Synthesis Of A Glyburide Derivative And Application In A Heterogeneous Enzyme Immunoassay Using Enzymatic Cycling.

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Synthesis of a glyburide derivative and application in a heterogeneous enzyme immunoassay using enzymatic cycling

Stephon, Robert Lee, Ph.D.

Lehigh University, 1991

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Synthesis of a Glyburide Derivative and Application in a Heterogeneous Enzyme Immunoassay Using Enzymatic Cycling

by

Robert Lee Stephon

A Dissertation

Presented to the Graduate Committee

of Lehigh University

in Candidacy for the Degree of

Doctor of Philosophy

in

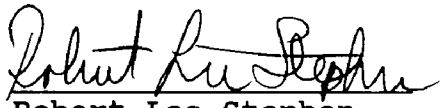
Chemistry

Lehigh University

September 20, 1991

CERTIFICATE OF PRESENTATION

This dissertation is respectfully submitted to the Graduate Faculty of Lehigh University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.



Robert Lee Stephon

A CERTIFICATE OF APPROVAL

Approved and recommended for acceptance as a dissertation in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Sept. 20 1991
Date

Ned D. Heindel
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ACKNOWLEDGMENTS

I would like to express gratitude to my committee members for their suggestions and guidance, as well as to Drs. Carl J. Lacey, Daniel Lima, and James Sturm for their expert help. I would especially like to thank Drs. Ned D. Heindel and Keith J. Schray for their patience and tutelage, Dr. Natalie Foster for her encouragement and enthusiasm, and Dr. Sam Niedbala for his support and friendship throughout this project.

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Abstract

The primary urinary metabolite of glyburide, 4-trans-[1-[[4-[2-(5-chloro-2-methoxybenzamido)ethyl]phenyl]sulfonyl]-ureido]cyclohexanol, has been synthesized de novo in six steps. Its hemisuccinate ester has been formulated to prepare an antigen that most closely resembles it.

The hemisuccinate ester was subsequently attached to bovine serum albumin via the protein's available lysine residues, and the resultant conjugate was used as an antigen to generate antibodies to glyburide in rabbits. The hemisuccinate ester was also conjugated to the surface carbohydrate residues of peroxidase. The purified antibodies were used in a solid-phase enzyme immunoassay for glyburide metabolites in urine, in which the drug-enzyme conjugate served as the color development catalyst. The immunoassay demonstrates crossreactivity with the primary metabolites of glyburide but not with metabolites of the closely related oral hypoglycemic drug, glipizide, or metabolites of other hypoglycemics such as tolbutamide or chlorpropamide.

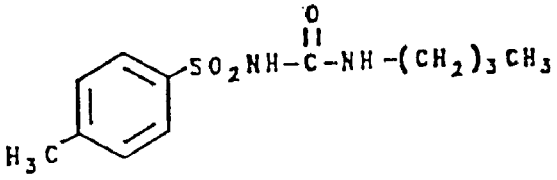
Several model systems were evaluated that may demonstrate greater sensitivity in a heterogeneous enzyme immunoassay for glyburide and other analytes in urine using the technique of enzymatic cycling. A cyclic derivative of nicotinamide adenine dinucleotide phosphate (2':3'-cyclic NADP+) was converted to β-NADP+ through hydrolysis of the cyclic compound by 3'-phosphodiesterase, 2':3'-cyclic nucleotide which was

bound to a solid surface by antigen-antibody interactions. The cofactor thus generated acts as a "trigger" that sets in motion a cyclic sequence of enzyme catalyzed events that produces many molecules of formazan, the reduced, colored form of p-iodonitrotetrazolium violet, for each molecule of β-NADP+ produced by the phosphodiesterase cleavage step. The kinetic parameters of this enzyme cycle are presented and applied with regard to the optimization of an enzyme immunoassay for 4-hydroxyglyburide in urine.

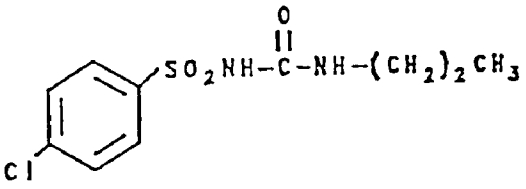
Introduction

Glyburide,1-[[4-[2-(5-chloro-2-methoxybenzamido)ethyl]-phenyl]sulfonyl]-3-cyclohexylurea (Micronase, The Upjohn Company), is a second generation orally active sulfonylurea drug used in the treatment of type II diabetes mellitus. Type II, or noninsulin-dependent diabetes mellitus (NIDDM), is characterized by pancreatic β-cell abnormalities and diminished insulin receptor responses. NIDDM affects approximately 2% to 4% of the population of the United States, or about fourteen times the number affected by type I, insulin-dependent diabetes mellitus. Pancreatic insulin secretion in NIDDM ranges from moderately impaired to slightly elevated; however, insulin responsiveness of the peripheral tissues, i.e., muscle, fat, and liver is consistently decreased. Such "insulin resistance" may be due to a receptor defect (either reduction in the number of insulin receptors in the periphery, or decreased receptor affinity), a postreceptor or postbinding defect such as abnormal coupling between receptor complexes and the glucose transport system, reduced transport protein activity, or some combination of these.[1] Failure in the control of NIDDM by exercise and dietary management indicates therapy with either a first or second generation sulfonylurea.

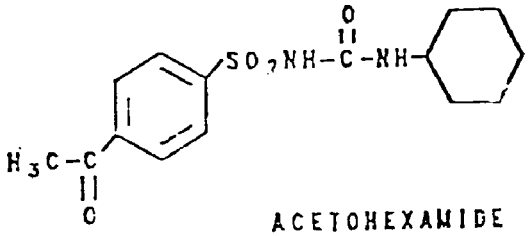
The first effective hypoglycemic sulfonylurea introduced in the United States was tolbutamide, followed by chlorpropamide, acetohexamide, and tolazamide (Figure 1).



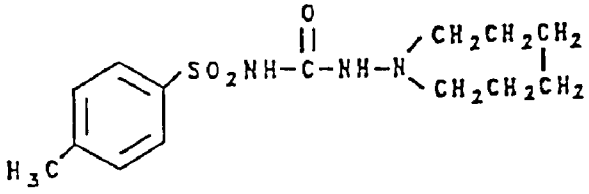
TOLBUTAMIDE



CHLORPROPAMIDE



ACETOHEXAMIDE



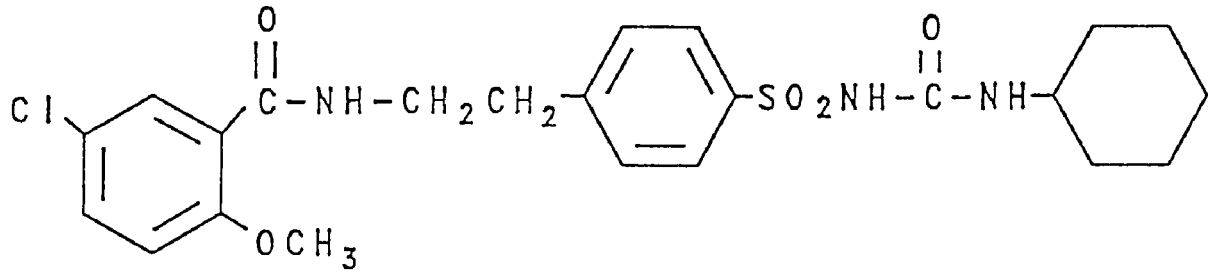
TOLAZAMIDE

Figure 1. Structures of tolbutamide, chlorpropamide, acetohexamide, and tolazamide

All of these drugs have been widely used for controlling noninsulin-dependent diabetes mellitus, and all suffer from some shortcomings. Tolbutamide has a short plasma half-life and must be administered several times a day, although even at this dosage some patients are still not adequately responsive. Chlorpropamide, on the other hand, has a half-life of approximately 25 to 42 hr and is potent enough that a single dose can cause severe and protracted hypoglycemia in some patients.[2] The challenge of finding a safe, effective, single daily dose of a hypoglycemic drug led to the development of the second generation sulfonylurea compounds, of which glyburide (Figure 2) has become one of the most widely used.

Changes in the molecular structure of the second generation sulfonylureas have profoundly influenced therapeutic potency. Per milligram, glyburide is about two hundred times more potent than tolbutamide, although their half-lives are comparable. This phenomenon can best be understood in the context of how these structural differences influence plasma membrane diffusion and serum protein binding characteristics.

The hypoglycemic actions of the sulfonylureas seem to be two-fold. These drugs enhance the secretion of insulin by the pancreatic islet cells and increase insulin's ablility to facilitate the entry of glucose into insulin-sensitive tissues.[3] At short times post-dosing, the greater effect is on insulin release. In the long term, however, the plasma



GLYBURIDE

Figure 2. The structure of glyburide

insulin may return to pretreatment levels. Thus, the sulfonylureas' continued hypoglycemic potency seems to lie more in their ability to enhance the effect of available insulin on tissues; in other words, to decrease insulin resistance. Both first and second generation sulfonylureas increase the insulin sensitivity of target tissue cells by either increasing the number of insulin receptors or decreasing the rate of receptor degradation.[4,5] The even greater increase in insulin sensitivity attributed to the second generation sulfonylureas appears to lie in the ability of these large, nonpolar, more lipid-soluble agents to more easily penetrate the cell membrane. Theoretically this should enhance the effect of insulin on intracellular glucose transport and metabolism; the polar, more water-soluble first generation drugs cannot easily diffuse into the cell. Furthermore, a disadvantage of first generation sulfonylureas is the anionic charge that they possess at physiological pH. This results in marked non-covalent protein (albumin) binding. Drugs bound in such a manner can be displaced by other anionic drugs, such as salicylates. The displaced sulfonylurea molecules may then attach to the target cell membrane, causing abrupt diffusion of glucose into the cell and an acute hypoglycemic episode. Because they have no significant electrical charge, second generation sulfonylureas like glyburide are not bound to albumin in the first place and cannot cause a similar reaction.

Glyburide is typically administered in doses of 1.25 to 10 mg per day, giving rise to peak serum or plasma concentrations of about 150-350 ng/ml within 4 hr.[6] The half-life of glyburide in plasma is approximately 10 hr;[7] hypoglycemic effects, however, may persist for 24 hr.[8] Glyburide is completely metabolized in the liver,[9] principally to 4-trans-hydroxyglyburide, and to a lesser extent is also converted to the 3-cis-hydroxy derivative.[10] Most urinary excretion occurs within the first 6 to 24 hr after oral administration of the drug.[11] Following administration of a single 5 mg dose of glyburide, approximately 30% to 50% of the drug is excreted in urine as metabolites within 24 hr; about 80% of the urinary excretion occurs as the 4-trans-hydroxy metabolite, 15% as the 3-cis-hydroxy metabolite, and 5% as a yet unidentified metabolite.[6]

While the increased efficacy of glyburide enables a single daily dose to supply the necessary hypoglycemic effect to most patients as compared to a drug such as tolbutamide, the lower dosages may give rise to such reduced quantities of the metabolites in urine that establishing patient compliance, or detection of the drug at all, becomes difficult. Assays for glyburide have included spectrophotometric, fluorometric, and colorimetric methods, all of which lack the requisite sensitivity for the detection of this drug in biological fluids.[12] Likewise, a liquid chromatography procedure exists for the determination of glyburide in human serum.[13]

Radioimmunoassay techniques are available for therapeutic monitoring of the drug in serum and plasma, using antisera raised to specific derivatives of the parent compound.[14,15] Screening for the presence of glyburide metabolites in urine, however, would provide a simpler, noninvasive technique for detection of the drug.

Enzyme-based immunoassays are typically less sensitive than radioimmunoassays but are preferred because they are generally safer and more economical.[16] The sensitivity of an enzyme immunoassay for the primary metabolite of glyburide (4-hydroxyglyburide) in urine may be increased by synthesis of an antigen that most closely resembles this structure (Figure 3), i.e., the antisera must be generated to the closest approximation of the established primary urinary metabolite.[17]

The first segment of this project entails a unique synthetic pathway to this target, followed by conversion of the 4-hydroxy derivative of glyburide to its hemisuccinate ester (Figure 4).[18,19,20] Secondly, this derivative is conjugated to bovine serum albumin (BSA)[21] for the production of antibodies to the metabolite, and to the enzyme peroxidase[22] (from horseradish) for use in a solid-phase enzyme immunoassay for glyburide metabolites in urine. The kinetics of enzymatic cycling using a model system that generates the cofactor β-NADP+ and cycles it between its oxidized and reduced forms is explored, and, lastly, an enzyme immunoassay capable of detecting sub-nanogram quantities of 4-hydroxyglyburide is

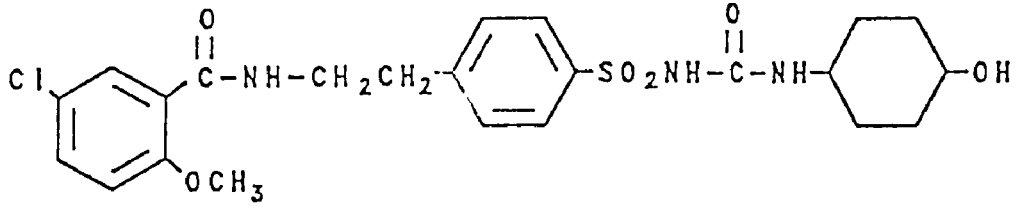


Figure 3. The structure of 4-hydroxyglyburide

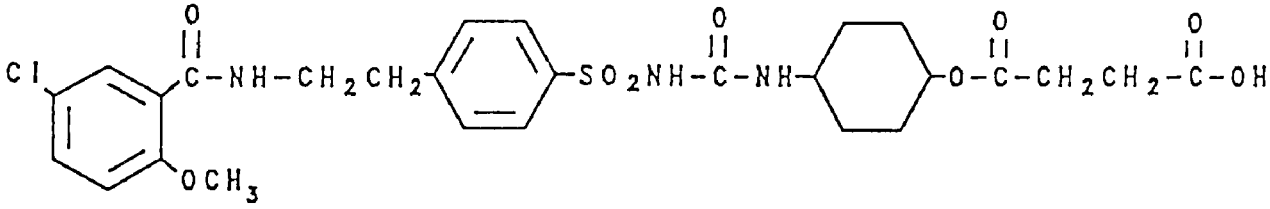


Figure 4. The structure of 4-hydroxyglyburide, hemisuccinate ester

developed with significantly increased sensitivity by using this enzyme-catalyzed cycle.

Enzymatic cycling was first developed for the analysis of the pyridine nucleotides nicotinamide adenine dinucleotide, NAD+/NADH, and their phosphorylated analogues, NADP+/NADPH.[23,24,25] The detection of these coenzymes is hindered by the low levels of each that exist in biological tissues. The technique of enzymatic cycling effectively amplifies the spectrophotometric signal caused by the oxidation or reduction of the cofactors themselves. For example, the reaction scheme developed for the analysis of NADPH (Figure 5) makes use of an enzyme/substrate combination that alternatively oxidizes and reduces the coenzyme with the concomitant production of a substance that is measured in a subsequent step. Under the appropriate reaction conditions (optimum pH, noncycling substrate concentrations greatly exceeding their respective Michaelis constants) each molecule of NADP+ formed from the oxidation of NADPH by glutamic dehydrogenase is capable of catalyzing the formation of 5,000 to 10,000 molecules of 6-P-gluconate. After the destruction of the cycling enzymes by heat or pH extreme the 6-P-gluconate is measured with additional NADP+ and 6-P-gluconate dehydrogenase.

Since the initial development of cofactor cycling, many variations of enzyme amplification schemes have been formulated. These include enzyme cascades, such as the

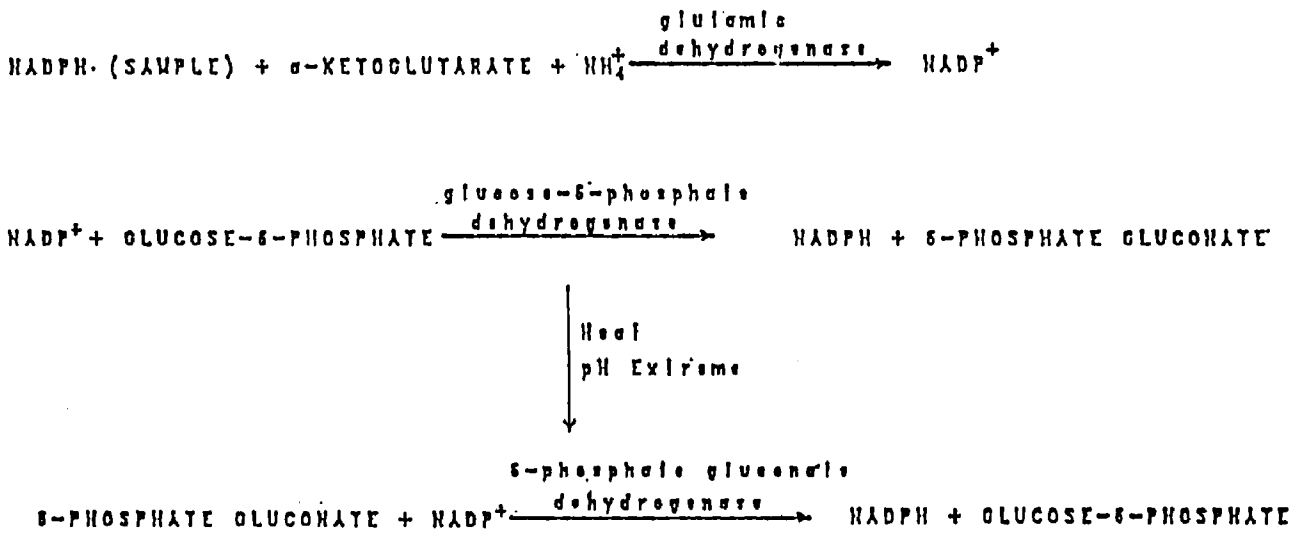


Figure 5. An enzymatic cycling technique for β-NADP+ or β-NADPH that uses the cycling enzymes glutamic dehydrogenase and glucose-6-phosphate dehydrogenase.

glycogen phosphorylase cascade (Figure 6)[26] and the sequential activation of protein factors responsible for the clotting of blood (Figure 7),[27,28] in which the final step of each cascade is a multiplicative function of each of the rate constants for all preceding steps in the cascade. Other enzyme cycles have included an enzyme amplified immunoassay adapted for electrochemical measurement, using an NAD+/NADH redox cycle coupled to an electrode via the active site of one of the cycling enzymes,[29] and a biochemical futile cycle in which glucose-6-phosphate is first converted to fructose-6-phosphate by glucose isomerase. Fructose-6-phosphate is converted to fructose-1,6-diphosphate by phosphofructokinase in the presence of ATP and then back to fructose-6-phosphate by fructose bisphosphate phosphatase. One molecule of inorganic phosphate (P_i) is liberated with each turn of this cycle. The inorganic phosphate formed can be subsequently detected by standard chemical means (Figure 8).[30] Most recently, an enzyme cycle has been employed in which a cofactor is generated <u>in situ</u> by an enzyme bound to an antibody immobilized on a solid surface. The generated cofactor cycles between its oxidized and reduced forms, producing one molecule of reduced formazan dye with each turn of the cycle (Figure 9).[31] The colored formazan is detected by simple spectrophotometric means, and the sensitivity of the assay can be regulated by the length of time the cofactor is permitted to cycle.

This dissertation discusses the use of new enzymes as

cofactor generators for cycling, including NAD+ kinase that converts β-NAD+ to β-NADP+, and 3'-phosphodiesterase, 2':3'-cyclic nucleotide, which converts 2':3' cyclic NADP+ to β-NADP+.

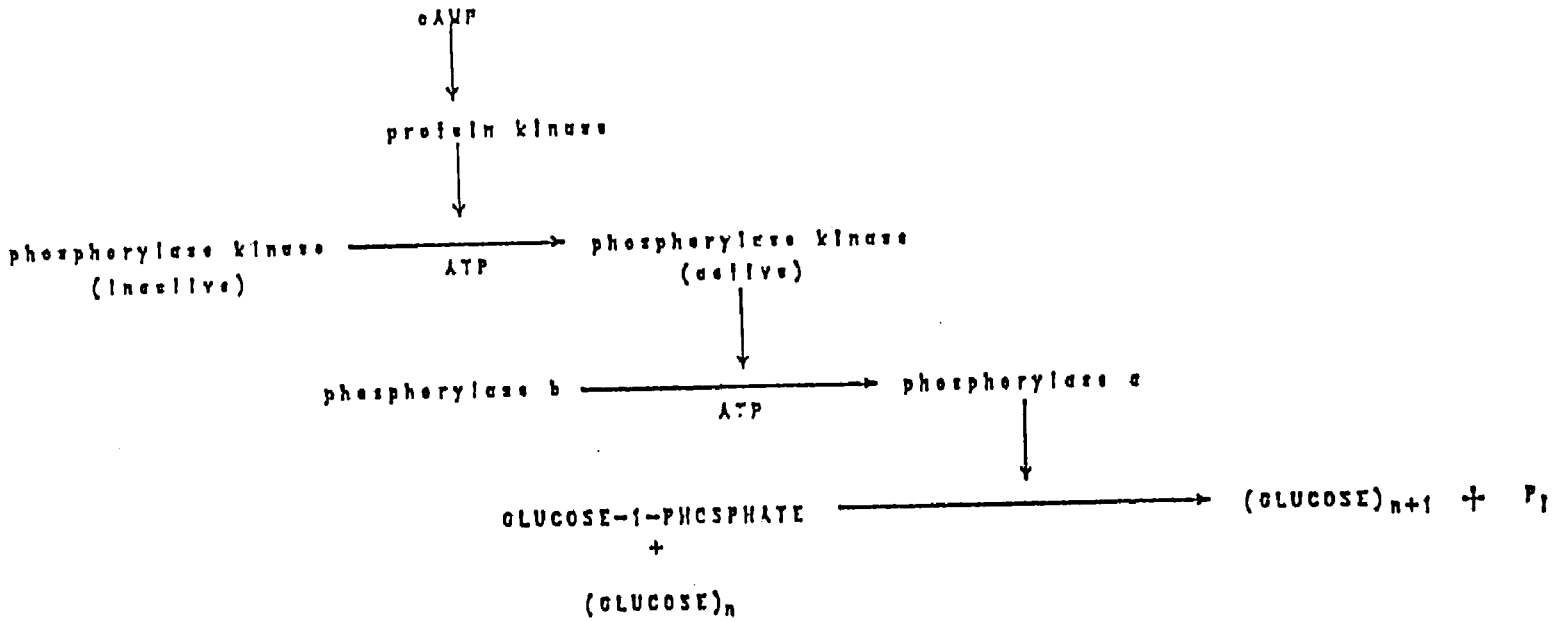


Figure 6. The cAMP-phosphorylase cascade

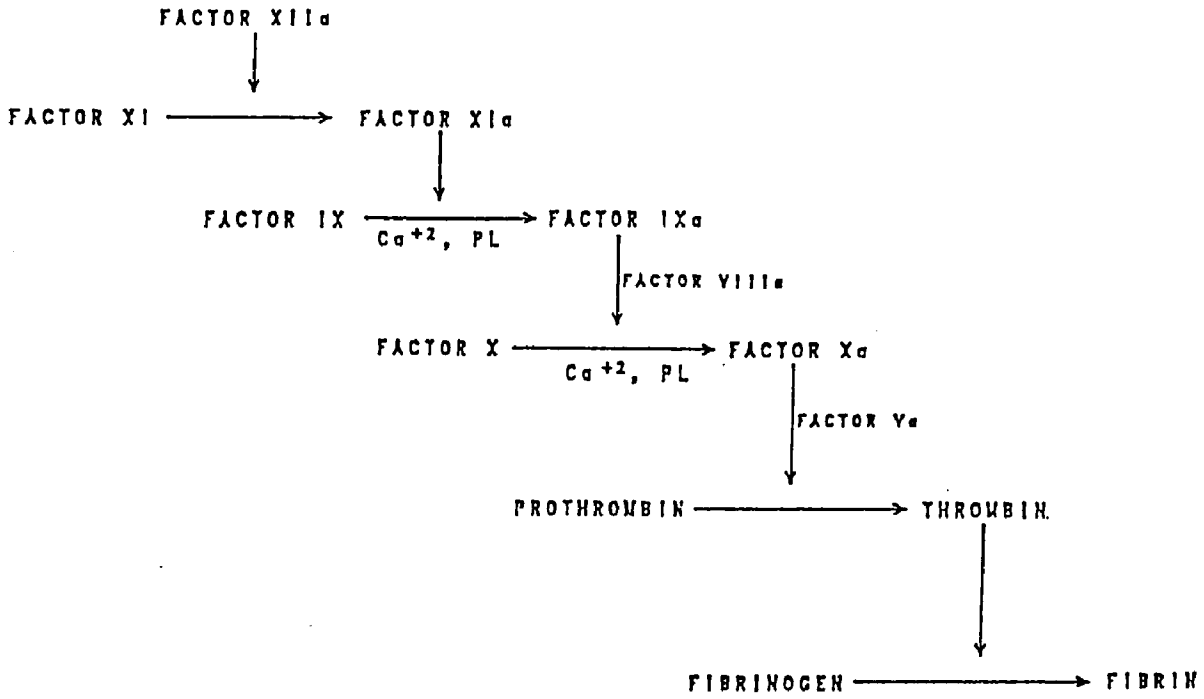


Figure 7. The blood-clotting cascade

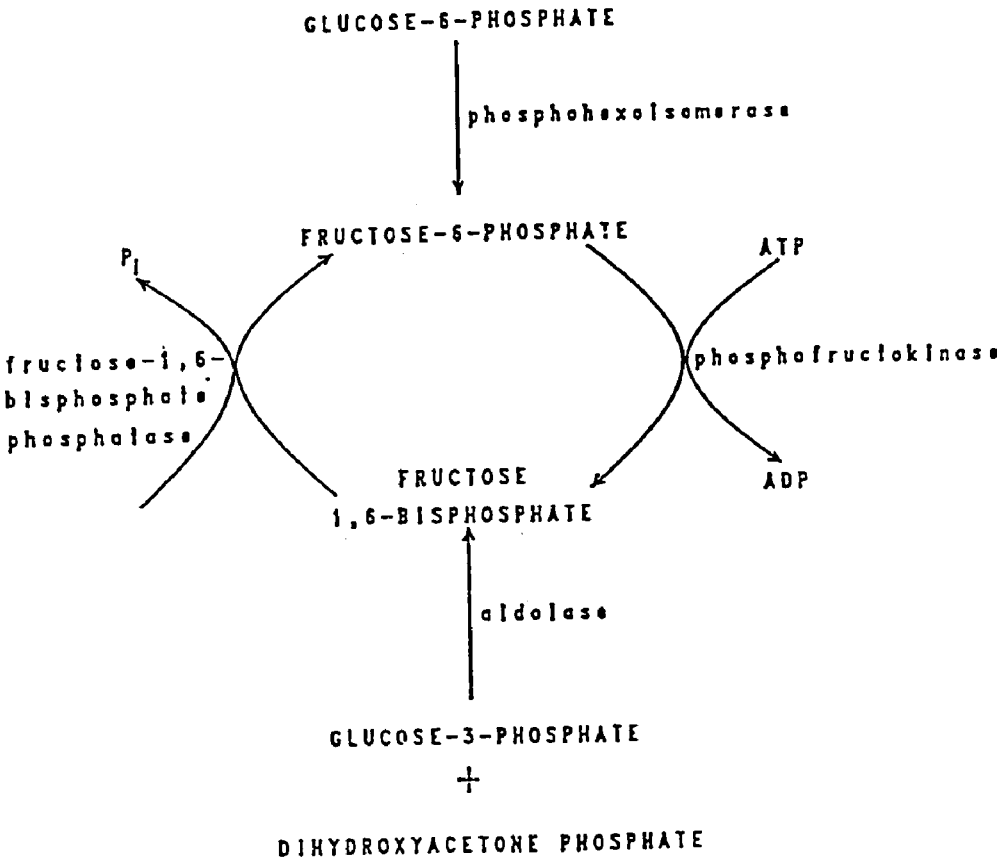


Figure 8. The fructose-6-phosphate, fructose-1,6-bisphosphate futile cycle

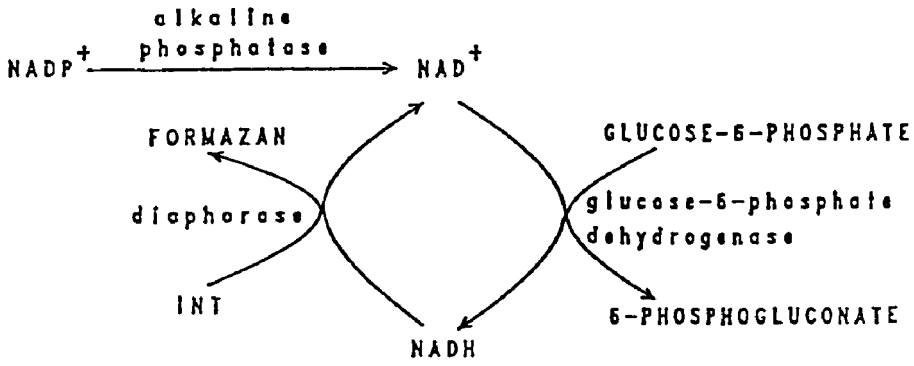


Figure 9. An enzymatic cycling procedure for β-NAD+ using alkaline phosphatase as the "trigger" enzyme, and glucose-6-phosphate dehydrogenase and diaphorase as the cycling enzymes

Chapter 1

Synthesis of 4-trans-[1-[[4-[2-(5-chloro-2-methoxybenzamido)ethyl]phenyl]sulfonyl]ureidocyclohexanol hemisuccinate ester

I. Introduction

Immunoassays for glyburide and its metabolites have already been developed using the hemisuccinate ester of the primary metabolite of glyburide, 4-trans-[1-[[4-[2-(5-chloro-2-methoxybenzamido)ethyl]phenyl]sulfonyl]ureido]cyclohexanol, or 4-hydroxyglyburide (Figure 10, R = OH) for the preparation of an appropriate antigen. In these cases, however, the 4-hydroxy analogue was obtained through rigorous extraction and purification procedures from the biological fluids of clinical subjects.[32,33] Other methods of obtaining the 4-hydroxy compound have included extraction and isolation of the metabolite following incubation of the parent drug with liver microsomes,[34] and from an aerobic culture of Streptomyces griseolus.[35]

Radioimmunoassays for glyburide and its metabolites have also been developed using antibodies raised to various derivatives of the parent drug itself. Royer's analyte model was prepared with a 4-acetic acid moiety as the attachment handle (Figure 10, R = CH_2COOH).[14] Synthesis of that target was accomplished by condensation of methyl 4-(isocyanato)-cyclohexylmethylcarboxylate with the known sulfonamide (Figure 11). This reaction scheme was, in fact, pioneered by Hsi who also employed this compound (bearing either a ^{14}C or a ^{3}H)

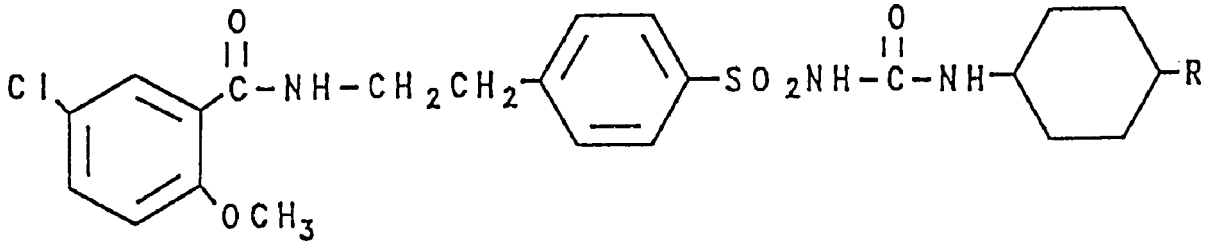


Figure 10. The precursor of various glyburide derivatives

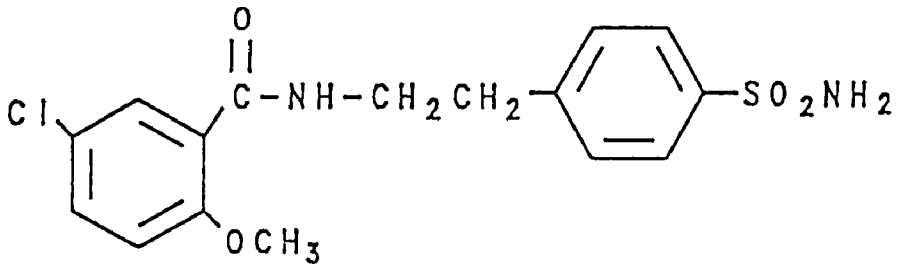


Figure 11. The structure of 4-[2-(5-chloro-2-methoxybenzamido)ethyl]-phenylsulfonamide

with cyclohexylisocyanate to prepare a radiotracer version of glyburide[36] by the very process employed commercially in the synthesis of the parent drug.[37] The synthesis employed herein yields the primary urinary metabolite of glyburide, 4-hydroxyglyburide. To avoid competing reactivities this synthesis avoids the use of an isocyanate. The 4-hydroxyglyburide is then linked to BSA following succinylation of the pendant -OH (Figure 10, R = $OCOCH_2CH_2COOH$). This pathway proceeds via the carbamate derivative of the sulfonamide followed by formation of the 4-hydroxycyclohexyl urea with (trans)-4-aminocyclohexanol.[38,39] This synthetic scheme was first used with (trans)-2-aminocyclohexanol to make the 2-hydroxy derivative of glyburide for subsequent use in animal metabolism studies but never used for the synthesis of a hemisuccinate ester in the development of an immunoassay for the drug or its metabolites.[40]

II. Experimental

A. Materials and Methods

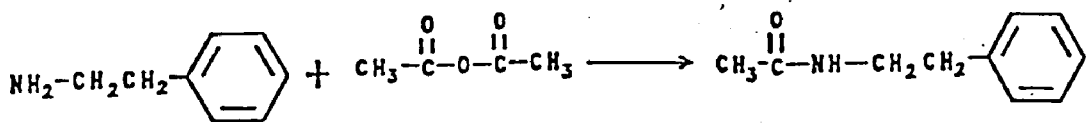
Melting points were determined on either a Fisher-Johns Melting point apparatus or a Melt-temp and are uncorrected. Elemental analyses were performed by Quantitative Technologies, Inc., Bound Brook, New Jersey. Infrared spectra (KBr and neat) were recorded on a Perkin Elmer 1420 spectrometer. ^{1}H-NMR spectra were recorded on a JEOL FX-90Q spectrometer operating at 89.55 MHz. Chemical shifts are reported in parts per million relative to tetramethylsilane

(TMS) and designated as follows: (multiplicity, number of protons, assignment, coupling constant (Hz)). The multiplicity is designated as follows: s=singlet, d=doublet, dd=doublet of doublets, t=triplet, q=quartet, m=multiplet, c=complex, br=broad.

All reagents and starting materials were purchased from the Aldrich Chemical Co., Milwaukee, WI unless otherwise noted and used without further purification. All solvents were ACS Reagent Grade or better unless otherwise noted.

B. Syntheses

1. N-(2-phenylethyl)acetamide.

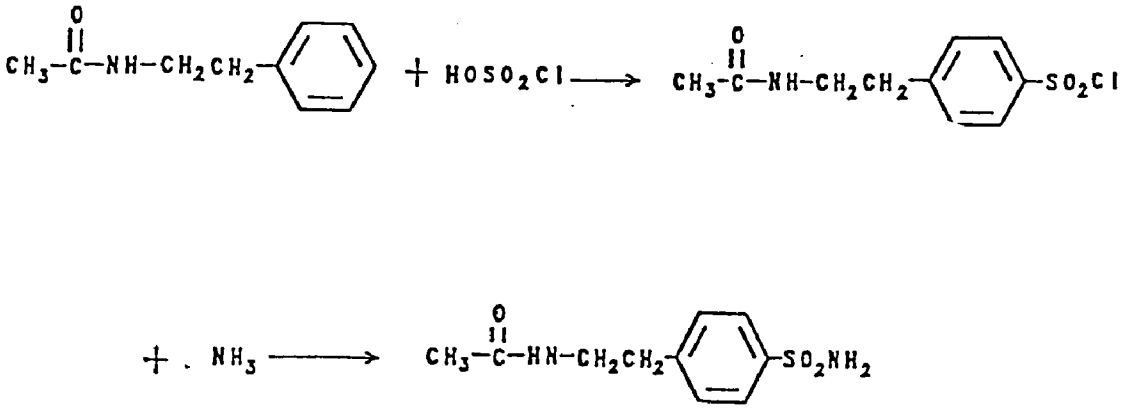


To 250 ml of 2-phenylethylamine (241 g, 1.99 mol) was added, over 1 hr with stirring, 94.0 ml of acetic anhydride (0.995 mol). The mixture was stirred and refluxed for 5 hr, and allowed to cool to room temperature. The crude product was dissolved in a minimum of water (1 l) and the pH adjusted to 9.5 with 5% aqueous NaOH. The crude product was extracted from the residual acetic anhydride/acetic acid with 3 x 50 ml of diethyl ether. The ether was washed with 3 x 50 ml of 5% aqueous HCl to remove any unreacted amine, dried over $MgSO_4$, and removed under reduced pressure. A yield of 155 g (48%) of a waxy yellow solid was obtained, m.p. 39-40° (lit. m.p. 51)[41]; ir (neat) 3300 (NH), 1650 (-CONH-) and 1550 cm^{-1} (-CNH-); ^{1}H-NMR (DMSO-d_6): δ 1.83 (s, 3H, CH_3), 2.72 (t, 2H, Ar-CH_2, J = 7.5 Hz), 3.22 (t, 2H, CH_2-NH, J = 7.5 Hz), 7.22 (m, 5H, Ar-H), and 8.00 ppm (br m, 1H, N-H).

Anal. Calcd for $C_{10}H_{13}NO \cdot 0.25\ H_2O$: C, 71.55; H, 8.11; N, 8.30

Found: C, 71.47; H, 7.92; N, 8.38

2. N-[2-(4-sulfonamidophenyl)ethyl]acetamide.



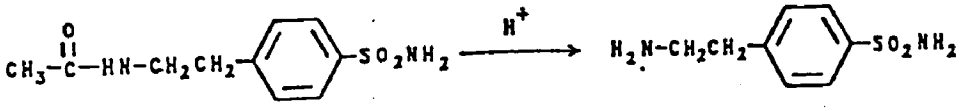
N-(2-phenylethyl)acetamide (150 g, 0.919 mol) was coated onto the inside surface of a 4 l erlenmeyer flask by gently warming the solid, adding the viscous fluid to the flask, and allowing the mass to solidify at 0°, with frequent swirling to ensure an even coating onto the glass surface. Chlorosulfonic acid (372 ml, 4.59 mol) was added in one portion, and the solution mixed for 0.5 hr. The solution was heated in a water bath for 1 hr and cooled to room temperature. The mixture was slowly poured, with stirring, onto 1000 g of ice in a 4 l beaker. The solid material formed was filtered under vacuum and washed with 50 ml of cold water. The crude N-acetyl-2-(phenyl-4-chlorosulfonyl)ethylamine was transferred to a 2 l beaker, and a solution of dilute ammonium hydroxide (100 ml concentrated NH_4OH + 100 ml H_2O) added. The mixture was heated

and stirred for 0.5 hr and allowed to cool to room temperature. The solution was acidified to litmus with 6M HCl, and the resulting solid collected by filtration and washed with cold water. A yield of 142 g (64%) of an off-white solid was obtained, m.p. 110-112° (lit. m.p. 172-173)[42]; ir (KBr) 3310 (NH_2), 3200-3100 (NH), 1610 (-CONH-), 1535 (-CHN-), and 1330 and 1145 cm^{-1} ($-SO_2N-$); ^{1}H-NMR (DMSO-d_6): δ 1.82 (s, 3H, CH_3), 2.81 (t, 2H, Ar-C<u>H</u>$_2$, J = 7 Hz), 3.32 (t, 2H, NH-C<u>H</u>$_2$, J = 7 Hz), 7.31 (s, 2H, NH_2), 7.43 (d, 2H, Ar-H, J = 4 Hz), 7.71 (d, 2H, Ar-H, J = 4 Hz), and 8.00 ppm (br m, 1H, N-H, J = 4 Hz).

<u>Anal</u>. Calcd for $C_{10}H_{14}N_2O_3S$: C, 49.57; H, 5.82; N, 11.56

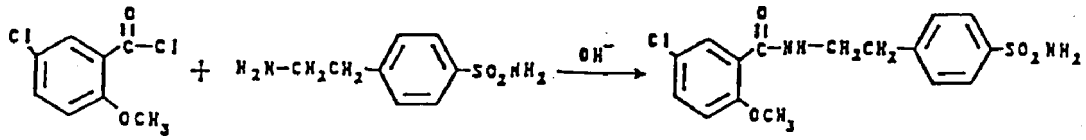
Found : C, 49.97; H, 5.73; N, 12.22

3. N-[2-(4-sulfonamido)]phenylethylamine.



To 100 g (0.413 mol) of N-[2-(4-sulfonamidophenyl)-ethyl]acetamide was added 100 ml of dilute hydrochloric acid (50 ml concentrated HCl + 50 ml water). The solution was stirred and refluxed for 3 hr, and the water removed under reduced pressure. The crude HCl salt of the product was dissolved in a minimum of 20% aqueous NaOH (50 ml) and filtered. The water was again removed under reduced pressure and the solid collected and dried. A yield of 65.5 g (79%) of an off-white solid was obtained, m.p. 250-255°(d) (lit. m.p. 147.5-149)[42]; ir (KBr) 3325 (NH_2), and 1330 and 1155 cm^{-1} (-SO_2N-); ^{1}H-NMR (DMSO-d_6): δ 2.70 (m, 4H, 2 x CH_2's), 3.25 (br s, 4H, 2 x NH_2's), 7.22 (d, 2H, Ar-H, J = 4 Hz), and 7.65 ppm (d, 2H, Ar-H, J = 4 Hz). Because the crude solid was isolated by total evaporation, sodium chloride would obviously by entrapped in the material. The combustion analysis showed that approximately 5 mol of NaCl were admixed per mol of the desired product.

4. **4-[2-(5-chloro-2-methoxybenzamido)ethyl]phenyl-sulfonamide.**

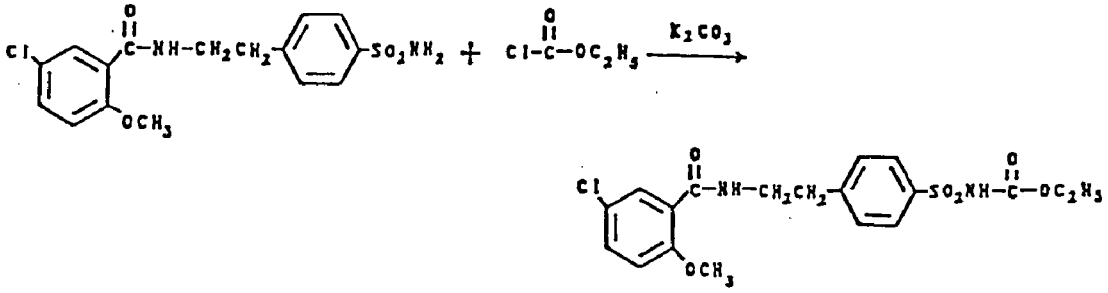


Thionyl chloride (97.7 ml, 1.34 mol) was added to 50 g (0.268 mol) of 5-chloro-2-methoxybenzoic acid; 0.50 ml of N,N'-dimethylformamide was added and the solution was stirred and refluxed for 3 hr. To 48.9 g (0.244 mol) of N-[2-(4-sulfonamido)]phenylethylamine in 100 ml of 10% aqueous NaOH in an ice bath was added 50.0 g (0.244 mol) of 5-chloro-2-methoxy benzoyl chloride over 1 hr with vigourous stirring. The resulting precipitate was isolated by filtration and washed with cold water. The crude product was recrystallized from 95:5 etharol:water containing 1% glacial acetic acid. A yield of 61.3 g (68%) of an off-white solid was obtained, m.p. 204-206°; ir (KBr) 3320 (NH_2), 1630 (-CONH-), 1530 (-CHN-), and 1320 and 1155 cm^{-1} ($-SO_2N-$); ^{1}H-NMR (DMSO-d_6): δ 2.95 (br t, 2H, Ar-CH_2), 3.55 (br t, 2H, NH-CH_2), 3.81 (s, 3H, CH_3O), 7.00 - 8.10 (m, 9H, Ar-H and NH_2), and 8.25 ppm (br m, 1H, N-H).

Anal. Calcd for $C_{16}H_{17}ClN_2O_4S$: C, 52.10; H, 4.61; N, 7.60

Found: C, 52.06; H, 4.66; N, 7.50

5. **Ethyl 4-[2-(5-chloro-2-methoxybenzamido)ethyl]-phenyl]sulfonamidecarbamate.**

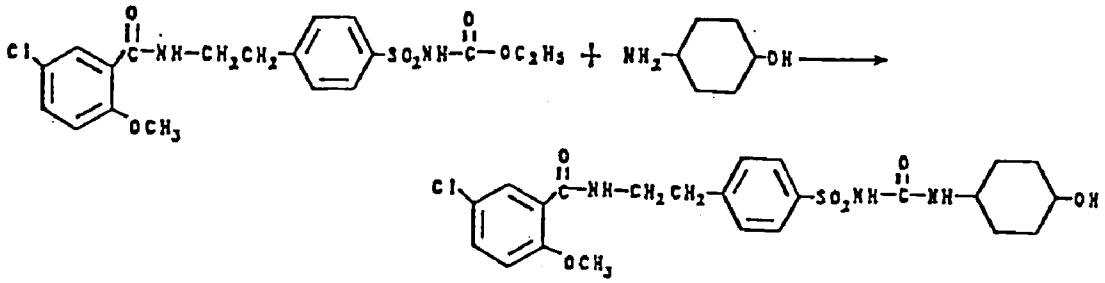


To a mixture of the sulfonamide (5.00 g, 13.6 mmol) and anhydrous potassium carbonate (4.89 g, 35.4 mmol) in 300 ml of acetone was added, over 3 hr with stirring, 1.7 ml (18 mmol) of ethyl chloroformate. The mixture was stirred and refluxed for 48 hr, allowed to cool to room temperature, and filtered. The resulting potassium salt of the carbamate was dissolved in a minimum of water (1 l), and any insoluble material was removed by filtration. The solution was acidified by the dropwise addition of concentrated hydrochloric acid, and the product precipitated. The crude product was recrystallized from 95:5 ethanol:water. A yield of 3.5 g (58%) of white crystals was obtained, m.p. 154-155°; ir (KBr) 3385 (NH), 1735 (-COCONH-), 1635 (-CONH-), 1535 (-CNH-), and 1350 and 1155 cm^{-1} ($-SO_2N-$); ^{1}H-NMR (DMSO-d_6): δ 1.10 (t, 3H, CH_3, J = 8 Hz), 2.95 (t, 2H, Ar-$C\underline{H}_2$, J = 6 Hz), 3.55 (t, 3H, NH-$C\underline{H}_2$ and N-H, J = 6 Hz), 3.82 (s, 3H, CH_3O), 4.05 (q, 2H, O-$C\underline{H}_2$, J = 8 Hz), 7.02 - 8.05 (m, 7H, Ar-H), and 8.32 ppm (br t, 1H, N-H).

Anal. Calcd for $C_{19}H_{21}ClN_2O_6S$: C, 51.76; H, 4.77; N, 6.36

Found: C, 51.28; H, 4.76; N, 6.18

6. 4-trans-[1-[[4-[2-(5-chloromethoxybenzamido)-ethyl]phenylsulfonyl]ureido]cyclohexanol.



(trans)-4-Aminocyclohexanol hydrochloride was converted to its free base and isolated by continuous extraction of a 10% aqueous NaOH solution with diethyl ether. To a solution of the carbamate (1.00 g, 2.28 mmol) in 50 ml of toluene was added, during 1 hr with stirring, a solution of the amine (0.30 g, 2.5 mmol) in 10 ml of toluene. The mixture was stirred and refluxed for 3 hr and cooled to room temperature. The toluene was removed under reduced pressure, and the product crystallized from 95:5 ethanol:water. A yield of 0.78 g (67%) of white solid was obtained, m.p. 195-203°; ir (KBr) 3400 (OH), 1720 and 1650 (urea HNCO carboxamide I and II bands), 1540 (-CHN-), and 1345 and 1160 cm^{-1} ($-SO_2N-$); ^1H-NMR (DMSO-d_6): δ 1.00-1.50 and 1.60-1.95 (m, 8H, cyclohexyl-H), 2.95 (t, 2H, Ar-$C\underline{H}_2$, J = 6 Hz), 3.42 (m, 4H, CH-O, CH-N, 2 N-H), 3.55 (t, 2H, NH-$C\underline{H}_2$, J = 6 Hz), 3.80 (s, 3H, CH_3O), 7.00 - 8.00 (m, 7H, Ar-H), 8.33 ppm (t, 1H, N-H, J = 4 Hz).

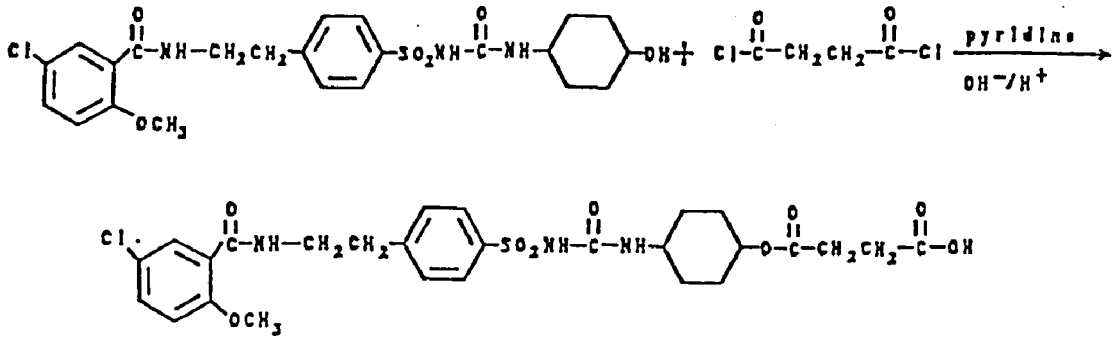
Anal. Calcd for $C_{23}H_{28}ClN_3O_6S$: C, 54.16; H, 5.54; N, 8.24

Found: C, 54.24; H, 5.40; N, 7.92

There was considerable difficulty in obtaining acceptable combustion analytical data. Multiple samples were prepared, recrystallized, and submitted. All samples submitted for analysis had identical TLC behavior (R_f of 0.61 on silica gel plates with a moving phase of heptane:ethanol:chloroform:water 33:33:33:1), identical melting ranges of 195-203°, and identical NMR spectra.

A low resolution mass spectrum was performed on a Kratos Mass Spectrometer Model 83-MS run in FAB mode at 4 kVolt source potential in a matrix of 3-nitrobenzyl alcohol. This run was designed to determine the unit mass of the parent ion (510 amu). Six subsequent high resolution runs calibrated against the 503 and 547 amu peaks of polyethylene glycol of ca 600 molecular weight were performed to determine the exact mass of that parent. The experimentally determined M/z was 510.1401 ± 0.005 amu. The unit's computer then calculated for this mass a "best-fit" formula which was determined as $C_{23}H_{28}ClN_3O_6S$ for which the theoretical mass would be 510.1466.

7. **4-trans-[1-[[4-[2-(5-chloro-2-methoxybenzamido)-ethyl]phenyl]sulfonyl]ureido]cyclohexanol hemisuccinate ester.**



To a mixture of 4-hydroxyglyburide (0.500 g, 0.980 mmol) and succinyl chloride (0.12 ml, 1.1 mmol) in 10 ml of warm anhydrous DMF was added, during 0.5 hr with stirring, a solution of pyridine (0.08 ml, 1 mmol) in 2 ml of DMF. The solution was stirred for 4 hr and the reaction mixture added, dropwise with stirring, to 10 ml of a saturated aqueous sodium bicarbonate solution chilled in an ice bath. After 0.5 hr the liberation of CO_2 ceased, and the pH was adjusted to 5 with concentrated hydrochloric acid. The product was isolated by filtration, washed with cold water, and recrystallized from 95:5 ethanol:water. A yield of 0.33 g (55%) of an off-white solid was obtained, m.p. 199-200°; ir (KBr) 3400 (NH), 1750 (-$OCOCH_2$-), 1725, 1710, and 1655 (-COOH, and urea HNCO carboxamide I and II bands), 1545 (-CHN-), and 1390 and 1165 cm^{-1} (-SO_2N-); ^{1}H-NMR (DMSO-d_6): δ 1.20-1.50 and 1.62-2.05 (m, 8H, cyclohexyl-H), 2.83-3.73 (overlapping m, 12H, 4 x CH_2's, CH-O, CH-N, and 2 N-H), 4.01 (s, 3H, CH_3O), 7.30 - 8.22 (m, 7H, Ar-H), and 8.55 ppm (br m, 1H, N-H).

Anal. Calc for $C_{27}H_{32}ClN_3O_9S.0.5\ H_2O$:

C, 52.38; H, 5.37; N, 6.79

Found: C, 52.03; H, 5.31; N, 6.87

Chapter 2

Generation of Antisera to the Glyburide Derivative

I. Introduction

The sensitivity and specificity of an immunoassay depend on the binding characteristics of the antibodies used.[43] These, in turn, are dependent on the quality of the immunogen used to obtain them; i.e., its size, degree of hapten incorporation, and purity. The goal of this section of this dissertation is to describe the preparation of a protein conjugate of the glyburide derivative that will elicit an appropriate immune response when injected into animals for the production and isolation of specific IgG antibodies to the 4-hydroxy metabolite of glyburide.

In general, the immunogenicity of a substance is directly related to its molecular weight.[43,44] Antigens with molecular weights of less than 1000 do not possess much potency as immunogens by themselves. Low molecular weight molecules like glyburide hemisuccinate ester combined with large proteins such as keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) become antigenic compositions. The quality of an antigen is also related to the size of the "bridge" between itself and a carrier protein; that is, the length of the chemical spacer between the protein and its hapten. An ideal spacer is about 4-6 atoms in length and improves the specificity of the resultant antibodies by ensuring that the hapten projects above the hydrated surface of the protein.

The site of the linkage is also of critical importance for the specificity of the antiserum. The most distinctive portion of the hapten should be the most remote from the linkage site and thus accessible to the animal's immune system in unaltered form.[43]

The antigen is typically injected as a water-in-oil emulsion in complete Freund's adjuvant. This mixture consists of mineral oil, a detergent, and heat killed Mycobacterium tuberculosis. The hydrophobic mineral oil remains at the injection site and delays absorption. The oil droplets are slowly removed by macrophages of the reticuloendothelial system allowing the antigen contained therein to be progressively released via a route which provides maximal exposure to the immune system. The detergent acts as an emulsifier so that the aqueous antigen suspension is contained within the droplets of mineral oil. The killed mycobacteria serve as a non-specific stimulus to the reticuloendothelial and immune systems. Only the initial injection and two or three booster injections require the use of this material. Subsequent booster injections make use of incomplete Freund's adjuvant, which lacks the bacteria. The type of animal used is generally dependent on the quantity of antiserum desired; i.e., a small animal such as a rabbit or guinea pig will not provide as much serum as will a sheep or goat. Consistent differences between species in their response to a given immunogen have not been demonstrated.[43,44]

The amount of immunogen used for injection is not critical, and may vary over a wide range. Usually, amounts on the order of 0.05 to 1 mg per injection into a rabbit, for instance, are enough to provoke an initial immune response. Doses greater than 1 mg may produce the condition of tolerance in which the recipient animal has no immune response to either the first or subsequent injections. Injection schedules may vary, though typically a booster injection containing about one-half of the initial amount of antigen is administered approximately 2-4 weeks after the first dose, then every 2 weeks after that, preceded by "test bleeds" to determine whether the specific antibody is present and whether sufficient titer exists to warrant the collection and purification of larger quantitites of blood from the animal.[44]

Antiserum may be used directly following its separation from the blood clot, although some purification procedures are often used. These procedures include partial separation of the IgG protein fraction from the serum by differential precipitation with ammonium sulfate. More more rigorous procedures such as ion exchange chromatography or affinity chromatography may also be used.[43,44]

Bovine Serum Albumin (BSA) contains a total of 59 lysine residues, out of which 30-35 possess ϵ-NH_2 groups that are available for conjugation. To prepare a potent immunogen approximately one-half, or 15-18 of these available lysines should be bound to drug molecules.[45]

Several methods are available for determining the number of haptens attached to a protein. Among these are measurement of the difference of ultraviolet spectra between free protein and protein containing haptens. The degree of hapten incorporation onto the protein is related to the increase in absorbance at a wavelength specific for the hapten, corrected for the amount of protein in the conjugate as measured by absorbance at 280 nm, the $wavelength_{max}$ of most proteins corresponding to the presence of aromatic amino acid residues (phenylalanine, tyrosine, tryptophan).[46,47] This technique is not adequate for the present case, however, because glyburide itself absorbs maximally the same wavelength region as BSA.

Another measurement technique involves the use of protein binding reagents. Certain electrophilic reagents attach covalently to free ϵ-NH_2 groups on proteins, forming conjugates whose color intensity is related to the quantity of free lysines present. Comparison of similarly treated BSA-hapten conjugates and free BSA thus provides a method for the estimation of the degree of hapten incorporation onto the protein.[48,49] In the present technique, an excess of 1-fluoro-2,4-dinitrobenzene is added to both BSA and to BSA to which glyburide hemisuccinate ester has been conjugated.[50] After correcting the observed absorbance for the amount of protein in each,[51] the number of lysine residues remaining free is calculated, and hence the number of lysines bound to glyburide hemisuccinate ester can be determined.

II. Experimental

A. Conjugation of the Glyburide Derivative to BSA

The glyburide hemisuccinate ester (100 mg, 0.164 mmol) was dissolved in 1 ml of DMF and 1 ml of a buffer consisting of 200 mM tris(hydroxymethyl)aminomethane, pH 6.0. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (42.6 mg, 0.180 mg) (Sigma) was added, the solution mixed vigorously, the pH adjusted to 6.0, and the mixture incubated at 2-8°C for 1 hr. A total of 111 mg (0.0164 mmol) of bovine serum albumin (BSA, Fraction V) (Sigma) was dissolved in 1 ml of the same buffer, mixed gently, and added to the EDC-activated hemisuccinate ester of glyburide over 0.5 hr with stirring. The pH of the solution was adjusted to 6.0 and the mixture incubated overnight at 2-8°C. The conjugate was purified by dialysis against 2 x 2 l of 200 mM Tris, pH 7.5, over 48 hr. The BSA-glyburide conjugate was lyophilized and stored in a brown bottle at 2-8°C.

B. Characterization of the Conjugate

BSA (10.0 mg) and 10.0 mg of BSA-glyburide conjugate (BSAG) were added to 5.00 ml of water in separate beakers and gently stirred. Protein dye reagent (5 ml of a solution composed of 100 mg Coomasie Brilliant Blue (Sigma); 50 ml 95% ethanol, 100 ml 85% phosphoric acid, diluted to 1.00 l with water and filtered through Whatman #2 filter paper)[52,53] was added to 0.100 ml of each solution in separate test tubes and mixed thoroughly. After 5 minutes the absorbance at 595 nm

vs. a reagent blank was measured and recorded. Aqueous sodium bicarbonate (1 ml of a 4.2% solution; 4.20 g sodium bicarbonate diluted to 100.0 ml with water) was added to the remaining BSA and BSAG solutions, and the pH of each adjusted to 9.0 with 100 mM NaOH. A 10 mg/ml solution (0.300 ml aliquot) of 1-fluoro-2,4-dinitrobenzene (20 mg FDNB, 2 drops 20% aqueous NaOH, diluted to 2.00 ml with water) was added to each, with stirring, and the pH of each readjusted to 9.0. The solutions were stirred for 2 hr, with the pH of each checked every 0.5 hr and readjusted to 9.0 if necessary. The BSA/FDNB and BSAG/FDNB solutions were then dialyzed against 3 x 2 l of 100 mM sodium bicarbonate buffer (16.8 g sodium bicarbonate, pH adjusted to 9.0, diluted to 2.00 l with water) over 48 hr. The absorbance of each at 360 nm vs. a reagent blank was measured and recorded. The number of free lysines in the BSAG conjugate was then determined as follows:

DATA

$A(BSA)_{595} = 1.384$ $\qquad$ $A(BSA)_{360} = 0.819$

$A(BSAG)_{595} = 1.041$ $\qquad$ $A(BSAG)_{360} = 0.157$

Calculations

A(BSA,adjusted for protein content) =

$$\frac{A(BSA)_{360}}{A(BSA)_{595}} = \frac{0.819}{1.384} = 0.592$$

A(BSAG,adjusted for protein content) =

$$\frac{A(BSAG)_{360}}{A(BSAG)_{595}} = \frac{0.157}{1.041} = 0.151$$

$$\frac{A(BSA,adjusted)}{(35\ free\ lys)} = \frac{A(BSAG,adjusted)}{(\#\ of\ free\ lys)}$$

$$(\#\ free\ lys) = \frac{A(BSAG,adjusted)\ x\ 35}{A(BSA,adjusted)}$$

$$= \frac{(0.151)\ x\ 35}{(0.592)}$$

= 9 free lysines on BSAG, if 35 free lysines were originally available on BSA

= 8 free lysines on BSAG, if 30 free lysines were originally available on BSA

The calculations indicate that there were approximately 26 or 27 molecules of glyburide hemisuccinate ester successfully bonded to the available lysine residues of bovine serum albumin. Between 15 and 18 bound haptens are required to make a sufficiently potent antigen; therefore, this conjugation was successful. The conjugate was provided to Lampire Biologicals, Inc., Pipersville, PA for the production of antibodies. The conjugate was also used for the production of glyburide antibodies using healthy, adult, New Zealand white rabbits provided by STC, Inc. (115 Research Drive, Bethlehem, PA) and Lehigh University. Identical injection schedules were followed for each animal population. The antibody production schedule at Lehigh University was abandoned, however, because of the expiration of the rabbit population.

C. Injection of the Immunogen

The following schedule was used for the injection of the BSA-glyburide conjugate into each of two adult, healthy, white New Zealand rabbits at Lampire Biologicals:

WEEK	QUANTITY INJECTED (PROTEIN)	SITE/MODE OF INJECTION
0	1 mg Comp. Freund's	Back/Intradermal
1	1 mg Comp. Freund's	Back/Intradermal
2	1 mg Comp. Freund's	Back/Intradermal
3	**NO INJECTION SCHEDULED**	
4	1 mg Incomp. Freund's	Back/Intradermal
5-6	**NO INJECTION SCHEDULED**	
7	1 mg Incomp. Freund's	Back/Intradermal
8-16	**NO INJECTION SCHEDULED**	
17	1 mg Incomp. Freund's	Back/Intradermal
18-19	**NO INJECTION SCHEDULED**	
20	1 mg Incomp. Freund's	Back/Intradermal
21-23	**NO INJECTION SCHEDULED**	
24	1 mg	Back/Intradermal

	Incomp. Freund's	
25-27	**NO INJECTION SCHEDULED**	
28	1 mg	Back/Intradermal
	Incomp. Freund's	

Test bleeds (5 ml whole blood in a glass tube without preservative) were received on week 6, week 12, week 18, and week 22. Production bleeds (25 ml) were received on week 25, week 29.

D. Antibody Purification Procedure

Whole blood collected in red-top Venoject containers was allowed to clot at room temperature for 1 hr. The serum was separated by centrifuging the specimens at 10,000 x g for 0.5 hr. Equal volumes of serum and a saturated solution of ammonium sulfate were stirred at room temperature for 2 hr. The mixtures were centrifuged at 10,000 x g for 0.75 hr, equal volumes of the resultant supernatant and saturated ammonium sulfate stirred again at room temperature for 2 hr, and centrifuged at 10,000 x g for 0.75 hr. The resultant supernatant was dialyzed against 2 x 2 l of 200 mM tris(hydroxymethyl)aminomethane, pH 7.5, over 48 hr. The antiserum was stored in glass containers at 2-8°C.

E. Results

The amount of protein in the purified antibody solution was measured and the protein was stored at 0°C.

Chapter 3

Heterogeneous Enzyme Immunoassay for Glyburide Metabolites in Urine

I. Introduction

A heterogeneous competitive enzyme immunoassay involves the competition between a free antigen (Ag) and an antigen labeled with an enzyme (Ag*) for binding sites on an antibody (Ab) that is capable of binding (Ag) and (Ag*) with equal affinity, such that the following equilibrium exists, according to the law of mass action for bimolecular reactions[54]:

$$(Ag + Ag^*) + Ab \underset{k_2}{\overset{k_1}{\rightleftharpoons}} Ag\text{-}Ab + Ag^*\text{-}Ab$$

Where $k_1 >> k_2$ for optimal performance. When the limiting variable in the above equation is the quantity of unlabelled antigen, typically a standard, control, or patient specimen, the quantity of (Ag*-Ab) measured in the label development step of the immunoassay is inversely proportional to the concentration of unlabelled antigen in the reaction mixture.

Glyburide is a second generation oral hypoglycemic drug used in the treatment of non-insulin dependent diabetes mellitus (NIDDM). The increased potency of glyburide gives rise to lower dosage requirements and may lead to decreased amounts of the drug and its metabolites in biological fluids. The characteristics of a peroxidase-coupled solid-phase enzyme immunoassay[55] for glyburide metabolites in urine is described, in which the antiserum has been raised to the primary 4-

hydroxy metabolite of the drug.

II. Experimental

A. Materials

Glyburide hemisuccinate ester was prepared as previously described. 4-Hydroxyglyburide and 3-hydroxyglyburide were obtained from the Upjohn Company (Kalamazoo, MI). 4-Hydroxyglyburide was also synthesized de novo. Peroxidase, from horseradish (EC 1.11.1.7), sodium borohydride, sodium periodate, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 1,6-hexanediamine, and 1-fluoro-2,4-dinitrobenzene (FDNB) were obtained from Sigma Chemical Company (St. Louis, MO). Dimethyl formamide (DMF) was from Aldrich Chemical Company (Milwaukee, WI). n-Propyl paraben was obtained from Mallinckrodt, Inc. (Belleville, NJ). o-Phenylenediamine (OPD) tablets were obtained from Beckman Instruments (Brea, CA). Bovine serum albumin (BSA, fraction V) was purchased from Intergen (Purchase, NY). Polystyrene microtiter plates containing 96 wells were purchased from Corning Glass Works (Corning, NY). The microtiter plate washer (EL 403) and plate reader (EL 311) were purchased from Bio-tek Instruments (Winooski, VT). Spectrophotometric grade acetonitrile, methylene chloride, and 2-propanol were purchased from Burdick and Jackson Labs. (Muskegon, MI). Solid-phase extraction columns (JETUBE #1805) were purchased from HAR-LEN Medical, Inc. (Pittsburgh, PA).

The HPLC system consisted of a Model 250 Binary LC pump,

a Model LC-235 diode array detector, and a Model 7125 injector with a 100 μl loop, all from Perkin-Elmer (Norwalk, CT). Samples were chromatographed on a 0.46 x 3.3 cm column packed with 3 μm particles of C18, also from Perkin-Elmer. The mobile phase consisted of acetonitrile added to a buffer of 440 mM ammonium phosphate, pH 7.0 (5.78 g of ammonium phosphate dibasic in 1 l of distilled water adjusted to pH 7.0 with 85% phosphoric acid). Chromatography was performed at 25°C with a mobile phase consisting of a gradient of 10% to 50% acetonitrile in the buffer over 0.5 hr. The flow rate was 1.5 ml/min and the effluent was monitored at 230 nm.

All other buffers and reagents were of analytical grade and used without further purification.

B. Binding of Anti-Glyburide Antibodies to a Solid Surface

The purified antiserum was diluted to a concentration of 10 μg/ml with phosphate buffered saline (PBS). The antibody solution (100 μl) was added to the wells of a polystyrene microtiter plate, the plates were incubated for 24 hr at 25°C, washed three times with distilled water, and incubated at room temperature for 48 hr with a Tris-saline buffer containing 1% BSA. Following a second wash step the plates were stored at 4°C until use.

C. Preparation of Glyburide-Peroxidase Conjugate

Glyburide hemisuccinate ester was also conjugated to horseradish peroxidase. The carbohydrate residues of the enzyme were first oxidized by treatment with sodium

periodate.[56] Following the formation of a Schiff base with 1,6-hexanediamine, sodium borohydride, and an excess of glyburide hemisuccinate ester in DMF were added along with EDC over 0.5 hr with stirring at 2-8°C. The conjugate was purified by exhaustive dialysis against 2 x 4 l of 200 mM Tris buffer, pH 7.5 for 72 hr.

D. Heterogeneous Enzyme Immunoassay for 4-Hydroxy-Glyburide in Urine

A stock standard of 4-hydroxyglyburide was prepared in ethanol to give a concentration of 1 μg/ml. The stock standard, stored at -15°C, was stable for at least six months. Urine standards consisting of 0, 50, 100, and 1000 ng/ml of 4-hydroxyglyburide were prepared by addition of the appropriate amounts of the stock standard to drug-free normal human urine. The assay was begun by the addition of 100 μl of glyburide-horseradish peroxidase conjugate and 10 μl of a patient specimen, standard, or control to a microtiter plate well coated with antibody to the drug. Binding competition proceeded for 0.5 hr at room temperature. The plates were then washed with 3 x 300 μl of distilled water, and o-phenylenediamine (OPD) in a citrate buffer containing 0.01% peroxide was added. The color reaction was allowed to develop in the dark for 0.5 hr and was stopped by the addition of 100 μl of 100 mM sulfuric acid. Absorbance at 492 nm was inversely proportional to the concentration of 4-hydroxy-glyburide in the sample.

E. HPLC Assay for Glyburide Metabolites

An unbuffered urine specimen (4 ml of patient specimen) was vigorously mixed with 100 µl (2000 ng) of a working solution of n-propyl paraben as an internal standard. The samples were added to solid phase extraction columns, and after one minute 8 ml of the extraction solvent composed of 9:1 methylene chloride:2-propanol was added to each column. The eluents were transferred to 12 x 75 mm glass tubes and evaporated to dryness at 40°C under nitrogen. The residue was reconstituted with 100 µl of acetonitrile, and 50 µl was injected into the instrument.

III. Results and Discussion

A. Precision and Detection Limit

The enzyme immunoassay exhibited good intra-assay and inter-assay precision over the range of a typical calibration curve (Table 1). The minimum detectable concentration of 4-hydroxyglyburide in the assay, defined as the mean determination of a zero standard (n = 10) plus 2 SD, was 1.7 ng/ml.

Table 1. Inter- and Intra-assay Precision of Enzyme. Immunoassay for 4-Hydroxyglyburide

Standard (ng/ml)	n	CV (%) intra-assay	CV (%) inter-assay
0	10	3.5	6.8
50	10	4.9	4.9
100	10	5.4	5.6
1000	10	5.7	7.9

B. HPLC Analysis of Positive EIA Specimens

Twenty (20) clinical urine specimens found to be positive by the immunoassay were subjected to HPLC analysis to confirm the presence of the metabolites themselves, and to determine the quantity and relative percentages of each urinary metabolite of glyburide. Figure 12 shows a typical HPLC chromatogram obtained from the extraction of one of the urine specimens. The 3-OH and 4-OH isomers of the metabolite were readily separable by the HPLC technique developed. Difficulties in the procedure, however, prohibited the generation of quantitative data.

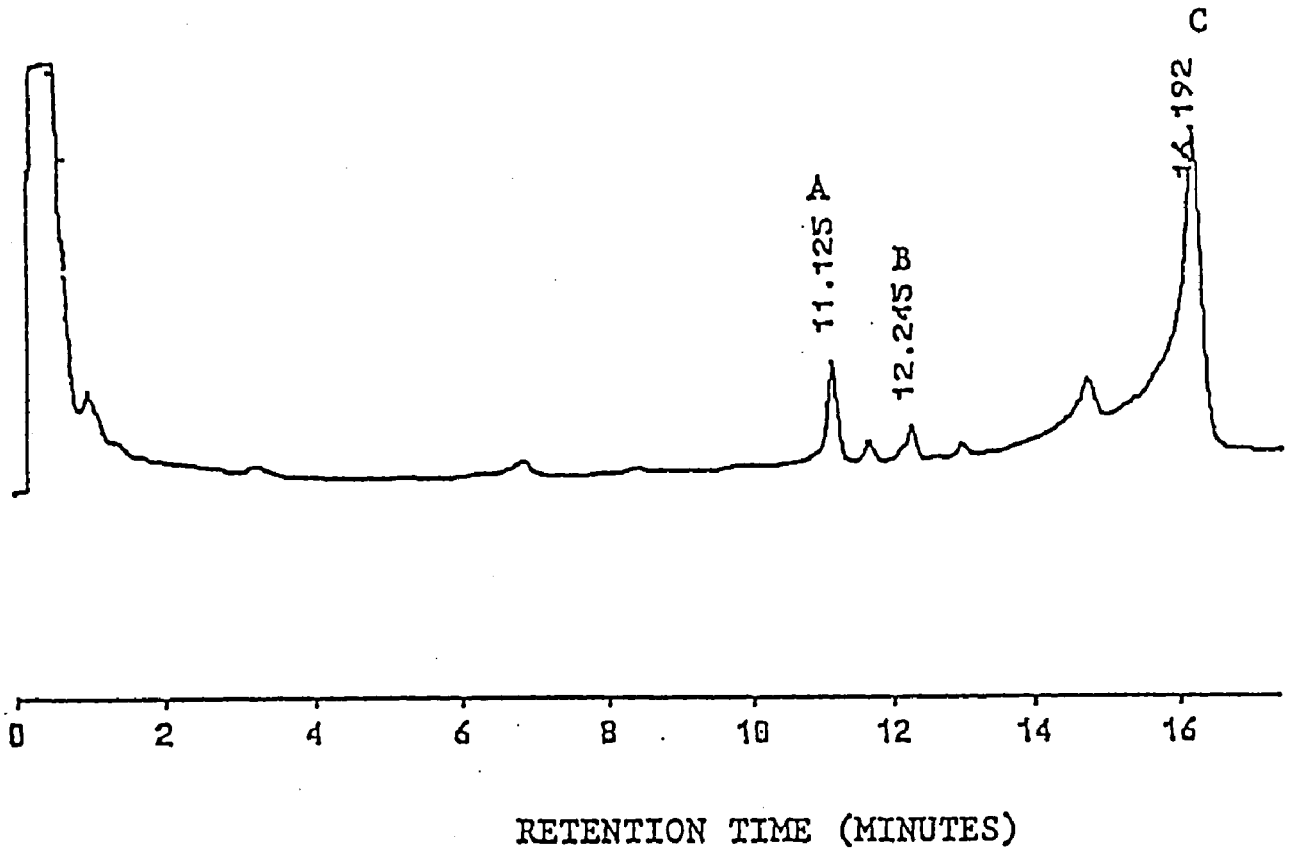
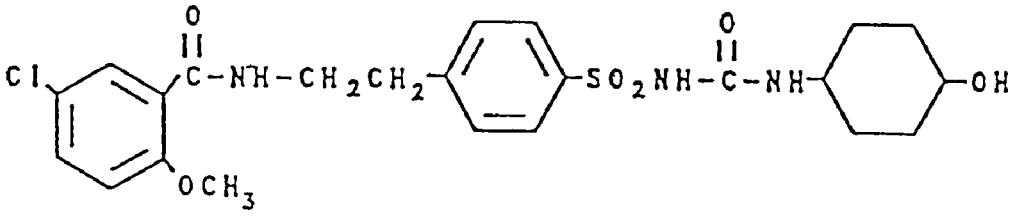


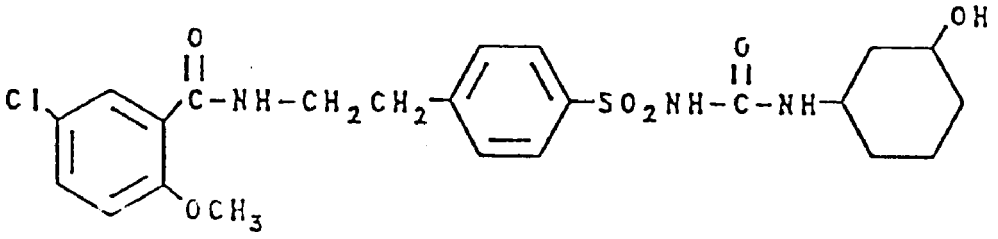
Figure 12. HPLC chromatogram showing (A) 4-hydroxyglyburide, (B) 3-hydroxyglyburide, and (C) n-propyl paraben, the internal standard.

C. Cross-Reactivity of the Assay

Although the antibodies used in this assay were raised against 4-hydroxyglyburide, the 3-hydroxy metabolite (Figure 13) is closely related that it would be expected to exhibit some level of positive response in the assay. The cross-reactivity of 3-hydroxyglyburide was measured by quantitating a standard containing 50 ng/ml of the metabolite against a typical binding curve (Figure 14) made from standards of 4-hydroxyglyburide. The quantity of 3-hydroxyglyburide calculated was 47.5 ng/ml, indicating a cross-reactivity of 95% at this level.



4-Hydroxyglyburide



3-Hydroxyglyburide

Figure 13. Comparison of the structures of 4-hydroxyglyburide and 3-hydroxyglyburide

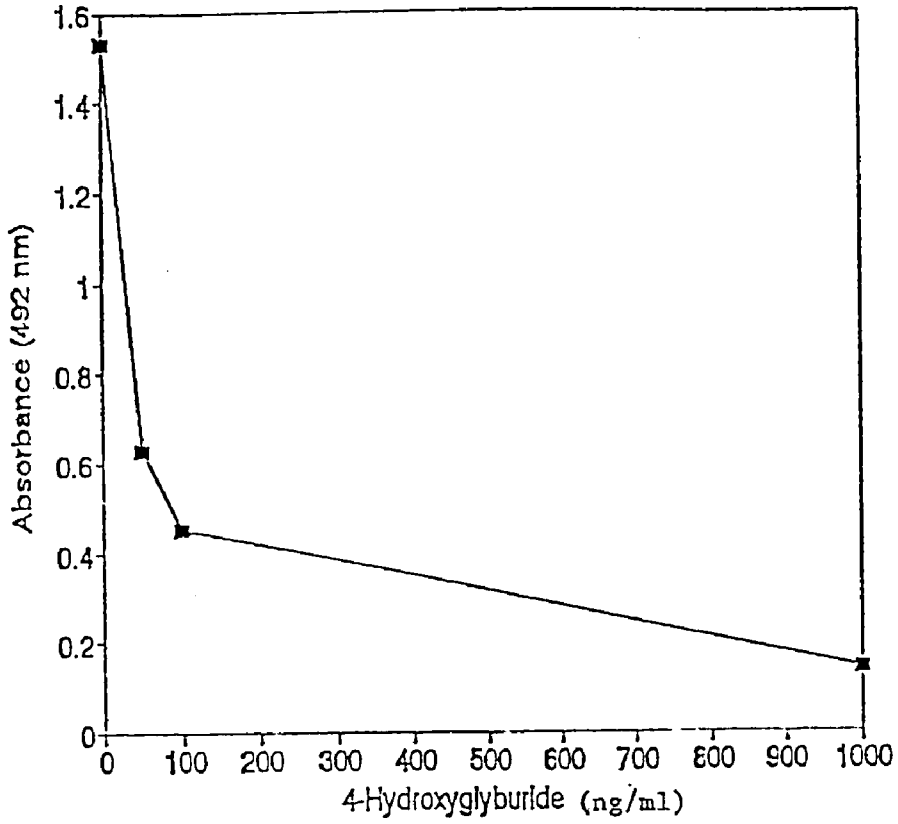


Figure 14. Binding curve for a heterogeneous EIA for 4-hydroxyglyburide, consisting of standards of 10, 50, 100, and 1000 ng/ml

Of the clinical urine samples tested, two were from patients that were currently taking glipizide, a closely related oral hypoglycemic drug (Figure 15). Although glipizide and glyburide are metabolized in a similar manner[8], these test results were negative by this assay.

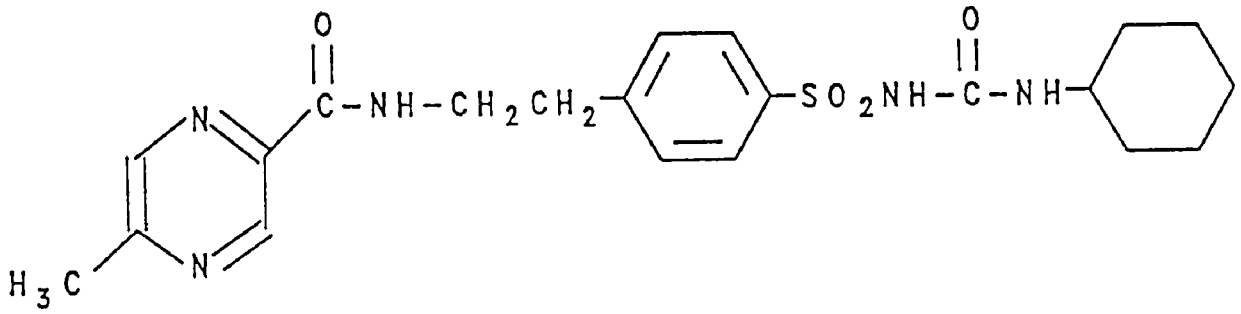


Figure 15. The structure of glipizide

Chapter 4

An Enzymatic Cycling Procedure for β-NADP+ Generated by 3'-Phosphodiesterase, 2':3'-Cyclic Nucleotide

I. Introduction

The technique of enzymatic cycling was first developed to increase the sensitivity of assays for cyclic nucleotides (NAD(H), NADP(H)) in biological fluids.[23] In this approach to amplification, the cofactor itself is alternately oxidized and reduced by two enzymes and their respective noncycling substrates This results in accumulation of a noncycling product whose quantity increases with cycling time. Hence, small amounts of cofactor can generate a more easily measured amount of product in a quantity that is related to the original amount of cofactor in the sample. One application of this technique is to increase the sensitivity of a modified competitive enzyme linked immunosorbent assay (CELIA) applicable to the low-level detection of a variety of antigens in biological specimens. There have been many enzymatic cycling procedures used to decrease the detection limits of enzyme immunoassays.[57,58,59] In this research project two enzymes, NAD+ kinase (EC 2.7.1.23) and 3'-phosphodiesterase, 2':3'-cyclic nucleotide (EC 3.1.4.37), were used to generate the cycling cofactor β-NADP+. NAD+ kinase was evaluated first for this purpose. The amount of colored product inducible by the actions of this enzyme was evaluated against two different cycling systems to optimize the sensitivity of the

immunoassay using this enzyme as a "trigger" in the production of β-NADP+. Following a competitive assay in which the enzyme conjugated to an antibody against 4-hydroxyglyburide competes with free antibodies for binding sites on an immobilized antigen, the substrate β-NAD+ and the cofactors 5'-ATP and $MgCl_2$ are added to the reaction mixture (Figure 16). The β-NADP+ thus formed from the catalytic action of NAD+ kinase cycles between its oxidized and reduced forms according to the kinetic parameters presently described for the cycling enzymes glucose-6-phosphate dehydrogenase and diaphorase and with the generation of an increased amount of chromophore.

NAD+ kinase, however, is a large, multimeric protein consisting of eleven subunits and having a molecular weight of approximately 270,000.[60,61] The specific activity of this enzyme indicates that one unit will phosphorylate 1.0 nmol (10^{-9} mol) of substrate β-NAD+ to β-NADP+ per minute at pH 7.5 and 37°C in the presence of 5'-ATP.[62,63] This turnover rate is approximately one thousand times slower than the rate of β-NADP+ production using the enzyme 3'-phosphodiesterase, 2':3'-cyclic nucleotide; moreover, the kinetic parameters of 3'-phosphodiesterase, 2':3'-cyclic nucleotide were measured at ambient temperature,[64] not 37°C.

A similar optimization scheme was followed using NAD+ kinase as well as with 3'-phosphodiesterase, 2':3'-cyclic nucleotide. The concentrations of the substrates for each of these enzymes, NAD+ and 2':3'-cyclic NADP+ were varied

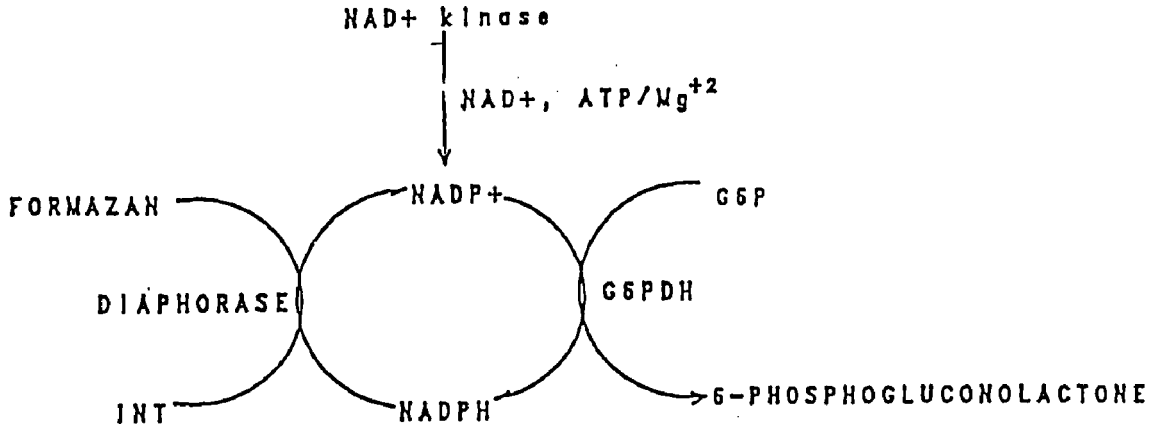


Figure 16. An enzymatic cycling procedure for β-NADP+ using NAD+ kinase as the "trigger" enzyme, and glucose-6-phosphate dehydrogenase and diaphorase as the cycling enzymes

in order to find a concentration of each that gave minimum background color and maximum speed of color development. The results (Figure 17) indicate that under optimum reaction conditions obtainable (i.e., temperature, pH, substrate concentrations)[65,66] the increase in sensitivity that was gained by enzymatic cycling using NAD+ kinase as the "trigger" for the cycling cofactor was approximately twenty-two fold greater than a noncycling assay for the cofactor, measured as follows:

$$A_{492nm}/A_{340nm}$$
$$= 1.553/0.072$$
$$= 21.6$$

The A_{492nm} was obtained by measuring the quantity of INT-formazan formed from the cycling process after 1 hr. The A_{340nm} was a measure of the reduction after 1 hr of an identical quantity of β-NADP+ as formed by the NAD+ kinase trigger. Identical concentrations of glucose-6-phosphate dehydrogenase and glucose-6-phosphate were used in both assays. Both buffer and temperature conditions in the two assays were identical; both were measured against reagent blanks.

Although the results indicate that enzymatic cycling with NAD+ kinase provides some increase in sensitivity over a non-cycling assay, this proved to be limited in two distinct ways:

(1) The enzyme glucose-6-phosphate dehydrogenase from

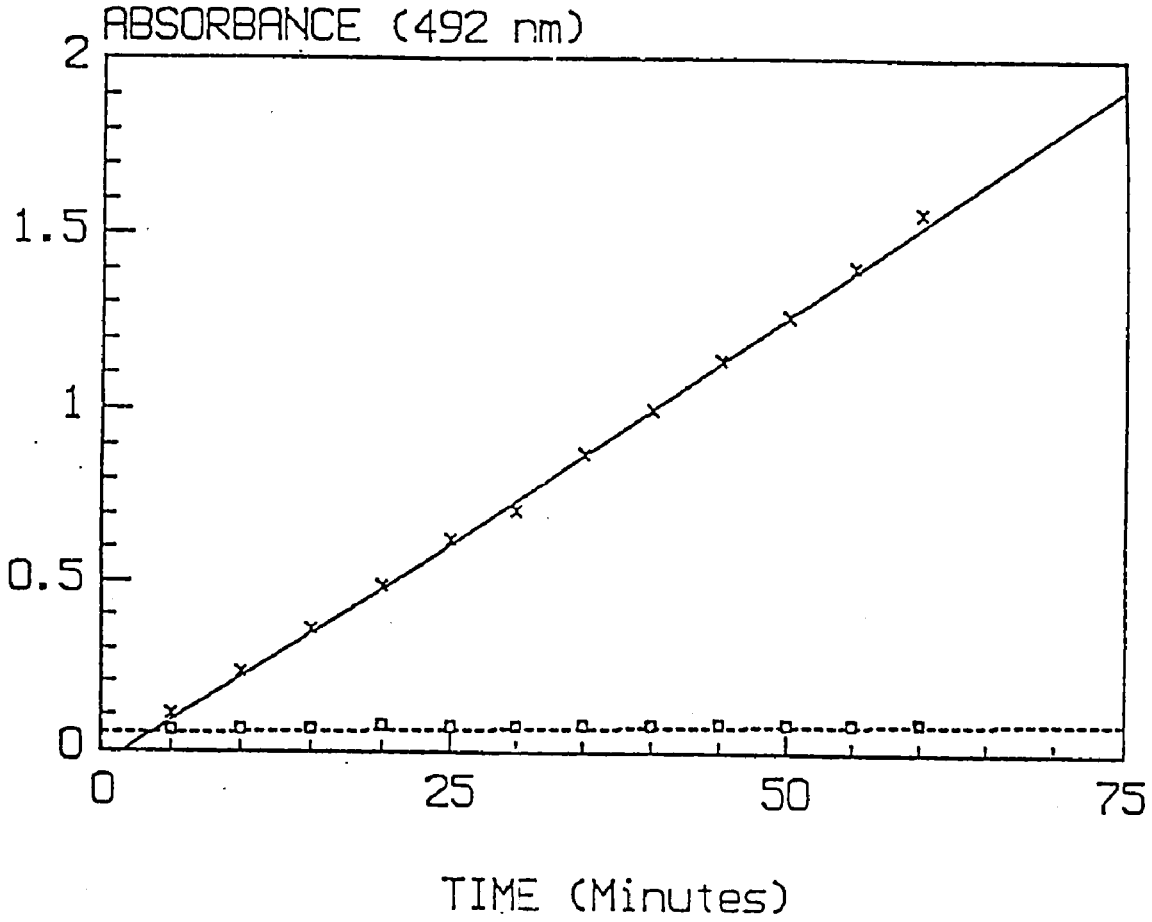


Figure 17. Comparison of a cycling procedure for β-NADP+ using NAD+ kinase with a non-cycling procedure for the cofactor

torula yeast, which is reported to be specific for β-NADP+, possesses some catalytic activity when β-NAD+ is its substrate. Also, the conversion of p-iodonitrotetrazolium violet (INT) to INT-formazan by diaphorase appears to be nonspecific for the cofactor involved; i.e., both β-NADPH and β-NADH were oxidized in this reaction, with the formation of colored product using either. Contaminating β-NADH, either from the preparation of β-NAD+ used as the substrate for NAD+ kinase or in one or both of the trigger/cycling enzyme(s) themselves, catalyzed the production of colored INT-formazan even in the absence of β-NADPH generated via the cycle. Searches for β-NADPH-specific diaphorase proved to be fruitless, with the possible exception of an enzyme isolated from spinach which was very difficult to purify and was not commercially available.[67,68]

(2) NAD+ kinase itself is an inherently weak enzyme in terms of specific activity. Although the majority of enzymes catalyze the formation of product in the micromolar range, this enzyme only catalyzes the conversion of 1 nmol of substrate to product, and only at an increased temperature (37°C).

Factor (1) precludes the use of greater quantities of the substrate β-NAD+ in any effort to "drive" the formation of β-NADP+ by saturation of NAD+ kinase. Factor (2) invariably leads to poorer sensitivity than could be achieved with a more vigorous enzyme. Such an enzyme could generate greater

quantities of cofactor initially. The greater amount of cycling cofactor would generate increased quantities of formazan over the same time period and lead to better sensitivity of the assay.

Because of these factors, a second variation in this cycling system was pursued.

This involved the use of a novel color development process that obviates the need for diaphorase in the reaction and should reduce background color development due to β-NADH acting as a primary substrate regardless of whether this cofactor was produced by glucose-6-phosphate dehydrogenase and β-NAD+. In this scheme the β-NADPH produced from β-NADP+ by glucose-6-phosphate dehydrogenase in the presence of glucose-6-phosphate acts as a cofactor in the reduction of oxidized glutathione, GSSG, by glutathione reductase. This reaction, which is specific for β-NADPH and GSSG, is in turn coupled to the reduction of 5, 5'-dithiobis-(2-nitrobenzoic acid), DTNB (Ellman's reagent), which forms the colored product thionitrobenzoic acid, TNB, and concomitantly reoxidizes the reduced glutathione, GSH.[69,70,71] Figure 18 shows the "double" cycling effect of this reaction. Furthermore, the Michaelis' constants of glutathione and β-NADPH for the cycling enzyme glutathione reductase, 6×10^{-5} M and 6×10^{-6} M, respectively, are smaller by comparison with the same constant values of the cofactor and INT for diaphorase.[72] Thus, less β-NAD+ would be used as a substrate for NAD+ kinase, and hence the risk of

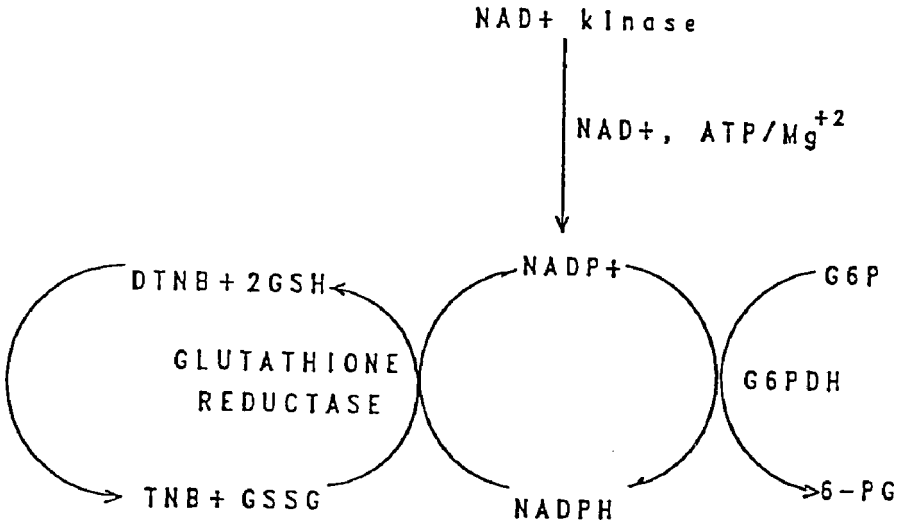


Figure 18. An enzymatic cycling procedure for β-NADP+ using NAD+ kinase as the "trigger" enzyme, and glucose-6-phosphate dehydrogenase and glutathione reductase as the cycling enzymes

background color development interfering with the sensitivity of the assay was reduced even further. This solution proved to be less than adequate, however, due to the nonenzymatic reduction of GSSG caused by nonspecific reducing agents in clinical urine specimens.[71] Substances such as glucose can react nonenzymatically with GSSG and lead to the formation of colored TNB. This nonspecific reduction of glutathione (GSSG) causes nonspecific background color development as well, and further reduces the sensitivity of the assay.

Finally, as determined by reaction of the enzyme NAD+ kinase with the amine labelling reagent 2,4,6-trinitrobenzene sulfonic acid[73,74] the protein itself is also deficient in the number of available lysine residues on its surface, whose amino groups are used for conjugation to various haptenic molecules or proteins. This property of the enzyme alone is what generally rendered it unsuitable for use as a label in the present type of heterogeneous immunoassay.

Thus, the physical and kinetic properties of the enzyme 3'-phosphodiesterase, 2':3'-cyclic nucleotide were extensively evaluated with regard to suitability for conjugation and β-NADP+ production. In the same type of immunoassay scheme, a conjugate to the enzyme 3'-phosphodiesterase, 2':3'-cyclic nucleotide (PDE), bound via an antibody to an immobilized antigen on a solid surface, is used to "trigger" the generation of a small quantity of β-NADP+ from 2':3'-cyclic NADP+ (Figure 19). The cycling nucleotide is again coupled

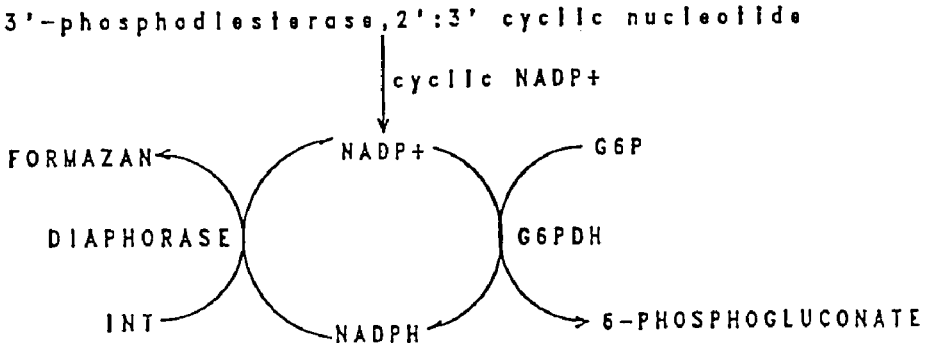


Figure 19. An enzymatic cycling procedure for β-NADP+ using 3'-phosphodiesterase, 2':3'-cyclic nucleotide as the "trigger" enzyme, and glucose-6-phosphate dehydrogenase and diaphorase as the cycling enzymes

directly to the production of a colored molecule, such as the formazan dye produced from the reduction of p-iodonitrotetrazolium violet (INT)[75]; i.e., a molecule of INT is converted to formazan with each turn of the cycle. One molecule of β-NADP+ liberated by PDE can therefore catalyze the production of many molecules of formazan dye.

Other enzymatic cycling assays utilizing NADP(H) or NAD(H) require the destruction of the cycling enzymes by either heat, pH extremes, or detergent treatment followed by measurement of an accumulated noncycling product in a separate reaction step.[76] The present method of detection eschews this additional step, is simpler and reduces the assay time. Likewise, the use of bound enzyme conjugates that generate NAD+ as the cycling cofactor similarly linked to the formation of a chromophore has been demonstrated only in assays for large macromolecules such as TSH by a sandwich technique.[77,78]

We used enzymatic cycling with 3'-phosphodiesterase, 2':3'-cyclic nuclotide to generate the cofactor β-NADP+ and the enzyme glucose-6-phosphate dehydrogenase and diaphorase to cycle the cofactor in order to increase the sensitivity of a direct enzyme-linked immunosorbent assay (ELISA) for the determination of small molecules, such as drugs, in biological fluids.

II. Experimental

A. Materials

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) from

torula yeast, diaphorase (EC 1.8.1.4) from C. kluyveri, 3'-phosphodiesterase, 2':3'-cyclic nucleotide (EC 3.1.4.37), p-iodonitrotetrazolium violet (INT), β-NADP+, β-NADPH, 2':3'-cyclic NADP+, and glucose-6-phosphate were purchased from Sigma Chemical Company (St. Louis, MO). All other buffers and reagents were of analytical grade and used without further purification. Spectrophotometric readings were obtained on a Perkin Elmer Lambda 3 spectrophotometer (Norwalk, CT).

B. Determination of Kinetic Parameters for G6PDH and Diaphorase

Michaelis' constants for both glucose-6-phosphate dehydrogenase (G6PDH) and diaphorase have been published[79]; however, both K_m and V_{max} values were remeasured to accomodate different temperature and buffer conditions used herein.

The kinetic constants for G6PDH were obtained in 100 mM Tris, pH 7.5. To 0.80 ml of buffer in a 1 ml quartz cuvette containing 2 mM glucose-6-phosphate and 10 mM magnesium chloride was added 0.10 ml of a series of β-NADP+ dilutions in Tris buffer. To start the reaction, 0.10 ml of the same buffer containing the enzyme was added. Absorbance readings were obtained at 340 nm against a reagent blank. A K_m of 6.9 μM for β-NADP+ and a V_{max} of 338 μmol/min/mg of enzyme were calculated from the data.

The kinetic constants for diaphorase were also obtained in 100 mM Tris, pH 7.5. To 0.80 ml of buffer in a 1 ml cuvette containing 10 mM INT in Tris buffer with 0.10% Tween 80 was added 0.10 ml of a series of β-NADPH dilutions in tris

buffer. To start the reaction, 0.10 ml of the same buffer containing the enzyme was added. Absorbance readings were obtained at 492 nm against a reagent blank. A K_m of 19 μM for β-NADPH and V_{max} of 15 μmol/min/mg of enzyme were calculated from the data.

C. Cycling Assay for β-NADP+

The cycling reagent consisted of 2 mM glucose-6-phosphate, 10 mM magnesium chloride, and 10 mM INT (with 0.10% Tween 80) in 100 mM Tris buffer, pH 7.5. Just before the start of the reaction various dilutions of β-NADP+ to give amounts ranging from 7×10^{-11} to 6×10^{-13} mol of the cofactor in 1 ml were added to the reaction mixture. The reaction was started by the simultaneous addition of 1 μg of glucose-6-phosphate dehydrogenase and 9 μg of diaphorase. Spectrophotometric measurements were obtained over a period of 0.5 hr. at 492 nm against a reagent blank.

D. Cycling Assay for 3'-Phosphodiesterase, 2':3'-Cyclic Nucleotide

3'-phosphodiesterase, 2':3'-cyclic nucleotide (PDE, isolated from brain white matter) readily hydrolyzes 2':3'-cyclic NADP+ to β-NADP+. The K_m for the cyclic nucleotide is 220 μM at pH 6.0 and 25°C.[80] Dilutions of PDE corresponding to 2 ng, 10 ng, and 20 ng of the enzyme in 50 mM (2-[N-morpholino]ethanesulfonic acid (MES) buffer, pH 6.5 were incubated at room temperature for 1 hr with 0.05 ml of a 400 μM 2':3'-cyclic NADP+ solution in the same buffer. The amount of β-NADP+ formed was determined by the same cycling assay

described above.

III. Results

A. Calculation of Optimum Cycling Enzyme Amounts

Optimal performance from a cycling system with a minimum expenditure of the cycling enzymes can be achieved by consideration of the kinetic behavior of each, in terms of K_m and V_{max}, in the presence of cycling substrate concentrations that are less than saturating. The reaction rates in this case are proportional to the quantity of substrate used, and the relevant kinetic factor for each enzyme is the apparent first order rate constant, $k = V_{max}/K_m$, where V_{max} is the reaction rate when the enzymes are saturated with β-NADP+ or β-NADPH, respectively. At steady-state the rate of reduction of β-NADP+ is equal to the rate of oxidation of β-NADPH, therefore $k_1[\beta\text{-NADP+}] = k_2[\text{NADPH}]$, where k_1 is the apparent first order rate constant for glucose-6-phosphate dehydrogenase and k_2 applies to diaphorase. The theoretical overall cycling rate can then be calculated as follows:[81]

$$k_c = k_1k_2/(k_1 + k_2) \quad \text{(equation 1)}$$

Under the present conditions, k_1 for G6PDH is equal to 49 min^{-1} and k_2 for diaphorase is 0.79 min^{-1}, when each are at a concentration of 1 μg/ml.

Calculations based on the use of various ratios of the cycling enzymes while maintaining a constant total amount of

protein produce a theoretical optimal ratio of the two enzymes in terms of the number of cycles per minute that can be attained (Table 2). These values were compared to experimentally determined cycling rates obtained by the addition of the two cycling enzymes, in various ratios, to the cycling reagent containing [β-NADP+] at a concentration below its K_m value for G6PDH. The results (Table 3) compare favorably with the theoretical cycling rate. A total protein content of 10 μg/ml in the assay was the minimum amount sufficient to obtain the necessary sensitivity and minimized the blank absorbance which is due to some lack of substrate specificity of G6PDH for β-NADP+. The cycling enzymes are apparently able to use the cyclic nucleotide as a substrate as well as β-NADP+ in this cycle. Higher concentrations of G6PDH appear to be the cause of increased background color and may reduce the overall sensitity of the assay.

B. Sensitivity of the Procedure for β-NADP+

Figure 20 shows the sensitivity and linearity of this cycling system for the measurement of low levels of β-NADP+. Using cycling enzymes at the concentration of 10 μg/ml and a cycling time of 0.5 hr, good linearity for β-NADP+ is demonstrated over a range of 6 to 700 x 10^{-13} mol of the cofactor.

C. Sensitivity of the Procedure for 3'-Phosphodiesterase, 2':3'-Cyclic Nucleotide

Figure 21 shows the results of the cycling procedure over 0.5 hr using β-NADP+ that was produced <u>in situ</u> by incubation

Table 2

Theoretical Cycling Rates with Different Ratios of G6PDH and Diaphorase

Total Enzyme Concentration (μg/ml)	Cycling Enzymes (μg/ml)		#cycles/min. (calculated)
	G6PDH	Diaphorase	
10.0	0.10	9.9	3.0
10.0	0.50	9.5	5.7
10.0	1.0	9.0	6.2
10.0	2.0	8.0	5.9
10.0	3.0	7.0	5.3
10.0	4.0	6.0	4.6
10.0	5.0	5.0	3.9
10.0	6.0	4.0	3.1
10.0	7.0	3.0	2.4
10.0	8.0	2.0	1.6
10.0	9.0	1.0	0.79

Calculations: The #cycles/minute were calculated from $k_c = k_1k_2/(k_1 + k_2)$, where k_1 and k_2 are the apparent first order rate constants for G6PDH and diaphorase, equal to 49 min^{-1} and 0.79 min^{-1}, respectively.

Table 3

Experimental Cycling Rates with Different Ratios of G6PDH and Diaphorase

Total Enzyme Concentration (μg/ml)	Cycling Enzymes (μg/ml)		Cycling Rate (mols Formazan produced/min.)	#cycles/min.
	G6PDH	Diaphorase		
10.0	0.10	9.9	0.033	2.4
10.0	0.50	9.5	0.092	6.8
10.0	1.0	9.0	0.085	6.3
10.0	2.0	8.0	0.075	5.5
10.0	3.0	7.0	0.063	4.6
10.0	4.0	6.0	0.052	3.8
10.0	5.0	5.0	0.041	3.0
10.0	6.0	4.0	0.031	2.3
10.0	7.0	3.0	0.022	1.6
10.0	8.0	2.0	0.009	0.71
10.0	9.0	1.0	0.005	0.32

Calculations: The #cycles/min. is related to the observed cycling rate by the formula [mols formazan produced/min.]/[mols NADP+]$_{added}$. The mols of formazan is determined by $c = A/\epsilon b$, where $\epsilon_{formazan} = 1.94 \times 10^4$ l x mols^{-1} x cm^{-1}, and b = 1 cm. [β-NADP+] in the assay mixture was 7×10^{-7} M ($K_m = 7 \times 10^{-6}$ M), reaction volume was 1.0 ml.

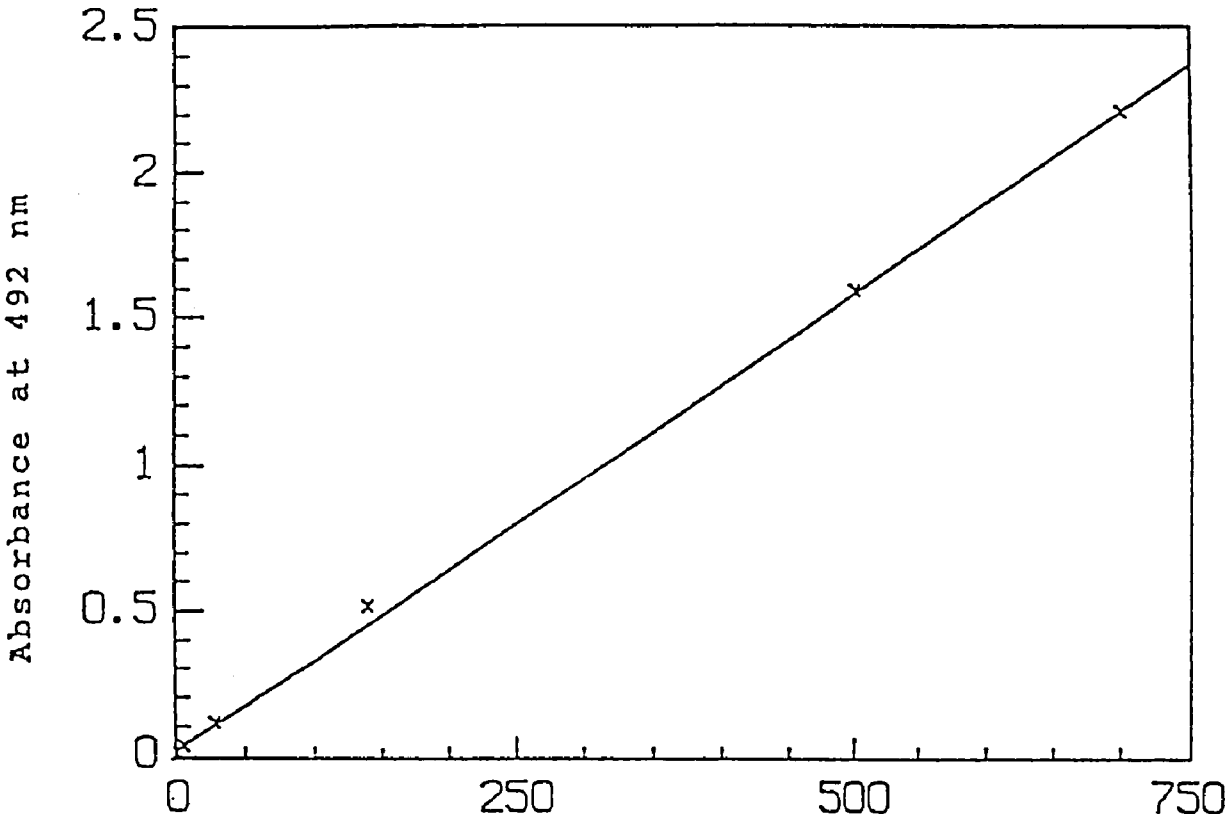


Figure 20. Standard cycling assay calibration curve for β-NADP+ (10 μg enzyme/ml) Sensitivity is to 6 x 10^{-13} mol of β-NADP+. Linearity extends to 700 x 10^{-13} mol of the cofactor

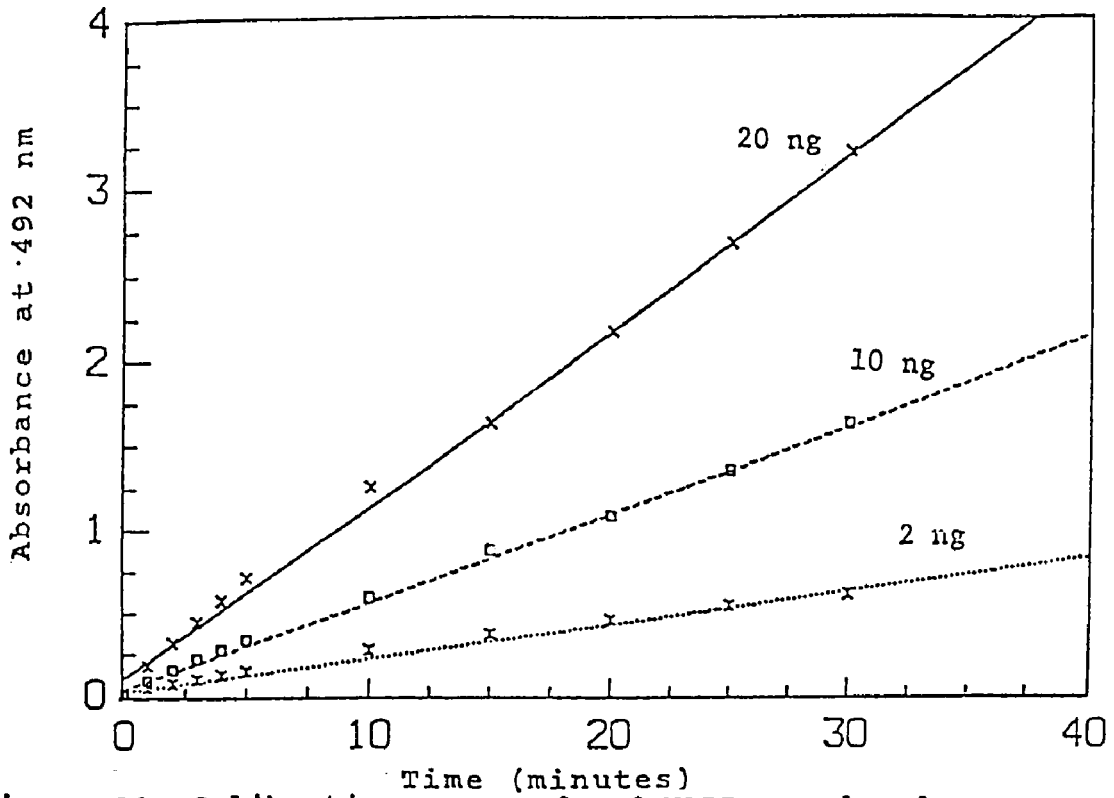


Figure 21. Calibration curves for β-NADP+ produced from various dilutions of 3'-phosphodiesterase, 2':3'-cyclic nucleotide. Graph A was obtained with 20 ng of PDE, graph B from 10 ng, and graph C from 2 ng of the enzyme. Incubation time of the enzyme with 400 μM 2':3'-cyclic NADP+ was 1 hr. Cycling time was 0.50 hr (10 μg enzymes/ml). Blank values were subtracted from each measurement

of 3'-phosphodiesterase, 2':3'-cyclic nucleotide (PDE) with saturating levels of 2':3'-cyclic NADP+ for a period of 1 hr. The results indicate that even low-nanogram amounts of the enzyme produce enough β-NADP+ to develop measurable quantities of colored prodcut during brief cycling times. For example, the absorbance at 492 nm due to the β-NADP+ produced by only 2 ng of PDE corresponds to about 180 x 10^{-13} mols of β-NADP+, easily measurable under the conditions proposed.

IV. Discussion

Small quantities of 3'-phosphodiesterase, 2':3'-cyclic nucleotide have been determined by means of an assay that uses glucose-6-phosphate dehydrogenase to specifically reduce the β-NADP+ produced by the action of PDE on the cyclic nucleotide.[69] While the torula yeast enzyme provides the lowest blank value of several dehydrogenases tested, blank activity, caused by 2':3'-cyclic NADP+ acting as a substrate for the cycling enzymes, can be appreciable in the presence of high levels of G6PDH. This problem is circumvented by maintaining the quantity of this enzyme at the minimum amount necessary to obtain the requisite sensitivity for the detection of β-NADP+. Blank values obtained over the course of the cycling assay correspond to approximately 75 x 10^{-13} mol of the cofactor. The dynamic range of the assay therefore extends from 75 to 700 x 10^{-13} mol of β-NADP+, although the sensitivity for the cofactor remains ten-fold greater.

Cycling rates of approximately 400/hr are sufficient to

detect sub-picomolar quantities of β-NADP+ with this assay. Previous cycling techniques have usually required much higher quantities of cycling enzymes and longer cycling times to achieve this level of sensitivity.[82] Since the detection limits of an EIA are strictly based on the extinction coefficient of the light absorbing molecule used,[83] the advantage of the present cycling system may be due to the larger molar absorptivity value (ϵ) of formazan (1.94×10^4 $M^{-1} \times cm^{-1}$) versus that of NAD(P)H (6.22×10^3 $M^{-1} \times cm^{-1}$) typically used in the detection step of other cycling systems.

When plotted as a function of the cycling rate, the amount of G6PDH (or diaphorase) that produces the fastest cycling rate can be determined by solving

$$\frac{\delta(\#\text{cycles/min.})}{\delta(\text{weight fraction of enzyme})} = 0 \quad \text{(equation 2)}$$

which gives the maximum of the hyperbolic curve obtained from the plot of the weight fraction of enzyme vs. cycling rate.[84] Figures 22 and 23 show the curves obtained for the calculated and experimentally determined cycling rates, respectively, using various ratios of the two enzymes. The quantity of G6PDH that produces the highest theoretical cycling rate (as determined by equation 2) is 2.2 μg/ml of the enzyme, in the reaction mixture containing 10 μg/ml of total enzyme, compared to the experimental value of 1.0 μg/ml.

3'-Phosphodiesterase, 2':3'-cyclic nucleotide has a

molecular weight of about 100,000, contains approximately 35 lysines and has a pI close to 10.[83] Although it is a hydrophobic protein, the enzyme may be conjugated to proteins of small molecules via the pendant ϵ-amino groups of its many lysine residues. The data indicate that even small quantities of this protein are enough to catalyze the formation of an easily measurable amount of β-NADP+ using the enzymatic cycling technique described. If required, lower detection limits would be readily obtainable by longer cycling times and/or longer periods of incubation of the cyclic cofactor with PDE. A competitive ELISA using 3'-phosphodiesterase, 2':3'-cyclic nucleotide as the enzyme conjugate is applicable to the low level detection of substances such as drugs (i.e., glyburide, LSD, alprazolam) in a variety of biological fluids.

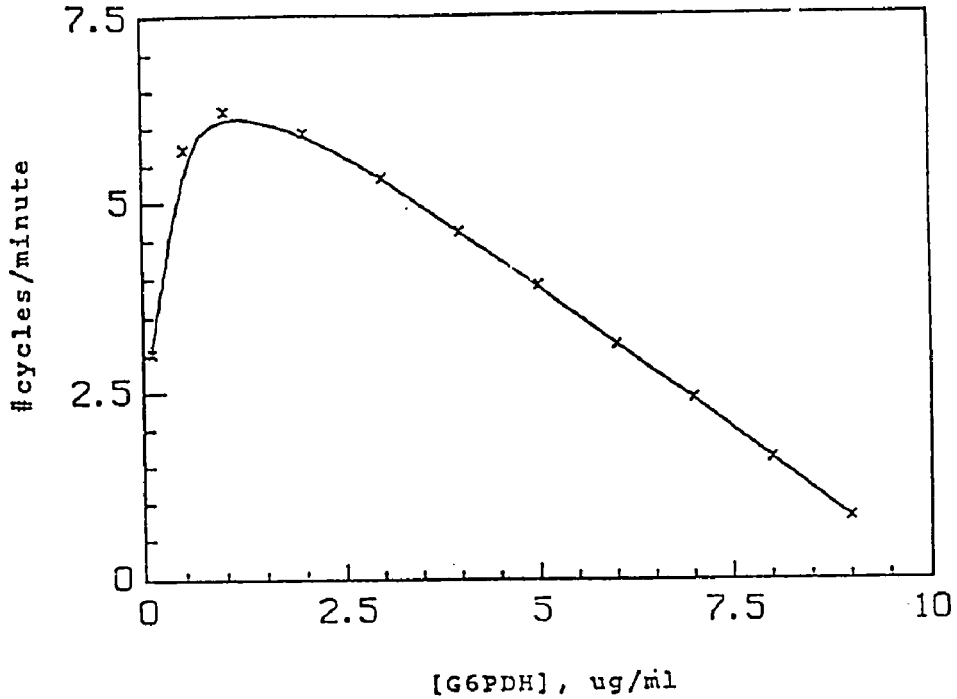


Figure 22. Theoretical hyperbolic curve obtained using the cycling rates calculated from $k_c = k_1k_2/(k_1 + k_2)$ for various ratios of G6PDH and diaphorase in the cycling assay for β-NADP+. Theoretical maximum cycling rate is obtained using 2.2 μg/ml of G6PDH and 7.8 μg/ml of diaphorase

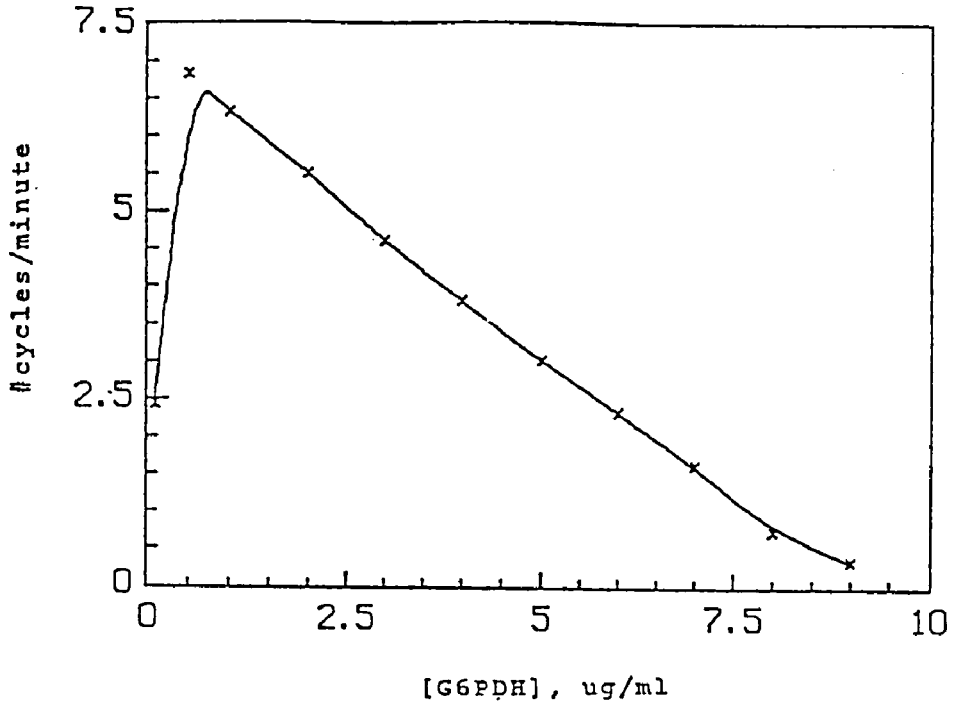


Figure 23. Experimental hyperbolic curve obtained by the addition of various ratios of G6PDH and diaphorase in the cycling assay for β-NADP. The maximum cycling rate is obtained using 1.0 μg/ml of G6PDH and 9.0 μg/ml of diaphorase

Chapter 5

Subnanogram Quantitation of Glyburide Metabolites in a Heterogeneous Enzyme Immunoassay Using Enzymatic Cycling

I. Introduction

The development of competitive binding assays based on the use of radiolabelled ligands as demonstrated by Yalow and Berson established a method for the low level detection of substances in biological fluids.[85] The introduction of enzyme labels to replace radioactive ones brought several advantages to the technique. Simple spectrophotometers could be used in place of expensive scintillation counters in the quantitative determination of antigen-antibody complexes. The danger of exposure to harmful radiation could be avoided, and radioimmunoassay reagents that have short shelf-lives and constitute hazardous waste materials could be replaced by less toxic enzymatic color development reagents.[86]

Immunoassays are widely used in clinical chemistry and toxicology for monitoring the levels of therapeutic drugs in serum and plasma and for the detection of drugs of abuse, primarily in urine specimens as part of a general screen for illicit substances. Current trends in the development of new immunoassay techniques are to move away from liquid-phase assays that involve radioisotopic labels and towards fast homogeneous or heterogeneous assays. The development of precise automated and semiautomated laboratory testing methodologies with detection limits that reach to nano- and

pico-gram levels is at the forefront of this technology.[87] The methods by which the sensitivity of competitive heterogeneous enzyme immunoassays can be improved are limited. Ultimately, the smallest amount of analyte that is detectable is dependent on the antibody and the label used for detection. Improved sensitivity may be seen with the use of highly active non-enzymatic labels such as I^{125} or H^3, as mentioned previously. The problem of finding an enzyme label that can be measured with comparable sensitivity may be solved by the use of highly active enzymes or by the use of enzymatic cycling.

This section of the dissertation describes a solid-phase enzyme immunoassay for the low-level detection of 4-hydroxy-glyburide that uses the optimized cycling parameters elicited in the previous section. Initially free antigen, from a urine sample, for instance, competes with excess antibody for limited binding sites on antigen immobilized on a solid phase. Following a wash step, antibody conjugated to PDE is added and subsequently binds to any free sites left on the immobilized antigen. The addition of substrate for PDE and the cycling enzymes, along with their substrates, enables the development of color. When only small amounts of free antigen are added to the reaction most of the excess antibody is able to bind to immobilized antigen, and there are fewer sites left for PDE-labeled antibody to bind. Hence, there is less color development when the addition of free antigen to the reaction

is small. Such a system serves as a model for the low-level determination of substances in biological fluids.

II. Experimental

A. Binding of BSA-Glyburide to a Solid Surface

The BSA-glyburide conjugate, as prepared for the generation of antibodies previously, was diluted to a concentration of 1 μg/ml with 50 mM tris buffer, pH 7.5 and 100 μl of it was added to the wells of a polystyrene microtiter plate. The plates were incubated for 24 hr at 25°C, washed three times with distilled water, and incubated at 25°C for 48 hr with a tris-saline buffer containing 1% BSA. Following a second wash step the plates were stored at 4°C until use.

B. Preparation of (Anti-Glyburide) IgG-Phosphodiesterase Conjugate

Sulfhydryl groups were first added to the amino functions of 3'-phosphodiesterase, 2':3'-cyclic nucleotide by reaction with 2-iminothiolane (Traut's reagent). The enzyme (1 mg) was added to 1 ml of 20 mM tris buffer, pH 8.0 containing 1 mM EDTA and 10 mM β-NADP+. The derivatizing reagent was added (1 mM in the same buffer), the pH of the reaction mixture adjusted to 8.0, and incubated at 0-4°C for 1.5 hr. The modified enzyme was purified by dialysis against 2 x 2 l of 20 mM Tris buffer containing 60% glycerol and 1 mM EDTA, pH 7.5 for 48 hr.

The amino groups of the antibodies were activated by reaction with m-maleimidobenzoyl-N-hydroxysulfosuccinimide

ester (Sulfo-MBS).[88] A solution of the protein (1 mg) was added to 1 ml of 20 mM tris buffer, pH 7.5. The derivatizing reagent was added (1 mM in the same buffer), the pH of the reaction mixture adjusted to 7.5, and the reaction mixture incubated at 25°C for 0.5 hr. The modified protein was purified by dialysis against 2 x 2 l of 20 mM Tris buffer, pH 7.5 for 48 hr.

The antibody-enzyme conjugate was formed by mixing the two modified proteins in equimolar ratios in a solution consisting of 1 ml of 20 mM Tris buffer, pH 7.5, and 1 ml of glycerol. The reaction was allowed to proceed for 0.5 hr at 25°C, and the conjugate was purified against 2 x 2 l of 20 mM Tris buffer containing 60% glycerol, pH 7.5, for 48 hr.

C. Competitive Enzyme-Linked Immunosorbent Assay (CELIA) for 4-Hydroxyglyburide in Urine

A standard of 4-hydroxyglyburide was prepared in methanol to give a concentration of 1 μg/ml. The standard, stored at -15°C, was stable for at least six months. Urine standards consisting of 0.01, 0.1, and 0.50 ng/ml of 4-hydroxyglyburide were prepared by addition of the appropriate amounts of the standard to drug-free normal human urine. The assay was begun by the addition of 50 μl of a solution of antibody (100 ng) and 100 μl of a standard, control, or patient specimen to a microtiter plate well coated with the BSA-drug conjugate. Binding competition of the antibody for free and bound glyburide proceeded for 0.5 hr. The plates were then washed with 3 x 300 μl distilled water, and 200 μl of the antibody-

PDE conjugate added. The plates were again incubated for 0.5 hr and washed with 3 x 300 µl distilled water. Substrate for 3'-phosphodiesterase, 2':3'-cyclic nucleotide was added (100 µl, 400 µM of 2':3'-cyclic NADP+ in 50 mM MES buffer, pH 6.5), and the production of β-NADP+ by the bound enzyme was allowed to proceed for 0.5 hr. The cycling reagent was added (100 µl containing 1 µg glucose-6-phosphate dehydrogenase, 9 µg diaphorase, 2 mM glucose-6-phosphate, and 10 mM magnesium chloride in 100 mM Tris buffer, pH 7.5) followed by the addition of 50 µl of 10 mM p-iodonitrotetrazolium violet (INT) in the same buffer, containing 0.10% Tween 80. Color development proceeded in the dark for 0.5 hr and the reaction was stopped by the addition of 50 µl of 100 mM sulfuric acid. Absorbance at 492 nm was directly proportional to the concentration of 4-hydroxy glyburide in the sample.

III. Results and Discussion

In this type of immunoassay, the extent of color development is <u>directly</u> proportional to the quantity of drug metabolite in the sample, due to the initial competitive binding step between free antigen (4-hydroxyglyburide) and excess antibody. As more free antigen is added, less excess antibody becomes available for binding to immobilized antigen on the solid surface. Therefore, more bound antigen remains exposed and available for binding antibody-labelled phosphodiesterase.

Initial experiments compared the extent of color

development in the assay using a urine specimen containing none of the metabolite and a spiked urine specimen containing 1 mg/ml (1,000,000 ng/ml) of 4-hydroxyglyburide. No difference in the intensity of color development was observed in several repeated trials. The lack of a positive result may be due to incomplete or poor conjugation between the phosphodiesterase enzyme and anti-glyburide antibody. All of the observed color development may have been due to nonspecific binding of the unconjugated enzyme to the microtiter plate test wells. The hydrophobicity of the phosphodiesterase makes conjugation and purification procedures difficult. Many trials were required to conjugate and purify the enzyme to the antibody without any observed precipitate formation which was presumably the denatured enzyme.

General Discussion and Conclusions

Three cycling systems were reviewed for this dissertation: cycling with β-NADP+ generated by NAD+ kinase and using diaphorase in the color development step, cycling with β-NADP+ generated by NAD+ kinase and using glutathione reductase in the color development step, and cycling with β-NADP+ generated by 3'-phosphodiesterase, 2':3'-cyclic nucleotide and using diaphorase in the color development step.

Of these three, the cycling system that uses the enzyme 3'-phosphodiesterase, 2':3'-cyclic nucleotide was chosen to be further developed for use in an enzyme immunoassay for glyburide metabolites with increased sensitivity. This enzyme has strong hydrophobic properties and has proved difficult to manipulate in terms of conjugation to haptens and other proteins because of the likelihood of its precipitation in purely aqueous buffer systems. By maintaining a buffer environment that includes at least 30% glycerol, however, the enzyme can be conjugated, and retains its activity. Unlike NAD+ kinase, this enzyme carries available lysine amino residues on its surface to which various haptens and/or other proteins can be attached. The enzyme NAD+ kinase has not proven useful for similar additions. The inherently slower turnover rate of NAD+ kinase is also a factor in its exclusion from further development in this immunoassay. It has been shown, however, that this enzyme, when coupled to enzymatic cycling with β-NADP+, is also capable of increased sensitivity

in an assay for the cofactor relative to a noncycling assay.

The heterogeneous enzyme immunoassay for glyburide metabolites in urine described herein uses the technique of enzymatic cycling that has been previously shown to impart increased sensitivity to a variety of enzymatic analyses. The cycle characterized is unique both in the technique by which the cycling species has been generated and with regard to the cycling enzyme system itself. This procedure may be used to increase the sensitivity of an enzyme immunoassay for virtually any low-molecular weight analyte and would be especially useful for the non-radiological determination of certain drugs/metabolites that have low pharmacological thresholds such as glyburide, LSD, or alprazolam, and therefore are present in low (subnanogram) quantities in biological fluids.

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