Studies on Staphylococcal Penicillinase

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SUMMARY

The induction of penicillinase in *Staphylococcus aureus* resistant to benzyl penicillin has been investigated. Inducers tested included several new penicillins derived from 6-aminopenicillanic acid, benzyl penicillin amide and cephalosporin C. Fifteen strains of *S. aureus* were examined, one in detail and the others using only two or three inducers. All strains possessed basal penicillinase and all were inducible, although maximum enzyme levels varied somewhat from one strain to another.

Hydrolysis of the penicillins by staphylococcal penicillinase was investigated by using supernatant fluid from a cephalosporin C-induced culture of *Staphylococcus aureus* 524 as the source of enzyme. Under these conditions all the penicillins except methicillin were inactivated, although at different rates. Hydrolysis of methicillin was only detectable at much higher enzyme concentrations. Hydrolysis of cephalosporin C was not observed.

The use of penicillins for the treatment of staphylococcal infections is discussed in the light of these results.

INTRODUCTION

Bondi & Dietz (1945, 1948) observed that penicillinase was produced by all penicillin-resistant strains of *Staphylococcus aureus* that occurred clinically. They concluded that penicillinase production contributed to penicillin resistance. Bondi, de Saint Phalle, Kornblum & Moat (1954) reported that enzyme formation was constitutive, but Geronimus & Cohen (1957, 1958) found that penicillinase was inducible by penicillin, although there was a fairly high level of basal enzyme. They were unable to detect its presence in culture supernatants. They showed that the increase in penicillinase activity on induction with penicillin was due not to an increase in the rate of transport of substrate to pre-existing enzyme but to an increase in concentration of the enzyme within the cells. The above work was carried out using benzyl penicillin as the inducing agent, but benzyl penicillin is so rapidly inactivated that unless repeated additions of small amounts of penicillin are made at intervals it is difficult to demonstrate induction.

Little is known of the variation in penicillinase production by different penicillinase-producing strains of staphylococci, and we used inducers which are not rapidly hydrolysed (gratuitous inducers) in order to study the maximum enzyme production of which the strains are capable. For these reasons a number of strains of penicillinase-producing staphylococci were studied, using cephalosporin C and 6-(2:6-dimethoxybenzamido)-penicillanic acid (methicillin) as inducers, and following the production of enzyme for 4 hr. Also, a limited study was made using benzyl penicillin and 6-aminopenicillanic acid as inducers and estimating the penicillinase after 2 hr.

METHODS

Organisms. The penicillinase-producing strain of *Staphylococcus aureus*, strain 524 SC (Rogers, 1953) was normally maintained in ampoules in the dried state and grown back in broth. Several nutrient agar slopes were inoculated from the broth culture, and after incubation overnight at 35° were stored at +2° until required. After 8 days a subculture was made on to fresh agar slopes and any remaining from the first batch were discarded.

We are grateful to Professor L. P. Garrod, St Bartholomew's Hospital, London, for a number of strains of staphylococci resistant to benzyl penicillin, isolated from human infections (Strains 41, 46, 50, 74, 75 and 82 to 90). The samples as obtained were freeze-dried and grown when required as above.

Media. The staphylococci were found to grow well in Hedley-Wright broth containing 2.5% (w/v) of sodium β -glycerophosphate and 0.1% (w/v) glucose (Rogers, 1954), or in casein hydrolysate medium containing 0.5% (w/v) glucose, 1 μ g./ml. nicotinic acid and 1 μ g./ml. thiamine (Rogers, 1945).

Induction tests

(a) *Staphylococcus aureus* 524 SC. The organisms on one of the stored agar slopes were washed off with 1 ml. of the appropriate medium and added to 50 ml. of the Hedley-Wright broth or the casein hydrolysate medium in a 250 ml. conical flask. The cells were then incubated at 35° on a graded shaker (1 foot throw, 86 cyc./min., Kantorowicz, 1951) until the opacity was equivalent to between 0.1 and 0.2 mg./ml. dry weight of cells. A 10% solution of gelatin was usually added at this stage to give a final concentration of 1% gelatin in order to reduce inactivation of the enzyme.

Conical flasks (25 ml.) containing the test substance at appropriate concentrations in 0.4 ml. of 0.01M-phosphate buffer of pH 7 were inoculated with 2.6 ml. of the culture of *Staphylococcus aureus*. The flasks were then placed on the shaker and incubated at 35° for 2 hr., after which 0.4 ml. 0.005M-8-hydroxyquinoline was added to each flask, and the contents chilled in ice. Since there was some variation in basal enzyme and induced enzyme from day to day, two control flasks were always used, one containing only culture and phosphate buffer and the other containing culture, phosphate buffer and 5 μ g./ml. cephalosporin C.

(b) *Other strains.* The supplemented Hedley-Wright broth (100 ml. in a 500 ml. conical flask) was inoculated with 0.4 ml. of a fully grown broth culture and incubated at 35° on the shaker. When the optical density was approximately equivalent to 0.125 mg./ml. dry weight of cells, 10 ml. of melted 10% gelatin was added, and then the inducer. For routine tests cephalosporin C (5 μ g./ml.) or methicillin (0.5 μ g./ml.) were used, since these concentrations were found to have a negligible effect on growth but gave strong induction of penicillinase. Samples were taken every hour for 4 hr. The proportion of free enzyme was also estimated in the 4 hr. sample by centrifuging a sample and assaying the supernatant.

Penicillinase assay. The manometric assay of Henry & Housewright (1947) was used under the conditions employed by Pollock (1952). The enzyme activity of the samples is expressed in units/ml. (Pollock & Torriani, 1953); one unit of penicillinase is the quantity of enzyme which destroys 1 μ mole of benzyl penicillin in 1 hr. under

standard conditions. The same technique was used to measure the rate of hydrolysis of the other penicillins using a known enzyme concentration.

Compounds tested. The compounds tested were benzyl penicillin (penicillin G), phenoxymethyl penicillin (penicillin V), phenoxyethyl penicillin (phenethicillin), phenoxypropyl penicillin (PA-248), 6-aminopenicillanic acid (6-APA), 6-(2:6-dimethoxybenzamido)-penicillanic acid (methicillin), 6-(2:4:6-trinitrophenyl)-aminopenicillanic acid (picramido-PA), benzyl penicillin amide and cephalosporin C. Benzyl penicillin amide was synthesized by the method of Holysz & Stavely (1950). It was an amorphous yellow powder, and was about 80 % pure by the hydroxamate assay (Ford, 1947). Picramido-PA was prepared by the reaction of equimolar proportions of 6-APA and picryl chloride in a mixture of acetone and 0.5 M phosphate buffer at pH 7. It was isolated and tested as the triethylamine salt. It was orange-brown and water soluble, and was 91 % pure assayed by the hydroxamate method. Penicillin G and penicillin V were obtained as the sodium and potassium salts respectively from Glaxo Ltd., Greenford, Middlesex. We are grateful to Pfizer Ltd., Folkestone, Kent, for gifts of 6-aminopenicillanic acid, phenoxyethyl penicillin and PA-248; to Beecham Research Laboratories Ltd., Brockham Park, Betchworth, Surrey, for a gift of methicillin (BRL 1241), and to Dr E. P. Abraham, F.R.S., Sir William Dunn School of Pathology, Oxford, for a gift of cephalosporin C sodium salt.

Preparation of enzyme for substrate studies

The supplemented Hedley-Wright broth (500 ml.) was inoculated with *Staphylococcus aureus* 524 SC and incubated at 35° on the shaker until the opacity was equivalent to 0.1 mg./ml. dry weight of cells. Cephalosporin C was added to give a concentration of 5 µg./ml. Sterile gelatin was added to a concentration of 1 % and the mixture returned to the shaker at 35° for a further 4 hr. 50 ml. 0.005 M-oxine was then added and the cells centrifuged at 3000 g for 20 min. The supernatant was decanted and stored at +2°. The mean of two penicillinase assays of this preparation was 28.4 units/ml.

RESULTS

Induction tests

The supplemented Hedley-Wright broth was a somewhat more satisfactory medium (for induction of enzyme) than the casein hydrolysate medium, and was used throughout. The addition of gelatin (Manson & Pollock, 1953) had little effect on the enzyme titre in the 2 hr. period studied.

The results of typical experiments on *Staphylococcus aureus* 524 SC with the various penicillins are shown in Table 1. Abraham & Newton (1956) and Pollock (1957), using *Bacillus cereus* 569 as test organism, had noted that an intact β -lactam-thiazolidine nucleus of the type found in benzyl penicillin seemed to be essential for induction, but this criterion has now to be extended somewhat since cephalosporin C has a β -lactam-dihydrothiazine nucleus (Abraham & Newton, 1961). Benzyl penicillin and phenoxymethyl penicillin did not give clear-cut increases in penicillinase. Growth of the organisms, however, was not affected by 25 µg./ml. Since both of these penicillins are rapidly inactivated by the basal enzyme present in the culture, the concentration of penicillin which arrives at the primary site of inducer action might be considerably different from that of the medium as a whole, and may

depend on a large number of variables. Phenoxyethyl penicillin behaved similarly to benzyl penicillin. Phenoxypropyl penicillin (PA-248) gave small but definite induction at $1\mu\text{g./ml.}$ Benzyl penicillin amide was insoluble in water, and was dissolved in a small volume of acetone and diluted with an equal volume of water. On dilution with culture this gave a colloidal suspension of the amide. It gave a slight induction, and at $20\mu\text{g./ml.}$ was somewhat inhibitory.

Table 1. *Induction of penicillinase in Staphylococcus aureus 524 SC by various penicillins and cephalosporin C*

The experiments were carried out in Hedley-Wright medium, as described in the text, and the density of the organism on adding inducer was about 0.1 mg./ml. dry weight. Enzyme levels were estimated after 2 hr. at 35° . Single additions of benzyl penicillin, phenoxymethyl penicillin and phenoxyethyl penicillin ($25\mu\text{g./ml.}$) gave enzyme increases of up to twice the basal level, which was of doubtful significance, but see Table 2.

Compound	Basal enzyme (units/ml.)	Range of inducer concentration giving optimal induction ($\mu\text{g./ml.}$), and enzyme level obtained (units/ml.)		Lowest concentration of inducer giving significant inhibition of growth ($\mu\text{g./ml.}$)
		Inducer	Enzyme	
Phenoxypropyl penicillin (PA-248)	3.4	1-5	8-11	25
Benzyl penicillin amide	2.9	2-10	9-11	20
6-Aminopenicillanic acid (6-APA)	3.8	10-50	40-60	50
Methicillin	3.0	0.5-2	50-65	2
Pieramido-PA	4.1	5-10	85-110	50
Cephalosporin C	1.0	10-50	30-50	100

6-Aminopenicillanic acid could be tested over a wide range of concentration since it has very low antibiotic activity compared with penicillin G (Batchelor, Doyle, Nayler & Rolinson, 1959). It is a much more powerful inducer than the preceding compounds tested. Good induction was obtained at $1\mu\text{g./ml.}$ and maximum induction at about $50\mu\text{g./ml.}$ Pieramido-PA was a strong inducer, giving the highest titres recorded in this series, possibly because its antibacterial activity was very low, and its rate of hydrolysis by the enzyme was also low. Methicillin is a strong inducer of penicillinase, as reported by Rolinson *et al.* (1960), and maximum induction under the conditions used here is shown at low concentrations in the range $0.5-2\mu\text{g./ml.}$ It is, however, an inducer at very low concentrations, e.g. $0.05\mu\text{g./ml.}$ At concentrations higher than $2\mu\text{g./ml.}$ growth of the organisms is inhibited. Cephalosporin C differs from the penicillins in that induction is hardly demonstrable at $1\mu\text{g./ml.}$, while at $5\mu\text{g./ml.}$ and above considerable induction occurs.

Enzymic hydrolysis of penicillins

The rates of hydrolysis of the penicillins at 30° by penicillinase from *Staphylococcus aureus* 524 SC are given in Table 2. All were tested under the same conditions using 2.8μ moles/ml. of the penicillin and 1.0 ml. of enzyme preparation containing 9.1 units/ml. The reaction mixture was buffered at pH 7. In each case where reaction took place the rate of reaction was constant over the period of measurement.

The rates given are the average of five experiments carried out under identical conditions on each penicillin.

The most readily hydrolysed penicillins are phenoxymethyl penicillin, benzyl penicillin and phenoxyethyl penicillin. It was reported by Garrod (1960) that phenethicillin was somewhat more effective against resistant staphylococci than phenoxymethyl penicillin, and the latter was slightly more active than benzyl penicillin. Since phenoxymethyl penicillin is hydrolysed even more rapidly than benzyl penicillin, it is possible that other factors, such as its relative acid stability, may play a part in determining its effectiveness against staphylococci. The low rate of hydrolysis of benzyl penicillin amide may have been due to it being in a colloidal state rather than in true solution.

Table 2. *Inducers of penicillinase in Staphylococcus aureus*

Compound	Induction	Rate of hydrolysis by <i>S. aureus</i> penicillinase relative to benzyl penicillin (all at 2.8 mm substrate concentration)
Benzyl penicillin (Penicillin G)	+ *	1
Phenoxymethyl penicillin (Penicillin V)	+ *	1.27
Phenoxyethyl penicillin (Phenethicillin)	+ *	0.92
Phenoxypropyl penicillin (PA-248)	+	0.60
6-(2:6-Dimethoxybenzamido)-penicillanic acid (Methicillin)	+	0.003†
6-(2:4:6-Trinitrophenyl)-aminopenicillanic acid (Pieramido-PA)	+	0.11
6-Aminopenicillanic acid	+	0.15‡
Benzyl penicillin amide	+	0.19
Cephalosporin C	+	less than 0.01

* Repeated additions needed to demonstrate unequivocal induction.

† Novick (1962); $V_{\max}$ is 0.03 (iodometric assay).

‡ Novick (1962), by iodometric assay.

On hydrolysis of 6-aminopenicillanic acid the carboxyl group forms part of an α -amino acid grouping, and is thus not sufficiently acidic to release CO_2 from the bicarbonate solution (Steinman, 1961 *a*). Its hydrolysis cannot therefore be measured manometrically, but it can be measured by the iodometric method (Perret, 1954).

Penicillinase production by different strains of staphylococci

All the fifteen penicillinase-producing strains behaved in much the same way. All of them were inducible with 6-aminopenicillanic acid, with cephalosporin C and with methicillin. Figure 1 shows the results of a typical experiment.

There was evidence of loss of enzyme activity in induced cultures of all strains. No way of preventing this loss of activity has yet been found, so that it is not possible to give exact figures for many of the parameters involved in enzyme induction. The following conclusions, however, seem to be fairly certain for induction with 6-APA, cephalosporin C and methicillin. Most strains had a basal enzyme activity equivalent to about 2–5 enzyme units per mg. dry weight of cells. The maximum level of activity generally occurs 2–3 hr. after adding inducer, with a drop at times later than this. Commonly by 4 hr. there has been a fall to three-

quarters or a half of the activity found at 2 or 3 hr. The maximum level of activity under the conditions used was generally 10–25 enzyme units/ml. of culture, equivalent to about 11–28 units per mg. dry weight of cells, although as noted in another section this could be increased by using higher levels of inducer than were used here.

Fig. 1. Penicillinase production with various inducers in *Staphylococcus aureus* strain 46. —○— No inducer; —△— cephalosporin C, 5 $\mu\text{g./ml.}$; ---□--- methicillin, 0.5 $\mu\text{g./ml.}$; —×— 6-amino-penicillanic acid, 5 $\mu\text{g./ml.}$

The inducing activity of 0.5 $\mu\text{g./ml.}$ of methicillin was approximately equal to that obtained with 5 $\mu\text{g./ml.}$ of cephalosporin C, while 5 $\mu\text{g./ml.}$ of 6-aminopenicillanic acid gave somewhat greater induction.

The level of the induced enzyme at 2–3 hr. after adding the inducer was usually 10–20 times the basal level. The lowest value for induced enzyme was four times that of the basal enzyme: it is thus clear that all the strains of penicillinase-producing staphylococci are inducible. However, high levels of enzyme are not pro-

duced by a single addition of benzyl penicillin. With all the strains the addition of 0.5 $\mu\text{g./ml.}$ of benzyl penicillin gave only a slight increase over the basal level of enzyme, being never more than twice that level; frequently the increase was undetectable by the technique used.

The amount of enzyme which was released from the cells was estimated 4 hr. after induction by assaying the supernatant fluid after centrifuging. It was generally found that about half the activity was cell-bound and half was extracellular. All the strains showed extracellular enzyme, but in preliminary experiments it was noted that very little cell-free enzyme was present earlier than 4 hr. after adding inducer. It may be noted, however, that the penicillinase of *Bacillus subtilis* is also mainly cell-bound in young cultures but mainly free after several hours (Pollock, 1961).

The findings above on penicillinase production in various strains of resistant staphylococci are in reasonable agreement with those of Wildhack, Ceci, Moat & Bondi (1960), Geronimus & Cohen (1957), Leitner & Cohen (1960), Rolinson *et al.* (1960), and Steinman (1961*b*).

DISCUSSION

It would appear from these results that all the strains of staphylococci resistant to benzyl penicillin can be induced to form penicillinase by treatment with the various penicillins. There does not seem to be any obvious difference in inducibility between the various resistant strains examined. Maximum induction obtained in a given time depends mainly on the initial concentration of the penicillin and on the rate at which it is hydrolysed by the enzyme as long as growth of the organism is unaffected. Other factors, such as the rate at which the inducer reaches its site of action in the bacteria, may have some slight effect, although this rate is generally assumed to be very rapid. Small amounts of benzyl penicillin were shown to be very rapidly fixed by an intracellular reaction in staphylococci by Rowley, Cooper, Roberts & Smith (1950). It is, however, possible that inducers of the cephalosporin C type may not be able to enter the cells so readily.

It is difficult with benzyl penicillin to demonstrate induction by a single addition of inducer. This is evidently because the benzyl penicillin is rapidly hydrolysed and after a short time the process of enzyme induction ceases. This is similar to most inducible enzyme systems, in which induction promptly ceases if the inducer is removed, and contrasts with the induction of penicillinase in *Bacillus cereus*, where enzyme production continues at a high rate after removal of the penicillin (Pollock, 1950). The efficacy of 6-aminopenicillanic acid, methicillin and cephalosporin C as inducers is evidently due to their resistance to inactivation by staphylococcal penicillinase. If a fairly constant concentration of benzyl penicillin is maintained by continuous addition, then induction continues and a high enzyme titre is obtained (Pollock, private communication). It follows from this that within a lesion in the animal produced by penicillin G resistant staphylococci, conditions for the induction of penicillinase may be very favourable no matter which penicillin is used for treatment. The penicillin will arrive at the site of infection continuously via the bloodstream so that even rapidly inactivated penicillins could induce considerable enzyme formation. Thus a non-inducing penicillin might therefore offer some clinical advantages. The rate of inactivation of the penicillin by the enzyme will largely

determine its effectiveness against the infection. In addition, when a penicillin is readily inactivated by the enzyme, the results of the usual *in vitro* tube-dilution sensitivity test can be very misleading as a guide to its clinical effectiveness, since the assay is commonly carried out using small and variable inocula, and the penicillin concentration at a given time will depend largely on the concentration of basal enzyme. Geronimus (1960) estimates that within a lesion the number of viable organisms could approach 10^{10} per gram of tissue, so that it is rash to predict the effectiveness of a penicillin when it is tested against inocula containing small numbers, such as 10^4 organisms/ml.

The most interesting of the true penicillins (i.e. excluding cephalosporin C) is methicillin. Its fairly low antibacterial potency compared to benzyl penicillin is offset by its resistance to staphylococcal penicillinase, making it just as effective against penicillinase-producing as against non-penicillinase-producing staphylococci. It is effective because it has an extremely low affinity for the enzyme (Novick, 1962). In clinical use the concentration of methicillin in the bloodstream, although therapeutically effective, is sufficiently low that enzymic inactivation (because of its very low affinity) is negligible. But it would not be necessary to change the enzyme affinity in order to develop clinically important resistance to methicillin; it would be sufficient if the ordinary staphylococcal penicillinase were to be produced in quantities about 1000 times greater than that found in most penicillinase-producing staphylococci. The use of penicillin has up to now selected for penicillinase-producing ability that is small but adequate from the point of view of the organism; it is possible that methicillin may be a more powerful selecting agent among the natural populations of staphylococci, either for a very high level penicillinase production or a changed penicillinase able to inactivate methicillin at low concentrations, or both. A watch should therefore be kept for the emergence of strains with a very high level of penicillinase production, as these would be clinically resistant to methicillin.

REFERENCES

- ABRAHAM, E. P. & NEWTON, G. G. F. (1956). A comparison of the action of penicillinase on benzylpenicillin and cephalosporin N and the competitive inhibition of penicillinase by cephalosporin C. *Biochem. J.* **63**, 628.
- ABRAHAM, E. P. & NEWTON, G. G. F. (1961). The structure of cephalosporin C. *Biochem. J.* **79**, 377.
- BATCHELOR, F. R., DOYLE, F. P., NAYLER, J. H. C. & ROLINSON, G. N. (1959). Synthesis of penicillin: 6-Aminopenicillanic acid in penicillin fermentations. *Nature, Lond.* **183**, 257.
- BONDI, A., JUN. & DIETZ, C. C. (1945). Penicillin resistant staphylococci. *Proc. Soc. exp. Biol., N.Y.* **60**, 55.
- BONDI, A. & DIETZ, C. C. (1948). The susceptibility of penicillinase-producing bacteria to penicillin. 1. Factors influencing susceptibility. *J. Bact.* **55**, 843.
- BONDI, A., DE SAINT PHALLE, M., KORNBLUM, J. & MOAT, A. G. (1954). Factors influencing the synthesis of penicillinase by *Micrococcus pyogenes*. *Arch. Biochem.* **53**, 348.
- FORD, J. H. (1947). Hydroxylamine method of determining penicillins. *Analyt. Chem.* **19**, 1004.
- GARROD, L. P. (1960). Relative antibacterial activity of three penicillins. *Brit. med. J.* **i**, 527.
- GERONIMUS, L. H. (1960). Inoculum size and apparent sensitivity of staphylococci to penicillins. *New Engl. J. Med.* **263**, 349.
- GERONIMUS, L. H. & COHEN, S. (1957). Induction of staphylococcal penicillinase. *J. Bact.* **73**, 28.

- GERONIMUS, L. H. & COHEN, S. (1958). Further evidence for inducibility of staphylococcal penicillinase. *J. Bact.* **76**, 117.
- HENRY, R. J. & HOUSEWRIGHT, R. D. (1947). Studies on penicillinase. II. Manometric method of assaying penicillinase and penicillin, kinetics of the penicillin-penicillinase reaction and the effects of inhibitors on penicillinase. *J. biol. Chem.* **167**, 559.
- HOLYSZ, R. P. & STAVELY, H. E. (1950). Carboxy derivatives of benzylpenicillin. *J. Amer. chem. Soc.* **72**, 4760.
- KANTOROWICZ, O. (1951). Shaking apparatus for the aeration of bacterial cultures. *J. gen. Microbiol.* **5**, 276.
- LEITNER, F. & COHEN, S. (1960). Effect of pH on constitutive and inducible penicillinase synthesis in *Staphylococcus aureus*. *Bact. Proc.* p. 150.
- MANSON, E. E. D. & POLLOCK, M. R. (1953). The thermostability of penicillinase. *J. gen. Microbiol.* **8**, 163.
- NOVICK, R. P. (1962). Staphylococcal penicillinase and the new penicillins. *Biochem. J.* (in the Press).
- PERRET, C. J. (1954). Iodometric assay of penicillinase. *Nature, Lond.* **174**, 1012.
- POLLOCK, M. R. (1950). Penicillinase adaptation in *B. cereus*: Adaptive enzyme formation in the absence of free substrate. *Brit. J. exp. Path.* **31**, 739.
- POLLOCK, M. R. (1952). Penicillinase adaptation in *Bacillus cereus*: An analysis of the three phases in the response of logarithmically growing cultures to induction of penicillinase formation by penicillin. *Brit. J. exp. Path.* **33**, 587.
- POLLOCK, M. R. (1957). The activity and specificity of inducers of penicillinase production in *Bacillus cereus*, strain NRRL 569. *Biochem. J.* **66**, 419.
- POLLOCK, M. R. (1961). 'Exo-enzymes' in *The Bacteria*, Vol. 4. Ed. I. C. Gunsalus & R. Y. Stanier. New York: Academic Press.
- POLLOCK, M. R. & TORRIANI, A-M. (1953). Purification et caractéristiques physicochimiques de la pénicillinase de *Bacillus cereus*. *C.R. Acad. Sci., Paris*, **237**, 276.
- ROGERS, H. J. (1945). The conditions controlling the production of hyaluronidase by micro-organisms grown in simplified media. *Biochem. J.* **39**, 435.
- ROGERS, H. J. (1953). Variant populations within a hyaluronidase producing culture of *Staphylococcus aureus*. *J. Path. Bact.* **66**, 545.
- ROGERS, H. J. (1954). The rate of formation of hyaluronidase, coagulase and total extracellular protein by strains of *Staphylococcus aureus*. *J. gen. Microbiol.* **10**, 209.
- ROLINSON, G. N., STEVENS, S., BATCHELOR, F. R., WOOD, J. C. & CHAIN, E. B. (1960). Bacteriological studies on a new penicillin—BRL 1241. *Lancet*, ii, 564.
- ROWLEY, D., COOPER, P. D., ROBERTS, P. W. & LESTER SMITH, E. (1950). The site of action of penicillin. 1. Uptake of penicillin on bacteria. *Biochem. J.* **46**, 157.
- STEINMAN, H. G. (1961*a*). Determination of the rate of enzymatic hydrolysis of 6-amino penicillanic acid. *Fed. Proc.* **20**, 232.
- STEINMAN, H. G. (1961*b*). Factors modifying induced formation of penicillinase in *Staphylococcus aureus*. *J. Bact.* **81**, 895.
- WILDHACK, J., CECI, C. N., MOAT, A. G. & BONDI, A. (1960). Relationship between penicillinase inducibility and penicillinase resistance. *Bact. Proc.* p. 129.