NUTRITION OF *LEPTOSPIRA POMONA*[1]

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Nutritional and other physiological studies on pathogenic leptospirae have been greatly impeded because animal serum, which is the most important constituent in the culture media employed, is required to produce good growth (Alston and Broom, 1958). Various animal sera can be used in the cultivation of leptospirae, but rabbit serum is one of the most satisfactory (Boyd, 1959). This present report is concerned with the growth-supporting activities of various fractions of rabbit serum and replacement of several of the fractions with better defined material.

MATERIALS AND METHODS

Leptospira pomona, Wickard, was used throughout this study. The organism was maintained in a medium containing 10 per cent pooled rabbit serum (H. F. Pelphrey and Son, P. O. Box 68, Rogers, Arkansas) in phosphate buffer. Cultures were transferred in duplicate every 7 days and a 1 per cent inoculum was used for each subculture. Cultures were incubated in the dark at 30 C. The medium was prepared by adding 2.0 g Na_2HPO_4 and 0.6 g KH_2PO_4 to 1,000 ml distilled water and adjusting the pH to 7.3 to 7.4. The phosphate buffer was dispensed in 9-ml amounts and autoclaved for 20 min at 121 C. One ml of pooled Seitz-filtered rabbit serum was added to the phosphate buffer as it was used.

Whole rabbit serum was fractionated by diluting it with an equal volume of distilled water and adding to the diluted serum that volume of a saturated solution of $(NH_4)_2SO_4$ which would give a final salt concentration of 50 per cent. At this salt concentration the serum globulins were precipitated from solution (Cohn et al., 1940). The supernatant liquid contained the albumin fraction which was precipitated at an $(NH_4)_2SO_4$ concentration of at least 70 per cent. These precipitates were dissolved in distilled water, dialyzed for 48 hr against running tap water, then against several changes of distilled water at 5 C. Dialysis was considered completed when the addition of a few drops of a solution of $BaCl_2$ to the fractions did not produce a precipitate. The ultrafiltrate fraction of rabbit serum was prepared by dialyzing serum against an equal volume of distilled water at 5 C for 3 days. All serum fractions were adjusted to a pH of 7.3 with either phosphoric acid or sodium hydroxide and sterilized either by Seitz filtration or by autoclaving. The protein concentration of the albumin and globulin fractions was determined by the Biuret method (Gornall, Bardawill, and David, 1949).

The following substances were tested as possible substitutes for the various rabbit serum fractions: Celite, charcoal, glycerol, dextrin, Dowex 50, Dowex 1, Amberlite IRC-50 and IR-45, soluble starch, PPLO serum fraction (Difco), crude egg lecithin, and commercial egg, animal, and soybean lecithin. These substances were sterilized by autoclaving except for the PPLO serum fraction which was purchased sterile.

Egg lecithin was prepared by the method described by Macfarlane and Knight (1941). Crude phospholipids (ether soluble-acetone insoluble) were extracted from lyophilized rabbit serum using acetone and ether. The crude phospholipid material was emulsified in 0.02 M phosphate buffer.

The resins were prepared by placing a quantity of the resin in a glass column and percolating 0.02 M phosphate buffer, pH 7.3, through it at a slow rate until the pH of the effluent buffer remained at 7.3. This usually takes from 3 to 4 days. When the resin was to be used, the phosphate buffer was drained from it and the resin was dried by spreading it in a thin layer on filter paper. It was found that it was best to determine the optimal concentration for growth supporting activity of each batch of resin processed. In the medium in which the albumin was replaced by the resin the best growth was obtained when the medium was dispensed in flasks to a depth of about 1 cm.

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Bacterial counts were made in a Petroff-Hausser counting chamber using dark field illumination and a magnification of 450×. Twenty counts of preparations from one sample showed that the average variation from the mean was 3.7 per cent as calculated by the coefficient of variability. The counts expressed in the paper are the average of single counts made from duplicate tubes. The size of the inoculum used throughout the nutritional studies was 1 per cent of a 5- to 7-day-old culture of *L. pomona* grown in the serum-phosphate medium. This represented from 1 to 2 × 10^6 cells per ml of final culture medium.

TABLE 1

Growth supporting activity of rabbit serum fractions for Leptospira pomona

Rabbit Serum Fraction Added to Basal Medium	No. Organisms per ml*
Basal medium†	$<10^5$
Rabbit serum	28.0×10^7
Globulin	$<10^5$
Ultrafiltrate	$<10^5$
Globulin + ultrafiltrate	$<10^5$
Albumin	4.1×10^7
+ Globulin	6.7×10^7
+ Ultrafiltrate	5.0×10^7
+ Globulin + ultrafiltrate	13.0×10^7

* Inoculum was 1 to 2 × 10^6 per ml; incubation at 30 C for 7 days; standard deviation ± 0.27 × 10^7.

† Basal medium is 0.02 M phosphate buffer.

RESULTS

The function of rabbit serum in the nutrition of *L. pomona* was investigated by dividing the whole rabbit serum into albumin, globulin, and whole serum ultrafiltrate, and testing each fraction and various combinations of the fractions for their growth-supporting capacity. The results (table 1) show that the globulin and ultrafiltrate fractions did not support growth separately or in combination with each other. The albumin fraction could support some growth. When the globulin or serum ultrafiltrate fractions were added to the albumin fraction they had a stimulatory effect on the growth of *L. pomona*, which they did not have in the absence of the albumin fraction. When the albumin, globulin, and ultrafiltrate fractions of rabbit serum were combined together good growth was obtained. The concentration of the albumin used could be varied greatly and still support good growth, whereas the best concentrations for the globulin and ultrafiltrate fractions were found to be within a much narrower range. The final concentration of these fractions in the medium calculated from their protein concentration was 0.4 to 0.5 per cent for albumin and 0.15 to 0.25 per cent for globulin. One ml of the ultrafiltrate fraction was used in 10 ml of medium.

Whole rabbit serum, when heated to 100 C for less than 1 min, no longer supports the growth of leptospirae (Chang, 1947). To extend this observation, the heat stability of the three fractions of rabbit serum was investigated. The criterion used

TABLE 2

*Heat stability of the various fractions of rabbit serum which support the growth of Leptospira pomona**

Rabbit Serum Fraction Added to Basal Medium	No. Organisms per ml†
Basal medium‡	$<10^5$
Rabbit serum (untreated)	27.0×10^7
Rabbit serum (treated)	$<10^5$
Albumin (untreated)	5.2×10^7
Albumin (treated)	$<10^5$
Albumin (untreated) + globulin + ultrafiltrate (untreated)	13.0×10^7
Albumin (treated) + globulin + ultrafiltrate (untreated)	$<10^5$
Albumin (untreated) + globulin + ultrafiltrate (treated)	13.6×10^7
Albumin (untreated) + globulin + ultrafiltrate (dried at 100 C)	12.0×10^7
Albumin (untreated) + globulin + ultrafiltrate (ashed)	4.4×10^7

* Treated = 121 C for 20 min.

† Inoculum was 1 to 2 × 10^6 per ml; incubation at 30 C for 7 days; standard deviation ±0.27 × 10^7.

‡ Basal medium is 0.02 M phosphate buffer.

for heat stability was exposure to 121 C for 20 min. This time and temperature was used for we are attempting to develop a medium which can be autoclaved. As would be expected (table 2), whole rabbit serum no longer supported growth when subjected to this heat treatment. The rabbit serum albumin was found to be heat labile and no longer supported growth after being autoclaved. The globulin fraction and the serum ultrafiltrate were found to be heat stabile. They retained their growth-supporting capacity after either being autoclaved or being evaporated to dryness. However, their activity was destroyed upon ashing.

An attempt was made to replace the albumin fraction of rabbit serum. It was found (table 3) that 4 per cent PPLO serum fraction (Difco), which is a fraction of bovine serum, would replace the rabbit serum albumin fraction. Again the need for all three serum fractions was demonstrated. The amount of growth obtained with PPLO serum fraction plus the rabbit serum globulin and ultrafiltrate fractions was similar to that obtained when all three rabbit serum fractions were used. Since the rabbit serum albumin fraction could be replaced with PPLO serum fraction, it indicated that the role of the albumin fraction was probably of a nonspecific type.

Because the possibility existed that the albumin fraction acted primarily as an adsorption agent, substances of known adsorptive powers were tested (table 4). These were Celite, charcoal, glycerol, dextrin, soluble starch, Dowex 50,

TABLE 3

Replacement of rabbit serum albumin with PPLO serum fraction and its effect on the growth of Leptospira pomona

Serum Fraction Added to Basal Medium	No. Organisms per ml*
Basal medium†	$<10^5$
Rabbit serum	21.0×10^7
Rabbit albumin + globulin + ultrafiltrate	13.5×10^7
PPLO serum fraction (4%)	3.2×10^7
+ Globulin (rabbit)	9.1×10^7
+ Ultrafiltrate (rabbit)	6.1×10^7
+ Globulin + ultrafiltrate (rabbit)	12.0×10^7

* Inoculum 1 to 2×10^6 per ml; incubation was at 30 C for 7 days; standard deviation $\pm 0.27 \times 10^7$.

† Basal medium is 0.02 M phosphate buffer.

TABLE 4

Replacement of rabbit serum albumin fraction with various substances and their effect on the growth of Leptospira pomona

Sample Tested When Added to Basal Medium* + Globulin + Ultrafiltrate	No. Organisms per ml†
Celite, 2%	$<10^5$
Charcoal, 4%	$<10^5$
Glycerol, 6%	$<10^5$
Dextrin, 0.02–0.2%	$<10^5$
Soluble starch, 0.1%	10.3×10^7
Dowex 50, 2%	$<10^5$
Dowex 1, 2%	$<10^5$
Amberlite IRC-50, 2%	$<10^5$
Amberlite IR-45, 2%	10.0×10^7
Amberlite IR-45, 2% without globulin + ultrafiltrate	$<10^5$
Rabbit albumin	12.0×10^7

* Basal medium is 0.02 M phosphate buffer.

† Inoculum 1 to 2×10^6 per ml; incubation was at 30 C for 7 days; standard deviation $\pm 0.27 \times 10^7$.

Dowex 1, Amberlite IRC-50, and Amberlite IR-45. The weakly basic resin, Amberlite IR-45 and soluble starch were the only substances of those tested which could satisfactorily replace the albumin fraction of rabbit serum. These substances without the rabbit serum globulin and ultrafiltrate fractions did not support growth.

A replacement for the globulin was investigated next. To study the function of the globulin fraction, it was further divided into two parts, a crude phospholipid (ether soluble, acetone insoluble) fraction and the protein portion of the globulin from which some of the lipid material had been removed. These two fractions were then tested separately and in combination with each other for their growth-supporting activity (table 5). Some of the growth-supporting capacity of the globulin fraction was removed upon extraction with organic solvents. It was found that this loss of activity was restored when the extracted crude phospholipid material was added to it. The crude phospholipid extract did support limited growth of *L. pomona* in the absence of the extracted globulin.

The data from the globulin extraction experiment indicated that the crude phospholipid material (ether soluble, acetone insoluble) was important in the nutrition of *L. pomona*. The crude lecithin fraction of egg yolk was next tested (table 6) as a replacement for the globulin fraction of

rabbit serum. This ether soluble, acetone insoluble fraction of egg yolk, when substituted for the globulin fraction of rabbit serum, supported good growth. The presence of both the resin and serum ultrafiltrate were required to give maximal growth. The concentration of the crude egg phospholipid material which produced maximal growth was 0.05 mg per ml of media. However, it has been found that the growth-supporting activity of the crude phospholipid material can vary greatly between different batches of eggs. Commercially prepared egg, animal, and soybean lecithin were substituted for crude egg phospholipid material without success.

DISCUSSION

The three fractions of rabbit serum, albumin, globulin, and whole serum ultrafiltrate, supported good growth of *L. pomona* when combined. The globulin and ultrafiltrate fractions did not support growth when tested separately, whereas the albumin fraction did support fair growth. The rabbit serum albumin fraction was the only heat labile fraction of the serum. Its growth-supporting activity was destroyed when it was exposed to a temperature of 121 C for 20 min. The exact temperature and time period required to inactivate this fraction was not investigated. The globulin and serum ultrafiltrate retained their activity after either autoclaving or being evaporated to dryness. Ashing did inactivate these two fractions.

The albumin fraction of rabbit serum can be successfully replaced with either a weakly basic resin (Amberlite IR-45) or soluble starch. The function of the albumin fraction is probably at least threefold, a source of amino acids (Fulton and Spooner, 1956; Gerhardt and Ball, 1959), a source of lipid material, and an adsorption agent. Helprin and Hiatt (1957) found that serum protein supplies and detoxifies fatty acids which stimulate the respiration of *Leptospira icterohemorrhagiae*. The primary function of albumin may be that of absorption to keep a substrate at a nontoxic level or to remove a metabolic product, since it could be replaced by a resin which by itself would not add any nutrients to the medium. It is difficult to assess the function of the weakly basic resin but it may be that it was adsorbing some negatively charged compounds such as fatty acids.

The globulin fraction of rabbit serum appears to function primarily as a source of lipid material, since if extracted with ether and acetone some of its growth supporting activity was lost. It was found that this lost activity was restored when the extracted crude phospholipid material was added to the extracted globulin. The extracted

TABLE 5

Acetone-ether extraction of rabbit serum globulin and its effect on the growth of Leptospira pomona

Sample Tested When Added to Basal Medium + 2% Amberlite IR-45 + Ultrafiltrate	No. Organisms per ml*
Basal medium†	$<10^5$
Rabbit serum‡	26×10^7
Globulin (unextracted)	13.8×10^7
Globulin (extracted)	8.5×10^7
Globulin (extracted) + extract	12.4×10^7
Extract	1.6×10^7

* Inoculum 1 to 2×10^6 per ml; incubation was at 30 C for 7 days; standard deviation $\pm 0.27 \times 10^7$.
† Basal medium is 0.02 M phosphate buffer.
‡ In basal medium only.

TABLE 6

Effect of crude egg lecithin on the growth of Leptospira pomona

Sample Tested When Added to Basal Medium	No. Organisms per ml*
Basal medium†	$<10^5$
Rabbit serum	24×10^7
Amberlite IR-45 (1%) + globulin + ultrafiltrate	10.2×10^7
Amberlite IR-45 (1%) + egg lecithin + ultrafiltrate	8.8×10^7
Amberlite IR-45 (1%) + egg lecithin (no ultrafiltrate)	4.2×10^7
Egg lecithin + ultrafiltrate (no Amberlite IR-45)	0.3×10^7

* Inoculum 1 to 2×10^6 per ml; incubation was at 30 C for 7 days; standard deviation $\pm 0.27 \times 10^7$.
† Basal medium is 0.02 M phosphate buffer.

crude phospholipid material did support limited growth of *L. pomona* in the absence of the extracted globulin. It is interesting that an egg yolk opacity producing factor (Johnson, 1958), found in the culture supernatant of leptospirae, caused a rapid production of turbidity when added to a solution of rabbit globulin, whereas it had little effect on rabbit albumin. This egg yolk factor may release the lipid material bound in the globulin fraction thus making it available for the growth of leptospirae. Kemenes and Lovrekovich (1959) found an agent which can loosen a thin layer of solid fat from a glass surface in some cultures of leptospirae. It is thought that this fat splitting enzyme is the same as the egg yolk opacity-producing agent, the only difference being in the sensitivity of the two test systems. The egg yolk test appears to be more sensitive since activity was found to be present in leptospiral cultures and under conditions which were negative according to Kemenes and Lovrekovich (1959).

Mifuchi and Kawata (1953) claimed that the addition of egg lecithin to Korthof's medium in place of serum would support the growth of *L. icterohemorrhagiae*. We found that the weakly basic resin (Amberlite IR-45) and the rabbit serum ultrafiltrate were required in addition to the egg phospholipid in order to obtain good growth. Fulton and Spooner (1956) found that the crude phospholipid fraction of rabbit serum and of egg yolk was active in stimulating the respiration of *L. icterohemorrhagiae*.

It has been possible to carry our *L. pomona*, Wickard, culture through serial transfers in the rabbit albumin, autoclaved globulin, and serum ultrafiltrate medium, and also in a medium containing soluble starch, rabbit serum globulin, ultrafiltrate, and red cell lysate. In the latter medium all components were autoclaved except the red cell lysate. Faine (1959) reported that hemoglobin may be a source of iron, an essential nutrient for leptospirae. In the medium containing albumin, presumably enough red cell lysate was present in the albumin to satisfy the requirements of the organism. Decreased growth was obtained in the starch medium when red cell lysate was omitted.

SUMMARY

Rabbit serum was divided into three fractions, albumin, globulin, and whole serum ultrafiltrate. When these fractions were tested separately the albumin fraction was the only one of the three that supported fair growth of *Leptospira pomona*. All three fractions were required to yield good growth. The albumin fraction was found to be heat labile (121 C, 20 min), whereas the globulin and ultrafiltrate fractions were heat stabile. They maintained their activity after either being autoclaved or evaporated to dryness. However, their activity was destroyed upon ashing. The albumin fraction was successfully replaced with the weakly basic resin, Amberlite IR-45, and soluble starch. The globulin fraction of rabbit serum appears to function primarily as a source of lipid material. Extraction of the crude phospholipid material from the globulin fraction decreases its growth-supporting activity. This lost activity was restored when the extracted material was added to the extracted globulin. Fresh crude egg yolk phospholipid material successfully replaced the globulin fraction of rabbit serum.

An *L. pomona* culture has been carried through serial transfers in the rabbit albumin, autoclaved globulin, and serum ultrafiltrate medium, and also in medium containing soluble starch, rabbit serum globulin, ultrafiltrate, and red cell lysate. In the latter medium all components were autoclaved except the red cell lysate.

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