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Ultra-microdetermination of Arginine by a Compound Microbiological Assay Method.* (20824)

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Most microbiological assay methods are based on the principle that an assay organism grows and produces acid proportionately to the concentration of an essential nutrient added at different levels to a basal medium lacking this substance but otherwise nutritionally complete. In amino acid assays the ratios between the moles of acid produced and nutrient added range from about 600:1 (glutamic acid) to 11,000:1 (tryptophan). Magnification in response evidenced by such ratios is a general characteristic of titrimetric microbiological assays.

From unpublished studies in this laboratory it became apparent that the magnification in response inherent in a given microbiological assay could be compounded by means of a 2-stage process in which the growth of a primary organism is regulated by the level of an essential nutrient added to the basal medium and that of a secondary organism by the level of an essential nutrient introduced into the basal medium as a metabolic product of the primary organism.

The determination of an amino acid by a compound microbiological assay procedure is described for the first time in this report. *Lactobacillus casei* 280-16 was selected as the secondary assay organism because of its response to D-lactic acid, a metabolic product of many assay organisms(1). The anticipated gain in sensitivity with this secondary assay was about 2000-fold since the quantity of L-lactic acid produced (available for titration) by *L. casei* 280-16 has been shown to be about 2000 times that of the D-lactic acid available for the growth of this organism(1). *Lactobacillus casei* ϵ (American Type Culture Collection No. 7469) was selected as the primary assay organism because it produces only relatively small amounts of D-lactic acid (1). The resulting relatively low effective magnifications (ratios between D-lactic acid produced and nutrient added) in primary assays with this organism seemed desirable in preliminary studies to avoid difficulties which might be anticipated in compound assays with the highest over-all magnifications. Arginine was selected as the test amino acid since it may be determined satisfactorily in biological materials by a standard (one-stage) assay with *L. casei* ϵ(2).

The compound microbiological assay method described in the present report for the determination of arginine with *L. casei* ϵ and *L. casei* 280-16 in combination has proved to be approximately 140 times as sensitive as the standard procedure while retaining the advantage of conventional macroscale manipulations.† Since other organisms commonly employed in standard assays produce proportionately much more D-lactic acid than

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L. casei ϵ, it is anticipated that compound assay methods may be made available in which the over-all increases in sensitivity are several thousand fold. Studies involving the determination of amino acids in biological materials readily obtainable only in small quantities would be greatly facilitated if microbiological assay procedures of high sensitivity were available. For example, 2000 specimens of *Leptinotarsa decemlineata* were required to furnish blood sufficient for the microbiological determination of 13 amino acids in the deproteinized plasma of this insect(3). One specimen would have sufficed if a compound assay procedure of 2000-fold over-all sensitivity had been available.

Preparation of standard. Aliquots of a stock solution containing 1.000 mg of L-arginine per ml‡ in 0.5 *N* hydrochloric acid were neutralized approximately to pH 7.0 immediately before use and diluted to give 2 working standard solutions containing, respectively, 10^{-4} and 5 x 10^{-4} mg of L-arginine per ml. From one to five 0.2 ml aliquots of the 2 solutions were pipetted§ into 13 x 100 mm soft glass test tubes in combinations yielding from 0 to 3 x 10^{-4} mg of L-arginine per tube in steps increasing by 2 x 10^{-5} mg per tube. Triplicate tubes were employed for each of the resulting 15 standard dosage levels. *Preparation of samples.* Protein samples were hydrolysed by refluxing them for 20 hours in 10 volumes of 8 *N* hydrochloric acid. An appropriate aliquot of each hydrolysate, neutralized approximately to pH 7.0 immediately before use, was diluted to contain approximately 2.5 x 10^{-4} mg of L-arginine per ml. One to five 0.2 ml aliquots of each sample solution were pipetted into test tubes by the same procedure as that employed with the standard solutions. Triplicate tubes were used for each of the resulting 5 sample dosage levels. A sufficient number of 0.2 ml portions of water were added to the sample and standard tubes to bring the volume in each to 1.0 ml, and the assay medium was added as described below.

Primary assay medium. Sufficient medium for 100 assay tubes was prepared by dissolving 37.5 mg of amino acid mixture,‖ 61.8 mg of vitamin-mineral mixture,¶ 30 mg of calcium acetate monohydrate, and 600 mg of glucose in 60 ml of hot water** followed by the addition of 15 ml of buffer solution,†† 17.5 ml of 3 *N* sodium chloride solution and sufficient water to make 100 ml. The medium was filtered and 1.0 ml was pipetted into each assay tube. The complete rack of assay tubes constituting an entire experiment was closely covered with a sheet of clean aluminum foil and autoclaved for 15 minutes at 15 lb. The tubes were allowed to cool without agi-

† The effective magnification in the primary assay with *L. casei* ϵ was expected to be only about 3% of that found by direct titration since only about 3% of the titratable acid produced by this organism is D-lactic acid(1). On this basis the over-all gain in sensitivity would be 60 fold (3% of 2000, the anticipated gain in sensitivity in the secondary assay). The actual gain was more than twice that predicted due to improvements in experimental conditions.

‡ The monohydrochloride (C. P. product of Amino Acid Manufactures). The stated concentrations are calculated for the free amino acid.

§ All repetitive pipettings were made with the aid of a Brewer automatic pipet (Baltimore Biological Co.).

‖ A dry stock mixture was prepared by grinding the following constituents together in a mortar: 9.28% each of DL-alanine, L-cysteine.HCl, L-glutamic acid, DL-leucine, DL-lysine.HCl, and DL-serine; 4.64% each of L-aspartic acid, glycine, DL-isoleucine, DL-phenylalanine, DL-threonine, DL-tyrosine, and DL-valine; and 2.37% each of L-histidine.HCl.H_2O, DL-methionine, DL-norleucine, L-proline, and DL-tryptophan.

¶ A dry stock mixture was prepared by grinding the following constituents together in a mortar: $MgSO_4$ (anhydrous) 47.5%, inositol 12.17%, 5.84% each of adenine sulfate, guanine hydrochloride, uracil and xanthine, choline chloride 4.87%, $FeSO_4$. $6H_2O$ 4.55%, $MnSO_4.H_2O$ 3.70%, 0.97% each of riboflavin and niacinamide, 0.49% each of thiamine. HCl and calcium d-pantothenate, pyridoxine.HCl 0.78%, 0.049% each of pyridoxamine.2HCl, pyridoxal.HCl and *p*-aminobenzoic acid, and 0.0024% each of biotin and folic acid.

** A small amount of relatively insoluble material was left undissolved in preference to heating the mixture excessively.

†† The aqueous solution contained ammonium acetate 11.53%, sodium acetate 11.72%, and 1.0% each of potassium monohydrogen and dihydrogen phosphates.

tation and were immediately inoculated as described below.

Primary inoculation. The stock culture of *L. casei* ε (A.T.C.C. 7469) was maintained in Yeast-Dextrose Agar (Difco product) stabs. Serial transfers were made at 3-week intervals and the resulting cultures were stored in a refrigerator after about 40 hours of incubation at 35°C. A liquid inoculum medium was prepared by dissolving 10 g of Bacto-Peptone (Difco product), 5 g of Bacto-Yeast Extract (Difco product), 206 mg of vitamin-mineral mixture,¶ and 20 g of glucose in 500 ml of hot water** followed by the addition of 50 ml of buffer solution†† and sufficient water to make one liter. This medium was covered with a layer of toluene and stored in a refrigerator. A 15-ml aliquot was placed in a 50-ml centrifuge tube. The tube was plugged with gauze-covered cotton, autoclaved 15 minutes at 15 lb, cooled without agitation, inoculated immediately from the stab culture and incubated for 18 hours at 35°C. The resulting growth was washed by centrifuging it (the cotton plug was secured at the top of the centrifuge tube with a rubber band), resuspending it aseptically in 15 ml of sterile 0.85% saline solution, and repeating the process 3 times. One-tenth ml of the final suspension was added aseptically to 100 ml of sterile 5.4% glucose solution, and 1.0 ml of the resulting mixture was pipetted aseptically into each (previously sterilized) assay tube. The aluminum foil cover, which had been removed (and the underside kept sterile) during the few minutes necessary to add the inoculum mixture to the assay tubes, was replaced carefully to avoid bacterial contamination and the rack of assay tubes was incubated in a precision water bath at 35°C for from 65 to 70 hours. At the end of this incubation period the resulting cultures (the amounts of growth obtained were insufficient to be evident by visual observation) were sterilized by steaming for 30 minutes at 100°C in the autoclave, cooled and immediately supplemented with the inoculated secondary medium as described below.

Secondary assay medium. Sufficient medium for 100 assay tubes was prepared in 2 parts as follows: Part I was prepared by dissolving 20.6 mg of vitamin-mineral mixture¶ and 2.4 g of N-Z-Case (an enzymatic digest of casein supplied by Sheffield Farms Co.) in about 40 ml of hot water** followed by the addition of 5 ml of buffer solution†† and sufficient water to make 50 ml. The resulting solution was filtered and autoclaved in a cotton stoppered flask for 15 minutes at 15 lb. Part II was prepared by dissolving 2.0 g of glucose in about 40 ml of hot water followed by the addition of 5.50 ml of *N* hydrochloric acid and sufficient water to make 50 ml. The resulting solution was autoclaved in a cotton stoppered flask for 15 minutes at 15 lb. The 2 parts were combined aseptically after cooling and were inoculated as described below.

Secondary inoculation. The culture of *L. casei* 280-16 described previously(1) was maintained in the manner described above for *L. casei* ε, and the inoculum suspension was prepared following the procedure described above for the primary inoculation except that only one washing with sterile saline was employed. Six-tenths of one ml of the final suspension of *L. casei* 280-16 was added aseptically to the 100 ml of sterile secondary medium, 1.0 ml of the resulting mixture was pipetted aseptically into each assay tube employing the same procedure as for the primary inoculation, and the rack of tubes was replaced in a precision 35°C water bath for an additional 40 hours of incubation.

Titration and calculation. At the end of the final incubation period growth was terminated by steaming the rack of tubes in the autoclave for 20 minutes at 100°C. The contents of each triplicate set of tubes were combined and rinsed into a 50-ml Erlenmeyer flask with an equal volume of water. Three drops of an 8 mg per ml solution of bromthymol blue in 50% ethanol were added to each flask and the mixtures were titrated to pH 7.0 (blue-green end point) with standardized approximately 0.1 N NaOH.‡‡ The standard titration values were plotted graph-

‡‡ This procedure of titrating the combined triplicate cultures was found to give nearly the same results with nearly the same probable errors as that of titrating the separate cultures and averaging the separately determined values.

TABLE I. Percentages of L-Arginine Found in Casein, Gelatin, and Silk Fibroin by the Standard and Compound Microbiological Assay Methods.* Eight trials.†

Casein		Gelatin		Silk fibroin	
Type of microbiological assay					
Standard	Compound	Stand.	Comp.	Stand.	Comp.
%					
3.72	3.26	8.84	8.19	1.04	.99
3.70	3.43	8.68	9.39	1.01	1.04
3.68	3.50	8.70	9.28	1.03	.97
3.68	3.67	9.02	9.93	1.04	1.15
3.62	3.65	8.78	8.92	1.00	1.08
3.65	3.58	8.77	8.75	1.01	.89
3.62	3.25	8.70	9.33	.99	1.10
3.68	3.58	8.76	8.57	1.02	.91
Averages					
3.67	3.49	8.78	9.04	1.02	1.02
Probable error of each trial calculated as % of avg					
.66	3.2	.84	4.1	1.2	6.1
Probable error of avg value calculated as % of avg					
.23	1.1	.30	1.4	.43	2.2
Ratio between probable errors					
1 : 4.8		1 : 4.9		1 : 5.1	

* All arginine values calculated on basis of moisture and ash-free protein. The casein and silk fibroin were the products described previously (4). The gelatin was a first extraction pigskin product of the Knox Gelatin Company. Values for arginine are in good agreement with those reported in the literature. Included among the latter are casein 3.7%, avg of values from 9 laboratories ranging from 3.6 to 3.9% (5); gelatin 9.0%, avg of values from 6 laboratories ranging from 8.0 to 9.3%; and silk fibroin 0.86%, avg of values from 5 laboratories ranging from 0.80 to 0.90% (6).

† Trials 1 through 4 were made simultaneously, employing the same sample and standard solutions, media and inocula. A separate standard curve included in each trial. Trials 5 through 8 were likewise made simultaneously (but not together with trials 1 through 4).

ically against the standard L-arginine dosages per tube. The amounts of L-arginine per tube corresponding to each of the 5 sample dosages in each case were estimated by interpolating the standard curve. The resulting arginine values were converted to g per 100 g of protein, and the values corresponding to the 5 dosage levels were averaged. If one of the 5 values differed from the value nearest it by an amount greater than the entire range of the remaining 4 values it was excluded in calculating the average.

The reliability of the compound microbiological assays was determined by comparing the results by this method with those obtained for the same samples by the standard microbiological assay method described previously (2). The results are given in Table I and Fig. 1 and 2.

Discussion. The experiments leading to the compound microbiological assay method presented above have been too numerous to describe in the limited available space. They have indicated, however, that apparently minor deviations from the described procedure may lead to entirely unsatisfactory results. Excessively high blanks and erratic results were avoided by autoclaving the bulk of the glucose separately from the other constituents of the basal media, employing not more than the indicated quantities of amino acid mixture or of inoculum in the primary medium, utilizing a secondary incubation period of not more than 40 hours, adjusting the acidity of the secondary medium to pH 5.7 optimum for the response of *L. casei* 280-16 to D-lactic acid, and guarding against contamination of reagents and glassware with traces of arginine. It became evident that more rigid control of conditions, more care in manipulations and greater precautions to prevent accidental contamination were required for successful assays by the compound (2-stage) than the standard (one-stage) method.

The component responses made use of in the compound microbiological assay of arginine are shown in Fig. 1 (Curve B) and 2. Curve B (Fig. 1) depicts the response of *L. casei* ϵ to arginine under standard assay conditions. Under somewhat modified conditions this response constituted the first of the 2 consecutive responses obtained in the compound assay. The dosages of arginine ranging up to 1.7×10^{-7} moles in Curve B may be seen to have effected the production of up to 3.1×10^{-4} moles of lactic acid per tube, and hence it is evident that an average magnification of about 1800 ($3.1 \times 10^{-4}/1.7 \times 10^{-7}$) times was attained over this response range. If it is assumed that the characteristics of this response were retained under the modified conditions employed in the compound assay, the average magnification achieved in the primary assay may be calculated to have been about

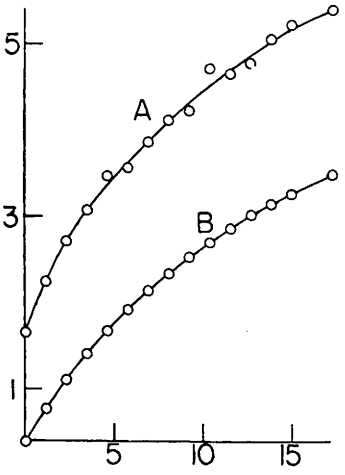

Fig. 1

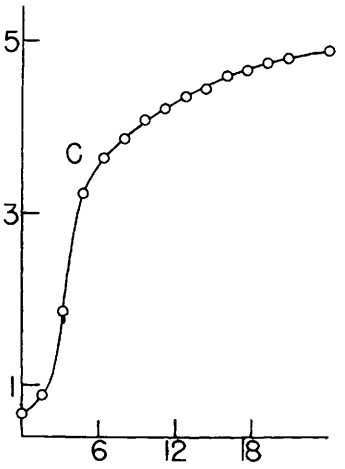

Fig. 2

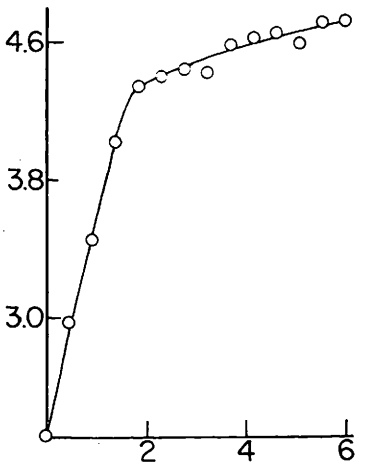

Fig. 3

FIG. 1. Arginine standard curves obtained by the compound (Curve A) and standard (Curve B) microbiological assay methods. The values on the vertical scale are the moles $\times 10^4$ of acid (calculated as lactic acid) produced per tube. The values on the horizontal scale are (Curve A) the moles $\times 10^{10}$ and (Curve B) the moles $\times 10^8$ of L-arginine per tube.

FIG. 2. Response of *L. casei* 280-16 to calcium D-lactate under conditions comparable to those employed in the compound microbiological assay of arginine. The values on the vertical scale are the moles $\times 10^4$ of L-lactic acid produced per tube. The values on the horizontal scale are the moles $\times 10^8$ (calculated as D-lactic acid) of calcium D-lactate per tube.

FIG. 3. L-leucine standard curve obtained by a compound microbiological method employing *L. arabinosus* 17-5 as the primary assay organism. The values on the vertical scale are the moles $\times 10^4$ of L-lactic acid produced per tube. The values on the horizontal scale are the moles $\times 10^{10}$ of L-leucine per tube. The data were taken from a paper presented by one of the present authors (M.N.C.) at the December 11th meeting of the Southern California Section, Soc. for Exp. Biol. and Med., Los Angeles, 1952.

54 (3% of 1800) times, since only D-lactic acid is effective in promoting the secondary response, and since this isomer constitutes only 3% of the lactic acid produced by *L. casei* ϵ(1). The response of *L. casei* 280-16 to D-lactic acid under the conditions employed in the compound assay is depicted in Fig. 2 (Curve C). Dosages of D-lactic acid up to about 7.4×10^{-8} moles may be seen (Curve C) to have effected the production of up to 3.1×10^{-4} moles of L-lactic acid per tube. The average magnification over this range in the secondary assay may therefore be calculated to have been about 4200 ($3.1 \times 10^{-4}/7.4 \times 10^{-8}$) times.

A typical arginine standard curve obtained by the described compound microbiological assay procedure is shown in Fig. 1 (Curve A). Dosages of arginine up to only about 1.2×10^{-9} moles were sufficient, under these conditions, to elicit the final production of up to 3.1×10^{-4} moles of L-lactic acid per tube, and hence the average resultant magnification over this range was about 260,000 ($3.1 \times 10^{-4}/1.2 \times 10^{-9}$) times. This value is in good agreement with the product (230,000) of the magnifications (54 and 4200) calculated for the 2 component assay stages. Owing to the high magnification of the compound assay, very small samples were sufficient for the determination of arginine by this procedure. The total amounts of sample (sufficient for triplicate determinations at each of 5 dosage levels) employed for each compound assay of arginine were as follows: casein 45 μg, silk fibroin 142 μg, and gelatin 18 μg. One hundred times these amounts were needed for the standard microbiological assays of this amino acid. The percentages of arginine found in casein, gelatin and silk fibroin in 8 consecutive trials by the standard and compound microbiological assay methods are listed in Table I. The probable errors calculated for the individual values were approximately 2 times greater for silk fibroin than for casein by either method, whereas those for gelatin

were between these 2 extremes. In spite of these relatively large differences a nearly constant ratio (about 5:1) was observed between the probable errors calculated for the values obtained by the compound method and those calculated for the values found by the standard method. The somewhat lesser precision of the compound method was also indicated by the poorer alignment of points on the standard curve (Curve A, Fig. 1) and by the poorer agreement between the values obtained by this method at different sample dosages (the mean deviations of the values obtained at 4 or 5 different sample dosage levels from their means averaged 7.4% with the compound method as compared with only 1.1% with the standard method for all the values listed in Table I).

The difference (4.9%) between the average values (3.67% and 3.49%, Table I) found for arginine in casein by the 2 assay methods was approximately 3.7 times greater than the sum of their probable errors (0.23% and 1.1%). Since the odds against a chance discrepancy of this magnitude between average values with these probable errors may be calculated to be about 78 to 1, the data appear to indicate a small systematic difference in the relative responses to arginine in casein hydrolysate obtained by the standard and compound microbiological assay methods. On the other hand, the difference (3.0%) between the average values (8.78% and 9.04%, Table I) found for arginine in gelatin by the 2 methods appears to be a chance discrepancy, and the values (1.02% and 1.02%, Table I) found for arginine in silk fibroin by the 2 methods were the same. It is evident that the described compound method would yield erroneously high arginine values for samples containing large amounts of D-lactic acid (the apparent activity of this substance due to its effect in the secondary assay may be calculated to be about 3% of that of arginine), but an error of this sort was not apparent in the above results. D-lactic acid may be removed from acidified sample solutions by extraction with ether, but it seems likely that this step would be required only in special cases.

It should be emphasized that applications of the compound microbiological assay principle do not appear to be limited to the determination of arginine, nor does the sensitivity attainable by the application of this principle appear to be limited to that attained by the method described in the present report. On the contrary, it seems likely that the present procedure could be adapted successfully to the ultra-microdetermination of any one of the amino acids essential for growth and acid production of *L. casei* ϵ merely by adding arginine to the amino acid mixture of the primary medium and omitting, instead, the amino acid to be determined. That primary assay organisms which produce higher percentages of D-lactic acid may lead to assays of greater sensitivity than those employing *L. casei* ϵ for this purpose was indicated by the results of preliminary experiments as well as by the theoretical considerations already mentioned. The results of one of these experiments concerning the response to L-leucine obtained by a compound microbiological method employing *Lactobacillus arabinosus* 17-5 as the primary assay organism is given in Fig. 3. The average magnification achieved over the linear portion of this response curve may be calculated to be 1,250,000 times, a value nearly 5 times that attained by the present arginine method and more than 1000 times that realized with the standard *L. arabinosus* assay of L-leucine.

Summary. It has been shown that the sensitivity of a microbiological assay method may be compounded by employing a secondary microbiological assay to determine the D-lactic acid component of the products obtained from the response of the original assay. Sensitivities of up to several thousand times those realized by the standard microbiological procedures have been shown to be possible theoretically in compound assays. The method described for the compound assay of arginine, designed purposely to be of relatively low sensitivity, was approximately 144 times as sensitive as the standard microbiological method for this amino acid. The average percentages of arginine found in casein (3.49%), gelatin (9.04%), and silk fibroin (1.02%), respectively, by the compound assay were found to be approximately the same as those (3.67%, 8.78%, and 1.02%,

respectively) by the standard method, although the probable errors ranged from 3.2% to 6.1% for the individual assays by the former method compared to 0.7% to 1.2% for those made by the latter method. The sample weights required per assay (each including triplicate determinations at each of 4 or 5 sample levels) were: casein 45 μg, gelatin 18 μg, and silk fibroin 142 μg. One hundred times these amounts were required for each assay by the standard microbiological method.

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Recovery of New Agent from Patients with Acute Respiratory Illness. (20825)

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An epidemic of acute respiratory illness occurred among the service personnel at Fort Leonard Wood, Missouri during the winter of 1952-1953(1). Influenza A′ virus was of etiologic importance in a majority of the cases during the mid-portion of the epidemic but not among the cases at the beginning or end of the outbreak. Approximately 20% of the non-influenzal patients presented clinical and X ray findings which were consistent with the diagnosis of primary atypical pneumonia (PAP) and many had acute respiratory disease (ARD)(2-5). However, in individual instances, it was difficult if not impossible to establish the etiology until the results of laboratory tests became available.

The present report is concerned with the isolation of a microbial agent, presumably viral in nature, which appears to have been of etiologic importance in the disease suffered by certain of the non-influenzal cases in the epidemic.

Materials and methods. Tissue culture. Tube and bottle cultures of HeLa cells† (human epidermoid carcinoma) were prepared by the general method of Scherer, Syverton, and Gey(6) except that tryptic digestion was not employed. The bottle (32 oz prescription size) and tube (16 x 150 mm) cultures were fed with nutrient fluid consisting of pooled human serum, 25 parts; chick embryo extract, 7 parts (2 parts 50% chick embryo extract and 5 parts 50% chick embryo extract ultrafiltrate); Hanks balanced salt solution, 68 parts, plus sufficient penicillin and streptomycin to give a final concentration of 50 units of each per ml. In order to remove the human serum contained in the nutrient fluid, the contents of the bottles and tubes were washed 3 times, immediately before use, with 20 or 1.0 ml, respectively, of 5% chicken serum in Hanks-Simms solution containing antibiotics. Finally, 30 ml (bottles) or 0.6 ml (tubes) of maintenance solution (10 parts pooled chicken serum; 5 parts 50% chick embryo extract ultrafiltrate; 85 parts Hanks balanced salt solution and antibiotics) were added to the

* The technical assistance of Vincent V. Hamparian, Sgt., U. S. A. is gratefully acknowledged.

† Bottle and tube cultures of HeLa cells and the nutrient and maintenance solutions were obtained from Microbiological Associates, Inc., Bethesda, Md.