MURIEL ROBERTSON

(*Frontispiece*)

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The 80th Birthday of Muriel Robertson

Muriel Robertson is a woman remarkable for many things: her research in little-explored fields and her capacity to size up problems; her encyclopaedic knowledge not only of biology but of the arts; her zest in life and her prodigious memory for things and people; her generous enthusiasm, occasionally interlarded with rather vehement criticism, for the work of others. She graduated M.A. from the University of Glasgow in 1905, and early showed her mettle by departing unchaperoned to Ceylon with a Carnegie Fellowship to study the life cycle of trypanosomes and other blood parasites in tortoises and leeches. On her return she became assistant to Professor M. A. Minchin at the Lister Institute, but in 1911, at the instigation of Sir Charles Martin, she was appointed Protozoologist to the Protectorate of Uganda under the Colonial Office. There she worked out the life cycle of *Trypanosoma gambiense* in the tsetse fly by elegant cytological work, and also investigated some aspects of trypanosomiasis in cattle in the field; but a bald statement of scientific work does scant justice to the phenomenon of a young woman bicycling through jungle, armed with her exquisite embroidery and a rifle for crocodiles, discoursing the while in Luganda to her assistants.

In the summer of 1914 Muriel Robertson returned to the Lister Institute and became actively involved in the remarkable collaborative work of British and French laboratories on the bacteriology of gas gangrene. She was Secretary of the first Anaerobes committee set up by the Medical Research Committee, and subsequently wrote the chapters on spore-bearing anaerobes in the Official Military Medical History and in the M.R.C. 'System of Bacteriology'. After further work on anaerobes she resumed work in 1930 on various aspects of protozoa, particularly with a classic description of the reactions of Tetrahymena to antisera, and in the 1939–45 war collaborated with Dr W. R. Kerr in investigations of trichomoniasis causing abortion in cattle. She was elected F.R.S. in 1947, and long after her official retirement continues her bench work indomitably.

On her 80th birthday, 8 April 1963, Muriel Robertson delivered the Marjory Stephenson Memorial Lecture of the Society, a fitting climax to her life-long interest in microbiology. She had had few formal students, but the many workers of all disciplines who have sat at her feet salute on her birthday one perennially young in outlook.

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Some Aspects of the Protozoa and their Way of Life

The Sixth Marjory Stephenson Memorial Lecture

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INTRODUCTION

I feel much honoured to have been invited to give the Marjory Stephenson Memorial lecture of 1963, but I must confess that I also approach the undertaking with considerable trepidation as it fills me with an acute sense of inadequacy.

In 1961 Professor Knight in his lecture on the growth of microbiology gave a very good account of Marjory Stephenson's own contribution and her influence on the development of the subject. She had a wide and catholic view of the study she had so much at heart. Microbiology, in her view, was to embrace the most various aspects of the investigation into the way of life found in micro-organisms.

I feel therefore that I may be allowed today to direct your attention to a large group of extremely diverse organisms collected under the general title of Protozoa.

This large phylum can be considered as made up of micro-organisms. Their size varies from minute creatures only a few microns in length to relatively large organisms, such as some of the ciliates, visible to the naked eye. It is rather futile to debate the question as to whether they are cells or not. They have, so to speak, the furniture of cells. The point, however, is that they are living creatures which carry out their complicated or relatively simple lives, as the case may be, in sequence. By that I mean that the stages follow each other in general in time, rather than being bound up in one organism the identity of which is laid down from the start. A protozoon divides and there are two but no parent remains; the parent does not die: it just disappears. A microgamete fuses with a macrogamete to form a zygote which ultimately divides up into a number of spores which carry on the cycle, but the zygote has disappeared. The span of life of the protozoa therefore is a debatable matter. Apart from violent death, it is either the period between divisions, or other forms of reproduction, or it is co-existent with the life of the planet from the time of the emergence of the organism.

The protozoa have been conditioned by the fact that they require a fluid or very moist environment. This need has influenced their nature and also limited their development. A great many protozoa produce cysts which are very resistant and can survive a considerable degree of desiccation; the degree of resistance, however, differs very much in different species. The protozoa have developed in the most versatile way and have, as parasites, penetrated into a variety of body fluids and tissues and also into unexpected environments. The parasitic Cnidospora, for example, live in the gall bladder of fishes and tortoises, and certain free-living amoebae were found by Dr Hindle in the hot springs at Dax in Southern France, at a temperature of 54° (Hindle, 1932). Dr Hindle collected these and made cultures of them; one of which he kindly gave to me. In my sample there was a small lively amoeba of the Hartmanella type. The technique for survival of the one I had was

characterized by a very remarkable readiness to encyst, showing that the range of conditions for active life was narrow.

Out of the immense variety of the biological capacity shown by the protozoa I propose to select a few examples of the ingenuity of their relation to their environment, and their pioneering spirit in the evolution of basic methods, which suggest patterns for the biological development found in many higher organisms.

The examples I propose to consider are:

(1) The character of the protozoan cell, the function of the nucleus and some of the varieties of nuclear division.

(2) The highly experimental approach of the phylum to sex.

(3) A consideration of some of their life-cycles and the capacity to rationalize certain of the complicated procedures, when a simpler method would appear to serve the purpose of survival.

It is obvious that these matters can only be treated rather schematically in the space at my disposal.

(1) The protozoan cell presents itself under a very large number of forms, but for the moment I want to consider the free-living less complicated types. Inevitably the prototype that comes to hand is one of the larger amoebae such as *Amoeba proteus*. The organism as seen under the light microscope is a gelatinous mass with various inclusions. It is contained within a double membrane called the plasmalemma which is only visible in an electron micrograph. The ground substance of protoplasm and its submicroscopic make-up is of such a complexity that no brief analysis is adequate; it is beyond the scope of this lecture and the competence of the lecturer. But it would seem to be responsible for the gel and sol activity of, for example, the pseudopodia and also for the internal movements so frequently observed in the protoplasm (Frey-Wyssling, 1948, 1955; Grell, 1956; Kopac, 1950; Landau, Zimmerman & Marland, 1954).

The peripheral membrane of the amoeba is under some interesting and obscure control and I have failed to find any explanation of the well-known fact that one can tell when an amoeba of this type is about to divide by observing that instead of rolling and lolling about it pulls itself together into a neat mulberry shape with prominent knobs. Moreover, it does this nearly always at night.

In the last decade Brandt (1958) and Schumaker (1958) have carried out studies on Amoeba. Brandt calls the form he worked with *Amoeba chaos chaos*, but in this country it passes by the less spectacular name of *A. proteus*, which is used by Schumaker. Brandt showed that the amoeba has a capacity to absorb protein through the plasmalemma or surface membrane. This occurs by the formation of tunnels into the cytoplasm by the invagination of the membrane. These tunnels become cut off and are broken up into droplets which are absorbed in the protoplasm. The process is known as pinocytosis. It was found to be induced by charged molecules which included many proteins and salts. In Schumaker's 1958 study the uptake, of two proteins, ribonuclease and cytochrome, was observed in great detail. His explanation of the results involved the binding of the protein molecules to the external membrane by a process which he considered to be pinocytosis. It would seem that these studies might form a step towards the understanding of the physiology of the large number of organisms which the protozoologists happily describe as saprophytic. These do not ingest food in an obvious

way and are contrasted with the holozoic organisms whose food is taken in by straightforward, often visible, methods and digested in food vacuoles (Steinert & Novikoff, 1960).

The protozoan cell, in addition to the nucleus, contains a number of formed elements and structures varying somewhat with the species, such as microsomes, the Golgi apparatus and various types of organelles. Those found most frequently in the protozoa comprise cilia, flagella, mitochondria, cytoplasmic membranes and plastids; these are essentially, as recently emphasized by Grimstone, similar in their fine structure to those of higher plants and animals (Grimstone, 1961). In spite of the importance of all the organelles, the general functioning of the cell is ultimately dependent on the nucleus, which contains the chromosome complex and the all important DNA. While this is in general true, researches by Brachet and his co-workers have shown that it is not immediately essential for the continued life of the cell. And it does not seem to be true that the nucleus is the only source of protein synthesis. He showed that in an amoeba divided so as to produce a non-nucleated and a nucleated half the oxygen consumption of the two fragments remained alike for at least 10 days. In the case of a unicellular alga *Acetabularia mediterranea* growth and protein synthesis proceeded at the same rate in both the nucleated and the non-nucleated halves during 2–3 weeks (Brachet, 1954, 1957).

In general the protozoan cell has one nucleus, but occasionally there may be more. These can be of the same structure and type as in the big amoeba Pelomyxa, to give only one example, which has a large number of similar nuclei. There can also, as in the ciliates, be a small micronucleus concerned with the heritable characters and active in nuclear recombination at conjugation, and the large trophic nucleus. This last is of a type with a different make-up from that found in the other groups of the protozoa and must be considered later.

The structure of the nucleus as shown in the light microscope in both living and stained preparations shows a considerable variety and they cannot all be gone into in detail. But in general there are two main types. One is the reticulate nucleus, in which the chromatin appears as granules or threads lying within the nuclear membrane often without a visible nucleolus. The other is the very frequent vesicular nucleus with a large karyosome now more usually called the nucleolus.

The chromatin, from which the chromosomes later appear in the more developed forms, is often, in the more primitive organisms, apparently diffused throughout the nuclear sap in the form of small particles which can be recognized by the Feulgen reaction. The chromosomes vary in the protozoa from well-defined structures comparable with those found in the metazoa to ill-defined aggregations of chromatin granules.

In this rapid review I propose only to describe the division of two small flagellates, both of which I have had the opportunity of investigating myself. In the resting state they both have typical vesicular nuclei but their mitotic figures are widely different. They are *Bodo caudatus* and *Heteromita globosa* (Robertson, 1927, 1928).

Bodo is the more primitive type and the chromatin is never visible in the form of recognizable chromosomes. It is a biflagellate with unequal flagella, each as usual with a basal granule. In addition, there is a kinetoplast which is Feulgen positive.

In the non-dividing nucleus there is a large dense nucleolus which does not stain in the Feulgen reaction and an arrangement of Feulgen-positive granules lying

between the nucleolus and the nuclear membranes. At division the single large nucleolus does not break up but keeps its identity throughout the process. In the early stage the nucleolus comes to lie at one side of the nuclear space and the chromatin, which now stains with much greater intensity, is aggregated on the opposite side, still within the nuclear space.

The nucleolus gradually moves into the chromatin mass, which gets pushed apart into two segments which come to lie at either end of the solid elongated bar of the nucleolus. This now elongates further, producing a dumb-bell figure, becoming increasingly thin at the centre, while the chromatin now lies as two separate masses at either end of the drawn-out nucleolus. The two nuclei finally draw apart and are now reconstructed in the original form. The kinetoplast has by now also divided and the body of the flagellate divides. The important point is that the nucleolus never disappears in this division, there is no equatorial plate nor are visible chromosomes or separate chromatin granules formed. In *Heteromita globosa*, which is also a biflagellate with two unequal flagella, the resting nucleus has again a large single nucleolus and the chromatin is arranged, as in Bodo, between the cell membrane and the nucleolus. At division, however, the nucleolus disappears and there is produced a perfect mitotic spindle, at the equator of which there are assembled small lumps of chromatin on an orthodox equatorial plate. At the apices of this figure the basal granules of the two flagella are found to have moved apart and are now functioning as centrosomes, one showing the shorter of the two unequal flagella and the other the longer one. The chromosomes, as we now call the Feulgen-positive little masses at the equator of the spindle, reduplicate and move apart to either pole, the nuclei are reconstructed in the usual way and the organism divides. The nucleolus reappears again as a large single structure in the centre of the nucleus. The centrosome-basal-granules now reduplicate themselves and the appropriate second flagellum grows out. In passing I should like to point out the double function of the basal granules of the flagella, both as basal granules of flagella and as centrosomes. This also occurs in the flagellated collar cells of certain sponges (Robertson, 1911*a*).

The point I wish to make concerning these two small flagellates is that one provides an example of a primitive form of nuclear division with a persisting nucleolus and the other a perfect type of mitosis with a disappearing nucleolus, which in all the broad essentials conforms to that found in higher types of organisms.

While I am considering the behaviour of some of the protozoan nuclei, I should like to mention some experiments carried out by Cleveland (1956).

He showed that in Trichonympha (one of the Polymastigote flagellates) the chromosomes could be completely destroyed by exposure to an appropriate concentration of oxygen (70–80 %) during the early period of gametogenesis when the chromosomes were reproducing themselves. This treatment does not damage the cytoplasm nor its organelles. The organism will live from three to five days and is capable of carrying on the differentiation towards gametogenesis as regards all the activities such as encystation, loss and renewal of the parabasals and flagella. The centrosomes or centrioles, as Cleveland prefers to call them, moreover function in a normal manner in the production of an achromatic spindle. Finally the cytoplasm can be effectively divided to produce two anucleate gametes; these of course

eventually die. It should be explained that in this species two gametes, a male and a female, which later escape, are formed within the one cyst.

These experiments throw an interesting light on the autonomy of the organelles and the achromatic structure from the immediate influence of the chromosomes and the DNA which they contain. I think the word 'immediate' must not be forgotten in considering these results.

(2) *Sex in the protozoa.* What we have to consider at this stage is not so much sex as what underlies it, namely the importance of biparental inheritance. As we find the protozoa today many species, both free living and parasitic, do not show any sign of it. What is meant by biparental inheritance is that at some stage in their life cycle there is a fusion of two reduced nuclei to make a new single one, thus obtaining the genetic material from two different sources.

Biparental inheritance, with the range of variation it implies in the possible recombination of the genetic material, has obvious potentialities in the creation of variants. It can also probably operate as a stabilizing agent for the species by the elimination of nuclear material at the reduction division.

The protozoa in which one finds biparental inheritance approach the matter in a number of very different ways. Some of the flagellates for example are haploid, that is to say they have only half the number of chromosomes all their lives except for a brief period during which they come together to form the zygote. Reduction to the haploid state occurs at the first nuclear division after syngamy. In these flagellates there is no observable difference between the two gametes and they are designated by the symbols + and − . There is, however, an important physiological distinction and clones made from one of the products of the first division of the zygote are unable to mate and form zygotes within the clone. It is only when gametes from a + clone meet gametes from a − clone that syngamy occurs and zygotes are formed.

One of the very simplest forms of syngamy occurs when as with *Polytoma uvella* the two haploid flagellates come together and, in a matter of minutes, both throw off their flagella and join by their anterior ends and fuse into a round shape and encyst, the two nuclei fusing as two small balls. The process can be observed over and over again by adding a little distilled water to a well-fed culture in decomposing hay and putting a few ml. into an open watch-glass. The syngamy and cyst formation gets going in about 10 min. The environment was changed, I may say in passing, by adding the distilled water and by the exposure to the air in the watch-glass. No doubt also the Polytoma were carrying some form of distinction, probably of the + and − kind.

We have been dealing with organisms that have a definite, almost a pedantic, outlook on biparental inheritance.

In Actinophrys, however, which is one of the beautiful freshwater heliozoa, there is a curious form of syngamy called paedogamy. This organism throws over the biparental idea altogether and carries on the process within the one cell. Bělař made a long and very excellent study of this organism in 1923 and 1924. Under certain conditions which he could control a single individual produces a cyst within which the organism, which is diploid, undergoes division into two; the two halves lying within the one cyst undergo two nuclear divisions of which the first is the reduction division whereby the diploid number of chromosomes is reduced to half.

In these nuclear divisions, as is usual, the unwanted halves degenerate and disappear. There is an interesting point raised by this disappearance of the surplus nuclei. It occurs at this stage in very many protozoan life cycles. The redundant nuclei are quickly digested in the protoplasm, but what difference is there in the definitive surviving nucleus in each gamete that prevents it sharing the fate of its fellows? The two now uninucleate organisms come together, the protoplasms fuse and the two nuclei unite into one diploid syncarion. Bělař found that one of the gametes after maturation was more lively than the other and he considered it to be the male gamete.

The German protozoologists of that period were much exercised by the bisexuality of the nuclear material. But it was never a very clear conception and I do not feel that it was very well established. The Actinophrys zygote secretes a protective cyst which upon being removed to fluid of lesser osmotic pressure germinates and the Actinophrys proceeds upon its active way again (Bělař, 1923, 1924).

Bělař's study is of particular value because he kept the organism under observation for a long time and he got a real insight into the way of life of this free-living Heliozoon. He found that syngamy could be induced or prevented according to the adjustment of the conditions of culture, and also that under uniformly favourable conditions Actinophrys could be cultivated indefinitely without syngamy or any other form of nuclear reorganization, beyond that of normal mitotic division. He actually kept a culture for 2 years and 8 months dividing normally, during which time there were 1244 generations without syngamy, and then he induced it again by the alteration of the external conditions. Here we have an organism with a definite if somewhat unusual type of syngamy, the expression of which is dependent upon the external stimulus of the environment, and which could be suppressed for an indefinite period without damage to the organism if the surrounding conditions were stabilized and kept uniformly favourable. This dependence on change in the environment emphasizes the point raised earlier that the protozoa carry out their lives in sequence in relation to the environment. Hartmann, in 1928, brought this aspect of the way of life of the protozoa to its logical conclusion and the life-cycle to the vanishing point, in his study of *Amoeba proteus* (Hartman, 1928). He maintained a single amoeba in a condition of active life without even permitting it to divide by cutting off a portion of the protoplasm every day. The regeneration of the protoplasm kept the cell in equilibrium for 130 days without division, when he gave up the experiment. Parallel experiments showed that these amoebae, if left undisturbed or if an insufficient amount of protoplasm had been eliminated, divided quite normally even after many days of this inhibited type of culture.

In tracing the emergence of the gamete types and the development of sex there is a great deal of material to choose from.

The method of association of two mature organisms in one reproductive cyst is found in certain parasitic forms, notably among the large group of the parasitic Gregarines, which are a subgroup of the Sporozoa. This is quite different from the paedogamy which we have seen in Actinophrys. In, for example, *Lipocystis polyspora*, parasitic in the fly *Panorpa communis*, after a phase of schizogony or asexual division, larger but apparently similar organisms are formed which are the gametocytes. They come together in pairs which form a single cyst but do not fuse into a single organism. In each organism the nucleus divides repeatedly, but the proto-

plasm does not. In each of the multinuclear gametocytes there now occurs a process whereby the nuclei bud off or are segregated into individuals consisting of one nucleus surrounded by a small portion of protoplasm. How this actually happens is one of the darker mysteries of protozoan physiology. The gametes from each of the gametocytes are now free in the common cyst; they fuse in pairs, one from each of the two gametocytes, and finally develop into sporozoites each containing 8 spores. Here, where the syngamy is perfectly clear, the differentiation of the sexes is nevertheless not obviously manifest, as the distinction between the final gametes is not well developed (Grell, 1938).

There are a number of interesting stages in the Coccidia, which are also a suborder of the Sporozoa, which show the development of the differences between the microgametes and the macrogametes, but I cannot go into all of them now.

Eimeria schubergi, however, parasitic in a centipede, for example, shows the final stage in the differentiation in the gametes. Here the macrogametocyte develops into a large macrogamete with finally one mature haploid nucleus. The microgametocyte produces a relatively large number of highly motile microgametes, one of which penetrates into the large macrogamete and contributes only the male nucleus, which becomes associated with the female nucleus on the fertilization spindle. The fertilized zygote now develops into the ookinete, in which four spores are developed, each with two sporozoites. The ripe sporocyst develops a very resistant cyst, which is evacuated to the exterior in the hope that it will be ingested by another centipede (Schaudinn, 1900).

The malarial parasite, also belonging to a subclass of the Sporozoa, has a life-cycle which in its broad outline resembles that of the Coccidia. It is more complicated, but also culminates in a macrogamete which is penetrated by a motile microgamete which delivers the male nucleus. This occurs in the gut of the mosquito which acts as the vector in transmitting the parasite.

The electron microscope studies by Professor Garnham and his co-workers of the sporozoites which are formed in the ookinete, and which are finally delivered from the salivary gland of the mosquito into the vertebrate host, are revealing a very complicated morphological make-up.

This is not part of my theme, but I should like to point out the latent morphogenetic capacity of the nuclear material which awaits the appropriate stimulus to come into action (Garnham, Bird & Baker, 1960; Garnham, Bird, Baker & Bray, 1961; Garnham, Bird & Baker, 1962).

To return to the type of syngamy exhibited by these organisms. We have here the full development of a female egg cell or macrogamete, which is responsible for the building material of the coming generation as well as contributing the female nuclear element, and a well-developed motile microgamete which plays a kinetic and purely nuclear role.

This again shows the pattern later found in so many of the higher forms, but I do not wish it to be considered that any of these arrivals at a mechanism found to be general in the metazoa has of necessity any philogenetic significance. My point is that the mechanism is arrived at by the protozoa and has also proved the appropriate one for the more developed organisms.

This matter has been well treated recently by Grimstone (1959), who sums up as follows: 'It is suggested that at the lower levels of organization exemplified by

biochemical characters, cell organelles, and early embryonic forms, similarities do not necessarily imply common ancestry, independent origin is equally probable'.

While we are dealing with sex there is one great class of the protozoa, namely the ciliates, which have arranged their nuclear outfit by dividing the trophic and sexual functions. Thus there are two nuclei in all the ciliates; the micronucleus, which in many types is very small, carries the genetic material which is responsible for hereditary characters, and the large macronucleus, which is responsible for the metabolism and trophic functions in general. The ciliates, instead of developing into male and female gametes which fuse as separate organisms to form a zygote, keep their identity and exchange a small nucleus which has been reduced to the haploid state by the usual process of maturation. The general arrangement of conjugation is familiar and need not be repeated here. The important point is that when the conjugating pair separate the exconjugates have each its old body, but a reconstructed new nucleus made up by the fusion of its own reduced nucleus with the wandering nucleus of the partner. The old macronucleus has been digested by this time. After some divisions (usually two) of the syncarion the definitive micronucleus is produced and in addition one of the micronuclei develops into the macronucleus by a process of chromosome reduplication without any spindle formation; thus producing a polyploid nucleus which may be of very large dimensions. The chromosomes in the formation of the macronucleus become aggregated into small bundles called genomes, each of which contains all the genetic material of the original nucleus. How the genomes are held together is not known (Piekarski, 1939, 1941; Grell, 1949, 1952, 1953; Fauré-Fremiet, Rouiller & Gauchery, 1957).

In this kind of polyenergid nucleus endomitosis takes place whereby the individual genomes divide without any spindle. This arrangement permits, first, the growth of the macronucleus, which in some of the larger species can be very large indeed; and, then, for its division without any elaborate mechanism. At cell division the mass shows a narrowing at the middle and in, for example, *Tetrahymena pyriformis* it elongates and divides, the two ends draw apart while the central junction is often left in the protoplasm, where it is digested. The development of the macronucleus and its fine structure is being actively studied at the present time. The work is of great theoretical interest, but it is not in a state as yet to be brought together in a survey of this kind.

With the curious passion for simplification which one encounters quite frequently in the protozoa, a group of ciliates—the Tetrahymena—are found, in both the wild state and particularly in cultivation in bacteria-free media, to dispense with the micronucleus. These strains never conjugate but they do not degenerate. The two species *Tetrahymena vorax* and *T. patula* L-FF, of which I have two amicronucleate strains, are dimorphic and also produce transparent (indeed they are rightly called invisible) cysts from which emerge eight small creatures known as tomites (Corliss, 1953; Williams, 1960, 1961). They manage all this and maintain their character without the sexual nucleus. The geneticists consider these amicronucleate types to be senescent forms, but they live happily for years and as I have said are frequently found wild in nature (Elliott, Addison & Cary, 1962). I have myself kept the monomorphic *T. pyriformis* strain GLR for a matter of 28 years and the two dimorphic forms each for 12 years.

The ciliates are definitely a tribe apart; they are extremely numerous and have an

extraordinary range of morphological types. Their development of cilia and organelles and their power of contraction are all remarkable. They are mostly free living, many of them are terrible predators and cannibals and in addition there are also a good many successful parasitic types.

(3) *Life-cycles.* I would like now to mention the life-cycles of some of the Trypanosomes. The appearance of the Trypanosome, as seen with the light microscope, is well known. The Trypanosomes belong to the group of the Trypanosomids, which is made up of four species: the Leishmania, which are without a flagellum for at least a good deal of their life; the Leptomonads with the kinetoplast at the anterior end and a free flagellum; the Crithidia with the kinetoplast still anterior or very near to the nucleus and the Trypanosomes proper with the kinetoplast at the posterior end and the flagellum running towards the anterior of the body with a free end.

The Trypanosomes are blood parasites and have a very wide distribution. They are present in fishes, in frogs and lizards and crocodiles and birds; in mammals from mice to elephants and in monkeys and man. There are, however, a few strange omissions. They have, so far as I am aware, not been found in newts in England and Europe, but are found in newts in America, where curiously enough they have not been found in fishes, at least in freshwater fish. This is probably due to idiosyncrasies in the vectors available for transmission (Barrow, 1953).

Trypanosomes were probably derived from leptomonad and crithidial forms inhabiting plant-sucking insects and various invertebrates. There is an interesting form called *Strigomonas oncopelti*, a leptomonad which is found in an African plant-sucking bug and in the latex channels of the plant on which the insect feeds. I mention it here because it has been a suitable subject for biochemical studies by Dr Newton.

The life-cycles of the Trypanosomes vary according to the nature of the vertebrate host and the type of transmitting agent, but the vast majority are transmitted by some blood-sucking vector and, before they are viable and, as it were, ripe for transmission to another host, they undergo a morphological change. They pass through a crithidial stage in which the kinetoplast has come to lie much nearer the anterior end of the organism. And upon regaining the trypanosome state they are once more ready to be delivered to the blood stream of the vertebrate host.

In, for example, the leeches which transmit the trypanosomes of tortoises and fishes, the whole story takes place in the intestine and the crop. The flagellates taken in with the blood go through a crithidial stage; some indeed become rounded off and go through a period of quiescence, but develop through a leptomonad phase and again into a crithidial state. All this group finally again assume the trypanosome shape, become very slender and elongated and pass from the crop into the salivary fluid in the proboscis and are injected into the fish (Robertson, 1911*b*).

In the Gambiense–Brucei group, the trypanosomes divide and multiply in the intestine of the tsetse fly, and, while they undergo a slight alteration in shape, do not go through the crithidial phase until they migrate forward into the proboscis and then into the salivary gland. Here, after a period of rapid multiplication as crithidia mostly attached to the gland wall by the flagellum, they turn into small free-swimming trypanosomes which are viable in the blood of the vertebrate (Robertson, 1913).

It should be mentioned here that the trypanosomes in the fish and the tortoises develop in almost 100 % of the leech intermediate host. Whereas the Brucei–Gambiense group complete the tsetse fly stage in at most 10 % of the flies and often in only 3 %. The tsetse fly, however, remains infective for the duration of its life.

At this point I must digress to remark that the electron microscope has revealed that the elegant simplicity of the trypanosome as seen in the light microscope masks a much more complicated structure. Dr Vickerman has lately shown that the kinetoplast in the blood stream form seems to be connected with a large mitochondrion passing forward towards the anterior end.

In the cultural form, which is thought to correspond, in general opinion, with the midgut stage in the tsetse fly, a second mitochondrion grows out from the kinetoplast towards the posterior end and the authors suggest that this pushes the kinetoplast nearer to the nucleus (Vickerman, 1962; Pittam & Vickerman, 1962). For the moment the deductions drawn from these morphological changes need further elucidation and it would be better if they were made from the investigation of the organism in the gut of the fly rather than from the cultural forms *in vitro*.

The cycles of different tsetse-borne African Trypanosomes vary in detail, but are all essentially of the same type, the final fly stage always taking place in the salivary fluid though not always taking place in the salivary gland. The connexion of a kinetoplast with the mitochondria has been recognized for some years, but its function in the cycle in the fly is a new suggestion (Clark & Wallace, 1960; Steinert, 1960).

But now we come to what can only be called a break out by the three subspecies *Trypanosoma evansi*, *T. equinum* and *T. equiperdum* of the Brucei group. These trypanosomes have been the subject of a detailed study by Dr Hoare and there is little doubt that this group is derived from the *T. brucei* type. But unlike all the other trypanosomes parasitic in mammals there is no intermediate host cycle. In the case of *T. evansi* the parasites are passed by direct mechanical transference of infected blood from one animal to another on the proboscis of Tabanid flies. *T. evansi* is found in horses, mules, camels, cattle and even in dogs. The subspecies *T. equiperdum*, which is parasitic in horses, is transmitted by direct contact at coitus. It has caused much loss among horses in Europe, especially in France, and it also spread into Canada, but it has been eradicated from that country about 40 years ago by the very remarkable work of a veterinary officer in 1915 (Watson, 1915). The whole elaborate business of the intermediate host cycle in the tsetse fly has simply been dropped and this triumphant group of trypanosomes has spread over vast areas beyond the distribution of any of the Glossina species. It causes a serious infection in North Africa, the Middle East, Asia, in India and China. In the guise of the subspecies *T. equinum* it has passed into the New World and is the cause of a disease in horses called Mal de Caderas in Brazil.

The behaviour of *Trypanosoma evansi* is a striking example of the short circuiting of a life-cycle when a simpler form of transmission meets the essential survival needs of a protozoan species.

Trypanosoma equinum has further simplified things by shedding the kinetoplast while, as is expected, retaining the blepharoplast or basal granule of the flagellum.

The akinoplastic trypanosomes seem to arise originally from individuals in *Trypanosoma evansi* infections, where at division the kinetoplast fails to divide so

that one of the products of division has a kinetoplast and the other has not. These last may disappear, being overgrown by the normal type, but in some strains fluctuating numbers of these mutant forms are found and in some cases a strain is found without any of the trypanosomes having a kinetoplast. The strains are perfectly viable and five of these were found in camels in the Sudan in the years 1934 to 1937 (Hoare, 1954, 1956). One of these strains has been maintained for 17 years in passage in mice and has never shown any return to the original condition.

The type of trypanosome known as *Trypanosoma equinum*, which is found in horses in the Argentine and other parts of South America, was first discovered in 1901. It is indistinguishable from the akinetoplast *T. evansi* strains found in camels in the Sudan. The disease in the New World was first described in Brazil in the middle of the last century derived, it seems, from imported horses. It is transmitted by direct passage by biting flies from animal to animal in the same way as *T. evansi*.

Occasional mutants without a kinetoplast appear in African *Trypanosoma brucei* strains, but they disappear and Reichenow has shown that the mutants do not survive in passage by the tsetse fly vector. This would seem to indicate that in the species transmitted cyclically by the tsetse fly these mutants are unable to survive. The loss of the organelle in *T. equinum* with the direct type of transmission has not inhibited the spread of the species, which has been established in South America for about a hundred years.

This akinoplastic condition can be produced artificially by exposing the trypanosomes to acriflavine and also to pyronin, as was first shown by Werbitski in 1910. It is interesting to find that *Bodo caudatus*, which as already shown is a free-living flagellate with a Feulgen-positive kinetoplast, when exposed to acriflavine produces up to 75% of akinoplastic organisms (Robertson, 1929); these however do not survive although they can divide once or twice. The drug, which is a bright yellow colour, is taken up by the kinetoplast *in vivo* and can be observed directly under the light microscope. As the strain becomes fast to the drug the colour ceases to be taken up *in vivo*. The inability of the Bodos to survive without a kinetoplast may perhaps be related to the presence of a large mitochondrion connected with the kinetoplast in these organisms lately described by Pitelka (1961). This is an electron microscope study and it is not known if all the species of Bodo have this character.

The trypanosomid *Strigomonas oncopelti*, which lives in the sap of an African latex plant and is transmitted by a plant-sucking bug, was found by Dr Newton to be quite insensitive to acriflavine and the drug is not taken up by the kinetoplast *in vivo*. Acriflavine will however penetrate into a dead organism staining the whole body (unpublished communication of Dr B. Newton, 1963).

I should like in conclusion to point out in what an interesting state the microbiological investigation of the protozoa is at this moment. The investigator can by means of the electron microscope observe the fine structure of the organism. He can follow the different stages of morphogenesis and in certain instances in time sequences which can be measured in minutes. It is true he does this by the despised methods of observation and description. He has powerful enzymes at his disposal; he can follow the uptake of labelled substances, and he can get some useful evidence from histochemistry.

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Antibiotics from *Aspergillus amstelodami*

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SUMMARY

A strain of *Aspergillus amstelodami*, which antagonized the growth of *Mycobacterium tuberculosis*, was shown to produce at least two antibiotic substances. A liquid medium was developed for their production, and cultural and assay procedures were defined. The antibiotics appear to be distinct from previously described derivatives of the true fungi and were named Amodin A and Amodin B. Both are active against certain Gram-positive and acid-fast organisms, but Amodin B has the wider antibiotic spectrum and is active against some Gram-negative organisms and strains of *Candida albicans*. Amodin A was produced in surface and submerged cultures, but in better yield in the latter; it was extracted and prepared as a crude product. It appears to be a moderately heat-labile peptide; though non-toxic to mice it did not prolong their survival when infected with *Staphylococcus aureus* or *M. tuberculosis* at the dosage of antibiotic used. Amodin B appeared only in surface cultures and was mainly present in the mycelium, from which it was liberated by dilute acid. It is relatively heat-stable and not inactivated by proteolytic enzymes. Amodin A production appears to be linked with the conidial mode of sporulation and Amodin B with the perithecial mode.

INTRODUCTION

A strain of *Aspergillus amstelodami* appeared as a contaminant which inhibited the growth of *Mycobacterium tuberculosis* on a plate of Lowenstein–Jensen medium which had been exposed to ultraviolet radiation. The strain is characterized by the production of relatively few perithecia but abundant conidial heads and shows a physiological abnormality which affects its growth from light inocula (Darling & McArdle, 1959). A culture (IMI 71295) has been deposited in the Commonwealth Mycological Institute, Ferry Lane, Kew, Surrey, England. The type of colonial variation may be of more than taxonomic interest because the different forms of sporulation may be associated with the formation of different antibiotics. We have found two antibiotics to be produced which we have named Amodin A and Amodin B.

METHODS

The fungus was maintained by mass inoculations of spores on Sabouraud medium or solid Czapek–Dox medium.

Production medium. Evans peptone (20 g.) and NaCl (5 g.) were dissolved in 900 ml. of distilled water, the solution was adjusted to pH 8·4, steamed for 1 hr. and filtered. To this was added 40 g. white table sugar, 7 ml. lactic acid (B.P.), 10 g. calcium lactate ($C_6H_{10}O_6Ca.5H_2O$) and 100 ml. Hartley's digest broth. The

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solution was adjusted to pH 6·0 with N-NaOH and sterilized by autoclaving at 112° for 30 min., during which the pH fell to 5·0.

Cultivation. Amodin A appeared in the metabolism solution of surface cultures, but was produced in greater quantities in submerged cultures in shaken flasks and on a larger scale in a deep fermentation unit. Shaken flask cultures were grown in 500 ml. Erlenmeyer flasks containing 125 ml. medium and three drops of lard oil to suppress foaming. The flasks were inoculated with about 10^8 spores and shaken on a reciprocating shaker with $3\frac{1}{2}$ in. stroke at 100 throws/min. Incubation was at 30° and the cultures were harvested on the 4th day. Deep fermentation was carried out in an adapted Hoover washing machine (Nixon, Borrow & Jefferys, 1959; details of technique will be supplied on request to the Department of Microbiology, The Queen's University, Belfast). Both methods gave similar results.

Amodin B was only produced in surface cultures. These were grown on layers of liquid medium about 4 mm. in depth. Roux bottles containing 85 ml. medium were seeded with about 10^7 spores/bottle from 4-day Sabouraud medium slope cultures. They were incubated at 32° and harvested on the 6th day. The metabolism solution was drained off and filtered through glass wool. The mycelium was then shaken with 30 ml. 0·1 N-HCl per bottle and the resulting acid solution similarly harvested. Solutions were stored at 4° at pH 4–6.

In the cultivation of the fungus, formation of Amodin A appeared to be related to the occurrence of conidial sporulation and formation of Amodin B to the occurrence of perithecial. Thus in submerged cultures, where only conidial sporulation took place, Amodin A alone was detectable. In surface cultures, where both modes of sporulation occurred, the antibiotics appeared in phase with the phenomena of sporulation; conidial sporulation was beginning on the 2nd day and maximal on the 4th corresponding with the production of Amodin A, while perithecial sporulation was beginning on the 3rd or 4th day and maximal on the 6th corresponding with the production of Amodin B.

Extraction of Amodin A. Several methods of extraction were investigated, and the following was found to be the most satisfactory. Manipulations were carried out in a cold room (4°) and solutions and reagents were chilled before use. The filtered metabolism solution to be extracted was adjusted to pH 5·2–5·6 with 0·1 N-NaOH and Norit charcoal (Savory & Moore) was added (4 g./l.). After adsorption for 1 hr. the charcoal was recovered by filtration or centrifugation and washed with 0·05 N-HCl, resuspended in 0·01 N-HCl and the suspension adjusted to pH 2·1–2·2. Acetone (4 l. per l. suspension) was then added and the mixture shaken for 30 min. The eluate was freed from charcoal by refrigerated centrifugation and the acetone removed by distillation under reduced pressure at temperatures near 40°. The concentrate was then shaken with twice its volume of diethyl ether and after separation the aqueous fraction was neutralized with N-NaOH. The ether layer was discarded and the residual ether in the aqueous fraction evaporated off under reduced pressure. The resultant clear yellow solution was freeze-dried.

Extraction of Amodin B. Metabolism fluid and the acid extract of the mycelium from surface cultures were concentrated by evaporation under reduced pressure, the temperature being kept below 75°. An unsuccessful search for a satisfactory extraction method was made. Charcoal adsorption with acetone elution and direct phenol extraction were tried. In the latter method enough phenol was used to

saturate the solution and leave a layer of such volume that there was 1 ml. for each 100–200 units of activity to be extracted. Recovery of Amodin B from the phenol layer was effected by shaking with ten times its volume of diethyl ether to remove the phenol and then adding 1 volume of distilled water.

Assay of Amodin A. *Corynebacterium fimi* (NCTC 7547), very sensitive to Amodin A, was used for its assay. Suspensions were prepared from 48 hr. cultures on meat digest agar slopes, washed off with normal saline and adjusted to Brown's opacity tube No. 8. The assay technique, based on a method used for penicillin (Knudsen & Randall, 1945), utilized the fact that with Amodin A, as with penicillin, the diameter of the zone of inhibition varies as the logarithm of the potency of the antibiotic, within the range of concentrations employed. Three replicate hole plates were used for each unknown. The medium consisted of Lab. Lemco, 1·5 g.; baker's yeast, 3·0 g.; Evans peptone, 6·0 g.; Davis agar, 15·0 g., in 1 l. distilled water, and dissolved by boiling. The solution was adjusted to pH 7·0 and then autoclaved at 126° for 20 min. The plates (4½ in.) received first a 15 ml. base layer and then an 8 ml. flood layer of the same medium inoculated with *C. fimi*, 4 ml. suspension/100 ml. After hardening overnight at 4°, eight 9 mm. diameter holes were punched in each. This provided one pair of holes for each of the four solutions used, i.e.: (1) lower concentration of standard; (2) lower concentration of unknown; (3) higher concentration of standard; (4) higher concentration of unknown (Fig. 1).

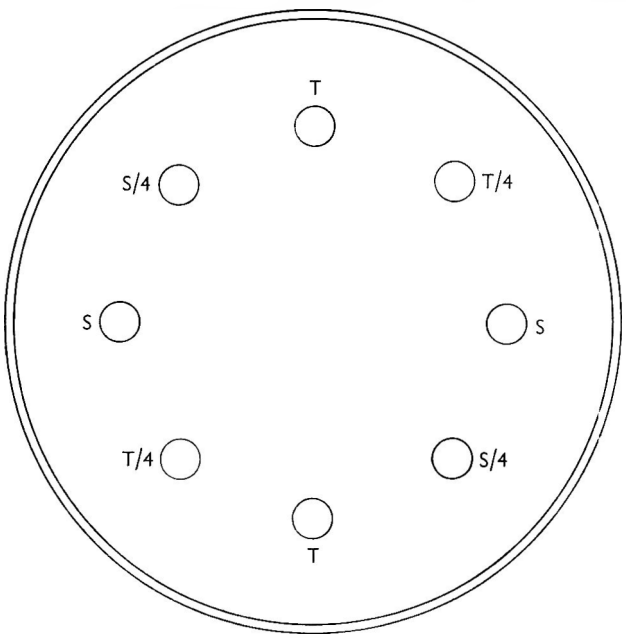

Fig. 1. Pattern of distribution of standard (S) and test (T) solutions on assay plates.

The dilution ratio (1/4), common to both the standard and unknown, was chosen in view of the gradual slope of the curve which relates the concentration of Amodin A with zone diameter. Potency of Amodin A preparations was referable to stable freeze-dried material having a potency of 100,000 units/g. One unit in 1 ml. of nutrient

agar was close to the minimal inhibitory concentration for *Corynebacterium fimi*. The standard solution was usually made to contain 100–200 units/ml., giving zones of inhibition about 25 mm. in diameter. Statistical examination of results underlined the need for close approximation in potency of the unknown and standard. To obtain this a preliminary trial was sometimes necessary. The solutions were put into the holes using a pipette delivering a measured 0·1 ml. Zone diameters were measured after incubation at 32° for 18–24 hr. Results were calculated according to standard statistical procedure for the evaluation of 4-point assays (Burn, Finney & Goodwin, 1950). Often the same solution gave a larger zone on one side of a plate than the other, due to inequality of agar depth. In the analysis of variance it was possible to eliminate a component from the sum of squares representing this potential source of error.

Assay of Amodin B. *Mycobacterium smegmatis* (NCTC 523) was sensitive to Amodin B and was used for its assay. Its use as a rapidly growing substitute for *M. tuberculosis* in the investigation of antituberculous substances was suggested by King, Knox & Woodroffe (1953). For the preparation of assay plates 48 hr. digest broth cultures were grown at 37°. After these had been agitated to disperse the growth, time was allowed for the larger clumps to settle and then the supernatant fluid was pipetted off and the opacity adjusted to Brown's opacity tube No. 2. *M. smegmatis* was relatively insensitive to Amodin A. Technique was similar to that used for Amodin A, but with a dilution ratio of 1/2 and nutrient agar as the medium and incubation at 37° for 48 hr. The unit of activity for Amodin B was such that 1·5 units in 1 ml. of nutrient agar gave the minimal inhibitory concentration for *M. smegmatis*. A concentrate prepared by evaporation under reduced pressure of an acid extract of surface culture mycelium was used as standard material. This and other solutions were very stable and were stored for several months without appreciable loss of activity.

Antibacterial activity. This was tested using agar dilution plates in the case of all organisms except *Mycobacterium tuberculosis*. Plates containing fourfold dilutions of Amodin A from 1600 to 25 units/ml. were streaked with dilutions of overnight broth cultures of the test bacteria, except in the case of pneumococci and streptococci, which were inoculated directly from cultures on blood agar. Inhibition of *M. tuberculosis* strains was tested in Dubos Tween albumin liquid medium. A range of dilutions of the antibiotic was prepared in 3 ml. volumes of the medium in bijou bottles and one drop of a 5-day culture of *M. tuberculosis* was added to each. The antibacterial activity of Amodin B preparations was tested similarly.

RESULTS WITH AMODIN A

Assay results with Amodin A. An example of the type of results obtained in assays of Amodin A is presented in Table 1. In this experiment two samples (A and B, each 0·05 g.) of Amodin A freeze-dried material were dissolved in 10 ml. distilled water. The standard was a solution of Amodin A containing 280 units/ml. It may be noted that fiducial limits are much wider for the sample B result (approx. $\pm 28\%$ of mean value) than for the sample A (approx. $\pm 7\%$). This is partly because the error sum of squares is greater in the case of the second group of plates, owing to uneven technique, and partly because there is a greater divergence of the sample solution potency from that of the standard. In general, with careful technique and

good approximation of unknown and standard potencies, fiducial limits approaching ± 5 % may be obtained.

Yield of Amodin A in submerged cultures. Amodin A began to appear in the metabolism solution between the second and third day and reached a maximum on the fourth day of incubation; e.g. in one fermentation a maximum value of 120 units/ml. was reached at the end of 3 days; at this time the pH value of the culture, which had dropped to 4·2 at 32 hr., had risen again to pH 4·9 and was still rising. The culture was harvested at 92 hr., by which time the pH was 5·2. The Amodin A concentration did not increase during the last 20 hr. of incubation, at least in the metabolism solution, but the delay gave time for the more complete utilization of nutrients, leaving less unwanted material in the harvested solution.

Table 1. *Assay results from two samples of Amodin A freeze-dried material*

(Sample A: Plates I–III. Sample B: Plates IV–VI.)

	Standard solutions, zone diameters in mm.		Sample solutions, zone diameters in mm.		
Plate	Diluted 1/4	Undiluted	Diluted 1/4	Undiluted	Relative potency (fiducial limits)
I	22·2	27·0	23·4	28·0	
	21·8	27·4	23·4	28·6	
II	22·0	27·0	22·2	28·0	1·36
	21·4	26·0	23·4	27·4	(1·25–1·45)
III	18·6	24·0	20·2	25·6	
	19·2	25·0	20·0	24·6	
IV	21·0	27·2	18·0	23·2	
	22·0	27·0	16·8	22·8	
V	21·6	27·6	17·6	22·2	0·36
	20·4	26·6	18·2	23·2	(0·28–0·48)
VI	22·0	27·0	16·0	21·8	
	20·2	26·4	16·0	23·2	

Results: sample A potency = 1·36 × 280 × 200 = 76,160 units/g. (fiducial limits = 70,000–81,200). Sample B potency = 0·36 × 280 × 200 = 20,160 units/g. (fiducial limits = 15,680–26,880).

Extraction of Amodin A. The solubility of Amodin A in aqueous acetone falls off sharply with increase of the acetone concentration to the region of 80 % by volume; this is the greatest concentration which can effectively be used for elution, but for routine purposes a lower concentration is preferable. In practice, the centrifuged deposit of washed charcoal at the beginning of the elution contains so much water that the addition of one volume of dilute acid and four volumes of acetone results in an effective concentration of about 76 % (v/v) acetone in water, which is satisfactory. There is advantage in adding the acid first because the charcoal readily suspends in it without aggregation, which occurs when the acetone is added first or ready-mixed with the acid. Also the pH value of the acid charcoal suspension is easy to test and to adjust to the value required. With this method, recovery of activity is virtually quantitative and a concentration of 10–20 times the potency in the starting fluid is achieved before freeze-drying. The figures for the last two extractions carried out on a 20 litre scale were 95 and 98 % recovery of the starting activity. Column methods tried were inefficient, giving recoveries less than 40 %.

Physical properties of Amodin A

Solubility. Amodin A is soluble in water, aqueous acetone, liquefied phenol, aqueous ethanol and methanol, slightly soluble in water-saturated *n*-butanol (300 units/ml.) and dry methanol (200 units/ml.) but insoluble in dry acetone, ethanol, *n*-butanol, ether, chloroform, ethyl acetate, amyl acetate.

Thermal stability. Metabolism solutions containing Amodin A lost activity at the rate of about 5–10 % monthly during storage at 4°. Concentrates stored in the cold at pH 2·0 were very stable and the monthly loss of activity was less than 2 %. Aqueous solutions were sufficiently stable at pH 1·0–8·0 to undergo manipulation at room and incubator temperatures over periods of several hours without appreciable inactivation. Aqueous solutions at pH 3·0–7·0 withstood immersion in boiling water for 6–8 min.; but over 90 % inactivation occurred after exposure to free-flowing steam for 20 min. Losses of activity observed during extraction and solvent tests suggested that Amodin A is more sensitive to heat when dissolved in aqueous acetone and alcohols than when in aqueous solution.

Dialysis. Cellophane sacs (British Cellophane PT 300) containing a solution of Amodin A (280 units/ml.) were suspended so as to rest on blocks of assay agar. After contact for 24 hr. the sacs were removed and the agar blocks and controls inoculated with *Corynebacterium fimi*. The results showed that Amodin A did not pass across the cellophan membranes. Solutions in such sacs did however lose activity, presumably by adsorption of the Amodin A on the cellophan surface.

Paper strip chromatography of solutions containing Amodin A was carried out by the descending method with butanol plus acetic acid as solvent. After overnight development, the strips were dried and cut longitudinally. One side was cut into 1 cm. pieces and placed on *Corynebacterium fimi* plates while the other was stained with Lissamine green. The Amodin A activity travelled as a broad colourless band having an R_F value of 0·47. The chromatograms showed several distinct bands one of which corresponded in position with the biologically active one.

Paper strip electrophoresis was done by the horizontal open strip technique (Grassmann & Hannig, 1952) using a potential of 120 V., current of 2·5 μA., and buffers ranging from pH 8·6 to pH 2·0. Twenty-five μl. of 20 % or 30 % (w/v) solution of freeze-dried Amodin A active material was applied to the starting line and the current passed for 16 hr. The strips were then dealt with in the same way as those from chromatography. It was found that mobility of the Amodin A was greater at pH 8·6 than at pH 7·5 and much greater at pH 2 than at pH 6. The distribution of the biological activity on all strips was that of a single homogeneous substance which tended to spread out as a rather broad band.

Adsorption chromatography was done on columns of neutral alumina with aqueous butanol as solvent. After a short run the column was sucked dry, extruded and cut into 1 cm. sections. Small amounts of these were plated on *Corynebacterium fimi* plates and the sections which showed antibiotic activity were eluted with 5 vol. acetone or methanol – 2 vol. of N/40 HCl, or were stained with Lissamine green. The biologically active fraction travelled down the column as a non-pigmented band which did not fluoresce in ultraviolet radiation. It travelled faster than the bands stained with Lissamine green and was separate from the lowest of these, though closely followed by it.

Starch electrophoresis (Kunkel & Slater, 1952; Stelos, 1956) showed that the antibiotically active fraction behaved as in paper strip electrophoresis at pH 2. Continuous paper electrophoresis in a Grassmann apparatus as modified by Holdsworth (1955) showed that the antibiotic material moved satisfactorily on the paper, but appeared in the collecting tubes below the point of application without any lateral separation from inactive constituents. The potential used (160 V.) was obviously inadequate.

Table 2. *Results of three ultracentrifuge experiments*

Potencies of the top and bottom contents are expressed as percentages of the mean potency. Fiducial limits are shown in parentheses.

Experiment	Top contents	Bottom contents
No. I (assay no. 84)	93 (76–112)	107 (90–126)
No. II (assay no. 86)	89 (81–96)	112 (101–120)
No. III (assay no. 87)	94 (87–101)	106 (98–115)
Means	92 (87–95)	108 (105–113)

Notes. In the case of assay no. 84 the wide fiducial limits are the result of lack of approximation in potency of the unknown and standard solutions. The fiducial limits for the means have been calculated on the basis of a combined variance estimate.

Ultracentrifugation. On three occasions solutions of about 1500 units/ml. potency, prepared from freeze-dried Amodin A material, were centrifuged in a Spinco preparative ultracentrifuge at 36,000 rev./min. for 1 hr. The top contents of the tubes were removed and assayed separately from the bottom and the washings of the emptied tubes were also tested. The results are shown in Table 2. In each case evidence of sedimentation was obtained, the bottom contents showing a concentration increase of the order of 10 % and the top contents a corresponding decrease. In Expt. 2 the results were significant at the 0·95 probability level. When the results of the three experiments were combined a mean figure of 8 % for the concentration change was obtained, with fiducial limits at 5 % and 13 %. In none of the experiments did assay of the tube washings show evidence of deposition.

Absorption spectrophotometry. Samples of solutions resulting from adsorption chromatography were examined spectrophotometrically over the range of wavelengths from 210 to 320 mμ but no absorption maxima correlating with the biological activity were found.

Effect of enzymes. Trypsin (Difco standard) in 0·25 % (w/v) concentration was incubated at 37° with Amodin A (200 units/ml.) at pH 7·1 and 4·1. After 2 hr. no activity remained in the sample at pH 7·1 and after 2·25 hr. at pH 4·1, less than 10 %. Papain (B.P.) at 0·5 % (w/v) produced a 70 % inactivation in 2 hr. at 37° at pH 4·6 and almost complete inactivation in 2·25 hr. at pH 6·3. Pepsin (B.P.) at 0·5 % (w/v) at pH 1·1 showed a barely detectable effect after incubation at 37° for 2 hr., whereas at pH 3·8 the activity was decreased by one-third in 2·25 hr. With

the same Pepsin and Amodin A concentrations, but incubation at 40° for 24 hr., inactivation was complete at pH 4·3 but 25 % of the control activity remained at pH 2·2.

Table 3. *Antibacterial spectrum of Amodin A*

Organisms (no. of strains in parentheses)	Concentrations giving partial inhibition (units/ml.)	Concentrations giving complete inhibition (units/ml)
Corynebacterium fimi (NCTC 7547) (assay test organism)	0·8	1·6
C. diphtheriae mitis (1)	Under 25	Under 25
C. diphtheriae gravis (1)	Under 25	Under 25
C. ulcerans (5)	100	400
Staphylococcus citreus (1)	25	100
S. aureus (Oxford strain)	10	25
S. aureus (2)	25	100
S. aureus (3)	100	400
S. aureus var. *albus* (1)	25	100
Sarcina lutea (1)	Under 25	Under 25
Streptococcus pyogenes (2)	Under 25	Under 25
S. viridans (3)	Under 25	Under 25
S. pneumoniae (5)	Under 25	Under 25
S. faecalis (7)	100	400
Bacillus anthracis (1)	400	Over 1600
B. subtilis (1)	1600	1600
B. megaterium (1)	100	400
Mycobacterium smegmatis (NCTC 523)	100	400
M. tuberculosis var. *hom.* (2)	30	60
M. tuberculosis var. *bov.* (1)	30	60
Mycobacterium sp. (NCTC 333)	400	1600
Neisseria catarrhalis (1)	100	400
Bordetella bronchiseptica (1)	400	1600
Haemophilus influenzae (1)	100	400
Escherichia coli (7)	400	Over 1600
Klebsiella pneumoniae (2)	Over 1600	Over 1600
Paracolon bacillus (1)	400	Over 1600
Proteus vulgaris (3)	400	Over 1600
P. vulgaris (1)	1600	1600
P. morgani (1)	400	1600
Pseudomonas pyocyanea (4)	1600	Over 1600
Salmonella typhi (1)	400	1600
S. typhi (11)	1600	Over 1600
S. paratyphi (1)	400	1600
S. paratyphi (11)	Over 1600	Over 1600
S. typhimurium (3)	Over 1600	Over 1600
Salmonella sp. (7)	1600	Over 1600
Shigella shigae (1)	400	1600
S. flexneri (1)	Over 1600	Over 1600
S. sonnei (1)	1600	Over 1600
Candida albicans (5)	Over 400	Over 400

In vitro *antibacterial tests of Amodin A preparations*

Tests of the antibacterial activity of Amodin A preparations are summarized in Table 3. The antibiotic-containing plates were read soon after the growth on the control plates had reached its maximum. The plates contained only a limited range of concentrations serving to distinguish those organisms which were relatively

sensitive from those which were relatively insensitive. As the units of measurement were arbitrarily chosen on the basis of biological effect, the results do not give any indication of the activity in terms of weight. The most active material prepared, still far from pure, had a specific activity of over 300,000 units/g. Amodin A preparations showed no antiviral activity when tested in tissue culture against type I poliovirus.

Development of resistance of staphylococci to Amodin A was studied with the Oxford strain and with a penicillin-resistant strain of pathogenic *Staphylococcus aureus* (M 381) isolated from a case of bovine mastitis and resistant to 10 units penicillin/ml. (Kindly supplied by Dr J. K. L. Pearson, Ministry of Agriculture, Stormont, Belfast.) Nutrient agar dilution plates were used and the organisms transferred several times by subculturing from the plate of highest concentration in the series which showed growth at each stage. Observations were made on the pattern of susceptibility to Amodin A in previously unexposed populations of staphylococci and on the emergence of resistant mutants during exposure. The results indicated that an inoculum of the order of 10^6 cocci from an unexposed strain of staphylococcus was composed of individuals showing a very wide range of susceptibility to Amodin A and containing organisms capable of giving rise to substrains several times more resistant than the parent strain. The results also indicated that, while second-stage mutants of resistance 16 times the original were readily selected, mutation at higher degrees of resistance did not often occur. But this last conclusion is tentative because, using impure Amodin A-containing material to prepare the plates, it is not possible to be sure there was not some other factor than Amodin A making its presence felt at the higher concentrations.

In vivo *tests with Amodin A preparations*

Toxicity. The acute intravenous toxicity of Amodin A was tested in mice with freeze-dried material containing 210,000 *Corynebacterium fimi* units/g. dissolved in distilled water to give 3% and 5% (w/v) solutions. These were injected intravenously in amounts varying from 0·1 to 0·5 ml.; only one dose was given. The mice were observed daily and weighed twice weekly. A few were sacrificed after 2 or 3 days, but the majority were observed for 4 weeks. Those sacrificed early were examined post mortem and liver, kidneys and spleen taken for histological examination. All 37 mice injected intravenously with a single dose of Amodin A solution survived. Of these, 5 received doses equivalent to more than 1 g. dry material/kg. body weight. The largest dose was 0·5 ml. of a 5% solution containing 5250 units Amodin A; two mice of 15 g. and one of 25 g. received this and for the former it represented a dosage of 1·67 g. dry material/kg. body weight. These animals were distressed and collapsed after injection but recovery was quite rapid and appeared to be complete. On post-mortem examination no evidence of toxicity was found apart from some cast formation in the renal tubules which was thought to have arisen from haemolysis caused by the injected solution.

The chronic toxicity (subcutaneous injection) was assessed in the course of experiments to test the effect of Amodin A on experimental infections with *Mycobacterium tuberculosis.* A group of 7 uninfected treated mice increased in average weight from 17·5 g. to 25 g. in the 19 days of the test, while in the same period a group of five uninfected untreated controls increased from 15·8 to 20·2 g. The treated

mice looked strong and physically normal with no sign of oedema; their appearance suggested that the injections had a favourable effect on growth.

Absorption and excretion. Urine from mice treated with Amodin A preparations was obtained at post-mortem examination and showed a concentration of between 100 and 200 units Amodin A/ml. urine; the blood concentration was about 10 units/ml. These findings suggest that Amodin A is absorbed from the subcutaneous tissues but is so freely excreted in the urine that a high blood concentration does not build up.

Mouse protection tests. The effect of Amodin A was tested in experimental infections with staphylococci and *Mycobacterium tuberculosis* in albino mice (strain VSBS) weighing 15–20 g.

Staphylococcus aureus strain PS/5 (kindly supplied by Professor E. T. C. Spooner, London School of Hygiene and Tropical Medicine) was used, which regularly produced multiple abscesses and death, usually within one week, after intravenous injection of 0·1–0·2 ml. of broth culture. Groups of inoculated mice were treated as follows: Amodin A solution 3640 units/ml.: subcutaneous dosage 0·2 ml. or 0·5 ml. daily; Amodin A solution 7500 units/ml.; subcutaneous dosage 0·5 ml. daily or 0·5 ml. 3 times daily. The Amodin A solution was first injected within 1 hr. of inoculation. No prolongation of life resulted in mice given any of the above treatments as compared with controls.

For the tests with *Mycobacterium tuberculosis* the Ravenel strain (var. *bovis*) was used (Donovick, 1950). A dose of 0·2 ml. of a 5-day culture in Dubos Tween medium and containing $3{\cdot}3 \times 10^8$ bacilli/ml. was inoculated intravenously into groups of mice which were then treated as follows: groups injected with $6{\cdot}6 \times 10^7$ bacilli given Amodin A solution 2500 units/ml., 0·2 ml. daily, or 0·2 ml. twice daily; groups injected with 8×10^6 bacilli given Amodin A solution 5000 units/ml., 0·2 ml. daily or 0·2 ml. twice daily. No prolongation of life compared with the controls resulted from any of the treatments.

RESULTS WITH AMODIN B

Yield of Amodin B in surface cultures. Amodin B was detectable in the metabolism solution from the fourth day of incubation and reached a maximum on the sixth day but the concentration was never more than 5 or 6 units/ml. and often was only 1 or 2 units/ml. Extraction of the mycelium with diluted HCl gave a greater yield than the corresponding metabolism solution showed. The acid solution usually contained from 6 to 12 units/ml. and it had a much lower content of unwanted material than had the metabolism solution.

Physical properties of Amodin B

Solubility. This was similar to that of Amodin A except that the solubility of Amodin B in aqueous acetone solutions did not fall off sharply as the acetone concentration increased above 80 % (w/v) and concentrations of 85 % and over could be used for elution.

Stability. Amodin B has greater stability than Amodin A and is more stable at pH 7·0 than at pH 2. With aqueous solutions at pH 5 to pH 7 and at 4° there was no detectable loss over periods of several months. When aqueous solutions at pH 7·0

or less were heated to 100° for 15 min., 5–10 % inactivation occurred; at pH 9·0 under these conditions the degree of inactivation was greater. Aqueous solutions did not withstand open evaporation on a water bath but were readily concentrated by evaporation under reduced pressure at temperatures below 75° at pH values between 5 and 7. Inactivation took place when dry material was treated with anhydrous methanol and ethanol.

Effect of enzymes on Amodin B. The effect of 0·125 % (w/v) trypsin at pH 7·0, papain (0·5 %, w/v) at pH 6·8 and pepsin 0·25 % (w/v) at pH 3·1 were tested on a solution containing Amodin B 15 units/ml. The mixtures of antibiotic preparation + enzyme were incubated for 24 hr. at 37°. No decrease of antibiotic activity by any of these treatments was observed.

Dialysis. A method similar to that used for Amodin A was used. The test solution contained 20 units Amodin B/ml. and the cellophan sacs were left in contact with the agar blocks for 24 and for 72 hr. After 24 hr. the test organism (*Mycobacterium smegmatis*) showed partial inhibition and complete inhibition after 72 hr. contact, indicating a slow passage of Amodin B through the cellophan.

Antibacterial activity of Amodin B. Freeze-dried powder prepared directly from the acid extraction of surface culture mycelium was used; it contained 750 units Amodin B/g. and also a little Amodin A. In the dilutions used the Amodin A would not have been in sufficient concentration to affect the results. Plates containing 25 units, 5 units and 1 unit of Amodin B/ml. were prepared. The test procedure was similar to that used for Amodin A; the results are shown in Table 4. Amodin B showed no antiviral activity when tested in tissue culture against type I poliovirus.

Examination of other strains of Aspergillus amstelodami *for antibiotic effects*

Three strains of *Aspergillus amstelodami* from the Commonwealth Mycological Institute (CMI) all gave evidence of antibiotic production; comparisons with our strain showed well marked differences however (Table 5). On solid media none of the CMI strains produced zones inhibitory to *Corynebacterium fimi*, which suggests that they produce little if any Amodin A. On liquid media the two CMI strains examined produced greater inhibitory activity on *Mycobacterium smegmatis* than did our strain, but it is not certain whether this was due to Amodin B or not—unlike the result with our strain the activity present in the metabolism solutions of the CMI strains was greater than that in eth acid extracts of mycelium. With the third test organism, a strain of Bacillus, the three CMI strains produced inhibitory zones on solid media but our strain did not, indicating that they produced some other antibacterial substance distinct from Amodin A and Amodin B.

DISCUSSION

The genus *Aspergillus* contains many antibiotic-producing species. All of the fourteen groups into which the genus has been classified (Thom & Raper, 1945) include one or more antibiotic-producing species (Brian, 1951). In the large *Aspergillus glaucus* group, nine species have previously been shown to produce antibacterial substances (Wilkins & Harris, 1942, 1945; Gill-Carey, 1949; Furtado, 1944; Gupta & Viswanathan, 1955) and *A. amstelodami* can be added as a further example. Gill-Carey (1949) examined a strain of *A. amstelodami* and did not observe

Table 4. *Antibacterial spectrum of Amodin B*

Organisms (no. of strains in parentheses)	Concentrations giving partial inhibition (units/ml.)	Concentrations giving complete inhibition (units/ml.)
Corynebacterium diphtheriae mitis (1)	1	5
C. diphtheriae gravis (1)	Under 1	Under 1
C. ulcerans (5)	1	5
C. fimi (NCTC 7457)	1	5
Staphylococcus aureus (7)*	1	5
S. citreus (1)	1	5
Sarcina lutea (1)	1	5
Streptococcus pyogenes (1)	Over 1	5
S. pyogenes (1)	Over 5	25
S. viridans (3)	Over 5	25
S. pneumoniae (1)	Over 1	5
S. pneumoniae (4)	Over 5	25
S. faecalis (7)	Over 5	25
Bacillus anthracis (1)	Over 1	5
B. subtilis (1)	5	25
B. megaterium (1)	Under 1	Under 1
Mycobacterium tuberculosis var. *hom.* (1)	1	5
M. smegmatis (NCTC 523) (assay test organism)	1	2
Mycobacterium sp. (NCTC 333)	Over 1	5
Neisseria catarrhalis (1)	Over 1	5
Bordetella bronchisepiica (1)	25	Over 25
Haemophilus influenzae (1)	Over 5	25
Escherichia coli (3)	5	25
E. coli (4)	Over 25	Over 25
Klebsiella pneumoniae 9 (2)	Over 25	Over 25
Paracolon bacillus (1)	25	Over 25
Proteus vulgaris (4) and *morgani* (1)	25	Over 25
Pseudomonas pyocyanea (4)	5	Over 25
Salmonella typhi (11)	Over 5	25
S. paratyphi (1)	5	25
S. paratyphi (1)	25	Over 25
S. typhimurium (3)	25	Over 25
Salmonella sp. (3)	5	25
Salmonella sp. (4)	25	25
Shigella shigae (1) *flexneri* (1) and *sonnei* (1)	Over 25	Over 25
Candida albicans (5) (on Todd & Hewitt agar)	1	5
(on Sabaraud agar)	8	16
Saccharomyces sp. (1) (on Todd & Hewitt agar)	Under 1	Under 1
(on Sabaraud agar)	1	5

* Including 'aureus', 'albus', Oxford and penicillin-resistant strains.

antibiotic activity, but this may illustrate the general principle that antibiotic-production is a strain, rather than a species, characteristic (Wilkins & Harris, 1945). Even when strains possess the capacity to produce antibiotics, the detection of this property depends to a considerable degree on successful selection of suitable growth conditions and test organisms. It is possible that antibiotic-production will prove a common finding among *A. amstelodami* strains.

The question arises whether Amodin A and Amodin B are identifiable with any previously characterized antibiotics. As far as can be seen, it would appear unlikely. Most antibiotics obtained from the true fungi have been extractable by such solvents

Table 5. *Comparison of strains for antibacterial effects*

Strain	Effect on *Corynebacterium fimi* (solid medium)	On *Mycobacterium smegmatis* (liquid medium)	On sporing bacillus (solid medium)
Our strain	+ +	+	−
IMI 57394	− −	+ +	+ +
IMI 17455	− −	+ +	+
IMI 16110	− −	Not examined	±

as chloroform and ether, and, of the few which are not, none corresponds in all its properties with either of the substances studied here.

Though pure samples of Amodin A and Amodin B have not been obtained it is possible to draw some conclusions about their chemical nature. In the case of Amodin A, its failure to dialyse, its relatively slow diffusion in agar and its behaviour in the ultracentrifuge indicate a fairly large molecule, and that this has a polypeptide structure is indicated by the inactivating effect of proteolytic enzymes. In the case of Amodin B, its dialysability and its relatively rapid diffusion in agar and its resistance to inactivation by proteolytic enzymes indicate a smaller molecule of a different character.

Regarding therapeutic potentialities, the mouse-protection tests carried out with Amodin A gave no indication of therapeutic activity at the level of dosage employed, but neither was there evidence of toxicity; in fact, uninfected mice receiving injections of Amodin A showed a greater weight gain and stronger physique than controls. The subject requires further study.

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The Effect of a Shift on the Frequency of Transduced *Salmonella typhimurium* Cells in the Early Period of Transduction

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SUMMARY

The frequency of transduced cells (*gal*$^+$) was reduced by the transfer of the *Salmonella typhimurium* recipient cells with adsorbed P22 transducing phage from a complete into a minimal medium. This reduction was most conspicuous immediately after the adsorption of the phage (3 min.) and then gradually decreased as transfer was delayed. After cultivation for 90 min. under conditions which permitted growth and multiplication of the cells the frequency of the transduced cells was practically no more influenced. The sensitivity of the potentially transduced cells to acriflavine was assayed soon after the adsorption of the phage. An early acriflavine-reversible phase was demonstrated in more than 50% of transductions. A close time relationship was found between shift-reversible and acriflavine-reversible phases.

INTRODUCTION

The final product of a stable transduction by the P22 phage is a recombined microbial cell which transfers the new marker obtained by transduction into all its progeny. It is assumed that the first step which leads to such a recombinant, i.e. the injection of deoxyribonucleic acid (DNA) of the transducing particles into the recipient cell, is analogous to the first stage of any phage infection. The second stage, i.e. the mechanism of the incorporation of the transduced fragment into the genetic apparatus of the recipient cell and the period during which the integration processes occur, is the main problem of the genetics of transduction.

Very little is known about what happens after the penetration of the transducing element into the recipient cell. In transduction of motility to non-motile Salmonella cells the first motile recombinants appear in about three generations after phage adsorption (Stocker, 1958). In studying the mechanism of transduction of galactose fermentation it was found that during the period in which the viable count of the culture increased *ca.* 32-fold the increase in the number of transductants was less, by a factor of 2–4 (Hubáček, 1961; Hubáček & Málek, 1962). The further results support our opinion that the potentially transduced cells divide at about the same rate as the rest of the population and during the first five divisions *gal*$^-$ (untransduced) cells are segregated (Hubáček, 1962). Delayed gene incorporation, which probably takes place during the first two divisions, and nuclear segregation may account for 'the lower number of divisions of transduced cells'.

This paper reports some experiments in which we succeeded in reducing the

frequency of transduced cells by a method which in its principle is similar to the 'shift-down' method (Maaløe, 1960), i.e. by transfer of a suspension of cells soon after the adsorption of the transducing phage from a complete to a minimal medium. The clarification of the nature of this effect might produce information about the processes leading to incorporation of the transducing element into the genetic apparatus of the cell. For the purpose of timing the period of incorporation the elimination of the free transducing element by acriflavine (Hirota & Iijima, 1957) has been followed.

METHODS

Bacterial and phage stocks. Strains of *Salmonella typhimurium* SW351, LT2 (Zinder & Lederberg, 1952) and SW351 R were used; SW351 (*gal*$^-$) is an auxotrophic mutant of strain LT22, lysogenic and carries P22 phage. The strain SW351 R is a prototrophic back mutant of the auxotrophic strain SW351. In liquid minimal medium it grows only slowly and on minimal agar produces only small colonies. Phage lysates used were P22 grown on LT2 (*gal*$^+$) strain and were made as described by Zinder & Lederberg (1952). The lysates were sterilized by filtration or by chloroform treatment. Prior to the experiment chloroform was removed by aeration for 30 min. at 37°. The sterility of the lysate was tested on nutrient agar. Phage titrations were made by the soft-agar-layer method (Adams, 1950).

Media. The phage was assayed in soft complete agar (0·7 %) laid over a complete bottom-layer agar. Bacterial recipients were grown on complete agar. Transductions were assayed on eosin-methylene-blue agar (EMB) with galactose. All the minimal and complete, liquid or solid, media used were as described by Lederberg (1950); in the minimal medium $MgSO_4$ was replaced by 0·01 % of $MnCl_2$, which was added in this concentration to all other media which were used for transduction. For the elimination of the transducing element by acriflavine Difco broth was used (Hirota & Iijima, 1957).

Transduction experiments. In all experiments high numbers of cells and phage had to be used because of the low frequency of the transduced cells (10^{-6}/recipient) which is characteristic for this type of transduction. 0·1 ml. of a 'starved' suspension of the recipient cells ($2·0–6·0 \times 10^{10}$ /0·1 ml.), obtained by washing the 48 hr. culture of SW351 or SW351 R strain from complete agar by saline, was mixed with 9·9 ml. of a phage suspension in a complete medium. This ratio has also been maintained when preparing a larger amount of the suspension of cells with the adsorbed phage. The titre of the phage stocks used in the experiments was $1·0–2·5 \times 10^{10}$ /ml., the multiplicity 1–6. The phage was adsorbed for 3, 10, 15, 30 or 60 min. at 37°.

The elimination of the transducing element by acriflavine. The phage was adsorbed for 10 min. under the conditions stated above. The cell suspension was then pipetted in 1·3 ml. amounts into flasks containing 63·7 ml. of the complete medium which were then incubated at 37° without aeration. At intervals from 0 to 150 min., 65 ml. samples were centrifuged in the cold; the sediment was then made up to 10 ml. and divided into two parts. One part of each sample was assayed for viable count and number of transductants. The cells in the other part of each sample were suspended in 35 ml. of Difco broth (pH 7·6) with acriflavine. After 8 hr. incubation at 37° without aeration the cells were centrifuged, washed with the complete

medium and the sediment suspended in 0·65 ml. of complete medium. Then the viable count and number of transductants were determined. From the viable count and number of transduced cells in the untreated control part of the sample (N_u) and after acriflavine treatment (N_a) the ratio $N_a:N_u$ was calculated. The stock aqueous solution of acriflavine (500 μg./ml.) was sterilized in an autoclave.

Viable count. Samples of 0·1 ml. were spread on pre-dried nutrient agar plates. The number of transduced (*gal*$^+$) cells was determined by spreading 0·2 ml. samples of the suspension on each of three pre-dried EMB galactose plates.

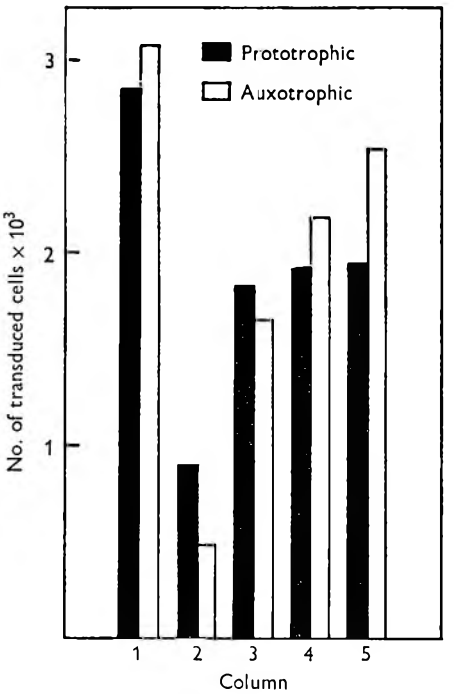

Fig. 1. The influence of various cultivation media on the frequency of the transduced cells. Two recipient strains of *Salmonella typhimurium* (*gal*$^-$) were used. The phage was adsorbed for 15 min. at 37° in complete medium and then 1 ml. samples of the suspension were pipetted into: complete medium (column 1); minimal medium (column 2); minimal medium with 10 mg./ml. casein hydrolysate (column 3); minimal medium with 5 mg./ml. of yeast extract (column 4); minimal medium with both yeast extract and casein hydrolysate (column 5). The cells in the samples were centrifuged in the cold, washed and suspended in 1 ml. of the above-mentioned media. The suspensions were exposed for 90 min. at 37° and the viable count and the number of transductants were determined. The columns of *gal*$^+$ transductants are expressed per $2·9 \times 10^9$ (auxotrophic) and $3·6 \times 10^9$ (prototrophic) cells in suspensions.

RESULTS

The influence of minimal medium on the frequency of transduced cells during the early phase following the adsorption of the phage. By transferring the recipient *Salmonella typhimurium* cells with the adsorbed P22 transducing phage from a complete into a minimal medium and subsequently incubating them for 90 min. at 37°, the frequency of transduced (*gal*$^+$) cells was reduced. The results of a single typical experiment are shown in Fig. 1. In the auxotrophic strain the difference in

the number of transduced cells between the complete and the minimal medium was more marked. The reduction of the number of transduced cells in the minimal medium was to a great extent abolished by supplementing with casein hydrolysate and/or yeast extract.

The object of later experiments was to establish for how long after the adsorption of the phage the sensitivity of the transduced cells to the minimal medium persisted. Figure 2*A* shows a plot of the whole number of cells and the number of transduced

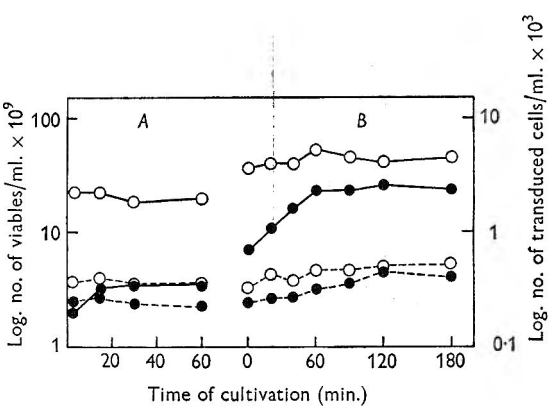

Fig. 2

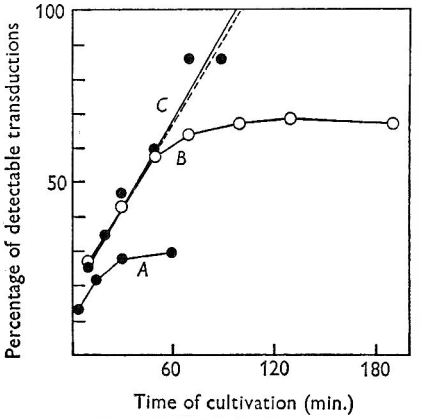

Fig. 3

Fig. 2. Total number of cells and number of transduced cells in complete and minimal medium during early periods of cultivation after phage adsorption. *A*, Recipient cells of strain sw351R (0·1 ml. suspension; $7{\cdot}3 \times 10^{10}$ cells) were mixed with phage (16·8 ml. of phage suspension in complete medium; $4{\cdot}2 \times 10^{11}$ of phage particles). After 3, 15, 30 and 60 min. of adsorption 1 ml. samples were taken. After washing the cells were suspended in 1 ml. of complete (○) or minimal medium (●), exposed for 90 min. at 37° and the viable count (---) and the number of transduced cells (—) then determined. *B*, Recipient cells of strain sw351R were mixed with the phage; after 10 min. of adsorption the suspension was diluted 5× with complete medium and incubated at 37°. Samples taken were washed in complete (○) or minimal (●) medium and resuspended in these media to original volume. In complete medium the viable count (---) and the number of transduced cells (—) were assayed at once; in minimal medium this was done after 90 min. exposure at 37°.

Fig. 3. The percentage of transduced cells detectable after 90 min. exposure in the minimal medium at certain periods after adsorption. *A*, Undiluted cell suspension (experimental conditions see Fig. 2*A*). *B*, Cell suspension diluted 5× (experimental conditions see Fig. 2*B*). *C*, After 10 min. adsorption the suspension of cells with phage diluted 50× with complete medium and incubated at 37°. At intervals samples were taken and chloramphenicol added (final concentration 15 μg./ml.). The cells were centrifuged and the sediment made up to 10 ml. and divided. One part was assayed at once for viable count and number of transductants. The cells in the other part were centrifuged, washed and suspended in 0·65 ml. of minimal medium; after 90 min. at 37° the viable count and the number of transduced cells were determined.

cells. Curve *A* in Fig. 3 indicates the percentage of transductants detectable after exposure to the minimal medium. The results of the experiment show that the sensitivity of transduced cells to shift from complete to minimal medium was highest at the onset of the transducing process (3 min. after the adsorption of the phage) and then decreased. However, after 30 min. exposure the sensitivity did not decrease any further. This phenomenon seemed to be due to the high concentration of cells in the adsorption mixture. In the next experiment the suspension of cells

with adsorbed phage was diluted 5 × in complete medium at the end of the adsorption period. In Fig. 2*B* the number of viable bacteria and the number of transduced cells are plotted; curve *B* in Fig. 3 represents the percentage of transductants detectable after exposure in the minimal medium. The results show that when the recipient cell suspension had been thus diluted the sensitivity of the transduced cells to transfer from the complete into the minimal medium at first decreased linearly but after 60 min. this decline stopped. Extrapolation of the linear part of the curve suggests that the transduced cells should, theoretically, become insensitive to the minimal medium after about 90 min. of cultivation.

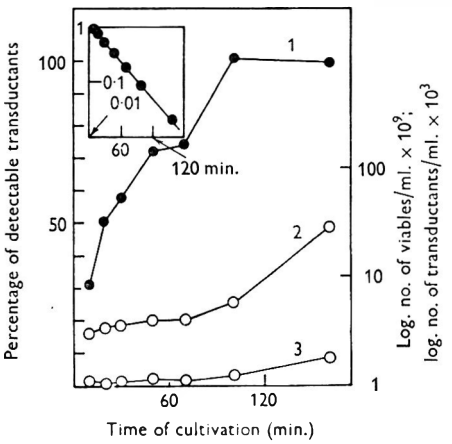

Fig. 4

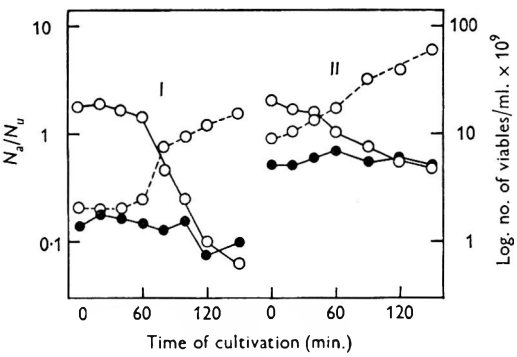

Fig. 5

Fig. 4. The percentage of transduced cells detectable after 90 min. exposure in minimal medium at certain periods of cultivation. Experimental conditions see Fig. 3*C*. The inset in the left corner represents the statistical evaluation of curve 1 by the least squares method and expresses the exponential relation between duration of cultivation and the fraction of transduced cells sensitive to minimal medium.

Fig. 5. The elimination of the transducing element by 8 hr. acriflavine treatment at certain periods after adsorption. I, II, number of experiment; N_u, number of cells in untreated control part of the sample; N_a number of cells after acriflavine treatment. --○--, log number of viable bacteria; —○—, log N_a/N_u of viable bacteria; —●—, log N_a/N_u of transductants.

To confirm this hypothesis experimentally the suspension with adsorbed phage was diluted 50 × with the complete medium. The treatment of these samples was more laborious, however, and even if it was carried out in the cold it required a certain stabilization of the system, at least during the initial transduction stages. From our experiments chloramphenicol was found to be most suitable for this purpose. It did not affect the frequency of transduced cells and did not otherwise interfere with the phenomenon studied here. Curve *C* in Fig. 3 shows the percentage of transduced cells which persisted after exposure in the minimal medium in relation to the time of preliminary cultivation in complete medium. This curve has a linear character and the sensitivity of transduced cells to minimal medium in fact disappeared after about 90 min. of cultivation. Figure 4 represents the percentage of transduced cells detectable after exposure in the minimal medium (curve 1) and also the number of viable bacteria (curve 2) and of transduced cells

(curve 3). It appeared again that the sensitivity of the transduced cells to the minimal medium virtually disappeared after about 90 min. of cultivation.

Elimination of the transducing element of the P22 phage by acriflavine. The timing of the fixation of the transducing element and especially the relationship between this integration process and the period of sensitivity of transduced cells to minimal medium were of the greatest interest here. First the optimal conditions for elimination of the transducing element of P22 phage had to be found. Experiments were carried out to test (*a*) the influence, 10 min. after adsorption, of various concentrations of acriflavine, (*b*) the possibility of an increased elimination by irradiation of the recipient cells after phage adsorption, and (*c*) the most suitable length of exposure to acriflavine. It had been found that the optimal concentration was 10–15 μg. acriflavine/ml. Irradiation of the recipient cells with adsorbed phage did not increase the effectivity of elimination; it was virtually identical when using the same concentration of acriflavine (10 μg./ml.) with or without irradiation of the cells. A suitable length of exposure of the cells to acriflavine was 8 hr. at 37° (Table 1).

Table 1. *The elimination of the transducing element by acriflavine after 10 min. of phage adsorption*

Suspension was irradiated 5 sec. at 50 cm. distance from Philip TUV lamp 30 W; 89 % of cells survived.

	Cell suspension with adsorbed phage					
	Non-irradiated			Irradiated		
Exposure to acriflavine (hr.)	No. bacteria $\times 10^9$/ml.	No. *gal*$^+$ cells $\times 10^3$/ml.	Elimination (%)	No. bacteria $\times 10^9$/ml.	No. *gal*$^+$ cells $\times 10^3$/ml.	Elimination (%)
0	5·6	4·0	0	5·0	3·8	0
4	7·2	2·7	32·5	5·3	2·3	39·3
8	7·0	2·1	47·5	5·1	2·0	47·7
20	2·5	0·24	68·0	1·7	0·2	70·0

In further experiments the relation between cellular growth and sensitivity of the transducing elements to acriflavine was assayed. The results of two such experiments are shown in Fig. 5. After 10 min. of phage adsorption the cell suspension (viable count in expt. 1 *ca.* 2×10^9/ml. and in expt. 2 *ca.* 8×10^9/ml., to follow the higher number of transductants) was diluted 50× with complete medium and incubated at 37°. At intervals samples were taken and divided into 2 parts. One part of each sample was treated by acriflavine for 8 hr.; the second part served as a control and was not treated by acriflavine. Before the assaying of viable count both suspensions were concentrated to the original volume.

In the early period up to 60 min. of incubation the number of viable bacteria after acriflavine treatment slightly increases ($N_a/N_u > 1$) and then the cells become sensitive and the viable count is reduced ($N_a/N_u < 1$). The number of transduced cells was most influenced in the initial phase of transduction; after 8 hr. exposure it decreased to about 80 % in expt. 1 and to about 50 % in expt. 2. In expt. 1 12·5 μg. and in expt. 2 10 μg. acriflavine/ml. were used. This early flavine reversible phase lasts during the 60 min. of cultivation. After this time it could hardly be judged when it ended because of the complication of the onset of sensitivity to acriflavine of the rest of the culture.

DISCUSSION

The above experiments have shown that it is possible to reduce the frequency of transduced cells by their exposure to minimal medium. The reduced yield of transductants was not paralleled by reduction in the total number of viable recipient bacteria. This sensitive phase of the potentially transduced cells to the minimal medium resembled the reversible phase as described by Ephrussi-Taylor (1958) in the transformation of pneumococcal cells.

What does the effect of the minimal medium on the frequency of transduced cells consist of? Our latest experiments show that the frequency of transductants was not influenced by the minimal medium itself. This effect was observed only after shift down from complete to minimal medium, but not when the cells after adsorption of the phage in minimal medium were transferred to minimal medium again (Hubáček, 1963). It may be assumed, therefore, that the reduced frequency of transductants after transfer from complete to minimal medium was not due to deficiency of some elements in minimal medium, but rather the result of disturbance in the regulation mechanisms in the cell. In this connexion the experiments of Maaløe (1960) are interesting; it was shown that after shift down repression of metabolic activity takes place.

Possible higher sensitivity of the pre-transductants to spreading (Goldstein, Goldstein, Brown & Shao-Chia Chou, 1959) was excluded by assaying the galactose-positive transductants in a soft agar layer. According to another interpretation the reduced frequency of transduced cells was not due to the death of the cells but rather results from the 'curing' of the cells from the transducing element. The nature of this event is unknown. Our preliminary experiments have shown that the number of transduced cells was reduced at 0° as well as 37°. The sudden chilling of the suspension had a stimulating effect and reduced the number of transductants in complete medium. The disturbance in the normal composition of amino acid pool in the starved cell seemed to be the primary cause of this event. But the change in the pool by itself probably did not cause the 'curing' of the cell from the transducing element. The presence of Mg^{2+}, Ca^{2+}, Mn^{2+}, cadaverine or putrescine in the minimal medium can reverse the effect of a shift and the frequency of transduced cells remains unchanged (Hubáček, 1963). This stabilization process could be demonstrated only at a higher temperature (22° or 37°). According to these results it might be speculated that the 'curing' process by itself consists at least of two stages. The first can be carried out at 0° and the transducing element need not be destroyed. The second one occurs only at higher temperature and may be inhibited by the presence of divalent cations. This hypothesis is being the object of further experiments.

From experiments on the curing effect of chloramphenicol on bacteria infected with different mutants of P22 phage it was concluded that the loss of phage element does not involve the elimination of the phage genome altogether and that the curing effect may be the result of damage to only some cistron or essential replica of it in the phage genome (Ting, 1960). The effect of a shift may be the same in this respect.

The results obtained from experiments with acriflavine showed an early flavine-reversible phase in more than 50 % of transductions but did not show exactly when it ends. However, they suggest a conspicuous relationship between shift-reversible and flavine-reversible phases. It may be assumed that the 90 min. phase of

sensitivity of transductants to transfer to minimal medium is the period in which the transduced element is not integrated into the genome of the recipient cell. In the course of multiplication of the potentially transduced cell in complete medium the fragment is apparently stabilized through integration with the bacterial genome. This period of cell growth required for incorporation agrees well with the results of the work of Jacob & Wollman (1957), who followed the ability of zygotes in which the DNA from one parent was labelled with ^{32}P to produce recombinants. Our results, however, are not in agreement with the opinion of Hartman & Kozinski (1962), who suppose that the fast stabilization of complete transducing elements toward ^{32}P decay is caused by the fact that the recombination events take place very rapidly following injection of this particle into recipient bacteria. Since the sensitivity of the DNA of *Escherichia coli* or Salmonella to destruction by ^{32}P decay is very similar to that of the phage (Stent & Fuerst, 1956) and as it is assumed that the integration of transducing element requires multiplication of the cells it is not likely that the act of recombination is responsible for the observed early stabilization.

The statistical analysis of the data were done by Mr M. Jílek, to whom thanks are due. The technical assistance of Mr J. König is gratefully acknowledged.

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Carbohydrate Metabolism of *Staphylococcus aureus*

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SUMMARY

The experiments described establish the importance of the pentose cycle in carbohydrate breakdown by *Staphylococcus aureus*. No Entner–Doudoroff pathway was found. Another feature is the extensive glucose effect: growth in glucose enhanced glycolysis suppressed the Krebs cycle, decreased the activity of the pentose cycle and suppressed the formation of many enzymes. Even the oxidation of pyruvic acid was decreased in glucose-grown organisms. The decrease of oxidative activity affected the cytochromes. The main products of carbohydrate oxidation were acetic acid and carbon dioxide. The accumulation of acetic acid indicated that the link between pyruvic acid and the citric acid cycle was weak.

INTRODUCTION

It is generally assumed that *Staphylococcus aureus* metabolizes glucose by glycolysis and subsequent oxidation of pyruvic acid (Elek, 1959). The breakdown of pyruvic acid has been studied by various authors but our knowledge about alternative pathways of glucose breakdown is scanty. Hancock (1960*a*) and Das & Chatterjee (1962) obtained indications that a pentose cycle is present; Fusillo & Weiss (1958) suggested a relation between the pattern of carbohydrate breakdown and resistance to antibiotics. Since there is a complete pentose cycle in *Sarcina lutea* (Dawes & Holms, 1958*a*) a detailed study of the carbohydrate metabolism of *S. aureus* seemed worthwhile. Five strains of *S. aureus* of different phage groups were used, one strain being studied in more detail. By Warburg experiments, enzyme studies with cell-free extracts and experiments with radioactive substrates, a general picture of the carbohydrate metabolism of *S. aureus* was obtained.

METHODS

Abbreviations. The following abbreviations are used: B-cocci, cocci grown in broth; G-cocci, cocci grown in broth + glucose; G-6-P, glucose-6-phosphate; 6-P-G, 6-phosphogluconic acid; F-1,6-P, fructose-1,6-diphosphate; Gald-3-P, glyceraldehyde-3-phosphate; R-5-P, ribose-5-phosphate; GSH, reduced glutathione; NAD, nicotinamide-adenine-dinucleotide (DPN); NADH, the reduced form of NAD; NADP, nicotinamide-adenine-dinucleotide phosphate (TPN); NADPH, the reduced form of NADP; PMS, phenazine methosulphate; TCA, trichloroacetic acid.

The strains of *Staphylococcus aureus* used were five propagating strains from the International Phage Typing System: 3A, 70, 77, 80, 187N with NCTC nos. 8319, 8352, 8356, 9789 and 9753, respectively. Strains of staphylococci were kept in stock as stab cultures in nutrient agar at 4°. After plating on blood agar single colonies were

inoculated to agar slopes which were used as working cultures for one week. Bacto nutrient broth (8 g./l. 0·7 % NaCl solution) was used as liquid medium. Conventional Warburg techniques were used. Suspensions of logarithmic-phase cocci were prepared from 400 ml. broth cultures inoculated with 0·25 ml. 18 hr. broth culture and incubated at 37° on a shaker until a standard turbidity (80 % absorbed light in a Moll extinctometer) was obtained. The cocci were centrifuged down, washed twice in phosphate buffer (pH 6·8) and re-suspended in buffer to obtain a 40-fold concentration (checked by turbidity). The quantity of cocci used in each Warburg experiment (as mg. dry weight) is indicated in the text.

Glucose was determined as described by Newburgh & Cheldelin (1955), ribose according to Mejbaum (1939) and pyruvic acid by the method of Friedemann & Haugen (1943) with xylene for the extraction. Acetic acid was determined as described by Rose, Grunberg-Manago, Korey & Ochoa (1954), acetylmethylcarbinol by the method of Westerfeld (1945), lactic acid by the method of Barker & Summerson (1941) and D (−)-lactic acid according to van den Hamer & Elias (1958).

For the preparation of cell-free extracts, suspensions of cocci were obtained as described. The cocci were washed twice in 0·85 % NaCl solution and suspended in 0·01 M-phosphate buffer (pH 7·0). A suspension (15 mg. dry weight/ml.) was mixed with twice its volume of ballotini (no. 11) and treated for 15 min. in a Mickle disintegrator at 4° (Hancock, 1960*b*). The mixture was freed from ballotini by centrifuging in a linen bag. The fluid obtained was centrifuged for 30 min. at 5000 *g*. The supernatant fluid was used as cell-free extract; it contained about 10 mg. protein/ml. Details about the determination of enzymes are given in the text and in Table 5.

For incubation with radioactive substrates Warburg vessels closed with a rubber stopper were used. The same conditions were used as in the determination of oxygen uptake. The vessels contained equiv. 4 mg. dry wt. cocci. After incubation for 2·5 hr. at 37°, 0·1 ml. of a solution of $NaHCO_3$ (106 mg./ml.) was added to the alkali in the centre well. The carbonate was precipitated as $BaCO_3$ for the determination of radioactivity.

One ml. of the coccal suspension was filtered through a Millipore filter (type DA), and the cocci washed 3 times with phosphate buffer.

After adding non-radioactive glucose, filtrate and wash-waters were oxidized with $K_2S_2O_8$ (Aronoff, 1960) and the CO_2 was collected as $BaCO_3$. The $BaCO_3$ was handled as described by Bosch (1955) and the radioactivity was determined on a layer of infinite thickness, using an end-window GM tube.

The cocci on the filter were dried with an infrared lamp and the radioactivity measured directly. It was shown in separate experiments that there was a constant ratio between the radioactivity of the cocci as measured directly and the activity of the $BaCO_3$ formed from the cocci by oxidation. All activities are thus presented as total activity in cpm, measured on $BaCO_3$.

The sum of the activities found in CO_2, filtrate and cocci gave the recovery. There was a systematic loss of radio-activity by peptization of some $BaCO_3$. Therefore the activities are expressed as % of recovery. When not all the substrate was used by the cocci the values were corrected for unused substrate.

RESULTS

Preliminary experiments. To obtain a first impression of the metabolic possibilities, the oxidation of glucose, gluconate, ribose, succinate and lactate by the five strains was studied by using suspensions of cocci grown in broth (B-cocci) and in broth +0·1 % glucose (G-cocci). The results are given in Table 1; a representative experiment is shown in Fig. 1 *a*, *b*. The ready oxidation of the five substrates by B-cocci of all five strains seems to indicate that a pentose cycle as well as a citric acid cycle is present. It is however remarkable that the oxidation of the intermediates of the pentose cycle (ribose, gluconate) and of the citric acid cycle (succinate) were nearly completely suppressed in G-cocci.

Table 1. *Oxidations by resting organisms of five strains of* Staphylococcus aureus

Warburg vessels contained equiv. 15 mg. dry wt. organism, 0·5 ml. 0·2 M-potassium phosphate buffer (pH 6·8); 10 μmole substrate were added from side-arm, total volume 2·4 ml. The centre well contained 0·1 ml. 20 % KOH; gas phase was air. All data are presented after subtraction of endogenous respiration. The initial rate of oxygen uptake is given in μmole O_2/hr. An asterisk * indicates that this rate was observed after an induction time. The total amount of oxygen used for the oxidation of 10 μmole substrate was recorded after 3 hr. Cont. means that the oxidation after 3 hr. was still going on; 0 that there is no oxidation at all.

	Broth-grown staphylococci strain					Glucose-grown staphylococci strain				
	3A	70	77	80	187N	3A	70	77	80	187N
Substrate	Initial uptake O_2 (μmole/hr.)									
Endogenous	5	5	4	7	8	3	1	2	2	1
Glucose	46	43	49	47	35	57	42	50	50	40
Gluconate	22	13*	17*	20*	5	1	1	2	0	0
Ribose	12	8*	3	13*	5	1	0	2	1	0
Succinate	22*	10	4	17*	18	0	0	1	0	0
Lactate	34	24	26	27	21	3	5	8	24	11
Acetate	0	0	0	0	0	0	0	0	0	0
	μmole O_2/10 mole substrate									
Glucose	29	21	27	20	22	21	18	21	21	20
Gluconate	45	31	30	31	Cont.	Cont.	Cont.	Cont.	0	0
Ribose	25	22	Cont.	27	Cont.	Cont.	0	0	0	0
Succinate	36	33	Cont.	Cont.	Cont.	0	0	Cont.	0	0
Lactate	19	19	13	15	25	10	8	10	10	10
Acetate	0	0	0	0	0	0	0	0	0	0

During the preparation of this paper Collins & Lascelles (1962) published analogous results, at least with regard to the suppression of oxidations of the intermediates of the citric acid cycle in glucose-grown *Staphylococcus aureus*. The dependency of oxidative possibilities on growth conditions thus required further study. Strain 3A was used for these experiments.

Citric acid cycle. Intermediates of the citric acid cycle were readily oxidized by B-cocci of strain 3A (Table 2). With G-cocci these substrates were not oxidized at all or only at an extremely low rate.

In cell-free extracts of B-cocci succinic dehydrogenase (Fig. 2) and malic dehydrogenase were found. Fumarase was shown by measuring the disappearance of the

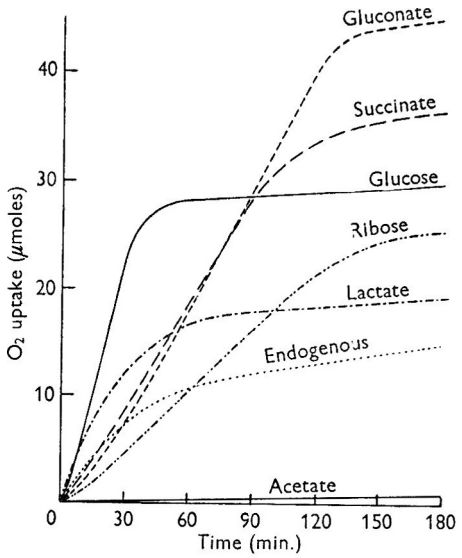

Fig. 1*a*

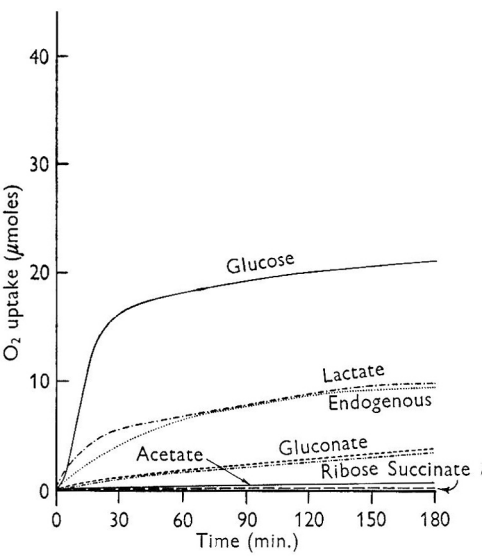

Fig. 1*b*

Fig. 1*a*. Oxidation of various substrates by resting, broth-grown cocci of *Staphylococcus aureus* strain 3A. Endogenous subtracted. Experimental details as in Table 1.

Fig. 1*b*. Oxidation of various substrates by resting, glucose-grown cocci of *Staphylococcus aureus* strain 3A. Legend as in Fig. 1*a*.

Table 2. *Oxidation of citric acid cycle intermediates and of amino acids by* Staphylococcus aureus 3A

Experimental details as in Table 1. An asterisk* indicates that the oxygen uptake showed an induction period. DL Forms of amino acids were used throughout except for the L isomer of ornithine. Leucine, valine, phenylalanine and tryptophan were not oxidized by either kind of cocci.

Substrate	Oxygen uptake (μmole O_2/hr.) by washed staphylococci of strain 3 A grown: Without glucose	With glucose
Endogenous	5	2
Oxaloacetic acid	14	1
Citric acid	2*	0
DL-Isocitric acid	3*	1
α-Ketoglutaric acid	4	0
Succinic acid	19	0
Fumaric acid	13*	1
Malic acid	13*	1
Serine	18	3*
Alanine	19	0
Ornithine	10*	1*
Proline	6*	0
Histidine	4*	0
Glutamic acid	34	16*
Aspartic acid	4*	1
Glycine	19	6*

extinction at 240 mμ due to the double bond in fumaric acid. Aconitase (Racker, 1950) and isocitric dehydrogenase (Siebert, Dubuc, Warner & Plaut, 1957) were not observed in our extracts. With G-cocci the case was entirely different: fumarase was not found in extracts of G-cocci and the activity of succinic dehydrogenase is markedly decreased (Fig. 2). These results seem to indicate that growth in glucose did inhibit the synthesis of some enzymes of the citric acid cycle (compare Table 5).

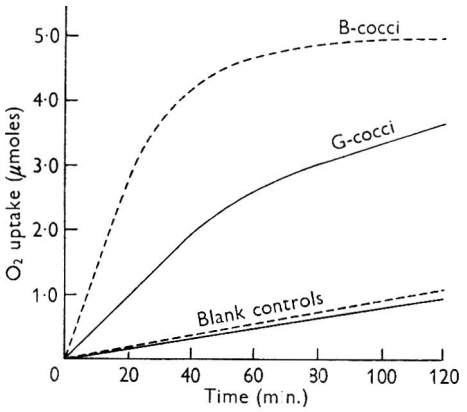

Fig. 2

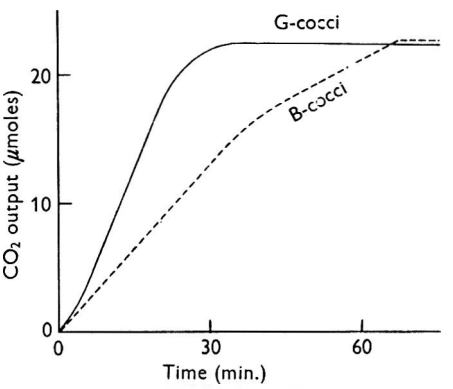

Fig. 3

Fig. 2. Determination of succinic dehydrogenase in broth-grown and glucose-grown cells of *Staphylococcus aureus* strain 3A. A Warburg vessel contained: 100 μmole phosphate buffer pH 7·7, 0·5 ml. cell-free extract (5 mg. protein) and 2 mg. phenazinemethosulphate. As substrate 10 μmole of succinate were added. The centre well contained 0·1 ml. 20 % KOH. Total volume 2·4 ml. Blanks not subtracted.

Fig. 3. Activity of glycolysis, under anaerobiosis, by resting, broth-grown and glucose-grown, cocci of *Staphylococcus aureus* strain 3A. The production of acid was followed by measuring the production of CO_2 in $NaHCO_3$ buffer. The Warburg vessels contained 15 mg. cells, expressed as dry wt., and 0·02M-$NaHCO_3$. From a side-arm 10 μmole glucose were added. Total volume 2·4 ml., gas phase $N_2 + 5$ % CO_2. Endogenous subtracted.

The oxidation of various amino acids by B-cocci and by G-cocci is shown in Table 2. In G-cocci the oxidation of amino acids was markedly decreased though glutamic acid was still oxidized appreciably. Furthermore, the oxidation of amino acids by G-cocci was always preceded by an induction period of about 20 min. These results suggest that staphylococci grown in broth + glucose do not use amino acids for energy production. The oxidation of glutamic acid in the absence of a functional citric acid cycle seems remarkable since glutamic acid is easily concentrated in the cell (Gale, 1951) and seems to be the main substrate for endogenous oxygen uptake (Ramsey, 1962; Strasters, unpublished). A ready induction or expansion of the enzymes of the citric acid cycle may be supposed.

Glycolysis. Under anaerobic conditions glucose was broken down to L(+)-lactic acid; in presence of arsenite this breakdown was quantitative (carbon recovery ~1·00). Without arsenite small amounts of carbon dioxide were formed and the carbon recovery was too low (presumably due to assimilation and to oxidation of pyruvic acid by traces of oxygen or other hydrogen acceptors). The presence of a glycolytic system is, however, established beyond doubt.

The activity of the glycolytic system differed for B-cocci and G-cocci; the latter were more active (Fig. 3). The enzymes of the glycolytic system were demonstrated

in cell-free extracts of both kinds of cocci (Tables 4 and 5). Phosphoglucoisomerase, phosphofructokinase and aldolase were of equal activity in both kinds of extracts, when determined separately. When the sequence of these enzymes together was estimated by studying the formation of triosephosphates from G-6-P the extract of G-cocci proved twice as active. Gald-3-P-dehydrogenase (Fig. 4) and lactic dehydrogenase were much more active in the extracts of G-cocci. Growth in glucose thus seems to increase some of the enzymes of the glycolytic system, thus explaining the enhanced glycolytic activity of G-cocci.

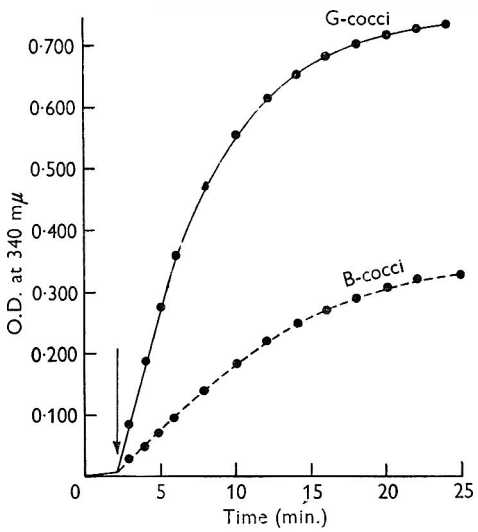

Fig. 4

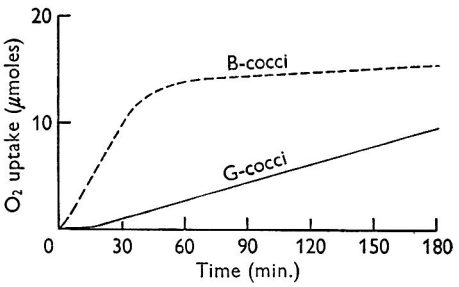

Fig. 5

Fig. 4. Determination of Gald-3-P-dehydrogenase in extracts of broth-grown and glucose-grown cocci by measuring the formed NADH in a Beckman DU spectrophotometer. A cuvette contained: 0·3 ml. cell-free extract (3 mg. protein), centrifuged at 70,000*g* to diminish the NADH oxidase, 60 μmole Na-diethyl-barbiturate, 30 μmole NaF, 10 μmole cystein-HCl, 30 μmole arsenate and 0·33 μmole NAD. At ↓ 6 μmole F-1,6-P are added. Total volume 2·7 ml.

Fig. 5. Oxygen uptake with 20 μmole Na pyruvate by resting cocci of *Staphylococcus aureus* strain 3A, grown with and without glucose. Endogenous subtracted. Experimental details as in Table 3.

Oxidative dissimilation of glucose. With glucose as a substrate oxygen uptake was greater for B-cocci than for G-cocci (Fig. 1*a*, *b*). In both cases CO_2 production equalled oxygen uptake (R.Q. about 1). Notwithstanding rigorous standardization the results of individual experiments were somewhat variable. For example, the oxygen uptake of G-cocci with 10 μmole glucose was 20·8, 17·9, 16·1, 17·1, 17·3, 19·6, 17·7, 17·8 μmole O_2 in eight experiments, with similar variations in CO_2 production. The production of 20 μmole CO_2 and uptake of 20 μmole O_2 would be consistent with glycolytic fission and subsequent oxidation of pyruvic acid to acetic acid and CO_2. The results of the determination of the reaction products are given in Table 3. No lactic acid, amino acids or other carboxy acids were found. Neither ethanol nor ketosugars were observed in the filtrate. The main products were CO_2 and acetic acid, but the amount of CO_2 was much more and the amount of acetic acid much less than would be consistent with the above hypothesis (see equation on

p. 227). A further oxidation of acetic acid by the citric acid cycle might be supposed as an explanation. However, even in presence of arsenite an appreciable amount of CO_2 was formed which cannot very well have been derived from pyruvic acid (compare Table 3).

Table 3. *Quantitative determination of products formed by the dissimilation of several substrates by resting* Staphylococcus aureus *cells of strain* 3A

The experiments were done as described in Table 1, but with equiv. 4 mg. dry wt. organism/vessel. In aerobic dissimilation the CO_2 was measured by the direct method. In anaerobic dissimilation the gas phase was N_2 and there was no KOH in the centre well. Where noted 5 μmole Na-arsenite/ml. were present. The amount of substrate was 10 μmole, but with pyruvate 20 μmole. After an incubation for 150 min. the cocci were centrifuged down and the products determined in the supernatant fluid. The values recorded are the mean of two determinations; endogenous values subtracted.

Cocci grown in broth with addenda	Gas phase	Sub-strate	Presence of arsenite	Compounds determined as μmole/vessel: Unused substrate	Acetic acid	Pyruvic acid	Lactic acid	AMC*	O_2	CO_2
Nil	Air	Glucose	−	0·1	6·2	0·0	0·0	0 0	27·8	27·7
Nil	Air	Glucose	+	3·3	0·2	7·9	0·0	0·9	8·7	6·6
Glucose	Air	Glucose	−	0·0	8·0	0·2	0·0	0·0	20·5	21·3
Glucose	Air	Glucose	+	0·1	0·6	12·0	0·0	1·9	12·8	10·4
Nil	N_2	Pyruvate	−	0·5	9·4	—	6·9	0·0	—	8·7
Nil	N_2	Pyruvate	+	15·6	1·8	—	1·7	0·6	—	2·1
Glucose	N_2	Pyruvate	−	0·7	9·4	—	7·7	0·0	—	8·2
Glucose	N_2	Pyruvate	+	18·5	1·0	—	1·1	0·3	—	2·3
Nil	Air	Pyruvate	−	0·0	11·1	—	0·0	0·0	15·2	24·8
Nil	Air	Pyruvate	+	15·5	2·9	—	0·1	0·5	0·3	2·7
Glucose	Air	Pyruvate	−	2·0	15·2	—	0·0	0·0	8·8	16·7
Glucose	Air	Pyruvate	+	18·8	1·5	—	0·3	0·1	0·6	0·2
Ribose	Air	Ribose	−	0·6	5·8	0·1	0·0	0·0	18·9	19·5
Ribose	Air	Ribose	+	6·5	2·1	3·2	0·0	0·3	2·2	0·8

* AMC = acetylmethylcarbinol.

It will be observed that the carbon recovery was too low. It will be shown below that this was partly due to assimilation, partly to diminution of endogenous metabolism by addition of substrate and, for a minor part, to the formation of other products. Thus the results described in this section suggest that a second system for the oxidation of glucose must exist. This system, presumably the pentose cycle, would then be much more active in B-cocci.

Dissimilation of pyruvic acid. Under anaerobic conditions pyruvic acid was mainly broken down by dismutation to lactic acid, acetic acid and CO_2 (Table 3). As far as final products are concerned, G-cocci and B-cocci behaved similarly. The rate of CO_2 production by G-cocci was, however, much slower (about 1/4) than that by B-cocci. In presence of arsenite the dismutation was slowed and a second mechanism which produced acetylmethylcarbinol and CO_2 took over. The activity of this mechanism was very dependent on the concentration of Mg^{2+} and thiamine. Under aerobic conditions (Table 3) pyruvic acid was oxidized to acetic acid and CO_2. The amount of CO_2 was in excess, especially for B-cocci, suggesting that some of the acetic acid was oxidized by the citric acid cycle, which was more active in B-cocci. The oxidation of acetic acid by the citric acid cycle when pyruvic acid is offered as a

substrate does not necessarily mean that this also will happen with glucose as a substrate since the (intermediate) concentration of pyruvic will be less in the latter case; indeed inhibition by arsenite was less complete with pyruvic acid as substrate (Table 3). The rate of pyruvate oxidation is much higher for B-cocci than for G-cocci (Fig. 5). Growth in glucose thus seemed to suppress pyruvic oxidation in accordance with the results of Sevag & Swart (1947).

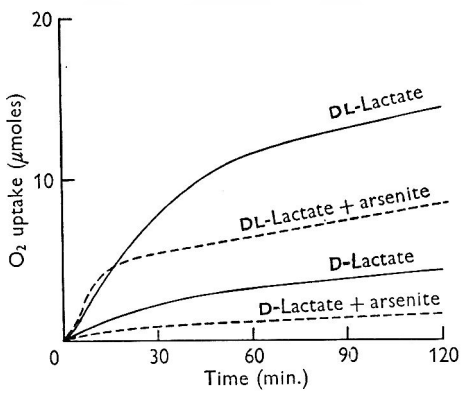

Fig. 6

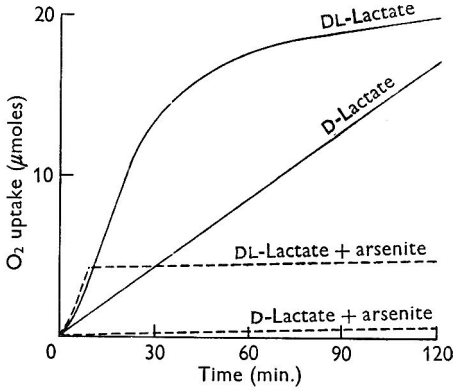

Fig. 7

Fig. 6. Oxygen uptake with 20 μmoles Li-DL-lactate and Li-D-lactate, with and without 5 μmoles arsenite/ml., by resting, glucose-grown cocci of *Staphylococcus aureus* strain 3A. The experiment is done as described in Table 1. Endogenous subtracted.

Fig. 7. Same experiments as in Fig. 6 with broth-grown cocci.

The dissimilation of lactic acid. DL-Lactate is oxidized rapidly by B-cocci and G-cocci in contradistinction to D-lactate, which is oxidized at a much slower rate (Figs. 6, 7). The results are in accordance with the presence of a L(+)-lactic dehydrogenase. As can be seen from Table 5 this enzyme is more active in G-cocci. The lower rate of oxidation in Fig. 6 was due to the suppression of the subsequent pyruvic oxidation in G-cocci. No racemase was present; the oxygen uptake, with or without arsenite, with D-lactate was completely different from the oxygen uptake with DL-lactate and only L-lactic acid was formed from glucose under anaerobiosis. Peculiarly, the oxidation of D-lactate was completely inhibited by arsenite. The mechanism of the D-lactate oxidation remains obscure.

The dissimilation of ribose and gluconate. Neither B-cocci, G-cocci nor ribose-grown cocci metabolized ribose anaerobically. This suggests that no phosphoketolase was present, as in heterofermentative lactobacilli (see also Table 5). G-cocci did not oxidize ribose (Fig. 1*b*); supplied ribose was recovered quantitatively. Ribose-grown cocci oxidized ribose with an R.Q. of about 1 and with acetic acid and CO_2 as main products (Table 3). Arsenite decreased the rate of ribose oxidation with accumulation of pyruvic acid and the formation of acetylmethylcarbinol. Since phosphoketolase was absent these results are best explained by supposing that ribose after phosphorylation was transformed by transaldolase-transketolase to hexosephosphate, which was then broken down (by the pentose cycle or by the glycolytic system) to pyruvic acid. The large excess of CO_2 with regard to acetic acid, in the experiment without arsenite, suggests oxidation of pyruvic acid by the citric acid cycle or a contribution of the dehydrogenases of the pentose cycle. The

accumulation of acetic acid in the experiment with arsenite is puzzling, considering the almost complete inhibition of acetic acid formation by arsenite in the experiments with glucose. With gluconate the oxygen consumption and CO_2 production by B-cocci were, respectively, 0·5 μmole and 1 μmole/μmole substrate higher than in experiments with ribose, as would be expected on the assumption that gluconate was phosphorylated and oxidized by 6-P-G-dehydrogenase to pentose phosphate. These results seem to indicate that transaldolase, transketolase and 6-P-G-dehydrogenase are present in *Staphylococcus aureus*, thus suggesting the presence of a complete pentose cycle.

Demonstration of the enzymes of the pentose cycle in cell-free extracts

Kinases. Kinases were demonstrated by the method of Colowick & Kalckar (1943). The results for B-cocci are given in Fig. 8 and for several other kinds of cocci in Table 4. Glucokinase and phosphofructokinase were always present. The systematic absence of fructokinase is remarkable considering the rapid oxidation of fructose. The absence of ribokinase and gluconokinase from G-cocci is conspicuous. The absence of these enzymes would alone be sufficient explanation for the suppression of the oxidation of ribose and gluconic acid by G-cocci. Growth on ribose induced the formation of ribokinase and gluconokinase.

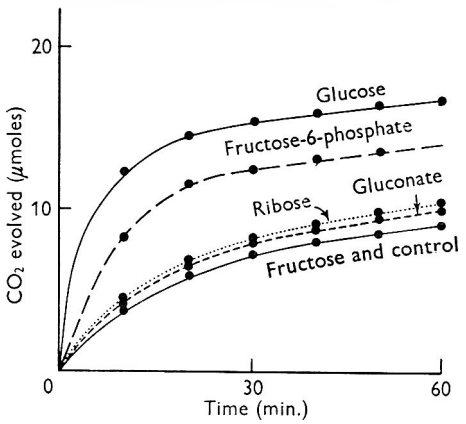

Fig. 8

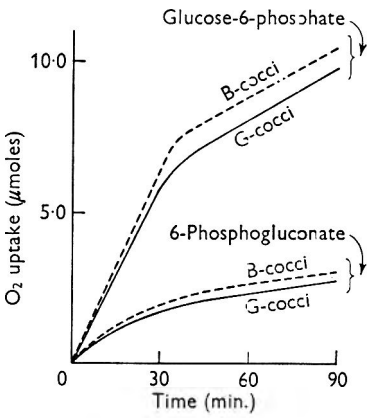

Fig. 9

Fig. 8. Determination of kinases in a cell-free extract of broth-grown cocci. Measured as CO_2 evolution from 0·02 M-$NaHCO_3$ buffer. A Warburg vessel contained: 0·5 ml. cell-free extract (5 mg. protein), 20 μmole ATP, 20 μmole $MgCl_2$, 25 μmole NaF and 10 μmole of the indicated substrates. Total volume 2·4 ml. Gas phase $N_2 + 5\%$ CO_2.

Fig. 9. Determination of G-6-P and 6-P-G-dehydrogenases in cell-free extracts of broth-grown and glucose-grown cocci of *Staphylococcus aureus* strain 3A by following the oxygen uptake. A Warburg vessel contained: 90 μmole Tris-buffer pH 7·8, 50 μmole $MgCl_2$, 0·5 μmole TPP, 5 μmole NADP, 2 mg. PMS and 0·5 ml. extract (5 mg. protein). The centre well contained 0·1 ml. 20% KOH. As substrate 10 μmole G-6-P or 6-P-G were added from a side-arm. Total volume 2·4 ml. Blanks subtracted.

Dehydrogenases. G-6-P and 6-P-G dehydrogenases were demonstrated by following NADP reduction spectrophotometrically and by O_2 consumption in Warburg vessels in the presence of phenazine methosulphate. Both dehydrogenases were equally active in B-cocci and in G-cocci (Fig. 9).

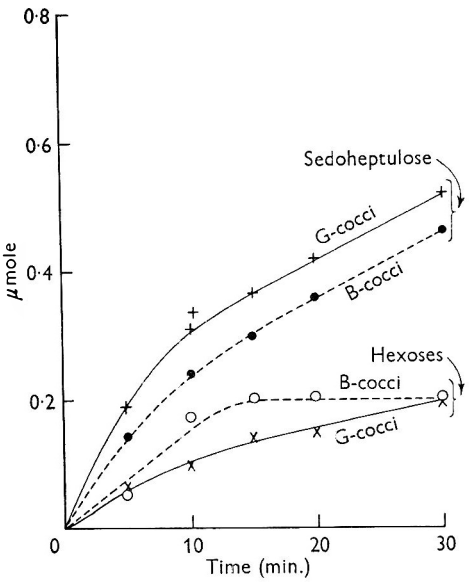

Fig. 10

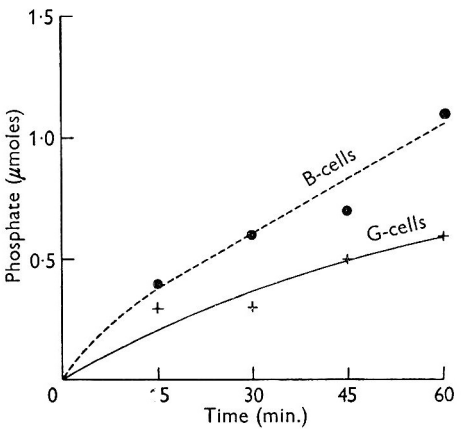

Fig. 11

Fig. 10. Formation of hexoses and sedoheptulose by cell-free extracts of broth-grown and glucose-grown cocci of *Staphylococcus aureus* strain 3A during incubation with R-5-P at pH 7·8. Incubation mixture contained 30 µmole Tris buffer pH 7·8, 1 µmole $MgCl_2$, 0·03 µmole TPP, 9 µmole R-5-P and 0·05 ml. extract (0·5 mg. protein). Total volume 1·0 ml. The reaction was stopped with 1·0 ml. 10 % TCA and centrifuged. Hexoses and sedoheptuloses were determined in the supernatant.

Fig. 11. Determination of hexosediphosphatase in cell-free extracts of broth-grown and glucose-grown cocci of *Staphylococcus aureus* strain 3A. The formation of inorganic phosphate from F-1,6-P is followed. The incubation mixture contained: 25 µmole borate buffer pH 9·5 and 0·4 ml. extract (4 mg. protein). As substrate 5 µmole F-1,6-P were added. Total volume 1·2 ml. The reaction was stopped by heating the mixture 5 min. at 100°. The inorganic phosphate was determined as described by Fiske & SubbaRow (1925). Controls subtracted.

Table 4. *Kinases which were determined in cell-free extracts of* Staphylococcus aureus *strain* 3A *grown in broth with* 0·1 % *of various carbohydrates. The kinases were determined as described in legend to Fig.* 8

	Staphylococci grown with				
	No addition	Glucose	Gluconate	Ribose	Fructose
Enzyme	Relative amounts of enzyme				
Glucokinase	++	++	++	++	++
Glueonokinase	±	−	+	+	−
Ribokinase	±	−	±	+	−
Fructokinase	−	−	−	−	−
Phosphofructokinase	+	+	+	+	+

++ = very active; + = active; ± = weakly active; − = absent.

Transaldolase, transketolase and the pentose isomerases. These enzymes were demonstrated by incubation of extracts with R-5-P, followed by spectrophotometric estimation of sedoheptulose and hexose phosphates according to Newburgh & Cheldelin (1955) and Kulka (1956). The results are presented in Fig. 10 for cell extracts at pH 7·8. At this pH value reduced glutathione (GSH) was without influence. With the same extracts at pH 6·0 in presence of GSH an even more rapid formation of sedoheptulose and hexoses was observed, but no activity was found without GSH. Again, a definite difference between B-cocci and G-cocci was not observed. The impression was obtained that a GSH-independent system with optimal activity at pH 7·8 and a GSH-dependent system with optimal activity at pH 6·0 are present in *Staphylococcus aureus*. There was no appreciable difference however between G-cocci and B-cocci.

Hexosediphosphatase was determined by liberation of inorganic phosphate from fructose-1,6-diphosphate; the results are shown in Fig. 11. B-cocci are the more active.

Table 5. *Presence of enzymes in cell-free extracts from cells of* Staphylococcus aureus *strain* 3 A *grown with and without glucose*

Enzyme	Activity in B-cells	Activity in G-cells	Determined as described by
Phosphoglucoisomerase	+	+	Jensen, Altschuller & Bard (1957)
Phosphofructokinase	+	+	Colowick & Kalckar (1943)
Aldolase	+	+	Sibley & Lehninger (1949)
Gald-3-P-dehydrogenase	+	+ +	Shankar & Bard (1956)
Lactic-dehydrogenase	+	+ +	Neilands (1955)
G-6-P-Dehydrogenase	+	+	Modification of van den Hamer (1960)
6-P-Gluconolactonase	+	+	.
6-P-G-Dehydrogenase	+	+	Modification of van den Hamer (1960)
TK-TA-Pathway	+	+	.
Hexosediphosphatase	+ +	+	.
Succinic dehydrogenase	+ +	+	.
Fumarase	+	−	.
Malic dehydrogenase	+	+	.
Glucokinase	+	+	Colowick & Kalckar (1943)
Gluconokinase	±	−	Colowick & Kalckar (1943)
Ribokinase	±	−	Colowick & Kalckar (1943)
Fructokinase	−	−	Colowick & Kalckar (1943)
Entner–Doudoroff pathway	(−)*	.	Vandemark & Wood (1956)
Phosphoketolase	(−)*	.	Heath *et al.* (1958)
Cytochromes	+ +	+	Chance (1952)

−, Enzyme absent; ±, weakly active; +, active; + +, far more active than + by the same enzyme; (—)*, grown with gluconate.

Phosphoketolase. By using the method of Heath, Hurwitz, Horecker & Ginsburg (1958) with ribose-5-phosphate as a substrate no acetylphosphate formation was observed in extracts of gluconate-grown staphylococci. The method was checked with good results with extracts of *Lactobacillus casei*, which we have found to possess very active phosphoketolase when grown with gluconate.

Phosphofructoketolase. Tested by the method of Schramm, Klybas & Racker (1958) a very small activity of this enzyme was found in extracts of gluconate-grown cocci. The method was checked with good results with extracts of *Acetobacter xylinum*. The presence of phosphofructoketolase in *Staphylococcus aureus* 3 A cannot

be excluded. The results of all enzyme studies are summarized in Table 5. The results indicate that all the enzymes of the pentose cycle are present in the *Staphylococcus aureus* strains used. They were in general equally active in B-cocci and in G-cocci with the exception of hexosediphosphatase.

Cytochromes. The decreased activity of the citric acid cycle in glucose-grown cocci raised the question whether the electron transfer system was also involved. The differential spectrum of oxidized and reduced coccal suspensions in 67 % glucose was measured spectrophotometrically according to Chance (1952); the quantity of cytochromes was estimated from the differences between maxima and minima in the differential spectrum (Fig. 12). From these data a decrease of the cytochromes of about 40 % in G-cocci compared with B-cocci was calculated. Growth in glucose indeed seems to decrease the activity of the cytochrome system.

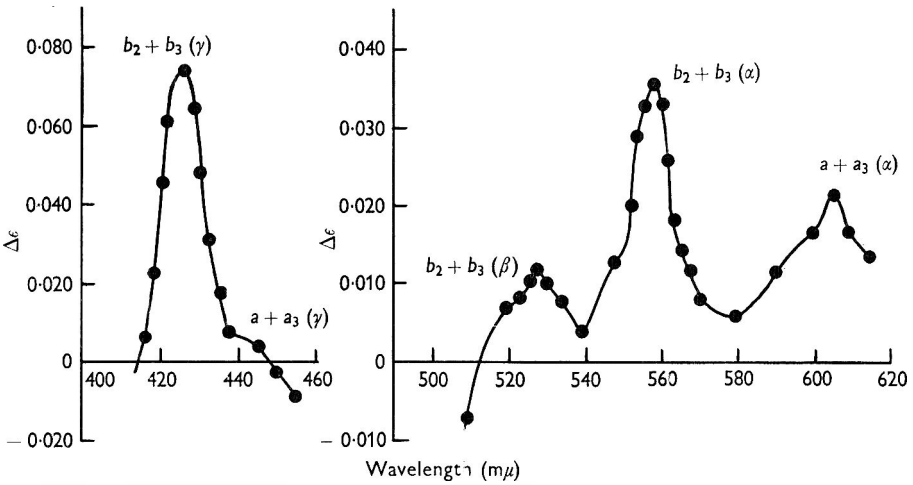

Fig. 12. Differential spectrum of cocci grown without glucose, suspended in 67 % glucose (15 mg./ml. cocci as dry weight).

Contribution of various oxidative systems in carbohydrate dissimilation

Evidence has been submitted for the presence of two mechanisms for glucose breakdown: the pentose cycle; glycolysis with subsequent oxidation of pyruvic acid, with or without involvement of the citric acid cycle. The participation of these systems in carbohydrate breakdown was studied by incubating suspensions of cocci with [1-^{14}C]glucose, [U-^{14}C]glucose or [1-^{14}C]ribose and determining the ^{14}C activity in the cells, in the surrounding fluid and in the CO_2. The results are summarized in Table 6. B- and G-cocci produce labelled CO_2 from [1-^{14}C]glucose. This is proof that the pentose cycle is engaged in glucose breakdown in both kinds of cocci, though more actively in B-cocci. With B-cocci, with or without arsenite, 64 % of the activity from [1-^{14}C]glucose was recovered as CO_2. This means that at least 64 % of the glucose went through the cycle. The flow through the cycle as percentage of actual hexosephosphate pool is probably greater (Wood & Katz, 1958; Dawes & Holms, 1958*c*) depending on the degree of recycling.

An estimation of the degree of recycling can be obtained by comparing the results with [1-^{14}C]glucose and [U-^{14}C]glucose. From the first C-atom 64 % was split off as

CO_2 as appears from the data with [1-^{14}C]glucose. This means that with [U-^{14}C]-glucose 64/6 = 11 % of the total activity was derived from the first C-atom. From 10 μmole glucose 6·2 μmole acetic acid were formed via pyruvic acid, corresponding to 6·2 μmole CO_2 or about 10 % of the total activity. From [U-^{14}C]glucose 11 + 10 = 21 % of the total activity would be expected in the carbon dioxide if no recycling occurred. Actually 53 % was found, leaving 32 % of the activity which must have been split off as CO_2 by recycling (at least 3 cycles).

Table 6. *Radioactivity in CO_2 and cells after oxidation of radioactive glucose and ribose*

Experimental details are described in Methods

The activity of the added [1-^{14}C]glucose and [1-^{14}C]ribose was 0·05 μC, of [U-^{14}C]-glucose 0·1 μC.

Kind of cells	Arsenite	Substrate	Activity of consumed substr. (counts/min.)	Recovery in %	% of recovery in CO_2	% of recovery in Filtrate	% of recovery in Cells
B	−	[1-^{14}C]glucose	27,851	96	64	27	9
B	+	[1-^{14}C]glucose	16,710	92	64	33	3
B	−	[U-^{14}C]glucose	50,532	95	53	32	15
B	+	[U-^{14}C]glucose	39,415	85	19	72	9
G	−	[1-^{14}C]glucose	27,851	98	47	43	10
G	+	[1-^{14}C]glucose	27,573	88	38	59	3
G	−	[U-^{14}C]glucose	50,532	98	39	48	13
G	+	[U-^{14}C]glucose	50,532	95	18	75	7
Ribose-grown	−	[1-^{14}C]ribose	34,044	99	58	25	17
Ribose-grown	+	[1-^{14}C]ribose	17,200	96	32	57	11

In presence of arsenite 16 % activity would be expected as CO_2 without recycling; 19 % was found. This seems to indicate that recycling was decreased in presence of arsenite.

With G-cocci the activity of the pentose cycle was much less; 47 % of the activity of [1-^{14}C]glucose appeared as CO_2, with arsenite only 38 %. Considering the decreased activity of the pentose cycle in G-cocci this difference might be due to some shuffling via transaldolase-transketolase (Stjernholm & Wood, 1960) resulting in some labelling of the carboxyl group of pyruvic acid from which labelled CO_2 could then be derived. In presence of arsenite the labelled pyruvic would remain in solution and less labelled CO_2 would appear. If this explanation be accepted it would mean that the amount of [1-^{14}C]glucose passing through the cycle would be nearer 38 % than 47 %.

Assuming no recycling, 20 % of the activity of [U-^{14}C]glucose would be expected in the CO_2, whereas 39 % was found. Also in G-cocci recycling seemed to occur; this also was suppressed in presence of arsenite.

The results with [1-^{14}C]ribose (Table 6) show that 58 % of the activity appeared as CO_2. Assuming conversion of ribose to hexosephosphate with subsequent glycolysis, two-fifths of the resulting pyruvic acid would be labelled in the carboxyl group and 3·3 μmole labelled CO_2 would be expected, or 33 % of the activity. Since 58 % was found it is obvious that at least 25 % of the [1-^{14}C]ribose was oxidized via the dehydrogenases of the pentose cycle. These results prove beyond doubt that the pentose cycle actively participates in carbohydrate breakdown by *Staphylococcus aureus*.

Carbon recoveries, assimilation

The low carbon recoveries in some of the previous experiments might have several explanations. The experiments with labelled substrates permitted some of these to be tested and some of the balance-equations could be corrected.

Assimilation. After incubation of cocci with [U-^{14}C]glucose the radioactivity of the cocci was 15% and 13% for B-cocci and G-cocci, respectively (Table 6). These values are a direct measure of the amount of substrate assimilated and show that assimilation was indeed one of the explanations of the low apparent carbon recovery. The radioactivity of the cocci after incubation with [1-^{14}C]ribose was a less reliable measure for the assimilation of ribose, since some randomization may occur by the transaldolase and transketolase in the pentose cycle. There is, however, no doubt that some assimilation took place.

Table 7. *Reduction of endogenous CO_2 production by substrate*

The amount of CO_2 produced from [U-^{14}C]glucose as calculated from the radioactivity in the CO_2 is compared with the amount of CO_2 as measured directly in Warburg vessels. Subtracting the first from the second gives the value for the true endogenous CO_2 production. Incubation time 150 min., 10 μmole of substrate. The substrate was used completely except for B-cells with arsenite, in which case 6·7 μmole were consumed. Cell density, 4 mg./ml.

			μmole CO_2			
Kind of cells	Arsenite	Carbon recovery	Calculated from activity	In Warburg with substr.	In Warburg without substr.	'True endogenous'
B	–	0·67	31·8	32·4	4·8	0·6
B	+	0·85	7·4	10·4	3·8	3·0
G	–	0·63	23·4	25·6	4·4	2·2
G	+	0·93	10·8	13·6	3·2	2·8

Suppression of endogenous metabolism. The supply of a substrate can decrease the endogenous O_2 uptake (Dawes & Holms, 1958*b*; Gronlund & Campbell, 1961). Under such circumstances the usual procedure of subtracting endogenous oxygen consumption or product formation from the experimental values (which was followed in our experiments) would be wrong, the final data being too low.

By comparing the activity of the CO_2 formed from [U-^{14}C]glucose with the total amount of CO_2 evolved the actual amount of CO_2 derived from endogenous sources can be calculated. This amount of CO_2 was always lower than the apparent value obtained in the Warburg vessel without substrate (Table 7). The difference depends on experimental conditions and was smaller in those cases where carbon recoveries were better. With the help of these data the carbon recoveries could be corrected.

Additional products. The radioactivity of the filtrate in the experiments with labelled glucose should tally with the amount of metabolic products (mainly acetic acid). With B-cocci it was found that after distillation of the filtrate to remove acetic acid the residue still contained labelled material equivalent to 9% of the total activity. After purification and chromatography three different substances with about equal radioactivity were shown; one of these was probably succinic acid.

Balance-equations. With the additional information about assimilation and suppression of endogenous metabolism, but excluding additional products, the balance-equations for glucose were corrected with the following results:

Broth-grown organisms:

10·0 glucose + 30·0 O_2 → 31·8 CO_2 + 6·9 acetic acid + 9·0 C assimilated; carbon recovery 0·91 (excluding additional products).

Broth-grown organisms with arsenite:

10·0 glucose + 13·6 O_2 → 11·0 CO_2 + 0·4 acetic acid + 11·7 pyruvic acid + 5·4 C assimilated; carbon recovery 0·96.

Glucose-grown organisms:

10·0 glucose + 22·2 O_2 → 23·4 CO_2 + 9·0 acetic acid + 0·2 pyruvic acid + 7·8 C assimilated; carbon recovery 0·83.

Glucose-grown organisms + arsenite:

10·0 glucose + 13·0 O_2 → 10·8 CO_2 + 0·7 acetic acid + 12·0 pyruvic acid + 1·9 acetylmethylcarbinol + 4·2 C assimilated; carbon recovery 1·00.

For ribose the suppression of endogenous metabolism was not known and the data about assimilation are questionable. The tentative equations are:

Ribose-grown organisms:

10·0 ribose + 20·1 O_2 → 20·7 CO_2 + 6·2 acetic acid + 8·7 C assimilated; carbon recovery 0·84.

Ribose-grown organisms with arsenite:

10·0 ribose + 6·3 O_2 → 2·3 CO_2 + 6·0 acetic acid + 9·1 pyruvic acid + 0·8 acetylmethylcarbinol + 5·5 C assimilated; carbon recovery 1·01.

The results are summarized in Fig. 13.

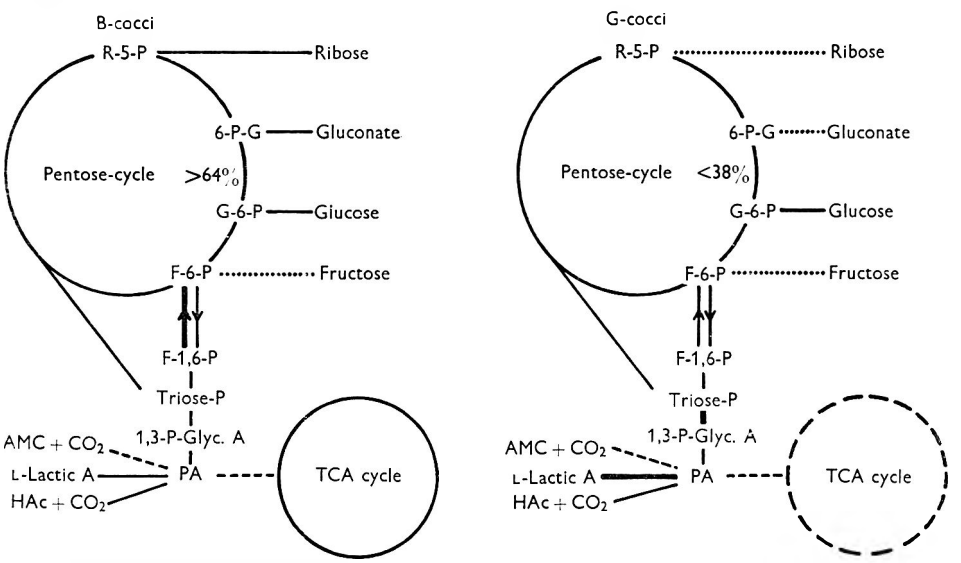

Fig. 13. Scheme of carbohydrate dissimilation by *Staphylococcus aureus*. ▬, Very active; —, active; ----, weakly active; ....., absent.

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Transfer of *colE1* and *colE2* During High-Frequency Transmission of *colI* in *Salmonella typhimurium*

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SUMMARY

In stock cultures of *Salmonella typhimurium* strain LT2 carrying the factor determining the production of colicine I, *colI*, together with other colicine factors, *colE1* or *colE2*, most of the small minority of bacteria competent to act as donors of *colI* also transmit their E factor. Most non-colicinogenic bacteria which acquire an E factor at the same time as *colI* become competent to transmit both factors. Similarly, bacteria carrying factors *colE1* and/or *colE2* accept *colI* normally and then usually become competent to transmit both *colI* and the E factor(s) they already carry. In experiments on the kinetics of colicine factor transmission by LT2 (*colE1*) (*colE2*) newly infected with *colI*, most acceptor bacteria that acquire any colicine factor acquire all three factors, within 5–20 min. for most pairs, but within 1 min. for a very few. When conjugation is interrupted within 5 min. of mixing, fewer acceptor bacteria acquire all three factors; all single-factor and two-factor classes are then represented, so the order of transfer of the three factors must differ in different pairs. Bacteria carrying *colE2* do not transfer this factor to donor bacteria from whom they acquire *colI*; but they become able to transmit *colE2* (and presumably *colI* also) within 15–20 min. of acquiring *colI*. Other observations support the hypothesis that in most competent donors carrying *colE1* and/or *colE2*, these factors multiply autonomously, as does *colI*.

INTRODUCTION

We have earlier shown (Ozeki, Stocker & Smith, 1962; Stocker, Smith & Ozeki, 1963) that in broth cultures of *Salmonella typhimurium* strain LT2 carrying the transmissible determinant for the production of colicine I (*colI*), only about one bacterium in 5000 is a 'competent donor', able to pair with, and transmit *colI* to an 'acceptor' bacterium of a subline not carrying *colI*; but that, by contrast, a high proportion (0·3–1) of bacteria which have just acquired *colI* are competent donors. Various observations led us to suspect that competent donors of *colI*, either arising spontaneously at low frequency in stock LT2 (*colI*) cultures or resulting from the recent acquisition of *colI*, transmitted not only *colI* but also *colE2* if they possessed this factor. Strains carrying *colE2* alone do not transmit this factor to any detectable extent; that is, no competent donors arise in cultures of LT2 (*colE2*). In the present paper we describe the transmission of *colE2* (and similarly *colE1*) by high-frequency colicinogeny-transferring (HFC) preparations—that is, by cultures containing a high proportion of bacteria newly infected with *colI* (Stocker *et al.* 1963). We show that most competent donors which carry *colE1* and/or *colE2* transmit

these factor(s) as well as *colI*, and report on the kinetics of their co-transmission. We have also investigated the segregation of these *col* factors amongst the progeny of newly infected bacteria and the time required for a newly infected bacterium to become a competent donor.

METHODS

The media, strains and techniques used were generally those of Ozeki *et al.* (1962) and Stocker *et al.* (1963). In this paper we are concerned only with the colicine factors *colI* and *colE2* originally derived from *Shigella sonnei* strain P9 and with *colE1* derived from *Escherichia coli* strain K12–30; these factors were introduced, alone or in combination, into sublines of *Salmonella typhimurium* strain LT2 as previously described. The original LT2 (*colI*) (*colE2*) or LT2 (*colI*) sublines were subsequently used as a source of these *col* factors, instead of *Shigella sonnei* strain P9. The subline carrying *colE2* alone, *cysD–36* (*colE2*), was obtained from the doubly colicinogenic subline *cysD–36* (*colI*) (*colE2*) by the rare spontaneous loss of *colI* (Ozeki *et al.* 1962).

HFC preparations were obtained by the procedure of Stocker *et al.* (1963). About 5×10^5 bacteria of a 'donor' subline carrying *colI* (and sometimes other colicine factor(s) also) and about 10^7 bacteria of an 'intermediate' subline not carrying *colI* (either non-colicinogenic or carrying colicine factor(s) other than *colI*) were inoculated into 10 ml. broth. After overnight incubation without aeration at 37°, to allow the 'epidemic spread' of *colI* amongst the intermediate population, the mixed culture was diluted tenfold in broth and incubated at 37° for 2 hr. (viable count then *c.* 7×10^8 bacteria/ml.). The ability of such an HFC preparation to transmit *col* factors to an acceptor strain, usually streptomycin-resistant, was determined as previously described (Stocker *et al.* 1963).

To determine the colicine or colicines produced by colicinogenic colonies, the colonies were picked or replicated to several plates, each of which after incubation was overlayered with a different colicine indicator strain—see Ozeki *et al.* (1962) for indicator strains used. When the only colicines involved were I and E2, which produce, respectively, narrow and wide inhibition zones, the plates after chloroform treatment were overlayered with soft agar inoculated with the two indicator strains CL104 (sensitive to colicines I and E2) and CL151 (sensitive to colicine I but not to E2) mixed in the ratio of 100:1. After incubation large turbid zones surrounded colonies producing only colicine E2; small clear zones surrounded colonies producing only colicine I; and zones with a narrow clear inner region and a wide turbid periphery surrounded colonies producing both colicines. When very few bacteria were colicinogenic, a sandwich plate technique (see Ozeki *et al.* 1962) made it possible to recognize the rare colicinogenic colonies without killing them; they were then subcultured to determine the colicines which they produced.

RESULTS

Preliminary observations. When strain LT2 (*colI*) (*colE2*) was incubated overnight with LT2 *col*$^-$, about 55% of the originally non-colicinogenic bacteria acquired *colI* only and 22% acquired both *colI* and *colE2* (Ozeki *et al.* 1962, Table 3*b*). On the hypothesis of the epidemic spread of *colI* amongst the non-colicinogenic bacteria initiated by the few 'competent donors' of the colicinogenic strain, this observation

indicated that: (i) some at least of the few competent donors in the LT2 (*colI*) (*colE2*) culture transmitted *colE2* as well as *colI*; (ii) that *colE2* was often transmitted with *colI* during the epidemic spread of *colI*; that is, that many bacteria newly infected with *colI* and *colE2* became competent to transmit both factors. Experiments on the transmitting ability of HFC preparations grown from an LT2 (*colI*) (*colE2*) donor and a non-colicinogenic intermediate strain confirmed conclusion (ii). In one such experiment the proportions of the intermediate strain which had acquired only *colI* or both *colI* and *colE2* were, respectively, 43 and 7 % after overnight incubation and 63 and 10 % after secondary incubation; this HFC preparation in 1 hr. transmitted *colI* alone to 47 % and *colI* with *colE2* to 10 % of the acceptor bacteria. No acceptor bacteria which had acquired *colE2* alone were observed in several experiments.

HFC preparations in which the intermediate carried colE1, colE2 *or both*

To test the ability of bacteria carrying *colE2* and newly infected by *colI* to transmit *col* factors, we made HFC preparations using strains carrying *colE2* as intermediate component. The usual proportion of intermediate bacteria were found to be carrying *colI* at the end of the overnight and 2 hr. incubation periods. Such HFC preparations transmitted colicinogeny to many acceptor bacteria, e.g. 40 % in 30 min. contact; most colicinogenic acceptor bacteria had acquired *colI* and *colE2*, a minority only *colI* or *colE2* (Table 1). Similar results were obtained with HFC preparations made with an intermediate strain carrying *colE1* or both *colE1* and *colE2* (Table 1). Thus when bacteria already carrying either *colE1* or *colE2* or

Table 1. *Transfer of colicine factors by HFC preparations in which the intermediate strain carried* colE1 *and/or* colE2

HFC preparations: In Expts. 1, 2, 3 and 6 the donor was *adeC-7 proA-46 fla*$^-$ (*colI*), and in Expts. 4 and 5 *cysD-36 fla*$^+$ (*colI*): in all experiments the intermediate was *cysD-36 fla*$^+$ carrying the indicated *col* factor(s). Acceptors: In Expts. 1, 2 and 3 *cysC-7 str-r*; in Expt. 4 *athC-5 str-r*; in Expt. 5 *cysD-36 str-r*; in Expt. 6 *adeC-7 proA-46 fla*$^-$ *str-r*.

Samples from a mixture of an HFC preparation with an acceptor culture were plated on streptomycin agar at the times indicated after mixing and the colicines produced by acceptor bacteria which had acquired colicinogeny were determined.

Expt.	*col factors* of intermediate strain	Time plated (min.)	Proportion acceptor bacteria producing colicine(s) shown (%)						
			I	E1	E2	I & E1	I & E2	E1 & E2	I & E1 & E2
1	*colE2*	10	2	–	1	–	4	–	–
		45	3	–	0	–	12	–	–
		120	9	–	0	–	40	–	–
2	*colE2*	30	2	–	0	–	40	–	–
3	*colE2*	60	0	–	2	–	52	–	–
4	*colE1*	30	0	0	–	17	–	–	–
5	*colE1*	60	2	1	–	23	–	–	–
6	*colE1* and *colE2*	25	0	0	0	3	1	0	6
		65*	0	0	0	2	0	0	16

0 = none detected: – = not applicable. * Mixture diluted 1/100 at 5 min. and a sample of the diluted mixture plated 60 min. later.

both E factors become competent donors on acquisition of *colI*, most of them become able to transmit all the factors they possess.

Kinetics of co-transfer of colI, colE1 *and* colE2. In *Escherichia coli* K12, the transfer of the chromosome from the Hfr to the F^- partner can be interrupted by blendor treatment, which separates the partners; the order of loci along the chromosome can be inferred from their time of entry (Wollman, Jacob & Hayes, 1956). Stocker and his colleagues (1963) studied the kinetics of transfer of *colI* from an HFC preparation by scoring the proportion of colicinogenic acceptor bacteria in blendor-treated samples taken at intervals from a mixture diluted soon after mixing, to prevent further pair formation. The period for completion of the transfer of *colI* varied in different pairs between 2 and 30 min., or even longer, although all

Table 2. *Kinetics of transfer of three factors from strain* LT2 (colE1) (colE2) *bacteria newly infected with* colI

HFC preparation: donor *adeC-7 proA-46 fla⁻ str-s* (*colI*) and intermediate *cysD-36 fla⁺ str-s* (*colE1*) (*colE2*). The percentage of intermediate bacteria carrying *colI* before and after the 2 hr. secondary incubation period in the three experiments was (*a*) 45 and 98, (*b*) 11 and 84, (*c*) 18 and 96. Acceptors: expts. (*a*) and (*b*), *adeC-7 proA-46 fla⁻ str-r col⁻*; expt. (*c*) *athA-4 phe-401 fla⁺ str-r col⁻*.

The mixture of HFC preparation and acceptor culture was gently diluted 1/100 into a number of tubes of broth at 5 min. (Expt. *a*) or 2½ min. (Expt. *b*). At intervals samples of the diluted mixture were treated in a blendor and plated on streptomycin minimal medium using the sandwich technique. In Expt. *c* the mixture was diluted 1/100 into one tube of broth from which all subsequent samples were taken. The colicines produced by at least 110 colicinogenic colonies from each sample were determined.

	Time plated (min.)	Acceptor bacteria colicinogenic		Colicinogenic acceptor bacteria, % producing colicines shown						
		No.	%	I, E1 & E2	I & E1	I & E2	E1 & E2	I	E1	E2
(*a*)	5	23/613	3·8	52	4	4	4	10	22	4
	15	29/575	5·0	83	7	7	0	3	0	0
	25	12/504	2·4	92	0	8	0	0	0	0
	45	21/525	4·0	76	0	14	0	10	0	0
	65	45/295	15·2	89	7	2	0	2	0	0
	25*	6/90	6·7	84	0	16	0	0	0	0
(*b*)	2½	35/3850	0·9	12	9	12	0	44	6	17
	5	14/4810	0·3	24	30	30	0	16	0	0
	10	63/4260	1·5	59	15	15	0	6	0	5
	20	63/3750	1·7	78	4	14	0	2	0	2
(*c*)	1	$130/2 \times 10^4$	0·65	1	0	2	13	1	18	65
	3	$272/10^4$	2·7	19	12	29	10	14	9	7
	3†	$365/8·5 \times 10^3$	4·3	30	16	19	6	13	4	12
	5	$311/10^4$	3·1	49	11	18	4	9	1	8

* Original undiluted mixture sampled after 25 min. incubation.
† HFC preparation treated in blendor before mixing with acceptor culture.

pairs had formed within 2 min. Interruption of conjugation in which three colicine factors were being transmitted from a competent donor carrying *colE1* and *colE2* and newly infected with *colI* was investigated, to test whether the three factors were transmitted simultaneously or successively; and, if successively, whether in a

constant or a variable order. In the first experiment (Table 2*a*) the mixture of HFC preparation and acceptor culture was diluted 1/100 into broth after 5 min.; at intervals samples were further diluted, treated in the blendor and plated. In the samples plated 15–65 min. after mixing, most acceptor bacteria which had acquired colicinogeny had acquired all three factors. In the sample plated at 5 min., i.e. immediately after diluting, acceptor bacteria with all possible single, double and triple combinations of the three colicine factors were present. Even in this sample, however, more than half the colicinogenic acceptor bacteria had acquired all three *col* factors. In a second experiment, therefore, the mixture was diluted, and the first sample treated in the blendor 2½ min. after mixing; in this sample the triply colicinogenic class then constituted only 12 % of the colicinogenic acceptor bacteria (Table 2*b*). In a third experiment (Table 2*c*) only 1 min. was allowed for pairing. In the first sample, taken immediately after mixing, the majority of acceptor bacteria which acquired colicinogeny (0·65 % of the acceptor population) acquired one colicine factor only, generally *colE2*, sometimes *colE1* and rarely *colI* (Table 2*c*). Amongst bacteria which had acquired two factors, bacteria carrying *colE1* and *colE2* were predominant. In the sample taken 3 min. after mixing the doubly colicinogenic types had become predominant; and after 5 min. the triply colicinogenic type was commonest.

The cell clumps characteristically found in HFC preparations are broken up by blendor treatment (Stocker *et al.* 1963). In part of the above experiment the HFC preparation was treated in the blendor before mixing, to ensure that any individual acceptor bacterium acquired *col* factors from only a single donor bacterium, rather than perhaps from more than one member of a clump of donor bacteria. This treatment rendered the donor bacteria temporarily non-motile by deflagellation (Stocker & Campbell, 1959), and a motile acceptor strain was therefore used to ensure a high frequency of collision and pairing (Stocker *et al.* 1963). The transfer of *col* factors (Table 2*c*) was not significantly different from that obtained from an untreated HFC preparation; it is therefore unlikely that the results of experiments in which this treatment was omitted were much affected by the acquisition by an acceptor bacterium of factors from more than one member of a clump.

Attempted separation of two functions of colI. Two characters are conferred on *Salmonella typhimurium* strain LT2*colI*; the abilities to produce colicine I and to transmit this and any other colicinogenic properties it possesses. If these two characters result from possession of two determinants, always transmitted together during ordinary uninterrupted pairing, then interruption at a very early stage might result in acquisition of one but not both of the postulated determinants. We therefore tested the transmission of colicinogeny by clones of a previously non-colicinogenic acceptor strain which had acquired the ability to produce one or more colicines during only 1 min. contact with an HFC preparation transmitting *colI*, *colE1* and *colE2* (Table 2*c*). Four clones producing colicine I (one producing colicine I only; two producing colicines I and E2; one producing colicines I, E1 and E2) all transmitted colicinogeny in the usual way during overnight incubation. Fifteen clones producing colicine E1 and/or E2 (nine E2; two E1; four E1 and E2) failed to transmit at all, or, in the case of strains producing colicine E1, transmitted to only a very low proportion of the acceptor bacteria. Thus in their ability to transmit these colicinogenic clones, derived from matings interrupted very early, behaved

like standard colicinogenic strains, and interruption which separated *colI* from other *col* factors during transmission did not separate the two functions of *colI*.

Segregation of newly acquired colicine factors. The method used to detect colicine production by acceptor colonies would give positive results with colonies in which only a proportion of the bacteria were colicinogenic. Many colicinogenic acceptor colonies were therefore streaked out and 5–10 colonies from each tested for colicine production. Nearly always all the subcolonies tested produced the same colicines as the unpurified clone. For instance, in one experiment with an HFC preparation of *colI* donor and *colE1 colE2* intermediate (Table 2*a*), we streaked out colicinogenic acceptor colonies selected as producing various combinations of colicines and as being derived from platings at 5–65 min. Segregation was detected in only 4 of the 55 clones, 3 amongst the 31 from samples plated only 5 min. after mixing and 1 amongst the 24 from samples plated 15–65 min. after mixing. Each of these 4 clones segregated two components, as follows: (I + E1 + E2) and (I + E1); (I + E1 + E2) and (I + E2); (I + E1) and *col*$^-$; (I + E2) and *col*$^-$. In another experiment with an HFC preparation of *colI* donor and *colE2* intermediate the two components of a segregating clone were (I + E2) and (I).

Acquisition of only some of the donor col *factors during uninterrupted pairing*

Even when the transmission of *colI* with E factor(s) from an HFC preparation to acceptor bacteria was allowed to continue undisturbed for longer than 15 min., up to 20 % of the acceptor bacteria which acquired any colicinogenic factor did not acquire all the factors (Tables 1 and 2). As segregating clones are rare it is unlikely that this minority resulted from segregation before sampling. These clones might, however, have resulted from separation by plating of some pairs in which transmission of the *col* factors was not complete. However, such bacteria were also found when artificial separation of paired bacteria by external forces was unlikely. We have reported earlier experiments in which a very few streptomycin-sensitive bacteria from an HFC preparation were added to a streptomycin-resistant acceptor culture and the tubes (after addition of streptomycin) incubated without shaking for many hours to permit the epidemic spread amongst the acceptor bacteria of *col* factors transferred by any competent donors inoculated (Stocker *et al.* 1963). In one experiment 116 tubes were inoculated with < 2 bacteria from an HFC preparation of *colI* → *colE2* type; 26 were positive, yielding colicinogenic acceptor bacteria. Production of colicines I and E2 was detected in 17 of these and of colicine I alone in 9. In similar experiments to determine the proportion of competent donors in an established doubly colicinogenic strain all of the 8 positive tubes amongst 21 inoculated with 3000–5000 donor bacteria yielded cultures producing both colicines I and E2.

Retention of colicinogeny by donor bacteria which transmit it

To explain our observations (Ozeki *et al.* 1962; Stocker *et al.* 1963; and above) on the epidemic spread of *col* factors introduced into a non-colicinogenic population we have assumed that a competent bacterium retains its *col* factor(s), and produces colicinogenic progeny, even after it has transmitted these factor(s) by conjugation to an acceptor bacterium. To check this we made an HFC preparation

with a streptomycin-resistant intermediate strain carrying *colE2*. As 18 % of the bacteria of an acceptor strain (streptomycin-sensitive and recognized by their nutritional character) acquired colicinogeny in 30 min. contact with this HFC preparation, we infer that about 18 % of the intermediate bacteria must have transmitted *colI* and *colE2*. However all of 380 colonies of the intermediate strain, obtained by plating at the same time, still produced colicine E2. The retention of *colI* by competent donors which transmit it could not be tested in the same way, since all HFC preparations necessarily contain a proportion (10–50 %) of bacteria of the intermediate strain which lack *colI* because they have not become infected with it. The experiment tests only the colicinogeny of competent donor bacteria which survive conjugation. However we have no reason, from viable counts or other evidence, to suppose that bacteria transmitting colicinogeny become non-viable; if effective conjugation were lethal to the donor partner it would be difficult to account for the epidemic spread of *col* factors.

Transmission of col *factors to colicinogenic acceptor bacteria*

An HFC preparation of a *colI* donor and a non-colicinogenic intermediate transmitted *colI* to about the same proportions of bacteria of a non-colicinogenic acceptor strain and of acceptor strains carrying *colE1* or *colE2*. Thus the possession of these latter factors did not interfere with the ability of bacteria to accept *colI* from an HFC preparation. We could not make a direct test of the transmission of *colI* from such an HFC preparation to bacteria already carrying *colI* because no distinguishable *colI* factors are available. But as *colE2* accompanies *colI* when it is transmitted from an HFC preparation of a *colI* donor and a *colE2* intermediate (Table 1), the transmission of *colE2* to non-colicinogenic acceptor strains and to acceptor strains carrying *colI* or other colicine factors could be compared. The proportion of acceptor bacteria carrying *colE1* which acquired *colE2* was the same as the proportion of non-colicinogenic acceptor bacteria (46 % in each case after 30 min. contact); but the proportion of bacteria already carrying *colI* which acquired *colE2* was tenfold less (5 % after 30 min. contact).

One-way transmission of col *factors*. To see if a colicinogenic acceptor bacterium could transmit its own *col* factors to a competent donor from which it acquired *colI*, we made an HFC preparation with a streptomycin-resistant, non-colicinogenic intermediate strain and mixed it with a culture of a streptomycin-sensitive acceptor strain carrying *colE2*. After 30 min. 7 % of the acceptor bacteria, distinguished by their nutritional character, had acquired *colI* from the HFC preparation; none ($< 0{\cdot}01$ %) of the streptomycin-resistant intermediate bacteria had acquired *colE2*, although about 7 % were presumably derived from those bacteria which had conjugated with and transmitted *colI* to acceptor bacteria carrying *colE2*. Therefore during transfer of *colI* from competent donor bacteria to acceptor bacteria there was no reverse transmission of *colE2* from acceptor to donor bacteria.

Time needed for a newly infected bacterium to become a 'competent donor'

When *colI* alone is transmitted at high frequency to a genetically marked acceptor strain it is impossible to determine the interval which elapses before these newly infected bacteria themselves become competent donors, because other competent donor bacteria, used to introduce *colI*, are necessarily present. Bacteria carrying

colE2 alone do not transmit this factor at a detectable rate, but soon after infection with *colI* transmit both *colE2* and *colI* at high frequency; therefore the time of onset in such bacteria of the ability to transmit *colE2* (with *colI*) could be used to determine the minimum time needed for bacteria newly infected with *colI* to become competent donors. An HFC preparation was made from a donor strain carrying *colI* and a non-colicinogenic intermediate strain, both streptomycin-sensitive. This HFC preparation was mixed with a culture of a streptomycin-sensitive strain carrying *colE2* as 'primary acceptor', together with a culture of a non-colicinogenic, streptomycin-resistant 'final acceptor' strain. After incubation for various times samples from the mixture were treated in a blendor, diluted and plated on streptomycin agar, and the proportions of final acceptor bacteria which had acquired *colI* or *colE2* were determined.

Table 3. *Time needed for strain* LT2 (colE2) *bacteria newly infected with* colI *to become competent donors*

Components of HFC preparation (all streptomycin-sensitive): Expt. 1, *cysD-36* (*colI*) and *cysD-36 col*$^-$; Expt. 2, *adeC-7 proA-46* (*colI*) and *adeC-7 proA-46 col*$^-$. Each HFC preparation contained about 90% of bacteria carrying *colI*. 4 ml. of the HFC preparation, 4 ml. of a broth culture of *cysD-36* (*colE2*) (streptomycin-sensitive primary acceptor) and 2 ml. of a broth culture of *adeC-7 proA-46 fla*$^-$ *str-r col*$^-$ (final acceptor) were mixed. After incubation for various times samples were treated in the blendor and drops of tenfold dilutions plated on streptomycin agar. The plates after incubation were overlayered to detect colicine I or E2 zones.

In Expt. 2 the proportion of *cysD-36* (*colE2*) bacteria which had acquired *colI* at various intervals was also measured, by scoring those bacteria which grew on minimal medium supplemented with cysteine.

	Expt. 1		Expt. 2		
	Proportion of final acceptor bacteria producing colicines			Proportion of final acceptor bacteria producing colicines	
Time (min.)	I	E2	Proportion of primary acceptor bacteria producing colicine I	I	E2
5	0·03	$< 10^{-6}$	0·03	0·007	$< 10^{-5}$
10	0·05	$< 10^{-6}$	0·07	0·06	$< 10^{-5}$
15	0·07	$< 10^{-5}$	0·14	0·06	$< 10^{-5}$
20	0·12	10^{-5}	0·14	0·06	$< 10^{-5}$
25	0·27	8×10^{-5}		0·04	$< 10^{-5}$
30	0·19	$2{\cdot}8 \times 10^{-4}$	0·16	0·06	10^{-5}
40	0·15	$1{\cdot}6 \times 10^{-3}$	0·20	0·09	$1{\cdot}6 \times 10^{-4}$

In one such experiment final acceptor bacteria producing colicine E2 were first detected, in a proportion of *c.* 10^{-5}, in a sample plated 20 min. after mixing; and in another experiment they were detected 30 min. after mixing (Table 3). In both experiments the proportion of final acceptor bacteria producing colicine E2 increased rapidly in the 5–20 min. after their first appearance. Thus some bacteria of the primary acceptor strain, already carrying *colE2*, became competent to transmit *colE2*—and presumably also *colI*—within 20 min. of infection by *colI*.

DISCUSSION

We have earlier shown that the transmissibility of *colI* in *Salmonella typhimurium* strain LT2 differs greatly from that of *colE1* and *colE2*. In the present paper we are concerned with the effects of one *col* factor on the transmission of another. The presence of *colE2* and/or *colE1* in a bacterium seems to have no effect on its behaviour in respect of *colI*. Thus in established LT2 (*colI*) lines a small proportion of the bacteria are competent donors of *colI*, whether or not the strain carries also *colE2* or *colE1* (Ozeki *et al.* 1962, Table 3*b*). Bacteria carrying *colE1* or *colE2* acquire *colI* by conjugation from competent donors as readily as do non-colicinogenic bacteria, and, like them, become competent donors of *colI* soon after (Ozeki *et al.* 1962, Table 3*c*; Table 1 above).

By contrast, *colI* greatly affects the behaviour of bacteria in respect of *colE1* and *colE2*. On the one hand the presence of *colI* in acceptor bacteria caused a tenfold reduction in acceptance of *colE2* from a *colI* → *colE2* HFC preparation, by some mechanism other than the prevention of pairing (Stocker *et al.* 1963); on the other hand, most bacteria which transmit *colI* transmit also *colE1* and/or *colE2* when they possess these factors.

The transmission of all their colicine factors by competent donors of *colI* possessing also *colE1* and/or *colE2* occurs in three situations. First, in established LT2 (*colI*) (*colE2*) and LT2 (*colI*) (*colE1*) lines all or most of the few bacteria which are competent donors of *colI* transmit also their E factors. Secondly, many bacteria which acquire *colI* and an E factor simultaneously from a competent donor themselves become competent donors of both *colI* and the E factor (see above, p. 233). Thirdly, when bacteria already carrying *colE1* and/or *colE2* are infected with *colI*, a high proportion of them become competent donors of both *colI* and the E factor(s) carried (Table 1).

When an HFC preparation, containing many LT2 (*colE1*) (*colE2*) bacteria newly infected with *colI*, was incubated for 25–65 min. with a non-colicinogenic acceptor culture, most acceptor bacteria which acquired colicinogeny acquired all three colicine factors (Table 1). The three different colicine factors in a competent donor might be joined in a linear linkage group, transferred sequentially into the acceptor bacterium, much as the chromosome, or part of it, of an Hfr bacterium is transferred into the F^- partner in *Escherichia coli* K12. In this case, and provided that transfer of the postulated linear linkage group always began at the same end (as in transfer of the Hfr chromosome), early interruption would permit transfer of only the factor at the leading end of the linkage group, and somewhat later interruption transfer of only the leading and the second factor in the group. In the kinetic experiments (Table 2) HFC preparations transmitted all three factors together with high probability when 1–5 min. was allowed for conjugation and a further 20 min. or more for transfer; even 5 min. after mixing, half or more of the acceptor bacteria which become colicinogenic acquired all three factors (Table 2*a*). But when samples were treated in the blendor and plated 1–5 min. after mixing, the proportion of bacteria which acquired all three factors amongst acceptor bacteria which acquired any factors was much less than one (Table 2*b*, *c*); acquisition of each single factor and each pair of factors was detected. Despite some variation between experiments and between samples in the same experiment in the relative frequencies of the

different single and double classes, this observation excludes the hypothesis of the transfer of *colI*, *colE1* and *colE2* in an unvarying sequence in all pairs. The data, however, do not exclude the possibility that the three factors in a competent donor are associated in a circular linkage group (cf. the model of Watanabe & Lyang (1962) for the arrangement of the units in the RTF plasmid) opening at different points in different bacteria and thus permitting sequential transfer with the order of transfer differing in different pairs (cf. the different types of Hfr mutants which can be obtained in an F^+ strain of *E. coli*). It is equally possible that the different *col* factors in a competent donor are unlinked and that during conjugation they are transferred independently, the probability of transfer during uninterrupted conjugation being high for each sort of factor and unaffected by transfer of any other sort.

The rapidity with which acceptor bacteria with all three factors become the commonest class indicates that in those pairs in which any factor is transmitted rapidly the transmission of all three factors is rapid. In the sample blendor-treated and plated 1 min. after mixing (Table 2*c*), of the acceptor bacteria which had acquired any colicine factor 81 % had acquired *colE2*, 32 % *colE1* and only 4 % *colI*; but the results are not sufficiently extensive to permit any conclusions about the relative speed of transfer of the different factors. The preponderance of the triple class in later samples suggests that all three factors are transmitted at about the same time in at least many of the pairs in which no factor is transferred early. Early interruption of mating did not result in acceptor bacteria acquiring the ability to produce colicine I without the ability to transmit colicinogeny, nor the reverse. This makes it unlikely that these two characters result from two distinct plasmids.

A minority of competent donor bacteria transmitted some but not all of their colicine factors not only in the kinetic experiments, where the interruption of conjugation by plating may have been responsible; but also in the experiments designed to determine the proportion of competent donors in HFC preparations, when many hours incubation in liquid presumably permitted the completion of conjugation. Furthermore, the epidemic spread of colicinogeny during 18 hr. incubation of a non-colicinogenic culture with a culture carrying both *colI* and *colE2* (or *colE1*) results in more acceptor bacteria acquiring *colI* alone than acquire both *colI* and the E factor concerned (Ozeki *et al.* 1962, Table 3*b*); this, too, indicates that some competent donors carrying *colI* and an E factor (or factors) transmit only *colI* when uninterrupted conjugation is permitted. Perhaps in some pairs transmission is interrupted by the spontaneous separation of the partners as reported in *Escherichia coli* K12 Hfr × F^- conjugation (Wollman & Jacob, 1958). Transmission of *colI* alone was not observed in a few experiments to determine the proportion of competent donors in an established LT2 (*colI*) (*colE2*) strain; perhaps transmission of *colI* without *colE2* by a minority of competent donors occurs only when the latter owe their infectivity to recent acquisition of *colI*. Transmission of *colE2* but not of *colI* by a minority of competent donors during uninterrupted conjugation perhaps also occurs; but would not be detected in these experiments because of the failure of the resulting LT2 (*colE2*) bacteria to initiate epidemic spread of *colE2* (Ozeki *et al.* 1962, Table 3*a*).

In competent donors and in acceptor bacteria about to become competent donors

because they have just acquired *colI*, all the colicine factors present, that is *colI* and any E factor(s), must be multiplying more rapidly than their hosts, and more than one particle of each factor must be present per cell. This we infer because: (i) transmission of *colE2* and *colI* by an HFC preparation does not result in the appearance of donor bacteria which have lost *colE2* (see above, pp. 236–7); (ii) nearly all bacteria newly infected with one or more *col* factors produce progeny all of which carry the factor(s) even when conjugation was interrupted before all the factors of the donor had been transmitted, so that presumably no more than one copy of a factor would have been acquired (Table 3) (cf. Alföldi, Jacob, Wollman & Maze, 1958); (iii) some bacteria carrying *colE2* become able to transmit it only 15 or 20 min. after they acquire *colI*, even though the bacterial concentration is so high that bacterial multiplication is very slow (Table 3). (The rapidity with which newly infected bacteria become infectious means that the possibility of a 'second round' of transmission in the acceptor population must be considered when a donor and acceptor population are incubated together for 30 min. or longer; but in many experiments of this sort the mixture was diluted soon after mixing, so reducing further collisions—and we think that in experiments in which mixtures were incubated for less than two hours, second-round transmission did not greatly affect the results.) The rapid 'autonomous' multiplication of *col* factors in competent donor bacteria suggests that in such bacteria the factors are not located on the chromosome—or at least that they are present elsewhere. We have no evidence for autonomous multiplication of the factors in bacteria which do not transmit—all bacteria in stock LT2 (*colE2*) lines and most in LT2 (*colI*) lines. It may be that the failure of these bacteria to transmit results only from their inability to conjugate. However, a recipient carrying *colE2* does not transmit this factor to its donor partner during conjugation in which it acquires *colI*; and transmission of chromosomal genes by colicinogenic derivatives of an LT2 Hfr stock (Zinder, 1960) is not always accompanied by transmission of the *col* factors of the donor (E. Dubnau, pers. comm.). This suggests that the failure to transmit *col* factors of bacteria which are not competent donors results from some cause beyond their inability to conjugate.

We have examined the behaviour of some further *col* factors, in HFC preparations and other cultures. One factor, *colK* derived from *Escherichia coli* K235, behaves much like *colE1* and *colE2*. In other instances, however, more complex interactions are found—for the presence of some *col* factors in an acceptor culture interferes with its acceptance of *colI*, and the presence of others partly prevents those bacteria which accept *colI* from becoming competent donors. These results will be described elsewhere.

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Deoxyribonucleic Acid Base Composition of Acetic Acid Bacteria

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SUMMARY

The base composition of purified DNA from 28 strains of acetic acid bacteria was determined. Most strains of the genus *Gluconobacter* clustered closely together at 60·6–63·4 % (guanine + cytosine) of total base. All strains of the *Acetobacter aceti* biotype lay within the range 55·4–64·0 % (guanine + cytosine). The close relationship and possible common phylogenetic origin of the genera *Gluconobacter* and *Acetobacter* is again stressed by these results. The base composition of DNA from acetic acid bacteria and from species of Pseudomonas was very similar, confirming the suspected close relationship between these groups. There is a noticeable agreement between the sequences of Acetobacter strains, arranged according to increasing % (guanine + cytosine) and arranged according to increasing enzymic equipment: strains with greater biochemical activity have on the whole also a higher % (guanine + cytosine) in DNA. The range of the compositional distribution of DNA molecules is on the whole broader in Acetobacter than in Gluconobacter. The results corroborate previous conclusions that both biotypes contain clusters of strains without species differentiation. A comparison of the paper chromatographic analysis with the method of thermal denaturation ('melting point') for estimating base composition of DNA showed that the latter method was to be preferred in routine analysis because of its ease, rapidity and reproducibility.

INTRODUCTION

DNA base composition appears to be a promising new tool in bacterial taxonomy (Lee, Wahl & Barbu, 1956; Belozersky & Spirin, 1958; Lanni, 1960; Sueoka, 1961; Schildkraut, Marmur & Doty, 1962; Marmur & Doty, 1962; Marmur, Seaman & Levine, 1963). The present knowledge in this field can be summarized as follows.

(1) Different 'strains' of the same well-established species have almost identical DNA base compositions. Thus, for seven strains of *Escherichia coli*, the molar content of guanine + cytosine (G + C) expressed as a percentage of the total base in the DNA was 50 % ± 0·4 (Marmur & Doty, 1962). Different strains of *E. coli* had 50 % (G + C), even when determined by different authors.

(2) Different 'species' which are known for morphological, physiological and other reasons to belong to the same genus, have identical or closely related % (G + C) values. This is well illustrated, for example, in the genera *Escherichia*, *Salmonella*, *Clostridium* and *Hemophilus* (Lee *et al.* 1956; Marmur & Doty, 1962). Exceptions are believed to be indicative of wrong classification. For example,

Catlin & Cunningham (1961) found all the strains of Neisseria they examined to have 49·5–51·5 % (G+C), except for *N. catarrhalis*, which had 40·0–41·3 %. They conclude that 'the inclusion of "catarrhalis" strains in the genus *Neisseria* appears illogical from the evolutionary point of view.' The suspected divergency within Proteus was confirmed by the results of Falkow, Ryman & Washington (1962), who found that *P. morganii* had a 50 % (G+C), whereas the other species had 39 %. On physiological and biochemical grounds *P. morganii* had previously been thought to be only remotely related to the other species 'vulgaris' and 'mirabilis' and a new genus *Morganella* had even been proposed for it (Fulton, 1943). The genus *Bacillus* offers another example where base composition tends to confirm the interpretation which was reached by quite different arguments. Base composition in Bacillus varies between 33 and 50 % (G+C); this is an unusually wide range for one genus. The explanation might be (De Ley, 1962) that Bacillus should not be considered a single genus, but a collection of genera or biotypes, which would form the bridge between the Enterobacteriaceae (about 50 % G+C) and the saccharolytic clostridia (about 27 % G+C). Bisset (1962) suggested that 'there is room for several genera in the family Bacillaceae'.

(3) Different genera, which are known or suspected for morphological, physiological, biochemical and other reasons to be closely related, also have base compositions which are very similar such as Escherichia, Salmonella and Shigella (Lee *et al.* 1956; Marmur & Doty, 1962), Proteus and Providence (Falkow *et al.* 1962). Unrelated genera have widely different % (G+C) values, the range being from 25 to 75 % (G+C).

(4) If the mean % (G+C) of the DNA of two strains is different by 10 %, there are few DNA molecules of the same (G+C) content common to both of them (Sueoka, 1961).

(5) Organisms with a closely related base composition are believed to be phylogenetically related (Sueoka, 1961) when they also display related morphological, physiological, biochemical and other characteristics.

However, up to now, the DNA base composition method has not been applied systematically to a wide set of strains of a group of bacteria other properties of which have been thoroughly investigated. The acetic acid bacteria seemed to be suitable objects for an application and test of the above conclusions. In view of the finding that Gluconobacter and Acetobacter each consist of a series of strains with a gradation of properties, it was proposed to regard all previous 'species' as variants within the biotypes *Gluconobacter oxydans* and *Acetobacter aceti*, which might phylogenetically be derived from the same common pool of ancestors (De Ley, 1961*a*). It was thus expected that the DNA base composition of Gluconobacter would be similar to that of Acetobacter, that the DNA base composition of all strains within each biotype would cluster in the same region, and that the base composition of acetic acid bacteria would be in the neighbourhood of that of Pseudomonas, namely 60–67 % (G+C), in view of their suspected close taxonomic relationship.

Two methods were used: (1) direct estimation of purine and pyrimidine bases after hydrolysis and paper chromatographic separation; (2) thermal denaturation ('melting point'). It was also the aim of the present work to compare the practicability of both methods for routine analysis.

METHODS

Organisms. We used the same strains as described previously (De Ley, 1961*a*) and will adhere to the nomenclature proposed in that paper. In addition, we also used *Acetobacter aceti* (strains *paradoxus* P1 and P2) and *A. aceti* (strains *peroxydans* 3 and 4) obtained through the courtesy of Dr J. W. M. la Rivière (Laboratory of Microbiology, Delft, the Netherlands), *Gluconobacter oxydans* strain *melanogenus* 116 (courtesy of Dr A. H. Stouthamer, Laboratory of Microbiology, Utrecht, the Netherlands) and *G. oxydans* strain *melanogenus* 49 (courtesy of Mr M. R. Kimmitt, H. P. Sauce Ltd., Birmingham, England). Repeated plating and re-determination of the properties of the strains according to the criteria of Frateur (1950) and De Ley (1961*a*) assured that only pure cultures were used. *Pseudomonas fluorescens* CCEB 488 was obtained through the courtesy of Dr O. Lysenko (Institute of Biology, Prague); *Agrobacterium tumefaciens* through the courtesy of Dr M. Bernaerts (Ministry of Economic Affairs, Brussels) and the *Escherichia coli* was a National Collection of Type Cultures (Colindale, London, Great Britain; NCTC) strain.

Mass cultures. All strains of acetic acid bacteria were incubated at 30° for 2 to 3 days on solid media in Roux flasks, except for the strains of 'peroxydans' and 'paradoxus', which needed 1 week. Strains of Gluconobacter and the 'mesoxydans' group of Acetobacter were grown on a medium containing (%, w/v): 10, glucose; 3, $CaCO_3$; 1, Yeastex (Ned. Gist en Spiritusfabriek, Brugge, Belgium); 2·5, agar. Most strains of the 'oxydans' and 'peroxydans' groups of Acetobacter were grown on 1·5% malt extract (Difco), 0·5% Yeastex, 3% (v/v) ethanol, 2·5% agar. *A. aceti* (*paradoxus*) was grown on 50% (v/v) red wine, 0·5% Yeastex, 1% DL-lactate Ca salt, 2% $CaCO_3$, 2·5% agar. Sometimes other media were used, e.g. with sorbitol instead of glucose. No differences in DNA base composition were then observed.

Pseudomonas fluorescens was incubated for one day at 30° in a liquid medium with 0·5% peptone (Difco) + 0·25% Yeastex and with passage of sterile air. Agrobacterium was grown in Roux flasks on a medium with 1% Yeastex, 1% glucose, 2% $CaCO_3$ and 2·5% agar for two days at 30°. *Escherichia coli* was grown for two days at 30° in Roux flasks on 0·5% peptone, 0·25% Yeastex and 2·5% agar.

Estimation of DNA base composition by paper chromatography. Organisms were harvested and washed as previously described (De Ley, 1961*a*). The pellet was extracted according to the method of Smith & Wyatt (1951). Two to three g. wet wt. living bacteria were suspended in 10 ml. N-NaOH and incubated at 37° for 20 hr. In some experiments the organisms were treated with 1% Na lauryl sulphate before NaOH extraction. No significant difference in the final yield was observed. After the extraction the residue was centrifuged down in a Servall SS–1 at 13.000*g* for 15 min. The DNA was precipitated by adjusting to pH 4 with acetic acid and adding one vol. of 95% (v/v) ethanol in water. The precipitate was centrifuged down and dissolved in 10 ml. dilute NaOH. Protein was removed by gel-formation with chloroform according to the method of Sevag, Lackman & Smolens (1938). The solution was shaken with 0·25 vol. chloroform and 0·1 vol. octanol for 30 min. and the chloroform and aqueous layers then separated by centrifugation at 2700*g* for 15 min. The upper aqueous layer was repeatedly treated with chloroform for 15 min. and centrifuged until almost no protein was observed at the liquid/liquid interface. DNA was again precipitated with ethanol at pH 4; the precipitate collected by

centrifugation, redissolved in 10 ml. dilute NaOH and the amount of nucleic acid and protein determined by measuring the extinction at 260 and 280 mμ according to Warburg & Christian (1942). DNA was again precipitated with ethanol at pH 4 and dried in a vacuum desiccator. It was hydrolysed with 72% (v/v) perchloric acid and the purines and pyrimidines of 10 μl. spots separated by paper chromatography with a mixture of 65% (v/v) *iso*propanol 2N with respect to HCl, as the solvent (Wyatt, 1951). After separation the bases were detected on the chromatograms by the photographic method of Markham & Smith (1949). The chromatograms were first dried overnight at room temperature. The source of ultraviolet radiation was a germicidal G.E. lamp; Gevaert Reflex Document paper was used for contact prints. The spots on the chromatograms were copied from the prints, cut out, eluted with 4 ml. 0·1 N-HCl (Wyatt, 1951) by standing for 15–20 hr. at 37°. Blanks of equal size were cut out at distances corresponding to the R_F value of the bases, from a strip of the same chromatogram, on which 10 μl. 2N-$HClO_4$ had been spotted between triplicate sample spots. The eluates of the bases were read against the corresponding blanks in a Beckman spectrophotometer (model DU) at the wavelengths indicated by Vischer & Chargaff (1948), except for cytosine, for which 274 mμ was used since this gave more reproducible results. The amount of each of the bases was calculated from the difference Δ between the extinction at the absorption peak and at 290 mμ. For test solutions containing 10 μg. bases/ml. 0·1 N-HCl Δ is: $\Delta_{\text{adenine}} = E_{262\cdot5} - E_{290} = 0\cdot900$; $\Delta_{\text{guanine}} = E_{249} - E_{290} = 0\cdot475$; $\Delta_{\text{thymine}} = E_{264\cdot5} - E_{290} = 0\cdot545$; $\Delta_{\text{cytosine}} = E_{274} - E_{290} = 0\cdot458$. Δ for cytosine was determined with a pure sample from Calbiochem (3625 Medford Street, Los Angeles 63, California, U.S.A.)

Estimation of DNA base composition by thermal denaturation. DNA was isolated according to the method of Marmur (1961). Thermal denaturation was followed at 260 mμ in a Beckman spectrophotometer (model DU) as described by Marmur & Doty (1962). To protect the photocell and the slit of the monochromator against excessive heating, which would result in inaccurate readings, it was found advantageous to use the following arrangement. The cell holder compartment was flanked at each side by the following set up: first two thermospacers for the circulating hot water from a Haake Ultra-Thermostat NB, followed by a 5 mm layer of cork and finally another thermospacer through which passed dropwise a current of tap water at 12°. To prevent traces of water vapour reaching the photocell (which would again result in erratic readings) a suitable well was machined into the side of the cell-holder compartment, which faced the photocell, and a circular quartz window was glued into the opening. The cell-holder compartment itself was insulated on all sides at the outside by cork. Tubing between the circulating water bath and the thermospacers has to be as short as possible and insulated by either plastic or asbestos. In this way the difference in temperature between the liquid in the cuvettes and the bath did not exceed 1·2°. The circulating water contained either 25% ethyleneglycol or 30% glycerol. The temperature in the cell-holder compartment was measured in a cuvette, containing water, tightly covered with a plastic cover, through which passed a calibrated thermometer. A suitable hole was drilled through the compartment cover. The thermostated cell holder compartment is schematically represented in Fig. 1. Blanks and the samples were measured in quartz cuvettes with ground glass stoppers. The T_m values were determined graphi-

cally according to Marmur & Doty (1962), T_m being the temperature at the midpoint of the transition of the absorbance–temperature curve.

Estimation of the range of base composition distribution of DNA. Assuming that the distribution of the compositional distribution of DNA molecules around T_m was Gaussian, the standard deviation σ of the distribution, expressed as % (G+C) and corrected for the natural transition width of adenine-thymine DNA, was determined for each strain from the absorbance–temperature curves according to Doty, Marmur & Sueoka (1959). The mean % (G+C) was calculated with Marmur & Doty's (1962) formula: % (G+C) = $(T_m - 69{\cdot}3)/0{\cdot}41$. The Gaussian distribution around the mean % (G+C) was calculated with the equation $y = 1/(\sigma\sqrt{2\pi})\exp(-x^2/2\sigma^2)$, in which x was expressed as % (G+C) around the mean. The results are represented graphically in Figs. 4 and 5. The ratio of the area under the y curve in any % (G+C) interval to the total area represents the fraction of the DNA molecules within this % (G+C) range of the total number of DNA molecules.

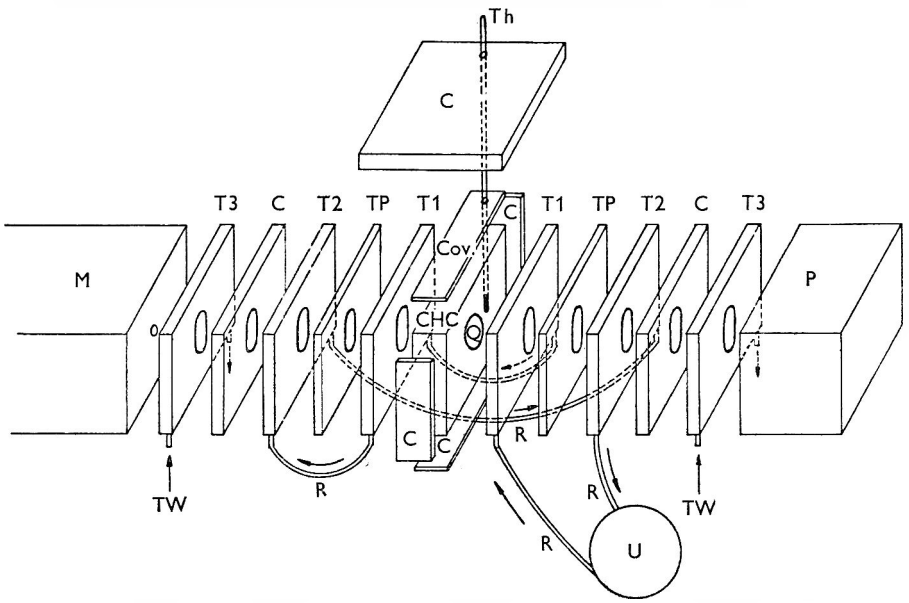

Fig. 1. Details of the thermostated cell holder compartment. M, Beckman monochromator (model DU); T3, thermospacer with circulating tap water (TW) at 12°; C, cork; T2 and T1, thermospacers with circulating hot water through insulated rubber tubing (R) from the ultra thermostat (U); TP, thermospacer plate; CHC, cell holder compartment with glued-in quartz window (Q); Cov., cell holder compartment cover; Th, calibrated thermometer; P, phototube housing from the Beckman spectrophotometer (model DU).

RESULTS

The results are summarized in Table 1. The T_m value is an average of at least two determinations; T_m values were in most cases reproducible to within 0·1°, exceptionally to 0·15°. σ was usually reproducible to about 0·25 % (G+C). The values given for the paper chromatographic method represent the average values of at least six estimations from two different extractions. The reproducibility of this method can be seen in Table 2, which illustrates the actual results obtained with three strains. The mean deviation on the ratio $(A+T)/(G+C)$ was 2·8, 2·9 and

3·7 % for these three examples, which is the common range of deviation in quantitative paper chromatographic extractions. When the base composition, expressed as % (G+C), as determined by the paper chromatographic method, was plotted against the T_m values of the same strains (see Fig. 2), the result was in good agreement with Marmur & Doty's (1962) formula % (G+C) = $(T_m - 69{\cdot}3)/0{\cdot}41$. Two examples of thermal denaturation curves are given in Fig. 3. The compositional distribution of DNA molecules is represented in Figs. 4 and 5.

Table 1. *Base composition, expressed as* % *(G+C), 'melting points' T_m values and standard deviation σ of the compositional distribution about the mean* % *(G+C) of purified DNA of various acetic acid bacteria*

(Methods: see text)

Strain	Paper chromatographic method % (G+C)	Thermal denaturation method		
		T_m	% (G+C)	σ
Biotype				
Acetobacter aceti				
liquefaciens 20	65·4	95·6°	64·0	1·5
xylinum 25	—	95·1°	62·8	2·6
xylinoides 4940	—	94·95°	62·4	1·5
estunensis E	—	94·85°	62·2	1·1
xylinum 8747	61·1	94·25°	60·7	2·25
mesoxydans var. *saccharovorans* 4	61·0	94·2°	60·6	1·6
pasteurianus 11	—	93·85°	59·8	1·75
aceti Ch31	59·5	93·8°	59·6	1·6
aceti var. *muciparus* 5	—	93·75°	59·5	1·5
mobilis 6428	58·8	93·5°	58·9	1·25
vini acetati 4939	—	92·9°	57·4	1·2
rancens 15	—	92·4°	56·2	1·1
cerinus var. *rosiensis* 22	56·5	92·2°	55·7	1·75
paradoxus P1	—	92·2°	55·7	1·1
rancens 23kl+	—	92·1°	55·4	0·9
paradoxus P2	—	92·1°	55·4	1·25
peroxydans 3	—	95·35°	63·4	1·5
peroxydans 4	—	95·35°	63·4	1·1
peroxydans 8618	61·0	95·0°	62·5	—
Gluconobacter oxydans				
viscosus 8131	62·1	95·35°	63·4	0·56
suboxydans 26	61·0	94·85°	62·2	0·5
capsulatus 4943	61·3	94·75°	61·9	0·5
suboxydans 3734	—	94·65°	61·7	0·63
gluconicum 4739	—	94·55°	61·4	0·38
melanogenus 49	—	94·4°	61·1	1·25
melanogenus 8086	61·0	94·2°	60·6	1·06
melanogenus 116	—	94·2°	60·6	0·87
suboxydans SU	58·1	92·8°	57·2	0·38

Three other bacteria were included as controls of the methods. *Pseudomonas fluorescens* 488 had a DNA base composition of 59·5 % (G+C), determined by paper chromatography, which is in good agreement with reported values for Pseudomonas in the literature (Lee *et al.* 1956; Marmur & Doty, 1962). *Agrobacterium tumefaciens* DNA had 58·8 % (G+C), also determined by paper chromatography. For other strains 58·2 and 58·8 % have been reported (Lee *et al.* 1956). For *Escherichia coli* a (G+C) % value of 50·0 was calculated from the T_m value.

Table 2. *Reproducibility of the chromatographic method for molar base composition determination*

(All the values given represent the average of three spots from one chromatogram. Methods: see text. Abbreviations: A = adenine; T = thymine; G = guanine; C = cytosine. As an evaluation of the method, the values A/T, G/C and A+G/T+C (which ought to be equal to 1), as well as A/G and T/C (which ought to be equal), are presented.)

Strain	A/T	G/C	$\frac{A+G}{T+C}$	A/G	T/C	$\frac{A+T}{G+C}$
Gluconobacter oxydans (*suboxydans* SU)						
Expt. 1	0·93	0·93	0·93	0·73	0·73	0·73
	0·96	0·93	0·94	0·75	0·73	0·74
	0·97	0·97	0·97	0·70	0·71	0·70
	0·97	0·96	0·96	0·72	0·71	0·72
Expt. 2	0·90	0·92	0·91	0·74	0·74	0·74
	0·96	0·98	0·97	0·70	0·71	0·70
Expt. 3	0·94	0·94	0·94	0·72	0·73	0·72
	0·98	0·96	0·97	0·70	0·71	0·70
	0·97	0·96	0·97	0·71	0·72	0·71
Acetobacter aceti (*liquefaciens* 20)						
Expt. 1	1·05	1·00	1·02	0·53	0·50	0·52
	1·05	1·02	1·03	0·53	0·51	0·52
	1·06	0·99	1·02	0·53	0·50	0·52
Expt. 2	1·06	1·05	1·06	0·56	0·56	0·56
	1·06	1·07	1·06	0·53	0·53	0·53
	1·05	1·01	1·03	0·55	0·53	0·54
	1·03	1·03	1·03	0·53	0·53	0·53
Pseudomonas fluorescens CCEB 488						
Expt. 1	0·93	1·08	1·02	0·65	0·75	0·70
	0·98	1·09	1·04	0·64	0·71	0·67
Expt. 2	1·01	1·12	1·07	0·65	0·72	0·68
	1·05	1·03	1·04	0·64	0·66	0·66
Expt. 3	1·03	0·95	0·98	0·72	0·67	0·69
	1·00	0·98	0·99	0·67	0·65	0·66

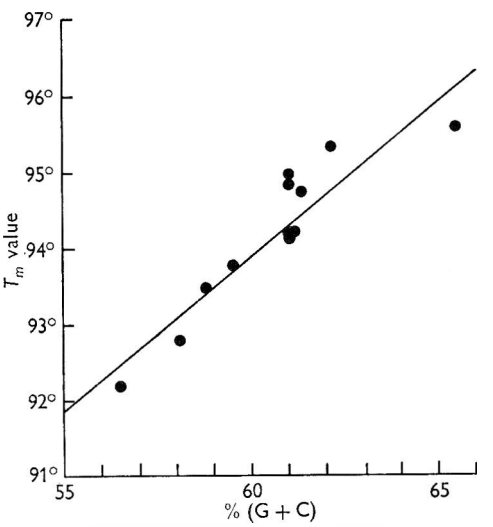

Fig. 2. Relationship between the 'melting point' T_m, determined by thermal denaturation, and the base composition % (G+C) determined by paper chromatography, of purified DNA from several acetic acid bacteria. The numbers are taken from Table 1. The curve was drawn from Marmur & Doty's formula $T_m = 0{\cdot}41$ (% G+C)+69·3.

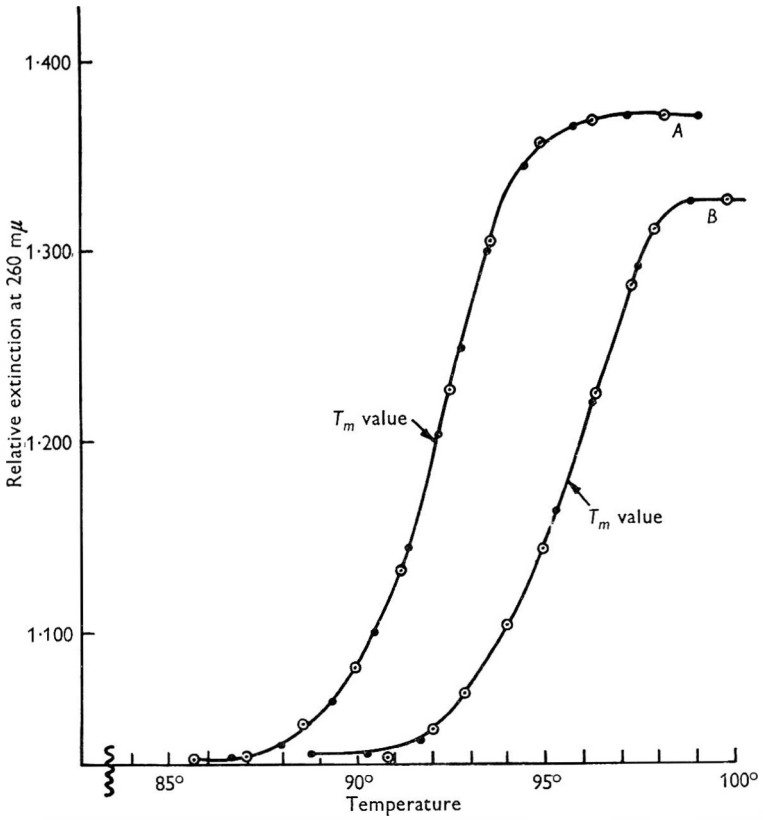

Fig. 3. Thermal denaturation curves of purified DNA of two strains of acetic acid bacteria. The examples were selected to represent the strains with the lowest T_m value (curve *A*, *Acetobacter aceti*, '*paradoxus*' P2) and with the highest T_m value (curve *B*, *A. aceti*, '*liquefaciens*' 20). All other strains of Acetobacter and Gluconobacter had T_m values within these limits. Each curve is the result of two different estimations. The results are expressed as relative extinction at 260 mμ, being the ratio extinction at temp. *t*/extinction at 25°.

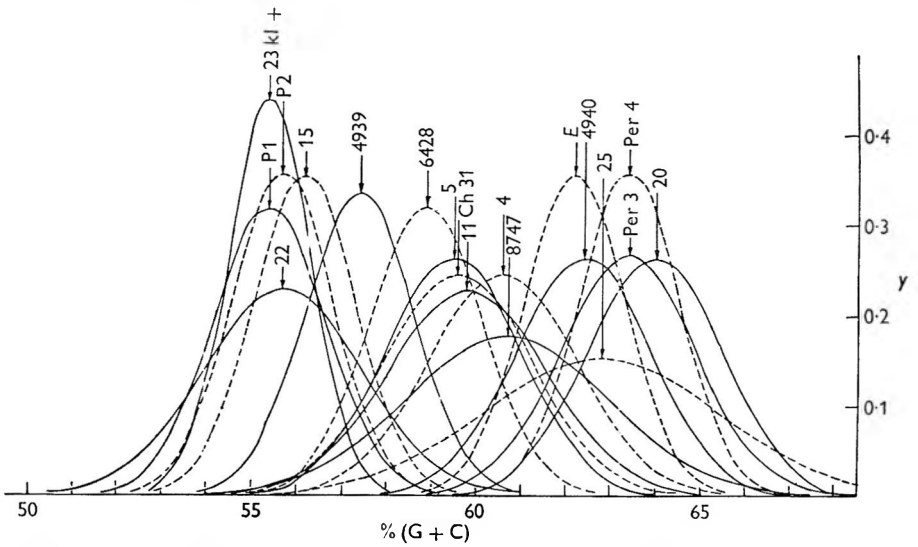

Fig. 4. Approximation of the compositional distribution of the DNA molecules of strains of Acetobacter. The symbols pointing to each curve are the strain numbers (see Table 1). The position of the arrows indicates the mean % (G+C). The curves were calculated as described in the text.

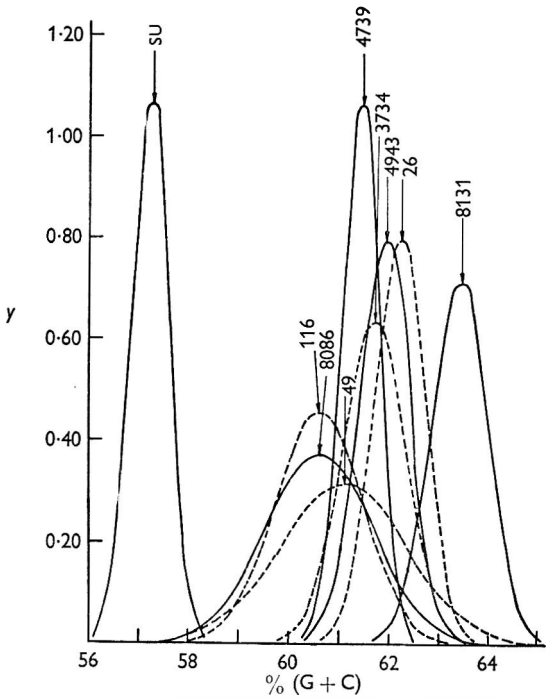

Fig. 5. Approximation of the compositional distribution of the DNA molecules of strains of Gluconobacter. The symbols pointing to each curve are the strain numbers (see Table 1). The position of the arrows indicates the mean % (G+C). The curves were calculated as described in the text.

DISCUSSION

All Acetobacter strains have base compositions of (G+C) between 55·4 % (strain 'paradoxus' P2, 'rancens' 23 kl+) and 64·0 % (strain 'liquefaciens' 20). Nearly all Gluconobacter strains lay in a narrow cluster from 60·6 % (strain 'melanogenus' 116) to 63·4 % (strain 'viscosus' 8131). Strain 'suboxydans' SU with 57·2 % was the only one to be somewhat removed from the central cluster of Gluconobacter (Table 1; Fig. 5). These data are in agreement with the conclusion of De Ley (1961*a*) that each biotype *Acetobacter aceti* and *Gluconobacter oxydans* can be regarded as a cluster of strains, without inner sharp breaks, thus making species differentiation unnecessary. This is also clearly illustrated in Figs. 4 and 5 by the continuously overlapping curves of the compositional distribution. Incidentally, the range of the distribution of DNA molecules of each strain is broader in Acetobacter (average σ 1·5) than in Gluconobacter (average σ for the melanogenus strains: 1·06; for the other strains: 0·5).

On the basis of physiological, biochemical and enzymic data De Ley (1961*a*) noted that the strains of the *Acetobacter aceti* biotype could be arranged in a smooth gradation from the most complex one to the one with the poorest enzymic equipment. A comparison of the sequence of Acetobacter strains of Table 1 with the sequence of Fig. 5 of De Ley's (1961*a*) paper shows a noticeable agreement: strains with greater biochemical activities have on the whole also a higher % (G+C). (De Ley & Schell, 1962, drew attention to the fact that the strain 'liquefaciens' 20 has to be removed

from Gluconobacter and belongs in the biotype Acetobacter, where it is the most complex representative.) In Gluconobacter the base compositions are so close together that they cannot be arranged in a sequence of statistical significance. In Table 1 and Figs. 4 and 5, some strains are not where they would be expected according to their position in the biochemical sequence. This holds for 'estunensis', 'pasteurianus' 11, 'suboxydans' SU and 'peroxydans' 3, 4 and 8618. However, it ought not to be expected that both sequences would agree completely. Indeed, the % (G+C) range encompasses the complete genotype and includes all properties, whereas the biochemical sequence was based mainly on carbohydrate metabolism. It might be that the strains 'estunensis', 'pasteurianus' 11 and 'suboxydans' SU possess or lack some properties which have so far escaped examination. The peroxydans strains were expected to have very low base compositions in the vicinity of 'paradoxus' and 'rancens' in view of their biochemical similarities with these strains. The base composition of the 'peroxydans' strains is unexpectedly high. Fig. 4 shows that the 'peroxydans' strains have in fact only very few DNA molecules in common with the 'paradoxus' and 'rancens' strains. A similar situation holds for strain 'suboxydans' SU, which is morphologically, physiologically and biochemically nearly indistinguishable from strain suboxydans 26, but nevertheless appears to have very few DNA molecules in common with it. Further work on the finer details of the DNA molecules in relation to the taxonomic position of these strains will be required.

As expected, Gluconobacter and Acetobacter strains have DNA with base compositions in the same range. These results support the hypothesis (De Ley, 1961*a*) which proposes that Gluconobacter and Acetobacter may have originated from a common pool of ancestors.

The DNA base compositions of the acetic acid bacteria are in the same range as those for Pseudomonas, which extend from 60 to 67 % (G+C) (Lee *et al.* 1956; Marmur & Doty, 1962). This stresses once more the suspected close relationship between the pseudomonads and the acetic acid bacteria. Phenotypically they have several features in common (Stanier, 1947; for a review see De Ley, 1961*b*). The similarity in the base compositions of the DNA molecules of both groups of bacteria also points to a possible genotypic relationship. It seems not unlikely that these bacteria derive from a common phylogenetic origin. The comparison between the methods of paper chromatography and thermal denaturation for the estimation of DNA base compositions for routine analysis showed that the latter procedure was to be preferred; it is easier, faster, less elaborate and yields more reproducible results.

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The Biosynthesis by Pneumococcus of a Non-Reducing Disaccharide from Uridine Diphosphoglucose

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SUMMARY

A particulate enzyme fraction from type VIII Pneumococcus obtained by centrifugation of disrupted organisms between 30,000 and 140,000*g* synthesized a non-reducing disaccharide from uridine diphosphoglucose (UDPG). The structure proposed for the material synthesized is glucopyranosyl-glucopyranoside, in which at least one of the linkages is of the β configuration. The reaction may be formulated as: UDPG + UDPG → glucopyranosyl-glucopyranoside. The reaction was not type-specific and was also found with particulate fractions from pneumococci of types II and III.

INTRODUCTION

The capsular polysaccharide of type III pneumococci is a polymer of cellobiuronic acid (β-1,4-glueuronosido-glucose); the biosynthesis of this polysaccharide from uridine diphosphoglucose (UDPG) and uridine diphosphoglucuronic acid (UDPGA) is well defined (Smith, Mills, Bernheimer & Austrian, 1960; Smith, Mills & Bernheimer, 1961). The mechanisms involved in the biosynthesis of the capsular polysaccharides of pneumococci of types I and VIII are being studied (Smith, Galloway & Mills, 1961; Mills & Smith, 1962); these investigations include the isolation and identification of small oligosaccharide molecules formed from uridine diphosphoglycosyl substrates. The first of these reactions, described here, is the biosynthesis of a non-reducing disaccharide from UDPG.

METHODS

Organism used. This was *Streptococcus pneumoniae* type VIII. The organism was grown in 1 l. lots of Difco brain + heart infusion medium (3·7 %, w/v; pH 7·4) containing 0·1 % neopeptone. After incubation of the culture for 16 hr., glucose was added to 1 % (w/v); the acid formed on subsequent incubation was neutralized with 3 N-NaOH. Incubation was at 36·5° and the organisms were collected by centrifugation at 20,000*g* for 20 min. at 3°, when 70 % of the added glucose had disappeared.

The particulate enzyme fraction. This was prepared as follows. The organisms harvested from 1 l. medium were suspended in 10 ml. 0·1 M-phosphate buffer (pH 6·5) and potassium thioglycollate added to 0·01 M. An equal volume of Ballotini No. 12 glass beads and two drops of *n*-octanol were added to the suspension and the organisms disrupted by shaking for 20 min. in a Mickle.

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disintegrator at 3°. The suspension, freed from Ballotini beads, was centrifuged at 30,000*g* for 15 min. and the supernatant fluid centrifuged at 140,000*g* for 1 hr. in the Spinco Model L preparative ultracentrifuge. The particulate material from the latter centrifugation was suspended in 2 ml. of 0·1 M-tris buffer (2-amino-2-hydroxymethylpropane-1,3-diol; pH 8·35) containing 0·01 M-potassium thioglycollate; this constituted the enzyme fraction.

UDPG labelled with ^{14}C in the glucose moiety was prepared as described by Smith *et al.* (1960). The specific activity of the UDPG was $1{\cdot}4 \times 10^5$ c.p.m./μmole.

Radioactivity of the samples. This was determined at infinite thinness in a microthin window gas-flow counter operated in the proportional region. Radioactivity on chromatograms was located with an automatic windowless gas-flow paper chromatogram scanner used in the Geiger region (Atomic Accessories Inc., Valley Stream, New York, U.S.A.).

Isolation of disaccharide. This was done as follows. After the specified incubation period the reaction mixture was heated in a boiling water bath for 2 min., cooled rapidly in ice, centrifuged, and the precipitate discarded. One g. Ultrasorb S.C. 120/240 (British Carbo Norit Union Ltd., West Thurrock, Grays, Essex) + 1 g. Hyflo Super-Cel (Johns Manville, New York City) were suspended together in 20 ml. 0·01 N-formic acid and the suspension poured into a chromatography column of 0·6 cm. diameter. The supernatant fluid from the reaction mixture was diluted with 3 vol. 0·01 N-formic acid and applied to the suitably packed charcoal-Super-Cel column which was then washed with 20 ml. 0·01 N-formic acid. The advisability of maintaining acid conditions during charcoal fractionation of sugars was described by Taylor & Whelan (1962). The sugars were fractionally eluted from the column with 100 ml. volumes of 5 % (v/v) ethanol in 0·01 N-formic acid, 15 % (v/v) ethanol in 0·01 N-formic acid and 5 % (v/v) pyridine in water, respectively. Each fraction was dried *in vacuo* and for analysis dissolved in 1 ml. distilled water.

Chromatography of sugars. This was done on Whatman no. 1 paper in: ethanol + ammonium acetate (pH 7·5; Paladini & Leloir, 1952); 2-butanol + acetic acid + acetone + water (Zilliken, Braun & György, 1955); *n*-butanol + ethanol + water + ammonia (Foster, Horton & Stacey, 1957); *n*-butanol + acetic acid + water (Partridge, 1948) at 44 + 16 + 40, by vol. The permanganate + periodate spray of Lemieux & Bauer (1954) was used for locating sugar spots.

Reducing sugars were estimated by the micromethod of Park & Johnson (1949).

Phosphorus micro-estimations were done by the method of Griswold, Humoller & McIntyre (1951).

β-Glucosidase was obtained from Sigma Chemical Co., St Louis, Missouri, U.S.A.

α-Glucosidase was prepared from baker's yeast by the method of Halvorson & Ellias (1958); the preparation so obtained was free from *β*-glucosidase activity.

RESULTS

Charcoal + Celite elution pattern

A reaction mixture consisting of: 9 μmoles ^{14}C-labelled UDPG, 1·5 μmole diphosphopyridine nucleotide (DPN), 45 μmoles $MgCl_2$, 2 ml. particulate suspension, 0·1 M-tris buffer (pH 8·35) to final volume 9 ml., was incubated at 32° for 60 min., and after heat inactivation was applied to a charcoal + Celite column. A typical

elution pattern is shown in Fig. 1. A small amount of radioactive material (0·2 % of total) was eluted with 5 % (v/v) ethanol in water and was shown to be glucose by paper chromatography. Thirty-five % of the total radioactivity was located in the 15 % (v/v) ethanol-in-water eluate (fraction A); the remaining activity was located in the 5 % (v/v) pyridine-in-water eluate (fraction B).

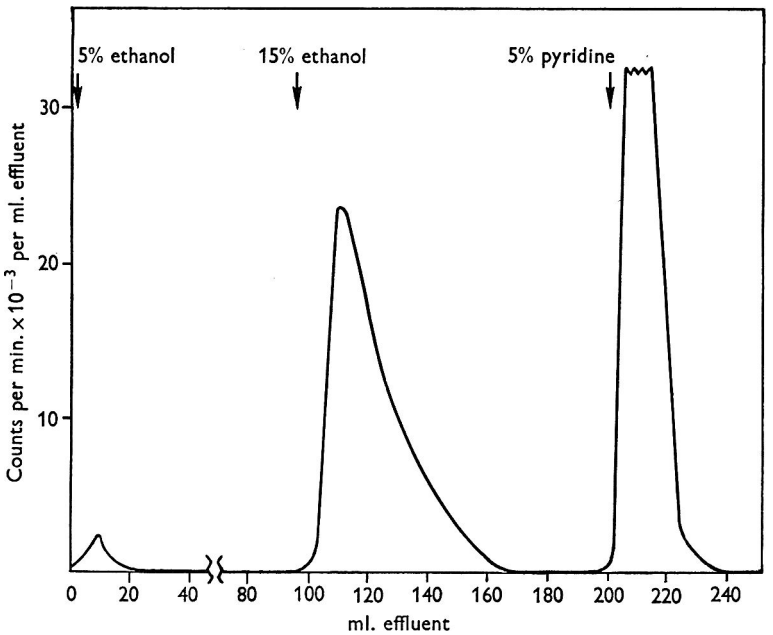

Fig. 1. Charcoal-Celite column elution diagram of radioactive products from the action of the particulate enzyme fraction on ^{14}C-UDPG. The aqueous alcohol used for elution contained 0·01 N-formic acid.

Analysis of fraction A

Paper chromatography of samples of fraction A in the various solvents gave a single radioactive component with the $R_{glucose}$ values shown in Table 1. The radioactive spot was insensitive to spray reagents for reducing sugars but was located with the permanganate + periodate spray (Lemieux & Bauer, 1954). The labelled material was hydrolysed with N-H_2SO_4 for 1 hr. at 100°, neutralized with an excess solid $BaCO_3$, the $BaSO_4$ removed by centrifugation and washed twice with 1 ml. distilled water and the combined supernatant fluids and washings were dried under reduced pressure. The hydrolysate was chromatographed on paper in the solvents described above; in all cases they showed glucose to be the sole component. The micromethod of Park & Johnson (1949) for the estimation of reducing sugars gave zero value on the unhydrolysed material. The liberation of reducing sugar after hydrolysis with N-H_2SO_4 is shown in Table 2. The labelled material was free from phosphorus as shown by the phosphorus micro-determination. No ultraviolet-absorbing material was detected in the labelled compound. That the unhydrolysed material showed no reducing power, its position in paper chromatographic and charcoal column analyses, and the liberation of glucose as sole product of acid hydrolysis, suggest that the compound is a glucopyranosyl-glucopyranoside.

Nature of the disaccharide linkage

The nature of the linkage was partially established by hydrolysis with β-glucosidase; 250 μg. of labelled disaccharide were incubated at 37° for 30 min. with 1 mg. β-glucosidase and 0·02 M-tris buffer (pH 7·2) to final volume 1 ml. The reaction mixture was heated in a boiling water bath for 2 min, centrifuged, and the supernatant fluid concentrated *in vacuo*. Chromatography of the reaction products in ethanol + ammonium acetate (Paladini & Leloir, 1952), followed by a scan of the

Table 1. $R_{glucose}$ *values of isolated disaccharide in various paper chromatographic systems*

(Values for authentic disaccharides are included for comparison.)

	Solvents			
Sugar	Ethanol + ammonium acetate	Butanol + acetone + acetic acid + water	Butanol + acetic acid + water	Butanol + ethanol + water + NH_4OH
	$R_{glucose}$ values			
Disaccharide	0·83	0·66	0·74	0·32
Lactose	0·87	0·55	0·61	0·32
Maltose	0·91	0·66	0·71	0·46
Cellobiose	0·91	0·61	0·71	0·43
Gentiobiose	0·90	0·53	0·72	0·30
Trehalose	0·98	0·69	0·89	0·50

Table 2. *The liberation of reducing material from the disaccharide on hydrolysis with* N-H_2SO_4

(The theoretical total glucose content of 200 μg. was calculated from the radioactivity of the disaccharide and the specific activity of the UDPG used ($1·4 \times 10^5$ c.p.m./μmole).)

Time of hydrolysis (min.)	Reducing material as glucose (μg.)
0	0
10	49
20	101
30	150
60	200
90	200
120	215
Theoretical for disaccharide:	200

chromatogram with the windowless gas-flow scanner, showed the presence of glucose, unchanged disaccharide and a third component with an $R_{glucose}$ value of 0·35 (Fig. 2). This latter component was eluted from the chromatogram with water and had a reducing value equivalent to 15 % of the total glucose liberated by hydrolysis with N-H_2SO_4. α-Glucosidase had no effect on the disaccharide when incubated with it under the optimal conditions specified by Halvorson & Ellias (1958). The particulate enzyme fraction did not produce the disaccharide described when incubated with UDPG + ^{14}C-glucose, UDPG + ^{14}C-glucose-1-phosphate, ^{14}C-glucose alone or with free ^{14}C-glucose-1-phosphate alone.

Analysis of Fraction B

Paper chromatography of fraction B in the ethanol + ammonium acetate solvent of Paladini & Leloir (1952) showed a single ultraviolet-absorbing component with an R_F value identical with that of UDPG; all the radioactivity was associated with this one component. Hydrolysis of this material in 0·01 N-HCl as described by Paladini & Leloir (1952), and chromatography of the resultant sugars in the solvents listed above, showed the presence of both glucose and galactose. A sample of the hydrolysed material was chromatographed in *n*-butanol + pyridine + water for four successive periods of 16 hr. each, the chromatogram being dried between each run. Complete separation of glucose and galactose was achieved by this means and the ratio of glucose:galactose was 1:4 in terms of radioactivity.

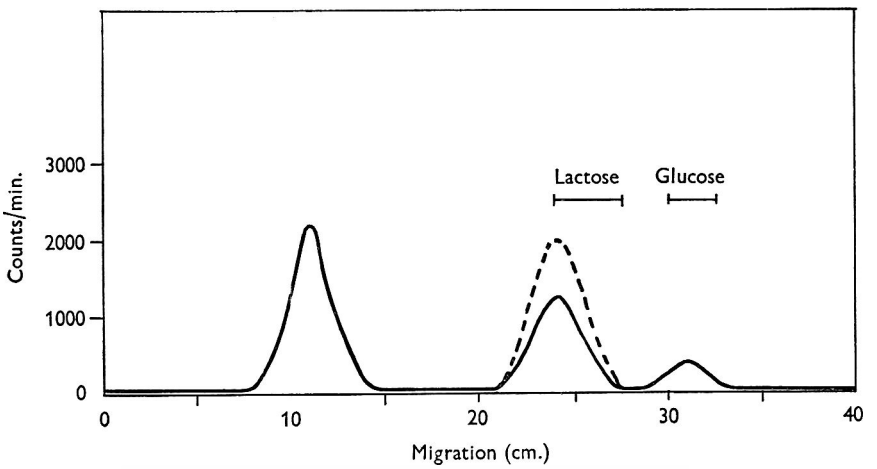

Fig. 2. Scan of radioactivity on chromatogram of the products of β-glucosidase action on the disaccharide. The glucose and lactose markers are indicated by bars. The broken curve is from the control reaction of the disaccharide without β-glucosidase.

DISCUSSION

The structure proposed for the material synthesized by *Streptococcus pneumoniae* type VIII in the reaction described is glucopyranosyl-glucopyranoside, in which at least one of the linkages is of the β configuration. The reaction involved in its formation may be formulated as follows:

UDPG + UDPG → glucopyranosyl-glucopyranoside
⇅
UDPGalactose

The disaccharide nature of the molecule may be deduced from the following observations: (1) The material is eluted from a charcoal-Celite column with 15% (v/v) ethanol in water under acid conditions (Taylor & Whelan, 1962). (2) The location of the compound on paper chromatography is that of a disaccharide unit, with R_F values closely approximating those of known disaccharides in most solvent systems. French & Wild (1953) showed that the number of monosaccharide units in a series of oligosaccharides determines their chromatographic position and that a

plot of log $R_F/(1-R_F)$ against the degree of polymerization gives a straight line. The non-reducing nature of the molecule, the liberation of glucose on acid hydrolysis, and the enzymatic cleavage with β-glucosidase, would suggest a C1 to C1 linkage within the molecule, where at least one of the glucosyl residues is linked in the β-configuration.

The component with an $R_{glucose}$ value of 0·35 in ethanol+ammonium acetate, which was produced on incubation with β-glucosidase, may be a higher reducing oligosaccharide obtained by transglycosylation. The transient formation of glucose oligosaccharides during the hydrolysis of cellobiose and other β-glucosides was shown by Crook & Stone (1957). Although the disaccharide is not attacked by α-glucosidase, it cannot be inferred that both linkages are thus of the β configuration. Halvorson & Ellias (1958) showed that α-glucosidase does not affect trehalose (α-1-glucopyranosyl-α-1-glucopyranoside).

Synthesis of the disaccharide is specific for UDPG, as UDPG in combination with glucose or glucose-1-phosphate will not produce the same material. Other disaccharides and oligosaccharides are, however, produced by such reactions, the natures of which are currently under investigation. Neither free glucose nor glucose-1-phosphate, single or in combination, will produce the disaccharide.

The conversion of UDPG to UDPGalactose by the particulate enzyme fraction is of interest as a demonstration of UDPGalactose-4-epimerase activity associated with particulate material. It should be noted that the equilibrium is in favour of UDPGalactose production as distinct from the reactions discussed by Kalckar & Maxwell (1958). The biosynthesis of the disaccharide is not specific for any one type of pneumococcus and has also been achieved with particulate material from pneumococci of types II and III. It is suggested that the reaction may be involved in the formation of pneumococcal cell-wall polysaccharide as distinct from the cellular or somatic polysaccharide, which does not contain glucose as a constituent sugar (Smith, Mills, Harper & Galloway, 1957).

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The Classification of 'Bacterium salmonicida'

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SUMMARY

Forty-two strains of 'Bacterium salmonicida', six of a non-pigmented fish pathogen and forty-two Aeromonas strains were compared morphologically, culturally, biochemically and metabolically. The results, which were computed electronically, showed a distinct difference between 'B. salmonicida' and the Aeromonas species. On the grounds of this variation in morphology, culture and biochemistry, it is suggested that 'B. salmonicida' be removed from the genus *Aeromonas* and given a generic place in the family Pseudomonadaceae. The name *Necromonas salmonicida* is suggested as an alternative to 'B. salmonicida'.

INTRODUCTION

The causal organism of furunculosis of the Salmonidae was originally called 'Bacterium salmonicida' (Lehmann & Neumann, 1896), though Marsh (1902) later cited it as 'B. trutta'. Since its systematic position is in doubt, the former name will be used here, though it is an illegitimate name. Recently, however, some workers, including Griffin, Snieszko & Friddle (1953*a*), Eddy (1960, 1962), Ewing, Hugh & Johnson (1961), and Schubert (1961), have classified the organism as one of the genus *Aeromonas*. The strains of this organism isolated at the Marine Laboratory, Aberdeen, did not fall into the genus *Aeromonas* when classified according to Skerman (1957) because of weak gas production from glucose. Several strains of this organism have now been collected and examined to determine whether they belong to the same species and are in fact members of the genus *Aeromonas*. The organisms were compared with another fish pathogen and some organisms belonging to the genus *Aeromonas*.

METHODS

Strains. The cultures of 'Bacterium salmonicida' and other organisms were obtained from the sources shown in Table 1. All the cultures were incubated at 22° unless otherwise stated, and all the results were replicated. One loopful from a 2-day nutrient broth culture was used as the inoculum.

Morphology. Two-day and 7-day cultures in nutrient broth and on blood agar were examined by phase-contrast microscopy for size, shape, granulation and configuration, and the same cultures were examined after staining by Gram's method. Motilities were examined in 2-day peptone water cultures and preparations from 2-day nutrient agar slopes stained by Kirkpatrick's method were examined for the presence of flagella.

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Cultural characteristics. The growth on Yeastrel agar of the following composition: Oxoid Lab-lemco, 5 g.; Yeastrel, 7 g.; Difco peptone, 9·5 g.; NaCl, 5 g.; Davis agar, 30 g.; distilled water, 1000 ml.; pH 7·0 and that in nutrient broth was recorded at 2 and 7 days. All the cultures were inoculated into peptone water and examined for flocculation after incubation for 7 days at 5°.

Table 1. *The sources of the organisms used in this survey*

No.	Source	Date	Donor
0–9	*Salmo salar* and *S. trutta*. Scottish waters	1960	—
10	*S. salar*, R. Itchen	1960	Dr M. H. Hughes, P.H.L., Winchester
11	*S. salar*, Bournemouth	1960	Dr G. J. G. King, P.H.L., Bournemouth
12–18	*S. salar* and *S. trutta*, Exeter area	1960	Dr W. J. Ryan, P.H.L., Exeter
19	*S. salar*, R. Wye	1954	NCTC 9378
20–25	*S. salar*, Welsh rivers	1959 1960	The Director, P.H.L., Carmarthen
26–27	*Coregonus pollan*, Lough Neagh	1960	K. U. Vickers, Min. Commerce, Belfast
28–29	Bonashamm	1959	Dr O. Ljungberg, Stockholm
30–31	*Esox lucius*, Minnesota	1959	Dr P. Economon, Minnesota
32–33	*Oncorhynchus kisutch* and *O. tschawytscha*, U.S.A.	1960	Dr J. W. Wood, Seattle
34–40	*Salmo* and *Oncorhynchus* spp. U.S.A. NCMB 833–839. CDC RH 39, 40, 67–69, 72, 78	—	Dr J. M. Shewan, Aberdeen
41	American strain E.B. 382	—	Dr O. Lysenko, Czeckoslovakia
42–75	*S. salar*, *S. trutta* and *Perca perca*, Scottish waters	1959 1960	—
76–81	*S. trutta*, Scottish waters	1961	—
82–86	Human and animal sources (Ewing *et al.* 1961), NCIB 9233, 9237, 9238, 9239, 9240	—	Dr J. M. Shewan, Aberdeen
87–90	Human sources (Caselitz & Günther 1960), NCIB 9244, 9245, 9248, 9249	—	Dr J. M. Shewan, Aberdeen

Carbohydrate utilization in peptone medium. The fermentation of carbohydrates was examined in 1% Difco peptone water with Andrade's indicator. The media were sterilized by Tyndallization and the gas production recorded as positive when more than one-tenth of the liquid in a Durham tube was displaced by gas. For a comparison of gas production in the presence of different peptones and from glucose sterilized by filtration and in buffered peptone, standard 50 mm. Durham tubes were used. The displacement of gas was measured to the nearest 0·5 mm. and the volumes expressed as a percentage. Hugh & Leifson's medium (1953) was used to determine the method of carbohydrate metabolism. The lactose sugars were read at 7 and 40 days; the remainder of the sugar tests were read only at 7 days.

The following tests were carried out as described by *Mackie & McCartney* (1960) unless otherwise stated.

Methyl red and Voges-Proskauer tests were performed in 7-day glucose phosphate peptone water cultures. Acetylmethylcarbinol was detected by Barritt's modification.

Indole production was tested by adding Ehrlich's rosindol reagent to 7-day peptone water cultures.

Nitrate reduction. After incubation for 7 days, Griess-Ilosvay reagents were added to nitrate broth cultures (*Topley & Wilson's Principles*, 1946).

Hydrogen sulphide production was tested with lead acetate strips and ZoBell & Feltham agar (1934).

Urease activity was studied in 7-day cultures in Christensen's agar (Christensen, 1946).

Ammonia production from peptone. Nessler's reagent was added to 2-day peptone water cultures (*Topley & Wilson's Principles*, 1946).

Catalase activity was tested by emulsifying a loopful of 2-day nutrient agar culture in 20 vol. hydrogen peroxide (Sneath, 1956).

Oxidase activity was tested by Kovacs method (1956).

Haemolysis and caseinolysin were tested on Eddy's medium (1960).

Liquefaction of coagulated serum was tested on Loeffler's medium after 14 days.

Liquefaction of gelatin was recorded after 7 days in nutrient gelatin.

Litmus milk was examined at daily intervals up to 14 days for acid production, clotting, proteolysis and reduction of litmus.

Aesculin hydrolysis was tested on the medium of Sneath (1956) after 4 days.

Phosphatase activity was tested on Barber & Kuper's medium (1951) after 2 days.

Starch hydrolysis was tested on nutrient agar containing 0·4% starch by flooding the plate with Lugol's iodine after incubation for 1 day.

Lecithinase production in egg-yolk medium was examined on Esselman & Liu's medium (1961) at 14 days.

Presumptive test for 'Bacterium salmonicida'. Griffin, Snieszko & Friddle (1953*b*) cultured organisms on a Trypticase-Yeastrel agar for 18 hr. On flooding the growth with *p*-phenylenediamine the development of a black coloration was taken as indicative of the presence of 'B. salmonicida'. The test was carried out as described by the authors above.

Growth in citrate was recorded after 7 days in Koser's medium.

Gluconate oxidation was tested by the method of Sneath (1956).

Production of 2,3-butanediol was determined after 1, 3 and 7 days' growth in glucose medium by Bullock's method (1961).

Phenylalanine and malonate tests were done by the method of Shaw & Clarke (1955).

Cellulase activity was estimated by the digestion of filter-paper strips in half-strength peptone water after 7 days (Skerman, 1957, see p. 1019).

Sensitivity to antibiotics. Evans Senstests were applied to the seeded surface of nutrient agar plates and the results read after incubation for 1 day. The vibriostatic agent, 0-129 (Shewan, Hodgkiss & Liston, 1954) was incorporated in filter-paper discs and applied to the agar and read as above.

Growth at different temperatures was examined on solid media and the results recorded after 1 day at 22° and 37° and after 7 days at 5°.

Growth in various salt concentrations was examined in peptone water with 1, 3 and 8% NaCl after 7 days' incubation.

Computation of the results. The results were tabulated, omitting the thirty features which were identical for all the strains examined. One hundred and eight features were treated as alternatives and three as quantitative ones. The statistical relationship was calculated by Mr J. C. Gower, who used the Rothamsted computer programme (Elliott 401 Computer programme F 39, Rothamsted Experimental Station,

1960). The formula for the similarity S_{ij} of two strains i and j is expressed as follows.

$$S_{ij} = \frac{t_{ij}}{n_{ij}} \times 100,$$

where t_{ij} is the total number of positive and negative matches and n_{ij} is the total number of features compared for the two strains i and j.

RESULTS

When the results were computed, it was found that they split into two definite groups at $S = 75$ (Mr Gower, personal communication). These consisted of (*a*) organisms 0–41 and 76–81, and (*b*) 42–75 and 82–90; i.e. the pigmented and non-pigmented 'Bacterium salmonicida' formed group a and the Aeromonas strains group b. Increasing the S value divided the organisms further until, at $S = 90$, the five groups shown in Fig. 1 emerged. As can be seen in Fig. 1 the strains have been rearranged and from now on the groups will not be expressed in numerical sequence, but in the rearranged order found in Fig. 1. Group I, 77–79, corresponded to the non-pigmented fish pathogen recently described and thought to be an achromogenic variant of 'B. salmonicida' (Smith, to be published); group II, 0–40, to 'B. salmonicida' and groups III, 48, 71; IV, 83–86; and V, 66–65, to the Aeromonas strains. The mean similarities of each group of organisms were calculated and these are shown in Table 2. It is evident that each group has a high similarity within itself (except in the case of group V). Groups I and II have a similarity to each other of 76%, while only one of 47·5–64% to the other three groups. Likewise the latter three groups have a similarity of almost 70% to each other.

Table 2. *The mean similarities of the groups*

	Group I	Group II	Group III	Group IV	Group V
Nos.	77–79	0–40	48, 71	83–86	66–65
Group I	95·6	—	—	—	—
Group II	75·6	92·1	—	—	—
Group III	47·5	51·3	94·3	—	—
Group IV	55·1	64·0	76·0	90·6	—
Group V	59·0	63·0	68·9	77·9	77·1

Table 3. *The mean similarities of the subgroups of Group IV*

	Group IVa	Group IVb	Group IVc	Group IVd
Nos.	83–87	49–84	47–90	43–86
Group IVa	97·25	—	—	—
Group IVb	88·35	93·96	—	—
Group IVc	86·90	89·22	90·33	—
Group IVd	80·80	81·83	81·59	85·23

The intra-group similarities for each member of groups I and II were calculated and they were found to lie between 93·5 and 95·8% in group I and 87·2 and 94·6% in group II. In group II all but 7 of the 42 strains had a mean similarity of 90% or over.

Groups I and II do not resolve further but group IV can be subdivided into 4 groups as shown in Table 3. Group IVa consisted of 3 NCIB cultures but none of the other groups showed any homogeneity with regard to source or pathogenicity.

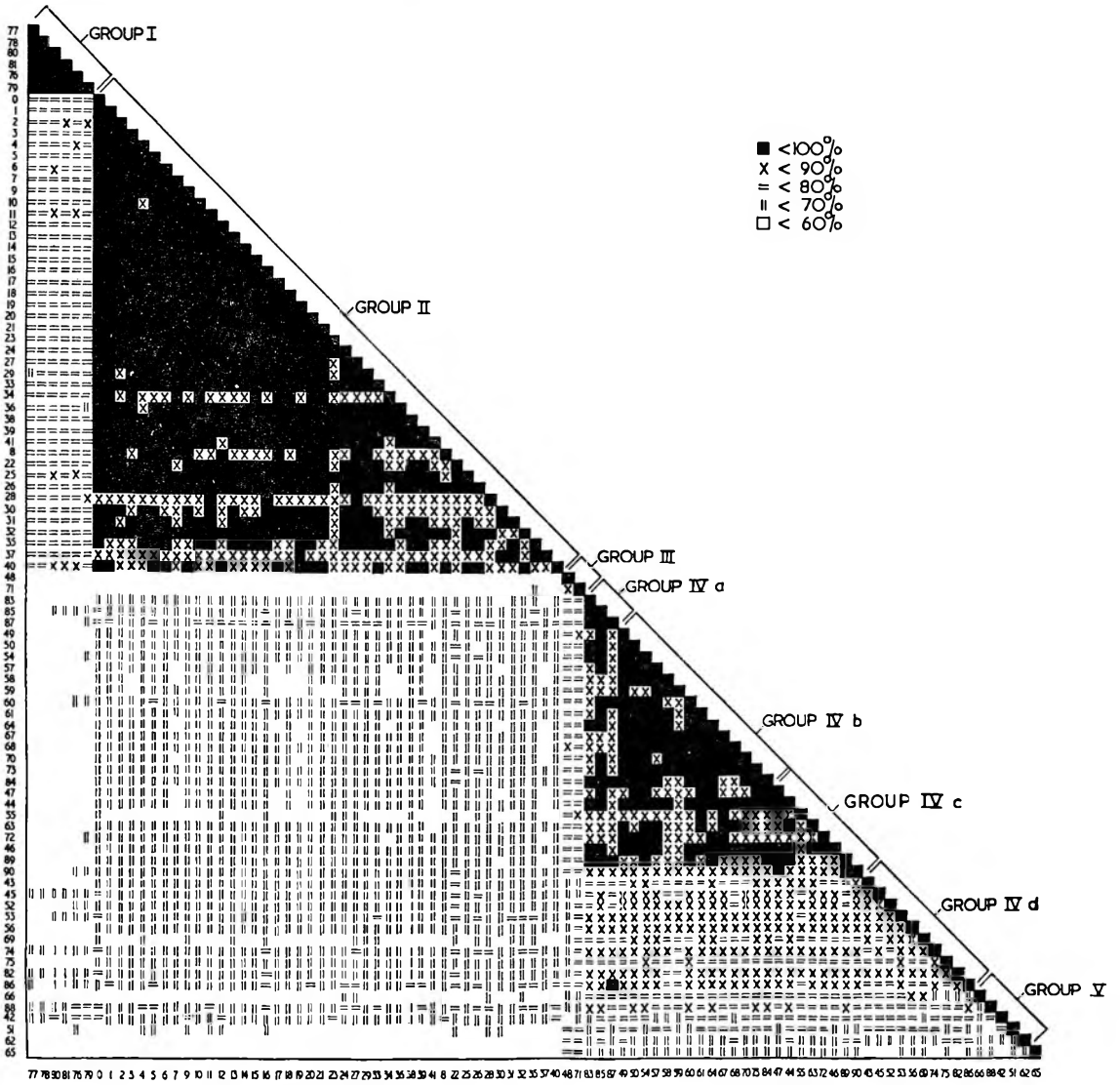

Fig. 1. Diagram of the relationship of the 91 strains used in the survey.

Table 4. *The average percentage of liquid displaced by gas in Durham tube*

Peptone Sugar	Difco T.G.	Oxoid T.G.	Evans T.G.	Difco T.M.	Difco F.G.	Difco B.G.
'B. salmonicida'	7·6 (78)	7·0 (80)	10·8 (79)	13·6 (78)	4·6 (78)	0·1 (78)
Aeromonas	19·0 (6)	21·0 (6)	61·6 (6)	43·6 (6)	9·8 (5)	10·4 (6)

T.G., Tyndallized glucose; T.M., Tyndallized mannitol; F.G., Filtered glucose; B.G., buffered glucose. Numbers in parentheses denote the number of tests.

The gas production of 'Bacterium salmonicida' in glucose and mannitol was compared with that of the group IV Aeromonas strains. The results in Table 4 show that, in Difco peptone, 'B. salmonicida' produced less than 10 % gas when the glucose medium was Tyndallized and even less when the glucose was Seitz-filtered or the medium buffered with 1 % phosphate. Oxoid peptone gave much the same results but with Evans peptone the gas production was 10·8 % with glucose. With the Aeromonas strains, the gas production was greater than 10 % in all cases, except with filtered glucose when the amount was 9·8 %. When the glucose was replaced by mannitol in the Difco peptone medium, 'B. salmonicida' produced a liquid displacement of 13·6 %.

The results of the morphological, cultural, biochemical and metabolic tests are shown in Table 5. In addition to those shown, all the organisms produced acid from mannitol, maltose and fructose, but had no action on dulcitol and inulin; they were fermentative in Hugh & Leifson's medium; methyl red negative; produced ammonia from peptone water; did not attack cellulose; grew in 1 % but not in 8 % salt (except for slight growth of strain 54); were resistant to penicillin, bacitracin, novobiocin, oleandomycin and 0-129; were sensitive to streptomycin and chloramphenicol and they all grew at 22°.

DISCUSSION

The non-pigmented group (I) is very homogeneous (93·5–95·8 % similarity) and this is not surprising since all the strains came from the same source. Another set of cultures has now been examined and found to have the same reactions as those recorded here but the results could not be included in the computer analysis.

The next group (II) 'Bacterium salmonicida' is also extremely homogeneou s as, with the exception of 7 strains, the mean similarities lie between 90 and 94·6 %. This is particularly striking because the organisms were isolated from fish from Scotland, England, Ireland, Wales, Sweden and the United States, and from *Salmo salar*, *S. trutta*, *S. gairdnerii*, *Coregonus pollan*, *Crassius auratus*, *Esox lucius*, *Oncorhynchus kisutch* and *O. tschawytscha*. Further, the age of the culture does not appear to influence its properties as the NCTC culture had been isolated in 1954, whereas the others were mainly isolated in 1959 and 1960. The 7 exceptional strains, which have mean similarities of 87–90 %, are not from any one source or species of fish, so that the strains of 'B. salmonicida' examined here cannot be separated into subgroups on the basis of source of specimen.

The Aeromonas strains were much more heterogeneous and can be divided into no less than 6 subgroups. These have, within themselves, quite a close similarity but these groups were not found to correlate with pathogenicity of the organisms or with the source of the specimen.

From the tables of results used in the computer analysis, it was possible to choose a typical strain for a group, i.e. a strain which has all the majority characteristics of the group. In the case of 'Bacterium salmonicida', one strain, 20, was found to have all the majority features so it is suggested that it be used as the neotype. It conforms to the description of 'B. salmonicida' given by Lehmann & Neumann (1896) in that it is a non-motile, short rod, incapable of growth at 37°, able to produce a brown pigment in agar and liquefy gelatin, to grow on the surface and in the

depth of solid media and to produce disease in salmonid fish. Strain 20 would therefore appear to be equivalent to the original 'B. salmonicida' strain.

Ewing *et al.* (1961) suggested ATCC 14174 as the neotype of the species and it was examined in this series where it was designated strain 34. The strain was found to have a mean similarity of 89·4% as compared with 94·52% for strain 20 and so did not have all the majority features of the group. In particular, strain 34 was found to be resistant to aureomycin, terramycin and tetracycline—a feature not recorded by Ewing *et al.* (1961). This resistance to the above antibiotics was only found with one other strain (36), and it suggests that these two strains had been isolated from a stock of fish which had been previously treated with antibiotics. Such strains would therefore be mutants rather than wild types so, for this reason, strain 34 was thought to be an unfortunate choice as the neotype.

Strain 20 along with strains of high (92·26) and low (87·2) similarities have been lodged with the National Collection of Marine Bacteria at Torry Research Station, Aberdeen, where they have been given the numbers NCMB 1102, 1103 and 1104 respectively. (1103 is strain 2 and 1104 strain 28 of this series.)

When undisturbed cultures of newly isolated strains of 'Bacterium salmonicida' are examined in broth, the growth is found to resemble that of the streptococci in that the liquid is clear and flocculi are adhering to the sides of the test-tube. This macroscopic appearance is not so evident after subculture but it can be reproduced in laboratory cultures by incubation at 5°. Lehmann & Neumann (1896) also record the similarity of 'B. salmonicida' to the streptococci in its growth in broth, which remains clear except for a delicate growth on the walls of the test-tube near the surface of the liquid. Microscopic examination of liquid cultures of 'B. salmonicida' reveals a characteristic appearance. The non-motile cocco-bacilli are found to be in short chains and clumps composed of these chains. This appearance is in complete contrast to that of the motile Aeromonas strains where single or pairs of organisms are scattered at random over the field.

Culturally, 'Bacterium salmonicida' differs from the Aeromonas strains in its production of a brown water soluble pigment and in the friable nature of its colonies. This latter friability is stable over a large number of subcultures.

In previous descriptions of 'Bacterium salmonicida' or *Aeromonas salmonicida*, the organism has been described as producing very little gas from glucose (Arkwright, 1912) or as a gas producer (Griffin *et al.* 1953*a*; Eddy, 1960, 1962; Ewing *et al.* 1961; Schubert, 1961). All the hundreds of strains of this organism isolated at the Marine Laboratory in the 9-year period 1953–62 produced very little or no gas from glucose but gave good gas production from mannitol. As gas production from glucose is a characteric feature of the aeromonads, the amount of gas present in the Durham tube was measured. Table 4 shows that 'B. salmonicida' produced a displacement of more than 10% only when Evans peptone was used in the medium. When the aeromonads are considered, it is noted that the greatest liquid displacement also occurred in the presence of Evans peptone but in this case the amount was 60%. This represented a threefold increase in the amount of gas produced in the presence of Difco or Oxoid peptones. The corresponding increase for 'B. salmonicida' was about 0·5 so it would appear that 'B. salmonicida' is not a vigorous gas producer. Buffered glucose was used to ascertain whether the lack of gas production was due to a decrease in the pH value of the medium inhibiting the fermentation process but

Table 5. *The results of the morphological, cultural, biochemical and metabolic observations*

	Non-pigmented group	'Bacterium salmonicida'	Aeromonas strains
Morphology			
On blood agar	Even-staining, round ended, Gram-negative cocco-bacilli, length less than twice width. Granular in phase-contrast preparations	Even-staining, round ended, Gram-negative cocco-bacilli, length less than twice width. Granular in phase-contrast preparations	Even-staining, round ended, Gram-negative bacilli, length greater than twice width. Granular in phase-contrast preparations
In nutrient broth	Even-staining, round ended, Gram-negative cocco-bacilli, length less than twice width. Non-granular pairs, chains and clumps were seen in phase-contrast preparations. (79 had no clumps)	Even-staining, round ended, Gram-negative cocco-bacilli, length less than twice width. Non-granular singles, pairs, chains and clumps were seen in phase-contrast preparations. (12 and 28 had neither chains nor clumps)	Even-staining, round ended, Gram-negative bacilli, length greater than twice width. In phase-contrast preparations, the organisms were non-granular at 2 days but granular after 7 days. They were arranged in singles and pairs. (42, 51, 57, 69, 71, 82, 83, 85, 86, 87, 89 were organized in chains or clumps)
Motility and flagellation	−	−	+ Polar
Culture			
On Yeastrel agar	Raised, round, entire, greyish yellow, translucent, friable colonies. No pigment	Raised, round, entire, greyish yellow, translucent, friable colonies. Brown water soluble pigment	Round, raised, greyish yellow, entire, translucent, non-friable colonies. No pigment
In nutrient broth	Clear to slight turbidity; flocculent growth with deposit and ring	Slight turbidity; flocculent growth in majority; deposit and ring	Uniform turbidity; non-flocculent growth; deposit and surface growth
In peptone water at 5°	No growth	All flocculent; no turbidity. (8, 34, 35, 40 no floccules)	No flocculation but uniform turbidity
Biochemistry (the numbers in parentheses are the exceptions)			
Acid from glucose	+	+	+
Gas from glucose	−	−	+ (42)
Acid from lactose, 7 days	−	−	− (43, 65)
lactose, 40 days	−	−	+ (50–53, 65, 75)
sucrose	+	−	+ (87)
raffinose	−	−	− (48, 55, 62, 65, 66, 69, 71)
dextrin	+	+	+ (51, 65)
arabinose	−	+ (30, 31, 37)	+ (42, 43, 45, 51–53, 56, 62, 66, 69, 74, 75, 82, 90)
rhamnose	−	−	− (62, 83)
trehalose	+	− (6, 8, 22, 26, 28, 40)	+ (46, 62)
amygdalin	−	−	− (66, 69)
galactose	+	+	− (70)
xylose	−	− (3, 14, 15, 17–19, 21, 23, 24, 29–31, 41)	− (48, 62, 65, 71, 75)
salicin	−	− (10, 15, 25–27, 31–40)	+ (42, 43, 45, 51, 52, 72, 82)
glycogen	+	+	+ (48, 51, 62, 65, 71)
starch	+	+	+ (48, 49, 51, 57–59, 63, 65–68, 72)
glycerol	+ (77)	+	+ (65, 72, 74, 75)
sorbitol	−	− (30)	− (44, 46, 48, 49, 51, 55, 58, 59, 61–63, 68, 70, 71)
cellobiose	−	−	− (42, 43, 51–53, 55, 56, 62, 65, 66, 69, 71, 75, 82)
inositol	−	−	− (48, 62, 71)
Voges-Proskauer test	−	−	+ (74, 75, 86, 88)

Indole production	−	−	−(43, 56, 66, 69)
Reduction of nitrates	+	+	−(42–45, 47, 48, 51–53, 56, 62, 65, 69, 71, 82–90)
Production of hydrogen sulphide	−	−	−(43–47, 50, 52, 54–57, 59, 63–67, 69, 83, 84, 88, 89, 72)
Urease	−	−	−(65, 75)
Catalase	−	+	+
β-haemolysis	−	+	+(45, 48, 51, 62, 65, 66, 71)
Caseinolysin	+	+	+(42, 51, 56, 62, 65, 66, 69)
Liquefaction of serum	−	+	+(65, 66, 88)
Liquefaction of gelatin	No growth	+	+(65)
Litmus milk	Proteolysis (78)	Proteolysis and reduction. (6, 31 proteolysis only; 19, 28, 35, 37 no action)	Proteolysis and reduction. (48, 71 clot, proteolysis and reduction; 62 no action; 51, 66 clot and reduction)
Kovacs oxidase test	+	+	+(48, 51, 65, 71)
Aesculin hydrolysis	No growth	+(37; no growth 19)	+(43, 45, 52)
Phosphatase	−(76)	+(11)	+(62)
Starch hydrolysis	+(79)	+	+(47, 48, 62, 65, 71)
Lecithinase	−	+(28, 32, 40, 41)	+(62, 65, 66, 86, 88, 89)
Presumptive 'Bacterium salmonicida' test	−	+(11, 28, 30, 37)	−(48, 51, 65, 71, 88)
Gluconate oxidation	−	−	+(42, 43, 45, 51, 69, 74, 75)
2,3-butanediol production	−	−	+(69, 74, 75, 82, 88)
Phenylalanine dehydrogenase	−	−	−(62, 86, 88, 90)
Malonate test	−	−	−(65, 66)
Metabolism (the numbers in parentheses are the exceptions)			
Growth at 5°	+	+	+(42, 64, 88)
37°	−	−	+(43–45, 48, 49, 52–54, 56–63, 66–69, 73, 88)
in 3 % NaCl	⊥*(78–80)	⊥(7, 25)	+(⊥88, −43, 45)
Koser's medium	−	−	+(83, 88, 90)
Sensitivity to terramycin, aureomycin and tetracycline	+	+(34, 36)	+
Sensitivity to erythromycin and novobiocin	−	−(37)	−
Sensitivity to neomycin		−	−(42–44, 47, 89, 90)

* ⊥ = slight growth.

A full description of NCMB 1102

This organism is a round-ended cocco-bacillus which grows in singles, pairs, and clumps of chains in nutrient broth. It is non-granular in broth but granular on blood agar when viewed by phase contrast but in both cases exhibits even staining with Gram's stain. In nutrient broth it produces slight turbidity, a deposit and a ring and flocculation is restored by culture at 5°. On nutrient agar, the colonies are round, raised, greyish yellow, translucent, entire and friable. A brown water soluble pigment is produced. It produces acid and gas from mannitol, maltose, dextrin, glycerol, glycogen: acid only from glucose, fructose, arabinose, galactose, and starch and has no action on sucrose, dulcitol, lactose, raffinose, rhamnose, trehalose, amygdalin, xylose, sorbitol, inulin and ethanol. It is fermentative in Hugh & Leifson medium. It does not produce acetylmethylcarbinol or 2,3-butanediol; it is methyl red negative; does not produce indole; reduces nitrates to nitrites; does not produce hydrogen sulphide; has no urease; produces ammonia from peptone; has a catalase; is β-haemolytic; has a caseinolysin; liquefies gelatin and coagulated serum; causes proteolysis and reduction of litmus milk; has an oxidase; hydrolyses aesculin; has a phosphatase and a lecithinase; hydrolyses starch; does not grow in Koser's medium; has no action on gluconate, phenylalanine or malonate; grows in 1 and 3 but not in 8 % NaCl; grows at 5° and 22° but not at 37°; is resistant to penicillin, erythromycin, neomycin, bacitracin, novobiocin, oleandomycin and 0-129 and sensitive to streptomycin, chloramphenicol, terramycin and tetracycline.

this was not so. The filtration of the glucose did not result in any increase in gas production so heating of the glucose did not have a detrimental effect on gas production. With Aeromonas strains it should be noted that even with Seitz-filtered glucose or buffered glucose the displacement of the liquid was 9·8% or 10·4% so that definite gas production would be recorded in almost every case. The results confirmed the fact that 'B. salmonicida' produced more gas from mannitol than from glucose.

In addition to an aeromonad being a vigorous gas producer, it is supposed to produce 2,3-butanediol from glucose. This characteristic has not been very assiduously applied to the genus in the past, probably owing to the lengthy procedure required to determine the compound. Bullock (1961) suggested a very simple tube test employing piperazine and potassium ferricyanide after oxidation with potassium iodide; if the organism does produce 2,3-butanediol, a bright blue colour develops. This was tested on organisms which had been proved chemically to produce 2,3-butanediol. The results showed good correlation for both the known positive and negative species so this test was employed in the study. In not one of the non-pigmented group or of the 'Bacterium salmonicida' strains could the compound be detected nor did they produce acetylmethylcarbinol—the intermediate compound. This is not in agreement with Liu (1962), who stated that one strain of 'B. salmonicida' did produce 2,3-butanediol when tested by the method of Neish (1952). Of the 42 strains of *Aeromonas*, all but five (69, 74, 75, 82, 88) produced 2,3-butanediol. Two of these strains (69, 82) did, however, produce the intermediate compound so the degradation of glucose may have been incomplete in these instances. By contrast, one strain, 86, appeared to have degraded the glucose completely as in this case no acetylmethylcarbinol was detected although 2,3-butanediol was present.

Griffin *et al.* (1953*a*) in their monograph, suggested that their results might justify transferring 'Bacterium salmonicida' to the genus *Aeromonas*, but they did not give any reason for this change, which has now been implemented (Snieszko, 1957). Eddy (1960, 1962) stated that *A. salmonicida*, despite its biochemical properties and lack of motility, should be retained in the genus *Aeromonas* but as a separate species. The present worker's results differ from those of Eddy (1960, 1962) in gas production from glucose, the fermentation of certain sugars and the phosphatase reaction. The findings of Ewing *et al.* (1961) differ from those given in this paper mainly in gas production and in fermentation of various sugars; these authors also found some phenylalanine dehydrogenase activity, weak or absent catalase activity and a positive methyl red test. They also thought that *A. salmonicida* warrants species rank but not that at present it should be removed from the genus *Aeromonas*. Schubert (1961) does not discuss the classification of *A. salmonicida*. He merely compares it with *A. hydrophila* and his results with the exception of the sugar reactions are in close agreement with those recorded here.

From the results of this survey, it can be seen that 'Bacterium salmonicida' differs from the *Aeromonas* strains in size, shape, configuration and lack of motility, in its friable colony and brown water soluble pigment formation, in its flocculent growth in broth, its weak gas production from glucose, in its lack of late acid production from lactose, in its ability to produce acid from sucrose, in its failure to produce acetylmethylcarbinol or 2,3-butanediol; in its ability to reduce nitrate

to nitrite and in its inability to grow in Koser's medium. This means that, when Kluyver & van Niel's definition (1936) is applied, 'B. salmonicida' could not be included in the genus *Aeromonas* owing to its lack of vigorous gas production, lack of motility and inability to produce 2,3-butanediol. Despite the fermentative nature of this organism, it appears to be more closely related to the Pseudomonadaceae than to the Enterobacteriaceae as it produces a water soluble pigment, is oxidase positive, does not produce acid from lactose, is unable to grow at 37° and is highly proteolytic. It conforms to the definition of the family Pseudomonadaceae (Winslow *et al.* 1917) so it is suggested that a new genus be formed in the Pseudomonadaceae to accommodate 'B. salmonicida' and its non-pigmented anaerogenic variant. As both the pigmented and the non-pigmented strains are capable of producing necrotic lesions and in some cases complete liquefaction of the internal organs of salmonids, the generic name *Necromonas* is proposed. This name is formed from the Greek noun necros a dead body and the Greek noun monas a unit. *Necromonas* would therefore be defined as M.L. fem. n. necrosis (producing) unit. To distinguish the non-pigmented strain from *N. salmonicida* the species name *N. achromogenes* is suggested.

Necromonas could be defined as a non-motile cocco-bacillus found in short chains and clumps of chains. It produces a friable colony and a brown water soluble pigment or no pigment on agar. In broth the growth is flocculent; it is oxidase positive; its action on glucose is fermentative but little or no gas accompanies the acid production; it does not produce 2,3-butanediol; it has no action on lactose, it is unable to grow at 37° and it is a fish pathogen.

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The Serological Identification of Streptomycetes by Agar Gel Diffusion Techniques

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SUMMARY

The agar gel diffusion technique was used to investigate the serological relationships between species and strains of *Streptomyces*. A method is described for the rapid production of antisera which show specific reactions and also a multiplicity of cross-reactions. The technique has been used for the identification and comparison of antibiotic-producing isolates of streptomycetes from soil.

INTRODUCTION

The taxonomy of the genus *Streptomyces* is complex and at present confused. Classifications based on morphology, biochemical characteristics and antibiotic production have been described and recently reviewed by Waksman (1961), but no comprehensive serological method has been accepted for identifying species of Streptomyces. Previous serological studies of this genus have used conventional precipitation, agglutination or complement-fixation techniques (Aoki, 1935, 1936*a*, *b*; Ludwig & Hutchinson, 1949; Slack, Ludwig, Bird & Canby, 1951; Okami, 1956; Kuroya, Katafiri, Sato & Mayama, 1958) with the result that, although the antisera showed some species specificity, frequent cross-reactions occurred between apparently unrelated species. Somatic antigens common to several strains of Streptomyces have been demonstrated by haemagglutination (Hata, Ohki, Yokoyama & Koga, 1953; Yokoyama & Hata, 1953), and recently the presence of group and specific antigens in other species was confirmed by Douglas & Garrard (1958) by passive haemagglutination. The difficulties encountered in this earlier work can be attributed mainly to the multiplicity of cross-reactions. An alternative technique, the agar gel double-diffusion technique (Ouchterlony, 1948), has been used to examine complex antigenic systems in bacteria and this paper describes its application to the genus *Streptomyces*.

METHODS

Organisms. Details of the organisms used in this study are given in Table 1, with the appropriate strain number and culture collection initials, i.e. NCTC (National Collection of Type Cultures, Colindale, London, England), NCIB (National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland), NRRL (North Regional Research Laboratories, Peoria, Illinois, U.S.A.), IMRU (Institute for Microbiology, Rutgers University, New Jersey, U.S.A.) or B/FD (Research Department, Boots Pure Drug Co. Ltd, Nottingham, England). All of these organisms had been maintained as freeze-dried cultures for varying periods.

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Preparation of antigens. Cultures were prepared by inoculating spores or vegetative organism into 500 ml. flasks containing 100 ml. sterile PYG broth (%, w/v: peptone, Difco, 0·5; yeast extract, Difco, 0·5; glucose, 1; Casamino acids, Difco, 0·1; NaCl, 0·5; pH 7·0–7·2). Good growth of streptomycetes was usually obtained after incubation for 3–4 days on a rotary shaker at 28°; bacteria were harvested after 2 days at 30°. Organisms were collected by centrifugation, washed twice in saline and resuspended in saline containing 0·01 % (w/v) thiomersalate. The concentration of the resulting suspensions, which were used for immunizing rabbits and in the diffusion plates was standardized to 60 mg. dry weight organism/ml.

Table 1. *Organisms used*

Strain	Source	Strain	Source
Streptomyces albus 3525	NCTC	*Streptomyces* sp. 2288	NRRL
S. albus B1337	NRRL	*Streptomyces* sp. A 9257	Soil isolate
S. antibioticus 6124	NCTC	*Streptomyces* sp. B 4049	Soil isolate
S. albogriseolus 1305	NRRL	*Streptomyces* sp. B 2886	Soil isolate
S. aureofaciens 2209	NRRL	*Streptomyces* sp. L 1652	Soil isolate
S. coelicolor B1260	NRRL	*Bacillus subtilis* 8236	NCTC
S. erythreus 903	B/FD	*Candida albicans* F 1/52	NCTC
S. erythreus 8594	NCIB	*Corynebacterium pyogenes*	B/FD
S. fradiae 474	B/FD	*Dermatophilus dermatonomus*	B/FD
S. griseus B/FD 196	IMRU	*Mycobacterium* sp. 607	B/FD
S. lavendulae 3440-8	IMRU		
S. lavendulae 3516	IMRU		
S. lavendulae 3531	IMRU		
S. lavendulae 3542	IMRU		
S. lavendulae 9000	NCIB		

PYG broth was found to be a most useful medium for this work; in all cases it gave diffuse growth in shake-flask culture without the appearance of the pellets which so often occur in other media. The medium used did not appear to affect the antigenicity of the Streptomyces species significantly. Antiserum to *Streptomyces* sp. 2288 grown in PYG broth produced the same number of precipitation lines when tested against this strain grown in PYG broth, PG broth (% w/v: peptone, Difco, 1; glucose, 1) or NZA broth (%, w/v: NZ amine A, Sheffield Farms, 0·1; peptone, Difco, 0·5; yeast extract, Difco, 0·5; glucose, 1; NaCl, 0·5). However, although the qualitative response was similar, mycelia from PYG or NZA media reacted with lower antiserum concentrations than mycelia grown in PG medium.

Early immunization experiments were made with the above suspensions or reconstituted freeze-dried ones. However, some difficulty was experienced in injecting this material into rabbits, particularly by the intravenous route, and later material was homogenized before being used for injection by disrupting the organisms, without added abrasive, in the Hughes press (Hughes, 1951) at −30°. This may also have liberated additional antibody-determining groups. Evidence to support this was obtained when antiserum prepared against disrupted *Streptomyces antibioticus* 6124 was placed in the centre reservoir of a diffusion plate and alternate outer reservoirs filled with suspensions of normal or disrupted mycelium at equivalent concentrations. In addition to the six precipitation lines adjacent to the normal mycelium, two additional lines were observed between the disrupted mycelium and serum. However, in a similar experiment with *S. lavendulae* 3440-8, additional lines were not observed.

Immunization procedure. Rabbits (2·5–3 kg.) of either sex were fed on Diet 18 pellets and hay *ad libitum*. Intravenous injections of suspensions of homogenized organism were made into the marginal ear vein. When antigen was given intramuscularly it was injected into the flexor muscles of two rabbits twice, at an interval of 1 week. One ml. of a 50 % emulsion of Freund's adjuvant (Difco) containing the equivalent of 30 mg. dry wt. disrupted mycelium/ml. was used. Blood samples were taken from the marginal ear vein at intervals. When satisfactory antibody titres had been attained the rabbits were fasted overnight, anaesthetized with urethane (1·6 g./kg.) followed by chloroform and the animal exsanguinated by heart puncture. The blood was allowed to coagulate, refrigerated overnight and the serum separated by centrifugation. Sera were stored in a deep freeze at $-10°$ as some loss in titre was observed after freeze-drying.

Serological methods. A slight modification of the agar double-diffusion technique of Ouchterlony (1948) was used. The tests were made in 9 cm. Petri dishes containing 20 ml. agar medium. We found that, of several tested, the basal medium described by Crowle (1958) gave the clearest results. The medium contained 1 % 'ion agar' (Oxoid) dissolved in 0·2 M sodium barbital + hydrochloric acid buffer (pH 7·4) containing 0·01 % (w/v) thiomersalate. Circular reservoirs were made with a 6 mm. cork borer and sera pipetted into two central reservoirs. The mycelial suspensions were placed in six peripheral reservoirs (Fig. 2). The Petri dishes were incubated in a humid chamber at 26° and examined daily for the appearance of precipitation lines. When fully developed (after 2–4 days) the lines were observed and counted by two independent observers.

Antibody titres were determined by the agar diffusion method which Thorne & Belton (1957) used for antigen titration.

RESULTS

The sera of normal rabbits did not react in agar with mycelial or whole culture preparations of any of the Streptomyces species used. A low antibody titre was sometimes obtained by repeated injections of shake-flask culture filtrate concentrated by freeze-drying. High antibody titres (about 1/128) were consistently obtained with mycelial preparations.

Two immunization procedures were compared for maximum antibody production. *Streptomyces lavendulae* 3440-8 disrupted mycelium was used in a comparison with two groups of three rabbits. One group was given a total of equiv. 60 mg. dry wt. antigen with adjuvant in two intramuscular injections with an interval of 1 week. Three weeks later equiv. 12 mg. dry wt. disrupted mycelium was injected intravenously. Rabbits in the other group received the same amount of antigen (equiv. 72 mg. dry wt.) intravenously in eight ascending doses over 4 weeks. Commencing 1 week later samples of serum from all the rabbits were obtained at intervals and the antibody titre determined. Although there was some variation between rabbits of the same group it could be concluded that the maximum titres occurred 6–7 weeks after beginning immunization. The method involving the use of adjuvant gave slightly higher titres and, since with it only three injections had to be made, this procedure was adopted in subsequent work. Serum from rabbits injected with Freund's adjuvant without added Streptomyces mycelium gave no precipitation lines when tested against a variety of Streptomyces strains.

Specificity of sera

Sera were prepared against seven established Streptomyces species and five strains of *Streptomyces lavendulae*. Generic specificity was investigated by testing these antisera against disrupted organisms of the following: *Bacillus subtilis*, *Candida albicans*, *Corynebacterium pyogenes*, *Dermatophilus dermatonomus*, *Mycobacterium* sp. 607. No precipitation lines were seen except one indistinct line against the

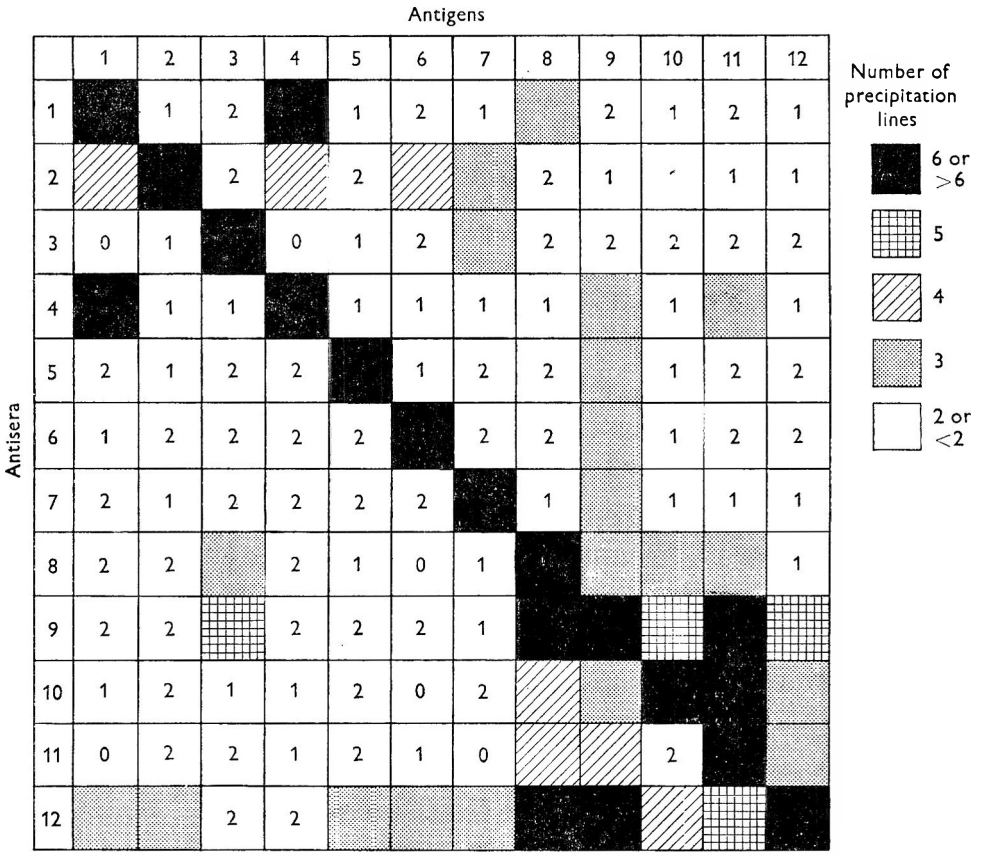


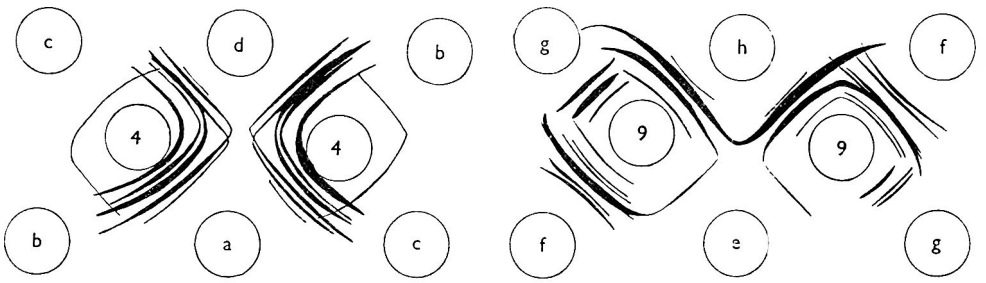
Fig. 1. Diagrammatic representation of antisera specificity shown by shading the squares according to the number of precipitation lines observed. Strains of *Streptomyces* represented as follows: (1) *S. albus* 3525, (2) *S. antibioticus* 6124, (3) *S. coelicolor* B 1260, (4) *S. erythreus* 903, (5) *S. fradiae* 474, (6) *S. aureofaciens* 2209, (7) *S. albogriseolus* 1305, (8) *S. lavendulae* 3542, (9) *S. lavendulae* 9000, (10) *S. lavendulae* 3440-8, (11) *S. lavendulae* 3531, (12) *S. lavendulae* 3516.

Mycobacterium strain. However, it was found that this was not related to the Streptomyces antibodies as sera from apparently normal unimmunized rabbits occasionally produced a similar line.

Each of the twelve antisera was tested at least twice against the twelve Streptomyces antigen preparations and the number and appearance of the precipitation lines recorded. The results are given in Fig. 1. The response of homologous pairs

was very marked and, in each case, at least six precipitation lines were observed. Also, the presence of many common antigens was indicated by the large number of cross-reactions. In many cases the cross-reacting lines were weak in comparison with the strong precipitation lines exhibited between homologous antigens and sera.

However, there was a very marked cross-reaction between *Streptomyces albus*, *S. erythreus* and their respective antisera. The diffusion plate showed a very strong pattern of identity, with many common precipitation lines (Fig. 2(*a*)); a detailed study of the two organisms was therefore undertaken. Their morphology, growth, pigment production and physiological reactions were very similar and quite different from the published descriptions of *S. erythreus* (Waksman & Curtis, 1916).



(A) (B)

Fig. 2. Agar diffusion plates (A) showing precipitation lines between antiserum to *Streptomyces erythreus* 903 (4) and (a) *S. albus* 3525, (b) *S. antibioticus* 6124, (c) *S. coelicolor* B1260, (d) *S. erythreus* 903; and (B) showing precipitation lines between antiserum to *S. lavendulae* 9000 (9) and (e) *S. fradiae* 474, (f) *S. lavendulae* 9000, (g) *S. lavendulae* 3440-8, (h) *S. lavendulae* 3531.

Both strains were found to be indistinguishable from another strain of *S. albus* (B 1337) and morphologically and serologically unlike a second strain of *S. erythreus* (8594). It was therefore concluded that the first culture labelled *S. erythreus* was in fact a strain of *S. albus* and was wrongly labelled in our collection.

Although the species were clearly differentiated according to the number of lines produced with a specific antiserum, the five strains of *Streptomyces lavendulae* were difficult to separate owing to marked cross-reactions; this is illustrated in Fig 2(*b*). Many strong precipitation lines, some of which are confluent, are seen between three *S. lavendulae* strains and the antiserum prepared against one of them. Only two faint lines were produced with this serum and a different species, *S. fradiae*. The cross-reacting strains of *S. lavendulae* do not show any obvious relationships to the groupings of the strains of this species proposed by Okami (1956).

Application to new isolates

The immunodiffusion method has been used on several occasions to assist in the identification of antibiotic-producing streptomycetes isolated from soil. Isolate A 9257, which produces the antibiotic antimycin, did not give any precipitation lines when tested against antisera to Streptomyces sp. 2288, another antimycin-producing strain (Leben & Keitt, 1948). This new isolate A 9257 was later shown to differ in morphology and physiological reactions from *Streptomyces* sp. 2283.

Another Streptomyces isolate, which produced an antibiotic similar to antimycin in a preliminary characterization, gave a strong pattern of identity when tested by the gel diffusion method against *Streptomyces* sp. 2288 antiserum and the homologous antigen preparation; the isolate was shown to be very similar to 2288 when grown on the usual identification media.

Isolate B4049 was found to produce antibiotics very similar to if not identical with the neomycin complex. Morphologically this isolate resembled *Streptomyces albogriseolus*, a species previously reported to produce neomycin (Benedict *et al.* 1954). *S. albogriseolus* antisera gave six precipitation lines with the homologous antigen and six lines with isolate B4049. The close serological relationship between these two strains was emphasized by the confluence of at least four of these lines; the two other lines were weaker and did not extend far enough to give evidence of confluence. However, when the Jennings triangular plate method (Jennings & Malone, 1955) was used, seven lines were observed for each antigen and each line was seen to be confluent. The two strains were therefore indistinguishable by this method. Further verification of their immunological identity was obtained by antibody absorption tests. Another isolate L1652, which produced neomycin, gave five precipitation lines against *S. albogriseolus* antiserum and none against antiserum to *S. fradiae*, a streptomycete used to produce neomycin commercially. Further evidence for the use of this technique to compare antibiotic-producing strains was reported by Cross (1962) for isolates producing oxytetracycline.

DISCUSSION

Because of their technical simplicity and ability to distinguish between antigenic molecules, immunodiffusion techniques are finding increased application to the taxonomy of microorganisms. To our knowledge, however, only three reports of the double-diffusion technique being used with Streptomyces have appeared. Wodehouse & Backus (1957) briefly reported antiserum to *Streptomyces aureofaciens* which produced nine precipitation lines when tested against homologous antigen, and suggested that the method is well adapted for use as an aid in differentiating species and strains within the genus. Bunch & Barth (1958), during a study on streams polluted by fermentation wastes, found that the presence of Streptomyces could be detected immunologically and that at least five antigens could be detected in undisrupted *S. lavendulae* mycelium. Guthrie, Roach & Ferguson (1962) have given preliminary details of the methods they have used in their work on the serology of aerobic aquatic actinomycetes.

In the work reported here we have shown that double-diffusion techniques have considerable value, not only for identifying Streptomycetes but for supplying supplementary characteristics to aid in the classification of this complex group of micro-organisms. A considerable degree of species specificity has been shown to exist. It is also possible that the technique may differentiate between the strains of some species (Cross, 1962). The antigens common to many species, which have caused difficulties in the application of conventional agglutination and precipitation tests, are also apparent.

The problem of deciding what weight should be given to antigenic structures in the application of immunodiffusion to Streptomyces taxonomy, as compared to

morphology and physiology, must await the results of a greater number of comprehensive studies as Shattock (1955) indicated in connexion with the application of serological techniques to bacterial taxonomy. It would also seem worth while to explore the use of isolated cell-wall antigens, following the work of Cummins (1962) on related genera, in an attempt to decrease the number of cross-reactions. From the more practical viewpoint, since the double diffusion method can easily and quickly show similarities between species, it seems that it may be useful in the identification of isolates producing new antibiotics. Ideally a bank of reference antisera would be needed, but, since activity is lost on prolonged storage and the production of a large number of sera is expensive in terms of time and materials, this may not be a practical procedure. Nevertheless, suitable antiserum to a new isolate may be produced in under 6 weeks. This can then be tested against type cultures and a relatively rapid serological identification of the new isolate obtained while more extensive conventional studies are being undertaken.

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Radiosensitivity in *Escherichia coli*

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SUMMARY

A radiosensitive mutant of *Escherichia coli* strain K12, W3747 was found to be similar to *E. coli* strains B and S with respect to survival following ultraviolet irradiation, plating-medium recovery, ability to 'reactivate' irradiated T1 phage, and in its cross-resistance pattern with radiomimetic chemicals. In all these respects the radiosensitive *E. coli* strain K12, AB1186, isolated by Howard-Flanders, was found to resemble the Hill radiation hypersensitive strain B_{s-1}, and, except in the ability to reactivate irradiated T1 phage, strain B_{s-2}. Strains $W3747_s$ and AB1886 acquired sensitivity to radiomimetic chemicals concomitantly with radiation sensitivity. Strains B_{s-1} and B_{s-2} on becoming hypersensitive to radiation did not become correspondingly hypersensitive to radiomimetic chemicals. In fact, they acquired a small degree of resistance to these chemicals, relative to strain B, and a substantial increase in resistance to 1-methyl-3-nitro-1-nitrosoguanidine and proflavine. These results are discussed in terms of known genetic loci implicated in radiation sensitivity and resistance.

INTRODUCTION

Escherichia coli strains B and S are naturally sensitive to ultraviolet (u.v.) radiation and X-radiation but sensitivity can be demonstrated only when they are plated, following irradiation, on complex media (Roberts & Aldous, 1949; Alper & Gillies, 1960; Hill & Simson, 1961; Woody, Mandell & Greenberg, 1961). Many different radiation-resistant mutants of *E. coli* strains B and S have been isolated (Witkin, 1947; Alper & Gillies, 1960; Greenberg, Mandell & Woody, 1961*a*) and these exhibit concomitant resistance to a variety of chemical agents such as nitrogen mustard (Bryson, 1948; Woody *et al.* 1961), nitrofurazone (Szybalski & Nelson, 1954; Hill & Simson, 1961; Woody-Karrer & Greenberg, 1963), Mitomycin C (Greenberg, Mandell & Woody, 1961*b*), nitrosoguanidines (Mandell, Woody & Greenberg, 1961), azaserine (Greenberg *et al.* 1961*a*). These radioresistant mutants have been differentiated on the basis of their cross-resistance patterns with radiomimetic agents. Mutants of *E. coli* strain B have also been isolated which are even more sensitive to radiation than is *E. coli* strain B itself. These include *E. coli* strain B_{s-1} (Hill, 1958), strain B_{s-2} (Hill & Simson, 1961) and strain B_{III} (Rörsch, Edelman, van der Kamp & Cohen, 1962). Unlike *E. coli* strain B, strains B_{s-1} and B_{s-2} do not form long filaments when treated with u.v. radiation. Furthermore, strains B_{s-1} and B_{III} exhibit a peculiar property when used as hosts for u.v.-irradiated coliphage T1: when irradiated T1 phage is plated on *E. coli* strains B_{s-1} and B_{III}, it is significantly more sensitive to the lethal effects of irradiation than when it is

plated on parent *E. coli* strain B. This has been taken to mean that *E. coli* strains B_{s-1} and B_{III} lack a mechanism, present in other strains of *E. coli*, which repairs the damage caused by u.v. radiation to T1 phage. On the other hand, *E. coli* strain B_{s-2}, like its parent, strain B, 'reactivates' irradiated T1 phage.

It would be of considerable significance if genetically controlled variations in radiosensitivity were demonstrable in *Escherichia coli* strain K12, if only because more is known about strain K12 than about strain B as a genetic tool. Most derivatives of *E. coli* strain K12 are radioresistant (Adler & Copeland, 1962; Howard-Flanders & Theriot, 1962) but strain K12 AB531 was reported by Adler & Copeland (1962) to be relatively less resistant than other K12 strains tested in their laboratory. However, the ultraviolet dose, about 600 ergs/mm.^{2}, reported to give one decade of kill in strain AB531 is in the same range as that reported for the most frequently occurring radioresistant mutants of *E. coli* strain s (Greenberg & Woody-Karrer, 1963). Howard-Flanders & Theriot (1962) isolated a mutant of strain K12 AB1157 (designated AB1886 by Howard-Flanders, Boyce, Simson & Theriot, 1962), which was very sensitive to u.v. radiation and which lacked the 'reactivating' mechanism for irradiated T1. Another radiation-sensitive mutant of strain K12 was isolated in our laboratory following treatment of strain K12 F'_{13} (w3747, Hirota & Sneath, 1961) with acridine orange (Cook & Greenberg, unpublished observations). This radiosensitive strain, $w3747_s$, can still transmit together, and at high frequency, the F factor and the ability to ferment lactose. A further description of this mutant was one of the purposes of the present work.

Since increased resistance to radiomimetic chemicals is an invariable concomitant of a mutation to radioresistance in *Escherichia coli* strains B and s (Greenberg & Woody-Karrer, 1963), another purpose of these experiments was to see whether mutations from resistance to sensitivity in *E. coli* strain K12 and from sensitivity to hypersensitivity in strain B were accompanied by a decrease in resistance to radiomimetic agents. Strains B, B/r, B_{s-1}, B_{s-2}, AB1886, AB1157, w3747 and $w3747_s$ were compared for the following properties: ultraviolet survival curves, plating-medium responses, 'reactivation' of irradiated T1 phage and cross-resistance patterns with radiomimetic chemicals. The results will show that in all these properties strains AB1157 and w3747 resembled strain B/r; strain AB1886 resembled strain B_{s-1} (but not B_{s-2}); and strain $w3747_s$ resembled strain B. There are, therefore, two kinds of mutation to radiosensitivity in *E. coli* strain K12. Both of these, significantly, are accompanied by increased sensitivity to radiomimetic chemicals, but the hyperradiosensitive mutants of *E. coli* strain B (B_{s-1}, B_{s-2}) show a decreased sensitivity to most of the radiomimetic chemicals.

METHODS

Strains of Escherichia coli *used.* The *E. coli* strains used, their derivation, relative radiosensitivities assayed on tryptone agar, and source are listed in Table 1.

Chemical compounds used. The chemicals used were: 1-methyl-3-nitro-1-nitrosoguanidine (which will be referred to as nitrosoguanidine, bought from the Aldrich Chemical Co., Milwaukee, Wisconsin, U.S.A., and recrystallized from ethanol); proflavine hydrochloride (obtained from the Allied Chemical and Dye Corp., New York, N.Y.); nitrogen mustard (a gift from Merck Sharp and Dohme, Rahway,

N.J.); Mitomycin C (supplied by the Cancer Chemotherapy National Service Centre, Bethesda, Maryland, U.S.A.); nitrofurazone (5-nitro-2-furaldehyde semicarbazone; a gift from Eaton Laboratories, Division of the Norwich Pharmacal Company, Norwich, N.Y.). All compounds were prepared in sterile distilled water immediately before use.

Table 1. *Summary of strains*

Strain of *E. coli*	Derivation	Relative radiosensitivity on tryptone agar	Source
B		Sensitive	Dr R. Hill
B_{s-1}	Strain B	Hypersensitive	Dr R. Hill
B_{s-2}	Strain B	Hypersensitive	Dr R. Hill
B/r (CSH*)	Strain B	Resistant	Dr H. I. Adler
S	Strain B	Sensitive	Dr A. D. Hershey
S/Mc lb (R_4)	Strain S	Resistant	
AB1157	Strain K12	Resistant	Dr E. Adelberg
AB1886	Strain AB1157	Sensitive	Dr P. Howard-Flanders
W3747	Strain K12	Resistant	Dr J. Lederberg
$W3747_s$	Strain W3747	Sensitive	

* Cold Spring Harbor.

Culture media. The media used contained per litre of distilled water:

(1) Tryptone agar: tryptone, 10·0 g.; glucose, 1·0 g.; sodium citrate, 2·0 g.; sodium chloride, 8·0 g.; agar, 12·0 g. (BBL, Baltimore Biological Lab., Inc.); adjusted to pH 7·0 or 7·8 with sodium hydroxide or to pH 5·5 with hydrochloric acid.

(2) Tryptone semi-solid agar: tryptone, glucose, sodium chloride, and sodium citrate at the same concentrations as in tryptone agar; agar, 6·0 g.; adjusted to pH 7·0 with sodium hydroxide.

(3) M9 agar: dibasic sodium phosphate, 5·8 g.; monobasic potassium phosphate, 3·0 g.; ammonium chloride, 1·0 g.; sodium chloride, 0·5 g.; glucose, 2·0 g.; magnesium sulphate ($0{\cdot}7H_2O$), 250 mg.; calcium chloride 14 mg.; 1 % gelatin solution, 10 ml.; agar, 8·0 g. (Ionagar, Oxo Ltd., London).

(4) Peptone broth: Bacto-Peptone, 10·0 g. (Difco Laboratories, Detroit, Michigan, U.S.A.); Difco Beef Extract, 3·0 g.; glucose, 1·0 g.; sodium chloride, 5·0 g.

Phosphate-buffered saline was 1 % sodium chloride in 0·02 M-phosphate buffer (pH 6·8).

Survival of u.v.-irradiated T1 phage. The u.v. radiation source was a single 15 W. General Electric germicidal lamp with a maximum output at 2537 Å. Calibrated with bacteriophage T2 according to the method of Latarjet, Morenne & Berger (1953), this lamp delivered 15·4 ergs/mm.^{2}/sec. at a distance of 51·5 cm. from the source. T1 phage, 5×10^6 particles/ml., was irradiated with a dose of 616 ergs/mm.^{2} and 0·1 ml. was mixed with 2·5 ml. of warm (48°) semi-solid tryptone agar containing one drop of the overnight culture to be tested. The semi-solid mixtures were then overlayered on to fresh tryptone agar plates and the number of plaques counted after overnight incubation at 37°. This is essentially the rapid method described by Rörsch *et al.* (1962) for differentiating between mutants capable of 'reactivating'

irradiated T1 phage and those unable to do so. When strains able to reactivate irradiated T1 phage were used as hosts, the plaques were confluent. When those unable to 'reactivate' irradiated T1 phage were used as hosts, a countable number of plaques were seen.

Survival of u.v.-irradiated bacteria. Cultures grown overnight in peptone broth were washed twice with buffered saline (pH 6·8) and exposed with gentle agitation in 50 mm. diameter Petri dishes containing 1 ml. of bacterial suspension (2×10^6 bacteria/ml.). Appropriate dilutions in cold phosphate-buffered saline were plated in duplicate on tryptone agar (pH 7·0) and on M9 agar, incubated at 37° for 24 and 48 hr. respectively, and counted. All manipulations subsequent to irradiation were done in subdued light to minimize photoreactivation. Sensitivity to u.v. radiation was calculated as the dose to give 10 % survivors.

Measurement of resistance to chemical agents. Cultures grown overnight in peptone broth were adjusted with a model 9 Nepho-colorimeter (Coleman Instruments Inc.) to $3{\cdot}5 \times 10^8$ bacteria/ml. and streaked on gradient plates according to the method of Szybalski & Bryson (1952). Gradient plates were made with tryptone agar (pH 5·5) except that pH 7·8 was used in tests with proflavine; M9 agar was used in tests with azaserine. The minimum inhibitory concentration (M.I.C.) was determined as follows: (length of solid growth/total length of streak) × maximum concentration (μg./ml.) of test compound.

RESULTS

Reactivation of u.v.-irradiated T1 phage. With the following hosts the plaques were confluent: *Escherichia coli* strains S, B, B_{s-2}, W3747 and W3747$_s$. Plates with *E. coli* strain B_{s-1} had 177 plaques; with strain AB1886, 145 plaques. To obtain numbers of plaques in this range for *E. coli* strains S, B, B_{s-2}, W3747 and W3747$_s$, the irradiated phage suspension had to be diluted 1/50. When this was done there were no significant differences in the number of plaques produced by each strain in this group.

Response to u.v. radiation. Figures 1, 2 and 3 show the survival curves following ultraviolet irradiation of *Escherichia coli* strains B, B_{s-1}, B_{s-2}, S, W3747, W3747$_s$, AB1157 and AB1886 when plated on tryptone agar and on M9 agar. The survival curves of the radioresistant parent strains AB1157 and W3747 (Fig. 3) indicate significant resistance, independent of plating medium, 860 ergs/mm.^{2} and 462 ergs/mm.^{2} respectively, resulting in 10 % survival.

Escherichia coli strains B_{s-1} and AB1886 (Fig. 1) were similar in that: (1) they were significantly more sensitive to u.v. irradiation than the other strains; (2) their survival curves were exponential to more than three decades of kill; (3) both showed a limited plating-medium response: there were more survivors at any dose when the bacteria were plated on M9 medium than when plated on tryptone medium, but the survivors on M9 medium were considerably fewer than those of strains B, B/r, AB1157 or W3747. On the other hand, *E. coli* strains W3747$_s$, B, and S (Fig. 2) were related in that: (1) their survival curves were identical when plated on complete medium; (2) they exhibited a marked plating-medium response: strain W3747$_s$ could not be distinguished from its parent strain W3747 when both strains were plated on M9 medium after u.v. irradiation.

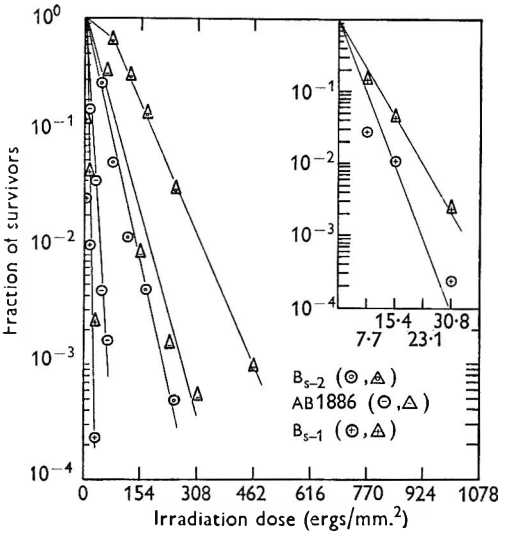

Fig. 1

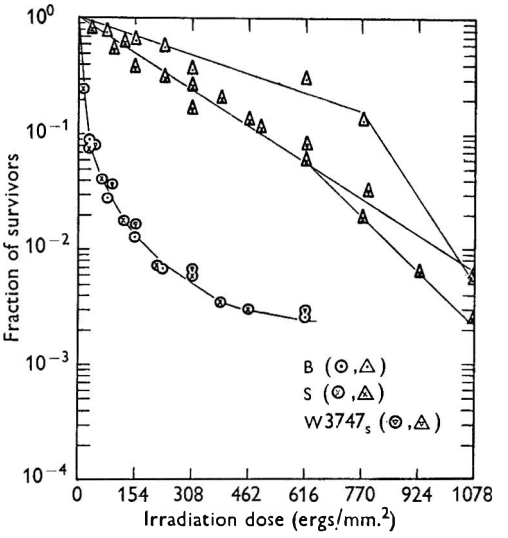

Fig. 2

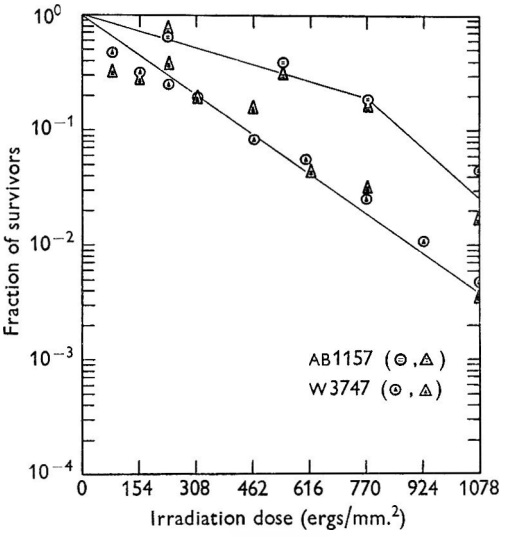

Fig. 3

Fig. 1. Survival of radiation-sensitive *Escherichia coli* strains B_{s-1}, B_{s-2}, and K12 AB1886 exposed to ultraviolet radiation and plated on tryptone agar, pH 7·0 (⊕, ⊙, ⊖) and M9 agar, pH 6·8 (▲, ▲, △). Each point represents an average of 3 independent determinations for strains B_{s-1} and B_{s-2} and 6 for strain AB1886. The insert in the upper right-hand corner indicates the plating-medium response of strain B_{s-1} on a more magnified scale.

Fig. 2. Survival of radiation-sensitive *Escherichia coli* strains B, S, and K12 $W3747_s$ exposed to ultraviolet radiation and plated on tryptone agar, pH 7·0 (⊙, ⊗, ⊛) and M9 agar, pH 6·8 (△, ▲, ▲). Each point represents an average of 5 independent determinations for each strain.

Fig. 3. Survival of radiation-resistant *Escherichia coli* strain K12 AB1157 and strain K12 W3747 exposed to ultraviolet radiation and plated on tryptone agar, pH 7·0 (⊜, ⊜) and M9 agar, pH 6·8 (▲, ▲). Each point represents an average of 2 to 4 independent determinations.

The survival curve for *Escherichia coli* strain B_{s-2} (Fig. 1) was exponential for more than three decades of kill, but it showed greater sensitivity than strain B only at doses higher than 154 ergs/mm.^{2} (The survival curves for strains B_{s-2} and B varied somewhat from those reported for these strains by Hill & Simson in 1961, perhaps due to a difference in experimental procedures or to a genetic drift in the bacterial populations: Greenberg & Woody-Karrer, in press.) Strain B_{s-2}, like strain B_{s-1}, exhibited a limited plating-medium recovery when plated on M9 medium.

Table 2. *Cross-resistance patterns of radiation-sensitive and radiation-resistant mutants of* Escherichia coli

	Test compound					
	NG*	NM	MC	NF	PF	AS
	Minimum inhibitory concentration (M.I.C.) for *Escherichia coli* strains B and S (μg./ml.)†					
	0·08 ± 0·01	16 ± 1	0·04 ± 0·01	0·15 ± 0·01	1·2 ± 0·2	0·004 ± 0·0005
Strain of *E. coli*	Fold increase in M.I.C.					
B	1	1	1	1	1	1
S	1	1	1	1	1	1
w3747$_s$	2·5	1·2	1·4	1·5	1·7	—
B_{s-1}	8	1·5	1	1·3	2·5	1
B_{s-2}	8	2·5	1	3·4	2·5	2·6
AB1886	17	0·5	1	1·5	1·7	—
B/r	45	20	23	19	3·8	15
S/MC lb (R_4)	35	16	16	14	2·5	15
w3747	35	16	11	14	2·5	—
AB1157	35	2·4	6	5·8	2·5	—

* The following abbreviations will be used: 1-methyl-3-nitro-1-nitrosoguanidine, NG; nitrogen mustard, NM; mitomycin C, MC; nitrofurazone (5-nitro-2-furaldehyde semicarbazone), NF; proflavine, PF; azaserine, AS.

† μg./ml. estimated from gradient plates (Szybalski & Bryson, 1952).

Response to radiomimetic chemicals. Table 2 shows the minimum inhibitory concentrations of five radiomimetic chemicals for each of the strains studied relative to *Escherichia coli* strains B and S. The following points should be noted: (1) The cross-resistance patterns of strains w3747, AB1157, and S/MC lb, a prototype of the most frequently occurring radioresistant class of mutants (R_4) isolated from *E. coli* strain S, were similar to that of strain B/r. All four strains were very resistant to all the agents tested. (2) The cross-resistance pattern of strain AB1886, indicating sensitivity as compared with that of strain AB1157, was very similar to those of strains B_{s-1} and B_{s-2}, the most significant feature being resistance to nitrosoguanidine and proflavine. (3) The cross-resistance pattern of strain w3747$_s$, indicating sensitivity as compared with that of strain w3747, most resembled those of *E. coli* strains B and S, being relatively sensitive to all the radiomimetic agents tested.

DISCUSSION

The evidence suggests that there are at least two different kinds of radiosensitive strains of *Escherichia coli* strain K12, one of which, strain w3747$_s$, is analogous to *E. coli* strains B and S, the other, strain AB1886, analogous to *E. coli* strain B_{s-1} (and probably to strain B_{III}, Rörsch *et al.* 1962). The u.v. survival curve of strain w3747$_s$ is identical with those of *E. coli* strains B and S when plated on tryptone medium, and all three strains exhibited a similar plating-medium response. Strains w3747$_s$, B and S were similar in sensitivity to the five radiomimetic agents tested, whereas strain w3747 was about as resistant to these compounds as the B/r and R_4 mutants of strain S. Finally, all three strains exhibited no measurable loss of ability to 'reactivate' irradiated T1 phage.

Escherichia coli strain AB1886, on the other hand, is very similar in many of its properties to *E. coli* strain B_{s-1}. Following u.v. radiation, the survival curves of strain AB1886 are similar to those of strain B_{s-1} in general shape, sensitivity, and plating-medium recovery. Strain AB1886 was sensitive, as compared with its radioresistant parent, to all of the radiomimetic agents studied, but was markedly less sensitive to nitrosoguanidine and somewhat less sensitive to proflavine than were *E. coli* strains B, S, or w3747$_s$. It is significant that *E. coli* strain B_{s-1}, even though more sensitive to u.v. radiation than its parent, strain B, was no more sensitive than strain B to most of the radiomimetic agents tested and was significantly more resistant to nitrosoguanidine and proflavine than *E. coli* strain B. Finally strains B_{s-1} and AB1886, in corroboration of reports by Ellison, Feiner & Hill (1960) and Howard-Flanders & Theriot (1962), did not reactivate u.v.-damaged T1 phage.

The locus which determines radiosensitivity in *Escherichia coli* strain B_{s-1} has not been mapped. However, Howard-Flanders *et al.* (1962) showed that the radiosensitivity of *E. coli* strain AB1886 is the result of a mutation (UV^R to UV^S) at a locus 0·37 the distance between the loci for arginine synthesis and arabinose fermentation; UV^R controls a mechanism, absent from UV^S mutants, which reactivates damage done by ultraviolet radiation.

The radiosensitive mutant strain B_{III}, of *Escherichia coli* strain B, isolated by Rörsch *et al.* (1962), is similar to *E. coli* strains B_{s-1} and AB1886 in its sensitivity to u.v. radiation and in its inability to reactivate u.v.-irradiated T1 phage. These investigators have shown that the radiosensitivity of mutant strain B_{III} is the result of a mutation (syn^+ to syn^-) at a locus between xylose fermentation and streptomycin resistance and closely linked to the former (Rörsch, Edelman & Cohen, 1963). The *syn* and *UV* loci, while on the same quadrant of the circular chromosome of *E. coli* as depicted by Jacob & Wollman (1961), do not map at the same place. It is possible that mutations at either of two different loci result in similar phenotypes, or that the linear arrangements of genes is not identical in at least this segment of the chromosome of *E. coli* strains B and K12.

Cook & Greenberg (unpublished observations) have demonstrated in crosses between radioresistant *Escherichia coli* strain K12 (Hfr 1 and 2) and *E. coli* strain S that there is a locus (which will be referred to as *RA* to distinguish it from *UV*) for radioresistance closely linked to the loci for lactose fermentation and resistance to phage T6. The nature of the sensitivity (RA^S) or resistance (RA^R) conferred by the gene at this locus is under further investigation. The evidence as yet unpublished

suggests that the sensitivity of strain w3747$_s$ is a result of a mutation at or near this locus. Its parent strain w3747 can transmit radiation resistance along with the ability to ferment lactose by F-duction to *E. coli* strain s1 (F^- Lac^-). *Escherichia coli* strain K12 w3747$_s$, while able to transmit together and at a high frequency its Lac^+ and F^+ characteristics, does not transmit radiation resistance to strain s1. However, the F′ episome of strains w3747 and w3747$_s$ is extremely unstable in *E. coli* strain s, rendering quantitative analysis difficult. A more promising approach to the genetics of the *RA* locus would appear to be to 'cure' strain w3747$_s$ of its episome, making it available as an F^- strain for recombination studies with donor strain K12s.

The fact that when *Escherichia coli* strain K12 becomes radiosensitive either by a mutation at the *UV* or the *RA* locus it also becomes sensitive to Mitomycin C, nitrogen mustard and nitrofurazone, can be interpreted to mean that some of the damage to the cell caused by these agents is similar to that caused by u.v. radiation. If the *UV* locus controls a repair mechanism this same mechanism might also repair damage done by radiomimetic chemicals. Howard-Flanders *et al.* (1962) suggested that UV^R reactivation acts on certain photoproducts associated with thymine in DNA. There is as yet no evidence that radiomimetic agents produce any changes in DNA thymine comparable to the thymine dimerization produced by u.v. radiation (Beukers & Berends, 1961). Nor is there any evidence that the damage done by radiomimetic agents can be photoreactivated as can thymine dimers (Setlow & Setlow, 1961; Wulff & Rupert, 1962). It is possible that the UV^R mechanism repairs other kinds of damage to DNA as well as thymine photoproducts. It is clear that the UV^R mechanism does not significantly repair damage done by nitrosoguanidine or proflavine since UV^S organisms are relatively resistant to these chemicals. In fact, in the mutation from *E. coli* strain B to strain B_{s-1}, in which sensitivity to ultraviolet radiation increases and sensitivity to Mitomycin C remains essentially unchanged, sensitivity to nitrosoguanidine and proflavine decreases. This is difficult to reconcile with the observation that in radioresistant mutants of *E. coli* strain B, resistance to all the radiomimetic agents increases along with increased resistance to radiation, both u.v. and X-ray (Greenberg & Woody-Karrer, 1963). It is also difficult at present to classify *E. coli* strain B_{s-2}, which resembles *E. coli* strain B_{s-1} in all its properties including a decreased sensitivity to nitrosoguanidine and proflavine, but is still able to reactivate u.v.-damaged phage. Strain B_{s-2} may represent another class of radiosensitive mutants.

The mechanisms responsible for the type of radiosensitivity observed in *Escherichia coli* strains B, s, and w3747$_s$ may result from the lack or loss of an enzyme able to repair kinds of damage different from that repaired by the *UV* locus. Apparently, for strain w3747 to exhibit the degree of radioresistance observed the *UV* and *RA* loci must both be functional. It is not yet clear what the genetic changes are which account for radiation resistance in the various radioresistant mutants of *E. coli* strains B and s (Greenberg & Woody-Karrer, 1963). The fact that there are many different radioresistant mutants of strains B and s would suggest that either mutations in a number of different modifiers of the *RA* locus or a variety of changes within the *RA* locus result in different radioresistant phenotypes.

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Sexual Polarity in *Streptomyces coelicolor*

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SUMMARY

A group of intersterile strains (R^-) have been identified in *Streptomyces coelicolor* Strain A 3 (2). They give fertile crosses with other strains (R^+) which in turn are fertile among themselves. In $R^- \times R^+$ crosses the contribution of R^+ markers to the recombinant progeny is usually small. The fertility factor(s), which cannot be eliminated by acridine dyes, may be transferred at high frequency in $R^+ \times R^-$ crosses, but the fertilized strains display a low fertility.

INTRODUCTION

Practically all the bacteria whose conjugation has been investigated have shown sexual polarity, that is, differentiation into donor (male) and recipient (female) strains. This phenomenon has usually been first observed when, after the performance of numerous crosses, some attempted cross has failed to give recombinant progeny (Lederberg, Cavalli & Lederberg, 1952). The sterility of a cross may be caused by various genetical as well as environmental factors, but as a rule it has been shown to be due to an encounter between two recipient strains.

Sexual polarity is manifested in various ways: the donor strain contributes fewer markers to the progeny (Hayes, 1952; Cavalli-Sforza, Lederberg & Lederberg, 1953); the donor strain may infect the recipient with its fertility factor (Lederberg *et al.* 1952; Hayes, 1953).

In *Streptomyces coelicolor* Bradley & Anderson (1958) reported the occurrence of some sterile crosses, but they gave no indication of the causes of the observed sterility. Hopwood & Sermonti (1962) and Sermonti & Hopwood (1964) observed in *S. coelicolor* A 3 (2) the occurrence of several sterile combinations of strains and a group of strains fertile in all combinations. This study extends these preliminary observations and in particular is concerned with the analysis of reciprocal crosses between strains of opposite 'sex'.

METHODS

The routine techniques used in the genetics of *Streptomyces coelicolor* were described in detail in a review paper by Hopwood & Sermonti (1962).

Strains. All the strains used in this work derive from the wild type of *Streptomyces coelicolor* A 3 (2) and have been obtained either as mutants after ultraviolet (u.v.) or X-ray treatment, or as recombinants from crosses between mutant strains. The symbols and phenotypes of the mutant alleles present in the strains used are shown

in Table 1. The order of the markers used here and the distances between them on the two linkage groups are as follows (Hopwood & Sermonti, 1962; Hopwood, personal communication, for location of *leu-1*):

$$\text{I}\quad \overset{l}{\underset{8}{\text{—}}}\ \overset{m}{\underset{3}{\text{—}}}\ \overset{h}{\underset{11}{\text{—}}}\ a; \qquad \text{II}\quad \overset{u}{\underset{9}{\text{—}}}\ \overset{p}{\underset{18}{\text{—}}}\ s.$$

The order of the markers in linkage group II with respect to those in group I is reversed as compared with the order arbitrarily chosen by Hopwood (1959) and adopted in later papers, for simplicity of description of some data.

Table 1. *Symbols and origins of the strains of* Streptomyces coelicolor *used*

Strains 4 and 8 are mutants of the wild type; these, and strains 109, 118 and 82, which are recombinants, were kindly supplied by Dr D. A. Hopwood. Strains 53, 44 and 43 are recombinants from the same cross (47 *hps* × 15 *m*). Strain 87 is a segregant from cross 109 *au* × 47 *hps*.

The meanings of the symbols are as follows: *arg* (*a*), requirement of arginine; *his* (*h*), requirement of histidine; *met* (*m*), requirement of methionine; *leu* (*l*), requirement of leucine; *phe* (*p*), requirement of phenylalanine; *ura* (*u*), requirement of uracil; *str* (*s*), resistance to streptomycin.

Fertility type					
R^+			R^-		
Code no.	Genotype	Abbreviated symbols	Code no.	Genotype	Abbreviated symbols
4	*his-1*	*h*	44	*his-1*	*h*
53	*met-2, phe-1, str-1*	*mps*	43	*met-2, his-1, str-1*	*mhs*
87	*arg-1, ura-1, str-1*	*aus*	118	*arg-1, ura-1, str-1*	*aus*
109	*arg-1, ura-1*	*au*	8	*his-12*	*h12*
82	*leu-1, met-2, arg-1, str-1, phe-1*	*lmasp*			

Media. A minimal medium (glucose asparagine agar) and two complete media were used; the latter are a sporulation medium and a reproduction medium (medium 1, according to Hopwood & Sermonti, 1962).

Crosses. Crosses were carried out by mixing the spores of two strains on slopes of reproduction medium, harvesting the spores of the mixed cultures after three or four days and plating these on selective media (minimal media variously supplemented). Characterization of the recombinants was done by replica-plating on diagnostic media.

Fertility tests. Various procedures have been adopted. The one most commonly used was as follows. Spores of the tester strain (usually 'sterile', see later) were streaked with a loop on a large area of a Petri dish containing well-dried reproduction agar medium. Spores of two or three strains to be tested were streaked separately side by side across the streak of the tester strain (Pl. 1). After incubation for 3–4 days the mixed culture was replica-plated on selective media: two media each selective for one of the parents, and one or more media selective for recombinants. The cross was considered fertile when on the latter media at least 24 recombinant colonies appeared in the area corresponding to the mixed culture, and sterile when recombinants were entirely absent and this sterility could not be attributed to imbalance between the two parent strains or to very

close linkage between the selected markers. Some 'sterile' crosses gave rise sometimes to a few minute colonies which turned out to be syntrophic growths (Pl. 1).

A more quantitative sterility diagnosis was obtained when the spores of an attempted cross performed by the standard procedure, then plated at high densities (at least 10^6 spores/dish) on a medium selective for unlinked markers, gave rise to no or very few recombinants. 'Fertile' crosses gave recombinants with frequencies of the order of 10^{-4} to 10^{-3} plated spores. These values appeared in approximately balanced mixed cultures (Table 2). The fertility test was always affected by some degree of uncertainty and subjectivity. In the present work strains have been used which showed the most extreme characteristics as regards the fertility test (Table 1).

Table 2. *Fertility and sterility in* arg-1, ura-1 (str-1*) × his-1 *crosses of* Streptomyces coelicolor *mutants*

	Parental types (%)		Recombinant types (per 10^6 spores plated)	
Selective medium (MM +)†:	ARG+URA	HIS	URA	ARG
Crosses	Frequencies of parental and recombinant types			
(strain A) (strain B)	(A)	(B)		
109 *au* R^+ × 4 *h* R^+	39	61	56·0	140·8
109 *au* R^+ × 44 *h* R^-	6	94	174·5	348·7
118 *au* R^- × 4 *h* R^+	11	89	51·0	180·0
118 *au* R^- × 44 *h* R^-	19	81	0·0	0·0
	89	11	0·0	0·0

* The character *str-1* has been ignored. Strain 109 is sensitive to streptomycin, strain 118 is resistant to streptomycin. Strain 87 *aus* R^+ gives results similar to 109 *au*.

† The substances added to the minimal media (MM) are indicated with the same symbols used to indicate the requirements (see Table 1; or resistance, Table 4), but in capital letters.

RESULTS

Fertile and sterile crosses

Some strains of *Streptomyces coelicolor* A 3 (2), when crossed with one another, did not produce any recombinant progeny or gave rise only to a very small number of recombinants (less than $1/10^6$ plated spores) on given selective media. The sterility diagnosis (see Methods) was, however, sometimes not absolute since crosses which did not give rise to any recombinants on one selective medium sometimes gave a small number of recombinants on another medium. Moreover, a combination of strains which had been sterile in some crosses could, after a few months storage of the strains, become moderately fertile. However, it has been possible to collect a small group of strains which gave the most regular negative response, which has been called R^- (R = recombination). These gave no or very few recombinants when crossed with one another, but were fertile when crossed with any other strain (R^+). Crosses between the various R^+ strains were also regularly fertile. The situation may therefore be summarized as follows: $R^- \times R^-$ = sterile or semi-sterile; $R^- \times R^+$ = fertile; $R^+ \times R^+$ = fertile. A range of strains with a degree of fertility intermediate between that of the R^- and R^+ strains used in this work (Table 1) probably exists.

Analysis of crosses between R^+ *and* R^- *strains*

In many bacteria, and in particular in *Escherichia coli* K12, the alleles of the male parent appear in the progeny with frequencies considerably lower than those of the alleles of the female parent (Hayes, 1953). In $Hfr \times F^-$ crosses the larger the distance between an allele of the *Hfr* strain and the origin of the chromosome the smaller is the frequency of this allele among recombinants (Wollman, Jacob & Hayes, 1956). However, estimation of allele ratios is made difficult by the obligatory use of selected markers which will appear in the progeny with frequencies of 100 %, affecting the frequency of linked alleles. Even when an unselected marker is not linked with a selected one, the allele ratio at the first locus varies according to whether the selected *Hfr* marker precedes or follows the selected marker in the process of transfer.

In *Streptomyces coelicolor* there are two linkage groups which appear to segregate independently. Hopwood & Sermonti (1962) gave good evidence indicating that these might correspond to two distinct chromosomes. We shall therefore provisionally assume that these are independent also in the process of transfer and integration. On this assumption, by selecting markers on one linkage group, we can obtain an estimate of the allele ratio at the loci on the other linkage group, among the recombinants from the mixed culture. In the present paper we shall mainly consider four-point crosses with two selectable alleles in repulsion in each linkage group. Recombinants have been detected on two media, on each of which two alleles in one linkage group were selected and the alleles on the other were non-selected and therefore available for analysis, which has been carried out by the routine procedure.

For each pair of linked genes four segregant classes occur, which are indicated in Table 3 by the four combinations of two symbols, R^+ and R^-. R^-R^- indicates the parental class with the R^- alleles; R^+R^+ the one with the R^+ alleles; R^+R^- the recombinant class deriving the left-hand (see map in Methods) allele from the R^+ parent and the right-hand allele from the R^- parent; R^-R^+ the complementary class. If we consider, for instance, the first linkage group in cross $R^+h \times R^-aus$:

$$\frac{R^+ \quad h \quad +}{R^- \quad + \quad a}$$

The four combinations of symbols will correspond to the four combinations of markers as follows:

R^-R^-	+	*a*
R^+R^+	*h*	+
R^+R^-	*h*	*a*
R^-R^+	+	+

The data in Table 3 permit the same kind of treatment that was developed in the analysis of heteroclones (Hopwood & Sermonti, 1962; Hopwood, Sermonti & Spada-Sermonti, 1963; Sermonti & Hopwood, 1964) except for the test of independence between the two linkage groups, which cannot be made. The almost regular excess of the R^-R^- class in respect to the R^+R^+ class points to a disturbance affecting the chromosomes derived from the R^+ parent, while the excess of the R^+R^- class in

respect to the R^-R^+ indicates a disturbance on the right side of the R^+ chromosome (probably accompanied in some cases by a disturbance on the left side of the R^- chromosome).

'Reciprocal' crosses $R^+ \times R^-$

The occurrence of strains identical as regards the genetic markers and behaving differently in the fertility test (Table 1) allows the performance of reciprocal crosses, for instance two crosses between strains *aus* and *h*, in one of which the *aus* strain is the R^- and in the other the R^+. Since the cause of sterility and fertility in various strains is not yet known, and sterility (or fertility) of the various strains may be due to different conditions, the reciprocity of crosses may be stated only with a certain degree of approximation. However, the data in Table 3 clearly show that R^+ markers are generally in defect. Out of a total of six crosses, implying analysis of twelve chromosomes, ten chromosomes have the male markers in a more or less pronounced minority (Table 3). The data in Table 3, first two columns, re-elaborated in Table 4 to make the position of the unselected markers more evident, clearly show that the different frequencies of the alleles cannot be attributed to the linkage of the unselected markers with the selected ones. In fact, the selective conditions are identical in the reciprocal crosses, while the ratio of the frequency among the R^+ and R^- alleles of the unselected linkage group is inverted. The deficiency of the R^+ markers must therefore be attributed to some other factor, most probably the fertility factor.

Table 3. *Frequencies of allele combinations at pairs of linked loci*, reciprocal crosses*

'Sex' of parents‡	Allele combinations (origin of alleles§)		Crosses† ... $\frac{h\ +}{+\ a}$ × $\frac{+\ +}{u\ s}$ Linkage group I		Crosses† ... $\frac{m\ +}{+\ h}$ × $\frac{p\ s}{+\ +}$ Linkage group II		Crosses† ... $\frac{m\ +}{+\ a}$ × $\frac{+\ p\ (s)}{u\ +\ (s)}$ Linkage group I		Crosses† ... $\frac{m\ +}{+\ a}$ × $\frac{+\ p\ (s)}{u\ +\ (s)}$ Linkage group II	
R^+ / R^-	Parental	R^-R^-	545	349	136	153	175	171		
		R^+R^+	118	148	5	1	19	10		
	Recombinant	R^+R^-	33	72	0	1	7	10		
		R^-R^+	20	30	0	0	6	4		
R^- / R^+	Parental	R^-R^-	381	211	76	*71*	102	*39*		
		R^+R^+	7	40	45	*101*	43	*178*		
	Recombinant	R^+R^-	2	27	0	16	2	16		
		R^-R^+	0	14	0	4	1	0		

* The segregation of markers of linkage group I was studied on a medium selective for markers of linkage group II and vice versa (see Table 4).

† The alleles of the parent strains are written as in the presumptive zygotes, one parent above and the other below the lines, which refer to the two chromosomes.

‡ The symbol above the line refers to the parent strain written above the lines beside crosses.

§ The symbols R^+ and R^- indicate the parent from which each allele derives. The first symbol refers to the left-hand allele of either linkage group, the second to the right-hand one (see text).

In two crosses, both involving the strain *mps* R^-, the R^- alleles on chromosome II are in a minority (Table 3; see figures in italics). The R^+R^- recombinants are still in excess over the complementary ones. In both cases the defect may be attributed to a single disturbance to the left end of the R^- chromosome, perhaps accompanied by a less pronounced disturbance to the right end of the R^+ chromosome.

Table 4. *Allele ratios in reciprocal crosses**

Selective media (MM +)†			HIS+ARG+STR		URA	
			Frequency of alleles (%)‡			
	Crosses§		Unselected markers	Selected markers	Selected markers	Unselected markers
Chromosome ...	I	II	I	II	I	II
R^+	*h* +	+ +	25 6	100 0	0 100	37 30
R^-	+ *a*	*u s*	75 94	0 100	100 0	63 70
R^-	*h* +	+ +	97 98	100 0	0 100	77 82
R^+	+ *a*	*u s*	3 2	0 100	100 0	23 18

* Detailed analysis in Table 3.

† See note to Table 2.

‡ Each number represents the frequency of the allele in the corresponding position (under 'crosses').

§ The alleles of the parent strains are written as in the presumptive zygotes, one parent above and the other below the lines, which refer to the two chromosomes.

In several cases when two strains differ in more than two markers on one linkage group, it is observed that the R^+ markers show frequencies gradually decreasing from the left to the right, in agreement with what is found in the four-point crosses. Table 5 shows an example in linkage group I. The order of the frequencies of the R^+ alleles corresponds very well to the order of the genes along the linkage group.

Table 5. *Gradient in allele frequencies on linkage group I*

Cross ... 82 *leu*-1 *met*-2 *arg*-1 *str*-1 *phe*-1 $R^+ \times 8$ *his*-12 R^-

Selective medium: (MM +)* ... LEU+MET+HIS+ARG−STR

Genotype				no.	Cross-over in region	Genotype				no.	Cross-over in region
+	+	*h*	+	448	—	+	*m*	+	+	10	1, 3
l	*m*	+	*a*	6	—	*l*	+	*h*	*a*	0	1, 3
l	+	*h*	+	36	1	+	*m*	*h*	+	1	1, 2
+	*m*	+	*a*	1	1	*l*	+	+	*a*	0	1, 2
l	*m*	*h*	+	2	2	+	+	+	+	0	2, 3
+	+	+	*a*	0	2	*l*	*m*	*h*	*a*	0	2, 3
l	*m*	+	+	15	3	*l*	+	+	+	0	1, 2, 3
+	+	*h*	*a*	0	3	+	*m*	*h*	*a*	1	1, 2, 3

	l	*m*	+	*a*
	+	+	*h*	+
Regions		1	2	3
Allele ratios	59	36	32	8
	461	484	488	512

* See note to Table 2.

Transmissibility of the R factor

Only a few preliminary data bearing on this point have been collected. Sterility segregates in some crosses, and in fact several of the R^- strains used in this work were obtained as recombinants from crosses, together with R^+ strains (Table 1). The large majority of segregants is, however, of the R^+ type (Hopwood & Sermonti, 1962). The fertility factor may be transferred at high frequency in $R^+ \times R^-$ crosses (Hopwood & Sermonti, 1962). Strains made fertile in this way, however, show a low degree of fertility and in crosses with R^- strains the markers of the fertilized parent do not show any clear tendency to be less frequent. Nothing is yet known about the nature of the fertility factor. We have frequently observed that sterile strains become moderately fertile with time, requiring continuous purification to preserve the character. All attempts to eliminate the R factor by means of acridine dyes, which have been effective in *Escherichia coli* (Hirota, 1960), have been unsuccessful.

DISCUSSION

The compatibility system in *Streptomyces coelicolor*, like that of the other bacteria showing sexual differentiation, involves a group of intersterile strains (R^-) and a group of strains fertile in all combinations (R^+). By analogy with *Escherichia coli* K12 (Hayes, 1953) we may suppose that the strains of the first group act as recipients (females) and those of the second as donors (males). The latter may presumably act also as recipients in $R^+ \times R^+$ crosses. The only evidence of a sexual polarity is based on the different contributions to the progeny of the markers of the two 'sexes'. The R^+ markers, as a rule, are less frequent. In this respect also *S. coelicolor* behaves in the same way as other bacteria (for review see Jacob & Wollman, 1961). The deficiency of the R^+ markers may be attributed to the incompleteness of the male chromosomes at their right ends, in all the R^+ strains examined so far.

The occurrence of a gradient in the frequencies of R^+ alleles, decreasing from left to right, points to a situation corresponding to that in *Hfr* strains in *Escherichia coli* K12. Occasionally the R^- markers are in defect. In this case the deficiency may be attributed to a loss at the left end of chromosome II, i.e. at the end opposite that at which the deficiencies in the male chromosomes are observed. This may be due to a tendency of the defective female chromosome to become incomplete at the left end, perhaps as a consequence of a post-zygotic loss. This shortage of markers of the recipient strains was observed by Lederberg (1949) in heterozygous strains of *E. coli* K12 and by Smith & Stocker (1962) in *Salmonella typhimurium*.

The infective nature of the fertility factor is far from clear. In $R^+ \times R^-$ mixed cultures it seems transmissible at high frequency, but the newly fertile strains display only a low fertility. This situation has a parallel in *Pseudomonas aeruginosa* (Holloway & Fargie, 1960). The failure of acridine dyes to render the R^+ strains infertile is further evidence of the integrated nature of the fertility factor in R^+ strains. Moreover, although the behaviour of the R factor in the progeny of crosses has not yet been extensively studied, there is some evidence that R^- strains and R^+ strains may be produced among recombinants of the same cross, thus indicating the possible segregation of the character. However, the fertility factor can pre-

sumably sometimes be lost spontaneously, or following irradiation, since all strains of *Streptomyces coelicolor* A3 (2) used in genetical studies, both R^+ and R^-, were derived from a single original culture (Hopwood, 1959), which had been purified at least once by the isolation of a single spore.

In conclusion we cannot yet decide whether the fertility factor(s) occur(s) in the free or in the integrated state in *Streptomyces coelicolor*, both conditions being probably present in the different strains. Since *Streptomyces coelicolor* has two linkage groups and perhaps two chromosomes, it will be of great interest to establish whether one or two fertility factors are involved in the process of genetic transfer and whether the markers of the two linkage groups behave independently during this process.

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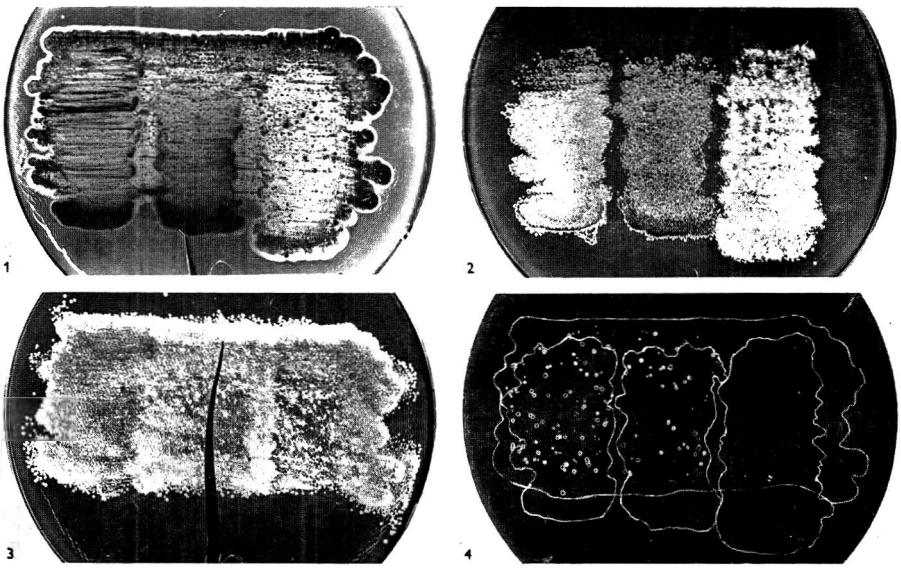

G. SERMONTI AND S. CASCIANO

(*Facing p.* 301)

EXPLANATION OF PLATE

Fertility test in Streptomyces coelicolor

Fig. 1. Mixed cultures on reproduction medium: the tester strain (118 *arg*-1 *ura*-1 *str*-1 R^-) was streaked first and the three strains to be tested (all requiring histidine; from the left, two R^+ and one R^-) were cross-streaked on the tester strain.

Fig. 2. Replica plate of the mixed cultures on to a medium supporting growth of the tested strain: minimal medium (MM) + histidine.

Fig. 3. Replica plate of the mixed cultures on to a medium supporting growth of the tester strain: MM + arginine + uracil.

Fig. 4. Replica plate of the mixed cultures on to a medium supporting growth of recombinants only: MM + arginine.

The white lines have been drawn to mark the zones corresponding to the tester and the tested strains. Observe recombinant colonies in the two fertile crosses (left and centre), while only sparse traces of abortive growth derive from the 'sterile' cross (right).

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Investigations about the Formation of Incomplete Forms of Fowl Plague Virus

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SUMMARY

During the formation of incomplete fowl plague virus, normal amounts of viral compounds are synthesized. The S-antigen, however, cannot be demonstrated within the cytoplasm in appreciable amounts by fluorescent antibody. The oligonucleotide pattern and the specific radioactivity of the ribonucleic acid (RNA) of incomplete forms are almost identical with those of standard virus. In contrast to chemically inactivated viruses no indication of multiplicity reactivation was found with incomplete forms. It is suggested that the incomplete forms are lacking in identical pieces of their genome, but contain that part of their RNA which codes for 'early protein', S-antigen and haemagglutinin.

INTRODUCTION

Incomplete forms of influenza viruses are spontaneously released from the host cell and occur when infectious viruses are passaged at a high multiplicity (von Magnus phenomenon) (von Magnus, 1954). They contain less ribonucleic acid (RNA) and ribonucleoprotein inner component (S-antigen) than complete virus (Ada & Perry, 1955; Lief & Henle, 1956; Rott & Schäfer, 1961). A low infectivity/haemagglutination ratio is a characteristic of incomplete forms. Their sedimentation constant is lower than that of standard virus and they have a higher lipid content (Uhler & Gard, 1954). The coat of the incomplete forms resembles those of standard viruses (Waterson, Rott & Schäfer, 1961; Barry, Waterson & Horne, 1962). They agglutinate erythrocytes like standard virus and also cause immunity and interference (von Magnus, 1954; Lief & Henle, 1956; Rott & Schäfer, 1961). The haemagglutinin isolated from them is identical with that from infectious virus particles (Waterson *et al.* 1961; Rott & Schäfer, 1961). They are to be distinguished from viromicrosomes, which are particles with a low infectivity/haemagglutination ratio obtained from infected cells (Rott & Schäfer, 1961).

Very little is known about the formation of incomplete influenza virus. The fowl plague virus, an influenza A virus, appears to be a suitable model to elucidate this problem, because it can be easily grown in tissue culture, where it produces incomplete forms under special conditions (Rott & Schäfer, 1960). The components of fowl plague virus appear in the host cell at different times and at different sites. The S-antigen, the carrier of viral RNA, can be demonstrated in the nucleus 2–3 hr. after infection whereas the haemagglutinin can be shown 1 hr. later exclusively in the cytoplasm. In later phases of the multiplication cycle the S-antigen emerges from the nucleus into the cytoplasm (Breitenfeld & Schäfer, 1957).

In the present paper a study is reported of the production of virus components, particularly RNA and S-antigen, under conditions promoting the formation of incomplete forms.

METHODS

Virus. The Rostock strain of fowl plague virus was used in all experiments. Standard virus was obtained by weekly passages of diluted virus (10^1 to 10^2 LD 50) in embryonated eggs.

Biological tests

Infectivity was determined by plaque test using chick embryo cells (Rott & Schäfer, 1960). Test material was diluted in ten-fold steps in buffered saline (pH 7·2; 0·02 M-phosphate), containing 0·72 % (w/v) NaCl and each dilution was plated in duplicate. Plaques were counted 3 days after infection.

Complement-fixation tests were done by a modification of the micromethod of Fulton & Dumbell (1949) described by Hennessen (1955). The complement-fixing activity of viral antigen was compared using convalescent serum from mice infected with influenza FM 1, and normal mouse serum. The convalescent serum reacts well with fowl plague S-antigen, but with neither the haemagglutinin nor the intact virus particles (Schäfer, 1957).

Haemagglutination (HA) tests were carried out by a standard method using plastic plates and a 1 % suspension of chicken erythrocytes (Davenport, Rott & Schäfer, 1960).

Tissue culture

Media and solutions

ES: Earle's saline + 100 i.u. penicillin per ml. + 100 μg. streptomycin.
LaYe: ES with 1 % lactalbumin-hydrolysate and 0·2 % yeast extract.
MM: Minimal medium: ES glucose free + 5 mM glucose + 2 mM glutamine (Eagle & Habel, 1956).
PBS: Phosphate buffered saline (Dulbecco & Vogt, 1954).
CS: Filtered calf serum.

Cells

In most experiments monolayers from 11-day-old chick embryos were employed, cultured in LaYe + 10 % CS.

For experiments with fluorescent antibodies monolayers of chicken embryo lung cells were prepared. Lungs of 16-day-old chick embryos were trypsinized, the cells distributed into Petri dishes, whose bottoms were fitted with microscopic slides, and cultivated in LaYe + 15 % CS.

Fluorescent antibody

The antiserum used was prepared by immunizing a rabbit with S-antigen isolated from fowl plague virus. The preparation of fluorescent labelled antibodies, and the tests for specificity, as well as the cytological technique, were performed according to Breitenfeld & Schäfer (1957).

RESULTS

Occurrence of von Magnus effect with fowl plague virus in tissue culture

For undiluted passages (UP) tissue cultures were used exclusively. Monolayers of primary cultures of chick embryo cells (1×10^7 cells/Petri dish) which had been incubated in 90 mm. Petri dishes for 24 hr. were washed once with PBS. The cells were inoculated with 2 ml. of infectious embryonic fluid, containing 5×10^8–1×10^9 pfu/ml. (1st UP), or with 2 ml. of undiluted tissue culture medium of the preceding passages (2nd–4th UP) harvested 14 hr. post infection (p.i.). After 30 min. adsorption at 37° the cells were washed three times with PBS and covered with 6 ml. of MM. The time of addition of MM was arbitrarily taken as the start of infection (time 0 p.i.). 14 hr. p.i. the MM was removed, centrifuged 10 min. at 3000 rev./min., and the supernatant used for the next passage. The pfu/HA ratio of the yield decreased continuously up to the 3rd UP (Fig. 1). This means that at least in the UP tested and with the conditions given above a von Magnus phenomenon occurred. These results are in accordance with those of former experiments (Rott & Schäfer, 1960).

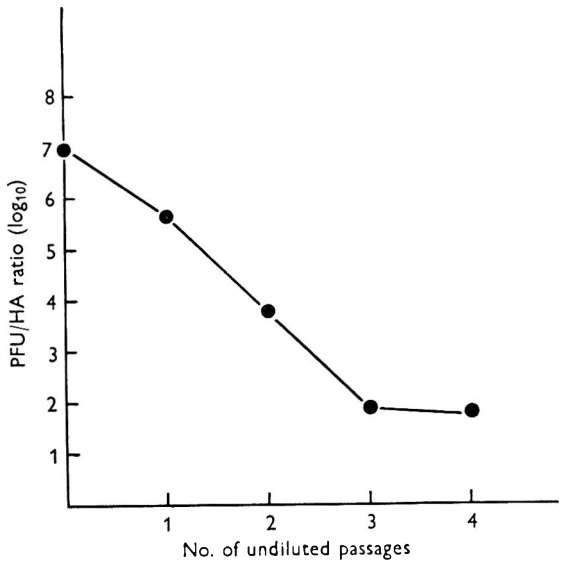

Fig. 1. Infectivity/HA ratio after undiluted passages of fowl plague virus in chick embryo cells.

Occurrence of different biological activities of fowl plague virus during undiluted passages

In order to obtain some information about the effect of the von Magnus phenomenon on the various stages of fowl plague virus multiplication, the appearance of the infectivity and of the haemagglutinating and CF activity was followed in the first 4 UP in the cells. At given intervals after infection of chick embryo cells with infectious embryonic fluid or undiluted culture medium, the MM was removed and the cell layers were washed twice with PBS. Then 2 ml. PBS containing 5% inactivated horse serum was added to each plate, and the plates were frozen at −40°. For each hourly sample, three assay plates were used. Before determining

the various virus specific activities, the cells were scraped and frozen and thawed three times to disrupt them. Cell debris was removed by centrifugation (3000 rev./min, 10 min.) and the supernatant assayed. In all undiluted passages examined, the infectivity decreased in the first 2 hr. It increased markedly in the 1st UP from the 2nd to 6th hr. p.i. This increase was less in the 2nd and the 4th UP. In the

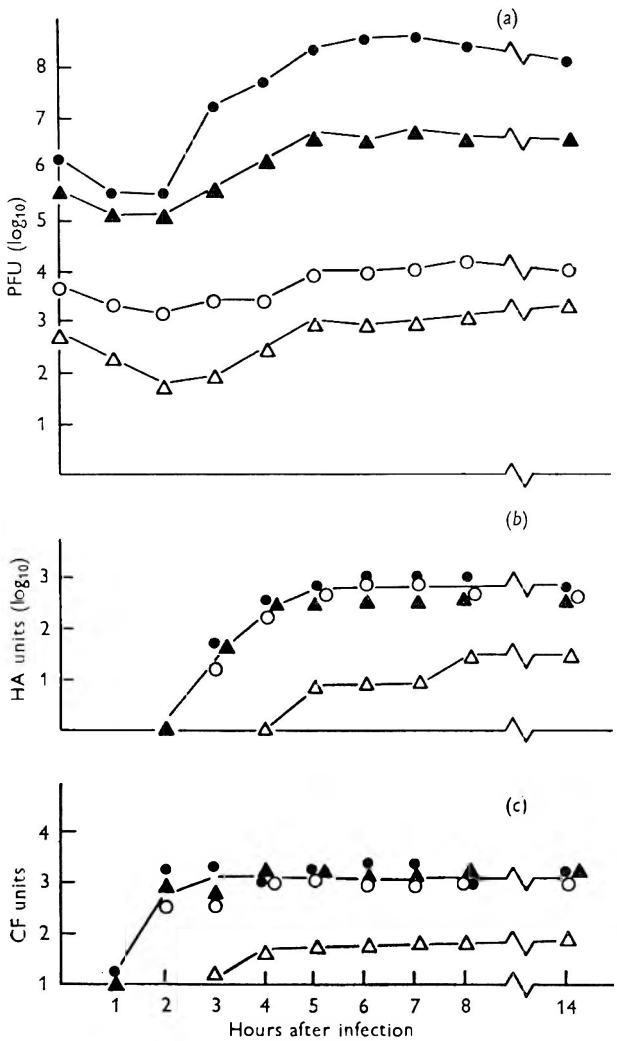

Fig. 2. Behaviour of infectivity, haemagglutinating activity and complement-fixing activity during undiluted passages in chick embryo cells. ●, ▲, ○, △ = 1st, 2nd, 3rd and 4th UP, respectively.

3rd UP, the infectivity remained at about the same level (Fig. 2*a*). Testing all UP in the plaque test the plaque yield decreased in proportion to the dilution. This means that no sign of multiplicity reactivation was observed. The plaque size, however, decreased with each successive UP, and it is possible that this occurs either by reason of interference caused by the increasing number of non-infective particles, or of a phenomenon discussed below. In comparison with the infectivity,

the production of the two viral components (the haemagglutinin and the S-antigen) in infected cells was not quantitatively different in the first 3 UP. In these UP the S-antigen titre increased first between 1 and 2 hr. p.i. and reached a plateau in the 3rd hr. after infection (Fig. 2*b*). The haemagglutinin (Fig. 2*c*) began to increase between 2 and 3 hr. p.i. The maximum value for haemagglutinin was found at 5 hr. p.i. The behaviour of the haemagglutinating and CF-activities in the first 3 UP corresponds well with the data obtained by Breitenfeld & Schäfer (1957). On the other hand, in the 4th UP S-antigen and haemagglutinin could not be demonstrated until 1 hr. later, and then in a lower concentration than in the first 3 UP. For our further studies viral materials derived from standard passages and the 2nd UP, respectively, were used.

Occurrence of viral RNA during undiluted passage

In order to determine whether the RNA of the S-antigen is formed as well as the protein in UP, a chemical RNA characterization was performed. This method, using the shift in the oligonucleotide pattern of the RNA of infected cells toward that of

Table 1. *Oligonucleotide pattern of RNA of cells infected with standard and second UP, of uninfected cells, and of purified virus RNA*

For experimental details see Scholtissek & Rott (1961). ^{32}P pulse from 1 to 4 hr. p.i. In Expt. 1 0·1 mC. ^{32}P/culture in Expt. 2 0·05 mC. ^{32}P/culture was used. In each case seven cultures were sampled. The counts in total RNA were calculated from the sum of the oligonucleotides determined.

Expt.	Oligonucleotide ratio	Uninfected cells	2nd UP	Standard passage	Virus*
1	Pfu/HA	—	2·5	6·2	—
	C/U	1·18	1·05	1·08	0·86
	C/AC	3·14	2·78	2·75	2·60
	C/GC	1·76	1·94	1·86	2·08
	Total counts in RNA	213,153	264,983	258,718	—
2	Pfu/HA	—	2·8	5·9	—
	C/U	1·25	1·09	1·04	0·86
	C/AC	3·27	3·04	2·98	2·60
	C/GC	1·79	2·02	2·03	2·08
	Total counts in RNA	102,238	121,576	122,345	—

* Taken from Scholtissek & Rott (1961), see also Table 2.

viral RNA labelled with ^{32}P, has been described in detail elsewhere (Scholtissek & Rott, 1961). ^{32}P pulses were given in this experiment from 1 to 4 hr. p.i. The amount of RNA synthesized in the cells infected with viral material of the 2nd UP did not differ significantly from that of cells infected with a dose of standard virus sufficient to infect all the cells (Table 1). This indicates that the RNA metabolism was similar during the formation of incomplete forms and after standard infection. In addition, there was no significant difference between the samples in the shift of the oligonucleotide pattern towards that of viral RNA. This is evidence that almost as much RNA, behaving chemically like viral RNA, was synthesized in the 3rd UP as in the standard passage.

Localization of S-antigen in cells during the formation of incomplete forms

Although viral RNA and S-antigen are produced in the cells under conditions of incomplete virus formation, the full amount is apparently not incorporated into the virus particles. This could result from an inhibition of the migration of the S-antigen out of the nucleus or, on the other hand, from a disturbance of the assembly of the viral components at the cell periphery. Accordingly, we examined the localization of the S-antigen with fluorescent antibodies at different times after infection during the 3rd UP and the standard passage. Embryonic chicken lung cells were used for this purpose, since they give a clearer picture than fibroblasts. Controls showed that the pfu/HA ratio decreased in lung cells infected with the 2nd UP to the same degree as in the chicken embryo cultures used for the growth experiments. From the 4th to the 10th hr. p.i. the cells attached to the slides were fixed, stained with S-antigen fluorescent antibodies and examined microscopically.

Nuclear fluorescence was already present to nearly the same degree 4 hr. p.i. in cells infected with the standard virus and the 2nd UP, indicating again that the speed of S-antigen production is nearly the same in both systems. A notable difference, however, was obtained with respect to the appearance of S-antigen in the cytoplasm in the late stages of infection. Plate 1, fig. 1 and 2, shows chicken lung cells at 10 hr. p.i. infected with standard virus and with the 2nd UP. The fluorescence of the 3rd UP preparation is retained in the nucleus, whereas that of the standard virus preparation has spread to the cytoplasm. It seems, therefore, that during the formation of incomplete forms the bulk of the S-antigen remains in the nucleus.

RNA composition of incomplete forms

Incomplete forms may incorporate cellular RNA instead of viral RNA (Morgan, Rifkind & Rose, 1962). In order to test this suggestion, virus of the 3rd UP was produced in the presence of ^{32}P and the oligonucleotide pattern of its labelled RNA determined. For the labelling of the virus preparations the ^{32}P was added immediately after infection. The viral material was collected 14 hr. after infection, purified by adsorption-elution with chicken erythrocytes, and by centrifugation. For details see Scholtissek & Rott (1961). There was no significant difference between the pattern of normal viral RNA and that of the 3rd UP (Table 2). The latter shows a somewhat higher variation, but it is completely different from the pattern of total cellular RNA. The radioactivity in the RNA of the incomplete forms was about one-third of that found in standard virus. These results correspond with earlier results which were concerned with the RNA and S-antigen content (Ada & Perry, 1955; Lief & Henle, 1956; Rott & Schäfer, 1961). Thus the specific activity in the RNA of standard and incomplete viruses is almost identical. The results suggest that at least the major part of the RNA contained in incomplete forms of the 3rd UP is not derived from the cellular RNA.

DISCUSSION

In the first undiluted passages of fowl plague virus in embryonic chicken cells, a von Magnus phenomenon is observed, i.e. the cells release virus with a low infectivity/HA ratio (see also Rott & Schäfer, 1960). In cells releasing incomplete virus the production of S-antigen and of haemagglutinin differs neither quantitatively nor

temporally from that formed in cells producing standard virus. In addition, chemically identifiable viral RNA is produced in nearly the same quantity in the two circumstances. Studies with fluorescent antibody to the S-antigen showed that this failed to appear in the cytoplasm when incomplete virus was being formed, as it does in cells producing standard virus. This retention of S-antigen within the nucleus may be one reason for the relatively low content of this antigen in incomplete virus, and for the fact that it never accumulates in appreciable amounts in the cytoplasm. These findings appear to conflict with those of Moffat, Holtermann & Hillis (1960), who found less fluorescence, rather than more, in the nuclei of cells infected with large inocula of influenza virus, and who suggested that in the von Magnus phenomenon there was less S-antigen formed, and that this was the reason why less was incorporated into the virus particles. However, they used calf kidney cells, and even with low inocula the virus released has a low infectivity/HA ratio. This suggests that the multiplication cycle in calf kidney cells is not strictly comparable with that in chick embryo cells. That the low content of S-antigen in incomplete virus released from chick cells cannot be ascribed to defective production of this antigen is supported by the fact that in chorioallantoic cells the amount of S-antigen found in the cells is nearly the same when incomplete virus is being produced as it is under standard conditions (Lief & Henle, 1956).

Table 2. *Oligonucleotide pattern of the RNA of purified virus, virus from the 3rd UP, and cellular RNA, respectively*

Pulse length of cellular RNA 3 hr. For the labelling of the virus preparations the ^{32}P was added immediately after infection. 3 hr. p.i. most of the viral RNA is synthesized (Scholtissek & Rott, 1961).

	Incomplete forms of the 3rd UP				Fowl plague RNA		Cellular RNA	
C/Oligo-nucleotide	Expt. 1	Expt. 2	Expt. 3	Expt. 4	3 expts.*	Greatest diversion from average value	9 expts.	Greatest diversion from average value
C/U	0·86	0·86	0·89	0·86	0·86	0·00	1·26	0·12
C/AC	2·72	2·28	2·84	2·46	2·67	0·11	3·07	0·40
G/GC	2·12	2·38	2·40	—	2·30	0·12	1·80	0·15
C/GAC (AGC)	3·02	3·58	3·19	—	3·50	0·20	2·40	0·45
C/AAU (AU)	1·44	1·49	1·47	1·42	1·60	0·15	2·45	0·16
C/GU	1·50	1·89	1·84	1·53	1·60	0·20	2·30	0·32

* Average of three experiments, determined together with the experiments of the 3rd UP. The values differ a little from those published earlier (Scholtissek & Rott, 1961).

The retention of fowl plague S-antigen within the nucleus has also been observed in chick embryo cells treated with p-fluorophenylalanine (FPA) (Zimmermann & Schäfer, 1960). The dose effect curve of FPA on the multiplication of fowl plague virus in chicken cells suggests that at low doses virus particles are produced with a low infectivity: HA ratio; this also suggests the formation of some kind of incomplete forms. Zimmermann & Schäfer (1960) suggested that FPA causes an inhibition of the transport mechanism by a disturbance of protein synthesis. It is possible that such a transport mechanism is lacking or diminished in some other types of cell, even without treatment by FPA. For example, in Earle's L cells the S-antigen is localized strictly to the nucleus (Franklin & Breitenfeld, 1959). Failure of the

transport mechanism may be a common factor in all these instances, including the von Magnus phenomenon. Nevertheless, despite the extremely low infectivity, incomplete fowl plague virus may contain as much as one third of the normal amount of S-antigen. Morgan, Rifkind & Rose (1962) suggested that cellular material might be incorporated into these forms; but it has been shown that the RNA corresponds in its oligonucleotide pattern and specific activity to that of viral RNA rather than host RNA.

The possibility that an entire content of genetic material is carried by a small number of particles, and that the remainder contain none, can be excluded by our results. On the one hand, the ability of second UP incomplete forms to induce the production of the normal amounts of S-antigen and HA within the cells, in spite of the thousand-fold lower infectivity, suggests that many particles can code for at least these two proteins. On the other hand, the number of infective particles is so low that if the genetic material were confined almost exclusively to them, there would be very much less RNA than the 30% in fact found. One possibility of unifying these observations is that the non-infective particles all lack at least one identical piece of RNA. If different particles lacked different pieces of RNA (i.e. of the viral genome) multiplicity reactivation should be demonstrable, but this is not the case. Complementation by multiple infection has not been observed. The piece of viral RNA which codes for the formation of HA must in any case be present, because the yield of HA is not significantly affected up to the 3rd UP. By way of contrast, chemically inactivated fowl plague virus, where the treatment might be expected to affect all parts of the genome, can be reactivated by multiple infection (Scholtissek, Rott & Schäfer, 1962; Schäfer & Rott, 1962), and the production of HA decreases rapidly and almost in parallel with the infectivity (Scholtissek & Rott, 1963).

These groups of observations are compatible with the idea that the synthesis of the RNA or, as suggested by Hirst (1962), pieces of the RNA, starts at one end. When incomplete virus is being produced, the RNA is removed from the template before its full length is completed. That part which codes for HA, S-antigen and 'early protein(s)' (Scholtissek & Rott, 1961) must be towards that end where synthesis begins. If one or a small number of fowl plague RNA molecules of normal length are introduced into the cell, production will proceed normally. However, the setting up of too many 'factories' in the nucleus, as in the first UP, could lead to the formation of shortened RNA because, perhaps, of a local lack of enough building blocks. Once these are produced the process could become in some sense *autocatalytic*. If such shortened pieces of RNA are less firmly attached to the template, and their progeny tend to release from it even earlier, shorter and shorter pieces would be produced. In the end, the RNA would be too short to code even for the HA, S-antigen and 'early protein', and the system would be exhausted. If this assumption is correct, then one should expect a diminution in the plaque size from one passage to the next, as has been observed. There are, therefore, two separate kinds of aberration to consider. First, that the virus genome itself becomes incomplete in the sense that some nucleotide sequences of the RNA, or of the pieces of RNA, are lost. Secondly, the transport out of the nucleus of the S-antigen formed within it is impaired. Our findings do not at present enable us to determine the relation of these two defects to each other.

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EXPLANATION OF PLATE

Localization of S-antigen by fluorescent antibody in chicken lung cells 10 hr. after infection. Fig. 1. Inoculated with standard virus. Fig. 2. Inoculated with the second UP.

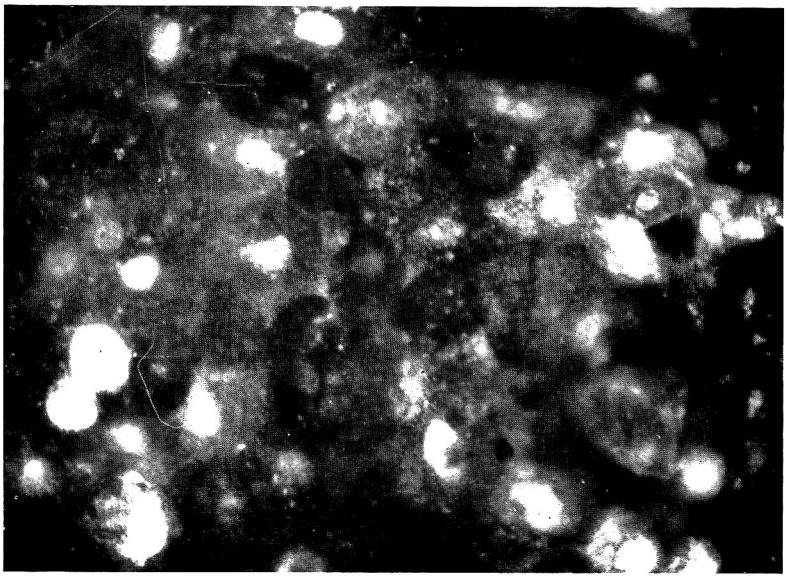

Fig. 1

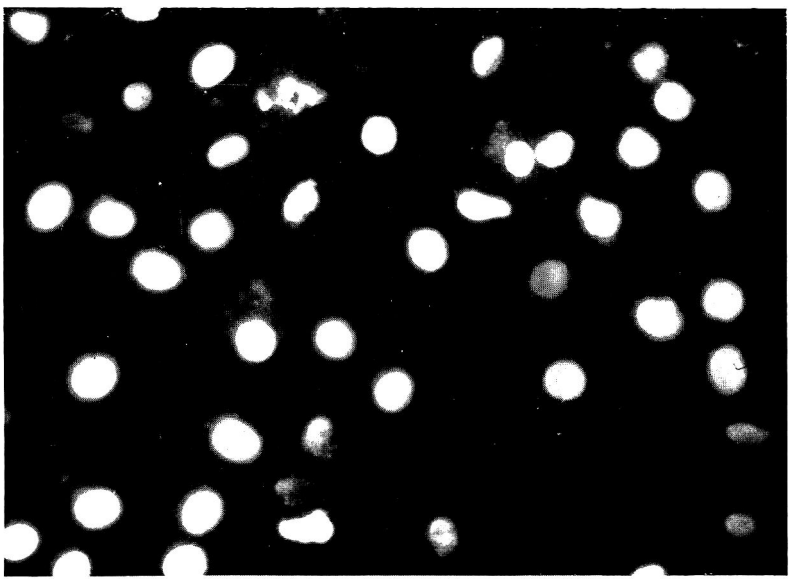

Fig. 2

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Sodium Azide Resistance in *Proteus hauseri*

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SUMMARY

The sodium azide resistance pattern of *Proteus hauseri* is not of the all-or-none unilocal type reported for *Escherichia coli* but rather of the obligatory multi-step or penicillin variety. Independently isolated 1st-step resistant variants possessed similar degrees of resistance to sodium azide. Some properties of azide-resistant variants of *P. hauseri* are described. In support of the above finding it was possible to transduce 1st-step (and only 1st-step) resistance into the wild-type by phage grown on either 1st-, 2nd-, 3rd- or 4th-step resistant organisms. It was also possible to transduce 2nd-step resistance into 1st-step resistant organisms by phage developed on independently isolated 1st-step resistant organisms or by phage from multi-step resistant variants. About 60 % of transduced genes expressed their phenotype in platings done immediately after the adsorption period. It is concluded that a number of loci, not closely linked, and possibly equipotent, control sodium azide resistance in *P. hauseri* and that resistance could be dominant to the wild allele.

INTRODUCTION

Three patterns of resistance development by bacteria to inhibitory agents have been observed (Bryson & Szybalski, 1955). With the obligatory single-step pattern (Manten & Rowley, 1953) organisms possessing maximal resistance may be selected by a single exposure to the agent. No intermediate degrees of resistance are encountered. Multistep resistance is a gradual step-wise process and requires repeated exposure and selection to obtain organisms with a high degree of resistance. This is the mode of development of resistance to penicillin (Demerec, 1948) and most other antibiotics (Bryson & Szybalski, 1955). In the facultative single-step pattern survivors possessing all grades of resistance may be selected by a single exposure to the inhibitor. This is the pattern of resistance elicited by streptomycin (Demerec, 1948). The discrete polygenic mechanisms governing the penicillin type of resistance were successfully dissected by the transformation studies of Hotchkiss (1951), the recombinant analysis of Cavalli (1952) and Cavalli & Maccacaro (1950, 1952), and the transduction studies of Banič (1959). The transformation experiments of Hotchkiss (1952) and the transduction studies of Watanabe & Watanabe (1959*a*) also confirmed the facultative one-step pattern governing resistance to streptomycin.

The inhibitory action of sodium azide on bacterial growth was known since the work of Loew (1891). Keilin & Hartree (1934) demonstrated that sodium azide was an inhibitor of catalase and the differential effect it had against the growth of many Gram-negative organisms as well as aerobes has been exploited in numerous selective media (Forget & Fredette, 1962). Sodium azide resistance was first mentioned as a marker in bacterial genetics by Lederberg (1947). Lederberg (1950) isolated an azide resistant mutant of *Escherichia coli* strain w478 on nutrient agar containing M/500 sodium azide. Cavalli (1952) claimed that sodium azide resistance in micro-

organisms was of the all-or-none type but also stated that 'azide can perhaps be said to belong to the group of drugs giving the streptomycin pattern of resistance'. Bryson & Szybalski (1955) stated that resistance to sodium azide was of the obligatory single-step pattern. Neither Cavalli (1952) nor Bryson & Szybalski (1955) referred to survival studies of micro-organisms exposed to different concentrations of sodium azide to support their claims, but both cautioned that the jump to azide resistance was small. Cavalli & Maccacaro (1950) and Cavalli (1952) crossed independently isolated azide-resistant mutants of *E. coli* and demonstrated that azide resistance was controlled by a gene locus linked to the threonine and leucine loci. No details regarding the isolation of the mutants were given and the concentration of azide used in the experiments was not stated. The concentration of sodium azide used in bacterial crosses (in various media) to select for the azide resistance marker was never greater than M/500, and Hayes (1957) and Jacob & Wollman (1961) used M/1600 and M/850 respectively. Bryson & Szybalski (1955) referred to unsuccessful experiments by P. E. Hartman to transduce azide resistance by means of bacteriophage, but Lennox (1955) was the first to report the transduction of a M/500 azide resistance marker, using phage P1 in an *E. coli* system. He did not directly select for azide resistance but showed that the marker could be jointly transduced with either the leucine or the arabinose marker. Coetzee & Sacks (1960*a*) reported the transduction of streptomycin resistance in *Proteus mirabilis*. An initial failure to transduce resistance to sodium azide stimulated the work reported here.

METHODS

Media. The composition of the MacConkey-type agar, soft agar and the broth employed are described by Coetzee & Sachs (1960*b*). Sodium azide (British Drug Houses) was added to the sterilized broth or to the molten agar at 47°. Azide media were prepared on the day of use. Dilutions of cultures were made in sterile 0·85 % (w/v) saline in distilled water. Solid media were inoculated with 0·1 ml. of dilutions of broth cultures and spread to dryness with a sterile glass rod. McIntosh and Fildes's jars (Mackie & McCartney, 1960) were used for anaerobic cultures. Cultures were incubated at 37° for 48 hr. unless otherwise stated.

Organisms. Proteus mirabilis strains 13, 193, 57 and phages 34/13, 14/13 *str-r* 12/57, 12/57 *str-r* were used throughout. In addition 4 strains of *P. vulgaris* freshly isolated from human faeces were used in survival-curve studies. Strains 13, 193, 57 and the phages have been described as well as the methods used for preparation of high-titre phage stocks and measurement of phage adsorption, and to detect lysogeny in transductants (Coetzee & Sacks, 1960*a*). Variants resistant to sodium azide are identified by *az-r* and a number indicating the step of resistance. Thus 13 *az-r*4 denotes a 4th-step azide resistant variant of strain 13. Catalase activity of cultures was qualitatively tested according to the method of Davis (1955). The flagellar stain and the hanging-drop motility method are described by Mackie & McCartney (1960). Flagellar antisera were prepared, adsorbed and tested according to Kauffman (1951).

Survival studies. An overnight broth culture of the wild-type organism was concentrated by centrifugations to *c.* 2×10^{10} viable organisms/ml. and plated on MacConkey agar containing different concentrations of sodium azide. Plates were

incubated and surviving colonies counted. A colony on the highest concentration of sodium azide was picked into 100 ml. broth and incubated overnight. Dilutions of this culture were made and plated on different azide concentrations. After incubation the selection process was repeated.

Growth-rate experiments. These were done by inoculating *c.* 10^5 organisms contained in 0·1 ml. of a dilution of an overnight broth or sodium azide broth culture into 100 ml. of broth or azide broth. Cultures were maintained at 137° in a water bath and sterile air bubbled through them. At intervals, dilutions were plated on plain or azide MacConkey and incubated.

Transduction techniques. Two tubes each containing 1·0 ml. of an overnight culture of the recipient organism (about 10^9 viable organisms/ml.) were centrifuged to clarity and the supernatants decanted. One ml. of transducing phage ($6–8 \times 10^9$ plaque-forming particles/ml.) was then added to one of the tubes. One ml. of either phage 34/13 *str-r* or 12/57 *str-r* (titre, 6×10^9 plaque-forming particles/ml.) was added to the other tube which served as the organism control. One ml. of each phage suspension was added to 3 ml. of broth in other tubes to confirm the sterility of the stock. After gentle agitation in a water bath at 37° for 20 min. 0·1 ml. from the first two tubes were plated on azide MacConkey and plain MacConkey and incubated. The latter plates were incubated for 5 hr. and then carefully overlayed with 4 ml. soft agar at 45° containing sufficient sodium azide to give the desired concentration after diffusion. After the agar had set, the plates were incubated. In certain experiments the contents of the tubes were also titrated on plain MacConkey for viable organisms. Phage sterility controls were plated on MacConkey agar after overnight incubation. The role of phage in lysates which yielded positive results was established according to methods previously used (Coetzee & Sacks, 1960*a*).

RESULTS

Stability of media. In view of the reports by Pike (1944), Diamond (1950) and Gerencser & Weaver (1959) on the instability of sodium azide-containing media the following experiments were done. MacConkey azide media were pre-incubated for periods of 1–8 days before inoculation with sensitive and azide resistant variants and further incubated. Simultaneously, freshly prepared azide media were also inoculated and incubated. In a series of three experiments the pre-incubated plates yielded similar counts to those on the freshly prepared azide media and proved their stability under test conditions. Forget & Fredette (1962), and workers quoted by them, also found azide-containing media (often prepared by more vigorous methods) to be stable for at least 7 days. As a matter of convenience freshly prepared media were nevertheless used for each day's experiments.

Survival in presence of sodium azide. Figure 1 shows a family of survival curves of organism 13 on graded concentrations of sodium azide. This composite presentation shows the resistance pattern of the organism to the particular inhibitory agent (Demerec, 1945, 1948). It is apparent from the survival curve of the wild-type organism that azide resistance is not of the obligatory single-step type (Manten & Rowley, 1953). Resistance is of the multi-step variety and the fact that the 2×10^9 wild-type organisms plated did not contain variants resistant to the highest concentrations of azide makes the streptomycin or facultative multi-step pattern

unlikely, and indicates that resistance to azide in this strain is of the obligatory multi-step or penicillin variety (Bryson & Szybalski, 1955). Variants surviving on M/217 azide were named 1st-step resistants. 2nd-, 3rd-, 4th-step resistant organisms were variants which grew on M/130, M/82, M/54 sodium azide respectively. 4th-step resistant variants were not resistant to concentrations much higher than M/54 but selection was not carried further. As in the case of penicillin (Demerec, 1945, 1948), the build-up of resistance was more rapid with each consecutive selection. A number

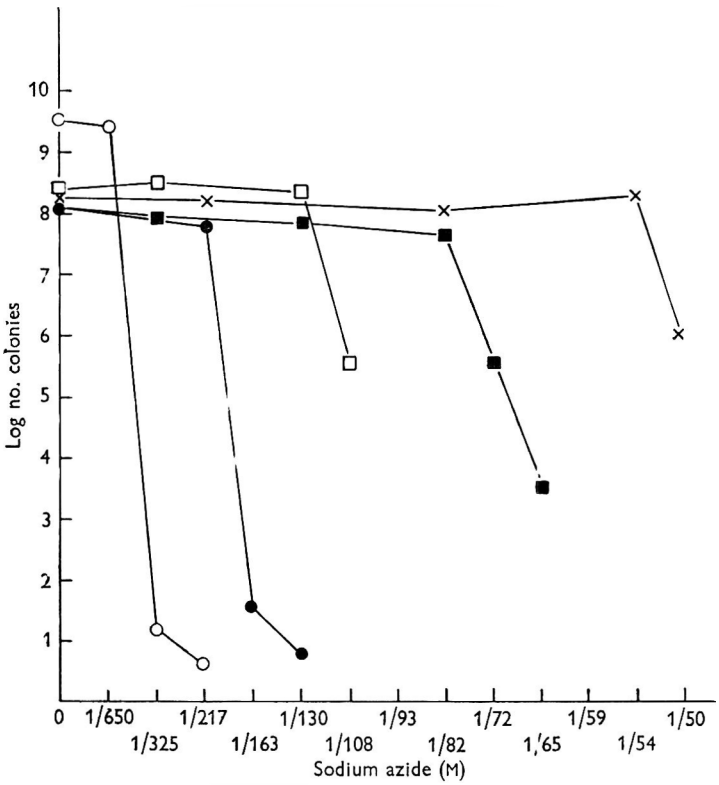

Fig. 1. Growth of *Proteus mirabilis* strain 13 on graded concentrations of sodium azide. A broth culture of strain 13 was concentrated to 2×10^{10} viable organisms/ml. and 0·1 ml. of suitable dilutions plated on different concentrations of sodium azide MacConkey. After 48 hr. incubation colonies were counted and one which grew on the highest concentration of azide picked into 100 ml. broth and incubated overnight. This culture was then again plated on MacConkey azide and the selection process repeated a number of times. ○—○, Wild type; ●—●, 1st-step resistant; □—□, 2nd-step resistant; ■—■, 3rd-step resistant; × — ×, 4th-step resistant variants.

of independently-isolated 1st-step variants of strain 13 had similar ranges of azide resistance. This also applied to 2nd-, 3rd- and 4th-step resistant variants and is in agreement with the findings of Demerec (1948) for penicillin-resistant mutants of staphylococci. Organism 193 was very sensitive to sodium azide and there were no survivors on M/500 azide. Selection, however, yield a family of curves which followed the resistance pattern of the other strains very closely. The four freshly isolated strains of *Proteus vulgaris*, as well as *P. mirabilis* strain 57, gave similar survival curves. Colonies representative of each resistance step were re-streaked on

azide media for purification before single colonies were inoculated on nutrient agar slants. After 24 hr. incubation they were stored at 4°.

Properties of resistant variants. Resistance to sodium azide appeared to be stable in that stock cultures maintained on nutrient agar and subcultured at about 2-monthly intervals for 2 years maintained their resistant properties. Variants of each degree of resistance produced colonies on media containing the corresponding concentrations of azide with diameters only about two-thirds of those of wild-type colonies on MacConkey agar. There was also a small variation in colony diameter among variants with the same degree of resistance, but no marked difference in colonial morphology was encountered between variants with different degrees of resistance. On plain MacConkey agar, all resistant variants produced colonies similar to but usually very slightly smaller than those of the wild-type. In azide media variants not only had long lag periods but also longer generation times than the wild-type. Similar growth differences from the wild-type were noted by Mitchison (1953) for streptomycin-resistant variants of *Escherichia coli* and by Mayr-Harting (1955) for a series of penicillin-resistant variants of staphylococci. In plain broth these differences were much smaller and variants and wild-types eventually attained similar maximum viable counts. Drug-resistant organisms are usually more slow-growing than the corresponding sensitive wild-types (Demerec, 1951; Michison, 1953; Watanabe, 1954; Mayr-Harting, 1955; Banič, 1959; Thornley & Yudkin, 1959) and the azide-resistant variants grew slower in the presence of the particular inhibitory agent than in its absence and so resembled the type III*b* streptomycin-resistant variants of *E. coli* described by Mitchison (1953), some of the *Staphylococcus aureus* penicillin-resistant strains of Mayr-Harting (1955), and the M_1 proflavine-resistant variants of *E. coli* of Thornley & Yudkin (1959).

Table 1. *Experiment to determine the effect of anaerobiosis on azide-resistant variants of strain* 13

Broth cultures of 13 *az-r1* and 13 *az-r4* were diluted to *c.* 10^3 viable organisms/ml. and 0·1 ml. plated on duplicate plates of azide-MacConkey and plain MacConkey agar. One of the azide and plain MacConkey agar sets were incubated anaerobically for 48 hr. The remaining plates were incubated aerobically for 48 hr.

	No. of colonies on azide-MacConkey agar									
	Anaerobic					Aerobic				
Variant	0	M/217	M/103	M/82	M/54	0	M/217	M/108	M/82	M/54
13 *az-r1*	243	0	0	0	0	221	234	0	0	0
13 *az-r4*	190	0	0	0	0	178	200	210	183	181

All resistant variants were motile, flagellated, and agglutinated with pure H antisera. 1st-, 2nd-, 3rd-step variants also swarmed, particularly at room temperature on MacConkey azide agar. No 4th-step variants were encountered which swarmed on M/54 azide MacConkey although they swarmed on plates containing M/82 azide. Sodium azide (M/650 in blood agar) was used by Snyder & Lichstein (1940) to inhibit the spreading of Proteus colonies.

Results presented in Table 1 indicate that azide resistant variants were unable to grow anaerobically on azide-containing MacConkey agar though they could do so on the azide-free medium. All azide resistant variants behaved in this manner. All

the variants produced catalase when growing aerobically on azide-MacConkey or anaerobically on plain MacConkey agar. There are no reports about the respiratory activity of azide-resistant variants, but Sevag & Shelburne (1942) found that the aerobic respiration of strains of streptococci and pneumococci was strongly inhibited by this chemical. Lichstein & Soule (1944*a*) and Forget & Fredette (1962) demonstrated that anaerobic organisms were more resistant to sodium azide than aerobic organisms. The former workers and Schäfer & Haas (1957) also observed that a series of Gram-negative bacteria and staphylococci were more sensitive to sodium azide when cultured anaerobically. There are no reports concerning the catalase activity of azide-resistant organisms, but Lichstein & Soule (1944*b*) showed that organisms which lacked catalase were most resistant to azide and these authors as well as Schäfer & Haas (1957) demonstrated that azide markedly inhibited the catalase activity of bacteria.

Table 2. *Transduction experiment with phage* 34/13 *az-r3 and strain* 13 *as recipient*

1 ml. of phage 34/13 *az-r3* (plaque-forming titre: 6×10^9/ml.) was mixed with a deposit of 10^9 recipient bacteria. 1 ml. of phage 34/13 *str-r* with the same titre was added to an equal number of the bacteria as a control. After 20 min. adsorption at 37°, 0·1 ml. of each was plated on MacConkey agar and incubated for 5 hr. The plates were then overlayed with 4 ml. soft agar containing sodium azide to give the desired concentrations after diffusion. Plates were then incubated for 48 hr. and the colonies counted. Phage controls were sterile.

	Concentration of sodium azide in MacConkey agar				
	M/500	M/325	M/217	M/163	M/130
	No. of colonies				
Test	+	294	333	9	0
Control	+	11	2	0	0

+ = Confluent growth.

Transduction experiments. Table 2 shows results of an experiment to transduce 3rd-step azide resistance into the wild strain 13 with phage 34/13 *az-r3*. The phage controls in this and other experiments reported were sterile. The test plates had numerous colonies growing on M/217 azide while the controls infected with phage 34/13 *str-r* had few colonies on concentrations above M/500. A further experiment with the same lysate but with 13 replaced as recipient by the wild-type strain 193 yielded even more clear-cut results in that the test had many colonies on M/217, whereas the M/500 control plates were barren. The transducing ability of the lysate was not affected by pre-treatment with deoxyribonuclease but transduction was completely abolished by the addition of a small volume of high-titre anti-phage serum which reduced the plaque-forming titre of the lysate to less than 10^5/ml. These experiments established the phage in the lysate as the genetic vector. It appeared that a phage lysate of 13 *az-r3* could only transduce wild-type strains to M/217 (i.e. 1st-step) azide resistance. Experiments were then done with the same recipients, but lysates prepared on variants 13 *az-r1*, 13 *az-r2*, and 13 *az-r4*. Again only 1st-step resistance was transduced. Table 3 shows an experiment in which variant 193 *az-r1* was treated with a phage lysate of 13 *az-r1*. The test plates had numerous colonies on M/120 while the control had few colonies above a concentration of M/217. This

result was taken to mean that a 1st-step resistant variant could be transduced to 2nd-step resistance by a lysate prepared on an independently isolated 1st-step variant. In a further experiment variant 13 *az-r1* was treated with the same lysate used in the first experiment reported—i.e. phage 34/13 *az-r3*. The control counts were 9 colonies on M/163 and 0 on M/120. The test had 217 and 196 colonies on these concentrations respectively. There was thus the situation where lysate 13 *az-r3* could transduce no more than 1st-step resistance in wild strains but could produce 2nd-step resistance from a 1st-step resistant organism. Similar results were obtained with *Proteus mirabilis* strain 57 and phage 12/57 system. In both systems it was possible to transduce 3rd-step sodium azide resistance into 2nd-step variants with lysates prepared on 4th-step resistant variants. Adsorption experiments showed that at least 95 % of the phage adsorbed within the 20 min. adsorption period. On this basis the transduction rate was about 10^{-7}/phage particle adsorbed. In all

Table 3. *Transduction experiment with phage* 34/13 *az-r1 and variant* 193 *az-r1 as recipient*

1 ml. of phage 34/13 *az-r1* (plaque-forming titre: 8×10^9/ml.) was mixed with a deposit of 1×10^9 recipient bacteria. 1 ml. of phage 34/13 *str-r* (plaque-forming titre: 6×10^9/ml.) was added to an equal no. of the bacteria as a control. After 20 min. adsorption at 37° 0·1 ml. of each was plated on MacConkey agar and incubated for 5 hr. The plates were then overlayed with 4 ml. soft agar containing sodium azide to give the desired concentrations after diffusion. Plates were then incubated for 48 hr. and the colonies counted. Phage controls were sterile.

	Concentration of azide in MacConkey agar					
	M/217	M/163	M/130	M/120	M/108	M/82
	No. of colonies					
Test	+	325	296	305	15	0
Control	+	8	0	0	0	0

+ = Confluent growth.

transduction experiments the number of transductants present on the directly inoculated azide agar was two-thirds of that on the double-agar-layer plates. Figure 2 shows that while about 60% of transduced *az-r* genes were immediately expressed, the greatest number of transductants was obtained by layering with azide-agar 4 to 5 hr. after plating. Reconstruction experiments with known numbers of azide-resistant organisms proved that resistant clones were not disturbed by overlaying with the soft agar (Coetzee & Sacks, 1960*a*). These experiments were verified by others in which transduction mixtures were plated on azide-MacConkey at intervals and also titrated for total viable counts. Although transductants were present on the earliest platings their numbers showed a disproportionate increase with the 4–5 hr. platings. No abortive transductants (Stocker, 1956; Ozeki, 1956) were encountered. All transductant clones tested proved to be lysogenic but no precautions (Coetzee & Sacks, 1960*a*) were taken to prevent secondary lysogenization occurring on the plates. Transductants did not adsorb transducing phage. This was attributed to lysogenic conversion (Coetzee, 1961). Transductants resembled the selected resistant variants in all other respects and have retained their resistance to sodium azide for more than a year.

Failure to appreciate the multi-step nature of sodium azide resistance in *Proteus*

mirabilis could account for our initial failure to transduce azide resistance: lysates of highly resistant variants were applied to wild-type recipient strains and selection was for transductants with equivalent resistance.

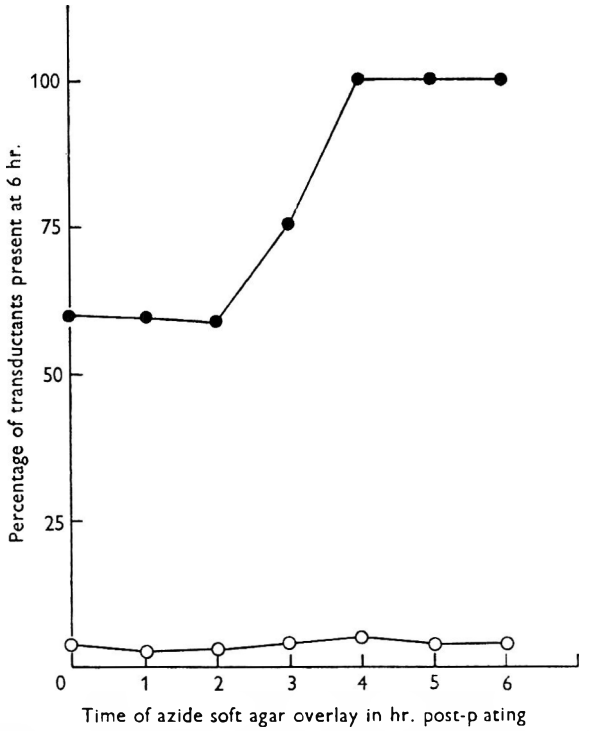

Fig. 2. Phenotypic expression of transduced genes. A transduction experiment was done with strain 57 and phage 12/57 *az-r4*. Phage 12/57 *str-r* was added to the organism control. After an adsorption period of 20 min. at 37°, 0·1 m.. of the test and control were spread on plain MacConkey. The plates were incubated. At intervals they were overlayed with 4 ml. of soft sodium azide-containing agar to give a final concentration of M/217 throughout. After further incubation for 48 hr. colonies were counted. The phage controls were sterile. ●—●, test; ○—○, control.

DISCUSSION

The resistance pattern of an organism to an antibacterial agent is a property of the particular agent (Bryson & Demerec, 1950). Szybalski & Bryson (1954) encountered an exception. *Mycobacterium ranae* may become completely resistant to chloramphenicol in a single step, whereas the resistance-pattern of other organisms to this agent is of the multi-step (penicillin) variety. Sodium azide resistance appears to be another exception. In *Escherichia coli* resistance to this agent is accepted as being obligatory single-step in nature (Cavalli, 1952; Bryson & Szybalski, 1955), while in *Proteus hauseri* it appears to follow a multi-step pattern. It was possible to transduce 1st-step (and only 1st-step) resistance into wild-type strains by phage grown on 1st- or multi-step resistant variants. 2nd-step resistance was transduced to 1st-step variants by phage produced on independently isolated 1st- or multi-step resistant organisms. 1st-step variants were fairly uniform in their degree of resistance to sodium azide. This is what would be expected if resistance to

sodium azide were governed by a number of more or less equipotent genes not closely enough linked to be jointly transduced (Banič, 1959). Due to lysogenic conversion (Coetzee, 1961), which prevented readsorption of transducing phage it was not possible to attempt the step-wise build-up of resistance to sodium azide by serial re-exposure of transductants to transducing phage as was done in transformation studies (Hotchkiss, 1951). The fact that resistant variants form colonies of practically the same size as the sensitive wild-type on plain MacConkey agar precluded the possibility of transduction of azide sensitivity into resistant variants as achieved by Watanabe & Watanabe (1959*b*) with a *str-s* marker in *Salmonella typhimurium*.

The four newly isolated strains of *Proteus vulgaris* gave sets of survival curves similar to those of *P. mirabilis* strains 13, 913, 57. No transducing phage was available to confirm a polygenic mechanism of azide resistance in the *P. vulgaris* strains, but there is no reason to suppose that they differ from *P. mirabilis*.

In the analysis of phenotypic expression of sodium azide resistance after conjugation in *Escherichia coli*, Hayes (1957) found that resistance was expressed within the zygotes after a lag of a few minutes and that expression was achieved in the whole population before the recombinants divided. He concluded that resistance to sodium azide was a dominant character in *E. coli*. In the present case it was found that 60 % of transduced resistant genes gained expression on platings done immediately after the adsorption period. The delayed expression of the remaining genes is difficult to explain. Morse (1959) met a similar situation with the transduction of Novobiocin resistance in staphylococci. Following his tentative explanation, it could be argued that, if azide resistance was dominant, the transduced genetic material could require a number of hours to penetrate the recipient organism and so explain the delay in expression. Alternatively, if the azide resistance gene requires a long time for phenotypic expression, it is possible that sodium azide might be slow acting. The transduced resistance genes would then find expression before the organism was inhibited, and so account for the transductants present on early platings. Mutant alleles are usually recessive to the wild-type (Lederberg, 1951; Watanabe & Watanabe, 1959*a*; Coetzee & Sacks, 1960*a*). The striking exception is the conclusive experiments of Hayes (1957) concerning sodium azide resistance in *Escherichia coli*. It is tempting to conclude that sodium azide resistance in *Proteus mirabilis* is also dominant but no decision can be made. The fact that no abortive transductants were observed could point to the recessive nature of azide resistance loci (Watanabe & Watanabe, 1959*a*; Coetzee & Sacks, 1960*a*). However, the absence of abortive transductants in various systems is not unknown (Holloway & Monk, 1959; Edgar & Stocker, 1961; Thorne, 1962) and they were also not encountered in transduction of the swarming characteristic in *Proteus mirabilis* (Coetzee, 1963).

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L forms of *Staphylococcus aureus*

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SUMMARY

All but four of twenty-five strains of *Staphylococcus aureus* exposed to concentrations of 60 μg. benzylpenicillin/ml. (or 100–500 μg. methicillin/ml. for penicillinase-producing strains) on nutrient agar media containing 3·5 % (w/v) sodium chloride and 10 % (v/v) horse serum, gave rise to typical L colonies. The L forms were subcultured on nutrient agar and in broth containing 3·5 % sodium chloride and were found to be completely resistant to the penicillins, cycloserine, ristocetin, vancomycin and cephalosporin, but to be sensitive to other antibiotics, often in slightly lower concentrations than the parent cocci. The L forms produced coagulase and, in two of three strains tested, were lysogenic although resistant to phage lysis. In studies of the transformation of staphylococci to L forms, it was noted that a variable but often very large proportion of the early L microcolonies failed to develop into typical colonies.

INTRODUCTION

During the last few years there has been considerable interest in the so-called 'L forms' of various bacteria. Although the term L form has been used with rather inadequate definition by many writers, there seems now to be general agreement that it is best used to refer to organisms, derived from typical bacteria, which can grow on suitable culture media but which lack an organized cell wall. This paper reports a study of organisms, derived from cultures of *Staphylococcus aureus* by exposure to penicillin in the presence of serum and a high concentration of sodium chloride, which are thought to lack the mucopeptide element of the normal cell wall. The production of L forms from staphylococci was described briefly by Dienes & Sharp (1956) and in more detail by Schönfeld (1959, 1961), Marston (1961*a*, *b*) and Mattman, Tunstall & Rossmoore (1961). The work reported here confirms many of the observations recorded previously and amplifies them with a study of the development of the L colonies and of the sensitivity of the L forms to a wide range of antibiotics; it also provides some additional evidence for the lack of an organized cell wall.

METHODS

Media. For most of the work described here, Difco Brain-Heart Infusion Broth or Agar was used, made up according to the makers' formula. For the standard 'L-form medium', there were added 3 % (w/v) sodium chloride (giving 3·5 % total NaCl), 10 % (v/v) normal horse serum and 60 μg. (100 units) benzylpenicillin/ml. Agar media were poured in 20 ml. volumes in 9 cm. plastic Petri dishes. After

drying to remove surface water the plates were inoculated and incubated at 37° enclosed in a polythene bag to prevent further drying.

Formation of staphylococcal L forms. For the isolation of L colonies from staphylococci it was found best to inoculate the agar media with 0·5 ml. of a 10-times concentrated suspension of the staphylococci grown overnight in nutrient broth.

Subculture of L colonies. For transfer of L colonies from one solid medium to another, blocks of agar with colonies on the surface were cut out and inverted on the new medium; they were pushed forward across the agar surface when growth appeared beneath them.

Growth was established in broth by the transfer of a block of agar carrying colonies to a screw-capped bottle containing 10–15 ml. of L-form broth. Latterly, following a suggestion from Dr W. J. Hijmans (Leiden, Netherlands) the agar block was disintegrated and a suspension of agar + colonies transferred to the broth.

Microscopic examination. The development of colonies on agar was observed by cutting out, at appropriate intervals, blocks of agar-carrying colonies, and inverting on them coverslips on which a drop of 1 % (w/v) ethanolic methylene blue had been placed; or alternatively on coverslips on which a mixture of 1 % (w/v) methylene blue and 1 % (w/v) azur II had been dried.

Strains. A great part of the work reported here was done with three strains of *Staphylococcus aureus*: PS 42E (NCTC 8413), PS 6 (NCTC 8403); 1444, a strain of phage type 3B/+ isolated in this laboratory. A number of confirmatory tests were made with other strains from the phage-propagating set (Blair & Williams, 1961) and with strains isolated here.

RESULTS

Characteristic L-form growth

The term 'L-form' is used here to refer to organisms growing on the special media to give the characteristic colonies described in previous work (e.g. Fig. 1 in Marston, 1961*a*); these colonies are up to about 2 mm. in diameter, are smooth, domed and have an entire edge. They are hard to the touch and microscopic examination shows that the centre part of the colony is formed deep in the agar. The colony spreads on the surface beyond the burrowing centre. The peripheral growth consists at first of spherical vesicles about 5–10 μ in diameter, which are later replaced by granular elements. The central part of the surface growth and the burrowing central growth seem to be made of small granular elements. Stained by methylene blue, the vesicles appear empty. With the methylene blue + azur stain some small granules were sometimes seen within the vesicles at their periphery. Prolonged incubation sometimes resulted in the formation of new burrowing foci at the periphery of the colony.

In broth culture, growth was in the form of microcolonies embedded in slimy material, shown by staining and enzyme digestion to consist largely of deoxyribonucleic acid. Microscopically the microcolonies consisted of clusters of vesicles and granular debris.

Transformation to L forms

The typical sequence of events observed when 10-times concentrated suspensions of an overnight staphylococcal broth culture were spread on a plate of the L-form medium was as follows. After incubation for 24–48 hr. there were usually no macro-

scopically visible colonies, but microscopic examination revealed numerous foci consisting of weakly staining discs or vesicles, each about 5–10 μ in diameter. After a further 48 hr. of incubation these foci were larger, consisting of more vesicles and a central granular area; differential focusing showed that some of the granules were in fact deep in the agar. After further incubation these foci grew and showed more extensive and often apparently multifocal penetration into the agar. Very often most of the foci subsequently degenerated and only a few formed typical L colonies. This description is based on several different experiments, three of which are recorded in Table 1. In control experiments with the staphylococci inoculated on to media containing penicillin but only 0·5 % sodium chloride, no similar foci or colonies developed.

Table 1. *Formation of L forms from staphylococci*

	Expt. 1	Expt. 2	Expt. 3
Viable cocci* ... inoculated	$0{\cdot}9 \times 10^7$	$1{\cdot}3 \times 10^7$	10^7
	Type of L-growth and number of colonies/sq.cm./culture plate		
Day 1	Vesicles, few with granules; $1{\cdot}7 \times 10^4$	0	Vesicles; $1{\cdot}8 \times 10^4$
Day 2	Vesicular with deep granules; $1{\cdot}1 \times 10^4$	Vesicular with deep granules; $0{\cdot}3 \times 10^4$	Vesicular with deep granules; $2{\cdot}1 \times 10^4$
Day 3	n.e.	n.e.	Vesicular with deep granules; $2{\cdot}7 \times 10^4$
Day 4	n.e.	Vesicular with deep granules; $0{\cdot}7 \times 10^4$	n.e.
Day 5	n.e.	n.e.	n.e.
Day 6	Typical L-form colonies; 0·5	Typical L-form colonies; 7×10^2	Typical L-form colonies; 3
No. L-form colonies/ primary focus	$0{\cdot}3 \times 10^{-4}$	1×10^{-2}	10^{-4}
No. L-form colonies/ coccus* in inoculum	6×10^{-8}	$5{\cdot}4 \times 10^{-5}$	3×10^{-7}

* = no. of cocci or clumps of cocci; n.e. = not examined on day indicated; 0 = no L foci seen.

The number of L colonies developing on the plates seemed variable, and many experiments were made to try to obtain more reproducible results, without success. Undoubtedly the degree of wetness of the agar is important, but the application of a piece of filter paper to the surface, found by Gooder & Maxted (1961) to enhance the growth of L colonies of streptococci, had no effect. Great variability was seen even when the experiment was carried out with a semi-solid agar layer.

Effect of different penicillin concentrations. In several experiments the standard inoculum of staphylococci was applied to a series of plates of the L-form medium which differed only in penicillin concentration (0·6–60 μg./ml.). After a few days of incubation the 0·6 μg. penicillin plates showed some colonies rather like those of staphylococci, but which were hard and partly embedded in the agar; they consisted of refractile bodies 1–2 μ. in diameter. These colonies appear to be similar to those described by Prozorovskii (1959) and Marston (1961*a*) as developing on media with a low penicillin concentration. Examination of the 0·6 μg. plates daily after inoculation showed that, in contrast to the usual sequence of events on 60 μg.

plates, the first foci to be visible were granules burrowing into the agar; vesicles were rarely seen. In only one of the five completed experiments (Table 2) were typical L colonies seen on 0·6 μg./ml. plates. With all penicillin concentrations from 3 μg./ml. upwards typical L colonies developed.

Table 2. *Change of staphylococci to L forms on media with differing penicillin concentrations*

Concentration of penicillin (μg./ml.)	Day of examination	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5
		No. and form of colonies				
0·6	1–3	$3·7 \times 10^4$ S+D: 89 % D: 11 %	17×10^4 All D	$1·9 \times 10^4$ All D	$3·9 \times 10^4$ D: 25 % S+D: 41 % S: 34 %	$2·7 \times 10^4$ RB: 100 %
	4–6	$0·8 \times 10^4$ All RB	16×10^4 All RB	4×10^2 All RB	$1·5 \times 10^4$ All RB	Semi-confluent L col.
60	1–3	$2·8 \times 10^4$ All S	6×10^2 All S+D	$9·1 \times 10^4$ All D	5×10^4 All S	$1·1 \times 10^4$ All S
	4–6	Semi-confluent All L col.	10^2 All L col.	$1·7 \times 10^2$ All L col.	$3·5 \times 10^4$ L. col 14 % S+D: 20 % Degenerate: 66 %	$0·4 \times 10^4$ All L col.

S = surface vesicles; D = deep granules; RB = refractile bodies, see text; L col. = fully developed L-form colony.

Little further study has yet been made of the colonies which developed on the 0·6 μg. plates, partly because of the difficulty of freeing them from staphylococci, but it seems that it is possible to carry them in subculture indefinitely and that they revert quantitatively to staphylococci in the absence of penicillin.

Proportion of staphylococcal strains transformed to L phase. Twenty-five different staphylococcal strains have been tested for the formation of L forms. Eighteen of the strains, including the three (PS 42E, PS 6 and 1444) on which most of the detailed tests were done were penicillin-sensitive and seven were penicillin-resistant; the latter were tested on agar-containing methicillin. All but two of the eighteen penicillin-sensitive and two of the seven resistant strains yielded L colonies, and many gave a profuse growth. The penicillin-resistant strains tested on methicillin agar tended to give fewer colonies than the sensitive strains tested on agar containing benzylpenicillin; a similar difference was found when sensitive strains were tested on methicillin agar.

Subculture of L forms

In most cases subculture of colonies by the block transfer method was successful, although the proportion of colonies which yielded growth was often small on the first subculture of newly transformed strains. Established strains gave a profuse growth of secondary colonies after overnight incubation.

Considerable difficulty was experienced in establishing cultures in broth by inoculation with agar blocks containing colonies, though three broth lines were initiated by this method. The use of disintegrated agar blocks, adopted recently,

appears more promising but still has not been consistently successful. Once established, the broth cultures were readily subcultured.

Growth in absence of penicillin. The nutrient agar and the broth cultures were carried for many subcultures in the L-form medium without penicillin. For example, a broth culture of the L form of staphylococcus PS 42E was established in July 1961, and after three subcultures in penicillin-containing media was transferred successively 23 times in penicillin-free media during the next 7 months. Subculture to appropriate media at frequent intervals yielded, except on the one occasion noted below, only typical L colonies and no staphylococci. Similar series of subcultures of the other two strains were made in the absence of penicillin. Agar-plate subcultures were also kept growing over the same period although, because the plates became contaminated from time to time, it was not possible to complete the entire series exclusively on penicillin-free media. A total of 16–20 subcultures on such media were, however, made.

Only on one occasion was there any evidence of reversion of these cultures to a normal staphylococcus. Cultures of three strains (two derived from PS 55 at the end of May, and one derived from PS 42E in June, 1961), each of which had had several transfers on penicillin plates, were transferred to penicillin-free media on 22 July; subcultures to penicillin-free media were made, for one strain on 31 July and for the other two on 18 August. All these subcultures yielded staphylococci which, though growing poorly and being coagulase-negative at first, subsequently regained a typical appearance and proved to be identical in phage type with the original parent strains of staphylococci. Further subcultures of the L-form broth cultures on penicillin-free media did not reveal staphylococci, and the above was the only occasion on which 'reversion' was observed. Since both strains of staphylococci were in use in the laboratory at the time, the appearance of staphylococci in the L form cultures might have been the result of coincidental contamination; but it seems more likely that the strains were at the time not yet fully stabilized in the L phase.

Several attempts were made later to demonstrate reversion by using L forms which had been through 30 or more subcultures, 20 or more of them in penicillin-free media. These L cultures were plated on media in which the serum was replaced with 5% yeast extract, or in which the sodium chloride was replaced by 1·3% or 3·7% di-sodium hydrogen phosphate. No evidence of reversion to staphylococci was obtained.

Characteristics of the L forms

Effect of antibiotics. The effect of antibiotics on the staphylococcal L forms was tested in four ways: (1) subculture of agar block cultures and of broth cultures of L forms to antibiotic-containing media; (2) subculture of broth cultures to L-form medium (without penicillin) and application of antibiotic-containing paper discs; (3) determination of minimal inhibitory concentration (MIC) of the antibiotics in L-form agar; (4) attempts to transform staphylococci to L forms on L-form agar which contained another antibiotic in place of penicillin. The results of these tests are shown in Tables 3 and 4.

Detailed examination of the antibiotics known to interfere with cell-wall metabolism is illustrated in Table 3. The L forms were resistant to the inhibitory action

of the penicillins, cephalosporin (PAC), cycloserine, vancomycin and ristocetin; but they were inhibited by novobiocin, bacitracin and crystal violet.

Table 3. *Effect of antibiotics on staphylococcal L forms*

(The reactions in this table are mostly based on tests with L forms from staphylococcus PS 42E, which is sensitive to all antibiotics used.)

Antibiotic (μg./ml.)		Growth from agar block subculture	Growth from broth subculture	Resistance to antibiotic disc	Change from staphylococcus to L form
Benzylpenicillin	6–60	+	+	+	+
Methicillin	6–60	+	+	+	+
Cycloserine	200–600	+	+	+	+
Cephalosporin (PAC)	100	+	+	+	+
Vancomycin	500–1000	+	+	+	−
Ristocetin	100–1000	+	+	+	−
Novobiocin	100–1000	−	−	−	−
Bacitracin	8–20	−	−	−	−
Crystal violet	10	−	−	−	−

Minimal inhibitory concentrations of several antibiotics for the L forms and for the parent staphylococci are shown in Table 4. For several of these antibiotics, the minimal inhibitory concentration for a staphylococcus was rather greater than that for the corresponding L form.

Table 4. *Antibiotic sensitivity of L-forms and their parent staphylococci*

	Staphylococcus strain			
	PS 42E		PS 6	
	Parent	L form	Parent	L form
	Minimal inhibitory concentrations (μg./ml.)			
Benzylpenicillin	0·1	> 1000	0·1	> 1000
Cycloserine	40	> 800	200	> 800
Cephalosporin	0·5	> 500	0·5	> 500
Vancomycin	2	> 1600	5	> 1600
Ristocetin	20	> 800	20	> 800
Bacitracin	1	80	5	5
Novobiocin	0·125	0·5	2	0·125
Tetracycline	0·5	1	2	0·125
Fucidin	0·125	1	2	0·5
Streptomycin	4	4	16	1
Neomycin	2	1	8	2
Kanamycin	4	1	16	2
Erythromycin	0·25	0·06	1	0·03
Chloramphenicol	5	1·25	5	2·5
Colomycin	> 1000	125	500	200
Sulphadimidine	> 1000	> 1000	> 1000	> 1000

Formation of L forms from staphylococci was achieved with methicillin, cephalosporin and cycloserine, though the yield of L colonies on methicillin plates was lower than on penicillin. There was a good yield of L colonies on plates containing 100 μg. cephalosporin/ml. L colonies from the methicillin, cephalosporin and cycloserine plates were subcultured easily on to penicillin plates.

Several attempts were made to change staphylococci to the L form with vancomycin but without success.

Phage sensitivity. Broth cultures of the L forms from staphylococci PS 42E, PS 6 and 1444 were spread on plates of L-form medium without penicillin, and drops of a preparation of the polyvalent K phage, containing about 10^8 phage particles/ml., were applied. There was no sign of inhibition or of lysis after overnight incubation. A similar test was made with the typing-phages active on the parent staphylococci; again there was no sign of any action.

Lysogenicity. Supernatant fluids from well-grown L-form cultures of the same three strains were spotted on to agar plates spread with cultures of staphylococci sensitive to the lysogenic phages of the parent strains. After overnight incubation the plates inoculated with the supernatant fluid of strain 1444 showed an identical lytic spectrum to that given by supernatant fluid of a broth culture of the parent staphylococcus. The L-broth culture of strains PS 6 and PS 42E did not show very convincing lysis. These tests were made with broth cultures which had been transferred in the L form for over a year. A further test was made with a newly isolated L-form culture from staphylococcus 42E; the supernatant fluid of this lysed one of the staphylococci (W 57) lysed by the parent, but not strains 1030 or 18042. It was thought that this loss of lysogenicity might be explained by the selection, through resistance to phage uptake by the L forms, of any non-lysogenic mutants that might have occurred. An attempt was made to reproduce the effect with staphylococci grown in broth containing 1 % sodium citrate to inhibit phage uptake. A streptomycin-resistant variant of staphylococcus PS 42E was transferred 15 times in citrate broth and then spread on a nutrient agar plate at a concentration calculated to give well-spread colonies. The plate was spread with a thin layer of agar and the surface irradiated with ultraviolet radiation to kill any surviving staphylococci; after overnight incubation to allow development of colonies, a broth culture of indicator staphylococcus 1030 was spread over the surface of the agar layer, and incubation continued for a further 24 hr. Plaques of lysis were seen in the indicator lawn corresponding to most of the colonies, but about 10 % of the colonies gave no plaques. These latter colonies were subcultured in streptomycin-containing broth and after further subculture were tested for lysogenicity for the indicator strain 1030; two non-lysogenic colonies were found in this way. It was of interest that both these colonies had apparently lost the prophage responsible for the lysis of strains 1030 and 18042 but retained that responsible for lysis of the third indicator strain, W 57. They thus resembled the L form in lytic activity.

Osmotic sensitivity. The L-form growth in a broth culture was centrifuged down and resuspended in: (*a*) water; (*b*) nutrient broth (containing 0·5 % w/v NaCl); (*c*) L-form broth. After 15, 30 and 60 min., samples from each were again centrifuged, resuspended in L-form broth and plated on L-form agar. The results of a typical experiment are shown in Table 5; the L forms were rapidly killed on suspension in water, more slowly in nutrient broth.

Several attempts were made to acclimatize the L forms to growth in lower concentrations of salt. It was possible to get growth in broth media containing only 1·5 % (w/v) sodium chloride, and on occasion to obtain satisfactory subculture from these cultures to agar media containing only 1·2 % (w/v) sodium chloride. The growth was never profuse but the colonies were typical in form.

Coagulase-production. Human plasma was added (10 %, v/v) to L-form broth medium and the tubes inoculated with a broth culture of three different L-form

cultures; characteristic clots were produced after incubation for 24–48 hr. at 37°; these clots were digested by a preparation containing streptokinase ('Varidase' Lederle Laboratories, N.J., U.S.A.).

Cell-wall components. A chemical analysis of L forms, and comparison with the parent staphylococci, is being made by my colleague Mr B. Pratt; the results will be published subsequently. In preliminary experiments it has proved impossible to detect any muramic acid in hydrolysates of three strains of L forms.

Table 5. *Osmotic sensitivity of staphylococcal L forms*

	Time of suspension		
	15 min.	30 min.	60 min.
Suspending medium	No. of viable units/0·02 ml.		
L form broth, 3·5 % NaCl	3000	3000	2000
0·5 % NaCl broth	1700	400	250
Water	70	15	15

Count of initial suspension = 2400 viable units per 0·02 ml.

Action of lysozyme. A broth culture of the L form of staphylococcus PS 42E was centrifuged down, and resuspended in nutrient broth containing 0·1 unit egg-white lysozyme/ml.; there was no change in turbidity. After 30 min. the suspension was recentrifuged, taken up in L-form broth and plated on L-form medium. A control, exposed to the broth but not to lysozyme, yielded 150 colonies; the lysozyme-treated suspension gave 206 colonies. In a similar experiment *Micrococcus lysodeikticus* was sterilized by 15 min. exposure to the lysozyme.

Filterability. A well-grown broth culture yielding about 500 L-form colonies from 0·02 ml. was filtered through an Oxoid membrane filter, and through a series of Gradocol membranes (kindly supplied by Dr F. Himmelweit) having estimated pore sizes of 450, 300, 140 and 69 mμ. A few L colonies (35 from 0·02 ml.) developed from the filtrate from the Oxoid filter (pore size about 700 mμ.) but from none of the others. A further series of experiments were made with broth cultures of various ages passed through Oxoid membrane filters but in these the viable count of the filtrate was lower and, with cultures aged 1–4 days, never exceeded 0·5 % of the original culture and was generally about 0·1 %. Membrane filters were also laid on the surface of L-form medium agar and inoculated with drops of staphylococcus culture and of an L-form broth culture. The staphylococcus and its L form grew on the surface of the filter, though less well than on the agar surface. After incubation for several days on the surface of the agar, the staphylococci and the L forms grew through the membrane to the underlying agar; but there was no indication that the L forms did so more readily than did the staphylococci.

Lyophilization. The L-form cultures have been successfully preserved by lyophilization, the centrifuged deposit from a broth culture being suspended in normal horse serum and freeze-dried in an Edwards Centrifugal Freeze Dryer.

DISCUSSION

It has thus been possible to obtain colonies which resemble those described for L forms from most of the staphylococci tested. Since the numbers of L-form colonies obtained in repeated experiments with single staphylococcal strains were very variable, it seems likely that the few failures were due to technical difficulties rather than to a real inability of some staphylococcal strains to be changed. This confirms the observations of Schönfeld (1961), as do the observations on the early stages of L-colony formation. Schönfeld did not, however, record the formation of large numbers of abortive L-form colonies. No explanation has been found for the irregularity of the appearance of L-form colonies. The observation that many abortive L-form colonies develop indicates that there are at least two stages at which lack of some factor required for growth or the presence of some growth-inhibitory factor might act. Schönfeld (1961) found that the number of L-form colonies was related to the square of the number of staphylococci in the inoculum; it was for this reason that a standard inoculum of a 10-times concentrated culture was used in the present work. In my hands the irregularity from one plate to another has been too great to permit useful statistical analysis in this respect. A similar irregularity has so far prevented proper attempts to discover whether the L forms may occur as mutants in normal cultures of staphylococci and merely be selected by penicillin + high NaCl concentrations. Our inability to obtain L-form colonies by exposing staphylococci to vancomycin or ristocetin, which are not inhibitory to growing L-form cultures, is perhaps some evidence against the mutational origin; likewise an observation that the yield of L-form colonies from staphylococci grown in L-form broth and plated on L-form agar is no higher than from staphylococci grown in nutrient broth, in which any L forms would have been destroyed.

The antibiotic sensitivity of the L forms conforms reasonably to expectation based on other information about the mode of action of various compounds on cell wall synthesis (e.g. Salton, 1960; Reynolds, 1961). It is interesting that the growth of the L forms is inhibited by bacitracin, which has recently been shown to inhibit staphylococcal growth in more ways than simply by interference with cell-wall synthesis (Smith & Weinburg, 1962); perhaps novobiocin can also be shown to have multiple effects. The apparently greater susceptibility of L forms than the parent organisms to several antibiotics is presumably due to greater ease of penetration in the absence of cell wall, and conforms well with Taubeneck's (1962) observations on L forms of Proteus.

Apart from the comparative sensitivity to various antibiotics, the conclusion that the staphylococcal L forms described here lack cell walls is supported by their insusceptibility to phage and their osmotic sensitivity. Reversion to staphylococci has not been convincingly observed so that, formally, there might be some doubt whether the colonies were truly derived from staphylococci. Their continuing coagulase production and, at least to a limited extent, their lysogenicity, as well as the fact that they have been isolated on many separate occasions from several different staphylococcal strains seems, however, to counter this objection. It seems that the partial loss of lysogenicity is due to the selection, by prolonged culture in the phage-resistant L form, of non-lysogenic mutants.

The stability of our penicillin-induced L forms of staphylococci is of interest in

view of the apparent regularity with which Schönfeld (1959) and Marston (1961*a*) were able to demonstrate reversibility of the L phase in their cultures. Perhaps the strains investigated in the present work differed from the strains of Schönfeld and Marston, especially in having been stabilized for a longer period in the L form; more probably we have not yet achieved the right growth conditions for reversion.

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Dry Liberation of Fungus Spores by Raindrops

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SUMMARY

Large transient increases in the concentration of some dry airborne spores coincident with the start of rain suggested that the first raindrops to wet surfaces might disperse spores other than in splash droplets or by wetting fructifications. Experimental collisions between glass beads or water drops and spore-bearing surfaces showed that both rapid air movement in advance of radially spreading splashes and vibration can suspend spores in air. Removal by air movement is most effective when large drops collide with surfaces carrying spores that are loose or raised above the surface.

INTRODUCTION

There are already three accepted ways in which rain influences the air spora. One, falling raindrops remove spores from suspension in the air, with an efficiency that depends on the dimensions of the spores, the diameters of the drops and the ease with which the spore surface is wetted (May, 1958; Davies, 1961). Although plant pathologists have neglected this 'rain-scrubbing' or 'wash-out' of particles from the air it is probably the most effective mechanism for depositing small particles (Hirst, 1959). Two, when raindrops collide with wet, spore-bearing surfaces, parts of the falling drop and the surface water film become incorporated in splash droplets that contain spores. Many plant pathogens are thus 'splash dispersed', but the process probably adds little to the number of airborne spores because most splash droplets are large and soon deposited (Gregory, Guthrie & Bunce, 1959). Three, wetting fructifications can initiate spore release, for example, ascospores are released by hydrostatic pressure (Ingold, 1953).

Evidence from spore trapping suggests that, when rain starts, there are often large transient increases in the numbers of some dry spores in air. Published reports mention *Cladosporium* sp. (Ainsworth, 1952; Gregory, 1954; Hirst, 1953, 1959; Rich & Waggoner, 1962), *Ustilago nuda* (Sreeramulu, 1962), *Pithomyces chartarum* (Meredith, 1962). Similar increases can accompany watering in glasshouses (Hirst, 1959). Our work shows that such increases could occur when the first drops of rain strike dry plant surfaces but does not disprove other suggested explanations (Ainsworth, 1952; Hirst, 1953; Gregory, 1954).

APPARATUS

The effects of water-drops colliding at terminal velocity with spore-bearing surfaces ('targets') were studied using a tripod ladder (Pl. 1, fig. 1) 25 ft. (7·6 m.) tall, fitted with a central vertical pipe 2·4 in. (6·0 cm.) diam. down which water-drops could be aimed accurately. The targets were placed within a vertical glass cylinder 5½ in. (14 cm.) diam. and 8 in. (20 cm.) high (Pl. 1, fig. 2). To delay removal, by the wind, of any spores liberated, the top of the cylinder was closed by a loose panel except for a 2 in. (5·1 cm.) diam. central hole through which the drops fell.

A small stationary-slide impactor (Gregory, 1954) was used to sample air within the sampling cylinder at 10 l./min. with its 2 × 14 mm. orifice directed tangentially half way up the wall of the cylinder. In some tests microscope slides coated with naphthol green B in gelatine (Liddell & Wootten, 1957) were exposed inside the cylinder to test whether spores were dispersed in splash droplets. Whenever possible the tests were standardized, and each comprised three 5 min. (50 l.) air samples, taken before, during and after the period when water-drop collisions occurred. The number of collisions had to be varied according to the density of spores on the targets. In some early tests collisions were continued throughout the 5 min. period, but in later tests 10 collisions at the beginning of the period was adopted as a standard. With some plant material only one drop could be used without making spore deposits uncountable. The large (5·0 mm.) drops were formed at the end of a glass tube, and smaller ones (3·0 and 2·0 mm.) at the end of a hypodermic syringe needle, the rate of dropping was controlled by a tap.

RESULTS

Preliminary tests

Water-drop collisions on wheat straws rusted by *Puccinia graminis* (Table 1 and Hirst, 1961) showed that, although some spores were splash-dispersed to the inner wall of the sampling cylinder (Pl. 1, fig. 2), most spores caught in the impactors were dispersed dry, a fact demonstrated by loading the impactor with slides coated with naphthol green B in gelatine and then over-coating, with 'Vaseline', one half of the area to be exposed beneath the orifice. Before counting, these slides were mounted in liquid paraffin. Uredospores arriving in water droplets were retained within characteristic stain-cleared circles on the gelatine area but few dry uredospores were caught on this half (Hirst, 1961 and Pl. 2, fig. 3). The portion coated with 'Vaseline' caught many more spores, for it retained those that arrived either dry or in droplets.

Tests in which 3·0 mm. diam. glass beads were dropped on rusted wheat straws stapled on cards, confirmed that mechanical shock accounted for much of the release (Table 1, A, B). Exposing the same straws later to 3·4 mm. water-drops released further spores from the part-exhausted specimens (Table 1, B). Half-embedding straws in wax (Table 1, C, D) decreased the number released by falling water-drops or beads, but subsequent exposure, to water-drops, of the specimens first exposed to beads resulted in a proportionally greater increase. This suggested that water-drops might have some other action besides mechanical shaking. Exposure of rusted straws taped securely to flexible plastic strips (pot labels) firmly clamped at one end (Table 1, E) showed that, when 3·4 mm. drops fell on straws on the upper surface of the strip, they released more spores than when the strip was inverted and the drops collided with the strip and the straws were underneath. This could have resulted from the joint action of shock and some other effect with straws uppermost (Table 1, E2) compared to the effect of shock alone, probably diminished when the straws were below (Table 1, E4). These phenomena were investigated in tests with *Lycopodium clavatum* spores 32 μ diam. uniformly deposited on inert surfaces.

Radial flow in the spreading drop after collision seemed another possible force.

Engel (1955) studied the impact of large (about 5·7 mm. diam.) drops falling at terminal velocity on inelastic surfaces, in connexion with the rain erosion of metals forming the skin of high-speed aircraft. After collision, drops spread radially on the surface up to 2·3 cm. radius, the whole process being completed in about 2 msec. The terminal velocity of the drops was 26·9 ft./sec. (820 cm./sec.). Momentarily in the first stages of collision the velocity of radial flow reached 8·6 times the terminal velocity and exceeded it until the drop was greater than 1 cm. in radius. Such rapid movement must disturb air within the laminar boundary layer and could transfer spores into turbulent air where they would be dispersed by the more usual processes of eddy diffusion.

Table 1. *Estimated total catch of* Puccinia graminis *uredospores in* 50 *l. air sample in preliminary tests (see text)*

Sample Treatment	(1) Before dropping	(2) First dropping	(3) After dropping	(4) Second dropping
A. 9 horizontal straws on card, 3·4 mm. drops	56	Water, 12,130	2	—
B. 9 horizontal straws on card, 3 mm. beads then 3·4 mm. drops	20	Beads, 11,918	1	Water, 843
C. 9 horizontal straws part embedded in wax, 3·4 mm. drops	3	Water, 573	4	—
D. 9 horizontal straws part embedded in wax, 3 mm. beads then 3·4 mm. drops	—	Beads, 2429	—	Water, 755
E. 3 horizontal straws taped to pot label, 3·4 mm. drops	—	Water Straws up, 629	—	Water Straws down, 39

Tests with Lycopodium spores

To assess the relative importance of these processes, it was necessary to have a series of reproducible deposits, each of a uniform density of dry spores and, at least in some tests, on surfaces resistant to mechanical shaking. Accordingly *Lycopodium clavatum* spores (32 μ diam.) were deposited uniformly (average density 700 spores/mm.^{2}) by quickly blowing a standard volume of spores into suspension at the top of a $5\frac{1}{2} \times 16$ in. (14×40 cm.) vertical cylinder and allowing them to sediment on either of two kinds of test surfaces. A cylindrical iron block 4·9 in. (12·5 cm.) diam., 1·5 in. (3·8 cm.) high and weighing 8·5 lb. (3·86 kg.) was used as an obstacle immovable and inelastic to water-drops. Celluloid discs of the same diameter but 0·012 in. (0·30 mm.) thick were rested on short mown grass to represent uniformly supported but slightly elastic targets. Between tests the block was washed and degreased; discs were washed and dried with a clean cloth in the first series of tests; in the second series, they were washed and treated with 'Cirrasol' on both surfaces to remove electrostatic charges.

In the first series of tests, air samples were taken to measure the effects of collisions by glass beads (3·5 mm. diam.) and water-drops of 2·4 and 5·2 mm. diam. In volume these were related as 1:0·32:3·27 but in mass as 1:0·13:1·31. In kinetic energy the glass bead was therefore reasonably similar to the larger water-drop. Table 2 shows that the large drop liberated between 6 and 10 times as many spores as the smaller, and that both released more from the inelastic surface than from the

elastic, presumably because some energy was wasted in locally depressing the celluloid. By contrast, beads liberated very few spores from the block, which they could not shake and deform as they could the celluloid disc. After applying 'Cirrasol', similar results were obtained but the catches were much smaller throughout, probably because some sticky 'Cirrasol' film remained. The results suggest that electrostatic forces were not important in determining the results of the first series. Photographs of Lycopodium deposits after collision show some of these effects clearly. The glass beads only slightly disturbed the spores on the steel block (Pl. 2, fig. 4), but on discs (Pl. 2, fig. 5) they re-arranged the spores, although without clearing the impact area (arrowed). The photograph also shows a smaller area cleared by the rebound impact of the bead, the track along which it rolled off the

Table 2. *Estimated total catch (hundreds of spores) in 50 l. air samples before, during and after collisions with Lycopodium spore-coated surfaces*

	Steel block			Celluloid disc (without 'Cirrasol'*)		
First series	Before dropping	During dropping	After dropping	Before dropping	During dropping	After dropping
2·4 mm. drops	2·6 ± 4·7	26·0 ± 22·7	0·3 ± 0·2	0·7 ± 0·6	13·3 ± 19·8	0·4 ± 0·6
5·2 mm. drops	0·2 ± 0·2	163·4 ± 64·7	0·3 ± 0·4	3·0 ± 7·2	126·9 ± 85·0	1·0 ± 1·7
3·5 mm. glass beads	2·2 ± 3·9	4·1 ± 2·6	0·3 ± 0·3	2·1 ± 4·4	32·1 ± 17·5	1·2 ± 2·5
				Celluloid disc (with 'Cirrasol')		
Second series				Before dropping	During dropping	After dropping
3·0 mm. drops	—	—	—	0·4 ± 0·1	3·3 ± 5·5	0
5·0 mm. drops	—	—	—	0·4 ± 0·1	17·3 ± 25·1	0·3 ± 0·5
3·5 mm. glass beads	—	—	—	0·2 ± 0·1	14·1 ± 15·0	0·1 ± 0·1

* Made by Imperial Chemical Industries Ltd.

disc and a system of concentric zones formed by shock waves travelling within the disc. On discs, drops of both sizes showed evidence of a fast radial splashing. The abrupt and toothed edge of the zone cleared by a 3·0 mm. drop (Pl. 2, fig. 6) suggests that either the splash cup was flatter or the radial wind less intense than with a larger (5·0 mm.) drop (Pl. 2, fig. 7). Within the cleared area both sizes of drop showed dense (white) accumulations of spores which had been wetted. When the surface dried, these adhered to the celluloid much more than dry spores, as shown by Pl. 2, fig. 8, where a disc exposed to a 5·0 mm. drop was inverted and tapped to remove the dry spores. Both Pl. 2, figs. 6 and 7, show a peripheral zone where some air-transported dry spores from within the cleared area have been re-deposited. This zone is best defined with the larger drop (Pl. 2, fig. 7) where microdensitometer measurements showed that it reached about $1\frac{1}{2}$ times the uniform density of spores. This halo is scarcely discernible around the impact area of 5·0 mm. drops on the steel block, probably because the inelastic surface produces stronger radial flows which carry the spores beyond the immediate halo area (Pl. 2, fig. 9). The numerical and photographic evidence combine to support the theory that both shaking and radial flow can be important in suspending dry spores in the air when water-drops strike dry surfaces. It remains to show how often they are.

Tests with fungi

Several fungi on plant material were tested to find whether collisions liberate spores. Where possible the tests were standardized, normally drops of 3·2 mm. diam. were used, 10 collisions were observed per test and 5 replicate tests were made. It was not possible to standardize the plant material, the density of spores it bore or the way it was supported during exposure to collisions. It seems best therefore to comment individually on the fungi listed in Table 3.

Phytophthora infestans–*on potato leaves*

Records from spore traps in infected potato crops do not indicate increased concentrations of spores in the air at the start of rain; indeed decreases are usual (Hirst, 1958). Special care was taken in testing *P. infestans* because we find that rapid humidity changes can release sporangia. Leaves with necrotic lesions 2–3 cm. diam. were collected in the evening and their petioles placed in water-filled specimen tubes taped to the inside walls of the 'sampling cylinders', which were closed, kept damp and shaded until after testing the following morning. (To minimize disturbance the impactor was inserted through the 2 in. hole in the top cover.) Separate specimens were used for each test or attitude. Collisions caused a small but definite increase in the number of spores released.

Pseudoperonospora humuli–*on hop leaves*

The leaves used came from plants grown in pots and kept moist in plastic bags until immediately before use; their petioles were fixed in modelling clay, which was stuck to the inner wall of the sampling cylinder so that the leaf attitude was either natural or inverted. Some spore release was expected without any water-drop collision, because the leaves had just been removed from a humid chamber to one into which dry air was continuously drawn (19 air changes occurred in the sampling cylinder every 5 min.). These spores are entirely restricted to the under surface (abaxial surface) of the leaves and more were caught from leaves in their natural attitude than from other leaves which were inverted. This is probably because spores were able to fall clear of their sporangiophores and the leaf surfaces when oriented naturally and so had a much greater chance of dispersal.

When drops struck the spore-free upper surface of leaves in their natural attitude, a small, black, eddying cloud issued from below the impact point. This is probably unique among these tests as the only time dry spores were liberated entirely as a result of shaking by water-drop collisions. Although the tests with leaves in natural attitudes produced more spores, the ratio between the catches of the before-, during- and after-dropping samples (1:4·9:0·4) was low compared to that (1:50·1:1·1) when the leaves were inverted and the collisions occurred on the spore-bearing 'under' (abaxial) surface. Presumably, this was because shaking was then accompanied by radial air-flow able both to liberate and disperse spores and almost to counteract the effect of inverting the leaves.

Peronospora parasitica–*on stems and capsules of* Capsella bursa-pastoris

The response to dropping was much larger than with *P. infestans*. The stems were laid horizontally, without fixing, on cards placed on short mown grass.

Table 3. *Estimated total catch (hundreds of spores) in 50 l. air samples taken before, during and after the water-drop collisions indicated*

Organism		Drop size (mm.)	Mean no. of drops/test	No. of tests	Before dropping	During dropping	After dropping
Phytophthora infestans	Natural attitude	3·0	10	10	0·8 ± 1·0	8·1 ± 7·3	0·4 ± 0·6
	Inverted	3·0	10	10	2·1 ± 5·4	7·9 ± 7·7	0·3 ± 0·3
Pseudoperonospora humuli	Natural attitude	3·4	1	5	51·7 ± 29·2	251·0 ± 201·2	23·4 ± 17·5
	Inverted	3·4	1	5	2·1 ± 2·4	104·3 ± 79·14	2·3 ± 1·3
Peronospora parasitica		3·0	10	10	0·7 ± 1·0	30·8 ± 64·5	0·2 ± 0·3
Oidium sp.	Conidia	3·4	114	10	0	13·4 ± 11·2	0
Venturia inaequalis	Conidia	3·4	75	10	0·6 ± 0·9	2·3 ± 2·8	0·4 ± 1·2
Puccinia graminis	Uredospores	3·4	42	9	0·3 ± 0·6	61·8 ± 66·8	0
P. striiformis	Uredospores	3·4	95	10	0	4·7 ± 3·2	0
P. obtegens	Uredo-natural attitude	3·0	10	5	0	11·4 ± 11·0	0
	Inverted	3·0	10	5	0	11·2 ± 10·3	0
	Teleuto-natural attitude	3·0	10	5	0	1·9 ± 1·7	0
	Inverted	3·0	10	5	0	3·2 ± 3·1	0
Ustilago nuda		3·4	1	10	51·3 ± 82·2	4025·2 ± 3295·0	33·7 ± 70·21
Cladosporium spp.	Mainly *C. herbarum*	3·4	70	10	12·7 ± 14·1	107·3 ± 35·0	7·4 ± 0·8
Alternaria spp.		3·4	12	5	0·1 ± 0·1	6·8 ± 2·4	0
Epicoccum spp.		3·4	12	5	0·5 ± 0·9	1·2 ± 0·6	0·1 ± 0·1
Pithomyces chartarum	Aleuriospores	5·3	1	10	0·4 ± 0·5	161·1 ± 131·4	0·4 ± 0·4

Oidium *spp.* (*probably* Erysiphe cichoracearum) *on potato leaves*

Chains of conidia formed on the upper leaf surfaces were dispersed by water-drop collisions. These leaves were also laid on thin cards and placed on short mown grass.

Venturia inaequalis–*on apple leaves stapled to light cards*

Only a small response, perhaps because the conidia are formed on very short stout annellate conidiophores. There is no doubt that they are easily splash dispersed.

Puccinia graminis–*on 3 in. lengths of rusted wheat straws stapled on cards placed on short mown grass*

Many of the uredospores in sori are already detached from their stalks. The tests show a large response which agrees with field observations that rain often increases the concentration of uredospores in the air (Hirst, 1961).

Puccinia striiformis–*as for* P. graminis *but on leaves of wheat*

The ratio between 'before-, during- and after-dropping catches' cannot be used for comparison with *P. graminis* but the much smaller catch 'during-dropping' supports observations that airborne spore concentrations of *P. striiformis* are seldom large, even among severely infected crops. It would be interesting to compare the uredospore production of these two fungi per unit area of pustule.

Puccinia obtegens–*uredo and teleuto sori on the under surface of thistle leaves*

Leaves were held in the sampling chamber as for *P. humuli*. Both leaf surfaces of each specimen were exposed to collisions, upper surfaces were exposed first in one half of the tests and lower surfaces first in the other. Collisions aided the release of both spore types regardless of leaf attitude.

Ustilago nuda—*on infected wheat ears*

Portions of smutted ear were laid, with rachis horizontal, on cards on short mown grass and each exposed to one collision. The large catch of spores before and after dropping reflect the ease with which these already-detached spores are dispersed, but there is no doubt that their release is much assisted by even a single water-drop collision.

Cladosporium *spp.*, Alternaria *spp. and* Epicoccum *spp.*

Spores of these saprophytes were released from leaves of ripe wheat plants stapled on to thin cards and laid horizontally on the base of the sampling chamber. Cladosporium and Alternaria, where spores are raised above the leaf surface, respond more than Epicoccum which has sessile spores.

Pithomyces chartarum—*propylene oxide-killed cultures on dead rye grass leaf*

There was a clear response to collision but the targets were more exposed than might be usual for litter in the sole of pastures.

Water-drop collisions on grass litter and short mown grass

Table 4 shows that Cladosporium concentration was increased several times by collisions with both medium and large drops. The same is probably true with

Botrytis and Epicoccum; but the total catches were much smaller. The slight increase with Alternaria on grass contrasts with its behaviour on wheat leaves and may indicate that it was infrequent on the litter samples tested.

Effect of drop size

Tests with a range of drop sizes on the release of three saprophytic genera from leaves of ripe wheat always showed increasing numbers of spores liberated as the size of drops increased. With Cladosporium $\log n+1$ spores of the total estimated catch was significantly correlated with drop size (Table 5).

Table 4. *Estimated total catch (hundreds of spores) in 50 l. air samples taken before, during and after water-drop collisions indicated*

Organism	Drop size (mm. diam.)	Mean no. of drops per test	No. of tests	Before dropping	During dropping	After dropping
Collisions on grass litter						
Cladosporium spp.	3·0	36	6	169·2 ± 214·6	547·1 ± 726·9	10·8 ± 6·6
	5·3	33	4	79·3 ± 99·9	1062·1 ± 1954·0	4·1 ± 5·2
Botrytis cinerea	3·0	36	6	0·6 ± 0·6	2·0 ± 2·0	0·1 ± 0·2
	5·3	33	4	0·4 ± 0·5	3·2 ± 5·7	0·1 ± 0·1
Alternaria spp.	3·0	36	6	0·2 ± 0·1	0·4 ± 0·4	0
Epicoccum spp.	3·0	36	6	0·7 ± 0·7	1·9 ± 1·3	0·1 ± 0·5
	5·2	33	4	0·2 ± 0·5	3·2 ± 5·8	0·1 ± 0·1
Collisions on short mown grass						
Cladosporium spp.	5·3	27	3	16·7 ± 26·3	76·3 ± 78·0	36·7 ± 34·2

Table 5. *Effect of drop size: estimated total catch (hundreds) in 'during dropping' sample*

Drop size	Cladosporium	Alternaria	Epicoccum
2·4	91·0 ± 104·7	2·1 ± 0·8	1·2 ± 0·6
3·0	111·8 ± 51·0	—	—
3·2	461·3 ± 294·8	6·8 ± 2·4	2·0 ± 2·3
5·2	1356·0 ± 32·4	25·7 ± 9·4	3·2 ± 2·4
Correlation coeff. 'r'	+0·7618	—	—
P	< 0·001		

Regression equation of drop size on $\log n+1$ catch of Cladosporium $y = 2{\cdot}567 + 0{\cdot}5124x$.

DISCUSSION

Tests with *Puccinia graminis* and Lycopodium deposits showed that water-drop collisions suspended dry spores in air in addition to dispersing others in splash droplets. Raindrops can disperse dry spores in two ways, by mechanical shaking, and by radial air movements that can exceed 100 m.p.h. for very brief periods and short distances of travel. Sometimes one effect is dominant, sometimes the other, but probably both usually operate. To avoid confusing the several ways in which rain can influence the air spora it seems desirable to have a brief way of referring to these processes and we suggest they be known as 'rain tap and puff'.

There are many reasons why our attempts to standardize tests with the various organisms only partially succeeded. These include differences in the presentation of the spores for dispersal; variations in the 'background' count of the air drawn

into the sampling cylinder (particularly important perhaps with Cladosporium); the great heterogeneity of the samples tested and differences in the directness of 'hits'; the degree to which spores were being liberated from the specimen in the sampling cylinder during tests, particularly important with Downy Mildews and Ustilago. These imperfections and the few replicate tests account for standard deviations of the same order as the means, but despite this inaccuracy there is seldom doubt that the collisions increased the catch. The smallest catch was usually in the 'after dropping' sample, presumably because much of the material was wetted and the supply of dry spores already partly exhausted. Comparisons of catches of different organisms and the ratio of before-, during- and after-dropping catches are often unreliable but usually support the expectation that the effect would be greatest with light dry spores already detached or borne at a distance from plant surfaces on structures from which they are easily sheared.

In addition to the published records and tests mentioned above, our spore trapping shows that the onset of rain at first increased but then decreased the concentration of many spore types including species of *Cladosporium*, *Alternaria*, *Epicoccum*, *Botrytis*, and *Erysiphe graminis*, *Uromyces fabae*, *Puccinia graminis*, *P. striiformis*, *P. recondita* and *Ustilago nuda*. The effect is particularly obvious with intense rain, when the proportion of large drops is great, but it is usually discernible in about half the rain periods. The resulting peak concentrations are often several times those occurring in dry weather, so they can contribute a large proportion of the total air-borne spore load within crops. However, continued rain will soon wash many of the spores from the air, so the effect is likely to be important only in spreading diseases locally. Because rain can fall at any time, it can disperse at night spores that usually have a day-time maximum, thus producing favourable infection conditions with more chance of persisting than during the day. It is possible, but unestablished, that these processes contribute to the characteristic smell of newly wetted soil.

We wish to thank Miss Maureen Thomson for technical assistance, Mr F. D. Cowland for the photographs, Dr J. R. Coley-Smith of the Department of Hop Research, Wye College, for co-operation in the tests on *Pseudoperonospora humuli* and Mr J. Drew Smith of the Ruakura Animal Research Station, Hamilton, New Zealand, for co-operation with the tests on *Pithomyces chartarum*.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Tripod ladder with central dropping tube.

Fig. 2. Sampling cylinder and spore trap in position at base of dropping tube.

PLATE 2

Fig. 3. Photomicrograph of portion of 'half-coated trap slide'. Above: portion where wet and dry spores are impacted on 'Vaseline'. Below: water droplet traces containing few uredospores on the area coated with Naphthol Green B in gelatine (see text).

Figs. 4–9. Photographs of *Lycopodium* spore deposits after exposure to single collisions. Fig. 4. Deposition on steel block exposed to one glass bead impact. Fig. 5. The same impact but on 'Celluloid' disc; note original strike (arrowed), rebound strike and track of bead rolling off the disc. Fig. 6. Result of 3·0 mm. drop falling on disc. Fig. 7. Result of 5·0 mm. drop falling on disc. Fig. 8. Deposit, similar to that in Fig. 7, after inversion and tapping, showing how spores are retained only where wetted, i.e. in peripheral splash droplets and central irregular area, into which residual water in impact area contracted. Fig. 9. Result of 5·0 mm. drop falling on block.

Fig. 1

Fig. 2

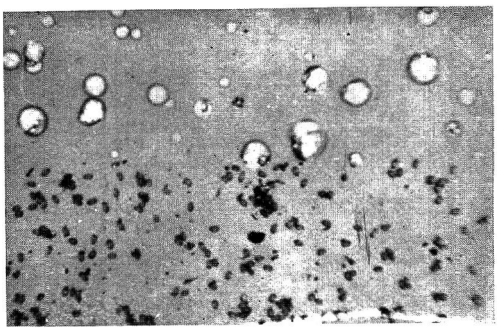

Fig. 3

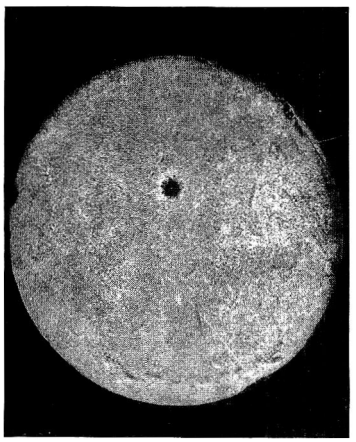

Fig. 4

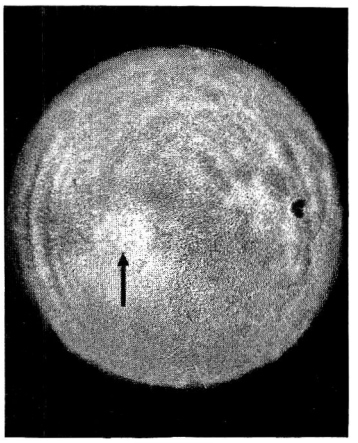

Fig. 5

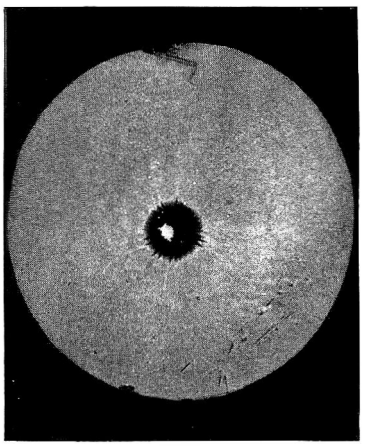

Fig. 6

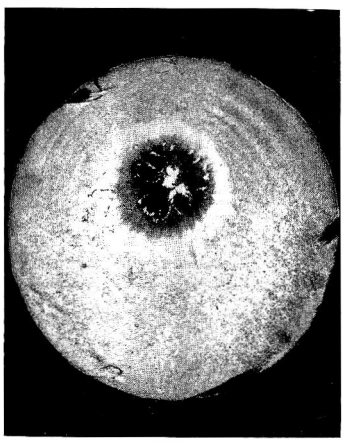

Fig. 7

Fig. 8

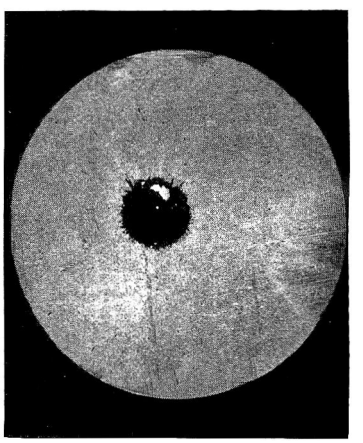

Fig. 9

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Observations on *Sphaleromantis tetragona*

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(*Received* 15 *July* 1963)

SUMMARY

Sphaleromantis tetragona Skuja, which has not been previously reported for Britain, is described with the use of the optical and the electron microscopes. The organism possesses an elaborate sheath which is not visible with the optical microscope; the sheath is probably made of organic material. Cell division and cyst formation are described. The cyst, not previously known, is described as seen with the optical microscope.

METHODS

The methods used for making preparations for the electron microscope were nearly the same as those described by Harris & Bradley (1958) but differed in some details. A drop taken from a concentrated suspension of living organisms was placed on a microscope slide, fixed by exposure to osmic acid vapour for 30 sec. and dried. A carbon film about 200 A thick was deposited on the dry residue and this preparation was mounted on copper grids for use in the electron microscope. The process of backing the film with plastic, previously used, was omitted. These grids were used without further treatment in making the direct micrographs. For the replicas the removal of the cell contents was necessary, but since the sheath is not made of silica no treatment with hydrofluoric acid was required. Equal weights of potassium permanganate and potassium dichromate were mixed and finely powdered; about 0·02 g. of this powder was mixed with about 0·2 ml. concentrated sulphuric acid and stirred until a dark green liquid was produced (chromic + sulphuric acid mixture used by D. E. Bradley in the procedure for making carbon replicas). The grids were floated one by one on a small quantity of this liquid and then washed in distilled water. I put one drop of the chromic + sulphuric acid mixture on a slide, floated the grid on this for 2 min., by which time it had begun to sink, removed the grid, dried it slightly on filter paper and transferred it to a drop of distilled water. I repeated this washing twice more and put the grid aside to dry.

OBSERVATIONS

Occurrence. *Sphaleromantis tetragona* was found on February 1962 in a small pond by the roadside at Arborfield Cross, Berkshire (National Grid 470,076 E., 160,067 N.). It was very numerous, very few other organisms were present in the water and it was therefore particularly suitable for study with the electron microscope. Cysts were found in large numbers on 28 February 1962. During 1963, up to the end of April, it has been seen three times, but each time in small numbers only and in a mixed flora.

Light microscopy. In its typical vegetative condition *Sphaleromantis tetragona* is a small flattened organism appearing square, oblong or trapezoid, with a depression in its anterior end and often also in its posterior end. The single flagellum is situated at the side of the anterior depression. No enclosing sheath is visible when the organism (living or fixed) is mounted in water. Cytological details, including the nucleus and contractile vacuoles, were not studied, but the chromatophore was examined in living and in osmic acid-fixed material. The chromatophore is U-shaped, one arm being broader and paler than the other; the narrow arm possesses the red eye spot or stigma (Fig. 1; 1–3).

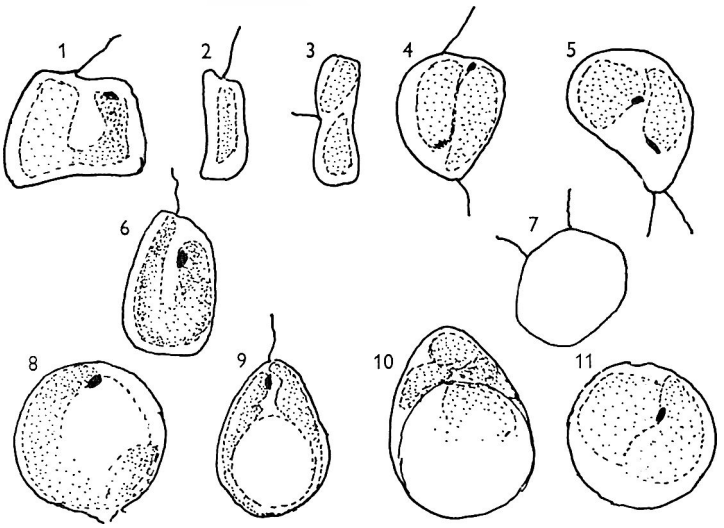

Fig. 1. 1–3: three views of organisms; 1, broad view; 2, edgewise, anterior/posterior axis; 3, edgewise, transverse axis. 4, organism with two flagella at opposite poles; 5, organism with two flagella close together; 6, trapezoid organism longer than broad; 7, outline of organism with two flagella somewhat separated; 8, spherical organism with large leucosyn body. 9–11, Stages in cyst formation: 9, large leucosyn body seen at rear of cell; 10, protoplast passing into cyst wall within cell membrane; 11, fully formed cyst. All ×2000.

The cyst is spherical to broadly oval, with an anterior pore. It appears to have a punctate wall but this appearance may be due to the presence of the punctate mother cell wall enclosing the cyst. Cysts were seen in large numbers in isolated organisms and among agglomerates of non-motile organisms embedded in mucilage.

Typical dimensions. The organisms anterior to posterior pole measured 6–6·5 μ; lateral measurements 3–4 μ, and 6–9 μ. Cyst 7–9 μ diameter, or when oval 9–9·5 × 7–8μ.

Comparison. This description is in general agreement with the descriptions given by Skuja (1939) and Bourrelly (1957). Skuja's measurements 10–12 × 7–10 × 2–3 μ show a slightly more flattened organism, elongated in the anterior-posterior direction; Bourrelly's measurements, 5 × 5 × 2·2·5 μ, show a smaller organism. These differences may denote different strains, different environmental conditions or different stages in the life cycle but are not, in my view, sufficient to constitute different species. Both Skuja and Bourrelly described two chromatophores, I

observed only one; this might be a difference of interpretation. Like many members of the Chrysophyceae the *Sphaleromantis tetragona* chromatophore has two strongly marked lobes which are joined by a narrow and somewhat obscure basal bridge. I agree with Bourrelly's statement that the organism appears to have a rigid membrane.

Developmental stages. When I first examined *Sphaleromantis tetragona*, organisms like those already described were present in very large numbers and any other forms which may have been present were not studied. A few days later another sample was looked at, many organisms were seen to have two flagella and in some of these organisms two red eye spots were clearly visible (Fig. 1; 4, 5, 7). The flagella were sometimes close together or only slightly apart, and sometimes at opposite poles. The organism had lost its characteristic flattened shape and had become ovoid or nearly so. In some organisms (Fig. 1; 4, 5) two chromatophores were clearly visible and neither was U-shaped. At this time it was assumed that these organisms were dividing, a process which I have watched in Mallomonas and in Synura, but unfortunately I did not watch the process on this occasion. Since reading the paper on conjugation in *Mallomonas fastigiata* var. Kriegerii by Wawrik (1960) I realize that what I describe above might be a stage in conjugation and not of division.

Electron microscopy. The rather rigid appearance of *Sphaleromantis tetragona* implies some sort of sheath or cell wall. No sheath can be distinguished with the optical microscope but with the electron microscope a very elaborate sheath is seen. This sheath covers the whole cell, including the flagellum. It may fit closely round the protoplasm (Pl. 2, fig. 5) or be separated from it (Pl. 1, fig. 3). The appearance of the sheath is best seen in replicas (Pl. 1, figs. 1, 2, 4). This sheath is a membrane ornamented with closely-set raised rings and scattered hairs which bear four terminal branches (which I call 'dendroid hairs') and also crimped or twisted threads. All of these seem to be built up of the same material, which has a dark line, either a ridge or an indentation, running along it. The flagellum sheath is best understood by comparing the replica shown in Pl. 1, fig. 1 with the more highly magnified direct electron micrograph shown in Pl. 2, fig. 6. These micrographs were all made from fixed material which was, of course, subsequently dried so that some flattening and distortion may have occurred. The flagellum (Pl. 2, fig. 6) shows a dark core presumably the 2+9 fibrils, and around this is the sheath. The sheath shows the usual rings and numerous dendroid hairs, here seen on two sides only, possibly because of flattening. In addition the flimmer are seen on both sides of the flagellum but I could not see their connexion with the core. The flimmer are unlike those of other Chrysophyte which I know, in that they point in different directions and often stick together in groups of from two to five or even more. The groups may divide and further out reunite or form fresh groups, but the individual flimmer threads can be traced through these groups. A certain amount of mucilage is present over the organism and in other parts of the background and this may have contributed to the unusual appearance by causing artifacts as the preparation dried.

Composition of the sheath. The rings, dendroid hairs and crimped threads appear to be made of organic substance, tough and possibly horny in texture. That they are not made of calcium carbonate was shown by treating some fixed material with acetic acid before preparing a replica; this treatment had no influence on the

appearance of the replica. Likewise the absence of silica is shown by treatment with hydrofluoric acid, which did not influence the appearance of the replica; compare Pl. 1, fig. 1 (prepared with hydrofluoric acid treatment) with Pl. 1, fig. 2 (untreated with hydrofluoric acid). The material did, however, dissolve in chromic + sulphuric acid mixture while associated silica scales were unaltered. Strong chromic + sulphuric acid dissolved the Sphaleromantis organisms, leaving no trace. Dilute chromic + sulphuric acid left some residue of a fixed dried microscopical preparation and a replica was made from this. At magnification ×3,000 nothing intelligible could be seen either of the form of the organism or of the sheath structure; but at magnification ×32,000, numbers of rings could be seen, mostly much corroded, though a few for some reason were only slightly affected, and the dendroid hairs and crimped threads had vanished.

I wish to thank Professor R. W. Ditchburn, F.R.S. and Dr T. Evans for allowing me to use the electron microscope in J. J. Thomson Physical Laboratory, University of Reading, and Mr G. Wood for making most of the preparations illustrated here and giving me technical help.

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EXPLANATION OF PLATES

Sphaleromantis tetragona

PLATE 1

Fig. 1. Replica of an organism with flagellum; ×8000.

Fig. 2. Replica of part of the sheath of the organism; ×32,000.

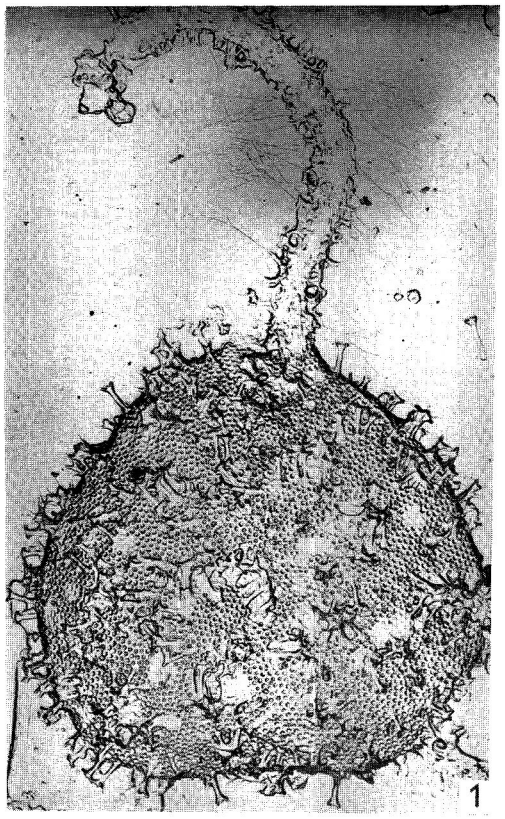
Fig. 3. Direct electron micrograph of an organism showing a loose sheath with its flagellum; ×12,000.

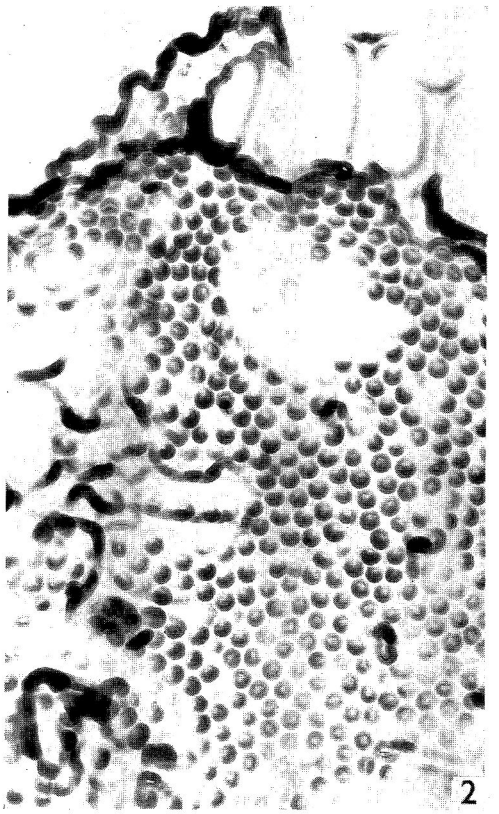
Fig. 4. Part of the sheath showing one dendroid hair and several rings; ×64,000.

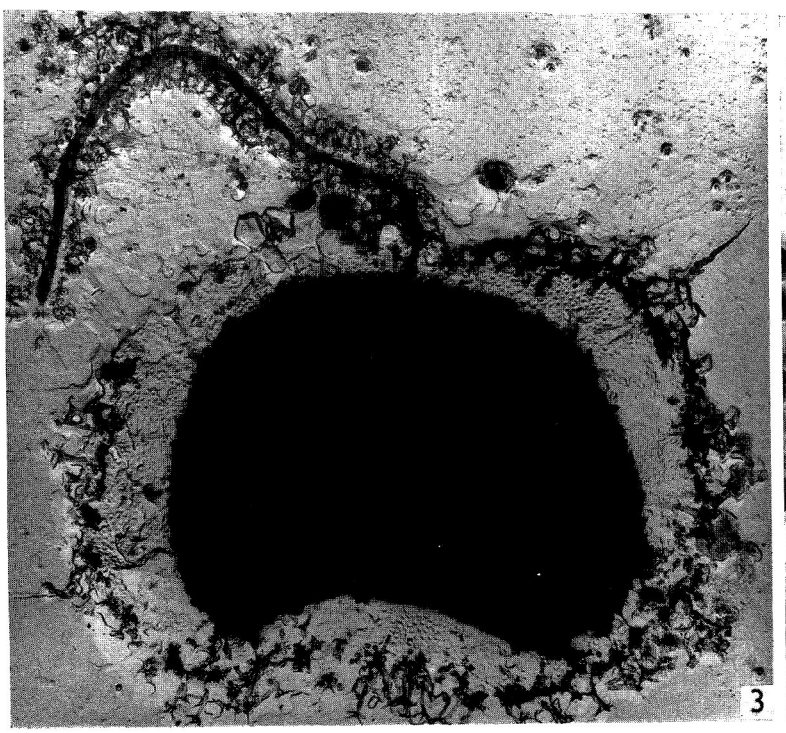
PLATE 2

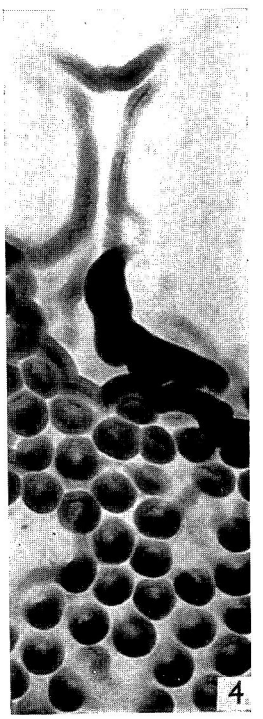
Fig. 5. Direct electron micrograph of an organism showing a closely fitting sheath with its flagellum; ×12,000.

Fig. 6. Direct electron micrograph of a flagellum. The two ovals are scales of a different organism ×26,000.









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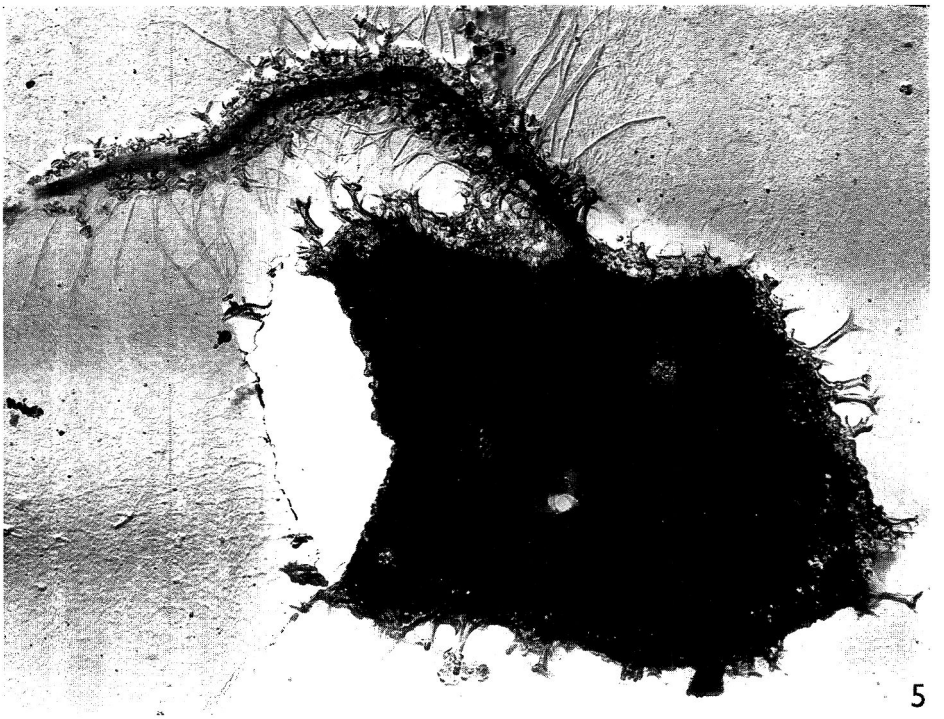
(*Facing p.* 348)



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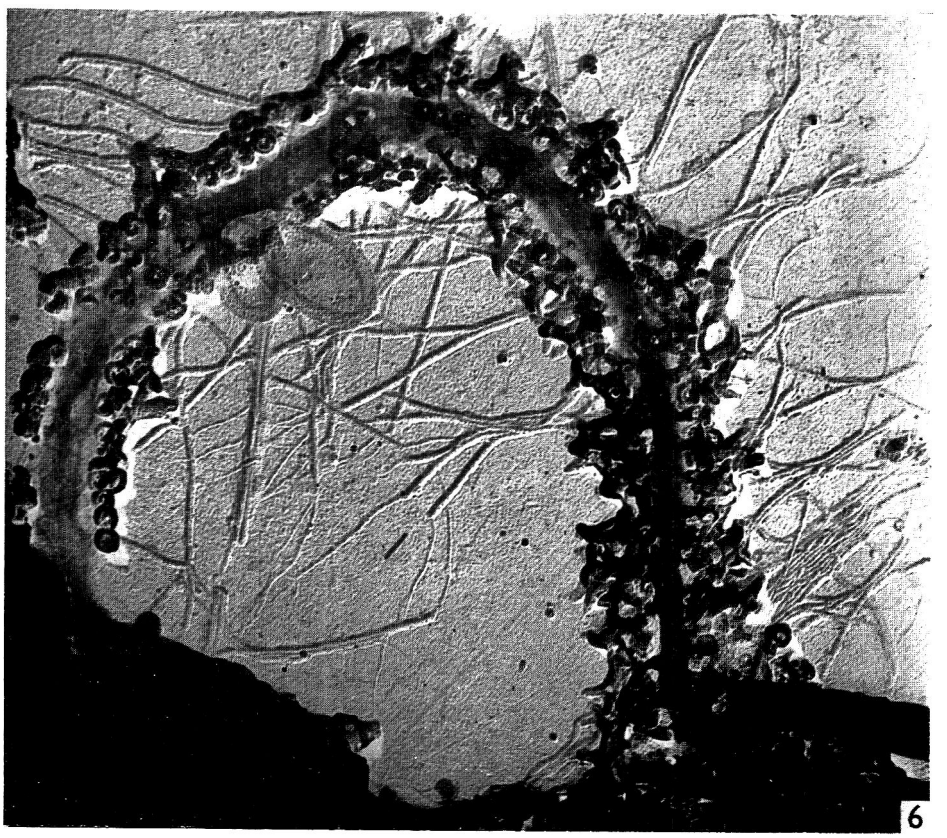

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