PROGRESS REPORT

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THE INFLUENCE OF X-RAYS ON THE KINETICS OF ERYTHROCYTE ENZYMES AS A BIOLOGICAL DOSIMETER

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Abbreviations used throughout this report are as follows:

ATP	adenosine triphosphate
ADP	adenosine diphosphate
AMP	5'-adenylic acid
F6P	fructose-6-phosphate
FDP	fructose-1,6-diphosphate
3PG	3-phosphoglyceric acid
R5P	ribose-5-phosphate
P^{32}	orthophosphate-P^{32}
CPM	counts per minute
RBC	red blood cells
KR-bicarbonate	Krebs-Ringer bicarbonate buffer
Tris	tris(hydroxymethyl)-aminomethane
glucose-U-C^{14}	uniformly C^{14}-labeled glucose
TAC	trichloroacetic acid
R_p	ratio of distances and paper chromatogram between a phosphate ester and inorganic-P^{32}.
R_g	ratio of distances on a paper chromatogram between a C^{14}-compound and glucose-U-C^{14}.

ABSTRACT

I. Formate Oxidation by Erythrocytes
D. A. Rappoport, J.A. Green and G.H. Gast
Archives of Biochemistry and Biophysics
In Press.

Oxidation of formate by human, dog, frog and avian erythrocytes were studied and the mechanism of this reaction was clarified. It was found that catalase combined with hydrogen peroxide to form a complex, catalase-peroxide, which was responsible for the oxidation of formate to carbon dioxide. All species investigated oxidized formate by this mechanism.

A copy of this manuscript has been forwarded to AFSWP, July, 1956.

II. C^{14}- Formate Metabolism by Isolated Tissues from X-Irradiated Rats.
D. A. Rappoport, R. A. Seibert and V. P. Collins
Submitted for publication to Radiation Research.

Minced test s, kidney, liver and spleen, and sections of intestine from rats treated with 5,000r total body X-rays, were incubated with C^{14}- formate 17 hours after irradiation. Similar preparations were made from unirradiated controls.

The spleen from irradiated rats exhibited a dramatic decrease in formate incorporation into all cell components; the liver showed enhanced formate metabolism while the test s and intestine did not show a significant change. The kidney showed enhanced formate utilization only in the lipid fraction.

Lack of uniform X-ray effect on tissue enzymes was noted, and the cause of both depression and enhancement in formate metabolism is discussed.

Copies of this report have been forwarded to AFSWP, July, 1956.

III. Preliminary Data on the Kinetics of Inorganic-P^{32} Uptake by the Soluble Fraction of Rat Erythrocytes and by Hemolysates.

Time studies were initiated on the esterification of inorganic-P^{32} by the soluble fraction of rat erythrocytes and by hemolysates under aerobic and anaerobic conditions, in presence and absence of glucose, adenosine and sodium fluoride.

The influence of Krebs-Ringer bicarbonate and tris buffers on the esterification of inorganic-P^{32} was investigated in the absence and presence of glucose, adenosine, and sodium flouride. This was tested with rat RBC-soluble fraction and with rat hemolysates. Some differences were noted in the type of phosphate esters formed, but the tris buffer facilitated incubation of samples hence it was chosen as the buffer for subsequent experiments. The presence of adenosine markedly enhanced the formation of phosphate esters as compared to the effect of glucose or glucose plus sodium fluoride.

Under aerobic conditions sodium fluoride did not affect the rate of formation of AMP and R5P, but it reduced the rate of formation of F6P and ATP, and enhanced the rate of the formation of 3PG.

To obtain complete anaerobic conditions, the RBC-soluble fraction was treated with carbon monoxide thus all oxyhemoglobin was converted to carboxyhemoglobin, and this eliminated a source of O_2 during incubations.

RBC-soluble fraction, in presence of adenosine, incorporated inorganic-P^{32} into F6P, 3PG, R5P, ATP, and ADP at similar rates under both aerobic (O_2 and 5% CO_2) and anaerobic conditions (CO). However, when glucose was added to RBC under anaerobic conditions (CO), the rate of

formation of F6P and 3PG increased continuously, while the rate of ADP and ATP formation increase only in the initial twenty minutes of incubation, but subsequently decreased. Also the formation of AMP was noted in the presence of glucose under anaerobic conditions.

Incubation of RBC-soluble fraction aerobically with adenosine markedly stimulated the rate formation of R5P, FDP, F6P, and ATP, but the rate of ADP formation remained the same. Only in the presence of adenosine did R5P, FDP and F6P form. In the absence of adenosine 3PG was synthesized at e slow rate.

A similar study has been initiated using glucose-U-C^{14} and KH_2PO_4. Although the products have been separated and their rate of formation noted, their identity remains to be established.

It has been concluded that the above preliminary data indicate that kinetic studies with RBC-soluble fraction and hemolysates are of sufficient interest and are enzymatically active to be pursued as an enythrocyte X-ray dosimeter.

CURRENT PROGRESS

Studies have been initiated on the kinetics of P^{32} uptake by rat hemolysates and RBC-soluble fraction as a possible erythrocyte X-ray dosimeter. Use of paper chromatography in the separation of phosphorylated intermediates greatly facilitated investigation on the type of the products formed, and also the rate of their formation. This report represents preliminary results on these studies. It is essential to note that the nature of the compounds reported is known only tentatively since they were identified by comparison to standards. Their exact identity is being established at this time.

Experimental. Blood was obtained from ether anaesthesized rats by heart puncture, and the pooled blood was centrifuged to separate the erythrocytes. After three successive washings with cold isotonic saline, the erythrocytes were lysed with an equal volume of distilled water. To prepare RBC-soluble fractions, the lysed erythrocytes were centrifuged in the cold at 10,000 rpm for 10 min., and approximately fifty percent of the lysed RBC remained as a soluble supernatant solution. The details of the incubation conditions are described under the respective table and figures referred to in the results.

The incubation mixtures were deproteinized with cold TCA to a final concentration of ten per cent. The clear supernatant solution was chromatographed on paper directly according to the procedure of Hauge and coworkers (J. Biol. Chem. 214, 1, 11 (1955)). Autoradiograms were made by direct contact of developed paper chromatograms with X-ray film. (See figures 1a through 9a). Phosphate esters were located on paper by means of the spray procedure of Hanes and Isherwood (Nature, 164, 1107 (1949)),

then the radioactivity in each spot was determined by means of a thin window Geiger-Muller tube.

Using known phosphorylated compounds as standards, the identity of the products from P^{32} incubations was only tentatively established. In experiments with glucose-U-C^{14}, the identity of the products is not known and this is under investigation at present.

Results. Comparative influence of KR-bicarbonate and tris buffers on P^{32} uptake is shown in Table 1. The effect of added glucose, glucose plus sodium fluoride, and adenosine are also shown in this table for both the RBC-soluble fraction and hemolysates, respectively. Some variation in the nature of the products is noted when either KR-bicarbonate or tris buffers are used. However, the most pronounced effect is obtained when adenosine is added in either buffer.

Under strictly anaerobic conditions, when carbon monoxide is used, and in the absence of glucose, the rate of formation of F6P and 3PG was rapid after an initial 20 minute lag period. (Figure 1).

Similarly, under aerobic conditions in absence of glucose, the formation of F6P and 3PG was also rapid but without a lag period. (Figure 2). The formation of ATP, ADP, and R5P was similar in both experiments. Under anaerobic conditions, in presence of glucose, the rate of formation of 3PG and F6P was enhanced, while ARP and ADP, showed a maximum rate of formation in 20 minutes which subsequently decreased. Here also the formation of AMP is noted although the rate of AMP formation is relatively low (Figure 3).

Incubation of RBC-soluble fraction with and without sodium fluoride is shown in Figure 4 and 5, respectively. In the absence of NaF F6P is

formed rapidly while 3PG is formed slowly (Figure 4); but in the presence of NaF, 3PG is formed rapidly and F6P is formed at an appreciably lower rate (Figure 5). The formation of ATP is somewhat enhanced in absence of fluoride (Figure 4), while R5P is formed at a slightly greater rate in presence of fluoride (Figure 5).

The influence of adenosine on P^{32} uptake was investigated. Figures 6 and 7 represent the rate of phosphate ester formation without and with adenosine, respectively. In absence of adenosine ADP is formed rapidly while ATP and 3PG are formed much more slowly (Figure 6). In presence of adenosine no 3PG is formed, but R5P and FDP are rapidly synthesized, and F6P appears at a much slower rate (Figure 7). The formation of ATP is also enhanced in presence of adenosine, although ADP is formed at approximately the same rate in presence or absence of adenosine (Figures 6 and 7).

In order to compare the products formed from glucose and P^{32} with those from glucose-U-C^{14} and K_2HPO_4, incubations of RBC-soluble fraction were made using glucose-U-C^{14}. Figure 8 shows the results of this preliminary study in the absence of adenosine, however the identity of these compounds remains to be established. Figure 9 shows the C^{14}-products formed in the presence of adenosine from glucose-U-C^{14}.

Discussion and Conclusions. Since the erythrocyte soluble fraction contains both the glycolytic enzymes as well as the hexosemonophosphate shunt, it was necessary to study the incorporation of P^{32} under both aerobic and anaerobic conditions. Under anaerobiosis only the glycolytic enzyme will function, while aerobically both the glycolysis and

shunt systems operate as evident from the products in Table 1.

Thus, use of CO with RBC-soluble fraction to insure absolute anaerobiosis shows mainly the formation of 3PG and F6P in absence of glucose (Figure 2), and in presence of glucose, the enhanced formation of these components is reflected by disappearance of the initial lag period (Figure 3). Formation of ATP and ADP can, however, be attributed to the glycolytic enzymes, such as those involved in the oxidative phosphoxylation of triosephosphate with the concomitant formation of ATP, while the kinase reactions responsible for utilization of ATP can thus form ADP. The rates of ATP and ADP formation do not necessarily imply de novo formation of these components in these experiments. Some of these compounds can be formed by exchange of phosphate with P^{32}.

Fluoride was used to partially inhibit glycolysis and facilitate accumulation of 3PG, and to observe the operation of the hexosemonophosphate shunt which is not affected by fluoride. Some enhanced formation of R5P was noted in presence of fluoride (Figure 5) and as predicted, in presence of fluoride F6P formed more rapidly and 3PG formed more slowly than in the absence of fluoride (Figure 4). Thus, the operation of the hexosemonophosphate shunt was enhanced by fluoride, since more intermediates were available for this system by the partial inhibition of glycolysis.

The influence of added adenosine is twofold; a) as a precursor for ADP and ATP, and b) as a source of ribose. Since more R5P is formed in presence of adenosine (Figure 7) and (Figure 6) also more FDP is formed due to conversion of pentose to hexose phosphate by the shunt

mechanisms. Also ATP formation is also enhanced in the presence of adenosine (Figures 6 and 7). Thus both of the above postulates are evidenced from the data.

Although glucose-U-C^{14} was utilized by RBC soluble fraction, it is too early to determine the nature of the products formed (Figures 8 and 9). This is being investigated at present.

From the above observations, the kinetic studies on P^{32} and glucose-U-C^{14} metabolism indicate that studies of X-ray effects on these systems should provide interesting and useful data.

Table I

Effect of Buffers and Additives on Inorganic P^{32} Uptake into Organic Esters by Rat RBC-Soluble Fraction and Hemolysate

RBC-SOLUBLE FRACTION

Buffer	Without Additives	10 uM Glucose	.04M NaF +10 uM glucose	10 uM Adenosine
KR-Bicarbonate	ADP*	ADP*	ADP*	ADP
	FDP*	FDP*	FDP*	AMP
	F6P*	F6P*	F6P*	FDP
			R5P*	F6P
			3PG*	RSP
				3PG
0.05M Tris	ADP	ADP	ADP	ADP
	AMP*	AMP*	AMP*	AMP
	FDP	FDP	FDP	FDP
	R5P*		R5P*	R5P
	3PG*		3PG*	3PG

HEMOLYSATE

KR-Bicarbonate	ADP*	ADP*	ADP*	ADP
	AMP*	AMP*	AMP*	AMP*
	R5P	R5P*	R5P*	FDP
	3PG*	3PG*	3PG*	RSP
				3PG
0.05M Tris		ADP*	ADP*	ADP*
		AMP	AMP*	AMP
		FDP	FDP*	FDP
		R5P*	R5P*	R5P
			3PG*	3PG

Incubated 2 ml. RBC solubles or hemolysate with buffer to a total volume of 4.5 ml. at pH 7.4. Contained 1 uM P^{32}-orthophosphate. One hour incubation in O_2-5% CO_2.

* - Present in trace quantities.

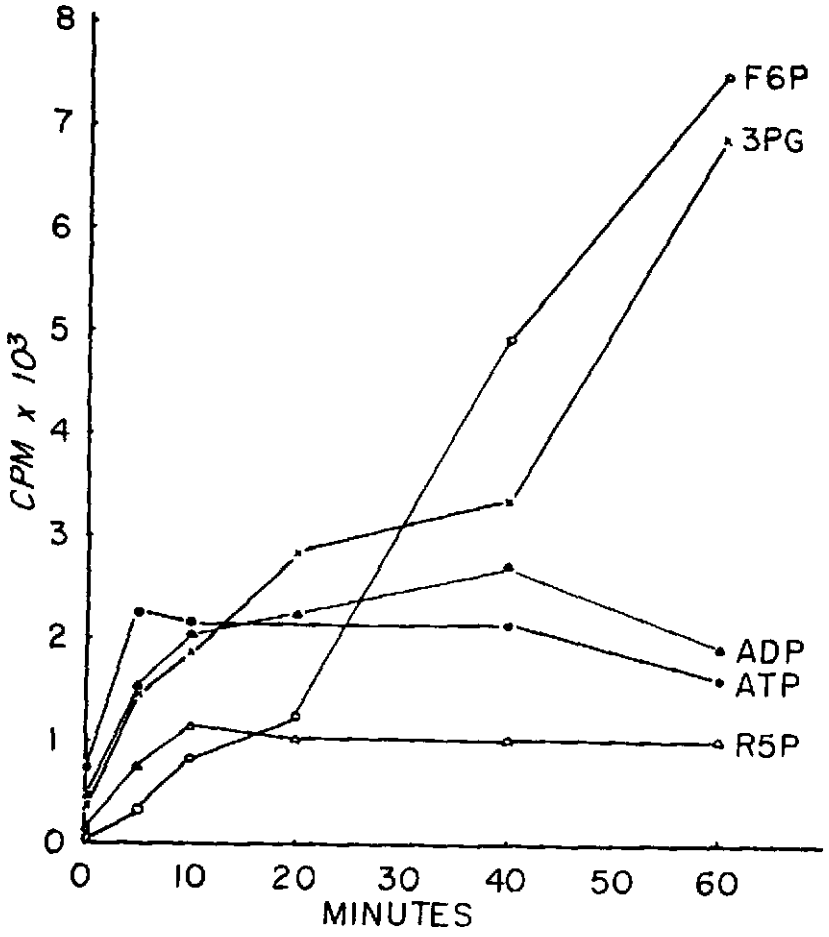

Figure 1. Formation of organic phosphate ester anaerobically in absence of glucose. Incubation mixture consists of 2 cc R.B.C. soluble fraction from 1:1 hemolysate in 0.05M Tris buffer pH=74, 10 uM adenosine, and 1 uM phosphate-$\underline{P}^{32}$ in a total volume of 3.7 cc. Incubation carried out in atmosphere of CO at 37°C after the RBC-soluble fraction was saturated with CO by bubbling CO through this fraction for 5 minutes.

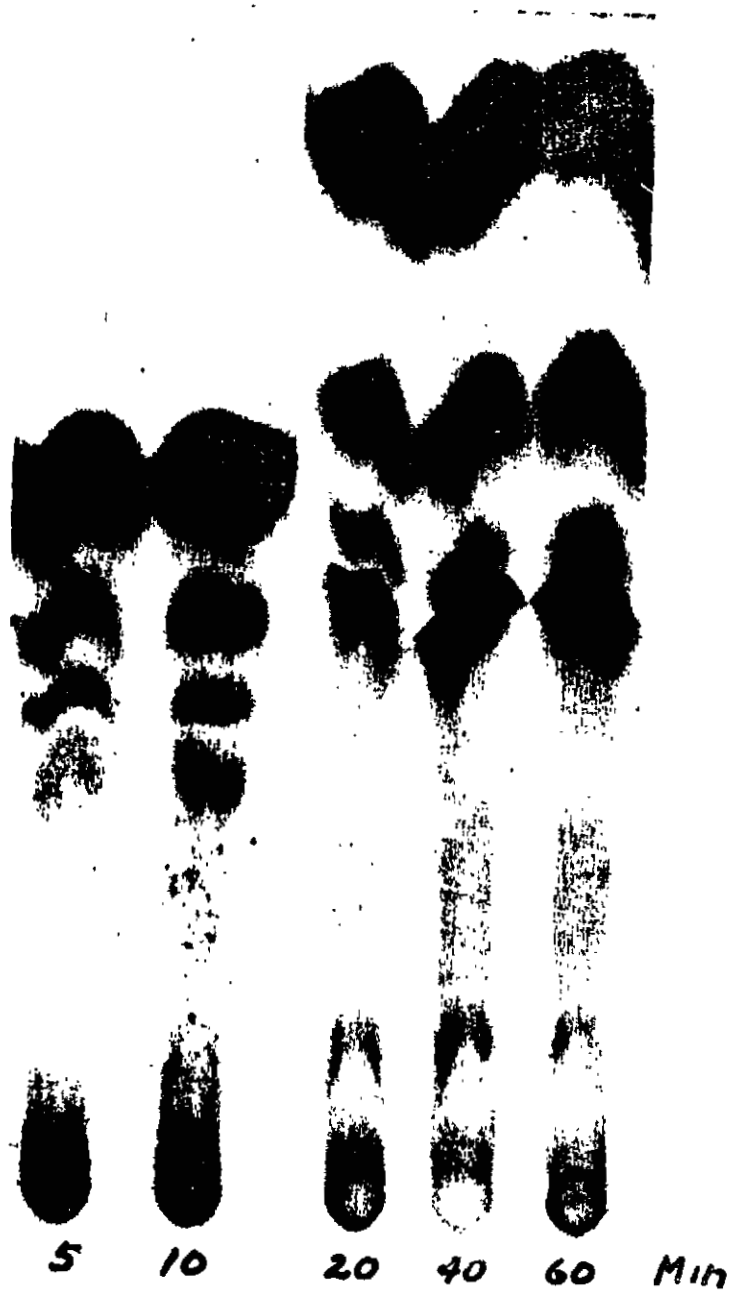

Figure 1a. Autoradiogram of products formed in experiment represented in Figure 1.

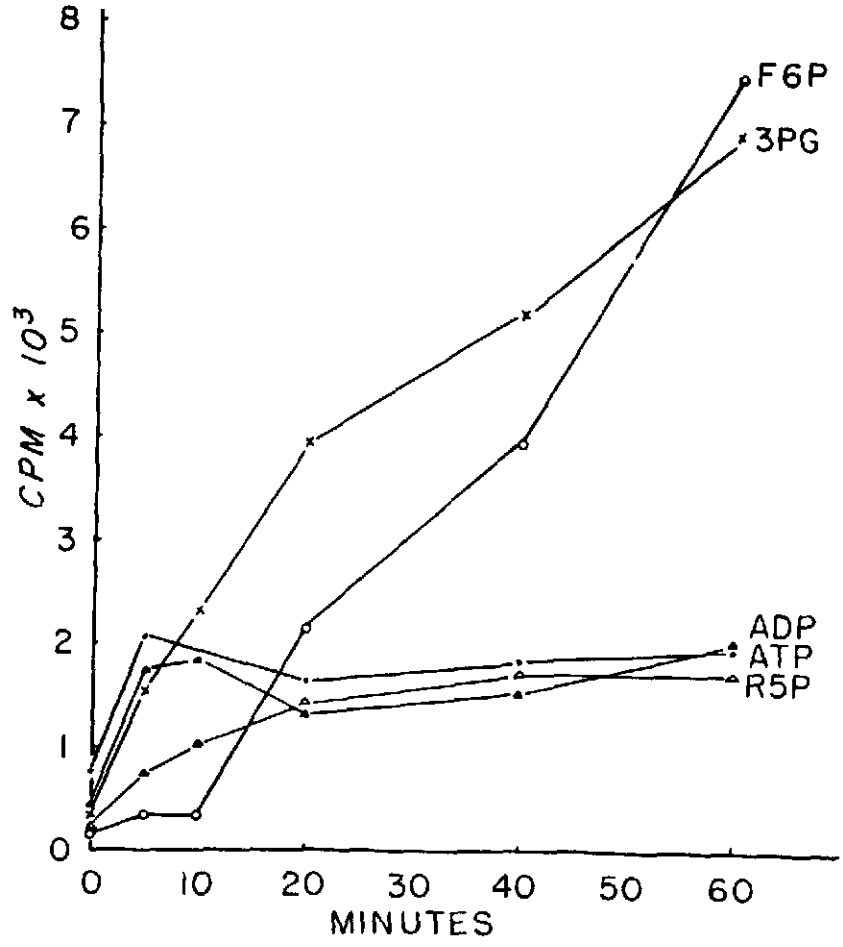

Figure 2. Formation of organic phosphate ester aerobically in absence of glucose. Incubation mixture is the same as Figure 1. Incubation carried out in atmosphere of O_2-5% CO_2 at 37°C.

Figure 2a. Autoradiogram of products formed in experiment represented in Figure 2.

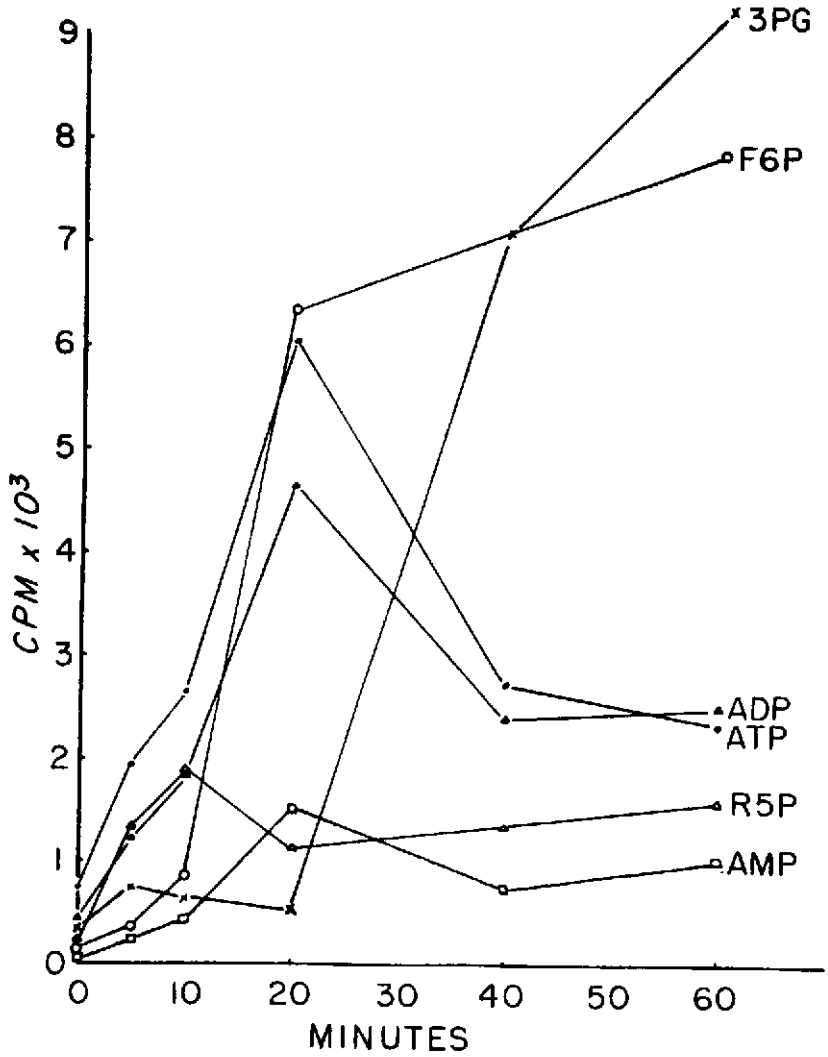

Figure 3. Formation of organic phosphate ester anaerobically in presence of glucose. Incubation mixture is the same as Figure 1 with the addition of 10 uM glucose. Incubation was carried out as in Figure 1.

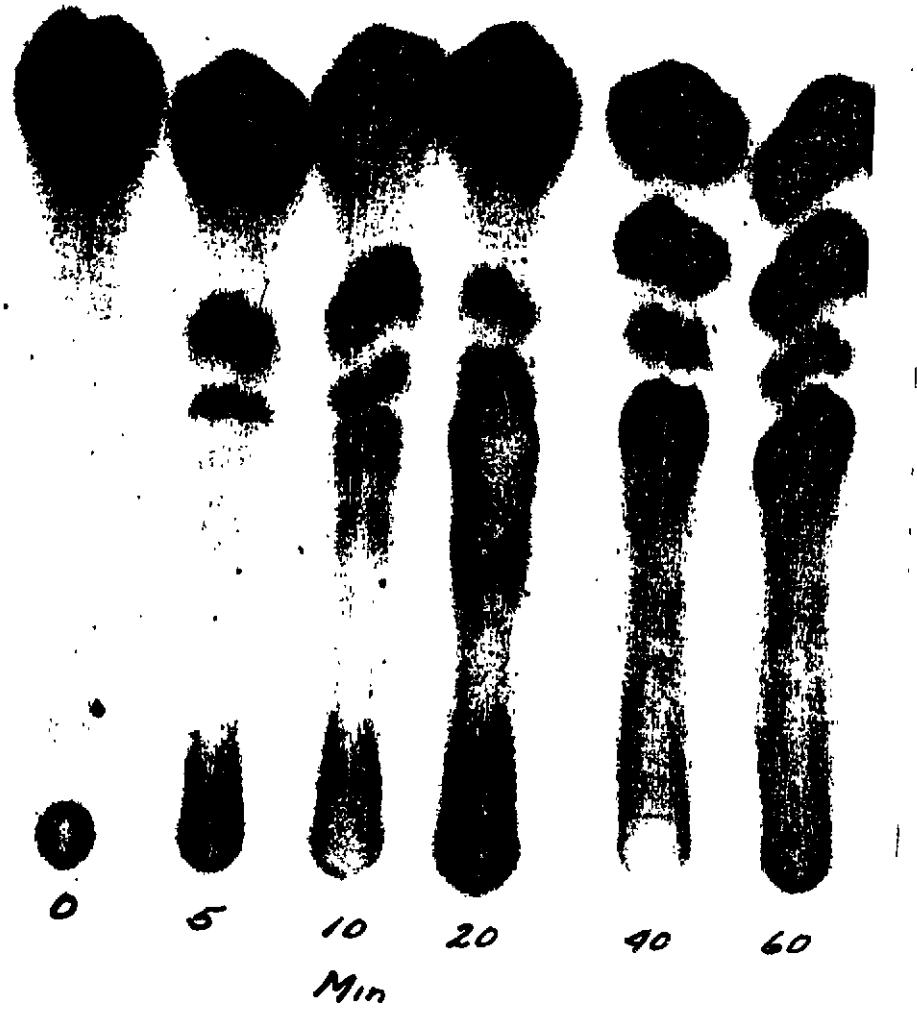

Figure 3a. Autoradiogram of products formed in experiment represented in Figure 3.

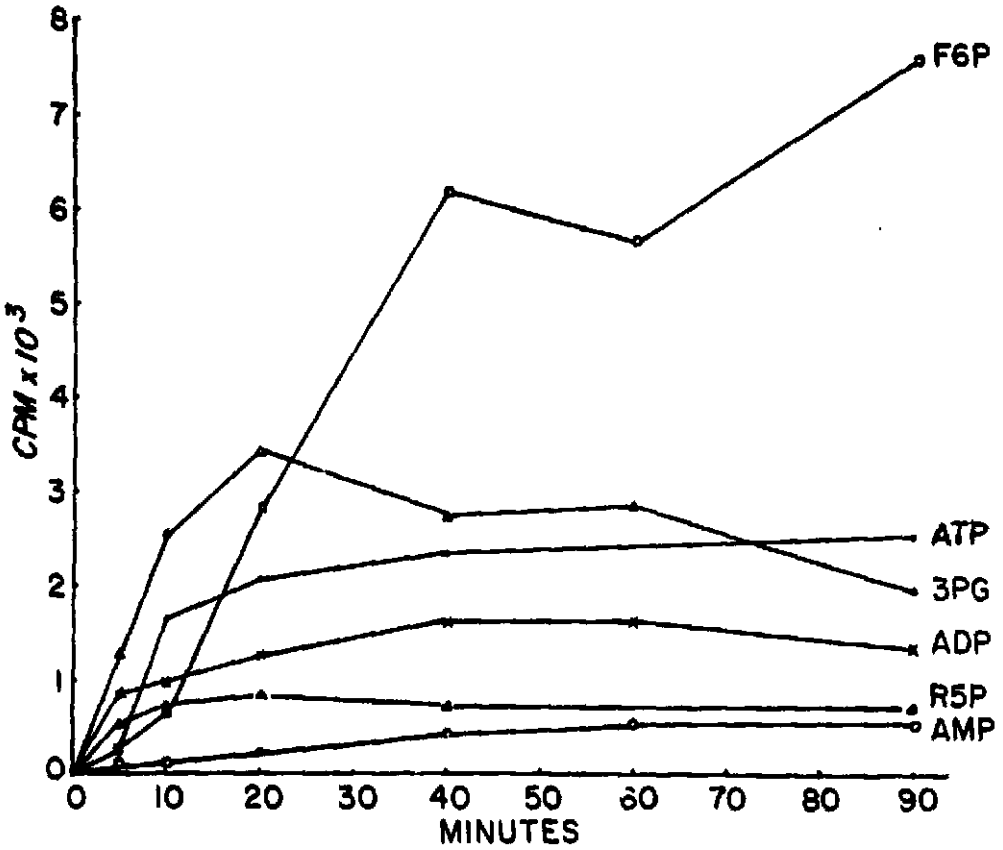

Figure 4. Formation of organic phosphate ester in the absence of NaF. Incubation mixture consists of 2 cc R.B.C. soluble fraction from 1:1 hemolysate in 0.05 M Tris buffer pH=7.4, 10 uM glucose, 10 uM adenosine, and 1 uM phosphate-P^{32} in a total volume of 3.8 cc. Incubation carried out in an atmosphere of O_2 -5% CO_2 at 37°C.

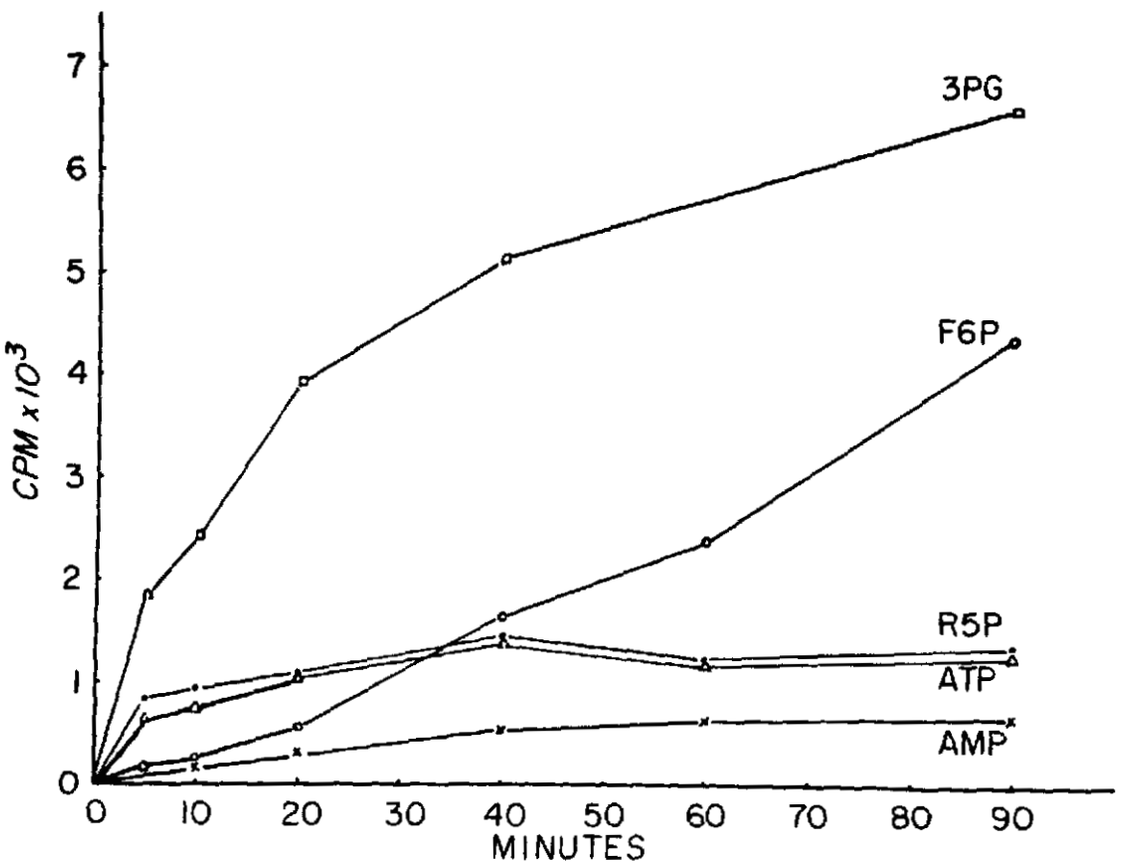

Figure 5. Formation of organic phosphate ester in the presence of NaF. Incubation mixture is the same as in Figure 4 with the addition of 0.04 M NaF.

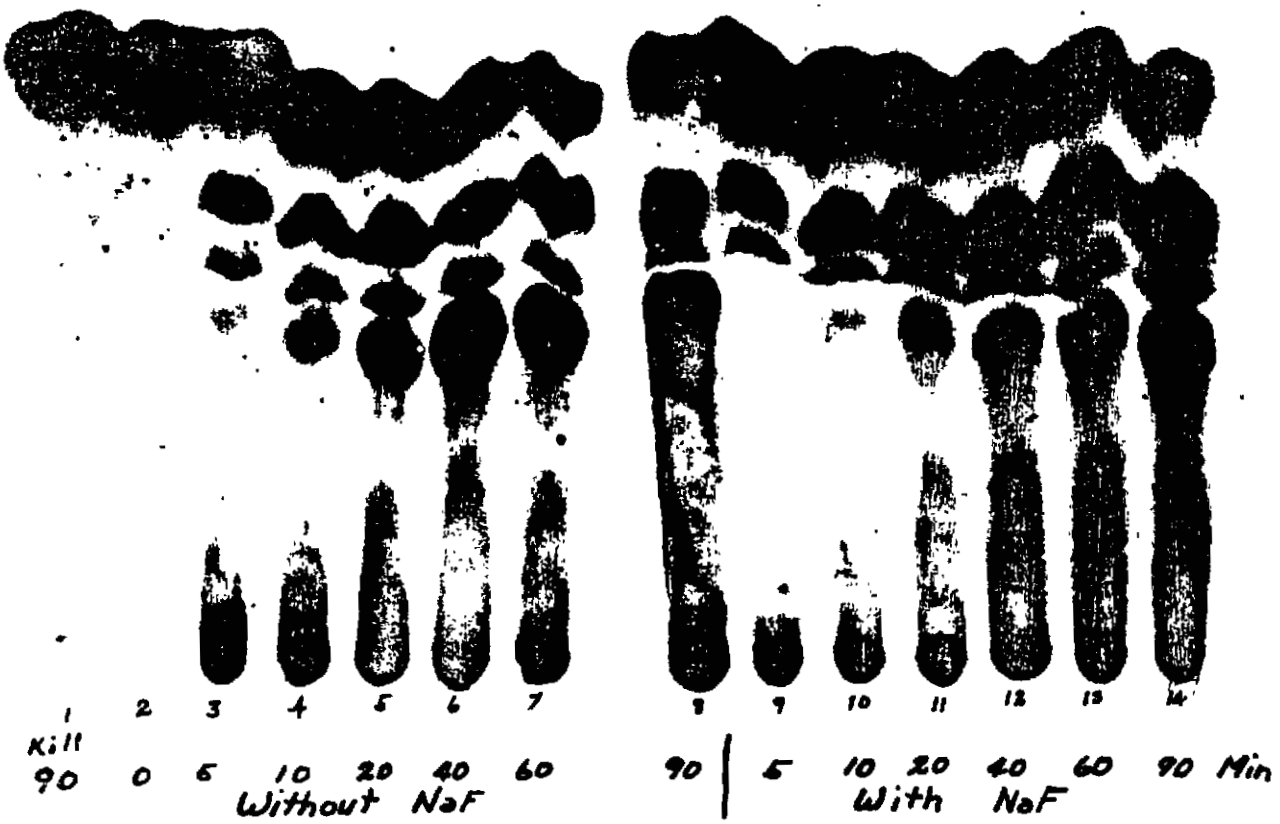

Figure 5A. Autoradiogram of products formed in experiments represented in Figures 4 and 5.

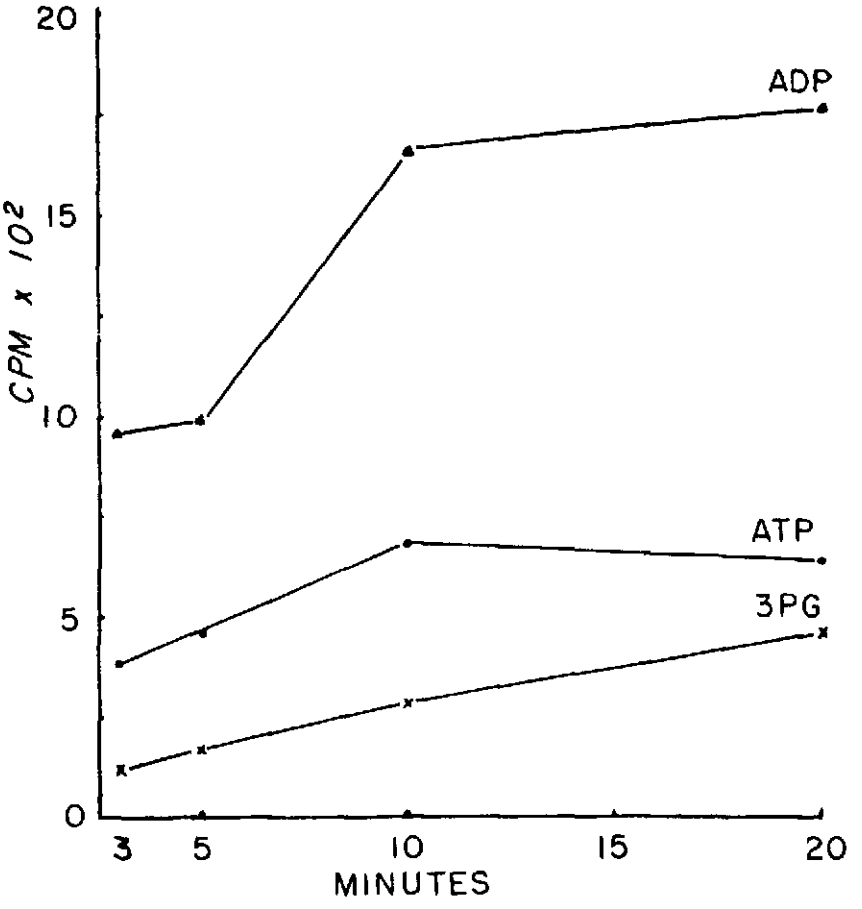

Figure 6. Formation of organic phosphate ester in absence of adenosine. Incubation mixture consists of 2 cc R.B.C. soluble fraction from 1:1 hemolysate in 0.05 M Tris buffer pH=7.4, 10 uM glucose, 1 uM phosphate-P^{32} in a total volume of 3.7 cc. Incubation carried out in atmosphere of O_2-5% at 37° C.

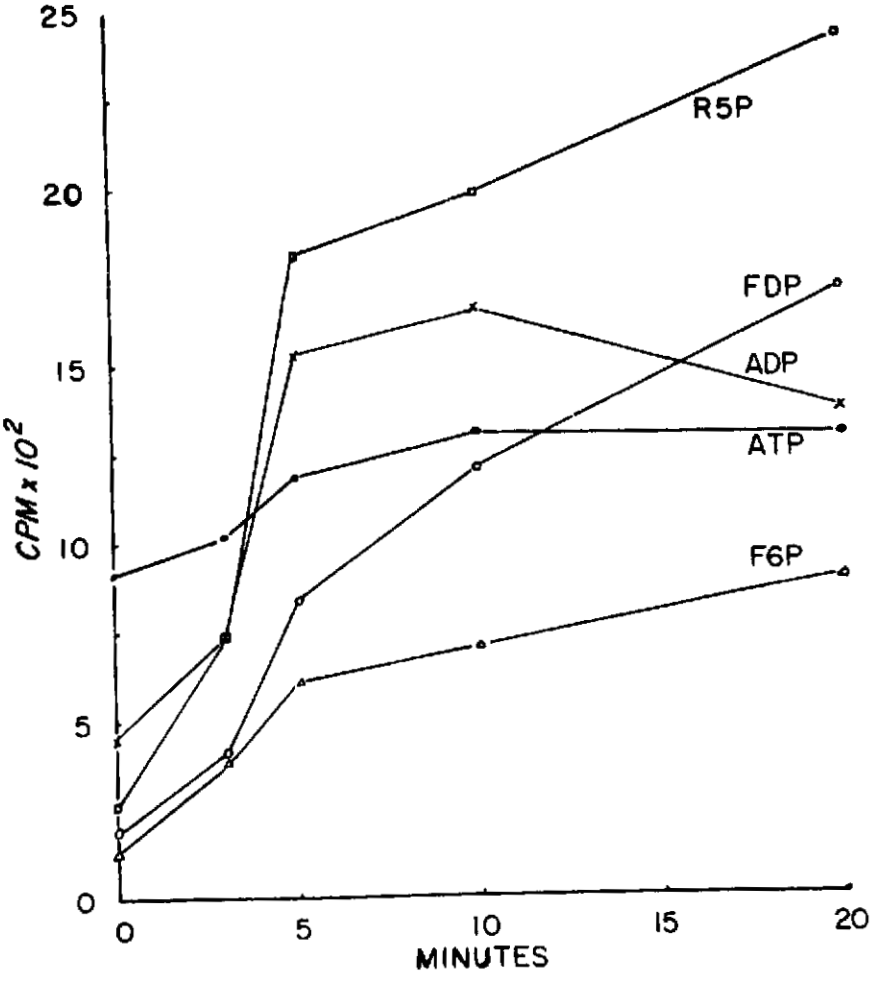

Figure 7. Formation of organic phosphate ester in the presence of adenosine. Incubation mixture is the same as Figure 6, with the addition of 10 uM adenosine.

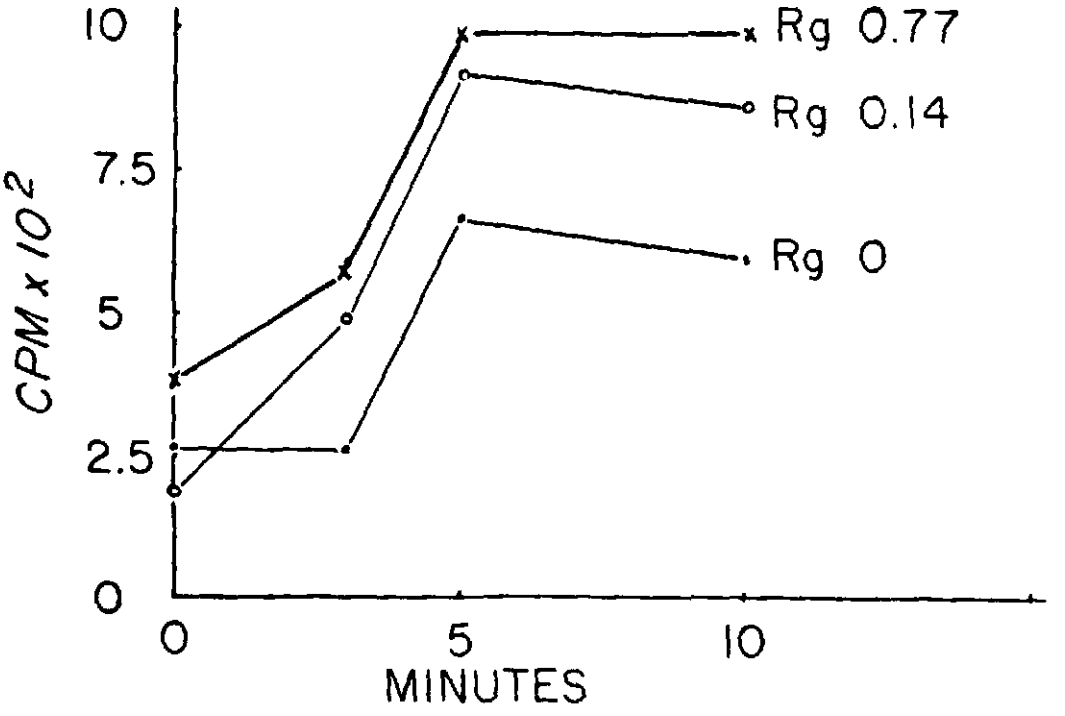

Figure 8. Formation of C^{14} - labelled products in absence of adenosine. Incubation mixture consists of 2 cc of R.B.C. soluble fraction from 1:1 hemolysate in 0.05 M Tris buffer pH=74, 1 uM K_2HPO_4 and 10 uM glucose-U-C^{14} in a total volume of 3.7cc. Incubation carried out in atmosphere of O_2 - 5% CO_2 at 37°C.

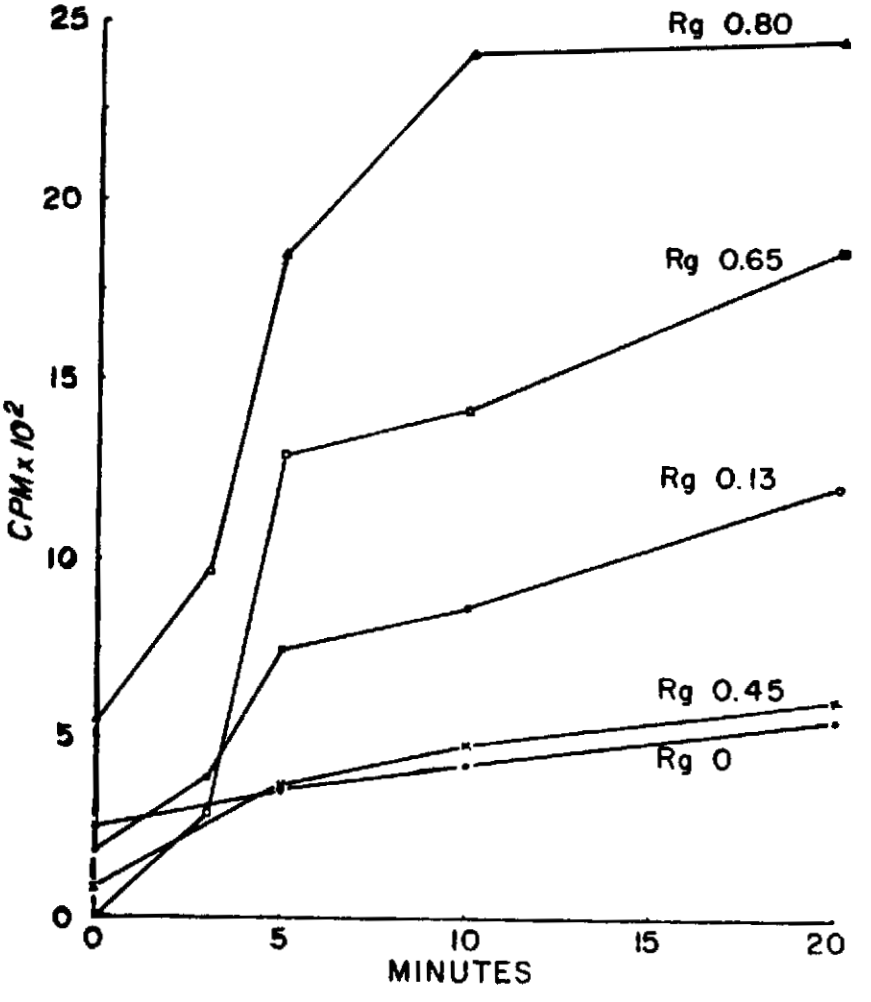

Figure 9. Formation of C^{14}- labelled products in presence of adenosine. Incubation mixture is the same as Figure 8 with the addition of 10 uM adenosine.

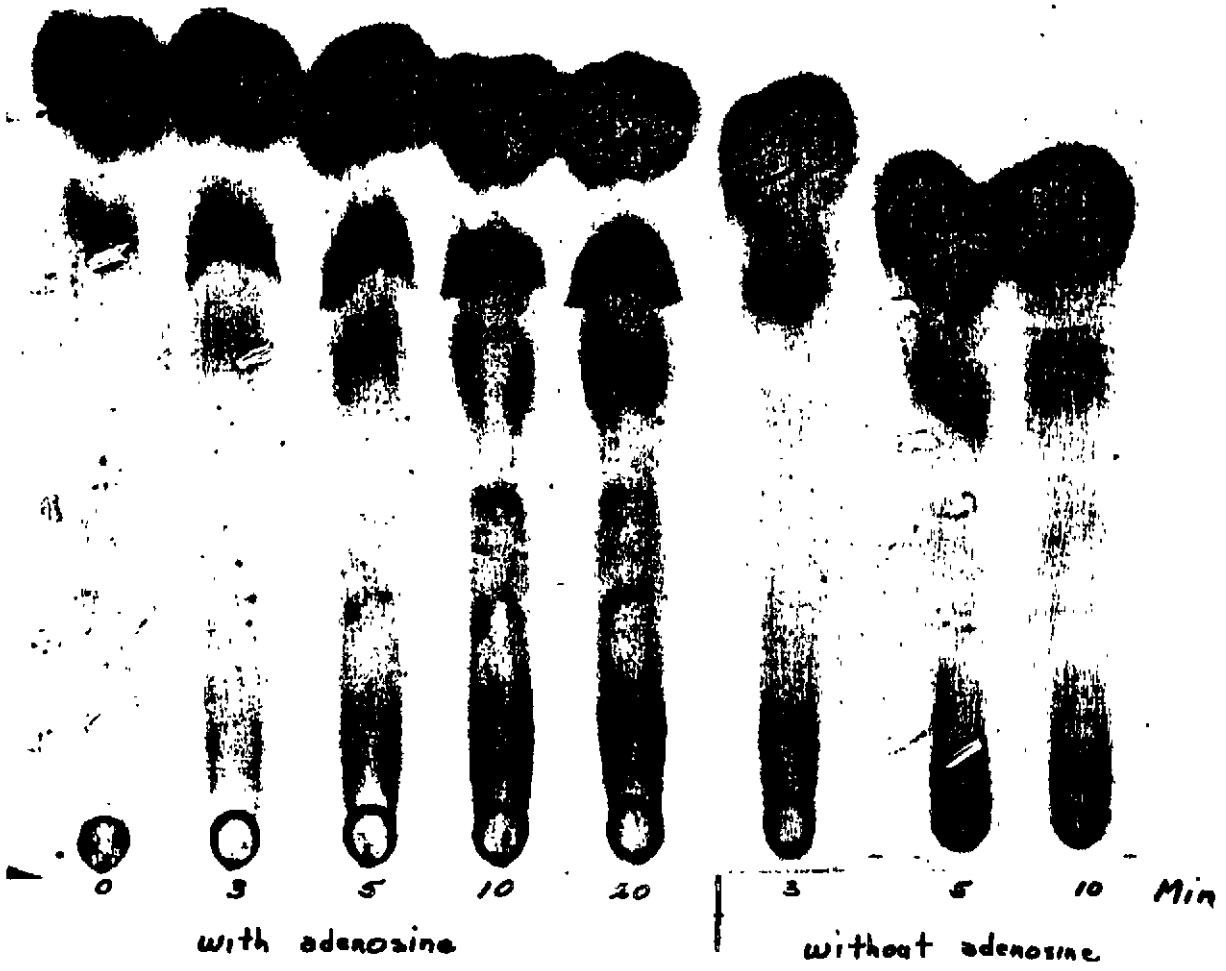

Figure 9A. Autoradiogram of products formed in experiments represented in Figures. 8 and 9.

FUTURE PLANS

In the immediate future RBC-soluble fractions and hemolysates will be used from total body X-irradiated rats (treated from 100 to 600r) at intervals from 1 day to 10 days after irradiation. The kinetics of P^{32} and glucose-U-C^{14} metabolism will be studied mainly: a) for the initial rate of metabolism from zero to 60 minutes, and b) for the rate of metabolism from 1 to 4 hours incubations. These results will be compared to the data from unirradiated controls.

It is also planned to carry out this study with glucose-1-C^{14} and glucose-6-C^{14} in order to simplify the interpretation of reactions due to glycolysis and the hexosemonophosphate shunt system.

The purpose of these investigations is to correlate X-ray dose with any changes in the rate of P^{32} and glucose metabolism, and to establish whether any correlation exists between varying post irradiation time intervals and notable changes in the rates of metabolism of the above substrates.

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   D. A. Rappoport, J. A. Green, and J. H. Gast.
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2. C^{14}-Formate Metabolism by Isolated Tissues from X-Irradiated Rats.
   D. A. Rappoport, R. A. Seibert, and V. P. Collins
   Submitted to Radiation Research.

3. Kinetics of P^{32} Metabolism by the Soluble Fraction of Rat Erythrocytes.
   D. A. Rappoport
   In preparation; to be submitted to J. Biological Chemistry.

4. Kinetics of Glucose Metabolism by Rat Hemolysates and RBC-Soluble Fraction.
   D. A. Rappoport
   In preparation; to be submitted to J. Biological Chemistry.