In the recovery experiments the esters were added to liver extracts in proportions similar to those found in determinations of these esters in livers of animals assimilating glucose or galactose. Approximately 80% of the added esters were recovered; there was no significant difference between the recoveries of hexose and phosphorus. The ratios 50° value/100° value were sufficiently close to the calculated values. As was to be expected, the standard errors of the means were relatively high in the experiments in which only 0.6–1.5 mg. ester P/100 g. liver was added. These facts have to be taken into consideration when experiments are designed. The limiting factors obviously are the methods used for the estimation of reducing power and of phosphorus. These difficulties are best overcome by increasing the quantities of liver used for the estimation but, unfortunately, in the rat at least, this cannot easily be done, particularly when other substances, as, for example, glycogen and hexose, have to be determined simultaneously.

SUMMARY

1. Glucose-1-phosphate and galactose-1-phosphate are determined in trichloroacetic acid extracts of rats' livers. After removal of interfering reducing substances, i.e. nitrogenous phosphoric esters, bile pigments, and glycogen, by means of mercuric acetate and HgS, the organic phosphates

are separated from inorganic P as water-soluble barium salts.

2. The reducing power of the organic phosphate fraction gives an approximate measure of the amount of hexose-6-phosphates present. The quantity of glucose-1-phosphate and galactose-1-phosphate taken together is determined by the increase in reducing power and the amount of inorganic P liberated after hydrolysis in a mixture of 0.05N HCl and 0.2N H₂SO₄ at 100° for 3 min. Incubation with 0.25N acid at 50° for 30 min. hydrolyses 28.6% of glucose-1-phosphate and 77.5% of galactose-1-phosphate. In a mixture of the two esters, the ratio 50° value/100° value is determined by the relative concentrations of the esters.

3. In experiments with addition of known quantities of ester to CCl₃.COOH extracts of liver, 84.5% of added glucose-1-phosphate (2.65–3.1 mg. P/100 g. liver), 79% of added galactose-1-phosphate (2.68–2.85 mg. P/100 g. liver), and 77% of added galactose-1-phosphate (0.6–1.5 mg. P/100 g. liver) were recovered. The ratios 50° value/100° value did not show any significant deviation from the expected values. The significance of the standard errors of the means of the results for the recovery of added material is discussed.

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An Improved Method for the Fractionation of Protein Mixtures by Electrophoresis

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The application of the electrophoresis technique to the problem of analysing protein mixtures was greatly refined by Tiselius [1937 *a, b*], who used the method particularly for the analysis of serum proteins. Since then it has been used by a variety of workers, Kekwick & Record [1940], for instance, having applied it to the isolation of the biologically active globulins from immune horse serum. Al-

though the method is of great value, only relatively small quantities of proteins can be separated by it and at a high cost. Moreover, attempts to increase the yields have met with considerable difficulties [Philpot, 1940].

In their purification of a variety of colloids by electrodialysis between two semi-permeable membranes, Pauli, Stamberger & Schmidt [1937] ob-

served the formation, in the fluid within the membrane cell, of superposed layers of different colloids [cf. Blank & Valko, 1928]. This is caused by the colloid particles' migrating to the membrane and concentrating at its surface. If the current density is not too great the colloidal particles are then more under the influence of gravity than of the electric field, and they move along the vertical face of the membrane, downwards if their specific gravity is greater than that of the dispersion medium, or upwards if it be less. These findings were applied to the concentration of rubber latices by Pauli *et al.* [1937].

It seemed that it might be possible to apply the method to the separation of protein mixtures. Thus, if the electrodialysis of a mixture were carried out in a buffer solution at the *pH* of the isoelectric point (I.E.P.) of one particular protein, under suitable conditions of current density and of time, the other proteins would move to one or other of the membranes and there concentrate in the lower layers, thus leaving the required protein in solution in the upper layers. This could then be removed from the cell by appropriate means. By repeating the process at different *pH*'s it should then be possible, by successive treatments, to resolve the mixture into a series of electrically homogeneous constituents. This process we designate 'electrodecantation'.

METHODS

Fig. 1 shows the elevation, through the middle of the glass parts, of the apparatus. It consists of two outer glass cells *A*, the circular open ends of which are covered by cellophane membranes *C*; the closed cells so formed are separated from

separating the outer cells from each other is 1.8 cm. Each electrode, *E*, consists of a circular carbon disk of diameter 9 cm. and is connected to a 12 V. battery by a carbon rod *F*, passing through the rubber bungs *D*; a voltmeter and an ammeter are included in the circuit. For clamping the apparatus together two vertical boards of hard wood, 18 × 22 cm., *G*, are used, the shorter edge being used as the base; these boards also serve as a stand for the apparatus. Each has a circular hole of diameter 7 cm., the lower edge of which is 11 cm. from the base, so that the neck of the cells *A* may be inserted. Four bolts, 15 cm. long, passing through the two wooden boards, are used to clamp the apparatus, by pressure on the outer surface of the cells *A*, heavy rubber washers being inserted between the boards and the glass surface of the cells *A* to prevent fracture; thin rubber washers of width 1 cm. are placed between cells *A* and *B*.

Holes in the cells *A*, through which the buffer solutions may be introduced or withdrawn, are provided at *H*, while holes for the introduction and removal of the protein solutions are provided in the middle cell *B* at *I*₁ and *I*₂.

Investigation of method

For test purposes a mixture of ovalbumin and haemoglobin was used. The ovalbumin was prepared by dissolving purified egg albumin (B.D.H., Ltd.) in distilled water, filtering and adding to the filtrate an equal vol. of sat. (NH₄)₂SO₄; after standing overnight at 0°, the mixture was filtered and dialysed against distilled water. When free from (NH₄)₂SO₄ the albumin solution was dialysed against a suitable buffer of the *pH* required in each particular experiment.

The haemoglobin was prepared by haemolysis of human red cells which had been washed well with isotonic saline. The concentrated crude solution was precipitated with sat. (NH₄)₂SO₄, dialysed first against distilled water, and then against buffer solutions of the required *pH*, as described for the albumin.

The buffer solutions used throughout consisted of suitable mixtures of 0.2*M* NaH₂PO₄ and 0.1*M* citric acid. The electrolysis was carried out at 0° at a potential of 1 V./cm. with a current of 0.8 amp.; the duration of each run was 6 hr. It was found by a series of experiments with haemoglobin solutions that at *pH* 5, after 6 hr. of electrodecantation, the upper one-third of the solution in the middle cell was free from haemoglobin; a similar result was obtained with egg albumin solutions at *pH* 7.

RESULTS

After the preliminary experiments, the separation of haemoglobin from egg albumin was investigated. Solutions of haemoglobin and egg albumin were

Fig. 1. Longitudinal section of electrodecantation cell.

each other by a short cylinder of glass, so that when clamped together, the middle cell *B* is formed. The inner radius of the ends of the cells *A*, which carry the membranes, is 11 cm., and the distance from the membranes to the inner surface of the rubber bungs *D*, through which the electrodes are inserted, is 9 cm.; the width of the glass cylinder

prepared as described, and these were finally dialysed against several changes of buffer at the approximate I.E.P. of haemoglobin, i.e. pH 6.8. Total N was determined on each solution, and the protein concentration was taken as $6.25 \times N$. Suitable volumes of each solution were then mixed together to yield a solution containing 1.1% haemoglobin and 0.9% albumin. The solution was submitted to electrodecentation at pH 6.8, under the conditions already described. After 6 hr. the middle cell (capacity 240 ml.) was emptied by running out twelve 20 ml. portions very slowly from the tap at I_2 . The total protein content of all the fractions so obtained was determined by Kjeldahl nitrogen estimation, the haemoglobin in each fraction being estimated colorimetrically against a standard prepared from the original haemoglobin solution. The concentration of albumin and haemoglobin in each fraction was then calculated. In Table 1 the first fraction (from the bottom of the cell) is numbered 12, while 1 represents the last 20 ml. from the top of the cell.

Table 1 shows that fractions 1-4 were completely free of albumin, and that a progressive increase in the albumin concentration occurred as the bottom of the cell was approached, the last fraction containing 2.5 g./100 ml. of this protein. In 6 hr. it is

Table 1. *Concentration of albumin and haemoglobin at different levels in the cell*

No. of sample	Albumin g./100 ml.	Haemoglobin g./100 ml.
1 (top of cell)	0.0	1.1
2	0.0	1.1
3	0.0	1.1
4	0.0	1.1
5	0.2	1.1
6	0.4	1.1
7	1.4	1.0
8	1.7	0.8
9	2.1	0.8
10	2.4	0.8
11	2.4	0.8
12 (bottom of cell)	2.5	0.8

possible thus to free one-third of the cell contents from albumin, and so to obtain one-third of the haemoglobin as an electrochemically homogeneous fraction. It had been anticipated that the haemoglobin concentration would remain constant in all 12 samples. Actually, as Table 1 shows, the fractions 1-6 retained the original concentration of 1.1 g./100 ml., fraction 7 was lower, i.e. 1.0 g./100 ml., while the remaining 5 fractions contained 0.8 g./100 ml. giving an average concentration of 0.96 g./100 ml., an apparent loss of about 13% of the original haemoglobin. This apparent loss was due to precipitation. When the haemoglobin con-

tent of each fraction was estimated on the cleared solution the precipitated haemoglobin remained unestimated; it was included, however, in the total N content, which was determined on a well-shaken sample. Thus in Table 1 the figures for albumin in the lower fractions are slightly higher than they should be.

The reason for the precipitation of a small fraction of the haemoglobin is not clear. The pH at which the electrodecentation was carried out was deliberately chosen to be slightly on the alkaline side of the I.E.P., and doubtless there was a slight migration of the haemoglobin under these conditions with a subsequent precipitation of a small part of it.

DISCUSSION

Although the methods still in the experimental stage, these results indicate that it should be applicable to many biological problems where the isolation of homogeneous protein fractions is involved. Thus, at the present time, it is being used for the separation of the biologically active globulins of immune horse serum. The apparatus is simple, and considerable quantities of material can be obtained rapidly at a low cost. There is, moreover, no theoretical reason why the apparatus should not be greatly enlarged, with a corresponding increase in output. The apparatus may be improved by the introduction of modifications to allow a continuous change of buffer solution in the outer cells, and by the provision of suitable taps, placed at different levels in the central compartment, to facilitate the removal of successive layers of the cell contents at the close of an experiment.

The optimum conditions of pH , time, and current density, for separation of different protein mixtures requires individual investigation.

SUMMARY

1. A preliminary description of a new method for the separation of protein mixtures is described.
2. The method, which depends on electrophoresis, possesses an advantage over the method of Tiselius in that it makes possible the preparation of considerable quantities of electrically homogeneous protein fractions.
3. The application of the method to the separation of mixtures of albumin and haemoglobin is described.

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The Reaction of Heparin with Proteins and Complex Bases

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Owing to its strongly acid nature, heparin forms stable salts with many proteins, examples of which have been reported by a number of workers, e.g. Reiner & Kopp [1928], Fischer [1931], Chargaff & Olsen [1937], and Scott & Fisher [1942]. The general nature of the reaction was first indicated by Fischer [1935] and Fischer & Astrup [1935] who studied its kinetics. While other examples of the reaction are now being reported, many authors appear to have overlooked these important contributions by Fischer, and in particular his finding that the reaction is a simple salt formation in which reversible dissociation of the salt occurs according to the mass law [Fischer & Astrup, 1935]. This latter point means that data may be analysed more thoroughly than hitherto, and that confusing results have frequently a simple physico-chemical explanation. During studies on heparin, the author has frequently confirmed Fischer's original observations in experiments carried out as controls for other studies. These findings, and the results of other experiments, are reported in this communication.

Three principles are available for the demonstration of the reaction of heparin with proteins. These are: (1) direct observation of the formation of the compound, e.g. precipitation of the gelatin-heparin compound when the two solutions are mixed; (2) the alteration in the properties of the protein, e.g. the shift in the isoelectric point (I.E.P.) of casein; the decrease in precipitability of serum globulin by alcohol, heat, and other precipitating agents [Fischer, 1935]; loss of activity by fumarase [Fischer & Herrmann, 1937]; (3) alteration in the properties of heparin, e.g. loss of dialysing power with egg albumin [Fischer, 1935] and loss of anticoagulant activity with protamines [e.g. Fischer, 1935; Chargaff & Olsen, 1937]. In our experience, it is necessary to choose a suitable method for each protein.

Failure to observe compound formation does not necessarily indicate that it has not occurred. Thus

when the heparin-protein compound is soluble under the conditions of the experiment (e.g. protamine in the presence of plasma) precipitation of the compound cannot be observed. The reaction can, however, still be detected by suitable indirect means (in this case by the loss of anticoagulant activity of the heparin).

Fischer indicated that a stoichiometric relationship existed between heparin and thromboplastin (and that presumably this extended to other proteins). Fischer & Astrup [1935] investigated this stoichiometric relationship more fully and found that the reaction could be represented as

$$\text{From the mass law, } \frac{C_{\text{protein}} \times C_{\text{heparin}}}{C_{\text{protein-heparin}}} = K.$$

Then, if a be the concentration of protein added, c the concentration of free protein, and b the concentration of added heparin, $a - c$ is the concentration of the product formed by the reaction, $b - (a - c)$ is the concentration of free heparin, and

$$K = \frac{c \{b - (a - c)\}}{a - c}. \quad (1)$$

Fischer & Astrup [1935] obtained satisfactory agreement with this equation for the mutual interaction of thromboplastin and heparin on hen plasma. The author has also found this to give good agreement for the reaction of heparin with casein, protamine, and toluidine blue, a non-protein basic substance. On the basis of these and other experiments, it seems likely that it is true of the reaction with all proteins, sufficient reversible dissociation taking place to have a significant effect on the kinetics of the reaction. The data of other authors show no disagreement with this view and tend to corroborate the general nature of the reaction. Actually, the earliest studies on heparin indicated that the biological action of heparin depended on such a reaction, since they showed that peptone