ally visible layers and corresponding to a network of cellulose chains making an angle of some 80° with each other. Complete understanding of such a structure is obviously impossible without an investigation of that of the *individual layers*—to decide whether even the finest layer has a structure similar to that of the whole wall. . . ." The developmental study which we have made does just this, and it shows, moreover, how the first and the successive layers are laid down.

Preston and Astbury still seem to insist upon their original evidence for one set of cellulose lamellæ in which the fibrils consist of a set of meridians. The arduous technique which leads to this conclusion was apparently carried out only upon one vesicle. Even so, the angles at which the chain directions crossed varied quite considerably at different parts of the wall and it seems that it would require more strict evidence to postulate a preferred direction as absolute as the system of meridians requires—evidence that should relate to more than one vesicle. Also, the evidence for the supposed poles seems not so convincing now as it might earlier have seemed to be. The areas in question gave an X-ray diagram which suggests disorder and random orientation, and this effect, through the whole thickness of the wall, might have arisen in other ways than the one suggested.

It is clear that the results of X-ray examination and of electron microscope studies of the same specimen should be compatible. It is granted that the X-ray method, and prominently so in the hands of Preston and Astbury, has yielded most valuable results. It is, however, still based on observations that have to be made over a relatively short length, which have to be extended over the wall surface as a whole, and it requires interpretations that are obviously complicated when dealing with a system composed of many successive layers each with its own fibrillar direction. Obviously what one does with the electron microscope is not to photograph one field but many such. The degree of integration which is obtained by the X-ray method, because it summarizes events over a given length, is obtained at least in part with the electron microscope by the taking of many pictures on many specimens and deciding from a very large number what is a typical condition and sequence of events. The electron microscope has, however, the great advantage that in each such field investigated the picture revealed has a degree of definition and precision that is not inherent in the X-ray method, which must average in each observation over a considerable area and many layers. Whereas in the electron microscope the several layers can be seen in sharp focus and the strand directions clearly discerned, the X-ray method only reveals this after interpretations that require to be made, and these, as is now evident, are subject to some uncertainty. For example, Preston and Astbury now suggest that the apparent prevalence of two layers in their work instead of three could be accounted for if one of these failed to show properly because two of them are more crystalline than the third. This is just the kind of problem that makes a survey of this sort difficult by the X-ray method, whereas the electron microscope seems to lead more directly to precision.

We can agree with Profs. Preston and Astbury that this, like any other problem of biology, admits of further study. Our purpose was to stress, however, that in these problems of molecular biology it is not enough to study the mature structure. A complete understanding must comprehend how the structure or organism grows and develops and, in achieving this, there is no substitute for the use in the investigation of the living material under such conditions that the changes that occur during growth can be observed. The application of this point of view to the problem of the *Valonia* wall has produced knowledge that did not arise from Preston's and Astbury's earlier work. This does not mean that the earlier results are to be rejected. It does mean, however, that knowledge grows by new observations, by the application of other techniques and by the different approaches that may be adopted by other investigators. It would be surprising and somewhat depressing, indeed, if sixteen years after the first major examination of the *Valonia* wall, made primarily upon a single vesicle, that this did not prove to be so.

In our view, the really challenging problems that these organisms still present are the following. What is the enzymatic machinery which permits the naked protoplasmic surface of the aplanospore so rapidly to build up cellulose in the manner that has now been described ? What is the carbohydrate substrate that is utilized ? What are the forces that control the first shift from randomly oriented fibrils to such strongly oriented parallel directions ? Having once achieved order in the first direction, what induces the organism to lay down its fibrils in the successive directions which seem to be required ? Even though there may be room for some difference of opinion concerning the minutiæ of the structure of the mature wall, these great problems still present their challenge and the way seems now open for them to be attacked.

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ZONE ELECTROPHORESIS OF MUSCLE EXTRACTS: SEPARATION OF PHOSPHOCREATINE, CREATINE, BETA-ALANINE PEPTIDES, AND NUCLEOTIDES

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IN the work reported here, the technique of electrophoresis in liquids supported by solid media has been applied to the separation of a number of non-protein nitrogenous constituents of mammalian muscle. The most important of these can be located on the supporting medium by ultraviolet fluorescence and can then be eluted for quantitative determination or for preparative purposes and purification. This affords a more specific and convenient method for analysis and isolation of some of the compounds studied than has hitherto been available.

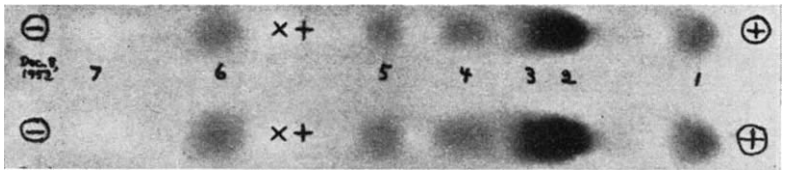

Fig. 1. Detection of rabbit muscle constituents by ultra-violet fluorescence after electrophoretic separation on filter paper.

0·7 μl. (upper half) and 1·0 μl. (lower half) of neutralized muscle extract deposited at +. Spots marked × are starting points corrected for electro-osmosis. 165 min. electrophoresis at 25° C. and 10 V./cm. in sodium succinate buffer – sodium chloride of *p*H 5·6 and μ 0·45.

1, phosphocreatine (as creatinine); 2 and 3, positions assigned to adenosine tri- and diphosphate, respectively; 4, adenosine monophosphate; 5, oxidized diphosphopyridine nucleotide; 6, creatine (as creatinine); 7, anserine and carnosine. Distance between centres of spots 1 and 5 corresponds to 90 mm.

Photograph taken with two 'Mineralight' lamps (2½ hr. exposure) and two 'Philora' lamps (5 min. exposure), all approximately 40 cm. from the filter paper. $F = 22$; Ilford Rapid Panchromatic plate, shielded from ultra-violet rays by Wratten *G* filter and 15-mm. mirror glass plate

The usual procedure in the present experiments was briefly as follows: Cold perchloric acid extracts of fresh skeletal and cardiac muscle were neutralized and concentrated to a volume corresponding to 40 gm. muscle per ml. For electrophoresis, 0·1–2 μl. was placed on or near the centre axis of an 8-cm. wide sheet of Munktell 20 filter paper; up to 250 μl. was used per 100 c.c. of Whatman cellulose powder saturated with buffer medium and packed tightly into an elongated 'Perspex' frame. The electrophoreses were carried out at potential gradients of 10 volts per cm. (filter paper) or about 3½ volts per cm. (cellulose powder) in the apparatus and with the precautions described by Kunkel and Tiselius[1], the filter paper being held tightly between two silicone-treated glass plates. The current ranged from 2 to 50 m.amp. Cooling and temperature control, when desired, were achieved by letting the lower of the two glass plates surrounding the filter paper rest on a large metal block, partly immersed in a water-bath of the proper temperature and by covering the upper glass plate with a metal trough filled with water of the same temperature. The duration of the electrophoresis runs on filter paper was ½–3 hr.; the cellulose powder experiments were run over-night. At the end of the runs the paper sheets, serving as supporting medium, and strips of paper pressed briefly against the surface of the cellulose powder packs, were dried in an air-stream at room temperature and inspected in ultra-violet light of around 254 mμ ('Mineralight' lamp of U. V. Products, Inc.). This served to reveal dark zones or spots on the fluorescent paper, due to adenine and hypoxanthine derivatives[2,3] and to preformed creatinine. The papers were then heated for a few minutes in a dry atmosphere at 120° C. for conversion of the non-visible phosphocreatine and creatine into creatinine. The heating also caused the appearance or intensification of light blue fluorescence due to anserine and carnosine, and possibly other peptides. It also gave rise to yellowish-white fluorescence, due to the presence of glucose[4], which was used as reference substance for estimating electro-osmosis, and of substances in the extracts, possibly also sugars (cf. below). This fluorescence was more intense in ultra-violet radiation of higher wave-length, such as that emitted by the Philips 'Philora' lamp.

A good example of the appearance in ultra-violet light of the centre portion of a dried and heated filter paper sheet after electrophoresis of an extract of skeletal muscle is furnished by the photograph reproduced in Fig. 1. This experiment was carried out at an estimated temperature of 25° C. in 0·175 *M* succinate buffer of *p*H 5·6, brought to ionic strength 0·45, by addition of sodium chloride. As can be seen from the photograph, satisfactory resolution of several of the ions studied was achieved. It was important at the *p*H employed and at the relatively high ionic strength and the resulting high current (28–30 m.amp.) to prevent heating in the paper in order to avoid decomposition of the highly acid-labile phosphocreatine. As is to be expected, this compound moves toward the anode at a high speed (spot 1 in the photograph). In addition to showing up as a dark spot in ultra-violet light, it could be revealed on the heated filter paper as creatinine by spraying with alkaline picrate[5], and as phosphate by treatment with acid molybdate reagent and hydrogen sulphide[6]. Further identification and quantitative analysis were afforded, following elution from the paper and treatment intended to convert phosphocreatine into creatinine, by the characteristic ultra-violet absorption curves and maxima of the latter compound[7]. Quantitative determination as the phosphate was not feasible because of insufficient separation at the *p*H's employed from free orthophosphate. The paper-chromatographic behaviour[8] of the eluate from the area occupied by spot 1 on the unheated paper served as a further check on the identity of the eluted substance with phosphocreatine.

The spots at positions 2, 3 and 4 in Fig. 1 have been assigned to adenosine triphosphate, adenosine diphosphate, and adenosine monophosphate, respectively, on the basis of evidence provided by the ultra-violet absorption spectra of the eluates, by tests for acid-labile phosphate groups, and by comparison with authentic samples with respect to speed of migration in the electric field at various hydrogen-ion concentrations. Due mainly to the choice of a not very favourable *p*H and to the large amount of adenosine triphosphate present, this compound did not separate from the diphosphate in the experiment shown. As has been reported by other authors[9,10], separation of the adenosine phosphates by electrophoresis is best undertaken at *p*H 3·7–4. Hypoxanthine derivatives were not seen in significant quantities in extracts of fresh skeletal and cardiac muscle, but were found to prevail in cardiac muscle excised ½–1 hr. after death of the animal (calf). Similar observations were made during analyses of extracts of brain and of the conductive tissue of the heart. The acid-extractable nucleotide content of the latter tissue was found to be only about one-fourth that of ventricular muscle.

The substance responsible for spot 5 in Fig. 1 was identified as oxidized diphosphopyridine nucleotide. A larger amount of this substance was prepared from the corresponding zone in the cellulose powder pack. The material isolated exhibited an absorption maximum in the ultra-violet at 260 mμ. Electrophoreses carried out with this material in the *p*H range 3·8–9·2 suggested that one was dealing with

an anion having about the size of a dinucleotide and carrying an average net charge not higher than unity. Its speed of migration toward the anode in this pH$_a$ range was therefore compared with that of authentic diphosphopyridine nucleotide, and was found to be the same. Like the latter compound, the zone 5 substance was split by standing in 0·5 N sodium hydroxide for 15 hr. at 37° C. chiefly into adenosine-5-phosphate and a moiety which, judging from its electrophoretic properties, is probably nicotinamide mononucleotide. Contrary to what might be expected from the report of Schlenck, von Euler and co-workers[11], nicotinamide and its riboside are not major products of this alkali treatment. Appearance of an absorption maximum at 340 mμ on reduction with sodium hydrosulphite[12,13] of the material eluted from zone 5 left little doubt as to the presence of diphosphopyridine nucleotide. Significant amounts of other substances containing nitrogen, ribose, or phosphate were not present in the eluate. In unfractionated perchloric acid extracts of calf heart muscle that has been contracting partly anaerobically for about one minute, oxidized diphosphopyridine nucleotide accounts, at pH 2, for 5–8 per cent of the absorption at 260 mμ, which is the absorption maximum of adenine and nicotinamide. In fresh rabbit skeletal muscle the corresponding percentage is lower.

Spot 6 in Fig. 1 was found to be due to creatinine formed from creatine by heating the filter paper, as evidenced by positive colour reactions on spraying the unheated and heated papers with α-naphthol-diacetyl[14] and alkaline picrate[5] reagents, respectively. The eluate from the unheated paper behaved like a solution of authentic creatine with respect to R_F values[15] and ultra-violet absorption[7,16], both before and after treatment designed to transform creatine into creatinine. Another test for creatinine given by spot 6 and also by spot 1, after electrophoresis in 0·05 M potassium phosphate buffer of pH 5·9, was the appearance of a yellowish-green fluorescence following application of butyric acid at high temperatures[17]. For some unknown reason, creatine present in and added to the muscle extracts migrated with a greater velocity towards the cathode than creatine applied separately, which at pH 5·6 is almost stationary.

Anserine and carnosine were identified as the components of fluorescent spot 7. The fluorescent material moved in the electric field with the same velocity as the authentic peptides. Carnosine was revealed on the paper as an intense red stain after spraying with alkaline diazobenzenesulphonate[18], while spraying with ninhydrin followed by heating produced a green coloration[19]. The eluate from the spot gave the same R_F values on the paper chromatogram as the authentic peptides[19,20], and yielded an ultra-violet absorption curve characteristic of imidazole compounds[21,22]. (Using a Hilger 'Uvispek' photoelectric spectrophotometer we find the absorption maximum of L(+)-carnosine to lie at a somewhat lower wave-length than was reported by de Gouveia *et al.*[22], namely, at 212 mμ (ε = 6,600), both at pH 3 and 11.) Because carnosine and anserine differ only slightly with respect to size, shape and ionization (see ref. 23), electrophoresis offers little chance for separating these compounds from each other. However, it makes possible their rapid separation from the bulk of the other muscle constituents.

The unmarked spots in Fig. 1 have not yet been identified. The bright fluorescent spot situated close behind phosphocreatine gave a positive reaction with the aniline hydrogen phthalate reagent[24], which suggests that the rapidly moving anion may be a phosphorylated sugar. Spraying of the filter paper with ninhydrin revealed, in addition to carnosine and anserine, a number of other ninhydrin-positive compounds present in appreciable amounts. Two of these, for which the heart of new-born animals is a good source, appear to be phosphorylated. This phase of the present work is not yet completed.

In some experiments, such as that represented in Fig. 1, requirements for determination of mobilities on paper were, in the main, fulfilled. Thus, evaporation of water from the liquid medium was held at a minimum, electro-osmosis was estimated, and other factors tending to disturb migration in the electric field, particularly adsorption of the ions under study on the paper, were taken into consideration. Making allowance for the electro-osmotic flow and for a 15–25 per cent retardation in the movement of the nucleotides, due to adsorption, and applying the correction factor of Kunkel and Tiselius[1] for non-linearity of the path of ionic migration through Munktell 20 filter paper, the following mobilities of some of the ions studied are obtained, at pH 5·6, ionic strength 0·45 and 25° C.: phosphocreatine, $-20{\cdot}6 \times 10^{-5}$; carnosine, $8{\cdot}8 \times 10^{-5}$; oxidized diphosphopyridine nucleotide, $-6{\cdot}4 \times 10^{-5}$; adenosine monophosphate, $-11{\cdot}5 \times 10^{-5}$; and adenosine triphosphate, $-17{\cdot}7 \times 10^{-5}$ cm.^{2} volt^{-1} sec.$^{-1}$. Mobility values for adenosine mono- and triphosphates calculated from filter paper experiments undertaken at ionic strength 0·1 and 0° C. were found to agree quite well with values[10] obtained under these conditions in free electrophoresis.

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