

ON THE BIOLOGICAL ASSAY OF THE CORTICAL  
HORMONE BY THE SURVIVAL METHOD, IN  
ADRENALECTOMIZED YOUNG RATS, AND ON  
THE INFLUENCE OF THE SALT CONTENT OF  
THE HORMONE EXTRACT

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For the biological assay of the cortical hormone methods have been devised which are based on the ability of this hormone to counteract the symptoms resulting from bilateral adrenalectomy. The first investigators used its life-extending properties as a criterion of its effect, death in the course of a few days being the inevitable result of bilateral adrenalectomy. With our present knowledge of the relation between the hormone and sodium metabolism, the sodium chloride effect must be excluded before the whole of a life-extending effect can be ascribed to the hormone.

Several methods have been proposed for the assay of the cortical hormone, based on the assumption that it will do away with other symptoms of cortical adrenal insufficiency. The growth of young rats after adrenalectomy has been used by Grollman and Firor [1933] in experiments that were, however, inconclusive. Similarly others have used the lowered resistance to various poisons and toxins; morphia has been suggested by de Meio and Lewis [1932], typhoid vaccine by Perla and Marmorston-Gottesman [1931*a*], and later histamine [1931*b*]. The lowering of functions as a result of adrenalectomy has also been employed; the greater tendency of rats' muscles to tire [Everse and de Fremery, 1933] and their defective temperature regulation [Widström, 1935].

The method which has probably been most elaborated is, however, the method described by Pfiffner, Swingle and Vars [1934]. These investigators determined the smallest maintaining dose for adrenalectomized dogs. It was required that the blood urea of the animals should

not rise 100 p.c., and that clinical symptoms such as vomiting must not occur. The maintaining dose was found to vary from animal to animal by about 100 p.c. From the Mayo Clinic, however, Kendall *et al.* [1934] stated that the variations were still greater. 1 mg. of Kendall's crystalline hormone could be estimated at 10-100 dog units, *i.e.* a variation of 1000 p.c.

Such great variations necessitate the use of many animals for each assay. If only for that reason a small experimental animal like the rat would seem to be more suitable, as was, indeed, admitted by Harrop, Pfiffner, Weinstein and Swingle in 1932. They mentioned a study by Kutz [1931] in which he recommended the use of the life-extending effect in young rats, and having found that none of them survived the operation for as much as 10 days, defined a rat unit as the amount of hormone which, injected daily, kept 50 p.c. alive for 20 days. They did not themselves advocate this method because various authors had given very different figures for the percentage mortality of young rats after adrenalectomy.

That a method based on the survival of adrenalectomized animals is the surest one must be taken for granted. Two fundamental objections may be urged against the other methods. The first is that if a lowered function is used as the index, it is not certain that this lowered function is due to the lack of the cortex and not to that of the medulla. This, according to Wyman and tum Suden [1932], is the case in the lowered resistance to histamine in the rat. The second objection is that in the relatively impure extracts examined there occur, in addition to cortical hormone, substances counteracting the changes produced by adrenalectomy. Eagle [1933] pointed out that a substance like choline, found in a well-known commercial preparation, did not prevent the death of adrenalectomized rats, though it might have other effects attributed to the hormone itself.

Hence the survival of small adrenalectomized laboratory animals should afford a simple method of biological assay of the cortical hormone, planned like those used in the study of vitamins. Bomskov and Bahnsen [1935] have used in this way adrenalectomized infantile mice. In what follows an account is given of a series of experiments with young rats, based on a study [1935*a*] in which I was able to show that an improved operative technique might in a certain strain of rats involve a mortality of 100 p.c.

## METHODS

*Experimental animals.* Young animals of a pure strain of white rats originally derived from the State Vitamin Institute in Copenhagen were used for all the experiments. They have lived through the last seventeen generations under very uniform conditions, on Gudjónsson's diet No. 4 for breeding animals. On this diet the rats thrive and multiply at a satisfactory rate. It contains about 10 p.c. water and 0.4 p.c. NaCl. This diet was used also during the experiments. At operation their age was 4-5 weeks and their weight about 50 g. After the operation the animals were kept in a thermostatic room at a temperature of  $25 \pm \frac{1}{2}^{\circ}\text{C}$ . They were weighed every day with an accuracy of 1 g.

*The operation.* Bilateral adrenalectomy was performed, as previously described [1935a], in one stage. The mortality in seventy-seven controls was 100 p.c. The duration of life after the operation was on an average 5.7 days and varied from 3 to 12 days. For twenty-seven animals which had been kept alive for 21 days after the operation by injections of the cortical hormone the mortality after stopping treatment was also 100 p.c., with an average duration of life of 5.9 days.

*The hormone extract employed.* The extract (Ecortan 110) was prepared according to Swingle and Pfiffner's method from the entire adrenals of hogs. A quantity of 70 kg. adrenal gland was worked up, and of this 54 kg. were used solely for this experiment. The extract of 100 g. corresponded to 1 c.c. It was kept in a refrigerator at  $2^{\circ}\text{C}$ ., as were also the dilutions employed. A residue determination showed 1.4 p.c., more than half of it, 0.9 p.c., being sodium chloride titrated as chloride. The adrenaline content was slight. No reaction for histamine was given by the isolated guinea-pig uterus.

*The method of injection.* The extract, like the physiological saline in the control experiments, was injected subcutaneously under the dorsal skin. It was given in a single daily dose; some few rats, however, were given their dose divided into two daily doses without any difference being observed in consequence.

#### I. THE INFLUENCE OF SUBCUTANEOUS INJECTIONS OF PHYSIOLOGICAL SALINE ON THE LIFE AND GROWTH OF ADRENALECTOMIZED YOUNG RATS

Since treatment with saline alone may prolong the life of adrenalectomized animals, including rats [Rubin and Krick, 1933], two series of experiments were carried out. In the first, thirty animals were given a single daily injection of 1 c.c. 0.9 p.c. saline. At the operation their

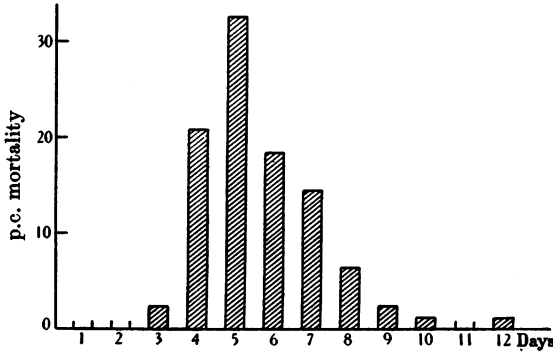


Fig. 1. Incidence of death of seventy-seven adrenalectomized young rats. Average weight at operation 51 g. Death on the average after 5.7 days [from Schultzer, 1935 a].

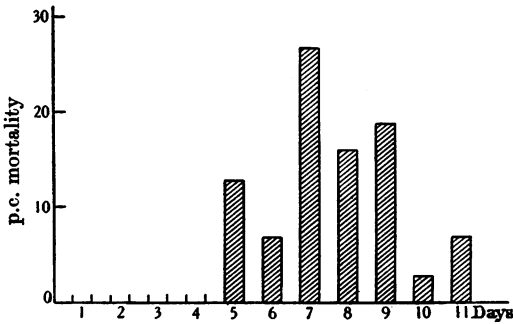


Fig. 2. Incidence of death of thirty adrenalectomized young rats, daily injected with 1 c.c. 0.9 p.c. sodium chloride. Average weight at operation 50 g. Death on the average after 7.7 days.

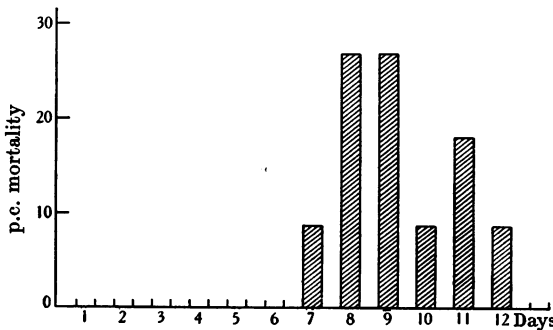


Fig. 3. Incidence of death of eleven adrenalectomized young rats, daily injected with 1 + 1 c.c. 0.9 p.c. sodium chloride. Average weight at operation 48 g. Death on the average after 9.3 days.

average weight was 50 g. The average duration of life after the operation was 7.7 days, all of them dying. Thus the period of survival was about 2 days longer than that of the controls previously mentioned, which had received no injections. At death their average weight was 51 g. In the second series eleven animals, average weight 48 g., were given two doses of 1 c.c. 0.9 p.c. saline daily, double the dose. The average duration of life was 9.3 days, 1.6 days longer than in the preceding series. Their average weight at death was 50 g. The percentage mortalities of the controls previously published and of these two series are given in Figs. 1-3. It is clear that even the smaller dose prolonged life and the larger one still more. But the effect is small compared with that of the injection of the hormone for 21 days described below.

Autopsy showed in the first series hæmorrhages of the gastric mucosa in 67 p.c., in the second series in 82 p.c., such as I have previously found [1935b] in non-treated adrenalectomized young rats.

The amount of physiological saline injected in the first series was equal to and in the second double the largest amount injected as a solvent for the hormone in the subsequent experiments. If, therefore, the extract is able to keep young rats alive for a relatively long period such as the arbitrary one of 21 days, it cannot at any rate be due solely to the sodium chloride in the extract. This disposes of the objection of Rogoff [1934] with regard to commercial preparations of the Swingle and Pfiffner type.

## II. THE INFLUENCE OF SUBCUTANEOUS INJECTIONS OF EXTRACT OF CORTICAL HORMONE (ECORTAN 110) ON THE VIABILITY OF ADRENAL-ECTOMIZED YOUNG RATS

A total of 146 adrenalectomized young rats were treated with the aforementioned hormone preparation. The great majority of them were females, the males being employed for other experiments. Previous work had shown that sex did not in any way affect the results in experiments of this kind.

The dose given varied; the largest corresponded to 100 g. of gland; the others were obtained by progressive halving of this, so as to correspond to 50, 25,  $12\frac{1}{2}$ ,  $6\frac{1}{4}$ ,  $3\frac{1}{8}$ , and  $1\frac{9}{16}$  g. of gland.

The doses between 25 and  $3\frac{1}{8}$  g. adrenal gland were given in three different volumes, namely 1,  $\frac{1}{2}$  and  $\frac{1}{4}$  c.c., the concentration of sodium chloride being the same in each case. Doses of a 100 g. were given only in a volume of 1 c.c., those of 50 g. in the two volumes 1 and  $\frac{1}{2}$  c.c., and that of  $1\frac{9}{16}$  g. only in the volume of  $\frac{1}{4}$  c.c.

Of the 146 young rats seventy-nine survived as long as the injections continued, that is 21 days; after the injections stopped they died on the 2nd-28th day, with an average survival period of 6.8 days. This is somewhat longer than in a group of twenty-seven rats previously described [1935*a*], where this period was 5.9 days. The mortality curve is shown in Fig. 4.

The great majority of the sixty-seven animals which did not survive the period of injection were among the seventy-seven animals which were given doses of less than  $12\frac{1}{2}$  g.; of these only seventeen did survive, whereas of the sixty-nine that received  $12\frac{1}{2}$  g. or more sixty-two survived.

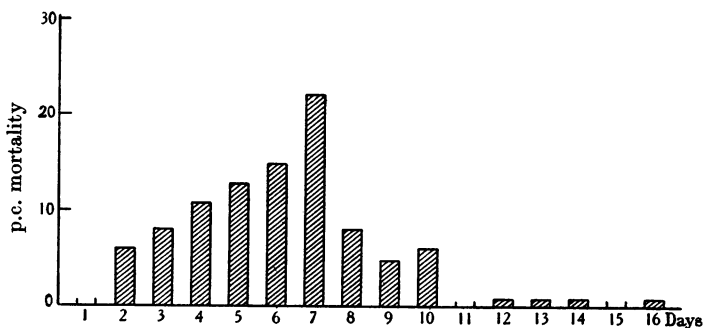


Fig. 4. Incidence of death of seventy-nine hormone-treated adrenalectomized young rats after discontinuance of injection of cortical hormone. Death on an average after 6.8 days. Two deaths, occurring on the 19th and the 28th day respectively, are not entered on the figure.

Table I shows the number and length of survivals for each dose and volume. The length of survival is somewhat shorter with the smallest doses, but with the others there is little difference in this respect. Fig. 5 shows the number of survivals with different doses without reference to the volume of saline in which the dose was contained, Figs. 6, 7 and 8 the influence of this volume. The amount of sodium chloride in 1 c.c. of saline has, as we have seen, some effect by itself: its effect when acting with the hormone especially in smaller doses is seen by comparing these curves: those in Figs. 7 and 8 where the volume was  $\frac{1}{2}$  and  $\frac{1}{4}$  c.c. respectively show a steeper decline.

Assuming that the reactions of adrenalectomized young rats to the cortical hormone shown in these curves is typical, it is clear that these animals can be used for assaying the hormone. If they are compared, it is seen that  $12\frac{1}{2}$  g. of the gland is the smallest dose which prevents their death after adrenalectomy when injected daily for 21 days. It is true that some few animals receiving this dose died; but this is the case

TABLE I. Average survival of animals in the various groups

Daily dose of adrenal gland g.	Volume of dose in 0.9 p.c. saline c.c.	Number of rats injected	Number of rats living through injection period of 21 days	Average weight at operation g.	Average weight after injection period g.	Average weight at death g.	Average survival period after discontinuance of injections days
100	1	8	7	51	63	59	7
50	1	7	7	47	61	60	7
	$\frac{1}{2}$	8	8	52	69	65	9
25	1	14	13	51	67	65	8
	$\frac{1}{2}$	5	4	52	71	71	6
	$\frac{1}{4}$	7	6	49	64	60	6
$12\frac{1}{2}$	1	8	7	52	67	64	6
	$\frac{1}{2}$	6	4	51	70	67	9
	$\frac{1}{4}$	6	6	48	65	64	8
$6\frac{1}{4}$	1	18	7	53	65	61	5
	$\frac{1}{2}$	8	2	54	67	62	5
	$\frac{1}{4}$	20	5	50	69	66	5
$3\frac{1}{8}$	1	8	3	48	57	55	4
	$\frac{1}{2}$	8	0	54	—	—	—
	$\frac{1}{4}$	8	0	50	—	—	—
$1\frac{1}{16}$	$\frac{1}{4}$	7	0	53	—	—	—

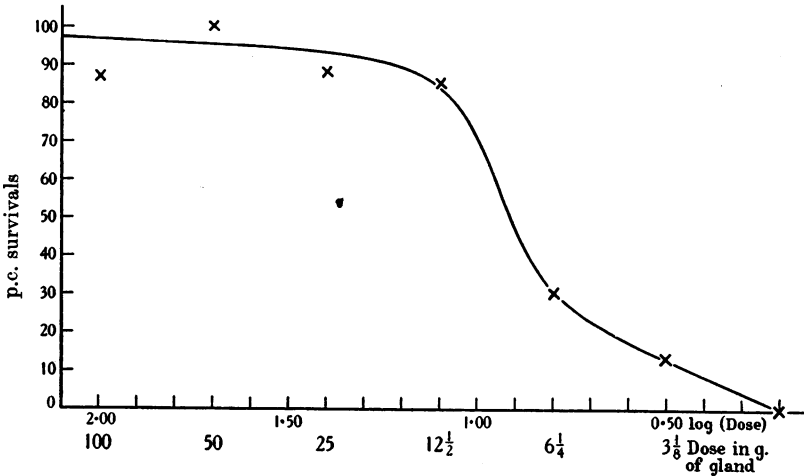


Fig. 5. Survivals on the different doses of adrenal gland. The volumes of the doses not taken into account.

too with animals receiving the eight times larger dose of 100 g. In both groups the number of survivals is above 80 p.c. (Fig. 5). This dose of  $12\frac{1}{2}$  g. protects too even when given in a volume of  $\frac{1}{2}$  or  $\frac{1}{4}$  c.c. (Figs. 7 and 8); but in these volumes especially the effect of reducing the dose

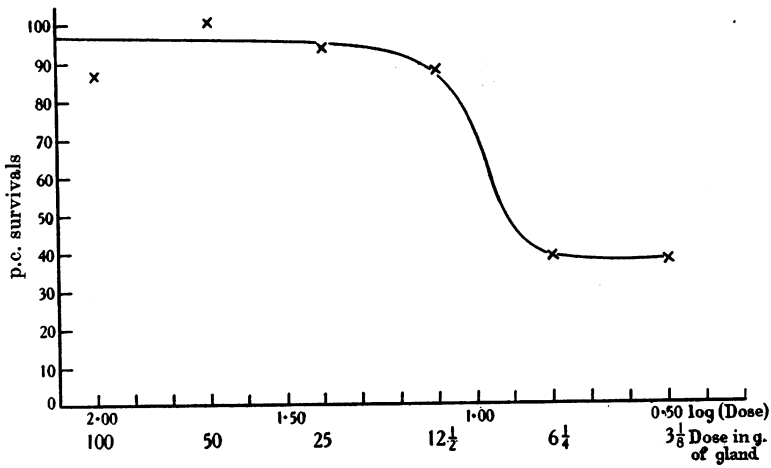


Fig. 6. Survivals of animals on the different doses of adrenal gland, all doses given in a volume of 1 c.c.

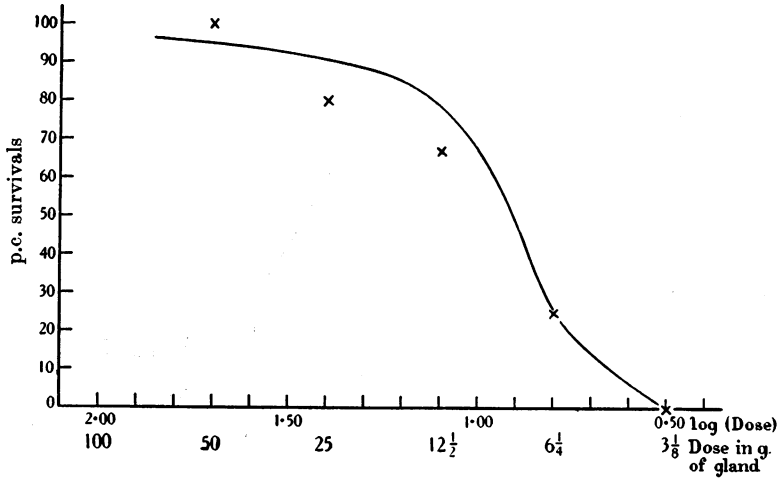


Fig. 7. Survivals on the different doses of adrenal gland, all doses given in a volume of ½ c.c.

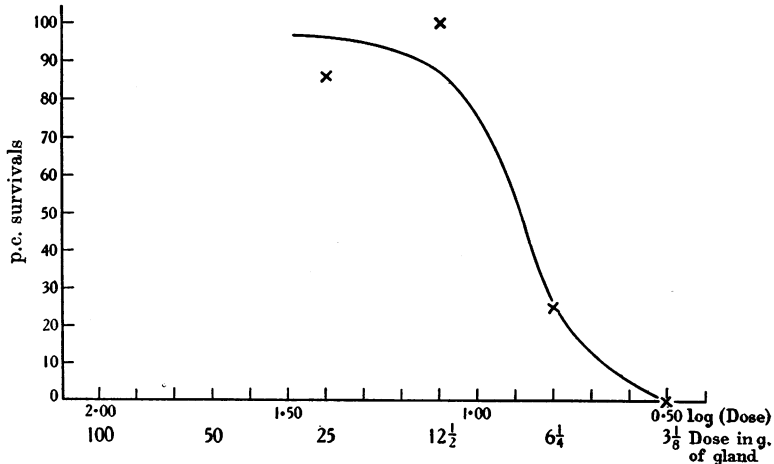


Fig. 8. Survivals on the different doses of adrenal gland, all doses given in a volume of ¼ c.c.



to  $6\frac{1}{4}$  g. is a sharp decline in the curve, and on again halving the dose no survivals occur at all. This steep decline of the curve for surviving animals is of essential importance for the purpose of standardization when only a limited number of animals is available.

### III. COMPARISON OF TWO DIFFERENT PREPARATIONS, ECORTAN 110 AND ECORTAN 156

When a few points of this curve can be determined in its characteristic part, *i.e.* where it declines, preparations can be compared with each other, as in the example which follows.

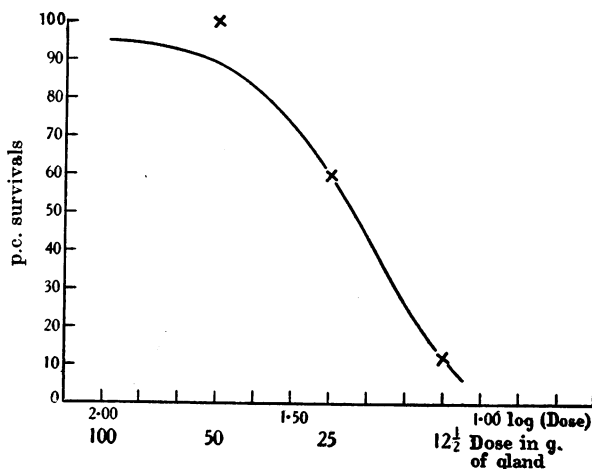


Fig. 9. Survivals on preparation Ecortan 156. Dose "50 g." in 1 c.c., "25 g." in  $\frac{1}{2}$  c.c. and "12 $\frac{1}{2}$  g." in  $\frac{1}{4}$  c.c.

The preparation 156 with which we are concerned is made like the preceding one, except that it is not finally purified by a permutite filtration, as indicated by Swingle and Pfiffner, but by ether extraction [Kendall *et al.*]. This latter purification appears to be more effective, and the adrenaline content of the preparation from being just determinable has been reduced to a mere trace. It was diluted so that 1 c.c. corresponded to 50 g. adrenal gland. For the assay seventeen animals were employed. Fig. 9 gives the curve for the percentage survivals on doses of 50, 25 and 12 $\frac{1}{2}$  g. adrenal gland, in volumes of 1,  $\frac{1}{2}$  and  $\frac{1}{4}$  c.c. respectively. A comparison between the logarithmic curves in Fig. 5 and Fig. 9 then shows that the dose designated 25 in Fig. 9 lies in potency between the doses designated  $6\frac{1}{4}$  and 12 $\frac{1}{2}$  in Fig. 5. It will be shown later how the relative potency can be calculated.

IV. THE INFLUENCE OF HORMONE INJECTIONS ON THE WEIGHT  
OF ADRENALECTOMIZED YOUNG RATS

Clinical experience shows that the cortical hormone is indeed able to tide patients suffering from Addison's disease over dangerous crises and prolong their life, but will not re-establish quite normal conditions, since the low blood pressure, the abnormal pigmentation, and other changes are either not affected at all or only in part. This may of course be because it is not only the cortex which is destroyed in this disease. It is therefore surprising that American investigators should state that adrenalectomized dogs under treatment with cortical hormone behave in all respects like normal animals, and that claims should be made in other communications that adrenalectomized young rats treated with hormone show a normal gain in weight.

These communications are, however, incomplete, or they speak of a gain in weight as normal when in reality it was decidedly abnormal. Kutz [1931] gives no figures nor growth curves for his young rats, and Grollman and Firor [1933] publish figures far below those generally regarded as normal.

In this laboratory normal young rats weighing 50 g. gain 72 g. in weight in the course of 21 days on Gudjónsson's diet No. 4. The animals treated with injections gained much less, those dealt with here on an average only 16 g. The highest figure was 34 g. in the 21 days of the injection period in a rat that had the dose of 25 g. daily. Only seventeen young rats in all gained 20 g. or more during the injection period. From Table I it is seen that there was no certain correlation between the size of the dose and the rate of growth during the injection period. On all doses, even those which resulted in the highest mortality, the gain in weight of the survivals ranged from 10 to 20 g. during the injection period. The only exceptions were the animals on a dose of  $3\frac{1}{2}$  g. adrenal gland, which gained only 9 g. This group, however, comprised only three animals, giving the figures 3, 10 and 14 g.

Hence, broadly it may be said that rate of growth during the injection period was independent of size of dose, since eight times the smallest protective dose gave no better result than that dose itself. The highest averages were found on doses of 25 and  $6\frac{1}{4}$  g. of gland, in both cases 19 g.

It has been held that by giving half the amount in each of two injections daily, instead of the whole in one, better growth is obtained. I have tried this on a few animals. The result, however, was negative. They

had a dose corresponding to  $12\frac{1}{2}$  g. of gland, and this dose was repeated 8 hours later each day. Four animals treated in this way gained 14, 8, 10 and 7 g. during the 21 days.

#### V. THE OCCURRENCE OF REMNANTS OF CORTICAL TISSUE AFTER ADRENALECTOMY

It is essential of course to make sure that the animals used for the estimation of the effect of the hormone die when the prolonged successful hormone treatment is stopped. Only then will it be safe to assume the complete absence of the adrenals which is indispensable. It is a well-established fact that small remnants of cortical tissue hypertrophy during hormone treatment [MacKay and MacKay, 1929; Carlson, 1931]. This appeared too from Gaunt and Gaunt's experiments in 1934. Gaunt had shown in 1933 that in a certain strain of rats the mortality following adrenalectomy was 95 p.c. In the same strain of rats they found that the mortality was reduced to 50 p.c. when the animals had first been kept alive for some time by hormone injections.

In my previous communication [1935*a*] I described an improved operative technique which gave in a group of seventy-seven young rats a mortality of 100 p.c., while twenty-seven young rats were kept alive for 21 days, like those dealt with in the present paper, by hormone injections. For this group too, the mortality was 100 p.c. when the injections were stopped, proving as I thought that in the given strain of rats the operation was really effective. Hormone treatment for 21 days must be considered sufficient to give any cortical remnants that may be left time to hypertrophy and then keep the animals alive after stopping the treatment. With the larger numbers, however, it turned out that a mortality of 100 p.c. after hormone treatment could not be maintained. In the preceding section an account was given of seventy-nine animals treated with preparation 110 and eight animals treated with preparation 156. As already mentioned, these eighty-seven animals died on cessation of the hormone treatment. But there were also some animals, eight in all or 9.2 p.c., not yet referred to, which did not die on stopping the treatment. Altogether I have operated upon 465 young rats with the improved technique, and fourteen have survived owing to remnants of cortical tissue. Of these 465 animals, 184 were treated with hormone for 21 days after the operation; and of these 184 animals, twelve did not die when the treatment had stopped, the larger numbers giving a somewhat lower percentage of such cases, viz. 6.5.

Dealing only with the eight animals in the experiments here described which did not die after cessation of treatment, most of them were already suspected of possessing cortical tissue when the injection period ended. Their growth rate was considerably above that of the other rats, as was previously pointed out [1935*a*]. During the 21 days of the injection period their weight increased on an average 42 g. (min. 21, max. 68 g.), as compared with the average figure of 16 g. for all the eighty-seven rats which died spontaneously after the injections were discontinued. These eight animals were killed after 3-4 weeks, when it was considered unlikely that spontaneous death would occur. For a good growth rate was maintained even then and in some progressively improved so as not to differ appreciably from the normal. On autopsy of these last no inconsiderable amounts of cortical tissue were indeed found. Suspicious bodies of tissue were cut across [1935*a*] and immersed in a dilute solution of silver nitrate. Blackening of the cut surface [Szent-Györgyi, 1928] was regarded as a proof that cortical tissue was present if it occurred within a minute. In three of the animals cortical tissue was found in this way, and they were the very animals that had shown the most rapid rate of growth, 68, 58 and 48 g. respectively, during the 21 days. It was not possible, however, to find any remnants of cortical tissue in the other five, in spite of the most careful search, probably because of the roughness of the method.

#### DISCUSSION

First, some facts of fundamental importance for the evaluation of the experiments may be referred to: to begin with constancy of the response in the strain of rats used. It was an extremely pure strain, and it had lived for many generations (at least seventeen) under quite uniform conditions and on the same diet: secondly, the great attention paid to all details of operative technique; and last, but not least, the fact that the same hormone preparation could be used throughout the experiment.

Conditions were thus favourable for a uniform reaction to the injections of the hormone. The only irregularity was due to the presence of remnants of cortical tissue. As shown in Section V they were found in only 6.5 p.c. of a large number. Such a relatively small proportion can readily be eliminated without inconvenience.

In estimating the effect of the cortical hormone the amount of saline in which the active principle was dissolved is important. In future assays in this laboratory we shall consider the advisability of giving all the

various doses in the same volume of saline,  $\frac{1}{2}$  c.c. or less. Or the effect of the saline may be entirely avoided by using a glucose solution as a solvent. It is true that the influence of sodium chloride was only noticeable when small doses were used in a large volume of saline (1 c.c.), but in a given case it may be of importance.

As previously pointed out, it is only the declining part of the curves giving the percentage survivals (Figs. 7, 8 and 9) which can be used in an assay. As long as there is no fixed standard of comparison, such as there is in the case of insulin, it is necessary to start from a curve obtained in the way indicated. The assumption that young rats under the given conditions show a uniform reaction to the cortical hormone is justified by the curves found, except where the influence of the saline asserted itself (Fig. 6).

Taking preparation 156 in somewhat more detail as an example, a fortunate choice of dosage yielded points in the declining part of the curve giving the percentage of survivals (Fig. 9). If only those points are chosen which represent the doses  $\frac{1}{2}$  and  $\frac{1}{4}$  c.c., they are comparable with the curve in Fig. 8, the shape of which expresses precisely the effect of doses of these volumes. The two curves have the same shape and show the same ratio of potency for 110 and 156 at both these doses. This ratio is found by measuring the height of the ordinate for the points to be examined in Fig. 9. In the curve in Fig. 8 the abscissa is determined for the points having the same ordinate. From the length of the abscissa, which is the logarithm of the size of the dose, the effect of the dose is calculated, measured in g. of gland in preparation 110. In this way it will be found that 25 g. of adrenal gland in preparation 156 have the same effect as 8.3 g. in preparation 110, and  $12\frac{1}{2}$  g. in preparation 156 the same as 4.9 g. in 110. Hence in this investigation the ratios between the effects of preparations 110 and 156 have been found to be 25 : 8.3 (=3) and  $12.5 : 4.9$  (=2.6) respectively. This agrees well with the fact that preparation 110 had been subjected to a less drastic purifying process involving less loss of active substance.

The rat survival method therefore furnishes a good basis for the biological assay of the cortical hormone. For survival under the given conditions really is a criterion of the effect of the cortical hormone, and the percentage of survivals, if less than 80, is in direct ratio to the size of the dose. In practice this means that a rough determination is made by means of some few experiments, so that doses can be chosen which yield percentages of survival ranging from 80 to 0. Then groups of animals (2-3) are given the doses considered suitable. As to how many

animals there should be in each group, this work gives no certain information. No doubt the larger the number of animals used the greater the degree of accuracy. It is, however, my distinct impression that by using only 6-8 animals in each group an accuracy below 50 p.c. may be attained.

#### SUMMARY

1. Young rats were adrenalectomized by an improved technique.
2. The influence of injections of 0.9 p.c. saline on the duration of life was studied. When 1 c.c. was injected once daily the average survival period in a series of thirty animals was lengthened from 5.7 to 7.7 days, when twice daily in another series somewhat more.
3. The percentages of survivals of seventy-nine hormone-treated animals were noted during a period of 21 days. The animals were daily injected with doses of a single preparation which ranged in the different groups from the equivalent of 100 g. of gland to 1/64 of that amount, and were given in most cases in three different volumes of 0.9 p.c. saline: 1,  $\frac{1}{2}$  and  $\frac{1}{4}$  c.c. respectively.
4. Curves show the percentages of survivals for different doses. With doses of  $12\frac{1}{2}$  g. or more, 80-100 p.c. survived. With smaller doses the curve declines steeply. As to the effect of the volume, the survival percentage for the smaller doses in the largest volume (1 c.c.) was decidedly higher than for the same doses in  $\frac{1}{2}$  or  $\frac{1}{4}$  c.c., a difference ascribed to the amount of sodium chloride.
5. By comparing with these curves those obtained with another preparation, tested on seventeen animals, the relative strength of the second preparation could be estimated with a fair degree of accuracy.
6. The gain in weight during the injection period bore no relation to the dose given, but was always less than half that normal for animals of that size. Dividing the dose into two daily injections did not give better growth.
7. Of animals kept alive for 21 days after adrenalectomy, 6.5 p.c. had to be discarded because remnants of adrenal gland hypertrophied during the period of observation.

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