While showing good radial sharpness, all spots are arced sufficiently to make decision as to certain indices and unit-cell dimensions difficult. The fibre-axis translation (b_0) is the only cell element clearly distinguishable. All non-meridional arcs of the first layer line, as well as diatropic reflexions on the first and second layer lines, indicate a value for b_0 of 5·3 A., as compared with the 5·65 A. suggested by Lotmar and Picken.

One may also ascertain that interferences comparable in sharpness and intensity with the others are not present at the position of the diffuse equatorial spot corresponding to the 'side-chain' spacing of the α-diagram. Diffraction in this region played a prominent part in the considerations of Lotmar and Picken and of Pauling and Corey. Our results throw considerable doubt on the correctness of the previously suggested unit cell, and also deny the validity of any structural conclusions which require, on the atypical muscle diagram, strong equatorial diffraction corresponding to spacings of about 10 A.

Crystalline proteins should show large cells the dimensions of which are functions of the large lengths and/or breadths of the molecules. Although the abnormal *Loligo* specimen yielded a single, partially oriented (equatorial) diffraction, corresponding to a spacing of about 50 A., this is independent of the Lotmar–Picken diagram, since on occasion similar diffraction has been observed in muscles failing to show the distinctive Lotmar–Picken phenomenon. The oriented rings observed in such cases are like those yielded by phospholipids[7], which are common tissue contaminants.

There remains the possibility, if protein is involved, that the wide-angle diffractions represent intramolecular diffraction from individual protein particles. This is excluded by the radial sharpness and the spotted character of the observed diffractions, which could not be produced by particles of size less than that reported for intra-myofibrillar filaments (breadths *c.* 100 A.)[8]. Several of the interplanar spacings observed for the Lotmar–Picken substance are as small as 2 A. or less, a magnitude which, though not impossible for a protein, is smaller than is usually encountered.

Additional indication as to the nature of the Lotmar–Picken substance was found in experiments employing solvents to disperse this material. The abnormal *Loligo* specimen was divided and one portion subjected to distilled water, another to benzene, for two days. After drying, neither specimen yielded the Lotmar–Picken diffractions, exhibiting only the normal α-diagram. Dispersion by water is rather inconclusive; but the solubility in benzene may narrow the possibilities. The water had weakened the intensity of the 50-A. ring mentioned above; but benzene removed it entirely. The more bulky sample of *Pecten* muscle was extracted with benzene, and upon concentration of the extract by evaporation of solvent the residue yielded a small-angle ring (43 A.) but no evidence of Lotmar–Picken diffraction.

Probably the Lotmar–Picken substance, while related in origin to the fibrous elements of muscle, is not a protein. It is more likely to be a small organic molecular species which usually remains hidden by complex formation or solid solution within or near the fibrous components. On occasions, the circumstances of which are unknown, this material may be released to form separate crystalline phases, much as the lipid components of the nerve myelin sheath separate from the protein upon drying[9]. While this view denies that the Lotmar–Picken diagram is of any direct significance for the problem of protein structure, the diagram may still retain considerable interest for conceptions of muscle structure.

[July 25.

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MOVEMENT OF PROTEINS ON FILTER PAPER

THE separation of proteins by chromatography has been described by several workers[1,2]. Recently, Franklin and Quastel[3,4] reported the separation of proteins on filter paper. They developed two-way chromatograms with a variety of solvents (sugars, amino-acids and sodium salts of weak dibasic or hydroxy acids). The importance of their work, and its possible value in clinical research, rendered it desirable to examine the movement of proteins on filter paper under the influence of different eluting solutions, and to follow the effect of the conditions employed on the appearance of the resulting chromatograms.

The factors concerned in the ultimate separation of a two-dimensional chromatogram are: (1) extension of the mixture from a single spot to form either an elongated band or a series of discrete spots along the origin of flow of the second dimension; and (2) separation of the components of this band to give the final pattern.

After certain preliminary experiments, it appeared doubtful whether either of these two conditions was fully met in the system under consideration, and further experiments were designed to study the

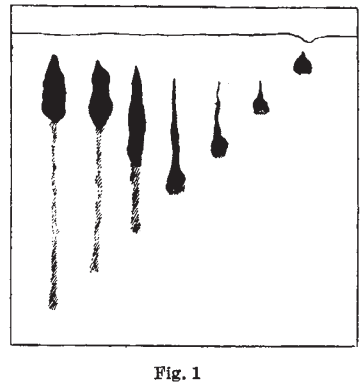

Fig. 1

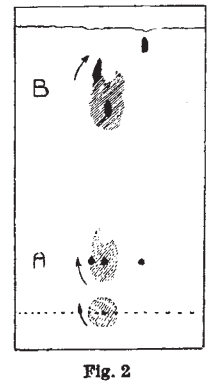

Fig. 2

Fig. 1. Diagram demonstrating the changing shape of protein spots at various intervals after the commencement of a chromatogram. The black area indicates the protein mass as demonstrated by ultra-violet fluorescence or iodine, the shaded area that which also stains blue with benzidine, showing that this indicator in part dissociates from the protein during the run

Fig. 2. Diagram demonstrating non-linear flow of solvent in neighbourhood of protein mass. When protein had reached *A*, spots of bromphenol blue were added at positions indicated. The relative movement of the component parts of the spot is shown by the positions of the bromphenol blue spots at *B*

mode of transport of mixtures of proteins such as occur in serum.

Following the conditions of Franklin and Quastel's experiment[4], serum and hæmin were mixed and 0·02 ml. dried on paper in a current of cold air. The chromatogram was developed by upward irrigation with 0·1 *M* glucose, lævulose, sodium glutamate, adipate malate or maleate solutions. To determine the shape of the protein mass after varying periods of time, spots were applied at increasing distances from the lower edge of the paper. When the solvent front had reached the uppermost spot and had flowed a distance of approximately 10 cm., the chromatogram was dried in a stream of cold air, and treated with benzidine (Fig. 1). There was a marked lag in the elution of the spot, leaving an indentation in the advancing front. Only after the solvent front had moved two or three centimetres ahead of the spot did elution commence. In further experiments small quantities of bromphenol blue were added to the chromatogram on either side of the mid-line of flow of the protein (Fig. 2). Material from the sides of the spots was eluted first and occasionally caused the production of "two separate fractions inside the finger" as observed by Franklin and Quastel[3]. Thereafter, owing to the inward flow of the solvent across the front of the spot, the tails fused and material from the rear was continuously carried to the front of the moving protein mass.

It was therefore apparent that the relative positions of the components of a mixture are dependent on certain factors not necessarily functions of its complexity. The protein is first segregated radially through the spot during drying, followed by peripheral elution. Secondly, infiltration and elution of the central portion which afterwards overtakes the material initially eluted causes the relative final positions of the fractions eluted from the centre and periphery of the original spot to depend on the distance the solvent front has travelled. It will be noticed that towards the end of a run of 20 cm., the protein is located as an oval spot only about twice the length of the original. Paper electrophoresis[5,6] of various portions of spots run under such conditions showed that at no stage in the process was there a linear distribution of the various components, albumin and globulin bands appearing in the final electrophoresis patterns of all areas of the spot. If by adequate manipulation of the conditions an elongated spot does result from the first run, this is again securely bound to the paper during drying, so that the developing solution is held back by the dried spot but eventually bursts through after its freely moving front has by-passed the spot by about 15 mm. The elongated protein trace is thus disrupted into a number of fractions. As many as eighteen have been observed immediately after the spot has been wetted out, if bromphenol blue is added to the protein initially. As the solvent front progresses, the faster-moving portions at either side flow inwards across the front of the protein, compressing the effective length of the complex of small spots, causing them to coalesce. Thus, the number of separate spots appearing on the final chromatogram depends on: (*a*) the length of the first run, since this affects the length of the spot; and (*b*) the length of the second run, since fewer spots are observed when this run is protracted.

It is also possible to show that the fractionation produced during the second run is not dependent on the existence of separate protein species in discrete positions on the paper. Spots of serum of varying length but containing the same volume were placed on the starting line, and developed with an appropriate solvent. The results showed great similarity to those obtained after a two-dimensional run.

Certain poly-hydroxy emulsifying agents were shown by Franklin and Quastel[4] to have the effect of increasing the number of fractions into which serum could apparently be divided. Since little or no separation may occur in the first direction, any increase in fractionation must be looked for in the second. This effect is apparently due to the greater degree of wetting-out occurring in those systems containing both protein and surface-active agent. Experiments with 'Span 20', sodium alginate and 'Tween 85' showed that the separation into small fractions could take place before the solvent front had passed the protein. There was therefore no tendency for the solvents to flow inwards and recombine the fractions; hence the greater number of fractions observed at the end of a run. For a similar reason the protein stayed with the front and did not lag behind.

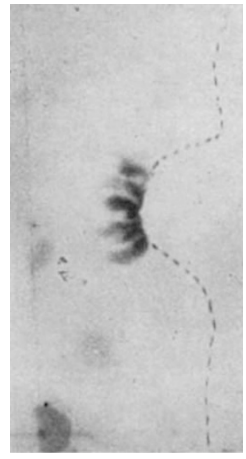

Fig. 3. Photograph of chromatogram obtained with 'Tween 80' and hæmin in the absence of any proteins. Solvent in both directions, *M*/100-fructose. Similar results are obtained with other combinations of solvents

There is therefore no reason for believing that the separate spots observed by Franklin and Quastel should be considered as individual chemical entities. Indeed, chromatograms of a mixture of the emulsifying agents 'Tween 80' or 'Tween 81' and hæmin without any protein produce an essentially similar pattern of spots (Fig. 3). Therefore, the inclusion of this type of compound may well increase the number of spots solely on its own account.

The conclusion to be drawn from this survey is as follows: during flow on a sheet of filter paper, the resistance of protein to transport in aqueous solution, coupled with the ease with which the solvent may by-pass the protein, will always tend to minimize the effectiveness of any possible separation of similar protein species.

The separation of proteins of quite dissimilar nature by this method, however, is, of course, perfectly practicable, as has been shown by Gross, Leblond, Franklin and Quastel[7]; but the patterns observed in complex mixtures such as serum are so dependent on the conditions of the experiment as to be pure

artefacts. Given adequate control, there is no doubt a good degree of reproducibility; but owing to differing interactions between various components of different mixtures, the patterns observed can have no direct correlation with the complexities of the mixture.

Since this work was completed, Swingle and Tiselius[8] have also expressed the opinion that the patterns observed under these conditions are artefacts dependent on the general properties of proteins in contact with paper; but owing to the fact that our observations may be of interest in further work on protein separation, we have felt it desirable that they should be placed on record.

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A technique for the two-dimensional chromatography of blood plasmas or protein mixtures on filter paper has been described by Franklin and Quastel[1] which differs in certain important respects from the well-known methods adopted in amino-acid paper chromatography. It was found that the additions of small quantities of surface active agents such as the 'Tweens' or 'Spans' seemed to facilitate the separation of plasma constituents and extend the protein 'pattern'. The technique adopted at present is to add 'Tween 85' or 'Tween 81' to the plasma and use hæmin as the protein marker. A mixture of an alcoholic solution of benzidine and hydrogen peroxide, made acid with acetic acid, is used for detecting the hæmin. Solutions of sucrose and of sodium potassium tartrate have been selected, after many experiments, as the most suitable developing solvents in the first and second dimensions respectively. The former solution appears to facilitate movement of certain protein hæmin complexes, as well as uncombined hæmin, in the first dimension; the latter solution allows movement of certain protein complexes in the second dimension while preventing movement of uncombined hæmin. There must not be overloading of the paper with protein; usually 0·02 ml. plasma is applied. The technique has been used in the study of blood plasma changes that occur consistently after heparin administration and after single high-fat meals[2] and in investigations of multiple sclerosis[3]. It has also been used profitably in an investigation of the combination between thyroxine and plasma constituents[4], in a study of the breakdown of casein by rennin and of the separation of rennin from crude rennet preparations[5], and in the demonstration of non-specific agglutinins for Brucella in bovine sera[11].

Drs. Hall and Wewalka point out that the upward flow of the plasma proteins from the original spot on the paper consists of a gradual elution by the ascending solvent and conclude "that the relative positions of the components of a mixture are dependent on certain factors not necessarily functions of its complexity". They also state, without giving the experimental evidence, that at no stage in the process is there a linear distribution of the various components, albumin and globulin bands appearing in the final electrophoresis patterns of all the areas of the spot (no surface-active agents were used). Thus, they intimate that no separation of protein constituents of the plasma takes place in the first-dimensional run. This conclusion leads them to imply that the fractionations seen in the second dimension are due simply to the mechanical or physical factors operating in this technique, and do not reflect any separations of a chemical nature. It is further claimed that the action of surface-active agents is only to prevent the coalescence of fractions which is observed in the second-dimensional run when no surface-active agent is present.

If these observations and conclusions are correct, it follows that the protein patterns of various plasmas should offer no great dissimilarities, since the patterns are allegedly not dependent on the chemical complexity of the plasmas but on the physical factors operating in the runs in the two dimensions.

Much experience has shown that the protein patterns obtained using our technique with normal plasmas are very similar to each other and may be reproduced with considerable regularity. On the other hand, the patterns obtained with many pathological plasmas differ very greatly from those given by the normal. These may also be duplicated with regularity in the same patient, so long as the clinical condition of the patient does not alter. There is now ample evidence which, it is hoped, will be published shortly, to show that the abnormal patterns observed in certain diseases revert to normal when the disease is successfully treated. Photographs of typical paper chromatograms of (*a*) normal plasma, (*b*) plasma of a patient suffering from cancer of the

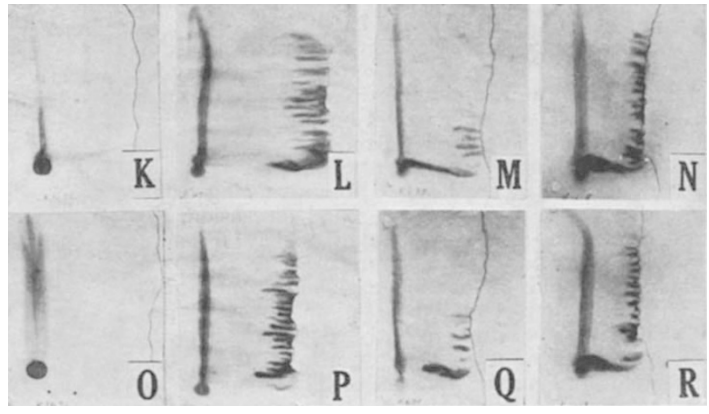

Fig. 1. *K*, Hæmin control (0·02 ml. of 0·3 per cent hæmin in 0·5 ml. of distilled water plus 0·02 ml. of 'Tween 81'); *L*, normal plasma (0·02 ml. of 0·3 per cent hæmin and 0·02 ml. of 'Tween 81' added per 0·5 ml. of plasma); *M*, plasma of a patient with cancer of the stomach (0·02 ml. of 0·3 per cent hæmin and 0·02 ml. of 'Tween 81' added per 0·5 ml. of plasma); *N*, plasma of a patient with lupus erythematosus (0·02 ml. of 0·3 per cent hæmin and 0·02 ml. of 'Tween 81' added per 0·5 ml. of plasma); *O*, same as *K* but with 0·01 ml. of 'Tween 85' instead of 'Tween 81' added per 0·5 ml. of plasma; *P*, same as *L* but with 0·01 ml. of 'Tween 85' instead of 'Tween 81' added per 0·5 ml. of plasma; *Q*, same as *M* but with 0·01 ml. of 'Tween 85' instead of 'Tween 81' added per 0·5 ml. of plasma; *R*, same as *N* but with 0·01 ml. of 'Tween 85' instead of 'Tween 81' added per 0·5 ml. of plasma