crystallized and tested against *Phycomyces blakesleanus*,[4] and no evidence of thiamin found.

Summary and Conclusions. Thiamin when added to a basal medium containing KH_2PO_4, $MgSO_4$ and with asparagin or NH_4Cl causes an increase in the growth of *Pityrosporum ovale*. With asparagin the increase is much greater than with NH_4Cl. However, if ethyl oxalacetate replaces the thiamin, growth in the NH_4Cl medium is just about equal to that in the asparagin medium. The addition of thiamin to the medium containing oxalacetate does not cause any further increase in growth. It would seem then that NH_4Cl and ethyl oxalacetate can be substituted for asparagin and thiamin in the cultivation of *Pityrosporum ovale*. It appears that if a suitable keto acid is supplied neither thiamin nor an amino acid is necessary. On the other hand, the asparagin may furnish both the carbon and nitrogen source needed by the organism, yielding ammonia and a carbon skeleton, probably oxalacetate. Thiamin increases growth when added to the asparagin by hastening the production of the carbon skeleton in some way. When this carbon skeleton is supplied as oxalacetate, thiamin is no longer necessary.

[4] Robbins, W. J., and Kavanogh, Frederick, *Proc. Nat. Acad. Sci.*, 1937, **23**, 499.

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Protective Tests in the Developing Chick Embryo With *Hemophilus influenzae* Type b.

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The assay of the protective power of immune sera versus *H. influenzae* type b is of some practical importance. Serum protection of the white mouse against infection with *H. influenzae* by the mucin method has been demonstrated.[1] However, this method seems not to have given very satisfactory results in routine use, and standardization of therapeutic anti-*H. influenzae* type b serum has been made by the determination of antibody nitrogen.[2] The identity of the precipitating antibody with the mouse protective power in the serum has been ascertained.[3]

The experience with the assay of the protective value of immune sera against *E. typhosa* and *Shigella*[4, 5] suggested an investigation whether a similar test could be developed for anti-*H. influenzae* type B serum.

The developing chick embryo has been previously found to be susceptible to infection with *H. influenzae*,[5,6] and the 6- to 7-day-old chick embryo has been employed as a test animal for the demonstration of the protective effect of sulfathiazole and sulfadiazine.[7] No reports have been found in the literature on the protective effect of immune sera against experimental infection of the developing chick embryo with *H. influenzae*.

Young cultures (5 to 6 h 37°C) either in a digest broth similar to the medium described by Silverthorne and Cameron[8] (but without agar) or in Levinthal broth were used through-

[1] Fothergill, L. D., Dingle, J. H., and Chandler, C. A., *J. Exp. Med.*, 1937, **65**, 721.

[2] Alexander, H. E., and Heidelberger, M., *J. Exp. Med.*, 1940, **71**, 1.

[3] Alexander, H. E., Heidelberger, M., and Leidy, G., *Yale J. Biol. and Med.*, 1944, **16**, 425.

[4] Weil, A. J., and Gall, L. S., *J. Immunol.*, 1941. **41**, 445.

[5] Weil, A. J., and McFarlane, J., *J. Immunol.*, 1944, **48**, 291.

[6] Gallavan, M., *Am. J. Path.*, 1937, **13**, 911.

[7] Altura-Werber, E., *J. Bact.*, 1943, **45**, 195.

[8] Silverthorne, N., and Cameron, C., *J. Pediat.*, 1942, **20**, 9.

TABLE I.

Protection by Immune Serum. Ten-day incubated chick embryos received upon the chorio-allantoic membrane a mixture of 0.1 ml culture dilution of *H. influenzae* type b (strain No. 62) diluted as indicated plus 0.1 ml serum in 5-fold decreasing dilutions. Ten embryos at each test level. Readings 4 days after infection.

Immune serum	Culture dilution	Microorganisms* (× 1000)	Serum dilutions at 50% endpoint of survival† ml	LD_{50} Virulence control microorganisms
No. 18R37, horse, refined	10^{-3}	48	0.004	2
	10^{-4}	4.8	0.0018	2
O, rabbit	10^{-3}	57	0.0083	4
	10^{-4}	5.7	0.0027	4
N, rabbit	10^{-2}	500	>0.01	2
	10^{-3}	50	0.001	2

* Calculated from plate count of 10^{-6} culture dilution.
† Calculated according to [9].

out the experiments. (Cultures incubated 18 hr do not loose virulence to an appreciable degree.) Dilutions were made in physiological saline solution buffered with M/50 phosphate to a pH of 7.2 to 7.4. We ascertained, in preliminary tests, that the viability of cultures remained unchanged in this fluid even in high dilutions for at least 2 hours. (The majority of bacteria will be killed within one hour if dilutions are made in distilled water.)

Infection can be effected by depositing bacteria on the chorioallantoic membrane and also into the amnion or into the yolk sac. Ten-day-incubated chick embryos proved to be maximally susceptible, the LD_{50} being between 1 and 10 micro-organisms. The use of younger embryos seems to be less advantageous from the point of view of their ability of using antibody to best advantage.

Most chick embryos die within 48 hours after infection. Thus, observation for four days gives an ample margin of safety. Bacteria could be recovered from the heart blood and the chorioallantoic membrane of the infected animals. Heart cultures of serum-protected animals were regularly sterile, while complete disappearance of viable bacteria from the chorioallantoic membrane was not always obtained.

Except for cutaneous bleedings, no gross pathological changes were observed in the embryo (see in this respect also[6]).

Sensitivity to infection decreases rapidly with the growth of the embryo, and the newly hatched chick is not affected by intraperitoneal infection with undiluted culture, that is, an infective dose in the order of 10^9 micro-organisms. In this respect, our experience follows that previously recorded for *E. typhosa*.[4]

As a rule, we used a strain No. 62, which had been obtained in 1939 from Dr. Dingle. A strain Rah, which was kindly sent to us by Dr. Alexander, proved to be equally virulent.

For the test of the protective value of immune serum, infection of the chorioallantoic membrane was routinely used. Immune serum injected into the yolk sac or amnion afforded protection, which was, however, restricted to infective doses not larger than 10^2 micro-organisms, even if serum was introduced 6 to 20 hours previous to infection.

In contrast to this, serum protection was found to be highly effective if the serum was dropped upon the chorioallantoic membrane, and it made no appreciable difference when the serum was applied anytime between 6 hr before and 4 hr after infection. After this time, no significant protective effect was observed. We found that it made no appreciable difference whether the serum was given separately or in admixture with the micro-organisms. Thus, simultaneous introduction of micro-organisms and serums in 0.2 ml total volumes was used in most of our tests, and the following data refer to this method of application.

Tests were made either by assaying a constant volume of serum against decimally decreasing dilutions of culture or—more often—by evaluating the effect of 4- or 5-fold decreasing volumes of serum versus one or 2

[9] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

dilutions of culture. Constancy of virulence was ascertained in each test by infecting control eggs with culture alone in decimal dilutions from 10^{-6} to 10^{-9}. A series of not-infected eggs were kept as viability controls; this is an important precautionary measure to insure the tests against interference by unforeseen damaging influences. 50% endpoints of survival were calculated according to the method of Reed and Muench.[9]

The chick embryos are rather uniformly susceptible to infection, but protection is subject to considerable individual variation. Thus, it was found to be advisable to use 8 or, better, 10 chick embryos at each test level. Normal horse and rabbit sera and, similarly, antipneumococcic horse and rabbit sera were found to be devoid of any appreciable protective value in volumes of 0.02 ml.

The immune sera employed in these tests consisted in the main of pooled sera from rabbits that had been treated with increasing volumes of *H. influenzae* type b suspension. Tests were also made with a refined and concentrated horse immune serum 18R37, that had been found by Dr. Heidelberger[10] to contain 1.25 mg agglutinin and 1.15 mg precipitin N/ml.

By way of exemplification, we give in Table I. the results of some of our experiments in which immune sera in 5-fold decreasing dilutions were tested against 2 dilutions of culture. It will be seen that small volumes of immune serum afford protection against infections in the order of 10^3 and 10^4 lethal doses. This latter level of infection is about the upper limit of effectiveness of immune protection. As can be seen from line 5 of the table, infection with well above 10^5 microorganisms is evidently so overwhelming that immune protection is of no avail anymore. This experience is paralleled by that with *Enterobacteriaceae*.[4, 5]

A similar method can also be used for chemotherapeutic experimentation; for instance, the LD_{50} of chick embryos treated by a single injection of 5 mg of sulfadiazine into the amnion immediately before infection with *H. influenzae* was found to be 120,000 microorganisms, the LD_{50} of the controls in this experiment being 2 microorganisms.

Summary. Ten-day-old chick embryos are maximally susceptible to infection with *H. influenzae* type b, the average LD_{50} being between one and ten microorganisms.

Protection against many thousand lethal doses can be obtained by immune serum.

A similar technic can also be employed for chemotherapeutic testing as exemplified by tests with sulfadiazine.

[10] Personal communication.

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Properties of the Etiologic Agent of Infectious Hepatitis.*

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Although *infectious hepatitis* has been transmitted experimentally to human volunteers by feeding[1] and inoculating parenterally[2-5] serum which is bacteriologically sterile, the exact nature of the icterogenic agent is yet to be defined. It is believed to be a virus.

During the past few years, numerous reports have appeared, describing the occurrence of jaundice in man following the administration of human blood products and indicating that the icterogenic agent (or agents) of such

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