SUMMARY

1. In the presence of growth-inhibitory concentrations of chloramphenicol, the incorporation of most L-[¹⁴C]amino acids into the protein fractions of *Bacillus megaterium* is inhibited to the extent of about 96%.

2. The incorporation of L-[¹⁴C]alanine into the cellular protein is only 50–85% inhibited by the same concentration of chloramphenicol. The residual incorporation can be accounted for largely by the uptake of ¹⁴C into the peptide fraction of the cell wall. However, when the L-[¹⁴C]alanine is presented to the cell for less than 2 min., the proteins of the membrane fraction are more highly labelled than are the cell-wall peptides.

3. In the presence of growth-inhibitory concentrations of both penicillin and chloramphenicol, L-[¹⁴C]alanine is incorporated into the protein of the cytoplasmic-membrane fraction, but not appreciably into the cytoplasmic protein or the cell-wall peptides.

4. In the presence of chloramphenicol, L-[¹⁴C]-aspartic acid is still converted relatively efficiently into the protein or peptide fractions of the cell-wall and cytoplasmic membrane. Radioautographs of these fractions show that the radioactivity is mainly associated with diaminopimelic acid.

5. It is concluded that the peptide components of the cell wall of *B. megaterium* are synthesized at sites on or closely associated with the cytoplasmic membrane.

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Loss of Excitability in Isolated Cerebral Tissues, and its Restoration by Naturally Occurring Materials

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Electrical pulses applied to cerebral tissues initiate large and rapid metabolic changes akin to those associated with increased cerebral activity *in vivo* (McIlwain, 1956b, 1959). Though general schemes linking impulse and metabolic response can be proposed, unidentified chemical factors are still involved. Previous attempts to identify such factors have been made by placing the tissue under adverse conditions so that metabolic response was lost, and then making specific additions to the

treated tissue with the object of restoring response. Often the adverse conditions led to defects which could not be made good in this way, but by applying electrical pulses to the tissue while it was being incubated in the absence of oxygen or of glucose, some success was obtained (McIlwain & Gore, 1953; McIlwain, 1956a). The partial restoration which was achieved was, however, obtained with known metabolites: with malic acid or fumaric acid, adenine or nicotinamide.

With the object of identifying new factors, a fresh beginning has been made by exposing cerebral tissues to other adverse conditions. In the belief that requirement for the metabolites just named was perhaps associated with incubation in the absence of substrates, in the present experiments we have employed methods in which tissue was placed in a normally adequate glucose-containing medium, but was kept in the cold or in the presence of adsorbents or of glycerol. The last-named procedure was adopted by analogy with glycerol treatment of muscle (Szent-Gyorgi, 1949). After such treatments, the tissue's excitability has been examined by placing it in fresh oxygenated glucose media, applying electrical pulses and measuring respiratory and other responses. Some experiments investigated the effects of increased concentrations of potassium salts, which also increase the respiration of normal cerebral tissues.

It proved relatively easy to induce in cerebral tissues a defect of the type desired, by standing them in cold fluid media. In subsequent experiments therefore various materials were added to the depleted tissue with the object of restoring its previous response to stimulating agents.

EXPERIMENTAL

Tissue metabolism

Tissues, media and stimulating agents. Guinea pigs were used unless otherwise stated; they were stunned by a blow on the neck, killed by bleeding and the brain was removed. Rabbits were killed by injection of air into an ear vein; human tissues were obtained at neurosurgery. In each case the cerebral cortex was promptly sliced at 0.35 mm. intervals with razor blade and a guide. Phosphate and bicarbonate media were based on those of Krebs (McIlwain, 1951; Rodnight & McIlwain, 1954); a medium buffered with 25 mM-2-amino-2-hydroxymethylpropane-1:3-diol (tris) at pH 7.4 was also employed, in which this buffer replaced the phosphate buffer of the phosphate medium; all contained 10 mM-glucose and were initially equilibrated with O_2 or (for bicarbonate media) with $O_2 + CO_2$ (95:5). An atmosphere of O_2 was maintained during respiratory measurements but not when the tissue was in cold medium (see below).

Electrical pulses were obtained and measured with the apparatus of Ayres & McIlwain (1953), and applied to tissues held in silver-grid electrodes in conical manometric vessels E (Ayres & McIlwain, 1953). Condenser pulses of exponential time-voltage relationship were employed, alternating at 100/sec., peak potential 10v and time constant 0.4 msec. When KCl was to be added during an experiment, it was placed initially in a side arm of a manometric vessel as an M-KCl soln., in quantity sufficient to give a final concentration of 36 mM- K^+ ions, which was chosen as the minimal concentration giving a maximal or near-maximal increase in respiratory rate.

Measurements. Respiration was measured manometrically by pressure readings each 5 min. in conventional

manometric apparatus and in that of Ayres & McIlwain (1953). Formation of lactic acid was determined at the end of manometric experiments by Barker & Summerson's (1941) method. In certain experiments in which tris media had been employed, the tissue's content of inorganic and creatine phosphates was determined after their separation by barium salts by the procedures described by Heald (1954). Data are given in terms of moist wt. of tissue.

In most experiments, respiratory response of the tissue to stimulating agents was determined by measuring respiration during 45 min., applying the stimulating agent (KCl or electrical pulses) and continuing measurements for a further 45 min. Glycolytic response to the stimulating agents was measured by comparing the lactic acid formation by tissues treated as just described with the formation by tissues to which no stimulating agent was applied. Typical experiments involved six tissue slices in separate vessels, some with added substances whose effects were being tested. For electrical stimulation, a specific electrode was used in each vessel. Experiments were planned so that the vessels with added substances in one experiment were without added substances in a duplicate experiment; in this way any variation due to electrode arrangements was randomized (variation was not large: Ayres & McIlwain, 1953). Electrical pulses applied as in the present experiments can cause uptake of O_2 in the presence of certain naturally occurring substances (Lewis & McIlwain, 1954). The materials used in the present experiments were therefore examined for such effects in the absence of tissue. No significant absorption of O_2 occurred on passage of pulses in the presence of blood serum, plasma or extracts of muscle and liver. A slight absorption occurred with pulses in the presence of an extract of brain (6 mg. dry wt./ml.) and correction was made for this in the values recorded in Fig. 3. In the presence of plasma fraction IV-4 (see below) a slight absorption of O_2 took place before pulses were applied. Absorption was equivalent to 1-2 μ moles/g./hr. when referred to an assumed slice weight of 50 mg. This value was sufficiently small to be ignored; it was not increased by pulses.

Exposure of tissue to adverse conditions. Standard conditions were: tissues were cut while wet with the medium to be used in the later part of the experiment, drained from excess of medium on a glass surface, trimmed to weigh about 45 mg., and weighed, and those which were to be exposed were placed in 3.5 ml. of the medium in 25 ml. conical flasks in a tray of ice-water which was put in a cold room. The flasks were loosely stoppered; the atmosphere was air. Other tissues which were prepared at the same time but which were not to be exposed were placed, after weighing, in fluid media in manometric vessels, O_2 was passed and metabolic measurements were made immediately. After the exposed tissues had remained in cold media for periods of 1-17 hr., they were removed with a mounted bent wire and medium was drained from them by drawing them up the side of the flask. The tissues were then placed in manometric vessels in fresh media (those to receive electrical pulses being first placed in the grid electrode), and the media were equilibrated with O_2 and respiratory measurements made.

For some experiments, uncut cerebral hemispheres were placed in screw-capped polythene containers of 30 ml. which contained filter paper moistened with the medium of the experiment, and which were kept at 0°. When slices

Table 1. *Respiration and glycolysis of guinea-pig cerebral cortex after keeping it under various conditions*

All slices weighed 45–50 mg. In Expt. 1 these were placed immediately in 3.5 ml. of phosphate–glucose media in manometric vessels; in Expt. 2 the slices were cut after 12 hr. and then similarly used. In Expts. 3–5, slices cut at zero time were placed in the medium 17 hr. later; those of Expt. 5 had been kept in 3.5 ml. of the same medium, but were removed from it to fresh media for the manometric experiment. For other details, see the Experimental section. Values are given $\pm$ s.d. with (in parentheses) the number of observations.

Expt. no.	Conditions of keeping at 0°	Time at 0° (hr.)	Respiration (μ moles of O ₂ /g./hr.)		Lactic acid formation (μ moles/g./expt.)	
			Ordinary medium	With 36 mM-K ⁺ ions	Ordinary medium	With 36 mM-K ⁺ ions
1	Control	0	59 $\pm$ 4 (9)	101 $\pm$ 9 (7)	28 $\pm$ 5 (6)	46 $\pm$ 6 (4)
2	As whole hemisphere	12	61 $\pm$ 6 (3)	97 $\pm$ 13 (3)	31 $\pm$ 4 (3)	41 $\pm$ 7 (3)
3	As slices, cut without medium	17	73 $\pm$ 9 (5)	90 $\pm$ 17 (5)	—	—
4	As slices, cut in medium and with approx. 50 μ l. of medium adhering	17	44 $\pm$ 9 (6)	52 $\pm$ 6 (5)	—	—
5	As slices, cut in medium and with 3.5 ml. of medium	12	47 $\pm$ 4 (8)	52 $\pm$ 6 (4)	30 (2)	35 (3)

were to be kept without medium, they were cut without contact with medium and weighed on a wire hook, which was subsequently suspended from the stopper of a 25 ml. conical flask above a small amount of medium which it contained; the flasks were kept at 0°.

Materials examined with exposed tissues

Preparations from blood. Human, guinea-pig and ox sera were prepared from whole blood allowed to clot at 37°. Before use in manometric experiments, CO₂ was removed as described by Warren (1944) and the pH readjusted to 7.4. Dried human serum was also employed; dialysis of the dried serum after reconstitution was carried out at 0–3° in cellophan tubing which was moved mechanically in 4 vol. of water, or other fluid medium, renewed at frequent intervals. The lactic acid of the serum was determined and found to fall from 5.0 to 0.2 mm in 12 hr. dialysis.

Acetone-precipitation of dialysed serum was carried out by adding about 50 ml. of the serum to 4 vol. of acetone at 0° with constant stirring. The mixture was centrifuged and the precipitate washed with further small quantities of acetone and dried in a vacuum desiccator containing paraffin wax. In one instance the precipitate was redissolved in water (6 g./100 ml.) and reprecipitated in the same way. Acetone was removed from the soluble fractions by vacuum-distillation and the residue freeze-dried. Extracts were also prepared from the dialysed serum by heating at pH 5.3 according to Hunter (1957). Chloroform extraction of serum samples or products was by repeated mechanical shaking with 2 vol. of CHCl₃.

By courtesy of Mr D. Purdie of Armour Laboratories, Eastbourne, we were supplied with ox-plasma fractions designated I, II, III-1, III-2, IV-1, IV-4 and V by Cohn *et al.* (1946), and prepared by ethanol precipitation at controlled pH. Albumin from fraction V was also obtained from L. Light and Co. Ltd.

Tissue extracts and other materials. Cerebral tissues were extracted in the cold as follows. The brain of a guinea pig or rabbit was removed rapidly and cut with a scalpel to blocks about 3 mm. thick; these were chopped in the machine of McIlwain & Buddle (1953) to prisms about 2 mm. $\times$ 0.2 mm. $\times$ 0.1 mm. These were suspended in 4 vol. of ice-cold medium buffered with tris and without glucose for

(a) 0.5 hr. and (b) 17 hr. The supernatant from centrifuging (1500 g, 10 min.) was used. A third type of extract (c) was made in a blender (2 min.) from the brain and 4 vol. of 0.9% NaCl. After standing 40 min. at 0° the mixture was centrifuged at 30 000 g for 10 min. at 0°.

Hot extracts were made from brain and from liver by cutting the organs with scissors to approx. 0.5 cm. cubes and dropping these into 4 vol. of water boiling in a beaker, so that the water continued to boil for 3 min. The mixture was then centrifuged at 1500 g for 15 min. We are grateful to Dr A. E. Bender (Bovril Ltd.) for supplying us with a sample of commercial meat extract and also of its desalted dialysate, both prepared as described by Wood & Bender (1957). Polyvinylpyrrolidone was kindly provided by May and Baker Ltd.; Nelson's Photographic Gelatin was used.

RESULTS

Conditions for obtaining loss of metabolic response to stimulating agents

A block of cerebral tissue a few grams in weight may be kept in a moist atmosphere at about 0° for at least 10 hr. without marked effect on the rates of respiration which are subsequently shown when it is sliced and incubated in glucose-containing media. Moreover, respiration is increased in nearly normal fashion when electrical pulses are applied to the tissue (McIlwain, Ayres & Forda, 1952). In the present experiments, similar stability has been shown in the tissue's glycolysis and in its respiratory and glycolytic responses to increased concentration of potassium salts. Thus increase in potassium salts from 6 to 36 mM, which increased the respiration of normal tissues by some 70%, increased the respiration of those kept at 0° for 12 hr. by about 60% (Table 1). The stability also extended to tissue sliced before it was kept at 0°, provided that the slicing was carried out in the absence of fluid media. Much greater loss of response to stimulation was, however, found when

the sliced tissue was kept in contact with glucose-containing media for comparable times. Respiration fell and after 17 hr. showed little response to potassium salts; glycolysis changed little but glycolytic response fell.

Keeping in fluid media. It appeared therefore that tissue might be altered in the way desired by keeping slices of cerebral cortex in glucose-containing media at 0°, and this was therefore done under a variety of conditions. The volume of medium with which tissue was kept could be altered considerably and still afford a similar fall in respiration. A 50 mg. slice moistened with only about 50 μ l. of medium gave an 18% respiratory response under conditions in which one placed in 3.5 ml. of medium increased respiration by 11% (Table 1). The change in 3.5 ml. of medium was progressive, as is shown in Fig. 1, and the fall in the stimulated rate of metabolism was, throughout, greater than those occurring in the absence of stimulating agent.

Loss of response was examined also in glucose media buffered with tris. This was done because the phosphate concentration in phosphate-buffered

media is much greater than in the tissue's normal environment, and makes difficult the determination of tissue phosphate (see below). Loss of response in tris-buffered media was found more rapid than in media with phosphate buffers. The tissue's response to potassium salts and to electrical pulses changed in comparable fashions (Fig. 2). Changes in the response of slices of the cerebral cortex from other animal species were also studied after they were kept at 0° for 5 hr. in tris-buffered media. Slices from the rabbit, which normally increased in respiration by 86% with applied pulses, after keeping increased by 43%; with those from man the increase before keeping was 71% and after keeping was only 12%.

Change in lactic acid formation on keeping tissues at 0° was studied, and results with guinea-pig tissues are included in Table 1 and in Figs. 1 and 2. Lactic acid formation in the absence of stimulating agents was relatively stable over the 17 hr. studied. Formation in the presence of electrical pulses or of 36 mM-KCl diminished, but to a lesser degree than did the tissue's respiration.

Media with added substances. In an attempt to obtain more rapid loss of the tissue's response to stimulating agents, adsorbents were added to the media in which the tissues were being exposed. This

Fig. 1. Change in respiration and in formation of lactic acid on keeping slices of guinea-pig cerebral cortex in phosphate-glucose media at 0°. After being kept for the period indicated by the abscissae, slices were placed in fresh glucose-phosphate media and incubated at 37° for the determinations recorded. Points give mean values of four to seven determinations (s.d. given by vertical lines). ●, Unstimulated tissues and those with 36 mM-potassium salts; ○, tissues with electrical pulses.

Fig. 2. Change in respiration and in formation of lactic acid on keeping slices of guinea-pig brain in tris-glucose media at 0°. Other details are as given in Fig. 1.

Table 2. *Exposure of guinea-pig cerebral cortex to adsorbents and glycerol*

Adsorbents were first treated with several volumes of medium for about 1 hr. until no further change in pH occurred, before adding them to fresh medium with the tissue. This procedure was adopted after several adsorbents had been found to alter the pH and Ca^{2+} ion content of the medium. The following adsorbents were examined under the conditions of Expt. 2, and induced no significant change in respiration and lactic acid formation: cellulose powder, silica, decolorizing charcoal and calcium phosphate. Values give the mean result, $\pm$ s.d. where applicable, of the number of experiments indicated in parentheses.

Expt. no.	Conditions of exposure	Respiration ($\mu\text{moles of O}_2/\text{g./hr.}$)		Lactic acid formation ($\mu\text{moles/g./expt.}$)	
		Ordinary medium	With 36 mM-K ⁺ ions	Ordinary medium	With 36 mM-K ⁺ ions
1	At 0°; 1 hr.	55 $\pm$ 3 (15)	88 $\pm$ 10 (8)	30 $\pm$ 5 (7)	50 $\pm$ 7 (7)
2	As 1; with alumina, 0.3 g.	48 $\pm$ 8 (12)	83 $\pm$ 11 (16)	28 $\pm$ 6 (6)	49 $\pm$ 7 (4)
3	As 1; with 20% (v/v) glycerol	57 $\pm$ 3 (4)	95 (2)	30 (2)	50 (2)
4	At 37°; 1 hr.	56 (2)	78 (2)	33 (2)	48 (2)
5	At 37°; 1 hr. with 20% (v/v) glycerol	58 $\pm$ 6 (6)	77 $\pm$ 8 (6)	34 (2)	54 (2)

had remarkably little effect on the loss of excitability (Table 2). Also, the presence of 20% (v/v) glycerol during depletion was without influence on the process at 0°. At 37°, loss occurred in respiratory response to KCl after 1 hr., but was again uninfluenced by glycerol (Table 2). These conditions induced relatively little change in glycolysis or in the glycolytic response to increased concentrations of KCl.

Tissues were also kept in media which contained blood serum, and were then transferred to ordinary media for metabolic experiments. In this way it was shown that loss of response to KCl in guinea-pig serum at 0° was the same as that occurring in bicarbonate-containing media; under both conditions respiratory rates of 46–47 $\mu\text{moles of O}_2/\text{g./hr.}$ after 17 hr. were unchanged when KCl (final concn. 36 mM) was added. The course of loss of response in bicarbonate medium was similar to that occurring in phosphate media and is shown in Fig. 1. Similar results were obtained after keeping the tissue in human cerebrospinal fluid. Loss of response in a tris-buffered medium with only 1.3 mM-KCl was similar to that occurring in the normal tris medium, which was 6.2 mM in potassium salts. The addition to tris media of mm-creatine, -adenosine and -guanosine together did not prevent the diminution of respiration or respiratory response.

Attempted restoration of response to stimulating agents

Loss of metabolic response to stimulating agents is seen to be gradual. In attempts to restore the response by addition of materials, a few sets of conditions were chosen for the initial loss of response (Table 3). These were: conditions A and B, after 5 and 17 hr. in phosphate medium, when respiratory response was lowered from its normal 80% to about 45 and 15% respectively; and con-

ditions C and D, after similar exposure in tris media, when loss was more severe. Substances examined for their ability to restore metabolic response were conveniently incorporated with the media of the subsequent experiments, in which metabolic responses by the tissues were examined. The added substances were thus (see Experimental section) incubated with the exposed tissue at 37° for 45 min. before application of the stimulating agent. The rates of respiration before and after stimulation were the data mainly used to judge the restoration of response caused by the added substance.

Table 3 shows that partial restoration of respiratory response has been achieved with added substances. After exposure for 5 hr. in phosphate media, dilutions of blood sera from guinea pig and from man increased respiration in the absence of stimulating agent and to a rather greater extent after stimulation. In an attempt to obtain greater differentiation between effects on stimulated and unstimulated respiration, tissue was examined after exposure for 17 hr. in phosphate media. Here again restoration of respiratory response was shown, but all increases were relatively small. They were smaller still after the tissue had been standing in tris media for 17 hr. The most suitable conditions for displaying recovery of response were conditions C of Table 3: keeping the tissue for 5 hr. in tris medium. Serum added under these conditions is seen to increase response both to electrical pulses and KCl.

These test systems showed that the ability of blood serum to restore respiratory response was not shared by a number of known substances (Table 4). Certain of these depressed the tissue's responses. In appraising the actions of the compounds and also of the preparations from natural sources described below it was especially necessary to be aware of any interactions, extraneous from the

present point of view, between the extract and the stimulating agents, and examination of this is described in the Experimental section. The substances examined (Table 4) are seen to include those previously found to have some ability to restore differently depleted cerebral tissues, and also a number of coenzymes, vitamins and intermediary metabolites. None was found effective in the present system. Also, as it was considered that the activity of blood serum might be related to its

colloid properties, polyvinylpyrrolidone and gelatin were examined but were ineffective.

Quantitative comparison and fractionation of natural materials. The preceding results showed that to identify a substance which restored respiratory response, fractionation of naturally occurring materials was likely to be required. For this purpose a quantitative assay was desirable, and Fig. 3 illustrates that which was adopted. Respiratory response of the exposed tissues to

Table 3. *Loss and partial restoration of respiratory response*

The responses recorded were to electrical pulses and to KCl, applied as described in the Experimental section. The control experiments, without added materials, were carried out under the conditions of Figs. 1 and 2. When materials (derived from guinea pigs) were added, they were incorporated into the fresh medium of the second part of the experiment, during which respiration was measured. Respiration values are means $\pm$ S.D. with the number of experiments in parentheses.

	Conditions of keeping at 0°		Materials added and amount (mg. dry wt./ml. of medium)	Respiration (μ moles of O ₂ /g./hr.)			Mean increase in respiration caused by stimulating agent			
							(μ moles of O ₂ /g./hr.)		(%)	
	Medium	Time (hr.)		No stimulation	With pulses	With K ⁺ ions	With pulses	With K ⁺ ions	With pulses	With K ⁺ ions
A	Phosphate	5	None	50 $\pm$ 5 (6)	68 $\pm$ 7 (4)	65 $\pm$ 4 (3)	18	15	37	30
			Serum, 28	62 $\pm$ 6 (3)	100 $\pm$ 9 (3)	—	38	—	61	—
B	Phosphate	17	None	43 $\pm$ 6 (15)	47 $\pm$ 7 (3)	49 $\pm$ 6 (5)	4	6	10	12
			Serum, 8.5	40 $\pm$ 4 (3)	—	50 (2)	10	—	25	—
C	Tris	5	None	42 $\pm$ 5 (15)	53 $\pm$ 8 (10)	55 $\pm$ 8 (5)	11	13	26	31
			Brain extract (a), 5.85	45 (2)	65 (2)	—	20	—	38	—
			Serum, 28	50 $\pm$ 6 (15)	80 $\pm$ 11 (15)	84 $\pm$ 4 (5)	30	34	60	68
D	Tris	17	None	39 $\pm$ 5 (15)	39 $\pm$ 6 (5)	40 $\pm$ 6 (10)	0	0	0	0
			Brain extract (b), 4.8	42 (3)	—	42 (2)	—	0	—	0
			Serum, 28	45 (2)	—	52 (2)	—	7	—	15

Table 4. *Materials examined for ability to restore respiratory response to cerebral cortex after the tissue had been kept at 0°*

Preparation of the tissue and examination of its response were carried out according to the conditions of Table 3 (B, C and D). The compounds examined under conditions D were employed individually as well as in the groups named, and gave the same result.

Materials examined (mm except when otherwise indicated)	Conditions of keeping at 0° (Table 3)	Results
Fumaric acid, 10, 20; glutamic acid, 10; glutamine, 2; succinic acid, 10, 40; nicotinamide, 2, 4, 20; thiamine, 1.5, 3; inositol, 0.5; ascorbic acid, 1; oxidized and reduced glutathione, 0.1; cozymase, 0.1; adenosine 5-phosphate, 1, 10; adenosine triphosphate, 1, 10	B	Response 15% or less with or without the added compounds
Fumaric acid, 5; glutamic acid, 5; oxidized glutathione, 0.1; cytidine, 1; γ -aminobutyric acid, 10; polyvinylpyrrolidone, 1.25%, 2.5%; gelatin, 2.5%	C	Response 28% or less with or without the added compounds
Muscle extract, 0.1–0.5%; desalted dialysate of muscle extract, 0.5–2%	C	Response 24% with or without added substances
Glutamine, 2; group i: glutamic acid, 2 and fumaric acid, 2; group ii: thiamine, 1, nicotinamide, 1, creatine, 1, adenosine triphosphate, 1; group ii plus fumaric acid, 2; group ii plus glutamic acid, 2; group ii plus group i. Hot extracts of rabbit and of guinea-pig brains (approx. 5 mg. of solids/ml.)	D	No response without additions; response 12% or less with added compounds

stimulating agents was expressed as a percentage of their respiration without stimulation and plotted against the quantity of added material.

On this basis, blood serum afforded the greatest restoration of response (Fig. 3, Table 5). Low concentrations of extracts from cerebral tissues or liver had activity equal to or greater than those of corresponding weights of serum, but in greater concentrations the liver and brain were inhibitory. The desalted dialysate of meat extract, described in the Experimental section, was without effect on the exposed tissue when included at 10 mg./ml. of medium. Many constituents of this dialysate have been identified (Wood & Bender, 1957). The meat extract before treatment contained too much salt for testing in the present system. Of the cerebral extracts, (a) and (b) (see Experimental section) were made by a process similar to that in which the tissue was kept in fluid and by which the test tissue lost its response; and at levels of about 6 mg. of solids/ml. they increased respiratory response from some 25 to 38 % under conditions *C* of Table 3. The extracts produced by blending the tissue greatly increased the respiration of normal as well as of depleted tissue, but had little effect in making good the respiratory response. After a few attempts to

Fig. 3. Effects of various concentrations of tissue preparations in restoring the respiratory response of guinea-pig cerebral cortex previously exposed at 0°. The sliced cortex was exposed under conditions *C*, Table 3, and respiratory response to electrical pulses was measured in tri-s-glucose media. Response is expressed as the mean percentage increase in respiration caused by the pulses, as in the last columns of Table 3. Serum was reconstituted from dried human serum; tissue extracts were prepared by method *c* (see Experimental section). $\blacktriangle$, Tissue with liver extract; $\circ$, tissue with brain extract; $\bullet$, tissue with serum.

Table 5. Action of blood sera and preparations therefrom on the respiratory response of cerebral tissues previously kept at 0°

Guinea-pig cerebral cortex was kept at 0° and examined under the conditions *C* of Table 3. Values are followed by s.d. and, in parentheses, the number of observations. The preparations were examined also for effect on the respiratory response of normal tissues (not kept at 0°), and were without action [mean response: 90 % $\pm$ 9 (16) in the absence of sera, and 82 % $\pm$ 11 (8) with sera].

Tissue respiration				
Material added	Dry wt. (mg./ml.)	Rates (μ moles of O ₂ /g./hr.)		Increase (%)
		Without pulses	With pulses	
None	—	44.5 $\pm$ 5 (39)	53 $\pm$ 7 (53)	20
Human serum	6	50	59	18
	12	50 $\pm$ 4 (4)	60 $\pm$ 5 (4)	20
	18.8	48 $\pm$ 4 (3)	65.5 $\pm$ 6 (3)	37
	24	61 $\pm$ 5 (3)	97 $\pm$ 7 (3)	59
	47	57.5 $\pm$ 4 (3)	82 $\pm$ 5 (3)	43
Ox serum	6	50 $\pm$ 3 (3)	60 $\pm$ 4 (3)	20
	12	51 $\pm$ 6 (3)	68 $\pm$ 7 (3)	33
	24	56 $\pm$ 5 (4)	88.5 $\pm$ 7 (4)	58
Guinea-pig serum	10	48 $\pm$ 6 (4)	68.5 $\pm$ 8 (4)	43
	20	50 $\pm$ 5 (4)	89 $\pm$ 10 (4)	78
Human serum, reconstituted, before dialysis	5	42.5 $\pm$ 5 (8)	49 $\pm$ 6 (12)	15
	10	47.8 $\pm$ 4.5 (10)	70 $\pm$ 5 (14)	44
	15	49 $\pm$ 6 (5)	75 $\pm$ 7 (5)	53
	20	50 $\pm$ 7 (10)	80 $\pm$ 8 (10)	60
	30	50 $\pm$ 6 (4)	75 $\pm$ 8 (4)	50
Human serum, reconstituted, dialysed	5	43 $\pm$ 6 (5)	55 $\pm$ 6 (6)	28
	10	47 $\pm$ 7 (10)	69 $\pm$ 8 (10)	47
	15	48 $\pm$ 6 (10)	73 $\pm$ 7 (10)	52
	20	50 $\pm$ 5 (8)	84 $\pm$ 8 (10)	68
Human serum, reconstituted, dialysed and acetone-precipitated	0	44 $\pm$ 5.8 (17)	51.3 $\pm$ 9 (13)	16
	5	39 $\pm$ 2.0 (5)	52 $\pm$ 4.8 (5)	34
	10	36 (2)	51 (2)	42
	15	39 $\pm$ 8 (17)	61 $\pm$ 4.8 (15)	57
	20	39 $\pm$ 3.2 (4)	64.5 $\pm$ 8 (5)	65

separate possible stimulating and inhibiting agents from brain and liver had been unsuccessful, blood serum was examined in more detail.

Activities of human, ox and guinea-pig sera were similar. After dialysis of human serum for periods up to 18 hr., the diffusible material, concentrated by evaporation, was found relatively inactive in restoring the tissue's response. Activity was retained, with little loss, in the non-diffusible fraction (Table 5). From the dialysed solutions it could be precipitated with acetone. Sera from different sources were found similarly active, and further information was sought by examining blood-protein fractions available commercially. These (Table 6; see Experimental section) were derived from ox plasma by the ethanol-fractionation of Cohn *et al.* (1946). Fraction IV-4 was found markedly more active than the others. Its activity was partly lost on heating (Table 6).

Tissue phosphates after loss and restoration of response

Of the many chemical characteristics of the tissue which could be relevant to the loss and restoration of its excitability, the levels of inorganic phosphate and creatine phosphate have been studied first. The brain *in vivo* contains about 3 μ moles/g. of each of these phosphates under

normal conditions (see McIlwain, 1959); in tissue removed for the metabolic experiments, but before other treatment, phosphocreatine is seen to be largely lost and much inorganic phosphate formed (Table 7). After incubation in glucose media, inorganic phosphate was restored to its normal level and a large part of the phosphocreatine re-synthesized. During the exposure of 5 hr. at 0° in tris-glucose medium, which was adopted for causing loss of response, some resynthesis of phosphocreatine and loss of inorganic phosphate also occurred (Expt. 2, Table 7). These processes were taken further on incubating the tissue after exposure, but neither proceeded as far as in the tissue which had not been kept at 0°. The serum which permitted further respiratory response also caused further resynthesis of phosphocreatine. Moreover, this effect of serum on the phosphates of the unresponsive tissue was the reverse of its effect on tissue incubated without having been kept at 0°. Here the inclusion of serum led to lower phosphocreatine and higher inorganic phosphate than were found in the same media lacking serum. (Of serum constituents, glutamic acid and glutamine have this effect: McIlwain, 1952.) These changes were such that, after incubation in tris-glucose media with serum, the phosphates reached similar levels whether or not the tissue had been kept at 0°.

Table 6. *Ox-plasma fractions and the respiratory response of tissues kept at 0°*

Guinea-pig cerebral cortex was kept at 0° and examined under the conditions *C* of Table 3. Values are from duplicate determinations except when followed by s.d. and, in parentheses, the number of observations.

Plasma fraction added	Dry wt. (mg./ml. of medium)	Respiration (μ moles of O ₂ /g./hr.)		Increase in respiration (%)
		Without pulses	With pulses	
None	0	46 $\pm$ 4 (13)	54 $\pm$ 6 (9)	17
I	7.5	58	67	15
	15	57	55	0
II	7.5	42	54	28
	15	43 $\pm$ 4 (5)	50.5 $\pm$ 3 (5)	17
	30	46 $\pm$ 5 (7)	60.5 $\pm$ 7 (5)	30
III-1	10	36	46	29
	20	45	49	9
III-2	10	47	59	26
	20	46	59	27
IV-1	5	44 $\pm$ 3 (4)	59 $\pm$ 4 (4)	34 $\pm$ 7
	10	54 $\pm$ 6 (5)	81 $\pm$ 7 (5)	50 $\pm$ 4
	20	49 $\pm$ 5 (5)	68 $\pm$ 4 (5)	39 $\pm$ 13
IV-4	5	51 $\pm$ 7 (6)	78 $\pm$ 8 (3)	52 $\pm$ 9
	10	45 $\pm$ 5.4 (6)	76 $\pm$ 8 (5)	67 $\pm$ 4
	20	43.5 $\pm$ 5 (5)	70 $\pm$ 10 (4)	62.5 $\pm$ 14
IV-4, after 2 min. at 100°	10	52 $\pm$ 6 (7)	66 $\pm$ 8 (6)	30
V	10	45 $\pm$ 4 (3)	66 $\pm$ 6 (3)	46
	20	54 $\pm$ 5 (3)	74 $\pm$ 3 (3)	38
Albumin	5	43 $\pm$ 4 (4)	55 $\pm$ 5 (4)	28
	10	45 $\pm$ 3 (8)	58 $\pm$ 7 (7)	29
	30	44 $\pm$ 4 (3)	59 $\pm$ 6 (3)	34
	60	48 $\pm$ 5 (3)	61 $\pm$ 6 (3)	27

Table 7. *Inorganic phosphate and phosphocreatine in treated tissues*

Guinea-pig cerebral cortex was prepared at room temperature. Incubation was for 45 min. in tris-glucose media at 37°. Reconstituted human serum was added where indicated, at a concentration giving 20 mg. dry wt./ml. Extraction of the tissue was done by grinding in 10% (w/v) trichloroacetic acid. Depletion was under conditions C, Table 3. Values are followed by s.d. and, in parentheses, the number of observations.

Group of Expts.	Treatment of tissue before extraction	Phosphocreatine (μ moles/g.)	Inorganic phosphate (μ moles/g.)
1	None	0.15 $\pm$ 0.01 (3)	7.2 $\pm$ 1.0 (3)
1	Incubated in glucose medium	1.64 $\pm$ 0.15 (6)	3.05 $\pm$ 0.36 (6)
1	Incubated with serum addition	1.06 $\pm$ 0.05 (4)	3.15 $\pm$ 0.7 (4)
2	Kept at 0°	0.43 $\pm$ 0.02 (4)	4.78 $\pm$ 0.58 (4)
2	Kept at 0°, incubated in glucose medium	0.85 $\pm$ 0.07 (7)	4.03 $\pm$ 0.24 (7)
2	Kept at 0°, incubated with serum addition	1.16 $\pm$ 0.14 (6)	3.86 $\pm$ 0.30 (6)
3	Kept at 0°, incubated in glucose medium	1.05 $\pm$ 0.2 (4)*	4.95 $\pm$ 1.5 (4)
3	Kept at 0°, incubated with plasma fraction IV-4 (5 mg./ml.)	1.35 (2)	3.4 (2)
3	Kept at 0°, incubated with plasma fraction IV-4 (10 mg./ml.)	1.55 $\pm$ 0.3 (5)*	4.24 $\pm$ 1.4 (5)

* In Expts. 3, comparison of the mean of the individual differences (in each experiment) caused by the addition of the plasma fraction, with the mean value for the slices not receiving the addition, indicated the effect of the addition on phosphocreatine to be significant with $P < 0.01$. The effect on inorganic phosphate was not significant.

Moreover, the serum fraction IV-4, possessing greatest ability to restore the tissue's respiratory response, was also found active in permitting resynthesis of phosphocreatine (Expt. 3, Table 7).

DISCUSSION

Under the conditions used in the present study, cerebral tissues have suffered greater loss in their respiratory response to electrical excitation than in their resting respiration, and a specific but unidentified material has been found of which a small quantity can restore response to excitation. It is relevant to consider possible relationships between the restorative material and the change undergone by the tissue on losing its ability to respond to excitation.

(1) Neural tissues excized and placed in media lose a variety of substances, some of which appear in the medium (Winterstein, 1929; McIlwain, 1958). In the present experiments, keeping the tissue in medium (at 0° for 5–17 hr.) is an essential part of the procedure causing loss of response, for tissue kept at the same temperature for the same time but without medium did not suffer loss of excitability. Loss occurred more slowly in a phosphate-buffered medium (30 mM-phosphate) than in a similar medium buffered with tris (2 mM-phosphate), and is seen to involve changes in tissue phosphates.

(2) Nevertheless, material which appeared in the medium when cerebral tissues were suspended in it did not restore excitability. Thus the medium does

not appear to be acting simply as a receiver of essential substances diffusing from the tissue; or, if it is so acting, the substances lost are not in a form in which they are readily re-assimilated by the tissue, for the defect induced in the tissue can be made good when other naturally occurring materials are added to the medium in which tissue is respiring.

(3) The material most successful in restoring tissue excitability comprises some 6% of the total proteins of blood plasma (Cohn *et al.* 1946). Its ability to restore the tissue does not receive a simple explanation in terms of its major protein constituents. The fraction IV-4 consists largely of α - and β -globulin. Fraction V, precipitated subsequently and less active in restoring excitability, contains much less of these globulins; but they are contained in fractions II and III, which are precipitated before fraction IV and which are relatively inactive in the test system we have used.

(4) As the metabolic response of cerebral tissues to excitation is greatly affected by the ionic composition of the tissue and of suspending media, it is relevant to note that Solomon, Lionetti & Curran (1956) have offered evidence for a cation-carrying substance in blood, and that Creese, D'Silva & Northover (1958) found that a non-diffusible fraction of blood serum aided the maintenance of the sodium content of isolated diaphragm at its normal (low) values. Also, Lindan, Quastel & Sved (1957) found serum fractions effective in antagonizing an inhibitory effect of chlorpromazine on cerebral tissues, and chlorpromazine was found by

McIlwain & Greengard (1957) to inhibit the metabolic response of cerebral tissues to electrical pulses. Lipids or lipoproteins are suspected of being involved in some of these actions, and the material is being further characterized.

SUMMARY

1. Slices of guinea-pig cerebral cortex have been exposed to a variety of adverse conditions with the object of diminishing their respiratory response to electrical stimulation, while maintaining relatively normal respiratory rates in the absence of stimulation.

2. The greatest differential effect occurred on exposure in tris-buffered glucose-containing media at 0° for 5 hr. These conditions lowered also the tissue's glycolytic response to electrical pulses and its respiratory response to high concentrations of potassium salts.

3. Restoration of the exposed tissue's respiratory response to electrical pulses was sought by adding a variety of substances to media in which the tissue was incubated. Additions included known metabolites, blood sera and extracts of brain, muscle and liver.

4. Blood sera from several animal species were the most active of these materials in restoring respiratory response. Dialysis and solvent precipitation suggested that the activity was associated with the serum proteins. Examination of plasma fractions obtained by the method of ethanol precipitation of Cohn *et al.* (1946) showed the most active fraction to be IV-4, which contains α - and β -globulin.

5. The inorganic phosphate and phosphocreatine of cerebral tissues have been determined after the chosen conditions of exposure and after incubation with blood-protein preparations, and relationships between the exposed tissue and restorative agents have been discussed.

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Studies on the Biosynthesis of Cholesterol

9. FORMATION OF PHOSPHORYLATED DERIVATIVES OF MEVALONIC ACID IN LIVER-ENZYME PREPARATIONS*

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After the discovery by Tavormina, Gibbs & Huff (1956) that mevalonic acid (3:5-dihydroxy-3-methylpentanoic acid) is efficiently converted into cholesterol by rat-liver homogenates, and after the

* Part 8: Gosselin & Popják (1959).

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demonstration that this acid furnishes the fundamental building unit in the synthesis of squalene (Cornforth, Cornforth, Popják & Gore, 1957, 1958; Dituri, Gurin & Rabinowitz, 1957) and hence of cholesterol (Isler, Rüegg, Würsch, Gey & Pletscher, 1957), we studied the enzymic transformations of mevalonic acid in liver preparations. It has been