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Substances with Acetylcholine Activity in Normal Rat Brain

By E. A. HOSEIN, P. PROULX AND ROUSHAN ARA

Department of Biochemistry, McGill University, Montreal, Quebec, Canada

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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 μ M) 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-

cessively with equal volumes of anhydrous ether until the pH of the aqueous layer was 6.5–6.8. The solution was concentrated *in vacuo* at 40° to 5 ml. and the pH adjusted to 8.5–9.0 by the addition of ammonia solution. Any precipitate formed was removed by centrifuging. Reinecke salt (2 ml. of a 5% solution in methanol) was added to the clear supernatant. After 2 hr., the precipitate formed was collected by centrifuging, washed with propan-1-ol and dissolved in a small volume of acetone–water (1:1, v/v) mixture (Bregoff, Roberts & Delwiche, 1953). The reineckate was then removed from the solution as described by Banister *et al.* (1953). The reineckate-free solution was evaporated under vacuum at 40° and the solid residue extracted twice with 5 ml. portions of methanol. These extracts were pooled, evaporated to 0.5 ml. under a stream of nitrogen and applied as a narrow band on strips of Whatman no. 1 filter paper, 6 in. × 20 in. The chromatograms were developed with water-saturated butan-1-ol for 17 hr. at 22°. The positions of the bands were detected on a test strip of the paper with 1% iodine in ethanol. The indicated regions of the paper were cut up and shaken with methanol to elute out the iodine-sensitive compounds. The eluates of the appropriate bands from the chromatograms, prepared from 12 such batches of brain extracts (each from 15 rats), were pooled and evaporated to dryness under nitrogen. The dry residues assumed to contain acetylcholine and propionylcholine were treated with 1–2 drops of 1% hydrochloric acid and 1 ml. of 10% tetrachloroaurate solution and kept for several hours in the refrigerator. The residues assumed to contain butyrylcholine and the CoA esters of betaine derivatives were first hydrolysed on a boiling-water bath for 10 min. with 1 ml. of 0.1N-hydrochloric acid, cooled and evaporated to dryness under a stream of nitrogen. The residues were extracted with methanol and the methanol extracts were again evaporated to dryness. Each dried residue was treated with hydrochloric acid and tetrachloroaurate as above except that the solution was heated for 10 min. on a boiling-water bath before storing it in the refrigerator (Carter, Bhattacharyya, Weidman & Fraenkel, 1952).

The tetrachloroaurates were collected by centrifuging, washed several times with 1% hydrochloric acid, or as described in the text, and dried overnight *in vacuo* over anhydrous calcium chloride. Melting points were determined on a Koffler apparatus.

Detection of acyl-CoA esters. The following methods were used to detect the presence of acyl-CoA esters in the unhydrolysed eluate: the hydroxamic reaction of Lipmann & Tuttle (1945); the bromophenol blue-complex formation described by Mitchell & Clark (1952) and Nowell & Wilson (1961); chromatography with propanol–formic acid–water (8:1:1, by vol.) (Nowell & Wilson, 1961); the measurement of the biological activity with a preparation of frog rectus abdominis, described below; and the measurement of the ultraviolet absorption at 232 m μ (Stadtman, 1952; Lynen, 1953; Mahler, Wakil & Bock, 1953) before and after alkaline hydrolysis. The acid hydrolysate was neutralized with 0.1N-sodium hydroxide and concentrated under nitrogen. It was examined quantitatively for ribose (Tauber, 1937) and for phosphate (Vogel, 1954). β -Alanine was determined by two-dimensional paper chromatography on 0.1 ml. of the concentrated hydrolysate with propanol–water (7:3, v/v) and phenol–water (3:1, w/v) as solvents and ninhydrin as detecting reagent (Hosein &

Smart, 1960). β -Mercaptoethylamine was detected on a similar chromatogram with sodium nitroprusside and ammonia solution (Hawk, Oser & Summerson, 1954). Adenine was detected by its absorption maximum at 260 m μ with the Beckman DK spectrophotometer (Kalkar, 1947).

Biosynthesis of CoA esters of γ -butyrobetaine and carnitine. These CoA esters were synthesized and characterized as described by Mahler *et al.* (1953), with γ -butyrobetaine or carnitine as the fatty acid derivatives. Purified fatty acid-activating enzyme from rabbit liver was incubated for 1 hr. at 37° with the betaine derivative (20 μ M), CoA (0.7 μ M), ATP (4 μ M), 100 μ M-glycylglycine buffer, pH 8, and Mg²⁺ ions (20 μ M). Control flasks contained no betaine derivative. Materials present in both the control and experimental flasks were separated by paper chromatography with water-saturated butan-1-ol and eluates from each band were tested for a positive hydroxamic reaction and adsorption at 232 m μ as above. The CoA esters used in all tests were from the band with R_f 0.55–0.65; no such material was found in the corresponding band of the chromatograms from the controls.

Preparation of 'free', 'bound' and 'total' acetylcholine fractions. A 10% tissue suspension from the brains of six pairs of rats was homogenized in cold aqueous eserine solution as above and then centrifuged at 18 000g for 20 min. in a HR1 International Centrifuge. The supernatant fluid, A, containing the 'free' acetylcholine, was decanted; the solid tissue residue was suspended in an equal volume of aqueous eserine, and 2 ml. of chloroform was added to it. This suspension was re-homogenized and kept for 2 hr. at room temperature or overnight at 4°. The suspension was then deproteinized by adding solid trichloroacetic acid to make a 5% solution and the precipitate formed was removed by centrifuging at 600g for 10 min. The supernatant fluid, B, containing the 'bound' acetylcholine, was then extracted three times with equal volumes of ether, and the pH of its aqueous layer was adjusted to 6.8–6.9 with 0.1N-sodium hydroxide. Supernatants A and B were evaporated under vacuum at 40° and the solid residues were extracted with 8 ml. of methanol. Any insoluble material was removed by centrifuging.

Two-thirds of the volume of each methanol extract was reduced to 0.1 ml. under a current of nitrogen and examined by paper chromatography as above. The remaining third of the methanol extracts (extract i) was reduced to dryness and suspended in 1 ml. of Locke's solution for biological assay.

Extracts containing the 'total' acetylcholine (supernatant C) were prepared from eserinated tissue suspensions treated with trichloroacetic acid immediately after the first homogenization. These extracts were processed as described above for supernatant B.

Assay of acetylcholine activity. Dried methanol eluates from chromatograms were resuspended in 1 ml. of Locke's solution. Portions of these solutions and of extracts i were assayed for acetylcholine activity with the eserinated preparation of frog rectus abdominis (MacIntosh & Perry, 1950). Assays were conducted in a 3 ml. bath after allowing the muscle to relax for 1 hr. in Locke's solution, under constant tension of 1 g. Further relaxation was allowed for another hour with Locke's solution containing neostigmine (μ M) before acetylcholine assays were started. Since the frog-rectus preparation varies greatly from one frog to

another, assays were conducted in duplicate or triplicate with different muscle preparations which responded within the range 30–70 μ g. of acetylcholine/3 ml.

In an attempt to obtain information on the relative distribution of the CoA esters of betaine derivatives in brain tissue, equal weights of cerebellum and cerebral cortex from rats were homogenized in aqueous eserine to make a 10% tissue suspension. These preparations were treated as described above for supernatant B.

RESULTS

Chromatograms of the material regenerated from the reineckate precipitate showed four distinct bands with R_f 0.05–0.15, 0.15–0.25, 0.25–0.35 and 0.50–0.70 respectively. Biological assay of the eluates from each of these bands showed that they all possessed acetylcholine-like activity.

Material with R_f 0.05–0.15. The R_f of the material in this band is similar to those of choline (0.1) and of acetylcholine (0.13) (Banister *et al.* 1953). Bregoff *et al.* (1953) have shown that, at pH 8.5–9.0, Reinecke salt causes the precipitation of both choline and acetylcholine. The crystalline tetrachloroaurate derivative formed from the dried eluate of this band (600 μ g.) contained material with two distinct melting points; some melted at 163° and the other at 223°. The tetrachloroaurate of acetylcholine has m.p. 165° and that of choline 230°. A mixture of equal amounts of the crystals of the tetrachloroaurates of choline and of acetylcholine had melting points similar to those obtained from the material in the eluates. Bands of the chromatograms were cut at R_f 0.05–0.1 and 0.1–0.15. The tetrachloroaurate from the zone with R_f 0.05–0.1 had m.p. 228° and mixed m.p. with authentic choline tetrachloroaurate 228–229°. The tetrachloroaurate from the zone with R_f 0.1–0.15 had m.p. 164° and mixed m.p. with authentic acetylcholine tetrachloroaurate 164°.

Material with R_f 0.15–0.25. The R_f of commercially available propionylcholine (California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.) is 0.21 (Banister *et al.* 1953), and its tetrachloroaurate has m.p. 253–255°. The tetrachloroaurate from the eluate of this band had m.p. 257° and mixed m.p. with authentic propionylcholine tetrachloroaurate 256°.

Material with R_f 0.25–0.35. The R_f of commercially available butyrylcholine (Mann Research Laboratories Inc., New York, N.Y., U.S.A.) is 0.29 in water-saturated butan-1-ol. The tetrachloroaurate prepared from the hydrolysed eluate had m.p. 230°, which was unchanged by admixture with authentic butyrylcholine tetrachloroaurate.

Material with R_f 0.50–0.70. The dried residue of the pooled methanol eluates of this band was dissolved in 2 ml. of water, and 1.5 ml. was hydrolysed

with 0.1N-hydrochloric acid. A portion (0.1 ml.) of the hydrolysate was examined by paper chromatography as described above. A single spot with R_f 0.1 was observed. An eluate of this material has no acetylcholine-like activity.

A portion (0.2 ml.) of the acid hydrolysate was adjusted to pH 8.5 with ammonia solution and 5% Reinecke salt solution was added as described above. The fine crystals formed were removed by centrifuging and the pH of the supernatant was adjusted to 1.2 with 6N-hydrochloric acid. This solution was stored at 0° for 3 hr. and the precipitate formed was collected by centrifuging. The two reineckate precipitates were examined by paper chromatography. No spot was observed with material precipitated at pH 8.5, but a single spot with R_f 0.1 was obtained from the material precipitated at pH 1.2. Bregoff *et al.* (1953) have shown that free or unconjugated betaines can be precipitated by Reinecke salt in acid. It seemed likely that the material precipitated in acid could contain unconjugated betaines. It contained no acetylcholine-like activity.

The remainder of the hydrolysate (1.2 ml.) was treated with 1 ml. of 10% auric chloride solution. After 1 hr. at 4°, crystals (X , about 400–500 μ g.) were separated by centrifuging and the mother liquor was kept at 4° for several hours, when crystals Y (about 50–100 μ g.) were formed. Crystals X were washed with 1% hydrochloric acid; some which dissolved at the third washing were recovered by evaporation as crystals X_1 . The residual crystals (X_2) were insoluble in 1% hydrochloric acid. The crystals, dried overnight at 22° *in vacuo* over anhydrous calcium chloride, had the following melting points: X_1 , 181°; X_2 , 211°; Y , 152°; all with decomposition. In every instance the odour of trimethylamine was detected from the decomposed material. The melting points were not changed by admixture with the tetrachloroaurates of γ -butyrobetaine, crotonobetaine and carnitine respectively. Bregoff *et al.* (1953) have shown that, at pH 8.5–9.0, Reinecke salt causes the precipitation of conjugated betaines. Since the material used on our original chromatograms was obtained by alkaline Reinecke salt precipitation, the betaines with R_f 0.1 found after hydrolysis were presumably derived from conjugated betaines.

The hydrolysate contained ribose and phosphate. Since these substances are not precipitated by Reinecke salt, they must have been part of the original unhydrolysed complex substances. β -Alanine, with R_f 0.3 in propanol–water and R_f 0.65 in phenol–water (Hosein & Smart, 1960), was detected in 0.05 ml. of hydrolysate. This amino acid is found in similarly prepared hydrolysates of CoA (Baddiley & Thain, 1951). A test with sodium nitroprusside to disclose the presence of β -mer-

Table 1. *Distribution of material with acetylcholine-like activity on chromatograms of brain extracts*

Supernatants *A*, *B* and *C* were prepared as described in the text. Activities, given as means, with ranges in parentheses and the number of experiments in square brackets, are expressed equivalent to $\mu\text{g.}$ of acetylcholine/g. of brain. The activity of supernatant *A* before application to the paper was 0.25 (0.17–0.33) [6] and that of supernatant *B* 0.05 (1.64–2.57) [6].

R_f of band	Probable component	Acetylcholine-like activity		
		Supernatant <i>A</i>	Supernatant <i>B</i>	Supernatant <i>C</i>
0.0–0.05	—	0.03 (0.02–0.03) [4]	0 [3]	—
0.05–0.15	Acetylcholine	0.21 (0.17–0.28) [5]	0.07 (0.05–0.09) [4]	0.32 (0.23–0.38) [3]
0.15–0.25	Propionylcholine	0.05 (0.04–0.07) [4]	0.08 (0.04–0.11) [4]	0.17 (0.12–0.19) [3]
0.25–0.35	Butyrylcholine	0.02 (0.01–0.04) [4]	0.05 (0.03–0.07) [4]	0.09 (0.05–0.11) [3]
0.35–0.50	—	0.01 (0 –0.02) [4]	0.03 (0 –0.08) [4]	—
0.50–0.70	Betaines (CoA esters)	0.01 (0 –0.02) [5]	2.16 (1.3 –3.0) [6]	2.41 (1.73–2.61) [3]
0.70–0.80	Betaines (CoA esters) (?)	0 (0 –0.01) [4]	0.40 (0 –0.9) [5]	—
0.80–1.0	—	0 (0 –0.01) [3]	0 (0 –0.02) [4]	—

captoethylamine was only very weakly positive. The presence of adenine was detected by its absorption maximum at 260 $m\mu$. The positive tests for ribose, phosphate, β -alanine and adenine suggest that the unhydrolysed eluate may have contained CoA. When 0.25 ml. of the unhydrolysed solution of the eluate of the band with R_f 0.50–0.70 was treated with hydroxylamine and ferric chloride, a positive reaction was observed, indicating the presence of a carboxylic acid derivative. The ultraviolet spectrum of material contained in 0.25 ml. of the unhydrolysed eluate showed adsorption peaks at 232 and 260 $m\mu$; only that at 232 $m\mu$ was decreased after hydrolysis, indicating thiol ester destruction. Material present in the eluate of this band gave a positive bromophenol blue-complex reaction, and examination of the complex formed by paper chromatography with the butan-1-ol-ethanol-water-acetic acid (32:8:12:1, by vol.) system, described by Nowell & Wilson (1961), revealed two spots with R_f values 0.36 and 0.42 when the paper was sprayed with 1% iodoplatinate. When 0.1 ml. of the concentrated eluate was examined in a second paper-chromatography system with propanol-formic acid-water (Nowell & Wilson, 1961), a band with R_f 0.55–0.65 appeared on staining with iodine. This material had acetylcholine-like activity which was destroyed by boiling in acid; it gave positive hydroxamic and bromophenol blue reactions; its absorption spectrum was identical with that of the original eluate and it had R_f 0.50–0.70 when examined again in the water-saturated butan-1-ol system.

The properties of enzymically prepared γ -butyrobetainyl-CoA and carnityl-CoA were compared with those described above for material in the eluate. These CoA esters had R_f values 0.58 and 0.62 respectively in water-saturated butan-1-ol, and 0.60 in propanol-formic acid-water. They both had acetylcholine-like activity, which was unaffected by cholinesterase but which was destroyed

by boiling in acid. Their ultraviolet-absorption spectra were identical and duplicated exactly that found in the eluate. This absorption spectrum for the CoA esters was totally different from those of CoA and ATP, each of which has a single maximum at 260 $m\mu$. Acetylcholine, choline and propionylcholine do not absorb in the ultraviolet.

Brain extracts were prepared as described above but without eserine. On examination by paper chromatography in water-saturated butan-1-ol, material was present with R_f 0.50–0.70 which had acetylcholine-like activity. This activity was destroyed by boiling in acid. The material present in this eluate duplicated exactly the properties described above for the original unhydrolysed eluate and the enzymically synthesized CoA esters of γ -butyrobetaine and carnitine.

Acetylcholine-like activity of the various bands on the chromatograms. The acetylcholine-like activity of the methanol eluates of each band from supernatants *A*, *B* and *C* are shown in Table 1. For the three supernatants, the acetylcholine-like activity found on the paper was higher than that observed before chromatography. On the basis of calculations made on the acetylcholine-like activity derived from the combined chromatograms of supernatants *A* and *B* and that of supernatant *C*, the acetylcholine content is about 10%, propionylcholine 5%, butyrylcholine 3%, and that of the CoA esters of betaine derivatives 75–80%.

By biological assay, the band with R_f 0.50–0.70 of the chromatogram prepared from rat cerebellum had activity equivalent to 4.1 $\mu\text{g.}$ of acetylcholine/g. of tissue, whereas that from the cerebral cortex had the equivalent of 2.0 $\mu\text{g.}$ of acetylcholine/g. of tissue.

DISCUSSION

The 'total' acetylcholine activity, in supernatant *C*, from fresh rat brain was of the same order as that of the combined supernatants *A* and

B, being equivalent to about 2.3 μ g. of acetylcholine/g. of tissue, whereas that recovered from the chromatograms of these extracts was equivalent to about 3.2 μ g. of acetylcholine/g. of tissue. Values of this order have been reported by Elliott, Swank & Henderson (1950).

Examination by paper chromatography and other analytical methods has shown that brain might contain at least three choline esters and three CoA esters of betaine derivatives. Since treatment of the tissue suspensions with chloroform at room temperature is known to promote autolysis, the possibility existed that the substances isolated could be artifacts. These substances were, however, found in trichloroacetic acid extracts (supernatant C) prepared under conditions which preclude autolysis. Butyrylcholine has been found in brain (Holtz & Schumann, 1954) but this could not be confirmed by Henschler (1956). The minute amount of this substance isolated in our experiments might provide an explanation for this failure.

Bellamy (1959) and Whittaker (1959) have shown that brain contains material with acetylcholine-like activity which is not affected by cholinesterase. Material believed to be the CoA ester derivatives of γ -butyrobetaine, crotonobetaine and carnitine was found in our experiments in brain extracts prepared without anticholinesterase. It is therefore unlikely that, in our experiments or those described in the literature, a choline ester was responsible for the residual acetylcholine-like activity, since it should have readily been destroyed by cholinesterase. In addition, choline could not be identified in the hydrolysate of eluates (in the band with R_F 0.50–0.70) prepared with eserine, although it was elsewhere on the chromatogram.

It is likely that brain extracts prepared as above might contain many substances which could contaminate the band with R_F 0.50–0.70 on the chromatogram. However, 18 amino acids found in proteins have a low mobility (R_F < 0.35) in water-saturated butan-1-ol (Fruton & Simmonds, 1954) and few, if any, of those remaining may have acetylcholine-like activity (Curtis, Phillis & Watkins, 1961). The ultraviolet-absorption spectrum and the mobility of certain nucleotides tested differ significantly from that obtained with the eluate. Spectrophotometric examination of the eluate (band with R_F 0.05–0.70) from the controls used in the biosynthesis of the CoA esters of γ -butyrobetaine or of carnitine showed that it contained neither CoA nor ATP. Examination of the biologically active material with paper chromatography in the propanol-formic acid–water system would have further reduced any contamination.

Hosein & Proulx (1960*a*, *b*) have shown that

water extracts made from the brains of animals treated with central-nervous-system stimulants or with electroshock contained the CoA esters of the betaine derivatives. These substances are not normally present in this fraction. Hosein & Ara (1961*a*) have found that supernatant A made from the brains of convulsing animals contained small amounts of the CoA esters of betaine derivatives, and that their concentration in supernatant B was decreased. Hosein & Ara (1961*b*) have also found that the amount of CoA esters of betaine derivatives of brain extracts from rats treated with ether or Pentobarbital is markedly increased (60%), whereas that of acetylcholine is unaltered. These results clearly indicated that, *in vivo*, under particular experimental conditions, the brain concentration of these CoA esters of betaine derivatives can be altered.

The results described in this paper emphasize that biological assays, with test objects sensitive to acetylcholine, performed on extracts from tissues (at least from brain), do not provide proof that all the active material in the extract is acetylcholine.

SUMMARY

1. Extracts of normal rat brain which are assumed to contain the 'total acetylcholine' content of the tissue were treated with Reinecke salt at alkaline pH to precipitate quaternary amines and other nitrogen compounds.

2. Chemical examination of the substances separated on the chromatograms suggested that choline, acetylcholine, propionylcholine, butyrylcholine and a mixture of the CoA esters of γ -butyrobetaine, crotonobetaine and carnitine were present.

3. Aqueous extracts of rat brain contained a mixture of acetylcholine, propionylcholine and butyrylcholine, of which acetylcholine contributed the greatest activity on biological assay with the frog rectus muscle. Deproteinized chloroform–water extracts showed that the CoA esters of the betaine derivatives were present exclusively in this fraction.

4. The choline ester fraction in brain extracts contained less than 20% of the total acetylcholine-like activity, and the CoA esters of the betaine derivatives contributed about 75%.

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Serum Proteins and the Livetins of Hen's-Egg Yolk

By J. WILLIAMS

Department of Biochemistry, University of Cambridge

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The water-soluble proteins of egg yolk comprise the livetin fraction (Plimmer, 1908). Many studies have shown that the fraction is heterogeneous. In moving-boundary electrophoresis there are three main components (Shepard & Hottle, 1949) which were called α -, β - and γ -livetins by Martin, Vandegaer & Cook (1957), and three main fractions were also found by paper electrophoresis (McCully, Maw & Common, 1959) and by ammonium sulphate fractionation (Gorini & Lanzavecchia, 1955). Chromatography on diethylaminoethylcellulose, on the other hand, gave ten components (Mandales, 1960). Onoe (1936) isolated a glycoprotein (vitellomucoid) from the livetin fraction and emphasized its distinction from ovomucoid. Schjeide & Urist (1959) concluded from studies with the ultracentrifuge that the egg-white protein, ovalbumin, is present in livetin in large amounts (45% of the total yolk protein), although the immunological

studies of Marshall & Deutsch (1951) showed only traces (0.2% of the total livetin protein). Some enzymes have also been found in the livetin fraction (Lineweaver, Morris, Kline & Bean, 1948).

Jukes & Kay (1932a) first noted an immunological similarity between serum globulin and livetin, which they thought to be a single protein, and suggested that livetin might be 'merely a translocated blood constituent' (Jukes & Kay, 1932b). Later immunological studies by Schechtman (1947), Knight & Schechtman (1954) and Nace (1953) supported this view; it had also been proposed by Shepard & Hottle (1949), who found the electrophoretic mobilities of the livetins to be similar to those of serum fractions.

The experiments described below were carried out primarily to characterize and identify the three bands of protein observed in paper electrophoretograms of livetin. The methods of immunoelectro-