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ON SIMPLER TESTS FOR PREGNANCY.

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It has long been desired that some objective means be available for the diagnosis of pregnancy, to supplement if not to replace the clinical means on which the diagnosis has until recently rested entirely. One such test is available, the (2)(51) Aschheim Zondeck test, with its minor modifications, first announced in 1928. This test has remained a standard one for (12) pregnancy, especially as modified by Friedman in 1928. The drawbacks, however, in the Friedman modification of the Aschheim Zondeck are very numerous, but the percentage of correct results is far in advance of any other pregnancy test thus far worked out. The inconveniences of the Aschheim Zondeck or Friedman test are as follows, (1) a large stock of female rabbits of 2300 - 3000 grams must be kept on hand, in relative isolation at considerable trouble and expense. (Friedman modification) (2) It requires at least 48 - 72 hours to get a reliable result. (3) Unless an animal is sacrificed for each test, a very considerable amount of time is required for operative procedure. (4) A certain number of animals die following the injection of the blood or urine. Much the same objections apply to the original Aschheim Zondeck test. Although rats are relatively inexpensive, the recommended use of several animals for each test increases the difficulty of keeping a proper stock on hand, and microscopic examination of the ovaries adds to the time of the test. For these reasons a simpler test for

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pregnancy is to be greatly desired, and preferably one which avoid the numerous complicating factors necessarily associated with biological tests.

A number of simpler procedures have actually been devised to meet these needs. These may be roughly divided into biological and chemical tests. Of the rapid biological tests the following have come out within the last year or two. (1) The intradermal injection of antuitrin S, first introduced by Gruskin (14) in January, 1936. (2) The Bercowitz test (3) published in 1933, which involves the application of the patient's serum to the conjunctiva and the observation of consequent pupillary changes. Of the chemical tests, the following have been most widely used: (1) The Kapellar-Adler (22) test for urinary histidine; (2) the Visscher-Bowman (42) test for prolan.

Of the chemical methods now in use, that of Kapellar-Adler is the oldest. The development of this test presents a very long and interesting evolution. It is essentially a test for histidine in the urine of pregnant women. Knoop (28) in 1908 was the first to introduce the chemical test used. Working with pure histidine he showed that on the addition of bromine, a rose red color developed. But, as he himself stated, the method was not sensitive in dilutions of beyond 1: 1000. It was shown by Hunter (21) in 1922 that the accuracy of the test was very dependent upon the exactly proper addition of bromine, and he was able to increase its sensitivity to a dilution of 1: 10 000, and to also secure a more permanent change of color by the later addition

of ammonia or ammonium carbonate. A violet color was obtained on the addition of ammonia, while in acid solutions the color varied from yellow brown to brown. If only very slightly alkalized with NaOH a transient pink tint resulted. In 1929 Voge (45) first made use of Knoop's original reaction for histidine in devising a pregnancy test. By his method 2.5 cc of first morning urine was heated to boiling with 1 cc of a 1/3 saturated solution of bromine in water; a transient rose color indicating the presence of histidine. Unfortunately the quantity of bromine used in this test could not be adjusted accurately enough and as pointed out by Weiss (49) the red tint is so fugitive as to be very easily overlooked. While some work which will be referred to later confirmed Voge's results, in general it may be said that this test proved unreliable.

In 1933 Kapeller Adler, (22)(24) making use of the works of the above writers, introduced her test. Actually this comprises two methods, one involving the quantitative purification of histidine and its later identification, the other, a much simpler qualitative determination to demonstrate its presence.

**Quantitative Method:** The first step accomplishes the precipitation out of the phosphates by adding barium carbonate. The precipitated phosphates are then filtered out and the excess barium removed by sulphuric acid. The solution is then neutralized and evaporated down to a small volume. Ninety percent alcohol is added until the solution becomes turbid, and to this alcoholic solution add one-third volume of ether followed by 100 - 150 cc of Hopkins'

reagent (10%  $\text{HgSO}_4$  in 5%  $\text{H}_2\text{SO}_4$ ). In this manner the histidine is precipitated out. Following its precipitation the histidine is washed in alcohol and ether and allowed to dry. It is then redissolved in dilute  $\text{HCl}$  and  $\text{H}_2\text{S}$  is run in. Next the  $\text{HCl}$  is evaporated off, in large part, on a water bath and the residue diluted with dilute  $\text{H}_2\text{SO}_4$  until each cubic centimeter represents 10 cc of the original urine. This is then tested with bromine and  $(\text{NH}_4)_2\text{CO}_3$  solution and the histidine content determined colorimetrically by comparison with a known solution.

The qualitative test is much simpler and quicker. To demonstrate the histidine a 1% solution of bromine in 33% acetic acid is added to the urine to be tested. After the urine has been first treated with  $\text{N}/10$   $\text{KMNO}_4$  solution until a pink color results which gradually fades, (in case it should remain, heat in the water bath and cool), the bromine solution is then added drop by drop until a weak yellow color appears. To avoid an excess of bromine a starch potassium iodide paper indicator can be used. The solution is then allowed to stand between five and ten minutes and 2 cc of  $\text{NH}_4\text{NH}_4\text{CO}_3$  solution are added and the whole heated in boiling water for five minutes. In a later modification of her test (26) she adds only one-half a cubic centimeter of the alkali solution and heats it only for one-half a minute. The solution is then cooled in cold water and a blue violet color is looked for.

Histidine has been found in urines of people in other than the pregnant state. In 1908 Engeland(10) was working with pooled urines and was able to recover small amounts of impure histidine.

This observation was later confirmed by Hefter (17) in 1925, who was able to extract the histidine in a pure state from pooled urines. Histidine has also been found in urines of people suffering from pernicious anemia. (38) Herrmans (18), in 1922 tried with no success to extract histidine from the urines of tuberculous patients, but in 1924 Reinwein (37) did find it in pooled urines of such cases. Hunter (20) in 1922 was able to recover it in urines of patients with measles, using the diazo reaction for its demonstration.

High values of histidine have been found to occur in the urine of patients having some liver pathology (20). In such conditions it is thought that the enzyme histidase, which normally changes the histidine to the imidazol compounds, (Kaufmann (27), is lacking in the liver and for that reason histidine appears in the urines. With regard to the occurrence of histidine in the urines of pregnancy, this was first reported by Honda (19). Naturally he could make no statements of its invariable presence in this condition. This rests mainly on the work of Vogels already cited, who, however, was uncertain whether he was dealing with histamine or histidine, and Kapeller-Adler, in articles already cited.

In explaining the presence of histidine in urines of pregnant women Kapeller Adler (25) in 1935 advanced the theory that there is an inhibiting agent which she calls anti-histidinase, that inactivates the ferment in the livers of pregnancy. However, this inhibition has never been observed in lower animals during their pregnant states. Her experimental work in support of these views

is as follows: In the livers of normal and diseased humans she found little variation in their capacity to deaminize histidine, but this power was practically completely lacking in the livers of pregnancy. Incidentally, she found none in placental extracts.

A reference was made above relating to the identification of histidine in urine by means of the diazo reaction. Pauly (35) was first to use this method in 1904. In 1914 Weiss and Ssoblew (47) confirmed this finding, but in 1917 Weiss (48) showed that the test was unreliable as a number of other substances also gave positive findings.

Another chemical test for pregnancy was introduced by Visscher and Bowman (42) in 1934. These investigators studied the chemical reactions of anterior pituitary substances which they introduced into normal urines. They were able in time to devise a purely chemical method for their detection, even in dilutions in which they occur in the urines of pregnancy.

The procedure of the test is a little more involved than that of Kapeller Adler, "To one cubic centimeter of first morning urine, as free from reducing substance as possible, are added the following solutions: 1 drop of 0.5% hydrogen peroxide, 5 drops of 1% aqueous phenyl hydrazine hydrochloride, 5 drops of 5% methyl cyanide and lastly, 5 drops of concentrated hydrochloric acid. The urine is then boiled for twenty-five minutes, and according to Visscher and Bowman a russet color with a flocculent precipitate represents a positive result; but Menken (33) in 1934 was able to find a flocculent precipitate in only one of his cases, and states that all of his positives gave a dust like and sometimes a more disperse precipitate. This was also true in all



of the cases which I ran.

In January, 1936, Gruskin (14) introduced an intradermal test for pregnancy in which he employed a solution prepared from placentas. This work was based upon his previous attempts at the diagnosis of cancers by means of serum reactions. In preparing the solution for the intradermal test Gruskin used only fresh placentas which he washed until they were practically free of blood. After this washing process was completed he ground the placental tissue to a pulp and placed it in acetone equal to three times its volume for twenty-four hours. The acetone was then poured off and the placental tissue allowed to dry. Following this he extracted it with .1 N NaOH and a buffer solution for twenty-four hours. The buffer solution was prepared in the following manner: 0.05 NHCl with 2.27 grams of  $\text{KH}_2\text{PO}_4$  per liter. The solution was then brought to a pH of 6.9. He preserved it with cresol and glycerine. In performing his test he introduced .1 cubic centimeter of the solution intradermally and .1 cubic centimeter of salt solution into the other arm as a control. In reading his tests he waited ten minutes and then looked for "pseudopodia" with or without formation of a wheal. A negative reaction consisted of an even margin in contrast to the irregular margin of the positive test. Gruskin reports a high percentage of correct reactions with the above test.

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E. Schwartz, in checking Gruskin's work, also reports very high percentages of correct results. Gruskin tried 191 cases with the following results, presented here in simplified form

from his detailed tabulation: Correct negatives,- 34; full term positive reactions,- 105; positive findings corroborated by clinical evidence,- 16; abortions, complete or incomplete, positive findings in 32; incorrect positives, 4, including one case of teratoma.

I performed my tests after the modification of Gilfillen-Gregg (13) who used commercial antuitrin S. These writers thought that because an anterior pituitary like substance is believed to be in the urine of pregnant women, they might not be sensitive to its intradermal application, and a non-pregnant woman might show a reaction to its presence. This is in brief the basis of the anterior pituitary injection as a test for pregnancy. These authors injected intradermally 2 minims of fresh antuitrin S. They used an extremely fine needle, and they used water to cleanse the skin because of their belief that alcohol made the antuitrin S less potent. After introducing the two minims intradermally they waited one-half hour before reading the reaction and if they got a slight reaction within this time they waited another half hour before drawing their final conclusions. They never observed the patient after one hour. They found that patients over thirty years of age did not react as rapidly as their younger patients. Patients near the menopause had reactions delayed up to three hours. A reaction (Non-pregnancy) consists of an area of erythema around the site of the injection measuring from 7 - 35 millimeters. A negative reaction consists of no erythema at the point of injection, except the reddened area over the bleb.

They found that a patient who is pregnant or who has aborted and retains some decidual cells will have no reaction to the antuitrin S. Reaction in the non-pregnant female usually begins within one to three minutes. Some cases of pregnancy showed a reaction for the first twenty minutes which promptly faded. Positive results were obtained in cases where there persisted dead or living decidual cells. In certain instances the Ascheim Zondeck was negative.

In carrying out my tests I found much the same conditions influencing my results. Seldom, however, was I able to read a reaction within the first minute or two. Some reactions, especially in males, were delayed as long as four to six hours.

There are a number of conditions which might conceivably influence the test. These have been pointed out by Lewis. (30) This author believes a histamine solution is responsible for the reactions of all irritating substances coming in contact with the skin. This substance can be liberated by electrical, mechanical, chemical, or thermal stimuli. These produce a triple response, local dilatation of the minute blood vessels, increased permeability of their walls, and arteriolar dilatation with the final formation of a wheal. Hare (16), in 1926, studied protein sensitiveness and agreed with Lewis in the latter's conclusions.

TABULATED RESULTS.

	Total No. Cases.	Total No. Pos. Cases.	Correct Pos. Cases.	% Correct Pos. Cases.	No. False Negatives.	% False Negatives.	Total No. Neg. Cases.	Correct Neg. Cases.	% Correct Neg. Cases.	No. False Positives.	% False Positives.	Voge Test.
Voge (46)	60	24	23	96%	1	31%	33	33	100%	0	0	Voge.
Dodds (7)	305	229	171	74.3%	58	25.7%	76	66	86.9%	10	13.1%	Dodds.
A. M. Young (50)	519	Not quoted	?	63%	?	39%	?	?	84%	?	16%	Young.
Siedman (40)	199	102	96	94%	6	5%	97	73	75%	24	25%	Siedman.
Eggers	100	50	33	66%	17	34%	50	46	92%	4	8%	Eggers.
KAPELLER - ADLER TEST												
Kapeller-Adler (26)	76	66	66	100%	0	0%	12	10	83%	2	16.6%	Kapeller-Adler.
Louros (32)	200	100	69	69%	31	31%	100	67	67%	12	12% questionable	Louros.
Stein (41)	154	100	88	88%	12	12%	54	51	94.5%	3	5.5%	Stern.
Brandsch (4)	292	172	144	84%	28	16%	120	106	89%	14	11%	Brandsch.
Foldes (11)***	185	?	?	100%	0	0	?	?	100%	0	0%	Foldes.
Ohligmacher ((34))	221	76	44	58%	32	42%	145	128	89%	17	11%	Ohligmacher.
Eggers	50	25	21	82%	3	12%	25	25	100%	0	0%	Eggers.

\*\*\* Foldes found positive Kapeller-Adler tests only in urines of high specific gravity; with pregnant urines of this type, he obtained positive findings even when these were diluted to sp. g. 1015. But the same was true, though to a limited degree, also of non-pregnancy urines. He does not consider the reaction reliable.

VISSCHER-BOWMAN TEST	Total No. Cases.	Total No. Pos. Cases	Correct Pos. Cases	% Correct Positives.	No. False Negatives.	% False Negatives	No. Negative Cases.	No. Correct Negatives	% Correct Negatives.	No. False Positives	% False Positives	Total % Correct
Visscher- (42) Bowman No. 1, 1934	19				4*							
Visscher- (44) Bowman No. 2, 1935	100	?	?	90%	?	10%	?	?	100%	0	0%	90% Pos. 100% Neg.
Dodds' (7) Visscher- Bowman test	317	100	90	90%	10	10%	80	72	88.8%	8	11.2%	100%
Menken, 1934 (33)	21	12	11**	90%	1	10%	9	9	100%	0	0%	95%
Dolff, 1935 (9)		54	49#	91%	5	9%		?	100%	0	0%	
Eggers	100	50	46	92%	4	8%	50	50	100%			

GRUSKIN AND  
SIMILAR SKIN TESTS

Gruskin's Test (14)##												
E. Schwartz (39)	237	155	149	96.13%	6	3.9%	66	60	90.90%	6	9.1%	
Eggers	100		###									81%

\* These were very dilute urines; on repetition, 3 later gave positive results; one patient left before the test could be repeated.

\*\* Four of these were early pregnancies, and with two the results were inconstant.

# Of 5 false negatives, 3 were not first morning specimens.

## See text.

### Only summarized results given here. This refers to the antuitrin S test.

In analyzing the above results, let us consider first the several tests for histidine. The Voge test, as has been pointed out, depends on a reaction which requires a nicely quantitative adjustment of bromine; at its best it is not especially sensitive, and the color reaction is so temporary that it may be overlooked easily. In view of these facts it is not surprising that the test has gained but little acceptance.

The Kapeller-Adler test for histidine is more sensitive and reliable. It provides for the accurate adjustment of bromine; its color changes are more pronounced and permanent, and the comparative results reflect its greater accuracy. However, Stern (41) has pointed out that the presence of other reducing substances, especially nitrites interferes with the test; also, it must be borne in mind that histidine may occur in the urine in conditions unassociated with pregnancy. For instance, Hunter, as has been previously mentioned, was able to demonstrate its presence in cases of measles, although in this connection it must be acknowledged that from his use of the diazo reaction, his findings are not directly comparable with those of the Kapeller-Adler method. Better evidence to a similar effect is offered by Krieger (29), who was able to demonstrate that the quantity of urinary histidine varied with the menstrual cycle. In general, it may be said of the histidine tests, and particularly of that of Kapeller-Adler, that they appear to offer a presumptive test for pregnancy, but that they are not sufficiently reliable for use in critical cases, the more especially since, as was shown by Kapeller-Adler herself (25), histidine in the urine of pregnancy is seldom demonstrable before the 4th week.

As to the Visscher-Bowman test, it has been introduced too recently to permit a final judgement. If the authors' contention is correct,

that the test is a chemical means of demonstrating in urine the presence of prolan, and if it is sufficiently sensitive to detect this in relatively high dilutions, the test should be as reliable as that of Ascheim-Zondek, in which the same substance is demonstrated by its biological reaction. The high percentage of correct results so far reported gives a promise of great usefulness for this test. Drawbacks which have already been found are the presence of albumen, which interferes with the test, and the presence in the urine of other reducing substances. (33).

The several biological tests mentioned are, with the exception of the Bercovitz test, also too recently introduced to permit any attempt at proper evaluation. The Bercovitz procedure, to which only passing reference has been made here, depends on pupillary changes which follow the introduction of the patient's serum to the conjunctiva, presumably through the content of the latter in prolan. The fact that it requires persistent observation over considerable periods, in conditions of absolutely constant light, makes the test a difficult one to conduct, and introduces a large personal factor.

There are also a number of objections to the Gruskin and antuitrin S tests. The injections must be performed with great care, to avoid even slightly excessive trauma, and to secure injection to exactly the proper depth; further, in my own experience, and as has been found by Gilfillen and Gregg, the time limits of the reaction are so variable as to introduce a definite source of possible misinterpretation.

Everything considered, of the several tests discussed here, that of Visscher-Bowman would appear to offer the greatest promise. Whether this promise will be fulfilled only time and extensive application will tell.

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