2. Comparisons with synthetic 3-hydroxy- and 4-hydroxy-pipecolic acids indicated that the isolated imino acid may not be 4-hydroxypipecolic acid, but 3-hydroxypipecolic acid. Strictly identical behaviour of natural and synthetic 3-hydroxypipecolic acid could not be demonstrated since the two substances differed in stereoisomeric composition.

3. 3-Hydroxy- and 4-hydroxy-pipecolic acids have been synthesized.

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Enzymic Phosphorylation of Creatine by 1:3-Diphosphoglyceric Acid

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Anaerobic glycolytic processes lead to the formation of phosphocreatine in skeletal muscle (Nachmansohn, 1928; Lundsgaard, 1931). High-energy phosphate compounds (1:3-diphosphoglyceric acid and phosphoenolpyruvic acid) are formed during glycolysis and phosphate is transferred from these substances to creatine, through the mediation of the adenylic nucleotides (Needham & van Heyningen, 1935; Needham & Pillai, 1937; Meyerhof, Schulz & Schuster, 1937; Innes, 1937) and creatine phosphokinase (Lohmann, 1934).

Needham & van Heyningen (1935) obtained direct evidence for the absolute requirement of adenylic nucleotides in the synthesis of phosphocreatine from phosphoenolpyruvate. However, when 1:3-diphosphoglyceric acid is the phosphate source, this requirement for adenosine diphosphate has only been assumed.

With the existing evidence, it could be assumed that phosphocreatine synthesis occurs in two ways: (a) as a two-stage reaction (reactions 1 and 2) connected by the adenylic system, or (b) as a single-

stage reaction (reaction 3) not requiring adenine nucleotides.

The apparent equilibrium constants of reactions (1) and (2) (Ogston & Smithies, 1948) are 5.1×10^3 and 2.3×10^{-3} . The equilibrium constant, either for the sum of both reactions or for reaction (3), may be calculated to be about 12, thus favouring the synthesis of phosphocreatine.

It is noteworthy that although myocardial tissue possesses a more efficient aerobic mechanism for the synthesis of adenosine triphosphate than does skeletal muscle, the molar concentrations of phosphocreatine and adenosine triphosphate in heart

muscle are approximately equal (Mommaerts, 1950), whereas in skeletal muscle the ratio is 4:1.

The transfer of phosphate from phosphocreatine to glucose or glycerol has been shown to occur without the mediation of the adenylic system (Meyerhof & Green, 1950; Morton, 1953). Break-down of phosphocreatine in dialysed brain extracts has been reported to occur without the addition of adenylic compounds (Tseitlin, 1953). Since this evidence suggests that phosphate transfer may occur without the addition of the adenylic system, the authors thought that phosphocreatine synthesis may also be possible under similar conditions. The lability of one of the substrates (1:3-diphosphoglyceric acid) and the possibility that reaction (3) may be only the sum of reactions (1) and (2) render the direct measurement of reaction (3) difficult. This report gives indirect evidence suggesting that (3) occurs. This work has been the subject of a preliminary communication (Cori, Abarca, Frenkel & Traverso-Cori, 1956).

EXPERIMENTAL

Special chemicals. 3-Phosphoglyceric acid was obtained from Schwarz Laboratories Inc., Mount Vernon, N.Y., U.S.A. All other substrates and cofactors were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. Fructose 1:6-diphosphate was purified as the strychnine salt (Sable, 1952) and treated with charcoal (see below) to remove any traces of nucleotides. Diphosphopyridine nucleotide (DPN) was further purified by ion-exchange chromatography (Kornberg, 1955). Brockmann's dry aluminium oxide (Williams, 1947) was obtained from E. Merck, Darmstadt, Germany. The charcoal used was Nuchar C-190, unground (West Virginia Pulp and Paper Co., Covington, Virginia, U.S.A.).

1:3-Diphosphoglyceric acid was prepared according to Negelein & Brömel (1939), but fructose 1:6-diphosphate, muscle aldolase and lactic dehydrogenase were used instead of the substrates and enzymes recommended in their method. The final product was also treated with charcoal. Acetyl phosphate was prepared according to Lipmann & Tuttle (1944). D-Phosphoglyceraldehyde-3-phosphate (Ballou & Fischer, 1955) was a gift from Dr Clinton E. Ballou.

Enzymic preparations from heart and skeletal muscle. Rats of either sex, weighing 200–250 g., were stunned and decapitated. The muscles of back and legs were excised and chilled. The hearts of 10–12 rats were pooled and chilled. The muscles were homogenized in a Waring Blendor at full speed with cold 0.01 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) hydrochloride buffer, pH 8, containing 0.01 M-KCl. A volume of 300 ml./100 g. of tissue was used, and extraction lasted 2 min. at 0°. For the hearts, homogenization was performed in a smaller cup (E. Machlett and Son, New York, U.S.A.) during 5 min. at 0°. The brei was strained through gauze and the extract clarified by centrifuging for 10–15 min. at 25 000 g at 0°. The sediment was discarded.

The proteins were precipitated by addition of solid

ammonium sulphate to 90% of saturation (63 g./100 ml. of extract) at 0°, and centrifuged for 10 min. at 25 000 g at 0° and redissolved in one-third of the original volume of the tris buffer-KCl mixture, pH 8.

Ammonium sulphate fractionation was carried out at 0° by addition of the solid salt whilst maintaining the pH at 8 with 2N-KOH. The precipitates were separated by centrifuging at 0° at 25 000 g for 10 min., and dissolved in a convenient volume of the tris buffer-KCl mixture, pH 8, and dialysed overnight against 50–100 vol. of the same solution at 4°. The enzyme solutions were heated for 4 hr. at 37° and clarified by centrifuging for 10 min. at 25 000 g at 0°. The protein concentration ranged from 7 to 60 mg./ml. In the description of results, the enzyme fractions will be defined by the lower and upper limits of ammonium sulphate (in percentage of saturation at 0°) used for precipitation.

In order to remove nucleotides (Taylor, Velick, Cori, Cori & Slein, 1948) the fractions were slowly stirred for 10 min. at 0° with 0.1–0.2 g. (dry wt.) of wet charcoal/ml. of solution and filtered through sintered glass. The charcoal had been washed previously with N-HCl and then with water until the washings did not produce turbidity in 0.1N-silver nitrate. Charcoal treatment affected the light absorption of the protein solution at 280 and 260 m μ in such a way that the ratio between the two optical densities tended towards or reached 1.75. This ratio is considered by Warburg & Christian (1941) to correspond to zero content of nucleic acids. The concentration of protein was decreased, but the enzymic activity/mg. of protein was not affected. Charcoal treatment reduces the stability of the enzyme preparations, which otherwise may be stored for several weeks at –25°.

Enzyme preparations for 1:3-diphosphoglyceric acid synthesis and for analytical purposes. Crystalline aldolase (Taylor, Green & Cori, 1948), crystalline triose phosphate dehydrogenase (Cori, Slein & Cori, 1948), lactic dehydrogenase (Kornberg & Pricer, 1951) and adenosine triphosphatase (Mommaerts & Parish, 1951) were prepared from rabbit skeletal muscle.

For some assay purposes it was found convenient to replace the crystalline muscle enzymes by a yeast preparation. This was a dialysed extract from the proteins precipitating at 35% (v/v) acetone concentration from a Lebedev juice (Warburg & Christian, 1939). Crystalline triose phosphate dehydrogenase (Krebs, 1955) and alcohol dehydrogenase (Racker, 1955) were obtained from yeast. Crude hexokinase (Sigma Chemical Co.) was purified by adsorption on alumina gel and ethanol fractionation (Berger, Slein & Cori, 1946). All these enzymes were also treated with charcoal as described above, in order to remove nucleotides. Glucose 6-phosphate dehydrogenase was obtained from baker's yeast (Negelein & Gerischer, 1936).

Incubation procedure. In the system testing formation of phosphocreatine from 1:3-diphosphoglyceric acid, the latter compound was generated *in situ* by means of a system composed of fructose 1:6-diphosphate, aldolase, triose phosphate dehydrogenase, DPN, inorganic phosphate, Mg²⁺ ions and cysteine. Creatine was the phosphate acceptor, pyruvate and lactic dehydrogenase were used to maintain DPN in the oxidized state. For some assays it was found more convenient to use yeast enzymes, which do not contain creatine phosphokinase, instead of muscle enzymes.

In these cases acetaldehyde was used as hydrogen acceptor instead of pyruvate.

The synthesis of phosphocreatine by enzyme preparations from skeletal muscle when added to the 1:3-diphosphoglyceric acid-generating system in the absence of added adenine nucleotides will be henceforth called 'direct' enzyme activity. This term will be used as opposed to creatine phosphokinase activity, which was assayed in the same manner but with fructose 1:6-diphosphate and the enzymes generating 1:3-diphosphoglyceric acid replaced by adenosine triphosphate (ATP). Incubation was carried out in open centrifuge tubes at 37° for 20 min. and the reaction was stopped with an equal volume of chilled 10% (w/v) trichloroacetic acid. The tubes were centrifuged for 10 min. at 0° and 3500 g and a measured portion of the supernatant was used for the different analytical procedures. A zero-time control was always provided, and results are always referred to it.

Phosphocreatine estimation. The procedure followed was modified from the procedure of Fiske & Subbarow (1929). A measured portion of the trichloroacetic acid supernatant (usually 1 ml., containing 0.2–1.5 μ moles of phosphocreatine and 12.5 μ moles of orthophosphate) was neutralized with N-KOH and phenolphthalein; 0.2 ml. of Fiske and Subbarow's calcium solution was added, and left for 10 min. at room temperature and centrifuged for 1–2 min. at 1500 g. The supernatant was poured into another centrifuge tube and the precipitate washed once with 2–3 ml. of water. To the combined supernatants and washing water, 0.2 ml. of calcium solution and 0.3 ml. of ethanol were added. After 10 min. at room temperature any precipitate was centrifuged off, the supernatant was poured into a 10 ml. volumetric flask and water to a volume of approximately 8 ml. was added. After addition of 1 ml. of 2.5% ammonium molybdate in 5N-H₂SO₄, the flask was left for 30 min. at room temperature (Kuby, Noda & Lardy, 1954). A volume (0.4 ml.) of reducing reagent (Fiske & Subbarow, 1925) was added and the volume of the flask completed. Colour was read after 10 min. at room temperature.

The main reason for the second addition of calcium was to reduce the high zero-time values which were obtained with the original procedure. This method was found to precipi-

tate also fructose 1:6-phosphate, adenosine diphosphate (ADP) and ATP. Since phosphocreatine might also coprecipitate, it was found advisable to check the reproducibility and accuracy of the method as follows:

Portions (1 ml.) of a trichloroacetic acid supernatant from an enzymic experiment (as described in Table 3 under 'complete system'), and containing 12.5 μ moles of orthophosphate and 1.25 μ moles of fructose 1:6-diphosphate, were carried through the analytical procedure described. Table 1A shows the results obtained in five identical samples. Known amounts of synthetic phosphocreatine, checked by the same analytical procedure, were added to another group of 1 ml. samples of the same supernatant. Table 1B shows that the amount of phosphocreatine determined in these mixtures corresponds to the amount of the synthetic compound added plus the average of phosphocreatine present in 1 ml. of supernatant.

Other analytical procedures. Total phosphorus, ADP and ATP were determined as described by Umbreit, Burris & Stauffer (1951), inorganic phosphorus according to Fiske & Subbarow (1925). Pentose was determined according to Meijbaum (1939). Acetyl phosphate was determined according to Lipmann & Tuttle (1945). The method of Warburg & Christian (1941) was used for protein determination. Phosphoglyceric kinase and enolase activity were determined spectrophotometrically (Bücher, 1955; Warburg & Christian, 1941). Activity of the different enzymes described above ('Enzyme preparations') was tested as described in the original methods cited.

RESULTS

Enzyme preparations obtained from rat skeletal muscle synthesize phosphocreatine by two different routes: (a) Creatine phosphokinase, with ATP, and (b) directly from 1:3-diphosphoglyceric acid in the absence of added ADP. The latter activity is referred to as 'direct' enzyme activity. No traces of ADP or ATP could be detected in the components of this system by organic phosphate or pentose determinations.

Enzymes from rat heart do not possess this 'direct' enzymic activity, although creatine phosphokinase may be found in comparable or larger amounts than in skeletal muscle (Table 2).

Requirements for the synthesis of phosphocreatine from 1:3-diphosphoglyceric acid. Omission of single components of the reconstituted system resulted in a marked decrease of phosphocreatine synthesis (Table 3). Maximum synthesis is obtained with Mg²⁺ ion concentrations of the order of mM (Table 4).

Addition of 0.5 μ M-ADP has no effect on the synthesis of phosphocreatine by the 'direct' system (Table 5); concentrations from μ M to 0.25 mM-ADP enhance the synthesis; higher concentrations are inhibitory.

Tests for the presence of adenosine diphosphate in the enzyme preparations. Since no organic phosphate or pentose could be detected in the enzyme preparations by chemical analysis, and since this

Table 1. *Determination of phosphocreatine phosphorus in trichloroacetic acid supernatants*

A: Determination of phosphocreatine phosphorus in five samples of the same trichloroacetic acid supernatant. B: Recovery of added synthetic phosphocreatine to a further five identical samples of the same trichloroacetic acid supernatant.

Addition		Amount of addition (μ moles)	Phosphocreatine found (μ moles)
A	None	—	0.76
			0.77
			0.77
			0.79
			0.80
	Average		0.77
B	Phosphocreatine	0.17	0.98
		0.34	1.11
		0.68	1.49
		1.02	1.80
		1.32	2.14

Table 2. *Difference between enzymes obtained from rat-heart and skeletal muscle*

All tubes contained, in a volume of 2 ml.: potassium phosphate buffer, pH 7.4 (50 μ moles of phosphate); $MgCl_2$, 10 μ moles; DPN, 0.5 μ mole; cysteine, 1 μ mole; potassium pyruvate, 10 μ moles; creatine, 100 μ moles. In addition, the tubes where creatine phosphokinase activity was measured contained 20 μ moles of ATP; the tubes where 'direct' activity was tested contained 5 μ moles of fructose diphosphate, 0.2 mg. of lactic dehydrogenase, 1 mg. of crystalline aldolase and 1 mg. of crystalline triose phosphate dehydrogenase. The enzymes from heart or skeletal muscle were assayed at levels between 0.6 and 8.0 mg. of protein/tube. The preparations are characterized by the limits of ammonium sulphate saturation used to precipitate them. Incubation was for 20 min. at 37°.

Enzyme preparation	Phosphocreatine formed (μ moles/mg. of protein)	
	Creatine phosphokinase	'Direct' activity
Heart muscle, 0-90 %	1.4	0
Heart muscle, 0-90 %	1.1	0
Heart muscle, 0-90 %	4.0	0
Skeletal muscle, 0-90 %	1.1	1.8
Skeletal muscle, 0-65 %	0.4	1.1
Skeletal muscle, 0-80 %	0.9	2.6

Table 3. *Requirements for the synthesis of phosphocreatine by the 'direct' system*

The complete tube contained, in a volume of 2 ml.: tris-HCl buffer (50 μ moles of tris), pH 7.4; potassium phosphate buffer, pH 7.4 (50 μ moles of phosphate); $MgCl_2$, 10 μ moles; DPN, 0.5 μ mole; cysteine, 1 μ mole; potassium pyruvate, 10 μ moles; creatine, 100 μ moles; fructose 1:6-diphosphate, 5 μ moles; lactic dehydrogenase, 0.2 mg.; aldolase, 1 mg.; triose phosphate dehydrogenase, 1 mg. Enzyme from skeletal muscle (fraction 40-90 %): 2.7 mg. Incubation was for 20 min. at 37°.

	Phosphocreatine (μ moles)
Complete system	2.6
Phosphate omitted	0.81
Magnesium omitted	0.39
Creatine omitted	0
DPN omitted	0.09
Fructose diphosphate omitted	0
Enzyme omitted	0.1

method could not rule out the presence of ADP in DPN or in fructose 1:6-diphosphate, experiments were designed to detect catalytic amounts of ADP. Both the enzyme from skeletal muscle and the enzymes used to generate 1:3-diphosphoglyceric acid contain from 1.4 to 4.2 units of phosphoglyceric kinase/mg. of protein. If any ADP should be present, this enzyme would phosphorylate it to ATP. By addition of glucose and hexokinase, the inorganic phosphate esterified in the oxidation of phosphoglyceraldehyde could be trapped. Table 6

shows that added ADP is essential if glucose is to be the phosphate acceptor.

Another test for the presence of minute amounts of ADP utilized hexokinase or myosin. Should the synthesis of phosphocreatine in the 'direct' system depend on the formation of ATP, then a reaction utilizing ATP such as the glucose-hexokinase system would inhibit the synthesis of phosphocreatine. Table 7 shows that the 'direct' system is not inhibited by glucose and hexokinase. Addition of 0.2 mM-ADP approximately doubled the formation of creatine phosphate, presumably by allowing creatine phosphokinase to function. The further addition of hexokinase and glucose reduced the phosphocreatine formation to about that found without the addition of ADP. Phosphocreatine synthesis in the presence of ATP is also decreased by hexokinase and glucose.

Similar results were obtained with myosin adenosine triphosphatase (Mommaerts & Parish, 1951), which did not affect the 'direct' activity but

Table 4. *Requirements of magnesium for phosphocreatine synthesis by the 'direct' enzyme*

All tubes contain, in a volume of 2 ml.: potassium phosphate buffer, pH 7.4 (50 μ moles of phosphate); DPN, 0.5 μ mole; cysteine, 1 μ mole; potassium pyruvate, 10 μ moles; creatine, 100 μ moles; fructose 1:6-diphosphate, 10 μ moles; lactic dehydrogenase, 0.2 mg.; aldolase, 1 mg.; triose phosphate dehydrogenase, 1 mg. Enzyme from skeletal muscle (fraction 40-90 %): 5.4 mg. Incubation was for 20 min. at 37°.

Concn. of Mg^{2+} ions added (mM)	Phosphocreatine formed (μ moles/tube)
0	1.25
0.25	4.4
0.50	5.5
1	6.3
2	6.3
5	6.6
10	6.6

Table 5. *Effect of different concentrations of adenosine diphosphate on the synthesis of phosphocreatine*

Conditions were as given in Table 2, but 2.1 mg. of a different preparation, fraction 40-90 % (skeletal muscle), was used.

$10^5 \times$ final concn. of ADP (M)	Phosphocreatine formed (μ moles/tube)
0	3.85
0.05	3.85
0.10	4.05
1	4.8
25	8.4
125	1.6
250	0.9
500	0.7

Table 6. *Requirements of adenosine diphosphate for the esterification of phosphate with glucose as acceptor*

All tubes contained, in a total volume of 2 ml.: tris-HCl buffer, pH 7.4, 50 μ moles; potassium phosphate buffer, pH 7.4 (8 μ moles of phosphate); $MgCl_2$, 10 μ moles; DPN, 0.5 μ mole; cysteine, 1 μ mole; potassium pyruvate, 10 μ moles; fructose 1:6-diphosphate, 5 μ moles. Triose phosphate dehydrogenase, 2 mg.; aldolase, 2 mg.; lactic dehydrogenase, 0.4 mg. Enzyme from skeletal muscle (fraction 40–90%): 2.7 mg. In column A, all tubes contained, in addition, 1.2 mg. of hexokinase and 20 μ moles of glucose; in column B they contained 100 μ moles of creatine. Incubation was for 20 min. at 37°.

$10^5 \times$ final concn. of ADP (M)	A Change of inorganic phosphate (μ moles)	B Appearance of phosphocreatine (μ moles)
0	0	2.6
1	-1.6	—
5	-1.9	—
10	-2.8	3.0
20	-3.9	—
50	-6.9	—

Table 7. *Effect of glucose and hexokinase on the synthesis of phosphocreatine*

All components were as described in Table 2; enzyme from skeletal muscle: 7.8 mg. of a fraction 40–90%; where indicated, 40 μ moles of glucose and 1 mg. of hexokinase were added. The results are expressed in μ moles of phosphocreatine formed/tube. Incubation was for 20 min. at 37°.

	'Direct' enzyme		Creatine phospho- kinase
	Without ADP	0.2 mm- ADP	
Without hexokinase	3.2	8.3	1.7
	3.2	6.3	—
Hexokinase and glucose added	3.2	4.5	—
	3.3	3.2	0.3

Table 8. *Synthesis of phosphocreatine with synthetic D-glyceraldehyde 3-phosphate*

All tubes contained, in a volume of 2 ml.: potassium phosphate buffer, pH 7.4 (50 μ moles of phosphate); $MgCl_2$, 10 μ moles; DPN, 0.5 μ mole; cysteine, 1 μ mole; acetaldehyde, 10 μ moles; creatine, 100 μ moles; D-glyceraldehyde 3-phosphate, 5 μ moles; triose phosphate dehydrogenase from yeast, 0.8 mg.; alcohol dehydrogenase from yeast, 0.6 mg. Enzyme from skeletal muscle (fraction 30–60%): 2.6 mg. Tube 3 contains, in addition, 0.5 μ mole of ADP. A volume (2 ml.) of cold trichloroacetic acid was added to tube 1 before the addition of the enzymes (zero time). Incubation was for 20 min. at 37°.

Tube no.	Phosphocreatine present (μ moles)
1	0
2	1.7
3	2.2

Fig. 1. Spectrophotometric assay of ADP. Optical density was measured at 340 $m\mu$ in a 1 cm. light path at 37°. The cuvettes contained, in a total volume of 3 ml.: tris-HCl buffer, pH 7.4 (300 μ moles of tris); $MgCl_2$, 10 μ moles; glucose, 30 μ moles; TPN, 0.5 μ mole; phosphocreatine, 12 μ moles (last addition); glucose 6-phosphate dehydrogenase, 2 mg.; hexokinase, 3 mg.; creatine phosphokinase (preparation from skeletal muscle; fraction 60–80% as in Table 15), 2.7 mg. Filtrate from the different tubes, as indicated in Table 8: 0.6 ml. At the point indicated by the arrow, 0.5 μ mole of ADP was added to cuvettes 1, 2 and 3. Temperature 37°. Numbers 1–3 correspond to those of the tubes in Table 8; numbers 4 and 5 contain no filtrate, but 0.6 ml. of water instead; 0.005 and 0.012 μ mole respectively of ADP was added at zero time to test the sensitivity of the method.

depressed creatine phosphokinase activity by 40%.

A still more critical test for ADP was performed with synthetic D-phosphoglyceraldehyde 3-phosphate as substrate and crystalline triose phosphate dehydrogenase and alcohol dehydrogenase from yeast. Table 8 shows that phosphocreatine is formed in this system in the absence of added ADP. Measured portions (3 ml.) of the trichloroacetic acid filtrates from this experiment were shaken five times with 15–20 ml. of ether to remove this acid. The aqueous phase was tested indirectly for ADP by following the reduction of triphosphopyridine nucleotide (TPN) spectrophotometrically at 340 $m\mu$. The assay procedure is based on the reduction of TPN by glucose 6-phosphate and its dehydrogenase. Glucose is phosphorylated with hexokinase, phosphocreatine and creatine phosphokinase only if ADP is present to catalyse phosphate transfer from phosphocreatine to glucose. As shown in Fig. 1, reduction proceeds only if the

Table 9. Requirement of adenosine diphosphate for the synthesis of phosphocreatine from 3-phosphoglyceric acid

The complete system contained, in a total volume of 2 ml.: potassium phosphate buffer, pH 7.4 (50 μ moles of phosphate); $MgCl_2$, 10 μ moles; creatine, 60 μ moles; potassium 3-phosphoglycerate, 40 μ moles; lactic dehydrogenase, 0.2 mg.; aldolase, 1 mg.; triose phosphate dehydrogenase, 1 mg. Enzyme from skeletal muscle (fraction 40–90%): 2.1 mg. Incubation was for 20 min. at 37°.

Addition	Amount of addition (μ mole)	Phosphocreatine formed (μ moles)
None	0	0
DPN	0.5	0
ADP	0.2	6.8
ADP	0.5	10.0

Table 10. Synthesis of phosphocreatine from 1:3-diphosphoglyceric acid and acetyl phosphate

The complete system contained, in a volume of 2 ml.: 1:3-diphosphoglyceric acid, 1.5 μ moles; $MgCl_2$, 10 μ moles; creatine, 20 μ moles in group A, 5 μ moles in group B. Enzyme from skeletal muscle (fraction 45–65%): 7 mg. The pH of the mixture was 7.2. Treatment with calcium for the precipitation of orthophosphate was performed at 37° to insure the hydrolysis of 1:3-diphosphoglyceric acid or acetyl phosphate. Incubation was for 10 min. at 37°.

	Phosphocreatine formed (μ moles)
A	
Complete system	1.5
Magnesium omitted	0.6
Creatine omitted	0
1:3-Diphosphoglyceric acid omitted	0
Enzyme omitted	0
1:3-Diphosphoglyceric acid replaced by 7 μ moles of acetyl phosphate	0
B	
Complete system	0.45
Complete plus 1 mg. of hexokinase and 30 μ moles of glucose	0.45
Complete plus 0.05 μ mole of ADP	0.83
1:3-Diphosphoglyceric acid replaced by 1.5 μ moles of ATP	0.07

amount of ADP present is of the order of 0.0017 μ -mole/ml. or more. The filtrates do not promote the reaction until ADP is added.

Nature of the possible precursor of phosphocreatine in the 'direct' reaction. The possibility that phosphoenolpyruvate is an intermediate in the 'direct' system was investigated. Preparations from skeletal muscle formed phosphoenolpyruvate, detectable by its absorption at 240 m μ , when 3-phosphoglyceric acid was used as substrate. However, the same preparations were unable to form phosphocreatine under the same conditions (Table 9). Addition of ADP elicited the synthesis of

phosphocreatine, which proved the presence of pyruvate kinase. DPN, aldolase, triose phosphate dehydrogenase and lactic dehydrogenase were added only to test them for ADP with this method.

On the other hand, phosphocreatine is synthesized from added 1:3-diphosphoglyceric acid in a mole to mole proportion (Table 10A). Hexokinase does not affect this reaction; ADP enhances it. Acetyl phosphate cannot act as phosphate donor, as shown in Table 10A. In this experiment approximately 40% of the initial acetyl phosphate was lost at the end of the incubation period, thus pointing towards the existence of acetylphosphatase in our enzyme preparations.

Formation of 1:3-diphosphoglyceric acid from phosphocreatine. Reversal of reaction (3) has been demonstrated by coupling it to the following reaction scheme:

Net change: phosphocreatine = creatine + phosphate

Triose phosphate dehydrogenase has to be added to catalyse reactions (5) and (6); the preparation from skeletal muscle catalyzes reaction (4). Table 11 shows the results of this arsenolysis.

Differences between creatine phosphokinase and the 'direct' system. All enzyme preparations from skeletal muscle contain creatine phosphokinase. Although the latter enzyme without any 'direct' activity has been prepared, it has not been possible to obtain the 'direct' enzyme system free from the

Table 11. Arsenolysis of phosphocreatine in the presence of 3-phosphoglyceric acid

The complete system contained, in a final volume of 2 ml.: tris-HCl buffer, pH 7.4 (20 μ moles of tris); potassium 3-phosphoglycerate, 40 μ moles; phosphocreatine, 4 μ moles; $MgCl_2$, 10 μ moles; reduced DPN, 0.2 μ mole; NaH_2AsO_4 , 2 μ moles; crystalline triose phosphate dehydrogenase from muscle, 0.5 mg. Enzyme from skeletal muscle (fraction 40–90%): 5.3 mg. Incubation was for 20 min. at 37°.

Incubation mixture	Disappearance of phosphocreatine (μ moles)
Complete system	1.05
Phosphoglycerate omitted	0
Reduced DPN omitted	0
Arsenate omitted	0.3
Magnesium omitted	0.14
ADP (2.5 μ moles) added	3.6

Fig. 2. Adsorption on aluminium oxide of the 'direct' enzyme and creatine phosphokinase. Assay of the enzymes was as indicated in Table 12. Curve A, 'direct' assay; curve B, creatine phosphokinase. Specific activities are those of the supernatant after treatment with the indicated amount of aluminium oxide.

Table 12. Effect of monoiodoacetate on the 'direct' enzyme and on creatine phosphokinase

The preparation was treated for 20 min. at $+1^\circ$ with different concentrations of potassium monoiodoacetate, pH 7, in the presence of the usual tris-HCl buffer-KCl mixture at pH 8. The inhibitor was then removed by dialysis against 1000 volumes of the same mixture. Untreated controls were dialysed in the same way. Incubation was for 20 min. at 37° . The assay system contains, in a final volume of 2 ml.: potassium phosphate buffer, pH 7.4 (50 μ moles of phosphate); $MgCl_2$, 10 μ moles; DPN, 0.5 μ -mole; cysteine, 1 μ mole; acetaldehyde, 10 μ moles; creatine, 100 μ moles. The test for 'direct' activity was performed with 8 mg. of yeast protein and 5 μ moles of fructose 1:6-diphosphate; creatine phosphokinase was assayed by replacing these components by 20 μ moles of ATP. Enzyme from skeletal muscle (fraction 40–60%): 3.5 mg.

Concentration of monoiodoacetate (mM)	Inhibition of enzymic activity (%)	
	'Direct' enzyme	Creatine phosphokinase
0.1	45	13
5.0	65	19
10.0	94	75

phosphokinase. However, some different patterns of behaviour of the two enzymes have been obtained. Supernatants of varying ratios of specific activities (Fig. 2) are obtained when a 40–60% ammonium sulphate fraction is treated in batches at pH 5.7 with different amounts of aluminium oxide.

The plot of creatine concentration against reaction velocity shows a different slope for creatine phosphokinase and for the 'direct' system (Fig. 3). No pH optimum has been observed for either enzyme; the activity increases steadily from pH 5.9 to 9.0.

The effects of monoiodoacetate, ammonium sulphate and heat inactivation are also different for creatine phosphokinase and the 'direct' system, as shown in Tables 12–14. The inhibition of creatine phosphokinase by monoiodoacetate is smaller than that reported by Ennor & Rosenberg (1954), but the conditions are different, since in our

Fig. 3. Effect of concentration of creatine on the rate of phosphocreatine synthesis. Assay was as described in Table 2, except for the concentration of creatine. Enzyme from skeletal muscle (fraction 40–90%): 7.8 mg./tube. Curve A, 'direct' assay; curve B, creatine phosphokinase assay. Assay time; 10 min. at 37° ; v , velocity of phosphocreatine synthesis (μ moles/min./ml.); s , concentration (M) of creatine.

Table 13. Effect of ammonium sulphate on the synthesis of phosphocreatine in the presence and absence of adenosine diphosphate

Assay was performed as detailed for the 'direct' enzyme in Table 12. Ammonium sulphate was added at pH 8 to the final concentrations indicated. Enzyme from skeletal muscle (fraction 30–60%) was used at a concentration of 0.7 mg./tube.

Final concn. of ammonium sulphate (M)	Inhibition of phosphocreatine synthesis (%)	
	No added ADP	ADP (0.25 mM)
0.025	78	4
0.05	88	34
0.10	100	97

Table 14. *Effect of heat on the 'direct' enzyme and on creatine phosphokinase*

The proteins were brought to a pH of 9 with KOH in the presence of the usual tris buffer-KCl mixture. Measured portions of the enzyme solution were heated at 45° for different times, chilled and centrifuged at 24 000 g at 0° for 10 min. The assay was performed as in Table 12. Amounts used: 1–3 mg. of enzyme from skeletal muscle (fraction 30–60 %).

Time heated (min.)	Phosphocreatine formed (μmoles/mg. of protein)	
	'Direct' enzyme	Creatine phosphokinase
0	1.25	1.15
2	1.45	2.2
4	0.95	3
6	0.52	3.3

Table 15. *Requirement of adenosine diphosphate for the synthesis of phosphocreatine in enzyme fractions from skeletal muscle*

Assay was as detailed in Table 12. The fractions were assayed at levels of 2.9 mg./tube for fraction 40–60 % and 0.45 mg. for fraction 60–80 %. The results are expressed in μmoles of phosphocreatine formed/mg. of protein.

Protein fraction (%)	10 ⁵ × Final concn. of ADP (M)	'Direct' enzyme	Creatine phosphokinase
40–60	0	2.8	0.52
60–80	0	0.015	5.5
	0.01	0	—
	0.10	0.28	—
	1.0	1.55	—
	10	4.10	—

experiments the inhibitor was removed by dialysis before assay to avoid inactivation of triose phosphate dehydrogenase.

A preparation rich in creatine phosphokinase (fraction 60–80 %) but practically devoid of 'direct' activity synthesizes phosphocreatine in a reconstituted system only if μM-ADP is added. The lower fraction (40–60 %), on the other hand, is capable of synthesis of phosphocreatine without the addition of ADP, although its creatine phosphokinase activity is considerably lower than that of the fraction 60–80 % (Table 15).

DISCUSSION

The results presented may be explained by assuming the existence in skeletal muscle of a 'direct' enzyme system capable of phosphate transfer from 1:3-diphosphoglyceric acid to creatine, without the mediation of ADP (reaction 3). Taking into account that all enzymes and substrates were treated with

charcoal to remove nucleotides and that DPN was further purified by ion-exchange chromatography, mediation of the adenylic system appears dubious. The presence of catalytic amounts of ADP was ruled out by the tests made on the enzyme preparations (p. 635).

The existence of such a 'direct' system does not necessarily involve the existence of a specific enzyme; creatine phosphokinase may catalyse both reaction (2) and reaction (3). The results described, however, suggest the existence of two enzymes. It may be argued that the pattern of fractionation on aluminium oxide may be due to separation of creatine phosphokinase from phosphoglyceric kinase. Direct assay proves that excess of phosphoglyceric kinase is present in the reconstituted system, and that all preparations from skeletal muscle also contain appreciable amounts of it. The experiments described in Tables 6 and 15 also prove the presence of phosphoglyceric kinase.

The concentration of ADP necessary to elicit synthesis of phosphocreatine in preparations devoid of 'direct' activity is the same as is required to increase the synthesis above the level obtained with preparations where the 'direct' system is present (Tables 5 and 15). This would be a measure of the minimum level of ADP required for creatine phosphokinase activity, and this finding also favours the hypothesis of two different enzymes. The enhancement of the synthesis of phosphocreatine by the addition of μM-ADP is probably due to transfer of phosphate from phosphoenolpyruvate to creatine, a process known to require ADP (Needham & van Heyningen, 1935).

The possibility that this direct phosphate transfer may be the transferase activity of a phosphatase (Morton, 1953) has not been ruled out. However, the experiments detailed in Table 5 show that our enzyme preparations do not liberate inorganic phosphate from the phosphorylated components of the system (DPN and fructose 1:6-phosphate). Acetyl phosphate cannot be used as a phosphate donor, but 40 % of it disappears after incubation for 10 min. It may be suggested that an acyl phosphatase (Harary, 1957*a, b*) could be the transferring enzyme. This seems not very likely, since the enzyme described by Harary (1957*b*) is not affected by monoiodoacetate and is quite heat-stable, whereas the 'direct' enzyme described in this paper is labile to both agents.

The enzyme catalysing reaction (3) could be named, if isolated, 1:3-diphosphoglycerate-creatine phosphotransferase. Its presence in skeletal muscle and its absence from myocardium is another functional difference between these two structures and might be interpreted as an adaptation to metabolic conditions which are not so dependent upon oxidative phosphorylation.

SUMMARY

1. An enzyme preparation has been obtained from rat skeletal muscle that transfers the carbonyl phosphate of 1:3-diphosphoglyceric acid to creatine without the addition of adenosine diphosphate.

2. The presence of adenosine diphosphate as a contaminant in the various preparations was excluded by the use of enzyme tests which would detect μM -adenosine diphosphate.

3. Enzymes utilizing adenosine triphosphate do not inhibit the direct transfer of phosphate from 1:3-diphosphoglyceric acid to creatine, whereas they inhibit creatine phosphokinase.

4. 1:3-Diphosphoglyceric acid was effective in the phosphorylation of creatine without the addition of adenosine diphosphate. Acetyl phosphate was ineffective.

5. The enzyme system described, and creatine phosphokinase, exhibit differences in their requirements for creatine, their heat stability and the inhibition caused by ammonium sulphate and moniodoacetate.

6. Heart-muscle preparations contain creatine phosphokinase but not the direct phosphate-transfer system.

7. The possibility of phosphate transfer directly from 1:3-diphosphoglyceric acid to creatine, and the existence of an enzyme different from creatine phosphokinase and from acyl phosphatase, are discussed. The name suggested for the new enzyme is 1:3-diphosphoglycerate-creatine phosphotransferase.

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