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The Preparation of Phosphoarginine: a Comparative Study

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Phosphoarginine was first isolated from fresh-water crab muscle as an amorphous barium salt $[\text{Ba}(\text{C}_6\text{H}_{14}\text{N}_4\text{O}_5\text{P})_2 \cdot 2\text{H}_2\text{O}]$ by Meyerhof & Lohmann (1928). No further work on this compound was reported until the publication by Ennor, Morrison & Rosenberg (1956) of an improved method for its isolation. This method took advantage of the fact that phosphoarginine forms a copper salt of limited solubility in water and a hydrochloride derivative that is insoluble in ethanol. The product differed in composition from that described by Meyerhof & Lohmann (1928) in that it corresponded to the formula $\text{C}_6\text{H}_{13}\text{BaN}_4\text{O}_5\text{P} \cdot \text{H}_2\text{CO}_3 \cdot \text{H}_2\text{O}$.

More recently, synthetic methods have been used to prepare phosphoarginine. Thus Thiem, Thoai & Roche (1962) developed a procedure that involved phosphorylation of arginine with phosphoryl chloride and separation by column chromatography of the monophosphorylated derivative. The latter was obtained as a crystalline lithium salt, $\text{C}_6\text{H}_{13}\text{Li}_2\text{N}_4\text{O}_5\text{P}$. Cramer, Schieffele & Vollmar (1962) have used more specific reactions to obtain the amorphous barium salt of phosphoarginine, $\text{Ba}(\text{C}_6\text{H}_{14}\text{N}_4\text{O}_5\text{P})_2 \cdot 3\text{H}_2\text{O}$. One involved the phosphorylation of N^α -benzyloxycarbonylarginine with bis-(*p*-nitrobenzyl) phosphorochloridate, and the other involved the transfer of the phosphorylated

amidino group of *O*-methyl-*N*-phosphorylisourea to ornithine.

In connexion with plans to carry out detailed kinetic studies of the reaction catalysed by ATP-L-arginine phosphotransferase (arginine kinase, EC 2.7.3.3):

it was essential to have relatively large amounts of pure phosphoarginine. Thus an investigation has been made of those methods which appeared capable of giving good and reproducible yields of pure product. The present paper reports the results of this investigation together with details of an improved method for the isolation of phosphoarginine from crayfish muscle. It also describes a procedure for the enzymic synthesis of the compound.

EXPERIMENTAL

Materials

Chemicals. Unless otherwise stated, AnalaR-grade chemicals obtained from British Drug Houses Ltd. (Poole, Dorset) were used. L-Arginine hydrochloride and ATP (Sigma Chemical Co., St Louis, Mo., U.S.A.) were used without further purification.

Charcoal was Nuchar C granular (West Virginia Pulp and Paper Co., New York, U.S.A.) which was treated by boiling

for 15 min. with $N\text{-HCl}$. It was then washed free of chloride, dried at 100° and sieved to obtain material of mesh size 40–100.

Dowex 50W (X8; H^+ form; mesh 200–400) and Dowex 1 (X4; Cl^- form; mesh 200–400) were supplied by Chemische Fabrik, Buchs, Switzerland. Dowex 50 (Na^+ form) was prepared according to the method of Moore & Stein (1951), and the same method was used to prepare the Li^+ form except that $0.4N\text{-LiOH}$ was used in place of $N\text{-NaOH}$.

Enzymes. Arginine kinase was prepared by the procedure of Morrison, Griffiths & Ennor (1957). ATP-AMP phosphotransferase (adenylate kinase, EC 2.7.4.3) was prepared according to the method of Colowick & Kalckar (1943), but only the 50–80% saturated $(NH_4)_2SO_4$ fraction was collected.

Determination of arginine. Free arginine was estimated by a slight modification of the method described by Rosenberg, Ennor & Morrison (1956), as indicated for the estimation of creatine by Morrison, O'Sullivan & Ogston (1961). The final volume was 3.0 ml. and readings were made at $535 m\mu$ after 30 min. at room temperature. Under these conditions, $0.1 \mu\text{mole}$ of arginine gave an extinction of 0.600 when measured in a cell of 1 cm. light-path in a Shimadzu spectrophotometer. Phosphorylated derivatives of arginine were estimated by the same method after hydrolysis in $N\text{-HCl}$ for 7 min. at 100° .

Determination of inorganic orthophosphate. This was determined by a slight modification of the method of King (1932). To the sample was added 2.0 ml. of 5% (w/v) ammonium molybdate in 15% (v/v) sulphuric acid and 0.5 ml. of the aminonaphtholsulphonic acid reagent. The volume was made to 10.0 ml. and readings were taken at $660 m\mu$ after 10 min. at room temperature. Under these conditions, $10 \mu\text{g.}$ of phosphorus gave an extinction of 0.115 when measured in a cell of 1 cm. light-path in a Shimadzu spectrophotometer. Labile phosphate was estimated by the same method after hydrolysis as described above.

Determination of nucleotides. The concentration of adenine nucleotides was determined by measuring the extinction of solutions at $259 m\mu$ and pH 7.0 (Bock, Ling, Morell & Lipton, 1956).

Determination of lithium and sodium. These were estimated by flame photometry and by spectrographic-absorption analysis. The determination of lithium by the colorimetric method of Thomason (1956) proved to be unsatisfactory in the presence of inorganic orthophosphate or organic material.

Determination of protein. Protein concentrations were estimated colorimetrically by the biuret method of Gornall, Bardawill & David (1949), with crystalline bovine serum albumin as the standard.

RESULTS

Isolation of phosphoarginine from crayfish muscle

The procedure outlined below is a modification of that described by Ennor *et al.* (1956) and eliminates the need for repeated precipitations of phosphoarginine as a copper salt, a step that other workers have experienced difficulty in reproducing. Further, a different species of crayfish (*Jasus verreauxi*) has been used since these are available

throughout the year. The tail muscle was collected as described by Ennor *et al.* (1956), but special precautions in relation to temperature are required if the muscle is to be stored (Table 1): at -18° there is a marked hydrolysis of phosphoarginine, but at -78° (in solid carbon dioxide) there is virtually none.

Unless otherwise stated, the following operations were carried out in a cold room at 4° and a MSE Major refrigerated centrifuge at 0° was used for all centrifugations.

Step 1: extraction of muscle. Frozen tail muscle was dropped into liquid nitrogen and ground to a fine powder, and 100 g. portions of the powdered material were extracted for 2 min. in a Waring Blendor with 450 ml. of 9% (w/v) trichloroacetic acid. The product was filtered on a Buchner funnel (with Whatman no. 531 paper) and the filtrate collected in a flask containing 30 ml. of $10N\text{-sodium hydroxide}$. The filter cake was re-extracted for 2 min. in a Waring Blendor with 200 ml. of 5% (w/v) trichloroacetic acid and the suspension filtered as before into the first alkaline filtrate. (The volume of $10N\text{-sodium hydroxide}$ was sufficient to make the pH of the combined filtrates greater than pH 9.) The gelatinous material that appeared after a few minutes was removed by centrifuging for 10 min. at $1000g$. A total of 500 g. of muscle was treated as described above, after which the supernatant solutions were combined and adjusted to pH 9.0 by the addition of $5N\text{-hydrochloric acid}$.

Step 2: precipitation of crude barium phosphoarginine. To the solution from step 1 was added with stirring 100 ml. of $1M\text{-barium acetate}$ and the suspension was re-adjusted to pH 9.0 by the addition of $5N\text{-sodium hydroxide}$. The precipitate was allowed to settle overnight and the bulk of the clear supernatant collected by siphoning. The remainder was collected by centrifuging for 10 min. at $1000g$ and the precipitate of insoluble barium salts discarded. The soluble barium salts were then precipitated by the addition with stirring of 4 vol.

Table 1. *Effect of storage on the phosphoarginine content of crayfish muscle*

Crayfish muscle, collected as described by Ennor *et al.* (1956), was placed in polythene bags and stored at either -18° or -78° (in solid CO_2). Samples (20 g.) of muscle were extracted twice with trichloroacetic acid (see the text) and the free and total arginine determined as described in the Experimental section.

Period of storage (days)	Bound arginine (%)	
	At -18°	At -78°
0	63.0	63.0
4	57.0	67.6
12	36.3	63.3
25	25.5	61.0

of 95 % (v/v) ethanol to the supernatant solution and, after adjustment of the mixture to pH 9.0 by the addition of *N*-sodium hydroxide, the precipitate was allowed to settle overnight. The slightly opalescent supernatant was removed partly by siphoning and finally by centrifuging for 10 min. at 1000*g*. The precipitate of barium salts was washed twice with 95 % (v/v) ethanol and once with ether, and dried *in vacuo* over anhydrous calcium chloride. The product can be kept at room temperature without an appreciable breakdown of phospho-arginine.

Step 3: extraction of water-soluble barium salts. The crude barium salts (16 g.) obtained in step 2 were extracted by stirring for 30 min. with 400 ml. of cold water. The supernatant, containing 83 % of the phosphoarginine, was collected by centrifuging for 10 min. at 1000*g* (solution 3*a*) and the residue re-extracted with the same volume of water and centrifuged as described above. The second extract, which contained 8 % of the phospho-arginine, was kept separate (solution 3*b*).

Step 4: removal of nucleotides by charcoal treatment. Acid-treated Nuchar C (40–100 mesh; 8 g.) was suspended in water and poured into a column (1 cm. diam.). After settling of the suspension under gravity, cotton wool was placed on top of the column and solution 3*a*, adjusted to pH 6.5–7.0 with 2*N*-hydrochloric acid, was passed through at the rate of 3 ml./min. This was followed by solution 3*b* which had also been adjusted to the same pH. Both effluents, which were free of material absorbing at 259 μ , were combined.

Step 5: crystallization of phosphoarginine. Barium was removed from the combined effluent solution by passage through a column (18 cm. $\times$ 2 cm.) of Dowex 50 (Na^+ form) at the rate of 3 ml./min. The column was then washed with 60 ml. of water. The effluents, which contained neither free arginine nor barium, were combined, adjusted to pH 8.0 with *N*-sodium hydroxide and concentrated to one-quarter of the volume on a Buchi rotary evaporator at 30° under reduced pressure. The concentrated solution was brought to pH 3.5 (methyl orange) by the addition of 2*N*-hydrochloric acid, and then 9 vol. of 95 % (v/v) ethanol was added with rapid stirring. After the addition of ethanol, the solution was readjusted to pH 5.0 (bromocresol green) with *N*-sodium hydroxide. The fine crystalline precipitate that formed rapidly was collected immediately by centrifuging for 10 min. at 1000*g*. The crystals were dissolved in 100 ml. of water, the pH brought to 7.0 with *N*-sodium hydroxide and the solution filtered. Recrystallization was carried out in the same manner and the product was washed twice with 95 % (v/v) ethanol and once with ether, and finally dried *in vacuo* over anhydrous calcium chloride at 0°.

The results of a typical preparation are summarized in Table 2. From 500 g. of crayfish muscle 3.7 g. of crystalline phosphoarginine was obtained; this represents a recovery of 66 %.

Though it was expected that the product obtained by the above procedure would be the monosodium salt of phosphoarginine, analysis for sodium indicated that no significant amounts were present. Indeed, when a column of Dowex 50 in the Li^+ form, rather than the Na^+ form, was used, the resulting product did not contain lithium. These results suggested that crystals of arginine phosphoric acid are obtained and, with the exception of the value for water, this conclusion was confirmed by elementary analysis (Found: C, 24.8; H, 6.2; N, 19.3; P, 10.7; arginine, 59.5; loss at 100°/15 mm. Hg, 6.8. Calc. for $\text{C}_6\text{H}_{18}\text{N}_4\text{O}_5\text{P}\cdot 2\text{H}_2\text{O}$: C, 24.8; H, 6.5; N, 19.3; P, 10.7; arginine, 59.5; water, 12.4 %). The C:N:H:P proportions from the above are 6.0:4.0:18.0:1.0, calculated on the basis of four N atoms/molecule. However, products with up to 6.6 C atoms/molecule have been obtained. It appears that the excess of C atoms is due to the presence of ethanol since the results could be fitted to a formula that assumed the presence of a fraction of a molecule of ethanol/molecule. Further, the excess of C atoms could be partly decreased by drying at 100°/20 mm. Hg, and even further, though not completely, decreased by drying at 100°/0.02 mm. Hg.

The arginine phosphoric acid contained trace amounts of free arginine even when freshly prepared. Although representing only a small percentage of the bound arginine, the amount nevertheless was sufficient to give relatively high blank values when used at the concentrations (2.0–10.0 mM) required in enzyme experiments. Moreover, the compound was not completely stable when stored at –10°. For these reasons, the barium salt was prepared as described below.

Table 2. Summary of yields of phosphoarginine in the various fractions obtained from an extract of crayfish muscle

Details are given in the Results section. The weight of muscle used was 500 g.

Fraction	Vol. or wt.	Phospho-arginine content (m-moles)	Yield (%)
Extract	3400 ml.	20.8	(100)
Crude barium salts	16.2 g.	20.4	98
Extract of barium salts	810 ml.	19.1	92
Extract after removal of nucleotides	790 ml.	15.1	73
Crystalline phospho-arginine	3.7 g.	13.7	66
Barium phospho-arginine	5.4 g.	12.3	59

Enzymic synthesis of phosphoarginine

Phosphoarginine may be prepared by enzymic phosphorylation of L-arginine by ATP in the presence of arginine kinase and the yield of product increased by the addition of adenylate kinase. The overall reaction may be represented by the equation:

The procedure developed was as follows: a reaction mixture containing 0.2 M-glycine, pH 10.0 (100 ml.), 0.2 M-magnesium chloride (1.0 ml.), 1 mM-EDTA, pH 8.0 (10.0 ml.), 0.1 M-L-arginine hydrochloride, pH 8.0 (100 ml.), 0.2 M-ATP, pH 7.6 (25.0 ml.), arginine kinase (120 mg.) and adenylate kinase (80 mg.) in a volume of 1 l. was incubated for 24 hr. at 15°. The phosphoarginine formed was isolated as described below and all operations, including centrifugations, were carried out at 2°.

Isolation of crude barium phosphoarginine. The incubation mixture was cooled to 2° and 20 ml. of cold 1 M-barium bromide was added with stirring. The precipitate which formed was removed by centrifuging for 10 min. at 1000g and discarded. Crude barium phosphoarginine was isolated from the supernatant solution as described in step 2 of the isolation procedure. Crystalline phosphoarginine was obtained from extracts of the crude barium salts (steps 3–5 of the isolation procedure) with a slight modification for the removal of nucleotides. Thus 9 g. of Nuchar was used to prepare the column and, after passage of the two extracts, it was washed with 200 ml. of water.

Table 3 summarizes the yields obtained at each stage of the preparation and indicates that the yield of phosphoarginine is 48 %.

Preparation of barium phosphoarginine

Because arginine phosphoric acid is unstable and since it has not been possible to prepare lithium, sodium or potassium salt by precipitation with ethanol at pH 8–9 (cf. Ennor *et al.* 1956), phosphoarginine was converted into a barium salt for storage. The resulting product is stable, even at room temperature, and can be quantitatively converted into a solution of any mono-cation salt containing no free arginine by passage through an appropriate ion-exchange resin. The method used for obtaining the barium salt was as follows. Arginine phosphoric acid (4 g.) was dissolved in 100 ml. of cold water and to the solution was added with stirring 20 ml. of 1 M-barium bromide. The pH was then adjusted to 8.2 with N-sodium hydroxide. No precipitate should form at this stage, but if one was present it was removed by centrifugation. The barium salt was precipitated by the addition with stirring of 4 vol. of 95 % (v/v) ethanol. After col-

lection by centrifuging for 10 min. at 1000g, the product was washed twice with 95 % (v/v) ethanol and once with ether, and dried *in vacuo* over anhydrous calcium chloride. The recovery of barium phosphoarginine was 90 % or better.

A number of samples of barium phosphoarginine prepared as described above have been analysed, and although a N:P ratio of 4.0 has been consistently obtained it has not been possible to ascribe a formula to the product. A typical analytical result is as follows: C, 16.4; H, 4.6; arginine, 38.0; Ba, 24.8; N, 12.4; P, 6.7; loss of weight at 100°/15 mm. Hg, 11.8 %. From these data the molar proportions, calculated on the basis that N = 4.0, for C:H:arginine:Ba:P:H₂O are 6.2:20.8:1.0:0.8:1.0:3.0. Similar results were obtained when barium phosphoarginine was precipitated under the conditions described by Ennor *et al.* (1956) and Cramer *et al.* (1962). Thus it would appear that the product obtained is a mixture of the hemibarium and monobarium salts.

The presence of one atom of barium/molecule would give rise to a compound possessing one unneutralized positive charge and thus it would be expected that the isolated product would contain an additional anion. The presence of the HCO₃⁻ anion was confirmed by the evolution of carbon dioxide on the addition of dilute hydrochloric acid to preparations of the barium salt, and this finding would account for the high molar proportion for carbon, which varied from 6.2 to 6.7. It should also be mentioned that the molar proportion for barium varied from 0.70 to 0.92. These results are in contrast with those of Ennor *et al.* (1956), who found that their product contained 7 carbon atoms and 1 barium atom/molecule.

Chemical synthesis of phosphoarginine

For comparison with the above methods of preparation and in an endeavour to simplify the pro-

Table 3. *Isolation of enzymically synthesized phosphoarginine: summary of the yields obtained in the various fractions*

Details are given in the Results section. The amount of L-arginine hydrochloride used was 1.74 g. (10 m-moles).

Fraction	Vol. or wt.	Phospho-arginine content (m-moles)	Yield (%)
Reaction mixture	1000 ml.	7.4	74
Crude barium salts	4.3 g.	6.2	62
Extract of barium salts	190 ml.	6.0	60
Extract after removal of nucleotides	370 ml.	5.3	53
Crystalline phospho-arginine	1.29 g.	4.8	48
Barium phospho-arginine	2.0 g.	4.5	45

cedure reported by Thiem *et al.* (1962), phospho-arginine was synthesized chemically by phosphorylation of arginine with phosphoryl chloride. The phosphorylation was carried out as described by Morrison, Ennor & Griffiths (1958) for the synthesis of phosphotaurocytamine.

L-Arginine hydrochloride (25 g., 0.12 mole) was dissolved in 20 ml. of water and 50 ml. of 10N-sodium hydroxide and transferred to a three-necked flask. To this solution was added a total of 38 ml. of phosphoryl chloride (0.43 mole) and 170 ml. of 10N-sodium hydroxide over a period of 1 hr. (3.0 ml. of phosphoryl chloride, followed immediately by 14 ml. of 10N-sodium hydroxide, was added every 5 min. to the rapidly stirred solution). The temperature of the mixture was maintained between -3° and 3° by periodic immersion in an ethanol-solid carbon dioxide bath and the pH kept at 13–14 (indicator paper). After the additions were complete, the mixture was stirred for an additional 30 min. while the pH was maintained at 13–14 by the addition of 10N-sodium hydroxide. The mixture was then filtered at 3° on a sintered-glass funnel and the residue extracted with 100 ml. of cold water by treatment in a Waring Blender. After filtration, the wash and filtrate were combined and allowed to stand overnight at 4° and pH 13.

The precipitate of inorganic salts that formed was removed by filtration at 4° and the pH of the clear filtrate adjusted to pH 7.6 with 5N-hydrochloric acid. (Analysis of this solution showed that 46% of the total arginine was present in bound form.) The volume of the solution was then reduced to approx. 80 ml. by evaporation at 30° under reduced pressure and, after cooling to 4° , the sodium chloride that was precipitated was removed by filtration. To the clear solution, adjusted to pH 6.2 with 2N-hydrochloric acid, was added 210 ml. of 1M-magnesium chloride. After readjustment to pH 9.0 with 3N-sodium hydroxide, the mixture was stirred for 1 hr. at 4° and the precipitate that formed was removed by centrifuging and discarded. To the clear supernatant solution was added 4 vol. of 95% (v/v) ethanol at 0° and the precipitate containing the water-soluble ethanol-insoluble magnesium salts collected by centrifuging for 10 min. at 1000g, washed twice with ethanol and once with ether, and dried *in vacuo* over anhydrous calcium chloride. The yield of crude synthetic magnesium phosphoarginine was 10.6 g.

The components of the crude synthetic magnesium phosphoarginine were separated by chromatography. The product (100 mg.) was dissolved in 20 ml. of water and passed through a column (1 cm. $\times$ 15 cm.) of Dowex 1 (Cl^- form) which was washed with water until the effluent was free of arginine. Elution was carried out with a linearly increasing gradient of sodium chloride (Parr, 1954)

by using two open connected vessels of equal cross-section which were kept at the same horizontal level. The mixing vessel contained 2 l. of water and the supply vessel contained 2 l. of 1.0M-sodium chloride. The sodium chloride concentration required to elute a particular compound was calculated by using the formula given by Peterson & Sober (1959). After collection of 20 ml. fractions at the rate of 1 ml./min., each fraction was analysed for total arginine and inorganic orthophosphate. The elution pattern is illustrated in Fig. 1.

For the purpose of identifying each of the components, from each fraction five tubes with the highest content of total arginine or inorganic orthophosphate or both were combined and analysed for both arginine and inorganic orthophosphate before and after acid hydrolysis. The results (Table 4) indicate that apart from arginine (fraction A), which is not retained by the column, the mixture contained inorganic orthophosphate (fraction E) and four phosphorylated derivatives of arginine. Since fraction B gave a molar ratio for bound arginine:bound phosphate of 1.0, and as it was eluted at the same concentration of sodium chloride as the product obtained by Thiem *et al.* (1962), it was concluded that fraction B contained the mono-phosphorylated derivative of arginine with the phosphoryl group substituted on the guanidino nucleus. On analysis fraction D behaved as though it contained free arginine and free inorganic orthophosphate, but the concentration of sodium chloride required for elution of this fraction precludes this possibility. Hence it appears that it contains N^{α} -phosphoarginine, which would react similarly to free arginine and be rapidly hydrolysed to inorganic orthophosphate in the presence of acid molybdate

Fig. 1. Fractionation on Dowex 1 (Cl^- form) of a solution of the water-soluble ethanol-insoluble magnesium salts obtained after the phosphorylation of arginine with POCl_3 . The arrow indicates the point at which elution was commenced. Other details are given in the text. ●, Total arginine; ○, inorganic orthophosphate.

Table 4. *Chemical analysis of the fractions obtained after chromatography of a solution of crude synthetic phosphoarginine*

The fractions correspond to those indicated in Fig. 1. Results are expressed as molar proportions.

Fraction	Free arginine	Total arginine*	Inorganic orthophosphate	Total phosphate*
A	1.0	1.0	0	0
B	0	1.0	0	1.0
C	0.1	1.0	0.4	1.1
D	1.0†	1.0	1.0‡	1.0
E	0	0	1.0	1.0
F	0	1.0	1.0‡	2.1

* Values obtained after hydrolysis for 7 min. in *N*-HCl.

† Estimate as free arginine as guanidino group is not substituted.

‡ The phosphoryl group at the *N*^α-position of arginine is hydrolysed in the presence of acid molybdate.

Table 5. *Comparison of the yields obtained by various methods for the preparation of phosphoarginine*

The yields were calculated in relation to the amount of the starting material indicated and on the basis that final product was barium phosphoarginine. It was assumed that lithium phosphoarginine was converted into the barium salt with a yield of 90%.

Method	Reference	Starting material	Yield (%)
Isolation	Meyerhof & Lohmann (1928)	<i>A. fluviatilis</i> muscle	—
	Ennor <i>et al.</i> (1956)	<i>J. lalandii</i> muscle	48.5
	Present work	<i>J. verreauxi</i> muscle	59.1
Enzymic synthesis	Present work	Arginine, ATP	45.2
Chemical synthesis	Thiem <i>et al.</i> (1962)	Arginine, POCl ₃	8.8
	Present work	Arginine, POCl ₃	13.0
	Cramer <i>et al.</i> (1962)	<i>N</i> ^α -Benzyloxycarbonylarginine	41.4
	Cramer <i>et al.</i> (1962)	Ornithine	36.5

(Winnick & Scott, 1947). The position of fraction F suggests that the compound eluted may be a diphosphorylated derivative of arginine, and the analytical values (Table 4) are consistent with the presence of an arginine derivative which is phosphorylated at the *N*^α-position as well as on the guanidino moiety. It would be expected that the *N*^α-P bond would be hydrolysed and estimated as inorganic orthophosphate in the King (1932) method. On the other hand, the N-P bond involving the guanidino group would be stable under these conditions, but would be hydrolysed in *N*-hydrochloric acid at 100° (cf. Morrison & Ennor, 1960). Fraction C would appear to contain a compound similar to that present in fraction B, but which is contaminated by some labile phosphorylated derivative of arginine.

On the basis of total bound arginine, the relative percentages of the phosphorylated derivatives of arginine were: fraction B (*N*-phosphoarginine), 41.5; fraction C (not identified), 12.8; fraction D (*N*^α-phosphoarginine), 25.6; fraction F (*NN*^α-diphosphoarginine), 20.1.

The phosphoarginine of fraction B was not isolated, but, on the basis of the yields obtained in the isolation procedure, it may be calculated that 25 g. of arginine would give 6.6 g. of the barium salt. This yield (13%) is similar to that obtained by Thiem *et al.* (1962).

DISCUSSION

A comparison of the various methods available for the preparation of phosphoarginine (see Table 5) shows that the best and simplest method is that described in the present paper for the isolation of the compound from crayfish muscle. Not only is a good yield obtained, but it is possible to obtain up to 5 g. of phosphoarginine in a single preparation. However, it is important that the muscle be stored at low temperature (−78°) after removal from the animal since at higher temperatures (−18°) there is a marked loss of phosphoarginine. When it is not possible to obtain muscle with a high phosphoarginine content, enzymic synthesis is the next best procedure. Only partly purified preparations of arginine kinase, free of phosphoamidase and adenosine-triphosphatase activities, are required and the preparation described by Morrison *et al.* (1957) meets these requirements. Further, this enzyme is not labile when stored at −10°. However, in this procedure it is not convenient to handle at any one time more than 1 l. of reaction mixture, which gives a yield of 1.3 g. of phosphoarginine.

The chemical methods for the synthesis of phosphoarginine as described by Cramer *et al.* (1962) are more specific than that with phosphoryl chloride, with the consequence that the yields are better. However, recalculation of the yields (Table 5)

reported by Cramer *et al.* (1962) on the basis of the amount of starting material shows that they are approximately the same as obtained by enzymic synthesis. Because of the need to prepare a number of intermediate compounds, the chemical method is more laborious. Though phosphorylation of arginine with phosphoryl chloride cannot be considered as a very satisfactory method for the synthesis of phosphoarginine because of the difficulty of chromatographing large amounts of material, nevertheless this procedure could be useful for the preparation of analogues of phosphoarginine. Such compounds may be used in specificity studies of arginine kinase.

In contrast with the report of Ennor *et al.* (1956), the product obtained when the lithium or sodium salt of phosphoarginine was precipitated with ethanol under acid conditions was the free acid rather than the hydrochloride. It should be noted that the analytical figures for nitrogen and phosphorus were similar to those reported by the above authors, but tests for Cl^- ions were negative. Attempts were made to obtain the dilithium salt of phosphoarginine under the conditions described by Thiem *et al.* (1962), but these were unsuccessful. At pH values above 5.0 either an oil or no precipitate was obtained. In any case, a dilithium salt with the composition reported would be improbable as such a compound would possess one unneutralized positive charge. In view of the failure to find either lithium or sodium in what were expected to be the monolithium or monosodium salts, and as analytical values for lithium were not given by the French group, some doubt must remain about the composition of the product that they obtained; the melting points of dilithium phosphoarginine (Thiem *et al.* 1962) and the free acid (Cramer *et al.* 1962) are 180° and $175\text{--}180^\circ$ respectively.

Phosphoarginine as a free acid is not completely stable and, moreover, fresh preparations contain sufficient free arginine to preclude its use in enzyme experiments. Therefore the compound was stored as a stable barium salt. The composition of the latter has not been elucidated, but appears to be a mixture of the hemibarium and monobarium salts. It also contains bicarbonate, which causes the values for carbon to be higher than those demanded by theory, and in this respect is similar to the products obtained by Meyerhof & Lohmann (1928), Ennor *et al.* (1956) and Cramer *et al.* (1962). Analysis of the analytical data reported for the alkaline-earth salts of the other phosphagens, e.g. phosphoglycocyamine (Fawaz & Seraidarian, 1946; Cramer & Vollmar, 1959), phosphotaurocyamine (Morrison *et al.* 1958) and phospholombricine (Ennor & Rosenberg, 1962), has indicated that these compounds also give values for carbon that are higher than the theoretical values.

SUMMARY

1. Methods are described for the isolation of phosphoarginine as a barium salt from crayfish muscle and for the enzymic synthesis of this compound from arginine and ATP in the presence of ATP-L-arginine phosphotransferase and ATP-AMP phosphotransferase.

2. The products obtained as a result of the phosphorylation of arginine with phosphoryl chloride have been identified.

3. Comparison of the various methods for the preparation of phosphoarginine has indicated that the isolation procedure gives the highest yield and is the most suitable for the preparation of relatively large amounts of the compound.

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