Migration of Histones from the Nuclei of Isolated Cerebral Tissues kept in Cold Media

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Slices of guinea-pig cerebral cortex, kept in buffered glucose media at 0° for 5–17 hr., lose their normal respiratory response to applied electrical pulses. This response can be restored to a considerable extent by Cohn fraction IV-4 of ox plasma (Marks & McIlwain, 1959). Dialysed acid or sodium chloride-ethanol extracts of whole brain and of isolated nuclei also inhibit the isolated tissue's response to electrical stimulation, without affecting normal respiration in the absence of pulses. Blood-plasma fraction IV-4 restores, almost completely, the respiratory response to stimulation of cerebral cortex slices treated with these extracts. Further, basic proteins such as histones from calf-thymus nuclei and the protamines, clupein and salmine, inhibit the respiratory response at concentrations as low as 0.1 mg./ml. It was concluded that the inhibitory material of the brain extracts was probably a histone, and that the loss of response in cerebral slices kept in cold media was due to a reaction of the tissue's histones with acidic sites or carriers that are essential to the metabolic response to electrical pulses (McIlwain, 1959).

The aim of the present investigation was to extract basic proteins from nuclei isolated from cerebral cortex slices after two types of treatment *in vitro*: (1) incubation under ordinary conditions, favourable for retention of respiratory response to electrical stimulation; and (2) keeping in cold media and then incubating. It was thought that such studies might demonstrate a loss of nuclear histones in the slices exposed to cold media. If this could be shown, studies on the localization of the site of reaction of basic proteins in extranuclear parts of the neurone should provide useful information on the biochemical events linking electrical stimulation and metabolism. To our knowledge the only study of histones from cerebral tissues, apart from preliminary characterization (Mirsky, 1943), is that of Palladin, Rashba & Shtutman (1951), who reported an arginine content of 12% and an isoelectric point of pH 10–11 for histones obtained from nuclei of the cerebral cortex of the cow and the dog.

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EXPERIMENTAL

Tissue, media, metabolism

Guinea pigs were used unless otherwise stated; they were stunned by a blow on the neck, the throat was cut to allow bleeding and the brain removed. Cerebral cortex slices, 0.35 mm. thick, were prepared promptly with a razor blade and guide, and after being weighed moist were transferred to phosphate media. Respiration was measured manometrically at 37.5° in the oxygenated phosphate media containing (mM): NaCl, 134; KCl, 5.2; CaCl₂, 2.8; KH₂PO₄, 1.3; MgSO₄, 1.3; Na₂HPO₄, 10.4; and glucose, 10; pH, 7.4 (Rodnight & McIlwain, 1954). Lactic acid was determined at the end of manometric experiments by Barker & Summerson's (1941) method. Large Warburg flasks (approx. 100 ml. capacity) were used so that a sufficient quantity of tissue slices could be obtained for the extractive procedures. Approx. 500 mg. of cortex slices were incubated with 15 ml. of buffered phosphate media in each flask. Slices to be exposed to cold were placed in the oxygenated media in Warburg flasks in a tray of ice-water which was kept in the refrigerator for 16 hr. The flasks were lightly stoppered and an O₂ atmosphere was not maintained. After exposure to cold, the medium was drained from the slices which were then suspended in fresh media, equilibrated with O₂, and respiratory measurements were made at 37.5°. The O₂ uptake of fresh slices, not exposed to cold media, was determined in parallel.

In studies with unsliced cerebral cortex, as much as possible of the white matter was removed by opening the ventricles and by blunt dissection which removed the layers of subcortical white matter.

Preparation of subcellular fractions

Nuclear fraction. All operations were conducted at 0 to 4°. The medium used for the isolation of nuclei was a slight modification of that of Allfrey, Mirsky & Osawa (1957) and consisted of 0.32 M-sucrose with 3 mM-CaCl₂ adjusted to pH 7.0 with NaOH. After brief preliminary dispersion in a glass homogenizer with a loose-fitting polythene plunger, homogenization was carried out in a Model 10 Emanuel-Chaikoff hydraulic tissue homogenizer (Microchemical Specialties Co.) with an orifice of 25 μ . This method gave high yields of intact nuclei, with almost no contamination by cell-bodies, and under conditions that could be precisely reproduced. Medium to tissue ratios (v/w) of 10 gave the best results. The homogenized tissue was filtered through one layer of cotton screen (300 mesh) and two layers of napped flannelette. The filtrate was centrifuged in a Spinco model L ultracentrifuge at 3500 rev./min. (800 g)

for 10 min. The sedimented nuclear fraction was washed four times by resuspending in the original volume of homogenizing medium and recentrifuging as before. The washed fraction was finally resuspended twice in 0.14M-NaCl, pH 7.0, for 10 min. and centrifuged. This procedure extracted nuclear globulins (Alfrey *et al.* 1957). The separation of the nuclei was controlled by examination with a phase-contrast microscope. To achieve better separation of nuclei from cellular particles from slices it was necessary to layer the homogenate on *m*-sucrose and centrifuge in a SW 25.1 rotor of the Spinco ultracentrifuge for 1 hr. at 24 000 rev./min. (50 000 *g*).

Other fractions. Sucrose-CaCl₂ media were unsuitable for preparation of mitochondrial and microsomal fractions because calcium salts increased a tendency of the particles to aggregate and to co-precipitate. These subcellular particles were isolated in media consisting of 0.32M-sucrose with 0.5 mM-EDTA (ethylenediaminetetra-acetic acid) at pH 7.0 and separated by differential centrifuging based on the method of Brody & Bain (1952). The following fractions were prepared: (1) 'nuclear', after centrifuging at 800 *g* for 10 min.; (2) large 'mitochondrial' after centrifuging at 5000 *g* for 10 min.; (3) small 'mitochondrial' after centrifuging at 20 000 *g* for 10 min.; (4) 'microsomal', after centrifuging at 105 400 *g* for 30 min.

Analyses. The nitrogen content of the various fractions was determined by a micro-Kjeldahl procedure. Deoxyribonucleic acid was determined by the *p*-nitrophenylhydrazine method of Webb & Levy (1955) with a deoxyribose standard.

Extraction and analysis of histones

Two procedures were used for the extraction of histones from isolated nuclei and other fractions of cerebral tissues.

Acid extraction. The procedure of Luck, Rasmussen, Satake & Tsvetkov (1958) for fractionation of calf-thymus histone was used with slight modifications. The washed nuclear and particulate fractions were suspended in 10 vol. of 0.2N-H₂SO₄ at 0°, agitated for 10 min. with a Vibromix stirrer, kept in the refrigerator for 40 min. and then stirred again for 10 min. The suspensions were then clarified by centrifuging at 31 000 *g* in the cold for 30 min. and the deposit was discarded. The histone sulphates were separated into arginine-rich and presumably lysine-rich fractions by ethanol precipitation in the cold according to the method of Ui (1956). To the clarified solutions absolute ethanol at -10° was added slowly to a final concentration of 20% (v/v), and the arginine-rich histone was allowed to precipitate overnight at -5°. The precipitate was removed by centrifuging at -5° and washed five times with 4 vol. of cold 95% ethanol. Almost all the precipitate dissolved in distilled water, and the little that did not was removed by centrifuging. The histone solution was then freeze-dried to a white powder that was freely soluble in distilled water. The lysine-rich histone was separated by adding absolute ethanol, to a final concentration of 50% (v/v), to the supernatant, after removing the arginine-rich histone, and again collected by centrifuging.

In some experiments the acid extract was dialysed against distilled water for 24 hr. before ethanol precipitation. When in trial experiments the histones were extracted as the chloride, the ethanol precipitates were more colloidal and difficult to remove completely by centrifuging. Therefore, all histones were extracted as the sulphate and con-

verted as desired into the chloride by adding an excess of 0.2M-BaCl₂, separating the precipitate by centrifuging, and then dialysing and freeze-drying the supernatant.

Salt extraction. This method was based on those of Crampton, Lipshitz & Chargaff (1954), Butler, Davison, James & Shooter (1954), Smillie, Marks & Butler (1955) and Hnilica (1959) except that the histones were separated from the deoxyribonucleoprotein by 0.2N-H₂SO₄. To the cold 0.14M-NaCl-washed nuclear fractions, NaCl was added (10 vol.) to give a final concentration of 1.5M. The suspension was gently stirred for 4 hr. at 0°. A strong gel of deoxyribonucleoprotein usually formed. The suspension was centrifuged at 50 000 *g* for 30 min. The residue was resuspended in a further 10 vol. of 1.5M-NaCl and the centrifuging was repeated. To the supernatant, N-H₂SO₄ was added to give a final concentration of 0.2N. A white precipitate formed. The mixture was kept for 1 hr. in the cold, and the deoxyribonucleic acid sulphate removed by centrifuging. The clear supernatant was dialysed against three changes of distilled water at 4°, filtered to remove insoluble protein, and the histone sulphates were separated into the arginine-rich and lysine-rich fractions by the ethanol precipitation method described above.

Hydrolysis of proteins. Weighed amounts (approx. 2 mg.) of the dried histone sulphates were hydrolysed in 2 ml. of 6N-HCl for 4 hr. at 110° in sealed tubes with 0.01 ml. of 12.5% (w/v) titanous chloride as catalyst (McIlwain & Hughes, 1944). The hydrolysates were neutralized with NaOH and the precipitate of titanous hydroxide removed by filtration. The precipitate was washed three times with water. The hydrolysate was then made up to standard volumes representing 100–200 µg. of protein/ml.

Determination of arginine. The arginine content of hydrolysates of cerebral histones was determined by the modified Sakaguchi method of Satake & Luck (1958) with *N*-bromosuccinimide instead of hypobromite as the oxidizing agent. A standard series of arginine solutions were run with each hydrolysate. This method gave reproducible colours, provided that the oxidizing agent was rapidly added and the colour read at 500 mµ 5–10 min. after development.

RESULTS

Histones from cerebral cortex

Before extracting histones from cerebral cortex slices incubated under chosen conditions, it was necessary to examine the extractability, yields and some properties of histones prepared from fresh un-sliced cerebral cortex. In addition, calf-thymus nuclei were extracted by the same procedure to check the reliability of the methods. Table 1 gives the data obtained. The arginine content of the histone sulphates from cerebral tissues was consistently less than from calf thymus. Salt extraction followed by acid treatment, dialysis and ethanol precipitation in the cold gave slightly higher arginine contents for histone preparations from cerebral tissues, but the yields of histone were lower. Dialysis of acid extracts before ethanol precipitation did not increase the arginine contents nor did reprecipitation with 5N-ammonia solution. The acid-extraction method, followed by fractional

Table 1. *Arginine content and yields of histone sulphates from cerebral tissues and calf thymus*

For details of separation of nuclear and other fractions, extraction of histones and arginine determination, see Experimental section. Method 1*a* refers to acid extraction followed by precipitation with 20% (v/v) cold ethanol. The method 1*b* refers to precipitation with 50% (v/v) final concentration of the cold ethanol supernatant from 1*a*. Method 2 refers to 1.5 M-NaCl extraction, acid treatment, dialysis and precipitation with 20% (v/v) ethanol. When the number of experiments was over three the arginine value is given $\pm$ s.d.; otherwise, each value gives the mean of duplicate determinations on material from one experiment.

Tissue fraction	Method	Yield (mg./g. wet wt. of tissue)	Arginine content (%)
Ox cerebral-cortex nuclei	1 <i>a</i>	2.6	10.9, 10.5
Ox cerebral-cortex nuclei	1 <i>b</i>	1.3	1.2, 1.4
Ox cerebral-cortex nuclei	2	0.5	10.5
Ox cerebral-cortex microsomes	1 <i>a</i>	1.4	5.8, 5.9
Guinea-pig cerebral-cortex nuclei	1 <i>a</i>	2.7	9.1 $\pm$ 0.3 (4)
Guinea-pig cerebral-cortex nuclei	1 <i>b</i>	1.1	1.6, 3.9, 3.2
Guinea-pig cerebral-cortex nuclei	2	0.7	10.6
Guinea-pig cerebral-cortex microsomes	1 <i>a</i>	1.6	4.2, 4.1
Human cerebral-cortex nuclei (right superior frontal lobe)	1 <i>a</i>	2.6	9.2
Calf-thymus nuclei	1 <i>a</i>	4.2	14.2
Calf-thymus nuclei	1 <i>b</i>	2.0	4.2

Table 2. *Distribution of nitrogen, deoxyribose and arginine of proteins extracted from subcellular fractions of unsliced guinea-pig cerebral cortex*

Homogenization of 3 g. of cortex was made with an Emanuel-Chaikoff apparatus in 0.32 M-sucrose with 3 mM- CaCl_2 , pH 7.0. Fractions were separated, acid was extracted and the proteins were precipitated by ethanol, 20% (v/v) at -5° (for details see Experimental section). The mitochondrial fraction was separated by a centrifugal force of 20 000 *g* for 10 min., the microsomal at 105 400 *g* for 30 min. The arginine values marked by an asterisk refer to the protein precipitated by 2.5 vol. of ethanol at -5° , removed by centrifuging, washed and freeze-dried. The arginine values are the means of two determinations.

Tissue fraction	N of fraction		Extracted protein		Deoxyribose of fraction	
	(mg./g. wet wt. of tissue)	(%)	Yield (mg./g. wet wt. of tissue)	Arginine content (%)	(μ g./g. wet wt. of tissue)	(%)
Whole homogenate	16.04	100	—	—	1820	100
Washed nuclei	4.47	28	—	—	999	55
Mitochondria	4.13	25	—	—	605	33
Microsomes	1.10	7	—	—	75	4
Supernatant	6.62	41	9.1	3.3*	91	5
Recovery	—	101	—	—	—	97
0.14 M-NaCl washings of nuclei	0.46	3	1.0	3.9*	—	—
First acid extract, nuclei	1.51	9	2.7	9.4	16	1
Second acid extract, nuclei	0.4	2	0.2	7.6	—	—
Acid extract, mitochondria	0.6	3	1.0	8.1	—	—
Acid extract, microsomes	1.13	6	1.5	4.2	—	—

ethanol precipitation in the cold, was the simplest method that gave satisfactory yields of arginine-rich histones, and this method was used for extraction of histones from slices. A minimum of 1.5 g. of guinea-pig cerebral-cortex slices (i.e. three hemispheres) was necessary to give an adequate yield of dry protein (i.e. 4 mg.). Basic proteins, extracted by acid from whole ox brain and from the guinea-pig 'microsome' fractions, consistently contained less arginine than the basic proteins obtainable from the separated nuclei. This finding was important as it enabled comparisons to be made between non-nuclear particulate fractions from slices treated in cold media (see below).

Table 2 shows a representative experiment in which fresh unsliced guinea-pig cerebral cortex was separated into subcellular fractions in sucrose-calcium chloride media, and the nitrogen, the deoxyribose and the arginine of the acid-extractable proteins were determined. When examined under phase contrast, the washed nuclear fraction showed little contamination with mitochondria. The mitochondrial fraction contained considerable numbers of small neuroglial nuclei and this is further reflected in the deoxyribose values. The first acid extraction of the nuclear fraction yielded proteins with the highest arginine content. Acid extraction of the mitochondrial pellet also yielded

basic proteins of high arginine content, but this was due to contamination by small nuclei. The basic proteins from the microsomal pellet were of low arginine content. Protein from the supernatant fraction, precipitated by 2.5 vol. of ethanol in the cold, contained 3–4 % of arginine.

Properties of cerebral cortex histones. Preliminary studies of the properties of the acid-extractable basic proteins were carried out to indicate that histones were in fact being prepared. The objective, at this stage of the study, was to obtain the mixed basic proteins and ensure that the reproducibility of the extraction procedures and the composition of the extracts were sufficient to enable comparisons to be made between extracts from tissue slices with or without pretreatment in cold media.

The undenatured, freeze-dried, cerebral cortex histones were white fluffy powders, soluble in water at neutral and at acid pH, and insoluble in 5N-ammonia solution. Their nitrogen content was approx. 17 %. On adding dilute alkali to a clear aqueous solution of the protein, clouding commenced at pH 9.0, and reached a maximum at pH 10.0 with no further turbidity above pH 10.5. The isoelectric point thus lies between pH 9 and 10.5. The solid proteins stain intensely with the dye Fast green FCF. The molecular weight of some components of the histones was large enough to prevent the proteins from passing through a cellophan dialysis membrane. Dialysis however did lead to losses of some components and to some formation of insoluble precipitates of material within the sac. The ultraviolet absorptions of guinea-pig and ox cerebral-cortex histones were determined in a Hilger spectrophotometer in 0.01N-hydrochloric acid. There was no absorption peak at 260 $m\mu$ and the ratio of absorption at 260 $m\mu$ to that at 280 $m\mu$ was 0.7. This indicates no contamination with nucleic acids. For ox cortex histone, $E_{1\text{cm}}^{1\%}$ was 5.4 at 280 $m\mu$ and 26.6 at 220 $m\mu$; for guinea-pig cortex histone, $E_{1\text{cm}}^{1\%}$ was 5.2 and 26.3 at 280 $m\mu$ and 220 $m\mu$ respectively. Cruft, Mauritzen & Stedman (1958) reported that $E_{1\text{cm}}^{1\%}$ was 6.035 at 275 $m\mu$ for 98 % pure β -histone from calf thymus.

A qualitative analysis of the amino acids in hydrolysates of the cerebral cortex histones was made by two-dimensional paper chromatography with pyridine-*n*-butan-1-ol-water (1:1:1, by vol.) and butan-1-ol-acetic acid-water (12:3:5, by vol.) as solvent systems. Of the basic amino acids, arginine gave the most intense spot with ninhydrin and Sakaguchi sprays. Other features of the amino acid composition were the absence of cystine and the occurrence of only trace amounts of methionine and taurine. Tyrosine was present. On alkaline hydrolysis there was no tryptophan and the Hopkins-Cole test was negative. Electrophoresis of guinea-pig cerebral-cortex histones in starch gels

(0.01M-acetate buffer, pH 5.0, 120V, 16 hr., 4°) revealed nine zones in the arginine-rich fraction and two zones in the lysine-rich fraction migrating to the cathode, with one faint zone migrating to the anode. It was apparent that the arginine-rich histones were a mixture of basic components.

Histones from cerebral cortex slices with and without exposure in cold media

The results in Table 2 show that the arginine contents of acid-extractable proteins from nuclei and microsomes, and of the ethanol-precipitable protein from the supernatant fraction, are characteristically different. If a migration of histones from the nucleus to some extranuclear site has occurred during storage in cold media it should be demonstrated by changes in the arginine content of the acid-extractable proteins from the subcellular fractions.

The results of three experiments on sliced cerebral cortex, incubated for 40 min. at 37.5° with and without prior treatment at 0° for 16 hr., are shown in Table 3. Considerable protein was lost from the slices to the phosphate media, both during incubation at 37.5° and on keeping in the cold. Ethanol-precipitated proteins from the incubation media had arginine contents which were similar for slices untreated and treated in cold media. If basic protein is lost from the nuclei of cold-treated slices, it does not appear in significant amounts in the incubation media but remains within the slice. Separation of homogenates of slices into subcellular fractions was considerably more difficult than the corresponding separation of unsliced tissue. The mitochondria were swollen, clumped together and adhering to the nuclei. They were thus co-precipitated with the nuclei on differential centrifuging. Four washings did not give clean nuclear preparations. The contamination of the nuclear fraction by mitochondria and other subcellular particles is shown by the high nitrogen values. The homogenates of slices were accordingly separated into three fractions: (1) a 'nuclear' fraction after centrifuging at 800 g for 10 min., (2) a 'particulate' fraction after centrifuging at 105 400 g for 30 min. and (3) a supernatant. The particulate fraction contained small mitochondria and microsomes but the yield of acid-extractable protein from this fraction was too small for measurements of arginine content to be made.

Table 3 shows that the basic proteins from the 'nuclear' fractions of cold-treated slices are both less in quantity and contain considerably less arginine than the same fractions from untreated slices. However, the arginine content of proteins precipitated from the supernatant is higher in cold-treated slices. These findings suggest that arginine-rich protein has been lost from the nuclei of these

slices and has in part appeared in the supernatant fraction. They do not rule out the possibility that the basic protein may have left the nuclei and become fixed to other subcellular particles. The nuclear fraction, derived from tissue kept in cold media, is contaminated with other subcellular particles and consequently if basic protein was attached to these particles they would be acid-extracted along with the nuclei. For this reason it was decided to attempt cleaner separations of the nuclei from sliced tissues by layering the 0.32M-sucrose homogenate over M-sucrose and centrifuging at 50 000 g for 1 hr. After centrifuging, the 0.32 M-sucrose layer and also

the M-sucrose layer were pipetted off, leaving a small pellet in the bottom of the centrifuge tube. Samples from the upper and lower layers and from the pellet were examined microscopically under phase contrast. The upper layer contained most of the nuclei with fragments of myelin and other membranes. The nuclei from slices that had been pretreated at 0° contained no nucleoli and were not granular in appearance, although the nuclei were intact and normal in shape. Nuclei from untreated slices contained nucleoli, but were swollen and showed a fine granularity to the nucleoplasm. The lower layer contained mitochondria, many small particles and

Table 3. Guinea-pig cerebral-cortex slices untreated and pretreated in cold media: respiration, arginine content of proteins from incubation media and subcellular fractions, and nitrogen distribution

Slices were prepared and respiration was determined with and without pretreatment at 0° for 16 hr. in buffered phosphate-glucose media. After respiratory measurements, slices were drained, homogenized and separated into the 'nuclear' fraction (800 g, 10 min.) and 'particulate' fraction (105 400 g, 30 min.), and the fractions were acid-extracted. The proteins were precipitated by ethanol (20%, v/v) at -5°, and their arginine was determined by a modified Sakaguchi reaction. For details see the Experimental section. Proteins in the incubation media, supernatant and NaCl washings were precipitated by 2.5 vol. of ethanol at -5°. Arginine values are the mean of duplicate determinations in three separate experiments. Respiration and lactate formation are expressed as mean values ±S.D. with the number of measurements in brackets.					
Material	A. From fresh slices incubated at 37.5° for 40 min.		B. From slices kept at 0° before incubation as in A		
	N of material (mg./g. fresh wt. of tissue)	Arginine of extracted protein (%)	N of material (mg./g. fresh wt. of tissue)	Arginine of extracted protein (%)	
Whole homogenate	14.6	—	13.2	—	
Medium of 0° exposure	—	—	2.8	3.4, 2.8, 3.5	
Incubation media	1.4	2.5, 2.3, 2.6	1.7	2.6, 2.1, 2.0	
Nuclear fraction	7.9	—	7.4	—	
Particulate fraction	1.7	—	1.0	—	
Supernatant	3.3	4.4, 3.3, 3.8	2.4	6.6, 6.1, 5.1	
0.14 M-NaCl washings	0.5	5.5, 6.0, 6.1	0.6	6.2, 4.7, 5.0	
Acid extract of nuclear fraction	2.1	8.6, 9.0, 8.3	0.9	5.8, 6.4, 5.5	
	A. From fresh slices incubated at 37.5° for 40 min.		B. From slices kept at 0° before incubation as in A		
	Respiratory rate (μmoles of O ₂ /g./hr.)	Lactic acid accumulating (μmoles/g./hr.)	Respiratory rate (μmoles of O ₂ /g./hr.)	Lactic acid accumulating (μmoles/g./hr.)	
Slices before homogenizing	57 ± 3 (6)	28 ± 5 (4)	42 ± 4 (6)	31 ± 6 (4)	

Table 4. Arginine contents of acid-extracted proteins from isolated nuclei and other particles of cerebral cortex slices untreated and pretreated by cold media

Slices of guinea-pig cerebral cortex (approx. 1.5 g.) after incubation at 37.5° for 40 min. were homogenized and layered on M-sucrose in the SW 25.1 rotor of a Spinco ultracentrifuge and spun at 50 000 g for 1 hr. The upper and lower layers were pipetted off. Under phase contrast, the upper layer contained mainly nuclei and the lower layer mainly small particles with a few nuclei. The lower layer was diluted to 0.3 M-sucrose and both layers were spun at 40 000 rev./min. for 30 min. in the 40 rotor. The pellets were extracted with 0.2 N-H ₂ SO ₄ , the proteins were separated, and the arginine content was determined as outlined in the Experimental section. Arginine values are the mean of two determinations.				
Layer extracted	Extracted material (mg.)		Arginine content (%)	
	Slices not exposed at 0°	Slices exposed at 0°	Slices not exposed at 0°	Slices exposed at 0°
Upper layer (mainly nuclei)	2.0	No protein obtained	8.7	—
Lower layer (mainly other particles)	1.6	1.5, 1.8	5.7	9.7, 9.4

a few small nuclei which had thus passed through the *m*-sucrose layer. The upper layer and the lower layer diluted to 0.3*M*-sucrose were centrifuged at 105 400 *g* for 1 hr., and the pellets acid-extracted. The results are shown in Table 4. No basic protein could be extracted from the layered nuclei from slices pretreated in cold media. Untreated slices contained nuclear basic proteins of arginine content similar to unsliced tissue (see Table 2). The particles, in the lower *m*-sucrose layer, from cold-treated slices contained basic protein of high arginine content. The basic proteins extracted from the lower layer particles from slices not subjected to cold media contained less arginine, approaching rather the values obtained for microsomes from fresh cortex (see Table 1).

These experiments indicate a complete loss of basic protein from the nuclei of cold-treated slices and the appearance of an arginine-rich, acid-extractable protein in the small-particle fraction. Further qualitative evidence for a loss of nuclear constituents in cold-treated slices was obtained by observing the effects of 2*M*-sodium chloride on isolated nuclei. When nuclei from fresh slices were suspended in 2*M*-sodium chloride and examined under phase contrast, a halo of extracted deoxy-ribonucleoprotein was visible (see Philpot & Stanier, 1956). Nuclei obtained from slices kept in the buffered phosphate-glucose media at 0° for 16 hr., and then suspended in 2*M*-NaCl, showed no nucleoprotein halos and did not form gels.

Combination of microsomal particles with histones

With the object of localizing more specifically the type of subcellular particles that might be involved

in the binding of histones released from nuclei under adverse conditions, properties of freshly prepared subcellular fractions from unsliced cerebral cortex tissue were studied. In particular, their ability to remove histones from solution was examined. In these experiments weighed samples from separated subcellular pellets were suspended in phosphate buffer (0.001*M*, pH 7.0) containing a known amount of histone and the suspension was clarified by centrifuging at 105 400 *g* for 30 min. Minimal salt concentrations were employed in these experiments to avoid precipitation or flocculation of the histone or the particles by the buffer alone. After centrifuging, measured portions of the supernatant solutions were hydrolysed and their arginine content was determined. The results are shown in Table 5. It was observed that when the microsomal fractions were suspended with histones from nuclei of the cerebral cortex itself or from calf thymus, flocculation occurred. The results in Table 5 show clearly that the microsomal fraction is the most active in combining with histones and removing them from solution.

Experiments were performed in which the microsomal fraction was suspended in protein solutions containing crystalline bovine albumin, γ -globulin (Cohn fraction II, Armour Laboratories) and purified gelatin at concentrations of 1 mg./ml. in phosphate buffer (0.001*M*, pH 7.0). After the suspension had been clarified by centrifuging at 105 400 *g* for 30 min. the protein content in a sample of the supernatant was determined. Under these conditions bovine plasma albumin and gelatin were not adsorbed by the particles and only 5% of the γ -globulin was removed from solution. These

Table 5. *Combination of subcellular particles from cerebral cortex with histones*

Subcellular fractions were separated from fresh cortex and homogenized in 0.32*M*-sucrose with 0.5*M*-EDTA, at pH 7.0, as outlined in the Experimental section. Samples (200 mg.) of the particulate pellets were suspended with histone solutions in 2 ml. plastic tubes for 10 min. Histone solutions contained 1 mg. of dried protein/ml. in *mm*-phosphate buffer at pH 7.0. The suspensions were centrifuged at 105 400 *g* for 30 min. in the 40 rotor of the Spinco ultracentrifuge. Samples of supernatants were taken for hydrolysis of the protein remaining, and arginine was determined in the hydrolysate. Arginine values lower than the control solution containing no added particles indicate removal of histone from solution.

Tissue fraction	Centrifugal force used in preparation		Arginine content of supernatant after suspension with 200 mg. of fraction (μ g./ml.)	
			Suspended with guinea-pig cerebral-cortex histone	
	<i>g</i>	min.	Suspended with thymus histone	
Nuclei	800	10	127	83
Large mitochondria	5 000	10	132	90
Small mitochondria	20 000	10	83	60
Microsomes	105 400	30	22	27
Histone alone	105 400	30	143	101

experiments indicate that the removal of proteins from solution by microsomal particles is to some extent specific for basic proteins and is not due to non-specific protein adsorption.

DISCUSSION

The suggestion (McIlwain, 1959), that histones leave their normal nuclear site when cerebral cortex slices are kept in buffered glucose media in the cold, has been shown in the present study to be correct. Nuclei from cold-treated slices yielded no arginine-rich basic proteins, but a particulate fraction from these slices containing predominantly small mitochondria and microsomes is considerably richer than one from untreated slices in these proteins. Further, freshly prepared microsomal fractions contain the particles most active in combining at neutral pH with histones and removing them from solution. It is concluded that in cold-treated slices the histones from the nuclei have combined with some component of the microsomal fraction. It appears likely that this combination is the basis of the loss of responsiveness to electrical stimulation, both in tissues kept in cold media and in normal tissues to which histones and protamines have been added as such.

The nuclear membrane is known to be permeable to some proteins (see Roodyn, 1959). Protamine and histone solutions have been observed to pass easily into isolated rat-liver nuclei (Anderson & Wilbur, 1952; Philpot & Stanier, 1957). Pollister & Leuchtenberger (1949) reported a 75 % loss of protein from guinea-pig nuclei immersed in physiological saline at 5° for 3 hr. without any changes in the microscopic appearance of the nuclear membrane. Recent studies of nuclear structure with the electron microscope have shown that the nuclear membrane has a fenestrated appearance and contains 'pores' and 'annulae' ranging from 250 to 1000 Å in diameter (Watson, 1954, 1959; Beams, Tahmisian, Devine & Anderson, 1957). If these structures are in fact true pores they are large enough to allow passage of proteins, particularly histones, if their associations within the nucleus are disrupted.

Histones are regarded as existing in the nucleus as salt-like compounds with deoxyribonucleic acid (for recent observations, see Crampton, 1957; Zubay & Doty, 1959). The ability to form gels, by cross-linkages between the long nucleohistone units, is a good indication of the integrity of the deoxyribonucleoprotein of the nucleus, and it has been shown that deoxyribonucleases prevent this gel formation (Dounce, 1955; Dounce, O'Connell & Monty, 1957). Isolated liver nuclei are permeable to these enzymes which affect their microscopic appearance (Anderson, 1953). Philpot & Stanier

(1956) showed that the optical and size changes, seen under phase contrast in nuclei placed in various media, were due to alterations in the nucleohistone gel and not to tonicity effects. The inability of nuclei from cerebral cortex slices kept in cold media to form nucleohistone gels, and the absence of granularity of the nucleoplasm, is further evidence for a dissociation of nucleic acids and histones. Whether this is due to a direct effect of cold, to the action of nucleases or is a result of the swelling of cerebral tissues in buffered, iso-osmotic glucose media (Pappius & Elliott, 1956) is unknown; more than one of these factors may be involved. Any specific localization requiring metabolic maintenance may be suspected to become defective in cold tissue, thus allowing the distribution of cell constituents to be conditioned to a greater extent by purely chemical affinities. The affinity for basic proteins of sites outside the nucleus is clearly shown by the results of Table 5. However, for development of the unresponsive state at 0° it was necessary that the tissue should be in contact with fluid media. The tissue under these conditions imbibes fluid, which may thus provide additional channels for movement of tissue constituents.

Of particular interest is the demonstration that microsomal fractions are the most active in binding histones. It is possible that the anionic sites, previously postulated (McIlwain, 1959) as being involved in the response of tissues to electrical excitation, are located in this fraction and are the entities inhibited by combination with histones and protamines. Basic proteins may be useful substances to aid in the characterization of the acidic, microsomal components. Basic proteins, particularly protamines, are known to form complexes with acidic substances of large molecular weight (e.g. insulin, heparin, phosphorylase and acidic mucopolysaccharides) and to interact with serum proteins (Portman & Holden, 1959; Rubini, Becker & Stahmann, 1953; Madsen & Cori, 1954; Bollet, Seraydarian & Simpson, 1957; Carroll, Callanan & Saroff, 1959; Truffet, de Gennes, Robert & Polonovski, 1958). The microsomal fraction of tissue homogenates, particularly of cerebral tissues, is heterogeneous but has been shown under the electron microscope to consist essentially of fragmented ergastoplasmic elements (Chauveau, Gautier, Moulé & Rouiller, 1955). In nerve cells the ergastoplasm is most conspicuous and is orientated in areas corresponding to the Nissl bodies. It consists of a continuous system of intracytoplasmic membranes, connected both to the nucleus and to the outer cell membrane, and small dense ribonucleoprotein particles closely associated with the membranes (Palade & Porter, 1954; Palay & Palade, 1955; Haguenau, 1958; Watson, 1955; DeRobertis, 1954). Evidence for the possibility that the acidic

sites responsible for binding basic proteins are located within these membrane structures is being sought.

SUMMARY

1. Histones were extracted from isolated nuclei of unsliced and sliced cerebral cortex and some of their properties studied. The arginine-rich histone contains at least nine components. The arginine content is 10–11%. Basic proteins from microsomal fractions contain 4–6% of arginine.

2. Nuclear histones are lost from nuclear fractions made from sliced cerebral cortex, which has been kept in cold buffered glucose media. Arginine-rich proteins can then be extracted from other subcellular particles of the tissue. Some basic protein also appears in the soluble supernatant proteins. No loss of histones occurred in nuclei from slices not subjected to cold media.

3. Isolated cerebral microsomal fractions bind histones extracted from cerebral cortex nuclei; they also bind calf-thymus histone.

4. It is suggested that the migration of the histone from the nuclei of slices kept in cold media and its combination with acidic microsomal constituents is involved in the concomitant loss of the tissues' response to electrical excitation.

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