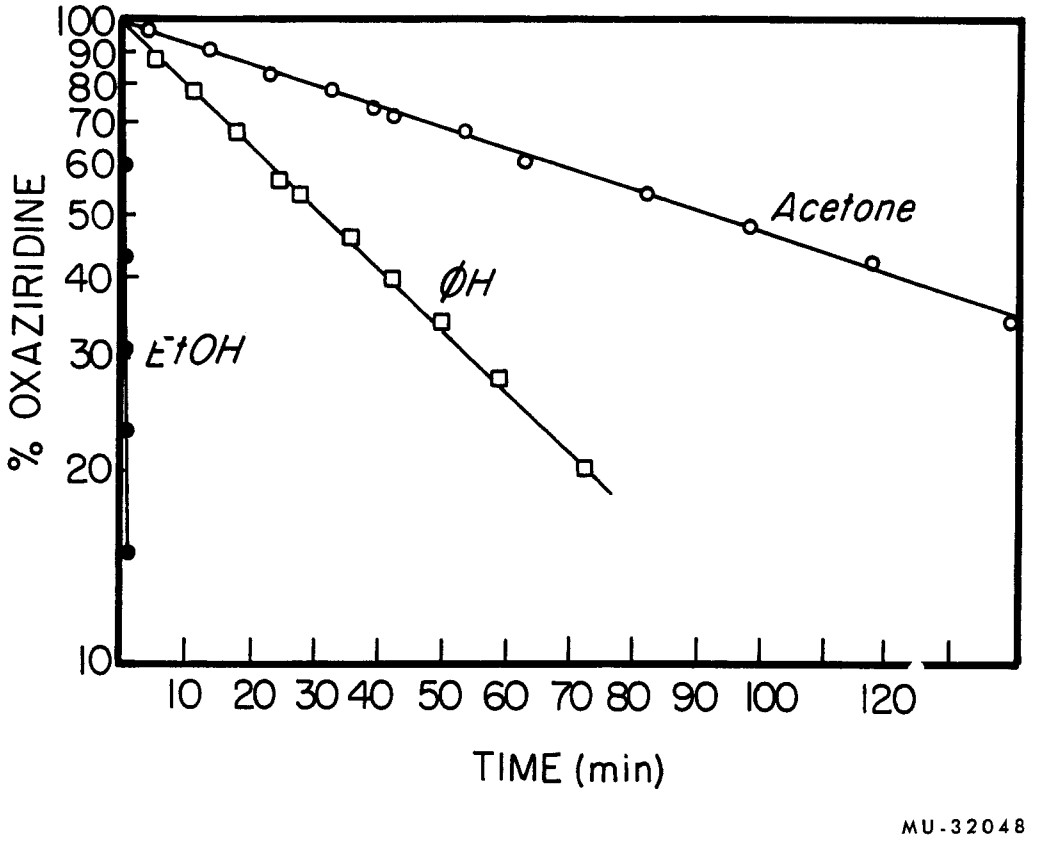
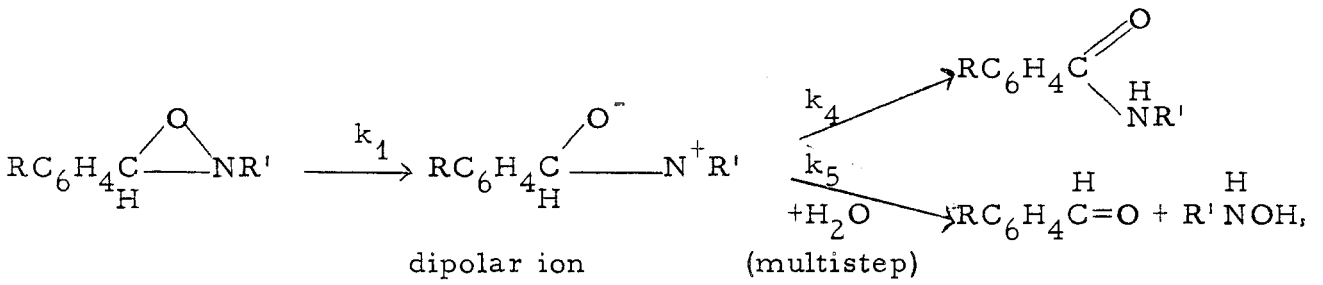


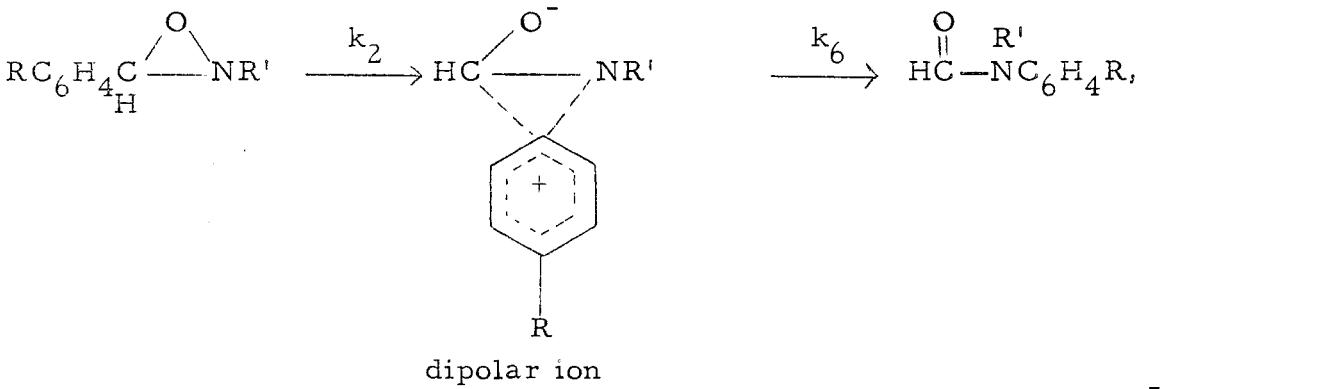
Fig. 16-10. Rates of disappearance of oxaziridine VII in absolute ethanol, benzene, and acetone.

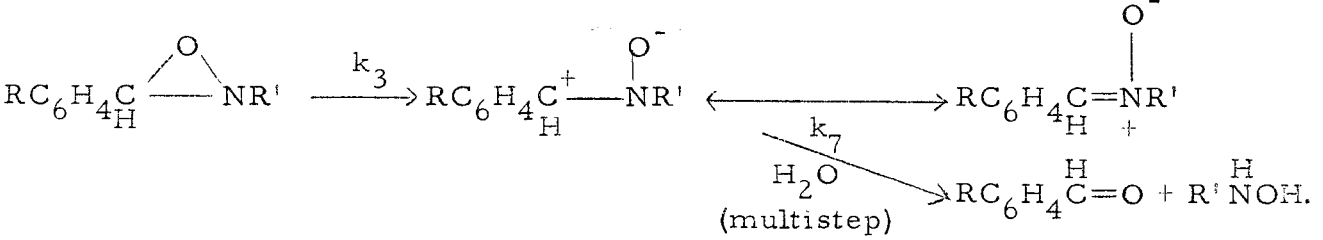
In ethanol, the active oxygen content, determined at different times after irradiation of nitrone V, correlated well with the amount of oxaziridine cation. Also, the amount of aldehyde present after hydrolysis with acid at different times after irradiation of nitrone VI in acetone and nitrone IV in benzene was consistent with the amount of oxaziridine cation VI and the amount of benzamide IV, respectively.

In Table 16-IV are given the overall rates of disappearance of oxaziridines (columns 3 and 4), assuming 100% formation of oxaziridine from nitrone, and the calculated rate constants of a number of primary rate-determining steps (k_1, k_2, k_3).

All the rates found experimentally could be expressed as first order in oxaziridine. A scheme that fits the first-order data observed, and that is consistent with the findings of a rate dependence on solvent, is given in the following chart.[14] Where there was aryl migration, there also was an increased rate of reaction. For this reason, participation of the aryl group[15] is shown in the primary rate-determining ionization step, k_2:







14. C. K. Ingold, Structure and Mechanism in Organic Chemistry (Cornell University Press, Ithaca, N. Y., 1953), 345-350.
15. S. Winstein, C. Lindegren, H. Marshall, and L. Ingraham, J. Am. Chem. Soc. 75, 147 (1953).

Table 16-IV. The rates of disappearance of oxaziridines in various solvents and rate constants of the primary rate-determining steps at 25°.

Oxaziridine	Solvent	Half-time (sec)	Rate of disappearance of oxaziridine $\frac{0.693}{t_{1/2}} \times 10^5$ (sec^{-1})	$k_1 \times 10^5$ (sec^{-1})	$k_2 \times 10^5$ (sec^{-1})	$k_3 \times 10^5$ (sec^{-1})
IV	Ethanol	3,600	19.3	15.6	3.7	--
	Acetone	95,500	0.73	0.66	0.04	--
	Benzene	50,400	1.38	1.32	0.06	--
	Isooctane	64,800	1.07	1.07	--	--
V	Ethanol	6,240	11.1	3.4	7.7	
	Acetone	228,000	0.30	0.28	0.02	
	Benzene	216,000	0.32	0.27	0.05	
	Isooctane	654,000	0.11	0.107	0.003	
VI	Ethanol	2	34,600	1,700	27,600	3,460
	Acetone	18,000	3.85	2.19	0.81	0.85
	Benzene	900	77.0	12.0	62	3.0
	Isooctane	960	72.3	12.0	54	6.3
VII	Ethanol	5	13,900	--	800	13,100[a]
	Acetone	5,560	12.5	--	--	12.5
	Benzene	1,860	37.3	--	--	37.3

a. This rate constant is for isomerization to the cis form. The rate constant for the isomerization of cis-nitrone VII to trans-nitrone VII was found to be 35×10^{-5} sec^{-1}; half-time was 1,920 sec.

The polar transition states lead to the formation of one or more of the intermediate dipolar ions as rate-determining steps.

In ethanol and acetone the rate of disappearance of oxaziridine (IV and V) was followed directly, and the observed rate constants were taken to be the sum of k_1 and k_2, assuming k_{-1} and k_{-2} to be negligible. In benzene and isooctane, the formation of the benzamide was used to follow the reaction.

For oxaziridine VI in all solvents except acetone, the rate-controlling step consists mainly of k_2, with small contributions from k_1 and k_3. In acetone the contribution of k_2 is reduced and that of k_1 and k_3 increased. For oxaziridine VII in all solvents, k_3 is the main contributor to the rate-determining step.

A side reaction that has been detected only in small amounts (5% or less) is the formation of imine. Recently the irradiation of N, α-diphenylnitrone (V) in concentrated methanol solution was reported to yield considerable quantities of imine, benzaldehyde, and nitrosobenzene.[16] Very probably there were traces of acidic material present in sufficient amounts to cause hydrolysis of the oxaziridine. As the N-phenylhydroxylamine was formed it could have been oxidized by the oxaziridine. This oxaziridine (V) is a very active oxidizing agent for potassium iodide. If the hydrolysis reactions are inhibited by control of acidity, as was the case in our studies, then the formation of the imine would be inhibited also. Besides imine in the ethanol reactions of oxaziridine IV, which was most subject to hydrolysis, there were found traces of a compound with λ_{max} of 415 mμ, which, with sulfuric acid, was converted to a form with λ_{max} of 535 mμ. This behavior resembles that of 4-phenylaminoazobenzene,[17] which might have been formed by a series of redox reactions and condensation from phenylhydroxylamine.[18] Similar types of compounds were noted to a small extent in the reactions of oxaziridine V in ethanol. With oxaziridine VI, imine was found to about the same extent as hydrolysis, both being very small amounts.

Discussion of Results

The initial product of irradiation of the nitrones is the corresponding oxaziridine.[10] The oxaziridine structure was established by (a) identity with the oxaziridines that could be synthesized from the corresponding imines, (b) active oxygen content, and (c) reactions similar to those found by Emmons for oxaziridines more stable than the 2, 3-diaryloxaziridines.[5a]

Oxaziridine Formation

The substituent difference between nitrone IV and nitrone VI caused a difference only in the apparent speed of oxaziridine formation upon

16. H. Shindo and B. Umezawa, Chem. and Pharm. Bull. 10, 492 (1962).
17. G. Badger, R. Buttery, and G. Lewis, J. Chem. Soc. 1954, 1888.
18. E. Bamberger, Chem. Ber. 27, 1548 (1894); 34, 61 (1901).

irradiation. The solvent effects on the ultraviolet absorption spectra of nitrones IV and VI are shown in Figs. 16-11 and 16-12. However, the solvent effects on the spectrum of nitrone V, which has no substituents on the benzene ring, are similar to those found for N-methyl-α-phenylnitrone.[19] The λ_{max} of the main absorption band of nitrone V and 320 mμ in isooctane, 317 mμ in acetonitrile, and 314 mμ in ethanol. Blue shifts occur with increasing polarity of the solvent similar to those found for pyridine N-oxide.[20] This observation indicates a less polar form for the excited state than for the ground state.[19] In order to obtain more information about the nature of the nitrone excited atate, physical measurements such as the emission spectra and the quantum yield as a function of wavelength and temperature are being determined.

Oxaziridine Rearrangement

Large solvent effects, indicating formation of charge in the transition state, provide evidence that the reactions of oxaziridines proceeded by ionic mechanisms.[14] The first step, and presumably the rate-determining one, was breaking of either the N–O or the C–O bond. When the C–O bond was broken, isomerization to the nitrone resulted. When the N–O bond was broken, rearrangement or hydrolysis reactions were initiated. The rearrangement reactions were typical 1, 2 shifts of a hydride ion, or of a group, with its pair of electrons to an electron-deficient atom.

In Table 16-V are summarized results of the work presented here and the literature data available on oxaziridine-bond cleavage according to oxaziridine type for both uncatalyzed and acid-catalyzed reactions. Assignment of the bond cleaved was made according to the nature of the products formed. Oxaziridines with 2-alkyl groups or 3-dialkyl groups were found to be sufficiently stable to be isolated. The 2, 3-diaryloxaziridines were stable enough in certain solvents to have absorption spectra measured. The 2, 3, 3-triaryloxaziridine was presumed to be only a very-short-lived intermediate.[21] N–O bond cleavage predominated when the groups on the carbon and nitrogen atoms were approximately equivalent. But when there was one aryl group on the carbon atom in excess of that on the nitrogen atom, C–O cleavage occurred. In the 2, 3-diaryl series, with one aryl group on both nitrogen and carbon atoms, C–O cleavage occurred to a major extent only when there was a strong electron-donating group on the carbon aryl group and an electron-withdrawing group on the nitrogen aryl group. Thus, in order for C–O bond breaking to occur, a high degree of stabilization of the developing carbonium ion relative to the nitrogen atom appears to be necessary.

The 2, 3-diaryloxaziridines described in this report were very reactive in comparison with the relatively stable 2-alkyl-3-aryloxaziridines.

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19. T. Kubota and M. Yamakawa, Bull. Chem. Soc. Japan 35, 555 (1962).
20. E. M. Kosower, J. Am. Chem. Soc. 80, 3253 (1958).
21. A. W. Johnson, J. Org. Chem., 28, 252 (1963).

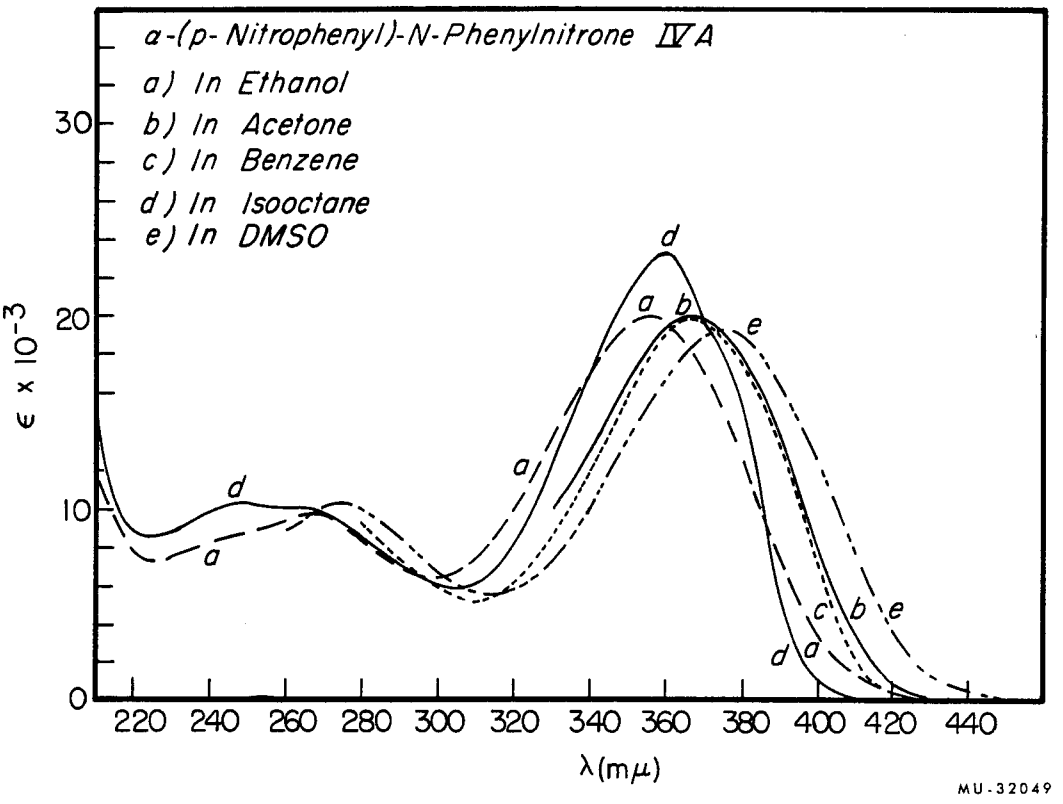

Fig. 16-11. Absorption spectra of nitrone IV in five solvents.

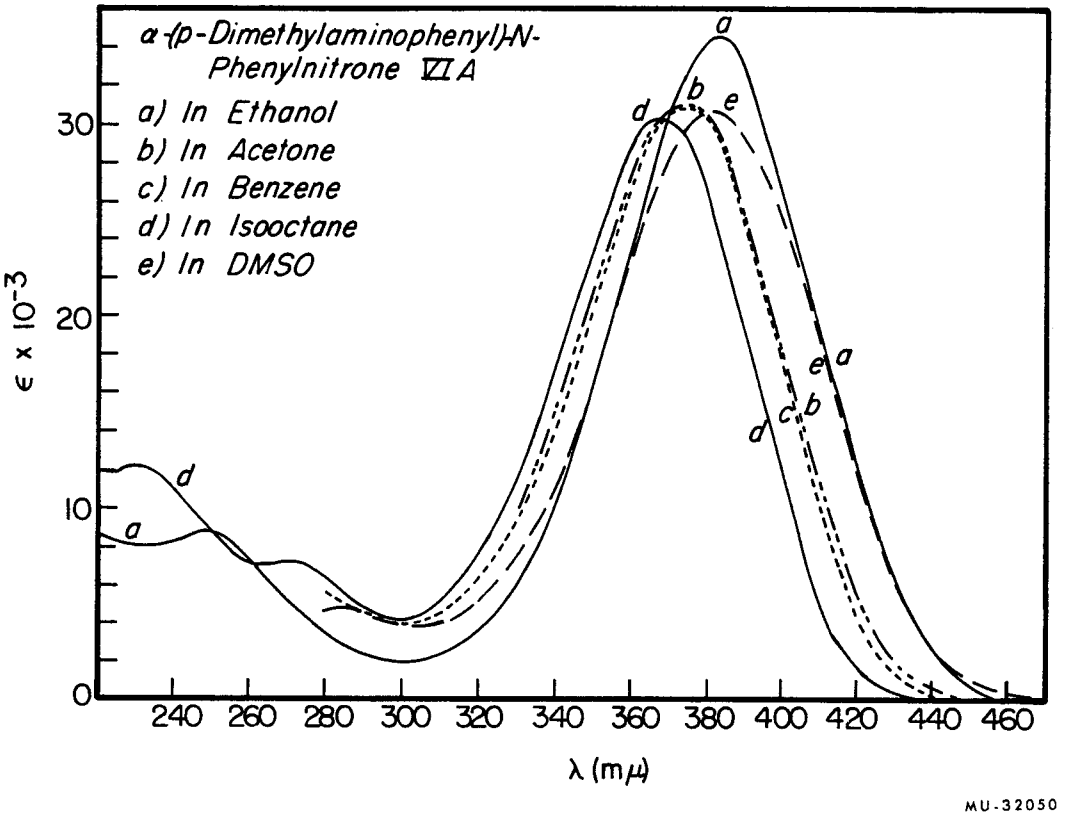

Fig. 16-12. Absorption spectra of nitrone VI in five solvents.

Table 16-V. Oxaziridine-bond cleavage in relation to oxaziridine type.

Oxaziridine Type	Method of Preparation	Bond Cleaved	
		Uncatalyzed reactions	Acid-catalyzed reactions[a]
2-alkyl, 3 aryl	Imine oxidation[b, c, d]	C-O	
2-alkyl, 3, 3-diaryl	Nitrone irradiation[e]	C-O	C-O
2, 3, 3-triaryl	Imine oxidation[f]	C-O	C-O
2, 3-diaryl	Nitrone irradiation[g, h, i]	N-O, C-O	N-O
2-aryl, 3-acyl	Nitrone irradiation[j]	N-O	
2-aryl, 3, 3-dialkyl	Imine oxidation[d]	N-O	
2, 3-dialkyl	Imine oxidation[b, c, d]	N-O	N-O
	Nitrone irradiation[k]		
2, 3, 3-trialkyl	Imine oxidation[b, c, d]	N-O	N-O

a. Sulfuric acid or hydrochloric acid solution.
b. W. Emmons, Ref. 5a.
c. L. Horner and E. Jürgens, Ref. 5b.
d. H. Krimm, Ref. 5c.
e. L. H. Sternbach, B. A. Koechlin, and E. Reeder, J. Org. Chem. 27, 4671 (1962).
f. A. W. Johnson, J. Org. Chem. 28, 252 (1963); presumed to be a very short-lived intermediate in the formation of nitrone.
g. L. Alessandri, Ref. 1; probably an intermediate in the rearrangement of the nitrone.
h. J. Splitter and M. Calvin, Ref. 8 and this report; spectrophotometrically detected.
i. L. Chardonnens and P. Heinrich, Ref. 3; probably an intermediate in the rearrangement of the nitrone.
j. F. Kröhnke, Ref. 7.
k. R. Bonnett, V. M. Clark, and Sir A. Todd, J. Chem. Soc. 1959, 2102.

This reactivity was attributed to the stabilization of the developing nitrogen cation by the aryl group in those oxaziridines (IV, V, and VI) that undergo predominantly N-O cleavage in both uncatalyzed and acid-catalyzed reactions. Oxaziridine VII was reactive also, but cleaved at the C-O bond to form the corresponding nitrone. The inductive effect of the m-nitro group in this compound evidently was strong enough to decrease stabilization of the developing nitrogen cation relative to the developing carbonium ion, which had additional stabilization due to the p-dimethylaminophenyl group. This latter group, also present in oxaziridine VI, did have an effect in that there was some C-O cleavage. Primarily, however, there was neighboring group participation[15] by the p-dimethylaminophenyl group, causing a greatly increased rate of reaction and migration of that group to the nitrogen atom. For oxaziridines IV, V, and VI in the solvents used, there was no linear relationship between log k_1 and the Hammett σ constants. The N-O cleavage rate was greater in oxaziridine IV than in V, but much greater than either of these was the rate in oxaziridine VI due to neighboring group participation.

Solvent Effects on Oxaziridine Rearrangements

Both ethanol and acetone as solvents caused effects that were attributable to the ionizing power and hydrogen-bonding ability of ethanol and the nucleophilicity of acetone. From Table 16-VI it can be seen that oxaziridines VI (k_2, k_6) and VII (k_3) are far more sensitive to the ionizing solvent ethanol than are IV and V (k_1k_4, k_1k_5, and k_2k_6). This indicates more complete ionization in the transition state for those oxaziridines in which electron donating substituents are present.[22] This is consistent with neighboring group participation by the p-dimethylaminophenyl group, stabilizing the developing nitrogen cation by dispersal of charge in the non-classical type of cation (k_2, k_6). In oxaziridine VII, the developing carbonium ion was stabilized by the p-dimethylaminophenyl group.

Table 16-VI. Sensitivity to solvent polarity of the oxaziridine ring opening.[a]

Oxaziridine	$\frac{R_{ethanol}}{R_{isooctane}}$	$\frac{R_{acetone}}{R_{isooctane}}$	$\frac{R_{benzene}}{R_{isooctane}}$
IV	18	0.68	1.3
V	100	2.7	2.9
VI	480	0.05	1.1
VII	370[b]	0.34[b]	---

a. The numbers are the ratio of the overall rates in the respective solvents from columns 3 or 4 of Table 16-IV.
b. Benzene used instead of isooctane; oxaziridine VII was insoluble in isooctane.

In oxaziridine V in ethanol, there was a large amount of phenyl migration (69%) in contrast to a much smaller amount (19%) of p-nitrophenyl migration in oxaziridine IV. The latter group is known to have a low migration aptitude in ionic reactions involving 1, 2 shifts.[23] Also, the sensitivity to the solvent ethanol was greater for oxaziridine V than for IV. This presumably is a result of greater neighboring group participation by the phenyl group than by the p-nitrophenyl group.

There was practically no aryl migration with oxaziridines IV and V in the other solvents used (benzene, isooctane, and acetone). This behavior shows similarity to that found by Winstein et al. in which phenyl participation increased in solvolysis reactions with increasing hydrogen-bonding power of the solvent.[24] A recent report stated that the ratio of phenyl to hydrogen migration in a pinacol rearrangement increased in going from formic acid to sulfuric acid.[25]

22. E. D. Hughes and C. K. Ingold, J. Chem. Soc. 1935, 252.

23. J. D. Roberts and C. M. Regan, J. Am. Chem. Soc. 75, 2069 (1953).

24. S. Winstein, M. Brown, K. C. Schreiber, and A. H. Schlesinger, J. Am. Chem. Soc. 74, 1140 (1952).

25. J. W. Huffman and L. E. Browder, J. Org. Chem. 27, 3208 (1962).

The acetone/isooctane ratio (Table 16-VI) appears anomalous, except for oxaziridine V. From scales of solvent polarity ("Z" values[20] and "Ω" values[26]), acetone would be expected to give rates somewhere between isooctane and ethanol. However, these solvent scales were not based on reactions that were known to involve formation of charge in the transition state leading to ionization. Where solvent scales (such as "Y" and K_{ion}[27, 28]) were derived from ionization reactions, either ether, benzene, or isooctane was not used[27] or the results with benzene were inconclusive.[28]

The "Y" values, which are based on ionization of t-butyl chloride, may not reflect cation solvation by acetone or similar solvents, inasmuch as the three methyl groups would sterically inhibit cation solvation.[29]

Norris and Haines noted that in the reaction of benzoyl chloride with ethanol, the solvents acetone and ether gave rates lower than in benzene or hexane, and much lower than in ethanol.[30]

Similarly, in this study the rates of disappearance of oxaziridines IV, VI, and VII were lower in acetone than in isooctane. In this group of oxaziridines, those that had increased sensitivity to solvent acidity (higher rates in ethanol) also had increased sensitivity to solvent basicity (lower rates in acetone) compared with isooctane. The greatest sensitivity to the basicity of acetone was in oxaziridine VI, in which the neighboring group participation by the p-dimethylaminophenyl group in the transition state was largely inhibited, resulting in much less migration of this group than in isooctane or benzene. Even in oxaziridine VII, in which there was no atom or group migrating, there was a much lower rate in acetone than in benzene.

For oxaziridine V, the rate in acetone was greater than in isooctane. However, for this compound the rate in benzene compared to isooctane was much greater than for oxaziridines IV and VI. As the sensitivity to basicity was reduced, the sensitivity to polarizability by benzene and acetone may have been increased. Recently it has been noted[31] that reactions that involve little charge separation in the transition state are more sensitive to the polarizability of the solvent. Ionization at the transition state would be expected to be less developed in oxaziridine V than in oxaziridines IV, VI, and VII in aprotic solvents.

Further work is in progress on the effects of solvent acidity, basicity, and polarizability on the rates of oxaziridine disappearance.

26. J. A. Benson, Z. Hamlet, and W. A. Mueller, J. Am. Chem. Soc. 84, 297 (1962).

27. A. H. Fainberg and S. Winstein, J. Am. Chem. Soc. 78, 2770 (1956).

28. S. G. Smith, A. H. Fainberg, and S. Winstein, J. Am. Chem. Soc. 83, 618 (1961).

29. A. Streitwisser, Chem. Rev. 56, 571 (1956).

30. J. F. Norris and E. C. Haines, J. Am. Chem. Soc. 57, 1425 (1935).

31. J. O. Edwards and R. G. Pearson, J. Am. Chem. Soc. 84, 16 (1962).

Experimental Procedures

All spectrophotometric measurements were carried out at 25°, using a Cary recording spectrophotometer, Model 11.

Materials

Reagent-grade benzene and spectro-grade isooctane (2, 2, 4-trimethylpentane) were distilled over lithium aluminum hydride. Reagent-grade acetone was distilled successively from anhydrous potassium carbonate and activated Linde molecular sieves (type 4A). Absolute ethanol was used without further purification. In the kinetic experiments, the solvents were used immediately after distillation. The glassware was rinsed with acetone and distilled water and dried. Glassware that had been in cleaning solution or in contact with aqueous sulfuric acid was not used.

Kinetic Experiments

Each nitrone in each solvent was irradiated for the minimum amount of time to achieve conversion to the corresponding oxaziridine. The rates of disappearance of oxaziridine in the dark were followed by various methods at 25°. The reactions were followed to at least ten half periods.

In Table 16-VII is a summary of the experimental conditions used, except for oxaziridine VI in ethanol. The following procedure was used to estimate the half time of reaction for this oxaziridine:

Five portions of nitrone VI in ethanol were irradiated as described in Table 16-VII, immediately analyzed at 268 mμ for VI-E and 382 mμ for VI-A, and then combined. After standing for 10 min to allow for completion of reaction, five drops of 0.1 N H_2SO_4 were added. Five other portions were irradiated, but immediately after irradiation a drop of 0.1 N H_2SO_4 was added to each portion with shaking. This was at approximately 7 sec. These five portions were combined, and after standing about 1 hr, spectra of both combined solutions were compared at 268 mμ for VI-E and 340 mμ for VI-F. The difference was consistent with about 89% completion of reaction at the time of addition of the acid. By extrapolation, the half time was estimated to be about 2 sec. From other determinations, analyzed at 382 mμ for VI-A, it was estimated that 4% isomerization occurred, with a 5-sec irradiation time. With more dilute dolutions, an optical density of 0.15, the photospots only 1.5 in. from the cell, irradiation at 1 and 2 sec, and analysis at 385 mμ for VI-A, the amount of isomerization was then about 10%. Correction for the nitrone not irradiated was estimated by comparison with samples irradiated slightly longer times. Extrapolation indicated a half-time for the reaction of about 2 sec. This also was in approximate agreement with the half-time estimated by irradiating portions of VI-A of optical density 0.6 for 2 sec and analyzing at 385 mμ for VI-A and 280 mμ for VI-B.

In following the rate of disappearance of oxaziridine VI in acetone, additional irradiation was done just before a determination and with the proper selection of analyzing wavelength, the oxaziridine cation only was measured. Correction was made for the isomerized oxaziridine. The

Table 16-VII. Experimental conditions used in determining the rates of disappearance of the oxaziridines.

Nitrone	Solvent and volume (ml)	Optical density at λ_{max}	Solution[a] thickness (cm)	Light source[b] and time of irrad	Analyzing wavelength (mμ)	First-order half-time (min)
IV	C_2H_5OH, 33	1.8	1	sun, 2 min	380[c] for IV-J	60
	CH_3COCH_3, 33	2.6	2	sun, 3 min	330 for IV-D	1590
	C_6H_6, 3.5	1.13	1	sun, 2 min	330 for IV-D	840
	iso-C_8H_{18}, 3.5	0.66	1	sun, 2 min	240, 280, & 320 for IV-D	1080
V	C_2H_5OH, 60	1.8	2	sun, 4 min	380[c] for V-J	104
	CH_3COCH_3, 40	2.0	1.5	sun, 5 min	360[d] for V-J	3800
	C_6H_6, 3.5	2.6	1	sun, 3 min	280 for V-D	3600
	iso-$C_8C_8H_{18}$, 3.5	1.8	1	sun, 2.5 min	280 for V-D	10900
VI	C_2H_5OH, 3.5	0.98	1	DxB, 5 sec	see text	0.033
	CH_3COCH_3, 60	2.1	1.5	sun, 45 sec	385[e] for VI-A & VI-J	300
	C_6H_6, 3.5	2.0	1	DxB, 15 sec	280, 380 for VI-B & VI-A	15
	iso-C_8H_{18}, 3.5	1.9	1	DxB, 15 sec	275, 367 for VI-B & VI-A	16
VII	C_2H_5OH, 3.5	0.27	1	DxB,[f] 2.5 sec	400 for cis-VII-A	0.083
	C_2H_5OH, 3.5	0.57	1	DxB,[g] 4 sec	412[h] for trans-VII-A	32
	CH_3COCH_3, 3.5	0.33	1	DxB,[i] 15 sec	390 for trans-VII-A	93
	C_6H_6, 3.5	0.83	1	DxB,[g] 10 sec	390 for trans-VII-A	31

a. The thickness of the layer exposed to the light source.
b. Two DxB photospots were 16 in. apart with the cell held in a small Pyrex beaker midway between. Sunlight was bright sunlight on a clear day.
c. Each sample of 3.5 ml was analyzed at the analyzing wavelength for base line. Then 1 drop of 10% H_2SO_4 was added with shaking and immediately analyzed at the same wavelength for IV-J or V-J.
d. Each sample of 3.5 ml was analyzed at the analyzing wavelength for base line. Then 1 drop of 0.1 $\underline{N}$ H_2SO_4 was added with shaking and immediately analyzed at the same wavelength for IV-J or V-J.
e. Each sample of 3.5 ml was analyzed at 385 mμ for VI-A, then irradiated 15 sec with two DxB photospots, analyzed again at 385 mμ for base line. Then 1 drop 0.1 $\underline{N}$ H_2SO_4 was added with shaking and immediately analyzed at 385 mμ for VI-J; correction was made for the amount due to oxaziridine from the reirradiated nitrone.
f. The two DxB photospots were 3 in. apart.
g. The two DxB photospots were 3 in. apart; cell in beaker of water.
h. Determination of the rate of isomerization of cis to trans nitrone; starting time 1 min after the start of the irradiation.
i. The two DxB photospots were 6 in. apart; cell in beaker of water.

interference avoided by this means was that due to the formation of nitrone VI and a small amount of imine VI as the reaction proceeded. For oxaziridine V in acetone and ethanol there was no interference with the oxaziridine cation determination at the wavelength used. However, in the oxaziridine IV determinations in ethanol, there was imine IV or some other compound that reacted with acid and absorbed appreciably in the region of the analyzing wavelength. The imine IV did not form a cation in detectable amounts, but the absorption of the imine itself, together with the fact that it hydrolyzed, caused some interference. Although oxaziridine IV in acetone could be followed by the cation-determination method, interference was quite troublesome, and better results were obtained by following the formation of the major product, IV-D. The formation of this product was also used in following the rate of disappearance of oxaziridine IV in benzene and isooctane. Similarly the formation of V-D was used in following oxaziridine V disappearance. The analyzing wavelength used was near the absorption maximum of V-D.

For oxaziridines VI and VII in benzene, the rate of disappearance of oxaziridine, as well as the formation of nitrone, was followed by using analyzing wavelengths that were in the region of their absorption maxima. In a similar manner the rate of disappearance of oxaziridine VI in isooctane was followed. Nitrone VII was insoluble in isooctane, so no data could be obtained for oxaziridine VII in isooctane. The rate of formation of nitrone was used in following the rate of disappearance of oxaziridine VII in acetone and ethanol.

When benzene saturated with water was used, the rates of disappearance of oxaziridines were greatly decreased. The addition of water to acetone resulted in increases in the rates.

α-(p-Dimethylaminophenyl)-N-(m-Nitrophenyl)Nitrone (VII-A)

A mixture of 0.5 g (0.0033 mole) of N-(m-nitrophenyl)hydroxylamine[32] and 0.51 g (0.0034 mole) of 4-dimethylaminobenzaldehyde was heated to a melt in a test tube and then kept at room temperature for 48 days. The product was then suspended in 150 ml of benzene and chromatographed on a column (13 × 1.8 cm) of heavy MgO with benzene. The first fraction from the colorless part of the column contained unreacted 4-dimethylaminobenzaldehyde. The second fraction of 100 ml from the orange-part of the column was treated with 300 ml of petroleum ether (bp 30 to 60°). The dark-red crystals were filtered; mp 170 to 172° (placed in melting-point block, hereafter referred to as "in," at 160°); yield, 0.173 g. The third fraction of about 100 ml, when treated as above, gave dark red crystals, mp 169 to 172°; yield, 0.058 g; combined yield 25%. The filtrate, when concentrated, yielded 175 mg of solid, mp 140 to 145°. On further purification, 0.046 g of material with a mp of 160 to 162°, and 0.070 g of mp 110 to 130°, were obtained.

An experiment was carried out with a solution of 1.95 g N-(m-nitrophenyl)hydroxylamine and 1.9 g of 4-dimethylaminobenzaldehyde in 10 ml

32. K. Brand and A. Modersohn, J. Prakt. Chem. 120, 160 (1929).

of 95% ethanol, which was allowed to evaporate to dryness. When, the next day the tarry material was treated with 10 ml of benzene, it solidified. The solid was filtered, suspended in another 10 ml of benzene, and again filtered. The red solid melted at 125 to 128°; yield, 2 g. The filtrate, upon evaporation, had a strong odor of aldehyde. Two more suspensions in 50-ml portions of benzene yielded filtrates that produced small amounts of red solid (mp about 160°) when petroleum ether was added. Further suspensions gave a product that melted at 129 to 130°; recrystallization from benzene did not raise the melting point. The absorption spectrum of this material was found to be a composite of the spectrum of the nitrone (mp 170 to 172°) and that of m-nitrophenylhydroxylamine in a 1:1 molar ratio. A mixture of 0.6 g of this low-melting product, 0.3 g of 4-dimethylaminobenzaldehyde, and 30 ml of 95% ethanol was heated to boiling and then allowed to evaporate. The resulting product was almost all nitrone, although some low-melting complex was still observed.

Numerous small runs were made with 0.1 g of m-nitrophenylhydroxylamine and 0.12 g (20% molar excess) of 4-dimethylamino benzaldehyde. Each was chromatographed on heavy MgO with benzene as eluent. Practically no condensation was observed when benzene or ethanol solutions were allowed to stand for several days. With no solvent, and upon heating at 100° for 45 min, nitrone was obtained; mp 165 to 168°; yield, 0.047 g (25%). Smaller yields were obtained with a lower or a higher temperature for 45 min or with a longer time (4 hr) at 100°. There was obtained the best yield after dissolving the material in 7.5 ml of 95% ethanol, using 0.1 g of 4-dimethylaminobenzaldehyde instead of 0.12 g, pouring the solution into an evaporation dish, and allowing the residue to stand for 3 days after the solvent had evaporated. The yield of nitrone was 0.096 g (52%), mp 165 to 168°.

For analysis, 0.4 g of crude material, melting at about 160°, was dissolved in 80 ml benzene and the nitrone precipitated by addition of 80 ml of petroleum ether. This procedure was repeated twice, yielding a product melting at 170 to 172° (in at 165°); λ_{max}, 390 mμ; ϵ, 27,300 in benzene; λ_{max}, 402 mμ; ϵ, 32,800 in absolute ethanol.

Anal. Calc for $C_{15}H_{15}N_3O_3$: C, 63.15; H, 5.30; N, 14.73.
Found: C, 63.37; H, 5.52; N, 14.92.

N-(p-Dimethylaminophenyl)-Formanilide (VI-E)

A solution of 150 mg of α-(p-dimethylaminophenyl)-N-phenylnitrone (VI-A)[4] in 450 ml of absolute ethanol was irradiated in seven lots, each one for 4 min, in a water-cooled glass container 1 cm thick, equidistant between two DXB photospots[33] 20 in. apart. After removal of the solvent, the remaining oil solidified, mp 65 to 68°; yield, 145 mg (97%). This was dissolved in 10 ml of 95% ethanol and precipitated by the addition of 30 ml of water, mp 72 to 74°; yield, 109 mg. This, then, was dissolved in 5 ml of 95% ethanol, charcoal was added, and the mixture was filtered. To the filtrate was added 10 ml of water; the solid was filtered, mp 74 to 75°; yield, 66 mg; λ_{max}, 268 mμ; ϵ_{max}, 21,800 in ethanol.

33. From General Electric Co.; previously RSP2.

Anal. Calc for $C_{15}H_{16}N_2O$: C, 74.97; H, 6.71; N, 11.66; mol wt 240.
Found: C, 75.06; H, 6.58; N, 11.60; mol wt 232 (cryoscopic method in diphenyl).

Additional product was obtained from the filtrate, as well as a small amount of p-dimethylaminobenzaldehyde. The infrared spectrum of this formanilide was similar to that of diphenylformamide.[34]

4-Dimethylaminodiphenylamine

A solution of 30 mg of N-(p-dimethylaminophenyl)formanilide, 3.5 ml of 95% ethanol, 2 drops of water, and 1 pellet of 85% potassium hydroxide (0.12 g) was heated at boiling for 15 min. The solution then was diluted with 10 ml of water; the resulting precipitate was filtered, mp 126 to 127°; yield, 23.5 mg (89%). The formanilide also could be hydrolyzed with alcoholic sulfuric acid. A mixture melting point with a sample prepared according to Fischer and Wacker[35] showed no depression. Both samples had λ_{max} of 290 mμ in ethanol.

The formanilide obtained from irradiation of 46 mg of nitrone VI was hydrolyzed as described above and then heated with 5 ml of 88% formic acid for 15 min. The residue from evaporation of the solution was dissolved in a small amount of absolute ethanol, treated with charcoal (no heat), and filtered. Water was added to the filtrate until it became cloudy. The resulting precipitate was filtered; mp 70 to 73°; yield, 15 mg. A mixture melting point with the formanilide obtained from irradiation of nitrone showed no depression.

4-Dimethylaminobenzanilide (VI-D)

A solution of 400 mg of α-(p-dimethylaminophenyl)-N-phenylnitrone (VI-A) in 900 ml of anhydrous ether was irradiated in twelve lots for 6 min each in the same apparatus described above. The solution was allowed to stand for 5 days in a stoppered flask and then was poured into evaporating dishes. After evaporation of the ether, 20 mg of feathery crystals was collected; mp 177-180°; mixture melting point with an authentic sample of 4-dimethylaminobenzanilide showed no depression.[36]

The remainder of the solid from the evaporating dish was suspended in ethanol and filtered; 66 mg of solid with mp 177 to 179° was obtained. On partial evaporation of the ethanol, additional crops of a solid material were obtained; 29 mg, mp 178 to 180°; 39 mg, mp 175 to 180°. Total solid obtained was 154 mg (39%). All the filtrate and some dark-colored material were diluted in ethanol and determined spectrophotometrically for additional 4-dimethylaminobenzanilide and other products. There was an additional 41 mg of 4-dimethylaminobenzanilide, bringing the total yield to 195 mg (49%);

34. F. Pristera, Anal. Chem. 25, 844 (1953).

35. O. Fischer and L. Wacker, Chem. Ber. 21, 2609 (1888).

36. G. Lockermann and W. Neumann, Chem. Ber. 80, 310 (1947).

there was also 43 mg 4-dimethylaminobenzaldehyde (17%) and 78 mg of N-(p-dimethylaminophenyl)formanilide (19.5%). The 4-dimethylaminobenzanilide has λ_{max}, 316 mμ and ϵ, 32,000 in absolute ethanol. A similar experiment in acetone yielded 40% of 4-dimethylaminobenzanilide, 25% of N-(p-dimethylaminophenyl)formanilide, 4% nitrone VI, 4% hydrolysis products. In both experiments the nitrone formed in the reactions was re-irradiated.

N,N-Diphenylformamide (V-E)

The N,α-diphenylnitrone (V-A) used was washed with 15% NH_4OH and dried. A solution of 50 mg of nitrone V in 460 ml of absolute ethanol was irradiated in seven lots, as described above, for 20 min each. After standing for 2 days, the ethanol was allowed to evaporate. The residue was treated with 50 ml of petroleum ether (bp 30 to 60°). This was decanted from the insoluble part and put on a column (3.5×1.8 cm) of two parts of silicic acid and one part of SuperCel. The petroleum ether was allowed to go through the column and then benzene was used to elute the yellow band and the N,N-diphenylformamide.

The first fraction contained the material from the yellow band along with some N,N-diphenylformamide. The absorption spectrum of the material from the yellow band appeared to be similar to that of the yellow color developed in nitrone V that had not been washed with NH_4OH and that had stood for 1 day in ethanol solution. The λ_{max} of the yellow material was approximately 415 mμ. This changed to 540 mμ in ethanolic-sulfuric acid and reverted to 415 mμ when neutralized. The second to fourth fractions yielded 22 mg of N,N-diphenylformamide, mp 57 to 60°. The column was immediately washed with ethanol, the solvent was evaporated, and petroleum ether was added to the residue. This was decanted from the insoluble material and evaporated, leaving 4.5 mg of N,N-diphenylformamide, mp 53 to 59°. Total yield of product was 53%. A second petroleum ether extraction of the original residue yielded 1.5 mg of benzanilide, mp 148 to 153°; a benzene extraction of the residue yielded 1.2 mg mp 100 to 140°; total yield, 5.4%. The crude N,N-diphenylformamide was purified by dissolving it in a small amount of ethanol and adding water dropwise until the solution became cloudy. After a few hours, the crystals were filtered; 4.5 mg, mp 67 to 68°; mixture mp with an authentic sample of N,N-diphenylformamide was 67.5 to 69°. A second crop weighed 4.5 mg; mp 61 to 64°; and residue, 3 mg, mp 58 to 61°.

Spectrophotometric determination of the original solution before isolation of the products showed about 12% unchanged nitrone V. When this portion was heated with 1 drop of concentrated H_2SO_4 for 1 hr just below the boiling point, the amount of diphenylamine found spectrophotometrically indicated about 65% yield of the N,N-diphenylformamide in the original solution. The remainder could be accounted for by hydrolysis of the oxaziridine to benzaldehyde and phenylhydroxylamine.

N,N-diphenylformamide (3 mg) was hydrolyzed by heating for 20 min in a solution of 1 ml ethanol, 2 drops of water, and one pellet of KOH. The solution was diluted with water and the solid filtered. After purification by dissolving the solution in ethanol and precipitation by addition of water, the

mp of the material was 50 to 51° and showed no depression when the material was mixed with an authentic sample of diphenylamine.

Irradiation of N, α-Diphenylnitrone (V-A) in Isooctane

A solution of 7 mg of N, α-diphenylnitrone in 70 ml of spectrograde isooctane was irradiated for 20 min between two DXB lamps as described above. After standing for a month, the solution was run through a column, 1.8×2.5 cm, of two parts of silicic acid and one part SuperCel. There was spectroscopic evidence of the presence of benzaldehyde in the isooctane eluted from the column. With benzene as eluent, three fractions containing benzanilide were obtained. After evaporation of the benzene, there was 4.4 mg of solid melting at 156 to 159° (identified by undepressed mixture mp and uv absorption spectrum). An additional 0.6 mg of benzanilide was present in ethanol washings of the evaporating dishes, as determined spectrophotometrically. The total yield was 5 mg (71.5%).

When a small amount of the concentrated solution before chromatography was heated with 1 drop of concentrated H_2SO_4 and 4 ml of ethanol, there was essentially no spectroscopic evidence of the presence of diphenylamine.

Irradiation of N, α-diphenylnitrone (V-A) in Acetone

An experiment similar to that above in isooctane was carried out in acetone. After standing 9 days, the solution was evaporated and then taken up in light petroleum ether. A 75% yield of benzanilide was obtained. There was spectroscopic evidence for a 5% yield of N, N-diphenylformamide.

N-(p-Nitrophenyl)-Formanilide (IV-E)

A solution of 1.3 g of 4-nitrodiphenylamine in 70 ml of 90% formic acid was heated 4 hr at 60° and then allowed to stand 6 days at room temperature. The absorption spectrum indicated a 78% yield of the desired formamide. When the solution was concentrated, crystals of the amine precipitated; they were filtered; yield 0.86 g, mp 130 to 132°. The filtrate was evaporated to dryness and the residue taken up in benzene and chromatographed on a column (11×1.8 cm) of 8 g silicic acid and 4 g SuperCel. The orange band of the amine was eluted with benzene, and after evaporation of the solvent, 0.10 g of amine remained. Total recovered amine was 0.96 g (74%). Six fractions, taken at the top of the orange band and above, yielded oils when the benzene was evaporated. After several days the oils solidified when seeded. The third, fourth, and fifth fractions seemed the most pure; they were combined, and had a total wt of 0.160 g. This product was further purified by solution in 13 ml of absolute ethanol and the addition of 17 ml water. A precipitate soon formed in the cloudy solution. It was filtered and dried in vacuo at 25°; yield, 0.122 g, mp 75 to 76°. After the addition of 50 ml water to the filtrate, 0.028 g of additional product, mp 75 to 76°, was obtained; the total yield was 0.150 g (10%). The uv absorption spectrum in absolute ethanol had two maxima: λ_{max} 312 mμ; ε, 11,300; λ_{max} 228 mμ; ε, 13,900; minimum, λ_{min} 265 mμ; ε, 3,600.

Anal. Calc for $C_{13}H_{10}N_2O_3$: C, 64.46; H, 4.16; N, 11.57.
Found: C, 64.42; H, 4.11; N, 11.43.

The first crystallization of this compound was effected by dissolving the oil in petroleum ether (bp 30 to 60°) and chilling the solution on dry ice until a precipitate formed. After the petroleum ether was decanted, the solid was air dried. The melting point was 50 to 56° after 1 day, but 2 months later was 74 to 75°. The compound appeared to be fairly stable in benzene and petroleum ether but was not stable in ether solution. The formation of resinous material probably was caused by peroxides in the ether.

A solution of 20 mg of the formanilide in 13 ml of 95% ethanol and 2 drops of concentrated H_2SO_4 was allowed to stand a week. Then 27 ml of water was added and the precipitate filtered. The yield of amine was 13 mg; mp 126 to 127°; mixture mp with 4-diphenylamine showed no depression. By adding more water to the filtrate an additional 3 mg of the amine was obtained.

Irradiation of α-(p-nitrophenyl)-N-Phenylnitrone (IV-A) in Ethanol

A solution of 20 mg of α-(p-nitrophenyl)-N-phenylnitrone in 350 ml of absolute ethanol was irradiated in 70-ml portions for 2 min each, as described above. After the solution stood for 2 months, it was evaporated. The risidue was treated with two 250-ml portions of petroleum ether (bp 30 to 60°) and the extract transferred to a column of 6 g silicic acid and 3 g SuperCel. Benzene was used as the eluent. The uv absorption spectrum of the eluted first yellow band was similar to that of the corresponding first fraction of the irradiated N, α-diphenylnitrone solution in ethanol. The second yellow band was of 4-nitrodiphenylamine, mp 121 to 124°, after elution and evaporation of the benzene. There was no depression of the mixture mp with an authentic sample. The yield of the amine was 1.4 mg, with 0.73 mg more determined spectrophotometrically from less-pure solid. The total yield was 12%. Ether was used to elute the N-(p-nitrophenyl) formanilide from the column. Determined spectrophotometrically, the yield of this was 4.3%. The petroleum ether–insoluble residue was dissolved in benzene and chromatographed on a very short silicic acid–SuperCel column with benzene as eluent. After evaporation of the solvent, 4.9 mg of p-nitrobenzanilide was obtained (mp 211 to 213°), with no depression of a mixture mp with an authentic sample. From spectrophotometric determination of other fractions, an additional 1.95 mg of p-nitrobenzanilide was obtained. The total yield was 34%.

Other experiments showed that if the irradiated solution were worked up after 1 or 2 weeks of standing, approximately the same yield of p-nitrobenzanilide was obtained. In addition, there was a 16 to 18% yield of N-(p-nitrophenyl)formanilide, but no 4-nitrodiphenylamine. Hydrolysis of the N-(p-nitrophenyl)formanilide with sulfuric acid in ethanol at 75° for 1 hr gave 4-nitrophenylamine. If the uv absorption spectrum of the known amounts of N-(p-nitrophenyl)formanilide and p-nitrobenzanilide was subtracted from the absorption spectrum of the solution before evaporation of the ethanol, the remaining absorption approximated that of p-nitrobenzaldehyde. The corresponding amount of N-phenylhydroxylamine that

should be present accounted for the difference. p-Nitrobenzaldehyde could not be isolated after the irradiated solution was evaporated. There was dark-colored material at the top of the column after elution with benzene.

If the ethanolic solution of nitrone IV were allowed to stand 24 hr before irradiation, or if all glassware contacting the solution were not previously rinsed with dilute ammonium hydroxide and distilled water, the yields of both N-(p-nitrophenyl)formanilide and p-nitrobenzanilide were decreased and that of p-nitrobenzaldehyde increased.

When a drop of concentrated sulfuric acid was added to a solution of 20 mg of α-(p-nitrophenyl)-N-phenylnitrone in 350 ml ethanol after irradiation as above, the yellow color of oxaziridine cation IV immediately appeared, and persisted for 40 min. When the solution was evaporated and chromatographed as above, there was found a 3.2% yield of 4-nitrodiphenylamine determined spectrophotometrically and a 16.5% yield of p-nitrobenzanilide isolated. Dark-colored material insoluble in benzene and ethanol was found, but no p-nitrobenzaldehyde could be isolated. The uv absorption spectrum recorded before evaporation was consistent with 80% hydrolysis. The H_2SO_4 was not added immediately after irradiation of each portion but was added after all the solution had been irradiated. Therefore some oxaziridine reactions could have taken place before hydrolysis.

Irradiation of α-(p-Nitrophenyl)-N-Phenylnitrone in Benzene

A solution of 4 mg of α-(p-nitrophenyl)-N-phenylnitrone in 70 ml of reagent-grade benzene was irradiated for 2.5 min as described above. After 6 days at room temperature the solution was chromatographed on a column (3×1.8 cm) of two parts by weight of silicic acid and one part SuperCel. After elution by benzene and evaporation of the solvent, there was obtained 2.4 mg of p-nitrobenzanilide, mp 211 to 212°. Mixture mp with an authentic sample showed no depression. Spectrophotometric determination of ethanol washings of the evaporating dishes yielded an additional 0.5 mg of p-nitrobenzanilide; total yield, 2.9 mg (72%). There was evidence of traces of N-(p-nitrophenyl)formanilide from the absorption spectrum of the first fraction. In a duplicate experiment, 2 ml of solution 6 days after irradiation was treated with 1 drop of concentrated H_2SO_4 and heated at boiling for 45 min, with volume maintained by addition of ethanol. Spectrophotometric determination indicated a 3.5% yield of N-(p-nitrophenyl)-aniline, 70% p-nitrobenzanilide, and about 25% p-nitrobenzaldehyde. When a 2-ml portion was evaporated and redissolved in ethanol, the absorption spectrum approximated that of p-nitrobenzanilide.

A solution of 4 mg of α(p-nitrophenyl)-N-phenylnitrone in 70 ml of benzene was irradiated as above and then several drops of concentrated H_2SO_4 were added. The acid layer at the bottom became yellow, but was colorless after 24 hr. The benzene solution was then chromatographed on a column (1.8×2.5 cm) of two parts silicic acid and one part SuperCel. The first fraction was obtained from the benzene of the solution. After evaporation there was 1.8 mg of a soft solid that consisted of about 90% p-nitrobenzaldehyde and 10% p-nitrobenzanilide determined spectrophotometrically. When rechromatographed with benzene as eluent, the second fraction after evaporation of the benzene yielded a solid (mp 96 to 99°) with no depression of a mixture

mp with p-nitrobenzaldehyde. The second fraction obtained by adding benzene to the column yielded 0.88 mg of p-nitrobenzanilide, mp 211 to 212°, and no depression of a mixture mp with an authentic sample. With that estimated from fraction 1, the total yield of p-nitrobenzanilide was 26%. The yield of p-nitrobenzaldehyde was 65%. Because of the insolubility of the added acid in the benzene, some rearrangement of the oxaziridine could occur.

Irradiation of α-(p-nitrophenyl)-N-Phenylnitrone in Acetone

A solution of 40 mg of α-(p-nitrophenyl)-N-phenylnitrone in 350 ml of reagent-grade acetone was irradiated as above in five 70-ml amounts for 3 min each. After the solution had stood 1 week, the acetone was evaporated, leaving a mixture of flakes of solid in an aqueous solution. The solid was filtered, yielding 29 mg. The solid was purified by dissolving it in benzene and chromatographing it on a 1-cm long column of two parts silicic acid and one part SuperCel with benzene as eluent. There was obtained 27.5 mg of p-nitrobenzanalide, mp 213 to 214°. There was no depression of a mixture mp with an authentic sample. Another 2.5 mg was accounted for by spectrophotometric determination from washings of the evaporating dish and filter funnel with ethanol. Total yield was 30 mg (75%). Ultraviolet absorption spectrum of the cloudy aqueous filtrate indicated the presence of p-nitrobenzaldehyde. Evaporation of this filtrate yielded 5 mg of resinous material.

2-Ethyl-3-(p-Nitrophenyl)Oxaziridine (I-B)

The α-(p-nitrophenyl)-N-ethylnitrone was prepared according to the method of Watt and Knowles.[37] However, the ether extract of the N-ethylhydroxylamine was not treated with dry HCl. A solution of 10 mg of nitrone I in 70 ml of acetonitrile was irradiated, as above, between two DXB photospots for 1 hour. Upon evaporation, a light yellow oil remained on the sides of the evaporating dish, with a small amount of solid on the bottom. After 1 day of standing some of the oil solidified. The part that was soluble in CCl_4 had an infrared spectrum identical to that of the oxaziridine obtained by peracetic acid oxidation of the corresponding imine.[5a] The uv absorption spectra also were identical. The yield of oxaziridine was 35%, with the remainder polymerized. The oil gradually polymerized upon standing and the polymer was insoluble in most solvents.

Irradiation of nitrone I in benzene gave mostly polymer. When nitrone I was irradiated in absolute ethanol there was considerable hydrolysis to p-nitrobenzaldehyde, as well as polymerization.

A solution of 7 mg of nitrone I in 62 ml of absolute ethanol was irradiated as above for 20 min and then immediately titrated for active oxygen,[38] which was found to be 63%.

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37. G. Watt and C. Knowles, J. Org. Chem. 8, 540 (1943).

38. S. Siggia, Quantitative Organic Analysis via Functional Groups, (John Wiley and Sons, Inc., New York, N. Y., 1949), p. 100.

2-(t-Butyl)-3-Phenyloxaziridine (III-B)

α-Phenyl-N-(t-butyl)nitrone was prepared according to the method of Emmons[5a] by isomerizing the corresponding oxaziridine. A solution of 10 mg of nitrone III in 50 ml of acetonitrile was irradiated for 2 hr in a quartz flask with 2 cm solution thickness, 14 in. above a Hanovia mercury arc lamp (Type 16200) with an SH medium-pressure arc bulb. At the end of 2 hr the uv absorption spectrum of the nitrone had completely disappeared. The solution was then boiled for 3 days, [5a] and the volume adjusted to 50 ml; the spectrum then showed 97% of the original nitrone. A reirradiation under the same conditions led again to complete disappearance of the nitrone spectrum.

A solution of 10 mg of the nitrone in 35 ml absolute ethanol was irradiated as above for 2 hr under slight vacuum and then titrated for active oxygen, which was found to be 90%. [38]

2-(t-Butyl)-3-(p-Nitrophenyl)Oxaziridine (II-B)

The α-(p-nitrophenyl)-N-(t-butyl)nitrone was prepared according to the method of Emmons. [5a] A solution of 30 mg of the nitrone in 204 ml of absolute ethanol was irradiated between two D×B photospots in three lots each for 25 min. After evaporation of the solvent, 50 ml of petroleum ether (bp 30 to 60°) was added to the residue and the insoluble material filtered. The filtrate was evaporated, leaving 12 mg (40%) of oxaziridine II, mp 56-59°. It was further purified by redissolving the material in petroleum ether, filtering, and evaporating the filtrate, mp 58 to 60°. A mixture mp with oxaziridine II, synthesized by peracetic acid oxidation of the imine according to the method of Emmons, [5a] showed no depression. The oxaziridine obtained by the irradiation method was titrated in ethanol solution for active-oxygen content and showed 95% active oxygen.

A solution of 10 mg of the nitrone in 62 ml of absolute ethanol was irradiated as above for 20 min and then immediately titrated for active-oxygen content. The value obtained was 90%.

Modified Active-Oxygen Determinations

The method[38] used by Emmons[5a] gave good results for oxaziridines I, II, and III. However, this method could not be used for oxaziridines IV and V without modification because the iodine was liberated from the potassium iodide almost instantaneously and then reacted very rapidly, presumably with the imine formed in the reaction. Dilution with water immediately after the addition of the potassium iodide quenched this reaction to a great extent. The active-oxygen content obtained by this modified method is probably low owing to incomplete liberation of iodine or reaction of the liberated iodine at the time of dilution. Because oxaziridines IV and V were not stable in ethanol solutions, the maximum active-oxygen content was obtained immediately after irradiation of the corresponding nitrones. Oxaziridines VI and VII were so unstable in ethanol solution that the active-oxygen content could not be measured. Details of the modified method used for oxaziridines IV and V are given below.

A solution of 8 mg of N, α-diphenylnitrone (V-A) in 69 ml absolute ethanol was irradiated for 17 min between two DXB photospots as described above. Two ml of water was added to one-half the solution as it was being chilled for 1 min in an ice bath. One ml of saturated KI solution was added, and was immediately followed by 100 ml of water. After 6 ml of acetic acid and 4 ml of starch indicator solution were added, the liberated iodine was titrated with 0.025 N sodium thiosulfate solution. Immediately after the irradiation, the active-oxygen content was 75% based on the nitrone used. After varying postirradiation times (solution kept at 23°), the following active-oxygen contents were found: 1/2 hr, 64%; 1 hr, 53%; 2 hr, 39%; 4 hr, 18%; 6 hr, 7%. For the last two determinations the end point was difficult to determine because of yellow reaction products.

A solution of 4 mg of α-(p-nitrophenyl)-N-phenylnitrone (IV-A) in 30 ml absolute ethanol was irradiated in a 600-ml beaker by direct sunlight until it was colorless (about 6 min). To this was added first 5 ml of water and then 1 ml of saturated KI solutions. A yellow color developed immediately. Just a few seconds after the addition of the KI solution 100 ml of water was added. After the addition of 6 ml of acetic acid and 4 ml of starch-indicator solution, the liberated iodine was titrated with 0.025 N sodium thiosulfate solution. A 69 to 72% active-oxygen content was found based on the nitrone used. A solution that stood 20 hr after irradiation had no active oxygen.

Yields Determined Spectrophotometrically (Table 16-I)

Various procedures were used to determine the yields spectrophotometrically. Small amounts (4 ml) of nitrone solutions (10^{-4} to 10^{-5} M) were irradiated by DXB photospots or sunlight until the nitrone completely disappeared. When there was no further change in the uv absorption spectrum (after standing in the dark), further irradiation was done to detect isomerization to the nitrone. Imines in the ethanol and acetone solutions were detected by their reactions to acid addition. The yields of the benzanilides and formanilides were determined through their specific optical absorptions. Yields of formanilides were determined also by hydrolysis with ethanolic sulfuric acid to the corresponding amines. The yields of hydrolysis products were estimated in ethanol solution from the amounts of aldehyde present. Spectrophotometric analysis was carried out on acetone and benzene solutions before and after evaporation of the solvent, and redissolving the residue in an equal volume of ethanol.

17. BIOSYNTHESIS OF THE OPIUM ALKALOIDS

Robert O. Martin and Henry Rapoport

Introduction

Since the primacy of thebaine in the biosynthesis of the hydrophenanthrene alkaloids—thebaine, codeine, and morphine—has been well established by use of intact poppy plants, attention has been turned toward establishing the immediate precursor of thebaine. The necessity of short-term exposures of single plants to $^{14}CO_2$ became apparent upon consideration of the high rate of incorporation of the isotope into thebaine (e.g., 10^4 to 10^5 dis per min per μmole after a half-hour exposure to 10 mC of $^{14}CO_2$). The thebaine concentration in the plant is only 0.01%.

In order to obtain a kinetic picture of the incorporation of $^{14}CO_2$ into various alkaloids (which are present in total amounts of less than 1 mg per plant), we sought an analytical system that could give rapid qualitative separations, quantitative data on microgram amounts of alkaloids, and simultaneous measurements of radioactivities. Sufficient data were in the literature to encourage the development of a gas chromatographic (VPC flow counting system. (VPC means vapor-phase chromatography.) No one piece of commercial apparatus was able to meet the many special restrictions imposed by resolution, detection, and counting of microgram amounts of the low-volatility compounds of the opium alkaloid series.

A rapid paper-chromatographic system has been developed to help corroborate the VPC data, as well as to allow isolation of small amounts of alkaloids.

Analytical Procedures

VPC Flow Counting System

A diagrammatic representation of the apparatus to be employed in the future is shown in Fig. 17-1. Some of the salient features of the system are summarized below:

1. Injector--down-stream type with changeable Pyrex-glass sleeve to reduce decomposition of sample resulting from contact with the hot stainless steel walls, as well as to facilitate cleaning.

2. Temperature-programmed oven--permits reduction in retention times for less-volatile compounds in a mixture, but does not produce any increase in column efficiency.

3. Column--1/8 in. O.D. × 2.3 mm I.D. × 3 ft stainless steel tube, coiled prior to filling, to prevent crushing of substrate support particles, and washed thoroughly with conc HCl, water, acetone, ethanol, and chloroform. Packing consists of Gas-Chrom Z (a silanized diatomaceous earth) coated with about 10% Dow UC-W-96 silicone gum. Particles of 65 to 80 mesh (obtained by sieving after coating) are blown into the

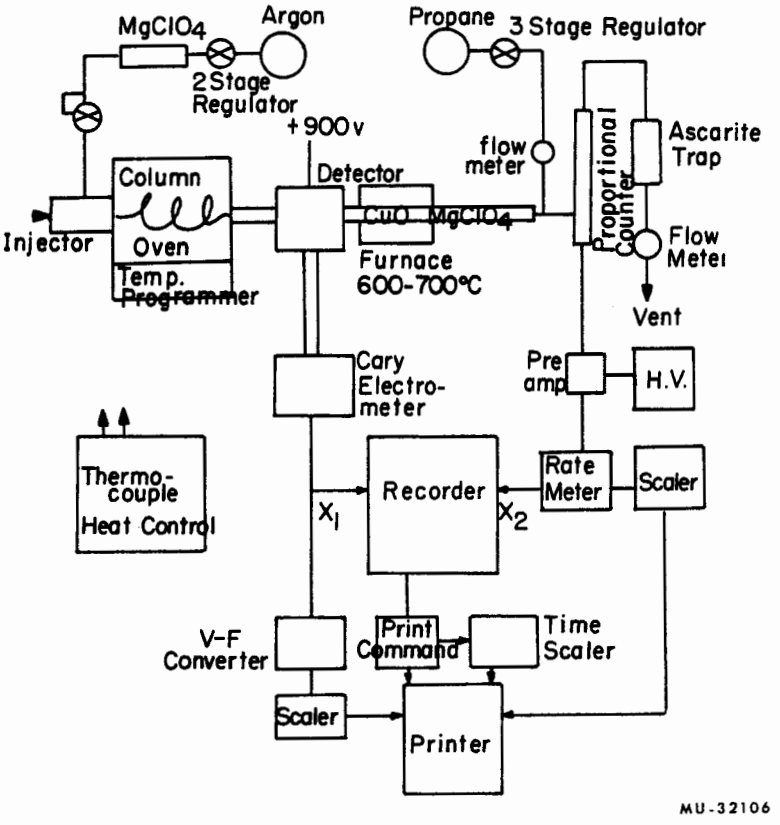

Fig. 17-1. VPC flow counting system.

column from a Lucite reservoir under 30 psi of N_2. Ends of the column are plugged with glass wool. The column is conditioned at 150° for 4 to 6 hr, then at 275° for 10 to 15 hr. It is necessary to avoid over-conditioning the column (25° above the maximum-use temperature is adequate), otherwise column decomposition products, which lead to undesirable absorption effects, may be formed. A column so prepared will permit a flow rate of about 60 ml/min at 160° C and 16 psi of argon.

4. Detector--argon triode ionization type with two 12-mC Pm^{147} sources to provide the excitation current. With 900 to 1200 V applied to the high-voltage electrode, positive ions are collected by a platinum-wire loop, and the resulting current (proportional to the concentration of contaminant) is measured with a Cary electrometer. The inside of the chamber is polished to a mirror finish to minimize condensation and decomposition of the alkaloids. All-metal gaskets and alumina insulators permit operating temperatures as high as 300° C.

   Sensitivity depends on the following main factors:
   a. Total number of beta particles in the chamber up to a saturation level (not yet determined).
   b. High voltage applied, up to a saturation level of about 1000 V.
   c. Chemical composition of compound being detected.
   d. Retention time (sharpness of peak) of compound being detected.

   The lower limit of thebaine detectable is about 0.1 μg. Reproducibility of response: 2% at 900 V over a few hours. (Not studied over longer periods.)

5. Combustion train--1/4 × 11 in. stainless steel tube, filled with copper oxide (wire form) at 600° to 800° followed by 1/4 × 6 in. stainless steel tube filled with 20 to 40 mesh $MgClO_4$ (at room temp).

6. Proportional-flow counter--Operated at room temperature. Sensitive volume: 40 ml. Plateau: 2.0 to 2.2 kV (argon-65 ml/min; propane--20 ml/min). Background: 35 to 40 counts/min (with 2 in. of lead shielding). Minimum detectable activity: 120 dis/min.

7. Data presentation:
   a. Graphic--on a Leeds and Northrup dual-pen recorder.
   b. Digital--by electronic print-out on paper tape of:
      (1) Scaler connected to proportional counter (activity).
      (2) Scaler actuated by voltage-frequency converter connected to electrometer output (mass-peak area).

Paper Chromatography on Al_2O_3-Impregnated Paper, Followed by Radioautography

A number of attempts with thin-layer chromatography (silica gel) gave rather poor separations, or none at all with protopine and cryptopine. The need for very dry plates, as well as the difficulties of preparation, handling, and radioautography, led to a few trial separations on an Al_2O_3-impregnated paper (S & S No. 967); these separations were so promising that within a week a good pair of solvents was found that would resolve a mixture of the following 10 alkaloids after a two-dimensional ascending run: reticuline, codeine, thebaine, laudanine, laudanosine, norlaudanosine, papaverine, protopine, cryptopine, and narcotine.

Prior to use, the paper is dried at 100 to 110° C for 3 to 4 hr and then stored over $MgClO_4$ in a desiccator. Eight-inch square sheets were first run ascending (2 to 3 hr) in diethyl ether (stored over Dowex-1 (OH^-) to keep it peroxide free). The paper was then vacuum dried in a desiccator for a half hour before turning the paper 90° and running it ascending in the second solvent, chloroform (shaken briefly with $MgSO_4$). Figure 17-2 illustrates the resolution obtainable with this solvent pair. Figure 17-3 shows the resolution obtained with ethylene dimethyl ether as the second solvent. Possibly a mixture of this solvent with chloroform would provide a good resolution of the phenolic alkaloids.

Other interesting combinations were (a) a one-dimensional run with ether in "chromatobox" or (b) a descending run with benzene (6 to 8 hr). Methylene chloride, ethylene dichloride, tetrahydrofuran, dioxane, methyl Cellosolve, acetone, methanol, and dimethyl formamide (DMF) might prove useful mixed with other solvents.

Maximum sensitivity of this method (when the iodo-platinate spray is used) appears to be about 5 μg for reticuline, codeine, thebaine, laudanine, protopine, and cryptopine; and 10 to 20 μg for norlaudanosine, papaverine, and narcotine (all three of which form a white or weak blue spot, rapidly fading to white on a pink background). Laudanosine reacts similarly to papaverine. Prior to spraying all 10 of the alkaloids absorb or fluoresce (depending on the concentration) in uv light. Laudanosine fluoresces at a concentration of less than 5 $\mu g/cm^2$.

Radioautography: The minimum ^{14}C activity detectable in 5 days using "duplitized" x-ray film is about 100 dis/min.

Testing of Extraction Scheme on Known Mixture of Alkaloids

A freshly prepared mixture of the 10 alkaloids listed on the previous page was divided into two parts, one of which was dried and stored under N_2, and the other extracted as far as the methylene chloride stage according to the scheme given earlier.[1] Using the VPC for analysis, 75% or more recovery was obtained for all compounds except narcotine and reticuline, both of which showed about 50% recovery.

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1. H. Rapoport, F. R. Stermitz, and D. R. Baker, J. Am. Chem. Soc. 82, 2765 (1960).

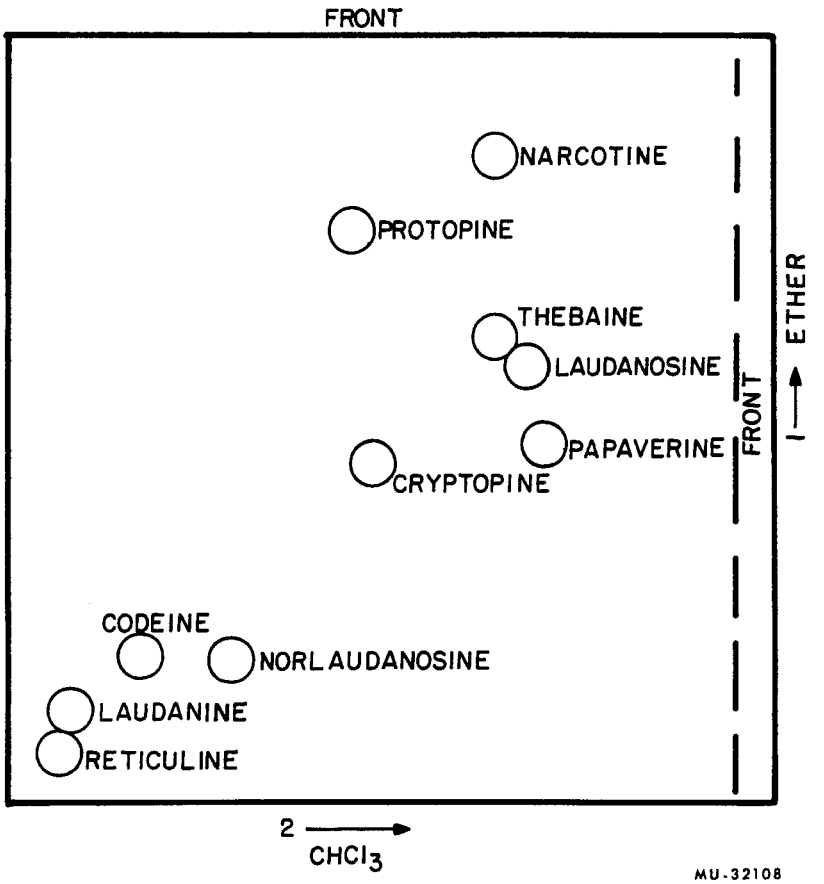


Fig. 17-2. Two-dimensional ascending chromatography on (Al_2O_3) - impregnated paper, run first direction in ether and second direction in chloroform with a mixture containing 10 to 15 μg each of 10 alkaloids.

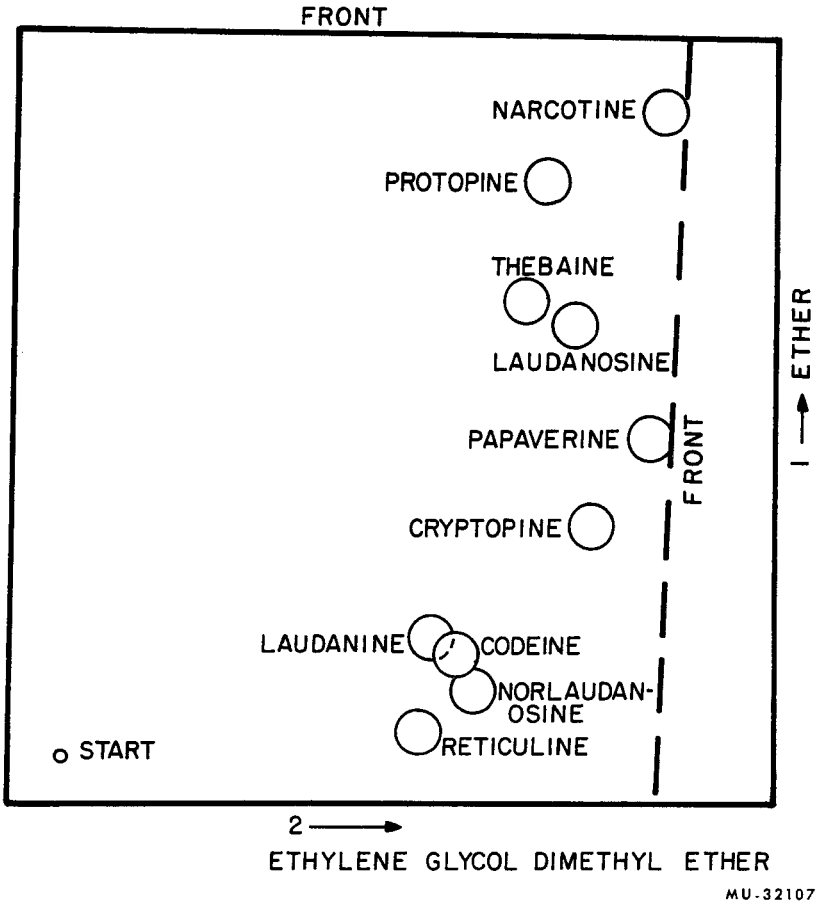


Fig. 17-3. Two-dimensional ascending chromatography on (Al_2O_3) - impregnated paper, run first direction in ether and second direction in ethylene glycol dimethyl ether with a mixture containing 10 to 15 μg each of 10 alkaloids.

Forty-Minute "Hot Run"

Fifteen plants were exposed to 33 mC of $^{14}CO_2$ for 30 min. Killing of the plants was begun by cutting them up and placing the pieces in a large Dewar flask (this took about 10 min), which was immediately removed from the exposure chamber and had liquid nitrogen added to it. As centrifuge trouble was encountered, the plant mash was filtered on a Buchner funnel. After the mash was reground a second time with butanol-benzene, it was strained and squeezed through four layers of clean cheesecloth. This latter procedure may prove to be the easiest and most rapid method for separating out the plant debris.

The plants were stunted in appearance, though all were within a few days of blossoming. This stunting was probably because two plants were growing in each pot, whereas only one plant is normally grown per pot. This may have accounted for the unusual alkaloid spectrum that was exceptionally low in minor alkaloids (the region between thebaine and narcotine on the VPC).

VPC analysis of the methylene chloride extract showed clearly the presence of only codeine, thebaine ($\approx$ 80 μg av/plant), and narcotine (700 μg av/plant), and a trace of protopine or cryptopine or both ($<$ 10 μg av per plant) and papaverine ($<$ 20 μg av/plant). The only measurable activity was in thebaine (approx 2×10^4 dis per min per mole) and narcotine (approx 400 dis per min per μmole). If papaverine had the same specific activity as thebaine, it would not have been detected due to the extremely small total amount available. It was difficult to assess with any accuracy the mass of the thebaine and codeine peaks because some other substance caused a negative peak in that region of the chromatogram. This latter phenomenon was also observed earlier with a plant that was worked up for practice. Tests using diluted aliquots of sample showed it was not due to overloading of the detector.

The above results were confirmed by chromatography on Al_2O_3 paper, followed by radioautography. Only codeine, thebaine, papaverine, and narcotine were detectable by the iodo-platinate spray, and only thebaine showed measurable activity.

Preparation of Reticuline

As the earlier supply of reticuline ($HClO_4$ salt) was getting low and was not pure (it showed several spots on paper chromatograms, especially when ethylene glycol dimethyl ether was the solvent), more material was prepared by hydrolysis of the dibenzyl derivative with 20% HCl for 1 hr at 150° C under slow N_2 bubbling. Attempted recrystallizations from isopropanol, water ethanol, and benzene failed. Attempts are being made to remove the last traces of HCl from the hydrolysate so as to render the salt insoluble in cold water.

Cracking of Alkaloids over Nickel Catalyst Followed by Hydrogen-Flame Detection

While more durable Pm^{147} sources for the argon detector were being devised, unsuccessful trials were made to crack alkaloids in the VPC effluent over a nickel catalyst (using H_2 as carrier gas), followed by detection of the lower hydrocarbons that should have formed from the flame ionization detector.

Summary

The two main microanalytical techniques (VPC flow counting and chromatography on Al_2O_3-impregnated paper, and radioautography) have been satisfactorily developed to be applied concertedly to the alkaloid biosynthesis problem. The practical lower limits of mass and radioactivity detection are (a) 0.1 μg and 120 dis/min (^{14}C) with the VPC system; (b) 5 to 20 μg and 200 dis/min for the paper chromatographic-autoradiographic method.

The traditional extraction scheme was tested with a mixture of 10 alkaloids, of which eight were recovered in about 80% yield.

Fifteen plants (stunted in appearance), after 40-min exposure to 33 mC of $^{14}CO_2$, produced an alkaloid spectrum unexpectedly low in minor constituents. The only two compounds with measurable activity were thebaine (2×10^4 dis per min per μmole).

Preparation of what is hoped will be a more homogeneous batch of reticuline is in progress.

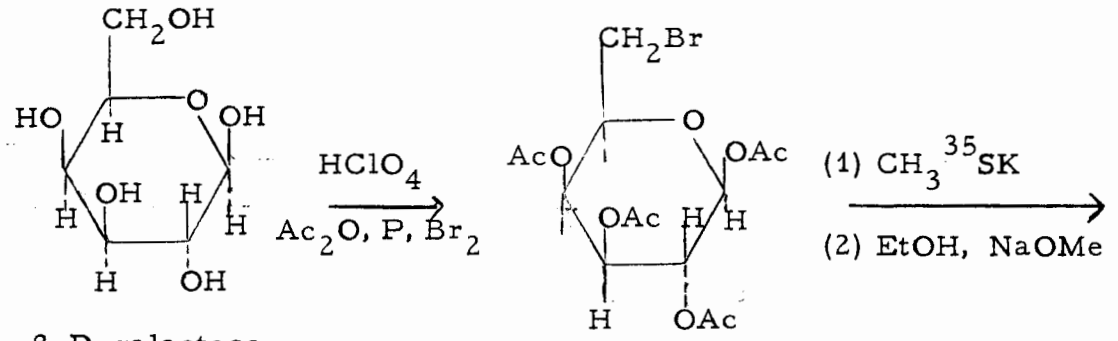
Catalytic cracking of column effluent, followed by hydrogen-flame detection, was abandoned after unsuccessful trials.

More durable, leak-proof Pm^{147} sources are being fabricated.

18. SYNTHESIS OF METHYL-β-D-THIOGALACTOSIDE-^{35}S

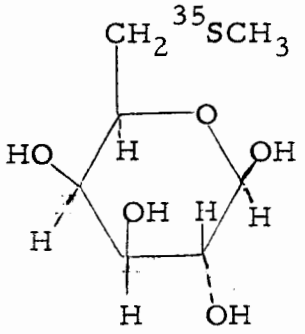
Sister Mary de Paul Palaszek and Richard M. Lemmon

For some forthcoming research on the mechanisms of enzyme induction and inhibition, it was desired to have available some ^{35}S-labeled methylthiogalactoside. The synthesis of this compound was undertaken according to the following reactions:



β-D-galactose

Acetobromgalactose



Methyl-β-D-thiogalactoside-^{35}S

The general procedure followed for the preparation of the acetobromgalactose was that of Bárczai-Martos and Kórösy[1]; this compound was then converted to the ^{35}S-labeled galactoside following the procedure of Helferich and Türk.[2]

Experimental Procedure

Acetobromgalactose

Acetic anhydride (200 ml, 2.1 moles) and $HClO_4$ (1.7 ml, 70%) were mixed at room temperature. To this solution was slowly added (addition time: 2 hr) 50 g (0.28 mole) of β-D-galactose (Pfanstiehl Chemical Corp., Waukegan, Ill.; mp 165°). The solution was maintained at 29 to 35° during

1. M. Bárczai-Martos and F. Kórösy, Nature 165, 369 (1950).
2. B. Helferich and D. Türk, Chem. Ber. 89, 2215 (1956).

the addition; it appears important to prevent any further rise in temperature, in order to hinder the formation of caramel. The solution was cooled to -10°, and 15 g (0.48 gram atom) of red phosphorus (amorphous powder) was added during 15 min. Bromine (90 g, 1.1 gram atoms) was added dropwise during the next 15 min; the temperature was kept at about 0°. Water (18 ml) was then added dropwise, with vigorous stirring, over 1/2 hr at the same temperature. The reaction mixture was allowed to stand in a stoppered flask at room temperature for 1.5 hr, then was dissolved in 150 ml of $CHCl_3$. The chloroform solution was poured into 400 ml of ice water, and the phases separated in a separatory funnel. The chloroform solution was suction-filtered, and twice extracted with equal volumes of ice water. The separated water phase was extracted with two 30-ml portions of $CHCl_3$, and these portions were added to the main $CHCl_3$ solutions.

The chloroform solution was dried overnight by $CaCl_2$, filtered through Celite, and the solvent was removed in a rotating evaporator. The highly viscous yellow residue was dissolved in 60 ml of Na-dried ether. However, no crystals appeared, even after the solution stood for 18 hr at 0°.

To a portion of the Et_2O solution was then added three volumes of petroleum ether (bp 30 to 60°). The two-phase system was heated to boiling and the cloudy upper phase (petroleum ether) removed by pipet and cooled to 0°. Colorless microcrystals (mp 74 to 78°) formed on the walls of the test tube, and the petroleum ether was decanted. A few of the crystals were added to the main ether phase. Again, microcrystals (10.4 g, mp 74 to 78° with softening at 67°) appeared at room temperature. To the ether filtrate was added a large excess of petroleum ether. The solid mass that separated was recrystallized from Et_2O, giving 36.3 g of crystals that melted at 73 to 79°, with softening at 69°. A second crop of crystals (about the same amount) was obtained from the ether filtrate. All of the crops of crystals were combined and recrystallized, giving 80.0 g, a yield of 63.5% from the galactose. This material, however, was impure, as was shown by its melting point of 74 to 77° (literature: 84°) and by the appearance of a brown discoloration on storage in a vacuum desiccator. Trituration of the material with water, followed by recrystallization from ether-petroleum ether, led to a purer product (mp 83 to 84°) with the elemental analysis given below. It was this purer product that was used in the subsequent preparation of the labeled galactoside.

Analysis for $C_6H_7BrO(OAc)_4$:

	C(%)	H(%)
Calc	41.00	4.66
Found	40.68	4.73

Methyl-β-D-Thiogalactoside-^{35}S

From a stock solution of 4.07 g K in 139 ml of MeOH, 2 ml (58.5 g atom K) was transferred (under a dry N_2 atmosphere) into a small round-bottom flask, which was then attached to a vacuum transfer line. The $CH_3{}^{35}SH$ (New England Nuclear Corp., 9 mC, sp act. 13 mC/mmole) was

contained in a small tube equipped with a breakoff seal. This tube was also attached to the transfer line. After the line had been evacuated to about 0.1 mm Hg pressure and the round-bottom flask immersed in liquid N_2, the breakoff seal was broken and the $CH_3{}^{35}SH$ quantitatively transferred into the flask. The flask was removed from the transfer line, warmed to -78°, opened, protected from moisture by a Drierite-filled tube, and to it was added 642 mg (1.56 mmoles) of acetobromgalactose (mp 83 to 84°). The flask was again stoppered (loosely), brought to room temperature, and agitated for about 5 min. The flask was then reattached to the vacuum line, refrozen in liquid N_2, and between 0.3 and 0.5 ml of unlabeled CH_3CH (5.4 to 9.0 mmoles) transferred into the flask through the line. Again the reaction flask was removed from the line, brought to room temperature, and agitated for a few minutes. The flask was then allowed to stand at room temperature for 1 hr.

The reaction mixture was then filtered through Celite, and nearly all the solvent removed by a rotating evaporator. The residue was dissolved in 5 ml of pyridine, and reacetylation effected by the addition of 5 ml of Ac_2O and heating for 12 min at 80°. (In practice reactions with unlabeled materials we found that we could not successfully isolate the final product without this reacetylation step.)

To the cooled reaction mixture were added 20 ml of benzene and 60 ml of water, and the two phases were separated. The benzene phase was washed successively with dilute $NaHCO_3$ solution, water, dilute $NaHSO_4$ solution, and finally with water. The benzene solution was dried with Na_2SO_4, decolorized with charcoal, and filtered. A yellow oil was obtained on evaporation of the filtrate. The oil was dissolved in a centrifuge tube in 3 ml of EtOH, a seed crystal was added, and the solution cooled to -78°. The material was left to crystallize at -10° over a weekend. The crystals were recovered after centrifuging, washing with cold EtOH, and drying in a vacuum desiccator. Their melting point was 102 to 103°. The yield was 94.7 mg (0.251 mmole), or 16% based on the acetobromgalactose. However, liquid scintillation counting showed that 28.1% (2.52 mC) of the starting activity (9 mC) was present in the tetraacetate, a not-unexpected result since the $CH_3{}^{35}SK$ was given a maximum opportunity to react before the unlabeled methyl mercaptan was added to the reaction mixture.

The labeled tetraacetate was hydrolyzed by dissolving it in 1 ml of absolute EtOH in a centrifuge tube and warming the tube to between 50 and 60°. One drop of a freshly prepared, 10% NaOMe solution (6.7 g Na in 87 ml MeOH) was added, and a yellow color developed. The solution was heated for 10 min at about 50° and then cooled to -15°. After some agitation of the solution, crystals appeared. The crystals were separated by centrifugation, and a second crop was obtained from the mother liquor. Both crops of crystals were combined and recrystallized from absolute EtOH overnight at -78°. The yield was 24.8 mg of crystals that melted at 175 to 176°. A mixed melting point with the authentic, unlabeled compound showed no depression.

19. EFFECT OF ACRIDINE ORANGE AND VISIBLE LIGHT ON THYMINE DIMER FORMATION AND DISRUPTION

Joan Friedman

The formation and disruption of thymine dimers have been found to play a role in the uv inactivation and photoreactivation of nucleic acids. In vitro, thymine dimers can be split only by irradiation with short-wavelength uv light, whereas the in vivo phenomenon of photoreactivation (with attendant breakage of dimers) occurs with visible light plus an enzyme. Visible light must be absorbed by the enzyme and transferred by some mechanism to the dimer, since the dimer absorbs only light of wavelengths less than 2600 Å. Nucleic acid stains, such as proflavine or acridine orange (both compounds absorb visible light and bind to nucleic acids), might provide a model for this type of energy transfer.

To investigate this possibility, we irradiated both thymine and thymine dimer with a photoflood lamp in the presence of acridine orange (AO), irradiated AO alone, and studied the effect of thymine and thymine dimer on the fluorescence of acridine orange.

I. Experimental Procedures

A. Visible-Light Irradiation of Acridine Orange

Acridine orange (Harleco) was purified by chromatography (on alumina) and recrystallization, as follows: About 1 g of AO hydrochloride was dissolved in 65 ml of distilled water, and the base precipitated by the dropwise addition of 3 M NaOH. The precipitate was filtered, washed, and dried, and then dissolved in about 15 ml of chloroform. The chloroform solution was filtered and dried over anhydrous sodium sulfate. This solution was then applied to an alumina column (neutral alumina of activity 3 was prepared from Woelm neutral alumina, activity 1, by washing with water and methanol, and drying, as described in Ref. 1). The column was 29 cm long and the inside diameter was 1.7 cm. Fifteen grams of alumina was used to chromatograph approximately 0.5 g of AO in chloroform. The AO was eluted with chloroform, leaving behind a dark band of impurities that could be eluted only by using glacial acetic acid. The chloroform was evaporated quickly on a rotating evaporator. The dye was then dissolved in dilute HCl, reprecipitated in base, washed, and recrystallized from ethanol-water.

A water solution of AO [6.56×10^{-6} M, calculated from the optical density (O. D.) at λ_{max} of 493 mμ, taking log ε as 4.81] was irradiated in a test tube with a lamp (G. E. RFL2 Photoflood) at a distance of 19 cm. The test tube was immersed in a beaker of water for temperature control, and the light was filtered with a bandpass filter (Corning 9782/4-96), which has its maximum transmission of about 78% at 490 mμ. The short-wavelength cutoff of the filter is at 350 mμ, and the long-wavelength cutoff is at

1. James Cason and Henry Rapoport, Laboratory Text in Organic Chemistry, (Prentice-Hall, Englewood Cliffs, N. J., 1962) 2nd Ed, Procedure a, p. 322.

620 mμ. In addition, the filter transmits some light in the infrared region, between 1.5 and 2.9 μ. The light intensity of the unfiltered lamp at 19 cm was measured with a Weston Illumination Meter (Model 756) and found to be 3600 ft-c. The beaker wall decreased the intensity by 300 ft-c. The first two times the experiment was done, a different sample was used for each period of illumination. The third time it was done, only one sample was used for the illumination and aliquots were withdrawn and their O. D. measured at various times. The O. D. measurements were made on a Cary Model 11 recording spectrophotometer.

B. Visible-Light Irradiation of Thymine Dimer in the Presence of AO

In one test tube, 100 λ of a solution of thymine dimer (about 0.1 mg/ml) was added to 2 ml of the AO solution (same solution as in Sec. I. A.). In a second test tube, 100 λ of the AO solution was added to 2 ml of H_2O. The solutions were irradiated side by side, as described above, for 2 min and again for 15 min. Difference spectra (one solution in the sample cell, the other in the reference cell of the spectrophotometer) were recorded before and after each irradiation.

Two solutions of AO plus water and AO plus thymine dimer, prepared exactly as above, were frozen in 5 ml beakers and irradiated from above, through the Corning filter, for 2 min. The solutions were then thawed and a difference spectrum obtained.

C. Visible-Light Irradiation of Thymine in the Presence of AO

One ml of thymine solution (≈ 0.02 g/l) plus 1 ml of AO (as above) in one test tube, and 1 ml of water plus 1 ml of AO in another test tube were irradiated, as described in Sec. I. A, for various times. Difference spectra were measured before and after irradiation. As a control, aqueous thymine solutions (4.34×10^{-4} M) were irradiated, one for 5 and another for 15 min under the same conditions.

One hundred fifty λ of thymine-2-^{14}C (Research Specialties Co., 8.94 μC/mg, 0.759 mg/ml, kept in frozen solution since March 1962) was added to 1 ml of 10^{-3} M AO solution (prepared by adding 26.6 mg of purified AO plus 100 λ of 1.0 N HCl to 100 ml of distilled water) and irradiated as in Sec. I. A for various times ranging from 5 min to 2.75 hr. Aliquots of 100 λ were withdrawn at these various times and spotted on small sheets of Whatman #4 oxalic-acid-washed filter paper. The papers were chromatographed in propanol-16 N NH_4OH-water (6:3:1 by vol) and n-butanol-propionic acid-water (equal volumes of butanol-H_2O, 86:14 by vol, and propionic acid-water, 60:40 by vol), dried, and radioautographed.

D. Effect of Thymine on the Fluorescence of AO

Acridine orange (solution described in Sec. I. C, second paragraph) was analyzed by the method of Weber[2] and found to contain a fluorescent impurity in the emission region of 560 to 570 mμ and absorption region of

2. G. Weber, Nature 190, 27 (1961).

490 to 500 mμ. Acridine orange has maximum absorption at 490 mμ and maximum emission at 530 mμ. Indeed, close examination of the traces of the emission spectrum showed a small shoulder on the long-wavelength side of the peak. Thymine (0.526 g/liter in distilled water) was used as a stock solution. Various dilutions, from 1/20 to 7/10, of this stock solution, were prepared and tested for their effect on the fluorescence of AO as follows: 2 ml of thymine solution were pipetted into a small Erlenmeyer flask; 20 λ of AO (about 10^{-4} M) were added and the micropipet flushed three times. The fluorescence of these solutions by excitation of 490 mμ were measured and compared with a solution of AO and water prepared the same way, at 10-mμ intervals from 490 mμ to 600 mμ. At each wavelength of emission, one sample was read as soon as possible after the other, so that errors due to fluctuation of the xenon lamp would not appear. In order to rule out the possibility that changes in fluorescence of the AO were due to pH changes, the fluorescence experiment described above was repeated, using 10^{-3} M acetate buffer, pH 5.4, instead of water. Since the reproducibility in preparing solutions was poor, accurate 2-ml volumetric flasks were used in both the acetate-buffer experiment and in a subsequent experiment using thymine in water. In this last experiment (thymine in water), a microburet was used to add the 20 λ of AO to the solution in a volumetric flask, instead of using a 20-λ micropipet as before.

E. Effect of Thymine Dimer on the Fluorescence of AO

Thymine dimer was prepared as described by Wulff and Fraenkel.[3] A solution of approximately 0.03 mg/ml showed no absorption at 265 mμ. Two experiments were performed. In the first, 2 ml of various dilutions of thymine dimer (≈ 0.3 g/liter) in water were pipetted into a small flask and 20 λ of AO (≈ 10^{-4} M) added to each. Measurements were taken as in Sec. I. D. In the second experiment, the dimer solution was assayed carefully by irradiating a solution of dimer with uv light until maximum regeneration to thymine occurred. The concentration of dimer thus obtained in the stock solution was 0.305 g/liter. The special 2-ml volumetric flasks were used in making up solutions, and the AO (same solution as in Sec. I. D) was added with a microburet.

II. Results and Discussion

A. Visible-Light Irradiation of AO

When AO is illuminated with 3300 ft-c in air, the absorption peak at 493 mμ drops sharply at first (O. D. drops 26% in the first 30 sec) and then decays slowly (see Fig. 19-1). The nature of the photoproduct is unknown, although Freifelder et al. noticed that oxygen seems to be required for this bleaching to occur.[4]

B. Visible-light Irradiation of Thymine Dimer in the Presence of AO

1. Unfrozen solution. Before irradiation, the dimer showed a slight peak at ≈ 260 mμ. This disappeared after 2 min of irradiation. The

3. Daniel L. Wulff and Gideon Fraenkel, Biochim. Biophys. Acta 51, 332 (1961).

4. David Freifelder, Peter F. Davison, and E. Peter Geiduschek, Biophys. J. 1, 389 (1961).

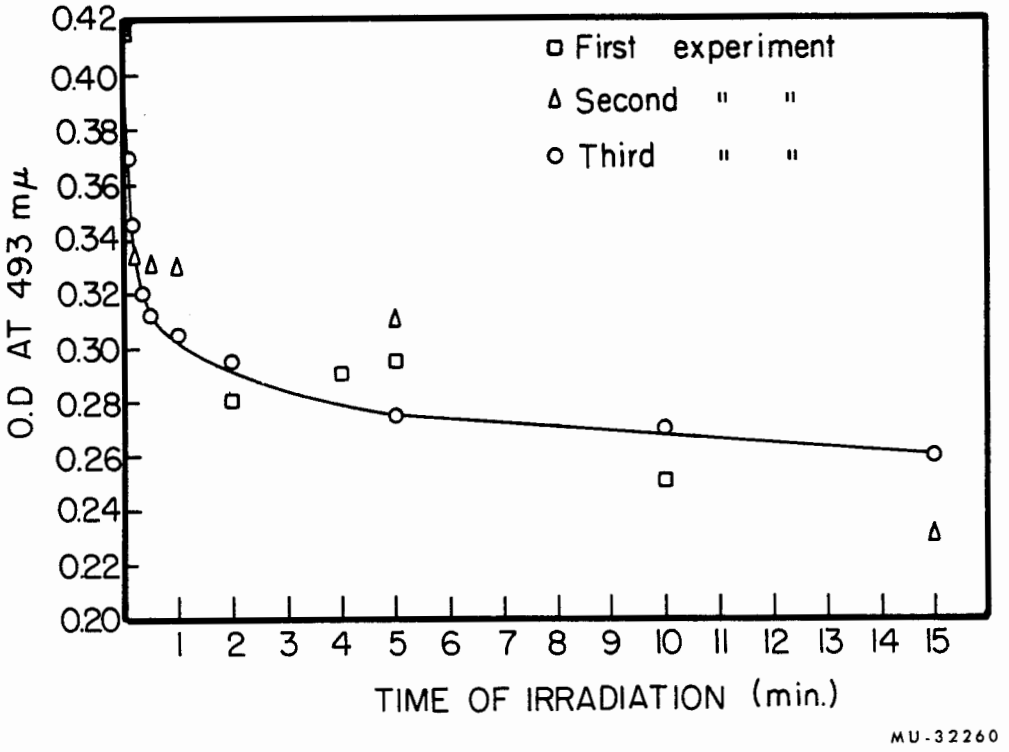


Fig. 19-1. Results of visible-light irradiation of an aqueous solution (about 10^{-4} M) of acridine orange.

difference spectra after 2-min and 17-min irradiation were identical. It appears that some thymine present as an impurity in the dimer may have disappeared upon irradiation. In any case, under these conditions dimerization was not reversed by AO and visible light.

2. Frozen solution. No increase in absorption at 265 mμ, which would indicate a reversal of dimerization, was observed. There was only a small decrease in absorption of the dimer and AO vs H_2O and AO at ≈ 270 mμ. As above, some thymine, present as an impurity, might have been destroyed. Also, since AO has an absorption peak at 273 mμ, this might reflect a slight difference in amount of AO destroyed in the two samples.

C. Visible-Light Irradiation of Thymine in the Presence of AO

1. A small decrease in the absorption peak of thymine was observed after 15 min irradiation. The O. D. dropped from 0.655 to 0.615, a decrease of 6.1%. Apparently, thymine is being converted to a non-uv-absorbing photoproduct through sensitization by AO, although this is happening rather slowly compared to the rapid photoreaction of AO itself. Fifteen-min irradiation of a five times as concentrated solution of thymine alone produced no change in the absorption spectrum.

2. Since the thymine-^{14}C used was rather old, several radioactive spots were observed. However, no radioactivity was detected in the region on the paper where the dimer should have been. The control and all nine irradiated samples showed the same pattern of spots, and since the ratio of radioactivity in the two main spots on each sheet was constant, it appears that no reaction took place. This is strange in view of the fact that the concentration of AO in this experiment was approximately 10^3 times that in (1) above.

D. Effect of Thymine on the Fluorescence of AO

Although there were experimental difficulties in obtaining reproducible preparations of the solutions, there seems to be a slight consistent increase in the fluorescence of AO in the presence of thymine over that of AO alone. There is no shift in the wavelength of the peak of fluorescence, however.

E. Effect of Thymine Dimer on the Fluorescence of AO

Thymine dimer also appears to increase slightly the fluorescence of AO. Usually added solutes may quench fluorescence but not enhance it. It is impossible to guess the nature of the interaction that brings about enhancement of fluorescence in this case.

III. Future Plans

Since all these studies were done in the presence of oxygen, it is possible that all the energy absorbed by the AO was used up in its reaction with oxygen. We propose to make further studies of the interaction between thymine dimer and AO and the dimer and proflavine in the absence of oxygen, in both the frozen and unfrozen states. The fluorescence results do not rule out the possibility that a loose complex between dimer and AO or proflavine might form in the absence of air or in frozen solution (or both) that might allow energy absorbed by AO to be transferred to the dimer.

20. SOME ASPECTS OF THE RADIATION CHEMISTRY OF DNA

Samuel Schrage and Richard M. Lemmon

Introduction

An understanding of the biological effects of ionizing radiation presupposes an understanding of the nature of the initial step of radiation damage. The unique genetic role of DNA has suggested its consideration as a "key molecule," i.e., the site of the primary radiochemical damage initiating the processes that may lead to mutations or to the death of the irradiated cell. Although experimental investigations have not supported the concept of its exclusive importance,[1] DNA has remained one likely candidate for the primary lesion, and studies of its radiation chemistry have suggested possible mechanisms for minor (mutations) and drastic (lethal) effects of ionizing radiation on biological systems. The observed effects (in vitro) of ionizing radiation on nucleic acid constituents (bases, nucleosides, nucleotides)[2] suggest a possible explanation of radiation-induced mutations; the effects on the polymer, DNA, are complex and depend on experimental conditions and, in particular, on the concentrations of oxygen and of water that may be present with the DNA. The pertinent features of the observations on DNA[3] will be mentioned below.

The present study was begun to obtain information on the relation between the effects of ionizing radiation on nucleic acid constituents and those on the polymer, and in particular on possible differences that might be observed, by autoradiography, between decomposition patterns of ^{14}C-labeled DNA when irradiated in the solid state and in aqueous solution. Observed changes[2] in the chemical nature of nucleic acid constituents that might explain radiation-induced mutations should be observable also when the polymer containing these constituents is irradiated. The state of aggregation of DNA and its environment during irradiation can be expected to affect not only its physical properties, such as chain length and molecular weight, but also the identity and relative abundance of possible decomposition products. It is assumed here that it is permissible to extrapolate effects observed with large radiation doses (required for the detection of radiation products) to the far smaller doses effective biologically. The subject of the radiosensitivity of DNA has been recently reviewed by Guild.[4]

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1. A. M. Kuzin, Intern. J. Radiation Biol. 6, 201 (1963).

2. Cyril A. Ponnamperuma, The Radiation Chemistry of Nucleic Acid Constituents (Ph. D. Thesis), UCRL-10053, June 1962.

3. A. J. Swallow, Radiation Chemistry of Organic Compounds (Pergamon Press 1960), New York, p. 233.

4. W. R. Guild, Radiation Res. Supplement 3, 257 (1963).

Experimental Procedures

We used a noncommercial sample of ^{14}C-DNA (specific activity 1.34 μC/mg; reported protein content 20%; radiopurity very satisfactory) that was generously supplied by Dr. Irving Goldman of Schwarz Bio-Research, Mount Vernon, N. Y. Samples of solid DNA (about 0.1 mg) or DNA solution (50 to 200 μl; concentration range 0.01% to 0.4%) were placed in 6 mm o. d. glass tubes that were then evacuated, sealed, and irradiated with Co^{60} gamma rays at a rate of approximately 10^6 rads/hr.

Before freezing the solutions and evacuating the tubes, we bubbled N_2 through the solutions for about 10 min to remove dissolved oxygen. Irradiated samples and unirradiated controls were hydrolyzed by adding, to the opened irradiation tubes, 20 μl of 70% $HClO_4$ to the solid[5] or to the residue left after evaporating the solution under vacuum. After the addition of the $HClO_4$, the tubes were kept in a steam bath for about 40 min. Their contents were then diluted with 10 to 25 μl of distilled water, and aliquots of appropriate activity were withdrawn and spotted on oxalic-acid washed sheets (9×11 in.) of Whatman No. 4 paper. After assay of the activity of the applied spot by GM counter, the sheets were subjected to descending chromatography, with n-propanol–16 N NH_4OH–water (6:3:1 by vol) in the first and n-butanol–propionic acid–water in the second dimension. [The second solvent system was prepared by mixing equal volumes of the following immediately before chromatography: n-butanol–water (86:14 by vol) and propionic acid–water (6.4 by vol)].

Autoradiographs were prepared by exposing x-ray films to the chromatograms. The identity of a labeled spot was determined by elution of the corresponding area of the chromatogram and subsequent co-chromatography with a sample of the ultraviolet-absorbing unlabeled compound with which it was supposed to be identical. Coincidence in position of the ultraviolet-absorbing area (as shown by the light spot on a "shadowgram")[6] and the radioactive area (as shown by the dark spot on the autoradiograph) confirmed the suspected identity.

The fraction of a decomposition product was assayed by counting (by GM tube in situ or by elution and liquid-scintillation counting) of the corresponding area of the chromatogram and comparison with the initial count at the origin of the chromatogram. In particular, the fraction of labeled material remaining at the origin was found by comparing the activity at the origin before and after chromatography.

Results and Discussion

Decomposition Products of DNA on Irradiation

The experimental results in the dose range of 10^5 to 10^7 rads do not show any readily observable decomposition products of DNA that can be

5. A. Marshak and H. J. Vogel, J. Biol. Chem. 189, 597 (1951).

6. C. A. Ponnamperuma, R. M. Lemmon, and M. Calvin, Science 137, 605 (1962).

ascribed to the effect of radiation. Autoradiographs of the hydrolyzate of the irradiated solid or solution are essentially identical with those of the hydrolyzate of the nonirradiated controls (Figs. 20-1 and 20-2). They show the expected separation of the hydrolyzate into the constituent bases—adenine, guanine, cytosine, and thymine (A, G, C, T)—but no noticeable darkening in other regions that would indicate other labeled degradation products of DNA exclusively attributable to radiation. The identity of the area labeled "X" (see Fig. 20-1(b)) that appeared on most autoradiographs of irradiated DNA solutions (and very faintly on autoradiographs of irradiated solid DNA) could not be determined. Nucleosides (adenosine, guanosine, cytidine, and thymidine), nucleotides (the deoxy-5'-monophosphates), and the degradation products listed below were tried without success.

Degradation product	Derived from
1. Hypoxanthine	A
2. Xanthine	G
3. 8-Hydroxyadenine	A
4. 8-Hydroxyguanine	G
5. 4-Amino-5-formamido-6-hydroxypyrimidine	A
6. 4,6-Diamino-5-formamido-pyrimidine	A
7. 2,4-Diamino-5-formamido-6-hydroxypyrimidine	G

In earlier work the above degradation products were found on the γ-radiolysis of aqueous solutions of adenine and guanine.[2] So far, we have not searched for radiolysis products of cytosine and thymine.

It should be stressed here that the relatively low specific activity of the DNA-^{14}C that we used diminished the immediate (few days' exposure) autoradiographic detectability of degradation products that were formed only to the extent of 1% or less of the decomposed DNA.

The decreasing G values found for the deamination of amino bases to hydroxy bases from the free base, through the nucleoside, to the nucleotide (Ref. 2, Table VII, p. 118) suggest an even lower yield in the polymer. A comparable relation between ultraviolet-induced spectral changes in nucleotides and nucleic acids has been investigated by Rushizky and Pardee.[7] The observed decrease in optical density on irradiation and the quantum efficiency were found to be much smaller in nucleic acids than in corresponding nucleotide mixtures. Lack of evidence for any of the above decomposition products on the autoradiographs should therefore perhaps be expected.

The incomplete hydrolysis (due to lowered $HClO_4$ concentration) of a DNA solution that is not evaporated before hydrolysis is illustrated in Fig. 20-2b.

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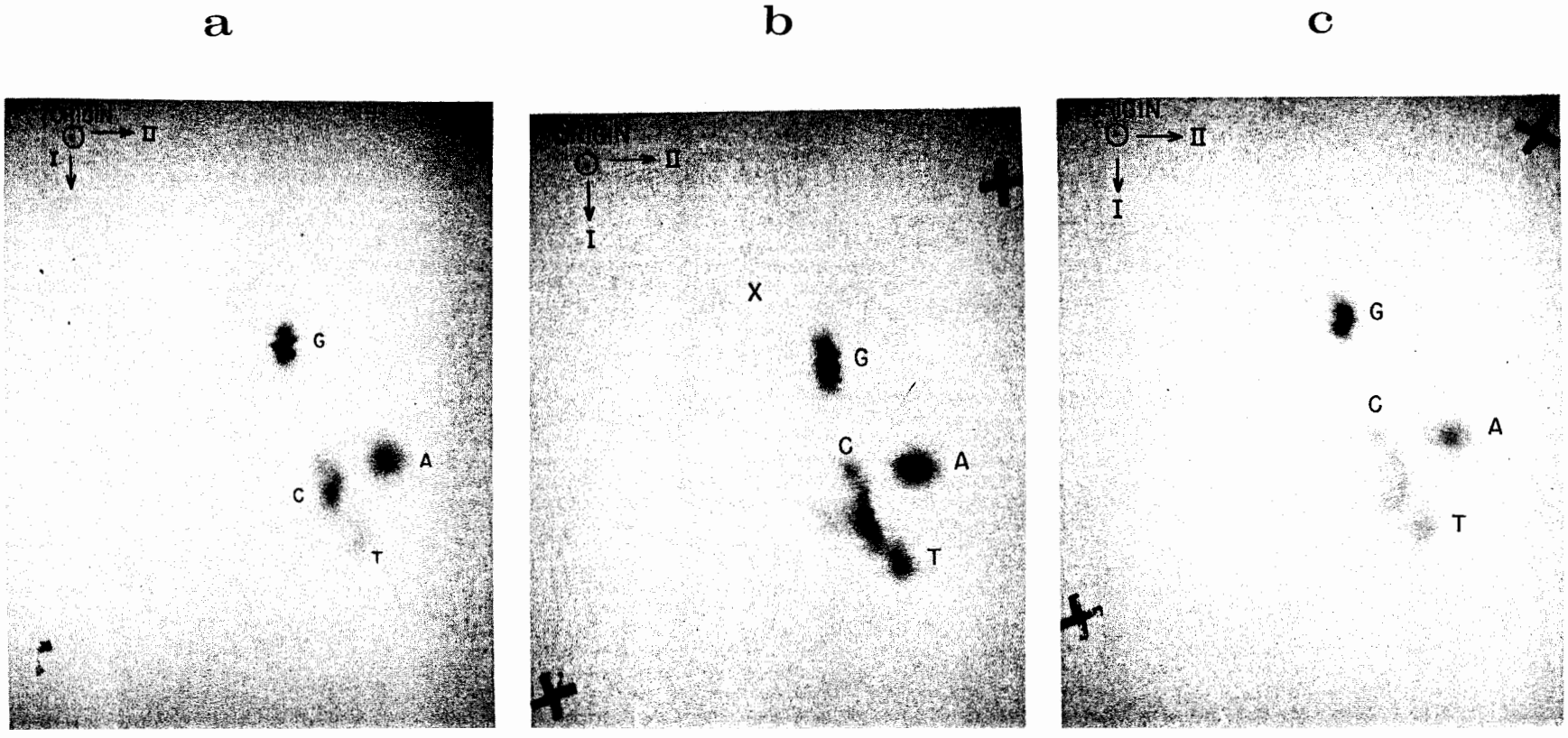
7. G. W. Rushizky and A. B. Pardee, Photobiochem. Photobiol. 1, 15 (1962).



ZN-4060

Fig. 20-1. Autoradiograph of hydrolyzate of solid DNA-^{14}C, I = propanol-NH_4OH solvent, II = butanol-propionic acid solvent. (a) unirradiated, (b) irradiated, 1.5×10^6 rads, (c) 2×10^7 rads.

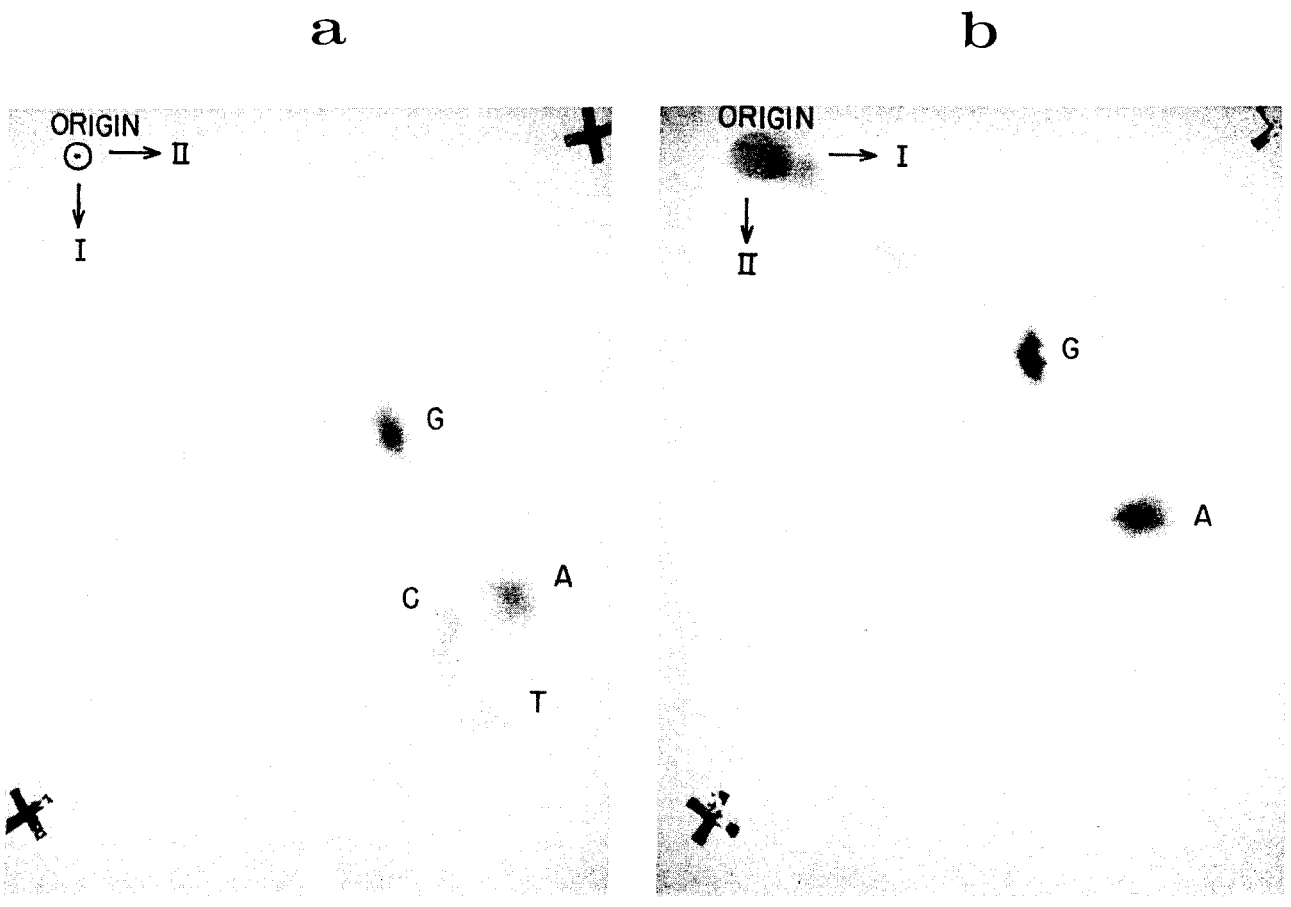


Fig. 20-2. Autoradiograph of hydrolyzate of unirradiated DNA-^{14}C solution (0.4%), I = propanol-NH_4OH solvent, II = butanol–propionic acid solvent. (a) evaporated prior to hydrolysis, (b) not irradiated prior to hydrolysis.

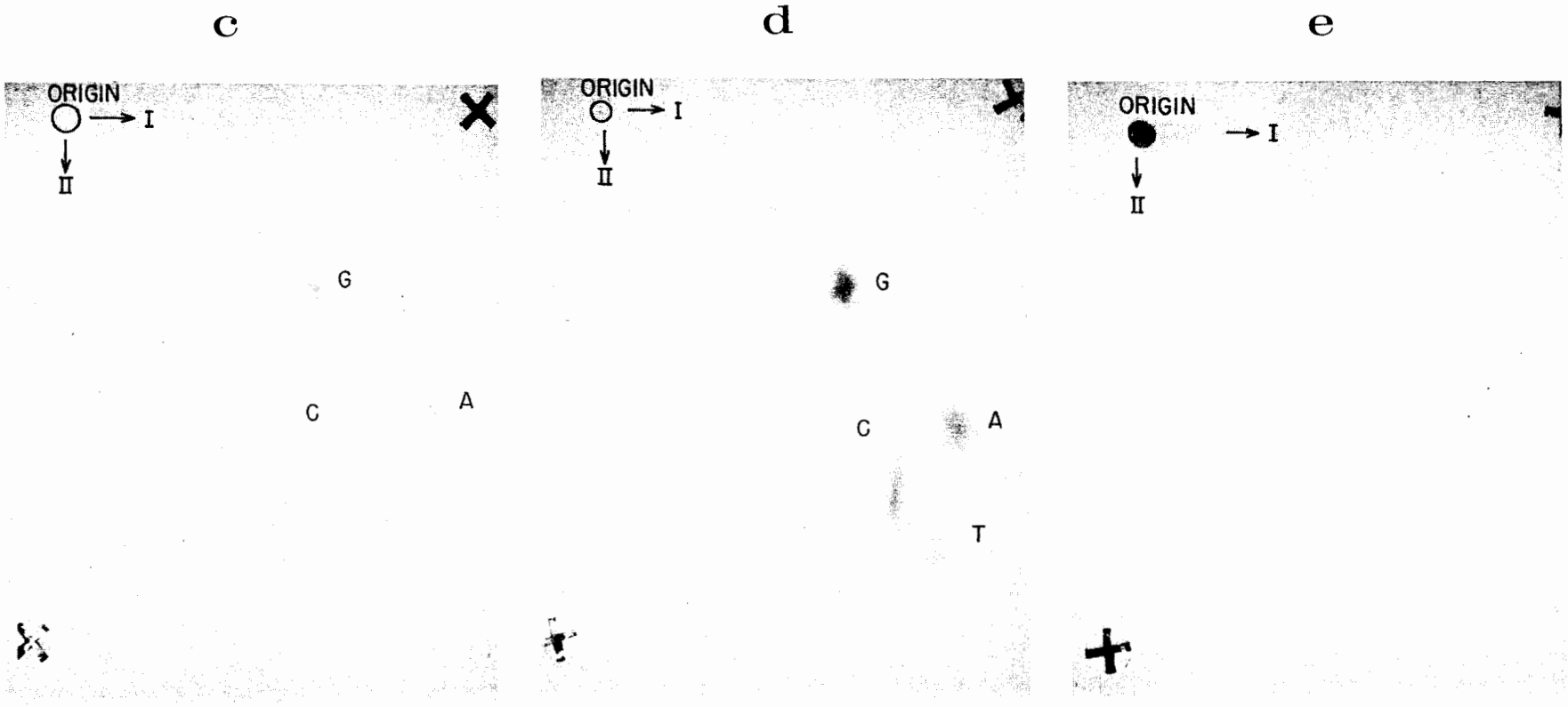


Fig. 20-2. Autoradiograph of hydrolyzate of irradiated DNA-^{14}C solution (0.4%), I = propanol-NH_4OH solvent, II = butanol–propionic acid solvent. (c) 2×10^5 rads, (d) 6×10^5 rads, (e) 2.8×10^7 rads.

JCRL-11046

Effect of Water

Autoradiographs of chromatograms of irradiated solid DNA and irradiated DNA solutions were essentially similar. This somewhat unexpected finding raised questions about the possible effect of water molecules present in solid DNA. (DNA contains about 20% water at a relative humidity of 50%). Care was therefore taken in the preparation of several samples dried at room temperature in a vacuum of 10^{-3} mm for 24 hr. The chromatographic analysis after irradiation showed that no differences could be found between these samples and others that had been kept over water for several hours.

The effect of hydration on the structure of DNA has been discussed by Falk.[8]

Observed Radiation Effects on DNA Solutions

Although solid DNA, whether irradiated or not, can be readily hydrolyzed by $HClO_4$ (as discussed), the extent of the hydrolysis of the residue from the evaporated irradiated DNA solution is found to depend on irradiation dose and on the concentration of the solution. The chromatographic pattern obtained on hydrolysis of irradiated solutions does not appear to change, but the fraction of labeled material remaining at the origin increases.

At higher doses the autoradiograph consists essentially of one dark spot corresponding to the origin of the chromatogram. No similar effects are observed with samples of solid DNA. Figure 20-3 shows the fraction of the original activity remaining on the origin of the chromatogram after two-dimensional chromatography as a function of the concentration of the solution and of the total dose. Although the trends seem significant, the numerical values probably depend on additional variables that were not satisfactorily controlled (see below for effect of oxygen). The relatively low specific activity of the DNA and the practical difficulty of expanding the useful concentration range of the solutions limited the investigation of the concentration dependence. Samples through which oxygen (instead of nitrogen) was bubbled for about 10 min and which were only partially evacuated before irradiation showed, on analysis, much smaller residual activity at the origin of the chromatograms than oxygen-free samples, or no residual activity at all. A possible explanation for the retention of an increasing fraction of ^{14}C-labeled constituents at the origin of the chromatograms is cross-linking produced by irradiation. The greater the total dose, and the lower the concentration, the greater this fraction would be expected to be. The resulting (intramolecularly linked) coils or (intermolecularly linked) branched polymers would be resistant to hydrolysis and therefore less likely to be moved from the chromatographic origin. Radiomimetic chemicals (e.g., nitrogen mustard) also appear to produce detectable cross-linking of DNA at concentrations lethal to cells.[9] The tempting suggestion that

8. M. Falk, K. A. Hartman, and R. C. Lord, J. Am. Chem. Soc. 84, 3843 (1962).

9. P. Alexander and K. A. Stacey, Proc. 4th Intern. Congr. Biochem., 9, 78 (Pergamon Press, New York, 1959).

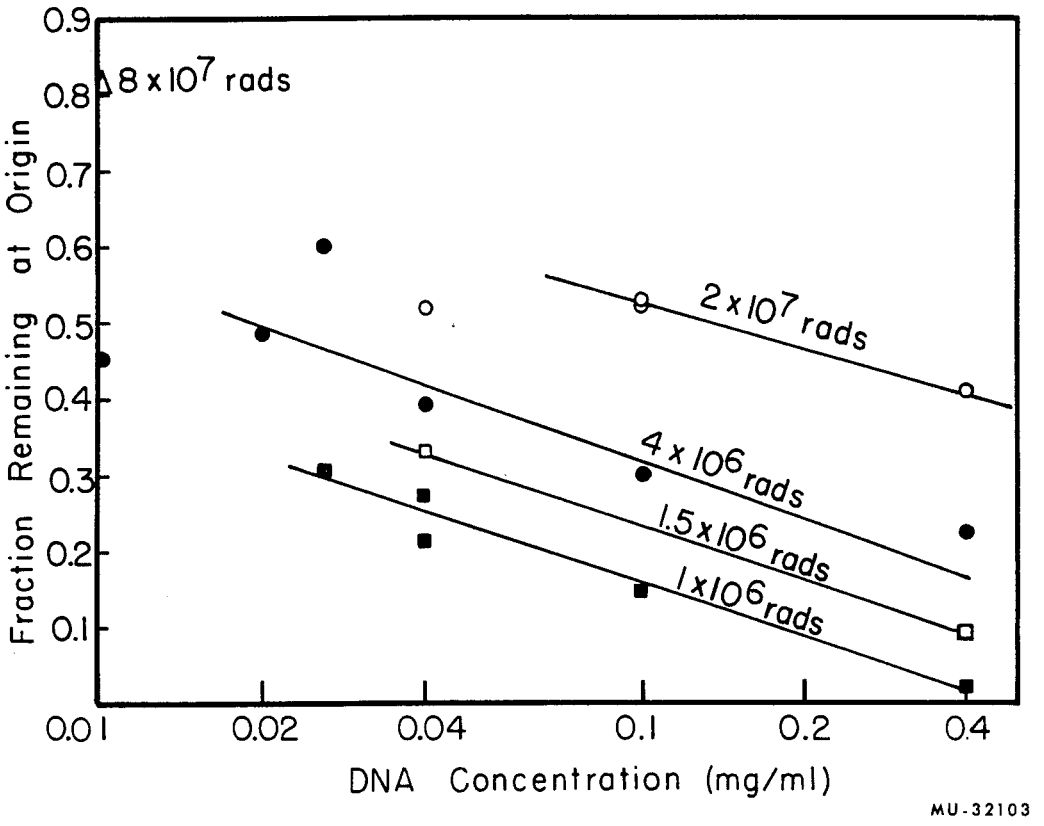


Fig. 20-3. Residual ^{14}C activity remaining at the chromatographic origin after irradiation of DNA-^{14}C solutions at different concentrations and different total doses of γ radiation.

cross-linking of DNA is important in initiating cellular damage, both on radiation and on treatment with radiomimetic chemicals, as well as some of the difficulties with this suggestion, is discussed by Alexander.[10]

Assuming the well-known cross-linking action of radiation[11] to be responsible for the reported observations with DNA solutions, the following explanation may be suggested for the surprising absence of similar evidence for irradiated solid DNA. The formation of cross-links is facilitated when the mobility of the molecule is increased. The mobility of DNA in solution allows coiling (itself facilitated by irradiation) that leads to the formation of intramolecular links. The resulting tightly coiled DNA is considerably more resistant to the hydrolysis procedure than the cross-linked molecules that must be present, at least to some extent, in irradiated solid DNA. The observed oxygen effect agrees with the expectation that the "active" sites produced by radiation are peroxidized and therefore no longer available for the formation of cross-links.[12]

DNA in Formamide Solution

It is of interest to investigate the radiation chemistry of DNA in a solvent in which it is not subject to the action of the hydroxyl radical. A series of irradiation exposures of a 0.21% solution of DNA in formamide furnished inconsistent values for the fraction of the hydrolyzed DNA remaining at the origin after chromatography. However, the fraction did not exceed 0.1 even at high doses. The lower concentration of "active sites" available for the formation of cross-links is apparently due to the absence of the action of the hydroxyl radical. A representative radioautograph is shown in Fig. 20-4. Identification of the products was not attempted, although the adenine and guanine spots are obvious. The structure of DNA in formamide solution is discussed in Ref. 13.

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10. P. Alexander, J. T. Lett, H. Moroson, and K. A. Stacey "Changes Produced by Ionizing Radiations and Some Related Agents in DNA in vitro and in vivo," in Immediate and Low Level Effects of Ionizing Radiations, A. A. Buzzati-Traverso, Ed., Symposium Supplement, Intern J. Radiation Biol. (Taylor and Francis, Ltd., London, 1960).

11. G. Adler, Cross-Linking of Polymers by Radiation, Science 141, 321 (1963).

12. Z. M. Bacq and P. Alexander, Fundamentals of Radiobiology 2nd Ed. (Pergamon Press, New York, 1961) p. 202.

13. G. K. Helmkamp and P. O. P. Ts'o, The Secondary Structure of Nucleic Acids in Organic Solvents, J. Am. Chem. Soc. 83, 138 (1961).

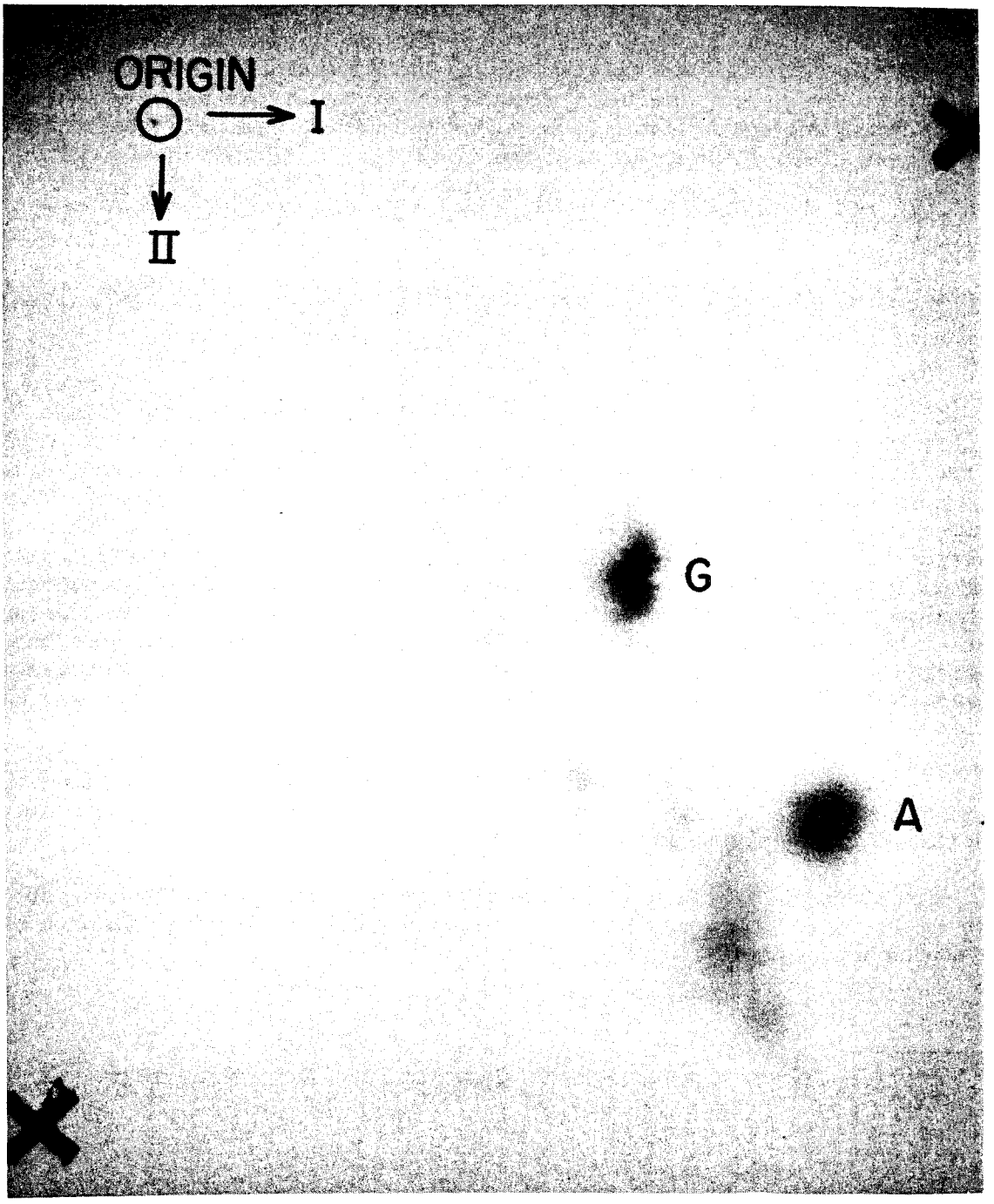

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Fig. 20-4. Autoradiograph of hydrolyzate of irradiated (6×10^5 rads) DNA-^{14}C solution (0.21% in formamide). I = propanol-NH_4OH solvent, II = butanol-propionic acid solvent.

21. NUCLEAR MAGNETIC RESONANCE

Melvin P. Klein

An analytical nuclear magnetic resonance (NMR) spectrometer (Varian Associates A-60) has been received and installed. This spectrometer has several design and operating features that encourage its use by personnel who are not NMR specialists. The radio-frequency oscillator is locked to the magnetic field strength so that the resonance condition is always maintained. This feature precludes the troublesome drifts encountered in spectrometers that stabilize the field and frequency independently. The spectral region of interest is calibrated so that chemical shifts and spin-spin coupling constants may be read directly from the chart recording. The synchronous motor and gear train that drive the chart recorder also drive the potentiometer that generates the field or frequency scan.

The sensitivity of this spectrometer (or any other) is inadequate to permit the direct study of very small quantities, or extremely dilute solutions, of large molecules. To increase the sensitivity we have applied the technique of continuous averaging.[1] The essence of this technique is the accumulation and storage of many scans through the spectrum. The storage device is a small digital computer. To operate successfully it is essential that each storage position, or address, correspond with a unique value of magnetic field. We have therefore modified the A-60 to permit switching from the internal scan to an external scan generated by the computer. This ensures a one-to-one correspondence between address and magnetic field. Use of this technique has enabled us to study solutions of chlorophyll as low as 10^{-4} M. The results of these experiments are included in the thesis of Anderson.[2]

A radio-frequency unit and probe for 25 Mc operation has been placed in operation. The unit is designed for P^{31} NMR. The first results obtained with this unit are given in Sec. 15 of this report.

Application of the continuous-averaging technique to the phosphorus NMR will permit study of a variety of biologically important phosphorus-containing molecules.

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1. Melvin P. Klein and George W. Barton, Jr., Rev. Sci. Instr. 34, 754 (1963).

2. A. F. H. Anderson, Some New Molecular Biology of Chlorophyll-a (Ph.D. Thesis), UCRL-10951, Aug. 1963.

22. STUDIES ON THE INHIBITION OF THE PHOTOREDUCTION OF FMN

G. K. Radda and Robert P. Foss

The photoreduction of flavin mononucleotide (FMN) and of its analogs without external electron donors, and by EDTA, [1] DPNH, [2, 3] and dihydrothioctic acid[4] has recently been described. It was shown that the first excited triplet state of the flavin participated in these reactions. [3]

In an effort to gain further understanding on the role of these photoreduction in vivo, the effect of a number of substances on the rate of photoreaction was investigated. The selection of these substances was guided by the following principles:

1. Since FMN is probably bound to proteins in photosynthetic systems, it was of interest to investigate initially the effect of several amino acids on the photoreduction.
2. It has been suggested[4] that the binding of the coenzyme to the protein in flavoproteins may be through a charge-transfer complex. It is also well known that FMN forms charge-transfer complexes with a number of donors in solution. [5] The influence of some of these types of substances on the photoreduction was therefore investigated.
3. Finally, the effect of some herbicides and other inhibitors of photosynthesis was also studied.

Results

The method for investigating the rate of anaerobic photoreductions was described previously. [1, 3] The reactions studied were the anaerobic photobleaching of FMN without an external reducing agent, and the photoreduction of FMN by diphosphopyridine nucleotide (DPNH) (and in one experiment by thioctic acid). We carried out all reactions in aqueous solutions, using phosphate buffers at pH = 7, and at ionic strength = 0.1. The concentration of FMN was between 0.2×10^{-4} and 0.5×10^{-4} M.

The reactions are inhibited markedly by substances such as serotonin and phenol (10^{-3} M) (Figs. 22-1 to 22-3). The effect of all the substances studied can be expressed as a % inhibition of the initial rate of photoreductions. These results are summarized in Table 22-I. For comparison

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1. G. K. Radda, in Bio-Organic Chemistry Quarterly Report, UCRL-10743, March 1963.

2. G. K. Radda, in Bio-Organic Chemistry Quarterly Report, UCRL-10934, July 1963.

3. G. K. Radda and Melvin Calvin, Inhibition of the Photoreduction of Flavin Mononucleotide, Nature (in press).

4. H. A. Harbury, K. F. Lanoue, P. A. Loach, and R. M. Amick, Proc. Natl. Acad. Sci. U. S., 45, 1708 (1959).

5. I. Isenberg and A. Szent-Györgyi, Proc. Natl. Acad. Sci. U. S., 44, 857 (1958); I. Isenberg, A. Szent-Györgyi, and S. L. Baird, Proc. Natl. Acad. Sci. U. S., 46, 1307 (1960).

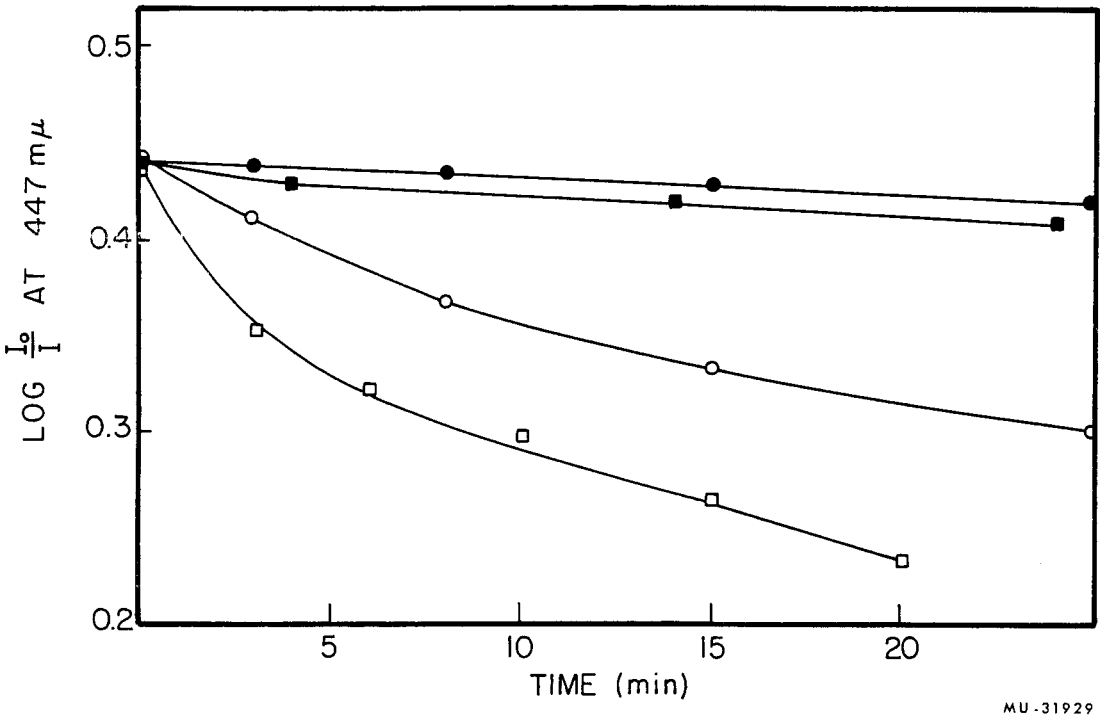

Fig. 22-1. Effect of inhibitors on photobleaching of FMN (pH = 7; phosphate buffer).
□ FMN
O FMN + caffeine (10^{-3} M)
▣ FMN + serotonin (10^{-3} M)
● FMN + phenol (10^{-3} M)

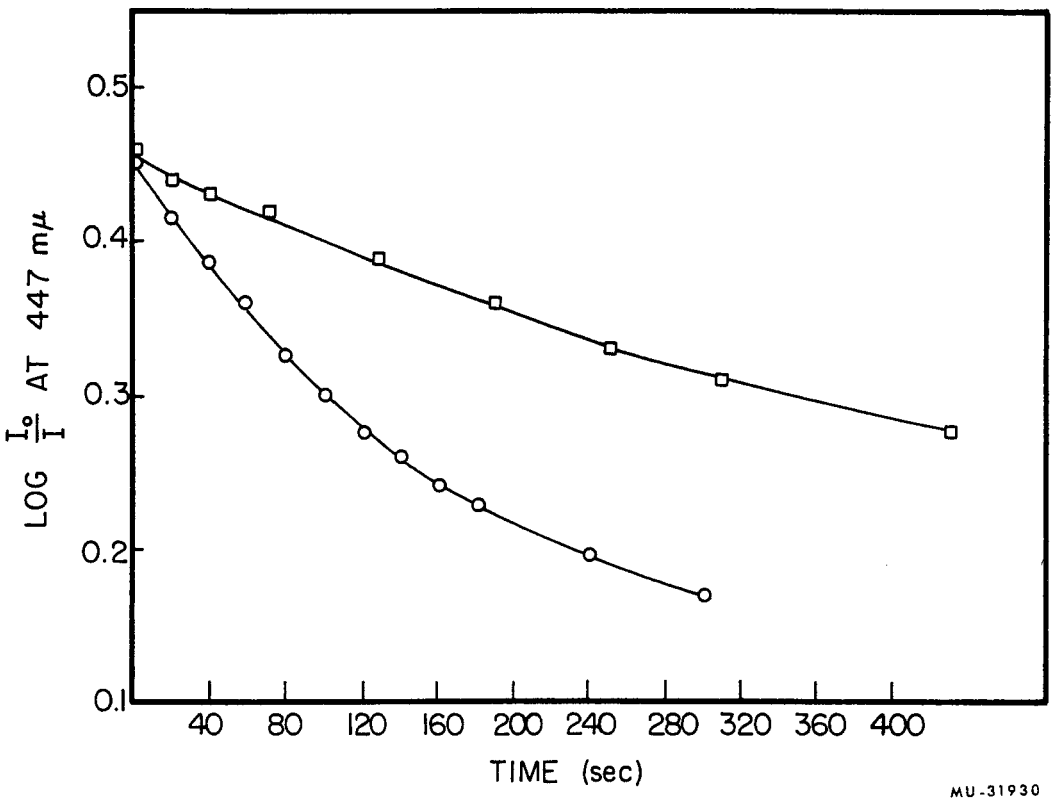

Fig. 22-2. Effect of serotonin on photoreduction of FMN by DPNH (0.5×10^{-3} M) pH = 7; phosphate buffer).
O FMN + DPNH
□ FMN + DPNH + serotonin (10^{-3} M)

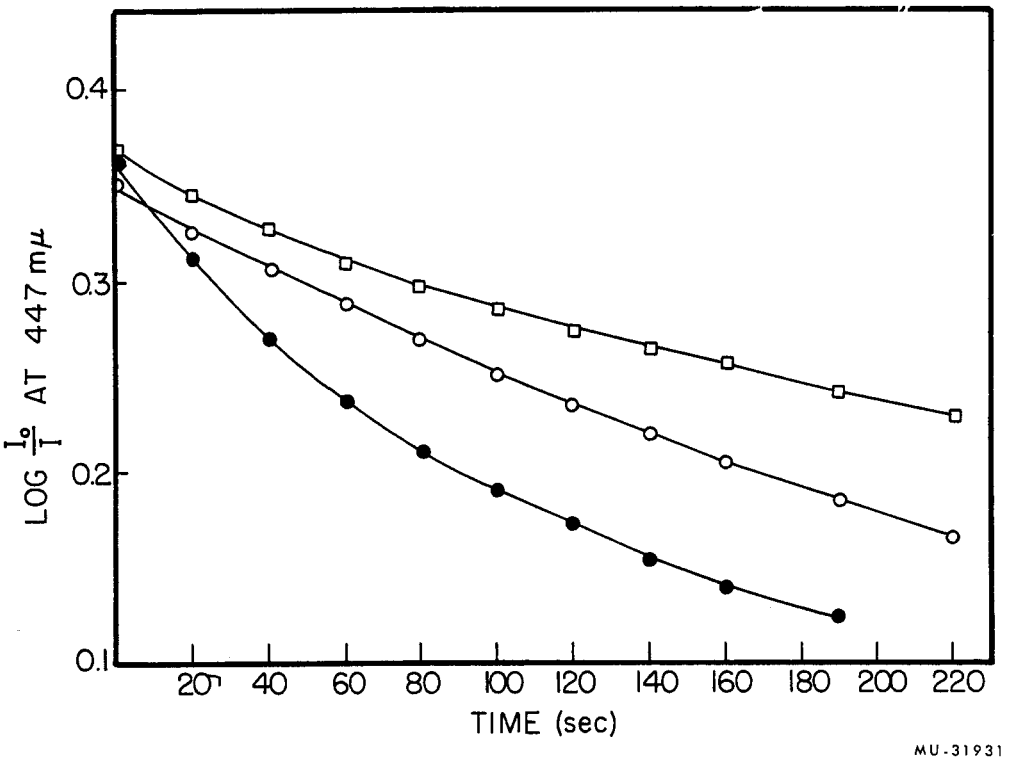

Fig. 22-3. Effect of dichlorophenylmethylurea (DCMU) and thioctic acid on photoreduction of FMN by DPNH (0.5×10^{-3} M).
● FMN + DPNH
O FMN + DPNH + DL-6-thioctic acid (10^{-3} M)
□ FMN + DPNH + DCMU (10^{-3} M)

Table 22-I. The effect of added substances on the initial rates of photoreduction of FMN.

Substance added	Temp (°C)	Effect on photobleaching (% inhibition)	Effect on photoreduction by DPNH (% inhibition)	Quenching of fluorescence (%)	Amount of charge-transfer complex formed (%)
Histidine 10^{-3} M	30	0	*	*	*
Phenylalanine 10^{-3} M	30	0	*	*	*
DL tryptophan 10^{-3} M	30	89	*	*	6.8[a]
L-tyrosine 10^{-3} M	30	90	*	*	*
Caffeine 10^{-3} M	30	65	*	1[b]	*
Muscle adenglic acid (10^{-3} M)	30	41	*	1.5[c]	*
Phenol 10^{-3} M	30	97	68	10[d]	*
Serotonin creatinine sulphate (10^{-3} M)	30	94	69	*	60[e]
Creatinine 10^{-3} M	30	0	*	*	*
Methyl viologen 10^{-3} M	17	0	*	*	*
DPN 10^{-3} M	30	*	0	*	*
DCMU 10^{-3} M	17	*	43	*	*
DL-6-thioctic acid 10^{-3} M	17	*	39	*	*
FAD (rel. to FMN)[f]	25	*	87	80[g]	*

*Not determined yet.

a. Ref. 5, equilibrium constant by absorption spectroscopy at pH = 7; temp 25 °C.
b. Ref. 6, calculated from dissociation constant measured by fluorescence at 17 °C, pH = 7, (quenching of fluorescence decreases with increasing temperature).
c. Ref. 6, calculated as in b at 17 °C for adenosine.
d. Ref. 7, measured directly at 15 °C, pH = 7.
e. Ref. 5, from equilibrium constant as in a.
f. FAD (rel. to FMN) in photoreduction by EDTA 92% inhibition.
g. Ref. 6, measured directly at pH = 7.

the inhibition by the adenine moiety in flavin adenine dinucleotide (FAD) compared to FMN are also given. In a number of cases the quenching of the FMN fluorescence under comparable conditions by the inhibitors has been reported, whereas in other cases the % of charge-transfer complex formed between the inhibitor and FMN can be calculated from the known equilibrium constants.[5,6,7] These are also included in Table 22-I.

When serotonin is added to a solution of $FMNH_2$ (FMN reduced photochemically prior to the addition of the inhibitor), there is no re-oxidation by the serotonin either in the dark or during illumination. On the other hand, when 2,4-dinitrophenol is added after FMN has been reduced, a slow increase in absorption at 445 mμ is observed in the dark. The total increase at this wavelength corresponds exactly to that expected on the basis of complete reoxidation of $FMNH_2$ to FMN.

The dependence of the % inhibition on the concentration of inhibitor has been measured for serotonin (Fig. 22-4). The temperature effect on the inhibition by serotonin is summarized in Table 22-II. It can be seen that the inhibition is more pronounced at lower temperatures.

Table 22-II. Effect of temperature on the inhibition by serotonin of the intramolecular photoreduction of FMN. (Serotonin concentration 10^{-4} M)

Temperature (°C)	Inhibition (%)
17	96
30	57
43	43

The phosphorescence spectrum of riboflavin in ethanol at 77°K is recorded in Fig. 22-5. The emission can be observed by eye and is seen as an orange glow lasting for about 2 seconds. When riboflavin is dissolved in a 1:1 (vol/vol) mixture of ethanol and phenol no such phosphorescence can be observed, although the glass still exhibits fluorescence.

The dark reduction of FMN by DPNH is entirely unaffected by serotonin under conditions in which the light-catalyzed reaction is inhibited.

Discussion

The observations described above can be briefly summarized as follows. Substances that are known to be good electron donors towards FMN (i.e., form charge-transfer complexes with it), or are expected to be good donors, are powerful inhibitors of the photoreduction. (The

6. G. Weber, Biochem. J., 47, 114 (1950).

7. K. Sakai, Nagoya J. Med. Sci. 18, 245 (1955).

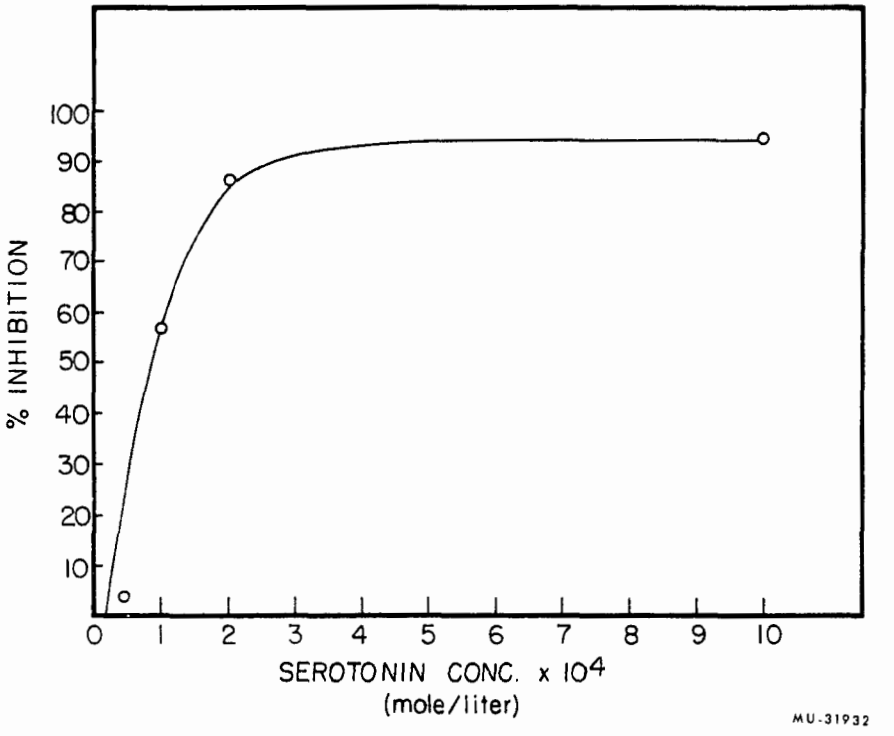

Fig. 22-4. Inhibition of photobleaching of FMN by serotonin (pH = 7; phosphate buffer). Effect of serotonin concentration.

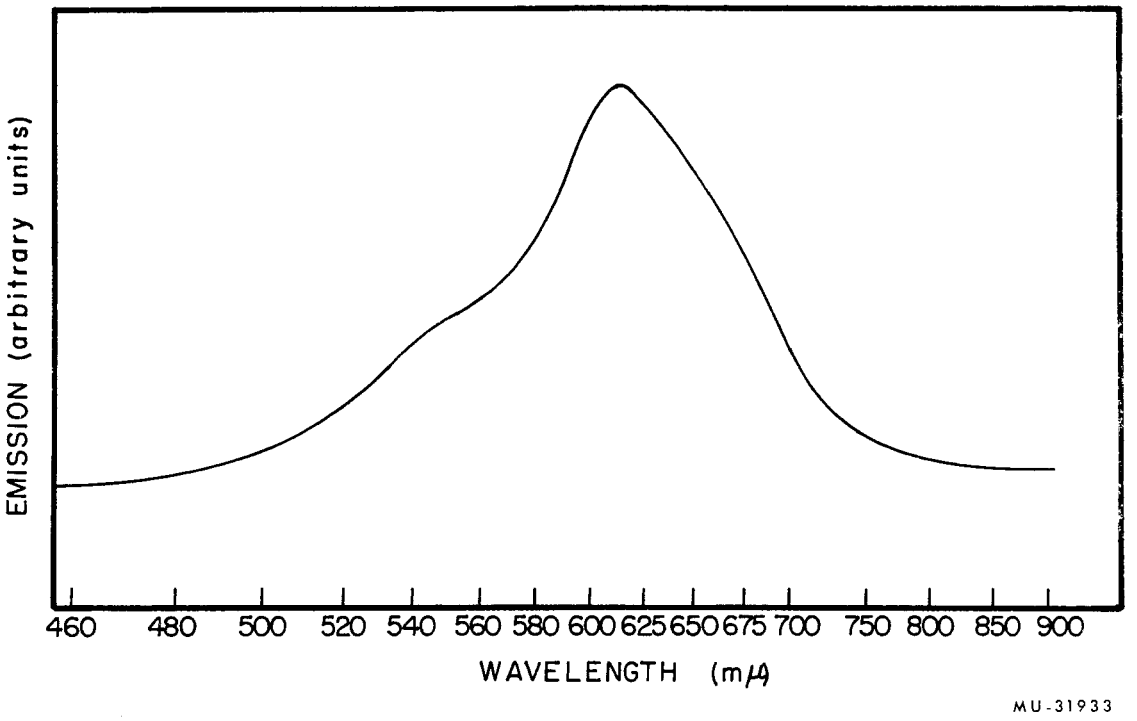

Fig. 22-5. Phosphorescence spectrum of riboflavin at 77 °K in ethanol.

following substances have been shown to form charge-transfer complexes with FMN: serotonin,[5] tryptophan,[5] caffeine,[4,6] phenol,[4] AMP,[8] and tyrosine[4]; phenylalanine is doubtful as there are two conflicting reports on its ability to form a complex.[4,5] On the other hand, good electron acceptors (e.g., methyl viologen, DPN) or substances that do not form charge-transfer complexes with FMN (e.g., histidine and creatinine[4]) have no effect on the rates. That the inhibition is not a result of the reoxidation of $FMNH_2$ by the substance either in the dark or in the light has been shown to be the case for serotonin.

This is probably also true for all the other inhibitors, except for 2, 4-dinitrophenol, as it is difficult to visualize any chemical reaction in which $FMNH_2$ would reduce the stable aromatic systems of the inhibitors. The fact that reduced thioctic acid reacts with FMN in the dark suggests that the reverse reaction between $FMNH_2$ and thioctic acid is not important, particularly since this reaction would be thermodynamically unfavorable (for thioctic acid $E^\circ = -0.286$ V,[9] and for FMN $E^\circ = -0.219$ V).[10]

A Possible Mechanism for the Inhibition. - It has been argued previously that the photoreduction of flavins proceeds through a long-lived triplet intermediate.[1,3] It is therefore reasonable to assume that in the absence of a reoxidation of $FMNH_2$ by the inhibitor, the inhibition is due to some interaction between FMN in the triplet state and the inhibitor. This is more likely since the effect of the inhibitors on the fluorescence of FMN is too small to account for the large decreases in rates. The same is true for the charge-transfer complex formed between FMN in its ground state and the inhibitor, the amount of complex formed being small when compared to the percent inhibition. Our hypothesis is further supported by the observation that the phenol effectively quenches the phosphorescence of riboflavin at 77° K.

The experimental results thus far obtained are in accord with a mechanism involving the formation of a triplet-state complex between excited FMN and the inhibitor. Such a mechanism has been proposed for the transfer of triplet-state energy from excited donors to appropriate acceptors in photosensitized and photoquenched reactions.[11] The mechanism may be written as:

$$\text{Donor}^* \text{ (triplet)} + \text{acceptor (singlet)} \rightarrow \text{complex}^* \text{ (triplet)} \rightarrow \text{donor (singlet)} + \text{acceptor}^* \text{ (triplet)} \quad (1)$$

$$\text{Donor}^* \text{ (triplet)} + \text{acceptor (singlet)} \rightarrow \text{complex}^* \text{ (triplet)} \xrightarrow{-E} \text{complex (singlet)} \rightarrow \text{donor (singlet)} + \text{acceptor (singlet)} \quad (2)$$

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8. P. Bamberg and P. Hemmerick, Helv. Chim. Acta 44, 1001 (1961).

9. W. M. Clark, Oxidation-Reduction Potentials of Organic Systems (Williams and Wilkins Co., Baltimore, 1960), p. 483.

10. H. J. Lowe and W. M. Clark, J. Biol. Chem. 221, 983 (1956).

11. R. P. Foss, Mechanism of Triplet Energy Transfer in Solution; Quenching of Excited States Benzophenone by Transition Metal Chelates, (Ph. D. Thesis) California Institute of Technology, 1963.

Reaction (1) describes the situation in which the lifetime of the intermediate complex is short compared to the time necessary for radiative or nonradiative decay of the complex, as a unit, to its ground singlet state. Reaction (2) represents the situation in which decay of the complex to its ground singlet state occurs before decomposition into ground-state donor and excited triplet-state acceptor takes place.

When very weak complexing between donor and acceptor occurs, such as in reaction (1), the efficiency of the acceptor to deactivate the excited donor decreases in a predictable manner as the triplet-state energy of the acceptor increases toward that of the donor.[11] However, even in the very weak complexing case, endothermic energy transfer may be large enough to compete significantly with other photochemical reactions of the excited donor. Strong complexing, on the other hand, allows deactivation of the donor to occur by direct radiative or nonradiative decay of the complex to its ground state (which may be considerably less stable than the excited-state complex), as described by process (2). Two possible explanations, based on the above mechanism, for our experimental results may be advanced:

A. Direct-energy transfer by process (1) between FMN and most inhibitors studied in this work seems highly improbable since the triplet-state energy of FMN is considerably lower than the triplet-state energies of most of the inhibitors. For instance, the maximum of the phosphorescence emission for FMN is 600 mμ compared to that for phenol, a very good inhibitor, with a maximum emission at 350 mμ.[12] Therefore, we suggest that the inhibition is due to the formation of a strong complex in the excited state, which may be viewed as a charge-transfer complex between the acceptor (in this case the FMN triplet) and the donor (the inhibitor). The formation of such a complex is reasonable, since FMN in the triplet state is a much better acceptor than in its ground singlet state (as evidenced by its ready reaction with hydrogen donors). The complex thus formed will lie at a lower energy level than the "free" triplet, and presumably its ability to return to the ground state by radiationless transition is more pronounced, thus resulting in its lesser reactivity towards the hydrogen donor.

B. The alternative way of looking at this phenomenon may be as follows. The charge-transfer complex formed between FMN in the ground state and the donor (which has a new absorption band on the long-wavelength side of the FMN spectrum) is itself the quencher of the FMN triplet. This quenching is the result of energy transfer from the FMN (triplet) to the charge-transfer complex (singlet), thus producing FMN (singlet) and charge-transfer complex (triplet). This latter triplet could in principle lie at a lower energy level than the FMN (triplet), and therefore the transfer is energetically permitted by reaction (1). The net result of this type of quenching is the same as the one described above.

The complex between the FMN (triplet) and the donor need not necessarily be of the charge-transfer type for cases in which the donor has a hydrogen capable of entering into hydrogen bonding (e. g., phenol). The

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12. G. N. Lewis and M. Kasha, J. Am. Chem. Soc. 66, 2100 (1944).

two forces (charge transfer and hydrogen bonding) may both contribute to the stability of the complex. Both explanations given above receive additional support from the observation that an increase in temperature decreases the efficiency of the inhibitor toward the deactivation of the excited FMN.

Significence of Inhibition Experiments in Relation to Photosynthesis - In assessing the significance of these inhibition experiments in relation to photosynthesis, the following facts seem particularly pertinent. Dichlorophenylmethylurea, which is known to inhibit oxygen evolution in photosynthesis, [13] has a fairly large inhibitory effect on the FMN photoreduction. Thioctic acid at a concentration level similar to that used in our experiments has been shown to have a definite effect on the early steps of photosynthesis, probably at the electron-transport level. [14] Finally, 2,4-dinitrophenol has been shown to have an inhibitory effect on cyclic photophosphorylation in the presence of FMN. [15] The fact that in our model experiments the inhibition does not appear to be specific, and that, in particular, certain amino acids are very effective inhibitors, at first seems to render the results described irrelevant to photosynthesis in vivo. If, however, the specificity of inhibition in vivo is not due to a chemical specificity but rather is a result of the ability of the inhibitors to penetrate to the relevant sites, the same type of chemical inhibition discussed in this work may be responsible for the specific effects in vivo.

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13. B. Kok and G. Hock, in Light and Life, ed. W. D. McElroy (Johns Hopkins Press, Baltimore, 1961), p. 397.

14. J. A. Bassham, H. Egeter, F. Edmonston, and M. Kirk, Biochem. Biophys. Res. Commun. 13, 144 (1963).

15. F. R. Whatley, M. B. Allen, and D. I. Arnon, Biochim. Biophys. Acta 32, 32 (1959).