The Subcellular Localization of Cerebral Phosphoproteins Sensitive to Electrical Stimulation

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1. On incubating cerebral-cortex slices at 37° in an oxygenated medium marked changes resulted in the subcellular distribution of proteins and phosphoproteins in the tissue. The protein content of the nuclear fraction more than doubled, whereas the yields of microsomal and supernatant proteins were both markedly decreased. The amount of phosphoprotein/mg. of protein decreased in the microsomal and supernatant fractions, but showed little change in the nuclear and mitochondrial fractions. The loss of microsomal protein could be partly prevented by rinsing the slices briefly in cold sucrose solution before dispersion; the altered subcellular distribution was apparently related to contamination of the dispersing solution with traces of salts from the medium. 2. The subcellular location of the phosphoprotein sensitive to the effects of electrical pulses applied to cerebral slices *in vitro* has been reinvestigated by two different procedures. Comparison between unstimulated and stimulated slices after incubation in the presence of [^{32}P]orthophosphate showed that phosphoprotein radioactivity increased on stimulation to a greater extent in a membrane-rich fraction than in a mitochondria-rich fraction, these being obtained by immediate density-gradient fractionation of the tissue dispersion. With fractions isolated by differential centrifuging the percentage increase in a combined mitochondrial and nuclear fraction was 5% as compared with 24% ($P < 0.02$) in the microsomal fraction and 30% in the original dispersion before fractionation. The sensitive phosphoprotein therefore appears to be located in structures sedimenting with the microsomal fraction, rather than with the nuclear fraction as previously claimed.

In a previous attempt to determine the intracellular location of the phosphoprotein that is sensitive to the effects of electrical excitation of cerebral tissue (Heald, 1957, 1959) cerebral slices were incubated with [^{32}P]orthophosphate, stimulated for 10sec., dispersed in sucrose solution and then centrifuged at 1500*g* for 10min. to yield a nuclear fraction and a post-nuclear supernatant containing mitochondria, microsomes and supernatant proteins. The specific radioactivities of the phosphoproteins in the two fractions were then determined and compared with the values for similar fractions from unstimulated slices. The major increase in phosphoprotein radioactivity was found in the nuclear fraction. A more precise location within the nuclear fraction was then explored on the basis of experiments with non-stimulated slices, in which it was assumed that the most rapidly metabolizing phosphoprotein within this fraction (as judged by incorporation of radioactivity under resting conditions) was identical with the protein sensitive to the effects of the pulses. This led to the suggestion that the nuclear fraction contains a phosphoprotein-rich structure involved in the response that is distinct from nuclei, mitochondria or microsomes (Heald, 1962).

Though it is clear that the increase in phosphoprotein radioactivity occurring as a result of stimulation is associated with particulate matter, there are several methodological reasons for reconsidering the suggestion of a more precise location within a novel subcellar fragment. First, it is difficult to accept, in the light of later work, that incorporation of [^{32}P]-orthophosphate into protein under resting conditions is necessarily a satisfactory measure of a protein involved in the response of the intact tissue to electrical excitation: metabolically active phosphoproteins occur in all parts of the cell (Trevor, 1963; Rodnight & Lavin, 1964*a*) and their rate of uptake of [^{32}P]phosphate no doubt depends on many factors. Secondly, interpretation of Heald's

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(1957, 1959) results is rendered difficult by the abnormally high amount of particulate matter present in the nuclear fraction, which contained over 45% of the total protein nitrogen of the tissue. This abnormal situation apparently arose from the application of an unusually high initial centrifugal force to dispersions of tissue that had been previously incubated in a saline medium. The latter treatment is known (as was recognized by the author) to result in a nuclear fraction severely contaminated with non-nuclear material (see, e.g., Wolfe & McIlwain, 1961), and with respect to the high centrifugal force it is shown in the accompanying paper (Trevor, Rodnight & Schwartz, 1965) that dispersions of fresh tissue submitted to the technique used by Heald (1957, 1959) yield a nuclear fraction containing considerable quantities of mitochondria, nerve endings and heterogeneous material probably of membranous origin. Added to the difficulties raised by cross-contamination is the observation (Trevor *et al.* 1965) that the nuclear subfraction, considered by Heald (1957, 1959) to contain the sensitive phosphoproteins is no richer in phosphoprotein than are membrane fractions derived from other sources, such as the microsomal fraction. In the accompanying study (Trevor *et al.* 1965) morphological examination also failed to produce evidence for the occurrence within the subfraction of a structure clearly distinct from contaminating nerve endings or membrane fragments of microsomal origin. It therefore seemed important to apply alternative fractionation techniques to this problem with the aim of obtaining more clearly defined fractions, by using as a measure of the sensitive phosphoprotein comparative observations on stimulated and unstimulated slices, rather than [^{32}P]orthophosphate incorporation under resting conditions. A preliminary report on this work has already appeared (Trevor & Rodnight, 1963).

EXPERIMENTAL

Preparation and incubation of tissue slices

Slices of guinea-pig cerebral cortex were prepared as described by McIlwain & Rodnight (1962) and incubated in the phosphate-free tris-buffered medium of Heald (1956). Slices were supported in quick-transfer holders incorporating the stimulating electrodes (McIlwain & Rodnight, 1962) and placed in beakers containing 5ml. of medium and incubated at 37·5° with continuous gassing with oxygen. After 30min., 0·2ml. of carrier-free [^{32}P]orthophosphate (10–20μc; The Radiochemical Centre, Amersham, Bucks.) was added and incubation continued for a further 3min. The slices were then rapidly removed and released into 50ml. of fresh oxygenated medium at 37·5°. After 10sec., the slices were again floated into transfer holders and placed in beakers containing 5ml. of fresh medium under the same conditions as the original incubation. Then, 2min. later, condenser discharge pulses from the generator of Ayres & McIlwain (1953) of frequency 100cyc./sec., duration 0·4msec. and peak voltage 15v were applied to the slices for a period of 10sec. They were then immediately released into 20ml. of 0·32M-sucrose at 0–2° to remove traces of saline and finally dispersed in either 0·32M-sucrose containing EDTA (0·5mM) at pH7·0 for subcellular fractionation, or in 10% (w/v) trichloroacetic acid. Control slices were treated identically except that the electrical pulses were not passed. Most experiments involved between six and 12 slices, and, since the manipulations detailed above could not be carried out simultaneously on all slices, they were individually subjected to the procedure in order of their preparation, the time-interval between dispersion or fixation of the first and twelfth slice being about 25min.

Fractionation of tissue dispersions

To compare the distribution of phosphoproteins from incubated slices with that found in fresh tissue, the procedure given by Trevor *et al.* (1965) was used except that the two mitochondrial fractions were combined. In experiments designed to localize the phosphoprotein fraction sensitive to electrical stimulation, one or other of two fractionation procedures was applied, both of which enabled the fractions to be fixed with acid denaturant within 90min. of dispersion in sucrose solution. In the first, a simplified density-gradient fractionation was used that involved layering of the dispersion of incubated tissue (100mg./ml.) over a discontinuous gradient consisting of 7ml. of 1·0M-, 8ml. of 1·2M- and 6ml. of 1·4M-sucrose and centrifuging at 10^5g for 75min. in the SW25 head of the Spinco model L machine. In the second procedure particulate fractions were obtained by centrifuging the dispersions for 15min. at $13{\cdot}3\times10^3g$-min. (F1) and then centrifuging the supernatant for 60min. at $10{\cdot}4\times10^4g$-min. (F2) in the no. 40 head of the Spinco model L centrifuge.

Analytical methods

Protein, succinate dehydrogenase and phosphorus. These were determined by the methods given by Trevor *et al.* (1965).

Phosphoprotein phosphorus. In preliminary comparative studies of subcellular fractions derived from fresh and incubated tissue, phosphoprotein P was measured as alkali-labile P (McIlwain & Rodnight, 1962). This method was found inadequate for reliable estimation of the specific radioactivity of the protein P owing to non-specific adsorption of ^{32}P by the insoluble residue. Since an absolute value for the concentration of phosphoprotein P in the tissue was not required, determination of the specific radioactivity of a fraction of the phosphorylserine released by partial acid hydrolysis of the insoluble residue was considered satisfactory. The procedure used was adapted from those of Schaffer, May & Summerson (1953) and Heald (1958). After extraction with 10% trichloroacetic acid, ethanol and chloroform–methanol (2:1,v/v), the protein residues derived from approx. 100mg. of tissue were hydrolysed for 6hr. in sealed hard-glass tubes with 2ml. of 2N-HCl at 100°. This period was chosen from studies on the time-course of the release of phosphorylserine from ^{32}P-labelled protein and observations on the breakdown of the phosphorylated amino acid to serine and phosphorus during the acid hydrolysis (Fig. 1); the quantity of phosphorylserine released was

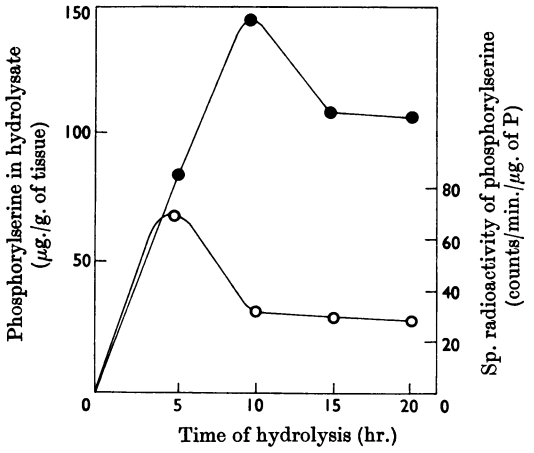

Fig. 1. Release of radioactive phosphorylserine during the hydrolysis of cerebral proteins. Four slices of guinea-pig cerebral cortex were prepared and incubated as described in the text. After 30min., 10μc of [^{32}P]orthophosphate was added to the medium and incubation continued for a further 10min. The tissue was then dispersed in 10% trichloroacetic acid and extracted with chloroform–methanol (2:1, v/v) and ethanol. The protein residue was divided into four portions by resuspension in ethanol and each was heated in 2ml. of 2N-HCl at 100° for various time-intervals. The concentration and specific radioactivity of phosphorylserine present in each hydrolysate was determined as described in the Experimental section. The values for the Figure were derived from three experiments. ●, Phosphorylserine present in hydrolysate; ○, specific radioactivity of phosphorylserine.

maximal at 10hr., and the specific radioactivity reached a peak after hydrolysis for 5hr. The hydrolysates were freeze-dried in the presence of NaOH pellets and conc. H_2SO_4 for 10hr. and the residues dissolved in 2ml. of 0·05N-HCl. They were then fractionated on columns (24cm. × 3cm.^{2}) of Dowex 50 (X12; H^+ form; 200–400 mesh) with 0·05N-HCl as the eluent at a flow rate of 15–25ml./hr. Radioactive inorganic phosphate was eluted from the columns between 20 and 50ml. and phosphorylserine between 70 and 100ml. Reproducibility was good and it was not necessary to extract nucleic acids from the tissue or to use carrier phosphorylserine. The fractions between 70 and 100ml. were evaporated to dryness at 40° in a rotary vacuum evaporator and the residues taken up in 4·5ml. of 0·05N-HCl. Two samples (2ml.) were transferred to small test tubes and again evaporated to dryness. Perchloric acid (2ml. of 12N) was added to each residue and one of each pair digested for 10min. to release P_i from phosphorylserine. The other sample served as a control for contamination due to free P_i, which never exceeded 10–15% of the phosphorylserine phosphorus. Approx. 1·8μg. of P was released from the phosphorylserine samples after digestion. The radioactivity of contaminating P_i contributed less than 20% of the total radioactivity derived from each phosphorylserine sample; all values quoted have been corrected for P_i radioactivity.

Determination of radioactivity. This was carried out after the measurement of inorganic phosphorus derived from the phosphorylserine residues. The blue solutions were diluted to 10ml. and radioactivity was determined in a liquid counter M6H (20th Century Electronics Ltd., New Addington, Surrey) with the Ecko scaler N530A (Ecko Electronics Ltd., Southend-on-Sea, Essex). The specific radioactivity is defined as counts/min./μg. of phosphorus and all measurements are expressed in this form.

RESULTS

Subcellular distribution of phosphoproteins after incubation of tissue slices. To determine the extent to which the subcellular distribution of phosphoproteins was changed after the incubation procedures, cerebral slices were immersed in oxygenated medium at 37·5° for 30min. and then dispersed in 0·32M-sucrose containing EDTA (0·5mM). The dispersion was separated into four primary fractions by differential centrifugation, and each was analysed for protein and phosphoprotein P. The results were compared with those obtained from corresponding fractions derived from fresh tissue (Table 1). During incubation there is a loss of 12–14% of the total tissue protein to the medium, and in addition the general distribution of particulate material is changed. The protein content of the microsomal and supernatant fractions is markedly decreased, changes in the latter fraction probably accounting mainly for the loss of total protein into the medium. The nuclear fraction is increased in protein content, presumably owing to contamination with mitochondria and microsomal components. Although there is some loss of phosphoprotein P during incubation, assumed to be due to enzymic dephosphorylation, the phosphoprotein P concentration of the nuclear fraction is unchanged. The extent to which dephosphorylation or modifications in the subcellular distribution of proteins contribute to the changes in protein P within the fractions cannot be assessed.

An alteration in distribution of protein after incubation was also observed in experiments in which brain slices were held in quick-transfer holders. Here the yield of microsomal protein was decreased even more, the protein content of this fraction being in the range 0–3mg./g. compared with approx. 12mg./g. for that from slices allowed to float freely in saline during incubation. No metabolic differences between free and held slices were observed during 30min. incubation as judged by phosphocreatine and potassium concentrations and lactic acid production. It was observed, however, that, when slices were released from their holders into 20ml. of ice-cold 0·32M-sucrose and then transferred after rinsing for a few seconds to the dispersion medium, the yield of microsomal protein approached 60% of that obtained from fresh tissue. It appears

Table 1. *Changes in the subcellular distribution of phosphoproteins after incubation of brain slices*

Three cerebral-cortex slices of approximate weight 100mg. were prepared from one hemisphere of each of two guinea-pig brains and incubated in Warburg vessels containing 5ml. of phosphate-free tris-buffered medium at 37° with O_2 for 30min. The six slices were then briefly drained of excess of medium and homogenized in 0·32M-sucrose to give a dispersion containing 100mg. of tissue/ml. A similar dispersion of fresh cortical tissue from the contralateral hemispheres was prepared at the time of cutting of slices. The dispersions were fractionated by differential centrifugation and phosphoproteins determined as alkali-labile P. Results represent the mean values derived from three distinct experiments.

Fraction and centrifugal force used (*g*-min.)	Protein (mg./g. of tissue)		Phosphoprotein P (mμmoles/mg. of protein)	
	Fresh tissue	Incubated tissue	Fresh tissue	Incubated tissue
Homogenate	94·3	83·0	9·4	7·4
Nuclear (8×10^3)	8·6	19·6	9·9	9·5
Mitochondrial (2×10^5)	38·6	34·6	6·0	6·5
Microsomal (7×10^6)	22·1	11·3	12·2	8·0
Supernatant	18·9	10·5	11·8	4·3

Table 2. *Effect of electrical stimulation on radioactivity of phosphoprotein in subcellular fractions isolated by a density-gradient procedure*

Six cerebral-cortex slices were prepared and incubated in quick-transfer holders as described in the text. Immediately after stimulation the slices were homogenized in 0·32M-sucrose to give a dispersion containing 100mg. of tissue/ml., which was layered over a sucrose density gradient and centrifuged at 125000*g* for 60min. The specific radioactivity of protein-derived phosphorylserine was determined in the fractions equilibrating over 1·2M- (*B*) and 1·4M-sucrose (*C*). Control slices were treated identically except that no electrical pulses were passed. The results are derived from three experiments.

Fraction	Protein (mg./g. of tissue)	Succinate-dehydrogenase activity (μmoles of succinate/mg. of protein/hr.)	Sp. radioactivity of protein [^{32}P]phosphorylserine (counts/min./μg. of P)	Percentage increase in radioactivity on stimulation (control=100)
B Control	40·5	3·5	12·1 (11·8, 12·1, 12·7)	173
Stimulated	39·5	3·1	21·1 (20·2, 20·4, 23·0)	
C Control	27·3	6·3	9·0 (8·3, 9·1, 9·6)	148
Stimulated	26·0	6·5	13·3 (13·9, 12·6, 13·5)	

that the presence of small quantities of saline in the dispersion medium results in gross changes in the distribution of particulate material. This conclusion was confirmed by an experiment in which small quantities of sodium chloride were added to a dispersion of fresh cerebral cortex in 0·32M-sucrose before fractionation. The final concentrations of sodium chloride used were 1·0, 3·4 and 5·8mM; the yields of microsomal protein/g. of tissue for the three concentrations were 15·1, 7·6 and 4·6mg. respectively. From these findings it was estimated that 0·1ml. of incubation medium carried with the slice or tissue holder to the dispersion medium (usually 5ml. of sucrose solution) would result in a 50% decrease in the yield of microsomal protein on subsequent fractionation. The difference in microsomal protein concentration derived from free and mechanically restrained slices apparently arises from the extra volume of saline carried to the sucrose between the tissue and the jaws of the holder. These observations led to adoption of the procedure of rinsing slices in sucrose immediately after incubation, followed by dispersion in fresh sucrose solution.

Stability of protein-bound ^{32}P. The stability of protein-bound ^{32}P of tissue dispersions in sucrose solution at 0–2° was determined after incubation and stimulation of cerebral-cortex slices under the usual conditions. The tissue was rapidly dispersed in either 10% trichloroacetic acid or 0·32M-sucrose, the latter being kept at 0–2° for various intervals before fixation with trichloroacetic acid. Fig. 2 shows the results of two experiments. Within 2min. of dispersion in sucrose the specific radioactivity of protein phosphorylserine from both control and stimulated slices decreased to approx. 70% of the value at zero time. Subsequently the phosphoprotein radioactivity changed only slowly. The difference in specific radioactivity between control and stimulated samples decreased by about 14% in 30min. In attempts to minimize this decline in

radioactivity, a number of agents were added to the sucrose solution including EDTA (5mM), iodoacetate (1mM), cyanide (1mM), mercuric chloride (0·1mM) and ferric chloride (0·1mM). None of these agents prevented a loss of radioactivity, although iodoacetate, EDTA and cyanide have been shown by Rose & Heald (1961) to inhibit the activity of a partially purified phosphoprotein phosphatase. However, without any additions to the sucrose solution, an effect of electrical stimulation on phosphoprotein radioactivity could still be detected after a period adequate to carry out at least a partial subcellular fractionation as described in the Experimental section.

Fractionation by density-gradient centrifugation. After the simplified density-gradient procedure described above, four particulate fractions were obtained and two of these (equilibrating over 1·2M- and 1·4M-sucrose) were analysed for protein, succinate-dehydrogenase activity and phosphoprotein radioactivity (Table 2). These fractions correspond approximately to subfractions *B* and *C* from fresh tissue. The percentage increase in phosphoprotein radioactivity is greater in fraction *B* than in fraction *C*. Though it is recognized that these fractions derived from incubated tissue are grossly cross-contaminated, the findings suggest that fraction *C* contains at least 60% of the total mitochondria of the tissue. These direct observations are therefore in agreement with the conclusion of Heald (1959) that the mitochondria are unlikely to contain the phosphoprotein that exhibits marked changes in metabolism during the brief passage of electrical pulses. However, the considerable increase in phosphoprotein radioactivity observed in fraction *C* (48%), together with the wide distribution of succinate dehydrogenase, indicates that cross-contamination occurs to a great extent during such a density-gradient procedure, and this necessarily limits interpretation of the findings.

Fractionation by differential centrifugation. Chemical analysis and electron microscopy of subfractions from fresh tissue (Trevor *et al.* 1965) suggested that, besides nerve endings and some mitochondria, subfraction *B* contained membrane material possibly of microsomal origin. In the density-gradient fractionation of incubated tissue, it is also evident that microsomal material could occur in fraction *B*. In view of the effect of saline on the subcellular distribution of particulate material, it is probable that the extent of contamination would be even greater than that occurring during fractionation of fresh tissue. It was therefore considered important to isolate a microsomal fraction relatively free of mitochondria and nerve endings. After dispersion of the tissue, two particulate fractions were obtained; one (F1) consisted of a combined nuclear and mitochondrial fraction and the other (F2) was considered to contain only microsomes. Each, together with a sample of the original dispersion, was analysed for protein, succinate-dehydrogenase activity and phosphoprotein radioactivity. To obtain sufficient material for these analyses several slices were pooled after incubation. Precautions were taken to ensure that this arrangement was representative in both control and stimulated tissue dispersions with regard to the origin of the slices (see Table 3). The protein content of the microsomal fraction is approx. 50% of that

Table 3. *Effect of electrical stimulation on radioactivity of phosphoprotein in subcellular fractions obtained by differential centrifuging*

Twelve cerebral-cortex slices were prepared from the brains of two guinea pigs and incubated in quick-transfer holders as described in the text. Immediately after incubation each slice was briefly rinsed in sucrose solution and dispersed in 3ml. of fresh ice-cold 0·32M-sucrose. The slices were treated sequentially in order of preparation, the last slice being dispersed 30min. after the initial slice. The dispersion was diluted to 100mg. of tissue/ml. and homogenization completed. The first, second and third slices from each hemisphere were equally represented in the control and stimulated parts of the experiment. Subfractions were prepared as indicated in the Experimental section. The experiment was then repeated in order to double the amount of material available for analysis. Results show the mean values derived from seven distinct experiments, followed by standard errors of the mean in the case of specific radioactivities.

Fraction	Protein (mg./g. of tissue)	Sp. radioactivity of protein [^{32}P]phosphorylserine (counts/min./μg. of P)	Percentage increase in radioactivity on stimulation (control=100)
Original dispersion	86·6	Control 17·8±1·9 Stimulated 23·1±2·0	130 (P=0·01)
F1: nuclear and mitochondrial	47·0	Control 15·3±2·3 Stimulated 16·1±2·9	105 (P=0·2)
F2: microsomal	11·2	Control 17·6±2·8 Stimulated 21·8±2·9	124 (P=0·02–0·01)

Table 4. *Effect of ouabain on phosphoproteins of electrically stimulated cerebral-cortex slices*

Three cerebral-cortex slices were prepared from one hemisphere of guinea-pig brain and incubated as described in the text. Ouabain was introduced to the medium together with radioactive phosphate and was present for a total contact time of 5 min. Immediately after stimulation the tissues were rinsed in ice-cold 0·32 M-sucrose and dispersed in cold 10% trichloroacetic acid. Control slices from the contralateral hemisphere were treated identically except that no electrical pulses were passed. The specific radioactivity of protein-derived phosphorylserine was determined by the method given in the Experimental section.

Expt. no.	Conditions	Concn. of ouabain (μM)	Sp. radioactivity of protein [^{32}P]phosphorylserine (counts/min./μg. of P)	Percentage increase in radioactivity on stimulation (control=100)
1	Control	0	14·5	—
	Control	5	15·9	—
	Stimulated	0	21·6	148
	Stimulated	5	19·3	121
2	Control	0	15·7	—
	Control	5	16·5	—
	Stimulated	0	24·0	153
	Stimulated	5	19·7	119

obtained in a corresponding fraction from fresh tissue and contains only 5–6% of the total succinate-dehydrogenase activity, indicating the presence of relatively few mitochondria. Differences between the phosphoprotein radioactivity of the fractions derived from control and stimulated slices were evaluated by a *t* test. No significant difference in phosphoprotein radioactivity after stimulation was observed in particulate fraction F1, containing nuclei and mitochondria. However, stimulation induced in both the original dispersion and in the microsomal fraction F2 a significant increase (*P* value between 0·01 and 0·02) in protein phosphorylserine radioactivity. In one experiment, which is not included in Table 3, the percentage increase in radioactivity on stimulation was 29% in the original dispersion, 1% in F1 and 100% in F2. The finding that the percentage increase in phosphoprotein radioactivity is lower in the microsomal fraction than the original dispersion may be related to the time of fractionation. The approximate time-interval between stimulation of slices and fixation with trichloroacetic acid was 15 min. for the dispersion, 30 min. for the combined nuclear and mitochondrial fraction and 75 min. for the microsomal fraction. It is possible that radioactive phosphorus is lost from phosphoprotein during the especially prolonged time-period necessary for separation of the microsomal fraction.

Electrical stimulation of cerebral-cortex slices, in addition to increasing phosphoprotein radioactivity, results in an increased flux of Na^+ and K^+ (Cummins & McIlwain, 1961). Ouabain, an inhibitor of ion transport, was introduced to slices at a final concentration of 5 μM, together with [^{32}P]phosphate. The agent was also present during the next 2 min. incubation in fresh medium before electrical stimulation, the total contact time being 5 min. Ouabain partially inhibits the increase in phosphoprotein radioactivity that normally accompanies the passage of electrical pulses (Table 4), but has little effect on the incorporation of [^{32}P]orthophosphate in the absence of pulses. These observations confirm the findings of Ahmed, Judah & Wallgren (1963).

DISCUSSION

Changes in the subcellular distribution of protein brought about by incubation of cerebral tissue in a saline medium are apparently related, at least in part, to contamination of the dispersing solution with inorganic salts. This effect of salts was only clearly evident when the slices were held in quick-transfer holders and the holders, with medium trapped between their jaws, were immersed in the sucrose solution. By removing the slice from its holder and briefly rinsing it, the yield of microsomal protein increased to that attained with free floating slices, drained of excess of medium in the usual way before dispersion. This yield is still some 50% below that obtainable from fresh tissue, and the remaining loss is probably due to irreversible penetration of salts from the medium into the tissue spaces (see Varon & McIlwain, 1961).

Density-gradient fractionation of whole dispersions of slices was adopted as a means of effecting a crude separation into particulate components in one step and in a shorter time than possible by differential centrifugation. In using unfractionated starting material for this technique it was recognized that only a very limited separation could be expected. In practice determinations of succinate dehydrogenase showed that there were twice as many mitochondria in the *C* as in the *B* fraction; on the other

hand, the increase in phosphoprotein radioactivity in fraction *B* was double that in fraction *C*. Thus, though these experiments do not suggest a specific location, they strongly suggest (with the proviso discussed below) that the mitochondrial phosphoproteins do not participate in the reaction, unless it is assumed that the mitochondria in the *B* fraction are a distinct population from those in the *C* fraction. That this is unlikely is shown by the experiments with differential centrifugation, where the phosphoproteins of a combined nuclear and mitochondrial fraction did not change in radioactivity on stimulation, whereas a statistically significant increase was found in the microsomal fraction, which contained only 5% of the total succinate-dehydrogenase activity. The discrepancy between these results and those of Heald (1959), who located the sensitive phosphoprotein in the nuclear fraction, is most likely explained by the greater abnormality in the distribution of subcellular components displayed in the earlier work, where rinsing of the slices before dispersion was apparently not adopted. The very high protein content of the crude nuclear fraction in Heald's (1959) experiments is therefore consistent with the suggestion that it contained a considerable proportion of the microsomal protein, which in the present work is shown to contain a phosphoprotein sensitive to pulses. Direct evidence for this suggestion is lacking, however, for, although in the present work virtually no microsomal protein was obtained from dispersions of slices when the quick-transfer holder containing the slices was immersed in the dispersing solution, the extent to which the lost protein is sedimented with the nuclear or mitochondrial fractions was not ascertained. On the other hand, Trevor *et al.* (1965) did observe membrane fragments, similar to those seen in the microsomal fraction by Hanzon & Toschi (1961), in electron micrographs of the nuclear fraction prepared from fresh tissue by Heald's (1959) procedure.

A further point requires comment. The results in Table 3 show that the increase in phosphoprotein radioactivity found in the microsomal fraction was of the same magnitude as observed in the original dispersions. This is rather disturbing, since the latter contained a relatively large amount of presumably inactive phosphoproteins, which might be expected to dilute the change in the active protein. Possibly, the induced increase in radioactivity of the microsomal phosphoproteins declines more rapidly than is suggested by the results of Fig. 2, which were obtained by analysis of whole tissue. Alternatively, or perhaps in addition, it could be postulated that certain non-microsomal phosphoproteins do in fact respond to the application of pulses, but are more labile during the fractionation procedure than are the microsomal phosphoproteins, and thus escape detection. A greater lability might

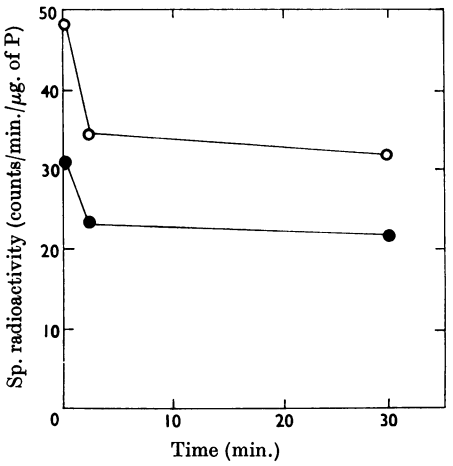

Fig. 2. Loss of phosphoprotein ^{32}P in cerebral tissue dispersions made in sucrose at 0–2°. Guinea-pig cerebral-cortex slices were incubated in phosphate-free tris-buffered medium and allowed to incorporate ^{32}P for 3 min. Then, 2 min. after transfer of the slices to fresh medium, electrical pulses were passed for 10 sec. and the tissue was then dispersed in either 10% trichloroacetic acid (zero time), or 0·32 M-sucrose at 0–2°. Protein was precipitated in the sucrose dispersions after 2 min. or 30 min. by the addition of trichloroacetic acid and phosphoprotein radioactivity was determined as described in the text. Control slices were treated identically except that no electrical pulses were passed. The values for the Figure were derived from two experiments. ●, Control; ○, stimulated.

conceivably be due to a more ready access to the enzyme phosphoprotein phosphatase (Rose & Heald, 1961). This enzyme was shown by Rose (1962) to have a bimodal distribution in cerebral tissue, 50–60% of the activity being in the soluble supernatant and 20–30% in the mitochondrial fraction, whereas only 3% was associated with microsomal material. Although in the present study fractionation was carried out in sucrose at pH 7·0–7·2 (the pH optimum for the partially purified enzyme being pH 5·5) and the medium contained EDTA, an inhibitor of the enzyme, it is possible that the enzyme continues to function with diminished activity. The presence of a considerable proportion of the enzyme in the mitochondrial fraction means that we cannot exclude at this stage the possibility that a mitochondrial phosphoprotein sensitive to pulses could be dephosphorylated by the enzyme during separation of the fraction.

Apart from the qualifying points discussed above the positive finding of a phosphoprotein sensitive to pulses in the microsomal fraction supports the suggestion, first put forward by Heald (1960), that a phosphoprotein is involved in the utilization of

phosphate-stored energy at the neuronal membrane. The precise functional processes concerned remain obscure, but the most promising working hypothesis is undoubtedly one of participation in some aspect of cation transport, such as mediation of the cation-stimulated adenosine-triphosphatase enzyme system (Skou, 1960). So far no unequivocal evidence has emerged for the direct participation of a protein phosphorylserine group in the adenosine-triphosphatase system, although under suitable conditions the phosphorylation of microsomal proteins by ATP is stimulated by Na^+ (Rodnight & Lavin, 1964*a*). Alternatively, supposing a direct participation of the phosphoprotein in the adenosine-triphosphatase reaction is eventually eliminated, it will remain conceivable that it plays a facilitating role in cation transport: for instance by modifying, in the course of its metabolism, the charge and conformation of membrane structures. It seems unlikely, however, that we are observing in these experiments the product of the cerebral enzyme system, studied by Rabinowitz & Lipmann (1960) and Rodnight & Lavin (1964*b*), that phosphorylates phosvitin and casein. Although this enzyme is stimulated by Na^+ and other univalent cations its wide subcellular distribution speaks against a specific role in membrane function. Finally, electrical pulses may modify the transport of substances other than cations across the neuronal membrane. A suggestion about phosphate ion transport and phosphoproteins has already been made by McIlwain (1963); moreover, there is evidence that glucose and amino acid assimilation may involve phosphorylated intermediates in the membrane (Rothstein, 1962).

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