The Biosynthesis of *N*-Phosphorylcreatine: an Investigation of the Postulated Alternative Pathway

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Before 1956 it was generally accepted that the sole pathway for the synthesis of *N*-phosphorylcreatine was the reaction catalysed by creatine phosphoryltransferase:

Although the results of isotopic experiments (Sacks & Altschuler, 1942; Harvey, 1955) had been interpreted as indicating the possible presence in muscle of a synthetic pathway for the formation of *N*-phosphorylcreatine not involving the adenine nucleotides, there was no direct evidence for such a reaction until the report by Cori, Abarca, Frenkel & Traverso-Cori (1956). These authors claimed that extracts of skeletal muscle catalyse the direct transfer of a phosphoryl group from 1:3-diphosphoglycerate to creatine to form *N*-phosphorylcreatine according to the equation:

In a more detailed report (Cori, Traverso-Cori, Lagarrigue & Marcus, 1958) it was suggested that only a single enzyme was involved. Furthermore, as supporting evidence for the non-participation of adenosine triphosphate, it was stated that there was no inhibition of *N*-phosphorylcreatine synthesis in the presence of either adenosine triphosphatase or glucose plus hexokinase and that no adenine nucleotides could be detected in the reaction mixtures at the end of the incubation.

Ennor & Morrison (1958) have pointed out that because of the ubiquitous distribution of the adenine nucleotides and creatine phosphoryltransferase, any demonstration of an alternative pathway for *N*-phosphorylcreatine synthesis must unequivocally exclude creatine phosphoryltransferase. Although Cori *et al.* (1958) took rigorous precautions to exclude adenine nucleotides from the reaction mixtures, they were unable to obtain a preparation free from creatine phosphoryltransferase. However, the new reaction has received some accept-

ance. Thus Cohn (1959) has cited it as being an exception to the general rule that ester phosphoryl groups are transferred to form O-P bonds, and Bock (1960) has pointed out that this reaction does not fit into his classification of phosphate-transfer enzymes. Reference has been made to the reaction by Joyce & Grisolia (1959) and by Padieu & Mommaerts (1960).

In the present work the reaction has been re-examined and an attempt has been made to ascertain whether in fact creatine phosphoryltransferase is involved. No specific inhibitor of this enzyme is known and the first approach was an endeavour to separate creatine phosphoryltransferase from the enzyme responsible for catalysing reaction (2). It has been found that relatively crude preparations from rat and rabbit skeletal muscle will catalyse the overall reaction described by Cori *et al.* (1958) under conditions similar to those described by these authors. Further fractionation, however, caused a loss of activity and, since this could be recovered in greater than additive amounts by recombination of fractions, it seemed that more than one enzyme was involved. Furthermore, it has been found that diphosphopyridine nucleotide (or reduced diphosphopyridine nucleotide), which was added by the above-mentioned authors as part of the reaction system, not only functions as part of the system but also gives rise to adenosine 5'-phosphate, which is converted into adenosine triphosphate under the experimental conditions. Evidence will be presented that a direct phosphoryl-group transfer is improbable and that the reaction described probably involves the participation of adenosine di- and tri-phosphate as well as creatine phosphoryltransferase.

EXPERIMENTAL

Materials

Abbreviations. In this paper the following abbreviations will be used: ADP, adenosine diphosphate; ADP ribose, adenosine diphosphate ribose; ammediol, 2-amino-2-methylpropane-1:3-diol; AMP, adenosine 5'-phosphate; ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; DPNH₂, reduced diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide.

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Chemicals. Unless otherwise stated nucleotides and substrates were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Commercial samples of DPN (purity, 95%) and enzymically prepared DPNH₂ (purity, 87–90%) were tested before use, as described below (Methods), for contamination by ATP or ADP. Some samples of DPN were purified by chromatography on Dowex 1 (formate) (Kornberg, 1957) and such samples were used to prepare DPNH₂ by chemical reduction (Beisenherz, Bücher & Garbade, 1955). ADP ribose was obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A. AMP was purified by chromatography on Dowex 1 (formate). The nucleotide was eluted with 0.25*N*-formic acid, adsorbed on acid-washed charcoal (see below) and recovered by stirring three times with isopentyl alcohol-water (10:90, v/v). The aqueous layer from the combined extracts was evaporated to dryness under reduced pressure and the product was crystallized twice from hot water. Before use, Nuchar C and Norit A were boiled for 15 min. with *N*-HCl, washed free of chloride and dried at 100°.

The barium salts of fructose 1:6-diphosphate and 3-phosphoglycerate and the silver-barium salt of phosphoenolpyruvate were converted into sodium salts by adding them as slurry to the top of a column of Zeo-Karb 225 (Na⁺) and washing through with water. The effluent was adjusted to pH 7.4 before making to the required volume and then treated with acid-washed charcoal. Phosphorylcreatine was prepared as the sodium salt (Ennor & Stocken, 1957). Sodium pyruvate was prepared from freshly distilled pyruvic acid, which was diluted with an equal volume of water, cooled and adjusted to pH 5 with 2*N*-NaOH. The sodium salt was then precipitated with acetone. Ammediol was obtained from Eastman Organic Chemicals, Rochester, N.Y., and neutralized with HCl. Hydroxylamine hydrochloride (British Drug Houses Ltd.) was neutralized before use with KOH. Calcium phosphate gel was prepared according to the method of Keilin & Hartree (1938).

Enzymes. Unless otherwise stated, frozen rabbit muscle was used as the source of the enzymes. Adenylic acid deaminase was prepared by the method of Lee (1957) to the stage of elution from calcium phosphate gel. Aldolase (Taylor, Green & Cori, 1948) and D-glyceraldehyde 3-phosphate dehydrogenase (Cori, Slein & Cori, 1948) were prepared in crystalline form. Both enzymes were twice recrystallized and stored as suspensions in 0.50 and 0.66 saturated solution of (NH₄)₂SO₄ respectively. Myokinase was prepared according to Colowick & Kalckar (1943), but only the 0.5–0.8 saturated (NH₄)₂SO₄ fraction was collected. Creatine phosphoryltransferase, prepared by method B of Kuby, Noda & Lardy (1954), was stored at 0° after freeze-drying. Before use, solutions containing 10 mg. of protein/ml. were stirred with acid-washed Norit A (1 g./10 ml.) for 15 min. at 0°, filtered and then stirred overnight with Norit A. Both treatments were then repeated. Fresh rabbit muscle was used to prepare myosin (Perry, 1955). Apyrase was prepared from potatoes by the method described by Van Thoi, Roche & An (1954) and was found not to attack AMP. Diphosphopyridine nucleotidase was prepared from mycelia of *Neurospora crassa* (Kaplan, 1955) and had an activity of 2300 units/mg. of protein. Alcohol dehydrogenase and hexokinase were commercial preparations obtained from Sigma Chemical Co. and lactic dehydrogenase was a sample from L. Light and Co. Ltd.

Rat- and rabbit-muscle preparations used in the early experiments were prepared as described by Cori *et al.* (1958)

with the exception that ammediol buffer was used. Subsequently the following procedure was adopted. All operations were carried out at 2° and centrifuging was done 15 min. after the additions of (NH₄)₂SO₄. The muscle was treated for 2 min. in a Waring Blendor with 3 vol. of 0.01*M*-ammediol buffer (pH 8) and the mixture was centrifuged at 1700*g* for 20 min. The extract was filtered through cotton wool to remove fat. Ammonium sulphate (330 g./l.) was then added slowly to the filtrate with mechanical stirring. The precipitate was removed by centrifuging and (NH₄)₂SO₄ (155 g./l.) was added slowly with mechanical stirring to the supernatant. The precipitate was collected by centrifuging, dissolved in 0.01*M*-ammediol buffer, pH 8 (0.12 vol. of original extract), and dialysed against the same buffer for 16 hr. (Fraction 1). The dialysed preparation was stirred for 15 min. with Dowex 1 (Cl[−]) (1 g./10 ml.) and then diluted to a protein concentration of 25 mg./ml. Ammonia solution (17*N*) was added to a saturated solution of (NH₄)₂SO₄ at 0° so that (on testing a dilution of 1 in 10) the pH was 7.4. This solution (1.5 vol.) was added dropwise with mechanical stirring to the enzyme solution obtained from the previous step and the precipitate was removed by centrifuging. The supernatant was brought then to 0.9 saturation by the slow addition of solid (NH₄)₂SO₄ (220 g./l.). The precipitate was collected by centrifuging, dissolved in ammediol buffer (0.4 vol. of Fraction 1). This preparation (Fraction 2) was stable for at least 2 months at 1–2°, but its stability decreased after treatment with charcoal. Fraction 1 has also been used to obtain some of the results reported in this paper and, unless otherwise stated, was stirred before use with Norit A (1 g./10 ml.) for 15 min. at 2° and filtered. Enzyme preparations have also been treated with Dowex 1 (Cl[−]) (100 mg./ml.) as the loss of protein is less than that obtained as a result of treatment with charcoal.

Methods

Creatine. The release of creatine from phosphorylcreatine was estimated by the method of Rosenberg, Ennor & Morrison (1956) after stopping the enzymic reaction by the addition of an alkali-ethylenediaminetetra-acetic acid mixture (Morrison, Griffiths & Ennor, 1957).

N-Phosphorylcreatine. The formation of phosphorylcreatine was demonstrated as creatine disappearance during incubation, and verified by recovery of creatine after hydrolysis for 9 min. at 65° at pH 1–2.

Protein. This was estimated either by the biuret method of Gornall, Bardawill & David (1949) or with the Folin-Ciocalteu reagent (Lowry, Rosebrough, Farr & Randall, 1951), crystalline bovine albumin being used as the standard for both methods.

Inorganic orthophosphate. This was determined either by the method of King (1932) or, if labile organic phosphorus compounds were present, by the method of Ennor & Stocken (1950).

3-Phosphoglycerate. This was estimated by the method of Bartlett (1959).

Ribose. This was estimated with the orcinol reaction as described by Hurlbert, Schmitz, Brumm & Potter (1954).

Diphosphopyridine nucleotide. DPN was estimated either chemically by the formation of the cyanide complex (Colowick, Kaplan & Ciotti, 1951) or enzymically with alcohol dehydrogenase by the procedure described by Bonnichsen & Brink (1955).

Reduced diphosphopyridine nucleotide. Oxidation of DPNH₂ was followed by studying the rate of decrease of absorption at 340 mμ in the Beckman model DK-2 ratio-recording spectrophotometer.

Nicotinamide. This was determined by measurement of the absorption at 262 mμ (ϵ 3.1×10^6) and distinguished from nucleosides or nucleotides containing nicotinamide by the increased absorption in 0.01 *N*-HCl (ϵ 5.28×10^6).

Hydroxamic acids. These were estimated by the method of Lipmann & Tuttle (1945).

Detection of low concentrations of adenine nucleotides. A linked enzyme system in which ATP and ADP act catalytically was used to detect traces of these compounds present as contaminants of other substrate nucleotide preparations. The method was also used to detect the formation of these compounds. The system involved reaction (1) and the following reaction:

The procedure was as follows: 10 μmoles of *N*-ethylmorpholine buffer, pH 7.0, 5 μmoles of CaCl₂, 2.5 μmoles of phosphorylcreatine and test material were incubated with 0.5 mg. of creatine phosphoryltransferase and 0.5 mg. of myosin for 20 min. at 38° in a total volume of 1.0 ml. and the release of creatine was determined. The method is capable of detecting 0.1–0.2 μm-mole of ATP, and is ten times as sensitive as the firefly-luminescence-assay method (Strehler & Totter, 1954) or the catalytic linked system, involving hexokinase and glucose 6-phosphate dehydrogenase, which was used by Cori *et al.* (1958). There was some inhibition of the release of creatine with added ATP at concentrations of AMP or DPN above 2.5 mM and this limited the assay to the detection of 1 part of ATP or ADP in 2500 parts of those compounds. No ATP or ADP could be detected in the purified samples of DPN and AMP, or in the commercial DPN used in this work. However, the commercial DPNH₂ and ADP ribose were contaminated by ATP or ADP to the extent of 0.1–0.2% and 0.05–0.1% respectively.

Assay of adenine nucleotide. The determination of AMP and ATP plus ADP was carried out with adenylic acid deaminase and potato apyrase (Kalckar, 1947) and the changes in extinction were followed in a Beckman DK-2 ratio-recording spectrophotometer in the presence of 0.1 *M*-sodium succinate buffer (pH 6.5).

Chromatography and electrophoresis. Nucleotides and nicotinamide were chromatographed on Whatman 3 MM paper by ascending chromatography in either (a) isobutyric acid–aq. NH₃ soln. (sp.gr. 0.88)–water (66:1:33, by vol.) or (b) ethanol–1 *M*-ammonium acetate, pH 7.5 (7:3, v/v) and were detected by visual inspection in ultraviolet light. Nicotinamide and its derivatives were also detected by the methods described by Kodicek & Reddi (1951). Nicotinamide and DPN could be rapidly separated (2–3 hr.) by electrophoresis either in 0.033 *M*-borate buffer, pH 8.9, or 0.033 *M*-phosphate buffer, pH 7.0 (Sundaram, Rajagopalan & Sarma, 1959). Dinitrophenylhydrazones were chromatographed by ascending chromatography in (c) 2-methylbutan-2-ol–ethanol–water (50:10:40, by vol.) (Ichihara & Greenberg, 1957) or by descending chromatography in (d) butan-1-ol–*N*-NaHCO₃ (1:2, v/v) with bicarbonate-washed papers (Seligson & Shapiro, 1952). Free acids were separated by descending chromatography in (e) pentan-1-

ol–5 *M*-formic acid (1:1, v/v) (Buch, Montgomery & Porter, 1952) and detected by spraying with bromophenol blue. Hydroxamic acids were chromatographed by descending chromatography in (f) butan-2-ol–formic acid–water (75:15:10, by vol.) (Hoagland, Keller & Zamecnik, 1956), (g) ethyl acetate–acetone–water (1:2:2.75, by vol.) (Micheel & Albers, 1956) and (h) butan-1-ol–acetic acid–water (4:1:5, by vol.) (Thompson, 1951) and were detected by the method of Lipmann & Tuttle (1945).

RESULTS

Because the release of creatine from phosphorylcreatine can be determined more readily than can the formation of phosphorylcreatine from creatine,

Table 1. *Requirements for the release of creatine from phosphorylcreatine in the presence of 3-phosphoglycerate*

Complete reaction mixture contained: ammediol buffer (pH 7.4), 100 μmoles; 3-phosphoglycerate, 4 μmoles; phosphorylcreatine, 2.5 μmoles; Mg²⁺ ion, 5 μmoles; DPNH₂, 0.25 μmole; arsenate, 1 μmole; enzyme, 2 mg. of a 0.64–0.75 sat. ammonium sulphate fraction of rabbit-muscle extract prepared as described by Cori, Traverso-Cori, Lagarrigue & Marcus, (1958). Volume, 1.0 ml.; temp., 38°; reaction time, 15 min. The enzyme preparation was stirred at 2° for 15 min. with Norit A (1 g./10 ml.) and filtered. The filtrate was stirred overnight with a similar amount of charcoal and again filtered.

Components	Creatine released (μmole/ml.)
Complete	0.315
Without arsenate	0.125
Without DPNH ₂	0
Without 3-phosphoglycerate	0
Without Mg ²⁺ ion	0.045

the initial studies were concerned with the reaction between phosphorylcreatine and 3-phosphoglycerate to form creatine and 1:3-diphosphoglycerate. It was pointed out by Cori *et al.* (1958) that this approach suffers from the disadvantage of the unfavourable equilibrium but that this can be overcome by the addition of triose phosphate dehydrogenase, DPNH₂ and arsenate. This results in arsenolysis of the 1:3-diphosphoglycerate formed and thus increases the release of creatine according to the following reactions:

With the additions referred to above, our results were similar to those reported by Cori *et al.* (1958). Thus charcoal-treated enzyme preparations from

either rat or rabbit skeletal muscle catalysed the release of creatine from phosphorylcreatine. The release of creatine required the presence of 3-phosphoglycerate and DPNH₂ and was increased by the addition of arsenate and Mg²⁺ ions (Table 1). In the present experiments, the rate of reaction was linear over the first 30 min. and was not affected by the addition of triose phosphate dehydrogenase, which, as was subsequently shown, was present in all muscle preparations. The rate of release of creatine from phosphorylcreatine was dependent upon the concentration of DPNH₂, and purified DPN was far less effective than DPNH₂ (Table 2). Since triose phosphate dehydrogenase catalyses equally well the arsenolysis of 1:3-diphosphoglycerate in the pre-

sence of either DPN or DPNH₂ (Racker & Krinsky, 1952), this result suggests that if 1:3-diphosphoglycerate is formed, then its formation is dependent on the presence of DPNH₂ and therefore probably does not occur by direct phosphoryl-group transfer.

In the absence of arsenate, DPNH₂ was oxidized when incubated with the enzyme preparation, 3-phosphoglycerate and phosphorylcreatine. The rate of this reaction was linear, as also was the release of creatine, but the amount of DPNH₂ oxidized at any time was greater than the amount of creatine released (Table 3). According to the reaction postulated by Cori *et al.* (1958) the amount of creatine released should be equivalent to the 1:3-diphosphoglycerate formed and, if the oxidation of DPNH₂ were due to the reaction 1:3-diphosphoglycerate + DPNH₂ → 3-phosphoglyceraldehyde + inorganic phosphate + DPN, the amount of DPNH₂ oxidized could not exceed the amount of creatine released.

Table 2. *Effect of oxidized and reduced diphosphopyridine nucleotide on the release of creatine from phosphorylcreatine in the presence of 3-phosphoglycerate*

Reaction mixtures contained: ammediol buffer (pH 7.4), 100 μmoles; 3-phosphoglycerate, 4 μmoles; phosphorylcreatine, 2 μmoles; Mg²⁺ ion, 5 μmoles; arsenate, 1 μmole; enzyme, 4.2 mg. of (A) and 5.6 mg. of (B), where A and B represent different preparations of Fraction 1 treated with charcoal as described in Table 1. Total volume, 1.0 ml. Tubes were incubated for 15 min. at 38°.

Nucleotide	Concn. (mM)	Creatine released (μmole/ml.)	
		A	B
DPN (purified)	0.25	—	0.027
	0.1	—	0.010
DPNH ₂	0.25	0.323	0.380
	0.1	0.144	0.160
	0.05	0.082	—
	0.025	0.043	—
	0.01	0.018	—
DPNH ₂ (prepared chemically from purified DPN)	0.25	—	0.35
	0.10	—	0.19

Table 3. *Relationship between the oxidation of reduced diphosphopyridine nucleotide and the release of creatine from phosphorylcreatine in the presence of 3-phosphoglycerate*

Reaction mixture contained: ammediol buffer (pH 7.4), 0.75 m-mole; phosphorylcreatine, 7.5 μmoles; Mg²⁺ ion, 15 μmoles; cysteine, 60 μmoles; 3-phosphoglycerate, 12 μmoles; DPNH₂, 0.3 μmole; enzyme, 1.2 mg. of a 0.6–0.9 sat. ammonium sulphate fraction of muscle extract treated with charcoal as described in Table 1. The total volume was 3 ml. and the reaction mixture was incubated in spectrophotometer cells at 18°.

Incubation time (min.)	Creatine released (μmole/ml.)	DPN formed (μmole/ml.)	DPNH ₂ oxidized (%)
10	0.029	0.05	52
20	0.058	0.096	100

Evidence for the requirement of more than one enzyme for reaction (2)

When the enzyme preparation (Fraction 1) was subjected to further fractionation with calcium phosphate gel, acetone or ethanol, the specific activity of each fraction obtained was less than that of the original solution. (The enzymic activity was determined by measuring the rate of creatine release from phosphorylcreatine in the presence of 3-phosphoglycerate, DPN, triose phosphate dehydrogenase and arsenate.) As activity could be recovered in more than additive amounts by recombination of two or more fractions, it seemed that more than one enzyme was involved and that the mechanism proposed by Cori *et al.* (1958) was probably incorrect.

If a direct phosphoryl-group transfer were operative, it would be expected that when phosphorylcreatine, 3-phosphoglycerate and hydroxylamine were incubated with the enzyme preparation (Fraction 1) a hydroxamic acid derivative of 1:3-diphosphoglycerate would be formed. However, under these conditions, no such derivative could be detected. The failure to obtain a hydroxamate was not due to the high KCl concentration associated with the hydroxylamine, for an equivalent amount of KCl did not inhibit the release of creatine with added DPNH₂. It is not known whether or not creatine was released in the presence of hydroxylamine because hydroxylamine interfered with the colorimetric determination of creatine.

The addition of DPNH₂ (0.25 mM) to the reaction mixture caused the formation of a hydroxamate, which was also obtained in the absence of phosphorylcreatine. The reaction could not be attributed to the ADP or ATP content (0.1–0.2%) of the DPNH₂ used, for these adenine nucleotides were inactive at 1 μM, although activity was obtained at

higher concentrations. When an enzyme preparation twice treated with charcoal was used, DPNH₂ could not be replaced by DPN, although the latter was active after chemical reduction (cf. Table 2).

Addition of DPN did bring about hydroxamate formation with a preparation treated once with charcoal. The reason for this difference is not known, but it may be related to the further removal of protein by a second charcoal treatment.

Reactions of 3-phosphoglycerate catalysed by the muscle preparation in the absence of phosphoryl-creatine

The hydroxamic acid derivative formed in the presence of 3-phosphoglycerate and either DPN or DPNH₂, in the absence of phosphoryl-group donors, was obtained under the conditions described in Table 4. As the enzyme preparation also contained 3-phosphoglycerate kinase, the hydroxamic acid derivative of 1:3-diphosphoglycerate was prepared under the same conditions with ATP (20 mM) replacing the DPNH₂ or DPN. At the end of the incubation period (2 hr.) an equal volume of ethanol was added and the mixtures were boiled. Cations were removed from the supernatant solution by addition of Zeo-Karb 225 (H⁺ form). The hydroxamates formed from 3-phosphoglycerate in the presence of DPN, DPNH₂ or ATP showed identical behaviour when chromatographed in solvents (*f*), (*g*) and (*h*), and the presence of phosphate in each was demonstrated after chromatography in solvent (*f*). In this solvent there was a clear separation of the hydroxamate (*R_F* 0.1) from inorganic phosphate (*R_F* 0.5). It was thus concluded that the muscle preparations formed 1:3-diphosphoglycerate in the presence of 3-phosphoglycerate and either DPNH₂ or DPN. This could be explained by the

conversion of some 3-phosphoglycerate into phosphoenolpyruvate, which would then act as a phosphoryl-group donor. Such an explanation is supported by the finding that hydroxamate formation with either DPN or DPNH₂ was inhibited by fluoride in the presence, but not in the absence, of phosphate (Table 4). The probable involvement of phosphoenolpyruvate was also shown by the disappearance of 3-phosphoglycerate in the absence of pyridine nucleotides (cf. Table 6) and the formation of an iodine-labile phosphorus compound (Schmidt, 1957).

The transfer of a phosphoryl group from phosphoenolpyruvate to 3-phosphoglycerate would result in the formation of pyruvate in addition to 1:3-diphosphoglycerate. That such a reaction did occur was confirmed by the isolation of pyruvate from those reaction mixtures which contained DPN or DPNH₂. The identity of the isolated material as pyruvate was confirmed by the similarity in chromatographic behaviour of the free acid and its 2:4-dinitrophenylhydrazone with that of an authentic sample of pyruvate and its hydrazone (see the Methods section). In addition, the melting point (214–215°) of the 2:4-dinitrophenylhydrazone derivative was identical with that of the authentic compound and was unchanged by admixture with the latter.

In the presence of pyridine nucleotides 3-phosphoglycerate gave rise to inorganic phosphate (Fig. 1). Although some phosphatase activity is apparent, the addition of DPN or DPNH₂ has a marked effect on the release of inorganic phosphate. The kinetics of the release of inorganic phosphate show that the initial rate is higher with DPNH₂ than with DPN and are consistent with inorganic phosphate being derived from 1:3-diphosphoglycerate.

Table 4. *Effect of fluoride on the formation of hydroxamate from 3-phosphoglycerate in the presence of oxidized and reduced diphosphopyridine nucleotide*

Reaction mixtures contained: nucleotide, 1 μ mole; 3-phosphoglycerate, 8 μ moles; Mg²⁺ ion, 5 μ moles; hydroxylamine, 0.2 m-mole; enzyme, 3.5 mg. of Fraction 1; either phosphate buffer (pH 7.4), 50 μ moles or ammediol buffer (pH 7.4), 100 μ moles; total volume, 1 ml. After 1 hr. at 38°, the apparent hydroxamate formation was determined by the method of Lipmann & Tuttle (1945). Results were calculated from the molecular-extinction value for the hydroxamate of 1:3-diphosphoglycerate as quoted by Krinsky (1959). Inhibition of the colour formation by components of the reaction mixtures and added fluoride ions was studied with succinic anhydride as a standard.

Buffer	Nucleotide	Fluoride added (μ moles)	Apparent hydroxamate formation (μ moles/ml.)	Inhibition of the colour formation (%)	Corrected hydroxamate formation (μ moles/ml.)
Phosphate	DPN	—	1.62	31.5	2.37
	DPNH ₂	—	1.71	—	2.51
	DPN	5	0.303	33.0	0.452
	DPNH ₂	5	0.176	—	0.262
	DPN	10	0.016	34.5	0.024
	DPNH ₂	10	0	—	0
Ammediol	DPNH ₂	—	2.08	17.0	2.51
	DPNH ₂	5	2.25	18.3	2.79

This compound decomposes spontaneously at pH 7.4 and 38°, but in the presence of triose phosphate dehydrogenase and arsenate a much more rapid breakdown occurs.

The conversion of 3-phosphoglycerate into 1:3-diphosphoglycerate, involving phosphoenolpyruvate, which occurs in the presence of DPN (or DPNH₂) and the muscle preparation, would also be catalysed by the enzymes of the glycolytic cycle in the presence of ADP or ATP. That the appropriate

glycolytic enzymes were present in the muscle preparation was demonstrated by the fact that there was a release of inorganic phosphate from 3-phosphoglycerate when DPNH₂ was replaced by either ADP or ATP (Table 5). The greater effectiveness of DPNH₂ can be explained by the requirement of triose phosphate dehydrogenase for either DPN or DPNH₂ in the arsenolytic reaction.

Reactions between phosphorylcreatine and 3-phosphoglycerate in the presence of oxidized and reduced diphosphopyridine nucleotide

Hydrolysis of phosphorylcreatine. A comparison of the amounts of inorganic phosphate formed from 3-phosphoglycerate and from 3-phosphoglycerate plus phosphorylcreatine (Table 6) shows that the increased amount of inorganic phosphate formed in the presence of phosphorylcreatine is equivalent to the amount of creatine released, indicating overall hydrolysis of phosphorylcreatine. In both cases the release of inorganic phosphate is markedly increased upon the addition of pyridine nucleotides and all the 3-phosphoglycerate disappears. In the absence of pyridine nucleotides there is also a loss of 3-phosphoglycerate, which is due to the formation of phosphoenolpyruvate and presumably 2-phosphoglycerate.

Fig. 1. Rate of release of inorganic phosphate (P_i) from 3-phosphoglycerate on the addition of oxidized and reduced diphosphopyridine nucleotide in the presence and absence of arsenate. Reaction mixtures contained: 3-phosphoglycerate, 5.5 μmoles; Mg²⁺ ion, 5 μmoles; ammediol buffer (pH 7.4), 200 μmoles; enzyme, 4 mg. of Fraction 1 in a total volume of 0.66 ml. Temp., 38°. Additions were: ○, DPNH₂ (1 μmole) and arsenate (1 μmole); △, DPN (1 μmole) and arsenate (1 μmole); ■, DPNH₂ (1 μmole); ●, DPN (1 μmole).

Table 5. *Effect of reduced diphosphopyridine nucleotide and adenine nucleotides on the release of inorganic phosphate from 3-phosphoglycerate*

Reaction mixtures contained: 3-phosphoglycerate, 8 μmoles; Mg²⁺ ion, 5 μmoles; ammediol buffer (pH 7.4), 100 μmoles; arsenate (pH 7.4), 1 μmole; nucleotide, 1 μmole; enzyme, 4 mg. of Fraction 1; volume, 0.56 ml. Tubes were incubated at 38°.

Nucleotide added	Inorganic phosphate released (μmole/tube)	
	15 min.	35 min.
None	0.05	0.067
DPNH ₂	0.272	0.381
ADP	0.128	0.239
ATP	0.127	0.159

Table 6. *Release of inorganic phosphate from 3-phosphoglycerate in the presence and absence of phosphorylcreatine*

Reaction mixtures contained: 3-phosphoglycerate, 4 μmoles; Mg²⁺ ion, 2.5 μmoles; ammediol buffer (pH 7.4), 100 μmoles; arsenate (pH 7.4), 0.5 μmole; enzyme, 2 mg. of Fraction 1. Additions were: DPN, 0.5 μmole; DPNH₂, 0.5 μmole; phosphorylcreatine, 2.5 μmoles. Volume, 0.4 ml.; temp., 38°; incubation time, 3 hr.

Additions	Phosphorylcreatine absent			Phosphorylcreatine present			
	Inorganic phosphate (μmoles)	Inorganic phosphate (μmoles)	3-Phosphoglycerate (μmoles)	Inorganic phosphate (μmoles)	Inorganic phosphate (μmoles)	Creatine (μmoles)	3-Phosphoglycerate (μmoles)
None	0.33	—	1.28	0.76	—	0	1.28
DPNH ₂	3.63	3.3	0	5.28	4.5	1.17	0
DPN	3.75	3.4	0	5.68	4.9	1.47	0

When 3-phosphoglycerate was replaced by pyruvate there was a release of creatine from phosphorylcreatine, but only in the presence of DPN or DPNH_2 (Table 7). The amount of creatine released in 20 min. was greater in the presence of DPNH_2 than in the presence of DPN. The addition of arsenate increased the rate of both reactions and this finding suggests that 1:3-diphosphoglycerate is formed.

The results indicate that either DPNH_2 and DPN may in some way be functioning as a source of ADP or ATP, or the pyridine nucleotides may be acting in a novel way as phosphoryl-group carriers. It is unlikely, on energy grounds, that the pyridine nucleotide phosphoryl carrier would be TPN. Moreover, although the addition of TPN resulted in a release of creatine in the presence of 3-phospho-

glycerate, the reaction rate was slower than with an equivalent amount of DPN.

Tests were carried out to determine if DPN and DPNH_2 underwent enzymic hydrolysis at pH 7.4. The results were negative, as judged by assay with both the cyanide and alcohol-dehydrogenase procedures, although the methods could not be relied upon to detect less than 3% of hydrolysis.

Table 7. *Effect of arsenate on the release of creatine from phosphorylcreatine in the presence of pyruvate*

Reaction mixtures contained: phosphorylcreatine, 2.5 μmoles ; Mg^{2+} ion, 5 μmoles ; ammediol buffer (pH 7.4), 100 μmoles ; sodium pyruvate, 10 μmoles ; enzyme, 4.5 mg. of Fraction 1 treated with Dowex 1 (Cl^-) (1 g./10 ml.). Additions were: arsenate (pH 7.4), 1 μmole ; DPN, 0.5 μmole ; DPNH_2 , 0.5 μmole . Total volume, 1 ml. Creatine was determined after 20 min. at 38°.

Additions	Creatine released ($\mu\text{mole/ml.}$)
None	0
DPN	0.055
DPN + arsenate	0.171
DPNH_2	0.176
DPNH_2 + arsenate	0.600

Fig. 2. Rate of release of creatine from phosphorylcreatine in the presence of diphosphopyridine nucleotide. Reaction mixture contained: ammediol buffer (pH 8.5), 100 μmoles ; Mg^{2+} ion, 5 μmoles ; phosphorylcreatine, 5 μmoles ; DPN (Sigma; purity, 95%), 2.5 μmoles ; enzyme, 8.3 mg. of Fraction 2. Volume, 1 ml., temp., 38°.

Table 8. *Stability of diphosphopyridine nucleotide under conditions leading to the diphosphopyridine nucleotide-dependent release of creatine from phosphorylcreatine*

Reaction mixtures contained: ammediol buffer (pH 8.5), 100 μmoles ; Mg^{2+} ion, 5 μmoles ; DPN, 2.5 μmoles . Additions were: phosphorylcreatine, 5 μmoles ; apyrase, 33 $\mu\text{g.}$; enzyme, 7 mg. of Fraction 2 (Expt. 1) or 8 mg. of Fraction 1 (Expt. 2). Volume, 1 ml.; temp., 38°; incubation time, 4 hr. (Expt. 1) or 2 hr. (Expt. 2). For the DPN assays 0.15 ml. samples were taken and the final volume in each case was 3.6 ml.

Additions	Creatine released ($\mu\text{moles/ml.}$)	DPN assay			
		Cyanide method ($E_{325 \text{ m}\mu}$)		Enzymic method ($E_{340 \text{ m}\mu}$)	
		With enzyme	Without enzyme (or boiled enzyme)	With enzyme	Without enzyme (or boiled enzyme)
Expt. 1					
None	—	0.578	0.583	0.592	0.602
Phosphorylcreatine	0.60	0.583	0.583	0.598	0.605
Expt. 2					
None	—	0.560	—	0.572	—
Phosphorylcreatine	0.48	0.554	—	0.570	—
Apyrase	—	0.554	—	0.564	—
Apyrase + phosphorylcreatine	5.0	0.560	—	0.565	—

Release of creatine from phosphorylcreatine in the presence of diphosphopyridine nucleotide

If the pyridine nucleotides were acting as phosphoryl-group carriers, creatine (or pyruvate) should be released when phosphorylcreatine (or phosphoenolpyruvate) is incubated in the presence of the muscle preparation with substrate amounts of DPN. Creatine was released under these conditions (Fig. 2), with no detectable release of inorganic phosphate or enzymic destruction of DPN. (DPN, rather than DPNH_2 , was used for these experiments as it was known to be free of contaminating adenine

di- and tri-phosphate.) The addition of apyrase, either in the presence or absence of phosphorylcreatine, did not bring about any detectable destruction of DPN (Table 8) but there was a quantitative release of creatine and inorganic phosphate from phosphorylcreatine that was dependent upon DPN. The amount of creatine released was affected by pH and DPN concentration (Table 9) but not by the Mg^{2+} -ion concentration.

These results are consistent with the formation of a phosphorylated derivative of DPN, which could be hydrolysed by apyrase, and efforts were directed towards the isolation of the compound.

Table 9. *Factors affecting the release of creatine from phosphorylcreatine in the presence of diphosphopyridine nucleotide*

Reaction mixtures (A) contained: Mg^{2+} ion, 5 μmoles ; phosphorylcreatine, 5 μmoles ; DPN, 2.5 μmoles ; enzyme, 14 mg. of Fraction 2; ammediol buffer (required pH), 100 μmoles . Volume, 1 ml.; temp., 38°. Creatine was determined at the end of a 3 hr. incubation period and the pH of duplicate tubes was checked after the same period. Results reported under B were obtained under the same conditions as described for A with the exception that ammediol buffer (pH 8.5) was used and the DPN concentration was varied.

A		B	
pH	Creatine released ($\mu\text{mole/ml.}$)	DPN ($\mu\text{moles/ml.}$)	Creatine released ($\mu\text{mole/ml.}$)
7.4	0.45	3.5	0.74
7.8	0.49	2.5	0.60
8.4	0.60	2.0	0.49
8.7	0.68	1.5	0.38
		1.0	0.29
		0.5	0.13
		0.25	0.10

The fractionation on Dowex 1 (formate) of the reaction mixture, after DPN and phosphorylcreatine were incubated with the muscle preparation at pH 8.5, was carried out as described in Table 10. The effluent from the Dowex 1 column was free of ribose and was identified spectrophotometrically and chromatographically as containing nicotinamide. Fraction 1 was identified chromatographically as containing DPN and treatment of this fraction with apyrase did not give rise to inorganic phosphate. Fraction 2 was free of acid-labile phosphate and was shown to contain adenine:ribose:phosphate in the ratio 1:2.02:1.8, indicating that the compound was ADP ribose. A comparison of the paper-chromatographic behaviour of this compound in solvent systems (a) and (b) with that of an authentic sample of ADP ribose showed that the two compounds were identical. The presence of ATP in Fraction 3 was shown by paper chromatography and enzymic analysis. Thus the amount of inorganic phosphate released by myosin was half that released by apyrase and by myosin plus myokinase. The product formed by the action of apyrase was AMP as judged by analysis with adenylic acid

Table 10. *Products formed as a result of the incubation of diphosphopyridine nucleotide with phosphorylcreatine*

Incubation mixture contained: DPN, 136 μmoles ; phosphorylcreatine, 250 μmoles ; ammediol buffer (pH 8.5), 4 m-moles; Mg^{2+} ion, 200 μmoles ; enzyme, 166 mg. of Fraction 2 in a volume of 40 ml. The mixture was incubated for 2 hr. at 38° and the release of creatine was 0.31 $\mu\text{mole/ml.}$ The reaction mixture was passed through a column of Nuchar C (3 g.), which was washed consecutively with 100 ml. of water, 60 ml. of 0.01 N-ethylene-diaminetetra-acetic acid (pH 7.0) and 100 ml. of water. Nucleotides were eluted with 250 ml. of isopentyl alcohol-water-ethanol (10:40:50). The organic solvent was removed under reduced pressure and the solution freeze-dried. The solids were redissolved in 50 ml. of water, adjusted to pH 8.5 by addition of N-NaOH and fractionated on Dowex 1 (formate) at 2°. The fractions were recovered from the eluting solution by adsorption on and elution from Nuchar C as described above.

Fraction	Eluting solution	Product identified	Recovery (% of total $E_{280\text{ m}\mu}$ material)
Effluent	Water	Nicotinamide	0.6
1	Formic acid (0.1 N)	DPN	81.0
2	Formic acid (4 N)	ADP ribose	10.5
3	Formic acid (4 N)-ammonium formate (0.4 N)	ATP	3.5

deaminase. Fraction 3 also contained, apart from ATP, some material which showed absorption at 260 μ m, but no attempt was made to isolate or identify this material.

The ATP could have arisen in three ways: by hydrolysis of a phosphorylated derivative of DPN during the fractionation procedure, or from contamination of the commercial DPN by compounds which can give rise to ATP under the experimental conditions or by non-enzymic hydrolysis of DPN to AMP with subsequent phosphorylation of the latter by phosphorylcreatine. To determine if ATP arose as a result of the breakdown of a phosphorylated derivative of DPN, incubation mixtures were analysed for ATP after incubation of DPN and phosphorylcreatine with the enzyme preparation for 2 hr. under the conditions described in Table 10. The high concentration of DPN precluded accurate analysis with the myosin-creatine phosphoryltransferase system, but accurate analysis could be obtained with adenylic acid deaminase and apyrase, as these enzymes are unaffected by the presence of DPN. The results showed that ATP or ADP was formed in amounts equivalent to about 10% of the added DPN. This assay also showed that AMP was formed in the absence of phosphorylcreatine and that the amount was equivalent to the ADP or ATP formed when phosphorylcreatine was present. If the muscle-enzyme preparation were omitted, there was no AMP formation, so the latter must have arisen enzymically during the incubation. The formation of AMP in the absence of phosphorylcreatine and of ATP in the presence of phosphorylcreatine was confirmed by chromatographic and enzymic analysis of the products isolated under the following conditions: at the end of the incubation period, the reaction mixture was heated for 1.5 min. in a boiling-water bath, cooled and the protein removed by centrifuging. To the supernatant was added 0.3 ml. of barium acetate (2M) and 20 ml. of absolute ethanol. After standing for 30 min., the precipitate of barium salts was collected by centri-

fuging and dried in a vacuum desiccator. The barium salts were converted into sodium salts by passage through a column of Zeo-Karb 225 (Na^+) as described above. (Assay of the supernatant solution with adenylic acid deaminase and apyrase showed that there was no increase in the total adenine nucleotides as a result of boiling the reaction mixture for 1.5 min.)

Since no enzymic hydrolysis of DPN could be detected in the reaction mixture it thus seemed that the AMP must have arisen from a contaminant of the DPN sample. Such a contaminant could be ADP ribose. The DPN used in these experiments had not been purified, for it had been shown not to be contaminated with ATP, ADP or AMP as judged by analysis with creatine phosphoryltransferase plus myosin and with adenylic acid deaminase. However, such tests would not give any indication of whether or not ADP ribose was present. When this compound was added the results were similar to those obtained in the presence of DPN (Table 11). The addition of DPNH_2 and TPN also gave essentially the same results. The yield of AMP in the absence of phosphorylcreatine and the yield of ATP and creatine in the presence of phosphorylcreatine were approximately the same on the addition of DPN, DPNH_2 and TPN, but were very much greater with an equivalent amount of ADP ribose. Under the same conditions, but with the amount of ADP ribose reduced to 0.3 μ mole, it was shown by chromatography that this compound completely disappeared. At the same time, 0.6 μ mole of creatine was released from phosphorylcreatine. (Because of the specificity of adenylic acid deaminase, the formation of AMP from TPN suggests that the phosphoryl group on the 3' position of the ribose moiety has been removed by hydrolysis.)

The above-mentioned results with ADP ribose could be accounted for by the reactions:

Table 11. *Formation of adenine nucleotides from pyridine nucleotides and adenosine diphosphate ribose*

Reaction mixtures contained: ammediol buffer (pH 8.5), 100 μ moles; Mg^{2+} ion, 5 μ moles; nucleotide, 2.5 μ moles; enzyme, 7.2 mg. of Fraction 2; where indicated, phosphorylcreatine, 5 μ moles. Volume, 1 ml.; temp., 38°; incubation time, 4 hr.

Nucleotide	Additions	AMP (μ mole/ml.)	ADP or ATP (μ mole/ml.)	Creatine (μ mole/ml.)
DPN (non-purified)	None	0.30	—	—
	Phosphorylcreatine	—	0.30	0.65
DPNH_2	None	0.31	—	—
	Phosphorylcreatine	(Trace)	0.28	0.68
TPN	None	0.348	—	—
	Phosphorylcreatine	—	0.33	0.68
ADP ribose	None	0.89	—	—
	Phosphorylcreatine	—	0.91	1.28

Release of creatine from phosphorylcreatine in the presence of purified diphosphopyridine nucleotide

The DPN sample used in the following experiments was purified by chromatography on Dowex 1 (formate). Analysis of the effluent indicated that it contained nicotinamide in an amount equivalent to 6% of the DPN. If it is assumed that nicotinamide arises as a result of the breakdown of DPN to ADP ribose, it may be concluded that the non-purified DPN is contaminated by 6% of ADP ribose. The purified DPN was also capable of bringing about the release of creatine from phosphorylcreatine at pH 7.4, but the amount of creatine released was less than that from an equivalent amount of the less pure commercial product (Table 12); the amount by which it was less was equivalent to twice the ADP ribose content of the less pure sample. It is clear then that some of the creatine released from phosphorylcreatine by the addition of commercial DPN is due to the presence of ADP ribose in the latter.

Table 12. *Release of creatine from phosphorylcreatine in the presence of purified and commercial diphosphopyridine nucleotide*

Reaction mixtures contained: ammediol buffer (pH 8.5), 100 μ moles; Mg^{2+} ion, 5 μ moles; phosphorylcreatine, 5 μ moles; enzyme, 7 mg. of Fraction 2; 2.5 μ moles of DPN. Total volume, 1.0 ml. Creatine release was determined after 4 hr. at 38°.

Nucleotide added	Creatine released (μ mole)
Sigma DPN (purity, 95%)*	0.74
Purified DPN	0.45

* 2.5 μ moles of this sample was shown to contain 0.15 μ mole of ADP ribose.

The activity of ADP ribose in bringing about the release of creatine from phosphorylcreatine suggested that the activity obtained with purified DPN may be due to the non-enzymic breakdown of the latter. Such a breakdown would not have been detected in previous assays, as comparisons were made with a standard DPN solution that had been incubated under the same conditions but in the absence of enzyme (cf. Table 8). Analysis of DPN at zero time and after incubation for 4 hr. (Table 13) showed that at pH 7.4 non-enzymic hydrolysis of DPN could not be detected. However, at pH 8.5 there was approximately 4–5% of hydrolysis. It would appear that the increased formation of AMP, ATP and creatine at pH 8.5 over that at pH 7.4 is due to the non-enzymic hydrolysis of DPN to ADP ribose. The lack of stoicheiometry at pH 7.4 between the amount of creatine released and the amount of ATP formed is presumably due to the limitations of the methods of assay.

The formation of AMP at pH 7.4 in the presence of the enzyme preparation, without detectable breakdown of DPN, could be accounted for by the relative insensitivity of the DPN assay, especially as analysis of the reaction mixture, by paper electrophoresis, after 4 hr. at pH 7.4 showed that nicotinamide (and therefore presumably ADP ribose) was formed from DPN. However, it is also possible that AMP may arise by enzymic hydrolysis at pH 7.4 of the α -isomer of DPN, which hydrolysis would not be detected by means of the cyanide and alcohol-dehydrogenase reactions. A check with purified *Neurospora* diphosphopyridine nucleotidase showed that the purified sample of DPN did contain 2.3% of the α -isomer and this could therefore make a contribution towards the amount of

Table 13. *Hydrolysis of diphosphopyridine nucleotide to adenosine 5'-phosphate and the conversion of adenosine 5'-phosphate into adenosine triphosphate in the presence of phosphorylcreatine*

Reaction mixtures contained: ammediol buffer, 100 μ moles; Mg^{2+} ion, 5 μ moles; DPN (purified), 2.5 μ moles; enzyme, 7.4 mg. of Fraction 2, which had been stirred at 2° with Nuchar C (50 mg./ml.); where indicated, phosphorylcreatine, 5 μ moles. Volume, 1.0 ml.; incubation time, 4 hr.; temp., 38°. For DPN assay 0.15 ml. samples were taken and the final volume was 3.6 ml.

Additions	DPN assay (enzymic method; $E_{340\text{ m}\mu}$)		Apparent DPN hydrolysis (μ mole/ml.)	Creatine release (μ mole/ml.)	AMP (μ mole/ml.)	ATP (μ mole/ml.)
	Zero	4 hr.				
pH 8.5						
None	0.710	0.680	0.106	0	0.246	0
Phosphorylcreatine	0.720	0.685	0.124	0.5	0	0.246
Boiled enzyme + phos- phorylcreatine	0.720	0.692	0.099	0	0	0
pH 7.4						
None	0.729	0.726	<0.02	0	0.068	0
Phosphorylcreatine	0.708	0.704	<0.02	0.247	0	0.076
Boiled enzyme + phos- phorylcreatine	0.710	0.713	<0.02	0	0	0

AMP formed. A third possibility is that even the purified DPN contains small amounts of ADP ribose. It has been found that whereas no ADP ribose could be detected by paper chromatography of freshly purified preparations of DPN, this compound could be detected after storage of the DPN either in solution or as a solid at 0° or -10°.

No detailed studies have been carried out with DPNH₂, but because this compound is more unstable than DPN it might be expected that non-purified samples would contain relatively larger amounts of ADP ribose. This could explain the greater activity of DPNH₂ over that of DPN in releasing creatine from phosphorylcreatine (Table 7) and inorganic phosphate from 1:3-diphosphoglycerate (Fig. 1). Chemical reduction of DPN may also give rise to ADP ribose as well as DPNH₂. If this were true, it would offer an explanation for the finding (Table 2) that purified DPN is active only after chemical reduction.

Release of creatine from phosphorylcreatine and formation of the hydroxamic acid derivative of 1:3-diphosphoglycerate in the presence of adenosine diphosphate ribose and adenosine 5'-phosphate

If the activity of the pyridine nucleotides is due to their non-enzymic conversion into, or contamination by, ADP ribose, which is then enzymically converted into AMP, it follows that similar results should be obtained when the pyridine nucleotides are replaced by AMP or ADP ribose. Indeed, this was found to be so. In the presence of 3-phosphoglycerate and the muscle enzyme, the addition of either of these compounds resulted in the formation of hydroxamic acids and, moreover, they were more effective than equivalent concentrations of DPN (Table 14). Higher concentrations of AMP inhibit the hydroxamic acid formation. The enzyme preparation was also capable of catalysing the overall reaction

The equilibrium of the reaction was completely to the right, as the amount of creatine released was twice that of the added AMP.

Synthesis of phosphorylcreatine from 1:3-diphosphoglycerate and 3-phosphoglycerate in the presence of oxidized or reduced diphosphopyridine nucleotide or adenosine 5'-phosphate

Enzyme preparations from rat and rabbit skeletal muscle were shown to synthesize phosphorylcreatine under the conditions described by Cori *et al.* (1958) (Table 15). It was also possible to confirm Cori's observation that the synthesis of phosphorylcreatine was not decreased by the addition of an ATP-utilizing system in the form of the glucose-

hexokinase system. It should be noted, however, that the reaction had ceased within 30 min. Kinetic studies were not attempted in view of the complexity of the system.

No tests have been made with 1:3-diphosphoglycerate but synthesis of phosphorylcreatine was achieved by the addition of either AMP or DPN to a system containing 3-phosphoglycerate and the muscle enzyme (Fig. 3).

Table 14. *Formation of hydroxamate from 3-phosphoglycerate in the presence of diphosphopyridine nucleotide, adenosine diphosphate ribose or adenosine 5'-phosphate*

Reaction mixtures contained: 3-phosphoglycerate, 8 μ moles; Mg²⁺ ion, 5 μ moles; ammonium buffer (pH 7.4), 100 μ moles; hydroxylamine (pH 7.0), 0.2 m-mole; enzyme, 7.4 mg. of Fraction 2, stirred for 15 min. at 2° with Nuchar C (50 mg./ml.); test nucleotide; volume, 1 ml. Tubes were incubated at 38° for 20 min. and hydroxamate formation was determined as in Table 4. The values have been corrected for 17% inhibition of the colour formation by the reaction mixture.

Nucleotide added	Concn. (mM)	Hydroxamate formation (μ moles/ml.)
DPN (purified)	2.5	0.474
	0.25	0.246
	0.10	0.043
ADP ribose	2.5	1.38
	0.25	0.474
	0.10	0.264
AMP	2.5	1.73
	0.25	3.32
	0.10	2.93
	0.05	2.57

Table 15. *Synthesis of phosphorylcreatine*

Reaction mixtures contained: phosphate buffer (pH 7.4), 25 μ moles; Mg²⁺ ion, 2.5 μ moles; cysteine (pH 7.4), 0.25 μ mole; pyruvate, 2.5 μ moles; fructose 1:6-diphosphate, 1.25 μ moles; creatine, 5 μ moles; lactic dehydrogenase, 0.125 mg.; aldolase, 0.25 mg.; D-glyceraldehyde 3-phosphate dehydrogenase, 0.125 mg.; rabbit-muscle enzyme, 3.75 mg. of Fraction 2. Additions were: DPN, 0.5 μ mole; glucose, 5 μ moles; hexokinase, 1 mg. All enzymes were stirred for 15 min. before use with Nuchar C (50 mg./ml.) and filtered. Volume, 0.55 ml.; temp., 38°.

Additions	Incubation time (min.)	Phosphorylcreatine synthesis (μ mole/tube)
DPN (Sigma)	90	0.48
	180	0.50
DPN (purified)	90	0.43
DPN (purified) + glucose	30	0.41
DPN (purified) + glucose + hexokinase	30	0.43

Fig. 3. Synthesis of phosphorylcreatine from creatine and 3-phosphoglycerate in the presence of adenosine 5'-phosphate or diphosphopyridine nucleotide. Reaction mixtures contained: phosphate buffer (pH 7.4), 50 μ moles; Mg^{2+} ion, 5 μ moles; creatine, 5 μ moles; 3-phosphoglycerate, 20 μ moles; AMP or DPN; enzyme, 7.4 mg. of Fraction 2 which had been stirred for 15 min. at 2° with Nuchar C (50 mg./ml.) and filtered. ▲, AMP, 0.25 μ mole; ●, AMP, 0.1 μ mole; □, DPN, 1.0 μ mole; ○, DPN, 0.25 μ mole; △, DPN, 0.1 μ mole. Volume, 1 ml.; temp., 38°.

DISCUSSION

The results reported in this paper clearly indicate that reaction (1) described by Cori *et al.* (1958) and catalysed by extracts of rabbit and rat skeletal muscle cannot be regarded as a direct phosphoryl-group transfer, catalysed by a single enzyme. The evidence for this is twofold. Fractionation of the extracts results in a loss of activity, which is restored by recombination of two or more fractions, and no hydroxamic acid derivative of 1:3-diphosphoglycerate is formed on incubation of 3-phosphoglycerate and phosphorylcreatine with hydroxylamine and the enzyme. The overall reaction does occur in both directions when either DPN or $DPNH_2$ is present.

Presumably it is because the pyridine nucleotides were added as an essential part of the test systems that Cori *et al.* (1958) were able to obtain these reactions. DPN (and probably $DPNH_2$) does not appear to function as an intact molecule, but rather by virtue of its non-enzymic hydrolysis to ADP ribose (or as a result of its contamination by this compound), which in turn is enzymically converted into AMP. The experimental results indicate that AMP is converted into ATP, presumably via ADP,

in the presence of either phosphoenolpyruvate or phosphorylcreatine. 1:3-Diphosphoglycerate may be involved directly in the conversion of AMP into ATP but, in any event, this compound would be converted non-enzymically into 3-phosphoglycerate, which would give rise to phosphoenolpyruvate. As ATP is formed and the appropriate glycolytic enzymes are present in the enzyme preparation, the synthesis of phosphorylcreatine from creatine and 1:3-diphosphoglycerate and the degradation of phosphorylcreatine in the presence of 3-phosphoglycerate can be accounted for by well-established reactions. Only catalytic amounts of adenine nucleotide need be formed because of the nature of the reactions.

It would seem that the failure of Cori *et al.* (1958) to obtain this overall reaction with heart-muscle preparations might well be ascribed to the absence of one or more of the enzymes required. Some preliminary experiments have shown that ATP is formed as a result of incubation of DPN (or $DPNH_2$) in the presence of phosphorylcreatine and partially purified preparations from rabbit- or pig-heart muscle, but no studies have been made of the overall reaction. It is also surprising that these authors failed to obtain phosphorylcreatine synthesis when DPN and 3-phosphoglycerate were incubated with a rat skeletal-muscle preparation.

The inability of Cori *et al.* (1958) to detect the presence of ADP and ATP in their incubation mixtures can be accounted for by the insensitivity of their assay procedure. The minimum amount of ADP (or ATP) which could be estimated by the method used was 0.005 μ mole. As the sample taken for estimation was equivalent to 0.3 ml. of the reaction mixture, then the presence of ADP (or ATP) at a concentration of 17 μ M would escape detection. Such a concentration of ADP was shown by these authors (Table 5 of their paper) to be effective in increasing the synthesis of phosphorylcreatine from creatine. It might also be mentioned that the values quoted by Cori *et al.* (1958) for the creatine-phosphoryltransferase activity of the muscle extracts are very low and in a number of experiments the values given for the amount of phosphorylcreatine synthesized in a 20 min. incubation period are taken to represent the rate of synthesis; they should, however, be regarded as the amount of phosphorylcreatine formed at equilibrium.

Strong evidence in favour of the non-participation of ADP and ATP was the finding by Cori *et al.* (1958) that whereas the synthesis of phosphorylcreatine from creatine and ATP was inhibited by the addition of glucose and hexokinase, that from creatine and 1:3-diphosphoglycerate was not. Because of the lack of kinetic studies, the interpretation of the results obtained is open to criticism.

There are no grounds for assuming that the affinity of hexokinase for ATP is greater than that of creatine phosphoryltransferase. Indeed, if the reverse were true then with high concentrations of creatine phosphoryltransferase (and very large amounts of this enzyme are present in extracts of skeletal muscle), no inhibition of the synthesis of phosphorylcreatine would be obtained. It would seem that the supposed inhibition of the creatine-phosphoryltransferase activity of the extracts could be explained as being due to the displacement of the equilibrium of the creatine-phosphoryltransferase reaction by the addition of glucose and hexokinase.

The conversion of AMP into ATP in the presence of either phosphoenolpyruvate or phosphorylcreatine implies that an enzyme(s) is present in the muscle extract which can convert AMP into ADP. Experiments with charcoal-treated preparations have shown that myokinase plus creatine phosphoryltransferase catalyse the overall reaction:

although either enzyme alone is without effect. In this connexion it is of interest that pyruvic kinase catalyses the conversion of AMP into ATP in the presence of phosphoenolpyruvate when the enzyme is contaminated with myokinase (Bücher & Pfeleiderer, 1955). Similar results were obtained by Chappell & Perry (1954) with creatine phosphoryltransferase, myokinase and phosphorylcreatine and confirmation of both these reactions has been reported by Molnar & Lorand (1960).

The hydrolysis of ADP ribose by the muscle preparation, and its failure to hydrolyse either DPN or DPNH₂, suggests that there may well be an enzyme which specifically hydrolyses ADP ribose. Jacobson & Kaplan (1957) have shown that extracts of pigeon liver contain a pyrophosphatase which hydrolyses ADP ribose but this enzyme also hydrolyses DPNH₂ although not DPN. A specific ADP ribose pyrophosphatase would provide a means of determining the contamination of both DPN and DPNH₂ samples by small amounts of ADP ribose.

SUMMARY

1. The reaction

does not involve a direct phosphoryl-group transfer catalysed by a single enzyme.

2. The overall reaction in both directions is catalysed by preparations from rat and rabbit skeletal muscle in the presence of either oxidized or reduced diphosphopyridine nucleotide.

3. The pyridine nucleotides function by virtue of their non-enzymic conversion into, or as a result

of their contamination by, adenosine diphosphate ribose, which undergoes enzymic hydrolysis to adenosine 5'-phosphate.

4. Skeletal-muscle preparations, treated with charcoal to remove adenine di- and tri-phosphate, catalyse the conversion of highly purified samples of adenosine 5'-phosphate into adenosine triphosphate in the presence of either N-phosphorylcreatine or phosphoenolpyruvate. Adenosine triphosphate is also formed when adenosine 5'-phosphate and N-phosphorylcreatine are incubated with myokinase and creatine phosphoryltransferase.

5. Rabbit skeletal muscle contains an enzyme which hydrolyses adenosine diphosphate ribose to adenosine 5'-phosphate, but which does not hydrolyse either oxidized or reduced diphosphopyridine nucleotide.

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Tricarboxylic Acid-Cycle Activity in *Streptomyces olivaceus*

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The tricarboxylic acid cycle occurs in mammals, plants and micro-organisms, although there is evidence indicating the occurrence of alternative pathways for terminal respiration (Krebs, 1954; Seaman & Naschke, 1955; Katagiri & Tochikura, 1958; Kornberg & Sadler, 1960). Amongst the genus *Streptomyces* this cycle appears to operate in *S. coelicolor* (Cochrane & Peck, 1953), *S. griseus* (Gilmour, Butterworth, Noble & Wang, 1955) and *S. nitificans* (Schatz, Mohan & Trelawny, 1955). Maitra & Roy (1959a) have shown that *S. olivaceus* utilizes both the pentose phosphate pathway and the glycolytic route for the catabolism of glucose to the stage of pyruvic acid. The present paper is concerned with the metabolism of pyruvic acid through the tricarboxylic acid cycle and related processes.

MATERIALS AND METHODS

Chemicals. Diphosphopyridine nucleotide (DPN⁺), triphosphopyridine nucleotide (TPN⁺), reduced diphosphopyridine nucleotide (DPNH), reduced triphosphopyridine

nucleotide (TPNH), flavin mononucleotide (FMN), flavin-adenine dinucleotide (FAD), horse-heart cytochrome *c* (mostly oxidized), silver-barium salt of phosphoenolpyruvic acid, sodium glyoxylate, phenazine methosulphate and crystalline bovine albumin were products of Sigma Chemical Co., St Louis, Mo., U.S.A.; reduced glutathione (GSH) and the disodium salt of adenosine triphosphate (ATP) were obtained from Schwarz Laboratories Inc., New York; DL- α -lipoic acid, *cis*-aconitic acid and DL-(+)-*allo*-isocitric acid from California Corporation for Biochemical Research, Calif., U.S.A.; L₃-(+)-isocitric acid was a generous gift from Dr H. A. Lardy; except where otherwise stated, the commercial variety of isocitric acid was used. Thiamine pyrophosphate was a gift from F. Hoffmann-La Roche and Co. Ltd., Basle, and antimycin A from Kyowa Fermentation Ind. Co., Tokyo. α -Oxoglutaric acid was a product of Fluka A.-G., West Germany, and oxaloacetic acid was obtained from Nutritional Biochemicals Corp., Ohio, U.S.A. Coenzyme A (CoA) was obtained from Pabst Laboratories, Milwaukee, U.S.A., and sodium fluoracetate from L. Light and Co. Ltd. All ¹⁴C-labelled compounds were from The Radiochemical Centre, Amersham, Bucks. Reduced cytochrome *c* was prepared either by reduction with a minimal amount of sodium dithionite and removal of the excess of reductant