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DENSITY DETERMINATION IN A PREFORMED GRADIENT OF CAESIUM CHLORIDE

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SUMMARY

A simple procedure for forming density gradients is described. By introducing the sample at a level close to the equilibrium position in a preformed gradient of CsCl, the time of centrifugation may be considerably shortened. The method applied to haemocyanin, MEF_1 poliovirus and Rift Valley fever virus gave densities of 1.32 and 1.23 g/ml, respectively.

INTRODUCTION

CsCl advocated as a density-gradient-forming substance by MESELSON *et al.*[1] has many advantages over sucrose[2]. CsCl has a higher diffusion constant; its solutions are far less viscous and most important of all, much steeper density gradients can be formed with CsCl than with sucrose.

In previous determinations of densities by the CsCl-gradient method, the material was either layered on top of a high concentration of CsCl (ref. 3) or mixed with conc. CsCl and centrifuged until equilibrium was attained[4,5]. In the latter method the substance to be examined finds its isodensity region during the period required for establishing sedimentation equilibrium by the CsCl. CRAWFORD[6] using both methods employed RbCl. Density-gradient centrifugation has been reviewed by BRAKKE[7].

The method described by MESELSON *et al.*[1] requires prolonged centrifugation which has obvious disadvantages. A modified method involving the use of a preformed gradient of CsCl and the introduction of the sample at a suitable level has now been investigated.

MATERIALS AND METHODS

The apparatus used to form the density gradient is shown in Fig. 1. It consists of a 5-ml syringe vertically adjustable by a rack and pinion. Both inward and outward movements of the plunger are controlled by a screw and nut and the tip of the fine needle is bent upwards.

The original solution of CsCl (pH 7.0) was prepared by dissolving 6 g of the salt (AnalaR, B.D.H.) in 10 ml of a phosphate buffer consisting of 0.02 M Na_2HPO_4 and 0.002 M KH_2PO_4. The solution was filtered through Whatman No. 1 paper and its

density was determined with the aid of a 10-ml volumetric flask at 20°. To form the gradient, solutions were prepared by mixing the original CsCl solution with the buffer in the ratios of 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1, undiluted original solution formed the 10th step in the gradient. To stabilize virus infectivity, 0.5% bovine albumin powder (Fraction V from bovine plasma) was added to each CsCl–buffer

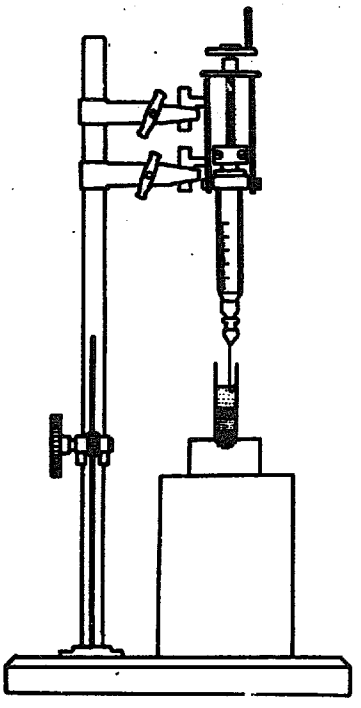

Fig. 1. Density-gradient-forming device. See text for details.

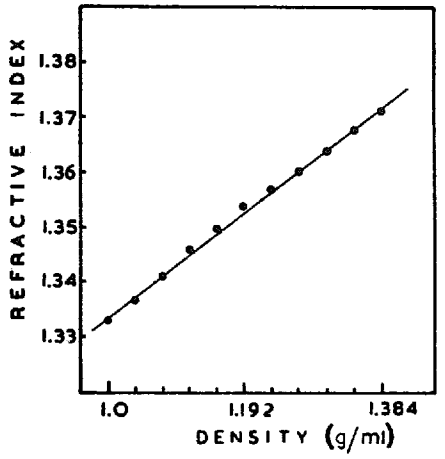

Fig. 2. Standard refractometric density curve of CsCl solutions.

mixture. The refractive index of the phosphate buffer, the original solution and of each CsCl–buffer mixture was measured in an Abbe refractometer at 20°. The values obtained were plotted against the densities of the mixtures and the resulting linear graph (Fig. 2) was used as a reference.

The density gradient was formed by drawing 0.4 ml of each mixture slowly into the syringe starting with the least dense. The contents of the syringe were then carefully transferred to a 12 × 50 mm centrifuge tube keeping the bent tip of the needle against the side of the tube. The syringe was raised with the rack and pinion during the introduction of the gradient so as to keep the needle tip just below the meniscus. Discontinuities in density and light refraction between the different layers were rapidly eliminated by diffusion. The sample was introduced at any desired level by substituting for one of the CsCl–buffer mixtures used to fill the syringe, a mixture of sample and CsCl having the same density as the mixture which it replaced. To hasten attainment of equilibrium the sample was introduced at a level close to its expected final position in the tube but results were confirmed by further experiments in which equilibrium was reached by movement of the sample both upwards and downwards in the gradient.

The tubes were centrifuged at 33000 rev./min for 5 h in the SW-39 rotor of the Spinco Model-LH ultracentrifuge at temperatures near 0°.

After centrifugation, the bottom of each tube was pierced with a fine metal pin specially made for the purpose and single drops were collected in a series of small test tubes which were immediately sealed with cellophane tape to prevent evaporation. Between 60 and 80 drops were obtained from each centrifuge tube. If the number was near 60, 3 drops were regarded as a fraction, if near 80, 4 drops. The drops constituting alternate fractions were pooled for virus titration. Individual drops collected between those that were pooled were used for determination of refractive index and thence, by interpolation in the reference graph, of the density gradient.

The fractions consisting of drops pooled for virus titration were diluted with 1.8 ml of saline containing 5% (v/v) rabbit serum, 200 units of penicillin and 0.2 mg of streptomycin per ml. This was regarded as a 1:10 dilution and further 10-fold dilutions were made in the same solution.

The following determination was made to test the technique:

(1) *The density of MEF_1 virus.* A 10% suspension of 4–5-day-old suckling mouse brains infected with the MEF_1 strain of Type-II poliovirus[8], was clarified by centrifugation at 10000 rev./min for 10 min and then centrifuged at 30000 rev./min for 90 min. The pellet containing the virus was resuspended in saline. The suspension was shaken up once with an equal volume of chloroform and the mixture centrifuged. The aqueous layer was recentrifuged at 30000 rev./min for 90 min. The pellet was redissolved in 0.3 ml serum–saline and the solution was mixed with an equal volume of the original CsCl solution. 0.4 ml of the mixture was introduced at the centre of a density gradient and centrifuged 5 h at 33000 rev./min.

After centrifugation, ten fractions of pooled drops from different regions in the tube were titrated in groups of six 3–4-week-old mice, each animal receiving 0.03 ml intracerebrally. Titres are expressed as the negative $\log_{10} LD_{50}$ per 0.03 ml (ref. 9). Infectivity units are the antilogarithms of the negative $\log_{10} LD_{50}$ value.

(2) *The density of Rift Valley fever virus.* Serum from mice infected with a pantropic strain of Rift Valley fever virus was mixed with an equal volume of the original CsCl solution and centrifuged in a density gradient. After centrifugation, serial 10-fold dilutions of alternate fractions were injected into groups of six 3–4-week-old mice, each mouse receiving 0.2 ml intraperitoneally. The titres are expressed as the negative $\log_{10} LD_{50}$ per 0.2 ml.

(3) *The density of haemocyanin.*

(a) Haemocyanin from *Burnupena cincta* (0.8%) which has a sedimentation coefficient of 90 S (ref. 10), was mixed with an equal volume of the original CsCl solution and centrifuged in a CsCl density gradient. After centrifugation, saline (0.5 ml) was added to every second drop. The relative haemocyanin concentrations in the drops were determined by the precipitin reaction in agar gel[11] with rabbit antiserum. The distance of the precipitin band from the antigen meniscus (Fig. 3) was measured with a microcomparator and the antilogarithm values of these measurements were plotted against the drop number in the region of the tube in which the haemocyanin was detectable[12].

(b) The density of *Jasus lalandii* haemocyanin ($s = 16$ S)[13] was determined in order to find out whether substances of low sedimentation coefficient reach their isodensity level during the 5-h period of centrifugation.

The haemocyanin was mixed with an original CsCl solution of density 1.167 g/ml to form CsCl–haemocyanin mixtures with densities of 1.14, 1.34 and 1.55 g/ml. The

haemocyanin–CsCl mixtures containing about 1% haemocyanin were introduced into separate density-gradient tubes in place of the CsCl–buffer mixtures with the same densities, *i.e.* the 2nd, 5th and 8th CsCl–buffer mixtures, respectively. The three tubes

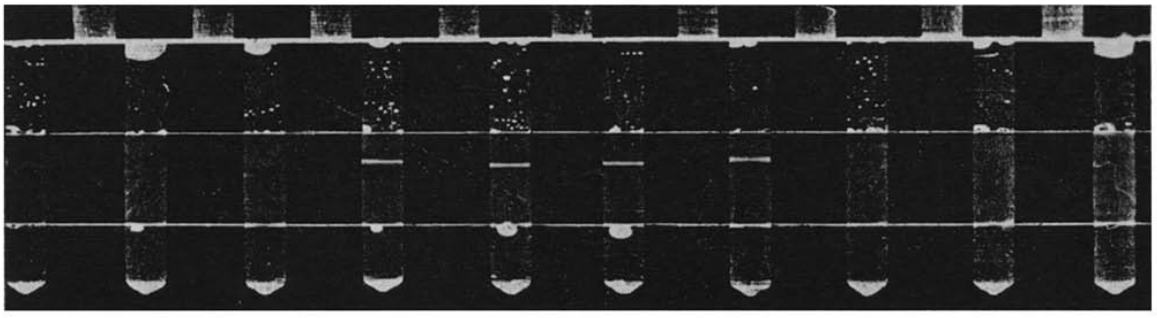

Fig. 3. Estimation of relative haemocyanin concentrations by precipitation in agar gel. Drop No. 16 (5th tube from the left) contained the highest concentration of haemocyanin.

were centrifuged for 5 h at 33000 rev./min. After centrifugation the contents of the tubes were divided into fractions which were examined by the methods described in Section 3a.

RESULTS

In presenting the results, the negative $\log_{10} LD_{50}$ values of the virus have been plotted as histograms and the antilogarithm values as smooth curves. The isodensity level of the virus was interpolated from the latter.

Proteins have a higher specific refractive increment than CsCl and therefore cause slight peaks in the curves relating density to position in the centrifuge tube. These peaks were disregarded in estimating the density of the region occupied by the substance under investigation.

(1) The results of the MEF_1 poliovirus centrifugation are given in Fig. 4. The zone of highest virus activity moved down from the centre of the tube (density region 1.2 g/ml), where the sample was introduced, to a density level of 1.32 g/ml.

In a second experiment a density gradient was formed from an original CsCl solution of density 1.732 g/ml. The virus suspension prepared as described above was mixed with an equal volume of CsCl with a density of 1.814 g/ml. The refractive index of the mixture was taken and its density interpolated from the reference curve. Since the mixture of MEF_1 virus and CsCl now had a density of 1.436 g/ml, it was introduced into the CsCl gradient in place of the 6th CsCl–buffer mixture. After centrifugation in the usual manner and titration of the samples, it was found that the zone having highest virus infectivity had in this case moved upwards in the tube to a density region of 1.32 g/ml.

(2) With Rift Valley fever virus infected mouse serum (Fig. 5), the region of peak virus infectivity occurred at a density of 1.23 g/ml.

In another experiment of which details will be presented elsewhere[14] purified Rift Valley fever virus was used instead of infected serum. A density of 1.23 g/ml was obtained confirming that the result of the experiment with serum was unaffected by the relatively high protein concentration.

The curve relating the infectivity of Rift Valley fever virus to position in the

density gradient shows considerable tailing which might have been due to some defect in the method or to variation in density among the virus particles. Mouse serum infected with Rift Valley fever virus was therefore introduced into the centre of a CsCl

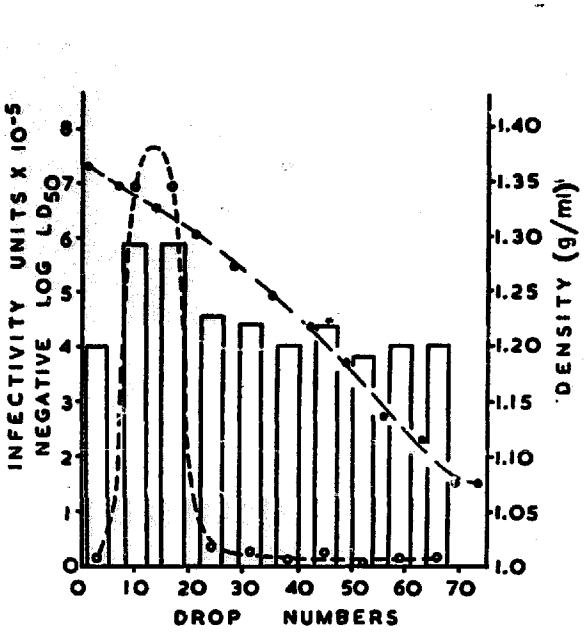

Fig. 4. Distribution of MEF_1 virus infectivity in a density gradient after centrifugation. The histogram represents the negative $\log_{10}$ LD_{50} values. The empty circles are the antilogarithm values. The full circles show the density distribution in the tube.

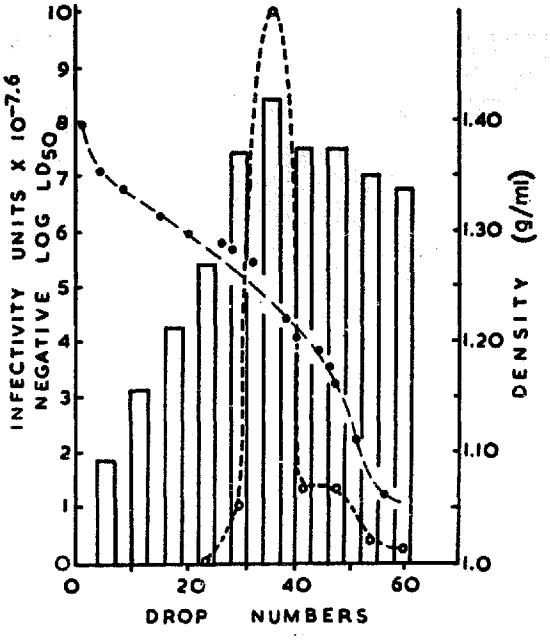

Fig. 5. Distribution of Rift Valley fever virus infectivity in a density gradient after centrifugation. The histogram represents the negative $\log_{10}$ LD_{50} values. The empty circles are the antilogarithm values and the full circles show the density distribution in the tube. The three points that fall off the smooth curve are due to the higher refractive index of the zone which contains the serum protein.

TABLE I

DENSITIES OF REGIONS CONTAINING *J. lalandii* HAEMOCYANIN BEFORE AND AFTER CENTRIFUGATION IN A CsCl GRADIENT

Tube No.	*Density of zone in which haemocyanin was introduced (g/ml)*	*Density of zone in which haemocyanin was found after centrifugation (g/ml)*
1	1.14	1.28
2	1.34	1.32
3	1.55	1.36
	Average	1.32

gradient in a centrifuge tube as described above, but instead of being centrifuged for 5 h, the tube was left at 4° for the same period. Sampling was done as before and eight samples of pooled drops from different density regions in the tube were titrated in mice. The infectivity curve so obtained (Fig. 6) shows that the tailing off of the histograms in Figs. 4–6 is probably caused by contamination during sampling.

(3a) *B. cincta* haemocyanin (Fig. 7) moved down in the gradient to a density level of 1.32 g/ml. The narrowness of the haemocyanin bands visible in the tube after

centrifugation (about 1 mm) compared to the width of the band before centrifugation (4 mm) suggested that the isodensity level was closely approached.

(3b) The density-gradient tubes containing *J. lalandii* haemocyanin were photo-

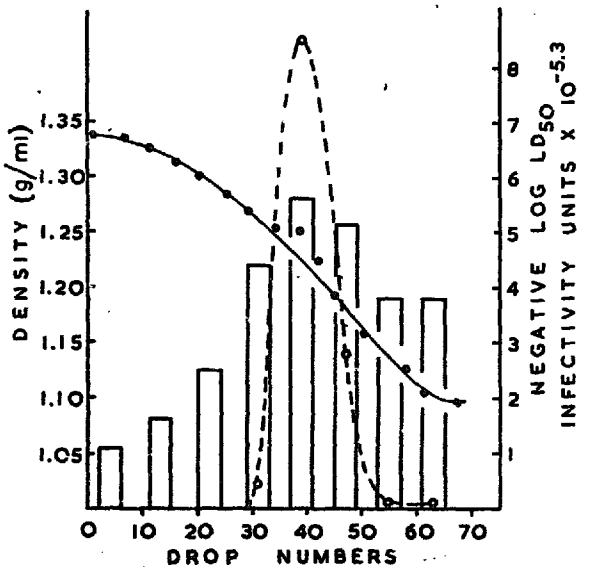

Fig. 6. Distribution of Rift Valley fever infectivity expressed as negative $\log_{10}$ LD_{50} values in a histogram. The empty circles are the antilogarithm values and the full circles show the density distribution in the tube. This tube had not been centrifuged but was kept at 4° for the same period as those which were centrifuged. The peak in the gradient curve corresponds to the peak of the virus infectivity.

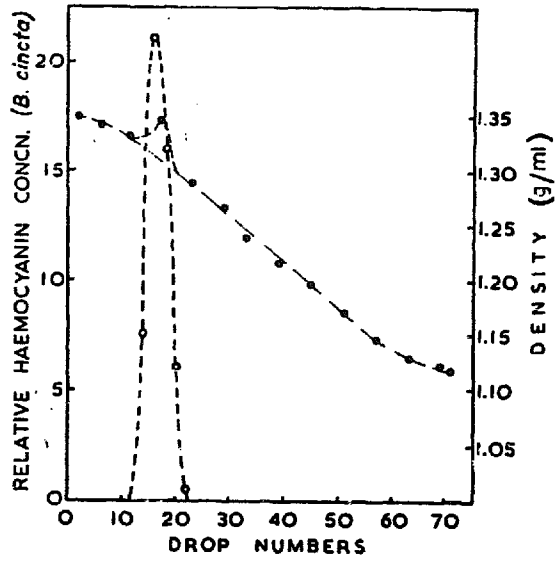

Fig. 7. Distribution of *B. cincta* haemocyanin (empty circles) after density-gradient centrifugation. The full circles represent the distribution of density in the tube. Note the peak in the refractive index gradient curve in the region of maximum protein concentration.

graphed before and after centrifugation. It may be seen in Fig. 8 that the bands condensed during centrifugation although those above and below the isodensity region (Tubes 1 and 3) did not reach equilibrium. The densities of the regions containing the haemocyanin before and after centrifugation are shown in Table I.

DISCUSSION

By means of centrifugation in a preformed gradient of CsCl, the density of *B. cincta* haemocyanin of sedimentation coefficient 90 S was found to be 1.32 g/ml in fair agreement with value of 1.35 found by SVEDBERG AND PEDERSEN[15] for *Helix pomatia* haemocyanin.

The new technique applied to MEF_1 poliovirus gave a density of 1.32 g/ml differing considerably from the value 1.56 obtained by SCHAFFER AND SCHWERDT[16]. These authors determined the viscosity and sedimentation rate in different concentrations of D_2O and obtained the density by extrapolation of the straight line relating the product of viscosity and sedimentation rate to solvent density. Hydrogen–deuterium exchange was assumed to be negligible but if any occurred it would account partly for the high density value.

Inserting a density value of 1.32 and a sedimentation coefficient of 156 S, which is the average value found for polio[10,17] in Stokes' equation, gives a particle size of

29.7 mμ whereas a density value of 1.56 would yield a particle size of 22.4 mμ. The former value is in closer agreement with the average diameter of MEF_1 virus (27 mμ) found by electron microscopy[17].

B. cincta haemocyanin which has a lower sedimentation coefficient than poliovirus, closely approached its isodensity level when centrifuged under the same conditions as MEF_1. It is to be expected that particles with higher sedimentation coefficients will approach their isodensity levels more rapidly and it is therefore probable that poliovirus reached this position under the conditions employed.

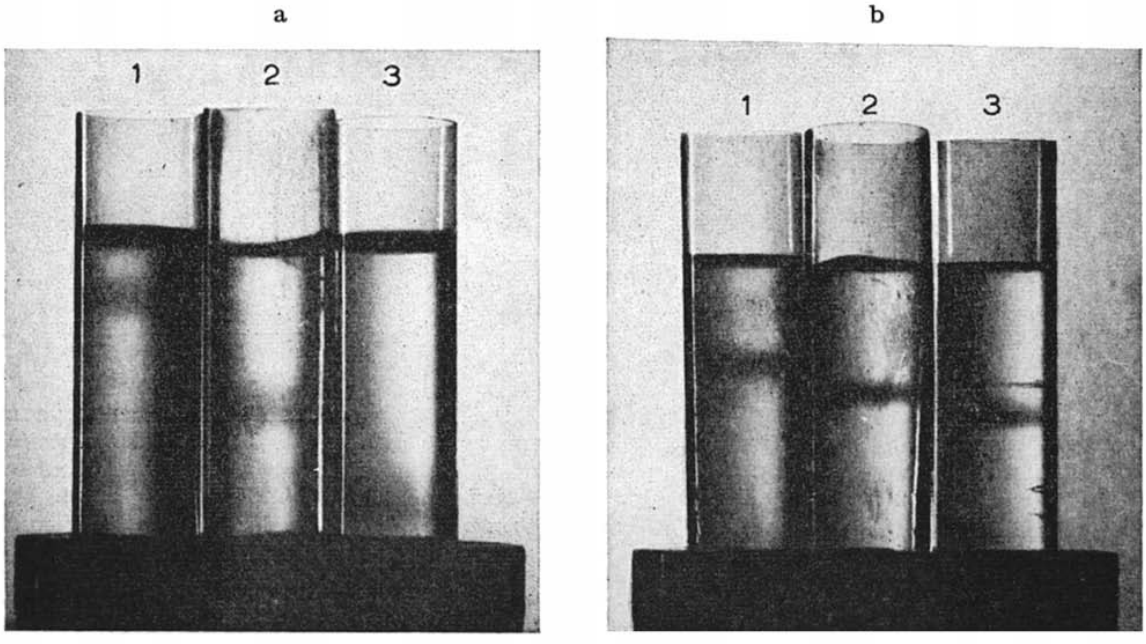

Fig. 8. Density-gradient tubes showing the position of *J. lalandii* haemocyanin before (a) and after (b) centrifugation at 33000 rev./min for 5 h. The haemocyanin band closest to equilibrium in Tube 2 is more condensed than the bands in Tubes 1 and 3 which have not yet reached the equilibrium zone.

A density value of 1.34 was estimated by MATTERN[18] for polio and Coxsackie viruses using the CsCl-gradient method of MESELSON *et al.*[1] and this value is in close agreement with that found for MEF_1 by centrifugation in a preformed gradient of CsCl. By the same method, Rift Valley fever virus was found to have a density of 1.23 g/ml.

J. lalandii haemocyanin which has a relatively low sedimentation coefficient (16 S) yielded the expected density of 1.32 g/ml when introduced very close to its isodensity level. The period of centrifugation was not sufficient for the haemocyanin to reach its isodensity region when introduced at levels remote from the equilibrium position. It is, however, of interest that the average of the densities of the regions in which the haemocyanin was found after centrifugation is equal to the true density of the protein in conc. CsCl.

Thus by means of a relatively simple device, it is possible to determine densities in a preformed gradient of CsCl by introducing the sample to be tested at a level close to its isodensity region. The correct densities of substances of low sedimentation coefficient may be estimated by introducing them above and below the expected isodensity region and finding the average of the resulting density values. This tech-

nique requires a relatively short time of centrifugation and would be specially useful for the examination of unstable viruses.

The drop method of sampling is not entirely satisfactory as it tends to broaden the zone where maximum infectivity is found. This is particularly evident in the region of the tube above the isodensity level of the virus. Sampling from the top is being investigated as an alternative method.

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REFERENCES

1 M. MESELSON, F. W. STAHL AND J. VINOGRAD, *Proc. Natl. Acad. Sci. U.S.*, 43 (1957) 581.
2 M. K. BRAKKE, *Arch. Biochem. Biophys.*, 45 (1953) 275.
3 R. E. F. MATTHEWS, *Nature*, 184 (1959) 530.
4 L. LEVINTOW AND J. E. DARNELL, *J. Biol. Chem.*, 235 (1960) 70.
5 B. ROIZMAN AND P. R. ROANE, Jr., *Virology*, 15 (1961) 75.
6 L. V. CRAWFORD, *Virology*, 12 (1960) 143.
7 M. K. BRAKKE, *Advan. Virus Res.*, 7 (1960) 193.
8 G. SELZER, M. SACKS AND M. VAN DEN ENDE, *S. African Med. J.*, 26 (1952) 201.
9 L. J. REED AND H. MUENCH, *Am. J. Hyg.*, 27 (1938) 493.
10 A. POLSON, A. EHRENBERG AND R. CRAMER, *Biochim. Biophys. Acta*, 29 (1958) 612.
11 A. POLSON, *Sci. Tools*, 5 (1958) 17.
12 A. POLSON AND M. H. V. VAN REGENMORTEL, *Virology*, 15 (1961) 397.
13 F. J. JOUBERT, *Biochim. Biophys. Acta*, 14 (1954) 127.
14 A. POLSON AND J. LEVITT, in preparation.
15 T. SVEDBERG AND K. O. PEDERSEN, *The Ultracentrifuge*, Clarendon Press, Oxford, 1940, p. 406.
16 F. L. SCHAFFER AND C. E. SCHWERDT, *Advan. Virus Res.*, 6 (1959) 159.
17 C. E. SCHWERDT AND F. L. SCHAFFER, *Ann. N.Y. Acad. Sci.*, 61 (1955) 740.
18 C. F. MATTERN, *Virology*, 17 (1962) 520.