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Isolated Nuclei from Cells of the Cerebral Cortex. Preparation and Enzyme Content

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Cell nuclei have previously been isolated from the liver and certain other animal tissues (Dounce, 1943), but little is known of the biochemical properties of nerve cell nuclei or of the part played by them in the economy of the cell. The nuclei of nerve cells differ from those previously obtained, in that they come from cells so highly specialized that they have lost their power of mitosis. It may therefore be expected that any biochemical characteristics found in them will reflect, not so much the special requirements of cell division as the part played by the nucleus in the normal functional activity of the cell. The relatively large size of the nerve cell nuclei and their prominent nucleolar apparatus have suggested that they may be specially concerned in the synthesis of the nucleoprotein and other metabolites required for the maintenance of the nerve axons (Caspersson, 1947).

The method now described for the preparation in quantity of isolated nuclei from the cerebral cortex is similar to that used by Dounce for liver: it depends on grinding the tissue with dilute citrate solution at pH 6·0–6·2 to cause extrusion of the nuclei from the cells, followed by repeated differential centrifugation at varying speeds to separate the nuclei from the cytoplasm and cell debris. The method is mild enough to avoid destruction of many of the enzymes present in the tissue, and it has enabled a study of the enzymes of the nerve cell nucleus to be made. It has been shown that these nuclei contain an active cytochrome oxidase, carbonic anhydrase, acetylcholine esterase and, contrary to some investigators who have used the Gomori histological method, active alkaline and acid phosphatases. These results give confirmation to the view that the nerve cell nucleus is metabolically active.

EXPERIMENTAL

Preparation of isolated nuclei

After removing the meninges from the brain, the grey matter was separated from the white by scraping with a blunt spatula. To 100 g. cortical tissue in a large mortar were added 240 g. crushed ice and 240 ml. water. The distilled water was previously brought to pH 7·0 by adding NaOH to neutralize any CO_2 dissolved in it. While grinding, citric acid solution (20 ml., 1·1 %) was added slowly, so that the pH (glass electrode) was kept at 6·0–6·2. Grinding was continued until the ice had melted (45 min.). The mixture (600 ml.) was then strained through four thicknesses of cotton gauze (20 mesh/in.) into four 250 ml. centrifuge bottles.

The centrifuging was carried out in eight stages with a Servall G/1 angle centrifuge, to which a fixed resistance of 177 Ω was connected in series to allow a wider range of low speeds to be obtained. At each stage the supernatant

solution was decanted and examined microscopically, after adding a trace of toluidine blue, to make sure that no nuclei were lost; it was then suspended in distilled water. After some preliminary trials, the conditions set out in Table 1 were found to be the best.

Table 1. *Conditions of centrifugations used in the isolation of cell nuclei*

(Columns 2, 3 and 4 give the rheostat position, duration and speed respectively of each centrifugation. At each stage the supernatant solution was discarded and the residue resuspended with a mechanical stirrer in the volume of water given in column 5. After the sixth centrifugation the suspension was strained through four thicknesses of gauze. After the seventh, the suspension was allowed to stand in a measuring cylinder for 45 min. The upper 95 ml., containing the nuclei, were carefully decanted from a small residue, consisting mainly of whole cells.)

Centrifugation no.	Position of rheostat (units)	Time (min.)	Max speed (rev./min.)	Water added to residue at end (ml.)
1	50	15	1600	400
2	50	10	1600	400
3	40	10	1450	400
4	35	5	1250	200
5	25	5	1000	200
6	15	5	650	200
7	20	5	800	100
8	60	10	1800	40

If the separation was found to be unsatisfactory at any stage, the residue and supernatant solution were recombined and the centrifuging was repeated with such modifications as might be necessary. The bucket-type centrifuge head could be used, but the optimal speeds and duration of the centrifuging were then different. With practice the method described worked successfully with rabbit, rat and human brains. It worked both on fresh tissue and on material which had been kept up to 3 days in an ice chest. The final suspension in 40 ml. distilled water was found convenient to use for most of the biochemical determinations which were carried out. It was kept in the refrigerator in a conical beaker, containing a few glass beads which assisted in producing a homogeneous suspension when required.

Microscopic examination, after staining with toluidine blue, showed the preparation to contain many nuclei from nerve cells and glial cells, with a few from the endothelial cells of the blood vessels. There were generally some capillary fragments present and occasional whole cells and nuclei with adherent cytoplasm, but it was estimated that such impurities could not have exceeded 5–10% of the weight. The nerve cell nuclei were about 40% of the total number present, but in view of their larger mean size the nerve cell nuclei would be expected to constitute the greater part of the dry weight. About 0·7 g. of nuclei (dry wt.) was obtained from 100 g. of fresh cortical tissue. In a series of ten human brains the nuclei represented on the average 4·8% of the dry weight of the cortical tissue.

Determination of enzyme activities

The alkaline and acid phosphatases were determined by the method of King & Armstrong (1934), using disodium phenyl phosphate as substrate. The rate of hydrolysis was followed with the reagent of Folin and Ciocalteu, using a photoelectric absorptiometer. For the alkaline phosphatase the hydrolysis was allowed to proceed for 15 min. at pH 9·0; for the acid phosphatase it was carried out at pH 4·9 for 1 hr. Activities are expressed in King-Armstrong units, or mg. phenol liberated/g. dry tissue at 37° under the standard conditions. For the cytochrome oxidase determination cytochrome *c* was prepared from heart muscle by the method of Keilin & Hartree (1938). The enzyme activity was then determined manometrically with ascorbic acid as a reducing agent as described by Schneider & Potter (1943). Activities are expressed as ml. O_2 taken up/hr./g. dry tissue.

Acetylcholine esterase ('true' or 'specific' cholinesterase) was determined by the titration method with acetylcholine as substrate and cresol red as indicator. For each determination three 25 ml. tubes were set up in a bath at 37°. The first tube contained 1 ml. 1% acetylcholine chloride, 0·5 ml. 0·04% cresol red solution, 12 ml. distilled water and 2 ml. of nuclei suspension. The second tube containing the same solutions with 0·1 ml of 1% eserine sulphate served as a control. The third tube, containing 7 ml. of 0·2M-boric acid in 0·05M-NaCl, 3 ml. of 0·05M-borax ($Na_2B_4O_7.10H_2O$) solution, 0·5 ml. of 0·04% cresol red and 3 ml. water, was used as a colorimetric standard for the titration, which was carried out at 15 min. intervals with 0·3N-NaOH. The enzyme activity was expressed as μmol. of acetylcholine hydrolysed/g. dry tissue/hr.

Carbonic anhydrase was determined by measuring the time taken for a solution of CO_2 to reduce the pH of a veronal buffer solution from 9·0 to 6·3 through the formation of bicarbonate ions. This method was suggested by Roughton & Booth (1946) who found that the more usual colorimetric method using Na_2CO_3 (Ashby & Chan, 1943) was unsatisfactory owing to the strongly inhibitory action of carbonate ions on the enzyme. In a large beaker containing crushed ice, eight tubes were set up, each containing 0·4 ml. veronal buffer (0·022M-veronal in 0·22M-sodium veronal) and 0·2 ml. 0·4% bromothymol blue solution. To four of the tubes were added 0·3 ml. of 1 in 3 dilution of the nuclei or tissue suspension and 4·6 ml. water. To the remaining four (control) tubes were added 4·9 ml. water. After mixing and allowing 30 min. or more for the temperature to fall to 0°, 5 ml. of water saturated with CO_2 at 0° were rapidly injected below the surface with a previously cooled 5 ml. glass syringe fitted with a wide-bore stainless steel needle. The CO_2 content of the saturated solution was approximately 0·07M as determined by titration with standardized $Ba(OH)_2$. The tube was rapidly stoppered, the contents mixed by shaking and the time was measured with a stop-watch for the colour to match that of a standard tube containing the same amount of tissue suspension in 1 ml. M/15-phosphate buffer pH 6·3, 0·2 ml. bromothymol blue solution and 9·3 ml. water. The mean time for the four control tubes and the four tubes containing enzyme was noted and enzyme activity was calculated as described by Roughton & Booth and expressed as mmol. CO_2 ionized/l./g. dry weight/sec. A small correction was made for the carbonic anhydrase content of the blood combined in the tissue; this was found by estimation by the benzidine method to be of the order of 0·05–1·5%. The blood content of the nuclear suspensions was negligible. Under the experimental conditions used, the time taken for the control tubes to change colour so as to match the standard was about 80 sec., while the time taken after the

addition of tissue or nuclear suspension was about 25–35 sec. Since all determinations were carried out in quadruplicate the reproducibility of the individual figures was good.

Calculation of the enzyme concentration in the cytoplasm

In attempting to assess the functional significance of the nuclei it was desirable to know the distribution of the enzymes studied between the nucleus and cytoplasm of the cells. A direct determination of the enzyme activity of cytoplasm would have been difficult, since the cytoplasm was greatly diluted by the method of extracting the nuclei, but it appeared possible to obtain approximate figures for the cytoplasm enzymes which would be sufficiently accurate to be of use by measurement of the enzymic activities of the isolated nuclei and of the whole tissue and calculation of the cytoplasmic values therefrom.

From determination of dry weights on 100 g. of fresh cerebral cortex in ten human brains it was found that the isolated nuclei represented approximately 5% (range 3·2–6·9%; mean 4·8%) of the dry weight of the whole tissue. The calculated enzyme content of 5% of the whole tissue at the level of activity in the nuclei was subtracted from the value found experimentally for the whole tissue. The value thus obtained for the enzyme content of the extranuclear material was then converted to an enzyme activity in terms of the dry weight of the extranuclear material. Taking the extracellular content of fresh human cerebral cortex to be 33% of the whole (Stewart-Wallace, 1939) and assuming this to resemble a colloid-free serum ultrafiltrate (Peters, 1935) containing only about 0·7% of dissolved solid (mainly NaCl), it could be calculated that in the grey matter of the brain the extracellular fluid contributes relatively little (about 2%) to the dry weight of the tissue. If it is taken that the enzymes examined are present mainly in the cells, which contain most of the dry weight of the tissue, the enzymic activity calculated for the extranuclear material would thus be expected to approximate to that of the cell cytoplasm. The figures given for the enzymic activity of the cell cytoplasm were calculated on this basis.

Stability of enzymes

For studying the distribution of enzymes between nucleus and cytoplasm, it was necessary that the enzymes should be relatively stable and able to stand up to the procedure involved in the separation of the nuclei from the tissue. Preliminary experiments were therefore carried out to test the stability of the enzymes chosen for investigation. The mean activity of alkaline phosphatase in whole tissue suspensions from five brains kept for 2–4 days at 1–4° in the refrigerator was 98·5% of the initial mean value. The mean activity for six preparations of nuclei kept for 2–5 days was 98·3% of the initial value, and further experiments showed no significant decrease in alkaline phosphatase activity in whole tissue and nuclei preparations kept for 17 and 19 days respectively. These results agreed with some previous observations of Fleischhacker & Yates (1936) on the stability of this enzyme.

Similar experiments to test the stability of the acid phosphatase of human brain showed a falling off to about 75–80% of the initial activity in five whole tissue suspensions kept for 4 days: but the activities found on keeping for a further period of 17 days in the refrigerator were still 78–80% of the initial values. The acid phosphatase was apparently more stable in the nuclei suspensions: four preparations showed no significant decrease in activity in 2–4 days and one showed an activity of 91·4% of the initial value after keeping for 19 days. Acetylcholine esterase is known to be a relatively stable enzyme (Glick, 1937), and no significant change of activity was found in the choline esterase activity of either nuclei or brain tissue suspensions kept at 1–4° for periods up to 20 days. Wilbur & Anderson (1948) reported no change in the carbonic anhydrase activity of whole rat brain preparations kept for several weeks at 0°: this was confirmed for nuclei and whole cell suspensions from human brain kept for periods up to 21 days at 1–4°.

Successive determinations of the cytochrome oxidase activity in preparations of human brain tissue and isolated nuclei showed a relatively rapid falling off in activity on keeping. Thus in two preparations of cerebral cortex the mean activity fell in 1 day to 76%, in 2 days to 61%, in 3 days to 48%, in 4 days to 37% and in 5 days to 29% of the initial value. It was also observed that determinations carried out on autopsy material obtained 24 hr. or more after death gave considerably lower cytochrome oxidase activities than were found in fresh brain biopsy specimens determined within 2–3 hr. of removal from the patients. The instability of this enzyme made it unsuitable for accurate studies of the distribution in the cerebral cortex, but in view of the importance of cytochrome oxidase in cellular oxidation reactions a few determinations on nuclei preparations are reported.

The human brains used in this investigation for the studies of enzyme distribution were taken at autopsy from subjects who had died from the intercurrent illnesses which are given in Table 3. There was no evidence in any of them of any abnormality of the brain. The autopsies were done 12–24 hr. after death and the biochemical work was started immediately. Except for cytochrome oxidase, it appeared that the enzymes chosen for investigation would not undergo any considerable loss of activity in this time.

RESULTS

The figures showed that alkaline phosphatase was present in relatively high concentration in the nuclei of cells of the cerebral cortex (Table 2). In every case the enzymic activity of the nucleus was greater than that of the whole tissue, or the activity calculated for the cell cytoplasm. The figures also suggested an apparent relation of the alkaline phosphatase activity of the nucleus to the age of the subject. Thus lower activities were generally found in the nuclei from the older subjects while higher activities were found in those from the younger subjects. The activity in the whole tissue and that calculated from the cytoplasm did not vary with the age to the same extent as in the nuclei. The acid phosphatase was generally less active than the alkaline phosphatase in the cell nuclei and it was more evenly distributed, so that the activities in nucleus and cytoplasm were similar. There was no

Table 2. *Distribution of alkaline and acid phosphatase between nucleus and cytoplasm of cells of human cerebral cortex*

(Units/mg. dry weight. Figures for nuclei and whole tissue were determined: values for cytoplasm were calculated. Causes of death are given in Table 3. In subject A. M. A., aged 6 days, the separation of grey and white matter was unsatisfactory and values for the cytoplasm could not therefore be calculated.)

Subject	Age (years)	Sex	Alkaline phosphatase			Acid phosphatase			Water content of whole tissue (%)
			Nuclei	Whole tissue	Cytoplasm	Nuclei	Whole tissue	Cytoplasm	
W. B. J.	59	M	27·0	5·2	4·1	11·4	13·1	13·2	84·6
L. G. H.	78	M	16·6	7·2	6·7	9·1	7·0	7·0	86·2
M. W.	69	F	14·6	7·1	6·7	8·3	9·1	9·0	86·3
H. E.	67	M	13·5	4·1	3·7	6·3	6·7	6·7	85·0
W. G. E.	57	M	19·4	9·4	8·8	18·0	13·1	12·8	83·7
T. E. J.	47	M	10·4	9·4	9·4	16·8	15·5	15·4	84·4
C. M.	51	M	14·6	8·3	7·9	10·2	14·5	14·7	83·7
R. M.	50	F	16·0	12·7	12·5	10·1	13·7	13·9	83·2
L. C.	3	M	68·5	12·1	9·1	10·7	10·8	10·9	85·5
H. B.	56	M	49·3	13·1	11·2	11·3	9·4	9·3	87·9
W. J. E.	48	M	40·2	9·2	7·6	10·4	7·2	7·0	84·5
H. A.	49	M	39·5	13·7	12·3	13·4	11·0	10·9	83·9
H. H.	46	M	44·7	6·1	4·1	13·1	9·8	9·6	83·1
A. C.	84	F	18·3	5·2	4·6	6·5	9·1	9·2	85·8
A. M. A.	6 days	F	54·8	11·4	—	5·8	10·1	—	—

Table 3. *Distribution of acetylcholine esterase and carbonic anhydrase between the nucleus and cytoplasm*

(Units/mg. dry weight.)

Subject	Acetylcholine esterase			Carbonic anhydrase			Cause of death
	Nuclei	Whole tissue	Cytoplasm	Nuclei	Whole tissue	Cytoplasm	
W. B. J.	287	266	265	33·1	96·7	100	Pulmonary congestion
L. G. H.	311	246	253	30·5	96·5	100	Coronary thrombosis
M. W.	286	248	246	33·2	77·2	78·4	Lobar pneumonia
H. E.	281	125	117	36·5	110	114	Coronary thrombosis
W. G. E.	317	220	215	34·5	76·6	78·8	Coronary occlusion
T. E. J.	260	218	216	44·4	146	151	Cardiac failure
C. M.	326	127	117	30·3	89·5	92·6	Rupture of aortic aneurism
R. M.	108	183	187	35·6	87·8	90·5	Renal tuberculosis
L. C.	51	235	245	24·3	67·4	70·0	Broncho-pneumonia
H. B.	231	169	166	19·7	88·5	92·1	Lymphatic leukaemia
W. J. E.	285	160	153	19·8	80·4	83·6	Carcinoma of bronchus
H. A.	279	212	208	27·5	78·1	80·8	Carcinoma of bronchus
H. H.	225	136	131	26·0	85·8	89·0	Carcinoma of bronchus
A. C.	164	240	244	—	—	—	Carcinoma of gall bladder
A. M. A.	90	870	—	57·1	29·8	—	Pneumonia

evidence of any relation to age. Acetylcholine esterase was found in considerable concentration in the isolated nuclei (Table 3). That this was the 'specific' or 'true' choline esterase was confirmed by preliminary tests with methyl butyrate as a substrate; this ester was not hydrolysed. In eleven of the fourteen brains examined, the activity was greater in the nucleus than in the cytoplasm. It is noteworthy that the activity in the nucleus was relatively low in the two infant brains included in the series. Carbonic anhydrase activity was considerably greater in the cytoplasm than in the nuclei. Cytochrome oxidase was found to be present in considerable concentration in the isolated nuclei from the cerebral cortex. In view of the instability of this enzyme little significance can be attached to the individual figures, but in the twelve brains examined, the mean activities were 32 units/mg. dry weight for the nuclei, 36 for the whole tissue and 39 calculated for the cytoplasm. Correction for the probable loss in activity on standing gave a value of approximately 50 for the fresh tissue. This agreed with the mean value of 48 units/mg. found for eight brain biopsy specimens taken from patients suffering from schizophrenia.

DISCUSSION

The observation that isolated nuclei from the cerebral cortex contain a high concentration of alkaline phosphatase may be related to the cytological

studies of Willmer (1942) and of Krugelis (1946), who reported that the chromosomes gave an intense reaction for alkaline phosphatase when tested by Gomori's histochemical method. Brachet & Jeener (1946) observed a certain parallelism between the intensity of the nuclear alkaline phosphatase reaction and the rate of phosphorus turnover in deoxyribonucleic acid, from which they concluded that the alkaline phosphatase may be responsible for phosphate exchanges in the deoxyribonucleic acid. It has recently been confirmed by Ross & Ely (1949) that alkaline phosphatase acts slowly on polymerized deoxyribonucleic acid as well as on the depolymerized form. The Gomori histochemical method has recently been subjected to a good deal of criticism (Martin & Jacoby, 1949). It is therefore of interest that the direct biochemical methods used in the present investigation have confirmed the high alkaline phosphatase activity of the nerve cell nucleus indicated by the histochemical method. The higher activity found in nuclei in the brains of infants would agree with the suggested function of alkaline phosphatase in relation to deoxyribonucleic acid metabolism, which is particularly fast in actively developing tissues.

The biochemical significance of acid phosphatase is not yet understood. The present studies indicated that it is present in similar concentration in the nucleus and cytoplasm of the nerve cell, though preliminary experiments on the stability of the enzyme suggested that the cytoplasm may contain two acid phosphatases which differ in their stability to keeping. Studies on the distribution of acid phosphatase by the histochemical method of Gomori have given divergent results and here the direct biochemical method would appear to be more reliable, for it has been shown that as much as 90 % of the enzyme may be lost during the preliminary process of embedding the tissue (Stafford & Atkinson, 1948).

A high concentration of cholinesterase was found in the cell nuclei. This does not appear to fit in with the views of Nachmansohn (1946) who has concluded, mainly from observations on squid axons, that cholinesterase is concentrated at the cell surface. From this, further conclusions as to the physiological function of the enzyme in neuronal conduction have been drawn.

For reasons already given, the figures obtained for the cytochrome oxidase activity represent minimal values. They show, however, that the nucleus is approximately as active as the cytoplasm in this enzyme and hence give support to the view that the cell nucleus is metabolically active.

The observations on carbonic anhydrase distribution can hardly be evaluated before more is known about the function of the cell nucleus and of the physiological significance of this enzyme. The present preliminary results are reported at this stage since some of them have a bearing on other work which is in progress elsewhere, and because this method of studying the isolated nuclei would appear to offer a new method of approach to the study of the chemical pathology of the brain in mental disease.

SUMMARY

1. A method is described for the preparation of isolated nuclei of cells of the cerebral cortex.

2. The distribution of enzymes between the nucleus and cytoplasm of cells of the human cerebral cortex was determined for (*a*) alkaline phosphatase, (*b*) acid phosphatase, (*c*) acetylcholine esterase, and (*d*) carbonic anhydrase.

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