ETIOLOGY OF YELLOW FEVER.

XII. Chemotherapy versus Serotherapy in Experimental Infection with Leptospira icteroides.

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The work of Ehrlich and Hata[1] on the therapeutic effects of arsenical organic compounds in various infections caused by spirochetal or protozoan organisms has led to considerable investigation by later workers, notably Jacobs, Heidelberger, Amoss, and Bull[2–5] and Pearce and Brown,[6] of the extent to which this kind of curative method is applicable. The present experiments were undertaken, not as research in chemotherapy, but merely as part of a routine study of experimental infection in guinea pigs with *Leptospira icteroides*, derived from certain cases of yellow fever.[7] It was of particular interest to ascertain how *Leptospira icteroides* would behave toward the two widely employed chemotherapeutic agents, salvarsan and neosalvarsan, and what difference, if any, there is between its behavior and that of the inciting agent of infectious jaundice in this respect, for the latter has been extensively studied by Inada, Ido, and their collaborators,[8] as well as

[1] Ehrlich, P., and Hata, S., Die experimentelle Chemotherapie der Spirillosen, Berlin, 1910.

[2] Jacobs, W. A., *J. Exp. Med.*, 1916, xxiii, 563.

[3] Jacobs, W. A., Heidelberger, M., and Amoss, H. L., *J. Exp. Med.*, 1916, xxiii, 569.

[4] Jacobs, W. A., Heidelberger, M., and Bull, C. G., *J. Exp. Med.*, 1916, xxiii, 577.

[5] Jacobs, W. A., and Heidelberger, M., *J. Biol. Chem.*, 1915, xx, 513, 659, 685; xxi, 103, 145, 403, 439, 455, 465.

[6] Pearce, L., and Brown, W. H., *J. Exp. Med.*, 1918, xxviii, 109.

[7] Noguchi, H., *J. Exp. Med.*, 1919, xxix, 547, 565, 585; xxx, 1, 9, 13, 87, 95, 401; 1920, xxxi, 135, 159.

[8] Inada, R., Ido, Y., Hoki, R., Kaneko, R., and Ito, H., *J. Exp. Med.*, 1916, xxiii, 377. Kaneko, R., and Okuda, K., *Verhandl. japan. path. Ges.*, 1916, vi, 49.

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by European investigators, especially Uhlenhuth and Fromme.[9] A large number of experiments with salvarsan and neosalvarsan, atoxyl, and arsacetin against *Leptospira icterohæmorrhagiæ* was also carried out in 1917, with negative results.[10] Being regarded by some as a spirochete, *Leptospira icterohæmorrhagiæ* was expected to yield to chemotherapy by means of salvarsan or its derivative, but Inada and Ido and others soon found that neither salvarsan nor neosalvarsan had any definite therapeutic value in infections with this organism. In this respect, at least, the organism discovered by Inada and Ido in infectious jaundice did not behave like the other pathogenic spirochetes, and this characteristic may be regarded as giving further support to the opinion that the leptospira group of organisms forms a type of its own and differs from the other pathogenic genera of Spirochætoidea. The experiments here reported concern the behavior of *Leptospira icteroides* toward the arsenical compounds, not only in the animal body, but also *in vitro*, and the effect upon the organism of salvarsanized serum. From the practical standpoint it seemed advisable to include also a brief comparison of the action of the arsenical preparations and that of anti-*icteroides* immune serum upon *Leptospira icteroides* in experimental infection and *in vitro*.

Effect of Salvarsan and Neosalvarsan upon the Course of Experimental Infection of Guinea Pigs Inoculated with Leptospira icteroides.

Several series of experiments were performed in order to ascertain whether administration of salvarsan or neosalvarsan simultaneously with the inoculation or shortly afterwards would in any way influence the development of the experimental leptospiral infection in guinea pigs. If the virulence of *Leptospira icteroides* for this animal were constant a few series of experiments only would have been sufficient to determine the point, but owing to the variable character of the pathogenicity of the organism for individual guinea pigs it was necessary to repeat similar experiments. In some series control animals survived or escaped infection, hence the interpretation of the effect of the drugs was rendered inconclusive. In the earlier experiments

[9] Uhlenhuth and Fromme, *Z. Immunitätsforsch., Orig.*, 1916, xxv, 418.

[10] Noguchi, H., unpublished results.

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the amounts of infecting material were near a single lethal dose, even subminimum lethal doses being used; *i.e.*, a quantity capable of producing in the majority of instances a mild infection with recovery. The mode of experiment was practically the same in all the series. In one instance an unneutralized solution of salvarsan was employed, otherwise salvarsan has been used as an alkaline solution. The injection of the infecting material was intraperitoneal and that of the drugs subcutaneous.

TABLE I.

Inoculation of Infecting Material into Guinea Pigs Followed by Injection of Drug.

November 27, 1919. The amount of infecting material used was 0.5 cc.

Amount of arsaminol-natrium.	Course of disease.	Result.	Remarks.
gm.			
0.03	Mild; 6 to 8 days.	Recovered.	Died in 15 days; no jaundice.
0.02	Moderate (+); 6 to 11 days.	"	No jaundice.
0.01	Severe (++++); 6 to 11 days.	"	Died in 13 days; jaundice fading.
0.005	Mild; 7 to 9 days.	"	No jaundice.
0.0025	Severe (++++); 6 to 11 days.	"	Killed in 11 days; typical lesions.
0.001	Died in 6 days.	Typical.	
0.0005	" " 9 "	"	
Control.	Severe (+++); killed in 6 days.	"	
"	Moderate (++); 7 to 8 days.	Recovered.	
"	Mild (±); 7 days.	"	

Series 1.—November 27, 1919. The infecting material consisted of a mixture of citrate blood (showing the leptospiras) of a guinea pig experimentally infected with Guayaquil Strain 1 and a rich culture of the same strain. Ten guinea pigs were inoculated intraperitoneally, each with 0.5 cc. of the mixture, and all but three (controls) then received subcutaneously a solution of arsaminolnatrium (a Japanese preparation of neosalvarsan), the quantities given ranging from 0.03 to 0.0005 gm. (Table I).

The amount of infecting material used in this series was near a single minimum lethal dose, as shown in the control animals and also in those which received the smallest amounts of the drug. It is noteworthy that in the three guinea pigs which received 0.03, 0.02,

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and 0.005 gm. of the drug, respectively, there was no jaundice at any period of the infection.

Series 2.—December 3, 1919. The infecting material consisted of a mixture of 1 cc. each of cultures of Guayaquil Strains 1 and 5, and 7 cc. of heart's blood from a guinea pig infected typically with Strain 1. 0.5 cc. of the mixture was inoculated intraperitoneally into each of sixteen guinea pigs. One group of eight animals was then inoculated subcutaneously with an unneutralized solution of salvarsan and the other group of eight with the solution of neosalvarsan in doses

TABLE II.

Inoculation of Infecting Material into Guinea Pigs Followed by Injection of Drug.

December 3, 1919. The amount of infecting material used for the treated animals was 0.5 cc.

Treated animals.			Untreated controls.	
Amount of drug.	Course of disease.		Test for virulence of infecting material used.	Course of disease.
	Salvarsan (acid solution).	Neosalvarsan.		
gm.			*cc.*	
0.03	Mild; recovery.	Mild; recovery.	1.0	Severe (++++); recovery.
0.02	No symptoms.	Severe (+++); recovery.	0.5	Mild; recovery.
0.01	" "	Mild; recovery.	0.2	Moderate (++); recovery.
0.005	" "	No symptoms.	0.1	No symptoms.
0.002	Died in 12 days; typical.	" "		
0.001	No symptoms.	" "		
0.0005	" "	Died in 13 days; typical.		
0.0002	" "	Severe (+++); recovery.		

varying from 0.03 to 0.0002 gm. There were four control guinea pigs which received varying doses of the infecting material and were not treated with the drug (Table II).

The results of these experiments suggest a possible slight protective effect of the drugs in some individuals, although, from the mildness of the infection in the control animals the survival of those individuals may also be explained on the basis of a natural resistance to *Lepto-*

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spira icteroides. It is of interest to note that more guinea pigs among those treated with salvarsan escaped the infection than among those treated with neosalvarsan. The indecisive character of the experiments made necessary another series with multiple minimum lethal doses. In Series 3 at least 50 minimum lethal doses were used for each guinea pig.

Series 3.—March 13, 1920. The infecting material consisted of a mixture of 5 cc. of a culture of Guayaquil Strain 1, 5 cc. of kidney emulsion, and 5 cc. of citrated heart's blood from a guinea pig inoculated with the same strain 4 days previously. In order to produce a fatal infection in guinea pigs 0.5 cc. of the mixture was inoculated intraperitoneally into each animal. As the protocol shows, 0.01 cc. of this material killed a control animal in 7 days with typical symptoms, and a much smaller quantity would have been sufficient to cause a fatal infection, although the minimum lethal dose was not determined in the present series of experiments.

Salvarsan was dissolved in sterile distilled water and a 0.1 N sodium hydroxide solution was gradually added until the precipitate first formed was completely redissolved. The final concentration of the drug was made 0.1 gm. per 10 cc. (stock solution). Neosalvarsan was dissolved in sterile distilled water in the ratio of 0.1 gm. per 10 cc. (stock solution). Further dilutions were made with 0.9 per cent salt solution.

The animals were inoculated with 0.5 cc. of the infecting material intraperitoneally, and within about 30 minutes varying amounts of salvarsan and neosalvarsan solution were injected subcutaneously. The amounts of the drug were 0.00005, 0.0001, 0.0002, 0.0005, 0.001, 0.002, and 0.003 gm. for guinea pigs of 350 gm., and each dose was contained in from 0.5 to 3 cc. of fluid, according to convenience in measurement. Table III gives the results.

Series 4.—March 18, 1920. Although it was still too early to know the results of the series of March 13, another series was begun on March 18 in which larger doses (0.01, 0.02, and 0.03 gm.) of salvarsan and neosalvarsan were used. The solutions of the drugs were freshly prepared from another set of ampules, and the infecting material consisted of a guinea pig kidney emulsion of Guayaquil Strain 1 of *Leptospira icteroides*. Other technical details were the same as in the previous experiments. Table IV gives the results.

The experiments of Series 3 (March 13) show that guinea pigs receiving at least 50 minimum lethal doses of a strain of *Leptospira icteroides* intraperitoneally and varying quantities of salvarsan or neosalvarsan subcutaneously within 30 minutes from the time of inoculation of *icteroides* undergo a typical course of leptospira infection, resulting in the majority of instances in death within a

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period the variations of which may be considered usual in such a series of experiments. With salvarsan there were two instances of recovery after a severe infection coinciding with elevated doses of the drug (0.001 and 0.003 gm.), while in animals receiving less of the

TABLE III.

Inoculation of Infecting Material into Guinea Pigs Followed by Injection of Drug.

March 13, 1920. The amount of infecting material used for the treated animals was 0.5 cc.

Treated animals.			Untreated controls.	
Amount of drug.	Course of disease.		Test for virulence of infecting material used.	Course of disease.
	Salvarsan.	Neosalvarsan.		
gm.			*cc.*	
0.00005	Died in 5 days; typical.	Recovery after a mild infection, with definite jaundice from Mar. 20–24, 1920.	0.5	Died in 6 days; typical.
			0.1	Died in 7 days; typical.
0.0001	" " 8 " "	Died in 5 days; typical.	0.01	Died in 7 days; typical.
0.0002	" " 5 " "	" " 6 " "		
0.0005	" " 6 " "	" " 7 " "		
0.001	Recovery after a typical infection, with intense jaundice from Mar. 19–25, 1920.	" " 9 " "		
0.002	Died in 6 days; typical.	Recovery after a mild infection, with jaundice from Mar. 19–23, 1920.		
0.003	Recovery after a typical infection lasting from Mar. 18–20, 1920.	Recovery after a severe infection, with intense jaundice from Mar. 18–23, 1920.		

drug death occurred in two instances 2 days earlier than in controls, a fact worthy of notice. With neosalvarsan the number of recovered animals was three, two with the largest doses of the drug (0.002 and 0.003 gm.) and one with the minutest dose employed (0.00005 gm.).

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All the rest died in from 5 to 9 days with typical symptoms. The results both with salvarsan and with neosalvarsan suggest that these arsenical compounds, when employed in certain quantities, may somewhat modify the severity of the infection and occasionally save a guinea pig from death, although failing to suppress the infection completely.

The results of the experiments of Series 4 (March 18) were less favorable than those of Series 3. Here, of the two controls one died in 12 days and the other recovered after a definite infection. Two

TABLE IV.

Inoculation of Infecting Material into Guinea Pigs Followed by Injection of Drug.

March 18, 1920. The amount of infecting material used for the treated animals was 0.5 cc.

Treated animals.			Untreated controls.	
Amount of drug.	Course of disease.		Test for virulence of infecting material used.	Course of disease.
	Salvarsan.	Neosalvarsan.		
gm.			*cc.*	
0.01	Died in 11 days; typical.	Died in 12 days; typical.	0.5	Died in 12 days; typical.
0.02	Had fever but no jaundice; recovery.	" " 12 " "	0.5	Recovery after a definite infection, with intense jaundice from Mar. 30–31, 1920.
0.03	Died in 11 days; typical.	Had fever but no jaundice; recovery.		

of the three guinea pigs treated, either with salvarsan or neosalvarsan, died in from 11 to 12 days, and the one animal which survived in each set had fever indicative of an abortive leptospira infection.

From the therapeutic standpoint neither salvarsan nor neosalvarsan is of any value in experimental infections of guinea pigs with *Leptospira icteroides*. In contrast, it may be interesting to include here a protocol illustrating the highly specific protective value of an anti-*icteroides* serum (horse). The amount of the immune serum required to protect a guinea pig from an infection with *Leptospira icteroides* is exceedingly minute.

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Contrasted Effect of Anti-icteroides Immune Serum in Vivo.

March 11, 1920. The experiments with the immune serum were undertaken with the same strain of *Leptospira icteroides* (Guayaquil No. 1) that was used in the chemotherapeutic experiments already described. The material was a mixture of the emulsions of kidney and liver from a guinea pig which was showing early symptoms of the leptospira infection. As the protocol shows, the minimum lethal dose of this emulsion was such that 1 cc. of a 1:10,000 dilution of it killed a guinea pig with typical symptoms in 12 days, and the same quantity of a 1:1,000 or 1:100 in 10 days. 0.5 cc. of the original emulsion killed a guinea pig in 8 days.

TABLE V.

Inoculation of Infecting Material into Guinea Pigs Followed by Injection of Anti-icteroides Serum.

March 11, 1920. The amount of infecting material used for the treated animals was 0.5 cc.

Treated animals.			Untreated controls.	
Anti-*icteroides* immune serum.		Course of disease.	Test for virulence of infecting material used.	Course of disease.
Amount.	Dilution.			
cc.			*cc.*	
1	1:100,000	Recovery after a mild infection.	0.5	Died in 8 days; typical.
			0.1	Recovery after a severe infection (exceptional resistance).
1	1:10,000	No symptoms.		
1	1:1,000	" " (suspicion of trace of jaundice on Mar. 20, which had disappeared the following morning).	0.01	Died in 10 days; typical
			0.001	" " 10 " "
			0.0001	" " 12 " "
			0.00001	No symptoms.
1	1:100	No symptoms.	0.000001	" "
1	1:10	" "		

For infecting guinea pigs to be treated with anti-*icteroides* serum, 0.5 cc. (about 5,000 minimum lethal doses) of the same emulsion was intraperitoneally injected. The different amounts of the anti-*icteroides* serum (collected from Horse 2 on February 25, 1920) were then injected, also intraperitoneally, within half an hour after the inoculation. The amounts of immune serum were 0.00001, 0.0001, 0.001, 0.01, and 0.1 cc. Table V is a record of the results.

According to a conservative estimate, therefore, the power of the immune serum is such that 1 cc. of a 1:10,000 dilution prevented an infection when the dose of the infecting material was 5,000 minimum

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lethal doses. In other words, 1 cc. of the serum had the power to protect a guinea pig of 350 gm. body weight against 50,000,000 (10,000 × 5,000) minimum lethal doses when administered intraperitoneally 30 minutes after intraperitoneal inoculation. The specific protective property of the immune serum is indisputably highly efficacious as compared with salvarsan or neosalvarsan, the value of which is at least doubtful. A point of considerable importance is that in certain guinea pigs receiving small quantities of salvarsan and neosalvarsan the period before death seemed to be shortened by 2 days (5 days), as compared with the average period in untreated control animals (7 days). It may be that the predilection of arsenic compounds for the renal tissues had a definite predisposing effect, due to chemical injury, upon this organ, which *Leptospira icteroides* also attacks particularly.

Direct Action of Salvarsan and Neosalvarsan upon Leptospira icteroides.

Ehrlich makes a special point of having found, in his quest for chemotherapeutic agents, that a preparation whose destructive power on a microbic organism is great *in vitro* may have no, or only a slight antagonistic effect when introduced into the animal body, or the relation between the effect manifested *in vitro* and *in vivo* may be the reverse. Ehrlich's efforts to find a chemotherapeutic preparation were principally directed toward its effect *in vivo*, since the pathogenic parasites with which he was working belonged to the class of protozoa or a class closely allied to it, and there were no virulent cultures on hand to be tested *in vitro* as well as *in vivo*. Certain arsenic compounds elaborated by Ehrlich and his coworkers displayed a highly sterilizing effect on various spirochetoid organisms when introduced into the animal body infected with them, while the direct effect of these preparations upon the same organisms *in vitro* was almost nil. Ehrlich interpreted the highly parasiticidal properties of these compounds as due to a certain modification (reduction) in the animal body of substances otherwise comparatively inert. Salvarsan and *Treponema pallidum* constitute a good example in point.

Leptospira icteroides is resistant to saponin,[11] a property which alone would serve to differentiate it from certain other groups of spiro-

[11] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 13.

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chetoid organisms (*Treponema* and *Spironema*). The fact that *in vivo* the leptospiras were scarcely influenced by the introduction of salvarsan or neosalvarsan constituted another point of dissimilarity between the leptospira and other spirochetal parasites. Hence the effect of salvarsan or neosalvarsan upon the leptospira from yellow fever cases *in vitro* presented a further interesting subject for study. Several experiments were also performed to determine the behavior of blood serum derived from a rabbit which had been injected intravenously 1 hour previously with salvarsan or neosalvarsan in considerable quantities; that is, a study was made of the effect of salvarsanized serum, as well as of drug solutions, upon *icteroides in vitro*.

TABLE VI.

Addition of Drug to Culture of Leptospira icteroides.

November 26, 1919.

Final concentration of arsaminolnatrium.*	Result.
1:2,000	All inactive and most have disappeared.
1:6,000	" " " some degenerated.
1:20,000	" "
1:60,000	" " thinner, and smoother.
1:200,000	" "
1:600,000	For the most part inactive.
1:2,000,000	" " " " " some still active.
Water (control).	" " " " active, a few inactive.

* The concentration resulting from the mixture of the drug solutions with the culture is given.

To each of several tubes containing 1 cc. of a culture of Guayaquil Strain 6 was added 1 cc. of different dilutions of salvarsan, neosalvarsan, or arsaminolnatrium. The mixtures were allowed to stand at room temperature and examined after 2 or 2½ hours, and again after 24 or 48 hours. As Tables VI and VII show, sufficient quantities of the drugs rendered the leptospiras immotile, and brought about general disintegration. The highest dilution of any of the drugs which still killed the organism was about 1:200,000.

In testing the leptospiricidal strength of the solutions of salvarsan or neosalvarsan, it was important to take note of the reactions of the solutions. Salvarsan, according to the usual practice, is first treated with sodium hydroxide solution until completely precipitated out

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at the point of neutral reaction. In order to obtain a clear solution it is necessary to add more alkali until the precipitate completely dissolves. At this point the solution is no longer slightly, but intensely alkaline. By reducing the alkalinity with hydrochloric acid to a reaction of about pH 8 a bulky precipitate is once more formed, and at pH 7 the entire substance flocculates out of the solution. In making a microbicidal titration a clear alkaline solution was used in ascending dilutions to eliminate the destructive effect of the reaction alone. It was found that the 1: 1,000 dilution was still too strongly

TABLE VII.

Addition of Drugs to Culture of Leptospira icteroides.

December 3, 1919.

Final concentration of the drug.	Salvarsan.		Neosalvarsan.	
	2½ hrs.	24 hrs.	2½ hrs.	24 hrs.
1:200	Precipitate (?).	Precipitate heavy.	All inactive.	All immobilized.
1:2,000	Some active; precipitate.	All immobile; precipitate.	" "	" "
1:20,000	Some active; precipitate.	All immobile; precipitate.	" "	" "
1:200,000	For the most part active.	All immobile.	" "	" "
1:2,000,000	For the most part active.	For the most part immobile; few active.	For the most part active.	Few active.
Saline (control).	All active.	All active.	All active.	For the most part active.

alkaline to be used (far beyond pH 8), the leptospiras dying rapidly in such a solution, while dilutions higher than 1:10,000 had a reaction which was practically that of the diluent (saline solution), pH 7. Neosalvarsan dissolved in distilled water in 1:100 dilution is a clear amber-yellow and shows to phenol red an intense red color; in diluting to 1:1,000 with saline solution its reaction approaches pH 7.2; in 1:10,000 dilution it is no longer perceptible to phenol red. In actual experiments, however, 1 cc. of each of the dilutions of the drugs was mixed with 1 cc. of a rich culture of one of the *icteroides* strains which

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had a reaction of pH 7.4. This mixing brought down the pH value of the lower dilutions and raised that of the higher. The reactions between pH 7 and pH 7.8 are well borne by *icteroides*, the optimum being near pH 7.2 to 7.4. A reaction beyond pH 8 or below pH 6.6 is unsuitable for the existence of the organism. The devitalizing action of salvarsan and neosalvarsan, even in optimum reactions, is rather slow, a contact of many hours being required before death ensues, as the protocol shows.

TABLE VIII.

Addition of Drugs to Culture of Leptospira icteroides.

March 18, 1920.

Final concentration of the drug.	Salvarsan.			Neosalvarsan.		
	Reaction.	After 15 min.	After 18 hrs.	Reaction.	After 15 min.	After 18 hrs.
1:200	Intensely alkaline.	Dead.		Slightly over pH 8.	Active.	Dead.
1:2,000	Intensely alkaline.	"		pH 7.2	"	"
1:20,000	pH 7.2	Active.	Dead.	pH 7.2	"	"
1:200,000	pH 7.2	"	"	pH 7.2	"	"
1:2,000,000	pH 7.2	"	" (?).	pH 7.2	"	Active.*
Saline control (no drug).	pH 7.2	"	Active.	pH 7.2	"	"

* All found dead after 96 hours; the control tube was lost through contamination.

To each of several tubes containing 1 cc. of the same culture of *icteroides*, Guayaquil Strain 5 (pH 7.4), was added 1 cc. of several different dilutions of drugs, 1:100, 1:1,000, 1:10,000, 1:100,000, and 1:1,000,000 of alkalinized salvarsan solution and neosalvarsan. The mixtures were allowed to stand at 26°C. and the contents examined under the dark-field microscope after 15 minutes and again after 18 hours. The results are recorded in Table VIII.

It is evident that *Leptospira icteroides* is highly sensitive to the action of salvarsan and neosalvarsan, but their action is comparatively slow, requiring many hours contact. The effects of the drugs in a culture medium are found to be approximately the same as in the case of direct mixing of culture and solutions.

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Various dilutions of the drugs were added to the usual medium (rabbit serum, 25 per cent, agar, 0.3 per cent, total volume, 6 cc.). The culture used was Guayaquil Strain 1, and the tubes were allowed to stand for 5 days at room temperature (26°C.). A medium containing salvarsan or neosalvarsan in a ratio of more than 1:200,000 was found to be unsuitable for the growth of the organism (Table IX). No attempt was made to determine quantitatively the exact leptospiricidal titers of the two drugs.

TABLE IX.

*Addition of Drugs to Culture Media.**

March 6, 1920.

Final concentration of the drug.	Salvarsan.	Neosalvarsan.
1:600	−	−
1:1,800	−	−
1:6,000	−	−
1:18,000	−	−
1:60,000	−	−
1:180,000	−	−
1:600,000	+	<+
1:1,800,000	+	+
1:6,000,000	+	+
Control.	+	+
"	+	+

* The medium consisted of rabbit serum, 25 per cent, agar, 0.3 per cent, total volume, 6 cc.

The changes in the color of salvarsan, when observed after 12 days standing, were noticeable in the tubes containing dilutions from 1:100,000 up, while with neosalvarsan the changes were not found in dilutions below 1:10,000.

Effect of Salvarsanized and Neosalvarsanized Serum.

The problem here was to determine the effect of the living body upon the drugs when the latter were introduced into the blood circulation. It has been assumed that in the animal or human body they are converted into highly spirocheticidal substances, as is said to be the case with respect to the organisms of syphilis, yaws, and relapsing fevers. It has already been shown that these drugs are practically without effect *in vivo* upon the course of the *icteroides* infection, and that they are highly destructive to the organism *in vitro*. It was this discrepancy which suggested the study of the salvarsanized serum.

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The mode of experiment was to inject a rabbit intravenously with salvarsan or neosalvarsan in a ratio of 0.05 gm. per kilo of body weight, the blood being drawn 1 hour after the time of injection. Two rabbits, weighing 1,500 and 2,000 gm. respectively, were injected on March 18, 1920, intravenously, one with salvarsan (alkaline), the other with neosalvarsan. After 1 hour they were killed for the blood. The clear serums were collected the next day and used in the active state in order to determine whether they were in any way different from a normal rabbit serum when mixed *in vitro* with the same quantity (1 cc. in each case) of a rich culture of Guayaquil Strain 5. The mixtures were kept in a thermostat at 28°C. during the period of observation. Table X gives the results.

The experiments demonstrated clearly the difference between the normal and the salvarsanized rabbit serums. All the leptospiras mixed with the latter died within 72 hours, while those in normal rabbit

TABLE X.

Effect of Salvarsanized, Neosalvarsanized, and Normal Rabbit Serum in Vitro.

March 18, 1920.

Duration of contact.	Salvarsanized rabbit serum, 1 cc., plus culture, 1 cc.	Neosalvarsanized rabbit serum, 1 cc., plus culture, 1 cc.	Normal rabbit serum, 1 cc., plus culture, 1 cc.
hrs.			
1	All active.	All active.	All active.
18	For the most part motile, but sluggish.	For the most part sluggish.	" "
48	No observation.	No observation.	No observation.
72	All dead and degenerated.	All dead and degenerated.	All active and multiplying.

serum steadily multiplied. It may be mentioned that the reactions of the drugged serums as well as the reaction of the normal serum were pH 7.4, hence the question of hydrogen ion concentration does not enter into the present comparative study. At the end of 18 hours the organisms were already less active in the drugged serums than in the normal serum.

It occurred to me that the cause of this slow leptospiricidal action of the salvarsanized and neosalvarsanized serums might be due to a gradual development of injurious substances by slow oxidation of the drugs. If this were the case we should find in these tubes a powerful and rapidly acting leptospiricidal substance at the end of 72 hours of exposure to the same experimental conditions. Two different experiments were carried out to ascertain this point.

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In the first experiment 0.5 cc. of a rich culture of the Merida strain[12] of *icteroides* was added to each of the three tubes containing salvarsanized, neosalvarsanized, and normal serum respectively. The results were similar to those observed with the Strain 5 culture; that is, there was no effect upon the organisms during the 1st hour; they were all active at the end of that time. After 24 hours the leptospiras in the salvarsanized serum were all dead, but there were many active survivors in the neosalvarsanized serum. After 48 hours, however, they were for the most part dead in the neosalvarsanized serum, but all were active in the tube containing the normal serum. In the second experiment three tubes containing 1 cc. of each of the serums were placed at 28°C. for 72 hours, then 1 cc. of the rich Merida culture was added to each. The mixtures were again put at 28°C. for the period of observation. Table XI gives the results of this experiment.

TABLE XI.

*Effect on Leptospira icteroides (Merida Culture) of Salvarsanized, Neosalvarsanized, and Normal Rabbit Serums after the Serums Had Stood for 72 Hours in the Incubator at 28°C.**

Experiment of Mar. 22, 1920.	Salvarsanized serum, 1 cc., plus culture, 1 cc.	Neosalvarsanized serum, 1 cc., plus culture, 1 cc.	Normal serum, 1 cc., plus culture, 1 cc.
After 1 hr.	All active.	All active.	All active.
" 18 hrs.	Some active.	Many active.	" "
" 48 "	All dead.	All dead.	" "

* There was a slight shift of pH value (to pH 7.8) in these serums on standing, but they were brought back to pH 7.6 by the addition of the culture.

These two series of experiments indicate that a certain antagonistic substance seems to have developed in the tubes containing the salvarsanized and neosalvarsanized serums during the period of 72 hours at 28°C., as shown by an earlier death and degeneration of the leptospiras than was the case with the fresh samples of these serums. The difference was especially definite in the effects observed at the end of 18 hours with the first series. On the other hand, in no instance was there any rapid immobilization or destruction of the organisms, the drugs having exerted no perceptible effect upon them even after an hour's contact. At all events, no rapidly leptospiricidal substances could be demonstrated in the salvarsanized or neosalvarsanized rabbit serum after exposure to the air for 72 hours at 28°C. The death of the leptospiras in these drugged serums was slow but certain.

[12] This strain was isolated from a case of yellow fever in Merida, Mexico, and will be described in a later paper (Noguchi, H., and Kligler, I. J., *J. Exp. Med.*, 1920, xxxii, in press).

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The question arises as to the form in which salvarsan or neosalvarsan existed in the blood serum of these two rabbits. This we do not know, but whatever its state, its ultimate concentration must correspond at most with a dilution of 1:20,000; that is, on the assumption that 0.05 gm. of the compound had diffused out in a space of 1,000 cc. In reality, the volume representing 1 kilo of body weight of the rabbit must be considerably smaller than 1,000 cc., hence the concentration of the drugs in the serum must have been stronger than 1:20,000. Other experiments (Table VII) showed that salvarsan or neosalvarsan added directly to a rich culture kills the latter within 24 hours; that is, in less time than that required with the salvarsanized or neosalvarsanized serum, which was at least 48 hours. Perhaps the arsenic compounds had undergone a modification in the animal body which converted them into substances operating much more slowly. As the animals did not urinate after the injection of the drugs up to the time of collecting the serum the slowness of action cannot be explained by elimination of the drug through the urine. Moreover, a dilution of 1:200,000 of the drugs when added directly to a culture caused the death of the latter in 18 hours. To summarize, then, serum drawn from rabbits at the end of 1 hour from the time of an intravenous injection of salvarsan or neosalvarsan in a ratio of 0.05 gm. per kilo of body weight possesses a slowly acting leptospiricidal property, which does not seem to be much increased in respect to rate of action by an exposure to the air for a period of 72 hours at 28°C.

Contrasted Effect of Anti-icteroides Immune Serum in Vitro.

Comparison has already been made of the chemotherapeutic value of salvarsan and neosalvarsan and the serotherapeutic value of anti-*icteroides* immune horse serum, showing the comparative inefficacy of these drugs and the highly potent specific protective property of the serum. Since the leptospiricidal power of the drugs was considerable *in vitro*, in sharp contrast with their lack of perceptible protective action *in vivo*, a similar comparison of the action *in vitro* of the immune serum and that of the drugs was of practical interest.

The same immune serum which was used in the experiments recorded in Table V was mixed with 1 cc. of a rich culture of Guayaquil Strain 5, the object being

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to determine how high a dilution of the serum still had leptospiricidal power in the test-tube. The maximum dilution which caused complete immobilization and subsequent degeneration within 1 hour was 1:20, while a 1:200 dilution caused considerable but incomplete agglutination and degeneration within 18 hours. No effect whatever was perceptible in a mixture containing the serum in a dilution of 1:1,000 or less. The results of the experiment are recorded in Table XII.

When we place side by side this low titer *in vitro* of the immune serum (1 cc. of a 1:100 dilution to 1 cc. of culture) and its protective titer *in vivo* (1 cc. of a 1:10,000 dilution to 0.5 cc. of culture, or 5,000 minimum lethal doses), it is easy to conceive at once how completely

TABLE XII.

Effect of Anti-icteroides Immune Serum upon Leptospira icteroides in Vitro.

March 19, 1920.

Anti-*icteroides* immune serum.		After 1 hr.	After 18 hrs.
Amount.	Dilution.		
cc.			
1	1:10 (final dilution 1:20).	All dead.	All degenerated.
1	1:100 (" " 1:200).	For the most part active.	For the most part agglutinated and degenerated, but some still motile.
1	1:1,000 (" " 1:2,000).	All active.	All active.
1	1:10,000 (" " 1:20,000).	" "	" "
1	1:100,000 (" " 1:200,000).	" "	" "
1	Saline control.	" "	" "

reverse is the relation that exists between the behavior of the immune serum on the one hand and that of salvarsan and neosalvarsan on the other toward *Leptospira icteroides in vitro* and *in vivo*.

SUMMARY AND CONCLUSIONS.

In several series of experiments guinea pigs were variously infected with different amounts of *Leptospira icteroides*, either in the form of culture, organ emulsion from infected guinea pigs, or a mixture of both. The infecting materials were of different grades of virulence; in some series the amount given was near a single lethal dose, in others a subminimum lethal dose was given, *i.e.* causing mild infection

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with recovery in the majority of animals, and in still others the animals were injected with at least 50 minimum lethal doses of a mixture of a culture and a highly virulent organ emulsion from a guinea pig. The animals were inoculated intraperitoneally, and within about 30 minutes each was injected subcutaneously with a different amount of salvarsan or neosalvarsan. The amounts injected were in most series 0.0005, 0.001, 0.002, 0.005, 0.01, 0.02, and 0.03 gm. per 350 to 450 gm. of body weight, and in one series, in addition to this dosage, 0.00005, 0.0001, and 0.0002 gm. were also tried.

Among the guinea pigs treated either with salvarsan or with neosalvarsan there were more recoveries than among the controls, but they were not in strict proportion to the amounts of the drugs injected. In the experiments with 50 minimum lethal doses of the infecting material there were several recoveries among those which received 0.001 to 0.002 to 0.003 gm., but all passed through a typical infection with all its symptoms. It is extremely doubtful, therefore, whether salvarsan or neosalvarsan mitigated the severity of the infection. The fact is noteworthy that in the same series of experiments the guinea pigs receiving 0.00005 and 0.0001 gm., or thereabout, of salvarsan died 1 to 2 days sooner than the controls, which died in 6 to 7 days. This suggests a possible earlier injury of the kidneys by the drugs, giving the leptospiras an easier and earlier access to, and localization in this organ. The inefficacy or dubious therapeutic value of salvarsan and neosalvarsan against the experimental *icteroides* infection of guinea pigs presents a close analogy to the observations already made by several investigators with *Leptospira icterohæmorrhagiæ*.

Several series of test-tube experiments were also made to determine the direct effect of salvarsan and neosalvarsan on *Leptospira icteroides* cultures. It was found, the injurious effect of alkalinity being eliminated, that the leptospiras remain motile for at least 1 hour in a concentration weaker than 1:10,000 of salvarsan or 1:1,000 of neosalvarsan. But they become gradually sluggish and succumb to the effect of the drugs at the end of 18 to 24 hours. The highest dilution which killed the leptospira in 18 hours was somewhere near 1:200,000.

When added to a culture medium, salvarsan and neosalvarsan both suppressed the growth of *icteroides* when their concentration in the

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medium was 1:200,000. Hence these two drugs are highly poisonous for *Leptospira icteroides*.

The serums derived from rabbits which received 0.05 gm. of salvarsan or neosalvarsan per kilo of body weight 1 hour before bleeding proved to be very different from a normal rabbit serum in their behavior toward *Leptospira icteroides*. In the salvarsanized or neosalvarsanized serums the leptospiras remained active for at least 1 hour but appeared somewhat sluggish at the end of 18 hours, and were all dead and degenerated when examined after 48 hours. On the other hand, the leptospiras mixed with normal rabbit serum lived well and multiplied during the same period of time and under otherwise identical conditions (at 28°C.) To these tubes another portion of culture was added to determine whether or not a rapidly detrimental toxic substance had appeared in the drugged serum while standing for 72 hours, but the organisms remained still active at the end of 1 hour, 24 hours being required to kill them. In another experiment the salvarsanized and neosalvarsanized serums, together with normal serum as a control, were first left standing for 72 hours, after which period a rich culture of *icteroides* was introduced. The organisms remained uninfluenced for 1 hour in all the serums, but at the end of 24 hours many of those in the drugged serums were dead, and none was left alive at the end of 48 hours. In normal serum they steadily increased in numbers and were all active.

It is evident, then, that salvarsan or neosalvarsan introduced intravenously into the body of the rabbit is present in some form in the blood serum drawn at the end of 1 hour. The substance present in such serum has a slowly operating injurious effect upon *Leptospira icteroides*. The action of the drugs seems to be slower after passage through the animal body than before. If this phenomenon were to take place also in the infected body injected with these drugs, it is obvious that in a rapidly evolving infectious disease like yellow fever the progress of the infection will be too rapid to allow the drugs to exert their beneficial effect upon the course of the disease.

In direct contrast to the behavior of salvarsan and neosalvarsan *in vivo* and *in vitro*, anti-*icteroides* immune horse serum in a dose of 0.0001 cc., or 1 cc. of a 1:10,000 dilution, protected guinea pigs from an infection with at least 5,000 minimum lethal doses of *icteroides*

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when injected simultaneously, but the same serum failed to exert any injurious effect upon the organism when mixed *in vitro* in a concentration weaker than 1:2,000. A rapid disintegration resulted with a concentration of 1:20 and almost complete agglutination and degeneration in 1:200.

The contrast between chemotherapy, as carried out with salvarsan and neosalvarsan, and serotherapy demonstrated with an immune serum is apparently of considerable practical significance.

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