Functional Tests of Adrenal Axis in Children with Measurement of Plasma Cortisol by Competitive Protein Binding

N. D. BARNES*, JENNIFER M. JOSEPH, SHELIA M. ATHERDEN, and
BARBARA E. CLAYTON

From The Hospital for Sick Children and the Institute of Child Health, London

Barnes, N. D., Joseph, J. M., Atherden, S. M., and Clayton, B. E. (1972). *Archives of Disease in Childhood*, 47, 66. **Functional tests of adrenal axis in children with measurement of plasma cortisol by competitive protein binding.** The competitive protein binding assay is the method of choice for estimation of plasma cortisol in children. Resting plasma cortisol levels in children without evidence of endocrine disease and with conditions affecting the hypothalamic-pituitary-adrenal axis are reported. These showed wide variation, and stimulation tests are therefore essential for diagnosis.

A normal response in the 30-minute tetracosactrin (Synacthen) test is defined as an increment of at least 10 $\mu\text{g/ml}$, with a final level of at least 25 $\mu\text{g/100 ml}$. 8 children with Addison's disease showed a minimal or negative response and 7 of 9 with hypopituitarism showed a subnormal response.

Similar criteria, an increment of at least 10 $\mu\text{g/100 ml}$, with a final level of at least 25 $\mu\text{g/100 ml}$ one hour after insulin, define a normal response to the intravenous insulin tolerance test. This test provides good discrimination between normal children and those with hypopituitarism, but negative results must be interpreted with caution.

Difficulties in the measurement of plasma corticosteroids have delayed the development of rapid, reliable adrenal function tests suitable for children. A test of the ability of the adrenal cortex to respond to adrenocorticotrophic hormone (ACTH) required full urine collections for several days (Clayton, Edwards and Renwick, 1963) and, in tests for the integrity of the hypothalamic-pituitary-adrenal axis, the measurement of groups of nonspecific urinary metabolites gave unreliable results in children (Clayton, 1968). The fluorimetric method for measurement of plasma 11-hydroxycorticosteroids (11-OHCS) has been widely used but has low specificity for cortisol (James, Townsend, and Fraser, 1967), and requires blood samples inconveniently large for paediatric work (Medical Research Council, 1971). In contrast, the competitive protein-binding method for plasma

cortisol described by Murphy (1967) is specific and so sensitive that very small blood samples can be used. This technique therefore seems likely to enhance considerably the ease and reliability of adrenal function testing in children. In this paper we describe a modified protein-binding method and report the results of plasma cortisol determinations obtained during investigations of the adrenal axis in children by the 30-minute tetracosactrin (Synacthen) stimulation test and the insulin tolerance test.

Subjects and Methods

Patients. All tests were performed on children undergoing investigation in The Hospital for Sick Children. When investigation was complete, those in whom there was no evidence of endocrine disease were classified as 'normal' controls. The criteria for a diagnosis of hypopituitarism were failure to secrete growth hormone in response to insulin, and/or Bovril stimulation combined with indirect evidence of thyrotrophic hormone and ACTH deficiency.

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*Present address: Mayo Graduate School of Medicine, Rochester, Minnesota 55901, U.S.A.

Plasma cortisol. Plasma cortisol was determined by the competitive protein-binding method of Murphy (1967) with a number of modifications, details of which are given in the Appendix.

The principle of the method is as follows: cortisol is transported in the blood stream mainly bound to a specific carrier protein, corticosteroid-binding globulin (CBG). A dilute solution of plasma is used as a CBG source and is equilibrated with tritium-labelled cortisol to form the assay solution. Cortisol is extracted from the sample under test with methylene dichloride, and the ability of this extract to displace radioactivity from the CBG in the assay solution is compared with that of cortisol standards. From this comparison, the cortisol content of the plasma sample can be estimated.

Synacthen stimulation test. After breakfast at 6 a.m. the child was allowed water only until the test was complete. Between 8.30 and 9.30 a.m. a blood sample was collected into heparin by finger prick or venepuncture. Tetracosactrin (Synacthen, Ciba), 0.25 mg/70 kg body weight, was then given by intramuscular injection and a second blood sample collected 30 minutes later.

Insulin tolerance test. The child was kept in bed under close medical supervision throughout the test. After an overnight fast a needle was placed in a vein and a blood sample taken. Insulin was then given by slow intravenous injection in a dose of 0.1 unit per kg body weight. A smaller dose was used if the child had a history of hypoglycaemia or was known to suffer from pituitary or adrenal insufficiency. Further blood samples were taken after 15, 30, 45, 60, and 90 minutes. The plasma cortisol levels fasting and 60 minutes after insulin are reported.

ACTH test. This was performed by the method of Clayton *et al.* (1963). The maximum output of total 17-hydroxycorticosteroids per 24 hours is reported. A value greater than 20 mg is normal.

Cortisol production rate (CPR). The method of Makin, Edwards, and Clayton (1968) was used. The normal value is 12.3 ± 2.8 (SD) mg/m² body surface area per 24 hours (J. Bertrand, personal communication, 1967).

Plasma ACTH. These levels were kindly determined by Professor J. Landon by radio immunoassay (Landon and Greenwood, 1968). The upper limit of normal in samples obtained between 8 and 10 a.m. is 70 pg/ml.

Results

Resting plasma cortisol. Fig. 1 shows individual resting plasma cortisol levels. The mean value was taken when more than one resting level was available for a single child. The mean resting value for blood samples taken between 8.30 and

FIG. 1.—Resting plasma cortisol values in children without evidence of endocrine disease and in those with conditions affecting the hypothalamic-pituitary-adrenal axis.

9.30 a.m. in 70 children without evidence of endocrine disease was 17.3 ± 7.8 (SD) µg/100 ml, in 15 children with hypopituitarism, 7.8 ± 4.4 µg/100 ml, and in 10 children with Addison's disease 12.4 ± 6.3 µg/100 ml. 3 children with Cushing's syndrome showed resting levels greater than 50 µg/100 ml. At midnight 8 children without evidence of endocrine disease showed a mean value of 3.1 ± 1.2 µg/100 ml, and one child with Cushing's syndrome, a level of 36.0 µg/100 ml.

Synacthen test. Table I shows the results of Synacthen tests in 38 children without evidence of endocrine disease, Table II those in 9 children with hypopituitarism, and Table III those in 8 children with Addison's disease. Fig. 2 shows the mean value ± 1 SD before and 30 minutes after Synacthen for each of these three groups.

Insulin tolerance test. Table IV shows the results of insulin tolerance tests in 13 children without evidence of endocrine disease, in 6 children

TABLE I
Synacthen Tests in Children Without Endocrine Disease

Case No.	Sex	Age (yr)	Plasma Cortisol ($\mu\text{g}/100\text{ ml}$)			ACTH Test (mg/24 hr)
			Resting	30 min After Synacthen	Increment	
1	F	0.2	32.0	60.0	28.0	19.6
2	F	0.6	6.2	28.6	22.4	
3	F	1.0	18.8	38.8	20.0	
4	F	1.2	17.0	51.0	34.0	
5	M	1.7	28.5	39.0	10.5	
6	F	2.0	18.8	46.0	27.2	
7	M	2.0	22.0	37.0	15.0	
8	F	2.5	18.0	30.0	12.0	
9	M	3.7	12.8	25.2	12.4	
10	M	3.8	26.8	46.8	20.0	
11	F	4.0	24.0	40.0	16.0	24.0
12	M	4.1	8.4	34.0	25.6	
13	M	4.3	16.0	28.0	12.0	
14	F	4.5	16.2	40.4	24.2	
15	M	4.5	30.8	60.0	29.2	
16	F	5.0	16.0	44.8	28.8	
17	M	5.1	4.5	31.3	26.8	
18	M	5.3	20.0	46.0	26.0	
19	M	6.0	5.6	23.6	18.0	
20	F	6.0	10.0	35.4	25.4	
21	M	6.1	7.0	29.0	22.0	22.0
22	M	6.3	15.0	31.4	16.4	
23	M	6.5	37.2	56.0	18.8	
24	F	6.7	18.7	43.4	24.7	
25	M	7.3	9.3	30.1	20.8	
26	M	7.3	12.8	39.0	26.2	
27	M	8.5	24.2	55.4	31.2	
28	M	9.1	22.6	55.4	32.8	
29	M	9.1	13.8	29.9	16.1	
30	M	9.9	34.0	45.4	11.4	42.0
31	M	10.0	16.0	51.6	35.6	
32	M	10.3	10.5	24.8	14.3	
33	F	11.5	32.8	48.0	15.2	
34	M	12.1	17.2	35.8	18.6	
35	M	12.3	15.6	35.2	19.6	
36	M	12.9	26.2	40.0	13.8	
37	M	13.3	12.5	25.0	12.5	
38	M	14.1	16.8	33.6	16.8	

TABLE II
Synacthen Tests in Hypopituitarism

Case No.	Sex	Age (yr)	Plasma Cortisol ($\mu\text{g}/100\text{ ml}$)			Comment
			Resting	30 min After Synacthen	Increment	
39	F	1.7	5.4	17.8	12.4	Idiopathic
40	M	2.8	12.0	27.4	15.4	Idiopathic
41*	M	3.1	18.8	34.6	15.8	Idiopathic
42	F	3.5	15.2	20.8	5.6	Idiopathic
43	F	4.1	6.8	16.8	10.0	Septo-optic dysplasia
44	F	7.3	4.0	16.4	12.4	Idiopathic
45	M	7.6	6.0	15.6	9.6	Idiopathic
46	M	7.7	4.5	19.7	15.2	Craniopharyngioma
47	M	14.6	6.0	14.0	8.0	Craniopharyngioma

*This patient received ACTH therapy until shortly before the test.

TABLE III
Synacthen Tests in Addison's Disease

Case No.	Sex	Age (yr)	Plasma Cortisol ($\mu\text{g}/100\text{ ml}$)			ACTH Test ($\text{mg}/24\text{ hr}$)	CPR* (mg/m^2 per 24 hr)	Comments
			Resting	30 min After Synacthen	Increment			
48	M	4.6	10.0	9.6	-0.4	4.5	5.7	Plasma ACTH C-terminal 4560 pg/ml N-terminal 3760 pg/ml Died; necropsy bronchopneumonia, cerebral sclerosis, total adrenal weight <1 g Plasma ACTH C-terminal 1800 pg/ml N-terminal 1825 pg/ml Sibs with cerebral sclerosis and Addison's disease†
49	M	9.8	14.8	12.4	-2.4	6.8		
50	M	12.7	8.8	6.0	-2.8	9.2	8.4	
51	M	6.4	14.9	13.5	-1.4	12.7		
52	M	6.8	23.4	23.4	0.0	4.8		Cerebral sclerosis with Addison's disease; unrelated
53	M	8.9	12.8	15.0	2.2			
54	M	11.0	5.8	5.6	-0.2			Idiopathic; adrenal antibodies present
55	F	13.2	18.0	20.5	2.5	6.6	14.7	Idiopathic

*Cortisol production rate.

†A fourth brother has Addison's disease and a fifth died from 'gastroenteritis' aged 6.

FIG. 2.—Mean plasma cortisol values ± 1 SD before and 30 minutes after Synacthen stimulation.

with isolated growth hormone deficiency, and in 9 children with hypopituitarism. Fig. 3 shows the mean value ± 1 SD before and 60 minutes after insulin for the normal and isolated growth hormone deficiency groups combined and for the children with hypopituitarism. In most instances the plasma cortisol was also estimated 15, 30, and 45 minutes after insulin but these values did not add further information.

Discussion

The competitive protein-binding assay enables plasma cortisol to be measured simply, rapidly, and

accurately. In normal plasma no significant difference was found when the cortisol value obtained by protein-binding was compared with that obtained by double-isotope dilution analysis (Bowman and De Luna, 1968; Iturzaeta, Hillman, and Colle, 1970) or by a specific thin-layer chromatographic and fluorimetric method (Nugent and Mayes, 1966). The protein binding was not affected by the presence of a number of synthetic steroids nor by a variety of drugs (Murphy, 1967). However, certain steroids, notably progesterone, 17-hydroxyprogesterone, and 11-desoxycortisol, compete for corticosteroid-binding globulin and, if present in abnormal amounts in the plasma, contribute to the apparent 'cortisol' value. The method is therefore not specific for cortisol in the first week of life, in congenital adrenal hyperplasia, in subjects taking metyrapone, and possibly in some other conditions with disordered steroid metabolism, such as adrenal carcinoma. In these situations the introduction of preliminary purification either by chromatography (Iturzaeta *et al.*, 1970) or by solvent partition (Murphy, 1967; Rivarola *et al.*, 1970) is necessary.

An important additional advantage of the method for paediatrics is the fact that the assay can be performed on very small quantities of plasma. Though we have preferred, for technical simplicity, to use 100 or 200 μl samples the estimation can, if necessary, be performed on as little as 10 μl .

In the fluorimetric measurement of plasma 11-OHCS a large and variable proportion (27–55%) of the total fluorescence was shown to be due to

TABLE IV
Plasma Cortisol Levels During Insulin Tolerance Tests

Case No.	Sex	Age (yr)	Plasma Cortisol ($\mu\text{g}/100\text{ ml}$)			Comment
			Resting	60 min After Insulin	Difference	
<i>Children without endocrine disease</i>						
5	M	1.7	36.0	50.0	14.0	Small normal
56	M	7.0	12.5	28.7	16.2	Small normal
57	M	8.0	10.0	27.8	17.8	Small normal
58	F	8.3	18.5	38.0	19.5	Small normal
59	M	9.1	13.0	29.0	16.0	Small normal
60	M	9.9	23.0	34.0	11.0	Small normal
61	F	10.0	12.0	28.0	16.0	Small normal
62	M	10.2	14.2	25.0	10.8	Small normal
63	F	11.4	15.0	29.0	14.0	Turner mosaic
64	M	11.9	39.0	57.4	18.4	Small normal
65	F	12.0	15.0	29.0	14.0	Small normal
66	M	12.1	24.0	35.2	11.2	Small normal
67	M	12.4	14.4	30.6	16.2	Small normal
<i>Isolated growth hormone deficiency</i>						
68	F	6.1	8.0	60.0	52.0	
69	M	9.0	10.8	27.6	16.8	
31	M	10.0	27.6	51.2	23.6	
70	M	10.2	8.8	40.4	31.6	
33	F	11.5	16.8	35.0	18.2	
37	M	13.4	15.6	34.0	18.4	
<i>Hypopituitarism</i>						
40	M	2.8	16.4	14.0	-2.4	Idiopathic
43	F	4.1	6.4	12.4	6.0	Septo-optic dysplasia
71	F	6.1	6.0	8.4	2.4	Craniopharyngioma
45	M	7.6	14.6	5.6	-9.0	Craniopharyngioma
72	M	7.7	5.8	5.8	0.0	Idiopathic
73	F	9.0	10.8	17.0	6.2	Craniopharyngioma
74	F	11.4	4.0	2.8	-1.2	Hypothalamic tumour
47	M	14.7	5.2	10.8	5.6	Idiopathic
75	F	15.0	3.8	2.6	-1.2	Craniopharyngioma

FIG. 3.—Mean plasma cortisol values ± 1 SD before and 60 minutes after intravenous insulin.

substances other than cortisol (James *et al.*, 1967). Values obtained by this method are therefore not comparable with those by protein binding. Few

normal values for plasma cortisol by protein-binding in children have yet been reported. Iturzaeta *et al.* (1970) gave a mean resting level in 46 children of 10.7 ± 4.8 (SD) $\mu\text{g}/100\text{ ml}$, and Rivarola *et al.* (1970), in only 6 children, gave a mean resting level of $11.9 \mu\text{g}/100\text{ ml}$. Results in the neonatal period were reported by Stevens (1970), but the specificity of the method is inadequate in the first few days of life because of the contribution of maternal progesterone. In the present series the resting levels in children without endocrine disease were variable, ranging from 4.5 – $39.0 \mu\text{g}/100\text{ ml}$, and the mean, $17.3 \mu\text{g}/100\text{ ml}$, was higher than those previously reported. However, these values were obtained in children undergoing investigation in hospital for a variety of acute and chronic conditions. The midnight levels in 8 children were uniformly low. The resting levels in 3 children with Cushing's syndrome were very high. The mean resting level in children with Addison's disease, $12.4 \mu\text{g}/100\text{ ml}$, was higher than the mean, $7.8 \mu\text{g}/100\text{ ml}$, in children with hypopituitarism. This difference is statistically significant ($P < 0.05$, Student's

't' test). The wide variation in resting levels in children without endocrine disease, the fact that cortisol secretion is normally episodic (Hellman *et al.*, 1970), and the observation that children with Addison's disease and hypopituitarism showed values within the normal range all emphasize that resting values alone are of little diagnostic use. Stimulation, and in the case of suspected adrenal overactivity, suppression tests are therefore essential.

The 30-minute Synacthen stimulation test is simple, rapid, and requires only three pricks. Synacthen is a pure substance which can be prescribed by weight and is only weakly antigenic. In this series a dose related to the weight of the child has been given which provides maximal adrenal stimulation but requires the measurement of very small doses for infants. A simpler dose regimen, such as 0.125 mg for infants up to 2 years and 0.25 mg for all older children, is therefore recommended. Studies of the Synacthen test with plasma 11-OHCS measurement in adults (Wood *et al.*, 1965; Greig *et al.*, 1969) and children (Job *et al.*, 1967; Davidson and Johnston, 1968) have demonstrated, and our results confirm, that it is a reliable screening test for impaired adrenal function. The criteria for assessing a normal response should be stringent so that partial degrees of hypoadrenalism will not be overlooked. For the reasons given above, the resting cortisol value is disregarded and suggested criteria for a normal response is an increment of at least 10 $\mu\text{g}/100\text{ ml}$ with a final level of at least 25 $\mu\text{g}/100\text{ ml}$. It has been shown that, though the resting cortisol level varies throughout the day, the level after stimulation is similar (McGill *et al.*, 1967). The suggested criteria will, therefore, apply at any time of day and the test is suitable for outpatient use. The final level in 2 children in the 'normal' group fell marginally below the proposed limit at 24.8 $\mu\text{g}/100\text{ ml}$ and 23.6 $\mu\text{g}/100\text{ ml}$. Neither showed evidence of endocrine disease, and both responded normally to the ACTH stimulation test.

Eight children showed a very small (less than 3 $\mu\text{g}/100\text{ ml}$) or negative response to stimulation. Primary hypoadrenalism was confirmed in 7 of them and was almost certainly present in the other child. 6 of these children suffered from the syndrome of cerebral sclerosis with Addison's disease (Forsyth, Forbes, and Cumings, 1971). Except in one case, the resting cortisol level was within the range found in 'normal' children. One child also had a normal cortisol production rate, a finding that has been reported previously in Addison's disease (Cope and Pearson, 1965). The two child-

ren in whom it was measured showed very high plasma ACTH levels.

Nine children with hypopituitarism were tested. All showed some response to stimulation but the increment was 10 $\mu\text{g}/100\text{ ml}$ or less in 4 and the final value was clearly subnormal in 7. Only 2 children, one of whom had recently received ACTH therapy, showed normal responses. An intermediate response may therefore provide a useful indication of the possibility of secondary hypoadrenalism. It is emphasized, however, that a normal response may occur. It has been suggested that a more prolonged stimulation test with depot Synacthen may give more reliable differentiation of normal from hypopituitary responses (Galvão-Teles, Burke, and Fraser, 1971).

These results suggest that partially atrophied adrenals, under maximal stimulation from endogenous ACTH, may be able to maintain a normal resting level and even a normal daily secretion of cortisol and yet be unable to respond further to stimulation from exogenous ACTH. In contrast, the adrenal in hypopituitary subjects has little resting 'drive' but responds, though more slowly than normal, to stimulation.

Of the tests which depend on the integrity of each limb in the hypothalamic-pituitary-adrenal axis the insulin tolerance test is probably the safest, most reliable, and least unpleasant for the subject (*British Medical Journal*, 1970). Close medical supervision is essential, as hypoglycaemic coma can occur, but if the insulin is injected and the blood samples are withdrawn through a single indwelling needle, the test is not disturbing, and symptomatic hypoglycaemia, if it occurs, can be reversed rapidly by intravenous injection of glucose. A further advantage of the insulin tolerance test is that it allows simultaneous assessment of the ability to secrete growth hormone. A fall in blood glucose to 50% of the resting level is considered an adequate stimulus. The greatest fall in blood glucose occurs approximately 15 minutes after injection of insulin, the cortisol response reaches a peak by 60 minutes, and the growth hormone response usually between 30 and 90 minutes. The 11-OHCS response to insulin-induced hypoglycaemia has been reported in adults (Jacobs and Nabarro, 1969) and children (Laron *et al.*, 1969). Our results confirm that the test provides good discrimination between normal children and those with hypopituitarism, and suggest that criteria similar to those proposed for the Synacthen test may be used to define a normal response, an increment of at least 10 $\mu\text{g}/100\text{ ml}$, with a value 60 minutes after insulin of at least 25 $\mu\text{g}/100\text{ ml}$. We have seen occasionally

negative responses to an adequate hypoglycaemic stimulus in children apparently without endocrine disease; negative results must, therefore, be interpreted with caution and confirmatory evidence obtained.

It is concluded that competitive protein-binding is the method of choice for measurement of plasma cortisol, and that combined with stimulation tests it offers a greatly improved degree of accuracy and reliability in assessment of the function of the adrenal axis in children.

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APPENDIX

Measurement of Plasma Cortisol

Procedure. Plasma (200 μ l) was extracted with 10 volumes of methylene dichloride (British Drug Houses Ltd. general purpose reagent, redistilled once). Duplicate aliquots of the extract and standards from 0–30 ng cortisol (Steraloids Ltd.) were evaporated to dryness and dissolved in 1 ml of a 4% solution of reconstituted dried plasma containing approximately 50,000 counts/min per ml tritiated cortisol. After incubation at 45 °C for 10 minutes and at less than 5 °C in an ice-bath for 30 minutes, 80 mg Florisil (Hopkin and Williams Ltd. 60–100 mesh) was added to each tube and the batch shaken mechanically for 30 seconds. 0.5 ml supernatant was removed and the radioactivity determined. A standard curve was prepared and the cortisol content of the unknown samples calculated.

Evaluation. The method proved simple and rapid (up to 30 samples in duplicate in 4 hours).

Recovery. Extraction of tritiated cortisol (Radiochemical Centre, Amersham) from plasma gave a recovery of 95 ± 1.7 (SD)%.

Precision. The coefficient of variation of repeated assays at concentrations of approximately 5, 10, 15, and 20 ng/100 ml were 2, 3, 6, and 5% respectively.

The coefficient of variation of values obtained on control plasma included in each assay over a 4-month period was 3%.

Accuracy. Plasma cortisol concentration was assessed in a number of plasma samples before and

after the addition of known amounts of cortisol. Recoveries were 101 ± 6 (SD)% with 10 ng added and 103 ± 4 (SD)% with 20 ng added.

Correspondence to Professor B. E. Clayton, Department of Chemical Pathology, The Hospital for Sick Children, Great Ormond Street, London WC1N 3JH.