THE INFLUENCE OF AMINO ACIDS ON THE GROWTH OF LEPTOSPIRA CANICOLA

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During our investigation (Rosenfeld and Greene, 1941) of various growth-promoting factors for leptospira, interest was aroused in the role of amino acids in the metabolism of these organisms. Experiments were begun to answer the following questions: Could an amino acid be substituted for the serum in the stock medium? Could the peptone in the medium be replaced by an amino acid? Would some particular amino acid added to the medium cause the leptospira to grow more abundantly? Were some harmful? Was the amount used important? If an amino acid did have an effect on growth, was this evident in the first transfer or only after several transfers?

MATERIAL AND METHODS

The culture medium. The medium of Schüffner as described by Smith and Tulloch (1937) and modifications of it were used.

The unmodified medium was prepared as follows: To 1,500 ml of tap water were added 1.5 grams of Witte's peptone. This was boiled and 300 ml of Ringer's solution and 150 ml of Sorensen's double phosphate buffer solution (pH 7.2) were then added. When the precipitation on continued boiling was complete, the mixture was cooled in the refrigerator, filtered, and the pH checked. The medium was bottled or placed in tubes for immediate use and autoclaved at 15 pounds for 20 minutes. For use, 0.3 ml of rabbit serum (passed through a Seitz filter) was added to 3-ml amounts and the tubes heated for 30 minutes in a water bath at 56 C.

The following variations of the medium were made: (a) Varying quantities of an amino acid were added. (b) Serum was omitted and the amino acid was incorporated or absent. (c) No peptone was used and the amino acid under consideration was present or absent. (d) Serum was excluded but otherwise the medium was similar to (c).

The largest amount of an amino acid was weighed and added to 100 ml of a medium. Dilutions of this were made to obtain the desired concentration of the amino acid. The quantities in mg per 100 ml of medium were, in most cases, 0.5, 5, 10, 25, 50, and 200. The solutions were tested for reaction, tubed, and autoclaved in the manner already described.

The first transfers of all variations of the medium pertaining to one particular amino acid were made on the same day. This was done by inoculating the content of each tube with 0.3 ml of a stock culture of *Leptospira canicola* grown for 7 days in the medium of Schüffner at 31 to 32 C. The same quantity of

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inoculum was used in subsequent transfers, which were grown for the same period and temperature. An amount of each type of medium was made for use in 5 transfers. Each transfer of one set of experiments shared the same serum but that used throughout the 5 transfers was not always from the same lot of serum.

TABLE 1

The influence of asparagine, aspartic acid, or leucine when peptone is omitted from Schüffner's medium

TRANSFER	THE NUMBERS OF ORGANISMS COUNTED IN MEDIUM CONTAINING VARYING MG OF AMINO ACID PER 100 ML						
	0	0.5	5	10	25	50	200
Asparagine							
1	78	89	131	153	118	157	117
2	51	61	44	71	83	64	33
3	45	42	64	53	66	64	71
4	53	59	125	158	157	165	131
5	54	60	115	159	146	150	123
Mean.....	56	62	96	119	114	120	95
Aspartic acid							
1	41	58	68	76	73	89	
2	21	32	16	25	32	28	
3	17	23	8	8	16	25	
4	29	35	24	28	42	60	
5	30	37	21	49	37	58	
Mean.....	28	37	27	27	37	52	
Leucine							
1	60	38	53	33	67	73	122
2	55	44	52	72	54	46	145
3	66	44	46	43	51	102	118
4	22	44	37	41	37	47	47
5	59	57	57	39	31	92	53
Mean.....	52	45	49	48	48	72	97

Asparagine: $F = 16$, $F_o = 3.38$.
Aspartic acid: $F = 19$, $F_o = 3.73$.
Leucine: $F = 8.7$, $F_o = 3.38$.

The substances studied. The amino acids (cp unless otherwise specified) used were: *dl*-alanine, *d*-arginine monohydrochloride, *l*-asparagine, *dl*-aspartic acid, *d*-glutamic acid, glycine, *l*(−) histidine, *dl*-leucine A.P., *l*(+)-lysine monohydrochloride, *dl*-methionine A.P., *dl*-phenylalanine, *l*(−) proline, *l*(−) tryptophane, *dl*-tyrosine and *dl*-valine. These products were obtained commercially, usually from the Amino Acid Manufactures, University of California, Los Angeles.

Computations. All counts were made on the seventh day of incubation. Transfers were made on the same day. Unless specified, a 1:4 dilution was made

TABLE 2

The influence of arginine or glutamic acid when added to Schüffner's medium

TRANSFER	THE NUMBERS OF LEPTOSPIRA COUNTED IN MEDIUM CONTAINING VARYING MG OF AMINO ACID PER 100 ML							
	0	0.5	5	10	25	50	100	200
Arginine*								
1	109				56	49	87	104
2	92				46	48	53	59
3	68				69	70	77	82
4	129				84	30	55	32
5	117				26	46	78	48
6	131				90	102	121	118
Mean	108				62	58	79	74
Glutamic acid*								
1	287	228	164	248	230	301		
2	291	288	211	184	197	222		
3	62	127	104	75	119	98		
4	282	331	316	256	225	194		
5	321	252	265	288	264	167		
Mean	249	225	212	211	207	196		

Arginine: F = 18.8, F_o = 2.70.
Glutamic acid: F = 5.01, F_o = 3.73.
* Culture not diluted.

TABLE 3

The effect of some amino acids when added to the medium of Schüffner as shown by the mean count over 5 transfers

MG PER 100 ML OF MEDIUM	ALANINE	GLYCINE*	LYSINE	METHIONINE	PHENYL-ALANINE	TRYPTO-PHANE	TYROSINE	VALINE
0	142	122	155	184	156	158	130	184
0.5	133	136	133	158	120	129	147	168
5	129	104	137	196	148	114	126	122
10	133	103	145	128	112	98	147	122
25	105	25	130	85	45	71	109	100
50	93	4	122	34	42	56	45	51
100				0.2				5
200	13	0	51	0	0		0.2	0.4

* Means of 4 transfers; the second was not counted.

by adding 0.5 ml of the culture to 1.5 ml of sterile physiological saline. The suspension was then heated at 56 C for 30 minutes to kill the organisms. This was done just prior to the enumeration.

A Petroff-Hausser counting chamber with a Neubauer ruling, a 10× ocular,

1.8-mm oil immersion objective, and a dark field were employed. The number of leptospiras in each of 80 small squares and the total number were noted. Then the mean number of the organisms for the 5 transfers was computed. However, when the leptospiras in all tubes of a particular modification of the medium died out by the second or third transfers, additional transfers were not made and no mean value was determined.

Observation of the means made it clear that some amino acids definitely influenced the growth of these organisms, some did not, and the effect of others was questionable. If the result was questionable, the effect of dosage was tested for significance by an analysis of variance. Such an analysis tests for dosage differences with the variation due to transfer being eliminated. The counts and means of these cases and the F and F_0 values are presented in tables

TABLE 4

The mean counts resulting when some amino acids were added to Schüffner's medium modified by excluding peptone

MG PER 100 ML OF MEDIUM	ALANINE	GLYCINE*	LYSINE	METHIONINE	PHENYL-ALANINE	TRYPTO-PHANE	TYROSINE	VALINE
0	66	59	56	34†	45	40	45	37
0.5	68	29	43	60	35	42	49	35
5	65	22	45	44	41	25	49	34
10	58	19	62	36	31	35	42	33
25	47	3	46	12	20	27	11	23
50	10	0.2	47	0.2	4	15	6	13
100				0.2				0.8
200	0.8	0	16	0	0.2		0.2	0

* Means of 4 transfers; the second was not counted.

† Based on 4 transfers; the fifth one was accidentally discarded.

1 and 2. The F value was considered significant when it exceeded the 1 per cent critical value, F_0.

No data are given when the addition of the amino acid to the medium was without effect. In cases in which the means indicated that the amino acid added to Schüffner's medium had an effect, these means are given in table 3 and no additional statistical analysis seems necessary. The parallel tendency of the amino acid to influence growth when peptone is omitted from the medium is shown by the recorded means in table 4.

OBSERVATIONS

The leptospiras died off by the second or third transfer in a medium without serum regardless of the presence of peptone or that of any of the amino acids studied.

When peptone was omitted from the medium of Schüffner, growth was maintained over the 5 transfers but the number of organisms was generally reduced to one third or less of that developing in the unmodified medium.

Table 1 shows that peptone can be replaced to a significant degree by asparagine, aspartic acid, or leucine, and that the amount of the acid used was important. It is also revealed that the replacement effect was evident in the first transfer. Of the dosages used, the best substitution was with 50 mg of aspartic acid, 10, 25, or 50 mg of asparagine, or 200 mg of leucine. However, in no case did the organisms grow so well as in the unmodified medium. With this concentration of leucine and no peptone, the number of leptospiras was about one half that in Schüffner's medium, and that with aspartic acid was one third. The most effective substitute for peptone was asparagine. The mean count at the 50-mg dose was 120 as compared to the mean 146 in the unmodified medium (table not given).

When varying quantities of asparagine, aspartic acid, leucine, histidine, or proline were added to the medium of Schüffner, neither stimulation nor interference in growth occurred. The largest concentration of aspartic acid was 50 mg, that of the others was 200 mg per 100 ml.

Depending upon the concentration, the following amino acids caused a reduction in growth of *L. canicola*: alanine, arginine, glutamic acid, glycine, lysine, methionine, phenylalanine, tryptophane, tyrosine, and valine (tables 2, 3, and 4).

Approximately 50 per cent fewer organisms were present when the concentration of amino acid per 100 ml of the medium (or the medium minus peptone) was 25 mg of methionine or phenylalanine, 50 mg of valine, and >50 mg of alanine or lysine. In Schüffner's medium without peptone, glycine and tyrosine produced the same reduction in smaller concentrations than those needed in the unaltered medium. The reverse was true for tryptophane.

A greater concentration of these supplements than those causing a 50 per cent reduction in the growth of the leptospiras generally resulted in such inhibition that few or no organisms were found in the fields examined.

DISCUSSION

Media used for growing *Leptospira icterohemorrhagiae* or *Leptospira canicola* require serum. As fresh serum is not always easily available for the relatively frequent transfer of the organism, it was hoped that some substitute for it could be found. Also, as growth in the regular media is not always consistent, it was desired to find some factor that would cause more abundant growth. That small amounts of nicotinic acid promoted the growth of *Leptospira* was reported by Yoshida (1939), by Ward and Starbuck (1941), and by Rosenfeld and the author in 1941. The latter workers also found that thiamine hydrochloride, nicotinic acid amide, or riboflavin in low concentration served as growth accessory substances. This activity was not so great as that of nicotinic acid. Nor would any of these factors replace serum.

In this study, regardless of the quantity used, none of the amino acids under consideration would serve in the place of the serum required for growth. Ono (1938) reported that none of the amino acids he investigated, singly or in combination, would substitute for serum.

Ward and Starbuck (1941) incorporated in a modified Noguchi semi solid

medium 0.01 per cent cysteine hydrochloride, glutamine, glycine, leucine, methionine, tyrosine, or valine. The leptospiras were not carried through several successive transfers in the media, but several experiments, consisting each time of 5 or 6 tubes of the various media, were performed. Cysteine hydrochloride accelerated growth but to a lesser degree than did nicotinic acid. Valine suppressed the organisms, and the other amino acids were without effect.

This work was already started at the time of that report. The liquid medium of Schüffner contains approximately 0.07 per cent peptone and a greater variety of salts than the medium used by Ward and Starbuck. Also, hemoglobin was not specifically added and did vary depending upon the amount in the lot of serum used. Varying dosages of each amino acid were used, generally the concentrations in the media were 0.5, 5, 10, 25, 50, and 200 mg per 100 ml. The organisms were followed through five successive transfers in each medium, except in that containing no serum. In the latter case the leptospiras died out by the second or third transfer and the tubes were discarded.

As supplements to Schüffner's medium, the amino acids did not increase the numbers. This finding does not agree with that of Ono, who states that asparagine or glutamic acid accelerated growth.

Here, in each experiment, Schüffner's medium minus peptone was more like the media used by Ono or by Ward and Starbuck. It was found that when peptone was omitted, none of the amino acids studied would serve so well in its place. However, asparagine, aspartic acid, or leucine substituted for peptone to some degree depending upon the quantity used. And asparagine used in concentrations of 10, 25, or 50 mg caused growth to reach more nearly the level attained in the unmodified medium of Schüffner. The results respecting the effect of asparagine agreed with the finding of Ono. He did not use aspartic acid. The other workers report leucine as having no effect. The amounts of leucine responsible for the greater growth in the absence of peptone in this report were 0.05 and 0.2 per cent. Leucine, in 0.01 per cent amount, had no effect, as was also found by Ward and Starbuck.

Histidine and proline were found not to influence growth. These are reported by Ono as "aiding" growth in contrast to "acceleration" caused by asparagine and glutamic acid. Other amino acids also found by him to aid the growth of *L. icterohemorrhagiae* were alanine, arginine, cysteine, glycine, lysine, and tryptophane. He stated that phenylalanine had no effect and that cystine and tyrosine hindered the organism.

The observations made here indicated that alanine, arginine, glutamic acid, glycine, lysine, methionine, tryptophane, tyrosine, or valine, when added to the medium of Schüffner or to that medium with peptone excluded, caused a reduction in the numbers of *L. canicola*. The result depended upon the amount of amino acid supplementing the medium and, in some cases, on the number of transfers made, because the harmful effect was not always evident in the first transfer. The influence of glutamic acid, lysine, or tryptophane was not definite until the second transfer. The discrepancy between no effect obtained by Ward and Starbuck and the harmful result reported here for the same amino acids can be accounted for by the greater dosage used in this study.

If the larger amounts of amino acids had decreased growth in all cases, it might have been said that it was due to the greater concentration of material in the medium regardless of what amino acid was present. But such was not the case. All of the amino acids studied were not detrimental to the organisms when added in amounts of 25, 50, or 200 mg. Also, when the concentration of solutes was lessened by the absence of peptone, the same harmful influence was apparent.

SUMMARY

Serum in the medium used for the cultivation of *Leptospira canicola* cannot be replaced by any of the amino acids studied.

If peptone is omitted from the medium, asparagine, aspartic acid, or leucine will substitute for it to some degree.

None of the amino acids tested accelerated growth when used as a supplement to the medium of Schüffner.

Asparagine, aspartic acid, histidine, leucine, and proline were without effect when added to this medium.

Alanine, arginine, glutamic acid, glycine, lysine, methionine, phenylalanine, tryptophane, tyrosine, or valine caused a significant decrease in the growth of *L. canicola* in the medium of Schüffner when the amount of the supplement was, in most instances, 0.025 per cent or greater.

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