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 re-investigated by two different procedures. Comparison between unstimulated and stimulated slices after incubation in the presence of [32P]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 [32P]orthophosphate, stimulated for 10 sec., dispersed in sucrose solution and then centrifuged at 1500 g for 10 min. 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 subcellular fragment. First, it is difficult to accept, in the light of later work, that incorporation of [32P]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, 1964a) and their rate of uptake of [32P]phosphate no doubt depends on many factors. Secondly, interpretation of Heald's
(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 5 ml of medium and incubated at 37-5°C with continuous gassing with oxygen. After 30 min., 0.2 ml of carrier-free \[^{32}P\]orthophosphate (10-20 \(\mu\)c; The Radiochemical Centre, Amersham, Bucks.) was added and incubation continued for a further 3 min. The slices were then rapidly removed and released into 50 ml of fresh oxygenated medium at 37-5°C. After 10 sec, the slices were again floated into transfer holders and placed in beakers containing 5 ml of fresh medium under the same conditions as the original incubation. Then, 2 min later, condenser discharge pulses from the generator of Ayres & McIlwain (1953) of frequency 100 cyc./sec., duration 0-4 msec. and peak voltage 15 v were applied to the slices for a period of 10 sec. They were then immediately released into 20 ml of 0-32 x-sucrose at 0-2°C to remove traces of saline and finally dispersed in either 0-32 x-sucrose containing EDTA (0-5 mm) at pH 7-0 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 25 min.

**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 90 min. of dispersion in sucrose solution. In the first, a simplified density-gradient fractionation was used that involved layering of the dispersion of incubated tissue (100 mg/ml) over a discontinuous gradient consisting of 7 ml of 1-0 x-m, 8 ml of 1-2 x- and 6 ml of 1-4 x-sucrose and centifuging at 10,000 g for 75 min. in the SW25 head of the Spinco model L machine. In the second procedure particulate fractions were obtained by centrifuging the dispersions for 15 min. at 13-3 x 10,000 g-min. (F1) and then centrifuging the supernatant for 60 min. at 10-4 x 10,000 g-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 phosphohydrolase.** 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 phosphoplyserine released by partial acid hydrolysis of the insoluble residue was considered satisfactory. The procedure used was adapted from that 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. 100 mg of tissue were hydrolysed for 6 hr. in sealed hard-glass tubes with 2 ml of 2 N-HCl at 100°C. This period was chosen from studies on the time-course of the release of phosphoplyserine 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 phosphoplyserine released was...
maximal at 10 hr., and the specific radioactivity reached a peak after hydrolysis for 5 hr. The hydrolysates were freeze-dried in the presence of NaOH pellets and conc. H$_2$SO$_4$ for 10 hr. and the residues dissolved in 2 ml. of 0-05N-HCl. They were then fractionated on columns (24 cm. x 3 cm.$^2$) of Dowex 50 (X12; H$^+$ form; 200-400 mesh) with 0-05N-HCl as the eluent at a flow rate of 15-25 ml./hr. Radioactive inorganic phosphate was eluted from the columns between 20 and 50 ml. and phosphorylserine between 70 and 100 ml. 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 100 ml. were evaporated to dryness at 40° in a rotary vacuum evaporator and the residues taken up in 4-5 ml. of 0-05N-HCl. Two samples (2 ml.) were transferred to small test tubes and again evaporated to dryness. Perchloric acid (2 ml. of 12 N) was added to each residue and one of each pair digested for 10 min. to release $P_1$ from phosphorylserine. The other sample served as a control for contamination due to free $P_1$, which never exceeded 10–15% of the phosphorylserine phosphorus. Approx. 1-8 μg. of $P$ was released from the phosphorylserine samples after digestion. The radioactivity of contaminating $P_1$ contributed less than 20% of the total radioactivity derived from each phosphorylserine sample; all values quoted have been corrected for $P_1$ 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 10 ml. and radioactivity was determined in a liquid counter M6H (20th Century Electronics Ltd., New Addington, Surrey) with the Ecko scaler N50A (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 30 min. 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 phosphorylserine 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 phosphorylserine protein during incubation, assumed to be due to enzymic dephosphorylation, the phosphorylserine 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 30 min. 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 20 ml. 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 100 mg were prepared from one hemisphere of each of two guinea-pig brains and incubated in Warburg vessels containing 5 ml of phosphate-free tris-buffered medium at 37°C with O2 for 30 min. The six slices were then briefly drained of excess of medium and homogenized in 0·32 M-sucrose to give a dispersion containing 100 mg 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.

<table>
<thead>
<tr>
<th>Fraction and centrifugal force used</th>
<th>Protein (mg/g. of tissue)</th>
<th>Phosphoprotein P (µmoles/mg. of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh tissue</td>
<td>Incubated tissue</td>
</tr>
<tr>
<td>Homogenate</td>
<td>94·3</td>
<td>83·0</td>
</tr>
<tr>
<td>Nuclear (8 × 10⁶)</td>
<td>8·6</td>
<td>19·6</td>
</tr>
<tr>
<td>Mitochondrial (2 × 10⁵)</td>
<td>38·6</td>
<td>34·6</td>
</tr>
<tr>
<td>Microsomal (7 × 10⁶)</td>
<td>22·1</td>
<td>11·3</td>
</tr>
<tr>
<td>Supernatant</td>
<td>18·9</td>
<td>10·5</td>
</tr>
</tbody>
</table>

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

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

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 0·32 M-sucrose before fractionation. The final concentrations of sodium chloride used were 1·0, 3·4 and 5·8 m m; the yields of microsomal protein/g. of tissue for the three concentrations were 15·1, 7·6 and 4·6 m g. respectively. From these findings it was estimated that 0·1 ml of incubation medium carried with the slice or tissue holder to the dispersion medium (usually 5 ml 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 ³²P. The stability of protein-bound ³²P of tissue dispersions in sucrose solution at 0–2°C 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 0·32 M-sucrose, the latter being kept at 0–2°C for various intervals before fixation with trichloroacetic acid. Fig. 2 shows the results of two experiments. Within 2 min. 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 30 min. In attempts to minimize this decline in
radioactivity, a number of agents were added to the sucrose solution including EDTA (5 mM), iodoacetate (1 mM), cyanide (1 mM), mercuric chloride (0.1 mM) and ferric chloride (0.1 mM). 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>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg/g. of tissue)</th>
<th>Sp. radioactivity of protein [32P]phosphorylserine (counts/min./ug. of P)</th>
<th>Percentage increase in radioactivity on stimulation (control=100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original dispersion</td>
<td>86-6</td>
<td>Control 17-8±1-9, Stimulated 23-1±2-9</td>
<td>130 (P=0-01)</td>
</tr>
<tr>
<td>F1: nuclear and mitochondrial</td>
<td>47-0</td>
<td>Control 15-3±2-3, Stimulated 16-1±2-9</td>
<td>105 (P=0-2)</td>
</tr>
<tr>
<td>F2: microsomal</td>
<td>11-2</td>
<td>Control 17-6±2-8, Stimulated 21-8±2-9</td>
<td>124 (P=0-02-0-01)</td>
</tr>
</tbody>
</table>

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 dispensed in 3 ml. of fresh ice-cold 0-32M-sucrose. The slices were treated sequentially in order of preparation, the last slice being dispersed 30 min. after the initial slice. The dispersion was diluted to 100 mg. 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.
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, 10% 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+ (Cummings & McIlwain, 1961). Ouabain, an inhibitor of ion transport, was introduced to slices at a final concentration of 5 μM, together with [32P]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 [32P]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 un fractionated 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 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 5.5–5) 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
mediates in the aimilation may has already role inmembrane there is over, U.S. ment, and casein. phosphatase reaction is of membrane formation role in

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