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THE JOURNAL OF GENERAL MICROBIOLOGY

The *Journal* will publish accounts of original research in general microbiology, i.e. the study of bacteria, microfungi, microscopic algae, protozoa, and viruses in their biological activities and, more particularly, the fundamental aspects of the study of these forms, including structure, development, physiology, genetics, cytology, systematics and ecology. Writers of papers on a specialized aspect of their subject should describe their work so that its relevance to their own science and to microbiology in general will be apparent to readers who may be unfamiliar with the particular aspect.

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'Easy reading's curst hard writing.'—*The Editors, J. gen. Microbiol.*

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Descriptions of new species of cultivable microbes should not be submitted unless an authentic specimen of a living culture has been deposited in a recognized culture collection

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Except for good reasons, micro-organisms should be designated by the names used in the works listed below. When other authorities are followed, they should be cited whenever obscurity might result from their use.

MICROFUNGI. *Ainsworth & Bisby's Dictionary of the Fungi*, 1961, 5th ed. (Kew: Commonwealth Mycological Institute.)

PLANT PATHOGENIC FUNGI AND PLANT DISEASES. *List of Common British Plant Diseases*, 1944. (Cambridge University Press.)

PLANT VIRUSES AND VIRUS DISEASES (1957). *Rev. appl. Mycol.* **35**, Suppl. 1–78.

BACTERIA. Author's preferences in naming are at present accepted provided that the designation is unambiguous and conforms with the International Bacteriological Code of Nomenclature (1949; *J. gen. Microbiol.* **3**, 444) and the Opinions issued by the International Committee on Bacteriological Nomenclature. If desired, a synonym may be added in brackets when a name is first mentioned.

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On Evidence Supporting a Deterministic Process of Bacterial Growth

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SUMMARY

Recent evidence supporting a proposed model (Koch & Schaechter, 1962) for the control of bacterial cell division is reviewed. Calculations from the published work of others are presented which show that the standard deviation of length of time between a cell division and Nth cell division does not increase, at least up to $N = 9$. This finding implies that each cell has an excellent clock, which is handed to the daughters without significant error, and that the observed fluctuations in age of cells at division are largely due to an additional fluctuation associated with cell division, but not timing it. In terms of our model, it is strong support for the deterministic growth of cell constituents and the equipartition of cell constituents at division. An expansion of the original model is considered which accounts for the difference between the original model and the finding regarding the correlation coefficients of the ages of mothers and daughters and that between sisters, and the skewed nature of the age-distribution curve. In the new version of the model, the assumption that there are random fluctuations in the critical size at division is replaced by the assumption that the fluctuations are not completely random but that there is a moderately positive correlation between the sizes of divisions of successive generations.

Several years ago we proposed (Koch & Schaechter, 1962) a model for bacterial cell division which took into account a number of the essential features of the statistical aspects of cell division as well as some of the known facts in the biochemistry of growth. To bridge the gap between the stochastical nature of cell division and the deterministic processes of protein synthesis, etc., the model had four basic postulates. First, that increase of bacterial constituents was substantially deterministic even at the single cell level. Explicitly, it was assumed that growth was exponential. Second, that there was a critical size which the cell must attain in order that division might ensue. Third, that there are random fluctuations, either about this critical time or a variable lag in the length of time when cell division would be apparent to the observer. Fourth, that cell division partitions the maternal cell, yielding daughters of identically the same size under certain conditions and nearly of the same size under other conditions. This model then could account for a number of the properties of growing populations observed by others, as well as experimental results from our own laboratory (Schaechter, Williamson, Hood & Koch, 1962).

Recently, mathematical consequences of this model have been greatly augmented

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by the work of Powell (1964). He has extended calculations based on this model to larger values of fluctuation and to fluctuation obeying more arbitrary distributions than were considered in our original publication or than seem to apply to the enteric bacteria.

However, of more vital import to this model would be further experimental justification of the four postulates on which it is based. Since the model was presented, two papers have appeared which contain data which we believe strongly support the concept that a highly deterministic process underlies the growth of micro-organisms and, in addition, that under certain conditions cell division leads to a precise equipartition of the cell contents.

A critical test for the simultaneous validity of both these assumptions, in that it would obviate the almost unavoidable experimental errors, would be the finding that standard deviation (S.D.) of the total time elapsed between one cell division and the cell division of all the Nth generation descendants would not increase with increasing N. Thus, independent of the details controlling the act of cell division, if the growth rate constant for protoplasm were constant at any time for all the cells, and if cell division divides the cell precisely in half, the variance for the interval between successive divisions should be the same, depending only on the chance fluctuations that determine the initial size of the first cell and the fluctuations that terminate the last division.

In our experimental work we did not make experiments over more than four generations because we felt that many cell divisions on a small region of a block of agar would lead to crowding which would interfere with the growth process and vitiate the conclusions. However, Kubitschek (1962) and Hoffman & Frank (1965) successfully made such experiments; in the former case to the fifth generation and in the latter case to the fourteenth generation. Data were presented in both their papers which permit computation of the desired quantities. These are shown in Table 1. It can be seen that the S.D. of total elapsed time from the division of the maternal cell on to the fifth generation in the case of Kubitschek's experiment and on to the ninth generation in the case of the Hoffman & Frank experiments proceeds without significant increase. This is true even though the microphotographs show that the organisms were extremely crowded by the end of the ninth generation. In the latter set of experiments, the overall mean interdivision time is not precisely given but from other data must be of the order of 21 min., so that with a coefficient of variation of 20%, the S.D. for a single interdivision time is about 4 min., which is not much less than the 5·69 min. fluctuation for 9 generations. This means that fluctuations in generation rate, together with the imperfections in equipartition at cell division, are much less than the coefficient of variation of the interdivision time divided by $\sqrt{N}$, and thus are less than $20\%/\sqrt{9}$, or 6% in a single generation.

This lack of increase in the S.D. with N is a strong support for the idea that the fluctuation is entirely due to a random chance at first division initiating the parental cell and to the random fluctuation terminating the last division, no matter how many intervening divisions with their concomitant fluctuations are involved. Thus, it follows that the growth must be highly deterministic and that, in addition, cell division under these experimental conditions is a very precise process, dividing the cell into very nearly identical daughters. The relationship for the increase of cellular constituents under usual laboratory conditions at the single cell level must

Table 1. *Standard deviation in total time interval to the Nth division of Escherichia coli*

	Data of Kubitschek (1963)						Data of Hoffman & Frank (1965).			
	Colony 1·1			Colony 2·3				Colony 63–3		
No. of generations	No.	Mean (min.)	S.D. (min.)	No.	Mean (min.)	S.D. (min.)	No. of generations	No.	Mean (min.)	S.D. (min.)
1	61*	26·28	6·54	62*	26·01	4·76	1	—	36·00	—
2	4	45·75	0·61	4	68·79	3·58	2	4	57·50	2·9†
3	8	70·47	7·54	8	93·19	3·24	3	8	79·75	5·26†
4	16	96·84	4·28	16	117·30	4·28	4	16	98·19	5·09†
5	31‡	124·43	7·31	32	143·24	4·64	5	32	119·09	3·69§
							6	64	140·55	4·03§
							7	123	162·94	4·46§
							8	231	186·33	6·32§
							9	451	217·89	5·69§
							10	772	230·71	7·69§

* All recorded interdivision times.

† Computed from the range and number of observations given by authors and tabulated ratio of the range to S.D. given in Pearson & Hartley (1958).

‡ One value omitted.

§ Values listed by authors as S.D. of mean, but must be estimates of S.D. from their indicated ranges and their Fig. 6.

be very nearly a pure exponential. This is stressed by the recent findings that extend the concept that DNA synthesis is essentially continuous at the single cell level (Pachler, Schaechter & Koch, 1965) and that there is exponential increase in DNA and other cell constituents within the cell cycle, as shown by the refined experiments of Cummings (1965) with synchronized organisms.

On the other hand, some of the predictions of the model are simply not generally obeyed. It is often found that the mother–daughter correlation coefficient is almost never as negative as $-0{\cdot}5$, that the sister–sister correlation is generally larger than $+0{\cdot}5$, and that the generation time distribution is positively skewed, in significant disagreement with the original model for precise equipartition at cell division. In fact, these were the findings in the experiments of Kubitschek quoted above. If it is assumed that these differences are not due to experimental artifacts, the original model must be refined. To do so, we must reject the concept of random fluctuation in the size of cells at division and replace it by a fluctuation in the critical size which is retained for a period extending over two or three generations. This assumption implies a moderate positive correlation in the sizes of cells at division of mother and daughter cells, as is the case (Errington, Powell & Thompson, 1965; Koch, calculations on unpublished data of Kubitschek). Thus, a deviation in generation time from the mean in one generation may not be made up on the average in the next generation, but is made up in the next two or three generations. This is a contribution equivalent to short-term memory that would tend to make sister–sister correlation somewhat higher and the mother–daughter correlation somewhat lower (more positive) than predicted by the original explicit model and which could account for the skewed generation time distribution as well.

To see this qualitatively, imagine that the critical size for all the cells in the culture is not random at all but varies periodically and systematically. For this limiting case, the sister–sister correlation coefficient would be $+1$, and the mother–daughter coefficient anywhere between $+1$ and -1, depending on the relationship between the doubling time and the period of the fluctuation. If the variation in the critical size is small compared to its mean, as we have shown, the generation time distribution will be normal. If a small amount of such a systematic fluctuation is 'mixed in' with a random fluctuation determining the critical size, then it is evident that the sister–sister correlation coefficient will tend to be greater than $+0{\cdot}5$ and the mother–daughter correlation coefficient will (in general) tend to be greater than $-0{\cdot}5$. The generation time distribution would still be normal.

The generation time distribution will be positively skewed if the persistent fluctuations are of a particular type. For example, if random fluctuations occur which quickly lower the critical size and are then followed by a slow return to normal, then the age distribution will be positively skewed. That the critical size is under physiological control is evident from the variation in cell size under different growth conditions. While such a model is required by the present data, further elaboration must await measurement of the distribution of the amounts of cell constituents amongst cells at the instant of cell division. A possible mechanism for the control of cell division which has these properties will be presented (Koch, in preparation).

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Approach to an Improved Taxonomy of the genus *Agrobacterium*

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SUMMARY

With a view to an improved taxonomy of the genus *Agrobacterium*, 45 strains, including representatives of all nomen-species, were used to investigate the following features: base composition and compositional distribution of pure deoxyribonucleic acid (DNA); type of flagellation; 3-ketoglycoside formation; phytopathogenicity for tomato and Datura. It is proposed to limit the genus *Agrobacterium* to two, or possibly three, species: (1) *Agrobacterium radiobacter* and its phytopathogenic variety *A. radiobacter* var. *tumefaciens*; (2) *A. rhizogenes*; and (3) possibly *A. pseudotsugae*. More work on the latter two species is required before they can definitely be accepted as separate species of this genus. The DNA of all the strains of the former two species has a T_m value in the narrow range of 93·8°–95·1°, corresponding with an average molar guanine + cytosine (GC) content of 59·5–62·8%. The variance σ of the compositional distribution of the DNA molecules ranges between 0 and 0·88% with an average of 0·24% (GC). The only available strain of *A. pseudotsugae*, with 67·7% GC, was completely out of this range and its chromosomal DNA was clearly different from that of the other two species. All strains of *Agrobacterium* proper were peritrichous, frequently with 5–6 flagella. All 8 strains of *A. radiobacter* and 24 of 28 strains of the variety '*tumefaciens*' converted lactose into 3-ketolactose; all the other strains were negative in this respect. Several arguments are advanced to include the strain *A. rubi* with *A. radiobacter* var. *tumefaciens* and to remove the species *A. stellulatum* and *A. gypsophilae* from this genus. The relationship between *Agrobacterium* and some other genera is shown graphically in a plot of mean similarity *versus* DNA base composition.

INTRODUCTION

The present taxonomy of the genus *Agrobacterium* is confused and varies from one textbook to another. Both *Bergey's Manual* (1957) and Prévot (1961) recognize *Agrobacterium* as a separate genus. The former text includes five phytopathogenic species (*A. tumefaciens*, *A. gypsophilae*, *A. pseudotsugae*, *A. rhizogenes*, *A. rubi*) and two non-phytopathogenic ones (*A. radiobacter*, *A. stellulatum*). Prévot (1961) recognizes only *A. tumefaciens*, *A. rhizogenes*, *A. rubi* and *A. pseudotsugae*, and includes *gypsophilae* in *Xanthomonas*. On the other hand, Krassilnikov (1959) does not mention the generic name *Agrobacterium* and includes the species '*tumefaciens*', '*rhizogenes*', '*gypsophilae*' and '*radiobacter*' in *Pseudomonas*; the other nomen-species are not mentioned. Dowson (1957) included the species '*tumefaciens*' and '*rhizogenes*' in *Erwinia*. The genus *Agrobacterium* as defined for example in *Bergey's Manual* (1957) is very heterogeneous and would contain both polarly and peritrichously flagellated organisms. The host-specificity range of some of the species (e.g. *A. rubi*) is poorly known and their classification is partially built on trifles such as small differences in nitrate reduction. A revision of this genus is thus much needed. Because DNA base composition and flagellation appeared to be useful features for an improved and simplified classification of the genus *Rhizobium* (De Ley & Rassel, 1965) we applied the same approach with a group of 45 agrobacteria, including representatives of each of the seven nomen-species. These organisms have few specific physiological and biochemical characteristics, except their phytopathogenicity and the production of 3-ketoglycosides from disaccharides and bionic acids. These features were also examined in our survey. Phytopathogenicity tests were limited to the use of tomato and Datura and were mainly intended as a control. The formation of 3-ketoglycosides was discovered in our laboratories (Bernaerts & De Ley, 1958; 1960*a*, *b*; 1961; 1963; Bernaerts, Furnelle & De Ley, 1963) and later confirmed elsewhere (Fukui *et al.* 1963; Fukui & Hochster, 1963*a*, *b*).

METHODS

Organisms. All the organisms used are listed in Table 1. Many of the strains were kindly given by the National Collection of Plant Pathogenic Bacteria (NCPPB: Ministry of Agriculture, Fisheries and Food, Hatching Green, Harpenden, England); the International Collection of Phytopathogenic Bacteria (ICPB; Department of Bacteriology, University of California, Davis, Calif., U.S.A.); the National Collection of Industrial Bacteria (NCIB; Torry Research Station, Aberdeen, Scotland); the Institut Pasteur, Paris, France; the Institut Agronomique de l'Etat, Gembloux, Belgium; the Laboratory for Microbiology, Technological University, Delft, the Netherlands. *Agrobacterium stellulatum* was obtained through the courtesy of Professor C. Stapp (Institut für Bakteriologie und Serologie, Braunschein-Gleismarode, Germany). Several strains were bought from the American Type Culture Collection (ATCC; Washington, D.C. U.S.A.). The strains *A. radiobacter* L/2/2/1 and M 2/1 were the ones with which 3-ketoglycoside production was first observed (Bernaerts & De Ley, 1958; 1960*a*, *b*).

Estimation of DNA base composition by thermal denaturation. Mass cultures of the organisms were prepared by growing them for about 2 days at 30° in Roux flasks on a medium containing (%, w/v): glucose, 1; yeast extract (Nederlandse Gist- en

Spiritusfabriek, Brugge, Belgium), 1; agar, 2·5. The bacteria were harvested and pure DNA prepared therefrom according to Marmur (1961). Thermal denaturation was determined with the recording thermospectrophotometer described previously (De Ley & Van Muylem, 1963). The midpoint of the thermal transition T_m and the variance σ expressed as % guanine + cytosine (GC) of the compositional distribution of DNA molecules were determined graphically from the recorded absorbance-temperature curves. The mean % GC was calculated with Marmur & Doty's (1962) formula, % GC = $(T_m - 69{\cdot}3)/0{\cdot}41$. A previous control of this formula with the acetic acid bacteria (De Ley & Schell, 1963) had shown it to be reliable in the present temperature range.

Flagellation. Since the flagella of Agrobacterium are easily detached during the ordinary staining procedure, electron microscopy was used. The methods were the same as in the previous paper (De Ley & Rassel, 1965) except that the suspending medium was much diluted, which decreased the background impurities, and that instead of 2%, 0·5% phosphotungstic acid was used, which produced sharper cell edges. Each strain was observed repeatedly at different growth stages.

Phytopathogenicity. A dense suspension of each bacterial strain was inoculated into the freshly wounded upper stem of two plants each of *Datura stramonium* and *Lycopersicon esculentum.* Tumour formation was recorded after 8–10 days. Tumourigenic strains always acted on both types of plants. No effort was made to test for the hairy-root disease attributed to *Agrobacterium rhizogenes.*

3-Ketoglycoside production was determined by the auxanographic method of Bernaerts & De Ley (1963).

RESULTS AND DISCUSSION

All the results are listed in Table 1.

General properties of Agrobacterium

There seems to be some hope that the knowledge of the % guanine + cytosine (GC) of the bacterial DNAs might partially help to clarify the present definition and classification of the genus *Agrobacterium.* All strains of *Agrobacterium radiobacter*, *A. tumefaciens* and *A. rhizogenes* had a T_m value in the very narrow range of 93·8°–95·1° (total range 1·3°) with a corresponding % GC of 59·5 to 62·8. Taken in conjunction with the other known morphological and physiological properties of these organisms, this indicates a possible very close genetic relationship.

All strains of these three groups are peritrichously flagellated, frequently with 5–6 flagella (Pl. 1). The largest flagella are about 4–5 times as long as the bacterium and have a diameter of 100–120 Å. Frequently 2–10 organisms stick together and single organisms are rare; this makes it sometimes difficult to count the exact number of flagella. One or two of the flagella are often subpolarly implanted. This is strongly reminescent of a similar situation encountered with *Rhizobium* (De Ley & Rassel, 1965) and may be another argument in favour of a close relationship between the two groups. Quite frequently the bacteria bear fimbriae-like structures which are longer, thinner (diameter 60–80 Å), more rigid and more fragile than the flagella.

Concerning the phytopathogenicity, it is seen from Table 1 that there were very few misnomers in *Agrobacterium tumefaciens*, except the strains '*rhizogenes*' NCPPB 5

Table 1. *Genotypic (expressed as 'melting point' T_m, °/o guanine+cytosine (GC) and variance σ of pure DNA) and phenotypic (tumourigenesis, type of flagellation, 3-ketolactose formation) properties of several strains of Agrobacterium*

For methods, see text. The peritrichous nature of the strains marked* was not as clear-cut as in the other cases.

Species name as received	Origin	Strain number	T_m in °C.	% GC	σ	Tumouri-genesis	Flagel-lation	3-keto-lactose forma-tion
		Proposed name: *Agrobacterium radiobacter* var. *tumefaciens*						
tumefaciens	NCPPB	398	93·9	59·9	0·62	+ + +	Peri	+ +
tumefaciens	Gembloux	C	94·1	60·3	0·25	+ +	Peri	+ +
tumefaciens	NCPPB	396	94·2	60·6	0·88	+ + +	Peri	+ + +
tumefaciens	ICPB	TT 111	94·2	60·6	0	+ + + +	Peri	+ +
tumefaciens	ICPB	TT 6	94·2	60·6	0·25	+ + +	Peri	+ +
rhizogenes	NCPPB	5	94·25	60·7	0	+ +	Peri	+ + +
tumefaciens	ATCC	11 158	94·3	60·8	0·31	+ +	Peri	+ +
tumefaciens	ATCC	143	94·35	60·9	0·31	+ +	Peri	+ +
tumefaciens	ATCC	4452	94·35	60·9	0·50	+ +	Peri	+ +
tumefaciens	ATCC	11 156	94·4	61·1	0·25	+ +	Peri	+ +
tumefaciens	Gembloux	A	94·4	61·1	0·12	+ +	Peri	+ +
tumefaciens	ATCC	11 157	94·5	61·3	0·12	+ +	Peri	+ +
tumefaciens	Inst. Pasteur	42 IV	94·5	61·3	0·6	+	Peri	+ + +
tumefaciens	Inst. Pasteur	B 6	94·55	61·4	0	+ + +	Peri*	+ + +
tumefaciens	NCPPB	4	94·55	61·4	0·25	+ + +	Peri	+ + +
tumefaciens	ICPB	TT 9	94·6	61·5	0	+ +	Peri	+ +
tumefaciens	ICPB	TT 107	94·7	61·8	0	+ +	Peri*	+ +
tumefaciens	Gembloux	tum 6	94·6	61·5	0	+ +	Peri	+ +
tumefaciens	NCPPB	925	94·7	61·8	0·19	+ + +	Peri	+ +
tumefaciens	Inst. Pasteur	S 1	94·7	61·8	0·2	+ +	Peri*	+ +
tumefaciens	ATCC	4720	94·8	62·1	0·13	+ +	Peri	+ +
tumefaciens	NCPPB	397	94·85	62·2	0·31	+ + +	Peri	+ + +
tumefaciens	Delft	F	94·9	62·3	0·25	+ +	Peri	+ +
radiobacter	Delft	G	94·9	62·3	0	+ +	Peri	+ +
rubi	ICPB	TR 2	93·8	59·6	0	+ + +	Peri	−
tumefaciens	NCPPB	223	94·7	61·8	0	+ +	Peri	−
tumefaciens	NCPPB	794	94·9	62·3	0·25	+	Peri	−
tumefaciens	ICPB	TT 133	95·0	62·5	0·25	+ + +	Peri	−
		Proposed name: *Agrobacterium radiobacter*						
radiobacter	NCIB	8149	94·15	60·5	0·25	−	Peri	+ +
tumefaciens	Inst. Pasteur	RV 3	94·25	60·7	0·81	−	Peri	+ + +
radiobacter	ICPB	TR 1	84·45	61·2	0·25	−	Peri	+ + +
radiobacter	Gembloux	S 1005	94·5	61·3	0·06	−	Peri	+ +
radiobacter	ATCC	4718	94·7	61·8	0·4	−	Peri	+ +
radiobacter	Own isolate	M 2/1	94·8	62·1	0	−	Peri	+ +
radiobacter	ICPB	TR 6	94·8	62·1	0	−	Peri	+ + +
radiobacter	Own isolate	L 2/2/1	95·0	62·5	0	−	Peri	+ +
		Proposed name: *Agrobacterium rhizogenes*						
rhizogenes	ICPB	TR 101	94·5	61·1	0·37	−	Peri	−
rhizogenes	ICPB	TR 107	94·9	62·3	0	±	Peri	−
rhizogenes	ICPB	TR 7	95·1	62·8	0·37	−	Peri	−
rhizogenes	NCIB	8196	95·1	62·8	0·66	−	Peri	−
		Doubtful cases						
tumefaciens	ATCC	11 095	94·4	61·1	0·62	−	Polar	−
tumefaciens	NCPPB	930	93·5	58·9	0·12	−	Peri	−
pseudotsugae	NCPPB	180	97·1	67·7	0·13	±	Peri	−
psygosphilae	NCPPB	179	92·4	56·2	2·5	−	Peri	−
stellulatum	Stapp	2216	96·4	66·0	0·63	−	Polar	−

and '*radiobacter*' G. There appeared to be none in '*radiobacter*'; we propose to relegate the strain RV 3 to '*radiobacter*' because it was not pathogenic.

The formation of 3-ketoglycosides appears to be specific for *Agrobacterium tumefaciens* and *A. radiobacter*; it was originally proposed as a quick and specific test for the crown-gall bacteria (Bernaerts & De Ley, 1963). The present examination of a much larger group of strains showed that 24 of 28 strains of *A. tumefaciens* and all 8 strains of *A. radiobacter* were positive. All other organisms were negative; nor did they produce 3-keto derivatives from maltose or sucrose. The test of Bernaerts & De Ley was used successfully by Béaud (1964) and Manigault & Béaud (1963). The present results further strengthen the practical applicability of this test: when an unknown strain, isolated from a diseased plant supposed to have crown gall, shows a positive test it belongs to the '*tumefaciens-radiobacter*' group; '*radiobacter*' is excluded because it is not pathogenic; the nature of the disease can thus quickly be established. Since 4 of 36 of our strains did not produce 3-ketoglycoside, a negative outcome of the test is no proof that no *A. tumefaciens* is involved; in this case one has to resort to the lengthy phytopathogenic identification. Grebner, Durbin & Feingold (1964) questioned the specificity of the test of Bernaerts & De Ley because a *Micrococcus* sp. produced 3-ketosucrose from sucrose. However, the amounts produced were so small that they were only detected by paper chromatography and the authors reported that the test of Bernaerts & De Ley was negative. Agrobacterium on the other hand converts frequently 75–100 % of the substrate into the 3-keto derivative (Bernaerts & De Ley, 1960*a*, 1961). Furthermore, Grebner, Durbin & Feingold did not state whether the micrococcus had its habitat in or on plant galls.

Agrobacterium rubi. According to *Bergey's Manual* (1957) the difference between *Agrobacterium rubi* and *A. tumefaciens* is based only on a few minor features (nitrite and indole production, rate of growth) and one major one (*A. rubi* infects only members of the genus *Rubus*). The following arguments show that it is unnecessary to keep *A. rubi* as a separate species and that it would be better to include it in *A. tumefaciens*. (*a*) A slight difference in nitrite and indole production and a slower rate of growth are of little value for species differentiation. (*b*) The following examples show that organisms labelled *A. rubi* are not specifically pathogenic for *Rubus* plants. Our strain is markedly pathogenic for tomato and Datura (this paper) and for sugar beet (Starr, 1946). Other strains are virulent for pinto bean (Drs Lippincott & Heberlein, personal communication). Typical '*tumefaciens*' strains are also pathogenic for these plants. McKeen (1954) also pointed out that it was almost impossible to distinguish between *A. rubi* and *A. tumefaciens*. (*c*) Transformations with DNA can be made between these two species (Klein & Klein, 1953), showing the close similarity between their chromosomes. (*d*) The % GC and the σ value of *A. rubi* DNA is nearly indistinguishable from the values for DNAs of the '*tumefaciens*' strains; these results are not opposed to the inclusion *A. rubi* into the '*tumefaciens*' group.

Agrobacterium pseudotsugae may be a species of *Agrobacterium*, but at present it is a doubtful case. The only strain available to us, NCPPB 180, had a T_m value of 97·1° and a very narrow compositional distribution of its DNA molecules. This shows that its chromosome has very little in common with *Agrobacterium* proper ('*radiobacter*' '*tumefaciens*', '*rhizogenes*'). Our strain was doubtfully tumourigenic for tomato, did not produce 3-ketolactose and its inclusion in *Agrobacterium* was already

doubted by Knösel (1962*a*). Two other strains were avirulent for pinto beans (Drs Lippincott & Heberlein, personal communication). More research on this group is required.

No decision can be taken about the taxonomic position of the strain *Agrobacterium tumefaciens* NCPPB 930. At best it might be a 3-ketolactose-negative *A. radiobacter* or *A. rhizogenes*.

Strains which are excluded from the genus Agrobacterium

Agrobacterium stellulatum was included in this genus because of one property only, namely that it forms star-shaped clusters. This property occurs with many genera, mostly tumour- or nodule-forming ones, such as *Agrobacterium*, *Rhizobium*, *Pseudomonas*, *Chromobacterium*, *Phyllobacterium* (Knösel, 1962*a*,*b*). Except for star-formation, these *A. stellulatum* organisms do not display any of the characteristics of *Agrobacterium*. It was reported to be polarly flagellate by Stapp & Knösel (1954); this was confirmed in the present work. It grows preferentially on sea-water media. We found it to be non tumourigenic for tomato and Datura, not to produce 3-ketolactose, and to have a T_m value of 96·5° which is decidedly out of the common range of the other strains. This organism should therefore be removed from the genus *Agrobacterium*; its flagellation, growth in sea-water media, T_m value and variance σ are not opposed to its inclusion in the marine pseudomonads. It may be that the strain '*stellulatum*' is closely related to the polarly flagellated *Pseudomonas savastanoi*, *P. tonelliala* and *P. rimaefaciens*; these also form stars (Knösel, 1962*a*).

'*Bacterium gypsophilae*' (Brown, 1934) was included in *Agrobacterium* mainly because of its tumourigenesis (Starr & Weiss, 1943). The strain '*gypsophilae*' NCPPB 179 is probably not an Agrobacterium because it has the following aberrant properties: (*a*) its T_m of 92·4° is completely out of the range of the other agrobacteria; the variance σ is unusually wide, 2·5 as against a range of 0–0·88 for the other strains; (*c*) this strain has an unusually high content of DNA, whereas the yield from species of *Agrobacterium* proper is rather poor; (*d*) our strain has a yellow pigment, in agreement with the original description (Brown, 1934), while other agrobacteria are not pigmented; (*e*) a strain of '*gypsophilae*' was not phytopathogenic for *Gypsophila paniculata* (Knösel, 1962*a*). The exact taxonomic position of the strains of '*gypsophilae*' remains to be established. Its low T_m value excludes its classification in the genus *Xanthomonas* and thus disagrees with Prévot's (1961) proposal; all xanthomonads have a T_m range from 95·4° to 97·6° (De Ley & Van Muylem, 1963; De Ley, Park, Tijtgat & Van Ermengem, 1965).

The strain labelled '*tumefaciens*', ATCC 11095, being polarly flagellate, cannot be included in the genus *Agrobacterium*. It was found to be non-pathogenic in the present work and by Drs Lippincott & Heberlein (personal communication).

An improved definition of and classification within the genus Agrobacterium

By combining the above results with the commonly known morphological and physiological features of these organisms an improved definition of and classification within the genus *Agrobacterium* is now possible. A temporary proposal may be as follows: Gram-negative, small short rods, motile by peritrichous flagella of number frequently up to 5–6. No detectable gas, and no or slight acid production

on ordinary media. No pigmented strains known. Optimal growth temperature 25–30°. Frequently pathogenic (hypertrophies). Many strains produce 3-ketolactose.

Key: I. Frequently pathogenic for angiosperms. T_m in the range 94·5° ± 0·8°, variance σ not above 1.

A. *Agrobacterium radiobacter*: non-pathogenic.
A. radiobacter var. *tumefaciens*: crown-gall bacteria.
Many strains of this species produce 3-ketolactose.

B. *A. rhizogenes*: hairy-root disease.

II. Pathogenic for gymnosperms. T_m about 97°.
A. pseudotsugae.

The reasons for including both *A. radiobacter* and *A. tumefaciens* in one species are as follows. (*a*) The values of T_m and of σ of both groups of organisms are indistinguishable, indicating a possible near-identity of their chromosomes. (*b*) DNA transforms a non-pathogenic *Agrobacterium radiobacter* into a pathogenic strain (Klein & Klein, 1953, 1956). (*c*) Artificial loss of pathogenicity in *Agrobacterium tumefaciens* can be induced by repeated subcultivation in amino-acid containing-media (Stapp, 1958, p. 93), or by ultraviolet irradiation (Béaud, Manigault & Stoll, 1963). (*d*) Both types are often lysed by the same phages (Roslycky, Allen & McCoy, 1962, 1963). (*e*) They are antigenically identical in the S phase (Coleman & Reid, 1945). (*f*) Numerical analysis reveals their identity; Graham (1964) even proposed to include both groups in *Rhizobium radiobacter.* If the proposal to include both species in one be accepted, it must be called *A. radiobacter* for historical reasons; its tumourigenic variety would then become *A. radiobacter* var. *tumefaciens.*

It is possible that *Agrobacterium rhizogenes* is also only a variety of *A. radiobacter.* Until this has been investigated further it seems advisable to keep *A. rhizogenes* as a separate species centre.

Organisms related to Agrobacterium

In *Bergey's Manual* (1957) the family Rhizobiaceae comprises three genera: *Rhizobium*, *Agrobacterium*, *Chromobacterium.* On the other hand, some authors assert that there is little or no relation between *Agrobacterium* and *Rhizobium.* Prévot (1961) separated the genera and put *Agrobacterium* between such unrelated groups as the Nitrobacteriae and the Thiobacteriaceae. Krassilnikov (1959), by including the agrobacteria in *Pseudomonas*, kept them removed from *Rhizobium.* The super-generic classification of these organisms is thus a matter of personal preference and not necessarily the reflexion of the natural relationship. The latter, however, can now be fixed by numbers. Ideally this should be possible by DNA-hybridization; for want of these data we attempted it by combining two numerical parameters: (i) the mean % similarity $\bar{S}$ derived from taximetric analysis (a quantification of phenotypic relationship) as published in the literature (ref. see Fig. 2); (ii) the % GC (a measure of their possible genetic relationship), from data obtained in the senior author's laboratory. The groups involved are:

(1) *Beijerinckia*,

(2) *Rhizobium leguminosarum*,

(3) *Agrobacterium* (or *Rhizobium*) *radiobacter*, *Agrobacterium* (or *Rhizobium radiobacter* var. *tumefaciens* (may or may not include the *rhizogenes* strains),

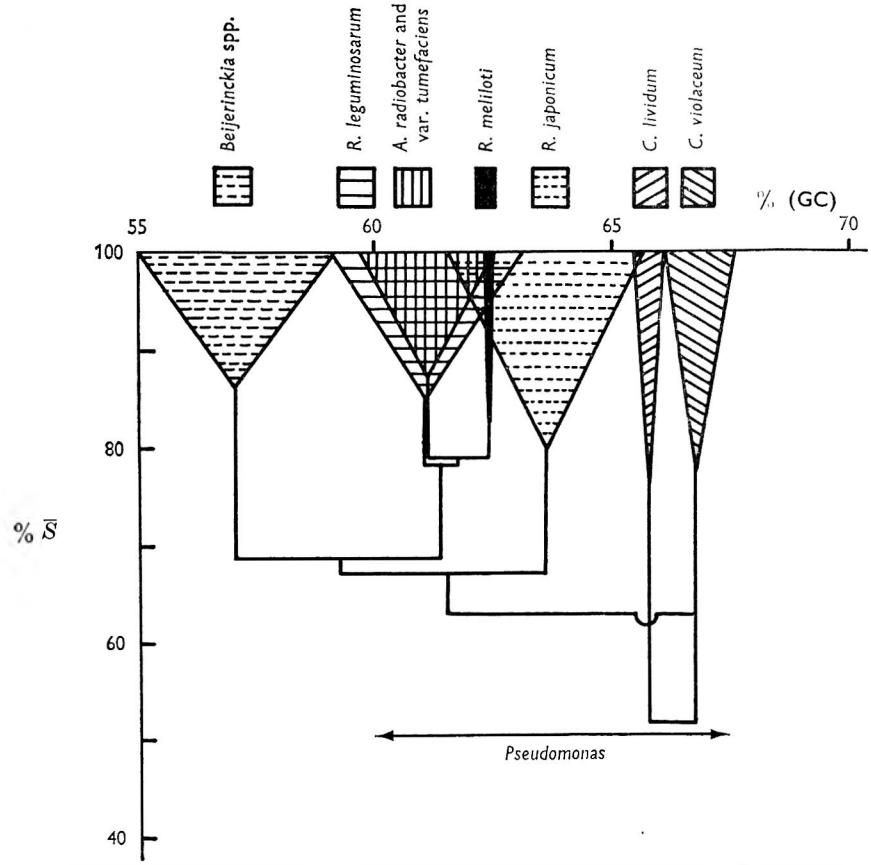

Fig. 1. Relationship between agrobacteria and some other organisms based on a genotypic (DNA base composition) and a phenotypic (mean % similarity $\bar{S}$) parameter.

Every group is represented by an inverted triangle, the top line representing the range of % GC and the bottom tip being the $\bar{S}$ value, arbitrarily put in the middle of the % GC range. Thus, strains of *Rhizobium japonicum* have a % GC ranging from 62 to 65·5 and an intra-group $\bar{S}$ value of 80. The $\bar{S}$ values of the chromobacteria were taken from Sneath (1957); 105 features of 20 strains of *Chromobacterium lividum* and 18 strains of *C. violaceum* were involved. A random selection was made of 7 lividum strains and 6 violaceum strains from Sneath's collection and used for the % GC determination (De Ley, 1964; De Ley & Van Muylem, 1963). The strains *C. violaceum* 9373 and *C. lividum* GA appear to be aberrant and are not included in the graph. The taximetric analysis by Graham (1964) involved 100 properties of 8 *Beijerinckia* strains, 32 strains of *Rhizobium leguminosarum*, 18 strains of *Agrobacterium radiobacter* and *A. tumefaciens*, 25 strains of *R. meliloti* and 27 strains of *R. japonicum*. We calculated the inter- and intra-group $\bar{S}$ values from this author's results. For the % GC determination 3 strains of *Beijerinckia* (De Ley & Park, 1966; De Ley, unpublished), 18 strains of *R. leguminosarum*, 2 of *R. meliloti* and 15 of *R. japonicum* (De Ley & Rassel, 1965), and 36 agrobacteria (this paper) were used. Although we did not use Graham's strains for the determination of % GC, yet our strains belong to the same named groups because: (i) they are culturally, morphologically, physiologically and pathogenically identical; (ii) Graham's taxonomic conclusions based on taximetric analysis, and ours based on % GC, are basically identical. Therefore the position of the triangles for these groups is only a close approximation and may undergo slight shifting when the same strains are studied by both methods. In order not to obscure the graph and as a measure of comparison, the % GC values of the pseudomonads (De Ley, Park, Tijtgat & Van Ermengem, 1965) are represented separately as a horizontal bar.

(4) *Rhizobium meliloti*,
(5) *Rhizobium* (or *Phytomyxa*) *japonicum*,
(6) *Chromobacterium* (or new generic name to be decided later) *lividum*,
(7) *Chromobacterium violaceum*.

Agrobacterium, *Rhizobium*, *leguminosarum* and *R. meliloti* all have a % GC value within the same range. Klein & Klein (1953) showed that the pathogenicity of *Agrobacterium tumefaciens* can be transfered to *Rhizobium leguminosarum*; similar results were obtained by Manil (1960). These results indicate a great similarity between the chromosomes of the two groups. As another phenotypic argument it may be mentioned that a strain of *R. leguminosarum* produces some 3-ketolactose from lactose (Bernaerts & De Ley, unpublished results). The inter-group mean similarity $\bar{S}$ of these organisms is high (Graham, 1964) and was calculated from this author's results to be about 79.

Beijerinckia indica is next closely related to the above groups, closely followed by *Rhizobium japonicum*.

Chromobacterium. The definition of this genus differs from one text to the other. *Bergey's Manual* (1957) considers only a few violet and bluish organisms and includes this genus in Rhizobiaceae. Prévot (1961) considered the chromobacteria in the same narrow sense as *Bergey's Manual* but removed them completely from both the agrobacteria and the rhizobia. In Krassilnikov (1959) Chromobacterium is only a collective noun, including a great variety of pigmented strains; it is, however, not a biological entity. We consider here only the violacein-producing biotypes *Chromobacterium lividum* and *C. violaceum* which are clearly separated both taximetrically (Sneath, 1957) and by their % GC (De Ley, 1964; De Ley & Van Muylem, 1963). Fig. 1 stresses Sneath's (1957) presumption that both groups deserve separate generic status. The production of violacein by both groups probably signifies (at least if the biosynthetic pathway for the pigment is the same in each) that in the course of evolution the pigment-producing system was formed first, followed only later by mutational diversification into the lividum and violaceum groups. From Graham's (1964) results, it can be calculated that *C. violaceum* shares an average of 60–66 % $\bar{S}$ with the other groups.

An unbiased opinion on the existence and the limits of a family Rhizobiaceae cannot be given; this may be more a matter of nomenclature than of classification. A more satisfactory taxonomic relationship is represented graphically in a plot of similarity *versus* DNA base composition.

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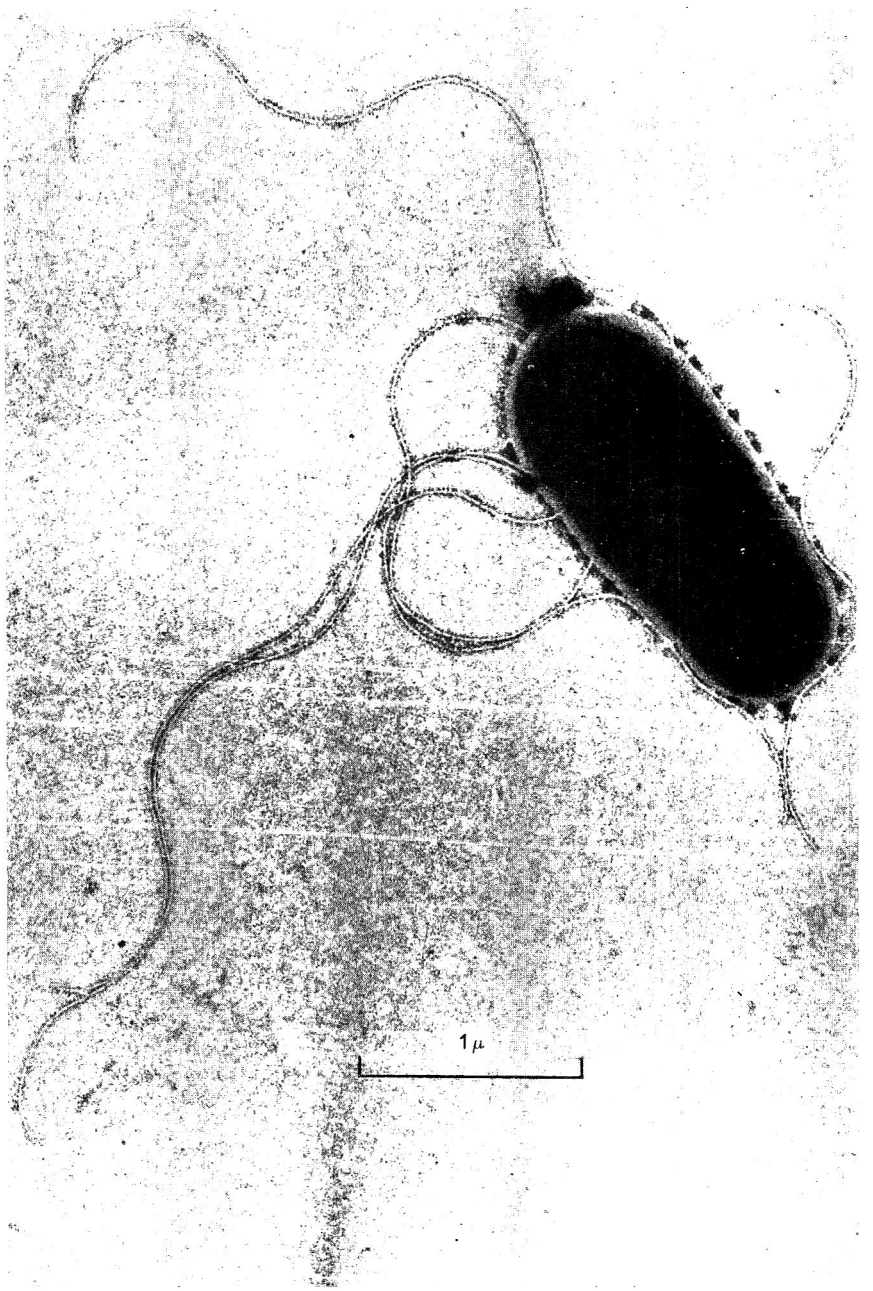

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EXPLANATION OF PLATE

Electron micrograph of *Agrobacterium radiobacter* var. *tumefaciens* strain c, showing the type of peritrichous flagellation.

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Changes in Morphology, Infectivity and Haemagglutinating Activity of Semliki Forest Virus Produced by the Treatment with Caseinase C from *Streptomyces albus* G.

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SUMMARY

Electron micrographs of negatively stained Semliki Forest virus were made before and after treatment of the virus with caseinase C from *Streptomyces albus* G. The virus appeared as roughly spherical (diameter 550–590 Å), covered with projections and sometimes bearing an appendix, which seemed to be a fold of the envelope. Treated with caseinase C in tris buffered saline, the virus lost its projections and its haemagglutinating activity but remained infectious. The site of haemagglutination, then, is probably located on the projections. Treated with caseinase C in phosphate buffer, the virus lost its infectivity as well as its haemagglutinating activity; the number of the remaining viral particles was not significantly modified. These structures had no projections; their envelope was degraded and sometimes completely destroyed. In this case, they had a smaller diameter (385–390 Å) than the projectionless particles obtained by treatment in tris buffered saline (410–460 Å). As the virus particles with degraded envelope still contained as much infectious RNA as the controls, it was thought that some degree of integrity of the envelope was necessary for the particle infectivity. Thus the site for infectivity appeared to be different from that for haemagglutination.

INTRODUCTION

Caseinase C, an enzyme produced by *Streptomyces albus* G, destroys the infectivity of lipoprotein-containing viruses such as Semliki Forest virus and influenza virus (Osterrieth & Dierickx, 1965; Reginster, 1965*a*). Influenza virus possesses, around its nucleoprotein, an envelope covered with spikes (Horne & Wildy, 1963). Sindbis virus, which is closely related to Semliki Forest virus, also possesses an envelope with projections (Mussgay & Rott, 1964). In both cases, the envelope is responsible for haemagglutination. Trypsin treatment of some strains of influenza virus cuts off the spikes and destroys the haemagglutinating activity (Borecky, Lackovic & Mrena, 1964). Caseinase C treatment at low ionic strength leads to similar results (Reginster, 1965*b*).

Semliki Forest virus treated with caseinase C loses its ability to agglutinate red blood cells (Osterrieth, 1965*a*, *b*). We have now treated Semliki Forest virus with caseinase C under two sets of conditions and have tried to correlate the observed modifications of the biological properties of the virus with changes in the morphology of the virus particles.

METHODS

Solutions. BBS = borate-buffered saline of Clarke & Casals (1958). TBS = tris-buffered saline (0·15 M-NaCl + 0·05 M-tris + 0·005 N-HCl) containing 0·1 % (w/v) bovine plasma albumin (Armour fraction V). GBSS = tris Gey's solution of Porterfield (1960) to which 0·1 % (w/v) bovine plasma albumin was added. PB = phosphate buffer (0·00284 M-Na_2HPO_4 + 0·0016 M-NaH_2PO_4; pH 7·0).

Growth medium for chick embryo cells: Porterfield (1960) tris growth medium in which the concentration of calf serum was increased to 20 % (v/v) and the chick embryo extract omitted.

Agar overlay medium: Porterfield (1960) tris overlay medium in which the final concentration of chick embryo extract was increased to 7·5 % (v/v), and the neutral red omitted.

Purified virus suspension. Our purification procedure is a modification of that of Cheng (1961). Two-day mice were inoculated intracerebrally with about 1000 plaque-forming units (p.f.u.) of Semliki Forest virus suspended in GBSS. After 24 hr, when some mice were dying, the brains were harvested and homogenized in BBS (pH 9·0). The suspension was clarified by centrifugation at 3000 *g* for 30 min. After addition of protamine sulphate (5 mg./ml.), the supernatant fluid was stored overnight at 4°. The next morning the suspension was again clarified by centrifugation at 3000 *g* for 45 min. The supernatant fluid was then centrifuged at 105,000 *g* for 1 hr and the resulting pellet resuspended in TBS. This suspension was treated for 30 sec. in a Raytheon sonic oscillator and clarified by centrifugation at 3000 *g* for 30 min. The supernatant fluid was then centrifuged at 105,000 *g* for 1 hr and the resulting pellet suspended in TBS. This final suspension, which will be referred to as 'stock virus', was distributed in vials, sealed, frozen on dry ice and stored at −70°.

Caseinase C. Throughout the experiments we used two stocks of purified caseinase C (Ghuysen, Leyh-Bouille & Dierickx, 1962). The enzyme was dissolved in distilled water and stored at −70°. One stock solution which contained 1·12 mg. protein/ml., had an enzymic activity of 1000 caseinolytic units/ml.; the other one contained 500 caseinolytic units/ml. One caseinolytic unit is defined as the amount of enzymic preparation which digests 50 % of the casein present in 0·5 ml. of standard solution (0·2 %, w/v, soluble casein in 0·05 M-K_2HPO_4) within 15 min. when incubated at 37° (Reginster, 1966).

Enzyme treatment. (i) *Treatment in TBS.* Stock virus in TBS was thawed and divided into two equal samples. Caseinase C was added (50 units/ml. when not otherwise stated) to the first one, and a corresponding volume of distilled water to the second one. After incubation at 37° for 1 hr, the two samples were titrated and samples taken for electron microscopy. TBS is a solution of high ionic strength and at pH 9·0. (ii) *Treatment in PB.* Frozen or fresh stock virus was centrifuged at 125,000 *g* for 1 hr. The pellet was resuspended in PB; the solution was divided into two equal portions. Caseinase C (20 to 125 units/ml.) was added to the first one and a corresponding volume of distilled water to the second one. After incubation at 37° for 30–90 min., the suspensions were titrated and samples taken for electron microscopy. Sometimes, the suspensions were centrifuged after enzyme treatment. PB is a solution of low ionic strength and at pH 7·0.

Haemagglutination. The technique of Clarke & Casals (1958), with goose red blood

cells and plastic trays, was used. One haemagglutinating unit (h.a.u.) is defined as the least quantity of virus giving partial haemagglutination.

Infectivity assay. Petri dishes (diam. 7·5 cm.) seeded with 3 to 4×10^7 chick embryo cells suspended in 10 ml. growth medium were incubated for 24 hr at 36° to obtain a monolayer. The growth medium was then discarded and the monolayer washed once with GBSS. Thereafter, the cells were inoculated with 1 ml. of serial 10-fold dilutions of viral suspensions and adsorption was allowed for 90 min. at room temperature. The excess of inoculum was sucked off and 7·5 ml. agar overlay poured on the cell sheet. The plates were incubated 48 hr at 36° and then stained with neutral red before counting the plaques.

Infectious-RNA liberation and assay. The virus suspensions were diluted 1/2 in a 4% (w/v) solution of sodium deoxycholate in phosphate buffered saline (pH 7·2; 0·02 M-phosphate + 0·30 M-NaCl) and incubated 30 min. at 2°. Controls were diluted in phosphate buffered saline without deoxycholate. Deoxycholate-treated virus and controls were also incubated in presence of ribonuclease (0·6 μg./ml.). The assay of these suspensions was done according to the method of Richter & Wecker (1963). The deposit of the overlay, the following incubation and the staining were done as described in the preceding paragraph.

Centrifugation experiments. One ml. of the caseinase C treated suspension and 1 ml. of the control were each mixed with 3·75 ml. of PB. To stop the destruction of infectivity, 150 μl. of 5 M-NaCl in distilled water and 125 μl. of a 4% (w/v) solution of bovine plasma albumin in distilled water were added to the treated suspension, and similarly to the diluted control. The virus suspensions were then centrifuged in the SW39 rotor of a Spinco preparative centrifuge at 125,000 ***g*** for 1 hr. This centrifugation was sufficient to sediment more than 90% of the virus particles in the controls. After centrifugation, the upper 4·8 ml. were set apart and called supernatant fluid. The lower 0·2 ml., called pellet, was diluted 5 times in phosphate buffered saline (pH 7·2; 0·01 M-phosphate + 0·15 M-NaCl). The total amount of haemagglutinating units, of virus plaque forming units (p.f.u.) and of deoxycholate liberable infectious RNA plaque-forming units (RNA-p.f.u.) present in the pellet and in the supernatant fluid were determined.

Electron microscopy. A sample of the virus suspension was mixed with an equal volume of potassium phosphotungstate solution (2%, w/v, in distilled water adjusted to pH 7·0; Brenner & Horne, 1959). One drop of the mixture was placed on a grid, already coated with Formvar (0·2%, w/v) and carbon. After 5 min., the excess of the suspension was blotted away. These preparations were examined with the Siemens Elmiskop 1, direct magnification ×40,000. The dimensions of the particles were measured on the final prints (×120,000), the magnification of which was controlled by the latex particles (diam. 126 mμ) present in some preparations. Each one of the given dimensions is the mean of at least 50 measurements, from 4 different sets of experiments. For counting the virus particles a mixture of virus and latex particles (Dow Chemical Co.) was sprayed from a commercial atomizer on the coated grids (Horne, 1961). The suspension to be sprayed was prepared by adding, in equal volumes, the latex suspension ($9·2 \times 10^{10}$ particles/ml.), the phosphotungstate (1%, w/v), a solution of bovine plasma albumin (0·4%, w/v in 0·005 M-phosphate buffer, pH 7·0) and the virus suspension the concentration of which was always chosen to give 2 to 5 virus particles/latex sphere (Miller & Ziegler, 1964).

Two hundred latex spheres and the corresponding number of virus particles were counted from at least 10 droplets of each preparation. The count was made directly on the screen of an Hitachi HS6 electron microscope (magnification ×10,000).

RESULTS

Characteristics of the stock virus

Haemagglutinating activity, infectivity and particle count. Our stock virus preparations were characterized by haemagglutination titres ranging from $10^{4.1}$ to $10^{6.6}$ haemagglutinating units (h.a.u.)/ml. and by infectious titres ranging from $10^{9.8}$ to $10^{11.7}$ plaque-forming units (p.f.u.)/ml. Two stock virus preparations were titrated five times. For one of these, $\log_{10}$ haemagglutinating titre was $5{\cdot}9 \pm 0{\cdot}5$ and $\log_{10}$ infectious titre $11{\cdot}5 \pm 0{\cdot}2$. For the other one, $\log_{10}$ haemagglutinating titre was $5{\cdot}6 \pm 0{\cdot}3$ and $\log_{10}$ infectious titre $10{\cdot}5 \pm 0{\cdot}2$.

The particle count was done on four stock preparations. Table 1 shows that in our experiments one haemagglutinating unit represented $7{\cdot}4 \times 10^6$ viral particles and that one plaque-forming unit contained on the average 22 virus particles.

Table 1. *Haemagglutinating activity, infectivity and particle count of Semliki Forest virus stock suspensions*

	$\log_{10}$ number of units/ml.		
Stock no.	Haemagglutinating	Plaque-forming	Physical count
18	5·6	11·2	12·5
19	5·9	11·4	12·7
20	5·9	11·3	12·7
21	5·6	11·2	12·6
Mean	5·75	11·27	12·62

Particle morphology. The images given by the different stock virus suspensions were very homogenous and showed only concentrated virus material (Pl. 1, fig. 1, 2); about half of the particles, penetrated by the stain, appeared 'empty'. The shape of the full virus particle was roughly spherical; some particles showed a lateral protrusion which sometimes took a tail-like appearance. The mean overall diameter of the particles was 575 Å (range 550–590). The surface of the particle and of its occasional protrusion was entirely covered with very thin (10–20 Å) projections. These projections were regularly spaced on the surface of the particles (centre to centre distance about 50 Å). They did not seem to design a simple fringe around the particles, but appeared to be connected one with the other at approximately half of their length, giving to the virus the general appearance of a sphere covered with a net. These projections were quite different from the spikes of influenza virus (Pl. 1, fig. 3). The thickness of the ring made by the projections around the particles is of 70 Å (range 50–80), and the mean diameter of the particles measured when the projections were not taken into account was 440 Å (range 410–460). The outer layer, supporting the projections, which was clearly visible on the empty-looking viral structures, was about 30 Å thick. Although the external structure of the virus was clearly the result of the repetition of similar structures, we could not distinguish any characteristic symmetry. Virus suspended in TBS or in PB and incubated at 37° did not show any modification of their morphology.

Effects of caseinase C treatment of virus in tris-buffered saline

Haemagglutinating activity, infectivity and particle count. When the virus stock was treated for 1 hr with caseinase C in TBS the infectivity of the virus suspension was only slightly decreased, but the haemagglutinating activity was almost completely destroyed. The particle count was not significantly modified (Table 2). Decreasing the concentration of caseinase C to 12 units/ml. or increasing it to 100 units/ml. did not change the % haemagglutinating activity that was destroyed. But, when the concentration of enzyme was 100 units/ml., the infectivity was then decreased to 30% of its original value.

Table 2. *Haemagglutinating activity, infectivity and particle count of Semliki Forest virus suspensions treated with caseinase C in tris-buffered saline*

In all experiments the amount of caseinase C was 50 units/ml. and incubation was at 37° for 60 min.

	Log_{10} number of units/ml.					
	Haemagglutinating		Plaque-forming		Physical count	
Expt. no.	Control	Treated	Control	Treated	Control	Treated
1	6·6	2·1	11·2	11·1	—	—
2	5·4	2·4	11·5	11·3	—	—
3	6·0	2·7	11·4	10·9	—	—
4	5·9	3·2	11·2	11·2	12·8	12·8
5	5·9	2·9	11·3	11·2	12·7	12·7
6	5·9	2·6	11·4	11·3	12·6	12·3
Mean	5·9	2·6	11·3	11·2	12·7	12·5

Particle morphology. Electron micrographs of the treated virus showed that the projections had disappeared (Pl. 1, fig. 4). The remaining structures had an average diameter of 435 Å (range 410–450), almost equal to the diameter of the untreated particles measured without taking the projections into account. Empty particles were still present in the preparations. On the micrographs, the tail-like protrusions are clearly shown to consist of a fold of the outer layer (Pl. 1, fig. 4, 5). This deformable layer will be, from now on, called envelope. As the envelope has the same thickness on the micrographs of control virus and of treated virus, it seems unlikely that the projections were merely flattened down by the treatment. They must have been removed or at least sufficiently modified as to become too thin to be seen in negatively-stained preparations. When the concentration of caseinase C was less than 25 units/ml. the projections were only partly removed, although the haemagglutinating activity was destroyed. When the concentration of enzyme was 100 units/ml. some of the virus particles showed incipient degradation of the envelope. Under the experimental conditions chosen, it can be concluded that caseinase C (50 units/ml.) does not modify the infectivity of Semliki Forest virus suspensions and does not decrease the number of virus particles. However, it destroys the haemagglutinating activity and removes or modifies the projections which normally cover the virus particle.

Caseinase C treatment of virus in phosphate buffer

Haemagglutinating activity, infectivity and particle count. Experiments in PB confirmed that the haemagglutinating activity was much more sensitive to caseinase C than was the infectivity. Table 3 shows that although in all our experiments about 99·9 % of the haemagglutinating activity was destroyed, the destruction of infectivity was irregular. The destruction of infectivity was more pronounced when the enzyme concentration and the incubation time were increased and when the initial infectious titre of the viral suspension was relatively low. The virus particles were counted in four experiments. The total number of virus particles was not much modified, although the infectious titre was decreased by the caseinase C treatment to less than about 10 % of its original value.

Table 3. *Haemagglutinating activity, infectivity and particle count of Semliki Forest virus suspensions treated with caseinase C in phosphate buffer*

All incubations were at 37°.

Expt. no.	Amount of caseinase C (units/ml.)	Time of incubation (min.)	Log_{10} number of units/ml.					
			Haemagglutinating		Plaque-forming		Physical count	
			Control	Treated	Control	Treated	Control	Treated
1	20	30	5·6	3·2	11·0	10·9	—	—
2	20	30	4·4	2·0	9·9	9·3	—	—
3	25	30	4·9	1·3	9·7	8·7	—	—
4	25	30	3·4	0·7	9·3	6·9	—	—
5	25	60	5·0	2·6	10·2	9·0	12·1	11·9
6	25	60	5·8	2·5	10·2	7·0	—	—
7	25	90	5·9	2·9	10·8	9·5	12·6	12·5
8	25	90	5·3	2·9	10·9	8·3	12·5	12·3
9	30	30	5·6	2·0	10·7	10·6	—	—
10	30	30	4·4	2·0	9·8	8·7	—	—
11	30	90	4·6	1·6	9·9	5·7	—	—
12	50	60	5·0	2·6	10·2	7·3	12·1	11·7

In one experiment we incubated at 37° for 60 min. samples of the virus suspension to which were added increasing amounts of enzyme (Fig. 1). The haemagglutinating titre was decreased to less than 1 % of its original value. This destruction was not increased by higher enzyme concentrations. The infectivity was decreased, and the degree of destruction was proportional to the enzyme concentration. The total number of particles was decreased to about 50 % of the original value. The treated preparations did not show empty particles. Thus, the decrease in number of particles was very likely the result of the destruction of these empty particles. The treated particles showed some tendency to form aggregates containing various numbers of particles. The total decrease in number of infectious units, counting an aggregate as an infectious unit, is represented by the curve VPa of Fig. 1. It is clear that if each aggregate of several particles behaved as an isolated virus infectious unit during the infectivity assay, the decrease in number of infectious units resulting from aggregation would not account for the loss of infectivity.

Particle morphology. The images obtained with treated preparations which showed

no decrease of infectivity were similar to those obtained from the virus treated with caseinase C in TBS. When the infectivity of the virus was destroyed by this treatment, the resulting particles appeared to be without projections and their envelope showed various degrees of alteration (Pl. 2). Sometimes pieces of envelope were seen isolated or in the process of being stripped off (Pl. 2, fig. 8). Some particles were devoid of envelope. Their mean diameter was 390 Å (range 385–395) and their surface did not show any morphological details. These naked particles will be called cores. Some debris were also visible on the micrographs in the form of hollow polygonal structures about 120 Å wide (Pl. 2, fig. 7).

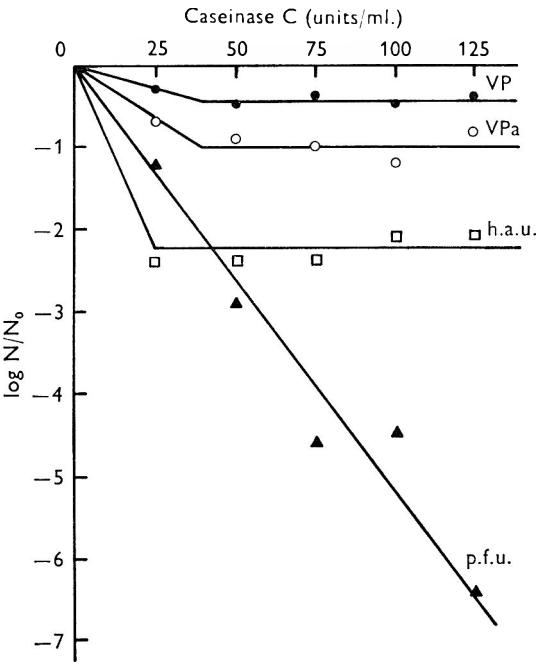

Fig. 1. Haemagglutinating activity, infectivity and particle count of Semliki Forest virus suspensions treated with various doses of caseinase C in phosphate buffer. N: number of plaque-forming units (p.f.u.)/ml. after treatment (or h.a.u. or VP or VPa). N_0: number of p.f.u./ml. before treatment (or h.a.u. or VP or VPa). VP: virus particles. VPa: virus particle agglomerations. Incubation was at 37° for 1 hr.

Centrifugation experiments

As the loss of infectivity could not be accounted for either by an aggregation of the particles or by a decrease of their total number, we looked for a release by the enzyme of infectious RNA or for a modification of the RNA infectivity. The caseinase C-treated and control viral suspensions of experiments 9, 10 and 11 (Table 3) were centrifuged at 125,000 *g* for 1 hr. Table 4 gives the distribution between supernatant fluids and pellets of the values of h.a.u., p.f.u. and RNA-p.f.u. (obtained after deoxycholate treatment). In control as well as in treated suspensions, the infectivity and the deoxycholate-liberated RNA infectivity sedimented likewise, 90 % being in the pellet. In controls, 90 % of the haemagglutinating activity was also collected in the pellet, but far less of the residual haemag-

glutinating activity sedimented from the suspensions treated with caseinase C. Small haemagglutinating structures were released by this treatment but there was no indication of infectious RNA release.

Table 4. *Distribution of total haemagglutinating activity, infectivity and infectivity due to RNA liberated by deoxycholate between supernatant fluids and pellets of centrifuged Semliki Forest virus suspensions after treatment with caseinase C in phosphate buffer (Table 3)*

The centrifugation was at 125,000 g for 60 min.

No. of expt. in Tabel 3	Viral suspension	Haemagglutinating units % in		Plaque-forming units % in		RNA plaque-forming units % in	
		Supernatant fluid	Pellet	Supernatant fluid	Pellet	Supernatant fluid	Pellet
9	Control	4	96	2	98	3	97
	Treated	18	82	10	90	9	91
10	Control	13	87	9	91	10	90
	Treated	>35	<65	14	86	12	88
11	Control	16	84	3	97	1	99
	Treated	25	75	⩾3	⩽97	7	93

Table 5. *Ratios of the virus infectious titre to the deoxycholate-liberated RNA infectious titre of Semliki Forest virus suspensions treated with caseinase C in phosphate buffer*

The suspensions were centrifuged at 125,000 g for 60 min.

No. of expt. in Table 3	Viral suspension	Log_{10} ratios of infectious titres of: Treated virus : RNA of treated virus	Control virus : RNA of control virus	Control virus : RNA of treated virus
11	Supernatant	0·1	4·7	4·7
	Pellet	0·2	4·3	5·2
	Original	0·3	4·7	4·4
10	Pellet	3·1	4·0	4·9
	Supernatant	3·3	4·0	4·8
9	Original	4·1	4·0	4·2
	Supernatant	4·2	4·4	4·7
	Pellet	4·2	4·3	4·0
		Mean	4·3 ± 0·3	4·6 ± 0·4

Samples of uncentrifuged suspensions, supernatant fluids and pellets were treated with sodium deoxycholate to liberate the infectious RNA which was then titrated. Ribonuclease tests, not included into the Tables, proved that the infectivity measured was destroyed by this enzyme and that infectious RNA did not interfere with the virus plaque-forming-units assay. Almost all the virus infectivity was destroyed by the deoxycholate treatment (Richter & Wecker, 1963; Osterrieth, 1964). When the caseinase C treatment destroyed a significant proportion of the infectivity, the p.f.u.:RNA-p.f.u. ratio was far greater in control than in treated suspensions (Table 5). The discrepancy between the p.f.u.:RNA-p.f.u. ratios of control and treated suspensions decreased together with the destruction of infectivity. When the infectivity had not been destroyed by the caseinase C treatment, the p.f.u.:RNA-p.f.u. ratio was similar in control and treated suspensions (expt. no. 9,

Table 3). In view of these differences, we also compared the virus infectious titre of the controls with the infectious RNA titre of the treated suspensions (Table 5, last column). These ratios were not significantly different from the p.f.u.:RNA-p.f.u. ratios of the controls.

Since in each experiment the ratios p.f.u.:RNA-p.f.u. of the treated suspensions were similar in supernatant fluids and pellets, we have an indication that the infectious RNA is not liberated by caseinase C treatment. Additional evidence is given by the fact that the ratios p.f.u. of controls:RNA-p.f.u. of caseinase C treated are similar to the ratios p.f.u. control:RNA-p.f.u. control. This observation also shows that the infectivity of the infectious RNA was not modified by treatment with caseinase C.

Thus when Semliki Forest virus was treated with a sufficient amount of caseinase C in PB, the haemagglutinating activity and the infectivity were both destroyed, the surface projections disappeared, and the envelope was degraded and eventually destroyed. After such treatment there was no proportionality between the particle count, the particle aggregation and the virus infectious titre. The infectious RNA was neither released nor inactivated.

DISCUSSION

Negatively-stained Semliki Forest virus appears to be formed of a roughly spherical core surrounded by a loose envelope which bears very thin projections. We cannot tell whether the nucleic acid within the core is free or protein-bound RNA, because the infectious RNA was liberated by deoxycholate treatment. The cores might be similar to the intracellular immature virus particles described by Mussgay & Weibel (1962) and Morgan, Howe & Ross (1961). They might also be related to the ribonucleoprotein extracted from infected cells (Wecker & Richter, 1962; Wecker & Schonne, 1961). Comparable cores have been described for Venezuelan equine encephalitis virus (Klimenko *et al.* 1965). Since we did not observe any structural details of the surface of the envelope nor of the core, we cannot give the name of capsid to any of the observed structures.

The projections and the envelope are sensitive to the action of caseinase C. Since we could not find any evidence for a liberation of RNA, we think that the cores are not sensitive to caseinase C in our experimental conditions.

Whether the projections are cut off, digested or only modified remains a matter for discussion, but they appear to harbour at least one site which is sensitive to caseinase C action. They are much more readily attacked by caseinase C than is the envelope which shows signs of enzymic disruption only when the enzymic treatment is done in phosphate buffer PB.

The haemagglutinating activity, similarly, is easily destroyed by caseinase C, but the destruction of infectivity requires the conditions which are necessary for envelope disruption. Haemagglutinating activity is also more readily destroyed by Pronase (Osterrieth, 1965*a*) than is infectivity. This is unusual; generally chemical destruction of the haemagglutinating activity also destroys the infectivity, e.g. the action of sulphydryl reagents on enteroviruses (Philippson & Choppin, 1961) and of proteases on group B arboviruses (Cheng, 1958).

The presence (or integrity) of the projections was not a pre-requisite for infec-

tivity. Since the projections and the haemagglutinating activity always disappeared together, although the latter more rapidly, it is reasonable to infer that the projections carry the site for haemagglutination. Tween + ether disruption of Sindbis virus, which disrupts the envelope without apparent modification of the projections, points out to the same localization of the haemagglutinating sites (Mussgay & Rott, 1964).

Mild caseinase C treatment done in phosphate buffer leads to the disappearance of the projections and of the haemagglutinating activity. Increasing caseinase C concentration or the time of the incubation brings a disruption of the envelope as well as destruction of the infectivity. Since neither a liberation of the RNA nor a decrease of its infectivity could be found, and as neither a decrease in number nor an aggregation of the virus particles could be held responsible for the loss of infectivity, we believe that the latter is the result of envelope disruption. Accordingly, the site for haemagglutination is different from the site for infectivity. Ether disruption of Murray Valley encephalitis virus also liberates from the virus a non-infectious structure which contains the RNA but which is not ribonuclease sensitive (Anderson & Ada, 1961). Smith (1964) suggested that the envelope of herpes virus may be necessary for its infectivity but Watson, Wildy & Russell (1964) suggested the opposite.

We define the envelope as the deformable outer layer of Semliki Forest virus which bears the projections and which can be destroyed by caseinase C. The viral envelope is described as the lipoprotein-containing outer layer of lipid-containing viruses, and it is supposed to be formed by some budding process through the host-cell membrane during the release of virus (Franklin, 1962; Knight, 1963; Mussgay, 1964). Lipases destroy the infectivity of arboviruses (Takehara & Hotta, 1961), and these are sensitive to ether (Andrewes & Horstman, 1949) and to deoxycholate (Theiler, 1957). It seems that any treatment, either proteolytic or lipolytic, which degrades to a certain extent the envelope of arboviruses, even if it does not disrupt the particle, destroys the infectivity. The importance of the envelope for the infectivity of Semliki Forest virus, and probably for arboviruses in general, is in agreement with the hypothesis which Cohen (1963) offered to explain the first steps of the infection of cells by myxoviruses: 'On close apposition of the virus cell surface, there is a fusion of the opposing lipoprotein membranes with establishment of continuity. In this way, the virus membrane envelope becomes incorporated in the cell membrane and the internal constituent of the virus becomes extruded in the cytoplasm.' The process of penetration into the cell is perhaps similar for arboviruses and myxoviruses, since these viruses share some common features. Both types of viruses are enveloped and covered with projections which are probably the site for haemagglutination, both are ether sensitive, both contain RNA. In phosphate buffer, caseinase C treatment destroyed the infectivity and the haemagglutinating activity of Semliki Forest virus and of influenza virus (Reginster, 1965*a*, *b*; Osterrieth, 1965*a*). Nevertheless, significant differences exist. The infectivity of influenza virus is more sensitive to caseinase C than is its haemagglutinating activity, whereas the reverse holds for Semliki Forest virus. The projections of these two viruses are morphologically different. The envelope of influenza virus is twice as thick (70–100 Å; Horne & Wildy, 1963) as that of Semliki Forest virus, and is not disrupted by a caseinase C treatment in phosphate

buffer which degrades the envelope of Semliki Forest virus. If the two viruses certainly share some chemical components in their outer layer, these appear to be located in different structures.

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EXPLANATION OF PLATES

Negatively stained preparations of Semliki Forest virus: magnification × 180,000.

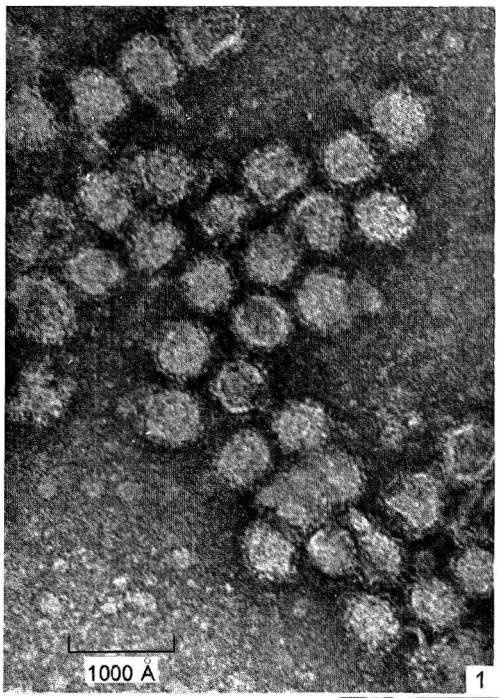
PLATE 1

Figs. 1–3. Untreated Semliki Forest virus particles (Figs. 1, 2) compared with influenza virus. (Fig. 3).

Figs. 4, 5. Semliki Forest virus particles treated with caseinase C in tris-buffered saline. The projections are removed (Fig. 4) and the appendix is shown to be formed by a fold of the envelope (Fig. 5).

PLATE 2

Figs. 6–8. Semliki Forest virus particles treated by caseinase C in phosphate buffer: the envelope is severely damaged (Figs. 6–8).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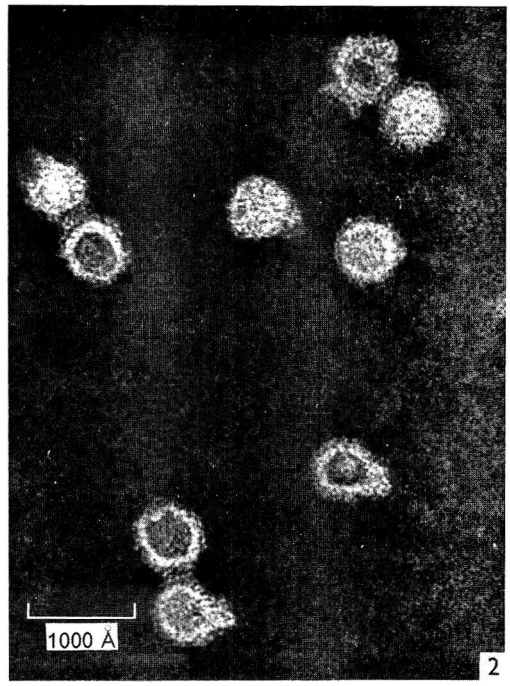
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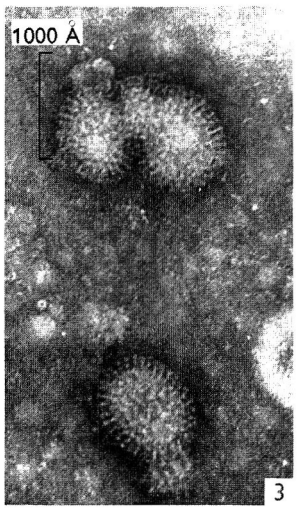
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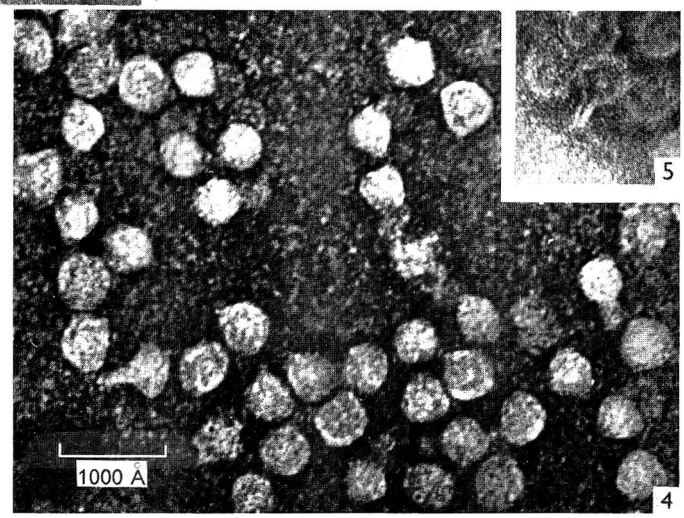
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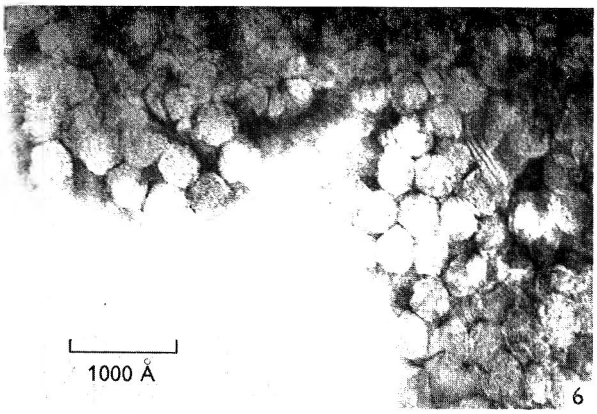
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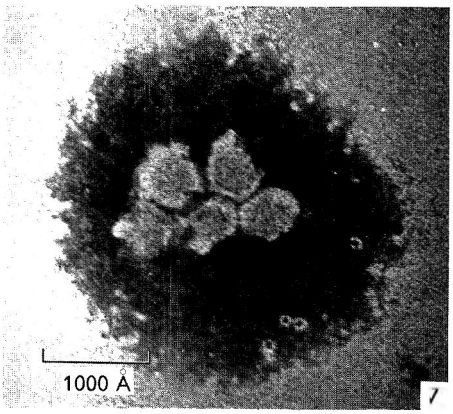
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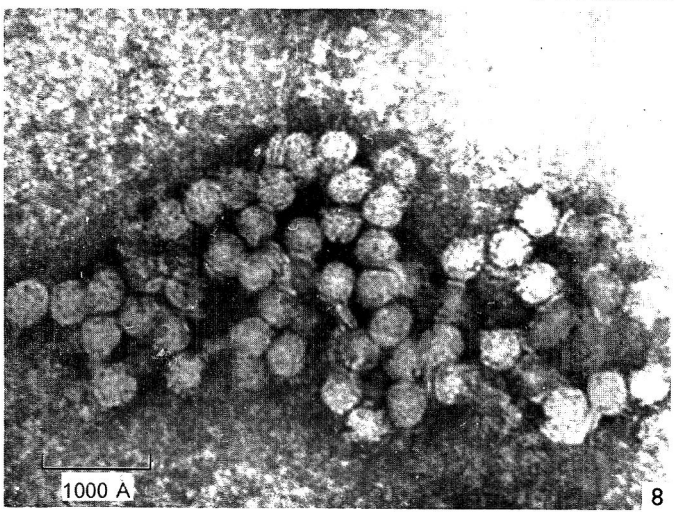
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Effect of Lactoperoxidase and Thiocyanate on the Growth of *Streptococcus pyogenes* and *Streptococcus agalactiae* in a Chemically Defined Culture Medium

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SUMMARY

A chemically defined culture medium was used to study the effect of purified lactoperoxidase and thiocyanate on the growth of several cultures of *Streptococcus pyogenes* and *S. agalactiae*. While not inhibited by either component alone, *S. pyogenes* growth was completely inhibited when both components were present in the medium. The growth inhibition was annulled completely by glutathione, thioglycollic (mercaptoacetic) acid or catalase. *S. pyogenes* glyceraldehyde phosphate dehydrogenase was inhibited by lactoperoxidase when hydrogen peroxide was present. The inhibition was annulled with cysteine and glutathione which suggested this dehydrogenase to be a possible site of inhibition. The inhibition was postulated to be a peroxidatic conversion of essential enzymic sulphydryl groups to inactive disulphide groups, thus interfering with the energy metabolism of *S. pyogenes*. With *S. agalactiae* cultures a delay in growth inhibition up to 6 hr resulted instead of complete growth inhibition. Catalase neutralized this effect. The extent of growth inhibition was greatest in those strains which were unable to adapt to an oxidative pathway for their energy supply. In becoming independent of the fermentative pathway, the cultures were no longer as sensitive to peroxidase, thiocyanate and hydrogen peroxide. The necessity for thiocyanate in the inhibitory system is not yet clear. Thiouracil and thiourea were ineffective replacements for thiocyanate.

INTRODUCTION

Streptococcus pyogenes (Lancefield, group A) and *S. agalactiae* (Lancefield, group B) have been used to study the naturally occurring bacterial growth inhibitors in milk (Auclair & Hirsch, 1953; Jones & Simms, 1930; Wilson & Rosemblum, 1952). *Streptococcus pyogenes* is a human pathogen which rarely infects the bovine udder, whereas *S. agalactiae* is one of the principal causes of bovine streptococcal mastitis. These organisms differ in their sensitivity to the natural inhibitors in milk and in their nutritional requirements. They are similar in morphology and under anaerobic conditions both are homofermentative lactic acid bacteria. Jones & Simms (1930) showed that *S. pyogenes* decreased in numbers when inoculated into raw milk, whereas *S. agalactiae* grew rapidly after a lag period of 6–8 hr. This was explained as an adaptation to the inhibitory factor during the lag phase.

Wright & Tramer (1958), using *Streptococcus cremoris* 972, and Portmann &

Auclair (1959), using *S. cremoris* 972 and *S. pyogenes* Richards, established lactoperoxidase as a component of the natural inhibitor complex in milk. The inhibitory action of lactoperoxidase for *S. cremoris* 972 has been confirmed in a whey ultrafiltrate medium supplemented with an amino acid mixture (Jago & Morrison, 1962). Reiter, Pickering & Oram (1964) showed that, in addition to lactoperoxidase and hydrogen peroxide, a thermostable, dialysable, acid-resistant substance present in milk was necessary for inhibition of lactoperoxidase-sensitive organisms. The latter substance was identified as thiocyanate.

The present report deals with the response of several strains of *Streptococcus pyogenes* and *S. agalactiae* to purified lactoperoxidase and thiocyanate in a chemically defined medium. Some preliminary studies on metabolic differences between *S. pyogenes* and *S. agalactiae* strains have been used to explain the difference in growth inhibition that was observed.

METHODS

Cultures. Seven cultures of *Streptococcus pyogenes* were used. All were obtained from Dr H. D. Slade, Department of Microbiology, Northwestern University Medical School, Chicago, Illinois. They represented the following serotypes: 3 *S. pyogenes* Richards, 19 *S. pyogenes* N 19, 6 *S. pyogenes* S 43, 1, 5, 25 and 30. Six strains of *S. agalactiae* 660–1, 50, 80, 142, 356, 383 were from our collection, and a seventh strain, Cornell 48, was obtained from Dr G. E. Morse, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania.

Lactoperoxidase preparation and assay. Lactoperoxidase (LPO) was prepared from fresh skimmed milk by the procedure of Morrison & Hultquist (1963). The purified enzyme was stored at 4° with toluene in the 0·5 M-sodium acetate solution used to elute the enzyme from the second resin column. The enzyme solution had a 412/280 mμ absorption ratio of 0·93. By assay, it contained 14·5 units lactoperoxidase/ml. and the activity changed very little during 3 months' storage.

Peroxidase activity was measured by a pyrogallol method. The reagents used were: 0·2 M-phosphate buffer (pH 6·9), freshly prepared 0·05 M-hydrogen peroxide, freshly prepared 1% aqueous pyrogallol, and a peroxidase standard containing 5 mg. horseradish peroxidase (Nutritional Biochemicals Corp., Cleveland, Ohio, 100 units/mg.)/100 ml. 0·01 M-phosphate buffer (pH 7·0). This stock solution was diluted 1/10 just before use in preparing a standard peroxidase activity curve with 0·01, 0·03 and 0·05 units. A standard curve was run with each assay.

The assay was performed by adding the reagents and sample to a Klett–Summerson colorimeter tube in the following order: 4·0 ml. buffer, sample, distilled water to 5·2 ml., 0·15 ml. hydrogen peroxide solution, and 0·15 ml. pyrogallol solution. The contents of the tube were mixed, held for 10 min. at room temperature, then 0·5 ml. 4 N-H_2SO_4 was added and the colour was measured with a Klett–Summerson colorimeter with a no. 42 filter.

The peroxidase activities of the lactoperoxidase preparation and whey samples were expressed in terms of horseradish peroxidase units. Whey samples from fresh milk contained 0·60–0·75 unit/ml.

Growth inhibition tests. The tests were carried out in a chemically defined medium (Mickelson, 1964), which was sterilized at 100° (steaming for 15 min.). Glutathione

(GSH), thioglycollic (mercaptoacetic) acid, catalase and ammonium thiocyanate (SCN^-) solutions were sterilized by filtration through an ultrafine sintered glass filter before adding to the medium. Lactoperoxidase was heated for 30 min. at 60° in a water bath, just before use, to expel toluene. Tests were done in a total volume of 4 ml. of medium including the added compounds in 13 × 100 mm. screw-capped tubes. Each tube was inoculated with a 16–18 hr culture of *Streptococcus pyogenes* or *S. agalactiae* grown in the chemically defined medium. The inoculum, 1–2% by volume of the test medium, was added so that the extinction reading of the inoculated medium was 0·05–0·08. After incubation at 37° measurements of growth were made in a Bausch & Lomb Spectronic 20 colorimeter at 550 mμ.

Manometric studies. Streptococcus pyogenes was grown in Brain Heart Infusion broth (Baltimore Biological Laboratories, Baltimore, Md., U.S.A.), which was supplemented with glucose to 0·8% concentration and 0·1 M-phosphate buffer (pH 7·0). *S. agalactiae* was grown in a medium containing 1% yeast extract (Difco, Detroit, Michigan), 0·4% Casitone (Difco), 0·8% glucose and 0·1 M-phosphate (pH 7·0). The phosphate buffer (M) was sterilized and added separately. Stationary and shaken cultures were grown in 50 ml. of medium in 125 ml. screw-capped Erlenmeyer flasks. The shaker cultures were grown in loosely capped flasks on a rotary shaker (New Brunswick Gyrotary Shaker Model s-3) at 120 rev./min. After incubation at 37° for 16 hr the cocci were harvested by centrifugation, washed once with distilled water, and resuspended to a concentration equiv. 10–12 mg. dry wt. cocci/ml. Four to five mg. cocci were used per respirometer flask in 2 ml. reactants. Respiratory activity was measured by conventional Warburg manometric procedure at 37°. When cyanide was used in the main flask compartment, M-KCN was substituted for 0·3 N-potassium hydroxide in the centre well.

Cell-free extracts of Streptococcus pyogenes s43, type 6 for measurement of triose-phosphate dehydrogenase were prepared from cocci grown in stationary culture for 12–14 hr in supplemented brain heart infusion broth. The cocci were harvested by centrifugation, washed once with 0·02 M-$NaHCO_3$, and the paste was mixed with an equal weight of alumina (No. A-305, Alcoa Chemicals, Aluminum Company of America, 1205 Alcoa Bldg., Pittsburgh, Pa.). The mixture was frozen in the deep freeze on a piece of glassine paper, then ground in a chilled mortar for 5 min. The ground paste was transferred from the mortar to a plastic centrifuge tube by slow successive additions of 1 ml. 0·075 M-phosphate buffer (pH 7·5), for each 50–60 mg. dry wt. cocci. The cell debris and alumina were removed by centrifugation at 3000*g* for 10 min. and the supernatant fluid was centrifuged at 20,000*g* for 20 min. The enzymic activity was present in the supernatant fluid which was either held at 4° when assayed on the same day as preparation or at −70° when held for several days. The protein content of the bacterial extracts was determined by a method described by Stadtman, Novelli & Lipmann (1951).

Peroxidase activity was determined by titration of the residual hydrogen peroxide after shaking coccal suspensions in Warburg flasks for 60 min. at 37° with 0·2 ml. 0·05 M-hydrogen peroxide in the presence and absence of 0·02 M-glucose. After adding 0·2 ml. 4 N-H_2SO_4 from a side arm at the end of the experiment to stop the reaction, the contents of each flask were transferred with washings into a centrifuge tube in a volume of about 8 ml. After centrifuging, each supernatant fluid was transferred to a 18 × 125 mm. tube, 0·5 ml. of 10% KI and 1 drop of 1% ammonium

molybdate were added and the iodine was titrated with 0·005 N-$Na_2S_2O_3$. Control flasks consisted of peroxide alone, peroxide+glucose, and peroxide+glucose acidified with 0·2 ml. 4 N-H_2SO_4 before adding cocci.

Lactic dehydrogenase (rabbit muscle, 2x, Nutritional Biochemicals Corp.) activity was measured by a decrease in absorption at 340 mμ due to oxidation of reduced diphosphopyridine nucleotide (NADH) with pyruvate as the substrate. Glyceraldehydephosphate dehydrogenase activity in cell-free extracts of *Streptococcus pyogenes* was measured by an increase in absorption at 340 mμ with glyceraldehyde-3-phosphate as the substrate. When purified rabbit muscle dehydrogenase (rabbit muscle, Calbiochem, A grade) was studied D-glyceraldehyde was the substrate (Colowick & Kaplan, 1959). Absorption measurements at 340 mμ were made with a Beckman DU-2 spectrophotometer. The enzymes were incubated at room temperature for 30 min. with the components of the inhibitory complex, singly and in admixture, before addition of substrate. Diphosphopyridinenucleotides, glyceraldehyde-3-phosphate, D-glyceraldehyde and catalase were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. The catalase was diluted to contain 5 units/ml. and sterilized by filtration before addition to culture media.

Flavinadeninedinucleotide (FAD) was determined in bacterial extracts by the enzymic method of DeLuca, Weber, & Kaplan (1956). Total flavin was measured on extracts of the cocci by absorption at 445 mμ. Extracts were prepared by suspending 0·10 g. lyophilized cocci in 10 ml. 0·01 M-phosphate buffer (pH 7·5) and heating in a boiling water bath for 10 min. After cooling, the extract was centrifuged for 20 min. at 20,000*g* to obtain a clear supernatant fluid. The extraction procedure was repeated with 3 ml. of the buffer and the supernatant fluids were pooled for analysis. Flavinadeninedinucleotide as % of total flavin was calculated according to DeLuca *et al.* (1956).

Glucose and lactic acid were determined by the anthrone procedure and the Barker & Summerson procedure, respectively, as described by Umbreit, Burris & Stauffer (1957). The contents of the Warburg vessels were transferred with washings into a graduated centrifuge tube. One ml. of 5% $ZnSO_4.7H_2O$ and 1 ml. of 0·3 N-$Ba(OH)_2$ were added and the volume was made up to 10 ml. The contents of the tube were centrifuged and analyses done on the clarified supernatant liquid.

RESULTS

Table 1 shows data comparing the effect of lactoperoxidase, thiocyanate and combinations of the two on the growth of five strains of *Streptococcus pyogenes* in the chemically defined medium. The lactoperoxidase and thiocyanate concentrations were in the range of those normally found in milk. Complete growth inhibition occurred with all strains tested in the presence of lactoperoxidase and thiocyanate. Either component alone had no effect on growth.

The growth inhibition of *Streptococcus pyogenes* N 19, as influenced by various concentrations of thiocyanate in the presence of 0·5 unit lactoperoxidase/ml., is shown in Fig. 1. At this concentration of lactoperoxidase, 2 μg. ammonium thiocyanate/ml. stopped growth for 24 hr. No growth occurred on continued incubation. The other four strains of *S. pyogenes* were also inhibited by these concentrations of lactoperoxidase and thiocyanate. Growth inhibition could be annulled completely

Table 1. *The effect of lactoperoxidase, ammonium thiocyanate singly and in admixture on the growth of five serotypes of Streptococcus pyogenes in a chemically defined medium*

Additions to medium	Growth of serotype*				
	19	1	5	25	30
None	0·70	0·60	0·64	0·79	0·69
LPO† 0·6 unit/ml.	0·65	0·57	0·67	0·80	0·66
NH_4SCN 15 μg./ml.	0·66	0·68	0·66	0·77	0·75
LPO 0·6 unit/ml., NH_4SCN 5 μg./ml.	0·07	0·08	0·08	0·08	0·08

* Growth measured as extinction at 500 mμ after 24 hr in 13 × 100 ml. culture tubes.
† LPO = lactoperoxidase.

by addition of 0·05 M-glutathione, 0·005 M-thioglycollic (mercaptoacetic) acid, or 0·1 unit catalase/ml. (Fig. 2). Lactoperoxidase (0·7 unit/ml.) was not active with thiouracil or thiourea (15 and 30 μg./ml.) when substituted for thiocyanate in growth inhibition tests with *S. pyogenes* N 19.

The sensitivity of *Streptococcus agalactiae* cultures to lactoperoxidase, thiocyanate or their combination was tested under conditions similar to those used for *S. pyogenes*. The *S. agalactiae* strains grew more rapidly than *S. pyogenes* strains and extinctions as high as 1·00 were sometimes obtained in the control tubes after 12 hr incubation. Complete growth inhibition was not obtained with any of the seven cultures tested. The growth inhibitory effect for *S. agalactiae* ranged from none with strain 50 to a maximum delay in growth initiation of 6 hr beyond that of

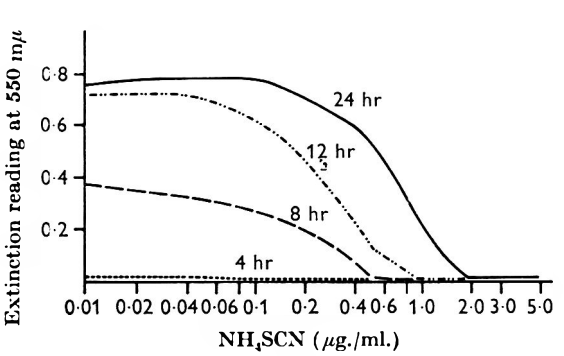

Fig. 1

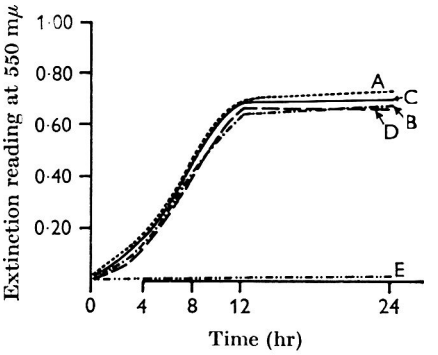

Fig. 2

Fig. 1. Influence of thiocyanate concentration in the presence of lactoperoxidase (0·5 unit/ml.) on the growth of *Streptococcus pyogenes* N 19 in a chemically defined medium.

Fig. 2. Growth inhibition of *Streptococcus pyogenes* N 19 in a chemically defined medium by lactoperoxidase and thiocyanate and its reversibility with glutathione, thioglycollic acid and catalase. Curve C = control; curve E = LPO, 0·5 unit/ml., + ammonium thiocyanate, 2 μg./ml.; curve B = same as (E) + catalase 0·1 unit/ml.; curve D = same as (E) plus 0·005 M-GSH; curve A = same as (E) + 0·005 M-sodium thioglycollate.

the control with strain 80, Fig. 3. Varying the thiocyanate concentration from 5 to 45 μg./ml. medium with a lactoperoxidase concentration of 0·6 unit/ml. had no effect on growth.

To determine whether the delay in growth initiation observed with certain

cultures of *Streptococcus agalactiae* was related to hydrogen peroxide formation, catalase was added to the chemically defined medium in the presence and absence of lactoperoxidase and thiocyanate. The addition of catalase (0·1 unit/ml.) not only abolished the delay in growth caused by the inhibitor complex but also caused a more rapid growth rate than in the controls which contained no lactoperoxidase or thiocyanate.

The decomposition of hydrogen peroxide by suspensions of *Streptococcus agalactiae* 50 grown on the shaker is shown in Table 2. The dry weights of cocci per flask

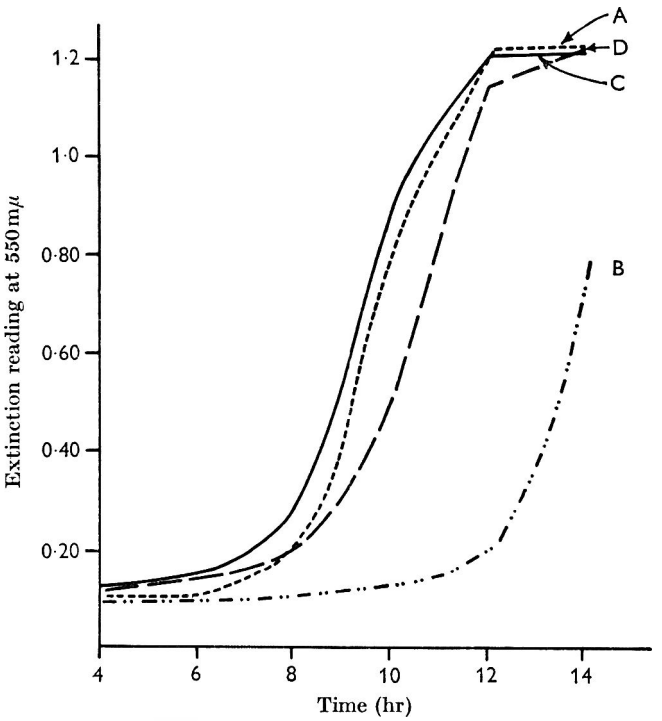

Fig. 3. Effect of lactoperoxidase, thiocyanate and their combination on the growth of *Streptococcus agalactiae* 80 in a chemically defined medium. Curve C = control; curve A = ammonium thiocyanate 15 μg./ml.; curve D = LPO 0·75 unit/ml.; curve B = LPO, 0·75 unit/ml., + ammonium thiocyanate 15 μg./ml.

were 20, 7·3, 6·6, 6 and 8·5 mg., respectively, for Expts. 1–5. There was no catalase activity that could be measured manometrically. *S. agalactiae* continued to respire in the presence of the hydrogen peroxide at 40% of the rate in the absence of peroxide. Correction was made for the oxygen produced from the decomposition of peroxide. The amount of hydrogen peroxide which disappeared during endogenous respiration was one-half to one-third that in the presence of glucose. With longer incubation and more respiration, there was more peroxide disappearance.

The second part of Table 2 shows results from two experiments with each of three cultures of *Streptococcus pyogenes*. Seven to 14 mg. dry cocci were used per flask. The respiration rate in the presence of peroxide with *S. pyogenes* s43 was only 10–15% of that when peroxide was not present. Hydrogen peroxide disappeared

from the reaction mixture when glucose was present and a small amount during the endogenous respiration. *S. pyogenes* Richards destroyed or utilized 85–100% of the hydrogen peroxide added. In Expt. 1 with *S. pyogenes* s43, the destruction of peroxide was by catalase action. From 9·1 μmole hydrogen peroxide 4·1 μmole oxygen were evolved when cocci were added to hydrogen peroxide in the absence of glucose.

Table 2. *Hydrogen peroxide decomposition by suspensions of Streptococcus agalactiae 50 and Streptococcus pyogenes cultures*

μmole H_2O_2 decomposed/mg. dry-wt. cocci/hr

	S. agalactiae 50				
Expt. no.	1	2	3	4	5
H_2O_2 + cocci	0·15	0·23	0·26	0·60	0·11
H_2O_2 + glucose + cocci	0·28	0·56	0·89	0·87	0·29
*H_2O_2 + glucose + cocci	0·037	0·054	0·053	0·08	0·034

	S. pyogenes culture					
	Richards		N 19		S 43	
Expt no.	1	2	1	2	1	2
H_2O_2 + cocci	0·14	0·11	0·11	0·068	0·39	0·12
H_2O_2 + glucose + cocci	0·80	1·36	0·12	0·35	0·39	0·45
*H_2O_2 + glucose + cocci	0·035	0·07	0·035	0·035	0·027	0·043

* Acidified before adding suspension of cocci.

The annullment of the inhibitory effect on *Streptococcus pyogenes* by glutathione and thioglycollic acid suggested that sulphydryl enzymes were involved and led to a determination of the effect of the components of the inhibitory complex on glyceraldehyde-phosphate dehydrogenase and lactic dehydrogenase activity. An active triosephosphate dehydrogenase was found in cell-free extracts of three different serotypes of *S. pyogenes*. Each of the enzymes required cysteine for activity. Each was affected in the same manner by hydrogen peroxide, lactoperoxidase and thiocyanate when these substances were tested singly or in combination. The enzyme was incubated for 30 min. at 25° with the components of the inhibitory complex before adding glyceraldehyde-3-phosphate to start the reaction. Tested singly neither substance had any inhibitory effect on triosephosphate oxidation. The rate of oxidation was always equal to or exceeded that in the control when lactoperoxidase, thiocyanate, or their combination was tested and slightly less than the control when hydrogen peroxide alone was tested (Fig. 4).

Complete inhibition of triosephosphate oxidation resulted when hydrogen peroxide and lactoperoxidase were present. Addition of 0·0036 M-cysteine completely annulled the inhibition, whereas 0·009 M-glutathione also annulled the inhibition but not as effectively (Fig. 5). The mixture of lactoperoxidase, thiocyanate and hydrogen peroxide inhibited the reaction 10–25%. Hydrogen peroxide and thiocyanate, without lactoperoxidase, caused erratic changes in extinction values immediately upon addition of the substrate, followed by a slower rate of substrate oxidation, which after 10 min. was equal to that of the samples in which only hydrogen peroxide was present.

Purified triosephosphate dehydrogenase from rabbit muscle was inhibited about

85% with one-tenth the concentration of hydrogen peroxide (0·000167 M) used in the reactions with the crude enzyme from *Streptococcus pyogenes*. The inhibition was completely annulled with 0·0027 M-glutathione.

Purified lactic dehydrogenase was inhibited by hydrogen peroxide (0·000167 M) but was not reactivated by glutathione. However, when the enzyme was incubated with the peroxide in the presence of 0·00267 M-glutathione, the enzyme was protected from inactivation.

The difference in response of the Lancefield Group A and B streptococci to the inhibitory complex indicated differences in their pathways of energy metabolism. To test this idea, the rate of glucose oxidation by suspensions of three strains of

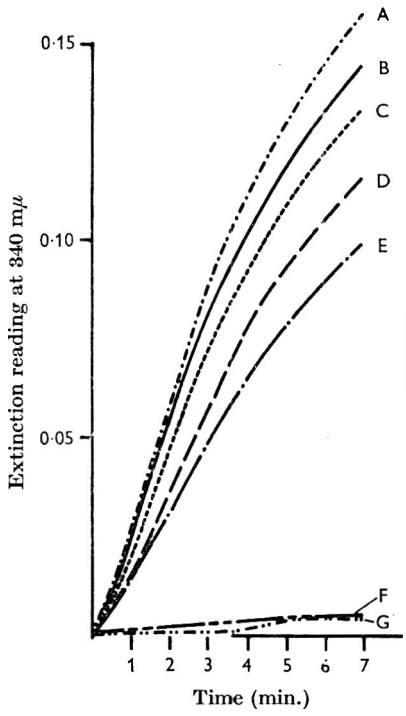

Fig. 4

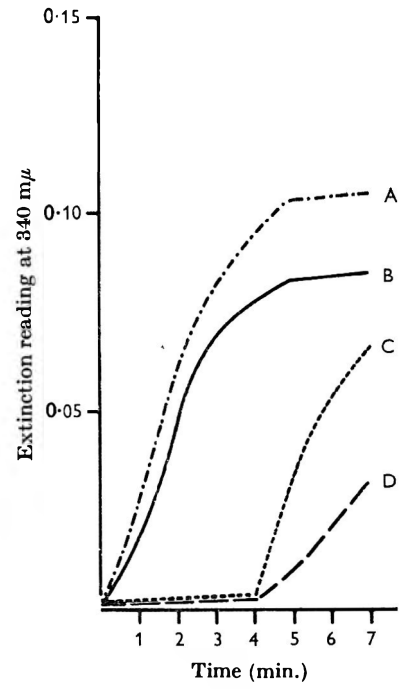

Fig. 5

Fig. 4. Effect of lactoperoxidase, hydrogen peroxide, thiocyanate, and their mixture on glyceraldehyde-3-phosphate dehydrogenase in extracts of *Streptococcus pyogenes*. Volume 3 ml. Reactants; 0·03 M-$Na_4P_2O_7$ (pH 8·4); 0·05 M-Na_2HAsO_4 (pH 8·4); DPN (6 mg./ml.) 0·10 ml.; 0·004 M-DL cysteine (pH 8·4); 0.006 M-glyceraldehyde-3-phosphate (Na); $1·66 \times 10^{-4}$ M-hydrogen peroxide; ammonium thiocyanate, 3·3 μg./ml.; lactoperoxidase, 0·6 units/ml.; bacterial extract, 0·25 ml. containing 3·25 mg. protein/ml. A = lactoperoxidase; B = ammonium thiocyanate; C = control; D = hydrogen peroxide; E = lactoperoxidase, thiocyanate, + hydrogen peroxide; F = lactoperoxidase + hydrogen peroxide; G = control minus cysteine.

Fig. 5. Inhibition of glyceraldehyde-3-phosphate dehydrogenase in extracts of *Streptococcus pyogenes* and reversal of the inhibition with cysteine and glutathione. Concentration of reactants same as in Fig. 4. Bacterial extract contained 5 mg. protein/ml. Reactivation by addition of 0·3 ml. 0·04 M-cysteine or 0·3 ml. 0·1 M-glutathione. Curve A = control; curve B = hydrogen peroxide; curve C = hydrogen peroxide + lactoperoxidase with 0·3 ml. 0·04 M-cysteine added 3 min. after starting reaction; curve D = same as C but with 0·3 ml. 0·1 M-glutathione added.

Streptococcus pyogenes and six strains of *S. agalactiae* grown in stationary and shaker cultures was determined (Table 3). The Q_{O_2} values with cocci grown in stationary cultures for all cultures were low. However, the *S. agalactiae* respiration showed a sensitivity to 0·001 M-KCN which was not evident with *S. pyogenes*.

Table 3. *Glucose oxidation by suspensions of Streptococcus agalactiae and Streptococcus pyogenes grown in stationary and shaken cultures*

Culture	Stationary culture		Shaken culture	
	Q_{O_2}	% inhibition (0·001 M-KCN)	Q_{O_2}	% inhibition (0·001 M-KCN)
S. agalactiae				
660–1	1·7	29	—	None
80	4·2	53	1·1	33
Cornell 48	3·8	38	5·3	24
142	0	0	20·0	45
356	4·0	62	50·0	83
383	0·19	60	101·0	84
50	2·3	29	124·0	83
S. pyogenes				
Richards	8·0	None	6·6	None
N 19	8·0 (6·5)	None	10·0	None
S 43	4·3	None	10·0	—

* μl./O_2/mg. dry-wt. cocci/hr.

Certain strains of *Streptococcus agalactiae* grown on the shaker developed the capacity of using oxygen as a hydrogen acceptor, whereas others either were not altered or were adversely affected by aeration. The strains which developed strong oxidative mechanisms for glucose, i.e. strains 50, 356, 383, also had their respiration inhibited more than 80% by 0·001 M-KCN. The strains which did not develop a strong glucose oxidizing system when grown with aeration were less affected by cyanide. *S. pyogenes* respiration was not affected by growth in shaker cultures. Endogenous respiration with all cultures was low, 1–5 μl./O_2/mg. dry-wt. cocci/6 hr.

The dependence of *Streptococcus pyogenes* and only partial dependence of certain strains of *S. agalactiae* on a fermentative pathway for energy is supported by other evidence. Suspensions of aerobically or anaerobically grown *S. pyogenes* converted glucose quantitatively to lactic acid. They had the same percentage of their total flavins as flavinadeninedinucleotide and their slow respiration was unaffected by 0·001 M-cyanide.

Suspensions of *Streptococcus agalactiae* grown aerobically had about 5 times more of their total flavin as the metabolically active flavinadeninedinucleotide than anaerobically grown cocci. As much as 85% of their respiration was inhibited by 0·001 M-cyanide and in the absence of cyanide as much as one-third of the glucose utilized was not accounted for as lactic acid. Anaerobically grown cocci converted glucose quantitatively to lactic acid and aerobically grown cocci were diverted toward a fermentative pathway when their respiration was blocked with cyanide. The behaviour of the less aerobic strain 142 was not so strongly affected by growth conditions or the cyanide (Tables 4 and 5).

Table 4. *Lactic acid yield from glucose used by suspensions of Streptococcus agalactiae in the presence and absence of 0·001M-cyanide*

Reactions run aerobically in respirometer flasks

Growth conditions *S. agalactiae*	Anaerobic		Aerobic	
	—	+ KCN	—	+ KCN
	Lactic acid %*			
50	85·0	89·0	62·8	79·0
383	92·5	98·5	67·0	85·0
356	105·0	105·0	72·0	88·2
142	83·0	104·0	86·0	92·5

* Lactic acid as % of glucose used.

Table 5. *Flavinadenine dinucleotide content as percentage of total flavins in aerobically and anaerobically grown Streptococcus agalactiae and Streptococcus pyogenes*

		FAD	Total flavin as riboflavin	% total flavin as FAD
S. agalactiae 50		*μg./g. lyophilized cocci*		
	Anaerobic	26·4	267·6	4·7
	Aerobic	113·0	223·3	24·1
S. pyogenes s 43				
	Anaerobic	54	95	27·2
	Aerobic	94·3	161·2	27·9

DISCUSSION

The data presented confirm the work of Reiter *et al.* (1963) who found that thiocyanate was necessary for growth inhibition of lactoperoxidase-sensitive streptococci. It is also necessary for the inhibition of *Streptococcus pyogenes* in the chemically defined culture medium. Neither lactoperoxidase or thiocyanate alone had any effect. That hydrogen peroxide is also necessary was demonstrated by a lack of inhibition when catalase or reducing agents were present. Reiter *et al.* (1964) found that inhibition of *S. pyogenes* (Pope) occurred in a semi-defined medium but not in heated milk to which lactoperoxidase was added. However, lactoperoxidase and thiocyanate inhibited the growth of *S. cremoris* 972 in a defined medium.

Annulment of the inhibitory effect for *Streptococcus pyogenes* with reducing agents confirms the results of others (Wilson & Rosemblum, 1952; Auclair, 1954) obtained in complex growth media. It suggests also that inhibition with lactoperoxidase and thiocyanate is the result of an oxidative change in which sulphydryl groups of enzymes are inactivated and then restored to activity by addition of a reducing agent.

Streptococcus agalactiae strains responded to the inhibitory complex in the chemically defined medium by a maximum lag of 6 hr in growth initiation. This

agrees with earlier work done in milk where *S. pyogenes* was found to die, whereas, *S. agalactiae* began to grow rapidly (Jones & Simms, 1930), after an extended lag period. *S. agalactiae* strains adapt to use oxygen as an electron acceptor to various degrees when grown on a shaker. Those strains which develop the strongest oxidative tendencies are least affected by the inhibitory complex. Strains 50, 356 and 383 developed the strongest oxygen uptake with glucose as a substrate and their respiration was 80–85% inhibited by cyanide. This result implies that iron-containing enzymes are used in their respiration and they perhaps have a good hydrogen peroxide tolerance. The peroxidase-like mechanism identical to that described for *S. faecalis* as a respiratory pathway is excluded, because it was not sensitive to cyanide (Seeley & Vandemark, 1951). The fact that *S. agalactiae* was not compeletely tolerant to peroxide was indicated by a more rapid rate of growth when catalase was added to the chemically defined medium and by a more rapid rate of respiration when catalase was added to coccal suspensions. Dr R. W. Brown of our laboratory (personal communication) has noted that the inhibition of growth of *S. agalactiae* by raw milk is overcome completely by catalase. The presence of hydrogen peroxide is necessary for the inhibitory system to function against *S. pyogenes* as it is for *S. cremoris* (Jago & Morrison, 1962). *S. pyogenes* cultures used in this work did not have their oxidative abilities enhanced by growth under aerated conditions nor was their slow rate of respiration affected by cyanide. Even in the presence of oxygen they are obliged to obtain their energy by the fermentative pathway.

The cultures of *Streptococcus agalactiae* and *S. pyogenes* which were studied here were able to respire slowly in the presence of 5 μmole hydrogen peroxide/ml. and to cause disappearance of the peroxide. Whether the hydrogen peroxide decomposition was non-enzymic and caused by its reaction with pyruvic acid resulting from glucose fermentation, or to peroxidase activity is uncertain (Greisen & Gunsalus, 1944). There exists in both organisms, therefore, a mechanism for destroying or utilizing hydrogen peroxide and preventing its accumulation. In addition to the catalase effect observed with *S. pyogenes* s43 (Table 2, Expt. 1), a similar observation was made with *S. pyogenes* Richards. The catalase activity, however, was not reproducible from one batch of cocci to the next as was the peroxidase activity. Inability to decompose hydrogen peroxide is not a suitable explanation for the sensitivity of *S. pyogenes* cultures to lactoperoxidase and thiocyanate.

The most striking metabolic difference found between *Streptococcus agalactiae* and *S. pyogenes* was the development under aerobic growth conditions of a cyanide-sensitive respiratory system in certain strains of *S. agalactiae*. Thus, they have an alternative oxidative pathway for obtaining energy from glucose which was not sensitive to lactoperoxidase and thiocyanate. Even though suspensions of some *S. agalactiae* strains did not develop increased respiratory activity when they were grown in shaken cultures, they did adapt after a delay to rapid growth in the defined medium in the presence of the inhibitor components. These cultures must also, though more slowly, develop an alternative pathway for their energy supply. The nature of the cyanide-sensitive respiration is under study.

Comparative studies of the effect of the components of the inhibitory complex (lactoperoxidase + thiocyanate + hydrogen peroxide) on glyceraldehydephosphate dehydrogenase activity and the growth of *Streptococcus pyogenes* suggest that this enzyme may be a site of inhibitor action. The inhibition of both appears to be an

oxidative reaction and annulment of the inhibition results from adding reducing agents such as glutathione or cysteine. The annulment of growth inhibition of *S. pyogenes* with glutathione, thioglycollic acid and catalase suggests that a sulphydryl enzyme is involved and that the inhibition is due to an oxidative change in which the –SH groups are converted to disulphide. Krimsky & Racker (1952) identified glutathione as the prosthetic group of glyceraldehyde-3-phosphate dehydrogenase from rat liver. If glyceraldehydrophosphate dehydrogenase is active only in the sulphydryl form, it is reasonable to believe that inactivation occurs through conversion of HS– to –S–S– which is restored to the active sulphydryl enzyme by glutathione. A very important function of glutathione might be to keep HS-enzymes, active only in the sulphydryl form, in the reduced form (Dixon & Webb, 1958). The lack of inhibition of the crude glyceraldehyde-3-phosphate dehydrogenase from streptococci by lactoperoxidase + hydrogen peroxide + thiocyanate suggests that the thiocyanate is competing with the oxidizable groups on the dehydrogenase in the bacterial extract.

The role of thiocyanate for inhibition by lactoperoxidase is not clear. Myeloperoxidase, but not horseradish peroxidase, in the presence of hydrogen peroxide oxidizes thiocyanate to sulphate and cyanide. Evidence for an unstable intermediate in the thiocyanate was obtained by measuring absorption at 235 mμ (Sörbo & Ljunggren, 1958). Horseradish peroxidase does not substitute for lactoperoxidase in the inhibition of *Streptococcus pyogenes* in the chemically defined culture medium nor with *S. cremoris* in a supplemented whey ultrafiltrate medium (Jago & Morrison, 1962). Thiouracil, thiourea and thiocyanate exist in two tautomeric forms, one containing a sulphydryl group and the other containing sulphur linked to carbon through a double bond; however, thiourea and thiouracil did not, under similar test conditions, replace thiocyanate in the growth inhibitor system for *S. pyogenes*.

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Addendum. Suspensions of three strains of *Streptococcus agalactiae*, 660–1, 80, and Cornell 48 oxidized glucose with Q_{O_2} values of 55, 26 and 22 respectively, if the cocci were harvested when 30–50% of the glucose in the growth medium had been used (6–10 hr incubation). Eighty to eighty-five % of this respiration was inhibited by 0·001 M-cyanide.

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A Method of Measuring the Sensitivity of Trypanosomes to Acriflavine and Trivalent Tryparsamide

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SUMMARY

Acriflavine-induced photosensitivity has been used to measure resistance in trypanosomes to certain aromatic arsenical drugs. Trypanosomes were incubated in a logarithmic dilution series of acriflavine and slides were prepared. After a standard exposure to light the % mortality as judged by mobility was calculated and converted into probits. Mean sensitivity and its standard deviation were estimated from graphs of probits *vs* log concentration of acriflavine. It is possible by a graphical method of analysis to distinguish between two or three populations even when these occur in the same infection. Numerical estimates of replicate analyses and analyses made on the same strain over a period of $2\frac{1}{2}$ years show that the system of measurement is stable. A range of 0·12 log units has been found in all measurements made over the period on the normal strain of *Trypanosoma brucei*. This may be compared with the lowest degree of stable resistance which is an increase of 1 log unit from the normal strain. Some factors which affect photosensitivity were examined, including temperature, action spectrum, concentration of drug, concentration of trypanosomes, time of incubation and the effect of oxygen. The method described is thought to be a compromise between that which is theoretically desirable and that which is simple to perform while still having an accuracy necessary for detailed studies of the development of drug resistance and of inheritance in trypanosomes.

INTRODUCTION

The discovery of the pathogenicity of African trypanosomes in the first years of this century led to a decade in which important aspects of the biology of the parasites were worked out. Many other features eluded workers then and, despite some intensive investigations in the intervening years, are still unknown. We have no certain knowledge, for example, of sexuality, of the details of the division cycle, of the mechanisms for inheritance or even of the site(s) of reproduction in the mammal. Two reasons for our deficiencies are clear. First, trypanosomes unlike bacteria do not grow readily in artificial culture, and when they do grow they change from the blood form to the insect form. Secondly, no reliable quantitative genetic characters have been found nor have accurate methods been available to assess characters such as drug resistance. Without satisfactory culture techniques it is difficult to obtain data on the division cycle, on nutritional requirements to develop gene maps and on the response of individuals to the action of drugs. The

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best that has been done is to study the action of drugs on trypanosome infections *in vivo* and *in vitro*, as a part of the search for new chemotherapeutic agents. In these studies resistance has been found to develop readily to most drugs. In the absence of reliable visual characters, drug-resistance characters offer an alternative approach. However, the established methods for drug testing give little information about the attributes of individual trypanosomes which comprise an infection. For example, when mice are infected with 10 million sensitive parasites and 10 resistant ones the course of infection differs little whether or not the mice receive drug treatment (Hawking & Walker, 1966). These matters have been discussed at length by Walker (1964).

A drug test method, if it is to be of use in genetical analysis, can be judged on five criteria. These are: (1) it should be sensitive and reproducible; (2) it should yield a mean value and distribution of sensitivity; (3) it should give comparable results over a wide range of sensitivity; (4) it should give data from which to distinguish and make estimates on two or more populations present in the same infection; (5) it should be applicable to drugs of different types. The method described in the present paper fulfils the first four criteria in respect of the single resistance character, namely resistance to aromatic arsenicals and amino-acridines.

Principles of the photosensitivity method. Acridine-induced photosensitivity in protozoa was first reported by Raab (1900) and elaborated by Tappeiner (1909) and Metzner (1924, 1927). Jansco (1931) observed that trypanosomes which were resistant to aromatic arsenical drugs, e.g. tryparsamide, were not as photosensitive as were normal trypanosomes. Jansco's observation confirmed the cross-resistance between acriflavine and aromatic arsenicals. Hawking (1938) measured the time in which normal and arsenic-resistant trypanosomes after incubation in acriflavine took to die when exposed to a bright patch of light in the centre of a dark-ground field. From the measurements it was apparent that variation existed between individuals. This was a simple method of estimating the mean value of a character in an infection and also the standard deviation of that character. However, it was necessary to use drug concentrations relevant to the degree of resistance in the strain being tested. Thus without further information relating the effect of light and the concentration of drug, comparison between strains was impossible. Further, owing to the difficulty in obtaining uniform illumination by dark ground a high value for the coefficient of variation was found.

In the method described here trypanosomes were incubated in various concentrations of acriflavine and then exposed to light. The time and intensity of exposure to light were made constant. After exposure the mortality rate was noted and its percentage calculated. The percentage mortalities were converted into probits before plotting a dose-response curve. For a single population the mean of individual sensitivities was given by the ED 50 and the reciprocal of the gradient was taken to be the standard deviation of that population. When two or more populations were present the dose-response curve became a characteristic 'S' shape. This will be described later, together with simple methods for analysis.

METHODS

During the development of the method to be described, several factors which affect the photosensitivity reaction were examined. From the results of this preliminary investigation on photosensitivity *per se* it was possible to derive the practical method. The preliminary studies may be recorded in outline both for the information gained about photosensitivity and also because it has been found useful to calculate deviations caused by the inevitable slight alteration of the conditions which occur when a biological technique is used routinely.

Basic equipment. An early Leitz monocular microscope was mounted on a base board together with a 30 W (6 V, 5 A). projection lamp, collector doublet lens, iris diaphragm, solenoid-operated camera shutter and the holder for a viewing filter. The normal microscope mirror was used and so also was the original aplanatic condenser. To make certain that no light from spurious reflexions or even light from the maximum aperture of the condenser reached the stage, a diaphragm was placed on top of the upper condenser lens. Slides were prepared from a suspension of trypanosomes after suitable incubation, by containing the organisms under a square cover slip ridged with petroleum jelly on three sides. It was of the greatest importance that no movement of the suspension should occur during the whole time a slide was being used. The optics above the stage were a Watson parachromatic objective ($\times 40$) and a Zeiss ($\times 12$) holoscopic ocular. The microscope tube was lined with black felt to decrease reflexions which otherwise impaired the image and caused the light meter readings to be unreliable.

Power for the projection lamp was obtained from a constant voltage transformer. As shown in the next section, the action spectrum for acriflavine-induced photosensitivity is in the blue-violet region. Tungsten filament lamps produce little energy in the blue-violet region and the amount is greatly dependent on the supply voltage. Other light sources such as high pressure mercury arc and 1000-W projection lamps were used but were less suitable than the low wattage tungsten lamp. The principal difficulty with the high-powered lamps was that the intense heat produced precluded the use of an efficient collector lens. In practice the most intense illumination was obtained by a short focal length ($f = 4{\cdot}7$ cm.), high aperture (4 cm. diameter) collector lens and a 30 W lamp.

The solenoid-operated camera shutter was controlled by a thyratron timer. The device could be pre-set to give accurate exposures between 1 and 14 sec.

The viewing filter protected the trypanosomes and the operator from damage by the intense light. The filter consisted of a dense negative (optical density = 2·35) and a deep red glass filter. The filter holder was arranged so that the filter could be slipped quickly in and out of the light path.

A light meter was used to standardize the intensity of illumination at the stage. When the optics were in Köhler illumination and normal focus was achieved, the eyepiece was removed and a $\times 6$ eyepiece with an attached photocell was inserted. The photocell (Mullard 90 AV) was chosen because its spectral response, through glass optics, has a peak sensitivity in the blue-violet region. A sensitive spot-galvanometer was used in the photocell circuit. The response of the photocell was tested and found to be linear to light intensity over the range of light intensities used.

Strains of trypanosomes and rodent hosts. A monomorphic virulent strain of *Trypanosoma brucei* (NIMR 2) group was used for the development of the method. Strain NIMR 2 was fully adapted to rats and to mice by rapid syringe passage in alternate hosts. *Trypanosoma rhodesiense* EATRO 118 was obtained from the blood of infected rats, it was isolated from man in 1959 at the East African Trypanosomiasis Research Organization. *Trypanosoma equiperdum* (NIMR1) was used in one experiment, this strain came from Dr J. D. Fulton and was known originally as Wellcome OE (Fulton & Spooner, 1959). NIMR numbered strains refer to the strains held by the trypanosome bank at the National Institute for Medical Research.

Male mice of the Parkes's strain were chosen because of the freedom from possible disturbance of the course of infection by the oestrous cycle of the female and also because this strain is extremely docile. Rats were of the hooded (black and white) strain and were all males for the same reasons.

Factors which affect photosensitivity

Simple tests of the effect of the temperature of incubation showed that no observable change in photosensitivity occurred between 4° and 37°.

The temperature during exposure to light greatly altered the trypanosome mortality. The effect was examined by using a water-warmed slide and thermistor measuring device. Water was pumped in a closed system which consisted of two copper coils immersed in beakers of water and a space between two large coverslips. The test trypanosome suspension was held between two coverslips and was placed on the upper coverslip of the warmed slide with liquid paraffin making a heat-conducting joint between the two glass surfaces. The temperature on top of the slide became that of the water within a few seconds. With this equipment it was possible to obtain accurately controlled and stable temperatures which could be altered to any new value within a minute or two, simply by adding hot water or ice to the beakers containing the heat-exchange coils. Observations of photosensitivity were made by the 'one concentration method' (see below, Analysis of results, for details) and the results adjusted according to the values found for each overlapping range in temperature. Experiments were made on different days. The results are expressed in Fig. 1. These show that relatively small changes, when compared with other factors, occurred up to 26°, but above this the photosensitivity increased more steeply. The plateau 35° to 41° and the sharp rise at higher temperatures are features about which nothing is known. The findings suggest that variations in 'laboratory temperature' (20°–22°) would be insignificant for practical purposes.

Action spectrum. Experiments of Tappeiner (1909), Metzner (1924) and Jansco (1931) showed qualitatively that the action spectrum was in the region of peak absorption of acriflavine (λ = 454 mμ). Ultraviolet radiation was used to find whether photosensitivity also occurred in the absorption band in this region. Light from a mercury vapour lamp passed through a Chance OX 7 filter and was focussed by quartz optics on to a quartz slide. No trypanosomes were killed after exposure for 2 min. in a concentration of acriflavine 1 μg./ml. It is concluded that ultraviolet radiation does not cause photosensitivity.

Drug concentration. The concentration of acriflavine was the variable chosen to be a measure of photosensitivity. The choice was made because the concentration can be known accurately and the procedure is easier, since logarithmic dilution

series can be made up once, whereas a logarithmic series of light exposures cannot accurately be made.

Acriflavine was insufficiently soluble in Ringer's solution, and all experiments were done with acriflavine dissolved in ion-exchange water. A dilution by volume of one part of stock drug solution was made with nine parts of trypanosome suspension. Thus all results refer to a slightly hypotonic suspension.

Time of exposure to light and drug concentration. The interaction of these two variables was examined by using the ED 50 values for time for exposure as the end-point. Trypanosome suspensions at several concentrations were used and the

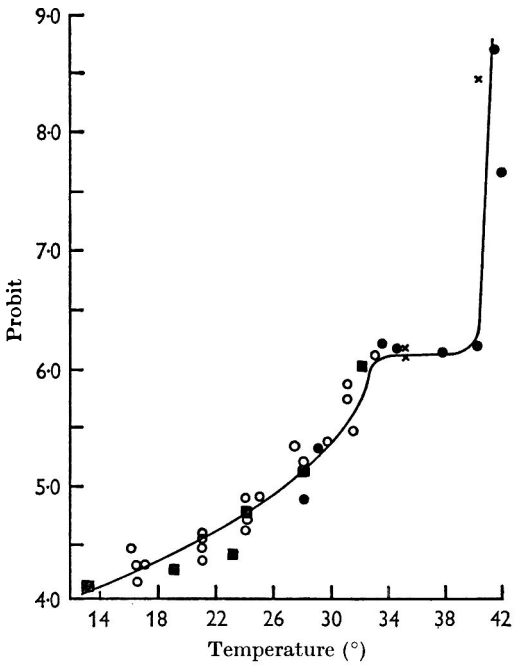

Fig. 1

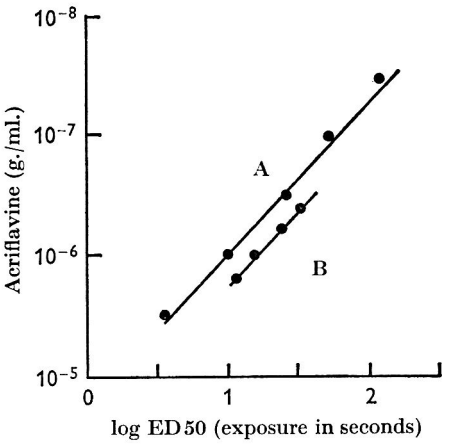

Fig. 2

Fig. 1. The effect of temperature on photosensitivity of organisms. Different symbols indicate experiments on different days. Three concentrations of acriflavine were $10^{-5\cdot8}$, $10^{-6\cdot0}$ and $10^{-6\cdot3}$.

Fig. 2. The effect of concentration of acriflavine on the ED50 measured in seconds (see text).

mortality observed after four or five different exposure times at each concentration. The logarithm of the ED 50 (in seconds) for concentrations is plotted against the logarithm of the concentration in Fig. 2. Line A was drawn through points obtained over a wide range of drug concentrations and times of exposure. Line B confirms the gradient of A, both are 1·3 over a narrower range. A systematic difference exists because of a change in the intensity of illumination between experiments.

The existence of a uniform relation between drug concentration and time of exposure was found for *Trypanosoma equiperdum* (NIMR 1). Similar, but less detailed experiments suggested that the gradient is characteristic of the strain. The gradients for *T. brucei* (NIMR 2) and *T. rhodesiense* (EATRO 118) were 1·0 and 0·91, respectively.

Time of incubation. Hawking (1934, 1938) found that uptake of acriflavine was very rapid, being virtually complete after a few minutes. This was not found in the present work, when spectrophotometric determinations of acriflavine remaining in solution were made. The results are shown in Fig. 3 and indicate that about 1 hr was required to reach equilibrium.

Measurements of how the photosensitivity changed with time of incubation were made by using the one-concentration method. The results are shown in Fig. 4 *a*, *b*. In both trypanosome strains the increasing photosensitivity had a value about equal to the steady value after 20 min. However, between 20 and 40 min. a peak sensitivity showed, only to fall to a steady value at 60 min. Exposures made after prolonged incubation (up to 9 hr) showed no real change in the response.

These results suggest that for a practical method 1 hr is the minimum period for incubation; after this it is not critical when the exposures are made.

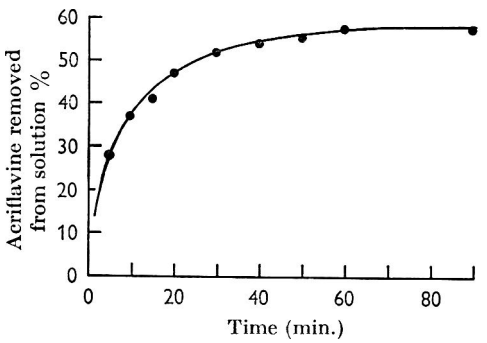

Fig. 3

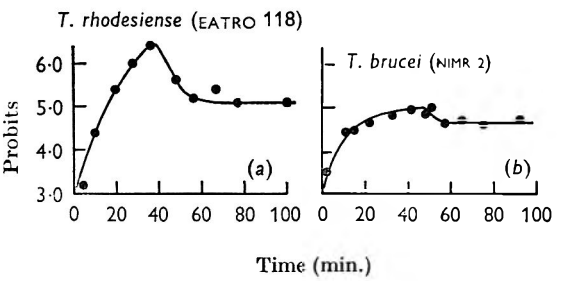

Fig. 4

Fig. 3. Spectrophotometric analysis of the rate of uptake of acriflavine by trypanosomes. Initial concentration $10^{-6\cdot0}$, trypanosomes 2000/μl.

Fig. 4. The effect of time of incubation on photosensitivity. Concentration for *Trypanosoma rhodesiense* EATRO 118 was $10^{-5\cdot8}$ and for *T. brucei* it was $10^{-6\cdot3}$.

The number of trypanosomes. Hawking (1938) observed that for a given concentration of drug when there were few trypanosomes these were more photosensitive than when there were many parasites. The present experiments confirmed this; ED 50 (in weight/volume units) was inversely proportional to the number of trypanosomes. It is therefore possible to correct mean values of photosensitivity for small departures from the standard number of trypanosomes.

The effect of serum and red cells. Serum and red cells are likely contaminants of trypanosome suspensions; their effects were examined by using the one-concentration method. The results from experiments with different concentrations of inactivated horse serum are given in Fig. 5. The results show that serum decreased the photosensitivity in a way roughly inversely proportional to the logarithm of concentration. For practical purposes it was necessary to use a medium which contained more serum than likely to be present as a contaminant. In this way the concentration of serum was standardized without detriment to the parasites by repeated centrifugation.

Red cells did not influence photosensitivity up to 5000/μl. This number is more than is desirable for other reasons.

The effect of pH value. This factor was investigated by using isotonic phosphate buffers between pH 5·8 and 7·7 (Hendry, 1948) and analysed by the one-concentration method at two times of exposure. The results, shown in Fig. 6, emphasize the need for the accurate control of pH value. It is convenient that pH 7·2 was both at the peak of sensitivity and also was a suitable value for good longevity of parasites in suspension (Lumsden *et al.* 1963).

The effect of oxygen. Oxygen is stated to have a profound effect on photosensitivity (Blum, 1941; Reid, 1960). Trypanosomes were incubated; (*a*) under liquid paraffin, (*b*) with the surface open to the atmosphere, and (*c*) with oxygen bubbled slowly through suspension. No differences were found between the treatments. In another

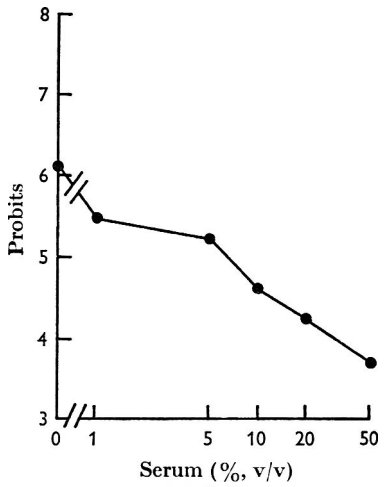

Fig. 5

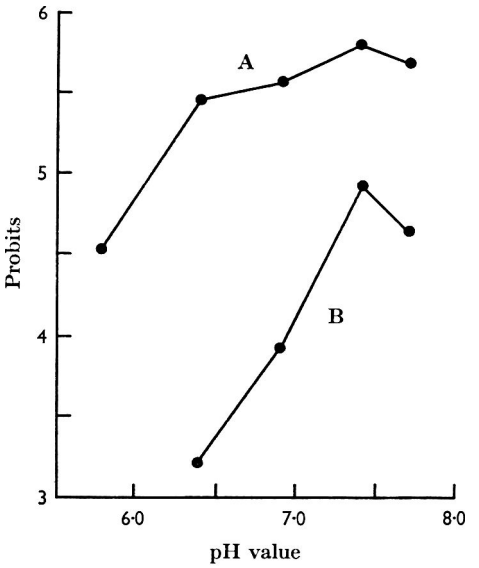

Fig. 6

Fig. 5. The effect of serum on photosensitivity.

Fig. 6. The effect of pH value on photosensitivity. A, exposure was 10·6 sec. and B, exposure was 5·5 sec.

experiment suspensions of trypanosomes, which consume oxygen vigorously, were kept in sealed slides for up to 9 hr. The photosensitivity, which was expected to decrease through lack of oxygen, remained constant or increased very slightly. It was concluded that the oxygen tension during incubation and exposure exerted little or no effect on quantitative photosensitivity.

The method for routine photosensitivity measurements

Details of the apparatus have already been described. From consideration of the changes caused by various factors (above) the final method may now be described.

The standard buffered medium consisted of: 50 ml. inactivated (56°, 30 min.) horse serum (Oxoid); 300 ml. Hendry's phosphate buffer (pH 7·2); 37·5 ml. isotonic glucose (53·4 g./l.); 10 ml. phenol red solution (1/1000 in saline); made up to 1 l. with Ringer's solution. This mixture was Seitz-filtered and put into 25 ml. containers; its keeping properties at 4° appear to be good.

Solutions of pure neutral acriflavine (3,6-diamino-10-methyl acridinium chloride; from Messrs Boots Ltd., Nottingham) were made in ion-exchange water because of the insolubility of the pure compound in balanced salts solutions. The initial concentration was 1/1000 (w/v) and serial tenfold dilutions were made. From each dilution were prepared nine others separated at a logarithmic interval of 0·1 until a continuous sequence $10^{-3\cdot0}$, $10^{-3\cdot1}$, $10^{-3\cdot2}$,...$10^{-4\cdot0}$, $10^{-4\cdot1}$,...$10^{-5\cdot0}$, $10^{-5\cdot1}$,...to $10^{-5\cdot9}$ and $10^{-6\cdot0}$ was obtained. These solutions served as stock for all further experiments. The rack of tubes was covered with a light-tight hood and stored at 4°. No change in the activity of the acriflavine has been observed during 3 years.

To avoid cumbersome wording, the dilutions and concentrations of acriflavine will be expressed as the positive value of the negative logarithm. Thus 1 μg./ml. becomes 6·0, and 0·5 μg./ml. becomes 6·3. The advantages of this system of notation, which is similar to the pH scale, were discussed by Walker (1964).

Trypanosome suspensions were prepared by centrifugation of heavily infected heart blood taken from young rats (60–100 g.). Heparin at the rate of 1 in 5000 (w/v) was used. The layer of trypanosomes was dispersed slightly and transferred to a quantity of the medium. The number of trypanosomes was adjusted from successive haemocytometer counts until there were about 10,000/μl.

Suitable acriflavine solutions were delivered in 0·1 ml. lots into each of a row of small glass tubes and 0·9 ml. of the trypanosome suspension was added to each tube. The tubes then were quickly shaken and the rack covered with a hood to exclude light. The preceding manipulations were all made in very dim light.

Incubation was for 1 hr at 20° to 22° (room temperature). Each tube was well shaken before a capillary tube was used to remove a small quantity of suspension from it. The drop of suspension was placed on a coverslip ridged on three sides with petroleum jelly. A slide (thickness 0·88–0·95 mm.) was lowered on to the coverslip. The preparation was examined by using the observation filter to check that it was not still flowing, and a field containing 40–60 trypanosomes was then located.

The microscope had previously been adjusted by using a standard slide to give a deflexion of 9 units on the galvanometer scale. Calculation, based on the manufacturer's data for the photocell, shows that about 0·064 lumens were passing into the eyepiece field of 372 μ diameter. Neglecting the absorption at the glass surfaces the intensity experienced by the trypanosome was roughly $5{\cdot}8 \times 10^{-7}$ lumens/μ^2 or, if the flagellum is considered as the only photosensitive organelle (Walker, 1961), then in 10 sec. the flagellum receives a total of $2{\cdot}2 \times 10^{-2}$ ergs or 5×10^9 photons in each μ of its length. This calculation uses the value 0·25 μ as the diameter of the flagellum (Dr K. Vickerman and Dr J. Williamson, personal communications).

Exposure without the observation filter in all routine experiments was for 10 sec. With the filter replaced the dead organisms were counted. A trypanosome was considered as dead when, after 1 min., no wriggling movements were seen. Experience showed that a few trypanosomes were motionless immediately after exposure but moved slightly within 10–30 sec. Prolonged observation of individual trypanosomes suggested that when no movement occurred after more than 30 sec. the organism would not recover motility. Two exposures were usually made for each preparation. The proportions of organisms killed were usually close in both exposures; but when they were not, a third or fourth exposure was made, and the proportions in close agreement were used for calculation. Data from each exposure of the number

killed and of the total number were added together and expressed as a percentage. The percentage was converted into a probit number by using tables, and the probit numbers plotted on the ordinate against drug concentration in logarithmic units. A complete dose-response curve was thus built up by using the number of drug concentrations necessary to cover the range of sensitivity of a given strain (Figs. 7, 8). While it is a simple matter to see an occasional living trypanosome among many dead ones and to count them accurately, the reverse situation is not so easy. This practical difficulty caused the data below probit 3·7 to be less accurate than other points.

Counts were made of trypanosome suspensions by adding 0·1 ml. of a 4 mg./ml. HCHO in isotonic saline with 0·5% methylene blue to 0·9 ml. of suspension. Ten large squares of an improved Neubauer haemocytometer were counted. These squares contained a total of about 400 trypanosomes. The expected standard error of counts was 5%.

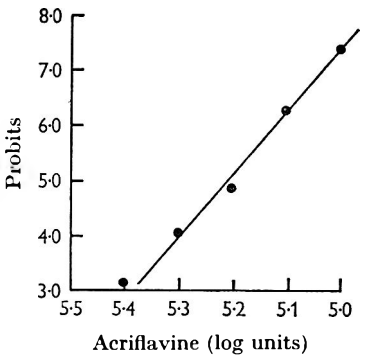

Fig. 7

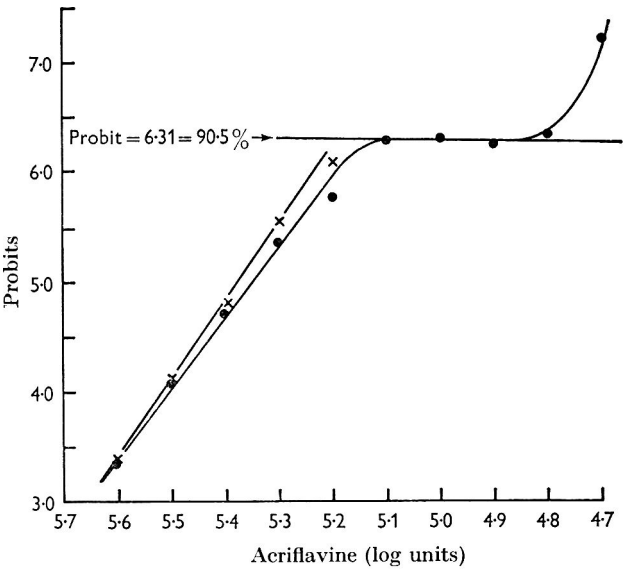

Fig. 8

Fig. 7. Results from the photosensitivity analysis of a resistant homogenous (log-normal) strain of *Trypanosome brucei* (NIMR2.) For calculation of log units of acriflavine see text p. 52.

Fig 8. Results from the photosensitivity analysis of a resistant infection of *Trypanosoma brucei* (NIMR2) which contained two populations (see Table 1). ●, Experimental points. ×, recalculated points for major population (see text).

Analysis of results

Analysis of data from routine tests was performed graphically by fitting the best straight line or curve to a set of points (Fig. 7). If the strain consisted of one population, the ED 50 value and standard deviation were read directly in the usual way. Correction for departure from the standard number of trypanosomes was made by adding or subtracting the difference between the logarithms of the number of trypanosomes and the standard (10,000/μl.). Thus when too few trypanosomes were present the ED 50 was larger, and it was necessary to subtract the correction for

number. This process is valid since both the difference in number and the drug concentration are expressed in logarithms to the base 10.

In the development of drug resistance several populations were found frequently in the same infection. A graph of probit *vs* log concentration appeared as a rounded step or steps (Fig. 8). The flat portion or point in inflexion between each steeply rising section of a curve indicated the probit which corresponded to the percentage of the total infection where one population ended and another began (Harding, 1949). Analyses of these inflexed curves were made graphically. When two populations were found the flat region of the curve was extrapolated to the ordinate and the probit obtained. The percentage mortality which corresponded to this probit indicated the proportion of the infection occupied by the more sensitive population. The percentage occupied by the less sensitive population was obtained by subtraction from 100. Because of the distortions introduced when two populations existed together it was necessary to recalculate the percentage mortality at each concentration and express the figure as a percentage of the relevant population. These new percentages were replotted as probits and the two straight lines analysed separately. Frequently one resistant population represented less than 5% of the total infection and it was impossible to give accurate evaluation to either ED 50 or standard deviation, although from the shape of the curve the existence of the population was certain. In such cases it was only possible to state the range of concentrations in which such a small population was found.

When tests were made by varying some factor and measuring the effect on photosensitivity the normal *Trypanosoma brucei* (NIMR 2) strain was used. This strain was known to give consistent results and to have a log-normal distribution of sensitivity which, of course, appeared normal because the drug concentrations were made up on a logarithmic scale. It was therefore possible to use the relation that for a given strain: $P_2 - P_1 = (C_1 - C_2)/s$, when P_1, P_2 are observed probit values, C_1, C_2 concentrations of acriflavine (in the logarithmic scale) and s is the standard deviation of sensitivity in the population. It follows that when one concentration is used the probit can be taken as indicating photosensitivity. This greatly decreased the number of observations needed and simplified the analysis.

When one factor caused a very wide variation in photosensitivity (e.g. the effect of temperature; Fig. 1) three concentrations were used. The final graph was constructed by taking one concentration as a standard and then calculating the difference between the probit values when temperatures overlapped. The difference was used to adjust the observations from the second and third concentrations in terms of the first concentration. The graph (Fig. 1) still bearing the probit scale looks absurd, for a probit value of 9·0 is impossible to measure directly.

RESULTS

Single populations

Typical results for a resistant *Trypanosoma brucei* (NIMR 2) are shown in Fig. 7. This straight line was fitted by eye after having made allowance for the loss of weight as points depart from the ED 50 (probit 5). The ED 50 from the graph was 5·21, standard deviation 0·09. The suspension of trypanosomes used in this experiment contained 39·3/μl. and this gives a correction of 0·01 to be subtracted from the

ED 50. The corrected mean photosensitivity for the experiment on this (resistant) strain was therefore 5·20; S.D. 0·09. These observations suggest this strain has a normal distribution of sensitivity and it is unlikely to have more than 4·5% of another population. Estimates of the maximum value for an unseen population are obtained from the Poisson distribution of the mean number of resistant trypanosomes per two fields when 100% mortality would be expected 19 times in 20. The percentage is found from: maximum percentage not seen $= 5/x$ when x is the total number of trypanosomes at the first concentration at which all are killed.

Two populations in a single infection

Trypanosome strain c 5 was obtained during an experiment on the development of resistance to aromatic arsenical compounds in *Trypanosoma brucei* (NIMR 2). A graph of the results is shown in Fig. 8. The flat region of the curve is at probit 6·31 and it represents 90·5% of the infection. A second analysis of the major population is also shown in Fig. 8. The features of each population are given in Table 1.

Table 1. *Analysis of the populations of a strain of Trypanosoma brucei* (NIMR 2) *with a bimodal distribution of sensitivity to acriflavine after exposure to a constant quantity of light*

	% of total	ED 50	Corrected ED 50	Standard deviation or range
Population A	90·5	5·375	5·35	0·14
Population B	9·5	4·75	4·73	4·7–4·8

See text for the units of the ED 50 and method of correction.

Since fewer trypanosomes are represented in population B, the accuracy of its ED 50 value was less than for population A. The standard deviation for population B cannot be calculated with certainty from such data. It is thought better to give a range which included 'most' of the population. It would be ideal if 'most' covered $\pm 2s$ (95%) of the population; but this is not possible and all attempts at pseudo-accuracy have been avoided.

Standard errors of the ED 50 can be found in the way adapted for probit analysis and comparison can be made between the populations. In practice a rule-of-thumb test, which is only applicable to strains with characteristics similar to those given above, is possible since the standard error of each ED 50 is given approximately by $s/\sqrt{(1{\cdot}5n)}$, where s is the standard deviation and n the mean number of trypanosomes per concentration present in the population being analysed. For populations as small as B (Table 1) no excessive claims for fine analysis arise when a figure of half the range is used for the standard error.

Accuracy of analyses of Trypanosoma brucei (NIMR 2) *populations*

Replicate analyses were made early in the development of the technique. The strain of *Trypanosoma brucei* (NIMR 2), was chosen because of its known sensitivity to arsenical drugs and because analyses had suggested it was a single population of normal sensitivity. The ED 50 values are compared in Table 2. Three analyses A, B and C were made one after the other. At each concentration two exposures were

made (numbered 1 and 2). The results from exposures 1 and 2 were combined to give data in A 1+2, B 1+2, C 1+2. The results from all exposures at each concentration were combined and analysed. This is shown in the row 'combined results'. In Table 2 the results given in the column headed 'graphical analyses' were obtained without correction for number of trypanosomes from the graphs. Arithmetical analyses were made by Miss M. V. Mussett, who used the usual re-iterative method for probits.

Table 2. *Table of ED 50 and s obtained by graphical and arithmetical methods of analysis of normally sensitive Trypanosoma brucei* (NIMR 2)

	Method of analysis				
	Graphical		Arithmetical		
Group	ED 50	*s*	ED 50	*s*	*P*
A 1	6·30	0·19	6·308	0·163	0·4
A 2	6·28	0·20	6·294	0·193	0·8
B 1	6·28	0·16	6·277	0·158	0·3
B 2	6·28	0·16	6·284	0·174	0·8
C 1	6·27	0·16	6·266	0·139	0·4
C 2	6·24	0·18	6·252	0·143	0·2
A 1+2	6·28	0·209	6·305	0·184	0·6
B 1+2	6·28	0·169	6·281	0·166	0·3
C 1+2	6·25	0·188	6·258	0·143	0·1
Combined results	6·287	0·181	6·285	0·172	0·8

s = standard deviation of population.
P = probability of being derived from a homogenous population (from χ^2 for linearity).

Table 3. *Results of photosensitivity analysis made on Trypanosoma brucei,* (NIMR 2)

Date	ED 50	Standard deviation
15 June 61	6·14	0·14
28 Aug. 61	6·07	0·11
2 Sept. 61	6·17	0·12
17 Oct. 61	6·05	0·16
26 Oct. 61	6·10	0·10
18 Nov. 61	6·06	0·07
15 Nov. 62	6·14	0·15
29 Nov. 62	6·08	0·17
7 Dec. 62	6·11	0·17
13 Dec. 62	6·12	0·09
27 Nov. 63	6·08	0·04
28 Nov. 63	6·12	0·10
29 Nov. 63	6·07	0·05
5 Dec. 63	6·08	0·15

The results analysed by either method show considerable uniformity even between the primary observations, A 1, B 1, etc., which represent a group size of only 40 trypanosomes. Since nearly 4 hr elapsed between the first and last measurements the temporal stability of the character is clearly indicated. The only consistent difference between the graphical and arithmetical methods of analysis appears in the estima-

tion of standard deviation. This difference is due to considerable loss of weight when points depart from probit 5. The discrepancy is small and for the present purposes unimportant.

Stability of drug sensitivity of Trypanosoma brucei (NIMR 2)

Photosensitivity observations made over some years are presented in Table 3. Although it is difficult to distinguish between instability of a character from instability of the system of measuring it, the results suggest stability in both the strain and the method. The figures given in Table 3 may be compared with the lowest degree of stable resistance which has a value of 5·2 units.

DISCUSSION

The results obtained by using acriflavine-induced photosensitivity show clearly that the method is capable of producing reliable estimates which fufil most of the criteria set out in the Introduction. It remains to discuss a few details which have not been tested by direct experiment and to comment on the reasons for adopting an elementary statistical treatment.

Oxygen tension has been shown to be without effect on photosensitivity despite biological removal or physical excess. For practical purposes, providing that the same manipulative procedures are followed, it would be impossible to exceed the extremes tested. However, removal of oxygen by addition of reducing compounds seems likely to interfere with the photosensitive reaction by direct action on the trypanosomes as well as by removing oxygen. Objections can be raised to an alternative method of removing oxygen from a suspension by a stream of nitrogen, because of the very low concentration of oxygen which would be expected to take part in the reaction. It remains therefore uncertain whether oxygen is required in the photosensitive reaction. The indication, bearing in mind the high oxygen consumption of trypanosomes (Brand, 1952), is that the organisms are themselves capable of removing oxygen from an environment. If this is correct then the stable photosensitivity found in slides sealed for many hours suggests that oxygen plays no part in the reaction.

It was not possible to investigate changes in photosensitivity which might occur with age or heaviness of infection, because of the difficulty of preparing trypanosomes from light infections. In several experiments it was necessary to use blood from rats which were only showing ten trypanosomes/ × 40 microscope field and from moribund rats with between 100 and 200 trypanosomes/ × 40 field. No difference was detected between these analyses and trypanosomes from an intermediate degree of infection. It is presumed that the age and severity of an infection has no appreciable effect on photosensitivity.

The reasons for adopting a graphical standard of analysis are that time is saved and that, because of the large number of trypanosomes in each group, even the simplest analyses themselves have small errors and the complex refinements for probit analysis on small groups are unnecessary. It should be added that it is not possible at present to control the light intensity better than 2%, with possibly wider fluctuations of the intensity of the active wavelength. This error may contribute to a variation of 0·03 units even in the same day.

For the purposes of example, if an average value for the standard deviation is

taken to be 0·1 units and if an analysis has 5 points on probits 3, 4, 5, 6 and 7, a significant difference ($P = 0{\cdot}05$) is found between means with a separation ranging from 0·043 when $n = 25$ to 0·020 when $n = 100$. It is clear that while extrinsic errors such as those introduced by the intensity of illumination may be as large as 0·03 units, no useful purpose is served by increasing n beyond 100. Although n can be made smaller than 100 in theory, two exposures at one slide have been found necessary, since each exposure serves to check the other. Thus data based on two exposures have a combined group size within the range 60 to 110 trypanosomes.

The magnitude of variation in the sequence of measurements on *Trypanosoma brucei* (NIMR 2) (Table 3) is clearly significant when rigorous standards are applied. Not enough experiments have been done to enable an analysis of variance to be made. The range in this sequence is 0·12 units and in the opinion of the writer a difference between means of 0·12 units may be a conservative estimate of a significant difference. However, when populations which have widely different sensitivities are present in the same infection competitive uptake of drug must be expected. The effect of this is to make resistant populations appear a little more resistant than when they appear alone. It is concluded that although the photosensitivity method is adequate for the purpose intended, rigorous statistical tests of the results are inappropriate because of some non-random variation in mean photosensitivities.

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The Assay, Extraction and Storage of Infective Ribonucleic Acid from Foot and Mouth Disease Virus

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SUMMARY

A plaque assay method is described for the titration of the infective ribonucleic acid (RNA) of foot-and-mouth disease (FMD) virus. The method depends on the dilution of the RNA in salt solutions of high molarity and the treatment of the BHK 21 cell monolayers used with M-NaCl. The method is reproducible and is at least as sensitive as other methods of titration. The extraction of RNA in the presence of EDTA gave titres corresponding to 0·1 % of the titre of the intact virus. RNA prepared from virus suspensions containing EDTA retained about 50 % of initial infectivity for 7 days at 4° but for longer periods it was best preserved in 70 % (v/v) ethanol at −20°.

INTRODUCTION

In any detailed study of the structure and multiplication of RNA viruses the methods used for the isolation and titration of the infective RNA are most important. With two exceptions, all the methods described for the titration of the RNA from FMD virus have given titres which varied between 0·1 and 0·001 % of the corresponding virus titre. In the first descriptions of the extraction of RNA from FMD virus (Brown, Sellers & Stewart, 1958; Mussgay & Strohmaier, 1958; Brown & Stewart, 1959), values of this order were obtained when the RNA was extracted with cold phenol and titrated by intramuscular or intracerebral inoculation of suckling mice or by observing the cytopathic effect (CPE) of the extract on pig kidney cell monolayers (Brown & Stewart, 1959). Hantschel (1963) and Hantschel & Veckenstedt (1964) also obtained similar RNA titres by intracerebral inoculation of mice. In 1960 Bachrach described a plaque assay method using calf kidney cell monolayers in which the highest RNA titres were only of the order of 0·001 % of those obtained with the virus. These values were of a similar order to those obtained for several other animal viruses. There were, however, two reports in which the titre of the RNA from FMD virus was considerably higher than those referred to above. Thomas & Leclerc (1959) claimed a very high titre in guinea pigs (namely, 10 % of that of the virus) for RNA extracted from guinea-pig or cattle virus, although they later reported (Thomas & Leclerc, 1960) that the titre was more usually of the order of 0·3 %. Thely *et al.* (1961) claimed that the titre of RNA in cattle was 88 % of that for the cattle virus from which it was prepared but it would seem wise to accept this exceptionally high titre with caution, especially as it would be almost impossible to preclude the presence of traces of ribonuclease in the method of titration used.

In many experiments over a number of years we had been unable to develop a plaque technique for the titration of FMD-virus RNA in pig kidney cells. Following the demonstration by Mowat & Chapman (1962) that BHK 21 cells are susceptible to FMD virus, the suitability of these cells for the assay of RNA was examined. As with pig kidney cells, very few plaques were obtained when the RNA was added in isotonic solution. When the cell sheets were washed with hypertonic solutions before the addition of the RNA (Koch, Koenig & Alexander, 1960) or the RNA was diluted in hypertonic solution (Alexander, Koch, Mountain & van Damme, 1958; Koch *et al.* 1960; Holland, Hoyer, McLaren & Syverton, 1960), more plaques were formed. These observations led us to make a more detailed study of the optimal conditions for plaque formation. In the present paper we also include experiments designed to improve the extraction and stability of RNA from FMD virus.

METHODS

Virus strains. Foot-and-mouth disease (FMD) virus strains of immunological types 0 (strain 1) and SAT 1 (strain Turkey 43/62) were used. The strains were maintained by passage in guinea pigs and in BHK 21 cells (Macpherson & Stoker, 1962). Virus from guinea pigs was collected as epithelium and vesicular fluid from the hind pads 24–48 hr. after infection and stored at −20° until required. The culture virus was collected as the supernatant fluid from cell monolayers incubated in Eagle's medium.

Extraction of RNA. Virus suspensions at 4° were shaken with an equal volume of phenol which had been equilibrated with one of the following: (*a*) 0·01 M- or 0·04 M-phosphate (pH 7·6); (*b*) H_2O; (*c*) 0·04 M-phosphate (pH 7·6) containing M-NaCl; (*d*) 0·04 M-phosphate or H_2O containing 0·01 % sodium ethylenediaminetetraacetate (EDTA). The two layers were separated by centrifugation at 2000 rev./min. for 2 min. and the aqueous layer extracted a second time with phenol. The phenol was removed from the aqueous layer by two or more extractions with redistilled ether and the dissolved ether removed in a stream of nitrogen. Each RNA extract was adjusted to pH 7·4–7·6 immediately after removal of the ether and, because of the stabilizing effect of M-NaCl on FMD-virus RNA (Thomas & Leclerc, 1959, 1960) and in view of our own results, this reagent was usually added. In some experiments M-NaCl, 0·01 % EDTA or 0·1 % sodium dodecyl sulphate (SDS) in 0·04 M-phosphate (pH 7·6) or bentonite was added to the virus suspension before phenol extraction.

Titration of virus. Most virus assays were made by the plaque technique in BHK 21 cell monolayers (Mowat & Chapman, 1962). As an alternative, tenfold dilutions of the virus suspensions were inoculated intracerebrally into groups of suckling mice (Skinner, 1951) and the end points calculated by the method of Reed & Muench (1938). A few assays were made in guinea pigs by intradermal inoculation of the hind pads and the end points calculated in the same way.

Titration of RNA. Infective RNA was assayed in animals or in cultured cells. For the animal tests tenfold dilutions in phosphate buffered saline (PBS) were inoculated intracerebrally into suckling mice or intradermally into the hind pads of guinea pigs. The RNA was also assayed by observing the cytopathic effect (CPE) produced in monolayers of BHK 21 cells. In these experiments care was taken to wash the

sheets of cells free from serum before adding the RNA but it was unnecessary to use hypertonic conditions. All washes were made with phosphate buffered saline (PBS) and the cell sheets incubated in Eagle's medium after the adsorption of the RNA. The monolayers were examined for CPE up to 72–96 hr after infection. The CPE led finally to the monolayers stripping from the glass.

RNA was also assayed by the plaque technique in BHK21 cells by the method developed in this work. It was again essential to wash the cells free from serum. The cell monolayers were therefore washed twice with PBS and then once with M-NaCl in 0·04M-phosphate (pH 7·6). This last solution was left on the monolayers for 5 min. Dilutions of the RNA in 2M-$MgSO_4$ in 0·1M-tris (pH 7·4–7·6) were then added and allowed to adsorb for 20 min. at 15–20°. After a final wash with PBS the monolayers were overlaid with agar made up in Eagle medium containing 10% ox serum and incubated at 37°. After 24–48 hr the cell sheets were stained with 1/10,000 neutral red to reveal plaques. An alternative method which we have found convenient is to fix the monolayers with buffered formalin (4%, w/v, HCHO in PBS) at room temperature and up to 48 hr later remove the agar and stain with methylene blue. The most reproducible results were obtained with monolayers which had been grown for 24–48 hr. Monolayers older than this were less sensitive and were more easily detached from the glass by the repeated washings required in the assay. This detachment was particularly noticeable when polystyrene Petri dishes were used.

RESULTS

Plaque assay for RNA

Since cells vary in their susceptibility to injury by hypertonic solutions (Koch *et al.* 1960; Sprunt, Koenig & Alexander, 1961), the ability of BHK monolayers to survive such treatment was first examined. The RNA titres following the use of different washing procedures and diluents for the RNA were then compared. The length of time required for pre-treatment of the monolayers was ascertained and the optimal time for RNA adsorption determined. Other factors, including the volume and pH of the inoculum, were also considered. When the best conditions had been found, the method was checked for reproducibility and the dose-response relationship determined. Finally, the assay was compared with other titration methods for RNA.

We found that BHK cells withstood treatment with hypertonic solutions provided that the time of contact was not excessive. Severe damage was caused when the monolayers were exposed to M-NaCl or 2M-$MgSO_4$ for longer than 30 min. Since increased damage occurred at 37° without any increase in the number of plaques (see Holland *et al.* 1960), the washing of cell monolayers and adsorption of RNA were always done at 15–20°.

Monolayers were washed 3 times to remove serum before the addition of RNA but the solution used for the third wash was varied. Very few plaques were obtained unless the final wash was made with a strong salt solution or the RNA was diluted in a solution of high molarity (Table 1, Expt. 1). The highest titres and least cell damage were obtained when the final wash was M-NaCl and the diluent for the RNA contained 2M-$MgSO_4$ (Table 1, Expts. 1–3).

Montagnier & Sanders (1962) obtained higher titres for encephalomyocarditis

virus RNA by subjecting the cells to hypotonic shock before adding the RNA in hypotonic solution, but the results in Table 1, Expt. 4, show that this technique was not applicable to our system.

However, pre-treatment of the monolayers with M-NaCl for periods varying between 1 and 15 min. did not alter the number of plaques subsequently developed and a standard time of 5 min. was found to be the most convenient.

Table 1. *Effect of diluent and pre-treatment of BHK monolayers on the titration of FMD-virus RNA. RNA extractions made with phenol saturated with 0·04 M-PO_4, pH 7·6*

Experiment	Diluent for RNA*	Pre-treatment	Titre (p.f.u./ml.)
1	PBS	3 × PBS	2·0
	0·04 M-PO_4	3 × PBS	0
	PBS	2 × PBS, 1 × M-NaCl	$2·6 \times 10^4$
	M-$MgSO_4$ in 0·1 M-tris	2 × PBS, 1 × M-NaCl	$1·0 \times 10^4$
	2 M-$MgSO_4$ in 0·1 M-tris	3 × PBS	$2·6 \times 10^4$
	2 M-$MgSO_4$ + 1 M-NaCl in 0·1 M-tris	3 × PBS	$2·7 \times 10^4$
	2 M-$MgSO_4$ in 0·1 M-tris	2 × PBS, 1 × M-NaCl	$2·3 \times 10^5$
	2 M-$MgSO_4$ + 1 M-NaCl in 0·1 M-tris	2 × PBS, 1 × M-NaCl	$2·8 \times 10^5$
	2 M-$MgSO_4$ in 0·1 M-tris	2 × PBS, 1 × 0·5 M-$MgSO_4$	$5·0 \times 10^3$
	2 M-$MgSO_4$ in 0·1 M-tris	2 × PBS, 1 × M-$MgSO_4$	$2·5 \times 10^4$
	2 M-$MgSO_4$ in 0·1 M-tris	1 × PBS, 1 × M-NaCl, 1 × 1 M-$MgSO_4$	$5·0 \times 10^3$
2	2 M-$MgSO_4$ in 0·1 M-tris	2 × PBS, 1 × M-NaCl	$1·9 \times 10^5$
	2 M-NaCl in 0·04 M-PO_4	2 × PBS, 1 × M-NaCl	$1·5 \times 10^5$
	M-NaCl in 0·04 M-PO_4	2 × PBS, 1 × M-NaCl	$1·9 \times 10^5$
	2 M-sucrose in 0·04 M-PO_4	2 × PBS, 1 × M-NaCl	$6·6 \times 10^4$
3	2 M-sucrose in 0·04 M-PO_4	2 × PBS, 1 × M-NaCl	$8·9 \times 10^3$
	2 M-sucrose in 0·04 M-PO_4	3 × PBS	$6·2 \times 10^2$
	M-NaCl in 0·04 M-PO_4	2 × PBS, 1 × M-NaCl	$1·6 \times 10^4$
	M-NaCl in 0·04 M-PO_4	3 × PBS	$2·0 \times 10^3$
4	2 M-$MgSO_4$ in 0·1 M-tris	2 × PBS, 1 × M-NaCl	$1·0 \times 10^5$
	2 M-$MgSO_4$ in 0·1 M-tris	3 × PBS	$1·8 \times 10^3$
	2 M-$MgSO_4$ in 0·1 M-tris	2 × PBS, 1 × 10 % PBS	$1·0 \times 10^2$
	2 M-$MgSO_4$ in 0·1 M-tris	1 × PBS, 1 × 10 % PBS, 1 × M-NaCl	$5·0 \times 10^4$

* Extracted from FMD virus strain Turkey 43/62 grown in BHK cells.

The initiation of infection by the RNA of FMD virus appears to be rapid (see Bachrach, 1960) and there was little increase in the number of plaques formed after the first few minutes of adsorption. As a routine the time allowed for adsorption was 15–20 min. since this allowed several titrations to be made consecutively. It was also advantageous to wash the monolayers with PBS following adsorption of the RNA since failure to do this often led to tearing of the cell sheet.

Throughout these experiments cell monolayers grown in 60 mm. Petri dishes were used. With plates of this size, inocula of 0·1–0·5 ml. could be conveniently spread over the monolayer during the adsorption of RNA. Changes in volume within this range did not appear to have a significant effect on the RNA titre.

The titration of RNA was also unaffected by changes in the range pH 7·0–7·6, irrespective of the diluent used, but least cell damage was caused between pH 7·4

and 7·6. For this reason all the solutions used for washing monolayers and diluting the RNA were buffered within this pH range.

The method finally adopted for regular use was to treat the cells with hypertonic saline followed by the addition of the RNA in 2 M-$MgSO_4$. The reproducibility of the method and the linearity of response between plaque number and dilution are

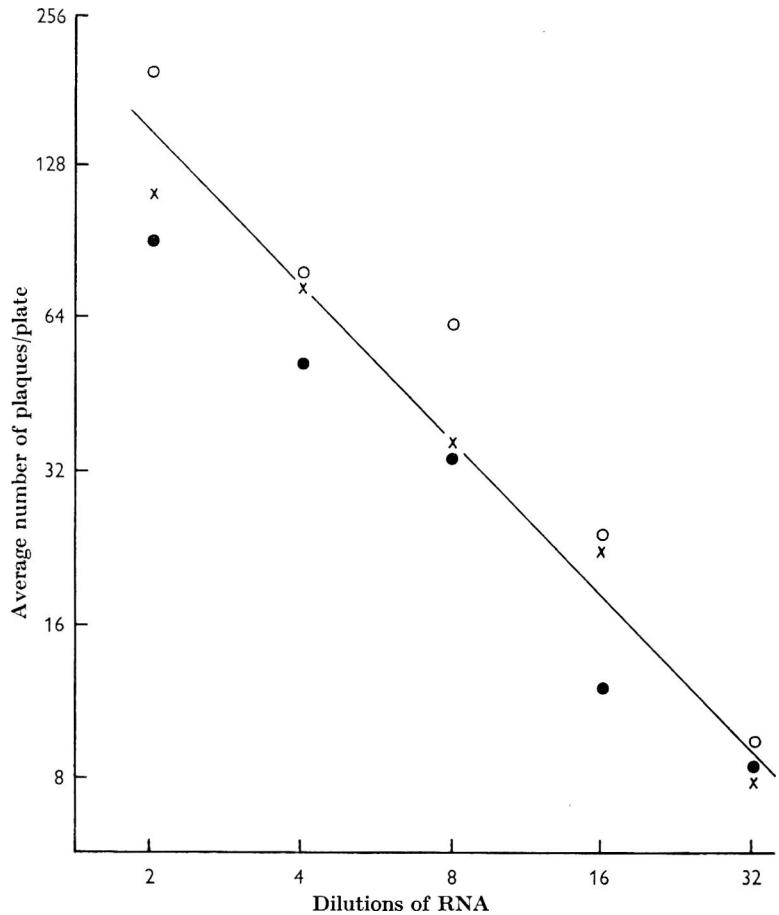

Fig. 1. Dose-response relationship for FMD-virus RNA (strain Turkey 43/62) on BHK 21 monolayers. Points were taken from three separate experiments.

shown in Fig. 1. Similar dose-response curves have been described for other infective RNAs (e.g. poliovirus RNA, Alexander *et al.* 1958; Koch *et al.* 1960; Holland *et al.* 1960; equine encephalitis-virus RNA, Colon & Idoine, 1964) and for FMD-virus RNA, using calf kidney cells (Bachrach, 1960). Outside a 16-fold dilution range and plaque counts 160–10, plaque formation was no longer a linear function of the RNA dilution and at very low dilutions inhibition may occur. This may be due to the aggregation of the RNA in the presence of 2 M-$MgSO_4$, as suggested by Holland *et al.* (1960), or to inhibition by cellular DNA and RNA present in the extract (Franklin, Wecker & Henry, 1959; Holland *et al.* 1960). The first possibility is contra-indicated in our system by the fact that RNA diluted in 2 M-$MgSO_4$ could be held for up to

1 hr at 0° without apparent loss in titre. Inhibition at low dilutions becomes of practical importance only when examining RNA samples of low infectivity (e.g. after prolonged storage) and was much less marked when 2 M-sucrose or M-NaCl was used as the diluent. Consequently, it was better to use one of these reagents for the titration of samples with low infectivity.

The plaque assay of FMD-virus RNA was compared with other methods of titration; typical results are presented in Table 2. The corresponding virus titrations are also given. For the strains used, the titres of RNA, but not always of virus, were equal to or higher than those obtained by *in vivo* methods. Consequently, it is misleading to express the RNA titre as a fraction of that of the intact virus without referring to the assay system used.

Table 2. *Comparison of the plaque method for FMD virus and its RNA with other methods of titration. RNA extractions made with phenol saturated with 0·04 M-PO_4, pH 7·6*

		Assay system		
	BHK monolayers			
Virus	p.f.u./ml.	log ID50/ml.	Mice (log ID50/ml.)	Guinea pigs (log ID50/ml.)
Turkey 43/62 passaged in BHK cells	Virus $2·0 \times 10^9$	9·5	8·8	—
	RNA $2·3 \times 10^5$	4·5	3·3	—
	Virus $4·0 \times 10^9$	—	9·1	—
	RNA $4·4 \times 10^5$	—	3·3	—
Turkey 43/62 passaged in guinea-pig plantar pads extracted with 0·04 M-PO_4	Virus $4·5 \times 10^7$	—	9·5	5·5
	RNA $1·0 \times 10^4$	3·5	4·1	3·5
Strain 1 passaged in BHK cells	Virus $9·4 \times 10^7$	—	8·2	—
	RNA $5·0 \times 10^3$	—	3·5	—

Extraction procedures for RNA

In these experiments the procedure described by Brown & Stewart (1959) was used as the standard method of RNA preparation with which other modifications were compared (Table 3). In contrast to Bachrach (1960), we found that the titres of RNA prepared with water-saturated phenol were somewhat lower than those of RNA prepared with buffered phenol, unless EDTA had been added. Since Bachrach (1960) had also reported that it was better to extract the RNA from solutions of low molarity, the effect of ionic strength on the extraction of FMD-virus RNA was then determined. We found that the titres of the RNA were unaltered by saturation of the phenol with 0·01 M- or 0·04 M-phosphate or by the addition of M-NaCl to the virus suspension before phenol treatment. These results, which were similar to those of Koch *et al.* (1960) with poliovirus RNA, suggested that ionic strength was of little importance during the extraction of FMD-virus RNA. Furthermore, despite the stabilizing effect of M-NaCl, there was no advantage in adding it to the virus suspension before extraction because the same effect could be achieved by the addition of M-NaCl after phenol treatment. Moreover, when the phenol used for the extraction had been previously equilibrated with M-NaCl, difficulty was experienced in keeping the phenol in solution and in separating the two phases.

Table 3. *The infectivity of FMD-virus RNA preparations extracted with phenol saturated with different solutions*

Virus	Additions to virus suspension	Additions to phenol	Mice (log ID50/ml.) Virus	Mice (log ID50/ml.) RNA	BHK monolayers (p.f.u./ml.) Virus	BHK monolayers (p.f.u./ml.) RNA
Guinea-pig pad suspensions in 0·04 M-PO_4	—	0·04 M-PO_4	8·5	4·2	—	—
	—	0·01 M-PO_4		4·2	—	—
	—	H_2O		3·3	—	—
	0·1 % SDS*	H_2O		2·8	—	—
Guinea-pig pad suspension in 0·04 M-PO_4	—	0·04 M-PO_4	8·1	3·0	—	—
	M-NaCl	0·04 M-PO_4		3·0†	—	—
	M-NaCl	0·04 M-PO_4 + M-NaCl		3·2†	—	—
	M-NaCl	H_2O		2·8†	—	—
	M-NaCl	0·01 % EDTA, H_2O		3·8	—	—
	0·1 % SDS, 0·01 % EDTA	0·01 % EDTA, H_2O		3·8	—	—
BHK suspension	—	0·01 M-PO_4	—	—	$1·4 \times 10^8$	$2·7 \times 10^3$‡
	0·01 % EDTA	0·01 M-PO_4		—		$1·2 \times 10^4$‡
	10 % bentonite suspension	0·01 M-PO_4		—		$3·1 \times 10^2$‡
BHK suspension	—	0·01 M-PO_4	—	—	$8·3 \times 10^7$	$4·7 \times 10^3$
	M-NaCl	0·01 M-PO_4 + M-NaCl		—		$4·7 \times 10^3$†
	0·01 % EDTA	0·01 % EDTA, H_2O		—		$7·4 \times 10^5$
BHK suspension	—	0·01 M-PO_4	—	—	$2·7 \times 10^9$	$1·6 \times 10^5$
	—	0·01 % EDTA, H_2O		—		$1·6 \times 10^6$

* Sodium dodecyl sulphate † M-NaCl not added to RNA after extraction. ‡ RNA dilutions in 2 M-sucrose.

Consequently, the overall recovery was decreased although the titre of the RNA was unaltered.

Two other ribonuclease inhibitors, bentonite and SDS, were also used. Bentonite (Fraenkel-Conrat, Singer & Tsugita, 1961), used in the preparation of FMD-virus RNA by Thely *et al.* (1961), was added to FMD virus suspensions before extraction with phenol saturated with phosphate. In every case the RNA titre was decreased. Unlike Bachrach (1960) we were unable to improve the RNA titres by adding sodium dodecyl sulphate to virus before extraction.

Following the results of Bachrach (1960) and Thely *et al.* (1961), the usefulness of EDTA in preparing FMD-virus RNA was examined. By the use of this reagent we were able to improve on Brown & Stewart's (1959) method. We found that, when 0·01 % EDTA in phosphate buffer (pH 7·6) was added to the virus before extraction, the yield of RNA was often about ten-fold higher than that obtained by the standard procedure. It was equally effective to extract the virus with phenol saturated with 0·01 % EDTA but increasing the EDTA concentration to 0·1 % in either the virus suspension or the phenol was of no advantage. The phenol supernatant layer after equilibration with EDTA was between pH 4·8 and 5·0 but the RNA titres obtained were no higher when the phenol was also buffered in the usual way. The RNA extracts were in the range pH 7·0–7·4 after ether treatment, in contrast to the values obtained when buffered phenol was used ($\geqslant$ 8·0).

The technique finally adopted was to add EDTA to the virus suspension to a final concentration of 0·01 % and to extract with phenol saturated with water containing the same concentration of the reagent. Even this method has at best produced only a ten-fold increase in RNA titre as compared with the standard procedure. This increased titre was presumably due to the stabilizing effect of EDTA on viral RNA first described by Fraenkel-Conrat (1957) for tobacco mosaic virus RNA and subsequently demonstrated for the RNAs of animal viruses by many authors (e.g. Ellem & Colter, 1961). Its usefulness for the storage of RNA is discussed in the next section.

Stability of infective RNA

Previous workers found that FMD-virus RNA is rapidly inactivated (e.g. Brown & Stewart, 1959; Bachrach, 1960; Hantschel, 1963). The experiments described below were designed to find convenient methods for retaining infectivity during laboratory manipulations and prolonged storage. We found that even in the presence of M-NaCl or 0·01 % EDTA, FMD-virus RNA lost 99 % of its infectivity in 24 hr at laboratory temperatures. It seemed essential to store the RNA at lower temperatures and we investigated its stability at 4° and −20°. In some instances ribonuclease inhibitors (bentonite or M-NaCl) were used to minimize the effect of traces of the enzyme which are known to survive the phenol extraction. Storage in the presence of EDTA was also examined. All titrations were made by the plaque method.

At 4° FMD-virus RNA could be stored successfully for several days provided that certain precautions were taken. The infectivity was lost within 3–4 days in the absence of EDTA, bentonite or M-NaCl but when any of these reagents was added the process was somewhat slower, about 20 % of the initial activity remaining after 7 days (Fig. 2). For periods up to 3–4 hr M-NaCl was useful to add to RNA extracts which did not contain EDTA. The most stable RNA preparations prepared in the

presence of EDTA retained about 50 % of the original activity after 7 days at 4° and some activity was still detectable after 7 weeks. FMD-virus RNA prepared in the presence of EDTA was also the most stable at −20°, but we were rarely able to achieve recoveries greater than 20 % of the initial infectivity after 2 days and 10 %

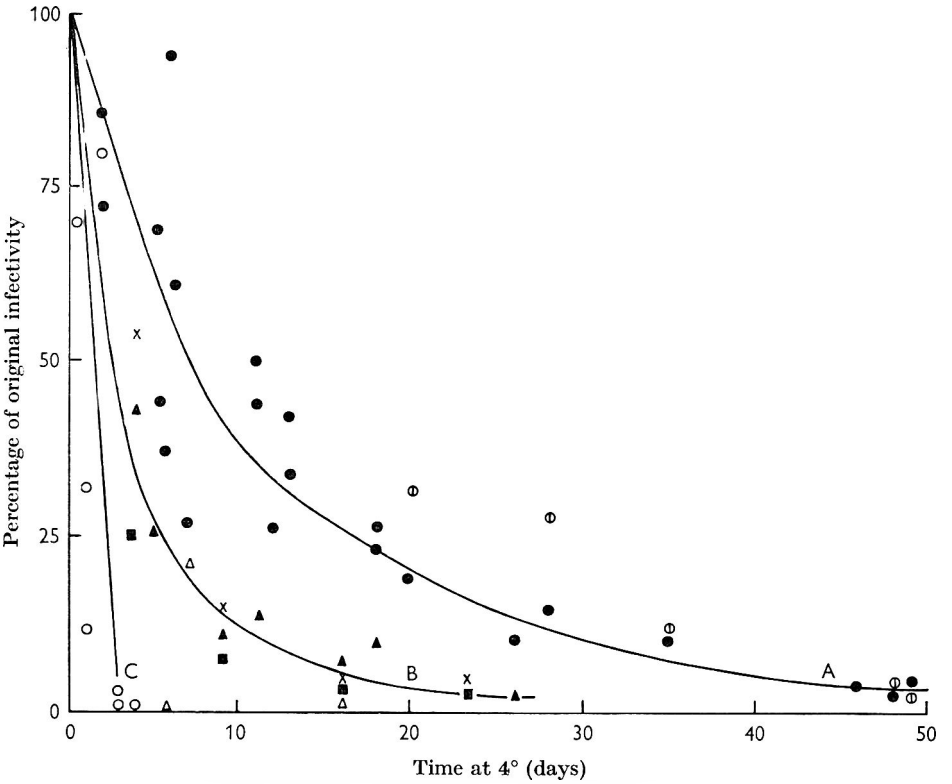

Fig. 2. Storage of FMD-virus RNA (strain Turkey 43/62) at 4°. Results are taken from eight experiments with virus grown in BHK 21 cells. A, the decline of infectivity of RNA prepared in the presence of EDTA: (●) with and (ⓘ) without M-NaCl added after extraction. B, the more rapid fall of infectivity of RNA prepared in the absence of EDTA but to which EDTA (△), M-NaCl (▲), M-NaCl+EDTA (×), or bentonite (■) were added before storage. C, the rapid decline of infectivity in RNA preparations which contained neither EDTA nor ribonuclease inhibitors ○.

after 5 days at this temperature, although the residual infectivity did not decline significantly over a further 7 days (Fig. 3). Addition of M-NaCl to preparations containing EDTA did not increase their stability at 4° or −20°.

The most effective method for preserving the infectivity was to add 2 vol. of ethanol and store at −20°. With this method all the initial infectivity could be recovered up to 6 weeks later. For the prolonged storage of FMD-virus RNA this appears to be the method of choice, although storage at 4° in the presence of EDTA is adequate for shorter periods.

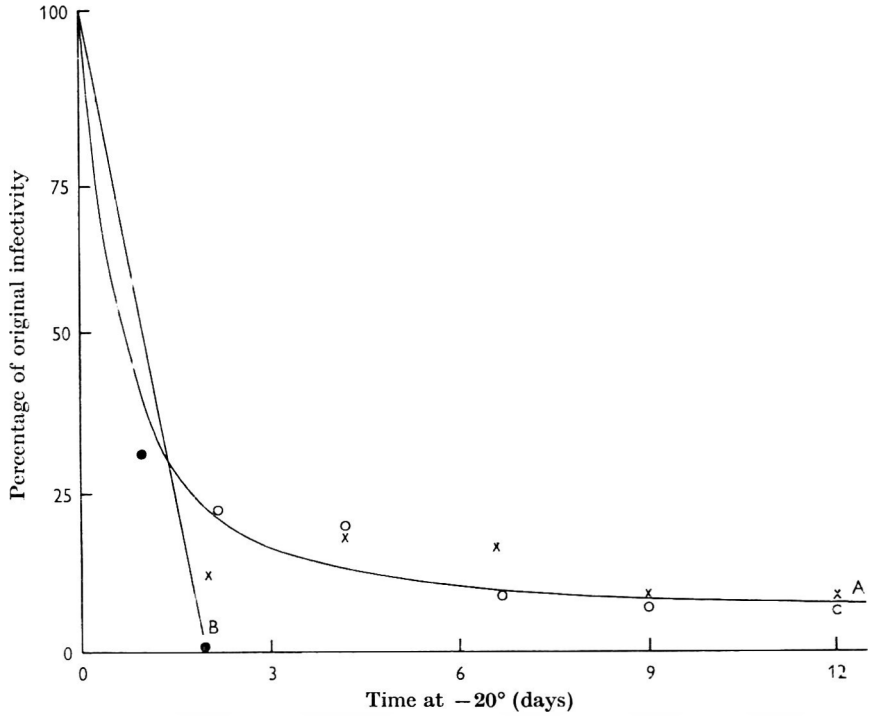

Fig. 3. Storage of FMD-virus RNA (strain Turkey 43/62) at −20°. Results are taken from two experiments with virus grown in BHK 21 cells. A, the decline of infectivity of RNA prepared in the presence of EDTA: (×), with and (○), without M-NaCl added after extraction. B, the more rapid fall of infectivity of RNA prepared and stored in the absence of EDTA and M-NaCl (●).

DISCUSSION

Although the RNA can be extracted from many viruses in almost quantitative yield, most workers agree that the infectivity of the extracts is usually only 0·1% or less of the infectivity of the virus suspensions from which they are prepared. Our best FMD-virus RNA titres were of this order, although the virus:RNA ratios are affected by the relative sensitivity of the assay system used for the virus and RNA respectively (Table 2). It appears therefore, either that the infective property of the FMD-virus RNA molecule is very easily damaged or that the infection of the cells is a much less efficient process than with the intact virus. With ^{32}P-labelled FMD virus Brown & Cartwright (1963) found that about 90% of the RNA is extracted from the virus by phenol and examination of this RNA by sucrose-density centrifugation showed it to be homogeneous, suggesting that all the molecules are potentially infective. The low infectivity may therefore be due to inefficiency in the process of infecting the cells. In our experiments with BHK monolayers less than 10% of the original activity was detected in the inoculum after 15–20 min. adsorption. This result might have been due to inactivation and not to adsorption of the RNA. It is difficult to exclude all traces of ribonuclease, and Norman & Veomett (1961) showed that inhibitors of ribonuclease, such as M-NaCl, do not completely inhibit the action of the enzyme at the cell surface. Holland *et al.* (1960)

and Sprunt *et al.* (1961) considered that the hypertonic solution used in the assay of poliovirus RNA played an active part in facilitating infection of cells by the RNA and did not act merely as a ribonuclease inhibitor. However, we have found that, although hypertonic conditions are necessary for the regular production of plaques by FMD-virus RNA in BHK cells, high salt concentrations were not essential for the initiation of infection. Cytopathic effects can be produced by the RNA in both BHK and pig kidney cells by using isotonic solutions and the titres given by this method in BHK cells are of the same order as those obtained by plaque assay. Furthermore, in contrast to Thomas & Leclerc (1960), we were unable in these experiments to improve titres of RNA in guinea-pig pads by using M-NaCl as the diluent instead of phosphate buffered saline. Therefore, the infection of cells by FMD-virus RNA, as opposed to the production of plaques, must be as efficient in isotonic as in hypertonic solutions. These results make the role of hypertonic solutions in the plaque assay of FMD-virus RNA even more difficult to interpret, but it seems probable that the low titres of the RNA were due to its inefficiency as an infecting agent rather than to its inactivation by ribonuclease at the cell surface.

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The Influence of Metal-complexing Agents on Citric Acid Production by *Aspergillus niger*

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SUMMARY

Addition of any of the chelating agents ethylenediaminetetra-acetic acid, diamino-cyclohexane-*N*,*N*-tetra-acetic acid or diethylenetriamine-penta-acetic acid at about 1·0 mM to a sugar + NH_4NO_3 + salts medium stimulated citric acid production by *Aspergillus niger*. The greatest stimulation (about tenfold) was obtained with EDTA (1·0 mM). In contrast, potassium ferrocyanide at 84 μM stimulated citric acid production about 20-fold. Ferrocyanide was effective only when present at the beginning of the mycelial growth period, whereas the chelating agents were effective at any time during the growth period. Stimulation of citric acid production by ferrocyanide could be repressed by transferring the mycelium to fresh medium without ferrocyanide, or by removal of the ferrocyanide precipitate in the medium. Analyses of the iron, copper and zinc contents of the mould mycelium are given. Ferrocyanide did not affect the copper and zinc contents of the mycelium: EDTA did not affect the iron, copper and zinc contents of the mycelium.

INTRODUCTION

Aspergillus niger produces large amounts of citric acid from sugars under certain conditions and this organism is used for the industrial production of citric acid. Crucial conditions in the fermentation are: the strain of the organism, the metal ion nutrition, and the physical environment of the mould. Perlman & Sih (1960) reviewed work on this process. The cultivation process is empirical and its mechanism not understood. One of the unexplained effects is the increase in citric acid yield caused by addition of a small amount of ferrocyanide to the medium. We chose the citric acid fermentation for a study of the effects of metal-chelating agents (as metal buffers) in culture media and have observed that certain chelating agents, like ferrocyanide, increase the yield of citric acid. The present paper reports these effects and discusses their significance. In a previous paper (Choudhary & Pirt, 1965*a*) the effects of metal buffers on the growth of *Aspergillus niger* were discussed.

METHODS

General. Details of media and cultural methods were described previously (Choudhary & Pirt, 1965*a*). The general procedure for the production of citric acid with *Aspergillus niger* was as follows: 25 ml. of medium M2 (carbon source, glucose at 150 g./l.) was placed in a 250 ml. conical flask, inoculated with conidia and incubated at 25° for 7 days on a rotary shaker. Potassium ferrocyanide, when

required, was added 24 hr after inoculation unless otherwise stated; other chelating agents were added before inoculation unless otherwise stated. In addition to the two strains of *A. niger*, Wis 72-4 and IMI-31821 (i), previously described (Choudhary & Pirt, 1965*a*) we also used the strain N-548 of Noguchi & Johnson (1961), supplied by Professor M. J. Johnson (Biochemistry Dept., University of Wisconsin). Media were sterilized by autoclaving; potassium ferrocyanide solution was sterilized by membrane filtration. The medium was initially at pH 3·5 and finally at about pH 2.

Wet ashing for trace metal analysis. Trace metals in mycelium and culture filtrate were determined after wet ashing. Cultures were filtered through filter paper in Buchner funnels. After thorough washing with de-ionized water the mycelium was transferred to a watch-glass using a glass rod, and dried overnight at 105°. Mycelium (0·8–1·0 g.) was placed in a 300 ml. Kjeldahl flask, 5 ml. HNO_3 and 1 ml. H_2SO_4 added and the flask heated gently so that the solution just started to boil. A further addition of 5 ml. HNO_3 was made as the solution began to char and when the oxidation had practically ceased, 1 ml. perchloric acid was added and the flask heated slightly more vigorously until white fumes began to form. Heating was continued until the solution was colourless. Samples of culture medium (10–15 ml.) were transferred to 300 ml. Kjeldahl flasks and evaporated to dryness. Wet ashing was then done by the procedure described above.

All the vessels and apparatus brought in contact with trace metal solutions for analysis were cleaned by standing for 2–3 hr in a boiling sulphuric acid + sodium nitrate mixture. The glass apparatus was then washed three or four times with glass-distilled water.

Estimations of Fe^{3+}, Zn^{2+}, Cu^{2+} *and* Mn^{2+}. Iron was determined by means of the α,α'-dipyridyl method of Jackson (1938). The digest was diluted with water to about 10 ml. and neutralized by dropwise addition of approximately 8 ml. concn. ammonium hydroxide (bromcphenol blue as internal indicator). The solution was then diluted to 100 ml. and a sample taken for the analysis.

To determine copper the Zincon method of McCall, Davis & Stearns (1958) was first tried but the reported colour was not developed. Instead, the diethyldithiocarbonate method of Eden & Green (1940) as modified by Arman (1957) was used. A sample of the neutralized digest was used for the analysis.

Zinc was determined by the dithizone 'mixed colour' method described by Sandell (1944). A sample of the neutralized digest was used for the analysis.

Manganese was estimated by the permanganate method of Sandell (1944), the un-neutralized digest of the mycelium or culture filtrate being used for analysis.

Other analyses. The CO_2 and O_2 in the effluent gas from the continuous-flow culture were analysed by the methods described by Pirt & Callow (1958). Samples of the culture filtrate were used for assaying citric acid and reducing sugar content. Citric acid was estimated colorimetrically by the method of Marier & Boulet (1958). The sample range was 20–200 μg. citric acid/ml.; the estimation was unaffected by glucose up to 2·0 mg./ml.

Reducing sugars (glucose and hydrolysed sucrose) were estimated by a modification of the ferricyanide method of Fujita & Iwatake (1931). The estimation was unaffected by the citric acid present in the sample. Sucrose was hydrolysed by the method of Pirt & Callow (1958).

Yield of citric acid (%) is expressed as: (g. citric acid produced/g. sugar utilized) × 100.

Chelating agents were obtained from Hopkin and Williams Ltd., Chadwell Heath Essex, England.

RESULTS

Action of chelating agents on citric acid production

Effect of concentration of chelating agent. The effect of chelating agents on citric acid production by *Aspergillus niger* was examined by growing the mould in medium M2 in shake-flasks with the addition of one of the following chelating agents: ethylenediaminetetra-acetic acid (EDTA), 1,2-diaminocyclohexane-*N*,*N*-tetra-acetic acid (CTDA), diethylenetriaminepenta-acetic acid (DTPA), nitrilotriacetic

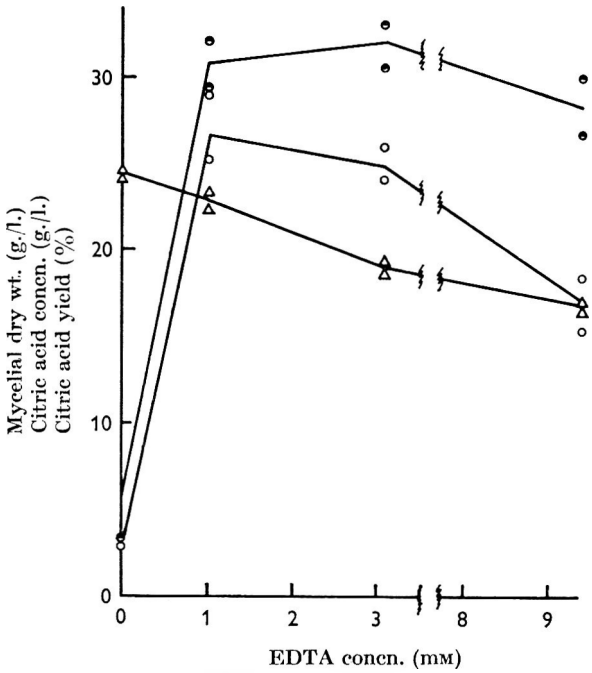

Fig. 1

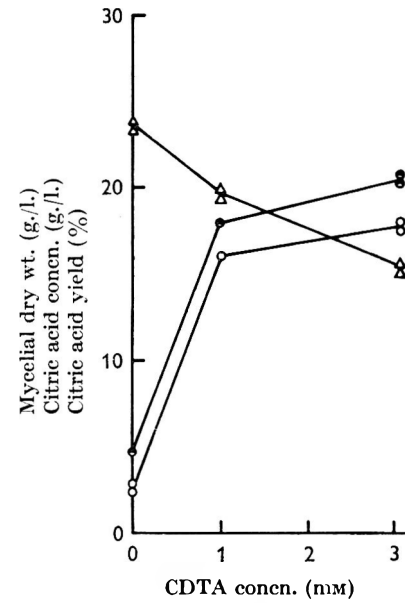

Fig. 2

Fig. 1. The effect of EDTA (0–9·4 mM; added at the time of inoculation) on citric acid production from glucose after 7 days by strain 72-4. Citric acid concn. ○; % citric acid yield, ◓; mycelial dry-wt. concn., △.

Fig. 2. The effect of CDTA (0–3·13 mM; added at the time of inoculation) on citric acid production by strain 72-4. Citric acid concn., ○; % citric acid yield, ◓; mycelial dry-wt. concn., △.

acid (NTA) and dihydroxyethylglycine (DHEG). The effects of EDTA, CDTA and DTPA at different concentrations are shown in Figs. 1, 2 and 3. Each of these chelating agents stimulated citric acid production, the maximum stimulation being reached with the chelating agent in the range 1–3 mM. Similar results were obtained with strain N-548. The results with strain IMI-31821(i) were qualitatively similar but the concentration and percentage yield of citric acid were only about one-third

of those obtained with the other two strains. The agents NTA and DHEG at concentrations up to 9·4 mM had no effect on citric acid production.

Effect of time of addition of chelating agent. The effect of adding EDTA or DTPA to shake-flask cultures at different times after inoculation with conidia was investigated. The effect of time of addition of 1·04 mM-EDTA with strain 72-4 is illustrated in Fig. 4. The results show that the chelating agents stimulated the production of citric acid when added at any time up to 72 hr after inoculation but maximum stimulation occurred with the addition between 0 and 24 hr. In a control medium (without added chelating agent) strain 72-4 produced 3·0 ± 0·5 g./l. of citric acid after 7 days with a 3% yield and the mycelial dry wt. was 22·0 ± 1·5 g./l.

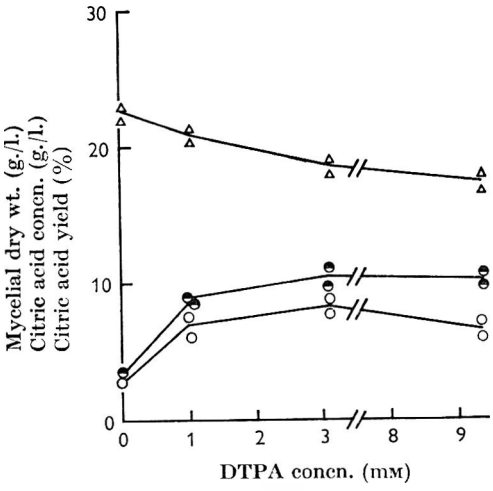

Fig. 3

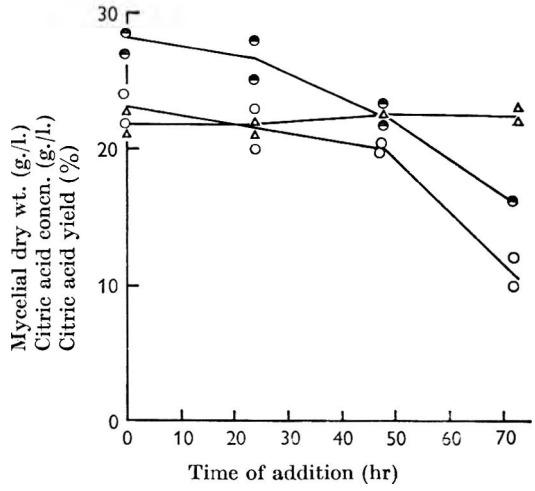

Fig. 4

Fig. 3. The effect of DTPA (0–9·4 mM; added at the time of inoculation) on citric acid production by strain 72-4. Citric acid concn., ○; % citric acid yield, ◓; mycelial dry-wt. concn., △.

Fig. 4. The effect of EDTA (1·04 mM), added at various times after inoculation, on citric acid production by strain 72-4. Citric acid concn., ○; % citric acid yield, ◓; mycelial dry-wt. concn., △.

Effect of increasing both ferric ion and EDTA concentrations. An experiment was made to see whether the stimulation of citric acid production was controlled by the Fe^{3+}:EDTA ratio. In a preliminary experiment it was found that maximum citric acid production occurred when the Fe^{3+}:EDTA molar ratio was about 1:30. In a second experiment the Fe^{3+} content of the medium was increased and the EDTA concn. was also increased to keep the Fe^{3+}:EDTA ratio constant at either of 3 values; 1:12·5, 1:25 or 1:37·5. The results are shown in Table 1. The iron present as a contaminant in the medium constituents was determined colorimetrically and allowed for in calculating the Fe^{3+}:EDTA ratio. The results show that the mycelial dry wt. was unaffected. At each Fe^{3+}:EDTA ratio, an increase in the Fe^{3+} concn. decreased the citric acid production. Therefore the Fe^{3+}:EDTA ratio alone did not control the citric acid production; some other factor must also have been involved. However, with the lowest Fe^{3+}:EDTA ratio there was some suppression of the inhibition caused by increased Fe^{3+} concn.

Table 1. *Effect of varying Fe^{3+} concentration and Fe^{3+}:EDTA ratio on citric acid production by strain 72-4*

	Fe^{3+}:EDTA molar ratio								
	1:12·5			1:25			1:37·5		
Fe^{3+} concn. (μM)*	Mycelial dry wt. (g./l.)	Citric acid (g./l.)	% citric acid yield	Mycelial dry wt. (g./l.)	Citric acid (g./l.)	% citric acid yield	Mycelial dry wt. (g./l.)	Citric acid (g./l.)	% citric acid yield
42	17·2	15·2	15·2	17·5	25·0	26·0	16·5	23·0	23·0
96	18·0	5·5	5·5	15·0	7·0	8·1	18·3	9·0	11·3
266	17·0	1·1	2·3	17·0	1·0	1·1	18·0	4·5	5·8
518	16·5	3·5	4·0	18·3	2·5	2·5	18·6	6·5	11·5

* Added iron + iron contamination (14 μM) in medium constituents.

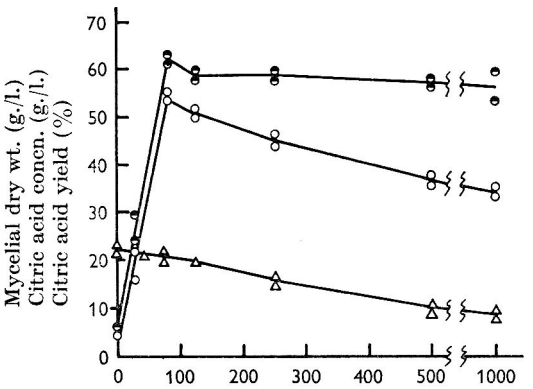

Fig. 5

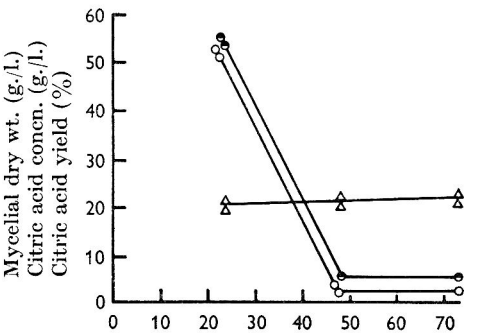

Fig. 6

Fig. 5. The effect of ferrocyanide concn. on the synthesis of citric acid from glucose by strain 72-4. The ferrocyanide was added 24 hr after inoculation with conidia and the culture duration was 7 days. Citric acid concn., ○; % citric acid yield, ◓; mycelial dry-wt. concn., △.

Fig. 6. The effect of time of addition of ferrocyanide (84 μM) on the production of citric acid from glucose by strain 72-4. Citric acid concn., ○; % citric acid yield, ◓; mycelial dry-wt. concn., △.

Action of ferrocyanide on citric acid synthesis

Effect of ferrocyanide concentration. Potassium ferrocyanide was added to the media in shake-flask experiments to characterize the ferrocyanide action and to compare it with that of the chelating agents. The influence of ferrocyanide concn. is shown in Fig. 5. The maximum concentration and percentage yield of citric acid were reached with 84 μM ferrocyanide. The decrease in citric acid production at higher ferrocyanide concentration may be attributed to the reduced amount of mycelium. Strain N-548 showed a similar response to ferrocyanide but produced less mycelium and consequently gave a lower citric acid yield (44 g./l.). When sucrose was used as the carbon source, even larger amounts of citric acid (80 g./l.) were produced by strain 72-4 in 7 days with a 65% yield. Apparently sucrose was converted more quickly than glucose into citric acid.

Effect of time of addition of ferrocyanide. The effect of adding the optimum amount

of ferrocyanide (84 μM) to the culture at different times is shown in Fig. 6. At 84 μM and pH 3·5, ferrocyanide inhibited the germination of conidia and hence could not be added initially. The most notable point is that the ferrocyanide unlike the chelating agents was without effect when added at 48 hr or later. Moreover, the mould grew in the small pellet form (Choudhary & Pirt, 1965*a*) only when the ferrocyanide was added at 24 hr, i.e. at the beginning of the mycelial growth period. It may be postulated that growth of the mould in the presence of ferrocyanide

Table 2. *Citric acid production by strain 72-4 in nitrogen-free medium M 2 with and without ferrocyanide*

Nitrogen source	Ferrocyanide concn. (μM)	Inoculum	Time of sample (days)	Mean mycelial dry wt. (g./l.)	Mean citric acid amount (g./l.)	% yield	Replicates
Absent	0	48-hr mycelium	3	9·5	3·3	7·0	2
			5	12·5	3·8	11·5	
			7	10·0	4·0	12·0	
Absent	84	48-hr mycelium	3	9·5	5·0	16·0	3
			5	9·2	8·0	21·0	
			7	9·6	8·5	20·0	
Absent	0	72-hr mycelium	2	9·5	5·0	16·0	3
			4	13·5	7·5	15·0	
			6	13·6	8·0	16·0	
Absent	84	72-hr mycelium	2	10·0	10·0	30·0	3
			4	12·0	14·0	30·0	
			6	14·0	15·0	29·0	
Present	0	Conidia	5	17·0	2·5	4·3	2
			7	20·8	3·0	3·3	
Present	84	Conidia	5	—	27·5	32·0	2
			7	20·0	60·0	50·0	

induces a mechanism for acid accumulation which is unaffected by subsequent removal of ferrocyanide. This hypothesis was tested in experiments with washed mycelium grown in the presence of ferrocyanide. Mould mycelium was grown in the presence of 84 μM-ferrocyanide for 48 and 72 hr; the mycelium was then filtered off in a chamber of sterile air (Harris-Smith, Pirt & Firman, 1963) and thoroughly washed with sterile distilled water. The washed mycelium was then aseptically transferred to 30 ml. of medium M2, with or without ferrocyanide (84 μM), and without NH_4NO_3 to prevent further growth. The flasks were then incubated on the shaker. As controls, unfiltered cultures were run in parallel, with and without ferrocyanide (84 μM). At intervals 4 ml. samples of culture were taken by pipette and analysed for citric acid, glucose and mycelial dry wt. The results (Table 2) show that for maximum citric acid production the nitrogen-free replacement medium needed to have ferrocyanide added. The omission of ferrocyanide from the replacement medium decreased the production of citric acid by about 50%. Some of the citric acid producing ability induced by the preliminary growth in ferrocyanide was retained. This result is interpreted to mean that the medium untreated with ferrocyanide contains some material which can reverse the change which ferrocyanide induces in the mycelium.

Influence of ferrocyanide precipitate on citric acid production. One hypothesis to

account for the role of ferrocyanide is that it makes the medium suitable for citric acid production by precipitating iron or other cations, thus causing the mycelium to be deficient in iron. It may therefore be expected that if ferrocyanide is added to the medium and the precipitate removed, this treated medium should give maximum citric acid production when inoculated. This hypothesis was tested in the following way. Conidia were germinated in medium M2 and the resultant mycelium after incubation for 26 hr was thoroughly washed with de-ionized water in sintered-glass crucibles placed in the cabinet with a sterile atmosphere. Part of the washed mycelium was used to inoculate medium M2 to which 84 μM-ferrocyanide had been added 24 hr previously. Another part of the washed mycelium was used to inoculate medium M2 after removing the ferrocyanide precipitate by membrane filtration 24 hr after the addition of ferrocyanide at 84 μM. The mould in the replacement

Table 3. *Effect of removal of ferrocyanide precipitate from medium M2 on the production of citric acid by strain 72-4*

Ferrocyanide concn. (μM)	Inoculum	Medium treatment	Time of sample (days)	Mean dry wt. (g./l.)	Citric acid: Mean concn. (g./l.)	Citric acid: % yield	Replicates
0	Conidia	None	7	22·5	3·0	3·0	2
84	Conidia	None	7	17·5	58·0	50·0	2
0	Washed mycelium 26 hr	None	6	23·0	2·0	2·0	2
84	Washed mycelium 26 hr	Ferrocyanide added 24 hr before inoculation	6	20·0	23·0	22·0	4
84	Washed mycelium 26 hr	Ferrocyanide added, ppt. removed after 24 hr and then inoculated	6	20·0	2·2	2·0	4

medium with ferrocyanide precipitate developed in the form of large filamentous but separate pellets. In the same medium with ferrocyanide precipitate removed the mould developed as a viscous conglomerate of large filamentous pellets. The effects on citric acid production and mycelial dry weight are shown in Table 3. The removal of the ferrocyanide precipitate from the replacement medium prevented stimulation of citric acid production. Some decrease of stimulation of citric acid formation resulted just from the transfer of the mould to the replacement medium. The important point established is that the presence of the ferrocyanide precipitate in the medium was essential for the stimulation of citric acid production. The function of the ferrocyanide precipitate may be to maintain a low concentration of Fe^{3+} or other metal ions in the medium.

Effects of metal-complexing agents on the trace-metal uptake by the mould

Experiments were made to determine whether or not there was a relation between the uptake of trace metals by the mycelium and citric acid production. Iron, copper and zinc in the mycelium and medium were estimated. The concentration of man-

ganese in the mycelium (1–2 g.) and in the filtrate (10–15 ml.) was below the amount (1 μg.) which could be estimated. Iron was not estimated in the cultures containing ferrocyanide.

Trace-metal analyses were done on mycelium and culture filtrates from cultures grown with and without EDTA, and with and without ferrocyanide. Citric acid production and the mycelial dry weights were determined, to correlate them with the trace-metal analyses. The results are given in Table 4. The excess of trace metal over that added represents the amount of metal contamination in the medium. The results show that EDTA did not significantly affect the uptake of Fe^{3+}, Cu^{2+} and Zn^{2+} by the mycelium. Also the ferrocyanide did not affect the Cu^{2+} and Zn^{2+} uptakes by the mycelium. Thus no relation was found between the trace-metal uptake by the mycelium and the citric acid production.

Table 4. *Trace-metal analyses of mycelium and medium from 7-day cultures of strain 72-4 in medium M 2 with and without metal-complexing agents**

Chelating agent (concn.)	Mycelial dry wt. (g./l.)	Citric acid		Repli-cates	Trace metals present†					
		Concn. (g./l.)	% yield		Mycelium (μg./g. dry wt.)			Culture filtrate (μg./l.)		
					Fe^{3+}	Cu^{2+}	Zn^{2+}	Fe^{3+}	Cu^{2+}	Zn^{2+}
None	22·4	3·0	3·0	5	46	5	19	1100	161	297
EDTA (1·04 mM)	20·8	25·0	26·0	6	48	6	21	1084	139	320
None	22·5	3·0	3·0	5	46	5	22	1100	161	350
Ferrocyanide (84 μM)	20·0	59·0	51·0	4	NA‡	5	21	NA‡	171	280

* When present, EDTA and ferrocyanide were added before or 24 hr after inoculation respectively.

† In each case the initial concentration of trace metals in the medium plus inoculum of conidia was (μg./l.): Fe^{3+}, 2120; Cu^{2+}, 242; Zn^{2+}, 676.

‡ No analysis.

The effect on citric acid production of omitting single trace metals from the medium, both with and without ferrocyanide, was examined (Table 5). Omission of iron did not affect the mycelial dry wt. but it caused the iron content of the mycelium to be markedly decreased. In the absence of added iron, ferrocyanide only slightly stimulated citric acid production. Also in the absence of added iron, ferrocyanide did not modify the mould morphology to the small discrete pellet form; the growth remained as in the control. It may be concluded that the minimum iron supply for maximum citric acid production is greater than the minimum iron supply for mycelium production. Copper was taken up by the mycelium to a much smaller extent than iron or zinc. The copper present as contaminant in the medium (182 μg./l.) was well above the maximum amount taken up by the mould, hence it is not surprising that omission of copper had no effect. Omission of zinc caused limitation of the mould growth by the zinc supply and greatly decreased the zinc content of the mycelium. Ferrocyanide did not stimulate citric acid production in zinc-starved mycelium. The morphology of the mycelium was in the form of large fimbriated pellets without zinc and ferrocyanide added. The presence of ferro-

Table 5. *Effect of omitting single trace metals from medium M 2 on citric acid production by 7-day cultures of strain 72-4 both with and without ferrocyanide*

Ferrocyanide concn. (μM)	Metal omitted	Mean mycelial dry wt. (g./l.)	Replicates	Citric acid		Trace metals present								
				Mean concn. (g./l.)	% yield	Medium with conidia inoculum (μg./l.)			Mycelium (μg./g. dry wt.)			Culture filtrate (μg./l.)		
						Fe^{3+}	Cu^{2+}	Zn^{2+}	Fe^{3+}	Cu^{2+}	Zn^{2+}	Fe^{3+}	Cu^{2+}	Zn^{2+}
0	None	20·0	4	3·0	3·0	1810	280	610	42	5	25	1000	220	165
84	None	19·0	4	60·0	56·0	NA	NA	NA	NA	7	23	NA	195	180
0	Fe^{3+}	21·6	4	2·5	3·0	428	229	610	14	8	27	196	170	NA
84	Fe^{3+}	21·0	4	6·0	7·0	NA	NA	NA	NA	6	28	NA	203	185
0	Cu^{2+}	22·5	2	2·0	2·0	1920	182	608	47	4	20	1100	91	213
84	Cu^{2+}	23·0	2	64·0	50·0	NA	NA	NA	NA	5	26	NA	98	200
0	Zn^{2+}	15·0	4	2·0	2·1	1971	250	270	51	4	14	1000	NA	53
84	Zn^{2+}	10·0	4	2·0	4·0	NA	NA	NA	NA	3	12	NA	NA	NA

NA = no analysis.

cyanide without added zinc produced the typical small discrete pellets usually obtained with added ferrocyanide. This result shows that growth in the small pellet form was not by itself a sufficient condition for maximum citric acid production.

DISCUSSION

The mechanism whereby *Aspergillus niger* produces citric acid in large amount under certain conditions is a problem of great interest. Citric acid is presumably a normal intermediate in the terminal respiratory metabolism of the mould and its massive excretion is apparently caused by a derangement of this metabolism. The conditions which favour the production of citric acid are: (1) special strains of the mould, (2) high oxygen supply, (3) a low pH value, about 2, (4) a special trace-metal balance. The latter condition is deduced largely by a consensus of opinion rather than from precise knowledge of the trace-metal balance. The stimulation of citric acid production by ferrocyanide may be interpreted as due to a favourable modification of the trace-metal balance. The stimulation by metal-chelating agents such as EDTA first reported by Choudhary & Pirt (1965*b*) similarly seems to be due to modification of the trace-metal balance. The observation that the Fe^{3+}:EDTA ratio should be kept low and that ferrocyanide does not stimulate unless iron is present in excess of that required for growth suggests that there should be an unlimited supply of iron but supplied at a critical concentration which is controlled by ferrocyanide or chelating agent. The stimulations by ferrocyanide and chelating agents differ markedly in the effect of the time of addition. Chelating agents can stimulate when added to the culture at any time up to 72 hr from time of inoculation or possibly later; on the other hand ferrocyanide stimulated only when added before 48 hr, that is, early in the growth period. This limitation of the ferrocyanide effect is evidence against the suggestion put forward by Martin (1955) that ferrocyanide has a direct action on the mould in stimulating citric acid production. The effect of the chelating agent at a later period, when most of the mycelium is formed, may be attributed to permeation of the lipid cell membrane by the undissociated acid and the binding of trace metals which have been taken up by the cell. It is assumed that ferrocyanide cannot do this. The lack of stimulation of citric acid production by the chelating agents NTA and DHEG may be attributed to the same properties which, as Choudhary & Pirt (1965*a*) suggested, prevent their having any action on mould morphology and on conidial germination.

Under the optimum conditions for citric acid production the mould always grew in the form of small discrete pellets like a suspension of sand particles. Perhaps this type of pellet formation should be included as the fifth special condition for citric acid production; it is, however, difficult to believe that this is anything other than a correlated effect caused by the same conditions which stimulate citric acid production.

The trace-metal analyses of mycelia indicate that the agents which stimulate citric acid production do not affect the total uptake of Fe^{3+}, Cu^{2+} and Zn^{2+} by the mould. The analytical method for Mn^{2+} was not sensitive enough to detect this element in the medium or mycelium. It would be interesting to do so in view of the observation of Noguchi & Johnson (1961) that Mn^{2+} may strongly inhibit citric acid production by *Aspergillus niger*; also the observation of Clark, Ito & Tymchuk

(1965) that ferrocyanide is particularly effective in removing Fe^{3+} and Mn^{2+} from the medium.

We also grew the mould in a stirred fermenter and in continuous-flow culture, but in these forms of culture citric acid was not produced in large amount and its production was not stimulated by ferrocyanide or chelating agents. In the stirred fermenter the mould grew in the viscous filamentous form. It thus appears that some other controlling factor remains to be discovered, or the agitation has to be of a type which permits formation of the right type of pellet morphology.

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Neurotoxin Release from *Shigella dysenteriae* by Phage Infection

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SUMMARY

Shigella dysenteriae organisms of a very young culture released neurotoxin into the medium within 1 hr after it was infected with T-phages. Two types of phage-associated enzymes were considered to be involved in this process. The first type was phage-bound enzyme, which was bound firmly with phage, worked together with phage, and depolymerized neurotoxin into smaller molecules. The second type enzyme was phage-induced lysin, so-called 'endolysin', which was separable from phage and had lytic activity on dead bacteria. The latter enzyme, however, had no direct effect on the amount of neurotoxin released from acetone-powder preparations of the bacteria into phosphate buffer, although endolysin lysed 25 % of the bacteria at 30° in 30 min. Young living *S. dysenteriae* organisms did not release neurotoxin into the medium under normal conditions, whereas the acetone-powder preparations of *S. dysenteriae*, i.e. dead organisms, released neurotoxin into the medium freely under the same conditions.

INTRODUCTION

Rough strains of *Shigella dysenteriae* produce an exotoxin which is a neurotoxic protein, whereas smooth strains of the organisms produce both a neurotoxin and an endotoxin which is closely associated or identical with somatic O polysaccharide antigen (Boivin, 1940). The existence of these two toxins has been the subject of controversy for a considerable time, but abundant experimental evidence leaves little doubt that both toxins exist (Engley, 1952). Many methods for production of Shiga neurotoxin have been described (Dubos & Geiger, 1946). Van Heyningen & Gladstone (1953) reported the production, purification and properties of a neurotoxin of *S. shigae*. Šourek & Raška (1963) purified Shiga exotoxin by preparative electrophoresis and gel filtration on Sephadex.

Li, Barksdale & Garmise (1961) reported that the complex of phage and *Shigella dysenteriae* released various biologically active macromolecules not ordinarily detected as extracellular products. The present paper explores the possible role of phage and phage-induced lysin in the release of neurotoxin by *S. dysenteriae*.

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METHODS

Organisms. Shigella dysenteriae wild type (strain 136-T) was isolated by Dr W. L. Barksdale from a case of Shiga dysentery in Tokyo in 1947. Strain 136-T forms smooth translucent colonies on Neopeptone agar, and is resistant to phages T_3, T_4, T_5 and T_7. From strain 136-T several rough mutants have been derived; among these are mutants 136-O and 136-R_4. These rough strains are capable of adsorbing and supporting the growth of more phages of the T series than was the wild type 136-T. Strain Sh 60 was obtained from Dr W. E. van Heyningen by Dr W. L. Barksdale. It is sensitive to phages T_2, T_3, T_4, T_6, and T_7, and is used for neurotoxin production.

Media. Tryptose agar consisted of Bactotryptose, 20 g.; glucose, 1 g.; sodium chloride, 5 g.; Bactoagar, 15 g.; thiamine hydrochloride, 0·005 g.; water to 1 l.; final pH 7·2; sterilized by autoclaving at 121° for 15 min. Plates were poured to the desired thickness of 5 mm. for the agar-diffusion method. Neopeptone broth consisted of Neopeptone (Difco) + 50 % (v/v) fresh beef infusion. PGT medium is the casein hydrolysate medium of Mueller & Miller (1941) as modified by Barksdale & Pappenheimer (1954). This medium was used for toxin production.

Bacteriophages. All phages of the T series were derived from stocks maintained by the late Mark H. Adams. Methods and materials for phage assay were as described by Adams (1959).

Acetone powder preparation of Shigella dysenteriae. Organisms were grown in Neopeptone broth at 37° for 18 hr, washed with cold saline by centrifugation and then washed four times with 10 vol. of cold acetone. Acetone from the final wash was decanted and the organisms were dried to a white powder in vacuum. Plates for endolysin assay were prepared by adding 0·5 ml. acetone powder (suspended in saline to an opacity reading of 25 at 590 mμ as measured in a Bausch & Lomb Junior spectrophotometer) to 2·0 ml. molten soft agar and plating as a soft agar layer according to the method described by Adams.

Preparation of toxin of Shigella dysenteriae. Organisms were grown in Neopeptone broth for 18 hr at 37°, and were centrifuged at 3000 rev./min. for 20 min. The sediment was resuspended in the casein hydrolysate (PGT) medium (de-ferrated) of Mueller & Miller in the concentration to opacity reading 9. The bacterial suspension was shaken at 35° for 18 hr at low speed (100 strokes/min.), then centrifuged at 6000 rev./min. for 30 min. in the cold (4°), and the supernatant fluid was filtered through a Selas filter (Selas Corporation of America, Dresher, Pennsylvania, U.S.A.). The filtrate was assayed for toxin by the agar-diffusion and flocculation methods.

Detection and assay of toxin. Detection of toxin was made by the agar-diffusion and the flocculation methods. Agar diffusion was particularly useful for the detection of specific antigen; Lf values of toxins were determined by flocculation.

Reference toxin. Purified Shiga neurotoxin was obtained from Dr W. E. van Heyningen of the Sir William Dunn School of Pathology, Oxford University. This toxin was used as a reference in the agar-diffusion and flocculation methods.

Antitoxin. The antitoxin used was refined dysentery antitoxin procured from Dr Mollie Barr of the Wellcome Research Laboratories, Beckenham, Kent, England; it contained 1400 units/ml.

RESULTS

Criterion for purity of toxin

The difficulty of complete separation of exotoxin and endotoxin of *Shigella dysenteriae* has been the main cause of controversies. For this reason, the reference toxin of van Heyningen and toxins prepared in our laboratory were tested against refined dysentery antitoxin by the agar-diffusion method. Filter-paper discs (13 mm. diam.) were placed on the agar plate as shown in Fig. 1. Diluted refined dysentery antitoxin (0·05 ml., containing 28 units) was placed on the centre disc. Reference toxin from van Heyningen containing 2 Lf units and toxins prepared in our Laboratory (A, B, C) were placed on surrounding discs. The plates were sealed with adhesive tape and kept at 30° for 2–3 days in a moist atmosphere. There were two precipitation lines between reference toxin and antitoxin; the inner line is later referred to as line 1, and the outer line as line 2. The refined antitoxin was then absorbed with *S. dysenteriae* organisms and tested against toxins by agar diffusion. After absorption of the antitoxin, only one precipitation line (line 1) was seen between reference toxin and absorbed antitoxin; line 2 had disappeared. It is apparent that the reference toxin contained two closely associated but different antigens, one having a common determinant with the somatic antigen, and the other being the neurotoxic antigen. Whether these two antigens were derived from a single large-molecule antigen or from a mixture of two distinct antigens is not known. However, further evidence indicates that these two antigens may be derived from a single large-molecule antigen. In the present paper, all determinations of neurotoxins are referred to line 1 between reference toxin and unabsorbed refined antitoxin.

Toxin release by phage infection in Shigella dysenteriae

Giant colonies of *Shigella dysenteriae* were grown on a tryptose agar plate incubated for 20 hr. One drop (0·03 ml.) of T-phage suspension containing 1×10^{10} plaque-forming units (p.f.u.)/ml. was put on each giant colony. Reference toxin and antitoxin were then added on the discs before plates were sealed and kept at 30° for 3–5 days for development of precipitation lines between antitoxin and phage-infected giant colonies. As can be seen from Fig. 2, phage-infected colonies released several kinds of macromolecules as well as neurotoxin.

Toxin release in phage lysates of Shigella dysenteriae

Shigella organisms grown in Neopeptone broth on a shaker at 37° for 4 hr in exponential phase were centrifuged down, washed with saline, and resuspended in PGT medium to an opacity reading of 5. The bacterial suspensions in PGT medium were infected with T-phages at a ratio of 1:5, and shaken at 37° for 1 hr. The lysed cultures were centrifuged for 30 min. at 4° and 9000 rev./min., and the supernatant fluids filtered through Selas filters. The filtrates were assayed for toxin by the agar-diffusion and flocculation methods. Bacterial suspensions without phage infection, as controls, were treated in the same way. The agar-diffusion plates showed that the neurotoxin (Fig. 1, line 1) of the reference toxin was further depolymerized into two smaller antigenic parts in the lysates. This indicated that the neurotoxin was an antigen of large molecular size which was further depolymerized

by the phage-associated lysate enzymes. The amount of toxin contained in these lysates was determined by flocculation, with the results given in Table 1. It is apparent that neurotoxin was released from very young *Shigella dysenteriae* organisms by phage infection.

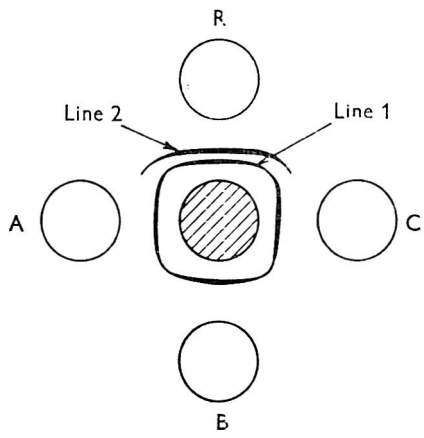

Fig. 1

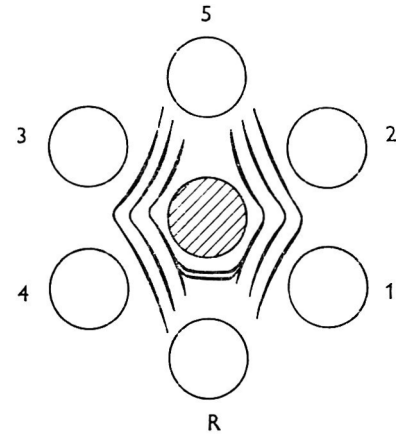

Fig. 2

Fig. 1. Precipitation reaction between antitoxin and toxin in agar plate. Centre disc contains antitoxin. Disc R contains reference toxin. Discs A–C contain 0·1 ml. of toxins prepared in PGT medium.

Fig. 2. Precipitation reaction between phage-infected giant colonies of *Shigella dysenteriae* and antitoxin. Positions 1–4 are giant colonies infected with phages T_1, T_3, T_5, T_6, respectively. Position 5 is uninfected colony. Centre disc contains antitoxin. R disc contains reference toxin.

Table 1. *Lf values of toxin in phage lysates of Shigella dysenteriae strains*

Lysates	Lf/ml.
Strain 136-T + phage T_1	15
Strain 136-O + phage T_1	20
Strain 136-R_4 + phage T_7	20
Strain Sh 60 + phage T_7	25
Strain 136-T without phage	Non-detectable
Strain 136-O without phage	Non-detectable
Strain 136-R_4 without phage	Non-detectable
Strain Sh 60 without phage	Non-detectable

Preparation of phage-induced lysin and its activity

Phage lysate was centrifuged at 3000 rev./min. for 20 min. and the supernatant fluid filtered through a Selas filter. The filtrate was ultracentrifuged in a Spinco Preparative Centrifuge at 40,000 rev./min. (143,000*g* maximum) for 4 hr. The phage in the supernatant fraction gave 4×10^4 p.f.u./ml., while the sedimented portion gave 2×10^{10} p.f.u./ml. The activity of this crude endolysin on acetone-powder preparations was that the lysin effected 25 % loss in turbidity of cell suspensions in 30 min. at 30°. The rate of lysis of the Shigella organisms by this lysin preparation is shown in Fig. 3, which illustrates the linear relationship between endolysin concentration and rate of lysis of acetone-powder preparations of *S. dysenteriae*.

The effect of endolysin on toxin release from acetone-powder preparations of Shigella dysenteriae

Acetone-powder preparations of *Shigella dysenteriae* were suspended in phosphate buffer (pH 7·0) to an opacity reading of 15. One ml. of endolysin preparation was added to 9·0 ml. of bacterial suspension; as a control 1 ml. of Neopeptone broth was added to 9·0 ml. of the bacterial suspension. These mixtures were incubated

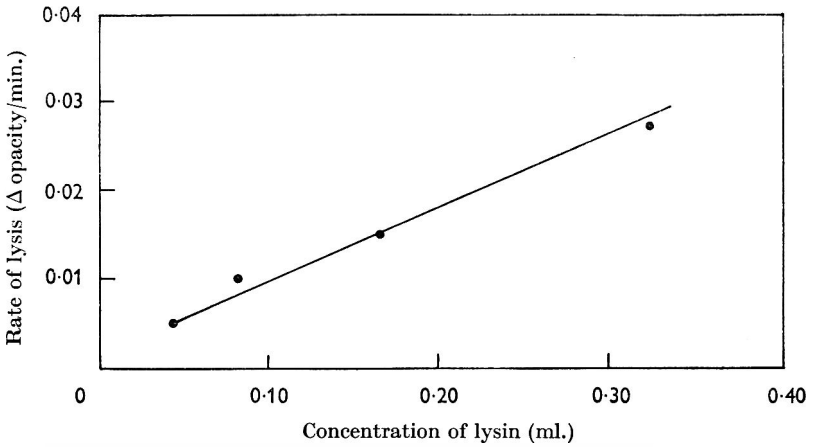

Fig. 3. Effect of lysin concentration on the rate of lysis of acetone-powder preparations of *Shigella dysenteriae*.

at 30° for 40 min., after which time the opacity readings were 10·0 and 13·4, respectively. The decrease of turbidity with the endolysin lysate at 30° in 40 min. was about 25 %. These mixtures were centrifuged in the cold (4°) and the supernatant fluids assayed for toxin by the agar-diffusion and flocculation methods. The agar-diffusion plate showed that both the endolysin lysate and the phosphate buffer contained neurotoxin, as well as another non-specific antigen. The amounts of antigenic substance in the endolysin lysate and the phosphate buffer were both 30 Lf/ml. It is apparent therefore that the endolysin did not have a direct effect on the amount of neurotoxin released from the acetone-powder preparations of organisms despite the lysis of 25 % of the organisms. This suggests that the neurotoxin was not directly released from the cytoplasm but was probably released from the surface of the cell wall. Examination of the plate showed that lines 1 and 2 of the reference toxin joined into one line of antigen released in phosphate buffer; this suggests that two antigens in the reference toxin might be depolymerized from a single antigen of large molecular size released in phosphate buffer (Fig. 4).

The effect of endolysin on living Shigella dysenteriae organisms

An overnight culture of *Shigella dysenteriae* was centrifuged, the sedimented organisms washed with cold saline once, and resuspended in phosphate buffer (pH 7·0) to an opacity reading of 15. One ml. of endolysin preparation was added to 9·0 ml. of bacterial suspension; as a control 1 ml. of Neopeptone broth was added to 9·0 ml. of bacterial suspension. These mixtures were incubated at 30°

for 40 min., then centrifuged at 4°, and the supernatant fluids assayed for toxin. No significant changes of the opacity reading were noticed, and no toxin was detectable, either by the agar-diffusion method or by the flocculation method. The results indicate that the endolysin was not effective in releasing toxin from living *Shigella dysenteriae* organisms.

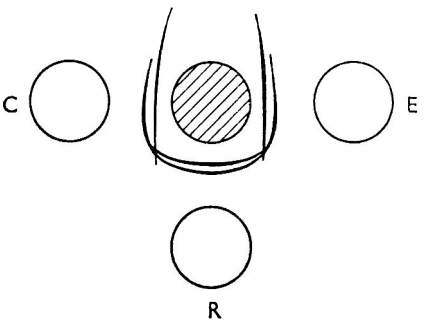

Fig. 4. Precipitation lines of endolysin lysate. Disc E contains 0·1 ml. of supernatant fluid of bacteria treated by endolysin. Disc C contains 0·1 ml. of supernatant of bacteria suspended in buffer. Disc R and centre disc contain reference toxin and antitoxin respectively.

DISCUSSION

Much has been written about the conditions for neurotoxin formation by *Shigella dysenteriae*, but there is no clear view about the mechanism of liberation of Shiga neurotoxin from the organisms. Thus it was interesting to see whether phage and phage-associated lysin in Shigella lysates might play some role in toxin release.

In the present work when *Shigella dysenteriae* and T-phages were brought together to produce infection and lysis, various kinds of biologically active macromolecules were released, including neurotoxin. Also, when *S. dysenteriae* organisms in a 4-hr culture were exposed to the action of a T-phage for 1 hr, only neurotoxin and its closely associated antigens were released into the supernatant medium. Neurotoxin released under this condition seems to be depolymerized, by phage-bound enzyme, from a single large-molecule antigen ordinarily extractable into phosphate buffer. The phage-bound enzyme or enzymes involved in this process are probably held within the phage, for the reason that lysate containing phage-induced lysin (endolysin) does not have the activity of depolymerizing neurotoxin. It is conceivable that the phage-bound enzyme may activate an autolytic enzyme or inhibit a depressor of an autolytic enzyme.

The endolysin, when incubated with acetone-powder preparations of *Shigella dysenteriae* at 30°, effected lysis of 25 % of the organisms in 30 min. The lysates contained neurotoxin; however, there was no difference in the amount of neurotoxin released under the effect of endolysin and that released into the phosphate buffer control from the acetone-powder preparations. This indicates that neurotoxin was not directly released from cytoplasm but rather released from the cell wall surface. Additional evidence for this idea is that, after the extraction of neurotoxin from acetone-powder preparations of *S. dysenteriae*, the opacity readings of the suspensions remained unchanged, and the organisms retained the normal shape of Gram-

negative rods when viewed in the microscope. Living organisms of growing *S. dysenteriae* suspended in phosphate buffer did not release detectable neurotoxin after incubation at 30° for 40 min. These findings suggest that the linkage between the neurotoxin and the cell wall is firm in actively growing organisms but, when these are dead (as in the acetone-powder preparations), the linkage is easily broken in phosphate buffer, probably because of the activity of some surface-attacking autolytic enzyme.

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The Nature of Self-Inhibition of Germination of Conidia of *Glomerella cingulata*

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SUMMARY

Conidia of *Glomerella cingulata* did not germinate under crowded conditions. This was not due to limiting effects of concentration of oxygen or carbon dioxide. The ill effect of crowding on germination was alleviated by adding large amounts of twice-crystallized bovine serum albumin. Nearly 88% conidia germinated in redistilled water when they were present in amounts less than 100/mm.^{2}; but on addition of the washings and exudates of conidia into such suspensions, germination as well as length of germ tubes was markedly decreased. Germination of conidia was increased by prolonged leaching of the conidia by soaking in redistilled water. More than 30% of the thoroughly leached conidia germinated in redistilled water under crowded conditions (3000/mm.^{2}), whereas less than 2% of the conidia sampled before leaching germinated under similar conditions. It is concluded that diffusible inhibitory compounds from the conidia appear to be responsible for the inhibition of germination. Several solubility classes of inhibitory compounds have been extracted from the cultures of *G. cingulata*. Among them, a basic fraction was relatively more toxic to *G. cingulata* than to *Bacillus subtilis*.

INTRODUCTION

The percentage germination of conidia of *Glomerella cingulata* has been found inversely proportional to concentration of conidia (Lingappa & Lingappa, 1965). Washed conidia do not germinate on laboratory media when dispersed in amounts greater than 3000/mm.^{2}, but they do germinate readily in redistilled water when there are less than 100 conidia/mm.^{2} It follows that the conidia are not nutritionally deficient. Nevertheless, addition of large amounts of certain nutrients resulted in germination of crowded conidia. Oxygen or carbon-dioxide concentration did not limit germination under those conditions. Studies on the nutrient requirements for germination of conidia of *G. cingulata* suggested that inhibitory metabolites from the conidia, rather than lack of nutrients, might be responsible for the inhibition of germination of crowded conidia. This possibility has been further examined in the present paper.

METHODS

The cultivation and germination procedures were the same as mentioned previously (Lingappa & Lingappa, 1965); the concentration of sucrose in the potato sucrose medium was 5·6% (w/v), not 8·5% (w/v) as there reported. For germination studies, conidia from culture slopes were washed three times by suspension in

water and centrifugation. Serial dilutions of washed conidia were dispersed in 1·5 ml. quantities into 35 × 10 mm. Petri dishes. The conidia settled quickly on the bottom of the Petri dishes. Therefore the concentrations of conidia were expressed in numbers per mm.^{2} The dilutions gave 30–3000 conidia/mm.^{2} The watery supernatant fluid obtained after initial and subsequent washing of conidia was passed through a sterilizing membrane filter in a micro-syringe holder (Millipore Filter Corporation, Bedford, Mass.). Conidia were produced in shaken liquid cultures which were set up by inoculating 10 ml. inoculum of about $1{\cdot}6 \times 10^8$ conidia/ml. into 150 ml. medium in 500 ml. Erlenmeyer flasks. Cultures were pooled in a large container, passed through a layer of cheesecloth to separate any mycelial balls, and duplicate samples taken to count and to determine the dry weight of conidia. The conidia were separated by centrifugation in an International Refrigerated Centrifuge at 10,000 rev./min. for 20 min. The separated conidia were washed once. The mycelia, conidia, and media were made acid or alkaline by addition of N-HCl or N-Na_2CO_3 and extracted in batches with reagent grade chloroform or dichloromethane (Fig. 2). The extracts were uniformly evaporated to dryness over filter-paper assay discs in a Flash-Evaporator (Buchler Instrument Inc., Fort Lee, New Jersey), at 40°, and at 0–20 mm. Hg pressure.

The biological activity of these extracts was tested as follows (Skinner, 1955): Half-strength potato sucrose medium, with 1·8% agar, was melted and cooled to 42°. The test organisms, which included conidia of *Glomerella cingulata* (10^6/ml.) and endospores of *Bacillus subtilis* (10^7/ml.), were uniformly suspended in agar and poured into Petri dishes, 12 ml. into 10 cm. diam. Petri dishes, or 20 ml. into 15 cm. diam. dishes. On these solidified seeded agar plates, assay discs containing the evaporated extracts were placed. Solvent control discs, containing the solvents or the residues left by evaporating amounts of solvents equal to the amounts used for extraction or fractionation, were also included in the assays. Because acids and alkalis used in adjusting the pH value of cultures give zones of inhibition, solvent extracts were carefully separated to avoid carry-over of emulsions. After incubation for 16 hr at 25°, the diameters of clear inhibition zones surrounding the assay discs were measured and recorded. Assay discs containing equal amounts of any one extract or fraction were assayed. In this way the activity of any one extract, or of a fraction, on *G. cingulata* could be compared with the corresponding activity on *B. subtilis*.

Extracts obtained from $CHCl_3$ or CH_2Cl_2 were further separated into five fractions solubility classes by following the procedure for separation of mixtures of water-insoluble compounds outlined by Shriner, Fuson & Curtin (1956; Fig. 3). These fractions were again assayed against test organisms. Preliminary chemical studies on these fractions were made following Shriner *et al.* (1956) and Openshaw (1955).

RESULTS

Effect of crystalline bovine albumin

Albumins inactivate many antimicrobial agents by binding, and therefore 4–9% serum albumin is included in antibiotic assays (Kavanagh & Dennin, 1963). Twice-crystallized bovine albumin (Nutritional Biochemical Corp., Cleveland, Ohio) was added to conidial suspensions to test whether the self-inhibitory metabolites of the conidia

were inactivated. Addition of albumin increased the germination of conidia in all conidial concentrations tested (Table 1). The effect of albumin was similar to that of Bactopeptone (Difco Laboratories, Detroit, Michigan). Peptone, however, was 3–5 times more effective on a weight basis than albumin in counteracting the inhibitory effects of crowding of conidia (Table 2). Soluble starch (B and A, Allied Chemical, New York, reagent grade) showed no effect at from 1 to 4 mg./dish.

Table 1. *Effect of bovine albumin, 25 mg./dish, on germination of conidia of Glomerella cingulata*

No. conidia/mm.^{2}	Germination (%)	
	In water	In albumin
30	84	93
60	64	86
120	39	80
220	19	64
400	10	37
950	4	22
1700	3	14
3100	1	10

Table 2. *Effect of albumin and of peptone on crowded conidia (3000/mm.^{2}) of Glomerella cingulata*

Bovine albumin		Bactopeptone	
mg./dish	Germination (%)	mg./dish	Germination (%)
0	1 (control)	1	5
5	2	2	15
10	12	4	32
20	25	8	57
40	56	16	78
80	87	32	95

Effect of exudates of conidia on the germination

Conidia grown at 27° on agar slopes were densely suspended in water to read a turbidity value of 1 at 400 mμ. This suspension was centrifuged and the supernatant fluid separated. The washed conidia were resuspended in water and the process was quickly repeated to obtain second and third washings. The cleaned conidia were suspended in half the original volume of water, to double the concentration of conidia, and designated double-strength suspension. This suspension was incubated in a 15 cm. Petri dish for 4½ hr at 27° and then centrifuged. The supernatant fluid (SE) contained all the materials which had diffused from the conidia. The washed conidia, so obtained, were diluted to 3000/mm.^{2} and allowed to germinate. These thoroughly leached conidia gave 30% germination in water, whereas corresponding samples taken from the same lot after the first wash gave only 2% germination.

The watery supernatant fluid from 1st, 2nd, 3rd washings and SE contained 960, 260, 60 and 80 μg. dry matter/ml., respectively. These fractions were autoclaved or passed through a sterilizing membrane filter and 0·05–1·6 ml. of solution added

to dishes containing 1·5 ml. dilute suspension of conidia; corresponding amounts of water or of 0·5 % (w/v) peptone solution were added to control dishes. These dishes contained about 30 conidia/mm.^{2} Addition of water alone did not affect germination of conidia (78–82 %), any more than 0·4 ml. peptone (2 mg.) solution increased germination and the addition of SE decreased germination (Fig. 1). Autoclaved SE and SE from double-strength suspensions of conidia were more toxic than SE from single-strength suspensions. The 1st, 2nd and 3rd washings contained small amounts of the medium carried over along with the readily extractable materials of the

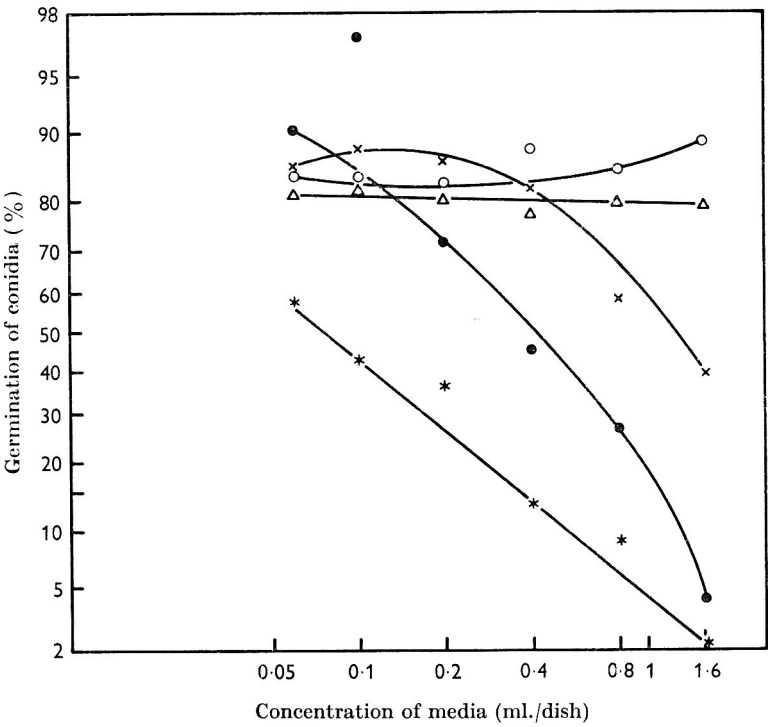

Fig. 1. Effect of adding exudates from conidia of *Glomerella cingulata* on the germination of conidia of *G. cingulata*. △——△, water; ○——○, peptone; ×——×, exudates from single-strength suspension of conidia; ●——●, exudates from double-strength suspensions of conidia; *——* , autoclaved exudates from double-strength suspensions of conidia.

conidia. Addition of more than 0·8 ml./dish of the washings into dishes containing washed conidia (30/mm.^{2}) resulted in 48–53 % germination as compared to 88 % in water, whereas less than 20 % conidia germinated when exudates from conidia (SE) were added. Not only the germination percentage but also the germ-tube length decreased on addition of SE (Table 3).

Conidial exudates were further examined; chloroform extracts were made of 45 ml. of SE after first adjusting to pH 10 and then to pH 4, and after these extractions the SE was adjusted to pH 7. These preparations were evaporated on filter-paper assay discs and placed on seeded agar plates. After incubation for 16 hr the diameters of zones of inhibition were recorded (Table 4). As compared to other fractions,

the chloroform extract of SE at pH 10 (SEC pH 10) was the most inhibitory, although other fractions also gave smaller zones of inhibition on *Glomerella cingulata*. The acidic fraction was more toxic to *Bacillus subtilis*.

Table 3. *Effect of exudates of conidia (SE) on germination and germ-tube growth of conidia (30/mm.²) of Glomerella cingulata*

Treatment (ml./dish)	Germination (%)	Germ-tube length (μ)
0·5 water	88	68
0·5 Peptone (2·5 mg.)	93	218
0·05 SE	91	167
0·1 SE	97	182
0·2 SE	72	179
0·4 SE	45	90
0·8 SE	27	45
1·2 SE	20	4

Table 4. *Assay of preparations from cultures of Glomerella cingulata*

	Assay organisms	
Preparations*	*G. cingulata* Inhibition zone (mm.)†	*B. subtilis*
SEC pH 4	7	15
SEC pH 10	30	15
SE remainder pH 7	12	20
MC (solid) pH 4	20	25
MC (solid) pH 10	8	0
SWC pH 4	7	20
SWC pH 10	15	25
SC pH 4	20	40
SC 1st extract, pH 10	25	10
SC 2nd extract, pH 10	15	25

* SE = spore (conidia) exudate, M = medium, SW = spore-washings, S = spores (conidia), C = chloroform extraction after pH adjusted to value noted before each preparation.

† Inhibition zone diameters (mm.) with 6·35 mm. diam. discs.

Activity of extracts from cultures

The above data indicated that some conidial-borne metabolites were able to inhibit germination of conidia. To obtain further evidence, direct solvent extraction of the fungus and fractionation of active extracts were attempted. The cultures of *Glomerella cingulata* were harvested on 3rd, 7th, and 14th days and extracted as shown in Fig. 2. The activities of the various preparations clearly indicated that substances obtained from the cultures which had been adjusted to pH 10 were relatively more toxic to conidia of *G. cingulata* than to endospores of *Bacillus subtilis* (Table 4). Extracts obtained from acidified preparations were also toxic to conidia but they were more toxic to *B. subtilis*. Obviously these extracts were crude mixtures of compounds belonging to the particular solubility class. Larger zones of inhibition were obtained from older than from younger cultures but those extracted from 7-day conidia gave greater activity than those from 3- or 14-day conidia (Table 5).

Fractionation and nature of the extract obtained under alkaline conditions

Acid and alkaline dichloromethane extracts were pooled and filtered through analytical filter paper to remove fine particles. The extracts were then dried under vacuum and assayed to make sure that they retained inhibitory activity against *Glomerella cingulata*. This dried pool was redissolved in dry diethylether, filtered and processed as in Fig. 3. Samples of the five fractions obtained were dried on assay

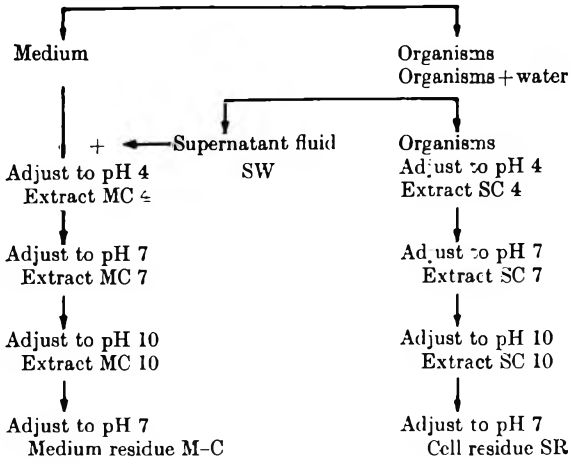

Fig. 2. Cultures of *Glomerella cingulata*: extraction procedure. pH adjusted with N-Na_2CO_3 or N-HCl. Extracted by 3 changes of $CHCl_3$ or CH_2Cl_2.

Table 5. *Activities of preparations from cultures of different ages of Glomerella cingulata*

	Assay organisms					
	G. cingulata			*B. subtilis*		
	Inhibition zone (mm.)†					
Age of cultures in days...	3	7	14	3	7	14
Preparations*						
MC pH 10	8	24	45	0	20	70
SC pH 10	10	20	10	0	15	0
MC pH 4	0	20	30	20	32	80
SC pH 4	12	15	15	25	30	25
SW pH 7	15	20	15	20	30	25

* MC = chloroform extract of media, SC = chloroform extract of conidia, SW = water extract of conidia (obtained by washing 3 times).

† Zone diameters in mm. with 6·35 mm. assay discs.

discs and assayed. The results indicated that all fractions had some inhibitory properties but the basic fraction was significantly more active against *G. cingulata* than against *Bacillus subtilis* (Table 6).

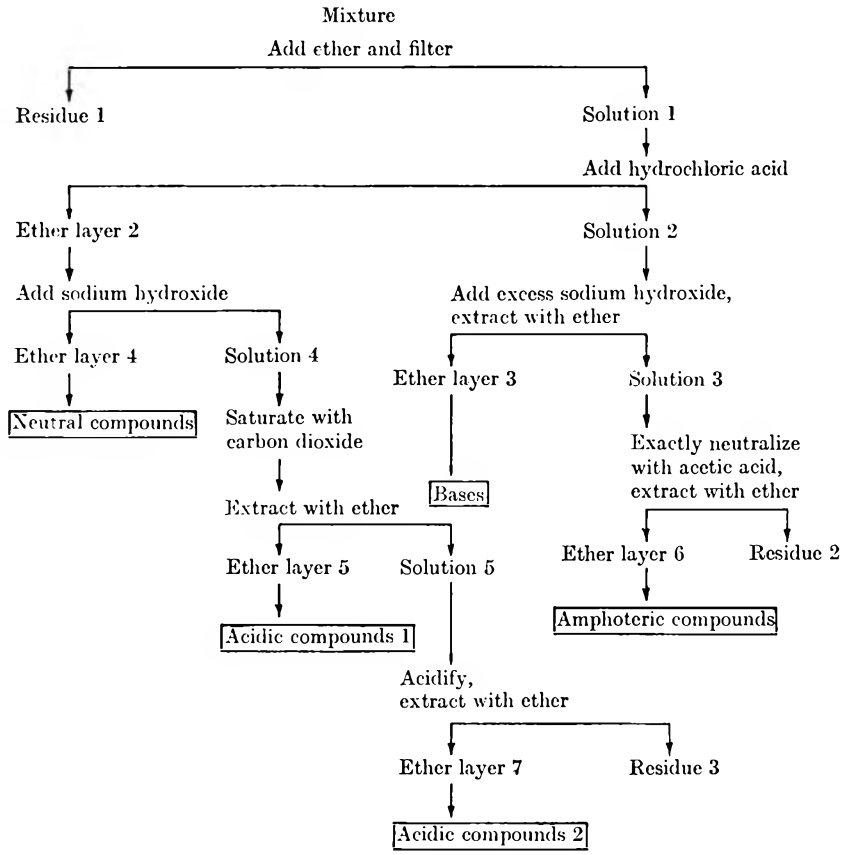

Fig. 3. Germination of conidia of glomerella: fractionation procedure.

Table 6. *Activity of fractions of crude dichloromethane extracts of cultures of Glomerella cingulata*

	Assay organisms*	
Fraction assayed	*G. cingulata*	*B. subtilis*
Base	25	8
Neutral compounds	7	0
Acidic 1	10	25
Acidic 2	20	10
Amphoteric	15	10

* Zone diameter with 3·2 mm. assay discs.

DISCUSSION

We found previously that the washed conidia of *Glomerella cingulata* did not germinate in water under crowded conditions (3000/mm.^{2}; Lingappa & Lingappa, 1965). Germination of crowded conidia was not increased by aeration but by the addition of peptone, or of amino acids. This ruled out the concentration of oxygen or of carbon dioxide as a limiting factor, and indicated that there was a need for exogenous supply of nutrients for germination of crowded conidia. Nevertheless,

the conidia germinated readily in redistilled water when dispersed in amounts less than 100/mm.^{2}. It was, then, suggested that the stimulatory compounds might be binding and inactivating some self-inhibitory metabolites of the conidia. This has now been further supported by the stimulatory effect of twice-crystallized bovine albumin on germination of conidia (Tables 1 and 2). High-percentage germination was obtained with 80 mg. albumin per dish, i.e. 4% albumin (Table 2). This does not give any indication of the nature of the inhibitor because albumins are non-specific binding agents. Various drugs, antibiotics, lipids and metal ions are bound and inactivated by albumins. Some substances, however, show greater activity in presence of albumin; serum albumin inactivated tyrocidine but the antibiotic activity of gramicidin was enhanced several fold by albumin (Dubos, 1949). The inhibitory effect of triacetin on *Candida albicans* was enhanced by albumin (Kubista & Derse, 1959).

Further evidence that diffusible inhibitors of the conidia might be responsible for inhibition of germination of crowded conidia was obtained by washing the conidia. Previously it was shown that washed conidia germinated better than the unwashed ones. At that time, it was not clear whether the repeated washing removed the inhibitors from the conidia or whether the conidia were picking up some contaminating trace elements from successive changes of redistilled water. Acid-washed and thoroughly water-washed Pyrex glass gives out to distilled water (μg./cm.^{2}/day at 20°) Si, 0·02; B, 0·002; Al, 0·0002; Na, 0·0005; K, 0·00001; and other elements, 0·00001 (data supplied by the manufacturer, Corning Glass Works, Corning, N.Y.). Therefore, instead of using many separate changes of redistilled water, the conidia were leached by soaking for 4½ hr and the supernatant fluid (SE) added to sparsely dispersed conidia (30/mm.^{2}). Addition of such exudates of conidia (SE) not only decreased the germination of conidia but also decreased the length of the germ tubes (Fig. 1; Table 3). In other words, the crowding effect could be reproduced for sparsely dispersed conidia by adding diffusates from dense suspensions of conidia. It follows that the washings contained some inhibitory substances leached from the conidia. Furthermore, 30% of the leached conidia germinated when dispersed at 3000/mm.^{2} in redistilled water as compared to 2% germination of unsoaked conidia. These results confirmed that the removal of diffusible inhibitors from the conidia enabled them to germinate under crowded conditions.

Extracts from conidia of *Glomerella cingulata* were also assayed on endospores of *Bacillus subtilis* to differentiate general antimicrobial components from self-inhibitors. Any product of an organism which is inhibitory to the organism producing it is considered to be a self-inhibitor. However, a self-inhibitor may or may not have other antibiotic properties. Fractions were obtained from *G. cingulata* some of which were more toxic to itself than to *B. subtilis* (Tables 4–6). More precise classification of these products into self-inhibitor or antibiotic will be possible only after purifying the compounds because the activities of crude extracts are not comparable on the basis of weight.

No attempt was made to identify the different fractions chemically. The chloroform extracts of acidified cells and media were tentatively identified as lipids, since glycerides, fatty acids, phospholipids and sulpholipids have been found in the lipids of conidia of *Glomerella cingulata* (Jack, 1964*a*, *b*). Self-inhibitory substances resembling short-chain fatty acids or their derivatives have been obtained from the

uredospores of *Uromyces phaseoli*, whose germination is also diminished by crowding (Bell & Daly, 1964). Fatty acids and their derivatives have antimicrobial effects (Scholefield, 1963). Spores of fungi are rich in reserve lipids which are actively utilized by the spores (Shu, Tanner & Ledingham, 1954; Lingappa & Sussman, 1959; Gottlieb, 1964). Some fatty acids are stimulatory to the spores (Farkas & Ledingham, 1959) while others are inhibitory (Lewis & Johnson, 1964). Triacetin, a neutral lipid, is as fungitoxic to *Candida albicans* as nystatin (Kubista & Derse, 1959). Triacetin, 10 mg./assay disc measuring 3·5 mm. diam., gave zones of inhibition 10 mm. diameter on both *G. cingulata* and *Bacillus subtilis*; tripalmitin was non-toxic to both organisms. Germination of spores of several bacteria is inhibited by rancid products of fats (Roth & Halvorson, 1952); these are so toxic that the inhibition of germination of spores of *B. subtilis* was proposed as a sensitive measure of rancidity of fats (Ramsey & Kemp, 1963). Chlorellin, a self-inhibitor of *Chlorella vulgaris*, was identified as a breakdown product of lipids (Spoehr *et al.* 1949; Scutt, 1964). Our results indicated that in comparable amounts the lipid fractions were relatively more toxic to *B. subtilis* than to *G. cingulata* (Tables 4 and 6), whereas the basic fraction was more toxic to *G. cingulata* than to *B. subtilis*. The selectively inhibitory basic fraction is the first such self-inhibitor reported in fungi. Occurrence of self-inhibitory metabolites and cellular constituents is clearly demonstrated in *G. cingulata* by these findings. The actual role of any combination of these inhibitors in the living organism is open to future studies.

The existence of self-inhibitors was known in fungi (Sussman, 1965; Cochrane, 1958). Recently, self-inhibitory products have been demonstrated in several fungi (Carlile & Sellin, 1963; Lingappa & Lingappa, 1964). Rotem (1963) found evidence for the existence of water-soluble, non-diffusible, and heat-labile self-inhibitors in the mycelium of *Alternaria pori f. sp. solani*. The mycelium of *Psilocybe paneoliformis* was reported to contain inhibitors which prevent fruiting (Urayama, 1960). Staling product in the cultures of several fungi grown in presence of 2,4-D and other phenoxy compounds has the attribute of a self-inhibitor (Naito, 1958). None of these self-inhibitors have been characterized. Until then the actual role of these compounds in the regular development and function of the organisms producing them will remain uncertain. It is inconceivable how self-inhibitors, if any are produced, can be without any regulatory function in the organism producing them. Self-inhibition of germination due to crowding might be of considerable survival value to the organism, preventing germination until well dispersed on suitable hosts or nutrient substrates.

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Growth of Sulphate-reducing Bacteria by Fumarate Dismutation

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SUMMARY

Desulfovibrio gigas and several strains of *D. desulfuricans* grew by fumarate dismutation in a sulphate-free medium. Two strains of *D. desulfuricans* grown in a chemically defined medium formed succinate, malate and acetate during fumarate dismutation. Sulphate reduction by these strains, though not by *D. gigas*, was almost completely inhibited in presence of fumarate as alternative electron acceptor. The anomalous behaviour of *D. gigas* was reflected to some extent by the hydrogen absorption coefficients for fumarate and sulphate reduction. Effects of fumarate media on the morphology of one strain are recorded.

INTRODUCTION

Under natural conditions the sulphate-reducing bacteria appear to utilize sulphate as terminal electron acceptor in the assimilation of organic substances, and have been shown (Postgate, 1951) to reduce certain other sulphur-containing anions in culture. Growth on pyruvate in the absence of sulphur compounds has been reported for strains of *Desulfovibrio desulfuricans* (Postgate, 1952) and *Clostridium nigrificans* (Postgate, 1963); Senez & Pascal (1961) and Baker, Papiska & Campbell (1962) obtained growth of various strains of *D. desulfuricans* on choline in absence of sulphate. Grossman & Postgate (1955) studied sulphate-free metabolism of malate and fumarate, not accompanied by growth, in *D. desulfuricans* strain El Agheila z. The present paper reports the discovery that *Desulfovibrio gigas* and a number of strains of *D. desulfuricans* are able to grow by dismutation of fumarate, i.e. by a Cannizzaro-type reaction which involves the simultaneous oxidation and reduction of the substrate.

METHODS

Organisms. Twenty-three strains of sulphate-reducing bacteria were obtained as freeze-dried ampoules from the National Collection of Industrial Bacteria (NCIB). The strain names are followed by the NCIB number.

(1) Mesophiles (growth temperature 30°).

(*a*) *Desulfovibrio gigas*, a fresh-water strain (NCIB 9332).

(*b*) *D. desulfuricans*, salt-water strains: El Agheila A, 8309; Norway 4, 8310; New Jersey sw-8, 8315; New Jersey sw-3, 8316; California 29:137:5, 8326; Texas 29:12:B, 8328; Australia, 8329; California 43:63, 8364; El Agheila 4, 8396; Venezuela, 8399; Aberdovey, 9492.

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(*c*) *D. desulfuricans*, fresh-water strains: Teddington R, 8312; Denmark, 8456; Woolwich, 8457; Byron, 8458.

(2) Thermophiles (growth temperature 55°). Strains of *Clostridium nigrificans*, all fresh-water strains: Holland CT, 8356; Delft 48T, 8357; Delft 3T, 8359; Delft 13T, 8360; Delft 15T, 8361; unnamed strains 8706 and 8788.

Maintenance of stock cultures. Cultures were raised from the freeze-dried condition at 30° or 55°, as appropriate, in the medium of Baars (1930) containing 1·0 g. Difco yeast extract/l. and 5 mM-cysteine hydrochloride. Sodium chloride, 25 g./l., was added for salt-water organisms. Details of the preparation, sterilization and pH adjustment of this medium are given elsewhere (Saleh, Macpherson & Miller, 1964). Stock cultures of the mesophilic strains were maintained in Postgate's modification of the medium C of Butlin, Adams & Thomas (1949; see Baker *et al.* 1962) containing cysteine, and NaCl where necessary. The thermophilic strains grew better in Baars's medium + yeast extract, in which medium stock cultures were therefore maintained. Subcultures were made weekly. Stock and experimental cultures were grown in Pyrex test tubes or Erlenmeyer flasks plugged with cottonwool and incubated in McIntosh & Fildes anaerobic jars under an atmosphere of N_2. Frequent tests were made on stock and experimental cultures for aerobic and anaerobic contaminants (Postgate, 1953).

Experimental media. Two types of media were used:

(1) A basal medium consisting of modified medium C with sodium lactate, Na_2SO_4 and $MgSO_4$ omitted, and containing 8 mM-$MgCl_2.6H_2O$. To this basal medium was added 50 mM-sodium lactate (L), 50 mM-sodium fumarate (F), or 50 mM-Na_2SO_4 (S), singly or in admixture. Since the basal medium contained yeast extract (Y), the various media derived from this basal medium were designated FY, LFY, FSY and LSY.

(2) A basal medium consisting of the chemically-defined medium of Macpherson & Miller (1963), also with lactate and sulphate omitted, and containing 0·25 mM-$MgCl_2.6H_2O$. Similar additions of lactate, fumarate or sulphate were made, the resulting media being designated F, LF, FS and LS.

$FeSO_4$ was added to all experimental media to 25 μM. This amount was sufficient for assimilatory sulphur and iron metabolism but insufficient for discernible growth by sulphate reduction to occur.

Chromatography. Dicarboxylic acids in culture filtrates were identified by descending chromatography on Whatman no. 1 paper using a *tert*-amyl alcohol + chloroform + water + formic acid (50 + 50 + 50 + 18·75, by vol.) solvent system or a di-isopropyl ketone + water + formic acid (50 + 50 + 13·3 by vol.) system. Oxaloacetate was searched for with a *n*-butanol + water + formic acid (95 + 100 + 5 by vol.) system (Magasanik & Umbarger, 1950) and a methylethylketone + acetone + water + formic acid (80 + 4 + 12 + 2 by vol.) system (Reio, 1959). Acetate, removed by steam distillation from the acidified culture filtrate, was identified by using *n*-butanol + 1·5N-NH_4OH (1 + 1 by vol.) solvent, and by the standard chemical tests (Feigl, 1960).

Sulphide estimation. For estimation of sulphide formed in cultures, H_2S in the gas phase of the anaerobic jars was first displaced by a stream of N_2, absorbed in 1% (w/v) cadmium acetate solution and the precipitated CdS determined iodometrically (Wilson & Wilson, 1959). Dissolved and precipitated sulphide was then removed

from the acidified and heated cultures by a stream of N_2 and estimated as above.

Manometry. Measurement of hydrogenase activity was made at 37° in the Warburg respirometer following the procedure of Littlewood & Postgate (1956) except that 15% (w/v) KOH was used as absorbent for H_2S and CO_2.

Dry weight determinations. Dry weights for the sulphide estimations were determined by centrifuging portions of cultures at about 15,000*g* for 10 min., washing and drying the bacteria at 105° to constant weight. For manometry, suspensions of organisms were standardized turbidimetrically and related to a calibration curve of strain Hildenborough (8303) for the purpose of calculating $-Q_{H_2}$ values.

RESULTS

Growth of various sulphate-reducing bacteria in fumarate media

Tubes of LFY and FY media were inoculated from stock cultures. The former medium was intended to test for the function of fumarate as terminal electron acceptor in place of sulphate, while growth in FY medium in absence of H_2 would indicate the simultaneous utilization of fumarate as electron donor, carbon source and electron acceptor. Where growth occurred, that in the fifth subculture into the experimental medium is shown rough-quantitatively in Table 1. Eleven mesophilic

Table 1. *Growth of sulphate-reducing bacteria in fumarate media*

Growth in the fifth subculture in experimental medium was recorded; that which was judged by eye to be about equal to growth in medium C was designated + + +. For explanation of symbols denoting media see Methods.

Organism	LFY medium	FY medium	Organism	LFY medium	FY medium
D. gigas	−	+ + +	*D. desulfuricans* fresh-water strains		
D. desulfuricans salt-water strains			Teddington R	+ + +	+ + +
El Agheila A	+ +	+ +	Denmark	−	−
Norway 4	+ + +	+ + +	Woolwich	−	−
New Jersey SW-8	±	+ +	Byron	+ + +	+ + +
New Jersey SW-3	−	+ +	*C. nigrificans*		
California 29:137:5	+ + +	+ + +	Holland CT	−	−
Texas 29:12:B	−	−	Delft 48T	−	−
Australia	+	+	Delft 3T	−	−
California 43:63	+ +	+ + +	Delft 13T	−	−
El Agheila 4	+ +	−	Delft 15T	−	−
Venezuela	−	+ + +	8706	−	−
Aberdovey	−	−	8788	−	−

strains dismuted fumarate; five of these grew less well or not at all in presence of lactate, for a reason not at present understood, while the converse was true for only one strain. No thermophilic strain grew in either medium. The trace amount of sulphate present was insufficient to support discernible growth when fumarate was omitted and lactate added.

Fumarate utilization in a chemically defined medium. To eliminate the possibility that fumarate dismutation was dependent on the presence of yeast extract, the two fresh-water strains which grew in LFY and FY media (Teddington R and Byron)

were later subcultured into both LF and F media. Heavy growth of both strains persisted through indefinite subculture in either medium.

Products of fumarate metabolism

Identification of metabolic end-products was done on filtrates of cultures of the fresh-water strains, since in these cases no desalting was necessary before chromatography. Succinate and acetate were present in F and LF cultures of Teddington R and Byron; traces of malate, confirmed by the colour reaction with ammoniacal $AgNO_3$ (Buch, Montgomery & Porter, 1952), were found in some cultures in F medium. Oxaloacetate was not detected. No growth of the Teddington R strain occurred in a chemically-defined medium containing succinate as sole carbon source, and sulphate; malate was dismuted in sulphate-free medium by this and certain other strains (Elford, Miller & Wakerley, in preparation).

Sulphide production during growth in presence of fumarate

It seemed of interest to determine whether sulphate reduction occurred in the presence of fumarate as an alternative electron acceptor. Strain Teddington R and *Desulfovibrio gigas* were inoculated into 200 ml. portions of FSY medium and into controls of LSY medium. The cultures were analysed for sulphide before reaching the maximum extinction attainable in FY medium by the strain concerned. The results of the analyses are given in Table 2.

Table 2. *Formation of sulphide during growth of sulphate-reducing bacteria*

Organism	Grown in FSY medium	Grown in LSY medium
	Sulphide formed (μmoles/mg. dry wt. bacteria)	
Desulfovibrio gigas	27·3	30·0
D. desulfuricans		
strain Teddington R	0·9	49·8
strain Byron	1·2	59·3

For explanation of symbols denoting media see Methods.

Uptake of hydrogen by organisms grown in fumarate media

The results of $-Q_{H_2}$ determinations for fumarate and sulphate reduction are shown in Table 3. A curious feature was that $Q_{H_2}{}^{\text{fumarate}}$ was more than ten times greater in strain Teddington R grown in LSY than in FSY medium. *Desulfovibrio gigas* has already been reported (Le Gall, 1963) to be hydrogenase-positive; its $-Q_{H_2}$ for sulphate reduction was markedly higher than those usually obtained with strains of *D. desulfuricans*.

Morphology and pigmentation of strain Teddington R in fumarate media

The description of strain Teddington R lodged with the NCIB states that it is non-motile, with a tendency to pleomorphism. In the present work, organisms from cultures in lactate+sulphate medium (LS) were found to be normal vibrios or short spirilla, of mean dimensions about $1{\cdot}8\mu \times 0{\cdot}7\mu$, and some showed the typical progressive motility of, for example, the Hildenborough strain (NCIB 8303). To rule

out contamination of our stock, cultures were raised from single organisms, both motile and non-motile, obtained from a culture in LS medium. A roughly similar proportion of motile to non-motile organisms was found in all these clones.

In LF medium the morphology was much the same as in LS medium. In F medium the organisms were rods of up to $3{\cdot}3\mu \times 0{\cdot}7\mu$, often with no detectable curvature, a few being motile. In FS medium the rods were longer (up to $6{\cdot}2\mu$), occasionally motile, with a tendency to form chains (non-motile) of up to four organisms. Only normal black colonies developed when these morphologically aberrant organisms were inoculated into Postgate's (1953) solid test medium.

Organisms from all these media showed a strong absorption band at about 553 mμ typical of reduced cytochrome c_3 in the Hartridge reversion spectroscope, and gave a positive reaction to the desulfoviridin test (Postgate, 1959).

Table 3. *Hydrogen absorption coefficients of Desulfovibrio organisms suspended in phosphate buffer*

	Desulfovibrio gigas		*D. desulfuricans* strain Teddington R	
	Hydrogen absorption coefficients			
Medium in which grown	$-Q_{H_2}^{SO_4^{2-}}$	$-Q_{H_2}^{\text{fumarate}}$	$-Q_{H_2}^{SO_4^{2-}}$	$-Q_{H_2}^{\text{fumarate}}$
LSY	677	240	20	1230
FSY	605	338	72	118
LFY	*	*	4	730
FY	*	*	9	890

* Not determined.
For explanation of symbols denoting media see Methods.

DISCUSSION

Grossman & Postgate (1955) reported growth of *Desulfovibrio desulfuricans* strain El Agheila z in an FSY-type medium; organisms suspended in phosphate buffer metabolized fumarate in absence of sulphate. Two strains have now been shown to grow by fumarate dismutation through indefinite subculture in a chemically-defined medium containing only trace amounts of sulphate; this nutritional pathway appears to be of common occurrence amongst the mesophilic sulphate-reducing bacteria, including the aberrant Norway 4 strain of *D. desulfuricans* (Miller & Saleh, 1964) and the recently-described species *D. gigas* (Le Gall, 1963). The detection of succinate, malate and acetate as end-products of growth suggests the existence of a metabolic pathway: fumarate → malate → lactate → pyruvate → acetate at the expense of reduction of some of the substrate to succinate (compare Grossman & Postgate, 1955). Hydrogen is not necessary for this sulphate-free growth. The mode of fumarate oxidation in sulphate-reducing bacteria is clearly different from that in *Acetobacter xylinum*, investigated by Benziman & Abeliovitz (1964), a characteristic of which is oxaloacetate accumulation.

Fumarate can act as an alternative electron acceptor to sulphate for growth reactions in Teddington R and Byron (the only strains of *D. desulfuricans* examined in this connexion) even when sulphate is present: sulphide production was almost completely suppressed in presence of fumarate, and succinate appeared among the

end-products of metabolism. Such preferential use of fumarate would be expected from theoretical considerations, though Grossman & Postgate (1955) found that small additions of fumarate to suspensions of strain El Agheila z organisms in phosphate buffer under H_2 increased the rate of sulphate reduction, in contrast to its effect on strains Teddington R and Byron in a nutrient medium under N_2. In the case of *D. gigas*, however, sulphide production per unit dry wt. of bacteria formed was roughly the same in FSY as in LSY medium (see Table 2), and a relatively small amount of succinate appeared on chromatograms of culture filtrates. The much lower values for $-Q_{H_2}^{SO_4^{2-}}$ than for $-Q_{H_2}^{\text{fumarate}}$ in strain Teddington R, and the converse for *D. gigas* (Table 3), may have a bearing on this observation, though Teddington R organisms grown in FSY medium actually had an unusually high $-Q_{H_2}^{SO_4^{2-}}$ value while only minute traces of sulphide were produced during growth in this medium. It appears that in the case of the sulphate reducers the behaviour of resting organisms towards oxidizable or reducible substrates may sometimes be quite different from that during growth.

The original description of strain Teddington R as non-motile is evidently incorrect; we have confirmed that it is pleomorphic, though not in lactate+sulphate medium.

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Energy Coupling During Sulphur Compound Oxidation by *Thiobacillus* sp. Strain C

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SUMMARY

The addition of either sulphide or thiosulphate to an aerobic suspension of a Thiobacillus was followed within 10 sec. by the synthesis of intracellular ATP. The ATP formation accompanying sulphide oxidation was strongly inhibited by 0·1 mM-2,4-dinitrophenol, but that accompanying thiosulphate oxidation was unaffected. The presence of carbon dioxide had no effect on ATP formation or on the steady-state concentration attained. 2,4-Dinitrophenol did not significantly affect oxygen uptake nor the kinetics of thiosulphate oxidation but it inhibited carbon dioxide fixation with either sulphide or thiosulphate as substrate. It is concluded that sulphur-compound oxidation can be coupled to two different types of phosphorylation, only one of which is sensitive to 2,4-dinitrophenol. In the presence of 2,4-dinitrophenol and with thiosulphate as substrate, carbon dioxide fixation was not limited by the availability of ATP but might be limited by the availability of reduced nicotinamide adenine dinucleotides.

INTRODUCTION

Organisms of the genus *Thiobacillus* obtain energy from the oxidation of reduced inorganic sulphur compounds. The coupling of this energy to synthetic reactions has long been thought to be mediated by high energy phosphate compounds (Vogler & Umbreit, 1942); recent work supports the view that ADP, ATP and reduced coenzymes are involved (Trudinger, 1956; Vishniac & Santer, 1957; Milhaud, Aubert & Millet, 1957; Johnson & Peck, 1965). Peck (1960, 1962) used cell-free extracts of *Thiobacillus thioparus*, and suggested that thiosulphate was first split reductively to sulphide and sulphite, the sulphite being oxidized subsequently to sulphate by reactions which produce ADP by a substrate-level phosphorylation, with adenosine 5′-phosphosulphate as an intermediate. This phosphorylation is insensitive to 2,4-dinitrophenol (DNP; Peck & Fisher, 1962).

By using $^{14}CO_2$-fixation as a measure of energy transfer in the presence or absence of DNP, we obtained results which suggested that both DNP-sensitive and DNP-insensitive phosphorylations accompany thiosulphate oxidation, but that DNP-sensitive phosphorylations are more important during sulphide oxidation (Kelly & Syrett, 1964*a*). We now present further evidence, from measurements of the ATP content of intact bacteria, for the occurrence of both DNP-sensitive and DNP-insensitive phosphorylation during sulphur-compound oxidation, and for the greater

importance of the former in sulphide oxidation. Our results also support the view that the reduced coenzymes necessary for CO_2-fixation are generated by an energy-dependent reduction by electrons from reduced cytochromes, similar to that shown in other autotrophs (Aleem, Lees & Nicholas, 1963) and recently reported for *T. novellus* by Aleem (1965).

A brief report of some of the present work has appeared elsewhere (Kelly & Syrett, 1964*b*) and the background to the problem was discussed more fully by Kelly (1965).

METHODS

The organism. Thiobacillus sp. strain c was grown as described previously in media containing 1·2% (w/v) $Na_2S_2O_3.5H_2O$ (Kelly & Syrett, 1964*a*). Organisms were harvested by centrifugation, washed and suspended in 0·1 M-sodium phosphate buffer (pH 7·0) to give dense suspensions usually containing about equiv. 3 mg. dry wt. organisms/ml.

Extraction and assay of ATP. Samples (2–3 ml. of dense suspensions were extracted with 0·2 ml. 14·5% (w/v) perchloric acid on ice for 10 min., then brought to pH 7·4 by the addition of 1·0 ml. of KOH of suitable concentration. The clear supernatant solution was pipetted from the perchlorate precipitate. ATP was measured by a firefly luminescence method (Strehler & Totter, 1952). The following were mixed in a 1·5 ml. cuvette at 20°: 0·5 ml. 0·1 M-sodium arsenate+sulphuric acid buffer (pH 7·4); 0·3 ml. distilled water; 0·2 ml. perchlorate extract; 0·04 ml. 20% (w/v) $MgSO_4.7H_2O$. The reaction was initiated by rapidly adding 0·1 ml. of an extract of firefly tails in 0·1 M-arsenate+H_2SO_4 buffer (pH 7·4) containing 2% (w/v) $MgSO_4.7H_2O$. The light emission peak was measured 20 sec. later in a 'Locarte' fluorimeter. The enzyme preparation and samples were kept on ice and allowed to warm only when assayed. Perchlorate depressed the luminescence, apparently by interfering with the enzyme reaction rather than by destroying ATP. In the presence of a constant amount of perchlorate, light emission was proportional to the amount of ATP in solution. ATP standards and samples were always assayed in the presence of equivalent amounts of perchlorate. Comparison of the ATP content estimated by this method with the content determined in similar samples extracted by boiling the organisms for 30 sec. in 90% (v/v) ethanol in water, showed no significant difference. Bacterial extracts contained no substances other than perchlorate which interfered with the assay. DNP caused no interference. Data supporting these statements have been given elsewhere (Kelly, 1965).

Simultaneous determination of ATP content and $^{14}CO_2$ fixation. When the ATP content only was required, suspensions of bacteria were aerated in a rapid sampling device (Syrett, Bocks & Merrett, 1964), or were shaken in 50 ml. conical flasks. When the ATP content and $^{14}CO_2$-fixation values were required simultaneously, bacterial suspensions (2–2·6 ml.) were shaken in 50 ml. conical flasks sealed with vaccine stoppers. Substrates ($KH^{14}CO_3$; $Na_2S_2O_3$; Na_2S) were injected into the flasks and the bacteria killed by injecting perchloric acid. Samples of the neutralized extracts were mixed with equal volumes of 5% (v/v) acetic acid in ethanol, and were used to estimate $^{14}CO_2$-fixation as described by Kelly & Syrett (1964*a*). The rest of the material was assayed for ATP. For time-course experiments it was better to set up a series of such flasks and to kill the bacteria after various time intervals

rather than to take a series of samples from a single flask with syringes. The latter method gave erratic values for ATP.

Gas exchange was measured by Warburg manometry.

Oxidation of radioactive thiosulphate was followed by the sampling and chromatographic methods described by Kelly & Syrett (1966).

Chemicals. $Na_2{}^{14}CO_3$ and $Na_2({}^{35}S.SO_3)$ were obtained from the Radiochemical Centre, Amersham, Bucks; ATP (disodium salt; $3\text{–}4H_2O$; 99–100 %) and desiccated firefly lanterns from the Sigma Chemical Company; hexokinase from L. Light and Co., Colnbrook, Bucks. Other reagents were 'AnalaR' or of the best quality marketed. Solutions of $Na_2S_2O_3$ and Na_2S were always prepared immediately before use; washed crystals of Na_2S were used.

RESULTS

ATP formation during sulphide and thiosulphate oxidation, and the effect of 2,4-dinitrophenol

The bacterial ATP content increased at least twofold within 10–20 sec. after the addition of sulphide or thiosulphate (Fig. 1; and Kelly & Syrett, 1964*b*). Low concentrations of DNP had little effect on the ATP increase during thiosulphate oxidation, while the same DNP concentrations severely depressed ATP formation after sulphide addition. Figure 1 demonstrates that this lower sensitivity of thiosulphate-dependent ATP formation to DNP inhibition was exhibited over a wide range of DNP concentrations. For example, 0·1 mM-DNP completely inhibited increase in ATP content when sulphide was oxidized, while the ATP formation with thiosulphate was unaffected by this concentration. Table 1 shows that treatment of the bacterial extracts with glucose and hexokinase virtually abolished the light emission, indicating the material determined by the firefly method was exclusively ATP.

Table 1. *Effect of treating thiobacillus extracts with hexokinase and glucose before assaying for ATP*

Samples taken at intervals after addition of thiosulphate to dense bacterial suspensions were extracted with perchloric acid (see text). Two series of cuvettes were prepared, each cuvette containing in 0·9 ml. 50 μmoles arsenate $-H_2SO_4$ buffer (pH 7·4); 10 μmoles $MgCl_2$; 0·2 ml. bacterial extract; 20 μmoles glucose. Series 1 received no further addition; series 2 received 0·5 mg. hexokinase. Both series were incubated at 23° for 16 min., then the ATP estimation was made as usual by adding 0·1 ml. firefly extract to each cuvette. Mean values from duplicates or triplicates are given.

Sample time after thiosulphate addition (min.)	ATP content (μmoles/g. dry wt. bacteria)	
	− Hexokinase	+ Hexokinase
Initial	1·57	0·18
0·33	4·17	0·23
1·5	4·00	0·23
3·0	3·42	0·17
6·0	4·68	0·18

ATP formation in the presence or absence of carbon dioxide

Initial experiments to measure ATP were made in the absence of CO_2. However, Fig. 2 shows that even the presence of CO_2 in high concentrations had little effect on the kinetics of the thiosulphate-dependent ATP increase. Since the presence of CO_2 did not abolish the increase in ATP content it was possible to measure simultaneously CO_2-fixation and ATP content of single samples taken over a period of time.

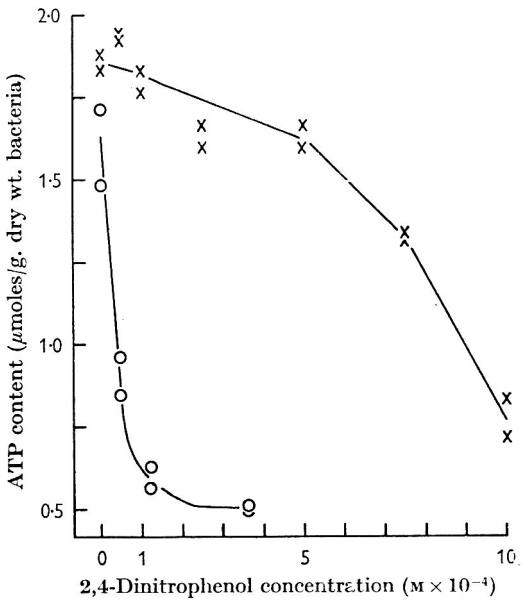

Fig. 1

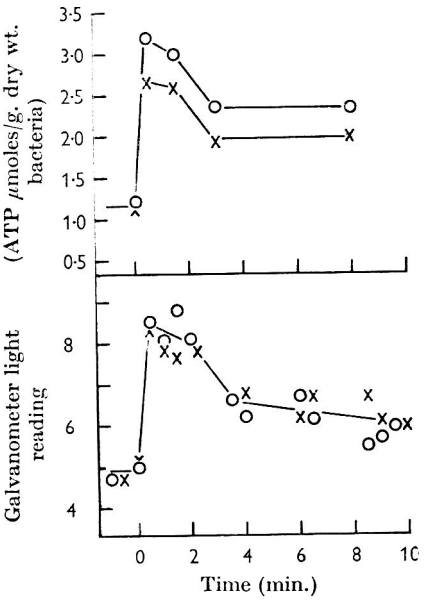

Fig. 2

Fig. 1. Effect of various concentrations of 2,4-dinitrophenol on ATP content of thiobacillus strain c oxidizing thiosulphate or sulphide. 2·7 mg. dry wt. bacteria/ml. were shaken at 21° for 15 min. with the DNP concentration indicated. 8·3 μmoles $Na_2S_2O_3$/ml. (–×–×–) or 0·83 μmole Na_2S/ml. (–○–○–) were added. $HClO_4$ was added to the thiosulphate series after 6 min. and to the sulphide series after 1 min. Endogenous ATP content (±DNP) was about 0·70 μmole/g. dry wt.

Fig. 2. Effect of carbon dioxide on ATP concentrations during thiosulphate oxidation. Upper graph: 4·6 mg. bacteria in 2·4 ml. phosphate (pH 7·0) shaken in sealed 50 ml. flasks at 21°. One series (–○–C–) had 0·4 ml. 40 % (w/v) KOH and a roll of filter paper in a centre well in each flask; the other series (–×–×–) had 4 μmoles $NaHCO_3$. $Na_2S_2O_3$ was injected at time 0. Lower graph: bacterial suspensions in a rapid-sampling device were aerated at 22° with CO_2-free air (–×–×–) or 1 % (v/v) CO_2 in air (–○–○–) and received $Na_2S_2O_3$ at time 0.

Simultaneous determination of $^{14}CO^2$ fixation and ATP content of bacteria oxidizing thiosulphate

The rate of $^{14}CO_2$-fixation during thiosulphate oxidation was not constant from zero time, but always increased to a steady rate 2–3 min. after substrate addition (Fig. 3). Since ATP formation was virtually instantaneous (Fig. 3) the phenomenon could not be attributed to a delay in thiosulphate metabolism. The addition of a second quantity of thiosulphate after the oxidation of the first resulted in renewed

CO_2-fixation at an instantaneously high rate (Kelly, 1965). Possibly, therefore, the initially low rate represented the time required to build up a steady-state intracellular concentration of CO_2-acceptor molecules, such as ribulose diphosphate.

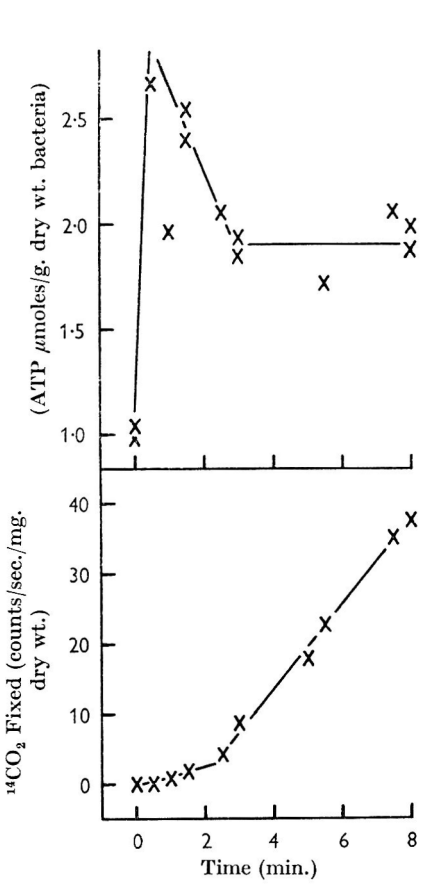

Fig. 3

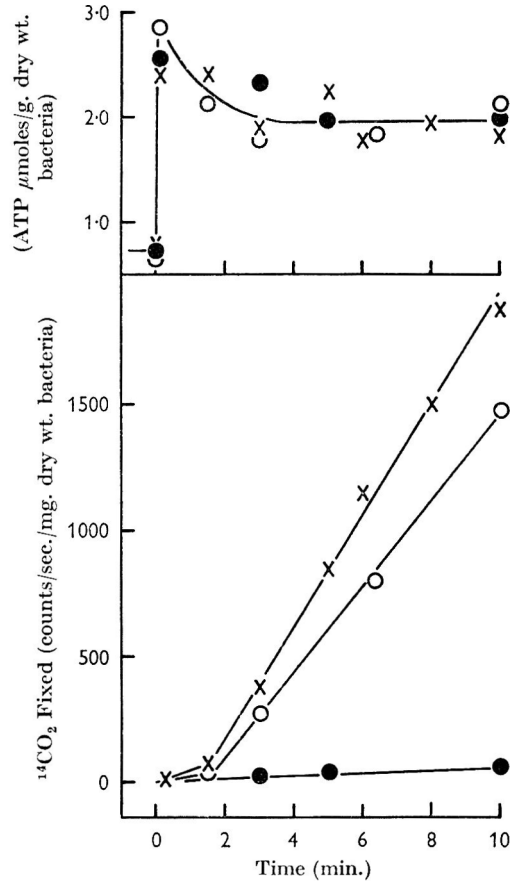

Fig. 4

Fig. 3. Simultaneous measurement of $^{14}CO_2$-fixation and ATP content during thiosulphate oxidation by thiobacillus strain c. Upper: ATP content; and lower: $^{14}CO_2$ fixed by dense bacterial suspensions shaken in sealed flasks into which $KH^{14}CO_3$ and $Na_2S_2O_3$ were injected at time 0. Temp. 21°; pH 7·0.

Fig. 4. Effect of 2,4-dinitrophenol on ATP content (upper graph) and $^{14}CO_2$ fixation (lower graph) by thiobacillus strain c oxidizing thiosulphate. Dense bacterial suspensions received $KH^{14}CO_3$ and $Na_2S_2O_3$ at time 0, after pre-incubation with no DNP (-×-×-), 5×10^{-5}M-DNP (-○-○-) or 5×10^{-4}-DNP (-●-●-). Temperature 21°; pH 7·0.

Effect of 2,4-dinitrophenol on $^{14}CO_2$-fixation and ATP content with thiosulphate or sulphide as substrate

DNP depressed both the initial low rate of $^{14}CO_2$-fixation and the subsequent steady high rate during thiosulphate oxidation (Fig. 4). The effect of DNP on $^{14}CO_2$-fixation bore no obvious relationship to the ATP content of bacteria oxidizing thiosulphate, since the initial rapid increase in ATP and the subsequent values were

not significantly altered by DNP (Fig. 4). Figure 5*a* confirms that no simple relationship existed between ATP content and $^{14}CO_2$ fixed after oxidation of thiosulphate for a set time, with a range of DNP concentrations. $^{14}CO_2$-fixation was 90% inhibited by a DNP concentration (0·2–0·3 mM) which only slightly lowered the ATP content. Even a DNP concentration which completely inhibited CO_2-fixation (0·75 mM) only decreased the increase of ATP content to 50% of the control level. However, the depression of the ATP content by DNP in bacteria oxidizing sulphide was similar to the relative inhibition of CO_2-fixation during

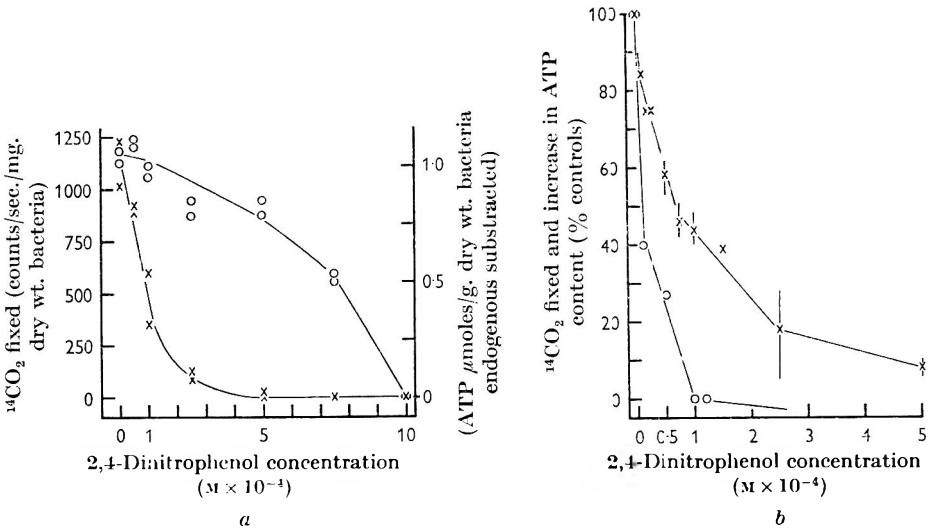

Fig. 5. Effect of various concentrations of 2,4-dinitrophenol on the ATP content (–○–○–) and $^{14}CO_2$ fixed (– × – × –) by Thiobacillus strain c after oxidizing thiosulphate or sulphide: (*a*) Thiosulphate oxidized for 6 min. ATP content in excess of the endogenous value of 0·8 μmole/g. dry wt. is shown. (*b*) Sulphide oxidized: ATP data are those obtained from two experiments after exposure of the thiobacilli to Na_2S for 60 or 90 sec. $^{14}CO_2$ data are derived from 1 to 7 separate experiments, and are values for *total* fixation during *complete* sulphide oxidation. Both values are expressed as % of control values. Vertical lines indicate the extreme values around the mean for $^{14}CO_2$ fixation. ATP values were calculated after subtraction of the endogenous ATP content in the absence of sulphide.

sulphide oxidation: Fig. 5*b* shows the inhibition of $^{14}CO_2$-fixation in bacteria allowed to oxidize equal amounts of sulphide completely. These values were derived from several experiments, and are to be compared with the ATP content of bacteria which were allowed to oxidize sulphide for a set time in two experiments. Clearly the formation of ATP during sulphide oxidation was at least as sensitive to DNP as was the coupled CO_2-fixation.

In a large number of experiments, DNP never inhibited oxygen uptake during sulphide oxidation. Thiosulphate oxidation by dilute suspensions was also unaffected or stimulated by DNP (Kelly & Syrett, 1963, 1964*a*). By using thiosulphate labelled in the outer ('reduced') atom with ^{35}S, 0·1 mM-DNP was found to have little effect on the kinetics of oxidation of the labelled atom by dense bacterial suspensions (in which oxidation was probably rate-limited by the concentration of dissolved oxygen). Figure 6 shows that labelled thiosulphate rapidly disappeared, with the parallel

formation of sulphate and the transitory accumulation of labelled trithionate, which was unaffected by DNP. The subsequent oxidation of the trithionate to sulphate was, however, slightly retarded by DNP (see Kelly & Syrett, 1964*a*).

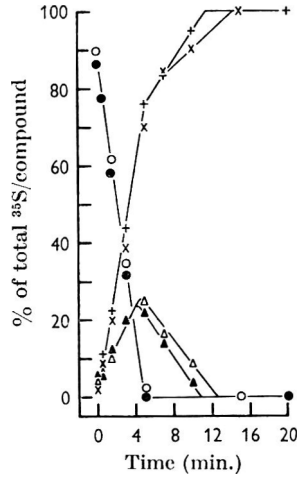

Fig. 6. Oxidation of $Na_2(^{35}S.SO_3)$ by thiobacillus strain c with and without 0·1 mM-DNP. 250 ml. flasks shaken at 26° contained in a final 12 ml. equiv. 33 mg. dry wt. bacteria; 1 mmole sodium phosphate, (pH 7·0); with or without DNP. 47 μmoles $Na_2(^{35}S.SO_3)$ (23 μc) were added at zero time. Samples (1 ml.) were pipetted into 1 ml. ethanol, centrifuged, and the supernatant fluids analysed chromatographically. With and without DNP, symbols are: $S_2O_3^{2-}$ –○–○– and –●–●–; SO_4^{2-} –×–×– and –+–+–; $S_3O_6^{2-}$ –△–△ and –▲–▲–.

Table 2. *Effect of 2,4-dinitrophenol on $^{14}CO_2$-fixation coupled to the oxidation of sulphur, sulphide and thiosulphate by thiobacillus strain c*

Warburg flasks contained in a final volume of 2·6 ml.: 5 mg. dry wt. bacteria; 200 μmoles sodium phosphate (pH 7); 6 μmoles $KH^{14}CO_3$ (4 μc); the indicated DNP concentrations. After pre-incubation for 50 min., 10 μmoles, $Na_2S_2O_3$, 2·5 μmoles Na_2S or 25 mg. elementary sulphur were added from the side arms. Temperature, 30°.

DNP concentration (M)	Initial rate of sulphur oxidation (μl./hr/flask)	Oxygen consumed in sulphur oxidation (μmoles/flask)	$^{14}CO_2$ fixed (counts/100 sec./flask)			$^{14}CO_2$ fixed/μmole or μg.atom substrate oxidized* (Counts/100 sec.)			($^{14}CO_2$ fixed/μmole or μg.atom substrate oxidized*) (%)		
			S°	S^{2-}	$S_2O_3^{2-}$	S°	S^{2-}	$S_2O_3^{2-}$	S°	S^{2-}	$S_2O_3^{2-}$
0	58	13·84	46,400	27,200	172,500	5,040	10,880	17,250	100	100	100
1×10^{-5}	58	13·80	26,800	—	—	2,920	—	—	58	—	—
5×10^{-5}	62	14·42	22,000	18,900	126,000	2,280	7,560	12,600	45	69	73
1×10^{-4}	54	13·17	13,600	—	—	1,540	—	—	31	—	—
2×10^{-4}	57	12·37	7,000	7,700	59,000	850	3,080	5,900	17	28	34

* Calculated for sulphur oxidation as 1·5 μmoles $O_2 \equiv 1$ μg.atom S°, from: $S^\circ + H_2O + \frac{3}{2}O_2 = H_2SO_4$.

Comparison of the effect of 2,4-dinitrophenol on $^{14}CO_2$-fixation with sulphide, thiosulphate or elementary sulphur as substrate

DNP had virtually no effect on the oxidation of elementary sulphur, but inhibited CO_2-fixation more than when sulphide or thiosulphate was oxidized (Table 2). The significance of this result in understanding the energy-coupling process is uncertain.

The relative amounts of CO_2 fixed during the oxidation of 1 μmole thiosulphate or sulphide and 1 μatom sulphur (calculated from oxygen consumed) were 100, 63 and 29, respectively, in this experiment.

DISCUSSION

These results show clearly that the addition of sulphide or thiosulphate to an aerobic suspension of *Thiobacillus* sp. strain c was followed within 10 sec. by the synthesis of ATP. But whereas the ATP synthesis which accompanied the thiosulphate oxidation was quite unaffected by the presence of 0·1 mM-2,4-dinitrophenol (DNP), that which accompanied the sulphide oxidation was strongly inhibited. These observations support the view that in this Thiobacillus, ATP can be synthesized by at least two different mechanisms, only one of which is inhibited by DNP. The sensitive mechanism appears to be the major one coupled to sulphide oxidation, at least in its early stages. At present the most probable interpretation is that the mechanism sensitive to DNP inhibition is similar to oxidative phosphorylation in mitochondria which is also inhibited by similar concentrations of DNP (Loomis & Lipmann, 1948). When thiosulphate is oxidized, the mechanism of ATP formation which is insensitive to DNP may well be that which Peck (1960) showed could accompany the oxidation of sulphite to sulphate by cell-free extracts of *Thiobacillus thioparus*. With these extracts, Peck showed that sulphite could be formed by the reductive cleavage of thiosulphate. Our results certainly suggest that the DNP-insensitive phosphorylation studied by Peck takes place in intact organisms and that therefore sulphite is an intermediate in thiosulphate oxidation. Whole organisms of *Thiobacillus* sp. strain c do not oxidize exogenous sulphite, possibly because it does not enter the organisms, so that the coupling of ATP formation to sulphite oxidation cannot be observed directly. From our results we cannot conclude anything about the origin of sulphite. It might be formed by reductive cleavage of thiosulphate, as Peck suggests, or by oxidation through a polythionate (London & Rittenberg, 1964).

ATP is required to drive the reactions of the Calvin cycle by which carbon dioxide appears to be fixed by Thiobacillus (Trudinger, 1956; Aubert, Milhaud & Millet, 1957). One might have expected, then, that in the presence of carbon dioxide the steady-state value of ATP would be lower. That this was not so presumably indicates that ATP formation coupled to thiosulphate oxidation was so rapid that the ATP concentration was maintained at its maximum value in spite of utilization by the carbon-dioxide fixation reactions.

When sulphide is the substrate, the effect of DNP is to decrease both the steady-state concentration of ATP and the rate of fixation of carbon dioxide and it is possible that the rate of carbon-dioxide fixation is limited by the amount of ATP available (Fig. 5*b*). But this cannot be so when thiosulphate is the substrate. Here the effect of DNP on carbon-dioxide-fixation, while less marked than when sulphide is the substrate, is nevertheless far greater than its effect on the steady-state level of ATP (Fig. 5*a*). In the presence of DNP the rate of carbon dioxide fixation must be limited by something other than the availability of ATP. This limiting factor could be the availability of reduced nicotinamide adenine dinucleotides, whose formation might require NAD(P) reduction with electrons from cytochromes reduced during thiosulphate oxidation (Aleem, 1965). Certainly, NAD reduction by reduced cyto-

chromes in mitochondria is dependent on ATP or a high energy compound (Chance, 1961; Danielson & Ernster, 1963) and is inhibited by DNP (Chance & Hollunger, 1963).

We have concluded earlier (Kelly & Syrett, 1964*a*) from the effect of DNP on carbon-dioxide-fixation that both DNP-insensitive and DNP-sensitive phosphorylations accompany thiosulphate oxidation. The decrease in the steady-state concentration of ATP at higher DNP concentrations with thiosulphate as substrate (Fig. 5), may indicate such a DNP-sensitive mechanism. However, the DNP concentrations producing the decrease are too high, in comparison with those affecting sulphide-dependent ATP synthesis, for this conclusion to be accepted with confidence. It is now clear, from the results presented in this paper, that the rate of carbon-dioxide-fixation is not a satisfactory index of ATP content when thiosulphate is the substrate. It may well be that thiosulphate oxidation is accompanied by both types of phosphorylation, but neither our results on carbon-dioxide-fixation nor those on ATP formation demonstrate this. Similarly, when sulphide is oxidized, it seems clear that the ATP formed initially is synthesized by a DNP-sensitive mechanism, but this does not rule out the participation of DNP-insensitive phosphorylation at a later stage in sulphide metabolism, perhaps at the level of sulphite oxidation.

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The Biosynthesis of Poly D-Glutamic Acid, the Capsular Material of *Bacillus anthracis*

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SUMMARY

Capsules of *Bacillus anthracis* only begin to be formed towards the end of exponential growth and appear first at the extremities of the cells. Once begun, capsule formation is not inhibited by tetracycline, so that capsular polypeptide is not synthesized like protein. Cultures were grown in broth + albumin in the presence of 0·015 M-HCO_3^- and 5 vol. CO_2 + 95 vol. air until capsulation began, and were then incubated in air in tetracycline broth (to inhibit subsequent enzyme formation): capsules continued to increase in size, which suggested that HCO_3^- made capsular synthesis possible but was not required for the formation of the polypeptide itself. Cultures transferred from air to adequate concentrations of CO_2 did not immediately become capsulated, whatever their stage of growth.

Mutants with altered nutritional requirements for capsulation were selected by phage α from wild-type strains grown either (1) on charcoal agar in air, so selecting for CO_2-independence (D mutants), or (2) on bicarbonate agar incubated in CO_2, so selecting for independence of an absorbent (F mutants). Both classes formed capsules in air ($\simeq$ 0·0001 M-HCO_3^-) and the capsular polymer apparently had the same chemical structure as that of their parent. No evidence was found that the mutants differed from their parent in being able to fix HCO_3^- more efficiently or in being able to utilize compounds normally derived from HCO_3^- that were present in the medium. They may therefore arise following mutation in regulatory genes controlling capsular synthesis.

Some D mutants grew very slowly in CO_2 but yielded CO_2-resistant mutants of which 66 were examined: 44 resembled the parental strain 2160S, and 22 were rough (C^-). CO_2-sensitive D mutants also gave rise to derivatives which formed rough colonies in air: some resembled strain 2160S; others were C^-; but some had a new phenotype in being rough in air, fully capsulated in 0·006–0·015 M-HCO_3^- and inhibited by 0·03 M-HCO_3^-.

Absorbents are required by wild-type strains to inactivate the long-chain fatty acids that occur in nutrient media. These acids are thought to interfere with the uptake or utilization of HCO_3^-, rather than with a later stage in capsular synthesis, because (1) they do not inhibit capsulation of D or F mutants, (2) they enable CO_2-sensitive D mutants to grow in CO_2, and (3) they probably interfere with toxin formation, which also requires CO_2. As toxin is protein it must be synthesized differently from capsular polypeptide, and the only stage common to both pathways is therefore likely to be HCO_3^- uptake.

INTRODUCTION

The capsule of *Bacillus anthracis* consists chemically of D-glutamic acid polymerized through γ linkages (Bricas & Fromageot, 1953; Housewright, 1962), while microscopically it appears as a uniform layer around fully capsulated organisms (Tomcsik, 1956). Capsules are formed by all pathogenic strains of *B. anthracis*, which become avirulent on mutating to the non-capsulated state, and large numbers of capsulated organisms are seen in blood taken from fatal cases of anthrax. One of the earliest observations concerning capsular synthesis was that virulent anthrax bacilli did not form capsules when subcultured on nutrient agar incubated in air (Bail, 1915), although they become capsulated when grown on serum agar (Sterne, 1937) incubated in air containing extra CO_2 (Ivánovics, 1937). Recently, however, serum was found to be replaceable by activated charcoal or anion-exchange resins, and its function is now thought to be the binding of long-chain fatty acids, like oleic and palmitic, which occur naturally in many culture media (Meynell & Meynell, 1964*a*). The amount of CO_2 required for capsulation varies with the pH of the medium in a way which shows that capsule formation depends not on the CO_2 concentration itself but on attaining a threshold concentration of bicarbonate ion (HCO_3^-) in the medium (Meynell & Meynell, 1964*a*). It is true that capsules are formed by organisms incubated in air + CO_2 on nutrient agar which contains $NaHCO_3$ without fatty-acid absorbents (Thorne, 1956), but capsule appears far later in the growth of the cultures and less is formed than when an absorbent is present. We have therefore suggested that the organisms either become phenotypically resistant to fatty acid as their growth rate falls or that they inactivate fatty acid as the population density increases (Meynell & Meynell, 1964*a*).

Bacillus anthracis also requires serum or charcoal as well as CO_2 for the synthesis of toxin and the protective antigen (Gladstone, 1948; Belton & Strange, 1954). Since the toxin is protein in nature, whereas the capsule is polypeptide and is not synthesized like protein (Meynell & Meynell, 1965), it seems likely that the only stage common to these biosynthetic pathways is the utilization of CO_2, and we therefore suggest that it is this stage which is inhibited by fatty acid. This hypothesis is also consistent with other evidence given below.

The steps which intervene between exposure to CO_2 and appearance of the capsular polymer have been studied by Housewright, Thorne and their collaborators (see Thorne, 1956, 1960; Housewright, 1962). Eastin & Thorne (1963) showed that ^{14}C in $^{14}CO_2$ rapidly appeared in compounds like aspartate and succinate, as in other genera (see Wood & Stjernholm, 1962), but the subsequent stages have not been defined (see Housewright, 1962, fig. 7, for a scheme based on this work). As regards the polymerization of glutamic acid to form the capsule, Hahn, Wisseman & Hopps (1954) concluded from studies with *Bacillus subtilis* which forms a mixture of poly D- and poly L-glutamic acid (Thorne & Leonard, 1958), that polymerization did not take place by the assembly of activated amino acids on ribosomes, as does protein synthesis, since polymer formation was not inhibited by chloramphenicol. This was confirmed by Leonard & Housewright (1963), who used a cell-free extract whose polymerizing activity was unaffected by chloramphenicol or ribonuclease. Leonard & Housewright also concluded from isotopic dilution experiments that poly D-glutamic acid is synthesized directly from L-, not D-glutamic acid. Our results

show that with *B. anthracis* the capsule-forming pathway is inactive during exponential growth and that capsules do not appear when tetracycline, with or without chloramphenicol, is added at this stage. However, when the organisms are approaching the stationary phase and capsulation has been initiated, it continues in the presence of these antibiotics, so that in *B. anthracis*, as in *B. subtilis*, polymerization does not occur in the same manner as protein synthesis.

Colonial variants eventually appear when virulent strains of *Bacillus anthracis* are repeatedly subcultured. Those of interest here fall into two main classes. The first class differs from the parental strain in forming capsules when incubated in air without added CO_2, which it does even on plain nutrient agar not supplemented with serum or charcoal. Mutation to CO_2-independence in respect of capsulation is therefore accompanied by indifference to fatty acid, which is consistent with our hypothesis that these acids interfere with HCO_3^- utilization rather than with a later stage in capsular synthesis. It also follows that mutants of this class can be selected in two ways from wild-type virulent strains: on charcoal agar incubated in air, to give CO_2-independent mutants (designated D mutants here) or on nutrient agar incubated in CO_2, to give fatty acid-indifferent mutants (designated F mutants). The second class of mutant discussed here comprises strains which are apparently unable to form capsules under any conditions and for this reason are used as avirulent live vaccines in veterinary practice (Sterne, 1937). These are designated C^-: about 50% revert to the virulent parental phenotype and 50% appear to be non-reverting (Meynell, 1963).

Table 1. *Colonial morphology of Bacillus anthracis strain 2160 s and its derivatives under various conditions*

	% CO_2 in atmosphere at pH 7·4*							
	0·03	0·6	1·2	2·5	5·0	10·0	20·0	40·0
	Colonial morphology							
Strain								
2160 s	R	R	R	R–M	M	M	M.In.	M.In.
C^- mutants	R	—	—	—	R	R	—	—
D mutants:								
CO_2-resistant	M	—	—	—	M	M	—	—
CO_2-sensitive	M	—	—	—	In.	In.	—	—
Derived from CO_2-sensitive D mutants:								
CO_2-sensitive C^-	R	R	R	In.	In.	In.	—	—
New class	R	R	M	M	M.In.	In.	—	—

* 0·03% CO_2 = air. 0·6% = 0·6 vol. CO_2 + 99·4 vol. air.

Colonial morphology: R, rough; M, mucoid; In. inhibited. All media incubated in additional CO_2 contain charcoal and $NaHCO_3$ (see Methods).

The first part of this paper discusses capsular synthesis by *Bacillus anthracis* strain 2160 s, which is wild-type in requiring CO_2 for capsulation (Meynell & Meynell, 1964*a*), and by one of its mutants, D 31. The second part considers the isolation and properties of mutants, some of which possess combinations of characters not previously described (Table 1). The degree of capsulation has been assessed throughout by microscopy and to this extent the results are qualitative. However, microscopy is essential for assessing the phenotypic heterogeneity of cultures and of individual

organisms. It will be seen below that not only do the organisms within a culture usually differ in respect of capsulation at a particular stage of growth but that when capsulation begins, it is normal for an individual organism to be capsulated at some parts of its surface and not at others.

METHODS

Culture media. The nutritional requirements of *Bacillus anthracis* strain 2160s, like those of many members of this genus, could not be defined completely, but included an absolute requirement for thiamine and either adenine, guanine, xanthine or hypoxanthine. A semi-defined medium (no. 2/6) was therefore used which contained 7 g. K_2HPO_4, 2 g. KH_2PO_4, 1 g. $(NH_4)_2SO_4$, 0·1 g. $MgSO_4.7H_2O$ and 0·55 g. trisodium citrate dissolved in 1 l. distilled water and supplemented with 10 g. Oxoid acid-hydrolysed casein, 2 mg. thiamine, 50 mg. adenine and 50 mg. tryptophan. The ability of strains to grow on different carbon sources was tested by adding 15 g. Oxoid Agar no. 3 to the salts mixture and by using smaller concentrations of supplements: 0·3 g. acid-hydrolysed casein, 2 mg. thiamine, 10 mg. adenine and 10 mg. each of tryptophan, methionine, glycine, valine, leucine and isoleucine. Carbon sources were included at a final concentration of 0·2 % (w/v). The plates were inoculated with drops of a saline suspension of each strain and, after incubation for 2 days, showed a dense area of growth if the carbon source was utilized; otherwise, only a thin grey film was seen.

Two undefined media were also used. No. 1/25 contained 1 g. Oxoid Lab-Lemco, 2 g. Oxoid Tryptone, 10 g. Oxoid Peptone, and 7 g. NaCl dissolved in 1 l. distilled water and adjusted to pH 7·4. This was used in liquid form for testing toxin formation, and in solid form for isolating mutants and scoring colonial appearances, since capsule formation was very pronounced on this medium. The second undefined medium was Oxoid Blood Agar Base on which capsulated colonies spread far less than on medium 1/25, an advantage in selecting mutants from rough outgrowths at their edges.

Media incubated in CO_2 contained that volume of a filtered molar solution of $NaHCO_3$ which was calculated to be in equilibrium with the chosen concentration of atmospheric CO_2 at pH 7·4. Thus, a final concentration of 0·03 M-$NaHCO_3$ was used with 10 % (v/v) CO_2 (see Meynell & Meynell, 1964*a*, fig. 1). One of two absorbents was also included to inactivate fatty acid; either 1/10 vol. of a filtered 7 % (w/v) of Bovine Serum Albumin Fraction V (Armour Laboratories) in distilled water, or 1/50 vol. of an autoclaved 10 % (w/v) suspension of charcoal MFC in distilled water (Hopkins & Williams, code 2997; this is finer than ordinary activated charcoal and does not settle in nutrient agar).

Bacterial strains and isolation of mutants. Bacillus anthracis strain 2160s is a non-toxigenic, asporogenous, derivative of the virulent strain 2160 (McCloy, 1958) and is wild-type in requiring additional CO_2 for capsule formation (Meynell & Meynell, 1964). D and F mutants were isolated by spreading 0·2 ml. of an overnight culture of strain 2160s in medium 2/6 on a plate of medium 1/25 which for the isolation of D mutants contained charcoal and was incubated in air, and for the isolation of F mutants contained $NaHCO_3$ and was incubated in 5 % (v/v) CO_2. After incubation for 4 hr, 0·2 ml. of a high titre stock of phage α, grown on strain 2160s, was spread over each plate (see Results), incubation continued for 18 hr, and the resulting

bacterial growth plated under the same conditions for isolated colonies. Each mutant arose independently since each of the overnight broth cultures of strain 2160s was inoculated from a different colony.

Inoculation and incubation of cultures. To avoid confusion, all inocula contained only non-capsulated organisms, for, if fully capsulated organisms are used, remnants of their capsules are seen on their progeny (Meynell & Lawn, 1965). Strain 2160s was therefore grown overnight in air in shaken liquid medium at 37°. Inocula of mutant D31 were prepared by incubating shaken cultures in air at 37° until they became faintly turbid, when they were transferred to room temperature (15–20°). The turbidity subsequently increased but less than 1% of the organisms showed signs of capsule next day. These cultures were diluted 1/100–1/400 in 30 ml. of medium 2/6 contained in plugged 250 ml. conical flasks carrying a horizontal side arm of $\frac{3}{4}$ in. diameter down which the culture could be tipped to measure its turbidity in a nephelometer (Evans Electroselenium Ltd.). This was set so that the $\frac{3}{4}$ in. ground glass standard gave a scale reading of 100; uninoculated liquid medium then gave a reading of about 12. A reading of 63 corrected for the blank corresponded to an extinction of 0·3 using a 1 cm. path and grey filter and to about 2×10^7 colony-forming units/ml. After inoculation, the flasks were incubated at 37° on a reciprocating shaker set at 100 strokes/min. with a 4 cm. throw. The necessary amounts of additional CO_2 for the gas phase were obtained either by placing cultures in anaerobic jars or by using stoppered flasks carrying inlet and outlet tubes and a second sidearm closed by a rubber ampoule-stopper, which allowed the culture to be sampled by needle and syringe without opening the flasks. The required concentration of CO_2 was obtained by replacing the appropriate volume of air by pure CO_2 at room temperature; thus, 5% CO_2 signifies 5 vol. pure CO_2 + 95 vol. air.

Cultures to be incubated in air presumed free from CO_2 were placed with their lids open in a glass jar, containing 20 ml. 20% (w/v) KOH, which was half-emptied five times and refilled with air passed through three wash-bottles containing 20% (w/v) KOH. In other experiments, open plates were incubated in a 3 l. desiccator containing 100 ml. 20% (w/v) KOH.

Staining methods. Samples of culture were fixed by addition to an equal volume of formalin (0·5%, v/v), and drops allowed to dry in air on flamed glass slides. A combined positive and negative stain, modified from Maneval (1934), was used; it consisted of 1 g. phloxine B (Edward Gurr or Hopkins and Williams), 2 ml. aqueous nigrosin (George Gurr), and 98 ml. distilled water. A few drops of stain were placed on the fixed film and the slide then set on edge on blotting paper and left to dry in air. The phloxine alone acted as a combined positive and negative stain which was not decolorized by nigrosin; it stained the bacterial cytoplasm and cell-wall pink, with the background a rather lighter pink. Nigrosin was added to improve the contrast. The capsular material therefore appeared unstained in relief.

The distribution of bacteria on the slides was markedly non-random because the capsulated organisms tended to congregate at the edges of the drops, possibly because they were held together by the strands of capsule seen in films stained by Ziehl–Neelsen's carbol fuchsin.

Toxin production. Each strain was grown for 20–24 hr at 37° in 4 ml. of medium 1/25 + albumin, held in a 100 ml. conical flask covered by a loose-fitting metal cap. For tests in the absence of added CO_2 the flasks were incubated on the shaker in

sealed anaerobic jars lined with blotting paper dipping into 50 ml. 20% (w/v) aqueous KOH lying on the floor of the jar. Although toxin formation is known to require more CO_2 than is present in air, toxin was nevertheless found in flasks incubated in jars filled with air alone, unless KOH was present, presumably because of CO_2 formed by the metabolism of the organisms (Puziss & Wright, 1959).

Toxin was detected by immunodiffusion in agar with the arrangement of reagents described by Lemcke (1964). The antitoxin (H/535, 8th immune) and control toxin preparation (B11 5/9) were kindly provided by Professor H. Smith, and were set up against wells filled with drops of the test cultures, with uninoculated medium as a negative control. Precipitin lines were visible after 2 or more days at room temperature; the results were recorded as + or −.

RESULTS

The physiology of capsule formation

Growth phase and the onset of capsulation. Strain 2160s growing in 5–10% CO_2 and its D mutants growing in air were examined at various stages of growth. No signs of capsule were seen until the culture was at least moderately turbid e.g. in liquid medium when the nephelometric reading (NR) reached 30, equivalent to about 10^8 *Escherichia coli*/ml. Individual organisms never became capsulated simultaneously, and heterogeneity was still evident even 12 hr later. Initially, capsule did not appear as a uniform layer, but in wedges placed at each side of the tip of a cell or at an intercellular junction where opposing wedges often joined so that the capsulated area was indented (Pl. 1; figs. 1, 2). Fragments of capsule are also seen in growing cultures when the inoculum consists of capsulated bacilli (Meynell & Lawn, 1965), but these are readily distinguished from newly formed capsule since at the ends of chains, they have a smooth convex outline and are not indented between adjoining organisms (Pl. 1, figs. 3–6).

The change in growing cultures that initiated capsule formation in late exponential growth has not been identified, but it was shown not to be the formation of a capsulogenic factor, which accumulated in the medium as the organisms grew, or a nutritional deficiency. Cultures were incubated in medium 2/6 for about 6 hr until nearly all the organisms showed wedges and the medium then sterilized by centrifugation and passage through a Seitz or a membrane filter. The filtrate was reinoculated in the usual way and the behaviour of the organisms compared with that of a control culture in filtered unused medium. However, neither the time of onset of capsulation nor its degree differed in the two cultures. Other causes were also considered. A change in pH value was not responsible, as even 18 hr cultures were only pH 7·4 compared to an initial value of 7·2. Oxygen deficiency seemed more likely, but transfer to an anaerobic environment did not induce capsulation. This was done in various ways. The culture (NR = 10) was tipped into the side-arm of the culture flask which was flushed with nitrogen for 3–15 min., sealed, and incubated at 37°. The growth rate halved thereafter and, although 8 hr later the culture was as turbid as in air, wedges were seen on less than 10% of bacilli while less than 5% were fully capsulated. Transfer to anaerobic jars gave similar results.

The effects of tetracycline or adenine deficiency on capsule formation. When tetracycline (30 μg./ml.) was added to growing cultures, the turbidity immediately ceased

to increase but the subsequent effect on capsule formation depended on the time at which tetracycline was added. When the culture was in early exponential growth (NR < 10) and the organisms were therefore uncapsulated, they remained uncapsulated. However, when the culture was approaching the stationary phase (NR > 120) and showed early uniform capsulation, the capsules subsequently increased in size during the next 2–3 hr. At intermediate times when wedges but not uniform capsulation were present, the addition of tetracycline led to the formation of chains containing both capsulated and non-capsulated organisms (Pl. 1, fig. 7).

Similar results were obtained when growth was limited by adenine deficiency. When adenine was omitted from medium 2/6, the turbidity increased exponentially until the NR $\simeq$ 20 (presumably because of contaminating purine and pyrimidine bases) and then increased linearly, and films again showed mixed chains containing fully capsulated and non-capsulated bacilli.

These observations suggested that capsulation results from two changes in the organism: activation of the capsule-forming pathway; and its functioning to produce sufficient capsular polymer to be visible under the microscope, a process which takes at least 60 min., judging from successive films of tetracycline-inhibited cultures. The two stages, which we refer to as 'activation' and 'polymerization' respectively, can therefore be examined, as in Table 2, by exposing cultures to tetracycline, since this prevents subsequent activation but not polymerization. A failure to form capsule might result from a defect in either function. Thus, if capsules appeared when organisms from non-capsulated cultures were subcultured in tetracycline-containing medium, it would suggest that the original absence of capsulation was due to failure of polymerization, not of activation (provided subculture excluded the presence of C^- mutants).

Effect of other antimetabolites. The effect of other antimetabolites was tested on cultures in which wedges of capsule were visible. Chloramphenicol did not stop the cultures growing completely, even at 250 μg./ml., and when tested simultaneously with tetracycline, it did not inhibit capsule formation. Nor did the L(+) *erythro* isomer of chloramphenicol inhibit capsulation, contrary to its effect on polymer formation by *Bacillus subtilis* (Hahn *et al.* 1954). Other agents without effect in the presence of tetracycline included benzylpenicillin (0·001 μg./ml.), sodium malonate or fluoroacetate (each at 0·002 M) and probably streptomycin (100 μg./ml.); but complete inhibition was produced by sodium azide (0·002 M), potassium cyanide (0·002 M) and 2,4-dinitrophenol (DNP; 0·001 M). Streptomycin, azide and cyanide were also tested alone; they immediately inhibited both growth and capsule formation. When DNP was tested alone, the turbidity continued to increase though the doubling time rose from 40 to 80 min., but more than 90 % of the organisms remained non-capsulated, even after incubation for 48 hr.

Nutritional requirements for capsulation. The minimal cultural requirements for capsule formation by mutant D 31 were very simple. The organisms became uniformly capsulated when they were grown in complete medium until wedges began to form, and were then washed twice by centrifugation and shaken for 2–4 hr in air in phosphate buffer (pH 7·4) containing L-glutamic acid (10 μg./ml.), glucose (0·1 %, w/v) and thiamine (2 μg./ml.). No capsules were formed when glucose or glutamic acid was omitted. Incubation in this medium did not cause activation, judging from

the fact that less than 100 % of organisms became capsulated. In other experiments cells were subcultured from complete medium before wedges had formed, and after incubation for 2 hr in the defined mixture were transferred to complete medium + tetracycline; capsules did not then appear.

Glucose was previously found to inhibit capsule formation by strain 2160s (Meynell & Meynell, 1964*a*) and other utilizable sugars have the same effect. However, this is not an instance of catabolite repression (Magasanik, 1961), since capsulation occurs normally when the medium is adequately buffered. The effect of acidity cannot be only to lower the HCO_3^- concentration in the medium because it also inhibits capsule formation by D and F mutants which do not require HCO_3^- (see below); it may therefore directly inhibit the capsule-forming pathway.

The last few generations of growth were also accompanied by a marked diminution in the length of the chains of organisms. Before this, each chain was often very long and contained perhaps 100 organisms, often plaited with other chains to form ropes which were visible as floccules suspended in the medium. As exponential growth came to an end, the number of organisms in each chain diminished and the culture became uniformly turbid.

Table 2. *The relation of HCO_3^- to capsule formation by Bacillus anthracis strain 2160s*

Culture grown initially in medium 2/6 + albumin incubated in:	Transferred to	Outcome
5 % CO_2*	5 % CO_2	Continuing capsulation
	5 % CO_2 + tetracycline†	Continuing capsulation
	Air‡	Capsulation subsequently prevented§
	Air + tetracycline‡	Continuing capsulation
Air	5 % CO_2	No capsules formed
	5 % CO_2 + tetracycline	
	Air	
	Air + tetracycline	

* 5 vol. CO_2 + 95 vol. air. Transferred when 10–50 % organisms showed wedges.

† 30 μg./ml. medium.

‡ Dissolved CO_2 was removed from the medium by adding the calculated volume of 0·25 N-HCl, and by repeatedly evacuating the culture vessel until the culture boiled.

§ Capsule present at the time of transfer was subsequently partitioned amongst the progeny (see Pl. 1, figs. 3–6; and Meynell & Lawn, 1965).

Bicarbonate ion and capsule formation by strain 2160s. Capsule formation by wild type strains of *Bacillus anthracis* depends not only on the growth phase of the cultures but also on the presence of a threshold concentration of HCO_3^- in the medium (Meynell & Meynell, 1964*a*). The function of HCO_3^- was therefore examined in a series of experiments in which strain 2160s was grown, for example, in CO_2 and then transferred to air, using tetracycline to inhibit subsequent growth and activation without affecting polymerization (see Table 2). The first half of Table 2 concerns cultures grown initially in CO_2 until 10–50 % organisms showed wedges. Continued incubation in CO_2 led to capsule formation by 100 % organisms in the usual way, whereas when tetracycline was present, less than 100 % became capsulated

(presumably because activation was not universal), though their capsules were often unusually large. When the organisms were incubated in air without tetracycline, growth continued but the cell-wall formed subsequently was not capsulated. Thus, in experiments where initially the organisms were fully capsulated, after further incubation in air for 2 hr without tetracycline none were fully capsulated and many showed non-capsulated regions as well as remnants of capsule (Pl. 1, figs. 3–6; Meynell & Lawn, 1965). However, in the fourth sample, containing organisms transferred from CO_2 to air with tetracycline, capsulation progressed, and the organisms resembled those in the second sample incubated in CO_2 with tetracycline. The fourth sample therefore showed that although HCO_3^- was required for activation of the capsule-forming pathway, it did not need to be present for capsule to appear. Assuming that the formation of visible capsule under these conditions reflects the synthesis of new polymer and not the secretion of polymer already formed at the time of transfer to air, it follows that HCO_3^- is not an essential precursor of the capsular material. When cultures are grown in $^{14}CO_2$ some of the label taken up by the organisms appears in the polymer (Eastin & Thorne, 1963), but this may simply mean that carbon derived from HCO_3^- becomes generally available for biosynthesis.

The second half of Table 2 concerns organisms grown initially in air and then treated in the same way as those grown initially in CO_2. In this case, no capsulation was seen, even in the samples transferred to CO_2 without tetracycline. Hence, the organisms could not respond immediately to HCO_3^-. In similar experiments samples were transferred at intervals to CO_2, and examined after incubation for various lengths of time. No signs of capsulation ever appeared rapidly (i.e. within 3 hr or less after transfer to CO_2), whatever the turbidity of the parent culture (NR = 10–120), although on prolonging incubation to 16 hr, capsules and wedges were formed, their incidence being greater, the younger the parent culture at the time the subculture was made. Since Eastin & Thorne (1963) have shown that CO_2 is fixed equally well by *Bacillus anthracis* whether it is previously grown in air or in CO_2, the failure of capsulation to begin immediately air-grown cultures are transferred to 5–10 % CO_2 showed that HCO_3^- or its derivatives do not act by inhibiting functions that otherwise prevent capsule formation. The long lag between transfer to CO_2 and the appearance of capsule is not inconsistent with induction of the capsule-forming pathway by HCO_3^-, for part of the lag represents the time required to form visible amounts of capsule. Moreover, although induction often occurs at the maximal rate, so that the enzyme content per bacillus increases hyperbolically to reach 50 % of its ultimate value after one generation, many cases are known in which induction occurs far more slowly (see Pollock, 1962, p. 162).

Effect of mutation on capsule formation

Isolation of D and F mutants from wild-type strains. The mutants described in the literature seem to have been isolated accidently when wild-type strains were subcultured in the laboratory. However, large numbers of mutants can easily be isolated even from virulent wild-type strains by using phage α described by McCloy (1958). This phage adsorbs to genotypically susceptible organisms only while they are non-capsulated: consequently, if it is added to a wild-type culture grown in air, the wild-type organisms are uncapsulated and are destroyed, whereas any mutants

forming capsules are unaffected and are thereby selected. This occurs despite the fact that the mutants are non-capsulated while growing exponentially: presumably, exponential growth stops sooner than phage multiplication in heavily inoculated plates.

The nature of D and F mutants. The chemical structure of capsular material formed by mutants of *Bacillus anthracis* in air appears to be identical with that formed by their wild-type parents in CO_2. Only a few strains have been examined exhaustively in this respect (see Bricas & Fromageot, 1960; Housewright, 1962), notably the virulent wild-type strain Vollum, its mutant, HM, which appears to resemble our D or F mutants (White, 1946), and its non-proteolytic mutant NP (Wright, Hedberg & Feinberg, 1951). None of 12 of our mutants yielded capsular material after autoclaving which differed from that of strain 2160s in mobility in low voltage electrophoresis (Torii, 1959). Also this material precipitated with $CuSO_4$ at pH 6·5 (Bovarnick, 1942) and, after complete hydrolysis by HCl, yielded a preponderance of glutamic acid. Traces of peptides and other amino acids were also present but to the same extent in parental and mutant preparations and were presumably derived from the broth-based nutrient agar used for growing the organisms.

The parent and its mutants therefore appeared to form the same product, but whereas the parent required CO_2, the mutants did not. In considering how this situation could arise, it should be recalled that the bicarbonate requirement for capsule or toxin formation cannot be relieved by supplementing the medium with compounds likely to be formed in CO_2 fixation. Those tested include aspartate, succinate, glutamate, oxaloacetate, citrate, fumarate, malate and orotic acid (Puziss & Wright, 1954; Meynell & Meynell, 1964*a*). The mutants might therefore differ from their parent in being able to utilize a CO_2-fixation product which occurs naturally in complex culture media. Alternatively, they might still be dependent on atmospheric CO_2 for capsule formation but be able to fix it more efficiently than their parent. If either of these explanations was valid, D and F mutants would be expected to form toxin as well as capsule in broth incubated in air. Since strain 2160s is non-toxigenic, this hypothesis was tested with strain 1444, a typical virulent wild-type strain susceptible to phage α (Meynell, 1963). Four D mutants were isolated and, for safety, a C^- mutant then isolated from each for tests of toxigenicity. Four C^- mutants were also isolated directly from the wild-type strain, 1444, as controls on the effect of mutation from C^+ to C^- on toxigenicity. None of the eight C^- mutants formed toxin on incubation in air (see Methods), although two of each group did so after incubation in 10 % CO_2. Therefore, the D mutants, though able to dispense with added CO_2 for capsule formation, did not at the same time become capable of toxin formation in air. Furthermore, any CO_2-fixation product such as aspartate which could be utilized by the mutants and not by strain 2160s could presumably be used also as a carbon source for growth, but the mutants were found to use the same carbon compounds as their parent; namely, aspartate, pyruvate, oxaloacetate, glucose, and galactose, but not citrate, fumarate, succinate, oxoglutarate or malate. Nor was there any evidence that the mutants formed capsules in air because they fixed atmospheric CO_2 more efficiently, since their colonies had the same appearance whether incubated in air or in air presumed free from CO_2. The remaining possibilities are either that the mutants synthesize a product normally derived from HCO_3^- which acts as an internal inducer (Table 2 shows that it is

not an essential precursor) or that they carry mutations in genes which regulate enzyme formation in the capsule-forming pathway (Meynell & Meynell, 1964*b*).

Response of D mutants to CO_2 and fatty acid. When grown in air, all D mutants were as capsulated on nutrient agar as on charcoal agar. However, they fell into different categories on incubation in 5–10% CO_2, for some grew as well as in air whereas others were inhibited. The former are therefore referred to as 'CO_2-resistant' and the latter as 'CO_2-sensitive'. A third type was inhibited by CO_2 on charcoal agar but not on plain agar. Although they appeared to be of three types, all D mutants may be basically the same and show differences which appear as qualitative because of the method of testing. If our hypothesis that fatty acids prevent CO_2

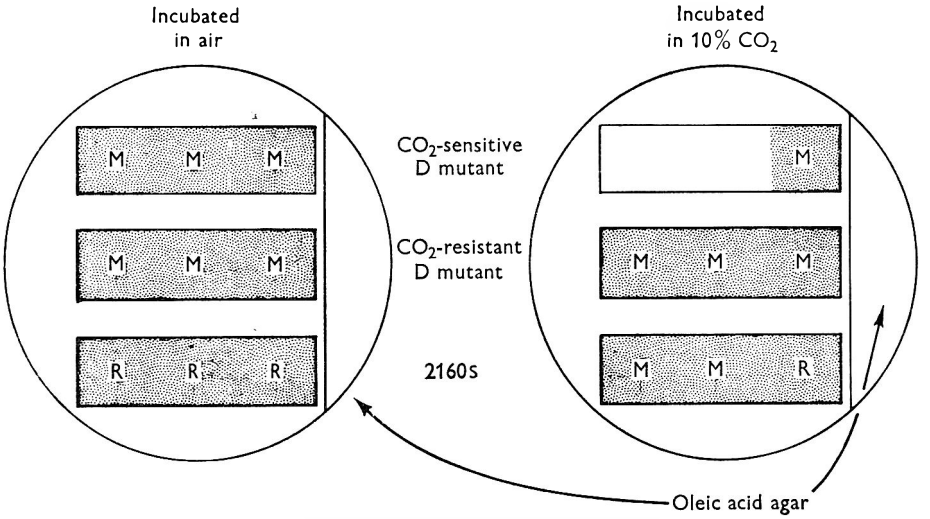

Fig. 1. The behaviour of *Bacillus anthracis* D mutants and of strain 2160s in air and in CO_2 (see text). Each plate had a ditch at one side containing 1 ml. of 5% (w/v) oleic acid in water + 5 ml. nutrient agar. M: mucoid growth of capsulated organisms. R: rough growth of non-capsulated organisms, which subculture showed to belong to the strain inoculated and not to consist of C^- mutants selected on the plate. The rectangles indicate the area inoculated, and stippling the area over which growth occurred.

utilization were correct, the CO_2-sensitive strains should be able to grow in CO_2 when fatty acids are present. The CO_2-resistant D mutants also provided a test of this hypothesis for, being CO_2-independent, they should be capsulated in the presence of concentrations of fatty acid which inhibit CO_2 uptake and therefore capsule formation by the parental strain, 2160s. All the D mutants were therefore compared with strain 2160s by lightly streaking them on plates of charcoal agar with a ditch at one side which contained oleic acid, one of the most abundant long-chain fatty acids found in agar and which is known to inhibit capsulation of wild-type strains of *Bacillus anthracis* growing in CO_2 (Meynell & Meynell, 1964*a*). The results after incubation in air and in CO_2 are shown diagrammatically in Fig. 1. CO_2-sensitive mutants formed a mucoid streak throughout the inoculated area in air, but in CO_2 grew only next to the ditch containing oleic acid, where again they were mucoid. The CO_2-resistant mutants grew throughout the inoculated area both

in air and in CO_2, and were also mucoid throughout. Strain 2160s was totally rough in air, while in CO_2 it was rough near the ditch and mucoid elsewhere. All these findings are consistent with inhibition of CO_2 uptake by fatty acid, which incidently appeared as a growth factor for CO_2-sensitive mutants incubated in CO_2. The intermediate type, which grew in CO_2 on nutrient agar but not on charcoal agar, presumably consisted of strains with only an intermediate degree of CO_2-sensitivity, and the fatty acid normally present in nutrient agar presumably restricted their uptake of CO_2 sufficiently to permit growth.

The effect of CO_2 on CO_2-sensitive D mutants. These mutants were originally identified by their behaviour on solid medium incubated in 10% CO_2, as their colonies instead of being perhaps 5 mm. in diameter after incubation for 20 hr were at the most pinpoint in size. This suggested that CO_2 was inhibitory but not rapidly lethal to the organisms, and when exponentially-growing cultures of the CO_2-sensitive mutant D14 were exposed to 10% CO_2, the colony count and turbidity both continued to increase, albeit very slowly (e.g. with a doubling time of 3 hr). Microscopy showed that the organisms appeared normal and also that they became heavily and uniformly capsulated in 1–1·5 hr, whereas control cultures incubated in stoppered flasks without additional CO_2 became capsulated later and heterogeneously, despite their considerably greater turbidity.

When CO_2-sensitive mutants were incubated on charcoal agar in CO_2 either they were completely inhibited or a few CO_2-resistant colonies of normal size were found at the primary site of inoculation. A total of 66 CO_2-resistant clones were isolated from 6 CO_2-sensitive D mutants: 44 resembled the parental strain, 2160s, in being rough in air and capsulated in CO_2 (Table 1); while the remaining 22 appeared to be rough under all conditions and were therefore classified as C^- mutants. No strains resembling CO_2-resistant D mutants were isolated.

Derivatives of CO_2-sensitive D mutants selected for rough colony formation in air. As some of the above CO_2-resistant derivatives were C^-, C^- mutants were next isolated from CO_2-sensitive D mutants grown in air to see if mutation from C^+ to C^- always led to CO_2-resistance. Of 61 rough outgrowths thus selected from 6 D mutants, 14 had the phenotype of strain 2160s, as their colonies were mucoid in 10% CO_2 but rough in air (Table 1); 24 appeared to be C^- and CO_2-resistant, like the C^- mutants selected by CO_2; and 23 grew in air but not in 10% CO_2 and were therefore at first recorded as C^- but CO_2-sensitive. However, when 11 of the latter were examined more closely, only 4 proved to be true C^- as the remaining 7 formed capsules in small concentrations of CO_2. Each of these 7 strains was grown in broth in air for 18 hr to give cultures containing chains of 2–4 bacilli. Viable counts were made by a drop method (Miles & Misra, 1938) on plates of charcoal agar which were then incubated in air and in either 0·6, 1·2, 2·5, 5 or 10% CO_2. Incubation in air or 0·6% CO_2 gave only rough colonies; in 1·2–2·5% CO_2 the colonies were fully mucoid and the colony count equalled that in air; but in 10% CO_2 the number of colonies of normal size was less than 0·2% of its former value, although a background of pinpoint mucoid colonies was seen whose count equalled the count on plates incubated in air. Hence, these strains had a new phenotype in that capsule formation required CO_2, though in concentrations about one-tenth of that required by strain 2160s, and growth was inhibited by higher CO_2 concentrations. Capsule-forming ability by these strains was sometimes detectable by microscopy after in-

cubation in air, for as many as 1 % of organisms might be capsulated although the colony appeared rough.

F mutants. These were defined as strains selected for their ability to form capsules in 5 % CO_2 on bicarbonate agar not supplemented with an absorbent. All of 14 strains examined were fully capsulated when grown in air or CO_2 on medium 1/5, whether or not it contained charcoal. These mutants may therefore correspond to D mutants able to grow under these conditions.

DISCUSSION

Two environmental factors evidently determine capsule formation by typical strains of *Bacillus anthracis*. The first is CO_2 (Ivánovics, 1937) or, to be more exact, the concentration of HCO_3^- in the medium (Meynell & Meynell, 1964*a*) whose function is to alter cellular metabolism so that capsule begins to be formed once the other cultural conditions are suitable (Table 1). Bicarbonate neither provides essential precursors for the capsular polymer nor inhibits reactions which interfere with its synthesis. The second environmental factor is an unknown change which occurs in the culture as the organisms approach the stationary phase. Once both factors are present, capsule formation begins and the effect of tetracycline shows that it comprises at least two stages—activation and polymerization.

One effect of activation is presumably to make glutamic acid available for polymerization; the results obtained with other systems suggest how this may occur. On approaching the stationary phase, the bacterial growth rate falls and less glutamic acid is incorporated in mucopeptide and other cell structures. At the same time, larger amounts of glutamic acid may be synthesized from α-oxoglutarate derived from the tricarboxylic acid (TCA) cycle (see Umbarger & Davis, 1962; Housewright, 1962; Huang, 1964) which is known to become more active in *Bacillus cereus* in the stationary phase (Goldman & Blumenthal, 1964). On this model, HCO_3^- promotes capsulation by being fixed in compounds whose presence further increases the activity of the TCA cycle, and hence the synthesis of glutamic acid. In support of this suggestion it may be noted that incubation in CO_2 increased the excretion of unpolymerized glutamic acid by a Bacillus strain studied by Chao & Foster (1959). Bicarbonate or its derivatives are only required to increase the activity of the TCA cycle and, once this is done, their presence is no longer essential for capsule formation when growth is prevented, as by tetracycline, since the cycle can presumably draw on other compounds for its syntheses. Any ^{14}C assimilated from $^{14}CO_2$ would be expected to appear in a variety of compounds associated with the cycle, and not to be confined to capsular polymer. The inhibitory effect on capsulation exerted by anaerobiosis (Sterne, 1937) also becomes comprehensible, since the TCA cycle is then largely inactive in *B. anthracis* and glucose is fermented to yield 2,3-butyleneglycol, acetylmethylcarbinol and a variety of organic acids, including lactic acid (Puziss & Rittenberg, 1957). Similar changes occur in *Micrococcus glutamicus*: during aerobic growth glucose is metabolized to form glutamate, whereas in anaerobic culture, lactic acid accumulates in place of glutamate (see Kinoshita, 1959).

A cell-free system capable of polymerization has been isolated by Leonard & Housewright (1963) but, unless it is non-specific, it seems unlikely to be present before activation occurs. A more probable sequence of events is that its formation is

induced by the accumulation of glutamic acid, just as polyphosphate is formed by an inducible system in *Aerobacter aerogenes* (Harold, 1964).

All the findings previously obtained with *Bacillus subtilis* (Hahn *et al.* 1954; Leonard & Housewright, 1963) and those given here for *B. anthracis* show that tetracycline and/or chloramphenicol permits polymerization of glutamic acid although inhibiting growth. Polymerization, unlike protein synthesis, evidently does not depend on the assembly of activated amino acids on ribosomes and, to this extent, it resembles the synthesis of other peptides like the antibiotics gramicidin (Winnick & Winnick, 1961) and tyrocidine (Mach, Reich & Tatum, 1963; Mach & Tatum, 1964), and those which occur in cell walls (Rogers & Mandelstam, 1962). Another sign that the amino acid sequence of these peptides is not specified like that of protein is that the composition of tyrocidin is partly determined by the constituents of the culture medium (Mach & Tatum, 1964). The structure of the polymer formed by *B. subtilis* is also determined by the environment, but here it is the ratio of poly D- to poly L-glutamic acid in the capsular material that changes and not the structure of individual polymer molecules (Thorne & Leonard, 1958). The polymerization of glutamic acid to form capsule has been discussed by Katchalski, Sela, Silman, & Berger (1964) in the same context as the formation of polyphenylalanine or polylysine in the model system for protein synthesis introduced by Nirenberg (see Nirenberg *et al.* 1963), but the present evidence strongly suggests that the analogy is misleading.

When capsulation begins, the ends of each cell become capsulated before the equator which is known to be the most recently synthesized region (Pl. 1, figs 1 and 2; Meynell & Lawn, 1965). The equator might be incapable of capsule formation (e.g. because glutamic acid is deviated to synthesis of cell-wall mucopeptide). Alternatively, every part of each cell might be capable of polypeptide synthesis but capsule is seen last at the equator because it is formed while the cells are still growing. The production of sufficient polymer to be recognizable as a capsule seems to take about 60 min., judging from the rate at which capsules appear in tetracycline-inhibited cultures. Since the doubling time is 40–60 min., the newly formed equator of each cell may not have time to produce a visible capsule before continuing growth and division cause it to be partitioned amongst the daughter cells, of which it forms the adjacent ends (Fig. 2). This explanation presupposes that when the growth of such cells is stopped by tetracycline, further incubation should lead to uniform capsulation because the equators should then have sufficient time to produce visible amounts of polymer. In fact, if tetracycline is added to a culture when wedges but not uniform capsules are present, the majority of cells do become uniformly capsulated 2 hr later, although a minority, which may, of course, have been killed by the antibiotic, still show wedges only.

The HCO_3^- requirement for capsule formation is lost on mutation but the chemical structure of the capsular polymer formed by D and F mutants in air appears to be identical with that formed by the wild-type in CO_2, so far as is known at present. Hence, the mutants appear to be constitutive in respect of capsule formation. The results suggest that the mutant phenotype does not arise from increased ability to fix atmospheric CO_2 or from accessibility to compounds which can replace CO_2, and the mutants presumably either form an internal inducer, which might be normally derived from HCO_3^-, or the enzymes concerned are synthesized in the

absence of an inducer. The mutants certainly resemble constitutive mutants in other systems. They are so readily isolated that they presumably arise by one mutation, rather than several. They differ from the parental strain only in not requiring CO_2, and the capsule is formed only at the end of exponential growth.

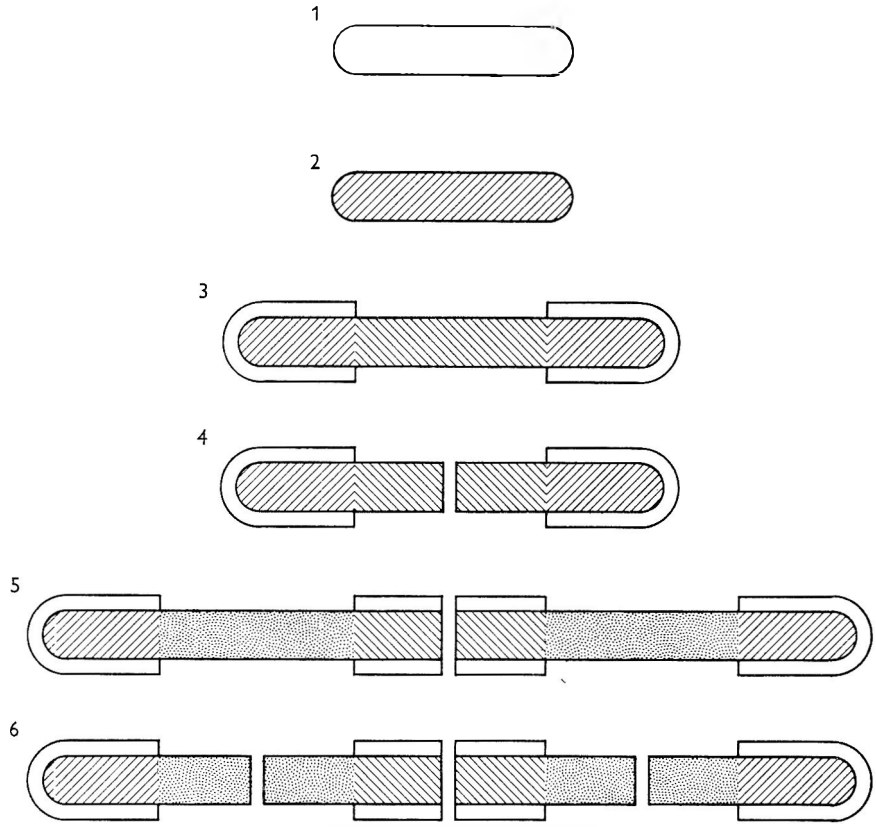

Fig. 2. An explanation for the non-uniform distribution of capsule on individual chains during the early stages of capsulation (see Pl. 1, figs, 1, 2). (1) Bacillus in which the capsule-forming pathway is inactive. (2) Bacillus in which activation has occurred. In this and succeeding drawings, the time at which each part of an organism is assumed to become activated is indicated by cross hatching and stippling. Although activation has occurred in 2, the organism does not become capsulated immediately because formation of sufficient polymer to be visible microscopically takes about 1 generation time. (3) The bacillus shown in (2) just before it divides. Elongation preparatory to division occurs by equatorial growth (Meynell & Lawn, 1965). The equator in (3) is therefore new and non-capsulated, while the extremities have had time to form a visible capsule. (4), (5), (6) The distribution of capsule during the second generation.

The amount of capsule formed in air is always greater than that formed by the parent in CO_2, judging from colonial appearances and stained films; this is compatible with constitutivity since constitutive mutants in general synthesize more of the appropriate gene-product than does the parent.

Although D mutants do not require CO_2 for capsule formation, they still respond to concentrations of HCO_3^- which induce capsulation of the parent. This is shown by the inhibitory effect of 5–10 % CO_2 on the CO_2-sensitive type of D mutant and

by the behaviour of certain CO_2-resistant D mutants whose colonies, following repeated subculture, were no longer fully mucoid on incubation in air but became so when additional CO_2 was present. This suggests a cause for the inhibitory effect of CO_2 on CO_2-sensitive D mutants. Their metabolism is evidently switched towards capsule formation, even in air, and the extra stimulus provided by additional HCO_3^- may therefore be thought of as dislocating cellular metabolism to a degree incompatible with normal growth. Growth of even the parent strain, 2160s, is inhibited by 20–40% CO_2 at pH 7·4, possibly for the same reason. Constitutive mutants of a given type generally differ in their rates of enzyme synthesis, which would account for the differing CO_2-sensitivity of individual D mutants. Those with the greatest activity of the capsule-forming pathway are presumably the CO_2-sensitive strains since these would be least able to tolerate further increase in its activity, while those with least constitutive production are presumably CO_2-resistant. The third class inhibited by CO_2 on charcoal but not on nutrient agar, is presumably constitutive to an intermediate degree and is able to grow when HCO_3^- uptake is lessened by the fatty acids present in nutrient agar. The D mutants, taken as a class, presumably possess every degree of constitutivity but fall into three groups because we can score their response to CO_2 in only three sets of conditions. Sensitivity to CO_2 also appears when *Mycobacterium tuberculosis* loses its requirement for CO_2 for growth. Such mutants are thought to assimilate CO_2 more efficiently than their parent owing to an increased production of biotin, and, as expected, biotin analogues enable them to grow in CO_2 although the same analogues inhibit the growth of their CO_2-requiring parent (Schaefer, 1957). The effect of these analogues on the growth of *M. tuberculosis* therefore bears a strong resemblance to the effect of fatty acid on capsulation by *Bacillus anthracis*, though the D and F mutants are unlikely to fix CO_2 more efficiently since they are capsulated even when incubated over KOH. It would be of interest to determine whether CO_2-sensitivity accompanies mutation to CO_2-independence in other genera. This may be of some practical importance if CO_2-sensitive mutants remain pathogenic, considering that incubation in CO_2 is used routinely in the bacteriological diagnosis of many infections.

Our suggestion that fatty acids interfere with the utilization of HCO_3^- (Meynell & Meynell, 1964*a*) can be verified with $^{14}CO_2$ (see Eastin & Thorne, 1963), and is now supported by several pieces of evidence. The fatty acids protect CO_2-sensitive D mutants from the inhibitory effects of CO_2 and evidently do not interfere with a relatively late stage in the biosynthetic pathway which leads to polymerization, for D mutants are capsulated on oleic acid ditch plates (Fig. 1). On the other hand, the dependence of toxin production upon the presence of an absorbent suggests that fatty acid also interferes with toxin synthesis. The only point common to toxin and to capsular synthesis seems likely to concern HCO_3^-, and it is therefore reasonable to suppose that this is the function which is affected by fatty acid. A mechanism by which fatty acids could inhibit HCO_3^- metabolism is implicit in the model advanced by Kodicek (1949, 1962) to explain their bacteriostatic action. He suggested that long-chain fatty acids penetrate cell membranes and disturb their structure together with any associated enzymic functions. On this view, membranes could be disordered to differing extents, ranging from a structural breakdown leading to lysis of cells like erythrocytes or protoplasts, to killing or bacteriostasis without lysis, and lastly, to inhibition of non-vital functions such as capsule formation or

sporulation (Hardwick, Guirard & Foster, 1951) without interference with growth. The inhibitory effects of phenols and other surface-active agents on the formation of flagella and Vi antigen (see Lacey, 1961) may arise in the same way.

A colony of *Bacillus anthracis* appears rough to the naked eye when it contains less than about 1 % of capsulated organisms. This occurs for two reasons: either the organisms are genotypically capable of capsule formation but the conditions are unsuitable, as with wild-type strains grown in air; or the capsule-forming pathway has become inactive following mutation, as in the C$^-$ mutants whose colonies are rough under all conditions. At least three classes of C$^-$ mutant might be expected, even with our present knowledge:

(1) Those in which HCO_3^- uptake or fixation is blocked. These should be recognizable because, if derived from wild-type, they should be non-toxigenic (as were 4 of 8 mutants of strain 1444) and, if derived from CO_2-sensitive D mutants, they should be CO_2-resistant (as were 24 of 61 mutants tested).

(2) Those in which the polymerizing enzyme is inactive and which consequently excrete either unpolymerized glutamic acid or a derivative into the medium. If derived from wild-type, excretion should occur only in CO_2, but if derived from a D or F mutant, excretion should occur also in air. Our colleague Dr M. W. McDonough examined supernatant fluids of cultures of mutant D31 and 8 of its C$^-$ derivatives by paper electrophoresis and found equal amounts of glutamic acid in each case (see Proom & Woiwod, 1950), but whether it consisted of the D- or L- isomer was not determined.

(3) Those blocked at intermediate stages which fix HCO_3^- but which do not excrete glutamic acid.

One class of rough colony obtained from CO_2-sensitive D mutants incubated in air had a novel phenotype in being rough in air, fully mucoid in far smaller concentrations of CO_2 than are required for capsule formation by strain 2160s, and inhibited by 10 % CO_2 (Table 1). We suggest that the second mutation decreased the rate of functioning of the pathway in the D mutant to a value slightly less than that which produced capsulation in air: the increased activity produced by 1·2–5 % CO_2 therefore produced capsulation, while the still greater stimulus provided by 10 % CO_2 increased the rate to an inhibitory degree.

There is little doubt that organisms growing in shaken broth cultures differ physiologically from those in infected animals. When an infection is eventually fatal, the organisms appear to increase exponentially until death occurs (Bonezzi, Cavalli & Magni, 1943; Tempest & Smith, 1957; Klein *et al.* 1961, 1963), yet they always appear capsulated (Cromarte, Bloom & Watson, 1947; Tempest & Smith, 1957), which is only to be expected since the capsule is essential for virulence (see Keppie, Harris-Smith & Smith, 1963). Initially, it seemed likely that exponential growth *in vivo* was analogous to growth in a chemostat with an essential nutrient present in limiting concentration, and that the consequent restriction of growth-rate led to capsule formation. Adenine was a reasonable possibility, for it is an essential growth factor for strain 2160s and is said to be almost absent from the tissues of certain animal species (Burrows & Bacon, 1958). However, adenine deficiency did not cause the cultures to become uniformly capsulated. A possibility still to be tested is that capsulation begins in broth cultures when the E_h value approximates to that of the tissues.

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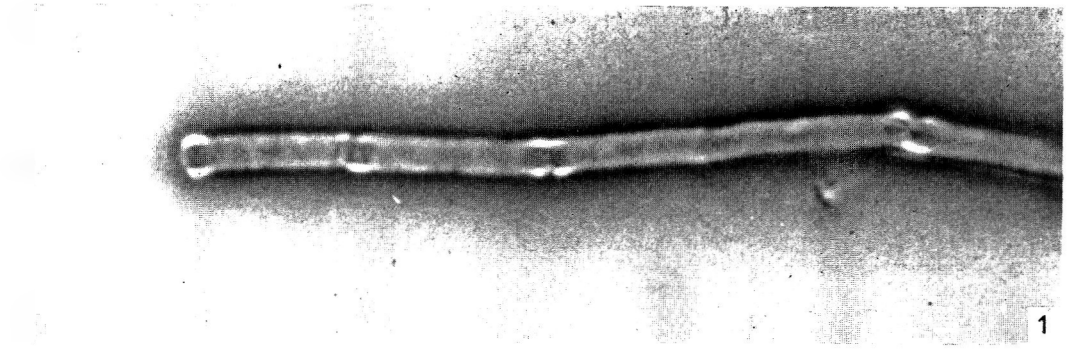
EXPLANATION OF PLATE

A CO_2-resistant D mutant, no. 31, of *Bacillus anthracis* strain 2160s, stained by phloxine + nigrosin after growth in air in medium 2/6.

Figs. 1, 2. Early capsulation, showing wedges at intercellular junctions and at the ends of cells. The capsule is indented opposite the junctions and does not surround the tips of the chains.

Figs. 3–6. Pieces of capsule inherited from an inoculum of capsulated cells (as described by Meynell & Lawn, 1965). Note that here, unlike figs. 1 and 2, the capsule has a smooth convex outline opposite intercellular junctions and surrounds the tip of each cell.

Fig. 7. Chains containing a mixture of capsulated and non-capsulated cells formed 2 hr after adding tetracycline to an exponentially growing culture.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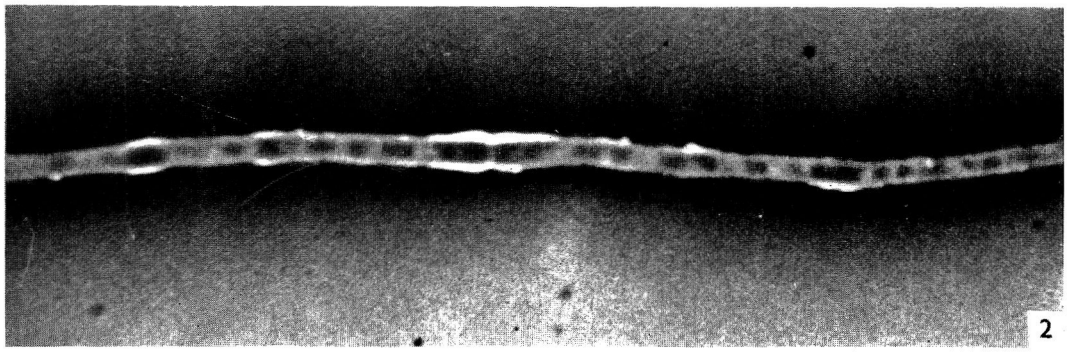
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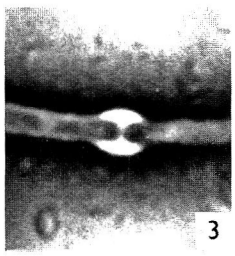
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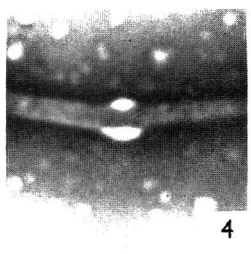
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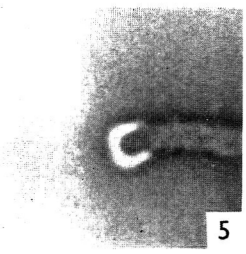
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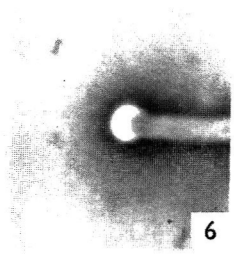
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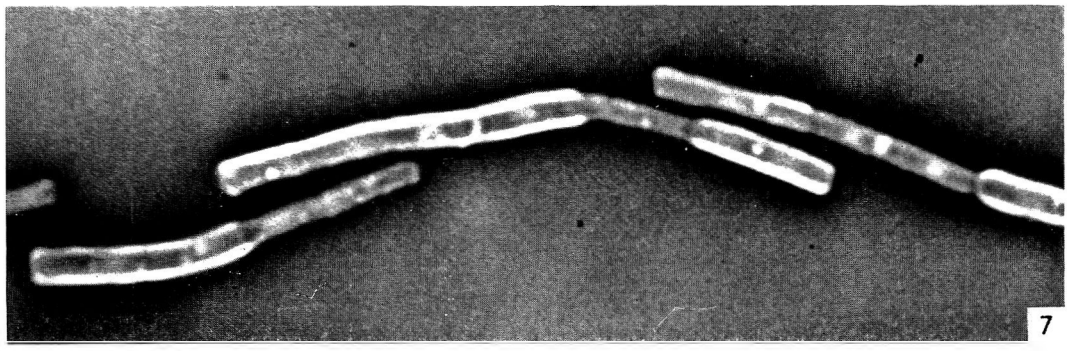
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Utilization of Sugars by Chlorella under Various Conditions

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SUMMARY

The consumption and utilization of a series of carbohydrates by a strain of *Chlorella pyrenoidosa* under different conditions of culture was examined. Only glucose, fructose and mannose were avidly consumed; galactose was consumed in small quantities. Light was highly stimulatory for the consumption of sugars. The carbohydrates assimilated were polymerized to starch; this induced gigantism in the organisms, a considerable increase in the dry weight, and inhibition of division.

INTRODUCTION

The various conclusions that have been arrived at about the nutrition and assimilation powers not only of related species of the genus *Chlorella*, but also within a given species, made us ponder on the great capacity of this organism to alter or change itself biochemically, thus making its taxonomic characteristics more complex. These considerations prompted us to make a study of the behaviour of our strain of *C. pyrenoidosa* when assimilating a series of sugars under different cultural conditions. Previously (Rodríguez-López, 1963) we showed that sugars induced gigantism in chlorella; but we felt that it would be interesting to know the behaviour of the alga towards the sugars studied; by growing cultures of it under various conditions, we investigated what sugars it could use, what quantities of these it consumed and how this consumption affected the yield, division and growth of the organism.

METHODS

An inoculum equivalent to 17 mg. dry wt. *Chlorella pyrenoidosa* was placed aseptically in 250 ml. Erlenmeyer flasks containing 100 ml. of basal culture medium A (Rodríguez-López, 1964). Each flask contained a 16 mM concentration of the sugar to be studied: glucose, fructose, mannose, galactose, xylose, arabinose rhamnose, sorbose, lactose, sucrose, maltose (Sigma Chem. Co.).

The cultures were grown in daylight and aerated with an 8 l./hr mixture of air containing 5 % (v/v) CO_2; other cultures were grown in daylight without aeration and still others in darkness without aeration. The temperature was maintained at 24° and all flasks were periodically shaken. The experiments lasted for 4 days and controls without sugar were run. Each experiment was repeated 10 times.

Consumption of carbohydrates was determined, by difference, by the Somogyi–Nelson colorimetric method (1944, 1952) (the sucrose being previously hydrolysed).

Growth of the cultures was measured by counting the number of organism with a Burker-ruled haemocytometer slide and the growth index (*GI*) determined:

$$GI = \frac{\text{No. organisms/mm.}^3 \text{ at end of experiment}}{\text{No. organisms/mm.}^3 \text{ at beginning of experiment}}.$$

The size of organisms was also measured by using a previously calibrated micrometer eye-piece.

The dry weights of organism were obtained by desiccation in an oven at 105° to constant weight. The results of consumption of carbohydrates and increase in dry wt. are recorded with reference to 100 mg. dry wt. alga.

RESULTS

Table 1 shows the consumption of the various carbohydrates under the various culture conditions. Of the sugars studied, only glucose, fructose and mannose were consumed in significant quantities when cultures were grown in daylight without aeration. The lowest consumption was by cultures kept in darkness. Galactose and xylose were also consumed, but in smaller quantities, especially the latter. None of the other sugars was consumed. The increase in the dry wt. of alga was proportional to the consumption of carbohydrates (Table 2). Nevertheless, the growth index presents very peculiar features, in accordance with the conditions under

Table 1. *Carbohydrate (mg) consumed in 96 hr by equiv. 100 mg. dry wt. of chlorella cultivated under various conditions, in a mineral medium with the addition of 16 mM concentration of a sugar*

Sugar added	In daylight, aerated, and 24°	In daylight, no aeration, and 24°	In darkness, no aeration, and 24°
Glucose	528 ± 26	1228 ± 86	426 ± 22
Fructose	441 ± 11	1162 ± 88	448 ± 35
Mannose	480 ± 33	1114 ± 40	346 ± 24
Galactose	102 ± 6	162 ± 31	152 ± 22
Xylose	47 ± 26	40 ± 22	0
Ribose	0	0	0
Arabinose	0	0	0
Rhamnose	0	0	0
Sorbose	0	0	0
Lactose	0	0	0
Sucrose	0	0	0
Maltose	0	0	0

Table 2. *Increase in weight (mg. dry wt.) of equiv. 100 mg. dry wt. of chlorella cultivated under various conditions, in a mineral medium with the addition of a 16 mM concentration of a sugar*

Sugar added	In daylight, aerated, and 24°	In daylight, no aeration, and 24°	In darkness, no aeration, and 24°
Glucose	454 ± 26	707 ± 57	235 ± 50
Fructose	444 ± 23	617 ± 43	231 ± 48
Mannose	450 ± 45	617 ± 58	246 ± 30
Galactose	307 ± 19	86 ± 10	51 ± 3
Xylose	215 ± 4	0	0
Ribose	222 ± 6	0	0
Arabinose	211 ± 5	0	0
Rhamnose	212 ± 3	0	0
Sorbose	216 ± 3	0	0
Lactose	255 ± 23	0	0
Sucrose	212 ± 4	0	0
Maltose	238 ± 17	0	0
Control	260 ± 27	47 ± 14	0

which the culture was kept (Table 3). Those cultures which contained assimilable sugar and were grown in daylight without aeration showed inhibition of growth to a marked degree when they contained either glucose, fructose or mannose. These experiments showed that only assimilated sugars produced giant forms (Table 3). Controls without sugar did not grow if placed in the dark.

Table 3. *Growth index over 90 hr of chlorella cultivated in a mineral medium with or without a sugar and under differing environmental conditions*

Sugar added	In daylight, aerated and 24°	In daylight, no aeration, and 24°	In darkness, no aeration, and 24°	Giant forms
Glucose	2·35	1·95	1·05	Yes†
Fructose	2·32	1·97	1·07	Yes
Mannose	2·39	1·92	1·06	Yes
Galactose	3·27	2·09	1·07	No
Xylose	4·78	2·14	0·98	No
Ribose	4·98	2·17	1·02	No
Arabinose	4·39	2·14	1·09	No
Rhamnose	5·04	2·20	1·00	No
Sorbose	5·08	2·20	0·97	No
Lactose	5·10	2·18	1·02	No
Sucrose	4·98	2·10	1·03	No
Maltose	4·90	2·13	1·00	No
Control*	5·44	2·21	0·98	No

* No sugar added.
† There are giant forms in all the cultures having glucose, fructose or mannose.

DISCUSSION

Of the various carbohydrates tested only glucose, fructose and mannose were consumed in relatively large quantities; these results differ from those of Samejima & Myers (1958). This supported our suspicion of the existence of biochemical modifications within the same species, that is to say that, as in other micro-organisms, including unicellular green algae, it reveals changes at the biochemical level induced by the environment and the cultural conditions.

Sucrose, which according to Milner (1948) is a component of chlorella, was not assimilable. This suggests the existence of barriers against penetration in the cell wall or cytoplasmic membrane.

The low consumption of xylose might have been due to impurities in the samples of this sugar. In the case of galactose, which was only used during the first 60 hr, perhaps a blocking mechanism is brought into play with the result that passage of this sugar is impeded.

Table 2 shows the high consumption of sugars in cultures exposed to light and not aerated as compared with that of those kept in darkness, so that light determined a difference in consumption. Griffiths (1961) referring to the light/cell-division relationship, suspected a 'participation of some photoreaction other than photosynthesis in the control of active cell division'. In our case also a factor dependent on light and distinct from photosynthesis, which acted as a stimulant of consumption of assimilable sugars, was shown.

The quantities of sugars consumed in the cultures kept in light and aerated

would in some way compensate for the autotrophic deficiences of the cultures chiefly during the hours of darkness at night, when also CO_2 was denied to them, for it is well known that chlorella is capable of using both forms of nutrition, autotrophy and heterotrophy, simultaneously.

The increase in dry wt. in the cultures is due in our case to the reserve substances accumulated, and are in direct proportion to the quantity of sugar consumed (Tables 1 and 2), which they later polymerize, transforming it into starch, as we demonstrated previously (Rodríguez-López, 1965); for while the normal forms of chlorella contain 23 % of total carbohydrates, the giant forms contain 84 %. So that if the alga takes from the medium glucose, fructose or mannose in quantities determined by the conditions of the culture, these sugars are polymerized by it into starch, which accumulates in the cell, causing a great increase in volume and dry wt. and producing giant forms, of measurements given by us (1963). The small quantity of galactose consumed increased the cell volume to only a very insignificant extent (Table 2).

The inhibition of cell division in all cultures exposed to light, whether aerated or not, in the presence of assimilable carbohydrates is explained (Table 3) by the desequilibrium of the consumption of these in relation to nitrogen. The lower the N/C consumption ratio, the greater the decrease in the synthesis of nucleic acids and protein; and as a result, cell division will be negatively affected.

Giant forms produced in darkness or in light are discoloured in part in comparison with normal ones. We have shown (1965) that this phenomenon, mentioned by Ikuko Shihira-Ishikawa & Eiji Hase (1964), is due to the high concentration of starch in the organism, which completely disorganizes the chloroplast and distributes the pigment in a greater cellular volume.

Where inhibition of cell division by assimilable sugars is really manifest is in cultures exposed to light and aerated. The growth in cell population remains at less than half the normal level. Under these conditions of autotrophic growth little gigantism is to be seen, despite the fact that the alga has optionally at its disposal an assimilable sugar as a source of organic carbon.

Thus, in this work with our strain of *Chlorella pyrenoidosa* we arrive at results similar to those obtained by Griffiths (1963) in *C. vulgaris* under the influence of glucose, that is to say, that under the conditions pointed out in our work, chlorella avidly consumes glucose, fructose and mannose, light being a very stimulating factor in the consumption of sugar. Inhibition of cell division, a notable increase in the dry wt. and significant gigantism are the results of this consumption; while if the cultures are maintained in normal autotrophic conditions, the metabolizing sugars alone will be used to make good the deficiencies, producing an increase in the dry wt., very little gigantism and markedly inhibited cell division.

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Sensitivity to Acid of the Type Antigens of *Streptococcus faecalis*

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SUMMARY

Extracts of *Streptococcus faecalis* cultures made with 0·01 N-HCl gave a precipitin reaction with type antisera. With 0·05 N-HCl, however, some cultures gave inactive extracts. Part of the type-specific polysaccharide is converted by the strong acid to a form in which it does not precipitate with homologous sera, but which specifically inhibits the reaction between antibody and unaltered antigen.

INTRODUCTION

During a study of *Streptococcus faecalis* cultures isolated from human sources we used the serological typing method described by Sharpe & Shattock (1952). We were able eventually to type 92% of the cultures, though we failed in nearly 40% when we used 0·05 N-HCl for extracting the antigen (Maxted & Fraser, 1964). This prompted a more detailed investigation of the optimal conditions under which the type-specific antigen can be separated from the bacteria.

METHODS

Organisms. Collections of cultures were received from four London hospitals. They had been isolated from a variety of human sources, including urine, faeces, blood and wound swabs. A further 11 strains from dental lesions were provided by Professor K. C. Winkler (Utrecht). In all, 267 cultures satisfied our criteria for *Streptococcus faecalis*; they belonged to group D, were resistant to 60° for 30 min., grew on tellurite media, reduced tetrazolium salts and did not ferment arabinose.

Antisera. Formalized vaccines of eight type strains (1, 3, 4, 5, 6, 8, 9, 19) were prepared from cultures provided by Dr M. E. Sharpe, by the method of Sharpe & Shattock (1952). Vaccine was given in 1 ml. doses intravenously to rabbits at 3- to 4-day intervals and test bleedings were taken from 3 weeks onwards. After 6 weeks the sera usually gave a strong precipitin reaction with homologous cultures in a ring test, and had a titre of not less than 1/128 by slide agglutination. Heterologous reactions were removed by absorption with suspensions prepared by heating a 48-hr broth culture at 70° for 30 min. and washing the organisms three times in saline. One volume of packed bacteria was added to 2 volumes of antiserum and the mixture incubated at 37° for 2 hr and left overnight at 4° before centrifugation.

Extraction with acid. The centrifuged deposit of bacteria from 50 ml. of 0·5% glucose nutrient broth culture was heated for 10 min. in a boiling water-bath with 2 ml. HCl of the requisite strength, and then neutralized with 0·05 N-NaOH.

Ethanol and acetone precipitation. After centrifugation, acid extracts were treated

with 2½ volumes of acid ethanol (1% HCl in 95% ethanol in water), and left overnight at 4°. The precipitate was removed and taken up in 2 ml. M/15 phosphate buffer (pH 7·6). The supernatant fluid from the ethanol precipitation was in turn mixed with 2½ vol. acetone and the resultant precipitate removed and dissolved in 2 ml. phosphate buffer (pH 7·6).

Extraction with streptomyces enzyme. The centrifuged deposit of bacteria from 50 ml. glucose broth culture was heated to 70° for 10 min. and then lysed by suspending in a crude streptomyces enzyme solution for 2 hr at 50° (Maxted, 1948).

Extraction with lysozyme. Overnight cultures in glucose broth were heated at 80° for ½ hr and centrifuged, the deposit washed three times with saline and suspended in lysozyme solution (1 mg./ml.) in saline, and adjusted to pH 7·5. After 3 hr at 37° the pH value was raised to 9·2, the fluid centrifuged, and the supernatant fluid readjusted to pH 7·5.

Mechanical disruption of bacteria. The centrifuged deposit from 250 ml. of glucose broth culture was suspended in 5 ml. phosphate buffer (pH 7·6) and shaken with ballotini No. 12 in a Mickle disintegrator. The cell-wall fraction was removed by high-speed centrifugation.

RESULTS

Extracts of 267 strains of *Streptococcus faecalis* were made by boiling with 0·05 N-HCl; 158 of them reacted with one of the eight type sera in the capillary precipitin test. The extracts of the 109 untypable cultures were treated with acid ethanol. The resulting precipitate was dissolved in buffer and tested with the type sera. A further 87 cultures then gave a type-specific precipitin reaction (Table 1).

Table 1. *Serological typing of Streptococcus faecalis strains from human sources*

	Number tested	Number typable	Type								Not typable
			1	3	4	5	6	8	9	19	
Routine method: 0·05 N-HCl extract	267	158	14	23	7	11	23	0	50	30	109
Acid extract non-reactive: retested after precipitation with ethanol	109	87	51	6	1	10	6	0	2	11	22
Totals			65	29	8	21	29	0	52	41	

Acetone-precipitates from these cultures were redissolved in buffer and added to solutions of the ethanol-insoluble material. This prevented the type-specific reaction. The failure of 0·05 N-HCl extract (pH 1·6) of some cultures to form a specific precipitate was therefore due to the presence of an inhibitory substance which was soluble in ethanol but precipitable by acetone.

When weaker strengths of hydrochloric acid were used for the initial extraction, the number of positive reactions was increased. Indeed, all cultures that were typable under any circumstances with one of the eight sera gave an active extract with 0·01 N-HCl (pH 2·4). This strength of HCl is therefore to be recommended for the routine typing method. Table 2 shows the serological reactions obtained with extracts made with a range of HCl concentrations. The two strains are of different serological types and show a marked difference in the resistance of their poly-

saccharide type antigen to acid hydrolysis. All typable strains also gave serologically active extracts with Streptomyces enzyme and with lysozyme. Some serologically active polysaccharide could be obtained simply by shaking the deposited culture with water.

Table 2. *Effect of strength of HCl on the extraction of active type-specific polysaccharide from Streptococcus faecalis*

	Type reaction of strain	
Extracted with	No. 28 type 1	No. 30 type 6
Water	++	++
HCl 0·01 N	++++	++++
0·03 N	+	+++
0·05 N	−	+++
0·1 N	−	+++
0·2 N	−	±

+ to ++++ = strength of precipitin reaction with homologous type serum.

Table 3. *Type-specific inhibition by 0·2 N-HCl extract of Streptococcus faecalis of precipitin reaction between 0·05 N-HCl extract and its homologous serum*

Equal volumes of undiluted serum and 0·2 N-HCl extract were mixed together and left for 1 hr. They were then tested with active (0·05 N-HCl) extract of an organism of homologous type with that of the serum.

Homologous type serum + HCl extracts of (undiluted)	Serum tested with 0·05 N-HCl extracts of type							
	1	3	4	5	6	8	9	19
	Precipitin reaction							
T1	−	++	++	+++	++	++	++	++
T3	++	−	++	++	++	++	++	++
T4	++	++	−	++	++	++	++	++
T5	++	++	++	−	++	++	−	++
T6	++	−	++	++	−	++	++	++
T8	++	++	++	++	+++	+++	+++	++
T9	++	++	++	++	++	++	−	++
T19	++	++	++	++	++	++	++	−

− to ++++ = strength of precipitation reaction with homologous type serum.

The effect of 0·05 and 0·01 N-HCl on individual cultures was reproducible, and it therefore seemed that the acid stability of the type-specific antigen was a constant character of a strain. Cultures which yielded active extracts with 0·01 N-HCl but not with 0·05 N were found in most serotypes, but were particularly common in types 1 and 5 (Table 1). With 0·2 N-HCl all extracts were serologically inactive, except that of the type strain of type 8.

The inhibition of the precipitin reaction by a substance extracted from the organism with strong acid was type-specific. An extract was prepared with 0·2 N-HCl from a representative of each serotype. Only the type 8 extract gave a precipitin reaction with the homologous serum. Mixtures were prepared of equal quantities of each of these extracts and each of the type sera. These were tested in capillary tubes

with an active (0·05 N-HCl) extract of an organism of type homologous with that of the serum. It will be seen (Table 3) that the 0·2 N extract inhibited the reaction between serum and the 0·05 N extract of homologous type, but with two exceptions not the reactions in other types. Type 5 showed a one-way cross with type 9, and type 6 with type 3, and there were corresponding cross-reactions in the inhibition test; and the 0·2 N-HCl extract of type 8, which was itself serologically active, did not inhibit the reaction between the 0·05 N extract of the same type and its homologous serum.

The distribution of type antigen in different parts of the bacterium was next examined. The cell-wall fractions of mechanically disrupted cultures were separated and treated with ribonuclease and deoxyribonuclease. They were then extracted with acids of various strengths and yielded type-specific antigen with the same acid sensitivity as that obtained from untreated organisms.

Table 4. *Ethanol precipitation and acid sensitivity of type-specific polysaccharide in the soluble cellular material obtained by mechanical disruption of Streptococcus faecalis*

	Culture							
	Acid resistant				Acid sensitive			
	Type 1			Type 3	Type 1			Type 3
	E1	E179	E137	E112	E10	E175	E171	E99
	Precipitin reaction*							
Control 0·05 N-HCl extract	+++	+++	+++	+++	–	–	–	–
Supernatant material from mechanical disruption:								
1st acid-ethanol precipitate	+++	+++	+++	+++	+++	+++	+++	+++
1st acetone precipitate	++	±	+	±	–	–	–	–
2nd acid-ethanol precipitate	+++	++	++	++	+++	++	++	++
2nd acetone precipitate	++	–	±	–	–	–	–	–
3rd acid-ethanol precipitate	+++	++	++	+	+++	++	++	+
3rd acetone precipitate	±	–	–	–	–	–	–	–
Acid-ethanol precipitate hydrolysed at 100° in 0·05 N-HCl/10 min.	–	–	–	–	–	–	–	–
Hydrolysed extract reprecipitated with acid ethanol	–	–	–	–	–	–	–	–

* + to +++ = strength of precipitin reaction with homologous serum.

However, the untreated supernatant fluid from the disrupted bacteria also gave a strong type-specific precipitation reaction. This finding contrasted with our experience with *Streptococcus pyogenes*, where the cell-wall antigens did not appear in the supernatant fluid from disrupted bacteria. Eight strains, four of which gave an active extract with 0·05 N-HCl and four of which did not, were disintegrated mechanically. Each supernatant fluid was precipitated first with ethanol and then with acetone. The two precipitates were each dissolved in buffer and re-precipitated three times to eliminate cross-contamination as much as possible, before being tested against the homologous serum (Table 4). The ethanol-insoluble fraction from all 8 strains gave a strong precipitin reaction with the corresponding type anti-

serum. There were some weak positive reactions between the serum and the acetone-insoluble fractions from the four strains which normally resisted acid hydrolysis, but these may have been due to material carried over from the ethanol-insoluble fraction, since the reactions disappeared after further re-precipitation with acid ethanol. None of the acetone-insoluble fractions had any inhibitory action on the precipitin reaction of the corresponding ethanol-insoluble fraction and its specific serum.

The ethanol-insoluble fractions from the supernatant fluids of disrupted suspensions were then treated with 0·05 N-HCl. All of them, whether derived from organisms which did or did not yield active extracts from whole organisms with acid of the same strength, were rendered inactive by this treatment (Table 4).

Centrifuged deposits of bacteria were therefore digested with trypsin to see whether a surface protein was protecting the cell-wall polysaccharide of some strains from the action of strong acid; no constant increase in susceptibility to 0·05 N-HCl resulted from this treatment.

DISCUSSION

Over nine-tenths of the group D streptococci we isolated from human lesions were *Streptococcus faecalis* (Maxted & Fraser, 1964). The success in typing 92% of *S. faecalis* cultures with eight sera suggests that this method might be useful in epidemiological studies of hospital infections with this organism. The manufacture of typing sera presents little difficulty, and the typing technique is easily done when 0·01 N-HCl is used for extraction.

Elliott (1960) and Sharpe (1964) both found that the type-specific carbohydrate of some strains of *Streptococcus faecalis* was destroyed by heating to 100° in the presence of strong acid; and Bleiweis & Krause (1965) showed, with one type 1 strain, that hydrolysis of the carbohydrate at pH 1·5 resulted in the formation of a dialysable inhibitor of the precipitin reaction between the type antigen and its homologous antibody. In our experience many cultures give serologically inactive extracts when treated with 0·05 N-HCl (pH 1·6). The effect of this strength of HCl appears to be to hydrolyze part of the type-specific polysaccharide to a substance which, although inactive in the precipitin reaction, retains the serological determinants of the antigen. It is therefore able to block the precipitation reaction between antibody and unhydrolyzed antigen. When sufficiently strong HCl is used (e.g. 0·2 N) the type-specific antigen of nearly all *S. faecalis* strains is rendered inactive, and in many cases the extracts consist almost entirely of acetone-insoluble inhibitory material.

There appears, however, to be a difference between the organisms which usually yield active extracts with 0·05 N-HCl and those which do not. The reason for this is not clear. Type-specific antigen obtained from the cell contents of disrupted organisms without chemical treatment appears to be uniformly sensitive to acid of this strength. This suggests that the antigen of the intact organism is protected in some way from the action of the strong acid.

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On the Antigenic Relationships of Certain Citrobacter and Hafnia Cultures

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SUMMARY

The biochemical and serological characters of 36 unclassified cultures are described, and according to these characters the organisms belong to the genera *Citrobacter*, *Aerobacter* and *Hafnia*. While studying the antigenic relationships of the O antigens the known antigenic relationship between some serotypes of *Citrobacter* and *Salmonella* was confirmed; new antigenic relationships with *S. locarno*, *S. kentucky*, *S. uccle* and *S. tranoroa* are described. The antigenic relationships of the Hafnia cultures with *Shigella flexneri* 4a and with two cultures with a provisional O group of Citrobacter were confirmed.

INTRODUCTION

The antigenic relationship between the genus *Citrobacter* and the genera *Salmonella*, *Arizona*, *Escherichia* and *Shigella* have been relatively thoroughly described, mainly in the monographs of Kauffmann (1941, 1954, 1961), Edwards & Ewing (1955, 1962), Sedlák & Rische (1961), Kampelmacher (1959). Much less is known about the antigenic relationship between the other genera of this family, Enterobacteriaceae.

The present work aims to improve our knowledge about the antigenic relationships of some of the less common enteric bacteria. We believe that knowledge of these relationships and their further systemic study will lead to a decrease of false diagnoses made on biochemical grounds. The antigenic diagnostic schemes for the groups Salmonella (Kauffmann, 1964), Arizona (Edwards, West & Brunner, 1948), *Citrobacter* (West & Edwards, 1954), as well as *Escherichia coli* (Kauffmann, 1954) form the basis for a further systematic study of relationships that are important both practically and theoretically.

METHODS

Cultures studied. The biochemical and antigenic properties of 36 cultures of Citrobacter were studied. All these strains were isolated from human pathological material during the period 1936–65. Their designations and origin are presented in Table 1.

Strains *Salmonella coli* 1–5, together with *S. ballerup* (Vi, z_{14}) and *S. arizona* (XXXIII: $z_4z_{23}z_{26}$) were originally listed by Kauffmann (1941) in the 1940 edition of the Kauffmann–White scheme and only later removed. *S. arizona* has become the test culture for serological group O1 of the present genus *Arizona* (Edwards, West &

Brunner, 1948) and *S. ballerup* one of the test cultures of the former 'Ballerup group' (Brunner, Edwards & Hopson, 1949), and is at present the test culture for the serological group O 29 of *Citrobacter* (West & Edwards, 1954).

Table 1. *Designation and origin of the cultures used*

Origin. designation of cultures	Originally described or identified as	Received from
2624/36	*S. coli* 1 (XXXI Vi...1, 5)	Prof. Dr F. Kauffmann, International Salmonella Centre, Statens Serum Institut, Copenhagen, Denmark
5396/38	*S. coli* 1 (XXXI...1, 5)	
491/36	*S. coli* 2 (XXXII...1, 5)	
5821/38	*S. coli* 2	
'Zurich'	*S. coli* 3 (IV, V, XII...z_{20})	
'KA'	*S. coli* 4 (IV, XXVII, XII...z_{21}	
'B'	*S. coli* 4	
LG 1099	*Citrobacter* (Sa O3, 10, 19)	Prof. Dr E. van Oye, National Salmonella-Shigella Centre, Brussels, Belgium
BR 184/6	*Citrobacter* (Sa O8, 20)	
Ci 47007	*Citrobacter*	Dr Lányi, State Institute of Hygiene, Budapest, Hungary
Ci 47009, 47010		
Ci 47012, 57015		
Ci 47018, 47020		
H 8997, H 9004, H 9011, H 9012, H 9013, H 9009, H 9020, H 9022, H 9024, H 9025, H 9008, H 9027	*Hafnia*	Dr E. Aldová, National Shigella Centre, Institute of Epidemiology and Microbiology, Prague, Czechoslovakia
P 6b	*E. coli* O67	Dr F. Ørskow, International Escherichia Centre, Statens Serum Institut, Copenhagen, Denmark
P 11c	*E. coli* O72	
H 310a	*E. coli* O94	
W 40	*E. coli* O122	

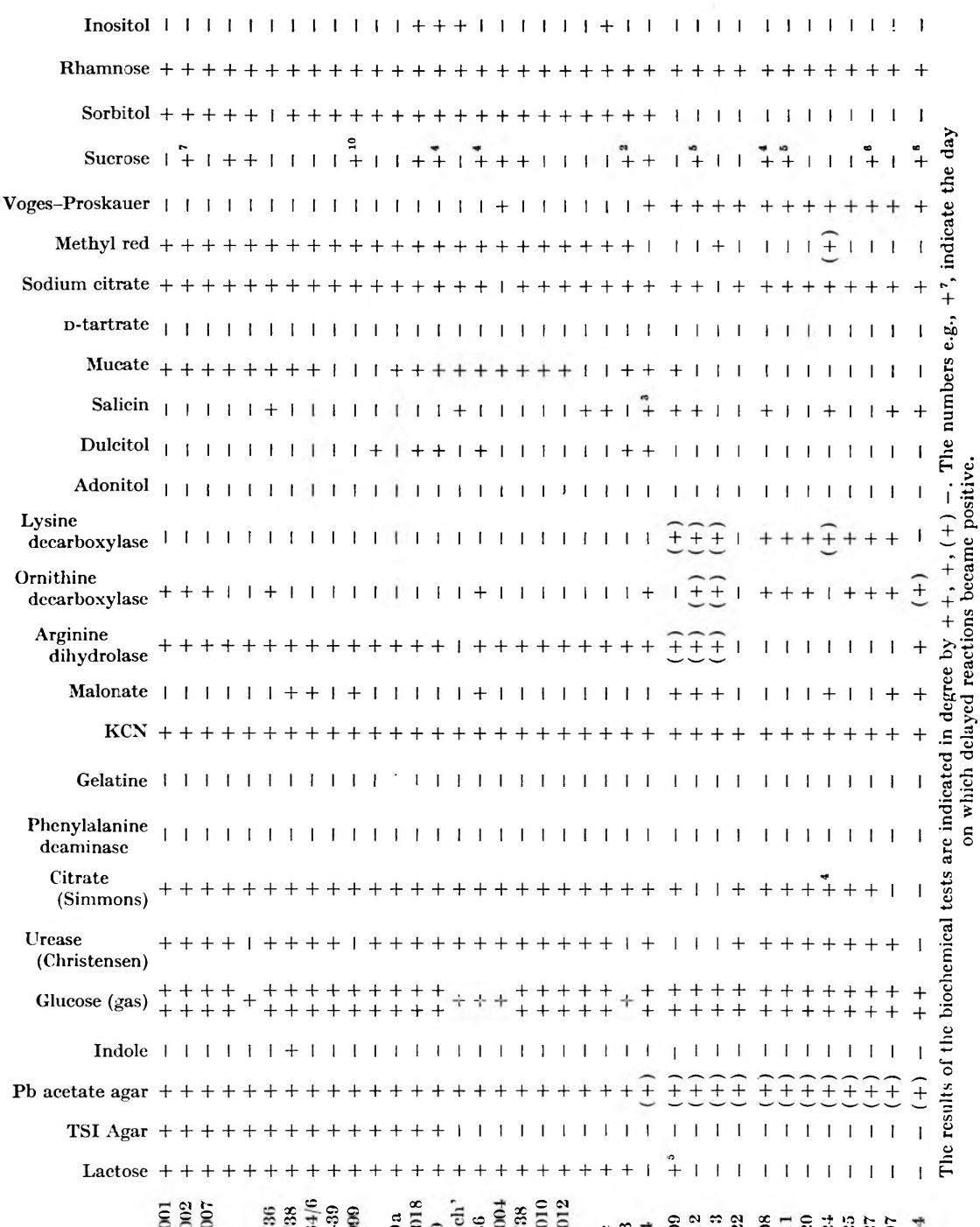
Cultures P 6b, 11c, H 310, W 40 are provisional test cultures of *Escherichia coli* serological groups O 67, O 72, O 94 and O 122 (Kauffmann, 1954), but have been characterized by Kauffmann as '...atypical in that they are indole negative, H_2S positive (ferric chloride gelatin) and ammonium citrate positive, i.e. they belong to the Bethesda group (or to *Escherichia freundii*)' (Kauffmann, 1954, p. 178). The other cultures were isolated by us much later (1963–65) and have not been previously described. The antigenic relationship of strains LG 1099 and 184/6 with *Salmonella anatum* and *S. kentucky* was shown by E. van Oye.

For the biochemical identification of the cultures the methods and culture media recommended by the *Enterobacteriaceae Subcommittee* (*Reports*, 1958) or by Kauffmann (1941, 1954), Edwards & Ewing (1955, 1962), or Kauffmann & Petersen (1956) were used.

Serological examination was done by the methods of slide agglutination and agglutination in test-tubes. Antigenic relationships were studied by using cross-agglutination and in some cases also absorption tests with the respective anti-O or anti-H sera of our own make.

After determination of the O antigens of our Citrobacter strains and their allocation to one of the serological O groups, we had at our disposal all the necessary

Table 2. *Biochemical properties of the organisms studied*



The results of the biochemical tests are indicated in degree by + +, +, (+) −. The numbers e.g., $+^{7}$, indicate the day on which delayed reactions became positive.

anti-O sera according to the original antigenic Citrobacter scheme of West & Edwards (32 serological O groups); in addition to these we had a further eight of our own anti-O sera (provisional new groups O 33–O 40; Table 4).

RESULTS

All the cultures corresponded to the basic taxonomic definition of the family Enterobacteriaceae in *Bergey's Manual* (1957) and to the definition of one or other of two genera of this family, i.e. *Citrobacter* or *Enterobacter*.

Table 3. *Antigenic relationships*

Designation of cultures	Serological O groups			
	Citrobacter West & Edwards O 1–O 32 prov. 033–041	*Salmonella* Kauffmann–White A–O 60	*Shigella*	*E. coli* (O 1–O 144)
Citrobacter				
2624/36, 5396/38 (*S. coli* 1)	—	O 48 (ident.) (*S. djakarta*)	—	—
491/36, 5821/38 (*S. coli* 2)	—	H 1, 5	—	—
'Zürich', (*S. coli* 3) 'B', 'Ka' (*S. coli* 4) Ci 47004	O 22	O 4, 5 (*S. paratyphi* B)	—	—
Ci 47001, Ci 47018, Ci 47019	O 12	O 57 (*S. locarno*)	—	—
LEO 429, W 40	Prov. O 36 (151)	—	—	O 122
BR 184/6	Prov. O' 38 (138 A)	O 8, 20 (*S. kentucky*)	—	—
LG 1099, Ci 47013	Prov. O 37 (153)	O 3, 10, 19 (*S. anatum*)	— *S. flexneri* 4a	—
Ci 47012	—	O 54 (*S. uccle*)	—	—
Ci 47010	—	O 55 (*S. tranoroa*)	—	—
Ci 47002	—	VI	—	—
P 6b	—	—	—	O 67 (ident.)
P 11c	—	—	—	O 72 (ident.)
H 310a	—	—	—	O 94 (ident.)
Hafnia				
H 8997, H 9009	Prov. O 37 (153)	—	*S. flexneri* 4a	Ec (O 1), O 13
H 9008, H 9011, H 9004, H 9020, H 9025, H 9027, H 9012, H 9013, H 9024	—	—	*S. flexneri* 4a	—

The biochemical characteristics of all the cultures are shown in Table 2. It is obvious from this Table that 24 of the cultures can, by their biochemical properties, be identified as typical members of the genus *Citrobacter*. Small deviations from the existing definition of the genus *Citrobacter* (Edwards & Ewing, 1962) were shown by

the indole-positive variant 5396/38 *Salmonella coli* 1, and the anaerogenic variants 'Zürich' *S. coli* 3, 491/36 *S. coli* 2, 'Ka' *S. coli* 4, Ci 47004 and Ci 47013. Only five cultures fermented sodium malonate and five cultures salicin.

Production of hydrogen sulphide (H_2S) was detected in all cultures after 24 hr incubation on lead acetate agar and in 14 cultures also on TSI agar.

All the other cultures belong to the genus *Aerobacter* (*Bergey's Manual*, 1957; Edwards & Ewing, 1962). Twelve of them could be included in the *Hafnia* group (Møller, 1954; Edwards & Ewing, 1962) and one of these (Ci 47014) corresponds to the definition of the species variously known as *Aerobacter cloacae* or *Cloaca cloacae* (Kauffmann, 1954), *Enterobacter* (Hormaeche & Edwards, 1959) or *Aerobacter* group A (Edwards & Ewing, 1962). In none of the cultures was H_2S production detected on TSI agar, not even after prolonged incubation. The production of small quantities of H_2S could be proved only on lead acetate agar after prolonged incubation at 22°. After incubation at 37° the MR and VP tests did not give clear positive or negative results. The results of these tests reported in Table 2 were after incubation at 22°.

Table 4. *Antigenic properties of 10 new provisional O-type cultures*

Original designation of studied cultures	O-type culture of provisional serological groups O 33–O 42 (relationship of O antigen)	Further serologically identical strains
P 6b	O 33 (*E. coli* O 67—)*	—
P 11e	O 34 (*E. coli* O 72—)*	—
H 310a	O 35 (*E. coli* O 94—)*	—
Leo 429	O 36 (*E. coli* O 122)*	KW 151, W 40
2624/36 (*S. coli* 1)	O 37 (*S. djakarta*—O 48)	5896/38 (*S. coli* 1)
BR 184/6	O 38 (*S. kentucky*—O 8, 20)	KW 138 A
LG 1099	O 39 (*S. anatum*—O 3, 10)	KW 153
Ci 47007	O 40 (*S. locarno*—O 57)	—
Ci 47010	O 41 (*S. tranoroa*—O 55)	—
Ci 47012	O 42 (*S. uccle*—O 54)	—

* These cultures have in the meantime been excluded from *E. coli*-type cultures.

The results of serological examinations are summarized in Table 3. In this table the antigenic relationships of all the cultures are expressed and in the case of Citrobacter cultures also their respective serological O groups according to the scheme of West & Edwards. All the Citrobacter strains were tested with Salmonella O sera 1–60, Shigella sera A I, A II, B, C I, C II, D, and coli sera O 1–144, as well as Citrobacter O sera 1–32. Some of the strains did not agglutinate in any of them and are not included in Table 3. The results shown in this Table are in complete agreement with the earlier known antigenic relationships of the genera *Citrobacter* and *Salmonella* (Edwards & Ewing, 1962; Kauffmann, 1941) as well as with the original statement of Kauffmann (1941) concerning the so-called *Salmonella coli* 1–4 cultures.

The antigenic relationship of the further Citrobacter cultures with *Salmonella locarno*, *S. kentucky*, *S. anatum*, *S. uccle*, *S. tranoroa*, *Shigella flexneri* 4a and *Escherichia coli* O 122 have, according to literature available to us, not yet been described. One of the Citrobacter strains (Ci 47002) with a Vi antigen is interesting. Its somatic antigen is entirely different from all other known Citrobacter O antigens including those of groups O 5 and O 29 (Ballerup group), which usually possess

Vi antigen (Kauffmann, 1954; West & Edwards, 1954). The antigenic relationship of another Citrobacter culture (47013, provisional O 37), with *S. flexneri* 4a and the very near relationship of Citrobacter strain Leo 419 (provisional O 36) with test strain *E. coli* O 122 also deserves mention.

In 11 strains of the *Hafnia* group very close antigenic relationship could be proved with *Shigella flexneri* 4a as was pointed out by van Oye & Ghysels (1961). Apart from that, antigenic relationship of two other Hafnia strains with Citrobacter of the provisional O group 37 was found.

DISCUSSION AND CONCLUSION

We have tried to show that by using suitable biochemical and serological methods differentiation of Citrobacter as well as Hafnia and *Aerobacter cloacae* strains within the framework of the family Enterobacteriaceae is feasible. Further, that systematic study of the antigenic relationships of the less-frequently isolated species of enteric bacteria has its theoretical as well as practical importance. In this connexion we met a well-known and often discussed problem, the taxonomic value of the MR and VP tests and H_2S production, which are of basic significance and importance in the taxonomy of the family Enterobacteriaceae.

It is well known that the results of MR and VP tests are significantly influenced by many factors; for example, the relative proportion and purity of the reagents, the size of the inoculum and the temperature and duration of the incubation (Kauffmann, 1956; Suassuna, Suassuna & Ewing, 1961; Hugh, 1965).

In the H_2S test there is a considerable difference in the sensitivity depending on the medium used (TSI agar, ferrous chloride gelatin, lead acetate agar, lead acetate test papers). The most sensitive indicator is lead acetate paper, but because of this quality it loses its diagnostic value, and even some Shigella strains will give slight and late positive results when grown on suitable media. Lead acetate agar is less sensitive, but with this it is possible to exclude the weakest H_2S; for example, some strains of *Salmonella typhi*, *S. choleraesuis*, *S. gallinarum-pullorum*, *Citrobacter*, *Enterobacter* and *Hafnia*. The sensitivity of ferrous chloride gelatin and TSI agar and all their known modifications is practically equal; they show only the production of relatively larger quantities of H_2S, as is usually produced by the majority of Salmonella, Arizona, Citrobacter, *Proteus vulgaris*, *P. mirabilis* cultures and perhaps by the newly described Edwardsiella strains (Ewing, Worther, Escobar & Lubin 1965). Kauffmann (1954) used ferrous chloride gelatin; Edwards & Ewing (1962) used TSI agar and stated: '. . . this medium corresponds very closely to the classical descriptions of the various groups of enteric bacteria. . . '. It is essential to know what culture medium was used for H_2S detection, and the results obtained by two methods (TSI agar, and lead acetate agar) are shown in Table 2.

In the genus *Citrobacter* we consider the extension and completion of the existing antigenic scheme of West & Edwards (1954) to be a logical aim. From our experience we are convinced that this is the only way to reach the correct conclusions on the part played by the different serological types of *Citrobacter* in the diarrhoeal diseases of man and animals (Sedlák & Rische, 1961). Our view of the Hafnia group is the same, and we agree with Dr E. van Oye (personal communication) who considers these cultures to be a likely cause of human dysentery.

The conclusions of our taxonomic study of 36 cultures of less common of Enterobacteriaceae can be summarized as follows:

The cultures described originally by Kauffmann (1941) as *Salmonella coli* 1–4 are typical Citrobacter cultures. Strains '*S. coli* 3 and 4' belong to O group 22 of the antigenic scheme of Citrobacter. Also a further four strains of *Escherichia coli* (O 67, O 72, O 94 and O 122) belong the genus *Citrobacter* and the test strain w 40 (O 122) is very closely related to the Citrobacter provisional test strain O 37.

An antigenic relationship has been described for the first time between Citrobacter group O 12 and *Salmonella locarno*, Citrobacter provisional group O 38 and *S. kentucky*, and Citrobacter provisional group O 37 and *S. anatum*; in two further Citrobacter strains their relationship with *S. uccle* (O 54) and *S. tranoroa* (O 55) has been newly described.

An antigenic relationship first described by van Oye in 11 Hafnia strains with *Shigella flexneri* 4a has been confirmed and a new relationship between two Hafnia strains and Citrobacter provisional O group 37 described.

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