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Author
Mayaudon, Jacques.

Publication Date
1955-06-01

UCRL 3016

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Contract No. W-7405-eng-48

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June, 1955

Printed for the U. S. Atomic Energy Commission

RIBULOSE-1, 5-DIPHOSPHATE AND CO_2 FIXATION FROM TETRAGONIA LEAVES EXTRACT[1]

Jacques Mayaudon[2]

Radiation Laboratory and Department of Chemistry
University of California
Berkeley, California

June, 1955

The presence in a cell-free extract of Chlorella of an enzyme required in the photosynthetic assimilation of CO_2 for the carboxylation of ribulose-1, 5-diphosphate[3] to PGA has been demonstrated. Later, Weissbach et al.[4] found a soluble extract from spinach which catalyzed the fixation of CO_2 into PGA in the presence of ribose-5-phosphate.

We have prepared an extract from Tetragonia expansa which has proved capable of catalyzing the reaction between ribulose-1, 5-diphosphate and HCO_3^- to form two molecules of 3-phosphoglyceric acid and which was free of all other enzyme systems capable of acting upon RuDP. The development of the procedure involved a number of stages, each of which is described in the following sequences. Finally, a number of properties of the enzymatic activity are given.

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1 The work described in this paper was sponsored by the U. S. Atomic Energy Commission.

2 Fellow of the Belgium Foundation, IRSIA, "Institut pour l'Encouragement de la Recherche scientifique dans l'Industrie et l'Agriculture, 1954-1955.

3 J. R. Quayle, R. C. Fuller, A. A. Benson and M. Calvin, J. Am. Chem. Soc. 76, 3610 (1954).

4 A. Weissbach, P. Z. Smyrniotis and B. L. Horecker, J. Am. Chem. Soc. 76, 3611 (1954).

I. CRUDE EXTRACT AND DIALYSIS

Fifty g (wet wt) of fresh leaves are ground in a blendor for 1 min with 50 cc of distilled water. To the supernatant from centrifugation (at 3000 rpm for 5 min), which has been filtered through filter paper, is added washed charcoal (5 g/100 cc extract). The pH is maintained at pH 7.0 by addition of 2N NH_4OH. The solution is centrifuged at 40,000 rpm for 10 min. The pale yellow supernatant is then adjusted to pH 7.0 with 2N NH_4OH. The solution can be kept in an ice bath for more than 1 week.

Chromatogram 1 shows a radioautograph representing what we get when 400 µl of this extract is incubated for 10 min with 50 µl RuDP (~0.1 µmole) and 50 µl 0.026 N $NaHC^{14}O_3$, 400 µc/cc.

The C^{14} fixed is distributed in five compounds: alanine, phosphoglyceric acid, phosphopyruvic acid, phosphoglycolic acid, and glyceric acid. The quantitative distribution of the activity counted on paper is given in Table I, line 2. Since only 40% of $C^{14}O_2$ is fixed in PGA, it is evident that many other enzymes are present that can transform either the initially formed PGA or some other products of CO_2 fixation as yet unknown.

As we know,[5] most of the enzymes of the glycolytic system are inhibited by dialysis. As we will see, the carboxylation is not inhibited by dialysis of the enzyme solution. We have tried to dialyze the extract against distilled water. Five cc of extract are placed in a cellophane bag and dialyzed against ~1 liter water with mild agitation -- the carbon-dioxide-fixation ability is not reduced by dialysis, but qualitatively, after 3 hours, we find a different distribution (Tables I; II, col. 2) in the C^{14} fixed. PGA appears to be the main product formed (~70% of C^{14} fixed).

However, it is not possible to continue to dialyze the leaf extract against water. The protein begins to precipitate and after 6 hours the carboxylation

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[5] E. H. F. Baldwin, "Dynamic Aspects of Biochemistry," first edition, Cambridge University Press, 1945, p. 346.

Table I

Distribution of C^{14} [a]

A. Reaction RuDP + $NaHC^{14}O_3$ (10 min)

Extract	Total counts fixed (cpm)	PGA	P-pyruvic	P-glycolic	Glyceric acid	Alanine	Other compounds	Chromato-gram
Crude extract	3500	1340 (38.3) [c]	127 (3.6)	207 (5.9)	1286 (36.7)	470 (13.8)	--	1
Extract dialyzed 3 hr against H_2O [b]	4100	2906 (70.8)	58 (1.4)	96 (2.3)	764 (18.6)	--	--	2
Extract dialyzed 6 hr against Fe^{+++} EDTA 10^{-5} M	3820	3190 (83.5)	--	--	243 (6.1)	--	--	3
Mg^{++} EDTA 10^{-5} M	4300	3553 (82.6)	--	--	518 (12.0)	--	--	4
Cu^{++} EDTA 10^{-5} M	2180	1667 (76.0)	--	--	144 (6.6)	--	--	5

B. RuDP (C^{14}) + $NaHCO_3$

Extract	Total counts (cpm)	Origin	PGA	P-pyruvic	Glyceric acid	Pentose mono-P	Other compounds	Chromato-gram
Extract dialyzed 6 hr against Mg^{++} EDTA 10^{-5} M	4700	250	2824 (60.0)	312 (6.6)	237 (5.0)	271 (5.7)	602 (12.8)	6

a In each tube 400 µl leaf extract, 50 µl RuDP (~0.1 µmole), 50 µl $NaHC^{14}O_3$, 0.026M (400 µc/ml) 50 µl 0.05 M phosphate buffer; time, 10 min; room temperature.

b 6 hours' dialysis against H_2O reduced the activity to zero.

c Percent distribution of C^{14}.

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Table II

Fractional precipitation of extracts for carboxydismutase[a]

Saturation $(NH_4)_2SO_4$	Dry Weight 10 cc in mg after dialysis from 50 g leaves, mg/10 cc	C^{14} fixed into PGA after 10 minutes	Vol used from 10 cc µl	Sp. Act. counts/min on paper 1 mg protein	Chromatogram
0-0.33	20	587	100	3,000	7
0.33-0.40	65	6024	50	15,000	8
0.40-0.50	10	571	100	600	9
0.50-0.75	15	0	0	0	10

[a] Same conditions as Table I

activity is lost. We have verified that the addition of some traces of Versene Fe^{++} or Mg^{++} at a very low concentration (10^{-5} M for instance) is enough to stabilize the protein, and the small amount of salt permits the use of paper chromatography for semiquantitative measurement.

The extract after 6 hours' dialysis against Mg^{++} [EDTA K] pH 7 shows 80% of the C^{14} fixed in PGA. Fe^{+++} EDTA is also a convenient stabilizer, showing a smaller amount of glyceric acid. However, the total count fixed is lower (Table I, line 4). [EDTA K] Al^{+++} and Cu^{++} (chromatogram 5; Table I, line 5) are detrimental and show appreciable decrease of the carboxylating activity.

When the extract that has been dialyzed for 6 hours against Mg^{++} [EDTA K] acts on radioactive RuDP with unlabeled $NaHCO_3$, the activity is distributed among many compounds (Table I B, Chrom. 6). PGA constitutes about 60% of the RuDP (C^{14}) added.

Results and Discussion

The carboxylation activity is not sensitive to dialysis as, for instance, is the malic enzyme. It seems that this carboxylation enzyme reacts as an independent system to fix carbon dioxide in ribulose-1, 5-diphosphate to give phosphoglyceric acid as the main product, but the reaction is not unequivocal. The several compounds formed from RuDP (C^{14}) indicate the presence of a variety of enzymes and suggest that further purification is desirable.

The metals in the Versene complex are not specific. The increase of free glyceric acid seen after dialysis against Mg^{++} [EDTA K] may be understood as a specific activation of phosphatase by Mg^{++}.

Summary

1. Enzymatic activity is found in the extract of Tetragonia expansa leaves; the extract is able to fix carbon dioxide on ribulose-1, 5-diphosphate.
2. The enzyme activity is insensitive to dialysis.
3. The main product of the reaction of RuDP with labeled bicarbonate and of labeled RuDP with bicarbonate is phosphoglyceric acid.

II. FRACTIONAL PRECIPITATION OF THE CARBOXYLATION ENZYME AND SOME PROPERTIES

A. Fractional Precipitation in Distilled Water

50 g (wet wt) of leaves are ground in a blendor for 1 min with 50 cc of distilled water. The supernatant from centrifugation at 3000 rpm for 5 min is filtered through filter paper and then centrifuged at 40, 000 rpm for 10 min. Charcoal need not be added if the precipitation with $(NH_4)_2SO_4$ is made immediately after the centrifugation. To the clear yellow supernatant is added a weight of $(NH_4)_2SO_4$ sufficient to achieve 0. 33 of saturation,[6] and pH is adjusted to 7 with 2N NH_4OH.

After centrifugation at 20, 000 rpm for 5 min, the clear supernatant is brought to 0. 4 saturation with $(NH_4)_2SO_4$, the pH is adjusted to 7, the precipitate is centrifuged out by centrifugation at 20, 000 rpm for 5 min and redissolved in 10 cc of 0. 05 M phosphate buffer pH 7. 0 and the solution is dialyzed 6 hours against one liter of solution of Mg^{++} [EDTA] (10^{-5} M). The operations are repeated again to obtain protein fractions between 0. 4-0. 5 SAS (saturated ammonium sulfate) and 0. 5-0. 75 SAS.

In Table II are given the relative weight of each fraction after dialysis and the radioactivity fixed on paper chromatograms after 10 min.

The enzyme activity is highest between 0. 33 and 0. 4 SAS and the specific activity in it is stronger than in the other fractions.

Properties

We find now that only phosphoglyceric acid appears as the final product of reaction if we incubate this enzyme extract with RuDP and $NaHC^{14}O_3$ (Table III). Without RuDP there is no fixation of $C^{14}O_2$. With labeled RuDP we find that 40% of the RuDP (C^{14}) appears in PGA. The yield of PGA is lower than for the crude extract. However, the enzyme extract 0. 3-0. 4 SAS contains fewer different enzymes. Besides PGA, only sugar monophosphates appear on the chromatogram. These, cut out, eluted from the

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[6] The saturation solubility used is 68. 5 g $(NH_4)_2SO_4$ for 100 cc initial solution volume.

Table III[a]

Substrate added	RuDP (C^{14})		Counts on origin after run (cpm)	PGA (C^{14})		Pentose monophosphate		Other compounds		Chromatogram
	Counts on origin before run (cpm)	RuDP(C^{14}) remaining		Activity on paper	% of transformed RuDP	Activity on paper	% of transformed RuDP	Activity on paper	% of transformed RuDP	
$NaHC^{14}O_3$ + enzyme	0	--	0	0	--	--	--	--	--	--
RuDP + $HC^{14}O_3^-$ + enzyme	0	--	0	6024	--	--	--	--	--	8
RuDP (C^{14}) + enzyme[b]	4650	2588	112	--	--	1880	95	96	5	12
RuDP (C^{14}) + HCO_3^- + enzyme[b]	4453	1751	0	1130	42	1415	52	186	4	25
RuDP (C^{14})[b] + boiled enzyme	4550	3894	303	--	--	300	--	61	--	26
RuDP (C^{14}) + [b] HCO_3^- + boiled enzyme[c]	4500	4277	226	107	--	416	--	117	--	27

[a] In each tube 50λ enzyme extract, same conditions as Table II.

[b] Time of incubation, 30 min.

[c] Solution boiled 5 min on steam bath.

paper, and run with selective carriers show that they are ribose and ribulose monophosphate (Chrom. 13 and 14). Further precipitation between 0.3 and 0.4 SAS does not remove the phosphatase activity.

B. Fractional Precipitation in Phosphate Buffer

We have found that the enzymatic extract can be prepared from Tetragonia expansa (New Zealand spinach) in a variety of ways so that it is free of other enzymes capable of transforming RuDP, if we make the preparation in phosphate buffer.

50 g (wet wt) of fresh leaves are ground in a blendor for 1 min with 50 cc 0.1 M phosphate buffer pH 7. The supernatant from centrifugation at 3000 rpm for 5 min is filtered through filter paper and then centrifuged twice again at 40,000 rpm for 10 min. To the clear yellow supernatant is added a weight of $(NH_4)_2SO_4$ sufficient to achieve 0.35 of saturation. The pH is adjusted, if necessary, to pH 7.0 with 2N NH_4OH and centrifuged at 20,000 rpm for 5 min. The pellet is discarded and the pale supernatant is brought to 0.39 saturation with $(NH_4)_2SO_4$. The precipitate is centrifuged out also for 5 min at 20,000 rpm. It is dissolved in 10 cc of 0.05 M phosphate buffer pH 7 and dialyzed 6 hours against one liter of solution (changed every hour) of 5×10^{-5} M Mg^{++} [EDTA K] at pH 7 or 10^{-4} M sodium acetate pH 7.

III. MECHANISM AND KINETICS OF THE CARBON DIOXIDE FIXATION

When it is incubated with RuDP and $HC^{14}O_3^-$, or RuDP (C^{14}) and HCO_3^-, the protein fraction 0.35-0.39 shows by radioautography that only phosphoglyceric acid is formed, according to the reaction[7]

$$\text{Ribulose-1, 5-diphosphate} + NaHC^{14}O_3 \rightarrow 2\ \text{PGA}, \qquad (1)$$

as is shown by the experiments given in Table IV.

Comparison of the data in lines 3 and 4 of Table IV shows that the ribulose-1, 5-diphosphate disappears to give phosphoglyceric acid, according

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7 J. A. Bassham, A. A. Benson, L. D. Kay, A. Z. Harris, A. T. Wilson and M. Calvin, J. Am. Chem. Soc. 76, 1760 (1954).

Table IV[a]

Substrate added	RuDP (C^{14})		Counts on origin after run (cpm)	PGA		Pentose monophosphate	Other compounds	Chromatogram
	Counts on origin before run (cpm)	RuDP(C^{14}) remaining		Activity on paper	% of transforming RuDP	Activity on paper	Activity on paper	
$NaHC^{14}O_3$ + enzyme	0	0	0	0	--	0	0	--
RuDP + $HC^{14}O_3$ + enzyme	0	0	0	6000	--	0	0	--
RuDP (C^{14}) + HCO_3^- + enzyme[b]	5600	639	274	4218	85	369	81	15
RuDP (C^{14}) + HCO_3^- + boiled enzyme[c]	5600	5142	~0	58	--	379	--	16

[a] In each tube 50λ enzyme extract, same conditions as Table I.

[b] Time of incubation 30 min.

[c] Solution boiled 5 min on steam bath.

to Reaction (1).

From Table IV and examination of Chromatograms 15 and 16, we infer that the enzyme solution is free of other enzymes capable of transforming RuDP, as the control (boiled enzyme) gives the same amount of pentose monophosphate. The kinetics of the reaction can now be studied, and it has been shown that the reaction is linear with the time for at least 16 minutes and is directly proportional to the amount of the enzyme added when ribulose-1, 5-diphosphate is not the limiting factor (Figs. 1 and 2).

The rate of the reaction is a function of $HC^{14}O_3^-$ concentration (Fig. 3). For this reason, all experiments are carried out with a constant concentration of $HC^{14}O_3^-$ so that it is in large excess.

IV. ACTION OF INHIBITORS

The action of some inhibitors has been tried. Table V A, B shows that 2, 4-DNP 10^{-4} M, 5×10^{-5} M chloromercuribenzoate and 10^{-3} M iodoacetamide (after 12 hours) are strong inhibitors and inhibit the reaction to about 60%. The Hg inhibition is reversed by cysteine. We infer the presence of the -SH groups in the enzyme for Reaction (1). Sodium azide, an inhibitor of the phosphorylated reaction, does not inhibit the carboxylation very much.

Discussion

It appears that the carboxylation in photosynthesis is due to an enzyme precipitating between 0.33 and 0.4 SAS. If the operations are carried out in distilled water the carboxylation activity is accompanied by a phosphatase activity giving ribose and ribulose-5-phosphate from RuDP. This phosphatase does not appear if the operations are carried out in phosphate buffer. In this case, the enzyme solution incubated either with RuDP and $HC^{14}O_3^-$ or RuDP (C^{14}) and HCO_3^- gives only PGA as product. The rate is proportional to the enzyme added and independent of the concentration of RuDP down to low values. The presence of HCO_3^- is necessary for the production of PGA (Table III). Furthermore, the rate of carbon fixation is approximately proportional to the concentration of HCO_3^-, even with very large excess of the ion. The

Table V[a]

Action of Inhibitors

A

Compounds	CPM	Inhibition	Chromatogram
Control	6024	--	8
NaN_3 (10^{-2} M)	4291	30	17
2, 4-DNP (5×10^{-4} M)	2950	50	7 (missing)
Chloromercuribenzoate (5×10^{-5} M)	2843	53	20
Chloromercuribenzoate (5×10^{-5} M) cysteine (10^{-3} M)	7012	--	19
Cysteine (10^{-3} M)	7529	--	21
Iodoacetamide 10^{-2} (1-10 min)	5150	--	18

B

Control	2333	--	22
Iodoacetamide (10^{-2} M) (after 12 hours)	990	58	23

[a] Same conditions as Table III.

-SH groups are functional, as is proved by the inhibition with iodoacetamide and chloromercuribenzoate, the latter inhibition being completely reversed by addition of cysteine. This last result is very close to the properties found by Fager[8] in the carboxylating enzyme associated with his suspension of chloroplast.

Summary

1. An enzyme able to fix carbon dioxide with ribulose-1, 5-diphosphate to give two molecules of PGA is found free of other enzymes from Tetragonia leaves.
2. The enzyme activity is the highest between 0.33 and 0.4 SAS.
3. The enzyme activity is sensitive to iodoacetamide and chloromercuribenzoate reversed by addition of cysteine. We infer the presence of -SH groups.

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8 E. W. Fager, Biochem. J. 57, 264 (1954)

LEGEND

Fig. 1 Linear relationship between the enzyme concentration and the rate of the carboxylation reaction.

Fig. 2 Linear relationship between the time and the rate of the carboxylation reaction.

Fig. 3 Influence of $NaHC^{14}O_3$ concentration on the rate of the carboxylation reaction.

In each flask:

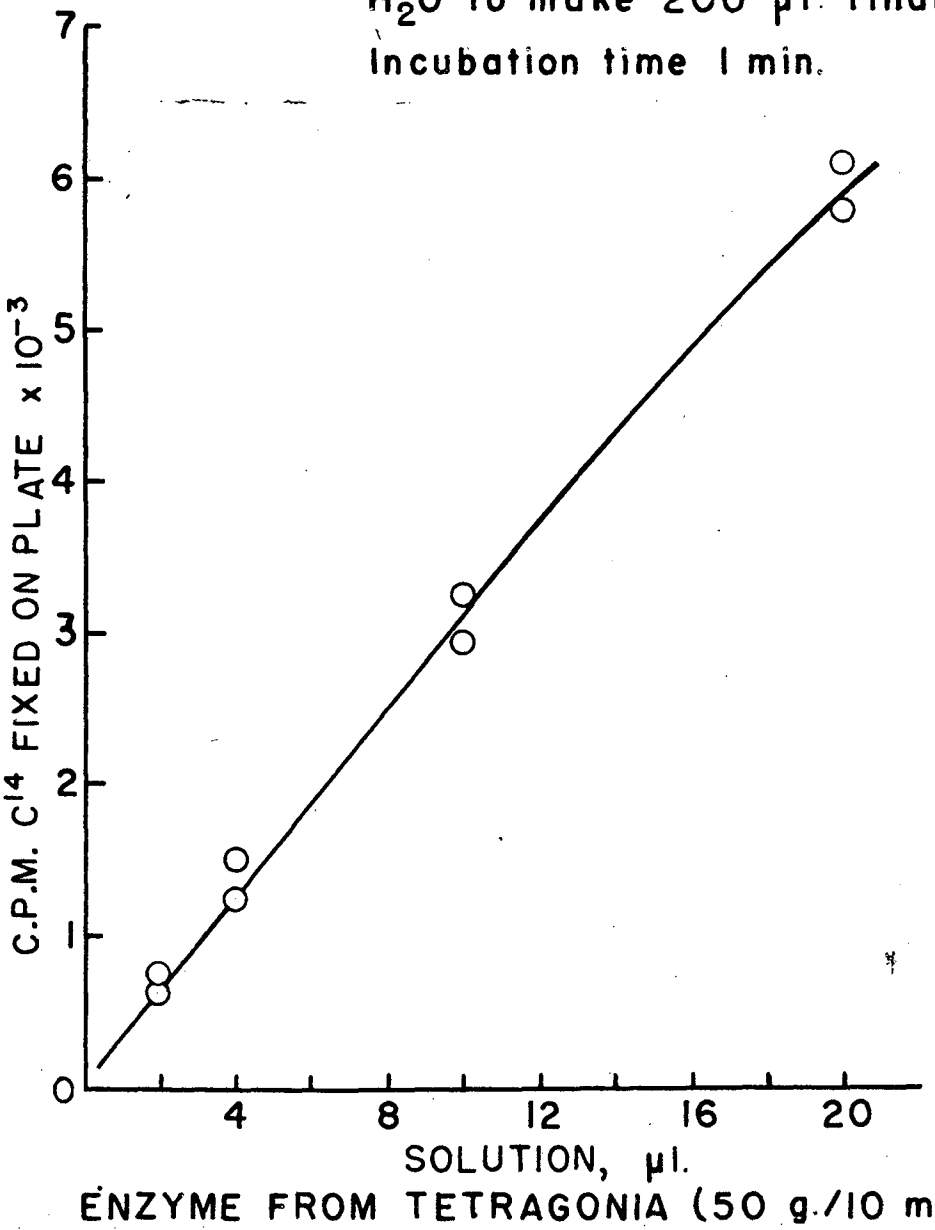
50 µl. $NaHC^{14}O_3$, 0.026N, 400 µc./ml

25 µl. RuDP ~0.05 µmoles

20 µl. phosphate buffer 0.05, pH 6.8

H_2O to make 200 µl. final volume

Incubation time 1 min.



ENZYME FROM TETRAGONIA (50 g./10 ml.)

Fig 1

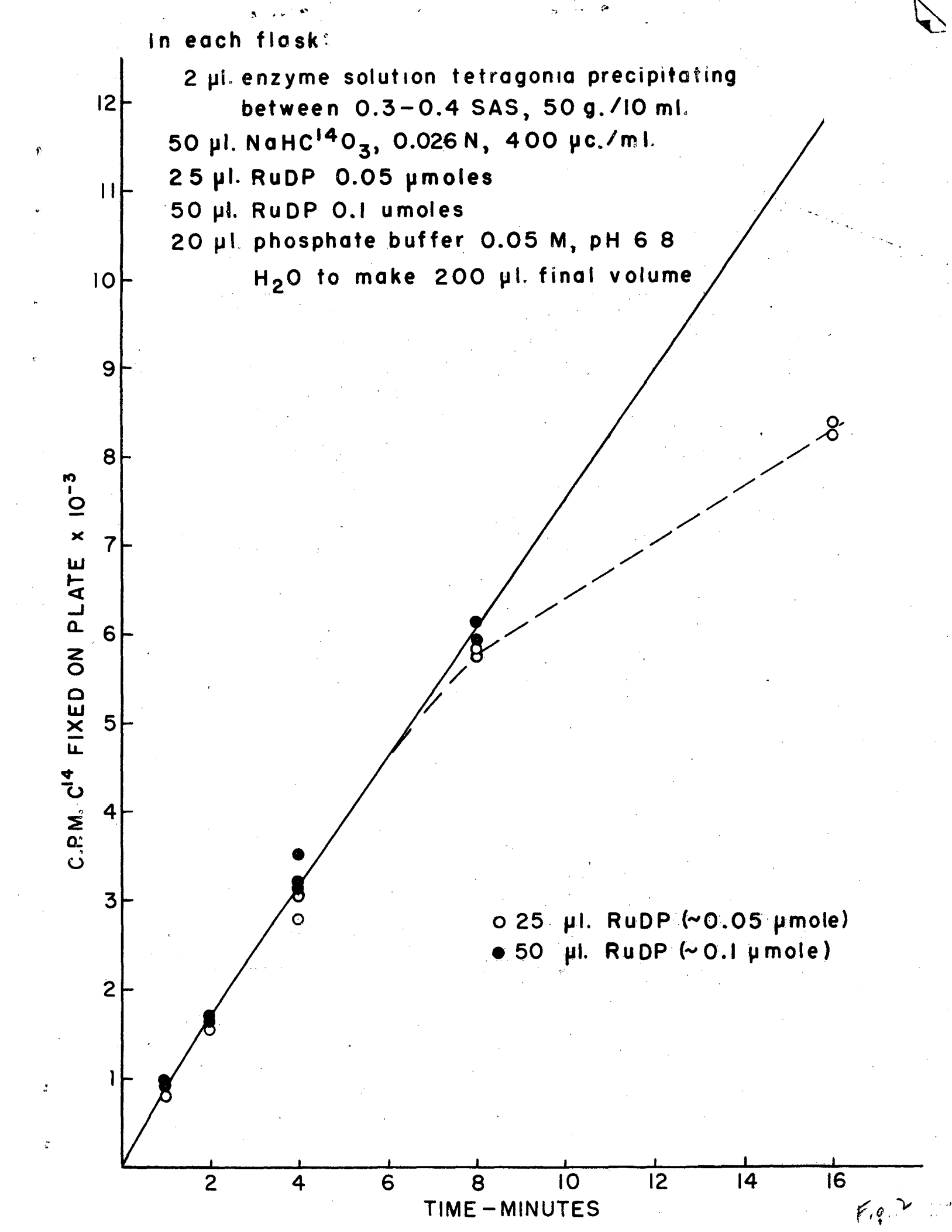

In each flask:
2 µl. enzyme solution tetragonia precipitating between 0.3–0.4 SAS, 50 g./10 ml.
50 µl. $NaHC^{14}O_3$, 0.026 N, 400 µc./ml.
25 µl. RuDP 0.05 µmoles
50 µl. RuDP 0.1 umoles
20 µl. phosphate buffer 0.05 M, pH 6 8
H_2O to make 200 µl. final volume
C.P.M. C^{14} FIXED ON PLATE x 10^{-3}
TIME – MINUTES
o 25 µl. RuDP (~0.05 µmole)
● 50 µl. RuDP (~0.1 µmole)

Fig. 2

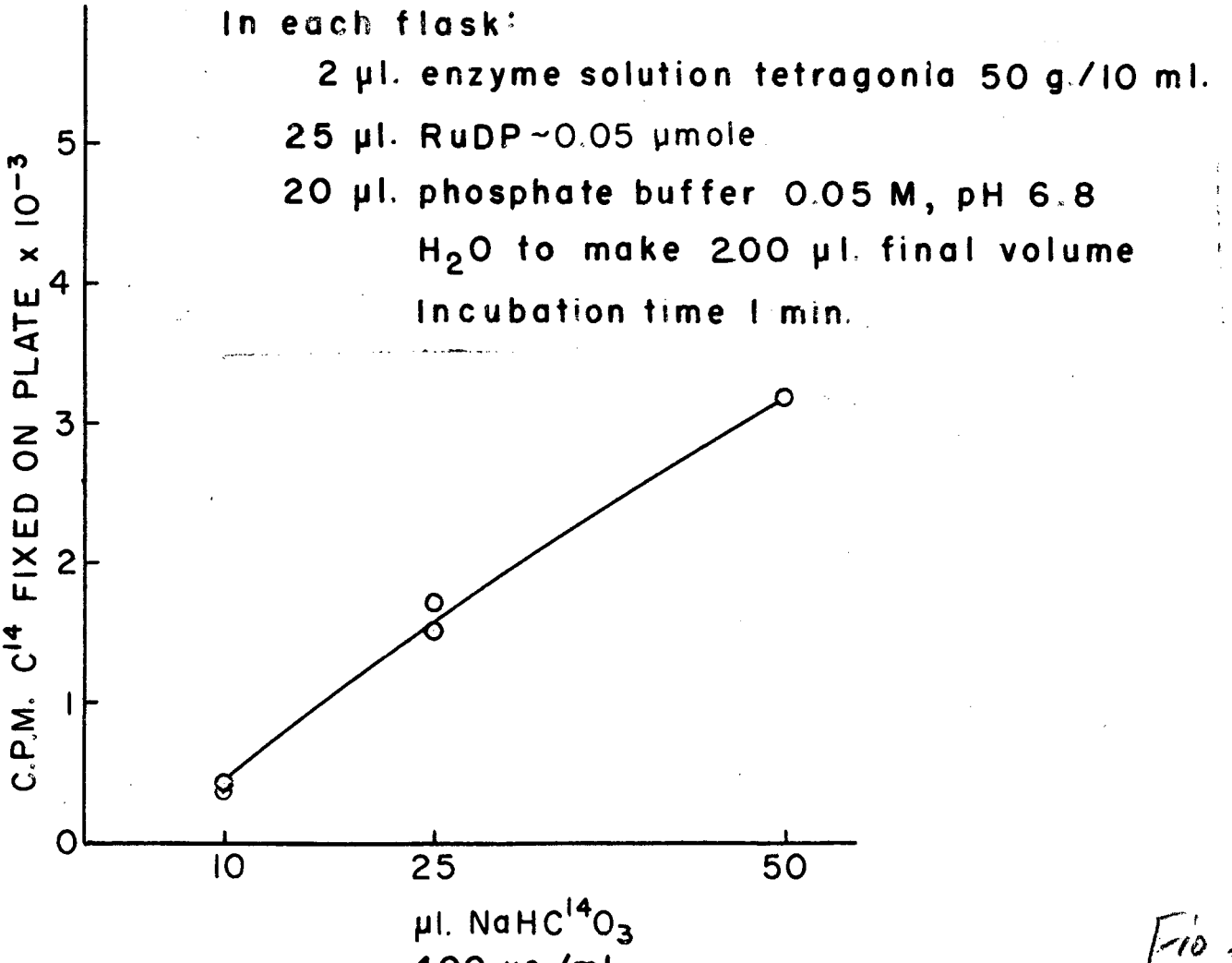
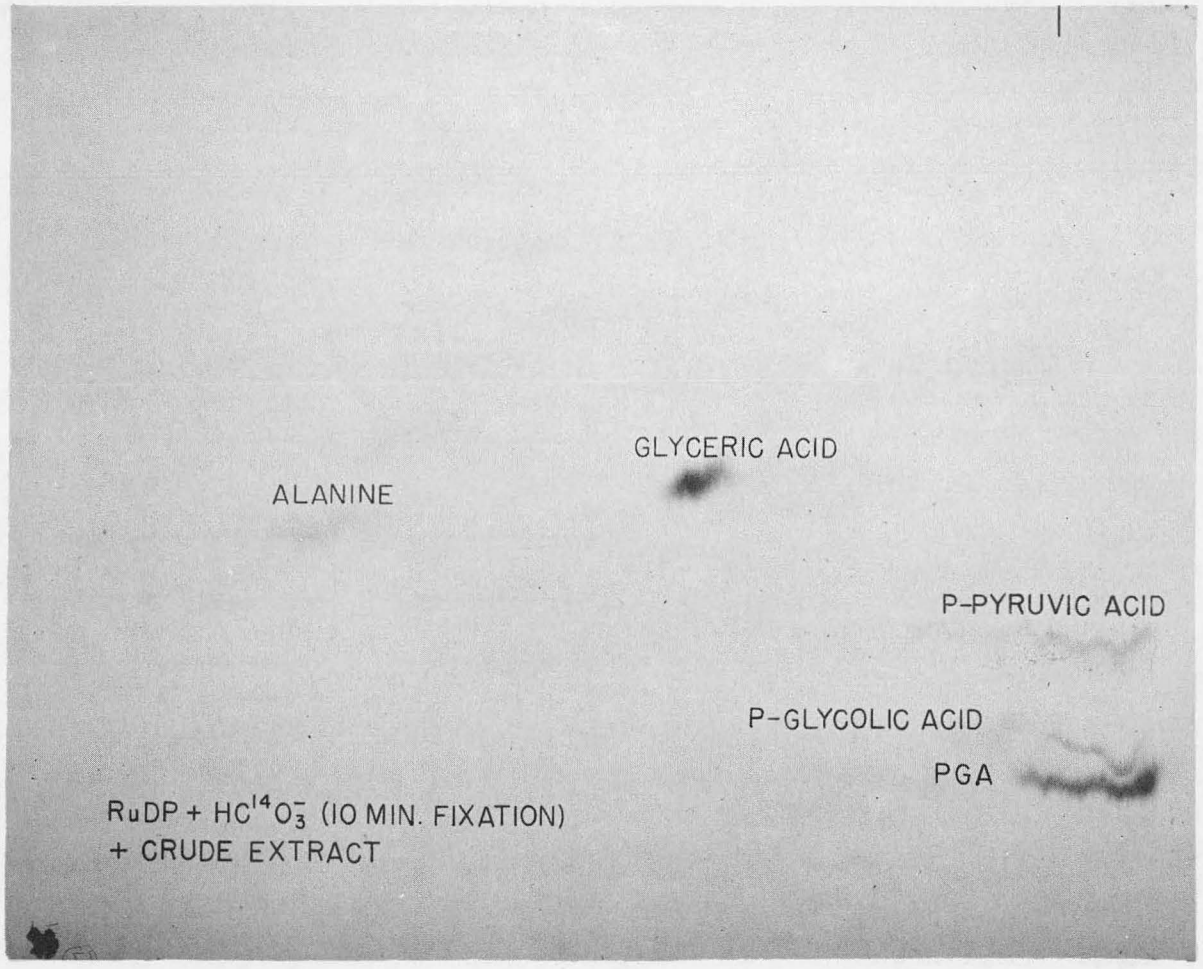
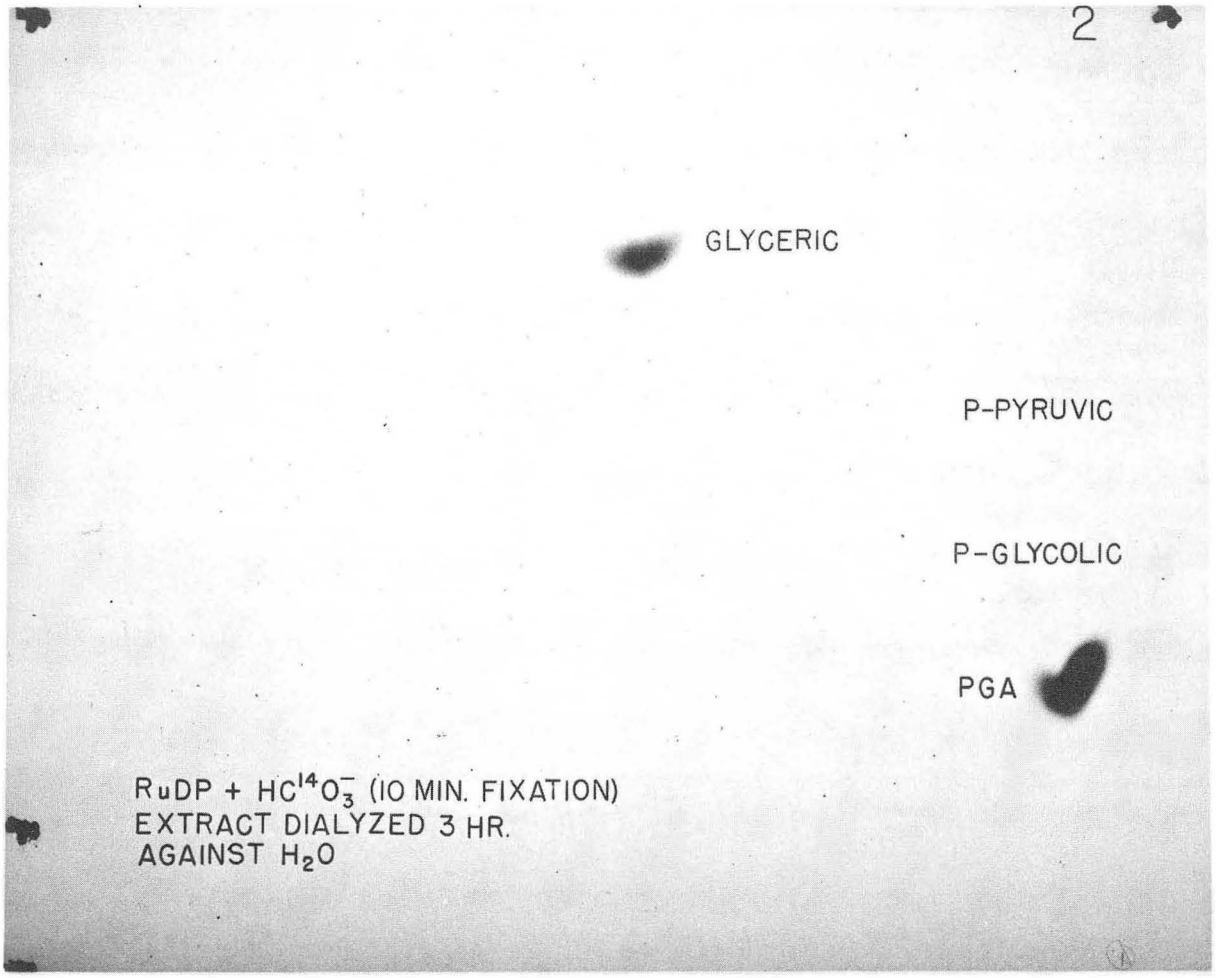

In each flask:
2 µl. enzyme solution tetragonia 50 g./10 ml.
25 µl. RuDP ~0.05 µmole
20 µl. phosphate buffer 0.05 M, pH 6.8
H_2O to make 200 µl. final volume
Incubation time 1 min.
C.P.M. C^{14} FIXED ON PLATE x 10^{-3}
0
1
2
3
4
5
10
25
50
µl. $NaHC^{14}O_3$
400 µc./ml.

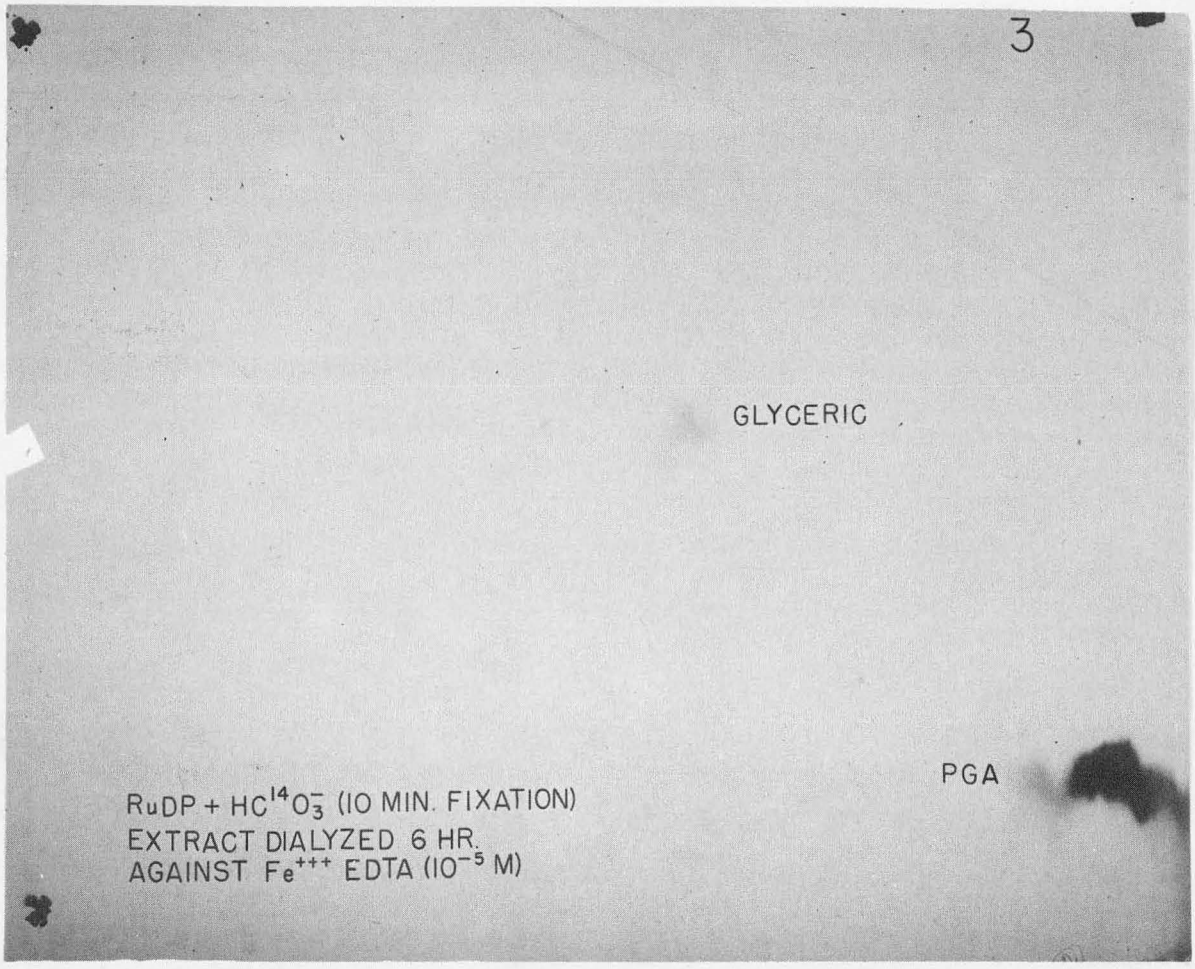
Fig 3


GLYCERIC ACID
ALANINE
P-PYRUVIC ACID
P-GLYCOLIC ACID
PGA
RuDP + $HC^{14}O_3^-$ (10 MIN. FIXATION)
+ CRUDE EXTRACT

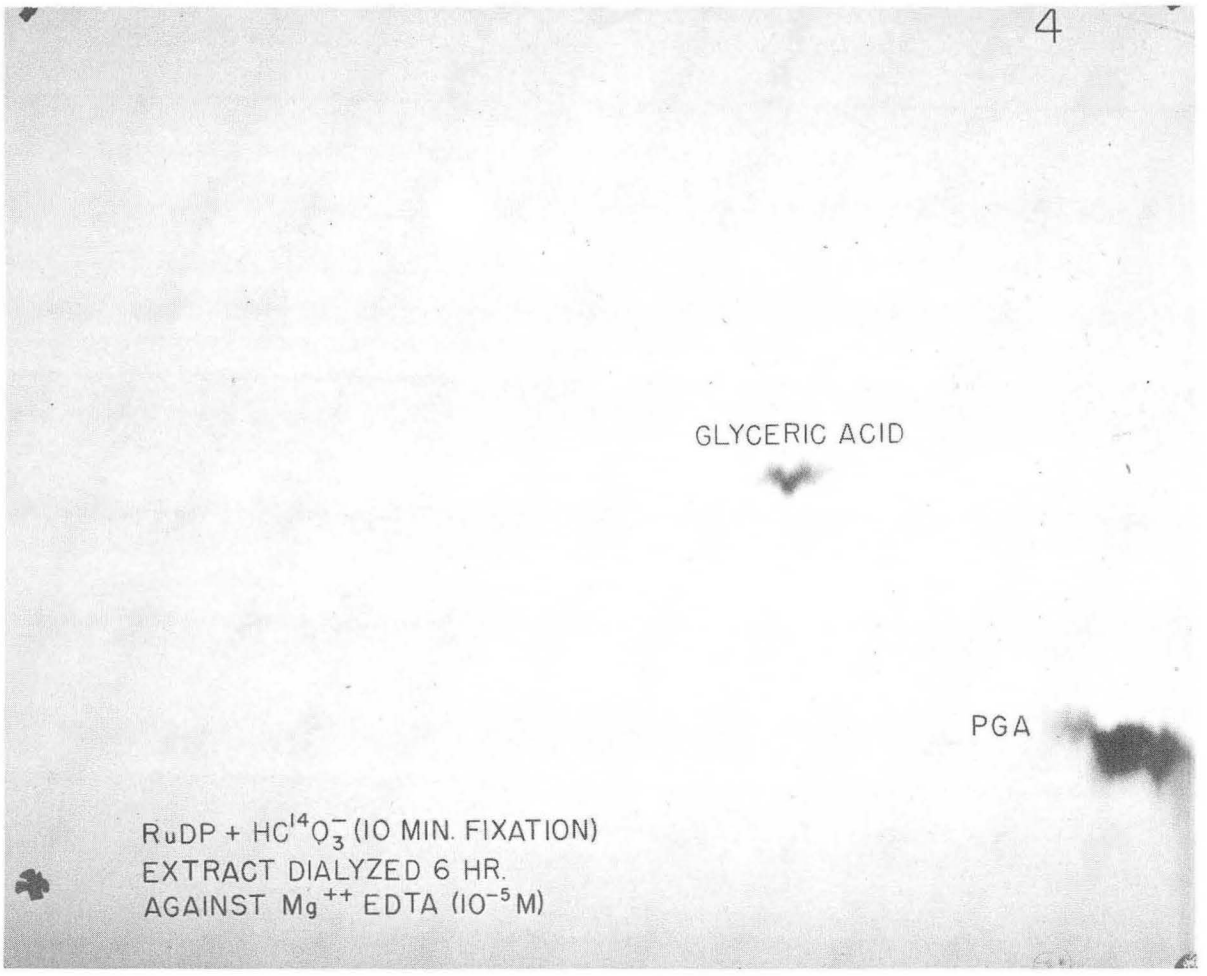
ZN-1312


2
GLYCERIC
P-PYRUVIC
P-GLYCOLIC
PGA
RuDP + $HC^{14}O_3^-$ (10 MIN. FIXATION)
EXTRACT DIALYZED 3 HR.
AGAINST H_2O

ZN-1313


3
GLYCERIC
PGA
RuDP + $HC^{14}O_3^-$ (10 MIN. FIXATION)
EXTRACT DIALYZED 6 HR.
AGAINST Fe^{+++} EDTA (10^{-5} M)

ZN-1314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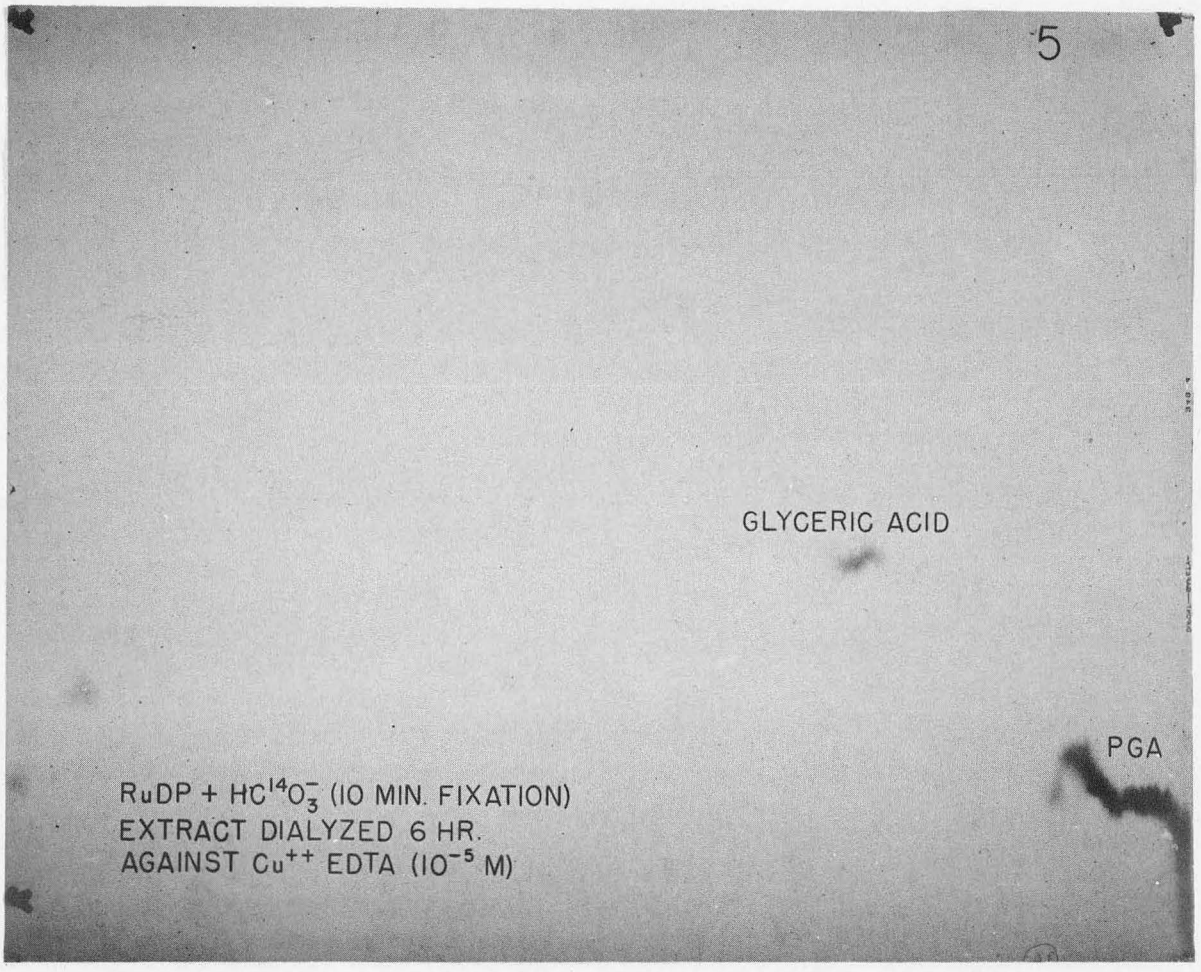
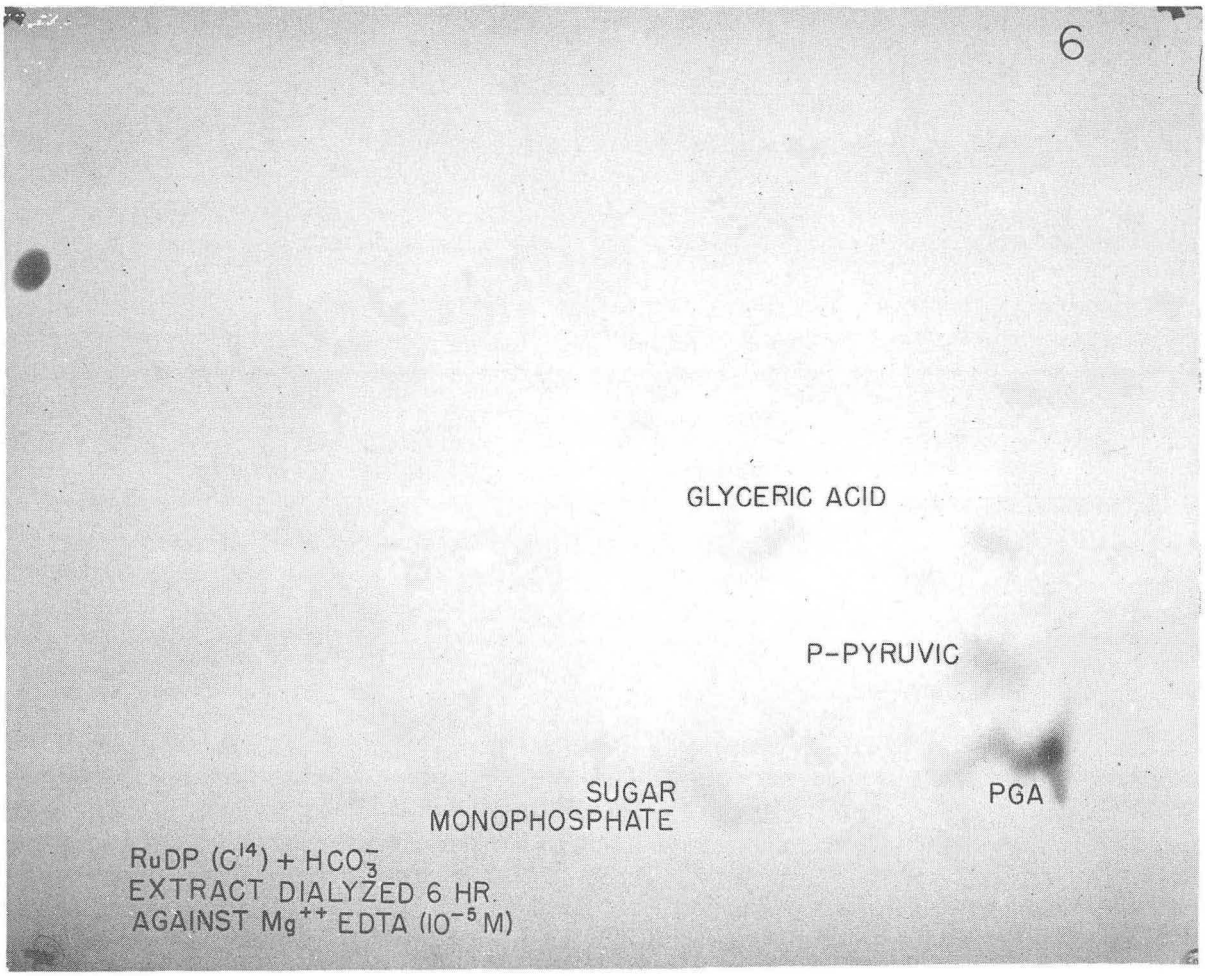
GLYCERIC ACID
PGA
RuDP + $HC^{14}O_3^-$ (10 MIN. FIXATION)
EXTRACT DIALYZED 6 HR.
AGAINST Mg^{++} EDTA (10^{-5} M)

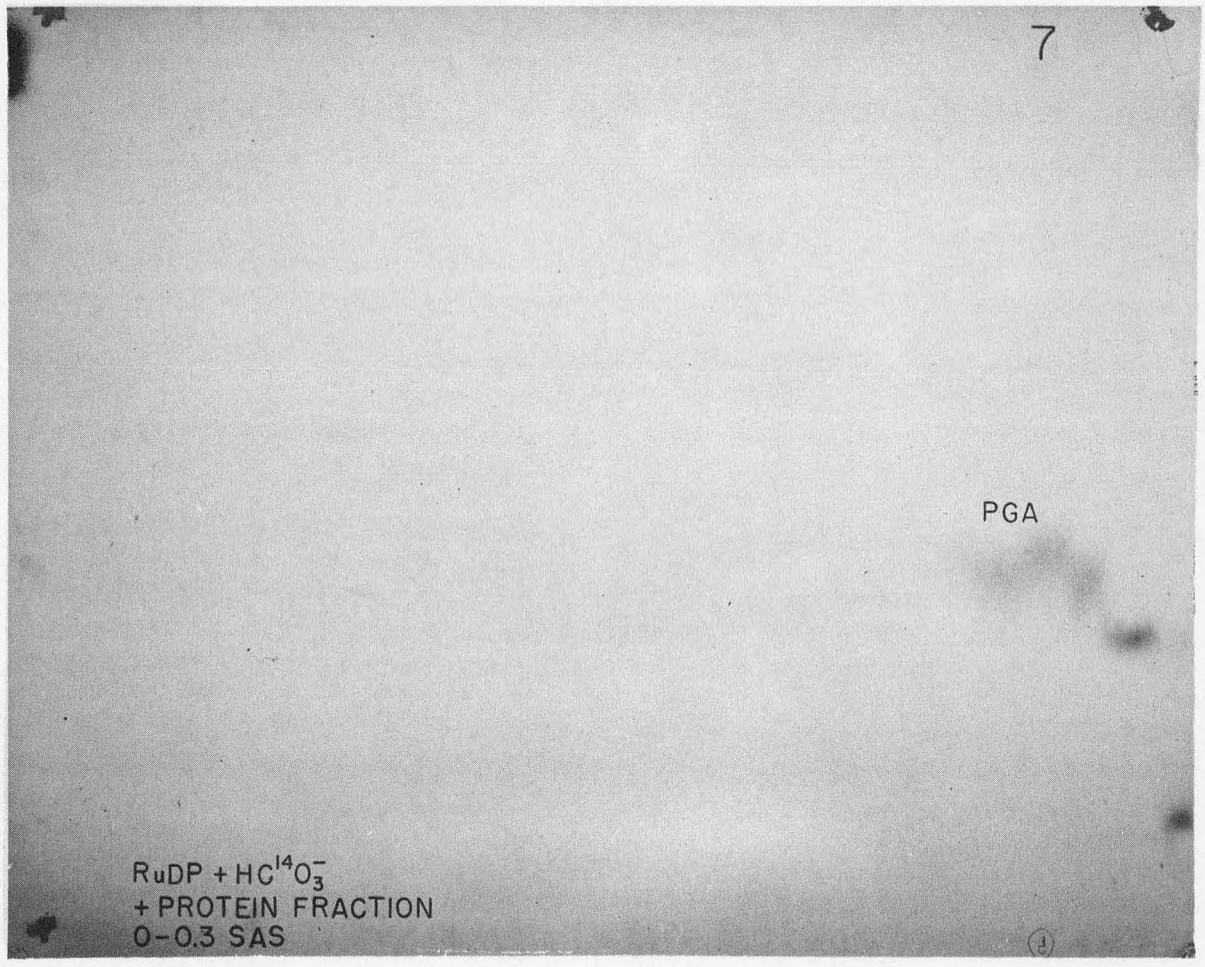
ZN-1315


5
GLYCERIC ACID
PGA
RuDP + $HC^{14}O_3^-$ (10 MIN. FIXATION)
EXTRACT DIALYZED 6 HR.
AGAINST Cu^{++} EDTA (10^{-5} M)

ZN-1316


6
GLYCERIC ACID
P-PYRUVIC
PGA
SUGAR
MONOPHOSPHATE
RuDP (C^{14}) + HCO_3^-
EXTRACT DIALYZED 6 HR.
AGAINST Mg^{++} EDTA (10^{-5} M)

ZN-1317



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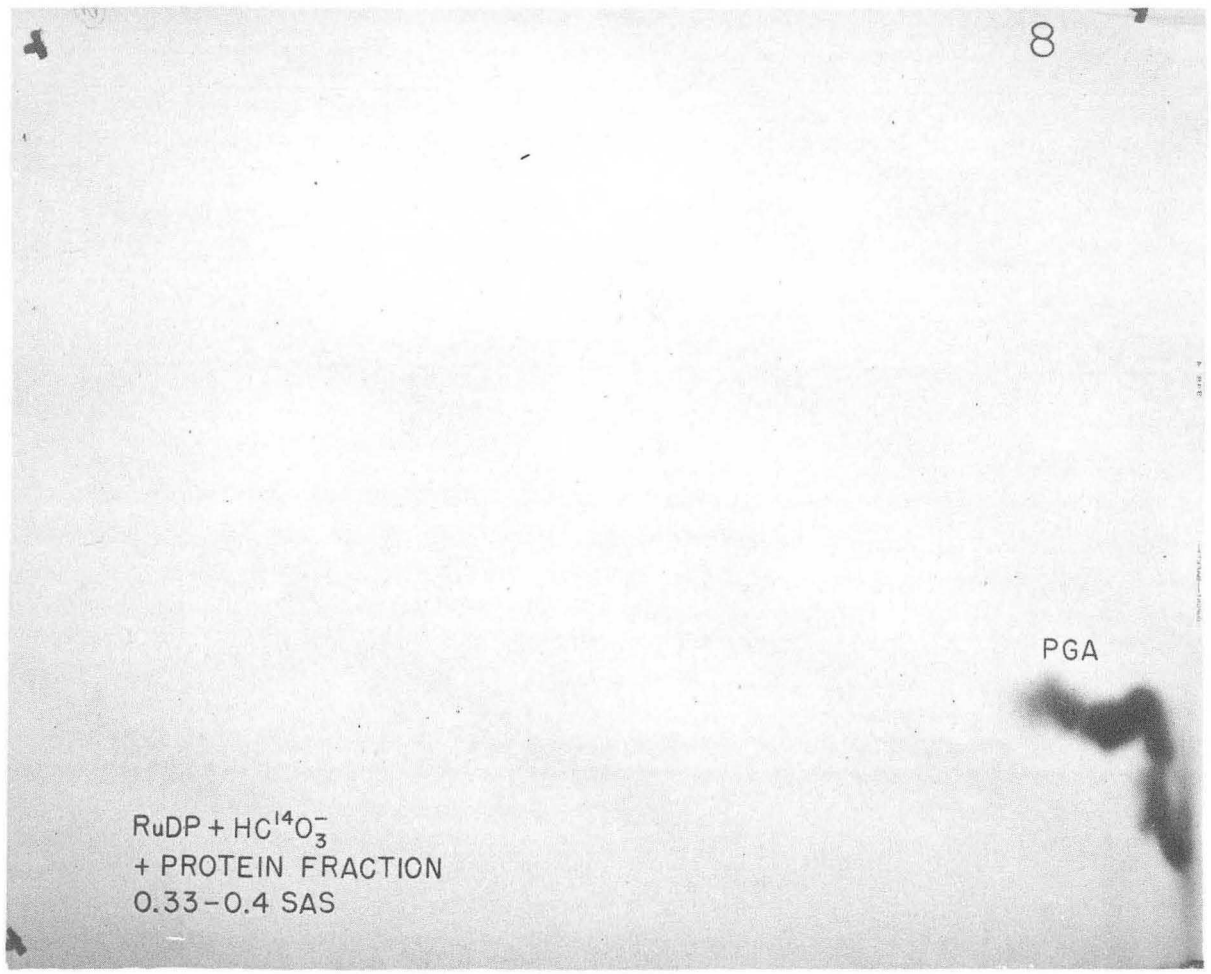

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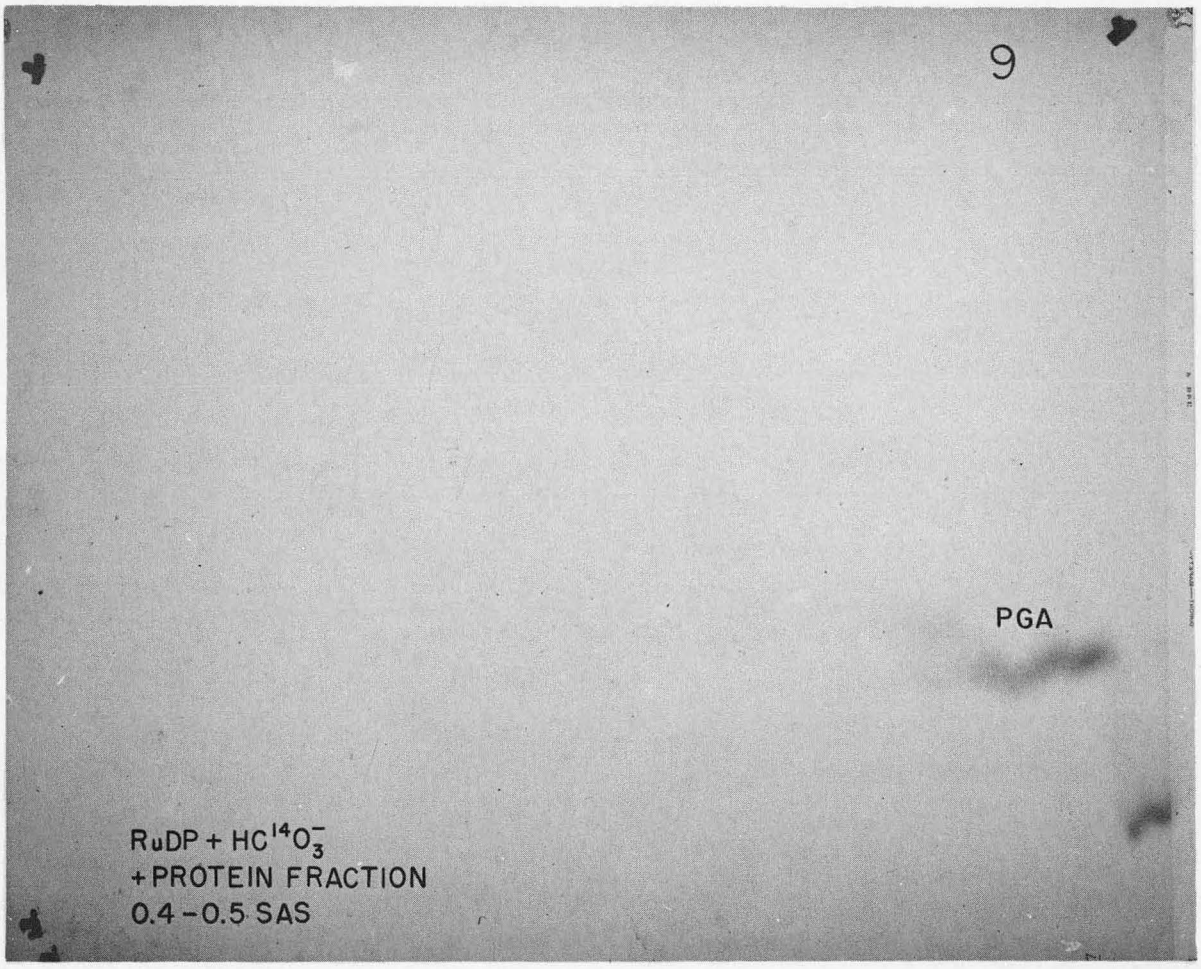

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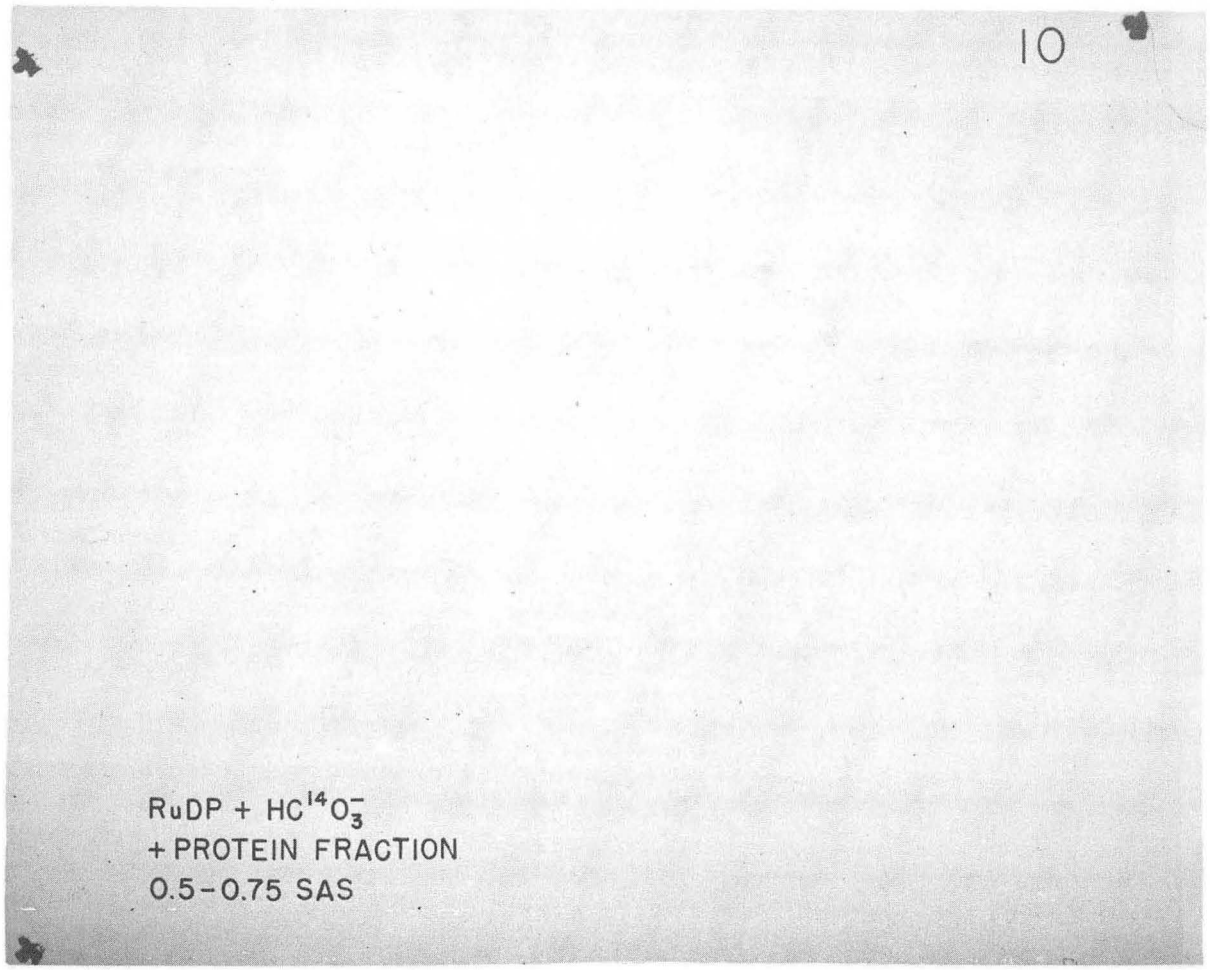

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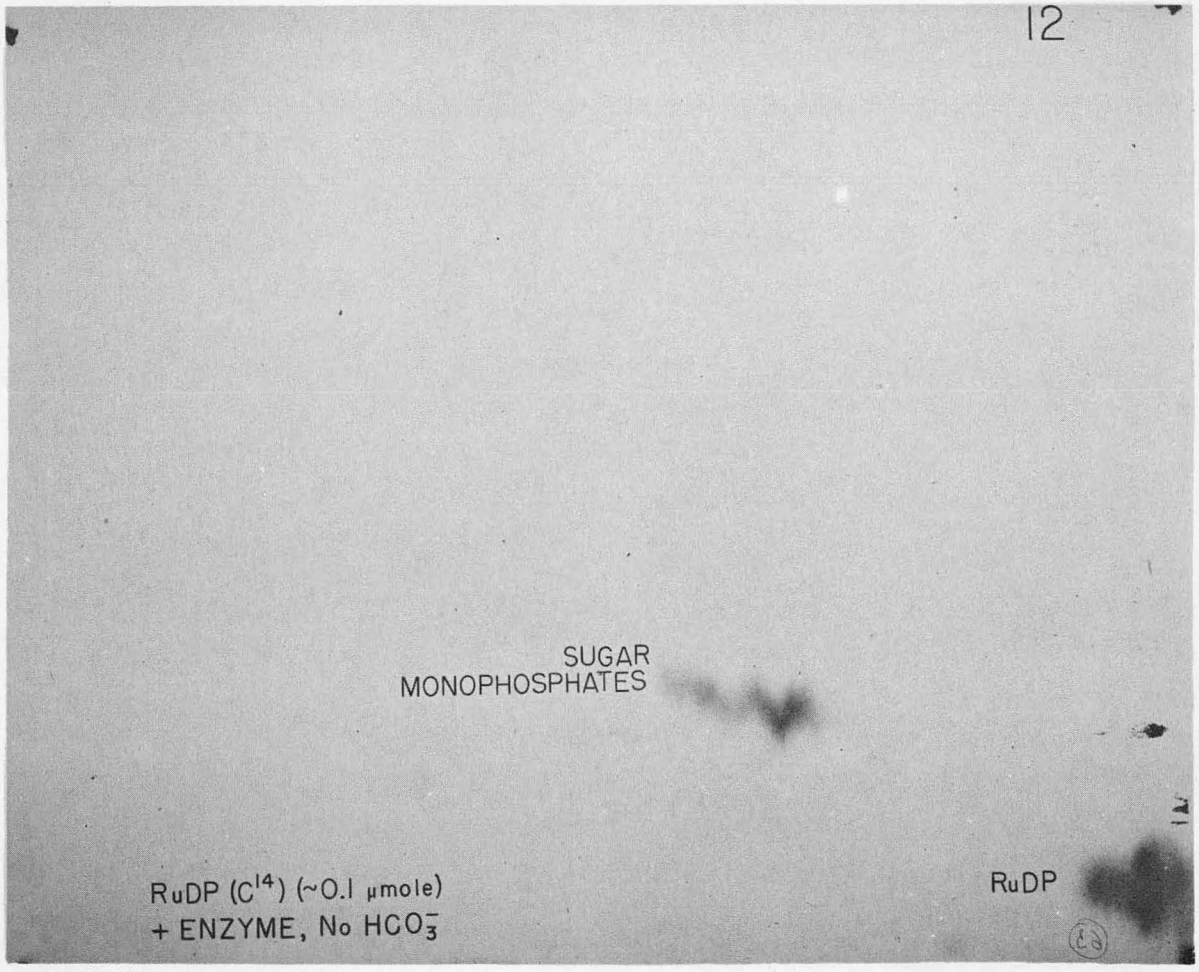
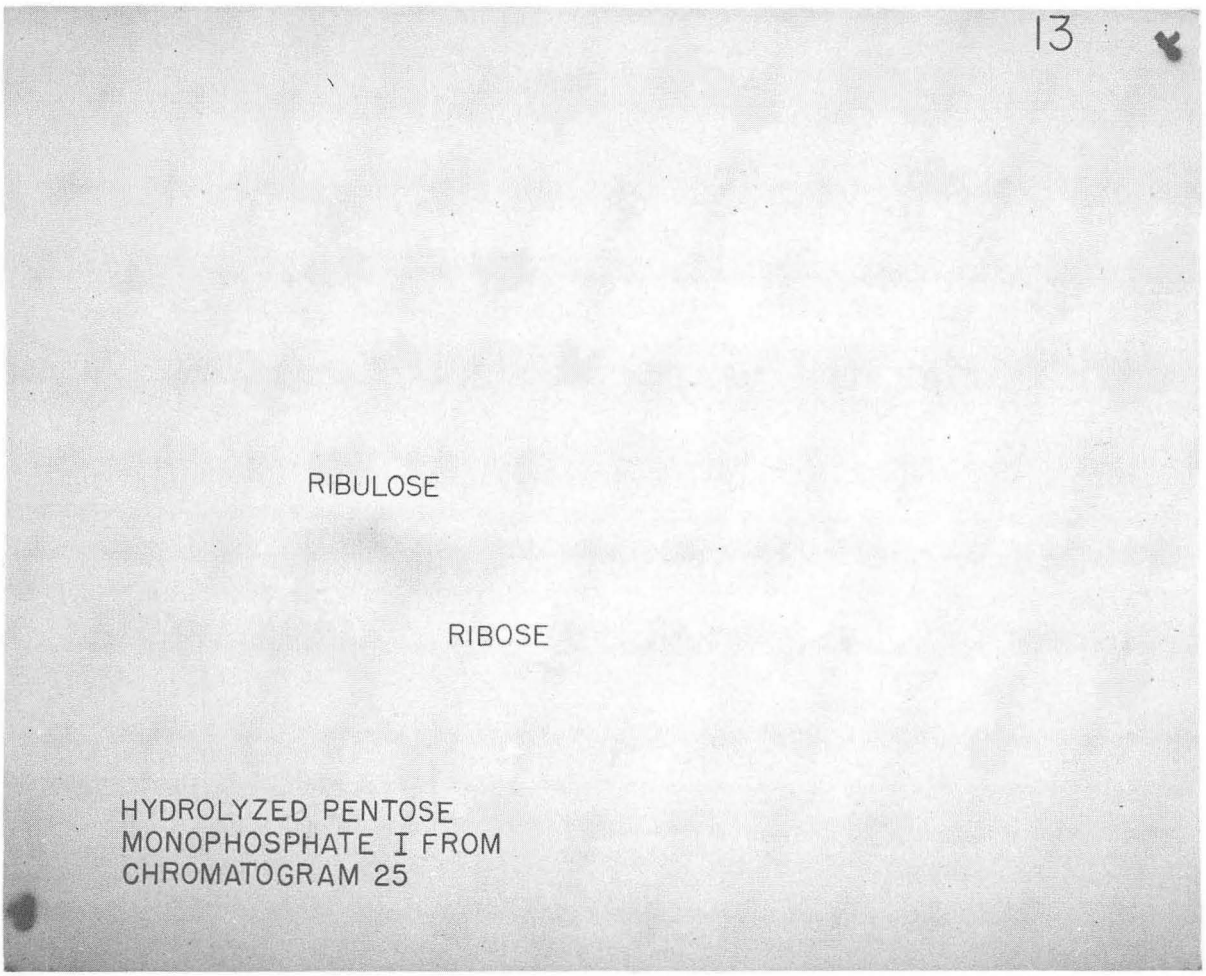
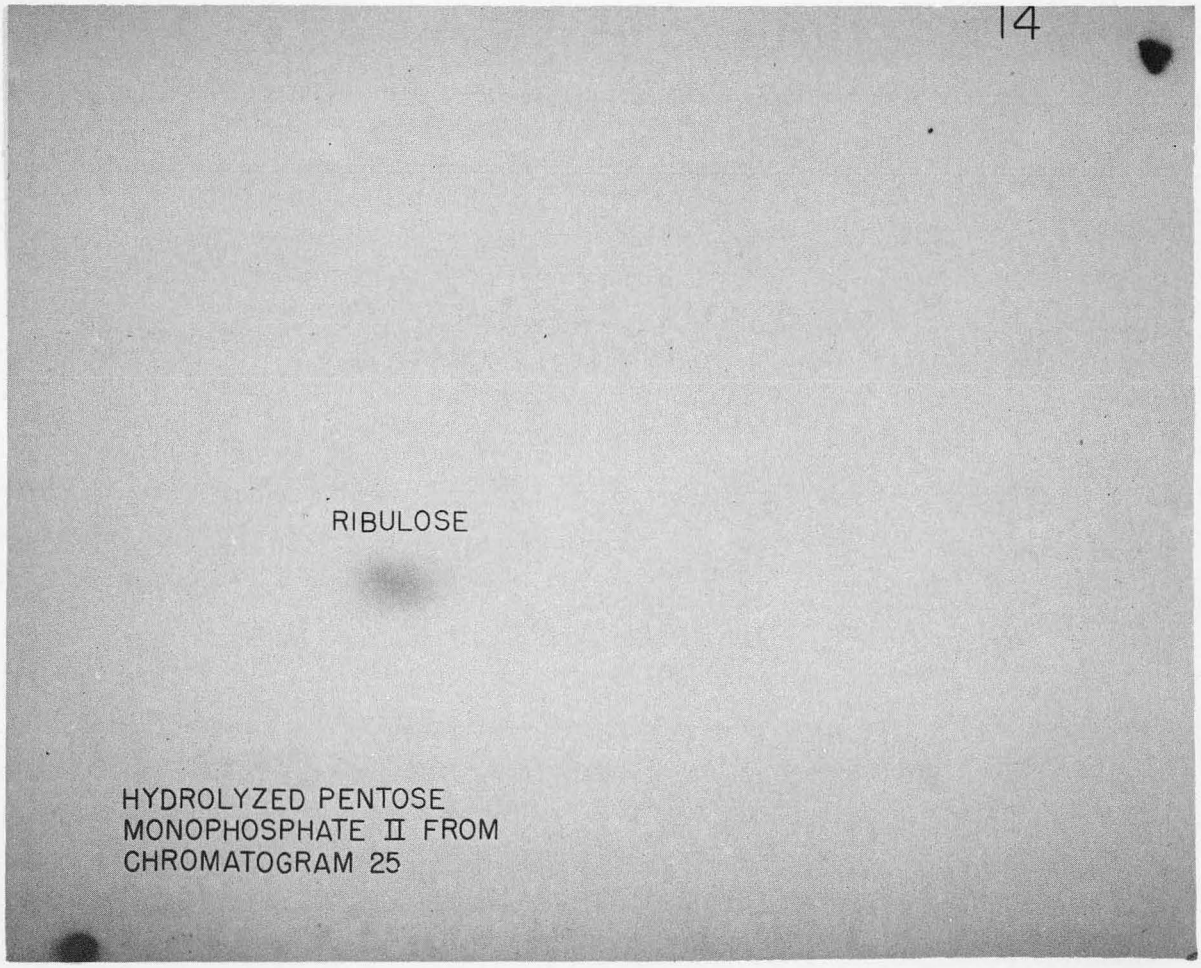

12
SUGAR
MONOPHOSPHATES
RuDP (C^{14}) (~0.1 μmole)
+ ENZYME, No HCO_3^-
RuDP

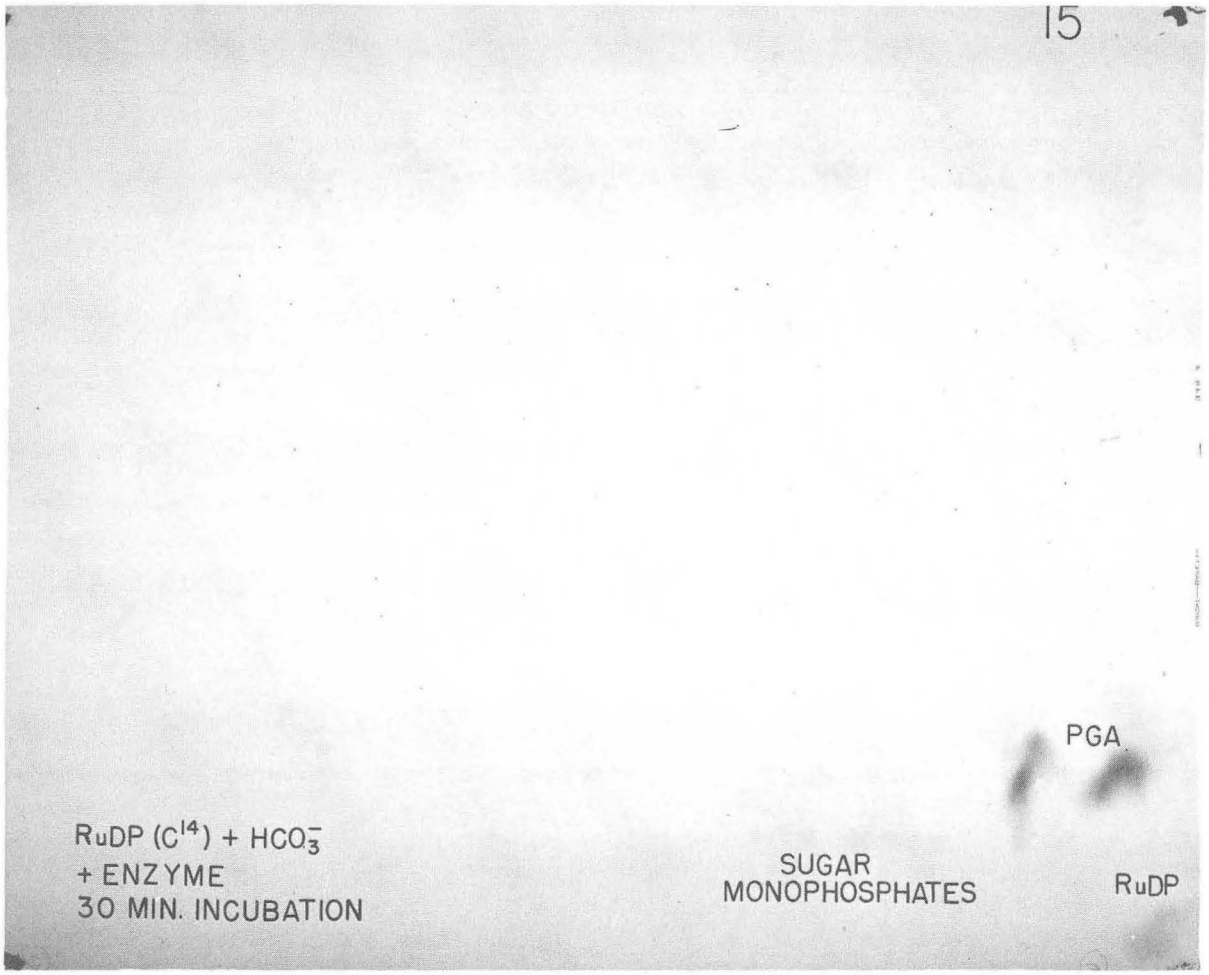
ZN-1322


13
RIBULOSE
RIBOSE
HYDROLYZED PENTOSE
MONOPHOSPHATE I FROM
CHROMATOGRAM 25

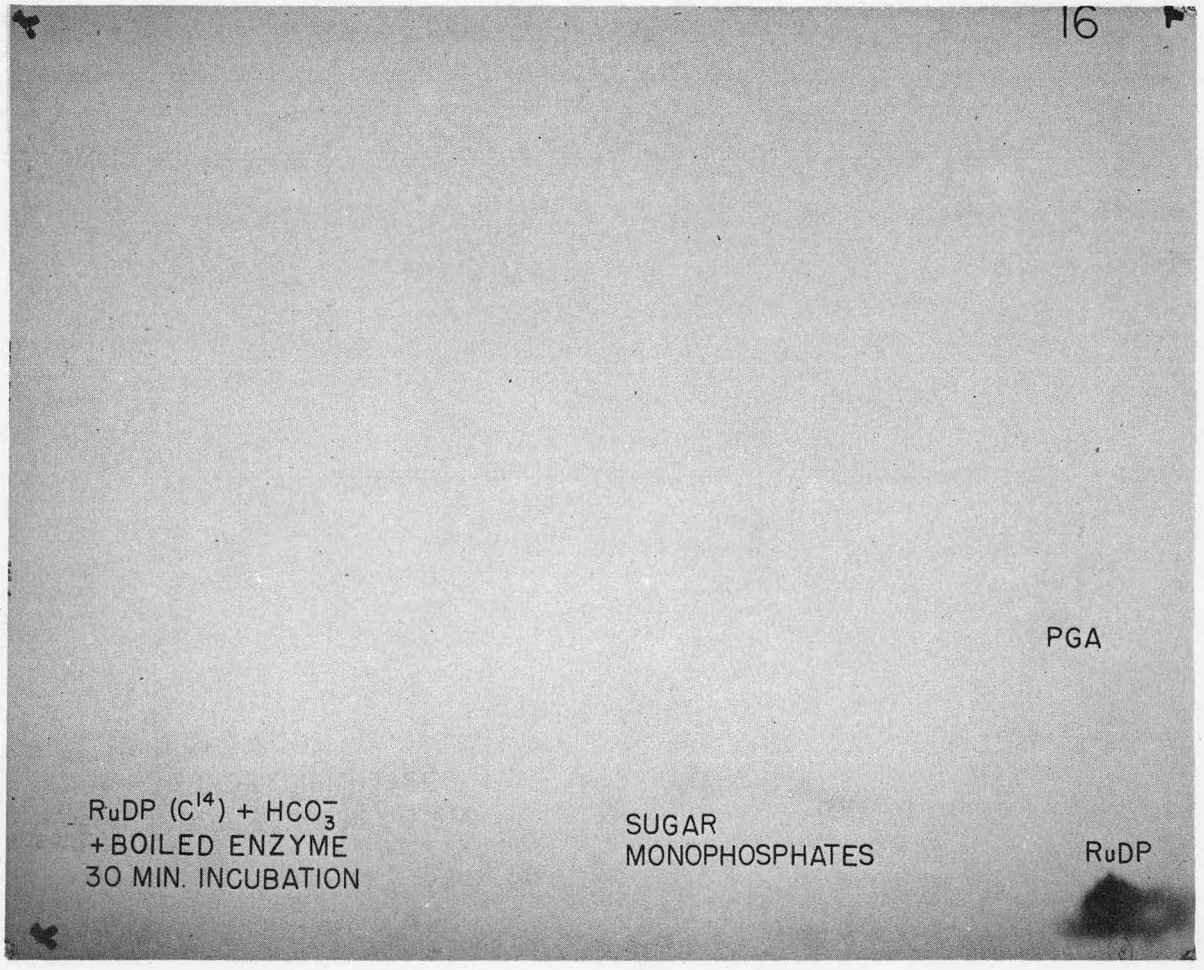
ZN-1323


14
RIBULOSE
HYDROLYZED PENTOSE
MONOPHOSPHATE II FROM
CHROMATOGRAM 25

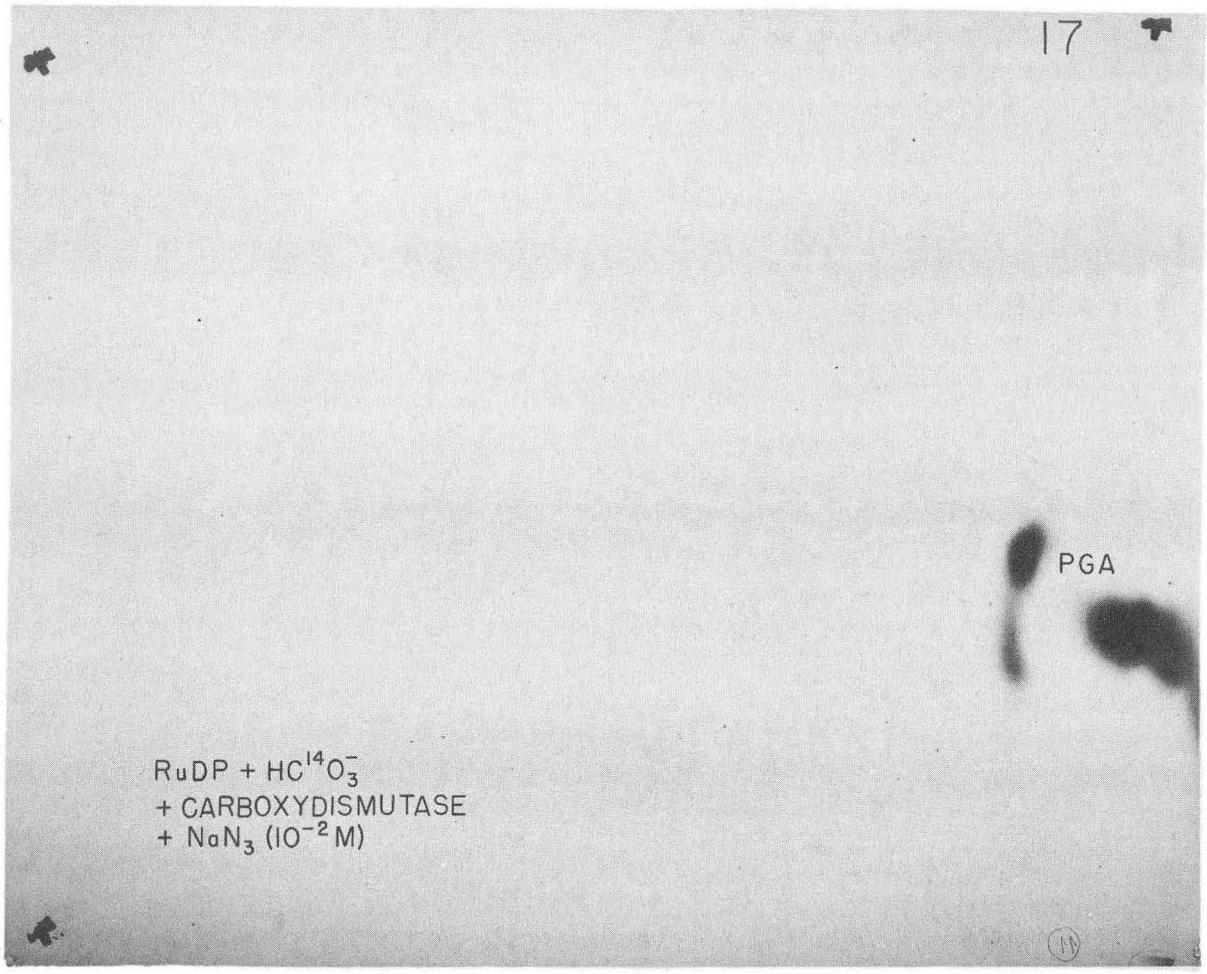
ZN-1324


15
PGA
RuDP (C^{14}) + HCO_3^-
+ ENZYME
30 MIN. INCUBATION
SUGAR
MONOPHOSPHATES
RuDP

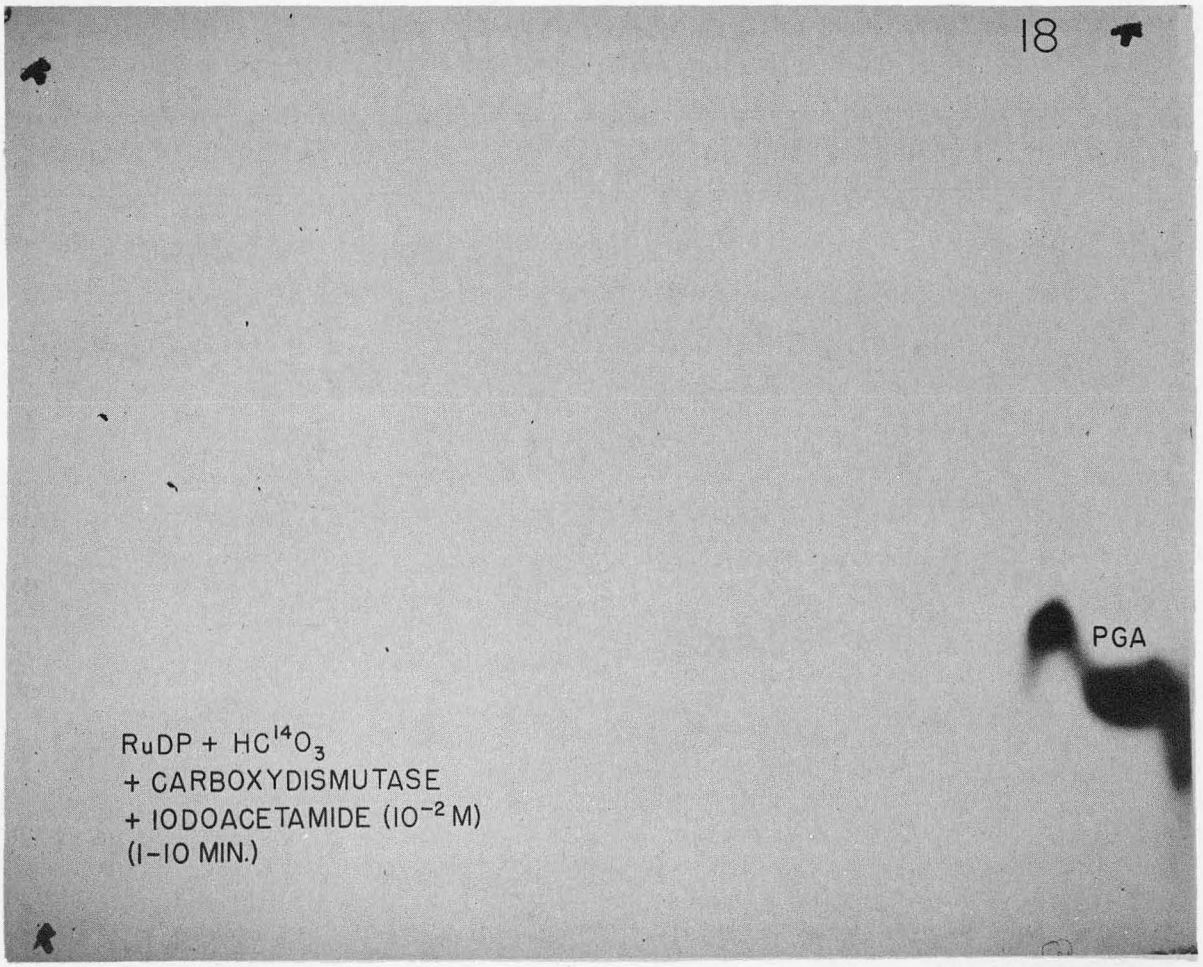
ZN-1325


16
PGA
RuDP (C^{14}) + HCO_3^-
+BOILED ENZYME
30 MIN. INCUBATION
SUGAR
MONOPHOSPHATES
RuDP

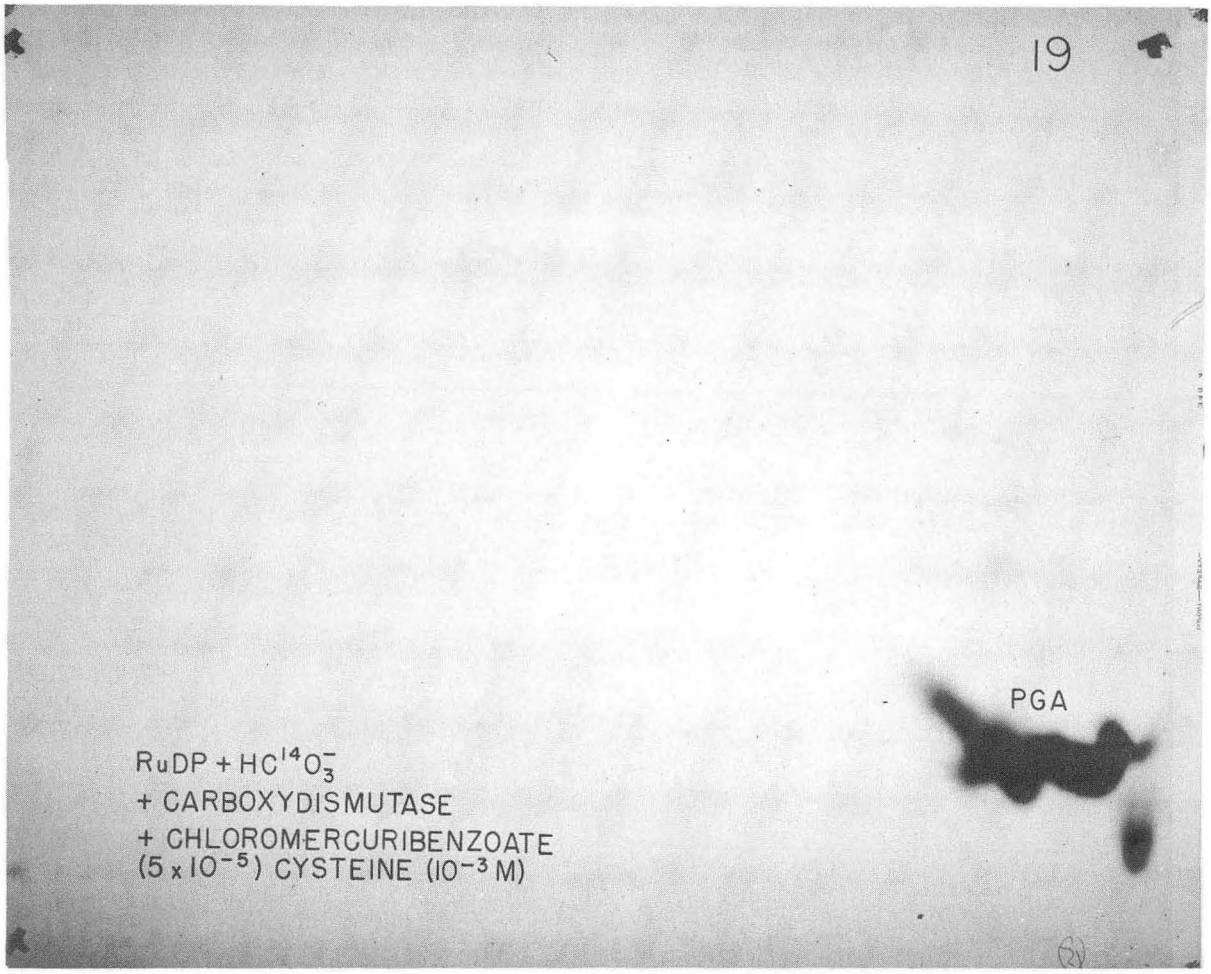
ZN-1326



ZN-1327


18
PGA
RuDP + $HC^{14}O_3$
+ CARBOXYDISMUTASE
+ IODOACETAMIDE (10^{-2} M)
(1-10 MIN.)

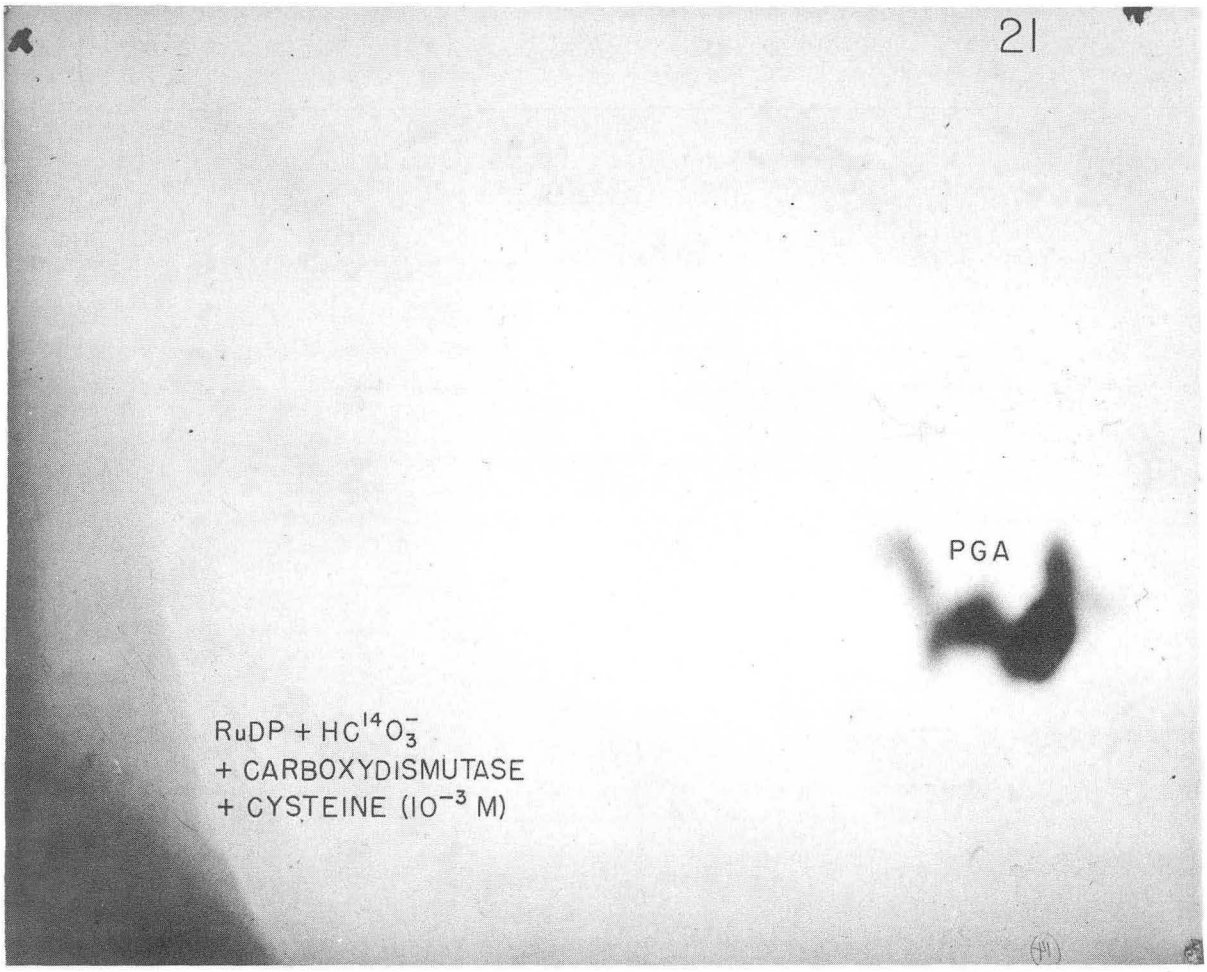
ZN-1328


19
PGA
RuDP + $HC^{14}O_3^-$
+ CARBOXYDISMUTASE
+ CHLOROMERCURIBENZOATE
(5×10^{-5}) CYSTEINE $(10^{-3}$ M)

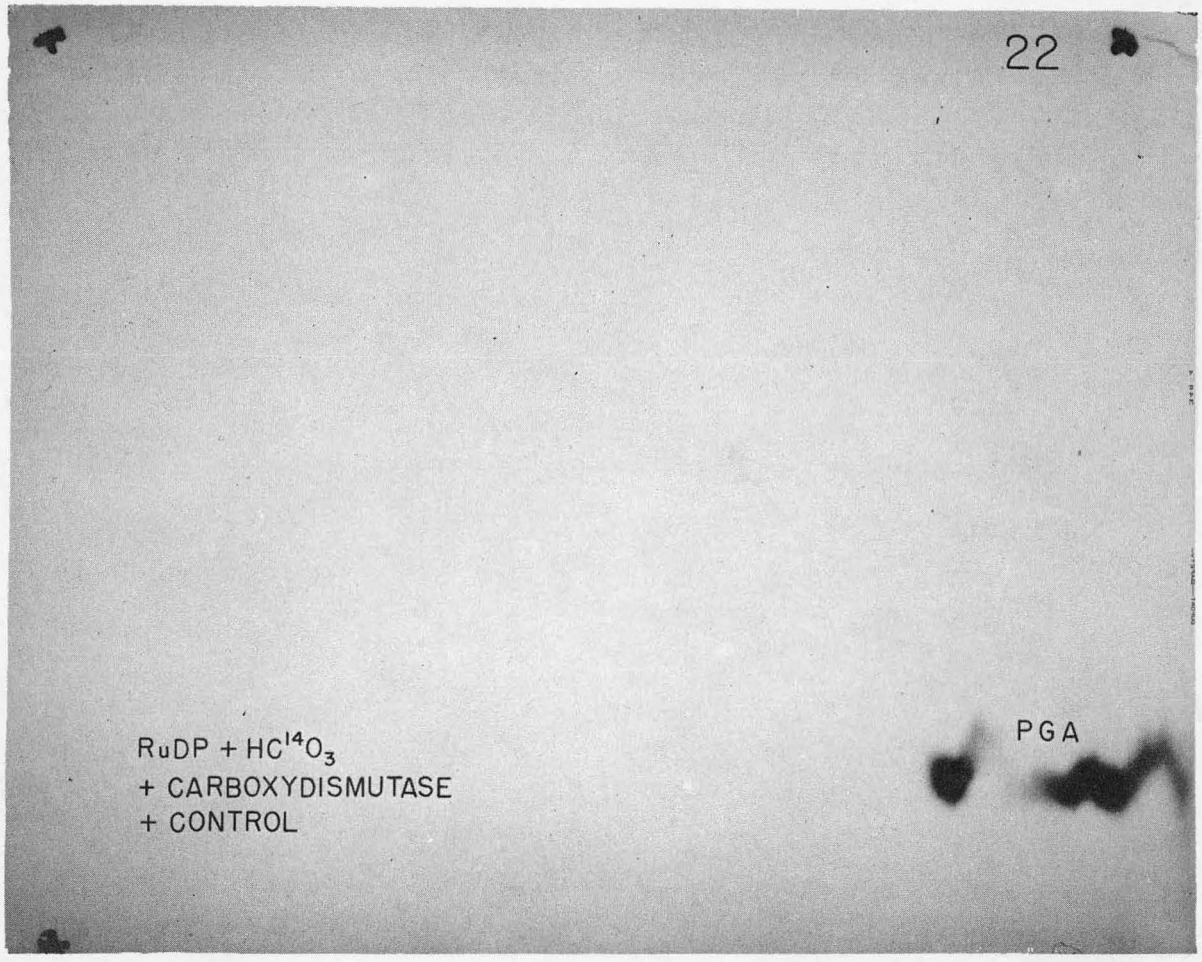
ZN-1329


20
PGA
RuDP + $HC^{14}O_3^-$
+ CARBOXYDISMUTASE
+ CHLOROMERCURIBENZOATE
(5×10^{-5} M)

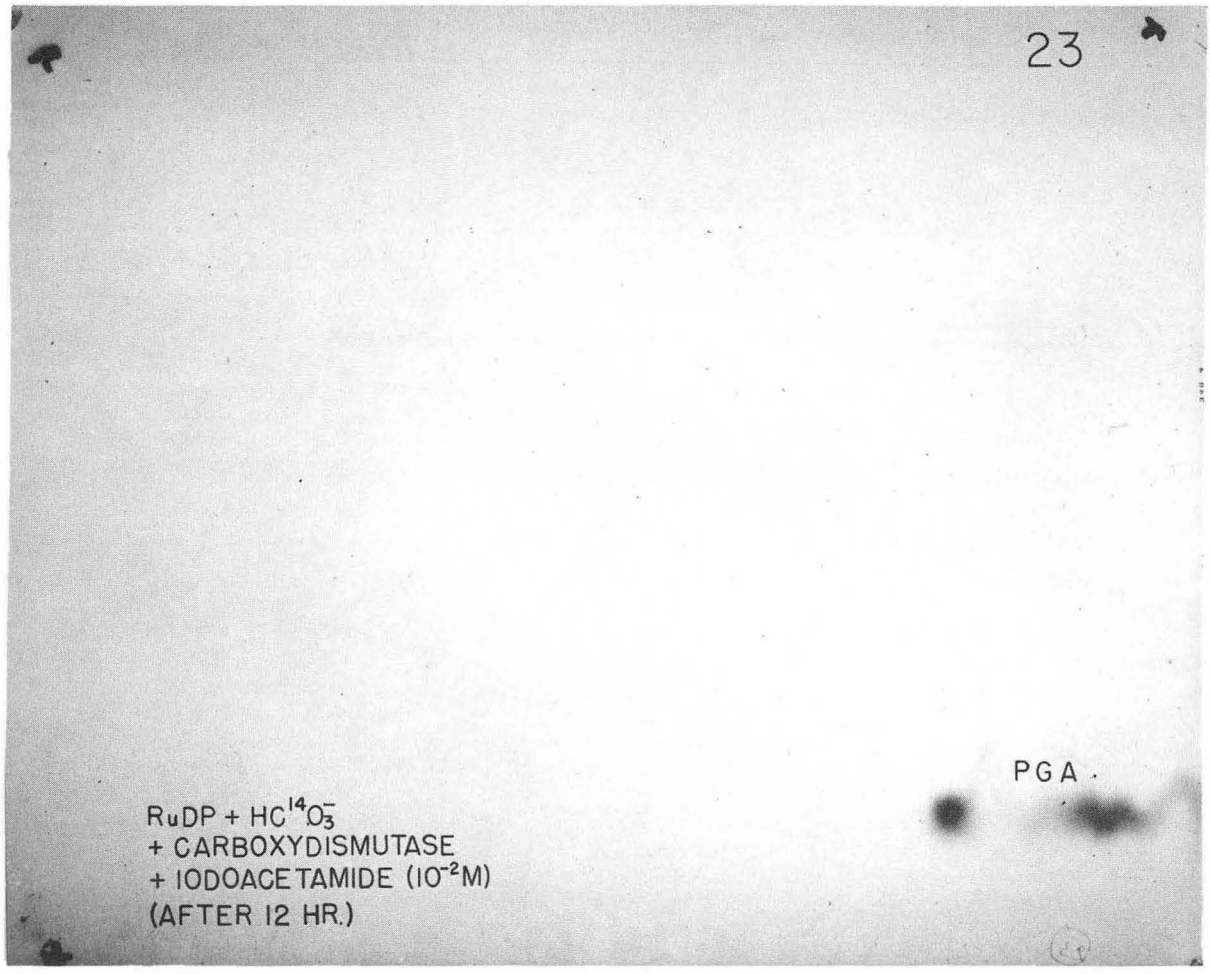
ZN-1330



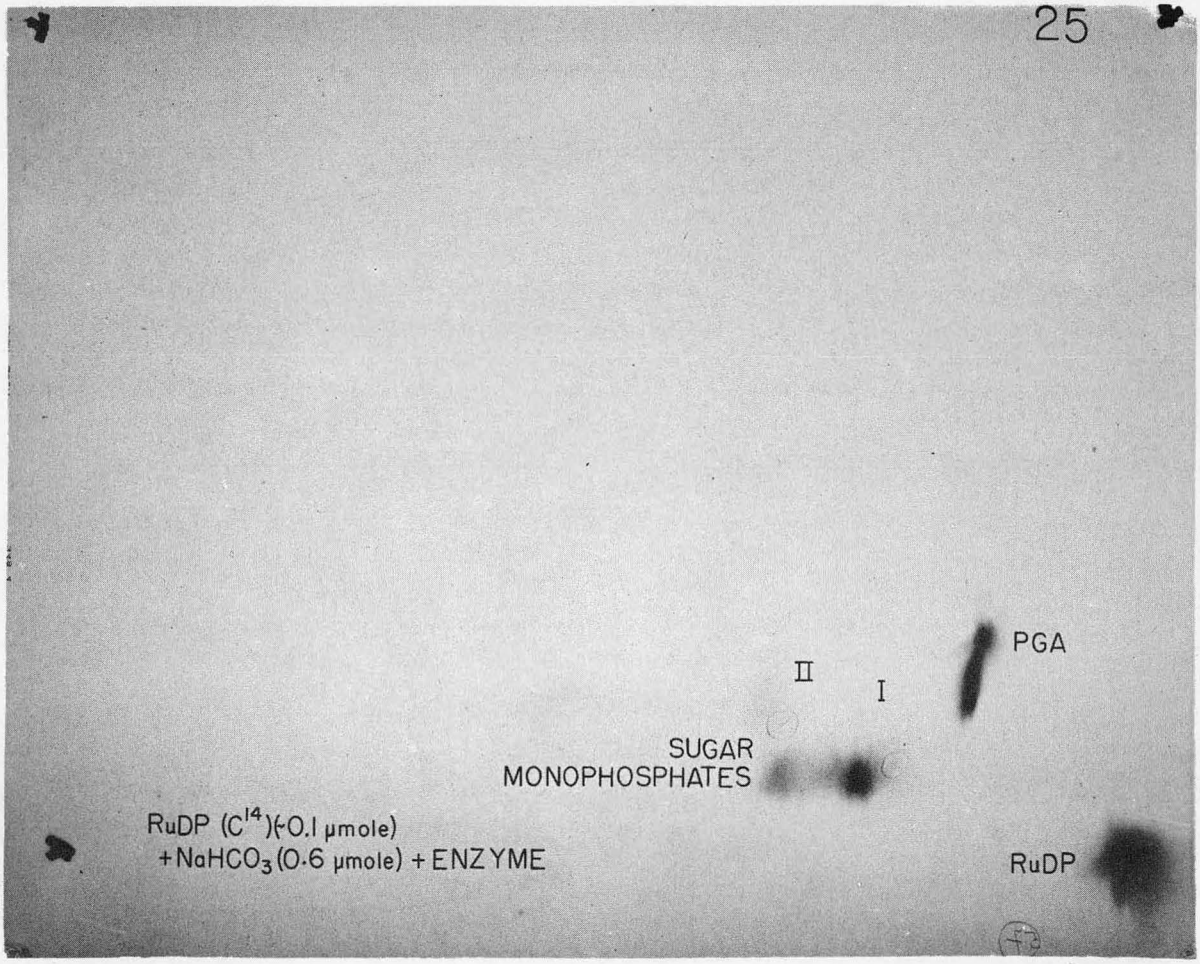
ZN-1331



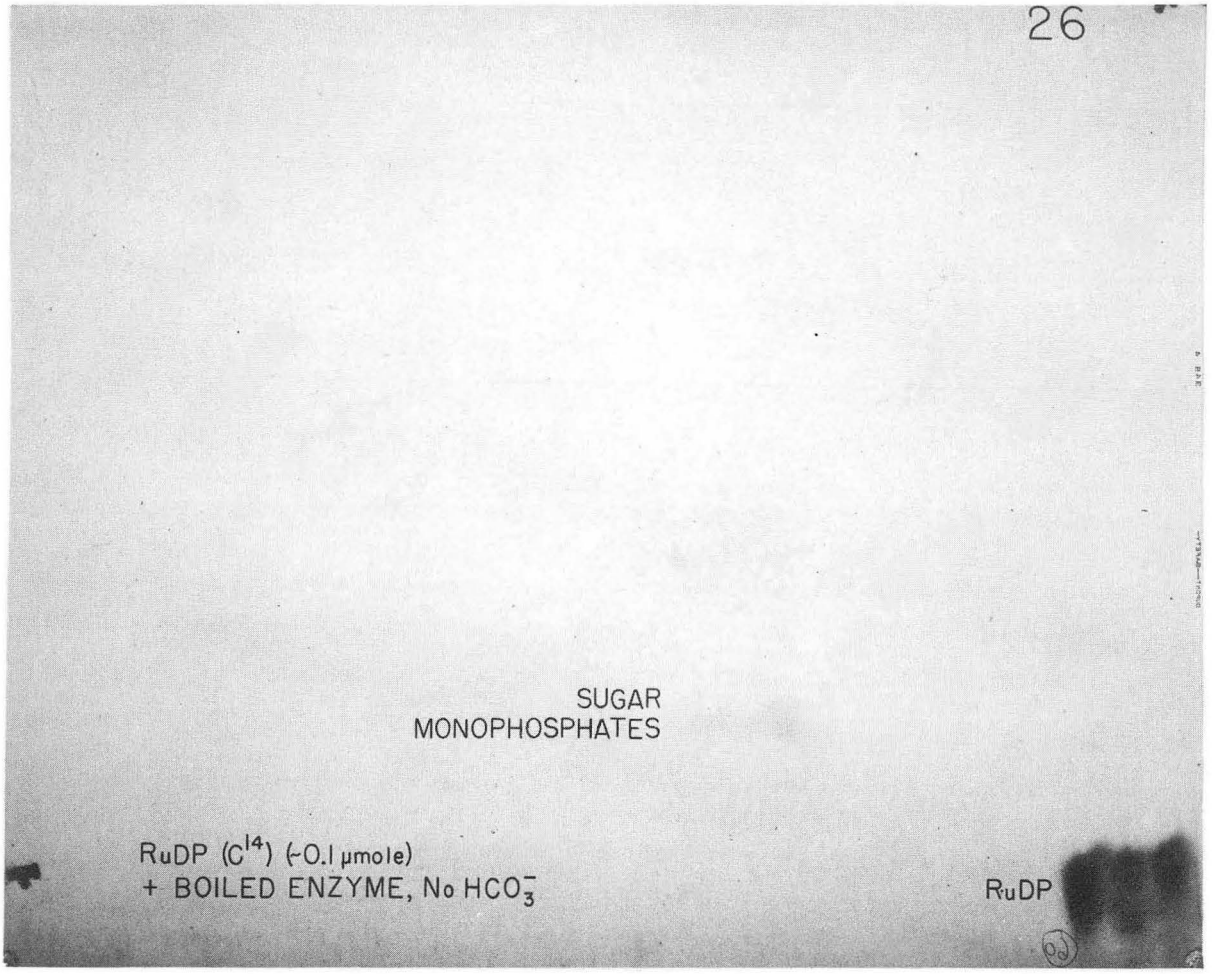
ZN-1332

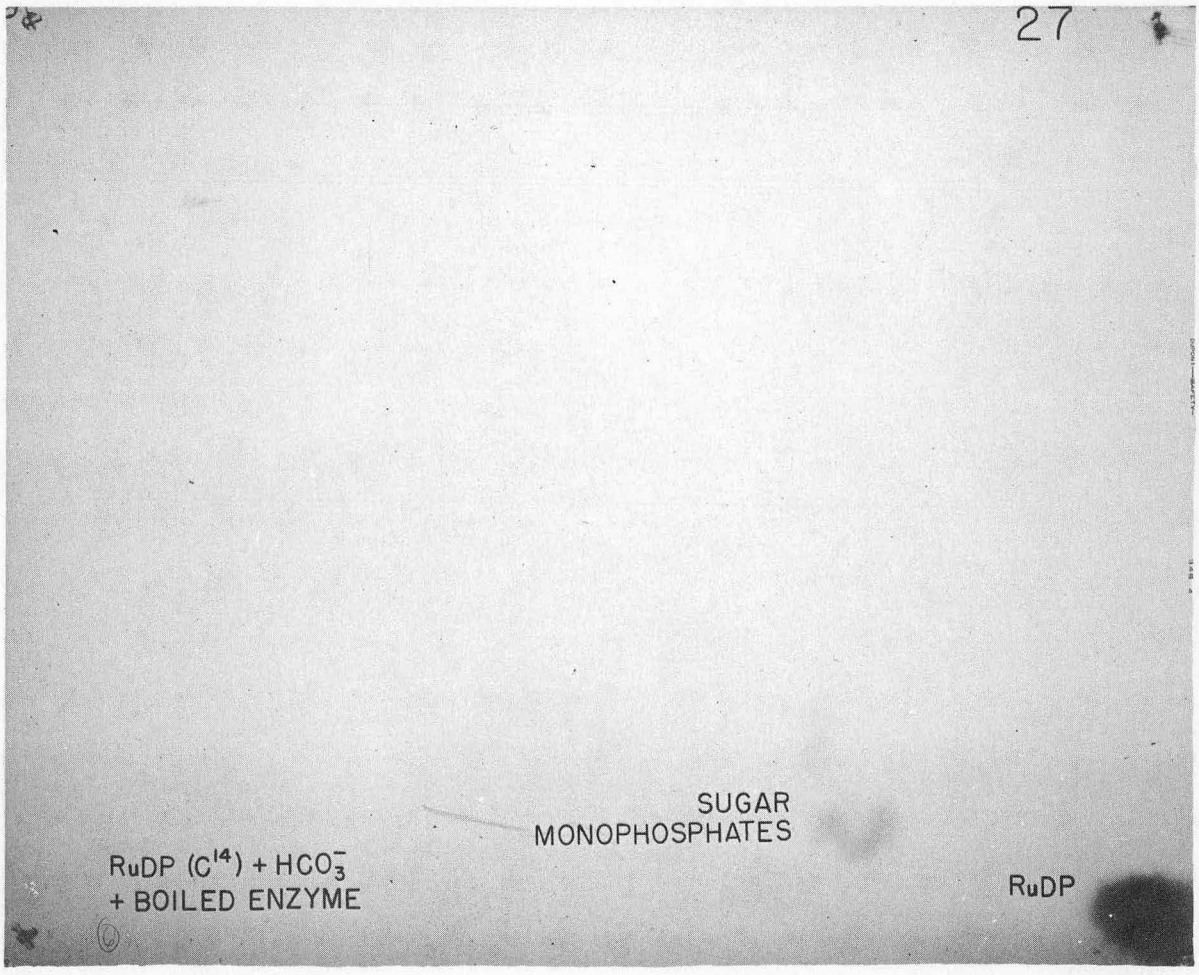

23
PGA
RuDP + $HC^{14}O_3^-$
+ CARBOXYDISMUTASE
+ IODOACETAMIDE (10^{-2}M)
(AFTER 12 HR.)

ZN-1333


25
PGA
II
I
SUGAR
MONOPHOSPHATES
RuDP (C^{14})(~0.1 μmole)
+$NaHCO_3$ (0.6 μmole) + ENZYME
RuDP

ZN-1334


26
SUGAR
MONOPHOSPHATES
RuDP (C^{14}) (~0.1 μmole)
+ BOILED ENZYME, No HCO_3^-
RuDP



ZN-1336