to ureteral excretion, the first question may be answered in the affirmative.

The method of preparation insures that the India ink observed in the collecting ducts has actually been present there *in vivo*. This was further confirmed by examining a number of sections from the same kidney. But it is quite clear from the microscopic examination that only some collecting ducts were filled. Since a number of injections of hormone were given to all rats before the injection of India ink, it is assumed that the hormone-containing solution, at least during some of the injections, penetrated as far up into the papilla as did the India ink. For these reasons it would seem safe to conclude that the hormone does not have any marked effect from the luminal side of the collecting ducts in the papilla of the rat, since such an effect would have caused an increased osmolality of the urine.

Summary. Unilateral, retrograde injection of arginine-vasopressin into the ureter of the hydrated rat did not result in a detectable increase in urine osmolality. The degree of penetration into the tubule was controlled by injection of an India ink solution and later histologic examination. Although only some collecting ducts could be assumed to be filled with hormone-containing solution the finding suggests that the hormone has no marked effect from the luminal side of the collecting ducts in the papilla of the rat.

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A Hemolytic Factor Associated with *Leptospira pomona* Cells.† (29705)

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A hemolysin associated with the culture supernatant fluids of leptospirae has been reported by several investigators(1,2,3,4). However, these workers reported very little hemolytic activity associated with the leptospiral cell. Valentine and Morter(5) reported considerable hemolytic activity associated with the cells of some isolates of *Leptospira pomona*. The partial purification and preliminary characterization of this hemolytic factor is the subject of this report.

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Materials and methods. The sources of the 19 isolates of *L. pomona* used in this study are given in Table I. Evaluation of the hemolytic factor of the cells and culture supernatant fluids of all isolates were undertaken and further studies were made of cellular hemolytic factor of strain P-7, the hamster lethal Wickard strain(6).

The *L. pomona* isolates were cultivated in Chang's medium(7) for 10 days at 28°C in Roux bottles. Each bottle was accepted for hemolysin evaluation if found free of contamination by test with fluid thioglycollate medium, tryptose broth in Castaneda flasks and blood agar plates (5% bovine blood).

L. pomona cells were concentrated by cen-

TABLE I. Hemolytic Activity of Growth Supernates *vs* Sonicated Cell Preparation and Kjeldahl Nitrogen of Sonicated Cell Preparations.

Isolate	Source	Supernatant fraction, hemolytic units	SCP	
			Hemolytic units*	Nitrogen†
P- 1	C‡	18	24	4.95
P- 2	C	192	92	5.09
P- 3	C	36	16	5.16
P- 4	D	200	52	5.36
P- 5	S	400	116	5.03
P- 6	H	0	8	5.16
P- 7	C	600	300	4.48
P- 8	C	600	300	4.95
P- 9	C	200	20	4.89
P-10	C	2	1	4.82
P-11	S	200	100	5.03
P-12	S	200	100	4.02
P-13	C	600	350	5.29
P-14	U	4	2	4.82
P-15	O	20	14	4.66
P-16	C	400	150	5.23
P-17	C	74	34	4.62
P-18	S	180	6	4.30
P-19	C	26	0	3.90

* Units of hemolytic activity expressed on adjusted total volume of 1 l for each preparation and are expressed per ml.

† mg of cellular nitrogen/l of culture.

‡ C, cattle; D, dog; S, swine; H, human being; O, sheep; U, unknown.

trifuging at a RCF = 34,800 *g* at 4°C. The packed cells were washed 3 times by suspending them in 20 times their packed volume of an isotonic phosphate buffer of pH 7.0 (4.6 g $NaH_2PO_4 \cdot H_2O$, 17.3 g $Na_2HPO_4 \cdot 7H_2O$ in 1 liter of distilled water) and centrifuging at a RCF = 34,800 *g* for 10 minutes. The washed cells were then resuspended in phosphate buffer to 1% of the original culture volume. The washed cells were disrupted by sonic vibration at 10 kc/sec for 10 minutes. Nitrogen determinations were carried out on each batch of the sonicated cell preparation (SCP) by the standard Kjeldahl procedure(8).

For purification a SCP was centrifuged for 1 hour at RFC = 34,800 *g* at 4°C. The resulting supernate, containing a large portion of the hemolytic activity, was used for the fractional precipitation. The precipitation was carried out in an ethylene glycol cold bath by adding cold (1°C) acetone dropwise to 100 ml of the supernatant (1°C) at a rate of 0.5 ml every 5 minutes, and after a lapse of 5 minutes for equilibration 0.5 ml more was added. As each level of the acetone was added, the temperature of the cold bath was gradually lowered from 1°C to −15°C. When the desired concentration of the precipitating agent was reached the mixture was allowed to remain in the cold bath for 30 minutes and then centrifuged at RCF = 34,000 *g* for 10 minutes at −10°C. The supernate was returned to the cold bath and the next level of precipitating agent was added. Acetone was added stepwise to concentrations of 20, 25, 30 and 35%. Each precipitate was dried under a vacuum and then resuspended to 25% of the original volume in phosphate buffer.

The hemolytic activity of the culture supernatant fluids was concentrated by precipitation with $(NH_4)_2SO_4$ to 40% saturation. After centrifugation at RCF = 4,000 *g* the precipitate was dissolved in phosphate buffer and then dialyzed at 4°C against tap water and then 0.85% saline. The dialysate was checked for complete removal of the $(NH_4)_2SO_4$ by qualitative testing with a 10% solution of barium chloride, concentrated by dialysis against 15% polyvinylpyrrolidone and adjusted to 10% of its original volume with phosphate buffer.

Protein of the precipitated fractions was determined by the biuret method(8) and presence of carbohydrates was tested for by the iodine test and by the reduction test using Benedict's solution(9).

Assays for hemolytic activity were accomplished by making 1:10, 1:100 and 1:1000 master dilutions of the hemolytic factor in phosphate buffer. Aliquots of the master dilutions were added to portions of saline in the assay tubes, to which 1.5 ml of 2% sheep erythrocytes(10) were added. The tubes containing the dilutions were then incubated for 30 minutes at 37°C and 1 hour at 4°C, centrifuged at RCF = 1,000 *g* for 5 minutes, decanted, and the optical density (O.D.) of the supernatant read at 540 mu.

Units of hemolytic activity were then determined from a standard curve, which was constructed by making a series of dilutions from 1.5:1 to 1:16 in 0.85% saline of a completely hemolyzed 2% sheep erythrocyte suspension (3 ml water + 1.5 ml of 2%

TABLE II. Purification of Hemolytic Factor of Soluble Portion of the SCP by Acetone Precipitation.

Material	Hemolytic activity, units/ml	Protein, mg/ml	Units/mg of protein	Index of purification
Total SCP	640	32.00	20	1.0
Phosphate supernate (soluble fraction)	160	1.00	160	8.0
0-20%	0	—	—	—
20-25	35	.21	170	8.5
25-30	72	.25	300	15.0
30-35	0	—	—	—

sheep erythrocytes). The O.D. of these dilutions was read on a spectrophotometer at 540 mu. One unit of hemolytic activity was arbitrarily defined as that amount of hemolysin which would lyse sufficient erythrocytes of the standardized solution to bring about an O.D. reading of 0.915. The standard curve was made by plotting O.D. readings against units.

Electrophoretic mobilities of the precipitates were determined on paper at buffer pH values ranging from 2.0 through 12.1. One tenth ionic strength buffers were used(11) at a constant current of 3 ma for 18 hours.

Results. Quantitative determinations of the cellular nitrogen from the growth obtained per liter of culture ranged from 3.90 to 5.36 mg of nitrogen (Table I). The average cellular nitrogen of 28 batches of *L. pomona* (all 19 isolates) was 4.83 mg per liter.

Hemolytic activity of the growth supernatant fluid is compared in Table I to the activity of the SCP prepared from the cells of that strain. The hemolytic units of the SCP or supernatant fraction were arithmetically adjusted to be the activity obtained from 1 liter of medium. Hemolytic activity ranged from 0 to 600 units in the supernatant fluids and from 0 to 350 units in the SCP's of the 19 isolates. Those *L. pomona* isolates having large amounts of hemolytic activity associated with the supernate, had in general large amounts of activity associated with the SCP. The hemolytic activity of the SCP of 2 strains, P-1 and P-6, was greater than that of the supernate.

Activity per mg of protein was increased from 20 units per mg of protein in the *L. pomona* (P-7) SCP to 300 units per mg of protein in the 25-30% acetone fraction (Table II). No activity was found in the 0-20% acetone fraction, 22% of the original activity of the SCP supernatant was found in the 20-25% fraction and 45% in the 25-30% fraction. Qualitative tests for carbohydrates were negative.

Electrophoretic mobility of the 25-30% acetone fraction could not be demonstrated at pH 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 11.7.

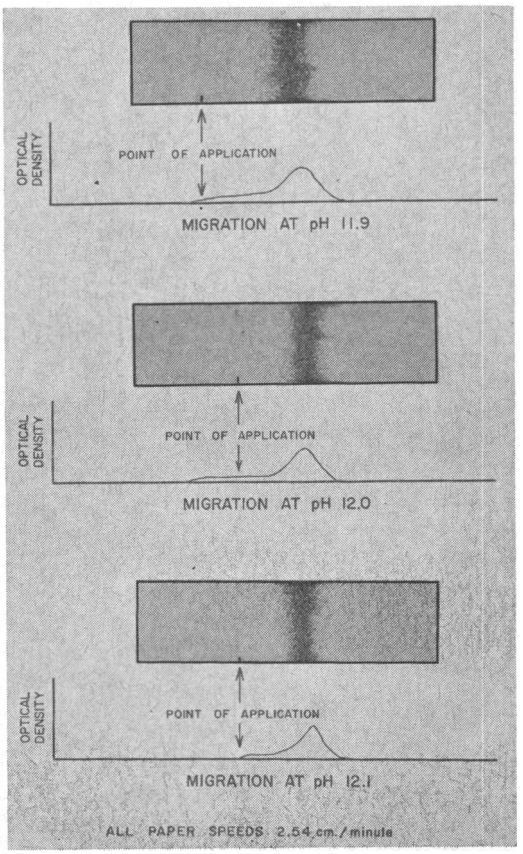

FIG. 1. Electrophoretic migration of 25-30% acetone fraction at pH 11.9, 12.0 and 12.1.

Migration was obtained at pH 11.9, 12.0 and 12.1 as a single moiety (Fig. 1).

Discussion. Evidence presented here shows that some isolates of *L. pomona* have considerable amounts of hemolytic activity associated with their cells. In general, the most activity was associated with the culture supernatant fluids; however, the amount of activity contained within the cells of many of the isolates cannot be considered as insignificant.

There is no reason to believe that the hemolytic factor of the cells is a different substance from the hemolysin of the growth supernatant fluids. However, further characterization of the cellular factor is needed in order to ally the 2 factors more closely.

Assuming the 2 factors to be identical, discovery of appreciable hemolytic activity associated with *L. pomona* cells offers an excellent source of the hemolytic factor for characterization and further purification. The SCP is not grossly contaminated by rabbit serum, tryptose and liver extract of the medium that are present in the supernatant fluids.

Summary. Considerable hemolytic activity has been found to be associated with the cells of some isolates of *L. pomona*. Purification of the hemolytic factor by acetone precipitation of a phosphate buffer soluble portion of the sonicated *L. pomona* cells increased the activity per mg of protein 15 times. The 25-30% acetone fraction was found to migrate as a single moiety using paper electrophoresis.

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Continuous Anaerobiosis for Cultivation of Spirochetes.* (29706)

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The anaerobic spirochetes, among which are numbered the causative agents of syphilis and other treponematoses, and "amphibionts" (1) indigenous to man and many animals, remain poorly understood principally because of difficulties of *in vitro* cultivation. No practicable means to grow authentic, virulent *Treponema pallidum* exists today apart from the living animal. The amphibionts, and avirulent "culture pallidum" spirochetes of uncertain provenance, can be grown *in vitro* with varying difficulty. A significant problem is their reluctance to form surface colonies on agar, hence to permit adequate purification. Several workers have reported that stock cultures of anaerobic spirochetes can be grown as surface colonies(2,1). Hardy, Lee and Nell(3) have recently described improved methods for this purpose, and have stressed nutritional factors as the principal determinants of such growth. It is recognized, however, that these microorganisms are exacting anaerobes, and that even brief exposure to atmospheric oxygen may inactivate them.

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