

and the same code of such diverse pursuits as controlled trials and exploratory observations.

I admit, as I said earlier, that it may appear impertinent for an unqualified camp follower to air such views, and particularly in this environment. There are just two things I would add in extenuation, and I hope that they may prove to be the only really categorical assertions of which I have been guilty in this lecture. The first is that from my associations with doctors in controlled trials I have learned that the better the statistician understands the doctor/patient relationship and the doctor's very real and unique ethical problem the better can he help to devise a trial that may be less than ideal experimentally but yet likely to be of some, and perhaps considerable, value to medicine.

Secondly, and still more important, I have learned that though the statistician may himself never see a patient—though indeed like Tristram Shandy's Uncle Toby he may live his life in doubt which is the right and which the wrong end of a woman—nevertheless, he cannot sit in an

armchair, remote and Olympian, comfortably divesting himself of all ethical responsibility. As a partner in a combined endeavour a full share of that responsibility will always lie with him. He must endeavour to acquire the ethical perception and code of honour that is second nature of those qualified in medicine. And above all he must learn to blend the objectivity and humanity that this lecture commemorates.

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## PREGNANCY DIAGNOSIS BY A ONE-STAGE PASSIVE HAEMAGGLUTINATION INHIBITION METHOD

BY

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There have been several reports on the detection and assay of human chorionic gonadotrophin (H.C.G.) by immunological methods. Brody and Carlström (1960) immunized rabbits with a purified preparation of H.C.G. and used the immune serum in a complement-fixation test for the presence of hormone in the urine of pregnant women. McKean (1960) demonstrated the feasibility of using a precipitin test with rabbit antiserum to detect H.C.G. in urine samples. A passive haemagglutination inhibition method for the same purpose was developed by Wide and Gemzell (1960), and was shown to be qualitatively accurate.

These observations were commented on by Butt, Crooke, and Cunningham (1961), who suggested that there was some lack of immunological specificity between H.C.G. and pituitary gonadotrophin (luteinizing hormone), and that the results of such *in vitro* tests should be accepted with reserve. Midgley, Pierce, and Weigle (1961) prepared rabbit antisera, using commercial preparations of H.C.G., and demonstrated that, although such antisera contained antibodies to antigens in normal human urine and normal human sera, it was possible to use such sera to demonstrate the presence of H.C.G. in serum and urine.

This communication deals with a one-stage haemagglutination inhibition system which has been developed and used to detect the presence of H.C.G. in urine in pregnancy, and with quantitative tests which have been carried out to assay the H.C.G. content of urine and of dried commercial preparations of hormone.

### Materials and Methods

#### Reagents

*Borate Succinic Acid Buffer, 0.05 M, pH 7.5.*—Sodium borate ( $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ ) solution was prepared con-

taining 95.5 g. of sodium borate and 37.5 g. of sodium chloride in 5 l. of distilled water. Succinic acid— $(\text{CH}_2\text{COOH})_2$ —solution was prepared containing 23.6 g. of succinic acid and 30 g. of sodium chloride in 4 l. of distilled water. Equal volumes of the two reagents were mixed and the pH was adjusted to 7.5 with a small volume of the sodium borate solution.

*E.D.T.A. Buffer pH 8.4.*—Disodium dihydrogen ethylene diamine tetracetate 17 g./l. in distilled water was adjusted to pH 8.4 with 2N sodium hydroxide.

*Borate Boric Acid Buffer pH 8.2–8.3.*—This was prepared from a mixture of 3 g. of sodium borate, 4.4 g. of boric acid ( $\text{H}_3\text{BO}_3$ ), and 7.6 g. of sodium chloride made up to 1 l. with distilled water.

#### Rabbit Antisera

Miscellaneous rabbits of about 2 kg. weight were injected with 1,500 units of H.C.G. (Leo) in 0.5 ml. of saline mixed and homogenized with an equal volume of complete Freund adjuvant (Difco). The mixtures were injected intramuscularly in the flank; the injections were repeated after 28 days, and the animals bled and serum separated 10 to 14 days later. The titres of the serum obtained by this method with H.C.G.-sensitized cell suspensions were usually 1/2,000–1/5,000.

#### Preparation of H.C.G.-sensitized Erythrocytes

Satisfactory preparations of sensitized preserved sheep erythrocytes have been made by two methods: (a) a modification of Ling's (1961) method and (b) an original method. The change to the second method was dictated by a desire to obtain a more stable product which was simpler to prepare.

*Method 1.*—Fresh sheep cells were washed three times in 20 volumes of saline and made up as a 1% suspension

in 5% sodium chloride, and to this was added 10% v/v commercial formalin (40% formaldehyde solution). The mixture was allowed to stand for 18 hours at room temperature and was then washed three times with 20 vols. of borate succinic acid buffer saline, and resuspended in the same buffer containing 0.2% formalin as a preservative. For sensitization the above suspension was washed three times with 20 vols. of borate succinic acid buffer and resuspended as a 1% suspension in E.D.T.A. buffer. The suspension (1 litre) was warmed to 37° C. and 5 ml. of a 2% solution of 1,3-difluoro-4,6-dinitrobenzene (D.F.D.N.B.) in dioxane was added. The mixture was kept at 37° C. for 30 minutes with occasional stirring. The cells were centrifuged, the supernatant was discarded, and the deposit was resuspended in 200 ml. of E.D.T.A. buffer, 3 ml. of a solution of H.C.G. containing 3,500 units in E.D.T.A. buffer being added. The mixture was incubated at 37° C. for 90 minutes with occasional stirring. The sensitized suspension was then washed four times with 100 vols. of borate succinic acid buffer and resuspended in 100 ml. (1/10 vol.) of borate succinic acid buffer containing 5 g. of sucrose and 10 ml. of sheep-cell-absorbed normal rabbit serum.

**Method 2.**—Fresh sheep cells were washed three times in 20 vols. of saline and a 1% suspension prepared in 5% sodium chloride solution containing 0.15 M phosphate buffer at pH 7 and 0.15% hydroquinone. The mixture was left for 15 minutes at room temperature and 10% v/v commercial formalin added, and the whole left for 18 hours at room temperature. The preserved cells were centrifuged and the deposit was suspended in borate succinic acid buffer. One litre of this 1% suspension was washed and resuspended in 200 ml. of E.D.T.A. buffer containing 3,500 units of H.C.G. The mixture was incubated at 37° C. for one hour with occasional stirring. The sensitized suspension was then washed four times with 100 vols. of borate succinic acid buffer and resuspended in 100 ml. (1/10 vol.) of borate succinic acid buffer containing 5 g. of sucrose and 10 ml. of sheep-cell-absorbed normal rabbit serum.

#### H.C.G.-sensitized Agglutinated Suspension

Sensitized cells prepared by either of the two methods described and at a concentration of 10% were diluted in saline to give a 1% suspension for testing. A series of dilutions of rabbit anti-H.C.G. differing by 100% over an appropriate range were prepared in borate buffer. These dilutions were pipetted in 1-ml. amounts into a series of 3 by  $\frac{3}{8}$  in. (7.5 by 0.9 cm.) round-bottomed tubes and 0.1 ml. of the 1% suspension of sensitized cells was added to each tube. The tubes were mixed by inversion and left at room temperature overnight. The highest dilution in the series which showed a fully agglutinated pattern—that is, a smooth carpet of cells covering the bottom of the tube—was taken as the titre of the serum. Since 1 ml. of this dilution of antiserum was required to agglutinate completely 0.1 ml. of the sensitized suspension, the volume of serum required to agglutinate the bulk of the 10% cell suspension prepared could be calculated and this amount added in 100 ml. of borate succinic acid buffer containing 5 g. of sucrose. This sensitized agglutinated suspension was filled out in 5-ml. ampoules in 1-ml. amounts and freeze-dried. The freeze-dried preparation was reconstituted in 5 ml. of borate boric acid buffer for use.

**Other Suspensions.**—Sensitized suspensions were also prepared with H.M.G.24 (post-menopausal urinary gonadotrophin) and N.H.S. (normal human male serum pool) by the second method.

**Control Suspensions.**—Control suspensions were prepared by suspending preserved suspensions (after treatment with D.F.D.N.B. or hydroquinone-formalin mixture) in 5% sheep-cell-absorbed normal rabbit serum in borate succinic acid buffer containing 5% sucrose.

#### Haemagglutination Inhibition Tests

All batches of test suspensions were standardized against a solution of H.C.G. containing 50 units/ml. Complete inhibition should be effected by 1 ml. of a dilution of 1/100–1/200 of this solution (0.5–0.25 unit of H.C.G.).

For clinical tests, urine samples were cleared by centrifugation and diluted 1/2, 1/5, 1/10, and 1/20 in borate boric acid buffer and pipetted in 1-ml. amounts into 3 by  $\frac{3}{8}$ -in. (7.5 by 0.9-cm.) round-bottomed glass tubes. Two series of dilutions were prepared. Then 0.1 ml. of the reconstituted H.C.G.-sensitized and agglutinated suspension was added to one series of tubes and unsensitized control cells to the other series. The tubes were inverted to mix and left overnight at room temperature and free from draughts or vibration. Each test series included a diluent control for both test and control suspensions.

The tests were read by observing the pattern of agglutination on the bottom of the tubes. Complete agglutination consisting of a smooth carpet of cells in the test series indicated that that contained less than one inhibiting dose of H.C.G. Different degrees of inhibition of agglutination resulted in the formation of dark rings of cells of different sizes (Fig. 1). The end-

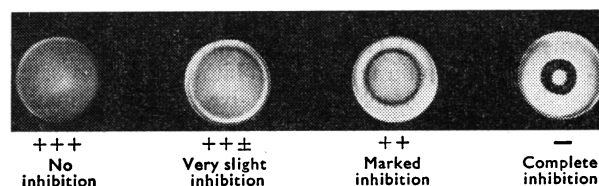


FIG. 1.—Typical haemagglutination inhibition patterns.

point of the test was taken as the highest dilution of urine showing unequivocal inhibition.

Control suspensions occasionally showed agglutination at the lower dilutions (1/2–1/5), due to non-specific agglutinins; this was most marked when tests were set up at a low temperature. When agglutination occurred with the control suspension the result at that dilution with the test suspension was ignored.

Typical results of such tests were as shown in Fig. 2. Quantitative titrations of urine samples and commercial H.C.G. solutions were carried out by diluting the materials to a suitable level (discovered by preliminary tests at wide differences of volume), pipetting the dilutions in volumes differing by 20% in a series of tubes and making up the final volume to 1 ml. with buffer. Sensitized suspensions were then added and the test was read as before. The tests were repeated an adequate number of times and the mean inhibiting volume of dilution was calculated. In each series of tests a solution of international standard H.C.G. was titrated in parallel and the potency of the unknown samples estimated by

calculation from the inhibiting capacity of the standard preparation.

Titration of H.C.G. in serum was carried out in the same way after a preliminary absorption of the serum. Normal serum contains non-specific inhibitors for passive haemagglutinating systems, but these inhibitors were removed by treatment with an equal volume of M/40 potassium periodate for half an hour at room temperature, followed by absorption with 1/10 vol. of packed washed preserved sheep cells to remove non-specific haemagglutinins.

**Male-toad Test**

By Scott's (1940) method 95 ml. of overnight urine was extracted and the kaolin fraction eluted to give

TABLE I.—Results of Haemagglutination Inhibition Tests on Samples of Urine from Clinically Diagnosed Pregnancies (All Confirmed at a Later Date), Tabulated According to Duration of Pregnancy

Duration in Weeks	6-8	9-12	13-16	17-20	21-24	25-28	>28	Total
Positive	15	39	33	8	7	14	8	124
Negative	—	1	1	—	—	—	—	2

TABLE II.—Non-pregnant Controls. All Negative, Tabulated According to Age-groups (Years)

Age-group	17-19	20-29	30-39	40-49	50-59	Total
No.	41	56	23	37	54	211

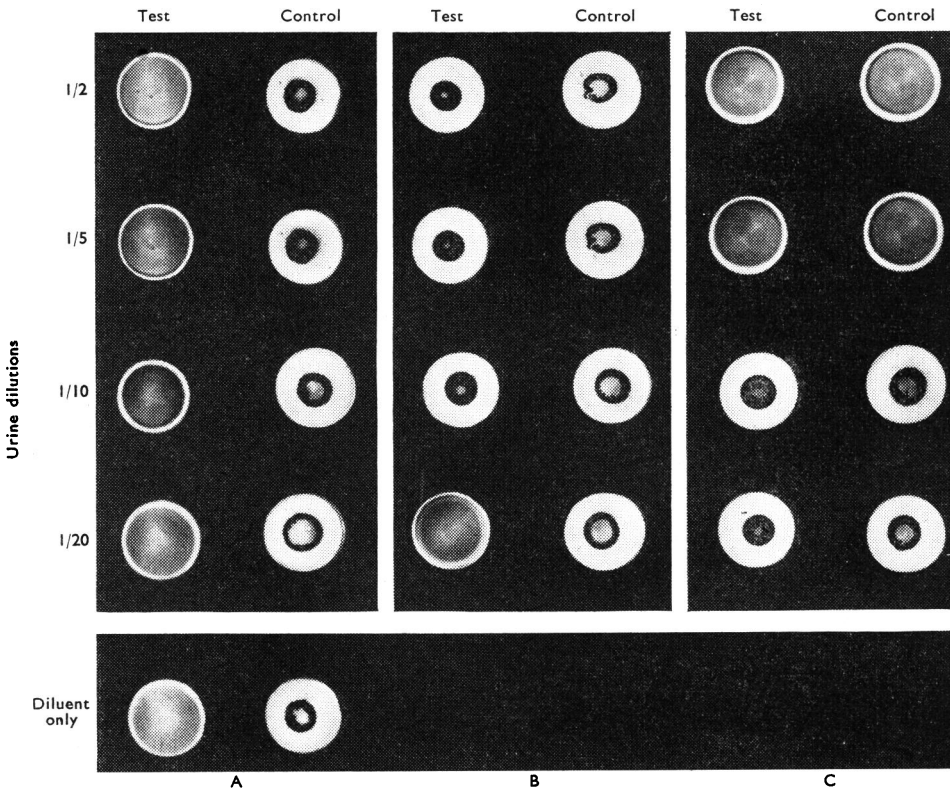


FIG. 2.—Typical appearance of haemagglutination inhibition test by the one-stage method. False agglutination as shown in C is uncommon.

a final volume of 5 ml. Then 2 ml. of the eluate was injected into the dorsal sac of the male toad *Bufo bufo*. The animal was catheterized four hours later and the specimen examined for spermatozoa. Each sample was tested once only.

**Bioassay for H.C.G.**—Quantitative estimations of H.C.G. were carried out by a standard method (*British Pharmacopoeia*, 1953).

**Tests on Urine Samples**

Morning specimens of urine from 126 women believed for clinical reasons to be pregnant were tested for the presence of H.C.G. by the haemagglutination inhibition method. The results are shown in Table I. The material was obtained from individuals whose pregnancies were of widely different duration, but there was no evidence

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that tests carried out in the third trimester were more likely to be negative.

Specimens were also tested from 217 members of the female staff at the Wellcome Research Laboratories, Beckenham. Six of these individuals were pregnant and gave positive results, while from the 211 non-pregnant individuals all samples were negative. These controls were taken from a wide range of age-groups (Table II).

In addition, 104 specimens were tested from female patients over 50 in the medical wards of Lewisham Hospital. No false-positive results were found in this series; however, 22 of the specimens caused a collapse of the agglutination pattern with in some cases up to 1/10 dilution of urine. This type of collapse occurs with normal human

serum (non-pregnant); it was distinguishable from true inhibition, since no true ring-pattern was formed and the sedimented cells had a gelatinous translucent appearance, often with an irregular edge (Fig. 3). Repeat tests on further samples from the same individuals were frequently negative. No satisfactory explanation of this phenomenon was found, and it was noticeable that in

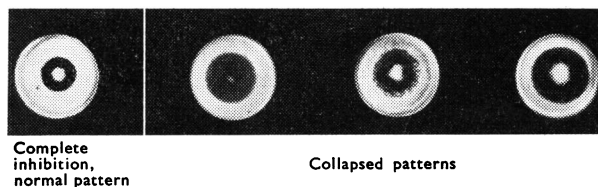


FIG. 3.—False inhibition (collapsed pattern) by non-specific inhibitors in serum or urine.

the control tests carried out on members of the W.R.L. staff, of whom 54 were aged over 50, this type of reaction did not occur, nor was it seen in the series of 388 comparative tests carried out on samples sent in for diagnosis. Many of the patients in the series giving collapsed patterns were suffering from pyuria and proteinuria; there was, however, no correlation between these findings and the results of the haemagglutination inhibition tests.

It was demonstrated that this type of false inhibition was entirely non-specific, since those samples of urine which produced this effect with the H.C.G./anti-H.C.G. systems affected other systems to the same degree. Sheep cells sensitized with bovine serum albumin and agglutinated with antbovine serum albumin (B.S.A./anti-B.S.A.) showed false inhibition due to the formation of "collapsed patterns"; other systems affected were: human - thyroglobulin/anti - thyroglobulin, human - growth-hormone/anti-hormone, diphtheria-toxoid/antitoxin, and tetanus-toxoid/antitoxin. This non-specific inhibitor in the serum samples could frequently, but not invariably, be abolished by treatment with M/40 potassium periodate. No false inhibition of this type was seen in the normal control serum, but a pool of samples of post-menopausal urine when concentrated by ultrafiltration in the cold to 1/50 vol. was found to give collapsed patterns up to a dilution of 1/5. This preparation likewise caused collapsed patterns in the unrelated haemagglutination inhibition systems previously mentioned.

A total of 388 samples sent in for pregnancy diagnosis by the male-toad test were tested in parallel by haemagglutination inhibition and the results compared at a later date (Table III). It was found that the haem-

TABLE III.—Comparison of Results of Pregnancy Tests by Haemagglutination Inhibition and the Male-toad Method

	No. of Samples	Positive Results	
		H.I.	Toad Test
Autumn, 1961 .. ..	210	98	84
Spring, 1962 .. ..	178	98	97
Total .. ..	388	196	181

agglutination inhibition method was at least as sensitive as the *in vivo* method, and investigation of the discrepancies at a later date confirmed its reliability (Table IV). It was found that a few false-positive results

TABLE IV.—Analysis of Discrepancies Between the Results by Haemagglutination Inhibition and the Male-toad Test for Pregnancy

	No.	Clinically Confirmed Results		
		Pregnant	Non-pregnant	Not Traced
Toad test positive H.I. test negative	12 (7+5)*	7	5	—
Toad test negative H.I. test positive	26 (20+6)	23	—	3
Total .. ..	38 (27+11)			

\* Figures in parentheses represent results for autumn of 1961 and spring of 1962.

had been recorded by the male-toad test and none by the haemagglutination inhibition method, and it was noticeable that the degree of correlation between these two methods was better in the spring of 1962 than it was in the autumn of 1961. It is well known that seasonal variation occurs in the sensitivity of the male toad to stimulation by H.C.G.

Examination of the titres (highest dilution of urine giving unequivocal inhibition) of the positive samples showed that 60% of the tests were positive at a dilution of 1/20 or more. Those discrepancies in which the toad test was negative and the haemagglutination inhibition test was positive showed a similar distribution, which suggested that the results were not the effect of a low concentration of H.C.G. in the untreated urine (Tables V and VI).

TABLE V.—Distribution of Titres of Haemagglutination Inhibition Positive Samples of Urine

Maximum dilution of urine causing inhibition	1/2	1/5	1/10	1/20 or more	Total
No. of samples .. ..	6	22	37	131	196

TABLE VI.—Distribution of Titres of Haemagglutination Inhibition Positive Samples of Urine Which Were Negative by the Male-toad Test

Maximum dilution of urine giving inhibition	1/2	1/5	1/10	1/20 or more	Total
No. of samples .. ..	1	6	2	17	26

### Tests on Serum Samples

Satisfactory results were obtained with tests on the sera of pregnant and non-pregnant women provided that the sera were first treated to remove anti-sheep haemagglutinins and non-specific inhibitors. Preserved sheep cells were found to agglutinate to a higher titre with normal human sera than did fresh sheep cells. These agglutinins could be removed by absorption with either fresh or preserved cells. It was also observed that this type of agglutination was masked by the presence of non-specific inhibitors which could be destroyed by treatment with an equal volume of M/40 potassium periodate.

### Quantitative Titrations of H.C.G. in Commercial Preparations

A series of comparative tests were carried out on samples of H.C.G. of widely different potency in terms of international units/mg. (Table VII). These prepara-

TABLE VII.—Comparative Assay of H.C.G. in Commercial Preparations by *in-vivo* and Haemagglutination Inhibition Methods

Sample	Bioassay		Haemagglutination Inhibition	
	Mean Potency	Limits of Error	Mean Potency	Range
D 59916 (u./mg.)	50.1	44.3-56.6	55.6	41.6-68.1
D 62053 .. ..	47.6	42.8-52.9	53.5	41.6-62.5
SM 2725 " ..	1.08	0.95-1.23	1.07	0.91-1.11
SM 2726 " ..	6.20	5.08-7.55	6.5	5.6-6.8
SM 2727 " ..	14.9	13.5-16.4	13.2	10.0-16.7
L 161055 (u./vial)	1,376	1,235-1,532	1,580	1,385-1,735
Proposed new international standard (u./ampoule) ..	4,998	4,707-5,308	5,550	4,545-6,250

tions were commercial products intended for human and veterinary use. The results of the tests appeared to indicate that the haemagglutination inhibition technique was capable of giving a reasonable indication of the potency of such preparations. However, these materials have undergone some purification procedures, and further tests were done with crude materials.

### Quantitative Titrations of H.C.G. in Urine Samples

Six samples of pooled urines from pregnant individuals were bioassayed in parallel with titrations by

the *in vitro* method. The results (Table VIII) showed considerable discrepancies between the two methods.

TABLE VIII.—Comparative Assay of H.C.G. in Pooled Urine Samples by *in-vivo* and Haemagglutination Inhibition Methods

Urine Samples	Bioassay		Haemagglutination Inhibition	
	Mean Potency (Units/ml.)	Limits of Error (Units/ml.)	Mean Potency (Units/ml.)	Range (Units/ml.)
1	29.7	25.3-35.1	40.6	31.3-50.0
2	33.4	27.5-40.7	57.0	41.6-55.6
3	46.4	39.2-54.8	38.2	27.8-41.6
4	68.3	49.6-94.1	48.2	38.4-55.6
5	56.8	51.8-62.2	37.9	27.8-41.6
6	41.3	36.7-46.5	27.9	20.8-35.7

**Non-specific Antigens and Antibodies**

Rabbit anti-H.C.G. was tested with sheep cells sensitized with H.C.G., H.M.G.24, and N.H.S. It was found that the antiserum contained agglutinins for all preparations. The cross-agglutinating titres were, however, lower than that found with H.C.G.-sensitized cell preparations. In addition, a rabbit antiserum to whole human serum agglutinated H.C.G.-sensitized cells to a low titre compared with the titre against N.H.S.-sensitized cells (Table IX).

TABLE IX.—Agglutination Titres Obtained with (1) Rabbit Anti-H.C.G. and (2) Rabbit Anti-N.H.S., Using Sheep Cells Sensitized with H.C.G., H.M.G.24, and N.H.S.

	Agglutination Titres with Sheep Cells Sensitized with:		
	H.C.G.	H.M.G.24	N.H.S.
Antiserum	..	..	..
Anti-H.C.G.	1/5,000	1/1,000	1/200
Anti-N.H.S.	1/50	—	1/10,000

TABLE X.—Agglutination Inhibiting Capacity of H.C.G., H.M.G.24, and N.H.S. for Sheep Cells Sensitized with H.C.G., H.M.G.24, and N.H.S. and Agglutinated with a Minimum Agglutinating Dose of Rabbit Anti-H.C.G.

Dilution of Inhibiting Antigen	Sensitized Suspensions Agglutinated with a Minimum Dose of Antibody		
	H.C.G.+ Anti-H.C.G.	H.M.G.24+ Anti-H.C.G.	N.H.S.+ Anti-H.C.G.
H.C.G. 50 u./ml.	1/2	—	—
	1/5	—	—
	1/10	—	++±
	1/20	—	+++
	1/50	—	+++
	1/100	—	+++
Diluent only	1/200	+++	+++
	1/500	+++	+++
	Neat	+++	+++
	1/2	+++	—
	1/5	+++	—
H.M.G.24 1 mg./ml.	1/10	+++	++±
	1/20	+++	+++
	1/50	+++	+++
	1/100	+++	+++
	1/200	+++	+++
	1/500	+++	+++
Diluent only	Neat	+++	+++
	1/10	+++	—
	1/100	+++	—
	1/1,000	+++	—
	1/10,000	+++	—
Diluent only	1/100,000	+++	+++
	Neat	+++	+++

Inhibition tests were carried out with H.C.G., H.M.G.24-, and N.H.S.-sensitized cells. Each of these suspensions was treated with a minimum agglutinating dose of rabbit anti-H.C.G. It was found that H.C.G. inhibited the agglutination of the H.C.G./anti-H.C.G. system in high dilutions, while H.M.G.24 and N.H.S. were ineffective in this way. Inhibition of the H.M.G.24/anti-H.C.G. system was effected by a moderate dilution of H.C.G. and a higher dilution of H.M.G.24; inhibition

of the N.H.S./anti-H.C.G. system was produced by relatively large amounts of H.C.G. and H.M.G.24, and in a dilution of 1/10,000-1/100,000 by whole human serum (Table X).

A number of urine samples were tested with the H.M.G.24/anti-H.C.G. system for the presence of the H.M.G. component. Of 53 samples from known pregnancies, 40 gave good inhibition of this system; and of 28 samples from non-pregnant individuals only five inhibited the system, and three of these individuals were over 45 years of age.

**Discussion**

It is evident from the studies of Brody and Carlström (1960), Butt *et al.* (1961), and Midgley *et al.* (1961) that non-specific antigens exist in commercial H.C.G. preparations and that antibodies to these are produced in rabbits. It appears, however, that pregnancy diagnosis can be carried out successfully and with a reasonable degree of accuracy by the methods described, and that with purified preparations of H.C.G. the agreement between the *in vivo* and *in vitro* methods of assay is satisfactory. It is of interest to speculate on the reason for the specificity of results in the presence of non-specific factors. It can be seen from Table IX that considerable cross-agglutination occurs when cells sensitized with H.C.G., H.M.G., and N.H.S. are each tested with anti-H.C.G. serum; however, the titres of the cross-agglutinating antibodies are relatively lower than those specific for the H.C.G. cells. Since it is the principle of the one-stage haemagglutination inhibition method to use only a minimum agglutinating dose of anti-H.C.G. for H.C.G. cells it follows that there will not be sufficient antibody in the mixture to agglutinate H.M.G. or N.H.S. cells; this observation is confirmed by the inhibition tests shown in Table X; H.M.G.24 and N.H.S. will not disagglutinate the H.C.G./anti-H.C.G. system.

It would appear also from the results of inhibition tests with commercial H.C.G. solutions and H.M.G./anti-H.C.G. and N.H.S./anti-H.C.G. systems that the commercial H.C.G. products contain components present in H.M.G. and N.H.S., and it is possible that this is in fact pituitary hormone in the case of the H.M.G. component. This possibility is reinforced by the observations made with the H.M.G./anti-H.C.G. system and urine samples; H.M.G.24 is, however, a relatively impure preparation, and the effects may be due to some urinary protein secreted in the tract. The N.H.S. component in commercial H.C.G. is likely to be simple serum proteins, since sensitization of cells with a mixture consisting predominantly of albumin and globulin is unlikely to produce a preparation agglutinable by antibodies to some antigen present in serum in relatively very small amounts.

These observations show that commercial H.C.G. contains an antigen not present in the other sensitizing agents, that antigen is in fact H.C.G., and that it is the major component in the H.C.G./anti-H.C.G. system.

When considering the production of an antiserum to a mixture of antigens it is of importance to remember that secondary antibodies tend to continue to rise in titre relative to the chief component if immunization is prolonged. It is therefore advisable to immunize rabbits with H.C.G. by a relatively short course of injections and to sacrifice potency to specificity.

It was surprising to find that the proportion of samples from known pregnancies which were negative

by haemagglutination inhibition was not obviously greater in the 17-→28 weeks group than that found in the 6-16 weeks group (Table I). It is well known that the excretion rate of H.C.G. in urine falls to a low level late in pregnancy. However, it has been observed by Wide and Gemzell (1962) that H.C.G. can exist in a physiologically inactive form in which it still combines with antibody, and that the apparent excretion rate of H.C.G., titrated by immunoassay, late in pregnancy is much greater than would be expected from past work carried out by bioassay. This could well explain the unexpected success of immunodiagnosis at late stages of pregnancy. It is of interest in this connexion that in those tests where a positive result was obtained by haemagglutination inhibition, and a negative result by the toad test method, there was apparently no reason to suppose that the levels of H.C.G. were unusually low (Tables V and VI). It may be that in these cases the proportion of physiologically active H.C.G. in the samples was low. In the light of Wide and Gemzell's observations with heated H.C.G. it is interesting that potassium periodate, which has been used to destroy non-specific inhibitors of agglutination in serum samples before assay for H.C.G., is also known to destroy the physiological activity of the hormone; it did not, however, affect the hormone's antibody-combining power in these tests.

#### Summary

A one-stage haemagglutination inhibition test for pregnancy has been described. Of 126 clinically confirmed pregnancies 124 were positive by this test, and there was no evidence of loss of accuracy even at a late stage of pregnancy. Non-pregnant controls totalling 211 individual samples from persons aged 17 to 59 were all negative by this test. Tests were carried out on 388 samples submitted for pregnancy diagnosis by the male-toad test and the results compared at a later date when clinical confirmation could be obtained. In 350 out of the 388 samples tested the results by haemagglutination inhibition and by the toad test were in agreement. The 38 discrepant results consisted of 12 tests where the toad test was positive and the haemagglutination inhibition test negative; of these subjects, seven were pregnant and five not pregnant on subsequent clinical examination. A further 26 tests were positive by the haemagglutination inhibition test and negative by the toad test, and clinical examination confirmed that 23 of these patients were in fact pregnant; three patients could not be traced. If it is assumed that those results in which both the toad test and the haemagglutination inhibition test were positive were correct, the overall accuracy of the haemagglutination inhibition method was 98.2% and the accuracy of the male-toad test 92.8%.

Although the accuracy of this test for the diagnosis of pregnancy was of a high order, quantitative assay of urine samples in parallel with bioassay was unsatisfactory. This could be due either to the presence of differing proportions of hormonally inactive but immunologically active H.C.G. in the different samples or to interference by non-specific antigens and antibodies.

It has been found that a biologically inactive form of H.C.G. produced by treatment of H.C.G. in solution with potassium periodate retained its original capacity to combine with antibody.

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## RICKETS IN IMMIGRANT CHILDREN IN LONDON

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We wish to draw attention to the prevalence of rickets among the children of certain immigrant communities living in London. All the cases of active nutritional rickets seen during the last five years in the paediatric departments of Guy's and King's College Hospitals are reported. There were 16 cases, and it is of great interest that only one was English; six were West Indian, five Greek Cypriot, one Nigerian, one Maltese, one Irish, and one Spanish. All the children were born in England except two, both of whom had lived in London for at least a year before rickets was diagnosed. Ten were girls and six boys. Their ages when first seen ranged from 9 to 38 months. All had clinical evidence of rickets, including skeletal deformity. Seven presented with bowing of the legs (Fig. 1), two with fractures, and seven with unrelated conditions.

Though no normal standards of body weight are available for non-indigenous children in London, a low level of nutrition in some of our cases was suggested by a body weight below the average for English children. Eight out of 14 whose weights were recorded were below the tenth percentile (Tanner and Whitehouse, 1959). A poor nutritional status was also suggested by the low haemoglobin levels, which in 11 out of 16 cases were between 4.5 and 10.5 g./100 ml.

All the children had biochemical and radiological evidence of active rickets. Serum calcium concentration