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ETIOLOGY OF YELLOW FEVER.

X. Comparative Immunological Studies on Leptospira icteroides and Leptospira icterohæmorrhagiæ.

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In a previous paper[1] it was reported that serum from convalescent yellow fever patients has a more or less marked antagonistic effect upon *Leptospira icteroides* derived from certain cases of yellow fever in Guayaquil, as manifested by a positive Pfeiffer phenomenon in the peritoneal cavity of the guinea pig. In a few instances the serum protected the guinea pig from a fatal infection with the organism. A similar result was obtained with the serum of guinea pigs which had recovered from a non-fatal experimental infection with the leptospira.

In the present paper the question of immunity will be more fully discussed, particularly with regard to agglutination, lysis, complement fixation, Pfeiffer's reaction, etc., with immune sera prepared in rabbits and horses by repeated inoculations of *Leptospira icteroides*. Experiments have also been carried out to determine the relation between this organism and *Leptospira icterohæmorrhagiæ* of infectious jaundice by means of cross-immunity reactions *in vitro* and *in vivo*. In the *in vivo* experiments not only passive, but also active immunity has been taken into consideration. A part of this study has already been published in a paper dealing with the leptospira isolated from wild rats and mice in Guayaquil[2] and will not be repeated here.

[1] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 9.
[2] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 95.

Production of Immune Sera.

Monovalent immune sera for each of four strains of *Leptospira icteroides* were prepared in rabbits by injecting the animals intravenously with 2 to 4 cc. of rich live culture, on rabbit serum medium, several successive times at intervals of 7 to 14 days. The animals showed a definite febrile reaction on the 4th or 5th day after the first injection but no other symptoms. Subsequent inoculations produced no perceptible reaction in most of the animals, although some died suddenly, probably owing to the development of anaphylaxis as the number of inoculations increased.

Polyvalent immune serum was produced in a horse by injecting intravenously (jugular vein) gradually increasing amounts of rich live cultures (horse serum medium) of five strains (Nos. 1, 3, 4, 5, and 6) of *Leptospira icteroides*. 20 cc. of the mixture of cultures were given as the initial injection, and subsequent injections were increased up to 200 cc. This dose was maintained for most of the time during immunization. The first inoculation caused a rise in temperature to 40°C. on the 3rd and 4th days, with considerable swelling along the inoculated side of the neck. The animal lost its appetite during the period but regained its normal condition within 5 days. There was no jaundice at any time. Subsequent inoculations caused no perceptible reaction. During a period of 65 days the horse received 2,495 cc. of mixed live cultures in fifteen injections. The following protocol gives the schedule of immunization.

Horse 2.—Feb. 6, 1919, 20 cc.; Feb. 11, 40 cc.; Feb. 15, 60 cc.; Feb. 19, 125 cc.; Feb. 24, 150 cc.; Mar. 1, 200 cc.; Mar. 6, 200 cc.; Mar. 11, 200 cc.; Mar. 15, 200 cc.; Mar. 19, 200 cc.; Mar. 24, 200 cc.; Mar. 28, 200 cc.; Apr. 2, 200 cc.; Apr. 7, 200 cc.; Apr. 12, 300 cc. First bleeding on Apr. 19, 1919.

Effects of Immune Sera upon Leptospira icteroides and Leptospira icterohæmorrhagiæ.

Monovalent immune sera were prepared in rabbits, as described, and experiments conducted to demonstrate the effects of such sera upon homologous and heterologous strains of *Leptospira icteroides* on the one hand and of *Leptospira icterohæmorrhagiæ* on the other.

The experiments were designed to throw more light on the relation that may exist, not only among different strains of these organisms, but also between *Leptospira icteroides* and *Leptospira icterohæmorrhagiæ* as distinct varieties. The effects of an immune serum are manifold, but we confined our observations to the phenomena of agglutination, immobilization, and disintegration of the organism when mixed with the immune serum *in vitro* and that of the reaction of Pfeiffer following the simultaneous inoculation of the organism and an immune serum into the peritoneal cavity of guinea pigs. Complement fixation tests were also made.

Agglutination.—The technique employed for the agglutination tests was as follows:

> Rich live cultures, grown at 26°C. for 2 to 3 weeks on rabbit serum medium (one part of serum plus three parts of 0.9 per cent sodium chloride solution), of each strain were selected. 1 cc. of each of a number of cultures was put into a series of small sterile test-tubes, and 0.2 cc. of the fresh immune serum to be tested was added to each tube. Controls with normal rabbit serum accompanied each series of experiments. The culture and immune serum were carefully mixed by gentle shaking and the tubes incubated at 37°C. (water bath) for 2 hours. Examinations of the contents were then made by means of the dark-field microscope.

With pronounced agglutination minute particles could be macroscopically detected. Another examination was made on the tubes after they had been left at room temperature for 96 hours, but the results were identical with those recorded after 3 hours, with the possible exception of a more granular appearance of some of the agglutinated cultures and in extreme instances a thin but definite grayish sediment at the bottom of the test-tubes.

In the present series of experiments there were available three monovalent antisera for *Leptospira icteroides*, comprising Strains 1, 5, and 6, and six for *Leptospira icterohæmorrhagiæ*, comprising the Japanese, British, American No. 1, Group 8, Group 11, and Group 30 strains. Against each of these nine monovalent immune sera were tested cultures of five different strains of *Leptospira icteroides*, Nos. 1, 3, 4, 5, and 6, and seven strains of *Leptospira icterohæmorrhagiæ*, Japanese, British, French, American No. 1, Group 8, Group 11, and Group 30.

A strong immune serum acting upon the homologous strain of *Leptospira icteroides* agglutinated the organisms quickly into rather large masses, in which they appeared tightly held together. Most of the organisms became immobile, gradually lost their elementary windings, and were soon transformed into stiff, irregularly granular filaments. If the serum was not strong enough to produce these changes, the agglutinated masses were for the most part degenerated, but with several apparently intact immobilized organisms at the periphery. In other instances the agglutinated masses contained a certain number of individuals which were still active, while in still others the organisms in agglutinated masses showed quivering motility in some part of their body. On the whole, agglutination is the first and more constant reaction observed and disintegration the secondary and less constant (Tables I and II).

Pfeiffer's Phenomenon.—To complete the observations, Pfeiffer tests were also performed with the immune sera and the various strains of *icteroides* and *icterohæmorrhagiæ*. The technique employed was that generally followed. 1 cc. of a given immune serum was mixed in a Petri dish with 1 cc. of a rich live culture and immediately injected into the peritoneal cavity of a normal guinea pig. The peritoneal fluid was withdrawn with a capillary pipette after 30 minutes and 2 hours and examined under the dark-field microscope (Tables I and II).

The reaction of Pfeiffer with the immune sera and homologous strains of *Leptospira icteroides* is prompt and complete. The organisms seem first to be agglutinated into large masses and then to be quickly disintegrated. The phenomenon may be complete within 15 minutes, so that no trace of the organisms can be seen in the peritoneal fluid. The same is true of *Leptospira icterohæmorrhagiæ* and the homologous immune sera. A decided increase of actively motile organisms was noticed in the guinea pig peritoneal cavity when a normal rabbit serum instead of a specific immune serum was used.

As shown in Table I, the five different strains of *Leptospira icteroides* reacted to each of the three monovalent immune sera. The intensity of agglutination and disintegration varied somewhat according to whether the strains were homologous or heterologous. Without

TABLE I.

Effects of Monovalent Anti-icteroides Sera upon Leptospira icteroides and Leptospira icterohæmorrhagiæ.

Serum.	Strain of *Leptospira icteroides*.																Strain of *Leptospira icterohæmorrhagiæ*.																	
	No. 1.			No. 3.			No. 4.			No. 5.			No. 6.			Japanese.			British.			French.			American No. 1.			Group 8.			Group 11.			Group 30.		
	Agglutination.*	Disintegration.*	Pfeiffer reaction.†	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.
Immune serum for *Leptospira icteroides*, Strain 1.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	±	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
	+	+		+	+		+			+	+		+	+		+																		
	+	+		+			+			+			+																					
	+	+																																
" " " " " " 5.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	+	+		+	+		+	+		+	+		+	+																				
	+	+		+	+		+			+	+		+																					
	+									+	+																							
" " " " " " 6.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	+	–	±	–	–	–	–	–	–	–
	+	+		+	+		+	+		+	+		+	+																				
	+			+	+		+	+		+			+	+																				
	+						+						+	+																				
Normal rabbit serum (control).	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

* The varying degrees of agglutination and disintegration are recorded by plus signs vertically placed; their absence by a minus sign.

† + indicates positive; ± doubtful; – negative.

TABLE II.

Effects of Monovalent Anti-icterohæmorrhagiæ Sera upon Leptospira icterohæmorrhagiæ and Leptospira icteroides.

Serum.	Strain of *Leptospira icterohæmorrhagiæ*.																						Strain of *Leptospira icteroides*.																	
	Japanese.			British.			French.			American No. 1.			Group 8.			Group 11.			Group 30.			No. 1.			No. 3.			No. 4.			No 5.			No. 6.		
	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.
Immune serum for *Leptospira icterohæmorrhagiæ*, Japanese strain.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−
	+	+		+			+			+	+		+	+		+			+	+		+																
	+	+								+	+		+	+					+	+																		
	+	+																	+																				
Immune serum for *Leptospira icterohæmorrhagiæ*, British strain.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	+			+	+		+	+		+			+			+			+																			
				+	+		+	+								+																							
				+	+																																			
Immune serum for *Leptospira icterohæmorrhagiæ*, American Strain 1.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	±		
	+	+		+			+			+	+		+			+	+		+																			
	+	+								+	+		+			+	+																						
	+									+	+					+																							
Immune serum for *Leptospira icterohæmorrhagiæ*, Group 8 strain.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	+	+		+	+		+			+			+	+		+	+		+	+																			
										+			+	+		+			+																					
													+	+																										
													+	+																										

Immune serum for *Leptospira icterohæmorrhagiæ*, Group 11 strain.	+	+	+	+	+	+	+	+	+	+	+	+	+	−	(?)	+	+	+	+	+	+	+	+	−	−	−	+	+	<	+	−	−	−	−	−	−	−	−	−
	+	+		+	+		+	+		+	+		+				+	+		+	+						+												
	+			+			+			+			+				+	+		+	+	.																	
																	+	+																					
Immune serum for *Leptospira icterohæmorrhagiæ*, Group 30 strain.	+	+	+	+	=	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
	+	+		+			+			+	+		+	+			+	+		+	+																	
	+	+		+			+			+	+		+	+			+	+		+	+																	
	+									+	+		+	+			+	+		+	+																	
Normal rabbit serum (control).	−	−	−	−	−	−	−	−	−	−	−	−	−	−		−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

exception the strongest reaction was obtained with the homologous and a less pronounced one with heterologous strains. Disintegration of the organisms was usually complete in the homologous but seldom so in heterologous combinations. Normal rabbit serum exerted neither an agglutinating nor a disintegrating influence upon any of the strains studied. On the contrary, the addition of normal rabbit serum to control tubes kept the organisms active for many days.

That these anti-*icteroides* sera did not agglutinate the various strains of *Leptospira icterohæmorrhagiæ* to any marked degree is also shown in this table. There were a few instances in which slight agglutination was observed, but none so marked as that which occurred with the strains of *icteroides*. In contrast to the results obtained with the *icteroides* strains, in no instance was there any disintegration of the *icterohæmorrhagiæ* organisms by an anti-*icteroides* serum. The Pfeiffer phenomenon was invariably positive with the anti-*icteroides* serum and the *icteroides* strains, but almost always negative when the anti-*icteroides* serum was tested with the *icterohæmorrhagiæ* strains. In two instances there was a suggestive reaction.

Table II presents the results obtained with six different monovalent anti-*icterohæmorrhagiæ* sera. The marked difference that exists between the *icterohæmorrhagiæ* strains and the *icteroides* strains is shown. Aside from slight variations, the *icterohæmorrhagiæ* strains reacted with the *icterohæmorrhagiæ* antisera quite generally and strongly and present a marked contrast to the *icteroides* strains, which reacted occasionally and never very strongly. The Pfeiffer phenomenon was positive in all combinations of the anti-*icterohæmorrhagiæ* serum with the *icterohæmorrhagiæ* group, but only occasionally and slightly with the *icteroides* group. The occurrence of a fairly marked agglutination in certain instances, such as, for example, anti-Japanese serum *versus* No. 1 *icteroides* strain or anti-group No. 11 serum *versus* No. 3 *icteroides* strain, is of considerable interest because of the occurrence of similar weak reactions among the combinations of anti-*icterohæmorrhagiæ* sera and certain heterologous *icterohæmorrhagiæ* strains. The only reliable differentiation in these instances would seem to be that of the Pfeiffer test. Beyond these few irregularities the behavior of the immune sera towards *icterohæmorrhagiæ* and *icteroides* seems to warrant the conclusion that the strains of

Leptospira icteroides and *Leptospira icterohæmorrhagiæ* form closely related but distinct groups.

Complement Fixation.—The technique used in the complement fixation tests was as follows:

Rich cultures of various strains of *Leptospira icteroides* and *Leptospira icterohæmorrhagiæ* were grown on rabbit serum media, killed by placing the culture tubes in a water bath at 60°C. for 10 minutes, and then used as antigens in the complement fixation tests. Graduated quantities (0.1, 0.05, 0.02, 0.01, 0.005 cc.) of each of the monovalent sera were mixed with a uniform quantity of the antigen (a quantity which had been found to be non-anticomplementary in several doses when tested with 0.1 cc. of complement), and to each tube was added 0.1 cc. of fresh guinea pig serum as complement. The mixtures were brought up to a total volume of 1.5 cc. for each tube by the addition of 0.9 per cent saline solution. After incubation at 37°C. for 1 hour, 0.1 cc. of a 20 per cent suspension of washed sheep corpuscles and 0.1 cc. of anti-sheep amboceptor (rabbit serum), representing three hemolytic units, were added to each tube, the contents thoroughly mixed, and once more incubated at 37°C. for 30 minutes. The results were read after standing for 1 hour. The actual quantity of each antigen used was 0.1 cc. of the killed culture, which exhibited only a slight anticomplementary property when used alone in quantities of from 0.4 to 0.6 cc.

In the majority of instances the reaction was maximum with 0.1 cc. of immune serum, and all tests with 0.005 cc. gave a negative result. The readings of the reaction obtained with 0.1 cc. of the antigens and 0.1 cc. of the immune sera are recorded in Table III.

Complete fixation took place when the immune sera were mixed with the homologous strains, both in the case of *Leptospira icteroides* and in that of *Leptospira icterohæmorrhagiæ*. Occasionally, especially among the *icteroides* strains, the fixation was as strongly positive with one or the other of the heterologous strains as with the homologous. There were a number of instances also in which a more or less definite fixation occurred when the anti-*icteroides* sera were mixed with the *icterohæmorrhagiæ* strains or the anti-*icterohæmorrhagiæ* sera with the *icteroides* strains, some reactions being as strong as ++. Generally speaking, however, there was only a limited degree of cross-reaction between *icteroides* and *icterohæmorrhagiæ*.

Variations in the fixation reaction were rather marked among the different strains of *icterohæmorrhagiæ*, according to the combinations of the immune sera and heterologous strains, some of which showed only a feeble fixation (+) with certain sera.

TABLE III.

Complement Fixation Tests.

Serum.	Strain of *Leptospira icteroides*.					Strain of *Leptospira icterohæmorrhagiæ*.								
	No. 1.	No. 3.	No. 4.	No. 5.	No. 6.	Japanese.	British.	French.	American No. 1.	American No. 2.	American No. 3.	Group 8.	Group 11.	Group 30.
Immune serum for *Leptospira icteroides*, Strain 1.	+*	+	+	+	+	+	−	−	−	−	−	−	+	−
	+	+	+	+	+									
	+			+										
	+			+										
Immune serum for *Leptospira icteroides*, Strain 5.	+	+	+	+	+	−	−	−	−	+	+	−	−	+
	+	+	+	+	+									+
	+	+		+										
	+			+										
Immune serum for *Leptospira icteroides*, Strain 6.	+	+	+	+	+	−	−	−	+	−	−	+	+	+
	+	+	+	+	+									
	+	+	+		+									
			+		+									
Immune serum for *Leptospira icterohæmorrhagiæ*, Japanese strain.	+	±	+	−	−	+	+	+	+	+	+	+	+	+
						+			+	+	+	+	+	+
						+								+
						+								
Immune serum for *Leptospira icterohæmorrhagiæ*, British strain.	−	−	−	−	−	+	+	+	+	+	+	+	+	+
						+	+	+				+		+
							+	+						
							+							
Immune serum for *Leptospira icterohæmorrhagiæ*, American Strain 1.	−	+	+	−	−	+	+	+	+	+	+	+	+	+
						+	+	+	+	+	+	+	+	+
									+		+			
									+		+			
Immune serum for *Leptospira icterohæmorrhagiæ*, Group 8 strain.	−	−	−	−	−	+	+	+	+	+	+	+	+	+
						+	+	+	+	+	+	+	+	+
									+		+	+		+
												+		

TABLE III—Concluded.

Serum.	Strain of *Leptospira icteroides*.					Strain of *Leptospira icterohæmorrhagiæ*.									
	No. 1.	No. 3.	No. 4.	No. 5.	No. 6.	Japanese.	British.	French.	American No. 1.	American No. 2.	American No. 3.	Group 8.	Group 11.	Group 30.
Immune serum for *Leptospira icterohæmorrhagiæ*, Group 11 strain.	−	+	−	−	−	+	+	+	+	+	+	+	+	+
		+				+		+	+	+	+	+	+	+
						+			+	+	+		+	
													+	
Immune serum for *Leptospira icterohæmorrhagiæ*, Group 30 strain.	−	−	−	−	+	+	+	+	+	+	+	+	+	+
						+		+	+	+	+	+	+	+
						+					+			+
						+								+

* Four plus signs vertically placed indicate no hemolysis, or complete fixation; three plus signs, about 25 per cent hemolysis; two plus signs, about 50 per cent hemolysis; one plus sign, about 75 per cent hemolysis. ± indicates almost complete hemolysis, and minus, complete hemolysis, or no fixation.

Protective Properties of Immune Sera against Leptospira icteroides.

Monovalent Immune Rabbit Sera.—Several monovalent immune rabbit sera, some specific for the *icteroides* strains and some for the *icterohæmorrhagiæ*, were tested for their protective properties against the experimental infection in guinea pigs with *icteroides* or with *icterohæmorrhagiæ*. For this purpose 1 cc. of culture of the strain to be tested was mixed with 1 cc. of immune serum and the mixture injected intraperitoneally into the guinea pig (Table IV).

It was found that Nos. 5 and 6 of the *icteroides* strains were prevented from causing any infection in guinea pigs when inoculated simultaneously, not only with their respective antisera, but also in combination with heterologous antisera. This would indicate that an immune serum prepared with one strain of *Leptospira icteroides* is equally protective against another strain of the same organism. That the protection was specific may be seen from the fact that these two strains of *Leptospira icteroides* caused either a fatal or a rather

TABLE IV.

Protective Properties of Monovalent Immune Rabbit Sera against Leptospira icteroides and Leptospira icterohæmorrhagiæ.

Serum.	Strain of *Leptospira icteroides*.		Strain of *Leptospira icterohæmorrhagiæ*.			
	No. 5.	No. 6.	Japanese.	French.	Guayaquil No. 8.	Guayaquil No. 30.
Normal rabbit serum (control).	One guinea pig died in 7 and the other in 6 days.	Died in 6 days.	Died in 9 days. Typical symptoms and lesions.	Died in 10 days. Typical symptoms and lesions.	Two guinea pigs died in 7 days. Typical lesions.	Two guinea pigs died in 8 days. Typical symptoms and lesions.
Immune Serum 945. Homologous with *Leptospira icteroides*, Strain 5.	Survived. No symptoms.	Survived. No symptoms.	Survived; severe infection with marked lesions.	Survived; severe infection.	Died in 9 days. Typical lesions.	Died in 11 days. Typical symptoms and lesions.
Immune Serum 942. Homologous with *Leptospira icteroides*, Strain 6.	Survived. No symptoms.	Survived. No symptoms.	Died in 9 days. Typical symptoms and lesions.	Survived; severe infection. Marked lesions.	Died in 8 days. Typical lesions.	Died in 10 days. Typical symptoms and lesions.
Immune Serum 911. Homologous with *Leptospira icterohæmorrhagiæ*, Japanese strain.	Died in 10 days. Typical symptoms and lesions.	Died in 10 days. Typical symptoms and lesions.	Survived. Slight lesions.	Survived. Examination showed a few lung lesions.	Survived. No symptoms.	Survived. No symptoms.

Immune Serum 947. Homologous with *Leptospira icterohæmorrhagiæ*, British strain.	Died in 9 days. Typical symptoms and lesions.	Died in 12 days. Typical symptoms and lesions.	Survived. Slight lesions.	Survived. No lesions.	Survived. No symptoms.	Survived; mild infection.
Immune Serum 952. Homologous with *Leptospira icterohæmorrhagiæ*, American Strain 1.	Died in 11 days. Typical symptoms and lesions.	Survived; mild infection.	Survived; mild infection.	Survived. No lesions.	Survived; mild infection.	Survived.
Immune Serum 904. Homologous with *Leptospira icterohæmorrhagiæ*, Guayaquil Strain 30.	Survived; severe infection.	Died of intercurrent disease.	Survived. Slight lesions.	Survived. No lesions.	Survived. No symptoms.	"

severe infection in guinea pigs when mixed with normal rabbit serum or with one or another of the antisera produced with different strains of *Leptospira icterohæmorrhagiæ*. It is noteworthy that the period of incubation when anti-*icterohæmorrhagiæ* serum and *Leptospira icteroides* are combined is somewhat prolonged. For example, with Strain 5 of *icteroides* the control animals with normal rabbit serum died in 6 or 7 days, while the guinea pigs receiving the anti-*icterohæmorrhagiæ* sera died between 9 and 11 days after the inoculation. This is also true of Strain 6, with which death occurred in 6 days in the control animal and in 10 and 12 days in the animals injected with the anti-*icterohæmorrhagiæ* sera. The symptoms and lesions were typical in all instances in which an infection ensued.

The results obtained by reversing the combinations, that is by mixing different strains of *Leptospira icterohæmorrhagiæ* with anti-*icteroides* immune sera, show also an unmistakable specificity of the protection afforded by these immune sera. A clear-cut specific protection is shown in the experiments with the Japanese and Guayaquil strains of *icterohæmorrhagiæ*; these three strains were effectively neutralized by their homologous immune sera, but not by any anti-*icteroides* immune sera, although the sera seemed to delay death in some instances. The French strain of *icterohæmorrhagiæ* was least virulent and did not cause fatal infection in guinea pigs when any one of the immune sera was simultaneously inoculated. The examination of the surviving guinea pigs for the lesions, particularly those in the lungs, after 24 days showed that the guinea pigs which received the anti-*icteroides* sera had numerous old hemorrhagic foci in the lungs, while none or only a few foci were found in those which were inoculated with the anti-*icterohæmorrhagiæ* sera.

Polyvalent Immune Horse Sera.—A horse was immunized with a mixture of cultures of four strains of *Leptospira icteroides* for a period of 65 days, during which 2,495 cc. of the cultures were injected intravenously as described in the protocol above. At the same time another horse, which had once been immunized for a period of several months in 1918 with various strains of *Leptospira icterohæmorrhagiæ*, was injected again, with *icterohæmorrhagiæ* cultures, comprising nine strains: Japanese (one strain), American (three strains), French (one strain), British (one strain), and Guayaquil (three strains).

The serum from each horse was then tested for its protective property in guinea pigs against some of the representative strains of *Leptospira icteroides* and *Leptospira icterohæmorrhagiæ*.

The procedure consisted in injecting into the peritoneal cavity of guinea pigs a mixture of a given quantity of culture (or liver emulsion) of a strain with 1 cc. of immune serum, either full strength or diluted, as indicated in Tables V and VI.

For each dose of the serum two guinea pigs were used in order to determine its titer as closely as possible. Only one guinea pig, however, was used in determining the protective titers of the anti-*icteroides* serum against the *icterohæmorrhagiæ* strains or *vice versa*. The amounts of culture (or liver emulsion) used in testing the corresponding antiserum were such as to approximate 500 minimum lethal doses, while for a cross-titration usually about 50 minimum lethal doses, or even 10 minimum lethal doses were chosen. The reason for reducing the number of minimum lethal doses in cross-protection experiments was that in several preliminary tests a larger quantity of culture was found not to be influenced to any great extent by a heterologous antiserum.

The anti-*icteroides* serum protected guinea pigs against approximately 500 minimum lethal doses of *Leptospira icteroides* in doses of 0.001 (for Strain 5) and 0.0001 cc. (for Strain 6). In other words, 1 cc. of this serum neutralizes about 500,000 to 5,000,000 minimum lethal doses of *Leptospira icteroides*, according to the degree of virulence of the culture. On the other hand, at least 1 cc. of the same serum was required to protect guinea pigs against 10 to 50 minimum lethal doses of the Japanese and French strains of *Leptospira icterohæmorrhagiæ*. 0.1 cc. of the serum averted a fatal outcome but failed to prevent wholly the infection. The difference in the protective efficacy of the anti-*icteroides* serum against *icteroides* and *icterohæmorrhagiæ* is striking.

The anti-*icterohæmorrhagiæ* serum exhibited also a marked specific protective property for the *Leptospira icterohæmorrhagiæ* strains, neutralizing at least 5,000,000 (for the French) to 500,000 (for the Japanese) minimum lethal doses per 1 cc. Its effect upon the *icteroides* strains was in each case feeble but distinct, since it protected guinea pigs completely against 50 minimum lethal doses per 1 cc.

TABLE V.

Protective Properties of Polyvalent Anti-icteroides Serum against Leptospira icteroides and Leptospira icterohæmorrhagiæ.

Anti-*icteroides* serum.	Strain of *Leptospira icteroides*.		Strain of *Leptospira icterohæmorrhagiæ*.	
	No. 5. 0.1 cc. of culture (about 500 M. L. D.).	No. 6. 0.1 cc. of culture (about 500 M. L. D.).	Japanese. 0.1 cc. of liver emulsion (about 10 M. L. D.).	French. 0.1 cc. of culture (about 50 M. L. D.).
cc.				
0.000001	Died in 7 days. Typical symptoms and lesions.	Died in 7 days.	Died in 7 days.	Died in 9 days.
0.000001	Died in 9 days. Typical symptoms and lesions.	" " 10 "		
0.00001	Survived.	" " 9 "	Died in 6 days.	Died in 10 days.
0.00001	Died in 9 days.	Survived.		
0.0001	" " 9 "	"	Survived (!).	Died in 8 days.
0.0001	Survived.	"		
0.001	"	"	Died in 14 days.	Died in 13 days.
0.001	"	"		
0.01	"	"	Died in 10 days.	Died in 13 days.
0.01	"	"		
0.1	"	"	Survived. Had fever.	Survived. Had fever.
0.1	"	"		
1.0	"	"	Survived. Had fever.	Survived. Had fever.
1.0	"	"		
Normal horse serum 1.0 cc. (control).	Died in 7 days. Typical symptoms and lesions.	Died in 7 days. Typical symptoms and lesions.	Died in 7 days. Typical symptoms and lesions.	Died in 8 days.

and prevented death, but not infection, when used in a dose of 0.1 cc. It will be seen, therefore, that the respective polyvalent antisera exert a powerful annihilating effect in guinea pigs upon their corresponding type organisms, but there also exists an undeniable, though

feeble, cross-protective reaction, which may be explained by assuming that the two groups of organisms are not altogether alien but are closely related to each other; they may even constitute two subspecies or races.

TABLE VI.

Protective Properties of Polyvalent Anti-icterohæmorrhagiæ Serum against Leptospira icterohæmorrhagiæ and Leptospira icteroides.

Anti-*icterohæmorrhagiæ* serum.	Strain of *Leptospira icterohæmorrhagiæ*.		Strain of *Leptospira icteroides*.	
	Japanese. 0.1 cc. of culture (about 500 M. L. D.).	French. 1 cc. of culture (about 500 M. L. D.).	No. 1. 0.1 cc. of culture (about 50 M. L. D.).	No. 5. 0.01 cc. of culture (about 50 M. L. D.).
cc.				
0.000001	Died in 6 days.	Died in 10 days.	Died in 8 days.	Survived (!).
0.000001	" " 7 "	" " 13 "		
0.00001	" " 6 "	Survived.	Died in 7 days.	Died in 8 days.
0.00001	" " 11 "	Died in 11 days.		
0.0001	Survived.	Survived.	Died in 8 days.	Died in 8 days.
0.0001	Died in 8 days.	"		
0.001	Survived.	"	Died in 7 days.	Died in 9 days.
0.001	"	"		
0.01	"	"	Died in 8 days.	Died in 15 days.
0.01	"	"		
0.1	"	"	Survived; fever.	Survived; mild jaundice.
0.1	"	"		
1.0	"	"	Survived.	Survived.
1.0	"	"		
Normal horse serum 1.0 cc. (control).	Died in 7 days.	Died in 7 days.	Died in 6 days.	Died in 7 days.

Active Immunity.

Guinea pigs vary considerably in their susceptibility to *Leptospira icteroides*. It sometimes happens that a culture which kills guinea pigs of average susceptibility in a dose of about 0.0001 cc. occasionally

fails to produce a fatal infection in a guinea pig in a dose as large as 0.01 or 0.1 or even 1 cc. Such refractory guinea pigs are rarely met with, but the fact that there exist certain unusually resistant individuals is of great interest. It has also been found that there are exceptionally susceptible individuals which respond to infection with an attenuated culture which no longer attacks the average guinea pig. For example, with certain cultures of *Leptospira icteroides* which had been repeatedly subcultured without passage through the guinea pig for many months, only one out of several animals inoculated with the same culture may come down with typical symptoms. In fact, when the first attempt to restore the virulence of the culture by animal passage failed, a second or third attempt with four or five guinea pigs each time was necessary to obtain a single positive result. These facts furnish possible explanations for certain paradoxical results which are sometimes encountered in determining the state of immunity.

In a discussion of active immunity we may distinguish between that which arises from recovery from a genuine infection and that which follows the inoculation of killed organisms. Animals which, after receiving an inoculation of a sublethal dose of a live culture, do not react definitely, may acquire a state of immunity similar to that of vaccinated animals.

The results with the guinea pigs which had recovered from a more or less pronounced infection after the inoculation of a culture or blood derived from a guinea pig dying of the typical infection with *Leptospira icteroides* will first be described. The tests consisted in the Pfeiffer phenomenon and the effect of the inoculation of different cultures in case of a negative Pfeiffer reaction.

Series 1.

Four guinea pigs were actively immunized with Strain 5 of *Leptospira icteroides*.

Guinea Pig Ch 1.—Nov. 27, 1918. Received 1 cc. of citrate blood from a guinea pig which had been infected with Strain 5 of *Leptospira icteroides* and showed the typical symptoms of *icteroides* infection. After the usual course of infection (fever, slight icterus, albuminuria) the animal became well within 2 weeks. Dec. 19. Received 1 cc. of culture of the same strain. No symptoms followed. Jan. 6, 1919. Received another injection of culture of the same strain, without any perceptible effect.

Pfeiffer Reaction.—Jan. 18. A rich culture of the same strain (1 cc.) was injected intraperitoneally. A prompt positive reaction was obtained. Jan. 22. The Pfeiffer reaction was tested with Strain 6, 1 cc. of a rich culture being used. Result positive. On the same day, 4 hours later, another Pfeiffer test was made on this animal with the Japanese strain of *Leptospira icterohæmorrhagiæ*, 1 cc. of culture being used. Result negative. This animal died in 23 days with the typical symptoms of *icterohæmorrhagiæ* infection.

Guinea Pig Ch 2.—The procedure with this animal was similar to that with Guinea Pig Ch 1.

Pfeiffer Reaction.—Jan. 18, 1919. Tested with 1 cc. of culture of the same strain. Result positive. Jan. 22. Pfeiffer test with 1 cc. of Strain 6 culture was positive. The same day, 4 hours later, tested with 1 cc. of French strain of *Leptospira icterohæmorrhagiæ*. Result negative. This animal died in 14 days with the typical symptoms of *icterohæmorrhagiæ* infection.

Guinea Pig Ch 3.—The procedure with this animal was similar to that with Guinea Pig Ch 1.

Pfeiffer Reaction.—Jan. 18, 1919. Test with 1 cc. of the same strain was positive. Jan. 22. Test with 1 cc. of the British strain of *Leptospira icterohæmorrhagiæ* was doubtful. The animal survived.

Guinea Pig Ch 4.—The procedure with this animal was similar to that with Guinea Pig Ch 1.

Pfeiffer Reaction.—Jan. 18, 1919. With 1 cc. of culture of the same strain test was positive. Jan. 22. With 1 cc. of culture of Strain 6 of *Leptospira icteroides* test was positive. The animal survived.

Series 2.

Three guinea pigs, out of a large number inoculated with Strain 6 of *Leptospira icteroides*, which suffered more or less severe infection and eventually recovered, were reinoculated with a culture of the same strain once or twice (as described in the protocols below) afterwards, but showed no characteristic symptoms, except a rise of temperature for a day in one (Guinea Pig C 2). Hence they were completely immune to the same strain and were used to test their resistance to certain other strains, including the Japanese strain of *icterohæmorrhagiæ*.

Guinea Pig C 1.—Dec. 3, 1918. Received 1 cc. of Strain 6 culture of *Leptospira icteroides*. The animal had typical fever and a trace of jaundice, but recovered in about 9 days. Two more injections of 1 cc. of the same culture were given, one on Dec. 19, and another on Jan. 6, 1919.

Pfeiffer Reaction.—Jan. 18. With 1 cc. of a rich culture of the same strain, a prompt and positive reaction. Jan. 22. Inoculated with 1 cc. of a rich culture of Strain 5 of *Leptospira icteroides*. Pfeiffer reaction positive. No symptoms within 1 month.

Guinea Pig C 2.—Dec. 11, 1918. Received 1 cc. of blood from a guinea pig showing typical symptoms (fever and slight jaundice). The animal eventually

recovered. Jan. 6, 1919. 1 cc. of a rich culture of the same strain was given. There was slight fever for a day, but no infection followed.

Pfeiffer Reaction.—Jan. 18. 1 cc. of a culture of the same strain was inoculated intraperitoneally, but no organism could be found in the peritoneal exudate after 30 minutes. Jan. 22. The animal was tested again for the Pfeiffer reaction, with 1 cc. of a rich culture of Strain 1 of *Leptospira icteroides*. There was a complete positive reaction within 30 minutes, and no infection followed the inoculation.

Guinea Pig C 3.—This animal was immunized in the same way as Guinea Pig C 2.

Pfeiffer Reaction.—Jan. 18, 1919. 1 cc. of a rich culture of the same strain was injected intraperitoneally. Reaction prompt and complete. Jan. 22. 1 cc. of a rich culture of the Guayaquil strain, Group 30, of *Leptospira icterohæmorrhagiæ* was inoculated intraperitoneally. When examined after 2 hours the organisms were partially agglutinated, but most of them were actively motile. On the 4th day the temperature rose and remained above normal for 3 days. There was a slight jaundice on the 9th and 10th days, which soon faded. The animal survived. In this instance there existed a mild infection with the *icterohæmorrhagiæ* strain.

Series 3.

Nov. 27, 1918. Three guinea pigs were inoculated with a culture of the Japanese strain of *Leptospira icterohæmorrhagiæ*. A second injection was given on Dec. 19, and a third on Jan. 6, 1919. The animals showed a definite but mild infection after the first inoculation, but recovered. No symptoms followed the second or third injection of the same strain.

Guinea Pig J 1. Pfeiffer Reaction.—Jan. 18, 1919. With a culture of the Japanese strain, test positive. The animal remained well.

Guinea Pig J 2. Pfeiffer Reaction.—Jan. 18, 1919. With a rich culture of Strain 5 of *Leptospira icteroides*, test negative. The animal survived, passing through a moderately severe *icteroides* infection.

Guinea Pig J 3. Pfeiffer Reaction.—Jan. 18, 1919. Pfeiffer reaction with a rich culture of Strain 6 of *Leptospira icteroides* not clear-cut. There was a tendency to formation of agglomerated masses, without immobilization or lysis of the organisms. The animal showed mild but typical symptoms of *icteroides* infection after 13 days, but eventually recovered.

Supplementary Experiment with Anti-icterohæmorrhagiæ Serum.

Jan. 16, 1919. A number of guinea pigs were inoculated with mixtures of a polyvalent anti-*icterohæmorrhagiæ* horse serum prepared by Inada and Ido and different strains of *Leptospira icterohæmorrhagiæ* and *Leptospira icteroides*, with a view to determining the protective property of this serum against the *icterohæmorrhagiæ* as well as the *icteroides* strains. 1 and 0.1 cc. of the serum were used, mixed with 0.5 cc. of a culture of each strain, and inoculated at once into the peritoneal cavity

of guinea pigs. The protective titer of the serum had previously been tested against the Japanese strain of *Leptospira icterohæmorrhagiæ* and the serum had been found to neutralize 1 cc. of the culture in a dose of 0.001 cc. The result of the present experiment showed that the guinea pigs which received the immune serum and cultures of the Japanese, French, American, and Guayaquil strains of *icterohæmorrhagiæ* survived, while the control animals without the serum died in 6, 9, 8, and 7 days respectively. There were no symptoms observed in the surviving animals at any time; they were completely protected by the serum in the doses given (1 and 0.1 cc.). All the guinea pigs inoculated with a culture of the British strain of *icterohæmorrhagiæ* with or without (one control) the addition of the immune serum survived; that is, the culture employed was apparently avirulent.

The result obtained with the strains of *Leptospira icteroides* was somewhat surprising. The strains used in this group were Nos. 5 and 6, which killed the control animals in 5 and 10 days respectively Both cultures, however, produced only a temporary febrile reaction when injected together with 1 cc. of the serum and a moderately severe but non-fatal infection with 0.1 cc.

All the guinea pigs surviving in this series of experiments were subjected on Feb. 10, 25 days after the first inoculation, to immunity tests with cultures of various strains. The guinea pig which had received on Jan. 16 the mixture of the Japanese strain and 1 cc. of anti-*icterohæmorrhagiæ* serum escaped infection and was inoculated with 0.5 cc. of a culture of Strain 5 of *Leptospira icteroides* on Feb. 10. It died with typical symptoms of *icteroides* infection in 7 days. The guinea pig which escaped infection with the mixture of the French strain and 1 cc. of anti-*icterohæmorrhagiæ* serum on Jan. 16 and was injected with 0.5 cc. of Strain 5 of *icteroides* on Feb. 10 died in 5 days with typical symptoms of *icteroides* infection. The guinea pig which escaped infection with the mixture of American Strain 1 and 1 cc. of anti-*icterohæmorrhagiæ* serum on the first injection and was inoculated with 0.5 cc. of Strain 5 of *icteroides* on Feb. 10 finally recovered after a moderately severe *icteroides* infection.

As already mentioned, the guinea pigs which were inoculated on Jan. 16 with 1 and 0.1 cc. of anti-*icterohæmorrhagiæ* serum, together with the cultures of Strains 5 and 6 of *Leptospira icteroides*, had a temporary febrile reaction or a moderately severe infection. These animals were injected on Feb. 10, 25 days later, with a culture of the Japanese strain. All except one, which showed only slight lesions in the lungs, died with the typical symptoms of *icterohæmorrhagiæ* infection in 8 to 9 days. This would seem to indicate that these animals were protected by the anti-*icterohæmorrhagiæ* serum from the fatal outcome of the *icteroides* inoculations, but they were not rendered immune against the Japanese strain of *icterohæmorrhagiæ* when tested 25 days afterwards. Also the anti-*icterohæmorrhagiæ* serum injected on the first occasion had no perceptible protective action against this strain after a period of 25 days.

SUMMARY AND CONCLUSIONS.

It has been previously reported[3] that a filterable microorganism belonging to the genus *Leptospira* has been recovered from the blood or organs of human beings suffering from the disease known as yellow fever in Guayaquil, and that the organism, which has been termed *Leptospira icteroides*, induces in certain experimental animals the characteristic symptoms and lesions observed in the patients from whom it was isolated. It has also been previously shown[1] that the serum from patients recovering from an attack of yellow fever in Guayaquil had the power to agglutinate and dissolve the organism when introduced into the peritoneal cavity of a normal guinea pig (Pfeiffer phenomenon). Moreover, the guinea pigs which had once been inoculated with the blood of yellow fever patients without succumbing to the infection, notwithstanding the fact that they had shown a definite febrile reaction after 4 to 5 days, were found to be refractory to a subsequent inoculation of a culture of *Leptospira icteroides*.[4] All these observations pointed to the possible relation of this organism to the disease known as yellow fever in Guayaquil. The demonstration of the filterability of the organism[5] and the transmission of the infection with the same organism by *Stegomyia calopus*[6] have further strengthened the probable etiological significance of the organism in yellow fever.

It was by no means a simple problem to determine the relation existing between *Leptospira icteroides* and *Leptospira icterohæmorrhagiæ*. An experiment reported in a previous paper seemed to justify the view that the two leptospiras[7] are closely related but not identical, yet it was necessary to exhaust various other modes of differentiation before the distinction between them was firmly established. The present paper continues this phase of the inquiry in further detail.

There have been taken up here the phenomena of agglutination, the reaction of Pfeiffer, complement fixation, the protective properties of various monovalent and polyvalent immune sera, and active

[3] Noguchi, H., *J. Exp. Med.*, 1919, xxix, 547, 565, 585; xxx, 87.
[4] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 1.
[5] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 13.
[6] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 401.
[7] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 95.

immunity. As the result of experiments in connection with these immunity phenomena the following data are presented.

Monovalent immune sera prepared by several successive injections in an animal naturally refractory to *Leptospira icteroides* possess the power to agglutinate *in vitro* not only the homologous strains, but also all other strains of *icteroides* tested. On the other hand, a slight effect, or none at all, has been observed when these immune sera have been mixed *in vitro* with various strains of *Leptospira icterohæmorrhagiæ*. A similar relation exists between the monovalent anti-*icterohæmorrhagiæ* sera and the various strains of *Leptospira icteroides*; that is, there is a slight agglutinating effect in some instances upon the *icteroides* strains, but it is never so strong as that occurring in tests against the *icterohæmorrhagiæ* strains. The Pfeiffer reaction gave a sharper differentiation between the two groups, for in most instances the phenomenon was specific for the group. There were occasional doubtful reactions, but not enough to warrant a confusion of the two groups.

Polyvalent immune sera, one specific for *icteroides*, and the other for *icterohæmorrhagiæ*, showed a high titer of neutralizing power for the cultures of the homologous groups. It was found, however, that the action of the sera is by no means absolutely specific, because the injection of a sufficient amount of the anti-*icteroides* serum apparently prevented a fatal outcome in a guinea pig inoculated with multiple minimum lethal doses of a culture of *Leptospira icterohæmorrhagiæ*, and *vice versa*. The specificity of the serum was demonstrated only when it was used in smaller quantities.

More or less specificity was shown by the complement fixation reaction, but it was not absolute. Weak fixation occurred when the anti-*icteroides* serum was mixed with one or the other of the *icterohæmorrhagiæ* strains and *vice versa*, and strong fixation occurred only when the antiserum was mixed with one of the *icteroides* strains. The question naturally arises whether or not this apparent specificity is due to the homology of the serum and not altogether to a difference in genus of the strains. In other words, it is justifiable to question whether all these variations in the degree of intensity of the reaction are not due to strain variations of the same genus. This question is not finally settled by the present investigation, in which only four

icteroides and nine *icterohæmorrhagiæ* strains have been carefully studied. Nevertheless, on the basis of the findings with these thirteen strains, it seems probable that *Leptospira icteroides* and *Leptospira icterohæmorrhagiæ* are closely allied but are nevertheless distinct in their immunological reactions. Perhaps the difference between the two may amount to that between subspecies or races. It has been pointed out earlier that the pathogenicity of the two is also distinct, inasmuch as *icteroides* produces chiefly icterus and nephritis and *icterohæmorrhagiæ* hemorrhage and nephritis, the icterus being less and the hemorrhage more prominent in the evolution of the latter infection.

In the study of active immunity—exclusive of vaccination—difficulty has been experienced in the evaluation of the results, owing to the existence of natural resistance to infection among guinea pigs. A guinea pig may recover from the inoculation of *Leptospira icteroides* and then resist a subsequent inoculation with a virulent strain of *Leptospira icterohæmorrhagiæ*, a condition simulating that brought about by the identity of the two organisms. However, the refractoriness of such an animal to *icterohæmorrhagiæ* may be due to its natural immunity to it. In the present study, therefore, only those guinea pigs were selected which had reacted typically—though in mild degree—to the *icteroides* infection, in order to determine whether they were subsequently immune to the inoculation of *icterohæmorrhagiæ*. Indeed, by this mode of experimentation it was found that the guinea pigs which had once passed through an attack of the *icteroides* infection were absolutely immune to a second infection with the same organism but reacted severely and sometimes fatally to a later inoculation of *icterohæmorrhagiæ*. Although there were a number of instances in which a previous infection with *icteroides* did not confer any perceptible immunity upon the guinea pigs against *icterohæmorrhagiæ*, another group of guinea pigs showed a considerable resistance to the *icterohæmorrhagiæ* infection as compared with those which had never been inoculated with *icteroides*. There is not much doubt, therefore, that an *icteroides* attack brings about, in some instances at least, a certain degree of resistance to the *icterohæmorrhagiæ* infection. Hence the study of the phenomena of active immunity strongly indicates that *icteroides* is closely related immunologically to *icterohæmorrhagiæ*.