the experimental animals was found to be most important. As this exceeded one hour, less favorable results were obtained.

The ideal demonstration of viability would have been reimplantation of the material grown in heterologous hosts back into the original human donor. This was not feasible, and so tissue culture was employed to provide confirmatory data. Pieces of 3 original epidermoid carcinomas were cultured in roller tubes and the sheets of epidermoid cells which grew out were compared with those obtained in tissue culture from the subcutaneous nodules of the first, second, and, in one case, third animal transplantations of the same growths; they were quite similar. Some mouse or rat fibroblasts were present but the typical epithelial cells predominated. One of these epidermoid carcinomas was highly anaplastic with many multinucleated cells. This same type of cell grew out in the tissue cultures from the original tumor and from the second transplant nodule. Unfortunately, no effort was made to culture material from the first transplant generation.

Summary. (1) Of 100 human neoplasms implanted subcutaneously in X-irradiated rats and mice as sieved or minced cell suspensions, 33 had no mitotic cells at the end of the first generation (8 days), 34 showed cellular proliferation at the end of the first generation but could not be transferred successfully to a second, and 33 were transplanted for 2 generations or more with evidence of active growth and vascularization. (2) Epidermoid carcinomas were most successfully propagated. (3) Failures were in part due to infection and to delay between surgical removal of the tumor and its implantation in the heterologous hosts.

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A Microbiological Method for the Determination of L- and D-Aspartic Acids.* (18855)

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It was shown previously(1) that D-aspartic acid supported only limited growth of *Leuconostoc mesenteroides* P-60 with relatively short incubation times in asparagine-free media. When low concentrations of L-asparagine were present, however, the growth response to D-aspartic acid was nearly the same as, or better than, that to L-aspartic acid. This suggested that *Leuconostoc mesenteroides* might prove useful for the determination of both aspartic acid enantiomorphs in hydrolysates of biological materials. The method which was subsequently developed for this purpose is described.

Experimental. The stock standard solution contained 4.00 mg of L-aspartic acid,‡ 1.00 mg of D-aspartic acid,‡ and 0.05 ml of 12 *N* hydrochloric acid per ml. It was stored at room temperature and diluted as required just before using. The diluted standard solutions were added in amounts of from 0.2 ml to 1.0 ml per tube to give a range of total aspartic acid concentrations of from 0 to 70 γ per tube in steps of 5 γ per tube.§ Each level was tested in triplicate. The standard was set

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1. Camien, M. N., and Dunn, M. S., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 74.

‡ These products, the same as those described previously(1), were kindly supplied by Dr. Max S. Dunn.

§ A 3 *N* NaCl solution was added, taking into account amount of NaCl formed in neutralizing HCl acid present.

up in the same manner for both L- and total aspartic acid assays, but since only 80% of the aspartic acid in the standard solutions was L-aspartic acid, the L-aspartic acid standard curve ranged from 0 to 56 γ per tube in steps of 4 γ per tube.

Samples. Dried cells of *Lactobacillus arabinosus* 17-5, *Streptococcus faecalis* R, and *Leuconostoc mesenteroides* P-60 were prepared according to the procedure employed previously(2). 1.5 g of each of these preparations and of a sample of casein ("vitamin test casein," General Biochemicals, Inc.) were hydrolysed by refluxing for 20 hours with 15 ml of 8 N hydrochloric acid. Each hydrolysate was filtered into a 100 ml volumetric flask, diluted to volume, and stored at room temperature. Aliquots were diluted further as required on the day of each experiment. Five levels of diluted hydrolysate, each in triplicate and ranging from 0.2 ml to 1.0 ml per tube, were tested for each sample.

Bromthymol blue was added during the preparation of diluted standard and sample solutions to facilitate neutralizing, and each final solution was prepared to contain 17.5 mg of sodium chloride¶ and 0.008 mg of brom thymol blue‖ per ml at about pH 6.2. A "blank" solution containing the same concentrations of sodium chloride and bromthymol blue was employed to bring the volume in each test tube to 1.0 ml before adding the medium.

The basal medium was the same as that employed previously(1), except that sodium chloride, norleucine, and norvaline were omitted and the concentration of each amino acid was increased by 50%. The basal medium was modified for the determination of L-aspartic acid by adding 60 mg of D-aspartic acid per 200 ml of medium (sufficient for 100 assay tubes, each containing 3 ml final volume after adding medium and sample) and adjusting the pH to 5.5. The basal medium was modified for the determination of total (L- plus D-) aspartic acid by adding 4.5 mg of L-asparagine monohydrate per 200 ml of medium and adjusting the pH to 6.5. 2 ml of medium were used per tube. The racks of tubes were covered with clean towelling, heated rapidly to 120° in the autoclave, held at that temperature for 10 minutes and cooled rapidly before inoculating.

The inoculum of *Leuconostoc mesenteroides* P-60 was the same as that employed previously(1), except that the growth from 7 ml of inoculum medium was resuspended in 100 ml of sterile saline, and 0.1 ml of this suspension was used to inoculate each tube. The inoculated tubes were incubated at 35°. The incubation period was from 39 to 42 hours for the L-aspartic acid determinations and from 63 to 66 hours for the determinations of total aspartic acid. The tubes were heated in the autoclave at 100° for 20 minutes to terminate growth. Titrations and calculations were carried out as described previously(3).

Discussion. Rather large changes in the "blank" response of *Leuconostoc mesenteroides* to L-aspartic acid were found to accompany variations in incubation time and temperature when the basal medium contained an excess of D-aspartic acid (Fig. 1). It was particularly desirable with the L-aspartic acid determinations, therefore, to be certain that the incubation temperature was precisely the same for each of the assay tubes and to terminate growth simultaneously in all the tubes at the end of a given experiment. The incubator used in the present experiments was found to produce a highly uniform temperature, although it seems likely that the reliability of a constant temperature water bath might be much superior in this respect. The incubation period for the L-aspartic acid determinations was kept relatively short (39 to 42 hours at 35°) to avoid excessively high "blank" titrations.

The initial pH of the medium was also found to influence the "blank" response to L-aspartic acid (Fig. 2). This effect did not

2. Camien, M. N., Salle, A. J., and Dunn, M. S., *Arch. Biochem.*, 1945, v8, 67.

¶ 1 ml of a 0.8 mg per ml solution of bromthymol blue in 5% ethanol was added per 100 ml of final dilution.

‖ The method of setting up the standard was analogous to that described previously(3).

3. Dunn, M. S., Camien, M. N., Malin, R. B., Murphy, E. A., and Reiner, P. J., *Univ. Calif. Publ. Physiol.*, 1949, v8, 293.

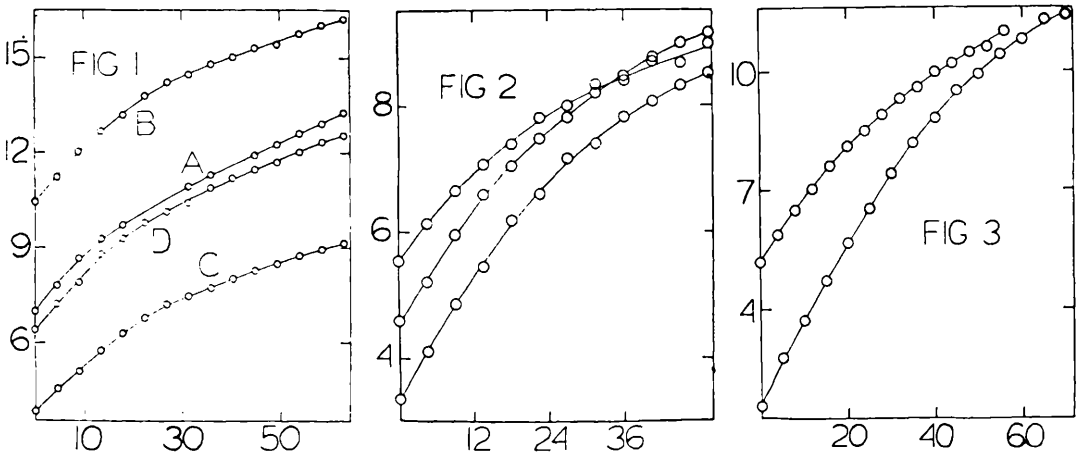

FIG. 1. Response to L-aspartic acid in medium containing 20 mg % D-aspartic acid. Curves A and B were obtained with 38 and 62 hr incubation, respectively, at 39°. Curves C and D were obtained with 38 and 62 hr incubation, respectively, at 33°. The values on the horizontal scale represent the γ of aspartic acid per tube, and values on vertical scale are calculated as ml of 0.01 N NaOH required to titrate 1 ml of culture.

FIG. 2. Response to L-aspartic acid with 38 hr incubation in media containing 20 mg % D-aspartic acid. Initial pH of medium was 5.5, 6.0, and 7.0, respectively, for the lower, middle, and upper curves. Coordinates are as in Fig. 1.

FIG. 3. Typical standard curves for determination of L-aspartic acid (upper curve) and total (L- plus D-) aspartic acid (lower curve). Coordinates are as in Fig. 1.

appear to result from destruction of nutrients in the medium during sterilization at the lower pH values since essentially the same results were obtained whether the pH was reduced before or after sterilization. An initial pH of 5.5 in the L-aspartic acid assay medium appeared to be best, and although the "blank" was relatively high even under these conditions (Fig. 3), the assays by this method were reproducible and accurate to a satisfactory degree (Tables I and II).

When the basal medium contained low concentrations of L-asparagine, mixtures of L- and D-aspartic acids were usually slightly more active than L-aspartic acid alone. For this reason a mixture of L- and D-aspartic acids was used rather than L-aspartic acid alone as standard in the determinations of total aspartic acid. By using a 4 to 1 ratio of L-aspartic acid to D-aspartic acid it was possible to use the same standard solutions for the total aspartic acid standard (range, 0 to 70 γ per tube) as for the L-aspartic acid standard (range, 0 to 56 γ per tube). Typical standard curves for L-aspartic acid and total aspartic acid determinations are shown in Fig. 3.

Recoveries of L-aspartic acid from samples containing no D-aspartic acid were low (because L-aspartic acid is less active than mixtures of L- and D-aspartic acids under these conditions) as determined by the total aspartic acid assay method (Table I). With this exception, however, satisfactory recoveries of L- and D-aspartic acids from mixtures of pure amino acids (Table I), from casein hydrolysate (Table I), and from hydrolysates of bacterial cells (Table II) were consistently obtained.

The aspartic acid values found at the different test levels were found to be reproducible to a satisfactory degree with both the method for L-aspartic acid and that for total aspartic acid. The mean per cent deviation from the mean was calculated for each "final" value (the mean of the individual values found at the 4** or 5 sample levels), and the average mean deviation from the mean (calculated from all the values obtained by these methods) was 2.1% with the method for L-

** Five sample levels were tested in each case, but when one of the 5 values was different from the value nearest it by an amount greater than the range of the other four values, this value was omitted in calculating the mean value.

TABLE I. Recovery of L- and D-aspartic Acids from Mixtures of Pure Amino Acids and from Casein Hydrolysate.

Added		Found					
		L-aspartic acid			Total aspartic acid		
L-aspartic acid	D-aspartic acid	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
		% in amino acid mixture*					
100		99.6	99.6	99.6	90.4	94.6	93.6
50	50	49.5	49.2	49.2	97.6	98.4	99.4
0	100	0	0	0	98.6	98.8	100.2
3.45	0	3.54	3.41	3.69	3.21	3.25	3.21
2.76	.69	2.98			3.48		
2.07	1.38	2.12			3.44		
1.73	1.72	1.71	1.74	1.81	3.45	3.40	3.48
.69	2.76	.71			3.40		
0	3.45	0	0	0	3.38	3.43	3.47
		g per 100 g of casein†					
0	0	7.61	7.54	7.61	7.88	7.95	7.71
8.10	0	15.41	15.73	15.31	15.48	15.89	15.60
0	8.10	7.44	6.99	7.22	15.79	15.87	15.89

* Mixtures containing 3.45% (total) aspartic acid contained also 6.9% each of DL-alanine, L-arginine · HCl, L-cystine, L-glutamic acid, L-histidine · HCl · H_2O, DL-isoleucine, L-leucine, DL-methionine, DL phenylalanine, L-proline, DL-serine, L-tryptophan, L-tyrosine, and DL-valine.

† N was determined in the hydrolysate and the casein concentration calculated as N × 6.25. The hydrolysate contained 13.17 g of soluble N per 100 g (uncorrected) of casein.

TABLE II. Recovery of L- and D-aspartic Acids from Hydrolysates of Bacteria.

	g per 100 g bacterial protein*			
	Added		Found†	
Bacteria	L-aspartic acid	D-aspartic acid	L-aspartic acid	Total aspartic acid
L. arabinosus	0	0	8.71 (8.49–9.17)	9.18 (9.07–9.33)
	9.15	0	18.58	17.92
	0	9.15	8.74	18.14
S. faecalis	0	0	7.04 (6.84–7.26)	9.19 (9.09–9.34)
	9.26	0	16.37	18.25
	0	9.26	7.03	18.13
L. mesenteroides	0	0	7.74 (7.64–7.89)	11.10 (10.83–11.34)
	9.4	0	17.07	20.40
	0	9.4	7.40	20.28

* N was determined in the hydrolysates and the protein calculated as N × 6.25. The hydrolysates of *L. arabinosus*, *S. faecalis* and *L. mesenteroides* contained, respectively, 9.72, 9.47 and 9.61 g of soluble N per 100 g of bacteria (dry wt, uncorrected for any residual moisture which might have been present).

† The values given for bacteria without additions of aspartic acid are averages of four independent determinations, ranges being given in parentheses. Other values represent single determinations.

aspartic acid and 1.3% with that for total aspartic acid.

Only small amounts of D-aspartic acid (the difference between L-aspartic acid and total aspartic acid) were detected in the hydrolysates of casein and *Lactobacillus arabinosus* cells (Tables I and II). Much more D-aspartic acid was found in the hydrolysates of *Streptococcus faecalis* and *Leuconostoc mesenteroides* cells (Table II), however, amounting to 23.4% and 30.3%, respectively, of the total aspartic acid. Although it is not impossible that such large amounts of D-aspartic acid may have been produced by racemization, it seems likely that the values found represent the approximate amounts of D-aspartic acid originally present in the protoplasm of these bacteria. That a relatively large amount of D-aspartic acid was found in the cells of *Leuconostoc mesenteroides* (Table II) lends support to the suggestion made previously(1) that D-aspartic acid may be an essential metabolite for this organism.

The average value, 7.85%, found for total

aspartic acid in casein (Table I) was considerably greater than that reported by Hac and Snell(4). However, the latter was corrected only for the moisture content of the casein sample, and the true value must have been somewhat higher, if impurities other than moisture were present in significant amounts. The values obtained in the present experiments were calculated on the basis of 16% nitrogen in casein, and the nitrogen was determined in the hydrolysate. This procedure would give high values either if the true value for nitrogen in casein were significantly less than 16% or if a significant amount of the casein nitrogen were insoluble in the hydrolysate (insoluble humin was removed by filtration before the nitrogen determination was made). The use of a more painstaking procedure did not seem warranted in the present experiments because the available casein sample was not one which had been carefully prepared and highly purified.

Summary. A microbiological procedure was developed for the determination of L- and D-aspartic acids with *Leuconostoc mesenteroides* P-60. Added L- and D-aspartic acids were recovered adequately from amino acid mixtures and hydrolysates of casein and bacterial cells. Only small amounts of D-aspartic acid were detected in casein and in *Lactobacillus arabinosus* cells, but in *Streptococcus faecalis* and *Leuconostoc mesenteroides* cells about one-fourth to one-third of the total aspartic acid was of the D- configuration.

4. Hac, L. R., and Snell, E. E., *J. Biol. Chem.*, 1945, v159, 291.

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A Modified Procedure for Determining Inulin Space.* (18856)

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Although the inulin space may be a somewhat vague entity, inulin has recently come into importance as a tool in the estimation of extracellular space. The inulin space is determined by dividing the total amount of inulin present in the animal by the plasma concentration after an equilibrium has been established between the plasma and the interstitial compartments. The simplest way to compute this quantity of retained inulin is to subtract the amount excreted up to the time of equilibration from the total amount given. Such a procedure has been criticized by Kruhøffer(1) on the grounds that the subtrahend is a relatively large fraction of the minuend and a small per cent error in either of these factors is magnified considerably in calculating the difference. Gaudino and Levitt(2) claimed that following a 2-hour inulin infusion, all the inulin is excreted within 4-6 hours. Therefore, they tried to obviate the difficulty inherent in the original method by computing inulin space as the ratio of the quantity of inulin excreted within 5 hours following termination of the infusion to the concentration of inulin in the plasma.

In using their method it was found that a much longer time was required to approach complete recovery. Therefore we have attempted to improve the accuracy and expediency of the inulin space determination by introducing a slight modification of the original method.

Methods. Thirteen experiments were performed on 10 female dogs under pentobarbital anesthesia. Pure dry inulin which was fructose and pyrogen free† was accurately weighed

* Supported by a grant from the U. S. Public Health Service.

1. Kruhøffer, P., *Acta physiol. scandinav.*, 1946, v11, 16.

2. Gaudino, M., and Levitt, M. F., *Am. J. Physiol.*, 1949, v157, 387.

† Pfanstiehl inulin which was checked repeatedly against recrystallized standards from different laboratories.