other hand, the mechanism of active Na extrusion is not the same as that of K uptake, for the former is largely Na specific and the latter largely K specific.

Deoxycorticosterone may be taken then to inhibit indirectly active Na extrusion by its effect on the active K uptake or on the K carrier.

SUMMARY

1. Sodium ions accumulated by the yeast cell during fermentation in a medium containing a high concentration of sodium can be actively excreted into a medium containing potassium ions with which the sodium ions exchange. In the absence of external potassium, sodium is excreted much more slowly with an accompanying anion.

2. Of eight crystalline steroids (cortisone, deoxycorticosterone, testosterone, progesterone, oestrone, methylandrostenediol, dehydroepiandrosterone and 17-hydroxycorticosterone) investigated for a possible effect on such sodium and potassium exchanges in the yeast cells, as well as on active potassium uptake during fermentation only three

(deoxycorticosterone, cortisone and 17-hydroxycorticosterone) showed significant effects; deoxycorticosterone showed the most pronounced effect, namely an inhibitory action on the active transport of sodium and potassium; cortisone also showed a slight inhibiting action whereas 17-hydroxycorticosterone had a small stimulating effect on the active transport.

3. Deoxycorticosterone does not inhibit appreciably the active excretion of sodium from yeast suspended in tap water and it is concluded that its marked effect on sodium excretion into a solution containing 0.1M-potassium chloride is on the potassium-carrier mechanism which has been interpreted in terms of a 'redox pump' (Conway, 1951, 1952*b*, 1953).

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REFERENCES

- Conway, E. J. (1951). *Science*, **113**, 270.
 Conway, E. J. (1952*a*). *Ciba Foundation Colloquia on Endocrinology*, **4**, 417. London: Churchill.
 Conway, E. J. (1952*b*). *Biochemistry of Gastric Acid Secretion*. Illinois, U.S.A.: Thomas.
 Conway, E. J. (1952*c*). *2nd Int. Congr. Biochem. Abstr.* p. 140.
 Conway, E. J. (1953). *International Review of Cytology*, ed. by Bourne, G. & Danielli, J. F. New York: Academic Press.
 Conway, E. J. & Hingerty, D. (1953). *Biochem. J.* (in the Press).
 Conway, E. J. & Moore, P. T. (1952). *2nd Int. Congr. Biochem. Abstr.* p. 141.
 Conway, E. J. & O'Malley, E. (1942). *Rep. med. Res. Coun., Ireland*.
 Conway, E. J. & O'Malley, E. (1943). *Rep. med. Res. Coun., Ireland*.
 Conway, E. J. & O'Malley, E. (1946). *Biochem. J.* **40**, 59.
 Conway, E. J. & Ryan, H. (1950). Unpublished observations.
 Rothstein, A. & Enns, L. H. (1946). *J. cell. comp. Physiol.* **28**, 231.

The Separation of the Phosphate Esters of Muscle by Paper Chromatography

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Knowledge about the phosphorus metabolism of muscle depends on the separation and determination of the phosphate esters in muscle extracts. In the early work, such as that done by Eggleton & Eggleton (1929), methods are described whereby the adenosine polyphosphate, creatine phosphate, orthophosphate and monophosphate ester fractions can be separated and determined. These methods have given valuable information about the large

changes in these esters in muscle after long periods of activity, but they are difficult to use, particularly for investigating short periods of activity in which the changes are small.

In this work the chromatographic method described by Hanes & Isherwood (1949), which has not so far been used for the esters in muscle extracts, has been examined. The esters in frog muscle have been compared with those in tortoise muscle and, to compare this method with the others, some of the early work on the changes during long periods of

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activity have been repeated. The method has been used in an attempt to obtain with tortoise muscle, following a suggestion of Hill (1949, 1950*b*), some information about the changes in the esters during short periods of activity.

EXPERIMENTAL

Chromatographic reference compounds

Adenosine triphosphate (ATP). Two samples were used. One, which gave a single spot on the chromatograms, was a gift from Dr J. Ottaway of the Biochemistry Department, University College, London. The other, a commercial one, described as 95% pure, was used in the recovery experiments (Table 2). It was found to contain small amounts of adenosine diphosphate (ADP) and orthophosphate. Other samples were examined but were found to contain up to four fractions.

Adenosine diphosphate. This was made from commercial ATP by the method of Lohmann (1935). It gave a single spot in the solvents used; 43% of the P was split off as orthophosphate on heating with $N-H_2SO_4$ for 10 min. at 100°.

Adenosine 5'-phosphate (AMP). This was a synthetic sample.

Creatine phosphate. This was a synthetic sample made by the method of Zeile & Fawaz (1938). It gave a single spot in the *n*-propanol/ammonia solvent. In the *tert*.-butanol/picric acid solvent it was unstable and usually gave a streak.

Orthophosphate, pyrophosphate, tripolyphosphate and hexametaphosphate. These were commercial samples.

Fructose 6-phosphate and fructose 1:6-diphosphate. These were gifts from Mr C. W. Parr of the Biochemistry Department, University College, London. Both gave single spots in the solvents used.

2:3-Diphosphoglyceric acid. This was a gift from Prof. M. Maizels. It was found by chromatography to contain about 20% of monophosphoglyceric acid.

Ethanolamine phosphate. This was a gift from Dr S. P. Datta of the Biochemistry Department, University College, London.

Technique for stimulation of muscle and preparation of extract

The muscles used. These were the gastrocnemius of the frog (*Rana temporaria*), the iliofibularis, the extensor iliotibialis and the sartorius of the tortoise (*Testudo graeca*), and the retractor pharynx muscle of the snail (*Helix pomatia*). The iliofibularis and extensor iliotibialis were once called the biceps cruris and the rectus femoris and are so described by Owen (1866, fig. 151, nos. 103 and 99). The sartorius is described as such by Owen (1866, fig. 151, no. 106). The weight of these muscles was usually between 300 and 1500 mg., except that of the snail which was 17 mg. In experiments in which a stimulated muscle was compared with an unstimulated one, the two pair muscles from the left- and right-hand sides of the same animal were used.

Fixation and stimulation of muscles. The muscles were fixed by freezing. Fixation in the stimulated state was done by suddenly immersing them in a mixture of acetone and solid CO_2 . This sudden freezing causes stimulation (see Lundsgaard, 1950) and makes it possible to fix the muscles

at the beginning of activity. Muscles which were fixed after a period of activity were frozen suddenly while the electrical stimulation was still being given. Frog muscles which have been frozen suddenly are fully contracted, but tortoise muscles which have not first been stimulated by other means are usually not, probably because they freeze before they have time to shorten.

Fixation in the unstimulated state was carried out by slow freezing (as suggested by Lundsgaard, 1950). The muscles were hung in a conical flask so that they did not touch the walls and then the flask was placed in a Dewar vessel containing solid CO_2 . The muscles took about 30 min. to freeze and did not show any sign of stimulation as long as they did not touch the cold sides of the flask. The protein residue from muscles fixed in this way was less opaque than that from those fixed by sudden freezing.

Preparation of muscle extracts. The muscles were broken up at about -70° and the powder dropped into 2-5 ml. of a 5% solution of trichloroacetic acid (TCA) in acetone (also at about -70°). Any lumps were broken up with a glass rod and the suspension was allowed to stand for about 1 hr. This was done to inactivate as much of the enzyme protein as possible at a low temperature so that spurious changes in the phosphate esters were lessened. The suspension was then slowly warmed up to room temperature with constant stirring. When this was reached the acetone was evaporated off, about 1 ml. of water was added and the powdered muscle extracted with the now aqueous TCA for about 1 hr. at 0°. Then the whole was washed into a graduated centrifuge tube and made up to the appropriate volume (1.5-4.0 ml. depending on the weight of the muscles).

Removal of calcium and magnesium from the extracts. Small amounts of calcium and magnesium in the extracts, which would spoil the chromatograms, were turned into soluble complexes with ethylenediaminetetraacetic acid (Walker & Warren, 1951). Usually 1 ml. of protein-free muscle extract was put into a graduated tube and 50 mg. of ethylenediaminetetraacetic acid added for every g. of muscle to which the amount of extract corresponded. It was then made alkaline with concentrated ammonia solution to lessen the breakdown of creatine phosphate, the total volume being brought to 1.1 ml. The ethylenediaminetetraacetic acid did not greatly change the distances travelled by the esters.

Chromatography

Choice of solvents. The most useful of the solvents suggested by Hanes & Isherwood (1949) have been found to be *n*-propanol/ammonia/water (60 ml. *n*-propanol:30 ml. conc. ammonia solution:10 ml. water) and *tert*.-butanol/picric acid/water (80 ml. *tert*.-butanol:20 ml. water:4 g. picric acid). Some chromatograms were run in a solvent of ethyl acetate/pyridine (water-poor phase from 100 ml. ethyl acetate:45 ml. pyridine:100 ml. water) but this was abandoned because orthophosphate often gave two spots.

The distances that spots of some phosphate esters and polyphosphoric acids travel in the two suggested solvents are given in Table 1, these distances being given in terms of that reached by orthophosphate. This is a more useful standard than the R_F value, particularly with the *n*-propanol/ammonia solvent, where the R_F values are difficult to measure because some of the solvent must be taken up on a pad of filter paper fixed to the bottom of the chromatogram. The values in Table 1 can be turned into the R_F values if they are multiplied by the R_F value for ortho-

phosphate. This is about 0.2 in *n*-propanol/ammonia and 0.7 in *tert*.-butanol/picric acid.

ADP travels the same distance as orthophosphate in the *n*-propanol/ammonia solvent and about the same distance as ATP in the *tert*.-butanol/picric acid solvent. It can be separated on a two-dimensional chromatogram in which the two solvents are used successively. ATP, orthophosphate, the creatine phosphate plus monophosphate ester fraction, the diphosphate esters, pyrophosphate and triphosphates are also separated on a two-dimensional chromatogram, but the individual mono- and di-phosphate esters are not.

Table 1. *Distances travelled by some phosphate esters in the chromatographic solvents used*

Substance	Distance travelled (in terms of that travelled by orthophosphate)	
	<i>n</i> -Propanol/ ammonia	<i>tert</i> .-Butanol/ picric acid
Monophosphoglycerate	1.7	0.75
Adenosine 5'-phosphate	1.5	0.78
Creatine phosphate	1.5	0.56
Ethanolamine phosphate	1.4	0.62
Fructose 6-phosphate	1.3	0.67
Adenosine diphosphate	1.0	0.20
Adenosine triphosphate	0.6	0.13
Pyrophosphate	0.6	0.48
2:3-Diphosphoglycerate	0.5	0.75
Fructose 1:6-diphosphate	0.5	0.67
Triphosphosphate	0.5	0.28
Hexametaphosphate	Streaks	Streaks
	0.0-0.6	0.00-0.50
Unknown A	3.5	—
Unknown B	1.7	0.20
Unknown C	0.9	0.40
Unknown D	0.6	0.60
Unknown E	0.5	0.10
Unidentified ester in snail muscle extract	1.0	0.65

Running of the chromatograms. Successive 5 μ l. volumes of the extract (total 50–200 μ l.) were put on the paper with a micropipette, the moisture being removed in a current of air. The paper was Whatman no. 4 filter paper which had been washed with 2*N*-acetic acid as suggested by Hanes & Isherwood (1949). The chromatograms were run at room temperature, usually for 16 hr. in *n*-propanol/ammonia and 8–10 hr. in *tert*.-butanol/picric acid.

Development of the chromatograms. The positions of the esters on the paper were found by spraying with the mixture suggested by Hanes & Isherwood (1949) (5 ml. 60% perchloric acid; 25 ml. 4% ammonium molybdate; 10 ml. *N*-HCl; water to 100 ml.), followed by heating in an oven at 85° for 10 min. and treatment with H_2S , the esters appearing as blue spots.

Two other tests could be done. When the chromatogram was sprayed with the reagent, orthophosphate appeared at once and creatine phosphate after 1 min. as yellow spots due to the formation of phosphomolybdic acid. The adenosine phosphates could be found by clipping to the chromatogram a piece of filter paper which had been dyed with proflavine and then looking at an ultraviolet lamp through the two layers, the radiation passing first through the chromatogram. The areas of the chromatogram in which the adenosine

phosphates were present absorbed the radiation so that the proflavine in the adjacent paper did not fluoresce and the adenosine phosphates appeared as dark spots on a fluorescent background. Neither of these tests could be done with chromatograms run in the *tert*.-butanol/picric acid solvent because the acid interferes.

Quantitative determinations. A standard area of the chromatogram, which included the spot of the ester to be determined, was cut out, put into a 100 ml. conical Pyrex flask and 5 ml. of *M*- K_2SO_4 solution which contained H_2SO_4 and a trace of $CuSO_4$ added. The whole was then evaporated to dryness, 1–2 ml. of conc. H_2SO_4 added and the paper oxidized by wet ashing, the oxidation being completed in about 5 min. The excess H_2SO_4 was then boiled off and the molten potassium hydrogen sulphate cooled and allowed to solidify. The solid was dissolved in water and heated at about 95° for 15 min. to turn all the P into orthophosphate. The sample was then transferred to a separating funnel, its total volume brought to 10 ml. and the P determined by the method of Berenblum & Chain (1938). A colorimeter which had been made by Dr E. J. Harris, the design of which was based on that described by Humphrey & Harris (1948), was used. The amount of K_2SO_4 added in this method is such that the final solution, when diluted to 10 ml., contains enough hydrogen sulphate to bring the hydrogen-ion concentration within the limits given by Berenblum & Chain, namely 0.1–2.0 *N*.

Other analytical methods

Determination of creatine phosphate and the monophosphate ester fraction. Creatine phosphate is not separated from the monophosphate esters on the chromatograms and determination of the P in the appropriate spot gives the sum of these compounds. If some of the extract is treated with 5% ammonium molybdate and *N*- H_2SO_4 for 30 min. at 20°, subsequent determination of the P gives a value for the sum creatine phosphate + orthophosphate. The value obtained for orthophosphate from the chromatogram can be subtracted from this to give a value for creatine phosphate, which can then be subtracted from the value obtained from the chromatogram for creatine phosphate + monophosphate esters to give one for the monophosphate esters.

Determination of the protein phosphorus. In most of the experiments in which tortoise muscles were used, the amount of P attached to the muscle protein was determined. The residue, after the first extraction with TCA, was washed five times with 5% TCA. The P in the residue was then determined by the method used for the P in the chromatogram spots.

RESULTS

Tests of the chromatographic method

The method was tested directly in two experiments. In the first, amounts of ATP and orthophosphate in the proportions found in normal muscle were put on the paper; in the second experiment amounts of orthophosphate, fructose 6-phosphate and fructose 1:6-diphosphate in the proportions found in muscles which have been poisoned by monoiodoacetate and then fatigued were used. The chromatograms were run in one direction in *n*-propanol/ammonia and in

the other in *tert.*-butanol/picric acid. The spots were then cut out and analysed for phosphorus. The results are given in Table 2 which shows that the values obtained agree with the known amounts put on the paper within 10%.

Table 2. *The recovery from two-dimensional chromatograms of mixtures of phosphate esters*

	Amount recovered from chromatogram ($\mu\text{g. P}$)			Amount applied ($\mu\text{g. P}$)
	A	B	C	
Expt. 1				
Orthophosphate	12.0	11.5		12.0
ATP	8.4	8.2		8.7
Total	20.5	19.7		20.7
Expt. 2				
Orthophosphate	20.4	21.2	20.4	19.1
Fructose 6-phosphate	12.0	10.7	13.6	13.5
Fructose 1:6-diphosphate	21.3	19.6	22.7	22.7
Total	53.7	51.5	56.7	55.3

Table 3. *Total phosphorus recovered from chromatograms of muscle extracts*

Type of muscle	Amount recovered from chromatogram ($\mu\text{g. P/g. of muscle}$)	Amount present in extract by direct estimation ($\mu\text{g. P/g. of muscle}$)
Frog	1526	1517
	1692	1609
Tortoise	901	942
	876	850
	1001	1073
	1192	1212

Comparisons of the total phosphorus in the muscle extracts with that recovered from the chromatograms are given in Table 3, which shows that the values agree to within 7–8%. The total orthophosphate in the extracts after 10 min. acid hydrolysis has been compared with the combined value for the labile phosphorus of ATP and ADP, orthophosphate and creatine phosphate and found to agree to within 15%. If the extracts contained radioactive orthophosphate, their activity agreed with that of the orthophosphate spot to within 10%.

The phosphate esters in muscle extracts compared with known esters

One-dimensional chromatograms of extracts of normal muscle show three well-defined spots. In the *n*-propanol/ammonia solvent these correspond to ATP, ADP + orthophosphate and creatine phosphate + monophosphate esters, and in the *tert.*-butanol/picric acid solvent to ATP + ADP, creatine phosphate + monophosphate esters and ortho-

phosphate. Two-dimensional chromatograms, in which the first solvent is *n*-propanol/ammonia and the second *tert.*-butanol/picric acid, show four spots. These correspond to ATP, ADP, orthophosphate and creatine phosphate + monophosphate esters. The ADP spot is usually faint. Its separation from ATP is sometimes unsatisfactory and then they must be determined together. Chromatograms of extracts of muscles which have been poisoned with monoiodoacetate and then fatigued show a spot corresponding to the diphosphate esters. This is not found with normal muscle, but analysis of the appropriate area of the chromatogram usually shows the presence of some phosphorus.

Some other fractions, which have not been identified, have been found, mainly in tortoise muscles. The two principal ones have been called unknowns A and B and are stable to the perchloric acid/ammonium molybdate reagent. With the present methods they can only be found by analysis of the appropriate area of the chromatogram. Three other fractions, which were shown up by the perchloric acid/ammonium molybdate reagent, were found in six muscles obtained from the same tortoise. These have been called unknowns C, D and E. The distances travelled by the unknowns on the chromatograms are given in Table 1.

Comparison of the amounts of the phosphate esters in frog and tortoise muscle

A histogram is given in Fig. 1 for the amounts of ATP and creatine phosphate + orthophosphate in the muscles of frog and tortoise. The frog muscles usually contain larger amounts than those of

Fig. 1. Histogram of the ATP and creatine phosphate + orthophosphate contents of frog and tortoise skeletal muscle.

tortoise. A single value of 156 $\mu\text{g. phosphorus/g.}$, which falls in the middle of the range of those for tortoise muscles, was obtained for the amount of ATP in the retractor pharynx muscle of the snail, which has about the same speed as tortoise muscle. There was no spot corresponding to creatine

Table 4. *Comparisons of the phosphate ester contents of pair frog muscles, one muscle having been stimulated, the other not*

Period of electrical stimulation (sec.)		Expt. 1 15	Expt. 2 10	Expt. 3 120	Expt. 4 Stimulated and fixed by sudden freezing
		Phosphate esters ($\mu\text{g. P/g. of muscle}$)			
ATP	Stimulated	437	299	288	392
	Unstimulated	504	294	284	363
	Difference	-67	+5	+4	+29
ADP	Stimulated	56	60	—	40
	Unstimulated	59	17	—	58
	Difference	-3	+43	—	-18
Creatine phosphate*	Stimulated	28	202	252	500
	Unstimulated	281	433	413	580
	Difference	-253	-231	-161	-80
Orthophosphate†	Stimulated	650	496	481	274
	Unstimulated	446	328	297	215
	Difference	+204	+168	+184	+59
Monophosphate esters	Stimulated	149	—	—	—
	Unstimulated	70	—	—	—
	Difference	+79	—	—	—
Diphosphate esters	Stimulated	6	19	—	2
	Unstimulated	3	21	—	0
	Difference	+3	-2	—	+2

* Expts. 2 and 3 include monophosphates.

† The results given in Expt. 3 are for a one-dimensional chromatogram run in *n*-propanol/ammonia. In this solvent ADP is not separated from orthophosphate. The other results are all for two-dimensional chromatograms.

phosphate on the chromatogram of this snail muscle, but a compound was present which had travelled the same distances as unknown C.

Comparison of stimulated and unstimulated frog muscles

Results from these experiments are given in Table 4. In Expts. 1-3 the stimulated muscles were tetanized with shocks from an induction coil before being fixed by sudden freezing. The changes found in them, namely a decrease in creatine phosphate and increases in orthophosphate and the monophosphate esters, are the same as those found in the early work on muscle. In Expt. 4 the stimulated muscle was both fixed and stimulated by sudden freezing. Under these conditions, the closest approximation to a single twitch which is possible with frog muscle, the stimulated muscle contained less creatine phosphate and more orthophosphate than the unstimulated one. In none of the experiments was there any significant breakdown of ATP into ADP.

Experiments with frog muscles poisoned with monoiodoacetate

In these experiments pair frog gastrocnemius muscles were poisoned by being soaked for 3 hr. in a Ringer solution containing 2×10^{-4} M-monoiodoacetate. It was necessary to take care in dissecting out the muscles because if they were damaged they developed rigor mortis after about 30 min. soaking. After soaking, one muscle was fixed without stimulation by slow freezing. The other was stimulated with an induction coil and made to carry out a rapid series of single twitches after about 150 of which it ceased to respond (cf. Lundsgaard, 1930a). It was then fixed before it had started to develop rigor.

The results of two of these experiments are given in Table 5. In two other experiments, only the ATP and diphosphate ester fractions were determined and the following changes were found, the amounts being expressed as $\mu\text{g. phosphorus/g.}$ In Expt. 3 the ATP decreased from 347 to 247 and the diphosphate esters increased from 20 to 391 on stimulation; in Expt. 4 the ATP decreased from 203 to 111 and the

Table 5. *Comparison of the phosphate ester contents of pair frog muscles poisoned with monoiodoacetic acid, one muscle having been stimulated to inexcitability but not to rigor, the other unstimulated*(From gastrocnemius muscles soaked 3 hr. in Ringer solution + 2×10^{-4} M-iodoacetate.)

	Phosphate esters (μ g. P/g. of muscle)					
	Expt. 1			Expt. 2		
	Stimulated	Unstimulated	Difference	Stimulated	Unstimulated	Difference
ATP	200	365	-165	60	238	-178
ADP	129	96	+33	135	57	+78
Creatine phosphate	116	351	-235	7	402	-395
Orthophosphate	361	399	-38	183	256	-73
Monophosphate esters	58	191	-133	—	—	—
Diphosphate esters	368	38	+330	224	38	+186
Unknown A	167	144	+23	—	—	—
Unknown B	126	109	+17	8	0	+8

Table 6. *Comparisons of the phosphate ester contents of pair tortoise muscles, both muscles being treated identically*

Fraction	Type of muscle								
	Iliofibularis			Extensor iliottibialis			Sartorius		
	Left	Right	Diff.	Left	Right	Diff.	Left	Right	Diff.
Phosphorus contents, expressed as μ g. P/g. of muscle									
Total extractable P	1100	1099	1	989	1172	183	1074	1148	74
Protein P	—	—	—	224	223	1	205	158	47
Phosphorus contents, expressed as % of total extractable P									
ATP	20.6	22.2	1.6	27.1	25.9	1.2	22.4	25.2	2.8
ADP	6.3	7.0	0.7	1.6	2.3	0.7	7.0	5.7	1.3
Creatine phosphate	31.1	29.6	1.5	30.3	32.7	2.4	21.0	22.0	1.0
'High-energy' P*	48.0	47.9	0.1	49.2	51.2	2.0	39.4	41.6	2.2
Orthophosphate	25.5	24.8	0.7	28.4	25.5	2.9	31.0	29.8	1.2
Monophosphate esters and adenylic acid	0.0	0.0	0.0	0.0	0.0	0.0	2.3	2.3	0.0
Diphosphate esters	4.2	5.0	0.8	0.0	0.0	0.0	0.7	0.6	0.1
Unknown A	8.1	6.5	1.6	6.8	7.7	0.9	8.8	8.2	0.6
Unknown B	4.2	5.0	0.8	5.8	6.0	0.2	6.8	6.2	0.6

* Sum of creatine phosphate + two-thirds ATP + one-half ADP.

diphosphate esters increased from 0 to 306. The increase in the diphosphate ester fraction observed in these experiments agrees with Lundsgaard's (1930b) conclusion that the esters which accumulate in these poisoned muscles consist largely of hexose diphosphate.

Comparisons of the phosphate ester contents of pair tortoise muscles treated alike

Pairs of tortoise muscles from the right and left sides of the same animal often differ in weight, sometimes by as much as 20%. To see if this difference was associated with any serious difference in the amounts of the phosphate esters in the muscles of a pair, some experiments were done in which both muscles were fixed in the unstimulated state by slow freezing. The results are given in Table 6. This shows that in two experiments a difference was found in the total amount of extractable phosphorus, which increased the differences in the amounts of the individual esters if these were expressed as μ g. phosphorus/g. The amounts of the

esters are more satisfactorily expressed as a percentage of the total extractable phosphorus, and Table 6 shows that if this is done, the differences between two muscles of a pair are not more than 10%.

Comparisons of stimulated and unstimulated tortoise muscles

In these experiments, following a suggestion of Hill (1949, 1950b), tortoise muscles have been used in an attempt to follow the changes in the phosphate esters during the early stages of activity. These muscles, being much slower than those of frog, are easier to fix at a given stage of contraction.

Muscles stimulated by sudden freezing. In these experiments the muscles were first cooled to 0°. One was stimulated and fixed at an early stage of contraction, when little or no shortening had occurred, by sudden freezing and the other was fixed without stimulation by slow freezing. The results of five of these experiments are given in Table 7. In an incomplete experiment, Expt. 6, the following

Table 7. *Changes in the phosphate ester contents of pair tortoise muscles, one muscle having been fixed and stimulated by sudden freezing, the other fixed by slow freezing*

Fraction	Type of muscle					Mean change	Mean content	
	Expt. 1. Ilio- fibularis	Expt. 2. Ilio- fibularis	Expt. 3. Ilio- fibularis	Expt. 4*. Extensor ilio- tibialis	Expt. 5. Sartorius		Stimu- lated	Unstimu- lated
	Length when frozen (% of resting length)							
Stimulated (frozen suddenly)	79	100	100	93	100			
Unstimulated (frozen slowly)	100	100	100	100	100			
Change in phosphate esters on stimulation								
	Difference (stimulated — unstimulated)							
	Expressed as $\mu\text{g. P/g. of muscle}$							
Total extractable P	- 89	+ 25	- 69	+ 120	- 191	- 40	967	1007
Protein P	—	- 2	+ 11	+ 32	- 11	- 3§	237§	240§
	Expressed as % of total extractable P							
ATP†	- 5.0	- 5.2	+ 3.2	- 2.8	+ 0.6	- 1.8	15.9	17.7
ADP	- 0.9	- 1.3	—	+ 0.2	- 0.3	- 0.6	4.8	5.4
Creatine phosphate	- 6.0	+ 0.1	- 2.5	- 0.8	- 2.3	- 2.3	15.1	17.4
'High-energy' P‡	- 9.8	- 4.0	- 0.4	- 2.6	- 2.1	- 3.8	28.1	31.9
Orthophosphate	+ 9.9	+ 3.6	- 4.7	- 4.0	+ 0.5	+ 1.1	32.2	31.1
Monophosphate esters and adenylic acid	+ 3.9	+ 1.6	+ 3.4	+ 1.2	- 0.4	+ 1.9	5.6	3.7
Diphosphate esters	- 0.1	0.0	+ 2.3	- 0.2	+ 0.6	+ 0.5	1.5	1.0
Unknown A	- 2.9	- 1.5	+ 2.3	+ 5.8	+ 2.2	+ 1.2	12.8	11.6
Unknown B	- 0.6	+ 2.7	- 3.9	+ 0.6	- 0.2	- 0.3	10.5	10.8
Unknown C	+ 0.2	—	—	—	0.0	+ 0.1	2.3	2.2
Unknown D	+ 1.4	—	—	—	0.0	+ 0.7	2.6	1.9
Unknown E	+ 0.2	—	—	—	- 1.0	- 0.4	1.9	2.3

* In this experiment the stimulated muscle was stimulated electrically just before being frozen.

† Expt. 3 includes ADP.

‡ Sum of two-thirds ATP + one-half ADP + creatine phosphate.

§ These three means include the results of one additional experiment which is described in the text.

differences, expressed as $\mu\text{g. phosphorus/g.}$, were found (value for stimulated muscle — value for unstimulated muscle): protein phosphorus, -42; ATP, -37; ADP, -12; creatine phosphate, -40; orthophosphate, -154; diphosphate esters, -4. The values for the protein phosphorus obtained in this experiment have been included in the means given in Table 7, but the other values have not because they cannot be expressed as a percentage of the total extractable phosphorus.

The results are somewhat erratic. The only clear-cut change on stimulation was a decrease in the 'high-energy' phosphate fraction (sum of two-thirds ATP + one-half ADP + creatine phosphate). In all experiments except one there was a decrease in creatine phosphate and ADP and an increase in the monophosphate esters. There was little sign of a change in the protein phosphorus. The decreases in ATP observed in Expts. 1, 2 and 6 are outside the usual variability found between pair muscles, whereas the changes in Expts. 3-5 are not, and many more experiments will have to be done to

decide whether there is a breakdown of the ATP of tortoise muscle in this kind of experiment.

Muscles stimulated electrically. The muscles were cooled to 0° . One was stimulated with condenser discharges at the rate of 6/sec. and after 5 sec., when it had just become fully contracted, it was fixed by sudden freezing while the stimulus was still being applied. The other, which was not stimulated, was fixed by slow freezing. The results of three experiments are given in Table 8. In two incomplete experiments, Expts. 4 and 5, the stimulated muscles were stimulated at a rapid rate with an induction coil, in Expt. 4 for 2 sec. and in Expt. 5 for 5 sec. The changes (value for stimulated muscle — value for unstimulated muscle, in $\mu\text{g. phosphorus/g.}$) were: in Expt. 4: protein phosphorus, -27; ATP, +12; ADP, -11; creatine phosphate, -8; orthophosphate, +62; diphosphate esters, +4; in Expt. 5: protein phosphorus, +8; ATP+ADP, +35; creatine phosphate, -56; orthophosphate, +70; diphosphate esters, +20. The values for the protein phosphorus obtained in these two experiments have

Table 8. *Changes in the phosphate ester contents of pair tortoise muscles, one muscle having been stimulated electrically for 5 sec. before fixation, the other not*

	Type of muscle				Mean content	
	Expt. 1. Ilio- fibularis	Expt. 2. Extensor ilio- tibialis	Expt. 3. Sartorius		Stimu- lated	Unstimu- lated
Length when frozen (as % of resting length)						
Stimulated	40	57	63			
Unstimulated	100	100	100			
Work done by stimulated muscle (g.cm.)	51	17	10			
Change in phosphate esters on stimulation						
Fraction	Difference (stimulated – unstimulated)			Mean change		
	Expressed as $\mu\text{g. P/g. of muscle}$					
Total extractable P	– 111	– 39	– 79	– 76	773	849
Protein P	+ 6	– 11	– 23	– 11†	220†	231†
Expressed as % of total extractable P						
ATP*	– 0.2	+ 2.0	– 2.8	– 0.6	26.7	27.1
ADP	—	+ 0.9	—	+ 0.9	7.2	6.3
Creatine phosphate	– 7.8	– 4.7	– 7.6	– 6.7	14.8	21.5
‘High-energy’ P†	– 7.8	– 2.8	– 9.4	– 6.7	36.1	42.8
Orthophosphate	+ 1.6	– 0.8	+ 3.3	+ 1.3	31.4	30.1
Monophosphate esters and adenylic acid	+ 1.7	+ 4.6	+ 4.8	+ 3.7	3.7	0.0
Diphosphate esters	– 0.1	0.0	– 0.3	– 0.2	1.7	1.9
Unknown A	+ 2.6	– 2.4	+ 0.2	+ 0.2	11.7	11.5
Unknown B	+ 2.2	+ 0.6	+ 2.5	+ 1.8	7.6	5.8

* Expts. 1 and 3 include ADP.
† These three means include the results of two additional experiments which are described in the text.
‡ Sum of two-thirds ATP + one-half ADP + creatine phosphate.

been included in working out the means given in Table 8, but the other values have not since they cannot be expressed in terms of the total extractable phosphorus.

The results show that after 5 sec. stimulation of tortoise muscle, creatine phosphate and the ‘high-energy’ phosphate fraction had decreased and the monophosphate esters and unknown B had increased. Orthophosphate had increased in all experiments except one. There was little sign of changes in the other fractions.

DISCUSSION

This work has shown that if the chromatographic method of Hanes and Isherwood is used in conjunction with other methods, the phosphate esters in muscle extracts can be separated into ATP, ADP, creatine phosphate, orthophosphate, the monophosphate ester fraction, the diphosphate ester fraction and up to five unidentified fractions, and quantitative values for these obtained.

The values for frog muscle are in good agreement with those obtained by Eggleton & Eggleton (1929), Lundsgaard (1930b) and Causey & Harris (1951) with other methods, which fact gives confidence in the chromatographic method. The values show that there is more ATP and creatine phosphate + ortho-

phosphate in frog muscle than in tortoise muscle, and this comparison of a fast striated muscle with a slow one shows the same trend as those of striated muscles with the slower smooth muscles. Thus Walaas & Walaas (1950) have shown that the slow unstriated uterine muscles of rats and rabbits contain much less ATP and creatine phosphate than the fast striated skeletal muscles.

The values for ADP in frog and tortoise muscle show that this usually forms 10–20% of the pyrophosphate fraction. This is higher than the values of 1–5% obtained by Bailey (1949) with rabbit muscle by enzymic analysis. No inorganic pyrophosphate, tripolyphosphate or metaphosphate was found in the extracts with the possible exception of a few of those of tortoise muscle, the unknown D in which may have been pyrophosphate.

The unidentified ester found in snail muscle was probably arginine phosphate but this has not been confirmed. The identity of the other unknown esters and their function in muscle is obscure. Cori & Cori (1931) state that about 70 $\mu\text{g./g.}$ of the phosphorus in rat-muscle extracts does not correspond to known phosphate esters and it is possible that this consists of the unknown esters now detected.

Current theories of muscular contraction postulate that the hydrolysis of ATP to ADP and ortho-

phosphate provides the energy for mechanical work (see, for example, Baldwin, 1952). To demonstrate this an increase in orthophosphate and either a decrease in ATP or an increase in ADP must be shown in the stimulated muscle, preferably in the same experiment. There was no such direct evidence, except in muscles fixed after exhaustion, until recently when experiments on the changes early in activity were published by Lundsgaard (1950) which suggested a decrease in the ATP + ADP fraction and by Mommaerts & Rupp (1951) which suggested a decrease in ATP and increases in ADP and AMP.

In the present work very little evidence for these changes has been obtained. The experiments in which stimulated and unstimulated unpoisoned frog muscles were compared show, in the stimulated muscle, only the decrease in creatine phosphate and the increases in orthophosphate and the monophosphate esters found by earlier workers (see, for example, Eggleston & Eggleston, 1929; Cori & Cori, 1934). Those in which exhausted and unstimulated frog muscles which had been poisoned with monoiodoacetate were compared show the changes found by Lundsgaard (1930*b*), namely decreases in creatine phosphate and the ATP + ADP fraction and an increase in the diphosphate ester fraction on exhaustion. Of the components of the ATP + ADP fraction, the ATP decreased and the ADP increased, but the results do not provide evidence for the hydrolytic breakdown of ATP during the exhaustion of the poisoned muscles because all the phosphorus which disappears from the ATP and creatine phosphate fractions reappears in the diphosphate ester fraction and not as orthophosphate. There were still appreciable amounts of ATP in the exhausted muscles, and their exhaustion cannot be due to the complete conversion of their supply of ATP into ADP which Lundsgaard's figures suggest.

The continued presence of ATP is no doubt due to the intervention of myokinase, which converts ADP into ATP and AMP. An equilibrium constant for this change in exhausted muscles can be obtained if the amount of AMP in them is assumed to be equal to the amount of ATP which has disappeared, allowance being made for the ATP which has gone into the ADP fraction. The constants calculated from Expts. 1 and 2 of Table 5 for $\frac{[\text{ATP}][\text{AMP}]}{[\text{ADP}]^2}$

are 0.6 and 0.1. These are of the same order as the value 0.444 which has been obtained by Eggleston & Hems (1952) with the isolated enzyme.

The experiments with tortoise muscles have given some idea of the reactions which take place in the early stages of activity, but further work will have to be done to confirm and extend them. The protein phosphorus, ADP, the diphosphate esters and unknown *A* have not been found to show any

significant trends. ATP, orthophosphate and unknown *B* behave erratically at the beginning of activity. After 5 sec. stimulation ATP shows no change and orthophosphate and unknown *B* show a tendency to increase. The monophosphate ester fraction increases from the beginning of activity and creatine phosphate and the 'high-energy' phosphate fraction decrease. The decreases in the 'high-energy' phosphates, usually in the creatine phosphate component, at the very beginning of activity support the hypothesis that the breakdown of these compounds provides energy for mechanical work and for heat production, which, as Hill (1950*a*) has shown, starts before the mechanical changes.

The amount of 'high-energy' phosphate which has been found to break down during the first 5 sec. of activity in tortoise muscles at 0° seems to be about 40 μg . phosphorus/g. Hill (1950*a*) has shown that the heat and work produced during this period is equivalent to about 2 mcal./g. which correspond to only about 5 μg . phosphorus/g. of 'high-energy' phosphate if this is assumed to break down hydrolytically. A similar, though smaller discrepancy, was noted by Mommaerts & Rupp (1951) between the amount of ATP broken down in their experiments and that expected from the energy production. Three possible explanations, which must be tested experimentally, can be put forward in the light of the present work to account for this discrepancy. These are: (1) that the phosphate esters do not change in the early stages of activity, the changes observed being entirely due to the difference in the rates of freezing and not to the stimulation; (2) that the 'high-energy' phosphates break down hydrolytically in the early stages and that much of the orthophosphate formed combines with glycogen to form hexose monophosphate, the discrepancy in the extent of the changes being accounted for, either by the difference in the rates of freezing, or by assuming that the suddenly frozen stimulated muscle takes some time to freeze completely and is in the active state for considerably longer than a few seconds; (3) that the change which provides energy for the production of heat and work is not necessarily the hydrolytic transfer of the phosphate of the 'high-energy' fraction to water, but may be a transfer to some acceptor with which it forms either a monophosphate ester or, in the case of muscles poisoned with monoiodoacetate, a diphosphate ester, the energy released being less than in a hydrolytic splitting.

SUMMARY

1. The method of Hanes & Isherwood (1949) for separating phosphate esters by paper chromatography has been used quantitatively in the study of these esters in muscle.

2. Tests with known mixtures of phosphate esters and the recovery of the phosphorus of muscle extracts from the chromatograms suggest that the individual values obtained with the method in its present form are reliable to within 10 %.

3. Trichloroacetic acid extracts of muscle have been found to contain certain phosphate esters not yet identified.

4. The pyrophosphate fraction present in the extracts is found to be entirely in the form of adenosine triphosphate and adenosine diphosphate.

5. Striated muscle from tortoise, which is about ten times slower than that from frog, contains less adenosine triphosphate and creatine phosphate + orthophosphate than the latter.

6. The chromatographic method has been used to repeat some of the classical experiments on the metabolism of intact muscle and the results of these experiments have been confirmed.

7. An attempt has been made to follow the changes in tortoise muscle during the early stages of activity. The most prominent ones are a decrease in the 'high-energy' phosphate (two-thirds adenosine triphosphate + one-half adenosine diphosphate + creatine phosphate), largely in the creatine phosphate component, and an increase in the monophosphate ester fraction.

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REFERENCES

- Bailey, K. (1949). *Biochem. J.* **45**, 479.
 Baldwin, E. (1952). *Dynamic Aspects of Biochemistry*, 2nd ed. p. 396. Cambridge University Press.
 Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.
 Causey, G. & Harris, E. J. (1951). *Biochem. J.* **49**, 176.
 Cori, G. T. & Cori, C. F. (1931). *J. biol. Chem.* **94**, 561.
 Cori, G. T. & Cori, C. F. (1934). *J. biol. Chem.* **107**, 5.
 Eggleston, L. V. & Hems, R. (1952). *Biochem. J.* **52**, 156.
 Eggleston, G. P. & Eggleston, P. (1929). *J. Physiol.* **68**, 193.
 Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
 Hill, A. V. (1949). *Nature, Lond.*, **163**, 320.
 Hill, A. V. (1950a). *Proc. Roy. Soc. B*, **137**, 268.
 Hill, A. V. (1950b). *Biochim. biophys. Acta*, **4**, 4.
 Humphrey, J. H. & Harris, E. J. (1948). *J. sci. Instrum.* **25**, 314.
 Lohmann, K. (1935). *Biochem. Z.* **282**, 109.
 Lundsgaard, E. (1930a). *Biochem. Z.* **217**, 162.
 Lundsgaard, E. (1930b). *Biochem. Z.* **227**, 51.
 Lundsgaard, E. (1950). *Proc. Roy. Soc. B*, **137**, 73.
 Mommaerts, W. F. H. M. & Rupp, J. C. (1951). *Nature, Lond.*, **168**, 957.
 Owen, R. (1866). *Anatomy of Vertebrates*. **1**, p. 235. London: Longmans Green and Co.
 Walaas, O. & Walaas, E. (1950). *Acta physiol. scand.* **21**, 1.
 Walker, D. G. & Warren, F. L. (1951). *Biochem. J.* **49**, xxi.
 Zeile, K. & Fawaz, G. (1938). *Hoppe-Seyl. Z.* **256**, 193.

The Turbidimetric Estimation of Hyaluronidase

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Both the formation of turbidity by the reaction between hyaluronate and protein and the kinetics of the enzymic depolymerization of hyaluronate are complex. Hence, the turbidity-reducing action of hyaluronidase cannot be interpreted by the existing theory of enzyme kinetics. However, the use of turbidity reduction as the basis of a procedure for the assay of hyaluronidase requires that the kinetics of the reaction and the effects of the concentration and mode of preparation of both the substrate and the enzyme upon the apparent enzymic activity be

known. In view of the incomplete and contradictory information available in the literature, these factors were investigated.

MATERIALS

Crude hyaluronidase was prepared by the Freeman, Anderson, Oberg & Dorfman (1949) modification of the techniques of Madinaveitia (1939) and Hahn (1943). Fresh bovine testes were extracted with dilute acetic acid, and the extract was fractionated with ammonium sulphate, dialysed and freeze-dried. The resulting product had 28 turbidity reducing units/mg. (Meyer, 1947). In one experiment, a purified preparation which had 200 turbidity reducing units/mg. was employed. This was kindly supplied by

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