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The Crabtree Effect: A Regulatory System in Yeast

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SUMMARY

When *Saccharomyces cerevisiae* is growing exponentially on glucose or fructose as carbon plus energy source, and in the presence of air, the glucose degradation proceeds mainly via aerobic fermentation. When the yeast is growing on mannose or galactose, degradation proceeds simultaneously via respiration and fermentation. This situation results from a repression of the of the respiratory enzymes synthesis by high fermentation rates. This regulatory system, called the 'Crabtree effect', consists actually of a repression of an energy source (respiration) by another energy source (fermentation). Various yeast strains were tested; the regulatory system was present in about 50% of them.

INTRODUCTION

For many years the 'Pasteur effect' was an important topic in biochemistry. The Pasteur effect is defined as an 'inhibition of the activity' of the fermentation pathway by respiration (see Slonimski, 1956). Recent work (Salas, Viñuela, Salas & Sols, 1965) pointed out the existence of a feed-back inhibition of the phosphofructokinase by citrate. If this allosteric inhibition is really responsible for the Pasteur effect, it means that the Pasteur effect is an inhibition of the fermentation pathway by an end product of aerobic glucose utilization. Nevertheless, it is important to keep in mind that all the studies of the Pasteur effect were made with suspensions of organisms. In other words, we do not know whether the Pasteur effect plays any important role during growth. We know that under certain well-defined conditions, i.e. the exponential growth of *Saccharomyces cerevisiae* in the presence of a high concentration of glucose, the Pasteur effect does not have the opportunity to work, because under these conditions the degradation of glucose proceeds via fermentation only. This phenomenon is known as the 'contre-effet Pasteur' or 'Crabtree effect' (after Crabtree, 1929).

It was shown by Swanson & Clifton (1948) for the first time that most of the aerobic growth of a yeast (*Saccharomyces cerevisiae*) on glucose proceeds via fermentation. A quantitative study of the aerobic growth of yeast made by Lemoigne, Aubert & Millet (1954) showed a diauxic growth on high concentrations of glucose: a fast growth involving intensive aerobic fermentation, followed by a slow growth involving oxidation of the accumulated ethanol. At lower glucose concentrations (5–10 mg./100 ml.) the absence of any noticeable diauxie suggested that the pathway used for the degradation of glucose might depend on the glucose concentration.

Slonimski (1956) studied the rate of respiratory adaptation in *Saccharomyces*

cerevisiae as a function of the glucose concentration. At low concentrations of glucose (below 6×10^{-3}M) the rate of respiratory adaptation increased as the concentration of glucose was increased. This relationship was to be expected since the aerobic fermentation of glucose proceeds very slowly at these glucose concentrations and severely limits the supply of energy to the organisms. But at concentrations of glucose higher than 6×10^{-3}M, the rate of adaptation to aerobic conditions decreased as the concentration of glucose was increased. Simultaneously, as the concentration of glucose increased, the rate of aerobic fermentation also increased. It is this inhibition of the synthesis of respiratory enzymes by the high fermentation rates which occur at high glucose concentrations which is known as the contre-effet Pasteur. Slonimski's work, which was done with suspensions of organisms, was followed by a more extensive study of the contre-effet Pasteur in a growing yeast (Ephrussi, Slonimski, Yotsuyanagi & Tavlitzki, 1956). Starting a culture in the presence of 3 % (w/v) glucose with organisms fully adapted to aerobic conditions, the following was observed: the rate of aerobic fermentation (Q_{CO_2} ferm.) increased sharply during the phase of exponential growth; at the same time the rate of respiration (Q_{O_2}) decreased to a low value. A few generations before the end of the exponential phase the glucose no longer saturated the fermentation system, and as a result the Q_{CO_2} ferm. decreased to a low value; simultaneously the Q_{O_2} increased (respiratory adaptation). This experiment showed clearly that the contre-effet Pasteur is an important part of the physiology of a yeast like *S. cerevisiae* growing in a high concentration of glucose.

In the following pages we shall use the term 'Crabtree effect' (Crabtree, 1929) rather than contre-effet Pasteur to define the 'inhibition of the synthesis' of the respiratory enzymes, in order to avoid confusion with the Pasteur effect, i.e. the 'inhibition of the fermentation activity' by respiration. The present work shows that under certain conditions the degradation of carbohydrates by an exponentially growing yeast may proceed through fermentation and respiration simultaneously. This situation occurs when mannose or galactose is used as carbon plus energy source instead of glucose, and seems to be the result of a partial but constant repression of the respiratory system. Several yeast strains belonging to different genera were tested for the Crabtree effect and about 50 % of them showed the effect. Fermentation and oxidation of glucose were compared in two strains, one of which showed the Crabtree effect and the other not; the comparison showed that the fermentation pathway was largely constitutive for the strain which showed a strong Crabtree effect.

METHODS

Growth medium. The medium used contained 10·9 g. KH_2PO_4, 5·0 g. Difco yeast extract and 30·0 g. hexose, made to 1000 ml. after adjusting to pH 6·5 with potassium hydroxide.

Hexoses. Hexoses of bacteriological purity were obtained from S. T. Gurr (136 New Kings Road, London, S.W. 6). Some batches of galactose had to be recrystallized in 80 % ethanol in water to remove glucose. The galactose from Merck was purified by two or three recrystallizations. Glucose contamination was detected by growing the 'petite' mutant of *Saccharomyces cerevisiae* on the sugar: diauxic growth was easily detectable because the petite mutant grows faster on glucose than on galactose.

Organisms. The strains of *Saccharomyces cerevisiae* used were diploid 'yeast foam' strains (normal and 'petite') originally from Dr B. Ephrussi's laboratory. *Candida tropicalis* IRC 121 was a strain isolated at the Institut de Recherches du C.E.R.I.A. All the other strains were obtained from collections.

Manometric determinations. To avoid modifications of the cell metabolism, the gas exchanges were measured directly in the culture, without centrifugation and resuspension. The manometric determinations were made on 2 ml. of culture containing 7×10^6 organisms/ml. Respiration (oxygen uptake) was measured in the presence of 0·3 ml. of a 20% KOH solution spread over a large surface of filter paper. Aerobic fermentation was measured under the same conditions but without KOH. The CO_2 evolved was considered to be the result of fermentation, assuming that the respiratory quotient ($Q_{CO_2}:Q_{O_2}$) of the concomitant respiration was unity.

RESULTS

Measurement of the limiting rates of fermentation of various hexoses

Using the petite mutant of *Saccharomyces cerevisiae* (respiration deficient) it is possible to measure the limiting rates of aerobic degradation of different hexoses without complications due to respiration. Data are presented in Table 1 for glucose,

Table 1. *Rates of fermentation and growth of Saccharomyces cerevisiae petite mutant on various hexoses*

Carbon source (3 g./100 ml.)	Aerobic fermentation (μl. CO_2/10 min./ 10^7 organisms)	Growth rate		Fermentation rate (% of glucose value)	Growth rate (% of glucose value)
		Generation time (min.)	Number of doublings/hr.		
Glucose	72·4	70	0·857	100·0	100·0
Fructose	70·6	70	0·857	97·5	100·0
Mannose	52·0	96	0·625	71·8	72·9
Galactose	30·0	139	0·432	41·4	50·4

fructose, mannose and galactose. Glucose and fructose had identical rates of fermentation. The rate that of degradation of mannose was somewhat lower (71·8% that of glucose) and that of galactose still lower (41·4%). The differences in growth rates of the petite mutant on these various sugars indicated that the rate of fermentation was the factor which limited growth. The rates of growth of the petite mutant on mannose and galactose were respectively 72·9% and 50·4% of the rate of growth on glucose, which is a clear and quantitative correlation between rate of fermentation and rate of growth.

Behaviour of the normal strain of Saccharomyces cerevisiae in the presence of various hexoses; rates of respiration, fermentation and growth

The results obtained with the petite mutant of *Saccharomyces cerevisiae* showed that the fermentation rates of mannose and galactose were slow and growth-limiting. The aim of the following experiment was to see whether a normal strain of *S. cerevisiae* was able to elaborate a respiratory system when mannose or galactose was the carbon plus energy source. Manometric determinations of the respiration and fermentation rates were made on growing cultures as described under Methods. The results are given in Table 2.

From a comparison of Tables 1 and 2, one can conclude that the maximum rate of fermentation of each of the various sugars (with the exception of galactose) were the same for both the normal and the petite strains. In the case of galactose the fermentation rate was lower for the normal strain than for the petite: this situation resulted probably from an inhibition of the fermentation by the respiration (Pasteur effect).

The respiration rate was low when the normal strain grew on glucose or fructose. The rate was twice as high when the strain grew on mannose, and four times as high on galactose. In other words, this yeast was able to elaborate a respiratory system when the rate of fermentation was sufficiently low.

Table 2. *Rates of fermentation, respiration and growth of Saccharomyces cerevisiae normal strain on various hexoses*

	Rate of aerobic fermentation		Rate of respiration		Rate of growth		
Carbon source	μl. CO_2/10 min./10^7 organisms	As % of glucose value	μl. O_2/10 min./10^7 organisms	As % of glucose value	Generation time (min.)	Number of doublings/hr.	As % of glucose value
Glucose	78·0	100·0	4·8	100·0	53	1·13	100·0
Fructose	69·0	88·4	6·1	127·0	53	1·13	100·0
Mannose	46·0	59·0	10·6	221·0	63	0·952	84·0
Galactose	15·3	19·6	20·0	416·0	72	0·833	74·0

Note. These experiments were repeated in the presence of a higher concentration of hexoses: 6 g./100 ml. The results were the same as those obtained at 3 g./100 ml.

The growth rate of the petite mutant on mannose or galactose was slower than the growth rate on glucose (Table 1). This was not the case for the normal strain, with which the growth rates on mannose or galactose were close to the growth rate on glucose (Table 2). This means that the increased respiration rate compensated for an insufficient fermentation rate.

Nature of the growth-limiting factor for Saccharomyces cerevisiae degrading a sugar by aerobic fermentation

The good correlation that exists between growth rate and fermentation rate on various sugars with the petite mutant of *S. cerevisiae* indicates that the rate of fermentation is the growth-limiting factor. The nature of this limitation might be either the rate at which intermediary metabolites are made available to the cell, or the rate of energy production. If one compares a yeast strain which degrades glucose by aerobic fermentation, like *S. cerevisiae* normal strain, with a strain which degrades glucose by respiration, like *Candida tropicalis* (an organism which has no Crabtree effect), one can eliminate the first possibility. Growing *S. cerevisiae* organisms have an aerobic fermentation rate of 78 μl. CO_2/10 min./10^7 organisms, which corresponds to a degradation rate of 1·74 μmoles glucose/10 min./10^7 organisms. *Candida tropicalis* grew at about the same rate with a respiration rate of 27·7 μl. O_2/10 min./10^7 organisms, i.e. a degradation rate of only 0·21 μmole glucose/10 min./10^7 organisms. It is thus clear that the growth-limiting factor for organisms when degrading a sugar by aerobic fermentation is not the rate of

intermediary metabolites synthesis but rather the rate of energy production. Therefore the Crabtree effect must be considered as the repression of an energy-producing system, respiration, by another energy-producing system, fermentation. Thus, when respiration occurs simultaneously with aerobic fermentation, as is the case with the normal strain of *S. cerevisiae* growing on galactose, the compensation that the respiration brings to the deficient rate of fermentation is actually an energy compensation.

Presence of the Crabtree effect in various yeast strains

The presence or absence of the Crabtree effect in various yeast strains, belonging to various genera (see Table 3) was determined as follows. Fermentation and

Table 3. *Presence of the Crabtree effect in various yeast strains*

Organism	μl. of gas/10^7 organisms/10 min.		Ratio, fermented glucose/ respirated glucose	Crabtree effect
	Respiration (O_2 uptake)	Aerobic fermentation (CO_2 evolved)		
Saccharomyces cerevisiae	4·8	78·0	49·0	+
S. chevalieri	1·2	90·8	250·0	+
S. fragilis	24·5	1·9	0·23	−
S. italicus	0·0	94·5	∞	+
S. oviformis	0·0	61·2	∞	+
S. pasteurianus	1·9	58·7	93·0	+
S. turbidans	3·6	68·2	57·0	+
S. carlsbergensis	0·0	68·2	∞	+
Schizosaccharomyces pombe	0·0	40·6	∞	+
Candida utilis	30·0	0·0	0·0	−
C. tropicalis	27·7	0·9	0·1	−
C. monosa	21·0	0·0	0·0	−
Trichosporon fermentans	15·5	0·0	0·0	−
Hansenula anomala	24·1	0·0	0·0	−
Debaryomyces globosus	12·3	22·2	5·4	+
Pichia fermentans	24·3	1·3	0·16	−
Schwanniomyces occidentalis	9·1	0·0	0·0	−
Brettanomyces lambicus	1·2	9·3	23·0	+
Torulopsis dattila	0·0	52·0	∞	+
T. sphaerica	25·7	3·5	0·4	−
T. glabrata	.	.	.	+
T. colliculosa	10·7	39·2	11·0	+
T. sake	13·3	0·0	0·0	−
Nematospora coryli	21·1	29·2	4·1	+
Nadsonia fulvescens	.	.	38·0	+

Note. The absence of a respiration value means that it was too low to be measured in the presence of the high rate of aerobic fermentation.

respiration rates were measured for each strain under the conditions described in Methods. The rate of respiration expressed as μl. O_2 uptake/10^7 organisms/10 min. was converted into μmoles degraded/10^7 organisms/10 min., assuming 1 μmole glucose degraded/6 μmoles O_2 taken up. The same conversion was made for the fermentation rate on the basis of one μmole glucose degraded/2 μmoles CO_2 released. The ratio of μmoles fermented glucose to μmoles oxidized glucose was taken as a measure of the Crabtree effect. *A priori*, the Crabtree effect can be considered to be positive when this ratio is > 1 and negative when the ratio

is < 1. The strains tested fell into two classes: (1) those in which the ratio was between infinity and 4; (2) those in which the ratio was between 0 and 0·4. Class 1 was thus positive (+) and class 2 negative (−) (see Table 3). About 50% of the strains tested showed no Crabtree effect, although most *Saccharomyces* strains do show it.

Comparative physiology of the Crabtree effect

The elaboration of the respiratory system in strains of *Saccharomyces cerevisiae* appears to be related to low capacity for aerobic fermentation. In view of the priority of fermentation and the suppletive character of respiration in *S. cerevisiae*, it was interesting to compare this organism with *Candida tropicalis*, which has no Crabtree effect. Table 4 shows that for *S. cerevisiae* the rate of glucose fermentation (in presence and in absence of oxygen) was almost independent of the way the yeast

Table 4. *Comparative physiology of the Crabtree effect*

		Rate of glucose fermentation			
		Saccharomyces cerevisiae		*Candida tropicalis*	
Condition of cultivation	Type of fermentation	μl. CO_2/10 min./10^7 organisms	As % of glucose value	μl. CO_2/10 min./10^7 organisms	As % of glucose value
Anaerobic with glucose	Anaerobic	88·7	100·0	64·7	100·0
Aerobic with glucose	Anaerobic	81·5	91·9	24·0*	37·0
	Aerobic	78·1	88·1	1·0	1·5
Aerobic with lactate	Anaerobic	22·8*	26·0	6·25*	9·6

* Initial rates (see Figs. 1 and 2 for the adaptation rate).

was previously grown, so long as glucose was present during the growth. Figure 1 shows that these fermentation rates remained constant as a function of time (fully induced). For *Candida tropicalis* the maximum rate of fermentation occurred only after anaerobic growth (Table 4); this rate was of the same order of magnitude as for *S. cerevisiae*, which means that the Crabtree effect was not related to the maximum capacity for fermentation. After growth of *C. tropicalis* on glucose in aerobic conditions, the rate of anaerobic fermentation was only 37% of the maximum. Adaptation to anaerobic conditions was quick: after 30 min. of adaptation, the anaerobic fermentation reached its maximum rate (see Fig. 2). Aerobic fermentation was almost non-existent for *C. tropicalis*, whatever the conditions of cultivation.

More rigorous conditions can be obtained when the strains are compared after growth on a non-fermentable substrate (lactate), and in the absence of glucose. Under these conditions the rate of anaerobic fermentation of glucose was decreased to 25·7% for *Saccharomyces cerevisiae* but the adaptation to anaerobic conditions was quick (Fig. 1). For *Candida tropicalis* the anaerobic fermentation of glucose was decreased to a very low rate (10%) and the adaptation to anaerobiosis was slow (Fig. 2). These observations are consistent with the opinion that the organisms which exhibit the Crabtree effect show a priority of the fermentation pathway over respiration. The fermentation system is largely constitutive in strains which

show a Crabtree effect. On the contrary, fermentation is used as an energy plus carbon source only under conditions of anaerobiosis in strains which do not exhibit the Crabtree effect.

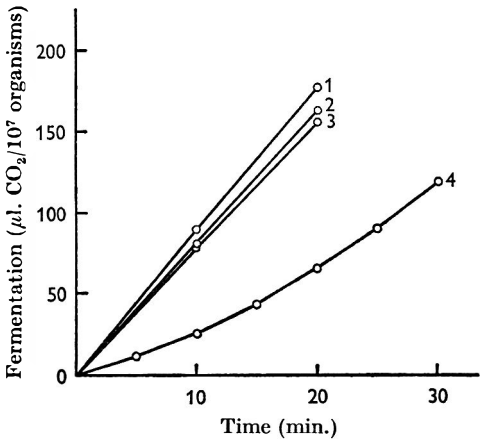

Fig. 1

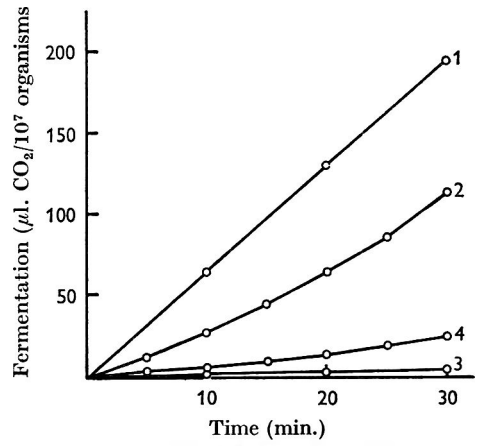

Fig. 2

Fig. 1. Aerobic and anaerobic fermentations of glucose by *Saccharomyces cerevisiae* after growth on glucose or lactate. Curve 1, anaerobic fermentation after anaerobic growth on glucose; curve 2, anaerobic fermentation after aerobic growth on glucose; curve 3, aerobic fermentation after aerobic growth on glucose; curve 4, anaerobic fermentation after aerobic growth on lactate.

Fig. 2. Aerobic and anaerobic fermentations of glucose by *Candida tropicalis* after growth on glucose or lactate. Curve 1, anaerobic fermentation after anaerobic growth on glucose; curve 2, anaerobic fermentation after aerobic growth on glucose; curve 3, aerobic fermentation after aerobic growth on glucose; curve 4, anaerobic fermentation after aerobic growth on lactate.

DISCUSSION

In the presence of air, a normal strain of *Saccharomyces cerevisiae* is able to grow with glucose, fructose, mannose or galactose as source of carbon plus energy at about the same rate for each substrate. Nevertheless, exponentially growing organisms have rates of aerobic fermentation which decrease from glucose to galactose (five-fold decrease) while the rates of respiration increase from glucose to galactose (four-fold increase). The slow rates of aerobic fermentation of mannose and galactose as compared with glucose might be the result of an inhibition of fermentation by the respiration which occurs simultaneously. In other words, the Pasteur effect might be responsible for the slow fermentation of mannose and galactose. But since we know that the fermentation rates of mannose and galactose are also slow for the petite mutant of *S. cerevisiae* (in which case there is no complication due to respiration) it is clear that the above situation does not result from the Pasteur effect. Thus, the high rate of respiration of galactose results from the low rate of aerobic fermentation of this sugar, and vice versa the very low respiration rate of glucose is the result of the high fermentation rate of this sugar. Now, the slow respiration rate of glucose may result either from an inhibition of the activity or from an inhibition of the synthesis of the respiratory enzymes. Spectroscopic observations made by Ephrussi *et al.* (1956) showed that aerobic growth of the

normal strain of *S. cerevisiae* in the presence of high concentrations of glucose resulted in an inhibition of the synthesis of the cytochromes *a*, *b* and *c*. The same conclusion is supported by our spectroscopic observations: the normal strain of *S. cerevisiae* growing exponentially on mannose or galactose is much richer in cytochromes than during growth on glucose. In other words, the high respiration rates observed during growth on mannose and galactose are the result of a de-repression of cytochromes synthesis.

One can further question whether the repression observed under conditions of high fermentation rates is the result of an accumulation of sugar degradation products or the result of an accumulation of adenosine triphosphate (or other form of energy). The experimental results show that even under conditions of de-repression of the respiratory system, i.e. during growth of the petite mutant on galactose, the rate of fermentative degradation of the sugar was still 0·67 μmole sugar/10^7 organisms/10 min., which is about three times the rate of degradation of glucose via respiration in *Candida tropicalis*: 0·21 μmoles glucose/10^7 organisms./10 min. It was thus not the shortage of intermediary metabolites that allowed a de-repression of the respiratory system when the fermentation rate was slow, but rather the shortage of adenosine triphosphate. This view is consistent with the low energy yield of fermentation as compared with the high energy yield of respiration. It would be useful to have further experimental confirmation; but as far as we know the Crabtree effect appears to be a repression of an energy-producing pathway (respiration) by another such pathway (fermentation).

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REFERENCES

Crabtree, H. G. (1929). Observations on the carbohydrate metabolism of tumors. *Biochem. J.* **23**, 536.

Ephrussi, B., Slonimski, P. P., Yotsuyanagi, Y. & Tavlitzki, J. (1956). Variations physiologiques et cytologiques de la levure au cours du cycle de la croissance aérobie. *C.r. Lab. Carlsberg, ser. physiol.* **26**, 87.

Lemoigne, M., Aubert, J. P. & Millet, J. (1954). La production d'alcool et le rendement de croissance de la levure de boulangerie cultivée en aérobiose. *Ann. Inst. Pasteur*, **87**, 427.

Salas, M. L., Viñuela, E., Salas, M. & Sols, A. (1965). Citrate inhibition of phosphofructokinase and the Pasteur effect. *Biochem. Biophys. Res. Comm.* **19**, 371.

Slonimski, P. P. (1956). Adaptation respiratoire: développement du système hémoprotéique induit par l'oxygène. *Proc. 3rd int. Congr. Biochem.* p. 242.

Swanson, W. H. & Clifton, C. E. (1948). Growth and assimilation in cultures of *Saccharomyces cerevisiae*. *J. Bact.* **56**, 115.

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The Crabtree Effect and its Relation to the Petite Mutation

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SUMMARY

Some yeast strains are able to give rise to 'petite' mutants upon treatment with euflavine while some other strains do not give rise to cytoplasmic respiration-deficient mutants. Nevertheless euflavine mimics the effect of the petite mutation in those strains which are unable to give petite mutants. There is a correlation between the presence of the Crabtree effect in a yeast strain and its ability to give petite mutants.

INTRODUCTION

A non-Mendelian mutation that results in loss of capacity for respiration in yeast was described by Ephrussi, Hottinguer & Chimenes (1949) and named 'petite' mutation. The mutation, which is irreversible, can be induced by some acridine dyes (Marcovitch, 1953). The simultaneous disappearance of several respiratory enzymes such as the cytochromes *a* and *b* and enzymes belonging to the tricarboxylic acid cycle, has been the object of numerous studies (Slonimski & Ephrussi, 1949; Slonimski & Hirsch, 1952; Slonimski, 1953*a*). The results of Linnane & Still (1956) indicated that the modification of the amount of enzymes of the tricarboxylic acid cycle, such as succinic dehydrogenase, probably arises from secondary effects of the mutation on control mechanisms. In other words, the primary effect of the petite mutation is an inability to synthesize cytochromes of types *a* and *b*.

The petite mutation has been studied almost exclusively in yeasts belonging to the genus *Saccharomyces*. A systematic study of the mutation in about twenty different strains of yeast, with euflavine as a mutagen, showed that some organisms were unable to give rise to petite mutants (De Deken, 1961*a*). These results led to the following observations: (1) the synthesis of respiratory enzymes in strains which did not give petite mutants was inhibited by a mutagenic acridine such as euflavine (De Deken, 1961*b*); (2) this inhibition, which was completely reversible, affects only the cytochromes *a* and *b*, i.e. those which are affected by the petite mutation. In the present work, a more detailed study has been made of the inhibition of the synthesis of respiratory enzymes by euflavine and of the spectrum of mutability to petite among various yeasts. An attempt has been made to define the causes of this distinction between those yeasts which give petite mutants and those that are unable to give petite mutants. The analogy between the Crabtree effect and the action of mutagenic acridines, i.e. the inhibition of the synthesis of some cytochromes, as well as the importance of the Crabtree effect for yeast physiology, i.e. separation

of the strains into fermentative and oxidative yeasts (see De Deken, 1966), suggested a correlation between the mutability to petite and ability to show the Crabtree effect. The results described below indicate that these two properties are indeed related.

METHODS

Growth medium. The medium contains 7·6 g. KH_2PO_4, 5·0 g. of Difco yeast extract/l. distilled water, adjusted to pH 6·0. The carbon sources were: glucose 30 g./l. or potassium lactate 0·1 M, or glycerol 0·1 M, or ethanol 10·0 g./l.

Reagents. The euflavine was isolated from acriflavine by the method described by Albert (1951). The proflavine was a commercial sample (British Drug Houses).

Organisms. The organisms were the same as the one described previously (De Deken, 1966).

Spectroscopic observations. The yeast samples were suspended in glycerol and the centrifuged pellet was plated between two Pyrex windows. The thickness of the yeast layer was 1 or 2 mm. The spectroscopic observation was made at the temperature of liquid air. For prolonged observation the sample was kept under vacuum in a metallic Dewar flask equipped with Pyrex windows. The sample was in thermal contact with the liquid air of the Dewar flask.

RESULTS

Yeast strains unable to give petite mutants

We noted previously (De Deken, 1961*a*) that some yeast strains were apparently unable to give petite mutants upon treatment by euflavine. If the synthesis of the cytochromes of such strains is reversibly inhibited by euflavine—in other words, if euflavine is also able to reach the site of cytochromes synthesis in these strains—then it is of interest to study such strains in order to find out what kind of genetic or physiological characteristic of the yeast strain is needed for the expression of the petite mutation. The ability of a strain to give petite mutants was tested in the following way. The yeast strain was grown with glucose as carbon source (see Methods) and treated with a known concentration of euflavine. The liquid cultures were incubated with shaking at the optimal temperature for each strain. The extinction of the culture was measured, and after five doublings of the optical density samples of the culture were plated on the same medium solidified with agar, but without euflavine. After growth, mutants colonies were identified by replication on a glucose medium and on a glycerol medium: respiration-deficient colonies were unable to grow on the glycerol medium. A strain was considered as unable to give petite mutants if no respiration-deficient colonies were detected after 20–25 generations of growth in the presence of a high dose of euflavine. The concentration of euflavine used in each case is given in column 2 of Table 1 and the number of generations passed in presence of euflavine is given in column 3 of Table 1. Column 4 of Table 1 shows the results obtained for 25 different strains. Even in the presence of euflavine concentrations as high as 25–50 μg./ml. (see column 2 of Table 1) about 50 % of the strains were unable to give respiration-deficient mutants. The mutation was more frequent among saccharomyces strains than among other yeasts.

Reversible inhibition of respiratory enzyme synthesis by euflavine in yeast strains unable to give petite mutants

The concentration of euflavine required to inhibit the synthesis of respiratory enzymes was measured in the following way. The organism was grown with a non-

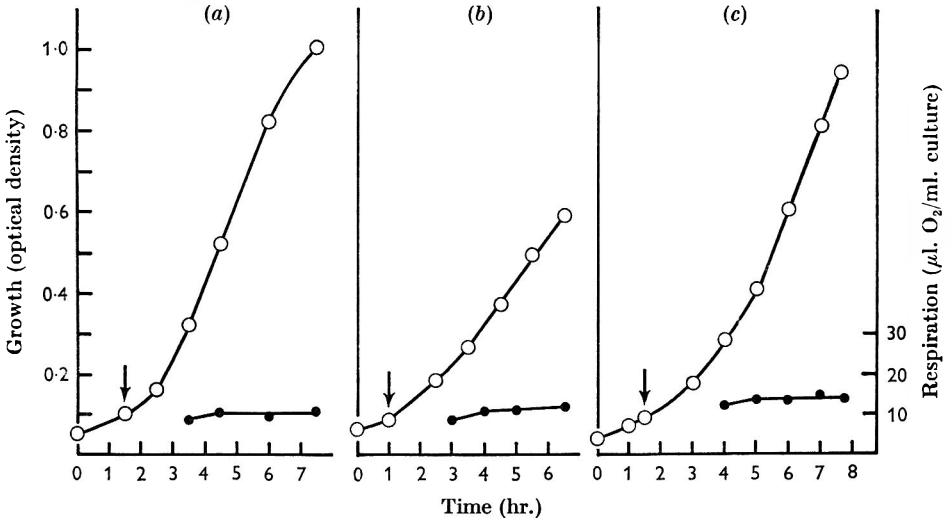

Fig. 1. Inhibition of the synthesis of the respiratory enzymes by euflavine. Organisms are growing on lactate. ○, growth; ●, respiration; *a*, *Candida tropicalis* with euflavine 2·5 μg./ml.; *b*, *Candida monosa* with euflavine 5·0 μg./ml.; *c*, *Candida pseudotropicalis* with euflavine 5·0 μg./ml. The time of addition of euflavine indicated by arrows.

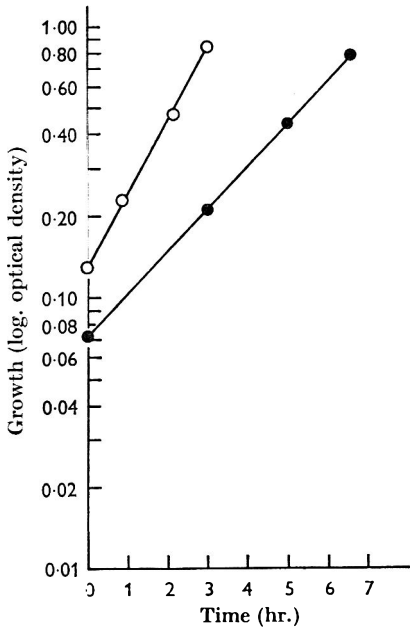

Fig. 2. Exponential growth on glucose in the presence of euflavine. ○, *Candida tropicalis* with euflavine 2·5 μg./ml.; ●, *Saccharomyces fragilis* with euflavine 2·5 μg./ml.

fermentable substrate (e.g. lactate, glycerol, ethanol) and euflavine was added to the culture at the beginning of the exponential phase of growth. When the concentration of euflavine inhibited the synthesis of respiratory enzymes, the rate of oxygen uptake/ml. culture became constant instead of increasing exponentially, and the growth became linear as a function of time. The experiment is illustrated by Fig. 1 for three different strains. In a control experiment (Fig. 2) it is shown that the same concentration of euflavine allowed exponential growth in the presence of

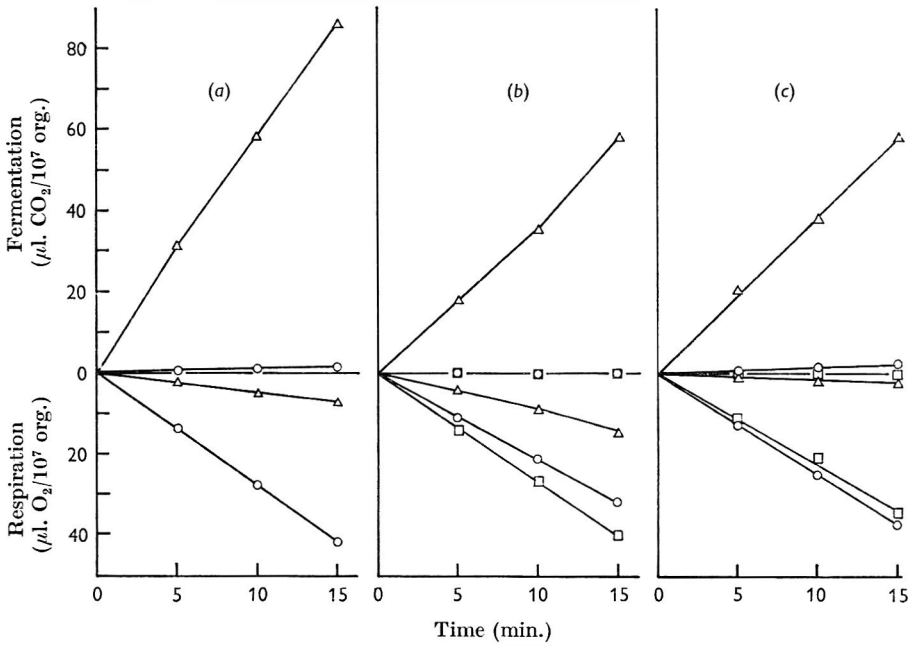

Fig. 3. Aerobic fermentation of glucose and respiration by organisms growing with and without acridines. ○, without inhibitor; Δ, euflavine, 2·5 μg./ml.; □, proflavine, 10·0 μg./ml.; (*a*), *Candida tropicalis*; (*b*), *Candida monosa*; (*c*), *Saccharomyces fragilis*.

a fermentable substrate; under these conditions the degradation of glucose proceeded via aerobic fermentation (Fig. 3) because of the lack of respiratory enzymes. The exponential growth on glucose plus euflavine in the absence of the respiratory enzymes synthesis shows also that the petite mutation, if able to occur, would not be lethal for the yeast strain. The results for 24 strains (column 1 of Table 1) show that a low concentration of euflavine (maximum 10 μg./ml.) inhibited the biosynthesis of respiratory enzymes in strains which did not give petite mutants as well as in strains which did give petite mutants. This means, at least, that euflavine was able to enter the cells and reach the site of synthesis of respiratory enzymes even in those yeast strains which did not give respiration-deficient mutants after treatment with euflavine.

Sensitivity of the site of respiratory enzymes synthesis to mutagenic and non-mutagenic acridines

Studies by Marcovitch (1953) indicated that some acridine dyes of molecular structure closely related to euflavine were not mutagenic. We know also that the

mutagenic acridines are the only ones able to inhibit the synthesis of respiratory enzymes in a *Saccharomyces cerevisiae* strain which does give petite mutants (Slonimski, 1953*b*). It was of interest to verify whether a non-mutagenic acridine, such as proflavine, did not inhibit the biosynthesis of respiratory enzymes in strains which are unable to give petite mutants. The aim of this experiment was to rule out the possibility of a general and non-specific inhibition of the synthesis of respiratory enzymes by acridines in strains which do not give respiration-deficient mutants.

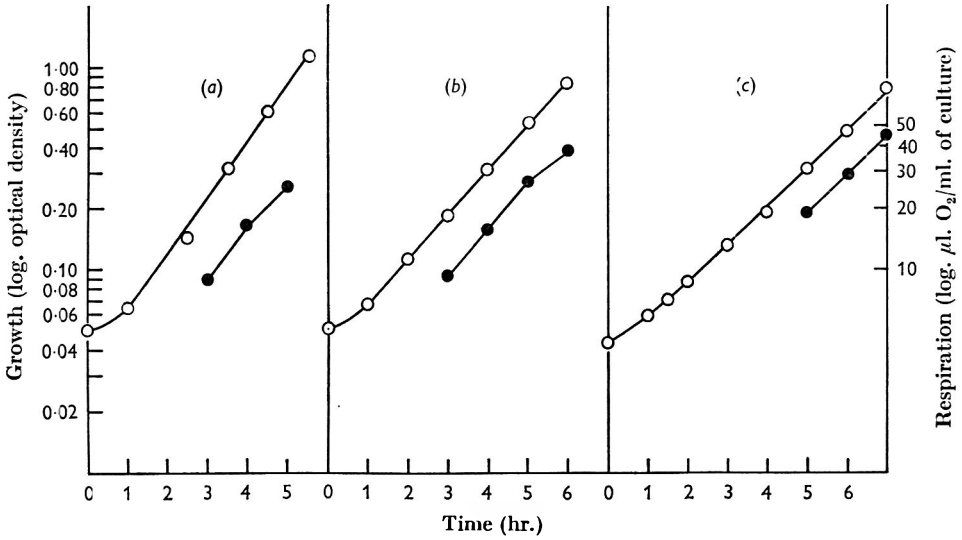

Fig. 4. Exponential growth and synthesis of respiratory enzymes in the presence of proflavine. ○, growth; ●, respiration; (*a*), *Candida tropicalis*; (*b*), *Candida monosa*; (*c*), *Saccharomyces fragilis*.

Three different strains were treated by proflavine, a non-mutagenic acridine, and the results are shown in Fig. 4. The experiment shows that proflavine allowed exponential growth and an exponential increase of the respiration rate in the presence of a non-fermentable substrate. Therefore a non-mutagenic acridine did not inhibit the synthesis of respiratory enzymes and it may be concluded that both kinds of yeasts, able or not to give petite mutants, are endowed with the same stereochemical characteristics as far as sensitivity to acridines is concerned.

Physiological function of the site sensitive to mutagenic acridines

It is well known that petite mutants are unable to synthesize the cytochromes of types *a* and *b*, but are still able to synthesize cytochrome *c*. It was of interest to know whether the effect of euflavine can mimic the petite mutation. Spectroscopic examination of *Candida tropicalis* (a strain unable to give petite mutants) grown in the presence of euflavine revealed that both cytochromes *a* and *b* are absent and that cytochrome *c* was still present. Moreover, we previously observed that the biosynthesis of cytochrome *c* in a petite mutant of *Saccharomyces cerevisiae* was also insensitive to the presence of euflavine (De Deken, 1961*b*). One may thus

conclude that both kinds of yeasts, able or not able to give petite mutants, have a euflavine-sensitive site which controls, in some way or another, the synthesis of cytochromes of types *a* and *b*.

The physiological characteristic common to yeast strains able to accomplish the petite mutation

Although euflavine is apparently able to reach the same site and to inhibit this site in the same manner in all the yeast strains tested, this action may or may not be followed by the mutation. Thus the accomplishment of the mutation has to be ascribed to a property of the strain itself. This property is probably genetic, but we

Table 1. *Relationship between Crabtree effect and petite mutation*

Organism (1)	Inhibition of the synthesis of respiratory enzymes: euflavine (μg./ml.) (1)	Induction of the petite mutation by euflavine			
		Euflavine (2)	No. of generations in the presence of euflavine (3)	Petite mutation (4)	Crabtree effect (5)
Saccharomyces cerevisiae	1·0	1·0	.	+	+
S. chevalieri	2·5	2·5	.	+	+
S. fragilis	5·0	10·0	19	−	−
		25·0	8	−	
S. italicus	2·5	2·5		+	+
S. oviformis	2·5	2·5		+	+
S. pasteurianus	2·5	2·5		+	+
S. turbidans	.	10·0	27*	±	+
S. carlsbergensis	2·5	2·5	.	+	+
Schizosaccharomyces pombe	2·5	2·5	.	+	+
Candida utilis	10·0	25·0	20	−	−
		50·0	8	−	
C. tropicalis	2·5	25·0	27	−	−
C. monosa	5·0	10·0	13	−	−
		25·0	10	+	
Trichosporon fermentans	2·5	25·0	22	−	−
Hansenula anomala	10·0	25·0	25	−	−
		50·0	17	−	
Debaryomyces globosus	10·0	5·0	.	+	+
Pichia fermentans	2·5	25·0	2	−	−
		25·0	5	+	
Schwanniomyces occidentalis	5·0	10·0	21	−	−
Brettanomyces lambicus	10·0	10·0	.	+	+
Torulopsis dattila	2·5	2·5	.	+	+
T. sphaerica	2·5	10·0	25	−	−
		25·0	8	−	
T. glabrata	5·0	5·0	.	+	+
T. colliculosa	5·0	5·0	.	+	+
T. sake	.	25·0	21	−	−
Nematospora coryli	5·0	5·0	.	+	+
Nadsonia fulvescens	.	10·0	23	−	+

* For some unknown reason the % of petite mutants among the population did not exceed 20% for this strain.

Note. It is obvious that the petite mutation would be lethal for a yeast unable to ferment glucose: strains able to ferment glucose were thus selected for this work.

may hope that the phenotypic expression of it (i.e. a physiological property) will distinguish the strains able to give petite mutants from those not able. Since the site of synthesis of cytochromes *a* and *b* is sensitive to euflavine and to a regulatory system (the Crabtree effect) at least in certain strains (see De Deken, 1966), we could investigate whether the property responsible for the Crabtree effect could also be the physiological property responsible for the accomplishment of the petite mutation.

The Crabtree effect was measured for all strains as described previously (De Deken, 1966). The Crabtree effect and the petite mutation may be compared in columns 4 and 5 of Table 1. The correlation between the two phenomena is fairly good. Among the 15 strains which exhibited the Crabtree effect, 14 gave petite mutants at low concentrations of euflavine. Among the 10 strains which showed no Crabtree effect, 8 never gave rise to respiration-deficient mutants. The two other strains (*Candida monosa*, *Pichia fermentans*) required very high doses of euflavine to bring about mutation—5 to 10 times greater than the concentration required to inhibit the synthesis of respiratory enzymes.

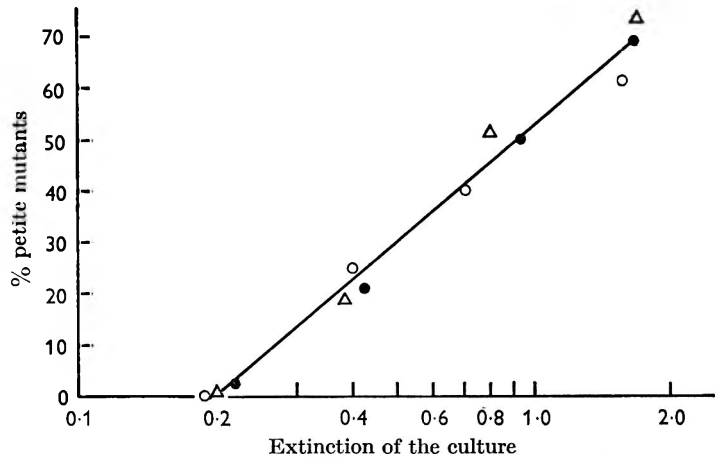

Fig. 5. *Saccharomyces cerevisiae*: differential rate of mutation induced by euflavine on different sources of carbon and energy, ●, glucose; Δ, galactose; ○, lactate. Euflavine (1 μg./ml.) was added when the culture had an extinction of 0·1 (Beckman C).

Saccharomyces cerevisiae: *relationship between the intensity of the Crabtree effect and the kinetics of the petite mutation*

It is possible to vary the intensity of the Crabtree effect, i.e. to vary the differential rate of respiratory enzymes synthesis, by modifying the nature of the carbon source (De Deken, 1966). The Crabtree effect is maximum with glucose (ratio 49·0), partial with galactose (ratio 2·2) and of course zero with a non-fermentable substrate such as lactate. The three different media were inoculated with *Saccharomyces cerevisiae* normal strain and treated with euflavine. Samples of the cultures were taken during growth and plated on a glucose medium without euflavine, to determine the proportion of petite mutants among the population. The percentage of petite mutants was plotted against extinction of the culture (Fig. 5) as measured with a Beckman C apparatus.

DISCUSSION

The cytoplasmic petite mutation implies that some genetic information is carried in the cytoplasm. It is reasonable to think that that this information is present in deoxyribonucleic acid located in the mitochondria (Schatz, Halsbrunner & Tuppy, 1964). Because of the irreversible character of the cytoplasmic petite mutation, we have to consider that this mutation results from a cessation of duplication of this cytoplasmic DNA rather than from a simple modification of this DNA. We know also that the petite mutant differs from the normal strain by having a modified structure of the mitochondria (Yotsuyanagi, 1962). It is thus reasonable to assume that the mitochondrial DNA contains information for some structural proteins of the mitochondria.

If it is obvious that a number of chromosomal genes are responsible for the structure of the cytochromes *a* and *b* (Sherman, 1963; Sherman & Slonimski, 1964), it is also quite possible that both the nuclear and the mitochondrial DNA are involved in the synthesis of the cytochromes *a* and *b*. The action of chloramphenicol on *Saccharomyces cerevisiae* suggests that the mitochondrial DNA determines the synthesis of the insoluble cytochromes, i.e. cytochromes *a* and *b* (Dr A. W. Linnane personal communication).

In the present article we came to the conclusion that euflavine mimics the petite mutation. Whether this inhibition is followed by mutation to petite or not is a characteristic of the given strain rather than a property of euflavine. The reversible inhibition of the synthesis of cytochromes *a* and *b* by euflavine may be understood as an interference of euflavine with the heterocatalytic activity of the mitochondrial DNA if we assume that this DNA is involved in the synthesis of the insoluble cytochromes.

We observed also that there is a correlation between the presence of the Crabtree effect in a given strain and the ability of this strain to give petite mutants, either spontaneously at a low frequency or in the presence of euflavine with a high frequency. The Crabtree effect is the phenotypic expression of a regulatory system involved in the synthesis of the cytochromes (De Deken, 1966). Therefore one can speculate that a genetic factor involved in the Crabtree effect is (at least under some conditions) an obstacle to the duplication of the DNA and helps euflavine to prevent the duplication. As a result of this situation the DNA replication becomes slower than the division rate of mitochondria and gives rise to mitochondria which lack the genetic information.

Two types of results reported in the present work seem to contradict the above hypothesis. (1) The differential rate of respiratory enzymes synthesis does not affect the mutation rate to petite. This means that the phenotypic expression of the Crabtree effect does not affect the rate of mutation but that the presence of the genetic elements involved in this regulatory system is important. (2) Two strains which show no Crabtree effect were able to give petite mutants. This may mean that the presence of only one genetic element involved in the regulatory system—either the moderating element or the acceptor site of the regulatory system—may be sufficient for the accomplishment of the petite mutation.

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REFERENCES

ALBERT, A. (1951). *The Acridines, Their Preparation, Physical, Chemical and Biological Properties and Uses.* London: Edward Arnold and Co.

DE DEKEN, R. H. (1961*a*). The dissociation of phenotypic and inheritable effects of euflavine in yeast. *Exp. Cell Res.* **24**, 145.

DE DEKEN, R. H. (1961*b*). Propriétés du site sensible à l'euflavine chez la levure. *Archs int. Physiol. Biochim.* **69**, 746.

DE DEKEN, R. H. (1966). The Crabtree effect: a regulatory system in yeast. *J. gen. Microbiol.* **44**, 149.

EPHRUSSI, B., HOTTINGUER, H. & CHIMENES, A. M. (1949). Action de l'acriflavine sur les levures. I. La mutation 'petite colonie'. *Ann. Inst. Pasteur*, **76**, 351.

LINNANE, A. W. & STILL, J. L. (1956). A re-examination of some problems of petite yeast. *Aust. J. Sci.* **18**, 165.

MARCOVITCH, H. (1953). Rapports entre la structure des acridines et leur activité en tant qu'agents inducteurs de mutants respiratoires chez la levure. *Ann. Inst. Pasteur*, **85**, 199.

SCHATZ, G., HALSBRUNNER, E. & TUPPY, H. (1964). Deoxyribonucleic acid associated with yeast mitochondria. *Biochem. biophys. Res. Commun.* **15**, 127.

SHERMAN, F. (1963). Respiration-deficient mutants of yeast. I. Genetics. *Genetics*, **48**, 375.

SHERMAN, F. & SLONIMSKI, P. P. (1964). Respiration-deficient mutants of yeast. II. Biochemistry. *Biochim. biophys. Acta*, **90**, 1.

SLONIMSKI, P. P. (1953*a*). *Formation des enzymes respiratoires chez la levure.* Paris: Masson.

SLONIMSKI, P. P. (1953*b*). A specific relation between enzymic adaptation and cytoplasmic mutation. In *Adaptation in Microorganisms*, Symp. Soc. gen. Microbiol. **3**, 76.

SLONIMSKI, P. P. & EPHRUSSI, B. (1949). Action de l'acriflavine sur les levures. V. Le système des cytochromes des mutants 'petite colonie'. *Ann. Inst. Pasteur*, **77**, 47.

SLONIMSKI, P. P. & HIRSCH, H. M. (1952). Nouvelles données sur la constitution enzymatique du mutant 'petite colonie' de *Saccharomyces cerevisiae*. *C.r. hebd. Séanc. Acad. Sci., Paris*, **235**, 741.

YOTSUYANAGI, Y. (1962). Etudes sur la chondriome de la levure. II. Chondriomes des mutants à déficience respiratoire. *J. ultrastruct. Res.* **7**, 141.

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Properties of Providence and *Proteus morganii* Transducing Phages

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SUMMARY

The properties of three transducing phages derived from providence strains NCTC 9207, 9246, 9290 and phage M derived from *Proteus morganii* NCTC 10041 are described. The providence phages are present in supernatant fluids of young broth cultures; phage M is ultraviolet-inducible. The 3 providence phages only attack and transduce into providence strains NCTC 9211, 9295; the action of phage M is restricted to *P. morganii* NCTC 2815. The phages transduce different markers at rates of $3 \times 10^{-7} - 1 \times 10^{-6}$/ phage adsorbed and are thus capable of generalized transduction. No abortive transductants were encountered. The providence phages are serologically related. Morphologically the phages resemble the salmonella transducing phage P22 but phage M differs in that it possesses a delicate collar round the short neck. The infectivity of the phages is Ca^{2+} independant. Phage M is more heat susceptible than the providence phages while its transducing particles are chloroform sensitive. Mutants of providence NCTC 9211, 9295 lysogenized by the phages are competent recipients in transduction experiments, while mutants of strain NCTC 2815 lysogenized by phage M yield no transductants when treated with suitable lysates of this phage. Small doses of ultraviolet irradiation of providence phages increase transduction rates. Phage M lysates similarly treated show no such stimulation. The phages have deoxyribonucleic acid base compositions similar to the organisms which they transduce and density gradient centrifugation reveals that transducing activity forms single peaks 2 or 3 fractions heavier than corresponding plaque-forming particles.

INTRODUCTION

Proteus and providence organisms display many intra- and inter-group differences (Ewing, 1958) and their classification is uncertain. Rauss (1962) advocated a tribe Proteae with genera *Proteus*, *Morganella*, *Rettgerella* and *Providencia*; *Topley & Wilson's Principles* (1964) excluded providence strains from a genus *Proteus* limited to '*vulgaris*', '*mirabilis*', '*morganii*' and '*rettgeri*' species. In an approach to this problem various gene loci of *Proteus mirabilis*, *P. morganii* and providence strains are being compared in this laboratory. With the former two organisms biochemical work is complemented with transduction studies using phages previously isolated (Coetzee & Sacks, 1960; Coetzee, 1966). To apply this technique in the providence work it was decided to search for transducing phages active on members of this group. The present paper describes properties of such phages and additional features of the *P. morganii* (Coetzee, 1966) transducing system.

METHODS

Media. In addition to the media previously used (Coetzee & Sacks, 1960) a minimal medium (Lederberg, 1950) was used for the selection of prototrophic transductants of providence strains. For transduction experiments with *Proteus morganii* the latter medium was supplemented with 0·0024% (w/v) L-cystine, 0·0001% (w/v), each, Ca pantothenate and nicotinic acid, according to Porter & Meyers (1945).

Phages and bacteria. The 23 temperate providence phages previously isolated (Coetzee, 1963*a*) and the *Proteus morganii* phage 10041/2815 (Prozesky, De Klerk & Coetzee, 1965) subsequently called phage M (Coetzee, 1966) were used. The general phage techniques including those of serology and determination of Ca^{2+} requirements for infectivity were according to Adams (1959). The organisms used for host-range experiments have been described (Coetzee, 1963*a*, *b*, *c*). One-step mutants resistant to 1 mg. streptomycin sulphate/ml. (*str-r*) were obtained as before (Coetzee & Sacks, 1960) and auxotrophic mutants of providence strains were selected by the penicillin method of Gorini & Kaufman (1960) after ultraviolet irradiation of washed suspensions to about 1% survival. Replication was from MacConkey agar to minimal medium; auxotrophs were identified auxanographically (Lederberg, 1950).

Ultraviolet radiation. The Hanovia sterilamp and the methods used to irradiate phage suspensions have been described (Coetzee & Sacks, 1960).

Preparation of phage lysates. Lysates with plaque-forming titres of about 5×10^9 p.f.u./ml. were prepared by the agar-layer method previously used (Coetzee & Sacks, 1960). Phage stocks were sterilized by membrane filtration (MF 30 Membranfilter Goettingen) or by adding 0·1 vol. chloroform. Purified preparations with titres of about 1×10^{12} p.f.u./ml. were obtained by differential centrifugation according to Prozesky *et al.* (1965).

Isolation of phage nucleic acid. The method of Mandell & Hershey (1960) modified by Davidson & Freifelder (1962) was used.

Isolation of bacterial deoxyribonucleic acid. Bacteria (3–4 g. wet wt.) were lysed with sodium lauryl sulphate and the deoxyribonucleic acid isolated according to Marmur (1961).

Estimation of base composition of deoxyribonucleic acid. Deoxyribonucleic acid was hydrolysed with 90% (v/v) formic acid at 175° for 30 min. (Wyatt, 1955). The bases were then separated on a two-dimensional chromatogram run on Whatman no. 1 paper by a descending technique. The solvents were first isopropanol + HCl (sp.gr. 1·18) + water (170 + 41 + 39 by vol.); then *n*-butanol saturated with water at 23° + 15 N-ammonium hydroxide (100 + 1 by vol.). The spots were made visible by u.v. irradiation (wavelength 254 mμ), quantitatively eluted and their base content estimated according to Wyatt (1955). Phosphate in nucleic acid preparations was estimated by the method of Bergold & Pister (1948).

Density gradient centrifugation of phages. A sample (0·3 ml.) of phage suspension (about 1×10^{12} p.f.u./ml.) was added to 3·0 ml. of a caesium chloride solution (Sheppard, 1962). The mixture was centrifuged at 27,000 r.v./min. for 18 hr in a Spinco model L preparative centrifuge with swinging-bucket rotor no. SW-39. Single drops were collected in 2 ml. broth from the bottom of the centrifuge tube

after puncture with a no. 22 syringe needle. The fractions were assayed for infective centres and transducing particles.

Electron microscopy. This was done as for proteus phages by the methods of Prozesky *et al.* (1965).

Transduction techniques. The 23 providence phages were screened for ability to transduce the *str-r* marker. The adsorption mixtures and controls were constituted as before (Coetzee & Sacks, 1960). After 20 min, at 30° the mixtures (or samples) were filtered through membrane filters (Iyer, 1962). The membranes were incubated on nutrient agar for 4 hr to allow for delay in phenotypic expression of the marker (Coetzee & Sacks, 1960) before transfer to the surface of MacConkey agar containing 1 mg. streptomycin sulphate/ml. Colonies were scored after 48 hr at 37°. With transductions of prototrophy to auxotrophic mutants the bacteria in adsorption mixtures were deposited by centrifugation after 20 min. The deposit was suspended in saline and samples plated on minimal media.

RESULTS

Three of the 23 temperate providence phages were able to transduce the *str-r* marker, The phages PL25, PL26, PL37 were present in the supernatant fluids of overnight broth cultures of providence strains NCTC 9207, 9246, 9290 respectively (Coetzee, 1963*a*). These 3 providence strains can be distinguished by means of their susceptibility to a series of lytic phages (Coetzee, unpublished results). These phages have identical host-ranges limited to providence NCTC 9211, 9295, on which they have equal efficiencies of plating and into which they transduce markers at about $3–9 \times 10^{-7}$/adsorbed phage. Strains NCTC 9211, 9295 have the same biochemical reactions and belong to providence biogroup 1 (Ewing, 1958). These strains can be distinguished by their reactions to the series of phages mentioned above. Phages PL25, PL26, PL37 had no action on any of the other strains of the family Enterobacteriaceae examined. Recipient controls in transduction experiments had less than 10 colonies on the membranes as compared with hundreds on test filters. Phage controls were sterile and transduction rates were not affected by previous treatment of phage lysates with chloroform or deoxyribonuclease (Coetzee & Sacks, 1960). Addition of potent antiphage serum, completely absorbed with recipient organisms, drastically decreased the transducing ability of phage suspensions. Phage M lysates differ from these providence phages in that the transducing particles of the former are sensitive to chloroform. Two days after chloroform treatment the transduction rate of the *str-r* marker had decreased from 1×10^{-6} to 5×10^{-9}/phage particle adsorbed, whereas the plaque-forming titre decreased by a factor of 2.

The 3 providence phages are more heat resistant than phage M. They undergo 95% inactivation at 70° for 15 min. as compared with 99·9% for the latter phage (Coetzee, 1966). Like phage M (Coetzee, 1966) the infectivity of the providence phages is Ca^{2+} independent. There was no difference between control titres and the titres recorded in the presence of 0·01 M-sodium citrate. Some of these data are presented in Table 1. The providence phages are related serologically but are distinct from phage M (Table 2).

The morphology of the providence transducing phages is shown in Pl. 1. They

possess polyhedral contours, short necks and base plates with a number of pins. Their average measurements are identical. The inter-apex length is 600 Å, tail length 160 Å and the base plate width is 210 Å. They lack the collar of phage M (Prozesky *et al.* 1965) and resemble salmonella transducing phage P22 (Anderson, 1960). Many of the capsids have an uneven rough appearance, suggestive of arrays of capsomeres; but neither the shape nor the packing arrangements of the latter could be determined. A short rod about 30 Å wide was sometimes seen projecting beyond the base plate of phage PL26 (Pl. 1, fig. 2). It may be nucleic acid; similar structures have been encountered with two non-transducing providence phages

Table 1. *Properties of providence and Proteus morganii transducing phages*

Phage	Derived from NCTC	Indicator and transduces into NCTC no.	$CHCl_3$ sensitivity of transducing particles	Transduction rate/ adsorbed phage	Ca^{2+} infectivity dependance	% inactivation 70° 15 min.
PL25	9207	9211 9295	—	3 to 9×10^{-7}	—	95
PL26	9246	9295	—	3 to 9×10^{-7}	—	95
PL37	9290	9295	—	3 to 9×10^{-7}	—	95
M	10041	2815	+	1×10^{-6}	—	99·9

Table 2. *Neutralization constants of providence and Proteus morganii phage antisera*

Dilutions of phage antisera were mixed with the phages at 37°. Phage was assayed at intervals. The proportion of surviving phage was plotted against time. Survivals were first-order reactions from which neutralization constants were calculated.

	Phage			
	PL25	PL26	PL37	M
	Neutralization constants ($min.^{-1}$).			
Serum				
Anti-PL25	120	30	20	0
Anti-PL26	20	110	10	0
Anti-PL37	10	20	180	0
Anti-M	0	0	0	80

(Prozesky *et al.* 1965) which have similar morphologies. *Clostridium perfringens* phage 80 (Vieu, Guélin & Dauguet, 1965) also has a structure which projects beyond the base plate; it is 65 Å in width and can be consistently demonstrated. It is thought to be a tail core.

A large number of different auxotrophs of providence strains NCTC 9211, 9295 were obtained. A few, 9211 *hi-2*, *try-1*, 9295 *ad-4*, *cys-10*, *leu-1* were selected for use because of their low revertant rates. These auxotrophs were transduced to prototrophy by the three providence phages produced on wild strains NCTC nos. 9211, 9295 or on other auxotrophs. The rates of transduction are similar to those of the *str-r* marker. No abortive transductants were detected.

No attempts were made to prevent secondary lysogenization of transduced clones (Coetzee & Sacks, 1960) and all colonies examined were lysogenized by the phage and resistant to lysis by any of the three providence phages. Unlike the *Proteus mirabilis* (Coetzee & Sacks, 1960; Coetzee, 1961; Böhme, 1963) and *P.*

morganii (Coetzee, 1966) systems, lysogenized providence prototrophic transductants which originated from auxotrophic recipients still yielded *str-r* transductants (albeit at lower rates) when treated with lysates prepared on *str-r* variants. This property allows a nice distinction to be made between the 3 providence phages (Table 3). Many other phages can transduce recipients previously lysogenized by them like P22 (Yura, 1956), P1 (Arber, 1960), B3, F116 (Holloway, Monk, Hodgins & Fargie, 1962) λ (Weigle 1957), φ80 (Matsushiro, Sato & Kida, 1964).

Table 3. *Transduction to streptomycin resistance of* NCTC *9211 and prototrophic* NCTC *9211 hi-2 transductants lysogenized with phages PL25, PL26, PL37, respectively*

In separate experiments NCTC 9211 *hi-2* was transduced to prototrophy by phages PL25, PL26 and PL37, produced on NCTC 9295 *str-r*. Prototrophic transductant clones were picked off and demonstrated to be lysogenized by the corresponding phage. These transductants are written 9211 (PL25), etc. Using these lysogenic transductants and the wild strain NCTC 9211 quantitative transduction experiments were done with the phage lysates previously used and selection for *str-r* transductants.

Recipients NCTC no.	Phages PL25	PL26	PL37
9211	811*	700	526
9211 (PL25)	89	325	371
9211 (PL26)	400	73	311
9211 (PL37)	327	361	49

* No. *str-r* transductants.

Table 4. *Base composition of deoxyribonucleic acids of phages and bacteria*

Deoxyribonucleic acid isolated from phage or organisms was hydrolysed with formic acid and the bases separated by paper chromatography in two dimensions. Ultraviolet absorbing areas were removed from the paper and eluted with 0·1 N-HCl. The concentration of each base was quantitatively determined from its molar extinction coefficient.

Source of DNA	No. analyses	Mean base content (moles/100 moles) Adenine	Thymine	Guanine	Cytosine	Guanine + cytosine (moles/ 100 moles)	Total bases /phosphorus (moles/ g. atom)
Phage M	7	25·7	25·4	24·6	24·3	48·9	97
Phage PL26	4	29·6	28·8	21·0	20·6	41·6	99
NCTC 2815	4	25·2	25·4	24·5	24·9	49·4	99
NCTC 9295	4	29·3	29·5	20·6	20·6	41·2	98

The differential effect of ultraviolet irradiation on the plaque-forming and transducing abilities of phages PL26 and M is illustrated in Fig. 1. The effect of this treatment on the transduction rate of phage PL26 is similar to that on phages PL25, PL37 and *Proteus mirabilis* transducing phages (Coetzee & Sacks, 1960) and many other transducing phages (Holloway *et al.* 1962). The initial stimulation in transduction frequency with PL26 was encountered for all three markers (*str-r*, *ad-4*, *hi-2*) tested. The behaviour of phage M was different. The transduction frequency of *ad-1*, *try-1*, *hi-1*, *str-r* (Coetzee, 1966) progressively decreased with time of irradiation of phage M. In this respect phage M resembles the *Pseudomonas aeruginosa* phage F116 (Holloway *et al.* 1962). Fig. 1 also shows that the plaque-forming ability of phage M was inactivated at a greater rate than that of phage PL26.

The deoxyribonucleic acid base composition of phage M, its recipient in transduction experiments *Proteus morganii* NCTC 2815, phage PL26 and one of its indicator organisms providence strain NCTC 9295 are given in Table 4. Each phage and host have practically the same guanine + cytosine molar content. Transducing phages λ (Schildkraut, Marmur & Doty, 1962) and P22 (Sinsheimer, 1960) also have the same base compositions as their bacterial partners. Exceptions are encountered with the *Bacillus subtilis* transducing phages where the nucleic acid of phage SP10 (Okubo, Stodolsky, Bott & Strauss, 1963) differs physically from that of its host, and phage PBS2 has a deoxyribonucleic acid which does not resemble that of *B. subtilis* chemically or in base content (Mahler, Cahoon & Marmur, 1964). The guanine + cytosine values obtained for the *P. morganii* and providence strains by the chemical,

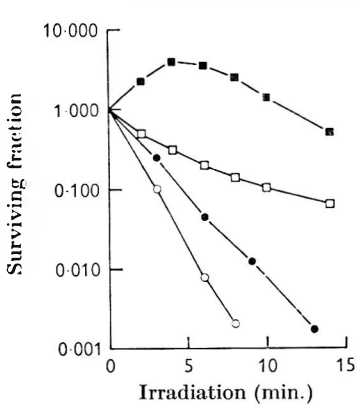

Fig. 1

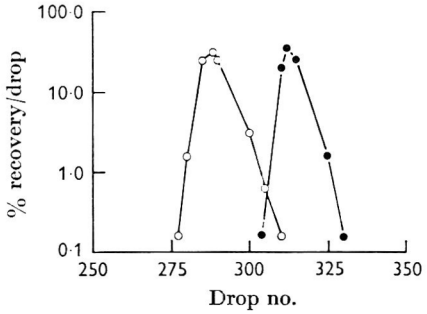

Fig. 2

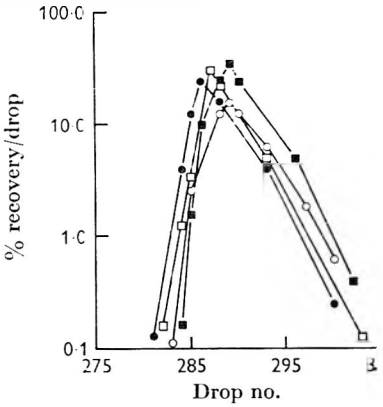

Fig. 3

Fig. 1. Effect of ultraviolet irradiation on plaque-forming and transducing abilities of phages PL26 and M produced on NCTC 9295, and 2815. Lysates were irradiated at 10 ergs/min.²/sec. At intervals samples were removed and used in quantitative transduction experiments with auxotrophs as recipients. The plaque-forming ability of the samples was also determined. Phage PL26: —●—, plaque formation; —■—, NCTC 9295. *ad-4* transductions to prototrophy. Phage M: —○—, plaque formation; —□—, NCTC 2815, *hi-1* transductions to prototrophy.

Fig. 2. Separation of phages PL25 and M by CsCl density-gradient centrifugation. A 3 ml. sample of a mixture of lysates was centrifuged and 420 drops collected in tubes containing 1 ml. broth, one drop/tube. The plaque-forming titre of the contents of tubes was assayed on NCTC strains 9211, 2815. Phage M, —○—; phage PL25, —●—.

Fig. 3. Separation of infective and transducing particles of phage PL25 by density-gradient centrifugation. Phage PL25 was prepared on strain NCTC 2925 *str-r*. A 3 ml. sample of phage and CsCl was centrifuged and 390 drops collected in tubes containing 2 ml. broth, one drop/tube. The plaque-forming and transducing titres were determined for the contents of tubes. Transductions of NCTC 9295 to *str-r* —●—, transduction of NCTC 9211 *hi-2* and *try-1* to prototrophy —○—, —□—, respectively. Plaque-formation, —■—.

methods used here correspond closely to the figures recorded by Falkow, Ryman & Washington (1962) for other strains of these organisms, which were determined by thermal denaturation of their deoxyribonucleic acids.

Figure 2 shows the distribution of particles collected after 18 hr centrifugation from a mixture of phages M and PL25. Each phage gives a distinct band and single peak of activity with the former displaced to the heavy side. The recovery of both phages was always above 90 %. Activities for transduction to wild type of NCTC 9211 *hi-2*, *try-1* and NCTC 9295 to *str-r* by centrifugal fractions of phage PL25 are pre-

sented in Fig. 3. Recovery of transducing particle input varied from about 30 % to more than 90 %, depending on the marker used. The curves are similar and show that transductions registered as single peaks coincident with, or up to 3 fractions more dense than, those of infectious centres. Experiments were also done with phage M samples transducing *str-r* or prototrophy to strain NCTC 2815 *ad-1*. Results were similar to those obtained with phage PL25. Transducing activity for the two markers coincided in a peak three drops more dense than the corresponding plaque-forming summit. The above results were reproduced with three independently prepared phage stocks of PL25 and two stocks of phage M. These results resemble those obtained with transducing phage P1 (Ting, 1962) and P22 (Sheppard, 1962). With the *Bacillus subtilis* transducing phage SP10 (Okubo *et al.* 1963) the curves representing the two types of activity were also single-peaked but were clearly separated by a number of fractions. Curves obtained here differ markedly from results recorded with *Escherichia coli* phages λ (Weigle, 1961) and $\phi 80$ (Matsushiro *et al.* 1964). Low frequency of transduction lysates of these phages exhibited a broader density band than the corresponding plaque-forming particles with several peaks or humps.

DISCUSSION

The three serologically related and very similar providence phages described here can transduce many different regions of the donor chromosome. They are thus capable of gernalized transduction (Campbell, 1964) like phage M (Coetzee, 1966). The rates of transduction correspond to those of other phages able to produce this type of transduction (Coetzee & Sacks, 1960; Hayes, 1964). As with the *Pseudomonas aeruginosa* (Holloway & Monk, 1959), *Staphylococcus pyogenes* (Edgar & Stocker, 1961). *Proteus mirabilis* (Coetzee, 1963*d*), *P. morganii* (Coetzee, 1966) and *Bacillus subtilis* (Thorne, 1962) transducing systems, no abortive transductants (Ozeki, 1956) were observed.

The finding of Holloway *et al.* (1962) and Prozesky & Coetzee (1966) that ultraviolet irradiation of *Pseudomonas aeruginosa* phage F116 and *Proteus mirabilis* phage 34/13 decreased the frequency of joint transductions may be taken in support of the theory (Jacob & Wollman, 1958) that this irradiation increases the frequency of exchange between the exogenote and bacterial chromosome. On these grounds the action of ultraviolet radiation on phage M transductions in decreasing the recovery of selected markers could be explained (Holloway *et al.* 1962) by assuming that phage M carries larger segments of bacterial chromosome than the providence phages and other phages which show an increased frequency of transduction with low doses of radiation. Unfortunately no joint transduction of markers has yet been found in the systems described here. Apart from the differential effect which ultraviolet radiation has on the plaque-forming and transducing properties of phage M lysates, chloroform affects the latter activity much more than the former property. Wilson (1960) encountered a similar phenomenon with *Escherichia coli* phage P1.

With the λ and P1 phage *Escherichia coli* transducing systems there is evidence (Campbell, 1957; Adams & Luria, 1958; Okubo *et al.* 1963) that markers from the donor bacterium replace homologous portions of phage chromosomes 'in some

kind of hybridization process' (Campbell, 1964) to form transducing particles. Because of the close correspondence of deoxyribonucleic acid base composition between phages and bacterial partners described here, it is possible that donor fragments are also integrated into the transducing phage chromosome by a process of substitution similar to that proposed for the *E. coli* systems (Hayes, 1964; Mahler *et al.* 1964). In *Bacillus subtilis* transducing systems, where there may not be enough genetic homology between phage and bacterium to allow pairing (Meynell, 1964) there is physical evidence (Okubo *et al.* 1963; Mahler *et al.* 1964) that here the bacterial donor fragments are not integrated into the viral chromosome but are packaged into the capsids of transducing particles by a process analogous to phenotypic mixing suggested by Zinder (1953).

In contrast to the distinction obtained between transducing and lytic particles by radiation and chloroform treatments, these two types of particle band very closely with single peaks in CsCl gradients of the lysates. These results may mean that, unlike λ (Weigle, 1961) and phage ϕ80 (Matsushiro *et al.* 1964) the providence and *Proteus morganii* transducing phages are formed by the bacterial donor fragment replacing a phage segment of nearly equal size. It was hoped to show that at least one of the temperate providence phages examined with activity extending to other members of the Proteus group (Coetzee, 1963*a*) would prove capable of transduction. As in the case of the *P. mirabilis* (Coetzee, 1963*d*) and *P. morganii* systems (Coetzee, 1966) the transducing phages discovered here have a very restricted host-range which excludes the possibility of inter-species hybridization.

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REFERENCES

ADAMS, J. N. & LURIA, S. E. (1958). Transduction by bacteriophage P1: abnormal bacteriophage function of the transducing particles. *Proc. natn. Acad. Sci., U.S.A.* **44**, 590.

ADAMS, M. H. (1959). *Bacteriophages.* New York: Interscience Publishers, Inc.

ANDERSON, T. F. (1960). On the fine structure of the temperate bacteriophages P1, P2, and P22. *Proc. Eur. Reg. Conf. electron microscopy*, **2**, 1008.

ARBER, W. (1960). Transduction of chromosomal genes and episomes in *Escherichia coli*. *Virology*, **11**, 273.

BERGOLD, G. & PISTER, L. (1948). The quantitative micro-determination of deoxy- and ribonucleic acids. *Z. Naturf.* **3***b*, 406.

BÖHME, H. (1963). Transduction of prototrophy to auxotrophic mutants of *Proteus mirabilis*. *Biochem. biophys. Res. Comm.* **10**, 60.

CAMPBELL, A. (1957). Transduction and segregation in *Escherichia coli* K12. *Virology*, **14**, 366.

CAMPBELL, A. (1964). Transduction. In *The Bacteria*. Ed. by I. C. Gunsalus and R. Y. Stanier, vol. 5, p. 49. New York: Academic Press Inc.

COETZEE, J. N. (1961). Lysogenic conversion in the genus *Proteus*. *Nature, Lond.* **189**, 946.

COETZEE, J. N. (1963*a*). Lysogeny in Providence strains and the host-range of Providence bacteriophages. *Nature, Lond.* **197**, 515.

COETZEE, J. N. (1963*b*). Lysogeny in *Proteus rettgeri* and the host-range of *P. rettgeri* and *P. hauseri* bacteriophages, *J. gen. Microbiol.* **31**, 219.

COETZEE, J. N. (1963*c*). Host-range of *Proteus morganii* bacteriophages. *Nature, Lond.* **199**, 827.

COETZEE, J. N. (1963*d*). Transduction of swarming in *Proteus mirabilis*. *J. gen. Microbiol.* **33**, 1.

COETZEE, J. N. (1966). Transduction in *Proteus morganii*. *Nature, Lond.* **210**, 220.

COETZEE, J. N. & SACKS, T. G. (1960). Transduction of streptomycin resistance in *Proteus mirabilis*. *J. gen. Microbiol.* **23**, 445.

DAVIDSON, P. F. & FREIFELDER, D. (1962). The physical properties of the deoxyribonucleic acid from T7 bacteriophage. *J. mol. Biol.* **5**, 643.

EDGAR, J. B. & STOCKER, B. A. D. (1961). Metabolic and genetic investigations of nutritionally exacting strains of *Staphylococcus pyogenes*. *Nature, Lond.* **191**, 1121.

EWING, W. H. (1958). The nomenclature and taxonomy of the Proteus and Providence groups. *Int. Bull. bact. Nomencl. Taxon.* **8**, 17.

FALKOW, S., RYMAN, I. R. & WASHINGTON, O. (1962). Deoxyribonucleic acid base composition of Proteus and Providence organisms. *J. Bact.* **83**, 1318.

GORINI, L. & KAUFMAN, H. (1960). Selecting bacterial mutants by the penicillin method. *Science*, **131**, 604.

HAYES, W. (1964). *The Genetics of Bacteria and their Viruses*. Oxford: Blackwell Scientific Publications.

HOLLOWAY, B. W. & MONK, M. (1959). Transduction in *Pseudomonas aeruginosa*. *Nature, Lond.* **184**, 1426.

HOLLOWAY, B. W., MONK, M., HODGINS, L. & FARGIE, B. (1962). Effects of radiation on transduction in *Pseudomonas aeruginosa*. *Virology*, **18**, 89.

IYER, V. N. (1962). Application of the membrane filter for the quantitative study of transformations with particular reference to phenotypic expression of an erythromycin-resistance mutation. *J. Bact.* **84**, 326.

JACOB, F. & WOLLMAN, E. L. (1958). Genetic and physical determinations of chromosomal segments in *Escherichia coli*. *Symp. Soc. exp. Biol.* **12**, 75.

LEDERBERG, J. (1950). Isolation and characterization of biochemical mutants of bacteria. *Meth. Med. Res.* **3**, 5.

MAHLER, I., CAHOON, M. & MARMUR, J. (1964). *Bacillus subtilis* deoxyribonucleic acid transfer in PBS2 transductions. *J. Bact.* **87**, 1423.

MANDELL, J. D. & HERSHEY, A. D. (1960). A fractionating column for analysis of nucleic acids. *Analyt. Biochem.* **1**, 66.

MARMUR, J. (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J. mol. Biol.* **3**, 208.

MATSUSHIRO, A., SATO, K. & KIDA, S. (1964). Characteristics of the transducing elements of bacteriophage ϕ80. *Virology*, **23**, 299.

MEYNELL, E. (1964). The significance of bacteriophage in bacterial classification. A review. *J. gen. Microbiol.* **36**, 461.

OKUBO, S., STODOLSKY, M., BOTT, K. & STRAUSS, B. (1963). Separation of transforming and viral deoxyribonucleic acids of a transducing bacteriophage of *Bacillus subtilis*. *Proc. natn. Acad. Sci., U.S.A.* **50**, 679.

OZEKI, H. (1956). Abortive transduction in purine-requiring mutants of *Salmonella typhimurium*. Genetic Studies with Bacteria. *Publ. Carneg. Instn.*, no. 612, p. 97.

PORTER, J. R. & MEYERS, F. P. (1945). Amino acid interrelationships in the nutrition of *Proteus morganii*. *Arch. Biochem.* **8**, 169.

PROZESKY, O. W. & COETZEE, J. N. (1966). Linked transduction in *Proteus mirabilis*. *Nature, Lond.* **209**, 1262.

PROZESKY, O. W., DE KLERK, H. C. & COETZEE, J. N. (1965). The morphology of Proteus bacteriophages. *J. gen. Microbiol.* **41**, 29.

RAUSS, K. (1962). A proposal for the nomenclature and classification of the Proteus and Providence groups. *Int. Bull. bact. Nomencl. Taxon.* **12**, 53.

SCHILDKRAUT, C. L., MARMUR, J. & DOTY, P. (1962). Determination of the base composition of deoxyribonucleic acid from its buoyant density of CsCl. *J. mol. Biol.* **4**, 430.

SHEPPARD, D. E. (1962). Density gradient centrifugation of bacteriophage P22. *Virology*, **17**, 212.

SINSHEIMER, R. L. (1960). The nucleic acids of the bacterial viruses. In *The Nucleic Acids*, Ed. by E. Chargaff and J. N. Davidson, vol. 3, p. 187. New York: Academic Press Inc.

THORNE, C. B. (1962). Transduction in *Bacillus subtilis*. *J. Bact.* **83**, 106.

TING, R. C. (1962). The specific gravity of transducing particles of bacteriophage P1. *Virology*. **16**. 115.

Topley and Wilson's Principles of Bacteriology and Immunity. (1964). 5th ed. Ed. by G. S. Wilson and A. A. Miles. London: Edward Arnold.

VIEU, J. F., GUÉLIN, A. & DAUGUET, C. (1965). Morphologie du bactériophage 80 de *Welchia perfringens*. *Ann. Inst. Pasteur*, **109**, 157.

WEIGLE, J. (1957). Transduction by coliphage λ of the galactose marker. *Virology*, **4**, 14.

WEIGLE, J. (1961). Densities of transducing λ bacteriophage. *J. mol. Biol.* **3**, 393.

WILSON, D. E. (1960). The effects of ultraviolet light and ionizing radiation on the transduction of *Escherichia coli* by phage P1. *Virology*, **11**, 533.

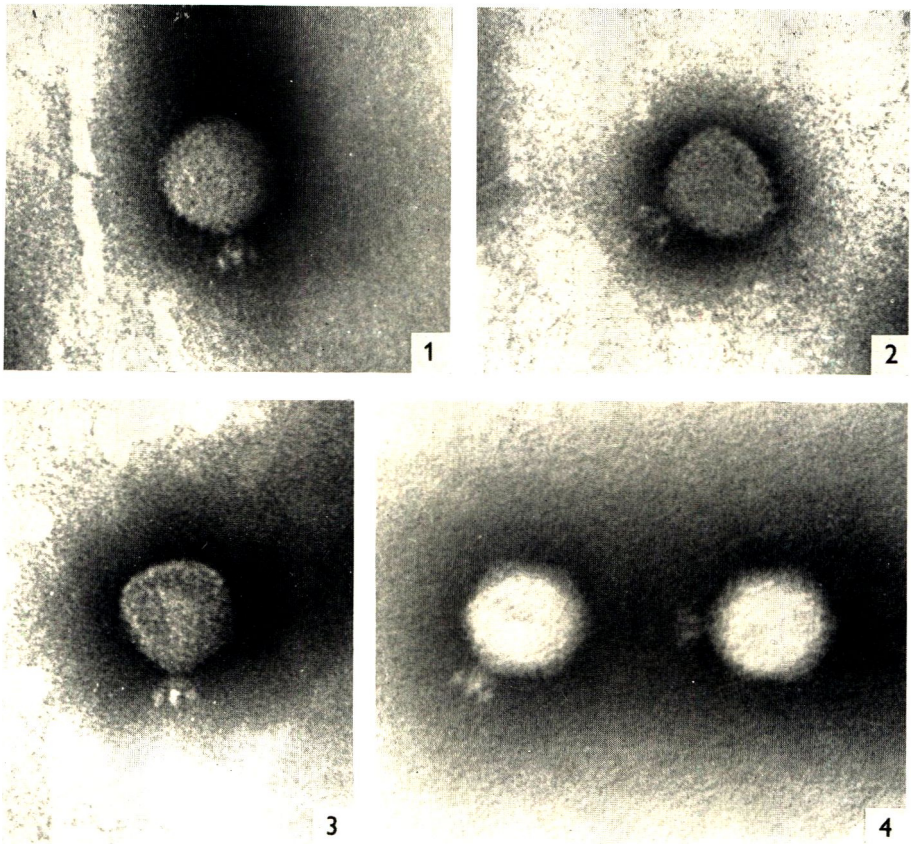
WYATT, G. R. (1955). Separation of nucleic acid components by chromatography on filter paper. In *The Nucleic Acids*, ed. by E. Chargaff and J. N. Davidson, vol. 1, p. 243. New York: Academic Press Inc.

YURA, T. (1956). Evidence of nonidentical alleles in purine-requiring mutants of *Salmonella typhimurium*. Genetic Studies with Bacteria. *Publ. Carneg. Instn.*, no. 612, p. 63.

ZINDER, N. D. (1953). Infective heredity in bacteria. *Cold Spring Harb. Symp. quant. Biol.* **18**, 261.

EXPLANATION OF PLATE

The magnification in all figures is $\times$ 270,000. All phages in ammonium acetate and phosphotungstate. Fig. 1, phage PL26; fig. 2, phage PL26; fig. 3, phage PL37; fig. 4, phage PL25.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J. gen. Microbiol. (1966), **44**, 177–193
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Some Properties of Three Related Viruses: Andean Potato Latent, Dulcamara Mottle, and Ononis Yellow Mosaic

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SUMMARY

Three similar and apparently previously unrecorded viruses were studied and found to be most like viruses of the turnip yellow mosaic group. Andean potato latent virus (APLV) was obtained from primitive cultivated potatoes collected in the high tropical Andes, dulcamara mottle virus (DMV) from *Solanum dulcamara* L. growing near Rothamsted, and Ononis yellow mosaic virus from *Ononis repens* L. growing in many parts of England. All three viruses are readily transmitted by sap inoculation; APLV and DMV are transmitted through the seed of infected plants; DMV is transmitted by the flea beetle *Psylloides affinis* Paykull. Plants infected by one of the viruses are not protected against infection by the others.

Purified preparations of these viruses have many common properties. Each virus has isometric particles 25–30 mμ in diameter, indistinguishable in appearance from one another and from the particles of turnip yellow mosaic virus. Preparations of each contain mainly two types of particles with sedimentation coefficients of about 115S and 55S, corresponding to infective nucleoprotein particles and non-infective 'empty' particles respectively. Each contains nucleic acid with a molar base composition of about G 16 % A 22 % C 33 % U 29 %. The three viruses are serologically related; antisera titres are 8–128 times greater with homologous than with the heterologous viruses. No serological relationship was found between these viruses and turnip yellow mosaic, wild cucumber mosaic, cocoa yellow mosaic, squash mosaic and red clover mottle viruses.

Plants infected with APLV or DMV when sprayed with solutions of 2-thiouracil or 6-azauracil produced fewer nucleoprotein particles and more 'empty' protein particles, than plants sprayed with water.

INTRODUCTION

In 1962 plants of Restharrow (*Ononis repens* L: Papilionaceae) showing a bright yellow mosaic were found growing on the cliffs near Tintagel in Cornwall, England. A virus was obtained from these plants by inoculating their sap to plants of several other herbaceous species; from these plants the virus was transmitted by sap to healthy *O. repens* plants, which then developed the bright yellow mosaic. This virus, which we called Ononis yellow mosaic virus (OYMV), aroused interest because its particles closely resemble those of turnip yellow mosaic virus (TYMV). A second virus, whose particles also resemble those of TYMV, and which is

serologically related to OYMV, was obtained from several clones of potatoes in the Commonwealth Potato Collection at the John Innes Institute, Hertford, England. These clones had been collected from the tropical Andes and had been tested for all the common potato viruses (McKee, 1964). In further tests, seedlings of a line of *Solanum stoloniferum* Schlechdt. et Bché, which is immune to potato virus Y (PVY), were inoculated with sap from 80 clones of Andean potatoes infected with PVY, to see whether any of the Andean isolates of PVY overcame this resistance. None of the inoculated plants became infected with PVY but some developed a mild mosaic, and were found to contain a virus, apparently unlike any of the many viruses previously found in potato, which we propose to call Andean potato latent virus (APLV). Attempts to transmit APLV from infected to healthy *S. stoloniferum* plants using the potato flea beetle *Psylloides affinis* Paykull failed, but a third virus, which was related to OYMV and APLV, was isolated from the *Solanum dulcamara* L. plants from which the potato flea beetles had been collected. This virus, which we called dulcamara mottle virus (DMV), was transmitted by the flea beetles.

In this paper we describe the occurrence and distribution of these three viruses, together with some of their properties, particularly those which seem useful for comparing them with other known viruses.

METHODS

The isolate of APLV, which we used for all our tests, was obtained from a clone of diploid potatoes (group Phureja) originally from Sogamoso, Colombia (Commonwealth Potato Collection No. 2853). It was maintained in *Nicotiana glutinosa* L. and *Solanum stoloniferum*. The stock isolate of DMV was obtained from naturally infected *Solanum dulcamara* and was kept in plants of *N. glutinosa*. The stock isolate of OYMV used came from an *Ononis repens* plant collected at Tintagel, Cornwall. These stock cultures and all test plants were kept in insect-free glasshouses at 16–24°.

The stability of the viruses in untreated sap from diseased plants was estimated as suggested by Bos, Hagedorn & Quantz (1960). They all proved stable and could be purified by several methods, but the following one was mostly used. Infected leaves were triturated mechanically in half their weight of pH 7·0 buffer containing 0·05 M-ascorbic acid and 0·1 M-disodium hydrogen phosphate. Chloroform and *n*-butanol, each one-quarter the volume of the buffer, were added and the mixture triturated further to form an emulsion which was centrifuged for $8 \times 10^4 g$ min. ($8000g$ for 10 min.), and the aqueous phase collected. The virus in this aqueous extract was further purified and concentrated by two or more cycles of differential centrifugation; $11 \times 10^6 g$ min. and $8 \times 10^4 g$ min. using 0·03 M-phosphate buffer pH 7·0 as the suspending fluid. All three viruses could be purified in other ways, for example, APLV was fully and reversibly precipitated in half-saturated ammonium sulphate solution or in 20% ethanol solution.

Purified preparations of the viruses were examined in a Siemens Elmiskop I electron microscope using the methods described by Nixon & Harrison (1959).

Preparations, suspended in 0·1 M-potassium chloride solution pH 7·0, were examined in a Spinco Model E analytical centrifuge using the schlieren optical system, and the sedimentation rates of the different components of the preparations

estimated by a graphical method (Markham, 1960). Each virus preparation was examined at 3–6 different concentrations (from about 0·1 to 5·0 mg./ml.), and the results obtained extrapolated to infinite dilution by calculating a regression line by the least squares method.

Nucleic acid was extracted from the viruses by incubating them at 18° in N-HCl for 12 hr. The base composition of the nucleic acids was estimated by a chromatographic method similar to that described by Markham (1955), using a tertiary butanol + HCl solvent, and the fluorescent screen described by Katz (1962) to detect the spots. Two or three estimates of the base composition were made on each of three or four separate preparations of each virus; the result given for each virus is the average of seven to eleven determinations. To check our application of the method we also estimated the base composition of tobacco mosaic and turnip yellow mosaic viruses, and obtained results within 5 % of those quoted by Markham (1959).

Antisera were prepared by injecting rabbits intravenously at weekly intervals with purified virus preparations (about 1 mg. virus/injection). Each rabbit was injected four times and was bled 10 days after the last injection. Quantitative tests for assessing the serological relationships between viruses were done by the tube precipitation method (Bawden, 1950). A micro gel diffusion method (Mansi, 1958) was used as a qualitative test for virus in plant saps. The serological relationships between the viruses were estimated by (1) determining the precipitation end-points of the antisera when titrated against purified preparations of the three viruses, and (2) the precipitation end-points of each serum after it had been absorbed with an excess of each of the viruses. First, the concentration of virus in each preparation was found by titrating it against its homologous antiserum, the preparation was then used at a constant concentration (approximately four times that of the precipitation end-point; about 20 mg./l.) to find the titres of the various antisera.

The viruses and antisera used in the serological and other tests were from various sources. The Virus Research Unit, Cambridge, kindly supplied the Cambridge strain of turnip yellow mosaic virus; also wild cucumber mosaic virus and its antiserum. The strain of cocoa yellow mosaic virus and its antiserum were those used by Brunt, Kenten, Gibbs & Nixon (1965). D. G. Robertson, Moor Plantation, Ibadan, Nigeria, kindly supplied the cowpea mosaic virus, and R. J. Shepherd, then at the Department of Plant Pathology, University of Wisconsin, U.S.A., the squash mosaic virus. The red clover mottle virus isolate was that described by Sinha (1960), and the antisera against turnip yellow mosaic, cowpea mosaic, red clover mottle, and squash mosaic viruses were all prepared at Rothamsted.

RESULTS

Biological behaviour

Symptoms shown by naturally-infected plants

Several of the primitive cultivated potatoes from which Andean potato latent virus (APLV) was isolated showed mosaic symptoms and leaf distortion, but these symptoms were apparently caused by other viruses (Table 1) for there were no symptoms common to all APLV-infected clones.

Solanum dulcamara plants infected with dulcamara mottle virus (DMV) showed

a very mild mottling and slight puckering of the leaves, especially those produced during cool weather (Pl. 1, fig. 1). Older leaves senesced prematurely.

Plants of *Ononis repens* infected with Ononis yellow mosaic virus (OYMV) showed a chlorotic mosaic (Pl. 1, fig. 2), brightest during the spring, but still evident even in the hottest weather. Infected plants were not noticeably stunted, their growth form was normal, they produced normal flowers without a colour break, and set viable seed.

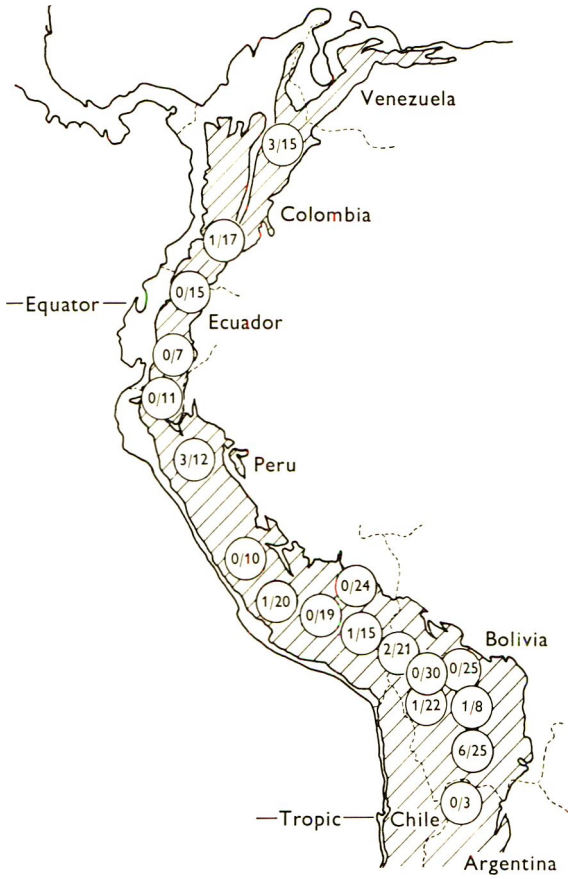


Fig. 1. Sites in tropical Andes from which potatoes infected with APLV were collected; at each site the numerator is the number of potatoes from that site found to be infected, denominator the total number of potatoes collected at that site.

Geographical distribution

In 1964 all the potato clones in the Commonwealth Potato Collection were tested serologically for APLV. Nineteen clones were found to be infected; all these came from a wide range of localities (Fig. 1 and Table 1) in the tropical Andes (John Innes Institute Report, 1960, 1963) where potatoes grow only at high altitudes. APLV was not found in any of the other potatoes in the Collection, which consisted of a further 280 clones from the tropical Andes, 132 clones from temperate Chile, and 131 stocks of cultivars from various sources including Great Britain, The Netherlands, and the U.S.A.

DMV was isolated from *Solanum dulcamara* plants from Streatley, Bedfordshire in 1964. The plants were growing in scrub-land on the slope of a chalk escarpment, and in hedgerows nearby. Sap from these plants was inoculated to *Nicotiana glutinosa* plants, which developed symptoms a week later. Sap from infected *N. glutinosa* and *S. dulcamara* reacted strongly in gel diffusion serological tests with DMV antiserum. About one-quarter of the *S. dulcamara* plants at Streatley were infected with DMV, but the virus was not isolated from a further 230 *S. dulcamara* plants collected from 31 sites in central and southern England (Fig. 2); some of the plants from three of the sites were infected with cucumber mosaic virus.

Table 1. *APLV-infected primitive potato clones; type, source, other viruses*

Type of potato*	Other viruses† isolated from the clone	Provenance	C.P.C. no.‡
Solanum tuberosum L. group Phureja (diploid)	X, L.R.	Nr. Pamplona, Colombia	2848
	X, Y	Sogamoso, Colombia	2853
	S	Nr. Firavitoba Colombia,	2865
	S, Y	Nr. Ipiales, Colombia	2942
S. tuberosum L. group Chaucha (triploid)	X	Huanucu, Peru	1777
S. tuberosum L. group Andigena (tetraploid)	X	Potosi, Bolivia	2771
	X		2775
	Y		2781
	—	Huaneabamba, Peru	3019
	X, S	Huaraz, Peru	3043
	S	Ayacucho, Peru	3054
	—	Nr. Acora, Peru	3125
	X	La Paz, Bolivia	3166B
	—	Oruro, Bolivia	3177A
	—	Sucre, Bolivia	3188
	L.R.	Nr. Potosi, Bolivia	3190
	S		3191
	X	Potosi, Bolivia	3195
S. tuberosum L. (group undetermined)	—	Batallas, Bolivia	3655

* Nomenclature that of Dodds (1962).
† X, potato virus X; Y, potato virus Y; S, potato virus S; L.R., potato leaf roll virus.
‡ Commonwealth Potato Collection number.

In 1963 and 1964 restharrow growing in many parts of England and Wales was inspected and tested for OYMV. Although restharrow has been recorded in all lowland areas of Britain (Perring & Walters, 1962) it is uncommon and difficult to find except on recently-stabilized sand dunes. We mostly inspected restharrow on coastal sand dunes therefore, and may have obtained an unrepresentative sample of the total restharrow population. Restharrow was sampled at 24 sites (Fig. 3), at each site 20–80 (average 32) shoots were collected at random. The shoots were inspected for symptoms, and sap from them inoculated to pea plants, all of which were later tested serologically for OYMV. OYMV was found in all parts of southern Britain, though perhaps it is more common in the south-west (Fig. 3). *Ononis spinosa* L., the only other common restharrow in Britain, mainly occurs in central, eastern, and southern England. Many plants of *O. spinosa* from six sites, some near infected *Ononis repens*, were tested for OYMV, but apparently none were infected,

though in glasshouse tests *O. spinosa* plants were readily infected when rubbed with sap containing OYMV.

Natural transmission

(*a*) *Seed.* In limited tests APLV and DMV, but not OYMV, were transmitted through the seed of infected plants. Seed from APLV-infected *Nicotiana clevelandii* Gray and *Petunia hybrida* Villm produced seedlings that looked healthy, but sap inoculation tests showed that the *N. clevelandii* seedlings were infected.

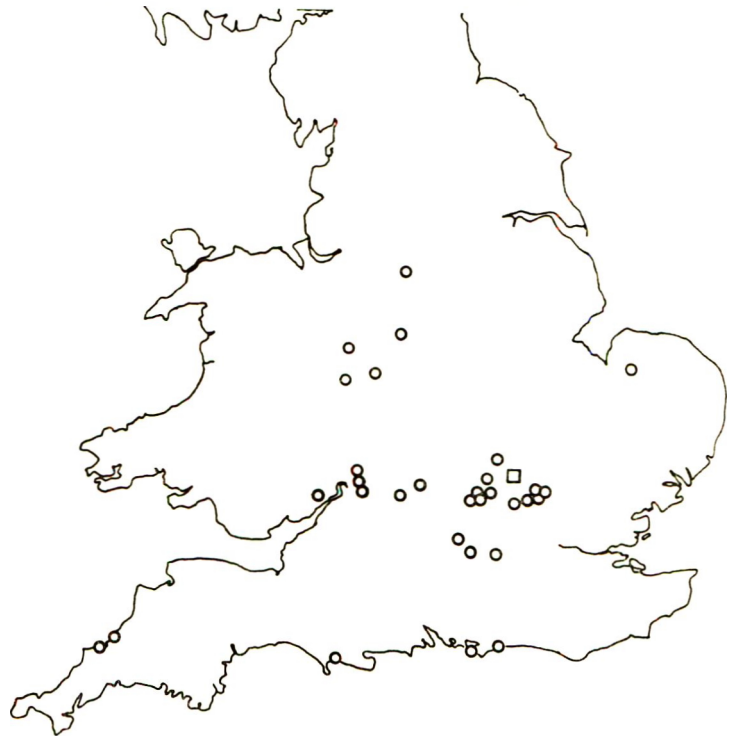


Fig. 2. Sites in England from which *S. dulcamara* shoots were collected; circles at sites from which no DMV was isolated, square at the site where DMV was found.

Seeds collected from infected *Solanum dulcamara* plants growing wild were germinated in the glasshouse, and the seedlings tested, either singly or in groups of two, four or eight by inoculating their sap to *Nicotiana glutinosa.* One infected seedling was found in the 441 seedlings tested, and it showed clear symptoms. Fifty seedlings grown from seeds collected from naturally-infected restharrow plants at Tintagel were tested by inoculating their sap to pea plants, but none was virus infected.

(*b*) *Insects.* The two related viruses turnip yellow mosaic and wild cucumber mosaic are transmitted by Halticid and Galerucid flea beetles (Markham & Smith, 1949; Freitag, 1952). APLV, DMV and OYMV in many ways resemble these viruses; experiments were therefore done to see whether they have similar vectors. During 1963 and 1964 insects were collected by sweeping and beating from restharrow plants at many sites. At most of the sites the pea aphid *Acyrthosiphon pisum* Harris and the Mirid bug *Macrotylus paykulli* Fall. were common, at a few sites the

Collembolan *Sminthurus viridis* L. was common, and the weevil *Apion ononis* Kirby was found at three-quarters of the sites where OYMV was found. The bug, the collembolan, and the weevil failed to transmit OYMV to healthy restharrow plants, though all fed on the restharrow test plants. A total of 100–200 individuals of each insect were used, either collected from diseased plants in the field and caged immediately on healthy test plants, or fed for various periods on diseased plants in

Fig. 3. Sites in England and Wales from which *O. repens* shoots were collected; black sector shows the proportion of shoots from each site infected with OYMV.

cages before being put on healthy plants. Several unidentified species of grasshopper were also found on the restharrow at most sites, and a single flea beetle *Crepidodera ferruginea* Scop. at one site; neither the flea beetle nor the grasshoppers fed on restharrow plants on which they were caged, and none of the plants became infected.

The potato flea beetle *Psylloides affinis* Paykull. also failed to transmit APLV from diseased to healthy *Solanum stoloniferum* plants, for the beetles, collected from wild *Solanum dulcamara* plants, would not feed on the *S. stoloniferum* plants. Several attempts were made to transmit DMV from diseased to healthy *S. dulcamara* plants by potato flea beetles collected from wild *S. dulcamara* plants. During the summer of 1964 about 150 first generation beetles were fed on an infected plant for one day and then caged on healthy plants, none of which became infected. In the autumn second generation beetles were collected, fed on an infected plant, and 48 of them caged in pairs on healthy seedlings, four of which became infected. In a third experiment, done later in the autumn when most wild *S. dulcamara* plants were almost

leafless, none of forty moribund beetles transmitted the virus. On each occasion at least one quarter of the beetles collected were caged on healthy test plants, but none of these became infected. In the spring of 1965 more beetles were collected from naturally-infected *S. dulcamara* showing symptoms. Four groups of twenty beetles were each caged on twenty *S. dulcamara* seedlings growing in a box. Two seedlings in one box and one in another became infected. When the experiment was repeated one month later, in hotter weather, none of the test plants became infected.

Experimentally-infected plants

Several different species of plants were inoculated with the three viruses. Most were inoculated by dusting their leaves with carborundum and then rubbing them with sap from either infected restharrow or pea (OYMV) or infected *Nicotiana glutinosa* (APLV or DMV). Some cultivated potato varieties were also grafted with scions of primitive potato or *N. glutinosa* infected with APLV or DMV respectively. All inoculated plants were tested 3–6 weeks later for infection by inoculating their sap to appropriate test plants; the presence of virus in plants showing symptoms was also checked serologically. Plants were most readily infected during cool weather and few became infected during hot summer weather; though APLV was transmitted by grafting at all times.

APLV infected more of the species inoculated than did OYMV or DMV (Table 2), though OYMV but not APLV or DMV infected several leguminous species. Only three species, all Solanaceae, were infected by all three viruses. None of the viruses infected *Brassica chinensis* L. or *Cucurbita pepo* L., which are susceptible to turnip yellow mosaic and wild cucumber mosaic viruses respectively, and only APLV infected *Chenopodium amaranticolor* Coste & Reyn, which is susceptible to cocoa yellow mosaic virus. It is interesting that APLV infected *Chenopodium quinoa* Willd., which is grown as a grain crop in the region of the Andes where the APLV-infected potatoes were collected.

The symptoms caused in the plants we found most useful or in other ways noteworthy are described below.

Andean potato latent virus. During hot weather infected *Nicotiana glutinosa* showed slight or no symptoms. In cool weather inoculated leaves showed indistinct chlorotic and necrotic local lesions. Systemically infected young leaves showed vein clearing (Pl. 1, fig. 3), those produced later were distorted and showed a chlorotic mosaic with necrotic flecking. APLV was transmitted by sap inoculation or grafting to many species of the Solanaceae, and infected all systemically, except *Solanum dulcamara*. In most, including the cultivated potato varieties, it caused slight or no symptoms. APLV infected two Chenopodium species causing indistinct necrotic and chlorotic flecking of the inoculated leaves and, occasionally, the tip leaves.

Dulcamara mottle virus. The symptoms in *Nicotiana glutinosa* during hot weather resembled those caused by APLV during cool weather, and in cool weather were much more severe; the tip leaves became distorted and crinkled because of necrosis of the leaf veins (Pl. 1, fig. 4). DMV caused more noticeable symptoms than APLV in most of the plants it infected, especially in *N. glutinosa* and *Nicotiana clevelandii* but, even when grafted with infected *N. glutinosa*, potatoes did not become infected. There was great seasonal variation in the susceptibility of *Solanum dulcamara* to sap

Table 2. *Hosts of APLV, DMV and OYMV*

		APLV	DMV	OYMV
Amaranthaceae	*Gomphrena globosa* L.	+	–	–
Apocynaceae	*Vinca rosea* L.	.	–	–
Chenopodiaceae	*Chenopodium amaranticolor* Coste & Reyn	+	–	–
	C. foetidum Schrad.	.	–	–
	C. quinoa Willd.	+	–	–
Cruciferae	*Brassica chinensis* L. 'Pe. tsai'	–	–	–
Cucurbitaceae	*Cucumis sativus* L. 'Everyday'	–	–	–
	Cucurbita pepo L.	.	–	–
Gramineae	*Hordeum vulgare* L. 'Proctor'	–	.	–
	Triticum aestivum L. 'Cappelle'	–	.	–
Papilionaceae	*Glycine soja* (L.) Sieb. et Zucc. 'Lincoln'	.	.	+
	Medicago sativa L. 'Du Puits'	.	.	–
	Ononis repens L.	.	–	+N
	O. spinosa L.	.	.	+
	Phaseolus vulgaris L. 'Prince'	–	–	–
	Pisum sativum L. 'Onward'	–	–	+
	Trifolium incarnatum L.	–	–	+
	T. repens L. 'S. 100'	.	.	+
	Vicia faba L. 'The Sutton'	–	–	–
	Vigna sinensis (L.) Endl. 'Paraguay 6'	–	.	–
Solanaceae	*Datura stramonium* L.	+	+	+
	Hyoscyamus niger L.	+	.	.
	Lycopersicon esculentum Mill. 'Kondine Red'	+	+	+
	Nicotiana clevelandii Gray	+	+	+
	N. debneyi Domin.	.	–	–
	N. glutinosa L.	+	+	–
	N. rustica L.	+	.	–
	N. tabacum L. 'White Burley'	–	–	–
	Petunia hybrida Vilm.	+	–	–
	Solanum chacoense Bitt.	+	.	.
	S. dulcamara L.	+	+N	.
	S. nigrum L.	.	+	.
	S. stoloniferum Schlechdt. et Behé.	+	.	–
	S. tuberosum L. gp Andigena	+N	.	.
	S. tuberosum L. gp Chaucha	N	.	.
	S. tuberosum L. gp Phureja	N	.	.
	S. tuberosum L. gp Tuberosum 'Arran Victory'	+G	.	.
	S. tuberosum L. gp Tuberosum 'King Edward'	G	.	.
	S. tuberosum L. gp Tuberosum 'Majestic'	+G	g	.
	S. tuberosum L. gp Tuberosum 'Pentland Beauty'	G	.	.
	S. verrucosum Schlechdt.	+	.	.
Umbelliferae	*Daucus carota* L. 'Sutton Intermediate'	–	–	–
	Pastinaca sativa L. 'The Student'	–	–	–

\+ Species infected by sap inoculation, sap giving positive serological reaction.
– Apparently not infected by sap inoculation.
N Natural host species.
G Species infected when cleft grafted with scions from infected *S. tuberosum* L. gp Andigena.
g Species not infected when cleft grafted with DMV-infected *N. glutinosa*.

inoculation with DMV. In several experiments during the summer a total of about two hundred *S. dulcamara* plants grown from one batch of seed were inoculated but none was infected. When, in the autumn, a few plants were grown from the same batch of seed and inoculated all were infected as were several hundred seedlings from three other batches of seed.

Ononis yellow mosaic virus. In cool weather the inoculated and tip leaves of *Pisum sativum* L. Onward developed a severe yellowing and necrosis and the plants died. In warm weather there was less necrosis, the plants recovered, and leaves produced later showed a brilliant yellow mosaic (Pl. 1, fig. 5). A few isolates from Devon gave milder symptoms even in cool weather. OYMV caused a more or less brilliant yellow mosaic in all its legume hosts. Of the three species of Solanaceae it infected, *Nicotiana clevelandii* showed a mild systemic mottling and necrotic flecking; only a few plants of the other two species became infected systemically and showed an indistinct chlorosis.

Plant protection tests

Tests were made to see whether plants systemically infected with one of the viruses resisted infection with one of the other two viruses using *Nicotiana clevelandii* for OYMV and either APLV or DMV, and *Nicotiana glutinosa* for APLV and DMV. About 3 weeks after plants were inoculated with one virus, leaves showing clear symptoms were inoculated with the second virus, and 2 weeks later sap from them was tested serologically for both viruses. None of the viruses protected plants against any other, and in each test the second virus infected already-infected plants as readily as comparable uninfected ones. The lack of protection was evident from symptoms, for the second virus produced more severe symptoms (usually necrotic lesions) on the already-infected plants than on the comparable uninfected ones.

Properties of the viruses in sap

Dilution end-point. The infectivity of sap from infected plants varied greatly. Usually the sap of inoculated leaves was most infective 2–4 weeks after the leaves had been inoculated, and was much more infective in winter than in summer. Sap of systemically infected leaves usually lost infectivity when diluted 10^{-3} to 10^{-5}, though in one experiment sap of *Nicotiana glutinosa* infected with APLV was still infective when diluted 10^{-7}.

Survival at different temperatures. Sap containing either DMV or OYMV retained some infectivity after 10 min. at 65°, but not after 10 min. at 70° (Table 3). APLV withstood higher temperatures: although most infectivity was lost after 10 min. at 75–80°, some remained after 10 min. at 90°, but not at 100°. The plants infected with virus surviving at 85° and 90° showed the same symptoms as those inoculated with unheated sap; and virus preparations made from plants infected with these three types of inoculum were indistinguishable in serological tests and in the electron microscope. The same result was obtained in 3 experiments. Sap containing APLV or DMV usually remained infective at laboratory temperatures (about 20°) for only 2–7 days, whereas sap containing OYMV was still infective after 2 weeks. Similar saps stored at +4°, −15°, or dried while frozen were still infective after six months.

Table 3. *Survival at different temperatures*

Temp. (°)*	Infectivity† of sap‡ APLV	DMV	OYMV
Unheated	100 % (18/18)	94 % (17/18)	93 % (26/28)
60	100 % (9/9)	92 % (11/12)	81 % (25/31)
65	100 % (9/9)	75 % (9/12)	40 % (4/10)
70	72 % (13/18)	0 (0/12)	0 (0/10)
75	100 % (9/9)	0 (0/12)	0 (0/28)
80	22 % (4/18)	0 (0/6)	— —
85	11 % (1/9)	— —	— —
90	11 % (1/9)	0 (0/6)	0 (0/4)
100	0 (0/9)	— —	— —

* Sap heated at indicated temperature for 10 minutes.

† Infectivity: percentage of plants infected; in parentheses, numerator is the number of plants infected, denominator is the total number of plants inoculated. Total of three experiments with each virus.

‡ Sap containing APLV and DMV from *Nicotiana glutinosa*, tested on the same species, sap containing OYMV from pea tested on either pea or *Trifolium incarnatum*.

Properties of purified preparations

Electron microscopy. Extracts of plants of *Nicotiana glutinosa* infected with APLV, of *Solanum dulcamara* and *N. glutinosa* infected with DMV, and of *Ononis repens* and *Pisum sativum* infected with OYMV all contained many approximately spherical particles, not found in comparable preparations from healthy plants. In the best electron micrographs the particles of all three viruses were indistinguishable in size and morphology from those of turnip yellow mosaic virus (Huxley & Zubay, 1960; Nixon & Gibbs, 1960) and cocoa yellow mosaic virus (Brunt *et al.* 1965). Some of the particles in negatively stained preparations were penetrated by the stain and were visible only as outlines, and the intact particles not penetrated by the stain showed the same arrangement of morphological subunits as turnip yellow mosaic virus (Pl. 1, fig. 6).

Centrifugation. In the analytical centrifuge purified preparations of the three viruses produced two boundaries, with sedimentation coefficients ($S_{20,w}$) for APLV of 52S and 112S, for DMV of 55S and 121S, and for OYMV of 56S and 114S (the standard errors of these estimates were from $\pm 1{\cdot}1$ to $\pm 0{\cdot}03$). The sedimentation rate was only slightly dependent on the concentration of the virus present, and increased by about 2–4S when the viruses were diluted from 5 to 0·25 mg./ml. These two components, similar to the two in preparations of turnip yellow mosaic virus (Markham & Smith, 1949), consisted of faster-sedimenting intact nucleoprotein particles and slower-sedimenting protein particles with the same external morphology as the intact particles. Some preparations of both DMV and OYMV also contained a third component with a sedimentation coefficient of about 95S. Such preparations were made from normal stock cultures of the viruses and had received the same treatment as the preparations which contained only two components.

Reichmann (1965) showed that, if various assumptions are made, the nucleic acid content of the nucleoprotein particles of a virus can be calculated, if preparations of the virus also contain 'empty' protein particles from the formula

$$P = (S_F/S_E - 1)/(S_F/S_E + 1)$$

where P is the percentage of nucleic acid in the 'full' nucleoprotein particles and S_F and S_E the sedimentation coefficients of the 'full' and 'empty' particles respectively. This formula applied to these three viruses gives a nucleic acid content of the nucleoprotein particles for APLV of 36·6%, for DMV of 37·5%, and for OYMV of 34·1%.

When centrifuged in sucrose density gradients the preparations of the viruses separated into two light-scattering layers. Some experiments comparing the properties of these layers were done using DMV, as this produces countable local lesions on *Nicotiana glutinosa* leaves. In one, samples were taken from both light-scattering layers, and from above them, between them and below them. The samples were dialysed against 0·03M phosphate buffer pH 7·0 and then examined in the electron microscope. The top light-scattering layer contained mostly particles penetrated by the phosphotungstate, whereas the bottom one contained mostly intact particles. The five samples were each rubbed on to 10–16 leaves of *N. glutinosa* and, starting with the uppermost sample, produced 0, 0·4 (top layer), 16, 94 (bottom layer), and 3·2 lesions per leaf. Thus the bottom layer sample was the most infective, contained most intact particles ($2{\cdot}5 \times 10^{13}$ particles/ml., 65% apparently intact, 35% empty, partly empty or damaged), and had the ultraviolet absorption spectrum of a nucleoprotein with a peak of absorption at 263–265 mμ wavelength. The other less infective samples contained fewer intact particles; for example, the sample of the top light-scattering layer, which gave 0·4 lesion/leaf, contained $1{\cdot}7 \times 10^{13}$ particles/ml., only 13% of which seemed to be intact, and had the ultraviolet absorption spectrum of a protein-nucleoprotein mixture with a broad peak of absorption at about 270 mμ wavelength. In gel diffusion serological tests, only samples from the light scattering layers and from between them gave lines of precipitate. Each sample gave only one line of precipitate and, in a suitably designed test, the lines from different samples were confluent.

Effect of base analogues on virus components. Francki & Matthews (1962) found that spraying plants infected with TYMV with 2-thiouracil solutions decreased the number of 'full' particles and greatly increased the proportion of non-infective 'empty' particles they contained. We sprayed *Nicotiana glutinosa* infected with either APLV or DMV with aqueous solutions (100 mgm./l.) of the five base analogues, 2-thiouracil, 6-azauracil, thioadenine, thiocytosine, and thioguanine and found that the first two, but not the last three, affected the amount of 'full' and 'empty' particles, which were extracted from the plants (Table 4). Spraying with thiouracil solution halved the total amount of virus produced, whereas azauracil increased it. Both analogues decreased the number of 'full' particles, and increased the relative proportion of 'empty' particles by about four times.

Nucleic acid analysis. The analyses (Table 5) show that the nucleic acids of the three viruses have similar base compositions. The large amount of cytidylic acid and small amount of guanylic acid accounts for the peak in the ultraviolet absorption spectrum being at 263–265 mμ rather than a shorter wavelength.

Serology. The three viruses are serologically different but are related to each other. Antigenically APLV and DMV have more in common with each other than with OYMV, and most of the tests using absorbed sera confirmed this conclusion (Table 6). However, the results of the tests with OYMV antiserum were anomolous, for absorbing OYMV antiserum with excess of either APLV or DMV decreased its

titre with OYMV from 1/512 to 1/128, whereas unabsorbed OYMV antiserum had a titre of only 1/16 when titrated against these two viruses.

Many of the tests showed that the antibodies common to OYMV and APLV antisera differed from those common to OYMV and DMV antisera. For example,

Table 4. *The effect of base analogues on APLV in Nicotiana glutinosa*

Spray*	Relative particle content of sap†			
	Total	'Fulls'	'Empties'	'E'/'F'
Water	100	77	23	0·3
2-thiouracil (100 mg./l.)	57	27	30	1·1
6-azauracil (50 mg./l.)	135	60	75	1·25

* Plants sprayed five times, starting 8 days after inoculation and then on alternate days. Plants harvested 3 weeks after inoculation.

† Virus purified by centrifugation, as described in text, and suspended in neutral 0·1 M-KCl. Relative amount of 'full' and 'empty' particles in the preparation estimated in the analytical centrifuge; all calculated relative to the total area of the two major peaks in the schlieren diagram produced by the virus preparation from water sprayed plants.

Table 5. *Molar base composition of the viruses*

Virus	Guanine	Adenine	Cytosine	Uracil
APLV	15·2 ± 0·5	21·6 ± 0·3	34·0 ± 0·3	29·2 ± 0·2
DMV	16·7 ± 0·3	22·6 ± 0·4	32·3 ± 0·3	28·5 ± 0·3
OYMV	15·6 ± 0·3	21·0 ± 0·1	34·2 ± 0·1	29·4 ± 0·3

Table 6. *Serological tests*

Antiserum	Absorbing antigen	titres*		
		APLV	DMV	OYMV
APLV	—	1024	64	16
	APLV	0 (4)	0 (2)	0 (2)
	DMV	512	0 (2)	4
	OYMV	1024	64	0 (2)
	DMV, OYMV	256	0 (8)	0 (8)
DMV	—	64	512	16
	APLV	0 (2)	256	8
	DMV	0 (2)	0 (2)	0 (4)
	OYMV	32	512	0 (2)
	APLV, OYMV	0 (8)	256	0 (8)
OYMV	—	16	16	512
	APLV	0 (2)	0 (2)	128
	DMV	8	0 (2)	128
	OYMV	0 (2)	0 (2)	0 (2)
	APLV, DMV	0 (8)	0 (8)	128
TYMV (1024†)	—	0 (2)	0 (2)	0 (2)
WCMV (1024)	—	0 (8)	0 (8)	0 (8)
CYMV (512)	—	0 (2)	0 (2)	0 (2)
RCMV (1024)	—	0 (1)	0 (1)	0 (1)
SMV (1024)	—	0 (1)	0 (1)	0 (1)

* Reciprocal of dilution end-point after 4 hr incubation at 37°. When no reaction least dilution tested given in parentheses.

† Homologous antiserum titre.

DMV antiserum absorbed with excess APLV had the same homologous titre as that absorbed with excess of both APLV and OYMV, whereas APLV antiserum absorbed with an excess of both DMV and OYMV had half the homologous titre of the same serum absorbed with DMV alone. Similarly, OYMV antiserum absorbed with DMV still reacted with APLV, whereas when absorbed with APLV it did not react with either DMV or APLV. Tests using sera absorbed with excess of both the other two viruses showed that about a quarter to a half of the antibodies in each serum were specific for the homologous virus.

The precipitation end-points of the sera were the same when titrated against different isolates of the homologous virus. Four isolates of APLV from potato clones were tested: C.P.C. Nos. 2828, 2942, 3190 and 3195 (Table 1), one OYMV isolate from Wales and another from Dawlish (Devon), and two isolates of DMV.

APLV, DMV and OYMV did not precipitate with antisera prepared against turnip yellow mosaic (TYMV), wild cucumber mosaic (WCMV), cocoa yellow mosaic (CYMV), red clover mottle (RCMV), and squash mosaic viruses (SMV) (Table 6).

DISCUSSION

These three viruses, which we will call the Andean potato latent group, seem not to have been described before. They are particularly interesting as a group in that though they are readily distinguished in serological tests and by their host ranges, they have particles with indistinguishable morphology, size, sedimentation behaviour and base composition. They also share many of their properties with some previously known viruses, especially those transmitted by beetles (Fig. 4). They most closely resemble the viruses of the turnip yellow mosaic group (turnip yellow mosaic, wild cucumber mosaic, and cocoa yellow mosaic viruses): the particles are of the same morphology, size, and sedimentation behaviour, though they have a slightly different base composition, and are apparently serologically unrelated to them. However, these two groups share so many of their properties that it seems reasonable to consider the APL group to be a subgroup of the TYM group.

The APL group also share some properties, for example, particle size and sedimentation behaviour, with the cowpea mosaic group of viruses (cowpea mosaic (CMV), bean pod mottle (BPMV), red clover mottle, squash mosaic and radish mosaic (RMV) viruses); but viruses in this group have a very different base composition and morphology (in electron micrographs they do not show thirty-two morphological subunits and have an angular outline).

Other viruses that have been transmitted by beetles include southern bean mosaic (SBMV), turnip crinkle (TCV) and cocksfoot mottle (CFMV) viruses. These differ from viruses of the APL, TYM and CM groups in that purified preparations seem to consist only of 'full' particles, which sediment as one component and contain about 20% nucleic acid. Only viruses within each of the first three groups share common antigens.

Transmission of tobacco ringspot virus (TRSV) by flea beetles was reported by Schuster (1963). This is interesting as TRSV and other viruses of the ringspot group (e.g. grape vine fan leaf and yellow mosaic, arabis mosaic, tomato black ring, and raspberry ringspot viruses) have particles of the same size, morphology, and sedimentation behaviour as viruses of the cucumber mosaic (CM) group. TRSV also

resembles viruses of the CM group in having uridylic acid as its most and cytidylic acid its least abundant nucleotide.

The beetle-transmitted viruses provide good examples of the characters that may be used either to group or separate viruses. Host-ranges and symptoms are usually not correlated with any specific features of the virus particles, or with their vectors, whereas various morphological and chemical features of the particles do seem to be correlated with one another and with the type of vector. These characters can provide the basis of a phenetic classification, which may be useful in the identification and prediction of properties of newly isolated viruses. With this objective we would distinguish the APL group by the following group of characters, which, though individually shared with various other groups of viruses, yet in combination seem to be unique.

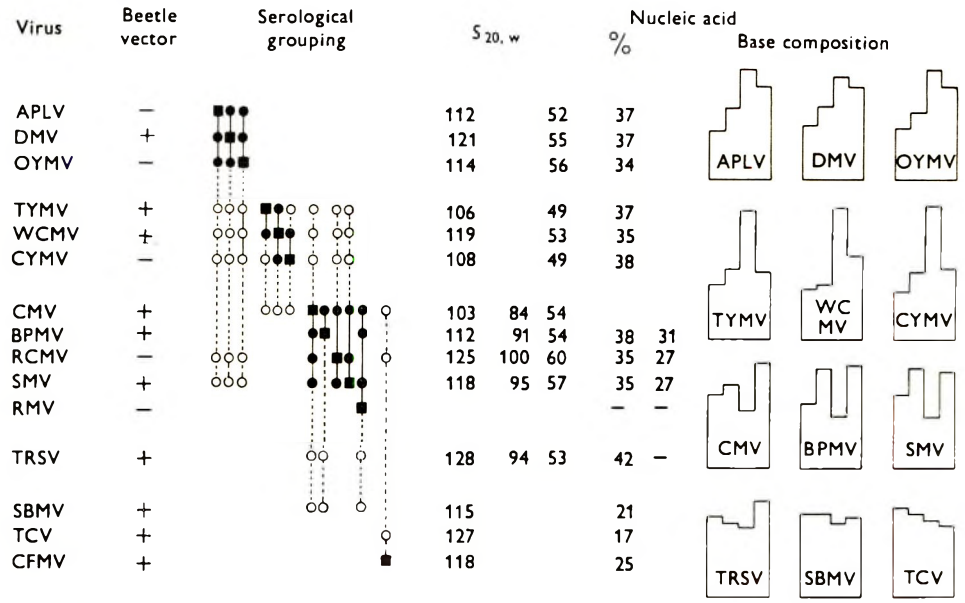


Fig. 4. Beetle-transmitted viruses. Virus name abbreviations in text. Serological relationships have been found between viruses joined by unbroken lines, but not between those joined with broken lines; antigen source marked with black square, antiserum circled. Nucleic acid molar base composition, left to right guanine, adenine, cytosine, uracil.

The data in Fig. 4 are from work in this paper and also Agrawal (1964), Bancroft (1962), Brunt, Kenten, Gibbs & Nixon (1965), Campbell (1964), Dorner & Knight (1953), Freitag (1952), Gibbs & Guissani (unpublished results), Leberman (personal communication), MacLeod (personal communication), MacLeod & Markham (1963), Markham (1959), Markham & Smith, J. D. (1951), Markham & Smith, K. M. (1949), Martini (1957), Mazzone, Incardona & Kaesberg (1962), Miller & Price (1946), Ross (1963), Shuster (1963), Semancik & Bancroft (1964), E. P. Serjeant (to be published), Shepherd (1963), Stace-Smith, Reichmann & Wright (1965), Symons, Rees, Short & Markham (1963), van Velsen (personal communication), Walters (1964*a*, *b*), Yamazaki & Kaesberg (1961).

(1) *Symptoms and host range.* Limited host range, causing mosaic symptoms in most hosts, necrotic symptoms in very few. No 'recovery' from infection.

(2) *Transmission.* At least one of the group is beetle-transmitted, two are occasionally seed-transmitted, and all are readily sap transmitted.

(3) *Particles*. Purified preparations of the virus contain two major components:

(*a*) Infective nucleoprotein particles, which are isometric, 25–30 mμ in diameter, with 32 major morphological subunits when examined in neutral sodium phosphotungstate and which contain about 36% RNA with approximate base composition G 6% A 22% C 33% U 29%.

(*b*) Non-infective protein particles of the same external morphology.

We are indebted to Dr R. Markham for advice on the nucleotide analyses, to Dr B. J. Selman, of the British Museum, who identified the insects, and to many other people who helped both technically and in the collection of samples.

REFERENCES

AGRAWAL, H. (1964). Identification of cowpea mosaic virus isolates. *Meded. LandHogesch. Wageningen*, p. 64.

BANCROFT, J. B. (1962). Purification and properties of bean pod mottle virus and associated centrifugal and electrophoretic components. *Virology*, **16**, 419.

BAWDEN, F. C. (1950). *Plant Viruses and Virus Diseases*, 3rd ed. Waltham, Mass., U.S.A.: Chronica Botanica.

BOS, L., HAGEDORN, D. J. & QUANTZ, L. (1960). Suggested procedures for international identification of legume viruses. *Tijdschr. PlZiekt.* **66**, 328.

BRUNT, A. A., KENTEN, R. H., GIBBS, A. J. & NIXON, H. L. (1965). Further studies on cocoa yellow mosaic virus. *J. gen. Microbiol.* **38**, 81.

CAMPBELL, R. N. (1964). Radish mosaic virus, a crucifer virus serologically related to strains of bean pod mottle virus and to squash mosaic virus. *Phytopathology*, **54**, 1418.

DODDS, K. S. (1962). Classification of cultivated potatoes. In *The potato and its wild relatives*. Ed. by D. S. Correll. Renner, Texas: Texas Research Foundation.

DORNER, R. W. & KNIGHT, C. A. (1953). The preparation and properties of some plant virus nucleic acids. *J. biol. Chem.* **205**, 959.

FRANCKI, R. I. B. & MATTHEWS, R. E. F. (1962). Some effects of 2-thiouracil on the multiplication of turnip yellow mosaic virus. *Virology*, **17**, 367.

FREITAG, J. H. (1952). Seven virus diseases of cucurbits in California. *Phytopathology* (abstr.), **42**, 8.

HUXLEY, H. E. & ZUBAY, G. (1960). The structure of the protein shell of turnip yellow mosaic virus. *J. mol. Biol.* **2**, 180.

JOHN INNES INSTITUTE (1960). *Ann. Rep. Innes Inst.* **51**, 26.

JOHN INNES INSTITUTE (1963). *Ann. Rep. Innes Inst.* **54**, 37.

KATZ, S. (1962). Locating ultraviolet-absorbing substances on paper chromatographs. *J. chem. Educ.* **39**, 34.

MACLEOD, R. & MARKHAM, R. (1963). Experimental evidence of a relationship between turnip yellow mosaic virus and wild cucumber mosaic virus. *Virology*, **19**, 190.

MANSI, W. (1958). Slide gel diffusion precipitin test. *Nature, Lond.* **181**, 1289.

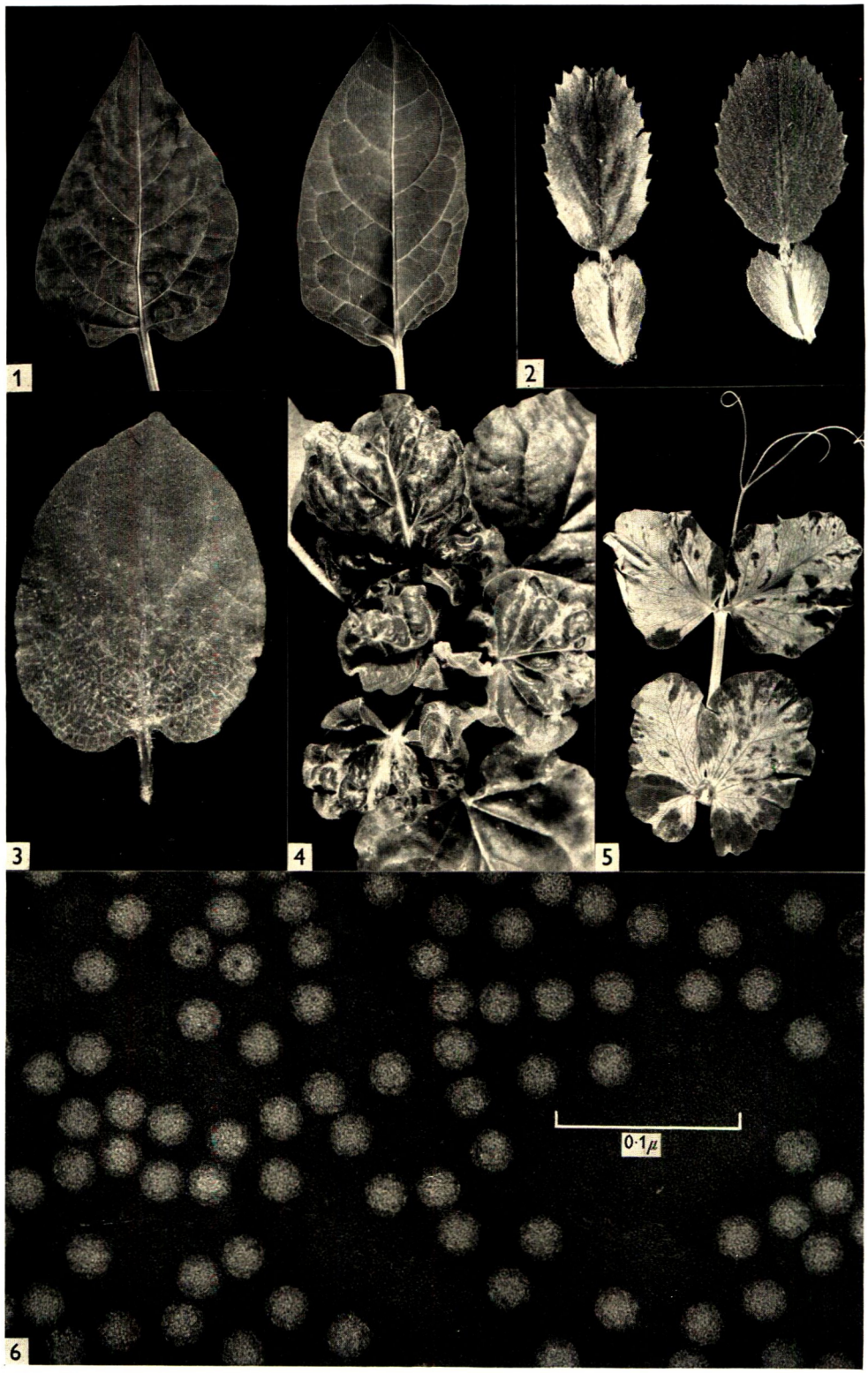
MARKHAM, R. (1955). *Modern Methods of Plant Analysis*. Ed. by K. Paech & M. V. Tracey, vol. 4, p. 246.

MARKHAM, R. (1959). In *The Viruses*. Ed. by F. M. Burnet & W. M. Stanley, vol. 2. chap. 2. New York and London: Academic Press.

MARKHAM, R. (1960). A graphical method for the rapid determination of sedimentation coefficients. *Biochem. J.* **77**, 516.

MARKHAM, R. & SMITH, J. D. (1951). Chromatographic studies of nucleic acids. *Biochem. J.* **49**, 401.

MARKHAM, R. & SMITH, K. M. (1949). Studies on the virus of turnip yellow mosaic. *Parasitology*, **39**, 330.



A. J. GIBBS AND OTHERS

(*Facing p.* 193)

MARTINI, C. (1957). The transmission of turnip viruses by biting insects and aphids. *Proc. 3rd Conf. Potato Virus Diseases*, p. 106.

MAZZONE, H. M., INCARDONA, N. L. & KAESBERG, P. (1962). Biochemical and biophysical properties of squash mosaic virus and related macromolecules. *Biochim. biophys. Acta*, **55**, 164.

MCKEE, R. K. (1964). Virus infection in South American potatoes. *Eur. Potato J.* **7**, 145.

MILLER, G. L. & PRICE, W. C. (1946). Physical and chemical studies on southern bean mosaic virus. *Archiv. Biochem.* **10**, 467.

NIXON, H. L. & GIBBS, A. J. (1960). Electron microscope observations on the structure of turnip yellow mosaic virus. *J. mol. Biol.* **2**, 197.

NIXON, H. L. & HARRISON, B. D. (1959). Electron microscopic evidence on the structure of tobacco rattle virus. *J. gen. Microbiol.* **21**, 582.

PERRING, F. H. & WALTERS, S. M. (1962). *Atlas of the British Flora*. London: Thomas Nelson.

REICHMANN, M. E. (1965). Determination of ribonucleic acid content of spherical viruses from sedimentation coefficients of full and empty particles. *Virology*, **25**, 166.

ROSS, J. P. (1963). Transmission of bean pod mottle virus in soybeans by beetles. *Pl. Dis. Reptr*, **47**, 1049.

SCHUSTER, M. F. (1963). Flea beetle transmission of tobacco ringspot virus in the lower Rio Grande Valley. *Pl. Dis. Reptr*, **47**, 510.

SEMANCIK, J. S. & BANCROFT, J. B. (1964). Further characterization of the nucleoprotein components of bean pod mottle virus. *Virology*, **22**, 33.

SHEPHERD, R. J. (1963). Serological relationship between bean pod mottle virus and cowpea mosaic virus from Arkansas and Trinidad. *Phytopathology*, **53**, 865.

SINHA, R. C. (1960). Red clover mottle virus. *Ann. appl. Biol.* **48**, 742.

STACE-SMITH, R., REICHMANN, M. E. & WRIGHT, N. S. (1965). Purification and properties of tobacco ringspot virus and two RNA-deficient components. *Virology*, **25**, 487.

STEERE, R. L. (1956). Purification and properties of tobacco ringspot virus. *Phytopathology*, **46**, 60.

SYMONS, R. H., REES, M. W., SHORT, M. N. & MARKHAM, R. (1963). Relationships between the ribonucleic acid and protein of some plant viruses. *J. mol. Biol.* **6**, 1.

WALTERS, H. J. (1964*a*). Transmission of bean pod mottle virus by bean leaf beetles. *Phytopathology*, **54**, 240.

WALTERS, H. J. (1964*b*). Transmission of southern bean mosaic virus by the bean leaf beetle. *Pl. Dis. Reptr* **48**, 935.

YAMAZAKI, H. & KAESBERG, P. (1961). Biophysical and biochemical properties of wild cucumber mosaic virus and two related virus-like particles. *Biochim. biophys. Acta*, **51**, 9.

EXPLANATION OF PLATE

Fig. 1. *Solanum dulcamara* leaves; left infected with DMV, right healthy.

Fig. 2. *Ononis repens* leaves; left infected with OYMV, right healthy.

Fig. 3. *Nicotiana glutinosa* leaf systemically infected with APLV.

Fig. 4. *Nicotiana glutinosa* plant systemically infected with DMV.

Fig. 5. *Pisum sativum* leaf systemically infected with OYMV.

Fig. 6. Electron micrograph of purified APLV bottom component mounted in neutral sodium phosphotungstate.

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The Effect of 8-Azaguanine on the Inducible Oxidation of Guanine by *Pseudomonas aeruginosa*

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(*Received 7 December* 1965)

SUMMARY

Pseudomonas aeruginosa NCTC 8203 was shown to metabolize guanine by deamination to xanthine, which was then oxidized to uric acid and further products. Guanine deaminase activity was present in non-induced bacteria but was 3–4 times greater in guanine-induced bacteria. Xanthine oxidase, and the uric acid oxidizing enzymes, were not found in bacteria grown in a minimal salts medium but were induced by adding guanine or xanthine to the culture medium towards the end of the growth period. When uric acid was added, only the uric acid oxidizing enzymes were induced. Enzyme induction also occurred when washed suspensions of the bacteria were incubated with guanine, xanthine or uric acid in Warburg flasks. 8-Azaguanine was deaminated more rapidly than guanine by non-induced bacteria and the rate was again greater with guanine-induced bacteria. 8-Azaguanine and 8-azaxanthine were not oxidized and did not induce the synthesis of deaminases or oxidases. 8-Azaguanine and 8-azaxanthine had no effect on the oxidation of guanine, xanthine or uric acid by fully induced bacteria. Equimolar concentrations of 8-azaguanine inhibited adaptation to the oxidation of guanine, xanthine and uric acid when added before the substrates. 8-Azaxanthine, under the same conditions, delayed adaptation to guanine and xanthine oxidation, and completely inhibited adaptation to uric acid. Mutant strains were isolated which were resistant to 10 mM-8-azaguanine and 5 mM-8-azaxanthine which completely inhibited growth of the parent strain. Another class of mutants was isolated resistant to 8-azaguanine and not resistant to 8-azaxanthine. Both classes of mutants were indistinguishable from the parent strain in their susceptibility to 8-azaguanine and 8-azaxanthine inhibition of adaptation to purine oxidation.

INTRODUCTION

The purine analogue 8-azaguanine is readily incorporated into the ribonucleic acid of some bacterial species (Smith & Matthews, 1957; Mandel & Markham, 1958), and prevents the synthesis of some inducible enzymes when added at the same time as the inducer (Creaser, 1956). We found that 8-azaguanine appeared to inhibit to some extent the synthesis by *Pseudomonas aeruginosa* of inducible permeases for some carbon compounds but not for others (Clarke & Meadow, 1959). We found that washed suspensions of the bacteria grown in the absence of succinate, which had decreased succinate permease activity, had an increased oxygen uptake with succinate as substrate in the presence of 8-azaguanine. One explanation for these results, was that 8-azaguanine was deaminated by the bacteria, thereby increasing the nitrogen compounds available for synthesizing succinate permease and oxidase. Bergmann, Ungar-Waron & Kwietny-Govrin (1964) have since shown

that 8-azaguanine is slowly converted to 8-azaxanthine by *P. aeruginosa* growing in minimal medium in the presence of 8-azaguanine. Pseudomonads are known to form inducible enzymes which enable them to metabolize various purines (Bachrach, 1957; Dikstein, Bergmann & Henis, 1957). In the present work we have followed the deamination and oxidation of guanine by *P. aeruginosa* and examined the effects of 8-azaguanine and 8-azaxanthine on guanine metabolism by induced and non-induced bacteria.

METHODS

Organism. The strain used was *Pseudomonas aeruginosa* NCTC 8203 maintained as described by Clarke & Meadow (1959).

Media. The bacteria were grown in a minimal salts medium with 20 mM-succinate as carbon source (Brammar & Clarke, 1964). The washed suspensions were prepared from bacteria incubated for 16–18 hr in 100 ml. batch cultures in 1 l. flasks shaken at 37° on a mechanical shaker. To obtain induced cultures, bacteria were grown for about 16 hr to an extinction reading at 670 mμ of about 0·9–1·2 (equiv. 0·5–0·6 mg. dry wt./ml.). Measurements of extinction were made with a Unicam SP 600 spectrophotometer (Unicam Instruments Ltd.). The appropriate purine was then added to give a final concentration of 2 mM and the incubation continued for a further 2 hr. The cultures were harvested by centrifugation and resuspended in 25 ml. 100 mM-phosphate or tris buffer (pH 7·2). The same minimal medium was used for determination of the growth-inhibitory concentrations of 8-azaguanine and 8-azaxanthine, with 1·2% (w/v) New Zealand agar added for plate cultures.

Enzyme activities. Oxygen uptake was measured by the conventional Warburg technique at 37° with air as the gas phase and 0·2 ml. 40% (w/v) KOH in the centre well. The main compartment of the Warburg vessel contained 1 ml. cell-free extract (equiv. 5 mg. dry wt. original bacterial suspension/ml.) or 1 ml. bacterial suspension (equiv. 2 mg. dry wt. bacteria/ml.). Phosphate buffer 100 mM (pH 7·2) was used unless stated otherwise, to give a final volume of 3·0 ml. Substrates or inhibitors were added from the side arm after equilibration for 20 min. Double side-arm flasks were used when required.

Deaminase activity was measured by estimating the ammonia released during aerobic or anaerobic incubation of cell preparations with the substrates, using the Conway microdiffusion technique for ammonia estimation followed by titration with 20 mM-HCl (Conway, 1957).

Cell-free extracts. Washed suspensions of bacteria, concentrated 10-fold in phosphate buffer, were disrupted using a bacterial press modified from that described by Milner, Lawrence & French (1950). Unbroken bacteria and debris were removed by centrifugation at 10,000*g* for 20 min.

Viable counts. Samples (0·1 ml.) of a suitable dilution of bacteria were spread on agar plates. Lemco agar (Clarke & Meadow, 1959) was used for counting the total number of viable bacteria and minimal agar + 8-azaguanine or 8-azaxanthine for counting the number of resistant bacteria.

Resistance tests. Minimal medium (5 ml.) containing 8-azaguanine or 8-azaxanthine was inoculated with about 10^4 bacteria and growth estimated by visual examination after 24 hr at 37°. Plate cultures were inoculated with 100–200 bacteria and the number and size of colonies determined after 24 hr at 37°.

Paper electrophoresis. Purines were separated by paper electrophoresis in 50 mM-sodium borate buffer (pH 9·2) and also in formic acid (1·5 M) + acetic acid (2 M) (1 + 1 by vol.) buffer (pH 2). Solutions were applied to Whatman no. 1 paper and subjected to 2·6 KV (80 mA) for 30 min. After drying, the purines were detected by their ultraviolet absorption and identified by comparison with authentic compounds.

Chemicals. Guanine, xanthine, hypoxanthine, uric acid, 8-azaguanine and 8-azaxanthine were obtained from L. Light and Co. Ltd. (Colnbrook, Bucks). These compounds are, except for uric acid, only very slightly soluble in water. The stock suspensions were prepared in a finely divided state by grinding the solid and heating it in a suitable volume of boiling water for 10 min.

RESULTS

Oxidation of guanine

Washed suspensions of *Pseudomonas aeruginosa* grown in the minimal medium were not able to oxidize guanine until they had been incubated with the substrate for some time. Chloramphenicol inhibited this adaptation but had no effect on the oxidation of guanine by fully induced bacteria (Fig. 1 *a*, *b*). The time taken for non-induced bacteria to reach a rapid rate of guanine oxidation could be varied by altering the conditions of the experiment. Bacteria suspended in phosphate buffer adapted more rapidly than those suspended in tris buffer. The carbon pool also affected the duration of the lag period and in some experiments with washed

Table 1. *Deamination and oxidation of guanine by Pseudomonas aeruginosa* NCTC *8203*

Values for 240 min. incubation in air in Warburg flasks, corrected for endogenous O_2 uptake 102 μl.

Guanine μmole/flask	O_2 uptake (μl.)	O_2 (μmole)	NH_3 (μmole)	O_2 : Guanine	NH_3 : Guanine
4	96	4·3	—	1·08	—
	102	4·5	—	1·14	—
8	205	9·1	8·7	1·14	1·09
	191	8·5	8·1	1·06	1·01
	187	8·3	—	1·04	—
12	294	13·1	11·9	1·09	0·98
	284	12·7	12·0	1·06	1·00
	290	12·9	12·0	1·08	1·00

carbon-starved bacteria grown in minimal medium containing 10 mM-succinate, no adaptation to guanine oxidation was detected. The bacterial culture had a low endogenous oxidation rate after 16–18 hr growth in the standard minimal medium and adaptation to guanine oxidation could also be measured with samples of unwashed bacteria taken directly from the culture medium. There was a wide variation in the time taken for complete adaptation to guanine by suspensions prepared under these different conditions. Washed suspensions prepared in phosphate buffer from bacteria grown under the standard conditions became fully adapted in 60–90 min. Washed suspensions in tris buffer and washed suspensions of

carbon-starved bacteria were found to take 120 min. or longer to become fully adapted.

Figure 1*b* gives the results of an experiment in which the bacteria had become fully induced during the 2-hr growth period in the presence of guanine. In other experiments the adaptation to guanine was not completed during this time so that bacteria harvested after 2 hr continued to adapt to guanine in the Warburg flasks. Incomplete adaptation to guanine oxidation was shown by non-linear oxygen uptake and partial inhibition by chloramphenicol. Cultures which had stopped growing because of exhaustion of the carbon source, and cultures which were well into the stationary phase, did not adapt to guanine oxidation as well or as rapidly as cultures which had not quite reached maximum growth.

It seemed likely that guanine was being metabolized by deamination to xanthine followed by the oxidation of xanthine to uric acid and other products. Guanine deaminase activity was present in non-induced bacteria and the activity was

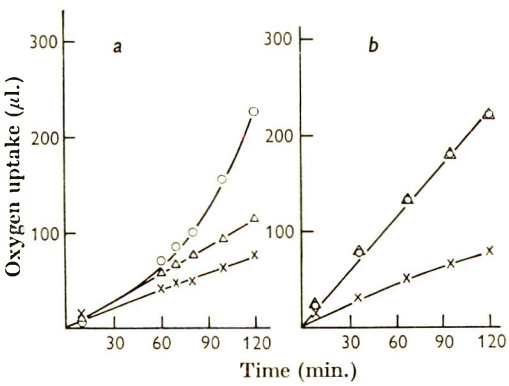

Fig. 1 (*a* and *b*)

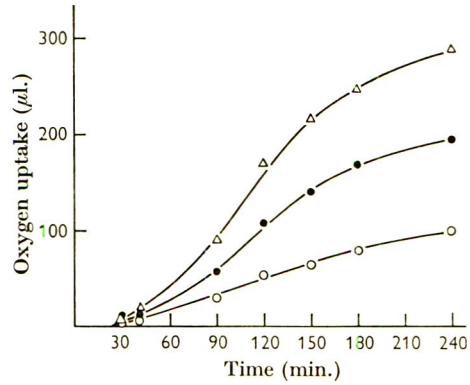

Fig. 2

Fig. 1 *a*, *b*. Effect of chloramphenicol on guanine oxidation by washed bacterial suspensions in phosphate buffer of (*a*) non-induced, (*b*) guanine-induced cultures of *Pseudomonas aeruginosa* NCTC 8203 equiv. 2 mg. dry wt. bacteria/flask. 100 mM-phosphate buffer (pH 7·2). Total vol. 3 ml. ○—○, guanine (10 mM); △—△, guanine (10 mM) + chloramphenicol (60 µg./ml.); × - ×, endogenous.

Fig. 2. Oxygen uptake by washed suspensions of *Pseudomonas aeruginosa* NCTC 8203 resuspended in 100 mM-tris buffer (pH 7·2). Equiv. 2 mg. dry wt. bacteria/flask. Total vol. 3 ml. ○—○, 4 µmole guanine; ●—●, 8 µmole guanine; △—△, 12 µmole guanine. Values for endogenous oxygen uptake have been subtracted.

about 25–30% of that of guanine-induced bacteria. This is considered in more detail in connection with the metabolism of 8-azaguanine (see Fig. 5).

The extent of deamination and oxidation of guanine was compared at low substrate concentrations (4, 8, 12 µmole/flask). Table 1 and Fig. 2 give the results of an experiment with non-induced bacteria resuspended in tris buffer (pH 7·2). The endogenous oxygen uptake (102 µl./240 min.) has been subtracted. Endogenous ammonia production was negligible. The lag period before any guanine oxidation was detected under these conditions was about 30 min., and after 240 min. the rate of oxygen uptake in the flasks containing guanine had decreased almost to the endogenous rate. The NH_3:guanine ratio after this time was 1:1 so that it could be

concluded that the guanine was completely deaminated by the end of the experimental period. After 240 min. the oxygen uptake was very slightly greater than could be accounted for by the oxidation of xanthine to uric acid.

In another experiment the ammonia production was compared with the oxygen uptake under conditions in which the substrate was present in excess (40 μmole/flask) by setting up several Warburg flasks in parallel to determine oxygen uptake, and removing single flasks at intervals for ammonia determinations. Up to about 180 min. the oxygen uptake was approximately equivalent to the ammonia released. This experiment was made with non-induced bacteria to avoid any substrate being carried over from the growth medium; it was therefore comparable to the conditions of the experiment in Fig. 1*a*. When washed suspensions of non-induced bacteria were incubated with guanine for longer periods the oxygen uptake reached values of O_2:guanine of over 3:1, which can only be accounted for by further oxidation of uric acid.

It seemed possible that under some conditions the guanine was being deaminated more rapidly than xanthine was being oxidized, particularly since the bacteria grown under all conditions had some deaminase activity but, as we found later, only the guanine- (or xanthine-) induced cultures had xanthine oxidase activity. Flask contents from an experiment done in the same way as that shown in Fig. 1*a* were analysed for purines after incubation for 6 hr. The bacteria were separated by centrifugation and extracted by boiling for 5 min. with 0·5 ml. Na_2CO_3 (1%, w/v). These extracts, and the supernatant fluid from the flask contents, were subjected to paper electrophoresis as described in Methods. Guanine was the only purine detected in the supernatant fluid, but extracts from bacteria which had been actively metabolizing guanine contained guanine and xanthine in approximately equal amounts. The method did not allow for the detection of uric acid. It was concluded that guanine was being deaminated more rapidly than the xanthine was being oxidized, and that even under the conditions when there was an apparent molar correspondence of ammonia release and oxygen uptake, at least a fraction of the uric acid produced was being further oxidized.

Adaptation to guanine, xanthine and uric acid

Non-induced cultures were able to adapt to the oxidation of xanthine and uric acid after incubation for some time with these substrates in Warburg flasks or in culture medium. The pattern of enzyme induction of bacteria induced with guanine, xanthine and uric acid, respectively, was examined. In a series of experiments 16 hr cultures were incubated for a further 2 hr with and without purine inducers. After this time the oxygen uptake of the washed suspensions was measured with these three compounds as separate substrates. Cultures induced with guanine or xanthine oxidized guanine, xanthine or uric acid without an appreciable lag (Fig. 3*a*, *b*). Those induced with uric acid were able to oxidize immediately only uric acid (Fig. 3*c*). As found previously with guanine adaptation, there was considerable variation in the adaptation to uric acid of bacteria grown under different conditions. Bacteria which had been in the stationary phase for some time were very poor at adapting to uric acid. Occasional batches of bacteria gave a higher rate of uric acid oxidation than that shown in Fig. 3*c*, but in no case did a uric acid induced culture show any

concomitant adaptation to guanine or xanthine. Figure 3*d* shows that washed suspensions of bacteria grown under these conditions without added purine inducers were induced to form the oxidative enzymes only after 60–90 min. of incubation. These results suggest that during adaptation to guanine, xanthine oxidase is induced by xanthine (and possibly guanine) and that the uric acid formed then induces the formation of enzymes for its own oxidation.

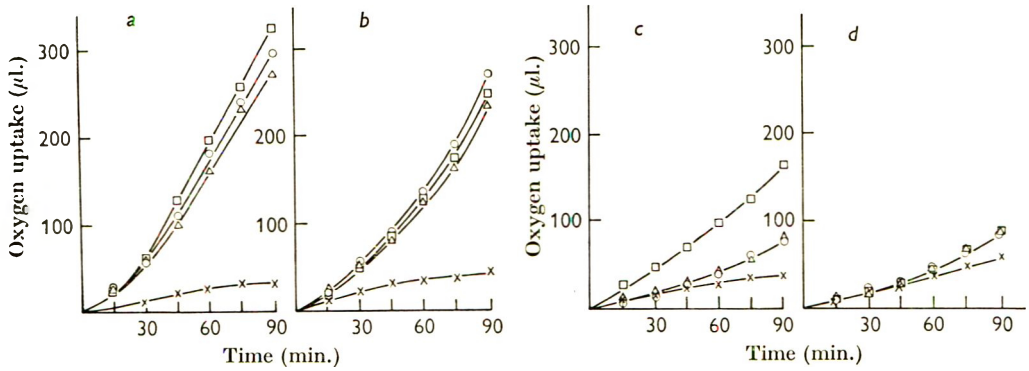

Fig. 3. Oxidation of guanine, xanthine and uric by washed suspensions of *Pseudomonas aeruginosa* NCTC 8203 in phosphate buffer. (*a*) guanine-induced; (*b*) xanthine-induced; (*c*) uric acid-induced; (*d*) non-induced. 100 mM-phosphate buffer (pH 7·2). Equiv. 2 mg. dry wt. bacteria/flask. Total vol. 3 ml. ○—○, guanine; △—△, xanthine; □—□, uric acid; × - ×, endogenous. Substrates 10 mM.

Cell-free extracts

The lag period before the oxidation of guanine, xanthine or uric acid by washed suspensions of non-induced bacteria might be due to the synthesis of deaminating enzymes or oxidative enzymes, or to the synthesis of permeases needed to transport the substrates into the cell. Cell-free extracts were therefore prepared and their oxidative abilities compared with those of whole bacteria (Fig. 4*a*, *b*). Chloramphenicol was added to all flasks to prevent adaptation during the experiment. Cell-free extracts from guanine-induced cultures oxidized guanine and xanthine at comparable rates though much more slowly than did the whole bacteria from which they were derived. Uric acid was not oxidized by these extracts (Fig. 4*b*). None of the extracts from non-induced cultures oxidized guanine, xanthine or uric acid. Induction by guanine and xanthine must therefore involve the synthesis of xanthine oxidase, though it may also include the synthesis of permeases for guanine and xanthine.

Metabolism of 8-azaguanine

Bergmann *et al.* (1964) found that after 24-hr growth of cultures of *Pseudomonas aeruginosa* in a minimal medium containing originally 8-azaguanine 100 µg./ml. they could detect only 8-azaxanthine in the medium and concluded that the 8-azaguanine had been slowly deaminated. We found that non-induced cultures of our strain of *P. aeruginosa* which were able to deaminate guanine were also able to deaminate 8-azaguanine. 8-Azaguanine was more rapidly deaminated than guanine by both induced and non-induced cultures incubated under anaerobic conditions

(Fig. 5). Non-induced cultures invariably possessed guanine deaminase activity, but the rate of deamination was very low so that rate-measurements were less accurate than those for induced cultures or for 8-azaguanine deaminase activity of induced and non-induced cultures. Increase in guanine deaminase activity was always accompanied by increase in 8-azaguanine deaminase activity, and it seemed reasonable to conclude that the same enzyme was responsible for the deamination of

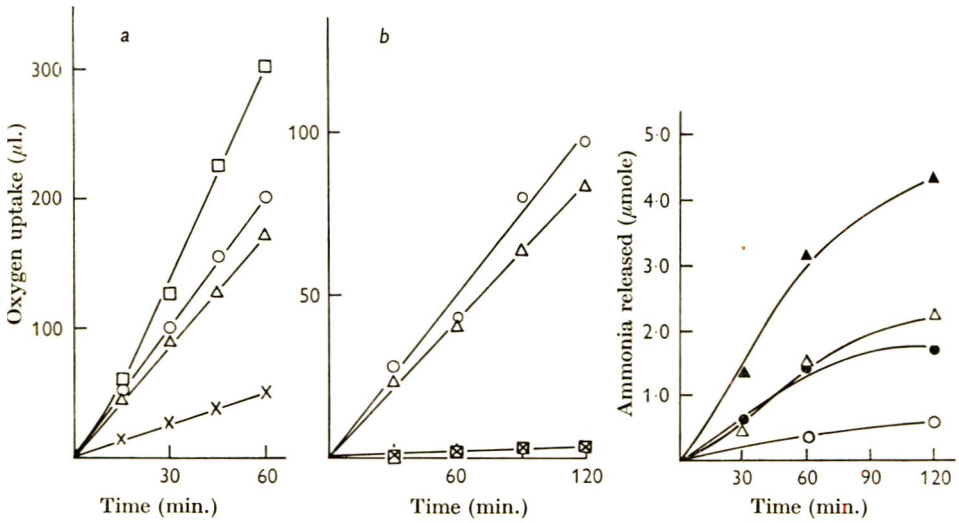

Fig. 4 (*a* and *b*)

Fig. 5

Fig. 4. Oxidation of guanine, xanthine and uric acid in phosphate buffer by washed suspensions and cell-free extracts of guanine-adapted *Pseudomonas aeruginosa* NCTC 8203. (*a*) whole organisms equiv. 2 mg. dry wt./flask; (*b*) cell-free extracts 1 ml. extract from equiv. 5 mg. dry wt. bacteria/flask. ○—○, guanine; △—△, xanthine; □—□, uric acid; × - ×, endogenous. Substrates 10 mM; chloramphenicol 60 μg./ml. present in all flasks. 100 mM-phosphate buffer (pH 7·2). Total vol. 3 ml.

Fig. 5. Anaerobic deamination of guanine and 8-azaguanine by washed suspensions of *Pseudomonas aeruginosa* NCTC 8203. Unadapted bacteria: ○—○, guanine; △—△, 8-azaguanine. Guanine adapted bacteria: ●—●, guanine; ▲—▲, 8-azaguanine. Experiment done in N_2 in Warburg flasks. 2·0 ml. bacterial suspension in 100 mM-tris buffer (pH 7·2); 10 mM substrates.

both purines. The enzyme is thus comparable with the rat liver guanase studied by Roush & Norris (1950). When washed suspensions of non-induced cultures were incubated with guanine in air for 2 hr the deaminase activity followed a typical induction curve due to increased synthesis of enzyme. This induction was prevented by chloramphenicol. The deaminase activity of non-induced bacteria incubated with 8-azaguanine was the same for washed suspensions incubated (*a*) aerobically, (*b*) aerobically in the presence of chloramphenicol, or (*c*) anaerobically. It appears therefore that 8-azaguanine does not act as an inducer for the deaminase, although it is a substrate of the enzyme.

Effect of 8-azaguanine and 8-azaxanthine on oxidation and adaptation

Since 8-azaguanine was deaminated by *Pseudomonas aeruginosa* NCTC 8203, it seemed possible that the 8-azaxanthine so formed might be oxidized by enzymes

analogous to, or identical with, those which oxidized xanthine. Washed suspensions of non-induced bacteria were therefore incubated with 10 mM-8-azaguanine or 10 mM-8-azaxanthine and the oxygen uptake followed manometrically. No increase in oxygen uptake over the endogenous rate was detected even after incubation for 6 hr. Organisms induced by adding guanine, xanthine, uric acid, 8-azaguanine or 8-azaxanthine to the culture medium 2 hr before harvesting, were also unable to oxidize 8-azaguanine or 8-azaxanthine. These two analogues were not therefore oxidized by the enzymes which metabolized guanine and xanthine, and there was no comparable induction of enzymes which oxidized 8-azaguanine or 8-azaxanthine.

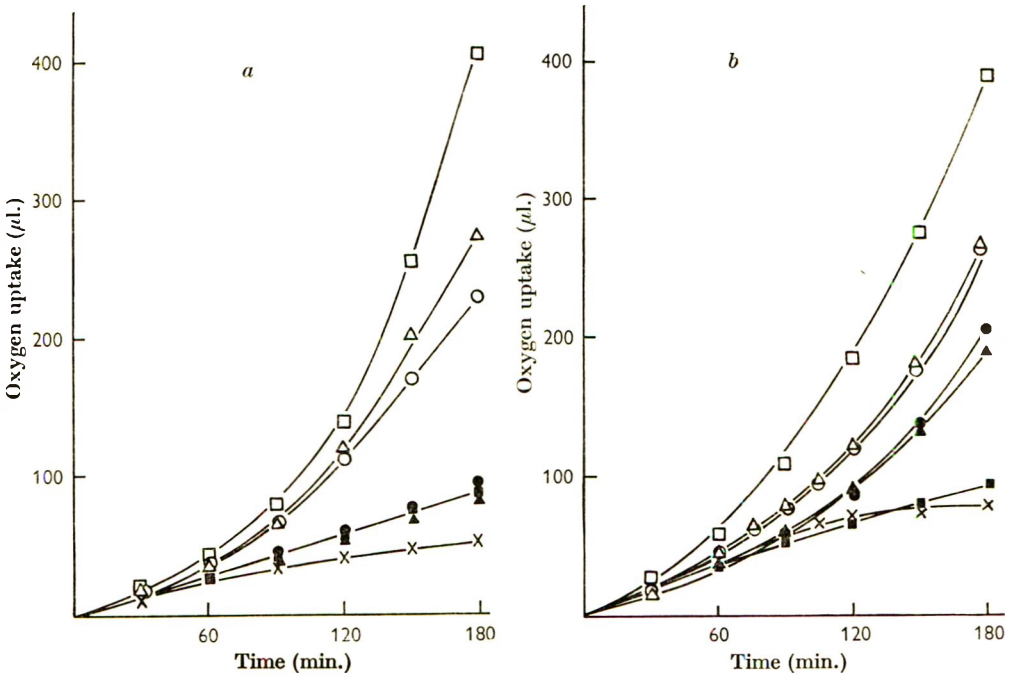

Fig. 6. Effect of (*a*) 8-azaguanine and (*b*) 8-azaxanthine on adaptation of washed suspensions of *Pseudomonas aeruginosa* NCTC 8203 in phosphate buffer. 100 mM-phosphate buffer (pH 7·2). Substrates and analogues 10 mM. Equiv. 2 mg. dry wt. bacteria/flask. Total vol. 3 ml. ○—○, guanine; ●—●, guanine + 8-azaguanine (or 8-azaxanthine); △—△, xanthine; ▲—▲, xanthine + 8-azaguanine (or 8-azaxanthine); □—□, uric acid; ■—■, uric acid + 8-azaguanine (or 8-azaxanthine); × - ×, endogenous.

The oxygen uptake of washed suspensions which actively metabolized guanine, xanthine and uric acid was unaffected by the addition of 8-azaguanine or 8-azaxanthine even in 100-fold excess. One explanation for this lack of inhibition might be that under the experimental conditions the aza-analogues do not enter the organism. However, the oxidation of guanine and xanthine by cell-free extracts was not affected by 8-azaguanine or 8-azaxanthine. These two compounds do not therefore act as inhibitors of xanthine oxidase or any of the enzymes which oxidize uric acid.

Experiments were made to determine the effect of 8-azaguanine and 8-azaxanthine on the induction process. Non-induced cultures were incubated in the Warburg

apparatus with 10 mM-guanine, equimolar 8-azaguanine being added at various times both before and during induction. A normal induction curve for guanine was obtained in all flasks except those in which the 8-azaguanine had been added before the guanine. Simultaneous addition of 8-azaguanine and guanine delayed induction slightly, but the final oxygen uptake was unchanged. The insolubility of the purines and their aza-analogues made it difficult to determine the absolute concentrations required for inhibition but, by decreasing the guanine concentration, it was possible to show that induction was inhibited only when 8-azaguanine was at least equimolar with guanine. Figure 6*a* shows the effect of 8-azaguanine, pre-incubated with the organisms for 20 min. before the addition of the other purine, on the adaptation to guanine, xanthine and uric acid. All three induction processes were inhibited by 8-azaguanine under these conditions. Adaptation to uric acid was less susceptible to 8-azaguanine than was adaptation to the other two purines and under some conditions, for example, in tris buffer or in growth medium, adaptation to uric acid was not inhibited at all by 8-azaguanine.

The incubation processes were affected quite differently by 8-azaxanthine under the same conditions (Fig. 6*b*). Adaptation to guanine and xanthine oxidation was delayed, but not inhibited, by equimolar 8-azaxanthine, while adaptation to uric acid was completely prevented. All three induction processes were considerably less susceptible to 8-azaxanthine than to 8-azaguanine, and in tris buffer adaptation to all three purines proceeded normally even after pre-incubation with equimolar 8-azaxanthine.

Growth and growth inhibition

For this strain (NCTC 8203) of *Pseudomonas aeruginosa* guanine is a poor carbon source but an excellent nitrogen source. It was thought that if 8-azaguanine was so readily deaminated it might be also utilized as a nitrogen source. This proved to be the case and although there was no growth at higher concentrations, good growth of plate cultures was obtained with a medium containing 20 mM-succinate as carbon source + 4 mM-8-azaguanine as nitrogen source. No growth was obtained without an additional carbon source. This result was somewhat surprising since both 8-azaguanine and 8-azaxanthine are known as growth inhibitors. In a minimal salts medium 50% inhibition of growth was obtained with 3 mM-8-azaguanine in liquid medium and with 8 mM in a solid medium. 8-Azaxanthine was growth inhibitory at much lower concentrations than 8-azaguanine; in minimal salts medium 50% inhibition of growth was obtained with 100 μM-8-azaxanthine in both liquid and solid media. Growth inhibition was annulled by adding excess guanine or xanthine.

Isolation of resistant strains

We were able to isolate resistant mutants of *Pseudomonas aeruginosa* NCTC 8203 by adding 8-azaguanine to the medium and using a heavy inoculum. Four flasks were set up containing 100 ml. minimal medium and inoculated with about 10^{10} bacteria. The results are given in Table 2. There was no growth lag for the control culture (A) but with 5 mM-8-azaguanine (D) the lag was 2 hr and with 10 mM (C) it was 12–14 hr. It was impossible to make accurate growth measurements for the flask with 20 mM-8-azaguanine (B) since there was some undissolved 8-azaguanine

in suspension; but after 24 hr the growth was comparable with that in the other flasks.

After 26 hr, samples were taken to determine the total viable count of the cultures and the numbers of resistant bacteria. The total count was similar for all four cultures. No resistant bacteria were found on the plates inoculated from the control flask, but a high proportion of the bacteria from flasks B and C were resistant to 10 mM-8-azaguanine and to mM-8-azaxanthine. Although no resistant colonies

Table 2. *Isolation of strains of Pseudomonas aeruginosa* NCTC *8203 resistant to 8-azaguanine and 8-azaxanthine*

	Flask			
	A	B	C	D
Concentration of 8-azaguanine added to medium:	0	20 mM	10 mM	5 mM
Duration of growth lag (hr)	0	(0)	12–14	2
Viable count after 26 hr (bacteria/ml. $\times 10^{-7}$)*				
Lemco agar	189	114	195	132
Minimal agar + 10 mM-8-azaguanine	0	131	85	0
Minimal agar + mM-8-azaxanthine	0	68	30	0
8-azaguanine resistance† 10 mM	0	+ + + +	+ + + +	0
1 mM	+	+ + + +	+ + + +	+
8-azaxanthine resistance† 10 mM	0	+	+ + +	0
1 mM	0	+ + + +	+ + + +	0
0·1 mM	+	+ + + +	+ + + +	+ + +

(0) Could not be determined; * Culture diluted $\times 10^6$, 0·1 ml. spread on plate; † Growth after 24 hr in liquid media.

Table 3. *Growth of resistant mutants of Pseudomonas aeruginosa* NCTC *8203*

AG mutants were obtained from single colonies on minimal medium + 8-azaguanine and AX mutants from minimal medium + 8-azaxanthine. For the viable counts cultures were grown for 18 hr in minimal medium, diluted 10^6, and 0·1 ml. spread on the counting medium.

	Counting medium		
	Lemco agar	Minimal agar + 8-azaguanine (10 mM)	Minimal agar + 8-azaxanthine (5 mM)
	Viable count (bacteria $\times 10^{-7}$/ml.)		
Mutant			
AG 1	188	184	181
AG 2	62	66	62
AG 4	64	56	0
AG 6	270	252	0
AX 1	72	96	99
AX 3	70	79	83

were found on the plates inoculated with about 10^2 bacteria from flask D, 2–3 resistant colonies occurred on plates inoculated with 10^4 bacteria from this flask, although again none were found on plates inoculated with the same number of bacteria from the control culture. It appeared that resistance to 8-azaguanine or 8-azaxanthine occurred very readily and that growth under these conditions selected resistant mutants. Suitable dilutions of the flask cultures were made for resistance tests in liquid medium; these confirmed that flasks B and C contained a high proportion of resistant bacteria. Several colonies were picked from the plates containing 8-azaguanine (AG strains) or 8-azaxanthine (AX strains) to test

whether a single factor was involved in resistance to both purine analogues. The single-colony isolates were inoculated into minimal medium and grown overnight. The cultures were then plated on Lemco agar and on minimal agar containing 8-azaguanine or 8-azaxanthine. It can be seen from Table 3 that there were two classes of mutants isolated from the 8-azaguanine plates. Mutants AG 1 and AG 2 grew equally well in the presence of concentrations of the two purine analogues which would inhibit the growth of the parent culture. Mutants AG 4 and AG 6 were resistant to 8-azaguanine but were not resistant to 8-azaxanthine. All the mutants isolated from plates containing 8-azaxanthine were resistant to both purine analogues; mutants AX 1 and AX 3 are typical cultures of this type. Representative mutants of each class (AG 1, AG 4, AX 1) were tested with guanine and 8-azaguanine in the Warburg apparatus. They behaved in the same way as wild-type organisms, adaptation to guanine oxidation being inhibited by pre-incubation with equimolar 8-azaguanine.

DISCUSSION

The experiments reported here are consistent with the view that the metabolism of guanine by *Pseudomonas aeruginosa* NCTC 8203 occurs by the same mechanism as in other strains of this species. Guanine is first deaminated to xanthine which is then oxidized to uric acid before breakdown of the purine ring system (Bachrach, 1957; Dikstein *et al.* 1957). The observed oxygen uptake in the presence of guanine results from its deamination to xanthine which is subsequently oxidized by xanthine oxidase and uric acid-oxidizing enzymes. In this organism some of the purine-metabolizing enzymes are inducible. Non-induced cultures showed no oxidizing activity towards guanine, xanthine or uric acid even in cell-free extracts, and whole organisms oxidized these substrates only after a lag period during which the oxidizing enzymes were being synthesized. The inhibition of induction by chloramphenicol confirms the idea that the induction period involved the synthesis of new protein. Our experiments do not distinguish between the synthesis of the oxidizing enzymes alone and the synthesis of the oxidizing enzymes plus any permeases.

The experiments on the specificity of induction produced further confirmation of the proposed metabolic pathway. Cultures pre-induced with uric acid oxidized this compound rapidly but only metabolized xanthine and guanine after a lag of 30 to 60 min. Those bacteria pre-induced by growth with xanthine or guanine, however, were capable of oxidizing all three substrates without lag. Since guanine deaminase is partly constitutive in these bacteria, these experiments could not be used to decide whether xanthine oxidase was induced only by xanthine or by both guanine and xanthine; but the uric acid oxidizing system was clearly induced by uric acid only and not by the other two purines. These enzymes appear therefore to follow a typical sequential induction path as first described by Stanier (1947).

The variation in the capacity of bacteria grown under different conditions to adapt to the oxidation of these purines suggests that the system may be subject to considerable repression by cell metabolites. Catabolite repression has been described for many systems but has been most intensively studied for *Escherichia coli* β-galactosidase (McFall & Mandelstam, 1963; Nakada & Magasanik, 1964; Loomis & Magasanik, 1965). In the case of *Pseudomonas aeruginosa* it may be that induction of purine oxidizing enzymes is repressed by purine derivatives. Although 8-aza-

guanine was a better substrate than guanine for the guanine-deaminating enzyme, it did not appear to act as an inducer for its synthesis and it was technically impossible to test whether it could prevent deaminase induction by guanine. There was no evidence of further metabolism of the azapurines beyond their deamination. This is supported by the fact that 8-azaguanine acted as a nitrogen source for growth but not as a carbon source.

The growth-inhibitory properties of 8-azaguanine have been ascribed to its incorporation into RNA. Smith & Matthews (1957) found that 100–500 μM-8-azaguanine inhibited growth of *Bacillus cereus* and could replace 40% of the guanine in its RNA. Growth of *Escherichia coli* and tobacco mosaic virus was inhibited by 8-azaguanine at higher concentrations and there was less incorporation of the analogue into their RNAs. Growth of *B. cereus* was inhibited by 100–500 μM-8-azaxanthine, which was incorporated into its RNA, but it had no effect on the growth of *E. coli* or tobacco mosaic virus. It has since been reported that 8-azaguanine can replace guanine in transfer RNA, and in template or messenger RNA (Levin, 1965; Weinstein & Grunberger, 1965). Rapid incorporation of 8-azaguanine into messenger RNA would be expected to give marked inhibition of the synthesis of enzymes being induced by exogenous inducers. But our earlier observations on permease induction suggested that the effect of 8-azaguanine on *Pseudomonas aeruginosa* was more complex. The experiments reported in the present paper confirm that this organism can deaminate 8-azaguanine at an appreciable rate and even use it as the sole nitrogen source for growth. The effect of 8-azaguanine in inhibiting induction of xanthine oxidase and the uric acid oxidizing system was shown only when it was added before the inducer and at an equimolar concentration. This inhibition might have been due to the incorporation of 8-azaguanine into the specific messenger RNA molecules, but the greater sensitivity of xanthine oxidase as compared with the uric acid oxidizing system is more compatible with direct competition with the inducer. Several cases have been reported of inducer analogues which prevent induction by substrate or non-substrate inducers. For example, phenyl-β-thiogalactoside prevents the induction of β-galactosidase in *E. coli* by methyl-β-D-thiogalactoside (Cohn & Monod, 1953) and the amide analogue cyanoacetamide gives competitive repression of amidase induction by acetamide and N-acetylacetamide in *P. aeruginosa* (Clarke & Brammar, 1964). The effect of 8-azaguanine and 8-azaxanthine on induction of the purine oxidizing enzymes might be due to competition with the purine substrates for inducer-binding sites. The relative differences observed in the effects of 8-azaguanine and 8-azaxanthine on the induction of xanthine oxidase as compared with the uric acid oxidizing enzymes would fit this interpretation.

Growth inhibition by 8-azaguanine and 8-azaxanthine is unlikely to be related to any effect on induction of the purine-oxidizing system. The isolation of some mutants resistant to 8-azaguanine but not to 8-azaxanthine, while other mutants were resistant to both analogues, suggests that more than one site of attack may be involved. Altered permeability is unlikely to be the main factor in the increased resistance of the mutants to the analogues since both types of 8-azaguanine-resistant mutants showed the same 8-azaguanine inhibition of purine oxidation as did the parent strain. Furthermore, the high frequency of isolation of strains resistant to 8-azaguanine and 8-azaxanthine suggests that more than one type of mutation

could confer resistance. Bergmann *et al.* (1964) found that their strain of *Pseudomonas aeruginosa* readily acquired resistance to 8-azaxanthine. One possible mechanism for this is suggested by the work of Gunther & Prusoff (1963) who found that certain azathymine-resistant mutants of *Streptococcus faecalis* had decreased efficiency of thymine utilization and assimilated 6-azathymine less well than did the parent culture. More than one change in enzymes concerned with purine utilization could therefore produce cultures resistant to growth inhibition by 8-azaguanine and 8-azaxanthine.

The results of the growth experiments with *Pseudomonas aeruginosa* NCTC 8203, together with the results of the deamination and oxidation experiments, support the idea that the azapurines compete with the normal purines at several different metabolic sites. The marked deaminase activity of this strain towards 8-azaguanine confirms the view that the stimulation by 8-azaguanine of oxygen uptake, with substrates such as succinate, can best be explained by the deamination of this compound which produces more ammonia for protein synthesis.

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REFERENCES

Bachrach, U. (1957). The aerobic breakdown of uric acid by certain pseudomonads. *J. gen. Microbiol.* **17**, 1.

Bergmann, F., Ungar-Waron, H. & Kwietny-Govrin, H. (1964). Action of 8-azaguanine and 8-azaxanthine on *Pseudomonas aeruginosa*. *Biochem. J.* **91**, 270.

Brammar, W. J. & Clarke, P. H. (1964). Induction and repression of *Pseudomonas aeruginosa* amidase. *J. gen. Microbiol.* **37**, 307.

Clarke, P. H. & Brammar, W. J. (1964). Regulation of bacterial enzyme synthesis by induction and repression. *Nature, Lond.* **203**, 1153.

Clarke, P. H. & Meadow, P. M. (1959). Evidence for the occurrence of permeases for tricarboxylic acid cycle intermediates in *Pseudomonas aeruginosa*. *J. gen. Microbiol.* **20**, 144.

Cohn, M. & Monod, J. (1953). Specific inhibition and induction of enzyme biosynthesis *Symp. Soc. gen. Microbiol.* **3**, 132.

Conway, E. J. (1957). *Microdiffusion Analysis and Volumetric Error*, 4th ed., p. 98. London: Crosby Lockwood and Son Ltd.

Creaser, E. H. (1956). The assimilation of amino acids by bacteria. 22. The effect of 8-azaguanine upon enzyme formation in *Staphylococcus aureus*. *Biochem. J.* **64**, 539.

Dikstein, S., Bergmann, F. & Henis, Y. (1957). Studies on uric acid and related compounds. IV. The specificity of bacterial xanthine oxidases. *J. biol. Chem.* **224**, 67.

Gunther, H. L. & Prusoff, W. H. (1963). Studies on the mechanism of resistance to pyrimidines in *Streptococcus faecalis*. *J. biol. Chem.* **238**, 1091.

Levin, D. H. (1965). Amino acid acceptor and transfer functions of s-RNA containing 8-azaguanine. *Biochem. biophys. res. Commun.* **19**, 654.

Loomis, W. F. & Magasanik, B. (1965). Genetic control of catabolite repression of the *Lac* operon in *Escherichia coli*. *Biochem. biophys. res. Commun.* **20**, 230.

McFall, E. & Mandelstam, J. (1963). Specific metabolic repression of three induced enzymes in *Escherichia coli*. *Biochem. J.* **89**, 391.

Mandel, H. G. & Markham, R. (1958). The effects of 8-azaguanine on the biosynthesis of ribonucleic acid in *Bacillus cereus*. *Biochem. J.* **69**, 297.

Milner, H. W., Lawrence, N. S. & French, C. S. (1950). Colloidal dispersion of chloroplast material. *Science*, **111**, 633.

Nakada, D. & Magasanik, B. (1964). The roles of inducer and catabolite repressor in the synthesis of β-galactosidase by *Escherichia coli*. *J. mol. Biol.* **8**, 105.

ROUSH, A. & NORRIS, E. R. (1950). Deamination of 8-azaguanine by guanase. *Arch. Biochem.* **29**, 124.

SMITH, J. D. & MATTHEWS, R. E. F. (1957). The metabolism of 8-azapurines. *Biochem. J.* **66**, 323.

STAINER, R. Y. (1947). Simultaneous adaptation: A new technique for the study of metabolic pathways. *J. Bact.* **54**, 339.

WEINSTEIN, I. B. & GRUNBERGER, D. (1965). Coding properties of s-RNA containing 8-azaguanine. *Biochem. biophys. res. Commun.* **19**, 647.

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Biosynthesis of the Antibiotic Nisin by Whole *Streptococcus lactis* Organisms

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SUMMARY

A reaction mixture is described consisting of a buffered solution of amino acids, salts, growth factors and glucose in which freshly harvested washed *Streptococcus lactis* incorporated radioactive tracers and synthesized nisin. Rapid nisin synthesis started after a delay of 30–60 min. but bacteria pre-incubated in the reaction mixture synthesized nisin without delay although the rate of protein synthesis remained the same as that of freshly harvested bacteria. Although growing *S. lactis* is sensitive to penicillin and mitomycin these antibiotics had no effect on nisin synthesis by washed organisms. Actinomycin D inhibited uptake of tritiated uridine immediately and inhibited nisin synthesis after a delay of about 60 min. Antibiotics which interfere with protein synthesis, e.g. chloramphenicol, puromycin and terramycin also interfered with nisin synthesis. The inhibition was immediate and occurred irrespective of whether the antibiotics were added at the beginning of an experiment or after 50 min. Nisin synthesis was more sensitive than protein synthesis. The data suggest that nisin synthesis occurs by a mechanism similar to that of protein synthesis.

INTRODUCTION

Polypeptide antibiotics frequently contain unusual amino acids; some of the constituent amino acids may be in the uncommon D configuration and at least some part of the molecule is cyclized. The physiological function of these substances is unknown. In the last few years the methods by which micro-organisms synthesize these unusual substances have interested many workers. Mach, Reich & Tatum (1963) and Mach & Tatum (1964) studied the synthesis of tyrocidine; the biosynthesis of polymyxin was studied by Paulus & Gray (1964); gramicidin S by Eikhom *et al.* (1963, 1964), Winnick, Lis & Winnick (1961); gramicidine by Okuda, Edwards & Winnick (1963); bacitracin by Bernlohr & Novelli (1963), Snoke (1961), Cornell & Snoke (1964), and Shimura, Sasaki & Sugawara (1964); mycobacillin by Banerjee & Bose (1964) and actinomycin by Katz (1960), Katz & Weissbach (1962), (1963) and Katz, Wise & Weissbach (1965). The conclusion reached by most of these workers is that polypeptide antibiotics are synthesized non-ribosomally; two recent studies with cell-free systems which synthesize gramicidin S further support this idea (Berg, Frøholm, & Laland, 1965; Yukioka *et al.* 1965).

However, Winnick and collaborators found that biosynthesis of gramicidin S,

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tyrocidine and gramicidin by whole organisms was prevented by inhibitors of protein synthesis (Winnick *et al.* 1961; Winnick & Winnick, 1961; Okuda *et al.* 1963; Uemura, Bodley, Adiga & Winnick, 1965). Later work with cell-free systems showed that ribosomes were essential for the synthetic process (Uemura, Okuda & Winnick, 1963; Bodley *et al.* 1964). Recently Winnick and co-workers isolated a gramicidin S 'messenger RNA' and determined some of its physical and chemical properties (Hall *et al.*, 1965; Sedat & Hall, 1965). These results suggest that gramicidin S is synthesized by a process similar to that of normal protein synthesis.

Synthesis of the polypeptide antibiotic nisin was of interest because it is not made by a Bacillus but by *Streptococcus lactis* (Mattick & Hirsch, 1947) and it appears to have an unusually large molecular weight (Chesseman & Berridge, 1959). Nisin is used in practice as a food preservative (Hawley, 1957; Gibbs & Hurst, 1964). The name 'nisin' describes a family of antibiotics, at least four of which have been partially characterized (Berridge, Newton & Abraham, 1952). Different strains of *S. lactis* are also known which produce different antibiotics (Hirsch & Grinsted, 1951; Hirsch, 1951*b*). The composition of nisin A was determined by Cheeseman & Berridge (1959); it contains 55 amino acid residues and the unusual amino acids lanthionine and β-methyllanthionine, but the optical configuration and sequence of amino acids are not known; it has no free terminal carboxyl or amino groups. Cheeseman & Berridge estimated the molecular weight of nisin A to be about 7000; this figure has recently been challenged by Bodansky & Perlman (1964). If the original estimate is correct, nisin, which is considered to be a polypeptide may be more complex than some of the small proteins, e.g. insulin, which has a molecular weight of 6000 (Sanger, 1959).

In the work described in this paper whole organisms of *Streptococcus lactis* were used to try to decide whether the synthesis of nisin is an enzymic or ribosomal process. The complex nutritional requirements and the vigorous acid production of this organism made it desirable to work with washed bacteria suspended in a reaction mixture of defined composition and, as far as possible, constant pH value. The effects produced by inhibitors of the synthesis of mucopeptides, protein, RNA and DNA (for review see Gale, 1963) suggest that the main mechanism concerned in nisin synthesis is similar to that of normal protein synthesis.

METHODS

Organisms. For nisin production *Streptococcus lactis* 354/07 (NCDO 497) was used. For nisin bioassay *S. cremoris* strain 1 P5 (NCDO 495) was used.

Media and culture conditions. The organisms were subcultured daily in a medium of the following composition (%, w/v): meat extract (Lemco), 1; yeast extract (Difco), 1; tryptone (Difco), 1; glucose, 1; NaCl, 0·5; Na_2HPO_4, 0·2; pH 7·0. Incubation was at 25° in deep tubes without shaking. Stock cultures were stored at 4° on slopes of the same medium with 1·5% (w/v) agar. New slopes were started at fortnightly intervals.

For preparing washed suspensions of *Streptococcus lactis* strain 354/07 the high nisin yielding medium of Hirsch (1951*a*) was used (medium 22). It contained (%, w/v): meat extract, 1; peptone (Evans), 1; glucose, 2·5; Na acetate, 1·5; Na citrate, 1·5; Na_2HPO_4, 0·5; and Ca pantothenate 1 μg./ml.

Chemicals. A.R. grade reagents were used. (U-^{14}C)-L-glutamic acid (6,4 mc/mM), (G-^{3}H)-DL-threonine (66 mc/mM and (5,6-^{3}H) uridine (22 c/mM) were purchased from the Radiochemical Centre, Amersham, Buckinghamshire. Penicillin, chloramphenicol, puromycin, terramycin and mitomycin were gifts from members of the department. Actinomycin D was a gift from Messrs Merck, Sharpe & Dohme, U.S.A.

Reaction mixture. The synthesis of nisin, incorporation of amino acids and protein synthesis were followed in a reaction mixture containing all the known growth requirements of *Streptococcus lactis*. This mixture contained amino acid mixture A (1·0 ml.), 10% (w/v) glucose (1·0 ml.), buffered salts solution B (1·0 ml.) purine + pyrimidine mixture C, (0·1 ml.), vitamin mixture D (0·1 ml.), tracer amino acids, bacteria equiv. 1 mg dry weight/ml. and inhibitors as required; total volume made to 10 ml. with distilled water. These various solutions had the following compositions. Amino acids, purines and pyrimidines were made up according to Gale & Folkes (1953). Amino acid mixture A contained each of the following at 2 mg./ml.: glycine, L-aspartic acid, tyrosine, tryptophan, phenylalanine, histidine, lysine, arginine, methionine proline, hydroxyproline, serine, cysteine, cystine, alanine, leucine, isoleucine and valine, the solution being adjusted to pH 7·0 with 2 N-NaOH. L-Glutamic acid and DL-threonine were made up as separate solutions at 2 mg./ml. and diluted 1/10 for use. (U-^{14}C)-L-glutamic acid was used at 50 μg./ml. with a specific activity of 2 μc/mg. (G-^{3}H)-DL-threonine was used at 100 μg./ml. with a specific activity of 30 μc/mg. Buffered salt solution B contained (%, w/v): 1, KH_2PO_4; 3·3, Na_2HPO_4; 1, NaCl; 0·7, $MgSO_4.7H_2O$; 15, trisodium citrate. Purine + pyrimidine mixture C contained adenine, xanthine, hypoxanthine, guanine, thymine, cytosine, uracil, each at 1 mg./ml. The vitamin mixture D was made up according to Niven (1944), and contained riboflavin, Ca-pantothenate, nicotinic acid, pyridoxine (each at 1 mg./ml.) thiamine (0·1 mg./ml.) and biotin 1 μg./ml.

Suspensions of organisms. Medium 22 was inoculated (10%, v/v) with an overnight culture of *Streptococcus lactis* 354/07, incubated at 30°, and the growth of the culture followed by its acid production. When the pH value had decreased to 6·0–5·8 (after about 6 hr) the organisms were harvested on the centrifuge, washed once with buffered salts solution B and finally suspended in distilled water at equiv. 10 mg. dry wt/ml.; 1·0 ml. of this suspension was used in 9 ml. reaction mixture. The dry weight of bacterial suspensions was estimated turbidimetrically with a Hilger spectrophotometer calibrated for the organism. Incubations were done at 30°.

Preparation of samples. For estimating nisin by bioassay the reaction was stopped by adding 10 N-HCl to bring the sample to pH 1·8–2·0; the tubes were then placed in a boiling-water bath for 5 min. and then centrifuged at 5000*g* for 10 min.; at pH 1·8–2·0 nisin is stable and remains entirely in the supernatant fluid (Hirsch, 1951*a*).

For estimating radioactivity and protein, the reaction was stopped by diluting with an equal vol. of cold 10% trichloroacetic acid (TCA).

For radioactivity estimations with a 'Panax' end-window Geiger counter (taking at least 300 counts) and the samples were prepared as described by Park & Hancock (1960). For estimations with a Nuclear Chicago liquid scintillation counter (System 724) TCA precipitates were filtered on 2-cm. membranes (Oxoid, London), washed

successively with 5 and 1 % acetic acid and finally with distilled water. The membranes were put into glass vials ($\frac{1}{2}$ in. $\times$ 2 in.), dried at 105° for 2 hr and then covered by 2·5 ml. toluene scintillation fluid (3·5 g. PPO, 2:5-diphenyloxazole, and 50 mg. POPOP, 1:4-bis-2-(4-methyl-5-phenyloxazolybenzene) in 1 l. toluene). The vials were then closed, placed in carrier bottles and counted to 10,000 counts or 4 min., whichever was quicker.

Fractionation of bacteria was done according to the method of Park & Hancock (1960).

Protein was estimated by the Lowry modification of the Folin & Ciocalteau method (Lowry *et al.* 1951).

Bioassay of nisin. The turbidimetric method of Berridge & Barrett (1952) modified to increase its sensitivity was used as the basis of the present method with which 1 mμg. nisin/ml. could be estimated with an accuracy of $\pm$15 %. Each result reported in this paper represents the average of at least three independent assays. The method was as follows.

Overnight cultures of the test organism *Streptococcus cremoris* (1 P5) were diluted with fresh medium and incubated at 30° for 1 hr. This step was repeated 2–3 times to get a vigorously growing culture; when a 1/10 dilution of such a culture had an extinction value of 0·1 at 600 mμ (in the Unicam S.P. 500 spectrophotometer) it was used to inoculate (1 %, v/v) a 1-l. volume of the same medium which was incubated for 1 hr at 30° (culture A). A volume (0·1 ml.) of sample containing nisin was added to 10 ml. of culture A and further dilutions in culture A were made as quickly as possible; the tubes were then incubated for 2$\frac{1}{2}$–3 hr at 30°. Growth was stopped by injecting into each tube 0·1 ml. of 0·004 % solution of thiomersalate (Berridge & Barrett, 1952). The extinction was measured in an S.P. 500 Unicam spectrophotometer at 600 mμ. Sterile precautions were unnecessary after the culture stages. A series of standards were set up in triplicate for each assay and values for the unknown samples were read from the curve. Figure 1 shows an example of the growth response curve obtained.

Nisin standard. A gift of purified nisin was received from Dr J. Tramer (United Dairies Central Laboratories, London). Dr B. Giles (Unilever Research Laboratory, Sharnbrook, Bedford) examined this material in a Spinco Analytical Ultracentrifuge Model E and reported that it behaved as a single substance. This purified nisin was further examined by high voltage paper electrophoresis (Shandon, London). Up to 1 mg. nisin/spot was used; the nisin spot did not give a ninhydrin reaction but the histidine residue which nisin contains was used to locate it with Pauly's reagent (Dent, 1947). The nisin spot was also located by bio-electrophoretograms. Neither test showed more than one spot. On the basis of the ultracentrifuge and electrophoretic data the sample was assumed to be pure nisin and the results of assays were expressed as μg./ml. in terms of this standard. The relationship of this material to the nisins A, B, C and D of Berridge *et al.* (1952) is not known. The nisin standard was dissolved at 100 μg./ml. in 0·05 N-HCl, dispensed in 0·1 ml. volumes in 15 ml. vials and stored frozen. For assay 10 ml. of 0·01 N-HCl was added to each vial and 0·1 ml. of the solution added to 10 ml. of culture A; further dilutions were then made. The first tube of the standard series thus contained 10 mμg. nisin/ml. and usually did not become turbid during the assay period.

Precautions. Many of the assays described below involved the estimation of nisin

in the presence of other antibiotics. Penicillin, oxamycin and terramycin introduced no complications since they were inactivated by the treatment (5 min. at 100° at pH 1·8–2·0) used for the extraction of nisin. Other antibiotics, particularly chloramphenicol, resisted this treatment and preliminary experiments were run to determine the maximum concentration at which these substances could be used without subsequently affecting the nisin assay. For example, the maximum permissible concentration of chloramphenicol in a reaction mixture was 20 μg./ml.

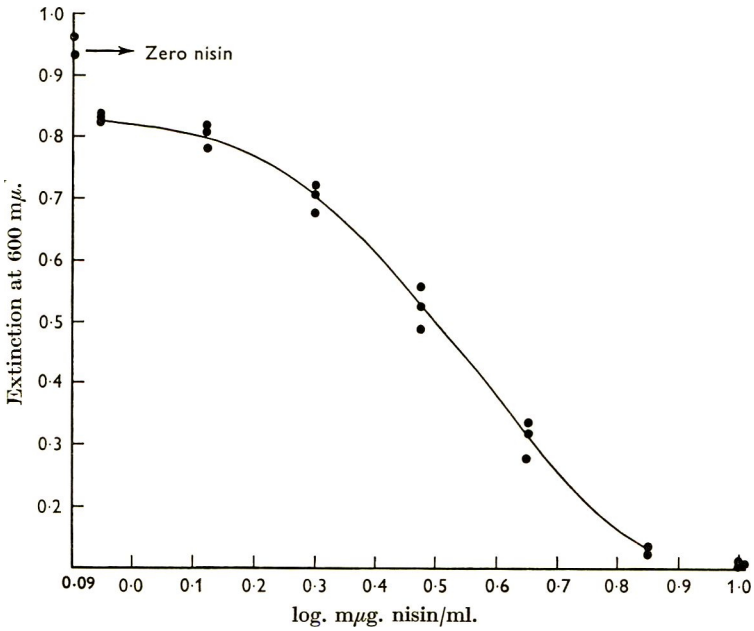

Fig. 1. Growth/response of standard in bioassay of nisin.

RESULTS

Time course of nisin synthesis

Washed *Streptococcus lactis* organisms grew slowly and synthesized nisin at an accelerating rate in the reaction mixture (Fig. 2). During the 120 min. experimental period the pH value remained above 6·0, dry wt. increased from 1·0 to 1·55 mg./ml., protein increased from 555 to 853 μg./ml., and the protein content of the bacteria remained approximately constant at 55%. The nisin content increased from about 1 μg./ml. to about 15 μg./ml. during the same period. The rates of protein synthesis and nisin synthesis were not linear; the rates increased particularly after 60 min. (Fig. 2). The total amount of nisin synthesized by suspensions of organisms prepared on different days varied considerably; at the end of the experimental period (120 min.) the nisin content ranged from 0·2 to 5% of the dry wt. of organism.

In another experiment a suspension of bacteria was divided into two equal volumes; one portion was incubated for 100 min. in the reaction mixture and then resuspended in fresh reaction mixture. Nisin synthesis and the incorporation of tritiated threonine of the pre-incubated and freshly harvested organisms were then

compared. The results (Fig. 3) showed that threonine was incorporated at the same rate by the two suspensions, but that nisin synthesis was more rapid in the pre-incubated bacteria and occurred without lag.

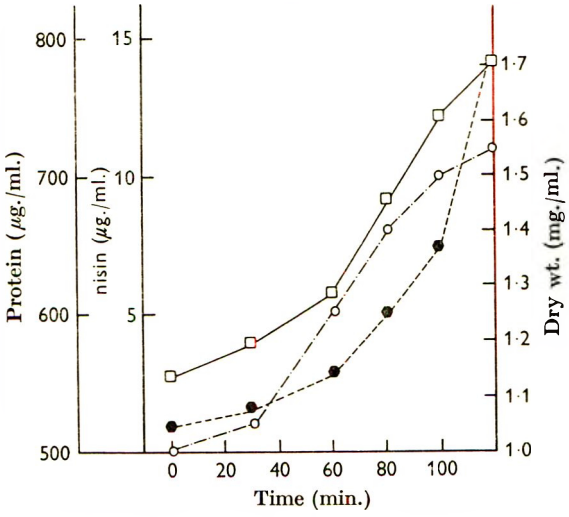

Fig. 2

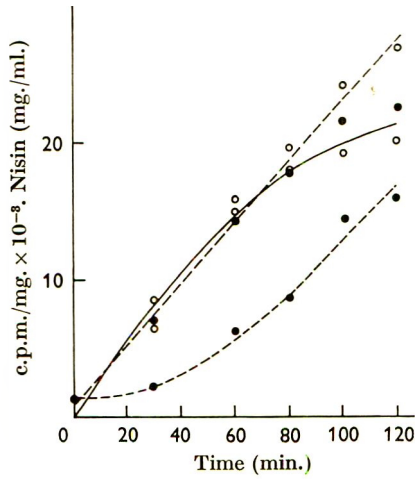

Fig. 3

Fig. 2. Changes in dry weight, protein and nisin synthesis of a washed suspension of *Streptococcus lactis* in the reaction mixture. Protein, □—; dry weight, ○–·–·; nisin, ●- - -.

Fig. 3. Nisin synthesis and incorporation of [G-^{3}H]-DL-threonine in freshly harvested and pre-incubated *Streptococcus lactis* organisms. Radioactivity, ——; nisin - - -; fresh organisms, ●; pre-incubated organisms, ○.

Incorporation of amino acids

The reaction mixture with organisms was incubated for 60 min. and the bacteria then fractionated by the method of Park & Hancock (1960). With (U-^{14}C)-L-glutamic acid 62% of the radioactivity was found in the fraction liberated by trypsin digestion; 28% was in the insoluble residue; and the remaining 10% was distributed between the nucleic acid and ethanol-soluble fractions. Glutamic acid does not specifically label the protein of *Streptococcus lactis* and was used only in early experiments on time-course studies with inhibitors of protein synthesis (e.g. as in Fig. 4). In later experiments protein was estimated by the Lowry *et al.* method or by incorporation of (G-^{3}H)-DL-threonine. Fractionation of bacteria incubated with labelled threonine showed that 88% of the radioactivity was in the trypsin-digestible fraction.

Effect of the antibiotics penicillin and cycloserine

Growing cultures of *Streptococcus lactis* are sensitive to penicillin; 0·05 μg./ml. causes inhibition in overnight growth tests (minimal inhibitory concentration, m.i.c., = 0·05 μg./ml.). Concentrations of penicillin up to 2 μg./ml. were without effect on nisin synthesis and incorporation of DL-threonine when tested in the reaction mixture; the time of addition of the antibiotic was also unimportant. Oxamycin which was tested up to 100 μg./ml. was also without effect.

Inhibitors of protein synthesis

Incorporation of (U-^{14}C)-L-glutamic acid and nisin synthesis were measured in time-course experiments, with and without inhibitors. Chloramphenicol has an m.i.c. of 5–10 μg./ml. and was tested at 5, 10 and 20 μg./ml. Results of a typical experiment in which chloramphenicol was added at time 0 or at 50 min. are shown in Fig. 4. Addition of chloramphenicol at time 0 caused a 94% inhibition of glutamic acid incorporation and 100% inhibition of nisin synthesis. When chloramphenicol was added at 50 min. further nisin synthesis was completely halted, but incorporation of glutamic acid continued at a slower rate. In similar experiments puromycin (m.i.c. about 30 μg./ml.) at 50 μg./ml. and terramycin (m.i.c. 0·5–1 μg./ml.) at 1 μg./ml. gave results similar to those obtained with chloramphenicol.

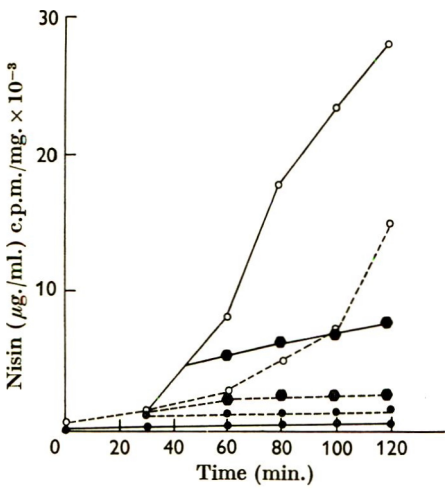

Fig. 4

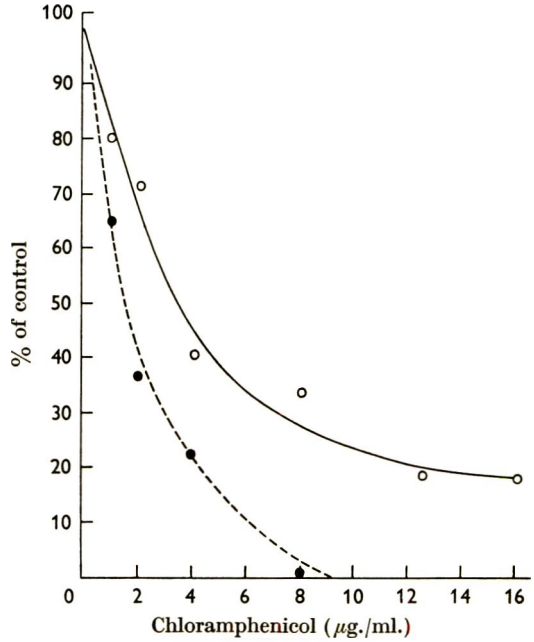

Fig. 5

Fig. 4. The effect of chloramphenicol (20 μg. ml.) on nisin synthesis and incorporation of [U-^{14}C]-L-glutamic acid by *Streptococcus lactis*. Radioactivity, ——; nisin; - - -; control, ○; chloramphenicol added at time zero, ●; at 50 min. ⬣.

Fig. 5. Protein and nisin synthesis by *Streptococcus lactis* in the presence of various concentrations of chloramphenicol, as % of control without antibiotic. Protein, ○—; nisin, ●- - -.

The controls shown in Figs. 2–4 all have the common feature that the rate of nisin synthesis increased after a lag of 30–60 min. To compare the action of antibiotics on protein and nisin synthesis, inhibitors were added after 60 min. to avoid this lag period: 20 ml. of the reaction mixture were incubated for 60 min. at 30° and then subdivided into 2 ml. portions containing a range of concentrations of inhibitors or with distilled water as a control. Incubation at 30° was continued for another hour and the protein (by method of Lowry *et al.*) and the nisin content

estimated. The increase in protein and nisin between 60 and 120 min. in the control tube was taken as 100% and the effect of inhibitors was expressed as a % of the control. Typical results obtained with the same three inhibitors are shown in Figs. 5, 6 and 7. In each case concentrations of inhibitor well below the m.i.c. were used. In all three cases the antibiotic was a more effective inhibitor of nisin synthesis than of protein synthesis. At an antibiotic concentration of one-tenth m.i.c. nisin synthesis was more sensitive than protein synthesis by a factor of 2 for chloramphenicol, 3·3 for terramycin and 10 for puromycin.

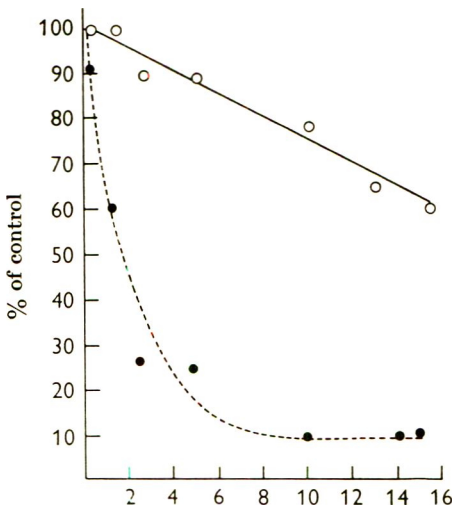

Fig. 6

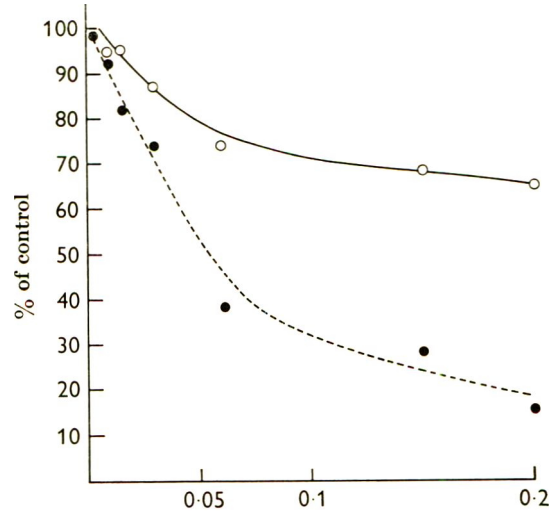

Fig. 7

Fig. 6. Protein and nisin synthesis by *Streptococcus lactis* in the presence of various concentrations of puromycin, as % of control without antibiotic. Protein, ○—; nisin, ●- - -.

Fig. 7. Protein and nisin synthesis of *S. lactis* in the presence of various concentrations of terramycin, as % of control without antibiotic. Protein, ○—, nisin, ●- - -.

Inhibitors of nucleic acid synthesis

Further time-course studies were made with mitomycin which inhibits DNA strand separation (Iyer & Szybalski, 1963). Nisin synthesis was insensitive to mitomycin (0·5 μg./ml.) although growth was inhibited by 0·03 μg./ml., the lowest concentration tested.

Actinomycin D, an inhibitor of RNA synthesis (Kirk, 1960; Hurwitz *et al.* 1962) was used at 10 μg./ml. In the 120 min. experimental period it had no effect on nisin synthesis when added at 60 min., but nisin synthesis was halted after 80 min. when the antibiotic was added at zero time. This experiment was repeated with tritiated uridine (1 μc./ml. reaction mixture) to assess the inhibition of RNA synthesis. In the first of such experiments (Fig. 8) the added isotope was all taken up by the control bacteria in the first 20 min. During the same period there was insignificant incorporation by the actinomycin-treated bacteria. Nisin synthesis in the control followed a linear course for 80 min., as in the previous experiment, and then increased. The actinomycin-treated culture synthesized nisin at a rate 60% that of

the control for 60 min., after which time nisin synthesis ceased altogether. In another similar experiment no nisin was synthesized during the first 40–60 min. and actinomycin completely blocked subsequent nisin synthesis; uptake of tritiated uridine was also inhibited (Fig. 9).

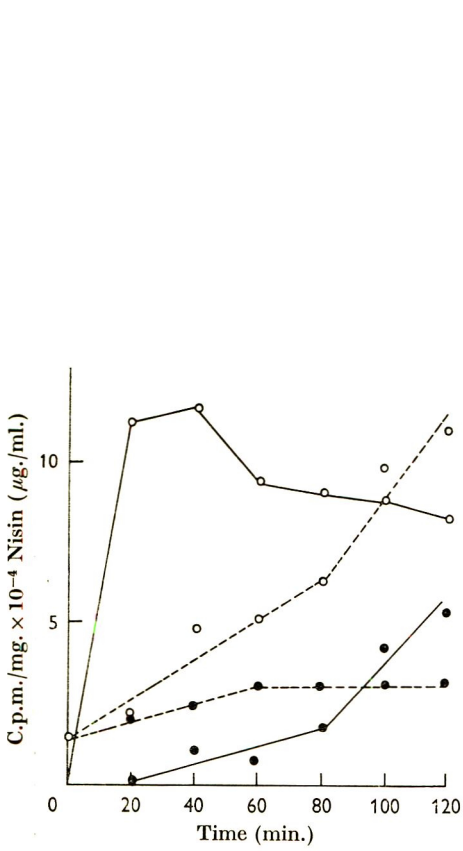

Fig. 8

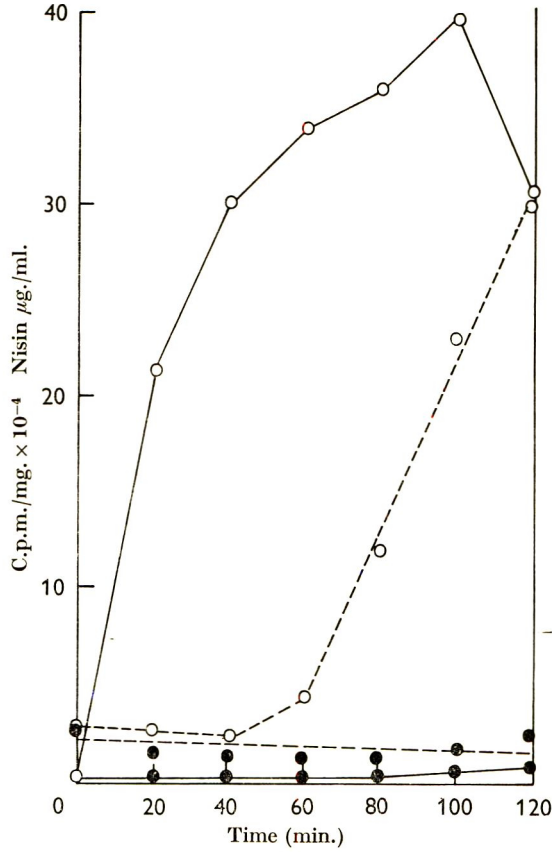

Fig. 9

Fig. 8. The effect of actinomycin D (10 μg./ml.) added at time 0 on nisin synthesis and incorporation of [5,6-^{3}H]-uridine by a suspension of *Streptococcus lactis* already synthesizing nisin. Radioactivity, —; nisin, - - -; control, ○; actinomycin-D treated, ●.

Fig. 9. As Fig. 8 but this suspension of *S. lactis* was not synthesizing nisin during the first 40 min. Radioactivity, —; nisin, - - -; control, ○, actinomycin-D treated, ●.

DISCUSSION

The evidence put forward in this paper favours the view that the major part of nisin synthesis occurs by a mechanism similar to that of normal protein synthesis. This view is based on the effect of inhibitors of protein synthesis (chloramphenicol, puromycin, terramycin) which also inhibited nisin synthesis. The effect observed might have been due either to nisin being synthesized ribosomally or to an indirect effect of the inhibitors on the formation of the enzymes concerned in nisin formation. In every case, therefore, the inhibitors were also tested under conditions when it

might be expected that the nisin-synthesizing enzymes were already formed. However, under these conditions there was also an immediate halting of nisin synthesis.

Actinomycin D when added early in the experiment inhibited RNA synthesis and nisin synthesis, suggesting that, as in the normal protein synthesis mechanism, messenger RNA is also involved in nisin synthesis. The results shown in Fig. 8 suggest that this RNA may have an unusually long half-life. The lack of effect of mitomycin suggests that the synthesis of nisin does not depend on newly formed DNA and that the code was already in the bacteria when they were harvested for experiment.

Penicillin did not inhibit nisin synthesis; cycloserine (oxamycin), an antibiotic known to interfere specifically with wall mucopeptide synthesis in *Staphylococcus aureus* (Strominger, 1962) was without effect on nisin synthesis. It does not appear likely that nisin is connected with the wall mucopeptide of the organism.

At present there is controversy about the mechanism of synthesis of gramicidin S which may be made ribosomally (Hall *et al.* 1965) or by some other means (Berg *et al.* 1965; Yukioka *et al.* 1965). However, the molecular weight of gramicidin S is only about 1350 according to Bodansky & Perlman (1964). A final proof that nisin is made ribosomally must await the development of a cell-free system derived from *Streptococcus lactis* as has been done with *Bacillus brevis* (Uemura *et al.* 1963; Okuda *et al.* 1964; Berg *et al.* 1965; Yukioka *et al.* 1965). The state of the organisms at the time of harvesting may be critical for preparations of this nature. For example, Fig. 3 here shows that freshly harvested bacteria and bacteria which had been incubated in the reaction mixture made protein at the same rate; the rate of nisin synthesis by the two suspensions was, however, very different. In the first 30 min. the pre-incubated bacteria made 6 μg. nisin/ml. reaction mixture and the freshly harvested bacteria made 1·2 μg. nisin/ml. reaction mixture. There is no simple explanation for this observation. It is also difficult to understand why, if nisin is made as other proteins on ribosomes, its synthesis should be more sensitive to chloramphenicol, puromycin and terramycin than other proteins (see, for example, Figs. 5, 6, 7). However, similar results have already been reported, for example, the preferential inhibition of β-galactosidase synthesis in *Escherichia coli* by chloramphenicol (Sypherd & Strauss, 1963) and puromycin (Sells, 1965) and of ornithine transcarbamylase synthesis by chloramphenicol (Browne & Rogers, 1963).

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REFERENCES

BANERJEE, A. B. & BOSE, S. K. (1964). Biosynthesis of mycobacillin, a new antifungal peptide. *J. Bact.* **87**, 1397.

BERG, T. L. FROHOLM, L. O. & LALAND, S. G. (1965). The biosynthesis of gramicidin S in a cell-free system. *Biochem. J.* **96**, 43.

BERNLOHR, R. W. & NOVELLI, G. D. (1963). Bacitracin biosynthesis and spore formation: The physiological role of an antibiotic. *Arch. Biochim. Biophys.* **103**, 94.

BERRIDGE, N. J. & BARRETT, J. (1952). A rapid method for the turbidimetric assay of antibiotics. *J. gen. Microbiol.* **6**, 14.

BERRIDGE, N. J. & NEWTON, G. G. F. & ABRAHAM, E. P. (1952). Purification and nature of the antibiotic nisin. *Biochem. J.* **52**, 529.

BODANSKY, M. & PERLMAN, D. (1964). Are peptide antibiotics small proteins? *Nature, Lond.* **204**, 840.

BODLEY, J. W., UEMURA, I., ADIGA, P. R., OKUDA, K. & WINNICK, T. (1964). Participation of ribosomes in the biosynthesis of gramicidins and tyrocidines. *Biochemistry*, **3**, 1492.

BROWNE, S. W. & ROGERS, P. (1963). Accumulation of repressor for ornithine transcarbamylase synthesis in *Escherichia coli* mediated by chloramphenicol. *Biochim. Biophys. Acta*, **76**, 600.

CHEESEMAN, G. C. & BERRIDGE, N. J. (1959). Observations on the molecular weight and chemical composition of nisin A. *Biochem. J.* **71**, 185.

CORNELL, N. & SNOKE, J. E. (1964). Biosynthesis of bacitracin and protein. *Biochim. Biophys. Acta*, **91**, 533.

DENT, C. E. (1947). The amino-aciduria in Fanconi syndrome. *Biochem. J.* **41**, 240.

EIKHOM, T. S., JONSEN, J., LALAND, S. & REFSVIK, T. (1963). On the biosynthesis of gramicidin S. *Biochim. Biophys. Acta*, **76**, 465.

EIKHOM, T. S., JONSEN, J., LALAND, S. & REFSVIK, T. (1964). Studies on the biosynthesis of gramicidin S in whole cells of *Bacillus brevis*. *Biochim. Biophys. Acta*, **80**, 648.

GALE, E. F. (1963). Mechanism of antibiotic action. *Pharmacol. Rev.* **15**, 481.

GALE, E. F. & FOLKES, J. P. (1953). The assimilation of amino-acids by bacteria. 14. Nucleic acid and protein synthesis in *Staphylococcus aureus*. *Biochem. J.* **53**, 483.

GIBBS, B. M. & HURST, A. (1964). Limitations of nisin as a preservative in non-dairy foods. In *Microbial Inhibitors in Food* ed. by N. Molin, p. 151. Stockholm: Almqvist and Wiksell.

HALL, J. B., SEDAT, J. W., ADIGA, P. R., UEMURA, I. & WINNICK, T. (1965). Gramicidin S messenger RNA. I. Isolation and characterization. *J. mol. Biol.* **12**, 162.

HAWLEY, H. B. (1957). Nisin in food technology. *Fd Mf.* **32**, 430.

HIRSCH, A. (1951*a*). Growth and nisin production of a strain of *Streptococcus lactis*. *J. gen. Microbiol.* **5**, 208.

HIRSCH, A. (1951*b*). Various antibiotics from one strain of *Streptococcus lactis*. *Nature, Lond.* **167**, 1031.

HIRSCH, A. & GRINSTED, E. (1951). The differentiation of the lactic streptococci and their antibiotics. *J. Dairy Res.* **18**, 198.

HUROWITZ, J., FURTH, J. J., MALAMY, M. & ALEXANDER, M. (1962). The role of deoxyribonucleic acid in ribonucleic acid synthesis. III. The inhibition of enzymatic synthesis of ribonucleic acid and deoxyribonucleic acid by Actinomycin D and proflavin. *Proc. natn. Acad. Sci. U.S.A.* **48**, 1222.

IYER, V. N. & SZYBALSKI, W. (1963). A molecular mechanism for mitomycin action: linking of complementary DNA strands. *Proc. natn. Acad. Sci. U.S.A.* **50**, 355.

KATZ, E. (1960). Biogenesis of the actinomycins. *Ann. N.Y. Acad. Sci.* **89**, 304.

KATZ, E. & WEISSBACH, H. (1962). Effect of chloromycetin and penicillin on the incorporation of amino-acids into actinomycin and protein by *Streptomyces antibioticus*. *Biochem. Biophys. Res. Com.* **8**, 186.

KATZ, E. & WEISSBACH, H. (1963). Incorporation of 'C' labelled amino-acids into actinomycin and protein by *Streptomyces antibioticus*. *J. biol. Chem.* **238**, 666.

KATZ, E., WISE, M. & WEISSBACH, H. (1965). Actinomycin biosynthesis. Differential effect of chloramphenicol on protein and peptide antibiotic synthesis. *J. biol. Chem.* **240**, 3071.

KIRK, J. M. (1960). The mode of action of actinomycin D. *Biochim. Biophys. Acta*, **42**, 167.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265.

MACH, B., REICH, E. & TATUM, E. L. (1963). Separation of the biosynthesis of the antibiotic polypeptide tyrocidine from protein biosynthesis. *Proc. natn. Acad. Sci. U.S.A.* **50**, 175.

MACH, B. & TATUM, E. L. (1964). Environmental control of amino acid substitutions in the biosynthesis of the antibiotic polypeptide tyrocidine. *Proc. natn. Acad. Sci. U.S.A.* **52**, 876.

MATTICK, A. T. R. & HIRSCH, A. (1947). Further observation on an inhibitory substance (Nisin) from lactic streptococci. *Lancet*, ii, 5.

NIVEN, C. F. JUN. (1944). Nutrition of *Streptococcus lactis*. *J. Bact.* **47**, 343.

OKUDA, K., EDWARDS, G. C., & WINNICK T. (1963). Biosynthesis of gramicidin and tyrocidine in the Dubos strain of *Bacillus brevis*. *J. Bact.* **85**, 329.

OKUDA, K., UEMURA, I., BODLEY, J. W. & WINNICK, T. (1964). Further aspects of gramicidin and tyrocidine biosynthesis in the cell-free system of *Bacillus brevis*. *Biochemistry*, **3**, 108.

PARK, J. T. & HANCOCK, R. (1960). A fractionation procedure for studies of the synthesis of cell-wall mucopeptide and of other polymers in cells of *Staphylococcus aureus*. *J. gen. Microbiol.* **22**, 249.

PAULUS, H. & GRAY, E. (1964). The biosynthesis of polymixin B by growing cultures of *Bacillus polymyxa*. *J. biol. Chem.* **239**, 865.

SANGER, F. (1959). Chemistry of insulin. *Science*, **129**, 1340.

SEDAT, J. W. & HALL, J. W. (1965). Gramicidin S messenger RNA. II. Physical and chemical properties. *J. mol. Biol.* **12**, 174.

SELLS, B. H. (1965). Puromycin: Effect on messenger RNA synthesis and β-galactosidase formation in *Escherichia coli* 15T. *Science*, **148**, 371.

SHIMURA, K., SASAKI, T. & SUGAWARA, K. (1964). Biosynthesis of bacitracin, I. Formation of bacitracin by a subcellular fraction of *Bacillus licheniformis*. *Biochim. Biophys. Acta*, **86**, 46.

SNOKE, J. E. (1961). Formation of bacitracin by protoplasts of *Bacillus licheniformis*. *J. Bact.* **81**, 987.

STROMINGER, J. L. (1962). Biosynthesis of bacterial cell walls. *Fed. Proc.* **21**, 134.

SYPHERD, P. S. & STRAUSS, N. (1963). Chloramphenicol promoted repression of β-galactosidase synthesis in *Escherichia coli*. *Proc. natn. Acad. Sci. U.S.A.* **49**, 400.

UEMURA, I., OKUDA, K. & WINNICK, T., (1963). Biosynthesis of gramicidins and tyrocidines in cell-free preparations from *Bacillus brevis*. *Biochemistry*, **2**, 719.

UEMURA, I., BODLEY, J. W., ADIGA, R. P. & WINNICK, T. (1965). Effect of antibiotics and ribonuclease on polypeptide and protein synthesis in different strains of *Bacillus brevis*. *Biochim. Biophys. Acta*, **95**, 86.

WINNICK, R. E. & WINNICK, T. (1961). Biosynthesis of gramicidin S. II. Incorporation experiments with labelled amino-acids analogues, and the amino-acid activation process. *Biochim. Biophys. Acta*, **53**, 461.

WINNICK, R. E., LIS, H. & WINNICK, T. (1961). Biosynthesis of gramicidin S. I. General characteristics of the process in growing cultures of *Bacillus brevis*. *Biochim. Biophys. Acta*, **49**, 451.

YUKIOKA, M., TSUKAMOTO, Y., YOSHITAKA, S., TAKITARO, T., SHUZO, O. & SHOHEI, O. (1965). Biosynthesis of gramicidin S by a cell-free system of *Bacillus brevis*. *Biochim. Biophys. Res. Com.* **19**, 204.

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Metabolism of o-Cresol by *Pseudomonas aeruginosa* strain T1

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SUMMARY

There is evidence that oxidation of *o*-cresol by *Pseudomonas aeruginosa* proceeds through 3-methylcatechol and 2-hydroxy-6-oxohepta-2,4-dienoic acid. 3-Methylcatechol has been characterized as a metabolite in growing cultures, and is oxidized by cells or cell extracts obtained from cultures grown on *o*-cresol but not by those grown on glucose. The rates of dissimilation of 2-hydroxy-6-oxohepta-2,4-dienoic acid are in excess of its rates of formation from 3-methylcatechol by extracts of cells. The catechol 2,3-oxygenase is formed in response to a variety of inducers and will cleave the ring of catechol and 4-methylcatechol also. The low specificity of the 2,3-oxygenase and earlier enzymic activities (hydroxylases), to both inducers and substrates, is discussed.

INTRODUCTION

Some bacteria metabolize methyl substituted benzenoid compounds by oxidation of this side chain, eventually, to a carboxyl group. This is then either lost as carbon dioxide and replaced by a hydroxyl group or retained until fission of the 'benzoic acid' formed occurs. The best documented example is the oxidation of *p*-cresol to *p*-hydroxybenzoate by the pseudomonad of Dagley & Patel (1957). They further showed that 2,4- and 3,4-xylenol were utilized by oxidation of the methyl group *para* to the phenolic function; this occurred also with the pseudomonad of Leibnitz, Behrens, Streigler & Gabert (1962). The oxidation of toluene by *Pseudomonas aeruginosa* also proceeds by oxidation of the methyl group (Kitagawa, 1956). On the other hand, Rogoff & Wender (1957) showed that 1-methyl- and 2-methylnaphthalene were metabolized without attack on the side chain and that 3-methyl- and 4-methylcatechol respectively appeared as products suggesting that the unsubstituted ring was cleaved first. 3-Methylcatechol has also been implicated as a bacterial metabolite of *m*-toluate (Ichihara, Adachi, Hosokawa & Takeda, 1962) and shown to undergo fission to 2-methylmuconate by a purified preparation of pyrocatechase from *Brevibacterium fuscum* (Nakagawa, Inoue & Takeda, 1963). This enzyme preparation will also oxidize 4-methylcatechol, and the u.v. characteristics of the product suggest that this is 3-methylmuconate. An alternative cleavage of 4-methylcatechol was suggested from the spectral data obtained by Dagley, Evans

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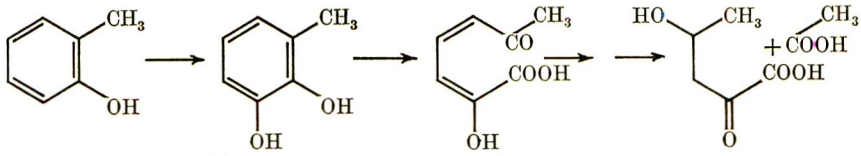
& Ribbons (1960), and this has been amply confirmed by Dagley, Chapman, Gibson & Wood (1964) who found that the 2-hydroxy-5-methylmuconic semialdehyde was the product. When, however, 4-methylcatechol was oxidized by the pseudomonad of Dagley & Patel (1957), grown on *p*-cresol, protocatechate was formed.

It was suggested in a previous report that *o*-cresol was not metabolized via salicylate and catechol since *Pseudomonas aeruginosa* strain T1, grown on *o*-cresol or on several other aromatic compounds, produced a non-specific catecho 2,3-oxygenase which cleaved the ring of 3-methylcatechol to yield keto-acids (Ribbons 1964). One mole of O_2 was consumed for each mole of 3-methylcatechol metabolized and CO_2 was not evolved. The details of the site of ring cleavage of several catechols, including 3-methylcatechol, have recently been elucidated and the subsequent route of degradation of the products of cleavage to pyruvate and other substances ascertained (Dagley *et al.* 1964). These observations are consistent with, but do not unequivocally establish 3-methylcatechol as an intermediary metabolite of *o*-cresol metabolism. Results reported here suggests that the reaction sequence, shown in Scheme 1, is responsible for the oxidation of *o*-cresol by *P. aeruginosa* T1.



o-Cresol — 3-Methylcatechol — 2-Hydroxy-6-oxohepta-2,4-dienoic acid — 4-Hydroxy-2-oxovaleric acid

Scheme 1

METHODS

Organisms. The strain used was *Pseudomonas aeruginosa* T1 and was kindly provided by Miss E. Pankhurst (Pankhurst, 1959). It was maintained on slopes of Lab-Lemco agar and subcultured every 6 weeks. Stock cultures were maintained at 4°. *Pseudomonas desmolyticum* (NCIB 8859) was maintained similarly.

Media. Cultures were grown on minimal salt media made by mixing sterile solutions (*a*) and (*b*) [3:2 (v/v)]. Solution (*a*) adjusted to pH 7·0 with NaOH contained (g./l.): KH_2PO_4, 9·0; $(NH_4)_2SO_4$, 2·0; solution (*b*) contained the carbon source and a trace element mixture (2·5 ml./l.). The final concentration of the carbon source was 0·1% when phenol, glucose or benzoate (neutralized to pH 7·0 with NaOH) were used and 0·05% when the cresols were used. The trace element mixture contained (g./l.): MgO, 11·12; $CaCO_3$, 2·0; ZnO, 0·41; $FeCl_3$, $6H_2O$, 5·4; $MnCl_2$, $4H_2O$, 0·99; $CuCl_2$, $2H_2O$, 0·17, $CoCl_2$, $6H_2O$, 0·24; H_3BO_3, 0·062; and concentrated HCl (58 ml./l.). Cultures of the organism were routinely subcultured in the specific liquid media (12·5 ml. in 50 ml. conical flasks), containing one of the carbon sources, every 2–3 weeks. *Pseudomonas desmolyticum* was grown on media of the same compositions except that naphthalene was added as carbon source (0·5 g./l.).

Growth and harvest. Inocula for experimental purposes were taken from liquid cultures containing a specific carbon source. A 24-hr culture (12·5 ml.) was used to inoculate 250 ml. of similar media contained in a 1 l. flask. Cultures were harvested

after overnight growth (16–17 hr) by centrifugation at approximately 20,000*g* for 1–2 min. at room temperature in a Servall SS-1 bench centrifuge. The pellets were washed twice, resuspended in 0·067 M-phosphate buffer, pH 7·1, filtered through glass wool and diluted to give a bacteria density of approximately 1·5–3·0 mg. dry wt./ml. Larger quantities of pseudomonads were obtained from 15 l. cultures in 20 l. Pyrex bottles fitted with spargers (porosity 1) for forced aeration. These were harvested on an air-turbine-driven Sharples supercentrifuge. These organisms were normally washed with water and stored as a pellet at −14° until required for the preparation of extracts. All cultures were grown at 30°.

Dry weight of bacteria. The extinction values of bacterial suspensions were measured at 570 mμ with a Unicam SP 600 spectrophotometer. A standard curve relating bacterial dry weight (dried at 105° for 24 hr) to extinction was linear up to $E_{570}=0{\cdot}6$ when the bacterial concentration was 325 μg. dry wt./ml.

Cell-free extracts. Twice washed organisms were disrupted with an M.S.E. ultrasonic disintegrator (Model 60W) for 4 min. below 5°. Bacterial debris was removed by centrifugation at 20,000*g*, for 15–20 min. at 0° (crude extract) and high speed supernatants were obtained after centrifugation at 109,000*g* for 90 min. in an M.S.E. 50 ultracentrifuge.

Gas exchange. Oxygen consumption and carbon dioxide evolution were measured by the direct method in constant volume respirometers at 30° (Umbreit, Burris & Stauffer, 1957). Dissolved oxygen was measured with a Clark oxygen electrode (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio, U.S.A.).

Spectrophotometric determinations. U.v. and visible spectra were plotted with the Beckman DB or Unicam SP 800 recording spectrophotometers. Assays of enzymic activity were conducted at 30° in both instruments. Infra-red spectra were obtained with a Perkin–Elmer Infracord.

Chemical estimations. Catechols were detected and estimated by the Evans (1947) test. Keto-acids were detected by preparation of their acidic 2,4-dinitrophenylhydrazones, the extraction of these into ethyl acetate and then into sodium carbonate solution (Friedemann & Haugen, 1943).

Chromatography. Thin-layer plates were prepared with Silica gel G (Merck) and developed with the following solvents: (*a*) benzene + acetic acid + water (80 + 20 + satd); (*b*) ether + light petroleum, boiling range 60–80° (7 + 3); (*c*) acetone + light petroleum, boiling range 60–80° (2 + 5); (*d*) xylene + chloroform (1 + 1). *o*-Cresol and 3-methylcatechol were detected in chromatograms by coupling with diazotized *p*-nitroaniline.

Buffers. 0·067 M-Phosphate buffer was prepared from KH_2PO_4 (9 g./l.) and neutralized to the desired pH value with 5 N-NaOH solution.

RESULTS

Figure 1 demonstrates the ability of whole organisms to oxidize a variety of aromatic compounds after growth on glucose or *o*-cresol. Catechol, 3-methylcatechol and 4-methylcatechol were all oxidized without lag after growth on *o*-cresol. Periods of adaptation to these substrates were apparent when the bacteria were grown on glucose. The total oxygen consumption during oxidation of 3-methylcatechol by whole organisms was generally greater than that observed during the oxidation of

catechol or 4-methylcatechol, by 0·5–1·0 mole oxygen per mole substrate. The reasons for this are not known. This observation is not a peculiarity of *o*-cresol grown organisms where 3-methylcatechol is a metabolite, but may be shown with organisms grown on phenol or *p*-cresol (amongst many other growth substrates) where 3-methylcatechol is unlikely to be an intermediate.

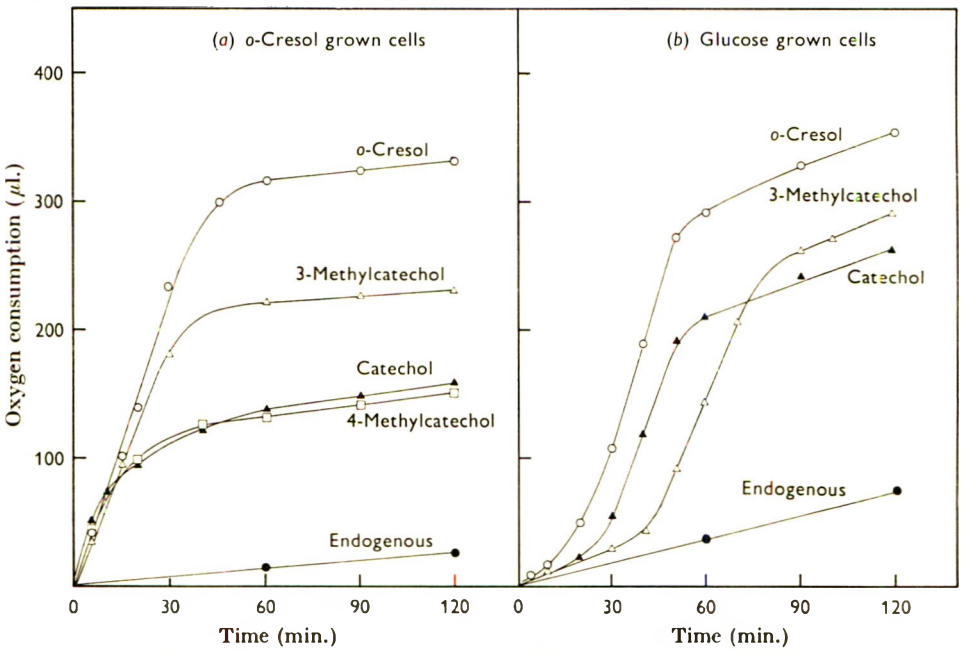


Fig. 1. Each Warburg flask contained 1·7 ml. of 0·067 M-phosphate buffer, (pH 7·0) and 1 ml. of cell suspension containing: (*a*) equiv. 1·71 mg. dry wt. bacteria, and (*b*) equiv. 3·02 mg. dry wt. bacteria; 0·1 ml. of a 25 mM substrate solution, or water was tipped in from the side-arm; the centre well contained 0·2 ml. of 20 % (w/v) KOH.

Experiments with whole organisms quickly led to the conclusion that enzymes induced by growth with specific aromatic compounds were also able to metabolize a variety of other, though related, aromatic structures. Thus it is shown in Table 1 that it was immaterial which of the four aromatic substances (*o*-, *m*- or *p*-cresol or phenol) was the inducer during growth; the induced organisms oxidized all the aromatic substances except benzoate without a lag period. On the other hand, organisms previously grown on glucose metabolized the aromatic substances only after a lag period, albeit short in some instances. Organisms obtained after growth on benzoate rapidly oxidized the catechols without lag; the response to *o*- and *m*-cresol was difficult to evaluate as very short periods of adaptation (3–10 min.) occurred; *p*-cresol and phenol oxidations by these organisms were, however, preceded by a distinct period of induction. The patterns of oxidation of aromatic substances by the enzymes induced in washed suspensions of bacteria harvested from glucose media, by exposure of these to aromatic substances, were similar to those shown in Table 1. A distinction therefore between possible pathways of *o*-cresol metabolism, via salicylate and catechol or via 3-methylcatechol, was impossible on the basis of differences in oxygen consumption. The inability of

salicylate and 2,3-dihydroxybenzoate to increase the rates of oxygen consumption beyond the endogenous rate may have been due to permeability barriers. Therefore direct demonstration of the formation of 3-methylcatechol from *o*-cresol was sought by the isolation of 3-methylcatechol.

A substance containing the *o*-dihydroxy groups (positive reactions in the Evans, 1947, test) frequently appears and later disappears in cultures containing *o*-cresol as sole source of organic carbon. Media which were not supplemented with Fe produced larger amounts of this catechol compound. This material was isolated and identified by the following procedures.

Table 1. *Oxidation of substrates by Pseudomonas aeruginosa with or without a lag period, after induction by growth*

Compounds tested as substrates	Growth substrates (inducers)					
	o-Cresol	*m*-Cresol	*p*-Cresol	Phenol	Benzoate	Glucose
o-Cresol	+	+	+	+	?	–
m-Cresol	+	+	+	+	?	–
p-Cresol	+	+	+	+	–	–
Phenol	+	+	+	+	–	–
Benzoate	–	–	–	–	+	–
Glucose	NT	NT	NT	NT	NT	+
Catechol	+	+	+	+	+	–
3-Methylcatechol	+	+	+	+	+	–
4-Methylcatechol	+	+	+	+	+	–

+, substrate oxidized without a lag period.
–, substrate oxidized with a lag period.
?, not evaluated; induction of enzymes to these substrates is often too rapid to demonstrate significant lag periods.
NT, not tested.

A 20 l. culture of *Pseudomonas aeruginosa* T1 containing approximately 160 mg. of the catechol was clarified at 60,000*g* and concentrated to approximately 500 ml. under reduced pressure, between 30 and 37°. The concentrate (pH 6·9) was extracted three times with an equal volume of ether. The ether extracts were dried over sodium sulphate and distilled to dryness, yielding a crystalline material. Thin-layer chromatography revealed two distinct zones which had the R_F values of *o*-cresol and 3-methylcatechol in four solvents (see Experimental). The *o*-cresol residue was removed by steam distillation and the dark brown residue dried under vacuum. After recrystallization from water it had a m.p. 66–68° which was not depressed when mixed with an authentic specimen of 3-methylcatechol. The product analysed C, 67·4; H, 4·73; (calculated for $C_7H_8O_2$, C, 67·8; H, 4·84). In addition, its ultraviolet and infrared spectra (Fig. 2 and 3), and the spectrum of the product of reaction in the Evans test, were in accord with authentic samples of 3-methylcatechol and unlike data obtained for catechol itself.

Extracts of organisms induced to metabolize *o*-cresol rapidly oxidized catechol, 3-methylcatechol and 4-methylcatechol with the consumption of 1 mole O_2/mole substrate (Table 2; the extra oxygen consumption by whole bacteria exposed to 3-methylcatechol was not observed with extracts). For catechol and 4-methylcatechol, yellow keto acids appeared with $\lambda_{max.}$ 375 and 379 mμ respectively, and later disappeared without further oxygen consumption or carbon dioxide evolution.

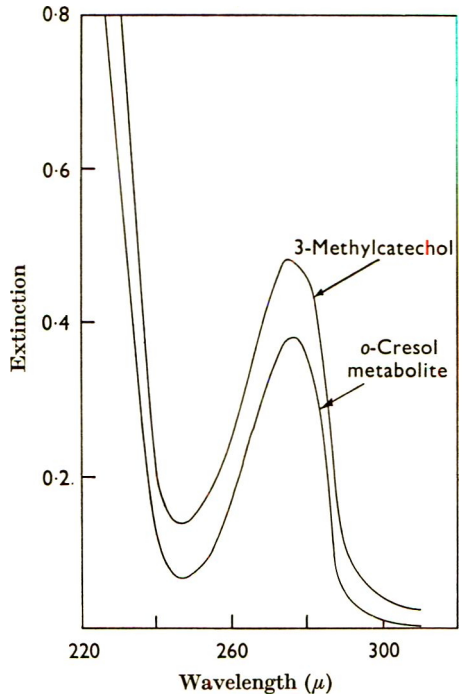


Fig. 2. Ultraviolet spectra of *o*-cresol metabolite and 3-methylcatechol in ethanol.

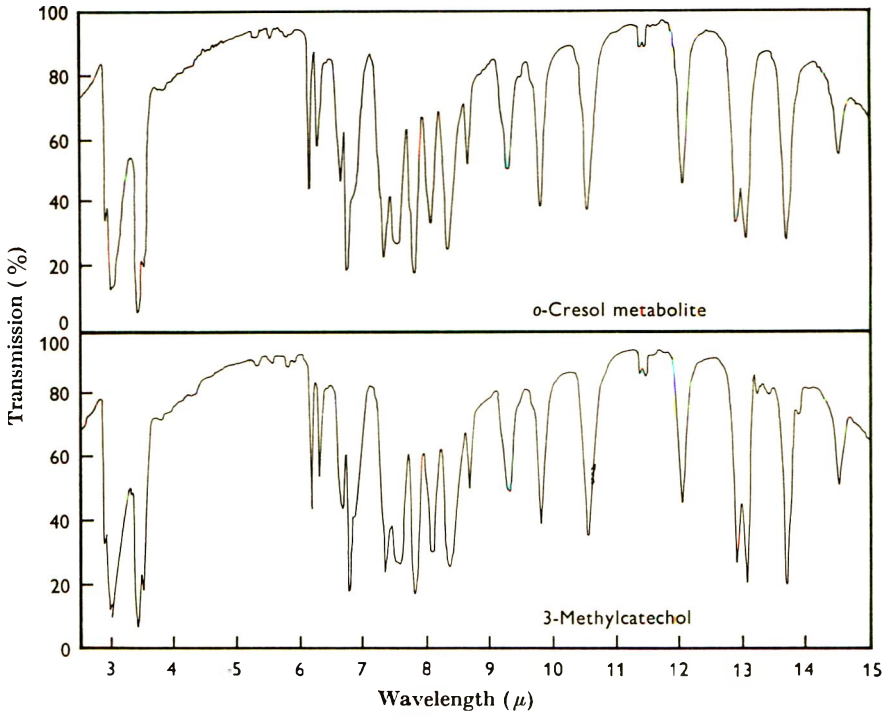


Fig. 3. Infrared spectra of *o*-cresol metabolite and 3-methylcatechol. Mulls in liquid paraffin B.P. were pressed between KBr discs and scanned with a Perkin–Elmer Infracord.

However, no coloured product appeared during 3-methylcatechol dissimilation by extracts. The presence of a catechol 2,3-oxygenase in these extracts makes the possibility of cleavage of 3-methylcatechol between positions 1 and 2, to form a colourless muconic acid, unlikely. The following experiments support this view. Cell-free extracts from *Pseudomonas desmolyticum* grown on naphthalene contain catechol 2,3-oxygenase which will oxidize catechol, and the two methyl substituted catechols with the intermediary formation of the yellow keto acids in all three cases. The product from 3-methylcatechol, 2-hydroxy-6-oxohepta-2,4-dienoic acid, which

Table 2. *Gaseous exchange during metabolism of catechol, 3-methylcatechol and 4-methylcatechol by cell suspensions and cell free extracts*

Preparation used	Substrate (μmoles)		Oxygen consumed (μmoles)	Carbon dioxide evolved (μmoles)
Cell suspension*	None		2·5	—
	Catechol	2·5	8·2	—
	3-Methylcatechol	2·5	11·8	—
	4-Methylcatechol	2·5	7·9	—
Cell free extract*	None	0·1	0·2	
	Catechol	10	9·8	0·25
	3-Methylcatechol	10	9·5	0·1
	4-Methylcatechol	10	9·6	0·34
Cell free extract†	None		0	—
	Catechol	0·5	0·49	—
		1·0	1·0	—
	3-Methylcatechol	0·5	0·49	—
		1·0	0·99	—
	4-Methylcatechol	0·5	0·5	—
		0·75	0·75	—

— Not measured; * Oxygen consumption measured manometrically; † oxygen consumption measured with an O_2 electrode.

absorbs maximally at 386 mμ at pH values greater than 7·2, is then metabolized slowly to acetate and to 4-hydroxy-2-oxovalerate (Dagley *et al.* 1964). Extracts of *Pseudomonas aeruginosa* T1, however, rapidly affected the latter reaction (Fig. 4). The product of 3-methylcatechol cleavage by extracts of an orcinol-grown pseudomonad was also degraded rapidly by extracts of *P. aeruginosa* T1. The failure to observe 2-hydroxy-6-oxohepta-2,4-dienoic acid as an intermediate of 3-methylcatechol oxidation is therefore not surprising since kinetic studies have shown that *P. aeruginosa* T1 extracts are able to degrade the intermediate at rates faster than it is formed (Table 3).

DISCUSSION

The pathway of metabolism of *o*-cresol by *Pseudomonas aeruginosa* T1 most probably involves hydroxylation of the nucleus, ring-fission of the 3-methylcatechol and subsequent fissions of the carbon chain by the pattern recently disclosed by the experiments of Dagley *et al.* (1964). It is worth examining the criteria used to establish that 3-methylcatechol is an intermediate: (1) whole bacteria oxidize this substrate without lag after growth on *o*-cresol but not after growth on glucose; (2) extracts of these bacteria oxidize 3-methylcatechol and further metabolize the products by established mechanisms; (3) 3-methylcatechol has been isolated as a

transient metabolite in *o*-cresol culture filtrates. Conclusions often drawn from the data supplied by methods (1) and (2) would establish 3-methylcatechol as a metabolite *o*-cresol. However, both catechol and 4-methylcatechol are metabolized by the *o*-cresol induced bacteria and extracts derived from them—facts which cast doubt on the validity of the conclusion that 3-methylcatechol is a metabolite of *o*-cresol.

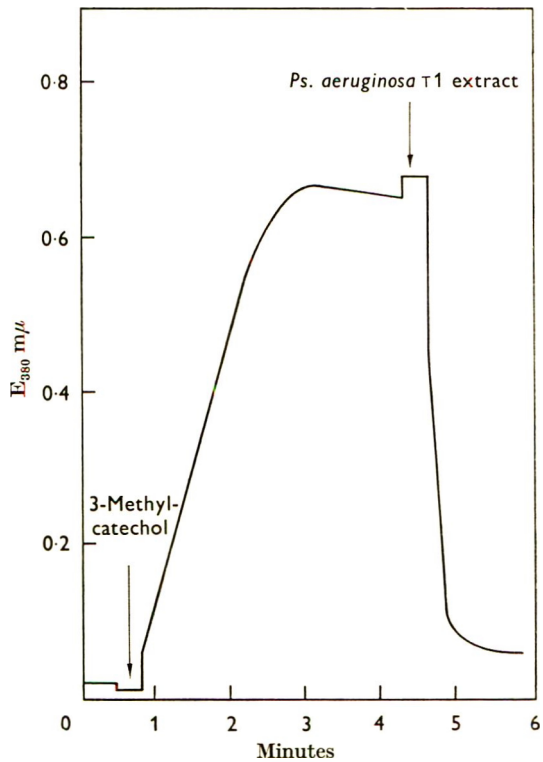


Fig. 4. Degradation of 2-hydroxy-6-oxohepta-2,4-dienoic acid by extracts of *P. aeruginosa* T1. The product of 3-methylcatechol cleavage was generated with extracts of naphthalene grown *P. desmolyticum*. The cuvette contained; 0·067 M-phosphate buffer, pH 6·9, (2·5 ml.); extract from *P. desmolyticum* (20 μl., 300 μg. protein); 10 μl. of 25 mM solution of 3-methylcatechol were added at the first arrow. The extract of *P. aeruginosa* T1 was added at the second arrow (10 μl., 120 μg. protein). Temperature 30°.

Table 3. *Rates of formation and dissimilation of the products of ring cleavage of catechol, 3-methylcatechol and 4-methylcatechol by extracts of Pseudomonas aeruginosa T1*

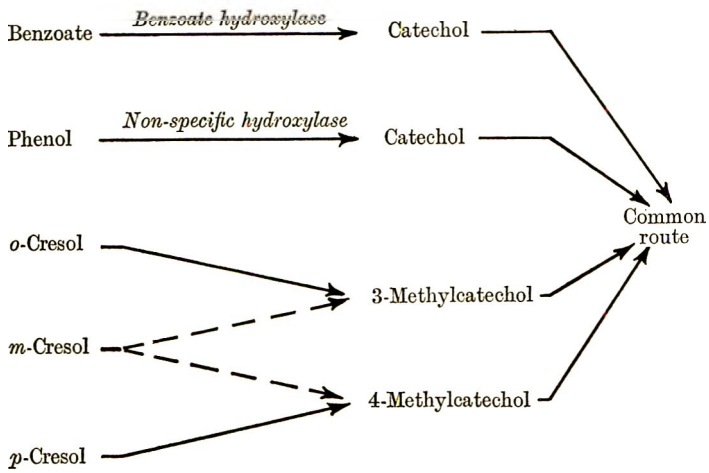
Substrate	Rate of O_2 consumption μmoles/min./ml. of extract		Rate of dissimilation of ring cleavage product μmoles/min./ml. of extract	
	Manometric	O_2 electrode		
Catechol	14	14·6	6·35	10·8
3-Methylcatechol	14	14·1	76·5	89
4-Methylcatechol	—	48·8	—	5·84

— Not measured.

Demonstration of the presence of enzyme activities in induced whole bacteria and extracts of them cannot be regarded as strong evidence for a metabolic route. Indeed Stanier (1950) clearly emphasized that the induced enzymes involved in the catabolism of these compounds must show high specificity for substrate and inducer, if the technique of sequential induction is to be used validly.

The isolation of 3-methylcatechol as an intermediary metabolite of *o*-cresol cultures of *Pseudomonas aeruginosa* T1 provides more satisfactory evidence for its role as the substrate of ring fission. It is possible that catechol may arise from 3-methylcatechol by oxidation to 2,3-dihydroxybenzoate and decarboxylation to catechol, which may then undergo ring cleavage. However, 2,3-dihydroxybenzoate is not oxidized by whole organisms or extracts and catechol is not formed anaerobically from this substrate, though enzymes capable of oxidizing 2,3-dihydroxybenzoate are easily obtained from other bacteria (Ribbons, 1965, unpublished). The existence of a metabolic route under these circumstances of poor specificity of the enzyme should, then, be confirmed by the detection of metabolites and their subsequent disappearance, e.g. by methods like those used by Bassham & Calvin (1957).

The low specificity suggested for the enzymes metabolizing catechol, the methylcatechols and the products derived from them is also exhibited by the inducer. The specificity of enzymes formed in response to any of the cresols or phenol further suggests that the hydroxylase does not discriminate these four substrates and does not metabolize benzoate. Benzoate however induces enzymes for its own catabolism to catechol which are quite distinct from those hydroxylating the cresols or phenol, although a common route for catechol oxidation is evident (Scheme 2).



Scheme 2

The route by which *m*-cresol is metabolized has not yet been determined, but 4-methylcatechol has been detected chromatographically as a metabolite of *p*-cresol.

Although these enzymes have not been studied in any detail the results so far obtained suggest that multi-enzyme systems are elaborated in response to a variety of structures and these multi-enzyme systems show low specificity. When more than one chemical structure is available for metabolism, the existence of such inducible non-specific multi-enzyme systems provides an economy of information

storage and retrieval, as well as of protein synthesis. Reaction sequences of this sort do not occur generally; but the biosynthesis of valine and of isoleucine are catalysed by the same enzyme sequence (Umbarger & Davis, 1962) and provide an analogy to the present catabolic scheme. More recently Gunsalus, Chapman & Kuo (1965) have studied reactions involved in bicyclic mono-terpenoid catabolism by pseudomonads and diphtheroids. They concluded that the enzymes catalysing reaction sequences to common intermediates are non-specific to both substrate and inducer. Low specificity in some instances would allow the exact order of three reactions in a sequence to be transposed without appreciable loss of efficiency. The multi-enzyme system involved in the degradation of the catechols has not been as extensively studied. Further purification of the enzymes involved may show more subtle differences in their control by catabolite repression or as isoenzymes. It is possible that the economic provision of a sequence of enzymes that will tackle a variety of similar structures is more widespread. Thus studies of the microbial metabolism of polynuclear hydrocarbons, naphthalene, phenanthrene and anthracene and methyl- or halogen-substituted naphthalenes have shown that end ring attack occurs with formation of 1,2-diols, cleavage between positions 1 and 8a, loss of a C_3 unit, presumably as pyruvate, and oxidation to a substituted salicylate and thence to the catechol containing one ring less (Evans, Fernley & Griffiths, 1965; Treccanni, 1963). This reaction sequence may also be catalysed by a non-specific multi-enzyme system that can be induced by a variety of similar structures. In the case of phenanthrene the first and second rings may be manipulated by the same enzymes.

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REFERENCES

BASSHAM, J. A. & CALVIN, M. (1957). *The Path of Carbon in Photosynthesis.* Englewood Cliffs, N. J. U.S.A.: Prentice-Hall.

DAGLEY, S. & PATEL, M. D. (1957). Oxidation of *p*-cresol and related compounds by a Pseudomonas. *Biochem. J.* **66**, 227.

DAGLEY, S., EVANS, W. C. & RIBBONS, D. W. (1960). New pathways in the oxidative metabolism of aromatic compounds by micro-organisms. *Nature, Lond.* **188**, 560.

DAGLEY, S., CHAPMAN, P. J., GIBSON, D. T. & WOOD, J. M. (1964). Degradation of the benzene nucleus by bacteria. *Nature, Lond.* **202**, 775.

EVANS, W. C. (1947). Oxidation of phenol and benzoic acid by some soil bacteria. *Biochem. J.* **41**, 373.

EVANS, W. C., FERNLEY, H. N. & GRIFFITHS, E. (1965). Oxidative metabolism of phenanthrene and anthracene by soil pseudomonads. *Biochem. J.* **95**, 819.

FRIEDEMANN, T. E. & HAUGEN, G. (1943). Pyruvic acid. II. The determination of keto acids in blood and urine. *J. biol. Chem.* **147**, 415.

GUNSALUS, I. C., CHAPMAN, P. J. & KUO, W. J. F. (1965). Control of catabolic specificity and metabolism. *Biochem. Biophys. Res. Comm.* **18**, 924.

ICHIHARA, A., ADACHI, K., HOSOKAWA, K. & TAKEDA, Y. (1962). The enzymatic hydroxylation of aromatic carboxylic acids; substrate specificities of anthranilate and benzoate oxidases. *J. biol. Chem.* **237**, 2296.

KITAGAWA, M. (1956). Studies on the oxidation mechanism of methyl group. *J. Biochem.* **43**, 553.

LEIBNITZ, E., BEHRENS, U., STREIGLER, G. & GABERT, A. (1962). Zur mikrobiellen Oxydation der *p*-ständigen Methylgruppe in Xylenolen. *Z. allg. Mikrobiol.* **2**, 81.

NAKAGAWA, H., INOUE, H. & TAKEDA, Y. (1963). Characteristics of catechol oxygenase from *Brevibacterium fuscum*. *J. Biochem.* **54**, 65.

PANKHURST, E. S. (1959). The biological oxidation of spent gas liquor. *J. appl. Bact.* **22**, 202.

RIBBONS, D. W. (1964). Bacterial metabolism of *o*-cresol. *Proc. 6th int. Congr. Biochem.*

ROGOFF, M. H. & WENDER, I. (1957). Methylnaphthalene oxidations by pseudomonads. *J. Bact.* **77**, 783.

STANIER, R. Y. (1950). Problems of bacterial oxidative metabolism. *Bact. Rev.* **14**, 179.

TRECCANNI, V. (1963). The bacterial metabolism of hydrocarbons. *Prog. indust. Microbiol.* **3**, 3.

UMBARGER, E. & DAVIS, B. D. (1962). Pathways of amino acid biosynthesis. In *The Bacteria*. Ed. by I. C. Gunsalus & R. Y. Stanier, Vol. 3, p. 167. New York: Academic Press.

UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. F. (1957). *Manometric Techniques*, 3rd edn. Minneapolis, Minn., U.S.A.: Burgess Publishing Co.

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Latent Effects of Haemolytic Agents

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SUMMARY

Although staphylococcal β-haemolysin does not lyse rabbit erythrocytes, it is absorbed by these cells and produces a prelytic change which can be recognized by facilitation of other haemolytic reactions. Combination of two haemolytic processes serves in general to detect latent effects of lytic agents or to accelerate their quantitative evaluation. β-Haemolysin apparently causes the same primary change in sheep and rabbit erythrocytes, namely enzymic splitting of sphingomyelin and related compounds, but only the sheep cells, when subjected to cooling, are able to undergo a second reaction, responsible for the release of haemoglobin.

INTRODUCTION

The effect of many haemolytic agents appears with considerable delay, indicating that the lytic process consists of at least two separate phases (Menk, 1932): (*a*) Attachment of the active compound to the red cell; and (*b*) the actual disruption of the cell wall, leading to the release of haemoglobin. The combination of the red cell with a haemolysin is often irreversible, i.e. brief exposure is sufficient to let the reaction continue even after the erythrocytes have been separated by centrifugation. However, in this case the extent of haemolysis is usually smaller than when the cells are left in continuous contact with the lytic agent (Cooper, Madoff & Weinstein, 1964; Reich, Bergmann & Kidron, 1965).

The interval between the attachment of a lytic substance and the actual escape of haemoglobin is apparently required to let the chemical reaction progress through the various layers of the membrane structure. Frequently, early alterations of membrane permeability can be recognized, e.g. by prelytic potassium release, before any morphological changes (swelling and disruption of cells) become visible (Davson & Danielli, 1938; Jacob & Jandl, 1962; Madoff, Cooper & Weinstein, 1964). In the present study, we shall show that such prelytic effects can also be demonstrated by facilitation of unspecific or specific lytic processes. In addition, the facilitation method uncovers interactions even in cases where the erythrocytes of a given species appear to be resistant to a haemolytic agent.

METHODS

Staphylococcal β-haemolysin or β-toxin. This material was purchased from Burroughs Wellcome, London. At a concentration of 0·005 international units (i.u.) per ml., it produced 50% haemolysis of sheep erythrocytes, when the hot–cold

method (Jackson & Mayman, 1958) was used. This concentration is designated as H_{50}. The lack of haemolytic action against rabbit erythrocytes, even when a concentration 100 times the H_{50} value for sheep cells was used, served to establish the absence of α-haemolysin in the commercial preparation.

Prymnesin. The purified toxin of *Prymnesium parvum* Carter was prepared according to the method described previously (Bergmann, Parnas & Reich, 1964). The material used in the present experiments had an H_{50} value of 3 μg./ml. when haemolysis was measured after 45 min. and of 1·1 μg./ml. when the reaction time was 150 min. This toxin thus differed from the preparation used in our earlier experiments, where the maximal lytic effect was attained after about 40 min. (Reich, Bergmann & Kidron, 1965). The change of behaviour (see Table 2) is probably due to a modification in the culture conditions of the phytoflagellate, which will be described elsewhere.

Ehrlich ascites carcinoma cells. These cells were collected from the peritoneal cavity of mice 7–11 days after inoculation. The cells were centrifuged at 4500 rev./min. during 5 min., the temperature being kept below +10°, and were washed twice with saline. One ml. of the sediment contained about 5×10^8 cells. The ascites cells were prepared for microscopic examination by fixation in methanol and staining with haematoxylin-eosin.

Erythrocytes. Blood was collected into glass tubes and shaken with glass beads. The erythrocytes were separated by centrifugation and washed with saline until the supernatant was colourless. Finally, they were diluted with saline (pH 7) to give a 7 % suspension.

Haemolysis. (*a*) *Test-tube method.* One ml. each of the 7 % suspension of erythrocytes and of the haemolytic agent, dissolved in saline, were incubated at 37° with 5 ml. of PBS (phosphate-buffered saline; Jackson & Little, 1957). The supernatant, obtained after centrifugation, was diluted with 2·5 volumes of bicarbonate buffer and the extinction measured at 540 mμ. Complete haemolysis of a standard sample was effected by treatment with distilled water.

The bicarbonate buffer contained 3 vol. of 0·1 % Na_2CO_3 and 1 vol. of 0·1 % $NaHCO_3$.

(*b*) *Fragiligraph* (Danon, 1963). This instrument, supplied by Elron Inc., Haifa, Israel, contains a semi-permeable cell, into which is placed the buffered suspension of erythrocytes. The outer compartment is filled with distilled water of 37°. The progress of the reaction is recorded automatically (see Fig. 2).

RESULTS

Absorption of β-haemolysin by 'resistant' cells

When erythrocytes are exposed to an agent like prymnesin, the ichthyotoxin from *Prymnesium parvum* Carter, the haemolytic action starts with a delay of about 15 min. (Reich *et al.* 1965). However, even short contact with the toxin is sufficient for binding. Thus, a solution containing 14 μg./ml. prymnesin was incubated at +5° with 4 batches of rabbit erythrocytes, each time for 2 min., i.e. a period too short to produce visible haemolysis. After the fourth batch of cells had been removed by centrifugation, the activity of the supernatant was tested against fresh rabbit cells at 37°. Incubation for 45 min. produced only 15 % haemolysis

indicating that the remaining concentration of prymnesin was less than 3 μg./ml., the H_{50} value for a 45 min. incubation period. Thus, during the 4 contacts, more than 80 % of the toxin had been removed from the solution.

A similar effect can also be demonstrated for staphylococcal β-haemolysin, although the latter has no lytic action against rabbit erythrocytes (Glenny & Stevens, 1935). A solution containing 0·17 i.u./ml. of β-haemolysin was brought into contact with 4 batches of rabbit red cells, each treatment lasting 15 min. After removal of the fourth batch, the activity of the supernatant was tested against sheep erythrocytes, revealing a drop to about one-third of the original concentration (Table 1*a*).

Table 1. *Absorption of β-haemolysin by 'refractory' cells*

(*a*) A solution of β-haemolysin, containing 0·17 i.u./ml., was incubated 4 times with a 1 % suspension of rabbit erythrocytes. Each treatment lasted for 15 min.; temperature 37°. After the fourth batch of cells had been removed by centrifugation, the activity of the supernatant was tested against sheep erythrocytes by the hot–cold method. Part of the β-haemolysin solution, which was not in contact with rabbit cells, but otherwise was exposed to the same experimental conditions, served as control.

	Dilution of supernatant	% Haemolysis
Control	1/34	50
Solution incubated with 4 batches of rabbit red cells	1/14	14
	1/10	48
	1/7	85

(*b*) A solution of β-haemolysin, containing 0·025 i.u./ml., was incubated at 37° with 3 batches of $1{\cdot}5 \times 10^7$ Ehrlich ascites tumour cells for 15 min., the cells being removed each time by centrifugation. The final supernatant was tested against sheep erythrocytes.

	Dilution of supernatant	% Haemolysis
Control	1/5	50
After treatment with 3 batches of tumour cells	1/2	25
	Undiluted	45

It has been shown that staphylococcal α-haemolysin affects not only erythrocytes, but alters also the permeability of rabbit kidney cells (Artenstein, Madoff & Weinstein, 1963) and of Ehrlich ascites tumour cells (Madoff, Artenstein & Weinstein, 1963). We have therefore examined the absorption of β-haemolysin by the latter. Incubation of β-toxin with 3 batches of ascites carcinoma cells, each time for 15 min., reduced the lytic activity against sheep erythrocytes to about one-fifth (Table 1*b*). Direct inspection of the stained tumour cells failed to reveal any visible morphological change.

Prelytic effect of β-haemolysin

The experiments described above show that β-haemolysin was effectively bound to cells which do not form 'substrates'. This type of absorption may represent a purely physical phenomenon, or the β-toxin may induce a 'prelytic' change in the membrane structure, which in itself is insufficient to cause disruption of the cells. If such a change does take place, it should reveal itself by enhancement of the effect of other haemolytic agents.

Osmotic haemolysis. When rabbit erythrocytes, suspended in saline of pH 7, are exposed to distilled water in the fragiligraph cell, haemolysis proceeds as shown

by curve *a* in Fig. 2. This process was characterized by two features: the lag period and the slope of the linear portion of the ascending branch. In the present experiments, we have selected the first parameter for the purpose of comparison.

Pre-incubation of rabbit red cells with β-haemolysin reduced the lag phase of osmotic lysis considerably. In Fig. 1 this effect is plotted as function of contact time. The lag period shortened progressively when exposure to a given concentration of β-toxin was prolonged. After 20 min. the value of the latency remained constant, indicating that the interaction of β-haemolysin with rabbit erythrocytes, a slowly progressing reaction, had been completed.

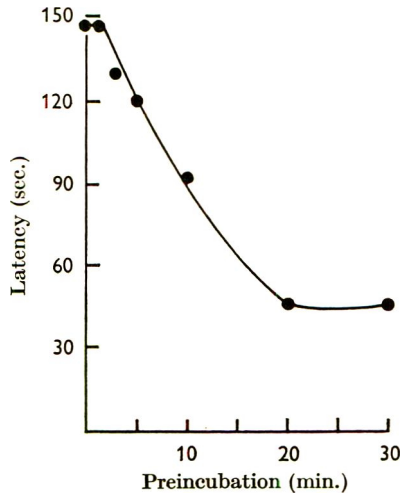

Fig. 1. Acceleration of osmotic haemolysis of rabbit erythrocytes by preincubation with β-haemolysin. A 1% suspension of rabbit red cells was incubated at 37° with 0·05 i.u./ml. of β-haemolysin, in the presence of PBS. Samples of this mixture were transferred at different times to the inner compartment of the fragiligraph and the progress of osmotic lysis was recorded. The latencies (ordinate), obtained from the fragiligraph curves, were then plotted as function of time of preincubation with β-haemolysin (abscissa).

Chemical haemolysis. Pretreatment of rabbit cells with β-haemolysin enhances the lytic action of other effective agents. This can be shown, for example, by exposing the erythrocytes first to the β-toxin for 20 min. and subsequently treating them with prymnesin. Table 2 clearly manifests the synergistic effect of β-toxin by considerable acceleration of the lytic process of prymnesin.

Is the prelytic effect of β-haemolysin reversible?

If the membrane alteration, induced in rabbit red cells by β-haemolysin, is not too profound, the process may be reversible. Rabbit erythrocytes were therefore incubated with β-haemolysin at 37° for 20 min. After separation from the lytic agent, the cells were washed 10 times, at intervals of 5 min., with PBS and after 1 hr the rate of osmotic lysis was measured. The result was the same as when the cells were transferred to the fragiligraph without washing. For instance, when the cells were incubated with 0·05 i.u./ml. for 20 min., the lag period in the fragiligram was reduced from 3 to 1·5 min. One hour later, when the cells had been washed 10 times, the lag period was still 1·6 min.

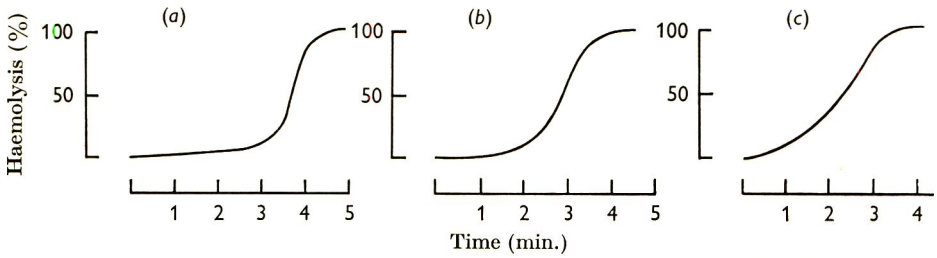

Fig. 2. Shortening of the latency of osmotic haemolysis of rabbit erythrocytes by brief incubation with prymnesin. *a*, Control; *b*, red cells left in contact with 3 μg./ml. of prymnesin for 30 sec.; *c*, preincubation with 3 μg./ml. of prymnesin for 3 min. The cells were transferred to the fragiligraph without further treatment.

Prelytic activity of prymnesin

The observations with β-haemolysin suggest that enhancement of osmotic haemolysis may serve as a general method to detect small concentrations of lytic agents or to shorten the necessary observation time. As an example we show in Fig. 2 the effect of brief incubation (for 30 sec. and 3 min. respectively) of rabbit erythrocytes with a concentration of prymnesin that causes 50 % haemolysis within 45 min. During the short contact of the red cells with this material there was no visible lytic action, but subsequent measurement in the fragiligraph revealed a marked acceleration of osmotic haemolysis.

Table 2. *Synergistic effect of pretreatment of rabbit erythrocytes with β-haemolysin on prymnesin-induced haemolysis*

A 1 % suspension of rabbit erythrocytes was incubated at 37° with 0·005 i.u./ml. of β-haemolysin for 20 min. The cells were centrifuged off and subsequently incubated with 1·1 μg./ml. of prymnesin at 37°. Red cells which had not been in contact with β-haemolysin served as controls. The progress of lysis was measured by the extinction of the supernatant at 540 mμ.

	% Haemolysis	
Time (min.)	Control	Cells after pre-incubation with β-haemolysin
5	3	9
10	3	20
15	5	32
20	6	53
30	13	79
45	26	89
60	46	95
75	68	100
100	83	—
150	97	—

DISCUSSION

Marks & Vaughan (1950), when studying the combined action of different staphylococcal haemolysins, observed additive as well as synergistic effects. The present experiments demonstrate that β-haemolysin, which is 'inert' towards rabbit erythrocytes, is nevertheless absorbed by these cells and induces a change

of membrane structure, which can be recognized by combination with another haemolytic process. The combination method does not only detect covert effects, but can reveal prelytic actions in general and thus may measure concentrations of haemolytic agents too small to produce by themselves rupture of the cell membrane, or it may shorten considerably the incubation time required.

Davson & Ponder (1938) have pointed out that two mechanisms are operative in haemolysis: (*a*) stretching and rupture of the lipid membrane, and (*b*) stretching of the protein mesh. The first effect is held responsible for the increased permeability of the red cell for cations, a phenomenon often encountered in the prelytic stage, while the second process may account for increased permeability for haemoglobin. The order (*a*) → (*b*) is assumed to hold for osmotic lysis. Since β-haemolysin has only a prelytic effect on rabbit red cells, the question arises whether its incomplete action can be related to one of the two steps required to accomplish full haemolysis.

Doery, Magnusson, Gulasekharam & Pearson (1965) showed that β-haemolysin exerts two enzymic activities, namely of sphingomyelinase and of lysophospholipase. Furthermore, Doery, Magnusson, Cheyne & Gulasekharam (1963) found that the sphingomyelin in the stroma of lysed rabbit erythrocytes is hydrolyzed by β-haemolysin qualitatively in the same way as sheep sphingomyelin. However, in spite of the susceptibility of the stroma-bound sphingomyelin in rabbit erythrocytes to enzymic attack, the treated cells do not release haemoglobin even when exposed to cold.

Similar phenomena have been described by MacFarlane (1950) for the toxins of various clostridium species. Thus the 'hot–cold' α-toxin of *Clostridium welchii* is much more effective against sheep than against rabbit erythrocytes. Haemolysis of rabbit red cells is not only weaker, but also is not altered by subsequent cooling. All clostridial toxins exhibit lecithinase activity and the enzymic rates can be compared by using egg lecithin as standard substrate. MacFarlane (1950) attempted to relate divergencies in haemolytic action to different rates of lipid hydrolysis. Indeed, sheep erythrocytes liberated acid-soluble phosphorus much faster when treated with α-toxin of *C. welchii* than when exposed to the γ-toxin of *C. oedematiens*, to which they are much less sensitive. However, when the phospholipids were first extracted from the sheep cells with alcohol-ether and then subjected to the hydrolytic action of the lecithinases, the same rate of splitting was found with either α- or γ-toxin. Apparently, the phospholipids, when incorporated into a specific membrane structure, change their susceptibility to enzymic attack.

One may be tempted to explain the different sensitivity of rabbit and sheep erythrocytes to staphylococcal β-haemolysin on a similar basis. Thus, Doery *et al.* (1963) assumed that the sphingomyelin in whole sheep cells is more easily accessible to the enzymes than the corresponding substrate in rabbit erythrocytes. De Gier & van Deenen (1961) found a marked difference in the lipid composition of the red cells of these two species. The sheep cells contain much less lecithin, but considerably more sphingomyelin and lysophosphatides than do rabbit erythrocytes. It is known that cells with a lower lecithin content undergo more rapid lysis in isotonic glycol (Jacobs, Glassman & Parpart, 1950). In a similar fashion, Doery *et al.* (1965) suggested that enzymic breakdown of sphingomyelin and related lipid components may cause more profound damage to sheep than to rabbit erythrocytes. Still, the differences in rate or extent of splitting of phospholipids cannot explain the effect of

subsequent cooling. As Oakley, Warrack & Clarke (1947) pointed out, 'something is involved in the haemolysis of these different types of red cells, besides the enzymatic attack on lecithin'. The nature of the cold reaction, which takes place in sheep erythrocytes but is absent in rabbit cells, remains one of the most intriguing problems of haemolytic mechanisms.

It should be recalled here that β-haemolysin exerts also an antagonistic action against many smooth-muscle stimulants (Anderson, James & Marks, 1954; Kelsey & Hobbs, 1954; Bergmann, Leon, Chaimovitz & Benzakein, 1961). This effect too may be ascribed to enzymic alteration of the lipid layers in the smooth-muscle membrane. However, the inhibitory action on the guinea-pig ileum can be reversed by repeated washings, in contrast to the irreversibility of the prelytic change in rabbit red cells.

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REFERENCES

Anderson, K., James, D. M. & Marks, J. (1954). The action of staphylococcal toxins on isolated rabbit intestine. *J. Hyg., Camb.* **52**, 492.

Artenstein, M. S., Madoff, M. A. & Weinstein, L. (1963). Studies of the biologic activity of purified staphylococcal alpha-toxin: I. The effect of alpha-toxin on cells in tissue culture. *Yale J. Biol. Med.* **35**, 373.

Bergmann, F., Parnas, I. & Reich, K. (1964). The action of the toxin of *Prymnesium parvum* Carter on the guinea-pig ileum. *Br. J. Pharmacol.* **22**, 47.

Bergmann, F., Leon, S., Chaimovitz, M. & Benzakein, F. (1961). A comparative study of staphylococcus haemolysins and colisan. *Br. J. Pharmacol.* **17**, 159.

Cooper, L. Z., Madoff, M. A. & Weinstein, L. (1964). Hemolysis of rabbit erythrocytes by purified staphylococcal alpha-toxin. II. Effects of inhibitors on the hemolytic sequence. *J. Bact.* **87**, 136.

Danon, D. (1963). A rapid micro method for recording red cell osmotic fragility by continuous decrease of salt concentration. *J. clin. Path.* **16**, 377.

Davson, H. & Danielli, J. F. (1938). Studies on the permeability of erythrocytes. V. Factors in cation permeability. *Biochem. J.* **32**, 991.

Davson, H. & Ponder, E. (1938). Studies on the permeability of erythrocytes. IV. The permeability of 'ghosts' to cations. *Biochem. J.* **32**, 756.

De Gier, J. & van Deenen, L. L. M. (1961). Some lipid characteristics of red cell membranes of various animal species. *Biochim. biophys. Acta*, **49**, 286.

Doery, H. M., Magnusson, B. J., Cheyne, I. M. & Gulasekharam, J. (1963). A phospholipase in staphylococcal toxin which hydrolyses sphingomyelin. *Nature, Lond.* **198**, 1091.

Doery, H. M., Magnusson, B. J., Gulasekharam, J. & Pearson, J. E. (1965). The properties of phospholipase enzymes in staphylococcal toxins. *J. gen. Microbiol.* **40**, 283.

Glenny, A. T. & Stevens, M. F. (1935). Staphylococcus toxins and antitoxins. *J. Path. Bact.* **40**, 201.

Jackson, A. W. & Little, R. M. (1957). Staphylococcal toxins. I. Factors affecting the hemolytic activity of alpha toxin. *Can. J. Microbiol.* **3**, 47.

Jackson, A. W. & Mayman, D. (1958). Staphylococcal toxins. IV. Factors affecting hemolysis by β-lysin. *Can. J. Microbiol.* **4**, 477.

Jacob, H. S. & Jandl, J. H. (1962). Effects of sulfhydryl inhibition on red blood cells. I. Mechanism of hemolysis. *J. clin. Invest.* **41**, 779.

JACOBS, M. H., GLASSMAN, H. N. & PARPART, A. K. (1950). Haemolysis and zoological relationships. Comparative studies with 4 penetrating non-electrolytes. *J. exp. Zool.* **113**, 277.

KELSEY, J. C. & HOBBS, B. C. (1954). Studies on the effect of staphylococcal culture filtrates on isolated rabbit gut. *J. Hyg., Camb.* **52**, 502.

MACFARLANE, M. G. (1950). The biochemistry of bacterial toxins. 5. Variation in haemolytic activity of immunologically distinct lecithinases towards erythrocytes from different species. *Biochem. J.* **47**, 270.

MADOFF, M. A., ARTENSTEIN, M. S. & WEINSTEIN, L. (1963). Studies of the biologic activity of purified staphylococcal alpha-toxin. II. The effect of alpha-toxin on Ehrlich ascites carcinoma cells. *Yale J. Biol. Med.* **35**, 382.

MADOFF, M. A., COOPER, L. Z. & WEINSTEIN, L. (1964). Hemolysis of rabbit erythrocytes by purified staphylococcal alpha-toxin. III. Potassium release. *J. Bact.* **87**, 145.

MARKS, J. & VAUGHAN, A. C. T. (1950). Staphylococcal haemolysin. *J. Path. Bact.* **62**, 597.

MENK, W. (1932). Zur Wirkung des Pararauschbrandhämolysins. *Zentbl. Bakt. ParasitKde*, Abt. I, Orig. **123**, 55.

OAKLEY, C. L., WARRACK, G. H. & CLARKE, P. H. (1947). The toxins of *Clostridium oedematiens* (*C. novyi*). *J. gen. Microbiol.* **1**, 91.

REICH, K., BERGMANN, F. & KIDRON, M. (1965). Studies on the homogeneity of prymnesin, the toxin isolated from *Prymnesium parvum* Carter. *Toxicon*, **3**, 33.

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Concentrations of Nicotinamide Nucleotide Coenzymes in Micro-Organisms

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SUMMARY

The results are presented of a survey of NAD and NADP concentration in twenty micro-organisms. They fell into three categories with respect to NAD concentration. (i) High NAD (> 4·5 μmoles/g. dry weight) in obligate anaerobes, members of the Lactobacillaceae and *Saccharomyces cerevisiae*. (ii) Medium NAD (1·0–3·0 μmoles/g. dry weight) in faculative anaerobes, photosynthetic bacteria and *Nocardia globerula*. (iii) Low NAD (< 0·9 μmoles/g. dry weight) in obligate aerobes.

Different categories were not found with respect to NADP concentration.

Consistent differences in NAD concentration due to conditions of aeration were not found, but growth on different substrates frequently led to changes in NAD concentration. The concentration of NAD in *Streptococcus faecalis* grown on gluconate was only 6% of the concentration in glucose-grown organisms. In *Pseudomonas oxalaticus* NAD concentrations when grown on formate or oxalate were 13 and 8 times, respectively, greater than the concentration in acetate-grown organisms.

NADP concentrations in *Leuconostoc mesenteroides* and *Streptococcus faecalis* were increased 2- to 5-fold by aeration. In *Nocardia globerula*, *Streptomyces griseus* and *Bacillus megaterium* the concentrations of NAD found after growth in complex media were 3–5 times greater than the amounts found after growth on minimal media. This effect was not observed with *Pseudomonas fluorescens*.

INTRODUCTION

Although there is a substantial amount of information on the concentrations of nicotinamide nucleotide coenzymes in animal tissues (see Greenbaum, Clark & McLean, 1965), relatively little is known of the concentrations in micro-organisms. Kaplan (1960) published, for NAD^+ and $NADP^+$ concentrations in eight species of micro-organism, figures which show certain tendencies. NAD^+ is almost always present in larger quantities than $NADP^+$. The concentrations of coenzyme in the four faculative anaerobes (*Escherichia coli*, *Aerobacter aerogenes*, *Photobacterium fischeri*, *Proteus vulgaris*) are all of the same order and are a little below the figure for *Saccharomyces cerevisiae*. The amounts found in the strict aerobes (*Pseudomonas fluorescens*, *Azotobacter agile*, *Mycobacterium butyricum*) are much lower than those found in the faculative anaerobes.

Takebe & Kitahara (1963) estimated the concentrations of nicotinamide

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nucleotide coenzymes in a variety of micro-organisms, mainly lactic acid bacteria. They did not observe the striking differences between strict aerobes and facultative anaerobes reported by Kaplan (1960), but they did find very high values for NAD among the lactic acid bacteria. They also found that there were significant differences between homofermentative and heterofermentative species; in general, NAD concentrations were higher and NADP concentrations lower in homofermentative species. Takebe & Kitahara (1963) also found that conditions of aeration and variations in substrate affected the NAD concentrations in *Escherichia coli*, *Lactobacillus plantarum* and *Saccharomyces cerevisiae* when growing on complex media.

The work of Kaplan (1960) and of Takebe & Kitahara (1963) suggested that there might be significant differences in nicotinamide nucleotide coenzyme concentrations between bacteria of different physiological types, and that the concentrations of these coenzymes might be affected by nutritional and cultural conditions. The present results are derived from a survey of NAD and NADP concentrations in a variety of micro-organisms made to confirm and extend the work of Kaplan (1960) and of Takebe & Kitahara (1963), and to find whether any physical or biochemical factors influence the intracellular concentrations of these enzymes.

METHODS

Micro-organisms, media and cultural conditions

Unless stated otherwise, cultures of all the micro-organisms used in this study were taken from the collection kept in this department.

Bacillus cereus (obtained from the Department of Bacteriology, University of Sheffield) was maintained on slopes of PMG agar (g./l.: peptone, 10·0; Marmite, 3·0; glucose, 10·0; NaCl, 5·0; agar, 10·0) and grown on the following medium: (g./l.) Difco peptone, 2·0; Marmite, 1·0; NaCl, 10·0; glucose, 10·0 or sodium acetate trihydrate, 10·0.

Bacillus megaterium was maintained on slopes of PM agar (g./l.: peptone, 10·0; Marmite, 3·0; NaCl, 5·0; agar, 10·0) and grown on the following medium (Holland, 1961; g./l.): NH_4Cl, 1·0; KH_2PO_4, 0·3; K_2HPO_4, 0·7; Na_2SO_4, 0·1; L-glutamate, 0·5; glucose, 10·0; (mg./l.) $MgSO_4.7H_2O$, 40; $MnSO_4.7H_2O$,5; $CaCl_2.2H_2O$, 2; $FeSO_4.7H_2O$, 2.

Bacillus subtilis obtained from the Department of Bacteriology, University of Sheffield, was maintained on PM agar slopes and grown on the acetate medium used for *B. cereus*.

Chlorobium thiosulphatophilum (National Collection of Industrial Bacteria) NCIB 8327 was maintained and grown on the thiosulphate medium of Larsen (1952).

Clostridium pasteurianum strain w-5 (originally obtained from Dr R. S. Wolfe, University of Illinois, Urbana, U.S.A.) was maintained by weekly transfer on 10 ml. tubes of potato broth medium (Jensen & Spencer, 1947). For estimation of coenzymes, *C. pasteurianum* was grown on 500 ml. quantities of the ammonium sulphate medium of Carnahan & Castle (1958) as modified by Lovenberg, Buchanan & Rabinowitz (1963), but with glucose (7·5 g./l.) instead of sucrose.

Clostridium welchii strain SR 12 was maintained on Robertson's meat medium (Oxo Ltd., London, E.C.4) and grown on the following medium: (g./l.) Robertson's

meat medium, 2 tablets; Oxoid tryptone, 20·0; Oxoid Lab Lemco, 2·0; glucose 10·0; (mg./l.) pyridoxine HCl, 10.

Escherichia coli strain 4071 was maintained on PM agar slopes and grown on the following media: *a*. Complex: (g./l.) Oxoid tryptone, 10·0; Difco yeast extract, 10·0; K_2HPO_4, 0·5; glucose, 3·0; *b*. Minimal: (g./l.) $MgSO_4.7H_2O$, 0·2; NH_4Cl, 2·0; Na_2HPO_4, 6·0; KH_2PO_4, 3·0; NaCl, 3·0. In experiments where growth was limited by substrate, the amounts of substrate added were: glucose, 570 mg./l., or succinic acid (neutralized by addition of NaOH) 750 mg./l. To ensure that oxygen did not become limiting, these amounts were calculated from the aerobic growth yields for *E. coli* determined by Whitaker (1961) to give a final $E_{610\,m\mu}$ of 0·6. In aerobic experiments where growth was not limited by substrate, or in anaerobic experiments, the glucose concentration was 10 g./l. *c*. Defined complete medium (S. R. Elsden, personal communication). The amino acid composition of this medium is based on the amino acid composition of *Escherichia coli* determined by Roberts *et al.* (1955). The medium contains in mg./l. medium *b* (above): aspartate, 206; lysine HCl, 199; methionine, 79; threonine, 86; isoleucine, 94; glutamate, 242; proline, 83; arginine HCl, 177; serine, 100; glycine, 93·5; cysteine HCl, 41; alanine, 176; valine, 100; leucine, 161; tyrosine, 59·6; phenylalanine, 84; tryptophan, 33; histidine HCl, 28·8; adenine, 81; guanine, 109; cytosine, 53·5; uracil, 47; thymine, 11·4; thiamin HCl, 0·5; pyridoxine HCl, 0·5; calcium pantothenate, 0·5; riboflavin, 0·5; nicotinic acid, 1·0; *p*-aminobenzoic acid, 0·1; biotin, 0·001; folic acid, 0·01.

Leuconostoc mesenteroides was maintained on PMG agar slopes, and grown on the medium of Takebe & Kitahara (1963) with glucose as substrate.

Leucothrix mucor (obtained from Dr T. Brock, Department of Bacteriology, University of Indiana, Bloomington, Indiana, U.S.A.) was maintained and grown on the media of Brock (1964).

Micrococcus denitrificans (Verhoeven strain; obtained from Dr J. G. Morris, Department of Biochemistry, University of Leicester) was maintained on the medium described by Kornberg, Collins & Bigley (1960) and grown on the medium of Kornberg (1958) with glycerol (5·0 ml./l.) as carbon source, and with $(NH_4)_2SO_4$ (5·0 g./l.) as nitrogen source instead of ammonium acetate.

Nocardia globerula (NCIB 8852) was maintained on PM agar slopes and grown on: *a*, the complex medium of Krebs & Bellamy (1960) or, *b*, the defined medium of Mencher & Heim (1962).

Peptostreptococcus elsdenii strain LC 1 was maintained and grown on the media described by Walker (1958).

Pseudomonas fluorescens strain KB 1 was maintained on PM agar slopes and grown on the following media: *a*, minimal: (g./l.) $(NH_4)_2SO_4$, 0·5; KH_2PO_4, 10·0; nitrilotriacetic acid, 1·0; substrate (glucose or sodium succinate), 5·0; stock salt solution (Bauchop & Elsden, 1960), 1·0 ml. The pH was adjusted to 7·0 with NaOH. *b*, Complex: (g./l.) peptone, 10·0; Marmite, 3·0; NaCl, 5·0; succinic acid, 15·0; NaOH to pH 6·6.

Pseudomonas oxalaticus strain ox 1 was maintained on PM oxalate (5 mM) agar slopes. It was grown on the medium of Johnson, Jones-Mortimer & Quayle (1964) with 100 mM formate, oxalate or acetate as substrate.

Pseudomonas saccharophila (obtained from Dr M. Doudoroff, Department of Bacteriology, University of California, Berkeley, California, U.S.A.), was maintained

and grown on the following medium: (g./l.) KH_2PO_4, 2·32; Na_2HPO_4, 6·25; NH_4Cl, 1·0; $MgSO_4.7H_2O$, 0·5; ferric ammonium citrate, 0·1; $CaCl_2$, 0·01; starch, 2·5. (M. Doudoroff, personal communication).

Rhodospirillum rubrum strain 1.1.1 and *Rhodopseudomonas palustris* strain 2.1.7 (both obtained from Dr J. Lascelles, Department of Biochemistry, University of Oxford) were maintained and grown on the malate + glutamate media of Lascelles (1959).

Saccharomyces cerevisiae was maintained on PMG agar slopes and grown at 30° on the following media: *a*, PMG; *b*, minimal: (g./l.) KH_2PO_4, 1·0; $(NH_4)_2SO_4$, 0·5; glucose, 10·0; nitrilotriacetic acid, 1·0; inorganic salt solution B (Barton-Wright, 1946), 10·0 ml.; vitamin solution (Wickerham, 1951), 10·0 ml.

Streptococcus faecalis (National Collection of Type Cultures) NCTC 6782 was maintained on PMG agar slopes. Cultures for estimating coenzymes were grown on the complex medium of Takebe & Kitahara (1963) with either glucose (20·0 g./l.) or gluconate (3·0 g./l.) as substrate.

Streptomyces griseus NCTC 7807 was maintained on PMG agar slopes and grown on: *a*, PMG; *b*, the medium of Mencher & Heim (1962).

Three cultures of each organism studied were always grown, harvested and extracted simultaneously. With some exceptions, it was found that the yield of bacterial paste from 300 ml. medium contained enough coenzyme for measurement under the conditions described below. Apart from the exceptions mentioned below, cultures were harvested 12–15 hr after inoculation. The lactic acid bacteria were grown at 37°, the photosynthetic bacteria at 26°–29°; all others at 30°.

Anaerobic cultures of *Clostridium pasteurianum*, *C. welchii*, *Escherichia coli*, *Leuconostoc mesenteroides*, *Peptostreptococcus elsdenii*, *Saccharomyces cerevisiae* and *Streptococcus faecalis* were grown in 500 ml. florence flasks. The obligate anaerobes were grown under an atmosphere of H_2, the facultative anaerobes under N_2.

Aerobic cultures of *Leuconostoc mesenteroides*, *Saccharomyces cerevisiae* and *Streptococcus faecalis* and of *Escherichia coli* in which substrate was not limiting, as well as all cultures of *Bacillus cereus*, *B. subtilis*, *B. megaterium*, *Pseudomonas fluorescens* and *P. saccharophila*, were grown in penicillin flasks which were agitated on a shaker with a rocking action. The rate of shaking was 40 strokes/min., with an amplitude of 20 cm.

Cultures of *Escherichia coli* grown aerobically on limited substrate, of *Pseudomonas fluorescens* grown on complex medium, and all cultures of *P. oxalaticus*, *Micrococcus denitrificans*, *Streptomyces griseus* and *Nocardia globerula*, were grown in 2 l. conical flasks, shaken at 200 strokes/min through a 1 in. circle in a Gyrotory incubator-shaker (Model G-25; New Brunswick Scientific Co., New Brunswick, New Jersey, U.S.A.). With *P. oxalaticus* grown on formate or oxalate, where growth was limited by increasing pH value as the substrate was used, 600 ml. medium were used to give sufficient bacteria.

Rhodospirillum rubrum and *Rhodopseudomonas spheroides* were grown for 36 hr in 1 l. Roux bottles in the light as described by Hoare (1963).

Leucothrix mucor was grown in 1 l. conical flasks on a Griffin wrist-action shaker (Griffin & George Ltd., Wembley, Middlesex).

Reagents

NAD, NADP (sodium salt), glucose-6-phosphate (disodium salt), alcohol dehydrogenase (yeast), glucose-6-phosphate dehydrogenase (yeast), and glutamic dehydrogenase (bovine liver) were brought from Boehringer Corporation (London) Ltd., London, W. 5. 2-Oxoglutaric acid was obtained from Koch-Light Laboratories, Colnbrook, Buckinghamshire. All other reagents were Analar, or of comparable purity.

Extraction of nicotinamide coenzymes

The procedure finally adopted, based on the methods of Bassham *et al.* (1959) and Takebe & Kitahara (1963), was as follows. The bacteria were harvested in a refrigerated centrifuge (15–20 min. at 5400*g*) and washed once with one-fifth of the original culture volume of ice-cold 50 mM phosphate buffer (pH 7·0; KH_2PO_4 + KOH mixture). The bacterial pellet was resuspended in 7–10 ml. 0·1M tris buffer, pH 8·2. Samples of this suspension were then taken to determine dry weight of organism. In the case of *Escherichia coli*, *Pseudomonas oxalaticus* and *Micrococcus denitrificans*, where curves relating extinction at 610 mμ (E_{610}) to dry weight already existed, dry weight was determined from the E_{610} reading of a diluted sample, measured in a Unicam SP 600 spectrophotometer. In the other cases, the bacteria in the sample were washed twice with water by centrifugation, resuspended in water, transferred to a tared weighing bottle and dried at 105° to constant weight.

The oxidized forms of the coenzymes were extracted by treatment with acid, the reduced forms by treatment with alkali. Acid and alkaline extraction was performed by adding HCl (1·5 ml. 0·33 N) or NaOH (1·5 ml. 0·33 N), respectively, to the suspensions, (3·0 ml.) in 20 × 150 mm. tubes. After incubation at 50° for 10 min., the extract was cooled to 0° and neutralized cautiously with either 0·45 ml. of N-NaOH or 0·45 ml. of N-HCl. In order to avoid local high concentrations, the acid or alkali was added at about 10 μl./sec., while the extract was stirred vigorously with a length of flexible polyethylene rod (3 mm. diam.) attached to an overhead stirrer. The neutralized extracts were centrifuged at 23,000*g* for 15 min. to remove insoluble material.

The oxidized forms of the coenzymes, extracted with acid, were then estimated by one of the methods described below. Before estimation, the reduced forms of both coenzymes (extracted with alkali) were oxidized enzymically by adding 10 μl. 2-oxoglutarate + NH_4Cl solution (containing 146 mg. 2-oxoglutarate and 53·5 mg. NH_4Cl/ml., adjusted to pH 7·0 with N-NaOH) and 10 μl. glutamic dehydrogenase (stock enzyme, diluted 5-fold with 0·1M phosphate buffer, pH 7·5) to each sample. After 15 min. at room temperature, 0·1 ml. 5 N-HCl was added to each tube, and the extracts incubated 15 min. at 50° to destroy enzyme. The extract was then neutralized by adding 0·09 ml. 5 N-NaOH, with the precautions described above and, when necessary, clarified by centrifuging for 15 min. at 23,000*g*.

The reliability of the extraction procedure was established by submitting standard solutions of oxidized and reduced forms of both coenzymes to the extraction procedure and measuring the recovery of the coenzymes spectrophotometrically (see below). The % recoveries were as follows: NAD^+, 98; NADH, 96; $NADP^+$, 98; and NADPH, 94. Further, it was shown that any reduced coenzyme present was

completely destroyed by acid extraction, and that the oxidized forms were destroyed during alkaline extraction.

Effect of extraction conditions on recovery of coenzymes. In preliminary experiments with *Saccharomyces cerevisiae*, by the procedure of Takebe & Kitahara (1963) it was found that the period of extraction was critical. Unless the extraction was carried out with extreme care, significant amounts of NAD were destroyed (Fig. 1). Since adequate regulation, both of temperature and of time of exposure of the bacterial, suspensions to heat under conditions 'near boiling' was difficult, the possibility of extracting the coenzymes at lower temperatures was investigated, and 50° was finally chosen as the extraction temperature. A time course of the release of NAD and NADP from *S. cerevisiae*, using the conditions of acid extraction described above, is shown in Fig. 2. Release of NAD(P) was complete in 8 min.; 10 min. was chosen as the standard time of extraction. There was no detectable loss of NAD(P) when the extraction time was extended to 15 min.

Some preliminary experiments were also done on the optimum concentration of acid for NAD extraction. The results are given in Table 1. The acid concentration chosen (equivalent to 0·5 ml. N-HCl in 4·5 ml. extract) was that giving maximum extraction at minimum acid concentration. The concentration of NaOH used in the alkaline extraction was chosen arbitrarily.

Table 1. *Effect of acid concentration on extraction of NAD from Saccharomyces cerevisiae*

N-HCl added (ml.)	Final HCl concentration (mN)	Final pH value	NAD recovered (μmoles/g. dry wt.)
1·0	222	0·8	3·74
0·5	111	1·2	3·79
0·25	55·5	1·7	3·59
0·15	33·3	2·8	1·74

Suspensions of *Saccharomyces cerevisiae* (3 ml.) in a final volume of 4·5 ml. were extracted and the NAD assayed spectrophotometrically as described under 'Methods'.

Estimation of nicotinamide coenzymes

Spectrophotometric method. The coenzymes were reduced enzymically (NAD with alcohol dehydrogenase, NADP with glucose-6-phosphate dehydrogenase), and the amount of coenzyme in the sample calculated from the increase in E_{340} and the molar extinction coefficient for NAD(P)H ($6{\cdot}22 \times 10^3$; P-L Biochemicals, 1961). The spectrophotometer used was a Unicam SP 500 (Unicam Instruments, Cambridge, England). The cuvettes used were glass, with a 1 cm. light-path.

The NAD assay system consisted of 1·0 ml. extract, 2·0 ml. pyrophosphate + semicarbazide buffer (Bonnichsen, 1962) + 10 μl. M-ethanol. After determination of the initial E_{340}, 10 μl. alcohol dehydrogenase (stock enzyme, diluted 10-fold with pyrophosphate + semicarbazide buffer) was added. The reaction was usually complete in 5 min.; the final E_{340} value determined 10 min. after addition of the enzyme.

The NADP assay system consisted of 1–3 ml. extract, 50 mM tris buffer (pH 8·3), to 3·0 ml. + 10 μl. 0·1 M-glucose-6-phosphate. After determination of the initial E_{340} value, 10 μl. glucose-6-phosphate dehydrogenase (stock enzyme, diluted 10-fold in

0·4 M triethanolamine buffer (pH 7·6; Hohorst, 1962) was added. In this case, the reaction was a little slower; the final E_{340} value was determined 15 min. after the addition of enzyme.

Fluorimetric assay. NAD and NADP were also assayed fluorimetrically by following the increase in fluorescence produced by the reduction of NAD(P)$^+$. The assay systems were the same as those used in the spectrophotometric assays, except that the total volume in the assay tubes was increased to 5·0 ml. by the addition of buffer and the amount of extract used was decreased to 0·02–0·1 ml. The fluorimeter used was that described by Dalziel (1962). Readings were taken directly from

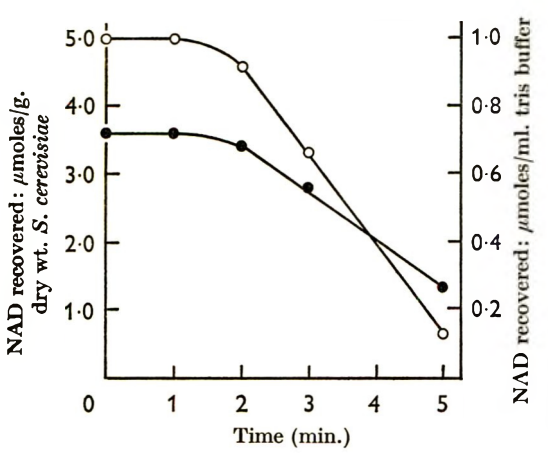

Fig. 1

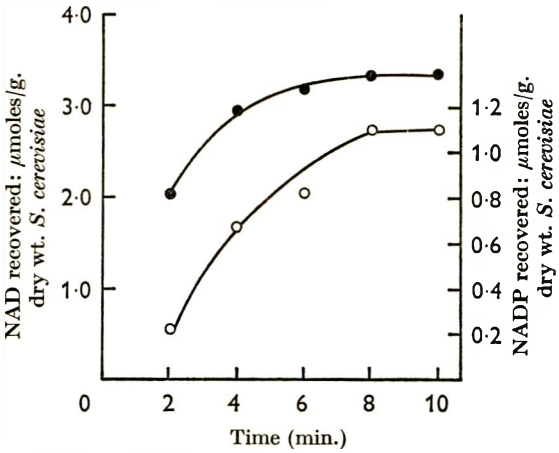

Fig. 2

Fig. 1. Destruction of NAD during extraction in boiling water bath. 3 ml. samples of 0·72 mM-NAD in tris buffer, pH 8·2; (○—○—○): 3 ml. samples of a suspension (25·0 mg. dry wt./ml.) of *Saccharomyces cerevisiae* in tris buffer, pH 8·2 + 1·5 ml. 0·33 N-HCl heated in boiling water bath. (●—●—●). Extracts neutralized and NAD assayed spectrophotometrically.

Fig. 2. Progress curve of extraction of NAD and NADP from *Saccharomyces cerevisiae* at 50°. 3 ml. samples of a suspension (25·0 mg. dry wt./ml.) of *S. cerevisiae* in tris buffer, pH 8·2+1·5 ml. 0·33 N-HCl extracted at 50°. Extracts neutralized and NAD (●—●—●) and NADP (○—○—○) assayed spectrophotometrically.

the millivoltmeter on the Vibron amplifier; the recorder was used only to indicate when the reaction was complete. The extracts varied considerably in their quenching properties and in inherent fluorescence. Increase in fluorescence was related to the quantity of NAD(P) present by addition of a known quantity of NAD(P)$^+$ as an internal standard.

RESULTS

Although the oxidized and reduced forms of the coenzymes were isolated and estimated separately, no significance was attached to the relative proportions of the two forms. The quantities of NAD(P) quoted refer to the sums of oxidized and reduced forms.

Survey of coenzyme concentrations. The results of a survey of NAD and NADP concentrations in various micro-organisms are given in Table 2. The bacteria studied apparently fall into three groups with respect to NAD concentration. No

Table 2. *Summary of NAD and NADP concentrations in various microorganisms*

Micro-organism	Medium	Conditions	NAD (μmoles/g. dry wt. organism)	NADP (μmoles/g. dry wt. organism)	No. of cultures assayed	Assay method
Streptococcus faecalis	Complex, glucose	Aerobic	10·59 ± 0·35	0·54 ± 0·05	6	S
S. faecalis	Complex, glucose	Anaerobic	10·06 ± 0·18	0·11 ± 0·02	6	S
Clostridium pasteurianum	Minimal, glucose	Anaerobic	8·91 ± 0·56	0·87 ± 0·08	6	S
Peptostreptococcus elsdenii	Complex, lactate	Anaerobic	7·25 ± 0·78	0·36 ± 0·11	6	S
Saccharomyces cerevisiae	Complex, glucose	Aerobic	8·26 ± 0·77	0·37 ± 0·07	6	S
S. cerevisiae	Complex, glucose	Anaerobic	6·70 ± 1·35	1·12 ± 0·05	6	S
Leuconostoc mesenteroides	Complex, glucose	Anaerobic	5·62 ± 1·35	0·30 ± 0·07	6	S
L. mesenteroides	Complex, glucose	Aerobic	4·90 ± 0·33	0·82 ± 0·10	6	S
Clostridium welchii	Complex, glucose	Anaerobic	4·91 ± 0·45	0·11 ± 0·01	6	S
Escherichia coli	Minimal, glucose (excess)	Anaerobic	2·67 ± 0·21	0·58 ± 0·21	6	S
E. coli	Minimal, glucose (excess)	Aerobic	2·44 ± 0·14	0·24 ± 0·07	6	S
E. coli	Minimal, glucose (limited)	Aerobic	2·56 ± 0·19	0·84 ± 0·21	6	S
E. coli	Complete (defined), glucose	Aerobic	1·98 ± 0·39	0·48 ± 0·07	3	F
Rhodospirillum rubrum	Malate + glutamate	Light, anaerobic	1·99 ± 0·04	0·43 ± 0·04	3	S
Nocardia globerula	Glucose + glycine + glutamate	Aerobic	1·96 ± 0·25	0·07 ± 0·03	6	S
Micrococcus denitrificans	Minimal, glycerol	Aerobic	1·33 ± 0·25	0·41 ± 0·22	6	S
Bacillus cereus	Complex, glucose	Aerobic	1·14 ± 0·35	0·12 ± 0·06	6	F
Chlorobium thiosulphatophilum	Thiosulphate	Light, anaerobic	1·11 ± 0·16	Not detected	3	S
Rhodopseudomonas palustris	Malate + glutamate	Light, anaerobic	1·09 ± 0·04	0·84 ± 0·24	3	S
Streptomyces griseus	Glucose + glycine + glutamate	Aerobic	0·89 ± 0·18	0·08 ± 0·05	6	S
Bacillus megaterium	Minimal, glucose	Aerobic	0·56 ± 0·14	0·11 ± 0·06	6	F
Pseudomonas saccharophila	Minimal, starch	Aerobic	0·40 ± 0·06	0·79 ± 0·01	3	S
Leucothrix mucor	Peptone	Aerobic	0·36 ± 0·08	0·10 ± 0·06	3	F, S
Bacillus subtilis	Complex, acetate	Aerobic	0·35 ± 0·14	0·70 ± 0·12	6	F
Pseudomonas fluorescens	Minimal, glucose	Aerobic	0·19 ± 0·08	0·04 ± 0·01	6	F, S

Extracts were prepared and coenzyme content assayed as described under Methods.
F = fluorimetric assay; S = spectrophotometric assay.
Values are presented in the form Mean ± standard deviation.

similar tendencies were observed with respect to NADP. The strict anaerobes and lactic acid bacteria had high concentrations of NAD (above 4·5 μmoles/g. dry weight). The faculative anaerobes had concentrations of between 1·0 and 3·0 μmoles/g. dry weight and the strict aerobes had, with one exception (*Nocardia globerula*), less than 1·0 μmole/g. dry weight. The three photosynthetic bacteria have NAD concentrations similar to those of the faculative anaerobes. *Saccharomyces cerevisiae*, which is metabolically a facultative anaerobe, had much higher concentrations of NAD than the bacteria of this physiological type.

Table 3. *Effect of anaerobiosis on NAD(P) concentrations*

Extracts were prepared and coenzyme concentrations measured spectrophotometrically as described under Methods. All cultures (six of each organism) were grown with glucose as substrate.

		Aerobic		Anaerobic	
		NAD (μmoles/g. dry wt. organism)	NADP (μmoles/g. dry wt. organism)	NAD (μmoles/g. dry wt. organism)	NADP (μmoles/g. dry wt. organism)
Organism	Medium				
Streptococcus faecalis	Complex	10·59 ± 0·35	0·54 ± 0·05	10·06 ± 0·18	0·11 ± 0·02
Leuconostoc mesenteroides	Complex	4·90 ± 0·33	0·82 ± 0·10	5·62 ± 1·35	0·30 ± 0·07
Escherichia coli	Minimal	2·44 ± 0·14	0·24 ± 0·07	2·67 ± 0·21	0·58 ± 0·21
Saccharomyces cerevisiae	Minimal	4·92 ± 1·27	0·27 ± 0·08	6·47 ± 1·12	0·31 ± 0·05
S. cerevisiae	Complex	8·26 ± 0·77	0·37 ± 0·07	6·70 ± 1·35	1·12 ± 0·05

Table 4. *Effect of complex medium on NAD(P) concentrations*

All cultures were grown aerobically. Extracts were prepared and coenzyme levels assayed as described under Methods. Coenzymes were assayed fluorimetrically in *Bacillus megaterium* (minimal medium). In all other cases they were assayed spectrophotometrically.

	Minimal medium		Complex medium	
	NAD	NADP	NAD	NADP
Organism	(μmoles/g. dry wt. organism)			
Nocardia globerula	1·96 ± 0·25	0·07 ± 0·03	5·23 ± 0·52	0·55 ± 0·10*
Streptomyces griseus	0·89 ± 0·18	0·08 ± 0·05	2·28 ± 0·10	0·18 ± 0·06*
Bacillus megaterium	0·56 ± 0·14	0·11 ± 0·06	1·52 ± 0·40	0·24 ± 0·11†
Pseudomonas fluorescens	0·27 ± 0·07	0·03 ± 0·01	0·07 ± 0·02	0·03 ± 0·01‡

* Grown on PMG medium; † grown on minimal medium + 0·1 % yeast extract; ‡ grown on PM-succinate.

Effect of conditions of growth on coenzyme concentration. The results of studies on the effect of anaerobiosis on NAD(P) concentration are summarized in Table 3. These results indicate that NAD concentration might differ between cultures grown aerobically and anaerobically, but no particular trend is discernible. NADP concentrations in *Streptococcus faecalis* and *Leuconostoc mesenteroides* were higher when the bacteria were grown aerobically than when they were grown anaerobically. This may reflect a greater use of the pentose phosphate pathway under aerobic conditions.

It was found with *Escherichia coli* that the addition of nicotinic acid (1·5 mg./l.) to minimal medium led to an increase in both NAD and NADP (NAD from

2·44 μmoles/g. to 9·44 μmoles/g.; NADP from 0·24 μmoles/g. to 0·45 μmoles/g.). With *Bacillus megaterium* the addition of 0·1 % yeast extract to minimal medium led to a 2-fold increase in NAD and NAD(P) (Table 4). NAD in *Nocardia globerula* and *Streptomyces griseus* was increased 2- to 3-fold on complex media, compared with the amounts found on minimal medium (Table 4). These increased amounts were not found when *Pseudomonas fluorescens* was grown on a complex medium. The effects on NAD(P) of growing *Saccharomyces cerevisiae* on complex and minimal media are shown in Table 3. Concentrations of NAD and NADP were generally lower when the organism was grown on a minimal medium than when it was grown on a complex one. The effects of growing *E. coli* under different conditions are shown in Table 2. It is not possible to detect any differences in NAD(P) concentration between *E. coli* grown in a completely defined medium with excess glucose and on a minimal medium with excess glucose, or between growth on a minimal medium with excess or with limiting glucose.

Table 5. *Effect of energy source on NAD(P) concentration*

Organisms were grown aerobically as described under Methods. *Escherichia coli* was grown on limiting substrate. Extracts were prepared and coenzymes measured as described under Methods. S = spectrophotometric assay; F = fluorimetric assay.

Organism	Substrate	NAD (μmoles/g. dry wt. organism)	NADP (μmoles/g. dry wt. organism)	No. of cultures assayed	Assay method
Streptococcus faecalis	Glucose	10·59 ± 0·35	0·54 ± 0·05	6	S
S. faecalis	Gluconate	0·66 ± 0·28	0·24 ± 0·01	3	S
Bacillus cereus	Glucose	1·14 ± 0·35	0·12 ± 0·06	6	F
B. cereus	Acetate	1·66 ± 0·28	1·42 ± 0·36	6	F
Pseudomonas fluorescens	Glucose	0·19 ± 0·08	0·04 ± 0·01	6	F, S
P. fluorescens	Succinate	0·27 ± 0·07	0·03 ± 0·01	6	F, S
Escherichia coli	Glucose	2·56 ± 0·19	0·84 ± 0·21	6	S
E. coli	Succinate	2·13 ± 0·26	0·70 ± 0·22	9	S
P. oxalaticus	Formate	3·59 ± 0·34	0·50 ± 0·25	6	S
P. oxalaticus	Oxalate	2·20 ± 0·37	0·87 ± 0·18	6	S
P. oxalaticus	Acetate	0·27 ± 0·07	0·41 ± 0·12	6	S

Some effects of substrate on NAD(P) concentrations are shown in Table 5. The NAD in gluconate-grown *Streptococcus faecalis* was about 16 times less than that found in glucose-grown organisms. The NAD found in gluconate-grown organisms is characteristic of a strict aerobe. When *Pseudomonas oxalaticus* was grown on acetate the NAD concentration was low, as would be expected of a strict aerobe; growth on formate results in a 13- to 14-fold increase, and on oxalate in an 8-fold increase in NAD over the concentration found in acetate-grown *P. oxalaticus*. The differences in NAD concentrations between glucose-grown and acetate-grown *Escherichia coli* or *P. fluorescens* are not significant. Only in the case of *Bacillus cereus*, where growth on acetate caused a 12-fold increase in NADP, was there any difference in NADP concentrations.

DISCUSSION

One of the objects of the work described here was to confirm and extend the work of Kaplan (1960) and Takebe & Kitahara (1963). The concentrations of NAD and NADP found by these authors are compared with those found in the present work in Table 6. In the case of the lactic acid bacteria, agreement between the values for NAD concentration of Takebe & Kitahara (1963) and those reported here is close. The amounts of NAD found by Takebe & Kitahara (1963) in *Saccharomyces cerevisiae* and *Escherichia coli* are lower, while their figures for *Bacillus subtilis* and *Pseudomonas fluorescens* are higher. The higher values might reflect the fact that these authors grew all their cultures on complex media. Comparison with the results of Kaplan (1960) is less easy, because his $NAD(P)^+$ concentrations are expressed as μg./g. wet weight, and because his bacterial extracts contained variable proportions of an unknown material other than NAD^+ or $NADP^+$ which reacted as oxidized nicotinamide nucleotide in his assay system; in addition, the method of extraction and assay was not described. Nevertheless, the concentrations of NAD found by Kaplan in four facultative anaerobes are of the same order as those reported here, as are the values for the strict aerobes. There is also good agreement between Kaplan and Takebe & Kitahara for the NAD concentration in *Saccharomyces cerevisiae*. In general, agreement between the different values reported for NADP concentrations is poor.

Table 6. *Comparison of NAD(P) concentrations with results of other authors*

	Present paper		Takebe & Kitahara (1963)*		Kaplan (1960)*	
	NAD	NADP	NAD	NADP	NAD	NADP
	μmoles/g. dry wt. organism					
Streptococcus faecalis	10·57	0·11	10·06	0·20	.	.
Leuconostoc mesenteroides	5·62	0·32	4·86–5·39	0·53–0·70	.	.
Saccharomyces cerevisiae	6·70	1·12	2·68–3·20	0·40–1·13	2·9	0·4
Escherichia coli	2·67–1·98	0·24–0·84	1·65	0·20	1·4	0·2
Aerobacter aerogenes	.	.	2·04	0·05	2·0	0·2
Achromobacter fischeri	.	.	.	.	2·2	0·3
Proteus vulgaris	.	.	.	.	1·9	0·2
Pseudomonas fluorescens	0·19	0·04	1·35	0·26	0·2	0·3
Bacillus subtilis	0·35	0·70	1·50	0·17	.	.
Azotobacter agile	.	.	.	.	0·6	0
Mycobacterium butyricum	.	.	.	.	0·5	0

* The values of Takebe & Kitahara (1963) were originally expressed as mg./g. dry wt. organism. Those of Kaplan (1960) were expressed as μg./g. wet weight. They have been converted to dry weight values on the assumption that the dry weight content of wet bacterial paste is 20 %.

These observations have been extended to show that the strict anaerobes and the Lactobacillaceae (which, though microaerophilic, have a strictly fermentative metabolism), the faculative anaerobes, and the strict aerobes, fall into distinct classes with respect to NAD concentration. There are two exceptions to this generalization. *Saccharomyces cerevisiae*, a faculative anaerobe, had NAD concentrations more typical of a strict anaerobe, and *Nocardia globerula*, a strict aerobe, had NAD concentrations as high as many facultative anaerobes.

The high NAD concentration found in the strict anaerobes seems to be associated with the absence of a haem-linked oxidation system. It was shown by Takebe, Shirakawa & Kitahara (1964) that in *Leuconostoc mesenteroides* at least 96% of the NAD remained in the supernatant fluid after a sonic extract had been centrifuged at 100,000*g* for 60 min. If the very small amount of NAD found here in strict aerobes was closely associated with the intracytoplasmic membrane, as the respiratory enzymes are in *Azotobacter agilis* (Pangborn, Marr & Robrish, 1962), and if most oxido-reduction reactions occurred close to the membrane, then the turnover of NAD could be very rapid and the effective concentration of NAD could be as high as it is in strict anaerobes.

Another object of the present work was to determine whether the concentration of NAD or NADP might vary with the conditions of cultivation. Takebe & Kitahara (1963) showed that NADP concentrations were generally higher in members of the Lactobacillaceae with a heterolactic fermentation pattern than those with a homolactic pattern. They suggested that this might be a reflexion of the greater utilization of the NADP-linked dehydrogenases of the pentose phosphate pathway in these organisms. Although NADP concentrations are in general at the limit of detection by the method used, it is possible to show that in *Streptococcus faecalis* growing on glucose there is a 3- to 5-fold increase in NADP in aerated cultures over the value in cultures grown under nitrogen. It is possible that under aeration there is an increased utilization of the pentose phosphate pathway in *Streptococcus faecalis*. This explanation cannot account for the similar increase in NADP found in *Leuconostoc mesenteroides*. In this organism the glucose-6-phosphate dehydrogenase reacts with both NAD and NADP, and 6-phosphogluconate dehydrogenase with NAD only (De Moss, 1955) and it has been shown (Kemp & Rose, 1964) that the hydrogen carrier for the reductive reactions of fermentation is NAD. It has not been possible to show any other consistent effects of aeration versus anaerobiosis or of minimal medium versus complex medium on NAD(P) concentrations.

The nature of the medium has been shown to have a considerable effect on NAD concentration. The addition of nicotinate (1·5 mg./l.) to a minimal medium caused a 4-fold increase in the NAD concentration in *Escherichia coli* and the addition of 0·1% yeast extract to a minimal medium led to a 2- to 3-fold increase in NAD concentration in *Bacillus megaterium*. In *Nocardia globerula* and *Streptomyces griseus* NAD after growth on complex media was 2–3 times higher than after growth on a minimal medium; NAD in *Pseudomonas fluorescens* was, in contrast, scarcely affected.

The nature of the carbon source may also affect NAD concentration. The dramatic decrease in NAD in gluconate-grown *Streptococcus faecalis* as compared with the value when the organism was grown on glucose was probably connected with the change-over from use of the glycolytic pathway in glucose-grown organisms to the ketogluconate pathway in gluconate-grown organisms (Gibbs, Sokatch & Gunsalus, 1955; Sokatch & Gunsalus, 1957).

The increased NAD concentration in *Pseudomonas oxalaticus* grown on formate may be explained by the fact that on formate the organism gains all its energy from the NAD-linked oxidation of formate, and that NADH is the reductant used in the reductive pentose phosphate cycle from which the biosynthetic pathways of the organism diverge (Professor J. R. Quayle, personal communication). The concentration

of NAD in oxalate-grown *P. oxalaticus* was some 8 times the value for acetate-grown organisms. During growth on oxalate the organism is still dependent on NAD-linked formate oxidation for its entire energy supply (Johnson *et al.* 1964), but the reducing agent in the early stages of biosynthesis is reduced NADP (Quayle, Keech & Taylor, 1961). This may account for the slightly higher concentration of NADP in oxalate-grown *P. oxalaticus*. In cases where growth on a different substrate did not induce a new catabolic pathway, as with *P. fluorescens* or *Escherichia coli* growing on glucose or succinate, NAD(P) concentration did not change.

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REFERENCES

Barton-Wright, E. C. (1946). In *Practical Methods for the Microbiological Assay of the Vitamin B Complex and Essential Amino Acids*. London: Ashe Laboratories.

Bassham, J. A., Birt, L. M., Hems, R. & Loening, U. E. (1959). Determination of the reduced and oxidized pyridine nucleotides in animal tissues. *Biochem. J.* **73**, 491.

Bauchop, T. & Elsden, S. R. (1960). The growth of micro-organisms in relation to their energy supply. *J. gen. Microbiol.* **23**, 457.

Bonnichsen, R. (1962). In *Methoden der enzymatischen Analyse*. Ed. by H.-U. Bergmeyer, p. 285. Weinheim, German Federal Republic: Verlag Chemie.

Brock, T. D. (1964). Knots in *Leucothrix mucor*. *Science*, **144**, 870.

Carnahan, J. E. & Castle, J. E. (1958). Some requirements of biological nitrogen fixation. *J. Bact.* **75**, 121.

Dalziel, K. (1962). Kinetic studies of liver alcohol dehydrogenase. *Biochem. J.* **84**, 244.

De Moss, R. D. (1955). Glucose-6-phosphate and 6-phospho-gluconate dehydrogenases from *Leuconostoc mesenteroides*. *Meth. Enzymol.* **1**, 238.

Gibbs, M., Sokatch, J. T. & Gunsalus, I. C. (1955). Product labeling of glucose-1-C^{14} fermentation by homofermentative and heterofermentative lactic acid bacteria. *J. Bact.* **70**, 573.

Greenbaum, A. L., Clark, J. B. & McLean, P. (1965). The estimation of the oxidized and reduced forms of the nicotinamide nucleotides. *Biochem. J.* **95**, 161.

Hoare, D. S. (1963). The photo-assimilation of acetate by *Rhodospirillum rubrum*. *Biochem. J.* **87**, 284.

Hohorst, H.-J. (1962). In *Methoden der enzymatischen Analyse*. Ed. by H.-U. Bergmeyer, p. 134. Weinheim, German Federal Republic: Verlag Chemie.

Holland, I. B. (1961). The purification and properties of megacin, a bactenocin from *Bacillus megaterium*. Ph.D. Thesis, University of Sheffield.

Jensen, H. L. & Spencer, D. (1947). The influence of molybdenum and germanium on nitrogen fixation by *Clostridium butyricum*. *Proc. Linnean Soc., N.S.W.*, **72**, 73.

Johnson, P. A., Jones-Mortimer, M. C. & Quayle, J. R. (1964). Use of a purified bacterial formate dehydrogenase for the micro-estimation of formate. *Biochim. biophys. Acta*, **89**, 351.

Kaplan, N. O. (1960). In *The Enzymes*. Ed. by P. D. Boyer, H. Lardy & K. Myrbäck, vol. 3, p. 105. New York, N.Y., U.S.A.: Academic Press Inc.

Kemp, R. G. & Rose, I. A. (1964). Coupling of reduced pyridine nucleotide in *Leuconostoc mesenteroides*. *J. biol. Chem.* **239**, 2998.

KORNBERG, H. L. (1958). The metabolism of C_2 compounds in micro-organisms. 1. The incorporation of [2-^{14}C]-^{2}acetate by *Pseudomonas fluorescens* and by a *Corynebacterium*, grown on ammonium acetate. *Biochem. J.* **68**, 535.

KORNBERG, H. L., COLLINS, J. F. & BIGLEY, D. (1960). The influence of growth substrates on metabolic pathways in *Micrococcus denitrificans*. *Biochim. biophys. Acta*, **39**, 9.

KREBS, H. A. & BELLAMY, D. (1960). The interconversion of glutamic acid and aspartic acid in respiring tissues. *Biochem. J.* **75**, 523.

LARSEN, H. (1952). On the culture and general physiology of the green sulfur bacteria. *J. Bact.* **64**, 187.

LASCELLES, J. (1959). Adaptation to form bacteriochlorophyll in *Rhodopseudomonas spheroides*: change in activity of enzymes concerned in pyrrole synthesis. *Biochem. J.* **72**, 508.

LOVENBERG, W., BUCHANAN, B. B. & RABINOWITZ, J. C. (1963). The chemical nature of clostridial ferredoxin. *J. biol. Chem.* **238**, 3899.

MENCHER, J. M. & HEIM, A. H. (1962). Melanin biosynthesis by *Streptomyces lavendulae*. *J. gen. Microbiol.* **28**, 665.

P-L Biochemicals (1961). *Ultra-Violet Absorption Spectra of Pyridine Nucleotide Coenzymes and Coenzyme Analogues*. Milwaukee, Wis., U.S.A.: Pabst Laboratories.

PANGBORN, J., MARR, A. G. & ROBRISH, S. A. (1962). Localization of respiratory enzymes in intracytoplasmic membranes of *Azotobacter agilis*. *J. Bact.* **84**, 669.

QUAYLE, J. R., KEECH, D. B. & TAYLOR, G. A. (1961). Carbon assimilation by *Pseudomonas oxalaticus* (OXI) 4. Metabolism of oxalate in cell-free extracts of the organism grown on oxalate. *Biochem. J.* **78**, 225.

ROBERTS, R. B., COWIE, D. B., ABELSON, P. H., BOLTON, E. T. & BRITTEN, R. J. (1955). Studies of biosynthesis in *Escherichia coli*. *Publ. Carnegie Instn*, no. 607.

SOKATCH, J. T. & GUNSALUS, I. C. (1957). Aldonic acid metabolism. 1. Pathway of carbon in an inducible gluconate fermentation by *Streptococcus faecalis*. *J. Bact.* **73**, 452.

TAKEBE, I. & KITAHARA, K. (1963). Levels of nicotinamide nucleotide coenzymes in lactic acid bacteria. *J. gen. appl. Microbiol.* **9**, 31.

TAKEBE, I., SHIRAKAWA, T. & KITAHARA, K. (1964). Quantitative studies of enzyme system of heterolactic fermentation. 1. Some aspects of cytoplasm of *Leuconostoc mesenteroides* as the site of heterolactic fermentation. *J. gen. appl. Microbiol.* **10**, 359.

WALKER, D. J. (1958). The purification and properties of the L-threonine deaminase of the rumen micro-organism LCI. *Biochem. J.* **69**, 524.

WHITAKER, A. M. (1961). The growth of micro-organisms in relation to their energy supply. Ph.D. Thesis, University of Sheffield.

WICKERHAM, L. J. (1951). Taxonomy of yeasts. *Tech. Bull., U.S. Dept. Agric.*, no. 1029.

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'Substrate-Accelerated Death' of Nitrogen-Limited Bacteria

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SUMMARY

'Substrate-accelerated death' (Postgate & Hunter, 1963*a*; 1964) occurred when a nitrogen-limited variant of *Aerobacter aerogenes* NCTC 418 (Postgate & Hunter, 1962) was starved at growth temperature (37° or 40°) in aerated saline buffers containing ammonium ion; it was not observed when the parent strain of *A. aerogenes* or *Escherichia coli* (MRE 162) was grown and starved under similar conditions. Sulphate ion increased the lethal effect of ammonium ion on the variant and magnesium did not abolish either the effect of ammonium or ammonium + sulphate ions. The *A. aerogenes* variant differed from the parent strain in morphology, colonial appearance on nutrient agar, biochemical and immunological reactions and ability to synthesize polysaccharide. In ammonium-limited medium at 37° or 40° at a dilution rate near 0·25 hr^{-1} the variant contained 3–5% and the parent strain 12–16% of dry weight as polysaccharide; in spent medium or phosphate buffer at 37° with added glycerol the rate of polysaccharide synthesis by the variant was about 25% that of the parent strain. When grown in nitrogen-deficient medium with excess glycerol in batch culture, populations of the variant containing 25% polysaccharide were obtained; the survival of the polysaccharide-rich variant was not affected by ammonium ion.

INTRODUCTION

Strange & Dark (1965) failed to observe 'ammonium-accelerated death' of *Aerobacter aerogenes* as reported by Postgate & Hunter (1963*a*, *b*, 1964). The respective tests differed in that Postgate & Hunter used a variant of *A. aerogenes* grown and starved at 40°, whereas Strange & Dark used the parent strain grown and starved at 37°. Re-investigation has confirmed these findings and shown them to be true whether the organisms were grown and starved at 37° or 40°. Evidently the apparent discrepancy was not due to differences in technique or interpretation of results but to a difference in the organisms themselves. During continuous growth at 40° under nitrogen-limiting conditions at a dilution rate of 0·25 hr^{-1} the variant contained much less polysaccharide than the parent strain did when growing under similar conditions but at 37°, and this difference was offered by Strange & Dark (1965) as a possible explanation for the divergent results. The present paper records a comparison of various properties of the variant and parent strains of *A. aerogenes*, including their survival in saline buffers with and without ammonium ion, and polysaccharide synthesis under various conditions. Some data obtained with strains of *Escherichia coli* are included.

METHODS

Organisms. Aerobacter aerogenes, NCTC 418 and a variant strain of this organism (Postgate & Hunter, 1962); *Escherichia coli*, strains MRE 162 (laboratory strain) and NCTC 8164.

Media and cultural conditions. Organisms were grown at 37° and 40° in chemostats (designed by Dr D. Herbert) with culture vessels containing 100 or 250 ml. medium (Postgate & Hunter, 1962; Strange & Dark, 1965). Defined nitrogen-limiting and carbon-limiting media described by Postgate & Hunter (1962) contained glycerol (2–10 g./l.) as the carbon energy source. Chemostats were inoculated with fully grown shaken batch cultures of the organisms grown in these media. The dilution rate was usually maintained near 0·25 hr^{-1}.

Viability determinations. These were made with slide cultures (Postgate, Crumpton & Hunter, 1961) or viable counts (Strange, Dark & Ness, 1961). The enriched agar medium used for both methods was described by Postgate *et al.* (1961) and contained 0·2% (w/v) glycerol or glucose.

Starvation. Bacteria were separated from the culture and washed twice in the appropriate saline buffer by centrifugation. The bacterial pellet was resuspended (at a concentration of $1–2 \times 10^9$ bacteria/ml.) in saline buffer. Volumes of washed bacterial suspension (0·2–0·6 ml.) were added to saline buffer with and without additions (final vol, 10–30 ml.) usually in test tubes (18 × 3 cm.); the test suspensions were aerated with sterile washed air through Pasteur pipettes. When experiments lasted more than 6 hr, suspensions were usually aerated in Dreschel type gas wash-bottles from which effluent air escaped through a water condenser fitted with a filter, or were agitated in Erlenmeyer flasks on a reciprocating shaker.

Saline buffers. Saline tris (Postgate & Hunter, 1962) contained 135 mM-NaCl, 4·8 mM tris buffer and 0·32 mM-EDTA (pH 7·0–7·2); saline phosphate 1 (Postgate & Hunter, 1962) contained 135 mM-NaCl and 6·7 mM sodium potassium phosphate buffer (pH 6·5); saline phosphate 2 (Strange & Dark, 1965) contained 108 mM-NaCl and 20 mM-potassium phosphate buffer (pH 6·5). 'Specpure' saline phosphate (pH 6·7–6·8) contained highly purified NaCl (108 mM), KH_2PO_4 (20 mM) and K_2CO_3 (5·4 mM) (Johnson, Matthey & Co., Ltd., Hatton Garden, E.C. 1) dissolved in glass-distilled water, and the solution was sterilized and stored in polypropylene bottles.

Analytical methods. Bacterial dry weights and total carbohydrate were determined as previously described (Strange & Dark, 1965).

RESULTS

Comparison of some properties of the variant and parent strains of Aerobacter aerogenes

The variant of *Aerobacter aerogenes* appeared and completely replaced the parent strain during continuous cultivation in glycerol-limiting defined medium at 37° and 40° in a chemostat (Postgate & Hunter, 1962). The ability of the variant to replace the parent strain in chemostats at dilution rates of 0·25–0·5 hr^{-1} was confirmed by mixing in a third chemostat samples from separate chemostat cultures of the two organisms and determining the relative proportion of each organism after various periods of growth (Fig. 1). At dilution rates above 0·5 hr^{-1}, the parent strain out-

grew the variant. As found by Postgate & Hunter (1962), the growth rate of the variant was lower than that of the parent strain during unrestricted growth in defined medium in batch cultures (Fig. 2).

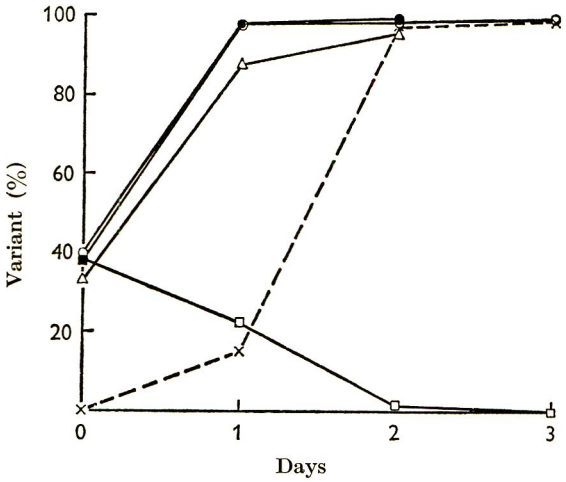

Fig. 1

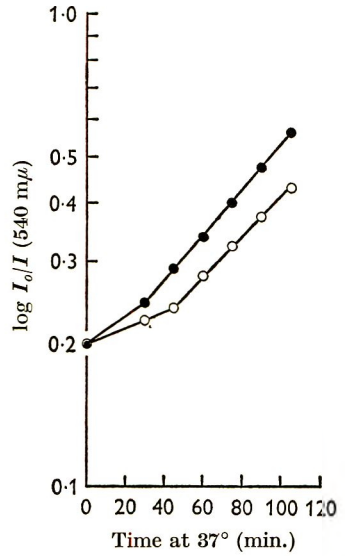

Fig. 2

Fig. 1. Growth of glycerol-limited parent and variant strains of *Aerobacter aerogenes* in mixed culture in chemostats at various dilution rates. Samples of cultures of the organisms growing separately in chemostats at the given dilution rate were mixed in a third chemostat and the proportion of each organism was determined by differential colony counts after intervals of growth at 37°. Proportion of variant organisms at dilution rates of 0·25 (○, ×), 0·3 (●), 0·5 (△) and 0·62 (□) hr^{-1}. One mixed culture (×) was seeded with 0·1 % variant.

Fig. 2. Comparison of the growth rates of parent and variant strains of *Aerobacter aerogenes* in defined medium at 37° with glycerol as the carbon energy source. Medium was inoculated with samples of glycerol-limited cultures (dilution rate, 0·5 hr^{-1}) and aerated at 37°. Turbidities of variant (○) and parent strain (●) cultures were measured in a spectrophotometer at 540 mμ.

Chemostats were inoculated with batch cultures of the variant grown from single colonies on solid medium, but after several days growth, samples of chemostat cultures produced three colonial forms on nutrient agar, all of which differed from parent strain colonies. The relative proportion of the different variant colonies varied from day to day but one type always predominated. The average length of variant organisms was greater than that of parent strain organisms (Postgate & Hunter, 1962). During the present work the parent strain did not alter in colonial appearance during continuous culture in either N-limiting or C-limiting media at 37° or 40° for 3 months.

The biochemical reactions of the variant in peptone water containing various carbohydrates differed from those of a typical *Aerobacter aerogenes*; in general, no gas or much less gas was produced and no acid was apparent with dextrin or starch. The biochemical reactions of pure cultures of the three colonial types of the variant differed slightly from each other.

Table 1. *Effect of ammonium ion on the survival of N-limited bacteria in saline buffers*

Bacteria growing in N-limiting medium (dilution rate 0·23–0·25 hr^{-1}) were washed and starved (3–5 × 10^7 bacteria/ml.) at 37° or 40° in aerated saline buffers with and without NH_4Cl. Viabilities were determined at intervals by slide culture (s.c.) or viable counts (v.c.). 'SP 1', 'SP 2' and 'ST' refer to saline phosphate and saline tris buffers (see Methods).

Organism	Growth temp.	Diluent	pH	Starvation temp.	NH_4Cl	Time (hr) 0	6	24	48	Viability assay
						Viability (%)				
A. aerogenes NCTC 418	37°	SP 1	6·5	37°	Nil	99	99	98	96	s.c.
					22 mM	99	99	95	94	
	37°	SP 2	6·5	37°	Nil	99	98	97	95	
					22 mM	99	99	93	89	
	37°	ST	7·0	37°	Nil	99	98	94	85	
					22 mM	99	99	93	82	
	40°	SP 1	6·5	37°	Nil	99	96	76	—	
					15 mM	98	93	80	—	
	40°	SP 1	6·5	40°	Nil	98	95	59	—	
					15 mM	98	92	58	—	
	40°	SP 2	6·5	40°	Nil	100	85	76	34	v.c.
					25 mM	100	88	75	33	
A. aerogenes variant	37°	SP 1	6·5	40°	Nil	97	93	44	16	s.c.
					25 mM	99	26	8	1	
	37°	SP 2	6·5	37°	Nil	98	93	35	6	
					25 mM	98	62	11	6	
	40°	SP 1	6·5	40°	Nil	98	73	—	—	
					25 mM	99	39	—	—	
E. coli MRE 162	37°	SP 1	6·5	40°	Nil	98	97	96	96	
					15 mM	98	94	94	95	
	37°	SP 2	6·5	37°	Nil	99	99	99	95	
					25 mM	97	99	97	97	

The pattern of precipitation lines produced by the variant in agar-gel double diffusion plates with antiserum produced in rabbits against the parent strain indicated that at least two antigens were absent from the variant or were present in much lower concentration.

Effect of ammonium ion on the survival of N-limited bacteria

Samples of bacteria from chemostat cultures of N-limited *Escherichia coli* MRE 162 and the parent and variant strains of *Aerobacter aerogenes* growing at 37° and 40° were washed and starved at 37° and 40° in various saline buffers (see Methods) with and without NH_4Cl (Table 1). The death rate of the variant, which was greater than that of the other organisms in buffer alone, increased in the presence of NH_4Cl.

Table 2. *Effect of magnesium and/or sulphate ions on 'ammonium-accelerated death' of the parent and variant strains of Aerobacter aerogenes*

Bacteria grown in N-limiting medium (dilution rate 0·23–0·25 hr^{-1}) were washed and starved (at 3×10^7 bacteria/ml.) at 40° in aerated saline phosphate 1 with and without additions. Viabilities of suspensions were determined at intervals by slide culture.

		Time (hr)				
		0	2	4	7·5	24
		Viability (%)				
Organism	Addition to diluent					
Parent strain	Nil	99	98	97	97	98
	15 mM-NH_4Cl	98	98	99	97	97
	15 mM-NH_4Cl + mM-Na_2SO_4	98	99	98	99	97
	15 mM-NH_4Cl + mM Na_2SO_4 + mM $MgCl_2$	98	98	98	98	96
Variant strain	Nil	98	98	95	85	—
	15 mM-NH_4Cl	97	97	84	58	—
	mM-$MgCl_2$	98	98	94	89	—
	mM-Na_2SO_4	98	98	94	90	—
	15 mM-NH_4Cl + mM-$MgCl_2$	98	97	94	68	—
	15 mM-NH_4Cl + mM-Na_2SO_4	98	92	50	27	—
	15 mM-NH_4Cl + mM-$MgCl_2$ + mM-Na_2SO_4	98	97	76	39	—

In contrast, the death rates of the parent strain *A. aerogenes* and of *E. coli* were unaffected by NH_4Cl. The effect of NH_4Cl on the variant increased with concentration up to 50 mM in 'Specpure' saline phosphate (Fig. 3). At higher temperatures (43–47°) the death rate of the parent strain was decreased by NH_4Cl (Fig. 4). Sulphate ion (mM) increased the effect of NH_4Cl on the variant (but not on the parent strain) starved at 37° or 40° (Table 2); magnesium ion (mM) slightly decreased but did not abolish either the effect of NH_4Cl or of NH_4Cl + sulphate ion on the starved variant (Table 2). Apparently continuous growth in N-limiting medium in a chemostat was necessary before the variant strain became susceptible to 'ammonium-accelerated death'. NH_4Cl had no effect on the survival of starved

stationary phase variant organisms grown in N-deficient medium in batch cultures (see below) and protected starved glycerol-limited variant organisms grown at a dilution rate of 0·25 hr^{-1}.

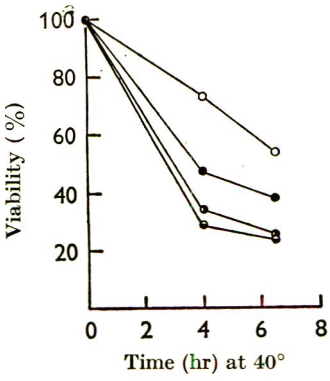

Fig. 3

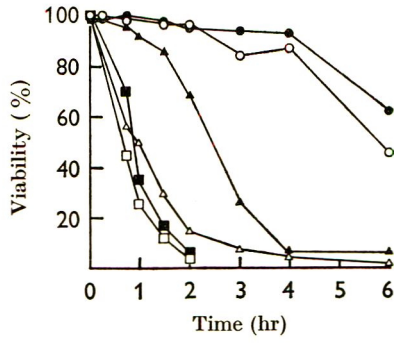

Fig. 4

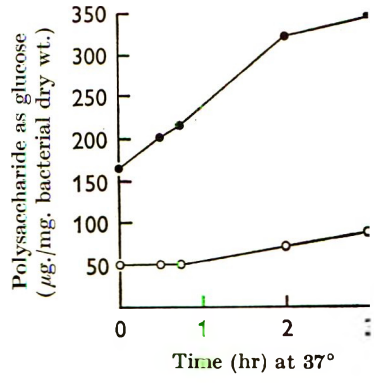

Fig. 5

Fig. 3. Effect of increasing NH_4Cl on the death rate of N-limited variant of *Aerobacter aerogenes* in 'Specpure' saline phosphate at 40°. Bacteria growing continuously at 40° (dilution rate, 0·25 hr^{-1}) were washed and resuspended at $4·4 \times 10^7$ bacteria/ml. in aerated saline phosphate with and without NH_4Cl at 40°. Viabilities (viable counts) at intervals of suspensions in buffer alone (○) and with 10 mM (●), 25 mM (◑), 50 and 100 mM-NH_4Cl (◒).

Fig. 4. Effect of NH_4Cl on the death rate of N-limited parent strain of *Aerobacter aerogenes* in saline phosphate at 43–47°. Bacteria growing at 40° in a chemostat (dilution rate, 0·23 hr^{-1}) were washed and starved (at 3×10^7 bacteria/ml.) in aerated saline phosphate 2 with and without NH_4Cl (10 mM) at 43° (○, ●), 45° (△, ▲) and 47° (□, ■); open symbols, buffer; closed symbols, buffer + NH_4Cl. Viabilities were determined at intervals by slide culture.

Fig. 5. Polysaccharide synthesis by washed suspensions of parent and variant strains of *Aerobacter aerogenes* in the presence of glycerol. Bacteria growing in N-limiting medium in chemostats (dilution rate, 0·25 hr^{-1}) were washed and resuspended (equiv. 580 μg. dry weight bacteria/ml.) in aerated 0·067 M-sodium potassium phosphate buffer (pH 7·0) containing mM-$MgSO_4$ and 0·5 % (w/v) glycerol at 37°. Samples were taken at intervals from suspensions of variant (○) and parent strain (●) for carbohydrate determination. Rates of polysaccharide synthesis in spent medium + glycerol were similar to those shown.

Polysaccharide synthesis by bacteria grown under N-limiting conditions in chemostats and in N-deficient medium in batch cultures

The previously reported polysaccharide content values of the variant and parent strains of *Aerobacter aerogenes* when growing in continuous culture at 40° and 37°, respectively, under N-limiting conditions (Postgate & Hunter, 1962; Strange & Dark, 1965) were confirmed: at a dilution rate near 0·25 hr^{-1}, the variant contained < 5 % polysaccharide and the parent strain contained 12–16 % polysaccharide. Increasing the glycerol concentration in the medium from 0·2 to 1·0 % (w/v) or decreasing the growth temperature to 37° had little effect on the polysaccharide content of the variant and did not abolish its 'ammonium-accelerated death'; increasing the growth temperature of the parent strain to 40° did not decrease the polysaccharide content nor make the organisms susceptible to ammonium ion during starvation. A comparison of the rates of polysaccharide synthesis by these organisms

in spent medium and in phosphate buffer (0·067 M) + mM-$MgSO_4$ containing glycerol was therefore made (Fig. 5). In 2 to 3 hr at 37° the polysaccharide synthesized by the variant was only 20–25 % of that produced by the parent strain. However, despite the slow rate of polysaccharide synthesis by the variant during and after continuous growth in N-limiting medium, in N-deficient medium containing 1 % (w/v) glycerol in shaken flask cultures at 37° for 20 hr, variant organisms containing 25 % or more polysaccharide were produced. The polysaccharide-rich variants of *A. aerogenes* survived much better in saline phosphate than did the continuously growing N-limited variants and its survival was unaffected by NH_4Cl during 96 hr at 37° (Table 3). Neither the parent strain *A. aerogenes* nor *Escherichia coli* NCTC 8164 grown in N-deficient medium in batch cultures, suffered 'ammonium-accelerated death'.

Table 3. *Effect of NH_4Cl on stationary phase variants of Aerobacter aerogenes grown in N-deficient medium (1 %, w/v, glycerol) in batch culture*

Washed bacteria (4×10^7/ml.) were starved in saline phosphate 2 with and without NH_4Cl in shaken flasks at 37°. Viabilities were determined at intervals by slide culture. Initially the bacteria contained 25 % total carbohydrate as glucose.

	Time (hr)			
	0	18	23	96
NH_4Cl	Viability (%)			
Nil	100	90	88	45
25 mM	100	94	90	44

DISCUSSION

The apparent disagreement between Postgate & Hunter (1964) and Strange & Dark (1965) about 'ammonium-accelerated death' of *Aerobacter aerogenes* was due to differences in the properties of the parent strain and the variant derived from it during continuous culture. The sensitivity of the N-limited variant to ammonium ion during starvation may be associated with a low content of polysaccharide reserve material as compared with that in the parent strain *A. aerogenes* and in other bacteria grown under N-limiting conditions (Holme & Palmstierna, 1956; Holme, 1957; Wilkinson, 1959). Starved polysaccharide-rich variant organisms, grown in N-deficient batch culture, were insensitive to ammonium ion. However, lack of polysaccharide does not explain the lethal effect of ammonium ion on N-limited variant organisms. Glycerol-limited variants grown at a similar dilution rate also contained little carbohydrate but did not suffer 'ammonium-accelerated death'. Apparently nitrogen limitation does affect the physiology of the organisms so that they may become sensitive to ammonium ion in the absence of a carbon source; perhaps when polysaccharide reserves are present, these supply a source of carbon and energy which allows balanced metabolism to occur, like that during normal growth. If this be true then 'ammonium-accelerated death' will not be observed with members of the Enterobacteriaceae which lay down polysaccharide reserves under conditions of nitrogen limitation.

REFERENCES

HOLME, T. (1957). *Bacterial Synthesis During Limited Growth. The Formation of Glycogen and of Nitrogenous Compounds in Escherichia coli* B *as Influenced by Nutritional Deficiencies.* Uppsala: Almqvist and Wiksells Boktryckeri Ab.

HOLME, T. & PALMSTIERNA, H. (1956). Changes in glycogen and nitrogen-containing compounds in *Escherichia coli* B during growth in deficient media. I. Nitrogen and carbon starvation. *Acta chem. scand.* **10**, 578.

POSTGATE, J. R., CRUMPTON, J. E. & HUNTER, J. R. (1961). The measurement of bacterial viabilities by slide culture. *J. gen. Microbiol.* **24**, 15.

POSTGATE, J. R. & HUNTER, J. R. (1962). The survival of starved bacteria. *J. gen. Microbiol.* **29**, 233.

POSTGATE, J. R. & HUNTER, J. R. (1963*a*). Acceleration of bacterial death by growth substrates. *Nature, Lond.* **198**, 273.

POSTGATE, J. R. & HUNTER, J. R. (1963*b*). The survival of starved bacteria. *J. appl. Bact.* **26**, 295.

POSTGATE, J. R. & HUNTER, J. R. (1964). Accelerated death of *Aerobacter aerogenes* in the presence of growth-limiting substrates. *J. gen. Microbiol.* **34**, 459.

STRANGE, R. E. & DARK, F. A. (1965). 'Substrate-accelerated death' of *Aerobacter aerogenes*. *J. gen. Microbiol.* **39**, 215.

STRANGE, R. E., DARK, F. A. & NESS, A. G. (1961). The survival of stationary phase *Aerobacter aerogenes* stored in aqueous suspension. *J. gen. Microbiol.* **25**, 61.

WILKINSON, J. F. (1959). The problem of energy-storage compounds in bacteria. *Exp. Cell. Res.* (Suppl.) **7**, 111.

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The Antigenic Relationship of Strains of *Trypanosoma brucei* Isolated in Nigeria

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SUMMARY

The relationships of eleven strains of *Trypanosoma brucei* were determined by agglutination tests with antisera to the basic and predominant antigens of the strains to assess (*a*) the relative values of these antigens for classification purposes, and (*b*) the antigenic diversity of *T. brucei* in Nigeria. The indications of the antigenic relationships of the strains obtained by comparisons based on both types of antigens were very similar, but comparisons of predominant strain antigens were easier to make and were applicable to more strains than were comparisons of basic strain antigens. Clones prepared from four strains isolated in one place in Nigeria were antigenically related, but clones of two other strains from the same area were antigenically distinct. Six strains from widely separated localities were antigenically different and produced few antigens in common.

INTRODUCTION

The numbers of antigenically distinct strains of the common species of pathogenic trypanosomes which can be isolated in the African continent, or in any well-defined geographical locality, have not yet been determined. The collection of such epidemiological information is still generally limited by the variability of trypanosomal antigens and, particularly in the cases of *Trypanosoma vivax* and *T. congolense*, by inadequate methods for isolating and growing strains in the laboratory. Knowledge of the properties of the antigens of *T. brucei*, *T. rhodesiense* and *T. gambiense*, which comprise the 'brucei' subgroup of trypanosomes (Hoare, 1957), has been greatly increased by several recent studies (e.g. Soltys, 1957; Weitz, 1960; Brown & Williamson, 1962; Cunningham & Vickerman, 1962; Seed, 1963), and work on antigenic variation has indicated two ways in which it may be possible to type the more pathogenic strains of these species (Broom & Brown, 1940; Gray, 1965*a*).

First, Broom & Brown (1940) found that although extensive antigenic variation occurred in a strain of *Trypanosoma brucei* in different infected animals, the trypanosomes transmitted by tsetse flies which had fed on such animals were serologically closely related. It has subsequently been shown that such cyclically-transmitted substrains of a strain have at least one antigen in common which has been called the *basic antigen* of the strain (Gray, 1965*a*). Preliminary results have shown that the basic antigens of strains from different localities are antigenically distinct (Broom & Brown, 1940; Gray, 1963), and it has been proposed that they may provide the basis for a serological comparison of strains (Brown, 1963; Gray, 1965*a*).

Secondly, each strain of *Trypanosoma brucei* also produces a group of variant antigens which have been described as *predominant antigens*. These trypanosomal antigens develop at an early stage of infection in animals infected with a strain either by tsetse flies or with strain variants transmitted by syringe (Gray, 1965*a*, *b*). The serological specificity of the predominant antigens of different strains has not yet been determined, but if they are strain specific they may provide a means of classifying strains which would avoid the difficulties associated with transmitting trypanosomes through tsetse flies (see Fairbairn & Culwick, 1950). It might also be possible to determine, at least in part, the antigenic relationships of many strains of low virulence, from which antigens for serological tests can only be prepared with considerable difficulty, by testing antisera to such strains for antibodies to predominant antigens of more virulent strains.

This paper describes experiments in which the serological relationships of eleven strains of 'brucei' subgroup trypanosomes were determined by comparing their basic and predominant antigens to explore these potential means of classification and to assess the degree of antigenic diversity of *Trypanosoma brucei* in Nigeria.

METHODS

Strains of trypanosomes

The strains consisted of polymorphic 'brucei' subgroup trypanosomes isolated from domestic livestock by injecting infected blood into rats. With the exceptions of strains 17/8 and 17/58 (see Table 1), the trypanosomes were very pathogenic for mice and rats, causing acute infections, massive parasitaemias and death after 2–3 days. The strains had all been subpassaged by syringe in mice and rats several times between original isolation and their selection for use in the present work. Stocks of the strains were preserved at $-80°$ during the experiments and further passage in mice and rats was limited to the procedures involved in preparing antigens for agglutination tests, agglutinating antisera, and in some instances, clones.

Two groups of strains were used in experiments.

(*a*) *Strains from one area.* Six strains were isolated from different animals during a survey of the incidence of trypanosomiasis in a herd of cattle kept for several years on a Government Farm at Raav, in Benue Province, Northern Nigeria (Fig. 1). Clones were prepared from each of these strains by the method of Inoki (1960) and used to assess the antigenic diversity of *Trypanosoma brucei* in a small area.

(*b*) *Strains from different areas.* Six strains, one of which (17/m) was also used in the preceding group, were obtained from different hosts infected in widely separated places in Nigeria (Fig. 1). Clones prepared from two of these strains, and ordinary populations of trypanosomes chosen at random from rodent-passaged lines of the other four strains, were used in experiments to assess the antigenic diversity of *T. brucei* in different areas of Nigeria.

Species of the trypanosomes. Although there have been several recent reports of attempts to differentiate between the three morphologically identical species *Trypanosoma brucei*, *T. gambiense* and *T. rhodesiense* by cultural, drug-sensitivity and physico-chemical tests (Lehmann, 1964*a*, *b*), it is still generally accepted that *T. brucei* can only be distinguished from the other two species with certainty by its

inability to infect man (Ashcroft, 1959). This requirement was satisfied with only one of the eleven strains used; strain 8/18 was non-infective for human volunteers (Godfrey, 1962), and was therefore an authentic strain of *T. brucei*. It may be assumed on epidemiological grounds, however, that the other ten strains were *T. brucei* because they were all isolated from domestic animals in Nigeria, where *T. rhodesiense* does not occur and *T. gambiense* has not been isolated from any naturally-infected vertebrate host other than man.

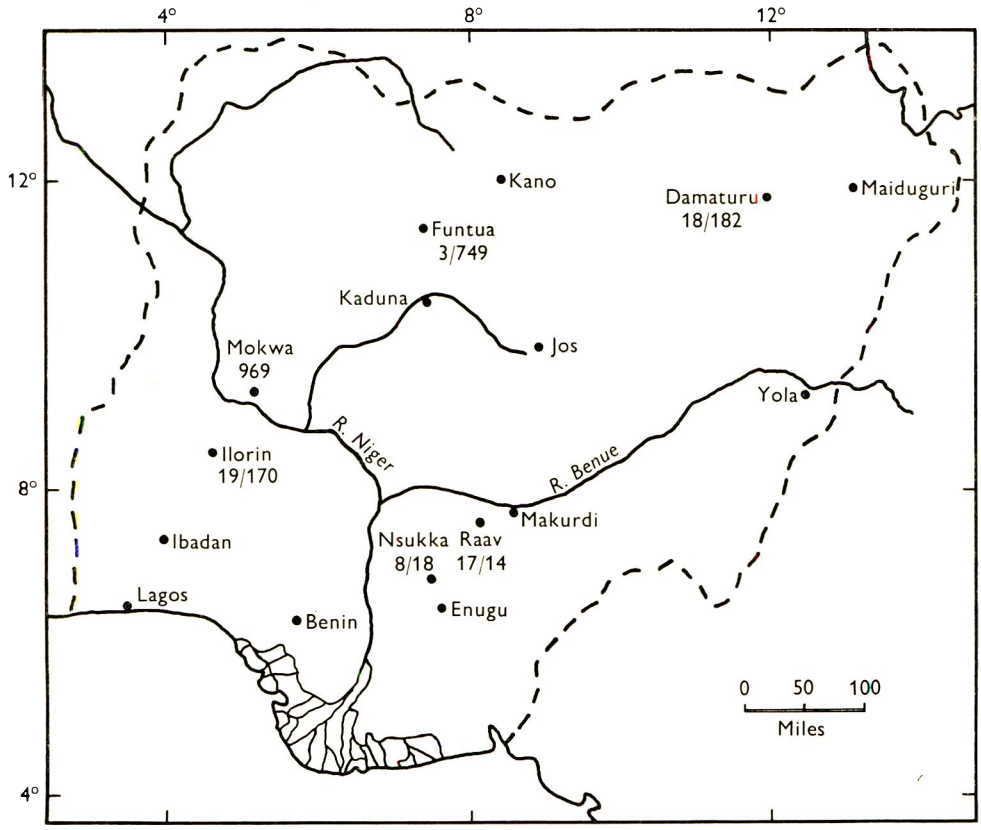

Fig. 1. Map of Nigeria showing places where strains of *Trypanosoma brucei* were isolated.

Determination of the antigenic relationships of the strains

The antigenic relationships of the strains were determined by agglutination tests using the method of Cunningham & Vickerman (1962) with minor modifications (Gray, 1962).

Antigens for agglutination tests. Attempts were made to infect *Glossina morsitans* and *G. palpalis* with nine of the strains to obtain trypanosomes with basic antigens for use as antigens in agglutination tests. No attempt was made to infect tsetse flies with the clones prepared from strains 17/8 and 17/58 because it was impossible to collect sufficient trypanosomes for agglutination tests on account of the low virulence of the strains for mice.

Glossina morsitans were infected with six of the nine strains and, in these instances,

antigens for agglutination tests were prepared by the method of Cunningham & Vickerman (1962), from cyclically-transmitted trypanosomes isolated during the first 5 days of infections from animals infected by the tsetse flies; these trypanosomes by definition thus possessed basic antigens of the strains. In the other three instances, cyclical transmission was not achieved and antigens for agglutination tests were prepared from syringe-passaged lines of trypanosomes in the hope that they possessed predominant antigens of the strains. The nature of the population of each strain from which an antigen was prepared is shown in Table 1.

Agglutinating antisera. Agglutinating antisera were prepared in rabbits as follows and stored at $-20°$ until required.

(*a*) *Antisera to strains from one area.* Antisera to the strains isolated from the cattle at Raav were prepared in two ways.

Method 1. Tsetse flies infected with the four more pathogenic clones (17/14, 17/30, 17/70, 17/96) were each fed once on separate rabbits from which sera were collected before infection and afterwards at intervals of 2–3 days for a month. Agglutination tests showed that sera obtained from these animals 10 days after they were bitten by infective flies had high titres of agglutinins to the basic antigens of the infecting trypanosomes.

Method 2. Suspensions of trypanosomes of the six clones were prepared from the blood of infected mice and used to infect rabbits. Each rabbit was given approximately 10 million trypanosomes of one of the clones as a single intravenous injection in 2·0 ml. of Alsever solution. Sera were collected from the rabbits before infection and afterwards at weekly intervals for a month. Thus, although it was not possible to prepare antigens for agglutination tests from clones 17/8 and 17/58 because of their low virulence for mice, enough trypanosomes were obtained to infect rabbits and thus to prepare antisera. Agglutinating antibodies to the variant antigens of trypanosomes persist at a high value in infected animals for several weeks and antisera obtained 3–4 weeks after infection contained antibodies to many of the antigens of the infecting strain including its predominant antigens.

(*b*) *Strains from different areas.* Antisera to the predominant antigens of this group of strains were prepared as described in *Method 2* above.

RESULTS

The antigenic relationships of strains from one area

The clones prepared from four of the six strains isolated in one area were antigenically related to each other (Table 2). The cyclically-transmitted trypanosomes of clones 17/14 and 17/30 behaved in a similar manner when agglutinated with antisera obtained from rabbits 10 days after infection by tsetse flies infected with the clones; these antisera contained antibodies to the basic antigens of the trypanosomes. The cyclically-transmitted trypanosomes of clones 17/70 and 17/96 differed from each other, and from those of clones 17/14 and 17/30, since 10-day antisera from rabbits infected by tsetse flies with clones 17/70 and 17/96 reacted mainly with the homologous trypanosomes. Although there were at least three antigenic types of trypanosomes in the cyclically-transmitted populations of clones 17/14, 17/30, 17/70 and 17/96, the clones were all able to produce similar antigens, because antisera obtained from rabbits 4 weeks after infection agglutinated the infecting clone and the three

Table 1. *The origins and characteristics of strains of Trypanosoma brucei*

Group of strains	Identity of strains	Origin of strains (see Fig. 1)	Original host	Date isolated	Passages in rats and mice between isolation and use in experiments	Population of strain used in experiments
Strains from one area	17/14	Raav	Ox	28. xi. 63	15	Cyclically-transmitted clone
	17/30	Raav	Ox	28. xi. 63	14	Cyclically-transmitted clone
	17/70	Raav	Ox	28. xi. 63	13	Cyclically-transmitted clone
	17/96	Raav	Ox	28. xi. 63	14	Cyclically-transmitted clone
	17/8	Raav	Ox	28. xi. 63	15	Syringe-passaged clone†
	17/58	Raav	Ox	28. xi. 63	14	Syringe-passaged clone†
Strains from different areas	969	Mokwa	Sheep	22. v. 61	19	Cyclically-transmitted line
	3/749	Funtua	Ox	22. iii. 61	*c.* 170	Syringe-passaged line
	8/18*	Nsukka	Pig	12. ix. 62	13	Cyclically-transmitted line
	17/14	Raav	Ox	28. xi. 63	15	Cyclically-transmitted clone
	18/182	Damaturu	Ox	8. ii. 64	8	Syringe-passaged clone
	19/170	Ilorin	Ox	24. iii. 64	8	Syringe-passaged line

* Authentic *Trypanosoma brucei*; † antigen not prepared.

heterologous clones. The antigenic similarities of clones 17/14, 17/30, 17/70 and 17/96 were confirmed by the reactions of antisera taken from rabbits 2 and 4 weeks after they had been infected with the clones by syringe (Table 3).

The clones prepared from strains 17/8 and 17/58 were not closely related to clones 17/14, 17/30, 17/70 and 17/96 because antisera from rabbits infected by syringe with clones 17/8 and 17/58 did not react strongly with the antigens of any of the other four clones (Table 3).

Table 2. *Agglutination of clones of Trypanosoma brucei prepared from strains isolated in one area in Nigeria by antisera from rabbits infected with the clones by tsetse flies*

Antigens prepared from clones	Titres of agglutinins in antisera to clones (as reciprocals of serum dilutions)			
	17/14	17/30	17/70	17/96
	antisera obtained 10 days after infection			
17/14	160	2560	80	—
17/30	80	1280	160	10
17/70	—	40	5120	—
17/96	—	—	—	320
	antisera obtained 4 weeks after infection			
17/14	160	1280	160	320
17/30	320	1280	80	160
17/70	320	640	640	640
17/96	160	80	40	2560

— = no agglutination.

Table 3. *Agglutination of clones of Trypanosoma brucei prepared from strains isolated in one area in Nigeria by antisera from rabbits infected with the clones by syringe*

Antigens prepared from clones	Titres of agglutinins in antisera to clones (as reciprocals of serum dilutions)					
	17/14	17/30	17/70	17/96	17/8	17/58
	antisera obtained 2 weeks after infection					
17/14	5120	5120	640	5120	—	—
17/30	5120	2560	640	2560	—	—
17/70	20	20	1280	80	80	40
17/96	10	20	10	5120	20	—
	antisera obtained 4 weeks after infection					
17/14	1280	5120	80	640	—	—
17/30	640	2560	80	640	—	—
17/70	320	320	160	160	40	10
17/96	320	—	160	2560	20	—

— = no agglutination.

The antigenic relationships of strains from different areas

The strains of *Trypanosoma brucei* from widely separated areas in Nigeria had few antigens in common. Antisera obtained from rabbits after 2 weeks of infection, which had high titres of agglutinins to the infecting strain, did not react with, or had much lower titres to, other strains (Table 4). Sera obtained from the rabbits 1–2 weeks later, when there had been ample time for antigenic variation in the strains

and for antibody production to predominant variant antigens, also, in general, did not react strongly with the antigens of heterologous strains. In each case the titre of agglutinins to the homologous strain had decreased and a noteworthy increase of antibody titre to a heterologous strain occurred in only one instance. The rabbit infected with strain 969 produced a high titre of agglutinins to strain 19/170, indicating that an antigen related to one of those of strain 19/170 had developed during the infection.

Table 4. *Agglutination of strains of Trypanosoma brucei isolated in different places in Nigeria by antisera from rabbits infected with the strains by syringe*

Antigens prepared from strains	Titres of agglutinins in antisera to strains (as reciprocals of serum dilutions)					
	969	3/749	8/18	17/14	18/182	19/170
	antisera obtained 2 weeks after infection					
969	5120	—	—	—	—	160
3/749	160	5120	—	—	—	—
8/18	—	—	5120	—	—	—
17/14	80	—	—	5120	—	160
18/182	—	—	—	—	1280	—
19/170	—	—	80	—	10	1280
	antisera obtained 3–4 weeks after infection					
969	320	—	—	20	—	10
3/749	40	1280	—	80	—	—
8/18	—	—	640	—	—	—
17/14	160	—	40	1280	—	320
18/182	—	—	—	—	160	—
19/170	640	—	40	10	—	160

— = no agglutination.

DISCUSSION

Although there are many difficulties associated with transmitting 'brucei' subgroup trypanosomes by tsetse flies in the laboratory (see Fairbairn & Culwick, 1950), comparisons of the basic antigens of strains, which are associated with cyclically-transmitted trypanosomes, may yet prove to be a useful method of determining strain relationships. It was found by this means that four clones prepared from trypanosomes isolated from different animals in one herd were antigenically related but not identical, and that three strains of trypanosomes isolated in different places in Nigeria were antigenically distinct. The former result supports the idea expressed earlier (Gray, 1965*a*), that it may be possible to trace strains in epidemiological studies by means of their basic antigens.

Comparisons based on the predominant antigens of trypanosomes, however, appear to offer a more practical and easier means of general classification than comparisons which depend on basic strain antigens. In every instance, indications of antigenic relationships of strains of *Trypanosoma brucei* from comparisons of their basic antigens were fully confirmed by agglutination tests with antisera to their predominant antigens, showing that predominant antigens, as well as basic antigens, are characteristic for each strain. It was also possible to determine, on the basis of predominant antigens, the antigenic relationships of three strains which could not be transmitted by tsetse flies and those of two strains which caused only

mild infections in mice. It became apparent when studying the relationships of four antigenically similar clones, that more work must be done to develop methods of finding whether strains which are closely related in terms of agglutination tests are antigenically identical or whether they only produce a number of variant antigens in common.

In view of the established capacity of trypanosomes for antigenic variation, and the numerous different antigens a strain can produce (e.g. Ritz, 1916; Osaki, 1959), it was of considerable interest that clones prepared from four strains of *Trypanosoma brucei* isolated in one area were antigenically related. This result is similar to those of Cunningham & Vickerman (1962) and Weitz (1962) who found that several strains of *T. brucei* isolated in South East Uganda had antigens in common. There may thus be some hope of success when attempts are made to immunize animals against a single trypanosomal species in a restricted area. In contrast to these findings, strains of *T. brucei* from widely separated places in Nigeria showed little antigenic relationship. Other authors have also found considerable antigenic differences between strains of *T. brucei* from different geographical localities (Broom & Brown, 1940), and the antigenic dissimilarity of the strains used in the present experiments is supported by experience in the field where it is generally recognized that strains encountered in different areas are liable to be immunologically distinct (see Hornby, 1941).

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REFERENCES

ASHCROFT, M. T. (1959). A critical review of the epidemiology of human trypanosomiasis in Africa. *Trop. Dis. Bull.* **56**, 1074.

BROOM, J. C. & BROWN, H. C. (1940). Studies in trypanosomiasis. IV. Notes on the serological characters of *Trypanosoma brucei* after cyclical development in *Glossina morsitans*. *Trans. roy. Soc. trop. Med. Hyg.* **34**, 53.

BROWN, K. N. (1963). The antigenic character of the *Brucei* trypanosomes. In *Immunity to Protozoa. A Symposium of the British Society for Immunology*, p. 204. Oxford: Blackwell Scientific Publications.

BROWN, K. N. & WILLIAMSON, J. (1962). Antigens of *Brucei* trypanosomes. *Nature, Lond.* **194**, 1253.

CUNNINGHAM, M. P. & VICKERMAN, K. (1962). Antigenic analysis in the *Trypanosoma brucei* group, using the agglutination reaction. *Trans. roy. Soc. trop. Med. Hyg.* **56**, 48.

FAIRBAIRN, H. & CULWICK, A. T. (1950). The transmission of the polymorphic trypanosomes. *Acta trop. Basel*, **7**, 19.

GODFREY, D. G. (1962). Experiments with *Trypanosoma brucei*. The authentication of isolation 8/18. In WILLETT, K. C. *The Annual Report of the West African Institute for Trypanosomiasis Research for 1962*, p. 14. London: Harrison and Sons Ltd.

GRAY, A. R. (1962). The influence of antibody on serological variation in *Trypanosoma brucei*. *Ann. trop. Med. Parasit.* **56**, 4.

GRAY, A. R. (1963). *Antigenic Variation in Some Trypanosomes of the Brucei Group*. Thesis, University of London.

GRAY, A. R. (1965*a*). Antigenic variation in a strain of *Trypanosoma brucei* transmitted by *Glossina morsitans* and *G. palpalis*. *J. gen. Microbiol.* **41**, 195.

GRAY, A. R. (1965*b*). Antigenic variation in clones of *Trypanosoma brucei*. I. Immunological relationships of the clones. *Ann. trop. Med. Parasit.* **59**, 27.

HOARE, C. A. (1957). The classification of trypanosomes of veterinary and medical importance. *Vet. Rev. Annot.* **3**, 1.

HORNBY, H. E. (1941). Immunization against bovine trypanosomiasis. *Trans. roy. Soc. trop. Med. Hyg.* **35**, 165.

INOKI, S. (1960). Studies on antigenic variation in the Welcome strain of *Trypanosoma gambiense*. I. Improvements in technique. *Biken's J.* **3**, 215.

LEHMANN, D. L. (1964*a*). Some physico-chemical means and a cultural method of differentiating *Trypanosoma rhodesiense*, *T. brucei* and *T. congolense*. *Ann. trop. Med. Parasit.* **58**, 9.

LEHMANN, D. L. (1964*b*). The selective action in culture of some trypanocidal drugs on *Trypanosoma rhodesiense* and *T. brucei*. *Ann. trop. Med. Parasit.* **58**, 189.

OSAKI, H. (1959). Studies on the immunological variation in *Trypanosoma gambiense* (serotypes and mode of relapse). *Biken's J.* **2**, 113.

RITZ, H. (1916). Über Rezidive bei experimenteller Trypanosomiasis. II. Mitteilung. *Arch. Schiffs-u. Tropenhyg.* **20**, 397. Abstracted in *Trop. Dis. Bull.* 1917, **9**, 341.

SEED, J. R. (1963). The characterization of antigens isolated from *Trypanosoma rhodesiense*. *J. Protozool.* **10**, 380.

SOLTYS, M. A. (1957). Immunity in trypanosomiasis. II. Agglutination reaction with African trypanosomes. *Parasitology*, **47**, 390.

WEITZ, B. (1960). The properties of some antigens of *Trypanosoma brucei*. *J. gen. Microbiol.* **23**, 589.

WEITZ, B. (1962). Immunity in trypanosomiasis. In *Drugs, Parasites and Hosts. A Biological Council Symposium*, p. 180. London: J. and A. Churchill Ltd.

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Variation in Content and Distribution of Magnesium, and its Influence on Survival, in *Aerobacter aerogenes* Grown in a Chemostat

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SUMMARY

The magnesium and RNA contents of *Aerobacter aerogenes*, growth-limited by Mg^{2+}, K^+, NH_4^+ or carbon source, in defined media at 35° increased with growth rate. The results support the view that the amounts of these constituents are functions of the growth rate and are inter-dependent. Up to 26 % of the total Mg^{2+} of bacteria freshly harvested from cultures containing excess magnesium was loosely bound to the bacterial surface; this adsorbed Mg^{2+} was removed by washing with 0·85 % (w/v) NaCl but was unaffected by distilled water. Mg^{2+}-limited bacteria had no surface-adsorbed magnesium. Surface-adsorbed Mg^{2+} stimulated polysaccharide synthesis, and affected the response of bacteria in saline buffer to stresses including starvation, heat-accelerated and substrate-accelerated death, and cold shock.

INTRODUCTION

When the growth rate of *Aerobacter aerogenes* cultures in a chemostat was limited by the supply of Mg^{2+}, the bacterial concentration varied with dilution rate (Tempest, Hunter & Sykes, 1965) which, assuming quantitative uptake of Mg^{2+} from the medium, indicated a variation in cellular Mg^{2+} content with growth rate. This assumption has now been confirmed by direct determination of the distribution of Mg^{2+} in Mg^{2+}-limited cultures by the Titan Yellow method of Gardner (1946) which, when used as described below, gave accurate and reliable results. Furthermore, it was found that the Mg^{2+} content of bacteria growth-limited by substances other than Mg^{2+}, increased with the growth rate. The Mg^{2+} associated with bacteria growth-limited by substances other than Mg^{2+} included some Mg^{2+} that was loosely bound; this 'adsorbed Mg^{2+}' could be removed by washing with salt solution (Strange & Shon, 1964). Adsorbed Mg^{2+} affected the response of the bacteria to subsequent stresses. The present paper records data which indicate a relationship between the intracellular Mg^{2+} and RNA contents of *A. aerogenes* grown under a variety of growth-limiting conditions and presents evidence which shows that surface-adsorbed magnesium is advantageous for the survival of the organisms in unfavourable conditions.

METHODS

Organism and cultural conditions. Aerobacter aerogenes (NCTC 418) was grown in 0·5 l. Porton-type chemostats (Herbert, Phipps & Tempest, 1965) in defined media containing growth-limiting concentrations of Mg^{2+}, glycerol (Tempest *et al.* 1965)

or K^+. The K^+-limited medium contained: Na_2HPO_4, $5{\cdot}0 \times 10^{-3}$ M; $(NH_4)\,H_2PO_4$, $4{\cdot}5 \times 10^{-2}$ M; $(NH_4)_2SO_4$, $2{\cdot}5 \times 10^{-2}$ M; citric acid, $1{\cdot}0 \times 10^{-3}$ M; $MgCl_2$ $1{\cdot}25 \times 10^{-3}$ M; $CaCl_2$ and $FeCl_3$, each $1{\cdot}0 \times 10^{-4}$ M; trace amounts of Mn^{2+}, Cu^{2+} and $NaMoO_4$. Glycerol was added to a final concentration of 30 mg./ml., and K_2SO_4 to $5{\cdot}0 \times 10^{-4}$ M. In addition, ammonium-limited and carbon-limited cultures of *A. aerogenes* were grown in defined media in a smaller chemostat (designed by Dr D. Herbert) as described by Postgate & Hunter (1962), but with a culture volume of 0·25 l. In each case, the dilution rate was progressively increased from 0·1 to 0·8 vol./hr and samples were taken (see below) at several intermediate 'steady state' growth-rate values. Exponential phase *A. aerogenes* organisms were harvested from shaken batch cultures of the organisms in a defined medium containing mannitol (Strange & Dark, 1962).

Survival studies. Washed suspensions of bacteria were subjected to heat stress at 48° as described by Strange & Shon (1964), to carbon substrate accelerated death (Postgate & Hunter, 1963; 1964) as described by Strange & Dark (1965), and to chilling at 0° as described by Strange & Dark (1962). Viabilities were determined with the slide culture method of Postgate, Crumpton & Hunter (1961), with an enriched agar medium similar to theirs except that glucose (0·2 %, w/v) replaced glycerol. Saline phosphate contained 0·108 M-NaCl and 0·02 M-potassium phosphate buffer (pH 6·5).

Analytical methods. Magnesium in bacteria and culture filtrates was determined by the Titan Yellow colorimetric method (Gardner, 1946). Mg^{2+} was extracted from bacteria as follows. Duplicate samples of culture (10 ml. containing equiv. 1–10 mg. dry wt bacteria/ml.) were centrifuged at 3000*g* for 10 min.; the packed cell pellets were suspended in a suitable diluent (see Results) and re-sedimented by centrifugation. The washed pellets were resuspended in ice-cold water (2·5 ml.) and cold 2 N-$HClO_4$ (2·5 ml.) added; after standing at 4° for 15 min., the suspensions were centrifuged and the clear extracts decanted into 10 ml. graduated test tubes. The pellets were re-extracted with cold N-$HClO_4$ (4 ml.) as before and the combined extracts diluted to 10 ml. with N-$HClO_4$. Total removal of Mg^{2+} from the organisms was confirmed by wet ashing the pellets followed by acid extraction and analysis for Mg^{2+}. The Mg^{2+}-content of $HClO_4$-extracts and culture filtrates was determined as follows. Reaction mixtures containing (final vol., 2·5 ml.) 1 m-mole $HClO_4$, 0·4 mg. gum ghatti, 0·15 mg. Titan Yellow and 2–12 μg. Mg^{2+} were prepared; colour was developed by adding 1 ml. 3 N-NaOH and measured after exactly 5 min. with a Bausch & Lomb 'Spectronic 20' spectrophotometer at 540 mμ. The amount of $HClO_4$ present in the reaction mixtures was important because colour formation decreased as its concentration increased. Under the conditions given, colour formed was directly proportional to Mg^{2+} concentrations between 2 and 12 μg. The presence of interfering substances in acid extracts was assessed by recovery of standard amounts of added Mg^{2+} from extracts. The only substance found seriously to interefere in the assay and which was present in significant concentration in some extracts was Polyglycol P-2000, a polypropylene glycol added to cultures to suppress foaming. This substance was quantitatively removed from extracts by two extractions with an equal volume of 'Analar' grade light petroleum (60–80° fraction). Bacterial dry weights, carbohydrate and RNA-contents were determined as previously described (Tempest *et al.* 1965).

RESULTS

Mg^{2+}-content of Aerobacter aerogenes as a function of growth rate and limiting substrate

Previous work (Strange & Shon, 1964) showed that *Aerobacter aerogenes* adsorbed Mg^{2+} from solutions of magnesium salts but this adsorption progressively decreased in the presence of increasing concentrations of Na^+ or K^+. It therefore seemed likely that bacteria separated from cultures containing excess Mg^{2+} would contain some Mg^{2+} adsorbed to their surface. This possibility was examined by washing samples of bacteria sedimented from cultures, with distilled water and parallel samples with 0·85% (w/v) NaCl, before acid extraction for Mg^{2+} assay. The results (Table 1) show that, with the exception of Mg^{2+}-limited organisms, the Mg^{2+} contents of water-washed bacteria were significantly higher than those of saline-washed bacteria.

Table 1. *Magnesium content of Mg^{2+}-, glycerol-, K^+- and NH_4^+-limited Aerobacter aerogenes grown at various dilution rates, and the effect of the washing procedure on adsorbed magnesium*

Samples of bacteria, separated from chemostat cultures, were washed with distilled water or 0·85% (w/v) NaCl and extracted with N-$HClO_4$ for Mg^{2+} assay (see Methods). For each growth limitation, figures in column (*a*) refer to Mg^{2+} content of water-washed bacteria, and in column (*b*) to Mg^{2+} content of saline-washed bacteria. All values are expressed as g. Mg^{2+}/100 g. dried organisms.

Dilution rate (hr^{-1})	Mg^{2+}-limited			Glycerol-limited			K^+-limited			NH_4^+-limited		
	(*a*)	(*b*)	(*a*/*b*)	(*a*)	(*b*)	(*a*/*b*)	(*a*)	(*b*)	(*a*/*b*)	(*a*)	(*b*)	(*a*/*b*)
0·1	0·101	0·101	1·00	0·151	0·130	1·16	0·148	0·124	1·19	0·192	0·175	1·10
	0·118	—	—	—	—	—	0·150	0·137	1·09	0·220	0·191	1·15
0·2	0·168	0·166	1·01	0·210	0·179	1·17	0·178	0·162	1·10	0·227	0·202	1·12
	0·156	—	—	—	—	—	0·182	0·156	1·17	0·264	0·233	1·13
	—	—	—	—	—	—	0·183	0·153	1·20	—	—	—
0·4	0·214	0·216	0·99	0·234	0·198	1·18	0·221	0·186	1·19	0·316	0·258	1·22
	0·220	—	—	0·244	0·208	1·17	0·223	0·186	1·19	—	—	—
	—	—	—	—	—	—	0·224	0·187	1·19	—	—	—
0·5	—	—	—	—	—	—	—	—	—	0·324	0·295	1·10
0·6	0·238	0·248	0·96	0·274	0·231	1·19	0·256	0·216	1·19	0·310	0·270	1·15
	0·246	0·249	0·99	—	—	—	0·240	0·208	1·16	—	—	—
	—	—	—	—	—	—	0·266	0·211	1·26	—	—	—
0·8	0·264	0·264	1·00	0·353	0·292	1·21	0·302	—	—	—	—	—
	0·284	—	—	0·319	0·300	1·06	—	—	—	—	—	—

Irrespective of the washing procedure, however, the concentration of Mg^{2+} in the organisms increased with the growth rate. The results also show that N-limited bacteria grown at low dilution rates contained significantly more magnesium than did bacteria growth-limited by other substances at equivalent dilution rates. It seems probable that the higher concentration of Mg^{2+} in slow growing N-limited bacteria was associated with the presence of a high concentration of bacterial glycogen. Determination of the RNA and Mg^{2+} contents of bacteria growth-limited by Mg^{2+}, K^+ and glycerol showed that the ratio RNA: Mg^{2+} varied only slightly with increasing dilution rate in the range 0·1–0·8 hr^{-1} (Table 2). The RNA content of Mg^{2+}-limited organisms growing at a dilution rate of 0·2 hr^{-1} decreased with

temperature (Tempest & Hunter, 1965) and, assuming complete uptake of Mg^{2+} from the environment, the Mg^{2+} content increased from 0·135 % (of bacterial dry weight) at 40° to 0·199 % at 25°; the corresponding increase in RNA content was from 9·4 to 14·9 %.

Table 2. *Cellular RNA : Mg^{2+} ratios in continuous cultures of Aerobacter aerogenes*

Samples of bacteria separated from chemostat cultures, were washed with 0·85 % (w/v) NaCl and extracted with ice-cold 0·5 N-$HClO_4$ and then with 0·5 N-$HClO_4$ at 80°. The RNA content of the 80° extract was determined by the orcinol method (see Methods). Similar samples of bacteria were assayed for Mg^{2+}.

Dilution rate (hr^{-1})	Mg^{2+}-limited organisms	Glycerol-limited organisms	K^+-limited organisms
	g. RNA/g. Mg^{2+}		
0·1	76	62	70
0·2	66	52	72
0·4	71	61	79
0·6	67	60	85
0·8	63	55	82

Effect of adsorbed Mg^{2+} on the physiology and survival of Aerobacter aerogenes

It seems probable that the Mg^{2+} which can be removed from glycerol-, K^+- or N-limited *Aerobacter aerogenes* by suspending the organisms in 0·85 % (w/v) NaCl solution is adsorbed to the surface layer of the organisms. The evidence supporting this assumption is that Mg^{2+} taken up by bacteria suspended in $MgSO_4$ solution is quantitatively removed by resuspension of the separated bacteria in 0·85 % (w/v) NaCl solution (Strange & Shon, 1964). The question arises whether this adsorbed Mg^{2+} serves a functional role in the organisms; various pieces of evidence suggest that it may well do so. For example (1) polysaccharide synthesis by washed suspensions of glycerol-limited *A. aerogenes* in the presence of glycerol was higher with bacteria washed with distilled water than with bacteria washed with saline (Fig. 1). (2) The death rate of water-washed mannitol-limited *A. aerogenes* in saline buffer (pH 6·5) at 40°, with or without mannitol (40 mM) was lower than that of saline buffer-washed bacteria (Fig. 2); the effect of the washing procedure on substrate-accelerated death (Postgate & Hunter, 1964) was very marked. (3) As shown previously (Strange & Shon, 1964), the washing procedure may affect the thermal resistance of bacteria; this was confirmed by heating water-washed and saline phosphate-washed mannitol-limited *A. aerogenes* in saline phosphate (pH 6·5) at 48°: the death rate of water-washed bacteria was lower than that of phosphate-washed bacteria (Fig. 3). To confirm that the lower heat resistance of saline phosphate-washed bacteria was due to loss of adsorbed Mg^{2+} during washing, bacteria were washed successively with saline phosphate (to desorb Mg^{2+}), mM-$MgSO_4$ and finally with distilled water (to remove excess $MgSO_4$). The survival characteristics of these bacteria were similar to those of water-washed bacteria (Fig. 3). Also, the presence of mM-$MgSO_4$ in saline phosphate during heating of saline phosphate-washed bacteria decreased the death rate (Fig. 3). (4) Sudden chilling caused loss of viability of suspensions of exponential phase *A. aerogenes* (Strange & Dark, 1962; Strange & Postgate, 1964) and it was found that the washing procedure affected

the susceptibility of the organisms to cold shock. Water-washed organisms were less susceptible to chilling in saline buffer (pH 6·5) than organisms washed with solutions of NaCl or sodium phosphate buffer (Fig. 4).

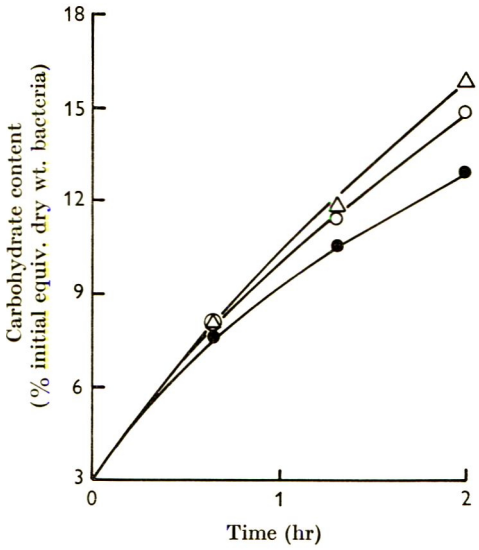

Fig. 1

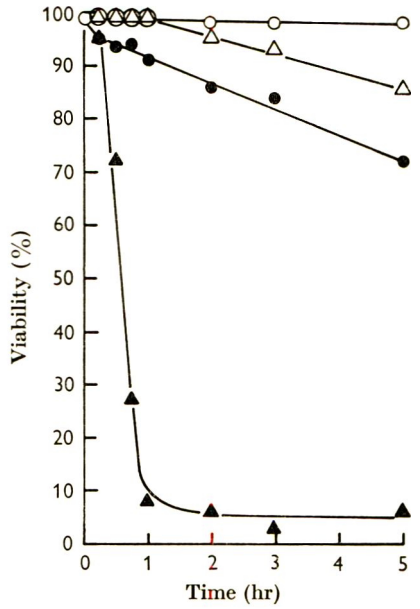

Fig. 2

Fig. 1. Carbohydrate synthesis in washed suspensions of glycerol-limited *Aerobacter aerogenes*. Organisms from a glycerol-limited culture (37°, dilution rate = 0·4 hr^{-1}) were separated from the culture by centrifugation (3000*g*, 5 min.) and washed either twice in water (○), twice in 0·85 % (w/v) NaCl(●) or once each, successively, in saline, 0·01 M-$MgCl_2$, water (△). Washed organisms were resuspended at a concentration equiv. 1 mg. dry wt. bacteria/ml. in 0·067 M-phosphate (pH 6·5) + 0·05 M glycerol and incubated at 37°, with aeration. Samples were analysed for carbohydrate as indicated in *Methods*.

Fig. 2. Survival of water-washed and saline phosphate-washed *Aerobacter aerogenes* at 40° in aerated saline phosphate (pH 6·5), with or without mannitol. Continuously grown mannitol-limited bacteria (dilution rate, 0·4 hr^{-1}) were washed twice with distilled water or saline phosphate and resuspended (about 4×10^8 bacteria/ml.) in aerated saline phosphate with and without mannitol (40 mM). Viabilities (slide culture) of suspensions of water-washed (○, ●) and saline phosphate-washed (△, ▲) bacteria. Open symbols, buffer; closed symbols, buffer plus mannitol.

DISCUSSION

The present results confirm previous reports that bacterial RNA (Herbert, 1961, Neidhardt, 1963) and magnesium (Tempest *et al.* 1965) increase with the growth rate of bacteria. The fact that the ratio of RNA:magnesium in *Aerobacter aerogenes* varied only slightly with dilution rate suggests that a large proportion of the magnesium was associated with the RNA. This view is strengthened by the finding (Tempest & Hunter, 1965) that when the RNA content of magnesium-limited bacteria growing at a fixed dilution rate was varied by altering the incubation temperature, a corresponding change in the magnesium content of the bacteria apparently occurred. Most of the cellular RNA is present in ribosomes which are

known to have magnesium as an integral part of their structure (Edelman, Ts'o & Vinograd, 1960; Rogers, 1964). However, proof that the magnesium requirement of bacteria increases with the growth rate mainly for the purpose of stabilizing an increasing number of ribosomes would require more precise data than those obtained here about the distribution of Mg^{2+} in bacteria. The present results show that in bacteria separated from environments containing an excess of magnesium, a proportion of their magnesium is loosely bound and removed by washing with saline, whereas magnesium-limited bacteria had no loosely bound magnesium associated

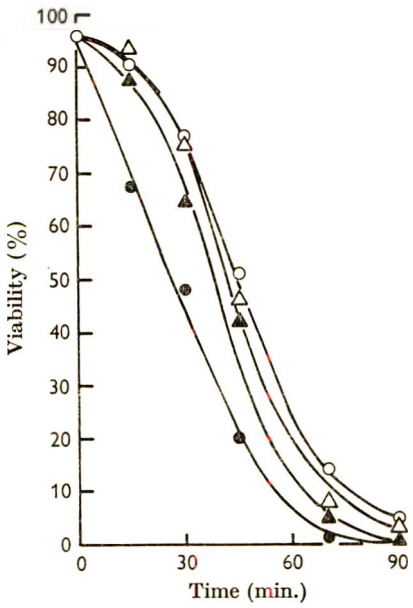

Fig. 3

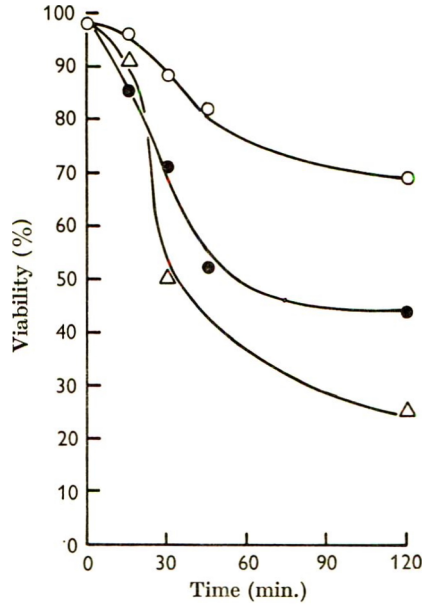

Fig. 4

Fig. 3. Survival of water-washed and saline phosphate-washed *Aerobacter aerogenes* at 48° in aerated saline phosphate (pH 6·5). Continuously grown mannitol-limited bacteria (dilution rate, 0·25 hr^{-1}) were washed: (*a*) three times with distilled water (○); (*b*) once with saline phosphate and twice with water (●, ▲); (*c*) successively with saline phosphate, 1 mM-$MgSO_4$ and water (△). The washed organisms were resuspended in water and samples were diluted 1/100 (final bacterial concentration about 3×10^8/ml.) in aerated saline phosphate, with (▲) and without 1 mM-$MgSO_4$, at 48°. Viabilities were determined by slide culture.

Fig. 4. Effect of washing procedure on the susceptibility of exponential phase *Aerobacter aerogenes* to chilling at 0°. Bacteria growing exponentially in shaken flasks of defined medium at 37° were separated and washed with distilled water (○), 0·15 M-NaCl (●) or 0·075 M-sodium phosphate buffer (pH 6·5) (△). Samples of the washed organisms suspended in the washing liquid were diluted 1/40 (final concentration about 3×10^7 bacteria/ml.) in saline phosphate (pH 6·5) at 0°. Viabilities were determined by slide culture.

with them. While adsorbed magnesium is evidently not necessary for growth, it did affect both the ability of the organisms to synthesize polysaccharide in the presence of glycerol and their resistance to various stresses. Therefore this adsorbed magnesium may have an important functional role in bacteria and this should be considered when preparing washed bacterial suspensions for studies of metabolic activity or survival.

We wish to thank our colleague Dr J. W. Dicks for some magnesium determinations and Mr T. H. Dunham for his skilled technical assistance.

REFERENCES

EDELMAN, I. S., Ts'o, P. O. P. & VINOGRAD, J. (1960). The binding of magnesium to microsomal nucleoprotein and ribonucleic acid. *Biochim. biophys. Acta*, **43**, 393.

GARDNER, R. J. (1946). Colorimetric determination of magnesium in plasma or serum by means of Titan Yellow. *Biochem. J.* **40**, 828.

HERBERT, D. (1961). The chemical composition of microorganisms as a function of their environment. In *Microbiol Reaction to Environment. Symp. Soc. gen. Microbiol.* **11**, 391.

HERBERT, D., PHIPPS, P. J. & TEMPEST, D. W. (1965). The chemostat: design and instrumentation. *Lab. Practice*, **14**, 1150.

NEIDHARDT, F. C. (1963). Effect of environment on the composition of bacterial cells. *Annu. Rev. Microbiol.* **17**, 61.

POSTGATE, J. R. & HUNTER, J. R. (1962). The survival of starved bacteria. *J. gen. Microbiol.* **29**, 233.

POSTGATE, J. R. & HUNTER, J. R. (1963). Acceleration of bacterial death by growth substrates. *Nature, Lond.* **198**, 273.

POSTGATE, J. R. & HUNTER, J. R. (1964). Accelerated death of *Aerobacter aerogenes* starved in the presence of growth-limiting substrates. *J. gen. Microbiol.* **34**, 459.

POSTGATE, J. R., CRUMPTON, J. E. & HUNTER, J. R. (1961). The determination of bacterial viabilities by slide culture. *J. gen. Microbiol.* **24**, 15.

ROGERS, A. (1964). The exchange properties of magnesium in *Escherichia coli* ribosomes. *Biochem. J.* **90**, 548.

STRANGE, R. E. & DARK, F. A. (1962). Effect of chilling on *Aerobacter aerogenes* in aqueous suspension. *J. gen. Microbiol.* **29**, 719.

STRANGE, R. E. & DARK, F. A. (1965). 'Substrate-accelerated death' of *Aerobacter aerogenes*. *J. gen. Microbiol.* **39**, 215.

STRANGE, R. E. & POSTGATE, J. R. (1964). Penetration of substances into cold-shocked bacteria. *J. gen. Microbiol.* **36**, 393.

STRANGE, R. E. & SHON, M. (1964). Effects of thermal stress on viability and ribonucleic acid of *Aerobacter aerogenes* in aqueous suspension. *J. gen. Microbiol.* **34**, 99.

TEMPEST, D. W. & HUNTER, J. R. (1965). The influence of temperature and pH value on the macromolecular composition of magnesium-limited and glycerol-limited *Aerobacter aerogenes* growing in a chemostat. *J. gen. Microbiol.* **41**, 267.

TEMPEST, D. W., HUNTER, J. R. & SYKES, J. (1965). Magnesium-limited growth of *Aerobacter aerogenes* in a chemostat. *J. gen. Microbiol.* **39**, 355.

J. gen. Microbiol. (1966), **44**, 281–292
Printed in Great Britain

The Classification of Micrococci and Staphylococci Based on their DNA Base Composition and Adansonian Analysis

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SUMMARY

Species of the genus *Staphylococcus* have a guanine+cytosine (GC) content in DNA within the range 30·7–36·4 moles %. All the Gram-positive cocci so far designated as micrococci and sarcinas which produce acid from glucose when grown under anaerobic or aerobic conditions and have a GC content in DNA within this range should be also classified as *Staphylococcus*. On the other hand species of the genus *Micrococcus* have a GC content in DNA within the range 66·3–73·3%. All the Gram-positive cocci hitherto designated as sarcinas and staphylococci which do not produce acid from glucose under aerobic or anaerobic conditions or only aerobically and which have a GC content in DNA within this range, should be also classified as *Micrococcus*. The range of GC content as outlined is approximate; it will be made more accurate (perhaps a little broader) when more data are available on the DNA base composition of the species belonging to the family Micrococcaceae.

On the basis of their DNA base composition the strains belonging to the genus *Micrococcus* are divided into three groups: group 1 (70·8–73·3 moles % GC); group 2 (67·5–69·5 moles % GC); group 3 (66·3–67·0 moles % GC). Similarly the strains belonging to the genus *Staphylococcus* have been divided into three groups: group 5 (36·4 moles % GC); group 6 (33·3–34·2 moles % GC); group 7 (30·7–32·7 moles % GC). Strains within each group are believed to be phylogenetically related. When subjected to Adansonian analysis, the strains of the individual groups were clustered into subgroups. Each subgroup comprised the strains with approximately the same DNA base composition and a high % similarity of physiological and biochemical characters. Strains clustered into subgroups are believed to be genetically closely related, some may be identical. The following subgroups are suggested: micrococcus subgroups 1a, 1b, 2a, 2b, 2c, 3a, 3b; staphylococcus subgroups 5a, 6a, 7a. Arabic numerals refer to the designation of the group.

The classification of micrococci presented in this paper is substantially the same as that of Baird-Parker (1965). The authors are of the same opinion as Baird-Parker (1965) that *Micrococcus denitrificans* should be reclassified with the Gram-negative genera.

INTRODUCTION

The study of phylogenetic relationships among bacterial species made on the basis of their DNA base composition leads to conclusive results only when it concerns species of the same morphology, i.e. usually the species belonging to the

same family. Even when two morphologically different bacteria belonging to different families have the same DNA base composition, it cannot be concluded that they are phylogenetically related. Their morphological difference suggests that they are phylogenetically distant. In this case similarity in DNA base composition does not express the fact that their DNA has a similar sequence of bases. On the other hand, organisms with the same morphology and DNA base composition are usually phylogenetically related. However, their phylogenetic relationship is of different degree and they may, therefore, differ from one another more or less physiologically and biochemically. Only the organisms that are known to have the same DNA base composition and high degree of similarity in physiological and biochemical characters may be considered to be closely related genetically. It is, therefore, believed that before separating a set of organisms into species it would be better to classify them into groups, each comprising the strains not differing in DNA base composition and having a high degree of similarity of physiological and biochemical characters. On the basis of these considerations we have attempted to classify bacteria originally included in the genera *Micrococcus*, *Staphylococcus* and *Sarcina*. The DNA base composition in these cocci has been recently determined by Rosypalová, Boháček & Rosypal (1966*a*, *b*). Rosypal, Boháček & Rosypalová (1966) and Rosypal & Rosypalová (1966). The strains with the same DNA base composition have been grouped together and then subjected to Adansonian analysis according to Sneath (1957) in order to cluster the strains which are similar not only as to DNA base composition, but also biochemically and physiologically. Such strains are believed to be closely related genetically.

METHODS

The strains studied were obtained from the Czechoslovak Collection of Microorganisms (CCM) and are listed in Table 1 under the name received by the CCM from other collections and under the new name assigned to them in the CCM. The base composition of DNA preparations from these strains were determined from their denaturation temperature (T_m) by Rosypalová, Boháček & Rosypal (1966*a*, *b*), Rosypal *et al.* (1966), Rosypal & Rosypalová (1966).

Each bacterial strain was tested for physiological and biochemical characters. For each strain, the following 67 properties were used in the computer analysis: grouping of organisms (packets, clumps); colonies on glucose yeast-extract agar (rough, smooth); pigment production on glucose yeast-extract agar (yellow, violet, pink pigments); dissociation in pigmentation; hydrolysis of starch, casein and gelatin; reduction of nitrates and nitrites; production of catalase, urease and lipase; sensitivity to chloramphenicol, chlortetracycline, terramycin, tyrothricin, nystatin, bacitracin and lysozyme; hydrolysis of aesculin and salicin; methyl red test; effects on milk; Gram-reaction; growth on Simmon's citrate agar; production of acid from carbohydrates (glucose, sucrose, galactose, fructose, mannose, lactose, xylose, glycerol, adonitol, sorbitol, mannitol); oxidation of carbohydrates in phosphate buffer at pH 7 (acetate, lactate, glycerol, mannitol, dulcitol, sorbitol, arabinose, xylose, rhamnose, glucose, galactose, fructose, sucrose, maltose, lactose, starch); oxidation of amino acids in phosphate buffer at pH 7 (glycine, alanine, threonine, leucine, lysine, histidine, arginine, proline, tryptophan, asparagine, glutamic acid).

Data concerning these tests and the methods used are given in papers by Kocur & Martinec (1962), Rosypal, Kocur & Hodák (1963), Rosypal & Kocur (1963), Rosypalová & Rosypal (1966).

The results of each test were scored as positive (+) or negative (−). Similarity (S) values for pairs of strains were calculated according to the formula (Sneath, 1957):

Table 1. *List of strains studied*

Number in Table 2 and Fig. 2	Number of the strain and the name under which it was received by the CCM	Number of strain and its new name given by the CCM
1	*Staphylococcus flavocyaneus* NCTC, 7011	*Micrococcus luteus* 247
2	*Micrococcus* sp. L.A. 9.1*	*M. luteus* 852
3	*M. flavocyaneus**	*M. luteus* 622
4	*M. flavocyaneus* L.A. 8.2*	*M. luteus* 851
5	*M. flavocyaneus* L.A. 8.1*	*M. luteus* 853
6	*M. flavus* ATCC, 400	*M. luteus* 210
7	*M. sodonensis* ATCC, 11880	*M. luteus* 144
8	*Sarcina subflava* ATCC, 381	*M. luteus* 559
9	*S. marginata* IFO, 3066	*M. luteus* 265
10	*S. flava* isolated by Kocur	*M. luteus* 309
11	*S. variabilis* IFO, 3067	*M. luteus* 266
12	*S. citrea*†	*M. luteus* 248
13	*S. pelagia* SIO	*M. luteus* 331
14	*Micrococcus luteus* ATCC, 398	*M. luteus* 810
15	*Staphylococcus afermentans* NCTC, 2665	*M. luteus* 855
16	*Sarcina lutea* isolated by Kocur	*M. luteus* 523
17	*S. lutea* isolated by Kocur	*M. luteus* 310
18	*Micrococcus lysodeikticus* 53–20‡	*M. luteus* 1335
19	*Sarcina aurantiaca* ATCC, 146	*M. luteus* 686
20	*Micrococcus cyaneus* L.A. 1.1*	*M. luteus* 856
21	*Staphylococcus roseus* NCTC, 7520	*Micrococcus roseus* 146
22	*Micrococcus roseus*§	*M. roseus* 905
23	*M. rubens* ATCC, 412	*M. roseus* 633
24	*Sarcina erythromyxa* ATCC, 187	*M. roseus* 706
25	*S. ventriculi* CRIPP	*M. conglomeratus* 208
26	*Micrococcus denitrificans* NCIB, 8944	*M. denitrificans* 982
27	*M. conglomeratus* ATCC, 401	*M. conglomeratus* 547
28	*M. candidus* NCIB, 8610	*M. varians* 1044
29	*M. citreus* NCIB, 8611	*M. varians* 1046
30	*Staphylococcus lactis* NCTC, 7564	*M. varians* 884
31	*Sarcina oliva*†	*M. varians* 250
32	*Micrococcus infimus* SIO	*M. varians* 313
33	*M. euryhalis* SIO	*M. varians* 315
34	*M. varians* M 3‖	*M. varians* 529
35	*M. cerolyticus* ATCC, 12559	*M. varians* 961
	Staphylococcus aureus NCTC, 8511	
	S. aureus lysogenic derivative of NCTC, 8511	

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IH, Institute of Hygiene, Warsaw, Poland; NCIB, National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland; ATCC, American Type Culture Collection, Rockville, Md., U.S.A.; NCTC, National Collection of Type Cultures, London, England; CRIPP, Central Research Institute of Plant Production, Prague-Ruzyně, ČSSR; SIO, Scripps Institution of Oceanography, La Jolla, California, U.S.A.; IFO, Institute for Fermentation, Osaka, Japan; CCM, Czechoslovak Collection of Microorganisms, J. E. Purkyně University, Brno, ČSSR.

$S = n_s/n_s + n_d$ where n_s is the number of features positive for both organisms and n_d is the number of features positive for one but not the other organism. The Mean Similarity expressed as 'Triangle Mean' ΔS was also calculated (see Sneath, 1957). The MINSK electronic digital computer was employed. Copies of the program may be obtained from the authors.

RESULTS

Taking into consideration that the values of moles % GC content in DNA determined in individual strains from the denaturation temperature T_m, are reproducible within the limits of ± 1 %, the whole set of strains can be divided into seven groups (see Fig. 1). In the sequels moles % GC means moles % guanine + cytosine in the given DNA preparation. Differences in moles % GC values within these groups are not considered to be significant. The borderlines between the respective groups are only approximate and may be modified as soon as new data on DNA base composition in micrococci and staphylococci are available. However, no substantial changes are to be expected. The following classification into groups is suggested:

Group 1, strains with a GC content within the range 70·8–73·3 moles %.

Group 2, strains with a GC content within the range 67·5–69·5 moles %.

Group 3, strains with a GC content within the range 65·8–67·0 moles %. As stated in the Discussion, the species *Micrococcus denitrificans* is not included in this group. The range of GC is therefore 66·3–67·0 moles %.

Group 4 is for the time being represented by only one strain with a GC content 54·2 moles % GC.

Group 5 is also represented by one strain with a GC content 36·4 moles % GC.

Group 6, strains with a GC content within the range 33·3–34·2 moles %.

Group 7, strains with a GC content within the range 30·7–31·1 moles %. This range is broader (30·7–32·7 moles % GC) when we take into account the data by Silvestri & Hill (1965).

When subjected to Adansonian analysis, the strains of individual groups were clustered into subgroups. Each subgroup comprised the strains with approximately the same DNA base composition and a high % of similarities in physiological and biochemical characters (see Table 2, Fig. 2). The intra-group and inter-group similarity values (ΔS or Triangle Mean) are given in Table 3. The success of the clustering is indicated by the intra-group S values. The subgroups were obtained at approximately 70 % ΔS (see Table 3).

Group 1 is divided into subgroups 1a ($\Delta S = 72$ %) and 1b ($\Delta S = 73$ %). The inter-group S value between these subgroups equals 59 % S.

Group 2 is formed of subgroups 2a ($\Delta S = 67$ %), 2b and 2c; subgroups 2b and 2c are represented by only one strain each. Subgroup 2b is very similar to subgroups 1a and 1b, though having a different moles % GC content.

Group 3 is formed of subgroups 3a, 3b and 3c, each represented by one strain. Subgroups 3a and 3b have great similarity to subgroup 1a, although differing from it by their DNA GC contents.

Groups 4, 5 and 6 have each only one subgroup: 4a, 5a and 6a ($\Delta S = 71$ %) and are quite dissimilar from all the above-mentioned groups and subgroups. There is a high percentage of similarity between subgroups 4a and 5a (inter-group S

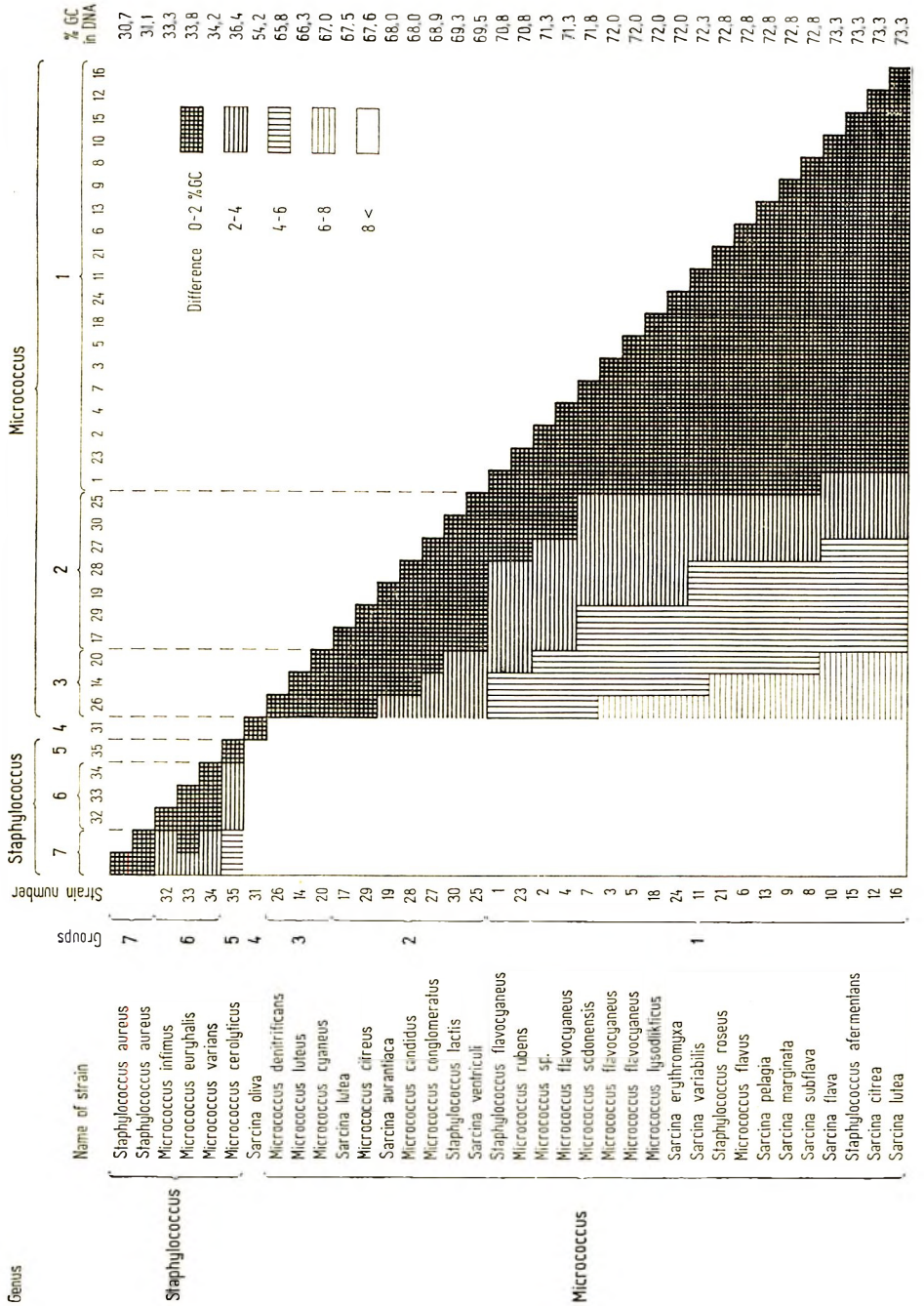

Fig. 1. Diagrammatic representation of the differences in DNA base compositions between the strains studied. The strains not differing each from another by more than 2 % GC are believed to have a similar DNA base composition. They form, therefore, a group of phylogenetically related organisms.

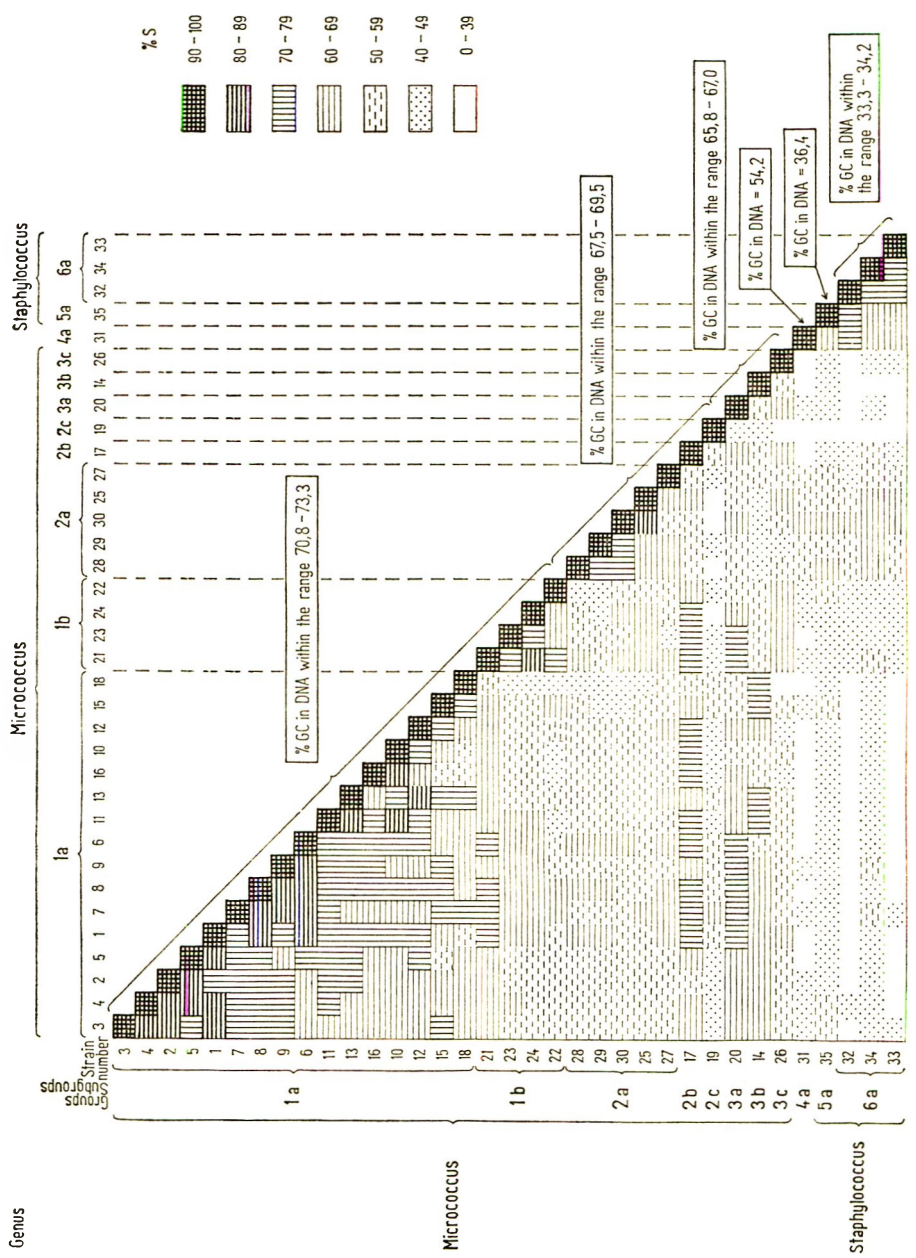

Fig. 2. Diagrammatic representation of the S-value table, prepared by shading the squares according to S-values between the strains.

Table 2. *The full table of S values (%) for micrococci, staphylococci and sarcinas after re-arranging the strains by the sorting procedure. The strains with similar GC contents in DNA were first grouped together and the members of each group were then sorted on the basis of their S values*

Name of strain as received	Groups	Subgroups	Strain number	3	4	2	5	1	7	8	9	6	11	13	16	10	12	15	18	21	23	24	22	28	29	30	25	27	17	19	20	14	26	31	35	32	34	33
GC in DNA within the range 70·8–73·3 moles %																																						
Micrococcus flavocyaneus	1	a	3	100																																		
M. flavocyaneus	1	a	4	89	100																																	
Micrococcus sp.	1	a	2	88	89	100																																
M. flavocyaneus	1	a	5	79	89	89	100																															
Staphylococcus flavocyanaeus	1	a	1	84	85	79	65	100																														
Micrococcus sodonensis	1	a	7	74	74	73	70	75	100																													
Sarcina subflava	1	a	8	71	76	70	76	82	81	100																												
S. marginata	1	a	9	71	72	70	68	73	81	83	100																											
Micrococcus flavus	1	a	6	68	69	68	73	82	86	84	84	100																										
Sarcina variabilis	1	a	11	67	72	70	76	77	71	78	73	79	100																									
S. pelagia	1	a	13	65	66	74	75	71	69	76	71	78	88	100																								
S. lutea	1	a	16	60	69	63	69	70	68	79	70	76	74	68	100																							
S. flava	1	a	10	68	68	63	68	78	68	74	69	79	83	72	84	100																						
S. citrea	1	a	12	65	62	64	70	71	69	76	67	73	76	81	68	77	100																					
Staphylococcus afermentans	1	a	15	73	64	67	59	65	73	75	75	67	65	73	58	61	73	100																				
Micrococcus lysodeikticus	1	a	18	65	61	69	61	58	70	62	67	64	62	70	55	58	65	74	100																			
Staphylococcus roseus	1	b	21	63	64	63	64	73	76	78	69	75	65	63	67	62	63	66	50	100																		
Micrococcus rubens	1	b	23	56	60	51	57	64	67	64	60	66	60	51	55	58	51	53	43	73	100																	
Sarcina erythromyxa	1	b	24	58	59	53	56	64	66	63	60	65	63	58	58	57	54	51	41	82	76	100																
Micrococcus roseus	1	b	22	59	56	54	52	53	63	61	53	55	49	54	48	47	54	66	49	70	69	68	100															
GC in DNA within the range 67·5–69·5 moles %																																						
Micrococcus candidus	2	a	28	54	55	53	58	66	57	66	59	64	59	57	57	56	57	55	42	63	53	52	49	100														
M. citreus	2	a	29	55	56	54	59	63	58	60	56	65	56	54	58	57	54	49	43	57	51	47	47	79	100													
Staphylococcus lactis	2	a	30	54	55	53	58	52	57	58	55	60	58	53	54	53	53	45	40	62	58	61	52	76	73	100												
Sarcina ventriculi	2	a	25	57	58	53	55	59	61	59	59	57	59	50	54	53	50	55	42	67	63	69	59	64	65	80	100											
M. conglomeratus	2	a	27	58	59	56	59	63	62	68	59	61	59	61	53	52	61	59	51	60	49	55	55	62	60	50	62	100										
Sarcina lutea	2	b	17	63	67	58	67	72	75	73	68	78	73	67	70	73	71	61	50	73	72	71	61	59	54	58	63	36	100									
S. aurantiaca	2	c	19	47	49	46	49	50	60	53	53	55	45	50	44	43	46	52	50	43	46	38	41	36	40	35	36	47	50	100								
GC in DNA within the range 65·8–67·0 moles %																																						
Micrococcus cyaneus	3	a	20	64	65	63	65	74	73	70	70	71	62	60	60	59	60	53	51	75	70	65	59	57	52	60	57	53	66	48	100							
M. luteus	3	b	14	65	66	69	61	62	65	67	62	59	72	76	55	58	65	74	74	58	50	53	58	49	43	46	49	51	58	44	51	100						
M. denitrificans	3	c	26	58	55	53	51	60	58	55	51	53	59	53	47	52	49	54	39	60	68	63	69	51	46	54	62	50	60	36	61	56	100					
GC in DNA = 54·2%																																						
Sarcina oliva	4	a	31	44	40	40	37	38	44	39	42	33	33	36	33	33	36	44	38	47	38	43	49	50	53	61	56	51	38	34	45	31	38	100				
GC in DNA = 36·4%																																						
Micrococcus cerolyticus	5	a	35	52	60	48	44	46	43	44	47	40	44	40	40	42	41	52	43	41	42	44	50	52	55	62	56	52	45	28	40	46	46	60	100			
GC in DNA 33·3–34·2%																																						
Micrococcus infimus	6	a	32	39	40	53	38	39	39	37	37	37	35	32	32	33	35	38	30	41	44	40	46	55	59	63	58	48	44	33	37	30	39	73	72	100		
M. varians	6	a	34	49	47	41	44	48	52	47	50	49	44	42	41	42	45	45	40	51	45	47	47	65	63	67	65	52	51	31	46	37	40	67	65	70	100	
M. euryhalis	6	a	33	40	39	36	33	37	40	35	38	35	33	33	30	29	30	39	30	43	40	42	48	54	55	60	61	54	40	30	35	37	43	61	63	73	70	100

value = 60%), 5a and 6a (inter-group *S* value = 67%), 6a and 4a (inter-group *S* value = 67%.

The strains of *Staphylococcus aureus* were not subjected to Adansonian analysis.

Table 3. *Intra-group and inter-group S values (% similarity) for all strains used in this study*

	Sub-groups	*Micrococcus* 1a	1b	2a	2b	2c	3a	3b	3c	*Staphylococcus* 4a	5a	6a	moles % GC in DNA
Micrococcus	1a	72											70.8–73.3
	1b	59	73										
	2a	56	56	67									67.5–69.5
	2b	68	69	58	100*								
	2c	50	42	39	50	100*							
	3a	64	67	56	66	48	100*						65.8–67.0
	3b	66	55	48	58	44	51	100*					
	3c	53	65	53	60	36	61	56	100*				
	4a	39	44	54	38	34	45	31	38	100*			54·2
Staphylococcus	5a	45	44	57	45	28	40	46	46	60	100*		36·4
	6a	39	45	59	45	31	39	35	41	67	67	71	33.3–34.2

* Single strains.

DISCUSSION

The genera Micrococcus and Staphylococcus. Drawing some general conclusions about the importance of DNA base composition for bacterial taxonomy, Sueoka (1961) pointed out that if the mean moles % GC of the DNA's of two strains is different by 10% there will be few DNA molecules of the same GC content common to them. Such strains are certainly phylogenetically distant and they should be classified as members of different genera, if it were the aim to include in the same genus only species more or less phylogenetically related. For this reason, the whole set of strains split into two branches obviously representing two distinct genera. The first branch is formed of groups 1, 2 and 3, i.e. strains with a GC content within the range 66·3–73·3 moles %. The second branch is formed of groups 5, 6 and 7, i.e. strains whose GC content ranges from 30·7 to 36·4 moles %. The same division into two branches is confirmed by Adansonian analysis, because all the strains of groups 5, 6 and 7 show a low percentage of similarities with respect to the groups 1, 2 and 3.

Silvestri & Hill (1965) arrived at the conclusion that Gram-positive catalase-positive cocci with low GC moles % values correspond to the genus *Staphylococcus*, while Gram-positive, catalase-positive cocci with high moles % GC values correspond to the genus *Micrococcus*. As outlined by Baird-Parker (1963, 1965) organisms placed in the genus *Staphylococcus* are characterized by ability to grow and produce acid from glucose when incubated under anaerobic conditions in a mineral salts yeast-extract agar medium containing bromcresol purple as pH indicator. On the other hand the genus *Micrococcus* includes cocci which are either unable to produce sufficient acid from glucose to change the indicator brocresol purple or which grow and produce acid from glucose only in the presence of air. The anaerobic fermentation of glucose is, therefore, recommended as a test for distinguishing the genus

Staphylococcus from *Micrococcus* (Subcommittee on Taxonomy of Staphylococci and Micrococci, 1965; Evans, 1965). Applying this test to the strains studied in this paper we have come to the following conclusions. (1) All the strains showing a high moles % GC were unable to produce acid from glucose under anaerobic conditions. Most strains did not produce any detectable acid from glucose under aerobic conditions. Only some of them produced acid from glucose when grown aerobically (strains of subgroup 2a). (2) The strains (group 7, subgroup 5a) with low values of moles GC content were able to produce acid from glucose anaerobically, while the others (subgroup 6a) only did so aerobically. On the basis of these results the following conclusions may be drawn.

Species of the genus *Staphylococcus* have a GC content within the range 30·7–36·4 moles %. All the Gram-positive cocci so far designated as micrococci and sarcinas which produce acid from glucose when grown under anaerobic or aerobic conditions and which have a GC content within this range should be also classified as *Staphylococcus*. On the other hand species of the genus *Micrococcus* have a GC content within the range 66·3–73·3 moles %. All the Gram-positive cocci hitherto designated as sarcinas and staphylococci which do not produce acid from glucose under aerobic and anaerobic conditions or only aerobically, and which have a GC content in DNA within this range, should be also classified as *Micrococcus*.

The range of GC content as outlined for the genera *Staphylococcus* and *Micrococcus* is only approximate. It will be made more accurate (perhaps a little broader) when more data are available on the DNA base composition of the species belonging to the family Micrococcaceae.

Classification of micrococci

In our opinion it is at present premature to classify micrococci into species until their physiology, biochemistry and DNA base composition are better known. It would be more convenient and less influenced by subjective speculations if for the time being taxonomists aimed to detect among the micrococci natural groups that were clearly defined physiologically as well as biochemically. Such an attempt to classify micrococci has been made by Hill (1959), Pohja & Gyllenberg (1962) and Baird-Parker (1963, 1965). On the basis of DNA base composition the micrococci we have studied may be divided into the following groups and subgroups.

Group 1. This group comprises strains with a GC content within the range 70·8–73·3 %. It consists of two subgroups.

Subgroup 1a is formed mostly of yellow pigmented organisms. Some strains are able to produce both yellow and violet pigments. These strains give yellow pigmented spontaneous mutants which have lost their ability to produce violet pigment and in some media they overgrow the parent culture (Rosypal *et al.* 1963). Thus, the ability to produce yellow pigment appears to be chacteristic of all the strains of this subgroup. Non-pigmented strains are exceptions (rare mutants). According to Kocur & Martinec (1962) all the strains included in subgroup 1a do not release detectable acid when grown in peptone water with glucose or other carbohydrates. Results obtained by Baird-Parker (1963, 1965) would indicate that the latter strains can, at least some of them, produce acid when grown in a mineral salts yeast-extract medium containing glucose. In comparison with other subgroups the ability to hydrolyze gelatin and the inability to oxidize mannitol in phosphate buffer at pH 7 may be of some importance for identification of this subgroup.

Considering all the above physiological characteristics, this subgroup 1a appears to correspond to Baird-Parker's Micrococcus subgroup 7 (Baird-Parker, 1965). According to Kocur & Martinec (1962) all strains included in subgroup 1a are considered to be identical with *Micrococcus luteus*. However, the type strain *M. luteus* ATCC 398 has a GC content different from that of all the strains of subgroup 1a (see also Rosypalová *et al.* 1966*a*, *b*). Since the name designation of the type species should not be changed, the name of this subgroup appears to be questionable for the time being.

Subgroup 1b is formed only of pink-pigmented micrococci, which are taxonomically well described. They obviously correspond to Baird-Parker's Micrococcus subgroup 8 and to the species named *Micrococcus roseus* (Evans, 1965; Baird-Parker, 1965; Kocur & Martinec, 1962). None of the strains belonging to this subgroup hydrolyze gelatin, but they reduce nitrates and oxidize mannitol and sorbitol in phosphate buffer at pH 7. They are not able to produce acid from glucose in peptone water.

Group 2. The strains belonging to this group have a GC content within the range 67·5–69·5 moles %. The following three subgroups are known:

Subgroup 2a is formed of yellow-pigmented strains. In contrast to other yellow-pigmented cocci they release acid from glucose when grown under aerobic conditions, but no acid is formed when they are incubated anaerobically; they oxidize sorbitol in phosphate buffer at pH 7. By these physiological characters they can be well distinguished from the other subgroups; they correspond to Baird-Parker's Micrococcus subgroups 5 and 6. As can be seen from Table 1 and Fig. 2 we included in subgroup 2a the strains which are considered by Kocur & Martinec (1962) as *Micrococcus conglomeratus* and *M. varians*.

Subgroup 2b. This is a yellow-pigmented strain which does not hydrolyze gelatin; in its other characteristics it resembles the strains of subgroup 1a. The similarity of this strain to the strains of subgroup 1a is evident from Fig. 2. The considerable physiological and biochemical similarity of this strain to the organisms of subgroup 1a has resulted in it being considered by Kocur & Martinec (1962) to be identical with the strains of subgroup 1a. The DNA base composition is significantly different from the strains of subgroup 1a, however, and place it in a separate group.

Subgroup 2c. This yellow pigmented strain differs from the strains of subgroup 1a not only in its DNA base composition, but also physiologically and biochemically; see the Adansonian analysis cf. Fig. 2.

Group 3. This group comprises strains with a GC content within the range 66·3–67·0 moles %. The species *Micrococcus denitrificans* is not included in this group. Therefore it consists of two subgroups:

Subgroup 3a. This is a violet pigmented coccus not producing a yellow pigment. Although having high *S* values with most strains of subgroup 1a, it differs from them by its DNA base composition and by ability to oxidize mannitol and dulcitol (Rosypalová & Rosypal, 1966; Rosypalová *et al.* 1966*a*, *b*).

Subgroup 3b. *Micrococcus luteus*, type species, ATCC 398. It has a GC content quite different from the strains of subgroup 1a and cannot be considered identical with them, as believed by Kocur & Martinec (1962). Its classification with the strains of subgroup 1a resulted from its showing some physiological and biochemical similarity to these strains.

'*Subgroup 3c*' formed of *Micrococcus denitrificans* is questionable. Baird-Parker

(1965) suggests that it should be reclassified with the Gram-negative genera and gives the following reasons for it: (1) it is Gram-negative and forms rods in young cultures, (2) it has a cell-wall mucopeptide which contains a wide range of amino acids characteristic of Gram-negative genera. For these reasons *Micrococcus denitrificans* cannot be included in the genus *Micrococcus* even if it has a similar GC content to that of other members of group 3. We must, therefore, correct the opinion of Rosypalová *et al.* (1966*b*), where this species was considered to be closely related to *M. luteus*. On the basis of the above-mentioned facts it shows no phylogenetical relationship to species of the genus *Micrococcus*.

Classification of staphylococci

It is not the aim of the present paper to deal fully with the classification of staphylococci. We touch upon this question only as it concerns the strains originally classified as micrococci and now classified as staphylococci. On the basis of DNA base composition we divided these strains into three groups. *Group 5* is represented by one strain (subgroup 5a) having 36·4 moles % GC. It is the strain *Micrococcus cerolyticus*, ATCC, 12559. Baird-Parker (1965) included it in his Staphylococcus subgroup V. It produces acid from glucose when grown under anaerobic conditions. *Group 6* consists of subgroup 6a, i.e. strains with a GC content 33·3–34·2 moles %. Since these strains produce acid from glucose only when grown under aerobic conditions, they have been so far classified as micrococci (*M. varians*). Their GC content places them, however, in the genus *Staphylococcus*.

Group 7 is formed of subgroup 7a and corresponds to the species *Staphylococcus aureus* (Baird-Parker's Staphylococcus subgroup I), whose GC content has been found to be 30·7 to 32·7 moles %. On the whole, it may be said that the range of GC content in DNA of the strains belonging to the genus *Staphylococcus* is so large that it will comprise more than the two species, so far recognizable, *Staphylococcus aureus* and *S. epidermidis*. This is also suggested by Baird-Parker's classification, in which staphylococci are divided into six subgroups (Baird-Parker, 1963, 1965). *Group 4* is formed of subgroup 4a represented by a strain with 56·4 moles % GC. It deserves particular attention because its DNA base composition is quite unlike that which has been found with the genera *Micrococcus* and *Staphylococcus*. It produces acid from glucose only when grown under aerobic conditions and is sensitive to several staphylococcal phages, but in DNA base composition it differs from both *Staphylococcus* and *Micrococcus*.

REFERENCES

BAIRD-PARKER, A. C. (1963). A classification of micrococci and staphylococci based on physiological and biochemical tests. *J. gen. Microbiol.* **30**, 409.

BAIRD-PARKER, A. C. (1965). The classification of staphylococci and micrococci from world-wide sources. *J. gen. Microbiol.* **38**, 363.

EVANS, J. B. (1965). Current views and problems relating to the taxonomy of the Micrococcaceae. *Int. Bull. bact. Nomencl. Taxon.* **15**, 111.

HILL, L. R. (1959). The Adansonian classification of the staphylococci. *J. gen. Microbiol.* **20**, 277.

KOCUR, M. & MARTINEC, T. (1962). Taxonomic study of the genus *Micrococcus*. *Folia Přírod. fakulty UJEP*, **3**.

POHJA, M. S. & GYLLENBERG, N. G. 1962. Numerical taxonomy of micrococci of fermented meat origin. *J. appl. Bact.* **25**, 341.

ROSYPAL, S., BOHÁČEK, J. & ROSYPALOVÁ, A. (1966). DNA base composition of *Micrococcus varians*. *Publ. Fac. Sci. Univ. J. E. Purkyně*. No. 471, 115.

ROSYPAL, S. & KOCUR, M. (1963). The taxonomic significance of the oxidation of carbon compounds by different strains of *Micrococcus luteus*. *Antonie van Leeuwenhoek*, **29**, 313.

ROSYPAL, S., KOCUR, M. & HOŠÁK, K. (1963). A study of violet-pigmented micrococci. Yellow-pigmented mutants of *Staphylococcus flavocyaneus*. *J. gen. Microbiol.* **32**, 189.

ROSYPAL, S. & ROSYPALOVÁ, A. (1966). A comment to the definition of the genera *Micrococcus* and *Staphylococcus*. *Publ. Fac. Sci. Univ. J. E. Purkyně*. No. 471, 105.

ROSYPALOVÁ, A., BOHÁČEK, J. & ROSYPAL, S. (1966*a*). Deoxyribonucleic acid base composition of violet cocci. *Antonie van Leeuwenhoek*, **32**, 105.

ROSYPALOVÁ, A., BOHÁČEK, J. & ROSYPAL, S. (1966*b*). Deoxyribonucleic acid base composition of some micrococci and sarcinae. *Antonie van Leeuwenhoek*, **32**. (in press).

ROSYPALOVÁ, A. & ROSYPAL, S. (1966). Some physiological properties of violet-pigmented micrococci. Their oxidation pattern and ability to grow in minimal synthetic medium. *Publ. Fac. Sci. Univ. J. E. Purkyně*. (in press).

SILVESTRI, L. G. & HILL, L. R. (1965). Agreement between deoxyribonucleic acid base composition and taxometric classification of Gram-positive cocci. *J. Bact.* **90**, 136.

SNEATH, P. H. A. 1957. The application of computers to taxonomy. *J. gen. Microbiol.* **17**, 201.

Subcommittee on Taxonomy of Staphylococci and Micrococci (1965). Recommendations. *Int. Bull bact. Nomencl. Taxon.* **15**, 109.

SUEOKA, N. (1961). Variation and heterogeneity of base composition of deoxyribonucleic acids: compilation of old and new data. *J. mol. Biol.* **3**, 31.

J. gen. Microbiol. (1966), **44**, 293–302
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Function and Location of a 'Germination Enzyme' in Spores of *Bacillus cereus*

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(*Received* 13 *January* 1966)

SUMMARY

An enzyme extracted from *Bacillus cereus* spores caused to germinate spores of this organism which had been sensitized by reagents which rupture disulphide bonds. Inactivation of the enzyme by thiol-blocking agents and by oxidation, and reactivation by reduction suggested that the enzyme's ability to germinate spores depended on thiol groups. No evidence was obtained to support the hypothesis that the enzyme was present in dormant spores in the oxidized inactive form and became reduced and active during germination. When spores were disrupted at pH 3 the enzyme was found to be bound to debris; probably on or within the central core of the spore. At pH 5·8 or below the enzyme remained bound to the debris, but at pH 7·0 the enzyme was irreversibly released when the ionic strength of the medium was high. In solutions of sodium phosphate less than 0·05 M, the enzyme remained mostly bound even at pH 7·0. It was largely released when the concentration of sodium phosphate was increased to 0·2 M. Extracts of germinated spores contained more unbound and less bound enzyme than extracts of ungerminated spores, suggesting that release of enzyme from a bound form occurred during germination of the spores.

INTRODUCTION

The germination of a bacterial spore involves the change of a stain-resistant phase-bright metabolically-dormant and heat-resistant organism into a readily-stainable phase-dark, metabolically-active and heat-sensitive form. This change can occur in as little as 30 sec. (Vary & Halvorson, 1965). This remarkable 'trigger' reaction can often be initiated by low concentrations of a single amino acid, riboside or sugar. It can also be simulated by what appear to be less natural means; i.e. by mechanical abrasion (Rode & Foster, 1960*a*), by surface active substances (Rode & Foster, 1960*b*), by alkanes (Rode & Foster, 1965), by enzymes like lysozyme (Gould & Hitchins, 1963), a spore enzyme (Gould & Hitchins, 1965) and by a heat-activated factor + a cofactor in *Bacillus cereus* strain T spore extracts (Vary, 1965). How a 'germinant' initiates the normal germination reaction is unknown. One possible mechanism of germination was suggested when it was found that lysozyme made spores germinate, probably by hydrolysing β 1–4 glycosidic bonds in mucopeptide in the spore cortex (Gould & Hitchins, 1963). To show germination by lysozyme, spores were first treated with reagents which ruptured disulphide bonds: this opened the spore coat structure, presumably by attacking the keratin-like

component of spore coats (Vinter, 1962; Kadota, Iijima & Uchida, 1965), and allowed lysozyme to reach lysozyme-sensitive mucopeptide in the underlying cortex (Gould, Georgala & Hitchins, 1964). Although lysozyme has not been detected in spores, extracts of spores of *B. cereus* contain an enzyme which, like lysozyme, can germinate sensitized *B. cereus* spores (Gould & Hitchins, 1965). Since this enzyme was normally present in *B. cereus* spores, and was able to germinate its spores, it seemed possible that this action within the spore was a prime event in normal germination. Within a dormant spore the enzyme clearly does not attack its substrate; however, on addition of the correct germinant the enzyme may act to cause rapid germination. The theory that germination involved depolymerization of spore mucopeptide by a lytic enzyme was originally proposed by Powell & Strange (1956). The purpose of the experiments described in this paper is to try to find whether activation, or release of the enzyme from a bound form does occur during germination.

METHODS

Production of spores. Spores of *Bacillus cereus*, strain PX were grown and cleaned, as described previously (Hitchins, Gould & Hurst, 1963).

Preparation of spore enzyme. Spore extract containing the enzyme was made by disrupting spores (equiv. 20 mg. dry wt./ml.) in water in a Braun tissue disintegrator (Shandon Scientific Co. Ltd., 6 Cromwell Place, London S.W. 7), whilst cooled to about 4° by a stream of carbon dioxide from a cylinder of liquid CO_2. Debris was then removed by centrifugation at 77,000*g* for 30 min. at 4°. Supernatant fluid was then used as crude enzyme. This could be partly purified by sequential precipitation in 0·1 M (pH 3·4) buffer (sodium citrate + phosphate) and in 60% saturated $(NH_4)_2SO_4$ (Gould & Hitchins, 1965).

Assay of spore enzyme. Sensitized spores were made by incubating spores (equiv. 10 mg. dry wt./ml.) in 8 M-urea + 25% (w/v) thioglycollic (mercaptoacetic) acid at 70° for 30 min. then washing 6 times with distilled water. Such spores were unable to germinate in the usual germinants (e.g. L-alanine, inosine, yeast extract) and must be presumed non-viable.

The enzyme was assayed by suspending sensitized spores (sufficient to give an extinction of about 0·3 at 580 mμ) in 0·1 M-sodium phosphate buffer (pH 7·0) containing the enzyme (usually 0·5 ml. of crude or partly purified spore extract). Unless otherwise stated, sodium thioglycollate (0·01 M-thioglycollic acid neutralized with NaOH) was included in each assay mixture to maintain the enzyme in a reduced form. The reaction mixture, in a total volume of 5 ml., was incubated at 37° and the extinction read at intervals. The extinction decreased when active enzyme was present. At the same time microscopical examination revealed that active enzyme caused phase-bright spores to become phase-dark. Decrease of extinction and phase darkening are changes characteristic of normal spore germination.

Preparation of spore debris containing cores. Spores in sodium citrate + phosphate buffer (0·1 M) at pH 3 were disrupted in the Braun tissue disintegrator at 4°. During disruption at this low pH value cores were released from within spore coats (Hitchins & Gould, 1964). The resulting core + coat mixture was washed with buffers (0·1 M) at pH 3 then at pH 5·8 by centrifugation, then resuspended in the appropriate buffer (see Results).

Chemicals and buffers. These were of reagent grade. Buffers (0·1 M) were sodium citrate + phosphate (pH 3·0) and sodium phosphate (pH 5·8–8·0). Sodium acetate (pH 5·8 and 7·0) was used whenever calcium chloride was added.

RESULTS

Germinative action of spore extracts

Sensitized spores incubated with spore enzyme became phase dark and shed dipicolinic acid as the extinction of the suspension decreased. The response was most rapid at about pH 7·8 and was similar to the response of spores to a germinant such as L-alanine or inosine (Gould & Hitchins, 1965), however, it occurred with sensitized spores which were non-viable. The enzyme did not cause germination of spores which had not been previously sensitized, in these respects germination induced by the enzyme was similar to germination induced by lysozyme or hydrogen peroxide (Gould & Hitchins, 1963).

Table 1. *Effect of ions on activity of the spore germination enzyme of Bacillus cereus*

Solutions were all adjusted to pH 7·0 with NaOH or HCl and the assay medium contained 0·1 M-sodium phosphate or acetate buffer (see Methods).

Ions added	Concentration (M)	Time for 30% decrease in extinction of sensitized spore suspension (min.)
NaCl	0·1	26
LiCl	0·1	40
KCl	0·1	33
CsCl	0·1	38
$MgCl_2$	0·1	33
$CaCl_2$	0·2	120
Chlorides of Ni^{2+}, Zn^{2+}, Fe^{3+}, Co^{2+}	0·1	> 200
Na ethylene diaminetetra-acetate	0·01	21
Na dipicolinate	0·01	30
$CaCl_2$ + sodium dipicolinate	Each 0·01	24

Stimulation of enzyme activity by ions

Tris buffer is known to sequester metal ions and it inhibits the enzyme (Gould & Hitchins, 1965). The effects of a number of ions and sequestrants on activity of the enzyme were therefore examined. Various ions (apart from heavy metals), ethylenediaminetetra-acetate (EDTA), and dipicolinate had small effects on activity of the enzyme (Table 1). Although the nature of the ions was relatively unimportant, their concentration affected the activity of the enzyme, which increased with increasing ionic concentration (Table 2). The enzyme was optimally active when the medium contained about 0·1 M-NaCl, Na_2SO_4 or K_2SO_4 or about 0·2 M-KCl.

Thiol groups and activity of the enzyme

The importance of thiol groups on the enzyme was suggested by its activation by thioglycollate (Gould & Hitchins, 1965). This was confirmed by inhibiting the

enzyme with *p*-chloromercuribenzoate, *N*-ethylmaleimide and Cu^{2+} and by inactivating it by oxidation with iodine or with oxygen (Table 3). Sodium thioglycollate annulled the inhibitions and reactivated enzyme which had been inactivated by oxidation or by thiol-blocking reagents.

Table 2. *Effect of concentration of ions on activity of the spore germination enzyme of Bacillus cereus*

Each solution contained 0·01 M-sodium phosphate and was adjusted to pH 7·0.

Ions added	Concentration (M)	Time for 30 % decrease in extinction of sensitized spore suspension (min.)
NaCl	0·02	> 200
	0·1	29
	0·2	30
KCl	0·02	> 200
	0·1	104
	0·2	45
Na_2SO_4	0·02	> 200
	0·1	27
	0·2	56
K_2SO_4	0·02	> 200
	0·1	28
	0·2	55

Table 3. *Inactivation of the spore germination enzyme by reagents which affect thiol groups*

Sodium thioglycollate was used at 10 mM. Iodine was dissolved in ×4 molar concentration of KI solution. After incubation of enzyme + iodine, excess iodine was removed by adding 82 mM-histidine.

Treatment of enzyme solution	Time for 30 % decrease in extinction of sensitized spore suspension (min.)	
	Thioglycollate absent	Thioglycollate present
No treatment	18	18
Oxygen at 2 atmospheres pressure, 20 min., 37°	87	67
Iodine solution, 30 min. 0° 0·034 mM	30	17
Iodine solution, 30 min. 0° 0·17 mM	> 200	38
p-Chloromercuribenzoate (mM) present during assay	> 200	20
N-Ethylmaleimide (mM) present during assay	> 200	Not tested
$CuSO_4$ (0·04 mM) present during assay	> 200	56

It seemd possible that the enzyme could be in an inactive oxidized form in spores and become reduced and active during germination. If this were so, extracts of ungerminated spores should contain enzyme in the inactive (oxidized) form. Freshly prepared extracts, however, always contained active enzyme, and the activity decreased during storage. It was still possible that the enzyme was in an inactive form in spores and became reduced and active during breakage, perhaps

because of a reducing action of other enzymes released into the extract. To avoid this, spores were broken at 4° and pH 3·0 or pH 10·6 at which extremes it was hoped that any enzyme activity in the extracts would be minimal. However, similarly active extracts were obtained in each instance, suggesting that the enzyme in dormant spores was already in the active reduced form. When extracts of ungerminated spores were compared with extracts of freshly germinated spores (95 % germinated after 5 min. in yeast glucose broth at 37°), no differences in the amounts of enzyme were found; it appeared that no change of inactive to active enzyme occurred during germination.

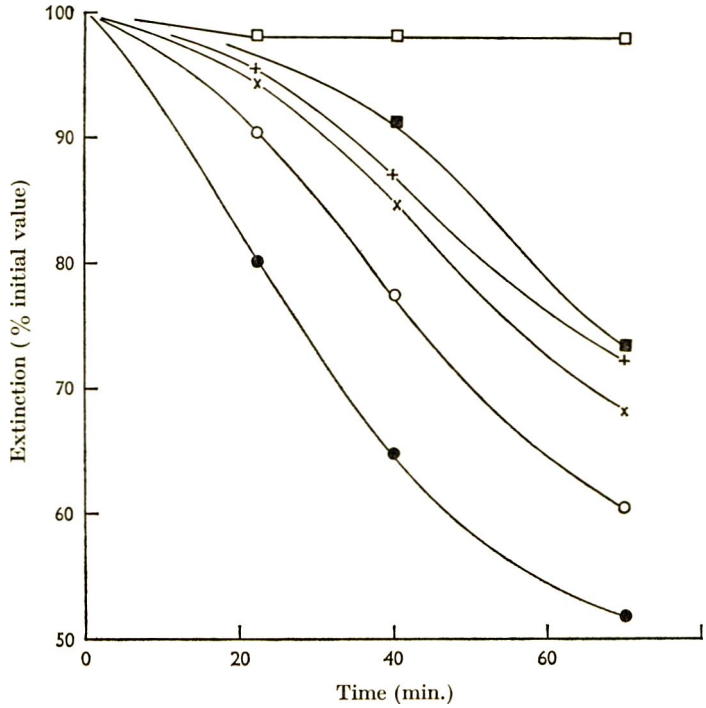

Fig. 1. Binding of spore germination enzyme to spore debris at different pH values. Debris was made by breaking spores at pH 3·0 as described in Methods. Samples were then extracted for 15 min. at 4° with 0·2 M-sodium phosphate buffers at the indicated pH values. Enzyme remaining bound to the debris was then released by ultrasonic treatment in sodium phosphate buffer (pH 8·0). The figure shows the extinction decrease of sensitized spores at 37° caused by this enzyme; from pellets extracted at pH 5·8 (●), pH 6·2 (○), pH 6·6 (×), pH 7·0 (+), pH 7·8 (■), enzyme-free control (□).

Location of the enzyme in spores

Spores broken at pH 3·0 yielded a mixture of spore coats, cores and insoluble matter (see Methods). After washing at pH 5·8 and resuspending in buffer at pH 5·8 the debris consisted of spore coats and cores free from dipicolinic acid. No enzyme was detected in the supernatant fluids during this procedure, although the isolated enzyme is soluble at pH 5·8. However, when the debris was resuspended at pH 7·0, enzyme was released from it and was detected in the supernatant fluid. When debris was subjected to ultrasonic treatment at pH 7·0 or 8·0 a further small release

of enzyme occurred. Figure 1 shows that enzyme which was bound to the debris at about pH 5·8 was released at higher pH values. This release of enzyme from a bound form was irreversible, i.e. when the pH value was decreased to 5·8 again enzyme was not re-absorbed by the debris during 1 hr at 4°. It therefore appeared that the enzyme was bound to some component of the debris, and that an increase in pH value weakened the binding so that enzyme was released.

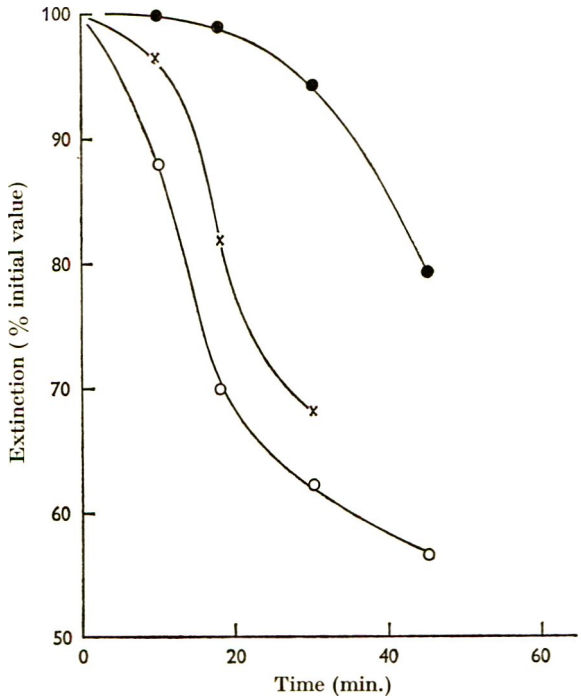

Fig. 2. Binding of spore germination enzyme to spore debris in media of low molarity. The figure shows the extinction decrease of sensitized spore suspension at 37° caused by enzyme extracted from spore debris with sodium phosphate solutions (pH 7·0) of different strength: 0·003 M extract (●), 0·2 M extract (○), 0·2 M extract of debris previously extracted with 0·003 M-sodium phosphate (×).

Debris was examined microscopically to find out to which component the enzyme was bound, Preparations at pH values of 3·0, 5·8 and 7·0 all contained fragments of spore coats. However, the preparations differed because at pH 3·0 the cores were phase bright and small (Pl. 1, fig. 1), at pH 5·8 cores were phase dark though still small (Pl. 1, fig. 2) and at pH 7·0 they were phase dark and swollen with indistinct outlines (Pl. 1, fig. 3). Ultrasonic treatment disintegrated most of the cores. When spores were disrupted at pH 5·8 no cores were formed, and the enzyme was not bound to the debris, which consisted of fragments of spore coat, but was found in the supernatant fluid. It seemed most likely that the enzyme was normally bound in or on the spore core, where it remained as long as the pH value did not exceed about pH 5·8. These experiments were made in 0·1 M buffers. When buffers with different molarities were used, the enzyme remained bound to debris even at pH 7·0 when the molarity was low. For example, Fig. 2 shows how the

enzyme was extracted from debris much more efficiently by 0·2 M than by 0·003 M sodium phosphate (pH 7·0). Thus, low pH value and low molarity favoured binding of the enzyme, and high pH value and high molarity caused its release.

Factors affecting binding of enzyme to spore debris

Apart from pH value and ionic strength, other factors affected the binding of enzyme to spore debris. For instance, the polyanionic sequestering agent ethylenediaminetetra-acetate (EDTA) decreased the amount of enzyme liberated in low molarity sodium phosphate, whilst the polyamine spermine caused a greater release (Fig. 3). Inosine, calcium and *n*-dodecylamine (a chemical germinant; Rode & Foster, 1960*b*), did not affect release during 5 min. at 4°. Incorporation of sucrose (15%, w/v) into the solutions did not prevent release of enzyme in 0·2 M-sodium phosphate solution, suggesting that release of enzyme was not due to osmotic lysis of some structure within which the enzyme was normally held.

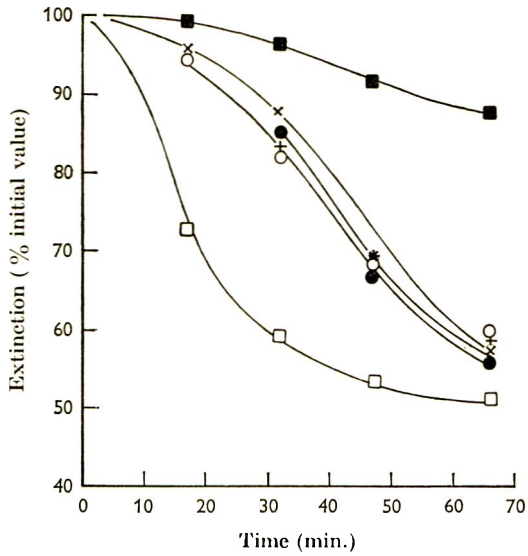

Fig. 3. Chemicals affecting release of spore germination enzyme from spore debris in media of low molarity. Debris was suspended for 5 min. at 4° in 0·02 M-sodium phosphate (pH 7·0) + the following chemicals: no addition (●), saturated *n*-dodecylamine (○), 4 mM-inosine (×), 17 mM-calcium dipicolinate (+), 20 mM-sodium EDTA (■) and 20 mM-spermine hydrochloride. (□) The figure shows that extinction decreased during subsequent assay of enzyme which was released in the presence of the various chemicals.

Binding of enzyme in ungerminated and germinated spores

Spores and germinated spores were compared as sources of the enzyme. It was found that during germination a change in the ratio of enzyme bound to debris in 0·02 M buffer (sodium phosphate, pH 7·6):enzyme free in 0·02 M buffer occurred. Table 4 shows this change in extracts made from ungerminated spores and from spores which were incubated in the germinant inosine until about 95% germination had taken place. The amount of bound enzyme had decreased and the amount of unbound enzyme had increased during germination of the spores.

Table 4. *Free and bound spore germination enzyme in ungerminated and germinated spores of Bacillus cereus*

Spores were germinated by incubation for 6 min. at 37° in 0·1 M-sodium phosphate buffer (pH 8·0) containing 5 mM-inosine. Buffer used to extract enzyme was sodium phosphate pH 7·6.

Source of extract	Time (min.) for 30 % decrease in extinction of sensitized spore suspension during assay of enzyme: Extracted by 0·02 M-buffer	Subsequently extracted by 0·2 M-buffer
Ungerminated spores	76	16
Germinated spores	38	31

DISCUSSION

The spore enzyme which will germinate suitably sensitized spores of *Bacillus cereus* is probably similar to spore 'S-enzyme' described by Strange & Dark (1957). This enzyme released soluble mucopeptide from spore walls. It did not lyse the same vegetative bacteria as did lysozyme, but it did attack spore walls and cell walls of some vegetative bacteria (Strange & Dark, 1957). Spore enzymes lysed chloroform-treated *Escherichia coli* (Work, 1959, Gould, 1962) suggesting that these enzymes attacked mucopeptide, although with a specificity different from lysozyme. The exact substrate(s) of spore lytic enzymes have not, however, been determined.

The fact that the enzyme in *Bacillus cereus* spore extracts can induce in sensitized *B. cereus* spores the changes which are normally recognized as germination, suggests that it might well have a primary role in germination. This is also suggested by the optimal germinative activity of the enzyme in media of ionic strength equivalent to about 0·1 M-NaCl, for Fleming & Ordal (1964) found that normal germination was also most rapid in media of about this ionic strength. Furthermore, removal of ions from the media inhibited germinative action of the spore enzyme, and is also known to inhibit normal spore germination (Rode & Foster, 1962).

Studies on the activation of spores by heat (Busta & Ordal, 1964) and by reducing agents (Keynan, Evenchik, Halvorson & Hastings, 1964) suggested that in spores which normally require some such form of activation in order subsequently to germinate rapidly, rupture of disulphide bonds in spore enzymes might be part of the activation process. It was therefore of interest to find that the *Bacillus cereus* enzyme studied in the present work was inactivated by oxidation and subsequently reactivated by reduction. Experiments to find in which form the enzyme existed in the ungerminated spore, however, all suggested that this was in the active reduced form, and therefore the enzyme did not require activation by reduction in order to attack its substrate.

Another method by which the enzyme within a spore might conceivably be made to attack its substrate during germination would be by release from a bound form (like lysosomal enzymes). It was therefore of interest to find that the enzyme was bound to spore debris, most probably to some part of the spore core. In our experiments, the enzyme was easily released from its bound form by increasing the pH

value or by raising the solute concentration. The only chemical tested which effectively caused release of the enzyme was the basic polyamine spermine which has strong affinity for electronegative structures, e.g. cell membranes (Mager, 1959). Spermine therefore probably released the enzyme by displacing it from its attachment to electronegative groups on or within the core. The ease with which enzyme was released by the various treatments suggests that its attachment is by weak electrostatic bonds or salt linkages and not by covalent bonding. Failure of EDTA to effect release of enzyme makes a role for calcium in linking enzyme to debris seem unlikely.

In an intact spore could some enzyme release mechanism operate during germination? If a germinant (e.g., L-alanine, inosine), or some product of its metabolism in a spore, initiated the release, then germination would quickly occur as freed enzyme attacked its substrate. The observation that during inosine-induced germination of spores an increase in the ratio of free:bound enzyme occurred, suggests that such a release of enzyme did take place. The substrate of the enzyme is probably the structural material of the spore cortex, which is predominantly mucopeptide (Warth, Ohye & Murrell, 1963). The importance of this mucopeptide in maintaining the dormant resistant structure of spores of various bacillus and clostridium species was emphasized by the observation of the germinative action of lysozyme (Gould & Hitchins, 1963), because lysozyme is known to hydrolyse mucopeptide. A search for an enzyme-release mechanism which could be initiated by a germinant or some product of its metabolism by a spore, will clearly be worthwhile. Apart from the possible role of the spore enzyme in germination, fractionation of spores and probable location of the enzyme in one structure (i.e. the core) is of interest. For instance, most enzymes in spores are somehow rendered not only heat-resistant but also dormant, i.e. they do not act upon added substrates. However, the spore enzymes which metabolize germinants cannot be dormant; they must be capable of acting on their substrates in the otherwise dormant spore in order to initiate germination. Further study should reveal whether enzymes, such as L-alanine dehydrogenase, alanine racemase, adenosine deaminase, ribosidase, etc., which one would expect to have a role in metabolizing germinants, are located in different regions of the spore to enzymes concerned with outgrowth of new vegetative cells.

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REFERENCES

Busta, F. F. & Ordal, Z. J. (1964). Heat-activation kinetics of endospores of *Bacillus subtilis*. *J. Food Sci.* **29**, 345.

Flemming, H. P. & Ordal, Z. J. (1964). Responses of *Bacillus subtilis* spores to ionic environments during sporulation and germination. *J. Bact.* **88**, 1529.

Gould, G. W. (1962). Microscopical observations on the emergence of cells of Bacillus species from spores under different cultural conditions. *J. appl. Bact.* **25**, 35.

Gould, G. W. & Hitchins, A. D. (1963). Sensitization of bacterial spores to lysozyme and to hydrogen peroxide with agents which rupture disulphide bonds. *J. gen. Microbiol.* **33**, 413.

Gould, G. W. & Hitchins, A. D. (1965). Germination of spores with Strange and Dark's spore lytic enzyme. In *Spores III*. Ed. by L. L. Campbell and H. O. Halvorson, p. 213. Ann Arbor, Mich: American Soc. for Microbiol.

GOULD, G. W., GEORGALA, D. L. & HITCHINS, A. D. (1964). Sensitization of bacterial spores to lysozyme and the use of fluorochrome labelled lysozyme to detect lysozyme substrate. In *Proc.* 3rd *Int. Symp. Fleming's Lysozyme.* Session 1*b*:61. Milan: Scuola Arti Grafiche O.S.F.

HITCHINS, A. D. & GOULD, G. W. (1964). Release of cores from bacterial spores by mechanical breakage in acidic media. *Nature, Lond.* **203**, 895.

HITCHINS, A. D., GOULD, G. W. & HURST, A. (1963). The swelling of bacterial spores during germination and outgrowth. *J. gen. Microbiol.* **30**, 445.

KADOTA, H., IIJIMA, K. & UCHIDA, A. (1965). The presence of keratin-like substance in spore coat of *Bacillus subtilis*. *Agric. Biol. Chem.* **29**, 870.

KEYNAN, A., EVENCHIK, Z., HALVORSON, H. O. & HASTINGS, J. W. (1964). Activation of bacterial endospores. *J. Bact.* **88**, 313.

MAGER, J. (1959). The stabilizing effect of spermine and related polyamines on bacterial protoplasts. *Biochim. biophys. Acta*, **36**, 529.

POWELL, J. F. & STRANGE, R. E. (1956). Biochemical changes occurring during sporulation in bacillus species. *Biochem. J.* **63**, 661.

RODE, L. J. & FOSTER, J. W. (1960*a*). Mechanical germination of bacterial spores. *Proc. natn. Acad. Sci., U.S.A.* **46**, 118.

RODE, L. J. & FOSTER, J. W. (1960*b*). The action of surfactants on bacterial spores. *Arch. Mikrobiol.* **36**, 67.

RODE, L. J. & FOSTER, J. W. (1962). Ionic germination of spores of *Bacillus megaterium* QM B1551. *Arch. Mikrobiol.* **43**, 183.

RODE, L. J. & FOSTER, J. W. (1965). Gaseous hydrocarbons and the germination of bacterial spores. *Proc. natn. Acad. Sci., U.S.A.* **53**, 31.

STRANGE, E. R. & DARK, F. A. (1957). A cell-wall lytic enzyme associated with spores of Bacillus species. *J. gen. Microbiol.* **16**, 236.

VARY, J. C. (1965). Heat-activated initiation of spore germination. *Bact. Proc.* p. 37.

VARY, J. C. & HALVORSON, H. O. (1965). Kinetics of germination of Bacillus spores. *J. Bact.* **89**, 1340.

VINTER, V. (1962). Spores of microorganisms. IX. Gradual development of resistant structures of bacterial endospores. *Folia Microbiol.* **7**, 115.

WARTH, A. D., OHYE, D. F. & MURRELL, W. G. (1963). Location and composition of spore mucopeptide in Bacillus species. *J. cell. Biol.* **16**, 593.

WORK, E. (1959). The action of a lytic enzyme from spores of a Bacillus on various species of bacteria. *Ann. Inst. Pasteur*, **96**, 468.

EXPLANATION OF PLATE

Phase-contrast micrographs of debris from spores of *Bacillus cereus* which have been disrupted at pH 3·0. The scale mark represents 5 μ.

Fig. 1. Debris in pH 3·0 buffer. The figure shows one unbroken phase-bright spore (*s*) and fragments of spore walls (*w*). A group of cores (*c*) liberated from within spore walls are phase-bright at pH 3·0.

Fig. 2. Debris in pH 5·8 buffer. An unbroken spore (*s*) and spore walls (*w*) appear as in Fig. 1. Cores (*c*) appear phase-dark at pH 5·8.

Fig. 3. Debris in pH 7·0 buffer. Cores (*c*) have now swollen and become pale and diffuse in outline.

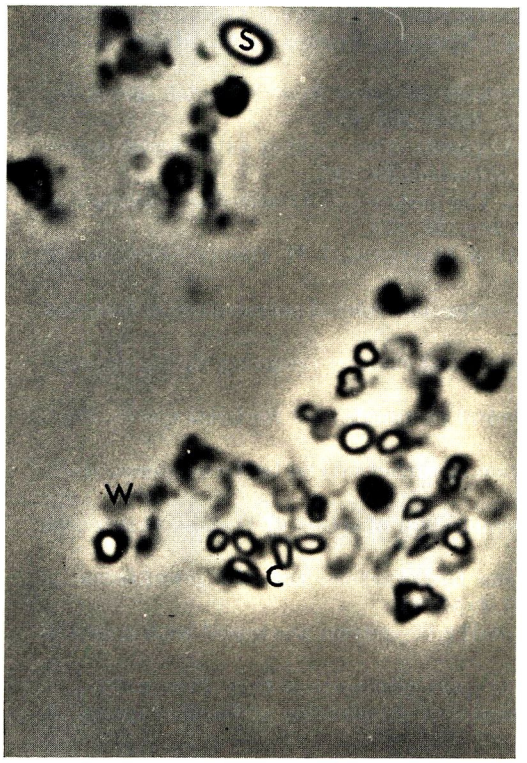

Fig. 1

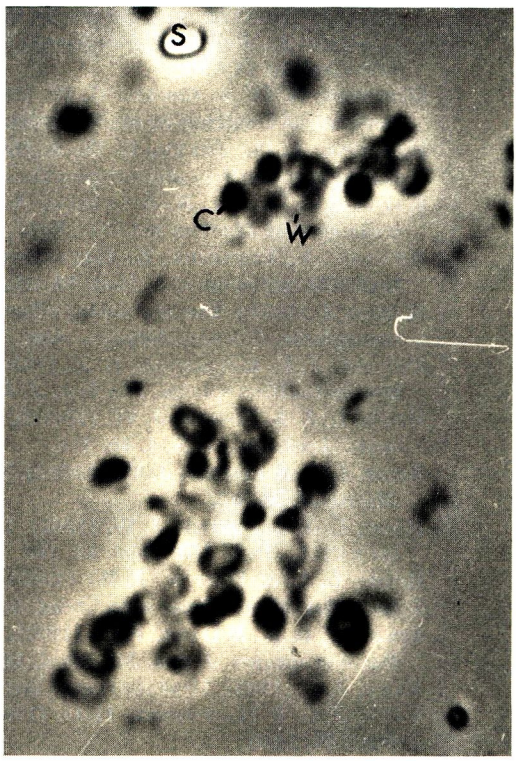

Fig. 2

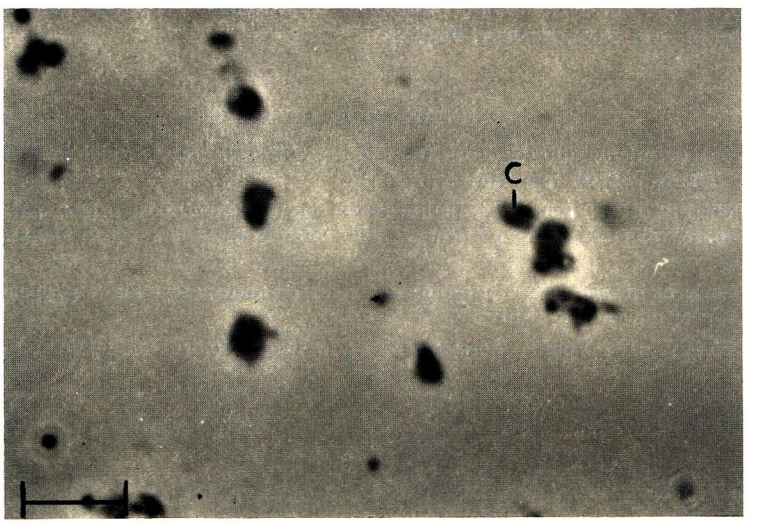

Fig. 3

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The Uptake of Aliphatic Amides by *Pseudomonas aeruginosa*

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SUMMARY

The uptake of aliphatic amides by *Pseudomonas aeruginosa* 8602 was studied by using 1-^{14}C-acetamide and 1-^{14}C-*N*-acetylacetamide (a non-metabolizable analogue). 1-^{14}C-acetamide was accumulated by the wild-type strains and by an amidase-negative mutant. The maximum ratio of internal to external concentration measured was 100:1. 1-^{14}C-*N*-acetylacetamide was concentrated by the wild-type strain and the maximum ratio of internal to external concentration measured was 80:1. No difference was detected in amide uptake as between induced and non-induced cultures of the wild-type or mutant strains. It is concluded that the organism possesses a constitutative permease for these amides. Cyanoacetamide had no significant effect on *N*-acetylacetamide accumulation by the wild-type strain at concentrations which repressed amidase synthesis. Repression of amidase synthesis by amide analogue repressors is therefore not due to inhibition of amide uptake by the bacteria.

INTRODUCTION

Pseudomonas aeruginosa 8602 produces an aliphatic amidase which is induced by several substrate and non-substrate amides. Brammar & Clarke (1964) studied the kinetics of amidase induction in exponentially growing cultures by using acetamide as a substrate inducer and *N*-acetylacetamide as a non-substrate inducer. Amidase was induced at a significant rate by 0·1–1 mM-acetamide or 1–10 mM-*N*-acetylacetamide, but there was no evidence from the kinetic data of an inducible permease system for accumulating amides within the organism. Induction by acetamide and by *N*-acetylacetamide was repressed by certain amide analogues, including cyanoacetamide and thioacetamide. The extent of repression by amide analogues in growing cultures was found to depend on the relative concentrations of the inducer and repressor amides. Carbon-starved organisms were induced to synthesize amidase by very low concentrations of inducers (10–100 μM) and it was possible to show competitive repression by cyanoacetamide of induction by acetamide or *N*-acetylacetamide (Clarke & Brammar, 1964). One of the possible sites of action of the amide analogue repressors is the point of entry of amides into the bacterial cell. We have now made a direct investigation of the permeability of *Pseudomonas aeruginosa* 8602 to amides by examining the uptake of 1-^{14}C-*N*-acetylacetamide by the wild-type strain and 1-^{14}C-acetamide by the wild-type strain and by an amidase-deficient mutant. The effect of cyanoacetamide on amide uptake was studied.

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METHODS

Organism. Our strain of *Pseudomonas aeruginosa* was originally obtained from the National Collection of Type Cultures as NCTC 8602. By subculture in acetamide + glucose medium we obtained strain 8602/A which was used in earlier investigations (Brammar & Clarke, 1964). We have renamed strain 8602/A *Pseudomonas aeruginosa* 8602, since it cannot now be confused with NCTC 8602 which is no longer maintained in the National Collection of Type Cultures. The parent strain *Pseudomonas aeruginosa* 8602 was maintained and grown as described by Brammar & Clarke (1964). The amidase-deficient strain am_{10} was isolated from strain 8602 after treatment with the mutagenic agent ethylmethane sulphonate. Strain am_{10} produced no detectable amidase under any of the conditions tested (Skinner & Clarke, 1965).

Media. The growth media used were those described by Brammar & Clarke (1964). The standard minimal salts growth medium contained 0·02 M-sodium succinate. For the carbon-starved bacteria sodium succinate was used as the carbon source at 0·01 M and acetamide at 0·02 M, which restricted growth to about equiv. 0·4 mg. dry wt. bacteria/ml.

Amidase assay. Enzyme assays were made by measuring the transferase activity with acetamide as substrate (Brammar & Clarke, 1964).

Amides. Acetamide was obtained from Hopkin & Williams Ltd. and purified by recrystallizing twice from ethanol. 1-^{14}C-acetamide (1 mC/m-mole) was obtained from Merck, Sharpe & Dohme of Canada Ltd. and purified by thin-layer chromatography on silica gel, in a similar way to that described below for 1-^{14}C-*N*-acetylacetamide. 1-^{14}C-*N*-acetylacetamide was prepared as follows. 1-^{14}C-acetamide (5 mg.) and Analar acetic anhydride (25 μl.) were heated at 130° in a sealed capillary tube attached to the bulb of a thermometer in an oil bath. After 4 hr the sealed capillary tube was slowly cooled and opened. The reaction mixture was removed into a capillary tube of finer bore and applied to a freshly activated silica gel thin-layer plate. The solvent used for chromatography was toluene + ethanol (80 + 20, by vol.) and unlabelled *N*-acetylacetamide was used as an adjacent marker in a parallel track. The marker amides were converted into hydroxamates by spraying with a saturated solution of hydroxylamine hydrochloride in 80% (v/v) methanol in water, containing NaOH 3% (w/v) and heating at 100° for 10 min. The hydroxamates were detected by spraying with a solution of $FeCl_3$ (10%, w/v) + HCl (0·1%, w/v) in 95% (v/v) methanol in water.

The radioactive areas were detected by radioautography with Kodak X-ray films (Blue Band or Royal blue). The radioactive area of silica gel corresponding to the *N*-acetylacetamide marker was removed with a spatula and the ^{14}C-*N*-acetylacetamide extracted with 1 ml. distilled water and stored at −30°.

Uptake of ^{14}C-amides. The ^{14}C-amides were made up to the required concentration with unlabelled amide and added to the bacterial incubation mixtures. Samples (1 ml.) were pipetted onto Oxoid 3 cm. membrane filters (Oxoid Ltd., London) or Millipore filters (V. A. Howe and Co. Ltd.), filtered under pressure and washed with about 10 vol. of ice-cold culture medium. The filters were dried at 80° for 15 min. and placed in phials containing 15 ml. scintillation fluid (0·04 g. 2-*p*-phenylene-bis-(5-phenyloxazole) + 4·0 g. 2:5-diphenyloxazole/l. toluene). The samples were

counted in a Nuclear Chicago liquid scintillation counter which gave a counting efficiency of about 60% under the conditions used.

The retention volume of the membrane filters was determined by filtering a 1 ml. sample of cell-free labelled medium. After each experiment the radioactivity in the medium was determined by application of a 10 μl. sample to a filter, drying and counting.

RESULTS

Uptake of 1-^{14}C-N-acetylacetamide by carbon-starved bacteria

The wild-type strain *Pseudomonas aeruginosa* 8602 was grown on limiting concentrations of acetamide to obtain induced bacteria, and on succinate to obtain non-induced bacteria. The uptake of 1-^{14}C-*N*-acetylacetamide by the washed

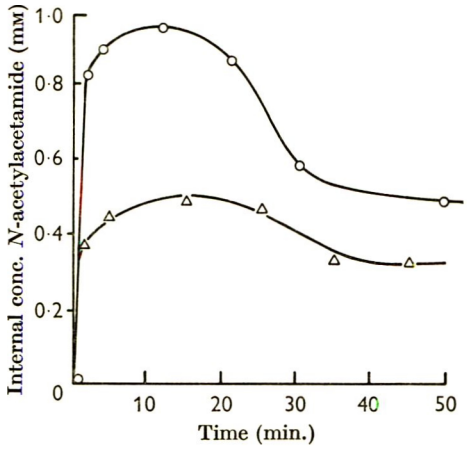

Fig. 1

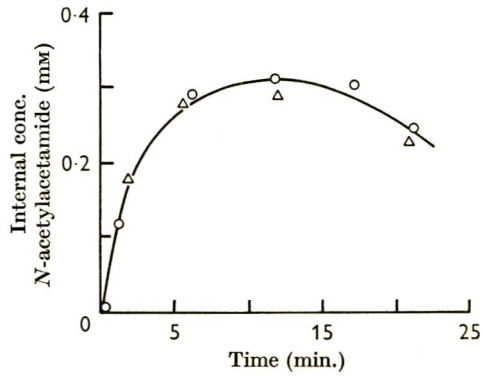

Fig. 2

Fig. 1. Uptake of ^{14}C-*N*-acetylacetamide by washed suspensions of *Pseudomonas aeruginosa* 8602 wild-type grown on limiting succinate. 10 μM-*N*-acetylacetamide. ○—○, incubated at 20°; Δ—Δ, incubated at 37°.

Fig. 2. Uptake of ^{14}C-*N*-acetylacetamide by washed suspensions of *Pseudomonas aeruginosa* 8602 wild type grown on limiting succinate. Incubation at 20° with 10 μM-*N*-acetylacetamide. ○—○, control; Δ—Δ, with mM-cyanoacetamide.

carbon-starved bacteria was followed at 37°, and samples were removed for estimations of radioactivity at intervals during 30 min. of incubation. Incorporation of radioactivity into the bacteria was rapid and there was no significant difference between the induced and non-induced bacteria. With 100 μM-*N*-acetylacetamide the radioactivity taken up by the bacteria rapidly reached a plateau value with both bacterial preparations. It was known from previous work (Clarke & Brammar, 1964) that acetamide-grown bacteria had a high amidase activity and that, under the conditions of the ^{14}C uptake experiments, succinate-grown carbon-starved bacteria were rapidly induced by *N*-acetylacetamide to form amidase. This experiment showed that there was no comparable induction of a permeability system for amide uptake since amidase-induced bacteria and non-induced bacteria behaved in similar ways.

The uptake of 1-^{14}C-*N*-acetylacetamide at 20° by succinate-grown carbon-starved

bacteria was compared with that at 37°. Figure 1 shows that the process was more rapid and reached a higher value at 20° than at 37°. An initial rapid uptake reached a plateau value which declined after about 15 min. to reach a new lower value. The internal concentration of *N*-acetylacetamide in the experiment at 20° was 80 times the external concentration at the plateau value and 40 times higher after about 30 min. At 37° the highest difference reached between the internal and external concentrations of *N*-acetylacetamide was about 40 times.

Cyanoacetamide, which represses amidase induction by *N*-acetylacetamide in carbon-starved bacteria had no effect on the uptake of *N*-acetylacetamide. Figure 2 shows the uptake of 10 μM-1^{14}C-*N*-acetylacetamide in the presence and absence of mM-cyanoacetamide. Under these conditions repression of amidase synthesis was virtually complete (Clarke & Brammar, 1964).

The uptake of 1-^{14}C-N-acetylacetamide by exponentially growing bacteria

The experiments with carbon-starved pseudomonads had shown that a concentration of cyanoacetamide which completely repressed amidase induction had no effect on the uptake of *N*-acetylacetamide by the bacteria. Similar results were obtained with growing cultures. A culture of *Pseudomonas aeruginosa* 8602 growing exponentially in succinate medium at 37° was induced with 1-^{14}C-*N*-acetylacetamide at about 8000 counts/min./ml. and diluted with unlabelled *N*-acetylacetamide to give a final concentration 5 mM. The culture was divided into two flasks to one of which cyanoacetamide was added to a concentration of 10 mM. Samples were removed at intervals for determinations of growth, amidase activity and radioactivity. The rate of amidase synthesis was 90% repressed but there was no significant difference in the uptake of ^{14}C by the bacteria.

Uptake of 1-^{14}C-acetamide

Acetamide permeation could not be properly studied in the wild-type *Pseudomonas aeruginosa* 8602 since it was impossible to separate the entry process from the further metabolism of acetamide. Acetamide was not hydrolysed by the amidase-deficient mutant strain am_{10} so that in this strain acetamide uptake reflects the transport process itself and not its coupling to intermediate metabolism. Acetamide uptake by strain am_{10} can be considered to be analogous to the uptake of a non-substrate inducer by the wild-type. 1-^{14}C-acetamide uptake was followed with wild-type and mutant strains. The cultures were grown on succinate medium and harvested after about 2 hr in the exponential growth phase; this provided the non-induced cultures. To obtain induced cultures, *N*-acetylacetamide to give a final concentration mM was added as soon as growth had started. Under these conditions wild-type bacteria are induced to synthesize amidase at a rapid rate. The bacteria were resuspended in succinate medium containing chloramphenicol 50 μg./ml. and the uptake of ^{14}C-acetamide followed at 25°.

Figure 3 shows the uptake of 1-^{14}C acetamide by strain am_{10}. The amount of radioactivity in the bacteria increased during the first 5 min. and then reached a plateau which was maintained for 10–25 min. There was no significant difference between cultures grown in the presence or absence of the enzyme inducer. Uptake of 1-^{14}C-acetamide by the wild-type gave similar results and again no significant difference was detected between induced and non-induced cultures. The values for

1-^{14}C-acetamide accumulation at the plateau by the wild type were approximately the same as those obtained with mutant strain am_{10}. It was calculated from the experiments with strain am_{10} that the internal concentration of acetamide reached a maximum of about 40 μmoles/g. dry wt. bacteria. This represents a ratio of internal to external concentration of 100:1.

Figure 3 also shows the results of typical experiments in which a 100-fold excess of non-radioactive acetamide was added to cultures of mutant strain am_{10} which had accumulated 1-^{14}C-acetamide. With both the induced and the non-induced cultures the radioactivity was displaced from the bacteria. The differences in the curves for induced and non-induced cultures are not significant. A similar experiment was made with an induced culture of the wild type but no displacement was detected. It was concluded that this was because the acetamide was being metabolized by the wild-type bacteria.

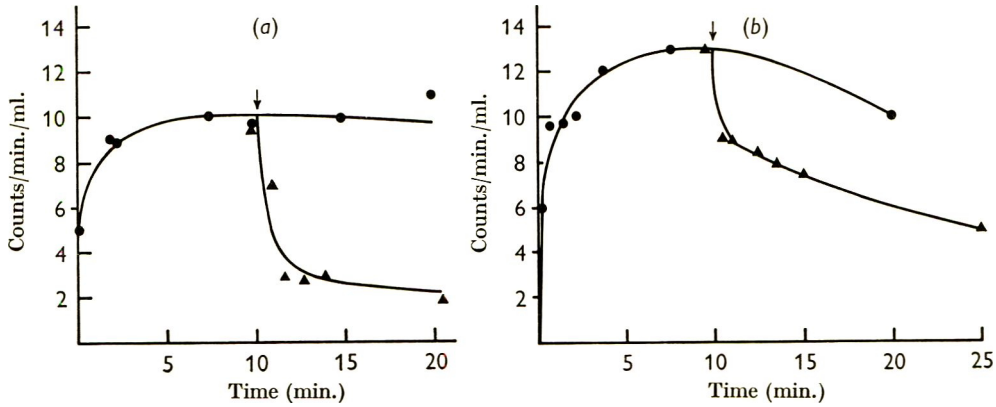

Fig. 3. Uptake of ^{14}C-acetamide by washed suspensions of *Pseudomonas aeruginosa* 8602 amidase-deficient mutant strain am_{10}. (*a*) non-induced bacteria, (*b*) induced bacteria. ●—●, 100 μM-^{14}C-acetamide; ▲—▲, 100-μM-^{14}C-acetamide, with 10 mM-^{12}C-acetamide added at the time indicated by arrow.

1-^{14}C-acetamide was recovered quantitatively and in an unaltered form from cultures of mutant strain am_{10}. Samples (1 ml.) of the incubation mixtures were taken at intervals after 1-^{14}C-acetamide had been added (5–25 min.), centrifuged, the pellets washed with ice-cold medium and disrupted by resuspending in 0·25 ml. ice-cold acetone 50 % (v/v) in water. Samples of the acetone extracts of the bacteria were counted and also subjected to thin-layer chromatography and radioautography. From each of the extracts only one radioactive spot was obtained which corresponded with the acetamide marker. The total count of radioactivity in 1 ml. of extract was 8400/min. and that of the acetamide spot on the chromatogram to which 0·25 ml. of the extract had been applied was 2000/min.

DISCUSSION

Considerable evidence has now been obtained for the existence in bacteria of stereospecific permeation systems which mediate the entry of organic compounds into bacteria. The term 'permease' was suggested to designate the specific proteins involved, and the most detailed studies of these systems have been made on

β-galactoside permease of *Escherichia coli* (Cohen & Monod, 1957; Kepes & Cohen, 1962; Fox & Kennedy, 1965). The existence of permease systems in *Pseudomonas aeruginosa* for the transport of intermediates of the tricarboxylic acid cycle was suggested by the work of Barrett & Kallio (1953), Kogut & Podoski (1953) and Clarke & Meadow (1959). Kepes & Cohen (1962) suggested that the following conditions should be the minimal requirement to demonstrate the existence of a permease: (1) accumulation of a natural substrate above the external concentration; (2) accumulation of a non-metabolizable analogue; (3) specific crypticity as opposed to normal utilization; (4) specific crypticity for a substrate analogue known to compete for metabolic enzymes *in vitro*. These conditions can only be fully satisfied when mutants are available which lack the specific permease and the specific enzymes of the metabolic pathway, In the present work we were concentrating on certain aspects of amide permeation. We wished to establish whether or not amides were concentrated by the bacteria, and if so whether the system was inducible and what effect repressors of amide induction had on the process.

^{14}C-*N*-acetylacetamide was concentrated by wild-type *Pseudomonas aeruginosa* 8602 above the external concentration and no difference was observed between non-induced bacteria and bacteria induced to form amidase by growth on acetamide. The uptake of 1-^{14}C-acetamide by an amidase-deficient mutant strain am_{10}, was similar to the uptake found for ^{14}C-*N*-acetylacetamide by the wild type. With strain am_{10} the initial rate of accumulation and the maximum internal concentration of amide again showed no significant difference between induced and non-induced cultures. Extraction of bacteria with aqueous acetone showed that the radioactivity was entirely due to free 1-^{14}C-acetamide. The accumulated 1-^{14}C-acetamide was displaced by adding excess of labelled acetamide. Induced and non-induced wild-type cultures were also shown to accumulate 1-^{14}C-acetamide. No displacement of radioactivity by unlabelled acetamide was detected with induced cultures of the wild-type strain, presumably because the acetamide was metabolized by the bacteria. These results satisfy the first two requirements of Kepes & Cohen (1962) for demonstrating the existence of a permease, i.e. the internal accumulation of a natural substrate and a non-metabolizable analogue above the external concentration. The identical behaviour of induced and non-induced cultures indicated that the amide permease was constitutive. Although both β-galactosidase and β-galactoside permease of *Escherichia coli* are inducible, Horecker, Thomas & Monod (1960) showed that galactose permease is constitutive while the enzymes for the initial steps in galactose utilization are inducible. Permeases for α-glucosides and amino acids are also constitutive in *E. coli* (Kepes & Cohen, 1962).

The effects of temperature on *N*-acetylacetamide uptake are similar to those described by Kepes & Cohen (1962) for the β-galactoside permease of *Escherichia coli*. The accumulation of thiomethyl-β-D-galactoside by a suspension of *E. coli* can be increased by a factor of 5 by lowering the temperature from 34° to 14°. We made no detailed studies on the kinetics of *N*-acetylacetamide and acetamide uptake by *Pseudomonas aeruginosa* 8602 at different external concentrations, but the maximum ratio of internal to external concentration found was 80:1 for *N*-acetylacetamide at 20° and 100:1 for acetamide at 25°. For *E. coli* β-galactoside permease, values have been calculated for maximum ratios of internal to external concentration of 5 for o-nitrophenyl-β-D-galactoside to 2000 for lactose.

From previous work it had been concluded that the amide analogue repressor cyanoacetamide competed directly with the inducer *N*-acetylacetamide (Clarke & Brammar, 1964). Enzyme induction by *N*-acetylacetamide is very sensitive to repression by cyanoacetamide. With exponentially growing cultures of *Pseudomonas aeruginosa* 8602, induction by 10 mM-*N*-acetylacetamide is 84% repressed by 100 μM-cyanoacetamide, while repression of induction by mM-acetamide requires a 10-fold excess of cyanoacetamide. We concluded that the three amides had affinities for an inducer binding site in the order acetamide > cyanoacetamide $\gg$ *N*-acetylacetamide. The most sensitive system to test whether this site was at the point of entry of amides into the organism was the effect of cyanoacetamide on the uptake of *N*-acetylacetamide. At concentrations of cyanoacetamide sufficient to repress enzyme synthesis completely no effect of cyanoacetamide on 1-^{14}C-*N*-acetylacetamide uptake by *Pseudomonas* 8602 was detected. We conclude that the site of competition cannot be at the point of entry into the organism, but must be at a site within it. The most probable site for competition of amide analogue repressors with inducer amide is the inducer binding site of a cytoplasmic repressor molecule produced by a regulator gene (Jacob & Monod, 1961).

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REFERENCES

Barrett, J. T. & Kallio, R. E. (1953). Terminal respiration in *Pseudomonas fluorescens*. Component enzymes of the tricarboxylic acid cycle. *J. Bact.* **66**, 517.

Brammar, W. J. & Clarke, P. H. (1964). Induction and repression of *Pseudomonas aeruginosa* amidase. *J. gen. Microbiol.* **37**, 307.

Clarke, P. H. & Brammar, W. J. (1964). Regulation of bacterial enzyme synthesis by induction and repression. *Nature, Lond.* **203**, 1153.

Clarke, P. H. & Meadow, P. M. (1959). Evidence for the occurrence of permeases for tricarboxylic acid intermediates in *Pseudomonas aeruginosa*. *J. gen. Microbiol.* **20**, 144.

Cohen, G. N. & Monod, J. (1957). Bacterial permeases. *Bact. Rev.* **21**, 169.

Fox, C. F. & Kennedy, E. P. (1965). Specific labelling and partial purification of the M protein, a component of the β-galactoside transport system of *Escherichia coli*. *Proc. natn. acad. Sci., U.S.A.* **54**, 891.

Horecker, B. L., Thomas, J. & Monod, J. (1960). Galactose transport in *Escherichia coli*. 1. General properties as studied in a galactokinase-less mutant. *J. biol. Chem.* **235**, 1580.

Jacob, F. & Monod, J. (1961). Genetic regulatory mechanisms in the synthesis of proteins. *J. mol. Biol.* **3**, 318.

Kepes, A. & Cohen, G. N. (1962). Permeation. In *The Bacteria*, vol. 4, p. 199. New York: Academic Press.

Kogut, M. & Podoski, E. P. (1953). Oxidative pathways in a fluorescent *Pseudomonas*. *Biochem. J.* **55**, 800.

Skinner, A. J. & Clarke, P. H. (1965). C_2 mutants of *Pseudomonas aeruginosa*. *J. gen. Microbiol.* **39**, viii.