GROWTH TEMPERATURES, VIRULENCE, SURVIVAL, AND NUTRITION OF LEPTOSPIRES*

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THE effect of elevated temperatures upon leptospiral growth *in vitro* is poorly understood. Replication of leptospires in many animal hosts probably takes place at 37°C or higher, yet efficient laboratory propagation demands incubation at 29–30°C. In some instances short initial periods of incubation at 37°C have been thought to favour isolation. A gap in our knowledge exists as to exact temperatures within host tissues and the substances in blood, brain, liver, and kidney that may protect pathogenic leptospires and allow them to replicate *in vivo*.

Since the lyophilisation of leptospires presents difficulties, animal passage is used to maintain virulence. Coghlan, Lumsden, and McNeillage (1967) reported the preservation of leptospires at –79°C, but did not examine virulence. Alexander *et al.* (1972) reported the preservation of *Leptospira interrogans* serotype *canicola* by the use of liquid nitrogen. Repeated subculture of strains is time consuming, expensive, and may result in loss of virulence (Turner, 1970).

“Polysorbate media” containing polysorbate 80 (Tween 80), albumin, ammonium chloride, thiamine and vitamin B₁₂ permit a better study of the nutritional requirements of leptospires than do the traditional media consisting of serum and buffered salt solution (Ellinghausen and McCullough, 1965; Ellinghausen and Sandvik, 1965; Ellinghausen, 1966, 1968*a*, 1968*b*).

Polysorbate media were used in this study, the objectives of which were (1) to survey the *in-vitro* growth patterns of leptospires at 37°C and their relationships, if any, to virulence, (2) to examine survival in semisolid medium over extended periods of storage at 23–25°C, (3) to discover the shelf life of semisolid and liquid medium, and (4) to determine the effect upon leptospiral growth of omitting certain constituents from the medium, or replacing them with other substances. The tests were made with laboratory strains and with leptospires from infected blood, liver, and kidney tissue.

MATERIALS AND METHODS

Media, diluent and buffer. The standard medium was the basic polysorbate 80 albumin medium described by Ellinghausen and McCullough (1965) and referred to in this paper as P-80 medium. This medium, unmodified or modified, was used in either semisolid or liquid form. Liquid medium was used in 10-ml volumes in 19 × 150-mm test tubes closed by 20-mm steel caps. Semisolid medium in 10-ml volumes in 20 × 125-mm screw-capped

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tubes was prepared with Difco Bacto or BBL (Baltimore Biological Laboratory) Agar. The basal medium without albumin was sterilised as previously described (Ellinghausen and McCullough, 1965). The 5 per cent. albumin (Bovine Serum Albumin Fraction V powder, Miles Laboratory, Kankakee, Illinois) supplement was sterilised by Millipore membrane filtration ($0.22\ \mu\text{m}$).

One per cent. bovine serum albumin diluent (BSAD) consisted of 1.0 g of Fraction V powder per 100 ml of 0.005-M phosphate buffer. The phosphate buffer (PB) contained 87 mg per l KH_2PO_4 and 664 mg per l Na_2HPO_4 .

Measurement of leptospiral growth. Growth was measured in liquid culture by means of either a Coleman 7 nephelometer or a Coleman 9 nephelocolorimeter. The medium had a light-transmittance value of 92 per cent. at 400 nm with a Bausch and Lomb Spectronic 20 colorimeter. The nephelometer was adjusted to zero with uninoculated medium and then calibrated with a Roessler 77.5 turbidity standard (Roessler and Brewer, 1967). Nephelometer readings were related to the total and viable counts in the following manner. Cultures and suspensions adjusted to give a nephelometer reading of 25 were diluted 1 in 10 and counted with a Petroff-Hauser counting chamber. The same adjusted cultures were also diluted decimally from 10^{-1} to 10^{-10} in BSAD; a 1-ml sample of each dilution was then cultured in 10 ml of semisolid P-80 medium. The correlation between Petroff-Hauser count and viability was routinely verified by this method, within the limits of accuracy imposed by 10-fold dilutions.

Tests for viability of cultures. Unless stated otherwise, 2-ml volumes of semisolid cultures stored at 23–25°C were carefully inoculated onto the surface of P-80 semisolid medium. Incubation was at 29°C until a definite subsurface zone of typical leptospiral growth ("Dinger's zone") appeared. After 90 days, all apparently negative cultures were carefully checked by dark-field microscopy before being discarded. No particular effort was made to protect cultures from the light. In the experiments described it should not be thought that all cultures were tested simultaneously.

Strains of Leptospira. Because all leptospireae are now regarded as belonging to a single species, the various organisms in this paper are referred to merely by giving the serotype followed by the strain in parentheses, e.g., *pomona* (Ohio). Most strains were from the culture collection of this laboratory. The strains used for production of standard antigen had been maintained from 1955 to 1961 by storing in Fletcher's medium at room temperature (23–25°C) and subculturing at 3-mth intervals. From 1962, P-80 semisolid medium was used and strains were subcultured no more frequently than once every 18 mth. The virulent strains mentioned later were all isolated after 1961.

Infected hamsters. Unless otherwise stated, weanling hamsters were given intraperitoneal inoculations of 1 ml of cultures that had reached their maximum turbidity. The cultures so used were at the end of the logarithmic phase of growth and were never older than 7 days. Their viable content was tested by the methods already described.

The MID or MLD for certain strains after periods of storage was determined. The hamsters were killed 14 days after inoculation and blood samples were taken. The kidneys were removed aseptically, placed in 2.5-ml sterile disposable syringes and expressed into 10-ml volumes of BSAD. These suspensions were shaken in screw-capped tubes and allowed to settle for 30 min. The tissue suspension supernate was usually then diluted decimally to 10^{-4} in BSAD and 1-ml volumes of each dilution were cultured in 10-ml volumes of semisolid P-80 medium.

In one experiment, *pomona* (MLS)-infected liver (1006 mg) and kidney (645 mg) were directly expressed through the tip of a 2.5-ml syringe into 10 ml of P-80 semisolid medium.

Microscopic agglutination (MA) test. Equal volumes of homologous antigen suspension were added to dilutions of hamster serum, the maximum final serum dilution being 10^{-6} . The antigen suspensions had a light transmittance of 57–63 per cent. Bacterial dry weights of antigen suspensions ranged from 50–68 μg per ml; these were determined by subjecting concentrated antigen suspensions to carefully controlled drying at $100^\circ\text{C} \pm 1.0^\circ$ for 24 hr. Cell counts averaged 200×10^6 cells per ml, but there was no uniform correlation amongst strains between cell count and turbidity. An incubation time of 1 hr at 25°C was used for the MA test.

Porcine blood infected with pomona (HCE). Citrated blood from a pig contained the strain in its twelfth serial passage. The blood was cultured in various media, the inocula being 0.3-ml volumes of decimal dilutions from 10^{-1} to 10^{-8} , prepared in BSAD.

RESULTS

Continuous subculture of leptospiral strains at 37°C, and inoculation of hamsters

Strains were tested for their ability to grow continuously at 37°C on repeated subculture at 7-day intervals in liquid P-80 medium from 1-ml inocula. Growth was measured nephelometrically, and viability at the end of the first subculture was tested by inoculating liquid P-80 medium and incubating at 29°C; viability at the end of the second subculture was tested similarly, except that semisolid P-80 medium was used. Of 27 strains, 23 were virulent or avirulent pathogens; four strains were saprophytes, namely *illini* (3055H), *biflexa* (SC3), *biflexa* (Patoc 1), and *biflexa* (CDC). The results are shown in table I.

All except two of the 23 pathogenic strains and two of the four saprophytic strains were viable at the end of the first subculture. By the end of the second subculture a further nine pathogenic strains had shown greatly reduced growth as estimated by turbidity measurements and they were no longer viable.

Table I depicts three main types of leptospiral growth pattern at 37°C. Some strains, e.g., *pomona* (HCE) rapidly died with no evidence of multiplication. Others, e.g., *pomona* (3341), showed depressed growth at the second subculture, with subsequent recovery. Still others, e.g., *illini* (3055-H) showed growth that was both continuous and vigorous. The pattern of growth amongst a number of *pomona* strains was quite variable, while all *grippotyphosa* strains were uniformly susceptible to growth inhibition at 37°C.

Serotype *pomona* (Ohio) grew well at 37°C, but a human isolate, *pomona* (HCE), considered to have been derived from *pomona* (Ohio) through an accidental infection, no longer had the ability to grow at 37°C.

Hamsters were inoculated with the 27 strains, 25 of which had been maintained in semisolid P-80 medium at 25°C and before inoculation were subcultured at 29°C three times at 7-day intervals in liquid medium, while *illini* (3055-H) and *biflexa* (SC3) had been grown continuously at 37°C.

Table I gives an indication of virulence as shown by the ability of the strains to infect the kidney, and by antibody response. Virulence could not be correlated with the ability to grow *in vitro* at 37°C.

Survival and virulence after storage in semisolid medium

Twenty-nine strains were tested; most of these strains have already been named in table I. Two millilitres of culture of each strain were inoculated into 10 ml of semisolid P-80 medium. Tubes were incubated at 29°C until growth appeared and they were then stored at 23–25°C. Viability tests were carried out at intervals.

All strains were viable 12 mth later, and of 28 strains tested after 18 mth storage only one, *grippotyphosa* (B-697), was non-viable. After 24 mth, each

of 21 strains was viable. Examination of a small number of cultures at a later date suggested that even more prolonged survival occurred; for example, *pomona* (strains DM₂H, and JB) were viable after 36 mth, and *pomona* (HCE) after 52 mth.

TABLE I

Lack of correlation between ability of leptospire to grow in continuous subculture at 37°C and virulence

Serotype and strain	Nephelometer readings of subculture numbers						Results of hamster inoculation	
	1	2	3	4	5	6	Kidney infected	Reciprocal MA titre
<i>pomona</i> (HCE)	23	2*	1	0	0	0	+ †	...
<i>pomona</i> (Ohio)	35	27	35	34	35	40	+	10,000
<i>pomona</i> (MLS)	28	1*	1	1	0	0	+	1,000
<i>pyrogenes</i> (Salinem)	18	1*	1	0	0	0	—	100
<i>autumnalis</i> (Akiyami A)	11*	1*	1	0	0	0	—	10
<i>grippotyphosa</i> (Moskva V)	20	1*	1	0	0	0	—	10
<i>grippotyphosa</i> (B-697)	40	1*	1	0	0	0	—	10
<i>grippotyphosa</i> (B-699)	41*	1*	1	0	0	0	—	10
<i>grippotyphosa</i> (ICS)	29	1*	1	0	0	0	+	1,000
<i>tarassovi</i> (Perepelicin)	17	5*	1	0	0	0	—	10
<i>georgia</i> (LT-117)	22	1*	1	0	0	0	—	10
<i>pomona</i> (DM ₂ H)	32	28	43	36	22	40	—	100
<i>pomona</i> (JB)	22	13	21	22	19	40	+	10,000
<i>pomona</i> (3341)	32	7	20	22	19	25	—	100
<i>pomona</i> (T-262)	38	16	34	27	27	40	—	10
<i>copenhageni</i> (Wijnberg)	26	12	21	8	28	28	—	10
<i>copenhageni</i> (M-20)	21	6	25	29	10	29	—	10
<i>canicola</i> (Ruebush)	20	26	20	2	0	0	—	10,000
<i>canicola</i> (Hond Utrecht IV)	29	26	25	30	23	30	—	10
<i>ballum</i> (S-102)	24	8*	0	0	0	0	—	10
<i>australis</i> (Ballico)	24	14	24	0	0	0	—	100
<i>wolffi</i> (3705)	32	25	31	33	23	35	—	1,000
<i>hardjo</i> (WR)	31	35	28	36	25	42	—	100
<i>illini</i> (3055-H)	80	80	80	80	80	80	—	10,000
<i>biflexa</i> (SC 3)	60	60	60	60	60	60	—	1,000
<i>biflexa</i> (Patoc 1)	20*	0*	0	0	0	0	—	100
<i>biflexa</i> (CDC)	4*	0*	0	0	0	0	—	10

* = Subculture non-viable after 7 days' incubation.

† = Hamsters died.

MA = Microscopic agglutination test.

Survivors killed and serum samples taken 14 days after inoculation.

After storage for 52 mth, *pomona* (HCE) was cultured for 7 days at 29°C in liquid medium and inoculated into groups of four hamsters in decimal dilutions ranging from 2×10^8 to 20 organisms. All the animals died.

After storage for 7 mth, *pomona* (Ohio) was similarly tested in pairs of hamsters. Doses of $3 \cdot 12 \times 10^8$ and $3 \cdot 12 \times 10^7$ each caused the death of one of a pair of hamsters within 8 days. No deaths occurred in pairs of animals inoculated with $3 \cdot 12 \times 10^6$, $3 \cdot 12 \times 10^5$, and $3 \cdot 12 \times 10^4$ cells, but when killed 26 days after inoculation each hamster had renal infection as shown by culture of a 10^{-4} dilution of kidney tissue suspension.

After 24 serial subcultures of 7 months' total duration in liquid medium at 29°C, *pomona* (Ohio) failed to cause death in pairs of hamsters inoculated with decimal-dose dilutions ranging from 1.36×10^8 to 1.36×10^4 leptospire, but cultures of 10^{-4} dilutions of kidney tissue suspensions at necropsy, 26 days after inoculation, invariably gave positive results. MA titers of 10^5 were found in all but two hamsters inoculated with 1.36×10^7 to 1.36×10^5 cells and killed 26 days later. The two hamsters inoculated with 1.36×10^4 cells had MA titers of 10^4 .

Keeping qualities of semisolid and liquid P-80 medium

Semisolid P-80 medium stored for 36 mth at 23–25°C supported growth of small numbers of *pomona* (DM₂H). Macroscopic growth was invariably visible after 3 days' incubation at 29°C in tubes inoculated with 2.2×10^7 cells. When the inoculum was reduced to 2.2×10^6 , 2.2×10^5 , and 2.2×10^4 cells, growth was visible at 6–9, 7–13, and 13–16 days respectively, except for the 36-month-old medium inoculated with 2.2×10^4 cells. In this tube a Dinger's zone did not appear, but darkfield examination after 24 days' incubation showed living organisms, and subculture in 3-month-old semisolid P-80 medium produced growth that was macroscopically detectable after 72 hr.

Liquid P-80 medium stored for 12 mth at 23–25°C was also tested with *pomona* (DM₂H). Decimal dilutions containing 22.6×10^6 to 2 organisms per ml were prepared in tubes containing 10 ml medium. After 21 days' incubation, the nephelometer readings of the eight tubes were, respectively, 48, 58, 60, 51, 51, 30, 5, and 0. After 28 days' incubation the corresponding readings were 48, 49, 52, 59, 58, 52, 54, and 54.

Survival of leptospire in BSAD

A suspension of *illini* (3055H), a robust organism, was washed twice in sterile PB and diluted decimally to extinction in BSAD. After the tubes had been held at 23–25°C for four days, viability was checked. Diluent which originally contained 2.46×10^8 to 2.46×10^5 cells per ml yielded viable organisms, but that originally containing 2.46×10^4 cells per ml or less was sterile. After 30 and 340 days' storage only those suspensions originally containing 2.46×10^8 and 2.46×10^7 cells per ml were viable. After 510 days' storage, suspensions originally containing 2.46×10^7 cells per ml were still viable.

Fourteen further strains or variants were similarly tested for their viability in BSAD, and also in PB, after storage for 7 days at 23–25°C. The results, shown in table II, demonstrated the superiority of BSAD.

In a similar experiment, nine other strains remained viable for at least 51 days in BSAD and PB when the original counts were high (30.8×10^6 to 20×10^6 cells per ml); the strains tested were *copenhageni* (M-20), *javanica* (Veldrat Batavia 46), *canicola* (strains Hond Utrecht IV, NADL-A13, and Moulton), *ballum* (S-102), *pyrogenes* (Salinem), *pomona* (HCE), and *ranae* (ICF).

Recent tests with *pomona* (DM₂H and JB) and *illini* (3055H) have shown that suspensions originally containing approximately 2×10^7 cells per ml BSAD were still viable after 17 mth at 23–25°C. Serotype *pomona* (JB) was still capable

of infecting hamsters; although death did not occur, isolations were made from blood, liver, and kidney at necropsy of the hamsters 28 days after inoculation.

Culture of infected blood in modified or incomplete forms of P-80 medium

A comparison was made of the growth-supporting properties of the semisolid P-80 medium and the following modified forms: standard medium without polysorbate 80; three media in which polysorbate 80 was replaced by either polysorbate 60, 40 or 20; three media in which either NH_4Cl , thiamine or vitamin B_{12} was omitted. When the 8 media were inoculated with 0.3-ml volumes of decimal dilutions of blood from a pig infected with *pomona* (HCE) the limiting dilutions of blood for growth in these media were shown to be 10^{-6} , 10^{-3} , 10^{-6} , 10^{-7} , 10^{-6} , 10^{-5} , 10^{-5} , and 10^{-3} respectively. Growth was assessed visually at intervals from the 10th to 90th day of incubation at 29°C . Thus, the omission from the standard medium of either its polysorbate or vitamin B_{12} content had a strikingly adverse effect on growth.

The possibility of continuous subculture at 29°C was also examined in relation to the eight media. Cultures from limiting dilutions of blood in each of the eight media were subcultured at 7-day intervals in the parent media. Continuous serial propagation in media lacking either polysorbate, NH_4Cl or thiamine did not give well-formed Dinger's zones and therefore growth was not readily detectable; a low level of growth did take place but was demonstrable only by subculture in complete semisolid P-80 medium. Continuous subculture with clear Dinger's zones proved possible in each of the four complete polysorbate media (P-80, P-60, P-40, P-20) and also, surprisingly, in medium lacking vitamin B_{12} .

Similar tests were made with eight comparable liquid media. When the inoculum was a 10^{-2} dilution of the infected blood, growth became visible after 10–12 days' incubation in all media, except that deficient in polysorbate; in this medium growth did not appear until the 17th day of incubation. Growth from a 10^{-6} dilution of blood took place in media lacking NH_4Cl or thiamine. In vitamin B_{12} -deleted medium macroscopically-detectable growth did not occur from dilutions of blood greater than 10^{-2} , but continuous subculture in the parent medium was successful.

Survival of pomona (HCE) in primary cultures made from porcine blood

Use was made of the positive cultures in semisolid media from limiting dilutions of blood in the previous experiment.

Cultures in the four polysorbate media and the polysorbate-deficient medium were stored for 7 months at $23\text{--}25^\circ\text{C}$ and then tested for viability. All were viable and, moreover, each grew with a Dinger's zone when transferred to its parent medium, except for the culture in polysorbate-deficient medium. In the latter, heavy growth as indicated by an obvious Dinger's zone did not develop but, probably because of the albumin content of the medium, viable organisms were present and could be detected by darkfield examination or subculture on complete media. Cultures from limiting dilutions of blood in the three media

deficient in NH_4Cl , thiamine or vitamin B_{12} were subcultured from one to three times in the parent media. Again, the final subcultures survived storage for 7 mth at 23–25°C.

Survival of 10 strains in modified or incomplete forms of P-80 semisolid medium

The following strains or variants were tested: *pomona* (DM_2H and JB), *grippotyphosa* (Moskva V; Raccoon, Ga.; Bobcat, Ga.; Squirrel, ICS; Swamp Rabbit, Ga.; Cottontail Rabbit, Ga.), *illini* (3055 hooked), and *illini* (3055 non-hooked). Approximately 2×10^6 viable organisms of each strain were inoculated into semisolid P-80 medium and into the seven modified or incomplete forms of P-80 medium already described. The eighty inoculated cultures were incubated at 29°C until growth appeared, and stored for 18 mth at 23–25°C. Subcultures in P-80 medium made to examine survival gave obvious Dinger's zones of growth within 30 days in all except three instances. The subcultures of the two varieties of *illini* (3055) stored in the eight different media gave visible growth simultaneously. There was some variation between the other subcultures in the experiment in respect of the time of first appearance of growth, but this seemed to be of a random nature and could not be related to strain differences or to differences between the eight media used. Two cultures required repeated sampling to show viability, and 79 recoveries were finally made from the 80 cultures.

Isolation of pomona (MLS) from liver and kidney tissue in complete or vitamin B_{12} -deficient medium

Semisolid P-80 medium, with and without vitamin B_{12} , was used in attempting isolations of *pomona* (MLS) from the liver and kidney tissue of hamsters infected four days earlier by intraperitoneal inoculations of a 10^{-4} dilution of liver tissue from a dying hamster.

The organism could be isolated from 0.1 mg of hamster-liver tissue, prepared by a dilution technique, regardless of the presence of vitamin B_{12} . Continuous serial subculture at 14-day intervals was possible in complete and B_{12} -deficient medium. Eleven months after original isolation the primary cultures, with or without vitamin B_{12} and kept at 23–25°C were still viable.

A 10^{-4} dilution of kidney tissue (0.006 mg) gave leptospiral growth in complete P-80 medium, but not in vitamin B_{12} -deficient medium.

Kidney tissue was also cultured in liquid P-80 medium, in which growth could be more readily quantitated. Culture of 0.64 mg of kidney tissue in vitamin B_{12} -deficient medium had, after 7 days' incubation, a nephelometer reading of 6, while in the complete medium the value was 42. These two cultures were subcultured in parent liquid medium and after three days the nephelometer readings were 0 and 28 respectively. With an additional four days' incubation the turbidity value was still 0 in non-supplemented medium and no further subculture was possible. By contrast, the nephelometer reading of the culture in complete medium had risen to 45, and continuous subculture producing heavy growth was possible.

DISCUSSION

No correlation between virulence and the ability of leptospires to grow continuously and well at 37°C *in vitro* was established. The three patterns of growth seen at 37°C indicate that different cultural populations occur. Serological classification does not necessarily correspond with temperature sensitivity: two *biflexa* strains were very heat sensitive whilst another strain grew well at 37°C.

Serotype *pomona* (Ohio) grew well at 37°C, but upon re-isolation as *pomona* (HCE) from an accidental human infection, it could no longer grow at this temperature. On the other hand, *pomona* (DM₂H) was capable of growth at 37°C both before and after passage through a human host.

Pasteurella pestis was found to be avirulent when cultured at 5°C (Panos and Ajl, 1963), but was virulent when grown at 37°C. The respiration of a leptospiral isolate from frog kidney (Ellinghausen, 1968*b*) diminished drastically when it was grown at 32.5°C instead of 29°C. Steinberg, Horswood, and Chanock (1969) attempted to obtain temperature-sensitive mutants of *Mycoplasma pneumoniae* in the hope that they would prove immunogenic against respiratory disease. Such approaches should be considered in relation to leptospiral diseases.

Manew (1969) reported that only two out of 138 leptospiral strains survived six months' storage in Fletcher's medium. The present study indicates that yearly subcultures in P-80 semisolid medium are likely to be satisfactory. The improved survival of leptospires in BSAD as compared with that in PB supports the finding of Schubert and Sulzer (1970) who noted the value of 6 per cent. bovine albumin in the preservation of leptospires.

The chemical contaminants in bovine albumin (Hanson and Ballard, 1968) such as fatty acids, citrate, lactate, pyruvate, aspartate, and alpha-keto-glutarate might account for its preserving activity. Chen (1967) used charcoal treatment to remove fatty acids from albumin, but albumin so treated was shown to be an inferior component of P-80 medium when tested with many strains of leptospires (Ellinghausen, unpublished data). Only by the use of minimal inocula can the value of albumin purified in different ways be assessed. Whether purification processes affect the ability of albumin to bind long-chain fatty acids or whether they remove traces of growth-stimulating substances is not known.

Leptospires stored for long periods in P-80 semisolid medium retained virulence, and one strain, *pomona* (HCE), was still lethal for hamsters in a dose of 20 organisms after storage for 52 mth. Semisolid P-80 medium was shown to have a shelf-life of at least 36 mth and this is probably significant in relation to the ability of the medium to preserve the viability of leptospires for long periods. No evidence of lipase activity in bovine albumin has been found (Ellinghausen and Sandvik, 1965) and this fact makes breakdown of the medium less likely. The long shelf-life of P-80 medium, both liquid and semisolid, is also of value in that it permits thorough testing of the quality of each batch before use.

Pearce and Lowrie (1972) have reviewed the possible mechanisms of localisation of bacteria in certain host tissues, paying due attention to nutritional factors which limit growth. The present paper includes studies on the general nutrition of virulent leptospiral isolates. The role of supplementary growth lipid in the form of polysorbate was immediately obvious, but one or two additional subcultures were necessary in medium lacking NH_4Cl or thiamine before the importance of these nutrients for maximum growth became apparent.

That growth can occur in P-80 medium from which certain nutrients are omitted suggests that the albumin component plays a vital role. Nutrient-deleted media could well provide an environment where growth is minimised, and this might be especially favourable for preservation of viability and virulence.

It was shown that *pomona* (MLS) isolated from liver had different vitamin B_{12} requirements from those of the same organism isolated from kidney. Differences in lipid requirements of leptospires isolated from brain, blood, kidney, liver, and urine await further study. Such a study may depend on further refinements in the purification of albumin.

The temperatures required by different isolates for maximum growth and virulence are still unpredictable. Advances have been made in understanding of the nutritional and metabolic requirements of leptospires, but how the latter affect leptospiral infections is unknown. Factors which permit the survival and multiplication of leptospires *in vivo* require deeper scrutiny.

SUMMARY

Some virulent and avirulent pathogenic leptospires and saprophytic leptospires grew continuously at 37°C , while others did not; variations amongst strains of the same serotype occurred and there was no correlation with virulence.

Each of 21 strains survived 24 months' storage in semisolid polysorbate medium (P-80 medium) without intervening subculture and one strain tested after 52 months was still viable and fully virulent. Semisolid and liquid P-80 media had a shelf-life of at least 36 and 12 months respectively at $23\text{--}25^\circ\text{C}$.

One per cent. albumin was shown to favour the survival of leptospires.

In experiments with modified or incomplete semisolid P-80 medium, various leptospiral strains grew and survived when polysorbate, 60, 40, or 20 were substituted for polysorbate 80, and when polysorbate, NH_4Cl , thiamine, or vitamin B_{12} were omitted. However, the omission of any polysorbate or of vitamin B_{12} had a strikingly adverse effect on growth. These modified media were used to culture blood infected with *pomona* (HCE). Continuous subculture eventually failed to produce readily-detectable macroscopic growth in the absence of either polysorbate, NH_4Cl or thiamine, but continuous subculture in the absence of vitamin B_{12} was successful. Nutrient-depleted P-80 media were still capable of maintaining viability and a low level of growth, probably because of the micro-nutrients in the one per cent. albumin component. Continuous subculture of *pomona* (MLS) from hamster liver was successful regardless of the presence of vitamin B_{12} in the medium, but vitamin B_{12} was essential for similar subculture of the same organism isolated from kidney.

REFERENCES

- ALEXANDER, A. D., LESSEL, E. F., EVANS, L. B., FRANCK, E., AND GREEN, S. S. 1972. Preservation of leptospires by liquid-nitrogen refrigeration. *Int. J. Syst. Bact.*, **22**, 165.
- CHEN, R. F. 1967. Removal of fatty acids from serum albumin by charcoal treatment. *J. Biol. Chem.*, **242**, 173.
- COGHLAN, JOYCE D., LUMSDEN, W. H. R., AND MCNEILLAGE, G. J. 1967. Low temperature preservation of *Leptospira*, preliminary communication. *J. Hyg., Camb.*, **65**, 373.
- ELLINGHAUSEN, H. C., JR 1966. The effect of aeration upon the growth of *Leptospira* serotypes. *Am. J. Vet. Res.*, **27**, 975.
- ELLINGHAUSEN, H. C., JR 1968a. Stimulation of leptospiral growth by glucose. *Am. J. Vet. Res.*, **29**, 191.
- ELLINGHAUSEN, H. C., JR 1968b. Cultural and biochemical characteristics of a leptospire from frog kidney. *Bull. Wildlife Dis. Ass.*, **4**, 41.
- ELLINGHAUSEN, H. C., JR, AND MCCULLOUGH, W. G. 1965. Nutrition of *Leptospira pomona* and growth of 13 other serotypes: fractionation of oleic albumin complex and a medium of bovine albumin and polysorbate 80. *Am. J. Vet. Res.*, **26**, 45.
- ELLINGHAUSEN, H. C., JR, AND SANDVIK, O. 1965. Tributyrinase activity of leptospires: fixed and soluble tributyrinase demonstrated by means of an agar diffusion test. *Acta. path. microbiol. scand.*, **65**, 259.
- HANSON, R. W., AND BALLARD, F. J. 1968. Citrate, pyruvate, and lactate contaminants of commercial serum albumin. *J. Lipid Res.*, **9**, 667.
- MANEW, C. 1969. A double-layer nutrient medium for the maintenance of leptospira strains. *Zentbl. Bakt. ParasitKde*, I Abt. Orig., **210**, 216 (in German).
- PANOS, C., AND AJL, S. J. 1963. Metabolism of microorganisms as related to their pathogenicity. *A. Rev. Microbiol.*, **17**, 297.
- PEARCE, J. H., AND LOWRIE, D. B. 1972. Tissue and host specificity in bacterial infection. In *Microbial pathogenicity in man and animals*, 22nd Symp. Soc. Gen. Microbiol., 1972, London, p. 193.
- ROESSLER, W. G., AND BREWER, C. R. 1967. Permanent turbidity standards. *Appl. Microbiol.*, **15**, 1114.
- SCHUBERT, J. H., AND SULZER, CATHERINE 1970. Preservation of *Leptospira* by storage at -75°C versus transferring every three months. *Bact. Proceed.*, **20**, 20.
- STEINBERG, P., HORSWOOD, R. L., AND CHANOCK, R. M. 1969. Temperature-sensitive mutants of *Mycoplasma pneumoniae*. I. In-vitro biologic properties. *J. Infect. Dis.*, **120**, 217.
- TURNER, L. H. 1970. Leptospirosis III. Maintenance, isolation, and demonstration of leptospires. *Trans. R. Soc. Trop. Med. Hyg.*, **64**, 623.