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Ernest O. Lawrence

Radiation Laboratory

STUDIES OF THE CARBOXYDISMUTASE SYSTEM AND RELATED MATERIALS

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Thesis

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TABLE OF CONTENTS

		Page
I.	Introduction	1
	A. General Aspects	1
	B. History	2
	C. Purpose of the Research	9
II.	Experimental Procedure	10
	A. Abbreviations	10
	B. Apparatus	11
	C. General Analyses	20
	D. Special Analyses	26
	E. Preparation of Ribulose-1,5-diphosphate	27
	F. Reaction of RuDP with Cyanide	33
	G. Hamamelonic Acid	35
	H. Preparation of Carboxydismutase	36
	I. Homogeneity Studies of Carboxydismutase Preparations	47
	J. The Carboxydismutase Assay	51
	K. Carboxydismutase Activity in the Presence of Digitonin	52
	L. Cofactors and Inhibitors	53
	M. Reproducibility of the Carboxydismutase Assay	54
	N. pH vs Carboxydismutase Activity	54
	O. Stability of Carboxydismutase	54
	P. Experiments with Carbonic Anhydrase	55
	Q. Other One-carbon Compounds as Possible Substrates	55
	R. Occurrence of Carboxydismutase	56

Page

S. Carboxydismutase from Chloroplasts Isolated in Nonaqueous Solvents 57

T. Attempts to Trap the Intermediate of the Carboxydismutase Reaction 58

III. Results and Discussion

A. Ribulose-1,5-diphosphate 60

B. Preparation of Carboxydismutase 69

C. Homogeneity of Various Carboxydismutase Preparations 85

D. The Carboxydismutase Assay 102

E. Enzyme Activity as a Function of Enzyme and Substrate Concentrations 132

F. pH Effect on the Carboxydismutase Activity 138

G. Stability of the Enzyme 140

H. Reversibility of the Carboxydismutase Reaction 141

I. Specificity of the Enzyme 142

J. Occurrence of the Enzyme 150

K. The Mechanism of Enzyme Action 155

IV. Summary 179

V. References 184

INTRODUCTION

General Aspects. Although each carboxylation (or decarboxylation) reaction results in an alteration of the structure of an organic compound by only one carbon atom, the role of this reaction in complex biochemical systems is no less an important one. Rarely is there a case in metabolism, where the substrate is not subjected to the action of a carboxylation or decarboxylation enzyme (carboxylase or decarboxylase, respectively) since ultimately, the substrate either may end as carbon dioxide and water or the substrate may take up carbon dioxide to form a new compound of higher molecular weight. Indeed, the list of known carboxylases and decarboxylases is large. Even the details of a given enzyme are so extensive that it is beyond the scope of this thesis to describe anything about the enzyme except for its possible relationship to carboxydismutase. There is, however, one general pattern that emerges from a casual examination of a list of enzymically catalyzed carboxylation reactions;* that is, one can catagorize them into three groups: (1) those reactions requiring extra energy; (2) those reactions requiring reducing power and (3) those reactions not requiring either extra energy or reducing power.[1,2] The last of the three categories may also be characterized as enolic carboxylation reactions.[2] Examples of each group of carboxylation reactions are, respectively, (1) the propionyl coenzyme A carboxylase system,[3] (2) the "malic" enzyme system[4] and (3) oxalacetic decarboxylase system.[5] Falling also within the last group is the reaction catalyzed by carboxydismutase. This reaction has the following well established stoichiometry:[6,7]

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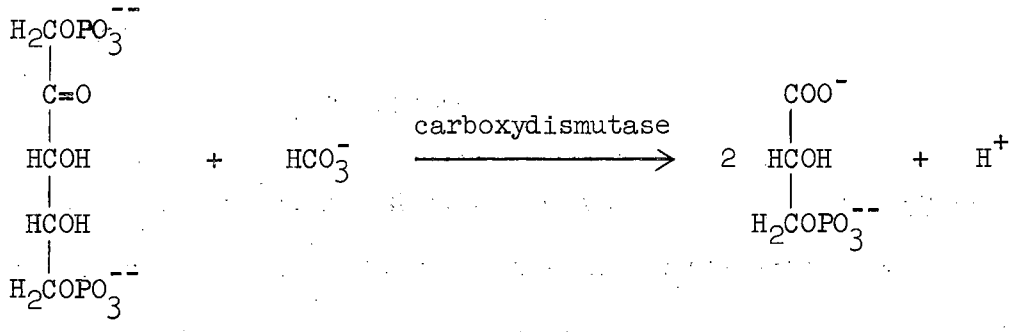
* Since decarboxylation reactions are related thermodynamically to carboxylation reactions, any discussion of the latter would automatically embrace that of the former. Henceforth no further reference will be made towards decarboxylation reactions unless specifically required.



ribulose-1,5-diphosphate 3-phosphoglycerate

From the above equation it is not immediately obvious what role this enzyme system plays in metabolism. All of the available evidence indicate that this system appears to be related to the primary carboxylation reaction of photosynthesis. The historical developments leading to the discovery of this enzyme system will illustrate this point.

History. Ever since carbonic acid was first implicated in photosynthesis by such pioneers as Senebier and Ingen-Housz at the end of the 18th century, the subject of photosynthesis in this respect has intrigued many scientists over the centuries. In fact, so enormous is the literature dealing with photosynthesis, that, in the words of Rabinowitch, '... a complete review of all papers on this subject, ... appears almost impossible.'[8] (A very illuminating account of the early history of photosynthesis may be found in this volume of Rabinowitch.) It was not, however, until the later half of the 19th century and the turn of the 20th century that the nature of the first stable product of carbon dioxide assimilation during photosynthesis was considered. The tacit assumption was that the primary product was organic in nature. From this point of view it is understandable that nearly a century elapsed before any hypothesis was proposed, for the chemistry of organic compounds was not conceived but several decades earlier by Wöhler (1828). Even so, many theories and speculations were proposed.[9] Among the compounds suggested as the first intermediates

of photosynthetic carbon dioxide fixation were organic acids (Liebig, 1843); formaldehyde (Baeyer, 1864); glycolic aldehyde (Fincke, 1914) and chlorophyll-carbon dioxide (Willstätter and Stoll, 1918). Many attempts were made to find these intermediates in plants, but the results were inconclusive. Feeding of some of the proposed intermediates to the plants also gave indefinite results. Finally, experiments on the isolation of enzyme systems capable of yielding the primary product of carboxylation were totally lacking.

Thimann, in 1938, returned to the organic acid theory of Liebig, but with a slight modification.[10] He suggested that an organic acid was the carbon dioxide acceptor. This acceptor underwent a second carboxylation followed by a photoreduction and then after undergoing various intramolecular changes, the acceptor was regenerated. This cyclic process was much like the Krebs cycle operated in a reverse manner. Again, little, if any, evidence was available in support of this idea.

With the advent of the radioactive isotope of carbon, C^{11}, the first major breakthrough was achieved. Ruben, et al.[11] allowed barley plants to assimilate $C^{11}O_2$, both in the light and in the dark, and found that the leaves incorporated radioactivity into the water soluble extract of the leaves. If, on the other hand, the barley plant was kept in the dark for 3 hours prior to the administration of $C^{11}O_2$, then very little radioactivity was detected in the water soluble extract. In a later paper by the same group of workers, the fixation of $C^{11}O_2$ by Chlorella was described.[12] By using standard techniques of organic chemistry, these workers established the fact that the compounds which incorporated the radioactive isotope possess similar chemical properties, whether formed in the light or in the dark. Thus a major portion of the radioactivity in the water-soluble extract of the algae was precipitated with Ba^{++} in

80% ethanol. Furthermore, on decarboxylation of the barium salt, a considerable percentage of the radioactivity was found in the resulting $BaCO_3$, indicating that the label was originally present in carboxyl groups. Application of the Schotten-Baumann reaction to the aqueous extract using benzoyl chloride followed by extraction of the resultant ester with chloroform showed that a substantial quantity of radioactivity resided in the chloroform layer. This fact indicated the presence of alcoholic groups in the labeled compounds. (Tests to demonstrate the presence of radioactive formaldehyde via its hydrazone were negative.) Although a large number of compounds were added as carriers, attempts to identify any one of them with the radioactivity (present in the water-soluble extract of Chlorella) failed. The problem, then, became one of the separation and identification of extremely minute quantities of radioactive substances. The requirements of this problem could not be fulfilled by the carbon-11 isotope because of its short half life (ca. 21 minutes) since any problem of this sort would usually be lengthy.

When the long-lived radioactive carbon isotope (C^{14}) became available after World War II, the answer to this problem was, at least in part, furnished. In 1948, Benson and Calvin described the first application of this isotope to the study of the effect of preillumination on the dark assimilation of carbon dioxide in Chlorella.[13] By means of ether extraction, adsorption column chromatography and ion exchange column chromatography, they obtained data which suggested that phosphoglyceric acid was the first stable intermediate of $C^{14}O_2$ fixation. These findings were confirmed by Gaffron and his group,[14] although initially, their results were in contradiction with those of Benson and Calvin.[15]

With the application of paper chromatography[16] coupled with the detection of the labeled material by radioautography,[17] the role of

phosphoglyceric acid as the first stable product of CO_2 fixation became increasingly clear. This method, permitting the rapid separation and identification of many compounds simultaneously, confirmed the previous findings obtained by column techniques. In fact, as Scenedesmus was exposed to $C^{14}O_2$, in the light, for progressively shorter times, the radioactive phosphoglyceric acid spot on the chromatogram became more and more dominant until for 5 seconds photosynthesis, about 90% of the tracer was found in phosphoglyceric acid with approximately 90% of the label in the carboxyl group.[18]

Having established the role of phosphoglyceric acid in photosynthesis, the next problem was to determine the chemical nature of the carbon dioxide acceptor. The fact that the first stable carboxylation product was a three carbon compound naturally implicated a two carbon compound as an acceptor. One of the very first of such compounds proposed was vinyl phosphate. That vinyl phosphate might serve as the CO_2 acceptor was based on two facts:[19] 1) a small spot (on the chromatogram) of 2-phosphoglyceric acid with a higher specific activity than the major spot, 3-phosphoglyceric acid, was found and 2) a small amount of C^{14} was lost from the aqueous alcoholic extract of Chlorella (after 1 minute photosynthesis in the presence of $C^{14}O_2$) when this extract was treated with 1 N HCl for 10 minutes at 80°C. Presumably the loss of radioactivity was in the form of acetaldehyde. The latter results, however, could also be accounted for by the decarboxylation of pyruvic acid[20] and phosphoenolpyruvic acid which are most likely to be present in the extract. The presence of four-carbon dicarboxylic acids in Chlorella extract was also consistent with the view that vinyl phosphate was the primary acceptor leading to 2-phosphoglyceric acid after carboxylation. When the role of malic acid was later shown not to be connected with the primary carboxylation reaction,[21] vinyl

phosphate was abandoned in favor of an alternate, as yet, unspecified C_2 precursor of phosphoglyceric acid.

With the application of the tracer technique for measuring steady-state concentrations of compounds in the metabolic pool, the first clue towards the primary carbon dioxide acceptor was revealed. The photostationary concentration of each compound was determined by exposing the algae to radioactive bicarbonate for a long period of time followed by extraction and separation of the compound in question with the aid of chromatography. The radioactivity incorporated into the compound was counted directly on the paper chromatogram. The specific activity of the initial $C^{14}O_2$ being known, it was then possible to calculate the concentration of the compound.

When such an experiment was carried out in the light followed by a dark period, all in the presence of $C^{14}O_2$, two marked changes occurred.[22] Thus with the onset of the dark period, the phosphoglyceric acid concentration suddenly increased while, concomitantly, the diphosphate (mostly ribulose diphosphate) concentration decreased. These results not only confirmed the fact that phosphoglyceric acid was the primary product of carboxylation, but also strongly suggested that ribulose-1,5-diphosphate was the acceptor.

This idea was strengthened by an experiment of another type.[23,24] Again, the algae was exposed to $C^{14}O_2$ until steady state was achieved when suddenly the carbon dioxide pressure was reduced to almost zero. The transient changes in the concentrations of many metabolites and notably those of phosphoglyceric acid as well as ribulose diphosphate were measured. The results showed a decrease in the concentration of phosphoglyceric acid with a concomitant increase in the concentration of ribulose diphosphate.

Furnished with these facts, the next problem was to obtain a cell-free extract of algae capable of yielding phosphoglyceric acid from $C^{14}O_2$ (or $HC^{14}O_3^-$) in the presence of ribulose diphosphate. This, in fact, was accomplished in 1954 in this laboratory by sonically rupturing Chlorella followed by centrifugation to obtain a cell-free preparation.[25] To this preparation was added labeled bicarbonate and ribulose diphosphate (isolated in a separate experiment by chromatography of an aqueous alcohol extract of Scenedesmus). In addition, experiments were also carried out with this cell-free extract using unlabeled bicarbonate and labeled ribulose diphosphate. In either case, the product obtained was radioactive phosphoglyceric acid (along with lesser quantities of organic acids). Where labeled bicarbonate plus unlabeled ribulose diphosphate were used, all of the tracer was located in the carboxyl group.

About the same time, Weissbach, et al. found in soluble spinach extract, an adenosine triphosphate and triphosphopyridine nucleotide dependent carboxylation system.[26] Thus, when this enzyme system was incubated with radioactive carbonate and ribose-5-phosphate in the presence of these cofactors, carboxyl-labeled phosphoglyceric acid was produced. If, however, ribose-5-phosphate-1-C^{14} was incubated with unlabeled carbonate, then the phosphoglyceric acid was tagged mostly (70%) in the β carbon of this acid; the rest of the radioactivity being in the carboxyl carbon.

It thus became apparent that the groundwork for future studies on this enzyme system was established. Indeed, many more publications on this subject were to follow -- on more refined studies of the spinach leaf enzyme system as well as on studies of similar enzyme systems derived from other sources. These studies are treated in their proper places in the ensuing sections of this thesis.

As a matter of historical interest, due credit should be given to Fager for the early discovery (1952) of an enzyme system from spinach leaf macerates capable of forming phosphoglyceric acid from carbon dioxide.[27] By 1954 (a paper submitted Oct. 31, 1953) he was able to characterize the carbon dioxide acceptor which led to this product, namely, that it was a relatively stable phosphate ester, but was not vinyl phosphate, phosphoglycolic acid, phosphoglycoaldehyde or ketose (fructose, sedoheptulose or ribulose) monophosphates.[28] The exact nature of the acceptor compound, however, remained obscure.

It should be noted that not all workers in the field of photosynthesis are in complete agreement with the idea that phosphoglyceric acid is the first product of carbon dioxide fixation or that ribulose diphosphate is the carbon dioxide acceptor. For example, Gibbs and Kandler, on the basis of the asymmetric labeling of glucose phosphates fromed during photosynthesis, suggested that the hexose diphosphate could not have arisen from the condensation of glyceraldehyde phosphate with dihydroxyacetone phosphate.[29] In support of this idea, Kandler then identified hamamelonic acid diphosphate with the early product of photosynthetic carboxylation.[30] This conclusion was reached by carrying out photosynthesis experiments with algae in the presence of radioactive bicarbonate and high concentrations of cyanide. Mortimer, working along the same lines, offered evidence for an alternate pathway for the photosynthetic carbon dioxide assimilation.[31] He conducted experiments with soybean leaves in the presence of labeled carbon dioxide and found that the level of glyceric acid was reduced to zero when hydrogen cyanide was added to the system. Finally, Gibbs found that the starch glucose was tagged mostly in the C_4 position when radioactive formate was fed to algae in the presence of 5% unlabeled carbon dioxide.[32]

Other experiments suggesting alternate pathways of carbon of a different nature were performed by Warburg[33] and Boichenko.[34] Thus Warburg, on the basis of fluoride inhibition studies, concluded that the decarboxylation of glutamic acid to form γ-aminobutyric acid and CO_2 is closely connected with the initial photosynthetic process. (This result and those of the preceding paragraph were discussed adequately in a recent paper by Calvin.)[35] The experiments of Boichenko, however, yielded results that implicate a nonprotein, noncarbohydrate, iron-containing CO_2 acceptor, which on carboxylation, resulted in an insoluble polyhydroxyacid. Whether the latter two pathways are related to a cyclic photosynthetic process remains a question. In any case, since the subject of this report deals only with the in vitro carboxydismutase system, any discussion of the investigations mentioned above will be considered only when it is relevant.

Purpose of the Research. The next thing to recognize are the objectives of the research described in this thesis. These are listed below:

1. To study the physical and chemical properties of ribulose diphosphate.
2. To purify extracts of Tetragonia expansa (New Zealand spinach) by several different methods and following this, to compare the purity of these preparations via their specific enzymic activity as well as their physical and chemical properties.
3. To study some properties of cell-free Chlorella extracts with respect to the carboxydismutase activity.
4. To study the effects of various additions (e.g. activators and inhibitors) on the carboxydismutase system.
5. Finally, to study the effects of the preliminary incubation of the activating metal ion and the substrates on the activity of carboxydismutase.

EXPERIMENTAL PROCEDURE

Abbreviations. The following abbreviations are henceforth used throughout this text.

General Abbreviations:

cpm	: counts per minute	mM	: millimole(s)
dpm	: disintegrations/minute	min	: minute(s)
eq(s)	: equation(s)	M	: molar
G-M	: Geiger-Mueller	N	: normal
g	: gram(s) or acceleration due to gravity	ppt	: precipitate
ir	: infrared	rpm	: revolutions/min.
kc	: kilocycles per second	soln	: solution
μC	: microcurie(s)	sp gr	: specific gravity
μg	: microgram(s)	sq	: square
μl or λ	: microliter(s)	supn	: supernatant liquid
μM	: micromole(s)	uv	: ultraviolet
ma	: milliamperes	hr	: hour
mg	: milligram(s)	O.D.	: optical density
mμ	: millimicrons	temp	: temperature
		xs	: excess

Special Abbreviations:

AS	: $(NH_4)_2SO_4$	DNP	: 2,4-dinitrophenyl
ATP	: adenosine-5'-triphosphate	EAMT	: Ehrlich ascites mouse tumor
CE	: chloroplast extract	EtOH	: ethanol
EDTA	: ethylenediamine-tetraacetic acid	FDNB	: 1-fluoro-2,4-dinitrobenzene
HmDP	: addition product of cyanide to RuDP (hamamelonic acid diphosphate and its isomer)	GSH	: glutathione

HOAc	: acetic acid	R_f	: ratio of distance travelled by spot to distance travelled by solvent front. Distance is measured from the origin to the densest portion of the spot.
MeOH	: methanol		
P_i	: inorganic phosphate		
μ	: ionic strength		
PGA	: phosphoglyceric acid	RMP	: ribose-5-phosphate
PrOH	: n-propanol	SAS	: saturated ammonium sulfate
PS	: photosynthesis	TDP	: threose-2,4-diphosphate
CoA	: coenzyme A		
OAc	: acetate	TPP	: thiamine pyrophosphate
PEP	: phosphoenolpyruvic acid	tris	: tris(hydroxymethyl) aminomethane
P-glycolic	: phosphoglycolic acid		

Apparatus. Homogenizers: Plant and animal tissues were ruptured with the aid of one of the following homogenizers: 1) blender, operated at full speed; 2) Virtis homogenizer, operated at 40,000 rpm; 3) Raytheon magnetostriction oscillator at 9 kc; 4) Potter-Elvehjem type homogenizer with a Teflon pestle; or 5) mortar and pestle, with or without sand. The sand was washed with warm HCl until the acid washing was colorless. It was then washed exhaustively with distilled water.

Centrifuges: The following centrifuges were employed for preparative purposes: 1) International Clinical Centrifuge (up to 2,500 X g), 2) International Portable Refrigerated Centrifuge, Model PR-2 (up to 2,500 X g); and 3) Spinco Ultracentrifuge, Model L (from 3,000 to 145,000 X g). All g values are maximum values. The homogeneity of enzyme preparations was studied in the Spinco Ultracentrifuge, Model E.

Electrophoresis Equipment: Electrophoresis of substances by the moving boundary method employed a Perkin-Elmer Tiselius Electrophoresis

Apparatus, Model 38. Studies by the zonal electrophoretic technique were performed with a horizontal paper strip apparatus.[36]

Radioactivity Counters: The radioactivity of samples were measured using a flow counter equipped with a thin window G-M tube. Precise countings were done in an assembly consisting of a Nuclear-Chicago scaling unit (model 183) with an automatic sample changer (Model C-110A) and a printing timer (Model C-111). The counter tube window for this arrangement was a Micromil* film and the quenching gas (Q gas) flowing through the system was composed of 99.05% He and 0.95% isobutane. Less precise counting was achieved with a different set-up; the counter tube being a modified G-M tube (Scott) fitted with a Mylar window[37] and flushed continuously with Q gas.

The samples were spread out on thin aluminum planchets in those cases where the samples were acidified with acetic acid and on glass planchets where the samples were acidified with HCl. The material was transferred to the planchet while on a rotating table,[38] with the aid of micropipets and to facilitate the spreading of the material, 1 or 2 drops of detergent (Aquet,** diluted 500 times) were added. (Some micropipets from 5, 10 and 15 µl, have been calibrated by weighing the volume of water delivered into a previously tared closed vessel. It was found that, in general, the reproducibility of the pipeting is within ± 4% of the mean value and the error of the mean value from that of the rated value falls also within 4%.) In general, the area covered by the radioactive material on the planchet was about 8.5 cm.^{2} The planchets were either counted with the Mylar window of

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* Supplied directly by the Nuclear-Chicago Corp.

** From the Emil Greiner Co., 22 N. Moore St., New York

the G-M tube flushed over the sample, or they were counted while the planchets were set in a recess approximately 2 to 3 cm from the window of the detector.

The counters were corrected for coincidence by making the appropriate serial dilutions of a high specific activity (1 μC/μM) amino acid solution, plating these solutions, and counting them. At the most, the amount of material on the planchet was of the order of 10^{-3} mg/sq cm and therefore, may be considered as "infinitely thin."[38] The correction thus obtained depended on the type of detector and scaler used and amounted to as much as 5% at 10,000 cpm for the Scott tube detector while for the automatic sample changer arrangement, no corrections were required.

Occasionally it was desirable to calculate the amount of product formed from a reaction involving labeled bicarbonate. This was achieved by determining the factor for converting cpm to dpm, providing that the stoichiometry of the reaction and the specific activity in μC/μM of the radioactive bicarbonate are known. A series of "infinitely thin" plates of $BaC^{14}O_3$ standard plates were prepared as follows: the $BaC^{14}O_3$ was precipitated from a $NaHC^{14}O_3$ solution of known specific activity, containing carrier amounts of Na_2CO_3, using $BaCl_2$ as the precipitant. The amount of Na_2CO_3 chosen was such that no more than 0.05 mg/sq cm of $BaC^{14}O_3$ was on the planchet for a given maximum aliquot portion of uniform suspension of $BaC^{14}O_3$. The precipitate of $BaC^{14}O_3$ was washed free of soluble salts with distilled water followed by ethanol. Different aliquot portions of a uniform (representative) suspension of the $BaC^{14}O_3$ were plated on aluminum planchets, with and without detergent, and then were counted in the usual manner. A stable reference standard (an "infinitely thick" $BaC^{14}O_3$ plate covered with a film of shellac) was also counted in

order to correct for daily variations in the counters. The results of the counting were plotted with the ordinate in terms of cpm per unit volume vs the abscissa in terms of the volume plated on the planchet. The true "infinitely thin" plate value was obtained by extrapolating to zero volume. A value of 1 cpm was found to be 4.6 $\pm$ 0.1 dpm when the stable reference standard gave a value of 4,200 cpm. It should be pointed out that exact specific activity of the bicarbonate used for the preparation of the $BaC^{14}O_3$ standards need not be known as long as the same bicarbonate was used in the reaction to be studied. Thus this method automatically compensated for any uncertainty in the specific activity of the labeled bicarbonate.

Spectrophotometers: The Cary Recording Spectrophotometer, Model 11 and 14, were used for both spectral analyses and quantitative determinations. In the latter determinations, the absorption of the sample was measured over a range of wavelengths. The Beckman Spectrophotometer, Model DU, however, was employed only for quantitative analyses and at a fixed wavelength. This instrument was particularly suited for measuring the absorption of eluates from column chromatography. All optical densities for both types of spectrophotometers were read in cells of 1 cm light path. Where monochromatic light was not absolutely essential, as in the colorimetric determination of phosphates or proteins, the Klett-Summerson Photoelectric Colorimeter was employed. Infrared spectrum analyses, from 2 to 15 microns, were carried out in an Infra-Red Recording Spectrophotometer, Model AB2 (Baird Associates, Inc.), equipped with a NaCl prism. The samples for this instrument were made into KBr pellets.

Fraction Collectors: Eluates from chromatographic columns were collected in test tubes or graduated centrifuge tubes placed in a fraction collector. The fraction collectors were of two types: 1) time operated, the time per fraction being kept constant and 2) siphon operated, the volume per fraction being kept constant.

Gradient Elution Apparatus: Aside from the usual stepwise gradient concentration techniques for the elution of chromatographic columns, a particularly effective way is to use a continuously changing concentration for this purpose. Such a continuous gradient was achieved in a specially designed apparatus. This apparatus consisted of a reservoir which contained a solution of concentration A flowing into a mixing vessel at a rate r_1. In turn, the solution in the mixing vessel flowed out (into a column) at a rate r_2. The amount of material x in a volume V of the liquid in the mixing vessel at any time t is described by an equation of continuity:[39]

$$dx/dt = Ar_1 - (x/V)r_2 \qquad \text{eq. I}$$

which becomes, when $x/V = C$; the concentration:

$$V(dC/dt) + C(r_1 - r_2) = Ar_1 - Cr_2 \qquad \text{eq. II}$$

One special case arises when $r_1 = r_2 = \text{constant} = R$. Under these conditions, V = constant also and the solution of eq. II is

$$C = A - (A - C_o)e^{-(R/V)t} \qquad \text{eq. III}$$

where C_o is the initial concentration of the material in the mixing vessel. Equation III is then a description of an exponential gradient. The experimental arrangement to achieve this type of gradient[40] is to connect a separatory funnel (the reservoir), with an airtight seal, to an Erlenmeyer flask (the mixing vessel). Flush at the bottom of the flask is attached a horizontal side arm (the outlet tube). The solution within the mixing vessel is constantly stirred with a magnetic stirrer. The concentration of the solution emerging from the outlet obeys eq. III.

When $r_1 \neq r_2$ the solution to eq. II is given by the expression:

$$\ln[(A-C)/A - C_o)] = [r_1/ (r_2 - r_1)]\ln(V/V_o) \quad \text{eq. IV}$$

where V_o is the initial volume in the mixing vessel.

An interesting case is when $r_2 = 2r_1$, eq. IV reducing to

$$C = A - (V/V_o)(A - C_o) \quad \text{eq. V}$$

In terms of the flow rate, r_1, where $V = V_o - r_1 t$, eq. V has the form:

$$C = C_o + (r_1 t/V_o)\ (A - C_o) \quad \text{eq. VI}$$

(A similar equation was also derived and given elsewhere.)[41] Under these conditions the concentration at the outlet tube varies linearly with time. The device to achieve the condition where $r_2 = 2r_1$ is shown in the lower left hand section of Fig. 1. It is imperative that the reservoir K and the mixing vessel L are identical in size and shape. Another apparatus which can accomplish the same goal is described by Lederer.[42]

The system shown in the upper right hand corner of Fig. 1 is a screw-driven syringe, providing pressure into the elution apparatus. With this system a pressurized-controlled flow rate is furnished. This system is particularly suited for certain types of chromatographic columns where the flow rates are apt to vary during the development. Under these circumstances eluates are collected in a time-operated fraction collector, the volume collected in each tube being nearly equal.

To test the characteristics of the system shown in Fig. 1, the following experiment was carried out: Water (50 ml) was added to the mixing vessel L and a dichromate solution (50 ml, prepared by mixing 0.5 ml of a saturated $K_2Cr_2O_7$ solution to make 50 ml of aqueous solution) was added to the reservoir K. To the outlet O of this system was attached a powdered

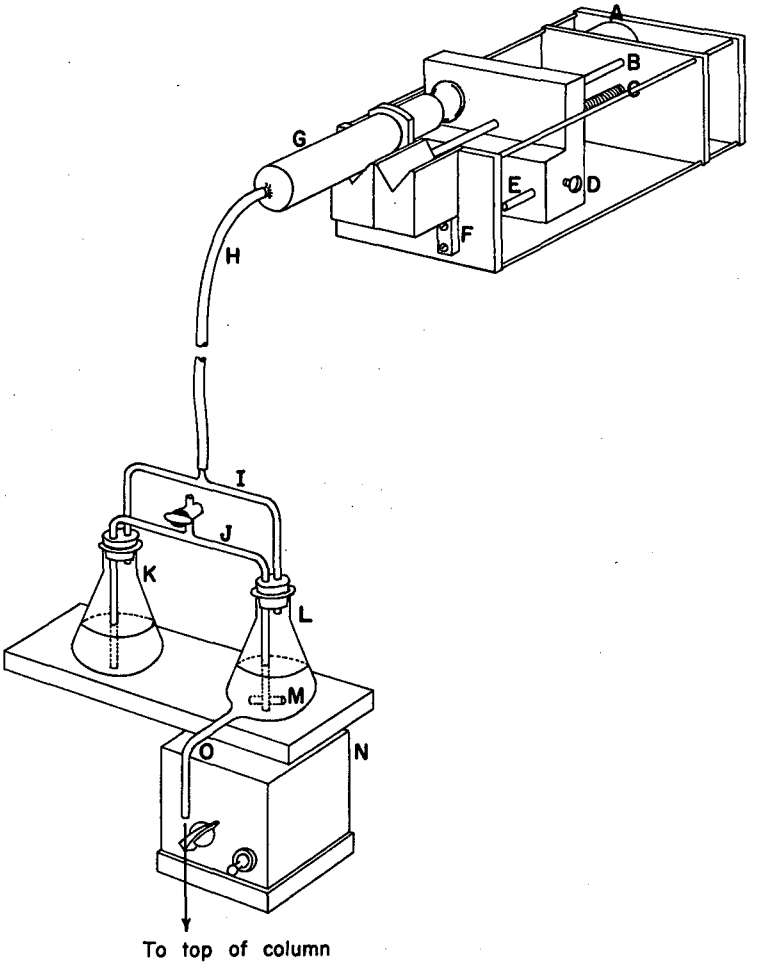

Fig. 1. Controlled flow linear gradient elution apparatus. A = drive motor; B = guide rod; C = feed screw; D = drive plate; E and F = limit switching mechanism; G = syringe; H = rubber tube; I = glass tube; J = siphon with stopcock; K = reservoir; L = mixing vessel; M = magnet; N = magnetic stirrer; and O = outlet tube. Full details of the operation of the flow controller are given elsewhere.[43]

glass column (1 cm diameter X 20 cm high). Since C_o, the initial concentration of dichromate ion in the mixing vessel, is zero, eq. VI reduces to the following expression:

$$C = (Ar_1/V_o)t,$$

where C is the concentration of dichromate ion in the mixing vessel at time t, A is the concentration of dichromate ion in the reservoir, r_1 is the flow rate from the mixing vessel and V_o is the initial volume of the water in the mixing vessel.

The siphon was formed by drawing the liquid up the siphon tube J with reduced pressure applied through the opened stopcock. After the stopcock was closed, the magnetic stirrer M and N and the flow controller A-G were switched on. The solution from the column was collected using a time-operated fraction collector. The flow controller was adjusted to give a flow rate of about 3 ml/20 minutes. The amount of dichromate collected in each tube containing 3 ml was determined spectrophotometrically at 372 mμ by diluting 50 μl of the fraction with 5 ml of 0.05 N KOH. The volume collected in each tube was also measured. The results are shown in Fig. 2. As expected, from tube no. 4 (retention volume of the column) to tube no. 27 (where the liquid in the mixing vessel was almost completely exhausted), the concentration of the dichromate varies linearly with time and the volume per tube is constant to about ± 0.1 ml.

Conductivity Meter: After protein is precipitated with a given precipitant, it is often necessary to reprecipitate it in order to achieve further purification. The quantity of precipitant occluded in the first precipitate, however, must be taken into account. This was done by measuring the conductivity of a standard volume of buffered solution containing a small amount of the redissolved protein. For this purpose, a Conductivity Bridge, Model RC, Industrials Inc., Jersey City 5, New Jersey, with a

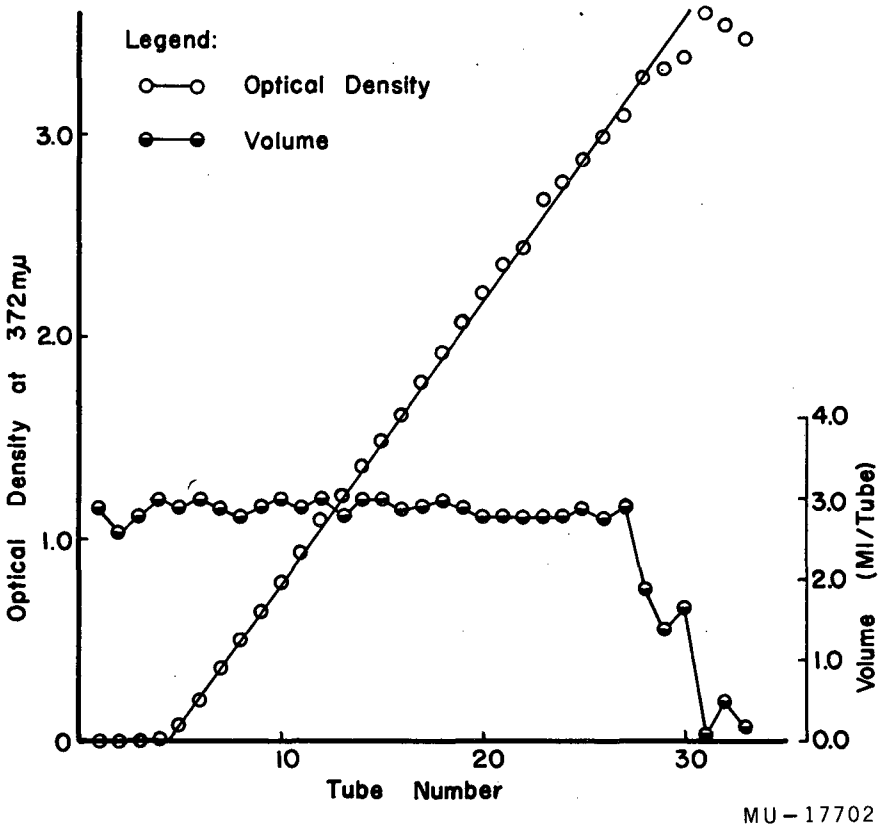

Fig. 2. Characteristics of the linear gradient elution apparatus. See text for explanation.

conductivity cell (cell constant = 1) was employed. The measured conductivity was compared with a standard curve of conductivity vs concentration of precipitant, the latter being dissolved also in a standard volume of the same buffer used with the protein. Corrections were then applied for the occluded precipitant before reprecipitation.

Rotary Dialyzer: It was often necessary to remove salts and low molecular weight material from a protein solution. This was accomplished by dialyzing the protein solution against a very dilute buffer. The dialysis bag was made of Visking cellulose acetate tape; the sample to be dialyzed was placed within the bag and then both ends were knotted. To insure complete dialysis, a rotary dialyzer was used, Fig. 3. This dialyzer not only mixed the solution within the bag by the tumbling action of the small glass rod A, but also provided stirring outside the sac by the sweeping motion of the dialysis bag while the glass shaft D is rotating. The external dilute buffer was changed periodically, i.e., at least once every two hours.

Rotating Evaporator: Solutions of relatively large volumes were concentrated with a Rinco rotating evaporator (Rinco Instrument Co., Greenville, Ill.).

General Analyses. Paper Chromatography: After a reaction was completed, the usual method was to separate the products by paper chromatography. For routine work Whatman No. 4 oxalic acid-washed paper was used, with phenol-water and butanol-propionic acid-water as developing solvents.[44,45] Before preparing the phenol solvent system, the phenol was either distilled directly or with aluminum turnings and sodium bicarbonate.[42] The former treatment produced a water phase when saturated with phenol, which had a pH of <3 while the latter treatment gave a water saturated with phenol

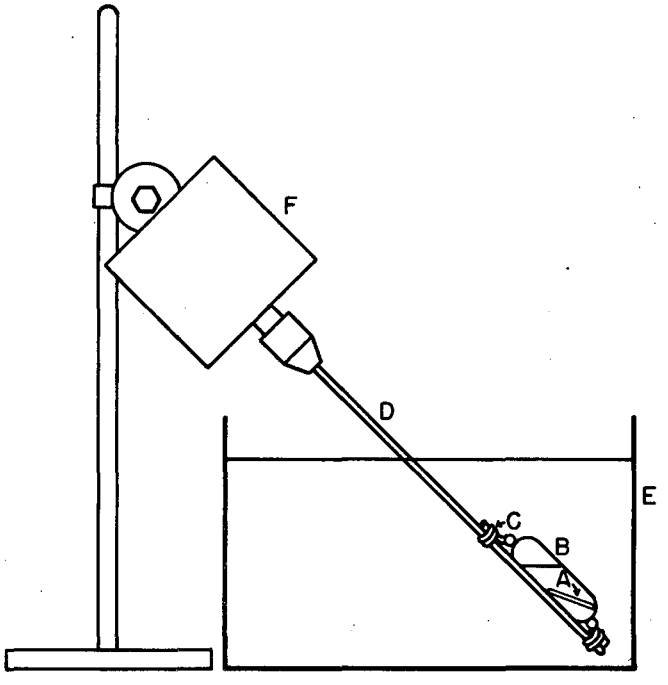

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Fig. 3. Rotary dialyzer.
A = glass rod; B = dialysis sac containing protein solution; C = rubber band; D = glass rod; E = large glass vessel containing very dilute buffer; F = motor operated at 5 to 6 rpm.

phase that had a pH of about 6. Since large amounts of salt caused streaking of the material on the chromatogram, samples containing no more than 3 to 4 μM of salt were applied at the origin of a two-dimensional chromatogram.

Phosphorylated esters were further separated using an ethanol-formic acid-sodium molybdate solvent system on Whatman No. 1, HCl-EDTA washed paper.[46] Three other possibilities were explored for solvent systems for separating phosphate esters. These were 1) the Bandurski acid solvent system containing methanol-formic acid-water, 2) the Bandurski alkaline solvent of methanol-ammonia-water, and 3) the propanol-ammonia-water system.[44] The supporting medium for the Bandurski solvent systems was Whatman No. 1, HCl-EDTA washed paper while for the propanol system, it was oxalic acid-washed Whatman No. 4 paper.

For all of the above-mentioned chromatography, the temperature during the development of the chromatogram was about 20 to 25°C. The papers were then dried under forced draft at room temperature.

Dinitrophenyl derivatives of amino acids were separated by two dimensional chromatography on unwashed Whatman No. 1 paper using toluene-2-chloroethanol-pyridine-ammonia as the first developing solvent and 1.5 M phosphate as the second solvent.[47] Good resolution of the compounds was obtained only when the temperature was greater than 25°C.

If the samples chromatographed were radioactive, the compounds on the chromatogram were located by exposing the paper to DuPont x-ray film, Type 507E, single coated Kodak x-ray film, double coated Kodak Blue Screen x-ray film or double coated Kodak No Screen x-ray film. The latter, although most sensitive for the detection of β-rays, produced a marked background fog. For all films, a spot of 300 cpm per sq cm required about 2 to 3 days exposure.

The identity of the chromatographically separated compound was established tentatively by its R_f. The radioactive compound was then eluted and cochromatographed with the authentic marker compound (ca. 150 μg of marker) for confirmation. Exact coincidence of the radioactivity with the marker compound was taken as a criterion for identity.

The identity of phosphate esters was further confirmed by treating the eluates suspected to be phosphate esters with acid phosphatase purified from Polidase-S (Schwartz Laboratories, Inc.) or with acid prostatic phosphatase.* The enzymic hydrolysate was cochromatographed with authentic marker compounds in the manner indicated in the preceding paragraph.

The acid phosphatase from Polidase-S was purified according to the method of Cohen.[48] A suspension of Polidase-S (10 g) in 100 ml of distilled water was made to 83% SAS by adding 63 g of $(NH_4)_2SO_4$. The mixture was allowed to stand at 0°C for ca. 10 minutes and was centrifuged at 36,000 X g for 5 minutes. The supernatant liquid was decanted and made to 100% SAS with an additional 12 g of AS. After standing for ca. 30 minutes at 0°C, the mixture was centrifuged at 36,000 X g for 5 minutes. Actually, not all of the AS was soluble and therefore, only the mixture containing the precipitated protein plus the supernatant liquid was centrifuged. This was done by gently swirling the mixture, allowing the heavier undissolved crystals of AS to settle, and then rapidly decanting the supernatant suspension into the centrifuge tube. The supernatant liquid obtained after the centrifugation was discarded and the precipitate was dissolved in a small volume of distilled water (ca. 5 to 10 ml). The resultant solution was dialyzed vs distilled water at 4 to 8° C for 12 to 14 hours, the distilled water being changed at least three times

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* Kindly supplied by Prof. H. A. Barker.

during this period. The dialyzed protein solution was lyophilized, yielding several hundred mg of light brown fluffy powder.

Eluates with a volume of 500 µl or less of radioactive spots from chromatograms were treated with 25 to 50 µl of enzyme solution for 12 to 16 hours at 37°C. The enzyme solution consisted of 1 mg of lyophilized protein per ml of pH 5.0, 0.2 M sodium acetate buffer containing 0.01 M $MgCl_2$.

The authentic marker compounds (added as carriers) were located on the chromatograms by spraying or dipping the papers with specific reagents. Free sugars and sugar acids (i.e., polyhydroxy acids) were detected with a methanol solution of ammoniacal silver nitrate spray.[49] This spray was rather insensitive for the detection of the sugar acids, often requiring at least 5 to 10 minutes heating at 100° C to develop the color of the spot. A more sensitive technique was to dip the chromatogram into an acetonic solution of silver nitrate and then to spray the paper with ethanolic NaOH.[50] In this case, no heat was required to generate the color for either the free sugars or the sugar acids. Organic phosphates were located by exposure of the paper chromatogram to sunlight after spraying it with an acidified ammonium molybdate reagent.[51] The inorganic phosphate was distinguished from the organic phosphates by its yellow color prior to exposure to sunlight; all other phosphates giving a blue color only after exposing to sunlight. The positions of amino acids were determined by spraying the chromatogram with an ethanol solution of ninhydrin.[52] Particularly effective was the modification of this spray, using a little collidine, whereby the amino acids were distinguished by their different hues.[53]

The dinitrophenyl amino acids were detected visually either under ordinary white light or ultraviolet light. Under uv, the dinitrophenyl derivatives appeared black instead of yellow and very minute amounts of

material can be detected under this condition. Tentative identity of the DNP-amino acid was established by its R_f, and confirmed by parallel chromatography of the eluted DNP-amino acid with synthetic DNP-amino acid.

Paper Electrophoresis: Both Whatman No. 1 and No. 4, either oxalic acid-washed or unwashed, were used as supporting medium in electrophoresis. The paper was usually 19 cm wide and 55 cm long. Buffers were generally one of the following: 1) pH 3.8, 0.1 M formic acid-ammonium formate; 2) pH 8.3, 0.1 M $NaHCO_3$; 3) pH 8.6, barbital, $\mu = 0.05$; and 4) pH 9.5, 0.1 M ammonium hydroxide-ammonium formate. The formic acid and ammonium hydroxide buffers were especially suited for electrophoresis where the sample was to be recovered or where the paper was to be sprayed with an indicator. Buffers which deposited a residue on the paper after drying gave a high background color when the paper was sprayed with reagents for the detection of sugars and phosphates. Samples were applied on the paper strip after the paper was equilibrated with the buffer for about 30 minutes. No more than 25 µl of solution was applied per spot on the origin line, except where a band of material was applied. Along with the samples, two colored markers were employed to indicate the extent of the migration of the ions. The marker solutions were 1) a saturated aqueous solution of picric acid and 2) an aqueous solution of potassium dichromate (2.4 g/10 ml). Five µl of each marker solution was applied on the paper.

Each electrophoresis was operated at a constant potential, generally from 600 to 700 volts. The current was allowed to vary from 10 to 50 ma, depending on the buffer and the temperature. The temperature of the paper was controlled by forcing cold tap water through the cooling plate (about 20° C) or by circulating ice water through the same plate (about 3° C). Each electrophoretic run was conducted for at least 2.5 hours. After the completion of the run, the paper was removed carefully; the

ends were blotted and finally hung on a rod to dry under a forced air draft at room temperature.

By comparing the relative mobility of the unknown compound to those of reference compounds or of the colored marker compounds, one tentatively identified the compound. Its identity was further established by chromatography of the eluted compound using the routine chromatography system. Final confirmation was supplied by cochromatography of the eluted material with authentic marker compounds as described in the previous section under Paper Chromatography.

Special Analyses. Total Phosphate: This method is described by Allen[54] and consisted of the complete hydrolysis of the substance followed by the colorimetric determination of the liberated phosphate by reaction with ammonium molybdate.

Inorganic Phosphate: This analysis involved the reaction of ammonium molybdate with inorganic phosphate under very mild conditions (pH 4).[55] The color was compared with that of a standard curve of inorganic phosphate.

Protein: The amount of protein in a solution was measured in three different ways: 1) dry weight, 2) colorimetric method, and 3) uv absorption. The dry weight method required the complete dialysis of the sample prior to drying. The samples were then lyophilized or evaporated to dryness over P_2O_5 under reduced pressure. The colorimetric method was based on the measurement of the color developed by a combination of the reaction of the Folin-Phenol reagent with the tyrosine residues of the protein and of the biuret reaction.[56] The uv absorption method employed the absorption of light at 275 or 280 mμ which is due to the tyrosine and tryptophane groups in the protein.[57] It is necessary at some stages of the purification, to correct for the absorption of nucleic acids often found associated with the protein.[57]

Ammonia: The method of Nessler[58] and of Moore and Stein[59] were applied for the determination of ammonia. Both methods are colorimetric measurements, the former being based on the reaction of free ammonia with the complex HgI_2 and yielding a finely divided yellow precipitate of $Hg_2NI.H_2O$. This precipitate is kept in uniform suspension by the presence of gum ghatti. The Moore and Stein method produces a blue-purple color as a result of the reaction of reduced ninhydrin with free ammonia and oxidized ninhydrin.

Chlorophyll: The method consisted in the extraction of chlorophyll-containing material with 80% aqueous acetone, clarification of the extract by centrifugation and measuring the light absorption of the supernatant liquid between 640 and 700 mμ. The details of this procedure are described by Arnon.[60]

Acyl Phosphate: Quantitative analysis of these compounds was carried out by reacting them with NH_2OH resulting in hydroxamic acids, followed by development of the color with $FeCl_3$.[61] The color of the reaction product of the sample was compared with that of succinic anhydride.

Preparation of Ribulose-1,5-diphosphate (RuDP). RuDP was prepared either by extraction from algae or by enzymic methods. RuDP-C^{14} was prepared solely by extraction from algae. While both of these methods have been published elsehwere, the detailed procedure for each of these methods is given below for the sake of completeness and convenience, along with a few slight modifications.

Extraction from Algae:[62] Freshly harvested algae, either Scenedesmus obliquus or Chlorella pyrenoidosa, (1 to 1.5% suspension) in ca. 20% diluted nutrient, was allowed to undergo photosynthesis in 1 to 4% CO_2 at 5 to 10,000 foot-candles from 15 to 30 minutes. To prepare unlabeled RuDP, N_2 was bubbled vigorously through the suspension in order to flush

out the dissolved CO_2. After exactly 30 seconds the algal suspension was rapidly drained into either cold (25° C) or hot (boiling) alcohol to a final concentration of 80% ethanol. The mixture was centrifuged at 1,500 X g for 10 minutes. The precipitate was extracted with a small volume of warm 20% ethanol and the mixture was centrifuged at 36,000 X g for 5 minutes. The extraction with 20% ethanol was repeated and the supernatant liquids obtained after each centrifugation were combined. The latter was evaporated under reduced pressure to a volume less than 1 ml. RuDP was separated from the other sugar phosphates by paper chromatography using phenol-water as solvent. Generally, the aqueous alcohol extract was applied as a band on the paper and the chromatogram was allowed to develop from 36 to 42 hours. Radioactive RuDP obtained from chromatograms of other photosynthesis experiments with algae and radioactive bicarbonate, was spotted in three places along the origin line as markers. The RuDP area, located by radioautography, was cut out, washed with absolute ethanol, and eluted with water. The eluate was either used directly or concentrated to a smaller volume. The yield per g of wet algae of RuDP is a maximum of one μM.

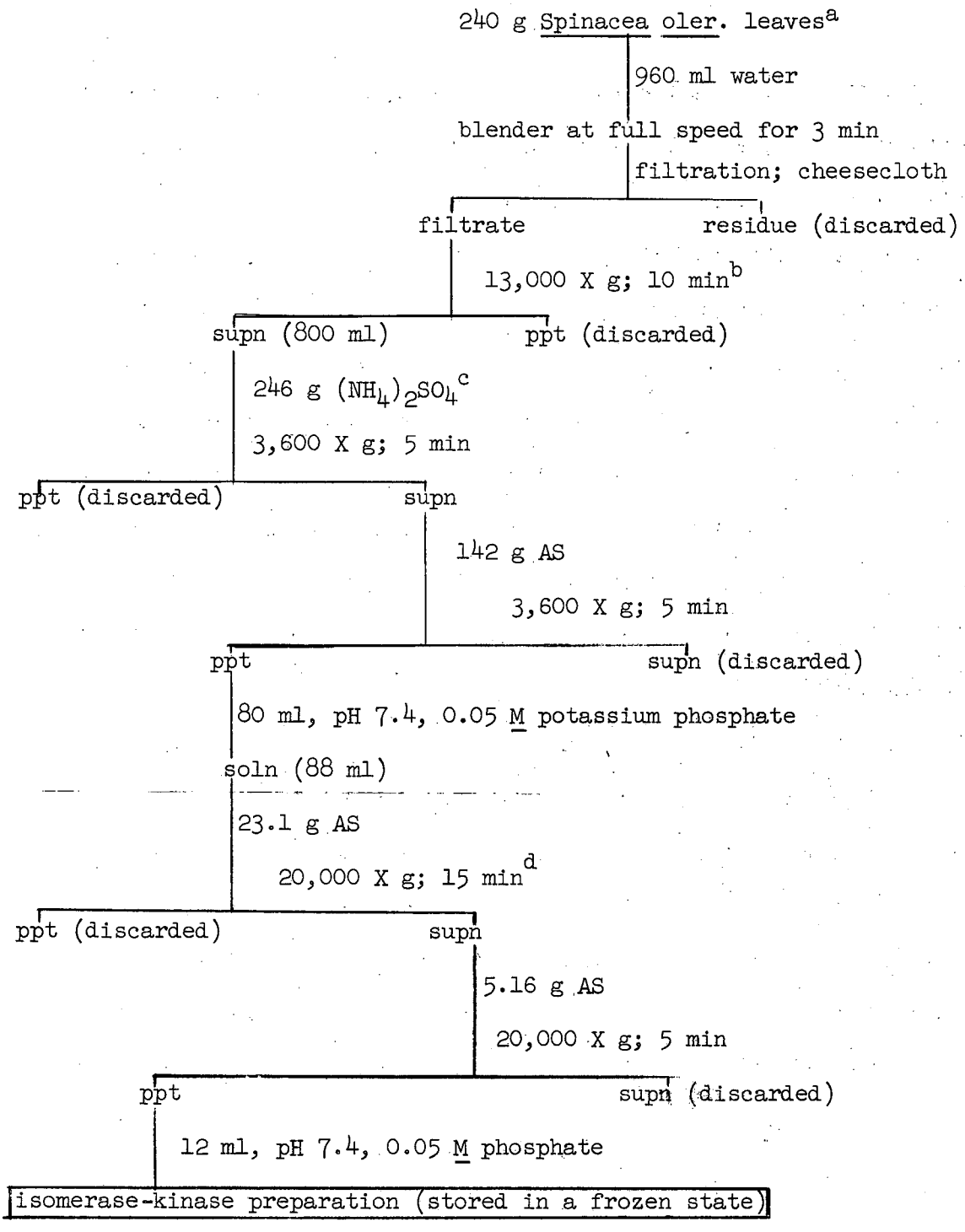
For the preparation of RuDP-C^{14}, the algae was preilluminated as in the method for unlabeled RuDP. Prior to injection of $NaHC^{14}O_3$ into the algal suspension, the CO_2 was removed and N_2 was flushed through the suspension for 1 minute in order to sweep out any dissolved CO_2. Radioactive bicarbonate was added (0.026 N, 400 μC/ml), and photosynthesis was carried out for 2 to 3 minutes. Approximately 0.025 mM of bicarbonate was used per ml of wet packed cells per minute. At the end of the 2 to 3 minutes, N_2 was bubbled rapidly through the suspension for 30 seconds and the algal suspension was quickly killed by dropping it into four volumes of room temperature alcohol. Subsequent treatments were identical to those described for the preparation of unlabeled RuDP. In addition to the

paper chromatography of the 20% alcohol extract, paper electrophoresis was performed on a small aliquot of this extract. Conditions of electrophoresis were: 1) formic acid-ammonium formate buffer, 0.1 M pH 3.8; 2) temperature, tap water-cooled; 3) potential, 750 volt; 4) current, 28 to 35 ma, and 5) duration of run, 3 hours. The purity of the RuDP-C^{14}, separated and eluted after paper chromatography, was investigated by treatment with acid prostatic phosphatase. The enzymic hydrolysate was chromatographed in two-dimensions using the phenol-water system and the butanol-propionic acid-water system. The bands of radioactive substances in the paper electrophoretogram were eluted, and were tentatively identified by subsequent chromatography using the usual two-dimensional solvent systems.

Enzymic Method for the Preparation of RuDP:[63,64] This method depends on the conversion of ribose-5-phosphate (RMP) to RuDP in the presence of adenosine triphosphate (ATP) and an enzyme mixture containing phosphopentose isomerase and phosphopentose kinase. The preparation of the isomerase-kinase enzyme mixture is outlined in the flow diagram Fig. 4.

The isomerase-kinase mixture was assayed prior to its use for large scale RuDP preparation. This was accomplished by following the rate of acid liberation using the pH stat.[65] The test solution contained 11 μM of ATP, 10 μM RMP, 10 μM $MgCl_2$, and 5 μM cysteine in 10 ml. The pH was adjusted to 7.40 and held constant during the reaction by the addition of 0.1 M NaOH. The reaction was started by the addition of the enzyme mixture (150 μl). A control without RMP was also done.

Large scale preparation of RuDP was achieved by reacting the isomerase-kinase mixture with the same components as those given in the assay technique, except that the quantities involved were on a large scale. In a typical preparative experiment, the procedure was as follows:



a) All operations were carried out near 0° C.

b) Spinco Ultracentrifuge, Model L, rotor number 21

c) After each addition of ammonium sulfate (AS), the solution was adjusted to pH 7.0 with 2 N NH_4OH.

d) Rotor number 40.

Fig. 4. Flow Diagram for the Preparation of the Isomerase-Kinase Mixture.

The barium salt of RMP (4 mM, 1.83 g) was dissolved in 40 ml of water and 40 ml of 0.15 M potassium sulfate was added. The mixture was centrifuged and the residue was washed with water (20 ml). To the combined washings and supernatant liquid, 6 mM ATP, 0.4 mM cysteine and 4 mM $MgCl_2$ were added and the volume was made up to 300 ml. The reaction was carried out in the pH stat at room temperature under an atmosphere of N_2 gas; the pH was adjusted to 7.40 and maintained constant by the addition of CO_2-free NaOH (1.8 N). To start the reaction, the isomerase-kinase preparation was added (5 ml). When the uptake of alkali (1.5 ml) had slowed down (requiring in this case, 37 minutes), the reaction was stopped by the addition of 50% trichloroacetic acid (20 ml). Acid-washed Norit A (120 g) was used to remove the nucleotides. The solution was centrifuged (1,000 X g for 10 minutes) and the residue was washed with four 200 ml increments of water. To facilitate the centrifugation of the Norit A, the solution was layered with a small amount of alcohol. Barium acetate (4 ml of 1 M) was added to the combined supernatant liquid and washings, and the pH was adjusted to 6.4 with saturated barium hydroxide. The precipitate was removed by centrifugation and washed with water (50 ml). The supernatant liquid and washings were combined and an equal volume of ethanol was added. RuDP precipitated in a gelatinous form; it was collected by centrifugation, washed with 80% ethanol and dried *in vacuo* over P_2O_5. Yield of the crude material: 1.75 g.

An attempt was made to purify this preparation of RuDP by fractional alcohol precipitation. Thus, the barium salt of RuDP (46 mg) was triturated with water (20 ml) and the solution was centrifuged to remove the insoluble material. The latter fraction (50 mg) was washed with 50% alcohol (5 ml) and dried *in vacuo* over P_2O_5; it consisted mainly of inorganic phosphate. The addition of ethanol (5 ml) to the supernatant liquid precipitated 22 mg of material.

Column chromatography of RuDP was achieved in a column 0.8 cm diameter x 27 cm length. The ion exchange resin used was anion exchanger, Dowex 1 in the formate form. The resin was freed of fines by decantation and converted to the formate form with 2 N formic acid. Elution was carried out with an apparatus which provided a linear gradient of ammonium formate, (see description under Apparatus).

RuDP (100 mg Ba salt) was dissolved in water (30 ml) with the aid of a little Dowex 50 (H^+ form) and the pH was adjusted to 6.0 with KOH. The solution was centrifuged and the supernatant liquid was run through the column. The column was washed with water and subjected to gradient elution. Samples were collected every 5 minutes in a time-flow fraction collector. The rate of flow was 0.44 ml per minute. Fractions containing phosphate were located by qualitative test. Aliquot portions from each fraction were spotted on strips of filter paper, dried and sprayed with the Hanes-Isherwood reagent.[51] The fractions were then assayed for total phosphate and RuDP. The method used for the determination of RuDP is based on the fixation of $C^{14}O_2$ by RuDP in the presence of the enzyme, carboxydismutase. It was assumed that the latter is specific for this substrate and that the enzyme preparation is sufficiently pure so that no other fixation is possible under the conditions of this assay. The reagent solution was prepared by mixing $NaHC^{14}O_3$ (200 µl of 0.036 N, 400 µC/ml), $MgCl_2$ (80 µl of 0.01 M in 0.04 N HCl), tris buffer (20 µl, pH 7.83, 1 M with respect to tris), and carboxydismutase (50 µl of a New Zealand spinach preparation fractionated between 32 and 35% saturated ammonium sulfate and containing 6 mg of protein per ml). An aliquot from each column fraction (5 µl) was placed on a planchet and the reagent solution (15 µl) was added. To reduce evaporation, the planchet was placed on top of a large moist filter paper in a covered vessel and allowed to incubate at room temperature. After two hours the reaction was stopped by

the addition of acetic acid (10 μl of 6 N) and the planchet was prepared for counting in the usual manner.

Once the RuDP was located, it was recovered from the eluate by adding 500 μl of barium acetate (1 M) plus 95% ethanol to make a final concentration of 50% ethanol. The mixture was cooled in an ice bath and the precipitate was collected by centrifugation. The precipitate was washed successively with 50% ethanol (20 ml), 95% ethanol (10 ml), absolute ethanol (10 ml) and dried *in vacuo* over P_2O_5.

Reaction of RuDP with Cyanide.[66] Formation of Carboxyl-labeled Hamamelonic Acid Diphosphate and its Isomer (HmDP): Two separate reactions were performed, one at a calculated pH of 6 and the other at a calculated pH of 11. For each reaction the monobarium salt of RuDP (12 mg) was triturated with water (1 ml) and Na_2SO_4 (40 μM). The residue of $BaSO_4$ was removed by centrifugation. For the reaction at pH 6, the residue was washed with 0.1 M HCl (200 μl) while for the reaction at pH 11, the residue was washed with water (200 μl). The supernatant liquid and washing for each reaction were combined and treated with 1.3 mg of $KC^{14}N$ (20 μM, 1 μC/μM) for 16 hours at room temperature.

Formation of Unlabeled HmDP: The monobarium salt of RuDP (117 mg) was triturated with water (10 ml) containing K_2SO_4 (75 mg). The insoluble material was removed by centrifugation and KCN (0.24 M, 900 μl) was added to the supernatant liquid. After 18 hours at room temperature the solution was centrifuged and the small amount of residue was discarded. The supernatant liquid was cooled in an ice bath and barium acetate (1 M, 600 μl) was added. The copious precipitate was collected by centrifugation, washed successively with 5 ml portions of 80% and 90% ethanol and dried under reduced pressure over P_2O_5; yield of the first crop being

120 mg. A second crop of 15 mg was recovered by adding two volumes of ethanol to the supernatant liquid.

The first crop was contaminated with $BaSO_4$ which was removed by triturating the total amount (120 mg) with HCl (0.1 M, 10 ml) followed by centrifugation. The insoluble residue was washed with water (5 ml); it weighed 42 mg and consisted mainly of $BaSO_4$. The supernatant liquid, combined with the water washing, was adjusted to pH 7.1 and then cooled in an ice bath. The precipitate was collected, washed with 50% ethanol (7.5 ml), 90% ethanol (5 ml) and dried *in vacuo* over P_2O_5; yield: 26 mg (product A). A further crop was obtained by adding an equal volume of ethanol to the supernatant liquid. This mixture was centrifuged, washed with 50% ethanol (4 ml), 90% ethanol (5 ml) and dried; yield: 22 mg (product B).

Both products A and B were cochromatographed separately with radioactive HmDP in the ethanol-formic acid-molybdate system and exact coincidence with the radioactivity and the phosphomolybdate color was obtained in both cases.

Identification and Characterization: Both the products of the reaction of RuDP with $KC^{12}N$ and $KC^{14}N$ were chromatographed in the following solvent systems: 1) Two dimensions in the general solvent systems, BuOH-propionic acid-water and phenol-water, 2) BuOH-propionic acid-water for 48 hours, 3) PrOH-NH_3-water, 4) EtOH-formic acid-aqueous molybdate, 5) MeOH-formic acid-water and 6) MeOH-NH_3-water.

Electrophoresis of both labeled and unlabeled HmDP was performed at pH 3.8, 8.3, and 9.5 using, respectively, formic acid, bicarbonate, and ammonium hydroxide buffers. The conditions were generally similar to those described previously under General Analyses.

Further characterization of the reaction products of RuDP with cyanide was achieved by dephosphorylating them with acid phosphatase or with HCl hydrolysis. In the latter case, HmDP was treated with 1 M HCl for 16 hours at 120° C in a sealed tube. (Heating with 0.1 M HCl at 103° C for 12 hours did not seem to dephosphorylate the products.)

Hamamelonic Acid. Preparation: Authentic hamamelonic acid was regenerated from the phenylhydrazide of hamamelonic acid* using the method of Hudson,[67] but modified for a smaller scale. Hamamelonic acid phenylhydrazide (50.3 mg, 176 μM) was mixed with $CuSO_4 \cdot 5H_2O$ (45.8 mg, 184 μM) and 1 ml H_2O. One to 2 μl of n-octanol was added to minimize foaming. The mixture was refluxed for 5 hours. Hydrogen sulfide, generated from a FeS-HCl mixture, was bubbled through the solution to remove the excess cupric ion. Sulfate was removed by adding an equivalent of $Ba(OH)_2$, 0.7 ml of saturated $Ba(OH)_2$ being required.

Both the starting material and the product were studied under paper electrophoresis (ammonium hydroxide buffer, pH 9.1) with an applied potential of 600 volts for 3 hours. The substances were also chromatographed two-dimensionally using the routine solvent systems with 150 to 300 μg of material applied on the origin of the paper.

The Lactone-Acid Interconversion: The formation of the lactone or conversely, the carboxylate ion of hamemelonic acid, in the presence of various buffers was studied. These buffers were as follows: pH 1 (calculated), HCl; pH 2.9, citrate; pH 4.6, acetate; pH 6.8, phosphate; pH 8.7, tris; pH 11.2, phosphate; and pH 13 (calculated), NaOH. In these studies 10 μl of hamamelonic acid solution (300 μg) was mixed with 50 μl of 0.1 M buffer and allowed to stand for 16 hours at 37° C. At the end of this

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* Kindly supplied by H. Simon through the laboratory of O. Schmidt.

period, each mixture was chromatographed in two dimensions with the general solvent systems except that the paper dimensions were reduced to one-half of the original size (i.e., 11.5 x 14 cm). As a result of this alteration, the sample on the paper was exposed to each solvent for only about 3 hours instead of the usual 8 to 10 hours.

Preparation of Carboxydismutase. Source materials for this enzyme were Tetragonia expansa* leaves and Chlorella pyrenoidosa cells. The occurrence of this enzyme was also studied in other plant and animal tissues, but not extensively. Three different methods were used for the purification of the enzyme from Tetragonia while the enzyme obtained from Chlorella was used only in the crude form for purification studies.

Enzyme from Tetragonia: Method I, the Crude Extract Method. In this method the enzyme was prepared by ammonium sulfate fractionation of the clarified crude extract of Tetragonia leaves.[62] The steps in this procedure are outlined in Fig. 5.

Further purification of the 32 to 35% SAS precipitate was attempted by using classical enzyme purification techniques. These studies were of an exploratory nature and are described below.

1) Heat Treatment: An ammonium sulfate fractionated enzyme solution (32 to 35% SAS ppt) containing 25 mg of protein in 2.5 ml was heated in a 56 to 58° C water bath for 4 minutes. A small glass rod was used to stir the solution. The mixture was cooled immediately to 0° C and centrifuged at 36,000 X g for 5 minutes. (The rotor was previously cooled to 0° C before centrifugation.) The supernatant liquid was assayed for activity. The final pH of supernatant liquid was 6.75. Protein content was determined on the basis of dry weight.

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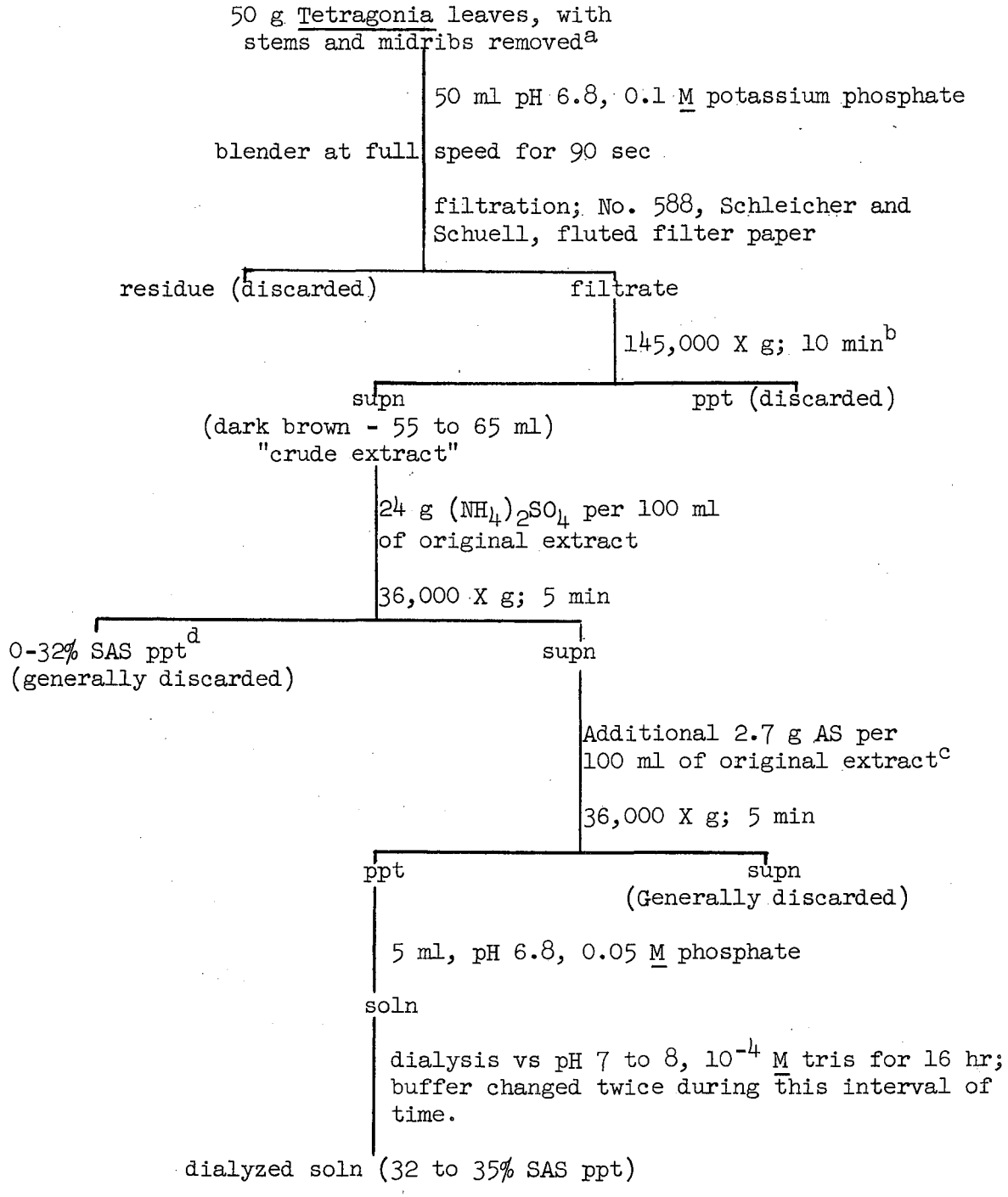
* New Zealand spinach, mostly commercial source.



a) All operations were performed at, or near, 0° C.

b) Spinco Ultracentrifuge, Model L, rotor number 40.

c) After each addition of ammonium sulfate (AS), the mixture was adjusted to pH 7 with 2 N ammonium hydroxide.

d) Fully saturated ammonium sulfate solution (100% SAS) was taken as 75.4 g $(NH_4)_2SO_4$ per 100 ml of initial volume of solution at 20° C.[68,69]

Fig. 5. Flow Diagram for Method I, the Crude Extract Method.

2) Ethanol Fractionation: Absolute ethanol was cooled to less than -40°C in a CO_2-acetone bath. The protein solution (32 to 35% SAS ppt) was cooled to 0° in an ice bath. The concentration of the latter was ca. 10 mg/ml. In the following operation it was essential to work at temperatures below 0°C. Cold ethanol (0.625 ml) was slowly added to 2.5 ml of chilled protein solution accompanied with gentle stirring. The precipitate was removed by centrifugation at 36,000 X g for 5 minutes. To the supernatant liquid was added 0.44 ml chilled ethanol. The precipitate was collected by centrifugation and resuspended in cold (0°C) 0.05 M, pH 6.8 potassium phosphate buffer. Most of the material appeared to be insoluble; it was removed by centrifugation and the supernatant liquid was subjected to assay. Protein content was determined on a weight basis.

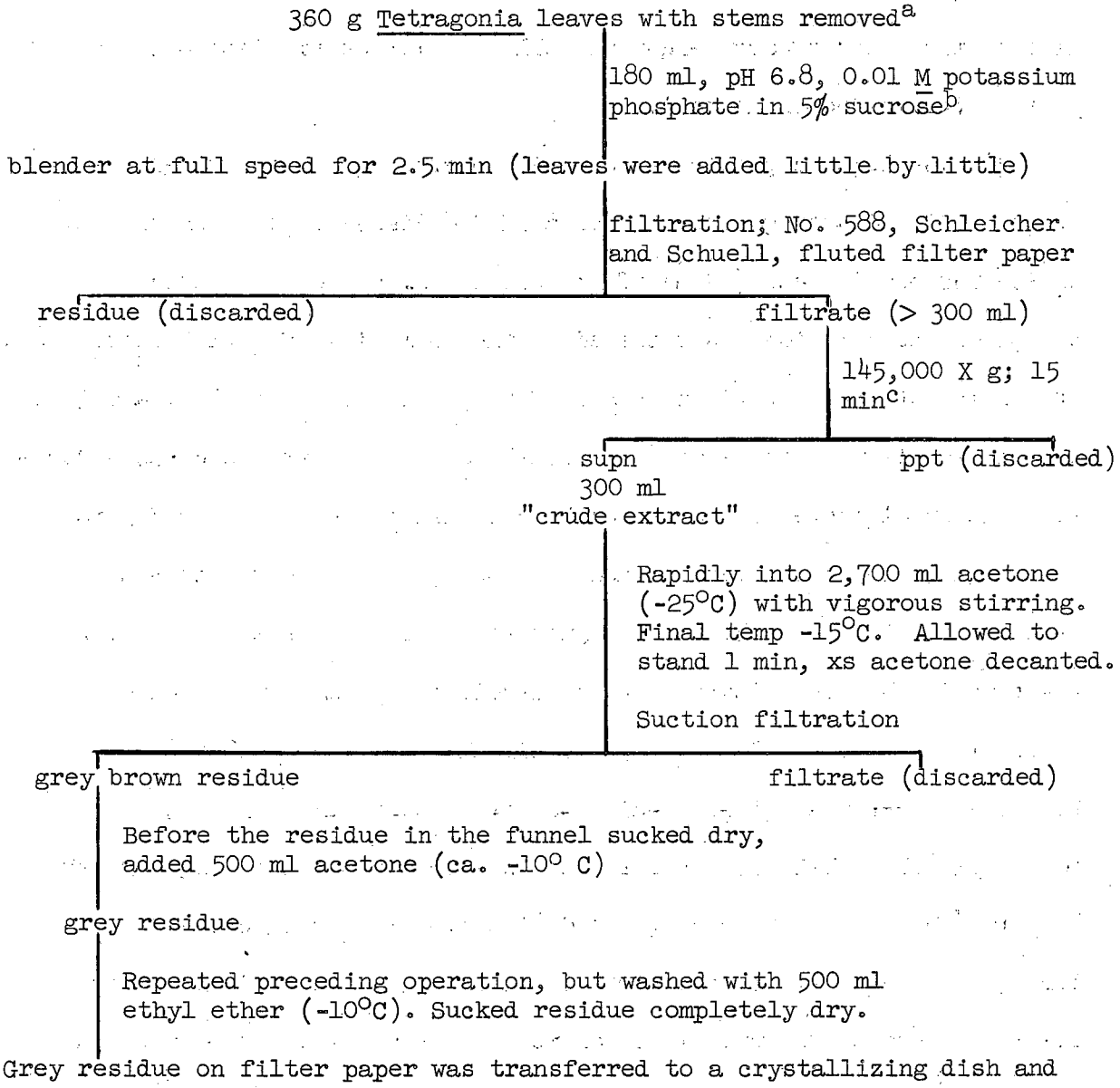
3) Acetone fractionation: To a solution of the 32 to 35% SAS precipitate (10 ml containing a total of 60 mg protein) was added 50 µl 2 M potassium phosphate, pH 6.8 and 0.5 g sucrose. Precooled acetone (-25°C) was added to this solution accompanied with vigorous stirring. The temperature rose to -15°C. The mixture was allowed to stand for several minutes. A turbid solution formed after this period. Centrifugation of the filtrate was carried out at -11° C at 1,500 X g for 10 minutes. The gummy precipitate was washed with 75 ml of -15°C acetone. A stirring rod was used to triturate the precipitate, but the latter was too gummy to handle well. The mixture was centrifuged again at 1,500 X g for 5 minutes. The precipitate, which was now partly powdery, but mostly still gummy, was washed once with 75 ml of cold ethyl ether (-15° C) and the mixture was centrifuged (1,500 X g; 5 minutes). The vessel containing the precipitate was quickly transferred to a desiccator and the material was dried over P_2O_5 under reduced pressure. Yield of acetone powder: 28 mg of apparently hygroscopic powder. Assay of this powder for carboxydismutase activity was

made on the basis of dry weight as well as colorimetric determination of protein (Folin-Phenol method).

4) Isoelectric precipitation: All operations were carried out at, or near 0°C. Starting with 8 ml of a pH 6.8 dialyzed solution of the 32 to 35% SAS precipitate (83 mg of protein determined colorimetrically), the pH of the solution was titrated to the following pH's with acetic acid of varying strengths (0.01 to 6 M): pH 5.6, 5.4, 5.1, 4.8, 4.5, and 3.3. A small sample was set aside (0.5 ml) for control. After each pH adjustment the precipitate was collected by centrifugation at 36,000 X g for 5 minutes. (No precipitate formed when the pH was adjusted to 3.3 although about 11% of the starting material was still present in this fraction.) The precipitates were redissolved separately in 2 ml of water with the aid of 0.01 N NH_4OH until the pH was between 6.8 and 8.2. The final supernatant liquid which was at a pH of 3.3, was also titrated to pH 8.8 with NH_4OH. All fractions were then dialyzed against pH 7.2, 10^{-4} M tris for 24 hours, the dialysis medium being changed twice during this interval of time. The solutions were stored in a frozen state and later assayed for protein colorimetrically and for carboxydismutase activity.

Method II, the Acetone Powder Method. This method involved the preparation of the acetone powder of the clarified crude extract of Tetragonia leaves, Fig. 6, followed by ammonium sulfate fraction of this powder, Fig. 7.

Method III, the Chloroplast Method. By this procedure the enzyme was obtained from the chloroplasts of Tetragonia. The chloroplasts were first isolated and then lysed osmotically to yield the chloroplast extract. This extract was fractionated with ammonium sulfate. The steps are given in Fig. 8 and Fig. 9.



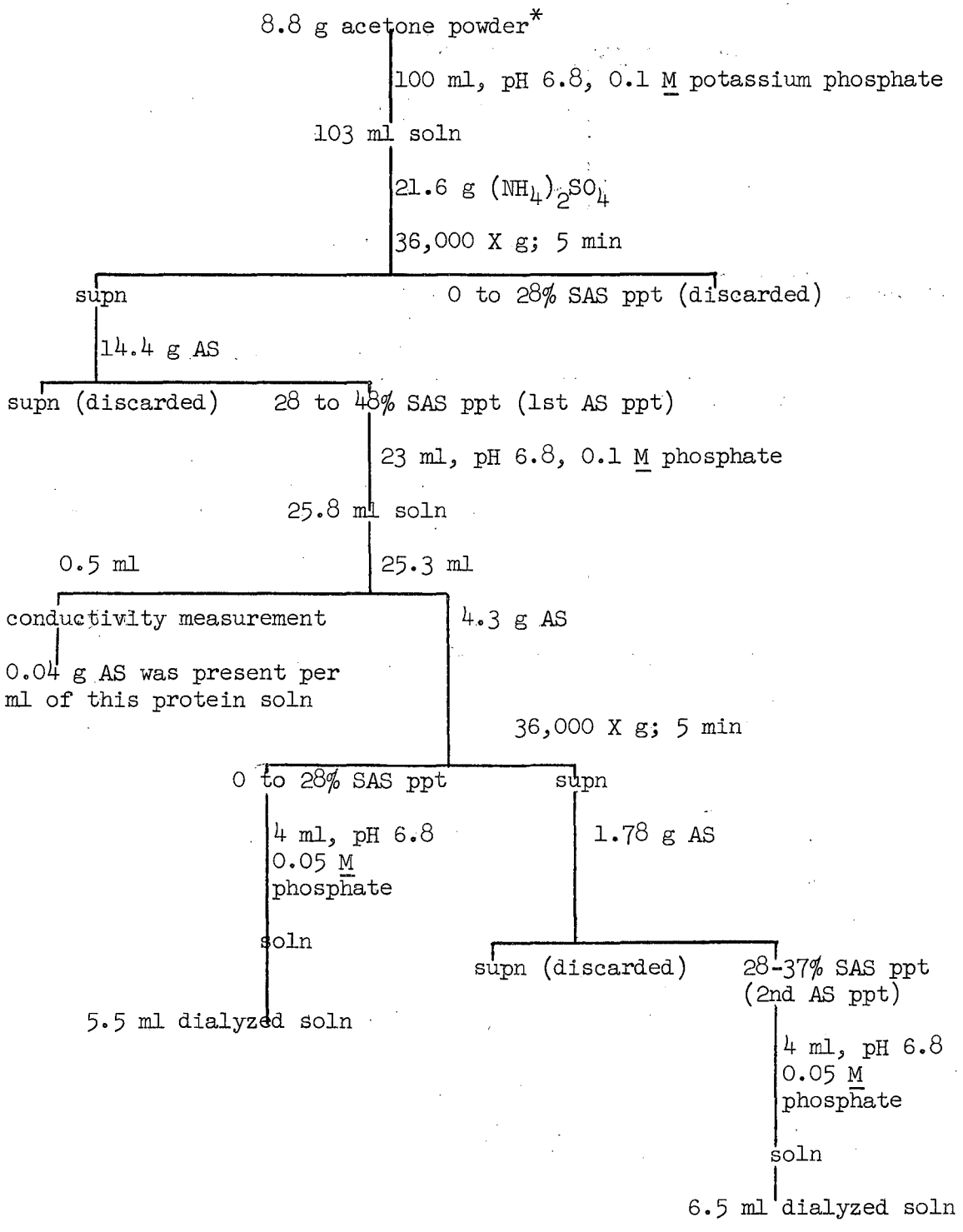
Grey residue on filter paper was transferred to a crystallizing dish and placed in a vacuum desiccator while in the cold room. The desiccator was evacuated with a water aspirator before allowing to rise to room temperature. The desiccator was then connected to a water aspirator at room temperature and the evacuation was continued until the grey residue became powdery dry (light grey in color): "acetone powder" yield: 8.9 g.

a) All operations were performed near 0° C, or otherwise at the temperatures indicated.

b) The addition of sucrose to the grinding medium, for preparing the acetone powder, was recommended by Prof. D. E. Green of the Enzyme Institute, University of Wisconsin.

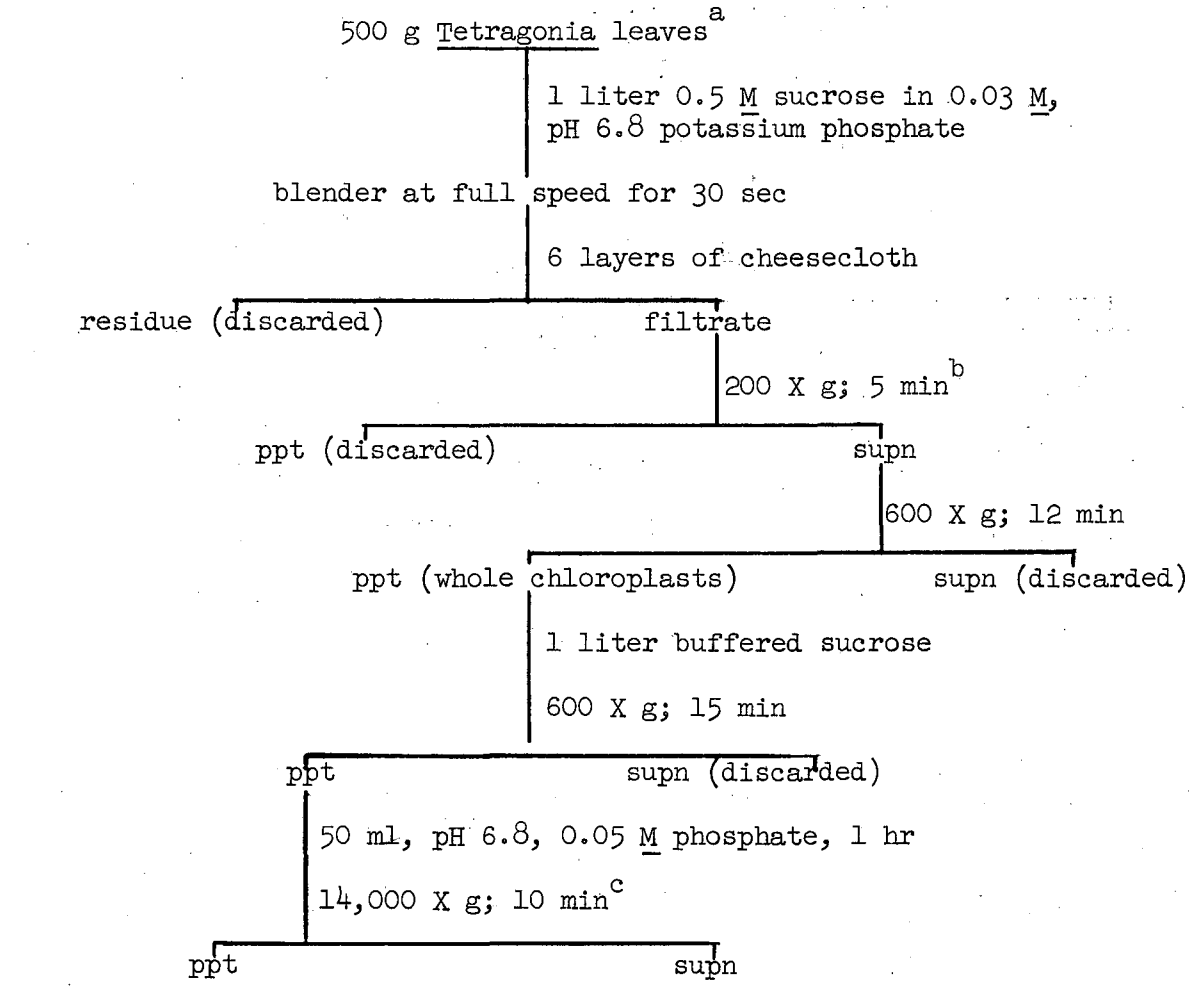
c) Spinco Ultracentrifuge, Model L, rotor number 40.

Fig. 6. Preparation of the Acetone Powder.



* All conditions and explanations for the abbreviations are given under Method I, the Crude Extract Method; unless otherwise noted.

Fig. 7. Ammonium Sulfate Fractionation of the Acetone Powder.

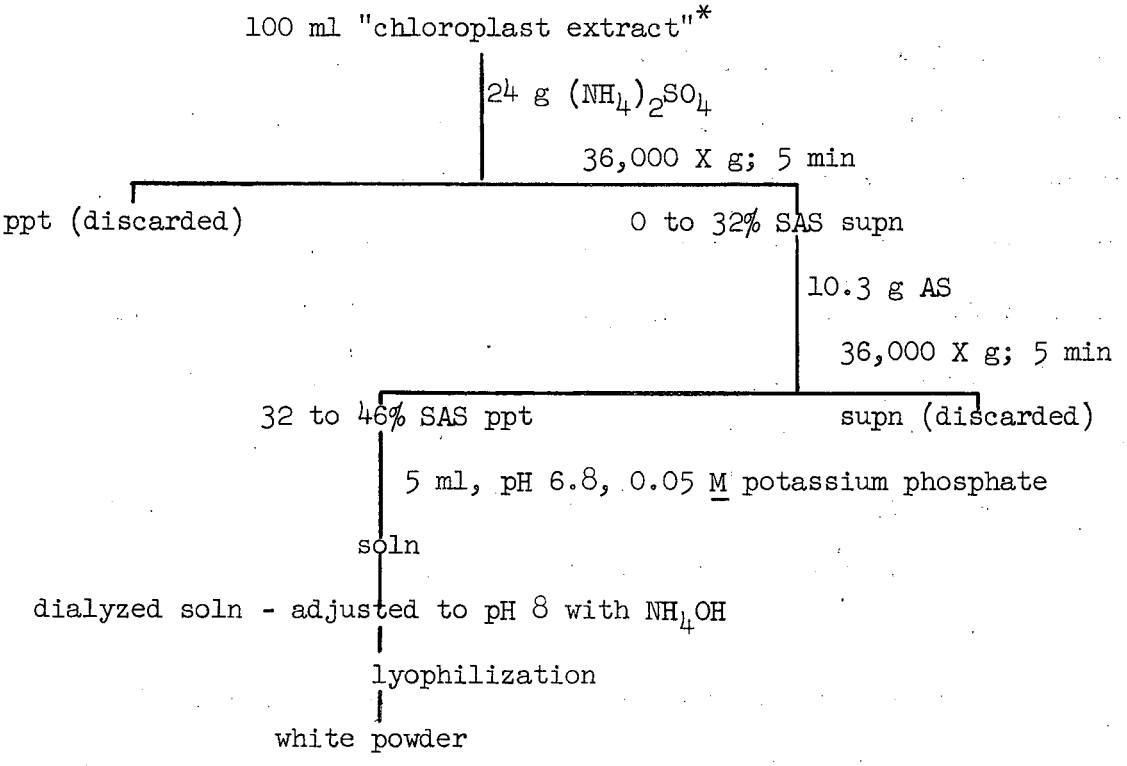


a) All operations were carried out at, or near, 0° C.

b) International Portable Refrigerated Centrifuge, Model PR-2.

c) Spinco Ultracentrifuge, Model L, rotor number 40.

Fig. 8. The Preparation of the Chloroplast Extract.



"32 to 46% SAS ppt", yield: ca. 50% of the initial amount of protein present in the "chloroplast extract." Enzyme solutions for typical assays contained 1 mg of powder per ml of pH 8, 0.001 M tris buffer.

* All explanations and conditions were identical to those in Method I except where otherwise noted.

Fig. 9. Ammonium Sulfate Fractionation of the Chloroplast Extract.

Enzyme from Chlorella Pyrenoidosa: 1) Preliminary studies. Eight ml of wet packed Chlorella cells, accumulated over a period of four days from the shaker culture flask, was suspended in 37 ml of 0.1 M potassium phosphate buffer, pH 6.8. The suspension was cooled to nearly 0° C and sonically ruptured for the following duration of time: 2.5, 5, 10, 20 and 30 minutes. At the end of each period, a 6 ml aliquot portion was removed and centrifuged at 145,000 X g for 30 minutes. The supernatant liquid was dialyzed at 4 to 8° C for 16 hours against 10^{-4} M sodium acetate which was adjusted to pH 7. Two changes of fresh dilute sodium acetate were made during this interval. The amount of protein was determined on a dry weight basis. Some of these preparations, after dialysis, were lyophilized yielding a fluffy white powder.

2) Preparative scale. In subsequent preparative experiments, the algae were accumulated over a period of several days; suspended in about 40 ml of pH 6.8, 0.1 M phosphate buffer, and ruptured sonically for 25 to 30 minutes. The supernatant liquid, after centrifugation at 145,000 X g for 30 minutes, was either dialyzed exhaustively (i.e. greater than 24 hours) against 10^{-4} M acetate at pH 7 and 0° C, or subjected to one ammonium sulfate precipitation (62 g/100 ml of original solution). In the latter procedure, the precipitate was collected by centrifugation at 36,000 X g for 5 minutes. The precipitate was redissolved in 0.01 M phosphate, pH 6.8. After removal of small amounts of insoluble material by centrifugation, the solution was dialyzed exhaustively and lyophilized; yield: 16 mg solid per ml of packed Chlorella cells (not treated with ammonium sulfate), 10 mg solid per ml of packed Chlorella cells (after ammonium sulfate precipitation).

3) Purification studies on the crude Chlorella extract. a) Preliminary experiment:[70] A solution of lyophilized crude Chlorella extract

(20.5 mg/10 ml distilled water, solution adjusted to pH 7.0) was made to 90% saturated ammonium sulfate (SAS) by the addition of solid ammonium sulfate. The pH of the mixture was adjusted to 7.4 with 2 N NH_4OH. The precipitate was collected by centrifugation at 36,000 X g for 5 minutes and then suspended in 100% SAS (50 ml, the latter being previously adjusted to pH 7.0 at 5° C using concentrated NH_4OH). This suspension was poured into the mixing vessel of a continuous exponential gradient elution apparatus in which the outlet was fitted with a loosely packed glass wool plug. Water (125 ml) was added to the reservoir. The liquid in the system was permitted to flow at free flow rate. Fractions were collected with a time operated fraction collector, set so that approximately 3 ml of effluent was collected for each tube. The time interval for each tube varied from 30 minutes initially down to 7.5 minutes at the end. The protein content in each fraction was determined by its optical density at 275 mμ.

b) Column technique for the ammonium sulfate fractionation of crude Chlorella extract: Lyophilized crude Chlorella extract (20.7 mg) was dissolved in 10 ml water and adjusted to pH 7.0. For a control, 0.5 ml was removed and frozen while to the remainder of the solution was added 6.65 g of ammonium sulfate. The pH of the suspension was adjusted to 7.0 with ammonium hydroxide (50 μl, 2 N). The precipitate was collected by centrifugation at 36,000 X g for 5 minutes and resuspended in 2 ml of 100% saturated ammonium sulfate solution which was previously adjusted to pH 7. The suspension was carefully added to an ion exchange column (XE-97, Amberlite, NH_4^+, 0.9 cm diameter X 20 cm length) which was washed previously with neutral 100% saturated ammonium sulfate. Previous experiments have shown that this ion exchange resin does not retain any protein from the crude extract of Chlorella at pH 7.0 when the salt concentration of the eluant is 0.1 M. When the meniscus of the suspension had dropped to the level of the top of the ion exchange resin, 3 ml of 100% saturated ammonium sulfate was added. A continuous exponential gradient elution apparatus was fitted to

the top of the column. Fifty ml of 100% SAS was added to the mixing vessel and distilled water (125 ml) was added to the reservoir. The flow of the liquid through the column was set for free flow. Fractions were collected in a siphon operated fraction collector, the volume collected per fraction being approximately 3 ml. The protein content was determined by uv absorption at 275 mμ. Only the peak tubes were tested for carboxydismutase activity.

Some exploratory experiments in the fractionation of the crude Chlorella extracts were undertaken. To test whether the enzyme derived from Chlorella is similar to the preparation obtained from Tetragonia, the usual classical technique for the ammonium sulfate precipitation was used. In a typical experiment, Chlorella accumulated over a period of several days, was sonically ruptured in phosphate buffer for 30 minutes. The exact details of this method are described under Preparative Scale. The supernatant liquid obtained after high speed centrifugation was fractionated with ammonium sulfate. Before this operation was initiated a small portion of this supernatant liquid was removed for dialysis and then set aside as a control. Dialysis was performed against 10^{-4} M NaOAc at pH 7 for approximately 42 hours. All operations were carried out at, or near, 0° C. In the sequence of operations for the purification, solid ammonium sulfate was used. The pH of the resultant solution was adjusted to nearly 7 with 2 N ammonium hydroxide. The precipitate obtained after each addition of ammonium sulfate was collected by centrifugation at 36,000 X g for 5 minutes. Each precipitate was redissolved separately with phosphate buffer (0.5 M, pH 6.8, 3 ml) and dialyzed according to the conditions given above. Protein was determined on a dry weight basis.

c) Treatment of the soluble Chlorella protein with lead acetate:[71] Lead acetate solution was prepared by dissolving 19 g of $Pb(OAc)_2 \cdot 3H_2O$ to make

100 ml of aqueous solution. The pH of this solution was adjusted to about 7.0 with 1 N NaOH. The lead acetate solution was allowed to stand for 2 days. The supernatant liquid (10 ml) was then diluted to 30 ml with water.

In the following experiment all operations were carried out at 0°C. Two ml of a water soluble extract obtained after sonic disruption of Chlorella containing about 12 mg of solid (determined on a dry weight basis after dialysis) was mixed with 0.6 ml of the diluted lead acetate solution and an additional 1.4 ml of water. The yellow precipitate was collected by centrifugation for 5 minutes at about 1,500 X g and redissolved with 2 ml of 0.2 M $NaHCO_3$. The mixture was bubbled with a stream of CO_2 for 2.5 minutes. A trace of n-octanol was added to minimize foaming. The mixture was centrifuged to remove the white precipitate of $PbCO_3$. After dialysis against 10^{-4} M NaOAc at pH ca. 7 for 44 hours, the supernatant liquid was tested for protein content (dry weight) and carboxydismutase activity.

Homogeneity Studies of Various Carboxydismutase Preparations. Physical Chemical Studies: Most of these studies were made on the preparation obtained by the Crude Extract Method. Ultracentrifugation was performed in potassium phosphate buffer at pH 6.67, ionic strength (μ) of 0.2.* The protein concentration was about 10 mg/ml. For electrophoresis, however, the protein concentration was about 5 mg/ml and the potassium phosphate buffers used in these experiments were of two different pH's and of two different ionic strengths, namely; pH 5.97, μ = 0.1; and pH 6.67, μ = 0.2.* Prior to the electrophoresis of the sample, the latter was

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* Experiments were generously carried out by K.K. Lonberg-Holm in the laboratory of Prof. H. K. Schachman.

dialyzed against the buffer for at least 18 hours at 4° C. The same protein fraction was studied under paper electrophoresis in barbital buffer, pH 8.6, $\mu = 0.05$ at 3° C for 6 hours. In this case unwashed Whatman No. 4 paper was used as the supporting medium. About 100 µl of protein solution containing a maximum of 960 µg of material was applied as a band 14 cm wide at the center of the paper (widthwise) after the paper was initially equilibrated with the buffer for at least 30 minutes. A varying potential was applied starting with 760 volts and ending with 650 volts; during all of this time, the current was maintained at 10 ma. At the end of the run, the paper was removed and strips 1 cm wide by 2.5 cm long (the length of each strip being parallel to the width of the paper), were cut from the moist paper while in the cold room (ca. 4 to 8°C). Each strip was placed into a test tube containing 1 ml of cold water. A stirring rod was used to press the strips beneath the water. After standing overnight at 4° C, a 500 µl aliquot portion was removed from each tube for protein analysis by the colorimetric method. The carboxydismutase activity was located by incubating 50 µl of the eluates with 40 µl of a buffered cofactor-substrate solution which consisted of the following: $NaHC^{14}O_3$, 26.6 µM at 400 µC; tris, 300 µM at pH 8.4; $MgCl_2$, 2 µM; and RuDP, ca. 0.5 µM; all made to a total volume of 1.55 ml with water. The reaction was carried out on a planchet in the form of a drop and placed in an atmosphere which was saturated with water. After 21 hours at room temperature, 20 µl of 6 N HOAc was added to stop the reaction. The mixture was spread on the planchet with the aid of 2 drops of detergent solution and counted.

Absorption spectra were taken of protein preparations at almost all stages of purification of the enzyme. The range under investigation was generally in the ultraviolet region (from 220 to 350 mµ). Protein concentration was about 0.6 mg/ml.

The homogeneity of the carboxylation enzyme prepared by the Acetone Powder Method was studied by the fractional solution method. The 28 to 39% SAS precipitate was divided into two portions, one for control and the other for the experiment. To each of these samples were added 0.1 ml of pH 8.4, 1 M tris and 9 ml of 93% SAS adjusted to pH 7.2. All operations with the enzyme were carried out near 0°C. The precipitated material was centrifuged at 36,000 X g for 5 minutes. The control sample was stored in the cold room (5° C) for the duration of the experiment. The supernatant liquid of the sample for the experiment was decanted. The precipitate was resuspended in 1 ml of 93% SASand added to a column (1 cm diameter X 20 cm length) packed with 0.1 mm diameter glass beads. The column was previously washed with 50 ml of 93% SAS. After the liquid level fell to the top of the column of beads, an additional 9 ml of 93% SAS was added and the linear gradient elution apparatus was connected. In the mixing vessel was placed 50 ml of 93% SAS and in the reservoir, 50 ml of distilled water. The flow rate was controlled to about 3 ml per 20 minutes. The time required to collect each fraction was set for 22 minutes. The protein concentration in each tube was determined by uv absorption at 275 mμ. (While these fractions were being read, the control was removed from the cold room and allowed to stand at room temperature.) Several fractions were assayed for enzyme activity. The protein concentration in tube was also determined colorimetrically with the Folin-Phenol reagent and the results were essentially similar to those obtained by uv analysis.

Organic Chemical Studies: N-terminal amono acids of the 32 to 35% SAS ppt (Fig. 5), the acetone powder (Fig. 6) and the 32 to 46% SAS ppt of the chloroplast extract (Fig. 9) were determined. The determination was based on the measurement of the N-dinitrophenyl derivatives of amino

acids obtained after reacting the protein with 1-fluoro-2,4-dinitrobenzene (FDNB) followed by hydrolysis of the resultant DNP protein.[72]

In a typical reaction, approximately 80 mg of protein was suspended in 2 ml of 0.2 M $NaHCO_3$ at pH 8.7. FDNB (0.05 ml) was added and the mixture was stirred vigorously using an air-driven stirrer. The reaction was carried out at 40° C. After 4 hours the reaction mixture was extracted three times with peroxide-free ethyl ether (prepared by shaking the ether with a slightly acidic solution of $FeSO_4$). The mixture was then acidified with 0.05 ml of concentrated HCl and re-extracted 3 X with ether. The precipitate was collected by centrifugation and finally, washed three times with water. All operations which included the extractions and the washings were done in a 12 ml centrifuge tube. The precipitate was dried under reduced pressure over P_2O_5. (The DNP acetone powder, originally greenish yellow, turned black on drying while the 32 to 46% SAS ppt, initially bright yellow, turned to orange yellow). The DNP derivative was hydrolyzed with 2.5 ml of 6 N HCl at 110°C for 16 hours in a sealed tube. The hydrolysate was diluted with water until the final concentration of HCl was 1 N. The solution was then extracted with ether until no more color was extracted. The ether extracts were combined, evaporated to dryness, and chromatographed. After chromatography the yellow spots were cut out, placed into test tubes and eluted with 4 ml of 1% $NaHCO_3$ at 50 to 60° for 15 minutes. The optical densities of the solutions were measured at 360 mμ.

Biochemical Studies: In order to test for contaminating enzymes in the purified carboxydismutase preparations, the standard assay method was used (see Carboxydismutase Assay below). The time of incubation was extended to several days instead of the usual 5 to 10 minutes. Toluene was layered on top of the incubation mixture to prevent bacterial growth. The products were studied by chromatography in the usual manner. The presence

of other enzymes were also detected by taking RuDP-C^{14} and unlabeled bicarbonate as substrates. In this case the RuDP-C^{14} was mixed with unlabeled RuDP so that the concentration of this substrate was nearly the same as that present in the standard assay mixture. The period of incubation was also extended to 2 or 3 days.

The Carboxydismutase Assay. There were three types of assays: 1) Assay I, where the enzyme was preincubated with the cofactor, one of the substrates and other additions, if any, for an unspecified period of time; 2) Assay II, where the enzyme was preincubated with all additions except one or two of the components for a known period and 3) Assay III, where the enzyme was not preincubated with any of the constituents of this enzyme system.

Assay I: The incubation mixture for this assay was prepared by mixing, in the order indicated, the following components: H_2O, to make 200 µl; $NiCl_2$, 0.4 µM; potassium phosphate, 2 µM at pH 6.8 or HCl, to make the final pH 7.0 when mixed with labeled bicarbonate; $NaHC^{14}O_3$, 1.3 µM at 20 µC; and the enzyme, about 25 µg of purified preparation or 100 µg of crude extract. RuDP, 0.1 µM was added to start the reaction. The mixture was incubated at 25°C for 10 minutes. The temperature was controlled to ± 0.1° using a thermoregulated water bath. The reaction was stopped by heating the reaction vessel with steam for 30 to 40 seconds followed by adding 50 µl of 6 N HOAc. A suitable aliquot portion (generally 1/10 of the total volume) was plated on an aluminum or a glass planchet and counted. No special precaution was taken to control the time of preliminary incubation of the enzyme with any of the components of the assay system.

Assay II: The constituents were the same as those in Assay I, except that in some cases, a $MgCl_2$ (2 µM) plus tris buffer (15 µM at pH 8.3) mixture was substituted for the $NiCl_2$ plus HCl or phosphate mixture. There

were two steps to this assay: 1) the preliminary incubation (preincubation) step and 2) the incubation step. In the former step all but one or two of the components of the assay system were mixed and allowed to stand either at 0° or 25° C for a predetermined time. At the end of this period the rest of the component(s) was added (the incubation step) and the complete system was incubated for a fixed time, (usually 5 or 10 minutes,) at 25° C. (In some cases where two components were preincubated with the enzyme, the first component was allowed to be in contact with the enzyme for a given time "a," followed by preliminary incubation with the second component for time "b." The first component would then be preincubated with the enzyme for a total time of a + b.) The reaction was stopped by adding HOAc and treated subsequently in a similar manner to Assay I.

Assay III: This assay, also termed as the non-preincubated or zero-time preincubated assay, contained the same components as Assay I or II. In this case, however, the metal ion and substrates were first mixed and the reaction was started by adding the enzyme to this mixture. Subsequent treatment of the reaction mixture was identical to that of Assay II.

Carboxydismutase Activity in the Presence of Digitonin. All operations were performed at, or near 0° C. A 0.5 M sucrose suspension (0.2 ml) of intact chloroplasts, prepared from Spinacea oleracea,[73] was mixed with an equal volume of digitonin. (The latter was the clear supernatant liquid obtained after suspending 0.5 g of digitonin in 12.5 ml of H_2O. The supernatant liquid was adjusted to pH 8.1 by adding 50 μM of pH 8.4 tris buffer.) An extra 5 ml of diluted digitonin (1:1 with water) was added to the chloroplast-digitonin mixture and after standing for 3 hours, the suspension was centrifuged for 30 minutes at 145,000 X g. The clear green supernatant liquid was assayed for chlorophyll, carboxydismutase activity, and colorimetrically for protein.

Assay III was employed for determining the enzymatic activity using Mg^{++} as cofactor. Between 30 to 50 μg of protein was added to each incubation mixture. Incubation time was 10 minutes.

For comparison a suspension of intact chloroplasts (0.1 ml) was mixed with 2.8 ml of pH 8.4, 0.01 M tris. The whole suspension was assayed for chlorophyll, protein and carboxydismutase activity.

Cofactors and Inhibitors of the Carboxydismutase System. The requirement for a cofactor(s) was demonstrated by heating the dialyzed crude extract of Tetragonia for 2 minutes over a steam bath, cooling and then adding it to an incubation mixture containing the 32 to 35% SAS ppt (Crude Extract Method). The conditions of Assay I were used. A reaction mixture having the heat treated enzyme, but no 32 to 35% SAS ppt, was used as a control. For comparison, an assay mixture was prepared which contained the 32 to 35% SAS ppt, but no heat treated enzyme.

The possibility that RuDP extracted from algae also contained an activator was considered. In this case, the RuDP was destroyed by hydrolyzing a solution of RuDP (ca. 75 μM) with HCl (150 μM) in a total volume of 150 μl for 4.5 hours at 100° C. The sample was taken to dryness under reduced pressure over P_2O_5. The incubation mixture was prepared in the same vessel; enzymically prepared RuDP was added as one of the substrates. A suitable control experiment for the presence of cofactors in the HCl was performed. Another experiment was designed to test for intact RuDP still present after the hydrolysis.

A variety of inorganic and organic substances, at different concentrations, were tested as cofactors or as inhibitors of the enzyme system. The effect of the added substance was readily measured by adding it to the standard incubation mixture and then proceeding in the usual manner for a carboxydismutase assay.

Reproducibility of the Carboxydismutase Assay.* For studying the reliability of the enzymatic assay, six incubation mixtures were prepared using a given enzyme solution and following the method of Assay III. Three of the incubation mixtures had no RuDP added to them (Controls) while the remaining three contained the completed system (Variables). To reduce systematic errors the mixtures were incubated in the following order: Control 1, Variable 1, Variable 2, Control 2, Control 3 and finally, Variable 3. After 10 minutes at 25° C the reactions were stopped by adding HOAc and 15 µl (from a total volume of 250 µl) of each mixture was plated on aluminum planchets (in triplicates) in the usual manner. The plates were counted in a Nuclear-Chicago counter with automatic sample changer. The plates prepared from the Variable and Control series were counted for 10,240 counts and 320 counts, respectively; the time required to reach these values being recorded. Each plate was counted three times.

pH vs Carboxydismutase Activity. Assay III was used for these experiments. The pH 8.3 tris buffer was substituted with each of the following buffers (15 µM): pH 5.95 and 6.22, sodium citrate; pH 6.59 and 6.71, histidine; pH 7.13, imidazole; pH 7.58, 7.98, 8.39, 8.82 tris; 9.21 and 9.52 histidine.

The Stability of Carboxydismutase. Enzyme preparations at various stages of purification were stored in the form of lyophilized powders and solutions. Storage temperatures ranged from room temperature to -20° C.

The effect of radiation on the assay system was also considered. In this case, in each incubation mixture, the amount of radioactive bicarbonate was varied from 1 to 20 µC while keeping the total bicarbonate concentration constant at 0.00665 M. Otherwise the assay conditions were those of Assay III (Mg^{++} as cofactor, 10 minutes incubation at 25° C).

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* The author gratefully acknowledges Y. Natori for this work.

Experiments with Carbonic Anhydrase. The action of carbonic anhydrase on the carboxydismutase system was studied by adding to the standard assay mixture (Assay III with Mg^{++} as cofactor and 10 minutes incubation at 25° C) 50 µg of the former enzyme. Incubation mixtures without carbonic anhydrase or RuDP served as controls.

Other One-carbon Compounds as Possible Substrates for Carboxydismutase.

Carbamyl Phosphate: Assay III was employed for these experiments, using 1.3 µM of dilithium carbamyl phosphate in place of $HC^{14}O_3^-$. Dilithium carbamyl phosphate was prepared according to the method of Lipmann.[74*] The rest of the constituents of the assay system were RuDP-C^{14} (0.04 µC), unlabeled RuDP (ca. 0.1 µM), Mg^{++} (2 µM), and an enzyme preparation via the chloroplast method (25 µg). Incubation conditions were 10 minutes at 25° C, after which the reaction was stopped by adding HOAc. One fifth of the total was applied on paper for chromatography in the usual two-dimensional solvent systems. The radioactive spots were then counted. Similar experiments with RuDP-C^{14} were performed on incubation mixtures containing unlabeled bicarbonate, but no enzyme; or containing unlabeled bicarbonate plus enzyme; or containing no bicarbonate and enzyme.

Formic Acid: In these experiments the assay system consisted of Mg^{++} tris, RuDP, radioactive bicarbonate or formate, and enzyme. The general procedure was similar to Assay III. The mixtures were incubated for 10 minutes at 25° C. In the formate experiments, 38 µM of C^{14}-labeled sodium formate (2.6 µC) was dissolved in 0.5 ml distilled water and 0.05 ml 1 N HCl was added. Nitrogen gas was streamed through the solution for 30 minutes at room temperature. The solution was neutralized with an equivalent of NaOH and then diluted to 1.0 ml. Fifty µl of this solution was used in the incubation mixture. To show that the radioactive formate contained no radioactive bicarbonate as contaminant, the following

control was prepared: Diluted formic acid (36 μM) was mixed with NaOH (40 μM) and 0.5 ml H_2O. $NaHC^{14}O_3$ (0.13 μM at 2 μC) was added to this solution along with 0.05 ml 1 N HCl. Nitrogen gas was bubbled through this solution for 30 minutes at room temperature, after which the solution was neutralized with 0.05 ml 1 N NaOH and then diluted to 1.0 ml. Fifty μl was added to the reaction mixture for the control. In all of the above incubation mixtures, after the required time of incubation, 50 μl of 6 N HCl was added to stop the reaction. A suitable aliquot portion was plated on glass planchets for counting. The enzyme used for this experiment was prepared by the Chloroplast Extract Method, and was about 25 μg per reaction mixture.

Two other experiments were performed as controls, one to insure that the enzyme was active by means of the standard assay with radioactive bicarbonate (instead of radioactive formate) and the other to show that the fixation of labeled formate was due to the presence of RuDP. The latter control was then one without added RuDP.

Occurrence of the Carboxylation Enzyme. The presence of the enzyme was studied in the following tissues: Nostoc muscorum, baker's yeast, rat liver, rat brain, lettuce leaves, mature avocado leaves, button mushrooms and Ehrlich ascites mouse tumor (EAMT) cells. Crude extracts were obtained by grinding the tissues in pH 8.4, 0.01 M tris at, or near, 0° C; except for EAMT cells which was homogenized in pH 6.8, 0.01 M potassium phosphate buffer. For rupturing Nostoc, yeast and EAMT cells a sonic oscillator was used. Rat tissues were ground in a Potter-Elvehjem homogenizer while mushroom, lettuce and avocado leaves were ground in a Virtis homogenizer. All homogenates were clarified by centrifugation at 145,000 X g for 10 minutes. Protein was determined colorimetrically with the Folin-Phenol reagent. Incubation mixtures were prepared as in Assay III with Mg^{++} as cofactor except that a maximum volume of extract was added to each incubation mixture (to make a final volume of 200 μl). Each reaction mixture

was layered with 50 µl of toluene and then incubated for 12 hours. The reaction was stopped by adding HOAc and prepared for counting in the usual manner.

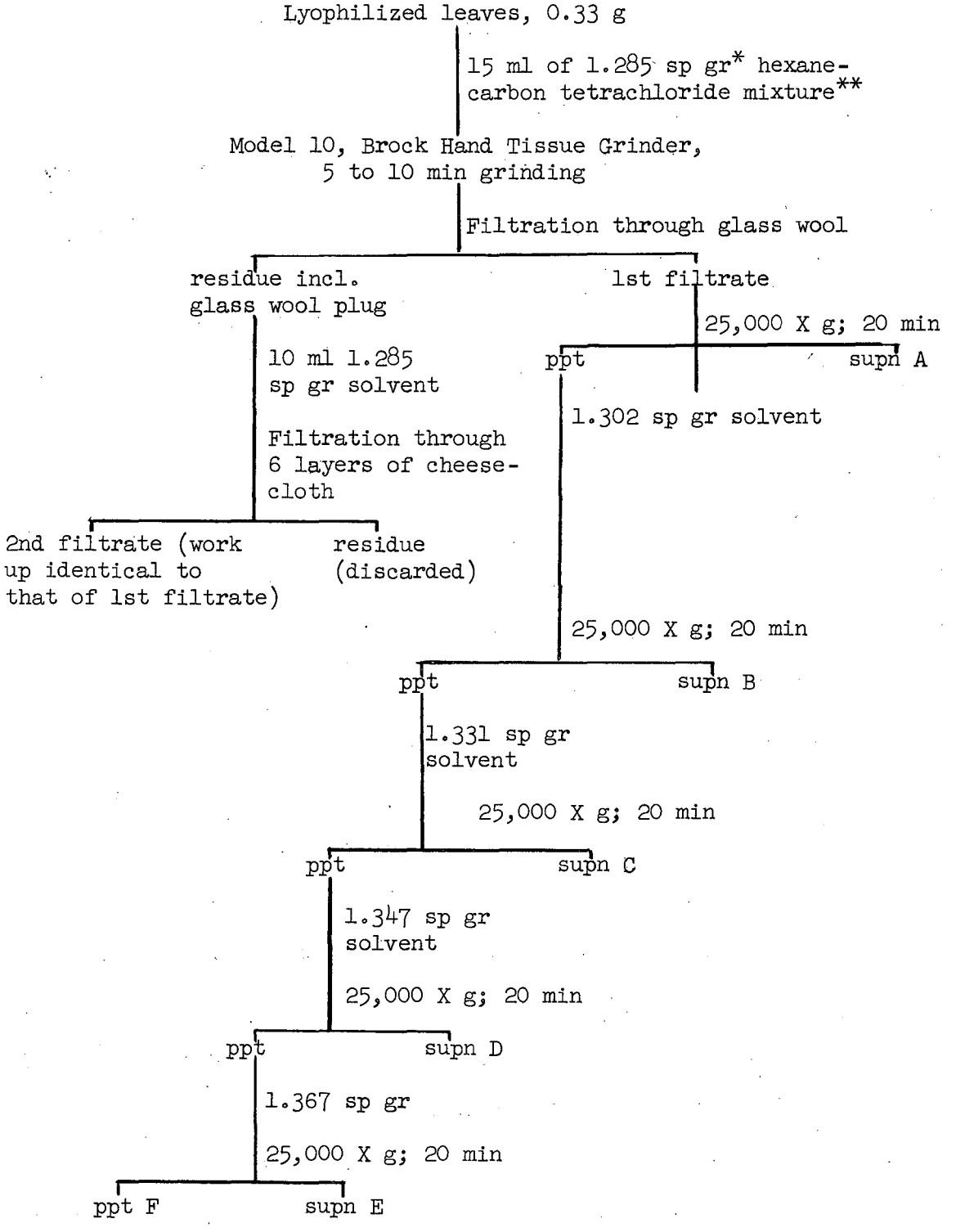
Carboxydismutase Activity from Chloroplasts Isolated in Nonaqueous Solvents. This method was developed by C. R. Stocking.[75] New Zealand spinach leaves, with stems removed, 100 g, were frozen in liquid nitrogen. The most convenient way was to wrap the leaves in cheesecloth so as to form a bag and then to dip it into a Dewar flask containing liquid nitrogen. As soon as the leaves were frozen, they were crushed, while in the bag, with several blows from a hammer. The crushed frozen bits of leaves were quickly poured into the bottom of a precooled (at -15° C) vacuum desiccator and lyophilized. Yield of lyophilized material: 6 g.

The scheme for the fractionation of the lyophilized material is given in Fig. 10.

The supernatant liquid obtained from each step of the fractionation was treated in the following manner: One-third, by volume, of the supernatant liquid was mixed with an equal volume of n-hexane. The precipitate was collected by centrifugation and resuspended in 50 µl of 0.5 M sucrose. The suspension was examined under the microscope at 900 times magnification (oil immersion). The remaining two-thirds of the supernatant liquid was treated in a similar manner except that the final precipitate was resuspended in pure n-hexane instead of sucrose. This suspension was transferred nearly quantitatively to a tared 3 ml conical centrifuge tube. The precipitate was collected by centrifugation, and the supernatant liquid was discarded. The precipitate was dried by standing overnight at room temperature. The tube plus residue was weighed giving thus, the weight of the precipitate. The precipitate was suspended in 0.01 M tris, pH 8.4 (1 ml) and analyzed for chlorophyll. Another portion was centrifuged

to obtain a clear supernatant liquid which was assayed for protein (colorimetrically) and for carboxydismutase activity (Assay III, with Mg^{++} as co-factor, 10 minutes incubation).

Attempts to Trap the Carboxylated Intermediate of the Carboxydismutase Reaction. So diverse were the conditions for these experiments that only an outline of them are given in the RESULTS AND DISCUSSION section. In general, these experiments fell into two categories; namely, those in which the method for inactivating the enzyme was varied, and those in which inhibitors were added to accumulate the intermediate.



* Specific gravity were measured at room temperature.

** All solvents were mixtures of n-hexane and carbon tetrachloride. Each ppt was resuspended in 10 ml of solvent in subsequent steps.

Fig. 10. Fractionation of Lyophilized Leaves of Tetragonia in Nonaqueous Solvent.

RESULTS AND DISCUSSION

Ribulose-1,5-diphosphate. RuDP from Algae: As stated in the INTRODUCTION section of this thesis, one of the objectives of this research was to study the properties of RuDP. Ordinarily, these studies would of necessity involve relatively large amounts of RuDP, but since algae yield only about 1 μM of RuDP per ml of packed cell,[24] these studies must be approached by employing more sensitive techniques. Towards this end, RuDP-C^{14} seemed to offer the best possibility.

The physical properties of RuDP generally include such properties as solubility, density, etc., but in these studies only the behavior of this compound towards paper chromatography and paper electrophoresis was considered. Such information was readily available without isolating the compound from the rest of the sugar phosphates present in the 20% EtOH extract of algae. Paper chromatography and paper electrophoresis of this extract gave the results shown in Fig. 11 and Fig. 12, respectively. Most conspicuous of the results is the predominance of the diphosphate area in both the chromatogram and the electrophoretogram (labeled as RuDP in the electrophoretogram). This area makes up 45% of the total radioactivity on the paper electrophoretogram. (It was not possible to give a true evaluation of the percentage of the diphosphate area on the paper chromatogram owing to the loss of many of the other radioactive compounds after 36 hours of development of the chromatogram. Some losses during the electrophoresis of the alcoholic extract also occurred, these compounds being cations.)

When the diphosphate area was eluted and rechromatographed in two dimensions for 48 hours in each direction, using the usual solvent systems, only one spot was generally obtained. On occasions, however, at least three spots were seen; the major one was RuDP and has an R_f greater than

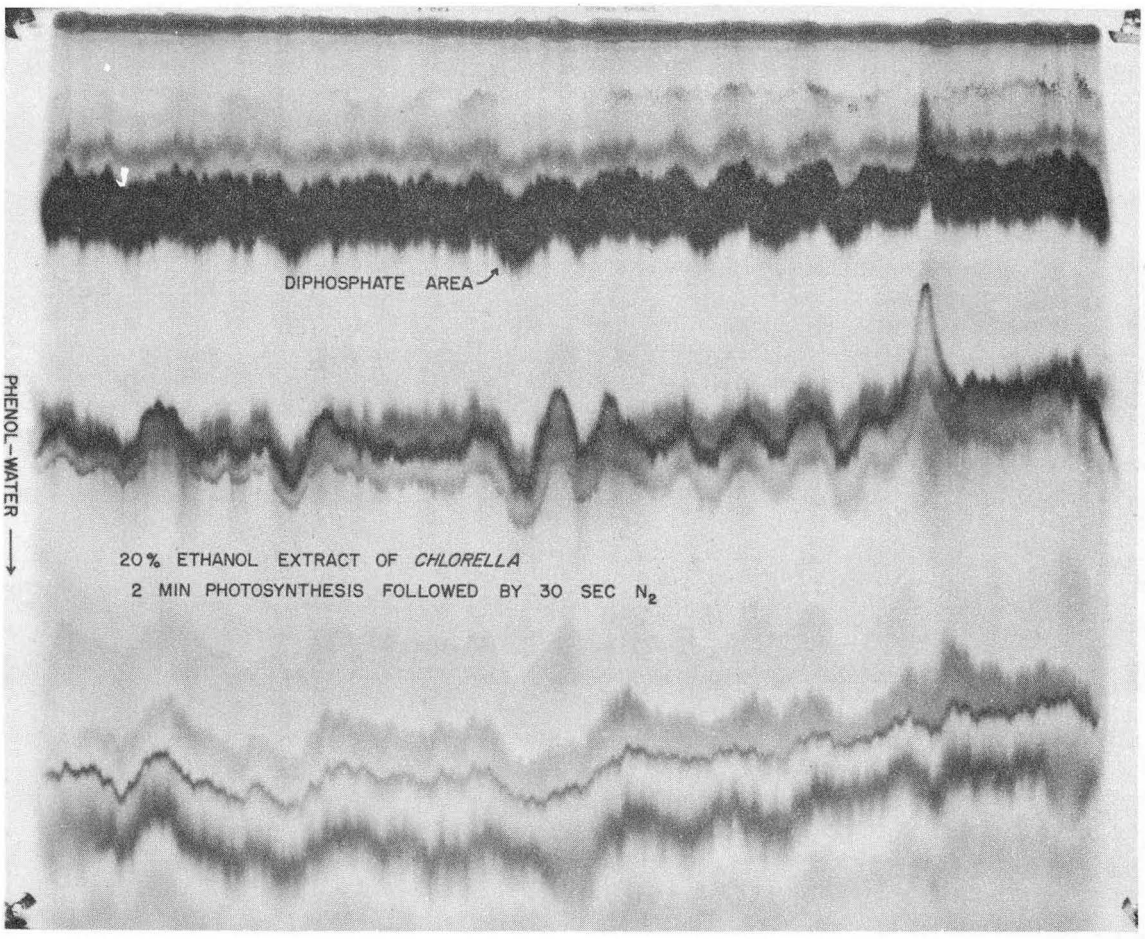


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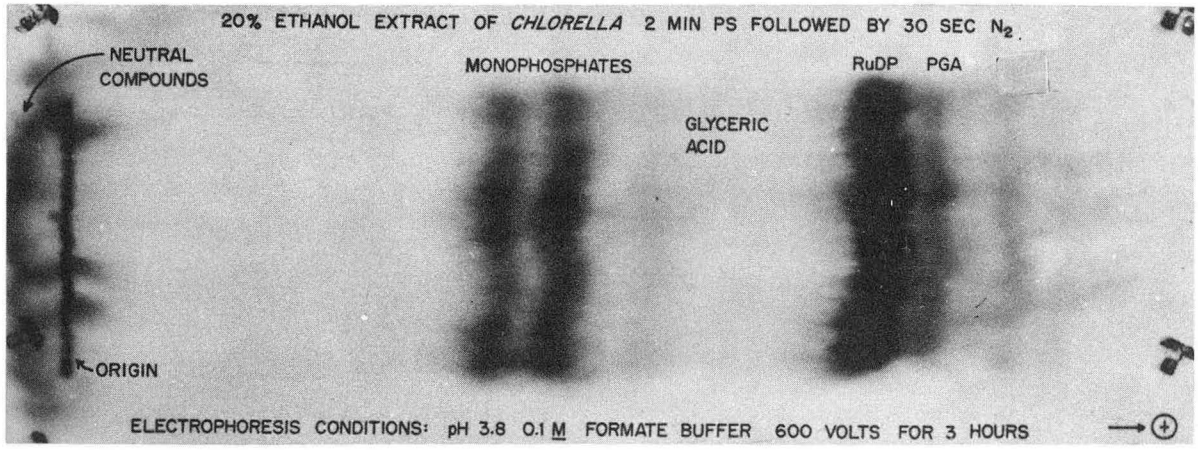
Fig. 11. Radioautograph of a chromatogram of a 20% EtOH extract of photosynthesizing Chlorella.



ZN-2575

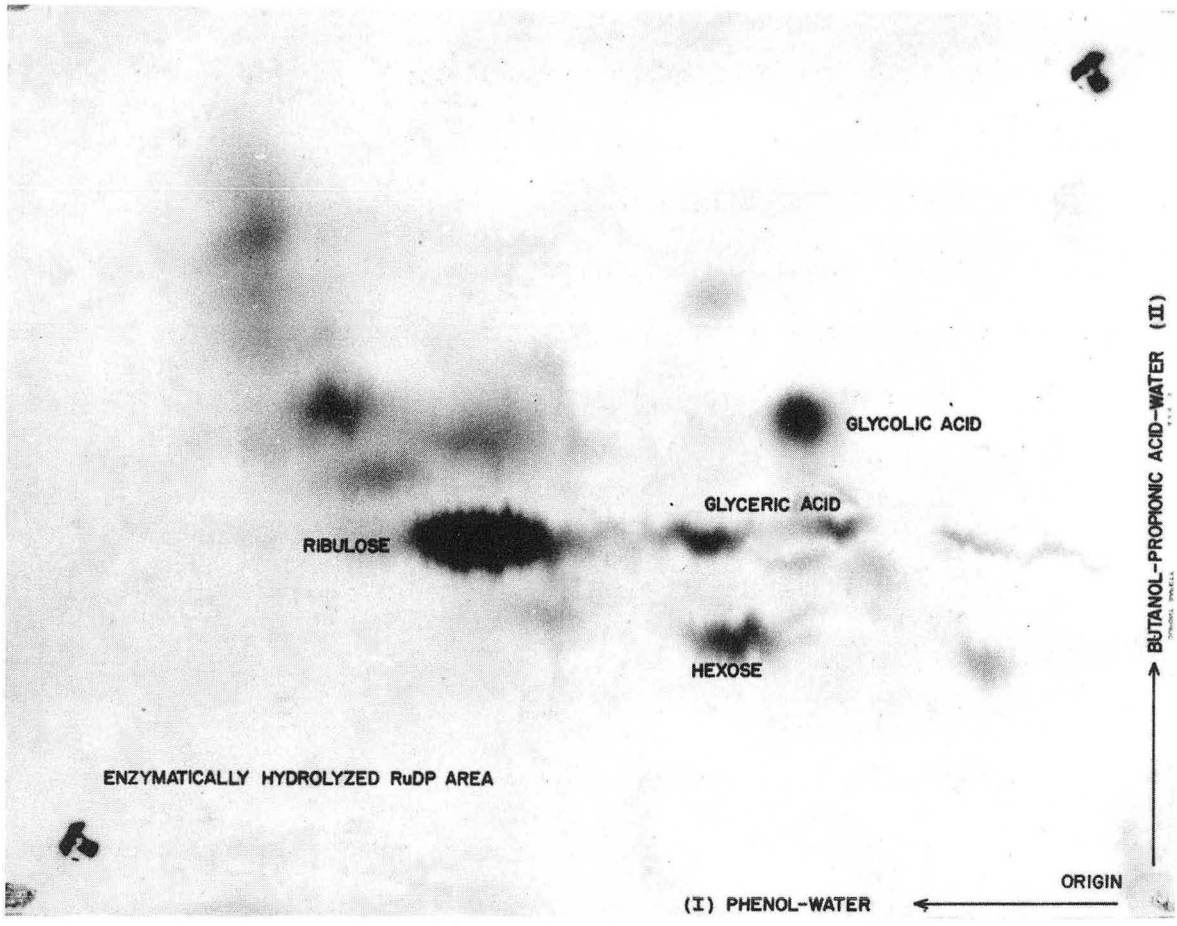
Fig. 12. Radioautograph of an electrophoretogram of a 20% EtOH extract of photosynthesizing Chlorella.

the two minor spots in the butanol-propionic acid-water direction and an R_f intermediate to the two minor spots in the phenol-water direction. (The other two spots were identified by Moses [76] as the diphosphate of a γ-keto acid and of hexose.)

Clearly, then, the diphosphate area of the paper chromatogram is not entirely RuDP, but rather a mixture of at least two other compounds. The findings of Goodman,[43] qualitatively, and of Bassham,[45] quantitatively, indicate that photostationary concentration of RuDP in the diphosphate region of a chromatogram exceeds 90%. The latter investigator found that photosynthesizing algae in the presence of 1% CO_2 has a steady state concentration of RuDP of 0.5 μM per ml of wet packed cells. On changing the CO_2 pressure from 1% to 0.003%, the amount of RuDP rose to twice the steady state level[24] (hence, the estimated yield under optimum conditions becomes 1 μM per ml of wet packed cells), but the percentage of RuDP in the diphosphate region was, at best, 80% of the total diphosphates present.

In the particular results shown in Fig. 11, RuDP constituted a minimum of 70% of the total diphosphates, as shown by dephosphorylation with acid prostatic phosphatase, Fig. 13. A minimum value was quoted because of the susceptibility of RuDP towards oxidative cleavage into PGA and phosphoglycolic acid.[77] There are also present in the above radioautograph, a dozen or more weak radioactive spots; in agreement with the results of Moses.[76] Among them are hexose, glyceric acid and glycolic acid.

Although some information on the properties of RuDP was gained from RuDP derived from algae, the amount of RuDP fell far short of the demands for further studies of the carboxydismutase system. It thus became desirable to obtain quantities of RuDP larger than that normally extracted from algae. A new approach was employed; namely, RuDP was prepared by means of an in vitro system of enzymes.



ZN-2576

Fig. 13. Radioautograph of a chromatogram of the diphosphate area dephosphorylated with acid prostatic phosphatase.

RuDP prepared by Enzymic Means: The principles of this method are outlined in the previous section, EXPERIMENTAL PROCEDURE. The yields were of the order of 1 or 2 grams of crude Ba salt for each gram of starting material (barium salt of ribose-5-phosphate). The crude Ba salt of RuDP was fractionated by alcohol precipitation and by column chromatography; full details of which are given in the experimental section. The results of the column chromatography are shown in Fig. 14. The elution pattern shows two well separated phosphorus-containing peaks. The first to emerge from the column contains only inorganic phosphate; the second peak is in the position expected for a diphosphate ester,[77] and the enzyme assay shows it to contain essentially all the RuDP. It is noteworthy that the RuDP and phosphate peaks do not coincide in the second peak, suggesting the presence of at least one phosphate ester in addition to RuDP. This impurity emerges from the column slightly ahead of RuDP. From the ratio of RuDP to organic phosphate, the quantity of this contaminant can be calculated. This constitutes the shaded area on the graph, Fig. 14, and represents about 20% of the material in the second peak. The fractions emerging between A and B (sample a) and between B and C (sample b) were pooled. They were recovered as the barium salt with the yield of sample a being 22 mg and sample b being 23 mg, starting with 100 mg of the crude Ba salt of RuDP.

Treatment of these samples with acid phosphatase (Polidase-S) gave, along with ribulose, a small quantity of ribose from sample a, while sample b gave only ribulose. Sample b is thus considered as a purified sample of dibarium RuDP. Further analyses of these samples supports this view, see Table I. Included in this table are the analyses of the crude preparation and those of the alcohol fractionated material.

Although the alcohol fractionated material had the highest percentage of organic phosphate, the inorganic phosphate content was

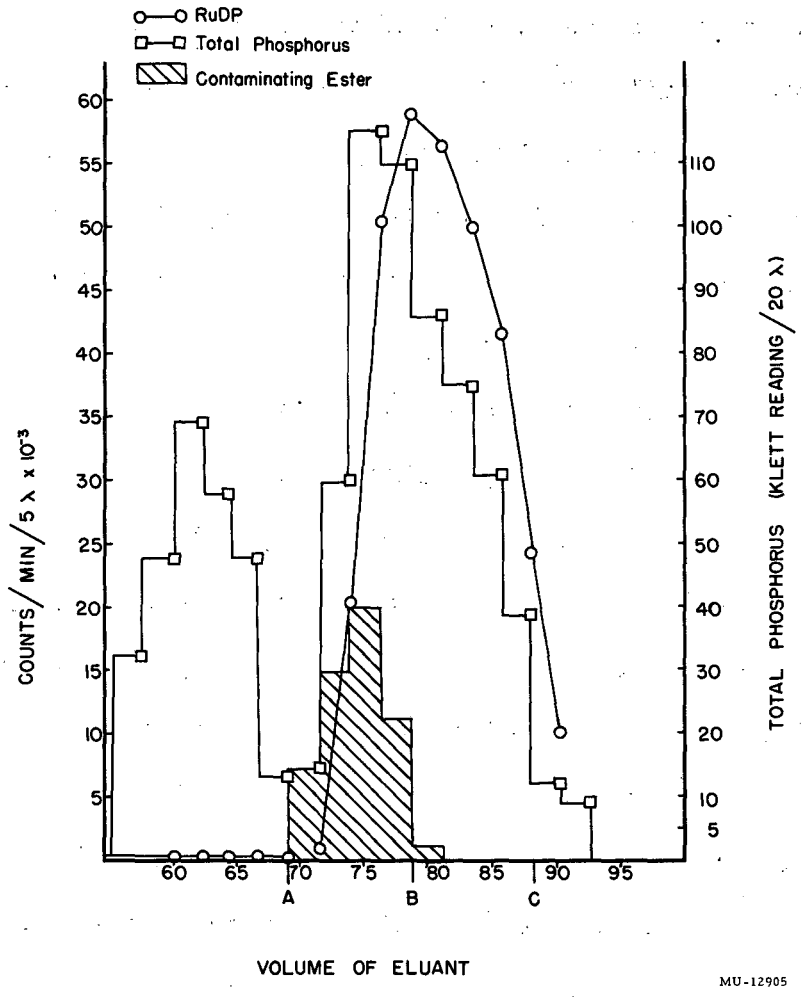


Fig. 14. Elution pattern of RuDP on a Dowex 1 formate column. Volume is in ml. Klett reading of 230 corresponds to 0.5 μM phosphate. The material was eluted by using a linear gradient elution apparatus with 100 ml of 5 M ammonium formate in the reservoir and 100 ml of water in the mixing vessel.

TABLE I

Phosphate Content and Purity of Various RuDP Preparations

Preparation	Inorganic Phosphate[a]	Organic Phosphate[a]	Ratio[b]	Purity[c]
Crude material	0.40	2.31	6	67
Alcohol ppt.	0.55	2.63	5	77
Column chromatography				
sample a	0.21	2.35	11	69
sample b	0.23	2.42	11	71

a) μM per mg of material.

b) Approximate molar ratio of organic phosphate to inorganic phosphate.

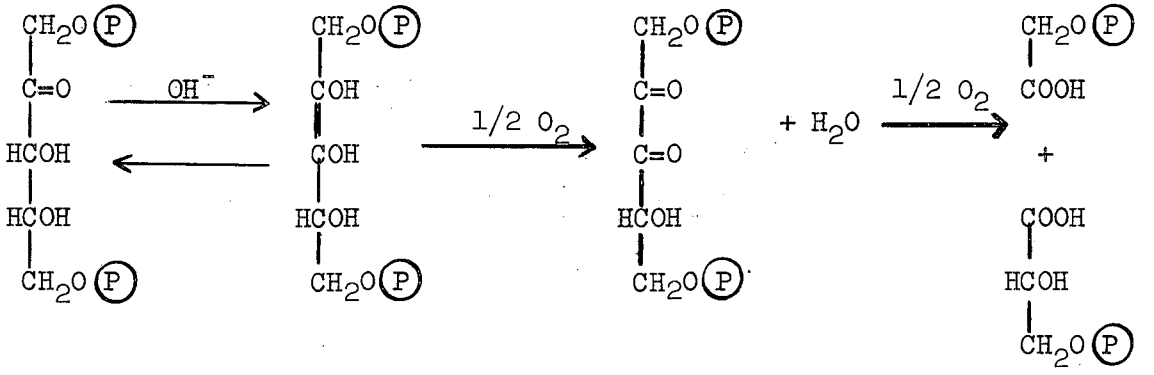
c) Percent by weight, assuming that all of the organic phosphate is RuDP.

correspondingly higher, thus giving a ratio of organic phosphate to inorganic phosphate of only 5. For this reason the purification of RuDP by alcohol precipitation was abandoned in favor of the ion exchange column chromatography technique.

Stability of RuDP: The problem of the stability of RuDP towards acid and alkaline hydrolysis has been adequately dealt with by a number of investigators.[43,63,78] The gist of their findings is that in both acid and alkali, the hydrolysis of both phosphate groups of the RuDP molecule proceeds at nearly the same rate. This result is manifested by the accumulation of only a small amount of ribulose monophosphate during the hydrolysis (in contrast to a large quantity of free ribulose). Furthermore, an analysis of the rate of hydrolysis displayed only a single first order kinetic curve.[43,63] One explanation for this phenomenon is the presence of the phosphate group on the 1 position which then renders the 5 position linkage more labile to acid.[63] Another proposal involves the migration of the ene-diol of RuDP from the 2,3 position to the 3,4 position resulting in a symmetrical ene-diol compound.[79] The latter must necessarily revert to the 2-ketopentose since the product of hydrolysis is ribulose. The latter mechanism could be tested by measuring the optical rotation of the RuDP before and after treatment with acid. In case the accumulation of this enolization product is small, the change in optical rotation should be correspondingly small and should correlate with the rate of the hydrolytic reaction.

RuDP also decomposes readily in aqueous solution in the presence of oxygen. This is evident in radioautographs of chromatograms in which the RuDP-C^{14} solution, after standing for a day, is rechromatographed. Not only is the original diphosphate present, but hexose monophosphate (arising from the hydrolysis of hexose diphosphate), pentose monophosphate (a hydrolysis product of pentose diphosphate), phosphoglyceric

acid and phosphoglycolic acid are found. In addition, when RuDP-C^{14} was dephosphorylated with acid phosphatase, the products were ribulose, hexose, glyceric acid as well as glycolic acid, Fig. 13, in accord with the previous findings. The latter two acids probably arose from the oxidation of RuDP, with a cleavage between carbon atoms 2 and 3.[77] In alkaline solution the oxidative cleavage of RuDP proceeds readily.[80] The mechanism of this reaction is probably one similar to the air oxidation of aldose to aldonic acid.[81] Applying this mechanism, the following would be the mechanism for the oxidative cleavage of RuDP:



Verification of this mechanism must await experiments along kinetic lines.

The Reaction of RuDP with Cyanide (HmDP): This topic is discussed in this section under the Mechanism of Enzyme Action.

The Paper Chromatographic and Electrophoretic Properties of RuDP: Rather than listing the behavior of RuDP towards paper chromatography and paper electrophoresis here, it is convenient to describe it elsewhere along with the chromatographic and electrophoretic properties of 3-PGA, 2-PGA, HmDP and inorganic phosphate. This table is given in this section on the Mechanism of Enzyme Action.

Preparation of Carboxydismutase. Enzyme from Tetragonia expansa: When a crude extract of Tetragonia is fractionated with ammonium sulfate (Method I), a purification of about 3 fold is achieved, Table II. The fraction

TABLE II

Purification of the Crude Extract

by Ammonium Sulfate Precipitation[a]

Material	Protein[b] mg	Specific Activity[c] units per mg	Total Activity[d] units	Purification[e]
Crude extract	800	0.087	70	1
33 to 35% SAS ppt	143	0.25	35	2.9

a) The Crude Extract Method or Method I, see experimental section.

b) Dry weight of solid per 100 g of fresh leaves.

c) Assay III; in each incubation mixture, in μM: $NiCl_2$, 0.2; $NaHC^{14}O_3$, 1.3 at 20 μC; RuDP, 0.08 of organic phosphate; either crude enzyme, 100 μg or partially purified enzyme, 25 μg. Final volume: 0.2 ml pH of incubation mixture: 7.2. Incubation time: 10 min. 1 unit is defined as 1 μM of carbon fixed during 10 minutes incubation at 25° C.

d) The product of mg of protein and the units per mg of specific activity.

e) Activity of the partially purified preparation relative to the crude extract.

precipitating between 33 and 35% saturated ammonium sulfate was chosen because the most active preparation precipitated in this concentration range.[62] Actually Mayaudon found that most of the activity resided in the material which precipitated in 33 and 40% saturated ammonium sulfate, but he chose to define 100% saturated ammonium sulfate as 68.5 g $(NH_4)_2SO_4$ per 100 ml initial volume of solution. If the solubility of ammonium sulfate is taken as 75.4 g per 100 ml,[68] (a criterion set by Dixon[69]), then these percentages become 30 and 36 respectively.

The results of Table II agree with those of Horecker,[6] and Ochoa[7] and Racker[82] who found that ammonium sulfate fractionation of crude extracts of Spinacea oleracea gave a 3-fold purification.

It was desirable to further purify the ammonium sulfate fractionated material using a variety of classical enzyme precipitation techniques. In each case the starting material was the 33 to 35% SAS precipitate and the purpose of the experiments was to test the feasibility of the various treatments for the purification. Table III lists the results of these experiments.

Only those fractions listed in Table III were assayed for carboxydismutase activity except for the experiment on isoelectric precipitation. For example, no effort was made to assay the material precipitated by heat, by ethanol in the range of 0 to 20% and 30 to 100%, or the supernatant liquid of the 90% acetone mixture. It is possible that these fractions have higher specific activity than the starting material (33 to 35% SAS ppt), but other workers have found that the treatments cited in Table III gave further purification of the ammonium sulfate precipitated material derived from Spinacea.[6,7,82] The only exception to this is the acetone treatment where, according to Ochoa,[7] acetone precipitation inactivates the carboxylase system.

TABLE III

The Effect of Various Precipitation Techniques on the Enzymic Activity

Treatment	Fraction Assayed	Recovery of Material %	Relative Specific Activity %
Heat	Supernatant	84[a]	70[c]
Ethanol	20 to 30% EtOH ppt	35[a]	72[c]
Acetone	90% acetone ppt	6[b]	40[d]
Isoelectric	pH 5.6 to 5.4 ppt	49[b]	59[c]

a) Based on dry weight determination relative to the starting material.

b) Based on the Folin-Phenol colorimetric determination relative to the starting material.

c) Percentage of the specific activity, in units per mg of protein, relative to the specific activity of the starting material. These samples were assayed by preincubating the enzyme with Ni^{++} plus $HC^{14}O_3^-$ for 10 minutes at 0° C and after adding RuDP, incubating the complete system for an additional 10 minutes. Full details are given in the experimental section under Assay II.

d) Same as c, except that Assay III was used.

On the other hand, all precipitates and the final supernatant liquid from the pH (isoelectric) precipitation experiment were assayed, but 80% of the recovered enzymic activity and over 60% of the recovered material resided in the fraction which precipitated between pH 5.6 to 5.4. In this study, the pH of the enzyme solution was decreased successively from an original pH of 6.8 to a final pH of 4.5.

In view of the partial inactivation of the carboxylation enzyme by these methods, Method I, the Crude Extract Method, was abandoned in favor of the Acetone Powder Method (Method II); the results of which are shown in Table IV.

Most conspicuous of this fractionation procedure is the 9-fold purification of the final ammonium sulfate precipitate over that of the crude extract. This value, however, must be regarded with some suspicion owing to the recovery of what appears to be more total activity in the acetone powder than in the starting material; namely, 27 units against 23 units, respectively. A more striking difference is found in another experiment where the acetone powder had a total activity of 89 units against 31 units for the crude extract. A point of interest here is that, in this case, 2.5 g of acetone powder was obtained from 100 g of fresh leaves. This is particularly surprising since the same amount of fresh leaves yields about a gram of nondialyzable solid, although, experimentally, at least 6 g of dried solid can be expected from 100 g of Tetragonia leaves when they are lyophilized. Even though the total activity of the sample is the product of its specific activity and its weight, it is unlikely that these two factors contribute largely to the discrepancy between the total activities of the acetone powder and the crude extract. Both of these factors were found to be reliable to better than $\pm$ 5% and hence, some other reasons must be considered. The results can be explained on

TABLE IV

Purification of Carboxydismutase by the Acetone Powder Method[a]

Material	Protein mg	Specific Activity[d] units per mg	Total Activity[e] units	Purification[f]
Crude extract	650[b]	0.035	23	1.0
Acetone powder	400[c]	0.067	27	1.9
1st AS ppt	44[c]	0.24	11	6.9
2nd AS ppt	6.5	0.33	2.1	9.4

a) Method II, see experimental section.

b) Weight determined colorimetrically with the Folin-Phenol reagent per 100 g of fresh leaves.

c) Dry weight of solid per 100 g fresh leaves.

d) Determined by Assay III: in each incubation mixture, in μM: $MgCl_2$, 0.1; tris, 15 at pH 8.4; $NaHC^{14}O_3$, 1.33 at 20 μC; RuDP, ca. 0.04; and enzyme, 30 to 50 μg. Final volume: 0.20 ml. Incubation conditions: 10 minutes at 25° C. 1 unit equals 1 μM of carbon fixed per 10 minutes at 25° C.

e) The product of mg of protein and the units per mg of specific activity.

f) Ratio of the specific activity of the sample to that of the crude extract.

the basis that the crude extract, from the time of its extraction to its assay, has lost a considerable amount of its activity while the acetone powder did not. (The acetone powder, when stored at room temperature for 6 weeks, remained fully active; whereas under such conditions, the crude extract would be completely inactive.) Alternatively, the crude extract may contain an inhibitor which is not precipitated by acetone thus accounting for the higher activity of the acetone powder. Further experiments are necessary for the answers to these questions.

In the two aforementioned methods for the preparation of carboxydismutase, one objectionable property was the intensely brown color of the enzyme solution. Often the enzyme contained a small amount of nucleic acid.[83] On further treatment of the ammonium sulfate fractionated enzyme, such as heat treatment, precipitation with ethanol, acetone or by isoelectric precipitation the brown color of the solution still persisted. In tobacco leaf extracts Wildman's group found that the brown color, which was due to the polyphenoloxidase system, produced a marked change in the properties of the soluble proteins.[84] For example, the pellet obtained after prolonged centrifugation at high speeds was very difficult to redissolve. Furthermore, the intense color often seriously hampered observations of the proteins during the course of the electrophoresis and the ultracentrifugation. When proteins were obtained from extracts which were prepared under 99.99% nitrogen atmosphere, all of these difficulties were eliminated.

It is known that the carboxylation enzyme is present in the water extract of spinach chloroplasts.[85,86] Moreover, this colorless extract contains a large portion of this enzyme and it was found to be

free of nucleic acids.[86] Accordingly, it seems that the best way to prepare this enzyme is to proceed via the chloroplast. Table V gives the results of the purification of the chloroplast extract.

It appears that the Chloroplast Extract Method offers no better purification than the Crude Extract Method, both giving a 3-fold purification. Numerous other experiments, however, have shown that the purification of carboxydismutase could be well over 20-fold. Thus, one experiment gave a chloroplast extract that had a specific activity 15 times that of the crude extract while another experiment gave, upon further fractionation of the chloroplast extract with ammonium sulfate, an additional 2 to 3-fold increase in the specific activity of the sample.

It should be noted that not all of the activity was extracted from the chloroplasts on the first washing with dilute buffer. In the experiment described in Table V, 75% of the total activity was recovered in the first washing. (In two other experiments, over 90% was recovered in this fraction). The remainder of the activity was distributed between the combined 2nd and 3rd washings (20%) and the particulate fraction (4%). The specific activities of these samples were 0.73 and 0.018 units per mg, respectively.

A point of further interest is the behavior of the chloroplast extract when it is fractionated with ammonium sulfate. Normally when a crude extract is fractionated in this manner (Method I), about 94% of the total recovered activity resided in the precipitate obtained between 30 to 36% saturated ammonium sulfate; (bear in mind the difference in criterion adopted in these experiments).[62] The remainder of the activity was located in the 0 to 30% SAS precipitate with less than 0.6% of the activity in the 36 to 45% SAS precipitate. On the other hand, the chloroplast extract when treated similarly, gave the following distribution of recovered activity: 0 to 32% SAS ppt, 3%; 32 to 35% SAS ppt, 40%; and 35 to 63% SAS ppt, 57%. The fact that over half of the recovered activity is precipitated in

TABLE V

The Purification of the Chloroplast Extract[a]

Material	Protein[b] mg	Specific Activity[c] units per mg	Total Activity[d] units	Purification[e]
Crude extract	1,100	0.46	460	1.0
Chloroplast extract (CE)	47	1.12	52	2.4
AS ppt of CE	26	1.43	40	3.1

a) The Chloroplast Extract Method or Method III, see experimental section.

b) Weight per 100 g of fresh leaves, determined colorimetrically with the Folin-Phenol reagent.

c) Determined by Assay III; in each incubation mixture, in μM: $MgCl_2$, 2; tris, 15 at pH 8.4; $NaHC^{14}O_3$, 1.35 at 20 μC; RuDP, 0.068; and enzyme, 24 to 30 μg. Final volume: 200 μl. Incubation conditions: 10 minutes at 25° C.

d) The product of the mg of protein and the units per mg, specific activity.

e) Ratio of the specific activity of the sample to that of the crude extract.

the range between 35 to 65% SAS indicates that the carboxydismutase derived from chloroplast extract is remarkably different from that of the crude extract. (The most active fraction was the 32 to 35% SAS ppt, however; the specific activity of this fraction was ca. 1.5 times that of the 35 to 63% SAS ppt.) More is discussed about this point later in this section under Homogeneity of Various Carboxydismutase Preparations.

The somewhat violent conditions in which leaves were ground; namely in a blender, would lead to an expectedly low yield of intact chloroplasts. It is therefore surprising that the yield of enzyme after the first ammonium sulfate precipitation is more or less comparable to the yield of the Crude Extract Method or that of the Acetone Powder Method. Thus, on the average, the yield per 100 g of fresh leaves after one ammonium sulfate precipitation was 110 mg, 50 mg, and 30 mg for the Crude Extract Method, the Acetone Powder Method and the Chloroplast Extract Method, respectively.

Many different conditions have been tried to increase the yield of the final ammonium sulfate precipitate. Since the major obstacle is the yield of intact chloroplasts, a variety of conditions were used to rupture the leaf cells and to prepare the chloroplasts. These are outlined below:

1. Leaves were ground with a mortar and pestle using sand or no sand.
2. Centrifugation speeds were varied for the isolation of the chloroplasts.
3. Leaves were ground in a blender for different time periods.
4. Leaves frozen in liquid nitrogen were ground in a blender at dry ice temperatures.
5. Chloroplasts were prepared by the method of Commoner, et al.[87] This method separates the chloroplasts from the rest of the debris and fragments by floating the chloroplasts in a hypertonic sugar solution.

6. Chloroplasts were prepared in isotonic salt solution (0.35 M NaCl) instead of sugar solution.

The results of these experiments indicate that the optimum conditions for maximum yield of protein in the chloroplast extract is, in fact, Method III. A full account of these experiments are described elsewhere.[88]

Carboxydismutase from Chlorella pyrenoidosa: The first experiments carried out on the carboxylation enzyme system containing RuDP and radioactive bicarbonate, used a preparation derived from the algae, Chlorella.[25] In these studies the crude enzyme was extracted from the algae by first rupturing them sonically in phosphate buffer and then removing the whole cells by centrifugation, thus resulting in a cell-free system. The yield of material was generally small and consequently, experiments were performed to determine the optimum condition for the large scale preparation of crude enzyme. Although exposure of the algae for long periods to an audio frequency oscillatory field is desirable to obtain maximum yield of protein in the extract, the activity of the enzyme in question is destroyed under such conditions. Hence when a cell-free enzyme system was obtained after varying lengths of sonication time, the amount of protein present in the extract ranged from 4.6 to 36 mg per g of cells after 2.5 to 30 minutes sonication, respectively. (These values are valid only for this particular oscillator since others of this type are sufficiently different to warrant determining their individual rupturing capacities.) The specific activity of the preparations varied from 0.055 units per mg protein after 2.5 minutes of oscillation to a maximum of 0.39 units per mg at 10 minutes (the yield at this juncture was 16 mg per g of algae) and then decreased to 0.27 units per mg at 30 minutes. It was decided to take a loss in the specific activity in favor of the recovery of the total activity (compare, for example, the total activity of 62 units for the 10 minutes oscillation sample with 97 units for the 30 minutes sample).

Thus, in the subsequent large scale preparation of the crude Chlorella extract, a period of 30 minutes was generally employed for the disruption of the algal cells.

The preparation of large amounts of crude enzyme of Chlorella, then permitted the uninterrupted studies of the carboxydismutase system during the winter months when the other source material, New Zealand spinach, was out of season. To show that the algal carboxylase is similar to that of Tetragonia, the behavior of the former to ammonium sulfate precipitation was studied. The results of this treatment are itemized in Table VI.

Nearly 91% of the recovered activity resided in the 28 to 41% SAS precipitate; this compared well with the result of Mayaudon[62] who found that 94% of the recovered carboxydismutase activity from Tetragonia leaves was located in the 30 to 36% SAS precipitate. From this standpoint, therefore, the enzyme extracted from these two sources are alike. One obvious difference, however, is the color of the crude enzyme preparation as well as the more purified material; that is the extracts from Chlorella are almost always colorless while those from Tetragonia are always dark brown. This suggests the absence of the polyphenoloxidase system in Chlorella, or at least the lack of one component of this enzyme system in these cells.

One disturbing aspect of the results shown in Table VI is the apparent recovery of total activity greater than that of the starting material (the crude extract). The recovered total activity is 0.89 units whereas the total activity of starting material is 0.48 units. The discrepancy can be ascribed to the loss of activity of the crude extract from the time of the rupture of the algal cells to the time of the assay. The loss is probably due to the action of some proteolytic enzyme present in the crude extract. Although the ammonium sulfate fractionated material also required

TABLE VI

Ammonium Sulfate Fractionation of the Crude Chlorella Extract

Material	Protein[a] mg	Specific Activity[b] units per mg	Total Activity[c] units	Purification[d]
Crude extract	124	0.0039	0.48	1.0
0 to 14% SAS ppt	2.5	0.00	0.00	0.0
14 to 28% SAS ppt	15	0.0056	0.08	1.4
28 to 41% SAS ppt	35	0.023	0.81	5.9
41 to 83% SAS ppt	50	0.00	0.00	0.0

a) Dry weight of nondialyzable solid.

b) Determined by Assay III; in each incubation mixture, in μM: $NiCl_2$, 0.2; HCl, 0.4; $NaHC^{14}O_3$, 1.3 at 20 μC; RuDP, ca. 0.05 (based on orcinol determination[63]); and enzyme, 25 μg. Final volume: 200 μl. Incubation conditions: pH 7.5, 10 minutes at 25° C.

c) The product of the mg of protein and the units per mg of the specific activity.

d) Ratio of the specific activity of the sample to that of the crude extract.

82

the same length of time for the preparation as the crude extract, it is probably during the course of the fractionation that these proteases were separated from the carboxylation enzyme and therefore escaped the degradative action. If the specific activity of the crude extract is recalculated on the basis of recovered total activity and recovered protein, purification then becomes 2.7 times, which is in good agreement with the results obtained for Method I (cf. Preparation of Carboxydismutase, Enzyme from Tetragonia expansa, in this section).

With the accumulation of large supplies of the crude extract of Chlorella, it was possible to explore the purification of the enzyme with various techniques. One such technique employed the method of fractional solution of the protein mixture using an apparatus similar to that of Zahl and Stahl.[70] The method is based on the following principle: After a mixture of proteins is completely precipitated in 90% saturated ammonium sulfate solution, the precipitate is washed with an ammonium sulfate solution of gradually decreasing concentration. (The latter is achieved by using a gradient elution apparatus.) The washings are collected in a fraction collector and each fraction is then assayed for enzyme activity as well as for protein concentration.

The details of this method, as applied to the crude extract of Chlorella, are given in the experimental section under Enzyme from Chlorella pyrenoidosa. Results of a preliminary experiment, using a glass wool plug to separate the washings from the protein precipitate, showed 5 peaks (uv absorption at 280 mμ), although there were some indications of leakage of protein through the plug owing to the partial solubility of the proteins in a relatively large volume of liquid. The method was then modified, a glass bead column or an inert ion exchange column being used as support medium and substituted for the glass wool plug. The results of this "ammonium

sulfate column chromatography" are shown in Fig. 15.

An elution pattern with five distinct peaks is evident. Assay of the peak tubes showed that the carboxydismutase activity was located in peaks 4 and 5, with the specific activity of peak 4 being three times that of peak 5. The specific activity of the starting material, however, was 1.3 times that of peak 4. Nevertheless, it appears that the method of fractional solution could serve as an alternate method for the purification of carboxydismutase. It should be pointed out that most of the activity (peak 4) was soluble in ammonium sulfate at concentrations less than 0.21 of saturation.

There are still some indications of a leakage of protein through the column, but to a much lesser degree than the experiment employing the glass wool plug. The leakage is apparent when a line is drawn so as to connect the minimums between peaks 1 and 2, 2 and 3, 4 and 5, and the trailing of material after peak 5.

So far ammonium sulfate, ethanol, acetone, heat treatment, and isoelectric precipitation have been applied to the purification of the carboxylation enzyme from Tetragonia, but it is often possible to remove undesirable proteins by using heavy metals as precipitants.[57] Lead ion was used, for example, in the purification of the Q-enzyme from potato.[71] The identical procedure was applied to the purification of the crude extract of Chlorella, the material precipitated by lead being retained for study. The results show that over 60% of the material was precipitated. When this precipitate was solubilized by removing the lead ion as lead carbonate, the resulting solution had a specific activity (Assay I) which was only 13% that of the starting material. The fault of this method may be attributed to one or a combination of many factors, among them being 1) the incomplete removal of lead ion, 2) denaturation caused by foaming during the streaming of CO_2 through the solution, 3)

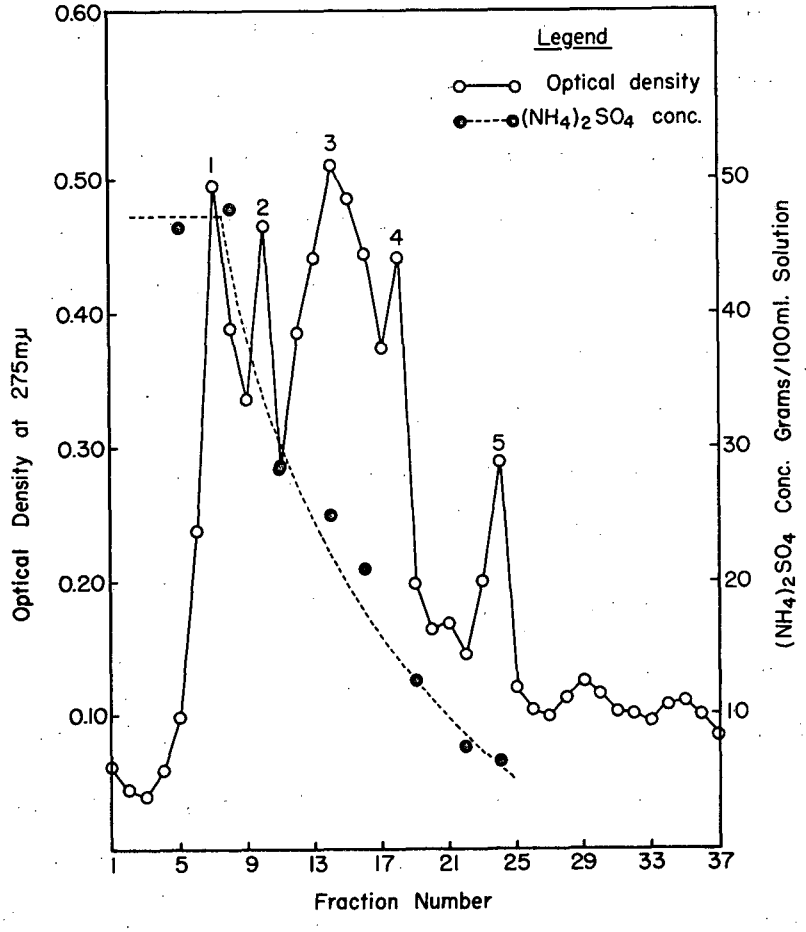


Fig. 15. Elution pattern of a crude extract of Chlorella using the method of fractional solution. One optical density unit equals 0.52 mg of protein per ml of solution. The peak tubes were assayed using Assay I. The incubation mixture contained, in μM: $NiCl_2$, 0.2; HCl, 0.4; $NaHC^{14}O_3$, 1.3 at 20 μC; enzyme, 25 μg (based on uv absorption at 275 mμ); and RuDP, 0.05 (based on pentose determination by the orcinol color). Final volume: 200 μl. Incubation conditions: pH 7.1, 30 minutes at 25°C. Column: 0.9 cm diameter X 20 cm length, Amberlite XE-97, NH_4^+ form. The exponential gradient elution apparatus: 50 ml 100% SAS in the mixing vessel and 125 ml water in the reservoir.

denaturation caused by the presence of added n-octanol used to reduce foaming, and 4) improper conditions for the precipitation of the carboxydismutase activity. (The last factor takes into account the possibility that the carboxylation activity might actually be in the supernatant liquid of the lead treated material.)

Homogeneity of Various Carboxydismutase Preparations. Further purification of carboxydismutase derived from Tetragonia greater than 10 to 20-fold proved to be unsuccessful (as in the case of the purification of extracts of Spinacea).[6] Nevertheless, it was of interest to investigate the purity of the preparations obtained by the various methods in order to determine any new course of action in the purification of this enzyme. The studies were approached from three sides employing physical chemical, organic chemical and biochemical techniques, the details of which are already indicated in the experimental section.

Physical Chemical Studies: Electrophoretic studies on the enzyme prepared by Method I, carried out in phosphate buffer at pH 6.7, $\mu = 0.2$ and at pH 6.0, $\mu = 0.1$; gave a single symmetrical peak in both cases. Moreover, the same result was obtained through ultracentrifugal analysis, confirming the findings of Mayaudon.[83] Actually, as indicated previously in the discussion on the preparation of carboxydismutase from chloroplasts, these observations were hindered because of the dark brown color of the enzyme solution. Analyses of the schlieren patterns of both the electrophoresis and the ultracentrifugation were therefore made with considerable difficulty. One other fact is available from the electrophoretic data; the mobilities suggest that the isoelectric point of the enzyme is in the region of 5. Consistent with this value are the results of the pH precipitation experiment where the bulk of the enzyme activity was found in the fraction precipitating between pH 5.6 and 5.4.

From the above data it appears that further purification of the carboxylation enzyme is hardly necessary. Even the starch electrophoresis data, in 0.1 M phosphate buffer at pH 6.9, showed no separation of the activity from the protein.[83] When paper electrophoresis was performed, however, on this ammonium sulfate fractionated enzyme; in barbital buffer at pH 8.6, $\mu = 0.05$, the activity clearly does not coincide with the bulk of the protein, see Fig. 16. It was not possible to calculate the specific activity of the fraction containing the maximum activity (+15 to +16 cm from the origin towards the anode) because the protein concentration was near the threshold of the colorimetric determination. The possibility that an artifact (owing to the denaturation of the protein) can arise from this type of experiment is not eliminated. For example, Horecker found that, in the free electrophoresis of spinach carboxylase conducted at pH 8.6, two schlieren peaks were observed near the end of the experiment, whereas when the electrophoresis was conducted at pH 7.7, only one schlieren peak was obtained. None-the-less, the method may be fruitful for further fractionation of carboxydismutase. Further experiments are required to fully evaluate this method.

The preparation obtained by the Chloroplast Extract Method was also analyzed ultracentrifugally. Since the preparation is colorless, the migration of the protein under the centrifugal field was easily observed. A picture of the schlieren pattern of the ultracentrifugal run is shown in Fig. 17. At least two components are evident, the faster one making up approximately 65% of the total area under the schlieren curve. Unless a study of the sedimentation rate as a function of protein concentration is performed, no correlation of the area under the curve to protein concentration can be made since anomalous boundary effects often arise during the ultracentrifugation of protein mixtures.[89] The schlieren diagram, however,

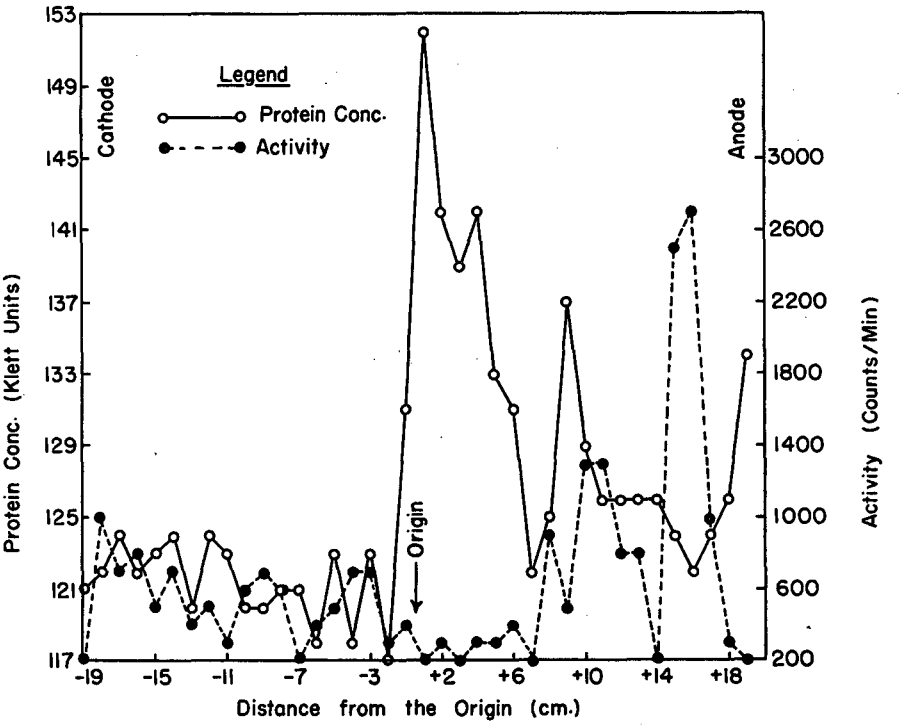


Fig. 16. Paper electrophoresis of an enzyme preparation purified by the crude extract method. Paper: unwashed Whatman No. 4. Temperature during the run: 3°C. Buffer: pH 8.6, μ = 0.05, barbital. Potential applied: 760 to 650 volts. Current: 10 ma. Duration of electrophoresis: 6 hours. Protein concentration: Determined colorimetrically with the Folin-Phenol reagent. Klett units are uncorrected for the blank reading (average value of 121). One Klett unit equals 0.6 μg of protein per 0.5 ml after correction for blank. Carboxydismutase assay: In each incubation mixture, in μM; $NaHC^{14}O_3$, 0.69 at 10.3 μC; tris, 7.7 at pH 8.4; $MgCl_2$, 0.052; RuDP, 0.0085 and enzyme, 50 μl. Incubation period; 21 hours at room temperature. Final volume: 90 μl. (The starting material, when assayed under these conditions gave 6,500 cpm per 5 μg protein.)

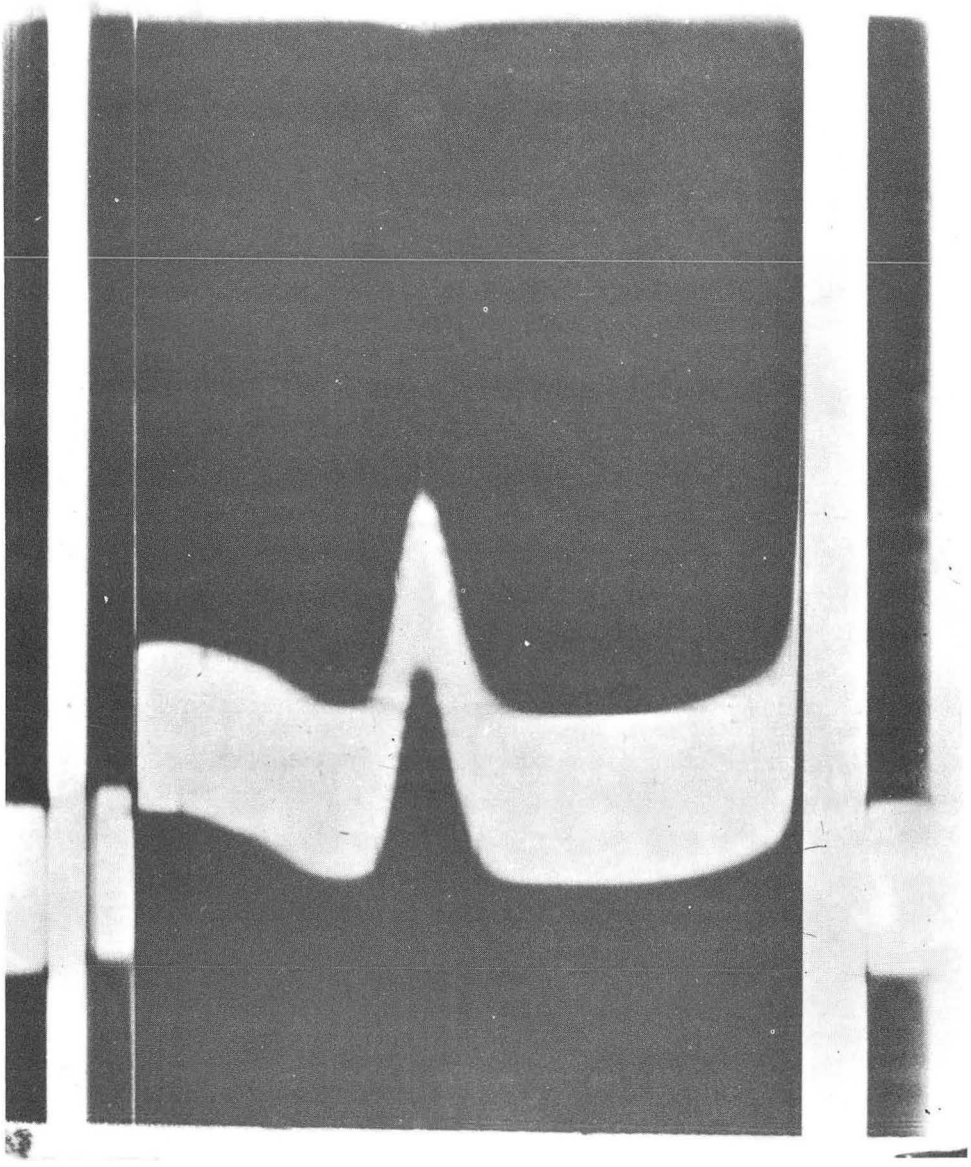


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Fig. 17. Ultracentrifuge Schlieren diagram of an enzyme fraction prepared by the Chloroplast extract method. Rotor speed: 30,531 rpm. Buffer: pH 6.8, 0.05 M potassium phosphate. Time: 48 minutes. Protein concentration: Ca. 5 mg per ml. Sedimentation is from left to right.

is identical to that obtained by Mayaudon in his studies with the enzyme prepared from Tetragonia by Method I.[83] Judging from the data of Mayaudon and Horecker,[6] it appears that the main peak is associated with the activity of the enzyme. The sedimentation coefficient, $S_{w,20}$, calculated from the data of this experiment; i.e., pH 8.4, 0.01 M tris; in 0.1 M NaCl; protein concentration, 11 mg per ml; is about 18 Svedberg units, in agreement with other investigators.[6,83,86] The diffusion coefficient, D, as measured by the rate of spreading of the boundary, is about 1.6×10^{-7} sq. cm per second. This number must be regarded as tentative because a small variation of the sedimentation coefficient with protein concentration may cause a large change in D. For example, gross errors over 50% may arise from artificial sharpening of the boundary.[90] This problem is also easily settled by determining the diffusion rate as a function of the concentration of protein. If the true D. is 5.5×10^{-7} sq. cm per second,[6] the molecular weight is estimated to be around 300,000 using the $S_{w,20}$ obtained in these experiments and assuming a partial specific volume of 0.75 ml per g of protein at 20° C. This molecular weight strongly suggests that this protein is either related to, or associated with the Fraction I protein found in soluble extracts of higher plant leaves when they are ruptured mechanically.[91] This subject is discussed fully by other workers.[83,86]

It was found that carboxydismutase prepared by ammonium sulfate fractionation of the crude extract of Tetragonia is a nucleoprotein and that the activity of this enzyme is not lost after the removal of the nucleic acid moiety.[83] In support of these findings are the results of the spectrophotometric studies on the various fractions of the cellular extracts, Fig. 18 through 20. These absorption spectra clearly indicate that as the enzyme becomes more purified (from the crude extract to the final ammonium sulfate-fractionated chloroplast extract), the nucleic acid content qualitatively decreases based on the shift in wavelength of the

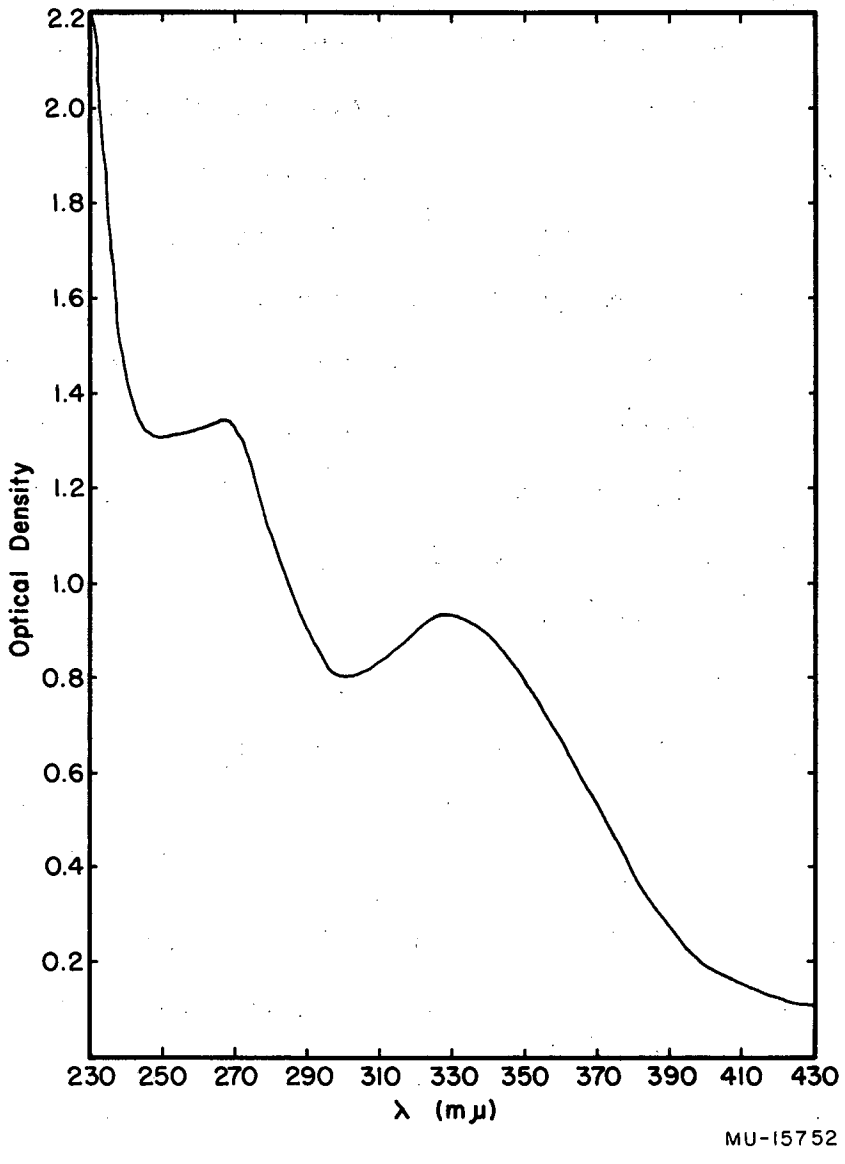

Fig. 18. Absorption spectrum of a crude extract of Tetragonia expansa. Concentration: 0.12 mg (dry weight basis) of solid per ml of pH 7, 10^{-4} M NaOAc. The crude extract was not dialyzed, but used directly for spectral studies. The dialyzed fraction gave optical densities at the absorption maximums about one-half those of the undialyzed material. Optical densities were measured in a cell of 1 cm path length.

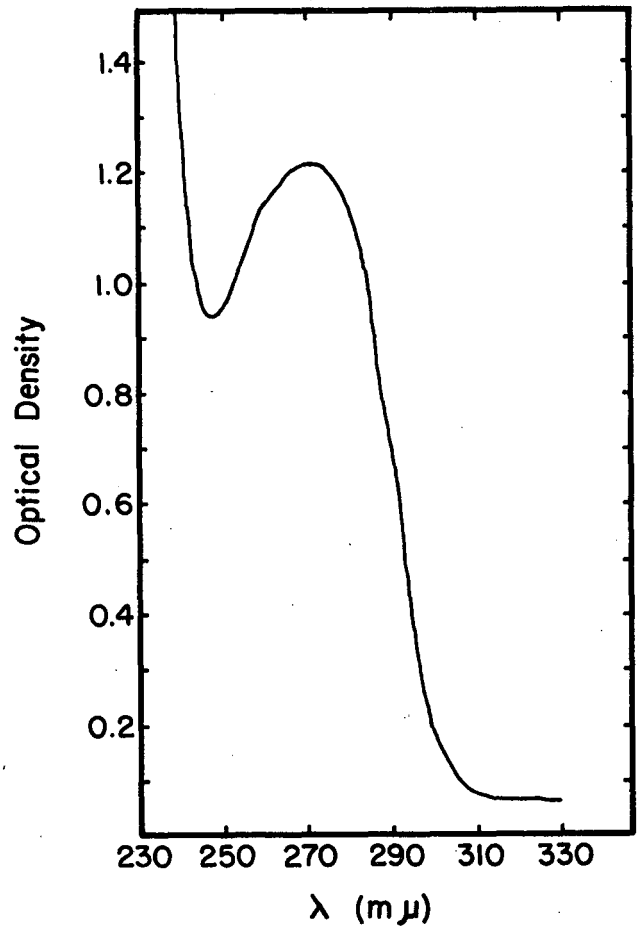

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Fig. 19. Absorption spectrum of a chloroplast extract of Tetragonia expansa. Concentration: 0.63 mg (determined colorimetrically with the Folin-Phenol reagent) per ml of 0.0125 M, pH 6.8 potassium phosphate buffer. Protein solution was undialyzed. Optical densities were measured in a 1 cm thick cell.

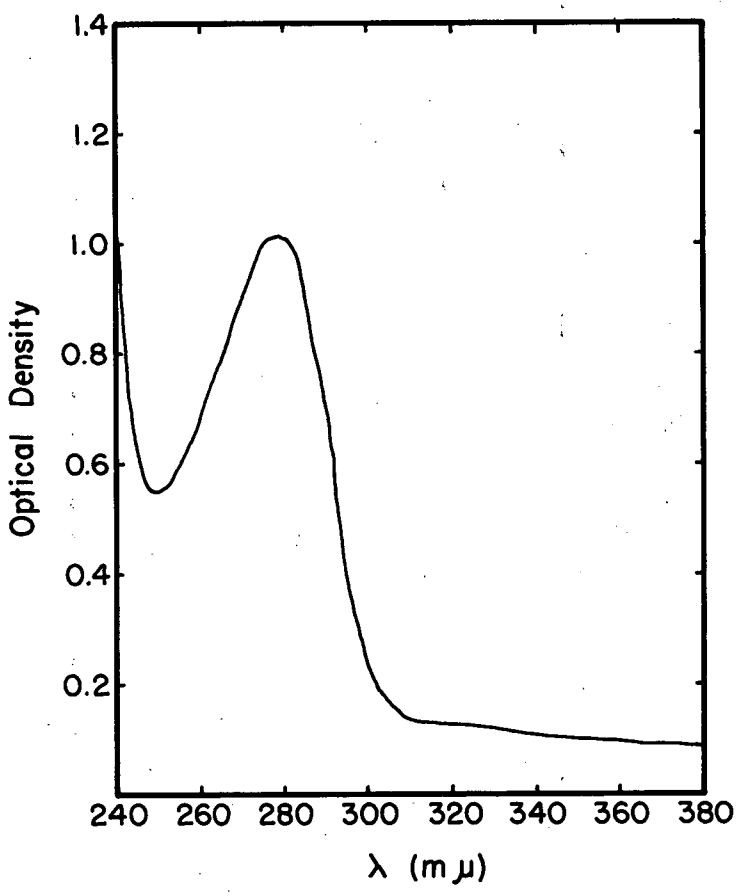

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Fig. 20. Absorption spectrum of an ammonium sulfate-fractionated chloroplast extract of Tetragonia expansa. Protein concentration: 0.62 mg (determined colorimetrically with the Folin-Phenol reagent) per ml of 0.01 M, pH 6.8 potassium phosphate buffer. Protein solution was undialyzed. Optical densities were measured in a 1 cm cell.

absorption maximum from 267 mμ to 277 mμ. A quantitative measure of the contamination of nucleic acids is indicated by the ratio of the optical density at 280 mμ to that at 260 mμ. When the ratio is greater than 1.6, the preparation is considered to be free of nucleic acids.[57] The ratios of optical densities are 0.83, 0.98 and 1.50 for the crude extract, the chloroplast extract and the ammonium sulfate-fractionated chloroplast extract of Tetragonia, respectively. These findings agree with the results obtained from studies on enzyme preparations from Spinacea; namely, that the carboxylation enzyme (and Fraction I) is free of nucleic acids.[86]

Note further that a second absorption maximum for the crude extract is present at 330 mμ, Fig. 18. This peak accounts for the brownish-yellow appearance of this solution. Actually, the more concentrated solution is brown, as are all fractions purified from the crude extract; for example, the ammonium sulfate precipitate of the acetone powder. In extracts of tobacco leaves this brown color is attributed to the polyphenoloxidase system.[84] Compatible with this notion are the spectra of some plant polyphenols which have absorption maximums in the region of 320 to 370 mμ,[92-94] although these polyphenols are not necessarily involved in the crude extract of Tetragonia.

So far three different instruments have been used to study the homogeneity of the enzyme preparations: electrophoresis, ultracentrifuge and spectrophotometer. A fourth approach was to study the solubility of the enzyme in ammonium sulfate, similar to the fractional solution technique applied for the purification of the crude extracts of Chlorella. In this particular case, however, a column packed with glass bead as a support medium was used and elution was achieved with an apparatus which provided a linearly decreasing concentration gradient of ammonium sulfate. Using an enzyme fraction prepared by the Acetone Powder Method (Method II), a separation of material from the enzymic activity was obtained, Fig. 21.

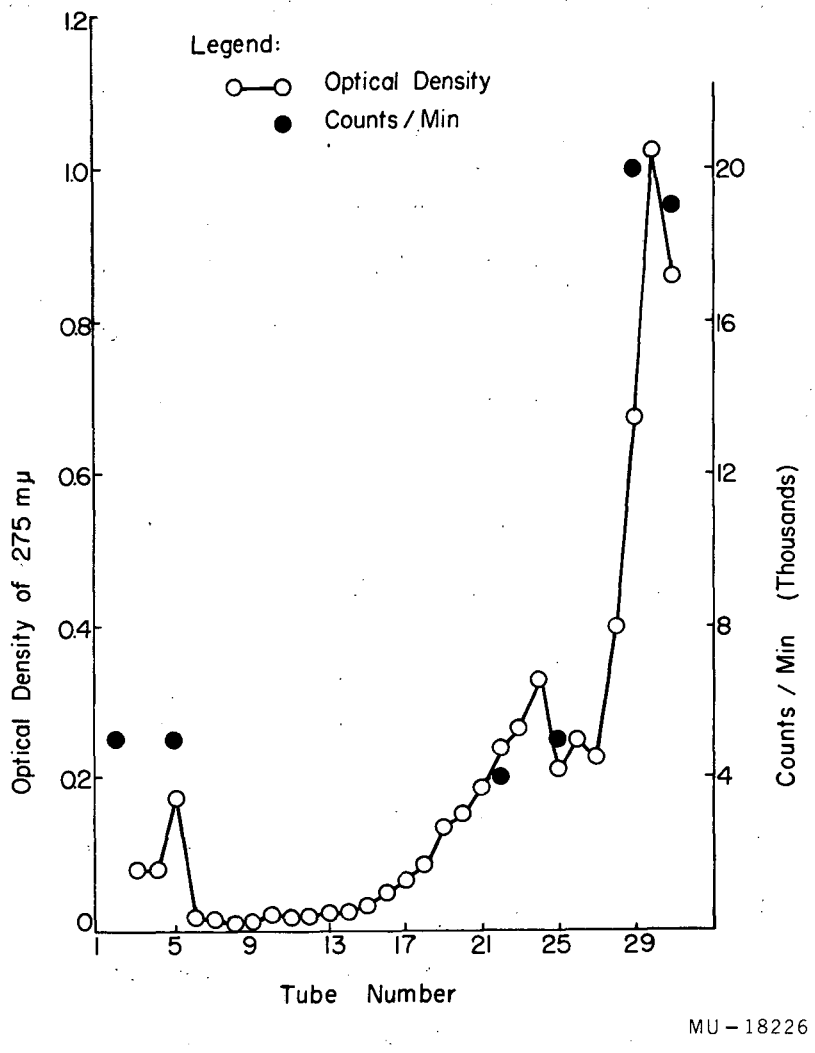


Fig. 21. Fractional solution of carboxydismutase from Tetragonia. Enzyme: 2nd AS ppt from the acetone powder method. Column: Packed with 0.1 mm diameter glass beads, 1 cm diameter by 20 cm length. Eluant: 93% SAS at tube no. 4, decreasing linearly to 0% SAS at tube no. 31. Optical density: 1 unit equals 0.43 mg protein per ml. Average volume per tube: 3.4 ml. Counts per minute: For 10 μg protein per 30 minutes incubation at 25°C. Incubation mixture contained, in μM: $NaHC^{14}O_3$, 1.35 at 20 μC; $MgCl_2$, 2; tris, 15 at pH 8.3; RuDP, ca. 0.04; and enzyme, ca. 10 μg (based on O. D. at 275 mμ). Final volume: 200 μl.

Whether this result is due to an artifact of the experiment such as the denaturation of the protein or, is in fact, real remains to be tested by further work. A doubt is cast on the results because of the higher specific activity of the control which was kept as a precipitate in the presence of saturated ammonium sulfate. In contrast to this, a lower specific activity resulted from a control where the enzyme was kept in solution for the duration of the experiment. For example, for the control in precipitate form and the control in solution form, values of 5,300 cpm and 800 cpm, respectively, were obtained. Thus, tube number 29 with an activity of 2,000 cpm could either represent an inactivation or a purification of about 2.5-fold, depending on the reference chosen.

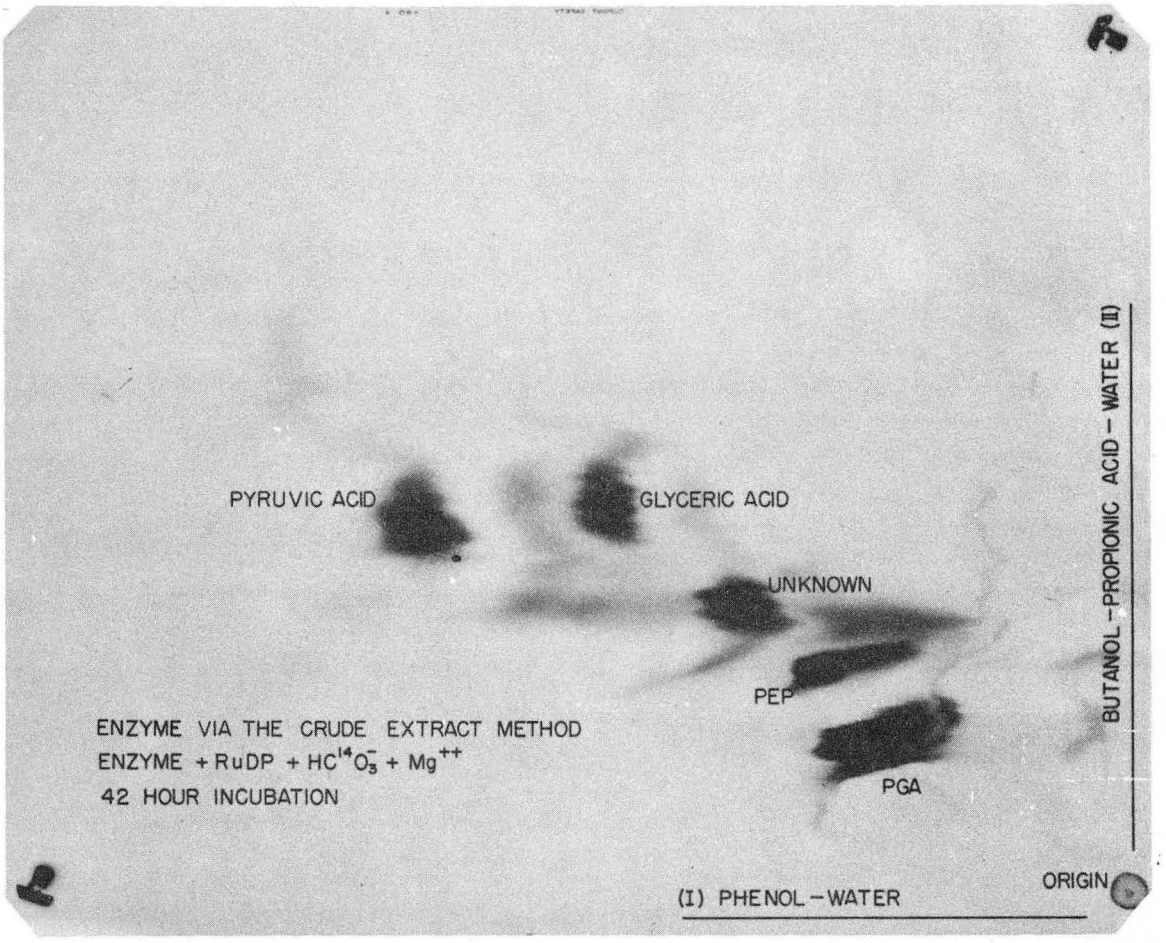
Often it is convenient to purify a protein by following the increase of its N-terminal amino acid content in the hope that its biological activity runs parallel to this increase. Such an experiment was attempted with the various preparations extracted from Tetragonia. Preliminary experiments employing the fluorodinitrobenzene method of Sanger[95] was applied to an ammonium sulfate fractionated crude extract (Method I). The results suggested that glutamic acid was the N-terminal amino acid and hence, was associated with the bulk of the protein along with the enzymic activity. Thus, after the usual treatment of the protein mixture with FDNB followed by quantitative paper chromatography, the results showed that besides a very large spot of 2,4-dinitrophenol (quantitative determination of this spot is not possible because of the volatility of this substance), a major spot of DNP-glutamic acid corresponding to ca. 1 μM per μM of protein of molecular weight 300,000 and minor spots of DNP-alanine, -glycine, -serine, and -threonine were detected. Later experiments, however, with the same technique applied to the acetone powder and the ammonium sulfate-purified chloroplast extract of Tetragonia,

96

showed that glutamic acid was not, in fact, related to the activity of the enzyme. This is evident from the relatively large content of DNP glutamic acid (0.32 μM per 100 mg protein) for the former while the latter had only 0.15 μM of DNP-glutamic acid per 100 mg protein. It is possible that the N-terminal amino acid is either glycine, proline, or cystine. Should this be the case, then the usual treatment would result in the destruction of these DNP-amino acid and concomitantly, yield dinitrophenol plus the amino acid.[72] The fact that a much larger quantity of dinitrophenol was obtained from the treated AS ppt of chloroplast extract then from the treated acetone powder is consistent with this view. At present no conclusion can be drawn from these experiments. Resolution of this problem will have to await the application of the Edman method for the determination of N-terminal amino acid.[96]

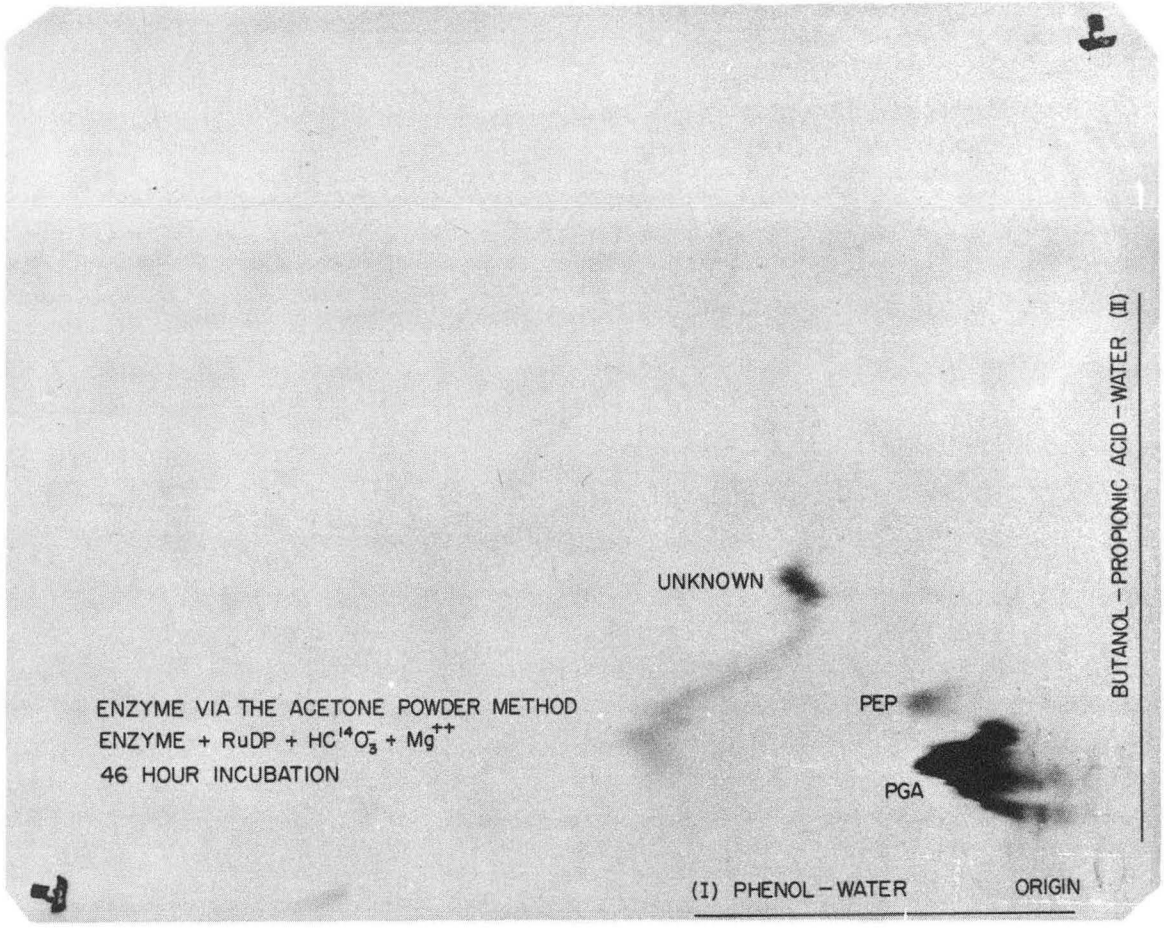
An extremely sensitive method for the detection of contaminating enzymes is available simply by allowing the standard assay mixture to incubate for several days instead of the usual 10 minutes. In this way, the product (PGA) and any radioactive bicarbonate are converted to other products if there are minute amounts of other enzymes present. Comparison of the radioautographs, Fig. 22 to Fig. 24 points clearly to the fact that, the ammonium sulfate-fractionated chloroplasts extract of Tetragonia contains the least amount of contaminating enzymes. Even in this preparation the presence of a small amount of enolase (and presumably phosphoglyceric acid mutase) is evident from the appearance of a small spot of phosphoenolpyruvic acid (PEP) after 32 hours incubation.

The acetone powder, twice precipitated with ammonium sulfate, produced after 46 hours incubation with RuDP and labeled bicarbonate, an additional compound, Fig. 23. This unknown compound was also obtained in major amounts when the ammonium sulfate-fractionated crude extract (Method I)



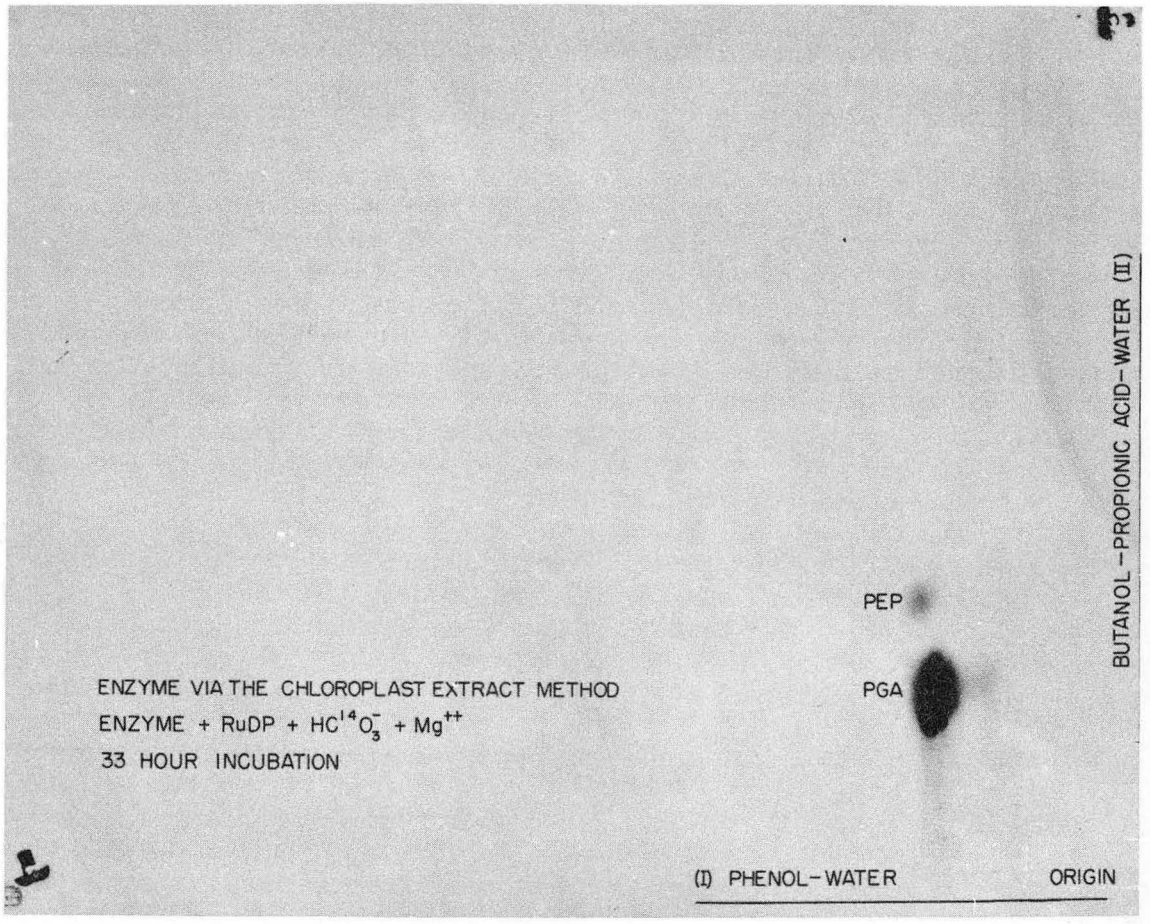
ZN-2578

Fig. 22. Products formed from 42 hours of incubation of ammonium sulfate-fractionated crude extract with RuDP and radioactive bicarbonate. Assay condition: Assay II at pH 7.0. In each incubation mixture, in μM: $NaHC^{14}O_3$, 1.8 at 20 μC; HCl, 0.8; $MgCl_2$, 0.2; enzyme (33 to 35% SAS ppt, Method I), 48 μg; and RuDP, ca. 0.08 of organic phosphate. Final volume: 200 μl (layered with toluene). Preincubation conditions: Enzyme with Mg^{++}, $HC^{14}O^-_3$ for 10 minutes at 0°C. Incubation conditions: 42 hours at room temperature. Amount on chromatogram: 180 μl of the incubation mixture on the origin.



ZN-2579

Fig. 23. Product of the action of an ammonium sulfate-fractionated acetone powder on RuDP and radioactive bicarbonate. Assay conditions: Assay III. In each incubation mixture, in μM: tris, 15 at pH 8.4; $MgCl_2$, 0.1; $NaHC^{14}O_3$, 1.33 at 20 μC; RuDP, ca. 0.05; and enzyme (28 to 37% SAS ppt, Method II) 59 μg. Final volume: 200 μl (layered with toluene). Incubation conditions: 46 hours at room temperature. Amount on the origin of the chromatogram: one-fifth of the total volume.



ZN-2580

Fig. 24. Products of the action of an ammonium sulfate-fractionated chloroplast extract on RuDP and radioactive bicarbonate. Assay condition: Assay III. In each incubation mixture, in μM: $MgCl_2$; tris, 15 at pH 8.3; $NaHC^{14}O_3$, 1.3 at 20 μC; RuDP, ca. 0.02; and enzyme (32 to 46% SAS ppt, Method III), 25 μg. Final volume: 200 μl and then layered with toluene. Incubation conditions: 33 hours at 25°C. On the origin of the chromatogram: 10 μl.

100

was tested, Fig. 22. On elution and rechromatography of the radioactive spot (the unknown compound), a minor labeled spot coincided with carrier aspartic acid, but the bulk of the radioactivity moved faster than aspartic acid in the butanol-propionic acid-water direction although the R_f of the major spot was the same as aspartic acid in the phenol-water direction. If the unknown compound was treated with KBH_4, the product did not cochromatograph with carrier malate. In fact, the R_f of the major radioactive spot was greater than aspartic acid in both solvent directions. (The R_f in the butanol-propionic acid-water direction was the same as the untreated spot.) It appears, therefore, that the compound was altered in some unknown manner after it was eluted and rechromatographed.

That PGA or PEP is the precursor of this unknown spot is indicated by the results in Fig. 23. Indeed, when PGA-C^{14} is incubated with the ammonium sulfate precipitated crude extract in the presence of unlabeled bicarbonate, the unknown spot was formed whereas when radioactive glyceric acid was added in place of PGA-C^{14}, the latter remained unchanged. One other fact is significant; that is, the unknown spot is not a phosphate ester as evident from the unaltered R_f of this compound when the original carboxydismutase incubation mixture was treated with acid phosphatase (Polidase-S). In this case, glyceric acid and pyruvic acid were produced, however.

In addition to the three spots obtained by incubating the enzyme prepared according to the Acetone Powder Method with RuDP and labeled bicarbonate, the enzyme prepared by the Crude Extract Method yielded a number of other spots, among them being a few minor unknown spots along with glyceric acid and pyruvic acid, Fig. 24. The presence of these compounds suggests that there is present in the incubation mixture (and therefore the enzyme system) some phosphatase activity. The indications are that

two types of phosphatase are operating, one in the acid region and the other in the alkaline region. By incubating the standard assay mixture, pH 8.4, for 10 minutes at 25° C in the usual manner and then merely adjusting the pH to ca. 9 with ammonium hydroxide, one can convert all of the PGA-C^{14} initially formed, to glyceric acid after an additional hour standing (determined electrophoretically). If the pH of the incubation mixture was changed from 8.4 to pH 4.0, after an initial 10 minutes of incubation at 25° C, a similar result was obtained. Consider, for example, two carboxydismutase incubation mixtures in which the enzyme (Crude Extract Method) was incubated for 82 minutes. To both mixtures was added acetic acid in order to stop the reaction, one to a final calculated pH of 2.7 and the other to a final calculated pH of 4.0. After standing an additional hour, the ratio of glyceric acid to the sum of glyceric acid plus phosphoglyceric acid was 0.07 for the former reaction mixture, while for the latter mixture the ratio was 0.56.

An experiment of another type can also shed light on the presence of other enzymes, but the observations were complicated by the innumerable spots arising from the decomposition of RuDP (see discussion on the stability of RuDP in a previous part of this section). The experiment was carried out as follows: RuDP-C^{14} was incubated with the enzyme preparation prepared by Method III (the Chloroplast Extract Method) in the absence of bicarbonate, but in the presence of the activating metal ion plus the buffer (tris). After 14 hours incubation the pattern, obtained after chromatography in the usual solvent systems, was quantitatively similar to that obtained from a reaction mixture containing no enzyme. This indicates that this carboxydismutase preparation is relatively free of enzymes capable of converting RuDP to compounds other than PGA. (Naturally, when RuDP-C^{14} was incubated with unlabeled bicarbonate, the

enzyme, Mg^{++} and tris buffer, PGA was formed along with a small amount of phosphoenolpyruvic acid.)

The Carboxydismutase Assay. Stoichiometry: In the INTRODUCTION it was noted that the stoichiometry of the carboxydismutase reaction was established by two investigators.[6,7] These workers found that from one mole of RuDP and one mole of bicarbonate, in the presence of the carboxylation enzyme, two moles of PGA plus one mole of hydrogen ion were formed. In the experiments described in this report, the activity of the enzyme was measured on the basis of the amount of acid-stable radioactivity fixed in an incubation mixture per unit time per unit weight of enzyme preparation. Hence, this assay assumed no particular stoichiometry. When it is necessary, however, to calculate the amount of product formed or the quantity of substrate consumed, the stoichiometry established by the above-mentioned investigators was assumed to apply to this case. It is at least definite that the product of the carboxylation reaction catalyzed by Tetragonia extracts, after 5 or 10 minutes incubation, is 3-phosphoglyceric acid. The R_f of this product in the usual two-dimensional chromatography as well as the molybdate solvent system corresponded to that of authentic 3-PGA. (After longer periods of incubation or using cruder enzyme systems, some 2-PGA is formed. This fact eliminated 2-PGA as the initial product of the carboxylation reaction.) Paper electrophoresis of this substance confirmed the fact that this substance has the same mobility as PGA. Chromatography of the radioactive material with the authentic 3-PGA gave exact coincidence. Further evidence was furnished when the labeled compound was enzymically dephosphorylated and found to cochromatograph exactly with glyceric acid. Finally, the radioactive product, purified by chromatography in the usual two-dimensional system, served as a substrate for the highly purified phosphoglyceric acid mutase.[97]

The selection of an incubation time of 5 or 10 minutes was because of two reasons. First, a high level of C^{14} was fixed without any sacrifice in the precision of measuring the incubation time. Second, the fixation during this time period fell within the linear portion of the fixation vs incubation time curve, when the enzyme was preincubated with the metal ion and HCO_3^-, Fig. 25. Although approximately 25% of the RuDP was consumed after 10 minutes incubation, the rate of fixation may be considered as essentially similar to the initial rate.

When the incubation was carried out under non-preincubated conditions (Assay III), a curve of fixation vs incubation time with a lag period was observed, Fig. 26. By extrapolating the linear portion of the curve to zero counts per minute, one obtains a lag period of about 5 minutes. This rather large period appears to be due to two factors: 1) the preincubation effect of the enzyme with the metal ion and one of the substrates and 2) the relatively large amounts of RuDP present in the reaction mixture (0.2 μM). At this concentration RuDP inhibits the enzyme. In the general assay system, where the amount of RuDP is only ca. 0.05 μM, the lag period is reduced to less than 1.5 minutes, hence contributing only about a 15% error in the assay. These results are in full accord with those found by Racker.[82] A more detailed discussion of both the inhibitory effect of RuDP in the enzyme system and the preliminary incubation effect on the enzyme are given later in this section.

Cofactors: When Mayaudon first partially purified the crude extract of Tetragonia and studied this system, he found it unnecessary to add cofactors to the assay mixture in order to have enzymic activity.[62] Since then, in the present experiments, the corresponding enzyme fraction (33 to 35% SAS ppt, the Crude Extract Method) exhibited little or no activity. The problem was resolved, in part, by boiling a dialyzed crude extract of

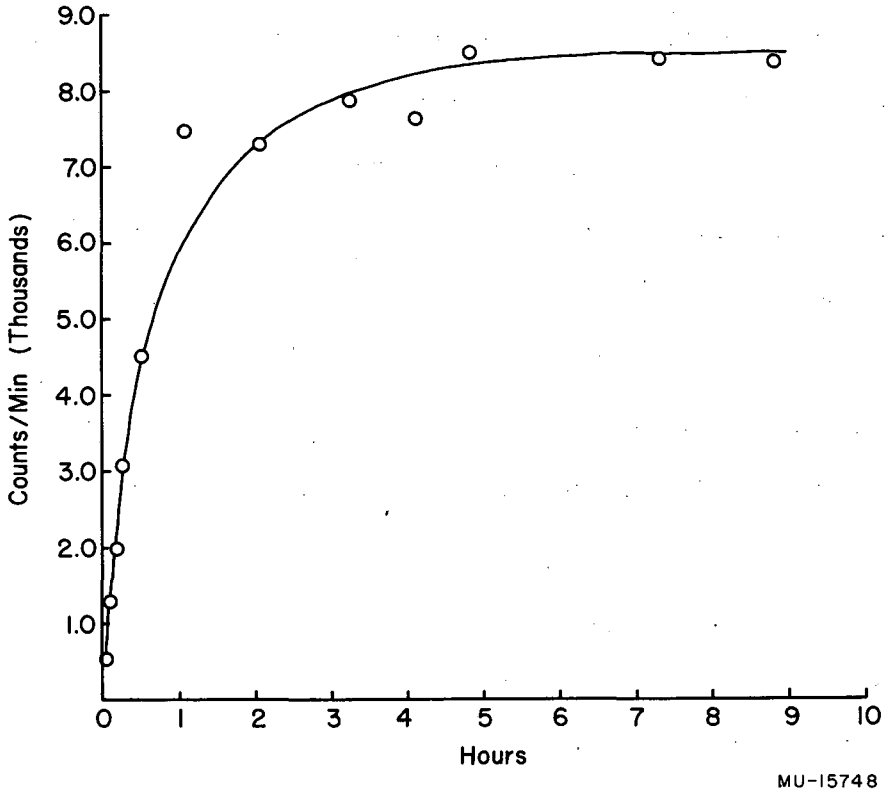


Fig. 25. Fixation of C^{14} from $NaHC^{14}O_3$ by the carboxydismutase system under preincubated conditions as a function of incubation time. Assay condition: Assay I (unknown preincubation time). In each incubation mixture, in μM: $NiCl_2$, 0.2; HCl, 0.4; $NaHC^{14}O_3$, 1.3 at 20 μC; enzyme (crude extract of Chlorella after 15 minutes of sonic oscillation), 100 μg dry weight; and RuDP, 0.04. Final volume: 200 μl. Incubation conditions; pH 7.1, 25°C. Plated: 6 μl and acidified with HOAc. Stable reference standard: 4,200 cpm.

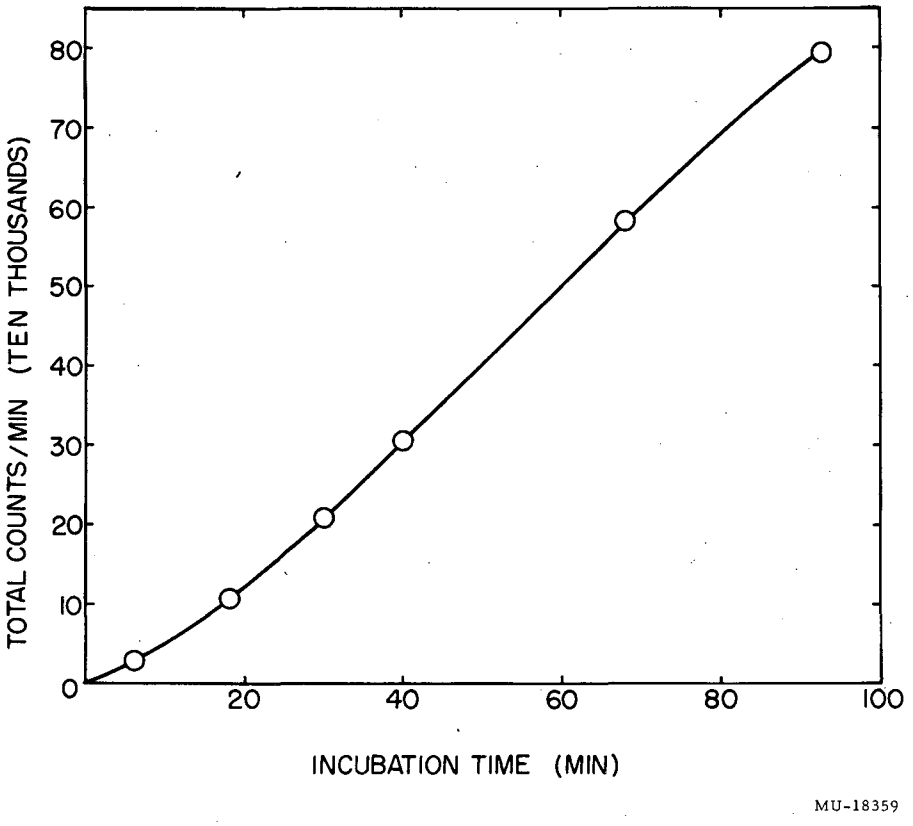


Fig. 26. The kinetics of C^{14} fixation by the carboxydismutase system under non-preincubated conditions. Assay condition: Assay III. In each incubation mixture, in μM: HCl, 1.6; $MgCl_2$, 0.8; $NaHC^{14}O_3$, 3.6 at 40 μC; RuDP, ca. 0.2; and enzyme (33 to 35% SAS ppt of the crude extract, Method I), 30 μg, colorimetrically. Final volume: 200 μl. Incubation conditions: pH 7.0, 25°C. Plated: 10 μl and acidified with HOAc. Stable reference standard: 4,400 cpm.

Tetragonia leaves and combining it with the ammonium sulfate fractionated material. The specific activity of this fraction was enhanced from 0.016 to 0.059 unit per mg protein, clearly indicating that a stable non-dialyzable cofactor was present in the crude extract. The question remains, however, as to why the enzyme system of Mayaudon was active without adding any cofactors whereas the same enzyme fraction was inactive in the present set of experiments under similar conditions. A close scrutiny of the constituents of the carboxydismutase system for both cases showed that the only difference was the source of RuDP, Mayaudon using RuDP derived from algae (in vivo) while RuDP in the present experiments was prepared enzymically (in vitro). That algal RuDP contained a cofactor for the carboxydismutase system was demonstrated by hydrolyzing it in acid and adding the hydrolysate to the enzymically prepared RuDP. This mixture, when tested in the usual manner for carboxydismutase assay yielded a doubling of the specific activity of the enzyme.

The stability of the cofactor(s) towards acid and heat treatment strongly implicated a metal ion as the activator of the enzyme. A host of metal ions from monovalent cations to tetravalent cations as well as complex forms of cations was tested in the assay. These are listed as follows: 1) Monovalent cations: LiCl, RbCl, $AgNO_3$, CuCl and $HgNO_3$; 2) Divalent cations: $BaCl_2$, $CdCl_2$, $CoCl_2$, $CaCl_2$, $CuSO_4$, $FeSO_4$, $MnSO_4$, $HgCl_2$, $NiCl_2$, $SnCl_2$, $ZnSO_4$, $SrCl_2Pb(NO_3)_2$ and $MgCl_2$; 3) Trivalent cations: $AlCl_3$, $Ce(ClO_4)_3$, $Fe_2(SO_4)_3$, $Fe_2(SO_4)_3$, $TiCl_3$ and $CrCl_3$; 4) Tetravalent cation: $SnCl_4$ and finally, 5) Complex ion: $VOSO_4$. All of these ions were tested at a final concentration of 2×10^{-3} M and 2×10^{-4} M. The final concentration of $VOSO_4$ was 2×10^{-4} M. Only Ni^{++}, Mn^{++}, Co^{++}, Mg^{++} and Cr^{+++} gave substantial fixation of the radioactivity, the most fixation being by Ni^{++} and decreasing in the order listed (Table VII). An

interesting feature of these experiments is the finding that neither Fe^{++} nor Fe^{+++} served as an activator at these concentrations; yet Horecker found that Fe^{++} (10^{-3} M) activates the enzyme system obtained from Spinacea oleracea.[6]

One other point of interest is the subsequent finding that the activation caused by the addition of Cr^{+++} is an artifact of the assay. Because the assay is based on the amount of acid-stable C^{14} fixed, the product was automatically assumed to be radioactive phosphoglyceric acid. Suitable controls, however, eliminated Cr^{+++} as an activator since a mixture of Cr^{+++}, $HC^{14}O_3^-$, and tris (or phosphate buffer plus enzyme), but no RuDP, yielded an acid-stable fixation product which is not PGA, but a compound having a composition of one carbon dioxide molecule to each molecule of chromic ion. The product is probably one similar to that of a carbonate-amine-metal complex.[98]

At this point, it seems appropriate to mention that glutathione stimulates the activity of the carboxylation enzyme 10 times over that of the control in preparations from Spinacea.[6] Furthermore, EDTA also has a similar effect. In this regard, the latter at 5×10^{-5} M final concentration, stabilized the enzyme derived from Tetragonia[62] and the present work has indicated that when the enzyme (crude extract) was dialyzed vs 10^{-4} M EDTA at pH 7 followed by incubation at 10^{-6} M EDTA, the enzyme was not only stabilized, but was still active even without adding metal ions to the incubation mixture. This suggests that the metal ion (or other cofactors) already present in the crude extract is bound rather tightly to some proteins in this extract.

A side experiment with digitonin was performed on the enzyme from both Tetragonia and Spinacea. When a half saturated aqueous solution of

TABLE VII

Fixation of Radioactive Carbon from Labeled Bicarbonate in the Presence of Various Metal Ions

Cation	Specific Activity, units per mg[a]	
Final conc[b]	2×10^{-4} M[c]	2×10^{-3} M[d]
Ni^{++}	0.062	0.083
Mn^{++}	0.023	0.035
Co^{++}	0.023	0.032
Mg^{++}	0.012	0.026
Cr^{+++}	0.012	0.015

a) Assay I, specific activity in units per mg (dry weight) of protein. One unit equals 1 μM of carbon fixed in 10 minutes of incubation.

b) Final concentration of metal ion in the incubation mixture.

c) In each incubation mixture, in μM: metal ion, 0.04; $NaHC^{14}O_3$, 1.3 at 20 μC; enzyme (32 to 35% SAS ppt of the crude extract of Tetragonia), 14 μg dry weight; and RuDP, ca. 0.1 of pentose (orcinol assay). Final volume: 200 μl. Incubation conditions: 10 minutes at 25° C.

d) In each incubation mixture, in μM: same as in c) except that metal ion, 0.4 and enzyme, 19 μg.

digitonin was used to extract the enzyme from intact chloroplasts of Spinacea, the specific activity (Assay III, Mg^{++} as cofactor) of the enzyme was 1.03 unit per mg of protein as compared with 0.58 unit per mg when tris buffer was used. This result appears not to be due to direct stimulation of the activity of the enzyme by the action of digitonin; for when the same solution of digitonin was added to a purified carboxydismutase system (Chloroplast Extract Method) of Tetragonia, no activation of the enzyme occurred. Excluding the possibility that the enzymes derived from the two different sources are quite different in their properties, these results could be interpreted in the following manner: 1) the digitonin stabilizes the enzyme by formation of micelles,[99] 2) the digitonin extracts some unknown cofactor from the chloroplasts and 3) the digitonin makes the enzyme more accessible to the substrates (e.g., greater permeability of RuDP through the chloroplast membrane to the enzyme).

Inhibitors: Among the inhibitors of the carboxydismutase system were the phosphorylated esters, Tables VIII and IX. Particularly effective were thiamine pyrophosphate (TPP), 3-phosphoglyceric acid (3-PGA) and threose-2, 4-diphosphate (TDP). These compounds inhibited the carboxylation enzyme by 40% at 10^{-4} M, 30% at 10^{-3} M and 55% at 10^{-3} M respectively. ATP at ca. 10^{-3} M concentration decreased the activity of the enzyme by only 15%. The nature of the TPP inhibition is unknown, but at least part of the effect may be due to a phosphate effect. This could be the primary effect although ATP does not possess as marked an effect on the activity as does TPP owing to a difference in assay conditions. The influence of PGA and TDP on the carboxydismutase activity seems to fall along a definite pattern. (Threose diphosphate was found to inhibit the triose phosphate dehydrogenase system of mammalian tissues.[100, 101] In The course of applying this inhibitor to a spinach chloroplast system, it

TABLE VIII

Some Organic Inhibitors of the Carboxydismutase System

Compound(s)	Assay Conditions*	Final Conc**	% Inhibition
TPP	Assay I, pH 7.4, Mg^{++}, 0.4 μM	1×10^{-4}	40
TPP plus thioctic acid	Same as above	1×10^{-4} ea. compound	65
Thioctic acid	Same as above	1×10^{-4}	10
ATP	Assay II, pH 7.0, Mg^{++}, 0.2 μM pre-incubated 10 min with enzyme at 0° C.	9×10^{-3}	95
ATP	Assay III, pH 8.3, Mg^{++}, 2.0 μM	9×10^{-4}	15
ATP plus glutamic acid	Same as above	9×10^{-4} and 8×10^{-4} resp.	15
EtOH	Assay II, pH 7.2, Ni^{++}, 0.2 μM pre-incubated 10 min. with enzyme at 0° C.	1×10^{-1}	20

* Details of the assay are given in the experimental section.

** Molarity of the inhibitor in the incubation mixture.

TABLE IX

Threose Diphosphate and Phosphoglyceric Acid as Inhibitors of Carboxydismutase

Compound	Assay Conditions*	Final Conc**	% Inhibition
TDP	Assay II, pH 8.3, Mg^{++}, 2.0 μM preincubated 2 minutes at 25° C with the enzyme	8×10^{-6}	10
TDP	Same as above	8×10^{-5}	10
TDP	Same as above	8×10^{-4}	55
TDP	Same as above, but preincubated only TDP with the enzyme	8×10^{-4}	35
TDP	Assay III, pH 8.3, Mg^{++}, 2.0 μM	8×10^{-4}	20
3-PGA	Assay II, pH 7.0, Ni^{++}, 0.2 μM preincubated 10 minutes at 0° C with the enzyme	1×10^{-4}	10
3-PGA	Same as Above	1×10^{-3}	30
3-PGA	Same as above	1×10^{-2}	80

* Other details of the assay are given in the experimental section.

** Molarity of the inhibitor in the incubation mixture.

was found that not only the dehydrogenase was affected, but also the total carbon dioxide uptake.[102] In view of these results, this compound was tested for its effect on carboxydismutase). A study of models of these compounds showed that these two compounds are similar in two respects: 1) the spatial configuration of the groups around the two bottom carbon atoms of these molecules and 2) the placement of the carbonyl group relative to the rest of the molecule. The fact that RuDP has a similar configuration as these molecules points strongly to a competitive action of these inhibitors for the active site of the enzyme. The position of the phosphate group attached to carbon atom one of RuDP relative to the rest of the molecule also resembles that of the number two carbon of TDP. It should be noted here that the inhibition by 3-PGA would not have been detected by the technique utilized by Horecker[6] and Racker[82] since both investigators assayed the activity by determining the total amount of PGA formed.

The reduction of activity by hydroxylamine is due to the formation of the oxime of RuDP. (See Table X) Ethanol affects the activity perhaps by partial denaturation of the enzyme. (Table VIII) Other organic inhibitors are described elsewhere,[6,25a, 62] among them being p-chloromercuribenzoate and iodoacetamide. Enzyme preparations from Chlorella, Tetragonia and Spinacea were studied in these cases.

The decrease of activity of the carboxylation enzyme owing to the presence of anions are shown in Table X. These inhibitions are not as large as those found by Horecker, probably because of differences in assay conditions. For example, phosphate and arsenate, both at 0.01 M, inhibit the carboxylation enzyme 25% and 35%, respectively. These values should be compared with the 70% and 100% inhibition by phosphate (0.01 M) and arsenate (0.02 M), respectively. That the reduction of enzymic activity is not due to an ionic strength effect alone, is demonstrated by the addition of Na_2SO_4 to the incubation medium.

TABLE X

Inorganic Inhibitors of Carboxydismutase

Compound	Assay Conditions*	Final Conc**	% Inhibition
NH_2OH	Assay II, pH 7.0, Mg^{++}, 0.2 µM, preincubated 10 min at 0° C with the enzyme, then RuDP treated with NH_2OH for 22 minutes at room temperature was added.	1×10^{-4}	10
NH_2OH	Same as above	1×10^{-3}	75
NH_2OH	Same as above	1×10^{-2}	100
NaF	Assay II, pH 7.0, Mg^{++}, 0.2 µM, preincubated 10 minutes at 0° C with the enzyme.	1×10^{-4}	0
NaF	Same as above	1×10^{-3}	15
NaF	Same as above	1×10^{-2}	60
K_2HPO_4***	Assay III, pH 8.3, Mg^{++}, 2.0 µM	1×10^{-2}	25
Na_2HASO_4***	Same as above	1×10^{-2}	35
Na_2SO_4	Same as above	1×10^{-2}	10

* Other details of the assay are given in the experimental section.

** Molarity of the inhibitor in the incubation mixture.

*** Adjusted to pH 8.3 by the addition of HCl or NaOH.

Note that fluoride ion at 0.01 M final concentration in the presence of Mg^{++} (0.001 M) inhibits carboxydismutase by 60%. This is expected for enzymes which are activated by Mg^{++}.[57] The effect on the enolase system is seen from the chromatograms of the fluoride inhibited carboxydismutase system. These chromatograms showed that no phosphoenolpyruvate is formed after ten minutes incubation (in contrast to the control where a trace amount of PEP was formed) and about 1% of the total acid-stable C^{14} fixed is in the form of PEP after 42 hours of incubation. These results indicate that fluoride inhibits the plant leaf enolase nearly completely under these conditions. A comparison of this inhibition with that of the yeast enolase system is not possible since the inhibition is dependent on the Mg^{++} concentration as well as the type of buffer used. (The activity of yeast enolase is decreased 50% by fluoride ion at 10^{-4} M in the presence of 2.7×10^{-6} M Mg^{++} and pH 6.7, 0.05 M phosphate buffer.)[103]

Of special interest are those experiments with additions which have little (i.e., ± 10% change) or no influence on the carboxydismutase activity. These compounds or mixtures of compounds are itemized in Table XI, along with their roles in photosynthesis or in carboxylation reactions.

The Preincubation of Metal Ions with Carboxydismutase, in the Presence and Absence of Bicarbonate: In the study of any enzyme, the problem of its assay is of paramount importance. For example the progress of the purification of the enzyme and the effect of various added substances on the activity of the enzyme both require an accurate, or at least, a reliable method of assay. Since the activity of carboxydismutase can be determined by measuring the amount of radioactive PGA formed from the carboxylation of unlabeled RuDP with $HC^{14}O_3^-$, any variation in the determination of the acid-stable fixation of the radioactivity must first be considered. Experiments showed that for a given reaction mixture, the variations in the

TABLE XI

Compounds Having No Effect on the Carboxydismutase Activity

Compound(s)	Final Conc (M)	Role in Photosynthesis or Carboxylation Reactions	References
Thioctic acid	2×10^{-4}	Cofactor for pyruvic carboxylase	1,2, 57
Biotin	1×10^{-4}	Cofactor for carboxylases	57,104
Avidin	1.5×10^{-5}*	Inhibitor for biotin dependent enzyme systems	104 105
Biotin plus avidin	1×10^{-4} 1.5×10^{-5}	----------	-----
Glutamic acid	8×10^{-4}	Possible early product of photosynthetic CO_2 fixation	33
Amino acid mixture (acid hydrolysate of protein)	1.8×10^{-3} total	----------	-----
6-Ethyl-8-thioloctanoic acid	1.25×10^{-3}	Inhibitor of photosynthetic O_2 evolution and CO_2 uptake	106
Azaserine	7 to 29 $\times 10^{-4}$	Possible inhibitor of transaminases in Scenedesmus	45
Thiamine	1×10^{-4}	Parent compound of cocarboxylase	-----
EDTA	1×10^{-4}	Metal ion complexing agent, activator of Spinacea carboxydismutase	6
Digitonin	8.6×10^{-5}	Natural surface active agent, used in extracting chloroplasts	107, 108

*Based on a molecular weight of 66,000. Specific activity: 2.5 units/mg. Actually avidin decreased the fixation by 25%, but addition of biotin did not reverse this result.

counts per minute are of the order of ± 5% or less. This fact indicates that the pipetting error, the plating error, together with the counting error, are relatively small compared with the error which is due to the reaction itself. In the latter case, when the acid-stable fixed radioactivity is compared from one reaction mixture to another, within a given day for a given enzyme solution, the variations may be as large as ± 33%, even though the conditions appeared to be identical. On the other hand when no metal ion is added to the incubation mixture, the enzyme has a low residual activity which yields a reproducible result (to within ± 5%).

These experiments indicate the 1) the enzyme is not stable over a period a day and/or 2) the enzyme requires a preincubation period with either the metal ion or the substrate or both. The first of these possibilities appears not to be the controlling factor because experiments have shown that the enzyme is quite stable over a period of a day even at room temperature (for greater details, cf., in a later section, Stability of the Enzyme.) With regard to the second possibility, it was found that in an effort to establish reproducible results in the assay technique, the order of addition of the enzyme to the incubation mixture markedly affected the enzymic activity. This observation led to the conclusion that the enzyme (crude extract of Chlorella) requires preincubation with the activating metal ion in the presence of bicarbonate in order to attain maximum activity, Fig. 27 and Fig. 28. From these figures it is apparent that the activity of the enzyme rises sharply to a maximum and gradually falls off as the preincubation time is increased. That the peak of activity varies as a function of the temperature of preincubation is evident from the curves showing a maximum of 65,000 cpm at 0° C preincubation temperature compared with 19,000 cpm at 25° C preincubation temperature. The results can be interpreted in two ways: 1) the time required to reach maximum activity is shifted from

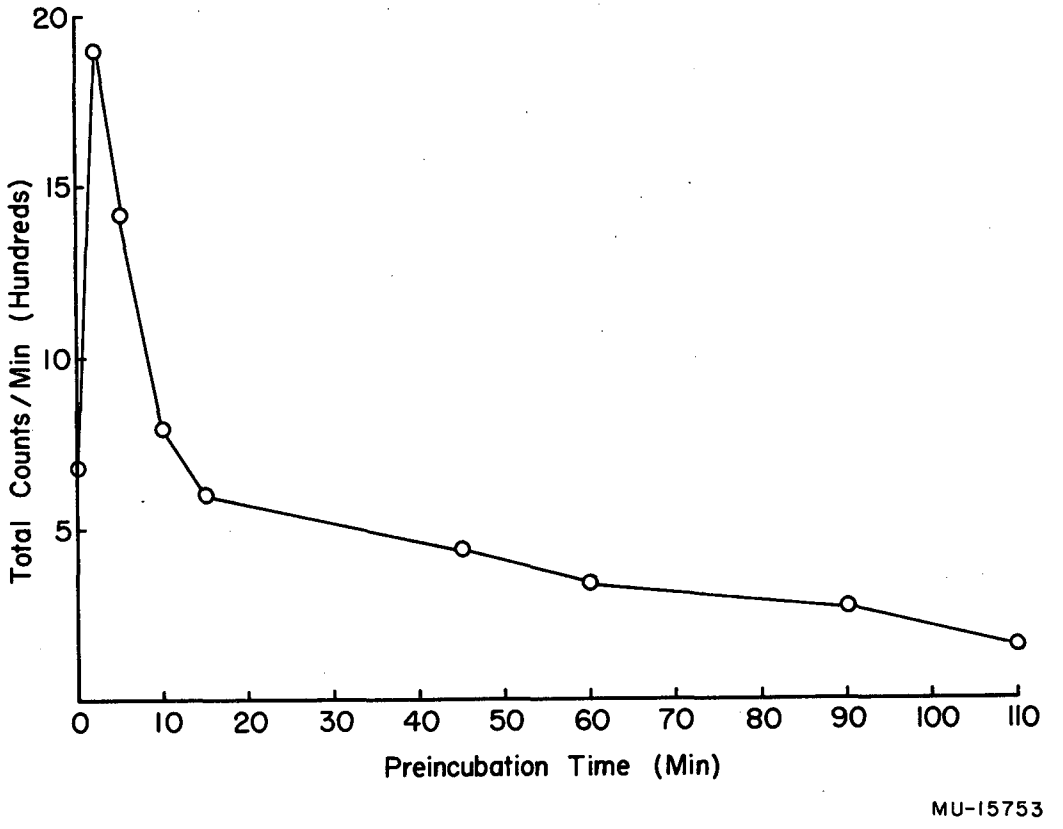


Fig. 27. The Preliminary incubation of a crude extract of Chlorella with Nickelous ion and bicarbonate at 25°C. Assay condition: Assay II. In each incubation mixture, in μM: HCl, 0.24; $NiCl_2$, 0.04; $NaHC^{14}O_3$, 0.26 at 4 μC; enzyme (crude extract of Chlorella), 20 μg and RuDP, ca. 0.05 of pentose (orcinol color). Final volume: 40 μl. Preincubation conditions: pH 7.1, 25°C. Incubation conditions: Same pH and temperature as before, 10 minutes. Stable reference standard: 4,200 cpm. The zero minute preincubation point employed the same assay conditions as above except that Assay III was followed (i. e., the enzyme was added last).

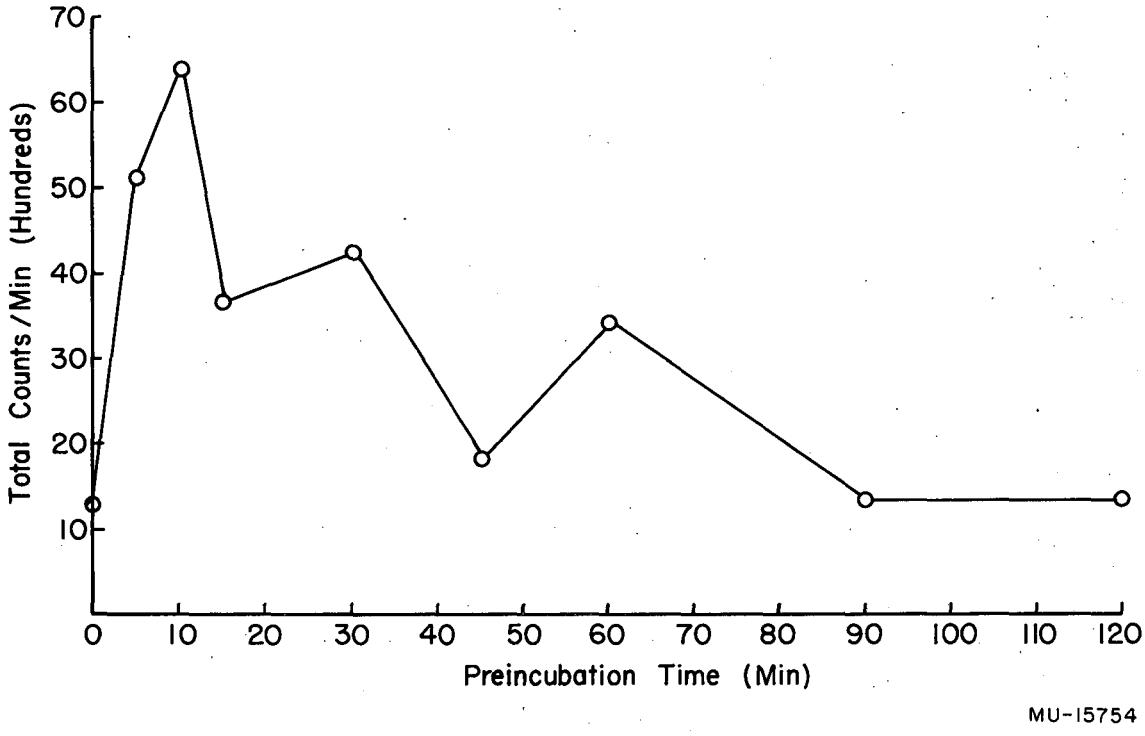


Fig. 28. The activity of the crude extract of Chlorella as a function of preincubation period with Nickelous ion and bicarbonate at 0°C. Assay conditions: Identical to those of Fig. 27, except that preincubation was at 0°C.

10 minutes at 0° C to less than 2 minutes at 25° C or 2) the enzyme is more active at 0° C than at 25° C preincubation temperatures owing to a rapid inactivation process at the latter temperature.

So far the curves shown are those of the crude extract of Chlorella; the preparations of Tetragonia (both the crude extract and the ammonium sulfate purified material) yielded essentially the same results. In the ammonium sulfate purified system, two main effects are observed when the metal ion concentration was increased: 1) the increase of the enzymic activity and 2) the shift of the activity maximum towards shorter preincubation times, Fig. 29. At a final concentration of 0.001 M Ni^{++}, the enzyme is saturated with respect to this cation, further increase of the metal ion concentration being ineffective for higher enzymic activity.

A consequence of these experiments was the finding that when the total incubation volume was decreased from 0.2 ml to 0.04 ml while keeping all of the components (i.e., RuDP, $HC^{14}O_3^-$, enzyme, Ni^{++} and H_2O) at the same concentrations, the maximum specific activity changed from 0.11 to 0.033 unit per mg. This phenomenon indicates that surface effects play a prominent role on the enzymic activity, perhaps by competitive adsorption of the metal ion or by a change in the pH of the reaction mixture.

Preliminary incubation of the enzyme with Mg^{++}, both in the presence and absence of bicarbonate, gave entirely different results from those of Ni^{++}, Fig. 30 and Fig. 31. No inactivation of the enzyme occurred at 0° C preincubation even after 60 minutes. Note further in Fig. 31, that Mg^{++} at 0.0001 M or less, activated the enzyme only to a small extent. Under these conditions then, the addition of cysteine to the incubation mixture had no effect. At 25° C preliminary incubation of Mg^{++} with the enzyme slowly decreased the activity as a function of time of preincubation, Fig. 32. Actually the first point was not at zero time, but at

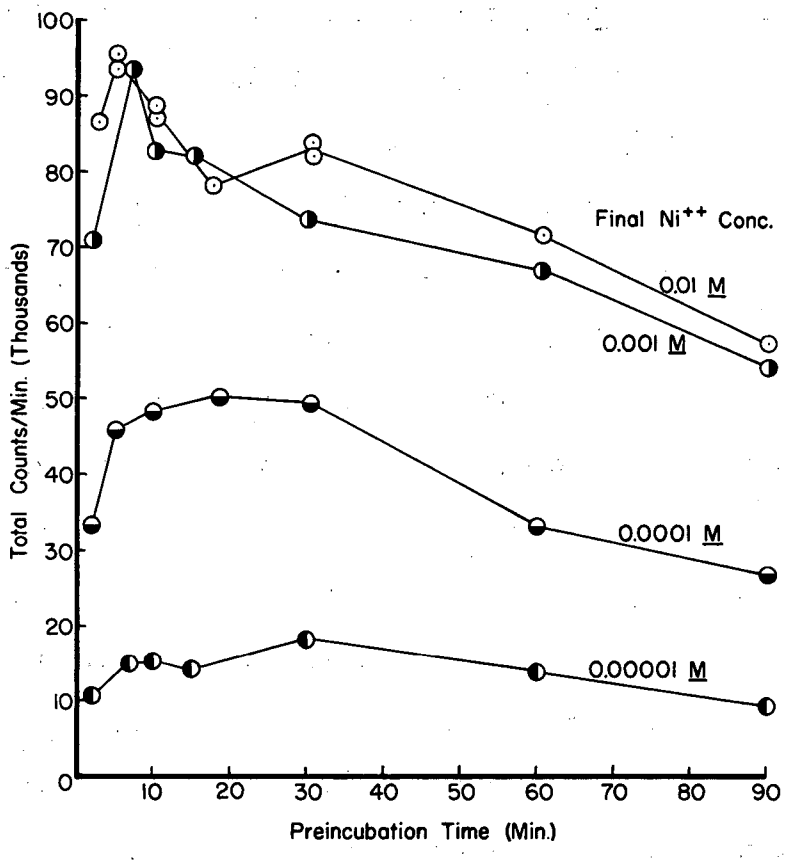


Fig. 29. The preincubation of carboxydismutase with various concentrations of Ni^{++} and bicarbonate at 0°C. Assay condition: Assay II. In each incubation mixture, in μM: $NiCl_2$, see curve above; HCl, 1.2; $NaHC^{14}O_3$, 1.3 at 20 μC; enzyme (32 to 35% SAS ppt of the crude extract of Tetragonia, Method I), 20 μg; and RuDP, 0.08 of organic phosphate. Final volume: 200 μl. Preincubation conditions: pH 7.1 and 0°C. Incubation conditions: 10 minutes at 25°C. Stable reference standard: 4,300 cpm.

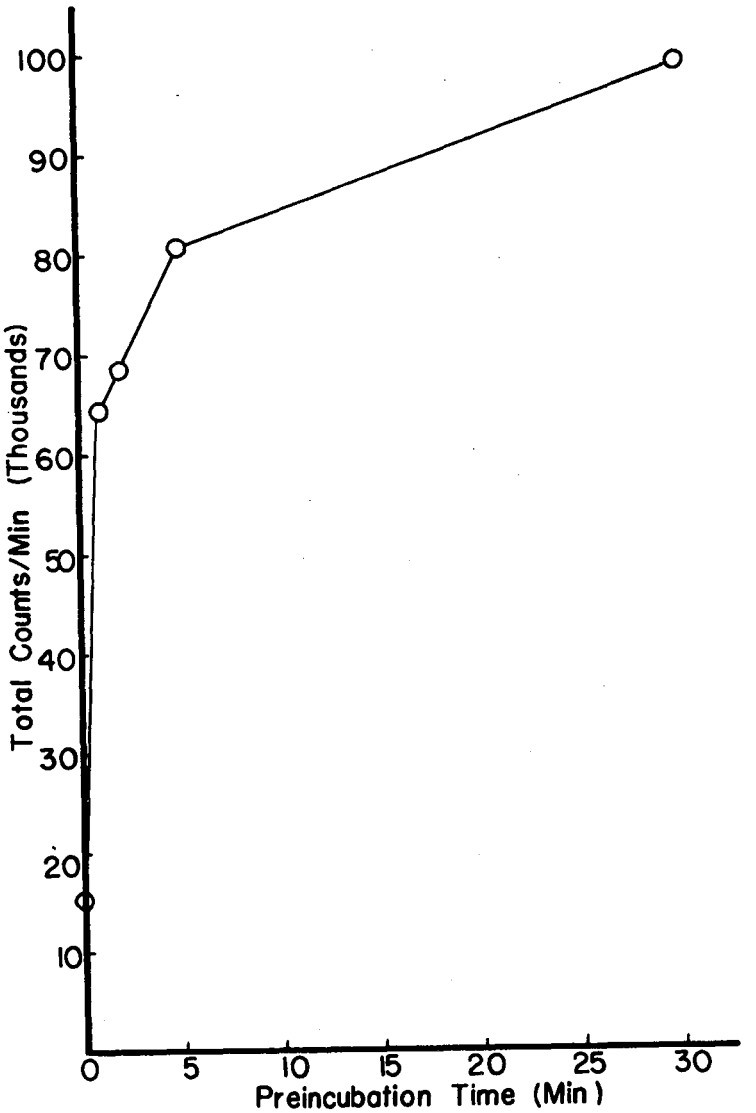


Fig. 30. The preincubation of carboxydismutase with Mg^{++} and bicarbonate at 0°C. Assay condition: Assay II. In each incubation mixture, in μM: $NaHC^{14}O_3$, 1.8 at 20 μC; $MgCl_2$, 0.2; HCl, 0.8; enzyme (32 to 46% SAS ppt of the chloroplast extract of Tetragonia, Method III), 25 μg; and RuDP, 0.03 (based on the carboxydismutase assay). Final volume: 200 μl. Preincubation conditions: pH 7.0 and 0°C. Incubation conditions: 5 minutes at 25°C. Stable reference standard: 4,600 cpm.

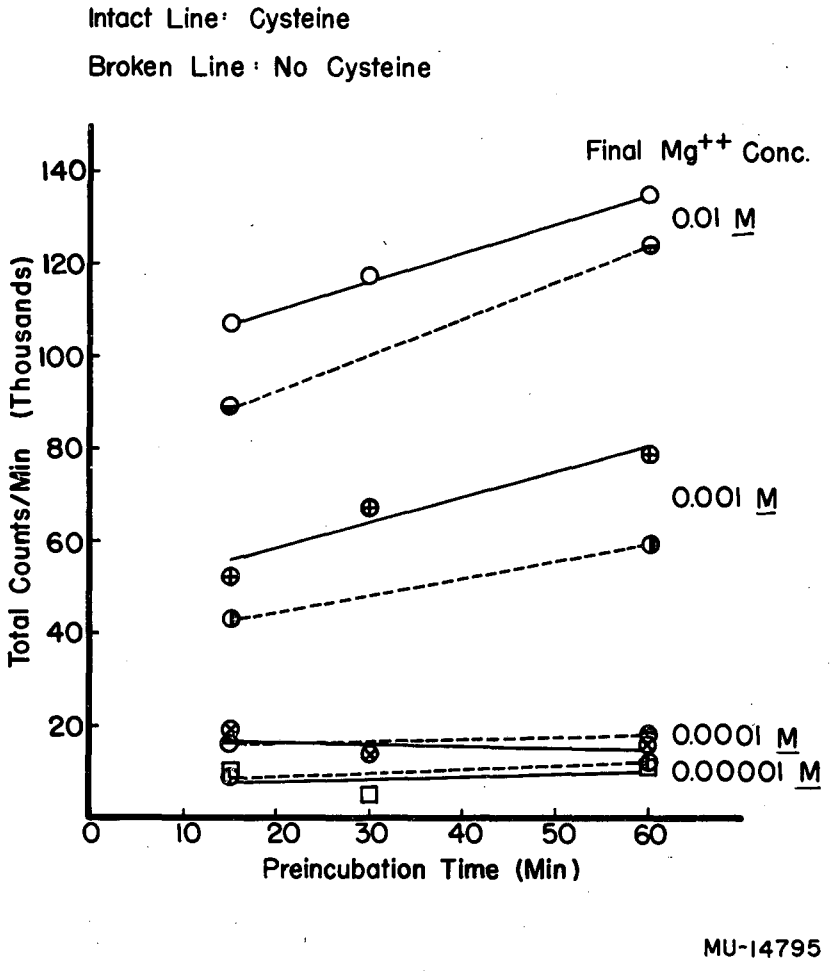


Fig. 31. The preincubation of carboxydismutase with different concentrations of Mg^{++} in the presence and absence of cysteine at 0°C. Assay condition: Assay II. In each incubation mixture, in μM: cysteine, 0.2; enzyme (32 to 46% SAS ppt of the chloroplast extract of Tegragonia, Method III), 25 μg; $MgCl_2$, see curve above tris, 0.5 at pH 8.0; RuDP, 0.03 (based on the carboxydismutase assay); and $NaHC^{14}O_3$, 1.8 at 20 μC. Final volume: 200 μl. Preincubation conditions: Enzyme with Mg^{++} or with Mg^{++} plus cysteine only, 0°C. Incubation conditions: 5 minutes at 25°C. Stable reference standard: 4,600 cpm.

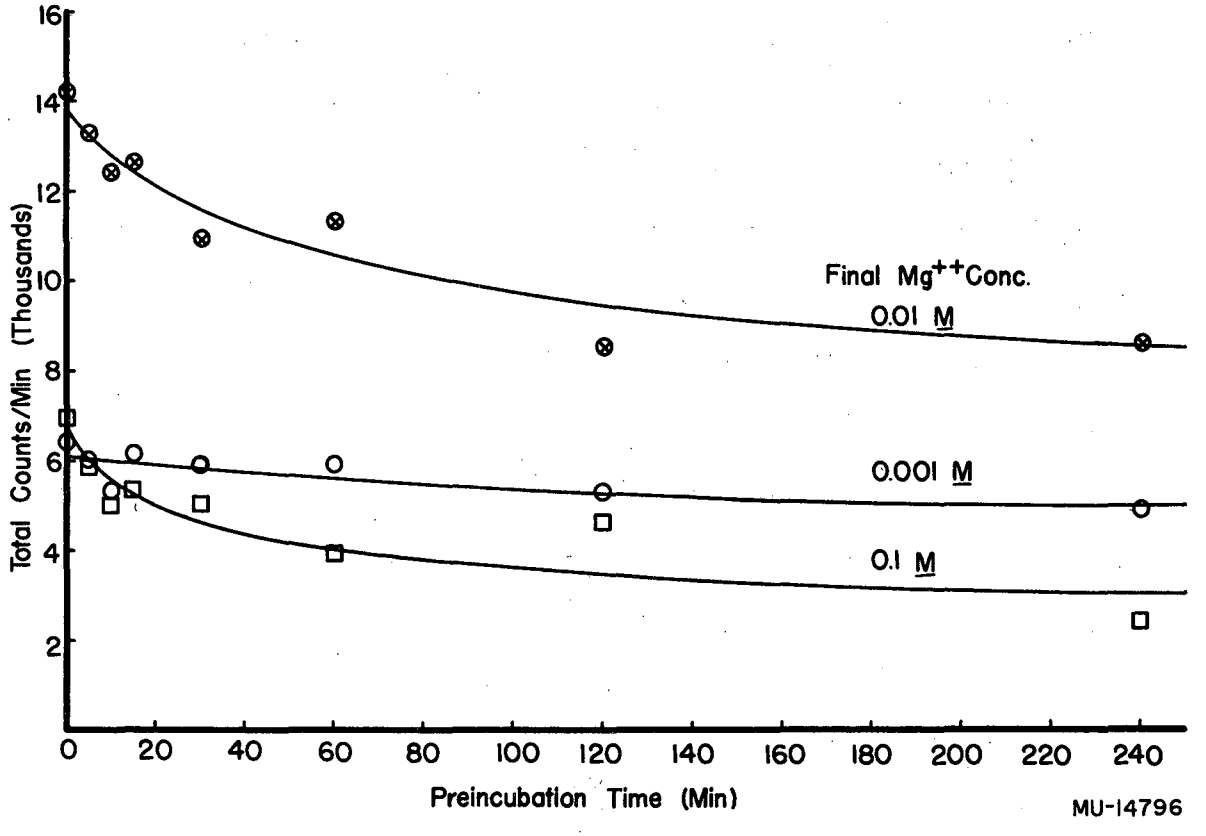


Fig. 32. The preincubation of carboxydismutase with various concentrations of Mg^{++} at 25°C. Assay condition: Assay II. In each reaction mixture, in μM: Enzyme (32 to 46% SAS ppt of the chloroplast extract of Tetragonia, Method III), 5 μg; $MgCl_2$, see curves above; tris, 2.0 at pH 8.0; RuDP, 0.2 (organic phosphate); $NaHC^{14}O_3$, 2.4 at 27 μC. Final volume: 200 μl. Preincubation conditions: Enzyme with Mg^{++} only 25°C. Incubation conditions: 10 minutes at 25°C. Stable reference standard: 5,600 cpm.

124

15 seconds. The possibility that maximum activity was reached within this first 15 seconds was considered. The results of an experiment designed to show this effect is given in Table XII. Indeed the true zero time preincubation gave an activity less than that of the 30 or the 60 minutes preincubation in both the 0° and the 25° C experiments. Although the conditions of these experiments were not identical to those described in Fig. 31 and Fig. 32 (where the enzyme was not preincubated with bicarbonate), the results were similar, in that the enzyme was being activated when preincubated at 0° C and was being inactivated when preincubated at 25° C after 60 minutes. The activation process at 25° C, therefore, is a very rapid one, requiring only a few seconds for maximal activity. From the temperature depencence of the preincubation effect, the process of activation by Mg^{++} appears to proceed first by the unfolding of the protein, similar to the denaturation of proteins. Hence, for a change of every 10° C, the denaturation of a protein may change as much as 10 to 100 fold while for ordinary enzymic reaction the change may only be of the order of 2 to 3 fold.[109] One can then speculate that the Mg^{++} is bound by the enzyme only after some weak bonds such as hydrogen bonds are broken.

The next problem to consider is the remarkable difference in the preincubation effects of Ni^{++} and Mg^{++}. Why should one ion give rise to only an activation effect while the other gives an activation followed by an inactivation effect? An explanation for the latter phenomenon is that Ni^{++} actually activates the enzyme by simply displacing the true metal cofactor from inactive sites of the enzyme to the active sites.[69] The ensuing inactivation process is accounted for by the subsequent displacement of the activating metal ion from the active site by Ni^{++} or by the reaction of essential sulfhydryl groups with Ni^{++}. (It may be argued that there is no metal ion present in the enzyme preparation since the fractionation involves

TABLE XII

The Preincubation of Mg^{++} and Bicarbonate with Carboxydismutase

(Total Counts per Minute)

Preincubation time, min.	0	30	60
Preincubation temp., 0° C.	110,000	170,000	182,000
Preincubation temp., 25° C.	110,000	172,000	153,000

Assay conditions: Assay II and Assay III. In each incubation mixture, in μM: $MgCl_2$, 2.0; tris, 15 at pH 8.3; $NaHC^{14}O_3$, 1.33 at 20 μC; enzyme (32 to 35% SAS ppt of the crude extract of Tetragonia, Method I), ca. 30 μg; and RuDP, 0.033. Final volume: 200 μl. Preincubation conditions: Enzyme with Mg^{++} and bicarbonate, at 0° and 25° C. Incubation conditions: 10 minutes at 25° C.

126

dialysis. It has been demonstrated, however, that dialysis does not remove all of the metal ion from crude extracts of Tetragonia, even when the preparations were dialyzed against EDTA. This conclusion is deduced from the fact that there is no loss of activity of the crude extract after dialysis). Thus it is possible that Mg^{++} is the true activating metal ion for the carboxydismutase system. Although Co^{++} and Mn^{++} are also cofactors for this enzyme, these ions may be eliminated as true activators since they (and Ni^{++}) do not stimulate the activity of an aged enzyme preparation, Fig. 33.* These negative results, however, may be due to partial denaturation of the protein because of its age or due to the formation of a tris-metal ion complex. (The formation of a tris-metal ion complex is evident from the color change of the metal solution when tris is added). While the competition between the enzyme and the tris for the metal ion can account for the nonactivation of the aged enzyme, experiments performed with this preparation in the absence of tris, using Ni^{++}, failed to activate the enzyme by more than 2.5 fold. A fresh enzyme, when treated similarly, is generally activated between 10 to 50 times. Finally, when an aged enzyme is preincubated with a mixture of two metal ions, Mg^{++} and Ni^{++} in the presence of tris, Mg^{++} activates the Ni^{++} treated system and conversely, Ni^{++} inactivates the Mg^{++} activated system, Table XIII.

According to the activation by displacement hypothesis then, nickelous ion (and presumably cobaltous and manganous ions) activates the enzyme by displacing the true cofactor (magnesium ion) from some inactive sites to the active sites. The inactivation by Ni^{++} after prolonged preincubation with the enzyme arises from the subsequent displacement of Mg^{++} from the active centers or by reaction of Ni^{++} with the essential sulfhydryl groups.

*Recently Racker found that Co^{++}, Ni^{++} and Mn^{++} activates RuDP carboxylase in Chlorella extracts only slightly. (111)

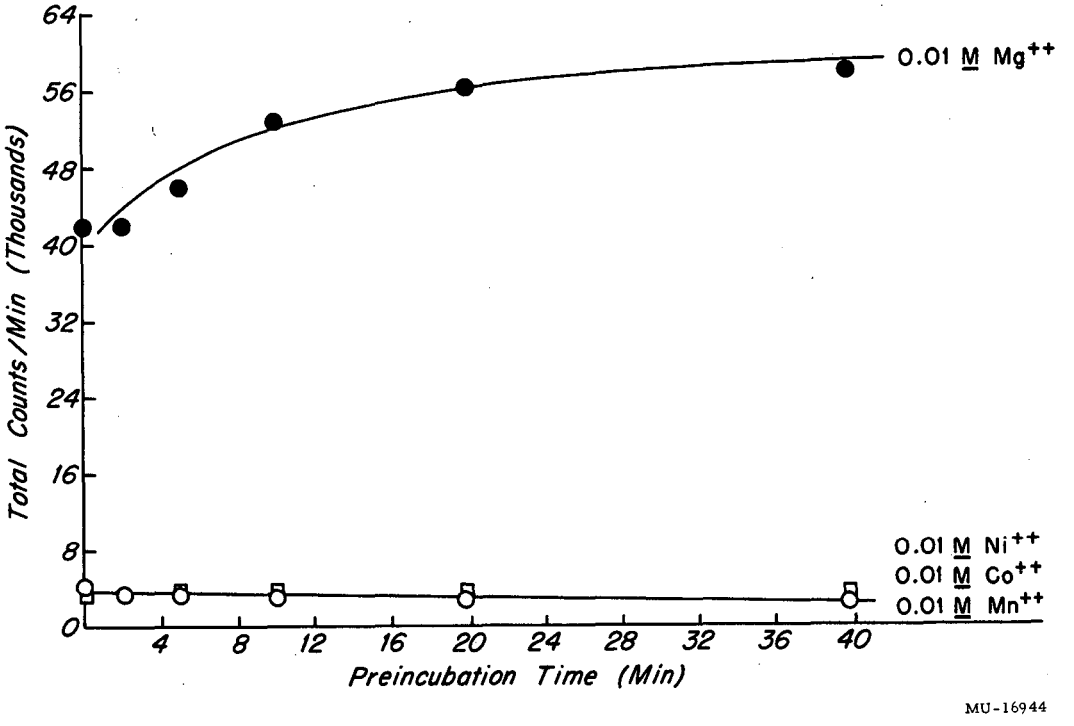


Fig. 33. The preincubation of an aged enzyme with various metal ions. Assay conditions: Assay II and Assay III. In each incubation mixture, in μM: Enzyme (32 to 46% SAS ppt of the chloroplast extract of Tetragonia, Method III, lyophilized powder stored at -15°C for 21 months), 25 μg; tris, 15 at pH 8.4; metal ion (chloride form), 2.0; RuDP, 0.048 (carboxydismutase assay); and $NaHC^{14}O_3$, 1.32 at 20 μC. Final volume: 200 μl. Preincubation conditions: Enzyme with metal ion only, at 0°C. Zero time preincubation (1st point): Method III, otherwise, same concentrations. Incubation conditions: 10 minutes at 25°C. Stable reference standard: 5,300 cpm.

128

TABLE XIII

The Effect of a Mixture of Metal Ions on the Activity of Aged Carboxydismutase

Pretreatment of the Enzyme	(units per mg) * Specific Activity
Mg^{++}, control (no RuDP)	0.000
Mg^{++} plus Ni^{++}, control (no RuDP)	0.000
No metal added	0.014
Ni^{++}, 10 minutes preincubation	0.009
Mg^{++}, preincubated 10 minutes	0.16
Mg^{++}, preincubated 10 min then added Ni^{++} with an additional 10 min preincubation**	0.051
Ni^{++}, preincubated 10 min then added Mg^{++} followed by an additional 10 min preincubation**	0.042

* Assay conditions: Assay II; 1 unit equals 1 μM of carbon fixed per 10 minutes of incubation. Weight is in dry weight of lyophilized enzyme. In each incubation mixture, in μM: Tris, 15 at pH 8.4; metal ion (chloride form), 1.0 each (see table); enzyme (32 to 46% SAS ppt of the chloroplast extract of Tetragonia, Method III, lyophilized powder stored at -15° C for 21 months), 25μg; RuDP, 0.048 (carboxydismutase assay); and $NaHC^{14}O_3$, 1.35 at 20 μC. Final concentration: 200μl. Preincubation conditions: Enzyme with metal ion only, at 0° C. Incubation conditions: 10 minutes at 25° C.

** The first metal was preincubated a total of 20 minutes while the second metal was preincubated for 10 minutes.

In the aged enzyme the true cofactor is bound more tightly than in the fresh enzyme or it is masked by a part of the protein which has unfolded after the partial denaturation so that the transition metal ions cannot displace the true cofactor off the inactive sites. That this denaturation may prevent these ions from entering the active centers is discounted by the fact that Mg^{++} can still activate the enzyme, the hydrated radius of the latter being larger than those of the hydrated transition metal ions.[112] The possibility that a complex between bicarbonate and the transition metal ion is larger than the hydrated or bicarbonate-Mg^{++} complex is not excluded, however.

Reproducibility of the Assay: Inasmuch as the carboxydismutase activity is largely affected by the effect of preincubation, the reliability of the general assay method was reinvestigated. In order to obtain a reproducible assay, the number of variables was minimized. For this reason the effect of preincubation was eliminated by conducting the assay under conditions where there was no preincubation (Assay III). The results are summarized in Tables XIV and XV. The control series (no RuDP added) is included to illustrate the extent of the counting error. When an overall average is taken of either the control series or the variable series, the mean error of the assay is not more than $\pm$ 3% from the mean value. Note that although the planchets are numbered serially from 1 to 9 for each series, the experiment was actually carried out in a random manner. Thus not only were the incubation mixtures, but also the plating of the samples randomized. In this way anysystematic error arising from the aging of the enzyme or the instability of the substrate may be discounted. A close scrutiny of the counting data indicates that a large part of the error was due to counting (see, for example, the control series). Each planchet of this series was counted for a total of 320 counts; the

130

TABLE XIV

Reproducibility of the Carboxydismutase Assay

Control Series (No RuDP)

Incubation mixture no.	I			II			III		
Planchet no.	1	2	3	4	5	6	7	8	9
Counts per min, 1st count	47	45	44	44	41	43	49	43	49
Counts per min, 2nd count	48	47	44	46	49	53	47	41	48
Counts per min, 3rd count	41	41	42	42	60	46	49	43	54
Mean value, counts per min	45	44	43	44	49	47	48	42	50
Mean error of mean value counts per min	3	2	1	1	6	4	1	1	2

Assay condition: Assay III. In each incubation mixture, in μM: $MgCl_2$, 2.0; tris, 15 at pH 8.3; $NaHC^{14}O_3$, 1.32 at 20μC; and enzyme (32 to 35% SAS ppt of the crude extract of Tetragonia, Method I, ca. 30 μg. Final volume: 200 μl. Incubation conditions: 10 minutes at 25° C. Termination of the reaction: 50 μl 6 N HOAc. Aliquot portion per aluminum planchet: 15 μl. Counting: Nuclear-Chicago scaler with automatic sample changer. Results were recorded in minutes per 320 counts.

TABLE XV

Reproducibility of the Carboxydismutase Assay

Variable Series (Complete Assay)

Incubation mixture no.	I			II			III		
Planchet no.	1	2	3	4	5	6	7	8	9
Counts per min 1st count	3,450	3,520	3,430	3,570	3,680	3,830	3,520	3,480	3,510
Counts per min 2nd count	3,340	3,590	3,440	3,590	3,660	3,810	3,500	3,410	3,510
Counts per min 3rd count	3,390	3,430	3,480	3,490	3,710	3,640	3,360	3,400	3,400
Mean value counts per min	3,390	3,510	3,450	3,560	3,680	3,760	3,460	3,440	3,470
Mean error of mean value counts per min	34	60	23	37	13	83	70	35	47

All conditions are similar to those given under Table XIV, except that 0.03 μM of RuDP was added and that each planchet was counted for 10,240 counts; the time required to reach this value was recorded.

time required to reach this value was then recorded automatically. This error is reduced by increasing the total number of counts to be recorded. Thus in the variable series, each planchet was counted for a total of 10,240 counts. Comparison of the percentage deviation obtained from the two series showed that the maximum deviation for the counting is ± 12% for the control series whereas for the variable series, it is ± 2%.

It was previously shown that some of the enzyme preparations can convert radioactive PGA into other compounds. If a volatile substance arises from this conversion (e.g. pyruvic acid), then a loss of radioactivity can be expected. In addition, labeled bicarbonate may be incorporated into compounds such as oxalacetic acid, which in turn is transformed into aspartic acid. It is felt, however, that errors arising from these two sources are partly self-compensating and that these errors are small, especially when the incubation period is 10 minutes or less. This is seen by chromatography of the products of the carboxydismutase reaction. In no case is the formation of products other than PGA and glyceric acid apparent when purified enzymes were used. Even crude enzyme preparations, after dialysis, yield more or less the same extent of contamination.

Dependence of the Carboxydismutase Activity on Enzyme and Substrate Concentrations. The rate of fixation of C^{14} by the carboxydismutase system follows a nearly linear dependence with respect to the enzyme concentration, Fig. 34. The slight departure from linearity can be accounted for by the presence of minute quantities of heavy metal ion present in the buffer solution or distilled water used in the incubation mixture, the enzyme being sensitive to these heavy metal ions. Alternatively, a similar effect can be obtained from an enzyme with a dissociable activator. Hence under concentrated conditions most of the enzyme is in the activated state

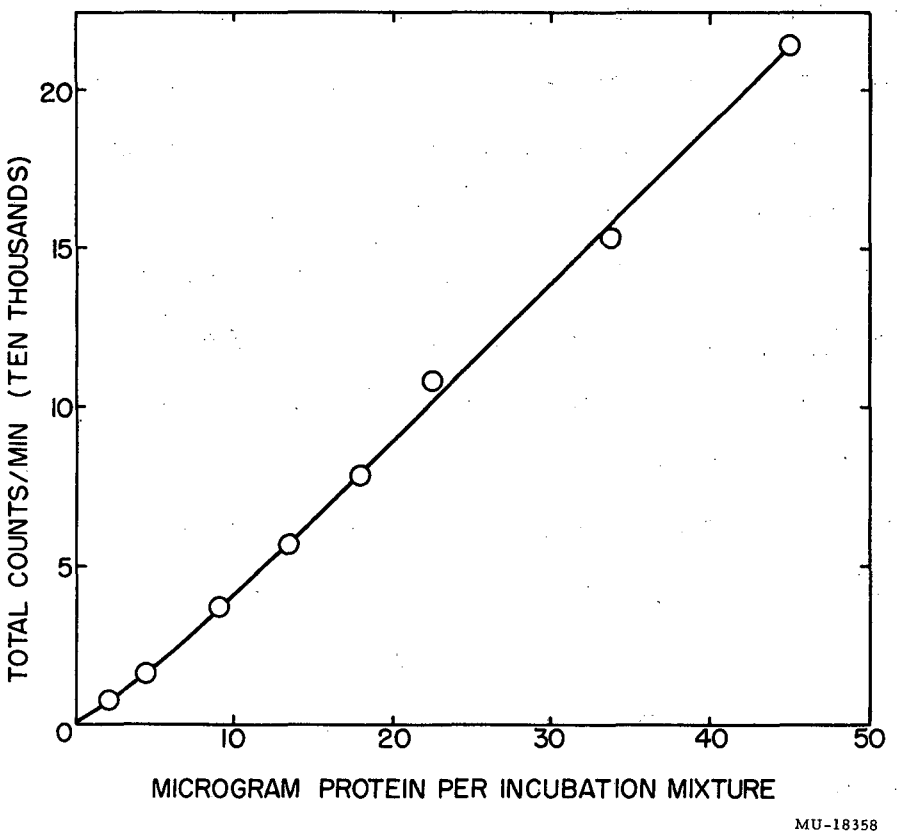


Fig. 34. Enzyme activity as a function of enzyme concentration. Assay condition: Assay III. In each incubation mixture, in μM: $NaHC^{14}O_3$, 1.35 at 20 μC; $MgCl_2$, 2.0; tris, 15 at pH 8.3; RuDP, 0.068 (carboxydismutase assay); and enzyme (32 to 46% SAS ppt of the chloroplast extract of Tetragonia, Method III), see curve above for concentrations. Final volume: 200 μl. Incubation conditions: 10 minutes at 25°C. Stable reference standard: 4,200 cpm.

while at low concentrations much of the enzyme is inactive because of the dissociation of the metal ion from the enzyme. A full account of these two cases is described elsewhere.[69]

The effect of bicarbonate concentration on enzyme activity is shown in Fig. 35. Since the extent of the reaction was very large, it was not possible to give an accurate value of the Michaelis constant for bicarbonate. The bicarbonate concentration at which the carboxydismutase reaction rate is one half the maximum rate is at least greater than 0.006 M. This value compares well with those quoted by other investigators for the enzyme system from Spinacea; viz., 0.01 to 0.02 M.[6,82] The significance of these results is discussed in a later section in terms of the specificity of the enzyme (see Specificity).

The influence of varying concentrations of RuDP on the carboxydismutase activity is shown in Fig. 36. The activity is proportional to the substrate concentration at levels less than 9×10^{-5} M and reaches a maximum at 1.35×10^{-4} M RuDP. Above this concentration, the enzyme is inhibited. Because of insufficient points in the lower concentration region, a double reciprocal plot of reaction velocity against RuDP concentration (according to Lineweaver and Burk[57]) provides no new information. Horecker's group, however, were able to analyze their kinetic data by this method. They found that, for the Spinacea carboxylase system, the K_s is 2.5×10^{-4} M and the maximum velocity of the reaction is about twice that of the observed maximum.[6] Whether these constants are the true Michaelis constant for the enzyme-RuDP complex and the true maximum velocity of the reaction, remains a question, since the mathematical treatment of this problem depends on a number of assumptions.

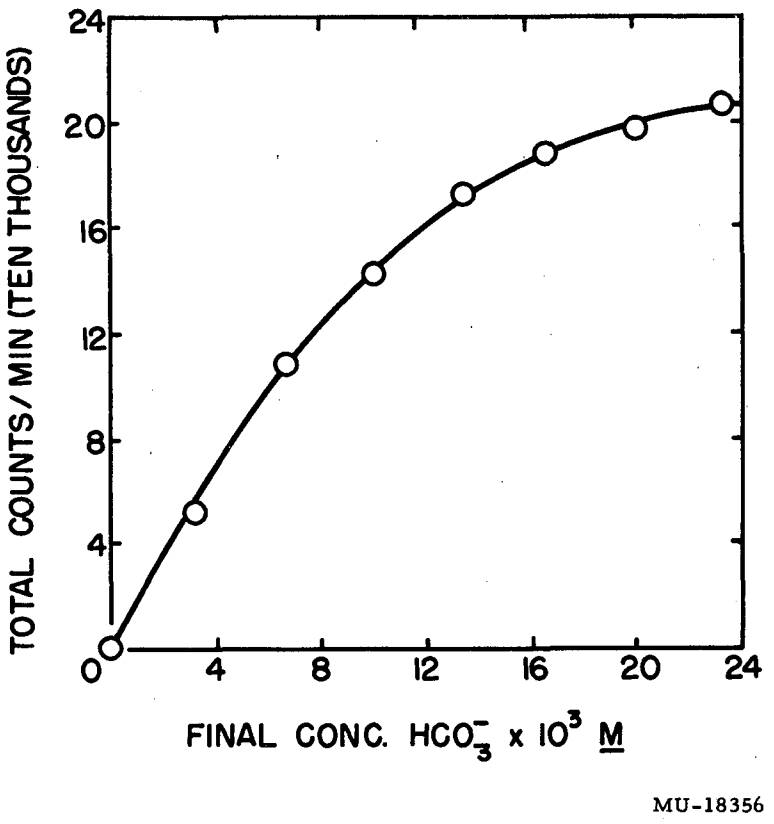


Fig. 35. The dependence of enzyme activity on bicarbonate concentration. Assay condition: Assay III. In each incubation mixture, in μM: tris, 15 at pH 8.3; $MgCl_2$, 2.0; RuDP, 0.033; $NaHC^{14}O_3$, see curve above 15 μC per μM; and enzyme (32 to 35% SAS ppt of the crude extract of Tetragonia, Method I), ca. 30 μg. Final volume: 200 μl. Incubation conditions: 10 minutes at 25°C.

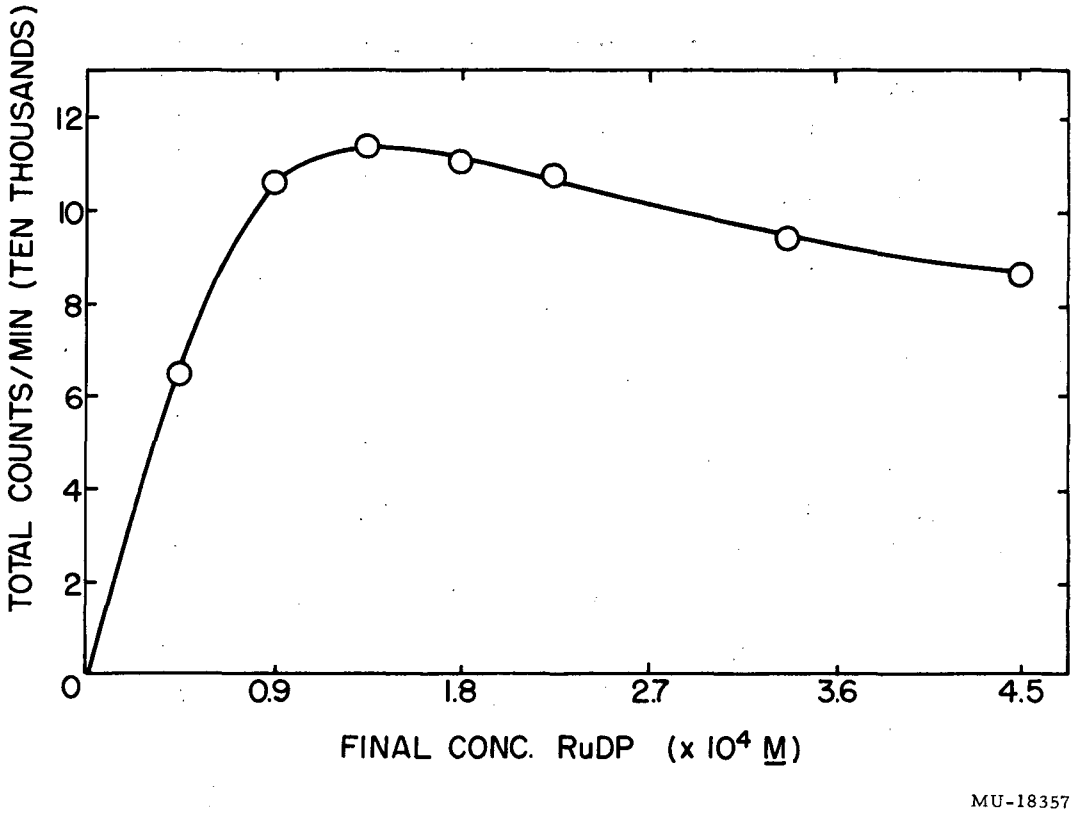


Fig. 36. The effect of RuDP concentration on the enzyme activity. Assay condition: Assay III. In each incubation mixture, in μM: $NaHC^{14}O_3$, 1.35 at 20 μC; tris, 15 at pH 8.3; $MgCl_2$, 2.0; RuDP, see curve (determined by carboxydismutase assay); and enzyme (32 to 46% SAS ppt of the chloroplast extract of Tetragonia, Method III), 23 μg (colorimetric determination). Final volume: 200 μl. Incubation conditions: 10 minutes at 25°C. Stable reference standard: 4,200 cpm.

Two mechanisms have been proposed to explain the inhibition of an enzyme by high concentrations of substrates; namely, the two point attachment theory[113] and the effective-ineffective enzyme substrate complex theory.[69,109,114] Both of these mechanisms require that the enzyme have two available sites to anchor the substrate. They differ only in that the former mechanism calls for the attachment of the substrate molecule to the enzyme at two centers on the protein (perhaps in a complementary fashion) for the enzyme-substrate complex to be active. On the other hand, in the latter mechanism, the substrate needs to be bound only to one site, but necessarily to the "right" or "effective" site in order that the enzyme-substrate complex be fully active. If the substrate is anchored to the enzyme at the "wrong" or "ineffective" site, then the enzyme is no longer active even when the ineffective-enzyme-substrate complex combines with a second molecule of substrate at the "effective" site.

When both mechanisms are interpreted mathematically, an identical expression is obtained, except that some of the constants are combinations of association constants of different reactions in the scheme of the mechanism:

$$1/v = 1/K_sV(S) + (K_1+K_s)/K_sV + K_2(S)/V \qquad \text{eq. VII}$$

where v is the initial velocity of the reaction at any substrate concentration (s), V is the maximum velocity of the reaction (proportional to the initial concentration of the enzyme), K_s is related to the association constant of the active enzyme-substrate complex, K_1 is the constant involving the association of the enzyme with a substrate molecule to form an inactive complex and K_2 is the constant involving the association

of inactive enzyme-substrate complex with a second substrate molecule. (The application of the steady-state method on one of these mechanisms also gave a mathematical expression of more or less the same form,[114] but it is beyond the scope of this thesis to describe, in detail, the differences).

Equation VII shows that at low substrate concentrations, a plot of 1/v vs 1/(S) and at very high substrate concentrations, a plot of 1/v vs (S) should give a straight line. In both cases the intercept is $(K_1 + K_s)/K_sV$, but the slopes of the straight line portion of the curves are $1/K_sV$ and K_2V respectively. Clearly, it is seen that a Lineweaver-Burk type plot does not yield a simple function such as the maximum velocity of the reaction or the Michaelis constant for the active enzyme substrate comples.

When the data shown in Fig. 36 is plotted with 1/v against (S), a straight line is obtained at high substrate concentrations. The intercept is 7×10^{-6} reciprocal counts per minute and the slope is 0.0104 $cpm^{-1}\underline{M}^{-1}$. The only information that these data show is that these mechanisms are consistent with the results of the kinetics. If the mechanism is true, then at very high RuDP concentrations the enzyme should be completely inhibited. Confirmation of this prediction must await experiments along this line.

The Effect of pH on the Carboxydismutase Activity. Carboxydismutase shows a typical bell-shaped curve of pH versus activity, with a pH "optimum" at 8.0, Fig. 37. Whether the loss of enzymic activity on both sides of the pH "optimum" is due to denaturation of the protein remains to be determined. The fact is that when the enzyme is subjected to isoelectric precipitation, a partial inactivation of the enzyme takes place. This suggests that, on the acid side of the "optimum," the low activity may be accounted for by the partial denaturation of the protein. The picture is not so clear on the alkaline side and needs to be worked out further.

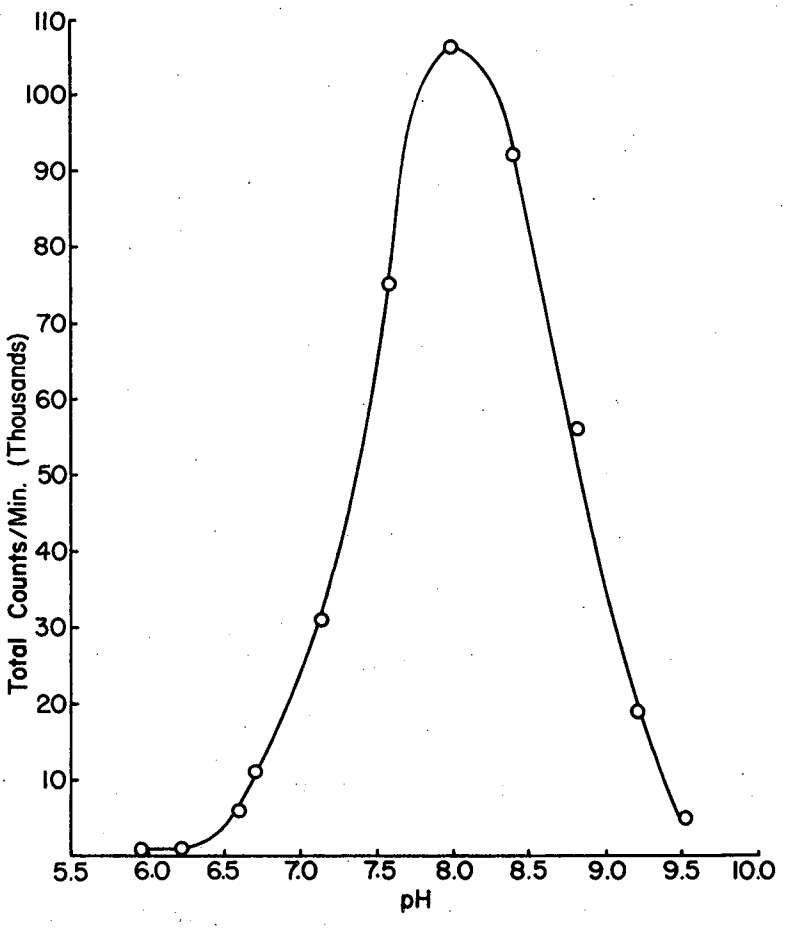


Fig. 37. The effect of pH on the activity of carboxydismutase. Assay condition: Assay III. In each incubation mixture, in μM: buffer, 15 (see experimental section for details); $MgCl_2$, 2.0; $NaHC^{14}O_3$, 1.33 at 20 μC; RuDP, 0.033; and enzyme (32 to 35% SAS ppt of the crude extract of Tetragonia, Method I), 30 μg. Final volume: 200 μl. Incubation conditions: 10 minutes at 25°C.

Stability of the Enzyme. It was mentioned in a previous part of this discussion that carboxydismutase is active after standing for one day at room temperature. Actually the stability of the enzyme is dependent on the stage of purification of the enzyme, the temperature at which the enzyme is stored, the state of the enzyme (solid or solution), the pH (cf. previous paragraph), and other factors associated with the stability of proteins. Generally if the protein is in a dialyzed crude stage and is a lyophilized powder or kept in a frozen state (in solution), the enzyme preparation is most stable. For example a frozen solution of a crude extract of Chlorella stored for more than 3 months at -20° C, remained fully active after one thawing. In fact a lyophilized powder of the same Chlorella extract, although standing at room temperature for greater than two years, was still active.

The remarkable stability of the enzyme is illustrated further by the following examples: 1) a solution of an unfractionated chloroplast extract of Tetragonia, stored overnight at room temperature was fully active; 2) a solution of an enzyme prepared by the Crude Extract Method, stored at -20° C for five months, retained 40% of its activity; 3) a lyophilized sample of this solution stored at -20° C for greater than 2 years, still had 38% of its original activity; and 4) a lyophilized preparation of the enzyme prepared by the Chloroplast Extract Method, stored under the same conditions as above, had 35% of its original activity.

Another factor to consider is the effect of radiation on the activity of the enzyme. For instance Boichenko and Zakharova found that when leaves of Primula obconica were exposed for 1 minute in the light to 3% CO_2 containing 0.08% $C^{14}O_2$, the total CO_2 assimilated was 0.026 mg. When the leaves were exposed to the same total concentration of CO_2, but in the presence of only 0.004% $C^{14}O_2$, the uptake of total CO_2 was 0.27 mg, a change

of 10-fold.[115] Their findings, however, do not agree with those of Holm-Hansen, et al.[116] Nevertheless in order to insure that no serious effects can arise from radiation on carboxydismutase, an experiment was conducted in which the enzyme was exposed for 10 minutes at 25° C to varying amounts of radiocarbon. In this experiment the total bicarbonate concentration was kept at 1.3 μM per incubation mixture and the radioactivity added was varied from 1 to 20 μC per incubation mixture. The results showed that in all of these reaction mixtures, the total carbon fixed was the same. It is conceivable that lower levels of radiocarbon might show some difference.

Reversibility of the Carboxydismutase Reaction. Since the assay is based on a measure of the radioactivity fixed, one way of detecting the radiocarbon is via radioautography. With this technique it is possible to detect as little as 10 counts per minute (when counted on the paper chromatogram) of a spot of about 1 to 2 cm^2 area after 6-weeks' exposure on X-ray film. Accordingly it seemed appropriate to apply this method for testing the reversibility of the carboxydismutase reaction. Despite the sensitivity of this method, however, there was no trace of labeled diphosphate (arising from the reversal of the carboxydismutase reaction) even when chromatograms of carboxydismutase reaction mixtures containing PGA-C^{14} (50,000 cpm, counted on paper) were exposed to X-ray films for greater than 2 months. The results are not too surprising when one calculates the amount of RuDP which can result from the reversal of the carboxylation reaction. Thus the concentration of RuDP that would be at equilibrium with 9.0×10^{-3} M bicarbonate, 1.4×10^{-4} M PGA and 1.0×10^{-8} M H^+ is calculated to be 2.5×10^{-15} M. If the specific activity of the labeled bicarbonate is about 15 μC per μM, and if the reversal of the reaction puts one tagged carbon atom into the resultant RuDP, then one

could expect only 1 count per 4 days; indeed a small number! These calculations are based on the free energy of the carboxydismutase reaction quoted by Bassham and Calvin;[45] namely, -8.9 kcal at pH 7.0. It is seen, therefore, that even with this extremely sensitive method of detection, the goal is far from being realized.

Specificity. A study of the specificity of this enzyme system must necessarily involve the two substrates, the CO_2 acceptor and the CO_2 donor. In the first category the compounds which have been tested as possible substrates were all sugar phosphates, among them being RuDP, ribulose monophosphate, RMP, hexose diphosphate and others.[6,25a] Only RuDP proved to be effective; xylulose-1,5-diphosphate failed to serve as a substrate.[117]

In contrast to the acceptor system, the donor system requires a special consideration. Because the reaction is normally carried out at the pH "optimum" (i.e., pH 8), most of the carbon dioxide in the incubation mixture is in the form of bicarbonate. This does not mean, however, that the actual carboxylating specie is bicarbonate. The pH "optimum" may only be a consequence of one or a combination of many factors; for example, the change in stability of the enzyme as a function of pH, the ionization of groups near the active sites of the enzyme and the ionization of the substrates. What then is the actual carboxylating specie? The answer to this question may lie in some comparisons of the *in vivo* and the *in vitro* maximal rates of CO_2 fixation and the CO_2 concentrations required for these rates.

So far the highest rate of CO_2 fixation by an *in vitro* carboxylase system was that found by Weissbach, *et al.*[6] In their assay system containing 0.05 M bicarbonate at pH 8.0, the rate of formation of PGA is 52 μM per 10 minutes incubation per mg of protein. These values are to be compared with those of the intact leaf, but first the conditions of the *in vivo* experiment must be normalized to those of the *in vitro* experiment.

In this laboratory it has been found that the maximum rate of CO_2 assimilation by intact leaves of Spinacea, in the presence of 0.8% CO_2 in air (down to 0.4% CO_2), is 190 μM per mg chlorophyll per hour.[118] With the chlorophyll to nitrogen weight ratio being between 0.32 and 0.35,[119] this rate becomes 62.5 μM of CO_2 fixed per hour per mg of nitrogen. If the plant protein is 15% nitrogen, the rate of fixation is then 9.4 μM of CO_2 per hour per mg protein. Since two moles of PGA are formed from each mole of CO_2, the rate of formation of PGA is 18.8 μM per hour per mg protein or 3.1 μM per 10 minutes. Weissbach et al., estimated that the carboxylation enzyme constitutes about 10% of the soluble protein of spinach leaves;[6] this value gives a rate of formation of PGA of at least 31 μM per 10 minutes per mg of protein.

If, however, 0.8% CO_2 is in equilibrium with a pH 8 buffer, the concentration of the resultant bicarbonate solution would be 1.1×10^{-2} M. This concentration of bicarbonate gives exactly half maximal velocity in the Weissbach et al., system or, in other words, 26 μM of PGA formed per 10 minutes per mg protein.* Therefore the rates of formation of PGA in the in vitro carboxydismutase system agrees quite well with that of the in vivo system.

The major fallacy of this argument is that the pH of the surrounding medium within the leaf cells may not be pH 8, but closer to pH 7. For example Arnon and Johnson found that the pH of the expressed sap of a variety of leaf tissues is not over 7.[120] (In this laboratory we have found that the pH of the expressed sap of Spinacea is 6.4). The unknown

*Racker recently found in spinach leaf homogenate, RuDP-carboxylase which can carboxylate 150 μM of RuDP per hour per mg of chlorophyll.[111] This number corresponds to 25 μM of PGA formed per 10 minutes per mg protein.

factor is the contribution of the organic acids (located in the vacuoles of the cells) towards the lowering of the pH of the expressed sap. If the pH of the medium surrounding the carboxylation enzyme is chosen to be 7.0, then under a 0.8% CO_2 atmosphere, the total concentration of the various carbon dioxide species would be 1.4×10^{-3} M (1.1×10^{-3} M bicarbonate and 2.7×10^{-4} M dissolved CO_2). When this value is substituted into the Michaelis-Menten equation along with the values for the Michaelis constant and the maximum velocity of the reaction ($K_s = 1.1 \times 10^{-2}$ M and V = 52 μM of PGA formed in 10 minutes per mg of protein), the rate of formation of PGA reduces to 5.8 μM per 10 minutes per mg of protein. Thus the rate is about 19% that of the intact leaf (or less, depending on whether the amount of soluble protein is substantially less than the total protein content of the leaves). This discrepancy is not too serious when one considers the handling that the carboxylation enzyme undergoes during its preparation.

A much larger discrepancy is present in the minimum concentration of bicarbonate required for the maximum rate of CO_2 uptake by the *in vivo* and the *in vitro* systems. This concentration is referred to as the saturation concentration and is 5.6×10^{-4} M in the leaf, corresponding to 0.4% CO_2 in equilibrium with a pH 7 medium; while in the isolated carboxydismutase system, the saturation concentration of bicarbonate is 2.2×10^{-2} M. Hence the *in vitro* saturation concentration is about 40 times that of the *in vivo* system. Although there is a host of reasons for this difference the simplest explanation could be that the dissolved CO_2 is the actual carboxylating specie. If one considers only the saturation concentration of dissolved CO_2, it would be 1.4×10^{-4} M for the *in vivo* system and 5.2×10^{-4} M for the *in vitro* system. These numbers are quite comparable, the

difference in the saturation concentration being accounted for by the differences in the nature of the isolated enzyme as compared with the enzyme in situ. Further evidence consistent with this hypothesis is found in the work of Park and Epstein.[121] By studying the ratio of the isotopes C^{13} to C^{12} in PGA, resulting from enzymic carboxylation of RuDP with bicarbonate of known isotopic composition, they implicated dissolved CO_2 as the actual carboxylating specie.

Because a small quantity of carbonic acid (H_2CO_3) is in equilibrium with dissolved CO_2, the possibility that the former is the actual carboxylating specie must be considered. Experiments with added carbonic anhydrase, an enzyme that catalyzes the rapid conversion of dissolved CO_2 to carbonic acid, showed no effect on the rate of fixation of radiocarbon from bicarbonate by the in vitro carboxydismutase system. These results, however, do not exclude the likelihood that the carboxydismutase preparation used in these assays already contained carbonic anhydrase.

It should be emphasized that all of the above discussion on the carboxylation rate of the isolated enzyme system are based on the data obtained by Horecker and his coworkers.[6] In the studies described in this report, no effort was made to obtain maximum rates. Hence the highest rate obtained so far is only about 4.7 μM of PGA per 10 minutes incubation per mg protein at a bicarbonate concentration of 0.0067 M. This rate is about one-fourth of that obtained by Horecker under equivalent conditions, the latter being corrected for by substituting into the Michaelis-Menten equation the appropriate parameters already quoted. The lower specific activity of this enzyme may be due to the lower specific activity activity of the starting material; see "Preparation of Carboxydismutase. Enzyme from Tetragonia expansa:" The most active starting material from Tetragonia has a specific activity of 1 unit, compared with 5.5 for the starting material from Spinacea (used by Horecker).

The disagreement in the carboxylation rates and in the bicarbonate saturation concentrations between the intact leaf and the isolated enzyme system can also be explained in terms of the denaturation of the enzyme, the lack of necessary cofactors and the possibility of another type of CO_2 substrate. In this last category is the so-called active CO_2 which has been considered as a natural substrate for the carboxydismutase system.[122] Initially various forms of active CO_2 have been suggested as intermediates in at least three apparently different enzymically catalyzed reactions. These reactions, all having an absolute requirement for ATP and bicarbonate, are the carboxylation of 1) propionyl CoA to form methylmalonyl CoA,[3] 2) β-hydroxyisovaleryl CoA to form β-hydroxy-β-methylglutaryl CoA,[123] and 3) β-methylcrotonyl CoA to form β-methylglutaconyl CoA.[104] For these reactions the active forms of CO_2 are, respectively, phosphoryl carbonate, adenyl carbonate, and a carboxylated biotin derivative.

Actually phosphoryl carbonate was suggested as the intermediate only for the propionyl CoA carboxylase system of animal tissues. Involved in the propionate metabolism of the bacteria, Propionibacteria, is another type of active CO_2, the carboxylated biotin compound.[124] Presumably, this biotin derivative is identical to the one proposed for the methylcrotonyl CoA carboxylase system.[104] In either case, these Co_2 intermediates have not been isolated from the propionate systems.

Adenyl carbonate has not been isolate also; however, its monoethyl ester has been synthesized and claimed to be a substrate for the "CO_2-activating" enzyme of the hydroxyisovaleryl CoA carboxylase system.[125] Recent results have cast doubts as to the validity of this claim as well as to the plausibility of this proposed active CO_2. It was found that ADP and a compound different from orthophosphate or pyrophosphate were formed by the "CO_2-activating" enzyme in the presence of ATP and bicarbonate

when NH_2OH was added.[126] It was later established that the compound was hydroxylamine phosphate.[2]

The third type of active CO_2, the carboxylated biotin, has actually been isolated from the enzymic reaction mixture, but only when biotin was added as a substrate in place of methylcrotonyl CoA.[104] The compound was stabilized by adding diazomethane to the incubation mixture and isolated as the dimethyl ester. This ester, when labeled with radioactive bicarbonate, coincided exactly with synthetic dimethyl ester of carboxylated biotin after chromatography on paper. (The synthetic material was obtained by treating the methyl ester of biotin with methyl chlorocarbonate, yielding the methyl ester of an allophanate-type derivative of biotin). Moreover upon mixing the labeled biotin derivative derived from the enzymic mixture with the synthetic compound, and then recrystallizing the material several times, constant specific activity was obtained, indicating that both synthetic and enzymically produced biotin derivatives are one and the same.

It is pertinent to metnion here another ATP dependent carboxylation system which involves biotin. This enzyme system forms long-chain fatty acids from acetate plus CoA (or acetyl CoA), ATP, bicarbonate, Mn^{++} and triphosphopyridine nucleotide.[127,128,129] No suggestions as to the nature of the active CO_2 have been forwarded other than the fact that biotin is required.[127,128]

Despite the fact that the role of the mixed anhydrides of carbonic and phosphoric or adenylic acids is in doubt for those enzyme systems mentioned above, it nevertheless seemed worthwhile to synthesize the ethyl esters of these compounds in order to test them in the carboxydismutase system. From the outset, however, the syntheses of these mixed anhydrides were totally without success.[130]

Consider, for example, the attempted synthesis of phosphoryl carbonate ethyl ester. An ethereal suspension of monosilver phosphate was treated with ethyl chlorocarbonate in a manner similar to the preparation of acetyl phosphate.[61] After isolating the silver salt of the product, it was analyzed for phosphate, silver, acid and alkaline-labile CO_2, C-CH_3, and acyl phosphate. Furthermore, elementary and infrared analyses were performed. The results show that, initially, the product was probably, in part, disilver phosphoryl carbonate ethyl ester, but it was rapidly converted to disilver ethyl phosphate. This conversion is similar to the decomposition of the ethyl esters of mixed carbonic-carboxylic anhydrides in which CO_2 and the ethyl ester of the carboxylic acid are produced.[131] Probably most of the monoethyl phosphate was formed during the reaction owing to, perhaps, too high a reaction temperature (i.e., greater than 0° C).

The synthesis of adenyl carbonate ethyl ester was attempted by first preparing monosilver adenylic acid. The latter was obtained by adding one equivalent of $AgNO_3$ to a solution of adenylic acid which was previously adjusted to pH 6.0. The precipitate was collected, washed with ethanol, acetone, and ether, and then dried at room temperature in the dark. The monosilver adenylic acid, suspended in ether, was treated with ethyl chloroformate. After removing the excess ethyl chloroformate by ether extraction, the product was precipitated with silver nitrate, washed with organic solvents, and dried in air in the dark. The product was analyzed as in the case of disilver ethyl phosphate. The results showed that the product is not monosilver adenyl carbonate ethyl ester, but disilver adenylic acid.

Although the methods given above fail to produce the desired compounds for studies in the carboxydismutase system, there is one active CO_2 compound that has actually been made; namely, carbamyl phosphate. This compound was prepared by reacting potassium phosphate with potassium cyanate.*[132]

* Prepared in this laboratory and generously supplied by Dr. J.A. Bassham for these experiments.

Phosphate and ammonia nitrogen analyses showed that the product of this reaction is 90% carbamyl phosphate. The latter was tested in an assay mixture containing RuDP-C^{14},Mg^{++}, and carboxydismutase. There were also three control assay mixtures; all containing RuDP-C^{14} and Mg^{++}: 1) control 1 with no enzyme, but with unlabeled bicarbonate, 2) control 2 with both enzyme and unlabeled bicarbonate, and 3) control 3 with enzyme, but no unlabeled bicarbonate. All mixtures were incubated 10 minutes at 25° C, using conditions of Assay III (cf. experimental section). The products were examined quantitatively after paper chromatography in two dimensions in the usual solvent systems. The results showed quite clearly that carbamyl phosphate produces no striking differences from that of bicarbonate alone. In fact, carbamyl phosphate forms less PGA than in control 3, but more than in control 1. There is one small difference in the chromatographic pattern of the carbamyl phosphate-containing incubation mixture. There is one extra spot in between the diphosphate and the pentose monophosphate regions. Incubation mixtures without carbamyl phosphate do not give this spot. It is not clear whether this compound is produced by enzymic action on RuDP (in the presence of carbamyl phosphate) or whether it is derived non-enzymically.

One other compound was tested as substrate for the carboxydismutase system. This compound, in a level more reduced than carbon dioxide, was formic acid. The fact that when carbon-labeled formic acid was fed to algae, the label was located mainly in the C-4 position of starch glucose suggests the importance of this compound in photosynthesis.[32] In addition, Quayle and Keech showed that in Pseudomonas oxalaticus, formic acid may have a role in the formation of the carboxydismutase activity.[133] When the bacteria was grown in medium containing oxalic acid as the sole carbon source, no carboxydismutase activity was present in the bacterial

extracts. On the other hand, when the bacteria was grown in medium containing formic acid, the bacterial extracts contained carboxydismutase. In the light of these experiments, it was of interest to test formic acid as a substrate for carboxydismutase. Carbon-labeled formate was incubated with carboxydismutase, Mg^{++} and RuDP for 10 minutes at 25° C. The same mixture without RuDP served as the control. It was found that no fixation of formate occurred. It was concluded, therefore, that carboxydismutase is specific for bicarbonate only (or dissolved CO_2)

Occurrence of the Enzyme. The occurrence of carboxydismutase in numerous plants and microorganisms has been studied extensively by Fuller and Gibbs.[85] Their results are compiled in Table XVI, Table XVII, and Table XVIII, along with the findings of this and other laboratories.

The most striking aspect about these tables is the lack of the carboxylation enzyme in mammalian tissues. Barrón, et al., however, have evidence suggesting the presence of carboxydismutase in certain carcinoma cells.[134] This conclusion was deduced from the fact that ribose-5-phosphate is utilized more rapidly anaerobically than aerobically, and that the uptake of this compound is accompanied by a simultaneous fixation of CO_2. Moreover when tagged carbon dioxide was used, all of the label was found in the carboxyl group of lactic acid. Apparently no more work was carried out by this group along this line, so that the above conclusions must be considered as tentative. An extract of mouse tumor, on the other hand, does not have any carboxydismutase activity, see Table XVIII.

The rather general distribution of RuDP carboxylase over a wide variety of higher plant tissues and microorganisms indicates that this enzyme is not strictly associated with the photosynthetic mechanism. It is noteworthy that buffered extracts of mature avocado leaves and Nostoc contain

TABLE XVI

Occurrence of Carboxydismutase in Extracts of Higher Plant Tissues

Description	Investigators	References
Spinacea oleracea leaves	Fuller and Gibbs, Weissbach *et al.*, Jakoby, *et al.*, Racker	85, 6 7, 82
Spinacea oleracea chloroplasts	Fuller and Gibbs, Lyttleton and Ts'o, Whatley, et al.	85, 86 135
Tetragonia expansa leaves	Mayaudon, *et al.*	62
Tetragonia expansa chloroplasts	Pon and Rabin	88
Rye grass (blades?)	Weissbach, *et al.*	6
Cabbage leaves	Racker	82
Tobacco leaves	Racker	82
Pea leaves	Fuller and Gibbs	137
Pea chloroplasts	Smillie and Fuller	138
Normal green barley seedlings	Fuller and Gibbs	85
Lettuce leaves	Pon	--
Valencia orange leaves*	Huffacker and Wallace	139
Valencia orange peels*	Huffacker and Wallace	139
Barley roots	Young	140
Pine pollens	Stanley and Young	141

*Present also in root and vesicles, but in small amounts.

TABLE XVII

Occurrence of Carboxydismutase in Extracts of Microorganisms

Description	Investigators	References
Chlorella pyrenoidosa	Quayle, et al., Kornberg et al.	25, 25a
Chlorella variegata, grown as an autotroph	Fuller and Gibbs	85
Chlamydomonas	Fuller and Gibbs	137
Green *Euglena*	Fuller and Gibbs	85
Astasia (a naturally colorless *Euglena*)	Fuller and Gibbs	85
Romeria	Fuller and Gibbs	137
Anacystis nidulans	Fuller and Gibbs	85
Hydrogenomonas vinelandii grown on an autotroph	Vishniac	85
Hydrogenomonas ruhlandii	Santer and Vishniac	142
Thiobacillus thioparus	Santer and Vishniac	143
Thiobacillus denitrificans	Trudinger	46
Thiobacillus thio-oxidans	Suzuki and Werkman	144
Rhodospirillum rubrum	Fuller and Gibbs	85
Escherichia coli, grown on xylose and carbon dioxide	Fuller and Gibbs	85
Micrococcus denitrificans, grown as an autotroph	Kornberg, et al.	145
Chromatium, strain D, grown both as an autotroph and heterotroph	Fuller and Gibbs	85
Pseudomonas oxalaticus (OX 1) grown in formate	Quayle and Keech	133

TABLE XVIII

Extracts of Organisms Containing No Carboxydismutase Activity

Description	Investigators	References
Ehrlich ascites mouse tumor cells	Lonberg-Holm and Pon	----
Seeds of *Tetragonia expansa*	Pon	----
Pea roots	Fuller and Gibbs	137
Tetrahymena gelii	Fuller and Gibbs	85
Euglena, bleached with streptomycin	Fuller and Gibbs	85
Escherichia coli, grown in glucose	Fuller and Gibbs	85
Barley seedlings, X-ray induced albino mutant	Fuller and Gibbs	85
Barley seedlings, genetic albino mutant	Fuller and Gibbs	137
Baker's yeast	Pon, Fuller and Gibbs	---,85
Mature avocado leaves	Pon	----
Nostoc muscorum	Pon	----
Button mushrooms	Pon	----
Rat liver	Pon	----
Rat brain	Pon	----
Neurospora crassa	Fuller and Gibbs	85
Chlorella variegata, grown as a heterotroph	Fuller and Gibbs	85
Pseudomonas oxalaticus (OX 1) grown in oxalate	Quayle and Keech	133

no carboxydismutase activity. It must be emphasized that the conditions for the assay of this enzyme is at pH 8 with Mg^{++} as the cofactor. It is possible that the enzymic carboxylation of RuDP to form PGA can occur in certain tissues at some other pH and may require some other metal ions as cofactor. Another possibility that must be considered is that a complete crude extract of plant tissues may contain an inhibitor of the carboxydismutase system. Hence a negative result cannot be construed as absolute proof for the absence of a carboxydismutase-like enzyme in the tissue concerned.

Of particular interest is the location of carboxydismutase within the cells of leaves. The specific activity of carboxydismutase in the extracts of Tetragonia chloroplasts being from 3 to 15 times that of carboxydismutase in the whole leaf extracts, Table V and Method III, implies that this enzyme is concentrated in the chloroplasts. While the total activity is found mainly in the whole leaf extract, see Table V, most of this activity probably originated from the chloroplasts since the isolation of intact chloroplasts depends on the grinding medium used and the method of grinding.[138] The problem of localization of the enzyme can be resolved by preparing chloroplasts from lyophilized leaf tissues in nonaqueous medium according to the method of Stocking.[75] Unfortunately a preliminary experiment along this line, using Tetragonia as the source material, failed to give a clear cut answer as to whether the carboxydismutase activity resides totally or partially in the chloroplasts or in the cytoplasm. The fault of this method seems to lie in the improper lyophilization of the leaves, the temperature being kept at 0° instead of the required -10° C. At the former temperature there is some danger of localized thawing and reformation of ice crystals, thereby breaking the structure of cellular materials. For this reason it

is subsequently difficult to isolate intact chloroplasts.

Fuller and Gibbs have shown that when chloroplasts were isolated from Spinacea leaves in 0.35 M NaCl, they contain about 70% of the total carboxydismutase activity of the leaf.[85] Moreover Smillie and Fuller have shown a clear association of the carboxydismutase activity with the chloroplasts of pea leaves.[138] This association was demonstrated by preparing the chloroplasts in a nonaqueous medium (according to Stocking) and then assaying them for carboxydismutase activity and chlorophyll content. The percentage distribution of carboxydismutase runs parallel to the percentage distribution of chlorophyll in the various fractions which precipitate in the nonaqueous solvents of different densities. Thus the percentage distribution of chlorophyll is highest in the fraction where the RuDP carboxylase activity (percent wise) is also highest. Furthermore a microscopic examination of this fraction showed it to contain the highest proportion of chloroplast.

The Mechanism of Enzyme Action. The Activation Step: An analysis of an enzymically catalyzed reaction must take into account two phases, the activation step and the reaction step. The former step involves, in its simplest form, the formation of the enzyme-substrate complex whereas in the more complicated tertiary system, the activation of the enzyme involves the sequence of combination of the various constituents of the enzyme system. For example a tertiary system containing a metal ion cofactor, M; a substrate, S; and an enzyme, E; may have at least two different sequences of combination:

Mechanism I	Mechanism II
1) $E + M \rightleftharpoons EM$	1) $M + S \rightleftharpoons MS$
2) $EM + S \rightleftharpoons EMS$	2) $MS + E \rightleftharpoons EMS$
3) $EMS \longrightarrow EM + P$	3) $EMS \longrightarrow E + P$

where P, EM and EMS are the product, the enzyme-metal ion complex and the enzyme-metal ion-sbustrate complex, respectively. The mathematical treatment of these two sequences gives the same results, namely:[69,114,146]

$$v = k\,(E)_o \,/\, \left[1 + K_m/(S) + K_m/K_{x,m}(M)(S)\right] \qquad \text{eq. VIII}$$

where v is the velocity of the reaction; k is the specific rate constant of the decomposition of EMS into EM or E plus P; K_m is the Michaelis constant; $K_{x,m}$ is the association constant for the formation of either EM or MS; and the terms in parentheses are concentrations of the respective constituents. It follows that it is not possible to determine the sequence of combination of the substrate or the metal ion with the enzyme from the usual Lineweaver-Burk analysis of the kinetic data.

It should be noted that there is a third mechanism for the formation of the enzyme-substrate-metal ion complex. This can be written as follows:

$$E + S \rightleftharpoons ES; \quad ES + M \rightleftharpoons EMS; \quad EMS \longrightarrow E + P + M$$

where ES is the enzyme-substrate complex and the rest are the same as above. This mechanism is distinguishable from Mechanisms I and II in that only the $K_m/(S)$ term in equation VIII above is altered to $K_m/(M)$. Clearly this is easily differentiated from the kinetics of Mechanisms I and II.

Despite the fact that a simple analysis of the kinetic data cannot tell the difference between Mechanisms I and II, several investigators

nevertheless attacked this problem along two lines. One approach was to compare the association constants of the metal ion for both the pure enzyme and the pure substrate with the kinetically determined constant.[146] The other approach was to measure the rate of the reaction in both the forward and reverse directions and obtain kinetically the value of $K_{x,m}$.[147] If the same value is obtained for the reaction in both directions, Mechanism I is favored, whereas if the values are different (corresponding to the difference in binding of the metal ion to the different substrates), then Mechanism II is favored. Both approaches have been applied for the study of the enolase system and it was found that the data are consistent with Mechanism I.

When the enzyme of the substrate is not pure or when the reaction does not proceed reversibly to any degree, the above-mentioned approaches are not applicable. Further complication is encountered when another substrate participates in the reaction. (Actually the reaction catalyzed by enolase in the reverse direction; that is, the conversion of phosphoenolpyruvate to 2-phosphoglycerate is a two-substrate reaction. The second substrate is water and therefore its concentration remains constant during the reaction). The carboxydismutase system falls within this category; namely, that both the enzyme and the substrate (RuDP) are impure, that the reaction is a two-substrate reaction and that the reaction is observed to be irreversible. In view of these complications recourse must be made to another approach for the study of the mechanism of activation of the enzyme. Fortunately there is one approach which can simplify the determination of the activation mechansim, providing that there is a time effect in the activation of the enzyme (the preincubation effect). In fact, in some cases, the time required for the activation of the enzyme may be extended over a measurable time interval by lowering the preliminary

incubation temperature. Such is the case for carboxydismutase; see for example the discussion of The Preincubation of Metal Ions with Carboxydismutase, in the Presence and Absence of Bicarbonate.

It should be noted that the existence of a time effect in the activation of an enzyme is not proof of the mechanism. Often the lag period, obtained when the enzyme is not preincubated with the metal ion, disappears when the enzyme is further purified. Furthermore the lag period may not be directly involved with the primary process of activation.[146] The interpretation of the preincubation effect, then, must be approached with due caution.

Regardless of this uncertainty, experiments were performed in which carboxydismutase was preincubated with Mg^{++}, RuDP, and $HC^{14}O_3^-$, in various sequences and combinations. The results are shown in Table XIX and Fig. 38. Clearly the enzyme is more active when it is preincubated with either metal ion or bicarbonate first than when it is preincubated with RuDP first, Table XIX. Moreover, in the presence of metal ion, the activity of the enzyme increases as a function of increasing time of preincubation with bicarbonate whereas the activity decreases as a function of preincubation time with RuDP, see Fig. 38.

One interpretation of this phenomenon is that the enzyme combines with the metal ion (or bicarbonate) first followed with bicarbonate (or metal ion) and then, finally, with RuDP. Whether the metal ion forms a complex with the enzyme before bicarbonate, or vice versa, is uncertain. The case where a magnesium-bicarbonate type complex combines with the enzyme (in effect, the simultaneous attachment of the metal ion and bicarbonate to the enzyme) is also not excluded. In the latter case the metal ion can serve as a connecting bridge between the free amino groups of the enzyme

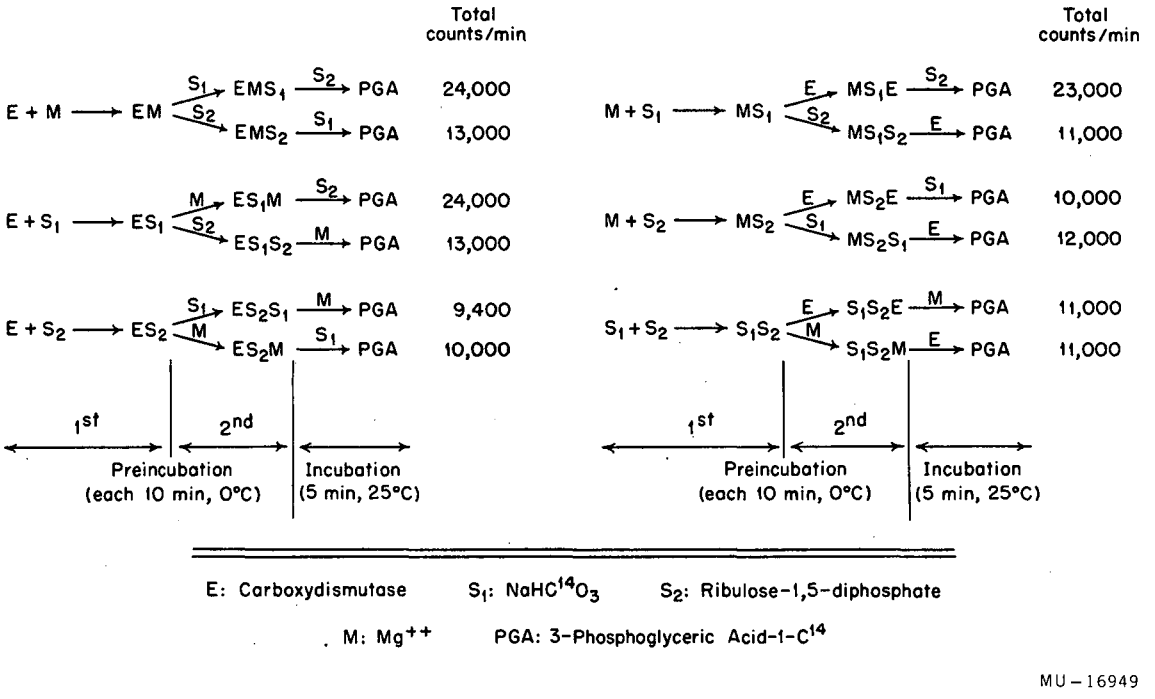


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Table XIX. The preincubation of carboxydismutase with substrates and cofactor Assay condition: Assay II. In each incubation mixture, in μM: $MgCl_2$, 2.0; tris, 15 pH 8.3; $NaHC^{14}O_3$, 1.35 at 20 μC; RuDP, ca. 0.07; and enzyme (32 to 46% SAS ppt of the chloroplast extract of Tetragonia, Method III, lyophilized powder stored at -15°C for 29 months), 25 μg. Final volume: 200 μl. Preincubation conditions: 1st component preincubated 20 minutes and 2nd component preincubated 10 minutes at 0°C with the third component. Incubation conditions: 5 minutes at 25°C. Stable reference standard: 4,600 cpm.

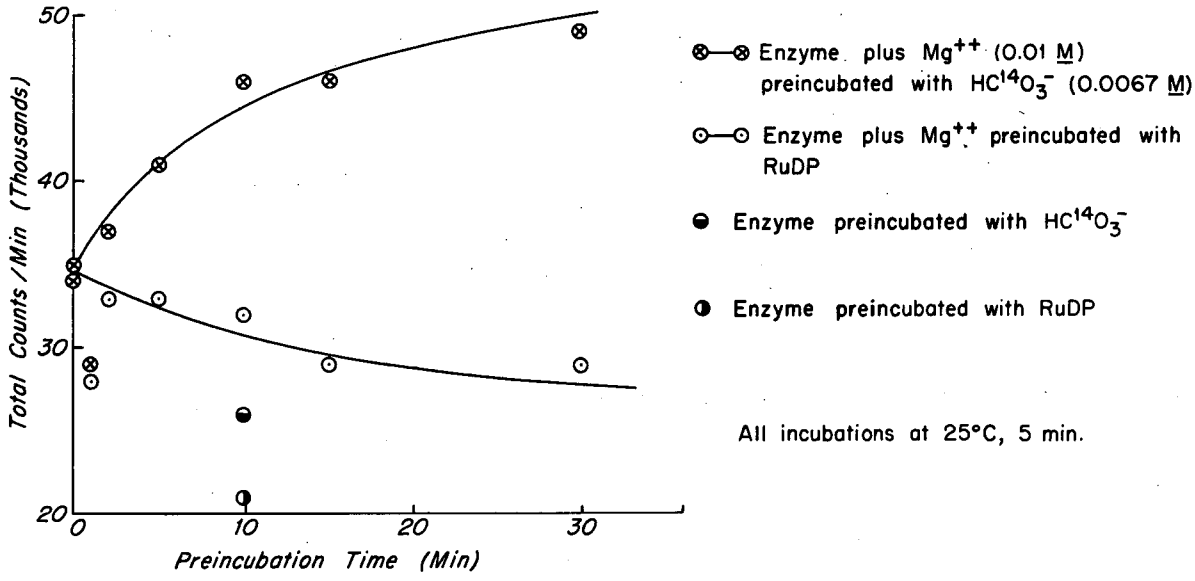


Fig. 38. The preincubation of carboxydismutase with substrates at 0°C. Assay condition: Assay II. In each incubation mixture, in μM: Enzyme (32 to 46% SAS ppt of the chloroplast extract of Tetragonia, Method III, lyophilized powder stored at -15°C for 29 months), 25 μg; $MgCl_2$, 2.0; tris, 15 at pH 8.3; $NaHC^{14}O_3$, 1.35 at 20 μC; and RuDP, ca. 0.07. Final volume: 200 μl. Preincubation conditions: Temperature, 0°C. Enzyme plus Mg^{++} case, enzyme preincubated 1 minute at room temperature with $MgCl_2$ and tris, then preincubated with one of the substrates; reaction started by addition of the second substrate. Enzyme case, enzyme preincubated with one substrate shown above; reaction started by addition of the other substrate plus $MgCl_2$. Incubation conditions: 5 minutes at 25°C. Stable reference standard: 5,800 cpm.

and bicarbonate, perhaps similar in many respects to an amine-cobaltous-carbonato complex.[98]

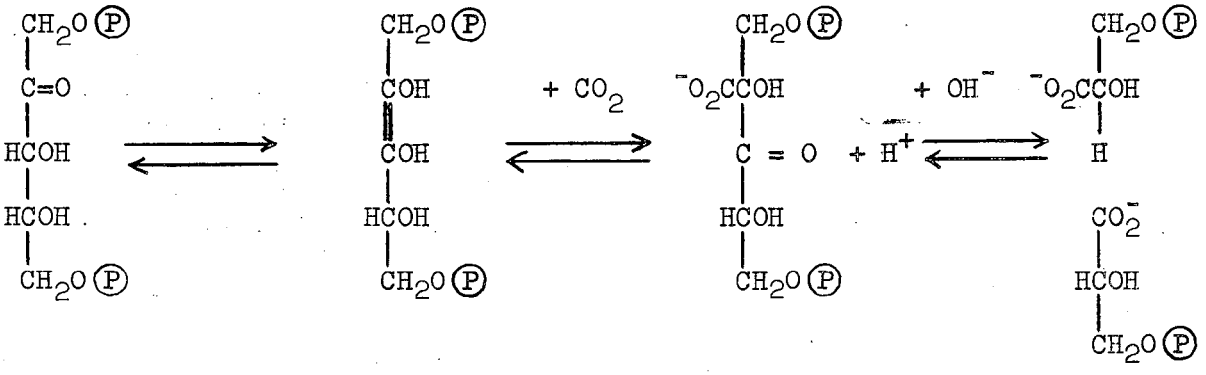
Another explanation for the activation phenomenon is that either the metal ion or bicarbonate activates the enzyme not by acting at the active sites, but at some sites adjacent to them. A conformation change of the enzyme is induced and this change is accompanied by the activation process.[2,146,148] It is perhaps noteworthy that this activation process by bicarbonate does not directly involve the bound bicarbonate for the carboxylation of RuDP. Once the enzyme is activated, the carboxylation can occur through the combination of the unbound bicarbonate with RuDP.

A third factor to consider is the presence of unlabeled bicarbonate in the reaction mixture. This is inevitable since the pH of the incubation mixture is 8.3. The amount of unlabeled bicarbonate present in the incubation mixture, excluding that already in the radioactive bicarbonate, was generally about 0.09 μM. This value represents about 7% that of the added labeled bicarbonate. If the unlabeled bicarbonate is bound to the enzyme, the preincubation effect may then be an exchange or displacement of the unlabeled bicarbonate by the radioactive bicarbonate. (Exchange reactions in native proteins proceed instantaneously,[149,150] but easily exchangeable hydrogens in native insulin requires at least 30 minutes for complete exchange with deuterium oxide at 37° C).[151]

The decrease of activity of the enzyme when it is preincubated with RuDP is due to substrate inhibition, the inhibition being larger the longer the enzyme is allowed to stand in contact with the inhibitor. Consider, for example, the inhibition of carboxydismutase by threose diphosphate, Table IX. At 8×10^{-4} M, threose diphosphate inhibits the enzyme by 20% when it is not preincubated with the enzyme while it inhibits the enzyme by 35% when it is preincubated with the enzyme for 2 minutes at

25° C. If the compound is preincubated with the more active enzyme-metal ion combination, however, then the inhibition is still larger, 55%. These experiments show that diphosphates inhibit the enzyme by competing for the active sites and for the activating metal ion.

The Mechanism of the Reaction: The mechanism of the carboxydismutase reaction was proposed by Calvin as proceeding through an enolization step followed by a carboxylation step and finally, a hydrolytic cleavage step.[45] These steps are shown as follows:



If this mechanism is true, then incubation of unlabeled RuDP in water labeled with deuterium or with tritium, whould yield in the absence of bicarbonate, RuDP which is labeled with deuterium or with tritium. On the other hand, if the incubation is carried out in the presence of bicarbonate, then the product PGA should be labeled with the tracer. Such an experiment has been carried out by Horecker's group and, in fact, the results were as predicted above.[136] One interesting fact derived from their results is that the enolization step appears to be enzymically catalyzed. It seems also that the carboxylation step requires the enzyme, since in the absence of the enzyme, some enolization of RuDP occurs. It is likely that the hydrolytic cleavage step is nonenzymic, such compounds as the β-keto acid being extremely unstable towards hydrolysis.

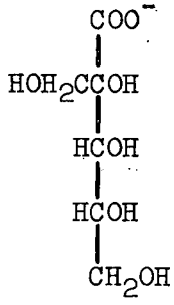
Although neither the enol form of RuDP nor the β-keto acid has been isolated from the in vitro system by Horecker's group, it is likely that these compounds are real intermediates of the reaction. Moses was able to detect, in chromatographic quantities in Chlorella, a γ-keto acid along with a compound tentatively identified as the lactone of the β-keto acid.[76] The γ-keto acid is apparently not involved in the primary carboxylation of photosynthesis since a decrease in the CO_2 pressure in the algae does not influence the concentration of this compound within the cell.[152] This fact suggests that the γ-keto acid is a stable end product arising irreversibly from either a direct carboxylation or from a precursor such as the β-keto acid. The lack of appearance of the β-keto acid may be due to the instability of this acid, it being decomposed by the subsequent work-up.

There were many attempts to demonstrate the existence of this β-keto acid in the isolated enzyme system. Conditions for the incubation were varied, among them being the use of high concentrations of PGA and RuDP as inhibitors to block the enzymic sites in the hope that the keto acid intermediate will accumulate. Another reaction was carried out in deuterium oxide in order to slow down the hydrolytic step. The reactions were stopped with a variety of agents, for example, strong acid (HCl), strong alkali (NaOH), weak alkali (NH_4OH), a reducing agent ($NaBH_4$, to convert the unstable keto acid to the more stable hydroxy acid), alcohols (methanol and ethanol), and even acid phosphatase (Polidase-S, to dephosphorylate the unstable compound, thereby, perhaps, making the product slightly more stable). Finally, a variety of analytical methods were used; some examples were long exposures of X-ray films to the carboxydismutase reaction product after paper chromatography, and paper electrophoresis

of the reaction product using bicarbonate as the buffer. In the latter method the pH of the buffer was the same as that of the incubation mixture. The incubation mixture was applied directly on the paper, previously equilibrated with this buffer, without stopping the reaction. It is interesting to note that radioactive bicarbonate, when subjected to electrophoresis in this buffer, gives a discrete radioactive spot moving towards the anode. Despite these rather extensive studies, they failed to produce any evidence for the existence of the postulated β-keto acid intermediate. Most likely, the amount of this acid is too small to be detected even by tracer techniques.

It is worthwhile to speculate further on the presence of the γ-keto acid found by Moses.[76] Apart from the explanations already proposed by this worker, it may be that this acid reflects the reservoir size of the related β-keto acid. If this is true, then the implications are that carboxydismutase *in vivo* is quite different from carboxydismutase *in vitro*. The rate of formation of the β-keto acid in the former case would be much more rapid than in the latter case; therefore accumulating keto acid and PGA, respectively.

If either the β-keto acid or the γ-keto acid is reduced with borohydride, a phosphorylated branch-chain hydroxy acid is formed. Dephosphorylation of the reduction product would produce hamamelonic acid and its isomers:



hamamelonic acid or 2-C(hydroxymethyl)-D-ribonic acid.

Related to this compound is the compound found by Kandler when he fed cyanide to algae during photosynthesis in the presence of CO_2.[30] This compound appeared in the diphosphate region of a two-dimensional chromatogram (butanol-propionic acid-water and phenol-water). When this diphosphate area was treated with acid phosphatase, two new spots appeared (in addition to other known sugars). Further characterization of the dephosphorylated material by rechromatography and paper electrophoresis at different pH's showed that these two spots were related, one being the acid and the other being the corresponding lactone. This compound was tentatively identified as hamamelonic acid.

Following the discovery of this new compound, Rabin, et al. suspected that it might have been an artifact of the experiment.[66] For example, ribulose can react with HCN to form the branch-chain cyanohydrin which, upon hydrolysis, yields hamamelonic acid.[153] It was conceivable, therefore, that the diphosphate of hamamelonic acid and its epimer (HmDP) can be obtained in a similar fashion. Indeed when RuDP was treated with cyanide, either at pH 6 or 11 for 16 hours at room temperature, the reaction proceeded to nearly 100%, allowing for impurities present in the crude RuDP starting material. The extent of the reaction was followed by taking a known aliquot portion of a radioactive cyanide addition product (HmDP-C^{14}), acidifying it with acetic acid, counting the plated sample, and comparing the counts per minute with the known specific activity of the starting labeled potassium cyanide.

Chromatography of HmDP-C^{14} in two dimensions using the routine solvent systems showed 87% of the radioactivity in the diphosphate region. When HmDP-C^{14} was chromatographed in butanol-propionic acid-water system for 48 hours, there was one main radioactive spot accompanied by a streak of

several minor spots. The R_f of the main spot is identical with that of RuDP. In the ethanol-formic acid-sodium molybdate system, a separation of RuDP from the major spot of HmDP-C^{14} was achieved, see Table XX. (The unlabeled HmDP was also cochromatographed in the latter solvent system with HmDP-C^{14}. Exact coincidence was obtained between the phosphomolybdate color and the radioactivity). In the alkaline solvent systems such as methanol-ammonia-water and n-propanol-ammonia-water, RuDP is unstable and therefore is destroyed during chromatography, one more evidence that HmDP is, indeed, different from RuDP. All of the solvent systems gave at least two spots when either HmDP or HmDP-C^{14} was chromatographed.

Before studying the properties of HmDP by electrophoresis, the conditions for the conversion of authentic hamamelonic acid to its lactone and salt or ionic form were determined. The acid was first generated from its phenylhydrazide by refluxing the latter in the presence of Cu^{++}. After 5 hours, all of the phenylhydrazide appeared to be removed as evidenced by three facts: 1) all of the starting material went into solution, the phenylhydrazide being relatively insoluble in water; 2) chromatography of the product in the routine solvent systems failed to show any sign of a spot with an R_f of the phenylhydrazide (ca. 0.9 and 0.7 in the phenol-water and butanol-propionic acid-water directions, respectively); and 3) the ammoniacal silver spray for reducing sugars gave a negative result, this spray being extremely sensitive for the detection of the phenylhydrazide since no heat is required for the test whereas for the detection of the salt of hamamelonic acid and its lactone (and even reducing sugars such as fructose) a temperature of 80° C for at least 10 minutes is required before a positive test is obtained.

TABLE XX

*P-Constants of Various Phosphate Esters during Paper Chromatography and Paper Electrophoresis

Solvent or buffer	MeOH-formic acid water	MeOH-NH_3-water	PrOH-NH_3-water	EtOH-formic acid-water-molybdate	Ammonium formate, pH 3.8
Paper (Whatman)	No. 1	No. 1	No. 4	No. 1	No. 1
Pretreatment of paper	EDTA-HCl washed	EDTA-HCl washed	Oxalic acid washed	EDTA-HCl washed	Unwashed
Compounds					
P_i	100	100	100	100	100***
RuDP	80	100?	---	30**	110
HmDP	80	90	30	40 & 20	110 & 120
3-PGA	90	110**	110	50	120
2-PGA	90	110**	100	60	---

*P-constant is defined as the migration of the compound relative to orthophosphate (P_i).[44] It was measured from the densest region of the spot to the origin and is given in percent. All percentages were rounded to the nearest ten. The P-constants of 3-PGA and 2-PGA for both methanol solvent systems are different from those quoted in the article by Benson. These differences are attributed to differences in temperature of chromatography and in the paper used.

**These compounds streaked in the direction of higher P-constants.

***All P-constants in this column are measured in the direction of the anode.

Hamamelonic acid exists as a lactone as shown by chromatography when the former is allowed to stand at pH 1 at 37° C for 16 hours. At pH 2.9 a second spot on the chromatogram is obtained, with an R_f corresponding to that of the ionic form, the ratio of lactone to salt being about 3 to 1 as estimated by the densities of the reduced silver -spots. A third spot appeared at pH 4.6, this one having the same R_f as that of the salt in the butanol-propionic acid-water direction and the same R_f as that of the lactone in the phenol-water direction. (This compound may be the free acid). Finally, at pH 6.8 or higher, only the salt form can be seen.

The rate of conversion of the ionic form of hamamelonic acid to lactone or vice versa cannot be determined by the experiments described above. Some clues, however, are provided by the behavior of these solutions towards chromatography. Thus the lactone obtained at pH 1 is not converted to the corresponding salt form during chromatography even though the mixture was exposed to solvents which had a pH of ca. 3 for greater than a sum total of 6 hours. (Recalled that at pH 2.9 the mixture showed the presence of both species, the lactone being the predominant one). Conversely, the salt obtained in the pH 6.8 (or higher) mixtures appeared not to be converted to the lactone in the presence of the same solvents. The conclusion is that the interconversion of the ionic form to the lactone, or the reverse, proceeds very slowly at room temperature at pH 3.

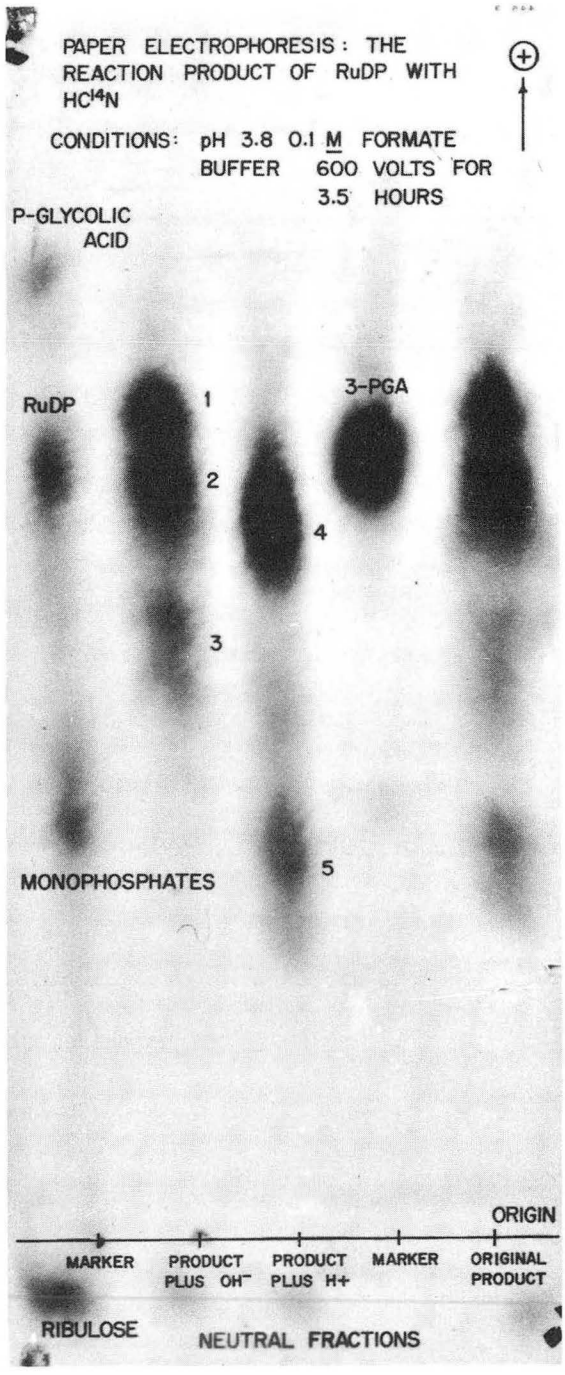
Having established that hamamelonic acid is all in the lactone form at pH 1, and all in the salt form at pH 7, after standing for 16 hours at 37° C, the reaction product of RuDP with radioactive cyanide (HmDP-C^{14} was treated similarly to obtain its lactone and salt forms. Actually HmDP-C^{14} was treated separately with 0.17 M HCl and 0.09 M NaOH for 16 hours at 37° C. (HmDP is not hydrolyzed by 0.1 M HCl at 103° C for 12 hours; it

is unlikely, therefore, that this material is hydrolyzed at the acid pH and conditions given above.) The acid and alkaline treated HmDP-C^{14} were subjected to paper electrophoresis at three pH's, giving the results shown in Fig. 39 to Fig. 41.

As in the paper chromatography of the radioactive cyanide reaction product (HmDP-C^{14}), paper electrophoresis of this product showed more than one radioactive spot. The major one has the largest mobility in the direction of the anode. It is worth indicating that RuDP and PGA have about the same mobility as the main spot of HmDP-C^{14}. In fact at pH 8.3 and 9.1, PGA has a mobility slightly greater than either RuDP or HmDP, although at these pH's the number of charges per molecule of PGA, RuDP, and HmDP are quite different, Table XXI. It appears that the mobility of these compounds is, to a large extent, governed by the charge to mass ratio of the compound.

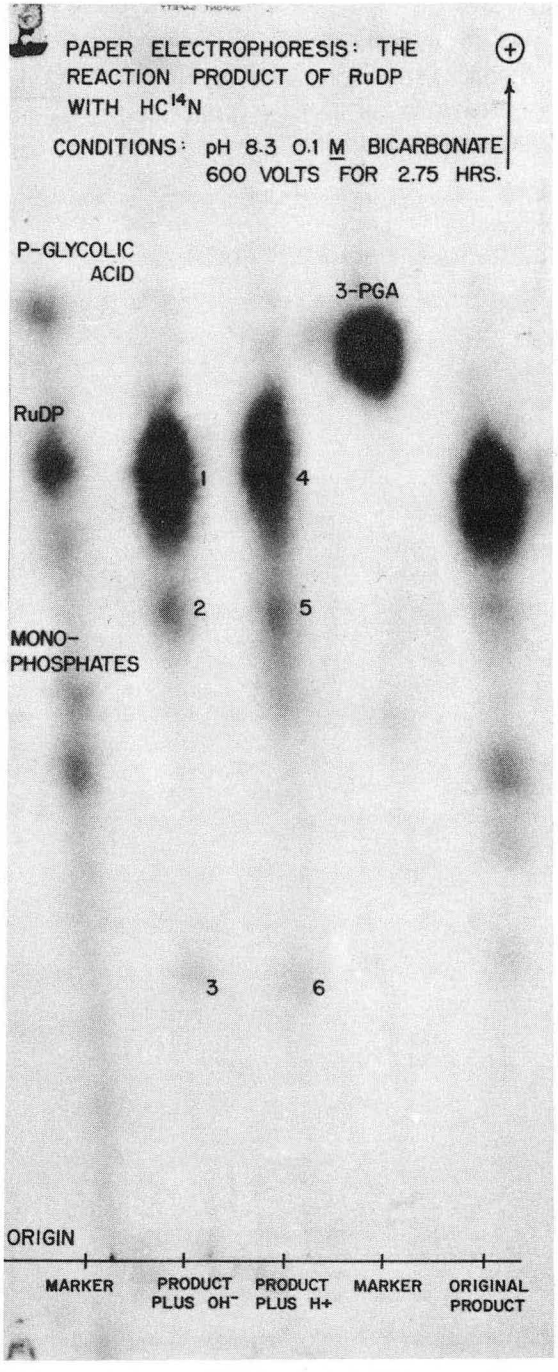
Although the reaction product of RuDP with cyanide cannot be distinguished from RuDP on purely mobility grounds, it is still possible to differentiate them by means of their phosphomolybdate colors, RuDP giving a purple color and HmDP giving a blue spot.

Both the reaction products of $HC^{14}N$ and $KC^{14}N$ with RuDP gave essentially the same complex electrophoretic pattern. Since the electrophoresis at pH 3.8 routinely gave the best resolution, only this electrophoretogram (Fig. 39) will be analyzed in detail. A casual examination of the electrophoretogram shows that the untreated HmDP (i.e., the original product) is a composite picture of the acid treated and the alkaline treated material. The mobilities of spots 1 and 2 being similar to that of RuDP suggest that they are diphosphates. That these spots are converted to one spot of lower mobility, spot 4, by acid treatment, means that the acid



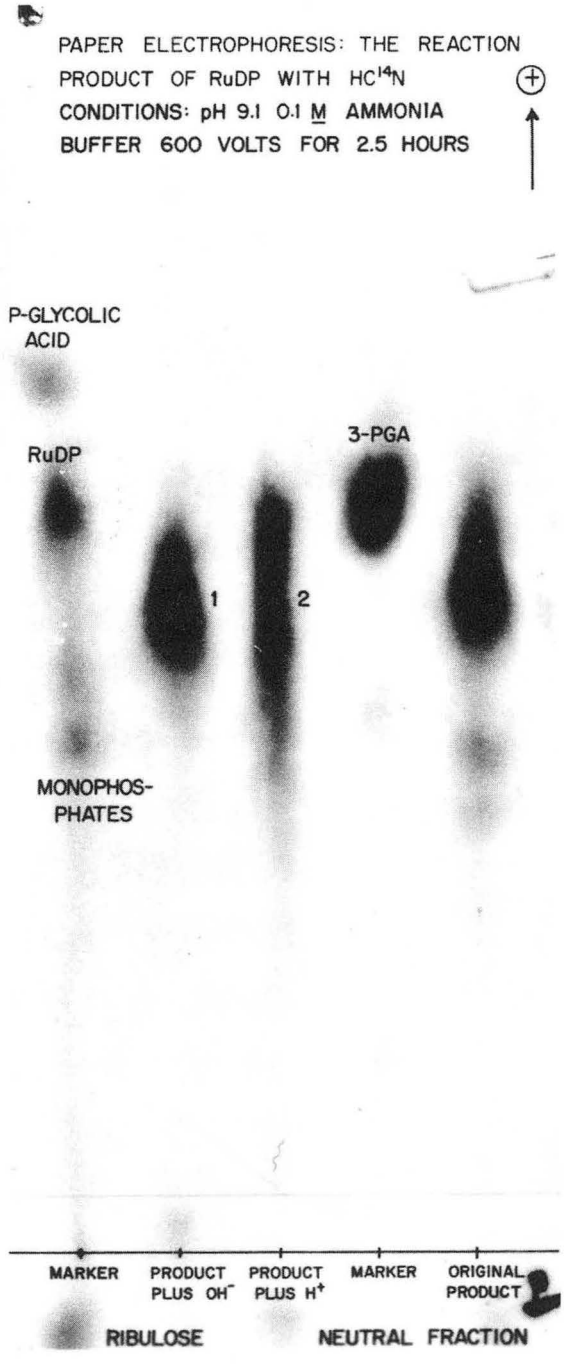
ZN-2581

Fig. 39. Radioautograph of the reaction product of RuDP with $HC^{14}N$ after paper electrophoresis at pH 3.8. Product plus OH^-: 0.33 μM HmDP-C^{14} in 55 μl 0.09 M NaOH. Product plus H^+: 0.33 μM HmDP-C^{14} in 60 μl 0.17 M HCl. Both kept at 37°C for 16 hours. Original product: HmDP-C^{14} (RuDP plus $HC^{14}N$), see experimental section. Amount of HmDP-C^{14} on the origin: ca. 0.06 μM. Electrophoresis conditions: pH 3.8, 0.1 M ammonium formate buffer at 600 volts for 3.5 hours. Temperature: cold tap water.



ZN-2582

Fig. 40. Radioautograph of the reaction product of RuDP with $HC^{14}N$ after paper electrophoresis at pH 8.3. All materials and conditions were identical to those in Fig. 39, except for the buffer and the time for the electrophoresis: pH 8.3, 0.1 M sodium bicarbonate buffer, 2.75 hours.



ZN-2583

Fig. 41. Radioautograph of the reaction product of RuDP with $HC^{14}N$ after paper electrophoresis at pH 9.1. All materials and conditions were identical to those in Fig. 39, except for the buffer and the time for the electrophoresis: pH 9.1, 0.1 M ammonium formate buffer, 2.5 hours.

TABLE XXI

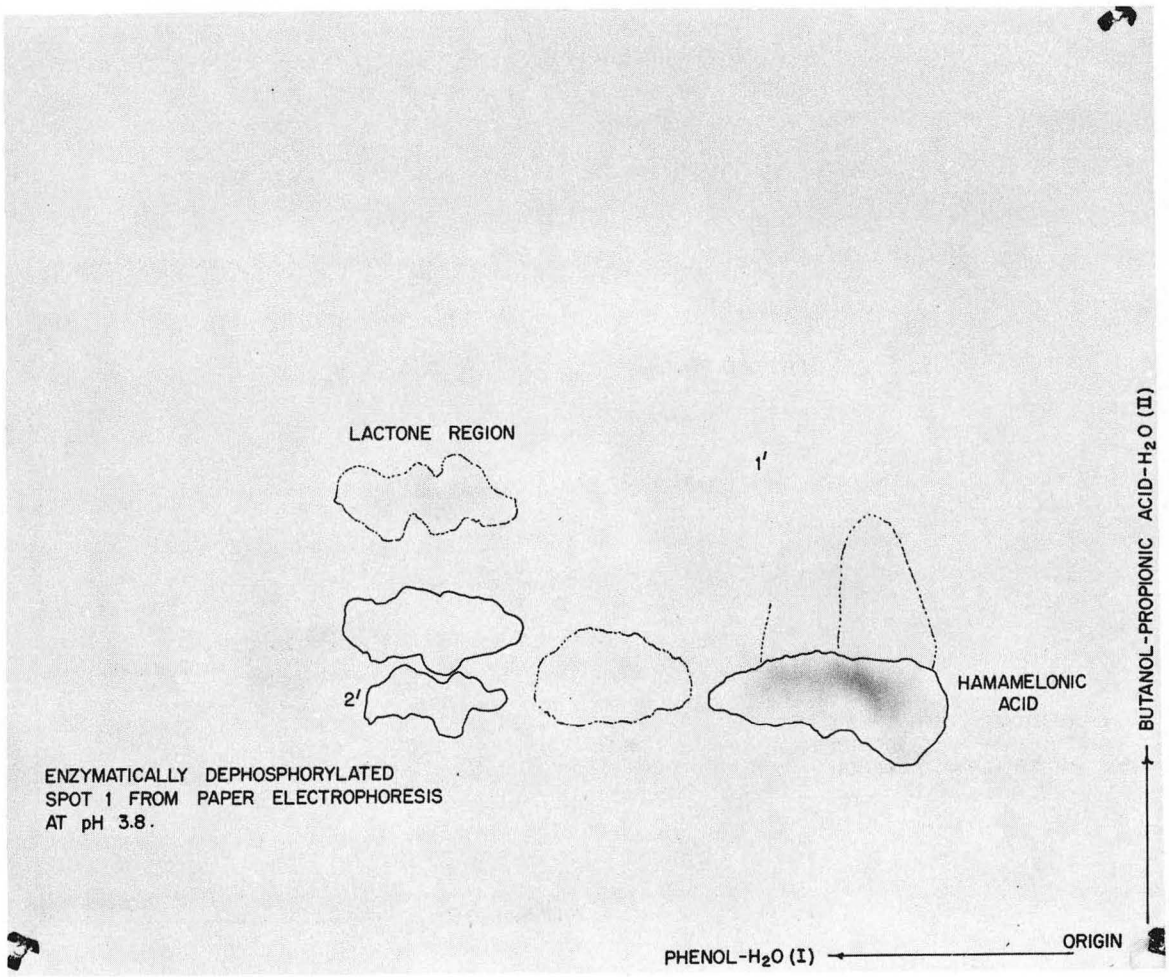
The Number of Charges and the Charge to Mass Ratios of Several Organic Phosphates at Two Different pH's.

Compound	pH 3.8 (Charges per molecule)*	pH 8 to 9 (Charges per molecule)*	pH 3.8 (Charges to mass ratio)	pH 8 to 9 (Charges to mass ratio)
3-PGA	1.5	3.0	0.0082	0.016
RuDP	2.0	4.0	0.0065	0.013
HmDP	2.5	5.0	0.0071	0.014

*For these calculations, the acid dissociation constants of the phosphate and carboxyl groups were assumed to be the same as those of orthophosphate and lactic acid, respectively.

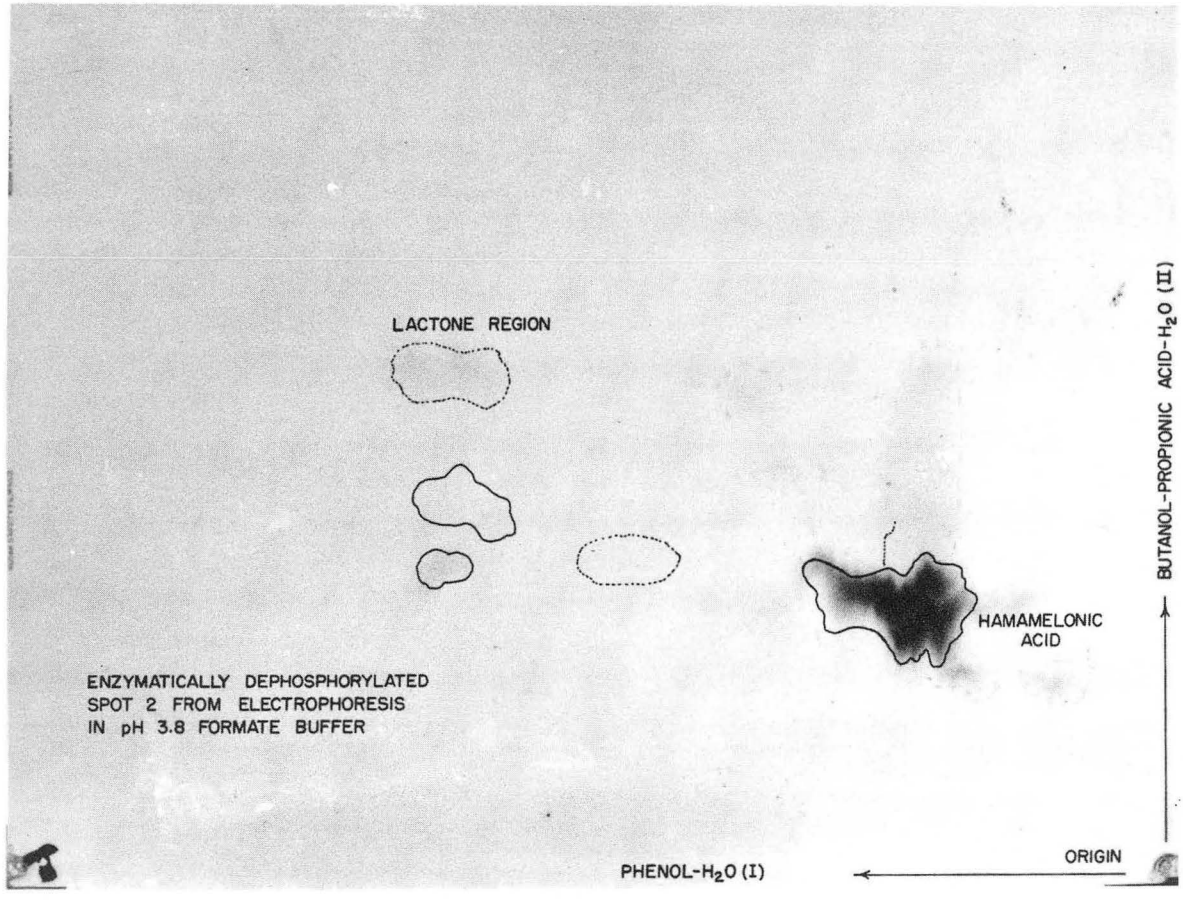
produced a change in the number of charges in these compounds. A similar result is obtained with spot 3, which upon treatment with acid yields spot 5, although in this case, the changes in the mobilities are much larger than in the previous case. The mobility of spot 5 corresponds quite well with that of the monophosphates. On the basis of the electrophoretic properties of these compounds, spots 1 and 2 could be the diphosphates of hamamelonic acid and its epimer, while spot 3 could be the monophosphates of gluconic acid and altronic acid, both arising from the reaction of cyanide with ribose-5-phosphate. The last compound probably originated from the hydrolysis of ribose diphosphate which was present as a contaminant in the crude RuDP, see discussion on RuDP Prepared by Enzymic Means. Although RuDP itself is as susceptible to hydrolysis as ribose diphosphate, little ribulose monophosphate is produced since the hydrolysis of either phosphate group proceeds at nearly the same rate. The main product of hydrolysis is then ribulose, which upon reaction with cyanide would yield the neutral cyanohydrin product. This is evident in Fig. 39, where in both acid and alkaline treated sample (also the original sample), the amount of neutral fractions remain unchanged. The possibility that spot 3 is hamamelonic acid or its epimer is excluded because acid treatment of this spot did not give a larger neutral fraction (corresponding to the lactone). Finally it can be said that spots 4 and 5 are the respective lactones of spots 1 to 3, thus accounting for the lower mobilities of the former.

Treatment of spots 1and 2 with acid phosphatase (Polidase-S) followed by chromatography of the hydrolysate gave the patterns shown in Fig. 42 and 43. Almost exact coincidence was obtained with the main radioactive spot and the salt of authentic hamamelonic acid for both hydrolysates of spots 1 and 2. Since authentic hamamelonic acid treated with pH 4.6 buffer for



ZN-2584

Fig. 42. Radioautograph of phosphatased Spot 1 from the paper electrophoresis at pH 3.8 of HmDP-C^{14}, after paper chromatography. The outlines of the marker hamamelonic acid and its lactone, along with other silver-positive material, have been drawn in. Dotted lines represent faint silver-positive material on the chromatogram. The zone labeled "Hamamelonic Acid" is actually the ionic form of this acid.



ZN-2585

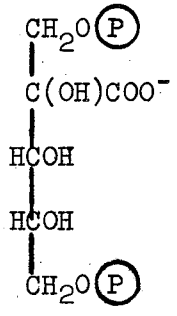
Fig. 43. Radioautograph of phosphatased Spot 2 from the paper electrophoresis at pH 3.8 of HmDP-C^{14}, after paper chromatography. Lines drawn in are described in Fig. 42.

16 hours at 37° C gave a salt to lactone ratio of 2 to 1 according to the density of their silver-positive spots, the conditions for the phosphatase action (pH 5.0 at 37° for 16 hours) should yield a slightly higher salt to lactone ratio. That this is the case is shown in both Fig. 42 and 43; however, the phosphatased spot 1 gave relatively more lactone than phosphatased spot 2. Numberous other spots (not lactone or salt) are also present in larger quantities in phosphatased spot 1 than in phosphatased spot 2, indicating that conditions are slightly different, e.g., pH of phosphatased spot 1 being slightly lower than that of phosphatased spot 2. Note that in Fig. 42, spot 1' and spot 2' are radioactive. Since the sole source of radioactivity was the cyanide used for the synthesis of HmDP-C^{14}, then the former spot may be the cyanohydrin of ribulose and the latter spot may be the free acid (i.e., the uncharged carboxylic acid of hamamelonic acid or its epimer and similar to the one present in the pH 4.6 mixture of authentic hamamelonic acid). The identity of the other nonradioactive spots is, at present, unknown.

One other fact can be deduced from these experiments, that is the facile hydrolysis of the intermediate cyanohydrin of RuDP. For example the cyanohydrin is present in minute quantities in the original RuDP-cyanide mixture. This is evident from Fig. 39, where spots 1 and 2, on treatment with acid, yield a spot of lower mobility, spot 4, indicating that the former spots have more charges than the latter. Furthermore, dephosphorylation of either spot 1 or spot 2 with acid phosphatase produces a radioactive spot which cochromatographed with the ionic form of hamamelonic acid and its epimer, Fig. 42 and Fig. 43. While these acids could arise from hydrolysis under the mild conditions (pH 5.0, 16 hours at 37° C) during the treatment by acid phosphatase, it is improbable

because the original reaction mixture already contained the diphosphates of these acids, spot 1 and spot 2 in Fig. 39. That spots 1 and 2 originated only after the action of alkali is discounted by the fact that the original reaction product gave both of these spots when subjected to paper electrophoresis, see right hand pattern of Fig. 39. Thus under the conditions of reaction of HCN with RuDP (pH 6, 16 hours at room temperature) hamamelonic acid diphosphate and its epimer are formed. (Note that the conditions for the preparation of hamamelonic acid are 1. reaction of ribulose with HCN at pH of ca. 8 for 1 day at 0° C and 2 days at room temperature followed by 2. hydrolysis with 1 M $Ba(OH)_2$ at 60° C for 4 to 8 hours,[154] or until no more ammonia is evolved.)

On the basis of available evidence, the ionic forms of these two compounds have the following tentative structure:



SUMMARY

An enzyme system has been studied from the standpoint of the purification and properties of one of its substrates, ribulose-1,5-diphosphate (RuDP), and the enzyme, carboxydismutase.

RuDP was prepared 1) by extraction from algae and 2) by conversion of ribose-5-phosphate (RMP) in the presence of adenosine triphosphate (ATP) and an isomerase-kinase mixture. RuDP-C^{14} was obtained also from algae by using radioactive bicarbonate as the source of carbon. The algal RuDP was separated from the other sugar phosphates by paper chromatography and recovered from the paper by elution with water. Studies with labeled RuDP showed that about 45% of the total radioactivity fixed by algae (2 to 3 minutes photosynthesis followed by 30 seconds flushing with nitrogen gas) was located in the diphosphate region of the chromatogram. Of this diphosphate area, at least 70% was RuDP. The enzymically prepared RuDP was separated from the starting material (RMP) and the intermediate ribulose-5-phosphate by aqueous ethanol fractionation of the barium salts of these substances, that of RuDP precipitating in 50% aqueous ethanol. At this stage, a yield of the order of 1 g of crude product (barium salt) was obtained per g of barium RMP. This preparation contained about 70% organic phosphate, the rest being inorganic orthophosphate. Chromatography of this crude material on a Dowex-1 (formate) ion exchange column gave a diphosphate fraction made up of 80% RuDP and 20 % ribose diphosphate. RuDP was found to be unstable, hydrolyzing easily to form as the main product, not ribulose monophosphate, but free ribulose. RuDP in aqueous solution also decomposed rapidly in air; the main products in this case were phosphoglyceric acid (PGA) and phosphoglycolic acid.

Source materials for carboxydismutase were Tetragonia expansa (New Zealand spinach) and Chlorella pyrenoidosa. The enzyme from Tetragonia was purified by ammonium sulfate precipitation of either 1) the clarified crude extract, 2) the acetone powder of the clarified crude extract, or 3) the water soluble chloroplast extract. On the average, the yield per 100 g of fresh leaves was 110 mg, 50 mg and 30 mg for the respective methods. The purification (the ratio of the specific activities, in units per mg protein, of the purified fraction to that of the clarified crude extract) was approximately 3, 10, and >10, respectively. Further purification of the ammonium sulfate fractionated crude extract was attempted using various classical techniques such as heat, acetone, ethanol and isoelectric precipitations, but all without success.

The enzyme from Chlorella was the clear supernatant liquid obtained after centrifugation of the suspension of sonically ruptured algae and was used directly without purification. This crude extract was found to have a behavior similar to that of Tetragonia when subjected to ammonium sulfate fractionation, the activity precipitating in the same ammonium sulfate range in both cases.

Homogeneity studies on some of the preparations obtained from Tetragonia, employing ultracentrifugal methods, spectrophotometric methods and enzymic assays, indicated that the most purified fraction was the ammonium sulfate precipitated chloroplast extract, although paper electrophoresis and N-terminal amino acid analysis suggested that this preparation was far from pure.

The course of the carboxydismutase catalyzed reaction was followed by counting the acid-stable, non-volatile fixation of radiocarbon from labeled bicarbonate when incubated in the presence of unlabeled RuDP and the enzyme.

The product of this reaction was shown to be unequivocally 3-PGA by paper chromatography. An alternate way for determining the extent of the reaction was achieved by incubating RuDP-C^{14} with unlabeled bicarbonate in the presence of carboxydismutase and following separation from other radioactive compounds by paper chromatography, the 3-PGA was counted.

Certain divalent metal ions were required for full activity of the carboxylation enzyme, among them being Ni^{++}, Mn^{++}, Co^{++} and Mg^{++} in decreasing order of their effectiveness. Addition of cysteine to the Mg^{++} activated system enhanced the activity of the enzyme only slightly.

A series of phosphorylated compounds served as inhibitors, these being thiamine pyrophosphate, 3-PGA, threose diphosphate, and ATP. Among the inorganic inhibitors were included hydroxylamine, fluoride, phosphate, and arsenate. Although avidin decreased the activity of carboxydismutase, the addition of biotin to this system did not restore the activity of the enzyme.

Preincubation of the enzyme with Ni^{++} in the presence of bicarbonate at 0° and 25° C gave a sharp rise in the activity followed by a gradual decrease in the activity of the enzyme as a function of preincubation time. On the other hand, when the enzyme was preincubated with Mg^{++} at 0° C (either in the presence of bicarbonate or the absence of bicarbonate), the activity of the enzyme increased abruptly and then leveled off as the preincubation period was increased. Preliminary incubation of the enzyme with Mg^{++} at 25° C, however, showed no increase in activity of the enzyme; instead a small decrease of activity was observed after long periods of preincubation. These results along with those of experiments carried out at 0° C indicated that the activation of the enzyme involved the unfolding of the protein prior to the binding of the metal ion. Furthermore, these

facts suggested that Mg^{++} is the true cofactor for carboxydismutase. Consistent with this notion is the fact that an aged enzyme preparation was only activated by Mg^{++}.

The activity of the enzyme was linearly dependent on enzyme concentration. Both bicarbonate and RuDP also exhibited a similar behavior at low substrate concentrations, but at high concentrations, the former gave a typical saturation curve while the latter showed a slight inhibitory effect.

A typical bell-shaped curve was obtained for the activity as a function of pH, the activity maximum being at pH 8. The low activity of the enzyme at lower pH's was attributed to the instability of the carboxylation enzyme on the acid side of the pH "optimum."

The carboxylation enzyme was stable for long periods of storage, the crude extract of Chlorella being fully active after storage for >3 months at -20° C and a lyophilized preparation of an ammonium sulfate fractionated chloroplast extract of Tetragonia having 35% of its original activity when kept at -20° C for >2 years. The activity of carboxydismutase was also stable towards C^{14} radiation.

The carboxydismutase reaction was found to be irreversible under the conditions employed.

Carboxydismutase required RuDP and bicarbonate as substrates; no other compounds served as substrates. Although the in vivo rate of fixation of carbon dioxide is within an order of magnitude of that of the in vitro rate, the saturation concentration of total CO_2 species for maximum rates of fixation in the in vitro system is 40 times that in the in vivo system. From this fact it was surmised that the actual carboxylating specie is dissolved CO_2, not bicarbonate.

The enzyme occurs in higher plants in the leaves, roots, peels, etc. In addition both photosynthetic and non-photosynthetic microorganisms contain this carboxylation activity. It is not clear whether all of the carboxydismutase activity resides in the chloroplasts of higher plant leaves, although it was deduced from specific activity considerations that the activity is concentrated there. The carboxylase activity does not seem to be in mammalian tissues.

The mechanism of enzyme action involves two steps, the mechanism of activation of the enzyme and the mechanism of the reaction. Preincubation studies with the substrates showed that when the enzyme alone, or when the enzyme plus metal combination is preincubated with bicarbonate, the activity of the enzyme increases as a function of preincubation time, but when preincubated with RuDP, the activity decreases as a function of preincubation time. One interpretation for these results is that the formation of an enzyme-metal ion-bicarbonate complex is required prior to the carboxylation of RuDP.

Although a diphosphate ester of a β-keto acid was postulated as the intermediate of the carboxydismutase reaction, all efforts failed to produce any evidence for its existence. These efforts included variations in conditions for the incubation, variations in conditions for terminating the reaction, and application of different analytical methods. The similarity of the reduced form of the dephosphorylated β-keto acid to hamamelonic acid led to an investigation of the reaction of cyanide with RuDP. This reaction, a facile one, led to a product which was tentatively identified as the diphosphate of hamamelonic acid and its epimer (and not the cyanohydrin).

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