The Preparation of Adenosinetriphosphate from Acetone-dried Muscle and a Chemical Method for the Preparation of Adenosinediphosphate

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During recent years adenosinetriphosphate has been shown to take part in an ever-increasing number of biochemical processes. It may therefore be worth while to record some findings on the isolation of adenosinetriphosphate from acetone-dried muscle and on some properties of its magnesium salt, which allow the preparation of adenosinediphosphate by chemical means.

In a preliminary communication Bielschowsky & Green (1943) gave a short account of the identification of the active principle present in a muscle preparation, 'myotoxin', described by Dyckerhoff, Schörcher & Torres (1939). Adenosinetriphosphate was found to be the agent responsible for the toxic action of myotoxin.

EXPERIMENTAL AND RESULTS

Analytical methods

Ribose was estimated by the method of Mejbaum (1939). The colorimetric readings were taken with an Ilford red filter and standard grey screen (King, Haslewood & Delory, 1937), calibrated against standard solutions of pure arabinose. The use of this procedure allowed accurate readings with a simple colorimeter (Klett), even when very small amounts of pentose had to be estimated. Lohmann's (1928) method of hydrolysis was used in the estimation of total, 7 min. and inorganic P by Briggs's method (1922). Nitrogen estimations were performed by a semi-micro-Kjeldahl method. Digestion was carried out with the addition of pure glucose and Cu selenite (Myrbäck & Euler, 1931). Ba was estimated as sulphate, Mg according to Tisdall & Kramer (1921). C and H estimations were carried out by Dr Weiler and Dr Strauss, Oxford.

Preparation of Dyckerhoff's 'myotoxin'

The myotoxin was obtained from rat, rabbit or ox muscle in the following way: muscle removed immediately after the death of the animal was minced with the greatest possible speed into five to seven times its weight of cold acetone with constant stirring. This process was completed within 10 min. of the death of the animal. The muscle mince was strained through muslin and resuspended in 7 vol. of fresh acetone. After removal of the acetone the process was repeated once more using 5 vol. of acetone. The muscle residue was then shaken with 3 vol. of ether which was filtered off with suction. The material was washed with 2 vol. of ether on the Büchner funnel, and after most of the ether had been removed, the residue was spread out on filter paper and the remaining ether allowed to evaporate at room temperature. The last traces were removed in a vacuum desiccator over $CaCl_2$. Finally the material was powdered, avoiding heating during this process. The dried muscle powder was stored in the refrigerator, where it seemed to remain stable for several months, and was used when required.

The muscle powder (250 g.) was thoroughly mixed with 1250 ml. of saline, extracted for 7 min. at room temperature on a shaker and immediately pressed through a cloth of fine texture. The extract was heated quickly and kept boiling for 7 min. It was then rapidly cooled to room temperature and the precipitate was removed by filtration (Whatman paper no. 54) and discarded. The clear filtrate was transferred into large measuring cylinders, placed in an ice bath, and 3 vol. of ice-cold methanol were added with stirring. After standing for 2 hr. the clear supernatant fluids were removed. The remaining suspension was spun down and resuspended in the centrifuge tubes with approximately 2 vol. of methanol and again centrifuged. The washing with methanol was repeated and followed by two washings with absolute ether. The white precipitate was transferred into wide capsules, quickly broken up and the ether evaporated in a vacuum desiccator (yield 3·25–3·6 g.).

Batches of 5 g. of this product were placed in a mortar and dissolved in 250 ml. of boiling saline, added in small portions. The solution was transferred to a fairly large beaker and boiled for 3 min. After rapid cooling to room temperature it was filtered (Whatman paper no. 5). After adding 3 vol. of cold methanol to the filtrate, it was left in the refrigerator for 2 hr. or longer. The resulting precipitate was washed and dried as described above. A white, amorphous, non-hygroscopic powder, soluble in water, was obtained which corresponds to 'myotoxin' (yield from 250 g. dry muscle powder, 2·3–2·5 g.).

The yield of this product can be increased by about 50% if the muscle residue after the saline extraction is re-extracted with about 600 ml. of saline and the two extracts pooled and worked up as described. Preparations different in appearance and properties from those obtained by cold saline extraction resulted from re-extraction with boiling saline as recommended by Dyckerhoff *et al.* (1939). The largest amount of muscle powder which could conveniently be worked up at one time in a laboratory with limited facilities was 250 g.

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Chemical examination of the white powder (myotoxin) showed that it contained 15–20% protein and 30% ash, the latter consisting mainly of magnesium pyrophosphate. Only traces of potassium and sometimes of glycogen were found. The orcinol reaction was strongly positive.

Isolation of adenosinetriphosphate from myotoxin

Separation of the protein from the non-protein components present in myotoxin was achieved by salicylic acid or 2N-hydrochloric acid.

To 1 g. of the preparation, dissolved in 50 ml. of water, a saturated aqueous solution of salicylic acid was added with stirring and ice cooling until the pH of the mixture was 4·5. After standing for 15 min. the protein precipitate was centrifuged off. From the supernatants the non-protein component was precipitated by acetone with a trace of solid NaCl. The resulting precipitate was washed once with acetone and once with ether in the centrifuge, then dried in a vacuum desiccator.

This product dissolved rather easily in water, at first forming droplets which then gave clear solutions of pH 6·6–6·8. All protein reactions were negative; sulphosalicylic acid, however, produced a faint turbidity in the solutions. The ash content of the preparation was, on the average, 25%. The orcinol reaction was strongly positive. After acid hydrolysis adenine was isolated as picrate, m.p. 279°. (Found: N, 29·1. Calc. for $C_{11}H_{10}O_8N_8$: N, 29·3%.) The molecular proportions of Mg : total organic P : 7 min. P were found to be 1·99 : 3·0 : 2·0; inorganic P was absent. These results suggested the presence of a magnesium salt of adenosinetriphosphate (ATP). This was confirmed by the preparation of the acridine salt of ATP (Wagner Jauregg, 1936). For this purpose the deproteinization with salicylic acid was not suitable, since an acridine salt of salicylic acid, m.p. 132°, was found to contaminate the nucleotide precipitate. Therefore 2N-hydrochloric acid was used for deproteinization. The acridine precipitates were collected on a Büchner funnel, washed once with ethanol-ether (1 : 1), followed by ether. After one recrystallization from water they melted at 210° with decomposition. These acridine precipitates could be fractionated to yield one compound which was insoluble in cold water and only very sparingly soluble in ethanol, and a second compound which was much more soluble in both these solvents. More than 90% was present in the form of the insoluble compound which, after one recrystallization from water, gave a satisfactory analysis for the acridine salt of ATP. (Found: N, 10·8. Calc. for $C_{10}H_{16}O_{13}N_5P_3 + 2C_{13}H_9N$: N, 11·3%.) The ratio total organic P : 7 min. P was 1·5. The melting point remained unchanged at 210° (decomp.).

Preparation of the barium salt of adenosinetriphosphate

Finely powdered myotoxin (5 g.) was dissolved in a mortar with 100 ml. of cold water, added in small quantities. Any insoluble material was removed either by filtration (Whatman paper no. 54) or centrifugation. All further steps were carried out at temperatures near 0° with ice-cold reagents. The clear solution was brought to pH 4·5 by adding glacial acetic acid drop by drop with constant stirring. The precipitate formed was spun down after 15 min. and discarded. To the clear supernatant a 25% (w/v) solution of Ba acetate in water was added in slight excess. The pH rose to about 6. The resulting precipitate was washed with water and purified as the Hg salt (Lohmann, 1931). The final Ba precipitation was carried out at pH 4·5–4·6. This second Ba precipitate was washed twice with water, 50% (v/v) ethanol, absolute ethanol and ether.

The barium salt obtained corresponded to $Ba_{1.5}$ ATP. The water content of the preparation varied in different batches; 4, more often 6, H_2O were found in the samples. Differences in the barium content were slight and probably due to variations in pH during the final precipitation, as reported by Kiessling (1934) for the barium salt of inosinetriphosphate. (Found: N, 8·5; P, 11·4; Ba, 26·5. Calc. for $C_{10}H_{13}O_{13}N_5P_3Ba_{1.5} + 6H_2O$: N, 8·6; P, 11·4; Ba, 25·2%.) The average yield was 3 g.

The magnesium salt of adenosinetriphosphate

Solutions of the magnesium salt of ATP could easily be prepared by treating the barium salt with magnesium sulphate. Such solutions containing between 30 and 50 mg. of Mg ATP/ml. were viscous, frothed easily and resembled solutions of polymers such as nucleic acids rather than solutions of mononucleotides. Difficulties arose when attempts were made to isolate the magnesium salt. Precipitation from aqueous solutions with ethanol or acetone was incomplete and the solubilities of the preparations thus obtained varied from batch to batch. Under apparently identical conditions of isolation preparations were obtained which were easily soluble in water, whereas others dissolved only on prolonged shaking and some were scarcely soluble at all. The yields of Mg ATP varied between 60 and 93%; from the less readily soluble preparations solutions were obtained which contained from 15–25 mg./ml. at 18°. Attempts to obtain soluble magnesium salts using the method of Neuberg & Sabetay (1925) also failed to yield uniform preparations.

It was outside the scope of this work to investigate why pure preparations of Ba ATP yielded magnesium salts which differed so widely in their physical properties, but one observation seems worth mentioning. When Mg ATP preparations obtained by methanol precipitation were redissolved in water a highly viscous mass remained undissolved. This viscous residue was easily soluble in 0·05N-

hydrochloric acid. Reprecipitation with methanol yielded a colourless, amorphous substance. Aqueous solutions of this substance were slightly opaque, their N : P : 7 min. P ratio was 10·02 : 5 : 2·95, values suggestive of diadenosine pentaphosphate, the dinucleotide which Embden (1932) suggested was present in heart muscle.

One of the reasons for the failure to obtain reproducible preparations of Mg ATP became obvious when the extraordinary instability of this compound in aqueous solutions was noticed. In initially clear solutions a slight turbidity appeared when they were left at room temperature (18°) for several hours. This turbidity increased progressively and a precipitate formed. A similar observation has been reported by Barrenscheen & Filz (1932). Precipitates appeared at once when solutions of Mg ATP were boiled. In both cases the precipitates consisted of colourless, slightly curved needles of varying length.

It seemed of interest to study the changes occurring in solutions of Mg ATP.

Dephosphorylation of the magnesium salt of adenosinetriphosphate

A clear, neutral solution of 500 mg. of Mg ATP in 15 ml. of water was placed in a water bath which was slowly heated, the boiling point being reached within 30 min. When the temperature had reached 50° the solution became increasingly turbid, flocculation was noticed at about 60° and a precipitate started to settle out at about 65°. The mixture was kept for 1 hr. in the boiling-water bath under a reflux condenser and then cooled with running water. In a filtered sample no more precipitate was formed on further heating. The precipitate was collected on a Büchner funnel, washed with hot water and dried over calcium chloride. The substance was only slightly soluble in hot water. It dissolved in dilute mineral acid, but could not be reprecipitated on neutralization. It contained only traces of inorganic P, the orcinol reaction was positive and organic and acid-labile P were found. The ratios of N : P : 7 min. P were 4·83 : 3·0 : 1·93, suggestive of slightly impure ATP. The yield was on the average about 25 % of the Mg ATP originally present.

The filtrate from this product contained large amounts of inorganic phosphate. On addition of dilute ammonia $(NH_4)MgPO_4$ was precipitated which, after one recrystallization, contained 5·9 % N (calc. N, 5·7 %). The filtrate from this precipitate contained only a trace of inorganic phosphate, the orcinol reaction was strongly positive and organic phosphate was present which could be partly hydrolysed in 7 min. For the isolation of the substance responsible for these reactions the solution was acidified with glacial acetic acid, and lead acetate solution was added until no further precipitate formed. After several washings with water the precipitate was decomposed with hydrogen sulphide. In the clear filtrate acetone produced an oily precipitate, which, after collecting on the centrifuge, was hardened with acetone and dried with absolute ether. The product showed signs of crystallization and melted with decomposition at 95–100°. It was extremely hygroscopic, the aqueous solution giving a strongly acid reaction. On analysis it contained N : P : 7 min. P in the ratio 5·4 : 2 : 1·04. These values suggest the formation of adenosinediphosphate.

The fraction of Mg ATP which precipitated out during the heating could be dephosphorylated in the same way by redissolving it in acid, neutralizing and reheating.

The amount of insoluble Mg ATP was found to decrease to about 10 % when the heating was applied to solutions of pH 4·5. At this pH heating for periods longer than 1 hr., gave rise to the formation of lower split products (Table 1).

Preparation of barium adenosinediphosphate

For the preparation of Ba adenosinediphosphate (ADP) the following procedure was adopted. $Ba_{1·5}$ ATP was dissolved in 0·2 N-HCl, the Ba was removed with the calculated amount of $MgSO_4$ (10 % (w/v) in water). To the Ba-free

Table 1. *Dephosphorylation in magnesium adenosinetriphosphate* (Mg *ATP*) *solutions at* pH 4·5

(Mg ATP solution prepared at 0°: $Ba_{1·5}$ ATP dissolved in 0·2 N-HCl, Ba removed with calculated amount of $MgSO_4$, N-NaOH added to pH 4·5. (1·088 mg. total P/ml., trace of inorganic P, N : 7 min. P = 5 : 1·94.) Samples (in individual graduated tubes) were placed simultaneously in a water bath at 20°. After having reached this temperature the first sample was removed, followed by others at specified times and temperatures. All were immersed in ice water, made up to volume and filtered.)

Time (min.)	Bath temp. (°)	Appearance of sample	(mg./ml. of filtrate) Total P	Inorganic P	Organic P	7 min. P	N	Ratio N : 7 min. P
0	20	Clear	1·09	0·05	1·04	0·65	0·82	5 : 1·80
18	92	Turbid	1·08	0·06	1·02	0·63	0·80	5 : 1·78
20	100	Precipitate	0·96	0·06	0·90	0·60	0·77	5 : 1·75
30	100	Precipitate	0·91	0·19	0·73	0·38	0·73	5 : 1·17
60	100	Precipitate	0·89	0·28	0·61	0·28	0·73	5 : 0·88
90	100	Precipitate	0·89	0·34	0·55	0·23	0·74	5 : 0·69
120	100	Precipitate	0·90	0·40	0·49	0·17	0·74	5 : 0·50

solution N-NaOH was added to bring the pH to 4·5. Of this solution 100 ml. were heated for 60 min. in a boiling-water bath, cooled and the precipitate filtered off. Table 2 shows the results of P and N estimations in this filtrate and also the values in the solution before heating was started. The filtrate was cooled in ice, acidified with 0·2N-HNO_3 and Lohmann reagent added in slight excess. The resulting precipitate, after washing, was resuspended in water, decomposed by H_2S, the filtrate from HgS aerated to remove excess H_2S. A saturated solution of Ba acetate in 50% (v/v) ethanol was added and finally absolute ethanol to bring its total concentration in the solution to 50%. The resulting Ba precipitate was washed with 50% ethanol, ethanol and absolute ether and finally dried in a vacuum desiccator.

Table 2. *Dephosphorylation in a magnesium adenosinetriphosphate* (Mg *ATP*) *solution used for the preparation of adenosinediphosphate* (*ADP*)

(Mg ATP solution prepared as in Table 1. Samples were taken before and after heating in a boiling-water bath for 60 min.)

	In solution at start	In filtrate after heating
Total P (mg./ml.)	0·93	0·81
Inorganic P (mg./ml.)	0·04	0·24
Organic P (mg./ml.)	0·89	0·57
7 min. P (mg./ml.)	0·59	0·27
N (mg./ml.)	0·68	0·66
Organic P/7 min. P	1·51	2·09
Ratio N : P	5·09 : 3	5·13 : 2

The Ba ADP retained $3H_2O$ on drying *in vacuo* at 50°. (Found: N, 10·3; P, 8·9; 7 min. P, 4·5. Calc. for $C_{10}H_{12}O_{10}N_5P_2Ba_{1·5}+3H_2O$: N, 10·2; P, 9·1; 7 min. P, 4·5%.) (Found, after drying over P_2O_5 at 113°: N, 11·1. Calc.: N, 11·1%.) Yield 80%.

To establish the identity of the chemically prepared Ba ADP with preparations obtained in the usual way by enzymic dephosphorylation of ATP some derivatives were prepared. The acridine salt, after one recrystallization from water had the correct m.p. 215° (Wagner Jauregg, 1936). (Found: N, 13·6. Calc. for $C_{10}H_{15}O_{10}N_5P_2+C_{13}H_9N$: N, 13·9%.) The barium salt was regenerated from the acridine salt. (Found, after drying at 50° *in vacuo*: C, 17·7; H, 2·5; N, 10·1; Ba, 29·3. Calc. for $C_{10}H_{12}O_{10}N_5P_2Ba_{1·5}+3H_2O$: C, 17·5; H, 2·7; N, 10·2; Ba, 30·1%.)

Adenine picrate was isolated from Ba ADP solution after hydrolysis in N-hydrochloric acid. (Found: N, 29·5. Calc. for $C_{11}H_{10}O_8N_8$: N, 29·3%.) Adenylic acid, prepared from Ba ADP according to Lohmann (1932) and isolated using the acetone method (Embden & Zimmermann, 1927) had m.p. 199°. The mixed m.p. with samples of adenylic acid prepared from muscle and from Ba ATP was identical. The Klimek & Parnas (1932) modification of the Böeseken reaction indicated the presence of a 5-nucleotide.

The ADP obtained by chemical means reacted with N-acid and with nitrite as would be expected, ADP having the constitution postulated by Lohmann (1935) and confirmed by Gulland & Farrell Walsh (1945).

The formation of inorganic phosphate when ATP solutions were heated to 100° could also be observed with salts other than the magnesium salt, e.g. the sodium and barium salts, but these salts were less suitable for the preparation of Ba ADP. Isolation of the pure compound was difficult in one case due to the solubility of the fraction of Na ATP which remained unsplit, and in the other due to the very low solubility of $Ba_{1·5}$ ATP at pH 4·5. The increase of inorganic phosphate in solutions of Na ATP and Ba ATP when heated to 100° for 60 min. is shown in Table 3.

Table 3. *Dephosphorylation in sodium and barium adenosinetriphosphate solutions at* 100°

(Ba ATP solution: saturated solution of $Ba_{1·5}$ ATP at 18°. Na ATP solution: prepared from $Ba_{1·5}$ ATP as in Tables 1 and 2.)

	Na ATP	Ba ATP
Total P (mg./ml.)	2·14	0·56
Inorganic P at start (20°) (mg./ml.)	0·11	0·01
Inorganic P after 60 min. in boiling-water bath (mg./ml.)	0·43	0·16

Inosinediphosphoric acid could be isolated by the same method from solutions of magnesium inosinetriphosphate, prepared from $Ba_{1·5}$ ATP according to Kleinzeller (1942).

Evidence for the presence of inosinetriphosphate in myotoxin preparations

When myotoxin solutions, after deproteinization with 2N-HCl, were treated with acridine, a precipitate was obtained, of which 90% consisted of the acridine salt of ATP, as already mentioned. The amounts of the ethanol-soluble acridine compound available were so small that purification by repeated recrystallization was not attempted. When this acridine compound was split by treating with sodium carbonate, the acridine filtered off by suction and the last trace removed by ether extraction, the ratio of total organic P : 7 min. P in the filtrate was 3 : 2 and on hydrolysis it yielded hypoxanthine which was isolated as the picrate. (Found: N, 25·8. Calc. for $C_{11}H_9O_9N_7$: N, 25·6%.) An acridine salt of inosinetriphosphoric acid, prepared from ATP, showed the same solubilities in water and ethanol as the ethanol-soluble fraction obtained from myotoxin.

Myotoxin as a source of adenosinetriphosphate

In order to test whether extraction of acetone-dried muscle with saline was a satisfactory method for the preparation of ATP the following experi-

ment was carried out. Saline and trichloroacetic acid extracts were simultaneously prepared from acetone-dried rabbit muscle. Pentose : 7 min. P ratios of 1 : 2·02 and 1 : 2·00, respectively, were found. The inorganic P content was 0·252 mg./ml. in the saline and 0·376 in the trichloroacetic extract. After leaving both extracts for 24 hr. at 15°, 0·243 and 0·405 mg. of inorganic P were found, figures which rule out enzymic activity in the saline extract. However, when the saline extract was heated slowly and then boiled with a total exposure to heat of 15–20 min. as in the preparation of myotoxin, the inorganic P rose to 0·286 mg./ml. of filtrate and a pentose : 7 min. P ratio of 1 : 1·53 was found, indicating a loss of ATP.

DISCUSSION

The work described in this paper formed part of an investigation on traumatic shock carried out under the aegis of the Medical Research Council during the years 1942–5, a full report of which has been deposited at the Library of the National Institute for Medical Research, London, N.W. 3.

The isolation of ATP from myotoxin led to the elaboration of a simple method for the preparation of pure ATP, applicable to small or large amounts of acetone-dried muscle. This material seems to be a good source of ATP. The inactivation of adenosinetriphosphatase by acetone, described by Barrenscheen & Lang (1932), may well explain the stability of ATP in such muscle preparations.

Traces of inosinetriphosphate were found to be present in some batches of ATP prepared by the method described. It seems likely that this compound was preformed in the muscle before immersion in acetone. Lohmann (1932) has shown that under certain conditions inosinetriphosphate can be formed in frog muscle by deamination prior to dephosphorylation.

A study of the magnesium salt of ATP led to some interesting observations. Evidence has been obtained of the presence of a polynucleotide precipitated by methanol from Mg ATP solutions. A preparation of the same composition was isolated by Embden (1932) from heart muscle and later confirmed by Ostern (1934).

Dephosphorylation in Mg ATP solutions occurs at room temperature and is accelerated by elevated temperatures. Suitable conditions have been found under which this breakdown of Mg ATP can be utilized for the preparation of adenosinediphosphate.

On the basis of the observations mentioned it seems doubtful whether Dyckerhoff's (1939) method of deproteinization as given for myotoxin is the best procedure for the preparation of ATP. The main constituent of myotoxin is Mg ATP which is easily dephosphorylated when its solutions are exposed to heat. The yield of ATP from saline extracts could probably be improved by a method of deproteinization avoiding high temperatures.

SUMMARY

1. A toxic principle present in the myotoxin of Dyckerhoff *et al.* (1939) has been identified as magnesium adenosinetriphosphate.

2. Traces of inosinetriphosphate have also been found in some batches of myotoxin.

3. A chemical method for the preparation of adenosinediphosphate (as barium salt) from magnesium adenosinetriphosphate is described.

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