# Short Communications

## Chitin in a Cambrian Fossil, *Hyolithellus*

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In a recent paper Poulsen (1963) suggested that the Cambrian fossil *Hyolithellus micans* (Billings) should be referred to the phylum Pogonophora, and remarked on the similarity of the inorganic constituents in this fossil and the tube of the living pogonophore *Galathealinum bruuni* Kirkegaard. Poulsen's material was obtained by elutriation of an unconsolidated Middle Cambrian clay from the Scandinavian island of Bornholm. He reports that 'the deposit...can only have been imperceptibly affected by diagenetic processes, and, accordingly much of the original...structure of the fossils is preserved.... The tubes of *Hyolithellus* consist mainly of an organic substance and some calcium carbonate.' Dr Poulsen was kind enough to present me with fragmentary material of *Hyolithellus*, so that I have been able to make a short investigation of the nature of the organic substances which have withstood 550 million years of fossilization.

The material was only slightly carbonized, some fragments of the organic portions being dark brown; others showed no sign of carbonization at all and remained a clear straw colour when cleared from the inorganic matrix. This operation was performed by treating for 5 min. with cold N-hydrochloric acid. The residue then consisted of particles of clay mingled with fragments of organic matter. No purpose was served by separating these, and the entire sediment underwent further treatment together. All procedures were repeated on portions of locust wing and on the tube of the present-day pogonophore *Zenkevitchiana longissima* Ivanov, to provide a control.

The organic fragments withstood treatment for 20 min. in saturated aqueous potassium hydroxide at 150°. After washing, these fragments were coloured brown by iodine in potassium iodide solution and turned reddish violet when this was replaced by dilute sulphuric acid. The alkali-treated fragments were soluble in 3% (v/v) acetic acid and in 75% (v/v) sulphuric acid. Both cellulose and chitin (and but few if any other organic structural materials) are relatively stable in hot alkali under these conditions, but the colour reactions and solubility in acids are properties shown only by chitin (Tracey, 1955). The fragments resisted solution in Schweitzer's cuprammonium reagent (in which cellulose dissolves), even after pretreatment with hot alkali or with a solution of chlorine dioxide in acetic acid ('diaphanol').

Chitin, which may be considered as a polymer of 2-acetamido-2-deoxy-α-D-glucopyranose (*N*-acetyl-D-glucosamine), yields D-glucosamine on acid hydrolysis and *N*-acetyl-D-glucosamine on enzymic degradation (Kent & Whitehouse, 1955). The fragments of *Hyolithellus* showed these reactions.

Fragments of the fossil were hydrolysed in a sealed tube in 6N-hydrochloric acid at 100° for 48 hr. The hydrolysate was dried over phosphorus pentoxide and potassium hydroxide, taken up in water and chromatographed on paper against D-glucose and D-glucosamine, by using six different solvent mixtures. A substance behaving like glucosamine was present, but no glucose or any other reducing sugar was detected.

A preparation with chitinase activity was obtained by steeping mushrooms (100 g.) with 35% (w/v) sodium chloride solution (100 ml.) at 4° for 48 hr. (Carlisle, 1960). This extract was purified and freed from materials of low molecular weight by passing it through a Sephadex column that had previously been buffered at pH 5·0. The resultant preparation had chitinase activity, but no detectable cellulase or proteolytic activity. Organic fragments of the fossil were digested with this preparation for 72 hr. Samples were concentrated *in vacuo* and chromatographed in butan-1-ol–ethanol–water (4:1:5, by vol.). *N*-Acetylglucosamine was shown to be present with Elson & Morgan reagents, but no glucose was detectable with aniline hydrogen phthalate or silver nitrate.

All these reactions were given equally by portions of locust wing and by the tube of *Zenkevitchiana*.

Chitin is invariably accompanied by protein, and structural proteins seem able to withstand fossilization. The acid hydrolysates were therefore chromatographed for amino acids. Six were found in the fossil and ten in *Zenkevitchiana*, of which alanine, serine and proline were identifiable in both with certainty. Locust wing gave a totally different pattern and lacked alanine. None of the hydrolys-

ates yielded any sulphur-containing amino acids. The structural protein of the pogonophore tubes may be a sclerotin, stabilized by quinonoid links as in the insect cuticle, rather than a keratin such as is found in vertebrates.

Several workers have claimed to demonstrate the presence of chitin in palaeozoic fossils, but (except for Arthropoda) there is no reason to believe any of these claims. Thus the oft-quoted statement of Kraft (1926) that Silurian graptolites contain chitin is based on grossly insufficient and inconclusive evidence. The material requires re-examination by modern methods, such as those that have been successfully employed in this investigation.

Brunet & Carlisle (1958) reported chitin in pogonophore tubes, and this was later confirmed by Rudall (1962) by X-ray-diffraction methods. The close similarity in organic constituents shown by *Hyolithellus* and recent pogonophore tubes reinforces the conclusion, based on the similarity of appearance and inorganic composition, that *Hyolithellus* is a pogonophore and that the Pogonophora are therefore to be counted among the oldest of animal phyla. These results also emphasize the enormous stability of structural proteins and chitin over a period of 500 million years under favourable conditions.

To summarize: (1) The organic constituents of *Hyolithellus* include chitin and protein, probably a sclerotin, which have persisted unchanged for 550 million years. (2) Acid hydrolysis yields D-glucosamine and half a dozen amino acids, including alanine, serine and proline. (3) Enzymic degradation with fungal chitinase yields *N*-acetylglucosamine.

I thank Dr V. Poulsen, of the Mineralogisk Museum, Copenhagen, Denmark, for his kind present of *Hyolithellus* material.

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## Removal of the *N*-Terminal Residue of a Peptide after Transamination

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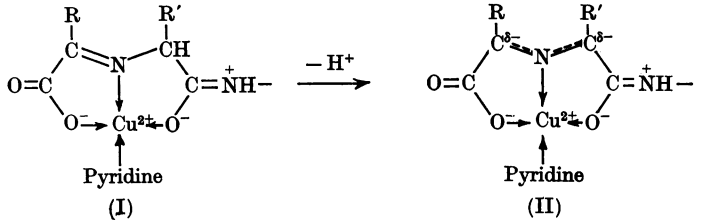
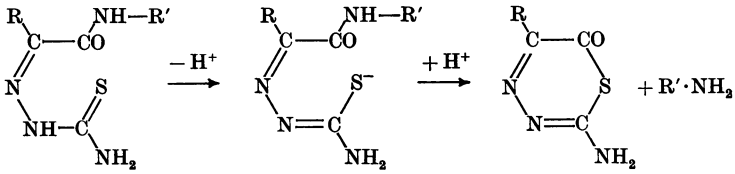
Dixon & Weitkamp (1962) obtained reductive amination of an oxo acid in peptide combination by transamination. The reverse reaction, the conversion of the *N*-terminal residue of a peptide into an α-oxo acyl residue, is now reported. It can be carried out under mild conditions if use is made of pyridine to evoke a large increase in the rate of copper-catalysed transamination (Mix & Wilcke, 1960; Mix, 1961*a*, *b*).

DL-Alanylglycine (0·1M) reacts at 20° in a solution of glyoxylic acid (1M), pyridine (10%, v/v) and cupric acetate (0·1M). After 15 min. the solution is chromatographed on a strongly basic resin (De-Acidite FF SRA 71, polystyrene beads containing quaternary ammonium groups, 100–200 mesh, 7–9% cross-linking) equilibrated and developed with a solution of acetic acid (0·5M), sodium acetate (0·5M) and toluene (0·03%). A column of 2 cm. × 60 cm. was used for a sample of 10 ml., i.e. for 1 m-mole of peptide. Oxo acids are estimated in the effluent by the $E_{367\cdot5\,m\mu}$ of their dinitrophenylhydrazones, which are extracted from ethyl acetate by a carbonate–bicarbonate buffer (cf. Lu, 1939; Friedemann & Haugen, 1943; Koepsell & Sharpe, 1952). A peak, $R_F$ 0·28, appears before the peak of glyoxylate. Sodium acetate is replaced by acetic acid in the portion of effluent that contains this product by passing it through a sulphonic acid resin (Zeo-Karb 225 SRC 13, 14–52 mesh, 8% cross-linking). Pyruvoylglycine (m.p. 88–89°) is isolated on evaporating the solution (yield after recrystallizing, 55%).

The mechanism generally accepted for the metal-catalysed transamination of amino acids (cf. Metzler, Ikawa & Snell, 1954; Mix, 1961*a*) is equally applicable to peptides. The electron-withdrawing property of the $Cu^{2+}$ ion facilitates ionization of the α-hydrogen of the amino acyl residue in the Schiff base formed with the oxo acid (I) and a proton may recombine on the other side in the ionized form (II).

The introduction of a carbonyl function into a peptide at a single locus opens possibilities of further specific reactions. In peptides that possess other types of amino groups it is likely that the transamination should be specific for those in α-

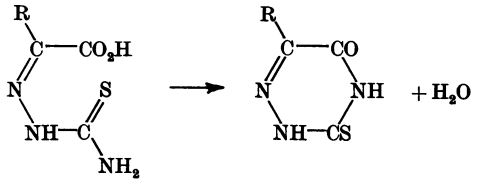



positions. Kurtz (1938) and Collman & Buckingham (1963) similarly selected α-amino groups by their preferential chelation with metal ions.

Since it could be useful to remove the *N*-terminal residue without other modification of peptide molecules, treatments unlikely to degrade peptides in general were tested on pyruvoylglycine for release of glycine. Incubation of its thiosemicarbazone (III) at 57° in a solution equimolar in carbonate and bicarbonate (pH 10) released 85% of its glycine in 5 hr., with a $t_{\frac{1}{2}}$ of about 45 min. The glycine was identified by electrophoresis on paper and estimated by reaction with ninhydrin.

A possible mechanism is: (III) → (IV) → (V). Such an attack on the terminal peptide bond by a favourably placed nucleophile (present in IV) is similar to the methods of Edman (1950, 1956), Holley & Holley (1952), Léonis & Levy (1951) and Collman & Buckingham (1963). The procedure described above may have uses denied to the first two methods cited if it leaves lysine residues unchanged and a peptide thus unmodified except for the loss of its terminal residue. The thiol ester (V) may be expected to hydrolyse to (VI) and be able to recyclize into a 5-hydroxy-3-mercapto-1,2,4-triazine (VII), e.g. 2-thio-6-azathymine (VII, R = $CH_3$) in the case of pyruvoyl-peptides (Bougault & Daniel, 1928):




Such compounds could serve to identify the terminal residue removed. The method proposed may be milder than that of McGregor & Carpenter (1962) who, after converting the terminal residue of a peptide into an oxo acyl residue with hypobromite, removed it with peroxide in 0·1 N-potassium hydroxide at 37°.

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# The Production of Methoxy-Substituted Fatty Acids as Artifacts during the Esterification of Unsaturated Fatty Acids with Methanol Containing Boron Trifluoride

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The use of boron trifluoride ($BF_3$) for promoting esterification of alcohols and organic acids was first described by Hinton & Niewland (1932). Mitchell, Smith & Bryant (1940) reported the use of $BF_3$–methanol for preparing methyl esters of organic acids, and Metcalfe & Schmitz (1961) advocated the use of this reagent for the preparation of methyl esters of long-chain fatty acids for gas–liquid chromatography. The composition of methyl esters prepared from mixtures of both saturated and unsaturated fatty acids in the presence of $BF_3$–methanol was found to compare favourably with that of esters of the same acids formed by treatment with either methanolic HCl or diazomethane (Metcalfe & Schmitz, 1961; Vorbeck, Mattick, Lee & Pederson, 1961).

Recently reagents containing $BF_3$ became available in Britain. Contrary to the experience of the foregoing workers (in the U.S.A.) who prepared their own reagents with $BF_3$ and methanol, the use of British reagents [$BF_3$–methanol (British Drug Houses Ltd.) and $BF_3$ 'etherate' (Hopkin and Williams Ltd.) to which methanol was added] does not yield pure methyl esters of unsaturated fatty acids. Gas–liquid chromatograms of methyl esters of the fatty acids of linseed oil and maize oil bore little resemblance to those of esters prepared by treatment of the respective acids with methanolic $H_2SO_4$ or diazomethane in that the proportion of $C_{18}$ unsaturated components was much decreased and spurious peaks were evident. The behaviour of $BF_3$–methanol towards a pure unsaturated fatty acid [oleic acid, 99%; Price's (Bromborough) Ltd.] was therefore investigated.

The acid (959 mg.) was treated with $BF_3$–methanol (15 ml.) in a manner similar to that described by Metcalfe & Schmitz (1961). After the removal of traces of unesterified acids on a column of alkali-treated silicic acid (McCarthy & Duthie, 1962) the methyl esters (939 mg.) were subjected to gas–liquid chromatography under conditions similar to those described by Duncan & Garton (1963); the chromatograms showed two main components which comprised methyl oleate and an unidentified component. The unidentified component had a 'carbon number' (Woodford & van Gent, 1960) of 19·1 on Apiezon L and 21·5 on polymerized ethylene glycol succinate. These values were unchanged by hydrogenation of the esters (5 mg.) in the presence of palladium catalyst. Thin-layer chromatography on Silica-Gel G (Merck A.-G., Darmstadt, West Germany) with 15% (v/v) ether in light petroleum (b.p. 40–60°) also resulted in the separation of the mixture into its two components. Accordingly the preparative separation of the methyl oleate from the unidentified component was effected on a column of silicic acid (A.R. 100-mesh powder; Mallinckrodt). Methyl oleate was eluted with 1% (v/v) ether in light petroleum. The material remaining on the column was completely eluted with 8% (v/v) ether in light petroleum. This fraction (283 mg. of a colourless oil), when subjected to infrared analysis (Unicam SP. 200 spectrophotometer), yielded a spectrum which conformed in general to that of a normal fatty acid ester, though the presence of an additional peak at 1100 $cm.^{-1}$ indicated that the material contained a methoxyl group (cf. Bellamy, 1958).

For reference purposes 12-methoxystearic acid was prepared from 12-hydroxystearic acid (obtained by catalytic reduction of ricinoleic acid) by using silver oxide and methyl iodide, as described for the preparation of α-methoxy acids (Kishimoto & Radin, 1959). The infrared-absorption spectrum of the methyl ester of the acid showed strong absorption at 1100 $cm.^{-1}$ and in all respects was closely similar to that of the unknown material.

Gas–liquid-chromatographic analysis on Apiezon L and polmerized ethylene glycol succinate showed that the methyl ester of 12-methoxystearate and the unknown component were completely inseparable, as did chromatography on thin layers of Silica-Gel G. It was therefore concluded that the unknown component was a methoxy derivative of methyl stearate which had resulted from the addition of methanol to the double bond of the oleic acid at the same time as the carboxyl group was being esterified. The product may thus represent a mixture of 9- and 10-methoxy derivatives of methyl stearate. That methoxyl groups were in fact present in the material was shown by the micro-Zeisel method though, for reasons not readily apparent and which were not investigated, the methoxyl value (9·0%) of the acid resulting from alkaline hydrolysis of the esterified product was lower than that required (9·9%) for a methoxystearic acid.

Three samples of $BF_3$–methanol (about 51 % of $BF_3$) obtained at different times from British Drug Houses Ltd. gave different yields of artifact from oleic acid, namely 30, 56 and 68 %; $BF_3$ 'etherate' (one sample) diluted with methanol to give 50 % of $BF_3$ gave 12 % of artifact. Whether or not the artifact-producing propensity is a function of the age of the reagent remains to be investigated, though Metcalfe & Schmitz (1961) stated that their reagent 'has an excellent shelf life and has been used up to 4 months after preparation'. It would, however, seem possible that the addition of an alcohol such as methanol to an olefinic bond may be catalysed by $BF_3$ regardless of the age of the reagent (cf. Meerwein, 1933).

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## The Rate of Turnover of Messenger Ribonucleic Acid in Rat Diaphragm Muscle

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Eboué-Bonis, Chambaut, Volfin & Clauser (1963) have shown that actinomycin D *in vitro* inhibits the synthesis of nucleic acid in isolated rat diaphragm muscle. If, as is now believed (Jacob & Monod, 1961), the synthesis of protein is dependent on the continuous formation of messenger RNA (m-RNA), the cessation of formation of m-RNA in the presence of actinomycin should result in a decline in the rate of incorporation of amino acids into protein, the steepness of this decline being dependent on the rate of destruction of the m-RNA molecules and thus providing a possible means of measuring their rate of turnover. In the experiments of Eboué-Bonis *et al.* (1963) the amount of amino acid incorporation occurring in a 2 hr. incubation period after treatment with actinomycin appeared to be normal. Little decline in the activity of the m-RNA would therefore seem to have taken place within this period. Fortunately, however, diaphragm muscle *in vitro* is able under normal conditions to incorporate amino acids into protein for at least 6 hr. (Manchester, 1961*a*) and it is thus possible to study the effect of actinomycin on amino acid incorporation over a longer period of time. Under such conditions a steady decline in the rate of incorporation is observed.

The technique for the incubation of diaphragm and preparation of protein samples was as described by Manchester (1961*b*). In the final stages the protein sample was dissolved in formic acid and a sample pipetted on to a stainless-steel planchet with concentric rings. The formic acid was removed by evaporation, the quantity of protein weighed and its radioactivity measured in a gas-flow counting system. The incorporation of [$^3$H]-orotic acid into nucleic acid was measured as described by Wool (1960) except that a scintillation-counting procedure was used. Actinomycin D (dissolved in ethylene glycol; 1 mg./ml.) was either present throughout the whole incubation period or, in other experiments, the hemidiaphragms were preincubated for 30 min. in the Krebs buffer at 37° in the presence of actinomycin but not the $^{14}$C-labelled amino acid and then transferred to fresh buffer containing $^{14}$C-labelled amino acid but no actinomycin. Similar results were obtained by either procedure. Equal quantities of ethylene glycol were added to all controls.

As described by Eboué-Bonis *et al.* (1963), actinomycin D at an initial concentration of 10 $\mu$g./ml. was found to prevent almost completely the incorporation of [$^3$H]orotic acid into nucleic acids, and in the experiments in which [$^3$H]orotic acid and inhibitor were added simultaneously incorporation was certainly over in the first 30 min. of incubation. A similar degree of inhibition was observed with a concentration of 2 $\mu$g./ml., and partial inhibition was obtained at 0·4 $\mu$g./ml. The incorporation of [$^{14}$C]glycine into protein of diaphragm is shown in Fig. 1; it can be seen that,

whereas in the absence of actinomycin incorporation continues at a reasonably constant rate for the whole of the incubation period, in its presence (10 $\mu$g./ml.) the rate of incorporation steadily declines. Similar curves have been obtained with [$^{14}$C]leucine and $^{14}$C-labelled algal-protein hydrolysate. Studies with [$^{14}$C]glycine show as much inhibition of incorporation in 4 hr. incubations with 2 $\mu$g. of actinomycin/ml. as with 10 $\mu$g./ml. Considerable though not quite as much inhibition is found with 0·4 $\mu$g./ml., thus paralleling the inhibition of nucleic acid synthesis. When incubation with actinomycin for 3 hr. precedes the addition of [$^{14}$C]glycine, the incorporation of $^{14}$C into protein in the subsequent 2 hr. is only about 7 % of that in the controls.

Thus in incubations longer than those carried out by Eboué-Bonis *et al.* (1963) an inhibitory action of actinomycin on amino acid incorporation into protein can be observed, and indeed in Fig. 1 a small inhibition can be seen even at 2 hr. If it is assumed that in the presence of actinomycin the quantity of active m-RNA falls off exponentially with time, then the quantity of m-RNA at time $t$ is $Ae^{-kt}$, where $A$ and $k$ are constants. If the rate of protein synthesis at time $t$, i.e. $dp/dt$, is proportional to the amount of m-RNA present, then $dp/dt = Be^{-kt}$, where $B$ is another constant, from which the amount, $p$, of protein formed since zero time (i.e. the amount of incorporation) is:

$$(B/k)\,(1-e^{-kt})$$

At zero time $dp/dt$ is equal to $B$, the rate of incorporation in the absence of actinomycin; $\ln(dp/dt) = \ln B - kt$, so that, by drawing tangents to the graph and plotting $\ln(dp/dt)$ against $t$, $k$ can be determined. The curve described for $p$ by substituting the values for $B$ and $k$ so obtained superimposes well on the experimental values observed. When half of the m-RNA has disappeared $dp/dt$ should equal $B/2$. For Fig. 1 the value of $t$ for which $dp/dt = \frac{1}{2}B$ is 2·5 hr.; in other experiments times ranging from 1·9 to 3·5 hr. have been obtained. Apparently, therefore, the half-life of m-RNA in diaphragm muscle appears to be as short as 2–3 hr., a figure which is rather low by comparison with what might be inferred from the time taken for actinomycin to interfere with protein synthesis in mouse fibroblast L cells (Reich, Franklin, Shatkin & Tatum, 1962) and in view of the capacity of reticulocytes to continue to synthesize protein for several days after nucleic acid synthesis has ceased (Lajtha & Oliver, 1960)—but these are both special cases. It could be that in diaphragm actinomycin interferes with metabolism in general, including the operation of protein synthesis, but the release of $K^+$ ions, a process sensitive to metabolic derangement (Creese, 1954), is not greatly affected by the inhibitor. Moreover, Staehelin, Wettstein & Noll (1963) have shown that the addition of actinomycin (10 $\mu$g./ml.) to a liver ribosomal system is without effect on the capacity of the system to incorporate amino acids into protein, from which it might be inferred that the inhibitor does not affect the stability and function of polysomal units once formed. However, Acs, Reich & Valanju (1963) have evidence that actinomycin can degrade RNA through an action independent of any interference with m-RNA synthesis. Such an action could obviously lead to a slowing of the rate of protein synthesis and a false estimate of the rate of turnover of m-RNA, a possibility that has to be considered in the present context.

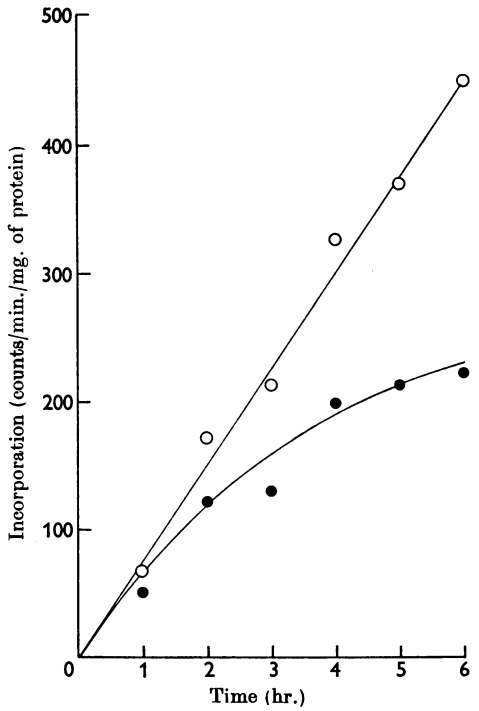


Fig. 1. Effect of actinomycin D on the incorporation of [$^{14}$C]glycine into the protein of isolated rat diaphragm. ○, Incorporation in the absence of actinomycin; ●, incorporation in the presence of actinomycin (10 $\mu$g./ml.).

I thank Dr A. Korner, University of Cambridge, for a gift of actinomycin D, and Miss J. Roberts for skilled technical assistance.

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## The Isolation of a Complex of Heavy Meromyosin and Adenine Nucleotide

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The formation of an enzyme–substrate complex between myosin and ATP has been inferred from measurements of free ATP concentrations during the myosin adenosine-triphosphatase reaction (Nanninga & Mommaerts, 1960*a*, *b*; Nanninga, 1962*a*). We now report the isolation of complexes of heavy meromyosin, bivalent metal ion and adenosine polyphosphate.

Myosin was prepared by the method of Mommaerts & Parrish (1951). Heavy meromyosin was prepared by a method similar to that described by Szent-Györgyi (1953), Gergely (1953) and Gergely, Gouvea & Karibian (1955), except that the bacterial protease Nagarse (Teikoku Chemical Industry Co., Osaka, Japan) was used instead of trypsin or chymotrypsin. The final purification step involved passage of the dissolved ammonium sulphate precipitate of the heavy meromyosin through a column of Sephadex G-200 (Pharmacia, Uppsala, Sweden) to remove ammonium sulphate, the remainder of the proteolytic enzyme, and other impurities. The heavy meromyosin, which was eluted in the first peak, was homogeneous as judged from sedimentation patterns obtained in the Spinco model E ultracentrifuge. It had a specific adenosine-triphosphatase activity 2–3 times as great as that of the myosin used as starting material. [$^3$H]ATP with a specific radioactivity of $8{\cdot}8 \times 10^7$ counts/min./$\mu$mole was obtained from Schwartz BioResearch Inc., Mount Vernon, N.Y., U.S.A. It was purified before use by paper chromatography.

Between 0·3 and 1·3 mg. of heavy meromyosin was mixed with 0·5 $\mu$mole of bivalent-metal chloride, 5 $\mu$moles of tris–HCl buffer and 0·05 $\mu$mole of [$^3$H]ATP, in a final volume of 0·5 ml., at pH 8·0 and at 2–4°. Where indicated, the mixture also contained 250 $\mu$moles of KCl. Immediately after the addition of [$^3$H]ATP the reaction mixture was placed on a column of Sephadex G-25 with a gel bed size 1 cm. diam. × 20 cm. high. The column was pre-equilibrated with 10 mM-tris–HCl buffer, pH 8·0. For experiments performed in 0·5 M-KCl, the column was pre-equilibrated with the same buffer containing KCl (0·5 M). After the reaction mixture had soaked into the column, elution was begun immediately with 10 mM-tris–HCl buffer, pH 8·0, or with the same buffer containing KCl (0·5 M). Fractions (1·0 ml.) were collected and the extinctions at 260 and 280 m$\mu$ were measured. In addition 0·1 ml. samples of each fraction were assayed for radioactivity in a scintillation counter. Internal standards of [$^3$H]-toluene served as controls on the counting efficiency. Model runs on the column with heavy meromyosin and bovine serum albumin showed that these proteins were eluted in peak 1 (tubes 8–10). On the other hand, ATP, ADP, [$^{32}$P]orthophosphate and [$^{32}$P]pyrophosphate were eluted in peak 2 (tubes 14–29). Fig. 1 shows the elution pattern obtained in an experiment with heavy meromyosin and [$^3$H]ATP. The protein peak coincided exactly with a radioactive peak (peak 1). These were clearly separated from the remainder of the radioactivity (peak 2). On the assumption that heavy meromyosin has a molecular weight of $3{\cdot}2 \times 10^5$ (Lowey & Cohen, 1962; Woods, Himmelfarb & Harrington, 1963), it was calculated that in this experiment peak 1 contained 1 molecule of nucleotide/5 molecules of heavy meromyosin. If 1 molecule of nucleotide combines with 1 molecule of enzyme, this corresponds to 20 % binding. When heavy meromyosin was omitted, or was replaced by bovine serum albumin, no radioactivity was found in peak 1. When [$^3$H]ATP was replaced by 0·2 $\mu$mole of [$^{32}$P]orthophosphate ($1{\cdot}0 \times 10^8$ counts/min.), peak 1 contained less than 1 molecule of orthophosphate/10 000 molecules of heavy meromyosin. This corresponds to less than 0·01 % of binding. The binding observed with ATP was not due to actomeromyosin, since the preparations used showed

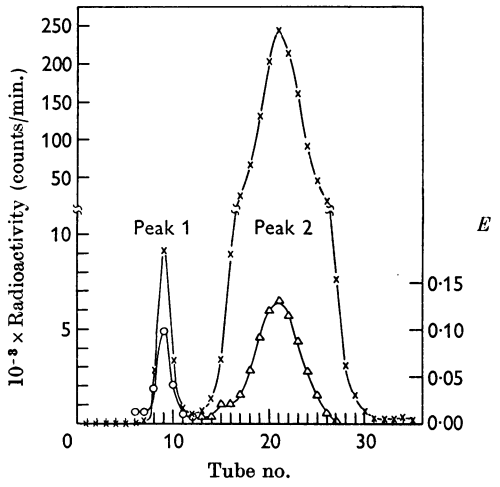


Fig. 1. Binding of adenine nucleotide by heavy meromyosin. The reaction mixture contained $MgCl_2$ (1 mM), KCl (0·5M) and heavy meromyosin (0·64 mg.). Other conditions are given in the text. ×, Radioactivity; ○, $E_{280\,m\mu}$; △, $E_{260\,m\mu}$.

no activation of adenosine triphosphatase by 5 mM-$MgCl_2$ at low ionic strengths (Leadbeater & Perry, 1963).

The binding of adenine nucleotide to enzyme in the presence of different metal ions was as follows: $MgCl_2$, 30%; $MgCl_2$+KCl, 20%; $MnCl_2$, 22%; $MnCl_2$+KCl, 15%; $CaCl_2$, 25%; $CaCl_2$+KCl, 4%; KCl alone, 4%. High concentrations of KCl decrease the yield of the complex. The decrease is greatest for $Ca^{2+}$ ions, in which case the amount of complex formed in the presence of 0·5M-KCl is no more than that formed in the control containing 0·5M-KCl but no $Ca^{2+}$ ions. The experiments with 0·5M-KCl have also been performed with myosin with similar results.

It was decided to determine the nature of the radioactive nucleotide in the complex. The experiment with 1 mM-$MgCl_2$ was repeated, except that the fractions that constitute peak 1 were collected in tubes containing 1 ml. of acetone. Unlabelled AMP, ADP and ATP (0·2 μmole of each) were added and the denatured protein was removed by centrifugation. The supernatant was neutralized and evaporated to dryness on a rotary drier. The residue was dissolved in water and passed through a small column containing equal parts by volume of acid-washed charcoal (Norite A) and cellulose powder (Whatman, coarse grade). When the mixture had soaked in, the column was washed with 2 ml. of water, and was then eluted with two lots of 2 ml. of ethanol–0·4N-ammonia (1:1, v/v). The eluates were evaporated to about 0·2 ml. on a rotary drier, spotted on Whatman no. 3 paper and subjected to ascending chromatography with isobutyric acid–ammonia–water as solvent (Krebs & Hems, 1953). The spots of AMP, ADP and ATP were eluted with water and counted. They contained 2, 47 and 51% respectively of the radioactivity.

The stability of the heavy meromyosin–nucleotide complex was examined as follows. An experiment was run with 1 mM-$MgCl_2$. The time at which fraction 9 emerged from the column was taken as zero time. Samples of this fraction were run through fresh columns of Sephadex G-25. The time at which the new fraction 9 emerged was noted in each case. After 23, 38 and 52 min. the radioactivity remaining in the new peak 1 was 64, 51 and 43% respectively of the original peak 1. This corresponds to a half-life for the complex of approximately 40 min. at 2–4°.

Kinetic studies provide evidence that ADP is also bound to myosin (Nanninga, 1962*b*). We find that [$^3$H]ADP also forms a complex with heavy meromyosin, but the degree of binding is less than with ATP.

These results provide evidence for the isolation of a complex between heavy meromyosin and ATP. It is possible that the complex is involved in the $^{18}$O-exchange reaction described by Yount & Koshland (1963). The requirement of bivalent metal ions for complex-formation suggests that the complex is chelated to the enzyme. Other types of bonding are not, however, ruled out.

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