Brit. J. Pharmacol. (1963), **20**, 95–98.

THE ASSAY OF CORTICOTROPHIN IN HYPOPHYSECTOMIZED AND IN HYDROCORTISONE-TREATED RATS

BY

A. ASHFORD AND J. I. M. JONES

From the Crookes Laboratories, Park Royal, London, N.W.10

(*Received August* 27, 1962)

Twenty-one pairs of parallel assays of corticotrophin are reported, in which results from hypophysectomized rats are compared with those from hydrocortisone-treated animals. The two methods show little difference in respect of potency, precision and limits of error.

The most widely used method for the biological assay of corticotrophin is that developed by Sayers, Sayers & Woodbury (1948), which measures the depletion of adrenal ascorbic acid in hypophysectomized rats. The procedure involves severe operational stresses and much skill, hypophysectomy being followed one day later by removal of one adrenal gland before injection of corticotrophin, to serve as a control, and removal of the remaining adrenal gland exactly 1 hr after injection. The difference in the ascorbic acid concentration in the two adrenal glands forms the criterion of response.

The first attempt to simplify this procedure was that of Munson, Barry & Koch (1948), in which the first adrenalectomy was omitted and both adrenals were removed after injection of corticotrophin and analysed together for ascorbic acid, the ascorbic acid concentration being inversely related to the dose of corticotrophin. This method still left some operational stress and the need for skill in hypophysectomy. Many attempts were made to avoid this operation by blocking the secretion of endogenous corticotrophin. After the original suggestion by Sayers & Sayers (1947) that certain corticosteroids, including cortisone, hydrocortisone (cortisol) and deoxycortone, blocked the secretion of corticotrophin, Hodges & Vernikos (1958) found that prednisolone and hydrocortisone (cortisol) were most effective in this respect. Dekanski & Harvie (1960) gave the results of forty assays in which hypophysectomy was replaced by injection of hydrocortisone acetate, and claimed the procedure to be one-and-a-half to two times as efficient as the original method, but they gave no results of assays on the same sample by both methods. Hamburger (1960) found all corticosteroids and their synthetic analogues to be inhibitory, with inhibition increasing in the order cortisone, hydrocortisone, prednisolone trimethyl acetate, prednisolone and dexamethasone. The last two gave complete inhibition in doses of 1.5 and 1.0 mg respectively per 100 g of rat body weight. Casentini, Hukovic & Tani (1957) found that fludrocortisone (0.9 mg per 100 g of rat body weight) completely inhibited the secretion of corticotrophin. Hamburger (1960)

TABLE

A COMPARISON OF THE RESULTS OF CORTICOTROPHIN ASSAYS

Potency ratios and slopes (b)

	Hypophysectomy						
						Regression significance	
Assay no.	$Potency_H$ (i.u./ml. or mg)	Fiducial limits (%)	Log. pot. ratio M_H	Variance S^2_H	s/b	t	P
1	22·5/ml.	65–153	−0·8279	0·3012	0·635	4·41	<0·001
2	0·433/mg	82–122	−0·2114	0·0192	0·357	7·36	<0·001
3	38·4/ml.	68–147	−0·0575	0·0738	0·753	3·71	<0·001
4	30·9/ml.	55–182	−0·3735	0·1756	1·043	2·57	<0·02
5	7·8/ml.	24–401	−1·3601	0·9426	1·411	1·85	0·07
6	128·0/mg	55–180	0·3523	0·1696	1·022	2·55	<0·02
7	117·0/mg	52–191	0·2285	0·2059	1·151	2·34	<0·05
8	111·0/mg	63–160	0·1553	0·1105	1·003	3·03	<0·01
9	0·43/mg	56–179	−0·3363	0·1713	1·144	2·55	<0·02
10	2·37/mg	49–202	−0·3429	0·2409	1·183	2·15	<0·05
11	37·1/ml.	82–121	−0·1096	0·0202	0·524	5·10	<0·001
12	22·2/ml.	72–140	0·1516	0·0543	0·609	4·35	<0·001
13	37·3/ml.	78–128	−0·1021	0·0325	0·728	4·59	<0·001
14	2·3/mg	66–152	0·0411	0·0677	0·863	3·37	<0·01
15	75·0/mg	62–162	−0·0872	0·1136	0·856	3·42	<0·01
16	103·0/mg	66–151	0·3642	0·0858	0·807	3·67	<0·001
17	91·0/mg	66–153	0·1809	0·0895	0·881	3·35	<0·01
18	127·0/mg	62–162	0·6731	0·1174	0·853	3·48	<0·01
19	106·0/mg	60–166	0·4070	0·1295	0·920	3·07	<0·01
20	110·0/mg	51–194	0·4551	0·2180	1·161	2·36	<0·05
21	89·0/mg	60–166	−0·1759	0·1268	0·925	2·80	<0·01
Arithmetic means		62–172			0·897		

$\Sigma W = 98{\cdot}7956$ ($W = 1/S^2_D$). $\Sigma WD = -6{\cdot}5698$. $\Sigma WD^2 = 7{\cdot}2301$ $(\Sigma WD)^2 = 43{\cdot}1623$.

$\bar{D}$ = Weighted mean of $D = \frac{\Sigma WD}{\Sigma W} = -0{\cdot}0665$. $S^2_{\bar{D}} = \frac{I}{\Sigma W} = 0{\cdot}01012 = 0{\cdot}1006^2$.

reported a single experiment in which injection of prednisolone and hypophysectomy gave the same responses and standard deviations, but the prednisolone-treated rats required more corticotrophin to produce the same depletion of ascorbic acid. The corticosteroid (1.5 mg per 100 g of rat) was administered 2 hr before the injection of corticotrophin, which is an advantage over the method of Dekanski & Harvie (1960) in which 6 mg of hydrocortisone acetate per 100 g of rat is given at 2 p.m. on the day before the assay and again at 8 a.m. on the day of assay.

The present paper reports a series of routine assays in which hypophysectomy was compared with inhibition by hydrocortisone of endogenous corticotrophin secretion, using the assay method of Munson *et al.* (1948).

METHODS

Assays were carried out on male albino rats (100 to 150 g of body weight) after they had been kept in a room maintained at a temperature of 27° C for 2 weeks.

On the day preceding the assay the animals were either hypophysectomized by the parapharyngeal route and given dextrose solution to drink overnight, or were injected intraperitoneally with hydrocortisone acetate (6.25 mg per 100 g of body weight) at 2 p.m. This dose was repeated at 9 a.m. next day. At 11 a.m. corticotrophin was injected subcutaneously into both hypophysectomized and hydrocortisone-treated rats, two dose levels being used.

1

FROM HYPOPHYSECTOMIZED AND CORTISOL-TREATED RATS

are based upon $\log_2$.

		Cortisol							
					Regression significance				
Potency_C (i.u./ml. or mg)	Fiducial limits (%)	Log. pot. ratio M_C	Variance S_C^2	s/b	t	P	$\frac{\text{Potency}_H}{\text{Potency}_C}$	D= ($M_H - M_C$)	S_D^2
24·6/ml.	57–177	−0·9129	0·1142	0·846	6·75	<0·001	0·915	0·0850	0·4154
0·175/mg	16–620	−1·1512	1·6355	1·818	1·42	<0·3	2·474	0·9398	1·6547
37·3/ml.	67–149	−0·1016	0·0783	0·802	3·57	<0·01	1·029	0·0441	0·1521
28·7/ml.	60–165	−0·4799	0·1231	0·827	3·14	<0·01	1·077	0·1064	0·2987
13·0/ml.	76–131	−0·6274	0·0371	0·515	5·97	<0·001	0·600	−0·7327	0·9797
126·0/mg	71–141	0·3373	0·0583	0·625	4·39	<0·001	1·016	0·0150	0·2279
126·0/mg	64–156	0·3372	0·0972	0·812	3·39	<0·01	0·929	−0·1087	0·3031
102·0/mg	48–206	0·0265	0·2599	1·387	1·97	0·06	1·088	0·1288	0·3704
0·51/mg	73–137	0·0276	0·0503	0·643	4·45	<0·001	0·843	−0·3639	0·2216
2·32/mg	70–142	−0·3705	0·0602	0·644	4·24	<0·001	1·021	0·0276	0·3011
46·2/ml.	65–153	0·2068	0·0891	0·800	3·43	<0·01	0·803	−0·3164	0·1093
18·8/ml.	69–198	−0·0876	0·1365	0·973	2·71	<0·02	1·181	0·2392	0·1908
42·2/ml.	62–164	0·0792	0·1173	0·875	2·92	<0·01	0·884	−0·1813	0·1498
3·0/mg	69–144	0·4307	0·0659	0·663	4·20	<0·001	0·767	−0·3896	0·1336
102·0/mg	60–168	0·3452	0·1326	0·974	2·90	<0·01	0·735	−0·4324	0·2462
120·0/mg	67–149	0·5795	0·0779	0·663	4·20	<0·001	0·858	−0·2153	0·1637
89·0/mg	80–122	0·1422	0·0220	0·410	6·77	<0·001	1·022	0·0387	0·1115
126·0/mg	67–149	0·6540	0·0778	0·640	4·34	<0·001	1·008	0·0191	0·1952
75·0/mg	75–133	−0·0933	0·0411	0·567	4·97	<0·001	1·413	0·5003	0·1706
96·0/mg	68–148	0·2703	0·0761	0·730	3·72	<0·001	1·146	0·1848	0·2941
102·0/mg	65–154	0·0247	0·0932	0·902	3·28	<0·01	0·873	−0·2006	0·2200
	64–176			0·815			1·032		

Homogeneity test, $\Sigma WD^2 - \frac{(\Sigma WD)^2}{\Sigma W} = 6{\cdot}7932$. χ (20 d.f.). P, 0·99=8·260.

The rats were anaesthetized with ether 3 hr later, and the adrenal glands were removed, trimmed free of fat, weighed and homogenized in 2.5% metaphosphoric acid. The homogenate was then centrifuged and ascorbic acid in the centrifugate estimated colorimetrically after addition of dichlorophenol indophenol.

Twenty-one pairs of parallel assays were made, assays 1 to 14 being with different production batches of corticotrophin against a high purity house standard assaying at 100 i.u./mg against the 2nd International Standard. Assays 15 to 21 were replicate assays of the proposed 3rd International Standard against the 2nd International Standard.

RESULTS AND DISCUSSION

The potencies obtained by each method and other relevant figures are given in Table 1. In general the two methods show little difference. If no difference exists, the ratio of the potency by one method to that by the other should be unity. Actually the simple arithmetic mean of these ratios is 1.032. Since most of the assays referred to different samples, detailed comparison was based on the evaluation of the individual differences (D) between the $\log_2$ potency ratios obtained by the two methods, the variance of these differences being the sum of the variances of the two comparable $\log_2$ potency ratios, i.e. $S_D^2 = (S_{MH}^2 + S_{MC}^2)$, where M_H and M^C are the $\log_2$ potency ratios with hypophysectomy and with hydrocortisone. The weighted

mean of these differences and its standard error were −0.0665 and 0.1006, so that the weighted mean does not differ significantly from zero ($t=0.657$ for 20 degrees of freedom). The mean index of precision (s/b) was 0.897 for the hypophysectomy and 0.815 for the hydrocortisone method, showing no significant difference between the methods. The validity of this statistical treatment depends on (1) the individual regressions being significant and (2) the values of D being homogeneous. Concerning the significance of regression, the probability P was <0.05 in most cases; notable exceptions were assay no. 5 (hypophysectomy) with $P=0.07$ and assays nos. 2 and 8 (hydrocortisone) with $P<0.3$ and 0.06. Omission of these assays does not affect the conclusions. The homogeneity of the differences in the potency ratios was tested by the formula $[\Sigma WD^2 - (\Sigma WD)^2/\Sigma W]$, W being the weight (equal to the reciprocal of the variance). The resulting value of 6.79 gave $P>0.99$ for χ^2 (20 d.f.) showing satisfactory homogeneity.

Since the work described above was completed, the technique has been altered to a single injection of 8 mg hydrocortisone per 100 g of rat 21 hr before the injection of corticotrophin. Similar satisfactory results are obtained, though no detailed parallel comparisons are available.

The authors are indebted to Dr D. J. Finney for his advice on the statistical treatment.

REFERENCES

CASENTINI, S., HUKOVIC, S. & TANI, F. (1957). Studi su di una nuova modalita' di dosaggio biologico dell'ACTH. *Boll. Soc. ital. Biol. sper.*, **33**, 909–912.

DEKANSKI, J. B. & HARVIE, M. I. (1960). The quantitative assay of corticotrophin using rats treated with hydrocortisone acetate. *Brit. J. Pharmacol.*, **15**, 95–100.

HAMBURGER, C. (1960). Assay of corticotrophin. Prednisolone pretreatment instead of hypophysectomy in the adrenal ascorbic acid depletion test. *Acta Endocrinol.*, **35**, 594–603.

HODGES, J. R. & VERNIKOS, J. (1958). A comparison of the pituitary inhibitory effects of prednisone, prednisolone and hydrocortisone. *Brit. J. Pharmacol.*, **13**, 98–102.

MUNSON, P. L., BARRY, A. G. & KOCH, F. C. (1948). A simplified hypophysectomised rat adrenal ascorbic acid bioassay method for adrenocorticotrophin (A.C.T.H.): specificity and application to preparative problems. *J. clin. Endocrinol.*, **8**, 586–587.

SAYERS, G. & SAYERS, M. A. (1947). Regulation of pituitary adrenocorticotrophic activity during the response of the rat to acute stress. *Endocrinol.*, **40**, 265–273.

SAYERS, M. A., SAYERS, G. & WOODBURY, L. A. (1948). The assay of adrenocorticotrophic hormone by the adrenal-ascorbic acid depletion method. *Endocrinol.*, **42**, 379–393.