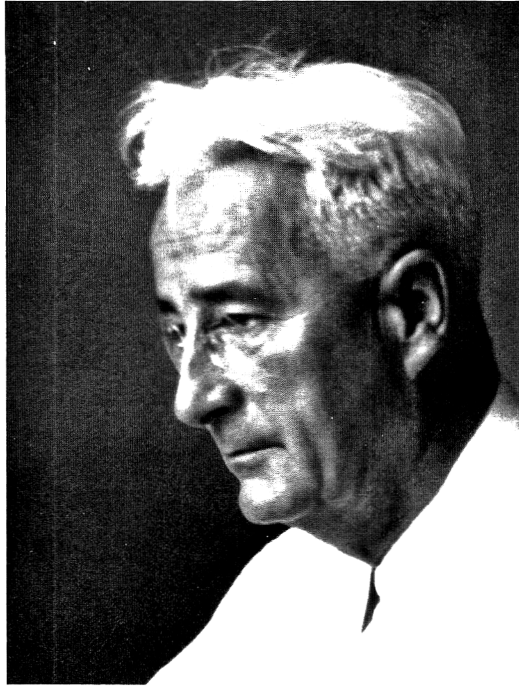

ERNST ALBERT GÄUMANN

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Printed in Great Britain

Obituary Notice

ERNST ALBERT GÄUMANN, 1893–1963

Professor Gäumann died on 6 December 1963, two months after his seventieth birthday. He had been presented on that occasion with a Jubilee booklet containing messages and recollections of earlier days from colleagues and former pupils from many countries; it is impossible to read that booklet without getting a vivid and unforgettable impression of the immense influence which he had on all who met him. With his death we lost a great mycologist, a leader of men, a man with a highly personal genius, and a genial if sometimes provocative conversationalist.

For almost forty years he was head of the world-famous Institut für spezielle Botanik der eidgenössischen technischen Hochschule, Zürich. Despite heavy administrative and teaching duties, both of which he took very seriously, throughout his life he carried on his own personal research, and encouraged that of his students and associates, with an almost unbelievable vigour.

The breadth of his mycological interests is shown by the books he wrote. He produced a succession of books on the morphology and taxonomy of fungi, all of which are still of great value to students and specialists; these include *Vergleichende Morphologie der Pilze* (1926), *Die Pilze* (1949) and *Die Pilze, Grundzüge ihrer Entwicklungsgeschichte und Morphologie* (1952). His works on special groups include one of the downy mildews. *Beiträge zu einer Monographie der Gattung Peronospora Corda* (1923), and the exhaustive, 1400-page long *Die Rostpilze Mitteleuropas* (1959), dealing with taxonomy, morphology, life-history and pathological significance of the European rusts.

In later years he devoted himself more and more to plant pathology. He felt that plant pathology had hitherto tended to be a mere collection of facts—of the kind 'this pathogen causes this disease of the following plants'—and that the time had come to make it a real science, dealing in general terms with problems of epidemiology, processes of infection and the biochemistry and physiology of host–parasite interactions. This generalized approach was first apparent in a book which he wrote as a young man in collaboration with his teacher, Professor Eduard Fischer: *Biologie der pflanzenbewohnenden parasitischen Pilze* (1929); it reached its final form in his *Pflanzliche Infektionslehre* (1946). This book can justly be claimed to have influenced the subsequent development of plant pathology in all countries; there is much in it with which one can disagree but as a stimulus to thought and experiment it is unsurpassed.

During the last twenty years, in collaboration with chemist colleagues in Zürich, he developed an active research group on phytotoxic metabolites of parasitic fungi and on antibiotics. Their work on lycomarasmin, fusaric acid and other phytotoxins and their relation to the etiology of wilt diseases was the forerunner of many similar investigations elsewhere, and opened up an important new field of investigation.

Gäumann received many honorary doctorates and other academic honours. He was an honorary or corresponding member of no fewer than 28 learned societies and academies. We in the Society for General Microbiology elected him an Honorary Member in 1959, and are proud that he was one of us.

P. W. BRIAN

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Death Mechanisms in Airborne *Escherichia coli*

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(*Accepted for publication* 23 *December* 1966)

SUMMARY

Differences in survival of *Escherichia coli* (strain B) sprayed from distilled water into air and into nitrogen as a function of relative humidity (RH) are reported. Two mechanisms which may contribute to death of airborne bacteria are described. In air one death mechanism occurring at low RH is attributed to the action of oxygen causing damage to flavin-linked enzymes as a result of free radical activity. Free radical suppressors are therefore expected to protect airborne *E. coli* B. Also, electron transport inhibitors like sodium azide, 2,4-dinitrophenol and potassium cyanide are shown to protect *E. coli* B against lethal effects of oxygen. An analogy is drawn with effects of oxygen on freeze-dried *E. coli* B. A second death mechanism of *E. coli* B in air occurs at higher RH's and is considered to result from the effect of aerosolization on RNA synthesis. The activation of RNAse as a possible protection to bacteria in the post-aerosolization medium is discussed.

INTRODUCTION

Because the effect of aerosolization upon bacteria survival is related in a complex manner to relative humidity (RH) of the atmosphere, earlier reports (Dunklin & Puck, 1948; Davis & Bateman, 1960; Bateman, McCaffrey, O'Connor & Monk, 1961) have suggested that bacterial sensitivity increases at certain critical degrees of dehydration. Later reports (Hess, 1965; Cox, 1966) have shown that other factors, e.g. spray fluid, oxygen content of the carrier gas and the collecting fluid, influence the disruption of vital structures and the unbalancing of metabolism. Kinetic studies indicate that: (i) the loss of viability of bacteria is not a simple process; (ii) first-order reactions do not apply; (iii) typically, death rate is initially high and subsequently diminishes progressively.

The possibility that overlapping lethal mechanisms exist, makes it extremely difficult to identify bacterial death by a given mechanism in a given set of conditions. One approach to the problem is the use of inert atmospheres to limit the effect of gaseous factors. Another is comparison between results of analogous studies, such as freeze-drying experiments, to derive support for evidence produced in aerosol tests. This paper reports results of such investigations.

METHODS

Growth of organisms

Cultures of *Escherichia coli* (strain B) were grown in 100 ml. of a chemically defined medium for 16 hr at 37°, using a shake flask technique. The medium consists of

1·0 % (w/v) glycerol; 0·5 % ammonium citrate; 1·0 % $K_2HPO_4.3H_2O$; 0·05 % $MgSO_4.7H_2O$; 0·05 % NaCl; 0·005 % ferric ammonium citrate; the pH being adjusted to 7·0, using dilute caustic soda solution.

Preparation of spray suspensions, survival estimation, apparatus and collecting fluids

The procedure was the same as described by Cox (1966) except that aerosols were generated by a 3-jet Collison spray and stored in a 500 l. rotating stainless steel drum. Aerosol samples were collected into phosphate buffer.

Technique for obtaining higher numbers of bacteria recovered from the aerosol

In order to detect measurable differences in their metabolism an adequate quantity of airborne bacteria must be recovered. Consequently the following modifications in spraying and collecting techniques were adopted: (i) the bacterial population of the spray fluid was increased tenfold to 10^{11} organisms/ml.; (ii) the 3-jet Collison spray was replaced by an 18-jet Collison spray; (iii) bacteria were sprayed into the drum for 2 min. instead of 1 min.; (iv) collection of the aerosolized bacteria was by a 55 l./min. subsonic impinger (K. R. May, personal communication) for 5 min. The bacterial population collected was measured turbidimetrically by an EEL spectrophotometer.

(^{14}C)-Uracil uptake

Suspensions containing 10^8 cells/ml. of aerosolized and non-aerosolized bacteria were incubated in separate shake flasks with 0·025 μc/ml. (^{14}C)-uracil (The Radiochemical Centre, Amersham, Bucks) at 37°. The suspending fluid consisted of the glycerol medium described previously. Samples were taken at regular intervals. The ice-cold 5 % (w/v) trichloracetic acid soluble matter was filtered through a 'Millipore' filter. After washing the residue on the filter with cold water, the radioactivity in the combined filtrate and washings was determined at room temperature by a 'coincidence' scintillation counting technique (Anderson & Smith, 1965). Reduction in the amount of radioactivity in the filtrate should correspond to the amount incorporated as ribonucleic acid. This was confirmed by extracting the ribonucleic acid from the residue on the filter with 5 % trichloracetic acid at 60° for 15 min. After filtering, the radioactivity of the filtrate was measured at room temperature.

Release into the medium of (^{14}C) from prelabelled s-RNA

(^{14}C)-labelled s-RNA was prepared by incubating a suspension of bacteria (10^{11} cells ml.) in the glycerol growth medium with (^{14}C)-uracil (25 μc./ml.) at 37° for 90 min. The bacteria were collected and washed three times with distilled water before being sprayed. After storing the airborne bacteria in the drum apparatus for a certain time they were collected into the usual growth medium. This suspension was incubated at 37° in a shake flask. A control suspension consisted of an identical quantity of (^{14}C)-s-RNA unsprayed bacteria. Samples were taken at regular intervals. Each sample was treated with ice-cold 5 % trichloracetic acid for 60 min., filtered, and the residue was incubated at 60° for 15 min. and again filtered. The radioactivity of this filtrate was determined at room temperature.

The synthesis of DNA

The effect of aerosolization on the synthesis of DNA in the bacteria was studied by measuring their uptake of (^{14}C)-thymidine. The procedure involved is the same as for the uptake of (^{14}C)-uracil.

The release into the medium of (^{14}C) from prelabelled DNA

The method of producing prelabelled (^{14}C)-DNA bacteria and measuring the effect of aerosolization on the release of (^{14}C) as a result of DNA degradation was the same as described previously for s-RNA.

The effect of air on the pyruvate metabolism of freeze-dried and aerosolized Escherichia coli B

Washed bacterial suspension of *Escherichia coli* B was freeze-dried in glass ampoules. These samples were then exposed to: (i) air; (ii) nitrogen at controlled RH in vacuum desiccators. Control of the RH was effected by saturated solutions of certain inorganic salts (Wexler & Hasegawa, 1955). After a certain exposure time the bacteria were resuspended in phosphate buffer.

Aerosolized bacteria were collected in phosphate buffer. Each suspension was made up of 5×10^8 bacterial/ml. in phosphate buffer, 10^{-3}M-$MgSO_4$ and 1000 μM-sodium pyruvate, before incubation at 37°. Samples were taken at regular intervals and their pyruvate content was measured by the Friedmann & Haugen method (1943).

The effect of air on the reduction of 2:4:6: triphenyl tetrazolium chloride by freeze-dried and aerosolized Escherichia coli B

A more concentrated suspension of bacteria was required to reduce the tetrazolium salt than was usually obtainable in the collecting fluid after aerosolization. Therefore the collected aerosolized bacteria were centrifuged and resuspended in one-fifth of the original volume of suspending fluid. The respective suspensions were made up of 5×10^8 bacteria/ml. in phosphate buffer, 5·0% (w/v) glucose and 0·1% (w/v) tetrazolium chloride (British Drug Houses Ltd.) before being incubated at 37°. The red insoluble formazan dye which developed was extracted from the cells by adding glacial acetic acid. The formozan was extracted from the aqueous phase by *n*-butanol and assayed spectrophotometrically at a wavelength of 540 mμ.

RESULTS

The percentage survivals of *Escherichia coli* B aerosolized at various RH values in air and in pure nitrogen and then collected after 10 min. into phosphate buffer are given in Fig. 1. Survival in nitrogen was much greater than in air, progressively so as the RH level fell. At high RH the survival pattern was extremely sensitive to small RH changes: for example, between the RH values of 75% and 82%, survival of *E. coli* B decreased from 36% to a minimum of 8%. At very high RH values the survival curves of *E. coli* B stored in air or in nitrogen tend to merge. This result is very similar to that found by Cox (1966). Figure 2 shows that the death rate was initially very rapid, being dependent on the RH, and was much faster in air than in

nitrogen. The initial rapid death rate was followed by a much slower secondary one which in some cases approached zero and which was much less dependent on both RH and gaseous composition of the atmosphere.

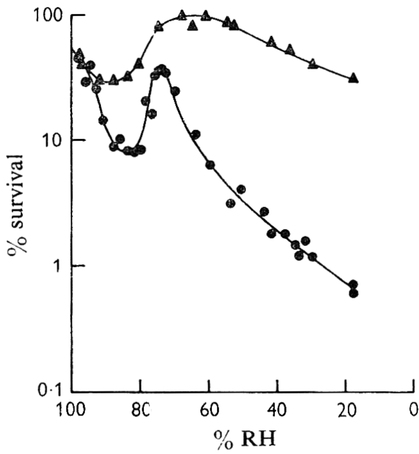

Fig. 1

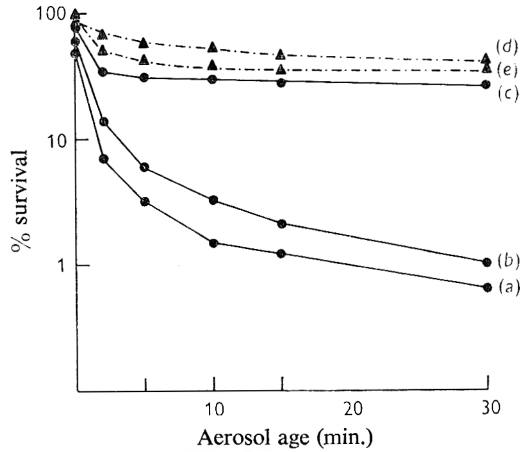

Fig. 2

Fig. 1. Survival of *E. coli* B at aerosol age of 10 min. —●—, sprayed into air; —▲—, sprayed into nitrogen.

Fig. 2. The death rate of aerosolized *E. coli* B. —●—, sprayed into air at (*a*) 35 %RH; (*b*) 50 % RH; (*c*) 97 % RH. ·—·—▲—·—·, sprayed into nitrogen at (*d*) 37 % RH; (*e*) 95 % RH.

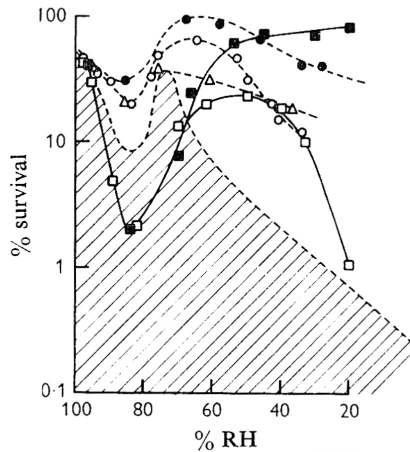

Fig. 3. Survival of *E. coli* B which have been incubated in the following chemicals: – – ○ – –, 2000 μM-NaN_3 (aerosolized into air for 10 min.); – – ● – –, 2000 μM-NaN_3 (nitrogen); —□—, 10,000 μM-cysteamine (air); —■—, 10,000 μM-cysteamine (nitrogen); – – △ – –, 500 μM-KNO_2 (air). The line above the shaded area shows the survival curve of *E. coli* B sprayed from distilled water into air.

The deleterious effect of oxygen on bacterial survival

In accordance with the findings of Hess (1965) and Cox (1966), oxygen was toxic to airborne bacteria. Furthermore, its toxicity progressively increased as the RH decreased below 70 %. Pre-incubation of *Escherichia coli* B with various inhibitors of

the electron transport system such as sodium azide, potassium cyanide and 2,4-dinitrophenol, before spraying, was found to increase the aerosol stability of *E. coli* B considerably, particularly at low RH (Fig. 3).

The detection of altered metabolism which might contribute to the death of airborne bacteria has been hampered by the problem of collecting enough material from the aerosol. Chemicals such as potassium nitrite and cysteamine which have been shown to protect freeze-dried bacteria and irradiated bacteria against the lethal effect of oxygen (Lion & Avi Dor, 1963; Baker, Ormerod, Dean & Alexander, 1966) have

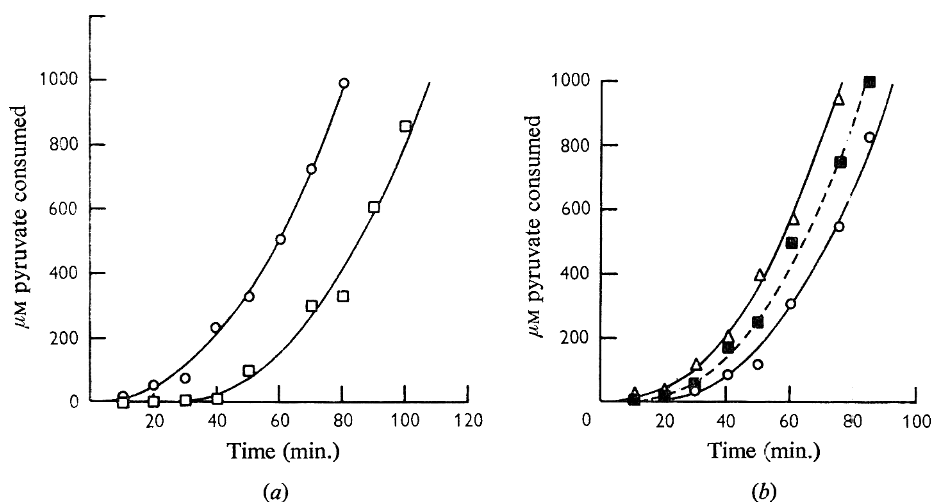

Fig. 4(*a*) The effect of air on the consumption of pyruvate by *E. coli* B which have been freeze-dried and exposed to air (—□—), or to nitrogen (—O—) for 4 hr before being resuspended in phosphate buffer containing 1000 μM sodium pyruvate and 10^{-3} M-$MgSO_4$. This suspension contains 2×10^8 bacteria/ml.

Fig. 4(*b*) The effect of aerosolization on the ability of *E. coli* B to consume pyruvate The bacteria were collected in phosphate buffer after being aerosolized into air (—O—) or into nitrogen (- - ■ - -) for 10 min. Unsprayed cells are used as control ——△——.

also been found to give some degree of protection to airborne *E. coli* B at low RH. Lion & Avi-Dor (1963), using a model enzyme system, were able to show that damage to a flavin-linked enzyme occurred on exposure of freeze-dried *E. coli* to oxygen. The effect of oxygen on the uptake of pyruvate by aerosolized and freeze-dried *E. coli* B (Fig. 4) may therefore be attributed to damaged flavin-linked enzymes connected with pyruvate metabolism (Benbough, 1965). Also, both aerosolization and exposure of freeze-dried *E. coli* B to air caused a great diminution in the reduction of tetrazolium chloride to formozan compared to results obtained by exposure to nitrogen.

Synthesis and breakdown of nucleic acids after aerosolization of Echerichia coli B

Data given on Fig. 5 indicate that the effect of aerosolization on the kinetics of the ability of *Escherichia coli* B to synthesize RNA was dependent on RH but was independent of the carrier gas. The RNA synthesizing ability of *E. coli* B decreases after being aerosolized into higher RH values. This diminution in the rate of RNA

synthesis appears to enhance the chances of the bacteria surviving in the aerosol and it may be attributable to mechanisms that degrade or release RNA from the bacteria. Loss of cellular RNA occurred to a maximal extent in bacteria which were aerosolized at 70 % RH (Fig. 6) which coincides with the RH where maximal survival occurs. Unequivocal evidence that differences in the rate of RNA breakdown in *E. coli* B aerosolized at various RH values may be attributed to fluctuations in the

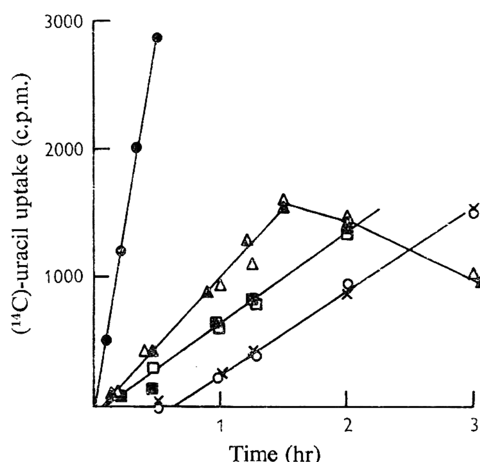

Fig. 5

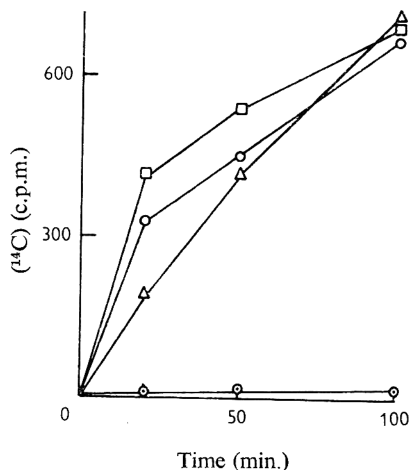

Fig. 6

Fig. 5. The effect of aerosolization on the uptake of (^{14}C)-uracil by *E. coli* B (10^8 cells/ml.). —●—, Control unsprayed bacteria. The following represent bacteria aerosolized for 10 min.: —▲—, at 35 % RH in air; —△—, at 35 % RH in nitrogen; —■—, at 72 % RH in air; —□—, at 82 % RH in air; —O—, at 98 % RH in air; —×—, at 98 % RH in nitrogen.

Fig. 6. The effect of aerosolization of *E. coli* B on the breakdown of (^{14}C)-s-RNA into a cold acid-soluble fraction. —⊙—, control unsprayed *E. coli* B (10^8 cells/ml.). The following represent results of bacteria aerosolized for 10 min. in air: —△—, at RH 95 %; —□—, at RH 66 %; —O—, at RH 35 %.

RNAse activity was extremely difficult to obtain. However, Webb (1965) has shown that the RNAse activity of *E. coli* dried at 80 % RH is nearly double those dried at 30 % RH. Support for the view that RNAse activity may contribute to aerosol stability may be obtained from the fact that: (i) Mg^{2+}-starved *E. coli* B, which have an enhanced RNAse activity (H. E. Wade, personal communication) have greater, aerosol stability than *E. coli* B grown in normal chemically defined medium (Fig. 7); (ii) *E. coli* strain 600 M.R.E. which has exceptionally small RNAse activity (Wade & Robinson, 1966) has a very low aerosol stability.

No apparent differences in the kinetics of DNA synthesis and breakdown in aerosolized *Escherichia coli* could be detected by the techniques in this paper (Fig. 8).

DISCUSSION

The kinetics of death rate show that oxygen and certain factors associated with the rate of dehydration contribute to the instantaneous death of airborne *Escherichia coli* B. The rapidity of death makes it virtually impossible to assess quantitatively

the interdependence of these lethal factors but this study indicates that the toxicity of oxygen increases in relation to the dryness of the air. At high RH values mechanisms lethal to airborne *E. coli* B must be independent of the presence of oxygen. The kinetics of death of *E. coli* B in air and nitrogen at high RH values were similar, indicating that other mechanisms contribute to their death, which is independent of the presence of oxygen (Fig. 2).

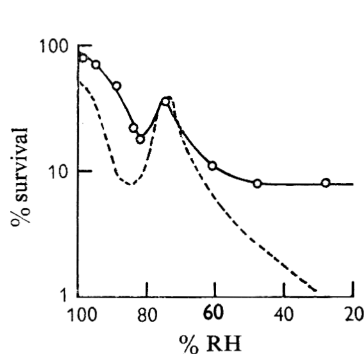

Fig. 7

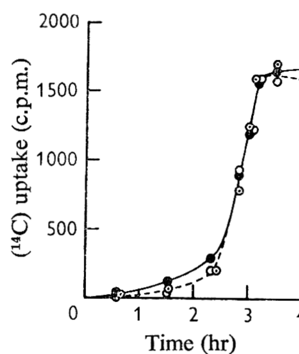

Fig. 8 (*a*)

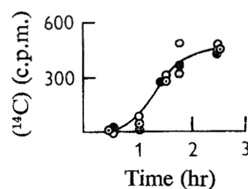

Fig. 8 (*b*)

Fig. 7. Survival of magnesium-starved *E. coli* B aerosol age of 10 min. in air. Interrupted curve, survival after growth in normal medium (from Fig. 1).

Fig. 8(*a*). The effect of aerosolization on the uptake of (^{14}C)thymidine by *E. coli* B (10^8 cells/ml.). —●—, control unsprayed bacteria. —○—, bacteria aerosolized at 35 % RH in air for 10 min.; —⊙—, bacteria aerosolized at 98 % RH in air for 10 min.

Fig. 8(*b*). The effect of aerosolization of *E. coli* B on the breakdown of (^{14}C)-DNA into a cold acid-soluble fraction. ●, control unsprayed *E. coli* (10^8 cells/ml.). The following represent results of *E. coli* aerosolized for 10 min. in air: ○, at 35 % R.H.; ⊙ at 98 % RH.

Indications that exposure of freeze-dried *Escherichia coli* B to oxygen-induced effects on the flavin-linked pyruvate oxidation similar to airborne *E. coli* B enable one mechanism of death of airborne *E. coli* B to be suggested. Contact between oxygen and freeze-dried *E. coli* results in the death of the bacteria and the formation of detectable free radicals (Lion, Kirby-Smith & Randolph, 1961; Dimmick, Heckley & Hollis, 1961). Most cellular oxidations are mediated by the pyridine nucleotide-flavoprotein-cytochrome *c*-cytochrome oxidase system, and recently Fox & Tollin (1966) have shown that the mechanism of flavoenzyme action involves radical formation. Consequently, the function of flavin radicals as mediators may well be interfered with as a result of (i) radical propagation; (ii) radical abstraction; (iii) radical combination with foreign radicals formed by the action of oxygen on freeze-dried or airborne *E. coli* B; or (iv) oxygen may combine directly with the flavin radicals to form lethal peroxide radicals. Decreasing the metabolism of bacteria is known to protect them against the lethal effect of oxygen (Goodlow & Leonard, 1961; Gerschman *et al.* 1954). Also, pre-incubation of the bacteria in metabolic inhibitors such as sodium azide, potassium cyanide and 2,4-dinitrophenol protects the bacteria to some degree. This must be associated with a decrease in the rate of flavin radicals formed, thus limiting the above lethal effects on airborne bacteria. Confirmation that the electron transport system of airborne *E. coli* B was damaged by oxygen was produced by the fact that bacteria which were aerosolized into air failed to reduce triphenyl tetrazolium chloride.

A free-radical scavenger, cysteamine, when pre-incubated prior to spraying, increases the viability of *Escherichia coli* B considerably at low RH values. The mechanism by which cysteamine protects is unknown but it may be related to its protective action against the effect of oxygen on irradiated *E. coli* (Baker *et al.* 1966).

At high RH values the extent of death of *Escherichia coli* B in air and in nitrogen were similar, indicating that other mechanisms independent of the presence of oxygen contribute to death. Cox & Baldwin (1966) demonstrated that the mechanism of death at high RH was different to that caused by oxygen. The considerable effect which aerosolization had on the s-RNA synthesis in *E. coli* B was found to be independent of the carrier gas used but changed considerably with changes in RH. Generally, as the RH increased, the initial rate by which aerosolized *E. coli* B can synthesize s-RNA gradually decreased. At low RH values (below 40 %) the initial synthesis of s-RNA suddenly ceases and a rapid breakdown of s-RNA takes place. At high RH values (above 85 %) an enhanced lag period occurred before s-RNA synthesis commenced. The sensitivity of airborne *E. coli* B to RH may be due to the great dependence upon RH of the effect of aerosolization on s-RNA synthesis. No adequate explanation can be afforded for the pronounced minimal survival of aerosolized *E. coli* B at the RH of 83 %, also found by Cox (1966).

Evidence in this report supports the view that ribonuclease activity may increase the aerosol stability of *Escherichia coli* B. This might be explained by structural modifications in the nucleic acids during aerosolization of the bacteria. These modifications, which might lead to some lethal biosynthesis of proteins, could thus be prevented by the destructive action of RNAse on the s-RNA.

The author thanks Mr I. H. Silver and Dr C. S. Cox for their interest and much helpful discussion and thanks Miss J. Soilleux for technical assistance.

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ห้องสมุด กรมวิทยาศาสตร์

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Effects of Stereoisomeric Isoleucines on Sporidesmolide Biosynthesis by *Pithomyces chartarum*

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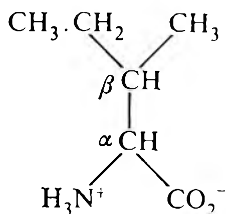
(*Accepted for publication* 31 *December* 1966)

SUMMARY

As well as the known sporidesmolides I, II and III, *Pithomyces chartarum* produces sporidesmolides or similar compounds which contain residues of *erythro*-isoleucine and α-hydroxyisocaproic acid. When *erythro*-L-isoleucine was added to the growth medium, synthesis of sporidesmolides I and III was inhibited and synthesis of isoleucine-containing sporidesmolides, mainly sporidesmolide II, was promoted. *Threo*-L-isoleucine in the medium was poorly utilized, but its presence resulted in production of a very complex mixture of sporidesmolides containing a higher proportion of isoleucine residues than the control. Epimerization of *erythro*-D-isoleucine occurred in the medium. Neither epimer was well utilized, and this isomer had little effect on sporidesmolide production or composition; it promoted some increase in the synthesis of isoleucine-containing sporidesmolides. *Threo*-D-isoleucine had effects qualitatively similar to but less pronounced than those of *erythro*-L-isoleucine, probably because of extracellular epimerization. The use of epimeric pairs of amino acids as tools for investigating biochemical processes involving inversion of configuration at an amino acid α-carbon atom is suggested.

INTRODUCTION

This paper describes some biochemical effects of α-amino-β-methyl-valeric acid (1), the trivial name for which is isoleucine. The α- and β-carbon atoms are both asymmetrically substituted, so that the amino acid exists in four stereoisomeric forms:



These are distinguished by the prefixes L- or D-, referring to the configuration about the α-carbon atom, and also by the prefixes *threo-* and *erythro-*, referring to the configurational relationship between the α- and β-carbon atoms (Vickery, 1963). Thus, the isomer that is a common protein constituent is *erythro*-L-isoleucine which, by inversion at the α- carbon atom only, is converted to its epimer, *threo*-D-isoleucine. In this paper the term 'isoleucine' is used without qualification when the stereochemical configuration is unspecified.

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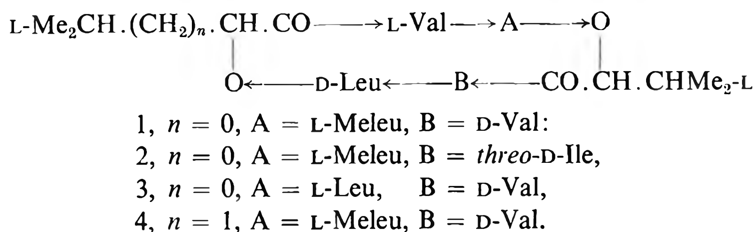
The fungus *Pithomyces chartarum* (Berk. & Curt.) M. B. Ellis (Ellis, 1960) produces a mixture of insoluble cyclodepsipeptides termed sporidesmolides (Done, Mortimer, Taylor & Russell, 1961) which are located as spicules on the surface of the spores (Bertaud, Morice, Russell & Taylor, 1963). Four pure sporidesmolides have been isolated.



When *P. chartarum* was grown in liquid surface culture on potato+carrot extract it formed mainly sporidesmolide I. This was shown by chemical degradation to be 1, a structure which has since been confirmed by synthesis (Shemyakin *et al.* 1965). A second component was also present which, although it was not isolated, was named sporidesmolide II and was tentatively assigned a structure in which the D-valine residue of 1 was replaced by a residue of a D-isoleucine isomer (Russell, 1960*a*, 1962). When *P. chartarum* was grown on potato+carrot extract to which optically inactive isoleucine had been added, its production of sporidesmolide I was inhibited, and the only compound that could be isolated was 2 for which in this paper the name sporidesmolide II is retained (Bertaud, Probine, Shannon & Taylor, 1965). Sporidesmolide II has been synthesized (Shemyakin *et al.* 1965), thus providing further confirmation that the isoleucine residue has the *threo*-D-configuration. A third compound, sporidesmolide III, is formed in small amount by *P. chartarum* growing on potato+carrot extract, and was shown to have structure 3 (Russell, MacDonald & Shannon, 1964). Sporidesmolide IV has not so far been obtained from *P. chartarum*, but is produced by the closely related *P. maydicus* (Sacc.) M. B. Ellis, (Ellis, 1960: Bishop *et al.* 1965). Its structure 4 was deduced by degradation (Bishop & Russell, 1964, 1967) and, together with that of sporidesmolide III, was confirmed by synthesis (Ovchinnikov, Kiryushkin & Shemyakin, 1966).

The aim of the experiments to be described was to determine the effects of each separate stereoisomer of isoleucine on the biosynthesis of sporidesmolides I, II and III. During the course of this work it became clear that these were not the only sporidesmolides produced by this organism, a conclusion also reached by Bertaud *et al.* (1965). Further work on this problem is clearly necessary. The present author is no longer working with sporidesmolides, but the results obtained so far appear to be interesting and are presented here.

METHODS

Organism. An isolate of *Pithomyces chartarum* derived from isolate 'C' (Done *et al.* 1961) was used. During the 4 years over which these experiments were conducted, the organism was maintained at 25° on potato glucose agar slopes by repeated subculture. From time to time it lost its ability to sporulate freely, and single-spore subculture from a vigorously sporing sector was used to obtain a suitably high-sporing isolate. It is therefore unlikely that the organism is now identical with isolate 'C', although the cyclodepsipeptides that it produces are unchanged.

Cultivation of organism. In one experiment the salts + glucose + asparagine medium of Ross (1960; Butler, Russell & Clarke, 1962) supplemented with yeast extract (0·1 %, w/v; 'Difco' brand), was used. In all others the medium was the potato + carrot extract of Done *et al.* (1961), containing dry matter 2% (w/v), reducing sugars 1 % (w/v; adjusted with glucose) and nitrogen 0·05 % (w/v; adjusted with ammonium sulphate). Amino acids were added before autoclaving. The methods used for sterilization of media and preparation of inocula (Done *et al.* 1961), and the general conditions of cultivation (Bishop *et al.* 1965) were as described.

Amino acids. Erythro- and *threo*-L-isoleucine were from Mann Research Laboratories, New York, N.Y.; *threo*-D-isoleucine was from California Biochemical Corporation, Los Angeles, California, and *erythro*-D-isoleucine from Koch-Light, Colnbrook, Bucks., England. Isoleucines of suitable purity were not readily obtainable. One sample of *erythro*-D-isoleucine, labelled 'chromatographically pure', contained at least four components. The isomers used were examined for optical purity by polarimetry, and for freedom from their epimers by ion-exchange chromatography (Spackman, Stein & Moore, 1958). None was completely pure by either criterion; a sample was accepted for use when it contained no amino acid other than isoleucine, when its specific rotation was such as to exclude the presence of more than 3 % (w/w) of an isomer of opposite configuration at the α-carbon atom, and when no more than 3 % (w/v) of its epimer (*threo-* in *erythro*-isoleucines, and vice versa) was detected. These limits represented the best quality obtainable for amino acids from commercial sources. The difficulties of obtaining steroeochemically pure isoleucines were discussed by Greenstein & Winitz (1961).

Analytical methods. Except where otherwise stated, details of or references to the analytical methods used in this work were those given by Bishop *et al.* (1965). *Cyclodepsipeptides* were determined by the method there described, except that pure diethyl ether was used. *Primary amino acids* were determined by ion-exchange chromatography, kindly done by Mr M. Hatton by the use of an EEL Automatic Analyser (Evans Electroselenium Ltd., Halstead, Essex). Quantitative paper chromatography of *N-methyl-leucine* and other amino acids was done as described for isoleucine by Riches, Rothwell & Russell (1966), with pure amino acids as standards. The sensitivity was lower for *N*-methyl-leucine (see Russell, 1960*b*), but satisfactory results were obtained in the range 0·05–0·2 μmole.

Hydrolysis of cyclodepsipeptides was done in concentrated hydrochloric acid + glacial acetic acid (1 + 1, by vol.) at 110° for 48 hr, and the excess of acid was removed *in vacuo* over potassium hydroxide. The molecular weight of sporidesmolide I is 638; to provide solutions of suitable concentration the hydrolysates were reconstituted to contain the products from 6·38 μg./ml. for paper chromatography of amino acids or 638 μg./ml. for ion-exchange chromatography. Alternatively, the dried residues were extracted with ether for investigation of the hydroxy acids by paper chromatography as previously described (Russell, 1962).

Partial hydrazinolysis of cyclodepsipeptides was done, and the products were examined by thin-layer chromatography, as described elsewhere (Russell, 1965*a*, *b*).

Infrared spectra were measured for paraffin mulls with an Infracord 137 spectrophotometer (Perkin-Elmer, Norwalk, Connecticut, U.S.A.), using cyclodepsipeptide samples dried *in vacuo* at 110°.

RESULTS

Pithomyces chartarum grew well on the standardized potato + carrot medium, or on the defined medium + yeast extract. Isoleucine isomers did not greatly affect growth. The 'natural' isomer, *erythro*-L-isoleucine, was slightly but consistently stimulatory, whereas the other, 'unnatural', isomers had a slight inhibitory effect (Table 1).

The effects of the various isomers on the number of spores produced in culture were similar to their effects upon growth. Thus, while the 'unnatural' isomers were without effect on sporulation, the presence of *erythro*-L-isoleucine in the medium resulted in a twofold increase in spore number (Table 2).

Table 1. *Effects of stereoisomeric isoleucines* on growth in Pithomyces chartarum grown in surface culture for 14 days at 25° on potato + carrot extract*

	Expt. no.						
	F†	54‡	59	76	77	90	
Isomer	Dry wt. (mg./ml.)						Mean (control = 100)
Control	4·8	3·95	2·7	6·5	6·5	6·4	100
Erythro-L	5·2	5·4	3·7	—	—	7·6	125
Erythro-D	—	—	—	5·7	5·6	5·1	85
Threo-L	4·0	5·2	2·1	—	—	5·1	93
Threo-D	3·4	3·1	3·2	—	—	7·4	95

* 5 mg./ml. † 21 days' growth. ‡ Defined medium + yeast extract.

Table 2. *Effects of stereoisomeric isoleucines* on sporulation in Pithomyces chartarum in surface culture for 14 days at 25° on potato + carrot extract*

	Expt. no.						
	F†	54‡	59	76	90	90e	
Isomer	Spores ($\times 10^{-5}$/ml.)						Mean (control = 10)
Control	40	19	7	44	26	26	100
Erythro-L	44	25	26	—	47	58	205
Erythro-D	—	—	—	49	24	—	102
Threo-L	29	23	10	—	25	—	108
Threo-D	26	13	7	—	36	—	93

* 5 mg./ml. † 21 days' growth. ‡ Defined medium + yeast extract.

Effect of stereoisomeric isoleucines on sporidesmolide yields

In estimating sporidesmolide yields, the ether used to purify the crude sporidesmolide residues was not previously saturated with the normal sporidesmolide mixture. The same modification was used, but by an oversight was not mentioned, by Bertaud *et al.* (1963). The object was to avoid contamination of those sporidesmolides being isolated with sporidesmolides produced under normal conditions, since these two were expected, and indeed were found, to differ in composition. Differences in composition might produce differences in ether-solubility. The method therefore did not necessarily

give the true yields of sporidesmolides, but it did allow a comparison of the present results with those of Bertaud *et al.* (1963).

When sporidesmolide yields were determined in this way, all four stereoisomeric isoleucines were inhibitory. The inhibition produced by the D-isomers was only slight, whereas the L-isomers had a marked effect. However, because of the solubility considerations mentioned above, it could not be assumed that the yields found were true yields. That they were, in fact, too low, was demonstrated by thin-layer chromatography of the ether extracts (Bishop *et al.* 1965) which showed the presence therein of sporidesmolides. These extracts were therefore evaporated to dryness and the residues extracted with *n*-hexane. Thin-layer chromatography of the hexane extracts showed that sporidesmolides were absent. The hexane-insoluble residues were dissolved in chloroform and purified by percolation through charcoal and aluminum oxide (Bishop *et al.* 1965), and from this treatment further amounts of sporidesmolides were isolated. Absence of other types of compound was verified by thin-layer chromatography. In Table 3 all the individual yields listed are uncorrected for ether-solubility. The mean yields, expressed as percentage of the control values, are given both uncorrected and corrected. The effect of the correction is small except for *threo*-L-isoleucine; sporidesmolides produced on media containing this isomer were more soluble in ether than in the other cases. The corrected yields also demonstrated that both D-isomers were very weakly, if at all, inhibitory to sporidesmolide biosynthesis.

Table 3. *Effects of stereoisomeric isoleucines* on sporidesmolide yield in Pithomyces chartarum*

	Sporidesmolides (mg./l.)† in expt. no.					Mean (control = 100)	
Isomer	F	59	76	77	90	Uncorr.†	Corr.‡
Control	109	54	140	145	279	100	100
Erythro-L	54	32	—	—	179	58	63
Erythro-D	—	—	153	135	163	87	92
Threo-L	22	3	—	—	75	18	29
Threo-D	83	52	—	—	215	83	95

* 5 mg./ml. † Results uncorrected for solubility in ether. ‡ Results corrected for solubility in ether.

Comparison of ether-soluble and ether-insoluble sporidesmolides by thin-layer chromatography revealed no difference in the number or relative intensities of the spots. It seems that the solubilities of individual sporidesmolides are not manifested independently, but depend on the composition of the mixture.

It has been established that in *Pithomyces chartarum* most if not all of the sporidesmolide mixture present occurs on the spores, and when spores are not formed, sporidesmolides cannot be detected in cultures (Bertaud *et al.* 1963). Inhibition of sporidesmolide biosynthesis by isoleucines might therefore occur by depression of sporulation, or by decrease in the amount synthesized per spore. The yields were therefore recalculated as mg. sporidesmolides/10^8 spores. The amount of sporidesmolides per spore in control cultures was very variable. Addition of either D-isomer did not inhibit sporidesmolide biosynthesis to a significant extent in terms either of total synthesis or of amount synthesized per spore. The depression produced by the

L-isomers resulted largely from a decrease in the amount synthesized per spore, and not from inhibition of sporulation (Table 4).

When the solubility correction was applied, *erythro*-L-isoleucine was found to inhibit sporidesmolide synthesis per spore more than *threo*-L-isoleucine.

The amounts of isoleucine remaining in the media at harvest were determined by paper chromatography. *Erythro*-L-isoleucine, a protein constituent presumably abundant in natural substrates, was completely utilized. It is doubtful whether any

Table 4. *Effects of stereoisomeric isoleucines* on sporidesmolides/spore in Pithomyces chartarum*

	Sporidesmolides/spore† in expt. no.					Mean‡	
Isomer	F	59	76	90	90e	Uncorr.	Corr.
Control	100	100	100	100	100	100	100
(mg./10^8 spores)	(2·7)	(7·7)	(3·2)	(10·7)	(10·7)		
Erythro-L	45	16	—	35	30	33	39
Erythro-D	—	—	98	63	—	81	85
Threo-L	27	4	—	28	—	30	49
Threo-D	117	96	—	55	—	89	102

* 5 mg./ml. † Expressed as a percentage of the value for control cultures. ‡ Results given uncorrected, and corrected, for solubility in ether.

Table 5. *Utilization of stereoisomeric isoleucines* by Pithomyces chartarum growing for 2 weeks at 25° on potato + carrot extract*

Isomer added	Amount utilized (%)	Composition of residual amino acid (%)	
		Erythro	*Threo*
Erythro-L	100	—	—
Erythro-D	29	79	21
Threo-L	20	1	99
Threo-D	82	6·5	93·5

* Initial concentration 5 mg./ml.

other isomer is normally encountered in more than traces by the organism. *Threo*-L-isoleucine and *erythro*-D-isoleucine were poorly but *threo*-D-isoleucine was well utilized (Table 5). A possible reason for this difference was revealed by analysis of the composition of the residual isoleucines using ion-exchange chromatography. Some conversion of *erythro*-D-isoleucine to a *threo*-isomer had occurred. No instance of inversion of an isoleucine isomer solely at the β-carbon atom is known; this change then may be presumed to result from inversion at the α-carbon atom, producing *threo*-L-isoleucine, an isomer which is also poorly utilized. If we postulate, to account for such an inversion, that *Pithomyces chartarum* produces an extracellular enzyme that racemizes D-amino acids then it is likely that *threo*-D-isoleucine in the medium would be converted by such an enzyme to its well-utilized epimer, *erythro*-L-isoleucine, and would thus appear to be well utilized. Some *erythro*-isoleucine was indeed present when cultures containing *threo*-D-isoleucine were harvested. Although the amount was less than was present as impurity at the outset, *erythro*-L-isoleucine is

well utilized and would not be expected to persist in the medium. The evidence is therefore in favour of its having been produced by epimerization of *threo*-D-isoleucine during the incubation period.

Analysis of the effects of stereoisomeric isoleucines on sporidesmolide composition

On unsupplemented growth media, *Pithomyces chartarum* produces a mixture of sporidesmolides. Mixtures were also formed on media containing amino acid supplements. No attempt was made to isolate the separate components, but physical and chemical methods were used to investigate the mixtures; the results gave evidence of their composition. Some notes on the methods used are given below; the results are presented in Table 6.

Melting point. Each known sporidesmolide has a characteristic melting point, but measurement of this constant is of little value in characterizing mixtures, which tend to melt at intermediate temperatures. Sporidesmolide II has a very much lower m.p. than sporidesmolide I, and a binary mixture with m.p. close to that of sporidesmolide II should contain little sporidesmolide I.

Specific rotation. The specific rotations of the known sporidesmolides are too similar for this constant to be used in analysing mixtures of unknown composition. The measurements provided good evidence that the mixtures consisted largely of sporidesmolides since all, including sporidesmolides from control cultures, gave values for this constant of about $-200°$ in chloroform solution.

Thin-layer chromatography. Sporidesmolides I and II were not resolved in the system used (Bishop *et al.* 1965) nor in any of several others tried. The method indicated the presence or absence of sporidesmolides III and IV, and permitted the degree of complexity of the mixtures to be assessed.

Infrared spectroscopy. All the known sporidesmolides have very similar infrared spectra. During this work it was found that each had a weak absorption maximum in the 11–12 μ region. The precise wavelength of this absorption maximum was characteristic of the particular sporidesmolide. Measurement of the whole spectrum was used to confirm the preponderance in the mixtures of compounds with the general sporidesmolide structure. Measurement of the position of the absorption maxima at 11–12 μ provided evidence as to the nature of the major components.

Amino acid analysis. Sporidesmolides I, II and III all differ in their amino composition (Table 6). Amino acid analysis of mixtures is therefore a guide to their composition. When these analyses were made by ion-exchange chromatography, significant quantities of *erythro*-isoleucine were detected. No sporidesmolide which contains this amino acid has yet been isolated. A *threo*-D-isoleucine residue is present in sporidesmolide II, and Shemyakin *et al.* (1963) reported that acid hydrolysis of sporidesmolide II was accompanied by slight epimerization of this amino acid. In the present work the ratio of *erythro*- to total isoleucine was different in hydrolysates of sporidesmolides from different treatments, although the hydrolysates were prepared at the same time and under identical conditions. Moreover, in one case 38 % of the total isoleucine was *erythro*-isoleucine. It was concluded that the presence of *erythro*-isoleucine in hydrolysates was not solely the result of epimerization during hydrolysis, but that contrary to a previous report based on paper chromatographic examination of hydrolysates (Russell, 1965*c*), *erythro*-isoleucine residues occurred in *P. chartarum* sporidesmolides.

Table 6. *Properties of sporidesmolides from Pithomyces chartarum grown on media containing stereoisomeric isoleucines*

Property	Sporidesmolide mixture from control culture	Sporidesmolide mixtures from cultures containing 5 mg./ml. of stereoisomeric isoleucines				Pure sporidesmolides			
		Erythro-L	*Erythro*-D	*Threo*-L	*Threo* D	I	II	III	IV
Melting point	252°	227°	256°	224°	194°	262°	228°	288°	233°
$[\alpha]_D$*	−198	−201	−201	−196	−202	−217	−228	N.D.‡	−212
R_F†	—	—	—	0·63	—	—	—	—	—
	—	—	—	0·57	—	—	—	—	0·57
	0·50	0·51	0·50	0·50	0·51	0·50	0·50	—	—
	—	0·34	—	0·39	—	—	—	—	—
	0·28	—	0·28	0·28	0·28	—	—	0·28	—
I.R.§	—	—	—	—	—	—	—	11·49	—
	—	11·52	—	—	11·52	—	11·52	—	—
	11·65	—	11·65	11·65	11·65	11·65	—	—	—
	—	—	—	—	—	—	—	—	11·68
Valine‖	1·91	1·36	1·69	1·77	1·72	2	1	2	2
Leucine‖	1·05	1·03	0·99	1·01	1·01	1	1	2	1
*Erythro*isoleucine‖	0·04	0·23	0·14	0·15	0·09	0	0	0	0
*Threo*isoleucine‖	0·08	0·70	0·23	0·38	0·34	0	1	0	0
N-Methyl-leucine‖	0·95	1·01	0·95	0·93	0·94	1	1	0	1
R_F hydrazides¶	0·39	—	0·37	0·38	0·37	0·36	—	N.D.	N.D.
	0·43	0·42	0·43	0·43	0·43	0·42	0·43		
	—	—	—	0·46	—	—	—		
HiV**	S	S	S	S	S	S	S	S	M
HiC**	W	W	W	M	W	0	0	0	M

* Specific rotation in angular degrees, in chloroform solution for sodium light, at room temperature (20–25°).

† In thin-layer chromatography (Bishop *et al.* 1965).

‡ N.D. indicates that the value was not determined.

§ Infrared spectral absorption maxima measured for paraffin mulls between 11 and 12 μ.

‖ Amounts of amino acids, in μmoles, formed by hydrolysis of 638 μg. of sporidesmolide mixture, or of 1 μmole of a pure sporidesmolide.

¶ R_F in thin-layer chromatography (Russell, 1956*b*) of the partial hydrazinolysis products of sporidesmolide mixture.

** Relative intensities of spots on paper chromatograms of hydroxy acids in sporidesmolide hydrolysates (Russell, 1962) having the same R_F values as α-hydroxyisovaleric (HiV) and α-hydroxyisocaproic (HiC) acids: S = strong, M = medium, W = weak, 0 = absent.

Hydroxy acid composition. Residues of α-hydroxyisovaleric acid are present in sporidesmolides I, II and III. When hydrolysates of control sporidesmolides were examined by vapour-phase chromatography (kindly done by Dr D. G. Bishop; see Bishop & Russell, 1967; the presence of α-hydroxyisocaproic acid was detected. This acid, one residue of which is present in sporidesmolide IV from *Pithomyces maydicus* (Bishop & Russell, 1967; Ovchinnikov *et al.* 1966) constituted up to 10% (w/v) of the total hydroxyacids of sporidesmolides from three different isolates of *P. chartarum*. Hydrolysates of the sporidesmolide samples were examined by paper chromatography, and the amounts of the two hydroxyacids were estimated visually. The method did not distinguish between α-hydroxyisocaproic and the isomeric α-hydroxy-β-methylvaleric acid.

Hydrazide analysis. Partial hydrazinolysis of sporidesmolide I gives the hydrazides of sporidesmolic acids A and B. These were resolved by thin-layer chromatography. The two hydrazides produced by partial hydrazinolysis of sporidesmolide II were not resolved, but gave a single spot with the R_F value of sporidesmolic acid B hydrazide. This technique was therefore useful in demonstrating the absence of sporidesmolide I.

Composition of sporidesmolides from individual treatments

Control cultures. By physical criteria the sporidesmolides from control cultures were identical with those previously isolated from *Pithomyces chartarum* grown on unsupplemented media. The amino acid analysis was similar to that previously reported (Russell, Synge, Taylor & White, 1962). The total isoleucine present in hydrolysates was 0·12 μmoles/638 μg., of which 33% was *erythro*-isoleucine.

Cultures containing erythro-L-isoleucine. The presence of *erythro*-L-isoleucine in the culture medium inhibited synthesis of sporidesmolides I and III. The infrared spectrum of the product isolated was identical with that of sporidesmolide II. A small amount of another compound was revealed by thin-layer chromatography, and hydrolysates contained more valine and less *threo*-isoleucine than would be released on hydrolysis of pure sporidesmolide II. The total isoleucine released was very much higher (0·93 μmoles/638 μg., 25% *erythro*) than in the controls. The evidence is consistent with the interpretation that *Pithomyces chartarum*, grown on medium containing *erythro*-L-isoleucine, produces mainly sporidesmolide II.

Cultures containing erythro-D-isoleucine. Sporidesmolides synthesized on medium containing this isomer were indistinguishable from those from control cultures except by amino acid analysis. Hydrolysates contained more isoleucine (0·43 μmoles/638 μg., 38% *erythro*) and less valine than control hydrolysates. The evidence is consistent with the interpretation that the organism produced the same sporidesmolides whether grown in the presence or in the absence of *erythro*-D-isoleucine, but that this amino acid altered slightly the proportions of the components in favour of those containing isoleucine, particularly one or both *erythro*-isomers.

Cultures containing threo-L-isoleucine. The presence of this isomer in the medium resulted in production of a complex mixture of sporidesmolides. This experiment demonstrated the value of thin-layer chromatography for investigating sporidesmolide mixtures. Five components were resolved by this means, whilst the infrared spectrum differed from that of the control sporidesmolides only in that the major peaks were broader. Sporidesmolides produced on this treatment liberated on hydrolysis con-

siderable amounts of isoleucine (0·53 μmoles/638 μg., 28 % *erythro*), and about twice as much leucic acid was present as in control hydrolysates. From the results of thin-layer chromatography, all four known sporidesmolides could have been present in the mixture in addition to two unknown ones.

Cultures containing threo-D-isoleucine. Thin-layer chromatograms of sporidesmolides from control and *threo*-D-isoleucine-enriched cultures were indistinguishable, and there was no difference in the m.p., in specific rotation or in the results of hydrazide or hydroxyacid analysis. However, the infrared spectrum of the product from treated cultures showed absorption maxima characteristic of both sporidesmolide I and sporidesmolide II. The hydrolysate contained considerable amounts of isoleucine (0·43 μmole/638 μg., 21 % *erythro*). I infer that *threo*-D-isoleucine did not affect the nature of sporidesmolides produced by *Pithomyces chartarum*, but that it altered their proportions in favour of those containing isoleucine, particularly of the *threo*-configuration.

DISCUSSION

When a D-amino acid is utilized by a micro-organism at a slower rate than its L-isomer it may be difficult to decide whether the D-isomer is itself absorbed, or whether it is first inverted by an extracellular enzyme. The present experiments provide an instance of how such extracellular racemization may sometimes be simply detected. Both *erythro*-D- and *threo*-L-isoleucine are poorly absorbed and, since they are epimers, the extracellular conversion of one into the other is readily detected by chromatography. This and other epimeric pairs could be useful in detecting the secretion of extracellular amino acid-racemizing enzymes, and more generally in investigating biosynthetic mechanisms in which inversion occurs or is suspected.

In the present instance, the effects of D-isoleucines on sporidesmolide biosynthesis are largely explicable as due to their slow extracellular conversion to the corresponding L-epimers. This is consistent with the observation that ^{14}C-D-valine was utilized by *Pithomyces chartarum* for sporidesmolide I biosynthesis with about one-third of the efficiency of the L-isomer, but that the distribution of radioactivity in sporidesmolide I was identical for both isomers (W. D. Bennett & G. W. Butler, personal communication).

The whole of the sporidesmolide I molecule, except for the *N*-methyl group, may be derived from L-valine and L-leucine supplied in the medium (Butler *et al.* 1962). Examples of antagonism, both between valine and isoleucine, and between leucine and isoleucine, have been reported (Meister, 1965). Each of the four stereoisomeric isoleucines might act as antagonist towards formation of any or all of the six residues of sporidesmolide I. Even if the effects of the D-isomers are due largely to their L-epimers, the possibilities are still numerous. The complexity of the situation is reflected in the effects of *threo*-L-isoleucine, in the presence of which at least five different sporidesmolides, or closely related compounds, were synthesized. Not only are the identities of these compounds unknown; it is also uncertain whether formation of any or all of them was induced by the amino acid, or whether they are all present, some in trace amount, in the sporidesmolide mixture normally produced by *Pithomyces chartarum*. The presence of *erythro*-isoleucine and α-hydroxyisocaproic acid in hydrolysates of control sporidesmolide mixtures suggests that the latter may be the case, but much more chemical work is necessary before the effects of *threo*-L-isoleucine can be interpreted.

A simple explanation of the effects of *erythro*-L-isoleucine is possible if we assume that biosynthesis of sporidesmolides I and II takes place by the same pathway; this is reasonable because they are homologues. On this hypothesis, the biosynthetic system will accept either L-valine for synthesis of sporidesmolide I containing a D-valine residue, or *erythro*-L-isoleucine for sporidesmolide II containing a *threo*-D-isoleucine residue. This is consistent with the known ability of *Pithomyces chartarum* to incorporate *erythro*-L-isoleucine into sporidesmolide isoleucine residues (Butler *et al.* 1962) and with the ability of L-valine completely to inhibit biosynthesis of sporidesmolides containing such residues (unpublished observations). With *erythro*-L-isoleucine present in excess, sporidesmolide II would similarly be synthesized to the exclusion of sporidesmolide I. In the presence of *threo*-D-isoleucine, extracellular inversion would produce only a slow supply of the *erythro*-L-isomer, so that a mixture of both sporidesmolides would result. This explanation, although attractive because of its simplicity, may well be wrong. The *Pithomyces chartarum* isolate used is almost certainly a heterokaryon (Dingley, 1962) and the effects of *erythro*-L-isoleucine, as well as of other isomers, might result from selection during growth. For this reason, little further progress in the study of sporidesmolide biosynthesis is likely until a high-sporing homokaryotic strain is obtained.

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Comparative Carbohydrate Catabolism in Corynebacteria

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SUMMARY

The catabolic pathways for the utilization of glucose and gluconate by one strain of each of five species of the genus *Corynebacterium* were examined. The relative participation of concurrent glucose pathways in each of these five strains was estimated. The findings indicated that *Corynebacterium hoagii* ATCC 7005 and *C. tritici* ATCC 11408 displayed catabolic behaviour similar to that of *Arthrobacter globiformis* ATCC 8010 in that the glycolytic pathway played a major role in glucose catabolism. In contrast, in *C. equi* ATCC 7698 and *C. sepedonicum* ATCC 9850, the glycolytic pathway and the pentose cycle pathway played almost equally important roles in glucose utilization. The catabolic pathway functioning in *C. xerosis* ATCC 7084 was distinct from these other strains of Corynebacteria and resemble that of some Acetobacter strains in that the pentose cycle pathway played a predominant role in the utilization of glucose and gluconate.

INTRODUCTION

The genus *Corynebacterium* was initially created by Lehman and Neumann (*Bergey's Manual*, 7th ed., 1957) to contain the diphtheria bacillus and other diphtheria-like organisms parasitic or pathogenic to animals. It has expanded its boundaries ever since, mainly on a morphological basis, to include many more species, from human, other animal, plant and soil sources. As it stands now in the 7th edition of *Bergey's Manual* (1957), the genus contains organisms of human, animal and plant origin having in common certain features, such as irregularity in shape, which varies from straight, curved, club-shaped or branched rods to almost coccoid organisms. Corynebacteria commonly show the so-called 'snapping division', the organisms show uneven and variable Gram strain and do not have endospores. Some soil organisms formerly in this group have obtained independence and emerged as a new genus *Arthrobacter*.

The species in the genus *Corynebacterium* were differentiated primarily on the basis of their habitat, along with some physiological characteristics. However, as stated in a footnote of *Bergey's Manual* (1957, p. 579), 'habitat relationships are used because comparative studies of the species in these groups are still completely lacking'.

Some studies have been made of the amino acid and carbohydrate composition of the cell wall of a few strains of corynebacteria (Orlova, 1954; Cummins & Harris, 1955, 1956; Mierzejewski, 1955; Rzucildo, *et al*, 1958) and on their nutritional characteristics (Lachance, 1960; Keddie, Leask & Grainger, 1966). Cummins & Harris (1959), while comparing Arthrobacter with Corynebacteria and other Gram-positive bacteria,

stressed the importance of cell-wall composition as basis of taxonomy; comparisons of amino acid composition might be of use in separating genera and of varieties of sugars and amino sugars in separating species. Keddie *et al.* (1966, p. 34) stressed that 'cell-wall composition afford a distinction between saprophytic strains of coryneform bacteria on the one hand and *C. diphtheriae* and related human and animal parasites on the other'.

Only a few strains of Corynebacteria have been investigated in regard to their carbohydrate metabolism. Hulanicka (1960) showed the existence of sedoheptulose and phosphopentose isomerase, epimerase, transketolase and transaldolase in *Corynebacterium diphtheriae* and concluded the pentose cycle (PC) pathway to be operative in this organism, although the extent of participation of the PC pathway in overall glucose catabolism is not known. The operation of the tricarboxylic acid cycle (TCA) pathway in this organism is reported by Jannes (1953) and Kornberg (1958). Zajic, De Ley & Starr (1956) reported the operation of the pentose phosphate (PP) pathway and TCA cycle pathway in a strain of *C. insidiosum*, and could not detect a specific dehydrase and aldolase which might convert 6-P-gluconate to pyruvate and triose-P, key enzymes in the Entner-Doudoroff (ED) pathway. Katznelson (1955, 1958) was unable to detect the formation of pyruvic acid from glucose, glucose-6-P, fructose-6-P and 6-P-gluconate in *C. michiganense*, *C. fascians* and *C. flaccumfaciens*. In these latter species, the presence of hexokinase, and glyceraldehyde-3-P dehydrogenase was not shown. Evidence for the presence of a poly-phosphate-glucose-phosphotransferase in *C. xerosis* was reported by Dirheimer & Ebel (1962).

In view of the lack of a direct comparative study of corynebacteria with respect to carbohydrate metabolism, the present work was undertaken with one strain of each of five species which originated from human, animal and plant sources.

METHODS

The organisms used were obtained from the American Type Culture Collection.

The strains studied were: *Corynebacterium hoagii* (ATCC 7005, human); *C. equi* (ATCC 7698, animal); *C. tritici* (ATCC 11408, plant); *C. sepedonicum* (ATCC 9850, plant); *C. xerosis* (ATCC 7084, human).

The stock cultures were grown on nutrient agar slopes and kept in a refrigerator at about 5°. Subcultures to new media were made at about 3-month intervals. *Corynebacterium sepedonicum* ATCC 9850 was grown only in liquid medium since its growth on nutrient agar was poor. The medium used for the growth of all Corynebacterium strains studied was that used by Lochhead & Thexton (1951) for the isolation of soil micro-organisms. The main source of carbon was either glucose or gluconate, at 0·15% (w/v). The carbon sources were sterilized separately, by autoclaving (120°, 30 min.), as well as some other medium constituents, and mixed aseptically after cooling. The media were adjusted to pH 6·8 with 0·1 N-HCl.

The organisms were grown in 250 ml. Erlenmeyer flasks containing about 130 ml. liquid medium, and incubated on a rotary shaker at 30° for *Corynebacterium xerosis*, *C. equi* and *C. hoagii*, and at 27° for *C. tritici* and *C. sepedonicum*. Three subcultures from the stock cultures were made at intervals before harvesting the organisms by centrifugation. The organisms so obtained were immediately resuspended in the growth medium containing no carbohydrates. Concentrations of organisms in these suspensions

were determined turbidimetrically, by means of a Klett-Summerson colorimeter, and adjusted to an extinction (640–740 mμ) corresponding to a concentration equivalent to 0·7–2·7 mg. dry weight organism/ml. Organisms used in experiments were harvested at the late stage of the exponential growth. Organisms grown in glucose medium or in gluconate medium were used.

The labelled substrates were: [1-^{14}C]glucose, [2-^{14}C]glucose, [3-^{14}C]glucose and [6-^{14}C]glucose were obtained from the National Bureau of Standards; [1-^{14}C]gluconate was bought from the Nuclear Chicago Corporation; [3, 4-^{14}C]glucose was prepared in this laboratory from liver glycogen of rats metabolizing $^{14}CO_2$ according to the method of Wood, Lifson & Lorber (1945); [2-^{14}C]gluconate, [3-^{14}C]gluconate and [6-^{14}C]gluconate were prepared from the corresponding labelled glucose samples by the method of Moore & Link (1940).

The radiorespirometric experiments were done according to the procedures described by Wang (1963). In a typical experiment, 10 ml. of a given suspension of organisms were placed in an incubation flask and allowed to utilize a prescribed amount of the labelled substrate under aerobic growth conditions. Respiratory $^{14}CO_2$ released by the organisms was swept through 10 ml. of an ethanolamine + ethanol solution (1 + 2, by vol.), which was replaced at hourly intervals. The experiment was terminated when the rate of production of respiratory $^{14}CO_2$ declined to an insignificant value, indicating that the given substrate had been exhausted from the medium. The vessels containing the suspensions were then cooled in ice for about 10 min., after which the organisms were separated by centrifugation. Both the washed organisms and the supernatant fluid thus obtained were assayed for radioactivity.

The radioactivity in respiratory $^{14}CO_2$ was assayed by means of liquid scintillation counting with a Packard Tricarb Spectrometer (Model 314-EX2) in the manner described by Wang (1963). The radioactivity of the organisms and the metabolic products in the media was determined by liquid scintillation counting with the thixotropic gel techniques (White & Helf, 1956). The efficiency of the liquid scintillation counter for different types of samples was determined by using the internal standards technique. Counts were made over a sufficient period to give a relative standard deviation no greater than 2 %.

RESULTS

All the Corynebacterium strains selected for the present work are capable of utilizing either glucose or gluconate as a sole source carbon. However, except in the case of *Corynebacterium sepedonicum* ATCC 9850 growth rates were generally better with glucose than with gluconate. Organisms grown in the glucose medium and the gluconate medium were used for the experiments, with ^{14}C-labelled glucose or ^{14}C-labelled gluconate as substrates. Regardless of the nature of the carbon source in the original culture medium, the radiorespirometric findings for a given substrate were essentially the same. However, with *C. hoagii* ATCC 7005, the utilization of gluconate by glucose-grown organisms was very sluggish, and hence, in this case only gluconate-grown organisms were used with ^{14}C specifically labelled gluconate as substrate.

The results of an experiment on the utilization of ^{14}C-labelled glucose or gluconate by the five Corynebacterium strains are given in Table 1. The data were those observed at the completion of substrate utilization which generally took 6 to 8 hr.

Table 1. *Results of radiorespirometric experiments on the utilization of glucose or gluconate by Corynebacterium strains*

Organism		Substrate		Peak respiratory $^{14}CO_2$ production		Substrate inventory (%)*			
Strain	Concentration of organisms (equiv. mg. dry wt/10 ml.)	^{14}C-Labelling	Concentration (mg./10 ml.)	Time occurred (hr.)	Extent (% hr.)	Respiratory CO_2	Organisms	Medium	Total
C. hoagii ATCC 7005	11	Glucose	4·0	2·0					
		C-1		2·0	20	44	36	12	92
		C-2		2·0	16	35	46	15	96
		C-3		1·8	22	45	37	12	94
		C-3, 4		2·0	25	50	28	11	89
		C-4†		2·0	27	55	19	10	84
		C-6		2·0	10	26	55	18	99
	11	Gluconate	1·5						
		C-1		0·8	44	82	3	14	99
		C-2		1·8	17	49	34	18	101
		C-3		1·8	18	43	16	39	98
		C-3, 4		2·0	23	57	20	25	102
		C-4†		2·0	30	71	24	11	106
		C-6		2·0	8	26	45	22	93
C. tritici ATCC 11408	7·0	Glucose	3·0						
		C-1		1·5	22	43	37	12	92
		C-2		1·6	10	22	50	17	89
		C-3		1·5	16	32	48	10	90
		C-3, 4		1·5	20	42	42	8	92
		C-4†		1·5	25	52	36	6	94
		C-6		2·5	6	19	52	20	91
	7·0	Gluconate	3·0						
		C-1		1·6	33	76	2	20	98
		C-2		2·2	11	28	55	10	93
		C-3		2·5	9	23	58	9	90
		C-3, 4		2·5	14	35	46	11	92
		C-4†		2·5	18	47	34	13	94
		C-6		2·8	8	20	53	18	91

Table 1 (*cont.*)

Organism		Substrate		Peak respiratory $^{14}CO_2$ production		Substrate inventory (%)*			
Strain	Concentration of organisms (equiv. mg. dry wt/10 ml.)	^{14}C-Labelling	Concentration (mg./10 ml.)	Time occurred (hr)	Extent (%/hr)	Respiratory CO_2	Organisms	Medium	Total
ATCC 7698 *C. equi*	15	Glucose	5·0						
		C-1		1·5	35	68	15	10	93
		C-2		1·9	29	59	22	11	92
		C-3		1·8	26	53	30	12	95
		C-3, 4		1·8	31	62	27	9	98
		C-4†		1·6	36	71	24	6	101
		C-6		1·3	24	45	30	16	91
	15	Gluconate	5·0						
		C-1		1·4	38	69	6	22	97
		C-2		1·4	29	58	26	7	91
		C-3		1·5	26	57	30	9	96
		C-3, 4		1·8	28	62	24	8	94
		C-4†		1·8	30	67	18	7	92
		C-6		1·6	18	40	36	14	90
C. sepedonicum ATCC 9850	8·0	Glucose	1·5						
		C-1		0·8	23	64	17	13	94
		C-2		1·0	21	51	34	13	98
		C-3		1·0	20	38	35	23	96
		C-3, 4		1·2	21	42	38	15	95
		C-4†		1·2	23	46	41	7	94
		C-6		2·5	7	27	45	20	92
	8·0	Gluconate	2·0						
		C-1		1·5	36	76	3	17	96
		C-2		1·8	27	59	18	11	88
		C-3		2·0	21	52	21	17	90
		C-3, 4		2·3	19	43	30	17	90
		C-4†		2·6	20	34	39	18	91
		C-6		2·8	18	48	28	16	92

Table 1 (*cont.*)

Organism		Substrate		Peak respiratory $^{14}CO_2$ production		Substrate inventory (%)*			
Strain	Concentration of organism (equiv. mg. dry wt/10 ml.)	^{14}C-labelling	Concentration (mg./10 ml.)	Time occurred (hr)	Extent (% hr)	Respiratory CO_2	Organisms	Medium	Total
C. xerosis ATCC 7084	27	Glucose	1·0						
		C-1		0·9	30	85	3	9	97
		C-2		0·9	22	68	14	7	89
		C-3		0·9	18	63	19	9	91
		C-3, 4		0·9	12	40	32	17	89
		C-4†		1·4	7	17	45	25	87
		C-6		3	3	14	56	21	91
	27	Gluconate	1·0						
		C-1		0·9	26	73	4	20	97
		C-2		0·9	23	65	10	23	98
		C-3		1·2	22	61	21	8	90
		C-3, 4		1·2	13	40	27	25	92
		C-4†		2·0	5	19	33	42	94
		C-6		2·0	4	16	50	24	90

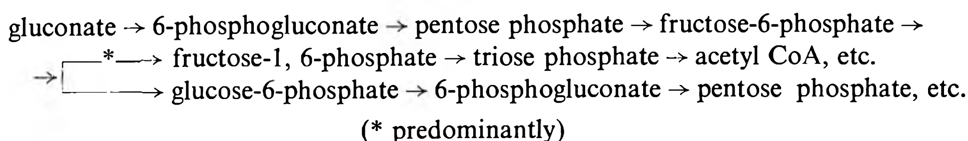
* Data presented under this heading are those observed at the end of each of the experiments.

† Data presented are obtained by calculation using data observed in the [3-^{14}C] glucose and [3,4-^{14}C] glucose experiments.

The data in Table 1 represent the average of at least four replicate experiments; the deviation among replicates was less than 5 %.

The analysis of the data with regard to the identification and estimation of glucose and gluconate pathways functioning in the various organisms relied on the following considerations. (1) Comparison of the extent of $^{14}CO_2$ production from the various labelled carbon atoms of the substrate at the end of utilization. (2) Comparison of the maximal rates of $^{14}CO_2$ production from labelled carbon atoms of the substrate (see Table 1, column headed 'peak respiratory $^{14}CO_2$ production'). (3) Since gluconate is a key intermediate in several glucose pathways, including the PP, the PC and the ED pathways, much information on the nature of the glucose pathway can be derived by a comparison of the over-all respirometric patterns observed in the glucose and gluconate experiments. Such a comparison assumes that the given gluconate behaved similarly to that derived from glucose catabolism. With this in mind, the information about glucose and gluconate pathways as found in the present work, can be summarized as follows.

Corynebacterium hoagii ATCC 7005. With glucose, the extensive conversion of C-3 and C-4 of glucose to respiratory CO_2 led to the conclusion that the Embden–Meyerhof–Parnas (EMP) pathway played a major role in this organism. The fact that the conversion of C-1 of glucose to CO_2 was significantly greater in extent than the conversion of C-6 provided evidence that the PP pathway was also operative. The operation of the latter pathway was further indicated by the findings in the gluconate experiments, where the conversion of C-1 of gluconate to CO_2 was prompt and extensive. However, pentose phosphate derived from the glucose via the PP pathway did not appear to be catabolized via the PC pathway. This conclusion was drawn from the fact that the extent and rate of conversion of C-2 of gluconate to CO_2 was considerably lower than that of the C-1 atoms. In fact, the rate and extent of conversion of gluconate carbon atoms other than C-1 to CO_2 appeared to reflect the operation of the sequential catabolic pathway previously described for the organism *Arthrobacter ureafaciens* ATCC 7562 *Arthrobacter globiformis* ATCC 8010 (Zagallo & Wang, 1962) which was given as follows:



(* predominantly)

Corynebacterium tritici ATCC 11408. Much of the discussion on the radiorespirometric findings with the *C. hoagii* ATCC 7005 also applies to *C. tritici* ATCC 11408. Glucose appeared to be metabolized by the *C. tritici* strain primarily via the EMP pathway and to some extent by the PP pathway; gluconate was metabolized via the PP pathway, and the pentose derived from gluconate was incorporated to fructose-6-phosphate and subsequently utilized via the EMP pathway or into cellular material instead of being engaged to any great extent in the pentose cycle reactions.

Corynebacterium equi ATCC 7698. The interpretation of the radio-respirometric pattern of the utilization of glucose by this organism was difficult. Superficially it appeared that the ED pathway, shown to function in pseudomonads (Stern, Wang & Gilmour, 1960), might have been operative in the *C. equi* strain since a close equivalence in catabolic behaviour (rates and extent of conversion of carbon atoms to respiratory

$^{14}CO_2$) between C-1 and C-4 of glucose and that between C-3 and C-6 of glucose were indicated by the radiorespirometric data. However, the maximal rate of $^{14}CO_2$ production from the C-6 of glucose occurred significantly earlier than that from the C-3 and the extent of conversion of C-4 of glucose to respiratory CO_2 was slightly greater than that of C-1. This led to a closer examination of the findings in the gluconate experiment. With gluconate as substrate, the catabolic equivalences, as indicated by the rates and extents of $^{14}CO_2$ production, between C-1 and C-4 of gluconate were not observed. Instead, the conversion of C-2 and C-3 of gluconate to respiratory $^{14}CO_2$ was only slightly less (in rate and extent) than that of the C-1 of gluconate. Since gluconate is a key intermediate of glucose metabolism in the ED pathway, it can therefore be concluded that in *C. equi* ATCC 7698 glucose was probably catabolized by way of the EMP glycolytic pathway and the PP pathway. The pentose phosphate derived from either glucose or gluconate via the PP pathway appeared to engage rather extensively in the PC pathway.

Another feature of the catabolic behaviour of *C. equi* ATCC 7698 was reflected in that the conversion of the C-6 of glucose and, to some extent, the C-6 of gluconate to respiratory CO_2 were extensive in rate and extent. These findings presumably related to the derivation of glyceral-dehyde-3-P, from either the EMP pathway or the PC pathway, being engaged extensively in the sequential operation of the triose-P isomerase and reversed aldolase reactions, leading to the formation of hexose phosphates. Such a mechanism would result in the transference of C-6 of glucose or gluconate to the C-1 position of re-formed hexose phosphates. Further catabolism of the re-formed hexose phosphates, by way of the PP pathway, would give a relatively more direct conversion of the C-6 of glucose or of gluconate to respiratory CO_2.

Corynebacterium sepedonicum ATCC 9850. Glucose appeared to be metabolized by this organism extensively by way of the PC pathway, as shown by the fact that, with glucose as substrate, the conversion of C-2 and C-3 of glucose was only slightly less quick and extensive as compared with that of C-1. Similar results were obtained in the gluconate experiments. Since the conversion of the C-4 of glucose to CO_2 was also quick and extensive, the EMP pathway was presumably also playing a role in the over-all catabolism of glucose.

Corynebacterium xerosis ATCC 7084. Both glucose and gluconate appear to be catabolized by this organism extensively, if not exclusively, by way of the PP and PC pathways. This conclusion was supported by the extensive conversion of C-2 and C-3 of glucose or gluconate to CO_2, with the extents of conversion only slightly less than that of the C-1 of either substrates. The slow conversion of C-4 and C-6 of glucose or gluconate to respiratory CO_2 indicated that the catabolism of the glyceraldehyde-3-P, derived from the PP and PC pathways, was much limited in extent. However, a significant amount of C-6 of these substrates was incorporated into cellular constituents, a fact which reflected the important role played by glyceraldehyde-3-P in the biosynthetic functions.

On the basis of the foregoing analysis of the data, the relative participation of concurrent primary glucose pathways in the various organisms can be estimated by the method of Wang & Krackov (1962). The definition of the term 'primary glucose pathway' is that defined by Wang & Krackov (1962) and by Wang (1964). The estimated values for the relative participation of concurrent pathways are given in Table 2. The pathway estimations presented in Table 2 represent only a crude assessment of the

relative participation of the concurrent pathways. This is so because the derivation of the equations for the pathway estimation, when applied to the organisms used in the present work, does not cover complicating factors such as the operation of the triose recombination pathway and the incorporation of intact glucose into cellular constituents, etc. Nevertheless, these complicating factors should not affect greatly the estimated values for participated pathways.

Table 2. *Estimated relative participation of concurrent glucose pathways in five Corynebacterium strains*

Strain	Relative participation of primary glucose pathways (% of the given substrate glucose)		Extent of pentose cycle operation
	EMP*	PP*	
C. hoagii ATCC 7005, human	78	22	Limited
C. tritici ATCC 11608, plant	70	30	Limited
C. equi ATCC 7698, animal	62	38	Extensive
C. sepedonicum ATCC 9850, plant	44	56	Extensive
C. xerosis ATCC 7084, human	15	85	Extensive

* The relative participation of the PP pathway in over-all glucose catabolism, as estimated by the method of Wang & Krackov (1962), was calculated in a direct manner and is hence a more reliable piece of information. On the other hand, the relative participation of the EMP pathway was estimated by difference and is subject to inaccuracies due to complicating factors. For *C. xerosis* ATCC 7084, it is possible that the EMP pathway did not play a role in the utilization of glucose.

DISCUSSION

Despite the classification of the five strains of organisms used in the present study in the genus *Corynebacterium*, the present findings indicate that there is marked differences in the catabolic mechanism of glucose catabolism between *Corynebacterium hoagii* ATCC 7005 and *C. tritici* ATCC 11408. Both organisms use markedly for glucose catabolism, the EMP pathway and to a lesser extent the PP pathway. The pentose phosphate derived from glucose via the PP pathway was not used to any significant extent in the PC relations. Such a catabolic behaviour resembles closely that found with strains of *Arthrobacter globiformis* ATCC 8010 and *A. ureafaciens* ATCC 7562. On the other hand, the organisms *C. equi* ATCC 7698, *C. sepedonicum* ATCC 9350 and *C. xerosis* ATCC 7084 displayed a significantly different catabolic behaviour with regard to both glucose and gluconate. In these three strains, the PP pathway played a more important role in glucose catabolism in comparison to the EMP pathway, and the pentose derived from glucose via the PP pathway was engaged in extensive catabolism via the PC pathway. In the case of *C. xerosis* ATCC 7084 the organism used heavily, if not exclusively, the PC pathway for glucose utilization. Such a catabolic behaviour resembles very much that found with several Acetobacter species (Kitos *et al.* 1958; Wang & Bjerre, 1961).

On the basis of the present findings, it appears that the five strains of the genus *Corynebacterium* studied can be grouped in three categories. The strains *C. hoagii* ATCC 7005 and *C. tritici* ATCC 11408 appear to belong to a category with catabolic behaviour for glucose and gluconate utilization resembling that of some of the

Arthrobacter strains typified by *Arthrobacter globiformis* ATCC 8010 (Zagallo & Wang, 1962). The strains *C. equi* ATCC 7698 and *C. sepedonicum* ATCC 9850 tested here resemble each other in that glucose is utilized by a more or less equal contribution of the EMP and PC pathways. The strain *C. xerosis* ATCC 7084 is distinct in its catabolic behaviour for both glucose and gluconate, with the PC pathway being the predominant route, as in some strains of Acetobacter and Gluconobacter.

The grouping of the Corynebacterium strains examined here by their biochemical behaviour, particularly their catabolic behaviour, does not seem to be related to the sources from which these organisms were initially isolated. Inasmuch as the cell wall compositions of the respective strains included in the present study are not known, it is not possible to correlate the present findings with the taxonomic classifications on cell wall compositions.

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The Short Forms and Long Forms of Proteus

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SUMMARY

Swarming long forms of Proteus arose from short forms at the edge of a colony on nutrient agar. Specialized small pre-swarmer forms were not seen. The long forms contained many nuclear units and did not have cross walls while actively swarming. Long forms were not found in liquid media. Chemical analysis of short and long forms showed that cell walls of the two forms had qualitatively the same amino acid composition and that inhibition of DNA synthesis was not responsible for the formation of long forms. Long forms differed from short forms in having no detectable amino acid pool, a characteristic that may be associated with excessive flagellar synthesis by developing long forms.

INTRODUCTION

In a previous paper (Jones & Park, 1967) it was mentioned that the swarming of Proteus is always associated with a change in morphology from short to long forms. Reports in the literature are not in agreement about the morphological events which lead to the formation of swarming long forms of Proteus on agar media. Klieneberger-Nobel (1947) reported that the long forms arose from small uninucleate organisms formed by division of the short, relatively wide organisms present during the early stages of growth on nutrient agar. These small 'pre-swarmer' forms were not observed by Moltke (1927), Russ-Münzer (1935), Kvittingen (1949*a*, *b*), Hughes (1957) or Hoeniger (1964). Kvittingen (1949*b*) and Hughes (1957) reported a high mortality rate amongst long forms. Hughes considered that swarming long forms of Proteus were morphologically indistinguishable from long forms induced by penicillin; this view was not held by Hoeniger (1964, 1966). Reports also differ about the formation of long forms in nutrient broth cultures. Russ-Münzer (1935) and Klieneberger-Nobel (1947) did not detect long forms in nutrient broth cultures but Kvittingen (1949*a*) reported them to exist in large numbers after growth for 6 hr at 37°.

Changes in morphology of micro-organisms have often been shown to be accompanied by alterations in the amount of specific cell components present or in their composition. Alterations in amino acid pools during formation of protoperithecia in *Neurospora crassa* (Barbesgaard & Wagner, 1959), during multicellular differentiation of *Dictyostelium discoideum* (Wright & Anderson, 1960) and during fruiting-body formation in *Myxococcus xanthus* (Dworkin, 1963) have been reported. Senthe-Shanmuganathan & Nickerson (1962) found differences in cell-wall composition as between triangular and ellipsoidal forms of *Trigonopsis variabilis*. Similarly, Bartnicki-

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Garcia & Nickerson (1962) showed that conversion of *Mucor rouxii* from filamentous to yeast-like growth resulted in a change in cell-wall composition.

Many agents which induce filament formation in Gram-negative bacteria interfere with some aspect of cell-wall formation (e.g. penicillin, Duguid, 1946; Rogers & Mandelstam, 1962) or with synthesis of deoxyribonucleic acid (e.g. phenethylalcohol, Berrah & Konetzka, 1962; nalidixic acid, Goss, Deitz & Cook, 1964; high hydrostatic pressure, ZoBell & Cobet, 1964).

The present paper describes morphological and cytological events during sequential stages of growth of Proteus on agar media and in liquid media and reports on a comparison of the chemical composition of cell fractions of short and long forms, which was done to see whether a chemical basis could be found for the differences between non-swarming and swarming organisms.

METHODS

Organisms. Proteus mirabilis NCDO 1881 was used for investigations on morphology and cytology of organisms. *Proteus vulgaris* NCDO 1882 was used for comparing the chemical composition of short and long forms. The maintenance of strains and the preparation of suspensions of organisms for inoculation were as described by Jones & Park (1967).

Media. The media used were as described by Jones & Park (1967).

Examination of cultures at sequential stages of growth. Twenty plates of nutrient agar dried for 1 hr at 37° were inoculated centrally with a drop (0·02 ml.) of a dense suspension of bacteria and incubated at 37°. One plate was withdrawn every 30 min. and the colony immediately examined with a microscope with a ×40 dry objective. After direct observation two impression preparations of segments taken from the centre to the edge of the colony were made on coverslips. One preparation was stained with dilute carbol fuchsin to show the gross morphology of the organisms, while the other was fixed in osmium tetroxide vapour for 3 min. and stained to reveal nuclear material by the acid Giesma technique (Robinow, 1942). Because after each 30 min. different plates were examined, different organisms were observed at each stage. The assumption was made that at any given time the cultures on all plates were at the same stage of development. From examination of the plates by eye it appeared that this assumption was valid, because swarms occurred at the same time on all plates used.

To follow the development of Proteus in liquid medium small volumes of nutrient broth in ⊥-shaped vessels were inoculated and incubated in a water bath at 37°. For each experiment one vessel was left stationary while the other was shaken in an attempt to make the degree of aeration similar to that of surface cultures on nutrient agar. Smears prepared from each vessel every 30 min. were stained with dilute carbol fuchsin and examined with the microscope.

Organisms from various cultures were examined for flagella after staining by the method of Rhodes (1958). Attempts were made to demonstrate cross walls in long forms of Proteus by the tannic acid + crystal violet method (Robinow, 1944) and the methods of Chance (1953) and Webb (1954). No cross walls were seen but, because the staining of walls of Gram-negative bacteria is often difficult, it was thought that the walls might be present but unstained. The plasmolysis method of Powell (1958) for detecting septation was therefore used to examine long forms from the moving edge of swarms.

Collection of short and long forms for chemical analysis. Proteus vulgaris NCDO 1882 was used for comparing the chemical composition of short and long forms. This strain was chosen because it swarmed on nutrient agar at 30° but not at 37° and so could also be used to compare short forms grown at a temperature suitable for swarming with short forms grown at a temperature which was not suitable.

To obtain a yield of short forms a sample of bacterial suspension (0·3 ml.) was spread over the surface of nutrient agar plates (diam. 9 cm.) which had been dried for 1 hr at 55°. After incubation for 4·5–5 hr or 24 hr at 30° or 37° organisms were harvested and washed twice in aqueous NaCl (0·5 M).

Long forms were collected from the swarming edge of colonies because this was the only way in which they could be obtained virtually free from short forms. In our hands the method used by Hoeniger (1965) to obtain long forms for electron microscopy gave a mixture of both forms. Nutrient agar plates (14 cm. diam.) were inoculated by placing a disc of filter paper (5·3 cm. diam.) soaked in a suspension of organisms at the centre of each plate. After incubation for 11 hr at 30° the second zone of each swarm was approaching the edges of the plates. The outer 5 mm. of this moving zone consisted almost entirely of long forms and was harvested by removing the nutrient agar from inside this zone, so leaving the outer 5 mm. of swarm and the uncovered agar surface intact on each plate. The swarming organisms were collected by washing them off the agar with NaCl (0·5M). The washings from several plates were pooled and centrifuged and the sediment stored at −20°. Long forms obtained over several weeks by many repetitions of this process were pooled, washed twice with aqueous NaCl (0·5M) and kept at −20°. The amount of long forms collected by this method was small (0·34 g. wet wt), but unwanted short forms were rare (Pl. 1, fig. 1).

Preparation of cell fractions. Because it was possible to collect only a small quantity of long forms, cell fractions were obtained in sequence from one batch of organisms. The sequence of Roberts *et al.* (1957) was used, but the methods for obtaining the fractions differed. The procedure is summarized in Fig. 1.

Analysis of cell fractions. Supernatant fluids which contained free amino acids were dried *in vacuo* over solid NaOH and the residues dissolved in small volumes of isopropanol+water (10+90, by vol.). Cell walls were hydrolysed in 6 N-HCl in tightly capped 1 oz. McCartney bottles for 20 hr at 105°. Hydrolysates were evaporated to dryness over a water bath, taken up in distilled water, again evaporated to dryness, and the amino acid residues dissolved in small volumes of isopropanol+water (10+90, by vol.). Free amino acids and cell-wall amino acids were identified by two-dimensional descending paper chromatography. Two solvent systems were used: (i) *n*-butanol+glacial acetic acid+water (60+15+25, by vol.), followed by ethanol+ammonia+water (90+5+5, by vol.), on Whatman no. 1 paper; (ii) *n*-butanol+methyl ethyl ketone+ammonia (sp.gr. 0·880)+water (50+30+10+10, by vol.; Wolfe, 1957) followed by *n*-butanol+glacial acetic acid+water (60+15+25, by vol.) on Whatman no. 4 paper. Positions of compounds were determined by dipping the papers in a solution of ninhydrin (0·5%, w/v) in acetone+water (95+5, by vol.). Amino sugars were detected by the silver nitrate reagent for sugars (Trevelyan, Procter & Harrison, 1950) and by the Elson & Morgan reagent (Smith, 1960).

DNA content was estimated by the diphenylamine method of Burton (1956) with herring DNA as standard, and RNA by the orcinol method of Ceriotti (1955) with D-ribose as standard.

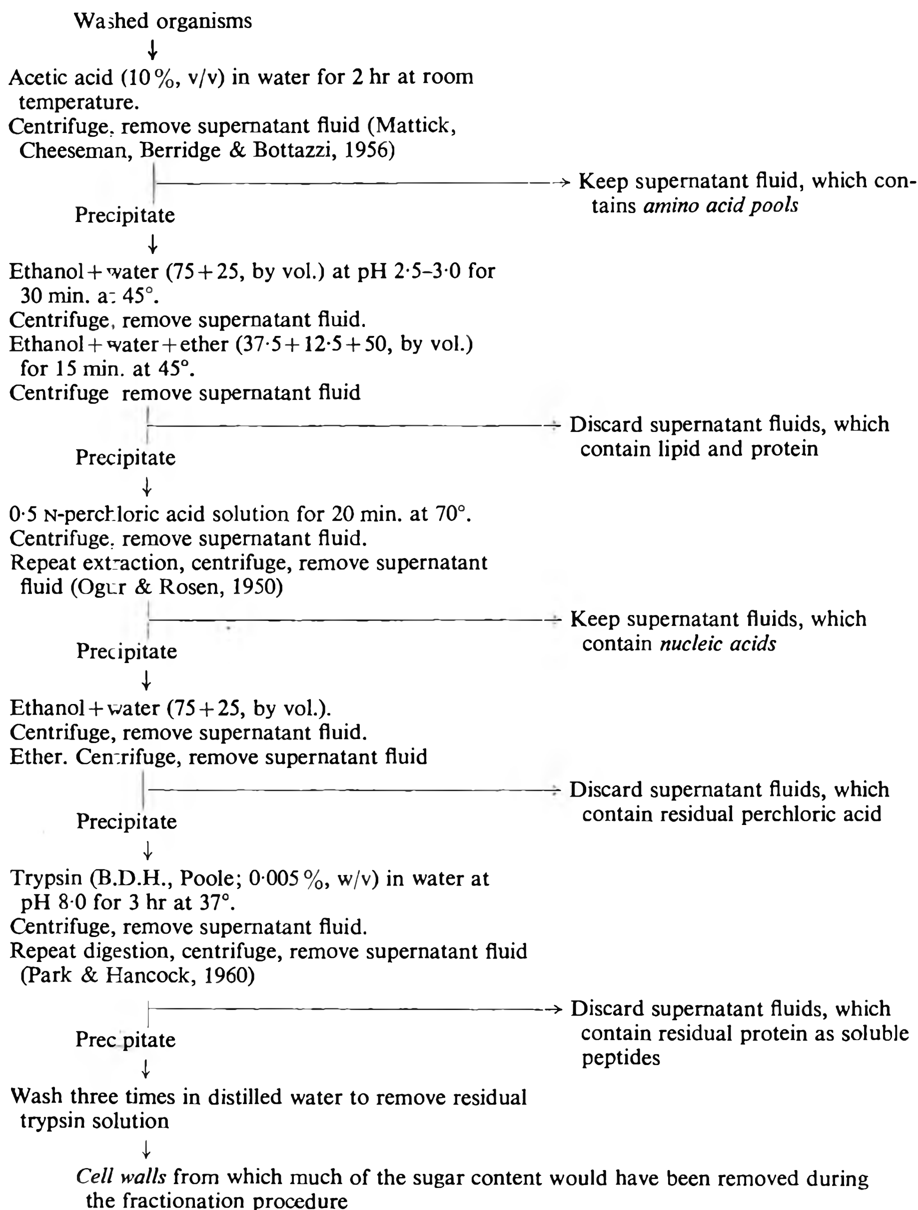

Fig. 1. Summary of the procedure for separation of cell fractions.

RESULTS

Morphology and cytology of organisms at sequential stages of growth

The stages of growth on nutrient agar before swarming were: (1) enlargement of the small coccoid organisms of the inocula to typical short forms each with 2–4 nuclear units; (2) multiplication of these short forms; (3) elongation of short forms at the edge of the colony after incubation for 3–3·5 hr to give long forms which

moved around each other. At stage 3 swarming then started and was visible to the unaided eye when the culture had been incubated for a total of 4 hr. The long forms moved across the nutrient agar surface *en masse* or occasionally in groups detached from the advancing edge. This observation agrees with that described by Hoeniger (1964). The development of a mass of small uninucleate 'pre-swarmer' forms as reported by Klieneberger-Nobel (1947) was not observed.

The number of nuclear units per organism depended on the length of the organism. Long forms were up to 70 μ long and had up to 46 nuclear units. The acid Giemsa technique thus indicated that DNA synthesis had not been inhibited (Pl. 1, figs. 2, 3). Cell-wall stains and plasmolysis experiments indicated that long forms had no cross walls; none were seen after staining for cell walls and on plasmolysis the protoplasm of each long form showed as only one unit. It was concluded that cell division was prevented at some stage before the formation of cross walls. Hoeniger (1966) did not see cross walls in Proteus long forms but did demonstrate separate units of protoplasm by plasmolysis followed by fixation and staining with thionin. Flagella appeared on the short forms after incubation on nutrient agar for 3 hr. The long forms which subsequently developed at 30° usually had more flagella per unit length of organism than did short forms, but detachment of flagella from organisms during the staining procedure made comparisons of numbers difficult. Hoeniger (1965), who used electron microscopy for observing the flagella of Proteus organisms, showed that long forms had more and longer flagella per unit length of organism than did short forms.

At no time during incubation for 10 hr, after 24 hr or after 48 hr were long forms seen in samples from cultures in shaken nutrient broth, in static nutrient broth, in static liquid minimal medium plus Casamino acids (1 %, w/v) or in static liquid minimal medium plus M/16 L-glutamic acid (Jones & Park, 1967); all these media, when solidified with agar, supported swarming. Long forms in liquid media have been reported by Kvittingen (1949*a*).

Chemical composition of cell fractions of short and long forms

Free amino acids were not found in the acetic acid extract from long forms. This indicated either that free amino acids were not present in long forms or that they had been lost during the collection procedure. Loss might have occurred because the cell envelope of long forms was naturally more permeable than was that of short forms or because it was made permeable by the freezing and thawing associated with the collection procedure. The free amino acids found in short forms are shown in Table 1. Preparations from short forms grown for 24 hr were added to chromatograms at five times the concentration of those from organisms grown for 4·5–5 hr because of the weak reaction with ninhydrin of the acetic acid extracts from organisms grown for 24 hr. There was no qualitative difference in the free amino acids obtained from cultures grown for the same time at 30° or 37°, but there was more amino acid material in organisms grown for 4·5 hr than in organisms grown for 24 hr. A larger variety of free amino acids is typical of young cultures of bacteria (Berridge, Cheeseman, Mattick & Bottazzi, 1957; Gregory & Mabbitt, 1957). The occurrence of β-alanine and lysine as the major free amino acids from 24 hr cultures is unusual. β-Alanine is found as a major component in fungi (Holden, 1962) but so far as we are aware in bacteria it has been found only in *Corynebacterium diphtheriae* (Work, 1949). The

presence of the amino sugar, which was probably glucosamine, is interesting since we have found no other reports of amino sugars in amino acid pools from bacteria or fungi. However, perhaps some of the 'unknown' spots often reported on chromatograms of amino acid pools are amino sugars.

Table 1. *Free amino acids obtained from short forms of Proteus vulgaris* NCDO *1882 grown on nutrient agar*

Increasing number of + signs indicates increasing amounts of amino acid determined visually by intensity of colour formed with ninhydrin. ± indicates colour just detectable. Because of differences in spot sizes between different amino acids on the chromatograms comparisons of concentrations made across the table are more valid than those made vertically. Amino acid pools from organisms grown for 24 hr were added to chromatograms at five times the concentration of amino acid pools from organisms grown for 4·5 hr.

Compound	Short forms collected after growth for 4·5 hr at 37°	Short forms collected after growth for 4·5 hr at 30°	Short forms collected after growth for 24 hr at 37°	Short forms collected after growth for 24 hr at 30°
Alanine	+++	+++	−	−
β-alanine	+	+	+++	+++++
Arginine	+	±	−	−
Aspartic acid	+	+	+	+
Citrulline or glutamine	+	+	−	−
Diaminopimelic acid	++	++	±	±
Glutamic acid	+++++	+++++	±	+
Glycine	++	++	−	−
Lysine	++	++	++	+++
Leucine or isoleucine	++	++	−	−
Methionine	+	+	−	−
Phenylalanine	+	+	−	−
Proline	±	±	−	−
Serine	+	+	−	−
Threonine	+	+	−	−
Tyrosine	±	±	−	−
Amino sugar	+++	+++	+++	++++

The results of nucleic acid estimations expressed as mg./g. wet wt organism, and as DNA/RNA ratios are given in Table 2. Expressing DNA as a fraction of RNA overcomes inaccuracies due to loss of organisms in the extraction procedures. There was not sufficient material from long forms for the results to be expressed in terms of wet wt organisms. Short forms from colonies grown for 4·5 hr and long forms gave the same DNA/RNA ratio. This similar ratio confirms the cytological finding that the formation of the long forms was not a result of inhibition of DNA synthesis. Short forms from colonies grown for 24 hr had a higher DNA/RNA ratio due to a decrease in the amount of RNA. This result is consistent with those reported for other washed resting bacteria (Mitchell & Moyle, 1951; Wade & Morgan, 1957).

The amino acids found in cell-wall hydrolysates (alanine, arginine, aspartic acid, diaminopimelic acid, glutamic acid, glycine, histidine, lysine, leucine and/or isoleucine, methionine, phenylalanine, proline, serine, threonine, tyrosine, valine, a trace of citrulline, an amino sugar) were, with one exception, the same as found by Kandler, Hund & Zehender (1958) in cell walls of *Proteus vulgaris*; however, Kandler *et al.* did not find arginine. We found no qualitative difference between the amino acid content of cell walls from short and long forms. Insufficient long-form material was available for a reliable quantitative estimation to be made.

DISCUSSION

Swarming long forms of Proteus were produced on nutrient agar, at the edge of colonies, but not in nutrient broth. The relationship between growth rate and swarming (Jones & Park, 1967) suggests that long forms occur only at the edge of a colony because it is there that nutrients are most readily available and organisms can grow fastest. It is not known why long forms occur on agar-solidified media but not in either static or agitated liquid media. It seems unlikely that the difference is due to the chemical nature of agar or to impurities in it. Hauser (1885) found long forms on

Table 2. *Estimations of DNA and RNA in short and long forms of Proteus vulgaris* NCDO *1882 grown on nutrient agar*

	DNA expressed as mg./g. wet wt organism	RNA expressed as mg. purine-bound ribose/g. wet wt organism	DNA/RNA ratio, i.e. mg. DNA per 1 mg. of purine-bound ribose of RNA
Short forms collected after growth for 4·5 hr at 37°	6·6	11·6	0·59
Short forms collected after growth for 4·5 hr at 30°	8·1	15·6	0·54
Short forms collected after growth for 24 hr at 37°	8·0	1·3	5·94
Short forms collected after growth for 24 hr at 30°	9·9	4·7	2·06
Long forms collected from second zone of swarm at 30°	*	*	0·64

* Insufficient material available for accurate weighing.

media solidified with gelatin and Jones (1966) found long forms on media solidified with either New Zealand agar (Davis) or purified agar (Difco). Jones (1966) also found that swarming occurred on agar media to which 0·5 % (w/v) charcoal or starch had been added to adsorb impurities such as fatty acids (Pollock, 1947), but Smith & Alwen (1966), who used 1 % (w/v) charcoal did inhibit swarming of Proteus. However, it seems that the important effect of agar so far as the swarming of Proteus is concerned is to change the physical state of the medium.

Thus long forms appear to develop because cell division is inhibited at some stage before the formation of cross walls, and not because synthesis of DNA is inhibited. Long forms differ from short forms harvested from young cultures on nutrient agar; the long forms have more flagella per unit length of organism and no free amino acid pool. The formation of long forms with many flagella in a Proteus culture on a solid medium may be considered as an event arising from a 'discontinuous biochemical process' similar to that postulated by Quadling & Stocker (1957) to explain the occurrence of rare motile bacteria in cultures of non-motile salmonellae. We suggest that the formation of long forms and the formation of many flagella are not independent processes but that one is dependent on the other. At an appropriate growth rate (Jones & Park, 1967) flagella synthesis or perhaps the synthesis of flagella-synthesizing sites becomes uncontrolled, and this results in a decrease in the amino acid pool, so that cell division is prevented by a shortage of amino acids or some other intermediate needed for the cell-division mechanism. The resultant long, heavily

flagellate forms are able to move over the surface and so spread to hitherto unpopulated areas of agar where synthesis of flagella ceases, and division is resumed to give short forms once more.

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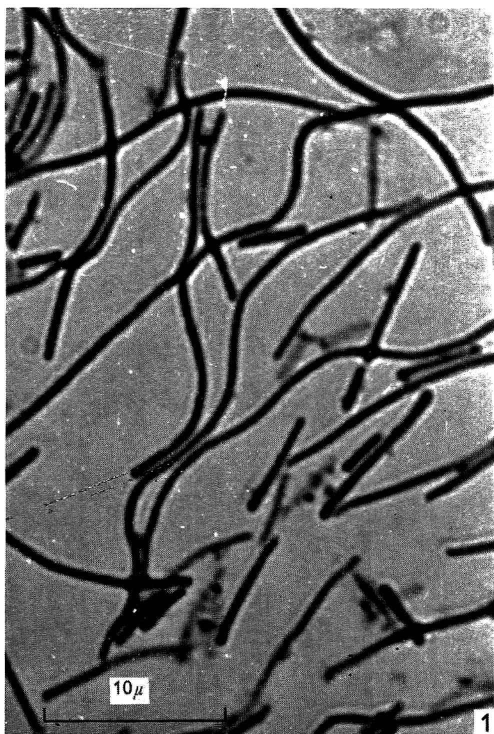

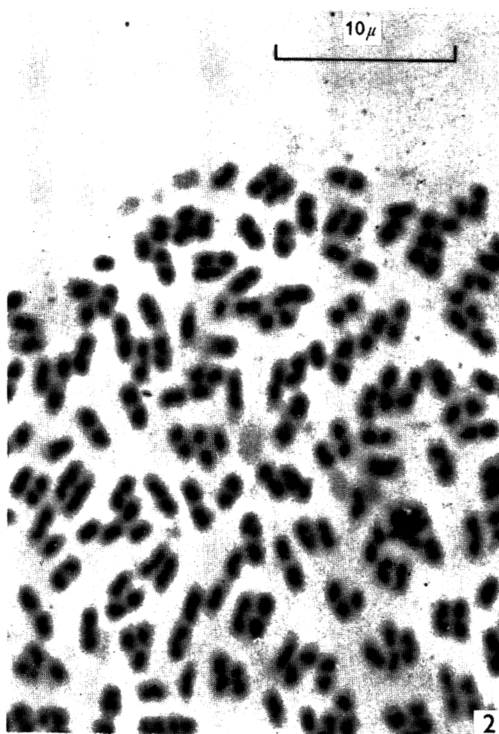

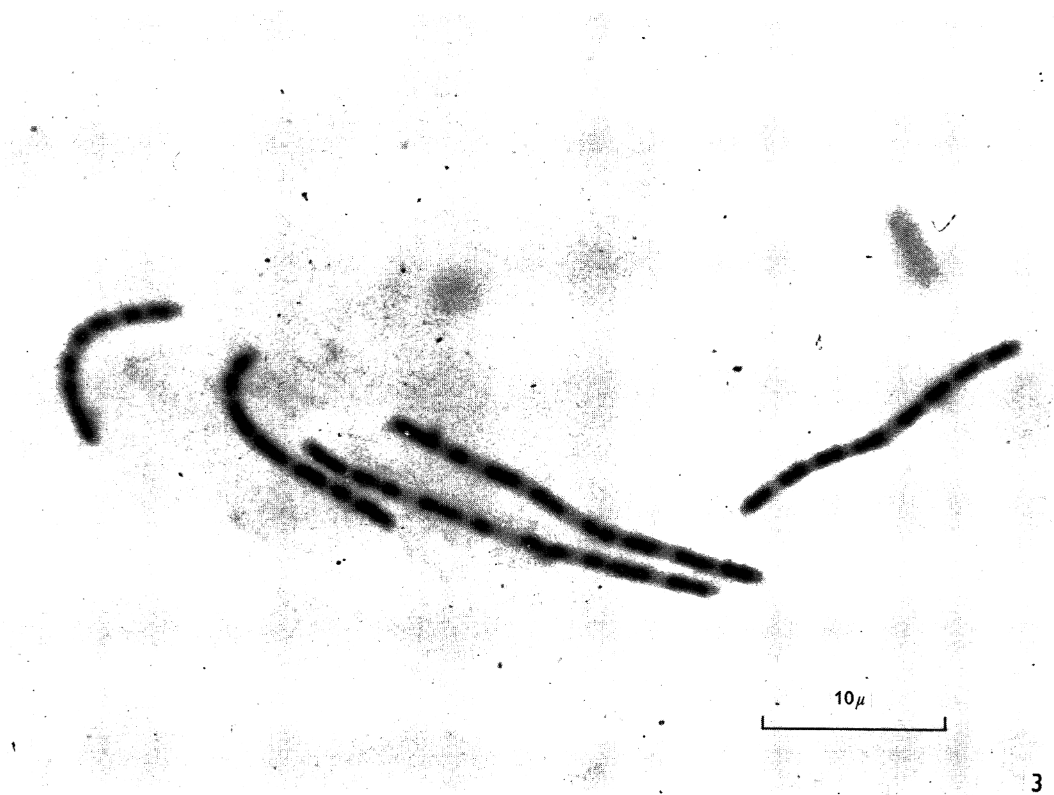

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EXPLANATION OF PLATE

Fig. 1. A representative sample of the harvest of organisms from the swarming edge of cultures of *Proteus vulgaris* NCDO 1882; stained with dil. carbol for 30 sec. The harvest consisted almost entirely of long forms. Scale on photograph.

Figs. 2, 3. Impression preparations of *Proteus mirabilis* NCDO 1881 grown on nutrient agar at 37° and stained by the acid Giemsa method to show nuclear material (dark areas). Fig. 2. Organisms from the edge of a culture after incubation for 2·5 hr: scale on photograph. Fig. 3. Organisms from the swarming edge of a culture after incubation for 4 hr: scale on photograph.

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The Influence of Medium Composition on the Growth and Swarming of Proteus

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SUMMARY

The ability of various compounds to support swarming of Proteus was determined by making additions to a minimal medium agar on which swarming did not occur. Swarming occurred when alanine, asparagine aspartic acid, glutamic acid, glutamine, proline or serine were present, either individually or together. It did not occur when certain other amino acids were added individually to minimal medium agar but did occur when these were added together. The ability of a compound to support swarming was correlated with its ability to serve as a carbon + energy source and with the stimulation of growth rate. The swarming phenomenon is discussed in the light of these findings.

INTRODUCTION

Many members of the bacterial genus *Proteus* exhibit the phenomenon of swarming when grown on solid medium (Plate 1). When inoculated at the centre of a plate of suitable medium and incubated aerobically at 30–37° the culture grows as short organisms for a few hours; then organisms at the edge of the colony thus formed elongate and spread over the surface, i.e. they swarm. Movement stops after 1–2 hr. Division of organisms at the edge of the colony then occurs, to give short organisms, and the cycle is repeated. All organisms beyond the edge of the initial colony constitute the swarm. The swarming phenomenon of Proteus has been described by many workers, with slight variations in their descriptions (Hauser, 1885; Moltke, 1927; Russ-Munzer, 1935; Dienes, 1946; Klieneberger-Nobel, 1947; Lominski & Lendrum, 1947; Kvittingen, 1949*a*, *b*; Hughes, 1957; Hoeniger, 1964; Jones, 1966). Two theories have been proposed to explain swarming. One is that local over-popuation exhausts the nutrients in the area and that the resultant gradient of nutrients at the periphery encourages outward growth and movement (Moltke, 1927; Russ-Munzer, 1935). The other theory is that swarming is a response to metabolic products which accumulate during growth of the non-swarming organisms (Lominski & Lendrum, 1947; Hughes, 1957).

There have been few studies of the effect of medium composition on swarming, apart from studies which involved additions of inhibitors to stop swarming, and all previous workers have used complex undefined media. We found that swarming of *Proteus mirabilis* and *P. vulgaris* did not occur on a solid medium prepared by adding agar to the minimal medium of Fildes (1938), although the organisms did produce

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flagella on this medium. It was therefore possible by making additions to Fildes medium to determine the ability of various compounds to support swarming. It became clear that many compounds would support swarming; the effect of these compounds on growth was therefore examined.

METHODS

Organisms. Several Proteus strains were isolated from swarms on blood agar plates obtained through the courtesy of Dr T. D. Martin from the Pathology Department, Royal Berkshire Hospital. Two strains of *Proteus mirabilis* were chosen for detailed study; these were deposited in the National Collection of Dairy Organisms (NCDO 1880, NCDO 1881). On nutrient agar both strains gave zoned swarms typical of Belyavin's (1951) 'A' phase ('Y' variant of Coetzee & Sacks, 1960). Maintenance was by subculture every 4 months on slopes of soft nutrient agar (see below) dispensed in screw-capped bottles. After incubation at 37° for 24 hr cultures were stored at room temperature.

Media. All media were prepared using demineralized water from a model 8E Deminrolit two-cylinder ion exchanger (Permutit, London, W. 4). Nutrient agar consisted of (%, w/v, in water) peptone (Evans), 0·5; Lab-Lemco (Oxoid), 0·5; NaCl, 0·5; New Zealand agar (Davis), 1·2; pH 7·2 before autoclaving (121°, 20 min.). For soft nutrient agar 0·8 % (w/v) agar was used. Nutrient broth was of the same composition apart from the omission of agar. The liquid minimal medium (MM liquid) for Proteus was that of Fildes (1938) but NaCl was incorporated to ensure sufficient electrolyte for swarming on solid media (Naylor, 1960; Sandys, 1960). Five solutions were prepared; Solution *a* was KH_2PO_4, 18·0g.; $(NH_4)_2SO_4$, 2·0g.; NH_4Cl, 2·0g.; sodium lactate (70 %, w/v, aqueous solution), 16·0g.; in 1800 ml. water; pH 7·6 before autoclaving (121°, 20 min.): Solution *b* was $Fe(NH_4)_2(SO_4)_2 . 6H_2O$,. 0·08 % (w/v) in N/50 HCl, sterilized by filtration through glass: Solution *c* was $MgSO_4 . 7H_2O$, 0·4 % (w/v) in water, sterilized by autoclaving (115°, 10 min.): Solution *d* was nicotinic acid, 0·012 % (w/v) in water, sterilized by autoclaving (115°, 10 min.): Solution *e* was NaCl, 24·0 % (w/v) in water, sterilized by autoclaving (121°, 20 min.). For use, solutions *a* (30 ml.), *b* (1·5 ml.), *c* (0·6 ml.), *d* (1·2 ml.), *e* (1·2 ml.) were mixed and made to 60 ml. with water or with aqueous solutions of test materials. To obtain a minimal medium agar (MM agar) the constituents of solution *a* (see above) were dissolved in 200 ml. demineralized water and added while hot to a hot solution of New Zealand agar (Davis; 43·2 g. in 1600 ml. water), after which the whole was sterilized by autoclaving (115°, 10 min.). For use, 30 ml. of this molten agar was cooled to 60°, the same amounts of solutions *b*, *c*, *d* and *e* as given above were added, and the volume made to 60 ml. with water or with aqueous solutions of test materials.

Plates were prepared by pouring about 15 ml. molten medium at 55° into glass Petri dishes (diam. 9 cm.) and drying open for 2 hr at 37°. They were used within 24 hr.

Inoculum. Organisms from a nutrient agar slope culture incubated at 37° for 24 hr were washed once in $\frac{1}{4}$ strength Ringer solution (50 ml.) and then suspended in 3 ml. $\frac{1}{4}$ strength Ringer solution to give a dense suspension. Each inoculum was tested to see that it consisted of organisms in the A(Y) phase (see above) by inoculation on two nutrient agar plates and examination after incubation to confirm that a zoned swarm was produced.

Determination of ability of various compounds to serve as carbon+energy sources and/or nitrogen sources in agar media. For each medium two plates per strain were inoculated by putting one drop (0·02 ml.) of bacterial suspension on the agar surface. After specified periods of incubation the amount of growth at the site of the drop inoculum was compared visually with that on a control medium appropriate to the experiment. The results obtained in this way agreed with results obtained in other experiments where the ability of a medium to support growth was ascertained by measuring the size of the well-isolated colonies, excluding any swarms, on streak plates.

Estimation of growth rate in liquid media. Samples of each medium (10 ml.) were placed in a conical flask (100 ml.) which had a side arm made from a test tube. Inoculum (0·5 ml.) was added and the flasks shaken at 110–120 cyc./min. in a water bath at 37°. At intervals of 30 min. flasks were removed for turbidity measurements and then quickly replaced. Measurements were made by tipping each flask to get the culture in the side arm which was then put in a nephelometer (Evans Electroselenium, Halstead, Essex). The instrument was adjusted at the start of each experiment to a sensitivity that gave a straight-line relationship over a wide range between turbidity and $\log_2$ dilution of a suspension of organisms. Generation times were obtained from readings within this range.

RESULTS

Survey of substances to determine their ability to support swarming

By using a modification of the auxanographic technique (Beyerinck, 1889, Pontecorvo, 1949), vitamin-free acid hydrolysed casein (Casamino acids, Difco), yeast extract (Difco), yeast nucleic acid (Koch–Lights) or a mixture of 10 water-soluble vitamins were tested for ability to support swarming. A small amount of one of these substances was put on a Proteus inoculum streaked across the centre of a plate of MM agar (Fig. 1) which was then incubated at 37°. Plates treated in this way were examined after 15 hr and 40 hr to see whether any swarming had occurred. Swarming occurred with Casamino acids and with yeast extract but not with the other substances. Examination of the swarms showed that they were similar to those produced on

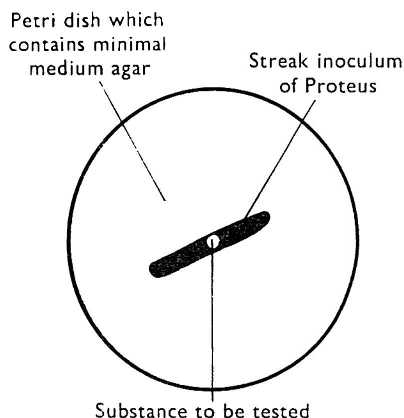

Fig. 1. Diagram to show the technique used to determine the ability of substances to support swarming.

nutrient agar; zones were present and the swarming edges consisted of long forms. Because the Casamino acids preparation was vitamin-free and because swarming did not occur with the mixture of 10 vitamins it was thought that swarming in the presence of Casamino acids or of yeast extract was due to the amino acid content of these preparations. A subsequent experiment showed that similar swarms occurred in the presence of a mixture of 22 amino acids (listed below).

A more detailed study was then made to determine whether the stimulation of swarming was due to any particular amino acid or group of amino acids. Aqueous solutions of single amino acids, adjusted to pH 7·2–7·8 with NaOH, were then tested at M/16 and M/128 of the L isomer by addition to MM agar before plates were prepared. When only DL-mixtures were available these were added to give the required concentration of L-isomer, since, although no other D-amino acids were tested, it had been shown that D-glutamic acid did not affect the ability of L-glutamic acid to support swarming. For each strain duplicate plates of each medium were inoculated by putting 1 drop (0·02 ml.) of inoculum on a disc of filter paper (diam. 5 mm.) which was at the centre of the plate. After 15 hr and 40 hr at 37° plates were examined and any swarms produced were measured along two radii from the edge of the filter paper to the edge of the swarm. No record was made of the number of zones per swarm. Swarms were produced on plates which contained L-glutamic acid, L-aspartic acid, DL-serine, L-proline, DL-α-alanine, L-asparagine or L-glutamine, but these were narrower than swarms on plates which contained all these 7 amino compounds together or Casamino acids. No swarming occurred on plates which contained any one of the following: glycine, DL-valine, DL-leucine, DL-isoleucine, DL-β-phenylalanine, L-tyrosine, DL-methionine, L-cysteine HCl, L-cystine, L-threonine, L-lysine HCl, L-histidine HCl, L-arginine HCl, L-hydroxyproline, DL-α-aminobutyric acid. However, swarms were produced when these latter amino acids, with the omission of α-aminobutyric acid (which inhibited growth) were incorporated together in MM agar at M/256 of each amino acid.

Ability of amino acids to serve as carbon + energy sources and/or nitrogen sources in agar media

The work described above showed that stimulation of swarming by the mixture of 22 amino acids was not due to any single amino acid or to a specific group of amino acids. It was thought that the amino acids must act in some non-specific way, so the possibility that there was a relationship between swarming and increased growth resulting from metabolism of added amino acids was considered. The various amino acids studied were examined for their ability to serve as carbon + energy sources and/or nitrogen sources when added to MM agar lacking sodium lactate and MM agar lacking NH_4Cl and $(NH_4)_2SO_4$, respectively. The results (Table 1) showed that only threonine and the 7 amino acids which supported swarming when added to complete MM agar acted as carbon + energy sources. Fifteen amino acids, including all those which served as carbon + energy sources, were each an effective source of nitrogen. Six amino acids supported slight growth, but no growth occurred with hydroxyproline. Swarming did not always occur on media which contained compounds that supported the largest amounts of growth but swarming sometimes occurred on media which did not support so much growth as did MM agar, on which swarming did not occur.

Growth rates and the ability of media to support swarming

The results of the previous growth experiments suggested that stimulation of swarming by amino acids was not associated with the total amount of bacteria produced. There was no correlation between the amount of growth at the site of inoculation and the production of swarms. Furthermore, when swarming did occur it did so before maximum growth had been reached at the inoculum site, i.e. at a time when the amount of growth was less than the maximum produced on media which did not support swarming.

Table 1. *Ability of* M/128 L-*isomer amino acids to serve as carbon and energy and/or nitrogen sources for Proteus mirabilis strains* NCDO, *1880 1881**

Compound added to appropriate basal medium	Amount of growth supported after 65 hr at 37° when compound was serving as:†			
	Carbon and energy source		Nitrogen source	
	Strain 1880	Strain 1881	Strain 1880	Strain 1881
DL-α-alanine	+++	+++	++++	++++
DL-serine	++	+++S	++++S	+++S
L-threonine	+	++	++++	++++
L-aspartic acid	+	+	++++S	+++S
L-glutamic acid	+++	+++S	+++S	++++S
L-proline	++++	++++S	++++	++++
L-asparagine	+	+	++++S	++++S
L-glutamine	+++	+++	++++S	++++S
Glycine	−	−	+++	+++
L-tyrosine	−	−	++	++
DL-tryptophane	−	−	++	++
L-cysteine HCl	−	−	++	++
L-cystine	−	−	++	++
DL-methionine	−	−	+++	+++
L-arginine HCl	−	−	+++	++
DL-valine	−	−	+	+
DL-leucine	−	−	+	+
DL-isoleucine	−	−	+	+
DL-β-phenylalanine	−	−	+	+
L-histidine HCl	−	−	+	+
L-lysine HCl	−	−	+	+
L-hydroxyproline	−	−	−	−
Controls				
sodium lactate	+++++	+++++	.	.
$NH_4Cl+(NH_4)_2SO_4$	.	.	+++	+++

* When tested as carbon and energy sources amino acids were added to MM agar from which sodium lactate was omitted. When tested as nitrogen sources they were added to MM agar from which NH_4Cl and $(NH_4)_2SO_4$ were omitted.

† Amount of growth, not including growth of any swarms, supported by amino compounds varied from ++++ (maximum growth) to − (same amount of growth as that on MM agar without sodium lactate or without NH_4Cl and $(NH_4)_2SO_4$ as appropriate).

Amount of growth supported when serving as carbon and energy sources cannot be compared with that supported when serving as nitrogen sources as in each case the amino compound supporting most growth was scored ++++ irrespective of the absolute amount of growth.

S, Swarming occurred.

The possibility that swarming was related to the rate of growth was investigated by using strain NCDO 1881. Because of the difficulties involved in measuring growth

Table 2. *Generation times of Proteus mirabilis strain* NCDO *1881 in various liquid media and occurrence of swarming on equivalent solid media*

Medium	Generation time (min.)	Occurrence of swarming on equivalent solid medium after 48 hr at 37°
Experiment 1		
MM liquid	108	−
MM liquid plus the following at M-16 L-isomer		
L-glutamic acid	66	+
L-proline	66	+
L-glutamine	72	+
Experiment 2		
MM liquid	120	−
MM liquid plus the following at M-128 L-isomer		
Mixture of DL-α-alanine, L-asparagine, L-proline, L-aspartic acid, L-glutamine, L-glutamic acid and DL-serine	57	+
Mixture minus glutamic acid	54	+
Mixture minus serine	60	+
Mixture minus glutamic acid and serine	54	+
Mixture minus alanine	54	+
Mixture minus asparagine	60	+
Glutamic acid	69	+
Serine	84	+
Experiment 3		
MM liquid	129	−
MM liquid plus the following at M-128 L-isomer		
DL-valine	126	−
DL-methionine	123	−
DL-isoleucine	117	−
L-lysine HCl	114	−
Glycine	108	−
Mixture of DL-α-alanine, L-asparagine, L-proline, L-aspartic acid, L-glutamine, L-glutamic acid and DL-serine	54	+
Experiment 4		
MM liquid	111	−
MM liquid casamino acids at (%, w/v)		
0·003	111	−
0·01	108	+i
0·03	96	+
0·09	81	+
0·27	60	+
Experiment 5		
Nutrient broth prepared without deliberately adding NaCl	68	−
Nutrient broth which contained 0·5% (w/v) added NaCl	44	+
Nutrient broth which contained 1% (w/v) added NaCl	42	+
Nutrient broth which contained 3% (w/v) added NaCl	60	−

+, Occurrence of swarming; −, absence of swarming; i, swarming was irregular and did not appear from every part of inoculum edge.

rates on solid media, growth rates in liquid equivalents of various agar media were measured. The assumption was made that the growth rates in aerated liquid media were comparable with those on the surface of corresponding agar media. The results from three experiments in which growth rates in MM liquid in the presence of various substances were compared with ability of these substances to support swarming on MM agar are given in Table 2 (Expts. 1-3). The growth rate in MM liquid which contained one or more substances known to support swarming was higher than the growth rate in MM liquid alone or in MM liquid which contained substances which did not support swarming. Another experiment (Table 2, Expt. 4) showed the effect of decreasing the concentration of Casamino acids, a preparation which supported swarming. The growth rate was decreased when the concentration of Casamino acids added to MM liquid was decreased from an amount which supported swarming on MM agar to an amount which did not.

Another system in which the relationship between growth rate and swarming could be studied was suggested by the work of Naylor (1960, 1964) and Sandys (1960) which showed that increasing or decreasing the concentration of NaCl in nutrient agar gave media which did not support swarming. We found that the growth rate in our nutrient broth (which contained 0·5 %, w/v, added NaCl) and in nutrient broth which contained 1 % (w/v) added NaCl was higher than in nutrient broth without any added NaCl and in nutrient broth which contained 3 % (w/v) added NaCl (Table 2, Expt. 5). These differences in growth rate could be correlated with the fact that swarms developed on nutrient agar which contained 0·5 % or 1 % (w/v) added NaCl but did not develop on nutrient agar which contained less or more NaCl.

Table 2 shows that there was not a specific value for growth rate which was exceeded in the liquid equivalents of all solid media which supported swarming. For example, nutrient broth without NaCl allowed a mean generation time of 68 min. and this medium when solidified with agar did not support swarming (Expt. 5): in contrast, MM liquid + 0·03 % (w/v) Casamino acids allowed a mean generation time of 96 min. and this medium when solidified with agar did support swarming (Expt. 4).

One other relationship was found between growth characteristics in liquid media and the development of swarms on comparable agar media. On various agar media swarms of different widths were produced although the corresponding liquid media supported similar growth rates. These differences in swarm widths were related to differences in the lengths of the lag phase in the various liquid media.

DISCUSSION

Despite suggestions that the swarming of Proteus is a response to a depletion of nutrients or to accumulation of toxic metabolic products there is little information on which to develop a satisfactory explanation for the phenomenon. The study of the phenomenon of swarming is difficult because swarming occurs intermittently and only on solid (agar, gelatin) media. Our studies and those of other workers (see Jones & Park, 1967) have shown that the occurrence of swarming of Proteus is always associated with the change from short forms to long forms and that long forms are not formed in liquid media (but see Kvittingen, 1949*a*).

Amino acid requirements for morphological changes of some micro-organisms have been reported. SentheShanmaganathan & Nickerson (1962) found that methio-

nine was required for the formation of the triangular form of *Trigonopsis variabilis*. Ensign & Wolfe (1964) found that lysine, asparagine, arginine or phenylalanine were required for the conversion of coccal forms to rod forms of *Arthrobacter crystallopoietes*. We found that swarming of Proteus, and therefore conversion from short to long forms, occurred when various amino acids were added to a minimal growth medium agar. The requirement did not appear to be for a particular amino acid and it seemed that the amino acids acted by stimulating growth rate. Naylor (1964) proposed a relationship between the total amount of cell material produced and the occurrence of swarming. He found that a medium which did not support swarming, i.e. nutrient agar prepared without deliberate addition of NaCl, did do so when other electrolytes or certain carbohydrates were added. He showed by growth studies on equivalent liquid media that addition of equi-osmolar concentrations of NaCl or dulcitol, additions which supported swarming on agar media, promoted greater amounts of growth of the strain of *Proteus vulgaris* used than did addition of an equi-osmolar concentration of glucose, which did not support swarming. An examination of Naylor's results reveals that besides increased total amounts of cell material produced, faster growth rates occurred when NaCl or dulcitol was added than when glucose was added; so it appears from these and our results that the important effect of added compounds is on the growth rate.

The correlation we found between growth rate and the occurrence of swarming of Proteus may provide an explanation for the change in the relationship of the syntheses of various cell components which leads to the formation of long forms. Sud & Schaechter (1964) showed that in media which supported high growth rate the amount of cell wall and cell membrane of *Bacillus megaterium* produced per unit of bacterial dry weight was less than when this organism was grown on media which supported lower growth rates. If a similar pattern occurs with Proteus it is conceivable that at high growth rates synthesis of these components decreases to such a degree that on agar media cell division does not keep pace with other processes and long forms are produced which can move over the surface. However, it is not yet clear how increases in growth rate bring about these changes. Perhaps substances required for cell division and for other growth processes, e.g. magnesium and potassium ions (Webb, 1949, 1953; Shankar & Bard, 1952) or amino acids, become limiting by insufficient rate of entry or of synthesis during the stage of rapid growth and are preferentially used for processes other than cell division. Or perhaps volatile toxic products like those postulated by Lominski & Lendrum (1947) and Hughes (1957) to explain swarming of Proteus may be produced more rapidly at high growth rates and so reach effective local concentrations only under these conditions. Volatile products of this kind were not detected by Jones (1966) and so this possibility was not investigated.

No threshold value can be given for growth rate beyond which swarms will occur, because growth rates in liquid media equivalent to nutrient agar which had been modified to inhibit swarming were higher than in minimal growth liquid media containing compounds which stimulated swarming. However, if swarming does result from a change in balance of synthesis of some cell components brought about by changes in growth rate the change in balance would be unlikely to occur at the same growth rate on media of widely different composition; the existing balance between synthesis of various components would be expected to be markedly different.

There remains to be considered the effect on swarming of changes in temperature. Since changes in temperature cause changes in growth rate it might be expected that the occurrence of swarming would be readily affected by changes in temperature. This is not so (Kvittingen, 1949*a*); media which support swarming do so over a wide temperature range. Some findings of Schaechter, Maaløe & Kjeldgaard (1958) may help to explain this apparent discrepancy. These workers showed that in a given medium the bacterial size and the composition (average mass, RNA, DNA, number of nuclei per organism) of *Salmonella typhimurium* were almost independent of temperature; that is, almost independent of changes in growth rate brought about by changes in temperature. Only changes in growth rate brought about by growth on different media resulted in changes in bacterial size and composition.

We wish to thank Mr N. G. J. Gruber for taking the photograph.

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EXPLANATION OF PLATE

Photograph of a swarm of *Proteus mirabilis* NCDO 1881 on a nutrient agar 9 cm. plate. The plate was inoculated at the centre with 1 drop of a suspension of organism and was incubated for 20 hr at 37°. × 1·3.

H. E. JONES AND R. W. A. PARK (*Facing p.* 378)

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Effect of Isoniazid on Biosynthesis in *Mycobacterium tuberculosis* var. *bovis* BCG

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SUMMARY

Radioactive incorporation techniques have been employed to investigate the effect of isonicotinic acid hydrazide (INH) on biosynthetic reactions in *Mycobacterium tuberculosis* var. *bovis* BCG. INH has no measurable effect on incorporation of ^{32}P phosphate, ^{35}S sulphate or ^{14}C glycine into whole cells, although it inhibits by 50% the incorporation of ^{14}C acetate. Incorporation of ^{32}P into trichloracetic acid (TCA) insoluble components of supernatant solutions from centrifuged disrupted bacteria appears to be inhibited soon after the cells are exposed to INH. Similar experiments with ^{35}S show a lag of 30–40 hr before incorporation is influenced by the drug. Chemical fractionation of organisms grown in the presence of ^{32}P show that the major effect of INH is exerted on incorporation into the ribonucleic acid fraction.

INTRODUCTION

In the fourteen years since isoniazid (INH) was first described (Fox, 1952) much research has been carried out to discover its mode of action and specificity, but the answer to these questions remains unclear. It is known that INH begins to be bactericidal to mycobacteria after a lag of one to two generation times (Middlebrook, 1952; Barclay, Ebert & Koch-Weser, 1953). In parallel with the decline in viability there was a diminution in acid-fast staining and neotetrazolium reducing activity (Koch-Weser, Barclay & Ebert, 1955). Schaefer (1954) demonstrated that INH is only active against tubercle bacilli when they were actively dividing and metabolizing in the presence of an energy source. These findings suggest that INH inhibits some aspect of biosynthetic metabolism and that the expression of this lesion is delayed. For example, the drug might inhibit the formation of an essential metabolite enough of which remains to permit growth for about one more generation. A number of enzyme systems have been shown to be affected by INH, among them those requiring pyridoxal phosphate as a co-factor, some involved in nicotinamide adenine dinucleotide metabolism, some amine oxidase enzymes, and catalase and peroxidase from various sources. However, it cannot yet be said that the antibiotic action of INH is definitely related to any of these systems. Many of the proposed sites of action have been reviewed by Krüger-Thiemer (1958). As INH is only active in growing bacteria it seemed that a study of the effect of this drug on biosynthetic reactions might throw light on the mode of action of INH.

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METHODS

Organism and media. The Copenhagen strain of *Mycobacterium tuberculosis* var. *bovis* BCG, sensitive to 0·05–0·1 μg. INH/ml. was obtained from Glaxo Laboratories Ltd., Greenford, Middlesex, through the kindness of Dr J. P. Ungar.

The strain was maintained by monthly subcultivation on Lowenstein-Jensen slopes. For use it was subcultured three times at intervals of 8 days in Dubos fluid medium (Dubos & Davis, 1946) containing 0·3% bovine albumen and incubated at 37°. Synthetic AFMR medium (Aldridge, Felton, Muchmore & Ramsay, 1959) containing glucose, ammonium chloride, salts and Tween 80, was dispensed in 100 ml. quantities in Roux bottles which were inoculated with 1·0 ml. of the third Dubos culture and incubated horizontally at 37° for 14–21 days.

Radioactive tracer techniques

The following isotopes were obtained from the Radiochemical Centre Amersham, Bucks: ^{35}S as sulphate, ^{32}P as orthophosphate, acetate-1-^{14}C (2 mc/mmole) and glycine-^{14}C (U) (2 mc/mmole). Diluted solutions of all isotopes in distilled water were sterilized by autoclaving at 120° for 10 min. Samples containing ^{14}C and ^{35}S were counted on nickel planchettes with an end-window Geiger tube, either at infinite thinness or corrected for self-absorption against standard self-absorption curves. ^{32}P was estimated in the same way or in a liquid counting tube containing 10 ml. solution.

Incorporation into whole bacteria. For experiments on incorporation of ^{14}C glycine, ^{14}C acetate and ^{35}S into whole bacteria, three 18-day cultures in AFMR medium received sterile INH to a final concentration of 10 μg./ml. and three control cultures were treated with a similar quantity of sterile distilled water. Each group of six then received either ^{14}C glycine (5 μc), ^{14}C acetate (0·45 μc) or ^{35}S sulphate (10 μc). At intervals during further incubation at 37°, 6 ml. samples from 2 of every test and 2 of every control culture were withdrawn aseptically. The organisms in each sample were washed, by centrifugation, in, successively, 0·1% solution of Triton WR 1339 (a dispersing agent) in 0·1 M-phosphate buffer (pH 6·8), Triton-phosphate buffer containing 0·1 M-non-radioactive acetate, glycine or sulphate and, finally, in Triton-phosphate buffer; their specific activities were then determined.

Incorporation of ^{32}P orthophosphate was followed in a similar fashion in 18-day cultures of BCG grown on AFMR medium. To each of 14 bottles was added 10 μc of ^{32}P orthophosphate. Half of the bottles also received sterile INH to 10 μg./ml. final concentration. One test and one control culture were harvested at various times by centrifugation. The organisms were washed successively in 0·1% Triton distilled water, 0·1% Triton in 0·1 M-phosphate buffer (pH 6·8), and finally 0·02% Triton distilled water before their radioactivity was assayed.

Incorporation into trichloracetic acid (TCA) insoluble compounds. Each 18-day culture on AFMR medium was given 10 μc ^{32}P orthophosphate solution and 10 μc of ^{35}S sulphate solution and isoniazid was added to half of the bottles. The organisms were harvested by centrifugation and were washed by centrifugation in, successively, Triton-water and Triton-0·1 M-phosphate buffer (pH 6·8) containing non-radioactive sulphate. The organisms were finally suspended in 25 ml. 0·02% Triton water and broken in the extrusion press (Milner, Lawrence & French, 1950). The broken sus-

pension was centrifuged at 30,000 *g* for 20 min. in an MSE-'17' refrigerated centrifuge and measured volumes of the opalescent supernatant were treated with an equal volume of chilled 10 % (w/v) TCA and held at 4° for 30 min. The precipitate was collected by centrifugation and washed in, successively, 5 % TCA containing 1 % non-radioactive phosphate and sulphate, 5 % TCA and 1 % acetic acid. The washed pellets were transferred to weighed nickel planchettes, dried, reweighed and counted with and without an aluminium screen to allow separate estimates of ^{32}P and ^{35}S.

Incorporation into various cell fractions. To obtain information on the effect of INH on incorporation of radioactive phosphate into various cell fractions, broken organisms were fractionated by the method of Winder & Denneny (1957) or by that of Schmidt & Thannhauser (1945). Using the Winder & Denneny fractionation scheme, organisms were grown, harvested, washed and broken as for experiments measuring incorporation into TCA insoluble components.

In experiments using the Schmidt–Thannhauser procedure, after normal growth on AFMR medium for 18 days, the organisms were harvested and resuspended aseptically in phosphate-free medium (pH 7·0). Each culture received 2 μg./ml. INH or sterile water in the first experiment. Each culture in the second experiment was treated with either 20 μg./ml. streptomycin (SM) or 1 μg./ml. INH or sterile water. There was no significant change in pH during the course of each experiment in any culture vessel. Organisms from each experiment were tested for bacterial contamination and were harvested, washed and broken as described above.

Analytical methods. Total phosphorus was measured after digesting the sample in perchloric acid by the technique of Strickland, Thompson & Webster (1956). Free phosphate was measured directly on material containing 0·1–1·0 μM-phosphorus by the method of Fiske & SubbaRow (1925). Acid labile phosphorus was measured similarly after hydrolysis of the sample in N-HCl for seven minutes at 100°.

Chromatography. Two-dimensional chromatography on products of nucleic acid hydrolysis was performed either in the solvent system described by Roberts *et al.* (1955) or in the system devised by Dorough & Seaton (1954). Roberts *et al.* used 70 % tertiary butanol in 0·8 N-HCl in the first direction and secondary butanol + water + formic acid (70 + 20 + 10) in the second direction. Radio-autographs were prepared on Ilford X-ray paper and contact prints were made of the chromatograms on Kodak reflex document paper, using an ultraviolet lamp with a Chance OX 7 filter.

The spots, located as described above, were cut from the paper and extracted with a small quantity of 0·1 N-HCl. The radioactivity of these solutions was estimated by making up to 10 ml. and counting in a liquid Geiger-counter tube. A paper blank from each chromatogram was treated in the same way. The absorbancy of the eluates at 250, 260, and 280 mμ was measured spectrophotometrically. The specific activity of each extract was expressed as counts/min./O.D. unit at 260 mμ or in terms of the O.D. at 260 mμ of the guanine spot on each chromatogram. The ratios of absorbancy at 250–260 mμ and 280–260 mμ, together with the location of the spot on the paper, were used to assist in establishing the identity of each spot.

The solvents employed by Dorough & Seaton (1954) were *n*-butanol – acetic acid + water (8 + 8 + 2) and acetone + *n*-butanol + water (8 + 1 + 1) in the first direction followed by isopropanol + ammonium sulphate + water (79 + 2 + 19) in the second direction. The chromatograms were run on Whatman no. 1 paper. Spots were located by ultraviolet photography. Quantitative estimations were similar to those described

above. The activity was eluted from blank and test spot by macerating the paper in 0·1 N-HCl and removing the paper fibres by centrifugation. These were washed and the supernatants combined and made up to 10 ml. for counting and measurement of ultraviolet absorbance. Blank deductions from each spot were corrected for the size of the spot using the weight of the paper as criteria of size. Specific activities were determined as described above.

RESULTS

Incorporation into whole cells

Incorporation of ^{35}S sulphate, ^{32}P orthophosphate and ^{14}C glycine was not affected significantly by 10 μg./ml. INH during 100 hr exposure in the sulphate and glycine experiments, and 50 hr exposure in the orthophosphate experiment. However, ^{14}C acetate incorporation was inhibited to about 50% of the control level by the same concentration of INH (Fig. 1). It is difficult to interpret the inhibition of acetate due to INH as it appeared long before growth of the organism was itself inhibited. It seems possible that this inhibition is not related to the bactericidal action of INH, since acetate is not needed for growth.

Incorporation into TCA insoluble cytoplasmic components

The effect of INH on the incorporation of ^{32}P orthophosphate and ^{35}S sulphate into TCA insoluble components of the cell-free fraction of disrupted BCG was next examined. The TCA precipitate contained the cytoplasmic protein and much of the nucleic acid components. Incorporation of ^{35}S sulphate and ^{32}P orthophosphate into these components of BCG was measured in preliminary experiments and it appeared that INH significantly inhibited ^{32}P incorporation immediately and inhibited ^{35}S sulphate incorporation after a lag period of 30–40 hr. This was investigated using both tracers in the same experiment; 18-day cultures of BCG were employed. Each vessel received either 10 μg./ml. INH or distilled water. In addition, 10 μc ^{32}P orthophosphate and 10 μc ^{35}S sulphate were added. A test and control culture were harvested at various times up to 140 hr. Since there was a certain amount of scatter in results from vessel to vessel, the results have been smoothed by plotting a moving average (Fig. 2). While incorporation of ^{35}S sulphate was hardly affected before 30–40 hr in the presence of INH, the drug appears to inhibit ^{32}P orthophosphate incorporation considerably as soon as the organism was exposed to INH. If ^{35}S incorporation is a measure of protein synthesis and ^{32}P incorporation is a measure of nucleic acid synthesis, it is possible that INH inhibits some aspect of nucleic acid synthesis soon after the organism is exposed to the drug and that this inhibition is followed by a cessation of protein synthesis after 30–40 hr. However, the fractionation procedure does not rule out the possibility of incorporation of either tracer into other TCA precipitable components which might be present in the supernatant; other fractionation procedures were therefore tested.

Fractionation by the Winder & Denneny method

The affect of INH on ^{32}P incorporation was further studied. After incubation for 40 hr with INH and ^{32}P phosphate, organisms were broken in the French press and subsequently fractionated as described by Winder & Denneny (1957). This fractionation procedure consists in extracting phospholipids with ethanol and ether + ethanol, followed by selective hydrolysis of ribonucleic acid (RNA), desoxyribonucleic acid

(DNA) and polyphosphate with 5 % TCA at room temperature. The specific activity as counts/min./μg. P was determined in duplicate for each fraction. The results are set out in histogram form (Fig. 3). It is evident that INH does not significantly inhibit incorporation of ^{32}P into phospholipids, phosphoprotein or inorganic polyphosphate.

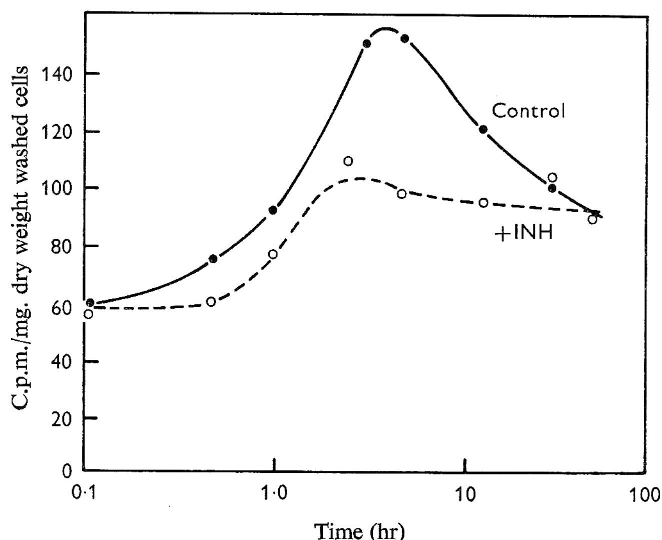

Fig. 1. The effect of INH on ^{14}C acetate incorporation into INH-sensitive BCG. Test and control cultures received 0·45 μc ^{14}C acetate at time zero. At the same time test cultures were treated with 10 μg./ml. INH.

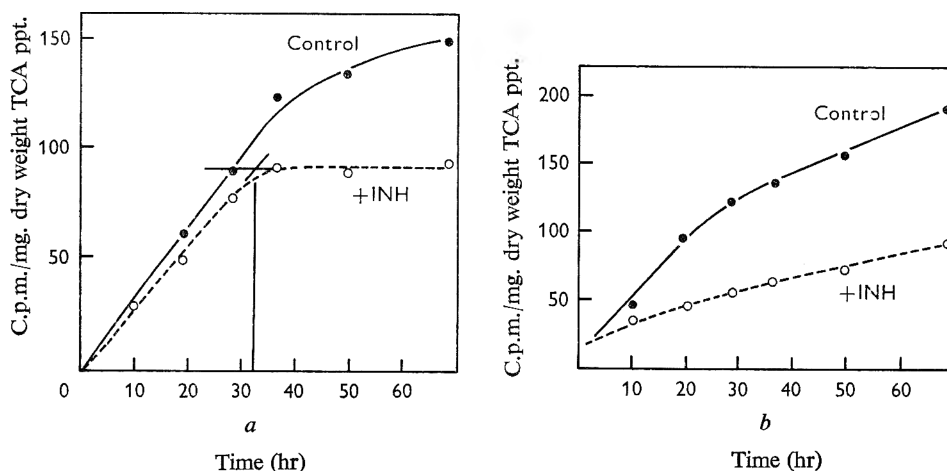

Fig. 2. The effect of INH on ^{32}P and ^{35}S incorporation into TCA precipitable cytoplasmic components of INH-sensitive BCG. At time zero all cultures received 10 μc ^{35}S sulphate and 10 μc ^{32}P orthophosphate. In addition test cultures were exposed to 10 μg./ml. INH. The data have been plotted as a moving average. (*a*) Incorporation of ^{35}S. (*b*) Incorporation of ^{32}P.

There is no detectable effect on fractions R_1 and R_2 which contain RNA, polyphosphate, and DNA purines. Any effect on nucleic acid synthesis in these fractions will however be obscured by the large excess of polyphosphate in each of these fractions

whose biosynthesis is not affected. There appears to be some reduction in specific activity in the TCA soluble component and in fraction D containing DNA or (mainly) apurinic acid and polyphosphate, but it proved impossible to determine the specific activity of nucleic acid components.

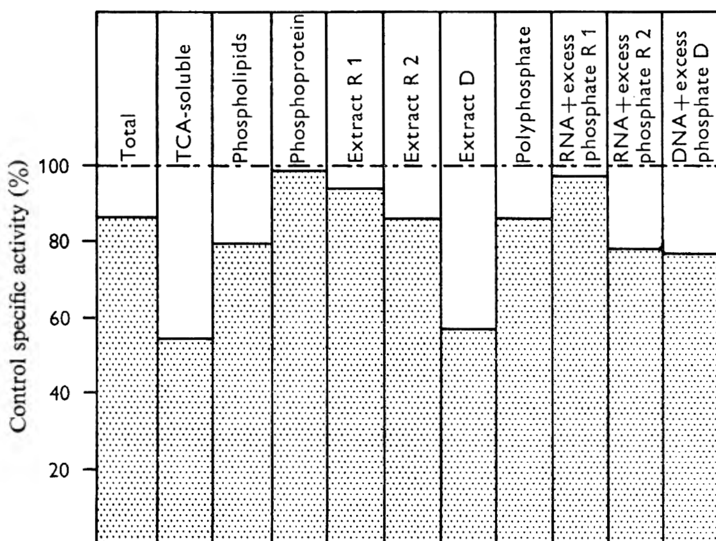

Fig. 3. The effect of INH on the specific activity of ^{32}P-phosphorus-containing fractions. Test and control cultures received 10 μc sterile ^{32}P orthophosphate. Test cultures were treated with 1·0 μg./ml. INH. After incubating at 37° for 40 hr, cells were fractionated according to the method of Winder & Denneny (1957). Fractions referred to in the histogram are those described by the above authors.

Fractionation by the Schmidt–Thannhauser method

INH-sensitive BCG was grown on AFMR medium for 16 days as usual, harvested under sterile conditions and resuspended in 100 ml. of phosphate-free AFMR medium at pH 7·0, so that each suspension contained the growth from 1½ Roux-bottle cultures. To each bottle was added 100 μc ^{32}P orthophosphate. Test cultures received sterile INH to 2 μg./ml. or an equivalent volume of sterile distilled water. All bottles were incubated at 37° for 68 hr. Each culture was checked for bacterial contamination and pH before harvesting. There was no detectable change in the pH of each culture.

^{32}P uptake was therefore studied in cells which were then washed, broken and treated according to Schmidt & Thannhauser (1945). In the first experiment chromatographic analysis of the nucleic acid derivatives was carried out using the solvent system of Roberts *et al.* (1955). The results are presented as a histogram (Fig. 4). It can be seen that while INH had very little effect on ^{32}P incorporation into either whole cells, acid soluble material, phosphoprotein or phospholipid it had a profound effect on the RNA fraction. Three ribonucleotides, guanylic acid, uridylic acid and cytidylic acid were tentatively identified by their ultraviolet absorption characteristics and by their position on each chromatogram. The specific activity of each of these ribonucleotides in organisms exposed to 2 μg. INH/ml. for 68 hr was 25% of the control activity, the greatest inhibition attributable to INH so far reported. It proved difficult

to obtain much DNA material and the products of acid hydrolysis of this fraction could not be identified. Although there is no agreement between the specific activity of each of the three spots (labelled X, Y and Z) isolated from the hydrolysed DNA fraction, their activities are all lower than the whole cell specific activity. It is probable that incorporation of ^{32}P into DNA is inhibited although it is impossible to decide in which order (if any) nucleic acid synthesis is affected by INH.

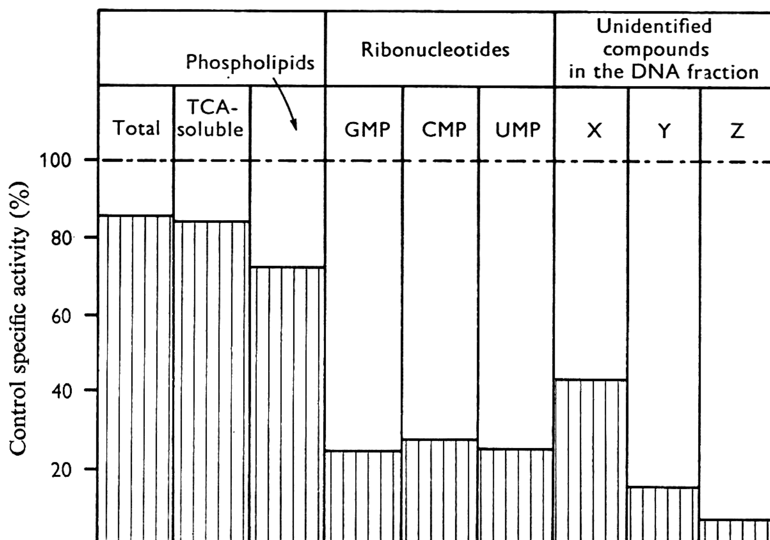

Fig. 4. The effect of INH on the specific activity of ^{32}P-phosphorus-containing fractions. Sixteen-day-old cells were washed and suspended in a phosphate-free medium. Test and control cultures received 100 μc ^{32}P orthophosphate. Test cultures were treated with 2 $\mu g./ml.$ INH. After 68 hr at 37° cells were fractionated according to the method of Schmidt & Thannhauser (1945).

This experiment was repeated and the effect of streptomycin (SM) contrasted with the effect of INH. It is known that while streptomycin exercises an immediate growth-inhibitory effect, INH acts only slowly after a lag period of 1–2 generation times (Koch-Weser *et al.* 1955). Organisms were fractionated as already described except that chromatograms of nucleic acid derivatives were run in the Dorough & Seaton (1954) solvent system. The results (Fig. 5) show that streptomycin inhibited incorporation of ^{32}P into all fractions tested. Incorporation into ribonucleotides was more reduced than in other fractions. However, in contrast to the effect of streptomycin, INH affected only the ribonucleotide fraction. In this experiment the specific activities in the control set appear to be low, but the relative order of inhibition in the treated sets is not affected.

DISCUSSION

The first experiments on the effects of INH on the incorporation of ^{14}C glycine, ^{14}C acetate, ^{35}S sulphate and ^{32}P phosphate into washed whole BCG organisms showed that, with the exception of incorporation of ^{14}C acetate, there was no significant change in the specific activity of INH-treated and control organisms. The three metabolites

glycine, sulphate and phosphate between them provide an indicator for the synthesis of most chemical fractions in mycobacteria. Hence it is probable that the site of action of INH is directed to a specific metabolic reaction, inhibition of which will have no immediate effect on overall biosynthesis. These results confirm the findings of several groups of workers on the effect of INH on the growth of mycobacteria. For example, it has been shown that growth of mycobacteria is inhibited only after a latent period of 1–2 generation times (Barclay *et al.* 1953; Schaefer, 1954). Mackaness & Smith (1953) have also shown that INH inhibits a specific biosynthetic reaction and that the expression of this inhibition occurs after a considerable delay.

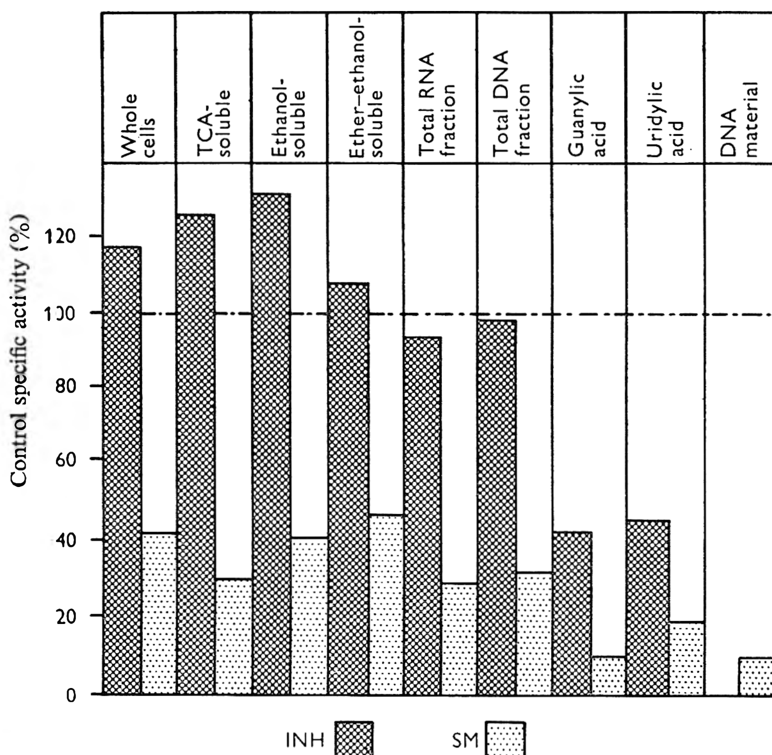

Fig. 5. The effect of INH and streptomycin (SM) on the specific activity of ^{32}P-phosphorus-containing fractions. Cells were treated as described for Fig. 4 with the exception that test cultures received either 20 μg./ml. SM or 1·0 μg./ml. INH.

With ^{14}C acetate the effect of INH was considerable. It was observed that during the first few hours of acetate fixation INH inhibits uptake by about 50 %. These results confirm the work of Meadow (1956) and Meadow & Knox (1956) who demonstrated that oxidation of acetate by washed cells of *Mycobacterium smegmatis* and BCG was inhibited by INH to 50 % of the control level. They concluded that an oxidation reaction within the mycobacterial cell was inhibited by the drug. This inhibition appeared to take place at widely differing INH concentrations although the amount of inhibition was always in the region of 50 %. The easiest interpretation of the data of these workers in the light of results with radioactive acetate is that INH inhibits not the oxidation but the uptake of the substrate. However, it is not easy to establish

the relationship of the effect to the antibiotic action of INH, as this inhibition appears to occur at once in the presence of INH and acetate is not necessary for the growth of these organisms.

Incorporation of ^{35}S sulphate into TCA-insoluble cytoplasmic components of BCG was not affected by INH over the first 30–40 hr; it then ceased completely. On the other hand, incorporation of ^{32}P orthophosphate into the same material was very greatly inhibited by INH as soon as the organisms were exposed to the drug. One explanation of these results seems to be that the delayed inhibition with ^{35}S sulphate is a consequence of the effect observed with ^{32}P phosphate. If the inhibited portion of the phosphate-containing TCA-insoluble fraction is nucleic acid and if the sulphate-containing component of the same fraction is protein it appears probable that INH inhibits some aspect of nucleic acid synthesis with the result that after a day protein synthesis ceases.

It was important to decide which phosphate compound was inhibited. The Winder & Denneny fractionations showed that INH had no significant effect on phosphate incorporation into several components, including polyphosphate, phospholipid and residual phosphoprotein. This procedure did not show the effect of INH on nucleic acid synthesis. The Schmidt–Thannhauser fractionation showed that INH had little effect on the specific activity of ^{32}P in phospholipids, TCA-soluble components and whole cells. However, a great reduction was noted with ribonucleotide and the products of hydrolysis of DNA.

A similar experiment using INH and streptomycin as a second control showed that in contrast to the action of INH, streptomycin inhibited biosynthesis of every component to 30–40 % of the control level although nucleic acid synthesis was affected even more. These findings are consistent with the results obtained by Barclay, Ebert & Koch-Weser (1953) and Mackaness & Smith (1953), who showed that INH inhibits growth of tubercle bacilli after a latent period corresponding to 1–2 generation times, whereas the bactericidal effect of streptomycin is immediate.

It is reasonable to conclude that INH first blocks some biosynthetic reaction which results in a cessation of nucleic acid synthesis. It is, of course, impossible to say that nucleic acid synthesis is the direct site of action of INH, although it is the earliest detectable effect of INH on the biosynthetic mechanisms in BCG reported so far. Following the blockage in nucleic acid synthesis, protein synthesis is inhibited, although this manifests itself after a lag period of 30–35 hr, which is similar to the delay in growth inhibition reported by the workers mentioned above. Finally, as a result of the cessation of protein synthesis, the synthesis of other cellular components is slowly affected as the concentration of most biosynthetic enzymes is reduced, either because they are diluted out or because they are broken down in the cell.

The results reported in this paper constitute part of a thesis presented to the University of London in 1962. Since this time the observation that INH inhibits some aspect of nucleic acid synthesis has been ably confirmed by Tsukamura & Mizuno (1962) and Gangadharam, Harold & Schaefer (1963) who used different analytical methods.

It is a pleasure to thank Professor R. Knox for his helpful suggestions and encouragement. This work was supported by a research grant from Guy's Hospital Endowment fund.

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The uptake and fate of Isoniazid in *Mycobacterium tuberculosis* var. *bovis* BCG

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SUMMARY

The incorporation of ^{14}C INH into sensitive and resistant bacteria has been investigated. Only organisms susceptible to INH took up the drug. Uptake was irreversible and became saturated at higher INH concentrations. Uptake was heat-labile, KCN-sensitive, partially inhibited by dinitrophenol and stimulated by SH group reagents. The activation energy of binding was of the same order as the activation energy of an enzyme catalysed reaction. Uptake at 37° was rapidly inhibited although at 4° it was not. The INH binding mechanism had many of the properties of catalase and peroxidase. It seems likely that all these functions were part of one enzyme which was missing from INH-resistant BCG.

Chromatographic studies on hot-water extracts of INH-treated BCG indicated that INH was converted into several unidentified products within the organism.

INTRODUCTION

The effect of isoniazid (INH) on the biosynthesis of a number of components of BCG has been examined in an earlier paper (Wimpenny, 1967). However, in attempting to elucidate both the mode of action and the specificity of INH as an anti-tubercular agent, the fate of the drug itself in the sensitive organism must be traced. Several papers have been published which throw some light on this aspect of the problem. Barclay, Ebert & Koch-Weser (1953) showed that *Mycobacterium tuberculosis* var. *hominis* strain H 37 RV sensitive to INH took up the drug, whilst a resistant variant did not. In later experiments (Barclay, Koch-Weser & Ebert, 1954) using the same organism, they investigated the uptake of ^{14}C isonicotinic acid, ^{14}C nicotinamide and ^{14}C nicotinic acid. Of these compounds, ^{14}C INH was bound and then only by the drug-sensitive strain. Organisms killed by heat or with formalin did not fix INH. These workers indicated that uptake of INH was greater at 5° than at 37° in living bacteria and concluded that INH was bound by physical adsorption. Youatt (1958*a*) confirmed that ^{14}C INH was bound only by sensitive mycobacteria. She showed that the drug was fixed only under actively metabolizing conditions and that uptake in sensitive mycobacteria was sensitive to cyanide, azide and heat. She found that *Staphylococcus aureus*, *Escherichia coli*, *Mycobacterium phlei*, *Proteus mirabilis*, *Bacillus megatherium* and *Candida albicans* took up very little INH. In addition Youatt showed (1958*b*) that the rate of breakdown of INH was greater in living BCG than in heat killed organisms.

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In contrast to the findings of Barclay *et al.* (1954) referred to above, Youatt found that ^{14}C isonicotinic acid was taken up equally well by sensitive or resistant BCG. She also observed that INH causes the accumulation of a yellow compound in the culture medium from sensitive cells treated with the drug. She tentatively identified this compound as a flavine and suggested that this might reflect a lesion in flavine synthesis.

Boone, Strang & Rogers (1957), at variance with the findings of Barclay and his co-workers, and with Youatt, have shown that INH-sensitive and INH-resistant *Mycobacterium tuberculosis* strain H37RV take up similar quantities of ^{14}C INH. Pyridoxal stimulates uptake of the drug in both strains of organism a hundred-fold.

This work was planned to investigate the uptake and fate of INH in sensitive and resistant bacteria in greater detail.

METHODS

Organisms and culture. Mycobacterium tuberculosis var. *bovis* BCG, both INH-sensitive and INH-resistant strains, were obtained from Dr J. P. Ungar of Glaxo Laboratories Ltd., Greenford, Middlesex, and were inhibited by 0·1 and by 10 μg./ml. INH respectively. These organisms were maintained and cultured as described by Wimpenny (1967). *Escherichia coli* NCTC 8164, *Staphylococcus aureus* strain Oxford NCTC 6571 and *Bacillus cereus* NRRL 569 were maintained on Dorset egg slopes and subcultured twice in digest broth before use. *Corynebacterium xerosis* (Guy's Hospital laboratory strain) was stored on blood agar and subcultured twice on digest broth before use.

The last four organisms were resistant to more than 1000 μg./ml. INH. For use these organisms were grown in 100 ml. quantities of digest broth in Roux bottles. They were incubated flat at 37° overnight and used.

Radioactive-tracer techniques. The following ^{14}C-labelled compounds were obtained from the Radiochemical Centre, Amersham, Bucks, England: D-glucose ^{14}C (U) 3 mC/mmole; adenine-8-^{14}C sulphate, 1 mC/mmole; glycine-^{14}C (U), 2 mC/mmole; isonicotinic acid hydrazide (carboxyl ^{14}C) 2 mC/mmole.

Aqueous solutions of these isotopes were sterilized by autoclaving at 10 p.s.i. for 10 min.

Samples were plated on to 1 cm. nickel planchettes and dried under an infra-red lamp. An end-window Geiger counting tube and Dynatron counting gear were used throughout. All experiments were performed in duplicate.

Incorporation experiments. INH-sensitive and INH-resistant BCG were grown in Aldridge, Felton, Muchmore & Ramsay (1959), AFMR, medium for 14–21 days. Measured samples of each culture were pipetted aseptically into sterile test-tubes. Each tube also received sterile ^{14}C carbonyl-labelled INH. Other additions of sterile distilled water were used to bring the final volume in each tube to 5·0 ml. The final concentration of INH was 0·36 μg./ml., which is equivalent to 0·025 μC/ml. isotope. Tubes were incubated as described in each experiment. The cells were then harvested by centrifugation. Cells were washed once in AFMR medium without glucose, then in AFMR medium containing 10 mg./ml. ^{12}C INH and finally in glucose-free AFMR medium. Experiments with other organisms were performed in an identical manner—except that they were grown in digest broth overnight and they were washed after harvesting at the end of the experiment in normal saline. They were washed again in

normal saline containing 10 mg./ml. ^{12}C INH. The organisms were finally washed in sterile distilled water, before counting.

Chromatography. Concentrated hot-water extracts of cells pretreated with ^{14}C INH were chromatographed on Whatman no. 1 paper in either 4+1 isopropanol+water or 5+1+4 butanol+acetic acid+water. Chromatograms were run overnight, dried and spots detected by ultraviolet photography and radio-autography.

RESULTS

Fixation of INH by various species of bacteria

The only organism to take up ^{14}C INH to any extent was the INH-sensitive strain of BCG. Resistant BCG took up about 5 % of the quantity fixed by the sensitive cells. The other bacteria took up a negligible amount of INH (Table 1).

Sensitivity to INH appears to be closely related to the ability of the organism to fix the drug.

Table 1. *The uptake of labelled INH by different species of bacteria*

Duplicate tubes received 0·36 μg. ^{14}C INH/ml. and were incubated for 24 hr at 37°.

	C.p.m./mg. dry cells
Mycobacterium tuberculosis var. *bovis* BCG	
Sensitive to INH	135
Resistant to INH	6·6
Escherichia coli	2·2
Staphylococcus aureus strain OXFORD	1·2
Bacillus cereus	0·4
Proteus vulgaris	0·8
Corynebacterium xerosis	1·6

The relationship between concentration of INH and uptake in sensitive and resistant NCG

These results have been expressed graphically and, in addition, theoretical curves of INH uptake have been plotted (Fig. 1). These were calculated on the assumption that the ratio of observed to calculated uptake was 1·0 at the maximum concentration tested, and that uptake was directly proportional to the amount of INH available at lower concentrations on all the other points on the graph. Theoretical curves were plotted for both resistant and sensitive organisms, so that a comparison could be made with the observed values.

Resistant organisms show no significant difference between the observed and the theoretical results. In sensitive organisms, however, the uptake of INH was greater at all concentrations lower than the maximum. Moreover, this difference increases as the concentration is decreased. In order to demonstrate this more clearly, the ratios of observed to calculated uptake are plotted in Fig. 2. At concentrations where it could be measured the observed-to-calculated ratio for INH uptake by resistant cells was approximately 1·0. However, this ratio rose to about 4·0 at low INH concentrations in sensitive cells. This demonstrated a qualitative difference in uptake by sensitive and resistant BCG and seemed to indicate that INH was concentrated and fixed in the sensitive cells by an active process.

INH fixation by sensitive and resistant BCG. Attempts were made to decide whether the mechanism of INH by sensitive cells was an enzymic or a physical process. If INH fixation was reversible in the presence of large amounts of non-radioactive INH, this would lend weight to the adsorption theory. If, however, INH was metabolized by some enzyme, it would no longer be able to exchange with free drug and it would be fixed within the cell.

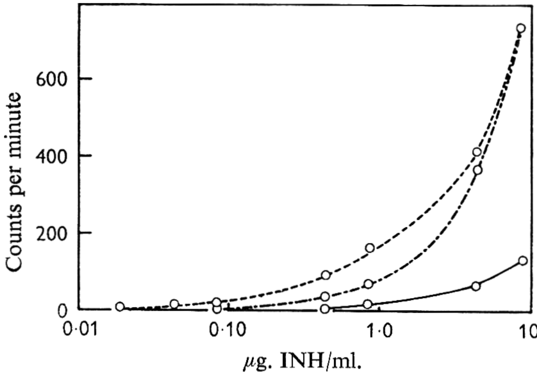


Fig. 1

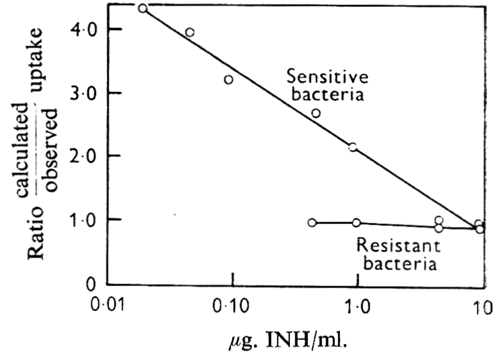


Fig. 2

Fig. 1. The effect of varying ^{14}C INH concentration on INH uptake by resistant and sensitive strains of BCG. ...... Observed uptake by sensitive bacteria - · - calculated uptake by sensitive bacteria; —, observed and calculated uptake by resistant bacteria.

Fig. 2. The ratio of observed to calculated INH uptake in INH-sensitive and INH-resistant BCG as a function of ^{14}C INH concentration.

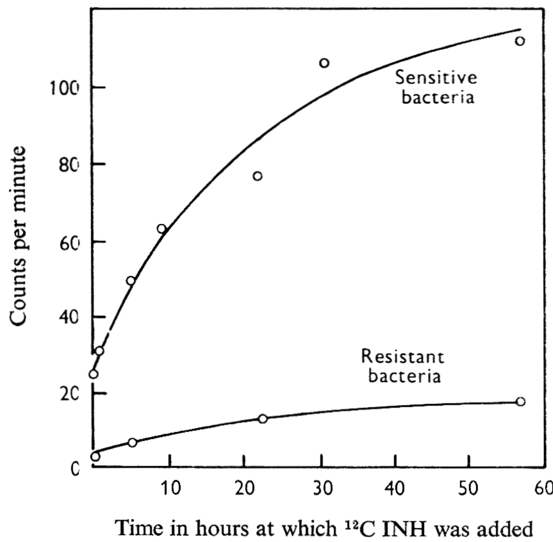


Fig. 3. ^{14}C INH fixation by sensitive and resistant strains of BCG. Cells of each strain were incubated with 0·36 µg./ml. ^{14}C INH At various times 100 µg./ml. non-radioactive INH was added. All tubes were incubated for a further 24 hr before the cells were assayed for radioactivity.

The results of this experiment appear in Fig. 3. It can be seen that INH was rapidly fixed by sensitive cells and was not subsequently released in the presence of high concentrations of non-radioactive isotope. There was only a small increase in irreversibly bound INH in the resistant cells over the same period.

The effect of some conditions, known to influence enzyme reactions, on INH uptake. It seemed desirable to confirm that an enzyme is involved in INH fixation by examining the effect of temperature and several chemical agents, which are known to affect enzyme reactions, on the fixation of INH by sensitive and resistant BCG.

Tubes of 18-day INH-sensitive and INH-resistant BCG cultures were set up as usual. Groups of duplicate tubes of both strains were treated. The results are shown in Table 2.

Table 2. *The effect of various conditions on the uptake of* ^{14}C *INH by sensitive and resistant strains of* BCG

Duplicate tubes received 0·36 μg. ^{14}C INH/ml. and were incubated for 40 hr at 37° except where otherwise stated.

Condition		Counts/min.	Comment
Control	Sensitive	135	Resistant uptake about 5 % of sensitive
	Resistant	6·6	
70° for 10 min.	Sensitive	2·7	Uptake heat labile, 80 % inhibited
	Resistant	26	Uptake appears to be slightly stimulated
4° incubation	Sensitive	172	Uptake 28 % higher than control
	Resistant	3·8	No change
10^{-2} M-KCN	Sensitive	7·4	Uptake completely inhibited
	Resistant	9·4	Uptake unaffected
10^{-3} M-PCMB	Sensitive	446	Uptake 3·3 times the control level
	Resistant	34	Uptake about 5 times the control level
4×10^{-4}M-2,4 DNP	Sensitive	65	Uptake about 50 % of control
	Resistant	7·4	Uptake unaffected

The control tubes behaved as described in Table 1 in that the resistant cells took up only 5 % of the amount of INH taken up by the sensitive cells.

Exposure to 70° for 10 min., however, resulted in a drop of 80 % in the uptake of INH by the sensitive cells, whereas the uptake in the resistant cells was slightly increased. Exposure to this temperature for this time commonly inactivates enzyme systems, and this result adds further evidence to the hypothesis that a thermo-labile system is directly involved in INH fixation. In resistant BCG, however, there appeared to be a slight increase in INH uptake, and this could be due to non-specific adsorption of INH on to intracellular constituents, as a result of increased permeability, following destruction of the cytoplasmic membrane.

'Incubation' at 4° for 40 hr resulted in an increase of about 30 % in uptake of INH by the sensitive cells, whilst the uptake of the resistant cells was not affected.

Parachloromercuribenzoate (PCMB) stimulated the uptake of INH in both sensitive and resistant BCG to about four times the control level.

Potassium cyanide blocked the uptake of INH by sensitive BCG completely, whilst the slight uptake by the resistant cells remained unchanged.

Cyanide is a common inhibitor of many enzymes, especially those requiring metal cofactors. This suggests once more that INH uptake is mediated by an enzymic process. Again the small amount of INH taken up by resistant cells is unchanged and was similar to the quantity taken up by cyanide-inhibited sensitive cells. This small amount was probably due to contaminating traces of INH adsorbed non-specifically to the cell.

2,4-dinitrophenol inhibited INH uptake in sensitive cells to 50% of the control level, but uptake by resistant cells remained unchanged. This drug uncouples oxidative phosphorylation which stops production of adenosine triphosphate (ATP) necessary for most energy-requiring reactions. Thus it is possible that an energy-requiring enzyme-catalysed reaction is involved in some aspect of INH uptake.

The effect of temperature on the rate of INH uptake

Sensitive BCG grown for 21 days on AFMR medium were incubated at 4°, 25° and 37°, in duplicate, for various times from 0 to 24 hr with ^{14}C INH. Uptake was followed over the first 24 hr and these results are shown in Fig. 4. It can be seen that the rate of INH uptake varied at the three temperatures tested. During the first 2 hr INH was fixed most rapidly at 37° and 25°. However, this fixation fell off quickly and by the 24th hr the order of total uptake at the three temperatures was reversed, the greatest amount of INH being fixed at 4° and the least at 37°.

This experiment was repeated over the narrower range from 0 to 2 hr, as it appeared that INH was fixed more rapidly at 37° than at either of the other temperatures tested during the first 2 hr. The results obtained are shown in Fig. 5.

It can be seen that the initial rate of uptake was lowest at the lower temperatures. The initial rates of INH uptake were measured at the three different temperatures and the log of this reaction velocity (expressed as $\log_{10}$ counts/5 min./hr) has been plotted against the reciprocal of the absolute temperature (Fig. 6). The slope of this graph can be used to determine the approximate activation energy of the reaction. This has been done four times and the mean figure for E appears to be 9900 cal./mole with a standard deviation of 2100 cal./mole, which is of the order of magnitude of the activation energy of a typical enzyme catalysed reaction. This distinguishes it from the very low or negative activation energies of physical adsorption.

Incubation at 37° and its effect on INH uptake at 4°. The fact that prolonged 'incubation' at 4° causes a greater uptake of INH than incubation at 37° for a similar period prompted the question: can organisms exposed to INH at 37° for various times subsequently take up more INH when incubated at 4° than organisms incubated at 4° all the time? Cultures were therefore incubated for various times from 0 to 20 hr at 37° with ^{14}C INH, and then stored for 24 hr at 4°. The results of this experiment appear in Fig. 7. It can be seen that, when the organisms were incubated at 37° and then held at 4°, they took up proportionately less INH the longer they were pre-incubated at 37°. Organisms held at 4° throughout the period of the experiment took up most INH of all.

The heat lability of the uptake system

Cultures of INH-sensitive and INH-resistant BCG were exposed to 60° for various times between 0 and 20 min. and tested for INH uptake by adding ^{14}C INH and incubating for 24 hr. INH uptake in sensitive organisms fell rapidly as the length of

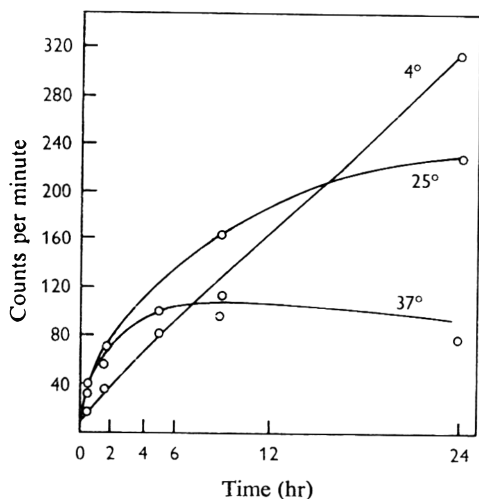


Fig. 4

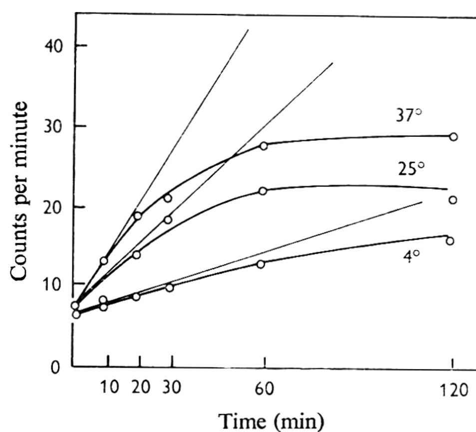


Fig. 5

Fig. 4. ^{14}C IHN uptake as a function of time, at 4°, 25° and 37° by INH-sensitive BCG. Each tube received 0·36 μg./ml. ^{14}C INH. At each sampling time 500 μg. ^{12}C INH was added to each tube.

Fig. 5. Uptake of ^{14}C INH at 4°, 25° and 37° in INH-sensitive BCG, over the first 2 hr of incubation with the drug. Each tube received 0·36 μg./ml. ^{14}C INH. At each sampling time 500 μg. ^{12}C INH was added to each tube.

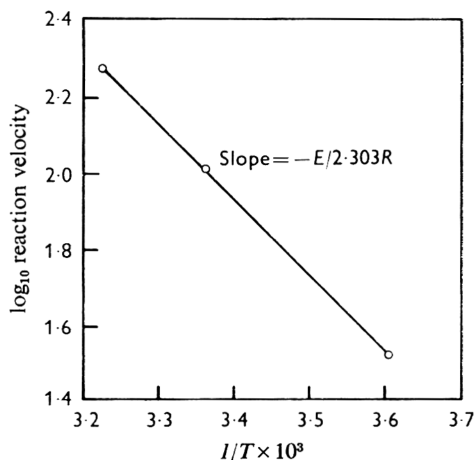
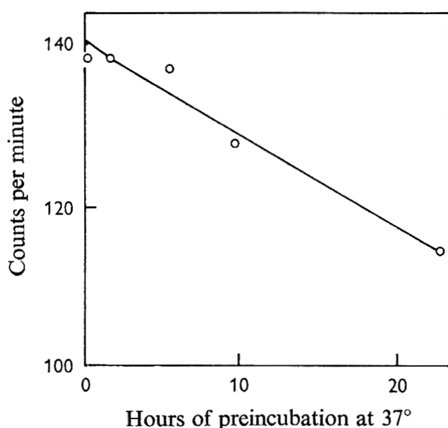


Fig. 6

Fig. 6. The log of the initial rate of ^{14}C INH uptake, determined from Fig. 5, plotted against the reciprocal of the absolute temperature.

exposure increased (Fig. 8). After about 10 min. at 60° the uptake by sensitive and resistant bacteria was about the same, and probably represents non-specific adsorption on to the same cellular component. However, about 80 % of the total INH uptake of sensitive bacteria was heat-labile as this was blocked after 10 min. exposure to 60°.

The effect of parachloromercuribenzoate (PCMB) on the incorporation of INH into tubercle bacilli. During the preliminary survey of the effects of various conditions on



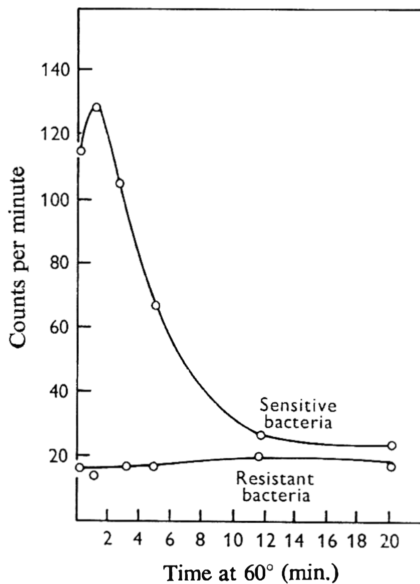


Fig. 7

Fig. 8

Fig. 7. Incubation of INH-sensitive BCG with ^{14}C INH at 37° and its effect on INH uptake at 4°. Each tube received 0·36 μg./ml. ^{14}C INH, and was incubated for various times at 37°, before being stored at 4° for 24 hr.

Fig. 8. The effect of exposure to 60° for various times on ^{14}C INH uptake in INH-resistant and INH-sensitive strains of BCG. Tubes were maintained at 60° in a water bath for various times, before being incubated for 24 hr at 37° with 0·36 μg./ml. ^{14}C INH. Cells were subsequently assayed for radioactivity.

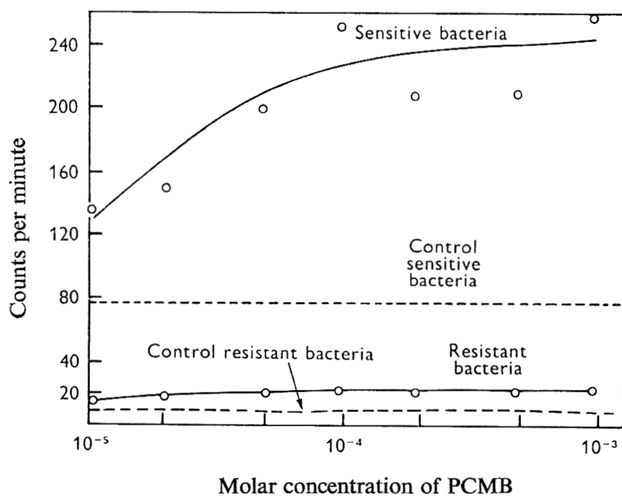


Fig. 9. The effect of various concentrations of PCMB on ^{14}C INH uptake by INH-sensitive and INH-resistant strains of BCG. Each tube received 0·36 μg./^{14}C INH and various concentrations of PCMB. After incubating at 37° for 40 hr the cells were assayed for radioactivity in the usual way.

the incorporation of INH into BCG it was shown that 10^{-3} M-PCMB stimulated INH uptake 3–4 times.

It was decided to compare the uptake of INH in INH-sensitive and INH-resistant BCG, treated with concentrations of PCMB between 10^{-5} and 10^{-3} M. These results (Fig. 9) show that with both sensitive and resistant strains INH uptake was stimulated by all concentrations of PCMB tested. The INH uptake that was observed in resistant cells, although small, was increased about twofold.

The effect of heat on INH uptake stimulated by PCMB. The effect of heat on ^{14}C INH uptake in sensitive BCG treated with PCMB was examined. The results (Fig. 10) show that the increased uptake due to PCMB treatment was as labile as the control uptake.

Table 3. *The effect of 10^{-3} M-PCMB on the uptake of normal metabolites by INH-sensitive and INH-resistant* BCG

Compound	BCG	10^{-3} M-PCMB	Counts/min.	Percentage of control
2 μC adenine	Sensitive	−	348	35%
		+	121	
	Resistant	−	689	7·5%
		+	52	
5 μC glycine	Sensitive	−	20,040	0·3%
		+	67	
	Resistant	−	21,111	0·9%
		+	194	
1 μC glucose	Sensitive	−	108	17%
		+	18	
	Resistant	−	163	15%
		+	25	

The effect of PCMB on the uptake of normal metabolites by INH-sensitive and INH-resistant BCG. The influence of PCMB on the uptake of three normal metabolites was examined; these were adenine, glycine and glucose. The results are presented in Table 3. It can be seen that in every case 10^{-3} M-PCMB inhibited the uptake of these substances. The effect was particularly noticeable with ^{14}C glycine.

PCMB interfered either with the uptake or the metabolism of three normal metabolites of INH-sensitive and INH-resistant BCG, in contrast to its effect on INH uptake.

The effect of other sulphyldryl (SH) group inhibitors on INH uptake. Two compounds were chosen: *N*-ethylmaleimide and iodoacetic acid.

The results are shown in Table 4 and from these it can be seen that both iodoacetic acid and *N*-ethylmaleimide stimulated INH uptake in both sensitive and resistant BCG. The increased uptake in sensitive bacteria was not as great as the stimulation observed with PCMB. However, the effect of both agents on INH uptake in resistant organisms was very marked.

The heat-lability of INH uptake in INH-sensitive or INH-resistant BCG *treated with N-ethlymaleimide or iodoacetic acid.* Stimulation of INH uptake by *N*-ethylmaleimide and iodoacetic acid was studied further by measuring the effect of heat on the system. With resistant BCG (Fig. 11) the enhanced INH uptake due to these reagents was

increased even further by heat treatment. However, with sensitive BCG (Fig. 12), although *N*-ethylmaleimide stimulated the uptake of INH, this uptake was also heat-labile. Examination of the latter part of the curve shows that uptake with or without the drug rose as the time of exposure to 60° increased, although uptake in the presence of *N*-ethylmaleimide was always higher. Comparison of this graph with Fig. 11 showing the heat lability of INH uptake in resistant organisms exposed to these drugs indicates that the shape of the latter part of the curve after 5 min. exposure to 60° is similar in both cases. It is probable that INH uptake follows a similar course in both strains of BCG except that a heat-labile mechanism binding INH exists in the sensitive organisms whilst this is not present in the resistant bacteria.

Table 4. *The effect of N-ethylmaleimide and iodoacetic acid on the uptake of isoniazid by sensitive and resistant BCG bacilli*

	IHN-sensitive BCG		INH-resistant BCG	
	Counts/min.	% of control	Counts/min.	Percentage of control
Control	120	.	8·6	.
Iodoacetic acid				
10^{-3} M	169	141	77	890
10^{-4} M	127	106	25	290
10^{-5} M	121	100	7·6	*c.* 100
N ethylmaleimide				
10^{-3} M	176	147	68	790
10^{-4} M	182	152	27	320
10^{-5} M	141	118	21	250

The effect of PCMB, N-ethylmaleimide and iodoacetic acid on INH uptake in three species of bacteria. Three organisms were chosen: *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus*. Cultures of each organism were treated with one of the three reagents or distilled water, and ^{14}C INH. Only negligible quantities of INH were taken up by each of the three organisms.

The extraction and chromatography of ^{14}C INH and its metabolites from INH-sensitive BCG. It seems likely that INH is converted by INH-sensitive BCG to some metabolite and this compound is responsible for the toxic effect of the drug in the bacterium. However, at present the nature of this substance is unknown.

It was decided to attempt the extraction of the radioactive INH and its derivatives bound by INH-sensitive BCG and to analyse this extract chromatographically.

Chromatographic examination of the hot-water extract of sensitive BCG treated with ^{14}C INH for 42 hr showed that less than 5 % of the activity could be accounted for as INH or any of three breakdown products—isonicotinic acid, isonicotinamide or diisonicotinylhydrazine. Most of this activity remained at the origin in both solvent systems (see Methods). It seems clear from this experiment that the fate of INH in INH-sensitive BCG was more complex than had hitherto been thought likely. It is obvious that a detailed chemical analysis of the metabolism of INH by tubercle bacilli is necessary and that this will throw more light on the mode of action of the drug.

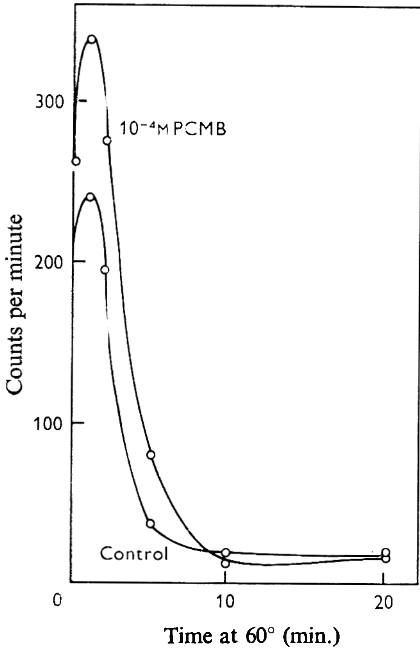


Fig. 10

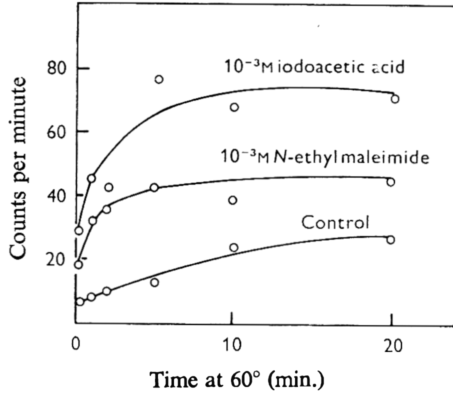


Fig. 11

Fig. 10. Exposure to 60° for various times on ^{14}C INH uptake in INH sensitive BCG, in the presence and absence of PCMB. Experimental details as for Fig. 8.

Fig. 11. The effect of heat on ^{14}C INH uptake in INH-resistant BCG treated with *N*-ethylmaleimide or iodoacetic acid. Experimental details as for Fig. 8.

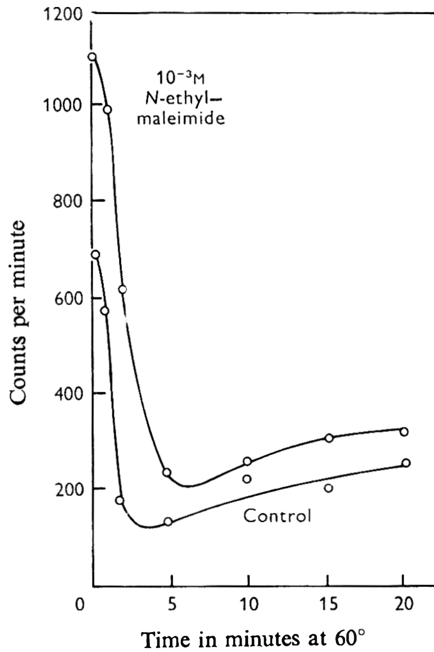


Fig. 12. The heat-lability of ^{14}C INH uptake in INH-sensitive BCG treated with *N*-ethylmaleimide. Experimental details as for Fig. 8.

DISCUSSION

The bulk of the evidence presented in this paper indicates the enzymic nature of INH uptake. This evidence will be discussed and a working hypothesis for INH action will be examined.

The relationship between the sensitivity of mycobacteria to INH and the mode of action of the drug requires accurate knowledge of the process by which the drug arrives at its site of action within the cell.

Early experiments were designed to correlate sensitivity to INH with uptake of the drug. These experiments showed that only sensitive organisms could take up INH. INH-resistant mycobacteria take up considerably less INH. Organisms normally naturally resistant fixed least INH of all. Whatever the mechanism of INH uptake therefore, the drug is found only in organisms which are sensitive to it. Uptake kinetics are qualitatively different when sensitive and resistant organisms are exposed to different concentrations of ^{14}C INH. Whilst the small uptake in resistant organisms is directly proportional to concentration, the high uptake in sensitive organisms becomes progressively saturated as the drug concentration rises. It seems likely that if INH was bound to the cell by non-specific physical adsorption the subsequent addition of high concentrations of non-radioactive INH would drastically reduce the amount of bound INH. In practice the binding of INH at 37° is irreversible. The sensitivity of INH uptake to different agents was examined. Uptake is sensitive to cyanide and partially sensitive to 2,4-dinitrophenol. Cyanide inhibits many enzyme systems, including those containing haem prosthetic groups.

2,4-dinitrophenol is a powerful uncoupler of oxidative phosphorylation and it is possible that INH uptake requires energy. This point has not been investigated further. In contrast to the effects of cyanide and 2,4-dinitrophenol, sulphydryl group reagents such as *p*-chloromercuribenzoate, *N*-ethylmaleimide or iodoacetic acid all stimulate INH uptake. This observation is clearest with PCMB. However, with all these reagents the additional uptake in sensitive cells is heat-labile though not in resistant organisms. Several explanations for this observation are possible. (i) SH-group reagents may non-specifically either assist in the binding of INH to the cell or alternatively increase the permeability of the cell to INH. The fact that uptake in sensitive though not resistant bacteria is heat-labile points to a greater degree of specificity than this explanation would imply. (ii) SH group reagents might inhibit a mechanism which metabolizes INH once it enters the cell and which under normal conditions itself limits the amount of INH that the sensitive cell can absorb. This latter is the preferred explanation in view of later experiments on INH uptake as a function of temperature. Earliest experiments showed, in agreement with Barclay and his co-workers, that incubation at low temperatures results in increased INH uptake. This was examined most carefully when INH uptake was measured as a function of time at these different temperatures. It was clear from these results that the initial rate of INH uptake was fastest at the higher temperatures. However, this rate rapidly falls off and at later times the picture is reversed and more INH appears in cells incubated in the cold. When the initial rate of INH uptake is expressed as a classical Arrhenius plot the energy of activation of the uptake process can be roughly estimated. In these experiments a figure of about 10,000 cal./mole is obtained which is of the order of magnitude of activation energy of an enzyme catalysed reaction. In common with the effect of SH-group reagents a higher INH uptake

is observed under the least physiologically suitable condition, in this case at 4°. It seems therefore that at 37° one component of the INH binding system is active and becomes rapidly and irreversibly saturated. This does not occur in the presence of SH-group reagents or at 4°. Careful examination of the sensitivity to heat of the INH uptake system by exposing cells to temperatures of 60° for various times shows uptake by sensitive cells to be quite labile. Uptake is reduced to the level in resistant cells after 10 min.

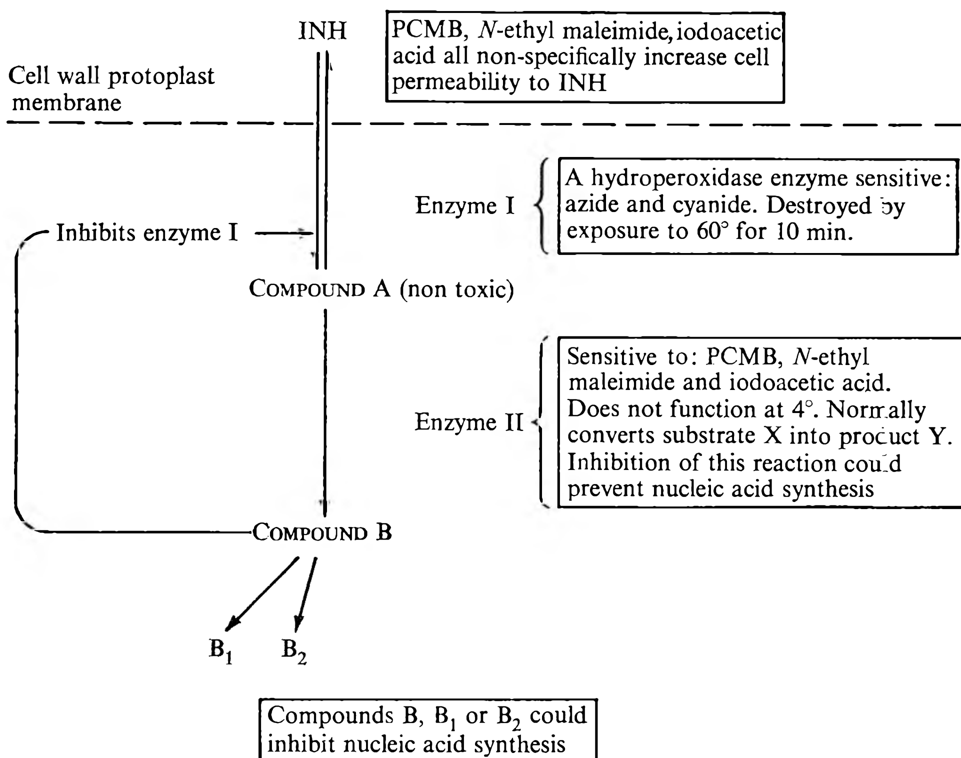


Fig. 13. A hypothetical scheme for the mode of action of INH.

Examination of all the available evidence leads to the conclusion that INH is bound in sensitive mycobacteria by a heat-labile protein molecule and that INH uptake has the activation energy of an enzymatic process.

On the basis of evidence derived from this paper and from other workers a tentative model for INH uptake is proposed (Fig. 13).

INH is taken up by sensitive mycobacteria at the active centre of enzyme I. Enzyme I is probably the hydroperoxidase enzyme which has been shown to be missing in resistant strains of mycobacteria (Middlebrook, Cohn & Schaeffer, 1954; Tirun-arayanan & Vischer 1959). This enzyme is heat-labile, sensitive to cyanide and also to azide (Youatt, 1958*b*).

INH reacts to form compound A which can be a metabolite of INH or free INH, and this is then released into the cell. Compound A is not in its own right toxic and is reversibly bound by the bacteria. At 37° compound A is converted into compound B by a second enzyme, enzyme II. The product of this reaction inhibits the uptake of

INH by enzyme I and compound B or another metabolite of compound B is toxic to the bacteria by interfering with nucleic acid synthesis (Wimpenny, 1967). The reaction catalysed by enzyme II is not reversible, does not take place at 4° and is probably inhibited by the SH group reagents PCMB, iodoacetic acid and *N*-ethylmaleimide. Enzyme I is not affected by any of these agents, and hence compound A accumulates to a greater extent in these than in control INH-treated cells. It is probable that the SH-group reagents also increase cell permeability to INH non-specifically in both INH-sensitive and INH-resistant BCG, as well as inhibiting the reaction of enzyme II. It may be that INH partially inhibits enzyme I although there is little evidence to confirm this. 80–90 % of the isotope can subsequently be extracted with hot water when INH-sensitive bacteria are incubated with ^{14}C INH. Chromatographic techniques show that a number of compounds other than INH or any of its normal breakdown products (isonicotinic acid, diisonicotinyl hydrazine or isonicotinamide) contain radioactivity. The most active of these remains stationary at the origin in two solvent systems. These unidentified compounds may be products of enzyme I or II or of subsequent reactions of these products. Resistant cells have lost enzyme I and hence cannot fix INH. However, as enzyme I has properties of catalase and peroxidase these organisms are now sensitive to H_2O_2. In these cells, even in the presence of INH, there is no compound A to serve as a substrate for enzyme II and the cells grow normally.

It is difficult to fit the results described in this paper and those presented by other workers into any simpler theoretical framework. The fact that the enzyme responsible for INH uptake in sensitive cells appears to be missing in resistant organisms makes it unlikely that this enzyme is one whose normal function in tubercle bacilli is necessary to the reproduction and growth of the cell. It follows that a second step must occur, the result of which is an inhibition of nucleic synthesis. But INH uptake at 37° appears to become rapidly saturated. Therefore, a metabolite of INH must inhibit the enzyme responsible in the first place for the uptake of the drug. This metabolite or another product of INH metabolism exercises its effect on nucleic acid synthesis. The formation of compound A (whether this is free INH or not) also explains the fact that INH is taken up to a greater extent at low temperatures and that this compound is reversibly bound to the cell (Barclay *et al.* 1954) because under these conditions the reaction catalysed by enzyme II cannot take place as compound B which normally inhibits INH uptake is not formed.

This report is prepared in part from a thesis presented to the University of London 1962, for the degree of Doctor of Philosophy. The author is grateful to Professor R. Knox for his constant help and encouragement, and to the Endowment Fund of Guy's Hospital for a research grant.

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Purification and Physico-Chemical Analysis of Fractions from the Culture Supernatant of *Escherichia coli* O78K80: Free Endotoxin and a Non-Toxic Fraction

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SUMMARY

The extracellular production of 'free endotoxin' from *Escherichia coli* serotype O78K80 is discussed. Isolation of purified toxic and non-toxic fractions from crude extracellular material and the physico-chemical properties of these fractions are described. Purified free endotoxin possessed similar properties to endotoxins extracted from the bacteria by conventional procedures; a purified non-toxic extracellular fraction corresponded to the so-called 'native hapten'. When dissolved in buffers containing sodium dodecylsulphate, the non-toxic fraction was not disaggregated, but the endotoxin produced sub-units of a smaller particle size than the non-toxic material. From these experiments it was concluded that the non-toxic material did not constitute simple sub-units of endotoxin.

INTRODUCTION

The term 'endotoxin' describes certain lipopolysaccharides of high molecular weight found in Gram-negative bacterial cell walls. Although Shear and his associates (e.g. Shear & Turner, 1943) have isolated tumour-necrotizing substances (endotoxins) from supernatant fluids of cultures of Gram-negative bacteria, most other workers have preferred to extract endotoxins from the bacteria themselves. An exception has been Work and her collaborators (Bishop & Work, 1965, Taylor, Knox & Work, 1966), who found endotoxic lipopolysaccharides in culture supernatant fluids of lysine-requiring mutants of *Escherichia coli* grown under lysine-limited conditions.

The release of large quantities of free endotoxin into the culture supernatant fluids of Gram-negative bacteria, under favourable growth conditions, has recently been described (Crutchley, Marsh & Cameron, 1967*a*). The present paper will deal with the fractionation of supernatant fluids of cultures of *Escherichia coli* serotype O78K80 and the subsequent physical and chemical characterization of the fractions; the accompanying paper (Crutchley *et al.* 1967*b*) will discuss the biological properties of these fractions.

Origin of toxicity. The published discussions of two symposia concerning endotoxins (led by Westphal *et al.* 1957 and Ribi *et al.* 1964) show that there is still a wide divergence of opinion about the chemical composition and physical properties of the moieties believed to be responsible for endotoxic activity. The German school of Westphal and his collaborators (Westphal, 1960; Westphal & Lüderitz, 1954; Westphal *et al.* 1957) concluded that lipid, possibly in conjugation with a polysac-

charide, was essential for toxicity; but a more recent investigation of endotoxins from rough Salmonella strains led them to suggest more strongly that the lipid moiety ('lipid A') was 'the factor decisive for at least some endotoxic effects' (Lüderitz & Westphal, 1966). They also believe that, with respect to endotoxic activity, combined polysaccharide may function only as a solubilizing agent. However, the American school of Landy, Ribi and their associates (Ribi, Haskins, Landy & Milner, 1961*a*; Haskins, Landy, Milner & Ribi 1961; Fukushi *et al.* 1964) have generally found very little lipid in their highly toxic fractions from several of the Enterobacteriaceae; all their lipid extracts, including lipid A, possessed less than 1 % of the biological activity of the parent endotoxins. The balance of their published evidence suggests that a carbohydrate moiety is implicated in the toxicity, although certain other factors may be involved (Ribi, Haskins, Landy & Milner, 1961*b*).

There have also been conflicting reports about the minimal molecular size of the complex required for full endotoxic activity. Most workers seem to favour a unit possessing a sedimentation coefficient of about 10S (cf. Ribi *et al.* 1964). Mild acidic hydrolysis (e.g. with 0·1 M-acetic acid for 90 min. at 100°) destroyed virtually all the toxicity of Ribi's endotoxin and produced a parallel decrease in the average sedimentation coefficient of the solute, although the 'acid hapten' obtained by this process ($s = 1{\cdot}4$S) retained certain antigenic characteristics of the parent endotoxin (Ribi *et al.* 1962). By using non-hydrolytic conditions of disaggregation, in solutions containing sodium dodecylsulphate (SDS), two groups of workers reached opposite conclusions: Oroszlan & Mora (1963) found that the endotoxic complex of *Serratia marcescens* dissociated into particles of about 3S, with significant concomitant decrease in the biological activity responsible for tumour damage; on the other hand, Beer, Staehelin, Douglas & Braude (1965) found that an endotoxic preparation from *Escherichia coli* could be treated with SDS without measurable loss of toxicity.

The above findings, and many other conflicting reports about endotoxins, may have arisen partly from the use of different bacterial species and serotypes, but probably mainly from the widely different experimental procedures used, particularly as regards extraction and purification. It is uncertain whether the extraction procedures commonly used to isolate endotoxins from bacteria (trichloracetic acid at 4°, Boivin & Mesrobeanu, 1933; phenol+water at 65°, Westphal *et al.* 1952; aqueous ether at 6–12° Ribi *et al.* 1961*a*) yield native endotoxins or degraded or aggregated derivatives of the parent materials. We have therefore developed a series of very mild extraction and purification procedures whereby endotoxic and other bacterial products may be isolated in large amounts from bacterial culture fluids without any significant change in the properties of the native substances.

METHODS

Acid-hydrolysed casein was obtained from Oxoid Ltd., ammonium sulphate from I.C.I., protein stains from G. T. Gurr (London), 'Ionagar for Electrophoresis' from Difco (Detroit, U.S.A.), Sephadex G 100 and Blue Dextran 2000 from Pharmacia (Uppsala, Sweden) and DEAE-cellulose DE 11 (1 m-equiv./g.) from Whatman (Balston, England). Yeast extract was prepared at the Wellcome Research Laboratories. All other chemicals were of Reagent or AR quality.

Preparation of crude endotoxin. Escherichia coli serotype O78K80 was grown in tanks for 42 hr at 35° in a vigorously aerated aqueous medium containing acid-hydrolysed casein (5%, w/v), sucrose (3%, w/v) and yeast extract (10%, w/v), adjusted to pH 6·5 (D. C. Edwards, unpublished). Before inoculation, the medium was clarified, heated to 85° and sterilized by Seitz filtration. This medium will be referred to as 'casein sucrose'.

The bacteria were removed from suspension by using a de Laval centrifugal separator and the supernatant fluid sterilized by Seitz filtration. Crude endotoxin I was precipitated from this supernatant fluid by addition of ammonium sulphate (about 700 g./l.) and allowing it to stand at 4° for 6 days. The brown flocculent precipitate (I) was recovered by centrifugation, resuspended and then dialysed thoroughly against distilled water. A small amount of insoluble material was removed by centrifugation and the endotoxin then reprecipitated at 4° by adding 65 g. AR ammonium sulphate per 100 ml. solution. The reprecipitated material (II) was treated as above to remove salts, clarified by centrifugation and freeze-dried to give a brown powder (Fig. 1).

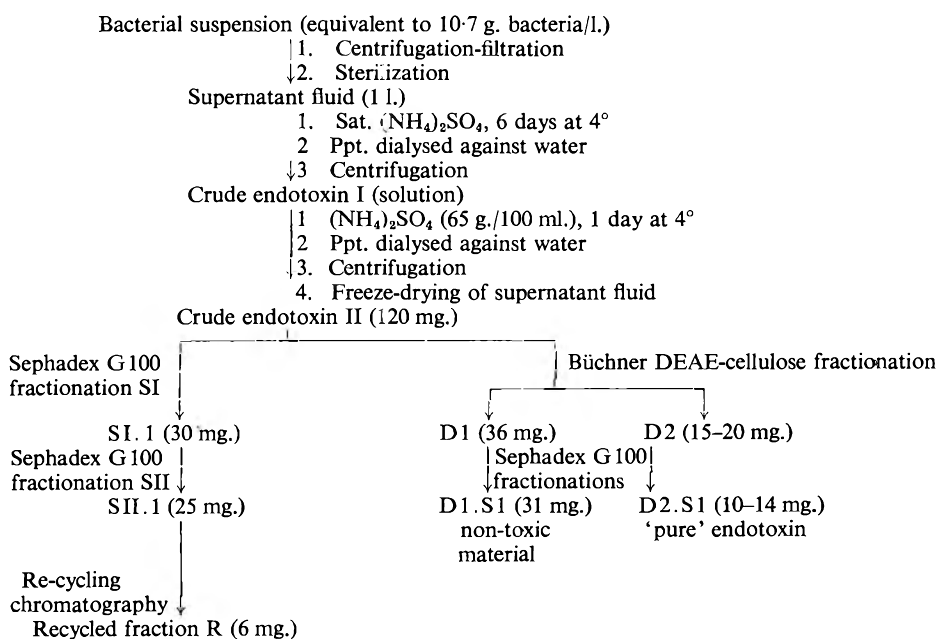

Fig. 1. The preparation and purification of endotoxin and non-toxic fractions from *Escherichia coli* O78K80. Yields of freeze-dried salt-free fractions per litre of sterile culture fluid are given in brackets after the appropriate fractions.

Purification of endotoxin

Recovery of purified fractions. Volatile buffers based on ammonium salts were used for all chromatographic separations in order to eliminate the necessity for extensive dialysis operations. Normally, the pooled chromatographic effluents were dialysed once against a 50- to 100-fold excess of distilled water, and the remaining traces of the volatile salts were removed during subsequent freeze-drying. This method has been used routinely to recover endotoxic fractions from large volumes of solution without loss in biological activity.

Gel filtration. Crude endotoxin II (40 mg.) dissolved in 3 ml. of 0·05 M-NH_4HCO_3 was applied to a Sephadex G 100 column (95 cm. × 3·28 cm. diameter), equilibrated with this buffer. Figure 2 shows the elution diagram for this fractionation (S I) for extinction readings at 230 mμ. (Endotoxin II showed a spectral maximum at 274–275 mμ. Readings were also taken at 230 mμ in order to gain a better assessment of the total solids eluted, including carbohydrate.) Endotoxic activity was shown to be associated mainly with the first peak, fraction S I.1 (Crutchley, Marsh & Cameron, 1967*b*): the bulk of the brown-pigmented material was found in fraction S I.6. Fraction S I.1 was purified further by a second fractionation (S II) on the Sephadex column described above, and the main endotoxic fracton S II.1 was reserved for further purification by recycling chromatography. In subsequent experiments, larger quantities (about 550 mg.) of the crude fraction II were applied to the Sephadex column but, because of the poorer resolution obtained, two further purification stages were required to achieve the same degree of purity as fraction S II.1.

Recycling chromatography. To obtain the highly purified fraction R (Fig. 1), partially purified endotoxin was repeatedly chromatographed on the long Sephadex G 100 column described previously. A solution of fraction S II.1 (125 mg. in 5 ml. 0·2 M-NH_4HCO_3) was recycled through the column by means of a peristaltic pump, and the effluent was continuously monitored for extinction at 253·7 mμ using an LKB Uvicord spectrophotometer and recorder. After six cycles through the column, the effluent was collected and its extinction measured at 230 mμ. All contaminating traces of nucleotide and peptide were removed during the course of the recycling process. The proportion of contamination by fraction S I.2 was also substantially decreased by the recycling procedure.

Ion-exchange chromatography. Since it had proved to be very tedious to resolve completely the major components of fractions S I.1 and S I.2 (Fig. 2) by gel filtration alone, chromatographic separation on columns of DEAE-cellulose and DEAE-Sephadex was investigated. Of these two anion-exchangers, DEAE-cellulose proved to be the more suitable in practice.

The first purification, utilizing a long narrow column of DEAE-cellulose, was applied to fraction S I.1. A high molecular weight, predominantly carbohydrate fraction, S I.D1. (identical with fraction D 1.S1 prepared by a slightly different method; Fig. 1), was found to be weakly bound to this column in dilute buffers, whereas most other material was strongly adsorbed. For subsequent large-scale separations, it was more convenient to carry out the first stage in the purification of crude endotoxin II by using a wide shallow DEAE-cellulose column, followed by further purification by gel filtration (Fig. 1).

A solution of crude endotoxin II from the salt-precipitation stages (600 mg. in 30 ml. of 0·005 M-NH_4HCO_3) was applied to a Büchner column (8 cm. × 10 cm diameter) containing DEAE-cellulose equilibrated to pH 8 and suspended in 0·005 M-NH_4HCO_3. Elution was performed successively with (i) 0·01 M-NH_4HCO_3 (1250 ml.) and (ii) M-ammonium acetate acetic acid buffer (ph 5·5, 500 ml.). A single peak of weakly ultraviolet-absorbing material (at 230, 260 and 275 mμ) was eluted with buffer (i), this was *fraction D1.* The bulk of the remaining material was eluted with buffer (ii), this was *fraction D2.* (In a subsequent experiment 0·2 M-NH_4HCO_3 was used as an intermediate buffer between (i) and (ii). This probably had the advantage of avoiding possible contamination of fraction D2 with traces of strongly adsorbed

D1, although the yield of D2 was lower due to its partial elution with the 0·2 M salt.)

Fraction D2 proved to be endotoxic and D1 non-endotoxic, but *both* were protective in mice against a subsequent challenge with *Salmonella typhi* 24 hr after injection of fractions D1 or D2 (Crutchley *et al.* 1967*b*). Fractions D1 and D2 were further purified by gel filtration on a Sephadex G 100 column (167·5 cm. × 1·2 cm. diameter); see Figs. 3, 4. Only the first fractions from each chromatographic experiment (D1.S1 and D2.S1, respectively), possessed the biological activities associates with fractions D1 and D2.

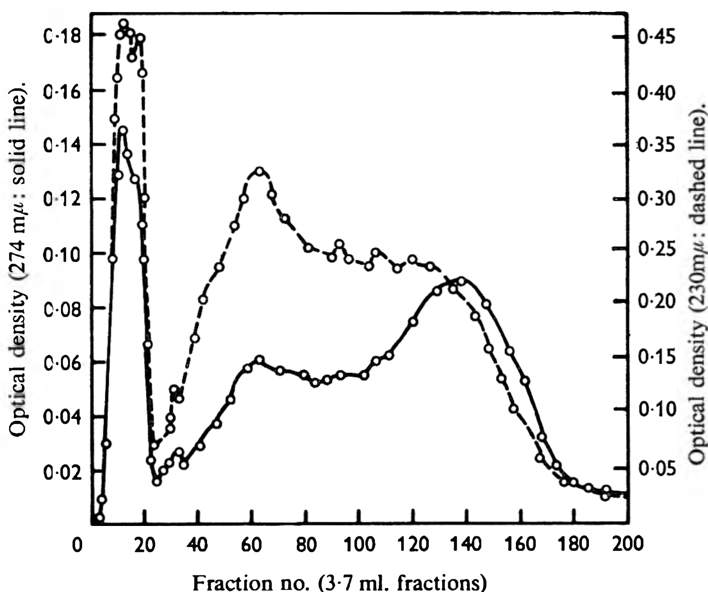

Fig. 2. Elution diagram for the Sephadex G100 fractionation (SI) of crude endotoxin II (40 mg. in 3 ml. of 0·2 M-NH_4HCO_3—elution with the same solvent).

Ultracentrifugal analyses. Crude and highly purified preparations were examined in a Spinco Model E Ultracentrifuge, using schlieren optics. Phosphate NaCl buffer (pH 7·8; I = 0·1) was used throughout as the solvent, except in certain experiments where sodium dodecylsulphate was added, 0·5 g./100 ml. of this buffer. Crude *Escherichia coli* O78K80 endotoxins, extracted from the parent bacteria of our culture by the aqueous phenol method of Westphal, Lüderitz, Eichenberger & Keiderling (1952) and by the aqueous ether method of Ribi *et al.* (1961*a*), were also examined in the ultracentrifuge for comparative purposes. The aqueous layer of the phenol-water extract was partially purified to the stage where nucleic acid was removed from the endotoxin by organic solvent precipitation. No further purification of the aqueous ether extract was performed beyond the stage of precipitating the crude endotoxin with 68 % EtOH-H_2O. Sedimentation coefficients for the higher molecular weight solutes in these preparations (Fig. 8) were calculated and corresponding concentrations were determined from traced magnified areas under the schlieren peaks by 'counting squares', using schlieren patterns from known concentrations of fraction D1.S1 as the standards. Appropriate corrections were made for differences in the schlieren-

phase plate angle and for radial dilution effects, but no correction was made for the Johnston & Ogston (1946) effect, since this was insignificant in most cases. The aqueous phenol extract, further purified by passage through Sephadex G 100 (Fig. 5), gave an almost identical elution pattern to that shown in Fig. 4 for fraction D2. The ultracentrifugal data obtained for the major fraction, P1, eluted at the void volume of the column, are also presented in Figs. 7 and 8 and in Table 1.

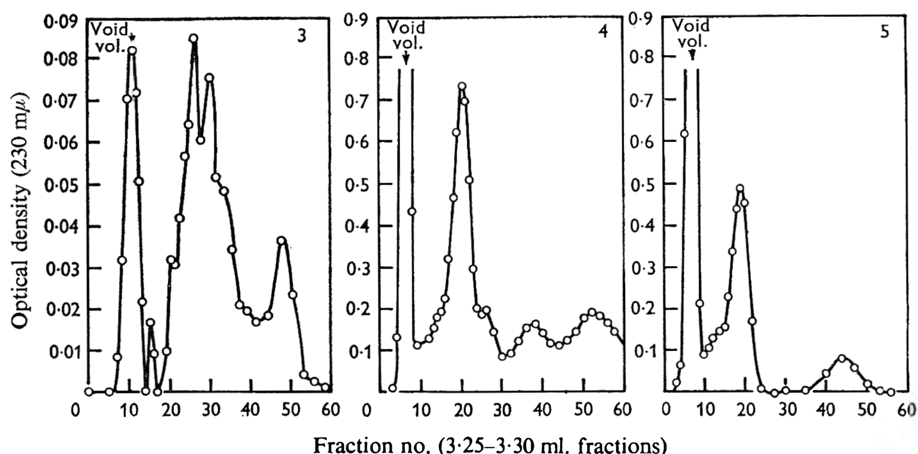

Fig. 3. Elution diagram for a Sephadex G100 fractionation of fraction D1 (20 mg. in 2 ml. of 0·2 M-NH_4HCO_3—elution with the same solvent).

Fig. 4. Elution diagram for a Sephadex G100 fractionation of fraction D2 (19 mg. in 2 ml. of 0·2 M-NH_4HCO_3—elution with the same solvent).

Fig. 5. Elution diagram for a Sephadex G100 fractionation of a partially purified aqueous phenol cell extract (20 mg. in 2 ml. of 0·2 M-NH_4HCO_3—elution with the same solvent).

Table 1. *Ultracentrifugal data for measurable schlieren peaks for buffered solutions (pH 7·8, I = 0·1) of aqueous phenol and aqueous ether extracts of Escherichia coli O78K80*

Calculated s^0_{20} and c values correspond (*a*) to endotoxic material, and (*b*) to non-toxic material.

Run no.	Fig.	Solute concn. (mg./ml.)	Speed (rev./min.)	Schlieren angle (°)	Aqueous phenol extract s^0_{20} (sec. 10^{13})	Aqueous phenol extract c (mg./ml.)	Aqueous ether extract s^0_{20} (sec. 10^{13})	Aqueous ether extract c (mg./ml.)
1	8*a*	6·0	59,780	45	(*a*) 11·2	—	17·5	—
					(*b*) 5·47	0·85	5·42	1·00
2	8*b*, *c*	30·0	59,780	70	(*a*) —	—	14·3	—
					(*b*) 2·94, 4·91	3·56, 1·04	3·16	2·96
3	8*d*	30·0	29,500	70	(*a*) 24·8	—	15·5	—
					(*b*) 2·97, 5·15	2·28, 2·05	3·19	4·01
4†	—	2·0	59,780	65	(*a*) (65)*	—	—	—
					(*b*) 5·82	0·59	—	—
5†	8*e*, *f* bottom patterns	6·0	39,460	65	(*a*) 12·9	—	—	—
					(*b*) 4·90	2·09	—	—

* Value obtained from a run at lower speed—probably corresponds to nucleic acid.

† Fraction P1 from phenol extract purified by Sephadex G100 chromatography.

All sedimentation coefficients were corrected to water at 20° (s^0_{20}) and, for the highly purified preparation D1.S1, a graph of s^0_{20} versus concentration (c) was plotted in order to determine $[s^0_{20}]_{c=0}$ (Fig. 7).

Other estimations of purity

Gel filtration. Highly purified materials were chromatographed on a Sephadex G 100 column (182 × 1·2 cm.), which was in routine use for the molecular-weight estimations of proteins (Andrews, 1964). The void volume of this and other G 100 columns was measured by using a solution of Blue Dextran 2000 (Pharmacia).

Electrophoresis. Cellulose acetate strip electrophoresis in barbitone buffer (pH 8·8, Kohn, 1960) and starch-gel electrophoresis at pH 8·5 (Smithies, 1955), in presence or absence of 6 M-urea, were used to investigate the purity of the endotoxic fractions. After electrophoresis at 175 V. for 1 hr, cellulose-acetate strips were stained with trifalgic acid or Ponceau S for proteins and with periodate-Schiff's reagent for carbohydrates (Kohn, 1960). Lipid was detected on drying the strips before staining, due to differences in the drying characteristics of cellulose acetate with adsorbed lipid. Lipid was not stained by the ozone–Schiff's method, presumably because of the absence of C=C bonds. Sliced starch gels were stained for protein with amido black 10B.

Immunodiffusion. The double diffusion method, in an agar gel supporting medium, was used to investigate the purity of fractions at each stage in the purification (Fig. 1). The preparation of antisera for these experiments is described in the companion paper (Crutchley *et al.* 1967*b*).

Spectrophotometry. Ultraviolet absorption spectra for aqueous solutions of purified fractions were automatically recorded on a Unicam SP 800 spectrophotometer.

Chemical analyses

Elementary analysis. Nitrogen was estimated by the micro-Kjeldahl method (Pregl, 1945) and phosphorus by a modified Gomori (1942) procedure with a sulphuric acid + hydrogen peroxide mixture as digesting agent.

Amino acids. Purified fractions D1.S1 (1·40 mg.) and D2.S1 (3·06 mg.) were hydrolysed with constant-boiling HCl (5·7-N) in vacuum for 24 hr. Amino acids were estimated by their colour reactions with ninhydrin, by using a Beckman-Spinco (Model 120) automatic analyser, with β-2-thienyl-DL-alanine added as an internal standard (Siegel & Roach, 1961).

Carbohydrates. Various classes of monosaccharides in purified fractions were determined by the sulphuric acid + cysteine procedure of Dische (1955). The hexosamine content for material which had been hydrolysed for 5 hr at 100° in 3 N- or 4 N-HCl (see Ribi *et al.* 1961*a*) was quantitatively estimated by the indole + HCl procedure (Dische, 1955) with appropriate non-deaminated controls, or by the short column of the Beckman-Spinco Analyser.

Fatty acids. Purified fractions D1.S1 and D2.S1 (3 mg.) were hydrolysed for 3 hr at 100° in 2 N-HCl. The fats were extracted directly from these hydrolysates with 3 × 1·5 ml. light petroleum (b.p. 60–80°). The pooled light petroleum phases were evaporated to dryness, saponified and examined as free fatty acids by gas-phase chromatography (Schmit & Wynne, 1965).

RESULTS

Ultracentrifugal analyses

The sedimentation patterns for crude endotoxin II (Fig. 1) indicated pronounced heterogeneity (Fig. 6*a*, *b*), but all preparations possessed a characteristic hypersharp peak ($[s^0_{20}]_{c=0}$ = 3·1 S). Fractions D1.S1 (Fig. 6*c*) and R (Figs. 6*d*, *e*, *f*) possessed

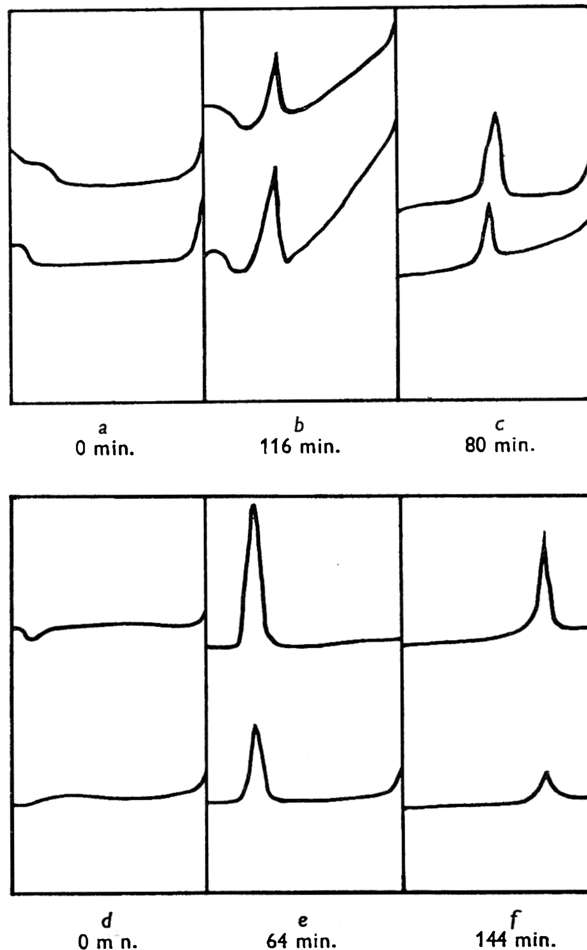

Fig. 6. Ultracentrifugal schlieren patterns of crude endotoxin II at 10 mg./ml. (*a* and *b*), fraction D1.S1 at 3·4 mg./ml. (*c*), and fraction R at 5·1 mg./ml. (top pictures, *d*, *e* and *f*) and at 2·55 mg./ml. (bottom pictures, *d*, *e* and *f*). All solutions prepared in phosphate-NaCl buffer (pH 7·8; I = 0·1): solutions shown in the bottom pictures of (*a*) and (*b*) and in the top picture of *c* contain, in addition, 0·5 g. of SDS per 100 ml. Speeds: 59,780 rev./min. (*a*, *b* and *c*) and 52,640 rev./min. (*d*, *e* and *f*). Temperature: 20° Schlieren angles: 50° (*a* and *b*), 65° (c), 45° (*d*, *e* and *f*).

only this peak together with occasional slight traces of higher molecular weight impurities. These two fractions, both non-toxic (Crutchley *et al.* 1967*b*), were identical by ultracentrifugal criteria; each exhibited the same variation of s^0_{20} upon *c*, yielding an extrapolated value of $[s^0_{20}]_{c=0} = 6{\cdot}7 \pm 0{\cdot}2$ S (Fig. 7).

Subsequent analysis of two endotoxic extracts of the present bacteria (aqueous phenol and aqueous ether extracts) showed heterogeneity and differences of composition between the two fractions (Fig. 8). Both extracts, however, possessed an endotoxic fraction of high molecular weight and also a component with similar sedimentation behaviour to that of non-toxic fraction D1.S1 at the same concentration (Table 1; Fig. 7). (In one aqueous phenol extract two hypersharp peaks were observed: Fig. 8*b–d*. Since no explanation can yet be given for this phenomenon, the data for these peaks has not been included on the graph shown in Fig. 7.)

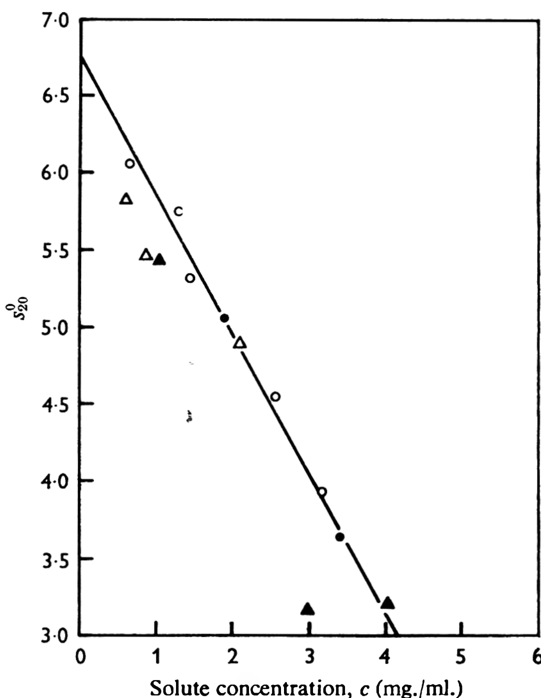

Fig. 7. Graph showing the variation of s^0_{20} with c for fractions D1.S1 and R. Calculated s^0_{20} and c values for analogous material in aqueous phenol and aqueous ether cell extracts were plotted after drawing the curve (Table 1).

Sodium dodecylsulphate disaggregated traces of high molecular weight material in crude endotoxin II (Fig. 6*a*) and in fraction R, but did not affect the sedimentation characteristics of the major peak of non-toxic material, other than slightly decreasing its sedimentation coefficient (e.g. $\Delta[s^0_{20}]_{c-0} \approx 0{\cdot}05$ S). This is probably a reflexion of small changes in ν and ρ for the solute and solvent, respectively.

The sedimentation patterns of the endotoxic fraction D2.S1 showed asymmetry and very rapid spreading of the main schlieren boundary (Fig. 9): probably these effects were due mainly to reversible association of endotoxin molecules and of their sub-units. In addition to the main schlieren boundary, a small quantity of material of very high sedimentation coefficient was observed to migrate across the cell as the centrifuge was accelerating up to the running speed. On adding sodium dodecylsulphate (SDS) (0·5 g./100 ml.) to translucent aqueous solutions of the toxic fraction, there was immediate clarification of the solutions, and a concomitant striking decrease

in the average sedimentation coefficient of the solute occurred (Fig. 9). The extent of disaggregation produced by SDS appeared to vary with different concentrations and with different preparations of purified endotoxin D2.S1. SDS also disaggregated the endotoxic component in the purified phenol extract P1, but had no effect on the non-toxic component (Figs. 8*e*, *f*).

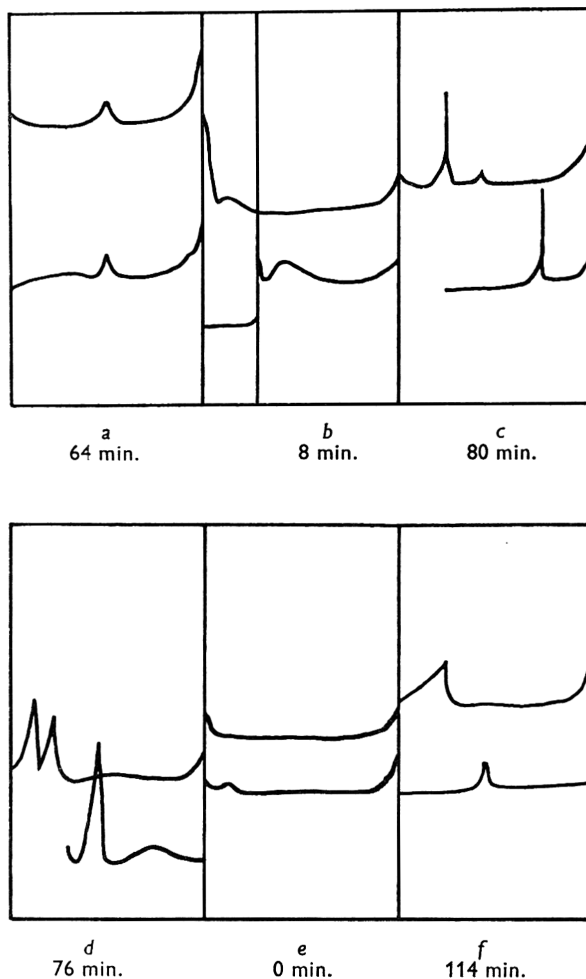

Fig. 8. Ultracentrifugal schlieren patterns of aqueous phenol (top pictures, *a–d*) and aqueous ether (bottom pictures, *a–d*) extracts of bacterial cells, and of purified phenol extracts P1 (*e*, *f*). All solutions in phosphate-NaCl buffer (pH 7·8; I = 0·1): solutions shown in the top pictures of (*e*) and (*f*) also contain 0·5 g: SDS per 100 ml. Other details given in Table 1.

Approximate $[s^0_{20}]_{c=0}$ values for the disaggregated material from fraction D2.S1 were 2·4 S and 4·1 S (Fig. 9*b*). Owing to the poor solubility and rapid boundary spreading of the main endotoxic peak in solutions without added SDS (Fig. 9*a*), no estimate of $[s^0_{20}]_{c=0}$ for this peak could be made. However, calculated values of s^0_{20} for 1 % and 2 % solutions were, respectively, 14·5 S and 13·2 S.

Estimations of purity

Gel filtration. Fractions D1.S1, D2.S1 and purified phenol extract (P 1) all gave single elution peaks upon rechromatography on a long Sephadex G 100 column; this indicated absence of any significant quantity of lower molecular weight contaminants. However, since these peaks emerged with the void volume of the column, small proportions of impurities of molecular weight higher than that of the bulk of the material (noticed in certain purified fractions during ultracentrifugation; Figs. 6, 8 and 9) would not have been detected.

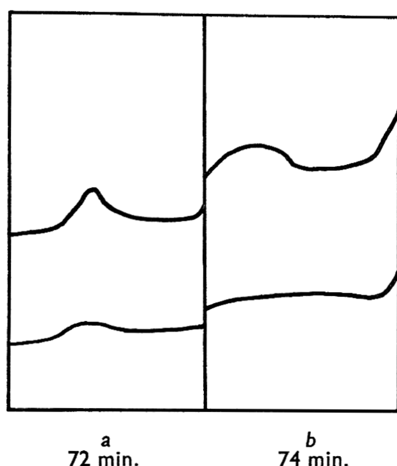

Fig. 9. Ultracentrifugal schlieren patterns of endotoxic fraction D2.S1 at 20 mg./ml. (top picture in *a*) and at 10 mg./ml. (bottom picture in *a*) in phosphate-NaCl buffer (pH 7·8; I = 0·1). In (*b*) D2.S1 at 3·0 mg./ml. (top) and 1·5 mg./ml. (bottom) was dissolved in this buffer containing 0·5 g. SDS per 100 ml. Speeds: 20,410 rev./min. (*a*); 59,780 rev./min. (*b*). Temperature: 20° Schlieren angles: 65° (*a*) and 55° (*b*).

Electrophoresis. On cellulose-acetate electrophoresis at pH 8·8, the endotoxic fraction D2.S1 migrated towards the cathode as a single band in which lipid, carbohydrate and peptide were detected: a trace of insoluble material remained at the origin. The purified phenolic extract, P 1, was largely retained at the origin (strong carbohydrate and weak protein staining); a lipid moiety of a similar negative mobility to fraction D2.S1 was also detected in P 1. Non-toxic fraction D1.S1 gave a single band of very low apparent negative mobility (possibly due to electro-osmotic flow of buffer) in which carbohydrate, lipid and protein were very weakly detectable. Starch gel did not prove to be a suitable medium for electrophoresis of the above samples, but was used for separation of lower molecular weight protein fractions present in crude extracts (e.g. endotoxin II).

Immunodiffusion. A sample of pooled horse antiserum against the whole extract of *Escherichia coli* O78K80 was of little value in estimating the homogeneity of purified fractions, since only one major and a trace of one minor precipitin line were formed against crude endotoxin II (Crutchley *et al.* 1967*b*). When examined against a potent rabbit antiserum to crude endotoxin II, little heterogeneity was observed in purified fractions R, D2.S1 and P1; D1.S1 did not give an immunodiffusion line with this

antiserum. A decrease in the number of precipitin lines was observed after each purification stage.

Spectrophotometry. The ultraviolet absorption spectra for aqueous solutions of purified fractions R, D1.S1, D2.S1 and P 1 (Fig. 10) indicated chemical differences between the fractions. Fractions R and D1.S1 possessed identical spectra, typical of carbohydrates; but D2.S1 (and crude also endotoxin II) showed an additional small absorption maximum at about 275 mμ, possibly due to aromatic amino acids. The maximum at 260 mμ for purified phenol extract, P 1, was almost certainly due to contamination by nucleic acid.

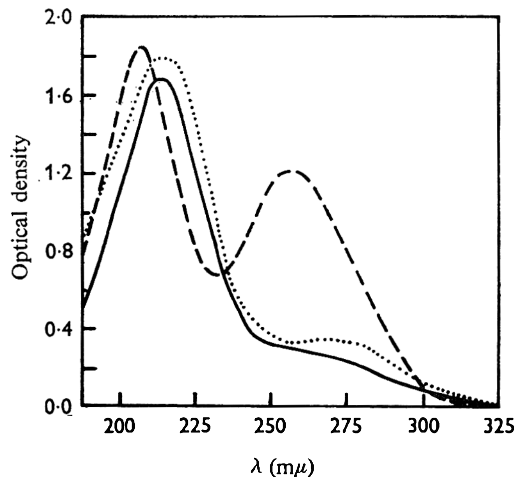

Fig. 10. Ultraviolet absorption curves for aqueous solution of purified fractions D1.S1 (1 mg./ml., solid line), D2.S1 (0·5 mg./ml., dotted line) and P1 (0·5 mg./ml., dashed line).

Chemical analyses

Quantitative chemical differences were found between fractions D1.S1 and and D2.S1 (Tables 2, 3). In particular, D2.S1 contained a higher proportion of long-chain fatty acids and a much greater proportion and variety of amino acids than did fraction D1.S1. Fraction D1.S1 was, however, much richer in amino sugar than was D2.S1; acid hydrolysates of these fractions showed that this sugar was chromatographically identical with glucosamine (long and short columns of the amino acids analyser used). High voltage paper electrophoresis of the hydrolysates in 0·1 M-borate buffer (pH 10; Michl, 1951; Foster, 1953), followed by staining with ninhydrin or $AgNO_3$+NaOH (Trevelyan, Procter & Harrison, 1950), confirmed that glucosamine was the only amino sugar in both the non-toxic fraction D1.S1 and the toxic fraction D2.S1.

The cysteine+sulphuric acid method of Dische (1955) showed some similarities in the spectra of fractions D1.S1 and D2.S1: hexose (and/or hexose derivatives), small quantities of heptose; but no significant quantity of pentose or 2-keto-3-deoxyoctulonic acid were detectable, from these spectra. More detailed analysis of carbohydrate and lipid components in fractions D1.S1 and D2.S1 is now under investigation.

Table 2. *Chemical analyses of purified fractions from Escherichia coli O78K80*

All results are averages of duplicate runs on similar samples.

Sample	N (%)	P (%)	Glucosamine (%)	Amino acid (%)	Fatty acid $\geq C_4$(%)
D1.S1	4·32	8·89	26·1 (27·8)*	1·2–1·4†	0·1
D2.S1	4·95	10·06	12·3	14·1–17·7	1·9

* Value obtained by the Dische (1955) procedure: other glucosamine contents obtained using the amino acid analyser.
† Limits defining 0–100% amino acid involved in chemical linkage.

Table 3. *Amino acid analyses of fractions D1.S1 and D2.S1*

Results are for 24 hr hydrolyses only.

	D1.S1		D2.S1	
	µmole amino acid/mg.	g. amino acid/100 g.	µmole amino acid/mg.	g. amino acid/100 g.
Glucosamine*	0·813	14·55	0·600	10·74
Lys.	ND†		0·087	1·27
His.	ND		0·029	0·45
NH_3	0·9007	—	0·765	—
Arg.	ND		0·065	1·13
Asp.	0·0151	0·20	0·211	2·31
Thr.	0·0129	0·15	0·104	1·24
Ser.	0·0318	0·33	0·086	0·90
Glu.	0·0132	0·19	0·152	2·23
Pro.	ND		0·059	0·68
Gly.	0·0168	0·13	0·157	1·18
Ala.	0·0146	0·13	0·178	1·58
½ Cys.	ND		ND	
Val.	ND		0·099	1·16
Met.	ND		0·020	0·30
Ile.	0·0045	0·06	0·062	0·81
Leu.	0·0045	0·06	0·124	1·52
Tyr.	ND			
Phe.	ND			
Totals‡	0·125	1·4	2·033	17·5

* Low results here due to extended period of hydrolysis.
† ND = None detectable (< 0·0005 µmole amino acid).
‡ Excluding glucosamine and ammonia.

DISCUSSION

Two essentially homogenous non-toxic fractions, D1.S1 and R, have been isolated from the extracellular material produced by *Escherichia coli* O78K80 by using two slightly different chromatographic procedures (Fig. 1). Apart from possible differences in trace impurities, these fractions were physically and chemically indistinguishable. Since higher yields were obtained by successive chromatography on DEAE-cellulose and Sephadex G 100, this method was chosen to prepare the non-toxic fraction in bulk. DEAE-cellulose offered a convenient tool for separating this weakly charged fraction from the endotoxic lipopolysaccharide-protein complex (D2.S1), which was strongly adsorbed at alkaline pH values, despite its apparent positive charge under these conditions (see electrophoretic experiments). The adsorptive capacity of DEAE-cellulose for the toxic complex may be partly due to a disaggregation of the complex

into negatively charged lipopolysaccharide and positively charged protein. A similar disaggregation of this complex occurred with Westphal's phenol + water extraction procedure (Marsh, unpublished observations.)

Aqueous ether or phenol + water extraction of the parent bacteria yielded components with similar physical characteristics to the non-toxic fraction D1.S1 (Figs. 7, 8). This fraction is almost certainly equivalent to 'native hapten' found in protoplasmic fractions (or whole bacteria) of several serotypes of *Escherichia coli* by Anacker *et al.* (1964), 1966). Both fraction D1.S1 and 'native hapten' possess not only similar sedimentation characteristics but also similar chemical and immunological properties, including the failure to stimulate the production of precipitating antibody in rabbits (Crutchley *et al.* 1967*b*). Jann's (1965) acidic polysaccharides, isolated from phenol + water extracts of *Escherichia coli*, also behaved in the ultracentrifuge similarly to non-toxic fraction D1.S1. The difference in toxicity between these two substances is probably due to the presence of endotoxin in 'acidic polysaccharide'; the charge difference between them may arise from a difference in the amounts of bound protein in the two substances.

Extracellular endotoxin (D2.S1) showed similarity in sedimentation behaviour to endotoxic preparations obtained from the parent bacteria by conventional extraction procedures. However, there was some evidence of a greater degree of aggregation in cell-wall endotoxins, particularly with the aqueous ether preparation. The higher peptide content of fraction D2.S1 as compared with other preparations of purified endotoxic lipopolysaccharide (Westphal *et al.* 1952; Ribi *et al.* 1964) is due to loosely bound protein which may be removed by phenol + water extraction (Marsh, unpublished observations).

By the mild fractionation procedures described in this paper, highly purified endotoxic, and non-toxic lipopolysaccharide-protein complexes were readily isolated from culture fluids of *Escherichia coli* O78K80, yielding substances which were essentially the same as those naturally occurring within the bacteria. The conventional procedures for extracting endotoxin from bacteria are more laborious than the method used here for obtaining free endotoxin. The vigorous extraction procedures which have been used previously (particularly those using phenol or trichloroacetic acid) probably lead to degradation of the endotoxic complex.

The relationship between fractions D1.S1 and D2.S1

Both Ribi *et al.* (1964) and Westphal *et al.* (1964) considered the toxic lipopolysaccharide unit of endotoxin to be an aggregate of polysaccharide sub-units held together by lipid moieties. This hypothesis is now further substantiated by the demonstration that sodium dodecylsulphate disaggregate both the free endotoxin liberated extracellularly (fraction D2.S1; Fig. 9*b*) and that extracted from the bacteria (fraction P1; Fig. 8*e, f*). Similar results were obtained by Oroszlan & Mora (1963) for endotoxin from *Serratia marcescens*. Disaggregation by sodium dodecylsulphate appears to be the mildest procedure available for obtaining 'haptenic' non-toxic sub-units from endotoxins.

The marked differences between the $[s^0_{20}]_{c=0}$ values for sub-units of endotoxic fraction D2.S1 and of the non-disaggregatable non-toxic fraction D1.S1, suggest that D1.S1 does not consist of simple sub-units of D2.S1. Differences of chemical composition between fractions D1.S1 and D2.S1 also support this conclusion. These

results show that fraction D1.S1 as a whole does not constitute a structural element of endotoxin (D2.S1). Immunological similiarity between the two fractions is probably associated with regions of chemical similarity, but this does not necessarily imply that part of non-toxic D1.S1 constitutes a structural element or a precursor of endotoxin (see Anacker *et al.* 1964). Therefore we find no justification, at present, in designating fraction D1.S1 as a 'native hapten' in the sense considered originally by Anacker and colleagues (1964). We reserve judgement on whether or not the non-toxic and endotoxic fractions are interrelated until more is known about the detailed structure of both substances.

We are particularly pleased to thank Mr J. M. Summerell for help with the ultracentrifugal experiments; also Dr A. W. Phillips, Dr Paley Johnson (Cambridge) and Mr A. W. Pink for making certain ultracentrifugal, amino acid, elemental and G.P.C. analyses, and Mr H. King for technical assistance. We are indebted to Drs M. Sterne and J. Cameron for helpful discussions.

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The Role of Tris in EDTA Toxicity and Lysozyme Lysis

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SUMMARY

Tris and EDTA combined to form a homologue which has far greater chelating ability than EDTA alone. The weaker serine and NH_4Cl homologues of EDTA replaced EDTA+tris in causing toxicity and permitting lysozyme lysis in *Azotobacter vinelandii*. Serine could effectively substitute for tris in these systems for *Pseudomonas aeruginosa*. These compounds had relatively little effect on *Escherichia coli*. The toxicity of EDTA+tris for these organisms could be alleviated by pre-incubation in physiological saline (0·15 M-NaCl). Subsequent lysozyme lysis was prevented by this treatment. The removal of metals from *A. vinelandii* cysts by EDTA+tris was also inhibited. The EDTA+tris+metallo chelate complexes were inactivated (dissociated) by saline.

INTRODUCTION

Many Gram-negative organisms which are resistant to lysozyme will lyse when ethylenediaminetetra-acetic acid (EDTA) and tris buffer (2-amino-2-hydroxymethyl-propane-1,3-diol) are also present in addition to lysozyme (Repaske, 1956, 1958; McQuillen, 1960*a*). Repaske first established a requirement for these compounds in this lytic system but did not determine the toxicity of the individual components. Socolofsky & Wyss (1961) were able to rupture cysts of *Azotobacter vinelandii* by using EDTA and tris. The released 'central bodies' were not viable and were lysed by adding lysozyme. Goldschmidt & Wyss (1966: to be published), while studying this toxic effect in greater detail, observed that the EDTA+tris system was toxic to vegetative organisms as well as to cysts even under conditions where cyst rupture did not occur (i.e. in 0·15 M-NaCl). EDTA suspended in water or 0·025 M-NaCl also ruptured the cyst exine, but the central bodies remained viable under these conditions. They also found that various other substances such as amines, amino acids and NH_4Cl could replace tris in producing toxicity in the presence of EDTA; these compounds also substituted for tris in the tris+EDTA+lysozyme system. These results are presented in the present paper. During this work, other bacteria were tested for their sensitivity to the EDTA+tris system (Goldschmidt, Goldschmidt & Wyss, 1967). Repaske (1958) noted that *Escherichia coli* was sensitive to his lytic system. Goldschmidt *et al.* (1967) found that male strains of *E. coli* (Hfr, male) were sensitive to the EDTA+tris system (65–95 % kill within 4 min.) while female strains and *E. coli* B/r (which is 'F°' and does not mate) were not killed. There are many reports in the literature discussing differ-

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ences in the surface configuration of male and female strains (Jacob & Wollman, 1961; Hayes, 1964; Maccacaro, 1955; Maccacaro & Comolli, 1956). Brinton, Gemski & Carnahan (1964) indicated that maleness is probably not a property of the cell wall but of specific pili projecting from it. These differences might play a role in EDTA + tris toxicity and subsequent lysozyme lysis. For these reasons, strains of different sexuality were also tested for their sensitivity to the EDTA + 'tris substitute' systems and subsequent lysozyme lysis under these conditions. *Pseudomonas aeruginosa*, which is very sensitive to EDTA, was also investigated.

METHODS

Media. Azotobacter vinelandii strain 12837 was grown at 33° on Burk medium (Burk & Lineweaver 1930) supplemented with 0·2 % butanol (as sole carbon source), 0·01 g. $MnCl_2$/l. and 2 % agar. The various strains of *Escherichia coli* (obtained from Dr E. Goldschmidt) were grown in Difco Penassay broth at 37°. *Pseudomonas aeruginosa* strain 160 (obtained from Dr C. Graber) was grown in Difco Penassay broth at 28°.

Harvesting procedures. Bacteria grown on agar were scraped off, resuspended in various diluents, agitated, strained through sterile cheesecloth and washed 2–3 times at room temperature by alternate centrifugation and resuspension in fresh diluent. Fifteen minutes in a Serval anglehead centrifuge Model SPX at 1400*g* sedimented the bacteria. Broth cultures were centrifuged and similarly washed.

Assay procedures. For the experiments concerned with EDTA toxicity and lysozyme lysis, organisms were suspended in the desired diluent and pipetted into optically calibrated sterile spectrophotometer tubes. The lytic agents were added so that the final volume of the reaction mixture was 4 ml. Lysozyme was always added immediately after EDTA. The action of EDTA and lysozyme was stopped by serial dilution in sterile 0·15 M-NaCl. The dilutions of *Azotobacter vinelandii* were streaked on solid Burk medium and incubated at 33°. *Escherichia coli* and *Pseudomonas aeruginosa* were similarly plated on Difco Penassay agar and incubated at 37° and 28° respectively. Per cent survival was determined by comparing the number of surviving colonies with the appropriate untreated controls. The changes in extinction (E) were determined by subtracting the extinction of the treated organism suspensions from that recorded for the untreated controls.

Extinction measurements. The Bausch and Lomb Spectronic 20, set at a wavelength of 600 mμ, was used to determine the extinction of suspensions.

Chemicals. The sodium salt of EDTA was dissolved in various diluents (NaCl, NH_4Cl serine, H_2O, etc.) depending upon the desired experimental conditions and adjusted to pH 8 with N-HCl and N-NaOH. Solutions of the sodium salt of dipicolinic acid (2,6-pyridinedicarboxylic acid: DPA) were similarly prepared. Tris was dissolved in de-ionized water and adjusted to pH 8 with N-HCl. Lysozyme was dissolved in water or in the various diluents. EDTA, lysozyme and DPA were freshly prepared for each experiment and sterilized by millipore filtration.

RESULTS AND DISCUSSION

Lysozyme lysis

The previous experiments of Goldschmidt & Wyss (1966) with tris substitutes posed a series of questions: Could various amino acids or NH_4Cl substitute for tris in preparing *Azotobacter vinelandii*, *Pseudomonas aeruginosa* and *Escherichia coli* for lysozyme lysis? Could these compounds similarly substitute for tris in causing EDTA toxicity in the latter organisms? If this occurred, was the toxicity also sex differentiated in *E. coli* as previously observed with EDTA + tris? (Goldschmidt *et al.* 1966). Could DPA substitute for EDTA in the lytic system?

The clearing of bacterial suspensions (measured quantitatively by the change in extinction) is commonly used to indicate the degree of lysis (Repaske, 1958; Socolofsky & Wyss, 1961; Herzberg & Green, 1964). Microscopic examination of our treated cultures supported these results. Lysis can be positively correlated with the decrease in extinction. As can be seen in Table 1, NH_4Cl could replace tris in the lytic EDTA + tris + lysozyme system for both *Azotobacter vinelandii* cysts and vegetative forms within the 4 min. experimental period. With the *Escherichia coli* strains, however (see Table 2), NH_4Cl or serine could not effectively substitute for tris in this brief time. Very little toxicity was noted, regardless of sex, although serine was slightly more toxic than NH_4Cl for the male strains. Only a slight decrease in extinction, if any, was noted when these compounds were substituted for tris.

Table 1. *Effect of various diluents on lysozyme lysis in Azotobacter vinelandii*

24 hr vegetative organisms and 14-day cysts washed twice and resuspended in the diluent. Final concentrations of reagents: 0·025 M-tris; 0·025 M-NH_4Cl; 0·25 mM-EDTA; 100 μg. lysozyme. Reaction time 4 min., pH 8·0. The change in extinction for the cysts in EDTA is due to rupture of the exine (outer shell) but not the central body (equivalent to the vegetative form). It is the additional lysis of the central body that is reflected in the EDTA + lysozyme column.

Cell type	Diluent	Survival in EDTA (%)	Change in extinction* EDTA	Lysozyme	EDTA + lysozyme
Vegetative	H_2O	65	0·040	+0·015	0·057
Cyst	H_2O	95	0·071	0·040	0·080
Vegetative	Tris	0·08	0·047	0·002	0·254
Cyst	Tris	0·01	0·294	0·035	0·464
Vegetative	NH_4Cl	0·34	0·036	0·012	0·202
Cyst	NH_4Cl	1·6	0·282	0·018	0·422

* The change in extinction represents a decrease compared to the untreated controls; an increase in extinction over that of the control is indicated by a +.

With *Pseudomonas aeruginosa*, serine could substitute for tris in preparing organisms for lysozyme lysis, but NH_4Cl had no effect (see Table 3). An increase in molarity (from 0·025 M to 0·05 M) increased both the amount of lysis obtained with serine and the speed of the reaction. There was no difference noted with NH_4Cl at the higher molarity even after exposure for 1 hr.

It is important to note that lysozyme lysis of male, female and B/r strains of *Escherichia coli* occurred regardless of the initial sensitivity to EDTA + tris when the

tris + EDTA + lysozyme system was used. This will be discussed in greater detail later in this paper. Neu & Heppel (1964) reported that *E. coli* B (similar to B/r) maintained a high degree of viability (60–90 %) when exposed to a sucrose + EDTA + tris mixture for 10 min.

Table 2. *Effect of various diluents on EDTA toxicity and lysozyme lysis in Escherichia coli*

Organisms washed twice and resuspended in the diluent. Final concentrations of reagents: 0·025 M-tris; 0·025 M-NH_4Cl; 0·025 M-Serine; 0·25 mM-EDTA; 100 µg. lysozyme. Reaction time: 4 min. pH 8·0.

Strain	Diluent	Survival in EDTA (%)	Change in extinction*		
			EDTA	lysozyme	EDTA + lysozyme
F^-	Tris	90	0	0·016	0·522
	NH_4Cl	85	0	0	0·033
	Serine	104	0·027	+0·068	+0·089
	H_2O	102	0·014	+0·180	+0·124
F^+	Tris	10	0·012	0·006	0·441
	NH_4Cl	100	0·036	0·036	0·036
	Serine	85	0·029	+0·013	0·054
	H_2O	100	+0·002	+0·029	0·007
Hfr	Tris	7	0·046	+0·008	0·316
	NH_4Cl	100	0·002	+0·016	0·075
	Serine	69	0·029	+0·081	+0·106
	H_2O	103	0·014	+0·101	+0·146
B/r	Tris	95	0·067	+0·068	0·308
	NH_4Cl	82	0·006	+0·005	+0·003
	Serine	80	0·014	+0·088	+0·089
	H_2O	100	0·003	+0·124	+0·097

* The change in extinction represents a decrease compared to untreated controls. An increase in extinction compared to the controls is indicated by a +.

The female strains of *Escherichia coli* eventually showed a decrease in viability after a lengthy exposure to EDTA + tris. When various strains were plated 2–4 hr later viability had decreased 30–60 %. During this time, viability in the males had decreased below 5 %. Therefore, both female and male strains were incubated for 2 hr with H_2O tris, serine or NH_4Cl and the various components of the lytic system (instead of the usual 4 min.). These data are presented in Table 4. There was no delayed lysis when NH_4Cl was substituted for tris, regardless of this longer exposure time. However, a considerable amount of lysis occurred in the absence of lysozyme when both male and female strains were incubated in EDTA + tris alone for this length of time. This explains the gradual loss in viability of the female strains. There was also some lysis with serine alone after a lengthy exposure (2 hr). Under these conditions, however, no additional lysis due to lysozyme was observed.

Pseudomonas aeruginosa was also lysed by 0·025 M-tris + EDTA. A small degree of lysis was observed at 4 min. However, longer exposure resulted in almost the same amount of lysis as found in the lysozyme system (see Table 3). It is also interesting to note that a higher molarity of tris (0·05 M) in combination with EDTA induced considerable lysis within the first 4 min. Herzberg & Green (1964) reported some lysis of

Salmonella typhimurium with EDTA + tris alone but found that far greater lysis occurred when lysozyme was also present in the system. Similar results with *P. aeruginosa* have been reported by Eagon & Carson (1965).

Table 3. *Effect of various diluents and time of exposure on lysozyme lysis in Pseudomonas aeruginosa*

24-hr shake-culture organisms were washed twice and resuspended in the diluent. Final concentration of reagents: diluents as shown in the table; 0·25 mM-EDTA; 100 μg. lysozyme. pH 8·0.

Diluent	Conc. (M)	Time of exposure (min.)	Control	Change in extinction*		
				EDTA	Lysozyme	EDTA + Lysozyme
Tris	0·025	4	0	0·078	+0·038	0 312
		60	0·033	0·158	+0·024	0 337
		120	0·058	0·182	+0·050	0 331
		180	0·042	0·294	0·005	0 350
		240	0·039	0·330	0·006	0 352
	0·05	4	0	0·133	0·056	0 338
		60	0·061	0·190	0·021	0 316
Serine	0·025	4	0	0·007	+0·062	0 006
		10	0·013	0·007	+0·056	0 083
		60	0·010	0·015	0·046	0 194
	0·050	4	0	0·017	+0·033	0 122
		50	0	0·040	+0·027	0 219
NH_4Cl	0·025	4	0	0·014	0	0 022
		60	0·013	0·017	0·008	0 051
	0·050	4	0	0·004	0·019	0 058
		50	0·019	+0·007	+0·019	0 076
H_2O		4	0	+0·026	+0·074	0 028
		60	0·004	+0·014	+0·112	0 057
		90	0·028	+0·064	0·026	0 030

* The change in extinction represents a decrease compared to the untreated controls at that time period; an increase in extinction over that of the control is indicated by a +. The change in extinction of the control represents the change from the initial (zero hour) reading.

Effect of dipicolinic acid (DPA)

DPA, another chelating agent, was tested for its ability to substitute for EDTA in inducing lysozyme lysis in these organisms. Other than the changes in optical density resulting from the initial rupture of the *Azotobacter vinelandii* cyst exine by DPA, there was no subsequent lysis of the central bodies when either the DPA + tris + lysozyme or DPA + NH_4Cl + lysozyme systems were tested. Similarly, *Escherichia coli* and *Pseudomonas aeruginosa* were not lysed when DPA replaced EDTA. Repaske (1958) had tested several other chelating agents such as versenol, 8-hydroxyquinoline and *O*-phenanthroline and noted an 'unusual specificity' for EDTA in his system. Gray & Wilkinson (1965*a*) also observed a similar specificity with *P. aeruginosa*. Since all the data comparing these organisms indicated that *A. vinelandii* and *P. aeruginosa* were somewhat more sensitive than *E. coli*, the degree of lysozyme lysis with the DPA system was also observed after a 1 hr exposure. No significant increase in lysis was noted with DPA + tris or DPA + lysozyme + tris after this longer exposure. One can

Table 4. *Effect of time on EDTA and lysozyme lysis of Escherichia coli*

Organisms were washed twice and resuspended in the diluent. Final concentrations of reagents: 0·025 M-tris; 0·025 M-NH_4Cl; 0·025 M-serine; 0·25 mM-EDTA; 100 μg. lysozyme. pH 8·0.

Strain	Time of exposure (min.)	Diluent	Change in extinction*		
			EDTA	Lysozyme	EDTA + lysozyme
F⁻	4	Tris	0·019	0·022	0·323
	120	Tris	0·236	0·026	0·402
	4	Serine	0·027	+0·068	+0·089
	120	Serine	0·106	+0·051	0·106
	4	NH_4Cl	+0·007	+0·001	0·055
	120	NH_4Cl	+0·008	0·018	0·054
	4	H_2O	0·014	+0·180	+0·124
	120	H_2O	0·023	+0·218	+0·063
Hfr	4	Tris	+0·008	0·024	0·219
	120	Tris	0·164	0·024	0·264
	4	Serine	0·029	+0·081	+0·106
	120	Serine	0·202	+0·034	0·079
	4	NH_4Cl	0·002	+0·016	0·075
	120	NH_4Cl	0·007	+0·011	0·079
	4	H_2O	0·014	+0·101	+0·146
	120	H_2O	0·029	+0·222	+0·080

* The change in extinction represents a decrease compared to the untreated controls. An increase in extinction over that of the control is indicated by a +.

conclude, therefore, that DPA cannot substitute for EDTA in preparing these organisms for lysozyme lysis.

Chelating ability of EDTA + tris

According to many reports in the literature (McQuillen, 1960*a*; Weidel, Frank & Martin, 1960; Herzberg & Green, 1964) the resistance of Gram-negative organisms to lysozyme lysis is due to the fact that the mucopolypeptide substrate is not accessible to the enzyme because the cell wall structure is more complex than in Gram-positive bacteria. The role of EDTA in mediating lysozyme lysis lies in its ability as a chelating agent to 'uncover' the mucopolypeptide layer either by removal of interfering ions or changing the surface charge or configuration. Once this has been accomplished, lysozyme can reach its substrate and lyse the cell walls (Weidel *et al.* 1960; Herzberg & Green, 1964; Voss, 1965). Leive (1965*a*, *b*) reported that EDTA treatment of *Escherichia coli* caused non-specific increases in permeability to such diverse compounds as actinomycin, *o*-nitrophenyl-galactoside and carbamyl phosphate. Other data of Goldschmidt & Wyss (1966: to be published) indicated that tris can act as a donor group to form a homologue with EDTA resulting in a more powerful chelating agent than EDTA alone. Martell & Calvin (1953*a*) also presented evidence for the increased stability and heightened activity of such 'EDTA-complexes' or 'homologues'.

Preliminary emission spectroscopic data (see Table 5) indicated that the removal of metals and subsequent toxicity of EDTA for *Azotobacter vinelandii* cysts was greatly influenced by the diluent used in association with EDTA. Magnesium, manganese and calcium were the metals affected to the greatest extent. In brief, EDTA + tris removed very large amounts of these three metals (the organisms were almost com-

pletely depleted and were not viable). No toxicity was observed in the absence of tris although magnesium was removed from the cysts in the H_2O+EDTA system (resulting only in the rupture of the cyst exine). Lysozyme lysis did not occur under these conditions. Eagon & Carson (1965) also reported the removal of calcium and magnesium from isolated cell walls of *Pseudomonas aeruginosa* when treated with tris + EDTA.

Table 5. *Effect of various diluents on the removal of metals from Azotobacter vinelandii cysts by EDTA*

14-day cysts washed twice and resuspended in the diluent. Final concentration of reagents: 0·025 M-tris; 0·15 M-NaCl; 0·25 mM-EDTA; 100 μg. lysozyme. Reaction time: [illegible] min. Emission spectrographs were obtained as follows. Treated cyst suspensions were centrifuged after EDTA treatment. The cysts were then placed in graphite cups, dried quickly and burned in an arc. The resulting spectrum was photographed. Metal concentrations were determined by density comparison with controls run on untreated cysts at various wave lengths on the plates. The degree of metal removal is indicated by a +.

	Diluents			
	Tris	H_2O	NaCl	NaCl + tris
Metals				
Magnesium	+ + + +	+ +	0	+
Manganese	+ + + +	0	0	+
Calcium	+ + + +	0	0	+
Toxicity (%)				
Cyst rupture	100	95	0	0
Survival	0·02	100	100	2·0
Lysozyme lysis (%)	90–100	None	None	5–10

+ + + + = No spectral line evident on spectrogram; 0 = spectral line same density as control.

Effect of NaCl on metal removal, EDTA toxicity and lysozyme lysis

According to reports in the literature the addition of salts render metallo + EDTA complexes unstable (Fleming, Ordal & Steinrauf, 1963; Wolin & Reichelt, 1964). Goldschmidt & Wyss (1966) also observed this effect with *Azotobacter vinelandii*. As can be seen in Table 5, very little metal removal was noted with EDTA + NaCl (0·15 M) and metals were removed to a much lesser degree with a EDTA + tris + NaCl system without previous incubation in NaCl, as compared to EDTA + tris alone. Lysozyme lysis was largely inhibited under these conditions even though the cysts were killed. When either *A. vinelandii* cysts or vegetative forms were shaken in 0·15 M-NaCl for several hours before exposure to EDTA + tris, 90 % survival resulted (see Table 6). Evidently, the increased incubation time in saline either allowed NaCl to permeate the cells or build up in high enough concentration at the cell surface as completely to dissociate or inactivate the metallo + EDTA + tris homologue. Gray & Wilkinson (1965*a*) reported an increase in survival of *Pseudomonas aeruginosa* from 0·01 % to 50 % when organisms were exposed to EDTA after washing in a 0·2 M-borate buffer instead of a 0·05 M-borate buffer. Their data might be explained on the basis of the dissociation of EDTA complexes at the higher molarity.

Escherichia coli B/r and Hfr strains and *Pseudomonas aeruginosa* were exposed to 0·15 M-NaCl for short periods of time in the presence and absence of tris. The degree of EDTA + tris toxicity and subsequent lysozyme lysis were observed. These data are reported in Table 6.

The addition of 0·15 M-NaCl to the EDTA+tris lysozyme system greatly inhibited lysozyme lysis in both strains of *Escherichia coli* as well as *Azotobacter vinelandii.* No lysis occurred in the *A. vinelandii* or B/r strains and only 10–30 % lysis was observed with the Hfr strain. Initial lysozyme lysis of *Pseudomonas aeruginosa* was also greatly inhibited when 0·15 M-NaCl was added (Table 6). Longer exposures (30 min. to several

Table 6. *Effect of NaCl on EDTA + tris toxicity and subsequent lysozyme lysis*

24-hr shake-culture organisms and 14-day cysts were washed twice and resuspended as indicated unless incubated between these steps in: (*a*) 0·15 M-NaCl for 15 min.; (*b*) 0·15 M-NaCl+0·025 M-tris for 15 min.; (*c*) 0·15 M-NaCl for 4 hr. Final concentrations: 0·025 mM-EDTA, 100 µg. lysozyme. pH 8·0. Reaction time: 4 min.

Organism	Diluent Systems: Wash	Diluent Systems: Resuspend	Survival in EDTA (%)	Change in extinction†: EDTA	Change in extinction†: Lysozyme	Change in extinction†: EDTA+ lysozyme
Escherichia coli						
Strain B/r	Tris	Tris	75	0·023	0·090	0·477
	H_2O	NaCl+tris	93	+0·022	+0·009	0·002
Strain Hfr G 6	Tris	Tris	11	0·046	0·067	0·316
	(*a*) H_2O	NaCl+tris	69	0·007	0·030	0·060
	(*b*) H_2O	NaCl+tris	19	0·010	0·060	0·095
	H_2O	NaCl+tris	25	0·001	0·011	0·075
Pseudomonas aeruginosa						
	Tris	Tris	13	0·086	+0·045	0·302
	Tris	NaCl+tris	23	0·002	+0·002	0·195
	NaCl	NaCl	86	0·002	0·004	0·050
	NaCl	NaCl+tris	51	0·021	0·029	0·150
	NaCl*	NaCl+tris	89	0·007	+0·006	0·083
	NaCl+tris	NaCl+tris	21	0·018	0·102	0·037
	H_2O	H_2O	100	+0·026	+0·074	0·028
	(*a*) H_2O	NaCl+tris	92	0·003	0·016	0·100
	(*b*) H_2O	NaCl+tris	58	0·008	0·010	0·173
	H_2O	NaCl	80	0·008	0·010	0·116
Azotobacter vinelandii cysts	Tris	Tris	0·01	0·294	0·035	0·464
	(*b*) H_2O	NaCl+tris	0·2	0·016	0	0·031
	(*c*) NaCl	NaCl+tris	92	0·004	—	—
	NaCl+tris	NaCl+tris	2	0·009	+0·001	0·029
Vegetative forms	Tris	Tris	0·1	0·047	0·002	0·254
	(*b*) H_2O	NaCl+tris	34	0	0·001	0·020
	(*c*) NaCl	NaCl+tris	88	0·007	—	—
	NaCl+tris	NaCl+tris	15	0·006	+0·001	0·018

* Organisms washed twice in 0·3 M-NaCl.

† The change in extinction represents a decrease compared to the untreated controls. An increase in extinction over that of the controls is indicated by a +.

hours) resulted in lysis in some cases, particularly with organisms washed in water or tris or pre-incubated in NaCl+tris. This lysis was actually not as extensive as that observed in the absence of saline; 0·3 M-NaCl afforded greater protection against EDTA+tris toxicity than 0·15 M-NaCl. Evidently the degree of dissociation of the EDTA+tris homologue in 0·15 M-NaCl was not high enough to prevent eventual lysozyme lysis in *P. aeruginosa* and higher concentrations might be required. However, lysis by tris+EDTA alone was completely inhibited in every case by 0·15 M-NaCl even after an exposure of several hours.

As can be seen in Table 6, when the Hfr strain of *Escherichia coli* (harvested in H_2O) was incubated for 15 min. in 0·15 M-NaCl before the other components were added, the survival in EDTA + tris + NaCl increased from 11 to 69 % while subsequent lysozyme lysis was 90 % inhibited. No doubt longer pre-incubation would have resulted in even greater survival. The B/r strain also showed an increased survival (from 75 % to 93 %) under these conditions. When tris was also present in the Hfr pre-incubation mixture, only an 8 % increase in survival was noted, while lysis was 70 % inhibited. An increase to 25 % survival was found when these organisms were exposed to this system without any previous incubation; here lysozyme lysis was still (77 %) inhibited. Thus, the degree of survival depended largely upon the conditions of pre-incubation with NaCl, while lysozyme lysis was inhibited as long as saline was present regardless of pretreatment. The data with *Pseudomonas aeruginosa* indicates a similar effect of 0·15 M-NaCl in increasing the survival of pseudomonads under these conditions (Table 6).

As already mentioned, EDTA + tris toxicity is sex-differentiated in *Escherichia coli*. However, subsequent lysis occurred when lysozyme was added (in the absence of NaCl) regardless of mating characteristics. This is another indication that the initial action of EDTA + tris is at the cell surface. Thus, the manner in which EDTA + tris 'prepares' organisms for lysozyme lysis does not necessarily produce lethality, since female and B/r strains are not killed by this step although they still become as susceptible to lysozyme lysis as the males. The toxicity observed with the male strains of *E. coli*, with *Pseudomonas aeruginosa* and *Azotobacter vinelandii* (which occurs within 4 min.), must be due to an additional action of EDTA + tris on other sensitive structures presumably within the organism or at the cytoplasmic membrane. There are many reports in the literature about the toxic effect of EDTA in the functioning of chromosomes and RNA (Kaufman & McDonald, 1957), on the inhibition of metallo-enzyme functions and on causing death by metal starvation (reviewed by Martell & Calvin, 1953*b*), etc. As previously mentioned, female strains of *E. coli* have surface characteristics different from those of the males (i.e. they behave as if they were more negatively charged and lack male specific pili). These differences may in some manner prevent additional EDTA + tris penetration to sensitive intracellular targets. In the males, evidently this penetration is not prevented since an 80–90 % kill was observed within 4 min. If the tris + EDTA homologue penetrated underneath the cell wall of the males via the hollow pilus, it would be very difficult to stop the toxic action by the usual method of adding NaCl to dilute and dissociate the complex. The continued intimate association of EDTA + tris would result in death probably by membrane damages, effect on chromosomes, etc. According to Dr C. C. Brinton (personal communication) this theory is entirely feasible.

The fact that NH_4Cl and/or amino acids can substitute for tris in producing toxicity to *Azotobacter vinelandii* and *Pseudomonas aeruginosa* but are not particularly effective with *Escherichia coli* indicates further differences in surface characteristics and sensitivity among these organisms. 'The sensitivity of an organism to EDTA should be dependent on the nature and strength of the bonds formed between the metal and the metal-binding components of the cell wall' (Gray & Wilkinson, 1965*b*). These authors considered *E. coli* to be only 'moderately sensitive' as compared with the 'exceptional' EDTA sensitivity of several other organisms such as *P. aeruginosa*.

Metals (both within and on the surface) are probably bound more tightly in

Escherichia coli. Serine+EDTA permitted lysozyme lysis within a few minutes of *Azotobacter vinelandii* and *Pseudomonas aeruginosa* but not of *E. coli*. A long exposure of several hours to tris or serine (+EDTA) was also needed here before lysis occurred in the absence of lysozyme, while the tris and serine homologues lysed *P. aeruginosa* after a brief exposure. These data indicated that the serine+EDTA homologue was a weaker complexing agent than tris+EDTA. The relative ineffectiveness of NH_4Cl for *E. coli* and *P. aeruginosa* indicated that it formed the weakest homologue of the three with EDTA.

The fact that NH_4Cl and serine formed weaker homologues with EDTA than tris might explain their failure to affect *Escherichia coli* to the same extent. According to Gray & Wilkinson (1965*a*), *Pseudomonas aeruginosa* is even sensitive to EDTA in borate buffer. The inhibitory effect of NaCl on EDTA+tris toxicity and subsequent lysozyme lysis also indicates the importance of the EDTA+tris complex in causing toxicity and preparing organisms for lysozyme lysis by virtue of its chelating activities. This supports the many reports in the literature which claim that the activity of EDTA is due primarily to its ability to form chelates. The EDTA+tris lysozyme system is commonly used in forming spheroplasts of Gram-negative organisms (McQuillen, 1960*a*). These spheroplasts can carry on limited synthetic functions such as enzyme synthesis and phage production, etc. (even an organism killed by ultraviolet radiation can carry out the latter function). According to Frazer (quoted in McQuillen, 1960*b*), however, these EDTA+tris spheroplasts never revert or divide and are 'nonviable' even though active synthetically.

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The Metabolism of Free Amino Acids by Washed Suspensions of the Rumen Ciliate *Entodinium caudatum*

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SUMMARY

Washed suspensions of *Entodinium caudatum*, grown *in vitro*, incubated anaerobically in the presence of penicillin and neomycin, incorporated single amino acids into the cell protein without conversion to any other amino acid. The ^{14}C-labelled components of the protozoal 'pool' and the medium were also investigated but no extensive catabolism of the amino acids observed. At low external concentrations the amino acids were taken up by an 'active' process, but above a critical concentration the amino acids entered the cell by passive diffusion and they have been divided into two groups depending on whether this critical concentration was approximately 0·0001 M or 0·001 M. The rate of amino acid uptake was not altered by the presence of inert particulate matter. Of the 75 % of the cell volume occupied by liquid, approximately two-thirds was freely available to amino acids.

INTRODUCTION

Although *Entodinium caudatum* can be grown *in vitro* in the presence of bacteria on a substrate of rice starch grains and dried grass (Coleman, 1960*a*) it has so far not proved possible to grow these protozoa in the absence of bacteria (Coleman, 1962). As a result, nothing is known about the amino acid metabolism of axenic rumen Entodiniomorphid protozoa although studies have been made by using protozoa prepared from *in vitro* cultures (Coleman, 1964*a*) and from the rumen and washed free from external bacteria. Protozoa from the rumen were used to show that ^{14}C was incorporated from ^{14}C-DL-alanine, DL-leucine and DL-valine by *Ophryoscolex caudatus* (Williams, Davis, Doetsch & Gutierrez, 1961) and *Epidinium ecaudatum* (Gutierrez & Davis, 1962) although with neither organism was the cellular distribution of the incorporated amino acid investigated. Warner (1964) found that rumen protozoa may contain a glutaminase and Abou Akkada & Howard (1962) with *Entodinium caudatum* obtained results consistent with this, but found negligible deamination of amino acids or uptake into cellular materials of the amino acids in casein or casein hydrolysate. However, these results were obtained by using non-radioactive methods, which would only detect large differences. In the present studies and those reported previously for glycine (Coleman, 1964*a*) the metabolism of ^{14}C-amino acids has been followed by using washed suspensions of *Entodinium caudatum* grown under standard conditions *in vitro* and an attempt made to determine, for 11 amino acids, the products of their metabolism and possible intermediates in this process, the kinetics of their incorporation by the protozoa and the permeability of the protozoa.

METHODS

Source of protozoa. The protozoa were grown and 'inoculum cultures' prepared and treated as described by Coleman (1962) except that inoculum cultures were treated each day with 15 mg. rice starch and about 10 mg. dried grass.

Preparation of protozoa for inoculation. The protozoa were taken from the inoculum cultures after removal of the surface scum and most of the supernatant liquid, and allowed to stand in 9 × 1 in. tubes until any grass present had sunk to the bottom. The supernatant fluid containing the protozoa was transferred to centrifuge tubes, the residual grass washed with salt solution B (Coleman, 1960*b*) and the washings added to the supernatant fluid. The protozoa were spun down and washed four times in salt solution B on a bucket-head centrifuge for 20 sec. from starting; the maximum speed was equivalent to 200*g*. The organisms were finally inoculated at a population density of 50,000–250,000/ml. incubation medium.

Incubation conditions for incorporation experiments. Except where otherwise stated the medium consisted of 1·8–8·2 ml. (chosen so that the final volume was 10 ml.) salt solution B (Coleman, 1960*b*), 0–6·0 ml. water, 0–6·0 ml. double strength salt solution B and 0·1–0·5 ml. ^{14}C-amino acid autoclaved (115° for 20 min.), all in a 15 ml. centrifuge tube. Standard salt solution contained 6·6 ml. salt solution B, 2·2 ml. water and the following additions. The concentration was altered by replacing the salt solution by water or the water and salt solution by salt solution of double the normal concentration. Immediately after removal from the autoclave the following additions were made aseptically: 0·2 ml. 1 % L-cysteine hydrochloride (neutralized and Seitz filtered), 0·2 ml. 5 % $NaHCO_3$ (Seitz filtered), 0·4 ml. penicillin G (25,000 units/ml.), 0·4 ml. 1 % neomycin sulphate solution and any other additions. After inoculation the tubes were gassed for 10 sec. with 95 % (v/v) N_2 + 5 % (v/v) CO_2, sealed with a sterile rubber bung and incubated at 39°. At the end of the experiment the protozoa were centrifuged and washed twice at 200*g* for 30 sec. in salt solution B.

Protozoal counts. The number of protozoa was estimated by the method of Coleman (1958). Only those protozoa which showed no signs of disintegration were counted.

Estimation of ^{14}C. ^{14}C in whole protozoa was estimated by washing the organisms with water onto an aluminium disc of area 4·7 cm.^{2} carrying a disc of lens tissue. The sample was spread by one drop of cetyltrimethylammonium bromide solution (5 mg./ml.) and fixed by one drop of polyvinyl alcohol (2 mg./ml.). The disc was dried at 40° and the ^{14}C estimated by using an automatic flow counter (Nuclear-Chicago Corp.) with an efficiency of about 20 %. Over 1000 counts at a rate greater than five times background were recorded for all fractions. Determinations were made with less than 0·5 mg. of material per cm.^{2} of disc. The amount of amino acid incorporated by protozoa was calculated from the known specific activity (usually 0·001–5·0 μC/μM) of amino acid added initially. For the estimation of non-volatile material in the medium 0·1–0·2 ml. was placed on an aluminium disc in the standard manner and then 0·1 ml. N-HCl added to remove the volatile material on drying down. To retain volatile compounds on the disc the HCl was replaced by 0·1 ml. 0·1 N-NaOH and the quantity present measured as the difference between the radioactivity determined in the presence of HCl and NaOH. As measurements of radioactivity on discs on which tracer quantities of theoretically non-volatile compounds such as ^{14}C-L-leucine had been plated out were lower after addition of HCl than after NaOH, the production of

volatile materials from amino acids was always corrected for 'volatile material' present initially and on incubation in the absence of protozoa. To determine the relative ^{14}C contents of spots on a chromatogram a thin mica end-window GM tube was placed directly on the spot, the position of which had been determined by radio-autography.

Fractionation of organisms. The protozoa after harvesting, washing as described above and resuspension in 2 ml. of water in a $5 \times \frac{1}{2}$ in. thin-walled test-tube were broken by immersion of the tube to the depth of liquid in the tube in a 80 kc/sec. 40 W. ultrasonic cleaning bath (KG 80/1, manufactured by Kerrys' of Chester Hall Lane, Basildon, Essex) for 15 sec. The homogenate was then centrifuged at 7000 *g* for 20 min. The supernatant liquid from this centrifugation is hereafter referred to as the 'broken cell supernatant fluid' and the pellet after washing once in water as the 'broken cell pellet'. This latter fraction contained all the viable bacteria in the homogenate.

These two fractions were further fractionated by a method based on that of Roberts *et al.* (1955). The fraction was treated with 5 % trichloroacetic acid (TCA) and allowed to stand at 4° for 30 min. The precipitate was centrifuged down and washed once with 5 % TCA. The supernatant fluid and the washing formed the 'cold TCA soluble fraction'. The precipitate was then extracted with 75 % (v/v) ethanol in water at 40° for 30 min. The residue was centrifuged down and the supernatant fluid formed the alcohol-soluble fraction. The precipitate was then extracted twice with 5 % TCA at 100° for 30 min. The supernatant fluids formed the 'hot TCA soluble or nucleic acid fraction'. The residue after further washing, once with acidified ethanol and once with ether, formed the 'residual protein fraction'. The TCA was removed from fractions by washing three times with ether before plating out for the estimation of ^{14}C. The alcohol-soluble fraction was further fractionated by the addition of equal quantities of water and ether and the two layers so obtained were separated. The aqueous layer after washing once with ether formed the 'alcohol soluble protein fraction' and the ether layer plus the washing formed the 'lipid fraction'.

The complete acid hydrolysis of any fraction was done by heating to 105° in 6 N-HCl for 16 hr in a sealed tube. At the end of this period the tube was cooled and opened and the acid removed on a boiling water bath in a current of air.

Paper chromatography. The following solvents were used: A, *sec*-butanol + formic acid + water (70 + 10 + 20, by vol.) at 30°; B, *sec*-butanol + formic acid + water (70 + 10 + 20, by vol.) at 4°; C, *sec*-butanol + formic acid + water (60 + 20 + 20 by vol.) at 4°; D, phenol + ammonia (sp.gr. 0·880) + water (80 g. + 0·3 ml. + 20 ml.); E, *n*-propanol + ethyl acetate + water (24 + 13 + 7, by vol.); F, ethanol + ammonia (sp.gr. = 0·880) + water (80 + 5 + 15, by vol.); G, *n*-butanol + acetone + water + di-ethylamine (10 + 10 + 5 + 2, by vol.; Hardy, Holland & Nayler, 1955); H, *n*-butanol saturated with 1·5 N-NH_4OH; J, *n*-butanol saturated with 1·5 N-NH_4OH/1·5 N-$(NH_4)_2CO_3$ buffer; K, *n*-butanol + cyclohexane + propylene glycol + water + ammonia (sp.gr. = 0·880) + morpholine (30 + 30 + 10 + 3·5 + 0·7 + 0·07, by vol.; Guillaume & Osteux, 1955).

Protein hydrolysates were chromatographed in two dimensions in solvents A and D (Roberts *et al.* 1955).

Identification of ^{14}C-labelled compounds in the medium and the protozoal 'pool'. As many of these compounds were present in small amounts and were often not detectable

on chromatograms by the use of conventional chemical sprays, their composition was elucidated by the following methods. The medium or the 'cold TCA-soluble fraction', after removal of the TCA with ether, was treated with Zeo Karb 225 (H^+) to remove free amino acids. The supernatant fluid was neutralized with 8 % (w/v) $Ba(OH)_2$ and the precipitate centrifuged down. If the presence of ^{14}C-labelled volatile acids was suspected, this supernatant fluid was evaporated to dryness in vacuum and the residue fractionated by paper chromatography. Otherwise the supernatant fluid was run through a column of Dowex 2 (OH′) which was then washed with water and any acidic compounds eluted with N-HCl. The eluates were evaporated to dryness in vacuum and the residues chromatographed. The basic compounds were eluted from the Zeo Karb 225 with N-NH_4OH and the eluate evaporated to dryness in vacuum.

The residues obtained from the resins as described above were chromatographed initially in solvent A and then the ^{14}C compounds present (as detected by radio-autography) eluted and re-chromatographed before and after hydrolysis in 6 N-HCl in solvents A–K and shown to run with the same R_F value as marker compounds. The identity of ^{14}C compounds provisionally identified by chromatography was confirmed by the 'fingerprint' technique of Roberts *et al.* (1955). For this method the unknown ^{14}C compound, in tracer quantities only, was mixed with a relatively large amount of the ^{12}C form of the compound with which it had been identified and the whole chromatographed in two dimensions. The ^{14}C spot was then detected by radio-autography and the carrier compound by a convenient chemical method. When the tracer and carrier compound are the same, then the pattern of the radio-autogram must agree in every detail with the pattern of the chemical spray.

The volatile acids produced during the metabolism of [U-^{14}C]-L-alanine were removed from the medium by steam distillation and then the distillate was neutralized, evaporated to dryness *in vacuo* and the residue chromatographed in solvent K which separated formic, acetic and propionic acids. The acidic material produced during the metabolism of [U-^{14}C]phenylalanine was extracted from the acidified medium with ether.

Chemicals. *N*-acetyl-DL-alanine and *N*-acetyl-DL-leucine were supplied by L. Light and Co. Ltd. Colnbrook. *N*-acetyl-L-glutamic acid was supplied by the British Drug Houses Ltd, Poole. *N*-formyl L-glutamic acid and α-*N*-formyl L-glutamine were synthesized by the methods of Tabor & Mehler (1954) and Borek & Waelsch (1953) respectively and *N*-formyl-DL-alanine by the method of Greenstein & Winitz (1961). The melting points and equivalent weights, obtained by titration with NaOH, of these last three compounds agreed with the values given in the literature.

^{14}C-compounds were supplied by the Radiochemical Centre, Amersham, Buckinghamshire. The specific activities of the compounds as supplied were, in μC/mg.; [U-^{14}C]-L-arginine, 113; [U-^{14}C]-L-aspartic acid, 226; [U-^{14}C]-L-glutamic acid, 262; [U-^{14}C]-L-isoleucine, 183; [U-^{14}C]-L-leucine, 320; [U-^{14}C]-L-lysine, 72; [U-^{14}C]-L-phenylalanine, 41; [U-^{14}C]-L-proline, 110; [U-^{14}C]-L-serine, 215 [;U-^{14}C]-L-valine, 59.

RESULTS

Washed suspensions of *Entodinium caudatum* prepared from growing cultures and incubated anaerobically in the presence of penicillin and neomycin incorporated ^{14}C from ^{14}C-labelled amino acids for 5 hr. Figure 1 shows the results obtained with

[U-^{14}C]-L-leucine. The amino acids could be divided into two groups depending on their rate of uptake by the protozoa when this was measured at an amino acid concentration of 0·01 mM in the presence of about 50,000 protozoa/ml. suspended in salt solution at 60 % of the standard concentration. The amino acids of group 1 which contained alanine, arginine, aspartic acid, glutamic acid and serine were incorporated at a rate of around 0·3–0·5 μg./10^7 protozoa/5 hr, and those of group 2 which contained isoleucine, leucine, methionine, phenylalanine, proline and valine at a rate of 3·0–4·0 μg./10^7 protozoa/5 hr. These results compare with a rate of 4·0 μg./10^7 protozoa/5 hr for glycine (Coleman, 1964*a*) under the same conditions.

The uptake of individual ^{14}C-amino acids present at 0·01 mM was also measured in the presence of 17 other ^{12}C-amino acids at 0·002 M. The complete 18 amino acid mixture contained L-alanine, L-arginine, L-aspartic acid, L-cysteine, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine and L-valine and the ^{12}C-form of the amino acid to be tested was omitted in each experiment. The addition of the 17 ^{12}C-amino acids had a variable effect; for example, they increased the incorporation of ^{14}C-alanine by 300 %, decreased that of ^{14}C-leucine by 75 % and had no effect on the uptake of ^{14}C-glutamic acid. A possible explanation of these results is provided below in the studies of the effects of individual ^{12}C-amino acids on the incorporation of ^{14}C amino acids.

Effect of amino acid concentration

At low amino acid concentrations (below 10^{-3} M) the rate of uptake of a group 2 amino acid was always greater than that for a group 1 amino acid, but over a certain critical concentration the rates for the two groups became similar. Above this concentration any increase in the amino acid concentration produced a proportionate increase in the amount of amino acid incorporated by the protozoa, i.e. the proportion of the original amino acid that was incorporated was independent of concentration. This is shown in Fig. 2, where the results are plotted as reciprocal of the ^{14}C incorporated over 3 hr against the reciprocal of the substrate concentration for serine (group 1) and leucine (group 2). It is apparent that there is a sharp break in both curves and that for serine occurs at a much lower concentration than that for leucine. Extrapolation of the right-hand part of the curve to the ordinate measures the maximum rate of uptake for this reaction at infinite amino acid concentration. The left-hand part of the curve always passes near to or through the origin. The results for all the amino acids tested are given in Table 1. This shows that the maximum rate for the right-hand part of the curve and the position of the break are much higher for group 2 than group 1 amino acids but that the slope of the left-hand part is similar for all amino acids. These results suggest that at high concentrations amino acids passed into the organisms by passive diffusion whereas at low concentrations below the break in the curve uptake could have been dependent on metabolic processes. The existence of a passive uptake is supported by the observations (*a*) that the proportion of the amino acid present, that was taken up, was independent of concentration and (*b*) that at infinite amino acid concentration an infinite amount would have been incorporated as shown by passage of the curves through the origin.

Effect of salt concentration on amino acid uptake

Figure 3 shows that within the limits of the salt concentration that the protozoa would tolerate without dying and at 0·01 mM amino acid concentration, there was little effect on the rate of uptake of group 1 amino acids but that the incorporation of

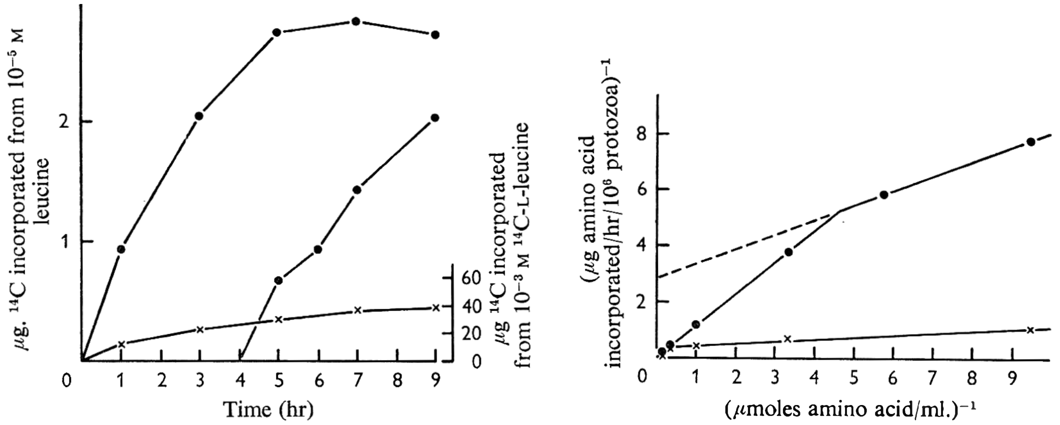

Fig. 1 Fig. 2

Fig. 1. Incorporation of 10^{-5} M (●—●) or 10^{-8} M (×—×) [U-^{14}C]-L-leucine added initially or after 4 hr by 10^7 *Entodinium caudatum* incubated anaerobically in the presence of 1000 units penicillin +400 μg. neomycin sulphate/ml.

Fig. 2. Effect of substrate concentration on the incorporation of [U-^{14}C]-L-serine (●—●) and [U-^{14}C] L-leucine (×—×) by *Entodinium caudatum* (48,000/ml.) in the presence of 1000 units penicillin +400 μg. neomycin sulphate/ml.

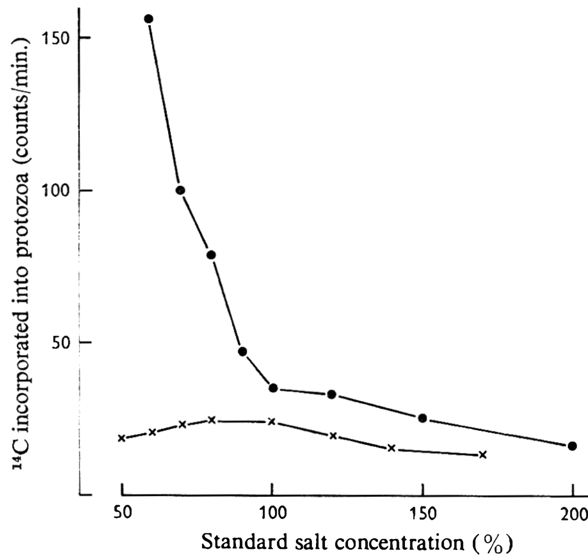

Fig. 3. Effect of salt concentration on the incorporation of ^{14}C from ^{14}C-labelled group 1 (×—×) or group 2 (●—●) amino acids (see text) by *Entodinium caudatum* incubated anaerobically in the presence of 1000 units penicillin +400 μg. neomycin sulphate/ml.

group 2 amino acids was markedly increased at low salt concentrations. The maximum velocity for the active uptake of phenylalanine (group 2) at 200 % of standard salt concentration was only 20 % of that at 50 % standard salt and the position of the break in the reciprocal uptake curve decreased from 0·003 M at 50 % to 0·0017 M at 200 % standard salt concentration.

Table 1. *The incorporation of amino acids by Entodinium caudatum*

Protozoa were incubated anaerobically at a population density of about 50,000/ml. in salt solution containing 1000 units penicillin and 400 μg. neomycin sulphate/ml. in the presence of 10^{-6}–2×10^{-2} M of each amino acid labelled with ^{14}C for 4 hr. The protozoa were then harvested, washed and their ^{14}C content determined. The quantity of amino acid incorporated was calculated from the known specific activity of the amino acid at each concentration tested, and the reciprocal of this value plotted against the reciprocal of the substrate concentration (Fig. 2). From these curves was calculated the maximum velocity of the active process (by extrapolation of the right-hand part of the curve to the ordinate), the position of the break in the curve and the slope of the left-hand part.

Amino acid	Group	Maximum velocity (μg./hr/10^6 protozoa)	Break at (mM)	Slope of left-hand curve (10^6 protozoa × hr/ml.)
L-Alanine	1	0·21	0·12	$1\cdot3\times10^3$
L-Arginine	1	0·73	0·22	$2\cdot2\times10^3$
L-Aspartic acid	1	0·09	0·10	$1\cdot8\times10^3$
L-Glutamic acid	1	0·09	0·20	$4\cdot2\times10^3$
L-Isoleucine	2	3·6	1·5	$1\cdot0\times10^3$
L-Leucine	2	2·88	2·5	$1\cdot2\times10^3$
L-Lysine	.	0·12	0·086	$1\cdot6\times10^3$
L-Phenylalanine	2	1·45	3·0	$3\cdot3\times10^3$
L-Proline	2	1·24	1·2	$1\cdot3\times10^3$
L-Serine	1	0·35	0·22	$1\cdot2\times10^3$
L-Valine	2	1·70	2·0	$1\cdot7\times10^3$

Effect of pH value on amino acid incorporation

Figure 4 shows the effect of variations in the pH value of the standard salts medium on the uptake of ^{14}C-isoleucine (group 2) at low and just above standard salt concentrations. At low salt concentration the optimum pH value was 6·8–7·0 but at the higher salt concentration there was no clearly defined optimum pH value.

Effect of particulate matter on the incorporation of amino acids

Holter (1965) showed that in *Amoeba proteus* which was taking up protein particles from the medium by pinocytosis only 5 % of the liquid in which the protein was suspended was taken up at the same time. Since *Entodinium caudatum* rapidly engulfs bacteria (Coleman, 1964*b*) and other particulate matter the effect of these materials on the incorporation of ^{14}C-amino acids from the medium was examined. Of the substances tested 0·02 % rice starch grains, 0·1 % casein, 10^8 polystyrene particles/ml. (approximately 2 μ in diameter) had no effect, whereas heated *Escherichia coli* and 10^8 sulphonated-polystyrene particles/ml. stimulated ^{14}C-leucine uptake by 10–40 % and live *E. coli* uptake by up to 100 %. In the presence of the live *E. coli* as much or more ^{14}C was found in the bacteria as in the protozoa after incubation for 3 hr but none was bound to the other particulate materials after these had been washed twice in salt solution. Nevertheless, since polystyrene particles stimulated when sulphonated but not when unsubstituted, it is possible that some amino acid molecules may be loosely

bound to the negatively charged sulphonyl groups even at pH 7 and be transported into the organism. It is considered that particulate matter *per se* does not increase the rate of amino acid incorporation, but that in the presence of charged particles or complicated particles such as bacteria, live or dead, some additional amount of amino acid was taken up associated with the particles.

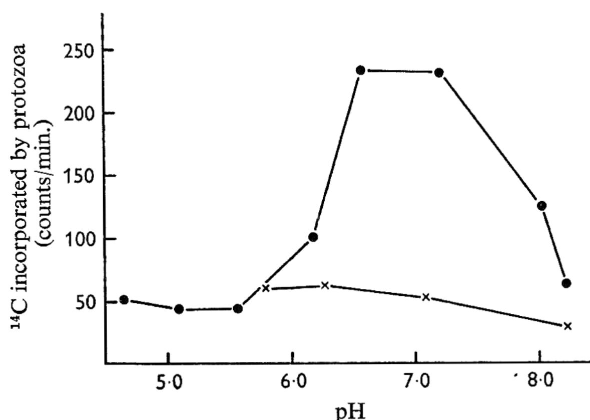

Fig. 4

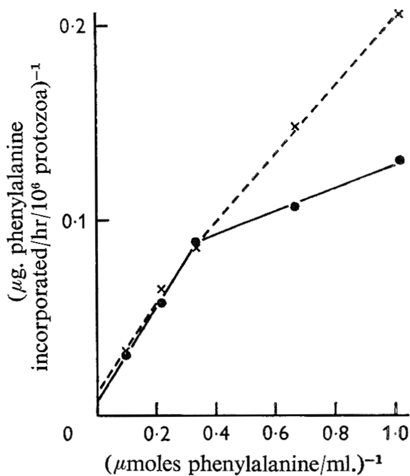

Fig. 5

Fig. 4. Effect of pH value on the incorporation of [U-^{14}C]-L-isoleucine (0·2 μg. and 40,000 counts/min.) by *Entodinium caudatum* (4·4 × 10^4/ml.) in the presence of 1000 units penicillin + 400 μg. neomycin sulphate/ml. Duration of experiment 3 hr. ●—●, 50 % of standard salt concentration; ×—×, 120 % of standard salt concentration.

Fig. 5. Effect of substrate concentration on the incorporation of [U-^{14}C]-L-phenylalanine by *Entodinium caudatum* incubated anaerobically in the presence of 1000 units penicillin + 400 μg. neomycin sulphate/ml. and in the presence (× ---- ×) or absence (●—●) of 0·01 M-DL-*p*-fluorophenylalanine.

Effect of p-*fluorophenylalanine on phenylalanine uptake*

If the uptake of amino acids consists of an active and a passive process, then the passive part should be extendable by the inhibition of the active process. In an attempt to find a suitable inhibitor the effect of various amino acid analogues on the uptake of their parent amino acids was investigated. *p*-Fluorophenylalanine was found to inhibit the incorporation of ^{14}C-phenylalanine and at 0·01 M-DL-*p*-fluorophenylalanine the same proportion of the added phenylalanine was incorporated at all substrate concentrations. Figure 5 is a reciprocal plot of the amount of ^{14}C-phenylalanine incorporated in 5 hr against phenylalanine concentration and shows that in the presence of *p*-fluorophenylalanine the discontinuity in the curve was almost completely abolished and that the slope of the curve was that of the passive reaction. Table 3 shows that in the presence of *p*-fluorophenylalanine the incorporation of ^{14}C from ^{14}C-phenylalanine into the protozoal protein was markedly decreased.

Gale (1947) showed that it was possible to distinguish between the uptake of amino acids into bacteria by an active or diffusion process by investigating the effect of a 10° alteration in temperature (the temperature coefficient = rate at $t+10°$/rate at $t°$). Davson & Danielli (1952) and Bull (1951) showed that the temperature coefficient for

a diffusion process should be 1·32, whereas that for an enzymic reaction was usually higher. To determine the temperature coefficient for the incorporation of ^{14}C-phenylalanine by *Entodinium caudatum* the uptake in the presence and absence of *p*-fluorophenylalanine was measured at 30° and 40°. In the presence of the inhibitor the temperature coefficient was 2·0 at all phenylalanine concentrations, and in the absence of inhibitor it was 3·4 and 1·9 at low and high phenylalanine concentrations, respectively. Although these results give further support to the suggestion that the mechanism of uptake at high phenylalanine concentrations and at all phenylalanine concentrations in the presence of *p*-fluorophenylalanine are similar, all the values for the temperature coefficients were much higher than those of 1·4 and 1·96 found by Gale (1947) for the uptake of lysine and glutamic acid, respectively, by *Streptococcus faecalis*. A possible explanation of this discrepancy may be found in the observation that at 30° the protozoa became almost non-motile and formed a tight pellet on the bottom of the tubes. At 30° the diffusion path for amino acid molecules from the bulk medium into the pellet and then inside the protozoa would certainly be much longer than at 40° where the protozoa were actively motile and were directing medium into their gastric sacs by organized ciliary activity. This effect of a 10° alteration in temperature would not occur in bacterial suspensions and hence the temperature coefficient obtained with protozoal suspensions could be larger.

Effect of amino acids on uptake of individual amino acids

In an attempt to find an explanation for the variable effect of a complete mixture of amino acids on the uptake of individual ^{14}C-amino acids the effect of single ^{12}C-amino acids at 0·01 M on the incorporation of individual ^{14}C-amino acids at 0·01 mM was investigated. Table 2 shows those amino acids out of L-alanine, L-arginine, L-asparagine, L-cysteine, L-glutamic acid, L-glutamine, glycine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine and L-valine which decreased or stimulated the incorporation of another amino acid by over 50 %. The majority of amino acids altered the incorporation of any particular amino acid by less than 10 %. For those experiments in which the effect of ^{12}C-L-cysteine was studied the cysteine was omitted from the standard medium and the traces of oxygen removed by bubbling O_2-free N_2 through the medium for 3 min. It is apparent that only those amino acids which are structurally related to the given ^{14}C-amino acid affected its uptake; this suggests that each amino acid may have a specific uptake mechanism. For all the amino acid mixtures listed in Table 2 the effect of the inhibitory amino acids tested at concentrations of the ^{14}C-amino acid from 0·01 to 20 mM but only with the pairs ^{14}C-phenylalanine + ^{12}C-*p*-fluorophenylalanine and ^{14}C-aspartic acid + ^{12}C-asparagine was the incorporation of the ^{14}C-amino acid independent of its concentration in the presence of 0·01 M inhibitor. This shows that only with these pairs was the active uptake reaction completely abolished by the inhibitor. However, the aspartic acid/asparagine effect might be an artifact as some of the asparagine was hydrolysed to aspartic acid during the experiment.

Intracellular products of amino acid metabolism

Table 3 shows the distribution in the major cell fractions of ^{14}C incorporated from ^{14}C-phenylalanine and is typical of that for any amino acid of group 2. Group 1 amino acids gave a similar pattern except that 40–50 % of the ^{14}C was in the cold TCA-soluble

fraction or cell 'pool'. To determine whether there had been any interconversions between the amino acids, the protein in the broken cell supernatant fluid and in the broken cell pellet were hydrolysed, the amino acids chromatographed on paper in two

Table 2. *Inhibition of the incorporation of individual ^{14}C-amino acids into Entodinium caudatum by other ^{12}C-amino acids*

Protozoa were incubated anaerobically at a population density of about 50,000/ml. in salt solution containing 1000 units penicillin and 400 μg. neomycin sulphate/ml. in the presence of about 0·2 μg. of individual ^{14}C-amino acids for 5 hr in the presence or absence of other ^{12}C-amino acids at 0·01 M. Only those ^{12}C-amino acids which decreased the incorporation of ^{14}C by over 50% under these conditions are listed below.

^{14}C-amino acid	Incorporation inhibited by:		Incorporation stimulated by:	
L-Alanine	L-serine	59%	None	
	L-cysteine	61%		
L-Aspartic acid	L-asparagine*	75%	None	
	L-glutamic acid	74%		
L-Glutamic acid	L-aspartic acid	58%	None	
	D-glutamic acid	52%		
	L-glutamine	70%		
Glycine	L-phenylalanine	73%	None	
	L-arginine	51%		
L-Isoleucine	None		L-valine	105%
L-Leucine	L-isoleucine	50%	L-valine	112%
	DL-*p*-fluorophenylalanine	76%		
L-Phenylalanine	DL-*p*-fluorophenylalanine*	95%	None	
L-Valine	None		L-leucine	98%
			L-isoleucine	91%

* In the presence of these ^{12}C-amino acids, the incorporation of ^{14}C was independent of the concentration of ^{14}C-amino acid.

Table 3. *Distribution of ^{14}C in the cell after the incorporation of [U-^{14}C-]-L-phenylalanine by Entodinium caudatum*

Protozoa were incubated anaerobically for 5 hr in salt solution containing 1000 units penicillin and 400 μg. neomycin sulphate/ml. in the presence of 240 μg. and 10 μC [U-^{14}C]-L-phenylalanine in the presence or absence of 0·01 M-DL-*p*-fluorophenylalanine. After incubation the washed protozoa were broken in a Potter homogenizer and the supernatant fluid and pellet fractions separated by centrifugation. These were further fractionated as described under Methods.

	Radioactivity (counts/min.)			
	Broken cell supernatant fluid		Broken cell pellet	
Protozoa incubated with *p*-fluorophenylalanine	−	+	−	+
Cold trichloroacetic acid-soluble fraction	600	350	350	100
Ethanol-soluble protein	1950	450	2750	650
Lipid	50	0	0	50
Nucleic acid	900	250	1650	100
Residual protein	4300	250	6500	100

dimensions and radio-autograms prepared. With each of the amino acids tested there was only one ^{14}C spot present and this chromatographed in the same position as a marker spot of the original ^{14}C-amino acid. No evidence was found of any amino acid interconversions in any of the cell fractions examined.

The cold TCA-soluble material in the broken cell supernatant fluid of protozoa which had incorporated ^{14}C from a group-2 amino acid labelled with ^{14}C contained only one radioactive compound, and this was always the free amino acid with which the protozoa had been incubated. With group 1 amino acids the pool usually contained two or more radioactive compounds, but as these were present in such small quantities only a few were identified. After incubation with ^{14}C-alanine the pool contained alanine and *N*-acetylalanine and after incubation with glutamic acid, glutamic acid and *N*-formylglutamine were present.

Incubation of protozoa with ^{14}C-L-leucine in the presence of high concentrations of salts (200 % of standard) decreased incorporation of ^{14}C into the protozoal protein by 92 % and into the pool by 75 % as compared with incubation in a low concentration of salts (50 % of standard). However, the only radioactive material in the pool or the protein was still leucine after incubation in high salt concentrations.

Extracellular products of amino acid metabolism

In this section the results are for the quantity of the extracellular products formed when measured after the metabolism of tracer quantities of the amino acids by approximately 10^6 protozoa suspended in 3 ml. medium for 4 hr. The results have been expressed per 100 μmoles carbon incorporated into the protozoa.

Alanine. The products were acetic acid 46 μmoles, *N*-acetylalanine 740 μmoles and *N*-formylalanine 100 μmoles. The latter two compounds were separated by chromatography in solvent C.

Glutamic acid. Acid-volatile products, 14 μmoles; *N*-formylglutamic acid, 250 μmoles; α-*N*-formylglutamine, 250 μmoles. The quantities of *N*-formylglutamic acid and *N*-formylglutamine were very variable and even after incubation for 50 hr were less than 5 % of the ^{14}C still present as glutamic acid. The absence of *N*-acetylglutamic acid was shown by chromatography in solvent B.

Leucine. Isovaleric acid 230 μmoles. The identification of this compound is reported in the following paper (Coleman, 1967). Attempts to demonstrate the formation of ammonia during the metabolism of 0·005 M-L-leucine by 10^6 protozoa/ml. were unsuccessful; there was no increase over the basal formation on the addition of the amino acid. Since *N*-acetylated amino acids were a common product of amino acid metabolism and since leucine and *N*-acetyl-leucine were not separated by chromatography in any of the standard solvent systems used previously (Coleman, 1964*b*), solvent A was used at 4° (i.e. as solvent B) instead of 30° to effect this separation. No ^{14}C-*N*-acetyl-leucine was detected after incubation of protozoa with ^{14}C-leucine.

Phenylalanine. Phenylacetic acid, 140 μmoles. The absence of phenylpropionic acid was shown by chromatography in solvent J. Since the protozoa used in these experiments contained bacteria (Coleman, 1962; White, 1966) it was possible that these were responsible for the formation of the fatty acids, despite the fact that penicillin and neomycin were present during the incubations. To test this possibility suspensions of intact and broken protozoa (which still contained viable bacteria) were incubated in the presence and absence of penicillin and neomycin with ^{14}C-leucine, and the amount of ^{14}C-volatile material produced was measured at intervals. After 6 hr the proportion of the initial ^{14}C-leucine which was rendered volatile was 3 % with broken protozoa under both conditions and 11 % and 22 %, respectively, with intact protozoa in the absence

or presence of the antibiotics, showing that the bacteria were not responsible for the formation of volatile material from leucine.

Permeability of Entodinium caudatum to amino acids

The results quoted above for the uptake of amino acids by *Entodinium caudatum* suggested that the protozoa were freely permeable to all amino acids and that the uptake of amino acids of group 1 and group 2 differed only in the active part of the process. To seek further evidence for this hypothesis an attempt was made to measure the proportion of the volume of a protozoon which was readily permeable to amino acids and other small molecules, by using a method based on that of Mitchell (1953). After the standard washing procedures the protozoa were made up in a very thick suspension such that on centrifugation the packed protozoal pad was approximately half the total volume. The experiments were made in 10 ml. graduated conical centrifuge tubes, the calibrations of which had been checked at 0·2, 0·5, 1·0 and 1·5 ml. Samples of 0·2 ml. 0·005 or 0·05 M-^{14}C-amino acid or other compound (1–10 μC/μM) and 1·0 ml. protozoal suspension or 1·0 ml. salts solution at the experimental temperature were mixed together rapidly in these tubes and 30 sec. later the whole was centrifuged for a total time of 45 sec. on a bucket-head centrifuge (final speed equivalent to 500 **g**). The volume of the protozoal pellet was measured and then the supernatant fluid removed and 0·1 ml. used for the estimation of ^{14}C. The time between mixing and the removal of the supernatant fluid was less than 5 min. When the experiments were done at 20° the protozoa were undamaged at the end, as determined microscopically, but when they were placed at 4° or 39° for 5 min. before mixing with the ^{14}C-amino acid there were signs of protozoal disintegration. For this reason, the time of exposure was kept to a minimum in experiments at 4° and 39°. Except where stated, all experiments were made at 20°. It was also necessary to use amino acids at at least 0·001 M because in the presence of tracer amounts only there was sufficient incorporation of ^{14}C to invalidate the results. The results were calculated as follows. The total amount of ^{14}C added to the system in the presence or absence of protozoa was calculated from the ^{14}C present in 0·1 ml. of medium in the absence of protozoa. From this value and the amount of ^{14}C present in 0·1 ml. supernatant fluid above the protozoa, the volume of liquid available to the amino acid in the presence of protozoa was calculated. The difference between this value and the total volume of liquid gave the volume impermeable to the amino acid and this was compared with the volume of the protozoa present. The percentage of the protozoal volume impermeable to the amino acid was given by

$$100 \times [\text{total volume} - (A/10B)]/\text{protozoal volume},$$

where, in counts/min., A = total amount of ^{14}C and B = ^{14}C present in 0·1 ml. supernatant fluid in the presence of protozoa.

Under these conditions $51 \pm 16\%$ (for 10 different batches of protozoa; range 31–72%) of the packed cell pad volume was impermeable to L-leucine and $59 \pm 13\%$ to L-aspartic acid. The amino acids L-alanine, L-glutamic acid, glycine, L-lysine, L-methionine and L-serine and also glucose, acetate and lactate gave similar results. In experiments with leucine the impermeable volume was not altered by halving the standard salt concentration, raising the temperature to 39° or lowering it to 4°. When the protozoal pellets in the above experiments were resuspended in salts medium and

the protozoa washed twice on the centrifuge, over 90 % of the intracellular ^{14}C was released. When the low molecular weight compounds were replaced by ^{14}C-*Escherichia coli* the impermeable volume was increased to 86 %. Since Conway & Downey (1950) showed theoretically that in a packed pad of spheres 26 % of the total volume was inter-particulate and since Roberts *et al.* (1955) found 10 % of intercellular water in a packed pad of *E. coli*, it is suggested that little of the protozoon was permeable to bacteria at 20°. However, it has been shown previously (Coleman, 1964*b*) that the gastric-sac volume of a protozoon as determined from the maximum number of bacteria that could be engulfed at 39° was $0{\cdot}8\text{–}2{\cdot}2 \times 10^4\ \mu^3$. The total volume of a protozoon was measured as $4{\cdot}7 \times 10^4\ \mu^3$ by centrifugation of a suspension containing a known number of protozoa for 5 min. at 500*g* and measuring the packed cell pad volume. Although this result includes the interstitial water surrounding each protozoon and is therefore too large, calculation from these results shows that the gastric sac, penetrable by *Escherichia coli*, when the protozoa were actively engulfing bacteria, occupied approximately 17–47 % of the volume of each protozoon, i.e. the impenetrable volume was 53–83 %, a similar value to that obtained for amino acids. To determine whether the impenetrability of 50–60 % of the protozoon was due to a permeability barrier or to the presence of solid material such as starch, the impermeable volume was measured in the presence of 500 μg. cetyltrimethylammonium bromide (CTAB)/ml. to break any permeability barriers and was found to be only 25 % of the protozoal volume. This result agreed with the finding that 74 % of the weight of a packed pad of protozoa was lost on drying in an oven at 105°, i.e. that 74 % of the pad was water. The starch present in the protozoa occupied 15–20 % of the protozoal volume as determined by centrifugation of sonically-treated protozoa for 30 min. at 3000*g* and measuring the volume of the white material at the bottom of the pellet. From the difference in the leucine-impermeable and CTAB-impermeable volumes it is apparent that 25 % of the cell volume was not permeable to amino acids or to bacteria. Since the protozoal ectoplasm never contained engulfed *E. coli* and was separated from the endoplasm by a distinct membrane (Coleman & Hall, 1966) it is tentatively suggested that the ectoplasm may not be permeable to amino acids in short-time experiments at 20°. Likewise, since ^{14}C-labelled compounds were found in the cold TCA-soluble pool of protozoa which had been incubated with ^{14}C-amino acids for several hours and then washed, it is apparent that compounds in some part of the protozoal cell, possibly the ectoplasm, did not rapidly diffuse into the medium.

DISCUSSION

Bryant & Robinson (1963) showed that many pure strains of rumen bacteria utilized ammonia in preference to amino acids for growth and when provided with amino acids as sole source of nitrogen incorporated little amino acid carbon into cellular materials. Since it is not possible to grow these protozoa in the absence of bacteria (Coleman, 1962) the nitrogenous compounds essential for growth cannot be determined directly, but from the absence of any amino acid interconversions it is suggested that *Entodinium caudatum* probably utilizes intact amino acids rather than forming them from carbohydrate and ammonia. Lewis (1955), who used crude suspensions of rumen bacteria, showed that some amino acids were extensively broken down to fatty acids, carbon dioxide and ammonia. More recently Lewis & Emery (1962) and

Menahan & Schultz (1964) showed that a common product from the metabolism of amino acids by crude rumen contents was an acid containing one carbon atom less than the parent amino acid, e.g. leucine was metabolized to isovaleric acid. The present results indicate that although *E. caudatum* catabolized amino acids very slowly the type of product was characteristic of that obtained with other rumen micro-organisms.

Although these experiments were made in the presence of antibiotics, it was possible that the intracellular bacteria, e.g. Bacterium 31 (Coleman, 1964*b*) which can grow with ammonia as sole nitrogen source and might therefore be expected to interconvert amino acids, might metabolize the amino acids before they could be incorporated into protozoal protein. However, the absence of any amino acid interconversions by either the broken cell supernatant fluid or the pellet fractions and of any volatile fatty acid production from leucine in the presence of broken protozoa suggest that the bacteria were of little importance under these conditions.

The results show that *Entodinium caudatum* has a definite but limited ability to concentrate all amino acids inside the organism. This active process, which is only important at low external concentrations, was inhibited by specific compounds (such as *p*-fluorophenylalanine on phenylalanine incorporation), was temperature-sensitive, was affected by salt concentration in the medium and had a finite maximum velocity which was dependent on the nature of the amino acid. At higher external concentrations the amino acids were taken up by a different process which was not inhibited by specific inhibitors, was less temperature-sensitive, was less affected by the salt concentration and had an almost infinite maximum velocity. The permeability studies made with thick protozoal suspensions at 20°, where the total ^{14}C-amino acid uptake was less than 15% of that at 39°, probably related to this latter passive process and showed that low molecular weight compounds in the medium could freely penetrate at least the endoplasm of the organism. Presumably the amino acids slowly diffuse into the organism under all conditions and the active uptake process is superimposed on this slow movement. For the amino acids discussed in this paper the rate of passive uptake always exceeded the rate of active uptake above 0·003 M, but for glycine (Coleman, 1963) the maximum velocity of the active process was over five times that for leucine and even at 0·1 M the active uptake was faster than the passive. It is of interest that *Streptococcus faecalis* (Gale, 1947) incorporated some amino acids, e.g. glutamic acid, by an active process at all concentrations, whereas lysine was taken up by a passive diffusion process at all concentrations.

Since all the measurements on the uptake of amino acids were of incorporation into the whole protozoon and since there was a comparatively small amount of ^{14}C found in the cell pool this suggests that it was the uptake mechanism that was the rate-limiting step in the passage of amino acids from the supernatant fluid into cell-protein. This barrier can be overcome by giving the amino acids as whole bacteria; it has been shown (Coleman, 1964*b*) that the same quantity of leucine was utilized much more economically by the protozoa when fed in the form of intact bacteria than when given as free amino acid.

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The Metabolism of the Amino Acids of *Escherichia coli* and Other Bacteria by the Rumen Ciliate *Entodinium caudatum*

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SUMMARY

Non-multiplying and growing cultures of *Entodinium caudatum* incubated anaerobically engulfed *Escherichia coli* bacteria specifically labelled with individual ^{14}C-amino acids and incorporated the amino acids into protozoal protein without conversion to any other amino acid. The protozoal cisternae 'pool' and the medium contained the free amino acid and with some amino acids the *N*-acetyl or *N*-formyl derivative in addition. The constituents of the pool were probably by-products of the metabolism of the bacteria and not intermediates between bacterial and protozoal proteins. There was no extensive catabolism of the bacterial amino acids by the protozoa, although some of the bacterial leucine, isoleucine and valine was broken down to isovaleric acid, α-methylbutyric acid and isobutyric acid, respectively. The addition to the medium of the ^{12}C-form of the ^{14}C-amino acid present in the *E. coli* decreased the incorporation of ^{14}C into the protozoa with half of the amino acids tested. The rate of loss of viability of various other bacterial species after engulfment by the protozoa was found to be independent of Gram-reaction, size or natural habitat.

INTRODUCTION

It was shown by Coleman (1964) that washed suspensions of *Entodinium caudatum* grown *in vitro* engulfed bacteria from the medium and that when the bacterium studied was *Escherichia coli* this was rapidly killed and digested. The digestion products were then liberated into the medium or incorporated into protozoal protoplasm. It was also shown that when the protozoa were offered two different species of bacteria, these were engulfed in the proportion in which they were present in the medium. The purpose of the present paper is to extend these studies by investigating the fate of these bacteria inside the protozoa and by the use of bacteria specifically labelled with ^{14}C-amino acids to elucidate the metabolism of these amino acids by the protozoa.

METHODS

Source of protozoa. Entodinium caudatum was grown and inoculum cultures prepared and treated as described by Coleman (1962) except that inoculum cultures were treated each day with 15 mg. rice starch and about 10 mg. dried grass.

Preparation of protozoa for inoculation. The protozoa were taken from the inoculum cultures in which they were present as a loose pellet at the bottom of the tube, after removal of the surface scum and most of the medium, and allowed to stand in 8×1 in.

tubes until any grass present had sunk to the bottom, leaving the protozoa in the supernatant fluid. This supernatant fluid was transferred to centrifuge tubes, the residual grass washed with salt solution B (Coleman, 1960) and the washings added to the supernatant fluid. The protozoa were spun down and washed three times in salt solution B which contained 0·03 % cysteine, on a bucket-head centrifuge for 20 sec. from starting; the maximum speed was equivalent to 200 *g*. For experiments with non-multiplying protozoa the organisms were finally used as inoculum to give a population density of $3-7 \times 10^4$ protozoa/ml. except in experiments on the constituents of the pool where concentrations up to 50×10^4 protozoa/ml. were used. For growth experiments $1-2 \times 10^4$ protozoa/ml. incubation medium were used.

Incubation conditions for engulfment of bacteria by non-multiplying protozoa. The medium consisted of 1·8–8·2 ml. (chosen so that the final volume was 10 ml.) salt solution B (Coleman, 1960), 0–6·0 ml. water, 0–6·0 ml. double-strength salt solution B and amino acids (if any) autoclaved (115° for 20 min.) in a 15 ml. centrifuge tube. Standard salt solution contained 7·4 ml. salt solution B, 2·2 ml. water, and cysteine and $NaHCO_3$ as described below. The concentration was altered by replacing the salt solution by water or the water and salt solution by salt solution of double the normal concentration. Immediately after removal from the autoclave the following additions were made aseptically: 0·2 ml. 1 % L-cysteine hydrochloride (neutralized and Seitz filtered), 0·2 ml. 5 % (w/v) $NaHCO_3$ (Seitz filtered), 0·1–2·0 ml. suspension of ^{14}C-bacteria in salt solution B (washed once and suspended at a concentration of 10^8-10^{10} bacteria/ml.) and any other additions. After inoculation the tubes were gassed for 10 sec. with 95 % (v/v) N_2 + 5 % (v/v) CO_2, sealed with a rubber bung and incubated at 39° except where otherwise stated.

Incubation conditions for metabolism of bacteria by growing protozoa. The medium and conditions used were those of Coleman (1964). At the end of experiments in which the uptake of ^{14}C-bacteria was being investigated the protozoa were centrifuged down and washed twice in salt solution B on a bucket-head centrifuge for 30 sec. from starting (maximum speed was equivalent to 300 *g*). Under these conditions less than 1 % of the free bacteria were sedimented with the protozoa. The washed protozoa were then plated out to determine radioactivity or, in experiments to measure the viability of intracellular bacteria, broken in a Potter homogenizer (Potter & Elvehjem, 1936) at room temperature until 98–100 % of the protozoa were broken (usually about 90 sec.). The residual bacteria were spun down from the first supernatant fluid obtained after removal of the protozoa and washed once at 2000 *g* for 10 min. on an angle-head centrifuge. Samples from the supernatant fluid after removal of the bacteria were placed on planchets with 0·1 ml. N-HCl or 0·1 ml. 0·1 N-NaOH for the estimation of the ^{14}C which was free in the medium. The radioactivity determined in the presence of HCl measured the non-volatile ^{14}C and the difference between the determinations in the presence of HCl and NaOH the volatile ^{14}C.

Sources of bacteria. See Coleman (1964). Bacteria 7G2, 21G13/1, 23G3/2 were isolated from inside washed protozoa (White, 1966).

Bacterial growth media. Escherichia coli was grown and maintained at 39° in C medium (Roberts *et al.* 1955) containing 0·2 % (w/v) glucose and aerated during incubation by passing sterile air into the medium through a cottonwool-plugged Pasteur pipette. To obtain bacteria uniformly labelled with ^{14}C, 0·05 μc [U-^{14}C]-glucose/ml. was added to the growth medium. To obtain *E. coli* specifically labelled

with one ^{14}C-amino acid the bacteria were grown in C medium + 0·2 % glucose + 0·02 μc ^{14}C-amino acid/ml. + those ^{12}C-amino acids (each at 0·015 M) required to prevent the passage of ^{14}C into other amino acids of the bacteria. The amino acids and other compounds added with ^{14}C-alanine were ^{12}C-aspartic acid, glutamic acid, leucine, serine, valine; with ^{14}C-glutamic acid were ^{12}C-arginine, aspartic acid, proline; with ^{14}C-glycine were ^{12}C-adenine, alanine, aspartic acid, glutamic acid, pyruvic acid, serine; with ^{14}C-lysine were ^{12}C-arginine, glutamic acid, isoleucine, proline, threonine; with ^{14}C-phenylalanine were ^{12}C-aspartic acid, glutamic acid, tyrosine; with ^{14}C-proline were ^{12}C-arginine, glutamic acid; with ^{14}C-serine were ^{12}C-adenine, alanine, aspartic acid, cysteine, glycine, glutamic acid, pyruvic acid; with ^{14}C-valine was ^{12}C-leucine; with ^{14}C-leucine and ^{14}C-isoleucine, no additions.

Other bacteria were grown at 37° for 16 hr in a medium that contained (per litre): salt solution (Coleman, 1958), 250 ml.; Difco yeast extract, 2 g.; Difco tryptose, 2 g.; glucose, 2 g.; [8-^{14}C]guanine, 14 μc (if any). *Serratia marcescens* and Bacterium 31 were able to grow on C medium (Roberts *et al.* 1955) containing 0·2 % glucose, and where it was required to label the bacteria with a ^{14}C-amino acid this medium was used. All the bacteria were grown aerobically in static culture in cottonwool-plugged flasks except for *Clostridium welchii* which was grown under CO_2 and *Escherichia coli* which was grown with forced aeration.

Nadsonia elongata was grown at 30° in cottonwool-plugged flasks for 15 hr in a medium that contained (per litre): $(NH_4)_2SO_4$, 2 g.; KH_2PO_4, 2 g.; Difco yeast extract, 5 g.; glucose 2 g.

Bacterial viable counts. Colony counts of *Escherichia coli* were made by serial 10-fold dilutions in C medium + 0·2 % (w/v) glucose (Roberts *et al.* 1955) followed by plating on C medium containing 0·2 % (w/v) glucose and 1 % (w/v) agar and incubation at 39°. Colony counts of other bacteria were made by serial 10-fold dilutions in medium which contained (per litre): salt solution (Coleman, 1958), 250 ml.; Difco yeast extract, 2 g.; Difco tryptose, 2 g.; glucose 2 g.; followed by plating on the same medium plus 1 % agar and incubation aerobically, except for *Clostridium welchii* which was incubated anaerobically at 39°.

Colony counts of *Nadsonia elongata* were made by serial 10-fold dilutions in its growth medium, followed by plating on this medium solidified with 1 % agar and incubation at 30°.

Total bacterial counts. The total number of bacteria in a washed bacterial suspension was estimated by counting an appropriate dilution in a Helber counting chamber of depth 0·02 mm. and square size 0·0025 mm^2.

Protozoal counts. The numbers of protozoa were estimated by the method of Coleman (1958). Only those protozoa which showed no sign of disintegration were counted.

Estimation of ^{14}*C.* ^{14}C in whole protozoa was estimated by washing the organisms with water on to an aluminium disc (planchet) of area 4·7 cm.^{2} and carrying a disc of lens tissue. The sample was spread by one drop of cetyltrimethylammonium bromide solution (5 mg./ml.) and fixed with one drop of polyvinyl alcohol (2 mg./ml.), the disc was dried at 40°, and the ^{14}C estimated by using an automatic flow-counter (Nuclear-Chicago Corp.) with an efficiency of about 20 %. Over 1000 counts at a rate greater than five times background were recorded for all fractions. Determinations were made with less than 0·5 mg. of material/cm.^{2} of disc. To determine the relative ^{14}C contents of spots on chromatograms a thin mica end-window GM tube was placed directly

on a spot, the position of which had been determined by radio-autography. $^{14}CO_2$ was determined by the method of Coleman (1956).

Fractionation of organisms, paper chromatography, identification of ^{14}C-labelled compounds in the medium and the protozoal 'pool', chemicals. See Coleman (1967).

RESULTS

Loss of viability of engulfed bacteria

Although it had been shown previously (Coleman, 1964) that many species of bacteria were engulfed by *Entodinium caudatum* and that *Escherichia coli* was rapidly killed after uptake, no studies were reported on the killing of other bacterial species. In an attempt to measure this loss of viability, the bacteria were first labelled by growth on a suitable medium in the presence of a ^{14}C-labelled compound which was usually [8-^{14}C]guanine where a complex growth medium was used. This tracer was used in preference to ^{14}C-glucose or a ^{14}C-amino acid under these conditions because these latter compounds gave poor labelling of the bacteria. The use of guanine-labelled bacteria had the disadvantage that more ^{14}C was liberated into the medium after prolonged incubation in the absence of protozoa, but provided that incubations of less than 3 hr were used, the amount released was small.

The number of bacteria engulfed was determined after incubation of the protozoa with bacteria of known specific activity by measuring the ^{14}C in the protozoa. The number of viable bacteria in the medium in the absence of protozoa was measured initially and at the end of the experiment to obtain an estimate of the loss of viability of the bacterium in the protozoal incubation medium. The number of viable bacteria present in the washed protozoa was measured before and after breakage of the protozoa in a Potter homogenizer (Potter & Elvehjem, 1936) initially, and after incubation in the presence or absence of bacteria. In all experiments corrections were applied for initial values and the bacteria present with unbroken protozoa, but these were usually less than 10% of the count in protozoa broken after incubation with bacteria.

The results obtained for average bacterial survival after incubation for 2·5 hr of protozoa with various species of bacteria are shown in Table 1. There was considerable variation between experiments with different batches of protozoa in the percentage survival of the same bacterial species, e.g. *Escherichia coli* survival varied from 0·7 to 13% and *Serratia marcescens* survival from 36 to 84%. It was apparent that the resistance of any bacterial species is not related to Gram reaction, bacterial shape or size or to the source from which the bacterium was originally isolated. The three members of the Enterobacteriaceae—*E. coli*, *Proteus vulgaris*, and *S. marcescens*—had widely different survivals inside the protozoa. The bacteria isolated from suspensions of *Entodinium caudatum* immediately after separation from the growth medium, namely Bacterium 31, Bacterium D, 7G2, 21G13/1 and 23G3/2 (White, 1966) had survival percentages of 7–82% and were therefore not particularly resistant to killing. It is possible, especially with Bacterium 31 which was found in larger numbers than the other bacteria inside the protozoa and which produced slime when grown in the presence of glucose, that bacteria which are found inside the protozoa may produce some kind of protective envelope in that environment.

Studies on the effect of the salt concentration in the medium on bacterial survival

showed in one experiment that when the salt concentration was altered from 100 to 50%, the number of surviving *Escherichia coli* decreased from 3·0 to 1·7% and that at 200% of standard salt concentration it increased to 7·5%. For *Serratia marcescens* the corresponding survivals were 30%, 68%, 69% at 50%, 100% and 200% of standard salt concentration. Variation in the population density of *E. coli* from $1\cdot2\times10^9$ to 19×10^9/ml. did not affect the survival of bacteria inside the protozoa. All the results quoted above probably give a high estimate of the number of bacterial survivors after 2·5 hr because the protozoa engulf bacteria throughout the incubation period and those which have just been engulfed obviously have more chance of survival than those engulfed at the beginning of the experiment.

Table 1. *The viability of bacteria engulfed by Entodinium caudatum*

Entodinium caudatum (approximately 5×10^4 protozoa/ml.) was incubated anaerobically in the presence of approximately 10^9/ml. ^{14}C-labelled bacteria of known specific activity for 2·5 hr. The number of bacteria taken up was calculated from the ^{14}C in the protozoa at the end of the experiment and the number of viable bacteria inside the protozoa from the difference in colony count before and after breakage of the protozoa in a Potter homogenizer. The viability of the bacteria in the absence of protozoa was also measured.

Micro-organism	Description	Proportion of engulfed micro-organism still viable after 2·5 hr (%)	Micro-organism survival in absence of protozoa (%)
Clostridium welchii	Gram + ve rod	<0·5	55
Escherichia coli	Gram − ve rod	0·7–12·5	100
Lactobacillus casei	Gram + ve rod	1·7	100
Proteus vulgaris	Gram − ve rod	23	90
Serratia marcescens	Gram − ve rod	60	70
Staphylococcus aureus	Gram + ve coccus	2	15
Streptococcus bovis	Gram + ve coccus (from rumen)	1–7	70
Bacterium D	Gram + ve coccus (from protozoa)	42	100
Bacterium 31	Gram − ve rod (from protozoa)	35	90
7G2	Gram − ve rod (from protozoa)	82	100
21G13/1	Gram + ve coccus (from protozoa)	7	27
23G3/2	Gram + ve rod (from protozoa)	8–70	95
Nadsonia elongata	A yeast	1	20

The metabolism of Serratia marcescens by Entodinium caudatum

Although *Serratia marcescens* and *Escherichia coli* are both short Gram-negative rods the former was far more resistant to killing by the protozoa than was the latter. Since *S. marcescens* tended to grow in small clumps it was possible that where each clump would give rise to one colony in the viable count assay, it would be necessary for only one bacterium in each clump to remain viable for the count to be unchanged. To investigate this further, the digestion of suspensions of *S. marcescens* or *E. coli* containing equal numbers of bacteria and labelled with ^{14}C-leucine was compared. Table 2 shows that when *S. marcescens* was engulfed: (*a*) less ^{14}C appeared in the supernatant fluid; (*b*) a smaller proportion of the total protozoal ^{14}C was present in the broken protozoal supernatant fluid (i.e. more ^{14}C was in the broken protozoal pellet which contained any intact bacteria); (*c*) ^{12}C-leucine free in the medium had

much less effect on the total ^{14}C found in the protozoa. These results are consistent with the hypothesis that *S. marcescens* was digested much more slowly than was *E. coli.*

Table 2. *The metabolism of Escherichia coli and Serratia marcescens labelled with ^{14}C-leucine*

Entodinium caudatum was incubated anaerobically in the presence of *Escherichia coli* or *Serratia marcescens* labelled with [U-^{14}C]-L-leucine (25,000 counts/min.) in the presence or absence of 0·01 M-^{12}C-L-leucine. After incubation for 11 hr the washed protozoa were broken in a Potter homogenizer and the supernatant fluid fraction was separated by centrifugation.

Time (hr)	Protozoa present	^{12}C-L-leucine present	^{14}C in protozoal fraction (counts/min.)	^{14}C in broken cell supernatant (counts/min.)	^{14}C in medium (counts/min.)
			Serratia marcescens		
0	+	+	1320	.	450
11	−	+	450	.	3970
11	+	−	11900	1700	4600
11	+	+	10700	970	9700
			Escherichia coli		
0	+	+	110	.	300
11	−	+	235	.	1180
11	+	−	5840	2600	8300
11	+	+	1960	830	11700

Effect of amino acids on the engulfment of Escherichia coli by Entodinium caudatum

In many of the experiments to be reported below the specific action of a free ^{12}C-amino acid on the incorporation of ^{14}C from *Escherichia coli* labelled with the ^{14}C-form of that amino acid was investigated, and it was therefore first necessary to study the action of amino acids on the engulfment of uniformly ^{14}C-labelled *E. coli.* When incubations were continued for 5 hr or longer the ^{14}C incorporated into protozoa under these conditions was altered by less than 10 % when the following amino acids were added at a concentration of 0·01 M: L-alanine, L-aspartic acid, L-arginine, L-asparagine, L-cysteine, L-glutamic acid, L-histidine, L-isoleucine, L-leucine, L-lysine, L-phenylalanine, L-serine, DL-threonine, L-valine. In contrast, 0·01 M-L-proline or glycine decreased the incorporation by 40 % after 30 min. or 5 hr of incubation. However, over the first 30 min. incubation 0·01 M-L-lysine or L-arginine stimulated the incorporation of ^{14}C by 50 % and 0·01 M-L-cysteine, additional to that in the basal medium, by 70 %. An increase in the concentration of L-arginine to 0·02 M and L-cysteine to 0·04 M increased this stimulation by a further 15 % and 20–150 %, respectively. Figure 1 shows the time-course for incubation under these conditions and shows that after the first hour the rate of ^{14}C incorporation was approximately the same whether in the presence or in the absence of arginine and cysteine. The proportion of the engulfed bacteria that had been rendered non-viable after 1 hr was the same under all conditions. The stimulation by L-cysteine was not due solely to its action as a reducing agent because 0·01 M-sodium thioglycollate (mercaptoacetate), which has the same E_0' as cysteine, produced only 60 % of the stimulation caused by the presence of 0·01 M-L-cysteine; 0·01 M-2-mercaptoethanol killed the protozoa.

Metabolism of ^{14}C-amino acid labelled Escherichia coli by non-multiplying Entodinium caudatum

Escherichia coli were prepared labelled with one ^{14}C-amino acid by growth on a glucose–ammonia+salts medium (C medium of Roberts *et al.* 1955) and tracer quantities of the relevant ^{14}C-amino acid+those ^{12}C-amino acids found by Roberts *et al.* (1955) or the present author's own experience to prevent the incorporation of ^{14}C into other amino acids.

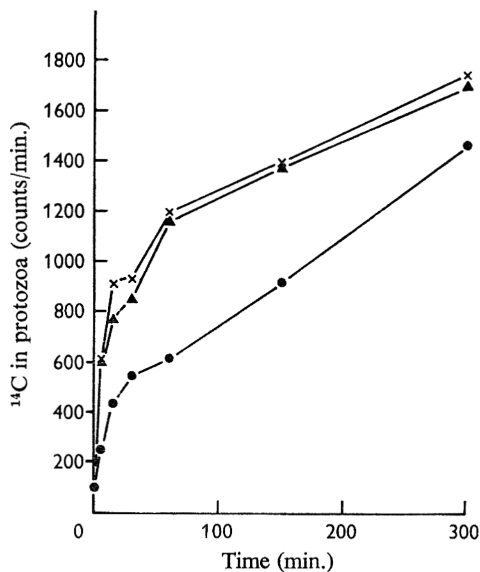

Fig. 1

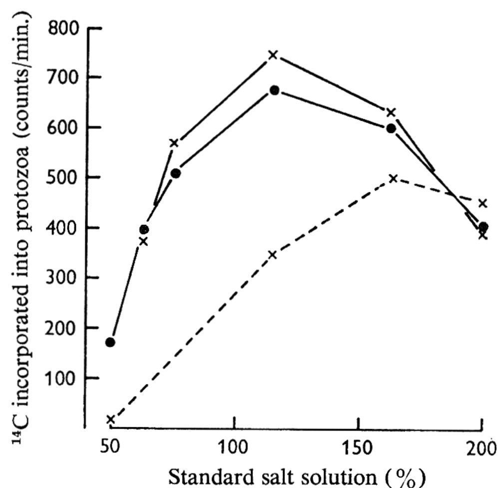

Fig. 2

Fig. 1. Effect of 0·04 M-^{12}C-L-cysteine (×—×) and 0·02 M-^{12}C-L-arginine (▲—▲) on the incorporation of ^{14}C into protozoa *Entodinium candatum* from uniformly ^{14}C-labelled *Escherichia coli* (12,300 counts/min.) (●—●).

Fig. 2. Effect of salt concentration on incorporation of ^{14}C from *Escherichia coli* labelled with individual ^{14}C-amino acids into *Entodinium caudatum*. ●—●, ^{14}C-leucine or ^{14}C-glutamic acid labelled *E. coli* incubated without added ^{12}C-amino acid. ×---×, ^{14}C-leucine labelled *E. coli* incubated in the presence of 0·01 M-L-leucine. ×—×, ^{14}C-glutamic acid labelled *E. coli* incubated in the presence of 0·01 M-L-glutamic acid.

Effect of ^{12}C-amino acids and salt concentrations. Figure 2 shows that the maximum incorporation of ^{14}C into *Entodinium caudatum* from ^{14}C-amino acid labelled *Escherichia coli* occurred at 120 % of the standard salt concentration. The shape of the curve was independent of which amino acid in the bacteria was labelled with ^{14}C. However, the effect on the total amount of ^{14}C incorporated by the protozoa of the presence of the ^{12}C form of the amino acid labelled in the bacteria with ^{14}C depended on the amino acid studied. Group 1 amino acids (alanine, arginine, aspartic acid, glutamic acid, serine, Coleman, 1967) had no effect or a slightly stimulatory one on the incorporation of ^{14}C, whereas group 2 amino acids (isoleucine, leucine, methionine, phenylalanine, proline, valine) decreased the incorporation at low salt concentrations, but had a slightly stimulatory effect at 200 % of the standard salt concentration.

These experiments were all made with ^{12}C-amino acids at a concentration of 0·01 M

and it has been shown (Coleman, 1967) that at this concentration the uptake of amino acids by the protozoa was not an active process. It was therefore of interest to determine whether low concentrations of a group-2 amino acid, where uptake of the amino acid by the protozoa was an active process, were as effective as higher concentrations in decreasing the incorporation of ^{14}C, at low salt concentration, from *Escherichia coli* labelled with the ^{14}C form of that amino acid. Figure 3 shows that, except at high initial bacteria:protozoa ratios, the same degree of inhibition was obtained at high and low free amino acid concentrations. This suggests that a group 2 amino acid entering the protozoa by either process was equally effective in decreasing the incorporation of ^{14}C from ^{14}C-amino acid labelled *E. coli*. At high bacteria:protozoa ratios and low

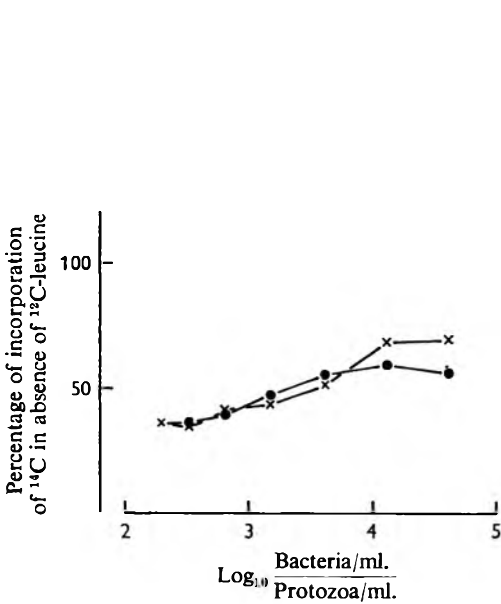

Fig. 3

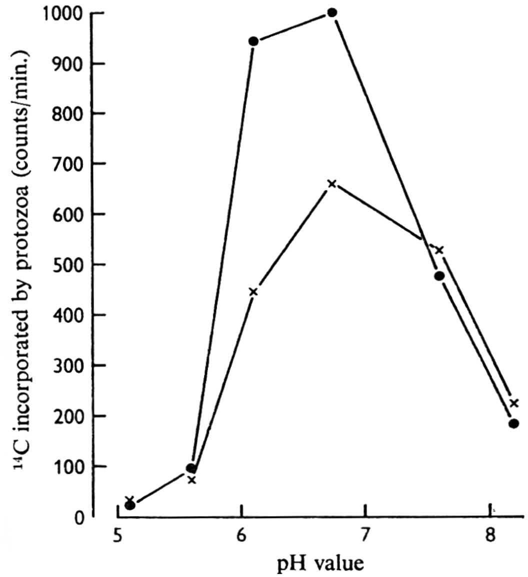

Fig. 4

Fig. 3. Effect of ratio of number of bacteria to number of protozoa present initially on incorporation into *Entodinium caudatum* of ^{14}C from ^{14}C-leucine-labelled *Escherichia coli* in the presence of 0·001 M (×—×) or 0·01 M (●—●) ^{12}C-L-leucine.

Fig. 4. Effect of pH value on the incorporation of ^{14}C from ^{14}C-isoleucine-labelled *Escherichia coli* by *Entodinium caudatum* incubated in the absence (●—●) or presence (×—×) of 0·01 M-^{12}C-L-isoleucine.

external amino acid concentrations, the quantity of amino acid entering the protozoa from the medium was probably not much greater than that entering by digestion of the bacteria. If it be assumed that amino acids from both sources mix, then under these conditions more bacterial amino acid would be incorporated into protozoal protein than at higher free amino acid concentrations.

Although it has been shown that these results could not have been the result of an effect of the amino acids on the rate of engulfment of *Escherichia coli*, confirmation was sought by investigating the effect of ^{12}C-amino acids on the incorporation into protozoal protein of ^{14}C-amino acids from uniformly labelled *E. coli*. In the absence of added free ^{12}C-amino acid all the amino acids in the protein of the broken protozoal supernatant fluid were labelled. In the presence of individual ^{12}C-amino acids of group

2 the incorporation of ^{14}C into that amino acid in the protozoal protein was markedly decreased, whereas with ^{12}C-amino acids of group 1, as would have been expected from the above results, the corresponding incorporation was little affected. It was also shown by this method that 0·01 M-^{12}C-glycine, which decreased the rate of engulfment of *E. coli* decreased the incorporation of ^{14}C into glycine to a greater extent than into aspartic acid or glutamic acid.

The effect of pH value. Figure 4 shows the effect of the pH value of the medium on the incorporation by the protozoa of ^{14}C from ^{14}C-isoleucine labelled *Escherichia coli*. At pH values above 6·0 the effect on this incorporation of the addition to the medium of 0·01 M-^{12}C-L-isoleucine decreased as the pH value of the medium increased. In contrast, a group-1 amino acid, e.g. glutamic acid, had no effect on the incorporation into the protozoa of ^{14}C from ^{14}C-glutamic acid labelled *E. coli* at any pH value tested.

The effect of temperature. For these experiments the protozoa were incubated for 3 hr in the presence of uniformly ^{14}C-labelled *Escherichia coli* and the incorporation of ^{14}C was measured at various temperatures under otherwise standard conditions. If the number of bacteria engulfed at 39° be assumed to be 100 then the number taken up was 72 at 43°, 18 at 30° and 2 at 25°.

The effect of an amino acid analogue. It has been shown previously (Coleman, 1967) that amino acids were probably taken up by the protozoa only by passage through the protozoal cell membranes. The finding that those amino acids which decrease the incorporation of ^{14}C from ^{14}C-amino acid labelled *Escherichia coli* were those which were incorporated most rapidly from the medium (i.e. group-2 amino acids) suggests that the bacteria may be taken up into the protozoal cytoplasm before digestion and evidence has been obtained for this hypothesis (Coleman & Hall, 1966). If this assumption is correct and if the uptake of a group-2 amino acid can be inhibited by an analogue then, in an experiment on the effect of this amino acid in decreasing the incorporation of ^{14}C from *E. coli* labelled with the ^{14}C-form of that amino acid, it may be possible to reverse this inhibition by the addition of the analogue. The only analogue which has been found to abolish completely the active uptake of an amino acid was *p*-fluorophenylalanine which inhibited the incorporation of phenylalanine. Unfortunately all concentrations of *p*-fluorophenylalanine decreased the rate of engulfment of *E. coli* and in the presence of 0·01 M-*p*-fluorophenylalanine the incorporation of ^{14}C from ^{14}C-phenylalanine-labelled *E. coli* was only 60 % of that in its absence. However, under these conditions, the further addition of 0·001 M-^{12}C-L-phenylalanine decreased the incorporation of ^{14}C by a further 0–10 %, whereas in the absence of *p*-fluorophenylalanine, phenylalanine decreased the incorporation by 67 %. Despite the inhibition of bacterial engulfment by *p*-fluorophenylalanine this result suggests that *p*-fluorophenylalanine might annul the inhibition of ^{14}C-uptake by phenylalanine.

Intracellular products of metabolism. Table 3 shows a typical distribution of ^{14}C in the major cell fractions obtained from protozoa which had metabolized *Escherichia coli* labelled with any group-2 amino acid in the presence or absence of the ^{12}C form of that amino acid free in the medium. Similar results were obtained by using *E. coli* labelled with a group-1 amino acid, except that the presence of the free amino acid in the medium had no effect on the amount of ^{14}C incorporated. As shown in Table 3, there was a marked increase in the amount of ^{14}C present in the cold TCA-soluble fraction of the broken protozoal supernatant fluid (the protozoal pool) when the

incubations were done in medium containing high concentrations of salt, and this was observed with *E. coli* labelled with any amino acid. Since the same quantity of ^{14}C-labelled bacteria was metabolized at both high and low salt concentrations, it is apparent that at high salt concentrations the ^{14}C was retained by the protozoa, whereas at low concentrations it was released into the medium.

To determine whether any of the bacterial amino acids were converted into other amino acids by the protozoa before incorporation into protozoal protein, the amino acids in the bacterial protein and in the broken protozoal supernatant fluid and pellet fractions were separated by two-dimensional paper chromatography and the position of radioactive spots determined by radio-autography. The protein of *Escherichia coli*

Table 3. *The metabolism of* ^{14}C*-valine labelled Escherichia coli by Entodinium caudatum*

Entodinium caudatum was incubated anaerobically in the presence of *Escherichia coli* labelled with [U-^{14}C]valine (53,500 counts/min.) in medium containing salts at 60 % (low salt) or 160 % (high salt) of the standard salt concentration in the presence or absence of 0·01 M-^{12}C-L-valine. After incubation for $5\frac{1}{2}$ hr the washed protozoa were broken by sonication and the supernatant and pellet fractions separated by centrifugation.

	Radioactivity (counts/min.)				
	Low-salt medium		High-salt medium		Initial *E. coli*
^{12}C-L-valine present	−	+	−	+	
Medium	15,000	17,100	11,800	11,700	·
Broken protozoal supernatant fluid	5,800	3,500	9,500	8,200	·
Broken protozoal pellet	5,700	2,100	7,800	5,000	·
Fractionation of broken protozoal supernatant fluid					
Cold trichloroacetic acid soluble	1,100	1,300	4,500	4,700	700
Ethanol-soluble protein	980	520	1,100	580	6,700
Lipid	330	160	230	240	850
Nucleic acid	220	40	175	80	1,800
Residual protein	2,700	1,400	2,800	2,600	40,000

which was prepared labelled with ^{14}C-alanine, arginine, glutamic acid, glycine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine or valine contained 90–100 % of the ^{14}C in the form of the one amino acid. The protein of both the protozoal fractions always contained only the same ^{14}C-amino acids as were present in the original *E. coli*, and the proportion of the total ^{14}C present in the principal amino acid was never less than that in the same amino acid in the bacteria. It was not possible to prepare ^{14}C-aspartic acid labelled *E. coli* which contained over 90 % of the total ^{14}C as aspartic acid, and although bacterial aspartic acid was apparently incorporated into the protozoal protein it was not possible to obtain unambiguous results. No evidence was obtained in any experiment for any amino acid interconversion by the protozoa.

The constituents of the protozoal pool. Most of the experiments on the constituents of the pool during the metabolism of ^{14}C-amino acid labelled *Escherichia coli* were made after incubation in the high salt concentration medium (200 % of standard) in order to take advantage of the larger quantity of ^{14}C present in this fraction under these

conditions. Figures 5 and 6 show the time-course for the appearance of ^{14}C in the cold TCA-soluble fraction during the metabolism of *E. coli* labelled with ^{14}C-glutamic acid (group 1) or ^{14}C-leucine (group 2). With the former, the ^{14}C in the pool continued to increase throughout the experiment, but with the latter it reached a constant value after 2 hr. The constituents of the pool during the metabolism of *E. coli* labelled with various amino acids were as follows.

Alanine. 65% alanine, 28% *N*-acetylalanine, 7% *N*-formylalanine. The relative proportions of these three compounds were very variable, and although the above result was typical, in one experiment there was 90% alanine and in another equal quantities of each compound. The properties were not altered by changes in the salt concentration or by replacement of the sodium propionate in the medium with sodium

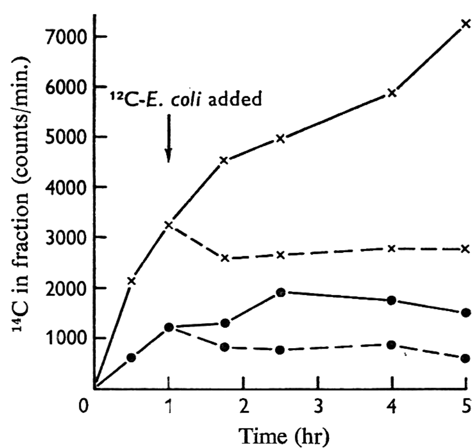

Fig. 5

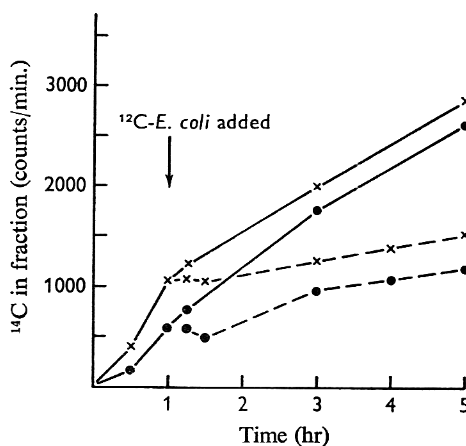

Fig. 6

Fig. 5. Effect of addition of tenfold excess of ^{12}C-*Escherichia coli* after 1 hr on the metabolism of ^{14}C-leucine labelled *E. coli* by washed suspensions of *Entodinium caudatum*. ●, ^{14}C in cold TCA-soluble fraction of broken protozoal supernatant fluid; ×, ^{14}C in protein of broken protozoal supernatant fluid; ---, ^{14}C in fractions after addition of ^{12}C-*Escherichia coli*.

Fig. 6. Effect of addition of tenfold excess of ^{12}C-*Escherichia coli* after 1 hr on the metabolism of ^{14}C-glutamic acid labelled *E. coli* by washed suspensions of *Entodinium caudatum*. ●, ^{14}C in cold TCA-soluble fraction of the broken protozoal supernatant fluid; ×, ^{14}C in protein of broken protozoal supernatant fluid; ---, ^{14}C in fractions after addition of ^{12}C-*Escherichia coli*.

bicarbonate. Attempts to formally identify the acetyl or formyl parts of amino acid derivatives were unsuccessful because (*a*) insufficient material was present for chemical identification and (*b*) the derivatives were not labelled by incubation of protozoa with ^{12}C-*Escherichia coli* and ^{14}C-acetate or ^{14}C-formate.

Glutamic acid. 35% glutamic acid, 65% *N*-formylglutamine.

Glycine. 100% *N*-acetylglycine.

Isoleucine, leucine, methionine, phenylalanine and valine. One compound only which was the amino acid labelled in the bacteria. No trace of any *N*-formyl or *N*-acetyl derivative was found.

Arginine and lysine. The pool obtained from protozoa which had metabolized *Escherichia coli* labelled with these basic amino acids contained at least four ^{14}C-

labelled compounds, one of which was the free amino acid. The other compounds were not identified, but were presumed to be acetyl or formyl derivatives of the amino acids.

Serine. No clearly defined spots were produced on chromatography.

To determine the metabolic function of these compounds in the protozoal pool, suspensions of *Entodinium caudatum* were allowed to metabolize *Escherichia coli* labelled with ^{14}C-leucine or ^{14}C-glutamic acid for 1 hr and then a tenfold excess of ^{12}C-*E. coli* was added to the medium and changes in the amount of ^{14}C in the pool measured (Figs. 5, 6). With both amino acids the addition of the ^{12}C-*E. coli* immediately stopped the incorporation of ^{14}C into the protozoal pool and the protein of the broken protozoal supernatant fluid. However, there was no sharp decrease in the amount of ^{14}C in the pool as might have been expected if the pool amino acids and

Table 4. *Products of the metabolism of the amino acids in Escherichia coli by Entodinium caudatum*

Protozoa were incubated anaerobically in the presence of *Escherichia coli* labelled with the ^{14}C-amino acids shown for 4–5 hr. The quantities of the various products in the medium were determined as described in the text and the results are expressed in moles/100 moles of carbon incorporated into the protozoa.

	^{14}C-recovered in:		
	Volatile acid	CO_2	Non-volatile material
[U-^{14}C]alanine	79	18	28
[1-^{14}C]alanine	24	62	28
[U-^{14}C]glutamic acid	6	5	26
[U-^{14}C]glycine	8	1	26
[U-^{14}C]isoleucine	38	6	28
[U-^{14}C]leucine	39	7	57
[1-^{14}C]leucine	32	133	31
[U-^{14}C]lysine	3	1	23
[U-^{14}C]phenylalanine	15	3	45
[U-^{14}C]proline	21	3	86
[U-^{14}C]valine	74	9	40

amino acid derivatives were on the pathway from bacterial protein to protozoal protein. It was not possible to use saturating concentrations of ^{14}C-labelled bacteria for these experiments and still be able to add a ten-times excess of ^{12}C-*E. coli* as the medium would be almost solid with bacteria, and under non-saturating conditions aberrant results may be obtained. It is possible that even if saturating conditions were used, that the bacteria might be killed and digested inside the protozoa in the order in which they were engulfed and then dilution of the digestion products of the ^{14}C-labelled bacteria with those of the ^{12}C-bacteria would not occur for some time. As there was a lag of 60 min. before significant quantities of non-volatile ^{14}C were liberated into the medium after the addition of ^{14}C-leucine labelled *E. coli* to protozoal suspensions, and as there was little decrease in the amount of ^{14}C incorporated in the presence of 0·01 M-^{12}C-L-leucine under these conditions for 60 min., it is suggested that the addition of ^{12}C-*E. coli* should effect the amount of ^{14}C in the pool within 90 min. of its addition if these pool constituents were intermediates. As the amount of ^{14}C in the pool in the experiment with ^{14}C-leucine labelled *E. coli* decreased by only 27 % in 90 min. and 50 % in 240 min., this is taken as evidence that the pool constituents were not intermediates between bacterial and protozoal protein.

Extracellular products of metabolism. On incubation of washed suspensions of *Escherichia coli* labelled with specific ^{14}C-amino acids with *Entodinium caudatum*, approximately half of the ^{14}C was incorporated by the protozoa and the remainder appeared as CO_2, volatile acids or as non-volatile materials in the medium (Table 4). The time-course for the metabolism of ^{14}C-amino acid labelled *E. coli* was similar to that shown previously for uniformly ^{14}C-labelled *E. coli* (Coleman, 1964). The molecular weight of the ^{14}C-labelled compounds in the medium was investigated by passage of the material through a column of Sephadex G50 (exclusion molecular weight 10,000). After 1–5 hr of incubation, only 11 % of the non-volatile ^{14}C in the supernatant fluid was recovered in the high molecular weight peak, showing that the bacteria were completely digested and not broken up into large fragments. The metabolites of *E. coli* labelled with individual ^{14}C-amino acids have been identified as follows.

Alanine. The volatile fatty acids produced from [U-^{14}C]alanine-labelled *Escherichia coli* have been identified by chromatography in solvent K (Coleman, 1967) as formic acid (20 %) and acetic acid (80 %) with an acetic acid:formic acid + CO_2 ratio of 1·9:1. Since two to three times as much CO_2 as volatile acid was produced in the presence of [1-^{14}C]alanine-labelled *E. coli* and since the acid was principally formic acid, this suggests that the carboxyl carbon of alanine was liberated as CO_2 or formate, and that the acetate was derived from C2 and C3. The non-volatile ^{14}C-material in the medium was 80–90 % alanine and the remainder was probably *N*-acetylalanine.

Glutamic acid. The non-volatile material in the medium was 50–70 % glutamic acid and 30–50 % *N*-formylglutamic acid or α-*N*-formylglutamine.

Glycine. The non-volatile material in the medium was 60–70 % glycine and 30–40 % *N*-acetylglycine. When ^{12}C-glycine was present during the incubation the 150 % increase in the amount of non-volatile ^{14}C present was due solely to an increase in the amount of glycine.

Isoleucine. The ^{14}C-volatile acid was identified as a C_5 acid by chromatography in Solvent K (Coleman, 1967). As it was not possible to resolve the four structural isomers of the valeric acid by paper chromatography the acid was chromatographed on a column of buffered celite (Bueding & Yale, 1951) and identified as either isovaleric or α-methylbutyric acid. To distinguish between these, equal quantities were added to a large excess of the ^{12}C form of each of these acids in turn and then the *p*-bromophenacyl esters prepared, recrystallized twice (Judefind & Reid, 1919) and the ^{14}C in the crystals determined. It was hoped that when the ^{12}C and ^{14}C acids were different no ^{14}C would appear in the crystals, but that when they were the same appreciable quantities of ^{14}C would be present. In the presence of ^{12}C-isovaleric acid and ^{12}C-α-methylbutyric acid, respectively, the recovery of ^{14}C was 7 % and 31 %, respectively, showing that the C_5 acid was probably α-methylbutyric acid.

As the formation of α-methylbutyrate from isoleucine must involve the loss of one carbon atom, an attempt was made to measure the amount of $^{14}CO_2$ produced during the metabolism of [U-^{14}C]isoleucine labelled *Escherichia coli*. Table 4 shows that the volatile acid:CO_2 ratio was 6·3:1 as compared with the theoretical value of 5·0:1 for a decarboxylation of isoleucine; this result is consistent with formation of α-methylbutyrate by this mechanism. The non-volatile material in the medium was ^{14}C-isoleucine.

Leucine. The volatile fatty acid produced from ^{14}C-leucine-labelled *Escherichia coli* was identified in the same way as that from ^{14}C-isoleucine-labelled *E. coli* except that

the ^{14}C recovered in the *p*-bromophenacyl esters was 35 % with ^{12}C-isovaleric acid and 4 % with ^{12}C-α-methylbutyric acid, showing that the volatile acid was isovaleric acid. The production of $^{14}CO_2$ from [U-^{14}C]leucine-labelled *E. coli* was also measured and the volatile acid:CO_2 ratio found to be 5·6:1 (Table 4) which agrees well with the theoretical value of 5·0:1 for the formation of isovaleric acid from leucine by decarboxylation. In an attempt to prove that it was carbon-atom 1 of leucine that was liberated as CO_2 a similar experiment was done with [1-^{14}C]leucine-labelled *E. coli* (Table 4) and under these conditions four times as much $^{14}CO_2$ as ^{14}C-volatile acid was produced, indicating that most of the CO_2 came from carbon-atom 1 of leucine. The non-volatile material in the medium was ^{14}C-leucine; no trace of *N*-acetylleucine was found.

Phenylalanine. Two non-volatile materials were present in the medium, one of which was extractable by ether from the acidified solution and shown to be phenylacetic acid. The non-extractable compound was free phenylalanine. The amount of phenylacetic acid produced from [U-^{14}C]phenylalanine-labelled *Escherichia coli* was deduced from the total non-volatile ^{14}C in the medium, and the fraction present as phenylacetic acid after separation of the two compounds by chromatography in solvent A or J. Under these conditions the phenylacetic acid:CO_2 ratio was 3·6:1 as compared with a theoretical value of 8:1 for the production of phenylacetic acid from phenylalanine by decarboxylation. This suggests that the phenylacetic acid may have undergone further metabolism.

Valine. The volatile fatty acid produced during the metabolism of ^{14}C-valine-labelled *Escherichia coli* was identified as isobutyric acid by the same methods as those used to identify α-methylbutyric acid as a product of ^{14}C-isoleucine metabolism. Table 4 shows that the volatile acid/CO_2 ratio was 8·2:1 which compared poorly with a theoretical value of 4·0:1.

Metabolism of ^{14}C-amino acid labelled Escherichia coli by multiplying Entodinium caudatum

It is possible that non-multiplying suspensions of protozoa may be unable to carry out biosynthetic reactions that normally occur in growing protozoa where new cellular material is being formed. To investigate this, *Entodinium caudatum* was allowed to grow in the presence of *Escherichia coli* labelled with ^{14}C-amino acids under the conditions described previously (Coleman, 1964) until the number of protozoa had at least doubled. In a typical experiment with *E. coli* labelled with ^{14}C-glutamic acid, the number of protozoa increased from 16,500/ml. to 34,200/ml. in 48 hr; 97 % of the bacteria were metabolized and 28 % of the ^{14}C was retained by the protozoa, the remainder appearing in the supernatant fluid. Of the ^{14}C in the protozoa 43 % was in the broken protozoal supernatant fluid (pool) after breakage of the protozoa and 90 % of the ^{14}C in this fraction was protein glutamic acid. When the protozoa were allowed to grow under the same conditions in the presence of 0·01 M-^{12}C-L-glutamic acid the incorporation of ^{14}C into the protozoa was decreased by 19 % as compared with 97 % when the amino acid studied throughout was leucine (Coleman, 1964).

The metabolism of *Escherichia coli* labelled with ^{14}C-alanine, arginine, glutamic acid, glycine, isoleucine, leucine, lysine, phenylalanine, serine or valine was studied under conditions which allowed protozoal growth, and for all these amino acids no evidence was obtained for any amino acid interconversions. In the presence of the

^{12}C-form of the ^{14}C-amino acid present in the bacteria ^{14}C incorporation into the protozoa was decreased by 20–50% for group-1 amino acids and by over 90% for group-2 amino acids. The greater effect with both groups under growth conditions as compared with non-multiplying protozoa was probably associated with the longer incubation times. This meant that the time taken for the bacteria to be engulfed, killed and digested was a much smaller proportion of the total time allowed and there was more time for equilibration with the free amino acid entering from the medium.

DISCUSSION

In their natural habitat of the sheep rumen, entodiniomorphid protozoa live in a medium which is poor in readily metabolizable compounds such as sugars and amino acids but contains up to 10^{10} bacteria/ml. (Bryant & Robinson, 1961). In view of this it is not unexpected that *Entodinium caudatum* should utilize amino acids much more economically from intact bacteria than from free amino acids in the medium. There has been speculation (Coleman, 1963) that *E. caudatum* might have been able to synthesize amino acids from carbohydrate which it engulfed as starch grains and ammonia which were present in rumen liquor. However, the present results indicate that *E. caudatum* probably satisfies its amino acid requirements from the digestion products of bacteria. Although the incorporations measured in experiments with non-multiplying protozoa may be the result of protein turnover those obtained with growing organisms were probably due to the synthesis of new protein. Other results with ^{14}C-labelled-starch grains have shown that little protein was synthesized from carbohydrate even by growing protozoa. The inability of *E. caudatum* to biosynthesize or interconvert amino acids is probably the result of a gradual loss of enzymes from the time when this protozoon, now found nowhere else, first invaded the rumen. This loss would have been brought about by growth on micro-organisms which probably contain all the low-molecular-weight nitrogenous compounds required by a living cell. The protozoa have, however, retained the ability to oxidatively deaminate some amino acids according to the reaction.

$$R.CH(NH_2)COOH + 2H_2O \rightarrow R.COOH + CO_2 + (NH_3) + (4H)$$

These results are in contrast to those of Abou Akkada & Howard (1962) who considered that non-multiplying suspensions of *Entodinium caudatum* prepared from the rumen did not deaminate amino acids or utilize casein for the synthesis of cellular constituents. However, these authors measured changes in the amounts of nitrogenous materials and this method is not sensitive to small changes in the amount of any compound present. In contrast to the slow catabolism of amino acids, starch grains are rapidly broken down to CO_2, H_2, formic, acetic, propionic and butyric acids (Abou Akkada & Howard, 1960), and since the protozoa in the present experiments contained starch grains a ready supply of 'active' acetate and formate was probably available for acetylation or formylation of amino acids. The exact reactions which occurred must have depended on the enzymes present and only the group-1 amino acids and glycine were ever found to be *N*-substituted. However, as no evidence was obtained that these pool constituents were intermediates in the synthesis of protozoal protein they were probably by-products of protozoal metabolism. Since the same compounds were found in the pool and the medium it seems probable that they gradually diffused out into the medium, especially if the salt concentration was low.

I wish to thank Mr G. A. Embleton for inserting permanent rumen cannulae into the sheep used to provide rumen fluid for the routine maintenance of the protozoa, the members of the Sub-Department of Chemical Microbiology, Biochemistry Department, University of Cambridge, for their helpful advice and criticism, and Miss B. Hanzl for valuable technical assistance.

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