1952

Beta-glucuronidase : a review of the literature and a study of its use as a screening test for gynecological cancer

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Submitted in Particular Fulfillment for the
Degree of Doctor of Medicine

College of Medicine,
University of Nebraska

December 13th, 1951
Omaha, Nebraska

I. Introduction

The subject of this paper was chosen with the purpose of obtaining some knowledge of cancer detection tests in general with emphasis upon one particular method. The value of a screening test for malignant growths in the human body has long been appreciated, but the inadequacy of medicine's discoveries is apparent from the hundreds of tests devised and the unsatisfactory results they give.

In order to better evaluate this review of the enxyme beta-glucuronidase, the qualities of an adequate screening test for any malignancy should be kept in mind.

These qualities may be listed without elaboration, for the importance of each is self-evident.

1. It must be specific.
2. It must be an objective test.
3. It must require only average training to permit its wide use.
4. It should require a minimum of special equipment and patient preparation.
5. The results should be obtainable in a short duration of time.
6. There should be a minimal number of false negatives; false positives are permitted on a slightly larger scale.

If a test meeting all 6 of the above requirements could be devised and used as we now use the Wasserman, Mantoux, etc. the early detection of cancer would be assured and many unnecessary deaths could be prevent-

ed almost daily. Unfortunately, such a test is not available, but perhaps by studying the principles of cancer growth, advances in biochemistry, and the progress of physiology, some man may make such a discovery.

The following discussion is designed to show how the primarily biochemical discovery of beta-glucuronidase developed into one of the most recent screening tests for gynecological cancer. A chronological discussion would have certain advantages, but by dividing this review into sections on chemical and physical properties of the enzyme, function, and clinical use of the discoveries, a more practical evaluation of the experimental work may be made.

II. Properties and Factors in Analysis

A. Chemical and Physical Properties

This phase of study in regard to beta-glucuronidase has not been investigated to the extent desirable, partically because of inherent technical problems and partially because of the recent emphasis on the clinical application of our existing knowledge.

First of all, it should be stated that we are apparently discussing not one but two separate enzymes, (1,2,3,) though this was not suspected originally (4) and has recently been denied. (5) Mills (1) first noted there were apparently two optimum pH for analysis of beta-glucuronidase in ox spleen and used this as evidence of the two

enzymes. Kerr at al (2) were able to substantiate Mill's work (1) by careful analysis of mouse liver and kidneys. Graph I is a reproduction from the work of Kerr at al which demonstrates their work in a concise form.

Sarkar and Summer (5) found this enzyme to be a protein (globulin) and that it was stable over a wide pH range below 50°C. At temperatures above 55°C, activity was markedly diminished. The remainder of this paper is concerned with enzyme-substrate concentrations and other material better considered under a later section.

Our only additional knowledge of the chemical and physical properties of this enzyme, other than what is revealed in the methods of assay is in regard to an anti-enzyme. The original work on this particular phase was by Fishman (6) who, after noting that mammalial plasma inhibited beta-glucuronidase, found high serum glucuronidase levels to be associated with low serum anti-glucuronidase levels. He further found increased amounts of serum glucuronidase of eclampsia to be associated with both low anti-glucuronidase and increased amounts of a "glucuronidase activating factor". Karunairatnum and Levvy (7) found that hydrolysis of phenylglucuronide by beta-glucuronidase was inhibited by saccharic acid, but not other closely related compounds, and apparently the conjugating function of the enzyme was unaffected. Whether these two separate findings are related or not remains to be seen.

B. Purification of Beta-glucuronidase Extracts

Fishman's original work of beta-glucuronidase (8) was an attempt to

make an extract of calf spleen containing a high enough concentration of the enzyme that it might be useful in the study of conjugated estrogens in urine. As an example of the work involved in such a study, table I is reproduced from his original paper showing his process. In 1946, Graham (9) altered the above procedure and was able to increase the purity of the extract 315 times as against the 140 times as seen in the table. This difference might, however, be accounted for on the basis of improved methods of analysis of the product.

Bernfeld and Fishman (10) produced an extract of powdered calf spleen which was 1200 times more active in beta-glucuronidase than the original substance. These workers made their calculations on the basis of mitrogen determinations and thought they had a practically pure extract. By assaying their purified extract, they found that one milligram dissolved in one cc. of water hydrolyzed o.165 mgm. of phenolphthalein glucuronide, the present substrate, at 37°C. However, Sarkar and Summer (5) have recently reported a 6000 fold increase in purity and even this may someday be surpassed.

There would be a very obvious advantage to having available beta-glucuronidase in a chemically pure state. First, it would permit a thorough investigation of chemical and physical properties which in turn would most likely answer many points now in debate. It would also forward the work on clinical use of determinations of this enzyme by giving us a definite, constant standard with which to compare assays.

C. Substrates and Their Preparation for Use in Assays

The basis for most all enzymatic determinations is the principle of allowing the enzyme in question to hydrolyze a substrate which is usually a salt, and then performing a test to determine either the amount of substrate present or the amount of one of the substances set free by the chemical reaction. The substrate used by Oshima (11) and Masamune (12) is not known due to their papers being unavailable, but both Marrian (13) and Fishman (8) used sodium menthol glucuronidate, a substance deemed satisfactory until the work of Talalay et al in 1946 (4) advocated the use of phenolphthalein mono-beta-glucuronide in the form of its cinchonidine salt. Fishman (14) had noted quite early that beta-glucuronidase had a greater affinity for some natural occuring glucuronides than it did for synthetic substances by determining the Michaelis-Menten constant. The use of such a substance, as estriol glucuronide for example, was and still is prohibited however by technical problems in its preparation as well as cost of production.

The preparation of this improved substrate was both expensive and time consuming, but the introduction of a reliable colorimetric method of assay which it permitted was invaluable. The workers injected rabbits with sodium phenolphthalein phosphate over a period of 17 days and collected the urine from these animals for 21 days. They found that 5 rabbits would excrete approximately 16 liters of urine during this time and from this 8.26 grams of the crude cinchonidine salt could be prepared. The crude salt was purified by crystallization from hot dioxane.

This method was later altered by Fishman et al (15) who acidified the urine of rabbits receiving injections of sodium phenolphthalein phosphate with 6 N HCl. They then extracted the substrate with washings of ethyl acetate, centrifuged the resulting solution, and evaporated it in vacuo at 50°C. To the solution was added a saturated solution of cinchonidine in ethyl acetate. The salt was crystallized by dissolving it in a mixture of hot methyl alcohol and ethyl acetate. The crystallization process was repeated again and the result was an increased yield of phenolphthalein mono-beta-glucuronide with a molecule of methylalcohol of crystallization.

These elaborate processes have been related for the purpose of demonstrating the extent to which men search for ideal reagents. The last-named method of substrate preparation is the one used most widely at present and was used in all determinations made for this paper. Many factors must be considered in choosing a substrate for enzyme experiments, a few of which are heat stability, pH stability, specificity for the enzyme, and the available means of analysis the substrate permits. When discussing the chemical assay of beta-glucuronidase additional advantages of the present substrate will be emphasized, but it may be stated that all the requirements are satisfied by phenolphthalein mono-beta-glucuronide.

D. Methods of Assay

1. Chemical Methods

As mentioned under the preceding heading, there is no satisfactory

method of determining the presence of beta-glucuronidase qualitatively or quantitatively. All assays up to the present time have been performed by allowing the enzyme and substrate to chemically act on each other and then testing for one of the products of the reaction.

Fishman's original method of assay (8) will be given here more for historical interest and comparison with our present methods. He allowed the enzyme to act on sodium menthol glucuronidate at a fixed pH for a set period of time and then halted the reaction by adding 10% trichloroacetic acid. The glucuronic acid liberated was measured by adding potassium ferricyanide which was reduced to ferrocyanide and titrated with a standardized ceric sulfate solution using setopaline C as an indicator. It was possible to take readings directly from the titration by first standardizing the ceric sulfate solution against a solution of glucuron, the latter being used due to its easier preparation in a pure state. Though this procedure is an indirect one and subject to many inherent sources of error even at best, it was satisfactory for the early preliminary work on beta-glucuronidase.

Talalay et al (4) offered a markedly improved method of assay when they showed the advisability of using phenolphthalein mono-beta-glucuronide as a substrate. These workers, besides their general method of assay, offered three facts which are important in present day analysis. First, they noted that the time of incubation of the enzyme-substrate mixture was immaterial to the final results since the process proceeded linearly with time. They also showed that 38°C. was the optimum temperature at which to carry out the test. Thirdly, they showed that a pH of

4.5 was optimum for making determinations. This last statement is in no practical conflict as yet with discoveries of two separate enzymes, one with an optimum pH of 4.5 and the other 5.5. Someday, it may be advisable to assay each of these fractions separately, but as yet there is no known advantage to such a procedure. Suffice it here to say that this method allowed the authors to obtain results which agreed within 4%. Since the time of publication of the above method however, Fishman et al (15) have offered a modification which is currently used by most investigators in the field.

According to this modification, into two Wassermann tubes were pipetted o.1 cc. of the substance being tested, 0.8 cc. of 0.1 M acetate buffer (pH 4.5), and 0.1 cc of 0.01 M phenolphthalein glucuronide. A third tube containing enzyme and buffer only served as the control. The digests were incubated for 15-24 hours after which 1.0 cc. of 5% trichloroacetic acid was added to each tube (1.0 cc of 10% trichloroacetic acid was added in the case of laked blood cells). The tubes were centrifuged for 10 minutes at high speed after which the contents were decanted into colorimeter tubes containing 2.5 cc. of an "alkaline reagent" (200 cc. of glycine buffer of pH 10.45 plus 50 cc. of 0.5 N NaOH in cases of digests treated with 5% trichloroacetic acid or 50 CC. of 1.0 N NaOH when 10% trichloroacetic acid was used). The resulting solution was brought to 6.0 cc by adding washings of the test tubes and read in a photolometer at 540 microns. The calculations used with this method are as follows:

Micrograms of phenolphthalein liberated X 1/hours incubation X 100/cc

of fluid analyzed equals units glucuronidase/100cc fluid

Laked Blood Cells

Micrograms phenolphthalein liberated X 1/hours incubation X vol. laked cells/0.1 X 100/vol. of original blood sample equals units of glucuronidase/100 cc. whole blood

Tissue

Micrograms phenolphthalein X 1/hours incubation X vol. of extract/0.1 X 1/grams of tissue equals units of enzyme/gram of tissue.

2. Histological Assays

The desirability of a histochemical test for beta-glucuronidase is self-evident, for by this means if it were a satisfactory method, we could gain knowledge as to the exact site of production of the enzyme, its method of activity in the body, and much other useful date. Friedenwald and Becker (16) developed such a process. Their method followed the basic ideas of enzyme analysis set forward earlier in that they allowed the tissue to be incubated in the presence of a glucuronide salt of an insoluble dye. As a check on themselves, they used two types of stains, and they checked their results by performing assays according to the method of Talalay et al. As to whether or not this method of analysis truly reveals the site of production of the enzyme, etc. is debatable at present, but this work has been studied by others. (17, 18)

III. Function of the Enzyme in Vivo

1. Formation of Beta-Glucuronidase by the Body

We actually know very little of the exact function of beta-glucuronidase, and much of our meager knowledge is questioned by various authors. That the enzyme is concerned with steroid metabolism, there can be little doubt, for it has been demonstrated by many authors. (19, 20, 31, 13) Friedenwald and Becker (16, 18) feel this enzyme plays an important role in mucoid metabolism, and many feel it is important in the fundamental process of growth. (2, 22) All of these assumptions are probably correct, and there may be many more functions of the enzyme, but other than this we can not narrow down the function of beta-glucuronidase in the body.

The site of production of beta-glucuronidase in the body is as yet unknown. We do know that it is found in many different organs, but as to which particular cells produce it or what part of any cell manufactures this enzyme we do not know. In regard to the latter statement, however, Friedenwald and Becker, (16) on the basis of histological assays of various rat tissues, claim the cytopam is the site of production and not the nucleus, while Campbell and Levvy (17) take an opposite stand.

The chemistry of formation of beta-glucuronidase is entirely unknown, there being no published work on the subject to date.

2. Relation of Beta-glucuronidase to Steroid Metabolism

As to the specific function of beta-glucuronidase in the body, it is only known that it is probably concerned with both hydrolysis (23, 24) and conjugation (24, 25) of many glucuronides. Fishman and Anlyan (26)

believe conjugation is the most important of these, while Karunairatnam et al (23) believe the opposite and still others believe the two functions to be of equal importance. (24) Nevertheless, there have been many interesting relationships demonstrated between the amount of this enzyme in the various body tissues and fluids and the level of steroid (estrogenic) metabolism. It is with these latter relationships that this section shall be concerned.

G. F. Marrian (13) first showed that there was a relationship between the estrogens and beta-glucuronidase in what is almost a "classical" paper in regard to this enzyme. In this paper, Marrian showed the similarity of effect of giving sodium estriol glucuronide and estriol orally despite the fact that the salt was water soluble and the estriol was ether soluble. The obvious solution to this apparent incongruous phenomenon was that some enzyme, beta-glucuronidase, had the ability to hydrolyze the salt.

A closer relationship of beta-glucuronidase to estrogenic function was demonstrated by W. H. Fishman and L. W. Fishman (27) and again by F. W. Fishman. (21) It was the purpose of these two papers to show that administering estrogens caused an increased activity of the enzyme only in the uterus. As an illustration of this work, table II is reproduced from this work.

Further evidence to support the concept of this enzyme being correlated with estrogenic levels of activity includes the paper by Odell and Fishman (19) who demonstrated a gradual increase in the level

of the enzyme in the endometrium during the first two-thirds of the menstrual cycle and a decline during the last third. Odell and Dieckmann (28) showed the serum level of beta-glucuronidase to increase 300-400% during a normal pregnancy, while Fishman (17) demonstrated the increased amount of the enzyme in blood cells during pregnancy. Presumably on the basis of estrogenic stimulation, Fishman and Anlyan (26) showed high levels of beta-glucuronidase activity during lactation. Another interesting relationship was brought out by Fishman et al (29) who found that normally 5 days post-partum the serum beta-glucuronidase level was only 50% of the level found at the time of delivery. Administering stilberterol, however caused the level to remain at 80% of that found at delivery.

All of the above mentioned work points toward a direct relationship between the level of estrogens and the amount of beta-glucuronidase. The only significant discrepancy in this concept results from two papers. Fishman and Anlyan (26) state that low levels of the enzyme are found in the genital organs following menopause or ovariectomy while Fishman et al (30, 31 and Fishman 32) state that high levels in vaginal fluid are associated with pan-hysterectomy and ovariectomy.

It is this close correlation of the amount of enzyme and the level of estrogenic activity that has formed the basis for the clinical use of the enzyme.

3. Beta-glucuronidase and Mucin Metabolism

Friedenwald and Becker (16, 18) have done the only work on the re-

lation of beta-glucuronidase to mucin metabolism, having first noted the correlation while developing their procedure for histochemical enzyme analysis. Their most significant paper on this subject (18) is the result of studying slides of various organs in the rat and rabbit, particularly ocular tissues. On the basis of the high activity in the ciliary epithelium of the eye, they offered this tissue as the site of production of the vitreous mucoid. They also noted the activity in the ducts of the sublingual gland and, assuming the relation of mucoid metabolism and enzyme to be a true one, advanced the theory that the mucoid elements of this gland's secretion are added as it passes through the ducts.

4. Beta-glucuronidase Levels and Growth Changes

It may be noted from much of the foregoing material that while the relation of this enzyme to estrogens and mucoid metabolism has been demonstrated, there is also evidence of a secondary relationship which may be the true one and far more important in the over-all picture. Additional evidence will be given in this section to support the view that beta-glucuronidase is not increased or decreased in amounts as function of hormonal stimulation per se, but only as that stimulation causes or impedes growth in the tissues.

Before considering this aspect of the function of beta-glucuronidase, it is interesting to note in retrospect that Fishman, (33) who has done more work on the subject than any other single individual, thought the

levels of the enzyme in any tissue to be dependent primarily on the level of the substrate in that tissue. In future studies as our knowledge of growth processes increases, this may be found to be basically true, but it shows that as late as 1940, the correlation with growth was unknown.

The works of Levvy et al (34) and Kerr and Levvy may (22, 35) best be considered as one, since their work was done to demonstrate one particular point and the work was done in only one laboratory. These people administered certain toxic agents such as borneol, menthol, etc. to rats who were permitted to live only long enough for the maximum effect of the drug to have occured. In each case, they closely correlated the histological appearance of tissue repair with the amount of enzyme. Since there was the possibility of the increased amounts of enzyme being caused by tissue damage, amounts of the drugs were given to a second group of rats sufficient to cause irrepairable damage. In this second group, enzyme levels were either unchanged or below normal in each case.

Another interesting observation was made by Fishman et al (15) who showed there to be little or no activity of beta-glucuronidase in the red blood cells or platelets. The significance of this observation may be realized upon recalling that these blood elements act only chemically, having no power of reproduction, growth, or other functions associated with living objects.

At the present time, this correlation of beta-glucuronidase with growth is regarded as the basic "function" with the effects of estrogenic

stimulation representing only one of many facets.

IV. Clinical Use of Beta-glucuronidase Assays

1. In Pregnancy

Due to the previously demonstrated correlation of the state of estrogenic stimulation and the level of beta-glucuronidase activity, one of the most obvious clinical uses to which this work might be put would be in the field of pregnancy. Considerable work has been done in using this enzyme in detecting abnormalities of pregnancy, but unfortunately the results have been disappointing. This does not, however, make the work less valuable to future investigators and the subject will be discussed here to show what has been done.

Bernard and Odell (36) worked with pregnant albino rats and noted several interesting phenomenon to occur, but in the light of the facts brought out the previous section, all the results are as one would suppose them to be. First, these men found that levels of the enzyme in the kidney, vagina, jejunum, lungs, thyroid and brain were unaffected by the pregnancy, which is in accord with previous knowledge, since these organs do not respond to stimulation of the hormones prevalent during pregnancy. They also noted that the placental site and spleen increased in activity, the former due to actual growth processes probably and the latter because of the increased function of blood forming elements. The fetus itself gradually decreased in enzymatic activity

as the growth rate decreased. Finally, the ovary and uterus (other than the placental site) decreased in amount of beta-glucuronidase activity, probably on the basis of a relative decrease in the amount of estrogenic stimulation. All these same facts are probably true in the human also. Odell et al (37) offered further information on what effects pregnancy has on this enzyme by noting that the activity was less in cord blood than maternal blood at the time of delivery. These men also found amniotic fluid to contain more enzyme than fetal urine but less than maternal blood.

McDonald and Odell (38) offered serum beta-glucuronidase determinations as a clinical test for pre-eclampsia with any determination of 20 gamma/cc. being diagnostic if the patient was part the thirtieth week of gestation. These men stated that differentiation of pre-eclampsia and hypertensive toxemia could be made using this test. This work was confirmed by the same men (39) in a subsequent paper, the second publication stating however that no information in regard to the severity or prognosis of the pre-eclampsis could be determined from the test.

Moore et al (40) later published the results of their determinations on serum levels of beta-glucuronidase in normal pregnancy, hypertensive toxemias, and renal toxemias. Statistically, these men could find no diagnostic value to the serum enzyme determinations in pregnancy and this is now the accepted viewpoint. Odell and Dieckmann (28) have recently reported that during toxemia, the activity is increased in only

two-thirds of the patients and even these results are inconsistent.

B. In Cancer

1. Serum Determinations

The only significant work on this phase of the present subject was done by Cohen and Husseby. (41) They, like others, (28) had noted there was no correlation between the serum beta-glucuronidase level and the presence or absence of a malignancy in the body. However, by giving estrogens (ethinyl estradiol and diethylstilbesterol) to patients with carcinoma of the breast, they could show an increase of approximately 50% in the serum enzyme level. The application of this discovery to cancer detection is not too probable at present, since breast carcinomas are stimulated by administration of estrogens and because the maximum increase in enzyme activity only occurs after 4 weeks. This work does, however, show that there are still many possibilities of clinical use of beta-glucuronidase assays.

2. Tissue Determinations

It has been generally agreed since the work of Fishman and Anlyan (42) that many malignant growths of the body, as well as metastatic nodes, (42, 43) show increased activity of beta-glucuronidase, but the applicability of this fact has been the source of many papers and considerable debate. The uses of assays of this enzyme to denote malignancy stems from the basic conception of its ability to reflect metabolic rate and growth in general, and it may be mentioned here that it is also be-

cause of this fact that the desired specificity is lost. Lorincz et al (44) have stated that tissue enzyme determinations are not to be correlated with cellularity as evidenced by nitrogen determinations, but this is not a commonly held concept.

Because of the easy accessability of gynecological carcinomas, the majority of work has been in conjunction with cervical lesions. Odell and Burt (20) found the enzyme activity to be significantly increased in genital cancer with the exception of those malignancies of the endometrium and ovary, and their results were marginal in cases involving vulva and vagina. They noted enzyme activity to increase from cervical os to endometrium, but found no difference between various sites of endometrium. This work has been supported by many others. (44, 28) It might also be noted here that there is general agreement that after irradiation of cervical carcinoma, the activity in both tissue (20, 44) and vaginal fluid (30, 32, 45) shows a significant decrease in amount.

3. Body Fluids

Two factors have combined to cause the many papers to be written on beta-glucuronidase in connection with vaginal fluid, the first being the desirability of developing a screening test for one of the most prevalent of carcinomas and the other being the relative ease with which this fluid may be obtained.

Odell and Burt (20) found they could detect cervical cancers by performing vaginal fluid assays and using 300 gamma/cc./hour as the divi-

ding line, the test being positive when results indicated more than this amount of activity was present. These workers reported 18% false positives, the principal causes of which were pregnancy, menstruation and vaginitis.

There are many factors influencing the level of enzymatic activity in vaginal fluid and it is interesting to consider here the lists of these factors as presented by some prominent workers in the field. Odell et al (45) give 5 principle factors, the first being radiation of the cervix. They further noted that blood contaminating the sample caused lower values to be obtained while bacteria and menstruation resulted in slightly increased values. In regard to temperature, they noted that storage of samples at 20°C. caused a rapid decrease in enzyme activity, but storage at 4°C. did not affect the results, providing the sample was not diluted. In addition to these variables, Kasdon et al (30) state that results are dependent on:

1. Patient's age
2. Method of sampling
3. Menopause
4. Trichomonas and other types of vaginitis
5. Hysterectomy
6. Pregnancy
7. Stage of menstrual cycle
8. Blood in the specimen

If one adds these two lists together, he may realize some of the technical

as well as practical difficulties in using beta-glucuronidase assays as a screening test for gynecoligical cancer. However, to demonstrate that all these variables are not without debate, it may be noted that Fishman (4) has recently stated that pregnancy, trichomonas infestation and WBC population are unimportant in enzyme determinations on vaginal fluid.

The question of the importance of bacterial and/or trichomonas infection in obtaining significant results has been considered in more detail in two papers of very recent publication.

Green et al (46) undertook the task of studying the enzymatic activity of pure cultures of organisms common to the vagina and found there to be no activity unless menthol glucuronide was added to the media, thus providing a substrate. Further analysis of the results of this work showed to the author's satisfaction that the level of enzyme activity was related, not to the number or species of bacteria, but to the growth rate of the culture.

Kasdon et al (47) found vaginal fluid activity above and below 300 gamma/cc./hour with equal frequency when the only abnormality was a trichomonas infection. These workers also tried, and failed, to produce beta-glucuronidase by incubating pure cultures of E. coli with sterile vaginal fluid. The conclusions to be drawn from this paper, as well as the one discussed previously, are limited to the fact that bacteria and trichomonas play no part in effecting the vaginal fluid

level of beta-glucuronidase.

At present, the subject of what value should be used as a "normal" value for vaginal fluid is somewhat disputed. Kasdon et al (30) stated that 300 gamma/cc./hour is too high a value and is the cause of the 20-25% false positives which usually result. They state that the normal value selected should be based on the known variables in the test and suggest an "80% value". This value would represent a level of enzymatic activity below which 80% of the population in an age group would be represented. These values were advocated by Fishman alone (32) and listed in a subsequent paper by Fishman, Kasdon, and Homburger. (31) These "80% value" normals are:

1. Age 2-40 ——————————<50 Units
2. Age 41-60 (pre-menopausal)——400 Units
3. Age 41-60 (post-menopausal)—750 Units
4. Age 61-90 ——————————800 Units

It should be noted that all work on this new normal was done using a new method of analysis in which the step of enzymatic inactivation was performed by heat instead of 10% trichloroacetic acid. This altered method of analysis may require correlation with previous methods to determine its full significance, but this 80% value may allow use of vaginal fluid beta-glucuronidase levels clinically after more experimental work.

There has been one published work on beta-glucuronidase activity in effusions of cancerous and non-cancerous origin written by Fishman

et al. (48) A brief summary of this work is best obtained by analyzing tables III and IV.

In order that a more thorough understanding of the subject might be had than that ordinarily gained from merely reviewing the literature, some of the clinical applications advocated by others were studied in the laboratory. No attempt was made to unearth new material, and no concentrated effort was made to prove or disprove the reports of others. However, it was hoped that by performing a few assays as they would be used in screening patients for gynecological cancer, an opinion of the value of this method might be made from first hand information. While this review concerns itself with only 86 cases, it is felt that there is enough variation in age, pathology, and other factors to fairly represent the test as it might be used by the practicing physician.

Material and Methods

All reports given in this review were given on patients admitted to University of Nebraska Hospital. The diagnosis of these patients is only given in regard to the presence or absence of gynecological malignancy, however, since that is all the test was designed to demonstrate. The ages of these women varied from 22 years to 73 years. All tests were performed without benefit of previous information in regard to symptoms, physical findings, or diagnosis.

The papanicolau smear reports are those reported on the patient's chart, being the result of study by the cytological cancer screening de-

partment. The pathology reports are likewise those given by the pathologists.

The method of analysis used was essentially that of Fishman et al and results are reported in gamma of phenolphthalein liberated per cc. of vaginal fluid per hour of incubation. In taking the samples, it was first suggested that 0.1 cc. of vaginal fluid be pipetted from the lip of a bivalve speculum into 3.0 of water, thus giving the required 1:30 dilution. This method was found to be nearly impossible however, so dilutions were made on the basis of weight determinations only. The only other modification of the basic method was in regard to incubation times. By shortening the incubation period to 3 hours instead of the 15-20 hours advocated originally, the photolometer readings were usually between 60 and 90, which is the most accurate range. This modification also permitted more determinations to be made more conveniently.

The abstrate used in making the determinations was not produced in the laboratory due to the time and expense involved as well as the complicated technical problems. All substrate was purchased from the Sigma Chemical Co. of St. Louis, Missouri.

V. Results

In this series, 85 cases are presented and the ability of beta-glucuronidase assays to correctly diagnose the presence or absence of female genital cancer is compared with both cytological and histological methods. The value of using an "80% value" in comparison with the previously used 300 gamma per cc. per hour is also considered. In all

cases, the histological diagnosis was assumed to be the correct one.

In analyzing Table VI, it is seen that by using 300 gamma per cc. per hour, a correct diagnosis was obtained in 43 of the 85 cases, or 50.5%, and using this same standard, there were 43.5% false positives and 6% false negative reports. In comparison, there were 58.8% correct diagnosis, 34.1% false positive reports, and 7.1% false negative reactions using Fishman's "80% value". These figures indicate results of considerably less value than those given in the literature and only slightly better than what might be had by pure chance. Technical inexperience, variation in equipment, variation in sampling, and many other factors might explain this discrepancy. However, the results can best be explained by realization that the test was taken from the "experimental" laboratory where procedures are more maticulous into the "clinical laboratory".

The papanicolaou smear techinque was performed on these same patients and a correct diagnosis was obtained in 62 of 84 cases, or 73.8%. There were 4.7% false negative reports, 2.3% false positive reports, and a total of 19.0% were reported as suspicious. The latter group may be further broken down; 13 cases, or 15.4%, of the total group reported as suspicious were reported as benign by the pathologists. Three cases of malignancy, representing 3.5%, were reported as suspicious.

The value of the cytological screening method is apparently quite better than beta-glucuronidase assays, but in comparing the two series here, let it be remembered that a "specialist" was reading the papanico-

laou smears. Nevertheless, a 20% difference in correct diagnosis, in this case, cannot be explained by difference in training alone.

The two methods may be compared in other ways than just the results they give. For instance, the taking of samples and preparation of material for performing tests is much easier in the cytological method than the enzyme assays. Performing the procedure is, however, simpler and less time consuming in case of the beta-glucuronidase determinations. Another factor to be considered is the relatively great amount of training required to read the papanicolaou slides as compared to that needed to perform the enzyme analysis.

VI. Summary

Beta-glucuronidase is an enzyme widely distributed in the cells of various tissues and performing the dual function of catalyzing the synthesis as well as the hydrolysis of glucuronide salts. In attempting to relate this function with known metabolic processes, it is only known that it is in some way concerned with growth and/or reproduction.

There is very little known about the chemical and physical properties of this enzyme, but it has been established that it is a protein, and stable to temperatures below 50°C. In performing assays, it has been found that most activity is found at pH 4.5 with a second peak at pH 5.5, this being some evidence for the existence of two such enzymes instead of one. Though the first attempt to purify beta-glucuronidase resulted in an increase of only 315 times the original concentration,

recent workers have concentrated samples up to 1200 times.

Most analyses for this enzyme are performed colorimetrically at the present time. This method requires the incubation of the tissue, body fluid, etc. with phenolphthalein mono-beta-glucuronide (the substrate) at 37°C. for 3 hours. At the end of that time, the reaction is stopped by adding trichloroacetic acid and then adjusting the solution to a slightly basic pH. The enzyme in the sample liberates phenolphthalein from the substrate and the amount of dye liberated is directly proportional to the amount of enzyme. The solution is then analyzed quantitatively for phenolphthalein colorimetrically.

A histological analysis for beta-glucuronidase has been developed and is extremely valuable in certain phases of research. The principle of analysis is much the same as the colorimetric method except that the substrate is the glucuronide salt of an insoluble dye. The dye is precipitated at the site of beta-glucuronidase in the cell.

No one has yet discovered the site of production of beta-glucuronidase and there has been no published work on the chemical processes involved in production of this enzyme.

Because many steroids (estrogens) are conjugated as glucuronides and because there seemed to be considerably more activity of beta-glucuronidase in the female genital organs than elsewhere, it was originally thought this enzyme was related to the level of estrogens in the body. There are several papers showing the relation of beta-glucuronidase

to stages in the menstrual cycle, menopause, pan-hysterectomy and oophorectomy. An indication of a broader relation was noted with development of the histological method of analysis which showed beta-glucuronidase to play a role in mucin metabolism. The concept of the relation to growth was developed after demonstrating that the amount of enzyme is increased in tissue repair but not tissue damage.

An attempt to utilize the existing information on beta-glucuronidase has been made in two fields; abnormalities of pregnancy and cancer studies. Advocation of blood serum beta-glucuronidase stemmed directly from the original concept of the relation of the enzyme to estrogens, but it was soon found that such tests did not differentiate the various toxemias of pregnancy as originally hoped.

Attempts to diagnose cancer, particularly cervical and vaginal types, with vaginal fluid determinations are still being made, though with decreased fervor. The literature reports up to 80% correct diagnoses using such determinations, but at this school the best percentage was 58.8%. It is felt by many workers now that the test is not specific for cancer but only indicates the level of metabolism; vaginal infections as well as simple cervical erosions causing false positive reactions. There is also considerable controversy on what degree of activity is normal and what is abnormal, just one more factor making it difficult to evaluate either the test or its results.

Since beta-glucuronidase studies on vaginal fluid were advocated as a screening method for diagnosing gynecological cancer, a comparison

was made between the efficiency of this method and the papanicolaou smear. A series of 85 cases were studied and the correct diagnosis was obtained with beta-glucuronidase assays in 50.5% or 58.8%, depending on which of two normal values was used as the standard. On these same cases, the cytology studies gave 73.8% correct diagnosis.

VII. Conclusions

1. The enzyme beta-glucuronidase in a protein promoting synthesis and hydrolysis of glucuronide salts.

2. The specific metabolic processes involving beta-glucuronidase are not known, but there is a relation to estrogenic and mucin metabolism as well as growth in general.

3. Serum assays for beta-glucuronidase can not differentiate abnormalities of pregnancy.

4. Vaginal fluid enzyme levels do not indicate the presence or absence of gynecological malignancy.

TABLE I

Hydrolysis was carried out at pH 5.4, 37.5°C., for eight hours; substrate, 0.0156 N sodium menthol glucuronidate

Step	Stage of Preparation	Activity *	Vol. of Extract	Total Activity **	Activity per mgm. protein nitrogen
A	Aqueous suspension of minced tissue	1.90	1685	8200	0.63
B	Supernatant sol. after centrifuging (A)	4.60		5200	0.66
C	Aqueous extract of acetone ppt. of (B)	3.45	1190	4100	2.41
D	Acidified extract	3.25	1100	3500	8.10
E	Evaporated concentrate	15.5	160	2500	5.96
F	Solution of ppt. obtained from (E) by ½ saturation with $(NH_4)_2SO_4$	20.0	112	2200	16.80
G	Product of fractional extraction of ppt. salted-out from sol. (F) with 35% s turated Amm. Sulf.	29.6	50	1500	45.50
	(G) repeated on sol. (G) with 40% $(NH_4)_2SO_4$	34.0	25	850	63.00
I	Sol. (H_1) combined with product of fractional extraction (37%) of insoluble residues from (G) and (H_1)	26.0	50	1300	87.00

* mg. of glucuronic acid liberated per 1 cc. of extract
** No. of cc. of extract times the number of mg. of glucuronic acid liberated per 1 cc. of extract
*** mg. of glucuronic acid liberated per mg. of nitrogen precipitated by 10% trichloroacetic acid

II

B-Glucuronidase Activity
Ovariectomized Mouse

B-Glucuronidase Activity						
Liver	Kidney	Spleen	Uterus	Vagina	Blood cells per ml.	Blood plasma per ml.
3.18	1.31	6.64	0.92	1.03	0.24	0.08
4.42	1.85	6.95	2.90	2.36	0.26	0.05
4.23	1.40	7.31	3.22	1.68	0.31	0.07
3.35	1.26	5.72	1.87	1.21	0.26	0.13
4.15	1.35	6.22	2.70	1.49	0.28	0.08
2.28	1.56	3.89	0.89	0.94	0.37	0.16
2.32	1.36	4.66	3.08	2.67	0.26	0.09
2.43	1.44	4.50	2.93	1.43	0.31	0.14
2.18	1.51	6.22	4.70	2.40	0.53	0.09
2.16	1.35	3.90	3.28	1.33	0.26	0.11

TABLE III

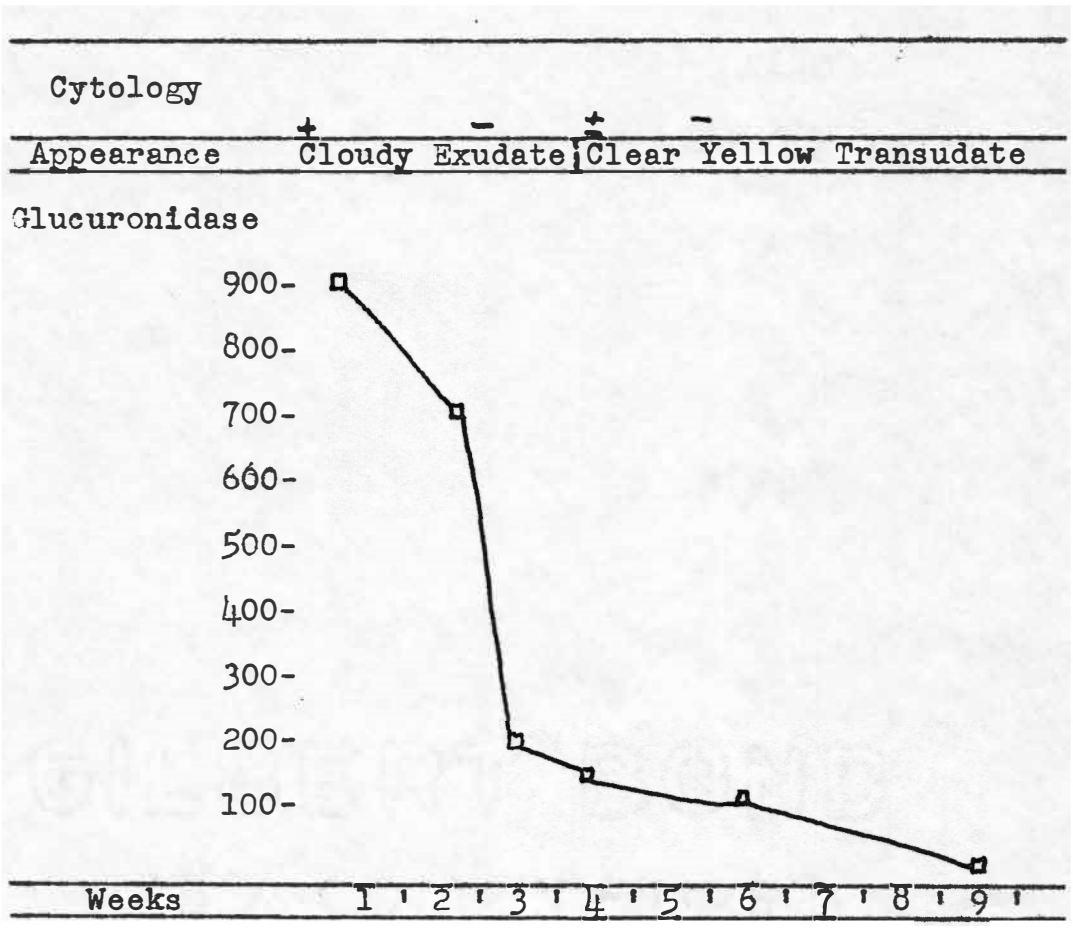

Cytology
+
-
±
-
Appearance
Cloudy Exudate
Clear Yellow Transudate
Glucuronidase
900-
800-
700-
600-
500-
400-
300-
200-
100-
Weeks
1 2 3 4 5 6 7 8 9

TABLE IV

Relation of Site of Cancer to Fluid Glucuronidase Values

Site of Cancer	Glucuronidase		
	No. of Analyses	Mean	Range
Cancer Group			
Breast	14	655	230-2725
Uterus	9	435	91-611
Lung	17	1655	160-8000
Gastro-intestinal tract	35	347	122-2105
Ovary	23	1187	262-3960
Miscellaneous	7	302	164-495
Non-Cancer Group	10	206	95-393

TABLE V

Identification Hospital #	Cytological Report	Vaginal Flaid Glucuronidase	Pathology Report	Age of Patient	Results		Menopause Passed
					80% Value	300Y/cc/hr	
105622	-	1190	B	49	-	-	No
106248	S	220	B	23	-	-	
104488	-	520	B	56	-	-	Yes
105818	-	420	M	29	-	-	
107255	-	1080	M	36	-	-	
104219	-	80	B	28	-	-	
106719	S	400	B	29	-	-	
107150	-	320	B	40	-	-	
105141	-	100	B	27	-	-	
106484	-	325	None	30	-	-	
107339	S	55	B	44	-	-	No
105476	-	350	B	42	-	-	No
106309	-	1400	M	53	-	-	Yes
104403	S	340	B	31	-	-	
106072	-	528	B	65	-	-	
106407	-	636	B	40	-	-	
107209	-	250	B	23	-	-	
104103	-	180	B	18	-	-	
105639	-	1150	M	71	-	-	
104641	-	665	M	60	-	-	Yes
105734	-	1300	M	46	-	-	No
106958	-	250	B	43	-	-	No
106958	S	1680	M	43	-	-	No
105137	-	900	B	73	-	-	
104689	-	1900	M	54	-	-	Yes
106630	-	540	B	37	-	-	
104716	-	840	M	43	-	-	Yes
106003	-	1800	M	56	-	-	Yes
105529	-	1500	B	36	-	-	
107315	-	920	B	50	-	-	No
104755	-	180	B	47	-	-	No
107011	-	250	B	41	-	-	No
106658	-	426	M	38	-	-	
106658	-	140	M	38	-	-	
105567	S	380	B	35	-	-	
105990	-	500	M	47	-	-	No
104293	-	300	B	32	-	-	

Table V (Continued)

Identification Hospital #	Cytological Report	Vaginal Flaid Glucuronidase	Pathology Report	Age of Patient	Results		Menopause Pas ed
					80% Value	300#/cc/hr	
106070	-	80	B	34	-	-	
107287	-	480	B	49	-	-	No
106120	-	1800	M	45	-	-	No
103972	-	100	M	68	-	-	
105028	-	720	M	65	-	-	
105750	-	40	B	24	-	-	
106192	-	1243	M	55	-	-	Yes
106386	-	90	None	24	-	-	
105026	-	100	B	42	-	-	No
105148	S	570	B	39	-	-	
104309	S	100	B	40	-	-	
104367	S	2880	B	70	-	-	
104214	-	Neg.	B	41	-	-	No
104737	-	940	B	67	-	-	
104298	-	320	B	19	-	-	
105905	-	330	None	62	-	-	
107282	-	320	B	33	-	-	
104110	-	600	B	42	-	-	No
105754	-	340	B	32	-	-	
107334	S	160	B	33	-	-	
105062	-	290	M	36	-	-	
104938	S	225	M	28	-	-	
105807	-	440	B	60	-	-	Yes
106024	-	1460	B	36	-	-	
104390	S	90	B	24	-	-	
106523	S	400	B	26	-	-	
105732	-	80	M	53	-	-	No
106682	-	570	B	45	-	-	No
104873	-	250	B	41	-	-	No
103717	-	395	M	68	-	-	
106408	-	1092	B	32	-	-	
104330	-	270	B	28	-	-	
107227	-	320	B	41	-	-	No
107127	None	860	None	35	-	-	
104390	S	85	B	24	-	-	
105008	-	Neg.	B	32	-	-	
105089	-	140	B	51	-	-	No
105066	-	80		45	-	-	No
106639	S	Neg.	B	33	-	-	
106718	-	300	B	46	-	-	Yes
105824	-	Neg.	None	35	-	-	
106057	S	340	M	31	-	-	
106983	-	300	B	52	-	-	No

Table V (Continued)

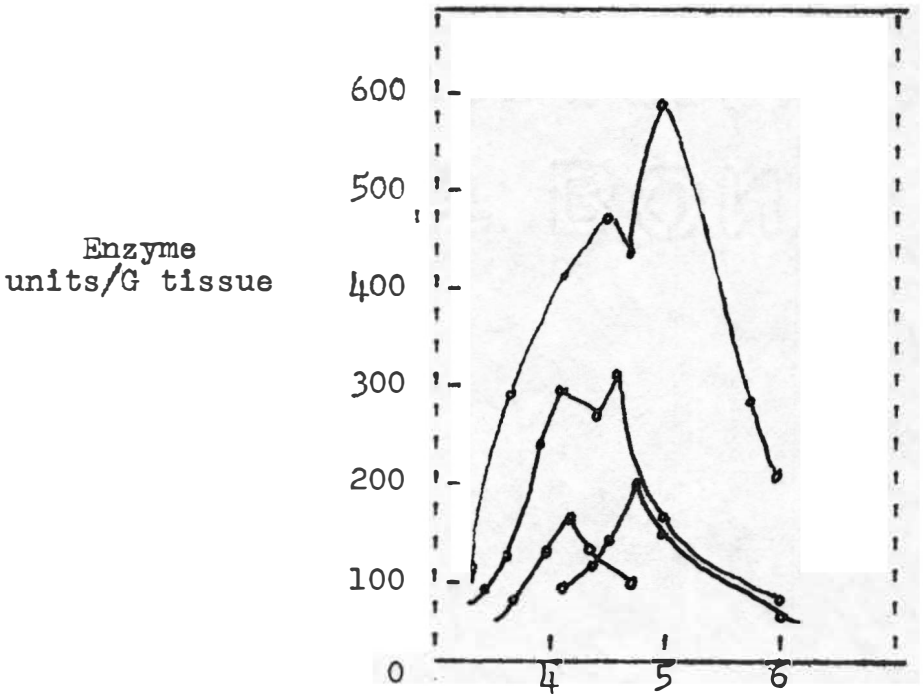
Identification Hospital #	Cytological Report	Vaginal Fluid Glucuronidase	Pathology Report	Age of Patient	30% Xylose	500 mg/sq. m.	Menopause Passed
104073	-	385	B	22	-	-	
106940	-	1250	M	71	-	-	
106790	-	480	B	19	-	-	
106292	-	2020	B	66	-	-	
107012	-	480	None	43	-	-	No

TABLE

Effect of Estrogens on
of Various Tissues of

Experiment Number	Estrogen (in aqu___ alcohol)	Total Dosage
1	Controls	0
	Estrone	0.5
	Estradiol	0.5
	Estriol	0.5
	Estriol	72.0
2	Controls	0
	Estrone	72.0
	Estradiol	72.0
	Estriol Glucuronide	72.0
	Stilbestrol	72.0

GRAPH I



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