showed that the production of skeletal lathyrism by AAN can be greatly augmented by simultaneous treatment with STH. Since neither the cartilaginous nor the bony proliferations induced by AAN are inflammatory in the usual sense of the word, this observation confirms our view that *corticoids can influence tissue reactivity to various agents, and not only to those which elicit typical inflammatory responses.* It is especially noteworthy that in the first experiment in which AAN intoxication was particularly acute and severe, concurrent treatment with STH not only failed to stimulate growth but actually inhibited it; despite this the hormone was most effective in augmenting the abnormal skeletal proliferation characteristic of lathyrism. In evaluating the possible hormonal participation in non-endocrine diseases (those not primarily due to hyper- or hypo-function of an endocrine gland) we must keep in mind that, under certain conditions, a hormone can influence a pathologic process without manifesting its classical endocrine effects.

In our opinion these observations—as well as our earlier experiments on the effect of cortisol upon AAN intoxication(1)—furnish additional support for the concept that the so-called "adaptive hormones" can participate in the development of the most diverse non-endocrine diseases. Special emphasis is laid upon the fact that this is true even of non-inflammatory tissue reactions to an exogenous irritant.

Summary. Experiments in rats indicate that somatotrophic hormone (STH) can greatly aggravate skeletal manifestations of experimental lathyrism that are produced with aminoacetonitrile (AAN). This is so even under conditions whereby STH fails to stimulate growth in length.

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Determination of L- and D-Glutamic Acids by Combined Microbiological Method and Racemization Procedure.* (22879)

MERRILL N. CAMIEN, ARTHUR YUWILER,† AND MAX S. DUNN
Chemical Laboratory, University of California, Los Angeles.

The microbiological assay of L-glutamic acid with *Streptococcus faecalis* R and of total (L- plus D-) glutamic acids with any one of several other organisms‡ has been reported previously as a provisional means of determining both L- and D-glutamic acids in protein hydrolysates(1). It has been found subsequently that utilization of D-glutamic acid by these assay organisms‡ (unpublished data)

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† Present address, Veterans Administration Center, West Los Angeles, Calif.

‡ *Leuconostoc dextranicum* (8086), *Leuconostoc mesenteroides* (8293), *Leuconostoc citrovorum* (797), and *Leuconostoc citrovorum* (7013). Numbers in parentheses refer to American Type Culture Collection catalog.

as well as by *Lactobacillus arabinosus* 17-5 (2,3) is markedly impaired in the presence of aspartic acid and that generally, therefore, the proposed method would be expected to yield erroneously low values for D-glutamic acid.§

An improved method for the determination of L-glutamic acid with *L. arabinosus* 17-5 is given in the present report. A sample treatment effecting complete racemization of glutamic acid is described as a means of estimating total glutamic acid from the L-glutamic acid content of treated material.

Methods. Assay samples, unless otherwise specified, were hydrolysed by refluxing them with approximately 10 volumes of 6 *N* hydrochloric acid for 24 hours, cooled, filtered and washed into volumetric flasks of not more than 3 times the initial hydrolysate volume, diluted with water to volume, and stored in glass stoppered bottles in a refrigerator. An aliquot of each hydrolysate to be assayed for L-glutamic acid was brought to pH 6.2 with a measured amount of standard 3 *N* sodium hydroxide solution and diluted to contain 80 to 120 γ of L-glutamic acid per ml. The diluted samples, in volumes of 0.2, 0.4, 0.6, 0.8 and 1.0 ml, were added in triplicate to 13 x 100 mm soft glass test tubes together with sufficient "blank" solution to make 1.0 ml per tube. Standard solutions of L-glutamic acid were similarly distributed to yield a 15 level standard curve ranging up to 120 γ per tube in steps of 8 γ. Standard, sample and "blank" solutions were all prepared to contain uniform concentrations of brom thymol blue (8 γ/ml) and of sodium chloride (3%, including that formed upon neutralization as estimated from the amount of sodium hydroxide employed) since this procedure eliminates possible deviations in results due to widely varying salt concentrations in the neutralized hydrolysates after appropriate dilution. The assay medium as prepared for use contained the following constituents in distilled water: vitamin-mineral mixture(4) 31 mg %, amino acid mixture‖ 1.05%, glucose 3%, buffer solution(4) 7.5 ml %, and L-glutamic acid 0.60 mg %.¶ The mixture was filtered (to remove the small amount of undissolved solids which remained after heating it briefly) and dispensed, 2 ml/tube, into the sample, standard and "blank" solutions, bringing the final volume/tube to 3 ml. The pH of the medium, considerably acid owing to its relatively high aspartic acid content, was left unadjusted. The charged tubes, supported in appropriate racks and covered with aluminum foil, were autoclaved 5 minutes at 122°, cooled, inoculated with *L. arabinosus* 17-5, incubated 64 to 68 hours at 35° in a water thermostat and steamed 20 minutes in an autoclave at 100° to terminate growth. Maintenance of the stock culture(5), preparation and use of the inoculum suspension(6), measurement of growth response(4), and calculation of assay results(4) were as described in the cited references. An aliquot of each hydrolysate to be assayed for total glutamic acid was neutralized and diluted to contain approximately 2 mg of glutamic acid per ml after adding a sufficient excess of base to make the final solution 2.5 *N* with respect to sodium hydroxide. Ten-ml aliquots of the resulting solutions were heated over night (16-18 hours) at 160° in 12 ml stainless steel bombs in which the residual air had been replaced with nitrogen. The treated solutions were

§ Satisfactory recoveries of both L- and D-glutamic acids from a known mixture of pure amino acids were reported(1), but the experimental mixture contained L-asparagine in place of L-aspartic acid, and it has subsequently been found (unpublished data) that L-asparagine has relatively little effect on D-glutamic acid utilization by the test organisms.

‖ A dry mixture containing the following percentages of amino acids was homogenized in a mortar and stored in a tightly capped bottle: DL-alanine 7.5, L-arginine monohydrochloride 7.5, L-asparagine monohydrate 0.75, L-aspartic acid 49.3, L-cysteine hydrochloride 1.5, glycine 0.75, L-histidine monohydrochloride monohydrate 0.75, DL-isoleucine 7.5, L-leucine 3.77, DL-lysine monohydrochloride 1.5, DL-methionine 0.75, DL-norleucine 1.5, DL-norvaline 1.5, DL-phenylalanine 3.77, L-proline 0.75, DL-serine 1.5, DL-threonine 1.5, DL-tryptophan 0.75, L-tyrosine 1.5, and DL-valine 5.66.

¶ Insufficient to promote appreciable growth in the "blank" tubes, but sufficient to eliminate the lag which otherwise appears in the low region of the standard curve.

TABLE I. L-Glutamic Acid and Total Glutamic Acids in Casein and Various Microorganisms.

	N.	L-glutamic acid				Total glutamic acid			
		Mean	Range	M.D.M.*	No. of determinations	Mean	Range	M.D.M.*	No. of determinations
		%				%			
Casein	15.60†	21.3	21.0-21.9	0.9	11	21.4	21.0-21.9	1.4	6
Lactobacillus arabinosus 17-5	10.51	9.3	9.0- 9.5	1.7	7	14.2	13.8-14.5	1.2	5
Streptococcus faecalis R	10.90	11.0	10.2-11.4	3.1	7	13.7	12.7-14.1	2.6	5
Leuconostoc mesenteroides P-60	10.89	8.1	7.9- 8.3	1.3	7	14.4	14.3-14.6	.6	5
Mycobacterium phlei 10142	8.54	10.9	10.8-11.0	1.1	2	12.7	12.4-13.0	2.4	2
Mycobacterium phlei 336/289B	9.53	19.7	19.6-19.8	0.5	2	22.0	21.8-22.3	1.1	2
Mycobacterium ranae	10.94	9.8	9.7- 9.9	1.0	2	11.2	10.9-11.4	2.2	2
Mycobacterium smegmatis O. V. 66831	10.71	9.6	9.6- 9.6	0.0	2	11.3	11.1-11.5	1.8	2

Casein sample was the same as that described previously(7). Mycobacterial preparations were duplicates of those described by Ginsburg, *et al.*(13). Lactic acid bacteria were produced in 18 liter cultures (incubated 48 hr at 35°) in inoculum medium described previously(6), harvested by centrifugation, washed with 0.85% saline solution, extracted successively with boiling water, hot acetone and cold ether, and air dried. The casein was hydrolysed as described in the text, and bacterial preparations hydrolysed as described by Ginsburg, *et al.*(13). The authors are indebted to Mr. Ginsburg for preparing the mycobacteria and hydrolysing all bacterial preparations. Glutamic acid values for casein are calculated as % of moisture- and ash-free material; all others are calculated as % of protein (N × 6.25).

* Mean percentage deviation of individual values from their mean.

† Corrected for moisture and ash; all other nitrogen values are uncorrected.

cooled, transferred to separate 100 ml volumetric flasks together with sufficient brom thymol blue and sodium chloride to yield final concentrations of 8 γ per ml and 3.0%, respectively, adjusted to pH 6.2 with hydrochloric acid, diluted with water to volume, and assayed for L-glutamic acid as described in the preceding paragraphs. Total glutamic acid was represented by twice the resulting L-glutamic acid values since the described heating with alkali was sufficient to convert either L- or D-glutamic acid to an equal mixture of the two forms. D-glutamic acid was represented by the difference between the L-glutamic acid found in the untreated hydrolysates and the total glutamic acid found in the corresponding samples after heating with alkali.

Results. Previously described methods for the determination of glutamic acid with *L. arabinosus* 17-5(7-11) have not been useful with samples containing appreciable levels of D-glutamic acid because this substance under the usual assay conditions promotes a response of the test organism quite different from that effected by the L-isomer. This problem has been eliminated in the present method, however, by enriching the assay medium with aspartic acid, thereby totally inhibiting response of *L. arabinosus* 17-5 to D-glutamic acid(2,3). Earlier methods specific for the L- form of glutamic acid have been based on the use of *Steptococcus faecalis* R, which cannot utilize D-glutamic acid under any conditions thus far tested(1,8), and additional methods could presumably be based on the use of *Leuconostoc citrovorum* 8082 (1), but in the authors' experience these bacteria, neither of which either grows as profusely or produces acid as abundantly as does *L. arabinosus* 17-5, yield less readily reproducible results than the latter organism.

Reproducibility and accuracy of the present L-glutamic acid assay method are apparent from the data given in Tables I and II in

TABLE II. Recovery of L- and D-Glutamic Acids Added to Hydrolysates of Casein and *Leuconostoc mesenteroides* P-60 Cells.

Sample	Trial	% glutamic acid‖ Added	Found	Recovered¶
Casein*	1	21.45 (L)‡	42.5 (L)	98.8
	2	21.45 " ‡	42.5 "	98.8
	3	21.29 (D)	41.0 (T)	92.1
	4	21.29 "	40.1 "	87.8
L. mesenteroides P-60†	1	7.929(L)§	16.22(L)	102.2
	2	7.929 " §	15.96 "	98.9
	3	14.31 (D)	27.49(T)	91.4
	4	14.31 "	27.93 "	94.5

* Values for glutamic acid added and found are calculated as g/100 g of moisture- and ash-free casein. Recoveries based on mean values, 21.3% and 21.4%, respectively, for L- and total glutamic acids in casein (Table I).

† Values for glutamic acid added and found are calculated as g/100 g of protein (N × 6.25). Recoveries based on mean values, 8.12% and 14.41% (rounded off to 8.1 and 14.4 in Table I), respectively, for L- and total glutamic acids in *L. mesenteroides* protein (Table I).

‡ When D-glutamic acid was added at this level in place of L-glutamic acid, resulting values found for L-glutamic acid, 21.4% and 21.6%, were not appreciably different from avg value, 21.3% (Table I), found in absence of added D-glutamic acid.

§ When 8.65% D-glutamic acid was added in place of L-glutamic acid, resulting values found for L-glutamic acid, 8.2% and 8.0%, were not appreciably different from avg value, 8.1% (Table I), found in absence of added D-glutamic acid.

‖ "Added" and "found" are calculated as g per 100 g of protein (either moisture- and ash-free casein or bacterial nitrogen × 6.25). Notations, L, D, and T in parentheses refer to L-, D- and total glutamic acids, respectively.

¶ Calculated as 100 times the quotient of amount recovered (amount found in recovery sample minus that found in original sample) divided by the amount added.

which it may be seen that L-glutamic acid values from repeated assays of the same samples were in close agreement (individual deviations from the mean values averaged less than 1.4% for all repeated assays) and that recoveries of L-glutamic acid added to hydrolysates were close to 100% (average deviation from 100 was 1.4% for all recovery experiments).

Total glutamic acid values were only slightly less readily reproducible (individual deviations from the mean values averaged less than 1.5% for all repeated assays) than those for L-glutamic acid, but it is apparent from the data shown in Table II that recoveries of D-glutamic acid were significantly low (average 91.4%, range 87.8-94.5). It does not seem likely that these low recoveries were due to incomplete racemization of glutamic acid in the samples prepared for total glutamic acid determinations since virtually complete racemization of pure glutamic acid is effected within 16 to 18 hours even at 145° (Fig. 1) and the test samples were heated at 160° for this period. Moreover, if racemization of glutamic acid in the casein sample had been incomplete, erroneously high values for total glutamic acid would have resulted, and such values would necessarily have been considerably higher than the corresponding values for L-glutamic acid. Actually, as may be seen in Table I, the mean values for L- and total glutamic acids in casein did not differ by as much as 0.5%. It seems likely, therefore, that some destruction of glutamic acid in the recovery samples occurred as a consequence of the racemization procedure, even though pure glutamic acid is stable to this treatment even at 170° for prolonged periods (Fig. 1).

The value, 21.3%, for L-glutamic acid in acid hydrolysed casein (Table I) appears likely to be correct within close limits because of the apparent reliability of the L-glutamic acid assay (Tables I and II) and because of the close agreement of this value with the average value, 21.7%, found upon repeated assays of the same hydrolysate by a mano-

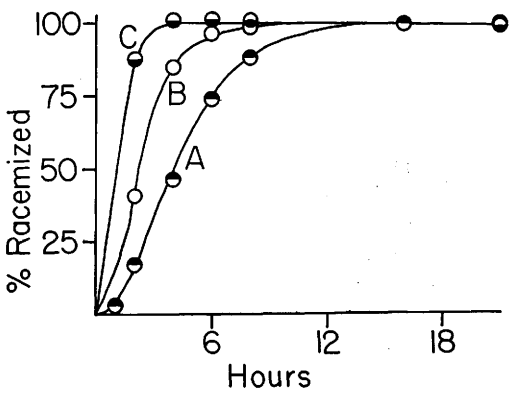

FIG. 1. Racemization of D-glutamic acid in 2.5 *N* sodium hydroxide under nitrogen at 145° (Curve A), 155° (Curve B), and 170° (Curve C). Degrees of racemization were calculated from amounts of L-glutamic acid (measured microbiologically) present in the mixtures (initially L-glutamic acid-free) after heating.

metric procedure employing a specific L-glutamic acid decarboxylase derived from *Escherichia coli*(12). The value, 20.7%, was reported earlier for L-glutamic acid in the same casein preparation(1).

It seemed likely, on the other hand, that the value, 21.4%, for total glutamic acid in the casein hydrolysate (Table I) might be somewhat low in view of the low recoveries of D-glutamic acid (Table II) and the higher values, 22.5%(7) and 22.3%(1), reported for hydrolysates of the same casein preparation analysed by procedures which would be expected to determine some or all of the D-glutamic acid in addition to the L-glutamic acid in this material. The value, 22.5%(7), might be expected to be somewhat high, however, since it was noted that *L. arabinosus* 17-5 was more sensitive in its response to racemic glutamic acid than to L-glutamic acid under the conditions employed(7), and the value, 22.3%(1), the average of values ranging from 21.3% to 23.1%, was not considered to be highly accurate. Moreover, our present value, 21.4% for total glutamic acid in casein, although 2.8% lower than that, 22.00%, reported by Chibnall and his coworkers(14), appears to be in accord with the results of these workers, quoted by Tristram(15), which indicated that only 0.7% of the total glutamic acid in acid hydrolysed casein is of the D-configuration.

The values, 9.3% and 14.2%, respectively, for L- and total glutamic acids in *L. arabinosus* 17-5 (Table I) are of the same order of magnitude as those reported earlier (6.7% and 13.1%, respectively, when recalculated as % of protein) for this species(1), although different and not strictly comparable cell preparations** were tested. The presence of a considerable amount of D-glutamic acid in cells of *L. arabinosus* 17-5 is therefore confirmed, and since the present cell preparation was subjected to extraction with boiling water, it is evident that the D-glutamic acid is tightly bound to insoluble material—probably proteins.

Significant amounts of D-glutamic acid were also found in the cells of *Leuconostoc mesenteroides* P-60 and *S. faecalis* R (Table I), an unexpected result with the latter organism, for which D-glutamic acid is neither utilizable nor capable of sparing L-glutamic acid utilization(1,8). This finding suggests that the cell wall of *S. faecalis* R is impervious to D-glutamic acid, which may nevertheless be produced intracellularly in this bacterium, probably through enzymatic racemization of the nutritionally essential L-glutamic acid.

The relative amounts of D-glutamic acid in hydrolysates of mycobacteria (Table I) are lower than in those of the lactic acid bacteria, ranging from 10% to 15% of the total glutamic acid in the former group as compared with from 20% to 44% in the latter. The values for L-glutamic acid in the mycobacteria are in close agreement (within 5%) with those reported previously for duplicate preparations(13)†† except in the case of *Mycobacterium phlei* 336/289B, for which the present value is 44% greater than that reported for the earlier preparation. The much higher percentage of L-glutamic acid in this strain than in the other strains listed in Table I is apparently correlated with its content of "cord factor"(13).

Summary. 1. An improved microbiological procedure, specific for the L- form of glutamic acid, was developed and shown to yield reproducible and accurate values for this amino acid in protein hydrolysates. 2. A sample treatment resulting in complete racemization of glutamic acid was developed to permit estimation of total (L- plus D-) glutamic acids from the L-glutamic acid content of treated materials. 3. L-Glutamic acid and total glutamic acids were determined in casein, 3 lactic acid bacteria, and 4 mycobacteria. D-Glutamic acid was negligible in casein hydrolysate but appeared to constitute 10% to 15% of the total glutamic acid in hydrolysates of mycobacteria and 20% to 44% in hydrolysates of lactic acid bacteria.

** The present preparation, but not the earlier one, was subjected to extraction with boiling water, and acetone extraction of the present material replaced the ethyl alcohol extraction which was employed in the earlier procedure.

†† Ginsburg *et al.*(13) employed the present method for the determination of L-glutamic acid, but unintentionally omitted an acknowledgement.

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Phenylketonuria VIII. Relation between Age, Serum Phenylalanine Level, and Phenylpyruvic Acid Excretion.* (22880)

MARVIN D. ARMSTRONG AND NIELS L. LOW (Introduced by Emil L. Smith)

Laboratory for Study of Hereditary and Metabolic Disorders and Departments of Biological Chemistry, Pediatrics, and Medicine, Univ. of Utah College of Medicine, Salt Lake City, and Utah State Training School, Amer. Fork, Utah.

In phenylketonuria the liver enzyme system which oxidizes phenylalanine to tyrosine is defective(1); and phenylalanine accumulates in the blood(2). Phenylalanine(3) itself, and its metabolites, phenylpyruvic acid (3), phenyllactic acid(3) and phenylacetylglutamine(4) are excreted. Previous attempts to relate blood or urine levels of phenylalanine(2,5,6) or phenylpyruvic acid(6,7) to the development of the mental deficiency which accompanies the biochemical defect have not shown a significant correlation, nor have blood levels of phenylalanine appeared to correlate well with phenylpyruvic acid excretion. In the course of recent work in this laboratory it became apparent that there is a rough correlation between the level of phenylalanine in the fasting serum of patients and their excretion of phenylpyruvic acid. Also, children under the age of 3 years generally have been found to have much higher blood levels of phenylalanine than have older patients, in most of whom a rather consistent level (30 to 50 mg/100 ml) is observed.

Methods. Serum phenylalanine determinations were made on samples collected in the morning before ingestion of food; this usually has allowed a period of 15 hours for clearance of excess dietary phenylalanine from blood. Modification of the method of Kapeller-Adler (8) described previously(9) has been used for these determinations; phenylacetylglutamine and β-phenyllactic acid do not interfere. Either the method of preparation of the TCA filtrate or determination itself may have led to higher values than have been reported recently(10). Our data for normal sera are in agreement, however, with those obtained by other workers(11), and, in any event, should be internally consistent, since all determinations were carried out in the same manner. It should be noted that the method of Kapeller-Adler is inherently inaccurate when sera containing low levels of phenylalanine are assayed; at the higher levels observed with most

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