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RESEARCH ARTICLE

Delivery of Sulfonylurea Anti-hyperglycemic Drugs across Phospholipid Membranes

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Abstract

The mechanism by which Sulfonylurea drugs cross the phospholipid membranes is not fully understood. A model has previously been proposed for the transport of long chain fatty acids which cross the phospholipid membrane by "free diffusion" mechanism. Fluorimetric assays have demonstrated that Sulfonylureas also cross the Phospholipid membrane by "free diffusion"to enter the lumen of small unilamellar vesicles (SUVs). Transport of Sulfonylureas causes a drop in pH inside the vesicles which results in a drop in fluorescence intensity of the trapped Pyranin dye in SUVs. This model is physiologically significant since ATP-sensitive K^+ ion channels serve as Sulfonylurea receptors in β-cells of the Islets of Langerhans and excitable cells such as heart and muscle cells along with dopaminergic neurons. To bind to the receptor, the molecules are required to cross the phospholipid bilayer either by free diffusion or with the help of transport proteins. In the absence of any known transport proteins, the proposed mechanism represents a viable model to explain the delivery of these compounds to living cells.

Introduction:

Sulfonylureas (Fig. 1) are used as drugs for the treatment of type II diabetes mellitus to stimulate the secretion of insulin from the pancreatic beta-cells. Type II diabetes mellitus is characterized by defects in insulin secretion as well as reduced insulin action.Two generations of drugs have been marketed so far to treat type II diabetes mellitus. Tolbutamide is an example of first generation of these drugs, whereas Glybenclamide is an example of second generation of these drugs. Glybenclamide is about two hundred times more effective than Tolbutamide. It is used to treat patients who cannot overcome hyperglycemia by dietary management and exercise. It can be used as the first drug of choice in these patients or as the replacement drug for those with primary or secondary failure during therapy with first generation sulfonylureas.[1)]

Sulfonylureas block ATP-sensitive K^+ channels in the β-cells of the Islet of Langerhans which results in the depolarization of the membrane and this leads to in an influx of Ca^{+2} and an increase in intracellular Ca^{+2} concentration. These series of events cause insulin release. Glybenclamide is about 10-100 times more effective than Tolbutamide for action on ATP-sensitive K^+ channels.[2)]ATP-sensitive K^+ channels are present in various tissues and may be associated with different cellular functions. In the heart, ATP-sensitive K^+ channels are activated during ischemic or hypoxic conditions and may be responsible for the increase of K^+ efflux and shortening of the action potential duration.

During aging, glucose intolerance gradually develops. This condition results in an increase in the postprandial blood glucose response while fasting blood glucose levels are often less increased. Treatment of elderly patients with type II diabetes mellitus focuses on reduction of hyperglycemic complaints and prevention of the development or progression of other secondary complications.[3)] The most common is

hypoglycemia. Hypoglycemia is usually associated with sulfonylureas with longer half-lives such as Chloropropamide and Glybenclamide. The hypoglycemic attacks can result in more serious conditions such as myocardial infarction or stroke. The sulfonylureas with shorter half-lives such as Tolbutamide and Gliclazide are relatively well tolerated and are the best choice to treat elderly patients.

"Free diffusion" is a spontaneous process that increases the entropy of a system and decreases the free energy.The transport process is influenced by the characteristics of the transport substance and the nature of the bilayer. Membrane proteins are not involved in Free diffusion. The diffusion velocity of a pure phospholipid membrane will depends on (i) concentration gradient, (ii) hydrophobicity, (iii) size, and (iv) charge, if the molecule has a net charge.In this study, fluorimetric assays have been used to demonstrate that sulfonylureas also cross the Phospholipid membrane into the lumen of small unilamellar vesicles (SUVs) by "free diffusion".

Materials:

Egg PC was purchased from Avanti Polar Lipids, Pelham, AL. Both tolbutamide and glybenclamide were purchased from Sigma Chemicals, St. Louis, MO. D_2O was purchased from Cambridge Isotope Labs, Boston, MA., Pyranin (8-Hydroxy-1,3,6-pyrenetrisulfonate) from Eastman Kodak Co. and the G-25 Sephadex gel was bought from Amersham Pharmacia Biotech, Uppsala, Sweden. The 5 mm NMR tubes used for the NMR experiments were from Wilmad, Buena, N.J., 3-(Trimethylsilyl) Tetradeutero Sodium Propionate (TSP) was from Wilmad, Buena, N.J., U.S.A. and fatty acid-free albumin used for the fluorimetric assays was from Sigma Chemicals, St. Louis, U.S.A. NMR experiments were performed on a Bruker DMX 500 spectrometer using a 5 mm triple probe. The fluorimetric assays were performed on a FluoroMax 2 fluorimeter. Mass spectra were acquired on a Finnegan Electrospray Ionization (ESI) mass spectrometer. Sonication of the samples was performed using a Branson 350 sonifier employing a micro-tip. pH measurements were made by the use of a Beckman Φ 71 pH meter fitted with a glass micro-electrode.

Methods:

Sample preparation for ^{1}H NMR experiments

The sample of Tolbutamide in solution was prepared by adding 3 mg tolbutamide (dry weight) to 1.8 mL 0.5% w/v KCl solution. It was visually observed that most of the added tolbutamide remained undissoved in solution and was observed as a white precipitate at the bottom of the solution.

The sample of 20 mole% Tolbutamide incorporated in liposomes (small unilamellarvesicles or SUVs) was prepared by co-sonication of 45 mg egg PC with 3.0 mg Tolbutamide in 0.5% w/v KCl solution. 2.25 mL of commercially available egg PC solution in chloroform was drawn by a pipet. 3.0 mg of Tolbutamide (dry weight) was added to it. The chloroform was evaporated under nitrogen and the sample was lyophilized for one hour. 1.62 mL of 0.5% w/v KCl and 0.18 mL D_2O was then added to the lyophilized sample and it was allowed to hydrate for two hours at 4 ^oC. The solution was then sonicated in an ice/water bath for one hour using a micro-tip sonicator and a 30% duty cycle. The sample was titrated by adding 1-2 μL of 1M KOH at a time.

The sample of 10 mole% Glybenclamide incorporated in SUVs was prepared by adding 146 μL of 21 mM solution of Glybenclamide in Dimethyl Sulfoxide (DMSO) to 0.9 mL of 0.5 w/v% KCl solution and 0.1 mL D_2O containing 25 mg PC pre-sonicated to form SUVs. The SUVs were prepared by the method already described before. The pH of the SUV solution was 10.63. The solution turned *milky* on the addition of DMSO and its pH dropped to 7.92. The pH was raised to 10.28 and the solution became translucent immediately.

Determination of pKa values by ^{1}H NMR

The pKas of Tolbutamide were determined both in solution and as 20 mole% additives to small unilameller vesicles (SUVs). The pKa of 10 mole% Glybenclamide was determined only in SUVs because of its insolubility in aqueous solution. All the pKa determinations were done by plotting chemical shift versus bulk pH.[4)]

1-D ^{1}H NMR experiments were performed at 20^{o}C in 10% v/v D_2O solution. 3-(Trimethylsilyl) Tetradeutero Sodium Propionate (TSP) was added to the samples as an internal standard. The experiments were performed on a Bruker DMX 500 NMR spectrometer using a "zgpr" pulse sequence. The frequency of the water pre-saturation pulse (O_1) was re-calibrated for each experiment. The downfield phenyl ring ^{1}H chemical shifts for both Tolbutamide and Glybenclamide were measured and plotted against the pH of the solution. Curve-fitting employing a "best fit" curve was used. The pH value corresponding to the mid-point of the sigmoidal curve was taken as the pKa of the compound.

Fluorimetric Assays

Pyranin-trapped small unilamellar vesicles (SUVs) were prepared according to established protocols.[5)]2.25 mL of commercially available egg PC solution in chloroform (Avanti) was drawn by a pipette. The mass of PC was estimated to be 45 mg. The chloroform was evaporated under nitrogen and the dried sample was lyophilized for one hour. At the end of the hour, 1.71 ml of 100 mM HEPES at pH 7.4 was added to it along with 90 μL of 0.5 mMPyranin solution (pH 8.2). The sample was centrifuged for twenty minutes in a low speed centrifuge. It was then sonicated for one hour in an ice/water bath by a Branson 350 sonifier using a 30% duty cycle and an output of 3. Pyranin was separated from the SUVs by the use of a G-25 Sephadex column containing 5 g of packing material. Pyranin was immobile on this column whereas the SUV solution eluted off with 100 mM HEPES at pH 7.4. The purified SUV solution was stored overnight at 4^{o}C before use.

Control Experiments

The effect of sonication on PC during the preparation of SUVs was checked by acquiring electrospray ionization mass spectra in the negative ion mode, before and after sonication of the sample. In the control experiment (Fig. 2 and 3), 2.25 mL of commercially available egg PC solution in chloroform (Avanti) was drawn by a pipette. The mass of PC was estimated to be 45 mg. The chloroform was evaporated under nitrogen and the dried sample was lyophilized for one hour. The lyophilized PC was suspended in deionized water and was then sonicated for one hour in an ice/water bath by a Branson 350 sonifier using a 30% duty cycle and an output of 3. The 1.8 mL solution was extracted with an equal amount of Chloroform. The chloroform solution was then analyzed by Electrospray Ionization Mass Spectrometry (ESI) in the negative ion mode. A similar sample was prepared for comparison but it was kept un-sonicated. Both the spectra were acquired using identical acquisition parameters. These spectra showed the presence of endogenous fatty acid in egg yolk PC (data not shown). The overall profile of all the molecular species present in the sample was very similar except for the appearance of a signal at an m/z ratio of 234.9 which was ignored as a contaminant.

To look at effect of dilution of the sample on the fluorescence intensity, 50 μL aliquots of 100 mM HEPES buffer at pH 7.4 were added to solutions of Pyranin-trapped SUVs containing pre-added 3 mole % Tolbutamide in one experiment and Glybenclamide in the other experiment. These experiments demonstrated that even when three times as much buffer was added as normally used for the fluorimetric assays(50 μL as compared to 15 μL), the change in fluorescence intensity was minimal.

The effect of the influence of the ionic strength of the buffer on the drop in fluorescence intensity was checked by exchanging the 100 mM HEPES buffer with 50 mM HEPESbuffer. It was found that the order of drop of fluorescence intensity still remained: Glybenclamide>Tolbutamide. The effect of the chemical nature of the fluorescent probe, pyranin, was also examined by exchanging it for a different fluorescent probe, BCECF (2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein) (Invitrogen). The order of the drop in fluorescence intensity was still: Glybenclamide>Tolbutamide.

Results:

pKa measurements

Sulfonylureas are weak acids. The pKas of Tolbutamide and Glybenclamide are reported as 5.43 and 5.3 respectively in solution.[6)]The sulfonamide group itself has a pKa of 10.1 in solution. When this group is next to a carbonyl group in a molecule, its pKa is lowered to a value between 5 and 6.5. It is known from previous studies that the effect of the ionization state of the molecule can be felt by a group

close to the ionizable group.[4)] For this reason, it was decided to measure the chemical shift of the phenyl ring hydrogens of both Tolbutamide and Glybenclamidelocated*ortho* to the sulfonamide group.

The pKa of Tolbutamide was determined both in solution and in the phospholipid bilayer at a ratio of 20 mole%. To make the determinations as comparable as possible, equal amounts (3 mg) of Tolbutamide were dissolved in solution in both the determinations (1.62 mL 0.5% w/v KCl and 0.18 mL D_2O). However, to determine the pKa of Tolbutamide in vesicles 45 mg egg PC was also added and co-sonicated with Tolbutamide. The titration in both the cases was done by adding 1-2 µL aliquots of 1 M KOH. The pH of the solutions was varied from the acidic to basic range and the chemical shift of the downfield phenyl ring hydrogen was monitored as a function of pH. It is already known from previous studies on fatty acids that the effect of pH change of the solution is manifested by a change in chemical shift of nuclei distant from the ionization center.[7)]

"Curve-fitting" the pH-dependent chemical shift of the phenyl ring hydrogen *ortho* to the sulfonyl group (upfield signal in Fig. 4) to a "best-fit value" provided sigmoidal curves in each case. The mid-points of the curves were used as the pKa values for each sample. It was seen that the pKaof Tolbutamide is 5.2 in solution whereas it shifts up to a value of 7.0 in the phospholipid bilayer. This result is consistent with the results reported earlier for Oleic Acid [7)] that show that the pKa of Oleic Acid shifts from 4.2 when bound to albumin to 7.5 when incorporated in 5 mole% PC vesicles. Like fatty acids, sulfonylurea derivatives are amphiphilic in nature and have limited solubility in HEPES buffer at pH 7.4.[8)] Similar to fatty acids, it is likely that in the presence of phospholipids, they become incorporated in the bilayer rather than co-existing as micelles or acid soaps in solution.

NMR experiments

NMR experiments were performed on Tolbutamide incorporated in SUVs. They provided evidence for the incorporation of this drug in the phospholipid bilayer. Fig. 4 shows the change in line shape and chemical shift values of the phenyl ring hydrogens of Tolbutamide at pH 3.30 as compared to pH 12.63. In the figure, the phenyl ring resonances of Tolbutamide are *broader* at pH 3.30 and sharper at pH 12.63. At a low pH Tolbutamide is protonated and is more soluble in the phospholipid bilayer. At a high pH, it is ionized and becomes at least partly soluble in solution.

To probe the ionization behavior of Glybenclamide by NMR, it was dissolved in Dimethyl Sulfoxide (DMSO) and added in 10 mole% ratio to pre-sonicated SUVs in 0.5 w/v% KCl. The ratio of DMSO to water in the final solution was 14.6% v/v. In the beginning, the pH of the SUV solution was 10.63. It dropped to 7.92 on the addition of DMSO and the solution appeared *milky*. The pH of the solution was raised again to 10.63 and it was observed that the solution turned transluscent. The chemical shift of the downfield phenyl ring resonance was monitored as a function of pH. Since, Glybenclamide has *two* phenyl rings. The ring next to the sulfonamide group was identified by chemical shift assignment of the molecule. Fig. 5 shows the signals of the phenyl ring of Glybenclamide which is next to the Sulfonamide group.

Fluorimetric assays

To monitor transport of sulfonylureas across the bilayer of the vesicles, 30 nanomole aliquots of sulfonylurea derivatives were added to a buffered suspension of vesicles with trapped Pyranin. The observed drop in fluorescence intensity, as shown in Figures 6 and 7, is due to a drop in pH inside the vesicles. Ten nanomoles of BSA (1/3 equivalents) were then added to the solution to extract the sulfonylureas from the vesicles. A partial recovery of the fluorescence intensity was observed. This is probably due to the fact that BSA does not have a very high affinity for these compounds.[9)] It reportedly has three binding sites for Tolbutamide with a K_d of 21 µM. It is unlikely that in the presence of the phospholipid bilayer of SUVs, these derivatives could form aggregates insolution at concentrations of 16.7 µM. The observed order for the drop in fluorescence intensity in 100 mM HEPES at pH 7.4 was observed to be: Glybenclamide>Tolbutamide

The order for the drop in fluorescence intensity may be due to the greater binding affinity of Glybenclamide as compared to Tolbutamide for the phospholipid bilayer. The fluorimetric response to different doses of Glybenclamide is shown in Figures 8 and 9. These figures show that due to the buildup of a pH-gradient across the phospholipid membrane, net flux of Glybenclamide occurs only up to a mole

ratio of fatty acid/PC of ~ 4%. This is due to the buildup of a pH-gradient across the phospholipid bilayer which opposes the transport of any further amount of these compounds into the lumen of SUVs.

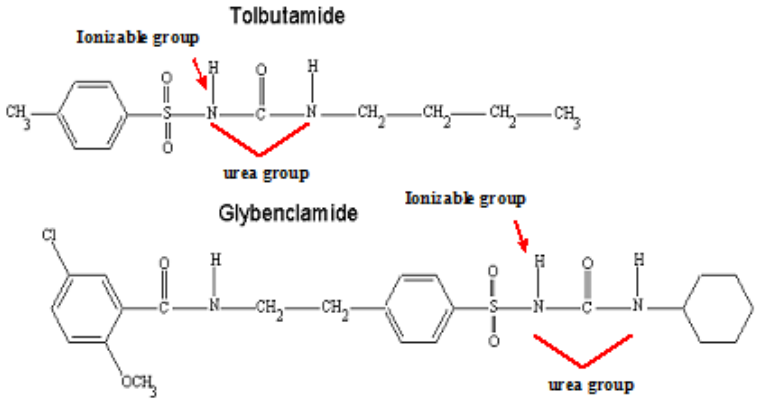

Fig. 1.Chemical structures of Tolbutamide and Glybenclamide showing that both the compounds possess an ionizable sulfonamide group. The basic structure of each molecule is of a urea group substituted by lipophilic substituents.

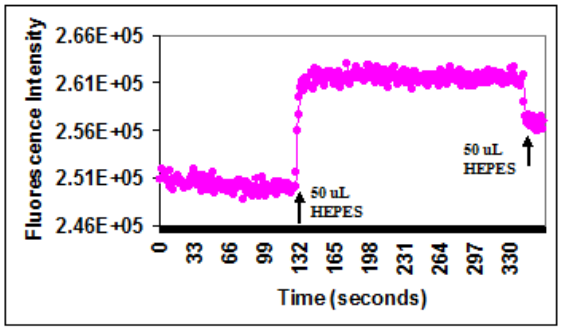

Fig. 2.Change in fluorescence intensity upon addition of HEPES buffer to SUVs containing 3 mole% Tolbutamide.

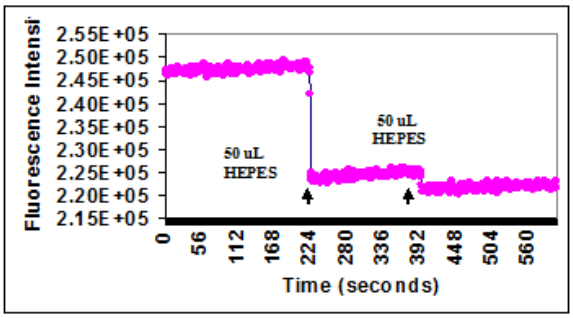

Fig. 3.Change in fluorescence intensity upon addition of HEPES buffer to SUVs containing 3 mole% Glybenclamide.

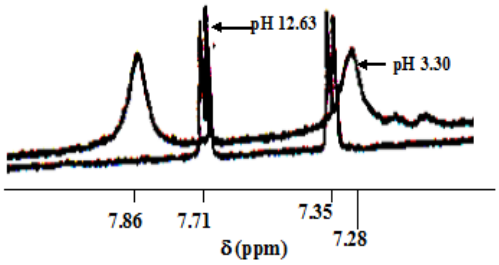

Fig. 4.Two superimposed 1-D ^{1}H NMR spectra of 20 mole% Tolbutamide incorporated in small unilamellar vesicles (SUVs) at pH 3.30 and 12.63 (downfield aromatic region of the spectra has been expanded).

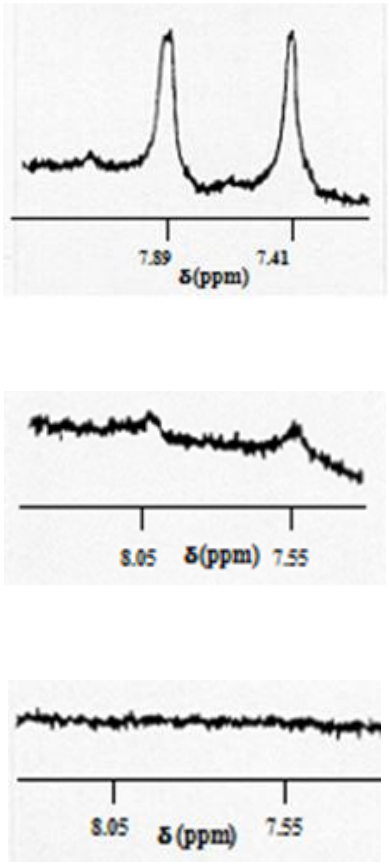

Fig. 5.Downfield aromatic region of the 1-D ^{1}H NMR spectra of 10 mole% Glybenclamide added to SUVs at pH 8.63 (top panel), pH 6.75 (middle panel) and pH 5.44 (lower panel).

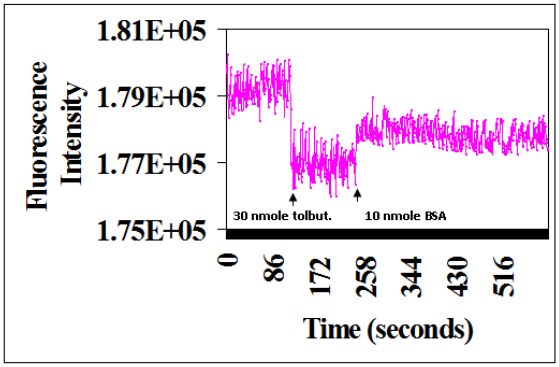

Fig. 6: Fluorimetric assay performed to see the effect of addition of tolbutamide to pyranin-trapped vesicles. A pH drop manifested by a drop in fluorescence intensity is observed. The fluorescence intensity did not recover fully after addition of bovine serum albumin (BSA).

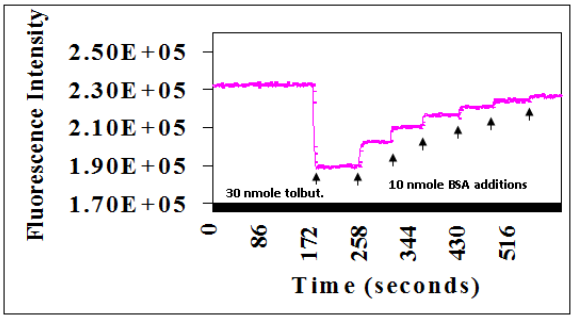

Fig. 7: Fluorimetric assay performed to see the effect of addition of Glybenclamide to Pyranin-trapped vesicles. A pH drop manifested by a drop in fluorescence intensity is observed. The fluorescence intensity does not recover fully even after repeated additions of BSA.

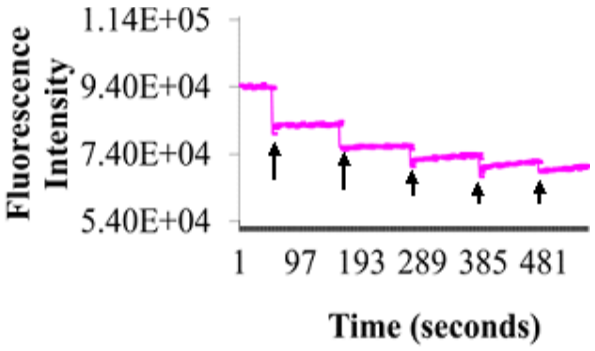

Fig. 8.Fluorimetric assay showing the dose-dependence of the drop in fluoresecence intensity on addition of Glybenclamide to Pyranin-trapped vesicles (*arrows indicate addition of Glybenclamide*).

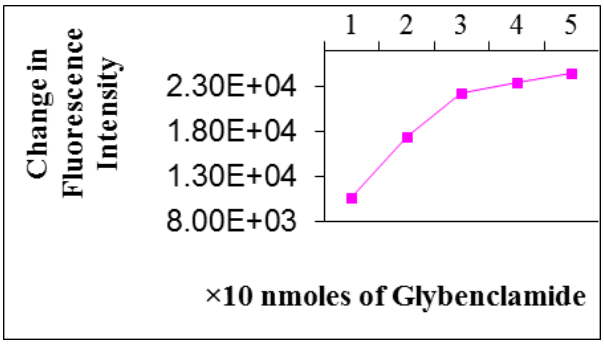

Fig. 9.Dose-response curve for the transport of Glybenclamide showing that a pH-gradient builds up that opposes the further transport of Glybenclamide in Pyranin-trapped vesicles.

Discussion:

In recent years, the "free diffusion" model originally proposed for the transport of fatty acids across the phospholipid bilayer has been questioned. Many studies have proposed a possible role of transport proteins for the uptake of fatty acids within cells.[10,11,12,13)] Sulfonylurea compounds are good candidates to test the validity of the "free diffusion" model since these compounds are known to be hydrophobic in nature and as this study demonstrated, possess an ionizable sulfonamide group. These derivatives are exactly the type of compounds for which the "Overton Rule" was formulated almost a hundred years ago. This rule states that the entry of a molecule inside a living cell is dependent upon the solubility of that molecule in the cell membrane surrounding the cell. This rule predicted the transport of some compounds into cell which include among others, cholesterol, bile acids and fatty acids. All these compounds are extremely hydrophobic in nature and are known to readily incorporate into the phospholipid bilayer and enter into the cell with half-lives of transport that range in value from a few milliseconds to a few seconds.[14,15)]

Sulfonylureas are weak acids due to the presence of an ionizable sulfonamide group. They have pKas ranging from 5-6.5 in solution. By conducting a pH-titration of a first-generation drug, Tolbutamide, its pKa was determined both in solution and when incorporated into small unilamellar vesicles (SUVs). It was found that the pKaTolbutamide undergoes an upward shift when it is present in the phospholipid bilayer. To monitor transport of sulfonylureas across the bilayer of the vesicles, 30 nanomole aliquots of sulfonylurea derivatives dissolved in ethanol were added to a buffered suspension of vesicles with trapped pyranin. The observed drop in fluorescence intensity was explained to be because of a drop in pH inside the vesicles due to the ionization of the sulfonamide group. On the basis of these experiments it was proposed that the "free diffusion model" [5,16)] originally proposed for long chain fatty acids can be used to explain the rapid transfer of sulfonylureas also. The proposed mechanism is also in conformity with the "Overton Rule".

Sulfonylurea compounds can experience four possible fates when they are added to aqueous solutions: (i) they can solubilize, (ii) they can form aggregates, (iii) they can become incorporated into the phopholipid bilayer, or (iv) they can form a monolayer at the air-water interface. Amphiphilic compounds like these typically have pKa values between 4 and 10.[17)]Theamphiphilic nature of such compounds is considered a pre-requisite for binding to the cell membrane. However, this still does not dictate that the compound will undergo an inward flip and enter the lumen. It was previously shown that such compounds experience an upward pKa shift upon binding to the phospholipid membranes.[18)] It also known that such amphiphilic compounds are oriented in the phospholipid bilayer in such a manner that the ionizable group remains exposed to the lipid-water interface while the hydrophobic portion of the molecule remains hidden in the bilayer.[19)]

The fluorimetric assays show that the pH drop inside the SUVs is greater upon the addition of Glybenclamide than on the addition of an equimolar amount of Tolbutamide. This is shown by the 1-D ^{1}H NMR experiments also. These experiments demonstrate that the membrane/water partitioning of Tolbutamide and Glybenclamide is pH-dependent. In HEPES buffer at pH 7.4 (used for the fluorimetric assays), both Tolbutamide and Glybenclamide are partially ionized. Tolbutamide prefers to bind to SUVs

in the neutral state whereas Glybenclamide prefers to bind to SUVs in the ionized form. Tolbutamide actually dissolves in solution when it is in the ionized state, whereas, Glybenclamide precipitates out of solution when in the neutral form. The fluorimetric assays demonstrate that due to the ionization of transported sulfonylureas, there is a drop in pH inside the lumen of the vesicles that results in a drop in fluorescence intensity of the trapped Pyranin.

Conclusion:

Both Tolbutamide and Glybenclamide can rapidly cross the phospholipid bilayer by a non-energy dependent "free diffusion mechanism." Transport of both these compounds by "free diffusion" across model membranes in the lumen of SUVs is accompanied by a drop in pH that results in a drop in the observed fluorescence intensity of the trapped Pyranin dye. Though the results do not disprove the existence of a carrier protein tofacilitate the transbilayer movement of these compounds across cell membranes, they do prove that the presence or absence of such a protein does not have to dictate the availability of these compounds to the β-cells of the Islets of Langerhans in pancreas.

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