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Nutrition of *Leptospira canicola*. II. A Chemically Defined Basal Medium Containing Purified Rabbit Serum Albumin.* (19215)

ALLAN SCHNEIDERMAN, MERIDIAN R. GREENE,† LEROY SCHIELER, LAWRENCE E. MCCLURE, AND MAX S. DUNN.

From the Departments of Bacteriology and Chemistry, University of California, Los Angeles.

It was shown by Greene *et al.*(1) that *Leptospira canicola* could be grown in a chemically-defined medium of amino acids, minerals, purines, pyrimidines and vitamins supplemented with dialyzed rabbit serum. Attempts to fractionate the dialysed serum by ammonium sulfate precipitation resulted in about 80% loss of the activity and the remaining activity was not concentrated in any fraction. In the present investigation an active fraction obtained by a modified procedure has been found to replace dialysed rabbit serum.

Experimental. The chemically-defined basal medium was prepared by introducing 0.5 ml each of solution A (36 mg % of each of the 19 amino acids employed previously)(1), solution B (vitamins, purines and pyrimidines in the concentrations given previously)(1) and solution S (autoclaved and filtered aqueous solution of the following salts at mg per cent concentrations given in the parentheses: Na_2HPO_4 (312), KH_2PO_4 (420), NaCl (1280), Na_2CO_3 (32), KCl (32), $CaCl_2$ (32)) into each 15 x 127 mm test tube. The tubes were autoclaved for 30 minutes at 15 lb pressure, 0.5 ml of solution G (36 mg % filter-sterilized aqueous solution of L-glutamine) and a selected volume of test material were added to each tube, each tube was inoculated with a culture of the test organism and each solution was diluted to 6 ml. The pH of the medium was 7.2-7.4. The transfer medium was prepared by adding 0.8 ml of filter-sterilized, dialysed rabbit serum‡ to each tube containing an autoclaved mixture of 0.5 ml of solution S, 3.96 mg of Witte's peptone and 4.3 ml of water. *Leptospira canicola*§ was transferred weekly by inoculating this medium with 0.4 ml of a 7-day culture of the spirochete and incubating the mixture for 7 days at 32°. The inoculum was prepared by centrifuging (2000 RPM for ½ hr) the 7-day growth, suspending the cells in a quantity of solution S sufficient to produce a turbidity of 30-40 de-

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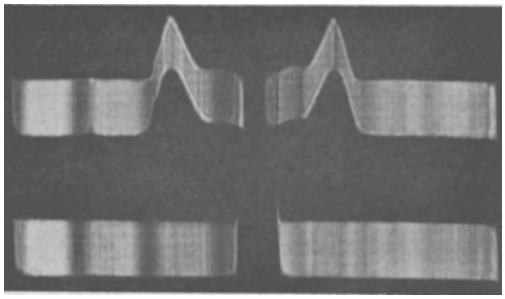
† Mrs. Gordon H. Ball.

termined with a Klett-Summerson photoelectric colorimeter equipped with a No. 42 blue filter. 0.4 ml of this suspension was added to each tube in subsequent experiments. Photometric readings were made at the time of inoculation and after 7 days incubation at 32° to determine the effect of test materials. The whole rabbit serum was fractionated in preliminary experiments by mixing varying amounts of solid ammonium sulfate with 5.0 ml aliquots of the serum and allowing the mixtures to stand overnight in the refrigerator. The suspensions were centrifuged, the supernatant liquids were decanted and each precipitate was suspended in an ammonium sulfate solution of the same concentration as that employed to obtain the precipitate. Each suspension was centrifuged, the sediment was dissolved in 0.85% saline solution and the mixture was diluted to 4.0 ml. Each supernatant liquid was diluted to 4.0 ml. Each sample (suspension or supernatant liquid) was dialysed against 0.85% saline to remove excess ammonium sulfate, and each dialysed sample was diluted to 5.0 ml. Precipitation of globulins was prevented by this treatment.

In larger-scale fractionations 225 ml of dialysed rabbit serum was dialysed (with intermittent stirring) against a solution of ammonium sulfate of a concentration such that at equilibrium a 42.5% saturated solution resulted. This slow (7 days) precipitation produced relatively large aggregates which could be separated conveniently by filtration. The suspension was filtered and the precipitate was washed with 25 ml of cold 42.5% ammonium sulfate solution. The filtrate (containing the active material) was dialysed for 7 days, while changing the water twice daily, to remove excess salts. The small amount of precipitate was removed by filtration and the filtrate was dialysed for 7 days against a solution of ammonium sulfate of a concentration such that at equilibrium a 71% saturated solution resulted. The suspension was filtered and the precipitate (containing the active material) was washed with 50 ml of cold 71% saturated solution of ammonium sulfate. The precipitate was dissolved in



Ascending Descending

FIG. 1. Electrophoretic patterns of ascending and descending boundaries of rabbit serum albumin. The pH was 8.41 using barbitol buffer and the ionic strength .1. The determination was made over about 6 hr at 200 volts and 4.5 milliamperes. The authors are indebted to Dr. Sidney Roberts, U.C.L.A. Medical School, for these determinations.

TABLE 1. Response of *Leptospira canicola* to Rabbit Serum Fractions.*

% ammonium sulfate saturation	Increase in optical density	
	Filtrate	Precipitate
14.2	31.6	‡
25.8	24.4	.0
42.5	23	.0
56.9	7.8	13.6
71 †	.0	17.6
Control (dialysed serum)	35	30
" (whole serum)	22.5	36.3

* 10% by volume of test material prepared in preliminary experiments was added to the chemically-defined basal medium. Filtrates and precipitates were assayed at different times.

† At higher concentrations the precipitate in the resulting viscous suspensions could not be separated by filtration or centrifugation.

‡ No precipitate formed.

‡ The authors are indebted to Mr. C. D. Boone (Puente, Calif.) for rabbit blood, Dr. E. J. Cohn for the crystalline human plasma albumin, the Lederle Laboratories, Pearl River, N. Y., for the crystalline sheep and crystalline bovine albumin, No. 2, and the Hyland Laboratories (Los Angeles, Calif.) for filtering and bottling the rabbit serum. For this purpose 100 ml aliquots of serum were placed in cellulose sausage casings (The Visking Corp. size 36 32) and the latter were suspended in 5-gal. bottles of distilled water. The bottles were allowed to stand in the refrigerator for 10 days during which the water was changed twice daily. The resulting dialysed serum was filter-sterilized and stored in sterile bottles in the refrigerator. The dialysed serum was added to media in amounts equivalent to a concentration of 10% whole serum.

§Strain No. 18 (Hond Utrecht IV) and *Leptospira icterohemorrhagiae*, Strain No. 20 (Wiznberg) were obtained through the courtesy of Dr. K. F. Meyer, University of California, San Francisco.

TABLE II. Response of *Leptospira canicola* to Rabbit Serum Albumin.

Albumin		Albumin	
Preparation No. 1,* ml/tube	Activity†	Preparation No. 2,‡ ml/tube	Activity†
.0	1	.0	0
.5	3	.2	8
1	6	.4	16
1.5	15	.6	33
1.7	19	.8	36
2	24	1	40
2.5	37	1.5	39
2.7	37		
3	38		
Control (dialysed serum)	34	Control (dialysed serum)	35

* Larger-scale preparation containing 2.75 mg N/ml.

† Optical density, each value average of 5 determinations.

‡ 6.9 mg N/ml.

water and reprecipitated twice in the same manner. Other preparations from as much as 2 liters of dialysed serum were made by analogous methods.

Results and discussion. The most active fraction of serum albumin was demonstrated to be electrophoretically homogeneous as shown by the patterns of the ascending and descending boundaries (Fig. 1).

As shown in Table I the growth of *Leptospira canicola* on the described chemically-defined basal medium with added filtrate decreased and with added precipitate increased with increasing concentrations of ammonium sulfate employed to fractionate proteins in the dialysed rabbit serum. According to the data in Table II growth of the spirochete increased with increasing concentration of the albumin fraction precipitated at 71% ammonium sulfate saturation. As indicated in Table III full growth of the organism was not maintained in serial subcultures on the chemically-defined medium lacking the amino acids (solutions A and G) but supplemented with the albumin. Since it was determined that 2.5 ml of the albumin fraction (Preparation 1) containing 6.9 mg of nitrogen was as effective in promoting growth of the spirochete as 0.8 ml of the dialysed rabbit serum (equivalent to 0.6 ml of the whole serum) containing 4.8 mg of albumin nitrogen, there was an approximate correlation between the activity of the albumin in the dialysed serum and its most active fraction isolated by ammonium sulfate precipitation. Although the active fraction may have been partially denatured the lack of exact equivalence in activity of these albumins may be explained, at least in part, if the estimate of nitrogen in the albumin of the dialysed serum were incorrect. In making this estimate it was assumed that the serum contained 5% albumin and the latter 16% nitrogen.

The activities toward *Leptospira canicola* of serum albumins obtained from bovine, horse, human and sheep bloods are tabulated

TABLE III. Response of *Leptospira canicola* Rabbit Serum Albumin in Different Media.

Group		Increase in optical density*						
		Initial culture	Subculture 1	2	3	4	5	6	7
A Albumin†	C-D M	31	33	26	27				
Control‡	C-D M	33	33	38	34				
B Albumin§	C-D M	32	28	25	10	26	29	30	27
	D-W	45	22	18	0				
	(C-D M)-(AA)	49	40	23	18	23	23	14	3
Control‡	S-B	34	34	29	33	35	31	31	30
C Albumin§	C-D M	19	21	35	39	30	32	39	
	S-B	33	41	46	47	44	41	47	
Control‖	S-B	22	31	43	26	36	36		

* Each value is the average of 2 determinations in Group A, 4 in Group B, and 6 in Group C. The groups were not run simultaneously.

† 2.5 ml (2.75 mg N/ml) of preparation No. 1 used per tube.

‡ Dialysed rabbit serum.

§ .8 ml (6.9 mg N/ml) of preparation No. 2 used per tube.

‖ Whole rabbit serum (different lots).

C-D M, chemically-defined medium; D-W, distilled water; S-B, stock base (same as transfer medium).

TABLE IV. Response of *Leptospira canicola* to Different Serum Albumins.

Albumin	Activity**
1. Crystalline bovine, No. 1*	0
2. human†	0
3. Control (dialysed rabbit serum)	36
4. Crystalline bovine, No. 2‡	10
5. sheep‡	12
6. Salt-precipitated sheep§	30
7. bovine§	9
8. horse	45
9. rabbit§¶	24
10. Control (dialysed rabbit serum)	33

* Alcohol-fractionated product obtained from Armour Laboratories, Chicago, Ill.

† Alcohol fractionated product obtained through the courtesy of Dr. E. J. Cohn, Harvard University.

‡ Alcohol fractionated product obtained through the courtesy of Lederle Laboratories, Pearl River, N. Y.

§ Prepared by the authors from whole blood obtained from the Los Angeles Wassermann Supply Co., Los Angeles.

Prepared by the authors from whole blood obtained from the Victory Slaughtering Co., Los Angeles.

¶ 1 ml (4.98 mg N/ml) used per tube.

** Increase in optical density (each value average of 5 determinations) after 7-days incubation at 32°. The chemically-defined basal medium was used with samples 1-3 and the stock medium supplemented with peptone and minerals with samples 3-8.

in Table IV. Generally, the crystalline and the alcohol-precipitated albumins lacked, or had low, activity while the salt-precipitated albumin fractions (except bovine) exhibited activity nearly comparable to that of the control (dialysed rabbit serum). Savino and Rennella(2) reported that hematin was a growth factor for *L. icterohemorrhagiae* but it seems unlikely that the growth effects observed in the present experiments are accounted for by heme compounds. It may be noted that traces of heme compounds were present in the inactive, as well as the active, fractions of the rabbit and horse sera.

In further experiments it was found that salt-precipitated horse or sheep albumin could substitute satisfactorily for dialysed rabbit serum or salt-precipitated rabbit albumin in supplementing a basal medium containing only peptone and salts. Subculturing of *L. canicola* was not attempted although it was determined that *L. icterohemorrhagiae* could be progagated satisfactorily by transferring it weekly over a 3-month period in the peptone-salts-rabbit serum albumin medium. It is of interest that Chang(3) was unable to maintain *L. icterohemorrhagiae* in a medium in which horse serum was replaced by crystalline bovine serum albumin.

Summary. *Leptospira canicola* has been cultivated satisfactorily on a chemically-defined medium supplemented with electrophoretically-homogeneous rabbit albumin separated from serum by ammonium sulfate fractionation. The results of experiments with crystalline bovine and human serum albumins and with salt-precipitated albumin fractions from bovine, horse and sheep sera have been presented.

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Rate of Potassium Exchange in the Rat Erythrocyte.* (19216)

JOHN M. WELLER† AND ISAAC M. TAYLOR.† (Introduced by A. Baird Hastings.)

From the Department of Biological Chemistry, Harvard Medical School, Boston.

Previous studies have shown that the human erythrocyte utilizes nearly a millimole of glucose for every millimole of potassium that enters the cell(1). The following experiments were undertaken to determine if a similar relationship existed in the metabolism of the rat erythrocyte.

Mature white rats of the Wistar strain were

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