of that of their heaviest littermate, and this is significantly fewer than the comparative figure for the normal group ($n = 1$, $\chi^2 = 172.3$). Of great interest is the fact that the frequencies for the normal and dwarf litters describe significantly identical trends from the 100-90.1% to the 60.0-50.1% class ($n = 4$, $\chi^2 = 6.0$), indicating that the factors influencing variability between these extremes are operative with equal effectiveness on both groups.

Conclusions. When the birth weight of a rabbit as determined by the routine procedure described is less than 50.1% of the weight of his heaviest littermate, a diagnosis of dwarfism is warranted. The birth weights of unusually small, nonviable animals derived from known transmitters of the abnormality fall below the 50.1% class, and these animals are, therefore, genetically true dwarfs in the sense that their abnormally low weights cannot be ascribed to environmental and nutritive factors operating in the interval between birth and the weight determination.

8187 C

Effect of Various Corticoadrenal Extracts on Diphtheria Toxin In Vivo and In Vitro.*

RAYMUND L. ZWEMER AND CLAUS W. JUNGEBLUT.

From the Departments of Anatomy and Bacteriology, College of Physicians and Surgeons, Columbia University.

This paper deals with the ability of various adrenal cortex extracts to raise the resistance of normal guinea pigs to injections of diphtheria toxin, and to inactivate the toxin *in vitro*. It is part of a research program[1] directed toward an understanding of the mechanism of natural resistance, and is an attempt to determine whether the adrenal cortex resistance factor can be isolated by the same chemical procedures used in extracting the vital hormone.

The 5 methods of corticoadrenal lipoid fractionation used are very briefly indicated on an accompanying composite chart (Table I). They have been studied by one of us (R.L.Z.) for a number of years and most of the essential principles have been reported by

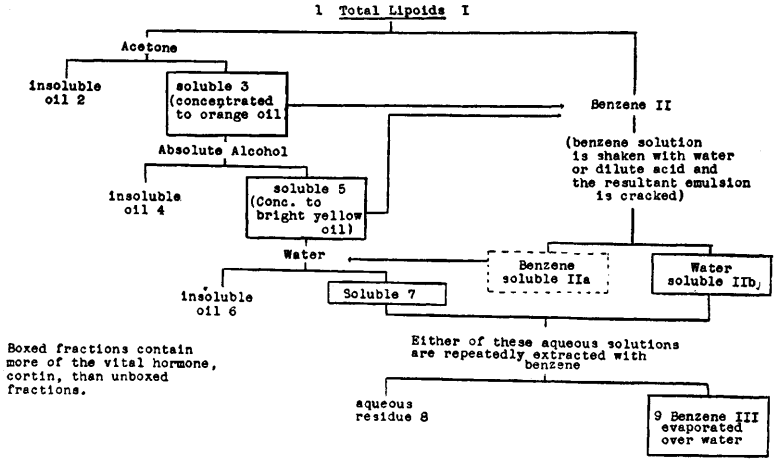
* Part of the expenses of this paper were defrayed by a grant from the Rockefeller Foundation.

1 Jungeblut, C. W., and Zwemer, R. L., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1229.

TABLE I.

ADRENAL EXTRACT FRACTIONATION.†

Five procedures are shown, the essential principles of which have been used by a number of authors[2–8] in preparing corticoadrenal extracts. The finely ground fresh adrenal glands are simultaneously extracted with benzene (80°-82°) and dehydrated with anhydrous sodium sulphate. The benzene solution is then concentrated to a thick oil. Details concerning the various steps are to be reported elsewhere.



workers on adrenal cortex extracts.[2–8] In addition to our own preparations, we used Eschatin (Parke, Davis & Co.) and 2 batches of corticoadrenal hormone kindly given us by Dr. Pfiffner of the Department of Biochemistry. One of his extracts had 100 dog units[9] and the other 40 dog units per cc.

The diphtheria toxin employed was from one batch of stabilized filtrate containing from 400-450 m.l.d. per cc., so that 1 cc. of a 1/200 dilution included at least 2 minimal lethal doses.

Over 150 guinea pigs were used as indicated in the tables and in the description of our results. Since many of the control animals accompanied the individual experiments with both corticoadrenal

† Many of these fractions were prepared in the Department of Anatomy by Messrs. L. Schwartz and Wm. Parsons during their work on another problem.

2 Hartman, F. A., and Brownell, K. A., *Am. J. Physiol.*, 1930, **95**, 670.

3 Swingle, W. W., and Pfiffner, J. J., *Am. J. Physiol.*, 1931, **96**, 165.

4 Zwemer, R. L., Agate, F. J., Jr., and Schroeder, H. A., PROC. SOC. EXP. BIOL. AND MED., 1931, **28**, 721.

5 Kutz, R. L., PROC. SOC. EXP. BIOL. AND MED., 1931, **29**, 91.

6 Grollman, A., and Firor, W. M., *J. Biol. Chem.*, 1933, **100**, 429.

7 Pfiffner, J. J., Vars, H. M., and Taylor, A. R., *J. Biol. Chem.*, 1934, **106**, 625.

8 Pfiffner, J. J., and Vars, H. M., *J. Biol. Chem.*, 1934, **106**, 645.

9 Harrop, G. H., Pfiffner, J. J., Weinstein, A., and Swingle, W. W., PROC. SOC. EXP. BIOL. AND MED., 1932, **29**, 449.

extracts and vitamin C, some of them were listed individually in the previous paper of this series.[1] In the present paper they are given in the first lines of Tables II, III and IV.

Intracutaneous tests with 1/50, 1/100, 1/200 and 1/500 m.l.d. of diphtheria toxin were made on 46 light colored, 350 gm. guinea pigs. Certain fractions of adrenal cortex extract, when given in various

TABLE II.

0 = negative
1 = very slight red (1 x 1 cm.)
2 = slight red (1½ x 1½ cm.)
3 = red (2 x 2 cm.)
4 = moderate necrosis
5 = heavy necrosis
ø = faded

Effect of Single Injections of Adrenal Cortex Extracts on Reaction of Normal Guinea Pigs to Small Intracutaneous Doses of Diphtheria Toxin.
(Corticoadrenal extracts were given 24 hours before toxin injections.)

Guinea pig No.	Extract fraction	Amount given cc.	Reaction after 24 hours 1/50 MLD	1/100 MLD	1/200 MLD	1/500 MLD	Reaction after 72 hours 1/50 MLD	1/100 MLD	1/200 MLD	1/500 MLD
Control average (16 animals)			3	3	2	2	5	4	3	2
1	1	1	2	2	2	2	5	5	4	2
2	3	2	0	0	0	0	5	4	2	2
3	3	2	0	0	0	0	5	3	2	1
4	3	2	0	0	0	0	5	5	2	2
5	3	2	1	1	1	1	5	4	1	1
6	3	2	1	1	1	1	5	4	2	1
7	3	2	2	2	1	0	5	5	1	0
8	3	1	1	0	0	0	5	5	4	2
9	3	1	1	1	1	1	4	3	2	ø
10	3	1	2	2	2	1	4	3	2	2
11	3	1	3	3	3	2	5	4	4	2
12	5	1	0	0	0	0	3	3	3	3
13	5	1	0	0	0	0	4	4	4	2
14	5	1	3	2	2	2	4	2	2	2
15	5	0.5	1	1	1	0	ø	ø	ø	0
16	5	0.5	3	2	2	2	4	4	3	2
17	7	3	1	1	0	0	4	4	2	2
18	7	2	2	2	1	1	3	3	1	ø
19	7	1	2	1	1	1	4	4	3	2
20	9	0.5	1	1	1	0	4	3	3	2
21	9	0.25	2	2	1	0	5	4	3	2
22	IIa	2	2	2	2	1	5	5	4	1
23	IIa	1	3	3	2	2	5	4	2	2
24	IIa	0.5	2	1	1	0	5	4	4	2
25	2	1	3	3	2	2	5	3	2	1
26	4	2	2	1	1	1	4	3	2	ø
27	4	1	2	2	1	0	5	4	4	3
28	4	1	3	3	2	2	5	4	4	2
29	6	1	3	3	3	3	5	5	5	3
30	8	1	3	2	2	2	5	4	3	2

Compare fractions 2 and 3; 4 and 5; 6 and 7; 8 and 9 for relative activity.

doses (depending upon their toxicity) 24 hours before toxin, diminished or prevented the characteristic skin reactions (Table II). Results with other fractions did not differ greatly from those observed in the 16 controls.

By comparing the extract fraction numbers in Tables I and II it can be seen that the toxin-inhibitory fractions are those possessing relatively larger amounts of cortin, the vital hormone of the adrenal cortex. They were administered both as oils and also as emulsions in water. The lack of protection shown by non-hormonal phospholipin or sterol fractions argues against neutralization of toxin by adsorption onto the surface of lipoid droplets.

Another control substance used, *i. e.*, olive oil, proved to have some anti-diphtheritic properties. This observation confirmed some recent work[10, 11] and seemed to favor the possibility of lipoid adsorption. However, naturally occurring, neutral fatty oils have a small amount of other lipoids mixed with them. Upon extraction with cold absolute ethyl alcohol these proved to have enhanced anti-diphtheritic properties, whereas, the large alcohol-insoluble fraction of olive oil was no longer effective. This is in harmony with a recent report[12] which states that crude lipoids on purification lose their power of assisting tissue immune reactions. Our observation may help to explain the variable results obtained when toxins are inactivated by admixture with lipoid emulsions.

The cell protective action of our aqueous corticoadrenal hormone preparations is supported by 6 experiments with Pfiffner's extract and Eschatin. These were apparently more effective when given 4 to 8 hours before the injection of the toxin. Since the time necessary for absorption into the body differs among the various oily and aqueous fractions used, it will obviously require a large number of tests with graded doses and time intervals for an exact comparison of relative potencies.

The negative skin reaction does not seem to be due to the possible presence of vitamin C in our extracts, since it requires 50 mg. to 100 mg. of cevitamic acid per day for 6 days[1] to bring about a comparable effect. However, the possibility of a synergistic action between cortin and Vitamin C has to be kept in mind.

In vitro tests. Sixty-three normal 250 gm. guinea pigs were injected subcutaneously with 2 m.l.d. of diphtheria toxin. The 24 control animals died within 72 hours. The 39 test animals received

10 Walsh, V. G., and Frazer, A. C., *Brit. Med. J.*, 1934, **1**, 424, 557.

11 Myers, G. N., *Brit. Med. J.*, 1934, **1**, 945.

12 Hanger, F. M., *J. Clin. Invest.*, 1934, **13**, 692.

1 cc. of toxin (2 m.l.d.) which had been mixed with 1 cc. of an adrenal extract fraction. The mixture had been allowed to stand at room temperature for ½ hour. More than 35% of the 28 guinea pigs receiving mixtures which contained cortin survived the test (Table III). Another 25% showed prolongation over the control

TABLE III.
Effect of 2 MLD of Diphtheria Toxin on 63 Normal 250 gm. Guinea Pigs, Alone and After Mixing with 1 cc. of Various Hormonal and Non-hormonal Fractions of Corticoadrenal Extracts.

No. of guinea pigs	Type of treatment	Died within 72 hr. %	Lived 4-9 days %	Survived %
24	Controls	100	0	0
11	Non-hormonal fractions (2, 4, 6, 8, IIa)	82	9	9
18	A. C. hormone fractions (1, 3, 5, 7, 9)	45	22	33
10	Pfiffner A. C. hormone extract	30	30	40

These results have been condensed for clarity in presentation. Some of our fractions and Pfiffner's extract were found to be effective when the amount used was the equivalent of 1 gm. of beef adrenal or 2.5 dog units.

period. Very potent hormone gave a better average than that shown, but we have included all animals used to determine the least amount that would inactivate 2 m.l.d. of toxin. This was found to be roughly equivalent to 1 gm. of beef adrenal. Eleven animals receiving non-hormonal fractions did not differ greatly from the controls. These results confirm previous *in vitro* studies[13] and favor the view that cell protective activity accompanies cortin in its chemical isolation.

Titration against 2 m.l.d. in vivo. Forty-five guinea pigs weighing 250 gm. were inoculated subcutaneously with 2 m.l.d. of diphtheria toxin, and corticoadrenal extract was injected intramuscularly (Table IV). From 100 to 200 times as many gram equiva-

TABLE IV.
Estimation of Beef Adrenal Gram Equivalent in Active Corticoadrenal Extracts Necessary to Counteract *in Vivo* the Effect of 2 MLD of Diphtheria Toxin on Normal 250 gm. Guinea Pigs.

No. of guinea pigs	Treatment	Died within 72 hr. %	Lived 4-9 days %	Survived %
24	Controls‡	100	0	0
29	A. C. extract 1 to 9 gm.	93	7	0
10	A. C. " 10 " 99 "	80	20	0
6	A. C. " 100 " 200 "	50	0	50

‡ Same controls as in Table III.

lents seem to be necessary in order to obtain survival of the animal under such conditions than is required for inactivation of the toxin

[13] Jungeblut, C. W., Meyer, K., and Engle, E. T., *J. Immunol.*, 1934, **27**, 43.

by direct contact with the hormone. Even with the most concentrated preparations only 50% of the animals survived. Weaker preparations sometimes delayed death for several days but in most cases were without effect.

These experiments indicate that extraction methods yielding the resistance promoting factor of the adrenal cortex parallel those used for the isolation of the vital hormone. Both end products are similar in certain of their chemical and physical properties. It would not be wise to speculate as to their identity, since even very potent adrenal cortex extracts contain several chemical entities.[14]

We do not believe that our active fractions contain a specific antitoxic substance, but rather a hormone which perhaps acts in a catalytic manner to decrease the permeability of cells to toxin. Best results are obtained when the maximal amount of hormone is in or available to the cells at the time of greatest demand. Coca, Russell and Baughman[15] felt that the natural resistance of the rat to diphtheria toxin was due to a property of its cells which prevented toxin from entering or becoming attached to them. This property diminishes after adrenalectomy.

Our work shows that injections of adrenal cortex extract give normal guinea pigs an increased cell resistance to toxin for one or 2 days. By the third day the toxin generally produces the characteristic necrosis. In some cases the potency of the adrenal cortex fraction was evidently so high that destruction of cells by toxin was negligible. Meanwhile other body mechanisms had probably either neutralized or otherwise disposed of the toxin. It must be noted that in the present experiments only single injections of extract were given. In counteracting the effects of other toxins[16] it was found that repeated daily doses were more effective.

[14] Wintersteiner, O., and Pfiffner, J. J., *J. Biol. Chem.*, 1935, **109**, C.

[15] Coca, A. F., Russell, E. F., and Baughman, W. H., *J. Immunol.*, 1921, **6**, 387.

[16] Zwemer, R. L., and Spence, M. J., in preparation.