PENICILLINASE PRODUCTION AND METAL-ION RESISTANCE IN *STAPHYLOCOCCUS AUREUS* CULTURES ISOLATED FROM HOSPITAL PATIENTS

K. G. H. DYKE*†, M. T. PARKER* AND M. H. RICHMOND‡

*Central Public Health Laboratory, Colindale Avenue, Colindale, London, and ‡Department of Molecular Biology, University of Edinburgh, West Mains Road, Edinburgh

THE genes determining the production of penicillinase by *Staphylococcus aureus* are usually located extrachromosomally on a plasmid (Novick, 1963), but in at least one strain (Asheshov, 1966) there is evidence that they form part of the chromosome. Other genes that have been found on the same plasmid as the penicillinase genes are those that determine resistance to metal ions and to erythromycin (Richmond and John, 1964; Novick and Richmond, 1965; Novick and Roth, 1968).

Resistance to mercury ions (Moore, 1960) is very frequently a character of staphylococcal strains that have become established endemically in hospitals, and mercury-resistant strains usually produce large quantities of penicillinase and are resistant also to antibiotics unrelated to penicillin (Richmond *et al.*, 1964). There are at least three immunologically distinct staphylococcal penicillinases (Richmond, 1965); "hospital" staphylococci generally form A-type penicillinase in considerable quantity, and a relatively large proportion of the enzyme is found extracellularly in such cultures (Dyke and Richmond, 1967).

Resistance to heavy metals other than mercury {viz. cadmium, arsenic (as arsenate), bismuth and lead} has been observed in *Staph. aureus* cultures, and the determinants for these resistances are sometimes on the same plasmid as the penicillinase and mercury-resistance genes (Novick and Roth).

In the present investigation, we attempted to establish a relation between the type of penicillinase and the resistance to several metal ions in strains of *Staph. aureus* that had been responsible for septic infections in hospital patients. At the same time, we tried to find out how frequently the penicillinase gene was carried on the chromosome, and whether genes conferring resistance to other antibiotics were located on the same plasmid as the penicillinase genes in a manner analogous to that which occurs in the resistance episomes of the enterobacteria (Watanabe, 1963).

METHODS

Cultures examined. The 96 cultures used in this investigation were those previously described by Dyke and Richmond, and had been isolated in 1963 as part of a continuing survey of *Staph. aureus* strains responsible for sepsis in hospitals in the London area. Approximately 100 consecutively isolated cultures had been received from each of eight hospitals,

Received 1 July 1969; accepted 27 July 1969.

† Present address: Microbiology Unit, Department of Biochemistry, University of Oxford.

‡ Present address: Department of Microbiology, University of Bristol.

and a strain was considered to be endemic if it was found three or more times in the series of cultures from one hospital. A "sporadic" strain was one that appeared only once in a collection of strains from a particular hospital. Only those strains that were lysed by at least one of the phages of groups I and III and were resistant to penicillin are included. Within these limits, one representative of each "endemic" strain from each of the eight hospitals and a culture of every "sporadic" strain from four of the hospitals, were studied.

Certain other cultures of *Staph. aureus* that were used in transduction experiments will be specified in the text.

Characterisation of strains. Cultures were phage-typed and tested for resistance to benzylpenicillin, streptomycin, chloramphenicol, tetracycline, erythromycin, oleandomycin and novobiocin by a disk test (Dyke and Richmond). Resistance to metal ions was determined on nutrient agar plates flooded with broth cultures, by means of paper disks containing 0.8 μ moles phenyl mercuric nitrate, 0.01 μ moles cadmium acetate or 0.8 μ moles sodium arsenate.

Degree of extracellularity and immunological type of penicillinase. These were determined as previously described (Dyke and Richmond).

Selection of penicillinase-negative variants. All strains were screened for loss of ability to produce penicillinase by the starch-iodine method (Dyke, Jevons and Parker, 1966), and where possible a single penicillinase-negative variant was selected.

Selection of cadmium-sensitive variants. A stationary-phase culture in casein yeast- (CY-) medium (Novick) was diluted 100 times in CY-medium and incubated at 43°C for 6 hr. The cultures were then diluted in CY-medium and spread on 5 CY-agar plates to produce about 50 colonies per plate. The plates were incubated for 16 hr at 37°C and then replicated on to CY-agar with added cadmium acetate to a final concentration of 50 μ M. After further incubation for 16 hr at 37°C, the plates were inspected and any colonies that had not grown on the cadmium plates were isolated from the master plates and tested for sensitivity to cadmium ions.

Transduction. Phage 80, no. NCTC9788, was propagated in lytic cycle on the donor bacterium by the soft agar layer method of Swanstrom and Adams (1951). The phage preparation was filtered, and the bacteria-free phage suspension was mixed with the recipient bacteria at a multiplicity of infection of 0.3 in the presence of 2mM-CaCl₂. Phage adsorption was allowed to occur for 30 min. at 37°C and further adsorption was prevented by addition of sodium citrate to a final concentration of 0.1M. The suspension was centrifuged and the pellet was resuspended in CY-medium and allowed to grow for 2 hr at 37°C before selection on plates containing CY-agar with added benzylpenicillin (0.1 μ g per ml) or 50 μ M cadmium acetate.

In some experiments, the phage preparation was irradiated with ultraviolet light as described by Asheshov.

RESULTS

Resistance to metal ions of penicillinase-producing Staph. aureus strains

Resistance to arsenate and cadmium was found in 70 of the 96 strains, and was always in association; 37 strains were mercury-resistant, and 34 of these were also resistant to arsenate and cadmium; 36 strains were resistant to arsenate and cadmium but sensitive to mercury (table I).

Strains that were resistant to all three metal ions were nearly always resistant to one or more antibiotics unrelated to penicillin (multiple-antibiotic resistant), and nearly two-thirds of them (24 of 34) were considered to have been endemic in hospitals in that they were representatives of groups of three or more identical cultures isolated from the lesions of patients in a hospital. With few exceptions, they had the phage-typing patterns of well-recognised "hospital staphylococci"; 22 of the 24 endemic strains were members of the 52/52A/80/81

complex (Asheshov and Winkler, 1966) or had the phage-typing pattern 83A or 84/85.

Just over one-third of the cultures (36 of 96) were mercury-sensitive but resistant to arsenate and cadmium. Although few of them (4 of 35) were multiple-antibiotic resistant, 10 were isolated under circumstances that suggested that they were established endemically in hospitals, and a number of them were recognisable as strains responsible for occasional outbreaks of sepsis in hospitals (5 had patterns including reaction with phage 29, 4 were lysed by phage 80 or phage 81 only, and one had the pattern 52A/79).

TABLE I
Metal-ion resistance of strains of Staphylococcus aureus

Sensitivity to			Number of strains			
Hg*	Asa	Cd	Total	"Endemic"	"Sporadic"	"Multiple-resistant"†
R‡	R	R	34	24	10	33
R	S	S	3	2	1	2
S	R	R	36	10	26	4
S	S	S	23	1	22	4

* Hg = Mercury; Asa = arsenate; Cd = cadmium.

† Resistant to one or more antibiotics unrelated to penicillin.

‡ R = Resistant; S = sensitive.

Only one of the 23 strains that were sensitive to all three metal ions appeared to have been endemic in a hospital.

Characteristics of penicillinase in relation to metal-ion resistance

The relation of metal-ion resistance to the amount and type of the penicillinase produced by the strains was next examined. A preliminary inspection of the results showed that the strains forming penicillinase of immunological type A fell into two well-defined groups, one in which a considerable proportion of the enzyme was found extracellularly and the other in which most of it was associated with the bacterial cells (fig. 1), and the line of demarcation appeared to be between 20 and 25 per cent. extracellular. Strains forming C-type penicillinase, on the other hand, showed no such differentiation; in nearly all cases the proportion of extracellular penicillinase was relatively high.

In table II, therefore, producers of A-type penicillinase, but not of C-type penicillinase, are divided into a group with high extracellularity (25 per cent. or more) and a group with low extracellularity (less than 25 per cent.). The table shows that the strains that were resistant to mercury, arsenate and cadmium all produced A-type penicillinase, that nearly all of them formed the enzyme in large amount, and that a considerable proportion of it was

extracellular. The two mercury-resistant but arsenate- and cadmium-sensitive strains had similar characteristics.

FIG. 1a.—A-type-penicillinase-producing strains.

FIG. 1b.—C-type-penicillinase-producing strains.

FIG. 1.—Extracellularity of penicillinase.

The percentage of the total penicillinase that is extracellular under standard conditions was determined as described in *Methods*.

Strains that were sensitive to mercury but resistant to arsenate and cadmium formed either A-type or C-type penicillinase, and in both cases the proportion of enzyme produced extracellularly was usually high.

Strains sensitive to all metal ions formed A-type or C-type penicillinase with about equal frequency, but produced much of the enzyme extracellularly only if the enzyme was C-type.

Endemic hospital strains of *Staph. aureus* were in general either mercury-resistant producers of A-type penicillinase, which usually formed large amounts of enzyme extracellularly (Dyke and Richmond), or were mercury-sensitive but arsenate- and cadmium-resistant producers of either A-type or C-type penicillinase. Although the latter strains nearly always produced a large proportion of the enzyme extracellularly, the total amount produced was in a number of cases not great. This point is clarified in table III, which shows the

TABLE II
Metal-ion resistance in relation to type of penicillinase formed by strains of Staphylococcus aureus

Type of penicillinase (and extracellularity)	Resistant to			Number of strains			
	Hg†	Asa	Cd	Total	"Endemic"	"Multiple-resistant"‡	High penicillinase§
A (high*)	R	R	R	33	24	32	31
	R	S	S	2	2	2	1
	S	R	R	12	5	2	4
	S	S	S	3	0	0	3
A (low)	R	R	R	1	0	1	0
	S	R	R	2	0	0	0
	S	S	S	10	1	2	3
C	R	S	S	1	0	0	1
	S	R	R	22	5	2	12
	S	S	S	10	0	2	3

* >25 per cent. penicillinase extracellular.

† Hg = Mercury; Asa = arsenate; Cd = cadmium.

‡ Resistant to one or more antibiotics unrelated to penicillin.

§ Penicillinase factor >0.5 (Richmond *et al.*, 1964).

|| R = Resistant; S = sensitive.

proportion of strains that produced large amounts of enzyme, and the proportion with high extracellularity, among "endemic" and "sporadic" strains. It appears that mercury-sensitive "endemic" strains, which were nearly always resistant to arsenate or cadmium, generally produced a penicillinase with a high degree of extracellularity, although less than half of them had penicillinase factors exceeding 0.5. Nevertheless, strains devoid of all metal-ion resistance, which may produce a considerable proportion of the enzyme extracellularly if it is of type C, were rarely present endemically in hospital. Thus an all-inclusive definition of the characters of the "endemic" strains must include both high extracellularity in penicillinase production and resistance to arsenate and cadmium.

Metal-ion resistance of penicillinase-negative variants

To isolate penicillinase-negative variants, a stock agar-slope culture that had been kept for several months was used to inoculate CY-medium; after

incubation overnight, starch plates were inoculated and were screened for variant colonies after incubation. In this way, penicillinase-negative variants were recovered from a single plate of some cultures, but with others it was necessary to examine a large number of plates. In all, it proved possible to obtain variants from 95 of the 96 cultures, but over 25,000 colonies of the remaining strain were screened without success.

A single colony of each penicillinase-negative variant was subcultured and tested for resistance to metal ions. Table IV shows the resistance of the original

TABLE III

Amount of penicillinase formed, and proportion of enzyme formed extracellularly, in "endemic" and "sporadic" strains of Staphylococcus aureus

Metal-ion sensitivity of strains	Penicillinase type	Proportion of			
		"endemic" strains		"sporadic" strains	
		with high* penicillinase production	with high† extra-cellularity	with high penicillinase production	with high extra-cellularity
‡Hg resistant	A(37), C(1)	23/26	26/26	10/12	11/12
Hg sensitive, Asa and Cd resistant	A	2/5	5/5	2/8	6/8
	C	2/5	5/5	10/17	15/17
Sensitive to Hg, Asa and Cd	A	1/1	0/1	5/12	3/12
	C	0	0	3/10	9/10

* Penicillinase factor >0.5 (Richmond *et al.*, 1964).

† >25 per cent. extracellular.

‡ Hg = Mercury; Asa = arsenate; Cd = cadmium.

cultures and of the variants. All strains that were mercury-, arsenate- and cadmium-resistant lost all three resistances, and strains that were resistant only to mercury lost this resistance, along with the penicillinase. Among the strains that were mercury-sensitive but resistant to arsenate and cadmium, however, the producers of C-type penicillinase behaved differently from the producers of the A-type enzyme; all 10 of the strains with A-type enzyme, but none of the 22 with C-type enzyme, lost these metal resistances along with the penicillinase.

These findings suggest that, in strains that are resistant to mercury, arsenate and cadmium, the genetic determinants of all three resistances are carried on the same plasmid as the penicillinase genes. In arsenate-resistant and cadmium-resistant strains that produce A-type enzyme, the metal-resistance genes are similarly linked with the penicillinase genes, but in producers of C-type enzyme the determinants of metal-ion resistance appear to have a different location.

Location of the cadmium-resistance gene in producers of C-type penicillinase

In order to find out whether the metal-resistance of producers of C-type penicillinase was located on a separate plasmid from the penicillinase gene, cadmium-sensitive variants were sought. All 22 of the cadmium-resistant producers of C-type penicillinase were examined by replica-plating on agar plates containing 50 μ M cadmium acetate and on CY-agar after growth for 6 hr at 43°C (May, Houghton and Perret, 1964). On the average, 355 colonies per strain were screened (range 185–961 colonies), but no sensitive variants were found.

TABLE IV

Association of metal-ion resistance with penicillinase production in strains of Staphylococcus aureus

Penicillinase type (and extracellularity)	Number of strains	Metal-ion resistance of original strains			Metal-ion resistance of penicillinase-negative variant		
		Hg†	Asa	Cd	Hg	Asa	Cd
A (high*)	33	R‡	R	R	S	S	S
	2	R	S	S	S	S	S
	11§	S	R	R	S	S	S
A (low)	1	R	R	R	S	S	S
	2	S	R	R	S	S	S
C	1	R	S	S	S	S	S
	22	S	R	R	S	R	R

* >25 per cent. of penicillinase extracellular.

† Hg = Mercury; Asa = arsenate; Cd = cadmium.

‡ R = Resistant; S = sensitive.

§ A penicillinase-negative variant was not obtained from one strain in this group.

The presumption that the cadmium-resistance determinant was located chromosomally was investigated by measuring the frequency of transduction of cadmium resistance after irradiating the transducing phage with graduated doses of ultraviolet light (Arber, 1960; Asheshov). The frequency of transduction of a chromosomal marker is stimulated by low doses of ultraviolet light, possibly because breakage of the phage-carried DNA induces repair enzymes in the recipient bacteria, and these repair enzymes facilitate recombination into the bacterial chromosomal DNA; on the other hand, there is no stimulation of the transduction of extrachromosomal DNA since no recombination is necessary for its establishment in the recipient, and the transduction frequency falls exponentially with increasing doses of ultraviolet light in parallel with loss of phage viability.

Phage 80 was propagated on strain no. 17855 which is a strain of phage-typing pattern 29/80, produces C-type penicillinase and is resistant to cadmium and arsenate but sensitive to mercury. The derived penicillinase-negative

variant of no. 17855 is resistant to cadmium and arsenate. The phage 80, propagated on no. 17855, was used to transduce either cadmium resistance or penicillinase to a penicillinase-negative variant of strain no. 9033. Strain no. 9033 is an A-type penicillinase-producing strain resistant to mercury, cadmium and arsenate, but the penicillinase-negative variant is sensitive to all three metal ions. Fig. 2 shows the effect of dose of ultraviolet light on this transduction. Penicillin resistance was transduced at a higher frequency than cadmium resistance, and the results suggest that in this strain the cadmium-resistance determinant is chromosomal, but the penicillinase gene is extrachromosomal. None of the penicillin-resistant transductants was resistant to cadmium or arsenate, and all the cadmium-resistant transductants were resistant to arsenate

FIG. 2.—The effect of ultraviolet irradiation on the frequency of transduction of cadmium resistance $\bigcirc$ — $\bigcirc$, and of penicillin resistance $\bullet$ — $\bullet$, from strain no. 17855 to strain no. 9033N.

ions but not to penicillin. At least in this strain it seems that the gene for metal-ion resistance is on the chromosome whereas the penicillinase gene is extrachromosomal.

Resistance of penicillinase-negative variants to other antibiotics

The 96 strains were all resistant to penicillin by virtue of the production of penicillinase. Fifty-three of them were resistant only to penicillin, and 43 were also resistant to one or more other antibiotics. The multiple-antibiotic resistant strains included 38 that were resistant to tetracycline, 28 that were resistant to streptomycin, and eight that were resistant to chloramphenicol; three had low-level resistance to erythromycin of the inducible type, and one had high-level (non-inducible) resistance to erythromycin and oleandomycin. Comparison of the antibiotic-resistance patterns of the parent penicillinase-producing strains with those of the derived penicillin-negative variants showed that, among the 44 multiple resistant strains, the only change other than loss of resistance to penicillin was that two strains had become tetracycline-sensitive. An examination of the original slopes of these two cultures showed that they contained a

large proportion of tetracycline-sensitive variants, only a few of which were penicillinase-negative. Further investigation indicated that the two resistances were lost independently, as described by May, Houghton and Perret. Thus, in no case is there any evidence of linkage of a gene determining resistance to one of the antibiotics tested to the gene determining penicillinase production.

Mitsubishi *et al.* (1965) described a strain of *Staph. aureus* in which the determinant of erythromycin resistance is present on the same plasmid as the penicillinase gene. This strain was non-inducibly resistant to high-levels of erythromycin, so it was decided to seek co-loss of penicillinase and erythromycin resistance in strains other than the one of this description included in the

FIG. 3.—The effect of ultraviolet irradiation on the frequency of penicillin resistance from the stable penicillin-resistant strain to strains no. 17855N ●—● and 9033N ○—○.

present survey. Five other penicillin-resistant strains with high-level non-inducible resistance to erythromycin were found among approximately 3000 strains isolated from lesions of patients in London hospitals in other years (Hewitt and Parker, 1967). Penicillinase-negative variants were selected from all five strains, but in none was there any alteration to the resistance to erythromycin.

Stable penicillin-resistance

Asheshov described one strain of *Staph. aureus* in which there is evidence that the penicillinase gene is chromosomal. We failed to obtain a penicillinase-negative variant from only one of the 96 strains, so that a chromosomal location for penicillinase must be uncommon. The strain with stable penicillinase had the phage-typing pattern 29/52/80 and was resistant to arsenate and cadmium ions but sensitive to mercury salts. Phage 80 was propagated on this strain and the phage preparation was irradiated with graduated doses of ultraviolet light and then used to transduce penicillinase into two

distinct penicillin-sensitive recipient staphylococci. The recipient bacteria were a cadmium-resistant but penicillinase-negative variant derived from strain no. 17855, and a penicillinase-negative, cadmium-sensitive derivative of strain no. 9033. The frequency of transduction of the penicillinase genes plotted against ultraviolet dose is shown in fig. 3. Strain no. 17855 was a much better recipient than strain no. 9033, but in both low doses of ultraviolet light stimulated transduction, indicating that the penicillinase gene was chromosomal. However, all 20 of the penicillinase transductants into strain no. 9033 were found to be cadmium-resistant, which suggests that the cadmium-resistance determinant is also chromosomal.

Asheshov obtained different results with her strain; the determinant for cadmium resistance was extrachromosomal and when penicillinase production and cadmium resistance were co-transduced both behaved as extrachromosomal markers.

DISCUSSION

Staphylococcal strains that have established themselves endemically in hospitals are almost exclusively members of phage-groups I and III, in which the penicillinase is always of immunological type A or C. The production of B-type penicillinase by members of phage-group II, and its relation to metal-ion resistance, will be described separately (Dyke, unpublished).

Our classification of staphylococcal strains as "endemic" and "sporadic" was somewhat arbitrary, and was based on the frequency with which they appeared in sets of cultures isolated consecutively from septic lesions in eight hospitals. This probably underestimated the number of "endemic" strains, because not every importation of a strain with "endemic" properties will necessarily be followed by spread of infection, and also because a strain first appearing late in the series might not have caused three lesions before the end of the annual collection of cultures. It was to be expected, therefore, that some of the strains classified as "sporadic" would have characters identical with those of the "endemic" strains.

Over two-thirds of the groups of endemic staphylococcal infections were due to easily recognisable "hospital staphylococci" that were resistant to several antibiotics, and also to mercury, cadmium and arsenate ions. They nearly always produced large amounts of A-type penicillinase, and a considerable proportion of the enzyme was extracellular (Richmond *et al.*, 1964; Dyke and Richmond, 1967). This association of the ability to produce large amounts of a "good" penicillinase with resistance to several antibiotics was not due to the presence of the genetic determinants for all the antibiotic resistances on a single extrachromosomal particle, as in the Enterobacteriaceae. With the single exception of the Japanese strain (Mitsuhashi *et al.*, 1965) in which a single plasmid determines the production of penicillinase and resistance to erythromycin, we have seen no evidence, in this or other series of cultures, of a genetic linkage between resistance to two unrelated antibiotics. It appears, therefore, that resistances in staphylococci are ecologically associated rather than genetically linked (Richmond *et al.*). Each new resistance acquired by a strain increases

the chances that it will persist long enough in the hospital environment to become resistant to further antibiotics, either by gene-transfer or by mutation.

A number of other strains also appeared to have a limited ability to cause endemic infection in hospitals. Nearly all of these strains were sensitive to mercury salts but resistant to cadmium salts and to arsenate, and most of them were sensitive to antibiotics other than penicillin. They formed less than one-third of the "endemic" strains and generally gave rise to rather smaller groups of infection than did the mercury-resistant strains; six of the 26 mercury-resistant, but only one of the ten mercury-sensitive but cadmium- and arsenate-resistant, strains caused ten or more septic lesions in any one hospital.

Penicillinase-producing strains that were sensitive to all three metal ions were relatively common in cases of sporadic sepsis, but rarely caused groups of related infections.

It was also observed that, in nearly all of the "endemic" mercury-sensitive strains, a considerable proportion of the penicillinase was extracellular, even though the total amount produced was not always great; but high "extracellularity" of penicillinase production alone will not explain the relative success of cadmium- and arsenate-resistant strains, because it occurs in nearly all strains that produce C-type penicillinase. Resistance to arsenate and to cadmium salts may have practical significance as a "marker" for strains of *Staph. aureus* with some ability to spread in hospitals, but the precise nature of the advantage it confers is not clear.

There is evidence of the in-vivo transfer of penicillinase plasmids from strain to strain of staphylococcus, probably mediated through phage (Novick and Morse, 1967). The frequency of transduction of plasmids is higher than that of chromosomal genes (Asheshov, 1966), and the interstrain spread of penicillin resistance might be more rapid if the determinant was on a plasmid than if it were part of the chromosome. It is to be expected, therefore, that plasmid-borne penicillinase would be more widespread in different strains of staphylococci than chromosomal penicillinase. The A-type penicillinase gene may at first have been chromosomal and closely linked to the genes for metal-ion resistance, and looping-out by the mechanism proposed by Campbell (1962) may have occurred because a mutation produced increased homology on either side of the region containing these genes. In this way, a plasmid might be formed which was capable of autonomous replication because it was a replicon with initiator and replicator (Jacob, Brenner and Cuzin, 1963).

C-type penicillinase genes are different in that they are rarely linked to metal-ion resistances; in this case the regions of homology on the chromosome that arose by mutation were perhaps such as to exclude cadmium and arsenate ions, so that they remained on the chromosome.

SUMMARY

Ninety-six penicillinase-producing strains of *Staphylococcus aureus*, all members of the phage-groups I and III, were examined for sensitivity to mercury, cadmium and arsenate.

Strains resistant to mercury, arsenate and cadmium were nearly always multiple-antibiotic resistant and produced large amounts of A-type penicillinase, much of it extracellular. They included representatives of a few widespread types of "hospital" staphylococci.

Other strains, that were sensitive to mercury but resistant to arsenate and cadmium appeared, though less frequently, to occur endemically in hospitals; they were generally sensitive to antibiotics other than penicillin and although they produced variable amounts of penicillinase, much of it was extracellular. Their penicillinase was either of the A-type or the C-type; in strains with A-type penicillinase the determinants for resistance to arsenate and cadmium appeared to be located on the penicillinase plasmid, but in strains with C-type penicillinase they appeared to be chromosomal. Penicillin-negative variants appeared relatively frequently in all but one of the cultures, suggesting that the genetic determinant for penicillinase production was on a plasmid; in one culture, however, there was evidence that it was chromosomal. Resistance to other antibiotics was not genetically linked with penicillinase production.

This work was completed while K. G. H. D. was a member of the Medical Research Council's staff at the National Institute for Medical Research, Mill Hill, London, N.W.7, and seconded to Colindale.

We wish to thank Mrs A. J. Raine for expert technical assistance.

REFERENCES

- ARBER, W. 1960. *Virology*, **11**, 273.
 ASHESHOV, ELIZABETH H. 1966. *Nature, Lond.*, **210**, 804.
 ASHESHOV, ELIZABETH H., AND WINKLER, K. C. 1966. *Ibid.*, **209**, 638.
 CAMPBELL, A. 1962. *Adv. Genetics*, **11**, 101.
 DYKE, K. G. H., JEVONS, M. PATRICIA, AND PARKER, M. T. 1966. *Lancet*, **1**, 835.
 DYKE, K. G. H., AND RICHMOND, M. H. 1967. *J. Clin. Path.*, **20**, 75.
 HEWITT, J. H., AND PARKER, M. T. 1967. *Ibid.*, **20**, 921.
 JACOB, F., BRENNER, S., AND CUZIN, F. 1963. *Cold Spring Harb. Symp. Quant. Biol.*, **28**, 329.
 MAY, J. M., HOUGHTON, R. H., AND PERRET, C. J. 1964. *J. Gen. Microbiol.*, **37**, 157.
 MITSUHASHI, S., HASHIMOTO, H., KONO, K., AND MORIMURA, M. 1965. *J. Bact.*, **89**, 988.
 MOORE, B. 1960. *Lancet*, **2**, 453.
 NOVICK, R. P. 1963. *J. Gen. Microbiol.*, **33**, 121.
 NOVICK, R. P., AND MORSE, S. I. 1967. *J. Exp. Med.*, **125**, 45.
 NOVICK, R. P., AND RICHMOND, M. H. 1965. *J. Bact.*, **90**, 467.
 NOVICK, R. P., AND ROTH, CHRISTINE 1968. *Ibid.*, **95**, 1335.
 RICHMOND, M. H. 1965. *Biochem. J.*, **94**, 584.
 RICHMOND, M. H., AND JOHN, M. 1964. *Nature, Lond.*, **202**, 1360.
 RICHMOND, M. H., PARKER, M. T., JEVONS, M. PATRICIA, AND JOHN, M. 1964. *Lancet*, **1**, 293.
 SWANSTROM, M., AND ADAMS, M. H. 1951. *Proc. Soc. Exp. Biol. Med.*, **78**, 372.
 WATANABE, T. 1963. *Bact. Rev.*, **27**, 87.