The Production of Acetylcholine by a Strain of *Lactobacillus plantarum*

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With an Addendum on the Isolation of Acetylcholine as a Salt of Hexanitrodiphenylamine

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SUMMARY: A strain of *Lactobacillus plantarum* has been isolated from Sauerkraut which can produce acetylcholine during growth and in washed suspensions. The conditions necessary for the production of acetylcholine are: (i) the presence of choline and (ii) the simultaneous fermentation of carbohydrate. The acetylcholine is formed inside the cells and subsequently passes into the medium. The amount formed can be about 5 μg. acetylcholine/mg. dry wt. cells/hr. The acetylcholine was identified by the usual biological tests and was also isolated and identified as the salt of hexanitrodiphenylamine. The mechanism of the acetylation has not been discovered. Cells grown in the absence of added pantothenate acetylate extremely slowly. The rate is restored by the addition of pantothenate to the cell suspension.

Instances of the production of acetylcholine by micro-organisms are not very numerous. Ewins (1914) isolated it as the platinic chloride from 1600 ml. of a liquid extract of ergot prepared according to the *British Pharmacopoeia* and hence attributable to production by *Claviceps purpurea*; the yield was not given. Keil & Kritter (1934) found 40 μg./ml. acetylcholine in Sauerkraut juice. From 3 l. of Sauerkraut they prepared a reineckate of acetylcholine from which 0·2 g. of a gold chloride compound was obtained. The same workers (1935) obtained from fresh white cabbage some choline which they suggested was the origin of acetylcholine in Sauerkraut, but they were unable to find choline in fresh cucumber, though acetylcholine was found in fermented cucumber; they stated that choline added to fermenting cucumber did not increase the yield of acetylcholine, which finally amounted to 25 μg./ml. estimated biologically. The organism was described as a lactic acid bacillus.

Keil & Gropp (1934–5) obtained 0·012 g. of a gold salt of acetylcholine from 2·7 l. of fermented cucumber juice. Its isolation from a silage of maize and sunflowers has also been reported (Keil & Pörtner, 1935).

Lactic acid bacteria producing acetylcholine in the fermentation of plant juices seem widespread. They have been isolated from: Sauerkraut; fermenting cucumber silage (Habs, 1937–8); fourteen kinds of fermented vegetables (Möller & Ferdinand, 1937–8); rat gut, human mouth and faeces (Habs, 1937–8).

It is impossible to decide from the data whether strains isolated from all these sources represent the same organism. Where bacteriological identification

has been attempted (Möller & Ferdinand, 1937–8; Habs, 1937–8), it seems that the organism is a lactic acid-producing bacillus, found most frequently in fermenting plant juices, having a temperature optimum below 37°.

In comparing the content of acetylcholine in fermented plant juices and in animal tissues the presence in the latter of an active choline esterase, an enzyme which has not so far been reported in the former, must be remembered. In animal tissues the spleen is among the richest in acetylcholine. Dale & Dudley (1929–30) extracted it from horse spleen with cold ethanol in amounts equivalent to 10 μg./g. wet tissue. From 32·34 kg. of spleen these workers obtained 64·5 mg. of acetylcholine as the dichloroplatinate. Chang & Gaddum (1933) estimated the acetylcholine content of a number of animal tissues and found the placenta and spleen to be the richest, containing about 30 μg./g. in both cases. Other tissues had only 1–4 μg./g. or less. Stedman & Stedman (1937) found 7 μg./g. of ox brain (wet weight), from 8 kg. of which they isolated it as the chloraurate. Thus fermented plant material yields acetylcholine in amounts greater than, but comparable with, spleen, i.e. about 60–80 μg./ml.

The present research was undertaken with the object of finding the origin of acetylcholine in fermented plant material and of studying its mode of production. As Keil's strain was not available to us it was necessary to isolate an acetylcholine-producing organism.

MATERIAL AND MEDIA

Sauerkraut

Sauerkraut was prepared as follows: About 1 kg. of finely shredded cabbage was put in a Kilner bottling jar with 20 g. NaCl in alternate layers, well pressed down and left at room temperature for 6–10 days; by this time the juice was at pH 3·8 and gave a positive reaction for acetylcholine (see below). At this stage the organism could be isolated; later the organisms tended to die off due to prolonged exposure to a low pH.

Cucumber juice

Fifty-one kg. of ridge cucumbers were peeled, minced and squeezed in a press; 25 l. of juice were obtained. This was filtered through pleated paper into 5 l. flasks and steamed for 3 hr. on three successive days. It was tubed and resterilized as required.

Media

Medium 1 (*glucose agar*). 330 ml. of a tryptic digest of casein (equivalent to 10 % casein); 10 ml. 10 % Marmite; 10 g. glucose; 30 g. agar; water to 1 l.

Medium 2 (*for heavy growth*). Inorganic medium (no. 3) 100 ml.; 330 ml. of a tryptic digest of casein (10 %); 10 ml. 10 % Marmite; 20 g. glucose (or other sugar); 10 ml. M-sodium acetate; 200 mg. cysteine HCl sterilized by filtration and added last; water to 1 l.

Medium 3. *Inorganic.* KH_2PO_4, 3·0 g.; $MgSO_4.7H_2O$, 2 g.; NaCl, 3 g.; $(NH_4)_2HPO_4$, 12 g.; $FeSO_4.7H_2O$, 0·03 g.; $CuSO_4.7H_2O$, 2·5 mg.; $MnSO_4.7H_2O$, 2·5 mg.; $ZnSO_4.7H_2O$, 2·5 mg.; water to 1 l.

Medium 4. *Synthetic.* Inorganic medium (no. 3) 100 ml.; glucose 20 g.; M-sodium acetate 100 ml.; *l*-asparagine 0·1 g.; *l*-glutamine 0·1 g.; *l*-cysteine 0·2 g.; *l*-tryptophan 0·02 g.; *l*-leucine 0·02 g.; *l*-isoleucine 0·02 g.; *l*-serine 0·02 g.; *l*-phenylalanine 0·02 g.; *l*-aspartic acid 0·02 g.; *l*-valine 0·02 g.; *l*-tyrosine 0·02 g.; *l*-alanine 0·02 g.;

l-threonine 0·02 g.; *l*-lysine 0·02 g.; *l*-methionine 0·02 g.; *l*-arginine 0·02 g.; *l*-histidine 0·02 g.; thymine 0·01 g.; adenine 0·01 g.; *l*-glutamic acid 0·02 g.; uracil 0·01 g.; hypoxanthine 0·01 g.; guanine 0·01 g.; to which were added the following growth factors: aneurin 200 μg.; riboflavin 200 μg.; calcium pantothenate 100 μg.; nicotinic acid amide 200 μg.; *p*-aminobenzoic acid 100 μg.; pyridoxin 100 μg.; folic acid concentrate equivalent to about 5 μg.; water to 1 l.

Medium 5. *Semisynthetic.* Inorganic medium (no. 3) 100 ml.; acid hydrolysate of casein (10 %) 100 ml.; glucose 20 g.; M-sodium acetate 100 ml.; *l*-tryptophan 200 mg.; cysteine HCl 200 mg.; growth factors as in medium 4; water to 1 l.

Medium 6. *Reaction solution* (does not support growth). McIlvaine's buffer pH 7·5 2 ml.; (M-sodium acetate 1 ml.*) 20 % glucose 1 ml.; 0·5 % choline HCl 1 ml.; ($MnSO_4 + MgSO_4$ each 0·4 % 1 ml.*) used with 1 ml. of a bacterial suspension about 20 mg./ml.; water to 10 ml. (* these items later found to be dispensable).

Neither the cucumber juice nor media 1–6 contained preformed acetylcholine.

Methods of estimation

Miscellaneous. The amount of growth and of cell suspension used were estimated by the use of a photoelectric turbidimeter, from the readings of which the dry weight was read off from a calibration curve constructed for the organism; results are recorded in terms of dry weight of cells throughout. Glucose was estimated by the method of Miller & Van Slyke (1936) and lactic acid by the methods of Friedemann, Cotonio & Shaffer (1927) and Friedemann & Kendall (1929), the latter after treatment with copper lime.

Volatile acids were estimated by the method of Friedemann (1938).

Acetylcholine. The method used was essentially that of Chang & Gaddum (1933), the exact procedure being that employed by Dr Feldberg, to whom we are indebted for instruction in using it. The procedure is as follows: The standard acetylcholine is prepared fresh each day and made up so as to contain 1 μg./ml. in slightly acid water.

Eserine stock solution consists of 1 g. eserine in 200 ml. of slightly acid water (0·002 N-HCl); 1 ml. of this solution is diluted afresh each day with 500 ml. frog Ringer.

The muscle is excised and put up in the glass chamber with frog Ringer and aerated with a steady stream of air bubbles for 1 hr.; the Ringer is then drained off and replaced by eserinized frog Ringer and aerated for a second hour. The muscle is then ready for use.

The standard acetylcholine and the solutions to be assayed should be kept in iced water. Acetylcholine standard solution (0·2–0·5 ml.) is made up to 10 ml. with eserinized frog Ringer and poured into the chamber and the muscle allowed to contract, the contraction being registered on a drum moving at about 10 mm./min. The period allowed for contraction is 1·5 min. The muscle is then washed once with frog Ringer and left at rest in eserinized frog Ringer for 5 min. The assays are repeated alternately with standard acetylcholine and the test solution till two adjacent contractions are obtained of the same height.

In order to eliminate error due to activating or sensitizing substances other than acetylcholine in the unknown solution, a portion of this should be boiled at pH 10, neutralized and a volume added to the acetylcholine standard equal to that used in the assay.

THE ORGANISM

Isolation

From the Sauerkraut prepared as described 1 ml. samples of juice were withdrawn at intervals and tested for acetylcholine. The test was positive on the 6th day, when the acetylcholine amounted to 2·6 μg./ml. The juice was then

heavily inoculated into cucumber juice which was incubated at 25° and tested for acetylcholine daily; a positive reaction was obtained on the 4th day when the concentration was *c.* 1·2 μg./ml. The culture was again subcultivated into cucumber juice and plated on to cucumber juice agar; two types of colony were obtained: large (*A*), and small (*B*); both were replated on to glucose agar (medium 1) plates; *A*-type colonies appeared in 20 hr. and *B*-type in 40 hr.; *A* organisms were Gram-positive streptococci and *B* organisms were Gram-positive short rods; when subcultivated into cucumber juice *A* gave no acetylcholine and *B* gave acetylcholine in 5 days.

Characteristics

The organism was kindly examined for us by Dr P. M. F. Shattock of the National Institute for Research in Dairying, Shinfield, near Reading, to whom we owe the following details:

Morphology: short rods with rounded ends usually in pairs, Gram-positive. Grows well aerobically at 30°, will not grow at 45°; growth stimulated by yeast extract. Very slow production of acid in litmus milk. Acid from sucrose, lactose, maltose, raffinose, salicin and aesculin. No acid from xylose. Provisionally identified as *Lactobacillus plantarum*.

A culture has been deposited in the British National Collection of Type Cultures, no. 7220, and in the American Type Culture Collection, no. 10241.

Other organisms tested for the production of acetylcholine

Twenty recently isolated strains of streptococci were grown in cucumber juice for 4–6 days and the medium then tested for acetylcholine; none was found. The following strains of *Lactobacillus* were kindly supplied by Dr Shattock from the National Institute for Research in Dairying, and were tested on the same medium after 48 hr. growth: *Lb. bulgaricus*, *Lb. helveticus*, *Lb. acidophilus* (Booth), *Lb. delbrücki* 4033, *Lb. delbrücki B*, *Lb. casei* 3253, *Lb. casei* Y.C.T.I., *Lb. pentoaceticus* (107), *Lb. plantarum* 3254 and *Lb. plantarum* 4125; all these gave no acetylcholine. *Lb. odontolyticus* gave 4·5 μg. acetylcholine/ml.; *Lb. plantarum*, our strain, gave 7·0 μg./ml. The *Lb. odontolyticus* was subsequently identified as *Lb. plantarum*.

Whether our organism is identical with Keil's we have not enough data to decide, but the very different temperature optima for acetylcholine production, below 25° for our organism and 30–35° for Keil's (Möller & Ferdinand, 1937–8), suggest that they are distinct, though it must be admitted that the temperature optimum for growth or any chemical reaction is not a stable characteristic.

General biochemical characteristics

The organism is a facultative anaerobe, it has no detectable catalase, cytochrome or cytochrome oxidase. In accordance with its lack of haematin enzymes its respiration as measured by the manometric oxygen uptake was of a very low order, Q_{O_2} 6–10, and was not materially influenced by age of culture.

The anaerobic oxidation of glucose by methylene blue (MB) was of a higher

order; the oxidation of sugars other than glucose was low or negligible (Table 1). When the organism was grown on the sugar in question, i.e. was adapted, the Q_{MB} (sugar) was higher (Table 2).

Table 1. *The oxidation of sugars by unadapted cells of* Lactobacillus plantarum *as measured by the reduction of methylene blue*

Growth medium 2 was used. Each Thunberg tube contained: 1·0 ml. bacterial suspension; 1·0 ml. phosphate buffer pH 6·5; 0·1 ml. 0·5 % methylene blue (MB); 1·0 ml. 0·5 % sugar or water; 1·8 ml. water. The end-point was read as 90 % reduction of the MB. $Q_{MB} = \mu l.\ O_2$ equivalent to MB reduced/mg. dry wt. cells/hr.

Sugar	Q_{MB} (sugar)	Q_{MB} (blank)
Glucose	50	2·8
Fructose	9·4	2·8
Maltose	28·1	9·4
Sucrose	9·7	6·8
Lactate	6·9	2·8

Table 2. *The oxidation of sugars by adaptea cells as measured by the reduction of methylene blue*

Cells grown on medium 2. Each Thunberg tube contained: 1·0 ml. bacterial suspension; 1·0 ml. phosphate buffer pH 6·5; 0·1 ml. 0·5 % methylene blue (MB); 1·0 ml. 0·5 % sugar or water; 1·8 ml. water. End point 90 % reduction of MB. $Q_{MB} = \mu l.\ O_2$ equivalent to MB reduced/mg. dry wt. cells/hr.

Sugar *A**	Q_{MB} (sugar)*	Q_{MB} (glucose)	Q_{MB} (blank)
Fructose	77	77	5·6
Galactose	71	79	14
Arabinose	6·9	35	2·5
Sucrose	25	73	3·4
Maltose	18·4	72	4·6

* Sugar to which organism was adapted.

The organism is more efficient as a fermenter than as an oxidizer. The fermentation was measured manometrically by the liberation of CO_2 by fermentation acid from bicarbonate buffer. The Q_{CO_2} of glucose increased with age of culture from Q_{CO_2} 120 for 20 hr., to Q_{CO_2} 191 for 44 hr. Fermentation of other sugars was low or negative with unadapted cells, but increased when the organism was grown on the sugar studied (adapted cells) (Table 3).

Temperature optimum for fermentation. The bacteria were grown for 2 days at 25° on medium 2. The fermentation was carried out in Thunberg tubes; each tube contained: 2·5 ml. phosphate buffer pH 7·0; 1 ml. bacterial suspension (2·5 mg./ml.); 1 ml. water; 0·5 ml. 0·5 % glucose in the hollow stopper. The tubes were evacuated and placed in the bath for 10 min. for temperature equilibration, the glucose was then tipped in. The tubes were removed at the end of 30 min. and the glucose estimated by the ceric sulphate method. The temperature optimum for fermentation is 40° (Fig. 1) and the pH optimum by the same technique 5·5–6·8 (Fig. 2).

Type of fermentation. The main product of fermentation was lactic acid, 71–83 % of the glucose fermented by washed suspensions of cells appearing as lactic acid; in these circumstances 20–30 % of the glucose was usually assimi-

lated by the cells. Since it was of importance to be certain whether this organism did in fact produce volatile acids these were specifically looked for.

A mixture of cells (280 mg.), 250 ml. phosphate buffer pH 6·5 and 2·5 g. glucose was fermented to completion at 25°, NaOH being added at intervals to keep the pH approximately constant. The cells were then centrifuged off,

Table 3. *The fermentation of sugars by unadapted cells (grown on glucose) and by adapted cells (grown on the sugar subsequently fermented)*

Growth medium 2 used for cultivation of cells. Each manometric cup contained: 1·5 ml. $NaHCO_3$, pH 7·2; 1·0 ml. bacterial suspension about 8 mg./ml.; 0·5 ml. 0·4 % glucose (or water) in side bulb. Temperature 37°; gas phase 5% CO_2 in N_2. $Q_{CO_2} = \mu$l. CO_2/mg. dry wt. cells/hr.

	Unadapted cells		Adapted cells		
Sugar	Q_{CO_2} (sugar)	Q_{CO_2} (blank)	Q_{CO_2} (sugar)	Q_{CO_2} (glucose) (for comparison)	Q_{CO_2} (blank)
Fructose	28·3	4·7	457	377	5·5
Galactose	2·0	1·0	226	198	1·9
Maltose	2·6	2·6	184	178	1·7
Sucrose	0	0	88	272	2·5
Lactose	0	2·6	309	329	0
Xylose	0	0	0	159	1·3
Arabinose	0	0	108	175	1·9
Hexosediphosphate	31	0	—	—	—

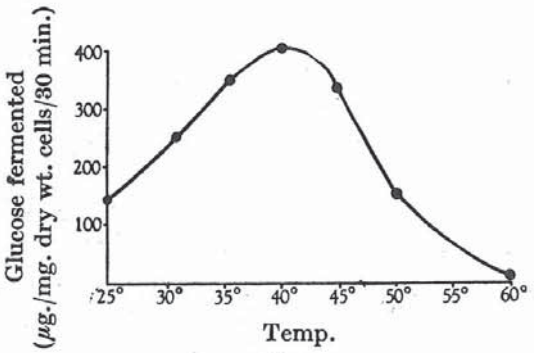

Fig. 1

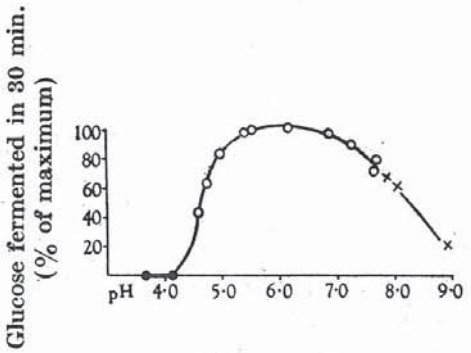

Fig. 2

Fig. 1. Effect of temperature on rate of fermentation of glucose.

Fig. 2. Effect of *p*H on rate of fermentation of glucose. ●—● acetate buffer; ○—○ McIlvaine's buffer; ×—× borate buffer.

the supernatant fluid filtered through Kieselguhr, made alkaline to phenolphthalein and evaporated *in vacuo* to 25 ml. Volatile acid was estimated in this volume by the method of Friedemann (1938). Volatile acid (6·46 mg. calculated as acetic acid) was obtained, equivalent to 0·35 % of the glucose fermented. This amount is within the limits of the experimental error and provides no evidence that acetic acid is a product of fermentation by this organism.

THE PRODUCTION OF ACETYLCHOLINE

Formation during growth of the organism

So far acetylcholine had only been produced by organisms growing in vegetable juices; it is, however, produced on media 1 (liquid) and 2, and in small quantities occasionally on 5, the medium best suited for the production being a mixture of equal volumes of cucumber juice and medium 2. The organism grown for 20 hr. on 1100 ml. of this medium produced 28 mg. acetylcholine and 490 mg. dry wt. of cells, the acetylcholine thus produced amounting to 25 μg./ml. culture and 57 μg./mg. cells. The acetylcholine was distributed between the cells and the medium. The total culture (cells and medium) contained 24·7 μg. acetylcholine/ml., of which 12·3 μg./ml. was in the supernatant. The washed cells added immediately to the fluid in the chamber containing the muscle gave no response; when, however, the cell suspension was slightly acidified, boiled and neutralized, it gave with the muscle an immediate response equivalent to 9·6 μg. acetylcholine/ml.; this response was abolished by boiling at pH 10.

This result is reminiscent of the synthesis of an acetylcholine precursor by brain tissue reported by Mann, Tennenbaum & Quastel (1938), who effected its liberation by the action of acid and of chloroform. We are inclined to think that in the case we were studying the acetylcholine was free in the cell, since it was liberated by the action at room temperature of surface active agents such as tyrocidin, aerosol O.T. and also by disrupting the cells by shaking with glass beads (Curran & Evans, 1942).

Thus 1 ml. cell suspension containing 18·4 mg. cells/ml., liberated on boiling 17 μg. acetylcholine/mg. cells; 1 ml. of the same suspension added to 1 ml. of tyrocidin solution (1 mg./ml.) and left at room temperature for 30 min. liberated the same amount of acetylcholine, while the control suspension left in water liberated 0·59 μg. acetylcholine/mg. cells. A similar result was obtained by the use of aerosol O.T. used in the same concentration and in the same conditions as the tyrocidin.

Ten ml. of a suspension of cells (20 mg./ml.) was shaken for 6 hr. in a mechanical shaker with glass beads in order to disrupt the cells (Curran & Evans, 1942); a similar suspension was left untreated. The control suspension before boiling gave a muscle response equivalent to 0·5 μg. acetylcholine/ml., after boiling 2·2 μg./ml. The disrupted cell suspension gave 2·42 μg. acetylcholine/ml. before boiling and 2·40 μg./ml. after boiling. Thus both surface-active agents and mechanical disruption liberated the acetylcholine from the cells.

Formation of acetylcholine in washed suspensions

The cells grown in any of the media described will, after centrifuging and washing, synthesize acetylcholine in medium 6, without further growth. It is seen from Table 4 that the non-proliferating cells continue to synthesize acetylcholine, which increases about threefold; it is also seen that, whereas at the start of the experiment 90 % of the acetylcholine was present in the cells,

at the end of the period 2 % was found in the cells and 98 % in the medium. A method for studying acetylcholine formation apart from cell multiplication having been arrived at it became possible to study the process in further detail.

Table 4. *The synthesis of acetylcholine by cell suspensions of* Lactobacillus plantarum

Cells grown on a mixture of equal volumes of cucumber juice and medium 2 at 25° for 20 hr. Cell suspension (3·9 mg. dry wt. cells/ml.) 1 ml. + 9 ml. reaction medium 6 incubated at 25°. Samples withdrawn at 0 hr. and 20 hr.

	Acetylcholine			
	μg./ml.	μg./mg. dry wt. cells	μg./ml.	μg./mg. dry wt. cells
Period of incubation (hr.)	0	0	20	20
Suspension before boiling, *A*	0·3	0·77	7·5	19·2
Suspension after boiling (total), *B*	3·0	7·7	9·4	24·0
B − *A* (stored in cells)	2·7	6·93	1·9	4·8

Relation between concentration of bacterial suspension and rate of formation of acetylcholine. The cells were grown as usual, centrifuged, washed and suspended in varying concentrations in the reaction medium containing 2 % glucose; incubation was at 25°. The rate of formation of acetylcholine was established by withdrawing samples and estimating acetylcholine at 0, 2 and 4 hr. for each concentration of cells used. Fig. 3 shows that in the conditions of this experiment the acetylcholine formed was proportional to the dry weight of cells present between 0 and 0·66 mg./ml.

Temperature optimum. Fig. 4 shows that the rate of synthesis was higher at 20° than at 37°; this is not in accordance with the optimum temperature for fermentation, which is 40°. As in most cases the experimental period was 2–4 hr. and never more than 20 hr., the experiments were carried out at 25° on account of the greater ease of maintaining constancy at this temperature.

Effect of anaerobiosis. In the expectation that anaerobic conditions would favour the reaction, aerobic and anaerobic conditions were tried; the aerobic reaction was carried out in small Erlenmeyer flasks, the anaerobic reaction in evacuated Thunberg tubes. The results are shown in Fig. 5. The difference is not very marked and subsequent work was carried out aerobically.

The effect of various constituents of the reaction medium. An experiment was carried out to determine which constituents of the reaction medium were essential to the synthesis of acetylcholine. Acetate and phosphate replaced McIlvaine's buffer, and glucose and choline were in turn omitted. From Table 5 it appears that in the absence of choline or of glucose no synthesis occurred, and that acetate buffer could replace citrate + phosphate mixture; also that added phosphate was not essential.

An analogous increase in the formation of acetylcholine due to the presence of glucose has been noted in the case of brain slices (Quastel, Tennenbaum & Wheatley, 1936); in this case the action was aerobic.

Concentration of glucose and age of culture. Fig. 6 shows that the rate of synthesis of acetylcholine was the same in 0·1 M- and 0·02 M-glucose, but that in

0·01 M-glucose there was a significant drop. The age of the culture was not of great importance in the synthesis (Fig. 7).

Correlation between acetylcholine synthesized, glucose fermented and change in pH. Fig. 8 and Table 6 show that synthesis proceeded smoothly so long as any glucose remained and then ceased, also that the ratio of acetylcholine synthesized to glucose fermented remained remarkably constant over the period of the experiment. Pyruvate could not replace glucose.

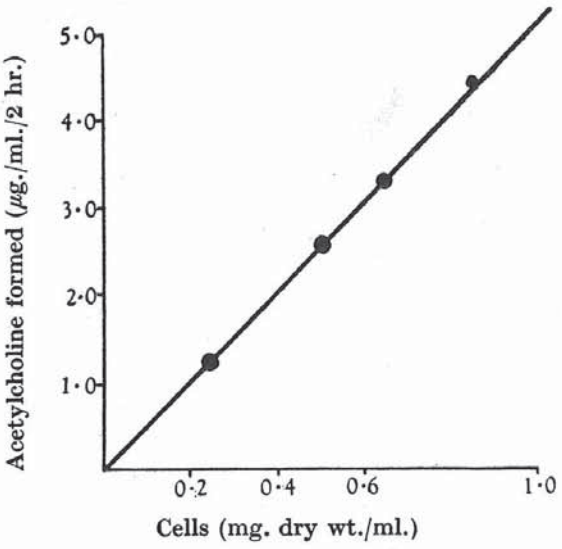

Fig. 3

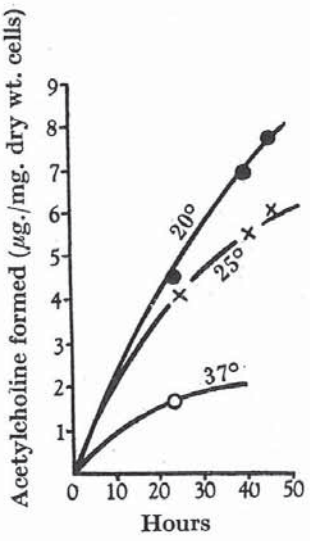

Fig. 4

Fig. 3. Effect of concentration of cells on rate of formation of acetylcholine.

Fig. 4. Effect of temperature on rate of formation of acetylcholine.

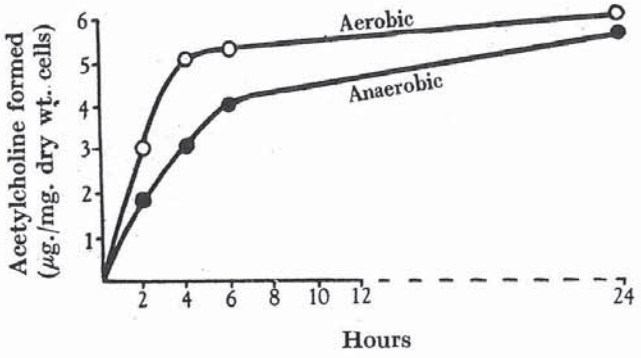

Fig. 5. Effect of anaerobiosis on rate of formation of acetylcholine.

The action of some inhibitors on the fermentation and on acetylcholine production is indicated in Table 7, and the action of certain phosphorylated derivatives on the synthesis of acetylcholine in Table 8.

Effect of the absence of choline from the growth medium

Medium 4 was devised to show whether the organism could grow without the production of acetylcholine. Cultures grown on medium 4 produced no measurable amount of acetylcholine in the cells or culture fluid. For example,

134 μg. acetylcholine/mg. cells was produced in the presence of choline and $<0{\cdot}17$ μg./mg. cells in its absence, the growth amounting to 0·091 and 0·092 mg./ml. in the two cases. When the mixture of amino-acids was replaced

Table 5. *The effect of various constituents of the culture medium on the formation of acetylcholine by* Lactobacillus plantarum

The cell suspension of the organism (grown as in Table 4) contained 10·8 mg. dry wt. cells/ml. Reaction mixtures as indicated below were made up with water to a final volume of 5 ml., and incubated at 25° for 20 hr.

Medium constituent	Composition of reaction mixtures (ml. of appropriate solutions)				
Sodium acetate, M	0·5	0·5	0·5	0·5	0·5
KH_2PO_4, M/15	0·2	0·2	0·2	0	0·2
$MnSO_4 + MgSO_4$, 0·2 %	0·5	0·5	0·5	0·5	0·5
Cysteine, 0·02 g./ml.	0·5	0·5	0·5	0·5	0·5
Glucose, 2 %	0·5	0·5	0	0·5	0·5
Choline HCl, 0·5 %	0·5	0	0·5	0·5	0·5
Cell suspension	0·5	0·5	0·5	0·5	0
	Acetylcholine μg./ml.				
Acetylcholine found in excess of that initially present in organism	20	0	0	24	0

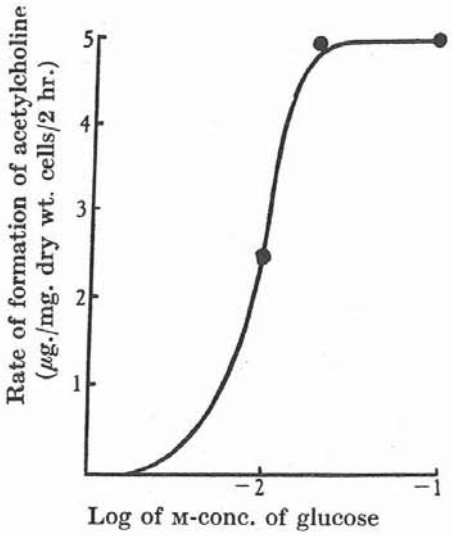

Fig. 6

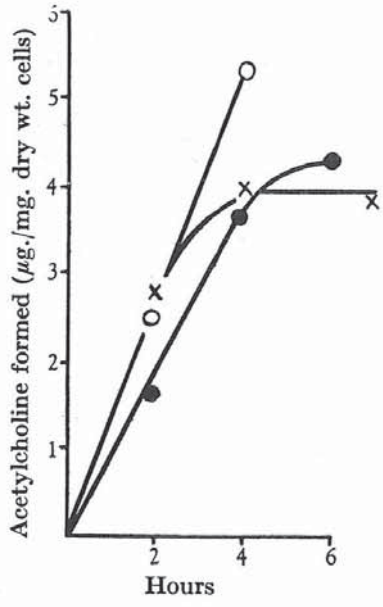

Fig. 7

Fig. 6. Effect of glucose concentration on rate of formation of acetylcholine.

Fig. 7. Effect of age of culture on rate of formation of acetylcholine. Age of culture ●—● 16 hr.; ×—× 20 hr.; ○—○ 24 hr.

by an acid hydrolysate of casein as in medium 5, the production of acetylcholine was either very low or nil, depending apparently on the sample of casein used in making the hydrolysate. When choline was added to this

medium in the usual concentration acetylcholine appeared, but the crop of cells was the same. When the cells in each case were centrifuged, washed and added to the reaction medium, acetylcholine was synthesized at approximately the same rate in both cases (Fig. 9).

Table 6. *The correlation between acetylcholine synthesized, glucose fermented and change in* pH

Organism grown on medium 5. Reaction medium; phosphate citrate buffer 10 ml.; $MnSO_4$ (4 %) + $MgSO_4$ (4 %) 5 ml.; choline HCl (0·5 %) 5 ml.; glucose (0·1 M) 10 ml.; cell suspension (6 mg. dry wt./ml.) 10 ml.; water to 50 ml. Incubation at 25°. Samples removed at times stated.

Period of incubation (hr.)	pH	Glucose		Acetylcholine formed	
		Present (mg./ml.)	Used (mg./ml.)	μg./ml.	μg./mg. glucose fermented
0	7·14	3·45	0·00	0·0	—
1	6·6	2·19	1·26	2·0	1·59
2	6·08	1·39	2·06	4·6	2·23
3·3	5·55	0·57	2·88	6·6	2·29
5·3	4·83	0·043	3·41	8·0	2·35
24	4·73	0·01	3·44	8·0	2·33

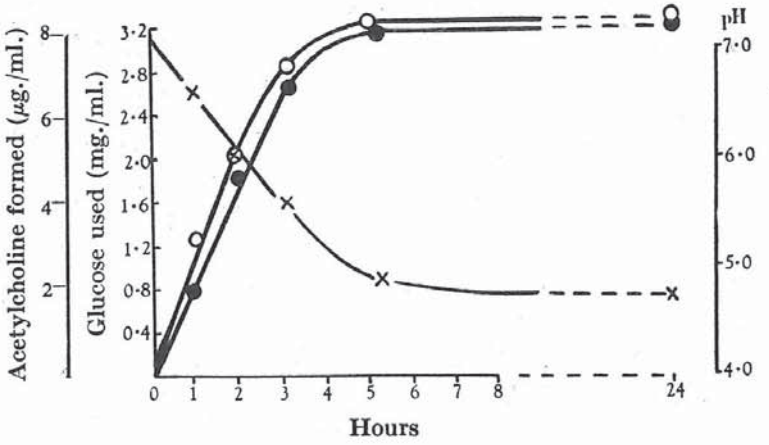

Fig. 8. Correlation between acetylcholine synthesized, glucose fermented and change in pH. ●—●, glucose fermented; ○—○, acetylcholine formed; ×—×, pH.

The proven origin of acetylcholine from choline in the reaction medium makes it highly probable that it has a like origin in cucumber juice and in ordinary complex laboratory media. Choline has been isolated from cabbage by Keil & Gropp (1934) but was stated to be absent from cucumber juice by Keil & Kritter (1935).

It was found possible to acetylate choline by the method of Fletcher, Best & Solandt (1935). This acetylation was only claimed by the authors to be very roughly quantitative (± at least 15 %). Its application to cucumber juice and to acid and tryptic digests of casein made it clear that these materials contained

Table 7. *The action of various inhibitors on fermentation and acetylcholine production*

	Inhibition (%) on	
	Fermentation	Acetylcholine production
Iodoacetic acid M/250	96	100
Na fluoride M/50	Increased lag	20
Phloridzin M/20	—	0
Na arsenite M/250	—	90
Na azide M/1000	—	0

Table 8. *The action of phosphorylated compounds on the synthesis of acetylcholine*

Experimental conditions as in Table 6, the substances shown being incorporated in the reaction medium. Time of incubation 3 hr.

	Acetylcholine (μg./ml.)
Glucose, 0·5 %	7·2
Glucose-1-phosphate, 1 %	6·0
Hexose diphosphate, 1 %	1·2
Na glycerol phosphate, 0·5 %	1·7
Na phosphoglycerate, 0·5 %	0·6 (neg.)
Blank	0·29

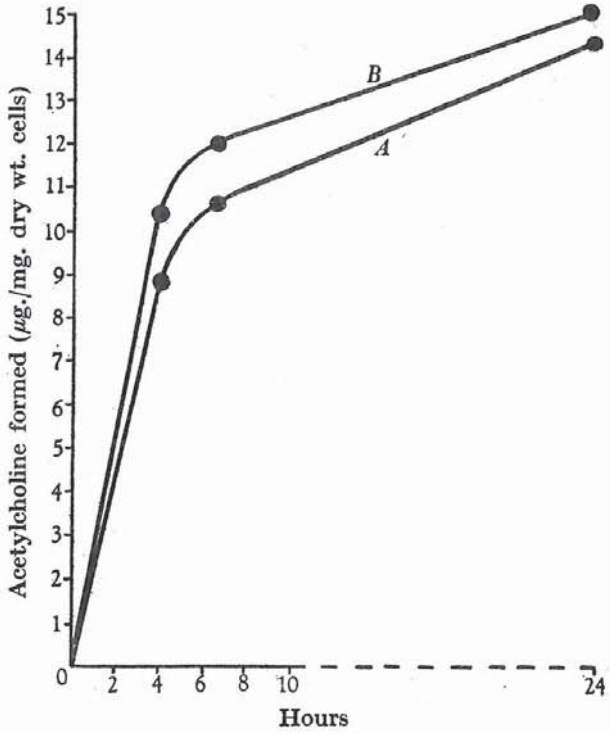

Fig. 9. Synthesis of acetylcholine by cells grown: *A*, without choline; *B*, with choline.

choline in sufficient quantity to account for the production of acetylcholine by the action of the organism in the presence of glucose (Table 9).

Table 9. *The acetylation of choline in various media by the method of Fletcher, Best & Solandt* (1935)

Choline HCl equivalent to 40 μg. acetylcholine in 5 ml. water was treated and acetylated according to the method of Fletcher *et al.* (1935), with the following results:

Choline taken (μg.)	Choline equivalent of acetylcholine obtained	
	μg.	% error
40	39	− 2·5
40	47	+17·3
40	45	+12·5
50	54·5	+ 9
50	43	−14

In a similar manner estimations of the choline content of cucumber juice and tryptic and acid hydrolysates of casein (10 %) were made:

	Choline (as acetylcholine) (μg./ml.)
Cucumber juice	75–80
Tryptic hydrolysate of casein (10 %)	40
Acid hydrolysate of casein (10 %)	20

Quantitative relation between choline present and acetylcholine formed

It will be noticed that in the reaction medium choline HCl was present in a final concentration of about 500 μg./ml.; the acetylcholine formed in the most favourable circumstances was of the order of 16 μg./ml. It should therefore be possible to diminish the concentration of choline added to one more nearly equivalent to the acetylcholine produced. An experiment of this type showed that a decrease in the concentration of choline from 400 to 16 μg./ml. diminished the rate of the reaction by about one-sixth. In order, therefore, for the reaction to proceed at something approaching maximum velocity a large excess of choline is necessary. It was thought that this might be due to the removal of choline by some reaction other than by acetylation. To test this the organism was grown for 40 hr. in medium 5 containing approximately 2·1 μg. choline/ml. as estimated after chemical acetylation; to this was added 40 μg. choline HCl/ml.; a control sample was acetylated and the acetylcholine estimated. The total initial choline was equivalent to 50 μg. acetylcholine/ml. After 40 hr. incubation the acetylcholine was 4·6 μg./ml. The whole culture was then acetylated, and the resulting total acetylcholine obtained was 53 μg./ml. This experiment showed that the choline originally present was recoverable as acetylcholine formed biologically *plus* that remaining in the medium.

We have not found conditions in which the rate of acetylcholine formation is proportional to the concentration of choline present, or independent of the concentration, or in which the whole of the choline present is acetylated.

Attempts to prepare active acetone-dried cells

It has been observed in studies on the production of acetylcholine by nervous tissue that homogenized brain synthesized acetylcholine in the presence of adenosine triphosphate (ATP) and fluoride and absence of carbohydrate or any source of energy other than ATP (Nachmansohn & Machado, 1943). Feldberg & Mann (1945–6*a*) prepared an active acetone powder from brain which, in the presence of ATP and NaF, acetylated choline, the rate of the reaction being increased by —SH compounds and by citrate. Feldberg & Mann (1945–6*b*) also showed that the rate of the reaction was increased by the addition of an 'activator', i.e. boiled juice prepared from acetone powder from brain or from fresh brain, liver, muscle or yeast; they were also able to replace the acetone powder by a saline extract of it.

It was considered desirable to show whether a similar preparation, active in the presence of ATP, could be prepared from our bacterial cells. The organism was grown for 2 days in 3300 ml. of medium 5; this medium was chosen in order to minimize the acetylcholine present in the cells. The total crop was 2·8 g. dry wt. This was spun down and washed once and shown to be active in the normal conditions. The cells were suspended in 15 ml. water and rubbed up with 30 ml. of cold acetone, filtered on a Buchner funnel, again rubbed up with acetone, filtered and dried in a vacuum desiccator; the yield of dry powder was 2·79 g. This powder, in amounts about 10 times the equivalent of fresh cells generally employed, was used in the experiment recorded in Table 10, kindly performed for us by Dr Feldberg in conditions identical with those used by him for acetone powder of brain. It is seen from the results (Table 10) that there was no evidence of any appreciable activity of acetone preparations of bacterial cells in the presence of ATP, with or without glucose or activator.

Thus we have so far obtained no information concerning the mechanism of the transfer of the energy derived from the bacterial fermentation to the acetylation of the choline.

The effect of pantothenic acid

In December 1946 at Cambridge Dr F. Lipmann of the Massachusetts General Hospital, U.S.A., gave an informal lecture on acetylations by liver tissue in which he had found the participation of a coenzyme involving pantothenic acid. Entirely as a result of this information (Lipmann, Kaplan, Novelli, Tuttle & Guirard, 1947) we performed the following experiment:

The cells were grown on medium 5 from which pantothenate was omitted. The washed suspension was divided into two portions, *A* and *B*. *A* was incubated in reaction medium 6, *B* in the same medium in which was incorporated calcium pantothenate, 10 μg./ml. The final concentration of the bacterial suspension was 0·96 μg./ml. Samples were withdrawn at intervals and the acetylcholine estimated. The results are shown in Fig. 10. The glucose used in 6 hr. was 875 μg./mg. dry wt. cells in *A* and 948 μg./mg. dry wt. cells in *B*. Our experiment should be regarded only as confirming Dr Lipmann's observations.

Table 10. *Inability of an acetone-dried preparation of cells of* Lactobacillus plantarum *to form acetylcholine under conditions which would have permitted its formation by homogenized rat brain*

Cells obtained from 2-day growth in medium 5, centrifuged off, washed and dried with acetone and used to set up the experiments detailed below.

	Composition of experimental mixtures							
	1	2	3	4	5	6	7	8
Phosphate buffer pH 7·2 ml.	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5
Saline (ml.)	2·0	2·4	2·0	0·4	—	2·0	—	—
KCl (mg.)	6	6	6	6	6	6	6	—
NaF (mg.)	3	3	3	3	3	3	3	—
Citrate (mg.)	15	15	15	15	15	15	15	—
$MgCl_2$ 4 % (ml.)	1	1	1	1	1	1	1	—
Cysteine (mg.)	0·45	0·45	0·45	0·45	0·45	0·45	0·45	0·45
Glucose (mg.)	—	—	—	—	—	—	25	25
Choline HCl (mg.)	—	2·5	2·5	2·5	2·5	2·5	2·5	—
ATP (mg. pyro-P)	0·4	—	0·4	—	0·4	0·4	0·4	—
Boiled rat brain (mg.)	—	—	—	80	80	—	80	80
Acetone powder (mg.) (from *Lb. plantarum*)	50	50	50	50	50	50	50	50
Total vol. reacting (ml.)	5	5	5	5	5	5	5	5
Amounts of acetylcholine found μg./mg. powder	1·0	1·1	1·1	1·3	1·5	1·0	1·4	1·2

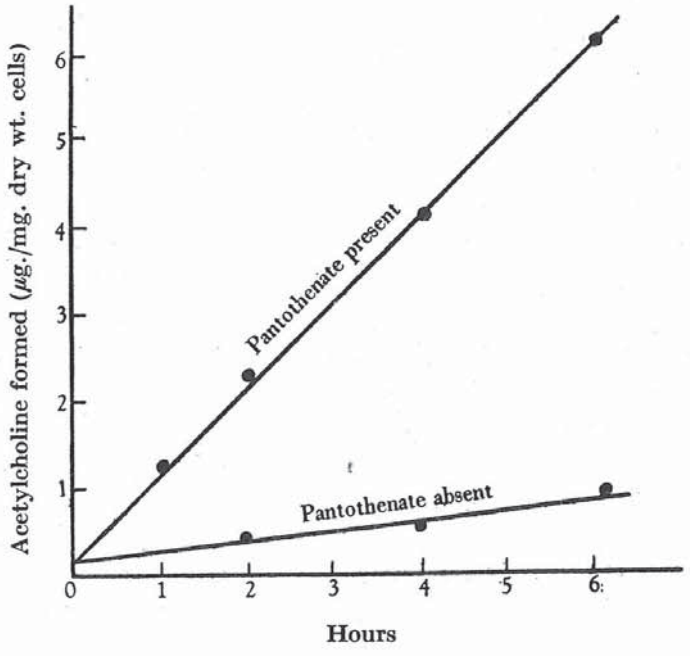

Fig. 10. Effect of added pantothenate on formation of acetylcholine by pantothenate-deficient cells in washed suspension.

Identification of acetylcholine

Our controls have invariably shown that the substance to which the muscle reacts is destroyed by boiling at pH 10. It was also destroyed by horse plasma and by human plasma and serum, and protected from these effects by eserine; the plasmas and serum were able to decompose added acetylcholine (Table 11).

Table 11. *The biological identification of acetylcholine; the effects of eserine and choline esterase-containing preparations*

The organism was grown on medium 2, boiled and neutralized to pH 7·0; this is the culture fluid. The experimental tubes containing the mixtures detailed below were incubated for 1 hr. at 37°, being made up to a total final volume of 2·0 ml. with water. Choline esterase: 1 = 50 % horse plasma; 2 = human plasma; 3 = human serum.

Constituent of mixture	ml. of solutions listed								
Culture fluid	1	1	0	1	0	1	1	1	0
Acetylcholine (10 μg./ml.)	0	0	1	0	1	0	0	0	1
Eserine 1 μg./ml.	0	0	0	0	0	0·2	0	0	0
Buffer pH 7·0	0	0	0	0	0·8	0	0	0	0
Choline esterase: 1	0	1	1	0·8	0	0	0	0	0·8
2	0	0	0	0·2	0·2	0·2	0	0·2	0
3	0	0	0	0	0	0	0·2	0·2	0·2
Water	1	0	0	0	0	0·6	0·8	0·6	0
Acetylcholine found (μg./ml.)	5	0	0	0	0	5	0	5	0

The biological tests being in accordance with the identification of the active substance as acetylcholine it was felt that corroboration by a chemical method was desirable. Mr K. Harrison undertook to do this, by the method detailed later (see Addendum), if sufficient material could be provided. Large-scale preparations were therefore undertaken. These were attempted in the first place by growing the organism in 1 l. batches in cucumber juice. It was found in all preparations of this volume that poor yields were obtained by using small inoculations, and that it was necessary to sow each 1 l. batch from a pilot preparation of a 20 hr. culture in 100 ml. of the same medium. From such a culture (cf. culture 1, Table 12) grown for 22 hr. a sample was withdrawn and the cells and total acetylcholine estimated. The whole culture was then centrifuged and the acetylcholine estimated in the supernatant. The cells were made up to 27 ml. with 0·01 N-H_3PO_4. A sample (0·5 ml.) was withdrawn for turbidimetric estimation and a similar sample for acetylcholine estimation after boiling in acid. In order to increase further the yield the cells were suspended in 250 ml. of reaction medium 6 and incubated 20 hr. (culture 2). A sample (1 ml.) was then withdrawn and the total acetylcholine estimated. The whole culture was then centrifuged and washed and the acetylcholine estimated in this second supernatant (2) and in the cells (2). The results of this experiment are given in Table 12.

It was hoped that in the precipitation method it would be possible to use the acetylcholine present in the total culture on cucumber juice after the removal of the cells. This was found to be impossible, as when the precipitating agent—hexanitrodiphenylamine—was added to the concentrated filtrate a tarry

precipitate was formed. For this reason the second process detailed above was resorted to, in which the supernatant from the growth medium was discarded and the cells resuspended in the reaction medium. Here again trouble was encountered owing to the presence of residual choline which also forms a salt

Table 12. *Method of preparation of large batches of cells and culture fluid for chemical identification of acetylcholine*

Culture 1. Cucumber juice (1000 ml.); inoculated with 100 ml. of 20 hr. culture grown on same medium, and incubated 22 hr. at 25°; acetylcholine estimated in sample of whole culture, cells and supernatant:

	mg.
Dry wt. of cells	490
Acetylcholine in total culture	28
Acetylcholine in total cells	6·48
Acetylcholine in total supernatant	15·4
Loss	6·1

Culture 2. Cells from culture 1 (less samples) suspended in 250 ml. of medium 6 and incubated for 20 hr. at 25°, similar assays performed.

	mg.
Acetylcholine in cells (start)	6·2
Total acetylcholine in culture after incubation	23·0
Acetylcholine in supernatant after incubation	8·8
Acetylcholine in cells after incubation (by difference)	14·2

with the reagent. Though the solubility of the choline salt is about 10 times that of the acetylcholine salt, the amount of residual choline may be 100 times that of the acetylcholine and the separation of the two would therefore be practically impossible.

The only feasible method therefore seemed to be to discard both supernatants 1 and 2 and to use the acetylcholine from the cells after reacting in medium 6. The following slightly modified procedure was therefore adopted. Medium 2 (1 l.) was sown with 100 ml. of a 20 hr. culture in the same medium. After 20 hr. incubation at 25° the culture was centrifuged and the supernatant discarded. The cells (*c.* 200 mg.) were resuspended in 50 ml. reaction medium 6 and left 4 hr. The cells were then spun off and the second supernatant discarded. The cells were suspended in 10 ml. of water + 1 ml. 2 M-H_3PO_4, boiled, and the acetylcholine estimated; the cell debris was centrifuged off and the supernatant used for the subsequent precipitation. In this manner 102 l. of culture were worked up in nine batches. This gave a total crop of 23 g. of cells and 111 mg. of acetylcholine.

DISCUSSION

The bacterial acetylation of choline bears some relation to that occurring in other tissues, viz. that the source of energy is fermentation; the organism being practically an anaerobe, the replacement of fermentation energy by that derived from oxidation is not to be expected. The mechanism of the transfer and the source of the acetyl radicle are at present obscure. Our failure to demonstrate the participation of ATP in the process is probably due to a failure

to attain the right conditions rather than to the existence of some mechanism of energy transfer in which this ubiquitous ester plays no part.

The significance of the mechanism in the cell economy is wholly unknown. It has to be remembered that this is an example of a cell mechanism which would not have been brought to light unless a very delicate method existed for the detection and measurement of the final product. The same mechanism may be used to bring about other acetylations in the cell about which we know nothing. The lack of correspondence between the concentration of choline required to permit maximal—or near maximal—acetylation and the amount acetylated indicates that there is some factor or factors remaining to be disclosed.

Addendum

The Isolation of Acetylcholine as a Salt (Hexylate) of Hexanitrodiphenylamine

By K. HARRISON

Ackermann & Mauer (1943) proposed the use of hexanitrodiphenylamine (dipicrylamine) as a precipitant for acetylcholine; the salt (or 'hexylate') with acetylcholine forms beautiful glistening red plates, m.p. 125° (corr.), soluble in water to the extent of 1 : 10,000 only. (It would seem that the uncorrected m.p. 183°, given by Ackermann & Mauer, is a typographical error.) Choline hexylate, m.p. 233° (corr.), is much more soluble in water.

To prepare the hexylate of acetylcholine the procedure was as follows: A warm filtered aqueous solution of the ammonium salt of hexanitrodiphenylamine ('Aurantia') was added to the neutralized extract obtained from the boiled suspension of cells previously described; after bringing the mixture to 60° and cooling in ice the hexylate was filtered off and recrystallized, first from 10% (v/v) ethanol in water and then from water. Authentic specimens of acetylcholine hexylate were obtained in similar fashion from neutralized acetylcholine chloride (Roche Products Ltd. and British Drug Houses Ltd.).

Owing to the high pharmacological activity of propionylcholine (Chang & Gaddum, 1933), it was deemed advisable to prepare propionylcholine hexylate also. Propionylcholine chloride was synthesized by the method of Cline (1934); analytical figures (Dr Weiler and Dr Strauss) and melting-points are summarized in Table 13. It will be observed that these hexylates (in common with other aromatic nitro-compounds) tend to give high figures for C and low figures for N, due to traces of NO_2 being estimated as CO_2; if the difference, C found $-$C calc., be transferred to N found, the analytical figures are very satisfactory.

Taking the evidence of melting-points together with the analytical results there seems to be no doubt that the substance produced by the organism used

in this work is acetylcholine, thus confirming the work of Keil & Kritter (1934). The recovery of acetylcholine in the form of hexylate from the neutralized bacterial extract amounted to about 75% of the acetylcholine determined

Table 13. *Melting-points and analytical figures for acetylcholine hexylate and propionylcholine hexylate*

	m.p. (corr.)
1. Acetylcholine hexylate (from acetylcholine chloride)	125°
2. Acetylcholine hexylate (from acetylcholine prepared from cells)	124°
3. Mixture of 1 and 2	124°
4. Propionylcholine hexylate (from propionylcholine chloride)	113°
5. Mixture of 2 and 4	Below 108° (indistinct)

Analytical results

	C	H	N%
1. Calc.	39·03	3·45	19·19
Found	39·3	3·35	18·8
2. Found	39·6	3·53	18·5
4. Calc.	40·1	3·71	18·74
Found	40·7	3·76	18·1

biologically. The high yield of hexylate provides further, if indirect, evidence that the bulk of the active substance is acetylcholine; although the possibility that traces of propionylcholine may also be present cannot be excluded, it seems to be remote.

The authors wish to record their thanks to Mr H. Mowl for help in the biological estimation of acetylcholine.

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