Preliminary Report on Coated Charcoal Immunoassay of Human Chorionic "Growth Hormone-Prolactin" and Growth Hormone.* (31420)

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Coated charcoal has previously been shown to provide a means for rapid separation of free molecules from the same molecules complexed to their carriers(1). This method of batch separation has the advantages of simplicity, rapidity, completeness of separation, and the small void volume of charcoal as compared to agents such as Sephadex. The coated charcoal technique has been successfully applied to assays for plasma iron and unsaturated iron binding capacity(2,3), as an *in vitro* test of thyroid function(4,5), for vit B_{12} and its binders (intrinsic factor, serum unsaturated B_{12} binding capacity)(1,6) and for insulin and insulin antibodies(7). These assays require the separation of free low molecular weight (iron), relatively low molecular weight (triiodothyronine, M.W. 651; vit B_{12}, M.W. 1355) and intermediate molecular weight (insulin, M.W. of the dimer approx. 12,000) substances from the same substances when complexed with their large protein or glycoprotein binders.

This communication extends the coated charcoal immunoassay technique to 2 hormones of relatively high molecular weight, human chorionic "growth hormone-prolactin" (CGP) and human growth hormone (HGH), using essentially the same procedure previously applied to insulin assay(7). This report presents the data mentioned elsewhere(7).

Materials. Buffer. Veronal-acetate buffer (Michaelis), pH 7.4 was prepared as described previously(7).

Charcoal suspension. A 5 g/100 ml suspension of Norit A neutral pharmaceutical grade charcoal (purchased from Amend Drug and Chemical Co., New York City) was prepared by adding the charcoal to buffer(7).

Dextran solution. 0.5 g of Dextran 250, purchased from Pharmacia, Inc. (Fair Lawn, N. J.), of 250,000 average molecular weight as determined by light scattering, was added to 100 ml of buffer.

Dextran-coated charcoal. Dextran-coated charcoal is prepared by mixing equal volumes of the charcoal suspension and dextran solution for 10 seconds. The product is stored at 4°C.

Human albumin. Normal serum albumin (human) USP, as Albumisol liquid, was purchased from Merck Sharp and Dohme. This product contains 12.5 g normal human serum albumin in 250 ml of buffered diluent. Commercial salt-poor albumin should not be used (7).

Albumin-buffer. Albumin-buffer is prepared by adding 7 ml of Albumisol to 93 ml of buffer to give an albumin concentration of 350 mg/100 ml.

Hormones and antisera. The unlabeled and labeled hormones and antisera (prepared in rabbits) were generously supplied by Drs. M. Grumbach and S. Kaplan. Unlabeled chorionic "growth hormone-prolactin" (CGP) and human growth hormone (HGH) were stored in albumin-buffer at 4°C in solutions of 10 and 1 ng/ml respectively. The I^{131}-CGP used had specific activities ranging from 90 to 145 $\mu c/\mu g$ and the I^{131}-HGH had a specific activity of approximately 355 $\mu c/\mu g$; both were prepared by a modification of the method of Greenwood *et al*(8).

Procedure for assay of radioiodinated hormones. Every newly prepared solution of I^{131}-CGP or I^{131}-HGH is assayed against the cold hormone standard using dextran-coated charcoal. The amount of radio-iodinated hor-

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TABLE I. Protocol for Radiodilution Curve Human Growth Hormone and Assay of Unknown Samples.

Sample	Albumin-buffer*	Control serum	2λ/ml I^{131}-HGH†	1 ng/ml cold HGH‡	1:50,000 anti-HGH§	1:10 $D^{250}CC$‖
1 14	3.0	.1	.5	—	.5	2.0
2 15	2.9	.1	.5	.1	.5	2.0
3 16	2.8	.1	.5	.2	.5	2.0
4 17	2.6	.1	.5	.4	.5	2.0
5 18	2.2	.1	.5	.8	.5	2.0
6 19	2.0	.1	.5	1.0	.5	2.0
7 20	1.5	.1	.5	1.5	.5	2.0
8 21	1.0	.1	.5	2.0	.5	2.0
9 22	—	.1	.5	3.0	.5	2.0
10 23	3.5	.1	.5	—	—	2.0
11 24	5.5	.1	.5	—	—	—
				Unknown serum		
12 25	2.9	.1	.5	.1	.5	2.0
13 26	3.4	.2	.5	—	—	2.0

Mix well. Incubate at 37°C for 4 hours.

Mix well. Centrifuge at 3000 rpm for 15 min. Count supernatants.

* 350 mg of albumin per 100 ml of veronal-acetate buffer (Michaelis), pH 7.4.
† Prepared by diluting I^{131}-HGH in albumin-buffer.
‡ Prepared by diluting non-radioactive HGH in albumin-buffer.
§ Prepared by diluting the anti-HGH antiserum in albumin-buffer.
‖ Prepared by mixing equal volumes of a 5.0 g % suspension of Norit A charcoal and a 0.5 % solution of Dextran of average molecular weight 250,000 (D 250).

TABLE II. Protocol for Assay of Serum Chorionic Growth Hormone-Prolactin.*

Sample	Albumin-buffer	2λ/ml I^{131}-CGP	Unknown serum	1:2000 anti-CGP	1:10 $D^{250}CC$
1 3	2.9	.5	.1	.5	2.0
2 4	3.4	.5	.1	—	2.0

* A radiodilution curve for the hormone may be prepared in a manner analogous to Table I, using 10 ng of non-radioactive CGP per ml of albumin-buffer in place of unknown serum. The reagents are prepared in a similar manner to those for assay of human growth hormone. Incubation time is identical for the assay of human growth hormone when our antibody is diluted 1:2000 with albumin-buffer.

mone present may be calculated from the following formula:

$$\text{ng } I^{131}\text{-hormone} = \text{ng cold hormone} \frac{B'}{B-B'}$$

where B = the net counts per minute of the supernatant containing antibody and I^{131}-hormone and B′ = net cpm of the supernatant containing antibody, I^{131}-hormone and standard cold hormone(6).

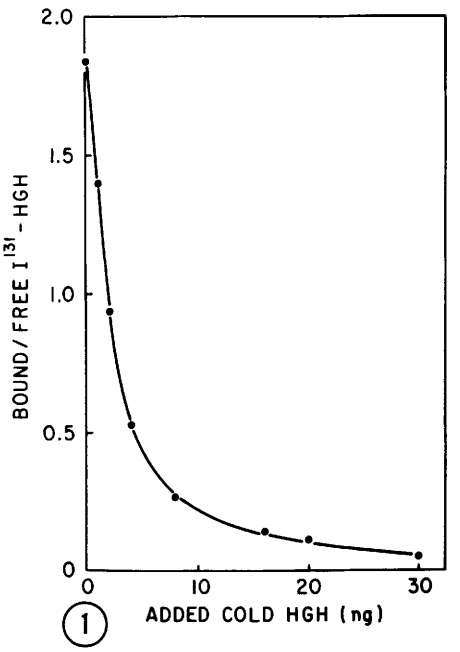
Method. Table I summarizes the assay procedure for HGH and Table II summarizes the assay procedure for CGP. All tests were done in duplicate in 10 ml test tubes. The reagents were added in the volume and sequence indicated. The tubes were incubated for 4 hours in a 37° water bath, after which 2 ml of dextran-coated charcoal was added to all tubes except the experiment standard. The tubes were then capped with Parafilm and mixed by repeated inversion for approximately 10 seconds. They were then centrifuged for 15 minutes at 3,000 r.p.m. and the supernatant containing bound hormone was decanted into counting tubes, leaving behind the button of sedimented charcoal containing the unbound hormone. The supernatant fluid was then counted in a well-type scintillation detector.

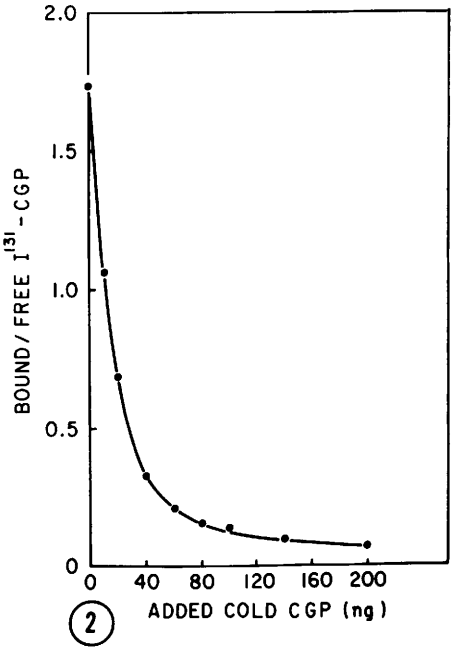
Calculation of serum HGH and serum and urine CGP levels(6). The level of hormone may be calculated from the formula:

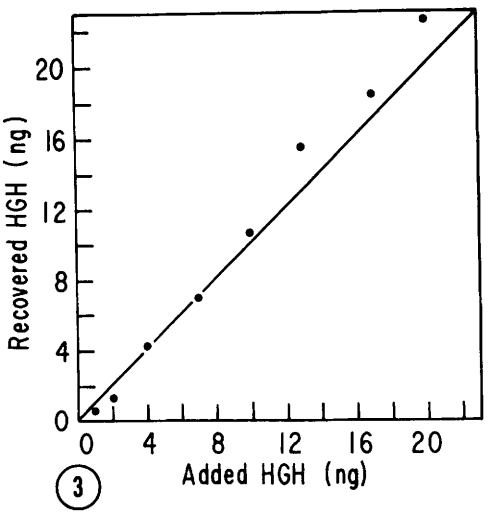
$$\frac{\text{ng hormone per}}{\text{0.1 ml specimen added}} = \text{ng } I^{131}\text{-hormone} \frac{B}{B''-1}$$

where B = net cpm of antibody control and B″ = net cpm of 0.1 ml of the unknown specimen added (serum or urine).

The net cpm was obtained by subtracting







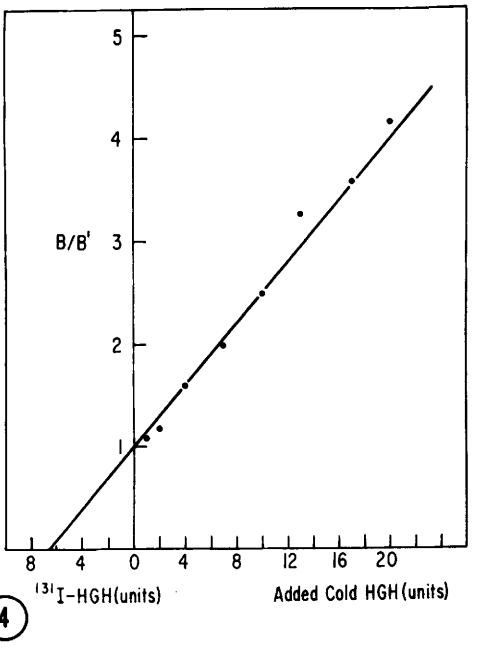


FIG. 1. Radiodilution curve obtained by charcoal immunoassay, in which the bound to free ratio of I^{131}-human growth hormone is plotted against the added human growth hormone.

FIG. 2. Radiodilution curve obtained by charcoal immunoassay, in which the bound to free ratio of I-^{131}CGP is plotted against the added CGP.

FIG. 3. Radiodilution curve obtained for human growth hormone replotted, replacing ordinate values with recovered unlabeled hormone.

FIG. 4. Data of Fig. 3 plotted to illustrate that charcoal immunoassay of growth hormone can be represented by a linear graph.

TABLE III. Comparison of Coated-Charcoal and Immunoelectrophoretic Assays for Human Growth Hormone.

Serum No.	Coated-charcoal assay (ng/ml)	Immunoelectrophoresis assay (ng/ml)	Double Ab
15	8	8	
16	62	38	84
17	100	>90	
19	16	26.5	32
20	10	15.5	13.6
23	6	<1	4 , 2.6
25	6	<1	2.4, 2.2
30	6	<1	
31	76	38	
32	10	3.5	
33	<2	<1	
34	10	<1	
35	2	<1	
36	2	<1	
37	10	<1	
38	4	<1	

the cpm of the appropriate supernatant controls from their respective tubes.

Results. Fig. 1 and 2 show radiodilution curves obtained for HGH and CGP, respectively, when the B/F ratio of hormone is plotted on the ordinate and the amount of added unlabeled hormone on the abscissa (6,10,11).

Fig. 3 shows a radiodilution curve obtained for HGH replotted, replacing the ordinate values with recovered unlabeled hormone (HGH).

Fig. 4 shows a replot of the data of Fig. 3, using the ratio B/B′ in place of B/F on the ordinate, where B = cpm of I^{131}-HGH bound by antibody and B′ = cpm of diluted I^{131}-HGH bound by the same quantity of antibody to demonstrate that radioimmunoassay of hormones may be represented by a linear graph(6,12).

Tables III and IV show corresponding data for HGH and CGP, respectively, using the coated charcoal assay and comparing the results with the chromatoelectrophoretic method (11).

Discussion. A quantity of antibody must be chosen with a maximal binding capacity equal to 60 to 80%(6) of the standard amount of radioiodinated hormone used in each test. This will provide an excellent bound to free ratio (B:F), theoretically ranging from 1.5 to 1 to 4 to 1. In practice, the level tends toward the lower range because of a significant amount of hormone damage (5-20%) in the preparation, handling and storage of the iodinated hormones.

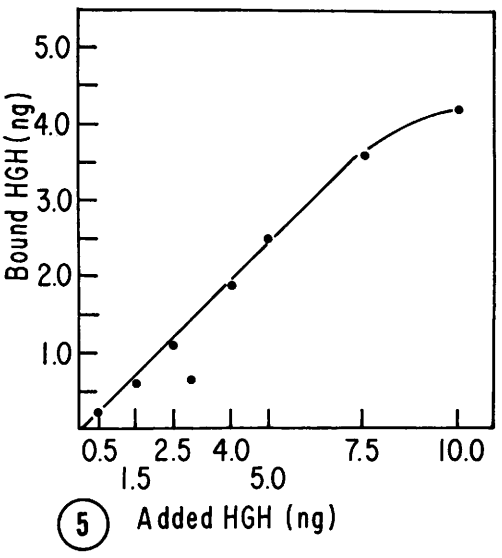
To determine the maximal HGH binding capacity of an antibody, a fixed quantity of antibody is incubated for 4 hours at 37°C in a series of tubes containing increasing quantities of labeled HGH. Dextran-coated charcoal is added at the end of incubation to separate free from bound HGH. The quantity of bound HGH is determined by counting the radioactivity in the decanted supernatant fluid after centrifugation sediments the charcoal. Fig. 5 shows the HGH-binding capacity of a 0.5 ml of a 1:100,000 dilution of rabbit HGH antibody serum, determined in this manner. The graph shows that the maximum binding capacity of 0.5 ml of a 1:100,000 dilution of this particular antibody is 3.75 ng of HGH.

The method can also be applied to determine the quantity of antibody needed to achieve binding of 60 to 80% of a given amount of labeled HGH. In this application, I^{131} is incubated at 37°C for 4 hours in a series of tubes containing increasing volumes of antibody serum in dilutions of 1:50,000. The quantity of HGH bound is determined as previously described. Fig. 6 shows the results of such an experiment, in which approximately 63% of the I^{131}-HGH is bound by 1.0 ml of a 1:50,000 dilution of anti-HGH antibody serum.

TABLE IV. Comparison of Coated-Charcoal and Immunoelectrophoretic Assays for Chorionic Growth Hormone-Prolactin.

Serum No.	Coated-charcoal assay (ng/ml)	Immunoelectrophoretic assay (ng/ml)
1	17	28.5
2	16	20.5
3	24	27.5
4	12	28.5
5	18	42.0
6	36	27.0
7	18	30.0
8	26	26.0
9	14	22.5
10	32	48.0
11	58	45.0
12	16	34.0
13	0	.037
14	3	2.3

In subsequent studies, we have found that 60 to 80% binding of a given amount of HGH may be achieved with *less* antibody if incubation is prolonged to overnight or over a weekend. To avoid damage to the labeled hormone, longer incubation is preferably carried out at 4°C. For consistently good B/F ratio, incubation with antibody should be 4 days at 4°C.



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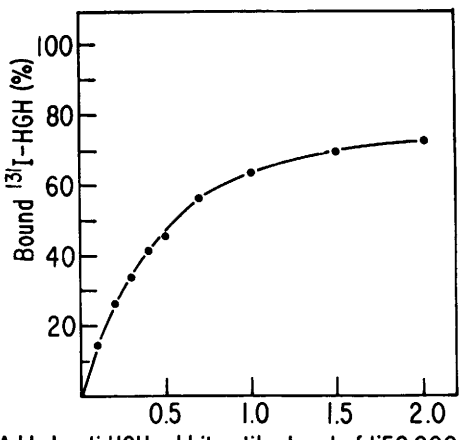

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FIG. 5. Growth hormone binding capacity of 0.5 ml of a 1:100,000 dilution of rabbit antibody to human growth hormone is depicted. Maximum binding capacity, represented by the plateau of the graph, is 3.75 ng of human growth hormone (for this particular antibody).

FIG. 6. Growth hormone binding capacities of increasing quantities of antibody after incubating with a fixed quantity of growth hormone. From such a graph, the quantity of antibody needed to bind 60 to 80% of the labeled growth hormone, at any given temperature and period time, is determined.

Preliminary studies of the application of coated charcoal to the assay of HGH and CGP indicate it is feasible. However, some samples of serum non-specifically exclude hormone from the coated charcoal, requiring further study before the procedure can be used with confidence for assay of the hormone in serum. It is for this reason 0.1 ml control serum is added to each tube for HGH assay.

The charcoal technique appears to offer advantages of simplicity over previous methods. Since it requires lower specific activity than prior methods, I^{125} may be used.

Summary. Charcoal premixed with dextran of average molecular weight 250,000 almost instantly adsorbs free human growth hormone or human chorionic "growth hormone-prolactin," but rejects antibody-bound hormone. The use of such dextran-coated charcoal makes simpler and more rapid the immunoassay of these hormones in biologic fluids, using radioisotope dilution with I^{131}-hormone and "biopsy" of the hormone pool by antibody to the hormone. The procedure yields a straight line graph when hormone added is plotted against hormone recovered.

Addendum: M. S. Wool and H. A. Selenkow recently published an abstract on the use of charcoal coated with Dextran 150 for assay of human growth hormone (Proc. 48th Meeting, Endocrine Society, Chicago, June 20-22, 1966, p. 123).

S. Reichlin and his associates at University of Rochester School of Medicine found excellent similarities of separation in comparison of the coated charcoal with the double antibody method for growth hormone assay. E. Knobil and his associates at University of Pittsburgh School of Medicine now have two papers in press in Endocrinology, describing studies on regulation of growth hormone secretion in the rhesus monkey using the charcoal method, which they find gives somewhat greater precision than the chromatoelectrophoretic procedure and is more rapid and convenient. They have run a number of

duplicate assays using both separation procedures and found that they yielded essentially identical results.

We are indebted to Drs. Melvin Grumbach, Selna Kaplan and Renee Wolter for constant advice and encourgement, as well as for supplying the hormones and their antibodies, and to John Farrelly for technical assistance. Dr. Grumbach and his associates performed the immunoelectrophoretic and double antibody assays. Dr. Wolter demonstrated linear increases in quantity of measured HGH using serially increasing quantities of acromegalic serum in a coated charcoal assay.

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Effects of Anthranil-, Benzo-, and Salicyl-Hydroxamic Acids on Mouse Brain Amines and L-DOPA Decarboxylase.* (31421)

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Anthranil-hydroxamic acid (AHA) lowers norepinephrine but not 5-hydroxytryptamine (5-HT) in rat brain(1). It was therefore of interest to determine the effect of benzohydroxamic acid (BHA) and salicyl-hydroxamic acid (SHA) on the concentration of these amines in brain. Mice were used in these experiments and the effects of the injection of the 3 hydroxamic acids were compared. Estimations of brain L-DOPA decarboxylase activity were also made.

Methods. AHA was obtained from Hynes Chemical Co., Durham, N. C.; BHA from Eastman Organic Chemical Co.; and SHA from Aldrich Chemical Co. Mice were injected intraperitoneally with 1 mg/g AHA and BHA, and 0.8 mg/g SHA in saline. Brains containing BHA or SHA were removed at intervals after injection and homogenized in 5 ml H_2O. Five ml 10% trichloracetic acid and 0.2 ml 10% $FeCl_3$ were then added. The mixture was filtered and the colored filtrate was read in the Coleman Spectrophotometer at 520 mμ. AHA was not extracted by H_2O so these brains were homogenized in 5 ml n-butanol and 2 ml acidic $FeCl_3$. This mixture was filtered and the colored butanol was read at the same wave length.

Mice were given hydroxamate intraperitoneally and then brain L-DOPA decarboxylase was measured manometrically(2). 0.28 g brain in M/15 phosphate buffer pH 7 was used with 3 μg pyridoxal phosphate and 4 mg DL-DOPA.

Two brains were pooled for each amine estimation. 5-HT assays are described elsewhere(1). Total catecholamines were measured in brain and cerebellum by the method of Potter *et al*(3) in mice receiving BHA and SHA. In this method brains are homogenized

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