

same ferrets proved susceptible 4 months later when they were injected with living canine distemper virus and died of distemper following the inoculation.

From these experiments one may draw the conclusions that inoculation of young puppies up to the age of one month with living distemper virus does not induce an attack of distemper. The nature of this insusceptibility is not known and may be due to inherited immunity, to immune substances taken in with the colostrum and milk or to some type of natural resistance. Five of the 6 mothers were immune to distemper at the time the litters were born. Administration by intradermal inoculation of living distemper virus to puppies during the first weeks of life in spite of producing no symptoms of disease seems to result in permanent immunity.

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#### Inactivation of Diphtheria Toxin *in vivo* and *in vitro* by Crystalline Vitamin C (Ascorbic Acid).\*

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For some years one of us (C.W.J.) has been actively interested in the study of natural resistance to infectious diseases, particularly in relation to poliomyelitis. This work has brought to light certain evidence which argues against the concept of universal latent immunization and favors acceptance of the view that the mechanism of natural resistance to this disease is purely physiological, depending essentially upon the function of the normal endocrine balance. While it is obviously very difficult in a problem as fundamental as this to determine precisely the particular rôle played by any one of the several glands of internal secretion, some experimental facts pointed very definitely to the significance of the anterior pituitary and the adrenal. It was also found that there existed a peculiar relationship between resistance to diphtheria and to poliomyelitis, suggesting the operation of a common protective factor in both diseases.

Meanwhile, the other author (R.L.Z.), on the basis of blood chemical and other studies, had been led to the belief that the ad-

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renal cortex was involved in the course and regulation of metabolic and toxic demands on bodily resistance. This paper presents the first phase of a joint research program designed to correlate the mechanism of natural resistance in some infectious diseases, particularly diphtheria and poliomyelitis, with adrenal function.

Implication of the adrenal in diphtheria is suggested by a great many diverse observations. It is a well established fact that the adrenal glands of susceptible animals suffer heavily in this intoxication and that the natural insusceptibility of the rat to diphtheria and other bacterial toxins can be broken down by bilateral adrenalectomy.<sup>1-6</sup> It has also been shown that this artificially created lack of protection can be corrected again by supplying the operated animal with the deficient factor, *i. e.*, cortical extract.<sup>7-10</sup> More recently it has been possible to demonstrate that cortical extracts have the capacity of inactivating diphtheria toxin *in vitro*<sup>11</sup> and can raise the natural resistance of normal animals to excessive toxic<sup>12</sup> and other<sup>13</sup> demands.

Various biologically important substances found in the adrenal cortex, and possibly present in the extracts, were tested to determine their contribution to the end result. One of these, Vitamin C, gave such interesting effects that it was studied more intensively. The data obtained from approximately 100 guinea pigs is herewith presented.

The experimental work proceeded along 3 main lines of investigation. First, Vitamin C,† in varying quantities, was combined with

<sup>1</sup> Scott, W. J. M., *J. Exp. Med.*, 1923, **38**, 543.

<sup>2</sup> Lewis, J. T., *Am. J. Physiol.*, 1923, **64**, 506.

<sup>3</sup> Belding, D., and Wyman, L. C., *Am. J. Physiol.*, 1926, **78**, 50.

<sup>4</sup> Jaffe, H. L., *Am. J. Path.*, 1926, **2**, 41.

<sup>5</sup> Marmorston-Gottesman, J., and Gottesman, J., *J. Exp. Med.*, 1928, **47**, 503.

<sup>6</sup> Marmorston-Gottesman, J., Perla, D., and Vorzimer, J., *J. Exp. Med.*, 1930, **52**, 587.

<sup>7</sup> Scott, W. J. M., and Bradford, W. L., *PROC. SOC. EXP. BIOL. AND MED.*, 1931, **28**, 428.

<sup>8</sup> Perla, D., and Marmorston-Gottesman, J., *PROC. SOC. EXP. BIOL. AND MED.*, 1931, **28**, 475.

<sup>9</sup> Hartman, F. A., and Scott, W. J. M., *PROC. SOC. EXP. BIOL. AND MED.*, 1931, **28**, 478.

<sup>10</sup> Perla, D., and Marmorston-Gottesman, J., *PROC. SOC. EXP. BIOL. AND MED.*, 1931, **28**, 650.

<sup>11</sup> Jungeblut, C. W., Meyer, K., and Engle, E. T., *J. Exp. Med.*, 1934, **27**, 43.

<sup>12</sup> Zwemer, R. L., and Jungeblut, C. W., (in preparation).

<sup>13</sup> Wolfram, J., and Zwemer, R. L., *J. Exp. Med.*, 1935, **61**, 9.

† We are grateful to Merck and Company for placing at our disposal a generous supply of Cebione, their natural crystalline Vitamin C preparation.

diphtheria toxin (2 m.l.d.) and the mixtures after standing one-half hour at room temperature were injected subcutaneously into guinea pigs. Second, guinea pigs were injected by the subcutaneous route simultaneously but in separate locations with Vitamin C and diphtheria toxin (2 m.l.d.). Third, guinea pigs received Vitamin C for several days so as to ensure a high storage of this substance, and were subjected subsequently to a series of intracutaneous injections of small doses of diphtheria toxin. All the guinea pigs used in Series I and II and their controls weighed from 230 to 280 gm. In Series III larger albino animals of about 350 gm. weight were employed. The diphtheria toxin used throughout this work represented one batch of stabilized filtrate, containing from 400 to 450 m.l.d. per 1 cc., so that 1 cc. of a 1:200 dilution was equivalent to at least 2 minimal lethal doses.

*Controls.* Each test was accompanied by one or more control animals receiving the toxin alone or in combination with 1 cc. of saline. All in all, 22 control guinea pigs were injected with 2 m.l.d. as calculated above. Without exception these animals died within from 24 to 72 hours with typical symptoms of diphtheria intoxication (Table I).

TABLE I.  
Injection of Normal Guinea Pigs with 2 m.l.d. of Diphtheria Toxin.

No. of G. Pigs	Dose of Toxin	Died within 24 hr.	48 hr.	72 hr.
22	2 m.l.d.	1	13	8

*Series I.* A total of 37 guinea pigs were used in the first series. While the dose of toxin was kept constant (2 m.l.d.), the amount of Vitamin C varied from 0.05 mg. to as much as 100 mg. In order to guard against the possibility of nonspecific acid destruction of the toxin in these tests in which toxin and ascorbic acid were brought into direct contact, the solution of Vitamin C was adjusted to pH 6.6 to 6.8 immediately before combining it with the toxin. This pH had no deleterious effect on the potency of the toxin as shown by control experiments in which animals receiving a mixture of 2 m.l.d. of toxin and lactic acid of the same pH died as promptly as the controls injected with toxin and saline. This observation, moreover, is in harmony with Walbum's conclusions on the degree of acid-resistance of diphtheria toxin.<sup>14</sup> As may be seen from Table II there is a very definite range within which inactivation of diphtheria toxin may regularly be obtained by Vitamin C. The optimal quan-

<sup>14</sup> Walbum, L. E., *Biochem. Z.*, 1922, **130**, 25.

TABLE II.  
Inactivation of Diphtheria Toxin by Vitamin C *in vitro*.

No. of G. Pigs	Dose of Vitamin C	Dose of Toxin	Died within 96 hr.	Died between 5 and 9 days	Survived
	mg.	m.l.d.			
5	100	2	3		2
4	50	2	3	1 (7d.)	
4	10	2	1	2 (5d., 6d.)	1
4	5	2			4
4	1	2			4
4	0.5	2			4
4	0.2	2		1 (7d.)	3
4	0.1	2	2	2 (6d., 9d.)	
4	0.05	2	4		

titles seem to lie between 0.5 mg. and 5 mg., larger or smaller doses of Vitamin C failing to protect with the same consistency. The failure of excessive doses to inactivate is of special interest since it seems to give evidence against a purely physical or chemical destruction of the toxin by this substance.

*Series II.* The second series comprises a total of 14 guinea pigs which were injected with 2 m.l.d. of diphtheria toxin and at the same time received a separate subcutaneous injection of different amounts of Vitamin C in acid solution, varying from 1 mg. to 200 mg. (Table III). These experiments show that under the

TABLE III.  
Inactivation of Diphtheria Toxin by Vitamin C *in vivo*.

No. of G. Pigs	Dose of Vitamin C	Dose of Toxin	Died within 96 hr.	Died between 5 and 10 days	Survived
	mg.	m.l.d.			
2	200	2	1		1
2	100	2	1		1
2	50	2	1		1
2	25	2	1		1
2	10	2	1	1	
2	5	2	1		1
2	1	2	2		

conditions of the test, approximately half of the animals survived which had received between 5 mg. and 200 mg. of Vitamin C. In contrast to the results obtained in the mixture tests there was no evidence of a relation between the dose of Vitamin C injected and the outcome of the experiment, except that with the smallest dose given both animals died. It is noteworthy that no greater regularity could be obtained in some additional tests in which the dose of Vitamin C was repeated the following day.

Some of the guinea pigs surviving in this and the first series

showed slight ulcerations at the site of injection and in some cases peripheral paralysis after an interval of approximately 2 weeks.

*Series III.* The above work made it clear that although inactivation of diphtheria toxin by Vitamin C is obtainable *in vivo*, yet our results lacked the precision observed with *in vitro* tests. It was therefore thought advisable to investigate the possibility of inducing an enhanced resistance against minute doses of diphtheria toxin in guinea pigs which had been allowed to store Vitamin C for 6 days before intracutaneous injection of the toxin.

A group of 13 guinea pigs were prepared by daily injection of various amounts of Vitamin C in either acid or partly neutralized solution. A modified silver nitrate treatment<sup>15</sup> of sections from the adrenals of some of the prepared animals demonstrated an increased reduction by ascorbic acid. The same method had previously shown

TABLE IV.

Effect of Vitamin C Storage on the Reaction of Guinea Pigs to the Intracutaneous Injection of Small Doses of Diphtheria Toxin.

Guinea Pig No.	Daily dose of Vitamin C for 6 days mg.	Reaction after 24 hr.				Reaction after 72 hr.				
		1/50 m.l.d.	1/100 m.l.d.	1/200 m.l.d.	1/500 m.l.d.	1/50 m.l.d.	1/100 m.l.d.	1/200 m.l.d.	1/500 m.l.d.	
Tests on Vitamin C Stored Guinea Pigs.										
1	100	0	0	0	0	2	2	1	0	
2	100	2	2	1	1	2	2	1	1	
3	100	0	0	0	0	2	2	2	0	
4	100	2	2	0	0	5	4	0	0	
5	100	2	2	2	1	4	2	1	1	
6	50	1	1	1	0	0	0	0	0	
7	50	2	2	1	1	2	2	1	1	
8	10	3	3	2	2	5	4	2	0	
9	10	3	2	2	2	2	2	0	0	
10	10	3	2	2	1	5	0	0	0	
11	1	3	2	1	1	4	0	0	0	
12	1	2	2	1	1	2	2	0	0	
13	1	3	3	3	2	5	2	1	1	
Tests on Control Guinea Pigs.										
14	—	3	3	3	2	5	4	2	2	
15	—	2	2	2	2	5	4	3	3	
16	—	3	3	2	2	4	4	4	2	
17	—	3	3	2	2	4	4	3	2	
18	—	2	2	2	1	4	3	0	0	
19	—	3	3	2	2	5	3	3	2	
20	—	2	2	2	1	5	5	4	3	
21	—	3	3	3	2	5	3	3	0	
22	—	3	3	3	2	5	4	3	3	
23	—	3	2	2	1	5	4	3	3	
24	—	3	3	2	2	5	4	2	1	
25	—	3	3	2	1	4	4	2	1	
26	—	3	3	2	2	5	5	5	4	

Key explained in the text.

<sup>15</sup> Moore, T., and Ray, S. N., *Nature*, 1932, **130**, 997.

a diminution of Vitamin C in this organ in the course of diphtheritic and poliomyelitic infection. The animals were shaved on the sides and 4 doses of diphtheria toxin, *i. e.*, 1/50, 1/100, 1/200 and 1/500 m.l.d. contained in a volume of 0.1 cc., were injected intracutaneously, two on each side. Readings were recorded every 24 hours for 5 days. For tabulation we have selected only the 24-hour and 72-hour results as being most significant and characteristic of the trend of events. Every experiment was controlled by the inclusion of one or 2 normal animals, the latter numbering 13 in all. To secure maximal accuracy in comparing the results in both groups of guinea pigs, the size and degree of redness as well as the intensity of necrosis for each individual animal are recorded in Table IV according to the following key: 0 = negative; 1 = v. sl. red (1 x 1 cm.); 2 = sl. red (1½ x 1½ cm.); 3 = red (2 x 2 cm.); 4 = mod. necrosis; 5 = heavy necrosis;  $\phi$  = faded.

It appears from Table IV that the storage of Vitamin C had a very pronounced effect either in inhibiting or completely suppressing the local reaction to diphtheria toxin. In the controls, the average reading after the first 24 hours was 3322, whereas most of the prepared animals had much lighter reactions. In 2 instances there was no trace of any reaction. At the end of 72 hours the average control reading was 5432 while the majority of the prepared animals had no necrosis even with the largest amount of toxin injected and exhibited much lighter reactions with the smaller doses. In keeping with the results of Series II, individual differences in response of the prepared animals sometimes seemed more important than the experimental variation in the amount of Vitamin C injected. As a rule, however, the larger doses apparently afforded better protection.

We conclude from these data that Vitamin C inactivates diphtheria toxin and helps to protect guinea pigs against the fatal outcome of diphtheria intoxication. Also that guinea pigs, injected with suitable amounts of this substance are rendered temporarily negative or definitely less sensitive to small doses of the toxin as determined in intracutaneous tests. The experiments therefore indicate an important part played by Vitamin C in the mechanism of natural resistance to diphtheria toxin.

After completion of this work it was found that a paper reporting similar results with Vitamin C and diphtheria toxin had been presented at the meeting of September 24, 1934, of the French Academy of Sciences, by E. Harde.<sup>16</sup>

<sup>16</sup> Harde, E., *C. R. Ac. Sc.*, 1934, **199**, 618.