

An Examination of the Levels of Human β -Glucuronidase in a Vohwinkel's
Syndrome Patient.

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Abstract

Vohwinkel's Syndrome is a rare autosomal dominant epidermolytic palmoplantar keratoderma (EPPK). In 1995, Peris et al described as: 1.) diffuse hyperkeratosis of the palms of the hands and the soles of the feet in which the skin takes on a "honey-comb" appearance. 2.) "star-fish" shaped keratotic plaques on the dorsal surface of the hands and feet, the wrists, knees, and elbows. 3.) fibrous constricting bands (pseudoainhum) at the interphalangeal joints that may eventually lead to autoamputation of that digit. 4.) the invariable appearance of the disease early in life. 5.) and familial incidence (autosomal dominant inheritance pattern).

Recently, it was discovered that one of the factors contributing to the disease was a molecular defect in a single protein located in the cornified cell envelope protein, termed loricrin (Mastrini, 1996). This defect seemingly impairs the differentiation process of the epidermal renewal system, by not allowing dead skin to slough off. The defect also contributes to barrier function impairment, causing affected patients to retain water in the keratinocytes. It was noted in 1988 by Camisa et al that Vohwinkel's Syndrome patients had an increased level in serum β -glucuronidase. β -glucuronidase is a lysosomal enzyme known to break down constitutive basement membrane proteins. It has been found in keratinocytes and Langerhan's cells. This study examined the levels of β -glucuronidase in epidermal skin punch biopsies, blood serum samples, and urine specimens from both diseased and non-diseased patients. A quantification of the levels of the enzyme was performed via a double antibody sandwich ELISA. It was hypothesized by this study that perhaps the increased levels of β -glucuronidase was compensatory for the molecular defect in loricrin.

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Introduction

The skin is a physical barrier at the interface between an organism and its environment-preventing water loss and withstanding mechanical, chemical, and microbial assaults (Roop,1995). To perform these functions, the outer layer of the skin (the epidermis) undergoes keratinization, a process in which epidermal cells progressively mature from basal cells which proliferate, differentiate and die as squamous cells of the stratum corneum. During the process of differentiation, certain genes are activated while others are down-regulated- leading to changes in both structural proteins and the expression and activation of enzymes that control posttranslational modifications, metabolic changes, and lipid synthesis (Roop, 1995). A defect in any one of these structural components or enzymatic processes has the potential to impair the barrier function of the skin and cause disease (Roop, 1995). There are several diseases that are the direct result of keratinization defects, usually by affecting the structural protein; keratin. Some examples include: epidermolytic ichthyoses , epidermolytic hyperkeratosis (EHK), epidermolytic palmoplantar keratoderma(EPPK), and ichthyosis bullosa of Siemens(IBS). Interestingly, only those diseases with defects in the upper layer keratins (EHK, IBS,EPPK) exhibit hyperkeratosis, or thickening of the stratum corneum (Roop, 1995). This study is an examination of a type of EPPK, known as Keratoderma Hereditaria Mutilans (KHM) or more commonly Vohwinkel's syndrome.

I. Vohwinkel's Syndrome:

A. History

In 1905, Hyde & Montgomery first reported three patients with hyperkeratosis of the palms and soles associated with ainhum-like constriction of the digits (Hyde, 1905). However, their descriptions of these patients were poorly described. It was in 1929 that K. H. Vohwinkel first described and named the rare skin disorder. He reported hyperkeratotic lesions on the palms and soles of a 24 year old woman and her 14 month old daughter. The mother had suffered since she was two, and eventually lost some of her digits due to spontaneous auto-amputation (Chang, 1981). The disease was formally called Keratoderma Hereditaria Mutilans (KHM), and soon became accepted as Vohwinkel's Syndrome. This rare dermatological disorder has only been reported clinically about thirty times to date, with the majority of these cases reported from northeast and central Ohio. It occurs in both sexes and its onset is early on in childhood. The pattern of inheritance has been established to be autosomal dominant. It is predominant among white or Caucasian people, although a similar hereditary palmoplantar keratoderma has been noted in African-Americans (Gibbs, 1966). Here, the classic KHM patterns seen on the elbows and knees are absent, along with a familial pattern of inheritance. The disease can be present in a **mild form**, which results in the appearance of hyperkeratosis. Hyperkeratosis, or accumulation of keratinocytes in the stratum corneum of glabrous (thick) skin. In normal glabrous skin, this layer, and the keratinocytes are continually sloughed off. In Vohwinkel's patients, the keratinocytes are

retained and accumulate, thereby causing the top layer to be thickened. It can also be present in a more **severe form** with: ainhum-like constriction of the digits that result in auto-amputation of that digit. It has also been associated with high frequency hearing loss, an unusual, pseudopelade-type alopecia; and transient, plantar bullae (Gibbs, 1966). In 1994, a female KHM patient was described, who along with normal symptoms of the disease also exhibited: craniofacial abnormalities that included a cleft lip and palate, microcephaly, and facial asymmetry (Sensi, 1994).

B. The Integumentary Layers of Glabrous Skin

Glabrous, or more commonly “thick skin” is characterized by its lack of hair follicles and sebaceous glands. This type of skin is found on the palms of hands, soles of the feet, knees, elbows, and fingertips. It is this type of skin that Vohwinkel’s Syndrome affects. The epidermis is a perpetually renewing tissue, comprised of five histologically distinct cellular layers, each with a distinct maturation state of the keratinocyte, the major cell type of the epidermis (Roop, 1995). Keratinocytes arise from stem cells in the basal layer [Keratin 5, Keratin 14], and move through a series of differentiation events [Keratin 1, Keratin 10] until they are sloughed into the environment (desquamation) (Roop, 1995). Thus, in normal epidermis, there is a balance between the process of proliferation and desquamation that results in a complete renewal approximately every 28 days. In the ichthyoses, the rate of desquamation may decrease, leading to epidermal cell retention (hyperkeratosis), or there may be an increase in proliferation, which further exacerbates the build up of skin cells in these patients (or both processes may occur simultaneously) (Roop, 1995).

The layers of human epidermis are as follows: from the deepest layer to the most superficial. First, stratum basale, stratum spinosm, stratum granulosm, stratum lucidum, and finally the stratum corneum.

Table1

Layer of Epidermis	Description
1. Stratum basale	Deepest layer of the epidermis. Here, active mitosis provides cells for the other layers.
2. Stratum spinosm	(Also called Prickel Cell Layer) This layer has several layers of polyhedral shaped cells that are actively mitotic.
3. Stratum granulosm	Here, two to four layers of flattened post-mitotic cells that contain keratohylaine granules are present.
4. Stratum lucidum	This is a clear layer. Here, there are no nuclei or organelles present in the squamous cells.
5. Stratum corneum	This is the outermost layer of dead, flattened, anucleate cells whose cytoplasm is replaced by keratin. This layer is constantly being shed in normal skin. It is thickened in Vohwinkel's patients.

The human epidermis is an extensive skin system whose main activity is to be a permeable barrier. This barrier system is located in the stratum corneum layer of the epidermis. Alterations in the layers composition are often associated with perturbations of barrier function, resulting in increased transepidermal water sequestration and desquamation, that result in a thickened, waxy, scaly, stratum corneum; just as we see in patients with KHM. It has been noted that Vohwinkel's Syndrome patients have an ability to retain water in the keratinocytes.

C. Appearance and Clinical Significance of Vohwinkel's Syndrome

Vohwinkel's syndrome is a very rare disorder characterized by a distinct histopathological and ultrastructural appearance. "Keratinocytes within the stratum corneum have a unique type of parakeratosis characterized by retention of condensed crenelated basophilic nuclei, the pathognomonic histopathological finding in Vohwinkel's Keratoderma (Mastrini, 96). The hyperkeratosis usually begins as patches of epithelia that grow until they form a thick yellowish plate over the palms and soles (Cole, 1984). The yellow color is directly caused by the thickening of the stratum corneum. This yellow color can be seen in the photographs in Figure 1. Normal conditions for these patients include thick, dry, and rough textured "thick" skin. These hard plates, also known as plaques, eventually become rigid and inelastic, predisposing them to the formation of fissures (Cole, 1984). Accompanying this maceration is infection, and the hyperkeratosis lose their plaque appearance and turn from yellow to gray-brown (Cole, 1984). Eventually, movement is restricted in these area's and once established persists through the lifetime of the patient.

i.) Clinical Diagnosis

Biopsy specimens of the affected areas usually demonstrate marked hyperkeratosis of the keratin layer (stratum corneum) with large keratohyaline granules (now called L-granules for the protein loricrin). Moderate acanthosis, which is present in the granular layer. Along with the presence of some cells in the upper layers of the epidermis with a highly vacuolated appearance (Krishnaram, 1986). It has also been

noted that in sections of skin biopsies, there are large numbers of Langerhan's cells present. Coincidentally, Langerhan's cells (dendritic cell), human keratinocytes, and melanocytes stain histochemically for β -glucuronidase (Camisa, 1988).

Lab work such as: complete blood cell count (CBC), hepatic enzyme levels, renal function tests, serum protein and fasting blood sugar levels are normal in most patients. Systemic examinations are usually normal as well. (Camisa, 1984).

The diagnosis of KHM is based on the characteristics modified from Peris et al, 1995, and are listed as follows:

1. Diffuse hyperkeratosis of the palms and soles, with a "honey-comb" appearance.
2. "Starfish-shaped" keratotic plaques on the dorsa of the hands and feet, and also involving the wrists, forearms, elbows and knees.
3. Fibrous constricting bands (pseudoainhum) at interphalangeal joints, which may cause autoamputation.
4. The invariable appearance of the disease early in life.
5. Familial incidence (autosomal dominant inheritance pattern).

The teeth, oral mucosa, and nails are unaffected and appear normal. Radiographic x-rays of the hands and feet reveal osteoporosis, demineralization of the bones, destruction of the interphalangeal joints, and tapering of the terminal phalanges (Presley, 1981).

The palmar lesions are usually the most severe in patients with KHM. The increased thickness in the epidermis is usually between 0.5cm to 2.5cm depending on the severity of the case (Landarzuri, 1991). The appearance of the palmar aspect of the hands of these patients is unique. They tend to look red, and this can be seen around the fingertips as observed in Figure 1. The fissures that were previously mentioned, seem to

divide the palm into blocks. The lesions usually end abruptly at the wrists and on the sides of the hands in a narrow red marginal zone or border (Landarzuri, 1991). Again, this can be observed in Figure 1. It is believed that on the palmar surfaces of the fingers there exists plaques that are divided at the interphalangeal joints by transverse fissures eventually leads to the constriction of the digits (Landarzuri, 1991). Thus causing the patient a great deal of pain and irritation. It also leads to a decreased joint mobility. Oddly, the dorsal aspect of the hand is usually not affected by these fissures, and as mentioned nails are also unaffected. However, as seen in Figure 2 the dorsal aspect of the hand does appear to be much thicker than normal skin.

Figure 1a is a photograph of the Vohwinkel's Syndrome patients hands. Notice the yellow tint and the redness on the fingertips.

Figure 1b is a photograph of the Vohwinkel's Syndrome patients hands. Notice the scaling of the dry skin and the yellowing in the skin folds. Notice also the red and thickened skin at the base of the wrist.



Figure 1a



Figure 1b

Figure 2a is a photograph of the Vohwinkel's Syndrome patients hands. Notice how thick the skin appears to be. Notice also the nails are unaffected and the yellow color is absent on the dorsal aspect.

Figure 2b is a photograph of the Vohwinkel's Syndrome patients hands. Notice the scaling of the skin of the palmar aspect is visible.



Figure 2a



Figure 2b

D. Molecular basis of the disease

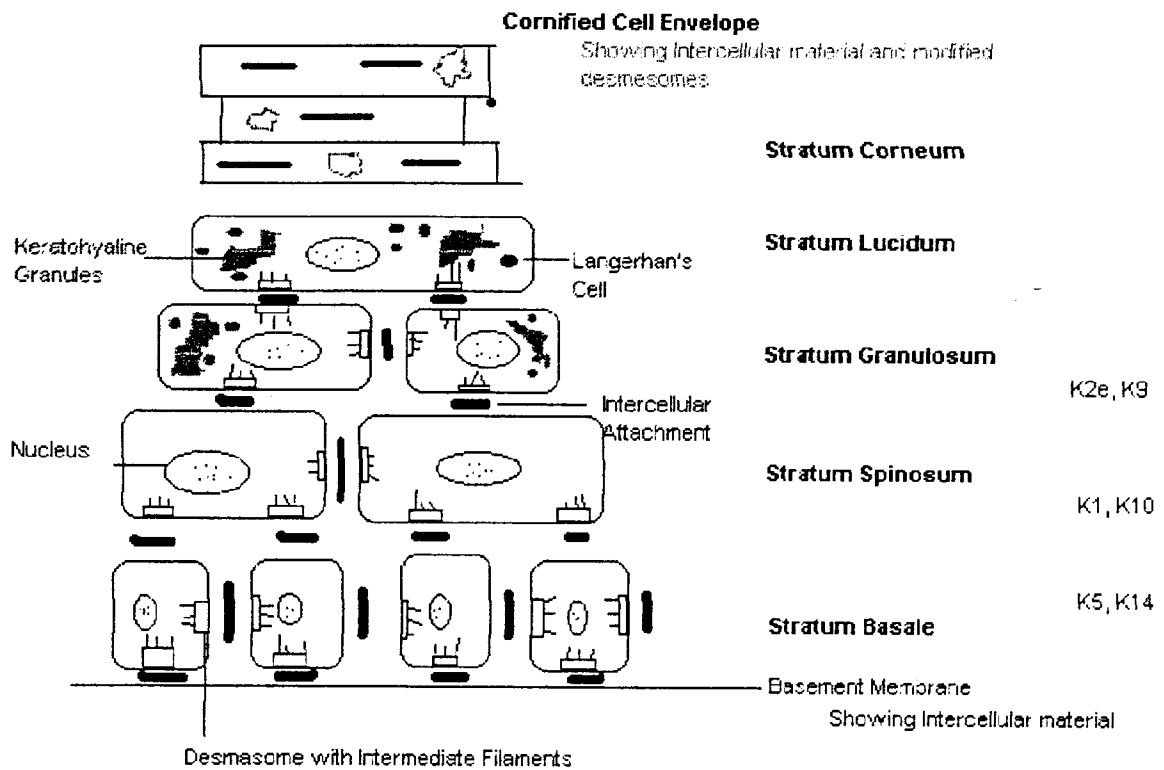
Keratins are the major structural proteins synthesized in keratinocytes. They assemble into a weblike pattern of intermediate filaments (IFs) that emanate from a perinuclear ring, extend through the cytoplasm, and terminate at junctional complexes called desmosomes and hemidesmosomes (Roop, 1995). Keratin IFs are essential for maintaining the integrity of the epidermis. Mutations in any of the six keratin genes result in four distinct epidermal diseases (Roop, 1995). Vohwinkel's Syndrome, a type of EPPK has a granular layer K9 defect. This mutation only affects the palmar and plantar epidermal regions of these patients. In the various keratin disorders, the IF networks collapse around the nucleus, preventing attachments between the filament-matrix complex and the inner surface of squames and alter interactions (desmosomal contacts) between neighboring cells, thereby potentially affecting desquamation (Roop, 1995). The various keratin's are labeled in Figure 3.

Huber et al 1995 identified mutations that result in loss of activity of transglutaminase K (TGK), an enzyme that cross-links proteins to form the cell envelope (CE), a specialized structure that replaces the plasma membrane of terminally differentiating keratinocytes. It consists of proteins cross-linked by covalent bonds into a rigid scaffold, with lipids covalently attached to its external surface. The cross-links between proteins are mainly disulfide bridges and highly stable covalent cross-links, catalyzed by transglutaminases, between glutamine and lysine residues (Roop, 1995).

It was discovered that lamellaer ichthyoses was a direct result of an abnormal cell envelope, which results from incomplete cross-linking of the major protein components (Roop, 1995).

If a defective cell envelope can cause ichthyoses , one might predict that the failure to synthesize the major protein components of the cell envelope, may result in the development of a similar disorder. In 1996, Christiano et al made a molecular breakthrough with Vohwinkel's syndrome. She found that the disease was linked to a molecular defect in the protein loricrin. Loricrin is a keratin protein that makes up the cornified cell envelope, along with involucrin, and other small proline rich proteins. Loricrin has been mapped to chromosome position 1q21 in the epidermal growth complex (EDC) (Mastrini, 1996). Essentially, an insertion of an additional glycine shifts the translation frame of the C-terminal Gly- and Gly/Lys- rich domains, which impairs the growth complex and barrier function in the epidermis of these patients.

Figure 3 depicts the layers that are present in “thick skin”. The diagram shows the location of the basement membrane along with the cornified cell envelope, where the protein loricrin is located. Also shown are keratohyaline granules, which are abundant in Vohwinkel’s Syndrome patients. Langerhan’s cells are phagocytic cells located in the upper layers of the epidermis.



K=Keratin's located in various layers of the epidermis.
 Each cell depicts a centrally located nucleus and desmosomes with attached intermediate filaments.
 In the upper layers, colored keratohyaline granules are depicted along with small Langerhans' cells.

II. β -Glucuronidase

A. Association history between the enzyme and Vohwinkel's syndrome

It was first noted back in 1988, that serum levels of β -glucuronidase were elevated in Vohwinkel's patients. Previous studies concentrated on enzyme activity, not total concentration. These studies involved a substrate kit sold by Sigma chemicals, and analyzed by spectrophotometer means. The normal range for the enzyme, by such a kit, in serum was 494-617 Units/100ml (Camisa, 1984). However, in the Vohwinkel's cases observed enzyme levels ranged from 706-922 Units/100ml (Camisa, 1988). Urine and skin studies have not been attempted in any capacity in Vohwinkel's patients prior to this study. In 1992, Dr. K.J. Ho developed an optimized double antibody sandwich ELISA, which measures total protein concentration of Beta-glucuronidase.

This ELISA protocol has never been used in Vohwinkel's Syndrome patients and normal ranges for the enzyme in the epidermis, serum, and urine have never been determined using this highly sensitive methodology.

B. The enzyme and its link to hyperkeratosis

It has been suggested that the activity of this enzyme reflects the degree of cellular proliferation taking place in that tissue or organ (Rutenberg, 1973).

Hyperkeratosis has been linked to an increase in enzyme levels of β -glucuronidase. An increase in its circulating concentration provides great clinical significance in many types of pathological process. It is also released into the synovial fluid in inflammatory joint diseases, like Rheumatoid arthritis (Jain, 1996). Similarly, metastasis of adenocarcinoma requires breakdown of the basement membrane, mediated in part by the release of β -glucuronidase from the cancer cells (Jain, 1996). It has also been shown that induction

of β -glucuronidase in the intestinal flora may be responsible for the pathogenesis of colon cancer (Jain, 1996). Hyperkeratosis, is a consequence of Vohwinkel's Syndrome.

β -glucuronidase is a hydrolytic enzyme encapsulated in a structure called a lysosome. Lysosomal vesicles are found in most eukaryotic cells. The vesicular hydrolytic enzymes are most active at a pH of around 5. This low pH optimum is a protective mechanism that prevents the enzymes from destroying the cell should leakage occur into the cytosol where the pH is neutral (Lenninger, 1993). Hydrolytic enzymes, including β -glucuronidase, are largely responsible for degrading glycosaminoglycans (GAGs). GAGs are extracellular compounds, and a cell must engulf them by invagination of the cell membrane (phagocytosis) forming a vesicle inside of which the GAGs can be degraded. The vesicle then fuses with the lysosome, forming a single digestive vesicle in which the GAGs are efficiently degraded (Lenninger, 1993). This ubiquitous enzyme is known to catalyze the breakdown of many connective tissue constituents (GAGs) like: dermatan sulfate, heparan sulfate, hyaluronic acid, and chondroitin sulfate. Chondroitin sulfate, a component of the ground substance of hyaline cartilage, is also found in the cornea, bone, and skin. Heparan sulfate is found in the lungs, arteries, cell surfaces, and the basal lamina. Dermatan sulfate is also found in the skin, blood vessels, heart, and heart valves. These molecules are all similar in that each core protein is bound covalently to many shorter glycosaminoglycan molecules.

Additionally, Hyaluronic acid has been reported to be synthesized by cultured epidermal cells and may also be present along with β -glucuronidase in the epidermal intercellular spaces (Camisa, 1988). By doing this, it is an essential enzyme for the

normal restructuring and turnover of these extracellular matrix components (Jain, 1996). The covalent attachments between glycosaminoglycans and core protein are glycosidic bonds between sugar residues and hydroxyl groups of Serine residues in the protein (Lenninger, 1993). Interwoven with these proteoglycans are fibrous proteins such as collagen, cornifin, *loricrin*, and elastin. This meshwork of extracellular proteins provides strength, resilience, a barrier, and directs the layers of the epidermis. As mentioned, β -glucuronidase is a lysosomal enzyme and breaks these glycolytic bonds. By breaking the bonds of larger molecules, smaller molecules are created. Since osmosis is a colligative property depending on the absolute number of particles in solution, the activity of the enzyme increases and the osmotic pressure created increases as well. The greater pressure allows water to flow into the cell much easier. Thus, an ability of the Vohwinkel's patients epidermis to retain water in the kerainocytes of the stratum corneum. However, why this occurs in Vohwinkel's patients has yet to be resolved.

C. Background on the enzyme

β -glucuronidase is a glycoprotein of about 332,000 Dalton (Jain, 1996). It is a homotetramer, comprised of four identical subunits. It is an enzyme that is highly abundant in the liver, spleen, pancreas, and other vital tissues including the *epidermis*.

The three dimensional structure of β -glucuronidase is of considerable biological and medical interest. Individual subunits are synthesized on membrane-bound ribosomes and translocated into the lumen of the rER. Structural studies on lysosomal enzymes such as β -glucuronidase are critical in understanding how these proteins are transported to the lysosomes from their sites of synthesis in the rough endoplasmic reticulum (Jain, 1996). Recent studies by Jain on β -glucuronidase showed that most lysosomal hydrolases carry

mannose-6-phosphate (M6P) on their oligosaccharide chains. M6P receptors present in the golgi bodies are responsible for the segregation and transport of lysosomal proteins. Exactly how the enzyme is sorted and packaged remains unknown. From the golgi bodies, the enzyme is transported to and incorporated within the lysosomes.

Materials and Methods

A. Sources of Beta- Glucuronidase

Three sources of β -Glucuronidase were collected. Samples were obtained at the same time and in the same manner for both individuals. They included: samples from the skin, the urine, and from blood plasma.

- i. Blood Samples: Blood was drawn from both Vohwinkel's and unaffected patients. The vacutainer® system was employed to draw the patients blood. A 26 gauge needle was inserted into the antecubital vein and 10cc of blood was drawn into evacuated red top tubes. The whole blood was placed on ice in collection tubes free of anticoagulants for thirty minutes, to begin coagulation of the red blood cells and to initiate the serum separation process. The samples were then separated by high speed centrifugation, discarding the red blood cells and retaining the resulting plasma. The serum was then maintained at -20°C .

- ii. Urine samples: Urine samples were obtained from both Vohwinkel's and unaffected patients. Approximately 90mls were collected in specimen containers. The samples were maintained at -20°C . One of the advantages of the ELISA is that the monoclonal antibodies are not inhibited by D-glucaro-1,4-lactone, a common inhibitor for β -glucuronidase in urine. Therefore, samples were not dialyzed against tap water.

iii. Skin samples: Skin samples were obtained from both Vohwinkel's and unaffected patients. The skin punch biopsies were performed by Dr. Brodell of Warren, Ohio using a 4mm skin punch. Two punches from the palms of the hands of each patient were obtained. The samples were frozen at -70°C in PBS until used. The skin was sectioned with a Damon Cryostat Microtome and then solubilized in 3% PBS/BSA. The samples were placed in microfuge tubes and refrigerated until further analysis. The skin samples were cross sectioned using a microtome cryostat procedure. Skin punch biopsies were harvested in 4mm sections, these were too large to analyze via the ELISA. To ensure that samples were small enough to be analyzed, a cryostat microtome was used.

B. Gel Filtration chromatography

The monoclonal antibodies were shipped in 45% saturated ammonium sulfate. Gel filtration, it is often used to "desalt" proteins. It was used to recover the antibodies. A sephadex column was prepared and used for recovery of both the primary and secondary monoclonal antibodies supplied by Dr. Ho (University of Alabama at Birmingham).

The procedure required the following solutions; TBS [58.44g of 0.5M NaCl, 4.84g Tris buffer (20mM Tris), adjust to pH of 7.5 with HCL], Sephadex G-25 mixture to a prepared column, and TBS/ antibody mixture. Approximately 0.5mls of the stock antibody was added to 0.5mls of TBS and the mixture went from cloudy to clear as the antibody was solubilized.

The same desalting procedure was performed for both the primary monoclonal antibody 7B6 and the secondary monoclonal antibody 6D2. The column was prepared using Sephadex G-25 powder in TBS buffer. The column was filled with the Sephadex

mixture. It was then washed thoroughly with TBS, this was done by filling the top of the column with TBS, using a glass pipette, while the bottom was held closed with a stopper. The stopper was then released to let the solution run through, and thereby wash the column. While the column was being washed, 30 microfuge tubes were labeled [1-30]. These tubes were used to collect the fractions of TBS solution containing the antibody. Once the column was washed and the tubes were labeled, the stock antibody solution sent by Dr. Ho was fractionated in half (0.5mls). Half of the antibody solution was added to another microfuge tube, this appeared as a white precipitate, and 0.5mls of TBS was added to the tube to bring the antibody into solution [now appears clear]. The remaining stock antibody was frozen again at -70°C . The antibody/TBS mixture was then carefully added to the top bed of the Sephadex column, while the bottom of the column remained stoppered, as to not miss any of the antibody coming off the column. The antibody was recovered in the labeled microfuge tubes. TBS was continually added to the top bed of the column as needed.

C. SDS-PAGE Gel

The SDS-PAGE gel is used to analyze a mixture of proteins. Proteins have different charge characteristics, to be able to separate them a massive negative charge is imparted on them. Sodium dodecyl sulfate, SDS, is an anionic detergent that denatures the proteins and gives all of the proteins in a mixture a negative charge. The proteins can then be separated based solely on their size. This is done by subjecting them to an electrical field in a polyacrylimide gel. The porous nature of the gel allows small molecules to pass through quickly, and larger molecules more slowly. This procedure is used to verify that the monoclonal antibodies were recovered from the gel filtration.

To run an SDS gel, the following are needed: resolving gel, overlay solution, acrylimide stacking gel, SDS electrode buffer, acrylimide stock, SDS sample buffer, resolving gel buffer, coomassie stain, and destain. The SDS PAGE gels were run to insure that our monoclonal antibodies were recovered from the desalting column. In this study a 10% resolving gel was used. A resolving gel consisting of 6.4g of glycerol, 24mls of resolving gel buffer at pH 8.8, 32mls acrylimide stock, 32mls H₂O, to which 240 µl of 10% ammonium persulfate and 96 µl TEMED are added. The resolving gel buffer consists of 18.17g tris, 8.20mls 3M HCL, and 0.40g SDS, and is brought to a total volume of 100mls with distilled H₂O. An overlay solution (enough for 15 minislab gels) was prepared by adding 6.0mls SDS running buffer, and 18.0mls distilled H₂O. A stacking gel (recipe makes 1 gel) consisted of 1.1mls stacking gel buffer at pH 6.8, 0.75mls of stacking gel acrylimide stock, 2.6mls H₂O, added to 30µl of 10% ammonium persulfate and 11µl TEMED. Stacking gel buffer consists of 6.05mls tris, 0.40g SDS, and 29.1mls 2M HCL and brought to 100mls with distilled H₂O (titrate to pH 6.8-7.0). The SDS electrode buffer consists of 6.05g tris, 28.84g glycine, and 2.00g SDS and bring up to 2000mls with distilled H₂O. The acrylamide stock was prepared using 14.6g acrylamide, 0.4g bis-acrylamide, and 36.0mls of distilled H₂O. The SDS- sample buffer consisted of 12.5g glycerol, 0.76g tris, 5.00mls 2-mercaptoethanol, 2.30g SDS, 12.3mls 0.5M HCL, and 71.0mls distilled H₂O (titrate to pH of 6.8-7.0). Coomassie stain was prepared by adding 225mls ethanol, 4g coomassie brilliant blue R-250 and stirred until dissolved completely with 225mls H₂O, and 50mls glacial acetic acid. Destain was made by adding 450mls ethanol, 450mls H₂O, and 100mls of glacial acetic acid.

The gels were composed of two types of acrylamide gels. The main gel is the resolving gel (sieving gel). On top of the resolving gel is a stacking gel which functions to concentrate the protein in the samples into thin zones, keeping the separating bands as thin as possible and thus improving resolution (Walker, 1997).

Mini slab gels were poured in sets of 10-12 gels in a casting chamber. Each gel was made in a glass sandwich consisting of two plates separated by two end spacers. The spacers determine the thickness of the gel, either 0.5 or 0.75mm thick gels were standard (Walker, 1997). The gels were assembled in the chamber. The resolving gel and overlay solution were also made, as previously indicated. The resolving gel was poured between the plates to about $\frac{3}{4}$ from the bottom of the glass plates. Then the overlay solution was gently pipetted over the resolving gel to insure that the top of the resolving gel will be straight across (Walker, 1997). The gel was then allowed to polymerize for at least an hour. The overlay solution is removed by aspiration and can be stored at 4°C for 1-2 months (Walker, 1997).

To run an SDS-Page gel, first a stacking gel was prepared and a comb was inserted to set up a standard well size. The gel was then overlaid with acrylamide, to insure the gel was sealed in place. The gel was allowed to polymerize for at least 30 minutes. Once polymerized, the comb was removed and the lanes in the stacking gel serve to accept the samples applied to them. The bottom and the upper chambers are filled with SDS-electrode buffer, making sure there are no bubbles trapped in the gel or wells (Walker, 1997). However, a bubble can be removed by pipetter fitted with a gel loading tip. About 1-10 μ l of sample was loaded into the wells using a pipetter with a loading tip. Molecular weight standards were used and are run in the end lanes, so that

the approximate molecular weights of proteins of interest can be determined (Walker, 1997). The electrode leads on the gel chamber are connected: red (+) to bottom electrode chamber and black (-) to the upper electrode chamber. These electrodes were also connected to a power source and run at 0.025 AMPS for about an hour and a half. The gel was then unloaded by prying apart the glass plates. At this time it is either stained or prepared for Immunoblot analysis. Immunoblot analysis discussion is reserved for the western blot section of materials and methods. To stain, the gel was placed in a petri dish with Coomassie stain for 15 minutes. It was then de-stained for about an hour. The gels were dried by placing them in a dialysis membrane overnight.

Samples recovered from the desalting column were labeled 1-30 as previously described. Ten fractions were selected to determine if Ab was present in them. This was accomplished by running the samples on an SDS-PAGE gel. Prior to loading the samples onto the stacking gel 20 μ l of sample from tubes 4-13 were placed in new microfuge tubes with 7 μ l 4x buffer. The following table indicates tubes labeled from desalting column in row 1, and lanes they were run on in the gel in row 2.

4	5	6	7	8	9	10	11	12	13
↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10

The results of the SDS-PAGE gel's from the purification of 7B6 and 6D2 can be seen in the results section. The antibodies were placed into new microfuge tubes based on the results of the SDS-PAGE gel. It was determined that tubes 9,10, and 11 of 7B6 contained the most antibody, these tubes were combined into a new 1.8 μ l microfuge

tube and labeled 7B6. This tube was used for all subsequent experiments. It was also determined that tubes 6,7, and 8 of 6D2 contained the most antibody, these tubes were combined into a new 1.8µl microfuge tube and labeled 6D2. This tube was used for all subsequent experiments.

D. Bradford assay

Protein concentrations were determined by a modification of the Bradford dye-binding procedure sold by Bio-Rad (Bradford, 1976). The Bio-Rad Protein Assay, is a simple colorimetric assay for measuring total protein concentration. The standard assay is used with samples having protein concentrations between 20-140 µg/ml . The Bio-Rad Protein Assay is based on the color change of Coomassie brilliant blue G-250 dye in response to various concentrations of protein. The dye binds to primarily basic and aromatic amino acid residues.

The protein standard used in our assay was an Immunoglobulin standard; IgG. The standard amounts in mg/ml included: 0,0.17,0.34,0.68,1.02,1.36. The standard assay which was run in test tubes included the following solutions; 990 µl H₂O (24 parts H₂O: 6 parts Bio-Rad dye) and 10 µl sample.

The Bradford assay was performed on the antibodies as follows. First, in a small beaker, 24 ml H₂O and 6 ml Bio-Rad dye were mixed together. Tubes were labeled for the standard protein samples and for the antibodies (7B6, 6D2). All of the samples were run in triplicate to generate a standard curve. To the labeled tubes, 10 µl of sample was placed in tubes along with 990µl of the dye mixture. The tubes were vortexed each time dye was added. The tubes were allowed to stand for 5 minutes and then read in a spectrophotometer at an optical density of 595 nm. The contents were pipetted out of the

tube and placed in a curvette tube in the spectrophotometer and then read. The protein assay generated a standard curve and from this we were able to determine the concentration of both 7B6 and 6D2, the data is in results section.

E. The ELISA assay

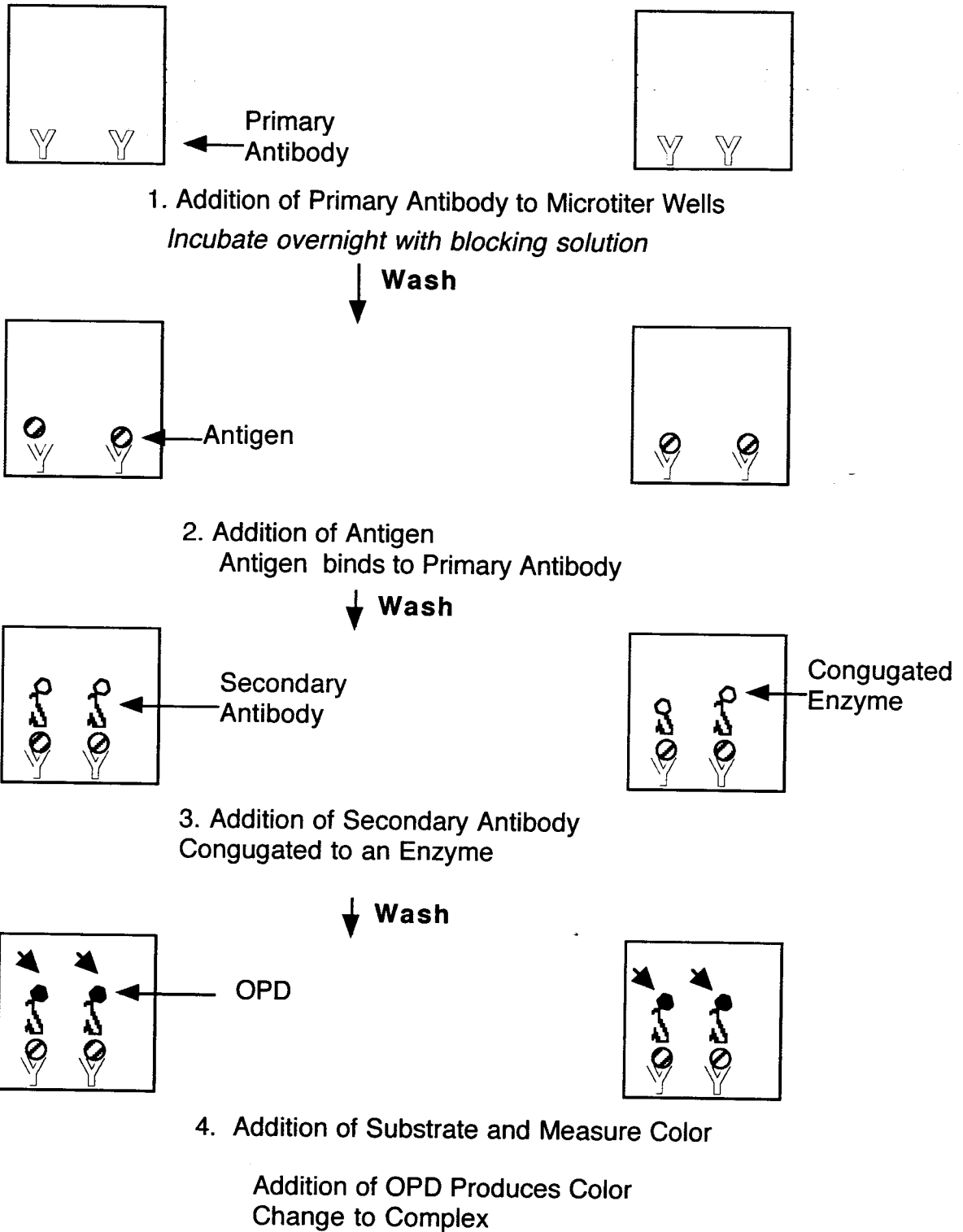
Enzyme Linked Immunosorbent Assay (ELISA) relies on a very specific interaction between a particular antigen molecule and an antibody. The ELISA used for the detection of β -glucuronidase was a sandwich ELISA. A sandwich ELISA allows for the detection or quantitation of an antigen. This technique involves a primary antibody being applied to a plate first and then the antigen, followed by a secondary antibody conjugated to an enzyme for color detection. The purpose of this procedure was to quantitate the absolute concentration of human β -glucuronidase in a Vohwinkel's syndrome patient. Figure 4 depicts how the sandwich ELISA was run. The procedure was optimized by Dr. Ho to only recognize human β -glucuronidase, not any bacterial forms of the enzyme.

To run an ELISA the following solutions were used. A 3% BSA/PBS with 0.02g of sodium azide, was the blocking solution, it blocked all unbound sites on the wells of the plate after the primary antibody was applied to the plate. The solution was prepared by adding 3 grams of bovine serum albumin (BSA) in 100 ml of 1X PBS (10ml of 10X stock buffer in 90ml of distilled H₂O). To this mixture 0.02 grams of sodium azide are added and the solution is mixed with a magnetic stirring apparatus. A solution of PBS with 0.0005% Tween 20, served as the detergent and was used for all washes in between the incubations. This solution was made up in large quantities due to the amount that was needed. Preparation included: 500 ml of wash solution; 450 ml of distilled H₂O, 50

ml of 10X stock PBS, mixed with 2.5 ml of Tween 20. A 3% BSA/PBS solution, was used to obtain the appropriate concentrations of 6D2 and Streptavidin-HRP. Adding 3 grams of BSA in 100 ml of 1X PBS (10 ml of 10X PBS and 90 ml of distilled H₂O). A freshly prepared substrate solution was used to create a color change that could be measured by an ELISA reader. It was prepared by adding 25 ml of phosphate citrate buffer at a pH of 5 (1.2g/ 100 ml of sodium phosphate and 2.94g/100 ml of sodium citrate were added to 100ml of 1X PBS this was stored in the refrigerator) to this 10mg of OPD an 10 μ l of H₂O₂ was added at the appropriate time. A 2.5M H₂SO₄ stop solution was prepared. It was prepared by adding 2.45g/100ml of 1X PBS. The antibodies were also placed in solutions for the ELISA. 7B6 had a concentration of 1.31 mg/ml, 20 μ g/ ml concentration was needed: $1.31/0.02= 64.5$. The 7B6 was prepared in 4ml amounts which was determined as sufficient amounts for 60 wells; $4\text{ml}/64.5=62.0 \mu\text{l}$ in 3.999mls of 1X PBS. 6D2 had a concentration of 4.99 mg/ml, 10 μ g/ml was needed: $4.99/0.1= 499$. The 6D2 was prepared in 4ml amounts as well; $4\text{ml}/499=8.0 \mu\text{l}$ in 3.998 mls of 3%BSA/PBS. Streptavidin-HRP had a concentration of 1.0 mg/ml and 10mg/ml was needed: $1.0/0.1=10\mu\text{l}$. The Streptavidin-HRP was prepared in 4ml amounts; $4\text{ml}/10= 4.0\mu\text{l}$ in 4.0 ml of 3% BSA/PBS.

Figure 4 is a schematic drawing depicting the double antibody sandwich ELISA used in this study, as outlined by Dr. Ho (1992). The squares are representative of the reactions occurring in each well of the polystyrene microtiter plate.

Double Antibody Sandwich ELISA



All blood, and urine samples (obtained on three separate occasions) were examined in triplicate by the ELISA. Due to the nature of the process under which the skin punch biopsies were obtained, the amount of this tissue was limited. Therefore, skin was analyzed at one time. All samples obtained for the ELISA protocol were run in triplicate lanes to ensure the average was statistically viable.

In a 96 well high binding polystyrene microtitration plate 50µl of the first monoclonal antibody was added to the wells, 7B6 IgG (20 µg/ml in PBS). The plate was incubated for one hour. All incubations were all carried out at room temperature. The plates were washed four times with 1X PBS in between all incubations. The wells were then filled to the top with 3% BSA/PBS containing 0.02% sodium azide (blocking solution), and incubated overnight, at this point the plate can be frozen until future use by removing the blocking solution, and storing at -20°C. The microtiter plate was removed from storage, and washed four times with PBS with 0.0005% Tween 20. 50 µl of specimen was then added to the wells, and the plate was incubated for 2 hours. It was recommended that a serial 2-fold dilution with 3% BSA/PBS should be made undiluted urines, seras, or other body fluids. So, a two-fold serial dilution was performed on all samples. 50µl/well of biotinylated 6D2 IgG (10 µg/ml) in 3% BSA/PBS was added, and incubated for 1 hour. 50 µl/well of Streptavidin-HRP (10 µg/ml) in 3% BSA/PBS was added, and incubated for 2 hours. 50µl/well of freshly prepared substrate solution (10 mg OPD in 25 ml phosphate citrate buffer, pH 5, with 10µl of 30% H₂O₂) was added and incubated for 20 minutes. The reaction was stopped by adding 50 µl/well of 2.5M H₂SO₄ and the color developed is read at a wavelength of 490 nm in a microtiter reader.

After the data was received from the ELISAs, it was quantitated using a Statistical program and demonstrated via graphs. The graphs displayed were all generated via Word Perfect's Quattro Pro Data analyzer version 6. All data was entered into this spreadsheet program and analyzed for the averages, standard deviations, and paired student t-tests. The averages collected were graphed by 2-D linear line graphs and can be seen in the result section.

F. Immunoblot Analysis- Western Blot

This technique also employed the use of monoclonal antibodies. It involved the electrophoretic transfer of protein bands to a PVDF membrane. Once transferred to this membrane, the proteins were further analyzed by probing with the primary monoclonal antibody 7B6 and an anti-goat secondary antibody. Chemiluminescent methods were then utilized for visualization of the blots via an autoradiographic piece of film. The purpose of this procedure was intended to be both quantitative and qualitative in nature. However, the antibodies were not as sensitive for this type of detection, and quantitation was not possible. It did provide qualitative evidence, enabling visualization of the antibody bound β -glucuronidase complex.

The solutions used in a western blot were prepared. Tris buffered salt (TBS) consisted of 58.44g of 0.5M NaCl, 20mM Tris base, adjusted to a pH of 7.5. A blocking solution of: 5g of 5% Non-fat dry milk, and 100ml of TBS-T. TBS-T consisted of 2mls of Tween-20 and 1000mls of TBS. A transfer buffer was prepared: 57.6g of glycine, 12.1g tris, 800ml of methanol, and 3200ml of H₂O. Lastly, the antibody solution consisted of: [1:1000 dilution] 16mls of TBS, mls of blocking solution, for a total volume of 20ml and 20 μ l of 1^oantibody. The secondary antibody solution [1:3000 dilution] was

prepared by adding 22.5mls of TBS, 7.5mls of blocking solution, for a total volume of 30ml and 10 μ l of goat anti-mouse antibody. For chemiluminescent detection, a 1:1 dilution was prepared : 5mls of 4-chloronaphthol methanol-cold [luminol]and 5mls of 30% hydrogen peroxide was needed.

First, an SDS-PAGE gel of β -Glucuronidase was run from samples of both the Vohwinkel's Syndrome and unaffected patients. The main or resolving gel was poured in advance. The stacking gel was prepared individually at the time of use. The stacking gel served to load the proteins, the separation occurred in the resolving gel. The bottom and upper chambers of the gel apparatus were filled with SDS electrode buffer. 1-30 μ l of protein sample were loaded into each well using a pipetter. A molecular weight standard was included in each gel. The standard serves as a marker for approximate molecular weights. Next, the electrodes were then attached: the red (+) lead to the bottom chamber and (-) to the upper chamber. The leads were connected to the power supply, and the gel was run at 0.25 Amps. The gels were run for about an hour, watching to ensure the dye front didn't run off the gel. After the dye front reached the bottom, running conditions were complete.

The proteins were then electrophoretically transferred to PVDF blotting paper using the Genie apparatus. Genie is a semiwet blotting apparatus. The Genie tray was assembled from the bottom upwards. The negative electrode was attached to the bottom. Transfer buffer was added to the level of the Scotch Brite pad, being Careful to avoid air bubbles. At the same time, the membrane was hydrating in a small amount of transfer buffer, prior to placing it in its position in the apparatus. The gel was placed in the apparatus backwards to the way in which it was loaded. The membrane was placed on

top of the gel. The filter paper was placed on top of the membrane. Followed by a Scotch Brite pad and bubble screen. Finally, the plexiglass cover and the electrodes are connected. The Genie apparatus was placed into running position and topped off with transfer buffer. The apparatus was run at 24 volts for approximately thirty minutes to one hour. The blot was ready for further processing using antibodies. Subsequently, the gel was stained with Coomassie Brilliant Blue to make sure that all the protein bands recognized specifically by the antibodies were transferred to the membrane.

Immunodetection of B-Glucuronidase was then carried out using a mouse anti-human primary antibody and a goat anti-mouse secondary antibody. The immunoblotting procedure was performed as follows: The PVDF blot was placed in blocking solution and incubated with gentle shaking for 1 hour at room temperature. After blocking, incubation with the primary antibody, 7B6 was carried out for 1 hour with gentle shaking at room temperature. Next, all unbound Ab was washed from the PVDF blot. The blot was washed 3 times, for 5 minutes in 100 ml of TBS-T with gentle agitation. Then, the blot was incubated for 1 hour at room temperature with a secondary antibody goat anti-mouse conjugated to horse-radish peroxidase. The PVDF blot was washed 3 times, for five minutes each in 100 ml of TBS-T and then two times with TBS. This was to ensure all of the secondary Ab was removed from the blot. The detection was carried out via chemiluminescent means.

The visualization of the blot requires chemiluminescent detection. Preparation included mixing 1 part [luminol] 4-chloronaphthol methanol (cold) with 1 part 30% Hydrogen peroxide in TBS. The 1:1 ratio consisted of 5ml of luminol and 5ml of Hydrogen peroxide. The blot was incubated in the mixture for 1 minute. The rest of the

procedure was performed in the dark. The blot was placed between a plastic sheet and all bubbles were squeezed out. The blot was aligned with luminescent paint marks on the plastic to aid in orienting the blot. The blot was then exposed to a piece of x-ray film (time varies from 2seconds to 30 seconds), by laying the film of top of the blot. The film was placed in a container with GB-X developer by Kodak for about 1 minute, where any bands were able to be visualized. Next, the film was placed in Indicator Stop Bath for Kodak 30 seconds. The film was removed from the stop bath and placed into Fixative by Kodak for about 5 minutes. The film was then washed thoroughly with water for 20 minutes and air dried. Subsequently, the PVDF blot was stained in Coomassie Brilliant Blue to compare to the results we see on film.

The audioradiographic film was then scanned into a computer, the brightness and contrast levels were adjusted. The scanned image was then subjected to quantitation using NIH Imager. This allowed the quantitation of the bands via a densatometer. The Vohwinkel's diseased bands were compared to non-diseased bands. The resulting graphs can be seen in the results section.

Results

I. Antibody Preparation and Characterization

A. Gel Filtration Chromatography and SDS-PAGE

Gel filtration was used to 'desalt' the antibodies and thereby purifying them. The results of the Gel filtration chromatography as analyzed by included SDS-PAGE gels that enabled visualization of the recovered antibodies from the various aliquots. The gels were used to determine the location of the antibodies in the chromatography fractions. Figure 5a shows the gel that was run on the primary monoclonal antibody, 7B6. From this gel it was determined that tubes 9, 10, and 11 contained the most antibody. The tubes were combined together and placed into a new 1.8ml microfuge tube and labeled 7B6, this was used for all subsequent experiments. Figure 5b shows the gel that was run on the secondary monoclonal antibody, 6D2. From this gel it was determined that tubes 6, 7, and 8 of 6D2 contained the most antibody. The tubes were combined together into a new 1.8ml microfuge tube and labeled 6D2, this was used for all subsequent experiments.

Both of these SDS-PAGE gels were run following gel filtration and “desalting” of both monoclonal antibodies. 7B6, the primary antibody is depicted in figure 5a. Lanes 6,7 and 8 were determined to contain adequate amounts of protein and were used as stock for further experiments. 6D2, the secondary antibody is depicted in figure 5b. Lanes 3, 4, and 5 were determined to contain adequate amounts of protein and were used as stock for all subsequent experiments.

Both gels show what fraction the protein is located in, and also that the antibodies are rather pure. Both heavy chains and light chains can be seen, with nothing in between.

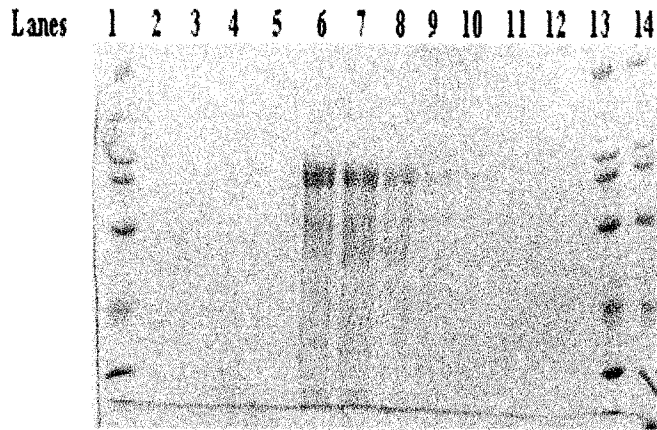


Figure 5a

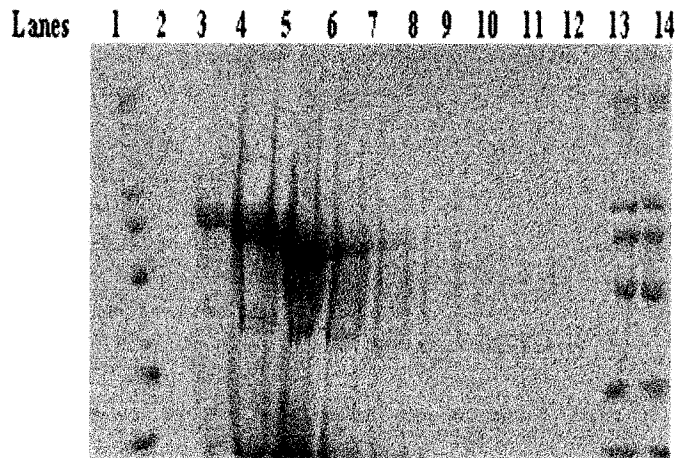


Figure 5b

B. Bradford Assay

Protein (antibody) concentrations of selected fractions were determined by the Bradford assay . The data from the standard Immunoglobulins were entered into Cricket and graphed on a scatter plot where linear regression was performed. The results from the values of the experiment are as follows: There was a 1:2 dilution performed on 7B6 and using the formula above the concentration was calculated to be 1.31 mg/ml. There was a 1:10 dilution performed on 6D2 and using the same formula the concentration was calculated to be 4.98 mg/ml. It was important that this procedure was performed prior to the running of any ELISA. The concentration of both 7B6 and 6D2 had to be determined to ensure that the proper amount was administered in all subsequent experiments.

II. Quantification of β -Glucuronidase levels in Blood, Urine, and Skin via an ELISA

A. ELISA

Standardization of the antibodies activity was determined by an ELISA in which standard β -Glucuronidase stock enzyme (provided by Dr. Sly at St. Louis University) was run in place of the antigens being tested. The protocol was carried out in the same manner and a standardized curve was generated. The graph is shown in figure 6.

The specific aim of this study was to quantitate the levels of human β -glucuronidase in a Vohwinkel's Syndrome patient and compare them to a standard (unaffected) patient. The hypothesis stated was that the levels of β -glucuronidase would be higher in the Vohwinkel's Syndrome patient, and the main way in which the quantification would be carried out was through a double antibody sandwich ELISA. Thus, the following graphs will demonstrate the results. There were three blood and three urine samples collected from both the diseased and non-diseased patients involved. Each time a sample was run, it was done in triplicate serial dilution's. The results of each plate were printed out by the ELISA reader. These numbers were then gathered and averaged together. The samples are arranged: blood 1, blood 2, and blood 3. Urine 1, urine 2, and urine 3. Due to the fact that the amount of epidermal tissue was limited, it was analyzed all at once. The results are arranged as: Vohwinkel's syndrome patient and skin non-affected patient.

Overall, the blood samples taken and analyzed supported the hypothesis that the levels of β -glucuronidase were higher in the Vohwinkel's Syndrome Patient.

The graph depicted in figure 6 was generated from the data collected using a stock standard enzyme (provided by Dr. William Sly of the University of St. Louis School of Medicine) in the double antibody sandwich ELISA as outlined by Dr. K.J. Ho (1992). The absorbance values were always read at 490nm. The standard enzyme was then used as a positive control for all subsequent ELISA experiments.

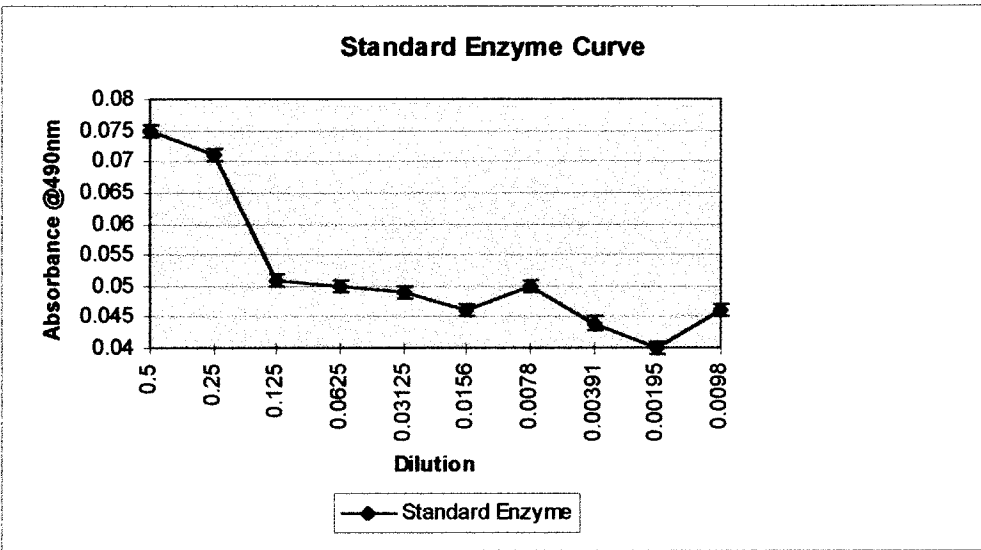


Figure 6

There were 3 blood samples taken from both the Vohwinkel's Syndrome patient and the non-affected patient. The samples were analyzed by the ELISA method. Each sample was run in triplicate. The optical density for each triplicate sample was then averaged. The averages appear on the following tables. Table 2a depicts data for non-affected blood samples. Table 2b depicts data for the Vohwinkel's Syndrome patient. Both positive and negative controls were included. The provided stock standard enzyme was used for the positive control. In the last well of the microtiter plate, no antigen (sample containing β -glucuronidase) was added. The samples were then diluted, using a serial dilution from 1:2 to 1:1024. The data was then graphed in figure 7.

Table 2a

Table of Results for Non-affected Blood Samples

In µg/ml

	Serial Dilution's											
	Positive Standard Enzyme 1:2	OD1	OD2	OD3	OD4	OD5	OD6	OD7	OD8	OD9	OD10	Blank (No Ag in well)
Trial 1	0.72	0.067	0.061	0.058	0.059	0.057	0.053	0.053	0.049	0.058	0.058	0.039
Trial 2	0.67	0.073	0.064	0.061	0.061	0.065	0.060	0.071	0.067	0.058	0.057	0.041
Trial 3	0.71	0.067	0.066	0.065	0.065	0.060	0.060	0.063	0.063	0.065	0.056	0.037

Table 2b

Table of Results for Vohwinkel's Syndrome Patient Blood Samples

In µg/ml

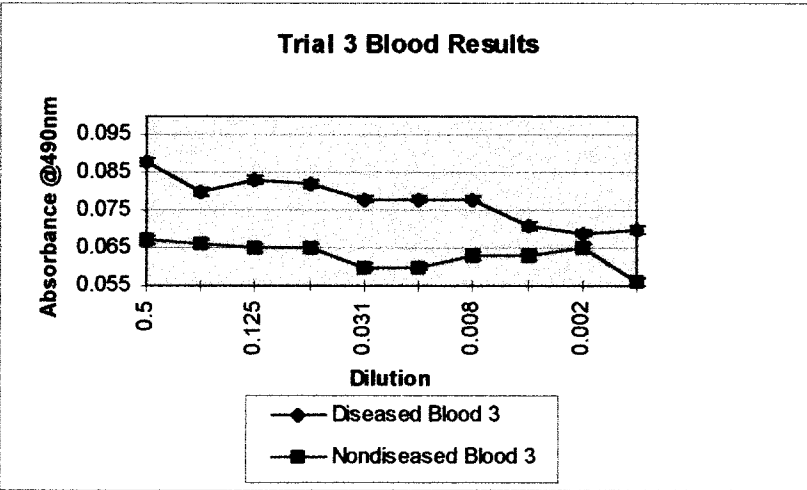
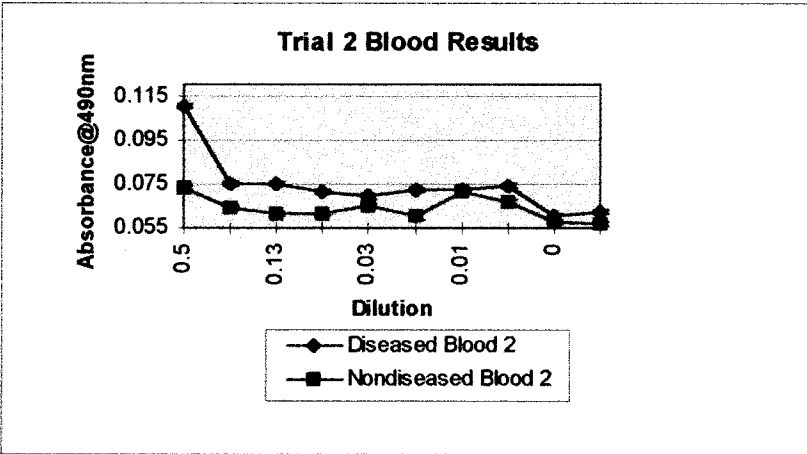
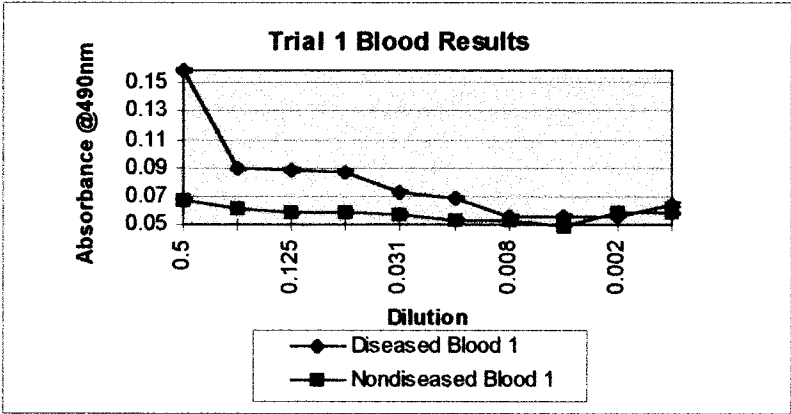
	Serial Dilution's											
	Positive Standard Enzyme 1:2	OD1	OD2	OD3	OD4	OD5	OD6	OD7	OD8	OD9	OD10	Blank (No Ag in well)
Trial 1	0.72	0.159	0.089	0.088	0.087	0.073	0.069	0.056	0.056	0.056	0.064	0.0037
Trial 2	0.69	0.110	0.075	0.075	0.071	0.069	0.072	0.072	0.074	0.060	0.062	0.042
Trial 3	0.71	0.088	0.080	0.083	0.082	0.078	0.078	0.078	0.071	0.069	0.070	0.039

The levels were consistently higher in the three samples. This can be seen in table 2a and table 2b. Table 2a shows the table of results for the averages of the non-affected blood samples that were collected. Table 2b shows the results for the averages of the diseased samples that were collected. To show comparisons directly, the data was graphed together. The samples analyzed were done in two-fold serial dilution's. The initial Vohwinkel's syndrome reading at a 1:2 dilution is $0.160\mu\text{g/ml}$ and non-diseased at $0.067\mu\text{g/ml}$. The affected value was about two times larger. Figure 7 shows a direct comparison of the blood results analyzed from the samples taken. It can clearly be seen that the absorbance readings were higher in the Vohwinkel's sample than in the unaffected sample.

The second blood samples obtained from were run under the same ELISA conditions. The 1:2 dilution value was $0.110\mu\text{g/nm}$, while the same dilution for the non-diseased sample was $0.073\mu\text{g/nm}$. The Vohwinkel's absorbance values are again, higher.

The third blood samples obtained from both were again run under the same ELISA conditions. This sample shows that the Vohwinkel's sample was again higher in its concentration of β -glucuronidase, but it was not as high as it was in the previous samples obtained. The 1:2 dilution value of the third blood sample was $0.088\mu\text{g/nm}$ for the Vohwinkel's sample and $0.067\mu\text{g/nm}$ for the unaffected sample. The graphs demonstrate that the diseased serum values were consistently higher than the non-diseased values, thus supporting the studies original hypothesis.

Figure 7. The following graphs depict the averaged data for each of the three blood samples obtained from both the Vohwinkel's Syndrome patient and the non-affected patient. The ELISA plates were all read at an optical density of 490nm. The dilution's, again, were serial dilution's from 1:2 to 1:1024.



Urine samples were also obtained on three separate occasions from both Vohwinkel's and unaffected patients. Each sample, again, was run under the same ELISA conditions. The overall average's of results obtained from these samples are shown in tables 3a and 3b. Table 3a refers to the averages obtained for the three unaffected urine samples. It should be noted that although the second sample appears quite high in the unaffected urine, that the Vohwinkel's sample was still higher. It should be noted that the urine levels don't seem to be as consistent with serum samples, and that it is probably due to the fact that the enzyme becomes less dilute and predictable in urine secretion. Camisa et al originally hypothesized that the high levels in serum were probably due to the epidermal leakage in capillaries. Indeed, the serum levels were higher in the Vohwinkel's diseased patient as Camisa noted in 1988. However, it was also thought that the levels would be increased in urine. If this was true, then another question should be posed: Is hyperkeratosis of the urinary tract also involved? Since, this study showed that over three random urine samples the levels of β -glucuronidase were higher, maybe that issue should be considered in future studies.

Figure 8 demonstrates graphically the results from the urine samples from tables 3a and 3b. The initial 1:2 dilution again confirms that the Vohwinkel's sample at $0.120\mu\text{g}/\text{nm}$ is much higher than the unaffected sample $0.088\mu\text{g}/\text{nm}$. The levels of β -glucuronidase seemed to be higher overall from the first samples. Again, the second values for the 1:2 dilution were $0.172\mu\text{g}/\text{nm}$ for the Vohwinkel's sample while the unaffected value was $0.128\mu\text{g}/\text{nm}$.

The third urine sample obtained again supported our hypothesis, although the values are not that high in either sample. Here, the 1:2 dilution for the Vohwinkel's patient was $0.072\mu\text{g}/\text{nm}$, while the unaffected was $0.061\mu\text{g}/\text{nm}$. Figure 8 shows that the absorbance values were consistently higher over the three trials. Thus, supporting the hypothesis that the Vohwinkel's Syndrome patients urine β -glucuronidase levels were higher than the unaffected levels.

There were 3 urine samples taken from both the Vohwinkel's Syndrome patient and the non-affected patient. The samples were analyzed by the ELISA method. Each sample was run in triplicate. The optical density for each triplicate sample was then averaged. The averages appear on the following tables. Table 3a depicts data for non-affected urine samples. Table 3b depicts data for the Vohwinkel's Syndrome patient. Both positive and negative controls were included. The provided stock standard enzyme was used for the positive control. In the last well of the microtiter plate, no antigen (sample containing β -glucuronidase) was added. The samples were then diluted, using a serial dilution from 1:2 to 1:1024. The data was then graphed in figure 8.

Table 3a

Table of Results for Non-affected Urine SamplesIn $\mu\text{g/ml}$

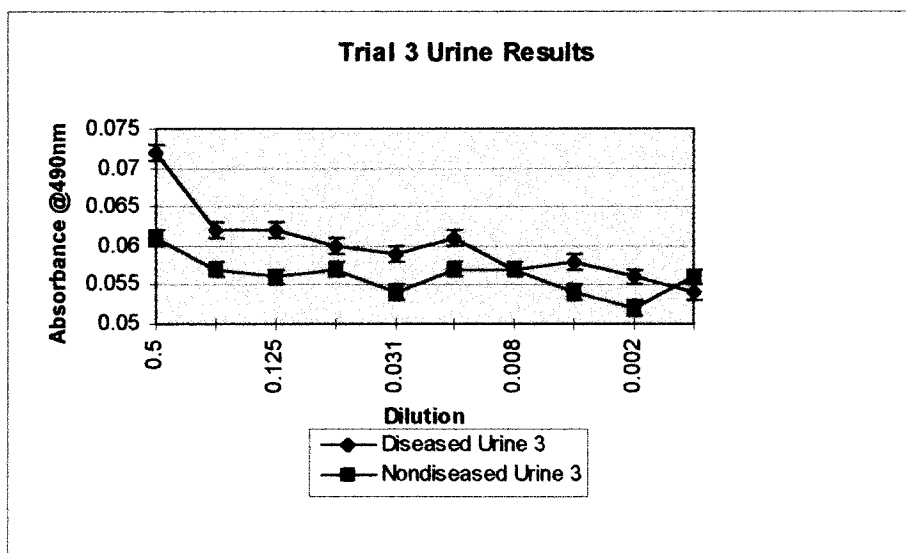
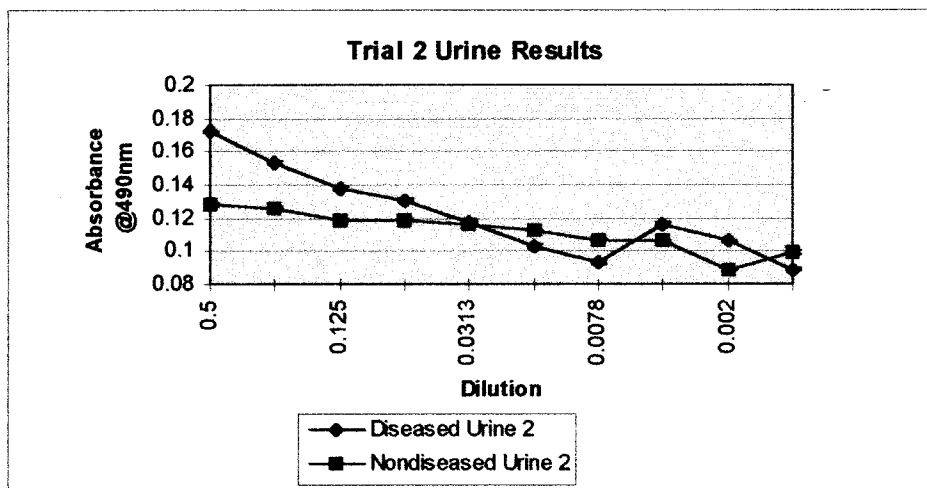
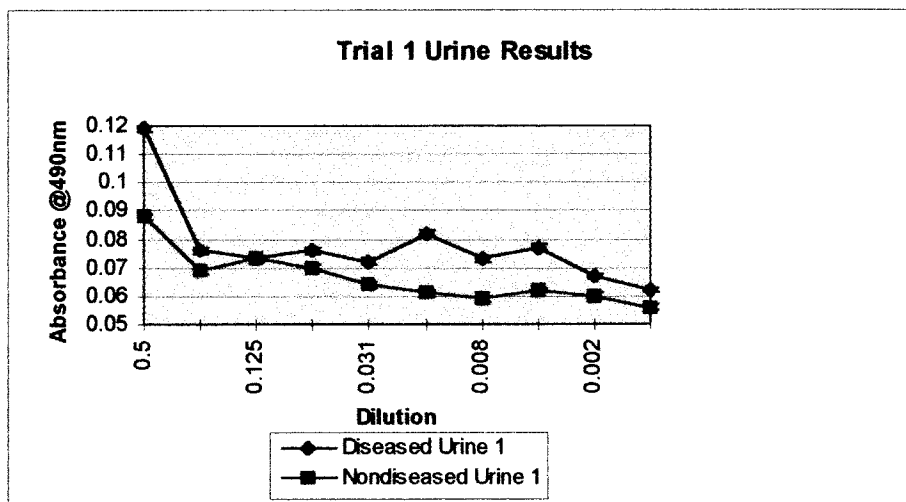
	Positive Standard Enzyme 1:2	Serial Dilution's										Blank (No Ag in well)
		OD1	OD2	OD3	OD4	OD5	OD6	OD7	OD8	OD9	OD10	
Trial 1	0.72	0.088	0.069	0.073	0.070	0.064	0.061	0.059	0.062	0.060	0.056	0.036
Trial 2	0.68	0.128	0.126	0.119	0.119	0.116	0.112	0.107	0.106	0.089	0.099	0.042
Trial 3	0.72	0.061	0.057	0.056	0.057	0.057	0.054	0.057	0.054	0.052	0.056	0.038

Table 3b

Table of Results for Vohwinkel's Patients Urine SamplesIn $\mu\text{g/ml}$

	Positive Standard Enzyme 1:2	Serial Dilution's										Blank (No Ag in well)
		OD1	OD2	OD3	OD4	OD5	OD6	OD7	OD8	OD9	OD10	
Trial 1	0.71	0.119	0.076	0.073	0.076	0.072	0.082	0.073	0.077	0.067	0.062	0.043
Trial 2	0.73	0.172	0.153	0.138	0.117	0.103	0.093	0.116	0.116	0.106	0.089	0.041
Trial 3	0.72	0.072	0.062	0.062	0.060	0.059	0.061	0.057	0.058	0.056	0.054	0.039

Figure 8. The following graphs depict the averaged data for each of the three urine samples obtained from both the Vohwinkel's Syndrome patient and a non-affected patient. The ELISA plates were all read at an optical density of 490nm. The dilution's, again, were serial dilution's from 1:2 to 1:1024.



Epidermal samples were obtained by a 4mm skin-punch biopsy performed by Dr. Brodell. Due to the procedure involved, there was a limited amount of tissue to analyze and all of the ELISAs were run following cryostat sectioning. The original hypothesis that Camisa et al introduced in 1988 was tested through this study. Was the epidermal levels of β -glucuronidase that much higher in Vohwinkel's patients that it was actually spilling over from the capillaries into the serum? The results of the ELISAs run on both the Vohwinkel's patient and the normal patient can be seen in tables 4a and 4b. This data was then subsequently converted into graphs and can be seen in figure 9. The levels of β -glucuronidase was substantially higher in the Vohwinkel's Syndrome patient, with absorbance values as high as 0.366 μ g/nm for the 1:2 dilution, while the unaffected value was only 0.130 μ g/nm. The data convincingly supports the hypothesis that the levels of β -glucuronidase were elevated in a Vohwinkel's Syndrome Patient and that the increased levels in the serum and in the urine could be due to epidermal spilling over.

To statistically compare the data obtained from this study, a paired t-test was performed on blood, urine, and skin from Vohwinkel's syndrome vs. unaffected individuals. To perform these statistical evaluations the mean and standard deviation for each trial were needed. This information was determined using data analysis tools on Quattro Pro version 6. This information was then used again in Quattro Pro to provide a value for the paired student t-test. All of the data derived can be seen in Table 5. In particular, the 1:2 serial dilution was examined for statistic viability. Next, the degree's of freedom were calculated

using the formula $[N-1=n]$, where N= the count and n= value used in the t-test formula. This number is then referenced in a significant limits of students t distribution, which can be found in any statistics book. A value above 2.101 at a 95% confidence level for a student t-test would statistically confirm that the data is not similar, as would be expected for the levels of β -glucuronidase in a Vohwinkel's syndrome patient vs. an unaffected individual. It can be seen in table 5 that all of the values determined by the student t-test in this study were above 2.101 and are therefore not similar.

There were skin punch biopsy samples taken from both the Vohwinkel's Syndrome patient and the non-affected patient. The samples were analyzed by the ELISA method. Each sample was run in triplicate. The optical density for each triplicate sample was then averaged. The averages appear on the following tables. Table 4a depicts data for non-affected blood samples. Table 4b depicts data for the Vohwinkel's Syndrome patient. Both positive and negative controls were included. The provided stock standard enzyme was used for the positive control. In the last well of the microtiter plate, no antigen (sample containing β -glucuronidase) was added. The samples were then diluted, using a serial dilution from 1:2 to 1:1024. The data was then graphed in figure 9.

Table 4a

Table of Results for Non-affected Skin Samples

In µg/ml

	Serial Dilution's											
	Positive Standard Enzyme 1:2	OD1	OD2	OD3	OD4	OD5	OD6	OD7	OD8	OD9	OD10	Blank (No Ag in well)
Trial	0.73	0.130	0.128	0.126	0.120	0.113	0.104	0.103	0.096	0.094	0.092	0.047

Table 4b

Table of Results for Vohwinkel's Patients Skin Samples

In µg/ml

	Serial Dilution's											
	Positive Standard Enzyme 1:2	OD1	OD2	OD3	OD4	OD5	OD6	OD7	OD8	OD9	OD10	Blank (No Ag in well)
Trial	0.74	0.366	0.363	0.358	0.355	0.355	0.349	0.347	0.338	0.277	0.243	0.049

Figure 9. The following graph depicts the averaged data for skin punch biopsies obtained from both the Vohwinkel's syndrome patient and the non-affected patient. The ELISA plates were all read at an optical density of 490nm. The dilution's, again, were serial dilution's from 1:2 to 1:1024.

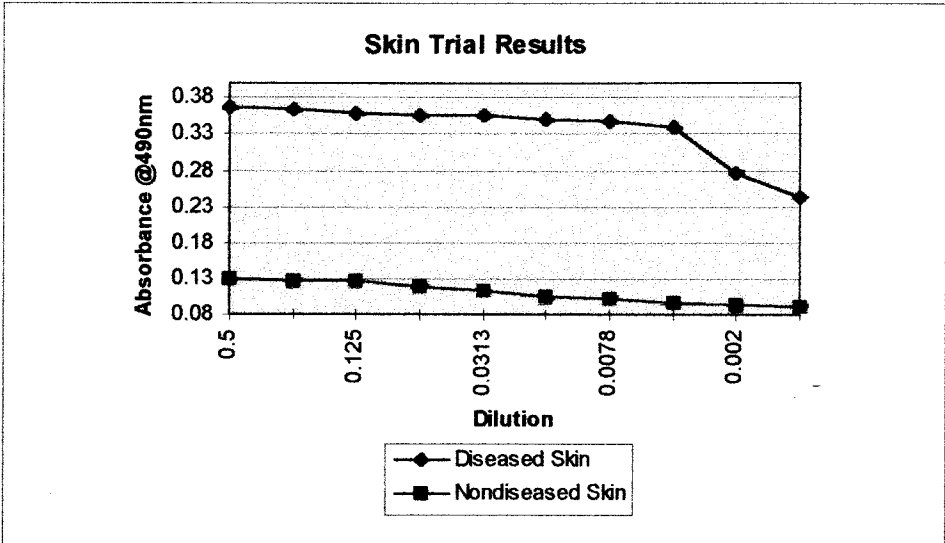


Table 5

Table of Statistical Correlation					
<u>Sample</u>	<u>Mean OD</u>	<u>Vohwinkel's Patient Standard Deviation</u>	<u>Mean</u>	<u>Non-affected Patient Standard Deviation</u>	<u>T-Test Result</u>
Blood 1	0.797	0.03081	0.0573	0.04923	2.63
Blood 2	0.074	0.013663	0.0637	0.005355	3.14
Blood 3	0.0777	0.00613	0.063	0.003399	9.04
Urine 1	0.0777	0.015521	0.0662	0.009354	4.03
Urine 2	0.1121	0.012133	0.1218	0.026678	2.31
Urine 3	0.0601	0.004932	0.0558	0.0024855	3.01
Skin-Punch Biopsy	0.3351	0.0411648	0.1106	0.014706	16.24
Standard Enzyme	0.0528	0.10922			

Paired Student T-tests were performed between the diseased and non-diseased samples obtained. Remembering that if the value obtained from the T-test is above 2.101, that the data is rejected as NOT being similar. If the value is below 2.101, then the data is accepted as being similar.

III. Quantification of β -Glucuronidase by SDS-PAGE and Immunoblotting

A. Western Blot Analysis

The purpose of this part of the study was to quantitate the levels of β -glucuronidase in both a Vohwinkel's syndrome patient and an unaffected patient. The double antibody sandwich ELISA provided a semi-quantification of the concentration of β -glucuronidase. Next, the samples were prepared and run on an SDS-PAGE gel and then electrophoretically transferred to a PVDF blot. The blot allowed us to take advantage of the monoclonal antibodies and detect only where β -glucuronidase was bound.

The gels were run on blood, urine, and skin samples. The samples were dissolved and solubilized in 1x sample buffer. Again, the gels were transferred to PVDF blots. The samples were then visualized through chemiluminescent detection and captured on x-ray film. The film was then scanned into a computer and analyzed via the NIH Imager, which essentially is a densitometer. This provided us with a direct quantification of the bands of samples analyzed.

The process of acquiring a gel with suitable bands to analyze using Immunoblot analysis proved tricky. Samples consisted of: a solubilized punch in 1x sample buffer, supernatant retrieved from mashing of the punch in 1X sample buffer, plasma in 1X sample buffer, and urine in 1X sample buffer. The original samples were diluted 1:1 with 1x sample buffer and run on a gel. The stacking gel was poured with a 14 well comb. The plasma, solubilized skin and the skin supernatant for both the diseased and non-diseased was overloaded (data

not shown). The samples were then run in lower amounts: skin volume of 5 μ l, plasma volume of 10 μ l, and urine volume of 15 μ l. The levels of the solubilized skin, skin supernatant, and plasma were again overloaded. This can be seen in figure 10. The samples were then diluted 1:10 with 1X sample buffer. The gel was loaded this time with 1 μ l of solubilized skin (KHM and non-affected), 1 μ l of skin supernatant (KHM and non-affected), 5 μ l of plasma (KHM and non-affected), and 30 μ l of urine (KHM and non-affected). The stacking gel was prepared using a 10 well comb. These amounts worked well and banding was achieved, as seen in figure 11. The same conditions were repeated and this time instead of Coomassie staining, the gel was electrophoretically transferred to a PVDF blotting paper. This blot was then prepared for chemiluminescent detection and then exposed to film in the darkroom. The film was first exposed at 30 seconds, and although banding was achieved there was a high level of background noise. The blot was re-exposed to film at 10 seconds and the noise was greatly reduced, figure 12. Thus confirming that the antibodies were in fact binding to β -glucuronidase. The film, also called an autoradiograph, was then scanned into a computer and subjected to NIH Imager. The program considered all the protein in the bands from the blot: both diseased and non-diseased for the serum samples. It confirmed that the β -glucuronidase was more concentrated in the Vohwinkel's Syndrome diseased patient, figure 13. The diseased band of interest registered 771.0 and the non-diseased band registered 703.0. Thus again confirming the hypothesis that the levels of β -glucuronidase in a Vohwinkel's syndrome patient were higher than the non-affected patient.

Figure 10 is a scanned image of an SDS-PAGE gel that was stained with Coomassie Brilliant Blue. The gel was obviously overloaded with protein sample.

Lane 1: Molecular weight standard
Lane 2: Non-diseased dissolved skin punch (5 μ l)
Lane 3: Non-diseased skin supernatant (5 μ l)
Lane 4: Diseased dissolved skin punch (5 μ l)
Lane 5: Diseased skin supernatant (5 μ l)
Lane 6: Non-diseased serum plasma (10 μ l)
Lane 7: Diseased serum plasma (10 μ l)
Lane 8: Non-diseased urine (15 μ l)
Lane 9: Diseased urine (15 μ l)
Lane 10-14: Blank

Figure 11 is a scanned image of a SDS-PAGE gel that was duplicated for the western blot, this time with the appropriate amount of protein sample loaded, and yielding good bands.

Lane 1: Molecular weight standard
Lane 2: Non-diseased dissolved skin punch (1 μ l)
Lane 3: Non-diseased skin supernatant (1 μ l)
Lane 4: Diseased dissolved skin punch (1 μ l)
Lane 5: Diseased skin supernatant (1 μ l)
Lane 6: Non-diseased serum plasma (5 μ l)
Lane 7: Diseased serum plasma (5 μ l)
Lane 8: Non-diseased urine (30 μ l)
Lane 9: Diseased urine (30 μ l)
Lane 10-14: Blank

Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14

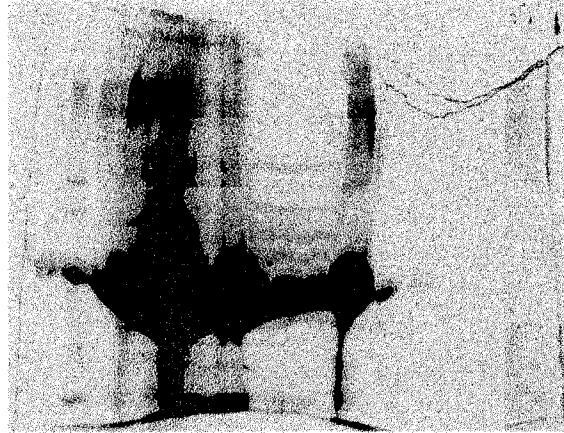


Figure 11

Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14

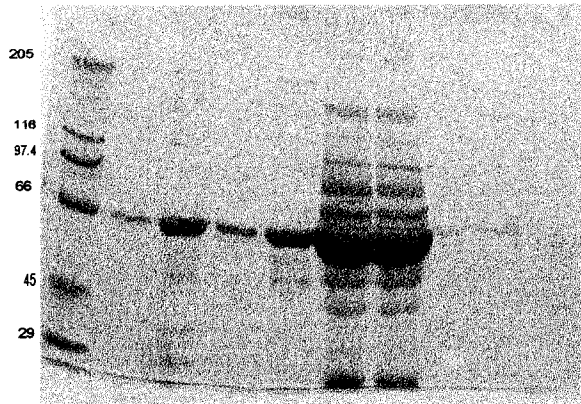


Figure 12

Figure 12

The gel pictured in figure 11 was also subjected to western blot analysis. Western blot analysis was performed using the monoclonal antibody specific for β -glucuronidase. The resultant PVDF blotting paper would only contain bands where β -glucuronidase was located, not all the protein that was present in the gel. The blot was subjected to autoradiography and is depicted in figure 12. The autoradiograph was exposed for about 10 seconds. Only the bands corresponding to the blood serum samples were apparent, they occur at about 103 daltons, as expected. The skin and urine samples were not visualized in the autoradiograph due to the poor binding of the secondary antibody with PVDF.

Lane 1: band corresponds with non- affected serum

Lane 2: band corresponds with Vohwinkel's syndrome serum

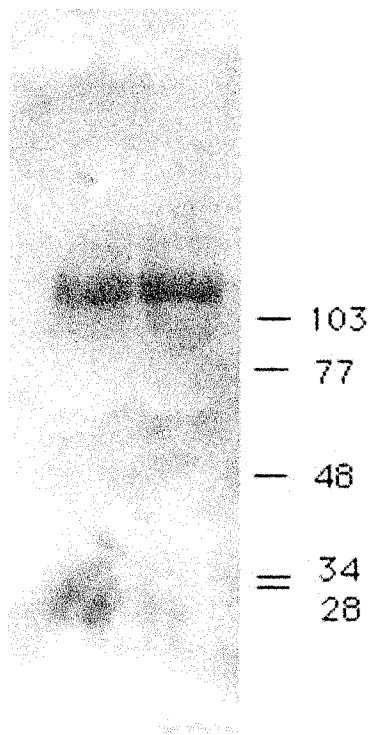
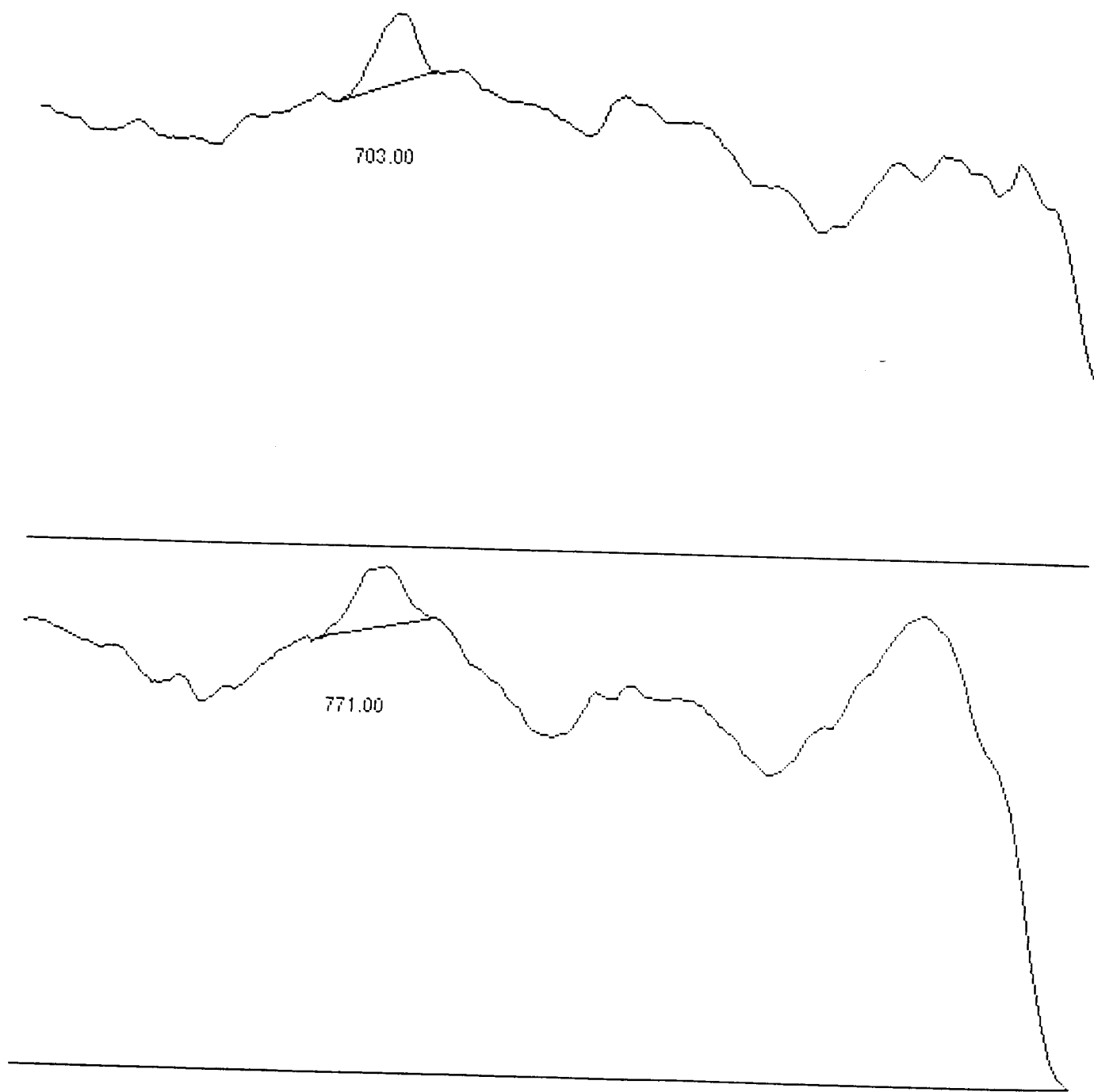


Figure13

The autoradiograph depicted in figure 12 was analyzed by scanning the autoradiograph through the NIH Imager. The peaks depicted correspond to the β -glucuronidase band of interest in the autoradiograph. The top corresponds to the non-affected patient. The bottom corresponds to the Vohwinkel's syndrome band of interest. It should be noted that the densitometer calculated the Vohwinkel's syndrome band to be of a higher concentration.

Non-affected Serum Sample



Vohwinkel's syndrome Serum Sample

Discussion

Vohwinkel's Syndrome, a particular type of EPPK, is characterized by hyperkeratosis of the stratum corneum. It was first reported back in 1929, by K.H. Vohwinkel. Since then only thirty cases have been reported. It is mainly characterized by hyperkeratosis of the stratum corneum. The Vohwinkel's Syndrome patient examined in this study has a mild form of the disease. Hyperkeratosis of both the palmar and plantar aspects of the hands can be seen in figures 1 and 2.

Little is known about this rare autosomal dominant disease. However, in the past decade more research has been done on Vohwinkel's Syndrome. In 1988 Camisa et al noted that the levels of human β -glucuronidase was increased in blood serum of afflicted patients. Camisa noted that perhaps this may be due to high amounts of the enzyme present in the skin, and that essentially the enzyme was 'spilling over' into the serum. The only way to determine if the enzyme levels were increased was to find a sensitive enzymatic assay. The assay involves a highly specific double antibody sandwich ELISA by Dr. Kang-Jey Ho (University of Alabama). The ELISA employed two monoclonal antibodies that were provided by Dr. Kang-Jey Ho. The antibodies were both mouse anti-human specific for B-glucuronidase. This assay enabled B-glucuronidase to be examined in blood, urine, and epidermal skin samples.

β -glucuronidase is a lysosomal enzyme. Several things are known about this enzyme. It is known to break down basement membrane constituents; namely glycosaminoglycans. It is also found in keratinocytes. It has also been linked to many diseases associated with hyperkeratosis. Many questions were asked in this study.

Were the enzyme levels increased? If so, how much? Are the skin levels that much more elevated over blood and urine? Why would this enzyme be increased so much? What function could it serve?

Other studies over the past decade included more information involving Keratin proteins and their direct role in the process of epidermal terminal differentiation. Along with this information Roop et al learned about the individual Keratin proteins that play an important role in the formation of the cornified cell envelope. Cornification of the epidermal squames is one of the last processes to occur prior to the cell being sloughed off. Christiano et al (1996) discovered that the keratin protein Loricrin, which is a member of the cornified cell envelope, is located on chromosome 21(1q21). She also discovered that Loricrin has a molecular defect in Vohwinkel's Syndrome patients (Mastrini, 1996). Chromosome position 1q21 is the location of the Epidermal Growth Complex. Thus, the mutation impairs both the structural lattice system by which transglutaminases cross-link these proteins for terminal differentiation and the barrier system that the stratum corneum provides. This molecular defect is believed to destroy the structural lattice system, which essentially traps the dead squames and prevents them from sloughing off. This causes Vohwinkel's patients to have a thickened stratum corneum with a yellow appearance. This thickening not only causes retention of the squames, but also sequesters water.

This study examined the levels of human β -glucuronidase in a Vohwinkel's Syndrome patient. It was accomplished by quantifying of the absolute concentration of the enzyme via an ELISA. It was also examined by western blotting. However, the sensitivity of the monoclonal antibodies in western blotting was shown to be too low to

yield good data. Only serum samples were detected through western blotting. This was still included in the results because it again showed that the levels of B-glucuronidase was elevated in Vohwinkel's syndrome patients. The results of the study show the levels of β -glucuronidase were elevated in a Vohwinkel's Syndrome patient. Levels were elevated in urine and blood serum, but especially in the epidermal skin punch biopsies. The increased concentration of the enzyme is demonstrated by the absorbance values detected by the ELISA reader. These values are reported in tables 2, 3, and 4. The data was then broken down in the various graphs that were generated in relation to each sample that was run. Finally, it was demonstrated by densitometry. It was obvious from the data obtained that the enzyme was in fact elevated. The hypothesis of this study was accepted.

Perhaps Camisa et al were correct in their suggestion that the elevation is directly caused by epidermal 'spilling over' into the serum and ultimately into the urine for secretion. The enzyme levels were the highest in the skin samples. The question remained: Why an increase in the levels of β -glucuronidase in these patients? Perhaps, it is compensatory for the molecular defect, or at least is attempting to compensate. If the molecular defect in Loricrin is impairing the cross-linking of the cornified cell envelope thereby preventing release of dead squames. Maybe, the enzyme is attempting to break down below the cornified level; at the basement membrane. Thereby releasing the dead skin. Another possibility involves the fact that loricrin is a keratin protein found in keratinocytes. Keratinocytes stain for β -glucuronidase. Therefore, β -glucuronidase may be increased inside the same cell as loricrin, attempting to break it down.

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June 6, 1995

Dr. Paul Peterson
Department of Biological Sciences
UNIVERSITY

Dear Dr. Peterson:

The Human Subjects Research Committee has reviewed and approved your proposal, "A Quantitative Histochemical Investigation of Keratoderma Hereditaria Mutilans (KLM)," (HSRC #95-38).

We wish you well in this study.

Sincerely,

A handwritten signature in black ink, appearing to read 'Peter J. Kasvinsky'.

Peter J. Kasvinsky
Dean of Graduate Studies

kb

c: A. Sobota, Chair, Biological Sciences
S. Ellyson, Chair, HSRC