83. Adenosine- and Inosine-nucleotides in the Phosphorus Metabolism of Muscle

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It is generally assumed that the adenosine-nucleotides play the main part in the intermediary P metabolism in muscle, whilst the inosine-nucleotides are of minor importance [Meyerhof, Lohmann & Meyer, 1931; Meyerhof & Lohmann, 1932; Lohmann, 1931*b*; 1932]. There has even been controversy about the existence of inosine-triphosphate, first prepared by Lohmann [1932]. In this paper the preparation of inosine-triphosphate, and the isolation of inosine-diphosphate, hitherto unknown, are reported. The results of a comparative study of the roles of adenosine- and inosine-nucleotides in some component reactions of muscle metabolism are described and discussed.

Experimental methods

(1) *Preparation of adenosine-triphosphate (ATP)*. The ATP was prepared from fresh horse muscle by the modification of Lohmann's method described by D. M. Needham [1942].

(2) *Preparation of inosine-triphosphate (ITP)*. The preparation of ITP by deamination of ATP with HNO_2 [Lohmann, 1932] was confirmed by Mozołowski & Sobczuk [1933] and Kiessling [1934], whilst Barenscheen, Braun & Filz [1933] contested the existence of the nucleotide, and more recently Wagner-Jauregg [1936] was unable to obtain a pure preparation. It was found in the present work that the deamination of ATP with HNO_2 proceeds smoothly without splitting off of inorganic P when the reaction is carried out in acetate buffer at pH approximately 4.

2 g. Ba-ATP were converted into the Na-salt and the solution was brought to a volume of 32 ml. 4.52 g. $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$, 10 ml. glacial acetic acid, and 20 ml. 60% NaNO_2 were added. The pH of the solution was 3.96 (glass electrode). After standing 5–6 hr. at room temperature the solution was cooled with ice and 2*N* NaOH was added until the pH was 6–6.5; 12 ml. 3*N* BaCl_2 were added, and the precipitation of the Ba-salt of ITP was completed by addition of 2 vol. of ethanol. The precipitate was spun off, suspended in 50 ml. H_2O , and 3*N* HCl was added to the ice-cold suspension to bring the final concentration of HCl to 0.1*N* (as described by Kerr & Seraidarian [1941] for the preparation of ATP). The insoluble fraction was centrifuged off, and re-extracted with 20 ml. 0.1*N* HCl. 1 g. urea was added to the combined centrifugates to destroy the remaining HNO_2 , and the liquid was kept in the ice-chest for 1–2 hr. The nucleotide was then precipitated by addition of 25 ml. 25% Ba-acetate and an equal volume of ethanol. The precipitate was spun off, redissolved in 0.1*N* HNO_3 as described above, and precipitated with Lohmann's reagent (as used for the preparation of ATP [Lohmann, 1931*a*]). The Hg-compound was decomposed with H_2S , and the nucleotide was precipitated twice as the Ba-salt by addition of excess Ba-acetate and an equal volume of ethanol to the solution of ITP in 0.1*N* HCl. The Ba-ITP was then washed twice with H_2O , twice with 50% ethanol, twice with absolute ethanol, once with ether and dried in vacuo over CaCl_2 . The yield varied between 50 and 60% of the theoretical amount. The compound contained 7 H_2O (found H_2O 14.3%; calculated 13.9%) which it lost by drying in vacuo over P_2O_5 at 100°. The anhydrous compound is very hygroscopic. Analysis: found 7 $_{\text{min}}$. P 7.62%; N (Kjeldahl) 7.07%; ratio N/7 $_{\text{min}}$. P 2.05; ratio total P/7 $_{\text{min}}$. P 1.50: calculated 7 $_{\text{min}}$. P 7.98%; N 7.20%; ratio N/7 $_{\text{min}}$. P 2.00; ratio total P/7 $_{\text{min}}$. P 1.50.

(3) *Preparation of myosin.* The myosin was prepared from rabbit muscle as described by Bailey [1942], but using 0.5M LiCl containing 0.03M NaHCO₃ as extractant, and reprecipitating twice from a 0.5M NaCl solution. For further details see Dainty, Kleinzeller, Lawrence, Miall, Needham, Needham & Shen [1942].

(4) *Preparation of dialysed muscle acetone powder.* Muscle extract [Meyerhof & Kiessling, 1935; see also D. M. Needham & Pillai, 1937] after standing 3 hr. at room temperature was dialysed 5 days at 0° against distilled water (to remove the nucleotides) and precipitated with 3 vol. ice-cold acetone. The precipitate was immediately washed with acetone, then with ether, dried, and stored. Before use a 5% suspension of the finely ground powder was prepared, and after a few minutes standing the liquid was centrifuged and the clear supernatant was used.

(5) *Preparation of muscle extract* [Ostern, Guthke & Terszakowec, 1936]. Fresh, minced rabbit muscle was extracted for 10 min. at 0° with 1.5 vol. water and strained through muslin. The extract was cleared by filtering through filter-paper pulp, solid NaHCO₃ was added to bring the pH to 7 (bromothymol blue), the liquid was dialysed for 6 hr. against running tap water, and left to autolyse at 20° for 90 min. The extract was kept in the ice chest and used within 48 hr.

RESULTS

(1) *Splitting of ATP and ITP by myosin.* It is generally assumed that the first step in the series of chemical changes accompanying muscle contraction is the breakdown of ATP to ADP and PO₄^{'''} [D. M. Needham, 1937; 1942], and recent work [Szent-Györgyi & Banga, 1941; D. M. Needham, 1942; Bailey, 1942; J. Needham, Kleinzeller, Miall, Dainty, Needham & Lawrence, 1942] added evidence in favour of the view [Engelhard & Liubimova, 1939] that the enzyme effecting this split of ATP is myosin itself. Since it was found [J. Needham *et al.* 1942] that inosine-triphosphate affects the flow-birefringence of myosin solutions to the same extent as ATP, the rates of splitting of PO₄^{'''} from ATP and ITP by myosin solutions were compared. Each test-tube contained 0.11–0.18 mg. myosin N (Kjeldahl), 0.1 ml. 18N CaCl₂, 0.5 mg. 7_{min.} P ATP or ITP (Na-salt), 1 ml. 0.1M buffer solution (glycine or veronal buffer), and the volume was adjusted to 2 ml. with 0.5M NaCl. After varying times of incubation at 37.5° the reaction was stopped by addition of 2 ml. 4% CCl₃COOH and inorganic P was estimated in an aliquot.

As shown in Fig. 1 and Table 1, ITP is split approximately 3 times as fast as ATP by myosin solutions. These results were obtained at pH 6.7 and 7.2 (veronal buffer), and at pH 9.1 (glycine buffer) with two different myosin preparations. As with ATP, myosin splits off only 50% of the 7_{min.} P of the ITP added.

(2) *Preparation of inosine-diphosphate (IDP).* As shown in Fig. 1 and Table 1, in the presence of myosin one PO₄ radical is liberated from each molecule of ITP. The inosine-diphosphate formed was isolated in the following way [see preparation of adenosine-diphosphate from ATP, Liubimova & Pevzner, 1941; Bailey, 1942]: 1 g. Ba-ITP (air-dry) was converted into the Na-salt and the solution was made up to 50 ml. A solution was prepared of myosin in 0.1M glycine buffer pH 9.1, containing 62.7 mg. protein and 200 mg. CaCl₂ in 150 ml. After bringing both solutions to 37.5° they were mixed and incubated for 10 min. at 37.5°. This time was found to be sufficient to bring about a 50% splitting of the 7_{min.} P of the ITP present (cf. Table 1). After incubation the myosin was precipitated by acidifying the solution with 2N HCl to pH 5, the mixture was centrifuged, and the clear ice-cold supernatant was brought to pH 6 with N NaOH. A slight precipitate, 70 mg. dry weight, which formed on addition of 3 ml. 25% Ba-acetate was centrifuged off, and after neutralization of the clear liquid to pH 7 the IDP was precipitated by excess of Ba-acetate and 50% ethanol. The precipitate was suspended in H₂O, 3N HNO₃ was added to the final concentration of 0.1N, and the IDP was reprecipitated from the clear

solution by excess of Ba-acetate and 50% ethanol. The precipitate was redissolved in 0.1*N* HNO₃ as described above and precipitated with Lohmann's reagent. The mercury compound was decomposed with H₂S, the clear filtrate freed of H₂S by aeration, and the IDP twice precipitated from 0.1*N* HNO₃ solution with excess of Ba-acetate and 50% ethanol. The Ba-IDP was washed with 50% ethanol until the washings were free of Ba⁺⁺

Fig. 1. Splitting of adenosine-triphosphate and inosine-triphosphate by myosin. 0.1*M* veronal buffer pH 7.2.

Table 1. *Splitting of ATP and ITP by myosin*

Exp. 1. 0.1 <i>M</i> veronal buffer pH 6.7 in 0.5 <i>M</i> NaCl.			Exp. 2. 0.1 <i>M</i> glycine buffer pH 9.1.		
Exp. no.	Myosin N in tube mg.	Initial 7 _{min.} P mg.	Duration of exp. min.	Inorg. P found mg.	
				ATP	ITP
1	0.18	0.500	5	0.053	0.194
			10	0.128	0.205
			15	0.142	0.208
			20	0.173	0.215
2	0.108	0.509	2	0.080	0.200
			3½	0.117	0.237
			5	0.156	0.255
			10	0.217	0.250
			15	0.246	0.259
			20	0.248	0.250

(tested with Na₂SO₄), with 75% ethanol, twice with 96% ethanol, once with ether, and dried in vacuo over CaCl₂. Yield 0.625 g., i.e. 68% of theory. Analysis: found 14.4% H₂O; 4.08% 7_{min.} P; 8.25% total P; 7.29% N (Kjeldahl): calculated for C₁₀H₁₁O₁₁N₄P₂Ba_{1.5}·6H₂O, 14.6% H₂O; 4.20% 7_{min.} P; 8.39% total P; 7.05% N. Ba in the anhydrous compound (Weiler): found 31.6%; calculated for C₁₀H₁₁O₁₁N₄P₂Ba_{1.5}, 32.6%.

(3) *Phosphagen formation in the presence of adenosine- and inosine-nucleotides.* Meyerhof & Lohmann [1932] have shown that the formation of phosphagen in muscle extracts proceeds also in the presence of ITP. In our experiments the rates of P transfer from

phosphoglycerate to creatine (Parnas reaction) in the presence of ATP, ITP, adenosine-diphosphate (ADP), and inosine-diphosphate (IDP) were compared. Acetone powder prepared from dialysed muscle extract (see above) was used as the enzymic system. Each tube contained 0.2 ml. 5% enzyme solution, 0.2 ml. 2% NaHCO_3 , 6 mg. creatine, 0.6 mg. Mg^{++} (as MgCl_2), 1.2 mg. P of Na-phosphoglycerate, and 0.29 mg. 7_{min.} P ATP or ITP (or equimolecular quantities of the corresponding diphosphates). The volumes of the

Fig. 2. The formation of phosphagen in the presence of ATP and ITP.

Table 2. *The formation of phosphagen in the presence of adenosine- and inosine-nucleotides*

Nucleotide	mg. 7 _{min.} P added	Duration of exp. min.	Phos- phagen P found mg.	Nucleotide	mg. 7 _{min.} P added	Duration of exp. min.	Phos- phagen P found mg.
ADP	0.181	5	0.192	IDP	0.181	5	0.009
		10	0.242			10	0.032
		15	0.318			15	0.048
ATP	0.291	15	0.287	ITP	0.292	15	0.058

contents of the test tubes were adjusted to 2 ml. with H_2O . After varying times of incubation at 40° the test tubes were transferred to a freezing mixture in order to stop the reaction, and the contents were deproteinized by addition of 2 ml. 8% trichloroacetic acid. The phosphagen content was estimated as described by Baldwin & D. M. Needham [1937]. In control experiments no phosphagen formation was found in the absence of ATP.

As shown in Fig. 2 and Table 2 the formation of phosphagen is 5–8 times as fast in the presence of ATP as in the presence of ITP. No appreciable difference was found in the rate of formation of phosphagen between the respective triphospho- and diphospho-nucleotides (see Table 2).

(4) *Phosphorylation of hexosemonophosphate.* It was shown by Ostern, Guthke & Terszakowec [1936], and Sakov [1941] that muscle extract contains an enzyme which transfers P from ATP to hexosemonophosphate (fructose-6-phosphate) with formation

of fructose-diphosphate. In our experiments the phosphorylation of hexosemonophosphate in the presence of ATP and ITP was examined; Robison ester (glucose-6-phosphate) was used, since the muscle extract contains an enzyme converting this into an equilibrium mixture of fructose- and glucose-6-phosphate. Iodoacetic acid was added to stop the reaction at the fructose-diphosphate and triose-phosphate stage. 5 ml. muscle extract, 4.5 ml. H_2O , 1 ml. Robison ester (6 mg. P), 0.3 ml. 0.5 *M* MgSO_4 , 0.2 ml. 0.1 *M* iodoacetate, 1.4 ml. 0.1 *M* veronal buffer pH 7.0, and 2 ml. ATP or ITP (2.50 mg. 7_{min} . P) were incubated at 20°. At varying intervals 2 ml. aliquots were pipetted into 0.6 ml. 10% CCl_3COOH and in aliquots of the centrifugate inorganic P and 7_{min} . P were estimated. As shown in Table 3, there is a rapid disappearance of 7_{min} . P in the presence of ATP, whilst only dephosphorylation of ITP is observed. No evidence was therefore obtained that ITP can transfer P to hexosemonophosphate. (It should be noted that the disappearance of 7_{min} . P in these conditions gives only a rough estimate of the ATP disappearing, since the hexose-diphosphate and triose-phosphate are to some extent split.) The rapid formation of NH_4^+ from ATP in presence of hexosemonophosphate, observed by Ostern *et al.* [1936], is therefore due to a deamination of the adenylic acid formed during the reaction.

Table 3. *Phosphorylation of hexosemonophosphate in the presence of ATP and ITP*

Time min.	2 ml. aliquots.			
	ATP		ITP	
	Inorg. P found mg.	7_{min} . P found mg.	Inorg. P found mg.	7_{min} . P found mg.
0	0.067	0.372	0.087	0.368
5	0.090	0.301	0.107	0.374
10	0.102	0.240	0.130	0.365
20	0.102	0.247	0.159	0.365

The results in Table 3 show that a rapid dephosphorylation of ATP and ITP takes place in the muscle extract. This is due to an enzyme which [Sakov, 1941] seems not to be identical with myosin. It was therefore thought of interest to examine the dephosphorylation of adenosine- and inosine-nucleotides in muscle extract. 5 ml. muscle extract, 0.1 ml. 0.1 *M* iodoacetate (to prevent rephosphorylation of the nucleotides by means of inorganic P), and 0.6 ml. 0.1 *M* veronal buffer pH 7.0 were incubated at 20° with 1 ml. of solutions of the respective nucleotides. 1 ml. aliquots were pipetted at varying intervals into 1 ml. 5% CCl_3COOH and in the deproteinized centrifugates inorganic P was estimated. As shown in Table 4, the formation of inorganic P increases linearly with time in the presence of ATP and ADP. ITP is split only partially, whilst IDP is not dephosphorylated at all.

Table 4. *Dephosphorylation of adenosine- and inosine-nucleotides in muscle extract*

Exp. no.	Time min.	mg. 7_{min} . P added	1 ml aliquots.	
			Inorganic P formed	
			ATP	ITP
1	5	0.170	0.018	0.016
	10		0.050	0.037
	15		0.073	0.048
	20		0.099	0.053
			ADP	IDP
2	5	0.106	0.018	0
	10		0.044	0
	15		0.065	0
	20		0.096	0

From these experiments it can be concluded that the dephosphorylation of ITP stops at the IDP stage. This suggests that the myokinase [Kalckar, 1942] which catalyses the reaction:

is strictly specific for the adenosine compounds.

(5) *Phosphorylation of glucose by yeast hexokinase.* The rate of phosphorylation of glucose by yeast hexokinase in the presence of ATP and ITP was measured. The enzyme solution was prepared by grinding 30 mg. of the dry powdered hexokinase with 4 ml. H_2O ; the suspension was left standing for a few minutes and spun off. The clear supernatant was used.

Test-tubes containing 0.8 ml. enzyme solution, 2.2 mg. $7_{\text{min.}} \text{P}$ of ATP or ITP, 2 ml. 0.4 *M* glucose, 2 ml. 0.1 *M* veronal buffer pH 7.8, 0.4 ml. 0.5 *M* MgSO_4 , and H_2O to bring the volume to 8 ml., were incubated at 37°. At varying intervals 1 ml. aliquots were

Fig. 3. Phosphorylation of glucose in the presence of ATP and ITP.

pipetted into 1 ml. 2*N* HCl. The $7_{\text{min.}} \text{P}$ was now estimated in the aliquots. The difference between the initial $7_{\text{min.}} \text{P}$ value and that after incubation indicates the amount of glucose phosphorylated.

As shown in Fig. 3, glucose is phosphorylated in the presence of ATP approximately 5 times as fast as in the presence of ITP.

DISCUSSION

The experimental results show that the ITP and IDP are considerably less effective than the respective adenosine-nucleotides in the P transfer reaction of Parnas, and in the phosphorylation of glucose by yeast hexokinase; no evidence of the transfer of P from ITP to hexosemonophosphate was obtained; on the other hand, myosin splits PO_4''' more readily from ITP than from ATP. Inosine-triphosphate has been isolated from frog muscle [Lohmann, 1932], hence it may be assumed that ITP can be formed during

muscle contraction. The formation of NH_3 during muscle contraction seems to be connected with the liberation of PO_4''' , although the views of different schools do not so far conform as to whether the formation of NH_4^+ precedes, is parallel to, or follows that of PO_4''' [see Mozolowski, Reiss & Sobczuk, 1932]. The mother substance of both NH_3 and H_3PO_4 is ATP. The higher rate of splitting of PO_4''' from ITP than from ATP by myosin solutions may thus have a bearing on the present conception of P metabolism in muscle during the first phase of contraction.

It is known that during aerobic muscle contractions the content of adenosine-nucleotides present in the muscle does not much decrease [Parnas, 1929]. If it is assumed that the inosine-nucleotides play a greater part in the P metabolism of muscle than was thought hitherto, their reamination would be expected. This reamination was postulated by Embden, Riebeling & Selter [1928] and Parnas [1929], but the suggestions advanced for its mechanism are controversial. Our attempts to find evidence of reamination of ITP or IDP to the corresponding adenosine compounds in muscle extracts have as yet been unsuccessful. Experiments were set up incubating ITP or IDP with aminoacids or aminoacid-amides in the presence of dialysed muscle extract (rabbit muscle extract as described by Meyerhof & Kiessling [1935] but not precipitated with acetone). This extract readily deaminated ATP, and the deamination was not inhibited by the presence of the added aminoacids. If reamination of the inosine-compounds had occurred, it would have been indicated by formation of NH_4^+ . In our experiments no NH_4^+ was formed when ITP or IDP was incubated together with alanine, glutamate, glutamine, aspartate, asparagine, or ornithine.

SUMMARY

1. The preparation of inosine-triphosphate (ITP) is described.
2. The rates at which PO_4''' is liberated from inosine-triphosphate and adenosine-triphosphate by myosin solutions are compared. Inosine-triphosphate is split approximately 3 times as fast as ATP.
3. The isolation of the hitherto unknown inosine-diphosphate (IDP) is described.
4. Phosphagen formation proceeds 5–8 times as fast in the presence of adenosine-nucleotides as in the presence of the inosine-compounds.
5. No evidence of transfer of P from ITP to hexosemonophosphate was obtained.
6. It is shown that the dephosphorylation of ITP in muscle extracts stops at the IDP stage. It is concluded that myokinase is strictly specific for adenosine-diphosphate.
7. The phosphorylation of glucose by yeast hexokinase proceeds approximately 5 times as fast in the presence of adenosine-triphosphate as in the presence of ITP.

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