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Nutrition of *Leptospira canicola*. III. Utilization of Vitamins and Amino Acids.* (20021)

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The present investigation is an extension of the authors' studies on the nutritional requirements of leptospira(1-4). A chemically-defined basal medium developed earlier was shown to be satisfactory for the cultivation of the spirochete when supplemented with dialyzed rabbit serum(3) or an electrophoretically-homogeneous rabbit serum albumin(4). Related studies on the utilization of amino acids and vitamins by pathogenic leptospira have been reported by other workers(5-11).

Experimental. The response of *Leptospira canicola*‡ to individual amino acids was determined by replacing the amino acid mixture in the previously described basal medium supplemented with rabbit serum albumin(4), with each of 22 amino acids in turn at 0, 10, 20, 40, 60, 80, 100, 150, 200 and 500 mg % concentrations. Seven replicate tubes were prepared at each concentration. The pH of the medium was 7.2-7.4. 0.3 ml of a suspension of *L. canicola*, prepared as described earlier(4), was added to each of 5 tubes with 2 tubes serving as controls. The final volume was 6 ml and the tubes were incubated for 7 days at 32°. Growth was measured as increase in optical density of the cell suspensions determined with the aid of a Klett-Summerson photoelectric colorimeter. In some experiments the inoculum was obtained directly from the 4th or 5th serial transfer of the organism grown on the medium (complete except for the omission of amino acids) in which growth had decreased progressively. The purpose was to determine whether or not the response of the spirochete had been affected by this treatment. Subsequently, an amino acid mixture simulating the composition of rabbit serum albumin was employed in an attempt to replace the albumin and the amino acid mixture of arbitrary composition used initially. The composition (Table I) of the electrophoretically-homogeneous rabbit albumin was determined by assaying for alanine by the method of Sauberlich and Baumann(12) and the 16 other amino acids by the procedures of Dunn *et al.*(13,14). All assays were performed in triplicate using 5 levels of sample and 15 levels of standard. The maximum deviation of the values at the

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‡ *L. pomona* (strain 9), *L. canicola* (dog Utrecht IV) and *L. icterohaemorrhagiae* (strain 20, Wijnberg Type AB) were obtained through the courtesy of Dr. K. F. Meyer, University of California, San Francisco. *L. ballum* (strain mouse 27), *L. sejroe* (strain Mallersdorf II) and *L. icterohaemorrhagiae* (biotype A, strain Kantorowicz) were obtained through the courtesy of Dr. J. W. Wolff, Institute for Tropical Hygiene, Amsterdam, Holland.

TABLE I. Amino Acid Composition of Rabbit Serum Albumin.

Amino acid	%*	Amino acid	%*
Alanine	4.1	Lysine	8
Arginine	5.3	Methionine	.29
Aspartic acid	7.5	Phenylalanine	11.9
Cystine	5.3	Proline	4.1
Glycine	2.3	Threonine	4.1
Glutamic acid	9.9	Tryptophan	.35
Histidine	3	Tyrosine	6
Isoleucine	3.2	Valine	6.6
Leucine	9.5		

* Determined by microbiological assay using methods described in the text. The rabbit albumin preparation analyzed contained 4.98 mg N/ml. Values are calculated to 16% N.

different levels of sample was 6%. Tests were also made on the effectiveness of asparagine (80 mg %) as a substitute for the amino acid mixture in the medium used to maintain the organism for 8 weeks in serial subculture and to determine which group constituents of the arbitrarily-selected purine-pyrimidine-mineral mixture(3) were essential. In these studies the concentration of each constituent was the same as that employed previously. The response of the organism to 12 vitamins (nicotinamide, nicotinic acid, riboflavin, pyridoxine, pyridoxamine, thiamine, biotin, choline, folic acid, inositol, pantothenic acid and *p*-aminobenzoic acid) was determined by adding each vitamin in turn to a chemically-defined medium less complex than used in the earlier-described experiments. This medium was an aqueous solution of Na_2HPO_4 312, KH_2PO_4 420, NaCl 1280, Na_2CO_3 32, KCl 32, $CaCl_2$ 32, and asparagine 80 (the numbers are mg % concentrations) supplemented with rabbit albumin. Thiamine chloride was tested at 0.001, 0.01, 0.1, 1.0, 10, 100 and 200 γ % concentrations and each other vitamin at 1, 10 and 100 γ % concentrations. Later, the growth response of *L. canicola* in the described basal medium, supplemented with rabbit albumin and 10 γ % of thiamine chloride, was followed for 2 months in weekly serial subcultures. This medium was also tested for its ability to maintain *Leptospira ballum*,† *Leptospira icterohaemorrhagiæ* (Biotype A and Strain 20),† *Leptospira pomona*,† and *Leptospira sejroe*.†

Results and discussion. As shown in Table II the growth of *L. canicola* was increased by a supplement of L-arginine • HCl (40-150 mg %), L-asparagine (40-200 mg %), L-aspartic acid (60-100 mg %), L-glutamic acid (40-500 mg %), or proline (10-500 mg %). The data are not shown for the remaining amino acids tested. L-Glutamine was without effect. Sixteen amino acids (Dl-alanine. L-cysteine • HCl, glycine, L-histidine • HCl, DL-isoleucine, L-leucine, DL-lysine • HCl, DL-methionine, Dl-norleucine, DL-norvaline, DL-phenylalanine, DL-serine, DL-threonine, DL-tryptophan, L-tyrosine and DL-valine) were not stimulatory and were definitely inhibitory at some level. It was also found (data not given) that the 5 amino acids (each tested at 40 and 80 mg %) listed in Table II stimulated the growth of *L. canicola* (inocula prepared from the amino acid deficient medium) as much or more on the experimental medium as on the peptone medium containing dialyzed rabbit serum.

Attempts to replace the rabbit serum albumin in the medium with an amino acid mixture simulating the composition of the albumin (Table I) were unsuccessful since concentrations of the amino acid mixture exceeding 10 mg % inhibited the growth of *L. canicola*.

It was observed (data not shown) that the basal medium containing rabbit albumin and asparagine (80 mg %) in place of the amino acid mixture permitted undiminished growth of *L. canicola* when transferred weekly over the experimental period (8 weeks). Although growth was depressed in the absence of the vitamin-purine-pyrimidine-mineral solution, only the vitamins appeared to be essential components of this mixture. It was found, also, that *L. canicola* could be transferred satisfactorily for 6 weeks (experimental period) in media containing glutamic acid (40 mg %), arginine (40 mg %), aspartic acid (80 mg %), or proline (80 mg %) in place of asparagine. *L. icterohaemorrhagiæ* could be maintained on the asparagine medium. The effectiveness of asparagine in replacing peptone in a medium containing serum has been reported by Greene(2) and Stuart (10) and it is of interest that Savino and Renella(9) grew *L. icterohaemorrhagiæ* on a chemically-defined medium containing asparagine.

TABLE II. Response of *Leptospira canicola* to Amino Acids.*

Amino acid	Increase in optical density — Amino acid concentration, mg %									
	0	10	20	40	60	80	100	150	200	500
L-Arginine • HCl	41	45	50	53	53	57	55	53	31	8
L-Asparagine	41	48	51	55	51	51	53	55	54	31
L-Aspartic acid	51	53	49	51	57	55	56	0	0	0
L-Glutamic acid	45	48	47	51	49	51	45	45	46	54
L-Proline	27	43	48	49	44	49	47	60	51	51

* Basal medium supplemented with rabbit serum described previously(4). See text for experimental conditions.

TABLE III. Response of *L. canicola* to Vitamins.

Vitamin	Increase in optical density* — Vit. conc., γ %			
	0	1	10	100
A				
Nicotinamide	14	13	16	16
Nicotinic acid	14	10	10	14
Riboflavin	14	9	8	14
Pyridoxine • HCl	14	8	12	14
Pyridoxamine • 2HCl	14	9	10	9
Thiamine • Cl	14	42	39	39
Control				
Complete vit. mixture			35	
Stock medium			44	
B				
Biotin	6	15	5	9
Choline • Cl	6	7	8	10
Folic acid	6	11	5	11
Inositol	6	6	7	5
Ca. dl-pantothenate	6	9	8	13
p-Aminobenzoic acid	6	5	10	10
Control				
Complete vit. mixture			32	
Stock medium			48	

* Avg of 4 tubes. A and B not conducted simultaneously. In A the basal medium of asparagine and salts contained 1.5 ml/tube of albumin preparation No. 3 (4.98 mg N/ml). Albumin preparation No. 4 (5.35 mg N/ml; 1 ml/tube) was used in B. Stock medium (Witte's peptone, salts, dialyzed rabbit serum).

The growth-enhancing activity of vitamins (tested singly) is shown in Table III. In further tests (data not shown) thiamine chloride in concentrations from 1 to 200 γ % was found to promote growth of *L. canicola* as effectively as the complete vitamin mixture. The spirochete was subcultured serially for 2 months in the medium containing only the albumin, salts, thiamine and asparagine. The observation that growth was not markedly affected by the omission of asparagine, in disagreement with the results reported earlier(4) showing the need for amino acids in serial subculture, may be explained (possibly) by antagonistic effects of certain B vitamins rather than by any need of the organism for particular amino acids. *L. icterohaemorrhagiæ* (Biotype A), *L. ballum, L. pomona, L. sejroe* and *L. canicola* were subcultured satisfactorily over the experimental period (14 weeks) in the stock transfer medium (Schuffner's medium with dialyzed rabbit serum) and in a medium composed of thiamine, asparagine, Ringer's solution, Sorensen's buffer and the rabbit albumin. In all cases the growth was good but somewhat less satisfactory in the latter than in the former medium. *L. icterohaemorrhagiæ* (strain 20) could not be subcultured longer than 7 weeks in the second medium. Brede and Lempfrid(15) have reported that 12 species and 23 pathogenic strains of leptospira could be grown in Korthof's medium in which rabbit serum was replaced by allantoic fluid from 13-day-old chick embryos.

It seems probable from the authors' experiments that serum albumin *per se,* rather than an amino acid or collection of amino acids liberated from this protein, is essential for *L. canicola* and other pathogenic leptospira. Although Marshall(6) has suggested that the primary role of the albumin may be to inactivate or adsorb some toxic substance, it seems unlikely from the present experiments that the albumin has this function. Whether the entire protein structure or a particular stereochemical unit is essential for growth of pathogenic leptospira remains to be determined.

Summary. It has been shown that the growth of *Leptospira canicola* on a chemically-defined medium lacking amino acids but supplemented with rabbit serum albumin is stimulated at certain levels of arginine, aspartic acid, glutamic acid or proline but is inhibited

by any one of 16 amino acids at some concentration. The rabbit albumin could not be replaced by a mixture of amino acids simulating the amino acid composition of the albumin determined by microbiological assay. Thiamine was found to be the only vitamin essential for the growth of *L. canicola*. *L. canicola, L. pomona, L. ballum, L. sejroe,* and *L. icterohaemorrhagiæ* could be subcultured on a simple medium containing only salts, thiamine, asparagine, and rabbit serum albumin.

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Physiologic Responses Attending Administration of Antimony, Alone or with Simultaneous Injections of Thyroxin.* (20022)

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Brady, Cowie, and also Maren and Otto have reported the high localization of antimony in the thyroid gland of the dog(1-3). Apparently this localization is not species specific since similar results were obtained by Smith(4) in the hamster, and by Kramer(5) in the rabbit. In this laboratory a strong affinity for antimony was demonstrated by the thyroids of rats exposed to the inhalation of finely divided antimony trioxide, and by rats fed a low level of antimony trioxide in their diet(6). The growth curve for the latter group of animals showed a slightly greater increase in body weight than the controls. The increase in body weight and the sluggish behavior of these animals, accompanied by the marked accumulation of antimony in the thyroid, were interpreted as a tendency toward hypothyroidism. If antimony did have antithyroidal activity when administered alone, one would expect that when given with simultaneous injections of thyroxin, their antagonistic actions should result in a nearly normal metabolic state.

In view of the above facts the following experiments were designed to determine the effect on thyroid function of antimony administered alone or in combination with thyroxin. Three experiments were conducted, 2 using albino rats and one using albino rabbits. The body weight and oxygen consumption were employed as measures of thyroid activity. The concentration of antimony in the tissues was determined in order to ascertain whether or not the presence of thyroxin influenced the pattern of distribution of antimony in the body.

Methods. Oxygen consumption was deter-

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