- Marinetti, G. V., Witter, R. F. & Stotz, E. H. (1957). *J. biol. Chem.* **226**, 475.
- Marples, E. A. & Thompson, R. H. S. (1958). *Biochim. biophys. Acta*, **30**, 187.
- Noguchi, S. (1944). *J. Biochem., Tokyo*, **36**, 113.
- Phillips, G. B. (1957). *Proc. nat. Acad. Sci., Wash.*, **43**, 566.
- Phillips, G. B. (1958). *Biochim. biophys. Acta*, **29**, 594.
- Saunders, L. & Thomas, I. L. (1958). *J. chem. Soc.* p. 483.
- Schmidt, G., Hecht, L., Fallot, P., Greenbaum, L. & Thannhauser, S. J. (1952). *J. biol. Chem.* **197**, 601.
- Shapiro, B. (1953). *Biochem. J.* **53**, 663.
- Tattrie, N. H. & McArthur, C. S. (1955). *Canad. J. Biochem. Physiol.* **33**, 761.
- van der Vies, J. (1955). *Biochem. J.* **60**, 671.
- Webster, G. R. (1954). *Biochem. J.* **57**, 153.
- Webster, G. R. (1956). *Biochim. biophys. Acta*, **20**, 432.
- Webster, G. R., Marples, E. A. & Thompson, R. H. S. (1957). *Biochem. J.* **65**, 374.

The Incorporation of ^{15}N into Adenine Nucleotides and their Formation from Inosine Monophosphate by Skeletal-Muscle Preparations

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Most vertebrate skeletal muscles contain an active enzyme specific for the deamination of adenosine 5'-monophosphate (Schmidt, 1928). When skeletal muscle is repeatedly stimulated or damaged this enzyme is responsible for production of the ammonia that was the subject of detailed investigations by the Parnas and Embden schools in the 1920's [see Needham (1932) for a review of this work]. Both groups of workers considered that resynthesis of the adenosine monophosphate from inosine monophosphate occurred in muscle and Parnas (1929) in particular favoured the idea of aerobic reamination at the expense of the tissue amino acids, although he failed experimentally to demonstrate this reaction (Parnas, 1933). Subsequent studies by Kalckar & Rittenberg (1947) confirmed that reversible deamination of the 6-amino group of adenine nucleotides could occur in living muscle. These workers showed that after the administration of [^{15}N]ammonium citrate to rats, significant incorporation of the isotope could be detected in the adenosine triphosphate isolated from skeletal muscle. With systems *in vitro*, Kleinzeller (1942) was, however, unable to demonstrate in muscle homogenates the reamination either of inosine diphosphate or of inosine triphosphate by incubation with amino acids. Not surprisingly, in view of the thermodynamical considerations (Tarver & Morales, 1951), Kalckar (1947*a*) failed to demonstrate the reversibility of the 5'-adenylic deaminase reaction. More recently, however, Nekhorocheff & Wajzer (1952) and Nekhorocheff (1955) have provided evidence that, in muscle, creatine can act as a precursor of the 6-amino group

of adenosine triphosphate, whereas Kometiani & Klein (1956) propose, on the basis of their experimental findings, β -alanine for this function.

The present paper is concerned with a study of the mechanism of reamination of inosine monophosphate in muscle systems. A partly purified enzyme preparation is described which catalyses the transfer of the amino group of aspartic acid to inosine monophosphate to give adenine nucleotides. Evidence is provided which indicates that the system may be similar to that described by Carter & Cohen (1955) for the synthesis of adenosine monophosphate in yeast.

A preliminary account of some aspects of this work has already been published (Newton & Perry, 1957).

METHODS

Whole-animal experiments. A total of 100 ml. of ammonium citrate solution containing 280 mg. of NH_3 nitrogen (62 atoms % excess of ^{15}N) was injected subcutaneously into adult rabbits in three equal doses at 2 hr. intervals. Animals were not fed but could move freely in their cages during the experiment. They were killed for the extraction of the muscle nucleotides 4 hr. after the final injection.

Preparation of homogenates. Back and leg muscles from a freshly killed normal rabbit were removed and chilled in 0.1*M*-KCl; they were freed from adherent moisture, minced and briefly homogenized with 4 vol. of a medium so that final concentrations in the homogenate were: 0.1*M*-KCl, 50 *mM*-glycylglycine-HCl, pH 7.5, *mM*- MgCl_2 , *mM*-adenosine triphosphate (ATP), 10 μg . of pyridoxal phosphate/ml. and 11 mg. of $^{15}\text{NH}_4\text{Cl}$ or the equivalent amount of nitrogen as [^{15}N]aspartic acid/100 ml. The homogenate was stirred at room temperature for 30 min., the reaction was stopped by the addition of 60% (w/v) trichloroacetic acid to give a final concentration of 7% and treated at 0°

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as follows. The precipitated protein was centrifuged down, washed twice with 5% (w/v) trichloroacetic acid, and the combined supernatant fluids were extracted ten times with ether to remove the acid. The clear solution was neutralized and stored at 0°.

Homogenate fractionation. Muscle from a freshly killed animal was chilled in 0.16M-KCl, freed from adherent moisture, minced and homogenized with 4 vol. of a medium containing 0.16M-KCl and 0.05M-2-amino-2-hydroxy-methylpropane-1,3-diol (tris)-HCl buffer, pH 7.4, (KCl-tris medium) in a Waring Blendor for 1 min. The soluble proteins were separated from other cell components by centrifuging at 15 000 g for 30 min. This fraction (soluble fraction) was used undiluted for enzyme incubations, whereas the sediment consisting of myofibrils, nuclei and some particulate material was resuspended in the KCl-tris medium to give a volume equal to that of the original homogenate.

Acetone-dried powder preparation. To the soluble fraction obtained as described above was added 2 vol. of cold acetone. The precipitated protein was washed at 0° by resuspension in acetone and centrifuging. This process was repeated once with acetone and once with ether and the dried powder was stored at -15°. Before use it was ground with ice-cold water (0.1 g./ml. of water) and dialysed overnight against distilled water at 0°. The insoluble residue was removed by centrifuging and the clear supernatant fluid used for enzyme assay.

For large-scale preparations the procedure was similar except that homogenization was carried out in 2 vol. rather than 4 vol. of KCl-tris medium.

Preparation of nucleotides from muscle extracts. The leg and back muscles were dissected from a freshly killed rabbit and immediately dropped into liquid nitrogen. While still frozen they were crushed to fine powder in a mortar, an equal volume of 10% (w/v) trichloroacetic acid was added and the suspension allowed to thaw. Liquid was separated from the precipitated protein by squeezing through muslin and the residue was again extracted with 2 vol. of 5% (w/v) trichloroacetic acid for 1 hr. at 0°. After the sediment had been removed by centrifuging, the combined supernatant liquids were clarified by filtration through cotton wool. Ten extractions with ether were carried out to remove most of the trichloroacetic acid from the aqueous phase, which was finally neutralized and stored overnight at 4°.

Separation of nucleotides. Nucleotides were separated by chromatography on columns of Dowex-1 (chloride form) by a modification of the method of Bergkvist & Deutsch (1954). The volume of resin was about 1.5 cm.³/mg. of phosphate. The resin was prepared for use by washing in sequence with methanol, 2N-NaOH and N-HCl; after the final acid washing it was washed with water until the eluate was chloride-free. The same resin was used for several experiments but was washed with NaOH and HCl between each experiment.

(i) Method (1). For complete fractionation of the adenine nucleotides the following procedure was used. (a) Nucleotides were placed on the column in a solution brought to pH 10.0 by the addition of aq. N-NH₃ soln. (b) The column was washed with water until the pH was 5.0 and the nucleotides were eluted in turn by stepwise application of the solutions (c)-(h): (c) 3 mN-HCl to elute adenosine monophosphate (AMP); (d) 5 mN-HCl to elute inosine monophosphate (IMP); (e) 0.01N-HCl-0.02M-NaCl to

elute adenosine diphosphate (ADP); (f) 0.01N-HCl-0.05M-NaCl to elute inosine diphosphate (IDP); (g) 0.01N-HCl-0.2M-NaCl to elute ATP; (h) 0.1-1.0N-HCl to elute inosine triphosphate (ITP), guanosine triphosphate (GTP), etc. Fractions (25 ml.) were collected and the elution was followed by determination of the ultraviolet-absorption spectrum of the eluent in the range 220-300 mμ. Elution was continued until the extinction of the eluate was less than 0.020. At least 1 l. of eluting fluid at each concentration was used. The identities of the nucleotides in the fractions were checked by paper chromatography in the isobutyric acid-NH₃ solvent described by Krebs & Hems (1953). The acidic and aromatic amino acids were also retained by the resin and could be eluted with dilute acid solutions. In the later work it was necessary to avoid contamination of the ADP and ATP fractions by these amino acids and so the columns were washed with large volumes of 0.01N-HCl before these fractions were removed, i.e. in place of (c) and (d) but before (e).

(ii) Method (2). When nucleotide solutions containing only a few components were to be analysed, and complete separation of the adenine nucleotides was not required, the following procedure was used. (a) Adsorption of the nucleotides at pH 10.0 as before. (b) Washing with 2 l. of water. (c) Elution with 1 l. each of solutions of increasing acidity: 1, 2, 3, ..., 9, 10 mN-HCl. Samples of 1 l. were collected, the u.v. absorption at 248 and 260 mμ was determined and the fractions were discarded. This washing removed AMP, glutamic acid, aspartic acid and IMP. (d) Elution with 0.1N-HCl to remove ADP and ATP. This eluate was collected in 5 ml. fractions and the nucleotide content determined as before.

Nucleotide solutions prepared by either method were neutralized and if the volume was large they were concentrated by freeze-drying, or by boiling if the sample was to be analysed immediately for ¹⁵N content. In pure fractions the nucleotide concentrations were estimated from their absorption in the ultraviolet with the extinctions determined by Kalckar (1947b).

Preparation of adenine from adenosine triphosphate. The ATP was hydrolysed by boiling in N-HCl for 1 hr. The adenine was precipitated as the silver salt, dissolved in 0.5N-HCl and recrystallized from the neutralized solution after removal of the precipitated AgCl and concentration to small volume in the water bath at 100°. After a further recrystallization from water the adenine moved as a single spot, identical with that obtained with a known sample of adenine, in the propan-2-ol-conc. HCl-water (68:17.6:14.4) system of Wyatt (1951). The preparation was negative to the ninhydrin test for amino acids.

Preparation of adenosine mono- from adenosine triphosphate. [¹⁵N]ATP, isolated by elution from a Dowex-1 column by 0.1N-HCl as previously described, was used for the preparation of [¹⁵N]AMP. The HCl concentration was reduced to about mM by the addition of Ag₂CO₃ and the neutralized solution of ATP was then hydrolysed to AMP by a method suggested by Dr M. Clark, which involved heating in 50% (v/v) pyridine at 100° for 6 hr. Aqueous pyridine was removed by distillation *in vacuo*, and the dry residue was taken up in water and again dried *in vacuo*. The nucleotide was then dissolved in water and separated from unhydrolysed ATP and ADP (about 10% of the total nucleotide) by chromatography on Dowex-1. After neutralization of the eluate containing the AMP it was

concentrated by freeze-drying. The AMP ran as one spot in the isobutyric acid- NH_3 system of Krebs & Hems (1953).

Liberation of the 6-amino nitrogen of adenosine mono- and tri-phosphate. AMP prepared as described above was deaminated by means of the specific 5'-AMP deaminase of rabbit myosin, by incubating together for 1 hr. at 30° : 1 ml. of myosin (2.7 mg. of protein/ml.) prepared as described by Perry (1955) and dissolved in 0.5 M-KCl, 0.5 ml. of m-succinate-NaOH buffer, pH 6.0, and 5 ml. of 14 mM-AMP. In some cases equivalent amounts of ATP itself were deaminated by incubation with 1 ml. of a 2% suspension of myofibrils, prepared as described by Perry & Grey (1956), in 0.1 M-KCl, 0.1 M-tris-HCl, pH 7.4, and 10 mM- MgCl_2 , in a total volume of 5 ml. Prior conversion into AMP was not necessary in this case because of the adenosine triphosphatase and myokinase present in the myofibrils together with the 5'-adenylic deaminase. After being deproteinized with 1 ml. of 60% trichloroacetic acid, the solution was made alkaline by the addition of 10 ml. of $\text{m-NaHCO}_3\text{-Na}_2\text{CO}_3$ buffer, pH 10.0. The NH_3 was distilled into 50 mM- H_2SO_4 in the apparatus described by Sprinson & Rittenberg (1948). The NH_3 -free solution remaining was digested with H_2SO_4 for the determination of the ^{15}N content of the IMP.

^{15}N -labelled ammonium salts. An aqueous solution of $^{15}\text{NH}_4\text{NO}_3$ supplied by the Eastman Kodak Co. (62 atoms % excess of ^{15}N in the ammonium group) was made alkaline and converted into citrate or chloride by distillation of the NH_3 evolved into a solution of the appropriate acid. For injection into animals labelled ammonium citrate was prepared from 1.8 g. of NH_4NO_3 , the solution was neutralized and the volume adjusted to 100 ml. It was sterilized by filtration through a Seitz filter and stored at $1-2^\circ$ until used.

L- ^{15}N Aspartic acid. This was prepared by the method described by Wu & Rittenberg (1949), with the following modification. Before the final recrystallization of the aspartic acid the solution was freed from contamination with copper salts by shaking with a solution of 8-hydroxyquinoline in CHCl_3 . When the ^{15}N aspartic acid was mixed with known aspartic acid it gave a single spot on chromatography in pyridine-acetic acid-water (50:35:15, by vol.) (Decker & Riffart, 1950) and in butan-2-ol-formic acid-water (70:10:20, by vol.) followed by phenol-water-conc. aq. NH_3 soln., sp.gr. 0.88 (80:20:0.3, by vol.). Electrophoresis on paper buffered with pyridine-acetic acid-water (1:10:89, by vol.), pH 3.5, for 1 hr. at 40 v under toluene (Sanger, personal communication) also gave a single spot. After degradation with H_2SO_4 the aspartic acid gave 95% of the theoretical nitrogen. Atoms % excess of ^{15}N , 59.2.

L- ^{15}N Glutamic acid. This amino acid was prepared from the hydrolysed protein of *Escherichia coli* grown in a medium containing $^{15}\text{NH}_4\text{Cl}$ as the only nitrogen source. The *E. coli* were grown in 1 l. of the C medium described by Roberts, Abelson, Cowie, Bolton & Britten (1955) containing 1 g. of $^{15}\text{NH}_4\text{Cl}$ (62 atoms % excess of ^{15}N) and 10 g. of glucose as energy source. After hydrolysis of the protein with 6 N-HCl for 24 hr. at 100° under reflux the barium salts of the aspartic acid and glutamic acid were precipitated as described by Jones & Moeller (1928). These salts were freed from barium by solution in 0.1 N- H_2SO_4 and the amino acids were separated by ion-exchange chromatography with Dowex-50 (HCl form) by a method described by Mr M. Rees (personal communication). Fractions containing

glutamic acid only were concentrated to a syrup from which crystals of glutamic acid-HCl were obtained. These were washed in ethanol and ether and dissolved in water.

The glutamic acid was chromatographically pure in a pyridine-acetic acid-water (50:35:15, by vol.) solvent system (Decker & Riffart, 1950), and on zone electrophoresis at pH 3.5 no trace of contamination by aspartic acid could be detected. Atoms % excess of ^{15}N , 54.

Preparation of samples for ^{15}N analysis. Solutions containing about 1 mg. of nitrogen were boiled in a 50 ml. Kjeldahl flask with 5 ml. of $\text{m-NaHCO}_3\text{-Na}_2\text{CO}_3$ buffer, pH 10.0, for 30 min. to remove contaminating NH_3 . Tests showed that this process removed quantitatively NH_3 added to nucleotide solutions without affecting the 6-amino group of the adenine ring. Digestion was carried out overnight with 2 ml. of 98% (w/w) H_2SO_4 and 0.3 ml. of the HgSO_4 catalyst recommended by Hiller, Plazin & Van Slyke (1949), and the NH_3 was distilled in the modified distillation apparatus described by Sprinson & Rittenberg (1948), into a solution of 50 mM- H_2SO_4 . After removal of a sample for estimation of the NH_3 by nesslerization (Johnson, 1941), the acid distillate was concentrated by boiling to about 2 ml. and finally stored in a sealed tube for analysis.

Controls containing unlabelled NH_4Cl were digested with each experimental series and their ^{15}N content was determined. This was always between 0.37 and 0.39 atom % ^{15}N , and was the figure used as 'normal' ^{15}N content.

Analysis of ^{15}N content. The samples were converted into nitrogen by the method of Sprinson & Rittenberg (1949) and the ^{15}N content was determined on a 180° mass spectrophotometer in this Department. A few samples were also analysed by Mr G. Dickinson at the National Institute for Medical Research, Mill Hill, and the values obtained agreed very well with those obtained with the Cambridge Instrument. The accuracy of the determinations was such that a standard error of ± 0.005 atom % ^{15}N was obtained.

RESULTS

A number of preliminary experiments were carried out in which IMP was incubated with various muscle preparations and nitrogen sources such as NH_4Cl , glutamic acid and aspartic acid. Aspartic acid in particular was selected in view of the similarities between the system under investigation and the amination of citrulline to give arginine (Ratner, 1947). Its likely role as precursor was further suggested by the results of AMP synthesis in yeast (Carter & Cohen, 1955), which were published during the course of this investigation. Incubations were carried out in the presence of active aerobic and anaerobic phosphorylating systems such as pigeon breast-muscle mitochondria isolated by the method of Chappel & Perry (1954) and rabbit muscle homogenates and acetone-dried powder extracts. Under these conditions it was hoped that any AMP formed from IMP would be trapped by phosphorylation to ADP or ATP, which nucleotides are not deaminated by the active 5'-adenylic deaminase present in the preparations.

With α -oxoglutarate as oxidizable substrate and the nitrogen sources mentioned above, IMP could not replace AMP as a phosphate acceptor for oxidative phosphorylation with pigeon breast-muscle mitochondria.

With the anaerobic systems and fructose diphosphate as substrate for glycolysis it was not possible to show appreciable uptake of inorganic phosphate with IMP as acceptor. There were indications that some AMP formation had occurred, however, for at times an increase in ATP level after incubation could be detected by paper chromatography.

Incorporation of ^{15}N into adenosine triphosphate in vivo

Rabbit muscle offers certain advantages for the study of the incorporation of ^{15}N into ATP and it was first necessary to confirm that the findings of Kalekar & Rittenberg (1947) with the rat were applicable to the rabbit. For this purpose [^{15}N]-ammonium citrate was injected into adult rabbits and the trichloroacetic acid extract of their skeletal muscles was chromatographed on a Dowex-1 column. Fig. 1 illustrates a typical elution chromatogram

Fig. 1. Chromatography of nucleotide fraction isolated from rabbit skeletal muscle after injection of [^{15}N]ammonium citrate; Dowex-1, method 1. Stepwise elution: (a) 3 mM-HCl; (b) 10 mM-HCl; (c) 10 mM-HCl-20 mM-NaCl; (d) 10 mM-HCl-50 mM-NaCl; (e) 10 mM-HCl-0.2 M-NaCl; (f) M-HCl. Maximum reading of ATP peak, E_1 cm. 34.5.

Table 1. *Distribution of ^{15}N in nucleotide fractions isolated from rabbit skeletal muscle after injection of [^{15}N]ammonium citrate*

Fractionation was carried out by method 1 (see Methods section). FAD, Flavinadenine dinucleotide; ITP, inosine triphosphate; GTP, guanosine triphosphate.

	Fraction no.	Identified compounds	Nucleotide N (% of total N)	Atoms % excess of ^{15}N
Expt. 1	1	AMP + glutamic acid + aspartic acid	22.5	0.091
	2	ADP + IMP + FAD	25	0.058
	3	Unknown	93*	0.512
	4	ATP	101	0.091
	5	ITP + GTP	92	0.072
Expt. 2		ATP amino group	—	0.153
	1	AMP + aspartic acid + glutamic acid	7	0.043
		AMP	8	0.027
	2	ADP + IMP + FAD	24	0.023
		ADP	102	0.023
	3	Unknown	100*	0.404
		Unknown + ATP	100*	0.097
	4	ATP	110	0.036
	5	ITP + GTP etc.	90	—

* Extinction of the unknown was assumed to be identical with that of ATP.

Table 2. *Incorporation of ^{15}N into adenine nucleotides by incubation of muscle fractions with labelled aspartic acid*

Incubations were carried out at 20° for 30 min. in 0.1 M-KCl, 10 mM-Sorensen phosphate buffer, pH 7.4, 2 mM-IMP, mM-AMP, mM- MgCl_2 , 2 mM-fructose 1:6-diphosphate, 0.002 % of coenzyme I, 10 μg . of pyridoxal phosphate/ml., 2 mM- ^{15}N -aspartic acid. The enzyme fraction represented 20 % of the total volume except in the acetone-dried powder experiment, when, in 22 ml. total volume, 20 ml. consisted of enzyme extract.

	Enzyme preparation	IMP	ADP + ATP	Atoms %	Activity*
		(μmoles)	(μmoles)	excess of ^{15}N in ADP + ATP	
Expt. 1	Whole homogenate	268	180	2.04	7.45
	Particulate	400	46	0.91	0.83
	Soluble	275	275	1.51	8.4
Expt. 2	Fresh soluble fraction	70	30	0.73	4.6
	Dialysed soluble fraction	37	11	0.90	2.68
	Aqueous extract of acetone-dried powder	39.5	16	0.36	1.08

* Activity is expressed as μg .atoms of ^{15}N incorporated into ADP + ATP/g. wet wt. of original muscle.

obtained under these conditions. From analysis of the fractions by spectrophotometry and paper chromatography it was clear that dicarboxylic amino acids were present in the fraction containing AMP and that IMP and flavin nucleotides were associated with the ADP. No amino acids could be detected in fractions eluted after the AMP.

Nucleotide fractions prepared in this way were analysed for their ^{15}N content. Table 1 contains the results of two experiments, in one of which the main fractions were further subdivided to improve the purity of the nucleotide samples. It can be seen from the results that ^{15}N was present in all the fractions containing adenine nucleotides. Although AMP could not be obtained free from other nitrogen-containing compounds, all the nitrogen could be accounted for by ADP and ATP in their respective fractions and both were significantly labelled with ^{15}N . The specific activity of these compounds was in both cases much higher than that obtained by Kalckar & Rittenberg (1947) for the adenosine polyphosphates isolated from rat muscle. A precise comparison between the degree of labelling of the various adenosine phosphates was not possible owing to the presence of contaminating substances particularly in the AMP fraction.

Incorporation of ^{15}N into adenosine triphosphate in vitro

Whole-muscle homogenates. When fresh rabbit muscle was homogenized at 0° in the appropriate medium (see Methods section) containing ^{15}N -aspartic acid, and subsequently incubated at 30° , appreciable incorporation of ^{15}N could be demonstrated in the ATP fraction isolated from the deproteinized extract.

The incorporation achieved, however, with $^{15}\text{NH}_4\text{Cl}$ alone as the source of nitrogen was barely significant, but the degree of labelling could be increased slightly by the addition of unlabelled aspartic acid and glutamic acid to the incubation

mixture. When aspartic acid replaced the NH_4Cl as the source of ^{15}N , the ATP recovered was highly labelled, e.g. 0.575 atom % excess, the content of ^{15}N being at least ten times that observed *in vivo* with $^{15}\text{NH}_4\text{Cl}$.

To confirm that the high content of ^{15}N found in the ATP fraction was not due to contamination with other compounds adenine was prepared from the fraction. On analysis this was shown to have a ^{15}N content only slightly less than that of the ATP fraction from which it had been prepared.

Fractionation of muscle homogenates. As a preliminary to purification of the enzyme system responsible for the incorporation of ^{15}N into ATP, rabbit-muscle homogenates prepared in the KCl-tris medium were separated into a so-called soluble fraction and the sediment obtained by centrifuging at 15 000 g for 20 min. These two fractions were then incubated independently with ^{15}N -aspartic acid, ATP, IMP and fructose 1:6-diphosphate, and the ^{15}N content of the ATP present in the medium after incubation was then determined as before. Both the soluble and the sedimented fractions gave rise to ATP of high ^{15}N content (Table 2). The breakdown of ATP due to dephosphorylation and deamination by the myofibrils in the sedimented fraction, however, was extensive, with the result that the yield of labelled ATP in this system was low. The total amount of ^{15}N recovered in the ATP isolated after incubation with the soluble fraction was 10 times greater than that recovered under similar circumstances with the sedimented fraction.

Table 2 includes also the results of experiments to show the effects of various treatments on the ability of the soluble fraction to incorporate ^{15}N from labelled aspartic acid into ATP. Both dialysis and precipitation as the acetone-dried powder led to some loss in activity. Considerable activity survived the latter treatment, however, and the acetone-dried powder provided a convenient source of the enzyme for subsequent studies.

Localization of ^{15}N in adenosine triphosphate

When ATP isolated from muscle, after administration *in vivo* of labelled ammonium citrate, was incubated with myofibrils, analysis of the isotope content of the NH_3 liberated showed that the ^{15}N concentration in the 6-amino group was about twice that in the molecule as a whole. This indicated that although there was greater specific incorporation into the 6-amino group than into the whole purine, some isotope had also been introduced into the adenine ring, presumably as a consequence of fresh synthesis occurring during the course of the experiment (Table 3). When muscle homogenates were used with ^{15}N aspartic acid as the nitrogen source the incorporation was confined practically entirely to the 6-amino group of the ATP. The results obtained in Expt. 2, Table 3, show that enzymic deamination of the AMP, obtained by degradation of ATP isolated from muscle homogenates after incubation, produced NH_3 4.9 times as highly labelled as the original AMP. Only about 2% of the total ^{15}N was present in the IMP left after deamination.

Properties of the acetone-dried powder extract

Acetone-dried powders prepared from rabbit muscle were not as active per gram of original

tissue as fresh homogenates, but they possessed the advantage of retaining activity after storage at -15° for periods up to 3 weeks. In addition, study of the reamination of IMP was facilitated with aqueous extracts of these powders because on a nitrogen basis the 5'-adenylic deaminase activity was only about 5% of that of whole-muscle homogenates; the adenosine triphosphatase activity was also much less. Usually acetone-dried powders were obtained from rabbit skeletal muscle but an active preparation was also made from pigeon breast muscle. An extract from the acetone-dried powder of rabbit heart gave a relatively small incorporation of isotope into ATP.

In many of the experiments with homogenates and extracts of acetone-dried powder, fructose 1:6-diphosphate and diphosphopyridine nucleotide were added to promote the phosphorylation by anaerobic glycolysis of any AMP formed. Results of an experiment designed to show the effect of omitting various components from the usual incubation system are presented in Table 4. Omission of fructose 1:6-diphosphate reduced the extent of incorporation only by a minor degree, and added ATP was likewise apparently not essential for the system. In fact the greatest incorporation obtained in this type of experiment, both from the point of specific activity and total incorporation,

Table 3. Concentration of ^{15}N in the 6-amino group of adenosine triphosphate

Incubation medium for the experiment *in vitro* contained 5 mM-IMP, 5 mM-ATP, mM- MgCl_2 , 2 mM- ^{15}N aspartic acid, 10 μg . of pyridoxal phosphate/ml., 0.1 M-KCl, 50 mM-Sorensen phosphate buffer, pH 7.4. Enzyme extract was 20% of total volume.

Expt.	Conditions	Nucleotide fraction or derivative	Atoms % excess of ^{15}N
Expt. 1			
	^{15}N Ammonium citrate injected <i>in vivo</i>	ATP* NH_3 (from ATP 6- NH_2 group)	0.091 0.153
Expt. 2			
	^{15}N Aspartic acid incubated with soluble fraction of muscle homogenate	AMP (from ATP) NH_3 (from AMP 6- NH_2 group) IMP (from AMP)	0.278 1.350 0.028

* Total N of this fraction was 3930 μg .atoms. From u.v.-absorption data it was estimated that 4000 μg .atoms of N were present as adenine nucleotide.

Table 4. Adenine and inosine nucleotides and the incorporation of ^{15}N into adenosine polyphosphates from aspartic acid by acetone-dried powder extracts

Incubation was for 1 hr. at 20° in 50 mM-Sorensen phosphate buffer, pH 7.4, 5 mM- MgCl_2 , 0.002% of coenzyme I, 5 mM- ^{15}N aspartic acid and, where added, 7 mM-IMP, 5 mM-ATP, 5 mM-AMP and 10 mM-fructose 1:6-diphosphate (FDP). Total volume, 10 ml. (including 5 ml. of acetone-dried powder extract).

Additions to basic medium	IMP (μmoles)	ADP + ATP (μmoles)	^{15}N in ATP (atoms % excess)	^{15}N incorporated (% of total)
AMP, IMP, FDP	69	49	0.293	2.4
AMP, IMP, FDP boiled enzyme	71	47	Nil	Nil
ATP, IMP	69	49	0.253	2.11
IMP, FDP	59	9	1.90	2.89
AMP, FDP	0	44	0.221	1.65
ATP	0	45	0.120	0.91

was obtained when IMP and fructose 1:6-diphosphate alone were added to the medium. The net synthesis of ATP in this system was, however, small. The complete system was heat-labile and the extent of incorporation rose with time. After 4 hr. 28.7% of the aspartic acid ^{15}N had been incorporated into the adenosine polyphosphate fraction, which had increased in amount with a complementary decrease in the IMP level compared with the concentrations of nucleotides at the beginning of the incubation (Table 5).

Precursors for the nitrogen of the 6-amino group of adenosine triphosphate

The most effective of the three possible precursors for the 6-amino group of the adenine nucleotides which were tested was aspartic acid. This applied both with homogenates and with extracts of acetone-dried powders of rabbit muscle. In the latter system there was no incorporation of ^{15}N from $^{15}\text{NH}_4\text{Cl}$ (Table 6) and the results obtained with this substance in the whole animal were

apparently due to prior conversion in the animal's tissues of the inorganic nitrogen into some other substance which could act as a direct precursor.

Nekhorocheff (1955) has reported that creatine and arginine can act as nitrogen donors for the reamination of IMP. As dialysed acetone-dried powder extracts could catalyse the reamination of IMP in the absence of added creatine or arginine it seems unlikely that these substances were acting as precursors in the studies reported here. Creatine could not be detected in extracts of the acetone-dried powders and the arginine content was always less than 5 $\mu\text{g./ml.}$

When [^{15}N]glutamic acid was used as the source of nitrogen, some slight but significant incorporation was observed. It was possible that in this system the glutamic acid was acting by transamination reactions to produce labelled aspartic acid from traces of oxaloacetate present in the extract. This last-named reaction did occur in the extracts, for incubation of the enzyme with glutamate alone produced traces of chromatographically identifiable

Table 5. *Time course of transfer of ^{15}N from aspartic acid into adenosine polyphosphates by an acetone-dried powder extract of muscle*

Incubation was carried out at 20° in 10 mM-Sorensen phosphate buffer, pH 7.4, mM- MgCl_2 , 0.002% of coenzyme I, 5 mM- ^{15}N aspartic acid, 5 mM-IMP, 5 mM-AMP, 10 mM-fructose 1:6-diphosphate, 10 $\mu\text{g.}$ of pyridoxal phosphate/ml. Total volume, 5 ml. (including 2.5 ml. of aqueous acetone-dried powder extract).

Incubation time (hr.)	IMP (μmoles)	ADP + ATP (μmoles)	^{15}N in ADP + ATP (atoms % excess)	Total incorporation (% of total ^{15}N)
0	22.2	(23.0)*	—	—
0.5	16.5	28	0.394	3.0
1.0	15.5	29	0.770	7.3
2.0	10.0	36	1.018	12.4
4.0	3.6	41	2.071	28.7

* Measured as AMP.

Table 6. *Glutamic acid, aspartic acid and ammonium chloride as source of ^{15}N incorporated into adenine nucleotides*

Incubations were carried out at 20°. Basic medium contained 5 mM-ATP, 5 mM-IMP, 10 mM-fructose 1:6-diphosphate, 10 mM-Sorensen phosphate buffer, pH 7.4, mM- MgCl_2 and 0.002% of coenzyme I. To this were added 5 mM-oxaloacetate or α -oxoglutarate, 5 mM- ^{15}N -aspartate, -glutamate or $^{15}\text{NH}_4\text{Cl}$ as indicated. Total volume, 6 ml. (including 3 ml. of acetone-dried powder extract), except in Expt. 3 where 2.5 ml. of extract was used and the total volume was 5 ml.

Expt. no.	Source of N	Incubation time (hr.)	IMP (μmoles)	ADP + ATP (μmoles)	^{15}N incorporated into ADP + ATP (atom % excess)
1	Aspartic acid	4	30	32.5	0.270
	NH_4Cl	4	26	35	Nil
2	Aspartic acid	4	30	33	0.280
	Aspartic acid + oxaloacetic acid	4	—	38	0.270
	Glutamic acid	4	32	30	0.003–0.013
	Glutamic acid + oxaloacetic acid	4	32	31	0.061
3	Aspartic acid	1	19	27	0.87
	Aspartic acid + α -oxoglutarate	1	22	25	0.447

aspartic acid. Further suggestions that glutamate was not acting directly as a nitrogen source, but rather through transamination reactions, were obtained by the following observations. With glutamate as the source of ^{15}N addition of oxaloacetate increased the incorporation of isotope into the adenosine polyphosphates, whereas with aspartate as the source of the isotope, incorporation decreased when α -oxoglutarate was added to the system.

Evidence for the existence of an intermediate in the reamination of inosine monophosphate by aspartic acid

It was first observed that a small fraction, not identical with any of the known inosine or adenine nucleotides, could be isolated by chromatography of the nucleotide extract from whole muscle obtained after the injection of [^{15}N]ammonium citrate. This small fraction (Table 1, fraction 3), which was eluted after ADP, consistently possessed much higher concentrations (usually greater than tenfold) of ^{15}N than any of the fractions containing the known adenine nucleotides. It had an absorption spectrum in the ultraviolet region with a maximum at $265\text{ m}\mu$ at pH 2.0. On analysis it was shown to possess the following characteristics. Hydrolysis in N-HCl at 100° for 1 hr. gave rise to adenine, which was identified chromatographically, and another compound which exhibited maximum absorption in the ultraviolet at $273\text{ m}\mu$. Attempts to characterize fraction 3 further were unsuccessful, due partly to the high salt content and partly to the small quantities available.

A similar compound was also found to accumulate after incubation of acetone-dried powder extracts with [^{15}N]aspartic acid, IMP and fructose 1:6-diphosphate. This compound was present in the combined ADP and ATP fraction (i.e. that eluted between 0.01 N- and 0.1 N-HCl). For example, in the experiment illustrated in Table 5 it represented 20% of the combined ADP + ATP fraction. Some acetone-dried powder extracts which possessed low ability to incorporate ^{15}N into ATP still promoted the formation of this substance, which was often more easily detected in such systems than in those rapidly catalysing incorporation of ^{15}N into ATP. The substance was not separated from IMP in the isobutyric acid- NH_3 system (Krebs & Hems, 1953), but satisfactory resolution was achieved in a system containing acetic acid-water-acetone (1:4:10, by vol.) and its formation could be semi-quantitatively followed under these conditions. The unidentified compound moved in front of IMP and the adenine nucleotides in this system. Elution of the unidentified spot from the chromatogram provided enough to measure the ultraviolet-absorption spectrum. It was characterized by a

maximum at $265\text{--}267\text{ m}\mu$ in 0.1 N-HCl which shifted to $270\text{ m}\mu$ at pH 13.0 (Fig. 2). Hydrolysis for 30 min. in N-HCl at 100° shifted the absorption maximum to $275\text{ m}\mu$.

In the usual incubation medium containing 10 mM-IMP , $10\text{ mM-aspartic acid}$ and $10\text{ mM-fructose 1:6-diphosphate}$ the new spot was readily detectable. Fructose 1:6-diphosphate was not an essential addition for its formation but in the absence of either IMP or aspartic acid it could not be detected on the chromatogram. Replacement of aspartic acid by glutamic acid also failed to produce a detectable spot.

On prolonged incubation of IMP with aspartic acid in the presence of the aqueous extract of acetone-dried powder of muscle the characteristic inosine nucleotide ultraviolet-absorption spectrum with a maximum at $248\text{ m}\mu$ shifted to one with the maximum in the range $258\text{--}265\text{ m}\mu$. A shift to the longer wavelength would be expected on formation of adenine compounds. This change in absorption spectrum suggested the possibility of following the reaction in semi-purified systems by a spectrophotometric method. The wavelength $280\text{ m}\mu$ was chosen as convenient for this purpose and a regular although small increase in absorption at $280\text{ m}\mu$ occurred as the reaction progressed. This increase did not occur in the absence of aspartic acid or IMP, and glutamic acid was not effective in replacing aspartic acid.

The experiments indicated that both IMP and aspartic acid were essential for the formation of an

Fig. 2. Ultraviolet-absorption spectra of unidentified compound formed on incubation of IMP and aspartic acid with an aqueous extract of acetone-dried powder of muscle. —, At pH 1.0 (0.1 M-HCl), max. $265\text{--}267\text{ m}\mu$; ---, at pH 13.0 (0.1 M-NaOH), max. $268\text{--}270\text{ m}\mu$.

Table 7. *Incorporation of ^{15}N into unidentified compound*

Incubation was for 4 hr. at 20° in medium identical with the basic medium described in Table 6. Total volume, 10 ml. (including 5 ml. of acetone-dried powder extract).

Nucleotide	Amount (μ moles)	Concn. of ^{15}N (atoms % excess)
IMP	26	—
ADP	1.1	1.320
Unknown	1.8	9.20
ATP	17	0.186

unidentified compound, which was probably an intermediate in the amination of IMP by this amino acid. In one case it was possible to isolate enough of the compound to carry out ^{15}N determinations (Table 7). The ^{15}N content was high, approximately one-sixth of that of the original aspartic acid, whereas the isotope level in the adenine nucleotides was much lower. This level of labelling of the unknown is close to the value which would be expected if it were adenylosuccinate. In this experiment, as in others carried out with systems *in vitro*, the ADP was more highly labelled than the ATP, which is consistent with the hypothesis that IMP is first reaminated to AMP which is subsequently phosphorylated in turn to ADP and ATP.

The properties of the intermediate isolated both in the experiments *in vivo* and *in vitro* are very similar to those of adenylosuccinate (Carter & Cohen, 1955), which was first shown to be an intermediate in the formation of AMP from IMP in yeast preparations. This compound can also be produced in yeast preparations by the incubation of fumarate and AMP. With muscle preparations, however, it was not possible to detect chromatographically the formation of the unidentified compound on incubation of fumarate and AMP.

DISCUSSION

In view of the findings reported above with the rabbit and pigeon and those of Kalckar & Rittenberg (1947) with the rat the active metabolism of the 6-amino group of adenine nucleotides is apparently a widespread reaction in skeletal muscle. Experiments with the aqueous extracts of acetone-dried powders indicate that in such systems the amino group of aspartic acid is an active precursor of the purine amino group whereas ammonium chloride is ineffective. The slight activity of glutamic acid is probably due to the transfer of its amino group to aspartic acid by transamination systems rather than to glutamic acid itself acting as a direct precursor.

As aspartic acid was the only nitrogen source

which needed to be added to dialysed enzyme preparations it seems unlikely that either creatine (Nekhorocheff & Wajzer, 1952; Nekhorocheff, 1955) or β -alanine (Kometiani & Klein, 1956) play a role in the reamination process studied here. The possibility cannot be excluded, however, that aspartic acid is decarboxylated to β -alanine by muscle preparations by some reaction not yet described. It is perhaps more likely that the results with β -alanine are explained by transfer of its amino group to aspartic acid by transamination reactions. Certainly in the yeast system for AMP formation from IMP (Carter & Cohen, 1955) it has been clearly shown that aspartic acid donates the amino group with the formation of adenylosuccinate as an intermediate. The widespread nature of this reaction is suggested from evidence which is accumulating with other systems (Abrams & Bentley, 1955; Liebermann, 1956). Significantly, in the rabbit-muscle preparations an adenine-containing compound highly labelled with ^{15}N and presumably an intermediate was formed. This compound contained 9.2 atoms % excess of ^{15}N , which is close to the expected value of 11.8 atoms % excess if it were adenylosuccinate. Also the limited study of the properties of the unknown compound which have been carried out are completely compatible with it being adenylosuccinate. Davey (1959) has published preliminary findings confirming the original indications (Newton, 1957; Newton & Perry, 1957) that adenylosuccinate is formed in the acetone-dried powder system we have used. In the work of Davey (1959) no details of the identification of adenylosuccinate are given other than the ultraviolet spectral change on incubation of IMP and aspartate in the appropriate system.

So far as one can conclude from these studies, the capacity of the muscle for reaminating IMP is much less than its potential AMP-deaminating ability. Indeed unless the 5'-adenylic deaminase activity of preparations was reduced in some way, e.g. by making acetone-dried powder, it was difficult to demonstrate an appreciable net formation of adenine nucleotides even when all requirements for the reaction were provided.

The extent to which deamination of AMP accompanies muscle contraction is far from clear. Reports that 5'-adenylic deaminase cannot be detected in certain muscles (Gilmour & Calaby, 1953; Anina, 1955) suggest that deamination need not accompany contraction. Experiments on whole muscle are more equivocal, for analysis of the chemical changes accompanying the rising phase of a single twitch in tortoise muscle has produced no evidence of IMP production (Mommaerts, 1955), although some difficulties in its detection are encountered in this muscle. Nevertheless Lange

(1954-55) could demonstrate an increase in IMP production during short periods of activity in frog rectus abdominus.

More recently by the analysis of changes in the ultraviolet absorption of light transmitted through a muscle fibre it has been concluded that a conversion of adenine into inosine compounds occurs during a single twitch (Wajzer, Weber, Lericque & Nekhorocheff, 1956). Although there is no doubt that IMP is formed in most skeletal muscles the precise stage in the cycle of chemical events when this occurs has yet to be determined. Nevertheless, when the nucleotide is produced it can be reconverted into AMP at the expense of amino acid nitrogen by enzyme systems endogenous to the muscle.

SUMMARY

1. The distribution of ^{15}N in the nucleotides of rabbit skeletal muscle after the administration of [^{15}N]ammonium citrate has been investigated.

2. Incubation of inosine monophosphate with [^{15}N]aspartic acid in the presence of homogenates or extracts of acetone-dried powders made from skeletal muscle produced labelled adenine nucleotides. In these systems no amination of inosine monophosphate took place with ammonium chloride as the nitrogen source and glutamic acid was only very slightly effective.

3. With muscle extracts practically all the isotope incorporated into the adenine nucleotides was localized in the 6-amino group. Nitrogen of both the purine ring and the 6-amino group of muscle adenine nucleotides was labelled after injection of [^{15}N]ammonium citrate.

4. Some properties of an aqueous extract of acetone-dried powder of muscle catalysing amination of inosine monophosphate by aspartic acid have been studied.

5. A compound, highly labelled with ^{15}N , has been isolated from whole muscle and from muscle extracts after incubation with inosine monophosphate and aspartic acid. Those properties of this substance which have been studied are identical with those of adenylosuccinate.

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REFERENCES

- Abrams, R. & Bentley, M. (1955). *J. Amer. chem. Soc.* **77**, 4179.
- Anina, I. A. (1955). *Ukr. biochem. J.* **27**, 137.
- Bergkvist, R. & Deutsch, A. (1954). *Acta chem. scand.* **8**, 1889.
- Carter, C. E. & Cohen, L. H. (1955). *J. Amer. chem. Soc.* **77**, 499.
- Chappell, J. B. & Perry, S. V. (1954). *Nature, Lond.*, **173**, 1094.
- Davey, C. L. (1959). *Nature, Lond.*, **183**, 995.
- Decker, P. & Riffart, W. (1950). *Chemikerztg.* **74**, 261.
- Gilmour, D. & Calaby, J. H. (1953). *Enzymologia*, **16**, 23.
- Hiller, A., Plazin, J. & Van Slyke, D. (1949). *J. biol. Chem.* **176**, 1401.
- Johnson, M. J. (1941). *J. biol. Chem.* **137**, 575.
- Jones, D. B. & Moeller, O. (1928). *J. biol. Chem.* **79**, 429.
- Kalckar, H. M. (1947a). *J. biol. Chem.* **167**, 461.
- Kalckar, H. M. (1947b). *J. biol. Chem.* **167**, 429, 445.
- Kalckar, H. M. & Rittenberg, D. (1947). *J. biol. Chem.* **170**, 455.
- Kleinzeller, A. (1942). *Biochem. J.* **36**, 729.
- Kometiani, P. A. & Klein, E. E. (1956). *Biokhimiya*, **21**, 389.
- Krebs, H. A. & Hems, R. (1953). *Biochim. biophys. Acta*, **12**, 172.
- Lange, G. (1954-55). *Biochem. Z.* **326**, 172.
- Liebermann, I. (1956). *J. Amer. chem. Soc.* **78**, 251.
- Mommaerts, W. F. H. M. (1955). *Amer. J. Physiol.* **182**, 585.
- Needham, D. M. (1932). *The Biochemistry of Muscle*. London: Methuen and Co.
- Nekhorocheff, J. (1955). *Proc. 3rd int. Congr. Biochem., Brussels*, p. 84.
- Nekhorocheff, J. & Wajzer, J. (1952). *Arch. Sci. physiol.* **6**, 275.
- Newton, A. A. (1957). Ph.D. Dissertation: Cambridge University.
- Newton, A. A. & Perry, S. V. (1957). *Nature, Lond.*, **179**, 49.
- Parnas, J. K. (1929). *Amer. J. Physiol.* **90**, 467.
- Parnas, J. K. (1933). *Bull. Soc. Chim. biol., Paris*, **15**, 1384.
- Perry, S. V. (1955). *Methods in Enzymology*, vol. 2, p. 582. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Perry, S. V. & Grey, T. C. (1956). *Biochem., J.* **64**, 184.
- Ratner, S. (1947). *J. biol. Chem.* **170**, 761.
- Roberts, R., Abelson, P., Cowie, D., Bolton, E. & Britten, R. (1955). In *Studies in Biosynthesis in Escherichia coli*. Washington: Carnegie Institute.
- Schmidt, G. (1928). *Hoppe-Seyl. Z.* **179**, 243.
- Sprinson, D. B. & Rittenberg, D. (1948). In *U.S. med. Bull. (Suppl.: Preparation and Measurement of Isotopes and Some of Their Medical Aspects)*, p. 82.
- Sprinson, D. B. & Rittenberg, D. (1949). *J. biol. Chem.* **180**, 707.
- Tarver, E. & Morales, M. (1951). *J. cell. comp. Physiol.* **37**, 235.
- Wajzer, J., Weber, R., Lericque, J. & Nekhorocheff, J. (1956). *Nature, Lond.*, **178**, 1287.
- Wu, H. & Rittenberg, D. (1949). *J. biol. Chem.* **179**, 847.
- Wyatt, G. R. (1951). *Biochem. J.* **48**, 584.