ACTIVE IMMUNITY PRODUCED BY SO-CALLED BALANCED OR NEUTRAL MIXTURES OF DIPHTHERIA TOXIN AND ANTITOXIN.[1]

By THEOBALD SMITH, M.D.,

George Fabyan Professor of Comparative Pathology in the Harvard University Medical School.

(*From the Antitoxin and Vaccine Laboratory of the Massachusetts State Board of Health.*)

In a former publication[2] I briefly called attention to the fact that an active immunity may be induced in guinea-pigs by mixtures of diphtheria toxin and antitoxin which, after subcutaneous injection, produce no local lesion recognizable during the life of the animal, no general disturbances indicated by loss in weight, and no paralysis. This acquired immunity remains in evidence at least two years.

This phenomenon is of both theoretical and practical importance. As the facts will show, it does not harmonize fully with current theories of the relation between toxins and antitoxins in mixtures. From the practical standpoint it offers a promising field for investigations in the active immunization of the human subject. If the latter reacts as does the guinea-pig, it should be an easy matter to confer a relatively high degree of active immunity, lasting at least several years, without any appreciable disturbances of health. The experiments recorded and tabulated below were planned with these two lines of inquiry in mind, and offer a basis for further investigations upon a very important subject.

METHOD.

The interpretations and inferences drawn from the results of this series of experiments are based upon the fact observed by a number

[1] Received for publication December 26, 1908.

[2] The degree and duration of passive immunity to diphtheria toxin transmitted by immunized female guinea-pigs to their immediate offspring. *Jour. of Med. Research,* 1907, xvi, 359.

of authors and by the writer, that an actively immunized female parent may transmit antibodies to the immediate young, who, receiving the immunity passively, soon lose it again. If active immunity of the mother reveals itself as passive immunity in the immediate offspring, the degree of passive immunity in the latter may be taken as a measure, relative only, to be sure, of the active immunity of the mother. In the series of experiments described below this principle was assumed to be true, and made use of throughout. Young female guinea-pigs received subcutaneously mixtures of diphtheria toxin and antitoxin, and the degree of passive immunity of their offspring was determined as a relative measure of the active immunity of the female parent.

Two other facts were fairly well established in the earlier paper quoted. Guinea-pigs receiving one, two or three doses of antitoxin subcutaneously in doses of three to five cubic centimeters did not transmit any immunity to their offspring several months later. Guinea-pigs which had recovered from a nearly fatal dose of toxin only did not acquire thereby enough active immunity to raise the normal resistance of their offspring appreciably. The first fact agrees with current knowledge that passive immunity is not transmitted to offspring unless perhaps the antibodies are injected during pregnancy. The second fact shows that there must be a certain degree of active immunity possessed by the mother to make any impression on the resistance of the offspring.[3]

The method which was followed throughout may be briefly described here to avoid needless repetition. Young female guinea-pigs whose genealogy was known were used for immunization. As a rule two or three of the same litter were chosen for the same experiment, to eliminate family differences. They received subcutaneously mixtures of diphtheria toxin and antitoxin of varying proportions. Each animal received but one dose before it was bred.

[3] Anderson (*Pub. Health and M. H. Service, Hygienic Laboratory Bull.*, 1906, No. 30) observed, independently of the writer, the absence of any transmitted immunity following the injection of toxin alone. He observed a certain degree of immunity in the young following the treatment of the mother with antitoxin alone. As all five injections, amounting to 4250 units, were made during pregnancy, and as the results of the tests of the second and third litters were irregular, the immunity of the mother was probably passive, and his data, therefore, do not invalidate the position here taken—that antitoxin alone does not confer active immunity.

The first litter was born three or more months after the injection of the mixture into the parent.

To test the resistance of the young, two procedures were available. One was to inject pure toxin and thereby determine the increase in the minimum fatal dose. The other was to inject mixtures of toxin and one antitoxic unit, and determine the increase of toxin in the L_+ dose, *i. e.*, the dose just necessary to kill the guinea-pig in the presence of one antitoxic unit. In the present work the second method was used exclusively.[4]

Since the passive immunity of the offspring gradually declines and practically disappears at the end of three months, the degree of immunity was determined as nearly as possible when the young were thirty days old. This rule was departed from in certain instances and allowance was made for it in estimating the resistance. In the fall and winter the growth of the young in weight is very slow, as compared with the spring and summer. Hence, there is much variation at this age in the weight of the animals undergoing the test, the extremes being 162 and 336 grams. The low weight did not, however, appear to seriously influence the resistance of the animals as shown by the immunity of litters of low and high weight belonging to the same mother.

The other method available for determining the active immunity of the treated mother was to test the antitoxic content of her blood[5] directly. For several reasons this method was not chosen. It would have been desirable to establish some relation between the antitoxic content of the mother's blood and the resistance of the offspring. This we hope to do in the near future. In the meantime some conception of the resistance of the mother was obtained by a final injection of a toxin-antitoxin mixture when the experiment had been concluded.[6]

[4] Repeated tests had shown that the minimum fatal dose is relatively high in the passively immune offspring. The precise numerical relation of this rise to that of the L_+ dose has not yet been studied.

[5] In the paper cited, one animal was subjected to this test which had received a nearly neutral mixture of toxin and antitoxin about one and one-half years before. The blood serum contained approximately $\frac{1}{4}$ unit per cubic centimeter.

[6] All the tests were made with one lot of toxin under toluol whose L_+ dose (minimum fatal dose in the presence of one antitoxic unit intimately mixed

In the course of the investigation several distinct questions presented themselves for solution, all of which are partly answered in the tabulated results given below. They may be briefly stated as follows:

1. What is the smallest amount of toxin added to one unit of antitoxin which confers enough active immunity upon a female so that it may be detected in the offspring three or more months later?

2. Is the immunity conferred by a non-neutralized mixture, *i. e.*, one which still produces local lesions, greater than that produced by a neutralized mixture, which produces no local lesion or paralysis?

3. Can the immunity produced by a single antitoxic unit plus a given amount of toxin be heightened by multiples of this mixture?

4. Does a toxin-antitoxin mixture confer as much immunity after standing several days as it does immediately after preparation?

5. Are there noteworthy differences, as regards the degree of acquired active immunity, following the same treatment among litters from different families, and among individuals of the same litter?

In the tabulated results given below, these five topics are not sharply separated, for some of the experiments throw light on several at the same time. They will not, therefore, be taken up in the order given here, but the experiments will be described more or less chronologically.

Before passing to the experiments proper, it is desirable to call attention to two minor questions bearing directly on them. One is the L_+ dose of adult guinea-pigs, since nearly all of the mothers after completion of the breeding experiments herein described were tested finally to determine their own resistance. In Table I three adult guinea-pigs, presumably of normal susceptibility, were tested. The L_+ dose for young pigs at this time was 0.21 c.c. The table

with it before injection) was 0.21 to 0.235 c.c. during the period of the entire experiment, lasting two years. The L_0 dose was 0.17 c.c. at the beginning of these experiments. At the conclusion of the last tests the L_0 dose was still 0.17 c.c.; 0.18 c.c. produced slight transient œdema and 0.19 c.c., transient induration, roughening of skin and slight loss of hair. Unless statement is made to the contrary, the toxin-antitoxin mixture was injected about 15 minutes after preparation. For the sake of uniformity the standard serum, issued by the Institute for Experimental Therapy under the direction of Professor Ehrlich, and used in the earlier experiments, was continued in the present series.

shows that for old adults it is about 0.30 c.c. This figure should be borne in mind in studying the resistances of the treated mothers.

TABLE I.

Adult Controls.

Designation.	Sex.	Weight.	Age.	Dose injected in c.c.	Result.	Remarks.
3053	Female.	580	4 years and 7 months.	.30 toxin + 1 unit antitoxin.	Dies in 3½ days.	Received 2 doses of antitoxin, about 5 c.c. each, early in life.
8093	Male.	672	5 months and 6 days.	.35 toxin + 1 unit antitoxin.	Dies in 2¾ days.	
4341	Female.	965	2 years and 2 months.	.30 toxin + 1 unit antitoxin.	Dies in 3½ days.	Received some antitoxin early in life.

The second matter relates to passive immunity transmitted, during the period of lactation, in the milk. The influence of lactation upon the immunity of the offspring in mice, so strikingly demonstrated in Ehrlich's now celebrated experiments with ricin and abrin, had not been satisfactorily defined in the case of guinea-pigs actively immunized with diphtheria toxin. In the series of experiments here described it became desirable to determine how far lactation was a factor. In Table II is given a crucial experiment, which goes to show that lactation plays no appreciable part in the passive immunity of the young, and that it may be neglected as a factor. No. 3896, a normal, untreated female, nursed the offspring of a highly immunized female, No. 4977, from the first day to the time

TABLE II.

Mother.						Offspring.						
Designation.	Estimated L_+ Dose When 30 Days Old, in c.c.	Dose Injected in c.c.	Result.	Second Dose Injected in c.c. (Period Between First and Second Dose).	Result.	Period Between Treatment of Mother and Birth of Litter.	Age in Days.	Weight in Grams.	Dose of Toxin + 1 Antitoxin Unit in c.c.	Result.	Estimated L_+ Dose.	Remarks.
3896	.215	—	—	—	—	—	31	210	.21	Dies in 5½ days.	.21	This litter was nursed by the highly immune mother No. 4977 from day of birth to day of test.
							36	213	.22	Dies in 2½ days.		
4977	"	.17 toxin + 1 unit antitoxin.	No lesion.	.40 toxin + 1 unit antitoxin (5 months and 27 days).	Trace of induration.	7 months and 1 month.	31	332	.30	No lesion.	.35 + +	This litter was nursed by the normal mother No. 3896 from the day after birth until the day of test.
							31	351	.35	Transient induration.		

of testing, thirty-one days after birth. Both young showed a very high degree of resistance. On the other hand, the offspring of No. 3896, nursed by the highly immune mother, No. 4977, from the day after birth until the day of the test, showed only the normal resistance.

ANALYSIS OF THE EXPERIMENTAL DATA.

On Table III are given all the facts pertaining to four female guinea-pigs treated with unsaturated and supersaturated mixtures of toxin and antitoxin. The treated guinea-pigs belong to two litters, A and B, respectively. They possessed, when treated, a certain degree of passive immunity inherited from treated mothers. This immunity is noted in Column 2 of the table. Much of this had probably passed away in Litter A, because the animals were treated when seventy-five days old.[7] The first of each pair received a mixture in which the toxin was in excess, for in each case an ulcer was produced. The second of each pair received a mixture containing two antitoxic units and, therefore, below the L_0 dose (.17 c.c.) for normal pigs, and probably considerably below this for these passively immune pigs. There was, therefore, no local or general effect produced by the injection.

Two litters each of the first, second and third, and three of the fourth animal were tested. These were born from three and a half to ten months after the single treatment of the mother.

In the table are given the age of the offspring, their weight, the dose injected and the result. From these data the L_+ dose for each litter is estimated and given in the eleventh column of the tables. This L_+ dose is approximate only. When it is a little higher than the figure given a single + is added. Where it is considerably higher two ++ are added. The effect of adding an excess of antitoxin to the toxin is well brought out in the passive immunity transmitted to the young. Bearing in mind that the L_+ dose of the toxin used was at this time 0.215 c.c. for normal animals, we observe that it is very high in the offspring of the first of each pair, and that it is but slightly above normal in offspring of the

[7] When the age is not given, the future mothers were treated when about 30 days old.

second. It is, in fact, nearly twice as high in the offspring of the first. It is of interest to note, however, that even a toxin to which a relatively large amount of antitoxin has been added and which is injected into guinea-pigs possessing some passive immunity still produces enough immunity just to appear in the young months later, as shown in the slight elevation of the L_+ dose.

TABLE III.

Mother.						Offspring.						
Designation.	Estimated L_+ Dose When 30 Days Old, in c.c.	Dose Injected in c.c. (Age of Pig).	Result.	Second Dose Injected (Period Between First and Second Dose).	Result.	Period Between Treatment of Mother and Birth of Litter.	Age in Days.	Weight in Grams.	Dose of Toxin + 1 Antitoxin Unit in c.c.	Result.	Estimated L_+ Dose.	Remarks.
								First litter				
4684	.35	.21 toxin + 1 unit antitoxin (75 days).	Small ulcer.	.45 toxin + 1 unit antitoxin (9 months and 11 days.)	No lesion.	4 months and 8 days.	24	285	.28	No lesion.	.28++	The L_+ dose for a normal guinea-pig of 250 grams = .215 c.c. toxin
							24	282	.24	ditto.		
								Second litter				
A						6 months and 15 days.	36	256	.38	Large ulcer.	.45+	
							40	265	.45	Dies in 5 days.		
								First litter				
4685	ditto.	.21 toxin + 2 units antitoxin (75 days).	No lesion.	.25 toxin + 1 unit antitoxin (9 months and 11 days).	Small ulcer.	3 months and 16 days.	29	265	.22	Moderate ulcer.		Toxin-antitoxin mixtures about 15 minutes old when injected.
							35	298	.24	Dies in 5 days.	.24	
								Second litter				
						6 months and 24 days.	40	301	.23	Dies in 2½ days.		
							36	272	.25	Dies in 2 days.	.23	
								First litter				
4788	.40	.27 toxin + 1 unit antitoxin (37 days).	Ulcer.	.70 toxin + 1 unit antitoxin (10 months and 23 days).	Small ulcer.	6 months and 22 days.	22	253	.30	Transient induration.	.35++	
							25	268	.35	ditto.		
								Second litter				
							31	192	.45	Dies in 2½ days.		
B							31	205	.50×	Dies in 4 days		× Lost ¼ of mixture during injection.
							31	206	.55[c]	Dies in 1½ days.	.50±	[c] Injection in part intraperitoneal.
							31	220	.60	Dies in 2½ days.		
								First litter				
4789	ditto.	.27 toxin + 2 units antitoxin (37 days).	No lesion.	.50 toxin† 1 unit antitoxin (10 months and 23 days).	Dies in 2½ days +	4 months and 26 days.	27	243	.215	Small ulcer.		† Injection partly intraperitoneal.
							27	281	.25	Large ulcer.	.28+	
							31	293	.28	Large ulcer.		
								Second litter				
						7 months and 4 days.	35	232	.25	Dies in 2½ days.		
							35	291	.30	Dies in 1½ days.	.24±	
								Third litter				
						10 months and 6 days.	34	264	.25	Dies in 2½ days.	.24	
							34	271	.30	Dies in 1½ days.		

The active immunity of the mothers was finally tested from nine to ten months after the early dose was administered. The difference

in the result between the members of each pair is shown in Columns 5 and 6, although that difference is not accurately defined by the test. The active immunity of the mother runs parallel to the passive immunity of the offspring.

On Table IV further experiments on the immunizing effects of balanced or L_0 doses are recorded. The three females of Litter C were of normal parentage, *i. e.,* not passively immune, as indicated in Column 2. The first received a little less than the L_0 dose of

TABLE IV.

Mother.						Offspring.						
Designation.	Estimated L_+ Dose when 30 Days Old, in c.c.	Dose Injected in c.c.	Result.	Second Dose Injected in c.c. (Period Between First and Second Dose).	Result.	Period Between Treatment of Mother and Birth of Litter.	Age in Days.	Weight in Grams.	Dose of Toxin + 1 Antitoxin Unit.	Result.	Estimated L + Dose.	Remarks.
								First litter				
4875	.215	.15 toxin + 1 unit antitoxin.	No lesion.	.45 toxin + 1 unit antitoxin (8 months).	Trans. oedema	4 months and 4 days.	28	278	.21	No lesion.		L_+ dose for normal guinea-pig of 250 grams = 0.215 c.c.
							28	259	.25	Transient swelling.		
							31	246	.30	Large ulcer	.35	
							35	271	.35	Dies in 4½ days.		
								Second litter				
C						6⅓ mos.	30	243	.35	Dies in 3½ days.		
							30	215	.35	Large ulcer.	.35	
								First litter				
4876	ditto.	.27 toxin + 2 units.	ditto.	ditto.	ditto.	3 months and 27 days	28	278	.215	No lesion.		
							28	272	.27	Small ulcer.	.30+	
							32	315	.30	Large ulcer.		
								Second litter				
						7 months and 3 days.	26	238	.35	Dies in 3 days.		
							26	217	.40	ditto	.35	
								First litter				
4877	d tto.	.38 toxin + 3 units antitoxin.	ditto.	ditto.	ditto.	4 months and 1 day.	28	255	.25	No lesion.		
							34	282	.30	Moderate ulcer		
							34	283	.35	Dies in 2½ days.	35	
							38	320	.35	Large ulcer.		
								Second litter				
						7 months and 9 days.	20	207	.35	Moderate ulcer.		
							20	211	.40	Large induration.	.35+	

toxin plus 1 unit antitoxin with no local or general reaction. The second received nearly double this dose of toxin, plus 2 units antitoxin; the third nearly triple the dose of the first plus 3 units antitoxin without any reaction. Taking the L_0 dose as 100 per cent., they received respectively 12, 20 and 24 per cent. less toxin than the L_0 dose. Two litters of each treated female were tested. The

results show a relatively high passive immunity of the young, practically the same in all. Taking the normal L_+ dose as 100 per cent., the actual L_+ dose was 35/21.5 or 163 per cent. of the normal.

The experiment does not inform us just what the effect of doubling and tripling the toxin-antitoxin dose is, as the actual doses of toxin were a trifle less for the second and third animal, but it does demonstrate the high degree of immunity transmitted by apparently balanced mixtures which produce no recognizable effect after injections. The relatively high active immunity of the three mothers is shown in Columns 5 and 6, and indicates a doubling of the L_+ dose.

The experiment reported on Table V is similar to that of Table IV, with certain differences to be noted. The three females of Litter D were born of an actively immunized mother, and their L_+ dose is estimated at .60 or 60/21 of the normal at the age of thirty days. They received doses in which there was enough toxin in excess for them to produce local ulcers. The doses are graded nearly as in Table IV, the second and third animals receiving respectively nearly two and three times the toxin-antitoxin mixture of the first. As a result the active immunity of the mothers after ten to twelve months is very high, for the L_+ dose is probably three times the normal for pigs of their weight (Columns 5 and 6). Their resistance is not the same, however, but increases from first to third. The same is true of the passive immunity of the offspring of which two litters from each mother were tested. The estimated L_+ dose rises from first to third group, and is very high in the third, corresponding to 63/21, or to about three times the normal.

A comparison of Tables IV and V shows that the unbalanced mixtures, or such as produce local lesions, give, as might have been anticipated, a much higher degree of immunity than those equivalent to or below the L_0 dose.

The experiment recorded on Table VI is somewhat like that of Table V. It was made to determine whether the three offspring of the same mother would acquire the same degree of immunity after treatment with the same dose. The litter was born of an immunized mother, and the degree of passive immunity at thirty days was estimated at .33 or 33/21 of the normal. At the age of fifty-eight

days much of this passive immunity must have disappeared, for in each the normal L_+ dose produced a larger ulcer. Tested again over nine months later the first was slightly less resistant than the others.

Only one litter of each was tested. The young of the second

TABLE V.

Mother.						Offspring.						
Designation.	Estimated L_+ Dose when 30 Days Old, in c.c.	Dose Injected in c.c. (Age of Pig).	Result.	Second Dose Injected in c.c (Period Between First and Second Dose).	Result.	Period Between Treatment of Mother and Birth of Litter.	Age in Days.	Weight in Grams	Dose of Toxin + 1 Antitoxin Unit in c.c.	Result.	Estimated L_+ Dose.	Remarks.
								First litter				
4799	.60	.28 toxin + 1 unit antitoxin (42 days).	Small ulcers.	.80 toxin + 1 unit (12½ months).	Large ulcer.	5 months and 19 days	29	290	.25	No lesion.		L_+ dose for a normal guinea-pig of 250 grams = 0.21 c.c.
							32	297	.30	Trans. induration.	.35++	
							32	287	.35	ditto.		
								Second litter				
						11 months and 7 days	33	173	.40	Large ulcer.		
							33	106	.45	ditto.	.50	
							33	199	.50	Dies in 3½ days.		
								First litter				
4801 D	ditto.	.50 toxin + 2 units antitoxin (42 days).	Large ulcer.	.80 toxin + 1 unit antitoxin (10 months).	Moderate induration.	6½ mos	28	254	.30	No lesion.		
							31	275	37	ditto.	.50+	
							35	265	.45	Small ulcer.		
							39	270	.50	ditto.		
								Second litter				
						9 months	30	206	.55	Dies in 7 days.		
							30	230	.60	Large ulcer.	.60	
							30	265	.65	Dies in 6½ days.		
								First litter				
4800	ditto.	.75 toxin + 3 units antitoxin (42 days).	Large ulcer.	.80 toxin + 1 unit antitoxin (13 months).	Slight transient oedema	5 months	32	267	.45	Trans induration.		
							32	274	.55	ditto.		
							36	240	.60	Small ulcer.	.65+	
							36	268	.65	ditto.		
								Second litter				
						12 months	33	178	.50	Trans. induration.		
							33	188	.55	Induration and loss of hair.	.65+	
							33	198	.60	Superficial necrosis.		

were slightly more resistant, but this may have been due in part at least to their greater weight when tested.

On the whole there is little difference between these identically treated animals, and it remains uncertain whether this slight difference is within the margin of error of the method or not. It was hoped that this experiment might reveal certain differences traceable to the father. For if the latter exerts any influence upon his

offspring it must be in transmitting a greater or less capacity for the production of antibodies under treatment. That he does not transmit any antibodies or passive immunity has been abundantly shown.

TABLE VI.

Mother.						Offspring.						Remarks.
Designation.	Estimated L_+ Dose when 30 Days Old, in c.c.	Dose Injected in c.c. (Age of Pig).	Result.	Second Dose Injected in c.c. (Period Between First and Second Dose).	Result.	Period Between Treatment of Mother and Birth of Litter.	Age in Days.	Weight in Grams.	Dose of Toxin + 1 Antitoxin Unit, in c.c.	Result.	Estimated L_+ Dose.	
4865 E	.33	.21 toxin + 1 unit antitoxin (58 days).	Large ulcer.	.60 toxin + 1 unit antitoxin (9 mos. and 11 days).	Small ulcer.	8 mos. and 13 days.	29	162	.30	Small ulcer.	.35+	
							34	163	.35	Moderate ulcer.		
4867	ditto.	ditto.	ditto.	ditto.	Trace of indura.	8 months.	28	248	.30	No lesion.	.40±	
							33	207	.40	Large ulcer.		
4868	ditto.	ditto.	ditto.	ditto.	ditto.	8 mos. and 11 days.	31	175	.35	Small ulcer.		
							36	176	.35	Moderate ulcer.	.35+	

The experiments detailed on Tables VII and VIII have both a theoretical and a practical bearing. Leaving aside the former, we may consider briefly the latter before referring to the tabulated results.

If the injection of a balanced mixture of toxin and antitoxin leads to an active immunity, what is the effect if such a mixture has stood for several days? If such mixtures should be found applicable to the active immunization of human beings, they would be of little value if they deteriorated very rapidly. Hence the tests were made with this point in view. The preliminary experiment made on three females of Litter F is given on Table VII. These animals were of normal susceptibility. They received a toxin-antitoxin mixture, which produced no local lesion.

The first received the mixture fifteen minutes after preparation. The second received a similar mixture after it had stood at 35° C. for one hour, and at room temperature for five and one-half hours. The third received a like mixture which had been kept at 35° C. for one hour, and at room temperature for twenty-two hours. Unfortunately this animal did not breed, but the final test on the three about six months later showed a trifling difference in resist-

ance among the three and in favor of the first animal. The offspring of the second, however, possessed a higher passive immunity than those of the first, proving that the toxin-antitoxin mixture had at least remained unharmed, if it had not been actually improved for immunizing purposes by the incubation.

To imitate conditions in practice more closely the tests recorded on Table VIII were made. Three females belonging to each of two litters, G and H, were treated with toxin-antitoxin mixtures,

TABLE VII.

Mother.						Offspring.						Remarks.
Designation.	Estimated L_+ Dose when 30 Days Old, in c.c.	Dose Injected in c.c.	Result.	Second Dose Injected in c.c. (Period Between First and Second Dose).	Result.	Period Between Treatment of Mother and Birth of Litter.	Age in Days.	Weight in Grams.	Dose of Toxin + 1 Antitoxin Unit	Result.	Estimated L_+ Dose.	
4977	.215	.17 toxin + 1 unit antitoxin.	No lesion.	.40 toxin + 1 unit antitoxin (5 mos. and 27 days).	Trace of induration.	4 mos.	33	275	.25	Transient swelling.		Toxin-antitoxin injected into No. 4977 15 minutes after mixing.
							33	294	.30	Large ulcer.	.30+	
F 4978	ditto.	ditto.	ditto.	ditto.	Slight induration.	4 months and 3 days.	31	247	.25	Slight induration.		Toxin-antitoxin mixture injected into No. 4978 after having been kept at 35° C, for 1 hour and at room temperature for 5½ hours.
							31	241	.30	Moderate ulcer.	.35+	
							37	245	.30	Large ulcer.		
							37	246	.35	ditto.		
4976	ditto.	ditto.	ditto.	ditto.	Moderate induration.	Did not	b	reed				Mixture injected into No. 4976 after it had been kept at 35°C. for 1 hour and at room temperature for 22 hours.

which had stood respectively fifteen minutes, two days and five days in a fluctuating room temperature, protected from diffuse light. In the dose given to Litter G the amount of toxin was 0.12 c.c. or about 12/23 of the L_+ dose. That given to Litter H was 0.15 c.c. or 15/23 of the L_+ dose, and it was somewhat below the L_0 dose. After the injection the animals were examined and weighed daily for several weeks, but no appreciable effect, local or otherwise, following the injection was noticed. One of the second litter (H) died before any young were born. Two litters from each of the remaining five mothers, as well as the mothers themselves, were tested to determine any existing immunity.

Several facts are clearly brought out in this table. The toxin-

antitoxin mixture, even when the toxin content is well below the L_0 dose, produces an active immunity in guinea-pigs after the mixture has been kept five days at 50°–70° F. The toxin in these mixtures

TABLE VIII.

Mother.						Offspring.						
Designation.	Estimated L_+ Dose when 30 Days Old, in c.c.	Dose Injected in c.c.	Result.	Second Dose Injected in c.c. (Period Between First and Second Dose).	Result.	Period Between Treatment of Mother and Birth of Litter.	Age in Days.	Weight in Grams	Dose of Toxin + 1 Antitoxin Unit in c.c.	Result.	Estimated L_+ Dose.	Remarks.
								First litter				
8090	.23	.12 toxin + 1 unit antitoxin.	No lesion.	.40 toxin + 1 unit antitoxin (8 mos. and 17 days).	No lesion.	4 months and 10 days.	26	260	.23	Transient induration.	.23++	Toxin - antitoxin injected into No. 8090 15 minutes after mixing.
								Second litter				
						7 months and 4 days.	30	336	.235	Slight trans. induration.		
							30	312	.26	Superficial necrosis.	.29+	
G							30	310	.29	Moderate ulcer.		
								First litter				
8088	ditto.	ditto.	ditto.	.45 toxin + 1 unit antitoxin (9½ months).	Trans. oedema	5 months and 4 days.	32	205	.26	Transient induration.		Toxin - antitoxin injected into No. 8088 after mixture had stood 48 hours at 50° –70° F.
							32	203	.28	ditto.	.28++	
								Second litter				
						8½ mos.	29	265	.26	ditto.		
							29	273	.28	ditto.	.32++	
							29	295	.32	Induration.		
								First litter				
8089	ditto.	ditto.	ditto.	.45 toxin + 1 unit (9 months).	Small ulcer.	4 months and 21 days.	25	220	.235	Transient induration.		Toxin - antitoxin injected into No. 8089 after mixture had stood at 50°–70° F. for 5 days.
							32	255	.25	ditto.	.27+	
							32	200	.27	Moderate ulcer.		
								Second litter				
						7 months and 25 days.	31	178	.24	Small ulcer.		
							31	202	.27	Moderate ulcer.	.30+	
							31	208	.30	Large ulcer.		
8080	ditto.	.15 toxin + 1 unit antitoxin.	ditto.		Dies of	infectious disease						Toxin - antitoxin injected into No. 8080 15 minutes after mixing.
								First litter				
8078	ditto.	ditto.	ditto.	.45 toxin + 1 unit antitoxin (9 months).	Large ulcer.	5⅔ mos.	30	311	.25	Large swelling.		Toxin - antitoxin injected into No. 8078 after mixture had stood 48 hours at 50° –70° F.
							30	305	.28	Large ulcer	.28+	
H								Second litter				
						8 months.	32	243	.25	Superficial necrosis.		
							32	241	.275	Large ulcer.	.30+	
							32	238	.30	Very large ulcer.		
								First litter				
8079	ditto.	ditto.	ditto.	.45 toxin + 1 unit antitoxin (8 months and 25 days).	Very large ulcer.	5½ mos.	32	225	.32	Dies in 2½ days.		Toxin - antitoxin injected into No. 8079 after mixture had stood 5 days at 50°–70° F.
							32	208	.27	ditto.	.26	
							37	260	.235	Large ulcer.		
								Second litter				
						7 months and 23 days.	34	270	.24	ditto.		
							34	263	.26	Dies in 4½ days	.26	
							34	263	.28	Dies in 2½ days.		

does not completely disappear as such, but exercises some influence in the body of the guinea-pig, although the latter is not injuriously affected by the immunization.[8] In the second place the active immunity of the mother and the passive immunity of the offspring is slightly higher, under the influence of the two-day mixture than under either the fifteen-minute or the five-day mixture.

This table, furthermore, clearly shows differences existing among families of guinea-pigs in the capacity to produce antibodies, *i. e.*, to become immune. Thus, Litter H received 25 per cent. more toxin in the toxin-antitoxin mixture than Litter G, yet the two females of Litter H were less actively immune and their young showed a lower degree of passive resistance than those of Litter G.

INTERPRETATIONS AND CONCLUSIONS.

The foregoing and earlier data taken together demonstrate that an active immunity lasting several years can be produced in guinea-pigs, by the injection of toxin-antitoxin mixtures which have no recognizable harmful effect either immediate or remote. They also show, what might have been anticipated, that under the same conditions mixtures which produce local lesions and which, therefore, contain an excess of toxin produce a much higher degree of immunity than the neutral mixtures, and that an excess of antitoxin reduces the possibility of producing an active immunity, and may extinguish it altogether. There is, therefore, a certain definite relation between the components of the mixture and the degree of immunity producible. Furthermore, toxin-antitoxin mixtures do not change materially within five days at room temperature. They are apparently more efficacious at the end of forty-eight hours than immediately after preparation. The experiments finally prove that a relatively high degree of active immunity can be induced by a harmless procedure, whereas the use of toxin alone leading to very severe local lesions is incapable of producing more than an insignificant protection.

The method, therefore, invites further tests in regard to its ulti-

[8] Experiments not yet completed indicate that some immunity is produced by toxin-antitoxin mixtures kept four days at room temperature, even when the toxin dose is but one-half of the L_0 dose.

mate applicability to the human being. Unless the subcutis of the guinea-pig reacts to toxin-antitoxin mixtures in a manner peculiar to itself, a practical, easily controlled method for active immunization can be worked out which should afford a larger protection than the serum alone and avoid the complications associated with horse serum. That proportion of toxin and antitoxin which would produce the highest desirable immunity consistent with the least discomfort would have to be carefully worked out for the human subject. From the nature of the immunity induced it is obvious, however, that such a method of immunization cannot take the place of a large dose of antitoxin in exposed individuals who must be protected at once. It would be applicable only as a general protective measure without reference to any immediate danger, since it would take several weeks, perhaps longer, to perfect the attainable immunity.

Passing to the theoretical aspects of the facts observed, we find no publications bearing directly upon the subject before us. Madsen[9] has, however, approached it very closely in his experiments on the immunization of animals with mixtures not fully balanced, or, in other words, in which the "toxones" were still free. He found that the injection of such mixtures in rabbits, goats and horses produces an active immunity. He makes the significant remark that perhaps in the immunizing capacity we may possess the keenest reagent for a poison which is not able to exert any toxic action in the body. This is fully borne out by the experiments described, for in these we pass beyond the visible spectrum, so to speak, of the toxin-antitoxin effects, and we are able to recognize toxic action only by the lasting immunizing effects.

Another publication which touches upon some phases of the same problem is that of Morgenroth on the union between toxin and antitoxin.[10] Morgenroth brought out the fact that a given toxin-antitoxin mixture is more toxic when injected directly into the circulation than when injected under the skin. Thus, an L_+ dose of 0.78 c.c. toxin + one unit antitoxin applied subcutaneously was of the same toxicity as 0.68 c.c. toxin + one unit antitoxin injected

[9] *Zeit. für Hygiene u. Infectionskrankheiten,* 1901, xxxvii, 251.
[10] *Zeit. für Hygiene u. Infectionskrankheiten,* 1904, xlviii, 177.

into the circulation. When the mixture had stood twenty-four hours this (L_+) dose was still 0.78 c.c. subcutaneously, but it had risen to 0.74 c.c. when introduced by the intracardiac route. The author makes two deductions from these results. He assumes that the velocity of reaction between toxin and antitoxin is slow, and that the union is not completed until the mixture has stood twenty-four hours. Hence, the L_+ dose of toxin injected into the blood is higher after twenty-four hours than immediately after mixing the toxin and antitoxin. He furthermore explains the fact that the subcutaneous L_+ dose remains the same whether the mixture is injected at once or after twenty-four hours, by assuming that in the subcutis of the guinea-pig there is a catalytic acceleration of the union of toxin and antitoxin.

In view of the writer's results it seems that not only immediately, but four to five days after the preparation of the mixture of toxin and antitoxin, there are still toxic substances available for the production of immunity in the body of the guinea-pig, when the dose of toxin in the mixture is far below the L_0 or neutral level. These toxins may be free, either because uncombined *in vitro,* or else because the mixture is partially dissociated *in vivo,* or there may be a third possibility. It is obvious that Morgenroth's investigations, however extensive and thorough, have not exhausted the subject, for both these inferences are incompatible with his. Perhaps his recent important studies on the recovery of toxin from its combination with antitoxin with weak acids may throw more light on this subject.[11]

The only conclusion which we may safely draw at this time is that the toxin-antitoxin mixture produces two sets of effects, essentially identical, however. One is visible, as injury (œdema, loss of hair, superficial and deep necrosis of skin, paralysis and death), and corresponds to the toxin spectrum of Ehrlich. The other is invisible and manifests itself only in degrees of active immunity. At what ratio of toxin to antitoxin in the mixture active immunity is no longer produced will vary somewhat with the guinea-pig used, but it is evident that traces of immunity are still transmitted to the young when the amount of toxin approaches half the L_0 dose.

[11] *Virchow's Archiv.,* 1907, cxc, 371.