appreciable synthesis of TPN from DPN and inorganic phosphate, using the kidney enzyme discussed above. These negative findings with the phosphatases underline the importance of the specific phosphokinase described by Kornberg (1950) which catalyses the synthesis of TPN from DPN, using adenosine triphosphate as the specific donor compound.

SUMMARY

1. Purified alkaline phosphatases from cow's milk and calf intestinal mucosa quantitatively convert TPN into DPN. DPN is not hydrolysed by either enzyme.

2. These results confirm that the third phosphate grouping of TPN is esterified (to ribose), since the action of both purified phosphatases is restricted to phospho mono-esters and amides.

3. Under the experimental conditions used, there was no measurable synthesis of TPN from DPN, either by enzymic transfer from phenyl phosphate by intestinal alkaline phosphatase or by non-enzymic transfer from phosphocreatine at pH 4.2.

4. By using alcohol dehydrogenase, followed by alkaline phosphatase, DPN and TPN can be estimated spectrophotometrically in the same solution.

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Phosphorylations in Rabbit Liver *in vivo*

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A study has been made of the time-course of the distribution of tracer phosphate among the acid-soluble P compounds of the liver, primarily to determine the extent to which correlations may be obtained *in vivo* with the known enzymic reactions involving these phosphorylated intermediates, and also to study some of the dynamic aspects of these processes. Such an investigation has been made previously (Sacks, 1951) in which the separations of the various P compounds present in a trichloroacetic acid (TCA) extract of liver were made on the basis of the solubilities of the barium salts and the relative ease of hydrolysis. Some of the desired separations were not possible by the methods then available; for example a separation of adenosine diphosphate (ADP) and adenosine triphosphate

(ATP) could not be made. As a consequence of the inadequacy of the methods, some of the findings were difficult to correlate with the known enzymic reactions. In particular, the results obtained failed to show that either glucose 1-phosphate (G 1-P) or ATP met the requirements of the postulates of Zilversmit, Entenman & Fishler (1943) for being the precursor of the P of glucose 6-phosphate (G 6-P).

The introduction of ion-exchange chromatography by Cohn & Carter (1950) for the separation of adenosine monophosphate (AMP), ADP and ATP, and the more recent development by Khym & Cohn (1953) of ion-exchange procedures for the separation of the phosphorylated sugars as the borate complexes, have made possible many of the

isolations necessary for such a study. These methods have been applied to experiments on rabbits, in order to obtain data for comparison with those of the previous experiments on rats. The results obtained have resolved some of the anomalies of the earlier data, so that a better correlation is possible between the findings *in vivo* and those of the known enzymic mechanisms. In addition, inorganic pyrophosphate (PP) is found to be present in significant amount in liver, and some functions of this compound have been suggested. Certain of the dynamic aspects of the metabolism of the phosphorylated intermediates have also been brought to light. Results on the time-course of the specific radioactivity have been obtained for orthophosphate, PP, AMP, phosphoglyceric acid (PGA), α -glycerophosphate (α -GPA), G 1-P, G 6-P, 4-carboxy-4-hydroxy-2-phosphoadipic acid (PHCA), and for each of the P groups of ADP and ATP. Some specific activity results have also been obtained for fructose 1-phosphate (F 1-P) and uridine 5'-phosphate (UMP). In each case except that of PHCA the identity of the compound on which radioactivity measurements were made, isolated by the ion-exchange procedure, has been established by parallel experiments in which the known pure compound has been adsorbed on and eluted from the ion-exchange resin, and by other appropriate tests.

EXPERIMENTAL

The experiments were carried out on young male New Zealand white rabbits weighing 1.7–2.4 kg. Free access to food and water was allowed up to the time of injection of the tracer phosphate, and access to water was allowed after the injection in the experiments of 4 and 6 hr. duration. The tracer phosphate was injected intravenously in dose of 50–100 μ C/kg. body weight, in 0.5–2.0 ml. of normal saline/kg. to which had been added 5 μ g./ml. carrier P as Na_2HPO_4 . Anaesthesia was induced just before the time of death by pentobarbital injected intravenously, supplemented by ether inhalation. At the appropriate time, a blood sample was drawn by cardiac puncture into a syringe containing heparin powder, and immediately thereafter the liver was excised and dropped into a large volume of a mixture of solid CO_2 and ether. The blood sample was centrifuged at 1° immediately after it was drawn, to minimize transfer of tracer P from plasma to corpuscles (Gourley & Gemmill, 1950), and the proteins of the separated plasma were precipitated by the addition of 9 vol. 5% TCA. Small volumes of the filtrate were taken for determination of P, and a large volume for the preparation of a sample for counting.

Further operations on the frozen liver were carried out in a cold room maintained at 1–2°. The extraction of the acid-soluble P compounds, removal of glycogen, and separation into 'alkaline-earth soluble and insoluble fractions' were carried out as previously described (Sacks, 1949). The various P compounds present in the 'alkaline-earth-insoluble' fraction were separated by ion-exchange chrom-

atography by a modification of the procedure described previously (Sacks, Lutwak & Hurley, 1954), made necessary because two of the nucleotide-containing eluates contained P compounds in addition to those responsible for the absorption in the ultraviolet shown by these solutions. The separations of the compounds present in the alkaline earth-soluble fraction were made initially by ion-exchange chromatography of the borate complexes, by a modification of the method of Khym & Cohn (1953).

Each P compound as isolated was converted into orthophosphate by the appropriate hydrolytic or wet-ashing procedure, the orthophosphate was precipitated as MgNH_4PO_4 and the precipitate separated by filtration through a sintered-glass filter funnel. The precipitate was then dissolved in a small volume of 5% TCA, the solution made up to suitable volume and samples were taken for determination of orthophosphate by the method of Fiske & Subbarow (1925). A suitable sample, containing 250–350 μ g. orthophosphate, was taken for conversion into phosphomolybdate for counting. Where necessary, carrier P was added to the solution before precipitation, to bring the weight of P in the counting sample within this range. The counting was done with a methane flow counter operating in the proportional range. The details of these procedures have been given previously (Sacks, 1951).

Separation of alkaline-earth insoluble compounds

All operations on this fraction in which acid solutions were involved were carried out in the cold room, except for the measurements in the spectrophotometer. The material was dissolved in the minimum volume of 0.2 N-HCl, and the solution freed from calcium and barium by passage through a column of Dowex-50 cation-exchange resin in the sodium form. The resulting solution was brought to pH 8–9 by the addition of dilute ammonia, and made up to a volume sufficient to reduce the chloride concentration below 0.01 M, the limiting value for complete adsorption of the anions on Dowex-1 resin (Cohn & Carter, 1950). Small samples were taken for determination of orthophosphate and of total P, and for adenine equivalent, by measurement of optical densities at 260 and 280 m μ . in a Beckman Model DU spectrophotometer. The solution was then passed over a column of Dowex-1 anion-exchange resin, 17–18 cm. high by 1.1 cm. in diameter, to adsorb the P compounds present. The resin used was the ordinary 10% crosslinked material, 200–400 mesh. The column was then washed with water, and the elution begun, at the rate of approximately 50 ml./hr., with 2 hr. collections made by an automatic fraction collector. Orthophosphate was completely eluted with 1 l. of the mixture of 0.01 M- $\text{K}_2\text{B}_4\text{O}_7$ and 0.025 M- NH_4Cl , used by Khym & Cohn (1953) in their work on the separation of the sugar phosphates as the borate complexes. The column was then buffered by 500 ml. of 0.01 M- NH_4Cl (Volkin & Carter, 1951), and the elution of the nucleotide fractions begun. The progress of the elution was followed by measurements of the optical density of the eluates at 260 and 280 m μ . Fig. 1 is a representative elution pattern, and gives the sequence of eluents used and the materials obtained with each eluent.

The eluate representing a single peak in the elution pattern was made alkaline with NH_3 , and the P compound or compounds present adsorbed on a short column of the Dowex-1 resin, 2–3 cm. high by 1.1 cm. in diameter. Those eluates in which the chloride concentration exceeded

0.01M were diluted two- to three-fold before this readsorption. The P compounds were then eluted from the short columns with small volumes of eluents of higher ionic strength and acidity than those used for the original separation. In this way a high degree of concentration was achieved, so that material originally contained in 1–2 l. was now present in 50–75 ml. of solution. 0.02N-HCl was used to elute the AMP and UMP from the short columns, and N-HCl for all the other fractions except ATP. For this the eluent was 0.02N-HCl plus 0.5M-KCl, to give directly the concentration of potassium ion for the optimum activity of ATPase.

Fig. 1. Elution pattern of nucleotide derivatives of barium-insoluble fraction of acid-soluble P compounds of rabbit liver. Continuous line, optical density of eluate at 260 mμ.; dotted lines, ratio of optical densities at 280 and 260 mμ. Elution peaks represent: A, AMP; B, UMP; C, mixture of compounds; D, ADP; E, mixture; F, ATP; G, mixture of polyphosphate derivatives. Peak C also contains PGA; peak E also contains PP, PHCA, and another compound of unknown nature.

Before the identity of peak B of Fig. 1 with UMP was established, this material was saved in the attempt to obtain enough material for characterization. The results on the radioactivity of UMP in Table 1 were obtained from the smaller number of experiments carried out after an authentic sample of UMP became available for comparison.

The concentrated eluates containing the AMP and UMP were wet-ashed with nitric and sulphuric acids for conversion into orthophosphate, which was then precipitated as $MgNH_4PO_4$.

The first 600 ml. of eluate obtained with 0.01N-HCl, peak C of Fig. 1, contained PGA and some of the nucleotide derivatives that Hurlbert, Schmitz, Brumm & Potter (1954) isolated from liver by ion exchange from Dowex-1 resin in the formate form with formic acid-ammonium formate eluents. These nucleotide materials were removed from the short-column eluate with acid-washed Norit charcoal (Fiske, 1934), and the filtrate, which showed no absorption in the ultraviolet, wet-ashed for conversion of the PGA present into orthophosphate.

The short-column eluate which contained the ADP was heated in a boiling-water bath for 20 min. to hydrolyse the one acid-labile P group. This was isolated as $MgNH_4PO_4$,

and the acid-stable P group then isolated from the filtrate by wet ashing.

The eluate obtained with 0.01N-HCl plus 0.04M-NaCl, peak E of Fig. 1, has a yellow colour, much more intense than can be accounted for by the quantities of riboflavin derivatives that may be present. It contains some of the nucleotide derivatives isolated by Hurlbert *et al.* (1954) and, with rabbit liver, three other P compounds, which do not absorb in the ultraviolet. The concentrated eluate from the short column containing this fraction was treated with Norit to remove nucleotides, and the filtrate neutralized to phenolphthalein with powdered barium hydroxide. The precipitate which formed was collected by centrifuging, washed with water, and treated with N acetic acid. The major portion dissolved, and the residue was assumed to be the barium salt of PHCA, on the basis that the dissociation constants of two of the carboxyl groups might be expected to be higher than that of acetic acid. This residue was wet-ashed for conversion into orthophosphate.

It was known from some preliminary experiments on rat liver that this eluate also contained PP. The identification as PP was made by parallel experiments with known PP, which showed the same elution characteristics from the resin and the same rate of hydrolysis by N-H₂SO₄ at 100°. A sample of the PP fraction from the liver of a rat which had been injected with tracer P was mixed with known PP, readsorbed and eluted from a Dowex-1 column, and all the ³²P present was found in a single fraction. This material was then precipitated, first as calcium salt, then as silver salt and finally as barium salt. Samples of each salt were converted into orthophosphate, and the same specific radioactivity was found for all three.

The fraction from rabbit liver was found to contain a considerable amount of a not easily hydrolysable P compound in addition to PP. Consequently the co-precipitation experiments were not carried out, as they would not have given significant results.

Fructose 1:6-diphosphate, if present, would also appear in this eluate (personal communication from Dr W. E. Cohn, confirmed with the known compound). Tests for fructose by the method of Roe (1934) on samples of the short-column eluate of this fraction, equivalent to 3–4 g. of liver tissue, were uniformly negative.

P^γ of ATP was hydrolysed by an ATPase preparation from rabbit muscle, with 0.01M-CaCl₂ and glycine buffer, pH 9.2. Over 95% hydrolysis of P^γ was obtained. In the precipitation of this as $MgNH_4PO_4$, the remaining ATP was adsorbed on the precipitate. The amount of this adsorbed P was determined, and allowed for in the weight of the counting sample taken, as the procedure for the preparation of the counting sample resulted in the hydrolysis of the labile P of ATP. No corrections to the radioactivity measurements were necessary as the differences between measured specific radioactivities of P^γ and P^β were within the standard deviation of the counts. P^β of ATP was isolated by hydrolysis, and P^α by wet-ashing.

Separation of 'alkaline-earth-soluble' compounds

This fraction was found to contain considerable AMP and UMP and traces of other nucleotide derivatives in addition to the sugar phosphates and α-GPA. When the solution of this fraction in dilute HCl was treated with Dowex-50 resin in the sodium form to remove Ba and Ca, then made slightly alkaline with NH₃, an appreciable portion of the

total P was not adsorbed on the Dowex-1 resin. This difficulty disappeared when the Dowex-50 was used in the hydrogen form. Some nucleotide material was adsorbed on the Dowex-50, but this was unimportant, as the nucleotide fraction was discarded.

The G 1-P and α -GPA were eluted together from Dowex-1 columns, 17–18 cm. high by 1.1 cm. in diameter, with the mixture of 0.01M- $K_2B_4O_7$ and 0.025M- NH_4Cl used by Khym & Cohn (1953). Their finding that there was considerable overlapping in the elution of these two compounds was confirmed. The entire volume of eluate containing these compounds was concentrated *in vacuo* to about 25 ml., at a bath temperature of 45–50°. The solution was stored overnight in the refrigerator, filtered from crystals of the tetraborate, and magnesia mixture added. Usually an appreciable amount of $MgNH_4PO_4$ was obtained, which was shown not to arise either from impurities in the eluting reagents or from hydrolysis of G 1-P. The latter point was established by the finding that the specific radioactivity of this P was much lower than that of the G 1-P. The P of G 1-P was isolated after hydrolysis at 100° for 15 min. in solution made 0.1N in HNO_3 . The P of α -GPA was isolated after conversion into glycolaldehyde phosphate and subsequent hydrolysis, by the periodate method of Leva & Rapoport (1943).

The elution of G 6-P was carried out with the mixture of 0.025M- NH_4Cl , 0.0025M- NH_4OH and 0.001M- $K_2B_4O_7$, used by Khym & Cohn (1953) for this purpose. The eluate was concentrated to a small volume, and the orthophosphate isolated after wet-ashing in the usual manner.

Tests with authentic F 1-P showed that the elution pattern of this substance was indistinguishable from that of F 6-P, and Khym & Cohn (1953) found that their elution did not effect a complete separation of F 6-P from R 5-P. For these reasons, no further attempts at differential elution were made after the removal of the G 6-P from the Dowex-1 columns. Instead, all remaining P compounds

present were stripped from the column by 50 ml. 0.1N-HCl, nucleotides were removed by Norit, and samples of the Norit filtrate taken for determination of pentose by the method of Mejbaum (1939) and of fructose by the method of Roe (1934). In many cases, most of the fructose found in the original solution of the alkaline-earth-soluble fraction could not be recovered. The ribose determinations indicated the presence of about 0.05 m-mole of R 5-P/kg. wet weight of liver.

Where enough F 1-P was present for an adequate counting sample, the solution was made N in nitric acid, and heated for 40 min. in a boiling-water bath (Tankó & Robison, 1935). This sufficed for complete hydrolysis of F 1-P. The hydrolysis of R 5-P under these conditions, about 5%, would give at most a 5% error in the specific radioactivity value for the P of F 1-P. In this case it was necessary to precipitate the orthophosphate directly as phosphomolybdate, and make the P determination after counting, because the small amount of P present could not be recovered quantitatively as $MgNH_4PO_4$. To avoid interference of chloride with the precipitation of phosphomolybdate, this was removed by the addition of slightly less than the calculated amount of $AgNO_3$. The P found on the precipitate agreed with the fructose value within 3–5%.

Isolation of the P of R 5-P was not attempted because of the small quantity present and the probability that other P compounds, such as propanediol phosphate and β -GPA, were present.

RESULTS AND DISCUSSION

The amounts of the various P compound found and the specific radioactivity measurements are given in Table 1. The value for AMP represents only that portion co-precipitated in the alkaline-earth-insoluble fraction. More AMP and UMP were

Table 1. Concentration of acid-soluble P compounds in rabbit liver, and time-course of specific radioactivities of P

Concentrations are as m-moles/kg. wet weight, mean and range. Radioactivity measurements as counts/min./mg. P, calculated to standard dose of 1×10^6 counts/min. injected/kg. body weight. Figures are means and standard error of mean.

Hr. after ^{32}P injection	...	...	...	0.5	1	2	4	6
No. of animals	...	...	...	4	5	5	4	4
P fraction	Concn.	Specific radioactivity						
Plasma inorganic	2.05 (1.63–2.87)	19450 $\pm$ 750	7700 $\pm$ 280	4100 $\pm$ 125	2275 $\pm$ 150	1350 $\pm$ 75		
Liver inorganic	5.36 (3.6–7.35)	3500 $\pm$ 170	3650 $\pm$ 240	2925 $\pm$ 200	2300 $\pm$ 150	1750 $\pm$ 115		
AMP	0.37 (0.15–1.00)	1100 $\pm$ 70	1350 $\pm$ 100	1575 $\pm$ 70	1550 $\pm$ 70	1350 $\pm$ 95		
Labile P of ADP	1.30 (0.80–1.80)	1950 $\pm$ 105	2450 $\pm$ 200	2400 $\pm$ 120	2250 $\pm$ 70	1750 $\pm$ 100		
Stable P of ADP	—	285 $\pm$ 14	625 $\pm$ 30	975 $\pm$ 90	1300 $\pm$ 95	1500 $\pm$ 60		
P γ of ATP	1.08 (0.35–1.42)	2275 $\pm$ 70	2575 $\pm$ 240	2350 $\pm$ 90	2425 $\pm$ 95	1575 $\pm$ 50		
P β of ATP	—	2275 $\pm$ 70	2550 $\pm$ 120	2475 $\pm$ 115	2275 $\pm$ 70	1700 $\pm$ 50		
P α of ATP	—	440 $\pm$ 30	1000 $\pm$ 75	1300 $\pm$ 115	1250 $\pm$ 100	1300 $\pm$ 170		
Pyrophosphate	0.41 (0.1–1.1)	2550 $\pm$ 210	2825 $\pm$ 210	2375 $\pm$ 310	2225 $\pm$ 60	1800 $\pm$ 60		
PGA	0.24 (0.10–0.45)	775 $\pm$ 35	1050 $\pm$ 90	1025 $\pm$ 50	1050 $\pm$ 30	920 $\pm$ 30		
PHCA	0.42 (0.05–1.20)	2250 $\pm$ 170	2650 $\pm$ 270	2300 $\pm$ 215	2200 $\pm$ 80	1450 $\pm$ 190		
G 1-P	0.20 (0.10–0.61)	1075 $\pm$ 90	2050 $\pm$ 120	2150 $\pm$ 115	1850 $\pm$ 150	1400 $\pm$ 150		
G 6-P	0.20 (0.10–0.60)	1575 $\pm$ 100	2050 $\pm$ 210	2250 $\pm$ 110	1925 $\pm$ 130	1650 $\pm$ 75		
F 1-P	—	—	1550*	1600†	—	1200 $\pm$ 65		
GPA	2.74 (1.50–5.45)	400 $\pm$ 50	525 $\pm$ 55	565 $\pm$ 40	600 $\pm$ 75	450 $\pm$ 60		
UMP	—	205†	190*	175*	—	485†		

* Figure obtained from a single experiment.

† Average of two experiments.

present in the soluble fraction, but quantitative results on the amounts of these two substances there present were not sought.

The presence of PP in such considerable quantity was unexpected. The possibility was considered that this substance might have arisen as an artifact, from decomposition of ATP in the slightly alkaline solution from which the P compounds were adsorbed on the Dowex-1 resin, or by the resin itself. However, when it was found that the specific radioactivity of the PP was higher than that of the P of ATP in the 0.5 and 1 hr. experiments, it became evident that such an artifact could not account for the entire quantity of PP found. The possibility that some PP arose in this way cannot be rigorously excluded.

The outstanding quantitative differences between these results and those obtained by the less specific procedures used previously (Sacks, 1949) are the smaller amounts of PGA, G 1-P, and G 6-P found in the present experiments. The average values for these compounds are less than one-third as great as those obtained by differential hydrolysis procedures. Part of the discrepancies may be accounted for by species differences, but it is evident that the hydrolytic procedure employed in the previous work did not effect complete separation of AMP and UMP from G 6-P, so that the values for the amount of this substance reported earlier included considerable amounts of P from these nucleotides.

Removal of tracer phosphate from plasma

The results on the removal of tracer P from plasma, shown in Fig. 2, have been analysed in the same terms of half-times of the two processes and fractions of the total quantity of tracer accounted for by each, as was done previously (Sacks, 1953) for data obtained on rats. The half-time of the rapid process, representing principally exchange-adsorption with P of bone, was found to be about 15 min., compared with the 20 min. found in the rats, and the 20 min. calculated from the data of Sacks & Culbreth (1951) on cats. The half-time of the slow process, considered to represent principally the overall exchange rate with the P compounds of the soft tissues, was found to have the same value of about 3 hr. that was noted in the earlier experiments on rats and cats. The quantity of tracer P removed from the plasma by this slow process in the present experiments was only one-eighth of the injected amount, compared with one-fifth in the cats, one-third in one strain of rats, and one-fourth in the other strain of rats. The maximum values for the specific radioactivities of many of the intracellular P compounds, e.g. PP, ADP, ATP, were reached at 1 hr. after injection of the tracer, thus indicating high turnover rates for these substances.

In the experiments on rats (Sacks, 1951) it was found that the specific radioactivity of plasma P was equal to the specific radioactivity of the labile P of the ADP-ATP fraction at the time when the specific radioactivity for this intracellular fraction was at its maximum. This is the relation anticipated from the postulates of Zilversmit *et al.* (1943) if all the extracellular P is equally available for turnover with the entire quantity of those intracellular P compounds formed directly from extracellular P, or from intermediates so formed that are present only in trace amounts. When such a relation is found, i.e. when the curve for the time-course of the specific radioactivity of a precursor intersects that of the specific radioactivity of the product at the time of maximum specific radioactivity of the product, it is permissible to conclude that there is no 'compartmentalization' of the product substance within the cell. In the present experiments, this condition is not present; the specific radioactivity of plasma P is higher than

Fig. 2. Time-course of specific radioactivity of plasma P. Solid line, experimental data; short dashes, extrapolation of observed values to zero time; longer dashes, extrapolation of slow component to zero time; dot and dash line, calculated fast component of removal of tracer from plasma.

that of any of the intracellular P compounds for at least 2 hr. after the maximum specific radioactivities of the latter compounds has been reached. As the ultimate source of the ^{32}P for the labelling of the intracellular P compounds is plasma ^{32}P , this finding implies either that during the first hour or so after the injection of the tracer, the specific radioactivity of the P in the extracellular phase available for turnover with intracellular compounds is considerably less than that of plasma P,

or that there is some 'compartmentalization', either structural or functional, of the intracellular P compounds. A third possibility is that both these factors apply.

The first of these alternatives implies that the transfer of phosphate ion across the capillary wall is a slow process, and does not take place by free diffusion, which would be rapid over the short distances involved. It is evident that the first few minutes after the intravenous injection of the tracer are required for mixing with the entire blood volume of the animal, and even if phosphate ion did diffuse freely across the capillary wall, some time would be required for equilibration of tracer P between plasma and extracellular phase of the tissues. However, the time factors in such processes are hardly long enough to explain the present results. Evidence for a slow, rather than a rapid, transcapillary movement of phosphate was obtained in the case of striated muscle (Sacks & Altshuler, 1942). In tracer experiments on cats, values were calculated for the specific radioactivity of the intracellular portion of the P, from the observed specific radioactivities and analytically determined amounts of plasma and muscle P, and the generally accepted values for the magnitude of the extracellular phase of muscle. These calculated values were negative for the first hour after injection of the tracer. The only acceptable alternative is that the passage of phosphate across the capillary wall into the interstitial fluid of the tissues is a slow process. Such an interpretation has been offered by Ennor & Rosenberg (1954) of their results on muscle. The other possibility they offer, that the extracellular phase of muscle is very much smaller than the measured 'chloride space' or 'sodium space', seems much less likely. The evidence for 'compartmentalization' is given below.

Mechanism of transport of phosphate across cell membranes

The measured values for the specific radioactivity of liver orthophosphate represent the mixture of intracellular orthophosphate present with both the interstitial fluid of the liver and the blood contained within the excised liver. It is impossible to arrive at a calculated value for the true intracellular orthophosphate of the liver which would have any significance, but it is evident that if free diffusion of phosphate ion took place across the cell membrane, isotopic equilibrium between orthophosphate of plasma and liver should be reached fairly rapidly. The observed values, showing great disparities between specific radioactivities of plasma and liver orthophosphate at 0.5 and 1 hr. after injection of the tracer, are not in keeping with the postulate of free diffusion. Furthermore, such a

postulate would require a specific anion-permeability of the tissue cells for phosphate, in contrast to the well-established impermeability to smaller anions such as chloride, as well as requiring that the evidence for slow transfer of phosphate ion across the capillary wall be discarded. The observed values are in keeping with the postulate of active transport first proposed by Sacks & Altshuler (1942). The finding in the present experiments that the PP is the intracellular compound which acquires the highest specific radioactivity, and does so soon after the injection of the tracer, suggests strongly that the formation of this substance on the cell membrane is a major mechanism for the transport of orthophosphate into the cell from the extracellular phase.

Mechanism for turnover of ATP

The rapid attainment of isotopic equilibrium between PP and the two labile P groups of ATP, and the further finding that these two labile P groups have reached equal specific radioactivities at the earliest time of sampling after the injection of the tracer, suggest strongly the possibility that the principal mechanism for the formation of ATP in liver is the reaction of AMP with PP. This is in contrast with the report by Cobey & Handler (1953) that in muscle P^{β} of ATP acquires the isotopic label much more slowly than P^{γ} . The breakdown of ATP into AMP and PP has been shown by Jones, Lipmann, Hilz & Lynen (1953) to furnish the energy for the acylation of coenzyme A, and is therefore an important step in the conversion of fatty acids into acetoacetic acid in the liver. A similar conversion of ATP into AMP and PP has been shown to take place in the activation of amino acids for protein synthesis in liver homogenates (Hoagland, 1955). The reversal of these two reactions, formation of ATP from AMP and PP, would account for the present results, and also allow for the conservation of the free energy of the PP bond.

The possible alternative mechanisms for the synthesis of ATP with the incorporation of ^{32}P are glycolysis, oxidative phosphorylation, and the myokinase reaction. If oxidative phosphorylation were to be an important mechanism for introducing ^{32}P into ATP, the intracellular orthophosphate of the liver would need to acquire a very high specific radioactivity shortly after the injection of the tracer. This could be achieved only by rapid and free diffusion of phosphate ion across the capillary wall and the liver cell membrane, both of which appear to be unlikely. The glycolytic pathway involves only P^{γ} of ATP, and therefore is unable to account for the appearance of ^{32}P in P^{β} , much less to account for the rapid attainment of equality of specific radioactivity by P^{γ} and P^{β} . Furthermore,

any ^{32}P acquired by P_γ through the glycolytic pathway would be derived from G 1-P, G 6-P and PGA, and the specific radioactivity of these three compounds is considerably lower than that of P_γ of ATP up to 1 hr. after injection of the tracer. These processes must be assumed to be operating, but their contribution to the incorporation of ^{32}P into ATP can hardly be significant in quantitative terms.

Significance of the myokinase reaction

Cobey & Handler (1953) obtained results which indicated a high apparent activity of myokinase in liver and kidney, but not in striated muscle. If the reaction catalysed by this enzyme, $2\text{ADP} \rightleftharpoons \text{ATP} + \text{AMP}$, were an important mechanism for the synthesis of ATP, then the specific radioactivity of the labile P of ADP should be at least equal to that of the two acid-labile P groups of ATP shortly after the injection of the tracer, and there should also be rapid attainment of equal specific radioactivities between AMP and the acid-stable P groups of ADP and ATP. Examination of Table 1 shows that neither of these is the case. The specific radioactivity of the acid-labile P of ADP increases more slowly than that of P^β and P_γ of ATP, and the AMP acquires the isotopic label at a much greater rate than do the acid-stable P groups of ADP and ATP. These results speak against any great importance of the myokinase reaction in the synthesis of ADP. However, the reversal of this reaction, the formation of ADP from AMP and ATP, is the most likely means of accounting for the presence of ^{32}P in the acid-labile P group of ADP. The energy for such a reversal could be derived from coupling with any of the oxidative stages in the metabolism of the liver.

Mechanism of formation of AMP

The results in Table 1 demonstrate that the rate of movement of the tracer into AMP is higher than that for the acid-stable P groups of ADP and ATP, and thereby rule out dephosphorylation of these two compounds as a principal mechanism for the formation of AMP. An alternative enzymic mechanism is available, namely the action of 5'-nucleotidase, which has been shown by Novikoff, Hecht, Podber & Ryan (1952) to be present in fairly high concentration in the cytoplasm of the liver cell. The operation of this enzyme in the direction of synthesis, again coupled with the energy supplied by oxidative reactions, could account for the observed findings. The P for this reaction could be derived from any of the intracellular reactions which liberate orthophosphate, e.g. ATPase, or from extracellular orthophosphate.

The localization of this enzyme in the cell is such as to make extracellular orthophosphate an alternative possible source. The situation with respect to UMP is similar, with a much lower turnover rate.

'Compartmentalization' of intracellular P compounds

The localization of enzymes in different structural elements of liver, e.g. nuclei, mitochondria and microsomes, is well established. The 5'-nucleotidase referred to above is present to only a small extent in mitochondria (Novikoff, Podber & Ryan, 1950), while the major portion of the ATPase activity is present within the mitochondria (Novikoff *et al.* 1952; Kielley & Kielley, 1953). It is therefore quite possible that there is a functional 'compartmentalization' of the intracellular P compounds, in the sense that a considerable part of the ATP may be present in the cytoplasm, not attached to any of the enzymes in mitochondria for which it is the substrate. If such be the case, although much of the AMP is attached to 5'-nucleotidase in the cytoplasm, the high rate of turnover of AMP relative to that of the acid-stable P groups of ADP and ATP could be accounted for. A similar explanation is possible for the specific radioactivity of the plasma orthophosphate remaining above that of the intracellular orthophosphate, which in turn remains above that of the intracellular P compounds for so long after these have passed their maximum specific radioactivity.

Origin of PGA

The specific radioactivity of the PGA is significantly less than that reported in the previous studies (Sacks, 1951). The difference is probably due to the more specific method for the separation of this compound from others resulting from the use of the ion-exchange resin. The low specific radioactivities found here suggest that a considerable fraction of the PGA is derived by oxidation of α -GPA, rather than through the operation of glycolysis. It should be noted in this connexion that PGA is the only 3-carbon member of the glycolytic pathway which has been found in the intact liver cell in the absence of enzyme poisons. The α -GPA, on the other hand, is primarily an intermediate in phospholipid synthesis, present in some ten times the concentration of the PGA. The relations of specific radioactivity between PGA, α -GPA and ATP can be accounted for on the assumption that PGA in liver arises both through glycolysis and from the oxidation of α -GPA. This oxidation may be the first step in the degradation of the GPA arising from the turnover of liver phospholipids.

Turnover of the sugar phosphates

The relations between the specific radioactivities of G 1-P and ATP found in the present experiments are similar to those previously reported on rats (Sacks, 1951). However, the values for G 6-P, instead of being far below those of G 1-P as in the earlier experiments, are now intermediate between those of G 1-P and the labile P of ATP. This finding disposes of the discrepancy between the tracer findings *in vivo* with respect to the possible precursor of G 6-P and the known enzymic mechanisms for the formation of this substance. The earlier, erroneous, result was due to the inclusion in the G 6-P fraction of large amounts of P of low specific radioactivity from AMP and UMP. The present results are readily interpreted in terms of the simultaneous occurrence of the phosphoglucomutase and hexokinase reactions, without regard to whether there is net increase or decrease in the amount of liver glycogen. Although no glycogen determinations were made in the present experiments, it was clear that the amounts of glycogen precipitated by the addition of ethanol to the TCA extracts were much less in the 4 and 6 hr. experiments than in those of shorter duration. The specific radioactivity relations found with respect to G 1-P, G 6-P and ATP are compatible with a cyclic process for the interconversion of blood glucose and liver glycogen. Evidence suggesting the existence of such a cycle is obtained from the data of Stetten & Stetten (1954) on the time-course of the distribution of the isotope in the liver glycogen of rats fed on glucose labelled with ^{14}C . In the first few hours the isotope was principally in the side branches of the glycogen molecule, but in rats killed more than 6 hr. after feeding the labelled sugar there was relatively more isotope in the central straight-chain portion of the glycogen than in the branches.

The specific methods used here for the isolation of G 1-P and G 6-P lead to the finding that these two compounds are present in the liver in approximately equal concentrations. This is quite different from the equilibrium in the phosphoglucomutase reaction, which was found by Colowick & Sutherland (1942) to be more than 90% on the side of G 6-P. The conditions of the present experiments are reasonably close to those of the physiological steady state with respect to these two compounds; it is therefore apparent that factors other than the purely thermodynamic ones operating on the isolated enzyme system govern the relative concentrations in the intact cell.

The high turnover rate of the P of PHCA is in keeping with that reported by Umbreit (1953) in rat liver. However, it is difficult to correlate the present data with the low turnover found by

Umbreit for the fraction of 'ester P', since this probably represents a mixture of compounds of varying turnover rates.

The few results it was possible to obtain on the turnover rate of F 1-P are inadequate to establish a precursor for this substance. If the mechanism involved is the transfer of P from ATP to fructose, as postulated by Leuthardt, Testa & Wolf (1953), then it would appear that this reaction is a slow one. The specific activity of the F 1-P in the 6 hr. experiments, for which complete information is available, indicates that isotopic equilibrium with P' of ATP has not yet been reached. Such a slow turnover is not unexpected, since no fructose was entering the liver.

The nature of the not easily hydrolysable P compound eluted from the resin together with PP has not been investigated. One possibility is that it is the 2:3-diPGA which Rapoport & Guest (1941) found to be present in rather large quantity in mammalian erythrocytes. The properties of this compound are such that it would be expected to follow PP closely in the elution from the resin. Assuming this composition for the compound found, the question remains open whether it is present intracellularly in liver or is merely a contaminant resulting from the presence of quantities of erythrocytes in the liver as excised. This question can be answered by comparing the specific radioactivities of the compound isolated from liver and erythrocytes.

SUMMARY

1. A time-course study has been made of the distribution of tracer phosphate between plasma P and the acid-soluble P compounds of rabbit liver. Ion-exchange chromatography has been used as the principal means of isolating the various P compounds.

2. The removal of the tracer has been found to follow the same biphasic exponential function as previously found in other mammalian species; the half-times of the two processes were found to be about 15 min. and 3 hr.

3. The slow process, which is considered to represent principally the exchange with the P compounds of the soft tissues, accounts for about one-eighth of the tracer removed from the plasma.

4. The results obtained suggest that there is a functional, if not a structural, 'compartmentalization' of some of the acid-soluble P compounds within the cell, so that the entire quantity of some of them may not be equally available for turnover processes.

5. Inorganic pyrophosphate has been demonstrated to be present in the liver and to have a higher turnover rate than any of the intracellular organic P compounds. The results are consistent

with the hypothesis that the formation of pyrophosphate on the cell membrane serves as a principal means for the entry of phosphate into the cell interior.

6. The results obtained also indicate that adenosine monophosphate (AMP) and uridine 5'-phosphate (UMP) may be formed by reactions at the cell membrane.

7. The principal mechanism for the formation of adenosine triphosphate (ATP) is indicated to be the reaction of AMP with pyrophosphate. The myokinase reaction and the glycolytic pathway appear to be of secondary quantitative importance in this respect.

8. The principal mechanism for the formation of adenosine diphosphate appears to be the reversal of the myokinase reaction.

9. The concentrations of glucose 1-phosphate (G 1-P) and glucose 6-phosphate (G 6-P) in the liver were found to be approximately equal, rather than with the G 6-P predominating, as required by the equilibrium of the phosphoglucomutase reaction.

10. The specific radioactivity relations between G 1-P, G 6-P and $P\gamma$ of ATP are such as to indicate that G 6-P is formed by the phosphoglucomutase and hexokinase reactions taking place simultaneously. This suggests that there is a continuous cycling between blood glucose and liver glycogen whether or not there is net deposition of glycogen in the liver.

11. The specific radioactivity relations of α -glycerophosphate (GPA), phosphoglyceric acid (PGA) and $P\gamma$ of ATP suggest that the oxidation of GPA rather than the glycolytic pathway is the principal means of formation of PGA.

12. The turnover rate of fructose 1-phosphate was found to be considerably lower than that of G 1-P.

13. The turnover rate of UMP is considerably lower than that of AMP.

14. The evidence suggests that the principal means for the formation of AMP and UMP is the action of 5'-nucleotidase, operating in the direction of synthesis by coupling with oxidative reactions.

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