through the sheath in the absence of axoplasm. The sea-water inside the axon was extruded after the immersion period and chemical determinations made. The rate of penetration of ACh was much more rapid in this case; equilibrium was reached between 5-10 minutes. At the end of the experiment, the axon sheath was tested for leaks by means of a water soluble dye.

In other experiments microscopically cleaned axons were immersed in 0.01 *M* ACh for 90 minutes and then washed for 10 seconds, 1 minute, and 30 minutes, in fresh aliquots of sea-water. The quantities of ACh determined in the washes were respectively: 4.4, 2.2, and 5.7 x 10^{-3} *M*. The 10 second wash was considered as surface contamination. The amount of ACh in the other washes was considered to represent a major portion of the ACh which had been distributed in the axon during the immersion period (molarities calculated from volume of axons).

Summary. The penetration of acetylcholine into the interior of the resting Squid axon, which had been immersed in ACh solutions, was demonstrated by its appearance in the extruded axoplasm. The outflow of ACh from resting axons previously immersed in ACh solutions was also demonstrated.

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Protein Separations and Interactions on Filter Paper.* (19762)

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Two technics are available whereby proteins may be caused to move on filter paper—chromatography and microelectrophoresis. The former method involves the movement of proteins by capillary ascent. A mixture of proteins diffuses from a spot into a streak by the action of an aqueous developing solvent (1). The latter technic, based on the Tiselius method, makes use of the different mobilities of the various proteins in an electric field(2). A combination of these two methods, chromatography in the first dimension followed by electrophoresis in the second dimension, provides a variation in the present 2-dimensional chromatography and makes it possible to study the separation and interaction of various proteins on filter paper.

Whatman No. 3 MM paper has been used throughout. The dry purified proteins are dissolved in 0.85% saline, and 0.04 ml aliquots are placed about 4 inches from the lower left hand corner of strips of filter paper approximately 8″ x 15″ in size. The spots are dried at room temperature, the papers formed into cylinders and stapled so that the edges do not touch, and then placed in shallow dishes containing the developing solvent. After the papers are thoroughly wetted by capillary action (about 1.5 hours), they are unstapled, rinsed quickly in veronal buffer solution (pH = 8.6), and placed in the electrophoresis apparatus.

The electrophoresis apparatus consists of a pair of plastic troughs (Perspex) 17″ x 4″ x 3¼″ each divided longitudinally into two compartments. Electrical contact between the

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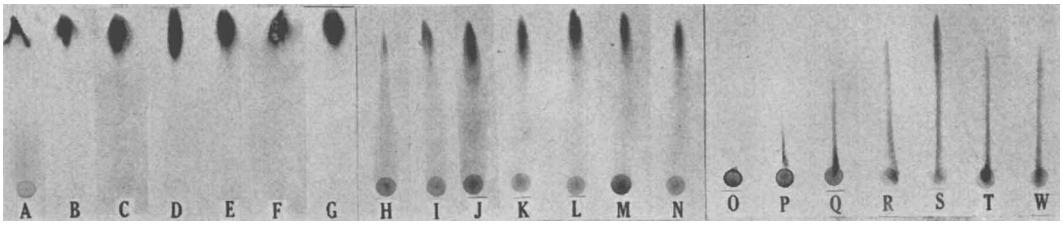

FIG. 1. Movement of proteins on filter paper at different hydrogen ion concentrations. A to G, .04 ml aliquots of a 3% solution of human serum albumin (Cohn Fraction V); H to N, .04 ml aliquots of a 2% solution of γ globulin (Cohn Fraction II); O to W, .04 ml aliquots of a 3% solution of fibrinogen (Cohn Fraction I). The following developing solvents were used: A, H, O, .05*M* potassium hydrogen phthalate pH 3; B, I, P, .05*M* potassium hydrogen phthalate pH 4.8; C, J, Q, .05*M* potassium hydrogen phthalate pH 5.4; D, K, R, .05*M* sodium citrate pH 6.1; E, L, S, .05*M* barbitone (veronal) pH 8.6; F, M, T, .1*M* sucrose unbuffered pH approximately 7; G, N, W, .1*M* sodium potassium tartrate unbuffered pH approximately 7.

buffer solutions in the outer and inner compartments is made through small wicks protruding through holes near the upper edge of the dividing wall. Carbon electrodes are placed in the outer compartments and connected through an ammeter and voltmeter to a source of DC from a selenium rectifier, with a variable AC input.

The filter paper strips are supported on a plastic frame fitted on to the troughs so that the ends of the papers dip into the buffer solutions in the two inner compartments of the troughs. The whole set-up is enclosed in a glass tank with a heavy glass lid resting on a rubber rim. Veronal buffer pH 8.6 consisting of 0.05 *M* sodium diethyl barbiturate and 0.01 *M* diethyl barbituric acid is used as the electrophoresis buffer solution. The filter paper strips are placed in such a position in the apparatus that the protein streak is about 3 inches from the surface of the liquid in the inner compartment of the negative electrode vessel.

A potential of 130 volts is applied across the papers for about 18 hours, after which the papers are dried, and the proteins stained with hot aqueous solution of Solvay purple (0.05%) containing 0.5% sulphuric acid(3). After about 5 minutes the chromatograms are washed with warm water to remove the excess dye and dried.

Effect of developing solvents at different hydrogen ion concentrations on movement of human serum albumin. The movement of albumin was investigated using 0.05 *M* phthalate buffer at pH 3, 4.8, 5.4, citrate buffer pH 6.1, veronal buffer pH 8.6, 0.1 *M* solution of sucrose and sodium potassium tartrate, both approximately pH 7. The position of albumin is virtually unchanged in all these developing solvents. All the albumin moves from the point of origin except with phthalate buffer pH 3. In this case some albumin remains at the origin. (Fig. 1, A to G.)

Effect of developing solvents on movement of γ globulin. Using the same developing solvents, γ globulin generally migrates to a position just below that of albumin, and a considerable portion remains fixed at the origin in all cases. At pH 3 in phthalate buffer, little protein moves up the paper as compared with that at pH 4.8, 5.4 etc. In veronal buffer pH 8.6, γ globulin moves to almost the same position as that of albumin. (Fig. 1, H to N).

Effect of developing solvents on movement of fibrinogen. In phthalate buffer pH 3 and 4.8, fibrinogen scarcely moves at all from the point of origin. At pH 5.4 there is a pronounced movement which becomes even more marked at pH 6.1 in citrate buffer. At pH 8.6 in veronal buffer, the movement is greatest, the fibrinogen flowing out from the point of application almost to the top of the paper, but with no well defined spot as is observed with albumin and γ globulin. The migrations in sucrose and sodium potassium tartrate solutions are similar to that in citrate buffer at pH 6.1 (Fig. 1, O to W).

Effect of developing solvents on the movement of albumin and fibrinogen using microelectrophoresis in the second dimension. In

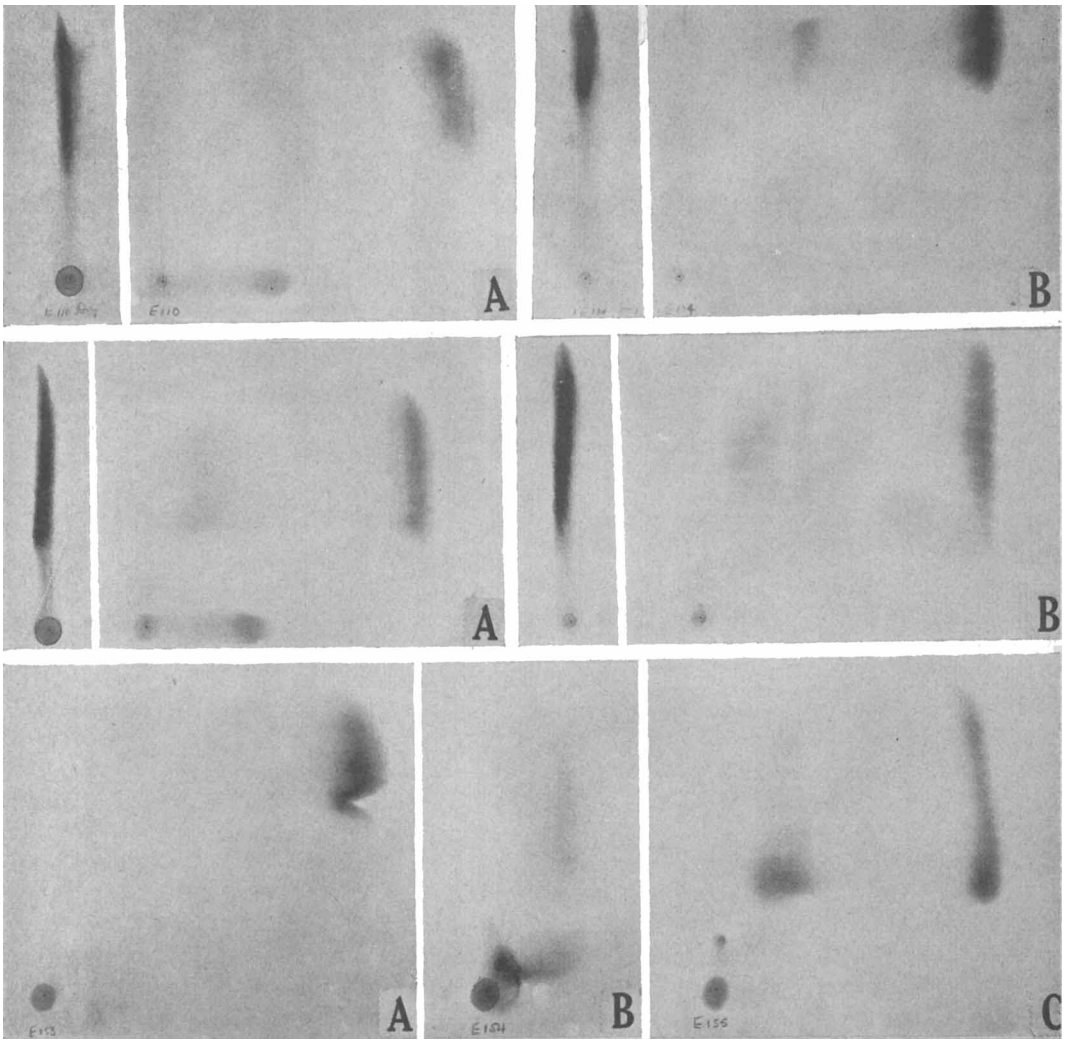

FIG. 2 (Top). Separation and interaction of albumin and fibrinogen on filter paper. A—.04 ml of a mixture containing 3% albumin and 3% fibrinogen chromatographed in the first dimension using phthalate buffer pH 4.8, showing the first dimensional streak, and the results of electrophoresis of this streak in the second dimension. B—Same as A, except chromatograph carried out in the first dimension using veronal buffer pH 8.6.

FIG. 3 (Middle). Separation and interaction of albumin, γ globulin and fibrinogen on filter paper. A—.04 ml of a mixture containing 3% albumin, 2% γ globulin and 3% fibrinogen chromatographed in the first dimension using phthalate buffer pH 4.8 showing the first dimensional streak and the results of electrophoresis in the second dimension. B—Same as A, except chromatograph carried out in the first dimension using veronal buffer pH 8.6.

FIG. 4 (Bottom). Effect of Tween 81 on movement of albumin and γ globulin using .1*M* sucrose solution in the first dimension and electrophoresis in the second dimension. A—.04 ml aliquot of a 3% solution of albumin containing .02 ml of Tween 81 per ml. B—.04 ml aliquot of a 3% solution of γ globulin containing .02 ml of Tween 81 per ml. C—.04 ml aliquot of a mixture containing 3% albumin, 3% γ globulin and .02 ml of Tween 81 per ml.

phthalate buffer at pH 4.8 fibrinogen, in contrast to albumin, hardly moves on the filter paper (Fig. 1, B and P). It should be possible, therefore, to separate albumin and fibrinogen by filter paper chromatography. When the two proteins are mixed and sent up the paper in phthalate buffer at pH 4.8, fibrinogen does not move, but albumin migrates as a more elongated band to a position below its usual one. The positions of these proteins

are easily detected by electrophoresis (Fig. 2A). Thus fibrinogen exerts a marked effect on albumin apparently without itself migrating, *i.e.* some interaction seems to take place without sufficient combination occurring to be visible under the present experimental conditions.

At pH 5.4, fibrinogen alone migrates to some extent, but mixed with albumin, there is a separation into two components; the one remaining fixed at the point of origin and the other moving up in apparent combination with albumin.

This apparent combination, or interaction, is complete in citrate buffer at pH 6.1. All of the fibrinogen moves up from the starting point. Some uncombined albumin, however, must also be present because the albumin band extends beyond the fibrinogen band. This same effect is observed using veronal buffer pH 8.6, (Fig. 2B) or solutions of sucrose or sodium potassium tartrate. A trace of fibrinogen is found at the origin when tartrate is used as the developing solvent.

Effect of developing solvents on the movement of albumin, globulin and fibrinogen. A restrictive influence of fibrinogen on the movement of γ globulin occurs when a mixture of the two are chromatographed on filter paper. This restriction seems to disappear when albumin is added to the mixture. At pH 4.8, all of the albumin travels with γ globulin, the albumin band extending above that of γ globulin. The fibrinogen remains at the origin as before (Fig. 3A).

At pH 6.1 in citrate buffer all the proteins move up on the paper. Fibrinogen and γ globulin move together, both their bands being shorter than that of albumin. The phenomenon is observed at pH 8.6 in veronal buffer, (Fig. 3B) and in solutions of sucrose or sodium potassium tartrate.

Thus it would appear that the presence of fibrinogen alters the chromatographic positions of both albumin and of γ globulin, separately or together, a phenomenon which would be explained by mutual interaction between the proteins. When the three are mixed, γ globulin moves with albumin.

Effect of Tween 81 in the movement of albumin and γ globulin. The addition of surface active agents such as Tween 81 (polyethylene sorbitan monooleate) to solutions of albumin and γ globulin appears to alter the rate of movement of the proteins and causes elongation of the first dimensional streak. Compare Fig. 1 F and M with Fig. 4, A and B). This occurs when using the various buffer solutions or solutions of sucrose or sodium potassium tartrate as developing solvents. The albumin band is elongated, but the greater change occurs with γ globulin. Its band extends from the point of application to the top of the paper, with the largest portion appearing at the origin. When the two proteins are mixed and the same quantity of Tween 81 added, the albumin band is extended considerably and the γ globulin moves up with the albumin to a new position. No γ globulin appears at the origin, but most of the γ globulin still appears in the lower part of the first dimensional streak.

The phenomena are consistent with the conclusion(1) that complex formation between the proteins and the surface active agent occurs, as well as interaction between the proteins themselves.

Conclusion. Using a combination of two technics, paper chromatography in the first dimension and paper microelectrophoresis in the second dimension, it is shown that protein separation may take place during capillary ascent of a mixture of proteins on filter paper and that protein interaction may also occur.

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