19. Elek, S. D., Fleming, P. C., *Lancet*, 1960, v2, 569.

20. Sutherland, J. M., *et al.*, *Am. J. Dis. Child.*, 1961, v102, 793.

21. Sonnenwirth, A. C., Greenman, M., Kallus, B. A., *J. Pediat.*, 1962, v61, 351.

22. Sabath, L. D., Postic, B., Finland, M., *Am. J. Med. Sci.*, 1962, v244, 484.

23. Klein, J. O., Sabath, L. D., Finland, M., *ibid.*, 1963, v245, 399.

24. Steers, E., Foltz, E. L., Graves, B. S., *Antibiot. and Chemother.*, 1959, v9, 307.

25. Haight, T. M., Finland, M., *Am. J. Clin. Path.*, 1952, v22, 806.

26. Novick, R. P., *Biochem. J.*, 1962, v83, 236.

27. Gilson, B. S. C., Parker, R. F., *J. Bact.*, 1948, v55, 801.

28. Grove, D. C., Randall, W. A., *Assay Methods of Antibiotics*, Medical Encyclopedia, Inc., New York, 1955, p14.

29. Lepper, M. H., Dowling, H. F., Jackson, G. G., Spies, H. W., Mellody, M., *Antibiot. Ann.*, 1956-1957, p640.

Received June 20, 1963. P.S.E.B.M., 1963, v113.

Studies on the Penicillinase of *Staphylococcus albus*.* (28564)

JAN O. KJELLANDER† AND MAXWELL FINLAND

Thorndike Memorial Laboratory, Second and Fourth (Harvard) Medical Services, Boston City Hospital and Department of Medicine, Harvard Medical School, Boston, Mass.

It has been demonstrated(1) that a large proportion of strains of *Staphylococcus albus (Staph. epidermidis)* from various clinical sources were resistant *in vitro* to penicillin G, and that about 10% of such strains were also resistant to methicillin and to some of the other new, semisynthetic penicillins that are considered to be resistant to degradation by penicillinase. The majority of these strains produced a penicillin G-beta-lactamase, but the mechanism of their resistance to methicillin appeared to be inherent in the bacterial cells. Further studies of the properties of penicillinase of *Staph. albus* are presented here and discussed in the light of current knowledge about the penicillinase of *Staph. aureus*(2-11).

Materials and methods. Bacterial strains. The 12 strains of *Staph. albus* (J1-J12) used are the same as those included in the previous study(1); they had been obtained in pure culture from the blood of patients at the Boston City Hospital. *Staph. aureus*, BCH 60/1, the strain that was used for comparisons was isolated in 1960 from a suppurative lesion; it was phage type 80/81/KS6, produced penicillinase, and had been used in earlier studies in this laboratory(2, 12). The susceptibility of these strains to penicillin G and methicillin as determined on antibiotic-containing agar by the inocula-replicating method of Steers, Foltz and Graves(13), is shown in columns 1 and 2, respectively, of Table I.

Preparation of penicillinase. Except where otherwise indicated the strains were grown for 24 hr at 37°C in brain-heart infusion broth (Difco), pH 7.2, containing methicillin, 1.0 μg/ml, as an inducer. Three preparations of these broth cultures were tested for penicillinase activity. One consisted of the culture sediment obtained after centrifugation at 3000 rpm for 30 min; the sediment was washed twice with Sörensen's phosphate buffer, pH 6.4, and extracted twice with acetone and then twice with ether at −20°C, as described by Gilson and Parker(3). The dried residues were resuspended in the original volume of buffer solution containing 0.5% gelatin and shaken vigorously with sterile glass beads; these suspensions will be referred to as "cell preparations." The culture supernatant was divided into 2 portions, one of which was passed through a Seitz filter, the other through a Millipore filter of 0.3 μ porosity. The 3 preparations from each strain were kept frozen at 20°C until used and showed no loss of enzyme activity during the 4

* Aided by a grant from Nat. Inst. Health.

† Present address: Bacteriol. Lab., Karolinska Sjukhuset, Stockholm, Sweden.

TABLE I. Penicillinase Activity of 12 Strains of *Staph. albus* and Their Susceptibility to Penicillin G and Methicillin.

Strain of Staph.	M.I.C.,* μg/ml		Penicillinase activity, units/ml at S=26.7 μM		V_{max}, cells, μmoles of penicillin G hydrolyzed per hr	K_m, cells, μM	Penicillinase activity, units/ml at S=M.I.C. (V mic)	
	Penicillin G	Methicillin	Cells	% of total			Cells	Whole culture†
albus: J1	1.56	1.56	1.2	94.1	4.0	66.7	.2	.3
J2	400	3.1	9.2	44.6	12.3	9.1	12.2	27.4
J3	3200	6.25	80.3	93.2	120.0	13.3	119.8	128.6
J5	1.56	1.56	1.0	93.3	2.4	40.0	.2	.3
J7	3.1	1.56	1.3	80.0	2.6	27.8	.6	.8
J9	50	3.1	8.0	61.9	12.8	16.4	11.4	18.5
J10	.39	6.25	.1	80.0	.3	71.4	.1	.1
J4	100	12.5	.5	80.0	1.3	40.0	1.1	1.4
J8	50	100	.2	87.0	.8	83.3	.5	.6
J6	1600	100	4.0	72.5	6.4	16.4	6.4	8.7
J11	>3200	100	22.9	40.0	40.0	20.0	39.9	76.6
J12	400	>100	6.5	77.7	10.0	14.7	9.9	12.7
aureus 60/1	>3200	3.1	18.2	38.0	25.0	10.0	24.9	65.7
Column No.	1	2	3	4	5	6	7	8

* Minimum inhibiting concentration.
† Sum of values for cell preparation + Millipore filtrate.

months of this study.

Determinations of penicillin G-beta-lactamase activity. Beta-lactamase activity was determined essentially as described by Novick(4), except that it was carried out at pH 6.4 (in Sörensen's buffer) and in a waterbath at 35°C. The concentration of substrate was 10 μg/ml (= 26.7 μmoles/l) unless otherwise indicated. Readings were taken on a Coleman Junior spectrophotometer at 620 mμ in selected, 19 × 105 mm round cuvettes. A typical experiment was conducted as follows:

To 80 ml of buffer solution was added 5 ml of 0.8 M iodine-iodide solution(4) and 5 ml of a heated 2% starch solution prepared according to Smithies(14). To each of 2 cuvettes was added 0.5 ml of the penicillinase preparation to be tested; this had been diluted in enough buffer solution to give a rate of decolorization of the starch-iodine that could be followed photometrically for 30-40 min. Nine ml of the starch-iodine-buffer solution, which had been kept at 35°C for at least 20 min, was added to one of the cuvettes; 15 sec later the same amount was added to the second cuvette and both were allowed to react for 15 min at 35°C. This period was chosen to allow the major part of the nonspecific absorption of iodine by the preparation to take place. In some instances, such as when whole cultures or undiluted Seitz filtrates were tested, the iodine content had to be 4 to 5 times higher, and the contact time 10 min longer because of the greater nonspecific absorption of iodine.

At the end of the preincubation period, 0.5 ml of a penicillin G solution, pH 6.4, and containing 200 μg/ml was added to one of the cuvettes that contained the penicillinase preparation and starch-iodine mixture, and after exactly 15 sec 0.5 ml of plain buffer solution was added to the second cuvette. Both cuvettes were left in the water bath for 10 min before any readings were taken. This period was shown, in preliminary experiments, to be necessary to allow for the initial, nonspecific absorption of iodine by the penicillin. At three 10-min intervals thereafter, photometric readings were taken, using the reading of the penicillin-containing cuvette to set the zero point. The decrease in optical density (OD) in this tube was thus read as an increase in the difference in OD between this cuvette and the one containing the penicillin-free color blank, which was read exactly 15 sec after the zero point had been set with the test cuvette.

On the basis of experiments carried out

with known concentrations of iodine and known amounts of penicillin G that had been totally hydrolyzed by staphylococcal penicillinase, a nomogram was constructed using the formula‡ given by Novick(4). From this nomogram direct readings could be made of enzyme activity (V), knowing the change in OD (Δ E) and the time (Δ t). The observed velocity of the reaction (v) was then corrected for the dilution of the preparation tested and expressed as the number of μmoles of penicillin G degraded/hr/ml of the original enzyme preparation. On the basis of 6 parallel determinations of v, the 95% confidence limits of this method were calculated to be $\pm$ 3.4%.

Determination of K_m and V_{max}. These 2 parameters as defined in the Michaelis-Menten equation(15), were determined only for the cell preparations. This was done by establishing the observed reaction velocities (v) at different levels of substrate concentrations (s). The s values chosen were 10.0, 5.0, 3.3, 2.5 and 2.0 μg/ml penicillin G, corresponding to 26.7, 13.4, 8.9, 6.7 and 5.3 μM/l. The reciprocals of v were plotted against the corresponding values of s in a Lineweaver-Burk(16) plot, and the values of V_{max} and K_m calculated directly from this graph, as suggested by Dixon(17). An example of such an experiment is shown in Fig. 1.

Enzyme induction. These experiments were carried out with broth cultures grown at 37°C for 6 hr with and without methicillin, 1.0 μg/ml. Activity of the enzyme in this instance was determined for the whole culture allowing for the increased amount of iodine and the longer preincubation period as noted above.

‡ $v = \frac{\Delta E}{lxPx\Delta t}$, where v = observed velocity of penicillinase activity in μmoles of penicillin G inactivated/hr; ΔE = difference in OD observed during the interval Δt (in hr); l = length of light path (diameter of cuvette); and P is the constant representing the change in OD of the starch-iodine solution resulting from addition of 1 μmole of penicilloic acid to 1 liter of starch-iodine, 40 μM with respect to the iodine. The value of lxP obtained in the standard experiments was 0.231.

Results. Enzyme activity. The penicillinase activity of the cell preparations is listed in column 3, Table I. The values varied widely from 0.1 (J10) to 80.3 units/ml (J3). The majority of the *Staph. albus* preparations showed activities of less than the 18.2 units per ml attained by the *Staph. aureus* preparation; the latter value was exceeded only by 2 preparations of *Staph. albus* (J3 and J11).

None of the Seitz filtrates had detectable enzyme activity, but some activity was found in the Millipore filtrates of all strains. Column 4 in Table I lists the activity of each cell preparation expressed as percent of total activity (cells + Millipore filtrate). In most of the strains of *Staph. albus* more than 75% of the total activity was found in the cell preparation, but in 2 of them (J2 and J11) the filtrates had essentially the same activity as the cell preparation. In the *Staph. aureus* almost 2/3 of total activity was found in the Millipore filtrate.

V_{max} and K_m. These values are recorded in columns 5 and 6 in Table I. As could be expected from the variations in penicillinase activity, the V_{max} showed a wide range of values from 0.3 (J10) to 120.0 (J3) μmoles penicillin G hydrolyzed/ml/hr; 10 of the 12 *Staph. albus* preparations yielded lower values than that of *Staph. aureus* (25.0 μmoles/ml/hr).

K_m values also varied, but not to the same extent. The lowest value for a *Staph. albus* preparation was 9.1 and the highest 83.3 μM of penicillin G; 6 of the preparations had K_m values of 20 or lower, approaching that of *Staph. aureus* which was 10.0 μM.

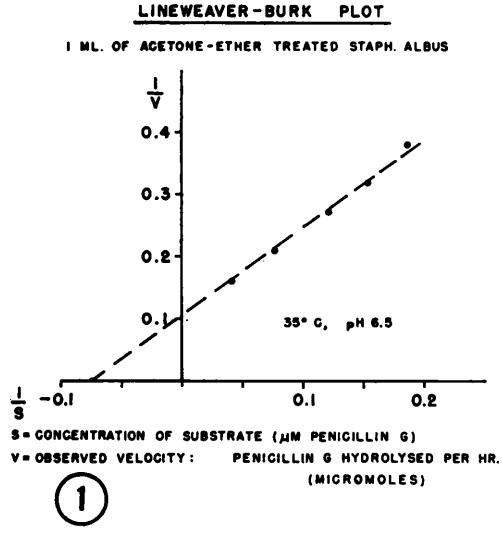
Induction experiments. The results obtained with strains of *Staph. albus* and with the strain of *Staph. aureus* are shown in Table II. All the enzymes were clearly inducible, since the activities in the methicillin-free broths were approximately 1% of those in the broths with methicillin.

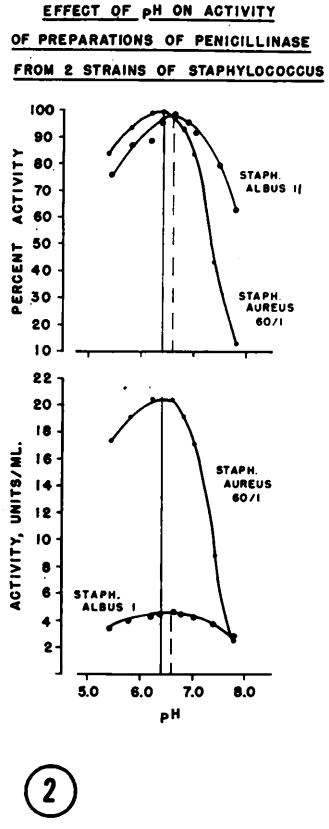
pH optimum. Fig. 2 depicts the results of experiments in which the cell preparations of *Staph. albus* J11 and of *Staph. aureus* 60/1 were tested for enzyme activity at different levels of pH. Although the shape of the two curves differs somewhat, the pH optima for

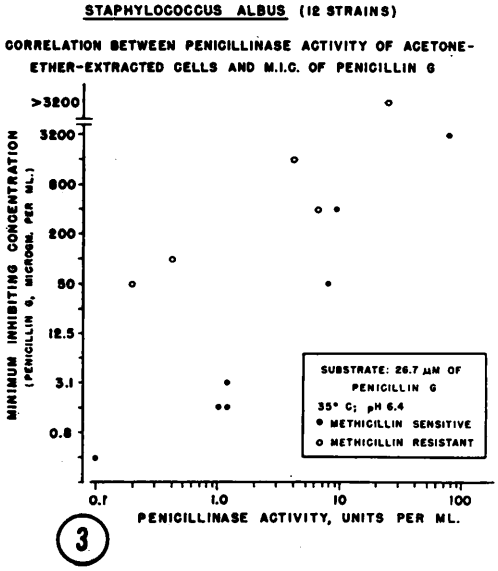
the 2 preparations were nearly the same, pH 6.4 and 6.6, respectively. The same experiment was carried out with 2 additional strains of *Staph. albus*, which showed pH optima of 6.4 (J3) and 6.5 (J9).

Correlation between enzyme activity and

FIG. 1. Lineweaver-Burk plot based on an experiment with the cell preparation of *Staph. albus*, J12, from which the following values are derived: $\frac{1}{V_{max}} = 0.1$; $V_{max} = 10.0$ μmoles penicillin G/ml/hr; $-\frac{1}{K_m} = -0.07$; $K_m = 14.7$ μM.







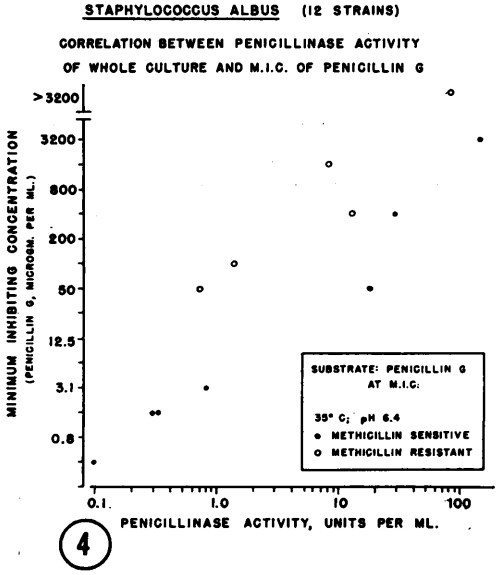


FIG. 1-4

TABLE II. Effect of Methicillin as an Inducer on Penicillinase Activity.

Staph. strain	Penicillinase activity of whole broth culture,* units† per ml: Not induced	Induced with methicillin, 1 μg/ml
albus J1	<.01	1.12
J3	.12	16.80
J11	.10	9.38
aureus 60/1	.16	10.08

* Cultures incubated for 6 hr at 37°C.
† μmoles penicillin G destroyed per hr.

MIC of penicillin G. In Fig. 3 the enzyme activities of the 12 *Staph. albus* cell preparations are plotted against the MIC of penicillin G on a log-log scale. Although there appears to be a fairly good correlation it was felt for reasons to be discussed below, that a better correlation might be obtained if the values for total activity (cells + Millipore filtrate) were used and the activity determined, not at a fixed substrate concentration (10 μg/ml or 26.7 μM), but at the substrate concentration corresponding to the MIC for the respective strains. These values are shown in Table I, column 8, and are plotted in Fig. 4. A better correlation is thus obtained, especially if the methicillin-susceptible strains and the methicillin-resistant ones are distinguished; the latter (5 strains with MIC $\geq$ 12.5 μg/ml) are shown in the figure as open circles and the former (7 strains, MIC $\leq$ 6.3 μg/ml) as solid dots. There is now a very good correlation between the 2 parameters within each group of strains.

Discussion. That penicillin G-resistant strains of *Staph. albus* produce penicillin beta-lactamase was shown previously(1). The present investigation was undertaken to characterize this enzyme further and to compare it with the better known enzyme *Staph. aureus*. The 2 enzymes were shown to be very similar in many respects. Both are found within the cells as well as extracellularly, and neither one passes through a Seitz filter(5). The higher activity of the Millipore filtrate as compared with the cell preparation of the *Staph. aureus* strain, the reverse being true for the corresponding preparations of the strains of *Staph. albus*, may possibly be explained by the differences in growth rate of these different types of bacteria. It was noted that *Staph. aureus* 60/1 grew rapidly, reaching the stationary phase within 8 hours at 37°C, as compared with 16-24 hr for the strains of *Staph. albus*. If, as suggested by Sabath and Finland(5), the extracellular enzyme represents enzyme liberated from disrupted cells, the growth rates would affect the distribution of enzyme in the direction indicated by present results. Other factors, such as structural differences in the 2 types of enzymes might, of course, also account for the findings.

The enzymes from the 2 types of bacteria also resemble each other in that they are inducible, and their pH optima appear to be nearly the same.

V_{max} values for the *Staph. albus* cell preparations varied over a 400-fold range, which is far more than can be explained by the error of the method. It might be argued that this variation does not necessarily reflect a true difference in the activities of the different enzymes, since the values are expressed in terms of volume of the original culture and not on the basis of weight or nitrogen content of the preparation, the variation possibly being due to differences in the amounts of enzyme tested. It seems unlikely that these explanations alone could account for such large differences, but the possibility cannot be totally excluded. However, inasmuch as the enzyme activities of the strains in this study correlated well with their susceptibility to penicillin G as shown in Fig. 4, it seemed more logical to determine the amount of enzyme activity actually produced by the strains within a specified time rather than to compare the preparation on a basis of weight.

The possible explanation offered for the differences in V_{max} values does not apply to the K_m values, since these are independent of amount of enzyme tested. Geronimus and Stansberry(10) reported a K_m value of 13 μM for the cell bound *Staph. aureus* penicillinase tested by them at pH 6.6 and 37°C and Novick(11) reported a value of 19 μM at pH 5.8 and 30°C. In the present study, the cell bound penicillinase was tested at pH 6.4 and 35°C and showed a value of 10 μM. These results are compatible and the differ-

ences might well be explained by the differences in experimental conditions. The *Staph. albus* strains show almost a 10-fold difference from the lowest to the highest value of K_m which is more than can be accounted for by the error of the method. Since the enzyme preparations tested were by no means pure, it is difficult to know whether this variation reflects a true difference in the structure of the enzymes, or is due to different locations of the enzymes in the cells or depends on the composition of the cells themselves which may affect the accessibility of the enzyme to the substrate.

In their early studies on *Staph. aureus* penicillinase, Spink and Ferris(6) found a good correlation between enzyme activity of the strains and their susceptibility to penicillin G. This was essentially confirmed by Gilson and Parker(3) who found some notable exceptions, on the basis of which Riewerts Eriksen and Hansen(7) concluded that there are probably factors "other than the amount of penicillinase produced (that) may be responsible for penicillin-resistance." For the *Staph. albus* strains used here, there was a fairly good correlation between their susceptibility to penicillin G and the enzyme activities when the latter was tested with a substrate concentration of 10 μg/ml or 26.7 μM (columns 1 and 3 in Table I and Fig. 3). There were, however, some exceptions; for example, strains J2 and J9 had approximately the same enzyme activities (9.2 and 8.0 units/ml, respectively) whereas their MIC values were appreciably different (400 and 50 μg/ml, respectively). On the other hand, the MIC of penicillin G for strains J8 and J9 was the same (50 μg/ml), but they differed markedly in their enzyme activities, 0.2 and 8.0 units/ml, respectively.

From the differences in K_m values obtained for the different preparations, it seems that testing of the penicillinase activities of the strains at a standard substrate concentration (10 μg/ml) tends to give somewhat misleading values. Activities of enzymes derived from strains with MIC values lower than the substrate concentration used in the assay tend to be too high, whereas the reverse would be the case with enzymes from strains with higher MIC values. It seems more logical to express the activities as those obtained at the highest substrate concentration at which the strains are still able to grow. The best estimate of this value is that of the MIC. When this was used as a basis for calculating enzyme velocities for the "whole cultures," the correlation was very good (Fig. 4). This modification also clearly indicated that the methicillin-resistant strains in this respect form a group separate from the methicillin-sensitive ones. The most likely explanation for this seems to be that, in addition to their penicillinase effect, the methicillin-resistant cells have a higher level of "inherent" or "natural" resistance to penicillin G than do the methicillin-sensitive ones. This is consistent with the findings of Barber(8) and of Wallmark(9), among others, who demonstrated that an increase in resistance to methicillin is paralleled by an increase in resistance to penicillin G. This permits the cells to produce their enzyme from a higher "base level" of penicillin G, which in turn leads to an increase in rate of hydrolysis.

Summary and conclusions. The properties of penicillin G-beta-lactamase from 12 strains of *Staph. albus (Staph. epidermidis)* were compared with those of *Staph. aureus* 60/1. The enzymes from both sources were similar in that: 1) they were inducible; 2) they were intra- as well as extracellular; the extracellular enzymes were filterable through Millipore filters of 0.3 μ porosity, but not through Seitz filters; 3) their pH optima were close (pH 6.4-6.6). The enzyme activities of acetone-ether extracted cells of 24 hr-induced broth cultures of *Staph. albus* varied from 0.1-80.3 units/ml (pH 6.4, 37°C); that of corresponding *Staph. aureus* cells was 18.2 units/ml. V_{max} values varied over a similar range. K_m values varied from 9.1 to 83.3 μM penicillin G for *Staph. albus* strains; K_m for *Staph. aureus* 60/1 was 10.0 μM. There was a close correlation between MIC and enzyme activity for *Staph. albus* strains, particularly when activity was measured at substrate conc. equal to MIC (Fig. 4). When thus compared, the strains fell into 2 distinct groups, one comprising 5 methicillin-resistant strains (MIC $\geq$ 12.5 μg/ml) and the other

the methicillin-sensitive ones (MIC $\leq$ 6.25 μg/ml). It is concluded that the level of resistance to penicillin G in *Staph. albus* is the result of both the activity of the penicillinase it produces and the level of its "inherent" or natural resistance, the latter being higher for the methicillin-resistant than for the methicillin-sensitive cells.

1. Kjellander, J. O., Klein, J. O., Finland, M., PROC. SOC. EXP. BIOL. AND MED., 1963, v113, 1023.
2. Sabath, L. D., Finland, M., *Nature*, 1962, v193, 272.
3. Gilson, B. St.C., Parker, R. F., *J. Bact.*, 1948, v55, 801.
4. Novick, R. P., *Biochem. J.*, 1962, v83, 236.
5. Sabath, L. D., Finland, M., PROC. SOC. EXP. BIOL. AND MED., 1962, v109, 478.
6. Spink, W. W., Ferris, V., *J. Clin. Invest.*, 1947, v26, 379.
7. Riewerts Erikson, K., Hansen, D., *Acta path. et microbiol. Scand.*, 1954, v35, 169.
8. Barber, M., in *Resistance of Bacteria to the Penicillins*, de Reuck, A. V. S., Cameron, M. P., Ed., CIBA Foundation Study Group 13, Little, Brown & Co., Boston, 1962, p89.
9. Wallmark, G., *ibid.*, p103.
10. Geronimus, L. H., Stansberry, M., *Bact. Proc.*, 1963, p93.
11. Novick, R. P., *Biochem. J.*, 1962, v83, 229.
12. Wallmark, G., Finland, M., PROC. SOC. EXP. BIOL. AND MED., 1961, v106, 78.
13. Steers, E., Foltz, E. L., Graves, B. S., *Antibiot. and Chemother.*, 1959, v9, 307.
14. Smithies, O., *Biochem. J.*, 1955, v61, 629.
15. Thorn, M. B., in *Biochemists' Handbook*, Cyril Long, Ed., D. van Nostrand Co., Inc., Princeton, N. J., 1961, p205.
16. Lineweaver, H., Burk, D., *J. Am. Chem. Soc.*, 1934, v56, 658.
17. Dixon, M., *Biochem. J.*, 1953, v55, 170.

Received June 20, 1963. P.S.E.B.M., 1963, v113.

A Diluent of Infectious Ribonucleic Acid of Japanese B Encephalitis Virus For Experiments with Chick Embryo Cell Monolayers.* (28565)

MASAHIRO NAKAMURA AND YOSHIE UENO (Introduced by T. Francis, Jr.)
Department of Microbiology, Kurume University School of Medicine, Kurume, Japan

Optimal conditions for extraction of infectious ribonucleic acid (RNA) of Japanese B encephalitis (JBE) virus and for plaque formation in chick embryo cell monolayers have been described(1), in which maximum number of plaques was formed when 1.0 M NaCl solution was used for a diluent of infectious JBE RNA. This report concerns a more efficient diluent than 1.0 M NaCl which was found in recent experiments.

Materials and methods. Brains of mice infected with the Nakayama strain (M-67 and -68) of JBE virus were homogenized with sterile distilled water. This suspension was used as a starting material. Infectious RNA was freshly extracted from this suspension just before each experiment by means of phenol extraction according to the methods of Gierer and Schramm(2) and Colter *et al.* (3) as modified by Nakamura and Ueno(1). Details of the procedure and extraction efficiency for RNA in relation to the starting virus have been given(1). That the infectivity of extracted RNA was not due to whole virus was demonstrated by the use of purified RNAase(1); this extracted RNA was very sensitive to RNAase treatment and showed a u.v. adsorption pattern characteristic of RNA, whereas the virus was not sensitive to RNAase. Chick embryo cell monolayers were prepared by seeding each bottle with 4 ml of cells obtained by trypsinization of 9-day-old chick embryos. The cells were suspended in 0.5% lactalbumin hydrolysate, 10% inactivated bovine serum, and 0.002% phenol red in Hanks' balanced salt solution (BSS) with antibiotics. After overnight incubation at 37°C, the cultures were used for assay of virus and RNA infectivities according to the plaque method of Porterfield(4). The cell monolayers were washed with phosphate buf-

* Aided by a grant from Nat. Inst. of Allergy and Infectious Dis.