3. No separation of the two activities was achieved by ammonium sulphate fractionation or by partial heat inactivation.

4. D-Glucose, D-galactose, D-glucono-1→4-lactone and D-galactono-1→4-lactone were competitive inhibitors with similar K_i values when measured against the two substrates.

5. Mixed substrate experiments supported the conclusion that one enzyme site is responsible for both activities.

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Carbohydrates in Protein

4. THE DETERMINATION OF MANNOSE IN HEN'S-EGG ALBUMIN BY RADIOISOTOPE DILUTION*

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There are a number of earlier reports that hen's-egg albumin contains carbohydrate (see Needham, 1931). Although Hofmeister (1890) and Hopkins & Pincus (1898) obtained the protein in crystalline form, it was not until Sørensen & Høyrup (1916) showed that the protein after three crystallizations was free from 'mucoids' that such observations could have real significance. As a result of experiments carried out with 10% barium hydroxide, i.e. under conditions that may have resulted in at least partial destruction of the carbohydrate, Levene & Mori (1929) expressed the opinion that egg albumin does not contain carbohydrate. That the Carlsberg workers did not share this view is evident from the careful work of Sørensen & Haugaard (1933), who critically examined the orcinol–sulphuric acid procedure for the determination of carbohydrates and showed that egg albumin contained mannose by following the rate of formation of the coloured product; their results suggested a content of 1·7% of this sugar (Sørensen & Haugaard, 1933; Sørensen, 1934). It was not established, however, how this carbohydrate was attached to the protein. In Part 1 (Neuberger, 1938) it was shown that the bonding was almost certainly covalent and that the mannose content of the protein was at least 1·2%, as shown by actual isolation of a derivative. The orcinol–sulphuric acid procedure has also been reported to give 1·8% mannose by Neuberger (1938), Hewitt (1938) and Kaverzneva & Bogdanov (1961), and 2·0% by Johansen, Marshall & Neuberger (1960). Montgomery (1961) has reported 2·0% as the mannose content using a phenol–sulphuric acid procedure (Dubois, Gilles, Hamilton, Rebers & Smith, 1956) on the whole protein. Later Lee & Montgomery

* Part 3: Johansen, Marshall & Neuberger (1961).

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(1961), using the same procedure, quoted values of 2·08–2·24% of mannose by direct analysis on the protein. However, if the protein were previously hydrolysed in N-sulphuric acid, somewhat higher values were obtained, which these authors interpreted as indicating the presence of 2·39% of mannose in egg albumin. Bragg & Hough (1961) have reported a value of 2·75%, using the benzidine–acetic acid procedure of Jones & Pridham (1954) after hydrolysis of the protein and separation of the sugar by paper chromatography.

Colorimetric methods on the whole protein, however, suffer from certain disadvantages. First, the controls for the assay consist of solutions of the free sugar of known concentration, and it is not certain that a polysaccharide gives a colour yield identical with that of equivalent amounts of the free sugar (cf. Vasseur, 1948). Secondly, different hexoses give different colour yields; thirdly, we cannot be sure that the whole of the colour is due to the presence of a sugar, particularly in those methods where relatively concentrated sulphuric acid is used. We have therefore developed a method which should be specific for determining mannose in proteins and have applied it to egg albumin. The method should be applicable to insoluble derivatives of other sugars (see, for example, Hirst, Jones & Woods, 1947; Mester & Major, 1956) in other proteins.

For a preliminary communication, see François, Marshall & Neuberger (1961).

EXPERIMENTAL

Orcinol–sulphuric acid procedure. This was a modification of Winzler's (1955) method as previously used by Johansen *et al.* (1960). To 1 ml. of sugar solution (or water for blank) was added 8·5 ml. of orcinol–sulphuric acid [7·5 vol. of H_2SO_4–water (3:2, v/v) added, after cooling, to 1 vol. of 1·6% (w/v) orcinol in water; this was prepared fresh daily]. After heating at 80°, usually for 15 min., the tubes were cooled and the extinction was read at 505 mμ with a Unicam spectrophotometer, SP. 600. The heating was continued for 45 min. for glucose and the glucosides, since the reaction is much slower than for galactose and mannose.

Hexosamine assays. These were all carried out on the hydrolysed glucosaminides by the method of Rondle & Morgan (1955) after taking the hydrolysates to dryness *in vacuo* over P_2O_5 and KOH at room temperature. The compounds used were:

(*a*) *Phenyl tetra-*O*-acetyl-β-*D*-glucosaminide.* Glucosamine hydrochloride (6 g.), anhydrous sodium acetate (8 g.) and acetic anhydride (40 ml.) were boiled for 2–3 min., cooled and poured into cold water. The solution was neutralized with $NaHCO_3$ and extracted with chloroform. The chloroform extracts were combined, washed with water, dried over Na_2SO_4 and evaporated under reduced pressure to a small volume.

The residual oil was heated on a boiling-water bath for 25 min. with 12 g. of phenol and 0·2 g. of toluene-*p*-sulphonic acid. The oil, after cooling, was added to a mixture of 100 g. of chloroform and 20 ml. of water and the chloroform layer extracted with one-twentieth volume of 1·25N-NaOH at 0°. The product was crystallized by the addition of ether and light petroleum (b.p. 40–60°), giving 5·6 g. of material (48% of the theoretical yield). It was hoped to *O*-deacetylate the compound by dissolving the material in 6 ml. of methanol containing 0·08 ml. of 1·15M-sodium methoxide and leaving it at room temperature for 2 hr. Precipitation of the material with acetic acid, however, gave phenyl tetra-*O*-acetyl-β-D-glucosaminide, which after crystallizing from 70% (v/v) ethanol had m.p. 203·5–204·5°, $[\alpha]_D^{20}$ −16·9° (*c* 1·169 in acetone) and −22·8° [*c* 0·83 in 50% (v/v) ethanol] (Found: N, 3·22. Calc. for $C_{20}H_{25}NO_5$: N, 3·3%). Leaback & Walker (1957) quote m.p. 204°, $[\alpha]_D^{18}$ −14·5° (*c* 1 in acetone).

(*b*) *Methyl* N*-benzoxycarbonyl-β-*D*-glucosaminide.* This was prepared by the method of Foster, Horton & Stacey (1957).

(*c*) *Other glucosaminides.* The other compounds were prepared previously: methyl *N*-acetyl-β-D-glucosaminide and methyl tetra-*O*-acetyl-D-glucosaminide, mainly α-isomer (Moggridge & Neuberger, 1938); phenyl *N*-toluene-*p*-sulphonyl-β-D-glucosaminide and phenyl *N*-propionyl-β-D-glucosaminide (Neuberger & Pitt-Rivers, 1939).

Radioactive mannose. Generally-labelled [^{14}C]mannose (0·01 mc; The Radiochemical Centre, Amersham, Bucks.) and 0·4 g. of mannose (British Drug Houses Ltd.), once recrystallized, were dissolved in warm methanol and crystallized by the procedure of Isbell, Frush & Schaffer (1955). The product was dried at 60°.

Hydrolysis of the protein. A weighed quantity (usually about 2 g.) of egg albumin was dissolved in cold water in which a known weight of the radioactive mannose (about 25–30 mg.) was also dissolved. The final protein concentration was approximately 2%. The solution was heated to boiling and with vigorous stirring sufficient constant-boiling HCl was added to make the final concentration 2N. The flask was heated at 100° for measured times. The solution was cooled and filtered, the filter being washed three times with water. The hydrolysate was neutralized with the weak anion-exchange resin Amberlite IR-4B (0·6 wet ml./m-equiv. of acid) and the sugars were washed from the resin with water. It was not possible to remove the acid by evaporation overnight at room temperature in a vacuum desiccator, since mannose is almost completely destroyed under these conditions.

The neutralized hydrolysate was reduced in volume under diminished pressure in a rotary evaporator at 40° to about 5–10 ml., the pH being between 5 and 6. Freshly distilled phenylhydrazine (0·3 ml.) was added, followed by acetic acid (about 0·2 ml.) to a pH of 5·5. After being kept at room temperature for 2 hr. and in the cold room (4°) for at least 3 hr. (usually overnight), the precipitate was filtered off and washed successively with 50% (v/v) acetic acid, water, ethanol and ether, and finally dried at about 50°. It was recrystallized by dissolving in hot pyridine (3 g./100 ml.) and filtered at about 50°, and 5 vol. of water was added at the same temperature. The open vessel was left in the desiccator over H_2SO_4 in the cold room overnight. The precipitate was filtered off and washed as before. The m.p. after one recrystallization was 190–191° (the product from pure mannose had m.p. 191–192°).

Radioactive counting. The radioactive samples were counted by mounting the dry powder (at least 25 mg.) on

1 cm.² polythene planchets for counting at infinite thickness (Calvin, Heidelberger, Reid, Talbert & Yankwich, 1949). A Geiger–Müller end-window counter was used.

RESULTS AND DISCUSSION

The orcinol–sulphuric acid method

In an earlier paper (Johansen *et al.* 1960) the estimation of the carbohydrate content of various preparations of egg albumin was described and values of 1·88–2·02 % were obtained. Repeated determinations of the carbohydrate content of a further preparation of the protein gave values of $2{\cdot}0 \pm 0{\cdot}1$ %, in agreement with the earlier results. In all these experiments the orcinol–sulphuric acid procedure as described by Winzler (1955) was used, except that the extinction was read at a wavelength of 505 instead of at 540 mμ. The reasons for this change were previously given (Johansen *et al.* 1960), and Fig. 1 shows the spectra obtained for maximum colour yield for glucose, galactose, mannose and for egg albumin. These spectra differ somewhat from those obtained by Friedman (1949), who used the conditions in the orcinol–sulphuric acid method employed by Sørensen & Haugaard (1933). Since in this method the amount of orcinol differs from that used in the present experiments, and because this has an effect on the amount of colour developed in a given period of time, we have determined (*a*) the rate at which the maximum colour is produced for the three hexoses, and (*b*) the maximum colour yield given by each of the sugars.

The rate at which the extinction at 505 mμ increases has been used to calculate the half-life for colour development. Some of these values are recorded in Table 1 together with those obtained with some glucosides. The similarity between the values obtained for glucose and the glucosides makes it likely that the formation of 5-hydroxymethylfurfuraldehyde is the rate-controlling step, the hydrolysis of the glycosidic bond being a much faster reaction. The amount of colour developed by the glucosides is in all cases determined solely by the number of glucose units and not by the type of bond linking them. These results completely endorse those of Sørensen & Haugaard (1933), who stated in connexion with their results on raffinose that 'the velocity with which the trisaccharide is hydrolysed is so great that it plays no part in the cause of the colour reaction'. It may be noted also that the rate of colour formation in the case of egg albumin is only slightly delayed relative to that of mannose.

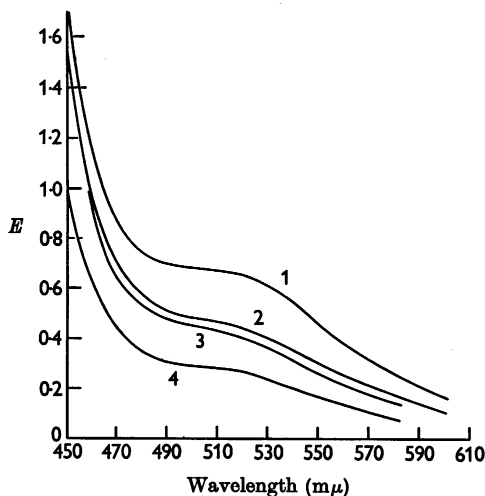

Fig. 1. Absorption spectra for the Winzler (1955) orcinol–sulphuric acid method as described in the text. 1, Egg albumin (14·2 mg.); 2, mannose (198 μg.); 3, glucose (202 μg.); 4, galactose (101 μg.). Heating time 15 min. for curves 1, 2 and 4; 45 min. for curve 3.

Table 1. *Rate and extent of colour development with the orcinol–sulphuric acid method on some hexoses and derivatives thereof*

Details of the method are given in the Experimental section. Up to about 500 μg. of each substance was used.

Substance	Sodium borate (μmoles)	Half-life of maximum colour development at λ = 505 mμ (min.)	Maximum molar colour yield based on glucose
Glucose	—	15·0, 14·0	1·00
Mannose	—	4·8, 3·6, 4·6	1·02
Galactose	—	5·8	1·12
Egg albumin	—	5·2, 6·0	—
Maltose	—	14·2, 15·0	2·0
Trehalose	—	15·5, 16·0	2·0
Methyl α-glucoside	—	16·8, 14·9, 15·5	1·0
Glucose + borate	24	14·5	—
Glucose + borate	47	12·5	—
Glucose + borate	94	8	1·00
Glucose + borate	188	7·5	1·00

Effect of borate on the orcinol–sulphuric acid reaction

The interaction of borate with sugars is well established and two quantitative colorimetric reactions for sugars are known to give enhanced colour yields in the presence of this anion. The Morgan & Elson (1934) reaction for *N*-acetylhexosamines was shown to be enhanced by the presence of borate (Aminoff, Morgan & Watkins, 1952), and a quantitative procedure making use of this observation was developed (Reissig, Strominger & Leloir, 1955). The colour reaction of carbazole with the products given by the interaction of uronic acids with sulphuric acid (Dische, 1947) has been shown to be considerably increased by the presence of borate (Gregory, 1960; Bitter & Ewins, 1961).

Gregory (1960) also indicated that borate enhanced the colour given by glucose in an orcinol–sulphuric acid procedure (Brückner, 1955). We examined, therefore, the effect of borate on the Winzler orcinol–sulphuric acid procedure, hoping to make the method more sensitive. However, our findings indicate that borate brings about no change in the maximum amount of colour produced by mannose, galactose or glucose. With glucose, however, it was found that the presence of borate caused an increase in the rate at which the colour was produced (Table 1).

Problems arising from the acid hydrolysis of protein-bound carbohydrate

Hydrolysis of a saccharide by acid to give the free sugars is known to cause losses of carbohydrate. This may be brought about by a direct degradation of the sugar (Newth, 1951; Rice & Fishbein, 1956), and by reaction of the sugar with amino acids (Gottschalk & Ada, 1956). Further, acid reversion of the sugar may play a part in giving low yields (Fischer, 1890), some of the products with mannose having been isolated by Jones & Nicholson (1958). The difficulty caused by acid reversion may be reduced by carrying out the hydrolysis in a solution where the sugar concentration is not greater than 2% (Jones & Nicholson, 1958).

The presence of carbohydrate increases the instability of tryptophan and the sulphur-containing amino acids on heating in acid (see Martin & Synge, 1945), although the stability of the remainder of the commonly occurring amino acids is unaffected by its presence (Dustin, Czajkowska, Moore & Bigwood, 1953). In view of this it was of interest to determine how the stability of mannose was affected by the presence of cysteine on heating in more dilute acid than is commonly employed in the hydrolysis of proteins. Such a comparison was carried out by heating a weighed quantity (about 50 mg.) of mannose in about 100 ml. of 2N-hydrochloric acid at 100° for 5 hr. The mannose remaining was then isolated, as the phenylhydrazone by the method described in the Experimental section, and weighed. The recovery of mannose from similar unheated solutions was about 90% for amounts of the hexose between 30 and 50 mg. By applying such corrections it was found that the loss of mannose on heating alone was about 23%, whereas in the presence of M-cysteine about 42% of the sugar was destroyed.

However, it is unrealistic to carry out a control determination of the loss of mannose on heating in acid with a synthetic mixture of the constituent amino acids of egg albumin. This would not approximate to the conditions prevailing at the beginning of the hydrolysis of the whole protein, when most of the constituent amino acids are combined in peptide linkage, and hence presumably unavailable for reaction with the free sugar which is liberated more readily than are the amino acids. By adding radioactive mannose at the beginning of the hydrolysis and estimating the radioactivity of the free mannose after various periods of time, it should be possible to estimate the maximum amount of the hexose liberated under the conditions of hydrolysis. Such a method should of course be specific for mannose.

The problem is still not solved with regard to mannose which might be combined with C-1 of glucosamine and which might not be liberated under the conditions of acid hydrolysis used (Moggridge & Neuberger, 1938; Gottschalk & Ada, 1956; Foster *et al.* 1957; Johansen *et al.* 1960). Although some glucosaminides do not give complete liberation of glucosamine (Moggridge & Neuberger, 1938; Foster *et al.* 1957), others are in fact almost completely hydrolysed under the conditions of acid hydrolysis employed (Table 2). It was previously reported that, under the conditions of acid hydro-

Table 2. *Liberation of glucosamine from some glucosaminides after hydrolysis under various conditions*

The temperature was 100°. Glucosamine was estimated by the method of Rondle & Morgan (1955).

		Time (hr.)				
	Concn. of HCl	0·5	1	2	3	4
D-Glucosaminide	(M)	Glucosamine liberated after hydrolysis (%)				
Phenyl tetra-*O*-acetyl-β-	1·2	82	101	102	—	101
	2·4	102	104	102	—	103
Phenyl *N*-propionyl-β-	1·2	76	89	95	96	95
	2·4	91	96	97	—	98
Methyl *N*-benzoxycarbonyl-β-*	1·2	53	73	80	81	82
	2·4	77	79	82	85	86
Methyl tetra-*O*-acetyl-α-	1·2	—	82	82	—	—
	2·4	—	85	—	—	—
Phenyl *N*-toluene-*p*-sulphonyl-β-	4·8	—	—	—	<2	—

* Foster, Horton & Stacey (1957) report 86% liberation of glucosamine in 1·16N-HCl.

lysis which we have employed in the present work, the greater part of the glucosamine is liberated (Johansen, Marshall & Neuberger, 1961). It seems likely therefore that most of the mannose will also be freed from glycosidic linkage.

Mannose content of the protein by isolation as the phenylhydrazone

The minimum possible content of mannose in the protein was measured by determining the quantity of mannose, isolated as the phenylhydrazone, after hydrolysing the protein for various times. The maximum amount of phenylhydrazone isolated corresponds to 1·77 %, as indicated in Fig. 2. These results were obtained by hydrolysing the protein (about 2 g.) and isolating the mannose as the phenylhydrazone in the usual way, assuming a 90 % recovery.

Mannose content of the protein by isotope dilution

The general method employed in these experiments was to hydrolyse egg albumin in 2N-hydrochloric acid in the presence of a weighed quantity of radioactive mannose. The mannose present after such hydrolysis was isolated as the (insoluble) phenylhydrazone and its radioactivity determined. From the number of counts obtained both from this isolated compound and also from the radioactive mannose used, the amount of mannose liberated from the protein was calculated. The D-mannose employed had an activity under the counting conditions used of 4898 counts/300 sec. (S.D. 43), and the phenylhydrazone prepared from it 3129 counts/300 sec. (S.D. 46). The ratio of counts in the mannose to that in its phenylhydrazone is 1·57, which may be compared with the ratio of the molecular weights of mannose phenylhydrazone to mannose of 1·50. The phenylhydrazone thus exhibits a slightly greater self-absorption than does mannose. In a typical experiment a mixture of 1·708 g. of egg albumin (corrected for ash and moisture) and 28·72 mg. of radioactive mannose was heated in 100 ml. of 2N-hydrochloric acid at 100° for 2 hr. The mannose phenylhydrazone produced from it had an activity of 1362 counts/300 sec., which became 1411 (S.D. 28) after one crystallization and 1430 (S.D. 13) after two crystallizations. These last two values correspond to 2·05 and 2·00 % respectively as the mannose content of egg albumin. By essentially similar methods, values of 1·94 % (1 hr. of hydrolysis), 2·02 % (3 hr.), 1·98 % (4 hr.) and 2·02 % (6 hr.) were obtained.

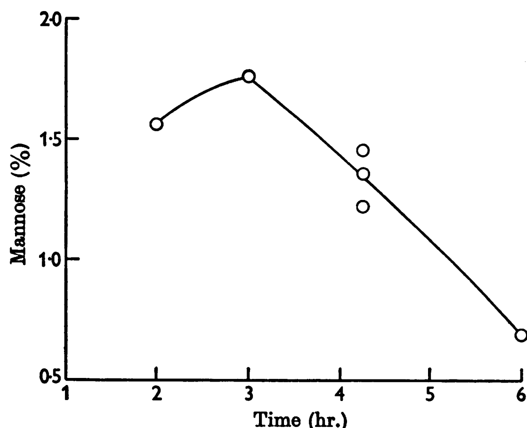

Fig. 2. Mannose isolated (as the phenylhydrazone) after hydrolysis of egg albumin in 2N-hydrochloric acid.

Thus the estimated content of mannose in the protein is about 2 ± 0·06 %. To assess whether this figure represents the true value, the reactions involved have to be considered: the liberation of the mannose from the protein (k_1) and the destruction of the sugar before (k_2) and after (k_3) liberation from glycosidic linkage, the k values representing rate constants. Neuberger (1938) and Nuenke & Cunningham (1961) have shown that the maximum liberation of reducing groups (which include mannose and glucosamine) from purified glycopeptides occurs in about 2 hr. in 1·5N-hydrochloric acid at 100°, 75 % of this maximum value being reached in 30 min.; in the present experiments 2N-hydrochloric acid was used. It is thus likely that under our conditions about half of the mannose was liberated in 10 min. We have no information of the rate (k_2) of destruction of bound mannose, but it is likely to be small compared with that of a sugar residue containing a free reducing group (k_3). The rate of the latter reaction, at least under the conditions obtaining 2 or 3 hr. after the beginning of the hydrolysis, can be approximately assessed from the results shown in Fig. 2. The half-life of the free mannose would appear to be about 120 min., the ratio of k_1/k_3 being about 12. It is not known whether the destructive effect of tryptophan and of the sulphur-containing amino acids is influenced by their being in peptide linkage, but it can be assumed that the rate of destruction of mannose (k_3) in the early stages of the hydrolysis is not greater than the approximate value suggested above, and may even be smaller for purely kinetic reasons.

In the isotope dilution experiments an amount y of radioactive mannose of molar radioactivity t is added to a solution of protein containing an amount x of covalently bound mannose. Let us suppose that after a period of hydrolysis of z min. all the bound mannose is liberated. Any sample of mannose phenylhydrazone isolated at z or more minutes after the beginning of the hydrolysis should have the same molar radioactivity s, and this value should give us x, as $x = s(x+y)/t$.

provided that the rates of destruction of free and combined mannose are the same or that the amount of free mannose destroyed before z min. can be neglected. Any destruction of mannose after z min. is of no significance. If neither of the conditions mentioned is met, the estimated value will be too high. It has been shown above that k_1 is much greater than k_3, and k_2 is certainly not greater than k_3, so that the amount of radioactive mannose destroyed, before complete mixing occurs with the mannose liberated from the protein, is likely to be insignificantly small.

General conclusion

The results which have been obtained by the orcinol–sulphuric acid procedure indicate a mannose content in egg albumin of about 2%. However, colorimetric methods might give erroneous results for reasons which have been discussed. With the isotope dilution method, the amount of mannose in the protein would also appear to be about 2%. The limitations in this method have been considered and the possibility was mentioned that the value thus obtained may be too high. The method used involving direct isolation is also subject to certain limitations. Recoveries of mannose might vary, depending on the other constituents of the medium from which the phenylhydrazone was precipitated. The maximum value obtained, however, by direct isolation was 1·77% and this must be the minimum content of mannose in the whole protein. Some of the sugar would be destroyed during hydrolysis so that the result would necessarily be lower than the true value. All these results together would point to the mannose content of the protein having a value between 1·77 and 2·0%. These values correspond to 4·5 and 5·0 residues respectively/45 000 g. of protein. It is likely therefore that egg albumin contains 5 residues of mannose/mol.

SUMMARY

1. Earlier results based on colorimetric techniques for the mannose content of hen's-egg albumin are critically discussed.

2. The mannose content of egg albumin by the orcinol–sulphuric acid method is about 2%. The effect of borate on this method has been examined.

3. A new procedure for the determination of mannose in a glycoprotein by radioisotope dilution has been developed and applied to egg albumin.

4. The maximum quantity of mannose liberated from the protein by 2N-hydrochloric acid by this method is about 2%. The possibility that this may not be the true content of mannose in the protein has been discussed.

5. The minimum mannose content of the protein by direct isolation as the phenylhydrazone has been shown to be 1·77%.

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Substances with Acetylcholine Activity in Normal Rat Brain

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Dale & Dudley (1929) isolated acetylcholine from ox spleen and Stedman & Stedman (1937) characterized it in ox brain, but the amount of acetylcholine isolated was considerably less than that estimated by biological assay to be present in the original extracts. Banister, Whittaker & Wijesundera (1953), on repeating Dale & Dudley's experiment, found that the acetylcholine activity of alcohol extracts of ox spleen could be separated into three components by paper chromatography in water-saturated butanol: 'S' component (R_F about 0·13), identified chromatographically as acetylcholine; 'M' component (R_F 0·21), identified as propionylcholine; and 'F' component (R_F 0·45–0·55) which was not identified. Whittaker (1956) suggested that this 'F' component was a mixture of acetylcholine, propionylcholine and an unknown zwitterion. Henschler (1957) concluded that the 'F' component was propionylcholine.

Beznak (1956) reported that rat hearts contained material which corresponded to Whittaker's 'F' component and concluded that it was acetylcholine which had formed a complex either with glass particles or with filter paper. Levy & Pierron (1959) observed that, in rat-brain homogenates treated with trichloroacetic acid, an acetylcholine–trichloroacetic acid complex was formed which greatly accelerated the mobility of the relatively slow-moving acetylcholine.

The acetylcholine content of brain is usually described as being present *in vivo* entirely in a bound form (Mann, Tennenbaum & Quastel, 1938; Elliott & Henderson, 1951). On homogenization with water, acetylcholine is easily separated into 'free' and 'bound' forms. The 'free' form is easily extracted during homogenization; 'bound' acetylcholine cannot be extracted by grinding with water or iso-osmotic sodium chloride, but can be extracted by treatment with fat solvents, organic or inorganic acids etc. (Mann *et al.* 1938; Elliott & Henderson, 1951; Crossland, 1951).

Other choline esters have been found in brain (Holtz & Schumann, 1954) and other tissues, and several authors have suggested the possibility that other substances acting like acetylcholine could be present (Banister *et al.* 1953; Hobbiger & Werner, 1948/1949; Hosein, 1960). Hosein (1960) found that the 'total' acetylcholine activity of brain extracts was due to several substances which, on chromatographic analysis, gave a pattern which resembled that obtained by Banister *et al.* (1953) with ox-spleen extracts.

In this paper, the provisional identification of several substances in rat-brain extracts which possessed acetylcholine activity is described.

METHODS

Extraction and analysis of acetylcholine from normal rat brain. Hooded rats (about 200 g.) were decapitated with a guillotine and the brains were rapidly removed, weighed and macerated in ice-cold aqueous eserine solution (10 μM) to make a 10% tissue suspension. The crude suspension obtained from 15 rats was homogenized in an Ace (all-glass) hand homogenizer, surrounded by crushed ice. Chloroform (5 ml.) was added, and the mixture was re-homogenized and kept at 10° for 2 hr. The suspension was then re-homogenized, and solid trichloroacetic acid was added to it to give a 5% solution. After 2 hr. at 10°, the mixture was centrifuged at 600g for 10 min. and a clear protein-free supernatant obtained. This solution was extracted suc-