4. Roots and ganglia appear to contain comparable amounts of enzyme inactivating substance P.

5. In low concentrations, roots and ganglia rapidly inactivate bradykinin; the enzyme concentration is greatest in dorsal-root ganglia and least in the ventral roots.

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Lactate Dehydrogenase as a Cytoplasmic Marker in Brain

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The glycolytic enzymes except hexokinase are found predominantly in the soluble cytoplasmic fraction of brain homogenates. However, about 20% of the total is recovered in a particulate fraction sedimenting at 13000*g* for 15 min. after the removal of nuclei and cell debris. The particulate fraction of one of these glycolytic enzymes, lactate dehydrogenase, was largely separated from a typical mitochondrial enzyme (succinate dehydrogenase) by centrifuging in a dense medium (Johnson, 1960). It was suggested that the particulate lactate dehydrogenase represented cytoplasm entrapped in incompletely broken axons.

A large proportion of brain acetylcholine exists in bound form in the sucrose homogenate and is present mainly in a fraction sedimented at 16000*g* for 60 min. In this case also a substantial separation of bound acetylcholine from succinate dehydrogenase can be achieved by density-gradient centrifuging (Whittaker, 1959). Electron-micrographs have shown that the subfraction richest in bound acetylcholine contains a high proportion of particles that have been identified as pinched-off nerve endings (Gray & Whittaker, 1962). These structures would be expected to contain cytoplasm and therefore lactate dehydrogenase also, if this enzyme has a cytoplasmic localization. This expectation has now been confirmed by experiments in which similar intracellular distribution patterns have been obtained for particulate lactate dehydro-

genase and bound acetylcholine in guinea-pig brain homogenates. Comparative studies have also been done on the extent of release of the bound components by mildly disruptive procedures.

A preliminary account of this work has appeared (Johnson & Whittaker, 1962).

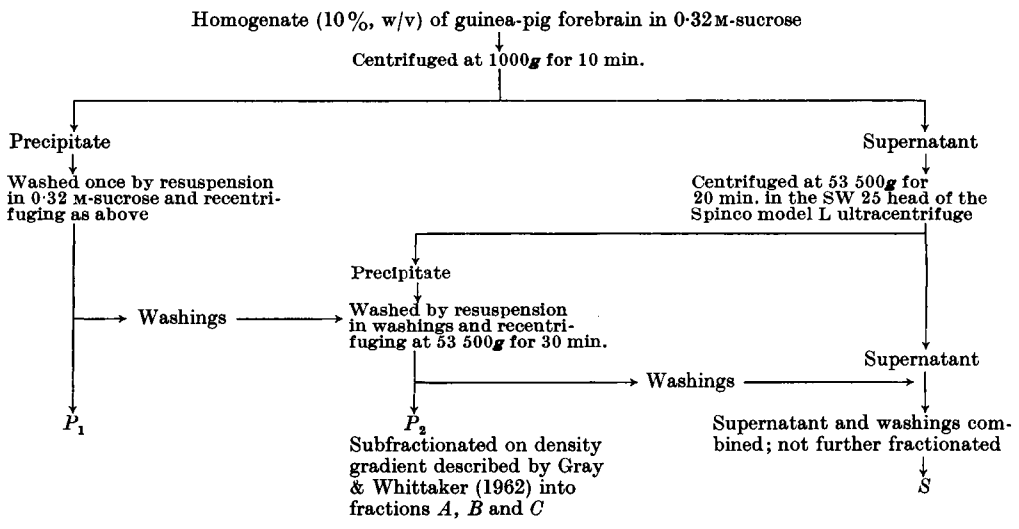
METHODS

Preparation of subcellular fractions. Four guinea-pig forebrains each weighing about 3 g. were separately homogenized in sucrose iso-osmotic to plasma (0·32 M) and the homogenates combined. The homogenizing and fractionation procedure (Scheme 1) was as described by Gray & Whittaker (1962) with one modification in order to obtain a washed P_2 fraction (mitochondria, nerve-ending particles, and myelin and small membrane fragments) in a reasonable time. The homogenizer pestle rotating at 840 rev./min. gave a somewhat coarser dispersion than is obtained at 1100 rev./min.: this presumably indicates a larger proportion of unbroken cells and accounts for the higher percentage of succinate-dehydrogenase activity found in the P_1 fraction (Table 1) than was reported by Aldridge & Johnson (1959) and Johnson (1961).

The separation of the P_2 fraction into *A* (light; myelin fragments), *B* (medium; mainly nerve-ending particles) and *C* (heavy; mitochondria) fractions by centrifuging into a discontinuous sucrose density gradient was as described by Gray & Whittaker (1962). The semi-continuous density gradient was made up by pipetting successive 3 ml. portions of sucrose (1·4, 1·2, 1·1, 1·0, 0·9 and 0·8 M respectively) over 1·5 ml. of 1·6 M-sucrose in a Lusteroid tube fitting the SW 25 head of the Spinco model L centrifuge. Two identical gradients were prepared at 0–2° and the boundaries allowed to disperse for 24 hr. before 8 ml. of the washed P_2 suspension (derived from about 5 g. of brain) was layered on top of each. Separation was achieved by centrifuging at 25000 rev./min. (53500 *g*) for 2 hr. as with the discontinuous density gradient (Gray & Whittaker, 1962). At the end of the run, the bottom of each tube was punctured with a no. 16 needle projecting 2 mm. from a no. 19 rubber bung and ground to a double-bevel point as described by Britten & Roberts (1960). Portions (3 ml.) were allowed to drip slowly into successive graduated centrifuge tubes. As nearly as possible, the equivalent layers from both density gradients were collected in the same tube. The actual volumes collected varied over the range 5·7–6·3 ml./tube.

Release of acetylcholine and lactate dehydrogenase by freezing and thawing. A P_2 pellet was suspended in 0·32 M-sucrose containing eserine (50 μM). After removal of a sample for assay, the remainder was frozen in a Pyrex tube by shaking the tube in a cold bath at −70°, and thawed in a stream of cold water. After the chosen number of treatments, a sample was removed and sedimented at 0–2° by centrifuging in an integral time–force field 30–50% greater than that used to prepare the fraction initially. The supernatant was decanted and the packed residue was suspended uniformly in 'frog Ringer' solution (composition: NaCl, 0·65%; KCl, 0·016%; $NaHCO_3$, 0·01%; $CaCl_2$, 0·012%) containing eserine (50 μM); this medium was convenient for the subsequent assay of acetylcholine (see below) and did not interfere with the assay of lactate dehydrogenase. Portions of supernatant and resuspended particles were used for assays.

Release in hypo-osmotic media. When the particles were to be treated with sucrose hypo-osmotic to plasma (i.e. more dilute than 0·32 M), the particulate material of the resuspended P_2 fraction, or of the *B* fraction, was sedimented by centrifuging in an integral time–force field 30–50% greater than that used to prepare the fraction, and the packed residue was suspended uniformly in sucrose of the chosen osmolarity containing eserine (50 μM) at 0–2°. After removal of a sample, the remainder was allowed to stand for the chosen time and the particles were resedimented in a time–force field similar to that used before



Scheme 1. Scheme summarizing preparations of subcellular fractions.

Table 1. *Distribution of succinate dehydrogenase, lactate dehydrogenase and acetylcholine among primary fractions of guinea-pig brain homogenate*

Experimental details are given in the text. Results are given as means of three experiments. The nomenclature of fractions is as in Scheme 1.

Activity	Percentage of total recovered activity in fraction P_1	P_2	S	Recovery (% of homogenate)	Homogenate activity/g. of brain
Succinate dehydrogenase	27	68	5	95	11 800 μl. of CO_2/hr.
Lactate dehydrogenase	11	20	69	100	44 μmoles of $NADH_2$/min.
Acetylcholine	16	64	20	93	11 μmoles of acetylcholine

but which was more effective because of the lower density and viscosity of the medium. The supernatant and residue were prepared for assay as in the freezing experiments.

Assays. The spectrophotometric assay of lactate dehydrogenase and manometric assay of succinate dehydrogenase were carried out as described by Johnson (1960). To obtain good recoveries of the succinate-dehydrogenase activity in particles that had been exposed in the density gradient to sucrose more concentrated than 0·32M, it was necessary to apply more elaborate rupturing treatment (Johnson, 1962) than that described by Aldridge & Johnson (1959) for primary fractions; the particles were dispersed in 10 mM-buffer (phosphate or other buffer as desired), pH 7·0, and allowed to stand at 0° for 2 hr.; the suspension was frozen and stored overnight at −20° to −70°: after thawing it was allowed to stand for a further hour at 0° before assay.

Acetylcholine was assayed with the frog-rectus-abdominis-muscle preparation as described by Whittaker (1959). Abnormalities in the muscle's response to stimulation in very dilute sucrose could not be compensated adequately, and in some lysis experiments the quantity of acetylcholine released was calculated from the difference between total and residual bound acetylcholine.

Electrophoretic separation of lactate-dehydrogenase isoenzymes. Electrophoresis of lactate dehydrogenase on agar gels was kindly carried out by Mr J. W. Boyd, as described by him (Boyd, 1962). For electrophoresis the initial homogenate and a high-speed supernatant obtained by centrifuging the supernatant from P_2 at 100000g for 60 min. were diluted 1 : 5 with ice-cold water and dialysed; the washed P_3 pellet was suspended in ice-cold water (2 ml./g. of tissue) and dialysed. Samples (5 μl.) of the dialysed fractions were placed on 0·8% agar in 0·07M-sodium barbiturate–barbituric acid buffer, pH 8·6, and submitted to electrophoresis at 30 mA and 130 v.

Electron-microscopic investigations. These were carried out by the negative-staining method as described by Horne & Whittaker (1962). Preparations were fixed at 0° by the addition of an equal volume of 10% (v/v) formaldehyde in 0·32M-sucrose, previously neutralized to pH 7·2 with 0·33N-NaOH. The mixture was diluted with 6 vol. of ice-cold aq. 1% (w/v) phosphotungstic acid previously neutralized to pH 7·2 with 2N-KOH, and transferred to grids with a micropipette. In this method, subcellular particles are seen as a whole, and not in section.

RESULTS

Distribution of acetylcholine, lactate dehydrogenase and succinate dehydrogenase in primary fractions. In the primary fractions obtained by differential centrifuging of the homogenate, the pattern of distribution of bound acetylcholine, lactate dehydrogenase and succinate dehydrogenase is shown in Table 1. It was similar to that previously established (Whittaker, 1959; Johnson, 1961), except that the P_1 fraction contained more succinate dehydrogenase and lactate dehydrogenase. This is probably due to the smaller proportion of cells broken by the slower-speed homogenization (see the Methods section). At this stage there is an obvious contrast between the distribution of lactate dehydrogenase and that of acetylcholine, lactate dehydrogenase being recovered mainly in the supernatant fraction and bound acetylcholine in the crude mitochondrial (P_2) fraction.

Table 2. *Distribution of succinate dehydrogenase, lactate dehydrogenase and acetylcholine after simple density-gradient separation of* P_2 *fraction from guinea-pig brain*

Experimental details are given in the text. Results are given as means of three experiments. The nomenclature of the fractions is given in the text.

Activity	Percentage of total recovered activity in fraction A	B	C	Recovery (% of P_2)
Succinate dehydrogenase	4	35	61	85
Lactate dehydrogenase	14	68	18	90
Acetylcholine	18	74	8	69

Distribution in subfractions of the P_2 *fraction.* Table 2 shows that, after density-gradient centrifuging of the P_2 fraction, there is a strong contrast between the distribution of succinate dehydrogenase on the one hand and those of lactate dehydrogenase and acetylcholine on the other, as previously reported for these pairs separately (Whittaker, 1959; Johnson, 1960). However, there is a close similarity between the distribution of lactate dehydrogenase and that of acetylcholine. The low mean recovery of acetylcholine is a cause of uncertainty in interpreting the results; it is considered that some loss occurs in all fractions during the lengthy manipulation, and also the assay of the

less active fractions is of limited precision since the whole sample available for bioassay is consumed in making only one measurement. In the one experiment in which eight subfractions were obtained after centrifuging over a semi-continuous gradient the recovery was better, although the same considerations pertain. Table 3 shows that the distributions of the two components are very similar, although relatively more lactate dehydrogenase is found in the denser layers. Studies by Michaelson & Whittaker (1962) and De Robertis, de Iraldi, Arnaiz & Salganicoff (1962) show that these layers contain large dense nerve-ending particles and are relatively deficient in acetylcholine.

Release of lactate dehydrogenase and acetylcholine from particulate fractions. The experiments described so far support the view that lactate dehydrogenase is a valid soluble cytoplasmic marker, its occurrence in particulate fractions reflecting the presence there of nerve-ending particles containing cytoplasm within an external membrane. Mildly disruptive treatments of brain particles such as freezing and thawing and resuspension in hypo-osmotic media cause release of only about 50% of the bound acetylcholine (Whittaker, 1959). Two explanations were offered: either acetylcholine exists in each particle in two forms, some less tightly and some more tightly bound, or the particles themselves vary in stability, about 50% of them surviving the various kinds of mildly disruptive procedure. It was thought that further information might be obtained by studying the release of lactate dehydrogenase and acetylcholine simultaneously in experiments of this kind.

With hypo-osmotic treatment such release of lactate dehydrogenase and acetylcholine as occurred was apparently almost completed within 10 min. The values shown in Table 4 refer to hypo-osmotic treatment for 10–70 min. and show, particularly at lower concentrations, that relatively more lactate dehydrogenase than acetylcholine is liberated. A similar extent of release occurred at any particular concentration when either the P_2 or B fraction was studied: thus about 50% of the acetylcholine and 75% of the lactate dehydrogenase was released from either fraction suspended in 60 mM-sucrose. Release of lactate dehydrogenase caused by repeated freezing and thawing of the P_2 fraction in 0·32 M-sucrose was likewise greater than the release of acetylcholine, although the contrast was much less marked (Table 5).

Table 3. *Distribution of lactate dehydrogenase and acetylcholine of* P_2 *fraction of guinea-pig brain in semi-continuous density gradient*

Experimental details are given in the text.

Fraction no.	Mean sucrose concn. (M)	Percentage of total recovered activity in fraction: Lactate dehydrogenase	Percentage of total recovered activity in fraction: Acetylcholine
1	0·32	2	5
2	0·55	12	16
3	0·85	13	19
4	0·95	17	16
5	1·05	18	21
6	1·15	19	16
7	1·3	15	6
8	1·5	4	1
Percentage of P_2 activity recovered		80	85

DISCUSSION

The observed distribution of lactate dehydrogenase in the primary fractions derived from guinea-pig brain homogenates and in the density-gradient experiments is most simply explained by the assumptions (1) that lactate dehydrogenase is a soluble cytoplasmic constituent, and (2) that the 20% or so which remains particle-bound reflects the occurrence in brain homogenates of particles

Table 4. *Release of lactate dehydrogenase and acetylcholine from* P_2 *and* B *fractions of guinea-pig brain after suspension in hypo-osmotic sucrose*

Experimental details are given in the text. The release of acetylcholine was calculated from the percentage remaining bound at the end of the treatment; the release of lactate dehydrogenase as a percentage of the sum of the soluble and particulate activities at the end of the treatment.

Concn. of sucrose (M)	Percentage release of activity: P_2 fraction: Lactate dehydrogenase: At 40 min.	P_2 fraction: Lactate dehydrogenase: At 70 min.	P_2 fraction: Acetylcholine: At 40 min.	P_2 fraction: Acetylcholine: At 70 min.	B fraction: Lactate dehydrogenase: At 10 min.	B fraction: Lactate dehydrogenase: At 60 min.	B fraction: Acetylcholine: At 10 min.	B fraction: Acetylcholine: At 60 min.
0·25	—	—	—	—	4	5	10	17
0·16	44	42	23	24	40	41	29	19
0·11	61	63	51	52	—	—	—	—
0·06	76	78	53	54	72	72	49	51

Table 5. *Release of lactate dehydrogenase and acetylcholine by repeated freezing and thawing of washed P_2 fraction in 0·32 M-sucrose*

Experimental details are given in the text.

No. of times frozen	Percentage of total activity released	
	Lactate dehydrogenase	Acetylcholine
1	32	16
2	38	24
4	44	34
8	54	47
16	54	46

formed by the tearing or pinching-off of nerve endings containing soluble cytoplasm within them. The identity of the particulate and soluble fractions of lactate dehydrogenase is shown by the fact that on electrophoretic separation on agar gel they separate into the same four isoenzymes (Plate 1*a*). It seems likely that the same explanation applies to the other glycolytic enzymes; the variation in the proportion of these which remain bound (7–28 %; Johnson, 1960) may reflect quantitative differences between the composition of nerve-ending cytoplasm and that of the mixed cytoplasm of the rest of the cells of the central nervous system.

If lactate dehydrogenase is accepted as a valid soluble cytoplasmic marker, the failure of up to 16 freezings and thawings to release more than 54 % of the enzyme from the P_2 fraction suggests that repeated freezing and thawing disrupts only a proportion of the outer membranes of nerve-ending particles. This suggests in turn that the 50 % release of acetylcholine under these conditions reflects, not two types of bound acetylcholine as previously favoured as an explanation (Whittaker, 1959), but a mixture of nerve-ending particles of varying stability. An examination of frozen-and-thawed preparations in the electron microscope after negative-staining (Plate 1*c*) confirmed the presence of nerve-ending particles with intact external membranes. Hypo-osmotic treatment, by contrast, caused up to 78 % release of lactate dehydrogenase under conditions which, again, released only about 50 % of bound acetylcholine. This points to the existence of a second permeability barrier to the outward diffusion of acetylcholine (not operative in the case of lactate dehydrogenase) which in part survives hypo-osmotic disruption but not disruption by freezing and thawing. This barrier could be the walls of the synaptic granules (Sjöstrand, 1953) or vesicles (De Robertis & Bennett, 1955), characteristic subcellular organelles of the nerve-ending cytoplasm about 500 Å in diameter which have been suggested as the storage sites of acetylcholine and other transmitters (De Robertis & Bennett, 1955; del Castillo & Katz, 1955). Electron-microscopic examination of P_2 preparations disrupted by hypo-osmotic treatment (0·06 M-sucrose) and subsequently negatively stained revealed few intact nerve endings, but numerous clusters of apparently intact synaptic vesicles were seen; these were often partially surrounded by a disrupted outer nerve-ending membrane (Plates 1 and 2, *d–f*). Such clusters are not seen in the frozen-and-thawed preparations. During hypo-osmotic treatment, the inflow of water into the nerve-ending particle could cause the outer membrane to rupture before the vesicular membranes were much affected. By contrast, freezing presumably affects both the external membrane and the vesicles simultaneously, and a smaller differentiation in the release of substances from the two cytoplasmic compartments is to be expected.

Additional evidence for the survival of synaptic vesicles after hypo-osmotic treatment of nerve-ending particles has been provided since the completion of this work by De Robertis, Arnaiz & de Iraldi (1962) and Whittaker, Michaelson & Kirkland (1963). The latter workers have separated vesicles from water-treated nerve-ending particles by a density-gradient procedure and have demonstrated the presence of bound acetylcholine in them.

SUMMARY

1. Previous conclusions on the distribution of succinate dehydrogenase, lactate dehydrogenase and bound acetylcholine in subcellular fractions of

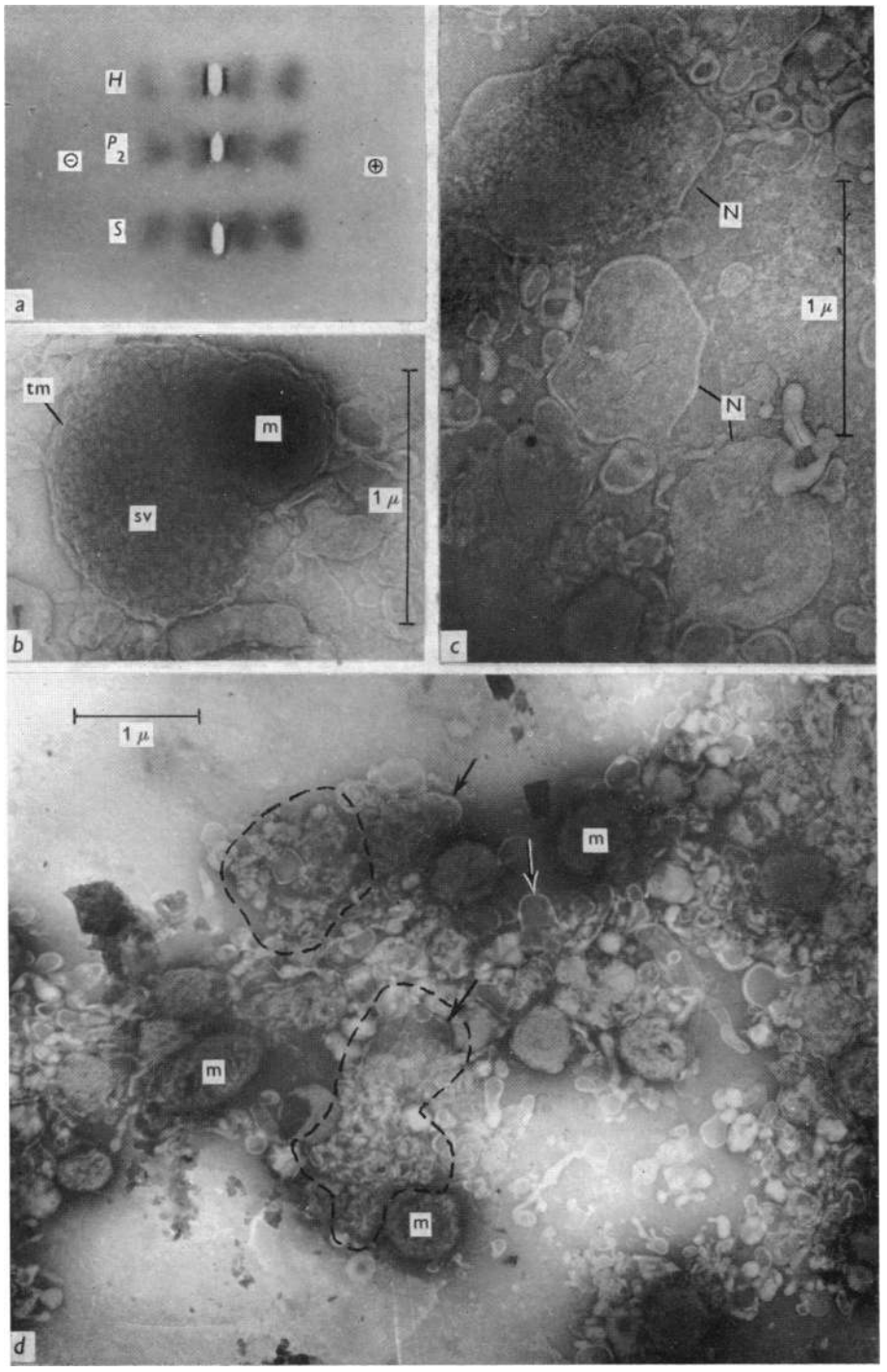
EXPLANATION OF PLATE 1

(*a*) Agar-gel electrophoretogram showing separation of homogenate (*H*), particulate (P_2) and soluble-supernatant (*S*) lactate dehydrogenase into four isoenzymes with identical mobilities. The white patches in the centre of each lane are the wells into which the fractions were placed before electrophoresis (the size of the wells is exaggerated by slight shrinkage of the agar gel).

(*b*) Normal nerve-ending particle as seen in negative-staining, showing synaptic vesicles (sv) and mitochondrion (m) enclosed within thin external membrane (tm). The vesicles and mitochondrion are seen through the external membrane.

(*c*) Electron-micrograph of negatively stained P_2 fraction after freezing and thawing 16 times. There are nerve-ending particles (N) with intact external membranes and absence of clusters of vesicles unconfined by external membranes as seen in (*d*).

(*d*) Electron-micrograph of P_2 fraction after suspension in hypo-osmotic medium (0·06 M-sucrose). There are masses of small vesicles (two such masses are encircled by dotted lines) unconfined by an external membrane, also free mitochondria (m). Traces of external membranes partially surrounding the vesicular masses are visible (at arrows).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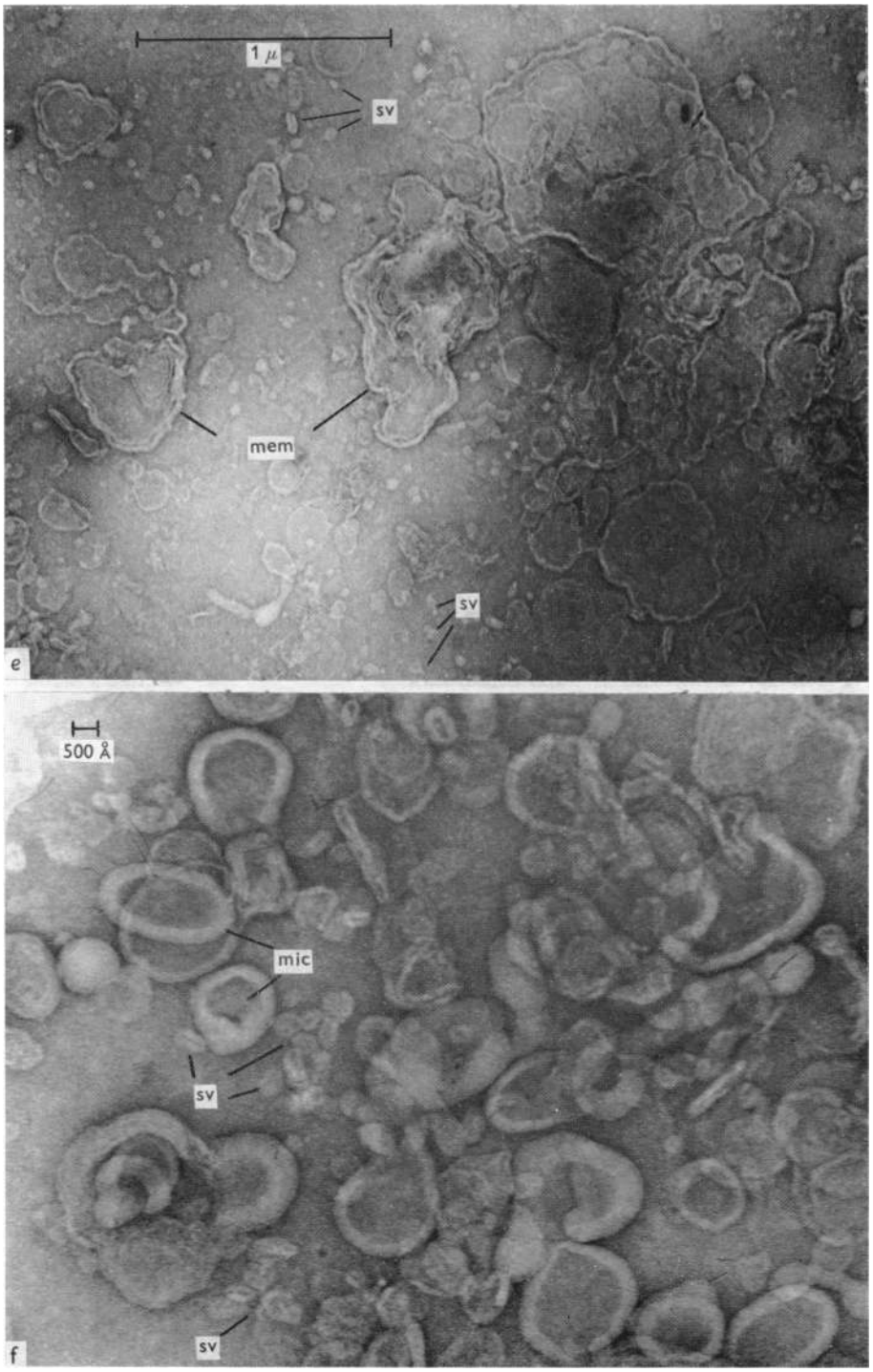

(*e*) and (*f*). A similar preparation to (*d*; Plate 1) after removal of larger particles by centrifuging at 10000*g* for 20 min. There are isolated vesicles 500 Å in diameter (sv), microsomes (mic) and disrupted external membranes (mem).

brain tissue have been confirmed by parallel studies.

2. Bound acetylcholine and lactate dehydrogenase were similarly distributed in density-gradient separations, and were located in the regions shown previously to contain the majority of the nerve-ending particles of the homogenate. Their location was clearly different from that of succinate dehydrogenase.

3. Repeated freezing and thawing liberated slightly more and treatment of the washed particles with sucrose hypo-osmotic with respect to plasma liberated considerably more lactate dehydrogenase than acetylcholine.

4. The results support the concept that acetylcholine and lactate dehydrogenase are both contained within the same nerve-ending particles, and that there is a further barrier within these particles that provides an extra hindrance to the release of acetylcholine; this barrier may be the membrane of the synaptic vesicles.

We are grateful to Mr J. W. Boyd for carrying out the agar-gel electrophoresis and for permission to publish the results. M. K. J. thanks Miss M. A. Jefferyes for skilled technical assistance.

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Preparation and Properties of Rat Transferrin

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The existence of two types of transferrin in the rat has been demonstrated (Beaton, Selby & Wright, 1961) by electrophoresis of the plasma in starch gel. Gordon (1962*a*) has shown that, when labelled with ^{131}I and reinjected, these two transferrins have closely similar rates of elimination. Their immunological identity has also been demonstrated (Neuhaus, Havez & Biserte, 1962). The rat transferrin used by Gordon (1962*a*) for metabolic investigation was purified first by electrophoresis in a block of Pevikon C870 (see below), after which it was iodinated with ^{131}I. The two transferrins with different mobilities were then separated by electrophoresis in starch gel. Further experiments showed, however, that the two types of transferrin could be separated more simply and in better yield by chromatography on diethylaminoethyl-Sephadex. The present paper describes a method of isolation in which electrophoresis in Pevikon, followed by this type of chromatography, is used. In addition some of the properties of the two types of transferrin thus prepared are reported. In the present paper these two transferrins are referred to as 'slow' and 'fast' on the basis of their electrophoretic mobility.

METHODS

Electrophoresis in Pevikon C870. The electrophoresis was carried out in general as described by Bocci (1962), by using Pevikon C870, obtained from Stockholms Superfosfat Fabriks A.-B., Stockholm, Sweden. Initially the rat serum was dialysed for at least 24 hr. against veronal buffer, pH 8·6 (75 mM-sodium diethylbarbiturate plus 15 mM-barbituric acid plus 5 mM-$NaHCO_3$). The buffer also contained sufficient Fe^{3+} ions to saturate all the transferrin present. The block (30 cm. × 17·5 cm. × 1·5 cm.) of Pevikon granules was made with the same buffer except that both the 5 mM-$NaHCO_3$ and the Fe^{3+} ions were omitted; 15 ml. of the dialysed serum was injected by syringe into a slit 8 cm. from the cathodic end of the block. Sufficient cooling was obtained by using a hollow metal plate on which the block