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*Loyola University Chicago*

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# An Enzymatic Assay for D-Glucaric Acid

# in

# Blood and Bile

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by

Nicholas M. Di Filippo

A Dissertation Submitted to the Faculty of the Graduate School of Loyola University in Partial Fulfillment of the Requirements for the Degree of Master of Science

February

1968

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# LIFE

Nicholas M. Di Filippo was born in Europe of American parents on November 1, 1939. He began his education in the public grade schools of Providence, Rhode Island, and completed grade school and high school in Joliet, Illinois. He is a graduate of Joliet Catholic High School and Loyola University's College of Arts and Sciences.

The author began his graduate studies in the department of Microbiology in September, 1965. He is married to the former Margaret Regina Hogan; the couple have three children: Lucy Anne (two years old), Thomas Jude (one year old), and Marie Regina (two weeks old).

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# Chapter I. Introduction

## A. Historical

In 1961, C. A. Marsh (22), then at the Rowett Research Institute in Scotland, was working with urinary β-glucuronidase inhibitors of unknown composition. When examining the dialysate from normal human urine, Marsh found that the potency of the inhibitor was enhanced by acid treatment and decreased by treatment with alkali; both treatments were reversible. He noted that these properties were consistent with the known specific inhibitors D-glucaro-1,4-lactone and galactarolactone. Although the presence of such compounds in mammalian body fluids had not previously been reported, Marsh suggested that D-glucaro-1,4-lactone could conceivably arise by oxidation of D-glucuronic acid or its 6,3-lactone and that D-glucuronic acid in turn could be formed *in vivo* through the breakdown of uridinediphosphate glucuronic acid. He later found that rat liver homogenates were able to convert D-glucurono-3,6-lactone into a product which inhibited rat liver β-glucuronidase, and, as in urine, this power was greatly increased by acid treatment and reversibly abolished by treatment with alkali. Partially purified enzyme preparations required the addition of nicotinamide adenine dinucleotide (NAD), which also potentiated the activity of crude preparations. It was not until 1962 that this NAD-dependent enzyme, forming D-glucaro-1,4-lactone from a lactone of D-glucuronic acid was named D-glucurono-lactone dehydrogenase by Marsh.

Since the discovery of the presence in mammals of the lactone of glucaric acid and the enzyme concerned with its formation, others have joined Marsh in expanding our knowledge of glucarate in relation to $\beta$-glucuronidase inhibition and the possible use of glucarate as a therapeutic tool in the prevention or treatment of ailments such as cholelithiasis (21), hyperbilirubinemia (3), and cancer of the bladder (9). A review book discussing these concepts and related topics has recently appeared, edited by Geoffry J. Dutton and entitled "Glucuronic Acid, Free and Combined" (11b).

B. Importance of D-glucaric acid

D-glucaric acid, also known as saccharic acid or glucosaccharic acid, the product of the reaction catalyzed by D-glucuronolactone dehydrogenase, is widely found in nature and has the following chemical formula:

Its importance in bacterial metabolism has been studied by Blumenthal (6,7), who found that when Escherichia coli was grown in glucarate, both resting-cell suspensions and cell-free extracts from this culture converted one mole of glucarate to one mole of pyruvate. From this starting point, the complete metabolic pathway for the conversion of D-glucarate to glycerate and pyruvate was soon elucidated. This pathway was also found in a variety of enteric

bacteria. Another pathway for D-glucarate degradation in pseudomonads leads to the formation of $\alpha$-ketoglutarate and $CO_2$ (44a).

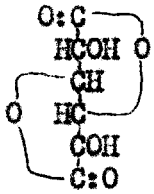
The importance of glucarate to the pathogenesis of gallstones in humans was recently considered by Naki (21). In the biliary tract, $\beta$-glucuronidase of bacterial origin, usually Escherichia coli, hydrolyzes bilirubin glucuronide into free bilirubin and glucuronic acid. Subsequently, the calcium in bile combines with the carboxyl group of the liberated bilirubin to form calcium bilirubinate, which then precipitates. But, bile contains glucaro-1,4-lactone, a normal inhibitor of $\beta$-glucuronidase (33,45). This lactone is the product of the reaction catalyzed by D-glucuronolactone dehydrogenase in the liver. Thus, glucaric acid may play two opposing roles in cholelithiasis: (1) it can support the growth of E. coli, which can then liberate bacterial $\beta$-glucuronidase, and (2) can inhibit the activity of the $\beta$-glucuronidase of bacterial origin, thus preventing calcium bilirubinate stone formation.

Anke (3) noted that bilirubin glucuronide formation was low in newborns and that the enzyme, $\beta$-glucuronidase, was elevated even in normal infants, thereby breaking down what little glucuronide was being formed. He considered suppressing $\beta$-glucuronidase using glucaro-lactones therapeutically, to counteract in the newborn a) a decreased production of the $\beta$-glucuronidase inhibitor, aggravating an already increased bilirubin load, b) as well as deficiency in the detoxicating mechanism in the absence of conjugation with glucuronic acid (24).

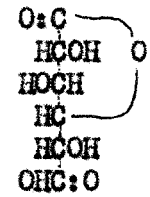
Boyland (9,10) tried to correlate the role of glucaro-1,4-lactone with the appearance and prophylaxis of bladder cancer. Although his attempt

to prevent the induction of bladder cancer in dogs with glucaro-1,4-lactone (10) was unsuccessful, Boyland has recommended oral administration of glucaro-1,4-lactone to men exposed to carcinogenic aromatic amines and to patients who have had bladder cancer removed or destroyed. Boyland believes this would reduce the amount of free carcinogenic aromatic amines in urine which would be released enzymatically from their glucuronides by the action of urinary β-glucuronidase.

Marsh has done a considerable amount of work on the urinary excretion of D-glucaric acid, and in 1963 definitely identified D-glucaric acid as a normal constituent of human urine (25). He found that the normal amount of glucaric acid excreted per 24 hr period was about 10 mg (using a more precise chemical analysis, Ishidate (15) has recently found that there is actually 15 mg excreted per day). This was the first demonstration of glucarate in mammalian fluids and since then glucarate has been detected and subsequently measured in human gallbladder bile (33,45). At present, the only known function of glucarate in humans is, as brought out previously, the ability to inhibit β-glucuronidase. However, glucarate may exist in a number of forms, and not all forms are equally inhibitory. According to Matsushiro (23) and Marsh (31), there are three possible lactones which can form from glucaric acid:


Glucaro-6,3-1,4-dilactone

Glucaro-6,3-lactone


Glucaro-1,4-lactone

Harigaya has studied the inhibitory effect on rat-liver β-glucuronidase by these various forms of glucaric acid, both in vitro and in vivo. In his in vitro studies, Harigaya (13) examined not only the various forms of glucaric acid, but also closely related compounds, and found that glucaro-1,4-lactone was the most potent inhibitor studied. He found that the inhibition of β-glucuronidase by acids derived from glucose and their lactones decreased in the following order: glucaro-1,4-lactone, glucaro-3,6-1,4-dilactone, glucaric acid, glucaro-3,6-lactone, glucuronic acid, glucono-γ-lactone, glucono-δ-lactone, gluconate, and glucuronolactone. The inhibitory potency of the last two compounds was found to be equal. From these results, Harigaya concluded that 3,6-lactonization causes a decrease, while 1,4-lactonization causes an increase in the inhibitory effect of the corresponding acid. Turning to in vivo experiments Harigaya (14) found the results to be somewhat different. The experiments were performed as follows: Various doses of glucarate or its lactones were administered by a stomach tube, the animals were sacrificed after 30 min and the activity of liver β-glucuronidase was then assayed. Thus, when glucaric acid and its lactones were orally administered to male mice, the potencies of inhibitory action of these compounds on liver β-glucuronidase were in the following order, beginning with the most potent: glucaro-3,6-1,4-dilactone, glucaro-1,4-lactone, glucaro-3,6-lactone and glucaric acid. Therefore, in contrast to the in vivo findings, the inhibitory effect was intensified, rather than decreased, by 3,6-lactonization. The fact that the effect of orally administered glucaro-1,4-3,6-dilactone was more powerful than glucaro-1,4-lactone

was not due to the quantitative transformation of the dilactone to the 1,4-lactone in the intestinal tract since it was found that the dilactone was transformed into the 3,6- and 1,4-lactones at equal rates. Thus, Harigaya concluded that the dilactone was more rapidly absorbed from the intestinal tract and more readily transported through the liver cell membrane than glucaro-1,4-lactone; when the two lactones were introduced directly into the blood, the relation was reversed. The in vitro inhibition of glucarate of β-glucuronidase from other sources, such as bovine liver (18), digestive gland tissue of the blue crab (37), bacteria (45), and urine has also been studied, and in each study, glucaro-1,4-lactone was found to be a potent inhibitor.

As emphasized throughout the previous discussion the only known value to humans of glucarate is derived from its ability to inhibit the enzyme β-glucuronidase. According to Plum (36), β-glucuronidase is a widely distributed enzyme, which hydrolyses the β-glycosidic bond between uronic acid and a variety of other moities, including steroids, hydroxylated aromatic compounds and sugar derivatives. This enzyme exists predominantly in a latent form in most tissues, being associated with lysosomes and microsomes. From this bound state it may be released as a free or non-sedimentable form during autolysis, ischemic necrosis, traumatic shock, experimental muscular dystrophy and hypervitaminosis A. Tissue activity of β-glucuronidase may be related to hormonal activity (11). According to Fishman (12), there is a dual localization of β-glucuronidase in endoplasmic reticulum and in lysosomes, and although he agrees with Plum that the enzyme is essentially inactive in the cytoplasm of cells, he suggests

the possibility that the enzyme may serve as a structural protein of the endoplasmic reticulum, a hypothesis that has yet to be tested experimentally. Fishman also points to a third localization of β-glucuronidase within body fluids, such as salivary, gastric and intestinal juices, bile, vaginal and spinal fluids, and urine (35), where the enzyme is active. The β-glucuronidase of bile appears to be extremely important in formation of gallstones of the bilirubin type. However, it may not be the endogenous β-glucuronidase which is of importance in this regard, but rather the β-glucuronidase of bacterial origin (19,20,21,41,43). According to Sato (42) the optimum pH for β-glucuronidase from E. coli is about pH 7. This is contrasted with tissue β-glucuronidase which has a pH optimum of 3.9 to 6.3. Since the pH of bile is usually near 7.0 this tends to favor the activity of bacterial β-glucuronidase while the activity of tissue β-glucuronidase "may be far from a maximum at normal pH of bile, since the latter is largely different from the optimum pH." The activity of serum β-glucuronidase in various disease processes has been widely studied (34,36,38,39) and appears to be intensified in atherosclerosis, epilepsy, and diabetes.

C. Present assays for D-glucaric acid in biological fluids.

Presently there are two methods for the quantitation of glucarate in two biological fluids, urine and bile. Both methods were first developed for testing the glucarate in urine and were later adapted to bile. No reports are available at present concerning either the qualitative or

quantitative detection of glucarate in other biological fluids such as blood, cerebrospinal fluid, serous fluid, etc.

The first method available for the determination of glucarate in urine was that of Marsh (25), based on the ability of glucarate to inhibit β-glucuronidase. The β-glucuronidase assay is carried out as follows: β-glucuronidase is obtained from livers of Lister strain rats. Biosynthetic phenolphthalein β-D-glucuronide, 0.63 mM, is the standard substrate for the enzyme assay, which is incubated for one hr. at 37 C in 0.125 M acetic acid-NaOH buffer, pH 5.2. The 4 ml incubation mixture includes 0.5 ml of enzyme solution, which liberates 35-40 μg of phenolphthalein in assays devoid of urine or other substances inhibitory to the enzyme. To estimate urinary glucarate, treated urine samples are added in known concentration to the buffered substrate immediately before the final addition of the enzyme, and the assay is carried out as usual. The urine is treated as follows: crude urine (pH 4.5-7.0) is treated at 100 C for 40 min. after adjustment with 3 N HCl to pH 2.0-2.2, followed by readjustment with NaOH to pH 4.0-4.5. This treatment is sufficient to rid the urine of β-glucuronidase activity, and after the readjustments there is no interference with the pH of the subsequent enzyme assay. To quantitate this method, Marsh selected an arbitrary unit, namely 1 "unit of inhibition" (U.I.) which he defined as "that quantity which produced 50% inhibition of rat liver β-glucuronidase in the standard assay at pH 5.2 with 0.63 mM phenolphthalein glucuronide as substrate." Thus, from a graph of inhibition against concentration of acid-treated urine, the U.I. present in a urine specimen can be measured.

In 1965, Ishidate et al. (15) developed a chemical method for the

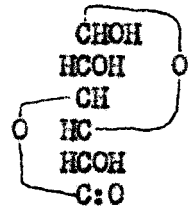
quantitative determination of glucarate in urine. Yagamuchi et al. (45) quickly adapted this improved method to measure the glucarate content of gallbladder bile. In essence, this was a chemical method based on an ion-exchange chromatographic separation of D-glucarate in urine or bile using an anion-exchange resin in borate form. The separation of D-glucarate was achieved by eluting the column with a borate-sodium sulfate solution. The D-glucaric acid thus separated was oxidized with periodic acid; the resulting glyoxylic acid was determined by a modification of the procedure reported by McFadden and Howes (45): The glyoxylic acid was condensed with phenylhydrazine to its phenylhydrazone and, in the presence of potassium ferricyanide and excess phenylhydrazine, eventually converted into 1,5-diphenylformazan, a colored substance. The latter was quantitatively determined spectrophotometrically at 520 mμ.

Although a considerable improvement over the method of Marsh, this method also had its disadvantages. Tartaric acid, L-gulonic acid, D-glucuronic acid, L-ascorbic acid and other hexaric acids could not be differentiated from glucarate. Another disadvantage appeared to be that there was no clear-cut separation of D-glucaric acid from the column (45).

D. Glucuronolactone conversion to D-glucaric acid; glucuronolactone dehydrogenase.

Glucuronolactone, the lactone of glucuronic acid and the substrate

for D-glucuronolactone dehydrogenase, has the following chemical formula:




The close structural similarity of D-glucuronolactone to D-glucaric acid lead Marsh (26) to speculate that D-glucuronolactone was a direct precursor of the D-glucaric acid produced biosynthetically. Although glucuronolactone has not been reported in mammalian tissue (11b), Marsh believes that the probable source of glucuronolactone *in vivo* is free glucuronic acid formed in tissues solely from UDP-glucuronic acid, either by direct action of phosphatases or by the hydrolysis of glucuronides formed by transfer of the D-glucuronic acid moisty to unknown acceptors. However, Marsh does not rule out the possibility that D-glucuronic acid may be obtained from dietary sources, for example, from the breakdown of acidic mucopolysaccharides. Kar (16) studied the action of goat liver microsomes and found that D-glucuronolactone could be converted to L-ascorbic acid, by the enzyme D-glucuronolactone reductase, a sulfhydryl-containing enzyme, which may compete with D-glucuronolactone dehydrogenase for its substrate. Baker (5) studied the metabolism of D-glucuronolactone-6-$^{14}C$ in man and found that, although a large portion of the dose was metabolized to respiratory $CO_2$, some *in vivo* conversion of D-glucuronolactone to ascorbic acid did occur as evidenced by radioactive ascorbic acid in urine. Unfortunately, he did not test for labelled glucaric acid, which presumably would have shown the activity not only of D-glucuronolactone reductase, but also of D-glucuronolactone dehydrogenase. The activity of this latter enzyme was studied by both Marsh (25)

and Ishidate (15) using different methods. When a dose of D-glucuronolactone was given to humans, an average of 16 per cent of it was converted into D-glucaric acid within a 24 hr period, as evidenced by an increase in urinary D-glucaric acid. This conversion was most likely due to the action of D-glucuronolactone dehydrogenase. However, Harigaya (15) questioned the formation of glucuronolactone, since oral administration of the latter was nearly without effect on the activity of liver β-glucuronidase. At any rate, the following is the probable mechanism of formation of glucaric acid lactones from glucuronolactones favored by most workers in the field (33):

$$\text{NAD} + \text{glucuronolactone} \longrightarrow \text{NADH}_2 + \text{glucaro-6,3-1,4-dilactone}$$

$$\text{glucarodilactone} \xrightarrow{H_2O} \text{glucaro-6,3-lactone} + \text{glucaro-1,4-lactone}$$

## Chapter II. Materials and Methods

### A. Materials

Blood was obtained by venipuncture and placed in heparinized tubes (1 ml of a 1% solution of heparin dried as a film inside tube). Bile was obtained from postmortem as well as surgical material. NADH and NADPH were obtained from the Sigma Chemical Company (NADH is grade III, disodium salt obtained from yeast; NADPH is the tetrasodium salt, type II, enzymatically reduced). Lactic dehydrogenase (crystalline suspension from rabbit muscle) and L-glutamic dehydrogenase (crystalline suspension from bovine liver) were also obtained from Sigma. Other chemicals, such as $MgSO_4$, tris(hydroxymethyl)aminomethane (Tris) and maleic acid, were obtained either from J. T. Baker Chemical Co. or Sigma. Pure potassium acid glucarate was obtained from D. C. Fish (prepared as the cyclohexylammonium salt) (11c). The Tris-maleate buffer was prepared by mixing 50 ml of a 0.80 M solution of Tris with about 45 ml of 0.8 M maleic acid solution, titrating with NaOH to pH 7.6. The volume was then made up to 100 ml. Deionized and distilled water was used throughout these studies.

### B. Growth of *Escherichia coli*

The medium for the growth of *Escherichia coli* strain CR63NA (8)

contained the following in one liter of distilled, deionized (DI) water: 8.0 g of D-glucarate, 16.5 g of anhydrous dibasic sodium phosphate, 1.5 g of monobasic potassium phosphate, 2.0 g of ammonium sulfate, 0.2 g of magnesium sulfate heptahydrate, 10 mg of calcium chloride dihydrate, and 50 μg of ferrous sulfate heptahydrate. A 5% inoculum of a starter culture grown for 24 hr in the same medium was used to initiate growth. The cells were grown for 16 hr (i.e., to the stationary phase) on a rotary shaker, after which they were harvested by centrifugation, resuspended in and washed twice with 0.05 M KCl.

C. Preparation of Enzymes

Each g (wet weight) of cells was suspended in 10 ml of 0.1 M Tris-maleate buffer, pH 7.6. After sonicating for 8 min with a Branson sonifier (position 6, 3 amp, 15 sec intervals for 8 min with a microtip). The supernatant fluid remaining after centrifugation for 10 min at 21,000 xg was used as the crude enzyme preparation. This crude preparation was partially purified using ammonium sulfate; all operations were performed at 0-4 C. To the 10 ml of supernatant was added 10 ml saturated $(NH_4)_2SO_4$ which had been neutralized with ammonia. After 15 min, with occasional stirring, the 0 to 50% $(NH_4)_2SO_4$ precipitate was removed by centrifugation, and 12 ml of saturated $(NH_4)_2SO_4$ was added to the 20ml of supernant fluid. After 15 min with occasional stirring the 50 to 70% $(NH_4)_2SO_4$ precipitate was removed by

centrifugation. The 50 to 70% pellet was suspended, without further treatment, in 5 ml 0.1 M Tris-maleate buffer and 0.1 ml of this partially purified enzyme fraction was used per cuvette in assaying for glucarate in blood or bile. This 50 to 70% fraction was found to contain all three enzymes, i.e., D-glucarate dehydrase, keto-deoxy-D-glucarate aldolase, and tartronate semialdehyde reductase, involved in glucarate metabolism by E. coli. The final protein concentration of the 0 to 70% fraction suspended in 0.1 M Tris-maleate buffer was 10.5 mg protein/ml. The enzyme could be stored at -20 C for more than a month.

D. Treatment of heparinized blood or gallbladder bile

Blood, obtained by venipuncture, was placed in heparinized tubes. Eight ml of the heparinized blood or bile sample were then pipetted into a 50 ml centrifuge tube to which was added 5 ml of a 1.8 N perchloric acid solution, giving a solution containing 0.62 ml blood or bile per one ml. After thoroughly mixing with a vortex, the protein was removed by centrifuging for 15 min at 15,000 xg at 0 C. Six ml of supernantant fluid were removed, to which 1.75 ml 3 M $KHCO_3$ were slowly added, giving a final solution containing 0.48 ml blood or bile per ml. After thoroughly mixing, the insoluble potassium salt of perchloric acid precipitated and the supernatant was decanted and frozen. During freezing the remaining potassium perchlorate becomes insoluble and immediatly after thawing, the perchlorate-free blood or bile solution

was decanted. The pH of this solution was between 8 to 9. The solution was then delactonized in a boiling water bath for a minimum of 30 min, after which it was cooled and ready for assay. If the solution could not be assayed within the next few hr, it was stored frozen until time of assay.

E. Assay

1. Assay for D-glucarate in absence of blood or bile:

A glucarate solution could be analyzed in two ways, one assay utilizing NADPH and another NADH. In using NADPH, two cuvettes were necessary, one serving as a control; their contents were as follows:

| | #1 | #2 |
|---|---|---|
| 0.4 M Tris-maleate, pH 7.6 | 0.50 ml | 0.50 ml |
| 0.08 M $MgSO_4 \cdot 7H_2O$ | 0.50 | 0.50 |
| NADPH (9.4 moles/ml) | 0.10 | 0.10 |
| Glucarate sample | ---- | 0.10 |
| $H_2O$ | 1.87 | 1.77 |
| | 2.97 ml | 2.97 ml |

The reaction was initiated by the addition of 0.03 ml E. coli enzymes to each cuvette. The reaction was followed at 340 mμ in a recording spectrophotometer for 90 to 110 min at 37 C.

The second method, utilizing NADH instead of NADPH was more sensitive since lactic dehydrogenase (LDH) was also added to the assay thus giving two moles of NADH converting to NAD per mole of glucarate present; again two cuvettes were necessary, one serving as the control:

| | #1 | #2 |
|---|---|---|
| 0.4 M Tris-maleate, pH 7.6 | 0.50 ml | 0.50 ml |
| 0.08 M $MgSO_4 \cdot 7H_2O$ | 0.50 | 0.50 |
| $H_2O$ | 1.82 | 1.72 |
| NADH (19.5 moles/ml) | 0.05 | 0.05 |
| Glucarate sample | ---- | 0.10 |
| LDH (0.5 mg protein/ml) | 0.10 | 0.10 |
| *E. coli* enzymes | 0.03 | 0.03 |
| | 3.00 ml | 3.00 ml |

The reaction was started by addition of the *E. coli* enzymes. The NADH conversion to NAD was calculated with $6.22 \times 10^6$ $cm^2$/mole as the extinction coefficient (6). The reaction was also followed at 340 mμ and at 37 C.

2. Assay for glucarate in blood

The blood was treated as outlined in section C, where each ml of the deproteinized sample contained 0.48 ml whole blood. The assay was carried out in three parts as follows:

Part I:

| | #1 | #2 | #3 | #4 |
|---|---|---|---|---|
| 0.40 M Tris-maleate, pH 7.6, ml | 0.50 | 0.50 | 0.50 | 0.50 |
| 0.16 M $MgSO_4 \cdot 7H_2O$, ml | 0.25 | 0.25 | 0.25 | 0.25 |
| $H_2O$ | 0.10 | 2.10 | 0.10 | ---- |
| Deproteinized blood, ml | 2.00 | ---- | 2.00 | 2.00 |
| NADH (19.5 moles/ml), ml | 0.05 | 0.05 | 0.05 | 0.05 |
| LDH (0.5 mg prptein/ml), ml | 0.10 | 0.10 | 0.10 | 0.10 |
| Glucarate (1 mole/ml). ml | ---- | ---- | ---- | 0.10 |

Part II:

After the LDH reaction has ceased, glutamic dehydrogenase (GLDH) was added:

| | | | | |
|---|---|---|---|---|
| GLDH, (2 mg protein/ml), ml | 0.05 | 0.05 | 0.05 | 0.05 |

Part III:

After GLDH reaction had ceased, the following were added:

| | | | | |
|---|---|---|---|---|
| NADH, ml | 0.02 | 0.02 | 0.02 | 0.02 |
| *E. coli* enzymes (10.5 ml protein per ml), ml | ---- | 0.10 | 0.10 | 0.10 |
| | 3.07 | 3.17 | 3.17 | 3.17 |

Cuvettes #1 and #2 were controls, and the changes in optical density (OD) at 340 mμ of both cuvettes were added and then subtracted from the changes in OD in cuvettes three and four. Cuvette #1 measured the conversion of NADH to NAD due to the presence of blood (no E. coli enzymes were present), while cuvette number two measured the NADH conversion to NAD due to any NADH oxidase which might have been present in the enzyme extract. Cuvette number three measured the amount of glucarate present in 0.96 ml whole blood, while cuvette number four measured the recovery obtained by adding a known amount of glucarate.

The first part of the assay measured the pyruvate initially present in the blood sample and converted it to lactate. This was necessary since, pyruvate was produced from glucarate by the addition of E. coli enzymes, and its conversion to lactate was again measured. The second part of the assay was necessary for the removal of α-ketoglutarate, since, it was found that glutamic dehydrogenase was present in the partially purified E. coli enzyme preparation. However, the amount of α-ketoglutarate normally present in 0.96 ml whole blood was not very significant (less than 2 μg); elimination of this step would result in less than a ten % error. The ammonium ions necessary for this reaction came from lactic dehydrogenase, which was suspended in an ammonium sulfate solution. This was the reason for having the reaction as the second portion of the assay. In the third portion, the NADH was again added to the assay to ensure an adequate amount of NADH for the E. coli enzymes, namely, D-glucarate dehydrase, keto-deoxy-D-glucarate (KDG) aldolase, and tartronate

semialdehyde reductase. However, the amount of NADH to be added varied, depending mainly on the amount of pyruvate initially present in the sample. Thus, in the assay for blood glucarate, two moles of NADH were converted to NAD per mole of glucarate present, one due to the reaction of tartronate semialdehyde going to glycerate, and the other due to the action of lactic dehydrogenase. The reaction was followed for 110 min at 37 C in a Gilford recording spectrophotometer.

3. Assay for glucarate in bile

Since gallbladder bile was deproteinized in the same manner as blood there was 0.48 ml of undiluted bile/ml deproteinized bile. Due to the opacity of the deproteinized bile as compared to that of deproteinized blood, it was often necessary to use less of the deproteinized bile. The assay for glucarate in the presence of bile is carried out as follows:

| | #1 | #2 | #3 | #4 |
|---|---|---|---|---|
| 0.40 M Tris-maleate, pH 7.6, ml | 0.50 | 0.50 | 0.50 | 0.50 |
| 0.16 M $MgSO_4$, ml | 0.25 | 0.25 | 0.25 | 0.25 |
| $H_2O$, ml | 0.70 | 2.10 | 0.60 | 0.50 |
| Bile solution, ml | 1.50 | ---- | 1.50 | 1.50 |
| NADPH (19.5 moles/ml), ml | 0.05 | 0.05 | 0.05 | 0.05 |
| Glucarate (1 mole/ml), ml | ---- | ---- | ---- | 0.10 |
| *E. coli* enzymes, ml | ---- | 0.10 | 0.10 | 0.10 |
| | 3.00 | 3.00 | 3.00 | 3.00 |

NADPH had to be substituted for NADH in bile since in some samples a cross-reaction converted any NAD present to NADH., It is not known what this reaction is. Thus, since NADPH had to be substitued, the lactic dehydrogenase reaction could not be utilized, since this enzyme is specific for NADH; therefore, one mole of NADPH was converted to NADP for every mole

of glucarate present. Here, as in blood, the reaction was followed for 110 min at 37 C in a Gilford Model 2000 automatic spectrophotometer.

## Chapter III. Results

### A. Results with glucarate in absence of blood or bile

A solution of glucarate could be measured accurately with recoveries close to theory using the procedure outlined. The following is an experiment which was performed essentially as in E.1. (see Materials and Methods) except that the final volume was 3.12 ml. In this experiment NADPH was used. There were three cuvettes, #1 is the control, #2 contains 0.1 moles of glucarate and #3 contains 0.2 moles of glucarate. The changes in OD were as follows:

| | #1 | #2 | #3 |
|---|---|---|---|
| 0 to 20 min | 0.042 | 0.210 | 0.397 |
| 20 to 30 min | 0.009 | 0.015 | 0.024 |
| 30 to 40 min | 0.006 | 0.012 | 0.012 |
| 40 to 50 min | 0.006 | 0.004 | 0.006 |
| 50 to 60 min | 0.006 | 0.006 | 0.006 |
| 60 to 90 min | 0.018 | 0.018 | 0.017 |

Therefore, taking 60 min as the end point, we have the following changes in optical density:

| | #1 = 0.069; | #2 = 0.249; | #3 = 0.445 |
|---|---|---|---|
| Subtracting the control: | | -0.069 | -0.069 |
| | | 0.178 | 0.376 |

Therefore, analyzing cuvette #2:

0.178/6.22=0.029; and 0.029x3.12=0.090 $\mu$moles of glucarate; actually added 0.100 $\mu$moles, and therefore have a 90% recovery.

Now, analyzing cuvette #3:

0.376/6.22=0.060; and 0.060x3.12=0.187 $\mu$moles of glucarate; actually added 0.200 $\mu$moles, and therefore have a 93.5% recovery.

B. Estimation of glucarate content of blood

The glucarate and the pyruvate initially present in twenty-three samples were as presented on the table on the following page. In Table 1, capital letters in the front column refer to the initials of the person from whom the blood sample was taken. Where there is a capital and a small letter, the "name" refers to the first two letters of the donor's last name since the first name was not available. The first twenty samples were taken from patients, the first three from Cook County Hospital and the next 17 from Hines Veterans Administration Hospital. The last three samples were taken from apparently healthy graduate students, and thus, may be considered as "normals." As yet, there is no explanation for the great difference obtained between "patients" (average 16 $\mu$gm glucarate/ml) and "normals" (average 66 $\mu$gm/ml). These findings were not correlated to the disease process of tested patients. At any rate, when all samples are considered as a group, values ranging from 6 to 67 $\mu$gm of glucarate/1ml (0.6 mg to 6.7 mg/100 ml) were obtained, while the pyruvate levels present varied from 4 to 13 $\mu$gm/ml (0.44 to 1.32 mg/100 ml).

Table 1. Glucarate and pyruvate content of human blood

| Name | Glucarate | | | Pyruvate | | |
|---|---|---|---|---|---|---|
| | ΔOD | 2X μmoles/ml | μgm/ml | ΔOD/3.00 ml | μmoles/ml | μgm/ml |
| WF | 0.082/3.14ml | 0.047 | 5.83 | ----- | ----- | ----- |
| EG | 0.134/3.21ml | 0.070 | 8.68 | ----- | ----- | ----- |
| MS | 0.226/3.31ml | 0.124 | 15.38 | ----- | ----- | ----- |
| GM | 0.298/3.19ml | 0.159 | 19.71 | 0.180 | 0.091 | 8.01 |
| Cl | 0.268/3.24ml | 0.149 | 18.47 | 0.136 | 0.069 | 6.08 |
| Re | 0.310/3.17ml | 0.166 | 20.58 | 0.224 | 0.116 | 10.22 |
| Ta | 0.208/3.17ml | 0.109 | 13.52 | 0.186 | 0.093 | 8.19 |
| Ca | 0.160/3.23ml | 0.084 | 10.42 | 0.134 | 0.066 | 5.81 |
| EB | 0.112/3.16ml | 0.059 | 7.32 | 0.094 | 0.050 | 4.40 |
| JA | 0.216/3.15ml | 0.111 | 13.76 | 0.106 | 0.053 | 4.67 |
| JK | 0.344/3.36ml | 0.192 | 23.81 | 0.148 | 0.075 | 6.60 |
| FS | 0.308/3.17ml | 0.165 | 20.84 | 0.160 | 0.081 | 7.13 |
| RW | 0.154/3.17ml | 0.079 | 9.79 | 0.152 | 0.076 | 6.69 |
| AJ | 0.138/3.17ml | 0.073 | 9.05 | 0.128 | 0.062 | 5.46 |
| Ba | 0.202/3.17ml | 0.105 | 13.02 | 0.152 | 0.076 | 6.69 |
| CD | 0.212/3.17ml | 0.111 | 13.76 | 0.172 | 0.084 | 7.40 |
| FM | 0.434/3.16ml | 0.230 | 28.52 | 0.110 | 0.053 | 4.67 |
| ER | 0.342/3.17ml | 0.181 | 22.44 | 0.294 | 0.146 | 12.86 |
| WW | 0.340/3.17ml | 0.181 | 22.44 | 0.198 | 0.100 | 8.81 |
| MW | 0.272/3.17ml | 0.144 | 17.85 | 0.192 | 0.094 | 8.28 |
| TF | 0.998/3.16ml | 0.528 | 65.47 | 0.303 | 0.150 | 13.21 |
| JW | 1.010/3.20ml | 0.540 | 66.96 | 0.302 | 0.150 | 13.21 |
| SB | .997/3.20ml | 0.533 | 66.09 | 0.293 | 0.147 | 12.94 |

The following table shows the per cent recovery of glucarate added directly to the assay cuvette:

| Sample | Glucarate added, μmoles | Endogenous glucarate, μmoles | Total glucarate found, μmoles | Glucarate recovered, μmoles | Recovery % |
|---|---|---|---|---|---|
| T.F. | 0.10 | 0.253 | 0.363 | 0.110 | 110 |
| J.W. | 0.10 | 0.259 | 0.369 | 0.110 | 110 |
| S.B. | 0.10 | 0.256 | 0.365 | 0.109 | 109 |
| M.S. | 0.10 | 0.060 | 0.166 | 0.106 | 106 |
| E.G. | 0.10 | 0.067 | 0.176 | 0.109 | 109 |
| Pooled A | 0.20 | 0.185 | 0.377 | 0.192 | 96 |
| Pooled B | 0.20 | 0.071 | 0.260 | 0.189 | 94 |
| Pooled C | 0.20 | 0.058 | 0.232 | 0.174 | 87 |

The recovery was generally close to 100%. Matched glass cuvettes were used to analyze the first five samples, while quartz cuvettes were used for the last three samples. From these and other experiments it appeared that the use of glass cuvettes unexplanably caused recovery to be 5 to 10% too high. Therefore, quartz cuvettes were used exclusively for all analyses following this observation. It was found that the per cent recovery drops if the last traces of potassium perchlorate have not been removed.

A number of compounds were employed to detect the presence of cross reactions in the assay. The following substances added directly to the assay cuvette did not interfere with the assay: 0.8 mg glucose, 1.2 mg L-tartaric acid, 0.2 mg potassium glucuronate, 0.09 mg gulonolactone, and 0.02 mg ascorbic acid. However, concentrations of ascorbic acid considerably above those normally found in blood, did interfere with the results, increasing the conversion of NADH to NAD:

(a) when 0.1 mg of ascorbic acid was added in cuvette #4(see Materials and Methods), i.e., replacing the glucarate in the recovery cuvette, the change in OD in cuvette #3 was 0.138 while that with ascorbic acid was 0.240.

(b) when 0.05 mg was added to #4, the change in OD of cuvette #3 was 0.154, while #4 was 0.228.

(c) when 0.03 mg was added to #4, the change in OD in cuvette #3 was 0.212 while that in #4 was 0.272.

(d) when 0.02 mg was added to #4, no difference was obtained. When the experiment was repeated a very slight difference (0.008) was obtained, the change in optical density being greater in cuvette #4. Thus, it appears that at the levels normally present in whole blood (0.4 to 1.5 mg/100 ml; Cecil and Loeb: Textbook of Medicine), ascorbic acid does not interfere with the assay and may thus be disregarded, although at higher concentrations its presence will significantly affect the assay.

C. Estimation of glucarate content of bile

A recovery cuvette was always run with each sample of bile, since the recovery in the presence of bile was uniformly less than 100%. The following data were obtained:

| Sample | Amount actual bile per cuvette | △OD | Glucarate per ml, μgm | Recovery % | Corrected glucarate per ml, μgm |
|---|---|---|---|---|---|
| A67-110 | 0.48 | 0.020 | 5.00 | 78 | 6.0 |
| A67-127 | 0.43 | 0.097 | 27.53 | 60 | 46.0 |
| A67-106 | 0.34 | 0.174 | 62.00 | 60 | 103.0 |
| A67-111 | 0.09 | 0.190 | 248.00 | 60 | 413.0 |
| A67-109 | 0.29 | 0.134 | 59.50 | 60 | 86.0 |
| A66-698 | 0.45 | 0.127 | 32.24 | 48 | 67.2 |
| A66-699 | 0.45 | 0.203 | 53.83 | 48 | 140.0 |
| C.C. | 0.44 | 0.096 | 25.70 | 87 | 29.5 |
| J.M. | 0.44 | 0.253 | 138.69 | 74 | 187.4 |

The first 7 samples were from autopsy material while the last 2 were from surgical cases. Previous discussions concerning cross-reactions with blood are equally applicable to bile.

D Blood glucarate analysis using NADH and NADPH

A pooled blood sample was divided into two parts. One part was analyzed using NADH as coenzyme, while the other portion used NADPH as the coenzyme. Since lactic dehydroganase cannot utilize NADPH, these experiments were performed to present added evidence that the substance measured by this assay was glucarate. The procedure for both experiments was as outlined on page 16, with the exception that in one experiment NADH was used, while in the other NADPH was used.

Using NADH (final volume 3.19 ml) the following changes in optical density were observed at the end point of 120 min:

| | | | |
|---|---|---|---|
| #1 = 0.152 | #2 = 0.210 | #3 = 0.720 | #4 = 1.470 |
| | +0.152 | -0.362 | -0.362 |
| | 0.362 | 0.358 | 1.108 |

Therefore, using the calculations explained on page 20, cuvette #3 gave a value of 23.96 μgm/ml, while cuvette #4 indicated that there was a 95.5% recovery (added 0.20 μmoles of glucarate and recovered 191 μmoles). Therefore, the actual level of glucarate (using NADH as coenzyme) for this sample was 25.06 μgm/ml.

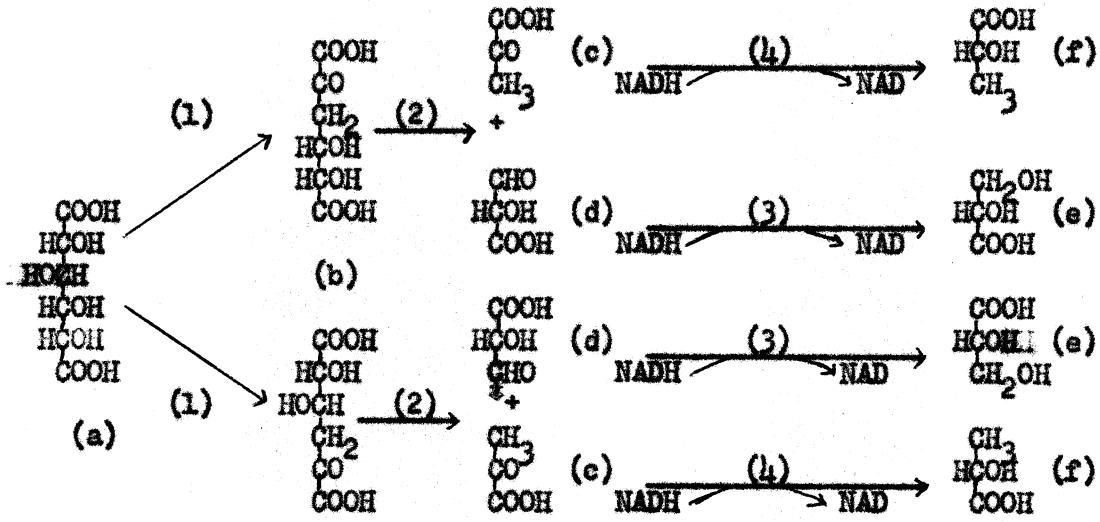
Turning now to NADPH (final volume 3.15 ml), the following optical density changes were recorded at 170 min:

| | | | |
|---|---|---|---|
| #1 = 0.120 | #2 = 0.126 | #3 = 0.410 | #4 = 0.732 |
| | +0.120 | +0.246 | -0.246 |
| | 0.246 | 0.164 | 0.486 |

Therefore, cuvette #3 gave a value of 21.08 μgm/ml, while cuvette #4 showed that there was an 82% recovery. Therefore, the actual level of glucarate in this sample, using NADPH as coenzyme was 25.71 μgm/ml. As can be seen these values are essentially identical.

## Chapter IV. Discussion

This thesis presents a new method of analyzing glucarate; its biochemistry is based on glucarate metabolism in E. coli as reported by Blumenthal and Fish (6):




(1) = D-Glucarate dehydrase

(2) = α-Keto-β-deoxy-D-glucarate aldolase

(3) = Tartronate semialdehyde reductase

(4) = Lactic dehydrogenase

---

(a) = D-Glucarate

(b) = α-Keto-β-deoxy-D-glucarate

(c) = Pyruvate

(d) = Tartronate semialdehyde

(e) = Glycerate

(f) = Lactate

D-Glucarate dehydrase, the first enzyme in this pathway, converts D-glucarate to keto-deoxy-D-glucarate; a specific aldolase than cleaves the latter compound into tartronate semialdehyde and pyruvate. Tartronate semialdehyde reductase and lactic dehydrogenase then act to yield glycerate and lactate, respectively. Thus, for every mole of glucarate which is metabolized, two moles of NADH are converted to NAD. D-Glucarate dehydrase, keto-deoxy-D-glucarate aldolase and tartronate semialdehyde reductase were partially purified by ammonium sulfate and all three enzymes were present in a 50 to 70% fraction of the cell-free extract of E. coli grown on glucarate as the sole carbon source. The biochemistry is slightly different when glucarate is analyzed in the presence of bile. In this case, NADPH must be substituted for NADH in the tartrenate semialdehyde reductase reaction, since, in some samples, a cross-reaction occurs converting any NAD present to NADH. The nature of this reaction is not known. Moreover, since LDH cannot utilize NADPH, LDH cannot be employed in the assay to determine pyruvate and only one mole of NADPH is converted to NADP per mole of glucarate present.

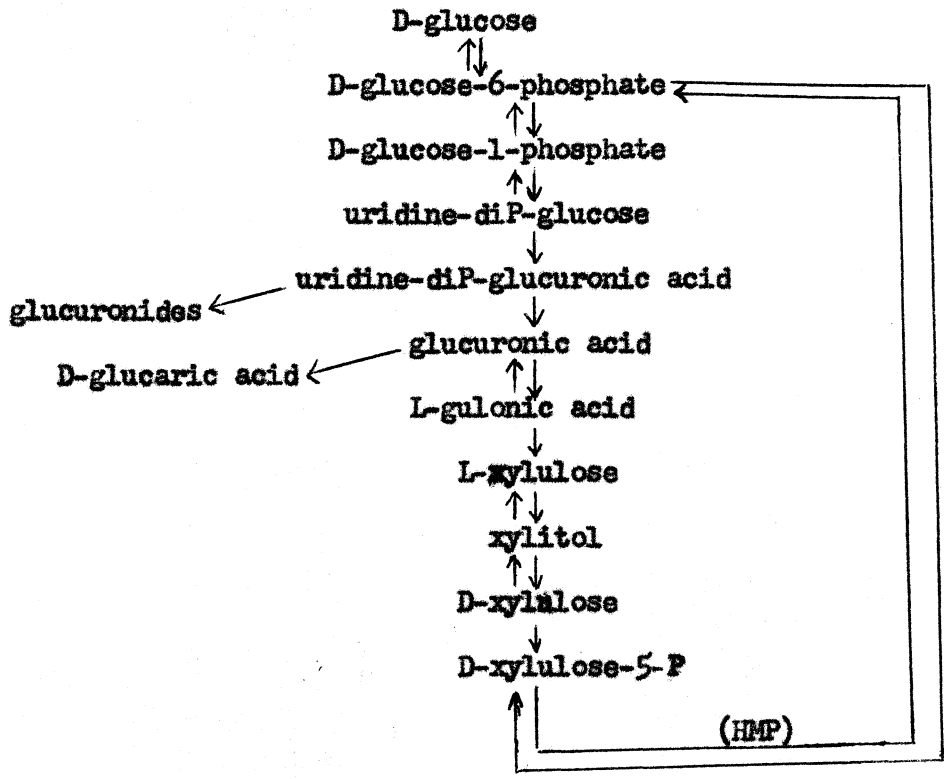
Glucose, L-tartaric acid, potassium glucuronate, gulonolactone, or ascorbic acid do not interfere with the assay. However, concentrations of ascorbic acid considerably above those normally present in blood did significantly affect the assay, increasing the conversion of NAD(P)H to NAD(P).

Employing the new enzymatic assay, the presence of glucarate in bile was confirmed; in addition, this new assay was employed in detecting

and measuring blood glucarate for the first time. The presence of glucarate in blood has not been previously reported; Marsh and Carr (30) stated "that the presence of D-glucaric acid could not be detected by us in normal rat serum." These investigators were testing for the presence of glucarate in serum using a method based on the ability of the 1,4-lactone of glucaric acid to inhibit β-glucuronidase. However, as Ishidate (15) stated, "this enzymatic assay was sometimes unsatisfactory for accurate determination" of glucaric acid. Although the method of Ishidate is a great improvement over that of Marsh, no one has, as yet, reported the presence of glucarate in blood.

The presence of glucarate in blood, as well as in bile and urine, is not unexpected since glucaric acid is formed in the liver as a result of the oxidation of glucuronolactone by D-glucuronolactone dehydrogenase (26). This enzyme is ubiquitous in nature (27), having been detected: a) in the liver and kidney of the rat, mouse, man, guinea pig, and pig; b) in the testes of the rat, mouse, and man; c) in the ovaries of the mouse; and d) in the brain (i.e., the thalamus) of rat and man. D-Glucuronolactone dehydrogenase, however, is not completely specific for D-glucuronolactone, since D-mannuronolactone also serves as a substrate (32,40).

Aarts (1,2) has studied the glucuronic acid pathway of glucose metabolism and its relation to D-glucaric acid. He offers the following summary of this pathway:




Since D-glucaric acid is not metabolized further, it can be considered as an end product of the pathway. The production of glucarate by this pathway may be greater in some people than in others. This can be seen in the results presented in this study for both blood and bile. It was found that values for blood ranged from 6 to 67 μgm/ml (23 samples) while the range for bile was 6 to 413 μgm/ml (7 samples). The latter range approximates that of Yamaguchi (45), who, using a chemical method following ion-exchange chromatography, reported values ranging from 38 to 634 μgm/ml bile (14 samples). As stated earlier, there have been no previous reports for blood glucarate.

It is not known presently what governs the body fluid levels of

glucarate. Yamaguchi, in referring to the studies on bile, stated that the "individual variation was thus quite large and this should be an interesting subject of future investigation." However, from the work of Marsh and Aarts, it is known that the physiological state of the person as well as the intake of certain drugs has a considerable effect on glucarate levels, as measured by D-glucuronolactone dehydrogenase activity as well as glucaric acid excretion in urine. According to Marsh (30) UDPG dehyrogenase and glucuronyl transferase are enzymes which are known to be deficient in fetal and infant liver tissues and "deficiencies of these enzymes appear to be one couse of the accumulation of unconjugated bilirubin in the newly-born, a condition causing neonatal jaundice which may lead to kernicterus, particularly in premature births. A lack of D-glucaro-1,4-lactone, due to low D-glucuronolactone dehydrogenase activity, in fetal and infant tissues could aggravate this pathological condition through a failure to suppress the hydrolysis of newly formed bilirubin glucuronide. Treatment of such cases with D-glucaro-1,4-lactone might thus have an alleviating effect." However, in view of the <u>in vivo</u> experiments of Harigaya (14), I believe that glucaro-1,4-3,6-dilactone would be a more effective therapy due to the greater ability of the latter compound to cross biological membranes. Marsh further states that the activity of D-glucuronolactone dehydrogenase is increased during pregnancy and may thus be a "physiological process for keeping the increased quantities of circulating steriod hormone in a conjugated state." Lastly, the D-glucuronolactone dehydrogenase activity of liver preparations from human cancer patients has been

found to be significantly reduced.

In addition to the physiological state, as mentioned above, the intake of certain drugs is also known to affect glucaric acid metabolism in humans. In vitro experiments by Marsh (28) showed that the conversion of D-glucuronolactone to D-glucaric acid was inhibited by barbital. In contrast, the results of his in vivo tests were surprisingly different (29), since he found that "glucaric acid excretion by rats treated with 50 mg barbital on three sucessive days increased 12-fold during the day after the initial dose, then remained almost constant." Moreover, daily administration of chloretone also elevated glucaric acid excretion, the distinction being that the excretion of the acid increased each day during the period of treatment (three days). At the end of this period, the animals were killed and liver D-glucuronolactone dehydrogenase activities measured; he found that "there was no significant difference between the enzyme activities of the drug-treated animals and those of controls." Based on this and other information, Marsh concluded that the elevated excretion of D-glucaric acid could be explained most easily by the increased availability of the precursor, D-glucuronic acid or its lactone, the common intermediate for the biosynthesis of L-ascorbic acid, D-glucaric acid, and L-xylulose. However in similar experiments with barbital, Aarts (2) found that stimulation occured within three hr after barbital administration to rats, and puromycin and actinomycin D were unable to block barbital-stimulated excretion of D-glucaric acid.

This indicated that the mechanism of stimulation does not involve increased synthesis of enzymes concerned in the glucuronic acid pathway. He concluded that a "fuller understanding of the mechanism of stimulation by barbiturate will require more knowledge" concerning in vivo D-glucuronic acid formation.

In summary, this thesis presents a new enzymatic assay for glucaric acid. The following was presented to show that the glucarate in blood and bile was actually being measured: 1) no cross reaction occurred with a number of compounds, 2) there was a close approximation of the glucarate levels in bile obtained by this assay to those levels presented by Yagamuchi (45), and lastly, 3) identical value was obtained on the same pooled sample of blood using NADH and NADPH as coenzymes (since LDH cannot utilize the NADPH the OD change using NADH is exactly double that produced using NADPH). Using this assay, the presence of D-glucaric acid in human blood was detected for the first time; the presence of glucarate in human bile was also comfirmed. Previously, glucarate was reported to be present only in human bile and urine. Using this new assay, a wide range of glucarate levels was found in both human blood and human gallbladder bile: human whole blood values ranged from 6 to 67 μgm glucarate/ml (0.58 to 6.70 mg/ 100 ml), while human gallbladder bile values ranged from 6 to 413 μgm/ml (0.6 to 413 mg/100 ml). As already mentioned, the latter values

approximated those of Yamaguchi (38 to 634 $\mu$gm/ml). The cause of such a wide range of glucarate concentrations is not presently known, although glucarate metabolism in humans is known to be affected by physiological conditions, disease and drugs.

A portion of this work was presentd at the American Chemical Society Meeting in Chicago, Ill., September, 1967; an abstract was published (11a).

## Chapter V. Acknowledgements

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