Site of Synthesis of the Peptide Component of the Cell Wall of *Bacillus megaterium*

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(*Received* 31 *March* 1959)

Gale & Folkes (1953) showed that single radioactive amino acids are still incorporated into the protein fraction of *Staphylococcus aureus* in the presence of growth-inhibitory concentrations of chloramphenicol. More recently, the bulk of this residual incorporation of amino acids has been found to represent synthesis of the hexosamine-peptide polymer which is a major constituent of the bacterial cell wall (Mandelstam & Rogers, 1958; Hancock & Park, 1958). Similar conclusions were reached by Crathorn & Hunter (1958) when studying the chloramphenicol-resistant uptake of [^{14}C]amino acids by *Bacillus megaterium*.

It was also shown that the initial uptake of L-[^{14}C]alanine in *B. megaterium* was located in the cytoplasmic (protoplast) membrane of the cell. It thus seemed likely that the peptides of the cell wall were elaborated at the same site as the mass of the cellular protein (Hunter, Crathorn & Butler, 1957; Butler, Crathorn & Hunter, 1958). More detailed observations on the uptake of alanine into the cell-wall protein are now given together with similar experiments on the incorporation of diaminopimelic acid.

METHODS

Organism. Strain KM of *Bacillus megaterium* was cultured as previously described (Butler *et al.* 1958). For all the experiments detailed here, the organism was grown on the 'C-glucose' medium of McQuillen (1955).

Experiments on the incorporation of [^{14}C]*amino acids by* B. megaterium *in the presence of chloramphenicol.* Bacteria grown overnight were harvested and re-incubated at 30° in fresh 'C-glucose' medium (1 mg./ml.) until they re-entered the logarithmic phase of growth. Chloramphenicol (100 μg./ml. unless otherwise stated) was added and the incubation continued for 15 min. before addition of the appropriate labelled amino acid.

Incubations were terminated by addition of an equal volume of ice-cold aq. 20% NaCl. Lysozyme (0·2 mg./ml.) was added and the bacterial suspension was gently shaken at 30° until the conversion into protoplasts was complete. The protoplasts were separated from the lysozyme digest by sedimentation. Membrane and cytoplasmic fractions were then obtained by adding ice-cold water to the protoplasts and sedimenting the membrane fraction at 10 000–20 000 *g* in the usual way (Butler *et al.* 1958).

Where the whole cell-wall fraction was required, the whole cells were sedimented from the suspension in ice-cold aq. 20% NaCl without any addition of lysozyme. The cells were then disintegrated ultrasonically as described below.

Preparation of cell walls from Bacillus megaterium. In our hands, the preparation of cell walls from *B. megaterium* by shaking with glass beads in the Mickle disintegrator (Salton, 1953) has given less satisfactory results than the method outlined below.

Whole cells (1·65 g.) were suspended in water (5 ml.) and subjected to ultrasonic radiation from an MSE–Mullard tissue disintegrator for five successive 30 min. periods. The temperature was maintained as low as possible throughout the treatment. At the end of each 30 min. period, whole cells were sedimented at 500 *g* and then resuspended for the next ultrasonic treatment. The combined supernatant fractions were centrifuged at 10 000 *g* for 10 min. and the sedimented cell-wall fraction was washed twice with water and twice with 0·06M-phosphate buffer (KH_2PO_4 brought to pH 7·0 with NaOH); each time the previous sediment was redispersed ultrasonically.

The crude cell-wall preparation obtained (244 mg.) was analysed by acid hydrolysis followed by two-dimensional paper chromatography. The normal cell-wall constituents of *B. megaterium*, namely, alanine, glutamic acid and diaminopimelic acid (Ingram & Salton, 1957), were the principal amino acids present but all the other common amino acids were detectable. The contaminating material could be removed by prolonged digestion with trypsin and ribonuclease in neutral phosphate buffer, but the yield of purified cell wall was then only 106 mg. from 214 mg. of the crude preparation.

Biosynthesis of [^{14}C]*diaminopimelic acid.* A lysine-requiring *Escherichia coli* mutant was grown on the minimal medium of Davis & Mingioli (1950) supplemented with 500 μc of sodium [1-^{14}C]acetate. Other conditions and time of harvesting the bacteria were as described by Work & Denman (1953). [^{14}C]Diaminopimelic acid was isolated from the culture medium by a modification of the procedure of Work & Denman (1953). The culture medium (300 ml.) obtained after harvesting the cells was adjusted to pH 3·0 with conc. HCl and boiled for 5 min. The slight precipitate obtained on cooling was spun off and the clear solution passed through a column of Amberlite 1R 120 (H form; 10·5 cm. × 2 cm.) previously washed until neutral. The column filtrate contained no ninhydrin-positive material. Elution of this column with aq. 2N-NH_3 soln. removed all the absorbed amino acids in the first 100 ml. After evaporation to dryness the amino acid mixture was taken up in 10 ml. of 1·5N-HCl and applied to a column of Zeo-Karb 225 (H form; 20 cm. × 2 cm.). The column was eluted with 1·5N-HCl and 25 ml. fractions were collected automatically. Fractions 17–22 were found to contain

diaminopimelic acid and on concentration yielded diaminopimelic acid dihydrochloride. Conversion into the free amino acid gave diaminopimelic acid (21 mg.) at a specific activity of 0·2 μc/mg. The overall radiochemical yield from sodium [1-^{14}C]acetate was 0·84%.

Paper chromatography and radioautography of hydrolysed protein and peptide fractions. These processes were carried out as described previously (Hunter, Brookes, Crathorn & Butler, 1959).

Materials. Generally labelled L-amino acids (5–9 mc/m-mole) and sodium [1-^{14}C]acetate (2 mc/m-mole) were supplied by The Radiochemical Centre, Amersham, Bucks. [1-^{14}C]Glycine (10·3 μc/mg.) was synthesized in these laboratories. All the labelled compounds were used without any dilution by non-labelled material.

Assays of radioactivity. Protein and other samples were prepared for assay of radioactivity as described previously (Crathorn & Hunter, 1957; Butler *et al.* 1958). End-window counting of solid ^{14}C samples was carried out as before (Crathorn & Hunter, 1957). The specific radioactivities of cell-wall 'protein' fractions were usually calculated by difference from the values obtained on the whole cell, the membrane and the cytoplasmic fractions. It was found that the calculated results agreed with those determined by direct isolation of the cell walls if the cell-wall 'protein' was assumed to account for 30% of the cellular protein, the membrane fraction 25% and the cytoplasmic fraction 45%. Errors due to adsorption, etc., are not important (see Hunter *et al.* 1959).

RESULTS

Effect of chloramphenicol on the incorporation of labelled amino acids into the protein fractions of the cell. In the presence of growth-inhibiting concentrations of chloramphenicol, it was found that the rate of uptake of several labelled amino acids into the protein of the whole cell was reduced to 2–4% of that in its absence. With L-alanine, which occurs in the cell wall, the observed inhibition in the amount of its incorporation into whole-cell protein was only 40–85%. The rate of uptake of glycine, which might perhaps undergo a limited metabolic conversion into a cell-wall constituent, was also inhibited by chloramphenicol to a slightly less extent than most of the other amino acids tested. Typical results are shown in Table 1. A closer examination of the results obtained with L-alanine showed that, although the bulk of the chloramphenicol-resistant incorporation was obviously located in the cell wall, the labelling of the membrane fraction was also inhibited to a much smaller extent than that of the cytoplasmic fraction.

A time study was therefore made of the uptake of L-[^{14}C]alanine into the various protein fractions in the presence of chloramphenicol. It revealed (Fig. 1) that at the earliest times the protein of the cytoplasmic membrane had the highest specific radioactivity. When incubations were prolonged beyond 2 min. the cell-wall protein was more heavily labelled.

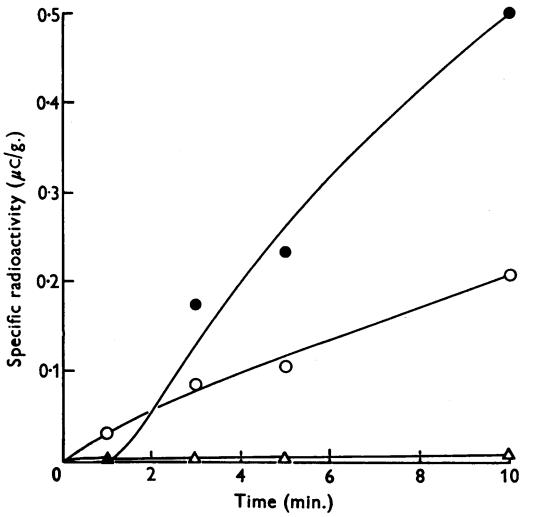

Fig. 1. Uptake of L-[^{14}C]alanine (10 μc) into the protein of the various cellular fractions in the presence of chloramphenicol. Conditions of incubation and sampling were as described in the text. Altogether 1 g. of whole cells was used in the experiment. ●, Cell wall (calc.); ○, cytoplasmic membrane; △, cytoplasm.

Table 1. *Uptake of labelled amino acids into the various cellular-protein fractions in the presence and absence of chloramphenicol*

The whole cells (250 mg. for each treatment) were incubated with or without chloramphenicol as described in the text. A portion (2 μc) of the [^{14}C]amino acid to be examined was added and the incubations were continued for a further 30 min. One-fifth of the suspension was then withdrawn and treated with trichloroacetic acid (to 5%), and membrane and cytoplasm fractions were prepared from the remainder of the suspension as described in the text.

[^{14}C]Amino acid added	Chloramphenicol	Specific radioactivity (μc/g.) of the protein		
		Whole cell	Membranes	Cytoplasm
L-Alanine	–	1·83	1·42	1·44
	+	0·33	0·20	0·02
Glycine	–	0·97	1·02	1·14
	+	0·073	0·059	0·038
L-Valine	–	1·89	1·94	2·54
	+	0·037	0·049	0·060

Supplementary experiments with L-[^{14}C]*alanine.* These were designed to test the hypothesis that the cell-wall peptides were initially synthesized within the cytoplasmic-membrane fraction. In the first place, cells labelled for 20 min. in the presence of chloramphenicol were washed and then 'starved' for 2 hr. by incubating them at 30° in a neutral phosphate buffer containing no labelled substances. They were then harvested and suspended in 'C-glucose' medium before re-incubating at 30°, chloramphenicol being present all the time. Radioactive assays showed that the radioactivity of the cell-wall peptides rose rapidly on re-incubation. However, the specific radioactivity of the membrane protein also rose slowly (Fig. 2).

In another experiment, whole cells were converted into protoplasts and then incubated at 30° for 20 min. with L-[^{14}C]alanine as described elsewhere (Hunter *et al.* 1959), but with the addition of chloramphenicol (0·1 mg./ml.) to the medium. The protoplasts were harvested and the radioactivity of the protein in the medium was determined with the aid of added 'carrier' serum albumin. However, there was at least ten times as much labelled protein in the membrane fraction as in the medium. A similar experiment was carried out with the isolated membrane fraction, again as described elsewhere (Hunter *et al.* 1959), with similar results. It was noteworthy, however, that the uptake of L-[^{14}C]alanine with membrane protein was inhibited to a lesser extent (80 %) by chloramphenicol than was L-valine (96 %), for instance.

In view of the known inhibitory effect of penicillin on cell-wall synthesis (Park & Strominger, 1957), cells were incubated as usual with L-[^{14}C]-alanine, in the presence of both chloramphenicol and penicillin. The incorporation of L-[^{14}C]alanine into the cell-wall peptides was stopped completely by the combination of antibiotics, but the incorporation of label into the membrane-protein fraction was not affected (Table 2).

To check the possibility that chloramphenicol might act by inhibiting cytoplasmic enzymes responsible for transferring labelled peptides from the membranes to the cytoplasm, membranes labelled in the normal way (Butler *et al.* 1958) with L-[^{14}C]alanine were incubated with cytoplasm treated with chloramphenicol (0·1 mg./ml.). Radioactivity was transferred to the cytoplasmic-protein fraction as usual (Table 3).

Incorporation of [^{14}C]*diaminopimelic acid and* L-[^{14}C]*aspartic acid in the presence of chloramphenicol.* Initial experiments showed that the bio-

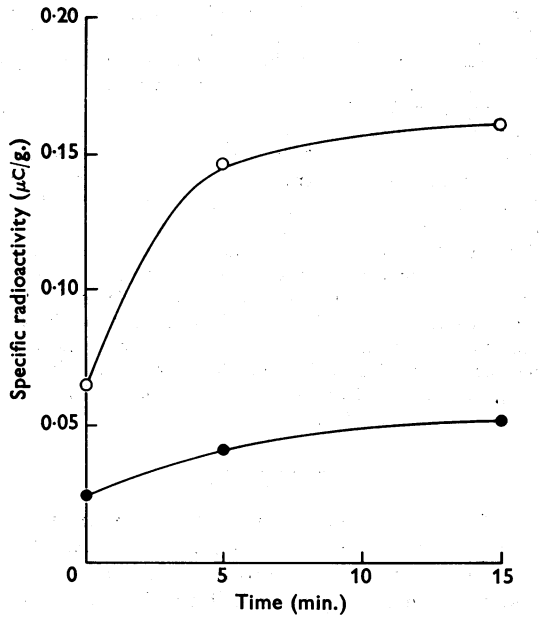

Fig. 2. Increase in specific radioactivity of protein from cell-wall and membrane fractions upon re-incubation of whole cells in 'C-glucose' medium. The cells (750 mg.) were initially labelled with L-[^{14}C]alanine (6 μc) in the presence of chloramphenicol as described in the text. They were then washed and suspended (1 mg./ml.) in 0·1 M-KH_2PO_4 (brought to pH 7·0 with NaOH) and incubated at 30° for 2 hr. in the presence of chloramphenicol (0·1 mg./ml.). They were finally resuspended (1 mg./ml.) in 'C-glucose' medium containing chloramphenicol (0·1 mg./ml.). ○, Cell wall (calc.); ●, cytoplasmic membrane.

Table 2. *Combined effect of penicillin and chloramphenicol on the uptake of* L-[^{14}C]*alanine into the protein fractions of the cell*

Cells (250 mg. for each treatment) were incubated for 15 min. as described in the text in the presence or absence of penicillin (0·5 mg./ml.) and chloramphenicol (0·1 mg./ml.). L-[^{14}C]Alanine (5 μc) was added in each case and the incubations were continued for a further 20 min. before terminating them as described in the text.

	Specific radioactivity of protein (μc/g.)		
Addition	Cell walls (calc.)	Cytoplasmic membrane	Cytoplasm
—	8·4	4·3	4·3
Chloramphenicol	4·1	1·6	0·04
Chloramphenicol + penicillin	0·0	1·9	0·05

Table 3. *Effect of chloramphenicol on the transfer of labelled protein from the membrane fraction to cytoplasm*

The membrane fraction was labelled *in vitro* with L-[^{14}C]alanine, and the transfer experiments were carried out according to Butler, Crathorn & Hunter (1958). Chloramphenicol was used at 1 mg./ml., and both treated and untreated cytoplasm were incubated at 30° for 15 min. before carrying out the transfer.

	Final radioactivity (mμc)		
	Membrane	Cytoplasm	Transfer (%)
Untreated cytoplasm	12·0	3·5	23
Cytoplasm treated with chloramphenicol	8·0	2·7	25

Table 4. *Incorporation of* L-[^{14}C]*lysine,* L-[^{14}C]*aspartic acid and* [^{14}C]*diaminopimelic acid into the whole cellular protein in the presence of chloramphenicol*

Whole cells (150 mg.) were treated with chloramphenicol as described in the text and divided into three parts, each of which was re-incubated with the amino acid concerned (1 μc). Samples were taken at intervals, treated with trichloroacetic acid and the protein was isolated and assayed in the usual way.

Incubation time (min.)	Specific radioactivity (μc/g.) of protein from cells treated with		
	L-[^{14}C]Lysine	L-[^{14}C]Aspartic acid	[^{14}C]Diaminopimelic acid
15	0·40	1·20	0·32
30	0·38	1·62	0·24
60	0·46	1·64	0·32
120	0·50	1·90	0·68
240	0·44	1·82	0·60

synthetic [^{14}C]diaminopimelic acid was not very efficiently incorporated by the whole cell. A comparison of the incorporation of L-[^{14}C]lysine and [^{14}C]aspartic acid, both possible biosynthetic precursors of diaminopimelic acid, with the incorporation of [^{14}C]diaminopimelic acid itself (Table 4) showed that in the presence of chloramphenicol the L-aspartic acid label was utilized much more readily than the ^{14}C of the diaminopimelic acid. This does not mean, of course, that the aspartic acid was incorporated more efficiently by weight than the diaminopimelic acid, which had a specific radioactivity about 100 times less than that of the aspartic acid. However, as [^{14}C]diaminopimelic acid of high specific activity was not available, a closer examination of the incorporation of [^{14}C]-aspartic acid was made.

A time study of the uptake of L-[^{14}C]aspartic acid into the different parts of the cell showed (Fig. 3) that, as with L-[^{14}C]alanine, the incorporation of aspartic acid was practically restricted to the cell-wall and membrane fractions. The cell-wall and membrane protein fractions were further examined by total acid hydrolysis, paper chromatography and radioautography. In all cases the principal radioactive component (about 90%) of the membrane and wall fractions was found to be diaminopimelic acid.

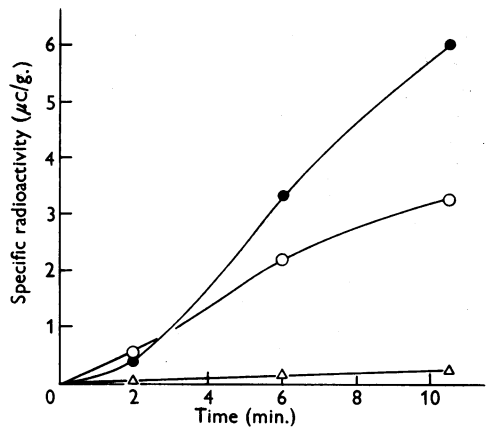

Fig. 3. Uptake of L-[^{14}C]aspartic acid (30 μc) into the protein of the various cellular fractions in the presence of chloramphenicol; 75 mg. of the whole cells was used. Conditions of incubation and the subsequent operations were as described in the text. ●, Cell wall (calc.); ○, cytoplasmic membrane; △, cytoplasm.

DISCUSSION

As more information became available about the composition of bacterial cell walls, it became obvious that the amino acids whose incorporation into the protein of *Staphylococcus aureus* was least

affected by chloramphenicol (Gale & Folkes, 1953) were also important constituents of the staphylococcal cell wall. Our results show that a similar situation exists in *Bacillus megaterium*, where the incorporation of L-alanine, a cell-wall constituent, and of L-aspartic acid, the biochemical precursor of diaminopimelic acid, are less affected by chloramphenicol than is the incorporation of other amino acids. With *S. aureus*, two groups of workers (Mandelstam & Rogers, 1958; Hancock & Park, 1958) showed that the bulk of the chloramphenicol-resistant uptake could indeed be accounted for by the incorporation of amino acids into cell-wall peptides. However, neither group of workers examined the labelling of the cytoplasmic-membrane fraction and the methods used would not have detected even a considerable amount of radioactivity in this fraction. With *B. megaterium*, our time studies of the uptake of L-[^{14}C]alanine into the various cellular fractions show that in the presence of chloramphenicol the protein of the cytoplasmic membrane fraction becomes significantly labelled. In fact, at the shortest times, the membrane protein carries a higher specific radioactivity than the cell-wall peptides. This is reminiscent of the situation with regard to cytoplasmic-protein synthesis (Butler *et al.* 1958), and suggests that the site of synthesis of the cell-wall peptides also lies within the cytoplasmic-membrane fraction.

Further support for this idea was difficult to obtain. Thus cells labelled with L-[^{14}C]alanine in the presence of chloramphenicol were 'starved' in phosphate buffer in the expectation that on reincubation in a full medium, labelled peptides would pass from the membrane fraction into the cell wall. However, the endogenous pool of L-alanine in the cell apparently remained so high that the amount of labelled material in the membrane protein actually continued to rise on reincubation. The specific radioactivity of the cell-wall peptide fraction certainly rose much more rapidly (Fig. 2), but this could be construed as no more than an indication of passage of labelled peptides from membrane to wall.

Nothing definite could be deduced from the experiments with protoplasts or the membrane fraction, although a lower degree of inhibition of the uptake of L-alanine into protein was observed. Only very small amounts of radioactive material passed from these fractions into the medium, which suggested that cell-wall receptors are necessary before labelled precursors are released from the membrane fraction.

Again, the experiments with penicillin were inconclusive. In one respect, however, the information obtained was valuable. It showed that the labelled peptide formed in the membrane fraction in the presence of chloramphenicol could hardly be due to the presence of residual wall mucopeptide not removed by lysozyme. Its formation was not affected by penicillin, which reduced the labelling of the cell-wall fraction itself to zero. Apparently penicillin, even in the high concentration used, does not inhibit the formation within the membrane fraction of precursors of cell-wall peptides. This finding is, however, in conformity with Park's (1958) suggestions on the mode of action of penicillin. He considers it 'probable that penicillin interferes with cell-wall synthesis by preventing the incorporation of the *N*-acetylmuramic acid peptide fragment into the wall'. However, results of our experiments on the transfer of labelled protein *in vitro* from the membrane to cytoplasm poisoned with chloramphenicol do make it unlikely that chloramphenicol has a corresponding action on the passage of peptides or proteins into the cytoplasm.

We decided that to obtain conclusive information it would be necessary to study the uptake of an amino acid present only in the cell-wall peptides. Diaminopimelic acid is apparently absent from the cytoplasmic proteins, but the initial experiments with the biosynthetic [^{14}C]diaminopimelic acid soon revealed that very large quantities of the labelled compound would be required to obtain useful results. However, it is known that L-aspartic acid is an immediate biosynthetic precursor of diaminopimelic acid in some organisms (work summarized and extended by Gilvarg, 1958), and it was soon found that the ^{14}C of L-aspartic acid was efficiently incorporated into the 'protein' fractions of both cell wall and membrane by *B. megaterium*. It is not certain from our experiments that L-aspartic acid is actually used more efficiently by the cell than the diaminopimelic acid, as we have no information about the relative 'pool' sizes of the two amino acids within the cell. But certainly we could obtain much heavier labelling of the protein fractions concerned when using the L-aspartic acid.

Conclusive evidence that aspartic acid was being converted by the cell into diaminopimelic acid was obtained when the proteins of membranes and cell walls, labelled with L-[^{14}C]aspartic acid in the presence of chloramphenicol, were hydrolysed and the products examined by chromatography and radioautography. With both membranes and cell walls the major labelled amino acid was diaminopimelic acid, which was estimated to contain approximately 90% of the radioactivity present in the hydrolysate. These results seem to give adequate confirmation of the hypothesis that the peptide components of the cell wall of *B. megaterium* are synthesized at sites on or closely associated with the cytoplasmic membrane.

SUMMARY

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

2. The incorporation of L-[^{14}C]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 ^{14}C into the peptide fraction of the cell wall. However, when the L-[^{14}C]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-[^{14}C]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-[^{14}C]-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.

The authors are grateful to Professor J. A. V. Butler for his encouragement and advice. They also wish to acknowledge the skilful technical assistance of Mr R. Goodsall.

Dr Pauline Meadow (University College London) was kind enough to carry out for us the incubations of the *E. coli* mutant with sodium [1-^{14}C]acetate.

This investigation has been supported by grants to the Chester Beatty Research Institute from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund and the National Cancer Institute of the National Institutes of Health, United States Public Health Service.

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

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(*Received* 9 *April* 1959)

Electrical pulses applied to cerebral tissues initiate large and rapid metabolic changes akin to those associated with increased cerebral activity *in vivo* (McIlwain, 1956*b*, 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, 1956*a*). The partial restoration which was achieved was, however, obtained with known metabolites: with malic acid or fumaric acid, adenine or nicotinamide.