were made. All species of spermatozoa tested, including those highly sensitive to an increase in osmotic pressure, were fully motile in 2·0 M glycerol. It is most unlikely that cells impermeable to glycerol would retain motility in the presence of such an excessive external osmotic pressure, and the retention of motility in the presence of high concentrations of glycerol was taken as evidence that the cells were permeated by this solute. Other neutral solutes can to some extent protect spermatozoa against damage by freezing and thawing (Smith & Polge, 1950), but none is as effective as glycerol.

SUMMARY

1. The spermatozoa of the rabbit, the bull, the fowl and the herring were subjected to freezing and thawing in media containing various concentrations of glycerol, and observations made of the resulting changes in motility of the spermatozoa.

2. These observations indicated that the damage suffered by the spermatozoa during freezing and thawing was caused by their exposure to excessive concentrations of salt when water was removed as ice.

3. The concentration of electrolyte above which irreversible damage occurred was characteristic for the spermatozoa of each species tested. The protective action of glycerol was due to its ability to prevent the salt concentration rising above this level.

We are indebted to Dr A. S. Parkes, F.R.S., for his interest and advice. Our best thanks are due to Dr Audrey U. Smith who made freely available the results of her wide experience in freezing living cells. We should also like to express our thanks to Mr J. H. S. Blaxter, of the Marine Laboratory, Wood Street, Torry, Aberdeen, who supplied the herring gonads.

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A Study of the Metabolism of Phosphorus in Mammalian Red Cells

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(*Received* 24 *June* 1954)

It has been well established that orthophosphate containing radioactive phosphorus ([^{32}P-]orthophosphate) present in the plasma enters the mammalian red cell by way of an exchange process without a net increase in the phosphate concentration within the cell (Hahn & Hevesy, 1941; Gourley, 1952.) The radioactive phosphorus thus taken up by the cell is present within the cell not only as orthophosphate but also in the form of organophosphate esters. One can therefore visualize two aspects of the phosphate metabolism of the red cell: (1) the extracellular–intracellular exchange process involving the transport of [^{32}P]orthophosphate across the cell membrane, and (2) the intracellular partition of phosphorus amongst orthophosphate and various organic phosphate esters.

This paper will deal with the intracellular partition of phosphorus as studied by means of the addition of [^{32}P]orthophosphate to the extracellular medium. It will be shown that the relationship of the relative specific activities (R.S.A.) of the intracellular phosphate esters to each other and to orthophosphate suggests that (*a*) orthophosphate enters glycolysis by way of the glyceraldehyde 3-phosphate dehydrogenase reaction, and (*b*) that the intracellular inorganic phosphate fraction is not the main source of the phosphorus incorporated into the ester phosphate.

In order to lend further support to the foregoing conclusions, measurement of the rate of disappear-

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ance of extracellular [^{32}P]orthophosphate and estimations of [^{32}P]distribution among phosphate ester components of the stroma were made.

METHODS

Between 0·05 and 0·15 ml. of 0·0007 N-$NaH_2{}^{32}PO_4$ (depending on the specific activity of the material) was added to 10 ml. of fresh, heparinized blood. In experiments lasting up to 6 hr. no glucose was added and no precautions were taken to ensure sterility, but in those lasting for longer periods sufficient glucose was added to raise the initial plasma concentration to about 400 mg./100 ml. and aseptic techniques were used. Incubation at 37·5° was carried out in siliconized glassware in order to minimize frothing. The blood was kept mixed and at nearly constant pH by bubbling a gas mixture containing 95% O_2 and 5% CO_2 slowly through a fine-bored glass tube.

Measurements of pH were made periodically on plasma samples of the incubating blood with a glass electrode. Over the course of 6 hr. no pH change was observed; over the course of 24 hr. the pH fell from a mean of 7·8 to 7·5.

At varying times suitable volumes of blood were removed and centrifuged at 2500 *g* for 15 min. The plasma was pipetted off and kept, and the cells washed first in an isotonic phosphate–saline solution (90 ml. 0·85% NaCl + 10 ml. 0·1 M-Na_2HPO_4) and secondly in isotonic saline, centrifuging being carried out at the same speed and for the same time at each washing. The 'buffy coat' was removed from the washed packed cells, which were finally frozen and kept in this state until further treatment.

The cells were then thawed and haemolysed by adding twice the volume of distilled water containing a trace of saponin, and 0·4 ml. of 0·2 M acetate buffer, pH 4·2. The haemolysate was spun in polyethylene tubes in a high-speed angle centrifuge (Servall) at 15 000 *g* for 20 min., the entire operation being performed in a cold room at 4°. The supernatant solution was carefully removed from the stroma and the latter washed twice more with the same acetate buffer; centrifuging was carried out as before. The stroma thus prepared was almost white. Both haemolysate and stroma were frozen, and on subsequent thawing 10% trichloroacetic acid (TCA) extracts were made by adding 0·1 vol. of a 100% (w/v) TCA solution to the haemolysate, and a known volume of 10% (w/v) solution of TCA to the stroma.

Chromatography. Phosphate esters were separated in the manner described by Caldwell (1953). The TCA extract (0·2 ml.) was dried on Whatman no. 4 paper (which had been washed previously with 2 N acetic acid) over an area 1 cm. or less in diameter, the whole process being carried out in a current of warm air. The chromatograms were run in *n*-propanol (60 ml.)–conc. NH_3 (30 ml.)–water (10 ml.) for about 16 hr. and *tert.*-butanol (80 ml.)–water (20 ml.)–picric acid (4 g.) for 5–6 hr. The esters could be located readily with a surface-scanning counter, and this method was adopted throughout. The following compounds were separated: adenosine triphosphate (ATP), adenosine diphosphate (ADP), 2:3-diphosphoglyceric acid (2:3-DPG), fructose 1:6-diphosphate, inorganic phosphate. The relative R_F values of these compounds are dealt with in detail in the papers of Hanes & Isherwood (1949) and Caldwell (1953).

Radioactivity assays. A standard area around each compound was cut out and the activity assayed with a thin-window Geiger–Müller counter. A similar area of paper known to be devoid of phosphate esters was assayed as background. The activity of the compounds was such as to give counting rates of 1000–3000 counts/min. Counting was continued for a sufficient length of time to give a statistical error of less than 3%. The radioactivity of the extracellular fluid was assayed by drying 0·05 ml. of the fluid on to a small area of filter paper and counting this in the same way.

Counts were also made occasionally on the supernatant fluid after the second washing of the cells, and it was found that practically all the radioactivity had been removed from the extracellular fluid; the activity was reduced to less than 0·5% of the original. Specific activities were corrected to a standard addition of 10^5 counts/min./ml. blood.

Chemical analyses. The area of paper containing each compound was ashed and oxidized with M-K_2SO_4 and conc. H_2SO_4 as described by Caldwell (1953), and orthophosphate was determined using a Kromatrol photoelectric colorimeter (Will Corp., Rochester, N.Y.) by the method of Berenblum & Chain (1938). For the haemolysates this method with 10 ml. of *iso*butanol was used. For the stroma

Table 1. *Reproducibility of findings on duplicate samples of blood*

R.S.A. = counts/min./μg. P.

	Sample *A*		Sample *B*	
Expt. 1	μg. P/ml. packed cells	R.S.A. at 2 hr.	μg. P/ml. packed cells	R.S.A. at 2 hr.
2:3-DPG	150	229	129	220
Inorganic phosphate	55·3	150	64·4	137
ATP	30·1	172	37·5	184
ADP	16·5	94	20·5	101
Fructose 1:6-diphosphate	15·0	67	12·0	75
	Sample *A*		Sample *B*	
Expt. 2	μg. P/ml. packed cells	R.S.A. at 4 hr.	μg. P/ml. packed cells	R.S.A. at 4 hr.
2:3-DPG	172	430	155	395
Inorganic phosphate	44·3	400	45·0	370
ATP	43·1	293	38·0	320
ADP	16·5	230	17·0	215
Fructose 1:6-diphosphate	11·1	100	9·3	115

the micromethod was used and separations were carried out in a small test tube; the final volume for colorimetry was 1·0 ml. As sodium arsenate was added during some of the experiments, separate tests were made to establish that this substance would not interfere with the colour produced in these analyses.

Reproducibility of methods. As interpretations of our results depend ultimately upon the reproducibility of the methods, tests were done on duplicate samples of the same blood incubated separately after the addition of the radioactive orthophosphate. It will be seen from Table 1 that the results based on chemical as well as on a combination of chemical and radioactivity determinations are reproducible within limits of 10–15%.

RESULTS

Phosphate compounds occurring in mammalian red blood cells. Many analyses of these compounds have been made by other workers, but with few exceptions the methods used are open to criticism, since they depend on salt precipitation and differential hydrolysis for the separation and characterization of each compound. The fractionation of cell extracts by means of the different solubility of the salts of phosphate compounds involves a number of errors principally due to coprecipitation and incomplete precipitation. Differential hydrolysis as a method for characterization of phosphate esters is particularly treacherous in red cell studies, since it has been shown that such criteria as the 7-min. hydrolysable phosphorus content are most inadequate (Mányai & Székely, 1954). At best such methods measure both ATP and ADP, and a small amount of hydrolysis of 2:3-DPG would introduce serious errors.

Thus such methods might give some guide as to the distribution of phosphate within the cell, but it is unlikely that they would achieve the unequivocal separation obtained by chromatography. Recently such separations have been made by Fleckenstein & Gerlach (1953), who used paper chromatography with solvents different from ours, and by Bartlett, Savage, Hughes & Marlow (1953), who used ion-exchange methods.

Precise separations can be achieved by both means and the results obtained differ to a considerable extent from those of earlier workers. The values obtained by us agree well with those obtained by the two authors mentioned above and are shown in Table 2. In this table also are shown the values obtained for other mammalian erythrocytes.

Extracellular–intracellular exchange of ^{32}P

Phosphate exchange in normal human red cells. Experiments were performed with a number of blood samples from different donors, and with samples from one donor at different dates. The rate of exchange of ^{32}P between plasma and cells was estimated by plotting the $\log_{10}$ of the residual ^{32}P activity of the plasma against time. A straight line is obtained if the points taken are within the first 4 hr. of incubation. After this time the intracellular and extracellular ^{32}P concentrations approach equilibrium and the slope flattens. These findings are in agreement with the earlier work of Gourley & Matschiner (1953), and indicate that ^{32}P transfer is a first-order process. A composite constant for the exchange process was calculated using the relation

$$K = 1/t \ln (C_0/C_t),$$

where t = hours, C_0 = extracellular radioactive phosphorus concentration at the beginning of the experiment and C_t = extracellular radioactive phosphorus concentration at time t. The constants thus obtained have been corrected to a standard haematocrit of 40%. Other authors have reported wide variations in the rate of ^{32}P exchange (Pertzoff & Gemmill, 1949), but it will be seen from Fig. 1 that the rates are closely reproducible if the conditions are constant. The rates of exchange in a single individual observed at a number of different times over the course of 6 months were also found to be constant (Fig. 1).

Table 2. *Chemical constitution, diameter/thickness ratios* (D/Th), *and rates of glycolysis of mammalian red cells*

	As μg. P/ml. of packed cells						
	ATP	ADP	2:3-DPG	Inorganic phosphate	K*	D/Th†	Glycolytic‡ rate
Man	25–80	12–25	120–180	35–64	110	3·7	15§
Dog	14–25	10–20	180–205	35–45	5	3·8	16§
Rabbit	20–35	15–25	180–240	50–70	90	3·7	18§
Cat	12–18	5–12	50–60	20–25	5	2·8	12·5‖
Ox	25–30	15–20	—	20–35	30	2·7	3·7§
Goat	20–25	12–18	—	17–26	18	2·0	8·6§

* As μmol. K/ml. packed cells. Data from Kerr & Daoud (1935).
† Data from Wintrobe (1952).
‡ As mg. glucose/hr./100 ml. of whole blood.
§ Data from Izzo (1949).
‖ Data from Hsu (1935).

Temperature dependence of phosphate exchange. The exchange of ^{32}P at three temperatures, 37·5°, 28·0°, 15·5°, all ±0·05°, was measured. At the lowest temperature there is only very slow penetration of ^{32}P. A composite diagram showing the $\log_{10}$ of the labelled phosphorus of the residual plasma plotted against time at the three temperatures is shown in Fig. 2. On the same figure is shown also the $\log_{10}$ of the transfer constant plotted against the reciprocal of its absolute temperature. From these values the activation energy of phosphate transfer has been calculated to be 19 800 cal./mol. This value is somewhat higher than 16 000 cal./mol. found by Gourley & Gemmill (1950), but both these values are 3–4 times that which would be expected for an exchange process resulting only from passive diffusion.

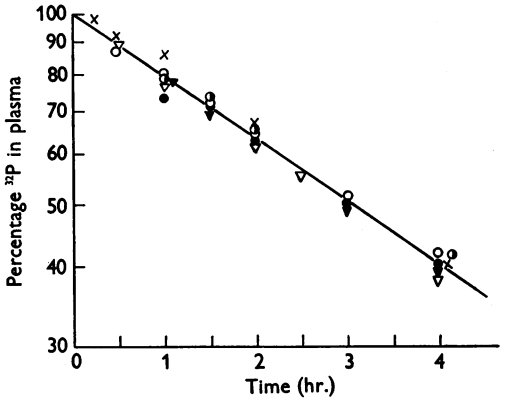

Fig. 1. Time course of ^{32}P exchange in different samples of blood and in one sample of blood on three different occasions. ●, TAJP, Dec. 1953; ▼, TAJP, Mar. 1954; ▽, TAJP, Feb. 1954; ○, LEY, Feb. 1954; ◑, LEY, Feb. 1954; ×, GM, Mar. 1954.

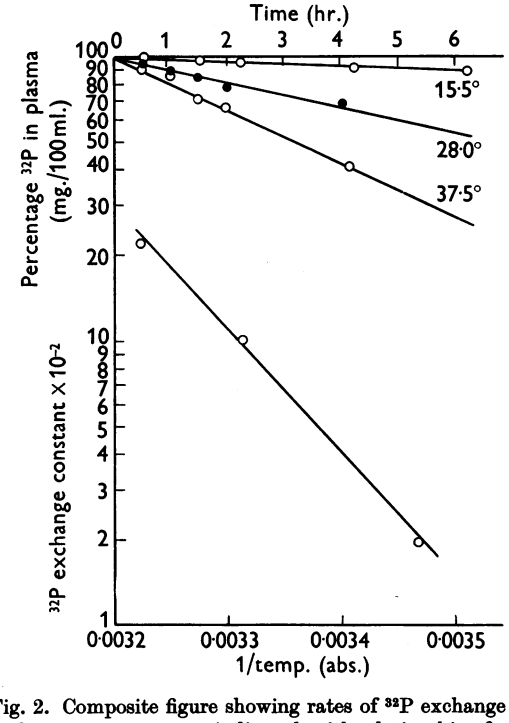

Fig. 2. Composite figure showing rates of ^{32}P exchange at three temperatures as indicated, with relationship of rate constants to the reciprocal of the absolute temperatures (lower figure).

At 3° the rate of exchange is negligible except during the first hour when a small but measurable exchange occurs. It is unlikely that this exchange is a result of inadequate temperature equilibration, since the blood was kept at 3° for 20 min. before the addition of [^{32}P]orthophosphate. Its short duration argues against the assumption that it constitutes a diffusion effect. It is conceivable that the effect (also observed by Gourley, 1952) is related to an adsorption process essentially physical in nature and unrelated to a metabolic transport phenomenon.

Effects of inhibitors. Either sodium fluoride or iodoacetate was added to blood after 2 hr. incubation at 37·5° (Fig. 3). In neither instance is the observed effect immediate, but occurs over the course of about 1 hr., producing in the case of iodoacetate a cessation, and in the case of sodium fluoride only a slowing of phosphate exchange. A similar latent interval was observed by Harris & Maizels (1951) on the effect of these inhibitors on sodium exchange. With both substances the plasma inorganic phosphorus rose by about 10 μg./ml., indicating that there was an escape of phosphorus from the cells.

Effects of different substrates

Adenosine. Dische (1938) observed that red cell haemolysates incubated with adenosine in the presence of sodium fluoride would take up phosphate and form ribose 5-phosphate. Later, Dische (1951) showed that this was converted into fructose

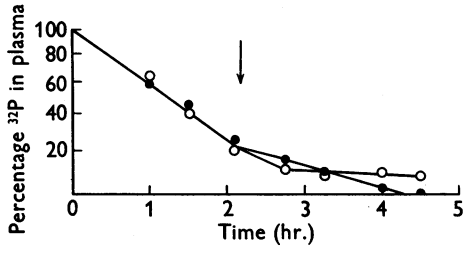

Fig. 3. The effect of the addition of sodium fluoride (1×10^{-3} M) and sodium iodoacetate (1×10^{-3} M) on phosphate exchange. Inhibitors added at time indicated by arrow. ○, blood with sodium iodoacetate; ●, blood with sodium fluoride.

1:6-diphosphate, hexose monophosphate, and triose phosphate.

As it was desirable to determine whether intact red cells could utilize adenosine, blood was taken from a donor early in the morning when his blood sugar was at a fasting level, and was incubated alone for 6 hr. At the end of this time the residual glucose concentration had fallen to 3 mg./100 ml. The blood was then divided into two parts to each of which [^{32}P]orthophosphate was added. To one part an adenosine solution in 0·9 % saline was added to give a final adenosine concentration of 0·005 M, and to the other a volume of isotonic saline equivalent to that of the adenosine solution. Incubation followed as usual and the results are shown in Fig. 4. The early fall in plasma ^{32}P-activity in the control sample was presumably due to the presence of the small amount of residual glucose. The [^{32}P] exchange is well sustained in the presence of adenosine with a rate constant (0·26 per hr.) slightly higher than that for glucose.

In order to show that the effect of adenosine resulted from the utilization of its ribose moiety, i.e. that this effect did not require the presence of a specific purine and that the primary reaction in the utilization of the *N*-riboside involved a phosphorylase, a similar experiment was performed with guanosine, and with adenosine and sodium arsenate. The addition of arsenate may be expected to result in the arsenolysis of adenosine or of glyceraldehyde 3-phosphate thus blocking phosphate exchange.

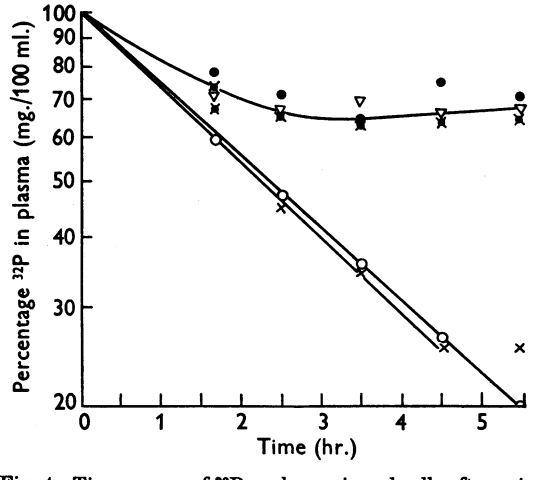

Fig. 4. Time course of ^{32}P-exchange in red cells after prior incubation for 6 hr., following the addition of different substrates. The plasma glucose at the onset of the experiment was 4 mg./100 ml. ×, cells with adenosine (5×10^{-3} M); ○, cells with guanosine (5×10^{-3} M); ✕, cells with adenosine (5×10^{-3} M) + sodium arsenate (5×10^{-3} M); ●, cells with glucose (200 mg./100 ml.) + sodium arsenate (5×10^{-3} M); ▽, cells with no added substrate.

Fig. 4 shows the inhibiting effect of arsenate in the presence of adenosine or additional glucose. Incubation of blood which contained adenosine as well as physiological amounts of glucose did not alter the rate constant, which remained close to that found for glucose alone.

One further experiment was performed with adenosine, the results of which will help to elucidate some of the findings described later in this paper. Whole blood was incubated with [^{32}P]orthophosphate and additional glucose, samples being taken after 6 and 24 hr. At the latter time the plasma glucose was still 150 mg./100 ml. The remaining blood was then divided into two parts, to one of which adenosine was added, and to the other 0·9 % saline. The changes in the extracellular fluid are shown in Fig. 5, where it will be seen that in the first 6 hr. the phosphate exchange was normal. At some time between 6 and 24 hr. ^{32}P escapes from the cells and this continues despite the presence of glucose in amounts adequate for glycolysis. The addition of adenosine however leads to a resumption of ^{32}P-uptake until a new equilibrium is attained at a lower level of ^{32}P-concentration.

Glutathione. The human red cell contains significant amounts of this substance, about 700 mg./l. of cells (Maizels, 1939). Addition of glutathione to red cells leads to an increase in the oxygen consumption of mature mammalian red cells and affects cation

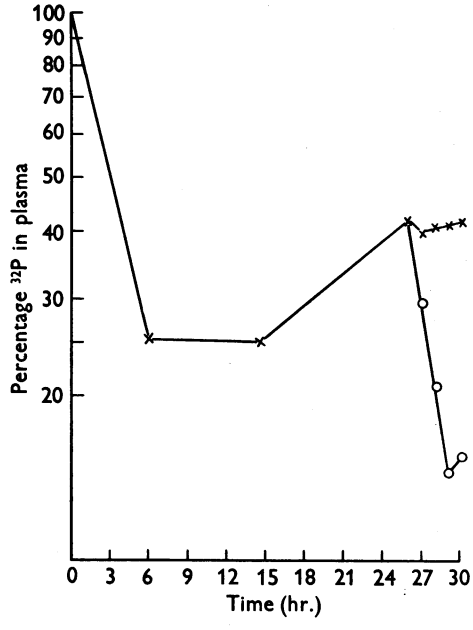

Fig. 5. Time course of ^{32}P-exchange over 30 hr. showing the effect of the addition of adenosine (5×10^{-3} M) at 27 hr. The plasma glucose concentration immediately before the addition of adenosine was 200 mg./100 ml. ○, blood with adenosine; ×, blood with no additional substrate.

exchange (Bruns, 1950). The addition of glutathione in final concentrations of 0·06 M together with 0·01 M-$MgSO_4$ was tried in our system, but no alteration in ^{32}P-exchange was observed.

Observations on other mammalian cells. Blood from five other mammals was obtained in order to assess any correlation which might exist between metabolism, chemical composition and shape (Table 2). Thus the ox and goat cell contain practically no 2:3-DPG, low concentrations of potassium, and are smaller in diameter and thicker than the human cell (Kerr, 1937; Fleckenstein & Gerlach, 1953; Wintrobe, 1952). The cells of both these species exhibit a low rate of phosphate exchange, as shown in Table 3, together with rate constants for dog, rabbit and cat red cells. Dog cells approximate closely to human cells in shape but have a greater content of 2:3-DPG and a very much lower content of potassium. Rabbit cells also have more 2:3-DPG and a similar shape, but their potassium content lies between that of dog and human cells. Dog, rabbit and human cells have approximately the same rate constants. The cat cells were obtained from an animal under barbiturate anaesthesia, and this may have played some part in the low rate of exchange that these cells showed (Pertzoff & Gemmill, 1949).

Intracellular phosphate partition

This aspect of the cell's phosphate metabolism has been studied by estimating the relative specific activity (R.S.A.) and total intracellular concentration of the various phosphate intermediates that have been separated. Provided that the cells maintain a steady state, an interpretation can be made of the rates at which glycolytic reactions are proceeding within the cell as well as in the stroma.

An analysis of the R.S.A. of a group of intermediates with respect to time is shown in Fig. 6. Up to 6 hr. the most active fraction is 2:3-DPG which bears a precursor relationship to ATP. With the exception of the first hour these compounds exhibit a considerably greater activity than orthophosphate. Gourley (1952) found that the isotope concentration of ATP and 2:3-DPG was higher than that of orthophosphate, but that contrary to our findings, ATP had a higher R.S.A. than 2:3-DPG. It will be pointed out later in this paper, as has been done elsewhere (Prankerd & Altman, 1954), that the relationships found here are what might be expected on the basis of our knowledge concerning the entry of inorganic phosphate into glycolysis at the triose phosphate dehydrogenase level. At equilibrium the R.S.A. of ATP, 2:3-DPG and inorganic phosphate are approximately equal, but those of ADP and fructose 1:6-diphosphate are appreciably lower.

It should be pointed out here that the R.S.A. of all compounds containing more than one phosphorus atom per molecule constitute merely the average R.S.A. of all phosphorus atoms present. No attempt has been made to differentiate between the R.S.A. of phosphorus atoms having different rates of turnover. Thus our data for the R.S.A. of ATP cannot be compared directly with those reported by Gourley (1952). If it is assumed that the adenylic acid phosphorus atom has an activity of approximately one-fourth or less of that of the two terminal phosphorus atoms during the first few hours of incubation, then an appropriate dilution factor would have to be applied to our R.S.A. values, raising them to a somewhat higher level. The R.S.A. of 2:3-DPG also constitutes an average of two phosphorus atoms differing widely in their R.S.A. Independent estimates have revealed that the ^{32}P-activity of 3-phosphoglyceric acid is extremely low, indicating that the radioactivity contribution of the phosphate group esterifying the hydroxyl group attached to C-3 of 3-phosphoglyceric acid is small. This would mean that R.S.A. of the other phosphate group is approximately 40 % higher than indicated. In view of these considerations a precursor relationship undoubtedly exists between the labile terminal phosphorus atoms of ATP and the 2-phosphate of 2:3-DPG. If similar considerations

Table 3. *Rate constants (per hour) for ^{32}P-exchange in various mammalian red cells*

Human cells incubated with adenosine and glucose, the others with glucose.

Man (+adenosine)	0·26	Cat	0·11
Man (+glucose)	0·24±0·01	Ox	0·07
Dog	0·23±0·01	Goat	0·07
Rabbit	0·21±0·01		

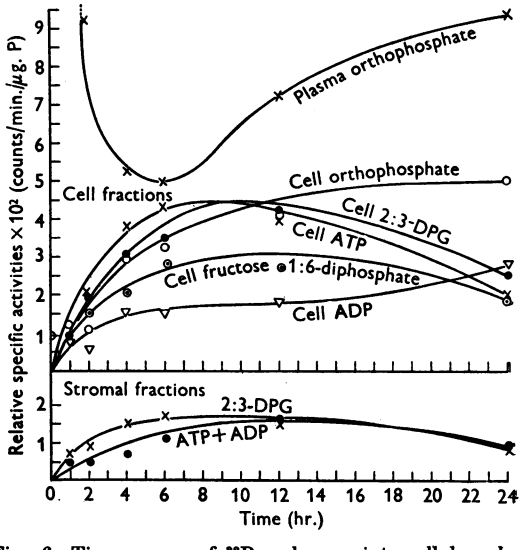

Fig. 6. Time course of ^{32}P-exchange, intracellular phosphate partition, and stromal phosphate fractions during 24 hr. after the addition of ^{32}P.

are applied to the data of Gourley (1952), such a precursor relationship is also present, although this was overlooked because of the failure to take into account the fact that the R.S.A. of 2:3-DPG consitutes the average isotope concentration of two phosphorus atoms of different activity.

When cells are incubated for periods longer than 6 hr. there is a fall in the R.S.A. of ATP and 2:3-DPG with a rise in that of orthophosphate, indicating that the system is no longer maintaining its original equilibrium. Some haemolysis occurs and the R.S.A. of plasma orthophosphate rises. In addition to the fall in ^{32}P-activity of the intracellular esters, there is also a fall in the total quantities of these esters, particularly 2:3-DPG, and to a less extent ATP; the amount and R.S.A. of the intracellular orthophosphate rises (Table 6). It is difficult to see how this fall in the R.S.A. values is brought about and possible mechanisms will be discussed later.

In Fig. 6 the R.S.A. of three compounds derived from the stroma are also shown. The amounts of these compounds are small and for this reason it has been impossible to separate ATP from ADP. The R.S.A. in the stromal fraction are much smaller than their intracellular counterparts.

Temperature dependence. The changes observed are depicted in Table 4. The turnover of compounds labelled with ^{32}P is greatly reduced at 15°, paralleling the findings for the exchange process. It appears that at low temperatures the turnover of ^{32}P in the 2:3-DPG fraction is reduced to such an extent that its activity no longer bears the precursor relationship that was observed at 37·5°.

Effects of inhibitors. It will be seen (Table 5) that sodium fluoride leads to a fall in the total amount of ATP with a more marked fall in its R.S.A., whereas 2:3-DPG tends to increase in amount with a slow but parallel rise in its R.S.A. This presumably occurred because phosphate exchange did not cease but was merely slowed; similar findings were observed by Rapoport & Guest (1939). Iodoacetate leads to a decrease in both amount and specific activity of 2:3-DPG and ATP. In both instances the intracellular orthophosphate rises in amount and activity coincident with the fall in the ester fractions. Considering the well known locus of action of these inhibitors, the changes are what might be expected.

Effect of different substrates

Adenosine. The intracellular and stromal fractions obtained in the experiments described earlier are summarized in Tables 6 and 7. The data in Table 6 demonstrate that adenosine, when added 24 hr. after incubation, brings about a regeneration of the 2:3-DPG and ATP contents of the cells and an increased incorporation of ^{32}P into these compounds. In spite of the fact that the plasma glucose concentration was still 200 mg./100 ml. after 24 hr. of incubation, analyses of those incubated portions

Table 4. *Effect of temperature on intracellular* ^{32}P*-turnover*

R.S.A. = counts/min./μg. P.

Temp. (°)		R.S.A. at varying times after addition of ^{32}P			
		0·5 hr.	2 hr.	4 hr.	6 hr.
37·5	2:3-DPG	85	222	486	375
	Inorganic phosphate	100	195	362	390
	ATP	45	168	400	370
	ADP	20	84	150	204
28·0	2:3-DPG	48	53	101	—
	Inorganic phosphate	119	179	168	—
	ATP	34	116	132	—
	ADP	25	56	68	—
15·0	2:3-DPG	3·5	5·1	—	13
	Inorganic phosphate	18	29	—	65
	ATP	14·5	16	—	29
	ADP	—	—	—	—

Table 5. *Effect of addition of sodium fluoride or iodoacetate after 2 hr. previous incubation with* ^{32}P

R.S.A. = counts/min./μg. P. Final concentrations: sodium fluoride = 0·01 M, iodoacetate = 0·03 M.

	Control		Sodium fluoride		Iodoacetate	
	μg. P/ml. packed cells	R.S.A.	μg. P/ml. packed cells	R.S.A.	μg. P/ml. packed cells	R.S.A.
2:3-DPG	160	220	179	380	123	196
Inorganic phosphate	55	210	84	290	85	380
ATP	29	120	15	40	10	20
ADP	15	80	29	85	21	75

Table 6. *The effect of the addition of adenosine to blood incubated for 25 hr. with [^{32}P]orthophosphate and an excess of glucose*

A = 25 hr. incubation before the addition of adenosine; *B* = following the addition of adenosine (0·005 M) after 25 hr. incubation; *C* = without the addition of any extra substrate at 25 hr. Plasma glucose after 25 hr. incubation = 200 mg./100 ml. R.S.A. = counts/min./μg. P.

		Hours of incubation with ^{32}P					
		6 hr.		12 hr.		25 hr.	
Expt.		μg. P/ml. packed cells	R.S.A.	μg. P/ml. packed cells	R.S.A.	μg. P/ml. packed cells	R.S.A.
A	2:3-DPG	148	330	84	356	39	240
	Inorganic phosphate	35	240	32	685	63	400
	ATP	58	210	35	230	25	200
	ADP	21	130	27	150	12	146
		26 hr.		30 hr.			
B	2:3-DPG	73	455	79·5	432		
	Inorganic phosphate	45	340	51	276		
	ATP	32	195	58·5	293		
	ADP	12	63	25·5	153		
C	2:3-DPG	30	232	45	184		
	Inorganic phosphate	66	326	61·5	440		
	ATP	30	310	27	124		
	ADP	16·5	180	23	268		

Table 7. *The effect of the addition of ribosides with and without arsenate, and glucose with and without arsenate, on blood which had been incubated for 6 hr. before addition of ^{32}P*

^{32}P and substrates all added at 0 hr. *A* = Adenosine (0·005 M) alone; *B* = adenosine (0·005 M) + Na_2HAsO_4 (0·004 M); *C* = glucose (150 mg./100 ml.) + Na_2HAsO_4 (0·004 M); *D* = guanosine (0·005 M) alone; *E* = no substrate or inhibitor added (molar concentrations of the substrates refer to plasma rather than whole blood). Plasma glucose at 0 hr. (i.e. after 6 hr. of incubation) before addition of substance = 4 mg./100 ml. R.S.A. (counts/min./μg. P) in brackets denotes stromal activities.

		Hours of incubation with ^{32}P and substrates					
		2 hr.		4 hr.		6 hr.	
Expt.		μg. P/ml. packed cells	R.S.A.	μg. P/ml. packed cells	R.S.A.	μg. P/ml. packed cells	R.S.A.
A	2:3-DPG	116	195 (52)	127	426	135	370 (95)
	Inorganic phosphate	45	185	48	416	45	400
	ATP	48	170 (0)	46·5	215	57	340 (54)
	ADP	31	135	25	95	36	66
B	2:3-DPG	78	95 (15)	55·5	64	27	30 (0)
	Inorganic phosphate	66	256	81	314	90	310
	ATP	16·5	24 (0)	25	110	27	40 (0)
	ADP	9·0	50	21	26	20	30
C	2:3-DPG	48	190 (0)	46·5	180	26	61 (0)
	Inorganic phosphate	48	360	79·5	364	117	344
	ATP	18	29 (0)	20	31	30	73
	ADP	16·5	19	13	19	21	22
D	2:3-DPG	117	120 (40)	156	193	150	340 (106)
	Inorganic phosphate	46	113	84	226	66	390
	ATP	45	109 (10)	27	190	36	224 (74)
	ADP	21	91	22	100	30	86
E	2:3-DPG	92	55 (20)	—	—	33	42 (25)
	Inorganic phosphate	54	265	—	—	61·5	225
	ATP	40	123 (5)	—	—	24	32 (10)
	ADP	21	61	—	—	16·5	40

of blood to which no adenosine was added after 24 hr. showed a progressive fall in 2:3-DPG with a decrease in the R.S.A. of this ester and of ATP. Such a finding suggests that although on incubation *in vitro* the red cell loses its ability to metabolize glucose after some 24 hr., the utilization of adenosine continues at a rate comparable to that prevailing during the first 4 hr. of incubation.

In Table 7 are shown the intracellular counterparts of the experiments in which the ribosides were added after all of the glucose had been metabolized during the preceding 6 hr. incubation period. In the absence of added ribosides, the concentration of 2:3-DPG decreased rapidly during the second 6 hr. coincident with a lesser decrease in ATP. At the same time the incorporation of ^{32}P into these esters was minimal. The effect of adenosine or guanosine in promoting the uptake of ^{32}P into both esters is dramatic and is accompanied by a net increase in 2:3-DPG. The addition of glucose in the presence of arsenate produces a different pattern of intracellular partition to that of adenosine in the presence of arsenate. In the former case the flow of ^{32}P into the 2:3-DPG fraction proceeds at a significantly greater rate and the R.S.A. of this fraction and also of ATP is higher than in the latter case. This difference may be attributed to the greater extent to which arsenolysis proceeds in the case of adenosine as compared with glyceraldehyde 3-phosphate. Similar changes were found in the stromal fractions.

Other mammalian cells. In the dog and rabbit the intracellular relationships are unlike those found in man. In the red cells of these animals the precursor relationship between 2:3-DPG and ATP is present, but the activity of orthophosphate exceeds that of either of these two esters. In ox and goat cells where no 2:3-DPG is present orthophosphate activity exceeds that of ATP. These changes are in part a result of greater orthophosphate activity and in part a result of diminished ester activity.

DISCUSSION

The uptake of orthophosphate by the cell and its transport across the cell membrane is thought to depend primarily upon glycolysis, particularly at the level of the oxidative phosphorylation of glyceraldehyde 3-phosphate. Such a concept points to an intimate relationship between phosphate uptake and glycolysis. It should be stated here that this does not imply the existence of a direct relationship between phosphate uptake and glucose transport, since the red cell membrane is peculiarly permeable to free glucose. The inhibition of phosphate uptake by inhibitors of glycolysis such as sodium fluoride and iodoacetate, as well as the finding that exhaustion of the cell's glucose supply leads to a cessation of phosphate exchange lend further support to the view that phosphate uptake and glycolysis are interdependent processes.

In order to elucidate the relationship between these two processes in the mammalian red cell, the following reactions deserve consideration:

If glycolysis is directly concerned with the uptake of phosphate by the red cell, a reaction into which

$$\text{glyceraldehyde 3-phosphate} + \text{DPN}^+ + \text{enzyme} \rightleftharpoons \text{3-phosphoglyceryl–enzyme} + \text{DPNH} + \text{H}^+. \quad (1)$$

$$\text{3-phosphoglyceryl–enzyme} + [^{32}\text{P}]\text{orthophosphate} \rightleftharpoons [1\text{-}^{32}\text{P}]\text{1:3-diphosphoglyceric acid} + \text{enzyme} \quad (2)$$

$$[1\text{-}^{32}\text{P}]\text{1:3-diphosphoglyceric acid} + \text{ADP} \xrightleftharpoons{\text{phosphoglycerokinase}} \text{3-phosphoglyceric acid} + [\textit{terminal}\text{-}^{32}\text{P}]\text{ATP}. \quad (3)$$

$$[1\text{-}^{32}\text{P}]\text{1:3-diphosphoglyceric acid} \xrightleftharpoons{\text{phosphoglyceromutase}} [2\text{-}^{32}\text{P}]\text{2:3-diphosphoglyceric acid}. \quad (4)$$

Rapoport & Luebering (1950)

orthophosphate can enter directly should be involved. Such a reaction is (2) which must be preceded by the oxidative step as represented by reaction (1) (Segal & Boyer, 1953; Oesper, 1954; Harting & Velick, 1954). From reaction (4) it is evident that 1:3-DPG is in equilibrium with 2:3-DPG, which is present in human red cells in amounts accounting for about half the total phosphorus content of the cell. Reaction (4) shows that the phosphate group in position 1 of 1:3-DPG, and in position 2 of 2:3-DPG are equivalent and that in accordance with reaction (3), the labelled phosphate group of 1:3-DPG is a precursor of the labile terminal phosphate group of ATP. From this sequence of events it may be predicted that 2:3-DPG should bear the same relationship as 1:3-DPG to ATP. Such a relationship has been verified by our experiments, and may, as we have seen, be extrapolated also from the results obtained by Gourley (1952), although this author had reached the opposite conclusion.

As to the site at which organic phosphate is linked to red cell glycolysis, our data, as well as Gourley's, suggest that phosphate is not participating in reaction (2) at the intracellular level, because the R.S.A. of intracellular orthophosphate is lower than that of both 2:3-DPG and ATP (except during the first hour). These findings indicate that

the intracellular orthophosphate may be derived from ATP as has been suggested by Rothstein (1954) for the yeast cell. These findings were not observed in cells of other species such as the dog and rabbit, suggesting that in these animals orthophosphate must be derived from other sources as well as ATP. It is possible that the diminished ATP activity in these cells is a reflexion of their low potassium contents, but further analyses are required to clarify these points.

On the premise that orthophosphate does not enter reaction (2) at the intracellular level, one must search for other sites at which it might participate in the reaction. Rothstein's suggestion that in the yeast cell phosphorylation is carried out on the cell surface seems most pertinent, and if this view can be extended to the red cell, keeping in mind that this cell differs markedly from the yeast cell with respect to glucose transport, then our findings related to the stromal fractions assume significance.

We have established that certain intermediates of glycolysis are present in the stromal fraction and that these incorporate ^{32}P derived from plasma orthophosphate. Although this might be thought to result from contamination with intracellular material, there are two points that make it unlikely. First, the stroma preparation was washed so thoroughly that any residual compounds must have been tightly bound; the completeness of the washing was confirmed by the absence of activity in the supernatant washings. Secondly, the R.S.A. values of the stromal intermediates do not parallel those found intracellularly, as they should do were they contaminants. It seems probable that these stromal intermediates indicate glycolysis having a specific function such as either the directed transport of phosphate, electrolytes, or glucose, or the supply of energy for the maintenance of the unusual biconcave shape of the human red cell. It was the last possibility that prompted us to investigate the stroma of the ox and goat cells, which are spherical. Virtually no activity could be detected in the stromal fractions from these species. More evidence is required before one can accept the hypothesis that glycolysis in the stroma is related to the cell's shape, but it may not be too remote to visualize the red cell envelope as possessing certain contractile properties, dependent upon a continuous regeneration of ATP.

Additional support for our concept of the active transport of phosphate into the red cell is gained from the magnitude of the energy of activation of this process, namely 19800 cal./mol. In general the activation energy of a passive diffusion process would involve only about 4000–5000 cal./mol. Our finding that phosphate exchange virtually ceases at 15° emphasizes the sensitivity of this process to a fall in temperature. This observation, together with those of Gourley (1952) and Halpern (1936), conflict with that of Hahn & Hevesy (1941), who found appreciable phosphate exchange at 0°. Probably a low order of passive phosphate diffusion occurs simultaneously with active transport, for Gourley & Matschiner (1953) found two exponential processes involved in phosphate exchange, signifying a more rapid process in the first half hour than at later times, and we have found a small degree of phosphate penetration into the cell during the first hour at 3° which did not increase subsequently.

Although probably of minor physiological importance there exists another mode by which phosphate may enter the red cell. The work of Dische (1938, 1951) with adenosine and guanosine made it clear that orthophosphate may be esterified by phosphorolysis of purine ribosides giving rise to the formation of ribose phosphates and thus to triose and hexose phosphates. The participation of adenosine and guanosine in mediating phosphate transport is well shown in the experiments in which ^{32}P-equilibrium is restored by the addition of the ribosides after 24 hr. incubation without them. After this period adenosine appears to be more effective in restoring the equilibrium than glucose, and it occurs at a time when the intracellular ATP has fallen to a level which is probably insufficient for the phosphorylation of glucose. Phosphorolysis of purine ribosides does not require ATP. These circumstances resemble those prevailing in Dische's experiments where ATP regeneration was blocked by the addition of sodium fluoride, and glycolysis inhibited without interfering with the phosphorolysis of the ribosides. As far as our experiments with these substances are concerned their phosphorolytic cleavage is thought to have two important consequences, (*a*) replenishing the depleted stores of 2:3-DPG by way of the formation of glyceraldehyde 3-phosphate formed from the enzymic breakdown of ribose 5-phosphate, and (*b*) forming fructose 1:6-diphosphate and hexose monophosphate in the manner described by Dische (1938, 1951). It thus appears that adenosine is more effective potentially than glucose as a substrate for glyceraldehyde 3-phosphate formation in the red cell, because the investment of energy, is smaller in the case of adenosine which does not require ATP for the phosphorylation of its pentose moiety. These concepts are pertinent to the interesting work of Gabrio, Stevens & Finch (1954) and Gabrio & Finch (1954) who showed an increased viability of red cells stored at 4° in acid–citrate–dextrose solution when purine ribosides were added. The role of these substances in phosphate exchange suggested by Dische's and our experiments could well explain Gabrio's observations.

A word of explanation is required for the falling R.S.A. of the intracellular 2:3-DPG and ATP between

6 and 24 hours. Such a fall would require the re-exchange of ^{31}P for ^{32}P in these esters. Since the compounds separated had all reached equilibrium by this time, such ^{31}P would have to come from an unequilibrated source. One such source might be the phospholipid fraction.

Explanation is also required for the rising extra- and intracellular orthophosphate activity after 24 hr. The former would appear to be due largely to a loss of orthophosphate from the red cells, since these cells resume phosphate exchange once a metabolizable substrate is added. Slight haemolysis and leucolysis also contribute to this rise in the R.S.A. of inorganic phosphate; any organic phosphates liberated in this way being broken down by the phosphatases of the plasma (Meister, 1947). No detailed examination of the number of leucocytes in the blood samples has been made, but it is unlikely that these cells contributed significantly to phosphate exchange in our system since the number of leucocytes present must have varied within the physiological range and yet the rates of phosphate exchange remained constant. This might appear surprising since leucocytes utilize glucose at a rate several times that of red cells (Hsu, 1935). However, anaerobic glycolysis by leucocytes may have been greatly inhibited, since the oxygen saturation of the blood during incubation may be expected to invoke the Pasteur effect. The rising intracellular orthophosphate in these experiments is most likely the result of the breakdown of adenosine diphosphate, and the action of phosphatases (Tsuboi & Hudson, 1953; Lennerstrand & Lennerstrand, 1940).

It is a provocative fact that some mammalian red cells contain great amounts of 2:3-DPG, whereas others contain almost none at all. Those cells which contain negligible amounts of 2:3-DPG also have a small diameter/thickness ratio (Table 2), and also have a low ATP/ADP ratio. One is tempted to associate these phenomena with the shape of the cell, that is, to postulate that spherocytosis is related to low energy stores within the cell. This possibility is supported by the low phosphate turnover in the esters of the stroma from those species with spheroidal red cells. 2:3-DPG is contained in most other cells of the body in minute quantities only, merely sufficient to fulfill the requirements for its coenzyme function in the conversion of 3-phosphoglycerate into 2-phosphoglycerate, a reaction catalysed by phosphoglyceromutase (Sutherland, Posternak & Cori, 1949). In this reaction 2:3-DPG is continually regenerated and is therefore only required in catalytic amounts. In the red cell it may be reasonable to regard 2:3-DPG as a potential energy source upon which the cell may draw for the regeneration of ATP by way of reaction (3).

If human red cells are incubated in their own plasma, the plasma glucose is used up in about 6 hr. Haemolysis does not begin for about 26 hr. If the cell is to continue to carry on its metabolic activities in the absence of glucose, it can only do so at the expense of its intracellular energy stores. This is borne out by the fact that 2:3-DPG continues to disappear at a rate faster than ATP during the 6–24 hr. incubation period. That the red cell may meet similar situations involving the exhaustion of extracellular substrate *in vivo* is possible if one considers the slow circulation through such a vascular organ as the human spleen where haemoconcentration may occur leading to a haematocrit as great as 80 % (Gibson *et al.* 1946). In addition to the large number of red cells per mole of available glucose, the large mass of generative lymphoid tissue will be competing for the same substrate. In such circumstances the red cell may be faced with 'relative starvation', the final effects of which will depend upon the duration of this deprivation. In a number of species there is a correlation between the size of their red cells, the cell's content of 2:3-DPG, and the cation gradients existing between the cells and plasma (Table 2). Small cells are less likely, for mechanical reasons, to stagnate in vascular organs (Whipple, 1941; Björkman, 1947; Young, Platzer, Ervin & Izzo, 1951) and the absence of high ionic gradients would lower the energy demands of such cells and, therefore, the necessary size of the storage depots.

Such considerations give a rational explanation of the biological variations of mammalian cells and may have some fundamental bearing on the understanding of the correct approach to red cell storage, and to the viability of the red cell in diseased states, a subject which will be discussed in a later paper.

SUMMARY

1. The exchange of ^{32}P between various mammalian red cells and their plasma has been studied, and the effects of temperature, inhibitors of glycolysis, and various substrates observed.

2. The dependence of ^{32}P-exchange upon glycolysis has been demonstrated and also the participation of purine ribosides in this process.

3. By means of paper chromatography of red cell extracts, the intracellular partition of ^{32}P has been studied, and a precursor relationship found between 2:3-diphosphoglyceric acid and adenosine triphosphate. The rationale of this has been discussed.

4. Glycolytic intermediates have been found in the stromal fractions of red cells and their possible significance considered.

5. The effects of temperature, inhibitors of glycolysis, and certain substrates on intracellular phosphate partition have also been investigated.

Adenosine has been found to form a substrate for regeneration of the essential energy stores of the red cell, even after prolonged incubation when it appears that glucose can no longer be utilized.

6. An attempt has been made to explain some of the biological variations in the red cells of certain mammalian species on a rational basis, and to intimate the importance of metabolism in the storage of red cells and their diseased states.

We wish to thank Mrs M. Murphy for helpful technical assistance. One of us (T.A.J.P.) wishes to thank Dr L. E. Young for his kindness in providing facilities for this research, and Professor M. Maizels, who initiated his interest in these problems. The authors are indebted to Professor Maizels, Drs A. Rothstein, K. Salomon, L. E. Young and E. J. Harris for helpful criticism and discussion in the preparation of this manuscript, and to Dr A. Dutton for advice on the statistical handling of the data. This paper is based in part on work performed under contract with the U.S. Atomic Energy Commission, at the University of Rochester, Atomic Energy Project.

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The Amino Acid Sequence in Bacitracin *A*

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(*Received* 30 *June* 1954)

Bacitracin *A* is the major constituent of a family of antibacterial polypeptides known as bacitracin (Craig, Gregory & Barry, 1949; Newton & Abraham, 1950). It has a molecular weight of about 1470 and has been reported to yield the following amino acids on hydrolysis: D-ornithine (Orn) (1), L-lysine (1), L-histidine (1), DL-aspartic acid (2), D-glutamic acid (1), L-cysteine (1), D-phenylalanine (1), L-leucine (1), L-isoleucine (2) (Craig, Weisiger, Hausmann & Harfenist, 1952; Craig, Hausmann & Weisiger, 1952, 1953; Newton & Abraham, 1953*a*,*b*). In addition, it yields a significant amount of a substance which runs slightly faster than isoleucine on columns of Dowex 50 (Craig, Hausmann & Weisiger, 1952). This substance has been detected independently by Porath (private communication)