both in the presence and absence of added ribonuclease.

4. A number of organic polyphosphates, phosphates, phosphites, phosphonates and fluorophosphonates were found to cause only slight inhibition of milk or intestinal phosphatase at 10^{-2} M concentration under the experimental conditions used. However, iodine and cysteine at 10^{-3} M concentration both caused strong inhibition of the two phosphatases. A possible mechanism for the inhibition by iodine is discussed.

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The Action of Purified Alkaline Phosphatases on Di- and Tri-Phosphopyridine Nucleotides

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The conversion of triphosphopyridine nucleotide (TPN) into diphosphopyridine nucleotide (DPN) was first reported by von Euler & Adler (1938). The nature of this reaction was not apparent until Kornberg & Pricer (1950a) showed that the third phosphate group of TPN was esterified at either the 2'- or 3'-position of the ribose moiety attached to

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adenine. Hydrolytic cleavage of this esterified phosphate group could thus account for the conversion of TPN into DPN.

It seemed likely that such a reaction would be catalysed by alkaline phosphatases which had been purified from cow's milk and calf intestinal mucosa (Morton, 1950, 1953a, 1954). It had been established that these enzymes acted on monoesters and amides of orthophosphoric acid, but not on diesters

or on pyrophosphates (Morton, 1955*a*). Hydrolysis by these enzymes would thus confirm the presence of a phosphate group in TPN esterified to ribose as proposed by Kornberg & Pricer (1950*a*). Hence the action of the purified enzymes on TPN was investigated, as well as the possible enzymic and non-enzymic synthesis of TPN from DPN by transphosphorylation from phosphocreatine and phenyl phosphate. Most of this work was carried out in 1950 and, although not previously published, has been described in detail elsewhere (Morton, 1952*a*).

MATERIALS AND METHODS

Enzymes

Alkaline phosphatases. These were purified from cow's milk (Morton, 1950, 1953*a*) and from calf intestinal mucosa (Morton, 1950, 1954). The method of storage and handling of these preparations is described in another paper (Morton, 1955*b*).

Alcohol dehydrogenase. This was crystallized from baker's yeast as described by Racker (1950). A crystalline enzyme from liver, kindly supplied by Dr E. C. Slater, was used in some of the initial experiments.

Lactic dehydrogenase of muscle. This was prepared from rabbit skeletal muscle essentially as described by Askonas (1951).

isocitric dehydrogenase. This was prepared from pig heart essentially as described by Graffin & Ochoa (1950).

Glucose 6-phosphate (G 6-P) dehydrogenase. This was a commercial preparation (Sigma Chemical Co.).

Buffers

Monoethanolamine. This was purified and buffers were prepared as described by Morton (1955*b*).

Aminotrihydrozymethylmethane (Tris). A commercial preparation (Sigma Chemical Co.) was used without further purification, and buffers were prepared as recommended by the manufacturers.

Substrates

Phosphocreatine. This was prepared according to Ennor & Stocken (1948).

Diphosphopyridine nucleotide. A number of different preparations have been used, with essentially similar results. Initially, preparations assaying 33% DPN, prepared by the late Mr J. Morgan, and 50% DPN, kindly supplied by Dr E. C. Slater, were used, and in later work a commercial preparation of 90% purity (Sigma '90', Sigma Chemical Co.).

Triphosphopyridine nucleotide. Two preparations, one assaying 19% TPN (kindly supplied by Dr E. C. Slater) and one of 48% TPN (Sigma Chemical Co.), have been used with similar results. Both preparations were free of DPN.

Methods

All measurements were made at room temperature (15–19°), using a Beckman spectrophotometer, Model DU, with 1 cm. quartz cuvettes. A value of 6.22×10^3 at 340 m μ . has been used for the molar extinction coefficient

of reduced DPN and reduced TPN (Horecker & Kornberg, 1948).

Estimation of DPN. This was measured by reduction with alcohol dehydrogenase essentially as described by Racker (1950). The test cuvette contained ethanolamine-HCl buffer (pH 9.3 at 20°) 0.142 mmole; aldehyde-free ethanol, 2 mmoles; and sufficient alcohol dehydrogenase to cause complete reduction of 0.02 μ mole DPN within 3 min. The final volume was usually 3 ml.

Estimation of reduced DPN. This was measured by oxidation with muscle lactic dehydrogenase. The cuvette contained, in 3 ml., 150 μ moles tris buffer, pH 8.6; 20 μ moles pyruvic acid; and sufficient enzyme to oxidize 0.02 μ mole of reduced DPN in about 5 min. At this dilution of enzyme there was no reaction with 0.1 μ mole reduced TPN in 15 min. Hence it was possible to measure reduced DPN in the presence of reduced TPN, as previously described by Kornberg (1950).

Estimation of TPN. This was generally measured by reduction with G 6-P dehydrogenase. The test cuvette contained, in 3 ml., 150 μ moles tris buffer, pH 8.6 at 20°; 30 μ moles G 6-P; and sufficient G 6-P dehydrogenase to cause complete reduction of 0.05 μ mole of TPN in about 5 min. In some cases TPN was reduced with *isocitric* dehydrogenase. The test cuvette contained, in 3 ml., glycylglycine-HCl buffer (pH 7.4; 100 μ moles); sodium DL-*isocitrate* (approx. 10 μ moles); manganous chloride (1.8 μ moles); and sufficient enzyme to cause complete reduction of 0.05 μ mole of TPN in about 10 min.

In consequence of observations to be described, TPN has routinely been estimated as follows. The test cuvette contains ethanolamine-HCl buffer and ethanol as described above for estimation of DPN, and, in addition, 30 μ moles magnesium chloride. Yeast alcohol dehydrogenase is added, and any change in optical density, due to DPN, is measured. Then 0.1 ml. of a solution containing about 10 μ g. purified intestinal alkaline phosphatase (Morton, 1954) is added, and the further change in optical density at 340 m μ ., due to the quantitative conversion of TPN into DPN, is measured. This gives an accurate estimation of TPN. The method has the advantage that both DPN and TPN may be determined in the one cuvette, and use is made of two enzymes which may be readily obtained in a highly purified and stable condition.

RESULTS

Conversion of oxidized TPN into DPN

This experiment was carried out by following the enzymic reduction of DPN at 340 m μ ., using alcohol dehydrogenase as described under Methods. As shown in Fig. 1, 0.16 μ mole of DPN added to the test cuvette was rapidly and completely reduced. When 0.17 μ mole TPN was added to a similar cuvette, however, there was no change in optical density (Fig. 1, curve *B*), up to 5 min. At this time, 0.17 ml. of a solution containing approx. 2.6 μ g. purified intestinal alkaline phosphatase was added. As shown in Fig. 1, curve *B*, there was an immediate increase in optical density. After 30 min. at room temperature (approx. 15°), the curve approached the theoretical value to be expected for quantitative conversion of the TPN into DPN.

Comparison of the curves *A* and *B* (Fig. 1) show that the alkaline phosphatase was limiting in the experiment represented by curve *B*.

The quantitative conversion of TPN into DPN shows that the purified phosphatase has no action on DPN. This was further confirmed by incubating

0.15 μ mole DPN with 2.6 μ g. intestinal phosphatase at pH 9.0 in ethanolamine-HCl buffer (0.04M) and magnesium acetate (0.01M) before addition to the test cuvette. The DPN was subsequently fully reduced by the alcohol dehydrogenase, confirming that the phosphatase has no action on DPN (Morton, 1955a).

The quantitative conversion of TPN into DPN has also been obtained using purified milk alkaline phosphatase instead of intestinal phosphatase. Since the use of alkaline phosphatase and alcohol dehydrogenase thus leads to the complete reduction of TPN, the method has been routinely used for the estimation of TPN (see under Methods). The substantial agreement with enzymic estimation of TPN with both isocitric dehydrogenase and G 6-P dehydrogenase is shown in Table 1.

Conversion of reduced TPN into reduced DPN

The estimation of reduced DPN was carried out spectrophotometrically as described under Methods. On addition of reduced DPN (0.22 μ mole) to the test cuvette containing muscle lactic dehydrogenase, it was rapidly and completely oxidized, as shown in Fig. 2, curve *A*. When reduced TPN was added to a similar cuvette, there was no change in optical density (curve *B*), up to 5 min. The addition of 0.1 ml. solution containing approx. 2.5 μ g. intestinal phosphatase caused rapid and complete conversion of the reduced TPN into DPN, as shown by curve *B* of Fig. 2. In this reaction, the muscle

Fig. 1. The conversion of oxidized TPN into oxidized DPN by intestinal alkaline phosphatase. The reaction mixture contained ethanolamine-HCl buffer, pH 9.3 (0.142 mmole), ethanol (2 mmole), alcohol dehydrogenase and, for curve *B*, magnesium chloride (30 μ moles) in 3 ml. Curve *A* shows the change in optical density after addition of 0.16 μ mole DPN; curve *B*, after addition of 0.17 μ mole TPN. At the arrow (curve *B*), purified intestinal phosphatase (approx. 2.6 μ g.) was added. The optical density for complete conversion of TPN into DPN is indicated by the line *Y*.

Table 1. Comparative estimates of TPN by different enzymic methods

TPN, as estimated originally from assay using isocitric dehydrogenase, was added to each cuvette, and the contents assayed by different procedures as described under Methods. The final volume in each cuvette was 3 ml.

TPN (μ mole)	Change in optical density at 340 m μ ., using:		
	Alkaline phosphatase with alcohol dehydrogenase	G 6-P dehydrogenase	isoCitric dehydrogenase
0.108	0.232*	0.228	0.233
0.197	0.405†	0.401	0.407
0.218	0.452†	0.451	0.450

* With milk alkaline phosphatase.

† With intestinal alkaline phosphatase.

Fig. 2. The conversion of reduced TPN into reduced DPN by intestinal alkaline phosphatase. The reaction mixture contained tris buffer, pH 8.6 (150 μ moles), pyruvic acid (20 μ moles) and diluted muscle lactic dehydrogenase. Curve *A* shows the change in optical density after addition of 0.22 μ mole of reduced DPN; curve *B*, after addition of 0.22 μ mole of reduced TPN. At the arrow (curve *B*), purified intestinal phosphatase (approx. 2.5 μ g.) was added.

lactic dehydrogenase was apparently limiting the rate. This resulted from dilution of the lactic dehydrogenase to the point at which there was no measurable reaction with reduced TPN.

Attempted transphosphorylation with oxidized DPN as acceptor

Both purified intestinal and milk phosphatases catalyse a direct transfer of the phosphate group from certain donor compounds to suitable acceptors (Morton, 1952*b*, 1953*b*). Synthesis of TPN by transphosphorylation to DPN was therefore investigated.

It has previously been shown that the transferase reaction catalysed by purified alkaline phosphatase is due to competition of the acceptor molecule with water (Morton, 1952*a*, 1953*b*). In attempting to obtain appreciable synthesis of TPN, therefore, it was necessary to use high concentrations of DPN.

In the initial experiment, the reaction mixture (0.25 ml.) contained phenyl phosphate (0.025*M*) as donor; magnesium chloride (0.01*M*); DPN (0.07*M*); ethanolamine-HCl buffer (0.04*M*) at pH 9.6; and intestinal phosphatase (about 1.5 μ g.). The control contained all components except phosphatase. After incubating for 30 min. at 38°, the tubes were heated to 100° for 30 sec., and TPN was estimated, using G 6-P dehydrogenase as described under Methods. There was no appreciable synthesis of TPN.

This reaction was repeated essentially as described above, but with the addition of G 6-P (0.04*M*) and G 6-P dehydrogenase to each tube, and with tris buffer (0.05*M*), at pH 8.8, in place of ethanolamine buffer. Under these conditions, any TPN synthesized should be immediately reduced. Moreover, even if TPN was formed and subsequently re-converted into DPN by hydrolysis, the reduced DPN would provide evidence of synthesis of TPN. However, there was no measurable formation of TPN after 30 min. incubation at 38°.

It has previously been shown that a non-enzymic transphosphorylation of glucose from phosphocreatine occurs at pH 4.0 (Morton, 1952*a*, *b*). It seemed desirable to investigate whether DPN could act as a phosphate acceptor to form TPN under similar conditions. For this purpose, 7.6 mg. of DPN (90 % pure) and 12.3 mg. of phosphocreatine were weighed into a small tube and 0.2 ml. of 0.5*M* sodium acetate buffer, pH 4.2, was added. After the components had dissolved, 0.1 ml. was removed immediately and held in crushed ice. The remaining solution was incubated for 30 min. at 38°, after which 0.1 ml. was removed. TPN was then estimated in both samples, using G 6-P dehydrogenase. There was no measurable synthesis of TPN.

DISCUSSION

It has previously been shown that the alkaline phosphatases purified from cow's milk and calf intestinal mucosa are true orthophosphomonoesterases, without action on phospho di-esters or pyro- or poly-phosphates (Morton, 1955*a*). The action of these purified enzymes, of defined specificity, on TPN as described here therefore leaves no doubt that TPN contains an esterified phosphate group which is readily hydrolysed, giving rise to DPN and inorganic phosphate.

The quantitative conversion of TPN into DPN with purified intestinal phosphatase (Fig. 1) was first observed in 1950, in collaboration with Dr E. C. Slater, shortly after the elegant formulation of the structure of TPN by Kornberg & Pricer (1950*a*). This study provided confirmation of the finding by Kornberg & Pricer that the third phosphate group of TPN was attached by an ester, rather than by an anhydride, link.

Kornberg & Pricer (1950*b*) observed that crude potato extracts, like yeast maceration juice (von Euler & Adler, 1938), would catalyse the conversion of TPN into DPN. That this reaction was due to a hydrolytic cleavage required investigation with purified enzymes of defined specificity as herein described. After this work had been completed, reports appeared of the conversion of TPN into DPN by partially purified prostate gland acid phosphatase (Kaplan, Colowick & Ciotti, 1952) and by kidney extracts (Sanadi, 1952). Sanadi (1952) partially purified the enzyme, which he designated as TPNase, although he pointed out that the properties of this enzyme closely resembled those of alkaline phosphatase. Consideration of the properties of Sanadi's enzyme with those of alkaline phosphatases (Morton, 1954, 1955*b*) leaves no reasons for doubting the identity with kidney alkaline phosphatase.

The failure to obtain synthesis of TPN from DPN by transphosphorylation was not unexpected. As shown previously (Morton, 1953*b*) both phosphatases exhibit a specificity toward the acceptor, and only some, but not all, compounds containing hydroxyl groups can compete with water for the transferred group from the donor compound. Both the structure and the size of the acceptor molecule seem important, since glucose and fructose, but not sucrose, act as acceptors for milk and intestinal phosphatases (Morton, 1952*a*, 1953*b*). DPN may well fail to act as an acceptor for similar reasons although it should be pointed out that the maximum concentration of DPN achieved in the present studies was well below that required to eliminate entirely the possibility of DPN acting as a phosphate acceptor to form TPN. It is noteworthy that Sanadi (1952) failed to obtain any

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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was largely carried out while holding a Travelling Scholarship awarded by the Gowrie Scholarship Trust of Australia. I am grateful to Dr E. C. Slater for his assistance with some of the experiments.

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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