NUTRITION OF *LEPTOSPIRA POMONA*

II. Fatty Acid Requirements

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Abstract

JOHNSON, R. C. (Fort Detrick, Frederick, Md.) AND N. D. GARY. Nutrition of *Leptospira pomona*. II. Fatty acid requirements. J. Bacteriol. **85**:976–982. 1963.—The albumin fraction of rabbit serum, obtained by $(NH_4)_2SO_4$ fractionation, supported good growth of *Leptospira pomona* Wickard when added to a medium containing phosphate buffer, NH_4Cl, and thiamine. Extraction of the albumin fraction with ethanol and ether (3:1) resulted in a loss of its growth-supporting activity, which could be restored upon the addition of the extract or various fatty acids. The growth-supporting activity of fatty acids containing 2 to 18 carbon atoms was investigated with the extracted albumin medium. The activity of the fatty acid was found to be related to the number of carbon atoms in the molecule. Maximal growth was obtained with palmitic, heptadecanoic, stearic, and oleic acids. The amount of growth of *L. pomona* increased at a fixed concentration of albumin as the molecular ratio of extracted albumin to the long-chain fatty acids increased from 1:1 to 1:6. At higher ratios, growth decreased sharply. Direct utilization of fatty acids during growth of *L. pomona* was shown by the incorporation of palmitic acid-1-C^{14} or -2-C^{14} into cellular material. A medium composed of 0.02 M Na_2HPO_4-KH_2PO_4 buffer (pH 7.4), 0.8% extracted albumin, 4×10^{-4} M palmitic acid, 10^{-3} M NH_4Cl, and 5 μg/ml of thiamine supported good growth. *L. pomona* was transferred ten times, and seven other serotypes were transferred five times, in this medium without any decrease in the amount of growth. Growth of *L. pomona* was initiated with approximately four organisms per ml.

Maximal growth of pathogenic leptospirae is usually obtained with media containing rabbit serum. The function of rabbit serum in the nutrition of *Leptospira pomona* was investigated by Johnson and Wilson (1960). Rabbit serum was divided into three fractions, albumin, globulin, and ultrafiltrate, and it was demonstrated that the combination of all three fractions was required for good growth. The ultrafiltrate fraction of rabbit serum was investigated by Johnson and Gary (1962), and a chemically defined substitute containing NH_4Cl and thiamine was formulated.

The lipid requirement of leptospirae has received the attention of several investigators. Helprin and Hiatt (1957) demonstrated that various fatty acids stimulated the respiration of *L. icterohaemorrhagiae* in the presence of "lipid-free" albumin. Woratz (1957) reported the growth of *L. canicola* in a serum-free medium containing fatty acids. Oleic acid was shown to be a growth factor for *L. pomona* by Vaneseltine and Staples (1961). Vogel and Hutner (1961) found that various esterified fatty acids promoted the growth of several strains of leptospirae in a chemically defined medium.

This report presents studies on the fatty acid requirements of *L. pomona* in an extracted albumin medium. The albumin fraction of rabbit serum, obtained by $(NH_4)_2SO_4$ precipitation, supported growth of *L. pomona* when added to a medium containing phosphate buffer, NH_4Cl, and thiamine. Extraction of the albumin with a lipid solvent resulted in a loss of its growth-supporting activity, which could be restored upon the addition of the extract or various fatty acids.

Materials and Methods

Leptospira pomona Wickard was the primary test organism used for the nutritional experiments. Stock cultures were maintained in a medium containing 10% pooled rabbit serum in

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0.02 M Na_2HPO_4-KH_2PO_4 buffer (Johnson and Wilson, 1960). Only approximately one-half of the expected growth was obtained with some lots of commercial rabbit serum. The addition of 10^{-3} M NH_4Cl to the medium corrected this inadequacy. Cultures were incubated at 30 C and transferred every 7 days.

The compounds tested were prepared in 0.02 M phosphate buffer (pH 7.4), and the experimental medium was adjusted to a final volume of 10 ml/tube with phosphate buffer. Fatty acids were tested in the form of their sodium salt. Other lipid materials tested were first dissolved in 95% ethyl alcohol, which was then dispersed in phosphate buffer. Lipid compounds were obtained from Nutritional Biochemical Corp., Cleveland, Ohio. Fatty acids were sterilized by autoclaving; α-ketoglutaric, malic, and pyruvic acids, and thiamine were sterilized by filtration through sintered glass.

Growth was measured turbidimetrically with a Coleman model 7 photonephelometer, as described previously by Johnson and Gary (1962). Several samples from each experiment were counted with a Petroff-Hausser counting chamber to verify the nephelometer readings. Each experiment was repeated. An inoculum of 0.1 ml of stock culture added to 9.9 ml of medium yielded an initial count of approximately 2×10^6 to 3×10^6 organisms per ml.

The albumin fraction of rabbit serum was obtained by $(NH_4)_2SO_4$ precipitation as described by Johnson and Wilson (1960). The albumin was lyophilized, and 5 g were extracted three times at room temperature with 400-ml samples of a 95% ethyl alcohol-diethyl ether (3:1) solvent (Bloor, 1914). The first extraction was for 16 hr and the second and third extractions were 2 hr each. The solvent was separated from the albumin by filtration. Extracted albumin was finally washed with a small volume of ether, to facilitate the drying process, and dried under vacuum. A stock solution of 5% extracted albumin was made in 0.02 M phosphate buffer (pH 7.4), sterilized by Seitz filtration, and stored at 4 C until used. Protein concentration of the albumin preparations was determined by the biuret method of Gornall, Bardawill, and David (1949).

Alcohol-ether extracts were evaporated to dryness, redissolved in 0.1 M NaOH, brought to a final volume of 100 ml with 0.02 M phosphate buffer, and adjusted to pH 7.4.

Uptake of palmitic acid was measured during growth of *L. pomona* in the presence of palmitic acid-1-C^{14} and -2-C^{14}. Carbon-14 counting was performed in a Nuclear instrument and a gas flow counter (model D47; Chemical Corp., Springfield, Mass.) at 1,200 v, employing a gas mixture of 98.7% helium and 1.3% butane. Counting periods of 5 min were used, and all counts were corrected for background.

RESULTS

As previously indicated, some lots of commercial rabbit serum were inadequate for optimal growth of *L. pomona* and could not be used for the preparation of satisfactory control media. The addition of 10^{-3} M NH_4Cl to the medium restored its full activity (Fig. 1). Growth was increased slightly by the incorporation of thiamine in the medium.

With the nutritional role of the ultrafiltrate of rabbit serum defined (Johnson and Gary, 1962), attention was directed to the albumin fraction (Johnson and Wilson, 1960). Rabbit serum albumin, when extracted with a lipid solvent (alcohol-ether, 3:1), loses most of its growth-supporting activity, which can be restored upon the addition of the albumin extract (Table 1). The albumin extract in the absence of the extracted albumin had very little activity, thus suggesting that the albumin fraction was functioning as a carrier of lipid material in a nontoxic state.

Specific fatty acid requirements were examined next by comparing the growth-supporting activity of fatty acids containing from 2 to 18 carbon atoms in a basal medium consisting of 0.8%

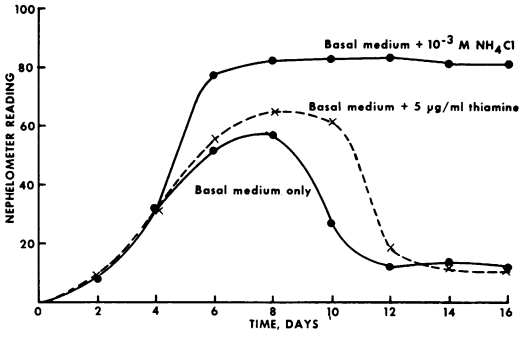

FIG. 1. *Growth response of Leptospira pomona to NH_4Cl and thiamine added to basal medium of 10% rabbit serum and 0.02 M phosphate buffer (pH 7.4).*

TABLE 1. *Ethanol-ether extraction of rabbit serum albumin and its effect on the growth of Leptospira pomona*

Basal medium* plus	No. of organsims/ml × 10^6
Unextracted albumin†	380
Extracted albumin	27
Extract	14
Extracted albumin plus extract	300

* Basal medium: 0.02 M phosphate buffer (pH 7.4), 5 μg/ml of thiamine, and 10^{-3} M NH_4Cl.

† Albumin preparations 1% protein .

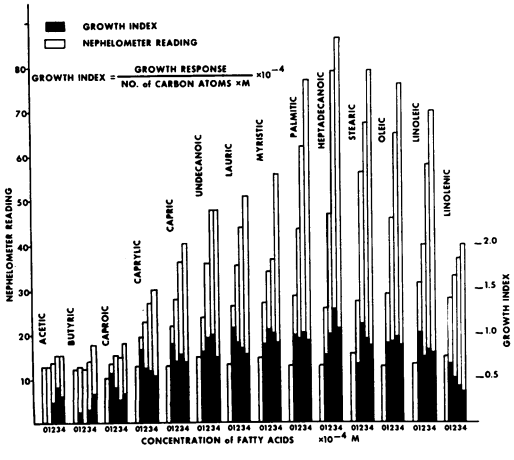

FIG. 2. *Growth response of Leptospira pomona to fatty acids added to basal medium of 0.8% extracted albumin, 10^{-3} M NH_4Cl, and 5 μg/ml of thiamine in 0.02 M phosphate buffer (pH 7.4).*

extracted albumin, 5 μg/ml of thiamine, and 10^{-3} M NH_4Cl in 0.02 M phosphate buffer (pH 7.4). The activity of the fatty acids was found to be related to the number of carbon atoms in the molecule (Fig. 2). Poor growth was obtained with the short-chain fatty acids, acetic, butyric, caproic, and caprylic; maximal growth was obtained with palmitic, heptadecanoic, stearic, and oleic acids. A correlation between the growth response and the number of carbon atoms in the fatty acid revealed an approximately equivalent growth response per carbon atom (growth index) with undecanoic acid through linoleic acid (Fig. 2). Fatty acids containing an odd number of carbon atoms supported growth as well as those containing an even number. A combination of palmitic, stearic, and oleic acids was no more active than the individual fatty acids (Table 2). All fatty acids were without growth-supporting activity in liquid medium in the absence of the extracted albumin when tested at the concentrations used in Fig. 2.

Correlation of structural changes of stearic acid on growth-supporting activity is presented in Table 3. The presence of one double bond (oleic acid) did not alter the extent of growth but did decrease the time required for maximal

TABLE 2. *Effect of combination of fatty acids on growth of Leptospira pomona*

Fatty acid added to basal medium*	Concn tested × 10^{-4} M	Nephelometer reading
None	0	16
Palmitic	3	63
Stearic	3	70
Oleic	3	69
Palmitic + stearic + oleic	3†	72
Control (10% rabbit serum)	—	109

* Basal medium: 0.8% extracted albumin, 0.02 M phosphate buffer (pH 7.4), 10^{-3} M NH_4Cl, and 5 μg/ml of thiamine.

† Concentrations: 10^{-4} M each.

TABLE 3. *Growth response of Leptospira pomona to structural changes in stearic acid*

Basal medium* plus	Concn tested × 10^{-4} M	Maximal growth	
		Nephelometer reading	No. of days
Stearic acid	3	66	17
Oleic acid	3	64	7
Linoleic acid	3	52	8
Linolenic acid	3	37	8
Dihydroxystearic acid	3	27	5
Stearic acid (methyl ester)	1†	28	6
Stearyl alcohol	3	10	—
α-Monostearin	2‡	71	21
Tristearin	1‡	23	20
None	—	16	8
Control (10% rabbit serum)	—	109	8

* Basal medium: 0.8% extracted albumin, 0.02 M phosphate buffer, 10^{-3} M NH_4Cl, and 5 μg/ml of thiamine.

† Higher concentrations inhibitory.

‡ Higher concentrations resulted in turbidity that interfered with nephelometry.

TABLE 4. *Utilization of palmitic acid-1-C^{14} and -2-C^{14} by Leptospira pomona*

Determination	Expt no.	Palmitic acid-1-C^{14}		Palmitic acid-2-C^{14}	
		Counts/min*	Original activity	Counts/min*	Original activity
			%		%
Initial count in medium†	I	2,556	100	4,162	100
	II	3,244	100	4,235	100
Final count in medium	I	429	17	995	24
	II	822	25	818	19
Final count in cells	I	762	30	2,344	56
	II	793	25	2,378	56
Count as volatile material‡	I	1,365	53	823	20
	II	1,629	50	1,039	25

* Counts per min per 0.1 ml of sample.

† Experimental medium: 0.82% extracted albumin, 0.02 M phosphate buffer, 5 μg/ml of thiamine, 10^{-3} M NH_4Cl, 3×10^{-4} M palmitic acid, and 10^{-6} M palmitic acid-1-C^{14} or -2-C^{14}.

‡ Volatile material equals initial count in medium minus final count in acidified medium.

growth by 10 days. The presence of two double bonds (linoleic acid) or three double bonds (linolenic acid) also decreased the growth time, but these were progressively inhibitory. Dihydroxystearic acid, stearyl alcohol, methyl stearate, and tristearin had little activity. α-Monostearin supported satisfactory growth but extended the growth period.

Utilization of palmitic acid during growth was measured by the incorporation of the C^{14} label of palmitic acid-1-C^{14} and palmitic acid-2-C^{14} into cellular material (Table 4). With palmitic acid-1-C^{14}, an average of 21% of the label remained in the medium, 28% appeared in the cells, and 52% as volatile material. Utilization of palmitic acid-2-C^{14} resulted in an average of 22% of the activity remaining in the medium, 56% in the cells, and 23% as volatile material. The amount of label appearing in the cells and in volatile material differed with the -1-C^{14} and in -2-C^{14} substrates, indicating that these carbon atoms were metabolized differently.

Other compounds tested for activity in the extracted albumin medium, with and without fatty acid, were glycerol (10^{-2} M), citrate (10^{-3} M), α-ketoglutarate (10^{-3} M), pyruvate (10^{-3} M), and succinate (10^{-3} M). None of the compounds demonstrated any growth-enhancing activity except glycerol, which supported a limited amount of growth in the absence of the fatty acid.

Johnson and Gary (1962) reported that NH_4Cl and thiamine would substitute for the ultrafiltrate fraction of rabbit serum in a dialyzed serum medium. The requirements for NH_4Cl and thiamine in the extracted albumin medium are shown in Table 5. Maximal growth of *L. pomona* was obtained when both compounds were added to the medium. When either compound was deleted from the medium, very poor growth occurred.

Utilizing the technique used by Oyama, Steinman, and Eagle (1953) with the Reiter treponeme and by Vaneseltine and Staples (1961) with *L. pomona*, an attempt was made to ascertain

TABLE 5. *Effect of palmitic acid, thiamine, and NH_4Cl on the growth of Leptospira pomona*

Additions to basal medium*	Nephelometer reading
Palmitic acid (4×10^{-4} M)	10
Palmitic acid + NH_4Cl (10^{-3} M)	10
Palmitic acid + thiamine (5 μg/ml)	16
Palmitic acid + NH_4Cl + thiamine	74
NH_4Cl + thiamine (no palmitic acid)	14
Control (10% rabbit serum)	98

* Basal medium: 0.8% extracted albumin in 0.02 M phosphate buffer (pH 7.4).

whether the only function of albumin was to reversibly bind fatty acids. A 10-ml portion of medium, containing 0.3% agar (Difco), 0.02 M phosphate buffer (pH 7.4), 5×10^{-5} M $CaCl_2$ and $MgSO_4$, 10^{-5} M Fe citrate, 10^{-3} M NH_4Cl, and 5 μg/ml of thiamine, was inoculated with 240×10^6 organisms, mixed, and overlaid with 1 ml of 4×10^{-4} M palmitic acid. A heavy band of growth occurred in this medium. No growth was evident in the control tubes overlaid with phosphate buffer. Diffuse growth occurred when extracted albumin was added to the palmitic acid overlay. Attempts to subculture the organism in the absence of the extracted albumin were unsuccessful.

Nutritional adequacy of the experimental medium containing extracted albumin in phosphate buffer, NH_4Cl, thiamine, and palmitic acid was investigated (Table 5). *L. pomona* also was successfully maintained through ten serial transfers in this experimental medium, with no reduction in the rate or amount of growth. The smallest inoculum that initiated growth was approximately four organisms per ml.

Various serotypes (Table 6) of leptospirae were tested to determine whether growth in this medium was restricted to *L. pomona* Wickard. All serotypes tested grew well in the experimental medium and were maintained through five serial transfers without any reduction in the rate or amount of growth.

TABLE 6. *Growth of serotypes of leptospirae in experimental medium**

Serotype	Strain	Nephelometer reading
L. australis A	Ballico	60
L. australis B	Zanoni	54
L. autumnalis (AB)	Akiyami A	56
L. bataviae	Unknown	60
L. canicola	Hond Utrecht IV	55
L. hebdomadis	Hebdomadis	51
L. icterohaemorrhagiae (AB)	M-20	51
L. pomona	Johnson	52
L. pomona	Pomona	47
L. pomona	Wickard	62

* Experimental medium: 0.8% extracted albumin, 4×10^{-4} M palmitic acid, 0.02 M phosphate buffer, 5 μg/ml of thiamine, and 10^{-3} M NH_4Cl.

DISCUSSION

Through the use of an extracted albumin medium and nephelometry, it was possible to assay the growth-supporting activity of various fatty acids. The relationship between the growth-supporting activity of the fatty acids and the number of carbon atoms per molecule is similar to the findings of Helprin and Hiatt (1957) on the stimulatory effect of fatty acids on the respiration of *L. icterohaemorrhagiae*. They also found that human plasma fraction V (albumin) was required to detoxify some of the fatty acids and that the amount of respiration was related to the number of carbon atoms present in a fatty acid.

Our results show that growth of *L. pomona* decreased as the molecular ratio of long-chain fatty acids to extracted albumin was increased from approximately 1:1 to 6:1. When the ratio was greater than 6:1, growth was usually inhibited and the medium became turbid. This indicated that each molecule of albumin was able to bind approximately four to six molecules of fatty acid, rendering them nontoxic for *L. pomona*. This binding capacity of the albumin was destroyed upon autoclaving. These data are in agreement with those of Davis and Dubos (1947), who found that one molecule of bovine serum albumin could bind three to six molecules of oleic acid sufficiently to prevent their bacteriostatic effect. Oyama et al. (1953) also reported that the molar ratio of 1:4 of bovine serum albumin to oleic acid produced maximal growth of the Reiter treponeme.

In the absence of albumin in a liquid medium, the growth of *L. pomona* was inhibited by the fatty acids, whereas the reversible binding of these compounds with albumin permitted good growth of this organism. The albumin "buffers" the fatty acids, keeping the concentration of free fatty acid below the bacteriostatic level, while the bound acid constitutes a reserve that replaces the fatty acid utilized by the bacteria (Davis and Dubos, 1947). The short-chain fatty acids, caprylic, capric, lauric, and myristic, supported satisfactory growth at ratios of albumin to fatty acid of 1:1 to 1:4. However, these fatty acids were more inhibitory than the long-chain fatty acids at higher ratios of albumin to fatty acid (1:5 and 1:6). The greater toxicity of these fatty acids may possibly be explained by: (i) the

short-chain fatty acids not being bound as tightly to the albumin as are the long-chain fatty acids (Teresi and Luck, 1952) and (ii) the fatty acids of medium chain length, C_8–C_{14}, having maximal inhibitory activity (Nieman, 1954).

Palmitic, stearic, and oleic acids appear to be the best substrates for the growth of *L. pomona* in that a maximal amount of growth per molecule is obtained. Stearic acid is the least satisfactory of the three because of its increased insolubility, which is manifest in the extended growth period. Vaneseltine and Staples (1961), using an extracted serum medium, found oleic acid to be a growth factor for a strain of *L. pomona*.

Direct utilization of a fatty acid by *L. pomona* was demonstrated by incorporating radioisotopes of palmitic acid in the growth medium and determining the uptake of the label by the cells. It was concluded that the organisms were utilizing the fatty acid and that 1-C^{14} and 2-C^{14} carbon atoms were metabolized differently by the organism, demonstrated by the difference in the amount of the label appearing in the cells and volatile materials. The fatty acids utilized during growth of this organism may be functioning both as energy and carbon sources.

Woratz (1957), using a gelatin-fatty acid medium, reported successful serial transfers of *L. canicola* with palmitic, stearic, and oleic acids but was unsuccessful with short-chain fatty acids. We successfully transferred *L. pomona* serially in the extracted albumin medium containing palmitic acid but had difficulty transferring the culture more than three or four times using lauric or myristic acids.

Diffusion experiments in agar medium (Oyama et al., 1953) were performed to ascertain whether albumin functioned only to detoxify fatty acids. The dense ring of growth that appeared in the first transfer in the absence of albumin indicated that the fatty acid (sole energy and carbon source) was available in adequate and nontoxic concentrations in the area where growth occurred. Evidence for albumin having functions other than the binding of fatty acids is the inability to transfer the organism more than once in its absence. Vaneseltine and Staples (1961) were also unsuccessful in maintaining serial subcultures of *L. pomona* in diffusion experiments in semisolid medium. The powerful binding capacity of albumin for ions and other compounds (Foster, 1960) indicates that it may also function by supplying essential ions or molecules for growth.

The nutritional requirements of leptospirae appear to be relatively simple. A medium containing extracted albumin, fatty acid, NH_4Cl, thiamine, and inorganic salts satisfies the growth requirements of many of the serotypes of pathogenic leptospirae. However, it must be noted that strains of leptospirae employed in this study were adapted to growth in vitro, and may differ considerably in nutritional requirements from the serotypes that are maintained only with great difficulty, if at all, in the usual serum-containing media (Alexander et al., 1962).

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