results suggest that the speed of reaction (2) is greater than that of reaction (1).

The problem of the differentiation between intra- and extra-cellular inorganic P in tissue extracts therefore remains unsolved and, as a consequence, it is not possible to determine the absolute turnover rate of any organophosphorus compound which has inorganic P as its immediate source of phosphorus.

SUMMARY

1. Two methods of determining the specific radioactivity of intracellular inorganic phosphate have been investigated.

2. Experimental evidence and arguments have been brought forward to suggest that either (a) the volume of the extracellular space in skeletal muscle is much less than 12 %, or (b) the speed with which inorganic phosphate is equilibrated between the two phases, plasma and extracellular fluid, is slower than has been supposed.

3. It is concluded that with the present state of knowledge it is not possible to determine the specific radioactivity of intracellular inorganic phosphate and hence the *in vivo* turnover rate of organophosphates for which inorganic phosphate is the source of phosphorus.

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An Investigation into the Turnover Rates of Organophosphates

2. THE RATE OF INCORPORATION OF ^{32}P INTO ADENOSINE TRIPHOSPHATE AND PHOSPHOCREATINE IN SKELETAL MUSCLE

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The previous paper in this series (Ennor & Rosenberg, 1954) dealt with the determination of the specific radioactivity (counts/min./ $\mu\text{g.}$) of intracellular inorganic phosphorus, and it was concluded that, with the techniques at present available, it was not possible to determine this value. It is generally assumed that the intracellular inorganic phosphorus is the precursor of the 'energy-rich' phosphorus of phosphocreatine (PC) and of adenosine triphosphate (ATP), and it follows that in the absence of a value for the specific radioactivity of this fraction it is not possible to determine the turnover rate of these compounds.

In the work reported here we have therefore determined the turnover rates of each of the phosphate groups of ATP and the phosphate of PC relative to one another. Whilst such a procedure does not permit the assignment of a definite value to the amount of, for example, ATP which is formed in unit time, it does give a qualitative idea of the speed with which each of the phosphorus atoms is replaced.

This paper is also concerned with the reliability of the results achieved by the perfusion technique of Kalckar, Dehlinger & Mehler (1944) which is essentially similar to the slice-washing methods

adopted by Furchgott & Shorr (1943). Neither Furchgott & Shorr (1943) nor Kalckar *et al.* (1944) have produced any reliable evidence to support their contention that whilst washing of tissue slices or perfusion of a limb was being carried out no change occurred in the specific radioactivity of the organophosphates. Some preliminary evidence has been published (Ennor & Rosenberg, 1952*a*) suggesting that the results of Kalckar *et al.* (1944) are erroneous, at least as far as the terminal phosphate group of ATP is concerned.

The subject-matter of this paper has been presented in preliminary form (Ennor & Rosenberg, 1952*d*).

EXPERIMENTAL

Enzymic preparations. Myosin was prepared as described by Bailey (1942), and myokinase according to the method of Colowick & Kalckar (1943) using the simplified procedure described by Rowles & Stocken (1950). Myosin was stored at 0° in 0.5M-KCl and the pH adjusted to 7.0 by the addition of solid NaHCO₃ after the final precipitate had been dissolved. The concentration of protein in the solution was such that 1 ml. contained about 0.8 mg. of protein N. It has been found that such solutions, containing one or two drops of toluene, retain maximum activity for 6–8 weeks. The myokinase was stored at –15° as the freeze-dried material under which conditions maximum activity is retained for over a year.

Tests on the activity of each were carried out by reference to an authentic sample of ATP, and nearly theoretical yields of P were obtained. It should be noted that it is not necessary to obtain complete breakdown of ATP since the specific radioactivities of the P groups were being measured and not the absolute amounts. It is, however, essential to be able to place reliance upon the specificity of the enzymes and to ensure this enzyme specificity tests were carried out at frequent intervals. For this purpose, recoveries were made of known amounts of ATP added to mixtures of organophosphates present in tissue extracts. Neither enzyme was able to act upon organophosphates other than on their true substrates, i.e. ATP and ADP, even though tests were carried out for periods up to 60 min.

Treatment of animals. The animals used throughout these experiments were well fed, healthy, albino hutch-rabbits of 2.1–2.4 kg. body weight. Surgical anaesthesia was induced by the intravenous injection of Nembutal (45 mg./kg.) immediately after which the appropriate dose of ³²P was given also intravenously. The procedure following injection of ³²P varied both as regards time of death and treatment of the tissues and will be specifically referred to later in connexion with the experimental results.

³²P. For details of the material used, see Ennor & Rosenberg (1954). The actual amount of ³²P injected varied (see text) but was such that the requisite amount was contained in 3–5 ml. of 0.85% (w/v) NaCl and contained about 1 mg. of inert carrier P in the form of NaH₂PO₄.

Preparation of tissue extracts. All extracts were prepared from the thigh muscles irrespective of whether the tissue had been initially dropped into the refrigerant, placed on the bench, stored in crushed ice or perfused. At the end of the experimental period the muscles in the last three categories were dropped into liquid O₂. The hard tissue was finely powdered by grinding in a porcelain mortar previously

chilled to the temperature of liquid O₂. A known weight (about 80 ± 0.5 g.) of the powdered material was then transferred to a Waring Blender and 200 ml. of ice-cold 10% (w/v) trichloroacetic acid (TCA) added. The mixture, when sufficiently thawed, was blended for 2 min. during which time the temperature never exceeded –1°. The resultant suspension was rapidly filtered by suction through a chilled Büchner funnel, and sufficient 5N-NaOH added to make the filtrate alkaline to phenolphthalein. The pad of precipitated protein was then returned to the Waring Blender and again blended with about 200 ml. of 5% (w/v) TCA for 15–20 sec. After filtration the two extracts were combined and sufficient 5N-NaOH was added to bring the solution to pH 7.3. The volume of the extract was then adjusted by the addition of water so that the equivalent of 70 g. of fresh muscle was contained in 500 ml. A 20 ml. sample of this extract was reserved for the determination of inorganic P and PC, and the remainder was used for the separation of the nucleotides.

Determination of the specific radioactivity of P in the inorganic and PC fractions. Determination of the inorganic ³²P was carried out on 1.0 ml. samples of the TCA extract, as described by Ennor & Stocken (1950). The radioactivity of the isobutanol extract (³²P) was determined by the method of Ennor & Rosenberg (1952*b*). The P of PC was determined by allowing 1.0 ml. samples to stand in 50 ml. separating funnels for 30 min. at room temperature in the presence of H₂SO₄ and ammonium molybdate, at concentrations given by Ennor & Stocken (1950). The resultant P was then extracted by the usual technique of shaking for 10 sec. and the colour intensity and radioactivity were measured. The difference between this P and the inorganic P is assumed to give phosphocreatine P, but it should be remembered that the P released by acid molybdate does not necessarily come entirely from PC (Weil-Malherbe & Green, 1951; Ennor & Rosenberg, 1952*c*). The possible effect of the hydrolysis of small amounts of other P compounds upon the results will be discussed later.

Determination of the specific radioactivity of the P groups of ATP. The usual technique of determining ATP by means of the P split by a 7–10 min. hydrolysis in N-HCl at 100° of a sample of the TCA extract, or of the insoluble fraction of the Ba salts prepared from such an extract, is of little value because of its obvious lack of specificity. For this reason, the main principles of the technique described by Kalckar (1944) were adopted. Such a method, provided the specificity and purity of the enzyme preparations are known, permits the accuracy, essential in radioactivity studies, to be gained. The actual technique employed was to precipitate the Ba salts from the bulk of the TCA extract, as described by Ennor & Stocken (1948). The suspension was separated by centrifugation, the supernatant fluid discarded, the precipitate suspended in water, and N-HNO₃ added. The solution was adjusted to pH 2.0 and an excess of 15% (w/v) Hg(NO₃)₂ in N-HNO₃ added. This produced an immediate precipitation of the Hg salts of the nucleotides, which were then separated by centrifugation and the supernatant fluid was discarded. The precipitated Hg salts were washed with 0.5% (w/v) Hg(NO₃)₂ in 0.1N-HNO₃ and again centrifuged. The supernatant fluid was discarded and the precipitate suspended in about 50 ml. of 0.1N-HNO₃ and gassed with H₂S until free of Hg²⁺. The suspension was filtered and the H₂S removed by vigorous aeration for 20 min. The solution of nucleotides was adjusted to pH 7.3 with N-NaOH. It is important to emphasize that throughout all these procedures,

which were carried out as rapidly as possible, the temperature of the solutions was maintained at 0° to minimize hydrolysis of the nucleotides.

Samples were then withdrawn from the nucleotide solution for determination of inorganic P. For this purpose, 5.0 ml. were treated as described by Ennor & Rosenberg (1952*b*). For the determination of the terminal phosphate group of ATP (ATP-P γ) the procedure and solutions described by Bailey (1942) were employed with slight modification. A 2.0 ml. sample of the nucleotide solution, 0.2 ml. of 0.1M-CaCl₂, 2.5 ml. of 0.1M glycine buffer (pH 9.0), 0.3 ml. myosin solution and 3.0 ml. water were used. The mixture was then incubated for 15 min. at 37°, at the end of which time 2.0 ml. of 25% (w/v) TCA were added and the solution was cooled to 0°. The protein was removed by filtration and duplicate 4.0 ml. samples withdrawn from the supernatant for determination of the radioactivity and concentration of the P, as described by Ennor & Rosenberg (1952*b*).

The middle P atom of ATP (ATP-P β) was removed by the concerted action of myosin plus myokinase. A 1.0 ml. sample of the nucleotide solution was treated with 0.2 ml. of 0.1M-CaCl₂, 0.3 ml. myosin solution, 0.3 ml. myokinase solution, 2.5 ml. of 0.1M glycine buffer (pH 9.0) and 3.7 ml. water. The resultant mixture was treated similarly to the myosin digest and, as before, duplicate 4.0 ml. samples of the protein-free solution taken for P radioactivity and concentration measurements. From the results of these determinations, the specific radioactivity of ATP-P γ and ATP-P β were calculated. Appropriate blank determinations were carried out and inorganic P was determined simultaneously with the determination of ATP-P γ and -P β .

The specific radioactivity of the P atom attached to the ribose of the ATP molecule (ATP-P α) was estimated after enzymic digestion of the nucleotides. For this purpose the bulk of the nucleotide solution was digested with a mixture of myosin and myokinase at 37° for 2 hr. in 0.1M glycine buffer at pH 9.0. The reaction was stopped by the addition of sufficient 25% (w/v) trichloroacetic acid to bring the concentration of this acid to 5%. The precipitated protein was separated by centrifugation and discarded. The adenylic acid in the supernatant fluid was isolated by the procedure described by Kerr (1941). The adenylic acid so obtained contained no ATP or ADP and an amount of inorganic P < 0.5% of the total P. It is believed that the product was pure, but as no further analyses were carried out there remains the possibility that traces of other P-containing compounds may have been present. This, however, would seem unlikely, having regard to Kerr's (1941) results and to the many steps involved in the isolation. Adenylic acid P was taken to be the difference between total P, as determined after wet-ashing, and inorganic P.

Corrections for decay of ^{32}P . All results have been corrected for the decay of ^{32}P . This has been done by reference to the counts/min. observed in the morning and afternoon of each day over the experimental period using an isobutanol extract of a solution of relatively high radioactivity, generally plasma. In this way the correction factor covered decay corrections and possible variations in the sensitivity of the liquid counter and electronic circuits.

Creatine. Free and total creatine (free creatine + creatine as PC) were measured in TCA extracts using the method described by Ennor & Rosenberg (1952*c*).

General precautions. In all cases, the time of counting has been such as to reduce the statistical error of counting to $\pm 2\%$.

It was noted that successive counts made on comparatively highly radioactive solutions (5000 counts/min.) led to a gradual increase in the background from a normal value of about 12 counts/min. to one of 30 or even higher. This is apparently owing, in part at least, to the action of SnCl₂, traces of which are present in the isobutanol extracts after reduction, on the glass walls of the liquid counter. As a result, the glass becomes etched and ^{32}P is adsorbed and is very difficult to remove, even by acid treatment and prolonged washing. The difficulty has been overcome by allowing conc. HF to remain in the liquid counter for about 5 sec. The counter is then washed free of acid and the resultant normal background remains below 20 counts/min. for some time before another HF treatment is necessary.

RESULTS

Because of the importance of determining the specific radioactivity of the intracellular inorganic P, the perfusion technique adopted by Kalckar *et al.* (1944) has been repeated and critically examined. The precise technique adopted in our experiments was to ligate the iliac vessels of one hind limb at a prescribed time after the injection of ^{32}P . The limb was then removed, wrapped in cellophan, and placed in crushed ice. This procedure occupied about 1 min. and immediately on completion both the abdominal aorta and the inferior vena cava were cannulated. The aortic cannula was connected through a cooling coil to a reservoir of ice-cold, P-free Ringer's solution. The reservoir was raised to 1.5 m. above the level of the animal and perfusion begun. Under these conditions, 1.5 l. of Ringer's solution were perfused through the hind limb in 30–45 min. On completion of the perfusion, the muscles were removed from the limb, and together with those from the control limb (which was packed in ice) dropped into liquid oxygen. TCA extracts of both sets of muscles were then prepared and analysis carried out.

The results (Table 1) suggest that perfusion reduces the specific radioactivity of the inorganic P and that it does not interfere with the specific radioactivity of either the β or the γ P atoms of ATP. This experiment, however, suffers from the same disadvantage as that described by Kalckar *et al.* (1944), i.e. that implicit in its design is the assumption that the control muscles, which were placed in ice for the duration of the perfusion, were adequate controls for the experiment and that no change in the specific radioactivity of any of the organophosphorus compounds can occur. There is, however, no experimental basis for such an assumption, and its validity has already been questioned in a preliminary report (Ennor & Rosenberg, 1952*a*).

The results of another experiment carried out on essentially similar lines as the previous one show some striking differences. In this experiment the thigh muscles from the control hind limb were removed as before but were divided across their

Table 1. *Specific radioactivities and concentrations of P in certain fractions of TCA extracts of perfused muscles and control muscles stored in ice*

(Rabbit, 2.4 kg. body weight, injected intraperitoneally with 0.5 mc ³²P and killed 30 min. later. ATP-P_γ and ATP-P_β refer to the terminal and middle P atoms of ATP, respectively.)

Phosphorus fraction	Perfused		Stored in ice	
	Specific radio-activity of P (counts/min./μg.)	Concentration (μg. P/ml. of extract)	Specific radio-activity of P (counts/min./μg.)	Concentration (μg. P/ml. of extract)
Inorganic P	5.6	71.2	10.8	77.7
ATP-P _γ	5.8	—	6.4	—
ATP-P _β	6.3	—	6.4	—

Table 2. *Specific radioactivities and concentrations of P in various fractions of TCA extracts of non-perfused muscle dropped immediately on excision into liquid oxygen, non-perfused muscle retained at room temperature (20°) and perfused muscle*

(Rabbit, body weight 2.2 kg., injected intravenously with 1 mc ³²P and killed 15 min. later. ATP-P_γ and ATP-P_β refer to the terminal and middle P atoms of ATP, respectively; PC-P refers to phosphocreatine-P.)

Phosphorus fraction	Immediately frozen in liquid O ₂		Stored at room temperature		Perfused	
	Specific radioactivity of P (counts/ min./μg.)	Concentration (μg. P/ml. of extract)	Specific radioactivity of P (counts/ min./μg.)	Concentration (μg. P/ml. of extract)	Specific radioactivity of P (counts/ min./μg.)	Concentration (μg. P/ml. of extract)
Inorganic P	55.5	22.8	30.4	57.0	15.7	20.7
PC-P	13.7	50.2	10.0	36.0	10.7	57.3
ATP-P _γ	16.1	—	11.5	—	10.1	—
ATP-P _β	6.7	—	9.5	—	8.8	—

long axes; one portion was dropped into liquid oxygen and the other placed on the bench at room temperature (20°) for the duration of the perfusion of the other hind limb. The muscles from the latter, together with the previous sample, were dropped into liquid oxygen at the end of the perfusion. It is apparent (Table 2) that there is no real difference between the perfused muscle and the muscle left on the bench in so far as the specific radioactivities of the organophosphate fractions are concerned. It would therefore seem to be of no consequence whether the control muscles are left at room temperature or stored in ice. There is, however, a very real difference between the specific radioactivities of the organophosphate fractions of these two sets of muscles and the muscles which were dropped immediately into liquid oxygen. In these latter the specific radioactivities of PC-P and ATP-P_γ are higher than in the other two samples, whilst that of ATP-P_β is lower. The specific radioactivity of the inorganic P fraction is lowest in the perfused muscle and highest in the sample which was dropped immediately into liquid oxygen. The former result conforms to expectations in view of the fact that some richly labelled inorganic P would be washed out in the perfusate. The intermediate position occupied by the specific radioactivity of the inorganic P in the

sample stored at room temperature is of interest. The fact that the concentration of inorganic P has more than doubled during the period over which the sample was left on the bench suggests that the observed fall in the specific radioactivity of the inorganic P was due to a dilution of the original inorganic P pool by inorganic P arising from the breakdown of relatively weakly labelled organophosphorus compounds. Similar findings, but varying somewhat in quantitative aspects, are shown in an additional experiment (Table 3). The concentration of inorganic P which is found in the perfused muscle is much the same as that in the muscle left at room temperature and much greater than that which was dropped into liquid oxygen. This result, which differs from that in Table 2, is typical of the bulk of the experiments which have been carried out. Two facts of interest are apparent from the results of the foregoing experiments. First, there is a marked difference between the specific radioactivities of ATP-P_β and ATP-P_γ, as illustrated by the analyses on the ATP isolated from the muscle dropped immediately into liquid oxygen; secondly, the effect of perfusion, or storage at room temperature or in ice, is to reduce the difference between the specific radioactivities of these two groups.

Table 3. *Specific radioactivities and P concentrations of various P fractions of TCA extracts of non-perfused muscle dropped immediately after excision into liquid oxygen, non-perfused muscle retained at room temperature (20°) and perfused muscle*

(Rabbit, 2.4 kg. body weight, injected intravenously with 1 mc ^{32}P and killed 15 min. later. ATP-P γ and ATP-P β refer to the terminal and middle P atoms of ATP, respectively; PC-P refers to phosphocreatine-P.)

Phosphorus fraction	Immediately frozen in liquid O ₂		Stored at room temperature		Perfused	
	Specific radioactivity of P (count/min./ $\mu\text{g.}$)	Concentration ($\mu\text{g. P/ml.}$ of extract)	Specific radioactivity of P (counts/min./ $\mu\text{g.}$)	Concentration ($\mu\text{g. P/ml.}$ of extract)	Specific radioactivity of P (counts/min./ $\mu\text{g.}$)	Concentration ($\mu\text{g. P/ml.}$ of extract)
Inorganic P	33.6	32.8	27.7	68.3	14.8	65.2
PC-P	15.5	52.5	9.4	39.0	5.8	58.8
ATP-P γ	14.0	—	9.5	—	5.6	—
ATP-P β	3.3	—	8.2	—	5.3	—

Table 4. *Specific radioactivities of various P fractions of TCA extracts of muscles dropped, immediately after excision, into liquid oxygen, retained in crushed ice for 1 hr. and perfused with 1.5 l. P-free Ringer's solution*

(Expt. 1 refers to results from thigh muscles of the same hind limb of one rabbit, and Expt. 2 to the thigh muscles from two hind limbs of another. The figures in parenthesis are the inorganic P concentrations in $\mu\text{g./ml.}$ of TCA extract.)

Expt. no.	Phosphorus fraction	Immediately frozen in liquid O ₂	Stored in ice
		Specific radioactivity of P fraction (counts/min./ $\mu\text{g.}$)	
1	Inorganic P	47.3 (49.7)	37.8 (59.8)
	PC-P	30.0	19.3
	ATP-P γ	27.6	20.0
	ATP-P β	15.2	31.2
		Immediately frozen in liquid O ₂	Perfused
		Specific radioactivity of P fraction (counts/min./ $\mu\text{g.}$)	
2	Inorganic P	32.4 (24.3)	13.9 (32.4)
	PC-P	14.6	7.4
	ATP-P γ	14.5	6.6
	ATP-P β	11.4	9.4

The degree to which the relationship between the specific radioactivity of ATP-P γ and ATP-P β may be altered by perfusion or storage in ice is illustrated in Table 4. In the case of the specific radioactivities of the inorganic P fraction, results similar to those described above have been obtained, i.e. perfusion of the muscle or retention in ice results in a fall in specific radioactivity. Similarly, either of these treatments results in a fall in the specific radioactivity of PC-P. The effect of both procedures on the specific radioactivities of the β and γ P atoms of ATP is of particular interest. It will be noted that in

the case of the muscles which were dropped, immediately on removal, into liquid oxygen, the specific radioactivity of the terminal (γ) P group is greater than that of the middle one. Perfusion, or retention in ice, has reversed this relationship so that the middle P group is now more strongly labelled. This phenomenon is one which has already been noted and wrongly interpreted by Kalckar *et al.* (1944) and its significance will be discussed later.

Quite apart from those changes which occur in muscle during perfusion, storage at room temperature or at 0°, and which only become obvious when ^{32}P is used, other changes are also evident. Perfusion has been used by Kalckar *et al.* (1944) with the idea of removing extracellular inorganic P. To some extent this latter will be removed and it would be anticipated that the residual inorganic P concentration in a perfused muscle would be somewhat lower than in a non-perfused muscle dropped, immediately on excision, into liquid oxygen. The data in Table 2 suggest that this is the case, but other results (Tables 3 and 4) have been obtained which clearly show that the concentration of inorganic P is actually greater in the perfused muscle than in the non-perfused and immediately frozen sample. Most of the results which we have obtained are in agreement with these latter, and it became of interest to determine the free and bound creatine contents of perfused muscles since an increase in inorganic P content is generally believed to have its origin in the breakdown of PC. This has been done in three instances on rabbits which were used for isotope experiments of the same type as those already described. The results (Table 5, Expts. 2 and 3) show that the perfused muscles actually have a higher concentration of inorganic P than the non-perfused. The creatine contents are also of interest. It will be noted that the differences between the free creatine contents in these muscles is no greater than one would expect as a result of normal variation from

Table 5. Comparison of the inorganic P and total and bound creatine contents of rabbit skeletal muscle either perfused, retained in crushed ice for 1 hr. or dropped immediately after excision into liquid oxygen

(All figures are expressed as $\mu\text{g. P}$ or creatine/ml. of extract, and are strictly comparable since all extracts were made up in the proportion of 14 g. muscle/100 ml.)

Constituent	Expt. 1		Expt. 2		Expt. 3	
	Immediately frozen in liquid O ₂	Stored in ice	Immediately frozen in liquid O ₂	Perfused	Immediately frozen in liquid O ₂	Perfused
Inorganic P	56.6	68.7	26.3	29.4	25.3	35.8
Total creatine	640	600	614	490	630	544
Bound creatine	272	276	246	216	326	267
% bound	42.5	46.0	60	56	48.4	50.8

Table 6. Specific radioactivities of the P atoms of ATP isolated from rabbit skeletal muscle at varying times after the injection of ^{32}P

Time after ^{32}P injection (min.)	Specific radioactivity (counts/min./ $\mu\text{g.}$)		
	ATP-P γ	ATP-P β	ATP-P α
15	14.0	3.3	—
	8.6	2.7	0.3
10	10.4	2.7	0.2
	17.7	2.2	—
5	11.2	1.9	—
	9.6	1.6	0.1

Table 7. Comparison of ratios of specific radioactivities of terminal P group/middle P group (P γ /P β) and of the middle P group/first P group (P β /P α) based upon data from Table 5

Time after ^{32}P injection (min.)	P γ /P β	P β /P α
15	4.2	—
	3.2	9
10	3.8	13.5
5	8.0	—
	5.9	—
	6.0	16

muscle to muscle. More important is the fact that the proportion of the total creatine which is phosphorylated is unchanged by perfusion. It must be considered unlikely, therefore, that the increase in inorganic P in the perfused muscle can have its origin in the breakdown of PC.

Other interesting conclusions can be drawn from a comparison of the results of Expts. 2 and 3 with those of Expt. 1 (Table 5). In the latter experiment, the results were obtained from the thigh muscles of the same hind limb. The muscles, on removal, were divided across their long axes; one part was dropped immediately into liquid oxygen, and the other placed in an ice bath for 30 min. As would be expected, the latter contained more inorganic P than the former, and it would be anticipated that this additional

amount of inorganic P would have arisen as a result of the breakdown of PC. That this is not so is proven by the fact that the PC content and the fraction of the total creatine which is bound is similar in both samples.

The foregoing results are believed to indicate that the perfusion technique is valueless as a means of assessing the concentration and specific radioactivity of the intracellular inorganic P, not only because of the changes which occur in inorganic P concentrations but also by virtue of the fact that serious alterations take place, during perfusion, in the specific radioactivity of the β and γ P atoms of ATP and in PC-P.

It is also clear that there is a difference in the speeds with which ATP-P γ and ATP-P β are replaced. The results (Tables 1-3) suggested that the ratios of the specific radioactivities of these two groups were not necessarily a true reflection of the relative rates at which these two atoms were replaced. Consequently, additional experiments were carried out wherein the time interval between the injection of ^{32}P and the removal of the muscles was shortened in an effort to reach a time less than that during which the terminal P atom of the entire ATP reservoir had been replaced.

In some of these experiments the specific radioactivities of each of the three P atoms were measured (Table 6), and it will be noted that the difference between the specific radioactivities of ATP-P γ and ATP-P β are of the same order as the differences between those of ATP-P β and ATP-P α .

When the ratios of the specific radioactivities of these groups are calculated (Table 7) a further relationship becomes apparent. As the interval between ^{32}P injection and death of the animal is increased, there is a decrease in the numerical value of these ratios. The indication then is that the 5 min. interval expresses more accurately than any other the speed with which ATP-P γ is replaced relative to ATP-P β and ATP-P α . Thus ATP-P γ is replaced 6-8 times more often than ATP-P β and this latter 16 times more often than ATP-P α .

DISCUSSION

The interpretation of the results in this paper rests largely upon the relative speeds with which each of the three P atoms of ATP are replaced. It is therefore desirable that the results relevant to this problem be discussed first.

Evidence has been adduced to show that there is a marked difference between the turnover rates of these P atoms. Absolute values cannot be assigned to these rates since it was shown (Ennor & Rosenberg, 1954) that measurement of the specific radioactivity of the intracellular inorganic P was not possible with existing methods. It is possible, however, to express the turnover rates of the P atoms relative to each other and these relative rates must be regarded as representing minimum values, since the shortest time interval investigated was 5 min. From the practical viewpoint it is very difficult to shorten this time and indeed such results, if achieved, would be subject to the criticism that considerable ^{32}P concentration gradients would be likely to exist throughout the plasma merely because of inadequate mixing. The results of the present experiments indicate that the terminal P atom of ATP is equilibrated with the intracellular inorganic P pool in 5 min. or less.

This high rate of replacement of the terminal P atom and the marked difference between this and the replacement rates of the other two P atoms of ATP is at variance with the conclusions of most other workers, but is in accord with modern concepts of intermediary metabolism. The relative rates are a clear indication of the nature of the preferred P acceptor in phosphorylations employing the adenylic acid system, i.e. that ADP is the preferred P acceptor, which is in agreement with the results of Kielley & Kielley (1951) and Slater & Holton (1952), which were obtained without the use of ^{32}P .

There is, in fact, no unanimity of opinion among workers who have used ^{32}P on the question of the rates at which ATP- $\text{P}\gamma$ and ATP- $\text{P}\beta$ are replaced, although it has been realized since the work of Korzybski & Parnas (1939) that the turnover rate of ATP- $\text{P}\alpha$ is much less than that of the other two P atoms. Most workers have been content to determine an average specific radioactivity of ATP- $\text{P}\gamma$ and ATP- $\text{P}\beta$ by measuring the specific radioactivity of the P released by acid hydrolysis, but the results obtained by such non-specific methods are of little value. Of those who have employed enzymic methods of analysis, Flock & Bollman (1944) have obtained differences in the specific radioactivities which were of a similar order to those reported here. However, those experiments of these authors, which were carried out at longer time intervals (15–60 min.), showed that differences still existed between the specific radioactivities of these two P

atoms and this is contrary to our findings. Kalekar *et al.* (1944) and Furchgott & Shorr (1943) have shown that differences existed, but these were small and their techniques were open to criticism (see below).

Perfusion of skeletal muscle has been shown to lead to marked changes in the specific radioactivity of each of the P fractions measured, and this fact alone completely vitiates the results of Kalekar *et al.* (1944). These authors were aware of the possibility that PC might break down during the course of the perfusion, but were totally unaware of the fact that considerable exchange also occurs. If it be assumed that no enzymic system will function at the temperature of liquid oxygen, then the specific radioactivities of the P fractions isolated from a muscle dropped, immediately after excision, into liquid oxygen must represent the specific radioactivities at the end of the experiment. It follows also that any departure from these values must take place as a result of the continuance of enzyme activity during the course of the perfusion, immersion in ice or storage at room temperature. If then, one regards the immediately frozen muscle as a control, the following changes are seen to occur during perfusion: (1) an increase in the concentration of inorganic P; (2) a decrease in the specific radioactivity of inorganic P; (3) a fall in the specific radioactivity of ATP- $\text{P}\gamma$, a rise in the specific radioactivity of ATP- $\text{P}\beta$ and a fall in the specific radioactivity of PC-P.

The decrease in the specific radioactivity of inorganic P has been interpreted by Kalekar *et al.* (1944) as due to the washing out from the tissue of highly labelled, radioactive inorganic P present in the extracellular fluid. These authors did not quote the concentrations of inorganic P which were present in their experimental material, but in our hands definite increases have been found which indicate that this interpretation is incorrect. Whilst some, perhaps all, of the extracellular inorganic P is undoubtedly removed by the perfusion, the results obtained on analysis of the muscles left in ice must be taken into account before an explanation of the lowering in specific radioactivity of inorganic P becomes possible. It will be noted (Table 4, Expt. 1) that the muscle sample dropped into liquid oxygen has a higher specific radioactivity and a lower concentration of inorganic P than has the portion dropped into ice. An increase in inorganic P concentration can only arise as a result of the breakdown of organo-P compounds within the muscle cell, and the fact that the specific radioactivity has fallen must be taken as proof that these compounds contained P of lower specific radioactivity than was present in the original inorganic P fraction. A similar explanation applies to the results obtained with perfused muscle (Tables 3 and 4), although in

these cases the fall in specific radioactivity of the inorganic P is partially due to the loss of the strongly labelled extracellular inorganic P consequent on perfusion. It can thus be inferred with some certainty that the specific radioactivity of the inorganic P in the perfused muscle is different from that of the intracellular medium. From the results obtained in the present experiments, it is not possible to fix the magnitude of the difference apart from indicating that the specific radioactivity of the inorganic P in the perfused muscle must be lower than that of the intracellular fluid. The high turnover rate of ATP-P γ suggests that the specific radioactivity of this P atom must be close to that of the intracellular inorganic P, and in this connexion the results (Tables 2–4) are of interest. It will be noted that in each case the specific radioactivity of ATP-P γ isolated from the muscles dropped immediately after excision into liquid oxygen is close to that of the inorganic P in the perfused muscle, but this would seem purely coincidence since the latter specific radioactivity was calculated from inorganic ^{31}P concentrations, which were certainly high owing to breakdown of organophosphate, and from inorganic ^{32}P values which were low for a similar reason. Such reasoning has as its basis the assumption that perfusion will wash out all inorganic P of extracellular origin, but this is unlikely (cf. ^{24}Na results of Ennor & Rosenberg (1954)) and the possibility exists that the vascular bed supplying small areas of muscle may have been occluded. If this were so, then the high specific radioactivity of the inorganic P in such an area would offset the specific radioactivity of the intracellular inorganic P, which would be low in value owing to the breakdown of weakly labelled organophosphate.

It is also of interest to note that no change has occurred in the concentration of PC in muscles which are either perfused or kept in ice. This result is unexpected since it is generally assumed that the increased levels of inorganic P arise from the breakdown of labile compounds such as PC. The fact that no changes occur in either the concentration of this compound or in the fraction of the total creatine which is phosphorylated may be taken as additional proof that the observed increases in the concentration of inorganic P arise from the breakdown of weakly labelled compounds.

The changes which occur in the specific radioactivities of ATP-P γ and ATP-P β may be explained by reference to Fig. 1, which graphically illustrates the relationship which must exist between the specific radioactivities of these various P atoms as a result of the marked differences which are present in the turnover rates.

Since ATP-P γ metabolizes more rapidly than ATP-P β and the latter more rapidly than ATP-P α , the relative specific radioactivities at an early stage

Fig. 1. Curves illustrating the interrelationship of the specific radioactivities of each of the three P atoms of ATP and inorganic P with time. (1) Intracellular inorganic P, (2) ATP-P γ , (3) ATP-P β , (4) ATP-P α . No precise significance should be attached to the heights of the curves, as the relative quantities of the materials have been chosen arbitrarily.

after the injection of ^{32}P will be as represented in Fig. 1 at, say, time x . Shortly after this the specific radioactivity of ATP-P γ falls, but if the specific radioactivities of each P atom are measured at any time up to say a the specific radioactivities of ATP-P γ > ATP-P β > ATP-P α and the magnitude of the differences will decrease until a is reached. At a the specific radioactivities of ATP-P γ is equal to that of ATP-P β and both are greater than ATP-P α but at time y the specific radioactivities of ATP-P γ < ATP-P β although both are still greater than ATP-P α . This relationship will hold until some time b is reached when the specific radioactivity of ATP-P γ is less than that of ATP-P β which now equals that of ATP-P α . At time z , a complete reversal of the specific radioactivity relationships will occur, when the specific radioactivity of ATP-P α will be greater than that of ATP-P β , which in turn will be greater than that of ATP-P γ .

Such relationships as those described above must occur if there are differences in the turnover rates of the P atoms concerned and if the specific radioactivity of the intracellular inorganic P falls rapidly. There is ample proof that the first condition is fulfilled and the experimental results (Table 4) afford proof that the second condition is similarly fulfilled. These results illustrate situations wherein the specific radioactivity of ATP-P γ is actually lower than that of ATP-P β , when muscle is allowed to remain in ice or is perfused. Since the specific radioactivity of PC-P closely follows that of ATP-P γ it follows that a similar behaviour may be expected and it will be noted that the specific radioactivity of PC-P is also lower than that of ATP-P β . Although no experimental results on PC-P were quoted by Kalcekar *et al.* (1944), these authors have observed

a similar phenomenon in frog muscle with ATP, that is, in one experiment the specific radioactivity of ATP- $P\gamma$ was less than that of ATP- $P\beta$. It is of interest to note that three different and incorrect explanations were advanced by these workers to account for the phenomenon.

It is generally believed that the turnover rate of PC-P is the same as that of the terminal P atom of ATP, and the present results support this belief. It should, however, be remembered that the method used for the determination of the specific radioactivity was based upon acid molybdate hydrolysis of the PC and that this is by no means specific. A recent publication of Ennor & Rosenberg (1952c) has shown that such treatment releases more P than can be accounted for on the basis of the PC present. The possibility therefore exists that in extracts from tissues containing ^{32}P in the organic form, some of the P which is released may arise from weakly labelled compounds, so that the specific radioactivity of the PC as measured may be lower than the true value. The converse, of course, is equally possible. Clearly, some specific enzymic method is desirable and experiments are in progress using creatine-phosphokinase for this purpose.

The present results, although giving no indication of the mechanisms by which the terminal P atom of ATP is replaced, do indicate that under the experimental conditions myokinase plays a relatively insignificant role. If this enzyme were an important factor in the regeneration of ATP, it would be expected that the specific radioactivities of ATP- $P\gamma$ and ATP- $P\beta$ would be equal; such, however, is not the case.

The speed with which ATP- $P\gamma$ is replaced in skeletal muscle under resting conditions is such that it precludes any attempt to measure differences in rates in working muscle. It would thus seem that the conclusions of Sacks (1940), Bollman & Flock (1943), Sacks & Altshuler (1942) and others with respect to this problem must be considered of doubtful value.

In view of the fact that metabolic exchanges of ^{32}P continue at a considerable speed when muscle is placed in ice, it becomes apparent that, in experiments involving the use of ^{32}P and perhaps other isotopes, steps must be taken to ensure that all metabolic activity is completely inhibited at the end of the experimental period. Results obtained with tissues cooled with refrigerants other than liquid oxygen or nitrogen, solid carbon dioxide:ethanol mixtures, etc., are therefore of questionable validity.

SUMMARY

1. The effect of perfusion and of cooling of skeletal muscle in ice on the specific radioactivity of the three phosphorus atoms of adenosine triphosphate, phosphocreatine phosphorus and inorganic phosphorus has been investigated. These procedures have been shown to result in marked

changes in the specific radioactivities of these compounds when compared with the specific radioactivities of the same compounds isolated from muscle dropped immediately into liquid oxygen.

2. The most important changes which occur are a breakdown of weakly labelled organophosphorus compounds with a dilution of the specific radioactivities of the intracellular inorganic pool, a fall in the specific radioactivity of the terminal phosphorus atom of adenosine triphosphate, a rise in the specific radioactivity of the middle phosphorus atom and a fall in the specific radioactivity of phosphocreatine phosphorus.

3. The relative turnover rates of the three phosphorus atoms of adenosine triphosphate and phosphocreatine phosphorus have been measured. It was concluded that the terminal phosphorus atom of adenosine triphosphate is replaced 6-8 times more often than the middle phosphorus atom, and that this latter is replaced about 16 times more often than the phosphorus atom attached to the ribose moiety of the molecule. Phosphocreatine phosphorus is replaced at about the same speed as is the terminal phosphorus atom of adenosine triphosphate, although some doubt exists as to the validity of the methods employed for this determination.

4. The need to ensure a complete cessation of enzyme action at the end of experiments which use isotopes has been stressed.

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