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The Breakdown of Adenosinetriphosphate in Extracts of Rabbit Muscle

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The claim, made in 1939 by Engelhardt & Lyubimova, that the structural protein, myosin, was itself the enzyme responsible for the dephosphorylation of adenosinetriphosphate (ATP) in muscle, has since been often contested. No evidence could be found to the contrary by Bailey (1942) and Needham (1942), but Polis & Meyerhof (1946, 1947) do not consider that myosin possesses, homogeneously, adenosinetriphosphatase (ATP-ase) activity. They contend that ATP-ase is absorbed on to myosin, like the potato apyrase in Kalckar's (1944) experiments. Various reviews of the controversy exist (Engelhardt, 1946; Polis & Meyerhof, 1947).

However, apart from this ATP-ase, which may or may not be myosin itself, Sakov (1941), a member of the Engelhardt school, reported a distinct non-myosin ATP-ase in aqueous extracts of muscle. The existence of such an enzyme is supported by calculations of Mommaerts & Seraidarian (1947) and Braverman & Morgulis (1948). The enzyme described by Sakov (1941) differs from myosin ATP-ase in solubility, pH optimum, and the extent of hydrolysis which it effects. It is not clear if this enzyme bears any relation to that reported by Lohmann (1928), who examined 'pyrophosphate' breakdown in a dilute salt extract of muscle, and described an ATP-ase with yet another set of properties. Sakov's (1941) non-myosin ATP-ase must further be compared with ATP-ases from tissues other than muscle, e.g. liver (Barrenscheen & Lang, 1932), nervous tissue (Reis, 1937), sperm (Lardy, Hansen & Phillips, 1945), potato (Kalckar, 1944) and various other animal tissues (Dubois & Potter, 1943).

In this paper, some experiments on the ATP-ase in aqueous muscle extracts, prepared according to the method of Sakov (1941), were performed with a view

to determining if the Sakov enzyme can, indeed, be considered distinct from myosin ATP-ase, and if it can be compared with ATP-ase from myosin-free sources.

METHODS

Incubations were carried out at either 25 or 37°; in all cases the reaction mixtures without the enzyme were incubated first for a few minutes and then the enzyme, at the required temperature, was added. The control and experimental tubes were deproteinized with a final concentration of 5% (w/v) trichloroacetic acid, and inorganic phosphate estimated by the method of Fiske & Subbarow (1925), using either a Finlayson single photocell, or a Hilger Spekker absorptiometer. The increase in inorganic phosphate was found to parallel the decrease of 7 min. P (inorganic P set free after 7 min. at 100° in the presence of N-HCl) in the reaction mixtures and therefore the ATP was undergoing simple hydrolysis.

ATP was prepared by the method of Needham (1942), and analysis for N and inorganic, hydrolysable and total P showed the purity to be over 95%. Synthetic muscle adenylic acid was kindly donated by Dr T. R. Mann of the Molteno Institute, Cambridge. The pH of the experiments was controlled with the glass electrode by determining the value of the mixture to be used, with water replacing the neutralized solution of ATP (or other substrate).

An attempt was made to keep as closely as possible to the quantities of material used by Sakov (1941), but since this author gives no details beyond indicating the range of the amounts used, the conditions adopted in this investigation differ slightly from experiment to experiment.

The enzyme was made by extracting chilled, fresh rabbit muscle twice with an equal volume of ice-cold water, and centrifuging after each extract; triple glass-distilled water was used for the majority of the extracts, solutions, and dialyses (the reaction mixtures contained less than 3×10^{-6} M total Cu). The muscle was disintegrated either by grinding with sand or homogenizing in the Waring blender. The

combined extracts were dialysed in the refrigerator for 3–24 hr. in a cellophan sac against 15 vol. of water; the duration of dialysis did not affect the enzyme activity. In the longer dialysis, the water was changed after 3 hr. After dialysis the extract had a pH of 5.5 and consisted of a clear red supernatant fluid and a white precipitate which could be centrifuged off in 5 min. at 3000 rev./min. For use in experiments, the precipitate was washed once with water equal in volume to the supernatant to which it corresponded; after centrifuging the precipitate was suspended in the same volume of water. Fractionation with $(\text{NH}_4)_2\text{SO}_4$ was carried out as follows: the ice-cold whole extract was treated with solid $(\text{NH}_4)_2\text{SO}_4$ and N-NaOH to give a final $(\text{NH}_4)_2\text{SO}_4$ concentration of 150 mg./ml. and pH 6.5. After standing 10 min. at 0° , the precipitate was centrifuged off for 10 min. at 3000 rev./min. and the supernatant treated as before to keep the pH at 6.5 while bringing the $(\text{NH}_4)_2\text{SO}_4$ concentration to 250 mg./ml. After standing 30 min. at 0° the precipitate had settled, and was removed by centrifugation. The supernatant fluid and the two precipitates were all made up to the volume of the whole extract originally taken, and dialysed overnight against 100 vol. of water; each developed a slight precipitate which was not removed before use. During the dialysis the pH of the enzyme suspensions fluctuated, the lowest level reached being pH 5.5; dialysis against 0.01 M-borate buffer (pH 7.0) prevented such an acidification, but did not materially alter the results.

Aneurin pyrophosphate (Merck) and L-cysteine (Wyeth) were used; the remaining reagents were from British Drug Houses, London, except for merthiolate (sodium ethyl-mercurithiosalicylate) which was kindly presented by Dr P. M. de Burgh, Department of Bacteriology, University of Sydney.

RESULTS

Molarity of the aqueous extract

In extracting myosin from muscle, a 0.5 M-salt solution is generally used. Myosin is partially soluble in much lower salt concentrations (Szent-Györgyi, 1947), and the molarity of an aqueous extract may be sufficiently high to extract some myosin. We therefore determined the molarity of the Sakov (1941) extract by the freezing point-depression method. However, the depression of the first extract, before dialysis, was 0.431, which corresponds to 0.12 M. It is unlikely that myosin would be present.

Distribution of activity

During dialysis of the fresh extract, a white precipitate appears. Sakov (1941) states that in his experiments, the extract, complete with its precipitated proteins, was used. It seemed of interest to study the distribution of activity between precipitated protein and supernatant fluid, since the former consists presumably of a salt-soluble protein, and may be myosin.

The activity was shown to be divided between the two fractions, but not completely recoverable. In a typical experiment (Fig. 1), where hydrolysis was

allowed to proceed for 15 min. at pH 7.3, the following percentage liberation of labile phosphate occurred: whole extract, 57; supernatant fluid, 9; precipitated proteins, 11%.

Fig. 1. Hydrolysis of ATP by fractions at different pH values. Time 15 min., temp. 25° ; 0.3 ml. enzyme in a final volume of 0.9 ml. containing 0.01 M-glycine and 45 μg. ATP. 7 min. P.

Hydrolysis of other substrates

Neither the whole extract nor its fractions had any action upon the phosphate bonds of glycerophosphate, yeast adenylic acid or phenyl phosphate. The whole extract and the supernatant fluid attacked aneurin pyrophosphate very slightly.

Table 1. *Hydrolysis of aneurin pyrophosphate and muscle adenylic acid*

(Enzyme (0.3 ml.) in a final vol. of 0.9 ml.; mixture contained 0.01 M-glycine buffer of pH 7.3 and 45 μg. adenylic acid P or 90 μg. aneurin pyrophosphate P. Values are % of total P liberated in 60 min. at 37°).

	Whole extract	Supernatant	Precipitate
Aneurin pyrophosphate	4.4	5.8	0
Muscle adenylic acid	24.0	9.8	0.8

Inorganic pyrophosphate was split by the whole extract and supernatant fluid (Fig. 2). The precipitated proteins did not hydrolyse inorganic pyrophosphate, and nearly all of the activity of the whole extract was, unlike the ATP-ase activity, recoverable in the supernatant fluid. It would seem also from the separation by ammonium sulphate fractionation (see below) that this pyrophosphatase is probably distinct from the ATP-ase.

Muscle adenylic acid was attacked slowly (Table 1). This hydrolysis is also apparent in the curves describing the action of the extract on ATP

(Fig. 3). It is possible that here the adenylic acid was first deaminated to inosinic acid which was then dephosphorylated.

Fig. 2. Hydrolysis of $\text{Na}_4\text{P}_2\text{O}_7$ by fractions at different pH values. Time 15 min., temp. 37° ; 0.3 ml. enzyme in a final volume of 0.9 ml. containing 0.01 M-glycine and $45 \mu\text{g. Na}_4\text{P}_2\text{O}_7\text{-P}$.

Fig. 3. Progress curve for the hydrolysis of ATP by the precipitate at pH 8.6 in the presence of 0.001 M- CaCl_2 and the whole extract at pH 7.0; 0.3 ml. enzyme in a final volume of 0.9 ml. containing 0.01 M-glycine and $45 \mu\text{g. ATP}$. 7 min. P.

Ammonium sulphate fractionation

Sakov (1941) found that 250 but not 150 mg./ml. ammonium sulphate precipitated the ATP-ase of the aqueous extract. In our experience, nearly all the ATP-ase activity was precipitated at 150 mg./ml. (Fig. 4). Using glycine buffer at 37° , neither the ammonium sulphate fractions nor the supernatant fluid showed any pyrophosphatase activity in the range pH 4–10; this indicates that the pyrophosphatase was destroyed by the treatment.

The purified system had a Q_P of 70–80 as compared with 30–40 for the whole extract. The Q_P , as defined by Engelhardt (1946), represents the $\mu\text{l. P}$ liberated/mg. dry wt. of enzyme preparation/hr.

Fig. 4. Hydrolysis of ATP by the $(\text{NH}_4)_2\text{SO}_4$ fractions. A, material precipitated by 150 mg. $(\text{NH}_4)_2\text{SO}_4/\text{ml.}$; B, material precipitated between 150 and 250 mg. $(\text{NH}_4)_2\text{SO}_4/\text{ml.}$; C, supernatant; material not precipitated by 250 mg. $(\text{NH}_4)_2\text{SO}_4/\text{ml.}$ Time 15 min., temp. 37° ; 0.3 ml. enzyme in a final volume of 0.9 ml. containing 0.01 M-glycine and $45 \mu\text{g. ATP}$. 7 min. P.

Effect of pH

In Figs. 5 and 6 are presented what we regard as typical pH curves for the whole extract. In no case were optima observed at either pH 4.8 or 6.0, the two values quoted by Sakov (1941) (see Discussion, p. 241). Borate buffer gave a little more activity than glycine at the same pH; borate was definitely better than acetate.

Although these buffers do not function well over the whole of the pH range tested, they were used because they were employed by Sakov (1941) and by other workers in studies on myosin ATP-ase. Each tube was adjusted to the required pH before incubation (see Methods, p. 238), and it is unlikely that the amount of inorganic phosphate produced during the reaction would change the pH significantly.

Progress curves

The whole extract produced inorganic phosphate equivalent to two groups from ATP in 30 min. at pH 7.0 (Fig. 3). It then slowly attacked the third group and effected some hydrolysis. As described above, muscle adenylic acid was itself hydrolysed slowly by the extract.

The precipitate removed the equivalent of two P groups at pH 8.6.

Ion effects

Table 2 shows the effect of activators and inhibitors on the whole extract, supernatant fluid and precipitated proteins. The most striking effect of

calcium and magnesium was on the precipitated proteins at pH 7 where marked activation occurred. With the whole extract calcium slightly inhibited, whereas magnesium slightly activated. This behaviour resembles, somewhat, that of the non-myosin ATP-ase described by Kielley & Meyerhof (1948a).

iodoacetate did not inhibit very markedly, while peroxide caused a slight inhibition, removable by cysteine (Ziff, 1944). Cysteine alone had only a slight activating effect. Cyanide inhibited to some extent, but glutathione activated, especially the whole extract and the precipitate. Fluoride inhibited the whole extract and precipitate.

Table 2. *Effect of activators and inhibitors*

(Enzyme (0.3 ml.) in a final vol. of 1 ml.; mixture contained 0.01 M-glycine or borate buffer, 0.01 M activator or inhibitor and 45 µg. ATP. 7 min. P. Incubated 15 min. at 37°. Figures are percentage of control.)

		P liberated as % of control			
		Whole extract	Super-natant	Precipitate	
pH	...	7.2	9.0	7.4	8.5
Ca		71	42	340	145
Mg		112	60	310	168
KCl		98	104	93	75
KCN		95	99	94	—
Cysteine		109	103	106	—
Glutathione		116	108	119	—
Iodoacetate		99	78	98	—
Merthiolate		81	101	35	22
H ₂ O ₂		92	—	—	—
Fluoride		88	109	89	—

Fig. 5. Effect of pH on the hydrolysis of ATP in 15 min. at 25° or 37°; 0.3 ml. whole extract in a final volume of 0.9 ml. containing 0.01 M-glycine and 45 µg. ATP. 7 min. P.

In an attempt to increase the efficacy of the SH inhibitors, experiments were carried out where the enzyme, buffer and inhibitor were pre-incubated together for an hour at 37° in the absence of substrate. Controls incubated with water instead of inhibitor showed that this procedure resulted in a loss in activity of all the fractions, but the subsequent degree of inhibition brought about by the SH reagents when substrate was added, was not increased when compared with the results set out in Table 2. For example, with the whole extract, merthiolate gave 90 % of the control, iodoacetate 100 % and peroxide 105 %.

DISCUSSION

Our object in this study was to consider if the ATP-ase in muscle extracts could be considered distinct from the ATP-ase of myosin and further, to endeavour to repeat the results of Sakov (1941).

It is unlikely that myosin ATP-ase is present in the aqueous extract and it could not account for the activity of the preparations. The salt concentration of the aqueous extract is 0.12M, at which strength myosin is to some extent soluble in potassium chloride but not in sodium chloride, magnesium chloride or calcium chloride (Szent-Györgyi, 1947). Activation of the precipitated proteins by calcium and magnesium (Table 2) resembles the specific activation of myosin ATP-ase which has often been described (Bailey, 1942; Singher & Meister, 1945; Mommaerts & Seraidarian, 1947), but this increase

Fig. 6. Effect of pH on the hydrolysis of ATP in 15 min. at 25° or 37°; 0.3 ml. whole extract in a final volume of 0.9 ml. containing 0.01 M buffer and 45 µg. ATP. 7 min. P. The breaks in the curves denote the points where borate replaced acetate.

Activators and inhibitors

The ATP-ase of aqueous extracts of muscle does not appear to be greatly dependent upon its sulphhydryl (SH) groups for activity. Merthiolate (except for its action on the precipitate) and sodium

in activity could be due to greater solubility specifically caused by the presence of these ions, potassium chloride being inactive in this regard (Table 2).

The effects of the SH inhibitors are again most striking on the precipitated proteins. Iodoacetate was not effective in inhibiting any fraction; the limitations of this compound as an SH inhibitor have been discussed by Singer & Barron (1944). The mercaptide-forming reagent, merthiolate, which is more effective than iodoacetate in combining with SH groups, inhibited the precipitated proteins greatly, but the whole extract only slightly. Neither the pH curve nor the progress curve on the precipitated proteins shows 'typical' myosin behaviour.

Even if some myosin were present, this alone could not account for more than a small part of the activity in a Sakov extract. The activity of the aqueous extract is apparently due to an ATP-ase which may be responsible for the 10% of ATP-ase activity which is not extracted from whole muscle as myosin (Needham, 1942). Such a non-myosin ATP-ase is called for by the calculations of Mommaerts & Seraidarian (1947) and Braverman & Morgulis (1948).

In attempting to repeat his results, we could not agree with Sakov (1941) as to the nature of this non-myosin ATP-ase. Not only did our ammonium sulphate fractionation differ, but, more importantly, we were unable to repeat his pH curve. Sakov presents two curves demonstrating the effect of pH on the 'mineralization' of ATP. In his Fig. 3, the dialysed muscle extract shows a peak at about pH 4.8 (about 17 μ g. P mineralized), a minimum at pH 7.0 (3 μ g. P) and a peak at pH 8.5 (8 μ g. P). However, in his Fig. 5, a single maximum is depicted at pH 6.2, giving 60–80 μ g. P depending on whether 48 or 2.3 hr. dialysis was used; in the legend it is stated that the 'liquid together with the precipitated proteins was used'. It is not clear whether this latter curve was obtained with the fresh extract or the protein precipitate obtained at 250 mg./ml. ammonium sulphate; there is no mention that the latter precipitate, when dissolved in water, was dialysed, or, if so whether a further precipitate appeared. In the 'Conclusions', Sakov simply states that there is present in the aqueous extracts a non-myosin ATP-ase with an optimum pH of about 6.0, but in his Table 5, this enzyme is referred to as the 'protein fraction from the aqueous extract'.

However, with quantities of reactants that fell within the range used by Sakov, and also with other concentrations, we could never detect an optimum at a pH of about 4.8 or 6, with either the whole extract or the ammonium sulphate fractions. On a few occasions there was a minor peak at about pH 6; but always the main optimum fell in the alkaline range. In this connexion, experiments on intact rigor muscle (Bate-Smith & Bendall, 1949) do not

support the contention that there is present an ATP-ase with an optimum at pH 6.

Our preparations appeared to remove all the labile P of ATP quickly, and then to attack slowly the third phosphate group; however, the work of Banga & Josepovits (1948) calls for caution in making such an interpretation of progress curves. Adenylic acid was also hydrolysed slowly, but this action on the third phosphate group may well be due to another enzyme present in the crude extract. Sakov (1941) considers the extent of hydrolysis one of the distinguishing features between this ATP-ase and myosin ATP-ase; yet his progress curve provides inadequate information, ceasing at 30 min., before even all the labile phosphate has been removed. On the other hand, Kalckar (1944) states that neither myosin ATP-ase nor water-soluble ATP-ase can attack ADP unless myokinase is present.

If myokinase, a water-soluble enzyme (Colowick & Kalckar, 1943), is present in the extract under investigation in this paper, it is of interest to consider if the characteristics of the ATP-ase activity could correspond to that which one would expect of (i) myokinase + myosin, or (ii) myokinase + the magnesium-activated ATP-ase recently described by Kielley & Meyerhof (1948*a*).

(i) Although it has been assumed that the low activity of the supernatant fluid, from which the proteins precipitated by dialysis had been removed, was due to interference with the system's stability, much of the behaviour of the whole and divided extract might have been explained by supposing that the vigorous hydrolysis of ATP in the whole extract was effected by myokinase (soluble) and myosin (precipitated). However, such a conjecture is excluded by the facts that (a) merthiolate hardly affects the whole extract, and (b) peroxide does not inhibit the whole extract, whereas Ziff (1944) and Mehl (1944) report its inhibition of myosin. This apparent non-SH character of the extract ATP-ase would prove an exception to the statement of Bailey (1947) that 'ATP, wherever it acts as substrate, does so with enzymes either of proven or suggested SH character...and may be deemed to have an affinity for some type of SH grouping to be found in proteins....'

(ii) The ATP-ase of Kielley & Meyerhof (1948*a*) also offers some points of comparison with the ATP-ases under consideration here. The Kielley & Meyerhof (1948*b*) enzyme, though stated to be extractable by water, was isolated by means of a 0.5M-potassium chloride salt mixture, and purified by ammonium sulphate fractionation, being precipitated at a concentration of 270 mg./ml. This concentration is close to that employed by Sakov (1941), but nearly double that employed in this paper. It is not possible to compare the extent of hydrolysis in our crude extract with that by the purified enzyme of

Kielley & Meyerhof. However, the pH optimum of the two enzymes lies in a similar range; and the magnesium activation and calcium inhibition reported in this paper, with regard to the whole extract, were observed by Kielley & Meyerhof in a greater degree. On the other hand, the instability and the apparent SH character of this latter enzyme are in direct contrast with the present findings.

If, then, muscle contains, apart from its specialized myosin ATP-ase, another enzyme breaking down ATP, can this latter be compared with ATP-ases performing a general function in other tissues? The assays performed by Dubois & Potter (1943) revealed ATP-ases in various muscular, glandular and nervous tissues, but these enzymes (studied in tissues homogenized in ice-cold water) showed optimum pH at about 9.2 with calcium exerting an activating effect. On the other hand, Barrenscheen & Lang (1932) investigated an aqueous extract of liver containing an ATP-ase with optima at pH 8.2 and 9; magnesium inhibited this extract. However, other workers have reported ATP-ases with optima in the region reported in this present paper. Lohmann (1928), using muscle extracts prepared with 0.9% (w/v) potassium chloride (which would certainly extract some myosin), obtained a pH curve symmetrical about pH 7.0. The ATP-ase from nervous tissue which Reis (1937) describes has, unlike that of Dubois & Potter (1943), an optimum pH at 7.7.5. Meyerhof & Wilson (1947) associate the 'apyrase' activity of brain with the solid tissue of homogenates, finding it to be destroyed by treatment with distilled water. Little is known of plant apyrases, but the ATP-ase in potato (Kalckar, 1944) has its pH optimum in the neutral and slightly acid range. ATP-ases are reported from various other sources, such as spermatozoa (Lardy *et al.* 1945) and the sea-urchin egg (Connors & Scheer, 1947). Among the invertebrates, oyster (*Saxostrea commercialis*) muscle has been investigated with regard to its content of myosin and other ATP-ases (Humphrey, 1950). This tissue, while possessing myosin ATP-ase of very low activity contains in addition a water-soluble enzyme capable of splitting two phosphate groups from

ATP; the myosin, unlike the latter enzyme, is of marked SH character. However, data are not available for most tissues to make possible a comparison between these enzymes and myosin on the basis of pH optimum, calcium-magnesium effects, and SH inhibitions.

SUMMARY

1. The adenosinetriphosphatase (ATP-ase) and other phosphatases in aqueous extracts of rabbit muscle, prepared according to the method of Sakov (1941) were studied. An inorganic pyrophosphatase was also detected.

2. The ATP-ase activity was divided between the proteins precipitated by dialysis and the supernatant fluid, but was not wholly recoverable in the separated fractions.

3. In contrast to Sakov's findings, nearly all the ATP-ase was precipitated at a concentration of 150 mg./ml. ammonium sulphate. This treatment destroyed the pyrophosphatase.

4. pH curves in glycine and acetate-borate buffers at 25° and 37° gave optima in the range pH 6.8–7.4 for the whole extract. In no instance were optima detected at Sakov's values of pH 4.8 and 6.0. Optima for the precipitated proteins, supernatant fluid, and 150 mg./ml. ammonium sulphate fraction were: 7.5–8.0, 9.4 and 8.0.

5. Two-thirds of the total phosphorus of adenosinetriphosphate was quickly hydrolysed, after which the remainder, presumably the third, non-labile phosphate group, was slowly removed. Muscle adenylic acid was itself slowly hydrolysed.

6. Calcium and magnesium induced a striking activation of the precipitated proteins.

7. Merthiolate, a mercaptide-forming reagent, inhibited the precipitated proteins strongly, but not the supernatant fluid. Glutathione and cysteine slightly activated the whole extract.

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The Carotenoids of the Gonads of the Limpets *Patella vulgata* and *Patella depressa*

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The ripe gonads of the gastropods *Patella vulgata* and *P. depressa* are bright pink in the male and olive-green to chocolate-brown in the female. A preliminary examination by Lönnberg & Hellström (1931) indicated the presence of carotenoids.

The present paper is concerned chiefly with the identification of the constituent carotenoids. It has been shown, however, that the ovaries contain, in addition, an unidentified pigment which is not a carotenoid.

EXPERIMENTAL

Extraction of material. The bodies of the limpets were cut away from the shells, and the gonads carefully removed and ground to a fine powder with acid-washed silver sand and anhydrous Na_2SO_4 . The resulting powder was then extracted with successive portions of peroxide-free ether (obtained by distillation of commercial ether over reduced iron) until the final extract was colourless. The extracts were combined, the solvent removed *in vacuo* at room temperature and the residue saponified under N_2 at room temperature for 24 hr. with 20% (v/v) ethanolic KOH. The unsaponifiable material was extracted with ether using a conventional method (Goodwin & Morton, 1946). The ether was removed *in vacuo* and the residue dissolved in a small volume of methanol and cooled in solid CO_2 ; considerable amounts of precipitated steroids were filtered off using a Büchner funnel packed with solid CO_2 . The methanol was removed *in vacuo* at about 30° and the residue, dissolved in light petroleum (b.p. 40–60°), subjected to chromatographic analysis. About 2000 limpets were analysed in this way during the autumns of 1948 and 1949. Male and female gonads were examined separately, but apart from the presence of pigment Y (see below, p. 246) in the ovaries, the results were identical.

Chromatography was carried out using alumina (Spence, Grade O) deactivated by suspending it in methanol for about 30 min. After filtering off the methanol the alumina was allowed to dry at room temperature by spreading it out on

large pieces of filter paper. If the alumina thus obtained was too weak for the purpose in hand it was reactivated by heating at 30–60° for 3–4 hr.

Using deactivated alumina and developing the column with light petroleum (b.p. 40–60°) containing 1–2% (v/v) of ether, four main zones separated in the following order of increasing adsorptive power: (I) yellowish-orange band, (II) broad red band adsorbed very close to (III), a narrow zone, and (IV) a yellow-orange zone.

A very small fraction which rapidly washed through the column was generally encountered; this behaviour, together with the absorption spectrum of the fraction, suggested the presence of α -carotene, but sufficient was never obtained to make identification certain.

Pigment I. This pigment, which was epiphasic to 90% aqueous methanol, was purified by repeated chromatography, using 1% (v/v) of ether in light petroleum (b.p. 40–60°) as eluant, to separate the pigment from traces of other pigments. The chromatographically homogeneous material was dissolved in methanol and the greater part of the colourless impurities removed by cooling in solid CO_2 and filtering while cold. After this final purification the absorption spectrum of the pigment was determined in light petroleum (b.p. 40–60°), CS_2 , and pyridine; in each solvent the curve was identical with that of an authentic sample of crystalline β -carotene. On chromatographing a mixture of pigment I and β -carotene no separation was achieved, thus confirming the identification.

Pigment II. This pigment was purified by adsorption followed by elution with 2–3% (v/v) ether in light petroleum. The adsorption curve showed only one peak in the visible region indicating a keto-carotenoid (Heilbron & Lythgoe, 1936). The data available indicated that pigment II was most likely to be either echinone (Lederer, 1935) or aphanin (Tischer, 1938, 1939), the former isolated from the gonads of *Paracentrotus lividus* L. and the latter from the fresh-water blue-green alga, *Aphanizomenon flos-aquae*. Tischer's (1939) suggested formula for aphanin is the same as that of Lederer (1935) for echinone; this will be discussed later. It was not possible to obtain *A. flos-aquae* in