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The Troubles of a Virus

The Seventh Marjory Stephenson Memorial Lecture

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The subject of my lecture concerns an aspect of microbiology rather different from that upon which Marjory Stephenson's great reputation was built. However the Marjory Stephenson lecturer may, I gather, deal with any side of the subject. I believe that the matters I shall discuss would have interested her and I hope also that my remarks may contain a few crumbs of interest for you.

Most people regard viruses simply as noxious parasites to be destroyed or at least thwarted, though perhaps virologists take a more lenient view, since upon the viruses their livelihood depends. But either way the viewpoint is distressingly anthropocentric; the only criterion seems to be their effect, direct or indirect, on that great hulking mammal, man. Nobody looks at matters from the point of view of the poor little virus, so many times smaller than man himself. After more than forty years' study of virology, I feel that I may try to get under the skin or capsid of a virus and from my seat in its nucleoid see how the world looks from a virocentric point of view.

It can too easily be imagined that a virus's life is an ideal one. A virion is equipped to penetrate the cell of its choice. It has the trick of divesting itself of the paraphernalia which helped it in that penetration and thereafter it can compel the cell it has entered to devote its resources to make more virions like itself. Within a short time—an hour or two in the case of some phages— it can become not only a mother but a grandmother and great-grandmother and that without any necessary introduction of the joys and sorrows associated with sex. Its progeny can go and do likewise, doubtless supposing that they can continue and duly inherit the earth. In fact, of course, they are limited, as all parasites are limited, by the supplies of susceptible hosts and by the ability of those hosts to react against them.

The first trouble they meet with is that they find themselves inadvertently stimulating the production of a hostile substance called interferon and this even while they are supposing themselves quite safe inside a cell. In consequence the cellular environment becomes much less favourable and their replication may be slowed down or even halted. They can, however, themselves react. Strains of viruses may develop, having less tendency to evoke interferon production and less susceptible to its action. I shall be suggesting later in this talk that variation in susceptibility to interferon plays no little part in affecting a virus's prospects.

The next trouble is that when enough virions have been shed into the environment they begin to stimulate antibody production. This reaction comes along a little later than the interferon production, but it is a menace which persists and increases

and may soon end the virus's prospects so far as the originally infected host is concerned. Here again is a trouble which viruses have to some extent learnt to overcome. First of all, of course, they can escape and manage in one way or another to reach fresh hosts. Before they have to do this, however, they may exploit the local possibilities a little further. They may penetrate the blood-brain barrier or reach the cornea or some other foothold where antibodies cannot so easily reach them. They do better in fact anywhere where they are superficially placed, in the skin or on mucous membranes. Antibodies may indeed be found in mucous secretions, but they are lower in titre and less effective than where there is readier interchange with the fluids of the blood.

Viruses may dodge antibodies in another way. Those of the pox and herpes groups may pass from cell to cell along fine intercellular channels and so extend the area of their dominion without having to leave the shelter of a cell and brave the antibody menace without. The virus of varicella spreads readily in such a manner in many sorts of tissue culture, but when one tries to demonstrate free virus in the culture fluids one commonly fails to do so. In the case of the virus of malignant catarrh of cattle the phenomenon is even more striking. Though it was long suspected that this was a virus infection, it was until recently impossible to demonstrate a free filterable agent. There is a bit of a mystery to be unravelled here. Both varicella and malignant catarrh readily infect fresh hosts; how, if they are so closely cell-bound, do they manage to do so? Some viruses of the myxovirus group are haemolytic *in vitro* and this haemolysis seems to be merely a manifestation of a cytolytic property. This seems to serve the purpose of allowing adjacent cells to become fused and to form a syncytium in which viruses can multiply. Here we have another ruse by virtue of which more virus can be built up in a focus in the body despite the presence of circumambient antibody.

But now suppose that our virus can make no more headway in its original host and is seeking fresh victims in ways to be discussed later. A time will come when those in its immediate environment are either dead or immune—in either case unavailable. How is this trouble to be surmounted? There is the geographical escape. An epidemic may be a migrating one. Outbreaks of fatal yellow fever amongst South American monkeys can be followed and have been found to spread as fast as 200 km. a month. In Africa, too, though yellow fever is an inapparent infection among the local monkeys, there is evidence that waves of spreading infection occur. Widespread epidemics among human beings, due to the Chikungunya and O'Nyongnyong viruses, show a similar pattern of movement. Nor, to come nearer home, do I need to remind you how influenza A virus travels from country to country.

The influenza virus exhibits two other features of interest. One may see phase-variation, the so-called P-Q variation, wherein less avid forms are produced, reacting poorly with specific antibody. Isaacs *et al.* (1954) have suggested that though the total antigenic composition of the less avid or Q phase may be unchanged, the components have been somehow shuffled, the virus being partly turned inside-out so that the antibody-reacting constituents are not so readily available at the virus's surface. The virus may well find this helpful to it, but of far greater significance for its future is the phenomenon known as 'antigenic drift'. Observations over the past thirty, and more conclusively, the last fifteen years, have shown that, after a period in which influenza has been virtually absent, new strains have appeared,

antigenically distinct from those prevalent earlier. As a rule they have been more closely related to their immediate predecessors than to those from further in the past. It has been shown experimentally that influenza passaged in partly immune mice or in the presence of amounts of antiserum inadequate for complete suppression, will encourage the emergence of antigenically novel strains. It was hoped at one time that if we knew the antibody-composition to be found in a human population, we could passage virus in the presence of similar antibody and see what turned up; thus it was hoped to predict what sort of a variant would appear with the next outbreak. I need hardly say that the influenza virus was smarter than we were and did not behave as had been guessed. What I have described as the 'flu virus's behaviour was true of the years up to 1957. Since the Asian or A2 virus appeared it has not varied as much as it had done before. Post-1957 strains do differ from each other but not very greatly: the A2 virus seems relatively more stable. Perhaps it is as a consequence that its visitations have been rather less frequent and widespread of late. Possibly production of the 1957 variant was a mistake from the virus's point of view.

Let us now consider what means are at a virus's disposal for reaching a new host. Though viruses are non-motile, they are equipped with homing devices which help them to attach to appropriate cells. These take the form of chemical substances with affinity for receptors on the cells' surface. The mechanisms involved have been particularly well studied in the case of influenza; here the readily observed reaction between the virus and receptors on the surface of red blood cells serves as a model of what is happening when the virus reaches a susceptible cell on the epithelium of the respiratory tract.

The homing devices require, however, the assistance of some outside agency to to bring virus into fairly close contact with the cell to be invaded. Often the host itself provides the mechanism; a sufferer from a cold sneezes and projects virus towards the next victim; a mad dog bites; a child's fingers pick up contaminated material; or infection is carried by the movement of wind or water. One of the most primitive and still highly successful methods depends on an alternation of hosts, especially when both of them are mobile. We are probably wise to consider so-called arthropod-borne infections of vertebrates as being essentially vertebrate-borne infections of arthropods. Overt disease in the arthropod is unknown in the case of arbovirus infections of birds and mammals; so it is more likely that the association of virus and arthropod is the older one. The virus then regards the mosquito- or tick-bitten vertebrate as a convenient means of finding its way to a new tick or mosquito. Much the same is probably true in the realm of plant-viruses, for the aphid-vectors of plant viruses (if we look at it that way round) are commonly unaffected. The matter is more doubtful in the case of some cicadellid-borne viruses, which may lead to harmful results in both plants and insects (Maramorosch & Jensen, 1963).

The arthropod-vertebrate cycle may not always be a sure and certain matter for the virus. Mosquitoes have a short life and may well perish before the ingested virus has had time to mature within them and reach their salivary glands ready for introduction into a new host. A mosquito which normally bites birds may at times take a meal from a horse or a man; a virus such as one of the North American equine encephalomyelitis viruses may then find itself in a host in which it can only multiply to a low titre; it has got into a blind alley. And what if one of the members of the

cycle disappears, as mosquitoes do during winter months? There has been much discussion as to how these equine encephalomyelitis viruses manage to overwinter, to carry on till mosquitoes re-appear in the spring. It has been suggested that migrating birds carry them south and then north again, or that they persist in occasionally hibernating mosquitoes or in bats or snakes. The most recent suggestion is that there is a hitherto unsuspected cycle in small rodents and their ectoparasites, for the viruses have been recovered from mice in New Jersey during winter months (Sussman, private communication).

A virus might be pardoned for thinking that this arthropod transmission was too chancy an affair and that it might be safer to try to reach one's next host directly by air transport, riding upon a sneeze. But here more troubles are encountered. Virus carried on the smallest droplet nuclei is exposed to the inactivating influences of dessiccation and exposure to light. It must reach its objective very quickly or perish. It is more stable if associated with a large particle which can somewhat protect it; but then it can easily fall too quickly to the ground. To succeed, it has to manage to board a particle of such intermediate dimensions that it avoids both these dangers. It must then be carried by the air current of an inhalation round the corner of the nostrils into the nose; to land prematurely on the external nares is to court destruction. Once within the nose it might expect to find safety at last. But not so; it probably lands upon a moving sheet of mucus upon which it gains an insecure foothold and which carries it inexorably backwards and then downwards to meet its doom down the oesophagus and into the acid cauldron of the stomach.

One inevitably wonders just how a respiratory virus ever manages to avoid these hazards; yet it certainly can do so. A local chilling and desiccation within the nose may temporarily halt the flow of mucus and give the virus its chance. Or perhaps the stream of mucus turns over and over as it goes, so that a few virus particles can seize an opportunity to snatch at a near-by cell before they are whisked away. Experiments on volunteers with rhinoviruses show that one cannot dilute virus-containing nasal washings very far and still produce colds. Rather large doses are necessary. The amount of virus which leads to a cold in nature must be much less than this. I shall try to account for this anomaly later.

The rhinoviruses which cause colds are labile in the presence of acid and cannot therefore survive passage through the stomach. Their relations, the enteroviruses, can withstand this and therefore have no trouble in reaching their habitat in the intestine. One can only guess whether they are acid-stable derivatives of the rhinoviruses, adapted to a life in the gut, or whether it is they who are the ancestors of the rhinoviruses which have discovered how to lodge in the nose and multiply there. The troubles of the enteroviruses are in any case different ones. For them faecal contamination is the key to success. Here they may fall between two stools. In an environment, let us say in a tropical village, where hygiene is practically non-existent, they can spread so readily that all children are infected at an early age. The enteroviruses then cause only infections of infants and are dependent for their continual existence on a continuous supply of fresh susceptibles. On the other hand, in a modern highly civilized community, faecal contamination cannot be relied on as a means of cross-infection and as our hygiene improves the enteroviruses will spread with greater and greater difficulty. Their victims will, however,

always include the visitor from civilization who penetrates into less hygienic parts of the world. On the whole, the enteric viruses seem to thrive pretty well.

Three of them, however, have made a grave error. The three serotypes of poliomyelitis have, apparently quite fortuitously and with no benefit to themselves, found themselves able to invade the central nervous system of man and cause paralytic disease. So hurtful has this been that man has been inspired to develop an effective polio vaccine which will, we hope, get rid of the paralytic disease. I do not know how a virulent poliovirus regards the prospect of being eliminated in favour of an anaemic avirulent man-made vaccine strain. With much the same disdain, I imagine, as a wolf might regard a Pekingese dog.

Passing over some less important routes of transfer of infection we come to vertical and transovarial transmission. This must seem an easy way out of a virus's troubles. Some viruses, in ticks, may be passed to a new generation in the ovum, never having to battle with the dangers of the world outside. The same is true of some viruses which are pathogenic for the larvae of Lepidoptera. In the case of lymphocytic choriomeningitis of mice, the phenomenon of immunological tolerance permits the virus to persist indefinitely in the blood and tissues of mice infected *in utero*. Infection of the young may be through the ovum, or, as with Bittner's virus causing mammary cancer in mice and in some other tumours and leukaemias of mice, it may be passed in the maternal milk. Some plant viruses, too, are transmitted in seed. Such vertical transmission is doubtless helpful to a virus, but notably so only when it supplements some other method of transmission. Unless the host species is increasing or extending its range, a pair of animals will on the average only give rise to two descendents in the next generation. Viruses transmitted only in a vertical way can therefore rarely hope to do more than maintain their numbers: they cannot conquer new worlds.

Some viruses have in the course of evolution found new ways of reaching fresh hosts. A few of these changes can be observed today or can be assumed to have taken place quite recently. The tick-borne encephalitis virus of Central Europe is at times transmitted by the milk of infected goats. The virus of Eastern equine encephalomyelitis, normally carried by mosquitoes, spreads among captive pheasants, probably as a result of pecking. The pox viruses seem to be adaptable creatures for some, such as smallpox, spread by the respiratory route, while the closely related ectromelia, at least sometimes, passes out in the faeces, and others—for instance, fowl pox and myxomatosis—are mechanically carried by insects. It is a reasonable hypothesis that many changes in routes of transmission have occurred in the past.

We now come to the greatest trouble which a virus must cope with. While it has to be sure of spreading to fresh hosts in the ways we have considered, it must not be too efficient or all its potential hosts will be eliminated by death or by becoming immune. Either it must spread only indifferently well, by a calculated inefficiency, or it must find some way of remaining latent until a new supply of susceptible hosts has become available. The virus's problem is of course the same as that of any parasite. The virtues of self-restraint may have to be learnt in a parasite's own interests.

'Inapparent infection' in the virus field has been defined (*Symposium*, 1957) as covering 'the whole field of infections which give no overt sign of their presence' and 'latent infection' as a special instance of inapparent infection in which 'the

infection is chronic and in which a certain equilibrium between host and parasite has been established'. Both truly latent and transient inapparent infections come into our story. It is a familiar idea that parasites and their hosts in the course of evolution become mutually tolerant. Instances of inapparent virus infection are accordingly far more numerous than those in which viruses cause overt disease. Arbovirus infections are always inapparent in arthropods and are usually so in their normal hosts—monkeys, rodents or birds as the case may be. They only come to our attention when fortuitously spreading to strange hosts. Enterovirus infections of man are usually inapparent; only the exceptional one 'goes beyond its bill' and by invading nervous or muscular tissues leads to overt disease. Polyoma virus in mice, and others which potentially give rise to leukaemia, are completely harmless in the ordinary way: it is man's deliberate interference with nature in his laboratory which permits them to cause malignant disease. One can pass other virus families in review and observe the same sort of thing. Those viruses are successful which have learnt not to damage their hosts and to spread, usually among young animals, wisely yet not too well. There are of course others which cause disease and use that disease as a means of spread: the encephalitis of rabies causes mad dogs to bite and the 'coughs and sneezes' caused by rhinoviruses and others 'spread diseases'.

A number of viruses, however, do not seem to have achieved the peaceful co-existence of the quietly successful ones, and these have gained their ends in other ways. For some a periodically activated latent infection has marked the road to success. The classical example, of course, is afforded by Herpes simplex. Primary infection usually takes place early in life or not at all; it often takes the form of a widespread stomatitis in children. Thereafter the virus settles down as a latent infection, often in the corner of the mouth or external nares, to be activated ever so often as a crop of blisters by various stimuli; these are different in different people, perhaps a common cold, menstruation or eating cheese. A little virus may be recovered at times from the saliva between episodes of fever blisters, but it seems fairly certain that it is the virus shed at the time of these episodes of activation which acts as the main source of infection.

Something of the same sort is seen with the related varicella virus. After an attack of chickenpox, virus may remain latent in the central nervous system, awaiting activation in the posterior root ganglia and a centripetal spread to cause the eruption of Herpes zoster. This may be the starting point of more varicella cases. Hope-Simpson (1965) has lately suggested that the whole sequence may have evolved for the benefit of the varicella-zoster virus; it can thus initiate a new chain of infection if a child-to-child infection-series has petered out.

New light has been thrown lately on the ecology of rabies. No longer can it be considered as an invariably fatal disease. In vampire bats it may exist as a latent infection, virus being present in salivary glands but not necessarily in the brain. There may also be virus in the brown fat and this may persist there during a bat's hibernation. Insectivorous and fruit-eating bats may also carry the virus. Recently 5% of bats in New Jersey were found to harbour the virus, though no cases of rabies in dog or man have been seen in that state for almost a decade (Sussman, private communication). At times the virus in insectivorous bats may cause encephalitis and they may then bite the human hand that touches them, or even attack unprovoked. In that the rabies virus does not necessarily kill infected bats, they

might seem to qualify as the original hosts of the virus. Quite a different view has been put forward by Johnson (1965). He regards bat rabies as part of an aberrant cycle and considers that the true hosts in North America are mustelids—skunks and ermines. The small spotted skunk was apparently known to backwoodsmen of the west as a vector of hydrophobia and was accordingly called by them 'phobey-cat'. From them the virus may spread to foxes and wolves in which it may cause devastating epizootics and may thus actually serve to eliminate the mustelids' competitors. Whichever view is correct, a sequence of alternating quiescence and activity is indicated and this might appear well suited, as with herpes simplex and zoster, to help in the perpetuation of the virus. The relative importance of bats, mustelids, foxes and dogs and other creatures in the ecology of rabies is, however, still under discussion.

Alternating quiescence and activity is of course a familiar feature of influenza. Over periods of a month or two it can cause outbreaks affecting millions and then, perhaps for two years, it may be hard to find. In between whiles, virus is only recovered from an occasional sporadic case, or perhaps serological evidence of its activity is detected. It seems that the virus's activity is at such a low ebb then that it can only infect the occasional unusually susceptible person. Then after a while a useful antigenic variant turns up or waning of herd immunity helps it to get going again.

We are beginning to get glimmerings of ideas concerning the periodical activity of colds—why they are more prevalent in winter and in certain climates. Despite efforts to produce some, there is no good evidence that rhinoviruses and epidemiologically similar agents spread any better in winter than in summer. Rather does it seem that an infection in winter is more likely to be clinically manifest. Simultaneous occurrence of colds over wide areas points to some activating stress which is likely to be of a meterological nature. I say this in spite of our own failure at Salisbury to show that chilling increases susceptibility to colds in individuals. It does not seem that one can explain matters on the supposition that some people carry a particular virus, as herpes is carried, and that this is periodically activated; for in one individual observed by Dr D. Hamre five successive colds were caused by five different rhinovirus serotypes. In a recent discussion of the subject (Andrewes, 1964) I have put forward a tentative hypothesis. According to this, cold viruses in small doses are constantly being passed back and forth between people, obtaining only temporary footholds, perhaps in quite small foci on the respiratory mucosa. These may be insufficient to cause generalized immunity and may be prevented from spreading widely on the mucosa by rapidly mobilized interferon or some other local defence. Only when this defence is breached as a result of some stress does a cold results. There is evidence that stress may inhibit interferon production. In a particular instance which has been published, interferon production was inhibited when mice were stressed by subjecting them to loud noise for 3 hr a day (Chang & Rasmussen, 1965). It is also clear that stresses of several kinds play a part in causing outbreaks of respiratory infections among service recruits (Pierce, Stille & Miller, 1963). If such an explanation should be correct it would indicate that cold viruses have evolved an ingenious mechanism for self-perpetuation. Despite the troubles I discussed earlier they seem to get around without much difficulty. If every happy landing led to a miserable cold, too many subjects would acquire

immunity, as they do after a cold—though only to that particular virus. But if it is only the people who chance to be in a receptive state who develop overt and therefore immunizing colds, the virus will find susceptibles available over long periods.

We do not yet know whether rhinoviruses are antigenically labile as influenza A is and whether such lability is a further aid to them. If, as now appears, there are at least eighty serotypes and probably many more, they may not need to vary. The ingenuity of a mechanism whereby they spread only to certain individuals may be an expression of their subtle efficiency rather than being calculated inefficiency such as I suggested earlier.

An alternation of activity and quiescence seems to be useful to a number of viruses and this may be helped by quite small variations in their properties. If, for example, environmental factors could switch them between states of greater and lesser susceptibility to interferon they might be able either to grow and spread more actively or, when it suited them, to go into temporary retirement.

An ideal virus could of course be one which directly benefited its host; and such viruses may actually exist. The maize hopper, *Dalbulus maidis*, normally feeds only on maize and a related grass species; on asters it dies. But when fed on asters already infected with aster yellows virus it thrives, and after a while is able to thrive also on uninfected asters, on carrots or on rye. The effect seems to be on the ability of the hopper to digest previously indigestible foods. Now aster yellows virus can be killed by moderate heat and such heat-treatment cures infected hoppers of their infection; but then, behold, their abnormally fine powers of digestion have gone also. Maramorosch & Jensen (1963) have suggested that we may be overlooking something of wide importance. If there were lots of beneficial viruses in the world, should we ever suspect that they existed?

I shall now re-assume my normal anthropocentric view of my subject. We have, I think, to admit that though viruses encounter many troubles, they are remarkably successful in overcoming them. If, however, we can manage to be temporarily virocentric and see where a virus's troubles lie, we may better be able, in the case of truly harmful viruses, to see that those troubles become even greater, so that we can become their masters.

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Inactivation of Influenza Virus by Caseinase C from *Streptomyces albus* G Culture-filtrate

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SUMMARY

After incubation for 1 hr at 37° with caseinase C (a purified fraction of actinomycetin), the neuraminidase activity of concentrated purified influenza virus PR 8 suspension was unchanged, whereas its infectivity and haemagglutinating activity were considerably decreased. After 4 hr, infectivity and haemagglutinating activity were still more decreased and most of the neuraminidase activity was destroyed. The ability to fix complement in the presence of specific antibodies was slightly decreased, whereas the ability to neutralize haemagglutination-inhibiting antibodies was not affected.

Influenza virus PR 8 suspension treated with enzyme caseinase C contained material sedimentable by centrifugation for 1 hr at 31,000 *g* and material which remained in the supernatant fluid under these conditions. Both materials fixed complement in the presence of control PR8 virus antiserum. The ability of control PR8 virus suspension to neutralize haemagglutination-inhibiting antibodies and its ability to react with strain-specific complement-fixing antibodies were related to material sedimentable by centrifugation for 1 hr at 31,000 *g*. Treatment of PR8 virus suspension by caseinase C destroyed its ability to produce antibody-fixing complement in the presence of control influenza virus PR8. But this treatment did not suppress ability to produce specific haemagglutination-inhibiting antibodies. Following this treatment of PR8 virus a new antigenic activity was shown: the antiserum to enzyme-treated virus not only fixed complement in the presence of enzyme-treated PR8 virus, but also in the presence of enzyme-treated Asian virus. Material which fixed complement in the presence of antiserum to enzyme-treated virus was sedimentable from enzyme-treated PR8 virus suspension by centrifugation at 31,000 *g*.

INTRODUCTION

Malchair (1958) reported that *Streptomyces albus* strain G culture filtrates obtained under suitable conditions, or actinomycetin, impaired the haemagglutinating activity and the infectivity of influenza virus by an enzymic process. In the present paper the action of a purified fraction of actinomycetin, caseinase C (Ghuysen, Leyh-Bouille & Dierickx, 1962; Dierickx & Ghuysen, 1962) on concentrated and purified influenza virus PR8 is examined, its effects on the following viral properties being compared: haemagglutination, infectivity, neuraminidase activity, ability to react with specific antibody, antigenicity.

METHODS

Viruses. Influenza A virus strain PR8 was purified and concentrated by one adsorption-elution cycle followed by centrifugation. Packed fowl red cells (2%, v/v) were added to infected allantoic fluid obtained after three passages of lyophilized virus. Agglutinated red cells with virus adsorbed were washed twice with cold saline and resuspended in a volume of saline equal to one-fifteenth of the original volume of allantoic fluid. After incubation for 4 hr at 37°, the suspension was clarified by centrifugation at 1500 *g* for 15 min.; the eluted virus was then sedimented by centrifugation at 26,000 *g* for 60 min. The pellet was resuspended in a volume of distilled water equal to one-hundredth of the original volume of allantoic fluid. The viral suspension, after removal of large particles by centrifugation at 5000 *g* for 10 min., was distributed in sealed vessels which were stored in solid CO_2, or were frozen in solid CO_2 and afterwards kept at $-70°$. This preparation is referred to as concentrated purified PR8 virus. A stock of a Belgian strain A2 virus isolated in 1963 was similarly prepared.

Caseinase C preparation. The caseinase C used in this work was obtained from Dr J. M. Ghuysen and Mr L. Dierickx; its purification will be reported elsewhere. By its action, casein becomes progressively less, and finally not at all, precipitable by trichloracetic acid. The presence in the digest of at least four compounds highly reactive to ninhydrin indicates the proteolytic activity of this enzyme preparation (Mr L. Dierickx, personal communication). The solution of caseinase C used had the same caseinolytic activity as a solution containing 150 μg. crystalline trypsin/ml. Two different stocks were used which behaved identically.

Enzyme treatment of virus. Virus, caseinase C preparation and phosphate buffer (pH 7, μ 0·1) were mixed in the following proportions by vol.: concentrated purified virus, 31; caseinase C solution, 5; buffer, 4. Virus and enzyme were thus finally in μ 0·01 phosphate buffer at pH 7. The mixtures were incubated at 37°.

Measurement of virus haemagglutinating activity. From a 10^{-2} dilution of virus suspension, several series of twofold dilutions were made in 0·5 ml. saline, on china or Perspex cup trays. To each cup was then added 0·5 ml. 0·5% (v/v) fowl red cell suspension. The haemagglutinating titre was calculated as the arithmetic mean of the reciprocal of the highest final dilution which gave detectable, although incomplete, haemagglutination in replicate series (most often four) after overnight incubation at room temperature. The amount of virus giving partial haemagglutination was taken as one haemagglutinating unit. When incomplete haemagglutination was not observed in a series, the titre was taken as the arithmetic mean between the highest dilution to give complete haemagglutination and the one immediately following which gave a negative result.

Measurement of virus infectivity. The technique with fragments of chorioallantoic membranes on the shell was used (Fazekas de St Groth & White, 1958). Sixteen twofold dilutions, the range of which included the ID50 dose were inoculated, with 10 cups/dilution. Two Perspex trays were put side by side on each of six aluminium shelves supported by a stainless steel frame and spaced at intervals of 2·5 cm. The ID50 dose was estimated graphically on logarithmic probit paper (Boyd, 1956).

Measurement of neuraminidase activity. The substrate was human urinary mucoprotein (Tamm & Horsfall, 1950). Free *N*-acetylneuraminic acid was esti-

mated by the sodium iodate+thiobarbiturate assay of Warren (1959; readings taken at 549 mμ). Each result was the average of four assays.

Measurement of the ability of virus to react with specific antibodies

Antiserum. Antiserum to influenza virus PR8 was obtained from a rabbit after intramuscular injections of virus purified by a single adsorption-elution cycle, the saline containing the eluted virus being cleared of red cells by centrifugation at 1500 *g* for 15 min., and immediately distributed in screw-cap bottles and stored at −25°. The rabbit was given 1·0 ml. injections of this suspension, the first five twice a week, the sixth 32 days after the fifth, and the seventh 24 days later. The rabbit was bled 7 days after the last injection. The serum was stored at −25° in screw-cap bottles.

Complement-fixation test. A micromethod in tubes was used (Sohier, Peillard, Gineste & Freydier, 1956) with complement constant at 2 units. Twofold dilutions of antiserum were tested in the presence of twofold dilutions of viral suspension. Fifty % haemolysis was taken as the end-point. Anticomplementary activity of rabbit serum was treated by diluting 1/10 in veronal buffer (Sohier *et al.* 1956) and heating at 56° for 30 min. An equal volume of guinea-pig serum diluted 1/2 in veronal buffer was added. The mixture was kept at 37° for 60 min. and afterwards heated at 56° for 30 min.

Absorption of haemagglutination-inhibiting antibodies. To titrate the ability of a virus suspension to absorb antibody from a given dilution of antiserum and to measure the limiting concentrations of virus which neutralized various concentrations of antiserum, the following two tests were used. (1) Twofold dilutions of virus were added to equal volumes of antiserum diluted 1/80; 60 min. later, twofold dilutions were prepared from these mixtures, using 0·25 ml. volumes; to each dilution was added four haemagglutinating PR8 units in 0·25 ml. and 60 min. later, 0·5 ml. red cell suspension. (2) Twofold dilutions of antiserum and of virus suspension were prepared. One-tenth ml. of each antiserum dilution was added to 0·1 ml. of each virus dilution. The mixtures were left for 60 min. before adding four haemagglutinating units of PR8 in 0·2 ml. and 60 min. later, 0·4 ml. of red cell suspension. Saline was used as diluent. Antisera were previously heated at 56° for 30 min. The four haemagglutinating PR8 units were in the form of diluted infected allantoic fluid. A 0·5 % (v/v) fowl red cell suspension was used. The tests were made at room temperature and read as soon as red cell sedimentation was complete in the diluent.

Antigenicity was tested by comparing two sera.

(*a*) *Sera.* Two guinea-pigs received six intraperitoneal injections of enzyme-treated or control PR8 virus suspension, respectively. The first five injections were done twice a week, the sixth 24 days after the fifth. The two animals were bled 4 days after the last injection. The stock of concentrated purified virus prepared for this purpose was obtained according to the technique described above, but by using two adsorption-elution cycles and a final centrifugation at 1500 *g* for 15 min. On each inoculation day 0·75 ml. of virus suspension was thawed. One half was incubated for 4 hr at 37° in the presence of caseinase C, the other half kept at 37° in phosphate buffer (μ 0·01; pH 7) for the same time. The effect of the enzyme was checked by a haemagglutination test. Control virus suspension diluted 1/10 in saline containing 1000 units penicillin G and 1000 μg. streptomycin sulphate/ml.

was inoculated into the guinea-pig for control antiserum. To the enzyme-treated virus suspension diluted 1/10 in saline containing antibiotics, 20 % (v/v) of packed fowl red cells were added; after 15 min. at room temperature, this mixture was centrifuged for 15 min. at 1500 ***g***. The supernatant fluid obtained after four such adsorptions was inoculated into the guinea-pig to produce antisera to enzyme-treated virus.

(*b*) *Inhibition of haemagglutination.* Twofold dilution series of sera previously treated by periodate to destroy non-specific inhibitors were prepared on china or Perspex cup trays. To 0·25 ml. of each serum dilution, four haemagglutinating units of virus in 0·25 ml. were added. After 60 min. at room temperature, 0·5 ml. of 0·5 % (v/v) fowl red cells were added. Readings were made as soon as red cell sedimentation was complete in the diluent (saline), the end-point being defined as the highest dilution at which no haemagglutination was observed.

RESULTS

Haemagglutinating activity

The haemagglutinating activity of concentrated purified influenza PR8 virus remained unchanged after incubation for 4 hr at 37°, in phosphate buffer (pH 7; ionic strength μ 0·01). In the presence of caseinase C, 90–99 % of the haemagglutinating activity was destroyed (Table 1). About 55 % of the destruction observed after 4 hr at 37° took place during the first hour of incubation (Table 3).

Table 1. *Haemagglutinating activity of concentrated purified PR8 virus (0·01 μ phosphate buffer pH 7), after 4 hr of incubation at 4° without caseinase C and at 37° with and without caseinase C*

Exp. no.	Haemagglutinating titre after incubation			
	Without caseinase C at		With caseinase C at 37°	
	4°	37°	Absolute	%
1	51,200	51,200	4,800	9·4
2	83,200	89,600	800	0·9
3	89,600	70,400	1,600	2·3
4	153,600	153,600	3,200	2·1
5	76,800	76,800	400	0·5
6	—	153,600	1,900	1·2

Infectivity

Incubation for 4 hr at 37° at pH 7 and μ 0·01 did not decrease the infectivity of concentrated purified PR8 virus. After the same length of time at 37° in the presence of caseinase C, the titre of the viral preparation was decreased to between 10^{-3} and 10^{-4} of its former value (Table 2).

Neuraminidase activity

The neuraminidase activity of PR8 virus was estimated on the basis of the amount of *N*-acetylneuraminic acid liberated, after incubation for 4 hr at 37°, by 1 part of viral suspension acting upon 9 parts of the mucoprotein solution (water containing penicillin 100 units/ml. and streptomycin 100 μg./ml.). This system contained 550 μg./ml. *N*-acetylneuraminic acid liberable by acid hydrolysis (0·1 N-H_2SO_4; 1 hr

at 80°). Under the conditions of our experiments, the relationship between the concentration of the viral suspension and the amount of free *N*-acetylneuraminic acid was given by the equation:

$$\log y = a \log x,$$

where a is a constant, x the concentration of the virus in percentage of the undiluted control, and y, the amount of free *N*-acetylneuraminic acid as % of the *N*-acetylneuraminic acid liberated after incubation for 24 hr in the presence of undiluted control virus suspension (about half of the *N*-acetylneuraminic acid freed

Table 2. *Infectivity of concentrated purified PR8 virus after 4 hr of incubation (phosphate buffer, pH 7, μ 0·01) at 4° without caseinase C and at 37° with and without caseinase C*

	Infectivity after incubation					
	Without caseinase C at				With caseinase C at 37°	
	4°		37°			
Expt. no.	$\log_{10}$ ID 50 in 1 ml.	Standard error*	$\log_{10}$ ID 50 in 1 ml.	Standard error*	$\log_{10}$ ID 50 in 1 ml.	Standard error*
1†	7·74	0·12	7·74	0·14	4·77	0·06
2†	7·85	0·09	7·92	0·08	3·64	0·17
3	8·12	0·15	8·12	0·13	—	—
4	—	—	7·44	0·14	4·11	0·12

* Finney (1947).

† Sample of the virus suspensions used respectively for Expts. no. 1 and 2 of Table 1.

Table 3. *Haemagglutinating activity, infectivity and neuraminidase activity of concentrated purified PR8 after incubation at 37° in the presence of caseinase C for different periods of time*

	Activity in percentage of control										
Length of incubation (hr)	Haemagglutination expt.					Infectivity expt.		Neuraminidase expt.			
	1	2	3	4	5	1	3	1	3	4	5
1	41	47	37	66	66	5·0	0·1	101	147	120	116
2	33	23	25	33	25	—	—	32	68	81	80
4	1	2	3	1	1	—	—	3	34	10	9
6	—	—	—	—	—	—	—	—	—	3	3

by acid hydrolysis). By this technique it was found that the neuraminidase activity of various dilutions of concentrated purified virus at pH 7, μ 0·01, was the same after incubation for 4 hr at 4° as at 37° (Fig. 1). After 4 hr at 37° in the presence of caseinase C the virus suspension liberated from 0 to 34 % of the amount of *N*-acetylneuraminic acid liberated by the control, which means that 75–97 % of the original neuraminidase activity had disappeared. After incubation for 1 hr the neuraminidase activity remained unchanged (Table 3).

Ability to react with specific antibodies

In the presence of rabbit antiserum, concentrated purified PR8 virus suspension previously incubated for 4 hr at 37° (pH 7, μ 0·01) fixed complement after overnight incubation at 4°. After an identical pre-incubation, but in the presence of caseinase

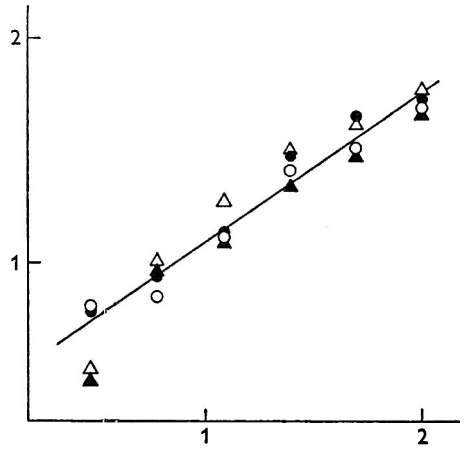

Fig. 1

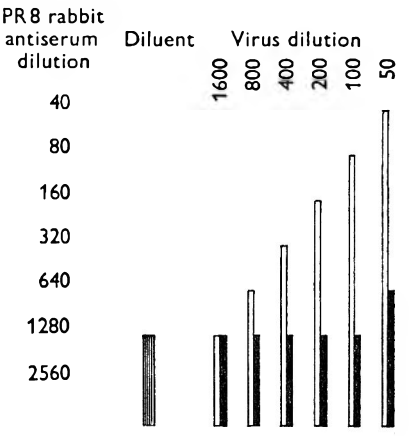

Fig. 2

Fig. 1. Neuraminidase activity of dilutions of concentrated purified PR 8 after 4 hr incubation at 37° or 4°, pH 7, μ 0·01. Ordinates: log y, where y is the amount of N-acetylneuraminic acid liberated after 4 hr incubation at 37° in % of the N-acetylneuraminic acid liberated by the undiluted virus suspension after 24 hr incubation at 37°. Abscissae: log x, where x is the concentration of the virus in % of the undiluted virus suspension. Expt. no. 1: 37°, ●; 4°, ○. Expt. no. 2: 37°, ▲; 4°, △.

Fig. 2 Neutralization by haemagglutination-inhibiting activity of anti-PR 8 rabbit serum by various dilutions of PR 8 virus suspension incubated at 37°, μ 0·01, pH 7 with or without caseinase C, further adsorbed by 20% fowl red cells or not. Shadowed band: haemagglutination in the presence of diluent alone. White bands: haemagglutination in the presence of various dilutions of concentrated purified PR 8 incubated at 37°, μ 0·01, pH 7. Pattern obtained by using control virus, virus treated by caseinase C or virus treated by caseinase C and further adsorbed by 20 % fowl red cells. Black bands: haemagglutination in the presence of various dilutions of concentrate purified PR 8 after 4 hr incubation at 37°, μ 0·01, pH 7 and adsorption on 20 % fowl red cell

Table 4. *Complement fixation, after overnight incubation at 4°, by concentrated purified PR8, untreated or treated by the caseinase C in the presence of anti-PR8 rabbit serum*

Virus	Virus dilution	Complement fixation in the presence of antiserum diluted 1/20	1/40	1/80	1/160
Control	1/2	+	+	+	0
	1/4	+	+	0	0
	1/8	+	+	0	0
	1/16	+	+	0	0
	1/32	+	0	0	0
	1/64	0	0	0	0
After enzymic treatment	1/2	+	+	0	0
	1/4	+	+	0	0
	1/8	+	+	0	0
	1/16	+	0	0	0
	1/32	0	0	0	0
	1/64	0	0	0	0

Average of the results obtained with samples of the virus suspensions used for Expts. nos. 1 and 2 of Tables 1 and 2.

C, the ability of the virus to fix complement in the presence of rabbit antiserum was slightly decreased, i.e. in the presence of serum diluted 1/20, the minimum active virus concentration was twice as high for treated virus as for the control, and conversely the serum titre measured in the presence of control virus diluted 1/2 was twice as high as that measured in the presence of the same dilution of treated virus (Table 4). This rabbit antiserum diluted 1/20 did not fix complement in the presence of standard soluble A antigen extracted from allantoic cells infected with PR8 virus.

After incubation for 4 hr at 37° in the presence of a caseinase C preparation, the ability of concentrated purified PR8 virus suspension to bind haemagglutination-inhibiting antibodies was not modified. By measuring non-absorbed antibodies, it was found that, when added to an equal volume of antiserum diluted 1/80, treated or untreated virus, diluted 1/200, caused the antiserum to lose 75% of its ability to inhibit haemagglutination. Virus preparations (treated or untreated) diluted 1/400 had no effect on the haemagglutination-inhibiting titre of the antiserum diluted 1/80. On the other hand, neutralization of haemagglutination-inhibiting antibodies was obtained by mixing various amounts of virus with various amounts of antiserum. The same limiting concentrations of treated and untreated virus suspensions neutralized each tested concentration (Fig. 2).

Ability of virus to adsorb on fowl red cells

Concentrated purified PR8 virus suspension previously incubated at 37° for 4 hr in the presence of caseinase C preparation was diluted 1/10 in saline and 20% (v/v) of packed fowl red cells added. After 15 min. at room temperature, this mixture was centrifuged for 15 min. at 1500 *g*. The supernatant fluid was tested for ability to absorb haemagglutination-inhibiting antibodies. The same limiting concentrations of both enzyme-treated virus suspension and adsorbed enzyme-treated virus suspension neutralized each tested concentration of antiserum (Fig. 2). Adsorption of control virus suspension by fowl red cells under the same conditions strongly decreased the ability of this suspension to neutralize haemagglutination-inhibiting antibodies (Fig. 2).

Antigenicity

Antiserum to control (untreated) virus and antiserum to enzyme-treated virus were examined for haemagglutination-inhibiting antibody and for antibody which fixed complement in the presence of standard soluble antigen and in the presence of enzyme-treated (*E* suspension: concentrated purified virus incubated at 37° for 4 hr in the presence of caseinase C) and control (*C* suspension: concentrated purified virus incubated for 4 hr at 37°); phosphate buffer pH 7, μ 0·01, virus suspensions of both influenza PR8 and Asian influenza (Table 5).

Antiserum to control virus and antiserum to enzyme-treated virus inhibited four haemagglutinating units of PR8 virus to 1/5120 and 1/320, respectively.

Antiserum to control virus fixed complement in the presence of enzyme-treated and control PR8 virus suspensions. The titre of antiserum to untreated virus in the presence of one unit of enzyme-treated PR8 virus antigen (*E* suspension diluted 1/2) was 1/80. Its titre in the presence of one unit of homologous antigen (*C* suspension diluted 1/8) was 1/320.

Table 5. *Complement fixation, after overnight incubation at 4° by various dilutions of concentration purified PR8 and Asian viruses, untreated or treated by caseinase C, in the presence of untreated PR8 virus guinea-pig antiserum or treated PR8 virus guinea-pig antiserum*

Virus	Virus diluted	Complement fixation in the presence of antiserum (guinea-pig)													
		Control PR 8 antiserum diluted							Enzyme treated PR 8 antiserum diluted						
		1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/10	1/20	1/40	1/80	1/160	1/320	1/640
Control PR 8	1/2	+	+	+	+	+	0	0	0	0	0	0	0	0	0
	1/4	+	+	+	+	+	+	0	0	0	0	0	0	0	0
	1/8	+	+	+	+	+	+	0	0	0	0	0	0	0	0
	1/16	+	+	+	+	+	0	0	0	0	0	0	0	0	0
	1/32	0	0	+	+	0	0	0	0	0	0	0	0	0	0
	1/64	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Control Asian	1/2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1/4	+	0	0	0	0	0	0	0	0	0	0	0	0	0
	1/8	+	+	0	0	0	0	0	0	0	0	0	0	0	0
	1/16	+	+	0	0	0	0	0	0	0	0	0	0	0	0
	1/32	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1/64	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Enzyme-treated PR 8	1/2	+	+	+	+	0	0	0	+	+	+	+	0	0	0
	1/4	+	+	+	0	0	0	0	+	+	+	+	+	0	0
	1/8	+	+	0	0	0	0	0	+	+	+	+	+	0	0
	1/16	+	+	0	0	0	0	0	+	+	+	+	0	0	0
	1/32	+	+	0	0	0	0	0	0	0	0	0	0	0	0
	1/64	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Enzyme-treated Asian	1/2	+	+	0	0	0	0	0	+	+	+	+	+	0	0
	1/4	+	+	0	0	0	0	0	+	+	+	+	+	0	0
	1/8	+	+	0	0	0	0	0	+	+	+	+	+	+	0
	1/16	0	+	0	0	0	0	0	+	+	+	+	+	0	0
	1/32	0	0	0	0	0	0	0	0	+	+	0	0	0	0
	1/64	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Antiserum to enzyme-treated virus fixed complement in the presence of untreated virus; its titre in the presence of one unit of enzyme-treated virus antigen (*E* suspension diluted 1/8) was 1/160.

Antiserum to untreated PR 8 influenza virus did not inhibit four haemagglutinating units of Asian influenza, neither did antiserum to enzyme-treated virus. However, it fixed complement in the presence of enzyme-treated and untreated Asian influenza virus suspensions, but its titre in the presence of either of these antigens did not exceed 1/20. Antiserum to enzyme-treated virus fixed complement in the presence of enzyme-treated Asian influenza virus suspension but not in the presence of untreated virus. The enzyme-treated virus antiserum titre in the presence of one unit of that antigen (*E* suspension diluted 1/8) was 1/320.

Neither control antiserum nor enzyme-treated virus antiserum fixed complement in the presence of standard soluble A antigen, or of caseinase C control solution.

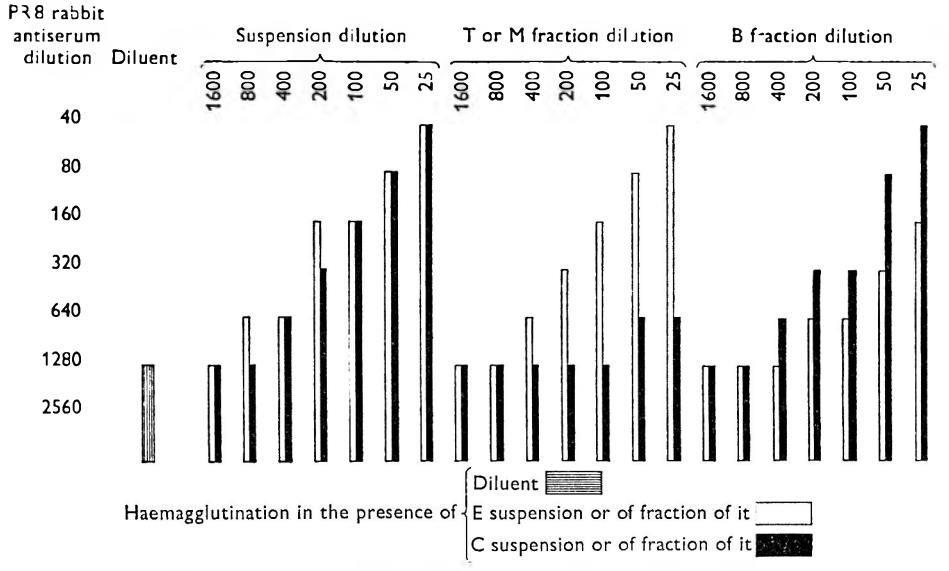

Fig. 3. Neutralization of haemagglutination inhibiting activity of anti-PR 8 rabbit serum by various dilutions of PR 8 virus suspension incubated for 4 hr at 37°, μ 0·01, pH 7, with or without caseinase C and by fractions of these suspensions obtained after 1 hr centrifugation at 31,000 *g* in swinging bucket. *E* suspension: enzyme-treated suspension; *C* suspension: control suspension; *T* suspension: 2 ml. top supernatant; *M* suspension: 2 ml. medium supernatant; *B* suspension: pellet resuspended in 1·25 ml. bottom fluid. *B* fractions dilutions expressed as dilution of material sedimenting by 1 hr centrifugation at 31,000 *g*. *T* and *M* fractions dilutions expressed as dilution of material non-sedimented after 1 hr centrifugation at 31,000 *g*.

Evidence for virus breakdown

PR8 *E* suspension, 0·7 ml., was diluted 1/10 in saline. Five ml. of this dilution were centrifuged for 1 hr at 31,000 *g* in swinging bucket rotor SW39 of a Spinco model L centrifuge. After centrifugation, the 5 ml. volume was carefully divided into three fractions with the aid of a syringe and needle. Four ml. of the supernatant fluid were removed as two fractions, 2 ml. of top supernatant (T) and 2 ml. of medium

Table 6. *Complement fixation after overnight incubation at 4° by various dilutions of concentrated purified* PR *8 suspensions treated or untreated by caseinase C and by fractions of these suspensions obtained after 1 hr centrifugation at 31,000 g in swinging bucket*

		Complement fixation in the presence of guinea-pig serum													
		Control PR 8 antiserum diluted							Euzyme-treated PR 8 antiserum diluted						
PR 8 antigen	Antigen concentration	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/10	1/20	1/40	1/80	1/160	1/320	1/640
C suspension	1/10	+	+	+	+	+	+	+	0	0	0	0	0	0	0
	1/20	+	+	+	+	+	+	0	0	0	0	0	0	0	0
	1/40	+	+	+	+	+	+	0	0	0	0	0	0	0	0
	1/80	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T from *C*	1/10	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1/20	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1/40	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1/80	0	0	0	0	0	0	0	0	0	0	0	0	0	0
M from *C*	1/10	+	+	+	+	+	0	0	0	0	0	0	0	0	0
	1/20	0	+	+	+	+	0	0	0	0	0	0	0	0	0
	1/40	0	0	+	+	0	0	0	0	0	0	0	0	0	0
	1/80	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B from *C*	1/2·5	+	+	+	+	+	+	+	0	0	0	0	0	0	0
	1/5·0	+	+	+	+	+	+	+	0	0	0	0	0	0	0
	1/10·0	+	+	+	+	+	0	0	0	0	0	0	0	0	0
	1/20·0	+	+	+	+	+	0	0	0	0	0	0	0	0	0
	1/40·0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1/80·0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E suspension	1/10	+	+	+	+	0	0	0	+	+	+	+	0	0	0
	1/20	+	+	+	0	0	0	0	+	+	+	+	0	0	0
	1/40	+	+	+	0	0	0	0	0	0	0	0	0	0	0
	1/80	0	+	+	0	0	0	0	0	0	0	0	0	0	0
T from *E*	1/10	+	+	0	0	0	0	0	+	0	0	0	0	0	0
	1/20	+	+	0	0	0	0	0	0	0	0	0	0	0	0
	1/40	+	+	0	0	0	0	0	0	0	0	0	0	0	0
	1/80	0	0	0	0	0	0	0	0	0	0	0	0	0	0
M from *E*	1/10	+	+	0	0	0	0	0	+	+	0	0	0	0	0
	1/20	+	+	0	0	0	0	0	0	0	0	0	0	0	0
	1/40	+	+	0	0	0	0	0	0	0	0	0	0	0	0
	1/80	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B from *E*	1/2·5	+	+	+	+	0	0	0	+	+	+	+	+	0	0
	1/5·0	+	+	+	+	0	0	0	+	+	+	+	0	0	0
	1/10·0	+	+	+	+	0	0	0	+	+	+	+	0	0	0
	1/20·0	0	0	0	0	0	0	0	+	+	+	0	0	0	0
	1/40·0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1/80·0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

E suspension: PR 8 suspension incubated for 4 hr at 37°, pH 7, μ 0·01 in the presence of caseinase C. *C* suspension: PR 8 control suspension. T: 2 ml. top

supernatant (M). To the remaining bottom 1 ml., 0·25 ml. saline was added and the pellet resuspended in this 1·25 ml. volume.

In this fraction the concentration of material sedimentable by 1 hr of centrifugation at 31,000 *g* was equal to 1/2·5 the concentration of this material in the *E* suspension, whereas the concentration of material not sedimentable by 1 hr at 31,000 *g* was 1/12·5 the concentration of this material in *E* suspension. Three fractions of PR8 *C* suspension were obtained following the same procedure. The three fractions of both *E* and *C* suspensions were tested for ability to neutralize haemagglutination-inhibiting activity of anti-PR8 rabbit serum and for ability to fix complement in the presence of antisera to enzyme-treated and untreated virus. The activities of these fractions were compared with the activities of *E* and *C* suspensions, the concentration of each fraction being expressed as dilution of material present in corresponding uncentrifuged suspension.

T, M and B fractions of PR8 *E* suspension (when the concentration of non-sedimentable material present in B fraction is allowed for) neutralized haemagglutination-inhibiting activity of anti-PR8 rabbit serum as well as did *E* suspension itself. B fraction only of *C* suspension neutralized haemagglutination-inhibiting activity of this antiserum to the same extent as did *C* itself. Nothing but traces of neutralizing material were found in T and M fractions of *C* suspension (Fig. 3).

Complement-fixing activity in the presence of antiserum to untreated virus was found in all three fractions of *E* suspension. In the presence of one unit of T fraction of *E* antigen (T from *E* suspension diluted 1/40) or one unit of M fraction of *E* antigen (M from *E* suspension diluted 1/40) the titre of control antiserum was 1/20, whereas in the presence of one unit of B fraction of *E* antigen (B from *E* suspension diluted 1/20) its titre was 1/80. Complement-fixing activity in the presence of antiserum to enzyme-treated virus was found in B fraction only of *E* suspension. The titre of this serum in the presence of one unit of B fraction of *E* antigen (B from *E* suspension diluted 1/10) was 1/80.

The complement-fixing activity of the control (untreated) suspension was bound to material sedimenting at 31,000 *g*: no activity was bound in T fraction of *C* suspension, traces in M fraction and maximum activity in B fraction (Table 6).

DISCUSSION

Kuroya *et al.* (1957) and Skryabin (1957) reported that some actinomycetes produce in their culture filtrates substances displaying antibacterial activity and activity against influenza virus. Nevertheless, to our knowledge, none of these antiviral substances has been purified and used for studies similar to those described here. In our conditions the action of the antiviral preparation on infectivity and haemagglutinating activity cannot be attributed to a modification of sensitive cells. Therefore, the discussion will deal with a direct action on the virus particle *in vitro*.

The enzyme preparation used in these studies is proteolytic, as shown by digestion of casein, and may be a trypsin-like enzyme. Trypsin is known to act on influenza virus; indeed it has been reported (Mayrow, Robert, Winzler & Rafelson, 1961) that the neuraminidase and haemagglutinating activities of influenza A virus can be separated after prolonged treatment at pH 7 by a high concentration of crystalline trypsin. However, the enzymic activity of the Lee strain of influenza B virus, can

be decreased by crude or purified trypsin at pH 7·2 or pH 8·5. The same effect was observed with the Melbourne strain of influenza A virus, after trypsin treatment at pH 8·5 (Stone, 1949). The haemagglutinating activity of influenza virus is known to be fairly resistant to trypsin treatment, its diminution requiring either prolonged incubation at 37° and pH 7 (Gresser & Enders, 1961) or pH 8–9 (Cleeland & Sugg, 1963). This behaviour was confirmed by incubating the concentrated purified PR8 virus at 37° for 4 hr in a solution of crystalline trypsin (pH 7, μ 0·01, 18·5 μg./ml. final volume) able to digest casein at the same rate as caseinase C at the usual concentration. After such a treatment. the haemagglutinating property of the virus was slightly increased. It seems therefore very unlikely that a trypsin-like proteolytic activity of the antiviral preparation is involved in the destruction of the haemagglutinating activity of the virus. The existence of a gradient in the sensitivity of the various viral properties towards the enzyme preparation under our conditions suggests either distinct enzymes responsible for their specific inactivation, or the progressive modification of the virus particle by a single enzyme.

The inability of treated virus to agglutinate red cells is associated with its inability to adsorb on red cells, which may account for the loss of infectivity of the virus particle. Production of haemagglutination inhibiting antibodies without antibodies fixing complement in the presence of virus particles, and appearance of a new antigenic activity together with evidence of breakdown of the virus raise several questions which are the subject of further investigation. Is the structure responsible for the production of antibodies which fix complement in the presence of viral particles destroyed? Is that structure, the one which is responsible for neutralizing haemagglutination-inhibiting antibodies and which is not sedimented at 31,000 *g*, the one which is also able to give haemagglutination-inhibiting antibodies and to react with strain specific complement-fixing antibodies that it cannot produce? Is the new antigenic activity detected in treated virus suspension, in that material which is sedimented at 31,000 *g*, the consequence of the unmasking of a pre-existing antigen or of a chemical change induced by the enzyme treatment? This antigen cannot be the soluble antigen since antiserum to enzyme-treated virus does not react with standard soluble influenza A virus antigen. Nevertheless, data support the hypothesis of the unmasking of an antigen present in both PR8 and Asian influenza virus particles. V antigens are known to possess some components cross-reacting within types in addition to strain specific ones (Henle & Wiener, 1944; Fulton & Dumbell, 1949). Sera obtained from animals or man after consecutive influenza infections show recall between distantly related strains (Henle, Lief & Fabiyi, 1958; Hobson & Pearson, 1961). Non-specific recalls are troublesome when preparing pure anti-S serum (Lief, 1963). Our control PR8 guinea-pig antiserum diluted 1/20 fixed complement in the presence of purified Asian influenza virus, but its titre in the presence of purified PR8 was much higher.

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Evidence that Initial Ultraviolet Lethal Damage in *Escherichia coli* Strain 15 $T^-A^-U^-$ is Independent of Growth Phase

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SUMMARY

A study was made of the kinetics of killing by ultraviolet (u.v.) radiation (2537 Å) and subsequent photo-reactivation (with black light) of *Escherichia coli* strain 15 $T^-A^-U^-$ (requires thymine, arginine, uracil) by using (*a*) logarithmic-phase cultures, (*b*) early-stationary-phase cultures, (*c*) logarithmic-phase cultures after 90 min. of incubation in the absence of arginine and uracil (−AU cultures). The stationary and −AU cultures showed the same enhanced resistance to u.v. killing. In all three cultures (1) the photo-reactivable sectors were the same, (2) the rate of photo-reactivation was the same function of u.v. dose, (3) the amount of light required for maximum photo-reactivation was a linear function of the u.v. dose. We conclude that the initial lethal lesions produced by a given u.v. dose were qualitatively and quantitatively the same in all three cultures, despite differences in u.v. survival. This implies that the stationary-phase cultures and the −AU cultures were more radiation resistant solely because they were better able to cope with the initial lethal damage. The survival curves can be satisfactorily described mathematically in terms of this model.

INTRODUCTION

Survival of irradiated logarithmic-phase bacteria can be altered by various metabolic inhibitors. For example, higher survivals may be obtained by (1) treatment with chloramphenicol before X-irradiation (Billen, 1959), (2) growth without a required pyrimidine, or amino acid, or both, before irradiation with ultraviolet (u.v.; Hanawalt, 1961) or X-rays (Billen, 1963), (3) plating on a medium containing chloramphenicol after irradiation with u.v. or X-rays (Alper & Gillies, 1960). In addition stationary-phase populations are often more resistant than logarithmic-phase populations to u.v. radiation (Durham & Wyss, 1956; Gates, 1929; Woodside, Goucher & Kocholaty, 1960) and to ionizing radiation (Stapleton, 1955). However, neither the changes in metabolic state nor the mechanisms by which such changes alter radiation-killing are understood.

We know that nucleic acid components are the principal absorbers of the u.v.

photons that kill *Escherichia coli* (Gates, 1930) and a variety of evidence strongly suggests that the initial lethal damage occurs predominantly and directly in deoxyribonucleic acid (DNA; see, for example, Wacker, 1963). Furthermore, selective inhibition, by u.v. irradiation, of DNA synthesis in *E. coli*, which has long been known (Kelner, 1953), is probably directly involved in bacterial killing (Setlow, Swenson & Carrier, 1963). Therefore it is reasonable to think that radiosensitivity changes produced by various metabolic treatments reflect changes in DNA or RNA synthesis. Polyauxotrophs of the thymine-deficient *E. coli* strain 15T$^-$ appear to be good specimens for studying the mechanisms of radiation killing, because (1) nucleic acid syntheses in these mutants can be studied in relation to other specific metabolic processes (see, for example, Maaløe & Hanawalt, 1961; Lark, Repko & Hoffman, 1963), (2) dramatic changes in radiation sensitivity of logarithmic-phase cultures of one poly-mutant, strain 15T$^-$A$^-$U$^-$ (requires thymine, arginine, uracil), are observed when protein and ribonucleic acid (RNA) syntheses are inhibited for various times before either u.v. irradiation (Hanawalt, 1961) or X-irradiation (Billen, 1963).

In the present paper we describe kinetics of (1) killing by far-ultraviolet (u.v.; 2537 Å) radiation of *Escherichia coli* strain 15T$^-$A$^-$U$^-$ in different metabolic states, and (2) the recovery from u.v. lethal damage after photo-reactivation by near-ultraviolet radiation (near u.v.). Analysis of the data indicates that pre-irradiation inhibition of protein and RNA syntheses in strain 15T$^-$A$^-$U$^-$ did not affect the production of initial lethal lesions by u.v. radiation. Consequently, another explanation must be found for the dramatic change in radiation sensitivity produced by this pre-irradiation treatment.

METHODS

Organisms and methods of culture. Escherichia coli strain 15T$^-$A$^-$U$^-$ (isolated by Barner & Cohen, 1958) was obtained from Dr R. B. Setlow. Some of its physiological and genetic properties were described by Kanazir *et al.* (1959). Stocks were maintained on refrigerated nutrient agar (Difco) slopes. Test cultures were grown in a liquid minimal medium [Davis mineral salts buffer, Lederberg, 1950, containing glucose 1·0% (w/v) as energy source; final pH 7·2] supplemented as required with thymine, 5–10 μg./ml.; arginine, 20–100 μg/ml.; or uracil, 10–20 μg./ml. Supplements are indicated as follows: fully supplemented, +T+AU; thymine only, +T−AU; arginine+uracil only, −T+AU. The amount of growth was estimated turbidimetrically at 490 mμ (E_{490}) by using a Photovolt 'Lumetron' 401 colorimeter. Early-stationary-phase organisms (90–120 min. after the end of exponential increase in extinction) were harvested from an overnight culture (16–18 hr at 37° in a shaker bath). For other growth phases, the overnight culture was diluted and incubated as required in the appropriate pre-warmed growth medium. To change nutrients, organisms were collected and washed on a membrane filter (Millipore, grade HA) and resuspended in the new pre-warmed medium.

The supplement concentrations used were adequate for growth; maximum growth in minimal medium (minimum lag phase; minimum generation time; maximum colony count) was obtained with the following minimum supplement concentrations: thymine, 5 μg./ml.; arginine, 20 μg./ml.; uracil, 10 μg./ml. (Figs. 1, 2). However, experiments with ^{14}C-labelled thymine showed that, at maximum viable titre, the culture grown in thymine 5 μg./ml. had used virtually all the thymine

in the medium; the thymine was not exhausted when a concentration of 10 μg./ml. was used.

It was also noted that thymine at 2 μg./ml. (used by a number of workers for growth of the $15T^-$ mutants) permitted maximum exponential growth rate, but produced a slightly longer lag phase (Fig. 1) and did not give maximum viable titre, because thymineless death occurred (Fig. 2) when thymine was exhausted from the medium before the end of the logarithmic growth phase.

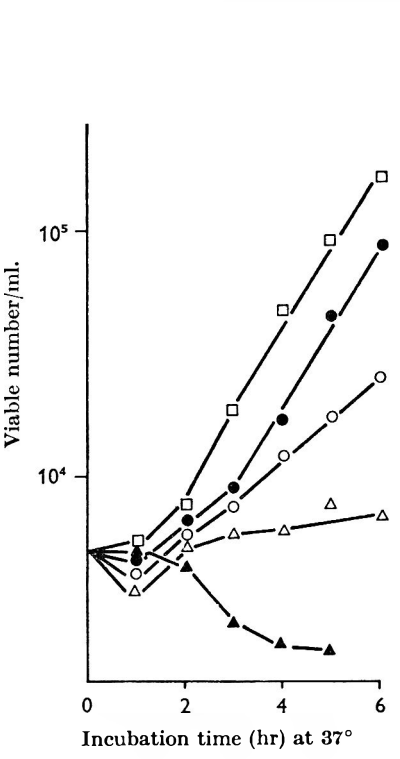

Fig. 1

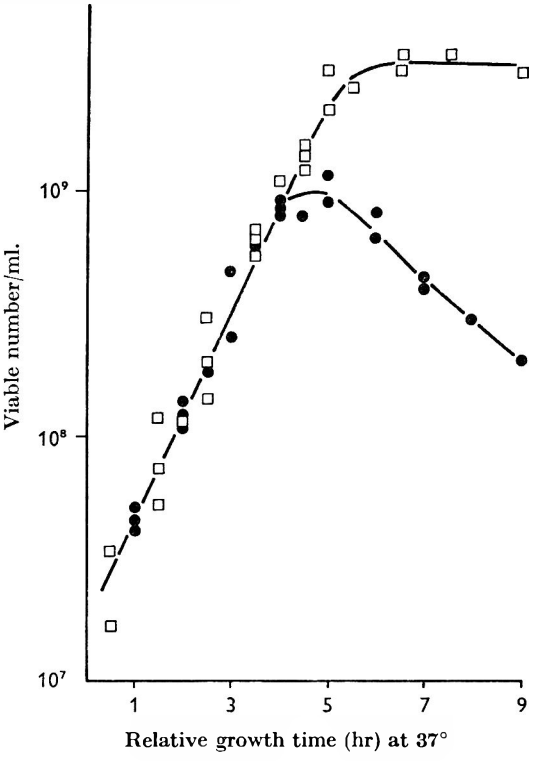

Fig. 2

Fig. 1. Effect of thymine concentration on aerated growth of early-stationary-phase *Escherichia coli* strain $15T^-A^-U^-$ diluted into minimal medium (arginine: 20 μg./ml.; uracil: 10 μg./ml.; thymine: □ = 10 μg./ml., ● = 2 μg./ml., ○ = 1 μg./ml., △ = 0·5 μg./ml., ▲ = 0·1 μg./ml.).

Fig. 2. Effect of thymine concentration on maximum viable titre attained by aerated growth of early-stationary-phase *Escherichia coli* strain $15T^-A^-U^-$ diluted into minimal medium (arginine: 20 or 100 μg./ml.; uracil: 10 or 50 μg./ml.; thymine: □ = 5 or 10 μg./ml., ● = 2 μg./ml.).

The viability of bacterial cultures was assayed by spreading measured volumes (usually 0·05 or 0·1 ml.) of diluted suspensions on nutrient agar plates, and counting the colonies after incubation for 16–24 hr in a dark humidified 37° cabinet.

Ultraviolet irradiation. For u.v. irradiation, bacteria were collected on a membrane filter, washed with several volumes of Davis mineral salts buffer, and resuspended and diluted in this buffer. One-ml. portions of the suspension (less than 10^6 bacteria/ml.) were irradiated at room temperature (22–23°) in the depressions

of a glazed ceramic spot-plate. To prevent unintentional effects by short-wavelength visible light, all u.v. irradiation experiments were done in a light-sealed laboratory illuminated by yellow fluorescent lamps (Westinghouse, F-40G0, 40 W., gold).

The u.v. source (2537 Å) was two General Electric 15 W. low-pressure mercury-vapour germicidal lamps (G15T8) in a desk-type white-enamelled reflector. Only the central 15 cm. length spans of the lamps were used. A small low-velocity exhaust fan was used to dissipate possible traces of ozone and to maintain constant temperature of the lamps.

The near-u.v. source used in photo-reactivation experiments was the central 14 cm. length spans of two General Electric 15 W. low-pressure mercury black-light lamps (F15T8-BLB; smooth spectrum 3100–4400 Å, peak at 3530 Å) in a desk-type white-enamelled reflector. Unirradiated bacterial controls exposed to the photo-reactivation source under the same conditions as for irradiated samples showed no loss of viability.

Incident dose rates were measured with a home-made meter (described by Jagger, 1961), and found to be 9 ergs mm^{-2}. sec^{-1}. at 60 cm. from the u.v. source and 94 ergs $mm.^{-2}$ $sec.^{-1}$ at 10 cm. from the photo-reactivation source.

RESULTS

Survival after ultraviolet irradiation

Fig. 3 shows u.v. survival data from thirteen experiments (three doses per experiment) for: (1) logarithmic-phase cultures; (2) early-stationary-phase cultures; (3) logarithmic-phase cultures grown for 90 min. in +T −AU medium (hereafter referred to as '−AU cultures'). The curves shown in Fig. 3 are plots of an equation whose form and derivation are presented in the Appendix to this paper. For the present, it is only necessary to note that the equation involves the following three constants and no other constants:

α is the negative of the slope at high u.v. doses. Regression lines drawn to the experimental data (which include about twice as many points as are shown in Fig. 3) give the value $\alpha = 0{\cdot}0199 \pm 0{\cdot}0076$ for the logarithmic-phase data, and $0{\cdot}0196 \pm 0{\cdot}0087$ for the early-stationary-phase and −AU data combined. The final slopes are therefore identical within experimental error.

$M+1$ is the ordinate intercept of the final slope. It has the value 5 for the logarithmic data and the value 273 for the early-stationary and −AU data combined.

r describes the rate at which the curves at low doses approach the final slope. It has the value $r = 0{\cdot}0183 \pm 0{\cdot}0013$ for the logarithmic data and $0{\cdot}0173 \pm 0{\cdot}0003$ for the early-stationary and −AU data combined. Therefore, these portions of the curves are similar (in the geometric sense) for all three cultures.

The fact that two of the three constants needed to draw these curves were invariant for logarithmic, early-stationary, and −AU cultures suggests that the three cultures were identical in some aspects of their photo-sensitivity. The derivation of the survival curve is based upon α being a function only of initial lethal damage, while $M+1$ and r are related to repair events. The constancy of α therefore suggests that the initial lethal lesions were the same in all three cultures, while the higher extrapolation number ($M+1$) of the early-stationary and −AU curves is consistent with greater repair occurring in these cultures.

Photo-reactivable sector

The results of a typical series of photo-reactivation experiments are shown in Fig. 4. Maximum photo-reactivation after each u.v. dose was taken as the survival corresponding to the highest point on the respective photo-reactivation curves (arrows in Fig. 4). Similar determinations were made for each of the three cultures.

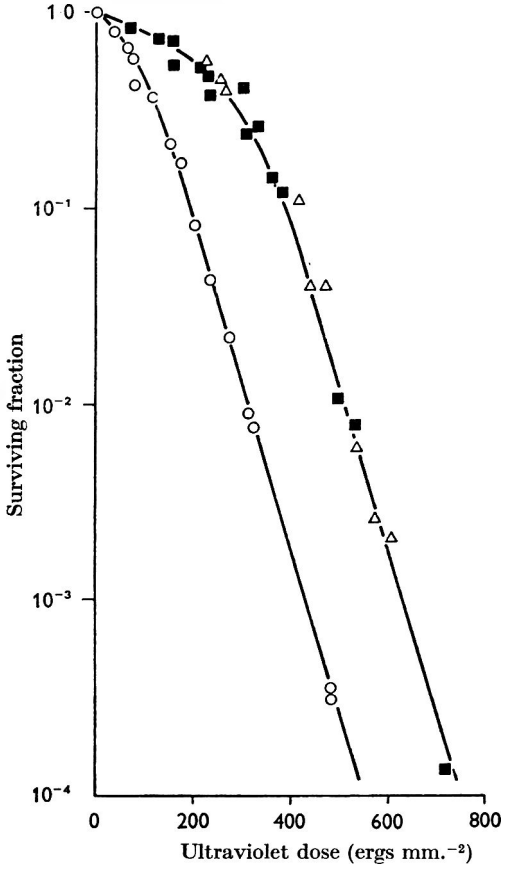

Fig. 3

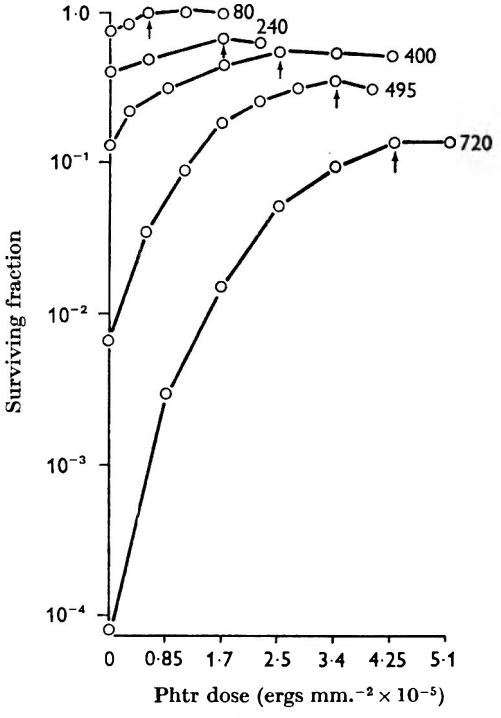

Fig. 4

Fig. 3. Ultraviolet survival of *Escherichia coli* strain $15T^{-}A^{-}U^{-}$ in different growth states (dark survival). Key: ○ = logarithmic phase (+T +AU); △ = early-stationary-phase (+T +AU); ■ = −AU (90 min. +T −AU).

Fig. 4. Photo-reactivation of a −AU culture of *E. coli* strain $15T^{-}A^{-}U^{-}$ after different doses of ultraviolet. Arrows indicate survival point corresponding to maximum photo-reactivation. Numbers on curves show ultraviolet dose in ergs mm.$^{-2}$.

Survival after maximum photo-reactivation (light survival) was plotted against the u.v. dose for each culture. The results of thirteen experiments with a logarithmic-phase culture are shown in Fig. 5. For −AU and early-stationary-phase cultures, the u.v. and light survivals were the same and therefore data were combined. Results of eighteen experiments are shown in Fig. 6.

The fraction of the u.v. dose which is apparently eliminated by photo-reactivation is called the photo-reactivable sector, a concept introduced by Dulbecco (1950). The numerical value of the photo-reactivable sector is

$$1 - \frac{\text{u.v. dose at a given survival}}{\text{u.v. dose giving same survival after maximum photo-reactivation}}.$$

The applications and significance of this concept were reviewed by Jagger (1958).

The photo-reactivable sector for each culture was calculated from the u.v. survival curves (see Fig. 3), and tested for regression on dose. There was no regression. Consequently, the respective photo-reactivable sectors are constant at

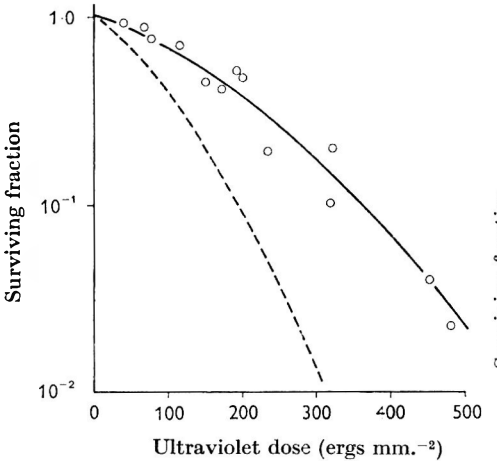

Fig. 5

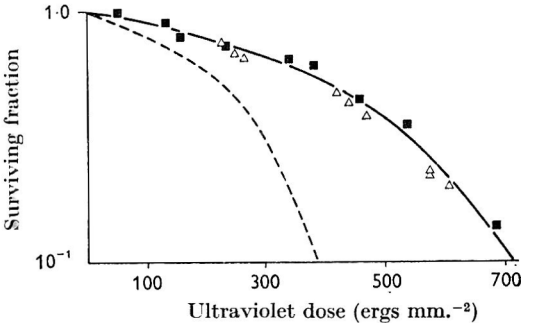

Fig. 6

Fig. 5. Ultraviolet survival of logarithmic-phase bacteria of *E. coli* strain 15T$^-$A$^-$U$^-$ after maximum photo-reactivation (light survival). Broken line is dark survival.

Fig. 6. Ultraviolet survival of early-stationary-phase and −AU bacteria of *E. coli* strain 15T$^-$A$^-$U$^-$ after maximum photo-reactivation (light survival). Broken line is dark survival. Key: △ = early-stationary-phase (+T −AU); ■ = −AU (90 min. +T −AU).

Table 1. *Photo-reactivable sectors for Escherichia coli strain 15 T$^-$A$^-$U$^-$ in different growth phases*

	Growth phase			Subtotal (stationary and −AU)	Total
	Logarithmic	Stationary	−AU		
Photo-reactivable sector	0·47	0·42	0·48	0·46	0·46
Standard deviation	0·10	0·03	0·04	0·05	0·07
Degrees of freedom	18	8	17	26	45

all doses. Table 1 shows the photo-reactivable sectors determined in forty-six experiments. Sectors were estimated from the data pertaining to each culture, as well as from lumping the stationary and −AU data (subtotal) and from lumping all the data (total). The photo-reactivation data were analysed for variance within and among the photo-reactivable sectors of the various cultures using the F-statistic method (Snedecor, 1956). The means were not different at the 1% level

of significance, showing that the photo-reactivable sectors were the same for all three cultures. This means that the spectrum of initial damages, as measured by photo-reactivability, was the same in the sensitive as in the resistant cultures. Thus, the initial lethal lesions were, in at least this respect, qualitatively the same in logarithmic-phase, −AU, and early-stationary-phase cultures.

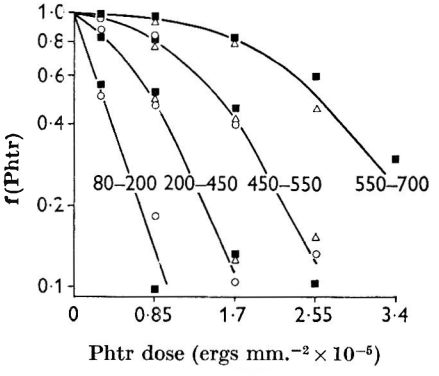

Fig. 7

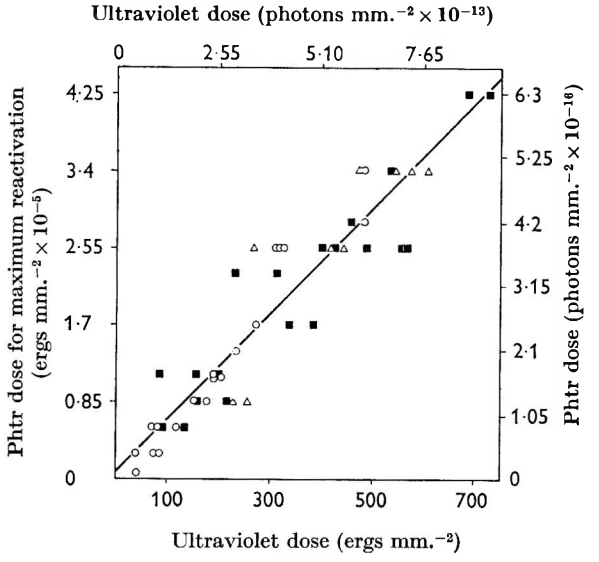

Fig. 8

Fig. 7. Photo-reactivability of ultraviolet-irradiated bacteria of *E. coli* strain 15 T⁻A⁻U⁻ in different growth states. The negative of the slope of the curve at any point describes the rate at which photo-reactivable cells are being photo-reactivated. Each point represents the average value of f(Phtr) for a given culture in a given u.v. dose range (numbers on curves, in ergs mm.$^{-2}$). Key: ○ = logarithmic phase (+T +AU); △ = early-stationary-phase (+T +AU); ■ = −AU (90 min. +T −AU).

Fig. 8. Dose of photo-reactivating light giving maximum photo-reactivation plotted against ultraviolet dose. Calculated average regression line is shown. Key: ○ = logarithmic phase (+T +AU); △ = early-stationary-phase (+T +AU); ■ = −AU (90 min. +T −AU).

Photo-reactivation rates

The photo-reactivation rates were determined by the method of Dulbecco (1950). At each survival, the value

$$\text{f(Phtr)} = 1 - \frac{P_x - S}{P_\infty - S}$$

(where P_x = viable bacteria after dose (X) of photo-reactivating light; P_∞ = viable bacteria after maximum photo-reactivation; S = viable bacteria after zero dose of photo-reactivating light), is plotted logarithmically against photo-reactivation dose. The negative of the slope of the resulting curve expresses the rate at which photo-reactivable bacteria are being photo-reactivated. Different shapes of the photo-reactivation rate curve are obtained for different u.v. dose ranges (see Fig. 7). This behaviour is not understood. To our knowledge, experiments of this type have not been attempted previously, which reflects the surprising paucity of kinetic data

on cellular photo-reactivation. For all three cultures, the photo-reactivation rate curves (Fig. 7) were identical for the same u.v. dose. Since photo-reactivation rate is a function of (1) the *number* of photo-reactivable lesions/cell, and (2) the *efficiency* of the photo-reactivation system, these data indicate that the amount of photo-reactivable damage might be the same in the sensitive as in the resistant cultures (see Conclusions). The regression of maximum photo-reactivation dose was linear for each culture. The average regression line is shown in Fig. 8, which also shows that, except at very low u.v. doses, about 700 incident photons of photo-reactivating light were required to reverse the damage done by each incident photon of u.v. radiation.

The positive sign of the Y-intercept of the regression line in Fig. 8 (which suggests that photo-reactivation at very low doses requires more light than might be expected) might not be significant. The 95% confidence interval of the Y-intercept, $0{\cdot}088 \pm 0{\cdot}224$ erg mm.$^{-2} \times 10^{-5}$, shows the true intercept could be zero or a small negative number. The probability that the sign of the Y-intercept is positive is borne out by recent experiments of ours (unpublished) which showed that the amount of light required for photo-reactivation in strain B_{s-1} (D_{37} of 1 erg mm.$^{-2}$) was not 1% but rather 20% of that required for photo-reactivation at the same survival value of strain B (D_{37} of about 100 ergs mm.$^{-2}$).

Conclusions

The shapes of the u.v. survival curves for logarithmic-phase, −AU, and early-stationary-phase cultures of *Escherichia coli* strain 15T$^-$A$^-$U$^-$ (Fig. 3) are consistent with the idea that the initial u.v. lesions are alike in all three cultures. The constancy of the photo-reactivable sectors (Figs. 5, 6) show that the initial lethal lesions were, as reflected by photo-reactivability, qualitatively the same at all u.v. doses in all three cultures. That the course of photo-reactivation was the same at any u.v. dose (curve shapes in Fig. 7) adds to the evidence that the initial lesions were qualitatively the same in all three cultures. The photo-reactivation rate was the same function of u.v. dose in all three cultures (Fig. 7), and the number of photons required for maximum photo-reactivation was the same linear function of u.v. dose in all three cultures (Fig. 8).

These findings (that photo-reactivation kinetics in all cultures were a function only of u.v. dose, and were independent of the survival obtained in the various cultures) can be accounted for only in two ways: (1) the photo-reactivation system was equally efficient in all cultures and the amount of photo-reactivable damage was the same in all three cultures; or (2) the photo-reactivation system had different efficiencies in the three cultures, but this was exactly matched by inverse differences in the amount of u.v. damage. With respect to the second possibility, one would hardly expect the numbers of lesions produced and the photo-reactivation efficiencies to differ greatly, if, as suggested above, the lesions were qualitatively the same in all three cultures. Furthermore, such an exact balance between number of lesions produced and photo-reactivation efficiency seems extremely unlikely. We therefore reject the second possibility on the grounds of *a priori* improbability, and conclude that the efficiency of photo-reactivation was the same in all three cultures. This would mean that the data of Figs. 7 and 8 reflect the production of a constant number of photo-reactivable lethal lesions for a given u.v. dose in all three cultures.

Since the photo-reactivable sectors were the same in all three cultures, it follows that, if the number of photo-reactivable lesions were constant for a given u.v. dose, then the number of non-photo-reactivable lesions was also constant. In other words, in all three cultures the initial u.v. lesions appeared to be quantitatively identical, and the photo-reactivable lesions quantitatively and qualitatively identical. It seems likely, therefore, that the initial u.v. lethal damage was the same in all three cultures. The data also showed that $-$AU and early-stationary-phase cultures were photo-biologically indistinguishable. This is discussed in the following paper (Ginsberg & Jagger, 1965), where it is concluded that the $-$AU culture is, in fact, an early-stationary-phase culture.

DISCUSSION

The greater radiation resistance of the stationary-phase cultures might be related to the presence of 'spare' genetic material, since inactivation of cells having 'extra copies' of a gene might require extra hits. Stapleton (1955), for example, suggested that the higher resistance of *Escherichia coli* B/r to X- and gamma-rays at different phases of the growth cycle might be correlated with the number of nuclear bodies/cell. Billen (1959) observed that the X-ray resistance of logarithmic-phase *E. coli* $15T^-$ was increased when the organisms were incubated with chloramphenicol (CAP), before irradiation under which conditions RNA and DNA syntheses continued while protein synthesis was inhibited. He found that the X-ray resistance did not change when thymine was withheld during the pre-treatment with CAP. Under these conditions, RNA synthesis continued but DNA and protein syntheses were inhibited. Billen concluded that the surplus DNA was probably responsible for the enhanced resistance during CAP treatment, and he cited data of other workers that are in agreement with this interpretation.

On the other hand, Gillies & Alper (1960) reported that in strains B or B/r of *Escherichia coli*, the DNA content at stationary-phase (where both strains are usually more resistant) was lower than in logarithmic phase.

Our own control data for *Escherichia coli* strain $15T^-A^-U^-$ showed that the amount of DNA per total number of bacteria was not significantly different in logarithmic-phase and stationary-phase cultures, and that if any difference did exist, there was slightly less DNA/organism in the stationary-phase cultures (Ginsberg, 1963; and unpublished data). Finally, differences in multi-cellularity and multi-nuclearity observed among cultures of *E. coli* strain $15T^-A^-U^-$ in different growth phases did not correlate with radiation sensitivity (Ginsberg, 1963). Consequently, we think it unlikely that the difference in radiation survival between cultures of the same strain irradiated in different growth states can be accounted for on the basis of differences in amounts of genetic material. A great deal of evidence (Boyce & Howard-Flanders, 1964; Harm, 1963; Setlow & Carrier, 1964) showed that metabolic repair of u.v. lethal damage can occur in bacteria. This suggests that differences in radiation survival of cultures in different growth phases or metabolic states might reflect *primarily* differences in the ability of the cells to cope with the initial damage, rather than differences in the damage itself. Our data indicate that, in at least one system, the initial u.v. lethal damage does not differ at all, and that, consequently, differences in survival reflect solely the ability of the cells to cope with this damage.

Setlow *et al.* (1963) reported that equal numbers of thymine dimers were initially produced by the same u.v. dose in two strains of *Escherichia coli* (B/r and B_{s-1}) which differed in their sensitivity to killing by a factor of several hundred. Though a different comparison (different strains) from ours (different states of one strain), their results and ours together support a possible general conclusion, namely, that most differences in radiation sensitivity of bacteria reflect differences in ability to cope with the initial lethal damage rather than differences in the damage itself.

The authors are indebted to Drs H. I. Adler, R. F. Kimball, R. B. Setlow, and G. E. Stapleton for their helpful comments on this manuscript. We are especially grateful to Dr Setlow for guidance in the presentation and discussion in the Appendix.

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APPENDIX

Derivation. We here derive an expression that describes the survival curves obtained in this study (see Fig. 3). When these curves are plotted as the logarithm of surviving fraction against u.v. dose, they have the following characteristics: (*a*) at low doses a constant negative slope, (*b*) at high doses a constant negative slope steeper than the initial slope, (*c*) in different cultures different initial slopes but the same final slopes. We assume that (1) a culture that experienced no intracellular recovery from lethal lesions would show a single-hit survival curve, (2) all deviations from this simple curve must then reflect recovery events, but (3) these recovery events rapidly diminish in importance at high doses, so that the final slopes of all the curves are the same and reflect the initial sensitivity (without recovery) of individual targets in the cell.

The surviving fraction, S, after dose D can be represented as the sum of (*a*) the fraction of the total population that has suffered no lethal damage ($e^{-\alpha D}$), and (*b*) the fraction of the total population that has suffered lethal damage ($1-e^{-\alpha D}$) but has a probability, P, of subsequent recovery. That is,

$$S = e^{-\alpha D}+P(1-e^{-\alpha D}). \tag{1}$$

It would be desirable at this point to break down fraction (*b*) of this population into component subpopulations, each of which sustained a given number of hits and which has a fixed probability of recovery. However, we may avoid the mathematical complexities of such analysis by following the empirical approach outlined below.

Since $e^{-\alpha D}$ is the fraction of the *total* population that has escaped initial lethal damage, $e^{-\alpha D}/S$ is the fraction of the *surviving* population that has escaped initial damage. Therefore, the fraction of the surviving population that has sustained initial damage (but subsequently recovered) is $1-(e^{-\alpha D}/S)$. This fraction for any

dose can be determined from the data, since α is the final slope of the curve (see assumption (3) above). Plotting of this fraction against dose for our data reveals a curve that rises exponentially from zero and approaches a plateau at high doses (the plateau beginning essentially at the point where the slope of the survival curve becomes constant). Expressing this mathematically,

$$1-\frac{e^{-\alpha D}}{S} = A(1-e^{-rD}), \tag{2}$$

where A is a constant representing the maximum value of the function, and r is a constant related to the rate at which the function rises. Solving equation (2) for S gives

$$S = \frac{e^{-\alpha D}}{(1-A)+A\,e^{-rD}}.$$

Let $A/(1-A) = M$. Then, substituting and rearranging,

$$S = \frac{(M+1)\,e^{-\alpha D}}{1+Me^{-rD}}. \tag{3}$$

Equation (3) may now be substituted back in equation (1) to obtain an expression for the probability, P.

In order to get an expression for the slope of the survival curve, we may take the logarithm of equation (3), and then the derivative with respect to dose

$$\ln S = \ln(M+1)-\alpha D-\ln(1+Me^{-rD}),$$

$$\frac{d(\ln S)}{dD} = -\alpha+\frac{Mr\,e^{-rD}}{1+Me^{-rD}}. \tag{4}$$

Discussion. At high doses, the denominator of equation (3) approaches unity, and S approaches $(M+1)\,e^{-\alpha D}$. Thus, on a semilogarithmic plot, the constant $-\alpha$ is the slope of the survival curve at high doses, and the constant $M+1$ is the Y-intercept of the extrapolated slope. With these two constants thus determined, one can then calculate the value of r from equation (3).

From equation (4), it can also be seen that, as the dose becomes very high the slope approaches $-\alpha$. At zero dose, the slope becomes $-\alpha+Mr/(1+M)$. Now, if M is small, the slope at zero dose approaches $-\alpha$. Thus, for small M, the curve approaches single-hit form (if M is zero, the curve is single-hit). For large M, the slope at zero dose, m_0, approaches $-\alpha+r$. Thus, there is a minimum initial negative slope obtainable, regardless of the value of M, and this minimum is determined by r, which can also be seen to represent the difference between the minimum initial slope and the final slope {i.e. $r = (m_0)_{\min.}-(-x)$}.

Equation (3) provides a satisfactory description of the data, both qualitatively (see above) and quantitatively (see text discussion of Fig. 3). The fact that two of the three constants are invariant for the three different cultures studied encourages us to believe that the fit of equation (3) to the data may be more than fortuitous, and that, consequently, it may be possible to arrive at reasonable interpretations of the constants (see below).

Equation (3) takes into account the radiation response of the population at both high and low doses without requiring assumptions of different kinds of initial damage. Also, it isolates mathematically the events involved in (*a*) induction of initial lesions, and (*b*) recovery from these lesions. It can readily be extended to the

case where the survival curve in the absence of recovery is of the multi-target type, instead of the single-hit type assumed here.

Haynes (1964) proposed an equation with constants rather similar to those in our equation, but he expresses the survival as a single exponential function, which includes all survivors. His approach is thus basically different from ours, in which the undamaged survivors and the damaged-but-recovered survivors are treated separately, recovery factors being applied only to the latter fraction.

Interpretation. The fact that equation (3) is derived in a reasonable way and provides a good description of the experimental data permits us to put a physical interpretation on the constants. The experimental curves bear a resemblance to multi-target curves, in that the final slopes are invariant. In multi-target analysis, the Y-intercept of the extrapolate of the final slope is the number of targets that must be inactivated in order to inactivate the cell. It therefore seems likely that the Y-intercept of the extrapolate of the final slope for our curves ($M+1$) is related to a critical target number. We suggest that M represents the average number of lesions that can be repaired in the time available for repair. Roughly speaking, cells that have sustained fewer than M lesions will probably recover, while those sustaining $M+1$ or more lesions probably will not recover. The maximum number of lesions that a cell can repair might be the product of (*a*) rate of repair, and (*b*) time to a repair-limiting point (e.g. onset of DNA synthesis). We have observed (unpublished data) a delay of about 60 min. in the incorporation of radioactive thymine into stationary-phase *Escherichia coli* strain 15T$^-$A$^-$U$^-$ subcultured in liquid medium. In parallel experiments, a logarithmic culture reached normal incorporation rate within 5 min. If the stationary culture ($M = 272$) repairs 272 lesions/organisms in 60 min., then the repair rate must be about 4·5 lesions/min. Assuming the same repair rate for a logarithmic culture ($M = 4$), the time to the repair-limiting point would be about a minute, which is consistent with the observed kinetics of resumption of DNA synthesis. Thus, the data are consistent with the interpretation that the difference in the survival curves for logarithmic and stationary cultures reflects, not a difference in the rate of repair, but a difference in the time available for repair.

We assumed at the outset that the constant α represents the final slopes of the curves. We suppose that, in the region where the curve approaches closely to this final slope, one begins to see the fraction of the population that sustained too many hits to be repaired within the time available for repair. Since a single unrepaired hit remaining in a cell will inactivate it, this fraction of the population dies with single-hit kinetics. That the final slopes are invariant suggests that the target cross-section is invariant with dose or mode of culture.

Equation (2) expresses the experimentally supported concept that 'the fraction of survivors that sustains initial damage and subsequently recovers' begins at zero, for zero dose, rises exponentially at low doses, and approaches a plateau at high doses. The constant r expresses the rate of this exponential rise, and is thus a measure of the degree of curvature of the survival curve as it makes the transition from the initial slope to the final slope. It can therefore be considered a measure of the rate of repair processes. That this constant is the same for logarithmic and stationary cultures in our system suggests that repair machinery is the same in both cultures, further supporting our suggestion that the essential difference in the response of the two cultures rests simply in the time available for repair.

Finally, we may note (see discussion of equation (4)) that, for a given repair rate (r), there appears to be a minimum initial negative slope obtainable, regardless of the value of M (which, in our interpretation, means regardless of the time available for repair). This suggests that some lesions are inherently *irreparable* by existing intracellular machinery. If this were not the case, then, for cultures that have a long time available for recovery, we would expect the survival curves to be simply of the multi-target type, with zero initial slope. That such survival curves are rarely seen for ultraviolet inactivation suggests that ultraviolet usually produces a significant fraction of damage irreparable by normal cell processes.

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Radiation Sensitivity and Growth Characteristics of an Arginine- and Uracil-Starved Culture of *Escherichia coli* strain 15 T⁻A⁻U⁻

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SUMMARY

A logarithmic-phase culture of *Escherichia coli* strain 15T⁻A⁻U⁻, in which protein and RNA syntheses were inhibited for 90 min. by withdrawal of arginine and uracil, was compared with a culture grown with all required nutrients present until 90–120 min. after the end of exponential increase in extinction (early stationary phase). The two cultures were indistinguishable by tests of radiation (ultraviolet, gamma) sensitivity, growth kinetics and morphology. It is concluded that the interruption of protein and RNA syntheses during active logarithmic growth forced the culture into the stationary phase.

INTRODUCTION

In the preceding paper (Ginsberg & Jagger, 1965) we showed that the ultraviolet (u.v.) resistance of *Escherichia coli* strain 15T⁻A⁻U⁻ (15tau) harvested in early stationary phase was identical with that of a logarithmic-phase culture in which arginine and uracil were withheld for 90 min. (−AU culture); furthermore, the −AU and early stationary-phase cultures did not differ from each other with respect to the nature or the number of initial u.v. lesions produced by a given u.v. dose. This suggests that organisms of a −AU culture might be in a physiological state similar to that of organisms from the early stationary-phase culture. The present paper gives evidence that the −AU culture and the early stationary-phase culture also did not differ from each other by tests of radiobiological response, growth kinetics, or morphology. It is concluded that interruption of protein and RNA synthesis in logarithmic-phase organisms of this strain forced them into the stationary phase.

METHODS

Bacteria, media and conditions of growth, and u.v. irradiation techniques were as described in the preceding paper.

Gamma irradiation. For gamma irradiation, organisms were washed and diluted in 0·067 M-phosphate buffer (6 g. Na_2HPO_4, 2·3 g. KH_2PO_4 per l. distilled water;

final pH 7). Gamma irradiation was performed on 5 ml. volumes of phosphate-buffer suspension of organisms in 5 × 100 mm. Pyrex test tubes. The gamma-ray source was a ^{60}Co 'Gammacell' Model 220 (Atomic Energy of Canada, Ltd), containing approximately 1200 curies of ^{60}Co (principal photon yield/disintegration: γ_1, 1·17 meV., > 99 %; γ_2, 1·33 meV., > 99 %) distributed in standard (U.S. Atomic Energy Commission) pencils around the cylindrical irradiation chamber. During irradiation, dose-gradient effects in the irradiation chamber were minimized by constant rotation of the sample tubes around the vertical axis of the cylinder while each sample tube revolved about its own vertical axis. All irradiations were done in air at room temperature. The exposure rate was approximately 1650 roentgens/min.

Microslide cultures for phase-contrast microscopy. Microslide cultures were prepared by the method of Adler & Hardigree (1964), with a drop of bacterial suspension containing about 10^8 bacteria/ml., which was flattened by a coverslip on to Difco nutrient agar. Growth and reproduction of individual bacteria could be observed for several hours by using the X97 oil-immersion objective of a Spencer AO phase (medium-dark contrast) microscope.

RESULTS

Photobiological similarity of −AU and stationary-phase cultures of Escherichia coli strain 15 T⁻A⁻U⁻

In the preceding paper (Ginsberg & Jagger, 1965) it is reported that both the u.v. survival and photo-reactivation curves were the same for −AU and early-stationary cultures of *Escherichia coli* strain 15 T⁻A⁻U⁻. Further confirmation of their photobiological similarity was obtained by the following experiment. Bacteria from each of the two cultures were inoculated into fresh growth media (+T +AU) and grown at 37° for 30 min. to mid-lag phase (half the time required for the beginning of exponential increase in viable numbers). Figure 1 shows: (1) the u.v. sensitivity of the lag-phase culture was the same whether grown from −AU or from early stationary-phase bacteria; (2) the photo-reactivable sectors were the same. The photo-reactivable sectors in these experiments had the same values as for logarithmic-phase, −AU and early stationary-phase cultures. The last observation shows that the photo-reactivable sector and, consequently, the spectrum of u.v. damage produced, did not change during transition from the u.v.-resistant (stationary-phase or −AU) to the u.v.-sensitive (logarithmic-phase) state. It also appears that the lag-phase survival curves had the same final slopes as the curves for logarithmic-phase, −AU, and stationary-phase cultures, suggesting that the nature of the initial lesions did not change during lag-phase transition, at least for the higher doses. The value of the ordinate intercept of the extended straight line portion of the lag-phase curves is about 20, intermediate between those for logarithmic-phase cultures (5) and −AU stationary-phase cultures (273).

Effects of ionizing radiation

Billen (1963) reported the increased resistance of −AU cultures of *Escherichia coli* strain 15 T⁻A⁻U⁻ to 250 k.v.p. X-rays. The following experiments were done to determine whether the gamma-ray survival curves were the same for −AU and

the early stationary-phase cultures. Bacterial suspensions in phosphate buffer were exposed in the ^{60}Co Gammacell. Survivors were assayed by colony formation at 37° on nutrient agar; the results are shown in Fig. 2; the survival curve of a logarithmic-phase culture is included for comparison. The same visually fitted curve describes the data for gamma-ray inactivation of either early stationary-phase or −AU cultures. The dose-reduction factor for early stationary-phase and −AU cultures compared to the logarithmic culture was constant (about 0·50) in the survival range studied.

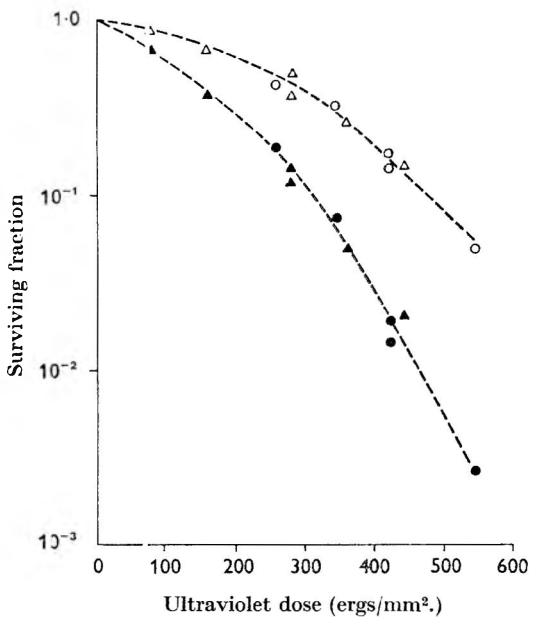

Fig. 1

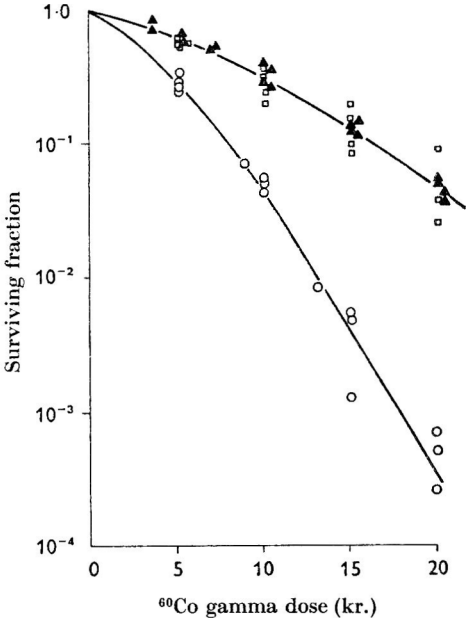

Fig. 2

Fig. 1. Survival of *E. coli* strain $15T^-A^-U^-$ after 2537 Å ultraviolet-irradiation (solid symbols) and after ultraviolet + maximum photo-reactivation (open symbols) of mid-lag phase cultures grown from early stationary-phase cultures (circles) and from −AU cultures (triangles).

Fig. 2. Survival of *E. coli* strain $15T^-A^-U^-$ after ^{60}Co gamma-irradiation of logarithmic-phase cultures (circles), early stationary-phase cultures (triangles), and −AU cultures (squares).

Growth studies

Growth of a logarithmic-phase culture of *Escherichia coli* strain $15T^-A^-U^-$ (in +T+AU supplemented medium) was interrupted at a viable count of 10^8 bacteria/ml. by withdrawal of arginine and uracil for 90 min. At the end of this time, growth was permitted to resume by adding arginine and uracil. The effects of these shifts on the curves of extinction and viable count are recorded in Fig. 3. Similar results were reported by Maaløe & Hanawalt (1961). Figure 4 shows the extinction and the viable count for a +T+AU culture entering the stationary phase and, subsequently, an early stationary-phase culture (90 min. after cessation of exponential extinction increase) beginning exponential growth. The similarities between Figs. 3 and 4 are striking. The slope of the extinction curve diminished abruptly when

arginine and uracil were withdrawn from a logarithmic-phase culture, and when a normal (+T +AU) culture entered the stationary phase. Extinction increased at a high rate almost immediately when arginine and uracil were added back to the −AU culture and when early stationary-phase bacteria were inoculated into fresh growth medium. The curve of viable numbers gradually decreased in slope after arginine and uracil were withdrawn from a logarithmic culture and as the +T +AU culture entered the stationary phase. Resumption of exponential increase in viability was delayed for about 60 min. after arginine and uracil were added back to the −AU culture, and after early stationary-phase bacteria were inoculated into fresh growth medium.

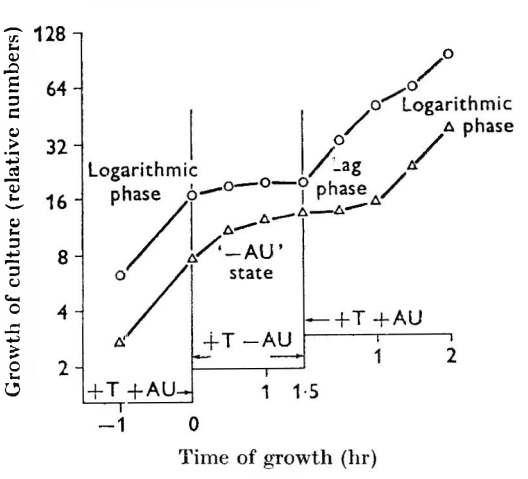

Fig. 3

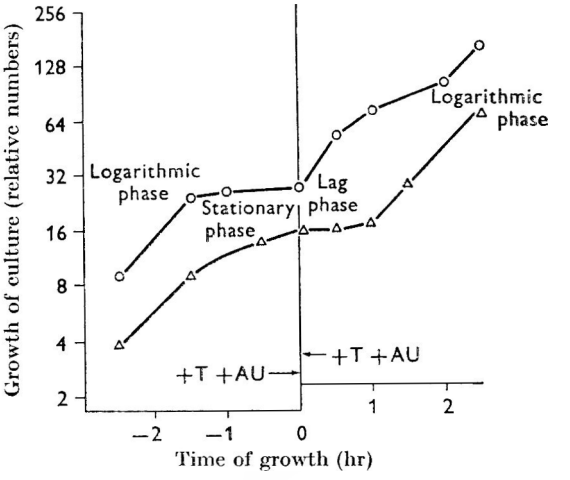

Fig. 4

Fig. 3. Growth of *E. coli* strain 15T⁻A⁻U⁻, as reflected by viable numbers (triangles) and extinction (circles). A culture in the logarithmic phase was interrupted by withdrawal of arginine and uracil for 90 min.

Fig. 4. Growth of *E. coli* strain 15T⁻A⁻U⁻, as reflected by viable numbers (triangles) and extinction (circles). A culture in supplemented (+T +AU) medium is shown during transition from logarithmic to early stationary phase, and from early stationary phase back to logarithmic phase, the latter being obtained by dilution at time zero into fresh medium.

Morphology of unirradiated organisms

Organisms from the early stationary-phase culture, and from a −AU culture, were inoculated on microslide nutrient agar mounts and observed for several divisions at room temperature by phase microscopy. No initial morphological differences were detectable between the organisms of the two cultures. The time course of increase in size during the lag phase and the time of onset of cell division were parallel in the two preparations. Initially the organisms were short, rod-shaped; the length : diameter ratio was about three. Double organisms were present in both preparations, but single organisms were predominant. In about 2 hr, organism length had doubled and a few organisms had begun to divide. At 3 hr, many organisms were about four times the original length, and more organisms were beginning to divide. By 4½ hr, microcolonies of 4–8 organisms each had formed, and almost all organisms were dividing. Central bodies were observed in most instances; and their number always appeared to be proportional to organism length.

DISCUSSION

We have reported in the preceding paper (Ginsberg & Jagger, 1965) that −AU and early stationary-phase cultures of *Escherichia coli* strain $15T^-A^-U^-$ were photobiologically alike. It is shown in the present paper that the −AU and early stationary-phase cultures returned to the u.v.-sensitive state in the same way when regrown in +T +AU medium, and that both cultures showed the same degree of sensitivity to gamma radiation. The data on growth and morphology indicate that the two cultures are identical. Thus, we conclude that inhibition of protein and RNA syntheses in the logarithmic bacterial culture forced the culture into stationary phase. Although the evidence presented (growth kinetics, morphology, radiation sensitivity) is sufficient to confirm our preliminary suggestion that a −AU culture is in a physiological state similar to that of the early stationary phase, it does not provide significantly new information about this state. We still do not understand why or how the usual transition from logarithmic to stationary phase is initiated (see Freter & Ozawa, 1963). We do not know for certain what changes, if any, occur in the quality and quantity of DNA in a bacterium entering stationary phase (Ginsberg, 1963). We have made a preliminary observation (unpublished) that the lag phase in a liquid subculture is about 20 min. longer for a 3–4 hr stationary-phase culture than for the 90–120 min. culture, and it is possible that the radio-sensitivities of cultures in these two portions of the stationary phase are different. Consequently, conclusions based on the experimental observations presented here, about the nature of a stationary-phase culture, would be highly speculative. The extent and detail of the gamma-ray experiment were limited to showing that the −AU and early stationary-phase cultures are alike in their survival response to ionizing radiation. However, the appearance of a constant dose-reduction factor over the survival range tested permits us to suggest, cautiously, that a situation analogous to that for u.v. killing could exist, namely, that (1) the initial damage by a given gamma-ray exposure is independent of growth phase or metabolic state, and, therefore, (2) the difference in survival is the result of differences in ability to cope with the damage. This notion is consistent with the experimental findings which led Glinos & North (1963) to conclude that a given X-ray dose produces the same initial damage in mammalian cells in logarithmic and in stationary-phase, even though the stationary-phase cells show a higher survival.

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The Life-Cycle of an Actinophage for *Streptomyces venezuelae*

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SUMMARY

The life-cycle of actinophage MSP2 in host *Streptomyces venezuelae* s13 was divisible into distinct stages by manipulating the cultural conditions and by the use of certain chemical compounds. Two types of attachment were observed: reversible and irreversible. Reversible attachment occurred instantaneously and was relatively independent of temperature and phage concentration. The controlling variables in extent of adsorption were the amount of host mycelium and ionic content of the medium. Irreversible attachment was time dependent and temperature dependent. Addition of acriflavine allowed phage attachment and injection to occur, but suppressed some stage of intracellular growth or maturation. Acriflavine prevented formation of viable phage, but phage components were demonstrated immunologically and electron-microscopically. Chilling of phage-infected cultures allowed lysis to proceed, with release of preformed phages; but no new phages were formed. These results indicate that the life-cycle of actinophage MSP2 is experimentally separable into attachment, injection, growth, maturation and lysis.

INTRODUCTION

Previous studies with actinophages MSP2 (Jones, 1963), MSP8 (Kolstad & Bradley, 1964) and MVP7 (Bradley, Lee & Jones, 1963) established procedures for routine propagation, concentration and purification of several litres of lysates containing 10^{11} plaque-forming units (p.f.u.)/ml. The specific action on phage growth of factors such as temperature of incubation and ionic content of the medium were not studied during the earlier works. It seemed probable that the life-cycle of actinophages, like coliphages (Stent, 1963), could be separated into five stages: attachment, injection, growth, maturation, lysis. It was reasonable to expect the composition of the medium and metabolic inhibitors to affect these stages differentially. This report shows that the life-cycle of actinophage MSP2 can be resolved into distinct stages by manipulation of the cultural conditions and by the use of certain chemical compounds.

METHODS

Stock cultures of *Streptomyces venezuelae* s13 were maintained at 30° on tomato-paste oatmeal agar (Gottlieb, 1961) by serial single colony subculture. Spore preparations were made by suspending in peptone yeast-extract broth (Bradley, 1959)

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the surface growth from 7- to 10-day stock cultures and homogenizing it with a tissue grinder. Vegetative mycelium of *S. venezuelae* was cultured in flasks of peptone yeast-extract broth inoculated with spore suspension to give 10^6 to 10^7 spores/ml. The flasks were shaken at 30° for 18 hr.

For studies of the attachment of phage to host, young vegetative mycelium was collected by centrifugation, washed twice with appropriate salt solution and suspended to give an extinction of 0·2 at 420 mμ (E_{420}) in the Bausch & Lomb Spectronic 20; this corresponded to about 10^8 colony-forming units/ml. Purified actinophage MSP2 was added to give 10^7 p.f.u./ml. Reversible attachment was determined by comparing the number of p.f.u. in the whole reaction mixture with the number in the supernatant fluid after centrifuging down the mycelium with attached phage. For irreversible attachment, appropriately diluted antiserum was added to the reaction mixture to neutralize the phage particles which were either unattached or only reversibly adsorbed to host mycelium. Hyperimmune antiserum was prepared by repeatedly injecting rabbits (Adams, 1959) with purified actinophage MSP2. The antiserum was absorbed with washed host mycelium. An alternative assay of irreversible attachment was the number of p.f.u. remaining attached to the mycelium after repeated washing with 0·15 M-NaCl. Viable actinophage particles were counted by the conventional soft-agar overlay technique (Jones & Bradley, 1962).

For growth studies, young vegetative mycelium was harvested by centrifugation, suspended in a solution 0·004 M in $Ca(NO_3)_2$ and 0·0015 M in NaCl. Purified actinophage MSP2 was added to give a multiplicity of about 10 phage particles/host colony-forming unit. After 30 min., the infected mycelium was collected, washed with 0·15 M-NaCl to remove free and reversibly attached phage, suspended in peptone yeast-extract broth and incubated with agitation. At intervals for 3–5 hr, samples were assayed for plaque-forming particles.

RESULTS

Reversible attachment

Attempts to obtain attachment of actinophage MSP2 to *Streptomyces venezuelae* mycelium in the actual culture fluid were unsuccessful; therefore young vegetative mycelium was grown in peptone yeast-extract broth, washed with salt solution and tested for ability to adsorb phage. Maximal attachment was obtained with solutions about 10^{-3} M in calcium, magnesium or manganese salts. Concentrations of NaCl exceeding 0·1 M inhibited attachment (Table 1). The attachment observed in inorganic salts medium with washed mycelium, with the free phage in the supernatant fluid used as the index of adsorption, was dependent upon host concentration but independent of phage concentration (Table 2). These results indicated that this adsorption was reversible. Reversibility was demonstrated directly by suspending the mycelium with attached phage in distilled water or in 0·1 M-NaCl (Table 3). Strains of *S. venezuelae* selected for resistance to actinophage MSP2 or to the closely related actinophage MSP8 retained the ability to adsorb MSP2. *Streptomyces rimosus*, a streptomycete naturally resistant to actinophage MSP2, had limited capacity to adsorb MSP2, whereas *Nocardia erythropolis* had none (Table 4).

Table 1. *Effect of cation content on attachment of actinophage MSP2 to washed mycelium of Streptomyces venezuelae s13*

S. venezuelae s13 was grown overnight in peptone yeast-extract broth, harvested by centrifugation, washed with appropriate salt solution and suspended to the original volume. Actinophage MSP2 was added and the reaction mixture shaken at 30° for 30 min. The whole mixture and the supernatant fluid after centrifugation were titrated.

Salt	Strength (M)	No. MSP2 particles/ml. Total	No. MSP2 particles/ml. Free	Proportion of phage particles attached (%)
$Ca(NO_3)_2$*	10^{-2}	$3{\cdot}8 \times 10^8$	$2{\cdot}8 \times 10^6$	27
$Ca(NO_3)_2$*	10^{-3}	$1{\cdot}0 \times 10^8$	$6{\cdot}0 \times 10^4$	94
$Ca(NO_3)_2$*	10^{-4}	$2{\cdot}8 \times 10^8$	$2{\cdot}2 \times 10^8$	21
$MgSO_4$*	2×10^{-3}	$1{\cdot}5 \times 10^8$	$1{\cdot}7 \times 10^5$	88
$MnSO_4$*	2×10^{-3}	$9{\cdot}5 \times 10^5$	$7{\cdot}0 \times 10^4$	93
NaCl†	2×10^{-1}	$1{\cdot}2 \times 10^8$	$1{\cdot}4 \times 10^8$	0
NaCl†	2×10^{-3}	$1{\cdot}3 \times 10^8$	$8{\cdot}0 \times 10^4$	94

* Salt solution also contained 2×10^{-3} M-NaCl.
† Salt solution also contained 2×10^{-3} M-$Ca(NO_3)_2$.

Table 2. *Effect of host and phage concentration on reversible attachment of actinophage MSP2 to host (Streptomyces veneuzelae s13) mycelium*

Washed mycelium of *S. venezuelae* s13 was suspended with phage MSP2 in 4×10^{-3} M-$Ca(NO_3)_2 + 10^{-3}$ M-NaCl and the reaction mixture was shaken at 30° for 30 min. Total phage and free phage in the supernatant fluid were determined.

Host concentration (E_{420})	Input phage titre	No. MSP2 particles/ml. Total	No. MSP2 particles/ml. Free	Proportion of phage particles attached (%)
0·40	$3{\cdot}0 \times 10^8$	$2{\cdot}8 \times 10^8$	$0{\cdot}3 \times 10^8$	89
0·40	$3{\cdot}0 \times 10^8$	$2{\cdot}2 \times 10^8$	$0{\cdot}2 \times 10^8$	90
0·40	$3{\cdot}0 \times 10^4$	$2{\cdot}9 \times 10^4$	$0{\cdot}1 \times 10^4$	97
0·28	$1{\cdot}0 \times 10^7$	$1{\cdot}0 \times 10^7$	$0{\cdot}3 \times 10^7$	67
0·12	$1{\cdot}0 \times 10^7$	$1{\cdot}0 \times 10^7$	$0{\cdot}6 \times 10^7$	40
0·03	$1{\cdot}0 \times 10^7$	$1{\cdot}0 \times 10^7$	$0{\cdot}9 \times 10^7$	10

Table 3. *Desorption of actinophage MSP2 from washed Streptomyces venezuelae s13 mycelium*

Washed mycelium was suspended in 10^{-3} M-$Ca(NO_3)_2 + 10^{-3}$ M-NaCl; phage was added and the reaction mixture shaken at 30° for 30 min. Thereafter, the mycelium was sedimented by centrifugation and suspended in the indicated desorption media. The desorption mixture was shaken for 30 min. at 30°. Adsorbed phage was measured by difference between titres of the total reaction mixture and the supernatant fluid.

Attachment phase no. MSP2 p.f.u./ml. Total	Attachment phase no. MSP2 p.f.u./ml. Free	Desorption medium	Desorption phase no. MSP2 p.f.u./ml. Total	Desorption phase no. MSP2 p.f.u./ml. Free
$1{\cdot}1 \times 10^6$	9×10^4	10^{-3} M-$Ca(NO_3)_2 + 10^{-3}$ M-NaCl	9×10^5	1×10^4
$1{\cdot}2 \times 10^6$	1×10^5	10^{-3} M-NaCl	8×10^5	7×10^5
$1{\cdot}1 \times 10^6$	7×10^4	De-ionized water	6×10^5	5×10^5
$1{\cdot}1 \times 10^6$	8×10^4	10^{-2} M-$Ca(NO_3)_2$	8×10^5	3×10^4

Irreversible attachment

In the previous experiments showing reversibility of attachment of phage to host, about 10 % of the adsorbed phage did not desorb, that is, a certain proportion were irreversibly attached to the host mycelium. The irreversibly adsorbed phage were not inactivated by antiserum directed against actinophage MSP2. Unlike the reversible phase, irreversible attachment was temperature dependent and time dependent (Table 5) and was not inhibited by 5 g. NaCl. (Table 6).

Table 4. *Specificity of attachment of actinophage MSP2 to mutants of Streptomyces venezuelae s13 and to other actinomycetes*

Washed mycelia of the indicated actinomycetes were suspended in 4×10^{-3} M-$Ca(NO_3)_2 + 10^{-3}$ M-NaCl; phage was added; the mixtures were shaken at 30° for 30 min. Total and free phage were assayed.

Organism	Phenotype	No. MSP2 p.f.u./ml. Total	No. MSP2 p.f.u./ml. Free	Proportion of phage particles attached (%)
S. venezuelae s 13	Sensitive to MSP2 and MSP8	$1{\cdot}1 \times 10^6$	$0{\cdot}1 \times 10^5$	99
S. venezuelae s 13 R 2	Resistant to MSP2 and MSP8	$1{\cdot}2 \times 10^6$	$1{\cdot}8 \times 10^5$	85
S. venezuelae s 13 R 8	Resistant to MSP2 and MSP8	$1{\cdot}4 \times 10^6$	$0{\cdot}8 \times 10^5$	94
S. rimosus	Resistant to MSP2 and MSP8	$2{\cdot}6 \times 10^6$	$1{\cdot}8 \times 10^6$	30
Nocardia erythropolis	Resistant to MSP2 and MSP8	$4{\cdot}7 \times 10^6$	$4{\cdot}7 \times 10^6$	0

Table 5. *Effect of time and temperature on attachment of actinophage MSP2 to washed mycelium of Streptomyces venezuelae s 13*

Washed mycelium of *S. venezuelae* s 13 was suspended in 4×10^{-3} M-$Ca(NO_3)_2 + 10^{-3}$ M-NaCl and equilibrated to the indicated temperature; phage was added to a final concentration of $1{\cdot}6 \times 10^6$ p.f.u./ml. and the reaction mixture shaken for 30 min. Reversible attachment was measured as the difference between total and free phage. Irreversible attachment was defined as resistance to specific antiserum.

Temperature	Assay	Time (min.) 5	10	30
		No. attached phage particles (p.f.u.)		
5°	Reversible	$1{\cdot}6 \times 10^6$	$1{\cdot}4 \times 10^6$	$1{\cdot}6 \times 10^6$
30°	Reversible	$1{\cdot}4 \times 10^6$	$1{\cdot}3 \times 10^6$	$1{\cdot}3 \times 10^6$
56°	Reversible	$1{\cdot}5 \times 10^6$	$1{\cdot}5 \times 10^6$	$1{\cdot}3 \times 10^6$
5°	Irreversible	10^2	3×10^3	5×10^3
30°	Irreversible	3×10^4	6×10^4	2×10^5
56°	Irreversible	$< 10^2$	$< 10^2$	$< 10^2$

Vegetative growth

The multiplication curve of actinophage MSP2 was determined by measuring the p.f.u./ml. in serial samples of infected mycelia. The latent period was 75–90 min., the rise period was from 90 to 180 min., and the burst size varied between 500 and 5000 p.f.u. (Fig. 1). To determine intracellular growth, samples of an infected culture were disrupted by ultrasonic treatment. The brief ultrasonic treatment required to rupture the mycelium did not damage mature phage particles, but decreased the

Table 6. *Effect of NaCl concentration on attachment of actinophage MSP2 to Streptomyces venezuelae s13*

Washed mycelium of *S. venezuelae* was suspended in solutions 0·004 M-$Ca(NO_3)_2$ and 0·1 M or 0·002 M in NaCl; phage was added. The reaction mixture was incubated at 30° for 30 min. Reversible attachment was determined as the difference between total and free phage. Phage not eluted by repeated washing with 0·15 M-NaCl were considered irreversibly attached.

NaCl (M)	No. MSP2 p.f.u./ml.		
	Reversible assay		
	Total	Free	Irreversible assay
0·002	$5·4 \times 10^6$	$1·8 \times 10^5$	$1·4 \times 10^6$
0·1	$5·4 \times 10^6$	$3·6 \times 10^6$	$1·2 \times 10^6$

Table 7. *Suppression of actinophage MSP2 replication in Streptomyces venezuelae s13*

Washed *S. venezuelae* s13 mycelium was infected in salt solution; attachment was stopped by antiserum; infected mycelium was sedimented and suspended in peptone yeast-extract broth (to give 2×10^8 p.f.u./ml.) with the indicated compound. After 4 hr, the amount of phage was assayed.

Treatment	Concentration (μg./ml.)	No. MSP2 p.f.u./ml.	Effect on host
Control, 30°	—	$1·4 \times 10^9$	None
37°	—	2×10^6	None
Acriflavine*	15	8×10^6	None
Endomycin†	200	$1·9 \times 10^7$	Inhibited
Novobiocin†	20	$2·1 \times 10^7$	Inhibited
Bacitracin‡	5§	3×10^8	Inhibited
Streptomycin‡	125	$1·3 \times 10^9$	None

* An equal mixture of euflavine and proflavine.
† From the Upjohn Company.
‡ From the California Corporation for Biochemical Research.
§ Units/ml.

Table 8. *Effect of acriflavine on actinophage MSP2 replication*

Washed *S. venezuelae* s13 mycelium was infected in salt solution; attachment was stopped by adding antiserum; infected mycelium was sedimented and suspended in peptone yeast-extract broth with acriflavine as indicated. Infected hyphae were disrupted by ultrasonic treatment.

Time (min.)	No. MSP2 p.f.u./ml.			
	20 μg. acriflavine/ml.		Control	
	Intact	Disrupted	Intact	Disrupted
0	3×10^7	1×10^7	$4·5 \times 10^7$	8×10^6
90	$2·5 \times 10^7$	1×10^7	$4·5 \times 10^7$	5×10^8
180	5×10^7	1×10^7	$7·7 \times 10^9$	5×10^9
270	9×10^7	2×10^7	4×10^{10}	2×10^{10}
270 (drug last 180 min.)	1×10^9	Not done	4×10^{10}	Not done
270 (drug last 120 min.)	$1·8 \times 10^{10}$	Not done	4×10^{10}	Not done

number of p.f.u. in newly infected cultures. The time between infection and the first appearance of mature phage intracellularly, that is, the eclipse period, was about 1 hr. The period of rise was 2–3 hr in cultures in which phage growth was interrupted by ultrasonic treatment. The maximal titre achieved was the same in artificially-lysed cultures as in cultures which had lysed normally.

Various factors were tested for ability to suppress phage replication. Only acriflavine (equal parts euflavine and proflavine) and elevated temperature sharply

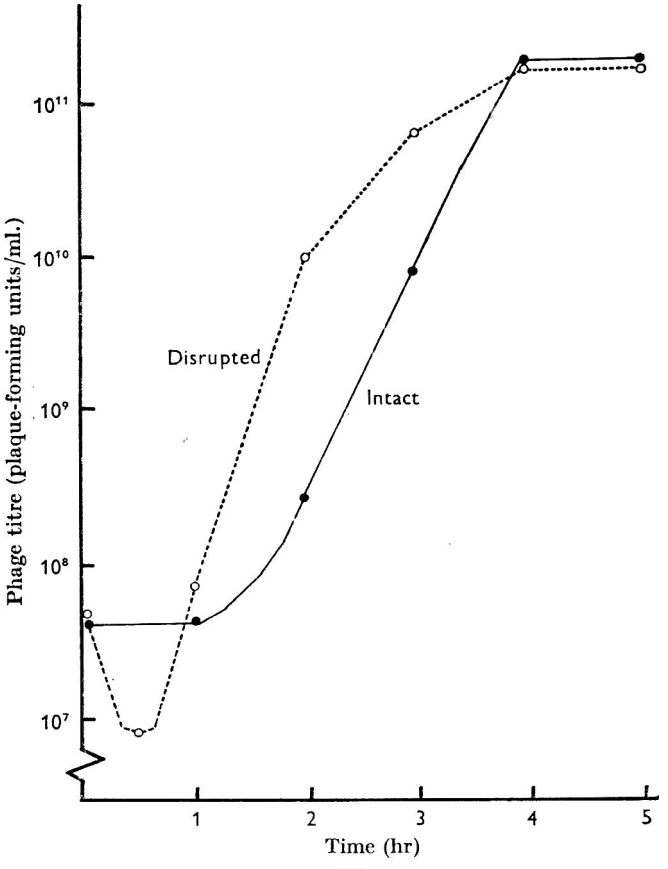

Fig. 1

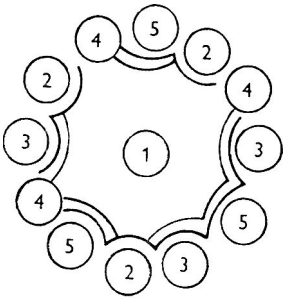

Fig. 2

Fig. 1. One-step growth curve (●———●) and intracellular growth curve (○———○) of actinophage MSP2 in host (*Streptomyces venezuelae* s 13) mycelium. Cultures were disrupted by ultrasonic treatment to determine intracellular phage.

Fig. 2. Diagram of agar gel diffusion plate of actinophage MSP2. Wells labelled 1 contained hyperimmune antiserum against actinophage MSP2; 2 contained purified, intact MSP2; 3 contained a fraction (35,000*g* sediment) from an acriflavine-treated, phage-infected *Streptomyces venezuelae* culture; 4 contained a comparable fraction (35,000*g* sediment) from an acriflavine-treated *S. venezuelae* culture without phage; and 5 contained purified MSP2 which had been disrupted by prolonged (2 hr) ultrasonic treatment.

inhibited phage growth without interfering with host growth (Table 7). Acriflavine did not kill intact phage, nor did it inhibit attachment or bactericidal action of phage upon the host. The intracellular development of intact phage was prevented

(Table 8). Infected host mycelium incubated for 90 min. without acriflavine and then for 3 hr with acriflavine, supported a 30-fold increase in phage. Apparently, delayed addition of acriflavine allowed release of preformed phage. Attempts to remove acriflavine by sedimentation of the host mycelium and its resuspension in fresh medium did not permit development of mature phage. The mycelium, once exposed to acriflavine, remained markedly yellow in spite of repeated washing.

Infected cultures, exposed to acriflavine, were disrupted by ultrasonic treatment and fractionated by alternate cycles of centrifugation at 8000*g* for 10 min. and at 35,000*g* for 30 min. The 8000*g* residue, which contained host cell walls, was discarded. The 35,000*g* sediment contained particles which resembled phage heads and tails when viewed in the electron microscope and which gave precipitate bands in diffusion gel experiments with antiserum directed against intact MSP2 (Fig. 2). Uninfected cultures, treated or untreated with acriflavine, were similarly processed, but did not yield particles which resembled phage components or give bands of precipitate when tested with antiserum directed against the actinophage MSP2.

Lysis

Incompletely lysed cultures often cleared and gave increased titres of phage during refrigeration. Accordingly, the effect of continuous and discontinuous chilling of phage-infected cultures was studied. Phage-infected cultures incubated continuously at 1° did not produce phage. The shift from 1° to 30° merely delayed the normal lytic cycle for 1 hr. The shift from 30° to 1° after 1 hr permitted a 100-fold increase in phage titre.

DISCUSSION

Two types of attachment of actinophage to host *Streptomyces venezuelae* s13 mycelium were observed. Reversible attachment was relatively independent of time, temperature and phage concentration. The principal controlling variable in the extent of adsorption was the amount of host mycelium. Irreversible attachment was time dependent and temperature dependent, clearly differentiating it from reversible adsorption. Also, reversible attachment was inhibited by high concentrations of NaCl, whereas the irreversible type of attachment was not. It is conceivable that reversible attachment is an ion-exchange reaction and irreversible attachment an enzymic process. To date, irreversible attachment has not been experimentally separated from the injection process.

Intracellular growth of actinophage MSP2 in *Streptomyces venezuelae* s13 was differentiated into an early phase which did not proceed at 1° and a later stage which was inhibited by acriflavine. Fractions of acriflavine-treated, phage-infected cultures contained particles which when viewed in the electron microscope resembled purified actinophage that had been disrupted by ultrasonic treatment (Anderson & Bradley, 1964) and by certain chemical compounds (Painter & Bradley, 1965). These same samples gave two precipitate bands with specific antiserum in diffusion gel experiments; these bands were immunologically identical to those obtained with the actinophage disrupted by ultrasonic treatment. Because these preparations contained empty heads and tails, acriflavine probably inhibited the assemblage of phage components into mature phage particles. The action of acriflavine on actinophage,

therefore, resembles that on T-even coliphages of *Escherichia coli* (DeMars, Luria, Fisher & Levinthal, 1953).

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With 1 *plate*
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The Role of NaCl in the Lysis of *Staphylococcus aureus* by Lysostaphin

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SUMMARY

Lysostaphin attacked both viable staphylococci and the mucopeptide portion of the staphylococcal cell wall. In the absence of salts, lysostaphin activity could only be recovered from the particulate portion of the lysed cell after centrifugation, whereas in the absence of salts its action on the mucopeptide resulted in a recovery of active material in both the sediment and the supernatant fluid. It appears from these observations that lysostaphin is complexed with its substrate and that NaCl is required to break the complex.

INTRODUCTION

Schindler & Schuhardt (1964) reported on the isolation of an organism of the genus *Staphylococcus* that produced a lytic agent, lysostaphin, active against all of 59 strains of staphylococci tested. The specificity for organisms of the genus *Staphylococcus* separated its mode of action from that of lysozyme. The authors also described an assay for determining the quantity of enzyme based upon the decrease in turbidity of a standard staphylococcal strain. Since the rate of lysis of a staphylococcal strain was a function of the concentration of lysostaphin present, it was possible to deviate from the standard assay micro-organism in these experimental procedures.

Schindler & Schuhardt (1965) purified and described some of the properties of lysostaphin and reported that it was a basic protein essentially devoid of cystine and cysteine and contained large quantities of aspartic and glutamic acid compared to the total amino acid content. The lytic protein had a sedimentation coefficient ($S_{20,w}$) of 2·35*S* and an isoelectric point between pH 10·4–11·4. The rate of lysis of *Staphylococcus aureus* by lysostaphin was dependent upon the concentration of NaCl present in the medium. This phenomenon paralleled the salt requirement of the muramidase lysozyme (Salton, 1957). By increasing the concentration of NaCl in the suspending medium from zero to 0·15 M, an increased rate of lysis of staphylococci was observed. Concentrations of salt above physiological values introduced parameters which affected both the bacterial cells and the stability of lysostaphin; thus such levels of salts were not evaluated. This investigation was undertaken to determine in what manner NaCl entered into the dynamics of the lysostaphin-substrate interaction.

METHODS

Micro-organism. Staphylococcus aureus, strain sv, the mouse-virulent variant of the Smith strain (Hunt & Moses, 1958) was grown in Trypticase soy broth at 37° under conditions of aeration and agitation to give a maximum yield of cocci/ml.

The cocci were harvested by centrifuging at 8000*g* for 15 min. and washing with 0·05 M-tris buffer, pH 7·5.

Preparation of cell walls. Sixteen g (wet weight) of washed *Staphylococcus aureus* (SV) were suspended in 1 M-NaCl and mechanically ruptured in a Nossil disintegrator (McDonald Engineering Co., Bay Village, Ohio) with no. 13 Ballotini beads. The equipment was operated for six 1 min. periods with the capsule kept under a constant flow of liquid CO_2 to minimize autolysin action. The suspension was passed through a coarse sintered glass filter to remove the glass beads and the walls were recovered by centrifugation as described by Perkins & Rogers (1959). The cell walls were treated with the enzymes trypsin, ribonuclease and pepsin according to the procedure of Morse (1962). Residual protein and lipid were chemically extracted by the method of Mandelstam (1962). The yield after drying *in vacuo* was 300 mg. The homogeneity of the preparation was checked in a Siemens Elmiskop electron microscope with a beam magnification of ×10,664.

Chemical analysis of cell wall. Total phosphorus was determined by the method of Fiske & SubbaRow (1925), and ribitol by thin-layer chromatography utilizing cellulose (Camag type D, Arthur Thomas, Phila., Pa.) at a thickness of 250 μ as the support and developed with *tert*-butanol + acetic acid + water (52 + 3 + 25, by vol.). The plates were sprayed with acetylacetone-*p*-dimethylaminobenzaldehyde reagent (Dawson, Elliot, Elliot & Jones, 1959) and observed for fluorescence under ultraviolet radiation (2570 Å).

N-terminal amino acids were determined by combining the treated cell walls under alkaline conditions with absolute ethanol containing 2,4-dinitrofluorobenzene. The mixture was placed on a shaker for 4 hr at room temperature. Excess 2,4-dinitrofluorobenzene was removed by continuous extraction with absolute ether until no further colour could be observed in the ether phase. The aqueous phase was brought to dryness and hydrolysed *in vacuo* with 6 N-HCl for 22 hr at 110°. The HCl was removed by drying *in vacuo* at 45° over KOH pellets and the presence of the coloured dinitrophenyl derivatives was determined by thin layer chromatography with Silica Gel G (Research Specialties Co., Richmond, Calif.) at a thickness of 150 μ as the support and developed with *n*-butanol + methyl ethyl ketone + water (2 + 2 + 1, by vol.). The plates were photographed through a 47 B filter on Kodalith film (Eastman Kodak Co., Rochester, N.Y.).

Total amino acids and amino sugars were determined on a Technicon Analyzer (Technicon Corp., Chauncey, New York) according to the instructions of the manufacturer.

Preparation of enzyme and determination of its activity. Lysostaphin prepared and purified by Mead Johnson Research Center, Evansville, Indiana, was dialysed for 24 hr against 1000 times its volume of tris buffer, pH 7·5, with frequent changes of the dialysing fluid. Samples of this material were assayed at 37° against a washed suspension of *Staphylococcus aureus* (SV) with NaCl added to give a 0·15 M concentration. Turbidimetric readings were recorded initially and at 5 min. intervals utilizing a Coleman Jr. Spectrophotometer operated at a wavelength of 540 μ. An equivalent sample of lysostaphin was added to a similar suspension of *S. aureus* with the exception that no NaCl was present. Turbidimetric readings were made as above; after 15 min. incubation the suspension was centrifuged in a Spinco Model L Preparative Centrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.)

at 33,000*g* for 15 min. at 4° and the sediment washed 3 times with tris buffer. The combined supernatant fluids from the washings were assayed for lytic activity against a suspension of *S. aureus* similar to that used in the initial enzyme assay. The deposit after centrifugation was resuspended in tris + saline buffer (pH 7·5) and turbidimetric readings made as previously described.

Cell walls suspended in de-ionized water were divided into two samples—one containing NaCl to a final concentration of 0·15 M and the other devoid of NaCl. Equal quantities of lysostaphin were added to each sample and turbidimetric readings recorded during the incubation period. The cell wall + lysostaphin mixture with no NaCl present was treated in the same manner after the 15 min. incubation as the viable cell + lysostaphin mixture.

RESULTS

Figure 1 indicated that in the absence of NaCl, minimum lysis of the staphylococci by lysostaphin occurred and the supernatant fluid of the suspension after centrifugation did not contain any lytic activity. A quantitative determination of lysostaphin as measured by lytic activity was obtained from the sediment after centrifugation and addition of NaCl, which was almost equivalent to the amount of enzyme initially added in the test.

Table 1. *Chemical analysis of 3684 μg. cell wall*

Wall components	μM	Ratio to glutamic acid	Calculated wt (μg) after correction for water of hydration
Aspartic acid	0·8	0·3	92·8
Threonine	0·2	0·07	20·4
Serine	2·0	0·7	176·2
Glutamic acid	2·8	—	364·3
Glycine	8·6	3	499·7
Alanine	5·6	2	451·4*
Lysine	2·6	1	335·9
Glucosamine	2·86	1	586·3†
Muramic acid	2·70	1	796·5†
			Total 3323·5

* One-half quantity is considered to be *c*-terminal amino acid and was not corrected for water of hydration.

† Adjusted on the basis of being *n*-acetyl derivative.

The method of purification of the cell walls resulted in a preparation that contained no detectable phosphorus or ribitol. *N*-terminal alanine, which one would expect to obtain from the labile *O*-alanyl ester linkage in teichoic acid (Armstrong, *et al.* 1958) was not present as dinitrophenylalanine. The amino acid analysis of the cell wall preparation (Table 1) showed the normal complement of amino acids and amino sugars reported in the staphylococci (Park, 1961) plus a small quantity of threonine and aspartic acid. The sum of the calculated weights of the constituents of the cell wall based upon the μM of material recovered after hydrolysis compared favourably with the initial dry weight of the cell walls used for the analysis.

The turbidimetric changes that took place when a suspension of cell walls was

mixed with lysostaphin is indicated in Fig. 2. In the absence of NaCl, a slight decrease in turbidity is exhibited during incubation. The saline suspension of the washed cell wall deposit obtained after centrifugation again exhibited a decrease in turbidity during incubation. The supernatant fluids obtained from the above centrifugation also contained active material as ascertained by its lytic action on washed *S. aureus* (sv). A total recovery of active material from the combined washings of the cell wall+lysostaphin suspension obtained before and after the addition of NaCl was not achieved.

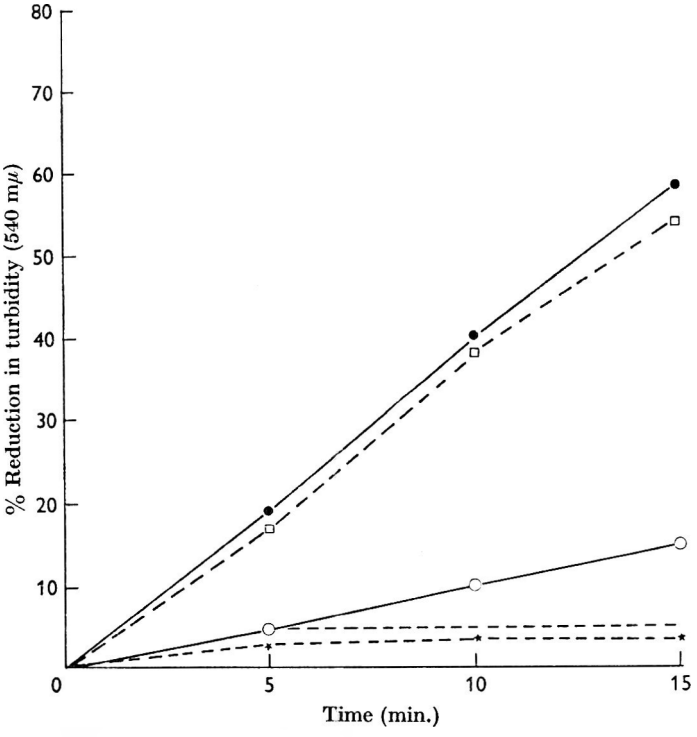

Fig. 1. Lysis of viable staphylococci by lysostaphin. ●———● cocci with lysostaphin + NaCl; ○———○ cocci with lysostaphin without NaCl present; - - - - cocci control with no NaCl present; ★———★ supernatant from cocci + lysostaphin mixture (without NaCl) added to viable cocci with NaCl present; □———□ deposit after centrifugation of cocci + lysostaphin mixture (without NaCl) resuspended with NaCl.

Electron micrographs (Pl. 1, figs. 1, 2) showed that the purified cell-wall preparation was attacked by the enzymic action of lysostaphin in that there was a conversion of the homogeneous cell-wall material to an irregular partially fragmented structure.

DISCUSSION

Since lysostaphin, by itself, does not sediment at 254,000*g* in a 4 hr period (Schindler & Schuhardt, 1965), the quantitative recovery of active lytic material from the washed deposit of a lysostaphin-viable *Staphylococcus aureus* mixture obtained after relatively low speed centrifugation indicates a complex being formed

between the enzyme and a component of the bacterial cell and that the addition of NaCl dissociated the complex. Weibull, Zacharias & Beckman (1959) found that under certain conditions lysozyme formed insoluble complexes with substances in the bacterial cell wall and the phenomenon which prevented lysis of the bacteria was interpreted in terms of a stable enzyme + substrate complex. Litwack (1960) in characterizing lysozyme and its substrate reported that complexes were formed with the teichoic acid of the cell wall as well as with the ribonucleic acids released from the lysed cells. He speculated that the stimulation of enzyme activity attributed

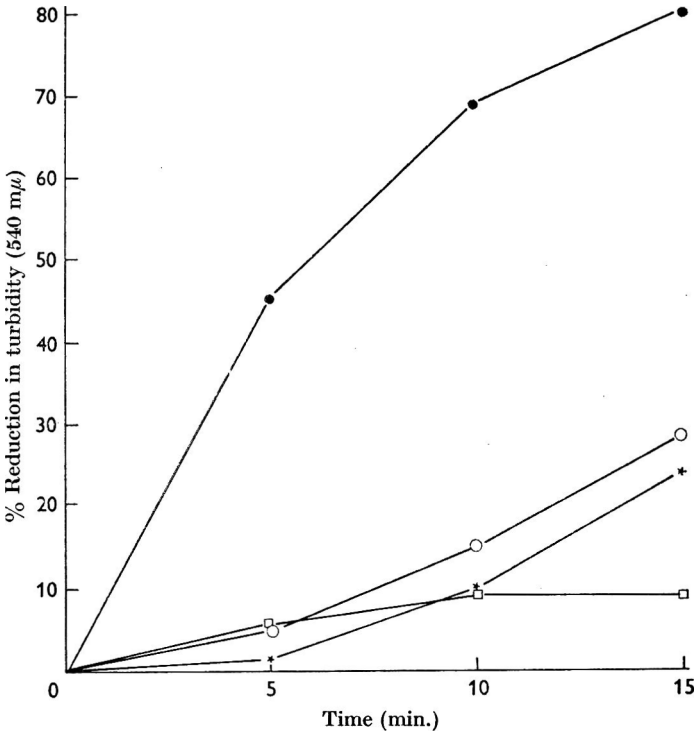

Fig. 2. Lysis of staphylococcal cell-wall mucopeptide. ●———● viable cocci with lysostaphin + NaCl; □———□ mucopeptide with lysostaphin without NaCl; ○———○ supernatant of mucopeptide + lysostaphin mixture (without NaCl) with viable cocci and NaCl; ★———★ deposit after centrifugation of mucopeptide + lysostaphin mixture (without NaCl) resuspended with saline. Mucopeptide control showed no change in turbidity.

to NaCl during lysis of whole cocci could be due in large part to the liberation of nucleic acid-bound enzyme by the increased ionic strength of the medium.

The absence of detectable teichoic acid and the quantitative recovery of amino sugars and amino acids from the purified cell-wall preparation indicated that the material used in these experiments was essentially the mucopeptide moiety described by Park (1961) and by Rogers & Perkins (1962) and any complexes formed by the enzyme would be with this substrate and not with other anionic derivatives in the bacterial cell.

Admittedly, the molar ratio of alanine to glutamic acid in our preparation was

less than that reported present in the uridine nucleotide that accumulated with penicillin-treated *Staphylococcus aureus* (Park & Strominger, 1957). It is not known whether this discrepancy was due to strain differences or loss of components in the purification procedure. The presence of lytic material in both the supernatant fluid and the sediment of the mucopeptide + lysostaphin mixture appears to be at variance with the results obtained when whole cocci are combined with the enzyme in the absence of NaCl. This anomaly suggests that the action of the lytic protein on the cell-wall mucopeptide results in the release of complexes of varying molecular weight some of which are small enough to remain in suspension after centrifugation. With viable cocci, on the other hand, adhering cytoplasmic material and proteins confer sufficient weight to the complexes released into the medium to result in their being deposited on centrifugation.

Since the lytic protein is cationic under the experimental conditions used it is conceivable that the complexes formed are with anionic derivatives of the cell wall and the dissociation of the bonds by NaCl is similar to that reported by Mora, Young & Shear (1959) on the complexing of cationic macromolecules.

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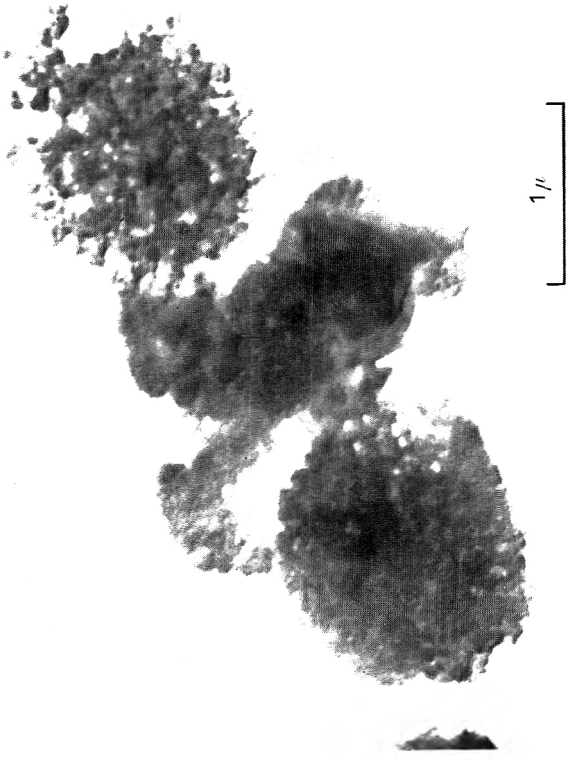

Fig. 2

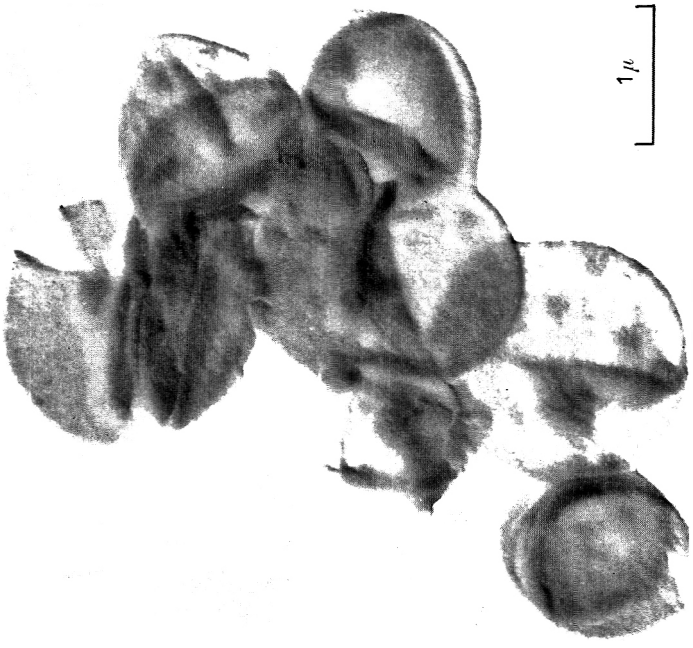

Fig. 1

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EXPLANATION OF PLATE

Cell wall mucopeptide before (fig. 1) and after (fig. 2) addition of lysostaphin. Shadow cast with platinum + palladium (80 + 20).

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With 1 plate
Printed in Great Britain

Bacteriophage Inhibition in Staphylococci

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SUMMARY

The inhibitory effect of high-titre preparations of phage 47 on certain strains of *Staphylococcus aureus* belonging to phage group III was produced with a phage:cell ratio of approximately 1:1. Staphylococci showing inhibition by preparations of phage 47 were converted to phage sensitivity by lysogenization with a phage derived from a strain sensitive to phage 47. Multiplication of phage 47 in lysogenized clones was compared with that in the original inhibited strains. This showed that inhibition was due to a phage/cell interaction in which most of the infected cocci were killed; a minority produced a few phage particles in smaller numbers with a longer latent period.

INTRODUCTION

In 1952 Williams & Rippon described the 'inhibitory' effect which may follow the application of undiluted high-titre phage preparations to certain strains of *Staphylococcus aureus*. The appearance produced on nutrient agar plates is a thinning of bacterial growth in the area on which the phage has been deposited, rather than the complete clearing seen with a phage-sensitive culture. When such a phage preparation is titrated out this 'inhibition' disappears, but no single plaques can be observed in the higher dilutions. Williams & Rippon stated that phage preparations from which the phage had been removed by absorption to heat-killed cocci, by filtration or by neutralization with antisera, no longer gave the effect. They concluded that the inhibitory agent could act only in the presence of phage. That the occurrence of inhibition might be influenced by the lysogenic state of the staphylococcus was indicated by the observations of Rountree (1959) and Rountree & Asheshov (1961) on staphylococci of phage type 80/81. These strains showed inhibition with phages 52 and 52A but could be converted to full sensitivity to these phages as a result of prophage substitution. The present paper reports observations on staphylococci of phage group III which show inhibition with phage 47. The observations indicate that the appearance of inhibition on nutrient agar is due to a phage/cell interaction, in which the majority of infected cocci are killed and only a minority produce new phage particles.

METHODS

Staphylococci. The strains of *Staphylococcus aureus* used were isolated from patients in this hospital; their phage reactions are shown in Table 1. Strain 009 was lysed by typing phage 47 at 1000 times its routine test dilution (RTD), but strains 2, 676 and 8503 showed inhibition. Clones, lysogenized with a serological type B phage (phage 009') from strain 009, were prepared. Strains 2, 676 and 8503 were lysogenic and carried other serological type B phages.

Phage stocks. Phage 47 stocks were propagated on staphylococcus propagating strain (PS) 47 in broth (Blair & Williams, 1961). Phage 009′ was prepared by ultraviolet induction of strain 009. Cocci grown in veal broth were centrifuged, washed and resuspended in Monod's buffer (1951) and then irradiated under a Westinghouse Sterilamp at a distance of 36 cm. for 30 sec.; they were then transferred to fresh broth, incubated at 37° for 1 hr and placed at room temperature until clearing occurred. Phages were filtered through Gradocol membranes of 800 $\mu\mu$ APD and tested for sterility before use.

Table 1. *Phage reactions of strains of Staphylococcus aureus used in the inhibition studies*

	Reaction with phages				
Staphylococcus	47	53 at RTD × 1000*	54	75	009′ at RTD
009	+	+	( )	+	−
2	(+)	+	( )	( )	+
676	( )	+	( )	( )	+
8503	( )	+	( )	( )	+
2 (009′)	+	+	( )	+	−
676 (009′)	+	+	( )	+	−
8503 (009′)	( )	+	( )	( )	−

* RTD = Routine Test Dilution.
+ = confluent lysis; ( ) = inhibition; (+) = inhibition with scattered plaques; − = no reaction.

Lysogenization. Staphylococcal strains were lysogenized by spotting undiluted phage onto a lawn of cocci. After incubation, a small quantity of the secondary bacterial growth in the area of lysis was streaked on blood agar plates. Single colonies were picked into broth and tested for the presence of the lysogenizing phage, for resistance to it and for alteration in phage-typing pattern. The lysogenized strains are numbered in the usual way, e.g. 2(009′) indicated strain 2 lysogenized with phage from strain 009.

Titration of inhibition. To determine the highest dilution of a phage preparation capable of giving inhibition on nutrient agar, tenfold dilutions were made in broth and quantities of 0·01 ml. were spotted onto the surface of phage agar plates previously flooded with a 4 hr broth culture of a strain known to give inhibition. The plates were incubated overnight at 30°. Care was required in the preparation of the agar plates and their flooding with the bacterial culture since variations in the depth of agar and the thickness of the bacterial lawn influenced the ease with which the end point of inhibition could be read. When the approximate titre of inhibition had been obtained, more closely spaced dilutions were tested similarly.

RESULTS

Relationship of phage titre to inhibition titre

Three stocks of phage 47 were examined to see what correspondence there was between the number of infective particles determined by plating on staphylococcus PS 47 and the highest dilution of the preparation that would produce inhibition on strain 8503. After titration on strains PS 47 and 8503 the stocks were centrifuged

in a Spinco Model L centrifuge at 20,000 rev/min. for 60 min. The supernatant fluids were removed and the pellets resuspended in one tenth the original volume of broth and titrated. If it is the phage particles or some substance associated with them that produces inhibition there should be correspondence between the titres. The results (Table 2) indicate fair correspondence, tenfold concentration of the phage producing a tenfold increase in the inhibition titre. The highest dilutions of phage giving inhibition contained from $2{\cdot}5 \times 10^5$ to $1{\cdot}8 \times 10^6$ phage particles/0·01 ml.

Table 2. *Relationship of titre of phage 47 on staphylococcal strain PS 47 to inhibition titre on strain 8503*

Phage preparation	Titre of phage on strain PS 47 (particles/ml.)	Inhibition titre/ml. on strain 8503	Minimum number of phage particles/0·01 ml. which produce inhibition
Stock 1 (*a*) Before centrifugation	$8{\cdot}6 \times 10^9$	1/100	$8{\cdot}6 \times 10^5$
(*b*) Precipitate after centrifugation	$8{\cdot}6 \times 10^{10}$	1/1600	$5{\cdot}5 \times 10^5$
(*c*) Supernatant fluid	$1{\cdot}1 \times 10^9$	1/20	$5{\cdot}5 \times 10^5$
Stock 2 (*a*) Before centrifugation	$6{\cdot}9 \times 10^9$	1/80	$8{\cdot}6 \times 10^5$
(*b*) Precipitate after centrifugation	$8{\cdot}0 \times 10^{10}$	1/1000	$8{\cdot}0 \times 10^5$
(*c*) Supernatant fluid	$1{\cdot}8 \times 10^9$	1/10	$1{\cdot}8 \times 10^6$
Stock 3 (*a*) Before centrifugation	$6{\cdot}5 \times 10^9$	1/160	$4{\cdot}0 \times 10^5$
(*b*) Precipitate after centrifugation	$2{\cdot}5 \times 10^{10}$	1/1000	$2{\cdot}5 \times 10^5$
(*c*) Supernatant fluid	$1{\cdot}6 \times 10^9$	1/20	$8{\cdot}0 \times 10^5$

Estimates were made of the numbers of cocci present on the agar plate at the time when the inhibitory phage preparation was deposited. An inoculum of 0·45 ml. of a 5 hr broth culture of strain 8503 was flooded onto an agar plate and a measured surplus of 0·1 ml. removed, leaving an inoculum of 0·35 ml. A colony count of the broth culture was made immediately before flooding. 0·01 ml. volumes of phage were deposited on the inoculated and dried plates. After incubation the diameters of 40 zones of inhibition were measured. From these, the mean area of the zones was calculated: it was one-thirtyseventh of the area of the plate. The approximate number of cocci within the area of the phage drop was calculated from these zone measurements and from the cell count. With an inoculum of $2{\cdot}6 \times 10^6$ cocci/zone, a phage:cell ratio of approximately 1:1 was sufficient to produce inhibition. There was thus no evidence that inhibition was due to lysis-from-without as a result of multiple infection.

Change from inhibition to phage sensitivity

Staphylococcus strain 009 which was sensitive to lysis by phages 47 and 75 carried a prophage. When strains 2 and 676 were lysogenized by phage 009′ they too became fully sensitive to phages 47 and 75, suggesting that the prophage state of the cocci might determine whether they showed lysis or inhibition (see Table 1). Plate 1 shows the appearances of the titrations of phages 47 and 75 on strain 676 before and after its conversion to phage sensitivity. On the other hand, strain 8503, when lysogenized with phage 009′ remained inhibited by phages 47 and 75.

The lysogenized clones of strains 2 and 676 still retained their original prophages, indicating that the mechanism of this conversion was not an obvious prophage substitution. Further evidence of the influence of phage 009′ on the phenomenon of inhibition was obtained by curing strain 2(009′) of prophage 009′. After ultraviolet irradiation of strain 2(009′) on citrate agar and replication of the surviving colonies on plates flooded with strain 2, one non-lysogenic colony was found among 900 lysogenics. On retesting with phage 47 this clone was inhibited.

It was concluded that at least in some cocci prophage 009′ acted as a genetic factor determining the phenotypic response to infection with phage 47. When this prophage was present in the cocci, infection of them with phage 47 resulted in lysis; in its absence they were inhibited by phage 47.

Phage replication in sensitive and in inhibited strains

Advantage was taken of the conversion of the strains 2 and 676 to phage sensitivity to compare the multiplication of phage 47 in these strains with that in the original inhibited strains and in strain 8503. Where appropriate, multiplication of phage 47 in strain PS 47 was used as a control.

Table 3. *Percentage adsorptions and adsorption rates of phage 47 on various strains of Staphylococcus*

Staphylococcus strain	Action of phage 47 at RTD × 1000	Phage adsorbed at (% adsorbed) 2 min.	5 min.	10 min.	Adsorption rate at ($K \times 10^{-9}$ ml./min.) 2 min.	5 min.	10 min.
8503	Inhibition	57·0	76·0	90·0	3·7	2·6	2·1
2	Inhibition	31·0	49·0	75·0	1·9	1·5	1·5
676	Inhibition	66·0	80·0	93·0	4·3	2·5	2·1
2(009′)	Lysis	46·0	59·0	77·0	3·3	1·9	1·6
676(009′)	Lysis	68·0	84·0	93·0	4·4	2·8	2·0
PS 47	Lysis	39·0	60·0	71·0	2·0	1·5	1·0

Adsorption rates. Rates of adsorption of phage 47 to its own propagating strain, to strains showing inhibition and to the lysogenized clones were determined. A broth culture was grown at 37° with aeration until the concentration was 1×10^8 cocci/ml. One ml. of the culture was mixed with 1 ml. of phage 47 (titre 1×10^8 particles/ml.) and 0·01 ml. 1 % (w/v) $CaCl_2$, both previously warmed to 37°, and the mixture held at 37°. At 2, 5 and 10 min. after mixing, 0·1 ml. samples were taken, diluted immediately to 1/100 in broth and cooled in an ice water bath. After centrifugation the numbers of phage particles in the supernatant fluids were assayed and the adsorption rate K was calculated. There were minor differences in the behaviour of the strains (Table 3), strains 676, 676(009′) and 8503 adsorbing the phage at a faster rate than did PS 47 and strains 2 and 2(009′). Lysogenization with phage 009′ had no effect on the adsorption rates in strains 2 and 676. It was concluded that inhibition did not involve processes concerned with the initial steps of phage infection and was not due, for example, to a deficiency of appropriate phage receptors on the surface of the cocci.

Recovery of adsorbed phage from inhibited cocci. The proportions of adsorbed phage

recoverable from the cocci as infective centres during the latent period were determined. Using the samples taken at 10 min. in the previous experiment, the centrifuge-deposited cocci were resuspended in 0·5 ml. broth and the number of infective centres assayed by plating 0·01 ml. volumes on strain PS 47. A smaller percentage of the adsorbed phage was recovered from the strains showing inhibition than from the phage-sensitive strains (Table 4). In strains 8503 and 676, only 16 and 14 % respectively of the adsorbed phage registered as infective centres as compared with 37 % in 676(009′) and 63 % in strain PS 47. Strain 2 differed from the other inhibited strains in that 30 % of the adsorbed phage was recoverable and that lysogenization with phage 009′ only increased this to 36 %, an insignificant difference. It should be noted that this strain 2 occasionally showed evidence of partial sensitivity to phage 47 in that very minute plaques were sometimes seen in higher phage dilutions on nutrient agar.

Table 4. *The recovery of adsorbed phage from staphylococci infected with phage 47*

Staphylococcus	Action of phage 47 at RTD × 1000	Phage added ($\times 10^6$/ml.) (a)	Phage in supernatant fluid ($\times 10^6$/ml.) (b)	Phage adsorbed ($\times 10^6$/ml.) ($a-b$)	Phage recovered as infective centres ($\times 10^6$/ml.)	Adsorbed phage recovered (%)
8503	Inhibition	130	12	118	19	16
2	Inhibition	100	25	75	23	30
676	Inhibition	150	10	140	20	14
2(009′)	Lysis	130	30	100	36	36
676(009′)	Lysis	140	9	131	48	37
PS 47	Lysis	100	29	71	45	63

Death of infected cocci within the inhibition zone on nutrient agar

To determine how many inhibited cocci were killed when infection took place on nutrient agar, the numbers of viable cocci within the zone of inhibition were assayed at 30 min. intervals up to 4 hr after the addition of the phage. The number of viable cocci from an uninfected area of the plate was also determined at the same time. An agar plate was inoculated with 0·35 ml. of broth culture containing $2{\cdot}5 \times 10^8$ cocci/ml. and dried. Drops (0·01 ml.) of undiluted phage 47 were deposited on the plate and allowed to dry. By using a sterile test-tube a circle of agar 8 mm. in diam. was punched out of the inoculated plate from within the area of the phage drop. A similar sample was taken from outside the area of phage infection. Each sample was placed in 1 ml. 0·85 % (w/v) NaCl, shaken 30 times, diluted, and 0·01 ml. volumes plated in quadruplicate on agar plates, the inocula being spread with a glass rod. After overnight incubation at 37°, 95 % of the calculated inoculum of an uninfected sample could be recovered by this method. The results (Figs. 1–4) showed normal growth curves for all uninoculated cultures. The colony count decreased continuously in strains 2(009′), 676(009′) and PS 57 after infection with phage 47. The inhibited strains showed a temporary decrease in the colony count within 60–90 min of infection, after which the numbers of cocci increased at rates similar to the growth rate of the uninfected cocci. The 'secondary growth' of inhibited strains was not due to selection of pre-existing phage-resistant mutants or to the presence of lysogenized cocci, since surviving cocci cultivated from the zone

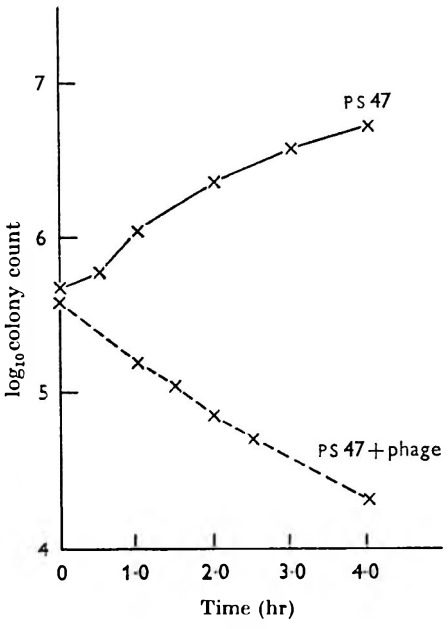
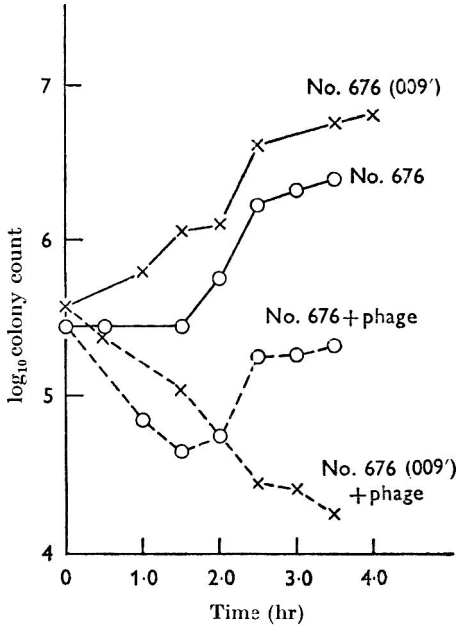


Fig. 1



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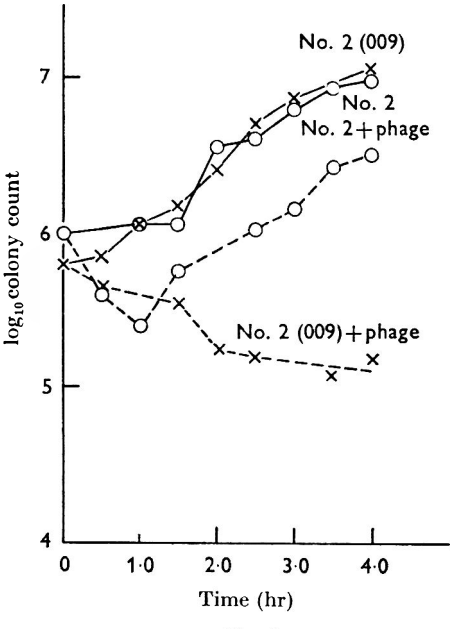

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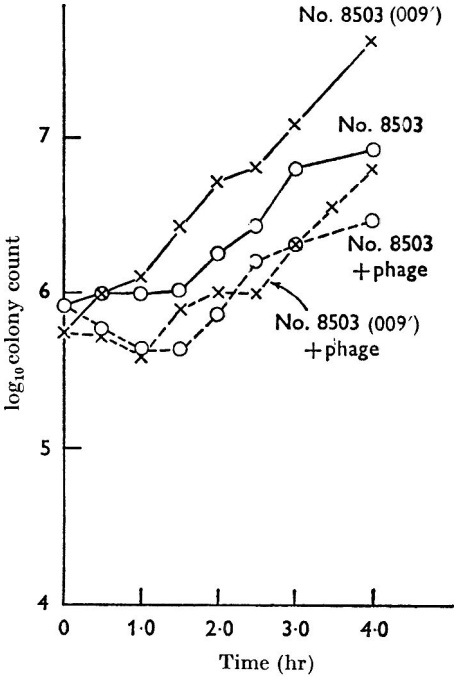


Fig. 4

Figs. 1–4. Colony counts of staphylococcal strains following infection with phage 47 on nutrient agar. ○ — — — ○, × — — — ×, cocci infected with phage 47; ○———○, ×———×, uninfected cocci.

Fig. 1. Staphylococcus strain PS47.

Fig. 2. Strains 676 and 676(009′).

Fig. 3. Strains 2 and 2(009′).

Fig. 4. Strains 8503 and 8503(009′).

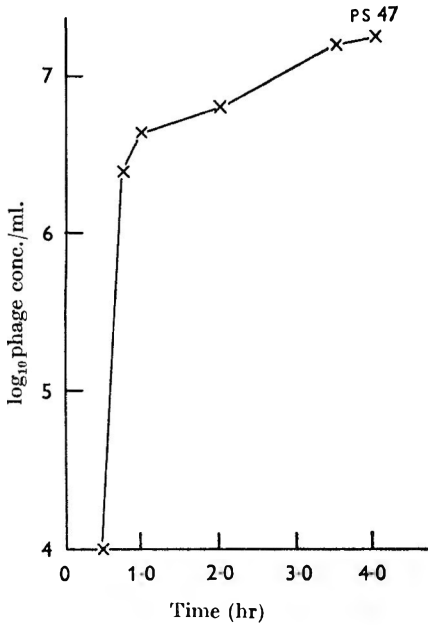


Fig. 5

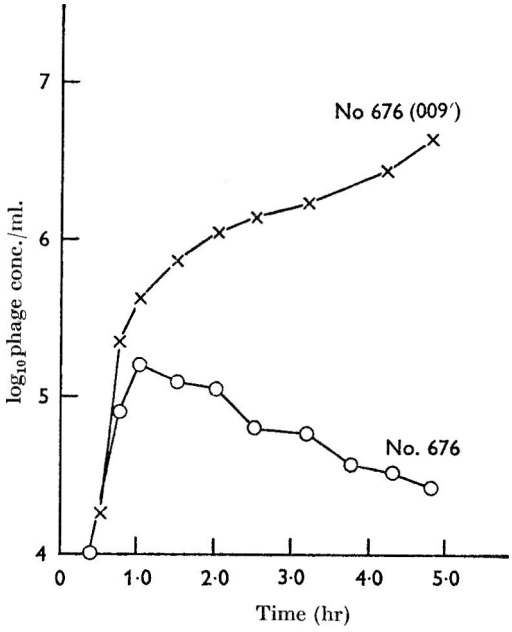


Fig. 6

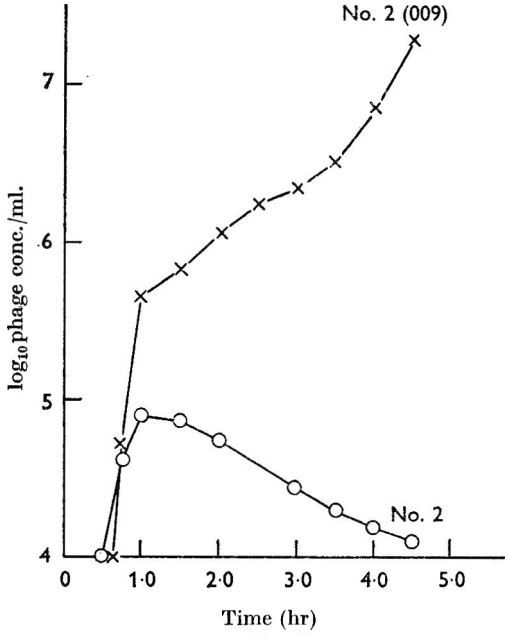


Fig. 7

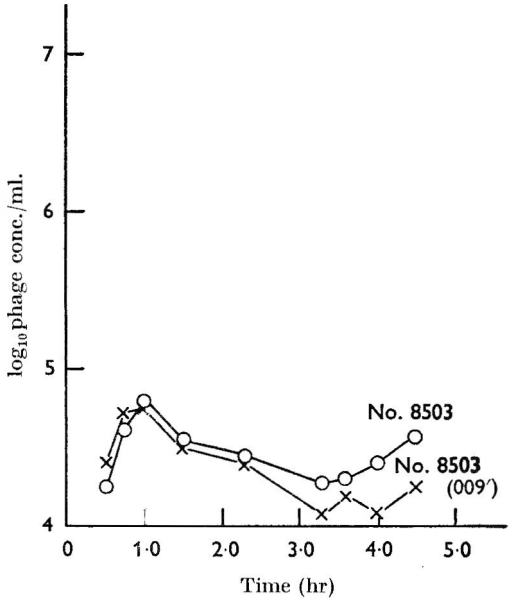


Fig. 8

Figs. 5–8. Production of phage 47 by strains of staphylococci grown in broth.

Fig. 5. Strain PS47.

Fig. 6. Strains 676 and 676(009′).

Fig. 7. Strains 2 and 2(009′).

Fig. 8. Strains 8503 and 8503(009′).

of inhibition still showed inhibition when retested with phage 47. It was concluded that death of cocci within 60–90 min. of infection was in part responsible for the appearance of inhibition on agar plates. In the fully sensitive cultures PS47, 2(009′) and 676(009′) the death of cocci continued throughout the period of observation, ultimately producing clear zones of lysis.

Phage production by inhibited strains

The production of phage 47 by sensitive, inhibited and converted staphylococcal strains was examined in broth. A broth culture was grown at 37° with aeration to a concentration of $2{\cdot}0 \times 10^8$ cocci/ml., 1 ml. of phage 47 was added to 1 ml. of culture to give a ratio of 3 to 4 phage particles/coccus. After 20 min. adsorption at 37° the mixture was cooled, centrifuged and the cocci resuspended in 2 ml. broth. Unadsorbed phage was neutralized by mixing the cocci suspension with antiserum and

Table 5. *The individual burst sizes of phage 47 in cells of various staphylococci*

Staphylococcus	Number of samples	Number of plates with no plaques	Number of plates	Total plaques	Plates with plaques: Plaques/plate
2	200	162	38	164	1 1 1 2 2 3 4 5 6 7 9 22 32 1 1 1 2 3 4 5 29 1 1 1 2 3 1 1 1 2 1 1 1 2 1 1 1 2
2(009′)	120	72	48	1412	1 1 2 4 11 21 40 66 75 80 92 112 1 1 2 5 12 21 44 68 76 84 1 1 3 7 12 21 47 69 1 1 3 12 21 48 1 1 15 22 48 18 24 52 27 53 27 58
676	120	103	17	106	1 1 3 4 7 9 12 32 1 1 3 13 1 1 15 1 1
676(009′)	110	83	27	683	1 3 5 10 20 34 45 57 77 81 93 2 3 6 12 20 39 46 2 3 13 29 2 16 29 17 18

holding at 37° for 10 min. A final dilution of 1/100 was made in broth, kept at 37° and 0·5 ml. samples taken at 30 min. intervals. After cooling and centrifugation, the numbers of phage particles in the supernatant fluid (that is, the numbers of new particles released) were counted.

Staphylococcal strain PS47 and the converted strains 2(009′) and 676(009′) gave high yields of phage (Figs. 5–8), there being increases of 3 log value after 4–5 hr. The inhibited strains, 2, 676 and 8503 showed an increase in the phage counts of one log or less between 40 and 60 min. Thereafter, there was a gradual decrease in

the counts which might be explained by the neutralizing action of the low concentration of antiserum still present in the diluted growth tubes. Except in the 8503 strains there was no evidence of further phage multiplication after the first burst.

The yield of phage 47 by individual cocci was determined in the inhibited strains 2 and 676 and in the converted strains 2(009′) and 676(009′). After infection and neutralization by antisera, the infected cocci were diluted in broth to give less than one coccus/0·01 ml. (at this dilution no effective antiserum should remain); 0·01 ml. samples were then distributed into separate tubes, held at 37° for 50 min. and the whole of each sample plated on strain PS 47. Table 5 shows the wide range of plaque counts observed. Those tubes giving one plaque were considered to have each contained an infected coccus whose burst was delayed until after the contents had been plated on strain PS 47. The average burst size was calculated from these data; for strains 2 and 676 plates showing one plaque were excluded; for strains 2(009′) and 676(009′) counts of three or less were excluded. The average burst sizes were greater in the converted strains than in the original strains; in strain 2(009′) it was 33·9 compared with 8·3 in strain 2, and in 676(009′) 30·3 compared with 12·2 in strain 676.

DISCUSSION

The behaviour of the inhibited strains of Staphylococcus examined in the present work provided evidence that the phenomenon of inhibition seen in phage typing is essentially a phage/cell interaction. A comparison of the behaviour of inhibited strains and their sensitive derivatives during the various steps of the phage cycle enabled some conclusions to be drawn as to the step or steps in the cycle at which inhibition operates. A ratio of approximately one infective particle to each coccus was sufficient to produce inhibition on nutrient agar, thus disposing of the hypothesis that lysis-from-without might be responsible for the appearance of inhibited cultures. Both inhibited and sensitive cocci adsorbed phage 47 at comparable rates, and about the same proportions of cocci were killed in the first round of infection. Thereafter, divergence in behaviour was observed. On nutrient agar plates showing inhibition, growth of the surviving cocci occurred, whereas on plates giving areas of lysis, the colony count of staphylococci decreased steadily, presumably as the result of the infection of cocci which had escaped the first round of infection. Phage growth curves in broth indicated that only a proportion of the infected cocci of the inhibited strains was able to produce infective particles, that the average yield from these productive cocci was lower than that from fully sensitive cocci and that lysis of some of these productive cocci was delayed. In other words, inhibition was not due to prophage immunity in which the multiplication of homologous infecting phage is completely blocked but to a partial incompetence of the cocci to produce infective phage. At what stage in the latent period this incompetence is expressed cannot be stated from the available data. That the infected cocci are no longer viable and that a proportion can produce complete phage although in decreased amount suggests a defect in maturation of the phage and/or in the production of an enzyme concerned in phage release. Study of infected cocci lysed by cyanide during the latter part of the latent period might provide information on these points.

When these facts are considered, an explanation of the appearance of inhibition on solid media is apparent. The number of cocci in initial contact with phage when

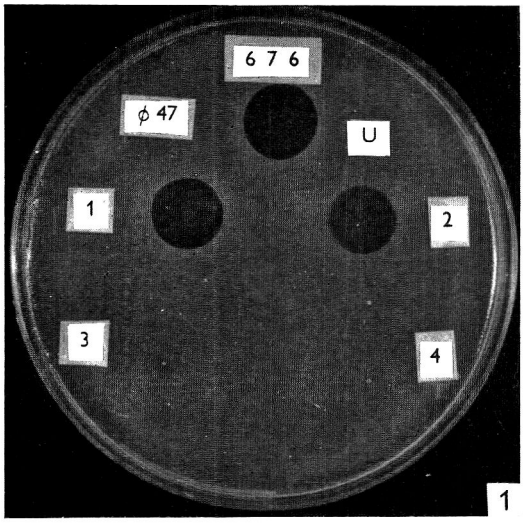
it is spotted on a nutrient agar plate is limited. When a phage preparation is used which contains sufficient infective particles theoretically to infect every coccus, 60 to 90 % of the cocci are killed but only 15 to 30 % produce fewer new particles, and some belatedly. The chances, then that these new particles may cause a second round of infection in cocci which are alive and growing, are small; they are more likely to encounter cocci that have been killed while many of the growing cocci that they do infect will be unable to make phage. In the cocci which survive initial infection growth and division occurs as in uninfected cocci. The final result is a bacterial zone of decreased density. When diluted phage is applied to such incompetent cocci in numbers such that there is one particle to every ten cocci or more, a plaque is unlikely to be formed and sufficient cocci will survive the initial infection to form a normal lawn of bacterial growth.

The three inhibited staphylococcal strains that were examined showed differences in behaviour which indicated that they were not identical strains. For example, in strain 2, a larger proportion of cocci was able to produce infective phage than did those of the other two strains; lysogenization with phage 009′ increased the average burst size but did not increase significantly the numbers of productive cocci. In strain 676, lysogenization with phage 009′ increased both the numbers of productive cocci and their average yield. On the other hand, lysogenization had no effect on strain 8503.

The effect of lysogenization with phage 009′ on strains 2 and 676 showed that in these staphylococci the ability to synthesize normal amounts of phage was linked with the presence of this prophage which had been derived from a phage-sensitive strain. This effect was not due to prophage substitution such as occurs in staphylococci of phage type 52/52A′/80/81 and in which the increased ability to produce phage is retained after the freshly inserted prophage is removed by ultraviolet irradiation. Curing of strain 2(009′) of its prophage 009′ infection caused it to revert to incompetence. This indicated that lysogenic conversion was the probable mechanism involved in this change. The genetic constitution of the cocci must also influence the consequences of such lysogenization since strain 8503, although apparently similar to strains 2 and 676 in many of its characters, showed no increased competence to produce phage 47 after lysogenization with phage 009′.

The phenomena described in the present paper differ from those described by West, Kelly & Shields (1963) who produced inhibitions of normally sensitive staphylococci by altering the salt concentrations in their media and thus altering the adsorption of the phages to their hosts.

This work was supported by a grant from the Australian National Health and Medical Research Council.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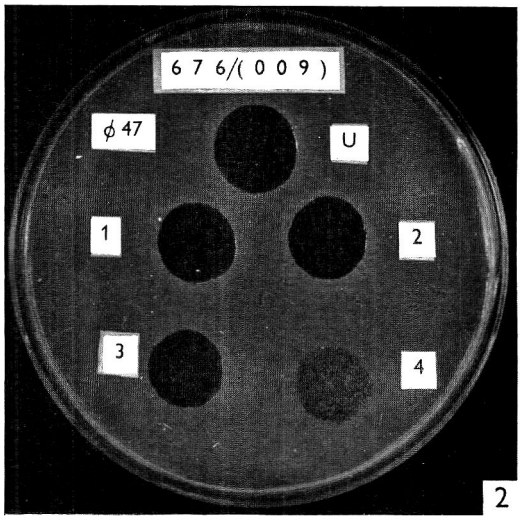
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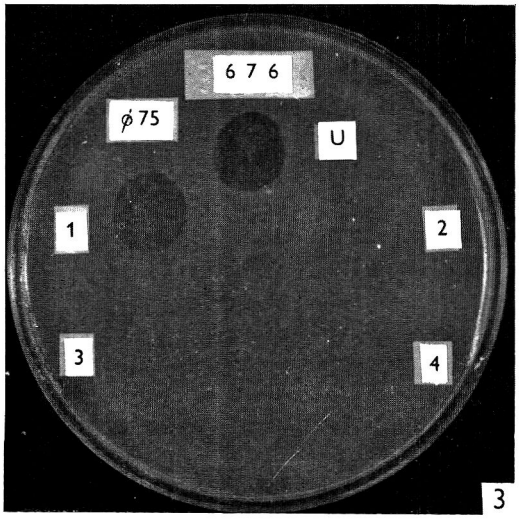
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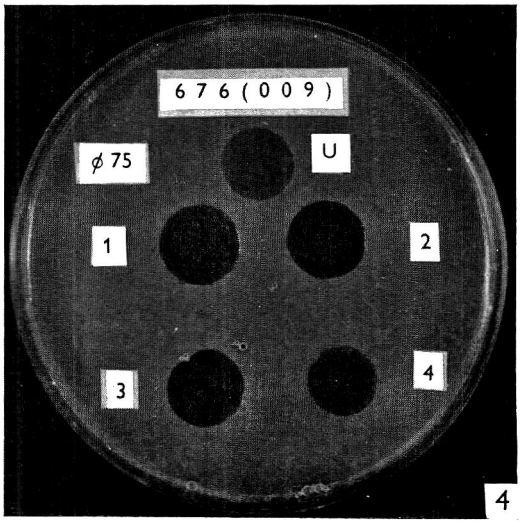
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EXPLANATION OF PLATE

Titration of phage 47 and phage 75 on Staphylococcus strains 676 and 676(009′). Serial tenfold dilutions of phage were deposited on inoculated agar plates; U = undiluted phage; 1 = 10^{-1} dilution; 2 = 10^{-2} dilution; 3 = 10^{-3} dilution; 4 = 10^{-4} dilution.

Figs. 1–2. Titration of phage 47.

Figs. 3–4. Titration of phage 75.

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The Effect of Bacterial Concentration on the Uptake of Labelled Arginine and Glucose by *Escherichia coli*

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SUMMARY

In a non-growing population of an arginine-requiring auxotroph of *Escherichia coli*, the uptake of [^{14}C]arginine and [^{14}C]glucose by the whole organisms, and the incorporation of [^{14}C]arginine in the acid-soluble fraction and total protein of the organisms, decreased with increasing bacterial concentration in a wide range (0·1–3·0 mg. dry wt. bacteria/ml.). It is concluded that the rate of transport/organism of arginine and glucose decreased with increasing bacterial concentration.

INTRODUCTION

In suspensions of several mammalian cell types (spermatozoa, reticulocytes, hepatic cells), the rate of incorporation of labelled amino acids into protein has been shown to decrease with increasing cell concentration (Bhargava, Bishop & Work, 1959; Bhargava & Bhargava, 1962; Bhargava, 1964; Bhargava & Bhargava, unpublished). With hepatic cell suspensions, this 'cell concentration effect' resulted from decreased permeability, due to enhanced cell contact with increasing cell concentration (Bhargava & Bhargava, 1962; Bhargava, 1964; Bhargava & Bhargava, unpublished). In the present paper we report experiments which show that in a resting population of an arginine-requiring auxotroph of *Escherichia coli*, *E. coli* 160–37, the rate of transport of [^{14}C]arginine and [^{14}C]glucose decreased as the bacterial concentration increased. This effect was more marked at lower than at higher bacterial concentrations. The results suggest that intercellular reactions may affect the rate of transport of nutrients in bacteria and thus indirectly influence intracellular metabolic activity.

METHODS

Organism. Escherichia coli 160–37, which requires either arginine or ornithine as a nutrient, was used. It was maintained on nutrient agar slopes. The organism was incubated statically at 35° in the following medium: NH_4Cl, 0·5 g.; $(NH_4)_2SO_4$, 0·5 g.; KH_2PO_4, 13·6 g.; $MgSO_4$, 20 mg.; $Fe(NH_4)_2(SO_4)_2.6H_2O$, 15·6 mg.; glucose, 20 g.; L-arginine hydrochloride, 0·1 g.; water to 1 l. The above medium without glucose and arginine is referred to subsequently as 'arginine and glucose-free (AGF)' medium.

Radioactive chemicals. Universally labelled [^{14}C]L-arginine and [^{14}C]glucose were obtained from the Radiochemical Centre, Amersham, Bucks, U.K.; they were diluted to the required specific activity with the unlabelled (reagent or analytical grade) analogue.

Incubation with the labelled compounds and the isolation of dry organisms. Bacteria from an exponentially growing culture (usually towards the end of the logarithmic growth phase) were harvested, washed twice in the cold with the AGF medium and then pre-incubated for 30 min. at 35°, without shaking, in the same medium. The bacteria were removed by centrifugation in the cold and resuspended in the AGF medium to give a slurry which was dispensed in a series of flasks (one for each bacterial concentration and each time point) which contained the appropriate amount of the AGF medium and the labelled compound to give the desired concentration of bacteria and compound in a total volume of 30–50 ml. The flasks were then incubated statically at 35°. At the end of the desired period, a set of flasks (one for each bacterial concentration) was removed and the contents centrifuged immediately in the cold. The deposited bacteria were washed in the cold, three times with the AGF medium containing a large excess of the unlabelled compound and once with distilled water. The washed bacteria were dried to constant wt. at 110°. There was no change in the extinction (E_{750}) or in the pH value of the bacterial suspension before and after incubation with labelled precursor compound.

Isolation of the acid-soluble fraction. Trichloroacetic acid (TCA, 10 %) was added in the cold to bacteria washed as above after incubation with the labelled precursor. The precipitate was removed by centrifugation and washed twice with 5 % TCA in the cold. All the TCA supernatant fluids were combined and the TCA removed by repeated extraction with ether. The aqueous fraction was evaporated to dryness. The residue obtained in this way was taken to contain all the free arginine and glucose (and their acid-soluble small molecular weight derivatives) of the bacteria.

Isolation of proteins. The washed TCA-precipitate of the bacteria, obtained as above, was treated with 5 % TCA at 90° for 30 min. to remove nucleic acids. TCA was removed by centrifugation and the sediment washed once with 5 % TCA. Lipids were removed from the residue in the usual way by treatment with organic solvents to give lipid-free and nucleic acid-free total protein of the bacteria. In some experiments the protein was isolated by treatment of the washed bacteria with 95 % ethanol in water at room temperature. Lipids were removed from the ethanol-insoluble precipitate in the usual way. Nucleic acids were not removed from the protein fraction isolated in this way.

Measurement of radioactivity. The acid-soluble fraction was plated as a solution in 40 % (v/v) ethanol in water, and the dry bacteria and protein as a slurry in 40 % (v/v) ethanol in water on 2 cm.^{2} aluminium planchets which were counted in a Phillips Geiger–Müller end-window counter with an efficiency of 6 % for ^{14}C. Appropriate correction was made for self-absorption where necessary. In experiments on the incorporation of [^{14}C]arginine and [^{14}C]glucose, a radioactivity of about 100 and 10 counts/min./mg. dry wt. bacteria, respectively, was obtained in the 0 hr samples. This radioactivity did not vary with concentration of bacteria and represented a small percentage of the incorporation obtained at the longer times. The 0 hr radioactivity has not been subtracted from the values reported for subsequent time points.

RESULTS

Uptake of [^{14}C]*arginine*

The total uptake of labelled arginine by whole bacteria proceeded linearly for about 30–60 min. after which it levelled off (Fig. 1). The period of linear uptake increased with decreasing concentration of bacteria. The rate of uptake also depended on the bacterial concentration; at any time point, progressively lesser amounts of [^{14}C]arginine were taken up per bacterium as the bacterial concentration increased (Fig. 2).

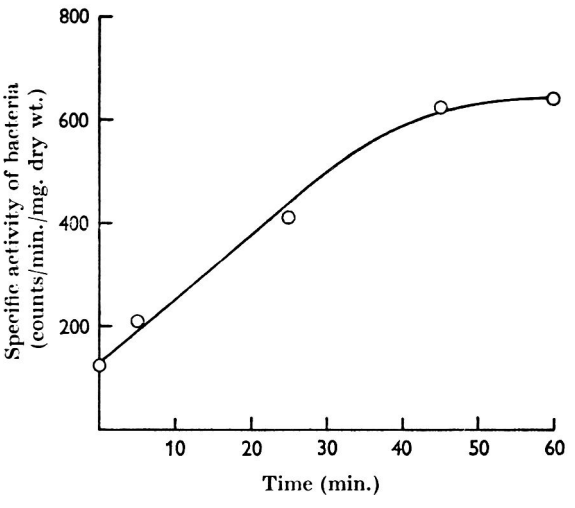

Fig. 1

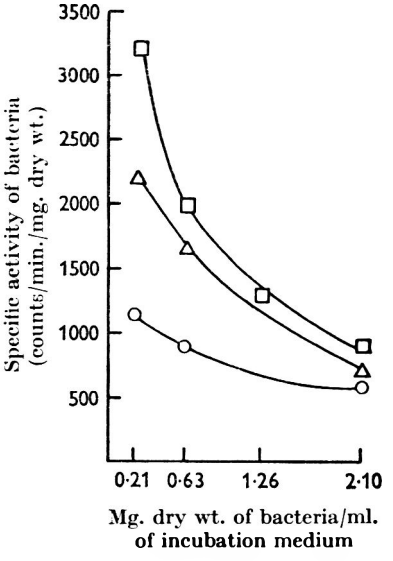

Fig. 2

Fig. 1. Incorporation of [^{14}C]arginine into organisms of a non-multiplying population of *Escherichia coli* 160–37. The concentration of bacteria was equivalent to 0·9 mg. dry wt. bacteria/ml. The bacteria were pre-incubated for 30 min. in an arginine-free and glucose-free salts solution before the addition of labelled arginine. The concentration of [^{14}C]arginine was 100 μg. (0·5 μC.)/ml.

Fig. 2. Changes, with the concentration of bacteria, in the quantity of [^{14}C]arginine taken up by the organisms of a non-multiplying population of *Escherichia coli* 160–37 on incubation with [^{14}C]arginine. The bacteria were pre-incubated for 30 min. in an arginine-free and glucose-free salt solution before addition of labelled arginine. The concentration of [^{14}C]arginine was 100 μg. (0·5 μC.)/ml. ○, 25 min; △, 40 min.; □, 60 min.

The radioactivity in the bacteria, following uptake of [^{14}C]arginine, would be expected to be primarily due to free arginine (or acid-soluble arginine derivatives) and arginine incorporated into protein due to protein turnover; the latter was shown (Mandelstam, 1958*b*) to occur to an extent of 5%/hr in resting organisms of the strain of *Escherichia coli* used in the present work. The radioactivity in the acid-soluble fraction, as well as in the protein fraction derived from unit dry wt. of bacteria, decreased with an increase in the concentration of bacteria at all the times studied (Figs. 3, 4). The dependence of the above uptake on bacterial concentration was more marked at longer times than at shorter times, as in the case of the total

uptake of [^{14}C]arginine by the whole bacteria. The incorporation of [^{14}C]arginine/unit wt. protein also decreased as the concentration of bacteria in the suspension increased (Fig. 5).

Uptake of glucose

The rate of uptake of [^{14}C]glucose by the bacteria also increased with decreasing concentration of bacteria (Fig. 6). As in the case of [^{14}C]arginine uptake, the period for which the uptake of [^{14}C]glucose proceeded linearly was longer at lower bacterial concentrations than at higher concentrations.

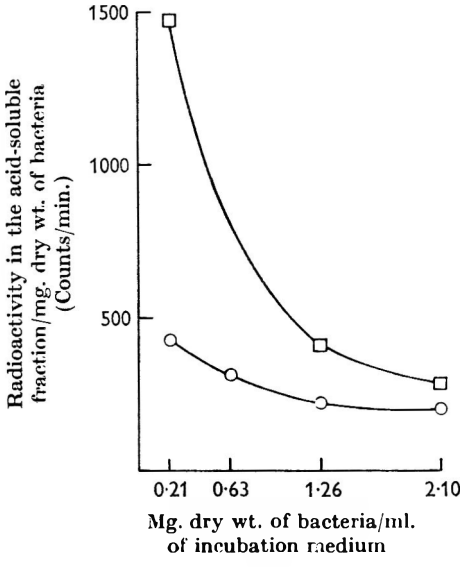

Fig. 3

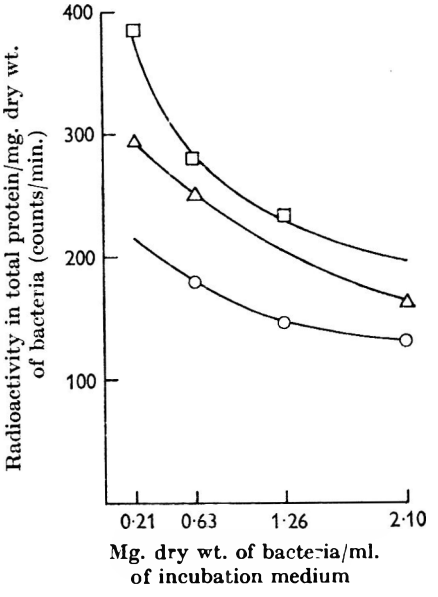

Fig. 4

Fig. 3. Changes, with the concentration of bacteria, in the quantity of [^{14}C]arginine accumulating in the acid-soluble fraction of a non-multiplying population of *Escherichia coli* 160–37 on incubation with [^{14}C]arginine. The bacteria were pre-incubated for 30 min. in an arginine-free and glucose-free salts solution before addition of labelled arginine. The concentration of [^{14}C]arginine was 100 μg. (0·5 μC.)/ml. ○, 25 min.; □, 60 min.

Fig. 4. Changes, with the concentration of bacteria, in the radioactivity incorporated in the total protein of a non-multiplying population of *Escherichia coli* 160–37 on incubation with [^{14}C]arginine. The bacteria were pre-incubated for 30 min. in an arginine-free and glucose-free salts solution before addition of labelled arginine. The concentration of arginine was 100 μg. (0·5 μC.)/ml. ○, 25 min.; △, 40 min.; □, 60 min.

DISCUSSION

In the present work, the bacteria were washed free from medium and then starved of glucose and arginine for 30 min. before either labelled arginine or labelled glucose was added. There was no increase in the extinction of the culture during incubation with the labelled precursor. Further, the uptake of glucose was nearly the same as reported for the same strain of *Escherichia coli* (*E. coli*, 160–37) in the maximum stationary phase of growth during which no formation of new organisms (as adjudged by synthesis of DNA) was observed (Kashmiri & Bhargava, 1965). It can, therefore, be concluded that there was no formation of new bacteria during the

period of incubation with the labelled precursor. The results clearly show that in such a non-multiplying population, the quantity of labelled arginine taken up in 20–80 min. by a unit population of the bacteria or their acid-soluble fraction or

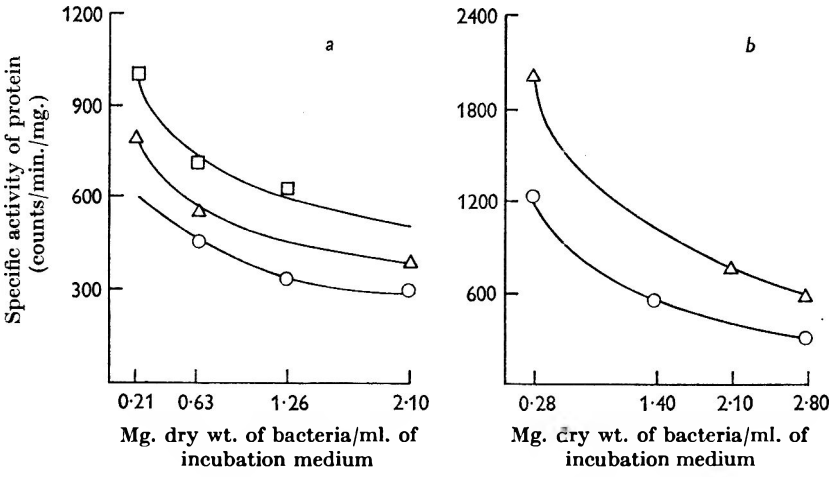

Fig. 5. Changes, with the concentration of bacteria, in the specific radioactivity of protein of a non-multiplying population of *Escherichia coli* 160–37 on incubation with [^{14}C]arginine. The bacteria were pre-incubated for 30 min. in an arginine-free and glucose-free salts solution before addition of labelled arginine. The concentration of [^{14}C]arginine was 100 μg. (0·5 μC.)/ml. The two figures *a* and *b* correspond to two different experiments. (*a*) ○, 25 min.; △, 40 min.; □, 60 min. In this experiment, the protein was isolated after treatment of the whole bacteria with TCA. (*b*) ○, 30 min.; △, 80 min. In this experiment, the protein was isolated following treatment of the whole bacteria with 95 % (v/v) ethanol in water.

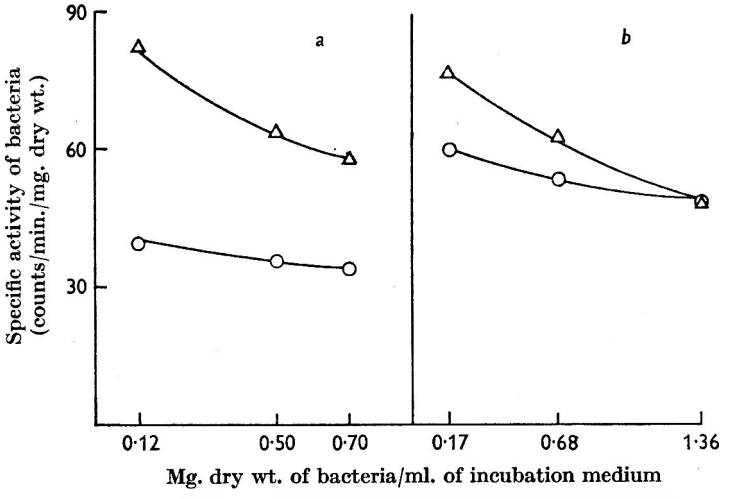

Fig. 6. Changes, with the concentration of bacteria, in the quantity of [^{14}C]glucose taken up by the organisms of a non-multiplying population of *Escherichia coli* 160–37 on incubation with [^{14}C]glucose. The bacteria were pre-incubated for 30 min. in an arginine-free and glucose-free salts solution before the addition of labelled glucose. The concentration of [^{14}C]glucose was 20 mg. (1 μC.)/ml. The two figures *a* and *b* correspond to two different experiments. (*a*) ○, 15 min.; △, 45 min.; (*b*) ○, 45 min.; △, 75 min.

protein, varied inversely with the concentration of bacteria. Such an inverse relationship might be due to a decrease in the specific radioactivity of extracellular arginine and/or a decrease in the rate of transport of arginine from the extracellular medium into the organisms, with increasing bacterial concentration.

The bacteria might secrete unlabelled arginine released as a result of intracellular protein turnover of 2–7 %/hr which has been shown to occur in resting populations of bacteria and yeasts deprived of an essential nutrient (Mandelstam, 1957, 1958*b*; Halvorson, 1958; Borek, Ponticorvo & Rittenberg, 1958; Urba, 1959; Mandelstam & Halvorson, 1960; Proctor, 1962). In such an event, increasing amounts of unlabelled arginine would be secreted with increasing bacterial concentration; this would cause the specific radioactivity of the arginine of the medium at any time point to decrease as the bacterial concentration increased. However, if it be assumed that the protein content of the bacteria is 75 %, that the protein contains 10 % arginine and that the protein turnover in the resting state is 5 %/hr (Mandelstam, 1958*b*), it can be calculated that the amount of unlabelled arginine released in 1 hr from the maximum concentration of bacteria used (equiv. 2·8 mg. dry wt. bacteria/ml.) could not exceed 10 μg./ml. culture medium. In fact, it has been shown that non-dividing *Escherichia coli* in which protein synthesis is blocked secrete amino acids to an extent of only 5 μg./mg. dry wt. bacteria/hr (Mandelstam, 1958*a*). The quantity of arginine found in the present work to be secreted by the bacteria could not therefore be sufficient to alter substantially the specific activity of the extracellular labelled arginine present initially in the medium (100 μg./ml.). This rules out the possibility that the observed effect of bacterial concentration might be due to secretion of unlabelled arginine by the bacteria and consequential decrease in the specific radioactivity of extracellular arginine.

It would, therefore, appear that there was a decrease in the rate of transport of arginine per bacterium, measured over a period of 20–60 min., as the concentration of bacteria increased. The reasons for this decrease are not clear. The obvious possibility that arginine became limiting at the higher bacterial concentrations is ruled out by the following considerations. The initial concentration of [^{14}C]arginine in the medium was 100 μg. ($1{\cdot}11 \times 10^6$ disintegrations or 67,000 counts/min.)/ml.; from this and the data of Fig. 2 it can be calculated that at the lowest bacterial concentration (equiv. 0·21 mg. dry wt. bacteria/ml.), just 1 μg. (1 %) of the labelled arginine was taken up by the bacteria/ml. medium in 60 min. This implies that only 10 % of the [^{14}C]arginine contained originally in the medium would have been consumed in 60 min., had the bacteria at the maximum concentration of equiv. 2·1 mg. dry wt. bacteria/ml. used in most experiments taken up labelled arginine at the same rate as was taken up by the bacteria at the lowest bacterial concentration used. The actual uptake of arginine by the bacteria at this concentration (equiv. 2·1 mg. dry wt. bacteria/ml.) in 60 min. was only 3 % of the [^{14}C]arginine present originally in the medium (Fig. 2). Thus, the concentration of arginine in the medium was sufficiently high to allow it to stay practically unchanged in 60 min. at all bacterial concentrations studied. The results obtained with [^{14}C]glucose (Fig. 6), which was present in the medium at 20 mg./ml., also indicate that the decrease in the rate of transport of the nutrients obtained with increasing bacterial concentration was not due to this nutrient becoming limiting.

One possibility would be that contact between bacteria, the frequency and

extent of which would increase with increasing bacterial concentrations, might decrease the permeability of the cell surface to some nutrients for the duration of the contact. In several mammalian cell systems cell contact has been shown to influence the visible movement of the membrane (Abercrombie & Ambrose, 1958) and it is conceivable that it may also affect the functional activity of the membrane in respect of the transport of nutrients. A cell concentration effect similar to that reported here for *Escherichia coli* has been observed with mammalian cell suspensions (Bhargava *et al.* 1959; Bhargava & Bhargava, 1962; Bhargava, 1964).

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The Stability of *Mycoplasma mycoides*

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SUMMARY

The morphology of *Mycoplasma mycoides* was well preserved after washing and suspension in buffered 0·4 M-sucrose solutions, but the survival of viable particles was no better, and the loss of ultraviolet (u.v.)-absorbing substances and the decrease of turbidity was no less than in hypotonic solution (0·01 M-tris HCl or 0·01 M-$Na_2HPO + KH_4PO_4$). The addition of Mg^{2+}, Ca^{2+}, spermidine or spermine (increasing order of activity) decreased the decrease of turbidity and loss of u.v-absorbing substances. Ca^{2+} and Mg^{2+}, but not spermine, increased the degree of survival of viable particles. Ethylenediaminetetra-acetate (EDTA; 0·01 M) increased the loss of u.v.-absorbing substances, and decreased the turbidity and degree of survival. Ca^{2+}, Mg^{2+} and spermine annulled the effects of EDTA on loss of u.v.-absorbing material and on the turbidity, but only Ca^{2+} prevented the lethal effect of EDTA. Filaments disappeared and cell volume increased when the organisms were transferred from hypertonic to hypotonic solutions; the shape changes were reversible.

INTRODUCTION

Smith & Sasaki (1958) showed that the viability of some Mycoplasma strains was much less affected by the osmotic pressure of the suspending medium than had been supposed. Butler & Knight (1960) confirmed this observation and showed also that metal-chelating agents (diethyldithiocarbamate, ethylenediaminetetra-acetate, 8-hydroxyquinoline), manganese dioxide and the use of de-ionized water of high quality increased the degree of survival in suspensions in dilute buffer solutions. In contrast, Ca^{2+} ions have a marked stabilizing effect on bacterial spheroplast suspensions in hypotonic solutions (Tabor, 1962) and spermine, spermidine, streptomycin and polylysine were very effective stabilizers, while monovalent cations, Mg^{2+} and the diamines 1,4-diaminobutane and 1,5-diaminopentane were relatively ineffective.

Razin & Argaman (1963) compared the susceptibility of mycoplasmas, bacterial L-forms, spheroplasts and protoplasts to lysis by osmotic shock, by alternate freezing and thawing and by surface-active compounds. The mycoplasmas and bacterial L-forms were more resistant to osmotic shock than were the bacterial protoplasts and spheroplasts but, like the protoplasts, the mycoplasmas were very sensitive to lysis by surface-active substances. Mycoplasma strains differed in their sensitivity to lysis by osmotic pressure changes, strains of *Mycoplasma mycoides* being among the more resistant ones (Razin, 1963). Razin (1964) showed that the Mycoplasma strains he examined resisted osmotic shock at 0°, but underwent rapid lysis at 37°. Divalent and polyvalent cations, in concentrations as low as 10^{-5} M, protected *Mycoplasma laidlawii* from osmotic lysis.

METHODS

Organisms. The strain V5 of *Mycoplasma mycoides* and the strain N of *M. capri* were used. The latter strain has been found not to form detectable amounts of polysaccharide (Plackett, Buttery & Cottew, 1963). The organisms were grown in supplemented BVF-OS medium (Plackett *et al.* 1963) and harvested when the turbidity of the cultures had increased to between one quarter and three quarters of the maximum expected value. Within this turbidity range, the organisms were highly filamentous and the ratio of colony count of viable particles to turbidity was approximately constant.

Reagents. The salts used were of AR quality; the water was glass distilled, but no special precautions were taken to prevent metal ion contamination. Spermine (grade B) and spermidine (grade A) were obtained from the California Foundation for Biochemical Research, and 1,4-diaminobutane from E. Light and Co. (Colnbrook, Bucks., England). Sucrose (M solution) was freed from cations by passage through Dowex 50 (H^+) column. The solutions containing ethylenediaminetetraacetate and divalent metal ions were adjusted to pH 7·4 with NaOH.

Procedure. Ten-ml. volumes of culture were chilled, and centrifuged at 15,000 rev./min. for 20 min. (Spinco, model L, rotor 40). The supernatant fluids were poured off and the walls of the tubes wiped dry with filter paper. The deposits of organisms were then washed twice by resuspension in 10-ml. volumes of the test solutions. The entire process, which occupied about 3·5 hr, was performed in the cold.

Counts of viable particles determinations were made by a plate colony count method after the suspensions had been kept about 1 hr at 0–2°, and again in most experiments after a further period of 1–3 days at 2°. The results are expressed as the % of the colony count of the original culture. Turbidity measurements were made at 650 mμ (Beckman Model DU, 1 cm. light path) on samples of the suspensions (warmed to room temperature) at the end of the storage period, and are expressed as % of the turbidity of the original culture. Leakage of u.v.-absorbing material was measured at 250 mμ in both of the supernatant fluids after washing, and also in the supernatant fluid obtained after centrifuging a sample of the suspension after storage. The sum of these measurements, corrected where necessary for the extinction of the washing fluid, are expressed as % of the extinction of an alkali lysate. For this, the unwashed pellet from 10 ml. culture was incubated at 37° with 1 ml. N-NaOH for 1 hr and the lysate diluted to 50 ml. for measurement.

The volumes of packed cell pellets were measured after centrifugation in graduated capillary-tipped tubes at 1850*g* for 18 hr at 2°. Polyvinylpyrrolidone (1%, w/v, in the suspending fluid) was used as an indicator for measuring the intercellular volume (Wetherell & Pollack, 1962).

Electron microscopy. Nine volumes of a solution of the same composition as that of the suspending fluid but containing in addition either 4% (w/v) formaldehyde or 5% (w/v) glutaraldehyde were added to 1 vol. of suspension of organisms. Phosphate buffer was used in the suspending fluid to avoid pH changes. After standing overnight at 2°, the organisms were washed and suspended in water. Droplets were air-dried on electron microscope grids before metal-shadowing. No differences in morphology between material fixed in formaldehyde or glutaraldehyde were

detected, but the impression was formed from dark field examination in a light microscope that both fixatives resulted in some shrinkage, and in an increase in the number of beaded filaments.

RESULTS

Effects on turbidity, loss of ultraviolet-absorbing material and survival

Mg^{2+}, Ca^{2+} and EDTA. There was a decrease in turbidity, a loss of u.v.-absorbing material from the organisms and a decrease in the colony count after washing the organisms in dilute tris or phosphate buffers (0·01 M, pH 7·4). The magnitude of

Table 1. *The effect of Mg^{2+} and Ca^{2+} on the stability of Mycoplasma mycoides*

Expt.	Divalent cation salts* (M concn.)	Turbidity (%)†	Extinction at 260 mμ (%)‡	Viable count (%) after stated period at 0–2°	
				1 hr	70 hr
1	None	50	44	13	0·3
	$MgSO_4$ (0·001)	78	28	15	0·2
	$MgSO_4$ (0·01)	84	24	60	21
	$MgSO_4$ (0·1)	95	25	57	41
2	None	60	35	49	8
	$CaCl_2$ (0·001)	70	27	57	2·5
	$CaCl_2$ (0·01)	87	20	44	27
	$CaCl_2$ (0·1)	88	23	45	19

* Salts added to 0·01 M-tris HCl (pH 7·4). † Percentage of culture. ‡ Sum of 260 mμ extinction of washings as % of alkali lysate.

Table 2. *The effect of Mg^{2+} and Ca^{2+} and EDTA on the stability of Mycoplasma mycoides*

Expt.	Additions* (M concn.)	Turbidity (%)†	Extinction at 260 mμ (%)‡	Viable count (%) after stated period at 0–2°	
				1 hr	70 hr
1	None	73	37	56	21
	EDTA (0·001)	71	50	56	23
	EDTA (0·01)	60	55	2·0	0·01
	$MgSO_4$ (0·01)	89	29	65	59
	EDTA (0·001); $MgSO_4$ (0·001)	89	29	48	45
	EDTA (0·01); $MgSO_4$ (0·02)	82	32	8	0·8
2	EDTA (0·01)	35	81	1·0	0·2
	$MgSO_4$ (0·01)	83	27	48	22
	EDTA (0·01); $MgSO_4$ (0·02)	80	33	22	2·1
	$CaCl_2$ (0·01)	97	24	47	32
	EDTA (0·01); $CaCl_2$ (0·02)	83	28	28	22
	$MgSO_4$ (0·01); $CaCl_2$ (0·01)	98	22	48	34
	EDTA (0·01); $MgSO_4$ (0·01) $CaCl_2$ (0·02)	83	28	31	24

* Additions to 0·01 M tris HCl (pH 7·4). † Percentage of turbidity of culture. ‡ Sum of 260 mμ extinction of washings as % of alkali lysate.

these changes was about the same for either buffer. Tris buffer was used for most of the experiments to be described. The effect of pH value was not investigated. The addition of $MgSO_4$ or $CaCl_2$ in concentrations of 0·01 M or higher decreased these

changes (Table 1), whereas ethylenediaminetetra-acetic acid (EDTA) at 0·01 M increased them, its effect on the loss of viability being greater after storage (Table 2). At one-tenth this concentration, EDTA had no effect on survival, but increased the leakage of u.v.-absorbing material. Mg^{2+}(0·01 g. ions/l.) prevented almost completely the decrease of turbidity, and the loss of u.v.-absorbing material which

Table 3. *The effect of polyamines on the stability of Mycoplasma mycoides*

Polyamine* (M concn.)	Turbidity (%)†	Extinction at 260 mμ (%)‡
None	71	21·4
Spermine (0·00001)	80	18·7
Spermine (0·0001)	86	13·5
Spermine (0·001)	89	11·7
Spermine (0·005)	95	11·7
Spermidine (0·0001)	78	17·0
Spermidine (0·001)	87	13·4
Spermidine (0·005)	97	13·3
1,4-Diaminobutane (0·005)	83	19·5

* Added to (0·01 M tris HCl (pH 7·4). † Percentage of turbidity of culture. ‡ Sum of 260 mμ extinction of washings as % of alkali lysate.

Table 4. *The effect of Mg^{2+}, Ca^{2-} and spermine on the stability of Mycoplasma mycoides*

Expt.	Cation additions (M concn.)	Turbidity (%)†	Extinction at 260 mμ (%)‡	Viable count (%) after stated period at 0–2°		
				2 hr	24 hr	48 hr
1	None	59	35	21	11	—
	$MgSO_4$ (0·01)	79	19	39	43	—
	$CaCl_2$ (0·01)	87	17	21	25	—
	Spermine (0·001)	82	14	20	3	—
2	None	68	32	42	19	11
	$MgSO_4$ (0·01)	90	20	75	81	70
	$CaCl_2$ (0·01)	92	16	41	47	41
	Spermine (0·001)	96	15	50	10	4
3	None	76	30	41	—	12
	$MgSO_4$ (0·01)	88	24	60	—	28
	Spermine (0·001)	95	18	49	—	6
	$MgSO_4$ (0·01)† Spermine (0·001)	104	17	38	—	32

* Added to 0·01 M tris HCl (pH 7·4). † Percentage of turbidity of culture. ‡ Sum of 260 mμ extinction of washings as % of alkali lysate.

occurred in the presence of 0·01 M-EDTA alone, but only partly prevented the effect of EDTA (0·01 M) on survival (Table 2). Ca^{2+}(0·01 g ions/l.) almost completely prevented the lethal effect of EDTA (0·01 M) as well as preventing effects on turbidity and on loss of u.v.-absorbing material (Table 2).

Polyamines. Spermine (0·0001 M) had about the same effect as 0·01 M-$MgSO_4$ or 0·01 M-$CaCl_2$ on turbidity and the loss of u.v.-absorbing material. Spermidine (0·001 M) had about the same effect as 0·0001 M-spermine; 1,4-diaminobutane had little effect at 0·005 M (Table 3). Spermine, in contrast to $MgSO_4$ and $CaCl_2$, did not

increase the survival of viable particles during storage, although the organisms survived in its presence when $MgSO_4$ was also added (Table 4). Spermine (0·001 M) was as effective as Ca^{2+} (0·01 g. ions/l.) in preventing the effects of 0·01 M-EDTA on the loss of u.v.-absorbing material and on turbidity (Table 5).

Table 5. *The effect of Ca^{2+}, EDTA and spermine on the stability of Mycoplasma mycoides*

Tris = 0·01 M-tris HCl (pH 7·4); P = 0·01 M-$Na_2HPO_4 + KH_2PO_4$ (pH 7·4); Suc = 0·4 M-sucrose.

Composition (M concn.)	Turbidity (%)*	Extinction at 260 mμ (%)†
Tris + $CaCl_2$ (0·01)	93	20
Tris + EDTA (0·01)	55	43
Tris + EDTA (0·01) + $CaCl_2$ (0·02)	80	23
Tris + EDTA (0·01) + spermine (0·001)	76	23
P + EDTA (0·01)	49	43
P + EDTA (0·01) + spermine (0·001)	75	24
P + Suc + EDTA (0·01)	34	51
P + Suc + EDTA (0·01) + spermine (0·001)	73	21

* Percentage of turbidity of culture. † Sum of 260 mμ extinction of washings as % of alkali lysate.

Table 6. *The effect of sucrose, NaCl and KCl on the stability of Mycoplasma mycoides*

Added solute (M concn.)*	Total solute concn. (molal)	Turbidity†	Extinction at 260 mμ (%)‡	Viable count (%) after 1 hr at 0°
None	0·03	78	22	45
Sucrose (0·25)	0·30	67	30	32
Sucrose (0·4)	0·50	61	25	36
NaCl (0·15)	0·30	65	35	42
NaCl (0·25)	0·50	65	35	30
KCl (0·15)	0·30	70	34	58
KCl (0·25)	0·50	61	33	30

* Added to 0·01 M-tris HCl + 0·01 M-$MgSO_4$. † Percentage of turbidity of culture. ‡ Sum of 260 mμ extinction of washings as % of alkali lysate.

Tonicity. In the absence of stabilizing cations, the decrease in turbidity and the loss of u.v.-absorbing material were greater when the organisms were washed and suspended in solutions containing sucrose (0·4 M) than they were in hypotonic solutions. In the presence of stabilizing cations the addition of sucrose made little difference. The effects of adding sucrose (0·25 M, 0·4 M) and of NaCl and KCl (0·15 M and 0·25 M) to tris + $MgSO_4$ (0·01 M) solution are shown in Table 6. The decrease in turbidity and the loss of u.v.-absorbing material were slightly greater, and survival about the same, in the presence of these solutes as they were in the suspension in hypotonic solution.

Effects on morphology

Observations were made by dark field microscopy and by electron microscopy of formaldehyde-fixed or glutaraldehyde-fixed suspensions of *Mycoplasma mycoides*. The cultures examined contained a high proportion of filamentous organisms, but

other forms were also present. The filaments were preserved after washing and fixing in solutions having a total solute concentration of 0·3 or 0·5 molal (dilute-buffer + sucrose or NaCl) followed by washing in water. There were no filaments in suspensions of organisms washed and fixed in hypotonic (0·03 molal) solutions. Electron micrographs of these suspensions showed electron-dense almost spherical forms, flattened forms and other material. The addition of stabilizing cations or of EDTA had no marked effects on the morphology of the organisms when suspended either in sucrose (0·5 molal) or in hypotonic (0·03 molal) solutions.

Table 7. *Effect of tonicity of suspending medium on the packed cell volume of Mycoplasma strains v5 and* N

Organisms were washed and suspended in 0·5 molal solution. 1 vol. suspension was added to 10 vol. solution of molality 0·03 or 0·5, and the suspensions were then centrifuged in graduated capillary-tipped tubes at 1850*g* for 18 hr at 2°. The cell volumes recorded are the pellet volumes from which the intercellular volumes (as determined by the amount of PVP in the pellets) were subtracted.

Strain	Age of culture (hr)	Molal solute concentration*	Cell vol. (μl.)
v5	40	0·07	41
		0·50	23
v5	18	0·07	18
		0·50	8·5
N	18	0·07	9·6
		0·50	5·4

* Composition of solution: 0·01 M-tris HCl (pH 7·4). + 0·01 M-$MgSO_4$ + 1 % w/v polyvinylpyrrolidone (PVP) + sucrose 0·02 M (total solute concentration = 0·07 molal), or 0·4 M (total solute concentration = 0·50 molal).

The effects of the tonicity of the suspending fluid on morphology, and the reversibility of these changes, are illustrated in Pl. 1, figs. 1–3; figs. 1*a* and 1*b* show the organisms after they had been washed and resuspended in 0·01 M-phosphate + 0·01 M $MgSO_4$ + 0·001 M-spermine + 0·4 M-sucrose solution (hypertonic solution); figs. 2*a* and 2*b* show their appearance after they had been washed in the hypertonic solution and then suspended in a solution of the same composition but lacking sucrose (hypotonic solution); figs. 3*a* and 3*b* show their appearance after they had been suspended successively in hypertonic, hypotonic and again hypertonic solutions. The flattened forms seen in Pl. 1, figs. 2*a* and 2*b*, may have been spherical in suspension and have collapsed during drying on the grids. Dark field examination of the unfixed suspension in a light microscope showed only what appeared to be spherical forms of various diameters with a maximum of about 1 μ. The rapidity of the shape changes was evident from the complete absence of streaming birefringence in the suspension in hypotonic solution and its immediate reappearance when the organisms were resuspended in hypertonic solutions. Measurements of cell volumes by using polyvinylpyrrolidone to measure the volume of the intercellular space in the cell pellets are shown in Table 7. Organisms were harvested from an exponential phase culture of *Mycoplasma mycoides* strain v5 which contained numerous filaments, from a stationary phase culture of v5 strain which did not contain filaments, and from an exponential phase culture of the *M. capri* strain N which consisted

mostly of short rod-like forms. The cell volumes were, in each case, about twice as great when the organisms were suspended in hypotonic solution as when in hypertonic solution.

DISCUSSION

These observations support those of others (Smith & Sasaki, 1958; Butler & Knight, 1960; Razin, 1963) that Mycoplasma organisms survive well in hypotonic solutions. They differ from those reported by Butler & Knight (1960) who found that, within an optimal concentration range, metal chelating agents, including EDTA, increased survival. In the experiments reported here, EDTA decreased survival and turbidity and increased the loss of u.v.-absorbing material, whereas Ca^{2+} and Mg^{2+} had a stabilizing effect. These conflicting results may be due to differences in concentrations of toxic metal ion impurities. The stabilizing effects of divalent cations and of polyamines for *Mycoplasma mycoides* are similar to those described by Tabor (1962) for bacterial protoplasts and spheroplasts and by Razin (1964) for *M. laidlawii*. Razin found that any of a series of divalent and trivalent cations as well as spermine and spermidine at 10^{-5} M and even lower concentrations, prevented lysis during incubation of suspensions of *M. ladlawii* at 37°. Spermine at 10^{-5} M also prevented death in a hypotonic medium. In the experiments reported here, spermine did not prevent death of *M. mycoides*. The observed interactions between Ca^{2+}, Mg^{2+}, spermine and EDTA may be the result of a competition between EDTA and the cell membrane for Ca^{2+} and Mg^{2+} on the one hand, and between Ca^{2+}, Mg^{2+} and spermine for common binding sites on the membrane on the other.

Mycoplasma mycoides responds to changes in the tonicity of the suspending solution by changes in shape and volume. The volume changes for individual undamaged organisms may be larger than those measured for the whole suspensions, because many of the organisms may have been damaged and their property of responding to osmotic pressure changes destroyed during the manipulations. In the case of the highly filamentous organisms of the v5 strain harvested from the 18 hr culture, the volume changes may have been considerably larger than the measurements shown in Table 7 for another reason. *M. mycoides* synthesizes a large amount of polysaccharide which is believed to form an extracellular capsular or slime layer closely surrounding the organisms in young cultures and later shed into the medium (Plackett *et al.* 1963). The suspension of organisms harvested from the younger culture would therefore contain a much larger amount of this extracellular material. The ratio of the volume to the number of turbidity units in the samples centrifuged was about four-fold greater for the suspension from the 18 hr culture than it was for the suspension from the 40 hr culture. The filaments have a very large ratio of surface area to volume. If a filament 0·1 μ in diameter and 7 μ in length and bounded by a membrane 85 Å in thickness were transformed to a sphere of the same surface area, the volumes within the external and internal surfaces of the membrane would increase by factors of 5·5 and 8, respectively; the diameter of the sphere would be 0·3 μ. This is about the diameter of the larger forms observed by dark field examination in a light microscope in unfixed suspensions in hypotonic solution. The plastic filamentous mycoplasmas therefore have the potential to increase their volume several-fold by dilution of their contents (that is to decrease their internal osmotic pressure) without an increase in surface area. This may help to explain their

relative tolerance to osmotic pressure changes compared with bacterial protoplasts, whose volume, within a narrower tonicity range, is inversely related to the osmotic pressure of the medium (Weibull, 1955) but which undergo lysis in dilute solutions. Razin (1963) found that the sensitivity of certain Mycoplasma strains to osmotic lysis decreased with the age of the culture. The older cultures would contain few filaments. Changes in size, shape, lipid composition of membrane, and internal osmotic pressure are doubtless important also in determining stability. All of these may change with age of culture.

I am indebted to Mr A. Abbot of the Walter and Eliza Hall Institute of Medical Research for the electron micrographs.

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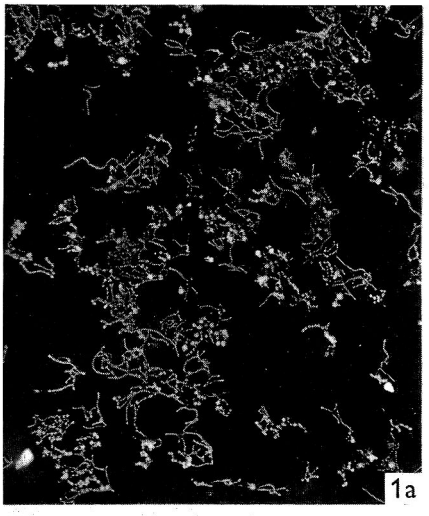
EXPLANATION OF PLATE

Figs. 1–3 illustrate the effect of the tonicity of the suspending medium on the shape of *Mycoplasma mycoides* organisms. Figs. 1*a*, *b* are of organisms washed and suspended in a solution of 0·01 M-phosphate (pH 7·4) + 0·01 M-$MgSO_4$ + 0·001 M-spermine + 0·4 M-sucrose (hypertonic solution). Figs. 2*a*, *b* are of organisms washed in hypertonic solution and then suspended in a solution of the same composition but lacking sucrose (hypotonic solution). Figs. 3*a*, *b* are of organisms suspended successively in hypertonic, hypotonic and then hypertonic solutions. The organisms for figs. 1–3 were then fixed for 18 hr at 0° in solutions of the same composition but containing 5% (w/v) glutaraldehyde, and washed and suspended in water. Gold-palladium shadowed. Figs. 1*a*, 2*a* and 3*a*, ×1500; figs. 1*b*, 2*b* and 3*b*, ×8600.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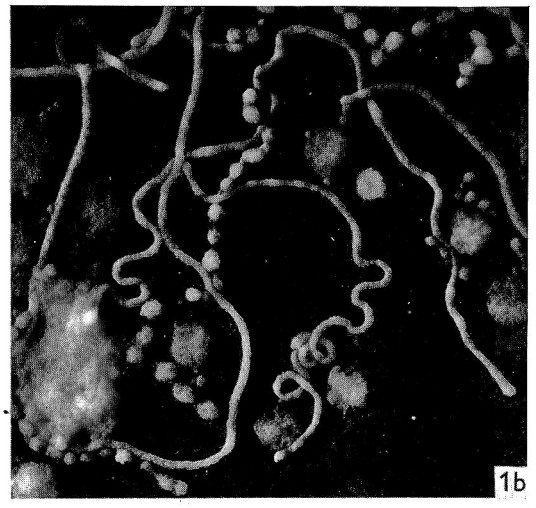
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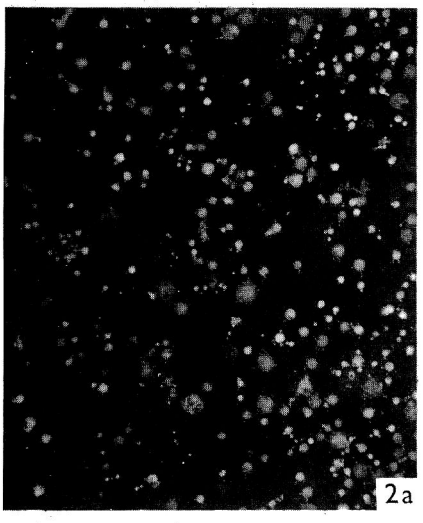
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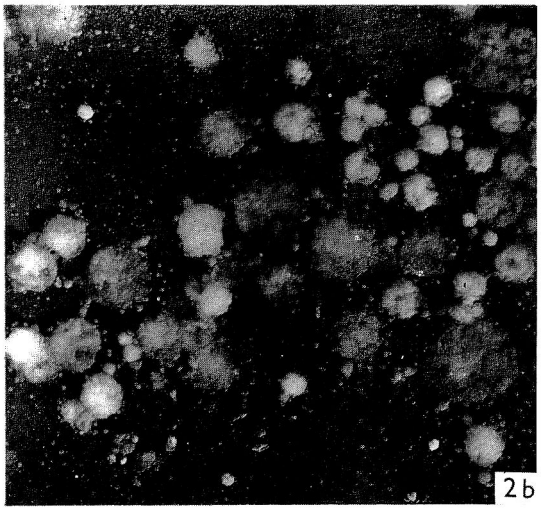
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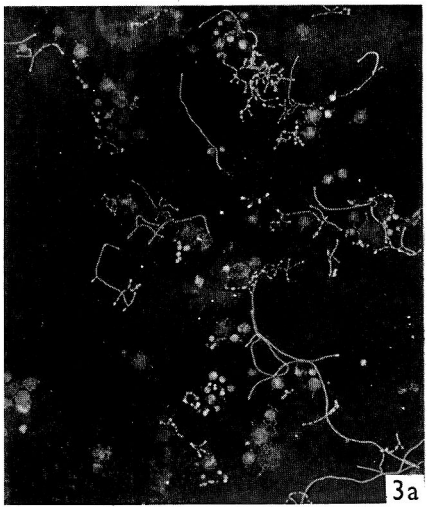
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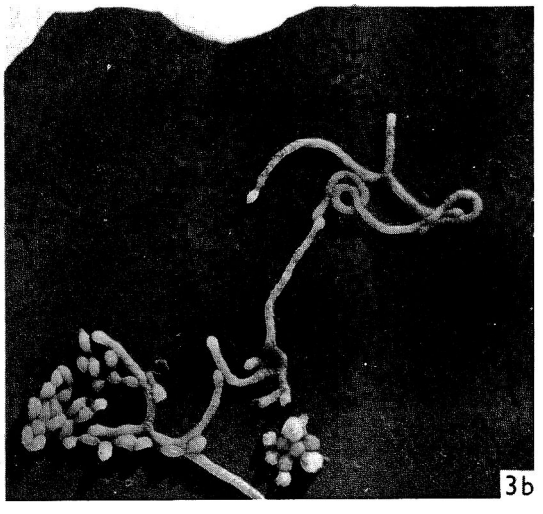
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Photoreactivation of Ultraviolet Induced Reciprocal Recombination, Gene Conversion and Mutation to Prototrophy in *Saccharomyces cerevisiae*

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SUMMARY

Strains of *Saccharomyces cerevisiae* heteroallelic for the adenine-2 locus, heterozygous for outside markers and homozygous for tryptophan-1, were ultraviolet (u.v.)-irradiated and the effect of post-treatment with white light determined. Mitotic gene conversion to adenine independence and mutation to tryptophan independence and to adenine independence were both decreased by post-u.v.-irradiation treatment with white light, whereas mitotic reciprocal recombination of outside markers was unaffected. The effect of varying the treatment with white light after a constant degree of exposure to u.v.-radiation produced a decrease in reversion frequency to a constant value at light exposures of 5 min. and more. The differing responses of reciprocal recombination and gene conversion are discussed.

INTRODUCTION

The effect of post-treatment with white light of ultraviolet (u.v.)-irradiated cells has been studied in bacteria (Doudney & Haas, 1958). Post-treatment has been shown to produce a decrease in the number of prototrophic revertants amongst the survivors of u.v.-irradiation. Changes of genotype during mitosis in diploid yeast may be due to one of three events: mutation, reciprocal recombination and gene conversion or non-reciprocal recombination (Roman, 1956*a*, *b*). Gene conversion in yeast was reviewed by Roman (1963) and Holliday (1964). Both gene conversion and reciprocal recombination, detected by the formation of homozygous recessive colonies in a heterozygous diploid, have been demonstrated to increase with u.v.-irradiation (James & Lee-Whiting, 1955; Roman, 1958), and it has been shown that a correlation exists between the occurrence of gene conversion and reciprocal recombination (Fogel & Hurst, 1963; Kakar, 1963). Roman & Jacob (1958) showed, however, that the proportion of reciprocal recombinants recovered among cells in which gene conversion had occurred decreased with increasing dosage of u.v. radiation.

Diploid *Saccharomyces cerevisiae* which is heteroallelic at the adenine-2 locus, $2n = ad_{2,1}/ad_{2,c}$ is red and requires adenine for growth. The symbols used here and throughout the text are those agreed to at the Carbondale Yeast Genetics Conference, 1963. The subscripts in $ad_{2,1}$ and $ad_{2,c}$ indicate that the mutations are independently isolated recombining alleles of the ad_2 locus. Gene conversion may occur during mitosis to give heterozygous diploids of either $ad_{2,1}/+$ or $ad_{2,c}/+$ genotype, with a corresponding change in phenotype from red adenine-requiring to white prototrophic. The double mutant heterozygote, $2n = ad_{2,1}-ad_{2,c}/+$ has not been

found (Cox, 1963). The present paper reports an investigation of the effect of white light as a post-treatment to u.v.-irradiation on gene conversion in such a heteroallelic diploid, on reciprocal recombination at outside markers from a heterozygous to a homozygous recessive condition and on mutation to prototrophy.

METHODS

Strains. The *Saccharomyces cerevisiae* diploids used were composed as follows:

$$98/2d \times 111/1d = a/\alpha,\ ad_{2.1}/ad_{2.c},\ hi_8/+,\ se/+.$$
$$J\,20\text{–}10 \times 126\,W5a = a/\alpha,\ ad_{2.1}/ad_{2.c},\ hi_8/+,\ +/me_2,\ +/se_1,\ tr_1/tr_1.$$

The haploids used were:

$$W71/1a = \alpha,\ ad_{2.c}\,se_1,\ tr_1;$$
$$J\,20\text{–}10 = \alpha,\ ad_{2.c}\,me_2,\ se_1,\ tr_1;$$
$$126\,W5a = a,\ ad_{2.1},\ hi_8,\ tr_1.$$

All strains are derived from cultures originally provided by Dr D. C. Hawthorne. The markers ad_2, se_1, and hi_8 are linked on chromosome XII as follows:

centromere-30-hi_8-15-se_1-23-ad_2 (Carbondale Yeast Genetics Conference, 1963).

Media. The complete medium used was described by Cox & Bevan (1962). It is a yeast-extract + peptone medium with 4% (w/v) glucose added; pH 6·7. The minimal medium was Difco Yeast Nitrogen Base without amino acids, solidified with Oxoid Ionagar, supplemented with growth factors as necessary and adjusted to pH 6·7.

Treatment. Before u.v.-irradiation, strains were grown for 2 days on complete medium agar, and suspended in saline at a titre of 10^7 organisms/ml. Samples (10 ml.) of this suspension were u.v.-irradiated in a Petri dish at 25 cm. from a Hanovia Model 11a ultraviolet lamp, generating almost entirely monochromatic radiation at 2537 Å. All manipulations were done in red light to avoid photoreactivation. For exposure to white light, 5 ml. samples were taken from the treated suspension and put into a boiling tube. The boiling tube was immersed in a cooled water-jacket with 5% (w/v) $CuSO_4$ as a heat filter. The white light source was two 200 W. Phillips Photoflood lamps set at right angles to each other. The following treatments were applied.

(1) Diploid *J 20–10 × 126 W5a.* Ultraviolet treatment for 0–90 sec., with and without post-treatment with white light for 5 min. The treated samples were scored for reversion to adenine independence, tryptophan independence, homozygosis at other loci and survival.

(2) Diploid *98/2d × 111/1d.* U.v.-treatment for 60 sec.; post-treatment with white light from 0 to 40 min. The treated samples were scored for reversion to adenine independence and survival.

(3) Haploid *W71/1a.* U.v.-treatment for 60 sec.; post-treatment with white light from 0 to 40 min. The treated samples were scored for reversion to adenine independence and survival.

(4) Haploids *J 10–10* and *126 W5a.* U.v.-treatment for 0–90 sec. The treated samples were scored for reversion to adenine independence and survival.

Method of testing for reversions and homozygosis. Reversions to adenine independence or tryptophan independence were detected by plating treated samples on minimal medium agar supplemented with amino acids but without adenine or

tryptophan. No attempt was made to distinguish between reversion and suppression due to mutations at other loci. Homozygosis was detected by replicating the colonies formed on complete medium agar by treated samples on suitably supplemented minimal medium agar.

RESULTS

The data on the survival and reversion to adenine independence in strain *J 20–10 × 126 W 5a* is expressed in Figs. 1 and 2. Fig. 1 shows that the effect of post-treatment with white light was to increase the % survival. Fig. 2 shows that the effect of post-treatment with white light was to decrease the % of revertant colonies amongst the survivors of u.v.-irradiation. These results indicate that the effect of white light post-treatment was to diminish the effect of u.v.-irradiation, whether measured as survival (about a ten-fold increase in the survival at u.v.-irradiation for 90 sec.) or as the frequency of revertant colonies (a decrease from about 0·2 to 0·1 revertants % survivors at 60 sec. u.v.-irradiation).

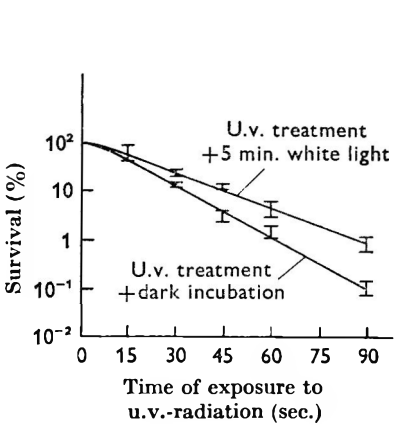

Fig. 1

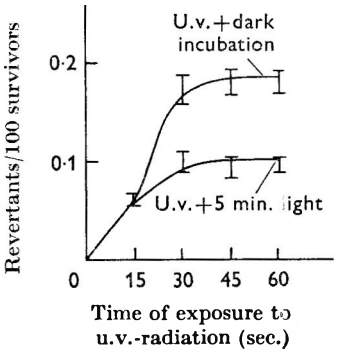

Fig. 2

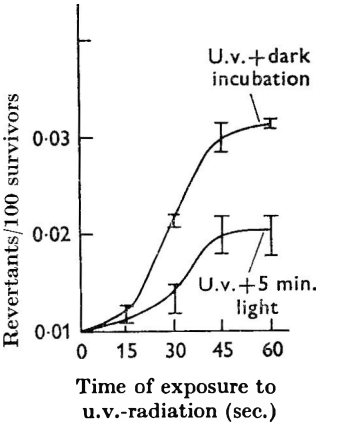

Fig. 3

Fig. 1. Survival values at various exposures to u.v.-radiation with and without 5 min. post-treatment with white light in *Saccharomyces cerevisiae* diploid *J 20–10 × 126W 5a*. The vertical bars show the 95 % probability limits.

Fig. 2. Reversion frequency expressed as % reversions to adenine independence/survivor at various exposures to u.v.-radiation, with and without 5 min. post-treatment with white light; *Saccharomyces cerevisiae* diploid strain *J 20–10 × 126W 5a*. The vertical bars show the 95 % probability limits.

Fig. 3. Reversion frequency, as % reversion to tryptophan independence/survivor, at various exposures to u.v.-radiation, with and without 5 min. post-treatment with white light; *Saccharomyces cerevisiae* diploid strain *J20–10 × 126W 5a*. The vertical bars show the 95 % probability limits.

Table 1 illustrates the effect of u.v.-irradiation on the parent haploid strains *126 W 5a* and *J 20–10*. These results indicate that in strain *126 W 5a* no increase in reversion frequency was measurable. In strain *J 20–10* reversion to adenine independence was increased from 0 to $0{\cdot}0042 \pm 0{\cdot}001$ % by 30 sec. exposure to u.v. radiation. Thus the increase in reversion to adenine independence in the diploid strain *J 20–10 × 125 W 5a* could not be accounted for on the basis of a sum of the frequencies of mutation to adenine independence at the ad_2 loci of the component strains.

The effect of post-treatment with white light for 5 min. on the number of cells revertant to tryptophan independence is shown in Fig. 3. White light produced a decrease in the number of revertant colonies at the various doses of u.v.-radiation (from approximately 0·02 to 0·01 revertants % survivors at 60 sec. u.v.-irradiation.

Comparison of Figs. 2 and 3 shows the close similarity between the effect of post-treatment with white light on the reversion to adenine independence and to tryptophan independence. In the reversion to adenine independence the process is that of gene conversion; reversion to tryptophan independence is by mutation.

Table 1. *Survival and reversion to adenine independence in Saccharomyces cerevisiae haploid strains J 20–10 and 126 W 5a.*

Strain	Ultraviolet irradiation (sec.)	Survival (%)	Revertants/100 survivors
126W 5a	0	100	0
	15	32·7 ± 4·0	0
	30	16·6 ± 0·35	0
	45	0·63 ± 0·097	0
J20–10	0	100	0
	15	50·5 ± 11·8	0·00146 ± 0·0002
	30	9·06 ± 1·02	0·0042 ± 0·001
	45	2·49 ± 0·19	0
	60	0·23 ± 0·049	0

Table 2. *The frequency of recessive homozygosis in Saccharomyces cerevisiae diploid strain J 20–10 × 126 W5a occurring after u.v.-irradiation, with and without post-treatment with white light*

Ultraviolet irradiation (sec.)	Colonies tested (no.)	U.v.-irradiation only. Homozygotes (no.)	(%)	U.v.-irradiation + 5 min. white light Homozygotes (no.)	(%)
Homozygosis for se_1, and hi_8, summed					
0	250	0	0	0	0
15	250	2	0·8	1	0·4
30	250	8	3·2	3	1·2
45	250	18	7·2	27	10·8
60	250	31	12·4	39	15·6
Homozygosis for all markers, summed ($hi_8 + se_1 + me_2$)					
0	250	1	0·4	1	0·4
15	250	3	1·2	4	1·6
30	250	18	7·2	13	5·2
45	250	27	10·8	42	16·8
60	250	62	24·8	75	30·0

Table 2 shows the occurrence of homozygosity at the se_1 and hi_8 loci on chromosome XII and the me_2 locus on another chromosome. The results from all three markers have been summed. There were no significant differences between the results for each marker. These results show that increases in u.v.-irradiation produce an increase in homozygosity by reciprocal recombination (from 0 at 0 sec. u.v.-iradiation to 24·8 % of survivors at 60 sec.). Post-treatment with white light had no significant effect on reciprocal recombination: after u.v.-irradiation for 60 sec.

there were 24·8 % homozygotes without white light post-treatment and 30·0 % of homozygotes with post-treatment.

The effect of a constant period of u.v.-irradiation followed by various periods of white light post-treatment on haploid and diploid strains is shown by Figs. 4 and 5. An increase in the duration of white-light treatment from 0 to 40 min. produced an increase in the number of surviving colonies. In the case of diploid strain *98/2d* × *111/1d*, survival increased from 3·76 ± 0·57 % at 0 min. to 23·8 ± 2·01 % at 40 min. post treatment. In haploid strain *W71/1a* survival increased from 0·475 ± 0·092 %

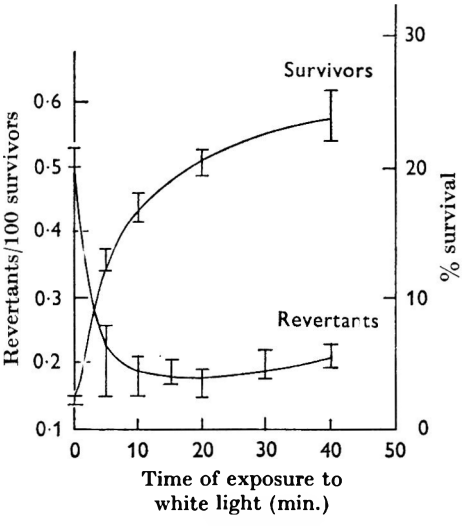

Fig. 4

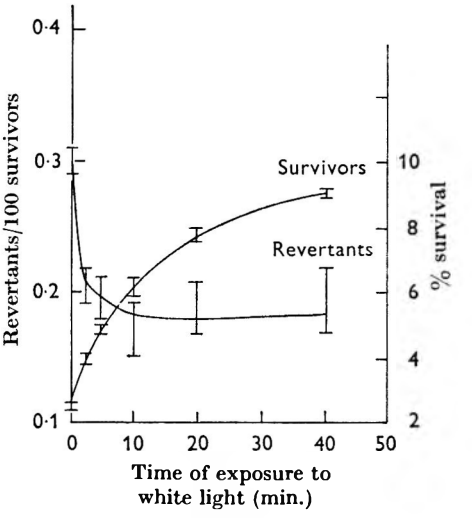

Fig. 5

Fig. 4. Survival and reversion to adenine independence after 60 sec. exposure to u.v.-radiation, with post-treatment by exposure to white light; *Saccharomyces cerevisiae* diploid *98/2d* × *111/1d*. The vertical bars show the 95 % probability limits.

Fig. 5. Survival and reversion to adenine independence at 60 sec. exposure to u.v.-radiation with post-treatment by exposure to white light; *S. cerevisiae* haploid *W71/1a*. The vertical bars show the 95 % probability limits.

at 0 min. to 6·93 ± 0·737 % at 40 min. post-treatment with white light. Reversion frequency can be seen from Figs. 4 and 5 to be decreased after 5 min. white light post-treatment from 0·5 ± 0·025 % to 0·2 ± 0·1 % in *98/2d* × *111/1d* and from 0·3 ± 0·01 % to 0·2 ± 0·02 % in *W71/1a*. Increases in the duration of light treatment above 5 min. did not further decrease the % of revertants amongst the surviving colonies.

DISCUSSION

The frequencies of reciprocal recombination, gene conversion to adenine independence and mutation to tryptophan and adenine independence were all increased by ultraviolet-irradiation. Both gene conversion to adenine independence and mutation to tryptophan or to adenine independence were decreased by post-treatment with white light. This decrease in reversion to prototrophy was accompanied by an increase in survival. This contrasts with the absence of any effect of post-treatment on reciprocal recombination, where the same frequency of recombinants

was obtained with and without post-treatment with white light. This difference in response between reciprocal recombination and gene conversion was also observed by Pittman (1961) in a diploid yeast heterozygous for adenine requirement (*AD/ad*) and heteroallelic for the gene controlling melezitose fermentation (*MZ*). Fogel & Hurst (1963) concluded from their results that reciprocal recombination and gene conversion were interdependent; but the present results indicated that the processes are distinguishable by their response to photoreactivation by white light. There are three possible explanations. One is that there is some reversible damage by u.v.-irradiation which is responsible for 90 % of killing, 50 % of gene conversion and mutation events and for no reciprocal recombination. Reciprocal recombination and the remaining 50 % of gene conversion and mutations are due to irreversible u.v.-irradiation damage. The second possibility is that photo-reactivation does not directly affect any of the processes involved in the changing genotype of the yeast cultures, but only survival. The results might be explained by assuming that u.v.-irradiation leaves a population of organisms with three components—alive, dead and those moribund but capable of revival by white light. At 60 sec. u.v.-irradiation the fraction capable of revival is about four times the size of the living fraction, but may contain a lower proportion of gene conversions or mutations. As organisms are recovered by photoreactivation, the proportion of revertants among all survivors would decrease. Reciprocal recombination, by contrast, may be assumed to be induced by u.v.-irradiation in a similar proportion of all fractions of the treated population, and therefore white-light reactivation will have no effect on the proportion of homozygous recessives recovered as the degree of survival increases.

The third possibility is that gene conversion and mutation require post-irradiation dark incubation for maximum expression, whereas reciprocal recombination does not. Witkin (1956, 1964) reported such an effect in the yield of back-mutations after post-irradiation treatment of bacteria. After 5 min. white light post-treatment of *Saccharomyces cerevisiae* strains *98/2d* × *111/1d* and *W71/1a*, a constant value of revertants/survivor resulted with further increases in light treatment, even though the degree of survival continued to increase. This suggests that u.v.-irradiation induces a fixed frequency of revertants at a given dose, which we may call $(a+b)$, where a is the frequency present after 5 min. post-treatment with white light and b is that frequency which requires dark incubation. The frequency $(a+b)$ will be distributed between survivors and non-survivors in the same ratio of a/b. After 5 min. post-treatment with white light, b revertants are lost and do not appear amongst the survivors. Further increases in the duration of white light treatment increases the number of survivors with a subsequent recovery of a revertants among them. Thus increases in survival with white light post-treatment greater than 5 min. will produce a constant percentage of revertants, since survivors are being recovered from the reversibly-damaged fraction of the sample which has a revertant cells.

These results show a remarkable similarity in the response of the processes of gene conversion and of mutation to u.v.-irradiation and photoreactivation. Gene conversion is usually thought of as a process of recombination and therefore distinct from mutation. It will be interesting to determine whether or not there is any basic similarity in the two processes.

One of us (J.M.P.) is indebted to the Department of Scientific and Industrial Research for a Research Studentship, during the tenure of which this work was done.

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Printed in Great Britain

The Synthesis of *p*-Aminobenzoic Acid and Folic Acid by Staphylococci Sensitive and Resistant to Sulphonamides

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SUMMARY

The synthesis of *p*-aminobenzoic acid and folic acid was investigated in three strains of Staphylococcus sensitive to sulphonamide drugs, and in four substrains resistant to sulphonamides. Another strain (*Staphylococcus aureus* R122) which was resistant to sulphonamides when isolated was also examined. A microbiological assay was used to measure the synthesis of *p*-aminobenzoic acid during the growth of organisms in a partially defined medium. *S. aureus* R122 formed about twenty times as much *p*-aminobenzoic acid as did any of the other strains, among which the synthesis showed only small variations and was not greatly affected by growth of the organisms in presence of sulphathiazole.

Folic acid was assayed as *Lactobacillus casei* factor. The response curve of the assay organism to extracts of every strain of Staphylococcus examined was the same; but it differed from the response to known forms of folic acid. The active material had little growth-promoting activity for *Leuconostoc citrovorum* or for *Streptococcus faecalis*. Washed suspensions of *Staphylococcus lactis* 2102 R formed about 10 times as much folic acid as the sensitive parent strain (2102) from glucose, *p*-aminobenzoate and glutamate; the sulphonamide-resistant substrain (2102 R) also formed more folic acid during growth. However, a second resistant *S. lactis* substrain (2102 R2) formed little more folic acid than did the parent strain. *S. aureus* R122 synthesized an amount of folic acid similar to that formed by *S. lactis* 2102 R. With two other strains (*S. aureus* H and JHM) the synthesis of folic acid was not increased when resistance was acquired. Washed suspensions of all the sulphonamide resistant substrains were able to synthesize folic acid in the presence of higher concentrations of sulphathiazole than were the sensitive strains.

INTRODUCTION

The study of the properties of drug-resistant staphylococci is valuable because of the frequency with which such strains occur in clinical infections (Jawetz, 1956; Harington, 1957). Resistance to sulphonamides was chosen for investigation because there is more detailed knowledge about the biochemical mode of action of these drugs than is the case with many of the antibacterial agents used in chemotherapy (Woods, 1952; 1958). Such information directs attention to the metabolism of *p*-aminobenzoic acid and folic acid, and suggests that the development of resistance may be related to changes in the metabolism of these compounds. Some sulphonamide-resistant strains of bacteria produce greater amounts of *p*-aminobenzoic acid during growth than do their sensitive parent strains (MacLeod, 1940;

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Landy, Larkum, Oswald & Streightoff, 1943) although this behaviour is not found in all cases of sulphonamide-resistance (Lemberg, Callaghan, Tandy & Goldsworthy, 1948; Yaniv & Davis, 1953). Lascelles & Woods (1952) studied the biosynthesis of folic acid (*Lactobacillus casei* factor) by *Staphylococcus lactis* 2102 and a sulphonamide-resistant substrain 2102 R. Folic acid was synthesized by washed suspensions of these organisms in a buffered solution of glucose + glutamate + *p*-aminobenzoate. In this mixture, and during growth, the sulphonamide-resistant strain synthesized about ten times as much folic acid as did the parent strain, and this synthesis was less sensitive to inhibition by sulphathiazole than was the synthesis by the parent strain.

In the present work, the synthesis of folic acid and of *p*-aminobenzoic acid in several strains of staphylococci has been examined to establish whether changes in the biosynthesis of *p*-aminobenzoic acid and/or folic acid generally accompany the development of resistance to sulphonamides.

METHODS

Organisms. Staphylococcus aureus strain JHM was isolated from a boil by Dr J. H. Marshall in this laboratory; from this a substrain (JHMR) was developed which was resistant to sulphathiazole. Strain H was the 'Oxford staphylococcus' (NCTC 6571); strain HR2 was a sulphonamide-resistant substrain developed from it by Dr June Lascelles. Strain R122 was a sulphonamide-resistant strain isolated from a patient by the Public Health Laboratory Service, Oxford. The sensitivities of these strains to sulphathiazole are shown in Table 1.

Table 1. *Relative resistance to sulphathiazole of various strains of staphylococci*

Organisms (about 10^5) were incubated for 50 hr at 37° in medium B (5 ml.) containing various concentrations of sulphathiazole, in tubes (150 × 19 mm.) sloped at about 5° above the horizontal.

Organism	Highest concn. of sulphathiazole permitting growth (M)	Relative resistance compared with parent strain
Staphylococcus lactis 2102	10^{-5}	—
2102 R	5×10^{-4}	50
2102 R2	5×10^{-4}	50
S. aureus JHM	5×10^{-6}	—
JHMR	10^{-4}	20
S. aureus H	5×10^{-6}	—
HR2	10^{-3}	200
S. aureus R122	5×10^{-4}	Resistant when isolated

Staphylococcus lactis 2102 was a laboratory strain, previously called *S. aureus* 2102 by Lascelles & Woods (1952). The organism formed golden coloured colonies on solid media, but it was coagulase negative and did not ferment mannitol. In these respects and in the production of acid and not acetoin from glucose it resembled more closely the characters ascribed to *S. lactis* n.sp. by Shaw, Stitt & Cowan (1951).

Strains 2102R and 2102R2 were sulphonamide-resistant substrains of 2102, the former developed by Dr June Lascelles.

All resistant substrains were obtained by serial subculture of the respective parent strains in lots of medium B + increasing concentrations of sulphathiazole. At each stage, a set of tubes containing a serial 1/2 dilution of sulphathiazole was inoculated from the tube of the preceding series containing the highest concentration of sulphathiazole which permitted growth in 2–3 days. The resistant strain was finally plated out and an isolated colony selected. Resistance developed more readily when only a small increment of concentration of sulphathiazole was used at each subcultivation after a short period of incubation.

Escherichia coli 273/384 (ATCC 9723a) was an X-ray induced mutant which required *p*-aminobenzoic acid for growth.

Stock cultures of all the above organisms were maintained on slopes of tryptic meat agar, subcultured monthly, incubated for 15 hr at 37° and stored at 2°.

Streptococcus faecalis R (ATCC 8043); *Lactobacillus casei* (*helveticus*) (ATCC 7469); and *Leuconostoc citrovorum* (*Pediococcus cerevisiae*; ATCC 8081) were maintained in Tryptose glucose agar stab cultures, subcultured monthly, incubated 24 hr at 37° and stored at 2°. With all organisms, four subcultures were taken at monthly intervals; each subculture was used as source of further subcultures.

Medium. All strains of staphylococci were grown in medium B which was based on acid-hydrolysed casein and modified from that of Lascelles & Woods (1952) by the replacement of DL-cysteine by L-cysteine hydrochloride (100 mg./l.) and by increasing the amount of KH_2PO_4 to 700 mg./l. and of $Na_2HPO_4 12H_2O$ to 10 g./l. This medium was prepared at double strength in glass-distilled water, and was brought to the desired volume with glass-distilled water and other additions before autoclaving for 7 min. at 115°. Glucose solutions were autoclaved separately.

Experiments with suspensions of organisms. Medium B (25 ml. after all additions) in a 180 × 32 mm. tube was inoculated with about 2×10^8 organisms from an agar slope. The tube was incubated for 14–16 hr at 37°, the tube being sloped at about 5° above the horizontal. Organisms were centrifuged and washed once with 0·02 M-phosphate buffer (pH 6·8 or 7·4; 25 ml.). Because of the large inoculum used the growth of none of the strains of staphylococci was inhibited by sulphathiazole at the highest concentration used (mM).

Mixture F for experiments on the synthesis of folic acid by suspensions of organisms was that described by Lascelles & Woods (1952). It contained glucose, glutamate and *p*-aminobenzoic acid in phosphate buffer and was prepared at double strength and subsequently brought to the desired volume with glass-distilled water and other additions.

Microbiological assay of p-aminobenzoic acid. The organism used was *Escherichia coli* 273/384, a mutant which needs *p*-aminobenzoic acid as a nutrient. The nutrient requirement is decreased by the presence of methionine, xanthine, thymine and vitamin B_{12} (Lampen, Jones & Roepke, 1949; de Haan, 1950); it was shown in the present work that these compounds were not present in material under assay in amounts sufficient to interfere. The minimal medium of Davis & Mingioli (1950) was used, modified by autoclaving the glucose in the medium. The sample, or standard amount of *p*-aminobenzoate, was added to double strength medium (2·5 ml.) in 150 × 19 mm. tubes, and the volume was made to 4·9 ml. with water. After

autoclaving for 7 min. at 115° the medium was inoculated with a suspension (0·1 ml.; about 2×10^6 organisms) of *Escherichia coli* 273/384 from an agar slope. The tubes were incubated sloped at about 5° above the horizontal for 48 hr at 37°; growth was assessed in an EEL photoelectric colorimeter (Evans Electroselenium Co. Ltd, Halstead, Essex) with a neutral density filter; the uninoculated medium being used to give the zero setting. Each assay included a standard response curve for *p*-aminobenzoate and each sample was assayed at two or three dilutions; every tube was set up in duplicate. The effective range of the assay was from 5 to 50 $\mu\mu$moles *p*-aminobenzoate, with an accuracy of $\pm 20\%$ (Fig. 1).

The precision of the assay was found by determining the statistic C, which measures the reliability of the assay slope (Bliss, 1956); a value of 1·03 was obtained. The more nearly C approaches unity the more precise is the assay, while an assay with a value of C greater than 2 is of little value. The validity of the assumptions that are made in this factorial analysis, namely, a linear relation between the response and the logarithm of the dose, and agreement in slope of the response curve for different samples, was confirmed statistically.

Samples for assay were obtained by heating the cultures of staphylococci for 5 min. at 100° and removing the organisms by centrifugation; the supernatant fluids were usually diluted tenfold. When sulphathiazole was also present in the samples it was separated from *p*-aminobenzoate by descending chromatography (12 hr at room temperature) on Whatman no. 1 paper, with *n*-butanol saturated with water as solvent. The position of *p*-aminobenzoate on the chromatograms was found by running a marker strip which was cut off and examined by bioautography with *Escherichia coli* 273/384. The part of the chromatogram that contained *p*-aminobenzoic acid was eluted with water. Recovery of known amounts of *p*-aminobenzoate was only about 50%.

Assay of folic acid. Material for assay of folic acid was prepared by autoclaving the reaction mixture (including organisms) for 10 min. at 115° with an equal volume of the appropriate assay medium at double strength. The supernatant fluid after centrifugation was used for the assay of various forms of folic acid by using three assay organisms.

The technique of Lascelles & Woods (1952) was used for assay of *Lactobacillus casei* factor. This assay organism responds to pteroylglutamic acid, pteroyltriglutamic acid and leucovorin (N^5-formyltetrahydropteroylglutamic acid), but extracts of all the staphylococci tested gave response curves which diverged in a similar manner from the response curve to pteroylglutamic acid (Fig. 2). Consequently the amounts of *L. casei* factor present in extracts was assessed against a reference extract as described by Lascelles & Woods (1952).

The assay for *Leuconostoc citrovorum* factor was that of Sauberlich & Baumann (1948) with the modifications introduced by Lascelles & Woods (1952). Synthetic leucovorin was used as a standard. *L. citrovorum* does not respond to pteroylglutamate and other unreduced forms of folic acid.

Streptococcus faecalis R responds to pteroic acid and N^{10}-formylpteroic acid (rhizopterin) as well as to pteroylglutamic acid and leucovorin. The medium was that of Rabinowitz & Snell (1947). Extract or rhizopterin solution was added to double-strength medium (2·5 ml.) in 125×16 mm. tubes, the volume brought to 4·4 ml. with water and the tubes were autoclaved for 7 min. at 115°; pyridoxal (0·1 ml.,

2·5 μM) sterilized by Seitz-filtration was then added. The inoculum was derived from a fresh stab culture in Tryptose glucose agar which was subcultured into Tryptose glucose broth and incubated 15 hr at 37°; this culture was diluted 1/100 and each tube of the assay inoculated with 0·5 ml. (about 2×10^7 organisms); for incubation the tubes were vertical in air for 40–44 hr at 37°. Standards containing rhizopterin (0·1–1·0 mμmoles) were set up in each assay; the curves for the response of the assay organism to synthetic rhizopterin and to staphylococcal extracts were similar (Fig. 3). The effective range of the assay was from 1 to 5 $\mu\mu$moles rhizopterin; the values for *S. faecalis*-factor activity are expressed in terms of this compound.

Chemicals. Pteroylglutamic acid and leucovorin were gifts from Dr E. L. R. Stokstad and rhizopterin was provided by Dr K. Folkers. Sulphathiazole was a gift from May & Baker Ltd, Dagenham, Essex.

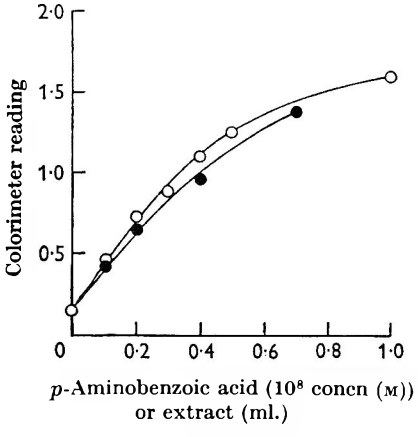

Fig. 1

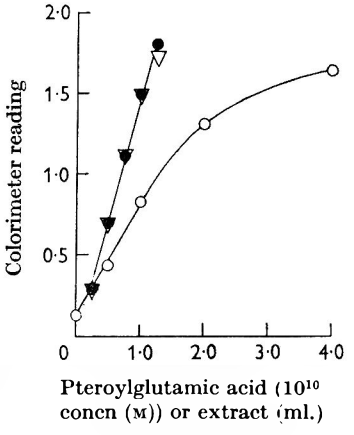

Fig. 2

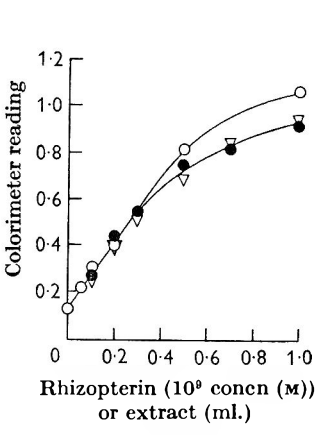

Fig. 3

Fig. 1. Response of *Escherichia coli* 273/384 to *p*-aminobenzoic acid and to a culture filtrate (of *Staphylococcus aureus* HR2) prepared as described in Methods. ○ = *p*-aminobenzoic acid; ● = culture filtrate. The assay organism was incubated at 37° for 48 hr before its growth was assessed.

Fig. 2. Response of *Lactobacillus casei* to pteroylglutamic acid and to extracts of *Staphylococcus aureus* H and HR2 prepared as described in Methods. ○ = pteroylglutamic acid; ▽ = *S. aureus* H extract; ● = *S. aureus* HR2 extract. The assay organism was incubated at 37° for 20 hr before its growth was assessed.

Fig. 3. Response of *Streptococcus faecalis* R to rhizopterin and to extracts of *Staphylococcus aureus* H and HR 2, prepared as described in Methods. ○ = Rhizopterin; ▽ = *S. aureus* H extract; ● = *S. aureus* HR2 extract. The assay organism was incubated at 37° for 40 hr before its growth was assessed.

RESULTS

Synthesis of p-aminobenzoic acid

The synthesis of *p*-aminobenzoic acid during growth of the various strains of Staphylococcus in medium B was investigated in the presence and in the absence of sulphathiazole. Incubation was for 14–16 hr, by which time growth had just reached the end of the logarithmic phase. The most striking feature of the results (Table 2) for growth in the absence of sulphathiazole was the very high synthesis of

p-aminobenzoic acid by *Staphylococcus aureus* R122; its concentration in the medium after growth was just over 10 μM. The resistant strains of *S. aureus* JHM and H did not synthesize appreciably different amounts of *p*-aminobenzoic acid than their parent (sensitive) strains. Still less *p*-aminobenzoic acid was synthesized by *S. lactis* 2102; in one of its resistant substrains (2102R2) the amount of synthesis was the same as for the parent, whereas in the other substrain (2102R) there was a five-fold increase. However, the extent of synthesis by strain 2102R was no greater than was found for the sensitive strains of *S. aureus*.

Possibly the remainder of the resistant strains synthesized greater amounts of *p*-aminobenzoate than their sensitive parent strains only in the presence of sulphonamides. Organisms were therefore cultured as before but in the presence of sulphathiazole; *p*-aminobenzoate and sulphathiazole were separated in the extracts by chromatography and the amount of *p*-aminobenzoic acid was assayed (Table 2). However, sensitive and resistant pairs of strains of *Staphylococcus aureus* synthesized rather similar amounts of *p*-aminobenzoate. Although the accuracy of the figures is low because of the poor recovery of *p*-aminobenzoate from the chromatograms, a ten-fold or greater difference in synthesis of *p*-aminobenzoate between sensitive and resistant strains (such as would be required to explain resistance) would have been detected easily.

Synthesis of folic acid

Lactobacillus casei factor. After harvesting from medium B, the content of *Lactobacillus casei* factor was about the same in all of the sulphonamide-sensitive strains (Table 3). In two of the resistant strains (*Staphylococcus lactis* 2102R, *S. aureus* R122) there was about five times as much factor, but in the other three resistant strains little more factor was present than in the corresponding parent strain.

Table 2. *Synthesis of p-aminobenzoic acid by sensitive and resistant strains of staphylococci*

Organisms were grown in medium B (25 ml.) for 14–16 hr and the cultures prepared for assay as described in the Methods section. The values for *p*-aminobenzoic acid with cultures containing sulphathiazole (mM) have been corrected for the known low recovery (about 50%) of *p*-aminobenzoate under these conditions (see Methods section).

Organism	Yield of *p*-aminobenzoic acid (mμg./mg. dry wt. of organisms) when cultures grown with Sulphathiazole	
	absent	present
Staphylococcus lactis 2102	35	—
2102R	150	—
2102R2	33	—
S. aureus JHM	170	420
JHMR	180	450
S. aureus H	210	120
HR2	240	50
S. aureus R122	4900	1400

Table 3. *Content of and synthesis of Lactobacillus casei factor by suspensions of staphylococci*

'Normal' staphylococci were harvested from medium B (25 ml.) after growth for 14–16 hr. For 'depleted' organisms the medium also contained sulphathiazole (5 μM for sensitive strains; 50 μM for resistant strains). The content of *Lactobacillus casei* factor was measured initially after suspension in mixture F. 'Depleted' organisms (0·2 mg. dry wt./ml.) were incubated at 37° for 5 hr in mixture F (final volume, 2·5 ml.) upright in 130 × 16 mm. tubes and the content of *L. casei* factor of the whole suspension again assayed.

L. casei factor is expressed below as pterolyglutamic acid.

Organism	*L. casei* factor (mμg./mg. dry wt. organisms)		
		'Depleted' organisms:	
	'Normal' organisms: Initial	Initial	After incubation in mixture F
Staphylococcus lactis 2102	35	3	18
2102R	200	8	110
2102R2	50	4	30
S. aureus JHM	30	4	10
JHMR	33	6	15
S. aureus H	28	6	13
HR2	28	6	12
S. aureus R122	130	8	80

Lascelles & Woods (1952) found that synthesis of *L. casei* factor by washed suspensions of *S. lactis* 2102 and 2102R occurred only in organisms which contained initially only small amounts of the factor; such organisms ('depleted') were obtained by growth in the presence of sub-inhibitory concentrations of sulphathiazole. In the present work normal organisms of all strains showed very slight synthesis of the factor. The finding of Lascelles & Woods (1952) that 'depleted' *S. lactis* 2102R synthesized about ten times as much *L. casei* factor as did its parent strain was confirmed (Table 3). *S. lactis* 2102R2, the other resistant substrain, also synthesized significantly more of the factor than did its parent, though the increase (1·7-fold) was much less than with strain 2102R. *S. aureus* R122, like *S. lactis* 2102R, had a relatively high content of factor after growth in medium B; suspensions of 'depleted' organisms also synthesized a comparatively large amount in mixture F (Table 3). No (sensitive) parent strain was available for comparison in this case. The resistant strains of *S. aureus* JHM and H, even after depletion, synthesized no more factor than the small amount formed by their parent strains. Incubation of the suspensions for longer periods in mixture F, growth of cultures in a richer medium, and treatment with higher concentrations of sulphathiazole (up to 1 mM) during growth did not appreciably increase the synthesis of the *L. casei* factor by these strains.

Lascelles & Woods (1952) found that the synthesis of *Lactobacillus casei* factor in mixture F by *Staphylococcus lactis* 2102 and 2102R was inhibited by sulphathiazole. Synthesis by the resistant strain was eight times more resistant to sulphathiazole than was that of the parent strain; the difference was 50-fold when growth was the criterion of sensitivity. It was pointed out that such a comparison was only realistic when it is known that all the substances necessary for the synthesis of the

Table 4. *Inhibition by sulphathiazole of the synthesis of Lactobacillus casei factor by staphylococci*

'Depleted' staphylococci were obtained (as in Table 3) and incubated in mixture F (as in Table 3) in the presence of the stated concentrations of sulphathiazole. *p*-Aminobenzoic acid was 10 μM throughout.

(20), etc. is the approximate percentage inhibition as compared with the control without sulphathiazole.

	Sulphathiazole (M)				
	0	10^{-6}	10^{-5}	10^{-4}	10^{-3}
Organism	*L. casei* factor formed after 5 hr in mixture F (mμg.*/mg. dry wt. organisms)				
Staphylococcus lactis 2102R	160	150 (10)	64 (60)	9 (90)	—
2102R2	44	40 (10)	18 (60)	5 (90)	0(100)
S. aureus JHM	5	6 (0)	3 (40)	2 (70)	0 (100)
JHMR	8	7 (10)	5 (30)	5 (40)	3 (70)
S. aureus H	7	6 (20)	5 (30)	4 (50)	0 (100)
HR2	8	7 (10)	6 (20)	7 (10)	4 (50)

* Expressed as pteroylglutamic acid.

factor by suspensions of organisms are present in optimal amounts. In the present work the sensitivity of the synthesis of *L. casei* factor to inhibition by sulphathiazole in suspensions of *S. lactis* 2102R2 resembled that of *S. lactis* 2102R (Table 4). In the experiments with *Staphylococcus aureus* JHM and H, and their resistant substrains, the amounts of the *L. casei* factor synthesized were low, but there was a progressive inhibition of this synthesis by increasing concentrations of sulphathiazole and the resistant strains were less sensitive to this inhibition than were the parent strains (Table 4).

Table 5. *Content and synthesis of Streptococcus faecalis factor by suspensions of staphylococci*

Normal and depleted staphylococci were obtained as in Table 3 and suspended in Mixture F. After removing a sample for assay of the initial *S. faecalis* factor, the organisms were incubated in mixture F (as in Table 3) and re-assayed for *S. faecalis* factor. The values in brackets were obtained after acid hydrolysis of the samples (see text).

	Normal organisms		Depleted organisms	
Organism	Initial	After incubation in mixture F	Initial	After incubation in mixture F
	S. faecalis factor (mμg.*/mg. dry wt. organisms)			
Staphylococcus lactis 2102	6	9	10	15
2102R	20	100	13	53
S. aureus JHM	0 (8)	5	2	3
JHMR	0	5	1	2
S. aureus H	0 (14)	0	1	2
HR2	0	5	1	2
S. aureus R122	3	8	2	9

* Expressed in terms of N^{10}-formylpteroic acid.

Streptococcus faecalis factor. A possible reason for the apparently low content of folic acid in extracts of *Staphylococcus aureus* JHM and H and their resistant substrains is that some of the folic acid might be present in a form to which *Lactobacillus casei* does not respond. *Streptococcus faecalis* responds to two compounds of the folic acid group, pteroic acid and rhizopterin, which are not active for *L. casei*; these compounds do not contain a glutamic acid residue. Results of determinations of the initial amounts (in organisms grown in medium B) of factors active with *S. faecalis*, and the amounts synthesized by washed suspensions are shown in Table 5. Only in the case of *Staphylococcus lactis* 2102R was there a striking synthesis by washed suspensions. The very low activity of most of the extracts of 'normal' organisms is noteworthy, since all the organisms had activity for *L. casei* (Table 3). Since there was good recovery of added rhizopterin from staphylococcal extracts it was unlikely that these extracts inhibited the assay organism. Most probably the folic acid in the extracts was a form to which *S. faecalis* did not respond although *L. casei* did. One such compound is pteroyltriglutamic acid, which on a molar basis is ten times as active for *L. casei* as for *S. faecalis* (Jukes & Stokstad, 1948). Extracts of two of the sensitive staphylococci were diluted with an equal volume of N-HCl, autoclaved at 115° for 10 min. and neutralized with N-NaOH; the activity of the extracts was considerably increased (Table 5). A quantitative conversion of material present in the extracts into factors active for *S. faecalis* was not attempted.

Leuconostoc citrovorum factor. Lascelles & Woods (1952) found that typical extracts of *Staphylococcus lactis* 2102R were much more active in supporting the growth of *Lactobacillus casei* than the growth of *Leuconostoc citrovorum*. This was confirmed in the present work, and it was also found that none of the extracts of any of the strains of *Staphylococcus aureus* (whether sensitive or resistant to sulphathiazole) had detectable activity as a growth factor for *L. citrovorum*.

DISCUSSION

The resistance of *Staphylococcus aureus* R122 to sulphonamides may be due at least in part to the synthesis of relatively large amounts of *p*-aminobenzoic acid. This organism synthesized twenty times as much *p*-aminobenzoic acid as did *S. aureus* strains JMH and H, and its growth was about one hundred times less sensitive to inhibition by sulphathiazole. A better comparison would be with a sensitive substrain of *S. aureus* R122 but this was not obtained. *S. lactis* 2102R showed a four-fold increase in *p*-aminobenzoate synthesis over the parent strain while its resistance to sulphathiazole had increased 50-fold. In other work where an increased synthesis of *p*-aminobenzoic acid was accompanied by sulphonamide-resistance there was not always a proportional relation between increase in *p*-aminobenzoate synthesis and change in resistance. For instance, the sulphonamide-resistant staphylococci described by Landy *et al.* (1943) synthesized about seventy times more *p*-aminobenzoic acid than did the parent strains, but showed a thousand-fold decrease in sensitivity to the drug.

All the staphylococcal strains examined here produced material which replaced folic acid for growth of *Lactobacillus casei* but which gave a response curve differing from that to synthetic pteroylglutamic acid (or other forms of folic acid available for testing; Lascelles & Woods, 1952). This *L. casei* factor may be a conjugate of folic acid with glutamic acid because it was not active in supporting the growth of

Streptococcus faecalis (which does not respond to known conjugates) in a medium deficient of folic acid, though it did support growth after hydrolysis with acid. *Staphylococcus lactis* 2102R, during growth and when incubated with *p*-aminobenzoic acid + glutamate + glucose, did form appreciable quantities of material which supported the growth of *S. faecalis*, and which gave a response curve resembling that to synthetic rhizopterin (N^{10}-formylpteroic acid). Possibly this response of *S. faecalis* represents the inefficient utilization of the *L. casei* factor synthesized by *S. lactis* 2102R. It seems more probable, however, that this *S. faecalis* factor was formed by the staphylococcus in addition to the *L. casei* factor; extracts of *S. aureus* R122 which were also rich in *L. casei* factor had low activity for *S. faecalis*.

Only *Staphylococcus lactis* 2102R and *S. aureus* R122 synthesized much more *Lactobacillus casei* factor than did the sulphonamide-sensitive strains of staphylococci either during growth or when suspended in a solution containing *p*-aminobenzoic acid + glutamate + glucose (mixture F). Nevertheless, in all the resistant strains the synthesis of *L. casei* factor in this solution was less sensitive to inhibition by sulphathiazole.

Sevag & Ishii (1958) gave evidence for a changed pathway of folic acid synthesis induced by sulphathiazole. Growth of a *p*-aminobenzoate-requiring mutant of *Escherichia coli* in the presence of sulphathiazole enabled the organisms to synthesize folic acid from 2-amino-4-hydroxy-6-carboxypterin and *p*-aminobenzoic acid. This ability was also shown by resistant organisms grown in the presence or absence of sulphathiazole, but not by sensitive organisms grown in the absence of sulphathiazole. It has not been shown whether this new pathway in *Escherichia coli* is less sensitive to inhibition by sulphathiazole.

Growth of organisms in the presence of sulphathiazole was necessary in the present work in order to obtain appreciable synthesis of folic acid by washed suspensions of organisms. The organisms were depleted of folic acid by growing with sulphathiazole; (they may accumulate precursors of folic acid); only glucose was required as an essential for synthesis of folic acid by these suspensions. Lascelles & Woods (1952) found that addition of 2-amino-4-hydroxy-6-formylpterin increased synthesis of folic acid by *Staphylococcus lactis* 2102 and 2102R in mixture F, but it was rather less effective than a mixture of amino acids which approximately doubled the synthesis of folic acid; other pterins, including 2-amino-4-hydroxy-6-carboxypterin, had no effect. There is however no evidence that sulphathiazole alters the pathway of folic acid synthesis in Staphylococcus. Although *S. lactis* 2102R synthesizes about ten times more folic acid than its parent strain, it does so from the same exogenous substrates, and the difference in the amount of folic acid formed is still shown during growth in the absence of sulphathiazole.

In general the results of the present work suggest that even within a group of closely related organisms there may be more than one mechanism by which resistance to sulphonamides is achieved, and that even two mechanisms may exist in the same organism. Thus one resistant strain (*Staphylococcus aureus* R122) was able to synthesize abnormally large amounts of *p*-aminobenzoic acid and was also able to convert this to folic acid more efficiently than were sensitive strains. Another strain (*S. lactis* 2102R) showed some (though less marked) increase in ability to synthesize *p*-aminobenzoic acid as compared with the parent (sensitive) strain, and

had a more marked ability than had *S. aureus* R122 to form folic acid. The other resistant strains (*S. aureus* JHMR and HR 2; *S. lactis* 2102 R 2) did not show significantly greater ability to synthesize *p*-aminobenzoic acid nor increased ability to convert it to folic acid. It seems likely, therefore, that yet another mechanism of resistance may operate in these strains. Some of these possibilities will be considered in the next paper (White & Woods, 1965).

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Biochemical Properties of Staphylococci Sensitive and Resistant to Sulphonamides

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SUMMARY

Four staphylococcal substrains resistant to sulphonamides were developed from sensitive parent strains by subcultivation in increasing concentrations of sulphathiazole. As compared with the parent strains, the growth of the resistant strains was 20–200-fold less sensitive to sulphathiazole in a semi-defined medium based on acid hydrolyzed casein than were the parent strains. Inhibition of growth by sulphathiazole was overcome competitively by *p*-aminobenzoic acid. The inhibition indices of the sulphathiazole-sensitive strains were about 10, and 100–1000 for the resistant substrains; the change in index was about proportional to the degree of resistance. The sulphathiazole-resistant strains were cross-resistant to other sulphonamides but were not resistant to *p*-nitrobenzoic acid. Not one of the sensitive or resistant strains was inhibited by aminopterin.

No change in the inhibitory effect of sulphathiazole occurred during growth of cultures in its presence, though the drug was partly converted to a second substance (of unknown structure). Resistant and sensitive pairs of strains formed similar amounts of unknown compound, so that it is unlikely that its formation has to do with resistance. Concentrated suspensions of organisms of all strains took up similar amounts of [^{35}S]-sulphathiazole when incubated in the growth medium. The attachment of the drug to the organisms was weak, but the uptake was not altered by the presence of *p*-aminobenzoic acid. The rates of aerobic growth and the nutritional requirements for anaerobic growth of sensitive and resistant strains were generally similar. The nutritional requirements for vitamins and amino acids varied among the sensitive strains, but there were only slight differences between resistant and sensitive pairs of strains.

INTRODUCTION

The syntheses of *p*-aminobenzoic acid and of folic acid (*Lactobacillus casei* factor) have been studied in three strains of staphylococci sensitive to sulphanomide-drugs and in five resistant strains (White & Woods, 1965). In only one of the resistant strains (*Staphylococcus aureus* R 122) was synthesis of *p*-aminobenzoic acid strikingly higher than in the sensitive strains, and only in this and one other resistant strain (*Staphylococcus lactis* 2102 R) was synthesis of folic acid increased above the value of the sensitive strains. All the resistant strains were able to synthesize folic acid from *p*-aminobenzoate, glucose and glutamate in the presence of higher concentrations of sulphathiazole than were the sensitive strains. Several biochemical

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mechanisms might contribute in the resistant strains to the decreased sensitivity to sulphathiazole of folic acid synthesis and of growth (Davis & Maas, 1952; Davis, 1957). To investigate some of these possibilities, the uptake and metabolism of sulphathiazole were examined. Other properties of sensitive and resistant strains were also compared to try to reveal further biochemical differences accompanying resistance.

METHODS

Organisms. The organisms and methods of cultivation were those used by White & Woods (1965). *Staphylococcus lactis* 2102R, 2102R2, *S. aureus* JHMR and HR2 were sulphonamide-resistant substrains of *S. lactis* 2102, *S. aureus* JHM and H, respectively; *S. aureus* R122 was a strain which was resistant to sulphonamides when isolated.

Media. These were prepared at double strength and brought to the desired volume with water and other experimental additions before autoclaving for 7 min. at 115°. Medium B was the semi-defined medium based on acid-hydrolyzed casein, which was described by White & Woods (1965). For anaerobic growth of staphylococci, medium B was supplemented with (mg./l.): $NaNO_3$, 170; uracil, 5; sodium pyruvate, 220; $NaHCO_3$, 1700. The sodium bicarbonate and pyruvate, sterilized by Seitz-filtration, were added to the autoclaved medium.

For the determination of amino acid requirements, the medium of Gladstone (1937) was used. To allow the amino acid content to be varied, each amino acid was made up in concentrated solution so that the addition of 0·1 ml. of each gave the final concentration required in 5 ml. of the completed medium.

Aerobic growth conditions. Inoculated medium (10 ml.) was incubated in optically matched 150 × 150 × 16 mm. ⊥-shaped glass tubes which were clamped in a rocking device and shaken at 37° in a water bath at 35 oscillations/min. with an excursion of 10 cm. (Morris & Woods, 1959). The inoculum was 0·2 ml. of a suspension in water of organisms (about 2×10^5/ml.) derived from an agar slope.

Semi-aerobic growth conditions. Medium (5 ml.) inoculated as above with 0·1 ml. of a similar suspension of organisms, was incubated at 37° in 150 × 19 mm. tubes, sloped at about 5° to the horizontal. This was the usual procedure for growth experiments, which were always set up in duplicate.

Anaerobic growth conditions. The usual conditions for growth experiments were followed, with the tubes incubated upright in a glass McIntosh and Fildes jar, in an atmosphere of 95% (v/v) hydrogen + 5% (v/v) carbon dioxide. The early stages of growth were observed through the sides of the jar, which was not opened at all until the end of an experiment.

Assessment of growth. Growth was estimated routinely by visual examination at intervals of about 12 hr during incubation. When quantitative results were required the turbidity of cultures was measured in 6 mm. diameter tubes with an EEL photoelectric colorimeter (Evans Electroselenium Co. Ltd, Halstead, Essex) with a neutral density filter; the uninoculated medium was used to give the zero setting. The cross-arms of the ⊥-tubes were made to fit the instrument, so that the tubes could be read in the colorimeter without withdrawing samples. Measurements of colorimeter readings in ⊥-tubes or in 6 mm. diameter tubes on the same culture agreed well. The relation between colorimeter reading and dry weight of organisms in water was linear for each strain of Staphylococcus, up to a reading of 3·0–3·5;

a reading of 1·0 was equivalent to 0·2 mg. dry wt. bacteria/ml. Very roughly, 1 ml. of a suspension reading 1·0 in the colorimeter corresponded to 10^8 colonies on peptone agar.

Growth of organisms for experiments with washed suspensions. Smaller quantities of organisms were grown in medium B (25 ml.) in 180 × 32 mm. tubes from large inocula as described by White & Woods (1965). Larger quantities were grown on the surface of tryptic digest of casein or peptone water, solidified with 2% (w/v) agar in Roux bottles, for 20 hr at 37°.

Chemical estimation of sulphathiazole. The method of Bratton & Marshall (1939) was used to estimate sulphathiazole at concentrations of 10 μM or higher. A standard curve was established relating concentration of solute to EEL colorimeter reading with green filter (OGR 1). Samples were usually diluted to give readings from 0·5 to 3·0 on the scale of the colorimeter.

Measurement of radioactivity. Solutions containing [^{35}S]-sulphathiazole were evaporated to dryness under an infra-red lamp on 15 mm. (internal diameter) planchets 1·5 mm. deep. The planchets were stamped out from aluminium sheet (0·1 mm. thickness) and were washed in acetone and water. A disk (15 mm. diameter) of Green's lens tissue No. 105 was put into a planchet before the solution was added. The disk caused the solution to dry evenly over the bottom of the planchet so that no correction for sample geometry was necessary. Counts were measured with an Ekco automatic scaler, Type N530.D (Ekco Works, Southend-on-Sea, Essex). An end-window Geiger-Müller tube Type EHM2/S (General Electric Co. Ltd, Magnet House, Kingsway, London) was connected to the scaler through a quenching probe unit, Ekco Type N558. There was no detectable effect of self-absorption provided the weight of dried sample was less than about 0·5 mg.; when necessary, samples were diluted to give weights on the planchet not greater than this. The relation between counts/min. (c.p.m.) and concentration of [^{35}S]-sulphathiazole was linear up to 3000 c.p.m. Sufficient counts were recorded for each solution to ensure that the probable error of the count was less than 2% (Francis, Mulligan & Wormall, 1954). Standard solutions of [^{35}S]-sulphathiazole were counted daily so that c.p.m. could be converted to mμmoles sulphathiazole.

The positions of radioactive substances on chromatograms were determined with an unshielded mica end-window Geiger–Müller tube which was connected to a scaler unit through a probe unit. The end-window of the Geiger tube was covered by a thin polythene disk with a hole 2 cm. square in the centre. Squares of this size were lightly drawn down the whole length of a chromatogram and the radioactivity of each square was measured for 2 min. by applying the counting tube window to the paper.

Chromatography. Descending chromatograms were run on Whatman No. 1 or No. 3 paper at room temperature. The solvents (parts by vol.) were: (i) *n*-butanol 80 + water 20 (ii) *n*-butanol 80 + sp.gr. 0·88 ammonia 3 + water 17, with mixtures (i) and (ii) after mixing the upper organic layer was used; (iii) methanol 35 + *n*-amyl alcohol 17·5 + benzene 35 + sp.gr. 0·88 ammonia 4 + water 8·5; (iv) methanol 35 + *n*-amyl alcohol 17·5 + benzene 35 + 2 N-hydrochloric acid to saturation. Mixtures (iii) and (iv) formed single phases (Ekman, 1948).

To detect diazotizable amines, chromatograms were allowed to dry after development, and the paper was then exposed for 10 min. to nitrous fumes generated from

sodium nitrite + hydrochloric acid. After a further 10 min. in air the chromatograms were sprayed with a solution of N-(1-naphthyl)-ethylenediamine dihydrochloride (100 mg./100 ml. water).

Measurement of uptake of sulphathiazole. The method was similar in principle to that described by Mitchell (1953) for measurement of the permeability of staphylococci to phosphate. Organisms were grown in Roux bottles, harvested, washed and suspended in water. The dry wt. of the suspension was determined, a measured volume taken and the organisms centrifuged down, then resuspended to a measured volume to give a concentration equiv. to about 100 mg. dry wt. bacteria/ml. in the solution (at double strength) used for investigation of permeability. This suspension (1 ml.) was pipetted into a 125 × 16 mm. tube, and sulphathiazole and other test solutions added to final volume of 2 ml. The tube was incubated upright at 37° and then weighed. The organisms were centrifuged and the supernatant liquid (E) was removed as completely as possible with a fine Pasteur pipette into a clean tube. The tube containing bacteria was reweighed after removing the excess solution from the inner wall with filter paper. In each experiment a tube was incubated in which the sulphathiazole was similarly diluted by the constituents of the incubation mixture, but without the organisms. The concentration of sulphathiazole in this solution (F) and in the supernatant liquid E was estimated by measurement of radioactivity or occasionally by the method of Bratton & Marshall (1939). The amount of sulphathiazole associated with the organisms at the end of the incubation, Z c.p.m., is given by $Z = A_F V_F - A_E V_E - \frac{1}{4} V_P A_E$, where A_E and A_F are specific activities (c.p.m./ml.) of supernatant liquid E and solution F, respectively; V_E and V_F are the volumes (ml.) of E and F respectively; V_P is the volume (ml.) of the centrifuged pad of organisms. The volumes of F and E were calculated from the weights and relative density (1·01) of these liquids. The interstitial volume for closely packed spheres was taken to be 25% of the total volume of the pad of organisms (Conway & Downey, 1950), which was also calculated from its weight and relative density (1·13).

Biochemical tests. Various biochemical tests were performed on the staphylococci, using the methods described in standard works of bacteriology (Mackie & McCartney, 1931; Kolmer & Boerner, 1945).

Chemicals. These were of Analytical Reagent quality when available. Aminopterin (4-aminopteroylglutamic acid) was a gift from Lederle Laboratories Division of the American Cyanamide Co. (Pearl River, N.Y., U.S.A.; Dr E. L. R. Stokstad). Sulphonamides were obtained from May and Baker Ltd, Dagenham, Essex. Sodium pyruvate was prepared by Mr R. W. Wakelin. Haematin was prepared by Dr June Lascelles by the method described by Lascelles (1956).

Synthesis of [^{35}S](sulphonyl)-sulphathiazole. 1. *p*-Acetaminobenzene [^{35}S]-sulphonyl chloride. Acetanilide (10 m-mole) was reacted with [^{35}S]-chlorosulphonic acid (30 m-mole, 7·2 mC) under conditions described by Gilman (1932). The product was used without further purification for the next step; yield 1 g. (14% conversion of [^{35}S]-chlorosulphonic acid).

2. N^4-acetyl-[^{35}S](sulphonyl)-sulphathiazole. *p*-Acetaminobenzene [^{35}S]-sulphonyl chloride (4·3 m-mole) was mixed at room temperature with 2-aminothiazole (10 m-mole) in 13 ml. dry ethyl acetate (Fosbinder & Walter, 1939). After 18 hr at room temperature the ethyl acetate was removed at 80°. Water (0·25 ml.) was

added and stirred thoroughly. To decompose any remaining *p*-acetaminobenzene sulphonyl chloride, 80 % (v/v) acetic acid (0·21 ml.) and water (1·9 ml.) were added and the mixture was warmed to 50° for 30 min. The suspension of N^4-acetylsulphathiazole was cooled to room temperature and left for 4 hr before the solid was collected and washed with cold water. Yield 0·9 g. (70 % conversion of *p*-acetaminobenzene sulphonyl chloride).

3. Sodium [^{35}S]-(sulphonyl)-sulphathiazole. Sodium hydroxide (0·36 g.) was dissolved in water in a 125 × 16 mm. tube; N^4-acetyl-[^{35}S]-(sulphonyl)-sulphathiazole (0·9 g.) and 'Norite' active charcoal (30 mg.) were added, and the volume was made up to 3·5 ml. The mixture was boiled vigorously for an hour, allowing the volume to fall to 3·1 ml. The mixture was filtered and the charcoal washed with water (0·15 ml.). The filtrate was brought to 80° and sodium chloride (0·7 g.) was added. The mixture was cooled to 20°, and the precipitate of sodium [^{35}S]-sulphathiazole was collected and washed twice with cold saturated sodium chloride solution (0·3 ml.).

4. Purification and isolation of [^{35}S]-(sulphonyl)-sulphathiazole. Damp sodium sulphathiazole was dissolved in water and the volume made up to 7 ml. 'Norite' charcoal (30 mg.) was added and the mixture was stirred for an hour at room temperature before filtering. The charcoal was washed twice with water (0·3 ml.). Concentrated hydrochloric acid was added to the filtrate, until the initial precipitate redissolved. Citric acid (15 mg.) and 'Norite' (15 mg.) were added and the mixture was stirred for an hour. The charcoal was filtered off and washed with water (0·3 ml.). A solution of ammonia was prepared from sp.gr. 0·88 ammonia (6·4 ml.) and water (12 ml.), and was added to the filtrate until it was alkaline to Congo Red, but acid to litmus. The suspension of [^{35}S]-(sulphonyl)-sulphathiazole was cooled and filtered. Yield 0·45 g. (58 % conversion of N^4-acetyl-[^{35}S]-(sulphonyl)-sulphathiazole.) Mixed melting point with authentic sulphathiazole 198–200°. The [^{35}S]-sulphathiazole resembled authentic sulphathiazole on chromatography with butanol + ammonia and with methanol + benzene + *n*-amyl alcohol + ammonia (R_F values 0·45 and 0·40 in the two solvents, respectively.) The [^{35}S]-sulphathiazole (mM) inhibited growth of *Staphylococcus aureus* JHM in medium B. This inhibition was overcome by *p*-aminobenzoic acid (0·1 mM) after 40 hr incubation. Similar results were obtained with authentic sulphathiazole.

[^{35}S]-Chlorosulphonic acid was obtained from the Radiochemical Centre, Amersham, Buckinghamshire. 2-Aminothiazole (specially purified material) was a gift from May and Baker Ltd, Dagenham, Essex.

RESULTS

Effects of inhibitors

Sulphonamides. The sensitivities of the various strains of Staphylococcus towards sulphathiazole, sulphanilamide, sulphadiazine and sulphapyridine were determined in medium B (Table 1). The highest concentrations of sulphadiazine and sulphapyridine that could be used were limited by the low solubilities of these substances in water. All the strains were most sensitive to sulphathiazole; sulphadiazine was the next most potent inhibitor and sulphanilamide the least effective. Resistance was not specific to the drug used to develop the resistant strain. The sensitivities

of the organisms to sulphathiazole were unchanged at the end of $2\frac{1}{2}$ years, during which time all the strains have been subcultured once every month, a total of about thirty transfers in the absence of drug.

Aminopterin. None of the strains of staphylococci was inhibited in medium B by aminopterin, even at the highest concentration tested (0·1 mM). Aminopterin was still inactive in the presence of just sub-inhibitory concentrations of sulphathiazole.

Table 1. *Sensitivity of the various strains of staphylococci to sulphonamides*

Organisms were grown in medium B at 37° in a set of tubes containing a serial 1/2 dilution of each drug, under the semi-aerobic conditions described in Methods. Values in brackets are times of incubation (hr) before growth occurred in the highest tolerated concentration of sulphonamide.

Organism	Highest concn (M) of drug permitting growth			
	Sulphathiazole	Sulphapyridine	Sulphadiazine	Sulphanilamide
Staphylococcus lactis 2102	10^{-5} (90)	*10^{-4} (90)	*10^{-4} (90)	5×10^{-4} (90)
2102R	5×10^{-4} (60)	*2×10^{-4} (20)	*2×10^{-4} (20)	*10^{-3} (20)
2102R2	5×10^{-4} (50)			
S. aureus JHM	5×10^{-6} (80)	*10^{-4} (80)	2×10^{-5} (80)	2×10^{-4} (80)
JHMR	*10^{-4} (60)	*2×10^{-4} (40)	*2×10^{-4} (40)	*10^{-3} (40)
S. aureus H	10^{-5} (80)	10^{-4} (70)	5×10^{-6} (80)	*4×10^{-4} (70)
HR2	*10^{-3} (60)	*2×10^{-4} (30)	*2×10^{-4} (30)	*10^{-3} (30)
S. aureus R122	5×10^{-4} (40)	*2×10^{-4} (30)	*2×10^{-4} (30)	*10^{-3} (30)

* Highest concentration tested.

Table 2. *Sensitivity of the various strains of staphylococci to p-nitrobenzoic acid*

Organisms were grown in medium B at 37° in a set of tubes containing a serial 1/2 dilution of *p*-nitrobenzoic acid, under the semi-aerobic conditions described in Methods.

Organism	Highest concn (M) of *p*-nitrobenzoic acid permitting growth after:				
	20 hr	30 hr	40 hr	50 hr	80 hr
Staphylococcus lactis 2102	10^{-4}	3×10^{-4}	10^{-3}	—	—
2102R	10^{-4}	10^{-3}	10^{-3}	—	—
2102R2	3×10^{-5}	3×10^{-5}	10^{-3}	—	—
S. aureus JHM	10^{-6}	10^{-4}	10^{-4}	10^{-3}	—
JHMR	10^{-6}	10^{-4}	10^{-3}	10^{-3}	—
S. aureus H	10^{-4}	10^{-3}	10^{-3}	2×10^{-3}	—
HR2	10^{-4}	10^{-3}	2×10^{-3}	2×10^{-3}	—
S. aureus R122	$< 2{\cdot}5 \times 10^{-4}$	$< 2{\cdot}5 \times 10^{-4}$	$< 2{\cdot}5 \times 10^{-4}$	$2{\cdot}5 \times 10^{-4}$	10^{-3}

p-Nitrobenzoic acid. *p*-Nitrobenzoic acid is an analogue of *p*-aminobenzoic acid which is inhibitory to some organisms especially in the presence of aspartic acid (Davis, 1951). In medium B (which contains aspartic acid) none of the strains of staphylococci was very sensitive to inhibition by *p*-nitrobenzoic acid (Table 2). Development of sulphonamide-resistance did not lead to any marked change in sensitivity to *p*-nitrobenzoic acid; *Staphylococcus aureus* R122 (the sulphonamide-resistant wild strain) was the most sensitive. Inhibition by *p*-nitrobenzoic acid (mM) was prevented by *p*-aminobenzoic acid, though the final amount of growth was

lower than in the absence of the inhibitor. There was no improvement of growth by addition of *p*-hydrozybenzoic acid, alone or together with *p*-aminobenzoic acid.

The inhibition index (Shive, 1950) is a useful concept in considering competitive inhibitions by analogues of metabolites. The value of K, the inhibition index, is given by $K = [I]/[S]$, where [S] is the metabolite concentration and [I] is the inhibitor concentration which gives a defined inhibition within a constant period of time. The concentrations [I] and [S] must be great enough not to be appreciably altered during the period of the experiment by the metabolic activities of the biological system. The inhibition by sulphathiazole of the growth of staphylococci in medium B was completely annulled by the addition of *p*-aminobenzoate. The inhibition indices were calculated from the concentration of *p*-aminobenzoate needed to permit half-maximal growth in medium B containing 2 mM-sulphathiazole after 40–45 hr incubation (Table 3). The inhibition indices of the various strains were roughly proportional to their degreees of resistance. As the time of incubation increased so the concentration of *p*-aminobenzoic acid needed to overcome inhibition decreased. Growth in these lower concentrations of *p*-aminobenzoate was as heavy as when sulphathiazole was absent, though there was a longer lag before growth began. With higher concentrations of *p*-aminobenzoic acid growth in the presence of sulphathiazole was as rapid as in the absence of the drug.

Table 3. *Relation between inhibition index and resistance of strains of staphylococci to sulphathiazole*

Organisms were grown at 37° under the semi-aerobic conditions described in Methods, in medium B containing 2 mM-sulphathiazole, together with serial 1/2 dilutions of *p*-aminobenzoic acid. The lowest concentration of *p*-aminobenzoate was determined that allowed at least half-maximal growth in 40–45 hr.

Organism	Inhibition index (K) after 40 hr	ΔK in resistant strain	Degree of resistance*
Staphylococcus lactis 2102	20	—	—
2102R	200	×10	×25
2102R2	200	×10	×25
S. aureus JHM	15	—	—
JHMR	330	×20	×20
S. aureus H	10	—	—
HR2	1000	×100	×100
S. aureus R122	660	×66†	×100†

* In medium B without *p*-aminobenzoic acid. † Compared to *S. aureus* H.

Metabolism of sulphathiazole

Cultures of the sensitive and resistant strains of staphylococci were grown from large inocula in medium B (25 ml.) in the presence of sulphathiazole (50 μM). After incubation (14 hr) the organisms were centrifuged down and the supernatant liquid heated in boiling water for 5 min. Sulphathiazole was estimated at the beginning and at the end of the incubation by the method of Bratton & Marshall (1939). These experiments showed no disappearance of sulphathiazole during growth.

Supernatant liquid from cultures grown in the presence of sulphathiazole (mM)

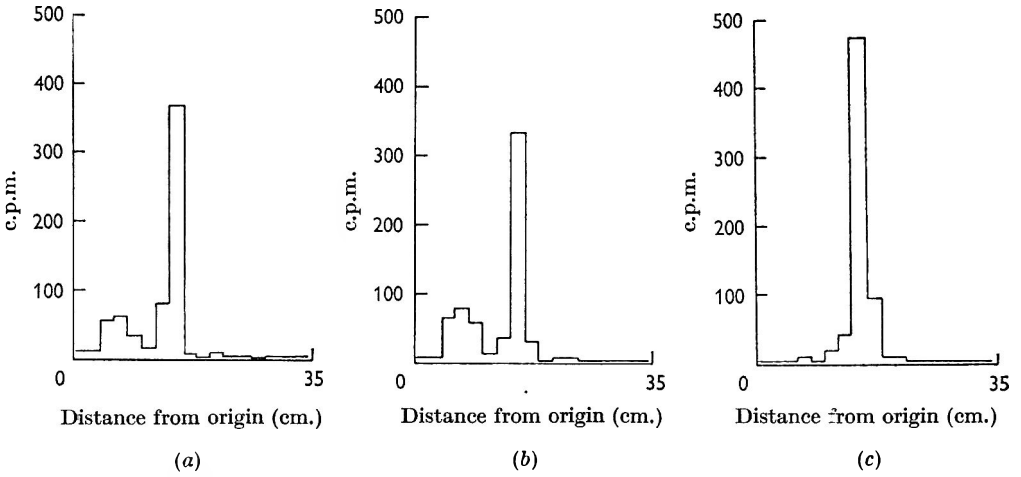

Fig. 1. Radioactivity on chromatograms of culture filtrates of *Staphylococcus aureus* H and HR2 grown in medium B for 14 hr in the presence of [^{35}S]-sulphathiazole (mM). Solvent: methanol + amyl alcohol + benzene + water + ammonia. (*a*) *S. aureus* H filtrate; (*b*) *S. aureus* HR2 filtrate; (*c*) [^{35}S]-sulphathiazole (mM) autoclaved in medium B then incubated 14 hr at 37°.

Table 4. *Chromatography of amino compounds related to sulphathiazole*

Descending chromatograms were run on Whatman No. 1 paper for about 6 hr at room temperature.

Substance	R_F value in: Solvent 1	Solvent 2	Solvent 3	Solvent 4
Sulphathiazole	0·43	0·43	0·55	0·53
p-Aminobenzoic acid.	0·24	0·23	0·10	0·10
4-Amino-5-imidazolecarboxamide	0·30	0·27	—	—
Sulphanilic acid	0·28	—	—	—
Sulphanilamide	0·49	—	—	—
Unknown compound	0·19	0·22	0·15	0·13

Solvent 1: methanol + amyl alcohol + benzene + water + ammonia; solvent 2: methanol + amyl alcohol + benzene + hydrochloric acid; solvent 3: butanol + water; solvent 4: butanol + ammonia + water.

were also tested for their potency in inhibiting growth of a sulphathiazole-sensitive strain (*Staphylococcus aureus* H) in medium B. The concentration of sulphathiazole in the diluted culture filtrate was taken to be the same as that of a standard solution of sulphathiazole which inhibited growth of the test organisms for the same length of time. Results from two or three dilutions of the filtrate gave values in very close agreement for the concentration of sulphathiazole in the undiluted filtrate. With all the sensitive and resistant strains of staphylococci tested there was no change in the inhibitory effect of the sulphathiazole after organisms had grown in its presence.

Chromatograms from cultures which had grown in the presence of sulphathiazole (usually mM) showed two spots of a similar brownish-red colour when diazotized and sprayed with the Bratton & Marshall reagent. The larger spot corresponded in position to the spot given by sulphathiazole itself (Table 4). The smaller spot was

chromatographically distinct from 4-amino-5-imidazolecarboxamide, *p*-aminobenzoic acid, sulphanilamide and sulphanilic acid; it was not present in cultures which had grown in the absence of sulphathiazole nor in autoclaved sulphathiazole-containing medium immediately after inoculation, or after incubation (14 hr) of uninoculated medium.

Table 5. *Radioactivity of* [^{35}S]*-sulphathiazole and unknown compound on chromatograms*

Organisms were grown in medium B at 37° (14 hr) in presence of [^{35}S]-sulphathiazole (mM). The unknown compound and [^{35}S]-sulphathiazole from culture filtrates were separated on chromatograms, and the paper was scanned for radioactivity, as described in Methods. Solvent: methanol + amyl alcohol + benzene + water + ammonia.

	c.p.m. 'Unknown'	c.p.m. Sulphathiazole	c.p.m. Total	% of total activity due to unknown
Staphylococcus lactis 2102	22	265	294	7
2102R	14	313	333	4
S. aureus JHM	82	423	512	16
JHMR	73	436	517	14
S. aureus H	155	457	687	23
HR2	204	404	666	31
S. aureus R122	95	181	289	33
*Sulphathiazole	21	611	699	3

* Incubated in uninoculated medium B at 37° (14 hr).

When liquid from cultures grown in the presence of [^{35}S]-sulphathiazole (mM) were chromatographed, radioactivity was found in the same positions as the two spots revealed by the Bratton & Marshall reagent, and nowhere else (Fig. 1). Only one peak of radioactivity was found after [^{35}S]-sulphathiazole was autoclaved and incubated in uninoculated medium. An approximate estimate of the proportion of the sulphathiazole that was converted to the unknown compound by the various strains during growth was made by comparing the number of counts found in each of the two peaks (Table 5). *Staphylococcus lactis* 2102 and 2102R formed much less of the unknown material than did the strains of *S. aureus*, but there was no correlation between the activity found in the peak due to the unknown compound and the sensitivity of the strain to sulphathiazole. For this reason, the chemical nature of the unknown compound was not investigated further.

Uptake of sulphathiazole by washed suspensions of staphylococci

The amount of sulphathiazole taken up by organisms during growth in medium B was so small as to be barely detectable even when [^{35}S]-sulphathiazole was used. The uptake was brought to a measurable value by using washed suspensions to increase the concentration of organisms. Suspensions were incubated in medium B for 1 hr usually in the presence of [^{35}S]-sulphathiazole (5 mM) and the uptake of drug determined as described in Methods. Chromatography of material extracted by boiling water from *Staphylococcus aureus* HR2 after incubation with sulphathiazole showed the presence of only one diazotizable amine, which was sulphathiazole itself. The uptake of sulphathiazole by all the strains, sensitive and resistant, was

about the same (Table 6). The uptake by *S. aureus* JHM and H and their resistant substrains was roughly proportional to the concentration of sulphathiazole in the medium during the incubation; the ratio: mμmoles sulphathiazole/x ml. organisms: mμmoles sulphathiazole/x ml. medium was fairly constant at different concentrations of sulphathiazole. A value greater than unity for this ratio indicates that the concentration of drug associated with the organisms is higher than that in the medium.

The values obtained for the uptake of sulphathiazole were compared with the amount of sulphathiazole that could be recovered from the organisms by washing with water or with a solution of sulphathiazole. The organisms which had been incubated for 1 hr with [^{35}S]-sulphathiazole (4 mM) were washed twice, either with water (2 ml.) or with unlabelled sulphathiazole (mM). Each washing procedure removed a similar amount of [^{35}S]-sulphathiazole from the organisms. This amount was the same as had been calculated to be taken up by the organisms (from the dilution of the [^{35}S]-sulphathiazole at the beginning of the experiment). These findings indicate that the sulphathiazole taken up by the organisms was not bound firmly, but exchanged very easily with the external medium.

Table 6. *Uptake of sulphathiazole from medium B by washed suspensions of staphylococci*

Suspensions of organisms were incubated for 1 hr at 37° in medium B with [^{35}S]-sulphathiazole. Uptake was assessed by measurement of the dilution of the radioactivity by the suspension of organisms (see Methods). Values are averages of duplicate determinations.

	Uptake of sulphathiazole (mμ moles/mg. dry wt. bacteria) Sulphathiazole (M) during incubation:			
Staphylococcus strain	5×10^{-3}	4×10^{-3}	$2{\cdot}5 \times 10^{-3}$	5×10^{-4}
S. lactis 2102	20 (1·3)	—	—	—
2102R	34 (1·5)	—	—	—
S. aureus JHM	23 (1·8)	15 (1·6)	10 (1·5)	3 (3·0)
JHMR	18 (1·4)	18 (1·8)	11 (2·0)	—
S. aureus H	20 (1·5)	—	10 (1·5)	—
HR2	22 (1·7)	—	11 (2·0)	—

Values in brackets are for the ratio mμmoles sulphathiazole/x ml. organisms: mμ moles sulphathiazole/x ml. medium.

Uptake of sulphathiazole in the presence of p-aminobenzoic acid

Suspensions were incubated as before in medium B for an hour at 37° with [^{35}S]-sulphathiazole (2·5 mM) and various concentrations of *p*-aminobenzoic acid. The uptake of [^{35}S]-sulphathiazole by the organisms was calculated from measurements of the radioactivity of the supernatant liquid. No difference was found in the uptake of sulphathiazole whether or not *p*-aminobenzoic acid were present (Table 7).

Growth in medium B

More complex media such as tryptic meat broth or peptone water did not give much heavier or more rapid growth of the staphylococci than was found in medium B. In all the media tested, *Staphylococcus lactis* 2102 and its substrains grew less well than any of the strains of *S. aureus*.

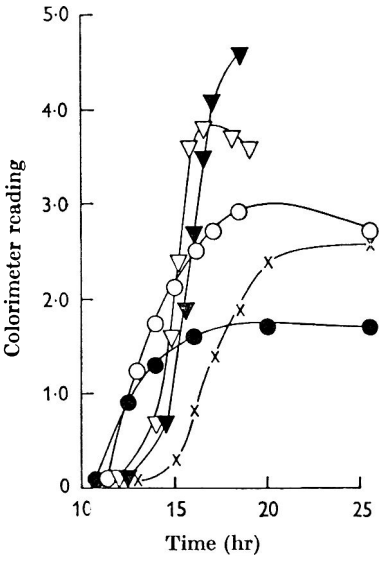

Fig. 2

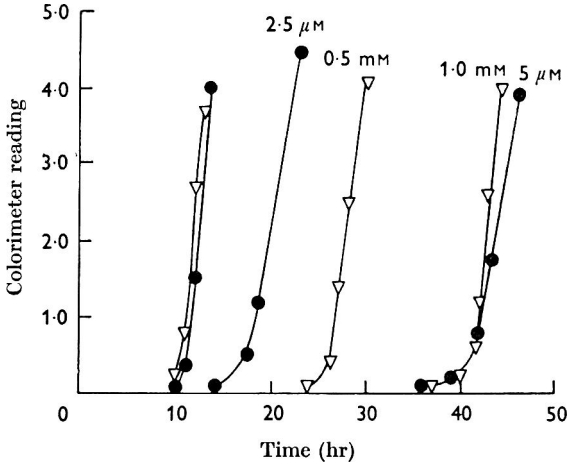

Fig. 3

Fig. 2. Aerobic growth of *Staphylococcus aureus* JHM and JHMR and of *S. lactis* 2102, 2102R and 2102R2 in medium B at 37°. ▽ *S. aureus* JHM, ▼ *S. aureus* JHMR, ○ *S. lactis* 2102, ● *S. lactis* 2102R, × *S. lactis* 2102R2.

Fig. 3. Aerobic growth of *Staphylococcus aureus* H and HR2 in medium B at 37° in the absence and presence of sulphathiazole (at the indicated concentrations). ● *S. aureus* H ▽ *S. aureus* HR2.

Table 7. *Uptake of* [^{35}S]*-sulphathiazole by staphylococci in the presence of p-aminobenzoic acid*

Suspensions of organisms were incubated 1 hr at 37° in medium B containing [^{35}S]-sulphathiazole (2·5 mM). Uptake was assessed by measurement of the dilution of the radioactivity by the suspension of organisms (see Methods). Values are averages of duplicate determinations.

Staphylococcus strain	Concn of *p*-aminobenzoic acid (M) during incubation	mμ moles sulphathiazole taken up/mg. dry wt. bacteria
S. aureus JHM	0	10
	$2·5 \times 10^{-5}$	12
	$2·5 \times 10^{-4}$	11
	$2·5 \times 10^{-3}$	12
S. aureus JHMR	0	11
	$2·5 \times 10^{-6}$	11
	$2·5 \times 10^{-5}$	10
	$2·5 \times 10^{-4}$	9
S. aureus H	0	10
	$2·5 \times 10^{-4}$	11
	$2·5 \times 10^{-3}$	13
S. aureus HR2	0	11
	$2·5 \times 10^{-5}$	14
	$2·5 \times 10^{-4}$	14
	$2·5 \times 10^{-3}$	12

Table 8. *Anaerobic growth of Staphylococcus lactis 2102, S. aureus* JHM *and* H *and resistant substrains*

Cultures were incubated anaerobically or semi-aerobically as described in Methods.

Medium	Strain 2102 a	2102 A	2012R a	2012R A	JHM a	JHM A	JHMR a	JHMR A	H a	H A	HR2 a	HR2 A
Medium B	0	1·1	0	0·7	1·2	2·2	1·2	2·1	1·1	2·9	1·4	3·0
Medium B + S.T.	—	—	—	—	1·3	2·8	0·8	2·6	0·7	2·9	1·3	2·8
Medium B + anaerobic supplement	0	1·5	0	1·2	2·4	3·1	2·6	3·2	3·3	3·3	2·9	3·9
Medium B + anaerobic supplement + S.T.	—	—	—	—	3·0	2·8	2·9	3·1	3·0	—	2·8	4·0
Medium B without glucose + anaerobic supplement	—	—	—	—	0·1	1·3	0·2	1·4	0·1	1·7	0·4	1·6
Medium B without glucose + anaerobic supplement + S.T.	—	—	—	—	0·1	1·3	0·2	1·5	0·2	—	0·4	1·4
Medium B + Difco yeast extract (0·1 % w/v)	0·6	2·4	0·6	2·3	—	—	—	—	—	—	—	—
Peptone water	0·3	1·0	0	1·0	—	—	—	—	—	—	—	—
Tryptic meat broth	0·2	2·0	—	—	—	—	—	—	—	—	—	—

a, Anaerobic; A, semi-aerobic. Numbers are colorimeter readings of turbidity of cultures after about 100 hr incubation.
S.T. Sulphathiazole μM and 0·1 mM for sensitive and resistant strains, respectively.

Aerobic growth. Growth curves for the various strains were measured in medium B, by using rocked ⊥-tubes. All strains of *Staphylococcus aureus*, whether resistant or not, grew at about the same rate and to about the same degree in the absence of sulphathiazole. Typical growth curves, for *S. aureus* JHM and JHMR are shown in Fig. 2. *S. lactis* 2102 and its resistant substrains grew to a lower final value; *S. lactis* 2102R2 consistently showed a lag before visible growth began, about 4 hr longer than the lag of the parent or the other resistant strain (Fig. 2).

When increasing concentrations of sulphathiazole were added to the medium, the lag phase was progressively lengthened. The final amount of growth was little affected by sulphathiazole with strains of *Staphylococcus aureus*; higher concentrations of sulphathiazole were required to prolong the lag phase with the resistant substrains than with the parent strains (Fig. 3).

Semi-aerobic growth. In medium B the lag phase before visible growth began was rather longer, about 15 hr, than under aerobic conditions, and maximum turbidity was not reached until about 30 hr. The highest amount of growth of strains of *Staphylococcus aureus* gave a colorimeter reading of 2·0–3·0, and 1·5–2·0 for *S. lactis* and its substrains.

Anaerobic growth. Staphylococcus aureus is a facultative anaerobe. A defined medium which supported the aerobic growth of *S. aureus* did not do so under anaerobic conditions unless carbon dioxide, pyruvate and uracil were present (Fildes & Richardson, 1937); the presence of nitrate was also advantageous. *S. lactis* 2102 and 2102R grew very poorly anaerobically in several media (Table 8), only in medium B supplemented with yeast extract was there appreciable growth. Lascelles (1956) described a strain of *S. aureus* which grew anaerobically in the presence of haematin. However, addition of haematin to medium B did not permit anaerobic growth of *S. lactis* 2102, indeed semi-aerobic growth was inhibited.

An absolute requirement for the supplement of uracil, pyruvate bicarbonate and nitrate was not found in *Staphylococcus aureus* JHM and H or their resistant substrains grown anaerobically in medium B, though addition of these compounds improved growth (Table 8). In the absence of glucose, however, anaerobic growth was very poor. Both sensitive and resistant strains were able to grow anaerobically in medium B in the presence of sulphathiazole (μM and 0·1 mM, respectively).

Nutrition

Amino acid requirements. Each amino acid was omitted in turn from a complete mixture which supported growth. The amino acids that were most often needed for growth were valine and arginine, which were required by all the strains of staphylococcus (Table 9). It was surprising that all strains were able to dispense with cystine and use sodium thioglycollate as main source of sulphur, since Gladstone (1937) reported that the requirement of staphylococci for cystine was the most difficult to abolish by 'training'. No changes in requirements for amino acids accompanied resistance to sulphathiazole in strains of *Staphylococcus aureus*, although there were differences in the requirements of the various parent strains. *S. lactis* 2102 grew only after 30 hr when aspartic acid was omitted and after 60 hr in the absence of glutamic acid. The growth of *S. lactis* 2102R was as rapid in the absence of either acid as it was in the complete medium (25 hr). When both amino acids were omitted at the same time, *S. lactis* 2102 did not grow after 6 days whereas 2102R grew after

2 days. *Staphylococcus lactis* 2102 R2 grew in the absence of aspartic acid as rapidly as in the complete medium, but glutamic acid was absolutely required for growth within 4 days.

Vitamin requirements. Each vitamin (nicotinic acid, thiamine, biotin) was omitted in turn from medium B. All the strains of *Staphylococcus aureus* required for growth nicotinic acid and thiamine, but not biotin; whereas *S. lactis* 2102 and its substrains required only biotin. These requirements were unchanged in the presence of sulphathiazole.

Table 9. *Amino acid requirements of Staphylococcus lactis 2102, S. aureus* JHM, H *and* R 122 *and sulphonamide-resistant substrains*

Organisms were grown semi-aerobically at 37° (see Methods) with each amino acid omitted in turn from an otherwise complete medium.

+ Amino acid required for growth; − Amino acid not required for growth; (+) growth delayed when amino acid is omitted.

Cystine (replaced by thioglycollate); hydroxyproline; isoleucine; lysine and serine were required by none of the strains.

	Strain							
Amino acid omitted	2102	2102 R	2012 R2	JHM	JHMR	H	HR2	R 122
Tryptophan	+	+	+	+	+	−	−	−
Tyrosine	+	+	+	−	−	−	−	−
Valine	+	+	+	+	+	+	+	+
Alanine	+	+	+	−	−	−	−	−
Threonine	+	+	+	−	−	(+)	(+)	−
Leucine	+	+	+	−	−	+	+	(+)
Methionine	+	+	+	(+)	(+)	(+)	(+)	(+)
Phenylalanine	+	+	+	−	−	+	+	−
Arginine	+	+	+	+	+	+	+	+
Histidine	+	+	+	+	+	−	−	+
Aspartic acid	(+)	−	−	+	+	−	−	+
Glutamic acid	(+)	−	+	−	−	−	−	−
Proline	−	−	−	+	+	+	+	−
Glycine	−	−	−	(+)	(+)	−	−	(+)

Biochemical tests. The strains were examined by various biochemical tests used routinely in bacteriology: sugar fermentation, formation of indole, Voges–Proskauer, methyl red test, nitrate reduction, gelatin liquefaction and hydrogen sulphide formation. No changes accompanying resistance were found though the different parent strains showed some variations in their properties. All strains except *Staphylococcus lactis* 2102 and its substrains were coagulase positive, and grew on solid medium containing 7·5% (w/v) sodium chloride.

DISCUSSION

Few differences other than their sensitivities to sulphonamides were found in the present work between resistant and sensitive strains of Staphylococcus. All the strains took up similar amounts of sulphathiazole from the growth medium. Whether this uptake was at a particular site in the organisms was not established, but since the presence of *p*-aminobenzoic acid had no effect on the uptake it is unlikely that much of the sulphathiazole was combining at a site where *p*-aminobenzoate also reacted. Noll, Bang, Sorkin & Erlenmeyer (1951) found that the uptake

of sulphathiazole by *Escherichia coli* was decreased by the presence of *p*-aminobenzoic acid in the medium. There was, however, a limit below which the uptake of sulphathiazole could not be decreased by high concentrations of *p*-aminobenzoate, therefore it was concluded that part of the sulphathiazole was free in the organism and that part was in competition with *p*-aminobenzoic acid for a receptor site. In the present work the method of measuring uptake of drug was not sufficiently refined for changes to be detected in the uptake by different parts of the organism. If much of the sulphathiazole associated with the organism were, for instance, absorbed at the cell wall, this would mask differences in the uptake of sensitive and resistant strains at other regions in the cytoplasm.

Other workers have reported that sulphonamide-resistant organisms do not take up smaller amounts of sulphathiazole than sensitive strains (Yegian & Budd, 1945). Wacker, Trebst & Simon (1957) found that a resistant strain of *Enterococcus stei* took up more sulphanilamide than did a sensitive strain, although the binding of the drug was less firm in the resistant strain. In the present work, sulphathiazole was not bound firmly by any of the strains and was easily removed by washing with water.

No evidence was found to support the view that the resistance of any strain to sulphathiazole was due to the conversion of sulphathiazole into non-inhibitory products. Sulphathiazole was partly converted to an unknown compound by growing staphylococci, but this took place in sensitive and resistant pairs of strains to similar extents. Also the proportion of sulphathiazole converted to unknown material was too small to explain the degree of resistance found in the sulphathiazole-resistant strains. Clear evidence was not obtained as to whether the unknown material was inhibitory after separation from sulphathiazole on chromatograms, perhaps because of contamination of the unknown with *p*-aminobenzoic acid, from which it was difficult to get good separation. Since culture filtrates showed the full inhibitory effect of the sulphathiazole added before incubation, it seems likely that the unknown material had inhibitory activity similar to that of sulphathiazole.

The rate of growth of sulphonamide-resistant staphylococci has been reported to be lower than that of sensitive organisms (Steers & Sevag, 1949; Beljanski, 1953). This was not found with any of the strains of *Staphylococcus aureus* used in the present work. *S. lactis* 2102 R 2 showed a lag 4 hr longer than did *S. lactis* 2102 and 2102 R, though the rate of logarithmic growth of all three strains was the same. Another difference between strain 2102 R and strain 2102 R 2 was that the former did not grow as heavily as the parent strain in the semi-defined medium B. This difference was not shown when strain 2102 R was first used, but developed over a period of three years with monthly subcultivation.

Although there were differences between one sensitive strain and another in the nutritional requirements for anaerobic growth, no evidence was obtained that the development of resistance or the presence of sulphathiazole affect anaerobic metabolism. Sevag & Steers (1949) found that a sulphonamide-resistant strain of *Staphylococcus aureus* was unable to grow anaerobically, whereas the parent strain could grow to a limited extent. Steers & Sevag (1949) also reported that two sulphonamide-resistant strains of *S. aureus* suffered a partial loss of ability to synthesize amino acids in comparison with the sensitive parent strains. In the present work

almost no differences were found between the amino acid requirements of sensitive and resistant strains of *S. aureus*. *S. lactis* 2102 grew only after a lag when aspartic or glutamic acid was omitted from the medium, whereas strain 2102 R grew normally in the absence of either acid, though not of both together. The greater ability of the resistant strain to synthesize glutamic acid might be related to the increase in the synthesis of folic acid, which contains glutamic acid in its molecule. Lam & Sevag (1957) gave some evidence that sulphathiazole inhibited transaminase activity in a sensitive strain of *S. aureus*. It may be that in strain 2102 R, transaminase activity has increased with the development of resistance, resulting in an increased rate of synthesis of aspartic and glutamic acid from oxaloacetic and α-oxoglutaric acids, respectively. These interpretations, however, are not valid for *S. lactis* 2102 R2 which has an absolute requirement for glutamic acid but is able to grow without added aspartic acid.

Two possible mechanisms of resistance that have not been explored are the presence in the resistant strains of an increased amount of a *p*-aminobenzoate-utilizing enzyme or a changed relative affinity of an enzyme for *p*-aminobenzoic acid and sulphathiazole in the resistant strains. If an increased number of receptor sites for *p*-aminobenzoate were present in a resistant strain, then all analogues of *p*-aminobenzoic acid should become less effective inhibitors, and cross-resistance to *p*-nitrobenzoic acid would be expected. In fact, no appreciable cross-resistance to *p*-nitrobenzoic acid was observed in any of the sulphonamide-resistant strains.

The second possibility, a change in the relative affinities of an enzyme for *p*-aminobenzoic acid and sulphonamides, is compatible with all the experimental observations. It accounts for the increase in the inhibition index in the resistant strains and for the decreased sensitivity of the synthesis of folic acid (White & Woods, 1965) in these strains to inhibition by sulphathiazole. A decreased affinity for sulphonamides might well be specific (Davis & Maas, 1952) and the affinity for other inhibitors of *p*-aminobenzoate utilization, such as *p*-nitrobenzoic acid, might be unchanged. The evidence for this mechanism of resistance is indirect, but its value is strengthened by the evidence that other mechanisms are not operating.

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Resistance to various Inhibitors in *Aspergillus nidulans*

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SUMMARY

Resistant strains of *Aspergillus nidulans* were obtained by one-step selection on high concentrations of various inhibitors; each strain was mutant in a different gene conferring resistance to actidione (*Act*), *p*-fluorophenylalanine (*pf*), teoquil (*te*), iodoacetate (*Iod*) or malachite green (*mg*). Some mutant alleles have been firmly, others tentatively, located. For comparative purposes attempts were made to find instances of multi-step or non-genic increases in resistance to malachite green and to teoquil, by prolonged exposure to low concentrations of inhibitor. No such increases were found.

Iod 1 is fully dominant, *Act 1* is semidominant, *pf 21*, *te 1* and *mg 1* are recessive. *mg 1* confers resistance to acriflavine to about the same degree as the non-allelic *acr 2* but these two mutant alleles do not show additivity. *pf 21* confers resistance to iodoacetate and also suppresses requirement for nicotinic acid (*nic 8*). *Iod 1* strains, which are not resistant to fluoroacetate, are able to use acetate as sole carbon source.

Nutritionally balanced heterokaryons, between *Act 1* and sensitive strains, show a gradual (and reversible) increase of the *Act* component on increasing actidione concentrations. Ultimately a plateau is reached; this presumably represents the nutritional limits of each particular combination of nutritional markers.

INTRODUCTION

Mutant strains of micro-organisms resistant to various agents have been fruitfully applied in diverse genetical and biochemical studies. However, the only detailed studies of resistance in *Aspergillus nidulans* are those of resistance to acriflavine (Roper & Käfer, 1957) fluoroacetate (Apirion, 1962) and *p*-fluorophenylalanine (Morpurgo, 1961*b*). The present work was undertaken to extend the range of resistant strains of this species and to make initial investigations on their promise as tools in a variety of studies. One-step mutants were deliberately selected as one aspect of the work. However, it would be of interest to compare one-step high resistance with multigenic or non-genic resistance to the same substance. *A. nidulans* is suitable for the study of extrachromosomal inheritance by the combined use of heterokaryosis, the sexual cycle and parasexual cycle; it is also a potentially favourable organism for the analysis of multigenic inheritance via mitotic analysis. At haploidization there is segregation, without crossing-over, of whole chromosomes; for a first analysis this decreases the number of segregating units to the haploid number of chromosomes. Attempts were made to increase resistance by prolonged exposure to non-inhibitory or slightly inhibitory concentrations of various agents.

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METHODS

The general techniques used throughout this work were those of Pontecorvo Roper, Hemmons, Macdonald & Bufton (1953). Incubation was at 37°.

Media. Minimal medium (MM), Czapek–Dox with 2% glucose. Complete medium (CM), a complex medium containing yeast extract, casein hydrolysate, hydrolysed nucleic acid, vitamins, etc. Solid media contained 2% agar.

Inhibitors. Actidione, iodoacetate, fluoroacetate, *p*-fluorophenylalanine (from Koch–Light & Co. Ltd, Poyle Trading Estate, Colnbrook, Bucks.), acriflavine (from British Drug Houses, Ltd. Poole, Dorset), malachite green (from G. T. Gurr, Ltd, New King's Road, London, S.W. 6); teoquil, a polymethylene *bis* isoquinolinium salt with anti-fungal properties (Collier, Potter & Taylor, 1955) was kindly supplied by Allen & Hanbury, Ltd. (Ware, Hertfordshire).

Organisms. Strains were taken from laboratory stocks maintained on complete medium CM. Mutant alleles, and their locations, are given by Pontecorvo *et al.* (1953), Roper & Käfer (1957), Käfer (1958) and Pontecorvo (1963*a*). Mutant types used widely in this work were: *y*, yellow conidia, *w*, white conidia; *pyro*, *bi*, *paba*, *ribo*, *cys*, *s*, *an*, *nic*, *ad* and *phen* designate, respectively, requirement for pyridoxin, biotin, *p*-aminobenzoic acid, riboflavine, cystine, thiosulphate, aneurin (thiamine), nicotinic acid, adenine and phenylalanine; *Acr1* and *acr2*, resistance to acriflavine.

Selection of one-step resistant strains. Resistant strains were selected by spreading conidia at high density (10^6 to 2×10^7/dish) on CM or supplemented MM with a concentration of inhibitor sufficient to suppress completely germination of sensitive conidia. Ultraviolet (u.v.) irradiation (usually to about 2% survival) was used in most cases. Putative resistant strains were isolated from the highest concentration of inhibitor to give colonies. To avoid clones only one colony was isolated from each plating.

Prolonged exposure to inhibitors. In an attempt to find multi-genic or non-genic adaptation, conidia were spread (20 to 50/dish) on a concentration of inhibitor which permitted full viability but less than full linear growth rate. In one series of experiments a concentration giving only 20% viability was used. Conidia were taken from a single colony, after 3–4 days of incubation for the next subculture. At intervals survival curves were determined for control and treated organisms.

Survival curves. These provided the most accurate measurement of resistance. They were determined by spreading conidia (20 to 50/dish) on increasing concentrations of inhibitor. About 400 conidia were tested at each concentration of inhibitor. Colony counts were taken at 3 days and expressed as a percentage of a control without inhibitor.

Genetic analysis. Techniques of analysis were, in general, those described by Pontecorvo *et al.* (1953). Location of a mutant allele to its linkage group via mitotic haploidisation (Forbes, 1959) was facilitated by the *p*-fluorophenylalanine technique (Morpurgo, 1961*a*; Lhoas, 1961).

RESULTS

Prolonged exposure to inhibitor

The *Aspergillus nidulans* strain *paba6 y*; *nic2* was serially subcultured on CM with 0·27 mg. malachite green/l. Viability was 100%, but at 70 hr the mean colony

diameter was 0·6 cm. as compared with 2·6 cm. on inhibitor-free medium. Many hundreds of colonies were examined throughout the experiment but no sectors of vigorous growth were seen. Survival curves were determined for cultures taken after 10, 20 and 30 subcultures; these were compared with a control serially subcultured without inhibitor. No significant difference in resistance was found between the two lines at any time. A further series of seven subcultures were undertaken with the strain *y*; *s12*; *pyro4*; *nic2* on CM with 0·4 mg. malachite green/l. The viability averaged 20%. Survival curves were determined after each subculture but again no significant change in resistance was found.

Only one series of subcultures was made with teoquil, with strain *y*; *s12*; *pyro4*; *nic2* grown on 0·5 mg. teoquil/l. The viability was 100%. After 15 subcultures no change in resistance was found.

Table 1. *Properties of some resistant mutants of Aspergillus nidulans*

Inhibitor	Mutant allele*	Location	Maximum sub-inhibitory concentration for†	
			Sensitive	Resistant
Teoquil	*te*1	Linkage group III; 25 units proximal to *s*12	1·5 mg.	3·0 mg.
Malachite green	*mg*1	Undetermined. Not in groups I, II or IV	0·6 mg.	1·0 mg.
Iodoacetate	*Iod*1	Left arm II; 25 units distal to *Act* 1	1·5 g.	3·0 g.
p-Fluorophenylalanine	*pf*21	Left arm I; 0·3 units from *ribo* 1	0·5 g.	5·0 g.
Actidione	*Act*1	Left arm III. 8 units from *phen* 4	0·7 g.	2·5 g.

* Lower case initial for recessive, capital for dominant or semi-dominant. *pf* alleles numbered from 21 to avoid confusion with mutants of Morpurgo (1961*b*).

† Maximum amount/l. complete medium permitting growth by point inoculation of conidia.

One-step mutants

Five strains of *Aspergillus nidulans*, each resistant to a different inhibitor, were selected. Details of these strains are shown in Table 1.

Degree of resistance. With the exception of the teoquil resistant strain, all the selected strains could be classified unequivocally by point inoculation of conidia to dishes containing concentrations of inhibitor which completely suppress the growth of sensitive strains. Early tests for sensitivity and resistance on teoquil were often ambiguous. This was due to fragments of mycelium in the inoculum of sensitive strains; such fragments have substantially higher resistance than do conidia; this was shown as follows. Plates of CM without inhibitor were heavily seeded with conidia of a sensitive strain *paba6 y*; *nic2*. An appropriate quantity of teoquil was added at a series of marked places at 1 hr intervals during the first 10 hr of incubation. After 2 days the inhibition zones were measured. There was a decrease in the inhibition zone from the addition at time 0 to that at 5 hr, when a plateau was reached. The sharpest decrease in inhibition, between additions at 3 and 5 hr, coincided with the germination time. Satisfactory classification of resistance to teoquil could be made only by testing drops of dilute conidial suspensions which were almost free from mycelium.

Genetic analysis. Meiotic analysis showed that, in each strain, resistance is determined by mutation in a single gene. Both meiotic and mitotic analyses were used to locate the mutant alleles (Table 1). Tests of dominance were made in heterozygous diploids and, in the cases of *Iod 1* and *Act 1*, in heterokaryons balanced with nutritional markers. The complete dominance of *Iod 1*, and semi-dominance of *Act 1*, held in both heterozygote and heterokaryon.

Resistance to more than one inhibitor. Each resistant strain was tested for increased resistance to all five inhibitors. Acriflavine was also included since it was known (Roper & Käfer, 1957) that mutations conferring resistance to acriflavine also confer resistance to malachite green. Fluoroacetate was included for comparison with iodoacetate. The only positive results were with *mg 1* resistant to acriflavine, and *pf 21* resistant to iodoacetate and slightly resistant to actidione. Ideally, to eliminate the possible effects of residual genotype, such tests should be carried out on the parent resistant strain and on the resistant recombinants derived from a cross to a sensitive strain. This was done for *mg 1* but not for *pf 21*.

Malachite green and acriflavine. Because of the cross-resistance effects it was of interest to determine whether acriflavine and malachite green were additive in growth inhibition. To test this, 64 plates of minimal medium were each seeded with 10^5 wild-type conidia. The plates contained various amounts of malachite green and acriflavine. At 65 hr the plates could be sharply classified on the basis of growth or no growth with the result shown in Fig. 1. The two compounds were additive in effect.

The possible interaction of *mg 1* and *acr 2* was also examined. Segregants from the cross *y*; *phen 4 mg 1*; *pyro 4* × *ad 15 paba 1 y*; *acr 2* were tested by point inoculation on complete medium with acriflavine: 10, 15, 20, 25, 30 and 40 mg./l. Of 68 segregants, 49 were resistant, 19 sensitive. This was consistent with a 3:1 ratio and negative interaction of *acr 1* and *mg 1* is excluded. The 49 resistant segregants would have the genotypes *acr* 2^+ *mg 1*, *acr 2 mg* 1^+ and *acr 2 mg 1*. These segregants showed a uniform degree of resistance indistinguishable from that of the parents. Substantial additivity of effect of *acr 2* and *mg 1* was, therefore, excluded.

Interactions of resistance and nutrition. It was shown (McIlwain, 1941; Roper & Käfer, 1957) that certain nutrients protect against inhibition by acridine; this was found to apply to malachite green. Conidia of sensitive and *mg 1* strains were suspended in supplemented minimal medium with concentrations of malachite green just sufficient completely to suppress growth. Various additions were made to the plates before incubation. Annullment of inhibition was read as +, ± or −, according to growth response around each addition. Responses were: thymus nucleic acid, biotin, riboflavine, $ZnSO_4$, $Fe_3(SO_4)_2$ and $CuSO_4$, all +; L-cysteine, hydrolysed casein and $MnSO_4$, all ±. Many other nutrients, including a mixture of 19 amino acids, gave no response. These results applied equally to sensitive and *mg 1* strains.

Only annullment by riboflavine was studied further. Conidia of *y*; *phen 4*; *pyro 4* or *y*; *phen 4 mg 1*; *pyro 4* were spread on dishes of supplemented minimal medium containing various concentrations of malachite green (0–0·6 mg./l.) and riboflavine (0–400 mg./l.). Colony counts were made at 3 days. For both strains riboflavine competitively annulled malachite green inhibition.

A different aspect of the interaction of nutrition and resistance was noted with

pf21. The cross, *pf21bi1*; *w3*; *pyro4* × *ribo1y*; *Act1*; *ni8*, yielded the following segregants with respect to resistance and requirement for nicotinic acid: sensitive, nicotinic acid independent, 88; sensitive, nicotinic acid-requiring, 93; resistant-nicotinic acid independent, 171; resistant, nicotinic acid-requiring, 0.

Segregation of resistance *versus* sensitivity was in the expected 1:1 ratio; among the sensitive segregants, nicotinic acid requirement *versus* independence segregates as expected. However, there was a total absence of the resistant and nicotinic acid-

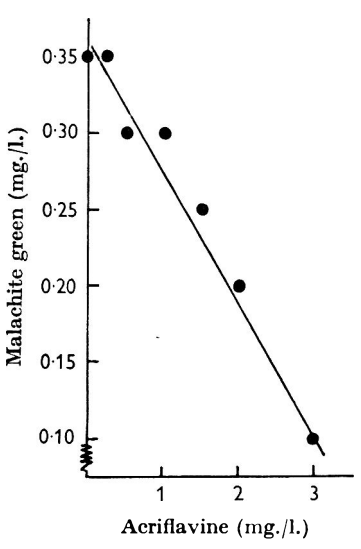

Fig. 1

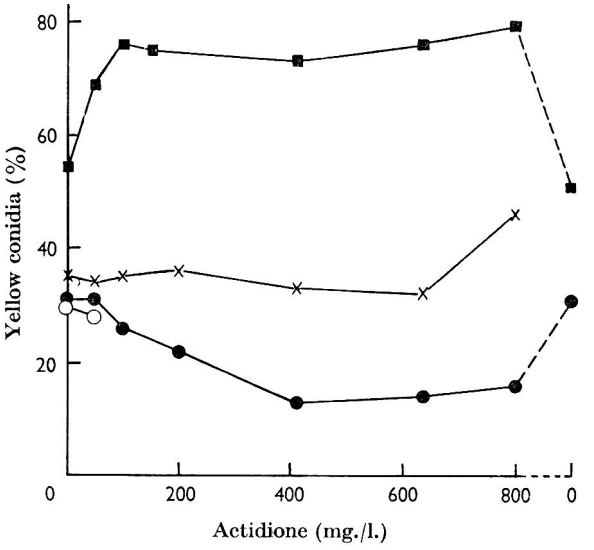

Fig. 2

Fig. 1. Additivity of acriflavine and malachite green in inhibition of growth of wild-type conidia of *Aspergillus nidulans*. Points represent limits of growth.

Fig. 2. *Aspergillus nidulans*; adaptive response of heterokaryons. ○, *ribo1 y*; *nic8* with *bi1*; *w3*; *cys2*; ■, *ribo1y*; *Act1*; *nic8* with *bi1*; *w3*; *cys2*; ●, *ribo1 y*; *nic8* with *bi1*; *w3*; *Act1*; *cys2*; ×, *ribo1y*; *Act1*; *nic8* with *bi1*; *w3*; *Act1*; *cys2*.

requiring class. The most likely explanation, in view of the otherwise normal phenotypic distributions, was suppression of *nic8* by *pf21*. Three resistant segregants, potentially carrying *nic8*, were outcrossed to sensitive nicotinic acid independent strains. In all three crosses a 1:1 ratio of sensitive:resistant was found among the segregants. From two crosses there were no segregants which required nicotinic acid. In the third cross, the resistant segregants were all nicotinic acid independent, while half of the sensitives required nicotinic acid.

Expression of Act1 in heterokaryons. Jinks (1952) was the first to demonstrate adaptive changes in the conidial ratios of heterokaryons in response to changes of media. The heterokaryons were synthesised from wild isolates of Penicillium. Some relatively complex situations have been explored in heterokaryons of *Aspergillus nidulans* (Pontecorvo *et al.* 1953; Pontecorvo, 1963*b*), but heterokaryons balanced on nutritional mutants are generally unsuitable for the exploration of a gradual adaptive change. *Act1*, because of its semi-dominance, suggested an alternative approach.

The following four heterokaryons were established on minimal medium:

1. *ribo 1y*; *nic 8+bi 1*; *w 3*; *cys 2*;
2. *ribo 1y*; *Act 1*; *nic 8+bi 1*; *w 3*; *cys 2*;
3. *ribo 1y*; *nic 8+bi 1*; *w 3*; *Act 1*; *cys 2*;
4. *ribo 1y*; *Act 1*; *nic 8+bi 1*; *w 3*; *Act 1*; *cys 2*.

Each heterokaryon was subcultured by transfer of mass hyphal tips, to dishes of minimal medium with various concentrations of actidione. Conidial ratios were determined for these heterokaryons at each actidione concentration. Conidia were randomly sampled from all areas of each colony; the ratio of white:yellow was determined by plating at least 400 conidia from each heterokaryon. The results (Fig. 2) show a clear adaptive response of heterokaryons 2 and 3. This response is reversed by subculture from actidione back to drug-free minimal medium. Slight differences between heterokaryons 2 and 3 in their degree of response may well be due to differences in residual genotype. The change in heterokaryon 4, at the highest actidione concentration, is probably due to sampling error. The plateau reached by heterokaryons 2 and 3 presumably reflect the nutritional limits of each heterokaryotic combination.

DISCUSSION

Multi-step or non-genic adaption. Malachite green was chosen for the major part of this work, its choice being dictated by the fact that one-step mutants were available for comparison. Furthermore, Wild & Hinshelwood (1955) and Bartlett (1959) had shown fungal adaptation to brilliant green whose structure is closely related to that of malachite green. Polygenic resistance is already known (Cavalli & Maccacaro, 1952; Hotchkiss & Evans, 1958). Non-genic resistance has been shown for several inhibitors in a variety of organisms (see for example Dean & Hinshelwood, 1952, 1954); although such adaptation has had a controversial history, it now appears likely that the pnenomenon can be accommodated by ideas such as those of Pollock (1953) and Jacob & Monod (1961).

In the present studies with malachite green and teoquil, neither multigenic or non-genic increase in resistance emerged at low concentrations of inhibitor. It is impossible to draw any substantial conclusion from such negative results; since the investigations were laborious and unrewarding they were not pursued further. A more profitable approach to multigenic resistance might well be through further selection from the one-step mutants but this has not yet been attempted.

One-step resistance. Resistant strains of *Aspergillus nidulans*, each mutant in a different gene, were readily obtained for each of the five tested inhibitors. Resistance to so diverse a range of substances shows one facet of the potential biochemical flexibility of the organism. Genetically determined resistance is undoubtedly achieved through any one of a variety of mechanisms; this is to some extent reflected in the different dominance relationships found in mutations to different or even to the same inhibitor (Roper & Käfer, 1957). No attempt has yet been made to pinpoint the biochemical effects of mutation in the present resistant strains though indirect preliminary evidence is available in some cases. Resistance to iodoacetate is of interest since iodoacetate inhibits a wide range of enzymes. Change in permeability seems a likely mechanism. However, *Iod 1* strains did not show increased resistance to fluoroacetate and they were able to use acetate as sole carbon

source; any change in permeability would need to be very highly selective. Resistance to *p*-fluorophenylalanine might arise through modification of an amino acid activating enzyme (Yanofsky, Helinski & Maling, 1961; Lewis, 1963) and this might also account for suppression by *pf21* of *nic8*. However, it seems reasonable to suppose that a mutation leading to such a change would be semi-dominant rather than recessive. An alternative mechanism of suppressor action, invoking change at the level of the ribosomes, is suggested by the work of Davies, Gilbert & Gorini (1964). Shikimic acid is a precursor of phenylalanine and nicotinic acid (Davis, 1955) and it is possible to suggest yet another mechanism of resistance and *nic8* suppression, based on the increased production of shikimic acid. Morpurgo (1961*b*) isolated strains of *A. nidulans* resistant to *p*-fluorophenylalanine and showed that some had a partial requirement for tyyrosine. From a comparison of linkage data (De Palma & Morpurgo, 1963) it seems very likely that Morpurgo's mutations and ours, which have no requirement for tyrosine, are allelic. If so, the *pf* locus poses a fascinating problem of pleiotropic effects differently expressed among non-identical allelic mutants. Any explanation of resistance determined by *pf21* must also take account of the unexpected cross-resistance to iodoacetate and actidione. Actidione appears to block either transfer of amino acids to ribosomes or polymerisation of amino acids (Siegel & Sisler, 1963). Both *pf21* and *Act1* show actidione resistance but do not yet give any pointers to the mechanisms of resistance. The *A. nidulans* mutant allele *mg1*, and the mutations studied by Roper & Käfer (1957), confer resistance to both malachite green and acriflavine. Malachite green and acriflavine are additive in inhibition of wild type and this might indicate a common sensitive site. The non-allelic mutations *mg1* and *acr2* are similar in the degree of resistance that each confers but they do not show any interaction in double mutants; this is in contrast to the situation in phage (Hessler, 1963). Annullment of inhibition by acriflavine or malachite green can be achieved with a variety of nutrients and such effects should be interpreted cautiously. Annullment of acriflavine inhibition by nucleic acid (McIlwain, 1941; Roper & Käfer, 1957) may be due merely to complexing of the nucleic acid and acriflavine outside the cell. On the other hand, competition between malachite green and riboflavine, both for sensitive and *mg1* strains, may reflect relief of inhibition of flavine containing enzymes. Inhibition of such enzymes by acridines is known (Haas, 1944; Hellerman, Lindsay & Bovarnick, 1946) and alterations in flavine containing enzymes is associated with acridine resistance in pneumococci (Gots & Sevag, 1948; Sevag & Gots, 1948*a*, *b*). Acridines have now assumed importance as mutagens for chromosomal and non-chromosomal elements and it would be particularly interesting to elucidate the mechanisms of resistance to them.

The main application of the *Aspergillus nidulans* mutants in the present work has been, for the present, in exploration. All the mutants, except *te1* which is laborious to classify, provide good selective markers for meiotic and mitotic analysis. The present work has shown the ease with which a variety of resistant mutants may be isolated; their differing dominance provides useful flexibility in a variety of genetic studies. *Act1*, a semi-dominant allele, has been used to show adaptive changes of conidial ratio in heterokaryons. Such changes could result from differential effects of actidione on the production of conidia by the component strains. But, since a plateau is reached in each adaptive response, it is far more likely that the conidial

changes reflect changes in nuclear ratio. Balanced heterokaryons of *A. nidulans* generally achieve a particular nuclear ratio dependent on the component strains (Pontecorvo *et al.* 1953; Clutterbuck, 1964). By use of the present system it should be possible to explore the nutritional limits of particular combinations of nutritional mutants. This could offer an approach, in *Aspergillus*, to the ideas of Kacser (1963) on rate-limiting reactions.

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The Properties of Phospholipase Enzymes in Staphylococcal Toxins

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SUMMARY

Two phospholipase enzymes have been identified in toxic preparations from *Staphylococcus aureus*, each having a mode of action like that of phospholipase C. One enzyme hydrolysed phosphatidyl inositol and lysophosphatidyl inositol, while the other hydrolysed sphingomyelin and lysophosphatidyl choline. The latter enzyme was always associated with β-haemolysin activity and it is concluded that β-haemolysin, sphingomyelinase and lysophospholipase are activities of one protein. A phospholipase A was also detected in a toxic preparation from an $\alpha\beta$ strain. A study of cultural conditions showed that this enzyme was produced under a variety of conditions, but only when the β-haemolysin activity was high.

INTRODUCTION

In preliminary communications (Magnusson, Doery & Gulasekharam, 1962; Doery, Magnusson, Cheyne & Gulasekharam, 1963) it was reported that toxic preparations from a strain of *Staphylococcus aureus* (*S. pyogenes*; strain B.S.M. 24) contained two enzymes which were separable by curtain electrophoresis. These enzymes hydrolysed the phosphatidyl inositols, (phosphatidyl inositol and lysophosphatidyl inositol) and sphingomyelin, respectively. It was also shown that the crude toxic culture fluid hydrolysed lysophosphatidyl choline. In each case, the mode of hydrolysis of the phospholipids was similar to that caused by phospholipase C.

The distribution of these activities in toxic preparations from a number of strains of *S. aureus* was examined. Inositol phospholipase was found in all the toxic culture fluids which contained α-haemolysin, while sphingomyelinase and lysophospholipase were found in all toxic preparations which contained both α- and β-haemolysins. No phospholipase activity was detected in culture fluids from non-haemolytic coagulase-negative strains.

Toxic preparations from $\alpha\beta$-haemolytic variants of *Staphylococcus aureus* which had arisen from parent α-strains were then examined. It was shown that the toxic preparations from the $\alpha\beta$-variants had acquired both sphingomyelinase and lysophospholipase activity. To examine the possible relationship between the phospholipase enzymes and the α- and β-haemolysins, the latter components were separated by fractional precipitation with $(NH_4)_2SO_4$ and chromatography on hydroxyl-apatite.

The present communication describes the mode of action, the properties and distribution of the phospholipase enzymes in staphylococcal toxic preparations. The

relationship of these enzymes to the staphylococcal haemolysins will be discussed. Evidence is presented to support the conclusion that β-haemolysin, sphingomyelinase and lysophospholipase are activities of one enzyme.

METHODS

Staphylococcal toxic preparations. The toxic preparations were culture fluids of the organisms grown for 4 days in saline within cellophane sacs suspended in Dolman's broth (Dolman, 1934). The whole culture unit was agitated on a shake table in a warm room at 37°; in a few cases, the shake table was not used, a mixture of oxygen + CO_2 (%, v/v 80 + 20) was gently bubbled through the contents of the sac. Toxic preparations from an $\alpha\beta$ strain (*Staphylococcus aureus*, strain B.S.M. 24) made by the latter method was used for the separation of enzymes by curtain electrophoresis.

Toxic preparations for separation of the haemolysins on hydroxylapatite columns were prepared in cellophane sacs, bubbling oxygen (%, v/v) + CO_2 (%, v/v) carbogen for strain CN 56 (α strain) and air for strain 1061–17 ($\alpha\beta$-haemolytic strain of bovine origin).

To examine the conditions which favoured the production of phospholipase A, toxic preparations were made from strains grown directly in Dolman's broth in static cultures (2–7 days incubation at 37°) and in 2–4 day cultures through which air or oxygen + CO_2 carbogen was gently bubbled.

Thiomersalate (0·01 %, w/v) was added to all toxic preparations.

Buffers. For quantitative estimations of enzymes, 0·3 M-ammonium acetate adjusted to the desired pH value with either 0·3 M-acetic acid or 0·3 M-NH_4OH was used. For chromatography on hydroxylapatite, a stock solution of 0·5 M-phosphate buffer (pH 6·7) was prepared according to Woodin (1959). Buffers of concentrations ranging from 0·05 M- to 0·25 M-phosphate were made by diluting the stock solution in water or 0·15 M-NaCl without further pH adjustment.

Paper chromatography. For phospholipids the methods of Marinetti, Albrecht, Ford & Stotz (1959) were used. For other compounds containing phosphorus, descending chromatography in the solvent ethylmethylketone + *n*-butanol saturated with water + *n*-butanol *n*-propanol + 98–100 % formic acid (9 + 5 + 1 + 4, by vol.; Gerlach, Weber & Döring, 1955) was used.

Substrates

Phosphatidyl inositol. A commercial preparation of soy bean phosphatidyl choline was used as the source of this phospholipid. This was shown by quantitative paper chromatography to have the following percentage (w/v) composition; phosphatidyl ethanolamine 22 %, phosphatidyl choline 28 %, phosphatidyl inositol 20 % and lysophosphatidyl inositol 3 % (Magnusson *et al.* 1962). For quantitative estimations the soy bean phosphatidyl choline was homogenized in an Omnimixer blender (1600 rev./min. for 5 min.). The emulsion was dialysed against water to remove soluble phosphorus compounds, and the non-diffusible phospholipid used at a concentration which represented 10 μg. soy bean phosphatidyl choline/ml.

Sphingomyelin. This was prepared from a crude extract of beef heart (Pangborn, 1951) and purified by the method of Rapport & Lerner (1958). When examined by paper chromatography, the product moved as a single component and failed to stain with ninhydrin.

Phosphatidyl choline. Crude egg phosphatidyl choline was purified by chromatography on silica gel (Saunders, 1957), and held in chloroform solution until required.

Lysophosphatidyl choline. Phosphatidyl choline was hydrolysed by phospholipase A prepared as described by Doery & Pearson (1961) and the resultant lysophosphatidyl choline purified as described by Saunders (1957). For quantitative estimations, aqueous emulsions of sphingomyelin, phosphatidyl choline and lysophosphatidyl choline were each used at a concentration of 5 mg./ml

Human plasma. Thiomersalate (0·02 %, w/v) and either cobalt or magnesium salts were added to citrated human plasma to provide a concentration (0·02–0·04 M) of Co^{2+} or Mg^{2+} in excess of that of the citrate ions.

Plasma phospholipids. These were extracted from freeze-dried citrated human plasma with successive lots of chloroform + methanol (2 + 1, by vol.). The solvent was removed from the pooled extracts in a Craig rotary evaporator, and the product dried *in vacuo* over phosphorus pentoxide.

Screening of toxic preparations for phospholipase enzymes. To detect inositol phospholipase, toxic preparations were placed in wells on 1 % (w/v) agar plates containing $MgSO_4$ (0·01 %, w/v), soy bean phosphatidyl choline (1 %, w/v) and thiomersalate (0·01 %, w/v). Plates were incubated at 37° for periods up to 48 hr. Opaque areas which surrounded the wells as well as control areas were removed and extracted twice with 10 ml. chloroform + methanol (2 + 1, by vol.) by shaking for 2 hr. The extracts were pooled and evaporated to dryness at 45° under a stream of air and reconstituted in chloroform + methanol (2 + 1, by vol.). The extract was analysed for phosphorus, and samples containing 10 μg. phosphorus were examined for phospholipids by paper chromatography. The relative loss of phosphatidyl inositol in toxin-treated substrate compared with controls indicated specific action on the phospholipid.

To detect sphingomyelinase, lysophospholipase and phospholipase activity, 0·5 ml. of the toxic preparation was mixed with 1 ml. human plasma, and shaken at 37° for periods up to 48 hr. The mixture was extracted twice with chloroform + methanol (2 + 1, by vol) by shaking for 1 hr. The pooled extracts were treated in the manner described for the detection of inositol phospholipase; loss of phospholipids indicated the presence of the respective enzymes. When a relative increase in lysophosphatidyl choline occurred, the presence of phospholipase A was indicated.

Estimation of inositol phospholipase, sphingomyelinase and lysophospholipase activities. Mixtures of 0·2 ml. toxic preparation, 0·5 ml. phospholipid emulsion and 0·5 ml. 0·3 M-ammonium acetate buffer (pH 7) containing 0·01 % (w/v) thiomersalate were shaken at 37° for 2·5 hr. After the addition of 0·25 ml. albumin (5 %, w/v) 0·5 ml. of trichloroacetic acid (TCA; 20 %, w/v) was added. The precipitate was removed by centrifugation at 70,000*g* for 30 min. in a preparative ultracentrifuge at 4°. Samples were evaporated in a boiling-water bath in a stream of air and analysed for phosphorus. Relative activities were expressed as μmoles phospholipid hydrolysed/ml. toxic preparation in 2·5 hr.

Determination of the end products of inositol phospholipase, sphingomyelinase and lysophospholipase

The product containing phosphorus. Inositol phosphate was detected by paper electrophoresis in the TCA supernatant fluid of the reaction mixture after removal

of the TCA by extraction with diethylether. Paper electrophoresis was done with a Spinco hanging strip cell, Model R, at 400 V. for periods up to 20 hr, with pyridine + acetic acid + water (25 + 1 + 225, by vol.) as the buffer. Dextran was used as a control for electro-osmosis. At the conclusion of the run, the paper was treated for the detection of phosphorus (Hanes & Isherwood, 1949). Inositol phosphate and glycerophosphoryl choline were used as markers. For sphingomyelinase and lysophospholipase, in each case a zero-time reaction mixture and one which had been allowed to proceed to completion, were freeze-dried, further dried *in vacuo* over silica gel, then extracted with chloroform. The residues were extracted with water. Samples of the chloroform and aqueous extracts were analysed for phosphorus and examined by paper chromatography.

Lipid reaction products. Reaction mixtures which had proceeded to completion were freeze-dried and the product extracted three times with 3 ml. light petroleum (b.p. 40–60°). The pooled extracts were dialysed in a rubber membrane against light petroleum as described by van Beers, de Iongh & Boldingh (1958). The diffusate was analysed for ester groups and free fatty acids. In addition, in the case of sphingomyelinase, the chloroform extracts of both a zero-time and a completed reaction mixture were analysed for nitrogen. In the case of lysophospholipase, ester group analyses were carried out on similar chloroform extracts.

Measurement of haemolytic activity. Erythrocytes from citrated rabbit blood or defibrinated sheep blood were washed 3 times with NaCl (0·15 M) and a suspension of them (2 %, v/v) prepared in this saline or the appropriate diluent. For the measurement of α-haemolysin, rabbit erythrocytes and crude toxic preparation were diluted in saline, but when purified toxic preparations were examined, both the toxic preparations and the erythrocytes were diluted in saline phosphate buffer (pH 7); Jackson & Little (1957). Equal volumes (0·5 ml.) of toxic preparation and rabbit erythrocytes (2 %, v/v) were incubated together for 1 hr, then centrifuged briefly to remove unhaemolysed erythrocytes. One drop of potassium ferricyanide (1 %, w/v) was added to each sample of supernatant fluid, and the extinction read at 630 mμ. A standard curve relating extinction and percentage haemolysis was obtained from appropriate dilutions of blood in water. For β-haemolysin, sheep erythrocytes and crude toxic preparation were diluted in saline. Purified toxic preparation was diluted in bovine serum albumin (0·2 %, w/v) in saline, giving approximately six-fold activation. Equal volumes of toxic preparation and sheep erythrocytes were incubated together for 1 hr, then held for 30 min. in an ice bath at 4° before estimating the percentage haemolysis in the manner described for α-haemolysin. For both haemolysins, the dilution of toxic preparation which produced 50 % haemolysis was determined. The reciprocal of this dilution was taken to be the number of units of haemolysin/ml.

Staphylococcal antitoxin. This was a commercial product prepared in these Laboratories by using a mixture of toxic preparations from α- and $\alpha\beta$-strains as immunizing antigens.

Antigenic components. These were detected on Ouchterlony plates in which toxic preparations at protein concentrations of 0·5–1 mg./ml. were diffused against staphylococcal antitoxin at appropriate dilutions.

Protein concentration. The extinction was measured at 280 mμ and calculation of the protein concentration was based on the assumption that $E^{0\cdot1\%}_{280\,m\mu}$ was 1·0.

Analytical methods. *Phosphorus* was estimated by the method of Brown (1954); *ester groups* by the method of Shapiro (1953); *nitrogen* by a micro-Kjeldahl procedure. *Fatty acid* was estimated by titration against NaOH (0·01 N), by using a Reyberg ultramicroburette with thymol blue as indicator. *Proteolytic activity* was detected in toxic preparations by a modification of the method of Levy (1952). Toxic preparations were placed in wells on plates containing gelatine (0·5 %, w/v) in saline agar. The plates were incubated at 37° for 24 hr, then a saturated solution of $HgCl_2$ in 10 % HCl was poured over the agar. Clear zones around the wells indicated proteolytic activity. *Lipase activity* was detected by the hydrolysis of maize oil in agar in plates by the method of Richou, Richou, Quinchon & Pantaléon (1960).

Separation of inositol phospholipase and sphingomyelinase by curtain electrophoresis. The toxin from *Staphylococcus aureus* strain B.S.M. 24, was dialysed against veronal buffer (pH 8·6; U, 0·02). Electrophoresis was carried out with the same buffer, in a Beckmann/Spinco continuous flow paper electrophoresis cell, Model C.P., for 44 hr, at 4°, 630 V and a current of 45 mA. The fractions were dialysed against NaCl (0·15 M) containing thiomersalate (0·01 %, w/v) and analysed for inositol phospholipase and sphingomyelinase.

Purification of α- and β-haemolysins from toxic preparations of strains CN 56 and 1061–17, respectively. Crude toxic preparation (culture fluid) was freeze-dried, reconstituted in water to 1/10 of the original volume and then centrifuged. By a procedure essentially that of Bernheimer & Schwartz (1963), the haemolysins in the supernatant fluid were partially purified by repeated precipitation with $(NH_4)_2SO_4$ and finally obtained in a fraction which was soluble in 40 % and insoluble in 60 % saturated $(NH_4)_2SO_4$. This fraction was then dissolved in the minimum volume of 20 % saturated $(NH_4)_2SO_4$ and dialysed against 0·05 M- or 0·1 M-phosphate buffer (pH 6·7). The dialysed solution (dialysis residue) was held for chromatography on hydroxylapatite.

Hydroxylapatite was prepared by the method of Tiselius, Hjerten & Levin (1956) with the modifications of Woodin (1959) and Jackson (1963). The hydroxylapatite columns were 1·8 cm. wide and optimum separations of components were obtained when 12–15 mg. protein were applied/cm. height of hydroxylapatite column. Elution was continued with increasing concentrations of phosphate buffer, and finally in the case of β-haemolysin with buffer in 0·15 M-NaCl. $E_{280\,m\mu}$ of eluates (2 ml.) was read, then thiomersalate was added to each eluate to 0·01 % (w/v). In the case of β-haemolysin, bovine serum albumin was also added (final concentration 2 %, w/v) as a stabilizer.

RESULTS

Mode of action of inositol phospholipase, sphingomyelinase and lysophospholipase

These enzymes were shown to have a common mode of action. With each enzyme, phosphorus was found in the TCA filtrate from the reaction mixture. With inositol phospholipase, the phosphorus was contributed by a single compound and identified by paper electrophoresis as inositol phosphate. Since this enzyme was inactive on purified phosphatidyl inositol, no quantitative phosphorus balance was attempted. With both sphingomyelinase and lysophospholipase, all the phosphorus of the chloroform extract of the zero-time reaction mixture was found in the aqueous

extract of the reaction mixture which had proceeded to completion (Table 1*a*), indicating complete hydrolysis of the substrates. With both enzymes the phosphorus in the latter extract was contributed by a single phosphorus-staining compound, identified by paper chromatography as phosphoryl choline.

Table 1. *End-product analysis of staphylococcal sphingomyelinase and lysophospholipase action*

(*a*) Distribution of phosphorus in zero-time and complete reaction mixtures (18 hr), expressed as a % of total phosphorus of the zero-time reaction mixture.

Enzyme	Reaction time (hr)	Phosphorus (%)	
		$CHCl_3$ extract	Aqueous extract
Sphingomyelinase	0	96	4
	18	2	93
Lysophospholipase	0	97	3
	18	8	100

(*b*) Lipid reaction products. Recovery of nitrogen and ester groups in chloroform extracts of zero-time and complete (24 hr) reaction mixtures.

Enzyme	Reaction time (hr)	Nitrogen (%)	Ester (%)
Sphingomyelinase	0	100	.
	24	110	.
Lysophospholipase	0	.	100
	24	.	88

When the reaction mixtures were dialysed against light petroleum, esterified fatty acids but no phosphorus were found in diffusates of reaction mixtures containing the phosphatidyl inositols or lysophosphatidyl choline. No free fatty acids were found. In addition, with lysophospholipase the ester group concentration of the chloroform extract of the completed reaction mixture (24 hr) was unchanged from that of the zero-time mixture (Table 1*b*).

In the case of sphingomyelinase, the nitrogen of the chloroform extract of the zero-time sample was completely recovered in the chloroform extract of the reaction mixture which had proceeded to completion. Since no free fatty acid was found and phosphoryl choline was the only reaction product containing phosphorus, the total recovery of the nitrogen in the chloroform extract is in keeping with the formation of N-acyl sphingosine. No estimation of the N-acyl group was attempted.

From the above observations, the following mechanisms, each like that of phospholipase C, are indicated:

phosphatidyl inositol + H_2O ⟶ diglyceride + inositol phosphate
sphingomyelin + H_2O ⟶ N-acyl sphingosine + phosphoryl choline
lysophosphatidyl choline + H_2O ⟶ monoglyceride + phosphoryl choline

Properties of the enzymes

Optimum pH value of reaction. Figure 1 shows the relationship between the μmoles phospholipid hydrolysed and pH value for each enzyme. It can be seen that the optimum activity of the inositol phospholipase was at pH 4·6, while the

maximum activity of the sphingomyelinase and the lysophospholipase was at pH 7·5.

Optimum temperature of reaction. Inositol phospholipase showed little change in activity between 20 and 40° but rapid loss of activity occurred at temperatures above 40°. Both sphingomyelinase and lysophospholipase showed maximum activity near 40°.

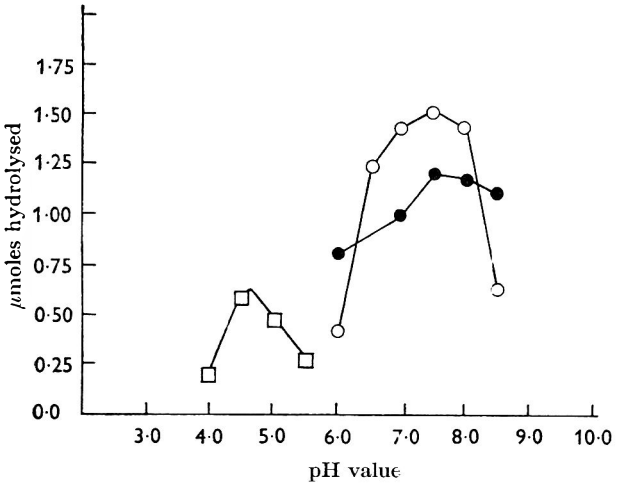

Fig. 1

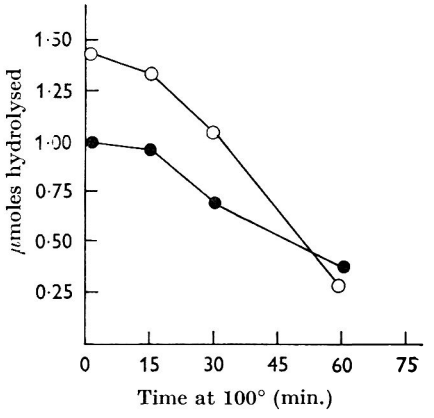

Fig. 2

Fig. 1. Effect of pH value of incubation mixture on the activity of enzymes. □, inositol phospholipase; ○, lysophospholipase; ●, sphingomyelinase.

Fig. 2. Stability of enzymes held at 100°. Toxic preparation at pH 7·2, was held in sealed ampoules at 100° for the times indicated and enzyme activity then determined. ○, lysophospholipase; ●, sphingomyelinase.

Stability to heat. Owing to the limited amounts of fractions available from the curtain electrophoresis of crude toxic preparations, it was not possible to determine the effect of heat on the inositol phospholipase. Figure 2 shows that after 1 hr at 100°, 33 and 20% of the sphingomyelinase and lysophospholipase activity, respectively, of the unheated toxic preparations remained.

Effect of enzyme concentration and time of incubation. The relationship between the concentration of enzyme and the μmoles phospholipid hydrolysed was linear for the three enzymes over the ranges used for these experiments, and became typically enzymic when excess enzyme was used. A linear relationship was found between time of incubation and μmoles phospholipid hydrolysed, over the region where the reaction was not limited by lack of substrate.

Inhibition of enzyme activity by staphylococcal antitoxin. Figure 3 shows the effect of commercial antitoxin on the activities of the enzymes. In all cases, the relationship between the amount of antitoxin used to inhibit the enzyme and the resulting enzyme activity was linear.

Effect of metal ions on activity of enzymes. Following the observation by Robinson, Thatcher & Gagnon (1958) that β-haemolysin was activated by Co^{2+}, the following tests were made. Both the sphingomyelinase and lysophospholipase were activated

by the addition of Co^{2+} or Mg^{2+}, Co^{2+} being more effective than Mg^{2+}; Ca^{2+} inhibited both enzymes. Figure 4 shows that with equimolar concentrations of Mg^{2+}, similar patterns of activation of both enzymes were obtained, the average maximum activation of the sphingomyelinase and lysophospholipase being 111 % (105, 105, 124) and 113 % (107, 111, 122), respectively. No such activation of the inositol phospholipase was found. Equimolar concentrations of Ca^{2+} inhibited the sphingomyelinase and the lysophospholipase to the same degree, the average maximum inhibition being 63 % (51, 69, 73) and 58 % (55, 58, 61), respectively.

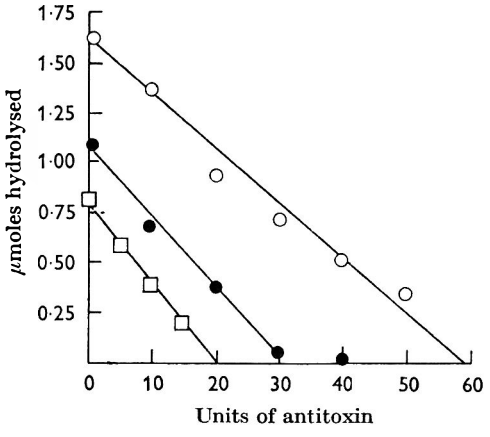

Fig. 3

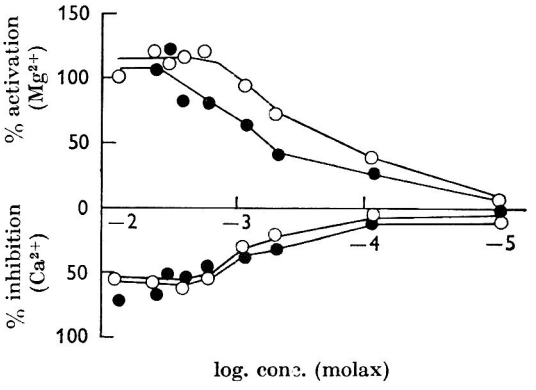

Fig. 4

Fig. 3. Inhibition of enzyme activity by antitoxin. Toxic preparation (0·25 ml.) and antitoxin (1 ml.; 0–50 units) were held at 37° for 45 min. and enzyme activity determined. □, inositol phospholipase; ○, lysophospholipase; ●, sphingomyelinase.

Fig. 4. Effect of the concentration of Ca^{2+} and Mg^{2+} on the sphingomyelinase and lysophospholipase activities. ●, sphingomyelinase; ○, lysophospholipase.

Table 2. *Distribution of inositol phospholipase, sphingomyelinase and lysophospholipase among strains of Staphylococcus aureus*

S. aureus strain	Source	Inositol phospholipase	Sphingomyelinase	Lysophospholipase
Coagulase-positive, haemolytic				
α-haemolytic	bovine (4)*	+	−	−
α-haemolytic	human (4)	+	−	−
αβ-haemolytic	bovine (7)	+	+	+
Coagulase-negative, non-haemolytic	milk (1)	−	−	−
	air (1)	−	−	−
	human (3)	−	−	−

* Figures in parentheses represent the number of strains examined.

Distribution of enzymes in staphylococcal toxins

Table 2 shows the distribution of phospholipase enzymes in toxic preparations from a number of αβ-strains and α-strains of *Staphylococcus aureus*, and also non-haemolytic coagulase-negative strains. It can be seen that inositol phospholipase C

was detected in strains which produced α-haemolysin, while sphingomyelinase and lysophospholipase were found only in strains which also produced β-haemolysin. No phospholipase activity was detected in culture fluids from the non-haemolytic coagulase-negative strains.

Table 3. *Distribution of enzymes in toxic preparations from strains of Staphylococcus aureus and their variants*

S. aureus strain	Haemolysin	Inositol phospholipase	Sphingo-myelinase	Lyso-phospholipase
No. II	α	+	−	−
Variant	αβ	+	+	+
No. 1873	α	+	−	−
Variant	αβ	+	+	+
Wood	α	+	−	−
Variant	αβ	+	+	+
No. 2063	α	+	−	−
Variant	αβ	+	+	+

Table 4. *Production of phospholipase A in toxic preparations obtained under different cultural conditions from strains of Staphylococcus aureus*

	Culture conditions			Haemolysin activity (units/ml.) Erythrocytes		
	Bubbled with					
S. aureus strain	O_2+CO_2 (80 % + 20 %, v/v)	Air	Not bubbled	Rabbit	Sheep	Phospholipase A
709	2-day incubation	.	.	128	350	+
	.	2-day incubation	.	12	100	−
	.	.	2-day incubation	8	40	−
	.	.	5-day incubation	12	150	−
1061–17	2-day incubation	.	.	128	350	+
	.	2-day incubation	.	12	100	−
	.	4-day incubation	.	600	600	+
	.	.	2-day incubation	3	40	−
	.	.	5-day incubation	32	280	−
	.	.	7-day incubation	12	600	+

Table 3 compares the distribution of phospholipase enzymes in toxic preparations from parent α-strains and their αβ-haemolytic variants which arose during normal subculturing. In all cases the variant which acquired the β-haemolytic characteristic also acquired sphingomyelinase and lysophospholipase activity. The inositol phospholipase was found in toxic preparations from both parent and variant strains. Phospholipase A activity in culture fluids of two strains grown under various conditions is shown in Table 4. The enzyme was detected only in culture fluids which had β-haemolytic activity equal to or greater than 350 units/ml.

Separation of inositol phospholipase and sphingomyelinase by curtain electrophoresis of toxic culture fluid from Staphylococcus aureus strain B.S.M. 24

The activity of inositol phospholipase and sphingomyelinase in the curtain electrophoresis fractions is shown in Fig. 5 where it can be seen that these enzymes were separated from one another, with some overlap. The fractions were not examined for lysophospholipase activity, but all subsequently examined preparations of sphingomyelinase have contained lysophospholipase activity also. The fractions containing inositol phospholipase were used for all the quantitative studies of this enzyme, since it was found only in very low concentrations in crude toxic preparations.

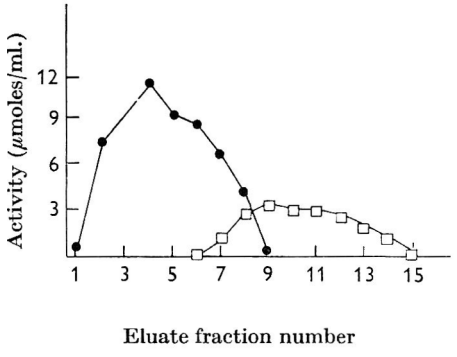

Fig. 5

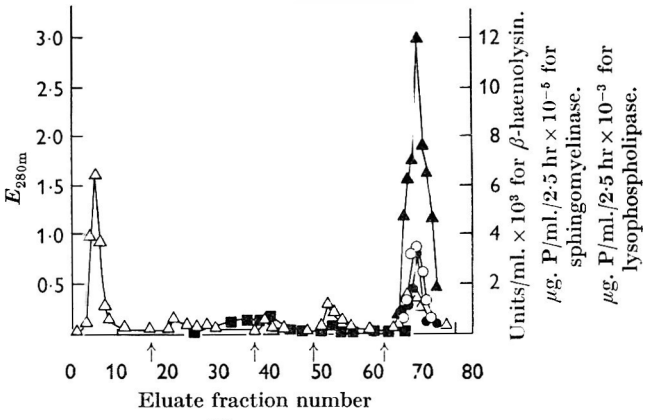

Fig. 6

Fig. 5. Activity of sphingomyelinase and inositol phospholipase in curtain electrophoresis fractions. Electrophoresis was done in veronal buffer (pH 8·6; U 0·02) at 630 V and 45 mA for 44 hr at 4°. ●, sphingomyelinase, □, inositol phospholipase.

Fig. 6. Chromatography on hydroxylapatite of 35 mg. protein of the $(NH_4)_2SO_4$ fraction from *Staphylococcus aureus* strain 1061–17. Eluting buffer, 0·05 M-phosphate (pH 6·7); at arrows eluents were changed successively to 0·075 M, 0·10 M, 0·15 M and finally to 0·25 M-phosphate (pH 6·7) + 0·15 M-NaCl. △, protein; ○, lysophospholipase; ●, sphingomyelinase; ■, α-haemolysin; ▲, β-haemolysin.

Purification of β-haemolysin from toxic culture fluid of Staphylococcus aureus strain 1061–17

Fractionation of β-haemolysin with $(NH_4)_2SO_4$ resulted in a fraction containing 10,000 units/mg., i.e. 25 % of the original activity. This fraction also contained 20 % of the original sphingomyelinase activity. Figure 6 shows the results of the chromatography of 35 mg. of this fraction in phosphate buffer (pH 6·7) on hydroxylapatite. This procedure was very similar to the one used by Woodin (1959) and Jackson (1963) and resulted in 50 % overall recovery of the protein. This was eluted in two main peaks and some minor ones. Proteolytic activity was eluted with the starting buffer, in this case 0·05 M, and the activity approximately followed the protein concentration. After the buffer was changed to 0·075 M, α-haemolysin was slowly eluted and continued to be eluted with slightly greater activity after the buffer was changed to 0·1 M, maximum activity being found in eluate fraction 42. When the eluting buffer was changed to 0·15 M a minor protein peak occurred and low concentrations of α-haemolysin continued to be eluted.

The second main protein peak was eluted with 0·25 M-phosphate buffer in 0·15 M-NaCl, maximum protein concentration occurring in eluate fraction 70. This peak contained the β-haemolysin, sphingomyelinase and lysophospholipase activities, all three of which followed symmetrical curves with maximum activity in eluate fraction 72 in each case. The β-haemolysin activity of this eluate was 37,000 units/mg., representing a four-fold purification. Sphingomyelin was hydrolysed more readily than was lysophosphatidyl choline. When the homogeneity of eluate fraction 72 was examined on Ouchterlony plates, a single line was obtained at a protein concentration of 1 mg./ml., but after 7 days a second diffuse line appeared. Thus a correlation, which had been indicated by a variety of approaches, has been established between sphingomyelinase, lysophospholipase and a highly purified preparation of β-haemolysin.

When the eluates in the minor protein peak eluted with 0·15 M-phosphate buffer were being studied, an additional phospholipase was identified when the eluates were incubated with plasma. Loss of phosphatidyl choline and an increase in lysophosphatidyl choline was readily detected with eluate fractions 44–66. This indicated phospholipase A activity and provided the first evidence that this enzyme was present in the toxic preparation. All the eluate fractions subsequent to no. 66 also hydrolysed phosphatidyl choline and, beginning with eluate fraction 68, hydrolysis of lysophosphatidyl choline also occurred in the region of β-haemolysin activity.

It has not been possible completely to define the region of phospholipase A activity. Slight hydrolysis of purified phosphatidyl choline was detected by paper chromatography after incubation for 48 hr with eluate fractions 44–60, with weak but maximum activity with eluate fractions 53–56. Ester group analysis of this system did not give consistent results. Without further evidence dependent on quantitative analysis, it is suggested that the hydrolysis of phosphatidyl choline in plasma, by eluates which contained lysophospholipase (eluate fractions 68–78), was due to the activation of trace amounts of phospholipase A by the continuous removal of the end-product by the highly active lysophospholipase. The possibility that the phospholipase A was produced by a contaminant organism was considered. However, after exhaustive tests for bacterial purity at each stage from toxin production to the final stage of purification, this possibility was eliminated. Lipase activity was not found in any chromatographic fraction.

Purification of α-haemolysin from toxic preparations from Staphylococcus aureus strain CN 56

Fractionation of α-haemolysin with $(NH_4)_2SO_4$ resulted in a fraction containing 45,000 units/mg., i.e. 25% of the original activity. The inositol phospholipase was detected qualitatively in this fraction. On chromatography of 173 mg. of this fraction in 0·1 M-phosphate buffer in the manner described for the separation of β-haemolysin, 50% of the haemolytic activity and 34% of the protein applied to the column were eluted in three well separated peaks. Two of these were eluted successively with the starting buffer and contained proteolytic and α-haemolysin activities respectively. Lipase activity was found in the third peak, which appeared on changing the eluting buffer to 0·15 M.

Considerable purification of α-haemolysin was achieved, the activity of eluate

fractions near the top of the peak being 72,000 units/mg. When the eluate containing the highest concentration of α-haemolysin was examined on Ouchterlony plates at a protein concentration of 0·5 mg./ml. a single reaction line was obtained; no other lines appeared after 7 days. However, a second diffuse line was seen when all the eluates on this peak were pooled, concentrated with some loss of activity and examined in a similar manner at 1 mg. protein/ml.

Exhaustive tests were made to trace the inositol phospholipase activity throughout the purification. The eluates of the three respective peaks were pooled, concentrated and tested for this enzyme. No concentration of the activity was detected. While the enzyme was detected in the eluates of the α-haemolysin peak treated in this way, the activity bore no relationship to the α-haemolysin activity. No enhancement of activity was obtained when various mixtures of these preparations were examined for the enzyme. In the absence of further purification and the failure to obtain enzyme activity when purified phosphatidyl inositol was used as substrate, no further work was done on this phospholipase.

DISCUSSION

The properties, the distribution in toxic culture fluids and the separation of the enzymes described above show that the inositol phospholipase, sphingomyelinase and lysophospholipase activities are those of two enzymes, each with a mode of action like that of phospholipase C. Whereas the inositol phospholipase was separated from sphingomyelinase by curtain electrophoresis, no separation of sphingomyelinase from lysophospholipase has yet been achieved. The curtain electrophoresis fractions which contained the inositol phospholipase were not homogeneous and further attempts to isolate this enzyme from toxic preparations by chromatography on hydroxylapatite were not successful. No correlation was found between the activity of this enzyme and that of a highly purified preparation of α-haemolysin or of any other component of staphylococcal toxic preparations.

The sphingomyelinase and lysophospholipase were present in crude toxic culture fluids in considerably higher concentrations than was the inositol phospholipase, and purification of the former enzymes by chromatography on hydroxylapatite was readily achieved. The distribution of these enzymes in toxic preparations from various sources and the effect of metal ions indicated a correlation between their activity and that of β-haemolysin. This was confirmed by the preparation of highly purified β-haemolysin which contained sphingomyelinase and lysophospholipase activity. Further, the sphingomyelinase and lysophospholipase activities of the chromatography eluates closely followed that of β-haemolysin rather than the concentration of protein, which itself was heterogeneous (Fig. 6). Thus until β-haemolysin and sphingomyelinase and lysophospholipase activities have been separated from one another, these may be regarded as activities of one protein. The identification of this enzymic activity with β-haemolysin is in keeping with the preferential haemolysis of sheep erythrocytes rather than rabbit erythrocytes by β-haemolysin, since the sheep cells contain a significantly higher concentration of sphingomyelin than do the rabbit cells (de Gier & van Deenen, 1961).

The properties of the staphylococcal phospholipase C enzymes make interesting comparisons with the phospholipase C enzymes from other sources. Unlike the

phospholipase C of *Clostridium welchii*, no preferential hydrolysis of phosphatidyl choline or phosphatidyl ethanolamine by the staphylococcal enzymes was observed. The optimal pH value for the staphylococcal enzymes, sphingomyelinase and lysophospholipase, was in each case near pH 7, which happens to be the optimum described for the phospholipase C of *Cl. welchii* (Macfarlane & Knight, 1941). The inositol phospholipase which in every case hydrolysed lysophosphatidyl inositol as well as the diacyl compound, had an optimum near pH 4·6, close to that of the enzyme in rat liver (pH 5·4) which also hydrolysed both these phospholipids (Kemp, Hübscher & Hawthorne, 1959).

In a former communication (Magnusson *et al.* 1962), a phospholipase A was reported in a staphylococcal toxic preparation from a pure α-strain CN 56 of *Staphylococcus aureus*. This observation could not be repeated and it was concluded that non-enzymic hydrolysis had probably occurred. The separation of a phospholipase A in fractions of a toxic preparation from an $\alpha\beta$-strain, reported in the present paper, was therefore of interest. In the light of the correlation just established between β-haemolysin and lysophospholipase, a toxic preparation containing a strong β-haemolysin activity would produce conditions favourable to the activation of a phospholipase A. The continuous hydrolysis of lysophosphatidyl choline by the lysophospholipase of β-haemolysin would favour the production of a phospholipase A, providing fresh substrate for the lysophospholipase.

Finally, like other phospholipase enzymes (Condrea, de Vries & Mager, 1962), those studied here were more active on native substrates than on purified phospholipids. It might well be that the relative activities of these enzymes *in vitro* might bear little relationship to those elaborated in the living animal.

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ADDENDUM

We thank Dr A. W. Jackson of Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Ontario, Canada, for kindly making available to us a small sample of purified β-haemolysin prepared by chromatography on hydroxylapatite. This was found on assay to contain 25 units. The phospholipase activity of this sample was examined, with plasma as substrate. It was found to contain both sphingomyelinase and lysophospholipase.

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