(A + B) through a methylene bridge does not allow fraction (C + D) to show this induced reactivity.

4. Acetylation of polar side chains in wool leads to a general reduction in the reactivity of cystine.

5. Treatment of wool with phenyl isocyanate leads to blocking of all the polar side chains without affecting the cystine. Such treatment almost completely inhibits subsequent reactivity of the cystine linkages.

6. It is suggested that the degree of reactivity of cystine linkages is largely governed by their accessibility to a reagent, close proximity of bulky side chains reducing reactivity through steric hindrance.

7. Other factors such as varying structural arrangement may be responsible for the variation in the nature of the products resulting from a particular reaction.

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Selective Inhibition of the Liberation of Extracellular Enzymes and Protein in Cultures of *Staphylococcus aureus*

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Most substances, such as amino acid analogues, which inhibit protein formation by bacteria, do so in a non-specific manner. That is, the formation of all protein ceases and growth stops when they are added to the culture medium. Any substance which acts upon the formation of a single protein or group of proteins is likely to be a valuable tool in studying biosynthetic processes. The present work describes the effect of some negatively charged polymers upon cultures of staphylococci. The liberation into the culture fluid of a group of extracellular enzymic proteins is inhibited, but growth of the organisms is not impaired. These extracellular enzymes are not essential for growth under the conditions chosen. Recently, considerable attention has been paid to

the ability of synthetic charged polymers, 'macroanions' and 'macroanions', to inhibit the action of formed enzymes (cf. Spensley & Rogers, 1954, for some references). These studies have not, however, been extended to the effects of macroanions upon living cells. An exception is the study by Katchalski, Bichowski-Slonmizki & Volcani (1953) of the growth inhibition of *Escherichia coli* and *Staphylococcus aureus* by the macroanions polylysine and polyarginine. Polyglutamic and polyaspartic acids, two macroanions examined by these authors, did not inhibit growth.

The ability of some macroanions to inhibit the liberation of at least two extracellular enzymes in cultures of staphylococci is proved in the work

described below but it is not yet possible to say with certainty that the polymer prevents the formation of these enzymes by the cells.

METHODS

Hyaluronidase and extracellular trichloroacetic acid precipitable protein in samples of the culture supernatant fluids were estimated and the results expressed as previously described (Rogers, 1954). Crystalline bovine serum albumin (Armour Fraction V) was used as the standard in the protein estimations. Growth was measured by the optical opacity of suitably diluted suspensions to light of wavelength 640 m μ . The results, expressed as mg. dry weight/ml. of culture, were interpolated from a standard curve relating dry weight to opacity; dry weights were determined by heating washed suspensions of the organisms to constant weight at 105°. Radioactive measurements on cell pastes were made by transferring the paste to 2 sq.cm. polythene disks, freezing and drying. The pad of dried organisms was then pressed flat with a plunger. A sufficient mass of organisms (>40 mg. for a 2 sq.cm. disk) was used to enable the pad to be considered of 'infinite thickness' in the calculations following the radioactive count. A thin end window Geiger-Müller counter was used and the minimum number of counts recorded in the experimental work was 2400; the error on such a count is about 2%. The amount of inhibitor fixed to the cells was calculated directly from the dilution of the labelled inhibitor.

Organisms and conditions of growth. The variant of *Staphylococcus aureus* strain 524, isolated previously by single cell manipulation (Rogers, 1953) which produces potent hyaluronidase has been used for the hyaluronidase work described here. This organism has been maintained in dried culture since its isolation. Intermittently the culture has been revived, overnight cultures plated on nutrient agar plates, and the enzyme-forming capacity of twenty individual colonies examined. When grown under the conditions previously described (Rogers, 1953) the enzyme potency still reaches 200-300 turbidity reducing units (t.r.u.'s) of hyaluronidase per ml. of culture supernatant. Frequently in the present work rather thick suspensions of the organisms in broth ($\approx$ about 1 mg./ml. dry weight) have been used and, under such conditions, growth is slow and non-logarithmic. The potency of enzyme formed is then less and more variable than with the much smaller inoculum hitherto used.

When coagulase formation was to be examined, *Staphylococcus aureus* strain Newman was used. The conditions for its cultivation were the same as those for strain 524 of the organism.

Two media have been used in the present work (a) Hedley Wright broth buffered with either 2.5% Na β -glycerophosphate or 0.1M Sørensen phosphate buffer at pH 7.0 and with a final glucose concentration of 0.25% (w/v); (b) the casein hydrolysate medium B (Rogers, 1945). Inorganic phosphate buffer has also been satisfactorily substituted for glycerophosphate in medium (b). The total omission of the extra buffer has been found to lead to a rapid destruction of hyaluronidase after its formation period. All the cultures were incubated at 35° in lightly plugged Erlenmeyer flasks of capacity 5 times that of the volume of culture, and shaken on a machine of the type described by Kantorowicz (1951) at the rate of 88 times per min. with a horizontal traverse of 8 cm.

Inhibitors

The hydroquinone-sulphonic acid-formaldehyde polymer (or, more briefly, 'polyhydroquinone-sulphonate') was prepared as previously described (Rogers & Spensley, 1954) and for the experiments discussed here only that portion of the initial crude polymer not dialysable through cellophan has been employed. For convenience this material is generally referred to below by the Code no. 53D. Two batches of this material, 53D_K and 53D_N, have been used for the work to be described. They both behaved identically. When labelled polymer was required the method of preparation was the same except that [¹⁴C]formaldehyde was used. A specimen of 53D having a specific activity of 0.305 μ C/mg. was then obtained. The radioactive formaldehyde was obtained by a slight modification of the method described by Arnstein (1951); we are indebted to Dr H. R. V. Arnstein for the use of the apparatus and for much helpful advice.

The polyphlorethin phosphate was kindly given us by Dr Högberg of Aktiebolaget Leo, Hålsingborg, and Dr J. E. Lovelock of this Institute provided the sample of sulphonated polystyrene. The styrene-maleic acid co-polymer was made available to us by the courtesy of Messrs Glaxo Laboratories Ltd.

A specimen of this last material was modified by sulphonation which was achieved by heating at 100° with an excess of concentrated sulphuric acid for 1 to 5 min., the product being then poured into water, neutralized and dialysed. The poly-*p*-aminophenolsulphonate was prepared from the known *p*-aminophenolsulphonic acid by condensation with formaldehyde in the manner already described for 53 (Rogers & Spensley, 1954); the product was dialysed against water before testing.

EXPERIMENTAL

In the first experiments various concentrations of 53D were added to unbuffered broth and casein hydrolysate media, which were then inoculated with 0.0015 of their volume of an 18 hr. culture of *Staph. aureus* strain 524. Final concentrations of the macroanion as high as 1 mg./ml. added to broth failed to affect the growth of the organisms, although 50 μ g./ml. was sufficient to reduce hyaluronidase formation by about 50%. The results obtained, however, were difficult to interpret because the addition of either the phosphate or glycerophosphate buffer to the medium greatly reduced the degree of inhibition and led to very variable results. In the absence of additional buffer, on the other hand, the hyaluronidase potency of the cultures dropped after the formation period and interpretation was therefore difficult, since the maximum potency measured might represent only the balance between destruction and formation. During this work it was noted that the organisms growing in the presence of inhibitor were brownish in colour, presumably owing to adherence of the inhibitor to the cells. It therefore seemed possible that if the cells were first treated with solutions of the inhibitor

and then washed, sufficient polymer might be left associated with the cells to inhibit enzyme formation.

Pretreatment of cells with 53D

Comparison was made of cells from an overnight culture and cells from a young (4.25 hr.) culture. In the latter the organisms were still multiplying logarithmically. Both cultures were grown in the broth-glycerophosphate medium from an inoculum of 0.0015 of their volume of an 18 hr. culture in the same medium. They were centrifuged at the end of the growth period and the cells washed with water. Each lot of cells corresponding to about 80 mg. dry weight was then resuspended in 20 ml. of 0.1 M Sørensen phosphate buffer at pH 6.2, and four equal volumes of 3.6 ml. measured. To these two sets of suspensions from the cultures sufficient of a sterile 10 mg./ml. solution of 53D in water was added to give the concentrations indicated in Table 1. The

Table 1. *Comparison of the effects upon hyaluronidase liberation of pretreating cells from a young (4.25 hr.) with those from an 18 hr. culture of Staphylococcus aureus with various concentrations of 53D*

The cells (13–16 mg. dry wt.) were treated with 4.0 ml. volumes of the stated concentrations of 53D dissolved in 0.1 M phosphate buffer at pH 6.2 for 15 min. at room temperature. After washing, these cells were resuspended in warmed buffered broth (20 ml. in 100 ml. flasks) giving a density equivalent to approx. 0.7 mg. dry wt./ml. Incubation was continued for 2 hr. at 35° with shaking.

Concn. of 53D (mg./ml.)	Hyaluronidase T.R.U./ml.		% inhibition	
	Y	O	Y	O
0	148	98	—	—
0.05	125	—	15	—
0.2	48	—	68	—
0.5	—	98	—	0
1.0	21	86	85	12
2.0	—	73	—	25

Y = young cultures, O = old cultures, — = no experiment made.

suspensions were allowed to remain at room temperature for 15 min., centrifuged and the cells washed with 5 ml. water. A small amount of inhibitor was present in the washing water but the cells which had been treated with inhibitor were still deeply stained, those from the young culture appeared to be darker than those from the 18 hr. culture. The eight samples of cells were then separately suspended in 20 ml. quantities of broth containing 0.1 M phosphate buffer at pH 7.0 already warmed to 35°. Incubation with shaking was continued for 2 hr. at 35°. At the end of this period the cultures were cooled to 0–4°, centrifuged at this temperature and

the hyaluronidase potency in the supernatant fluid estimated. Table 1 shows the results which were obtained. It is to be seen that, whereas cells from the 18 hr. culture are relatively little affected, by treatment with inhibitor, the logarithmically growing cells form little hyaluronidase at all after treatment with the 1 mg./ml. solution of the inhibitor.

In these experiments pretreatment was carried out at slightly acid pH because it was known that the combination between proteins and macroanions was less effective in alkaline solution. An examination was subsequently made of the effect of pretreating in 0.1 M phosphate buffers with pH ranging from 5.8 to 7.0. The pH used did not influence the degree of inhibition of enzyme liberation caused by the polymer. Therefore in subsequent tests the pH for pretreatment was maintained at 6.2.

The use of logarithmically growing cells was technically undesirable as a routine procedure, partly because of the large volumes of culture required to yield reasonable quantities of cells. Examination was therefore made of cells from an 18 hr. culture which were subsequently reincubated for a short period in broth. The technique used was as follows: cells from 50 ml. of an 18 hr. culture were centrifuged, resuspended in 100 ml. of fresh broth medium already warmed to 35°, and shaken at this temperature for 1 hr. The organisms were then removed by centrifuging and washed once with 20 ml. of water. Such cells, which have been used for the rest of the work to be described, are referred to as revived cells. In Table 2 the enzyme production by treated and untreated cells from the original 18 hr. culture is compared with the behaviour of treated and untreated revived cells. The conditions of treatment were such that half the cells from each culture were treated with 10 ml. of 0.8 mg./ml. 53D in 0.1 M phosphate buffer at pH 6.2. Both the treated and the control cells, which had been incubated with pH 6.2 buffer alone, were resuspended in 50 ml. amounts of buffered broth medium in 250 ml. Erlenmeyer flasks. These suspensions were shaken at 35°. Samples were taken from all four flasks at the indicated times, cooled to 0–4° immediately after withdrawal, centrifuged and hyaluronidase estimations made on the supernatant fluids. Growth was also estimated by the turbidity of the samples before centrifuging. The brown colour of the treated cells did not significantly affect the optical opacity measurements. Hyaluronidase formation by the revived cells is almost completely inhibited (Table 2) by treatment with 53D, and they thus behave similarly to cells from the logarithmically growing cultures. The cells from the 18 hr. culture are rather more affected than those used in the previous experiment but again form much more hyaluronidase after treatment with

Table 2. *Comparison of the effects of 53D upon hyaluronidase liberation and growth of staphylococci from an 18 hr. culture and of these cells after reviving*

Suspensions of the organisms in 0.1 M phosphate buffer at pH 6.2 were pretreated with 53D for 15 min. at room temperature. The amount of inhibitor used was about 100 $\mu\text{g.}/\text{mg.}$ dry wt. cells. The control suspension was treated with buffer alone. The cells were subsequently suspended in warmed buffered broth to give an opacity equivalent to approximately 1 mg. dry wt./ml. of culture and incubated for the stated times at 35° with shaking.

Time (min.)	Growth (mg./ml. dry wt. of cells)		Hyaluronidase (T.R.U./ml.)		% inhibition of hyaluronidase liberation
	Control	Treated	Control	Treated	
	18 hr. cells				
15	1.12	1.08	<2	<2	—
30	1.24	1.20	<5	<2	—
70	1.62	1.46	11	5.0	55
130	1.95	1.87	119	60	50
180	2.24	2.08	110	73	34
Revived cells					
15	1.28	1.37	<5	<2	—
30	1.48	1.58	<2	<5	—
70	2.08	2.04	80	<5	>95
130	2.50	2.45	123	10	92
180	2.54	2.70	123	11	91

inhibitor than the treated, revived organisms. The rate of growth of neither sets of cells has been affected by previous treatment with the polymer. It may be noted that, as the cells grow, the degree of inhibition, particularly with the cells from the 18 hr. culture, diminishes. It has been subsequently shown, using the labelled inhibitor, that this can be explained by a decreased concentration of inhibitor associated with the cells firstly due to growth of the cell mass and secondly due to the passage of some of the inhibitor from the cells into the culture fluid.

The effect of treating the revived cells with varying amounts of the polyhydroquinone-sulphonate was then examined. It did not seem to be important whether the same mass of cells was treated with a small volume of a concentrated solution of the inhibitor or with a larger volume of a more dilute solution. Either method of treatment inhibited the appearance of enzyme by about 90% providing not less than 50 $\mu\text{g.}$ of inhibitor/mg. dry wt. of cells had been added. The ratio of the total mass of inhibitor to dry wt. of cells in the treatment process appears to be the critical factor in the subsequent inhibition of enzyme liberation. Hence the amount of inhibitor added/mg. dry wt. of organisms has been quoted in the present work. When the volume of inhibitor solution to mass of cells was varied a 1 mg./ml. solution of 53D was used. When amounts of inhibitor less than 50 $\mu\text{g.}/\text{mg.}$ cells were used, the inhibition decreased until, at levels below about 2 $\mu\text{g.}$ of 53D/mg. dry wt. of cells, no effect was demonstrable.

Separate experiments were made to determine how much inhibitor was attached to the cells,

particularly at $\mu\text{g.}$ inhibitor/mg. cells ratios of about 0.05, a value which represents a saturation of the inhibiting effect. Labelled polymer was used for these measurements.

For technical reasons and also because it was thought that the closely applied layer of radioactive material on the cells might make the interpretation of results, in the present context, difficult because of radiation damage, cells which had been treated with labelled polymer were not used to examine the inhibition of enzyme formation. The labelled polymer had been made by the same chemical procedure as the unlabelled 53D and several separate batches of 53D inhibited to the same extent. There is, therefore, no reason to suppose that the behaviour, apart from possible radiation effects, of the labelled polymer in culture would be different from other batches of the material.

Revived cells were treated with the labelled inhibitor by the technique previously described. Samples of the cell suspensions were taken after pretreatment and washing, and were frozen and dried on polythene disks for radioactive estimation.

Table 3, which combines the results from experiments with labelled and unlabelled 53D, shows the relationship between the degree of inhibition of hyaluronidase liberation by 53D and the amount of the labelled inhibitor fixed to the cells. It will be seen that if the amount of inhibitor used for pretreatment is raised above the critical level, e.g. to 100 $\mu\text{g.}/\text{mg.}$ dry wt. of organisms, no more is fixed on to the cells. It appears that the maximum degree of inhibition of enzyme liberation is reached when the cell is saturated with inhibitor.

Table 3. *The influence of pretreating revived staphylococci with different amounts of polyhydroquinone-sulphonate upon the liberation of hyaluronidase and upon the fixation of the polymer on to the cells*

The cells were pretreated for 15 min. with either (1) 53 D (for effects upon enzyme liberation) or (2) labelled 53 D (for measurement of polymer fixation) under the usual conditions. After washing, cells (1) were resuspended in warmed buffered broth to give an opacity approximately equivalent to 1.0 mg./ml. dry wt. of cells and incubated with shaking at 35° for 2 hr. Cells (2) were washed and the cell paste put on to polythene disks for radioactivity measurements. The experiment numbers refer to experiments using separate batches of cells.

Weight of 53 D used/mg. dry wt. of cells (μ g.)	Inhibition of enzyme formation		Fixation of labelled 53 D/mg. dry wt. of organisms	
	Expt. no.	%	Expt. no.	μ g.
12	1	59	3	4
20	1	65	3	7
36	2	79	—	—
56	2	93	3	14
120	1	96	3	14
120	2	96	3	14

The inhibitor used in these studies was, as already stated, only that portion of the crude polymer which was retained in cellophan on dialysis and hence consisted solely of fairly large molecules. Furthermore, it is known (Diczfalusy *et al.* 1953; Spensley & Rogers, 1954) that macroanions inhibit enzymes such as hexokinase which are likely to be vital to the economy of the cell. It therefore seems reasonable since growth is not inhibited to suppose that the inhibitor remains on or near the surface of treated organisms and does not penetrate the cell wall and reach the vital internal enzymes. On this assumption a calculation can be made of the proportion of the cell surface occupied when inhibition of extracellular enzyme liberation is almost complete. Counts were made of the individual staphylococci in the suspensions, the ordinary haemocytometer cell technique being used. It was found that 1 mg. dry wt. of suspension corresponded to 2×10^{10} cells to which is attached 14×10^{-6} g. of inhibitor when saturated (see Table 3). Each cell therefore carries 7×10^{-16} g. of inhibitor. This corresponds to $\frac{6.1 \times 10^{23} \times 7 \times 10^{-16}}{224} = 1.9 \times 10^6$ actual

monomeric units of the polymer (6.1×10^{23} = the Avogadro number and 224 = 'molecular' weight of each monomeric unit of polymer). An estimate of the area occupied by a monomeric unit (kindly made for us by Mrs O. Kennard) is about 8×10^{-15} sq.cm. and hence the amount of inhibitor attached to one cell could cover an area of

$$1.9 \times 10^6 \times 8 \times 10^{-15} = 1.5 \times 10^{-8} \text{ sq.cm.}$$

A cell of 1 μ . diameter has a surface area (if a smooth sphere is assumed) of 3×10^{-8} sq.cm. Thus it appears from this inevitably rough calculation that, when the liberation of enzymes is almost completely inhibited, a considerable proportion of the cell surface may be occupied by a mono-molecular film of inhibitor.

Cell-associated enzyme

One possible explanation of the means by which such a layer of polymer could prevent the appearance of an extracellular enzyme would be that it makes the cell impervious to the hyaluronidase molecules but allows free transit of the smaller molecular weight metabolites necessary for maintaining growth of the organisms. If the passage of hyaluronidase from the cells into the culture fluid were prevented by the layer of polymer one would expect an increase in the potency of enzyme associated with the cells themselves during growth. It has been previously shown (Rogers, 1954) that during early stages of growth of shaken cultures of staphylococci, small amounts of enzyme accumulate in close association with the cells. An examination was made of the potency of this cell-associated hyaluronidase in both treated and untreated suspensions of revived cells after 75 min. incubation in broth at 35°. The cells were removed from the cultures, spun down, washed, resuspended in 0.1M phosphate buffer pH 7.0 and 0.2 % gelatin, frozen and thawed and broken by shaking with Ballotini beads according to the method previously described (Rogers, 1954). Hyaluronidase in the supernatant obtained by centrifuging the suspensions of frozen and broken cells and in the culture supernatants themselves was estimated. Table 4 shows that there is certainly no tendency for more enzyme to accumulate in association with the treated cells; rather the appearance of cell-associated enzyme is also inhibited. The

Table 4. *The effects of pretreatment of revived cells upon the accumulation of cell-associated hyaluronidase*

Cells were pretreated with 53 D at the rate of 100 μ g./mg. dry wt. of organisms or with buffer alone (control suspension) under the conditions previously described. They were then suspended in warmed, buffered broth to give an opacity of about 1.0 mg. dry wt./ml. of culture and incubated with shaking at 35° for 75 min. Free and cell-associated hyaluronidase in the cultures was determined as described in the text.

	Total units of hyaluronidase (T.R.U.)	
	Treated suspension	Control suspension
Extracellular hyaluronidase	655	8450
Cell-associated hyaluronidase	<28	562
% cell-associated hyaluronidase	<5	7

inhibition of the liberation of extracellular enzyme cannot, therefore, be explained simply as due to a 'bottling-up' of free enzyme inside the cells by the 'varnishing' coat of inhibitor.

Change in the cells during revival

In the earlier experiments it had been shown that cells from young cultures or cells from 18 hr. cultures which have been subsequently incubated for an hour in fresh medium can be more effectively treated with inhibitor than those from overnight cultures. It had also been noted that the cells from young cultures appear darker after pretreatment than those from 18 hr. cultures. It, therefore, seemed likely that during the process of 'reviving' the cells some change takes place which enables more of the inhibitor to be attached to the cell surface. An experiment was planned using labelled inhibitor to examine whether the process of revival did in fact involve a change and if so, to estimate the speed of this change.

An 18 hr. culture of the organisms (100 ml.) in the usual medium was centrifuged and the cells then resuspended in broth (100 ml.) at 35° containing 0.1 M phosphate buffer and 0.25 % glucose; a sample was taken immediately. The flask was subsequently shaken at 35° and samples taken after 5, 20, 30 and 60 min. All the samples were cooled immediately and centrifuged at 0–4°. The cell pastes so obtained were then treated with labelled inhibitor at the rate of 0.3 mg./mg. dry wt. of organisms, under the usual conditions, washed, transferred to disks, frozen and dried for radioactive determination. Table 5 shows that the cells immediately after resuspension fixed

Table 5. *The amount of inhibitor taken up by staphylococci from an 18 hr. culture resuspended in broth and incubated at 35°*

Cells from an 18 hr. culture were resuspended in warmed buffered broth to give an opacity equivalent to about 2.0 mg. dry wt. of cells per ml. of culture. A sample (20 ml.) was taken immediately and again at the indicated times during incubation at 35° with shaking. All samples were cooled to 0° and centrifuged. The cells were then washed and pretreated with ¹⁴C-labelled 53D at a rate of 300 µg./mg. dry wt. of cells, washed again and the pastes used for radioactivity determinations. The figures under Expt. no. refer to experiments with different batches of cells.

Expt. no.	Time (min.)	53D fixed/mg. dry wt. organisms (µg.)
1	0	6
2	0	7
2	5	10
1	10	14
2	20	12
1	30	14
2	60	14

6–7 µg. of inhibitor/mg. of dry cells and that after 1 hr. incubation in broth they were able to fix 14 µg. The value of 6–7 µg. of inhibitor is somewhat larger than the amount which would be predicted from the degree of inhibition of hyaluronidase liberation. When cells from 18 hr. cultures were treated with a saturating dose of 53D the inhibition of the appearance of hyaluronidase ranged from 25 to 50 % with different batches of cells. A degree of inhibition of 50 % using revived cells would correspond to the fixation of less than 4 µg./mg. dry wt. of cells (see Table 3). It is thus possible that factors other than the amount of inhibitor fixed on the surface account for the difference between cells from 18 hr. cultures and the so-called revived cells. Insufficient work has, however, been done to allow firm conclusions to be drawn.

The change which enables revived cells to attach increased amounts of inhibitor occurs rapidly. Table 5 shows that at 35° the amount of inhibitor fixed has reached its maximum in 10–20 min. The nature of the change in the cells responsible for this effect is unknown but presumably involves a cationic shift in the region of the cell surface. It is not without interest that the potency of cell-associated hyaluronidase also rises to its maximum at about 30 min. under the conditions used here.

The effect of other macroanionic inhibitors

A number of other macroanionic substances which were known to be strong inhibitors of isolated hyaluronidase were tested for their effects upon the liberation of this enzyme by staphylococci. Revived cells were used and the technique of pretreatment and the subsequent incubation in broth were exactly as described for the experiments with 53D. The level used for pretreatment was 100 µg./mg. dry wt. of organisms.

There appears (see Table 6) to be little correlation between the ability of the macroanions to inhibit formed hyaluronidase as shown by the inhibition index, and their power to inhibit the liberation of the extracellular enzyme in cultures. The styrene-maleic acid co-polymer, for example, which is the strongest enzyme inhibitor known at present is almost totally inactive on the organisms. Polystyrene-sulphonate, on the other hand, although rather a poor enzyme inhibitor is quite active in culture. Sulphonation of the styrene-maleic acid co-polymer slightly reduced its ability to inhibit the action of hyaluronidase, but converted the compound which was almost completely inactive in culture, into a strong inhibitor. This latter observation suggests that the strongly ionized sulphonate group may be necessary for the action of the inhibitors in culture. Such a group, however, is apparently not essential since the 'polygentisic acid' of Hahn & Frank (1953) which has no sul-

Table 6. Pretreatment of cells with various macroanionic inhibitors of hyaluronidase

Revived cells were pretreated with the compounds at a level of 100 µg./mg. dry wt. under the same conditions as those used previously for 53D. The treated organisms were then resuspended in warmed buffered broth to give a density equivalent to 1.0 mg./ml. and shaken at 35° for 2 hr.

	Hyaluronidase (T.R.U./ml.)			Inhibition (%)			Inhibition index*
	1	2	3	1	2	3	
Polyhydroquinone-sulphonate	13	—	15	74	—	88	46
Polystyrene-sulphonate	27	—	—	45	—	—	22
Polystyrene-maleic acid co-polymer	45	—	—	15	—	—	100
Sulphonated polystyrene-maleic acid co-polymer	—	56	38	—	53	68	73
Polyphloroglucinol phosphate	39	—	—	20	—	—	38
Polygentisic acid	—	43	64	—	64	46	30
Poly- <i>p</i> -aminophenol-sulphonate	—	106	69	—	12	41	40
No inhibitor	49	119	118	—	—	—	—

* Inhibition index is the inverse of the concentration of the compound required to inhibit 1 T.R.U. of testicular hyaluronidase by 50 % when tested under the conditions previously described (Rogers & Spensley, 1954).

phonate groups, is an inhibitor. The behaviour of the poly-*p*-aminophenol-sulphonate is of some interest. This compound inhibited the appearance of enzyme to a very variable extent. In two experiments the results obtained were 12 and 40 % inhibition. It was at first thought that the very low result was due to some simple error in the experiment since a third experiment again showed a 40 % inhibition by this compound, but further work showed that this substance demonstrated to a much greater degree a phenomenon shown by the polyhydroquinone sulphonate. Reference has already been made to the diminishing degree of inhibition of hyaluronidase appearance obtained when the cultures of the treated cells are incubated over a period of time. The inhibition caused by pretreatment with 53D, for example, fell from more than 95 to 91 % during 3 hr. incubation of pretreated revived cells, and from 55 to 34 % when cells from an 18 hr. culture were used (see Table 2). Cells treated with poly-*p*-aminophenol-sulphonateshowed a very much greater recovery of their ability to liberate enzyme with increasing time of incubation. In one experiment, for example, the degree of inhibition after 90 min. incubation was 44 %, whereas after a further 90 min. both the treated and control cells had formed the same amount of enzyme. It therefore seems possible that the one low result obtained for this compound reflects a variation in the rate of recovery of the enzyme-forming ability. It may be that the substitution of one hydroxyl group in the polyhydroquinone-sulphonate by an amino group is sufficient to reduce the strength of the sulphonic acid group below the value necessary for close adhesion to the cell during incubation of the cultures. Cells treated with polygentisic acid, however, did not show this phenomenon. None of the inhibitors had any significant effect upon the growth of the organisms.

The effect of 53D upon coagulase formation

Table 7 shows that 53D is as able to inhibit the formation of coagulase by *Staph. aureus* strain Newman, as it is the formation of hyaluronidase by *Staph. aureus* strain 524. The conditions of testing were the same as those used for examining hyaluronidase formation. Revived cells were used and the level of treatment was 50 µg. of inhibitor/mg. dry wt. of organisms. When the dose of inhibitor used was halved, the inhibition was approximately halved.

Table 7. The effect upon coagulase liberation of pre-treating revived cells of Staphylococcus aureus strain Newman with 50 µg. of 53D per mg. dry weight of organisms

The conditions for treatment of the cells and subsequent incubation in broth were as described in Table 2. Revived cells only were used.

Time of incubation (hr.)	Clotting time (sec.)*			
	Treated cells		Control cells	
2	2400	2400	270	300
4	900	840	170	170

* Time required/ml. of the culture supernatant to clot a mixture of fibrinogen and activator incubated at 37°.

The production of trichloroacetic acid precipitable protein

It was previously shown (Rogers, 1954) that when *Staph. aureus* strain 524 is grown in shaken culture on a casein hydrolysate medium, trichloroacetic acid precipitable protein is liberated into the culture fluid. The above results have shown that pretreatment of staphylococci with 53D inhibits the appearance of both hyaluronidase and coagulase to about the same extent. It, therefore, seemed

possible that the inhibitor was preventing the liberation of all extracellular proteins. In order to test this possibility revived cells were pretreated at a level of 50 $\mu\text{g.}$ of 53D/mg. dry wt. of organisms in the usual manner, and resuspended in the buffered casein hydrolysate medium at an initial density equivalent to approximately 1.0 mg. dry wt. of cells/ml. (Rogers, 1954). These suspensions were then shaken at 35° and sampled at the times indicated in Table 8. The samples were cooled to 0–4°, centrifuged at this temperature and the supernatant fluids used for hyaluronidase and trichloroacetic acid precipitable protein determinations, carried out as described under the Methods section.

normally in treated cells was sought by examining the rates of respiration and glycolysis of normal and treated cells using glucose as substrate.

Revived cells were treated with 53D at a level of 50 $\mu\text{g.}/\text{mg.}$ dry wt. of bacteria and washed under the usual conditions. This suspension and its control (cells treated with buffer only) were then measured into Warburg flasks and the rate of oxygen uptake and carbon dioxide production from 0.01M bicarbonate buffer was measured at 35°. The side arms of the flasks contained sufficient glucose solution to give a final concentration of 0.01M. Flasks containing no glucose were included to measure endogenous respiration. An atmosphere of

Table 8. *The effect of pretreating revived cells with 53D upon the liberation of trichloroacetic acid precipitable protein by Staphylococcus aureus 524*

After pretreating revived cells, previously grown in broth in the usual way, with 53D at the rate of 100 $\mu\text{g.}/\text{mg.}$ dry wt. of cells or with buffer alone (control cells), they were washed and resuspended in warmed buffered casein hydrolysate medium to give a density equivalent to 1.0 mg. dry wt. of cells. Incubation was then continued at 35° with shaking. Samples were taken at the indicated times, cooled, centrifuged and the hyaluronidase and trichloroacetic acid precipitable protein in the supernatants determined.

Time (min.)	Treated cells		Control cells		% inhibition	
	Hyaluronidase (T.R.U./ml.)	Extracellular protein ($\mu\text{g.}/\text{ml.}$)	Hyaluronidase (T.R.U./ml.)	Extracellular protein ($\mu\text{g.}/\text{ml.}$)	Hyaluronidase	Extracellular protein
30	0.7	4.0	5.6	5.0	87	20
60	2.1	8.5	21	15	90	44
90	4.4	17	38	37	88	54
120	7.5	24	38	40	85	40
180	5.6	36	34	63	84	43

The results obtained for the inhibition of the appearance of hyaluronidase confirm the previous results obtained using the broth medium. The treated cells secrete only about 10–15% of the enzyme produced by the control cells. The appearance of extracellular protein is also inhibited, but to a very much less extent; about 50–60% of the normal amount of trichloroacetic acid precipitable protein was found in the supernatant fluid from the culture of treated cells. The nature and origin of this protein which is still formed despite the almost complete suppression of some enzymes is a matter for further work.

Effect of treatment with 53D upon respiration and glycolysis

The impaired ability of treated cells to form extracellular enzymes and protein does not extend to the rate at which the total mass of bacteria increases in the cultures. Moreover, concentrations of up to 1.0 mg./ml. of 53D added to the culture medium failed to influence growth. This suggests that small metabolites can enter and leave the treated cells at an undiminished rate. Further proof that metabolism of small molecules proceeds

95% N_2 and 5% CO_2 was used for the glycolysis experiments and of air for measurement of respiration. Both the Q_{O_2} ($\mu\text{l. O}_2$ consumed/mg. dry wt./hr.) and the $Q_{\text{CO}_2}^{\text{CO}_2}$ ($\mu\text{l. CO}_2$ produced/mg. dry wt./hr.) for the treated and untreated suspensions agreed with each other within the limits of experimental error. The former were 97.5 for the treated suspension and 97.0 for the control, whilst the results for glycolysis were 57.5 and 52.5 respectively. Thus treatment with 53D, at a level which almost completely suppresses the appearance of hyaluronidase and coagulase and about 50% suppresses the appearance of extracellular protein, has no effect on the enzyme systems concerned with either oxidation or fermentation of glucose.

DISCUSSION

The present results are consistent with the idea that the macroanion polyhydroquinone-sulphonate can form a closely attached layer, which may occupy a considerable proportion of the surface of the staphylococcal cells. This layer does not interfere with the passage of the low molecular weight metabolites necessary for growth or for the maintenance of

oxidation and fermentation of glucose. It does, however, almost completely suppress the appearance of two enzymes or enzyme-like substances, hyaluronidase and coagulase, in the culture fluid and reduces the amount of trichloroacetic acid precipitable proteins liberated by about 50 %. The question to be decided is whether or not the inhibition phenomena are to be regarded as effects on formation or effects on liberation of the proteins by the cells into the culture fluid. At present little evidence has been obtained upon which a decision might be reached. It is almost certain that hyaluronidase is not simply retained within the cells. It is, however, still possible that if a secretion mechanism is inhibited, enzymes which accumulate inside the cells are rapidly destroyed, either by the mass action reversal of enzyme formation or by intracellular proteolytic enzymes. Another attractively simple hypothesis is that the layer of inhibitor on the cell surface combines with the enzyme as it is formed and prevents its secretion into the medium or its detection after rupture of the cell. If this is so, then the ratio of inhibitor to enzyme required for inhibition of activity must be of an entirely different order from that found for the inhibition of isolated enzyme. Even with the low protein content of the solution used for determining inhibition indices (i.e. 0.2 % gelatin) it requires about 0.03 mg. of 53D to half-neutralize one unit of hyaluronidase. In the cultures this dose of inhibitor fixed to the cells suppresses the appearance of approximately 170 units. It is, however, dangerous to argue from the inhibition of an enzyme already formed to the inhibition of one freshly formed by a cell in close proximity to the inhibitor. A more cogent argument against this hypothesis is provided by the observation (Pollock, unpublished results) that the liberation of penicillinase into the culture fluids of *Bacillus cereus* is also greatly inhibited by treating the cells with 53D. This enzyme is not itself inhibited by 53D and therefore is unlikely to combine with it. It is difficult in this example to argue that the layer of polymer is simply combining with the freshly formed enzyme.

If indeed the layer of inhibitor is interfering with the processes of enzyme formation this is a matter of great interest from many points of view. Not least of these is the clear suggestion implicit that some stage in the formation of extracellular enzymes may

occur in the region of the cell surface. Such a hypothesis is consistent with the very great ease with which the small amounts of hyaluronidase associated with the cell can be removed (Rogers, 1954), suggesting that extracellular proteins may be formed as a capsule around the organisms rather than diffusing from within the cells.

Irrespective of the explanation of the mechanism of action of macroanions, the phenomenon of suppression of the appearance of extracellular enzymes and presumably toxins, without stopping growth of the micro-organisms, may prove of interest in more biological studies of the role of these active extracellular substances in infective processes.

SUMMARY

1. Treatment of staphylococcal cells from young cultures or of those from 18 hr. cultures which have been re-incubated in broth (revived cells) with certain macroanions prevents the appearance of hyaluronidase and coagulase during subsequent growth. Increase in the mass of cells in the cultures is not affected by this treatment.

2. The appearance of trichloroacetic acid precipitable protein during growth is depressed by about 50 % by macroanionic treatment.

3. Previously treated 'young' or revived cells retain enough inhibitor to occupy a considerable proportion of the cell surface.

4. A rapid change occurs during the process of revival which allows the cells to retain increased amounts of inhibitor.

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