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The Choline Acetyltransferase of Human Placenta

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1. Various methods for the extraction of choline acetyltransferase (acetyl-CoA–choline *O*-acetyltransferase, EC 2.3.1.6) from immature human placenta (18–28 weeks of gestation) are described. 2. The crude enzyme was found to be stable at $-18°$ and $+4°$ under a variety of conditions. 3. Purification methods, including ammonium sulphate fractionation, gel filtration on various grades of Sephadex and DEAE-Sephadex fractionation, have yielded a preparation of high specific activity.

The role of ACh* in the nervous system has prompted a considerable amount of research into the localization, isolation and properties of choline acetyltransferase, the enzyme responsible for ACh synthesis (for reviews see Hebb, 1957, 1963). These investigations have been concerned almost exclusively with the enzyme obtained from nervous tissue and, although it has been purified to some extent (Berman, Wilson & Nachmansohn, 1953; Ebashi & Kumagai, 1954; Burgen, Burke & Desbarats-Schonbaum, 1956; Mehrotra, 1961), the enzyme has never been isolated in the pure state. Possibly the most active non-mammalian source of choline acetyltransferase is the blowfly brain (Smallman, 1956), which, in crude form, has been calculated (Hebb, 1957) to have a specific activity 0·3 μmole of ACh produced/min./mg. of protein. Crude extracts of squid-head ganglion have a specific activity about 0·1 and Berman *et al.* (1953) achieved a tenfold purification of this material. The highest specific activity so far reported from mammalian tissue is about 0·05 (Ebashi & Kumagai, 1954), obtained by partial purification of the enzyme from guinea-pig brain.

The presence of ACh in the human placenta has been known for many years (Chang & Gaddum, 1933), and the presence of choline acetyltransferase has been confirmed by Comline (1946). The concentration of the enzyme reaches a maximum between 18 and 24 weeks of gestation, the specific activity being then about fourfold that of the most active mammalian nervous tissue (Hebb & Ratkovič, 1962). A study of the extraction, properties and purification of the placental enzyme and a comparison of its specificity with the enzyme of nervous origin was of obvious interest. Choline acetyltransferase of low specific activity isolated from full-term placenta has been described by Kato (1960).

A comparative specificity study of the partially purified enzyme has appeared (Hemsworth & Morris, 1964) and the enzyme's molecular weight has been estimated (Bull, Feinstein & Morris, 1964). Although the pure enzyme has not yet been isolated, a preparation of high specific activity has been obtained and the present paper describes the extraction and properties of crude choline acetyltransferase from 18–28-week human placentae, together with a comparative investigation of purification methods with particular reference to ammonium sulphate purification and gel filtration.

* Abbreviation: ACh, acetylcholine.

EXPERIMENTAL

Materials

CoA was prepared from baker's yeast by the method of Hebb (1955); for details see Nordenfelt (1963). The preparation contained 300–400 Lipmann units/ml. Phosphotransacetylase (acetyl-CoA–orthophosphate acetyltransferase, EC 2.3.1.8) was prepared from dried cells of *Clostridium kluyverii* (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) according to instructions given by the Sigma Chemical Co., St Louis, Mo., U.S.A. (see Nordenfelt, 1963). Acetyl phosphate was either obtained from Worthington Biochemical Corp. or synthesized by the method of Avison (1955). Thioglycollic acid (Hopkin and Williams Ltd., Chadwell Heath, Essex) was redistilled *in vacuo* under N_2 and stored at $-10°$. All other chemicals were either of AnalaR quality or of the highest purity commercially available.

Solutions. Solution A: 0·05M-cysteine, neutralized to pH 6·4 with N-KOH, in 0·154M-NaCl. Solution B: 0·05M-thioglycollic acid, neutralized to pH 6·4 with N-KOH, in 0·154M-NaCl. Solution C: 0·02M-sodium phosphate buffer,

pH6·6, containing 0·1M-NaCl, 5mM-EDTA and 5mM-thioglycollic acid, neutralized to pH6·6 with N-KOH.

Estimation of choline-acetyltransferase activity

Incubation. The incubation medium contained a coupled enzyme system, similar to that described by Hebb, Krnjević & Silver (1964), with phosphotransacetylase, CoA and acetyl phosphate as an enzymic source of acetyl-CoA. The complete medium contained, in final volume 0·9ml.: choline chloride, 11·5μmoles; KCl, 160μmoles; acetyl phosphate, 9·2μmoles; $MgCl_2$, 4·9μmoles; L-cysteine–HCl, 23μmoles, neutralized to pH6·4 with N-KOH; eserine sulphate, 0·13μmole; sodium phosphate buffer, pH6·9, 12·8μmoles; CoA, 20–30 units; phosphotransacetylase, 0·25mg.; the choline-acetyltransferase preparation (0·1ml.).

The medium was incubated at 39° for 10min. to allow formation of acetyl-CoA and the choline acetyltransferase was then added. After a further period at 39° (usually 1hr., but 10min. with very active preparations) the incubation was stopped by the addition of 6 drops of Universal Indicator (British Drug Houses Ltd.) and 0·3N-HCl (0·5ml.). The acidified incubation mixture was quantitatively transferred to a 50ml. conical flask with 3×2ml. portions of frog Ringer solution and brought to 100° to destroy excess of acetyl phosphate. After cooling to room temperature, 0·5% (w/v) Na_2HPO_4 (1ml.) was added and the volume was adjusted to 20 or 40ml. with frog Ringer solution. The final pH was approximately pH4. The activity of the acetyl-CoA-generating system was checked at each incubation with standard rabbit-brain choline acetyltransferase prepared as an acetone-dried powder extract in solution A. The incubation medium (minus the choline acetyltransferase) could, for convenience, be stored at −18° for periods up to 4 weeks without loss of activity.

The range of enzyme concentrations over which the substrates used above produced a linear rate of ACh synthesis was determined by incubation of various dilutions of a highly active preparation. For subsequent estimation of unknown enzyme activity, preparations were diluted so that the rate of synthesis of ACh was within the linear range.

Assay of acetylcholine. The incubation mixture, prepared as described above, was assayed on the eserinized frog rectus abdominis muscle according to the procedure of Feldberg (1950), with the same precautions to control the effect of sensitizing substances on the rectus muscle. In addition (Feldberg, 1950), it was sometimes convenient to use a heat-treated inactivated enzyme for control incubations. All determinations were made in duplicate. Overall errors of estimation were computed to be within ±10% but duplicates rarely differed by more than ±5%.

Enzyme units. One unit of enzyme activity was defined as 1μmole of ACh synthesized/min. at 39° (m-units were used when appropriate). Specific activity was expressed as units/g. of placental tissue, units/g. of acetone-dried powder, units/ml. of extract or units/mg. of protein.

Protein. The protein content in column eluates was estimated by absorption at 280mμ (1mm. cells, Unicam SP.500 spectrophotometer). For measurements of specific activity in terms of protein concentration the ratio of the extinctions at 280 and 260mμ (Warburg & Christian, 1941) was measured or protein N was determined by the micro-Kjeldahl procedure.

Extraction of tissue

Placentae. Immature human placentae of 18–28 weeks gestation, produced either by spontaneous abortion or by clinical termination of pregnancy, were obtained as fresh as possible from the Maternity Hospital, Mill Road, Cambridge. If extensive blood clotting had not occurred the placentae were perfused with cold 0·154M-NaCl through the umbilical cord vessels until the tissue was cleared of as much blood as possible. The cord was removed and the adjacent chorionic and amnionic membranes were gently teased away from the required pulpy tissue. The tissue was usually processed immediately but could be stored at 0° for several days without appreciable loss of activity.

Extraction procedures. On a small scale, acetone-dried powders or homogenates were prepared. Acetone-dried powders were prepared as described by Hemsworth & Morris (1964) for brain tissue. The powders were extracted at a concentration of 100 or 50mg. of powder/ml. with cold 0·154M-NaCl either alone or in combination with other reagents added to study their effect on enzyme activity. The extracts were centrifuged (International type SB, rotor 225, 1380*g* for 10min.) and the supernatants were either stored at −18° or examined immediately for enzyme activity.

Homogenates were prepared from chopped tissue with cold solution B at a concentration of 100mg. of tissue/ml. in a hand-operated ball-type glass homogenizer. The homogenate was centrifuged as described above before estimation.

For extraction of whole placentae, where the weight of tissue was 100g. or more, the tissue was chopped and then homogenized at 4° in a Townson and Mercer top-drive macerator for 1min. with the aid of a sufficient volume (usually about 100ml.) of cold solution B to form a free-flowing slurry. The latter was then freeze-dried in the cold room (4°) and the resulting powder was extracted with solution B and centrifuged (Servall angle-head centrifuge, 12000*g* for 15min.). The centrifugate was re-extracted with sufficient solution B to give a final concentration 75mg. of powder/ml. and centrifuged again. The combined supernatants were stored at −18° immediately, or, in the later part of this investigation, subjected to fractionation with $(NH_4)_2SO_4$ between 15 and 30% (w/v) before storage. This method of extraction gave fairly reproducible activities from a large number of placentae.

Purification of enzyme

Ammonium sulphate fractionation. Preliminary experiments showed that at pH values much below 5 considerable losses of enzyme activity occurred and therefore salt fractionation below this pH was not studied. Extracts of freeze-dried tissue in solution B were adjusted to pH5, 6 and 7 respectively, and were progressively fractionated with $(NH_4)_2SO_4$ in 5% (w/v) steps between 15 and 30%. The pH was readjusted where necessary with 0·5N-acetic acid or 0·5N-KOH. Precipitation at each step was allowed to proceed for 1hr. at 4° before centrifugation (12000*g* for 15min.). The supernatants at each stage were sampled before further addition of $(NH_4)_2SO_4$, the precipitates resuspended in solution B and centrifuged; the insoluble material was discarded. After dialysis overnight at 4° against 3×2l. changes of solution B the samples were

recentrifuged and the supernatants then assayed for choline-acetyltransferase activity and protein content.

The effect of refractionation of enzyme preparations obtained by an initial 20–30% (w/v) $(NH_4)_2SO_4$ step at pH5 was also studied. The precipitates and supernatants were treated as described above.

Gel filtration. The various grades of Sephadex were allowed to swell in 0·154M-NaCl for periods from 1 day for Sephadex G-25 to 1 week for the most porous gel, Sephadex G-200. During this time 'fines' were removed by decantation. The gel beds were formed by allowing a slurry of gel particles to percolate under gravity into vertical glass chromatography columns fitted with porosity 1 sintered-glass disks and previously filled with 0·154M-NaCl. After 2–3cm. of gel had settled the excess of liquid was allowed to percolate through the lengthening gel bed. When the required bed height was obtained 0·154M-NaCl was percolated continuously through the column in the cold room. Before an experiment the columns were equilibrated with the eluting solvent (either solution B or solution C). Percolation of high-molecular-weight blue-dyed dextran (Pharmacia) through the columns prepared in this way produced compact and even bands and enabled the void volumes to be determined. For Sephadex G-75 gel filtrations two columns of dimensions 45cm. × 1·7cm. and 80cm. × 1·7cm. were employed to investigate the effect of column length on the efficiency of separation. Subsequently columns of dimensions 102cm. × 4cm. were used for Sephadex G-100 and G-200 filtration experiments.

The flow rates obtained with Sephadex G-75 and G-100, employing a small hydrostatic head, were about 13 and 3·2ml./hr./cm.^{2} respectively. It was found necessary, with Sephadex G-200 columns, to form a 3cm. layer of coarse Sephadex G-25 above the sintered disk before preparing the main Sephadex G-200 bed. This technique prevented the sinter from being progressively blocked by Sephadex G-200 gel particles and a flow rate, approximately constant over several experiments, of 1·6ml./hr./cm.^{2} was obtained.

Enzyme solutions were applied directly to the drained surface of Sephadex G-75 gel columns. With the more porous gels a 3cm. layer of Sephadex G-25 was formed on the top of the column and the enzyme preparation was carefully layered on to the gel surface under the buffer so as to give a very compact starting band. Where necessary, the density of the enzyme solution was adjusted by the addition of sucrose. Column eluates were collected in a Locarte fraction-collector with a constant-volume collecting device. Samples of the fractions were analysed for their protein and choline-acetyltransferase content and the remainder of the fractions were stored at temperatures below 0° (−10° or −18°). The active material was then thawed to 0° and concentrated.

Concentration of column eluates. A number of techniques including freeze-drying, pervaporation, addition of dry Sephadex G-25, Carbowax, $(NH_4)_2SO_4$ precipitation and ultrafiltration were investigated. The best methods were found to be ultrafiltration at 0° for enzyme preparations of intermediate specific activity and $(NH_4)_2SO_4$ precipitation (0–30%, w/v) at 4° for material of high specific activity. Any $(NH_4)_2SO_4$ which remained in the redissolved precipitate was removed by passage of the solution through a column (15cm. × 1cm.) of Sephadex G-25 equilibrated with solution C. The enzyme preparation was then stored at −70° or used immediately for further purification.

DEAE-Sephadex. This medium was used to purify further the enzyme obtained from Sephadex G-200 columns. The gel powder was allowed to swell in 0·1M-NaCl for 4 days, during which time the 'fines' were removed. The suspension was titrated to pH6·6 with 0·2M-sodium phosphate buffer, pH6·6, and then equilibrated with solution C. The enzyme preparation (usually 5ml.) from the Sephadex G-200 step, equilibrated with solution C, was applied to a column (15cm. × 1cm.) of the DEAE-Sephadex and eluted with the same solution. Fractions (10ml.) were collected and analysed as before. Active fractions were concentrated by $(NH_4)_2SO_4$ precipitation and stored at −70°.

Immunoelectrophoresis. A modification of the technique of Grabar & Burtin (1960) was employed, 1·5% (w/v) agar gel containing 0·1% (w/v) of sodium azide and veronal buffer, pH8·6 and I 0·025, being used. The buffer vessels contained the same buffer but with I 0·05. The electrophoresis was carried out for 2hr. at 7v/cm. Longitudinal slots were then cut along the migration axis and anti-human serum was placed in them. After diffusion overnight at room temperature the plates were examined for precipitin lines caused by the presence of serum proteins in the choline-acetyltransferase preparation. This technique was used in the later stages of the purification to follow the removal of contaminating serum proteins.

RESULTS

Incubation and assay. The incubation medium used in these experiments was designed originally for the determination of the small amounts of choline acetyltransferase from nervous tissue, but it will be seen from Fig. 1 that in the present

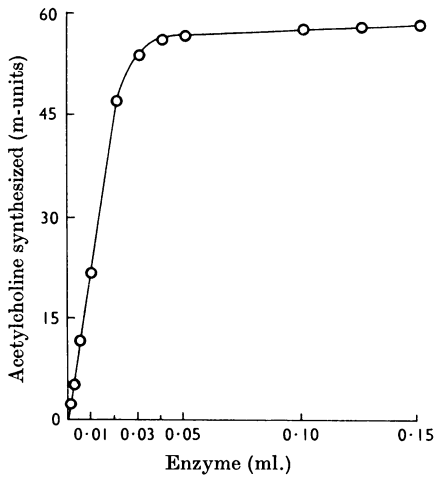

Fig. 1. Production of ACh by standard incubation medium. Various dilutions of a placental extract, partially purified by fractionation between 15 and 30% (w/v) $(NH_4)_2SO_4$, were incubated for 1hr. at 39°. The ACh produced was determined by bioassay. For details see the Experimental section.

experiments a linear relation between enzyme concentration and ACh synthesis was obtained up to 46 m-units of ACh/min. At intermediate values of specific activity the incubation medium was capable of a substantially linear ACh production for incubation periods up to 60 min. but for preparations of specific activity higher than 600 mμmoles/min./mg. of protein it was both necessary and convenient to incubate for 10 min. only.

Extraction procedures. The enzyme in homogenates of placental tissue had a higher specific activity than did extracts of acetone-dried powders, which were also more variable in their activity. Thus the specific activities of homogenates of two 25-week placentae were 1·25 and 1·07 units/g. of tissue, whereas the corresponding acetone-dried-powder activities were 0·80 and 0·37 unit/g. of tissue respectively. Control experiments showed that freeze-drying of homogenized placental tissue did not lead to loss of activity.

Table 1. *Stability of crude choline acetyltransferase at* −18°

Placental tissue, acetone-dried powders and various extracts of acetone-dried powders were stored at −18° over a period of 2 months and examined periodically for enzyme activity. Tissue was stored in stoppered test tubes and acetone-dried powders were stored in evacuated Thunberg tubes containing P_2O_5. The acetone-dried-powder extracts (50 mg. of powder/ml.) were prepared by extraction with cold 0·154 M-NaCl alone or in the presence of 0·05 M-cysteine, pH 6·4, or 0·05 M-choline. For estimation of enzyme activity, tissue samples were homogenized in cysteine in NaCl (100 mg. of tissue/ml.) and the acetone-dried powders were extracted with cysteine in NaCl (50 mg. of tissue/ml.). For enzyme estimation tissue homogenates were diluted 1:1 (v/v) with 0·154 M-NaCl; all other preparations were diluted 1:10 (v/v). For details of enzyme estimation see the text. Enzyme activity: 1 unit = 1 μmole of ACh synthesized/min. at 39°.

	Enzyme activity (units/g. of tissue)				
	Zero time	2 weeks	4 weeks	6 weeks	8 weeks
Tissue	1·07	1·05	1·03	1·31	1·18
Acetone-dried powder	0·36	0·37	0·40	0·38	0·25
NaCl extract	0·34	0·40	0·38	0·34	0·34
Cysteine–NaCl extract	0·43	0·48	0·43	0·39	0·43
Choline–NaCl extract	0·34	0·44	0·39	0·39	0·39

Table 2. *Effect of various reagents on the choline-acetyltransferase activity of placental acetone-dried-powder extracts at* 4°

Various extracts of placental acetone-dried powder (50 mg. of powder/ml. of extractant) were prepared at 4° with 0·154 M-NaCl alone, with 0·05 M-EDTA in NaCl and with 0·05 M-EDTA in NaCl containing 50 or 100 mM-cysteine, -thioglycollate, -2-mercaptoethanol, -2,3-dimercaptopropan-1-ol or -choline respectively. The solutions were adjusted to pH 6·4 before the acetone-dried powders were extracted. Samples (0·1 ml.) were withdrawn at various times, diluted 1:10 (v/v) with 0·154 M-NaCl and the choline-acetyltransferase activity was determined as described in the text. 1 m-unit = 1 mμmole of ACh synthesized/min. at 39°. The molarities under the heading 'Concn.' in the Table refer to the final concentration of the reagents added to the EDTA in NaCl solution.

		Enzyme activity				
		Initial (m-units/ml.)	Residual (% of initial)			
Extraction medium	Concn. (mM)		8 days	14 days	21 days	28 days
NaCl	zero	70	95	110	89	95
EDTA–NaCl	zero	81	82	67	35	27
Cysteine–EDTA–NaCl	50	81	104	104	123	111
	100	81	109	109	96	96
Thioglycollate–EDTA–NaCl	50	81	114	109	122	96
	100	81	110	107	105	96
2-Mercaptoethanol–EDTA–NaCl	50	88	100	92	83	71
	100	88	100	96	45	27
2,3-Dimercaptopropanol–EDTA–NaCl	50	81	91	50	50	30
	100	81	82	25	26	33
Choline–EDTA–NaCl	50	81	100	87	85	64
	100	81	109	82	59	61

Stability of stored crude enzyme preparations. Tables 1 and 2 show that the crude enzyme, stored under a variety of conditions at −18° and +4°, was generally very stable but the presence of 0·1M-mercaptoethanol or 2,3-dimercaptopropanol at both concentrations employed caused serious activity losses. The presence of 0·05M-EDTA caused a considerable loss of activity in the absence of any thiol-protecting agent (Table 2) but this loss was partially prevented by choline.

Stability at 39°. The effects of incubation of the unpurified enzyme at 39° for 2hr. under various conditions are shown in Table 3. This experiment was designed to accelerate inactivation of the enzyme and it will be seen that in the absence of cysteine and EDTA all activity was lost. These reagents present separately provided some protection but nearly 70% of the initial activity was preserved when both were present.

Fractionation with ammonium sulphate. The results are shown in Fig. 2. At all three pH values the highest specific activities were obtained in the protein which precipitated between 20 and 25% (w/v) ammonium sulphate. The most active material, however, was obtained at pH5·0 and the least active at pH7·0. Furthermore, the overall recovery at pH5·0 was 70% whereas at pH6·0 and pH7·0 the recoveries were 57% and 50% respectively. At pH5·0 relatively more enzyme was precipitated at 20% than at 30% whereas the reverse was found at the higher pH values.

The effect of refractionation of enzyme initially precipitated between 20 and 30% (w/v) ammonium sulphate at pH5·0 is shown in Table 4. In the small-scale experiment (*a*), rather more than a 20-fold increase of specific activity was obtained in the 26–

Table 3. *Effect of EDTA on choline-acetyltransferase activity at* 39°

Acetone-dried powders of placental tissue were extracted with 0·05M-cysteine, pH6·5, in 0·154M-NaCl or in 0·154M-NaCl alone, both at a concentration of 50mg. of powder/ml. After dilution (1:1, v/v) with 0·154M-NaCl or 0·10M-EDTA, pH6·5, the extracts were incubated at 39° for 2hr.; control samples were kept at 0° during this period. For estimation of choline-acetyltransferase activity 0·1ml. samples were incubated with the substrate medium as described in the text. 1m-unit = 1mμmole of ACh synthesized/min. at 39°.

Extract	Choline-acetyltransferase activity (m-units/ml. of extract)	
	Control (0°)	At 39°
NaCl	18·7	0
EDTA–NaCl	16·0	5·17
Cysteine–NaCl	46·0	5·17
Cysteine–EDTA–NaCl	38·4	26·2

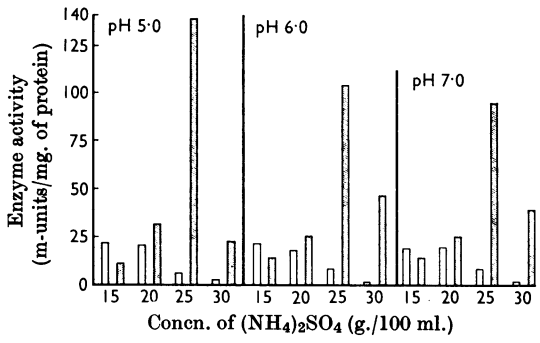

Fig. 2. Effect of pH on $(NH_4)_2SO_4$ fractionation. Extracts of freeze-dried tissue in solution B (940ml., 17mg. of protein/ml.) were adjusted to pH5, 6 and 7 respectively and fractionated with $(NH_4)_2SO_4$ in 5% steps from 15 to 30% (w/v) at 4°. The pH was readjusted where necessary with 0·5N-acetic acid or 0·5N-KOH. Precipitation at each step was allowed to proceed for 1hr. before centrifugation (12000*g* for 15min.). The supernatants at each stage were sampled before further addition of $(NH_4)_2SO_4$, and the precipitates resuspended in solution B and centrifuged; the insoluble material was discarded. All solutions were dialysed overnight at 4° against three changes of solution B, recentrifuged and the supernatants then assayed for choline-acetyltransferase activity (see the text) and protein N (micro-Kjeldahl). Enzyme activity: 1m-unit equals 1mμmole of ACh synthesized/min. at 39°. Specific activities: □, supernatants; ■, resuspended precipitates.

Table 4. *Refractionation of choline acetyltransferase with ammonium sulphate at* pH5·0

Two enzyme preparations [(*a*) 25ml., specific activity 130m-units/mg. of protein, 3·8mg. of protein/ml.; (*b*) 105ml., specific activity 152m-units/mg. of protein, 4·5mg. of protein/ml.] obtained from an initial precipitation between 20 and 30% (w/v) $(NH_4)_2SO_4$ at pH5·0 were refractionated in 2% (w/v) steps at pH5·0. The precipitates were extracted with 0·05M-thioglycollate, pH6·5, in 0·154M-NaCl, dialysed at 4° against three changes of the same solution and their choline-acetyltransferase activity and protein content were determined (see the text). Enzyme activity: 1m-unit = 1mμmole of ACh synthesized/min. at 39°.

$(NH_4)_2SO_4$ fraction (%, w/v)	Choline-acetyltransferase specific activity (m-units/mg. of protein)	
	(*a*)	(*b*)
0–20	—	83
0–22	150	—
20–22	—	277
22–24	100	394
24–26	351	347
26–28	390	70
28–30	18	8

28% fraction, whereas in the larger-scale experiment (*b*), a similar degree of purification was found in the 22–24% fraction. Enzyme preparations with a specific activity about 300 m-units/mg. of protein, obtained by the two-stage ammonium sulphate procedure, were very unstable and sometimes lost up to 50% of their activity in 2 weeks at −18°. Subsequent investigation indicated that preparations subjected to the first 15–30% (w/v) ammonium sulphate stage only were much more stable and could be stored at −18° without significant loss for several months. As a routine, placentae were therefore processed only to this stage before subjection to gel filtration.

Gel filtration

Fractionation on Sephadex G-75. The protein, enzyme-activity and specific-activity elution patterns resulting from (*a*), fractionation on a 45 cm. × 1·7 cm. column, and (*b*), refractionation on an 80 cm. × 1·7 cm. column, are shown in Fig. 3. Activity was associated with the trailing edge of the excluded protein in Fig. 3(*a*) and the specific-activity peak was displaced slightly farther to the right with a maximum of 79 m-units/mg. of protein, representing a purification factor of 2·75. The active fractions were pooled, concentrated by freeze-drying and the powder, dissolved in 2 ml. of solution B, was refractionated on the larger column. Although the displacement of activity from excluded protein was not greatly improved a further increase of maximum specific activity to 173 m-units/mg. of protein was obtained, giving an overall purification factor of 6·1 for the two columns.

Fractionation on Sephadex G-100. In a preliminary experiment, with a small column (45 cm. × 1·7 cm.), the choline-acetyltransferase activity was eluted once again in the trailing edge of the excluded protein; the relevant portion of the elution diagram is shown in Fig. 4(*a*). The preparation applied to this column had a specific activity 0·23 unit/mg. of protein, obtained by Sephadex G-75 fractionation. The most active fractions from this column lost 50% of their activity in 4 days at −10° but nevertheless a maximum specific activity 0·67 unit/mg. of protein was found in tube 17 subsequent to this loss. A typical elution diagram obtained from experiments with the larger column (102 cm. × 4 cm.) is shown in Fig. 4(*b*). A much clearer separation of activity from the excluded protein was found, illustrating the fact that column length is a very important factor in gel-filtration resolving power.

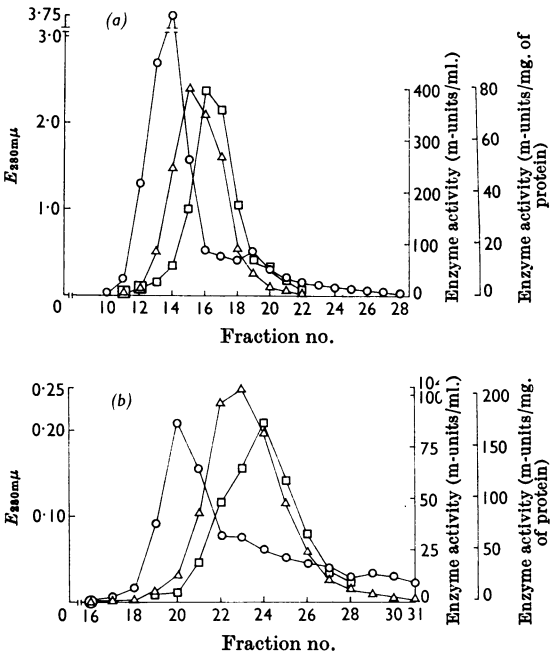

Fig. 3. Fractionation of choline acetyltransferase on Sephadex G-75. (*a*) Elution pattern given by $(NH_4)_2SO_4$-fractionated enzyme (2 ml., specific activity 28·4 m-units/mg. of protein). Column dimensions: 45 cm. × 1·7 cm. Fraction volume 5 ml. (*b*) Effect of refractionation of the concentrated (freeze-dried) activity (5 ml.) on a longer column (80 cm. × 1·7 cm.). For details of determination of enzyme activity see the text. Eluent: solution B. Fraction volume 5 ml. ○, E_{280}, 1 mm. cells; △, choline acetyltransferase (m-units/ml.); □, choline acetyltransferase (m-units/mg. of protein). 1 unit equals 1 μmole of ACh synthesized/min. at 39°.

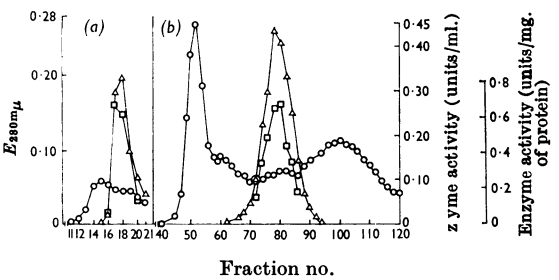

Fig. 4. Fractionation of choline acetyltransferase on Sephadex G-100. (*a*): Elution pattern of Sephadex G-75-purified material (5 ml.; 0·23 unit/mg. of protein). Column dimensions: 45 cm. × 1·7 cm. (*b*): Fractionation of $(NH_4)_2SO_4$-purified material (20 ml., 68·4 m-units/mg. of protein) on a large (102 cm. × 4 cm.) column. Eluent: solution B (see the text). Fraction volume 10 ml. ○, E_{280}, 1 mm. cells; △, choline acetyltransferase (m-units/ml.); □, choline acetyltransferase (m-units/mg. of protein). 1 unit equals 1 μmole of ACh synthesized/min. at 39°.

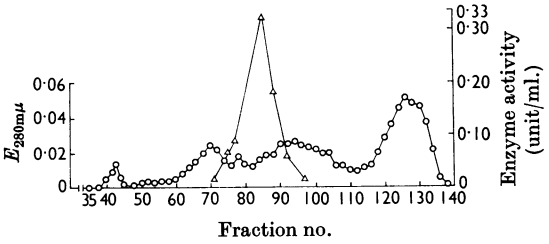

Fig. 5. Fractionation of choline acetyltransferase on Sephadex G-200. Elution pattern obtained from $(NH_4)_2SO_4$-purified material (24ml., 158m-units/mg. of protein). Column dimensions: 102cm. × 4cm. Eluent: solution C (see the text). Fraction volume 10ml. For determination of enzyme activity see the text. ○, E_{280}, 1mm. cells; △, choline acetyltransferase (unit/ml.). 1 unit equals 1μmole of ACh synthesized/min. at 39°.

The bulked activity (tubes 73–87) was 0·35 unit/mg. of protein and concentration of this material by precipitation with ammonium sulphate gave a further increase to 0·5. The recovery of activity was 70% and the concentrated material lost only 10% of its activity in 2 weeks at −18°. It will be noticed that material of similar specific activity prepared by the two-stage ammonium sulphate procedure was considerably less stable.

Fractionation on Sephadex G-200. The volume of enzyme preparation and the way in which it was applied to the column were critical. Volumes greater than 25ml. produced less separation of the major protein bands and inferior results were obtained if the applied enzyme layer was distorted or caused to diffuse during the layering procedure. The elution diagram of a satisfactory experiment is shown in Fig. 5. In this preparation there was very little protein of high molecular weight (> 200000) and the enzyme was eluted between two of the three main protein bands. The total activity eluted was about 90% and the combined peak tubes (74–90) contained 60% of the total. The ammonium sulphate-concentrated material had a specific activity 1·28 units/mg. of protein, representing an eightfold purification and an overall purification from crude extract of 48-fold. Similar results were obtained in several experiments; the highest specific activity achieved was 1·55 units/mg. of protein. In these experiments the enzyme activity was determined after 10min. incubation only.

Fractionation on DEAE-Sephadex. Immunoelectrophoresis and analytical ultracentrifugation of the active material derived from the Sephadex G-200 step revealed that the main contaminating proteins of serum origin were albumin and 7s globulin. Further experiments showed that human serum albumin was retained on columns of DEAE-Sephadex equilibrated with solution C, whereas choline acetyltransferase was not. A further two-fold purification resulted when Sephadex G-200-fractionated enzyme was treated in this way. Thus a specific activity 2·34 units/mg. of protein was found in the first 10ml. fraction eluted from a column to which material with specific activity 1·28 was applied. Recoveries were about 50–70% and subsequent immunoelectrophoretic analysis revealed that the contaminating albumin had been removed. The presence of non-serum protein in the $\alpha\beta$-globulin region (probably containing the choline acetyltransferase) was revealed after plates, subjected to electrophoresis but not immunodiffusion, were stained with Amido Black.

DISCUSSION

The purification of choline acetyltransferase was undertaken to facilitate the study of the enzyme present in nervous tissue and the choice of the human placenta, a non-innervated organ, as source material may seem surprising. However, the placental and rabbit-brain enzymes, for instance, have similar molecular weights (Bull *et al.* 1964) and have a similar specificity both with regard to *N*-alkyl analogues of choline (Hemsworth & Morris, 1964) and to their capacity to synthesize propionyl- and butyryl-choline (D. Morris, unpublished work). Further, the high concentration of the enzyme in the immature placenta and the ease with which it is released from the subcellular structure (Hebb & Ratkovič, 1962) make the placenta suitable for the present purpose.

It will have been noticed that tissue homogenates (Table 1) had a much higher activity than did extracts of acetone-dried powders. This is believed to be due to the ease with which the enzyme is released from the tissue; activity is probably lost in the first acetone wash. Brain choline acetyltransferase, which is only released from its binding sites with some difficulty, does not exhibit this difference of activity between homogenates and acetone-dried powders.

The crude enzyme, although very stable under most conditions, lost activity in the presence of some thiol-protecting agents but not others (Table 2). It is conceivable that dimercaptopropanol and mercaptoethanol caused fission of disulphide bonds necessary for the maintenance of the integrity of the enzyme. Mercaptoethanol also caused inhibition when it was substituted for cysteine in the incubation medium.

The effects of EDTA on enzyme activity (Tables 2 and 3) suggest that one or more thiol groups are present in the active enzyme. At low temperatures (0° and 4°) the inhibitory effect of EDTA can be

attributed to the removal, by this reagent, of protective metal ions. At 39°, where inactivation may be due, partly at least, to oxidation of essential thiol groups, removal of cations by EDTA would prevent metal-ion catalysis of this oxidative process both in the enzyme itself and in the added cysteine. The overall effect would be retention of activity. The presence of essential thiol groups in the choline acetyltransferase of the squid-head ganglion has been postulated by Reisberg (1957).

The higher specific activities obtained by refractionation with ammonium sulphate at pH 5·0 after the initial 15–30% ammonium sulphate step were not very reproducible and the preparations were very unstable compared with material of similar activity produced by Sephadex G-100 gel filtration. In the experiments illustrated in Table 4, the starting protein concentrations were very similar, namely (*a*) 3·8mg. of protein/ml., (*b*) 4·5mg. of protein/ml., but the highest specific activity occurred at different concentrations of ammonium sulphate. The small difference in protein concentration may, however, be sufficient to account for the results (Dixon & Webb, 1964). The refractionation step is included in this paper as a useful and rapid procedure when preparations of intermediate specific activity are required for immediate use.

It is evident that columns of Sephadex G-75 can equilibrate with molecules of molecular weight greater than the 40000 originally claimed by the manufacturers. A provisional value for the molecular weight of placental choline acetyltransferase, determined by sedimentation only, of 59000 has been found (Bull *et al.* 1964). Fig. 3 shows that the enzyme is not excluded from the inside of Sephadex G-75 gel particles. The greatly improved separation found with Sephadex G-200 illustrates the fact that equilibration of molecules between the internal and external volumes of the gels is by no means an 'all-or-none' phenomenon. $S_{20,w}$ of the enzyme is 4·6s, a value very similar to that of albumin. However, the elution volume from Sephadex G-200 columns is appreciably lower than that of albumin and this observation, when considered in relation to the sedimentation data of the two proteins, suggests that the placental enzyme may have a higher molecular weight and a markedly greater degree of asymmetry than albumin. The provisional molecular weight of 59000 is therefore likely to be too low because of the asymmetry of the enzyme. It is of interest that the molecular weight of rabbit-brain choline acetyltransferase, calculated from sedimentation and diffusion data, is 67000 (Bull *et al.* 1964). The frictional ratio of this enzyme is calculated to be 1·17, a value less than that of albumin. It is possible therefore that the choline acetyltransferase of placenta and rabbit brain may differ appreciably in shape. There have been reports that with proteins of different frictional ratios the elution volumes obtained with Sephadex G-200 correlate more closely with diffusion coefficients than with molecular weight (Laurent & Killander, 1964; Siegel & Monty, 1965).

The major problems associated with the purification of placental choline acetyltransferase are the presence of large amounts of serum proteins and the instability of the partially purified enzyme. Immunoelectrophoresis has greatly facilitated the design of procedures for the separation of the enzyme from serum proteins and most of the latter have now been removed. The different degrees of stability found with enzyme of similar specific activity but prepared by different procedures suggest that other proteins present may affect stability. No protection was afforded by the addition of diisopropyl phosphorofluoridate (0·1mM).

In conclusion, a sequential procedure involving ammonium sulphate precipitation, Sephadex G-200 gel filtration and ion-exchange chromatography on DEAE-Sephadex has resulted in 80–100-fold purification of human placental choline acetyltransferase. Further studies are in progress which, it is hoped, will lead to the isolation of the pure enzyme.

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