IN VITRO DIFFUSION STUDIES OF SOME DRUGS ACROSS THE CHORIOAMNION MEMBRANE OF FULL TERM HUMAN PLACENTA

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TO MY MOTHER

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ABSTRACT

There is ample evidence to suggest that the *in vivo* drug exchange between the fetus, mother and amniotic fluid occurs via the placental site as well as through the chorioamnion membrane.

In this work, the *in vitro* diffusion of different drugs through mounted human chorioamnion membrane was studied in an effort to elucidate the mechanism of transport of pharmacologically active agents across the above membrane.

An extensive study was made on the diffusion of procaine hydrochloride and sodium salicylate, from which it appears that procaine hydrochloride diffuses by a carrier transport mechanism in contrast to sodium salicylate which passes through the membrane by a simple diffusion process. The passage of tetracycline hydrochloride, dexamethasone-21-phosphate and sodium ampicillin were similarly studied.

All the drugs examined in this work were shown to diffuse at different rates. Cholesterol was found to be taken up by the membrane at a rate which followed zero order kinetics though very little of the steroid diffused through.

It was concluded that the human chorioamnion membrane could be characterized as a selective membrane to drug permeability.

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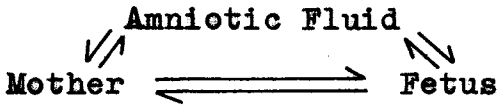
Introduction

The exchange of water and solutes between the mother and the fetus has been of great concern in the past. However, the problem received greater attention after the thalidomide tragedy in 1961. It is known that a cycle exists among the mother, fetus and amniotic fluid concerning water and electrolytes (1, 2), and such a cycle could be considered as a three compartment system (Scheme I)



Scheme I.

Several studies suggested that fetomaternal exchange is not limited to the placental site but may occur across the chorioamniotic membrane as well (3, 4, 5, 6, 7). When deuteriated water was injected into the maternal blood it was found to pass into the amniotic fluid, and conversely when injected into the amniotic fluid it appeared in the maternal blood (3). The possible routes by which such an exchange could have occured can be visualized from Scheme I. Thus, Gray and coworkers (8) reported that at least 25% and probably 50% of the amniotic fluid exchanged in humans occured via the placental site and the rest via the chorioamniotic membrane.

Although there is abundant evidence to suggest that

fetal micturation occurs in the uterus, most investigators believe, contrary to Hippocrates, that the amniotic fluid is not primarily composed of fetal urine (2). Later studies suggested that the amniotic fluid is in rapid exchange between the mother and the fetus. Flexner and coworkers (9) found that the amniotic fluid (about one liter during gestation) is completely replaced once every 2.9 hours, and this was in principle supported by Plentl and coworkers (3) who reported that the exchange rates between the mother and the amniotic fluid, were 600 ml per hour, in both directions.

The amniotic fluid increases in quantity up to the sixth or seventh month of gestation, after which it diminishes to about one liter at the end of pregnancy. It protects and allows the free movement of the fetus at the later stages of pregnancy. It contains less than 2% of solids, consisting of urea and other extractives, inorganic salts, a small amount of protein and frequently a trace of sugar. The general belief that some of the amniotic fluid is swallowed and ingested by the fetus is supported by the fact that epidermal debris and hairs have been found among the content of the fetal alimentary tract. Furthermore, when radiopaque substances were injected into the amniotic fluid they were found to concentrate in the fetal lungs, indicating absorption of substances in the fluid by the digestive tract (4).

The chorion, the vascular part of the chorioamnion membrane, expands by an obliteration caused by the amnion. It is made up of the primary villi extending from the chorion to the uterine wall. As the villi increase in size and ramify, many secondary villi grow out from its wall. As they grow and branch, chorionic mesoderm and angioblasts extend into them and multiply to keep pace with their growth. The isolated angioblasts, in the villi and in the chorion, multiply and develop isolated vascular channels which soon connect to form vascular plexuses that unite to form the vascular tree which finally connects with the blood vessels of the embryo.

The amnion, whose exact mode of origin in man is not known, is non vascular. It is made up of cuboidal cells separated by mesenchymal cells. Its outer surface is covered by a layer of mesoderm. At about the fourth week of gestation, the amniotic fluid accumulates and expands it until it comes into contact with the inner wall of the chorionic membrane and obliterates it. It also grows around the body stalk and yolk sac and then forms the covering of the umbilical cord (10).

There is ample evidence to suggest that the <u>in vivo</u> diffusion of solutes in a pregnant woman occurs through the three compartment system (Scheme I). Thus, a number of dye stuffs have been found to pass from the amniotic sac into the fetus and mother, in guinea pigs, white rats and humans (4).

Albano (11) while experimenting with humans, found that when phenosulphonaphthalein is injected into the amniotic sac near term, it has a disappearance half time of 7 hours. Dieckmann and coworkers (12) showed that congo red slowly disappeared from the amniotic cavity and promptly appeared in the maternal circulation. Conversely, quinine and barbituric acid were noticed to pass readily from the mother to the amniotic fluid (4). De Snoo (13) reported that methylene blue and saccharin were excreted in the urine of women to whom the substances were introduced into their amniotic sac. McGaughey and coworkers (7), Serr and coworkers (14) found equal concentrations of urea and uric acid in the mother and the fetus and higher concentrations between the amniotic fluid and the mother. This could be rationalized either by the fetal excretion of urea and uric acid or by an active transport from the maternal tissues into the amniotic fluid. On the other hand, in vivo studies with various carbohydrates suggested that their passage involved a specific transport mechanism similar in many respects to that of the erythrocyte (15, 16), and not a simple diffusion process. Intramuscular and intravenous injection of ampicillin and benzyl penicillin to humans, showed high levels of these drugs in the amniotic fluid, while chloramphenicol, streptomycin and tetracycline did not pass into this compartment (17). Moreover, Freda (6) suggested the passage, directly from the uterine wall into the amniotic fluid and vice versa, of blood group substances

A, B and O (H) despite their large molecular weight.

In vivo quantitative permeability studies across the placental membranes are difficult to conduct because the amount of solute transferred per unit time cannot be measured accurately, and the arteriovenous differences among the different solutes have not yet been determined (1, 16).

Although in vitro experiments permit quantitative studies on the permeability of substances through the chorion, amnion and chorioamnion membranes, very little has been done on a molecular level. Garby (4), performed transport studies across the human chorion with quinine, creatinine and some labelled ions. However he was not able to detect a potential across the chorionic membrane. Battaglia and coworkers (18) while performing in vitro studies on the human chorion as a membrane system found that D-Glucose was utilized and allowed to pass by the membrane without a reduction in the membrane activity, while D-arabinose passed across the membrane without chemical alteration, and inulin did not pass through. More recently, Battaglia and coworkers (16) reached the conclusion that the carbohydrate passage through the chorion laeve followed a simple diffusion process. Katz and coworkers (19) studied the in vitro relative transfer of estriol and its conjugates (estriol-3-sulfate and estriol-16-glucosiduronate) across the fetal membranes. Estriol was transported faster than its conjugates through the chorion and amnion when tested separately. Transfer of all the above steroids was more rapid

through the amnion than the chorion, and a homogenate of the latter when incubated with estriol-3-sulfate resulted in its hydrolysis. No hydrolytic activity was observed with the amnion. Battaglia and coworkers (20) while comparing the permeability of different layers of the primate placenta to D-arabinose and urea found no change in the permeability of the three membranes (chorion, amnion and chorioamnion) with gestational age. All three tissues were found to be more permeable to urea than D-arabinose.

From the above discussion there is relatively little and diverse evidence to support conclusions on the transport of substances via the chorioamnion membrane. Thus a simple diffusion process was suggested for quinine and creatinine (4) and carbohydrates (18); while in vivo results obtained with urea, uric acid (7, 14), and electrolytes on the chorioallantoic membrane of the pig (21) seem to indicate that an active transport is operative.

The present report deals with some attempts of obtaining more knowledge concerning the mechanism of transport of some pharmacologically important molecules across the chorioamnion membrane of the human placenta.

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Materials and Methods

Reagents and Drugs used

Brodie's solution (American Instrument Company, Inc. Silver Spring, Maryland.)

Calcium Chloride (Analar).

Cholesterol (E. Merck A.G. Darmstadt).

4-C^{14}-Cholesterol (The Radiochemical Center, Amersham, England).

Dexamethasone-21-Phosphate (Merck Sharp and Dohme, U.S.A.).

Dioxane, redistilled (Analar).

Magnesium Sulfate, Hydrated ($MgSO_4.7H_2O$) (Analar).

Oxygen, High Purity Dry, was bubbled through Barium Hydroxide and water traps (L'air Liquide, Beirut, Lebanon).

2,5-diphenyloxazol ("PPO") (Packard Instrument Company, 2200 Warrenville Road, Illinois).

Dimethyl - 1,4-bis {2(5-phenyloxazol)-benzene} ("Dimethyl - POPOP") (Packard Instrument Company, 2200 Warrenville Road, Illinois).

Potassium Chloride (Analar).

Potassium Cyanide (Analar).

Potassium Hydroxide (Analar).

Procaine Hydrochloride (E. Merck A.G. Darmstadt).

Sodium Ampicillin ("Penbritin", Beecham Research Laboratories, Brentford, England).

Sodium Chloride (Analar).

di-Sodium Hydrogen Orthophosphate, Hydrated ($Na_2HPO_4.12H_2O$) (Analar).

Sodium Salicylate (E. Merck A.G. Darmstadt).

Tetracycline Hydrochloride (Through the courtesy of Charles E. Frosst & Co. (Middle East) S.A.L.)

Kreb Ringer's Phosphate Buffer (pH 7.4) (22).

The buffer was prepared by mixing the following:

100 parts of 0.90% Sodium Chloride (0.154 M) solution.

4 parts of 1.15% Potassium Chloride (0.154 M) solution.

3 parts of 1.22% Calcium Chloride (0.11 M) solution.

1 part of 3.82% Magnesium Sulfate (Hydrated) (0.154 M) solution.

33 parts of 0.1M phosphate buffer solution pH 7.4. (made by adding 20 ml 1N Hydrochloric Acid to 35.8 g di-Sodium hydrogen Orthophosphate (Hydrated) and making it up to 1000 ml with distilled water.)

Scintillation Mixture (scintillation "cocktail").

The scintillation cocktail used contained a primary and a secondary scintillator and had the following composition:

4 g 2,5-diphenyloxazol ("PPO") (primary scintillator).

0.05 g dimethyl-1,4-bis {2(5-phenyloxazol)-benzene} ("Dimethyl POPOP") (secondary scintillator)

1000 ml Toluene

Instruments and Apparatus

Erlenmyer flasks, 10 ml (Corning).

Hypodermic needles Yale 20 2.5 long.

S & S analytical filter paper No. 597.

Spinal needles Yale 20 15.2 cm long.

Syringes

Durex Eva glass (Micromatic) 5 ml.

Vim Glass Tip Tuberculin Regular Syringe. One ml.

Volumetric flasks 25 ml, 50 ml (Corning).

Vials for scintillation (20 ml) were purchased from Packard Instrument Company, 2200 Warrenville Road, Illinois.

Apparatus Used for the Diffusion of Drugs

The apparatus (Fig. 2) consists of two cylindrical leucite blocks 80 mm in diameter and 36 mm thick. Into each of the opposing surfaces of each block a well 20 mm in diameter and 18 mm deep has been cut. A membrane when mounted forms a common wall between the wells. Surrounding each well a cylindrical chamber 9 mm thick and 25 mm deep serves as a constant temperature jacket which is connected with an inlet and outlet each 6 mm in diameter. Thus, when water at 38°C (pumped from a constant temperature bath by means of a centrifugal circulating pump) is circulated in this jacket, the temperature within the wells is 37°C.

Each well communicates through a chimney 7 mm in diameter and 92 mm in height with the atmosphere. At the top of each chimney a rubber septum is placed. Through the septum

a long spinal needle (No. 20 15.2 cm long) is put extending all through the chimney to the bottom of the chambers providing a means of introducing the buffer and drug to be studied, and for serial sampling of the drug at different intervals. When no sampling is performed, the spinal needles are connected to a gas tank (Oxygen) and the gas is allowed to bubble into the well's solution and subsequently escape through a short hypodermic needle (No. 20 2.5 cm long) which acts as a gas exit.

The apparatus throughout the experiment, is mounted at an upright position on a wooden base (Fig. 1).

In all cases two identical sets of the above apparatus were used.

Spectrophotometry

All spectrophotometric measurements were performed in a Perkin Elmer automatic recording spectrophotometer (Model 202) using one centimeter thick quartz cells.

Radioactivity Measurements

All radioactivity measurements were taken with a Packard Tri-Carb Scintillation Spectrometer (Model 3003) with a background of 12 counts per minute (cpm) and an efficiency of 70%.

Samples to be counted were introduced into appropriate scintillation vials and one ml of dioxane, followed by the addition of 17 ml of the scintillation mixture ("cocktail")

were added and counted for five minutes after thorough mixing.

Manometric Techniques Used for the Determination of the Chorioamnion Oxygen consumption

The procedure followed for the chorioamnion oxygen consumption was mainly that of Friedman and Sachtleben (22). Small pieces of the chorioamnion membrane (0.2 g) were cut and washed as described under "Preparation of Membrane". The pieces were introduced in precalibrated Barcraft-Warburg vessels of 15 ml capacity. One of the vessels was left empty to be used later as a thermobarometer. To the side well of each of the vessels containing the cut membranous pieces and into the vessel serving as a thermobarometer, 3 ml of the buffer or the drug solution were introduced. To the central well containing a piece of one cm^2 fluted filter paper, 0.1 ml of 10% potassium hydroxide solution was added. The vessels were then attached to the manometers, dipped in the constant water bath at 37°C and allowed to agitate for 10 minutes. Subsequently, the level of Brodie's solution in the right hand part of the manometer was adjusted to the 250 scale, the stopcocks turned to prevent atmospheric connection to the manometers, and immediately the level of the left hand part of the manometers was recorded. The vessels were then allowed to agitate in the water bath and readings were taken (at time intervals) on the left hand part of the manometer. The level

of the Brodie's solution in the right hand part was always adjusted to the 250 scale prior to every reading.

Thin Layer Chromatography (TLC).

All thin layer chromatograms, unless otherwise specified, were run ascending in pre-equilibrated chambers and were of 0.25 mm thickness. The plates were activated at 110°C for 30 minutes. The Silica Gel G that was used to coat the plates was purchased from E. Merck A.G. Darmstadt, Germany.

Following are the solvents and reagents used for the elution and development of the TLC plates:

Solvents (23a, 23b)

1. Chloroform-Methanol (80 + 10).
2. Methanol-Acetic Acid-Ether-Benzene (1+18+60+120).
3. Chloroform-Acetone- (Phosphate-Citrate buffer) (50+50+20).
4. Methylene Chloride.
5. Acetone-Methanol (50 + 80)

Reagents (23a, 23b)

1. Dragendorff's-reagent.
2. Ferric Chloride for hydroxamic acids and phenols.
3. U-V light after exposing plate for a few seconds to ammonia vapor.
4. Acetic anhydride-sulphuric acid (Liebermann-Burchard Test).
5. Iodine-Azide solution.

Preparation and Mounting of the Membrane

Full term, human placentas were dipped in a freshly prepared Kreb Ringer's Phosphate Buffer solution and directly sent to the laboratory. The placentas were then exposed in a plastic tray and with the help of fine surgical scissors two round neighboring pieces of the tissue (referred to as chorioamnion) representing a piece of the intact membranous portion of the placenta and consisting of chorion and amnion, were cut from the membrane portion opposite to the placenta. The membranes were inspected carefully to insure no macroscopic defects and then washed with the buffer from any blood that might have resided on them.

Subsequently, a membrane piece was mounted between the two wells of the apparatus in such a way as to expose the chorionic surface (representing the maternal side) to the right hand well, and thus the amniotic surface (representing the fetal side) was exposed to the left hand well. Into the right and left hand wells 6.5 ml of the drug solution and an equal volume of the buffer were introduced, at the same time, respectively. Water (at 37°C) was then circulated around the jackets surrounding the wells.

General Method of Sampling

Unless otherwise specified, samples were withdrawn from the right hand wells of each of the apparatus with a one ml tuberculin syringe, and were of such volume that at

the end of the experiment the level of the liquid in the wells remain fully covering the membranes. The samples were then diluted appropriately to allow accurate spectrophotometric measurements, taking into account that leached material from the membrane did not interfer with the measurements as was shown by base line spectra.

In all the cases studied, samples were taken at the end of the experiment from both chambers and were analysed by thin layer chromatography to insure that no alterations occured during the diffusion of the drug through the membrane.

General Discussion

Transport Phenomena Across Membranes

Two main processes are available for drug transport:

1. Passive Transport

In this kind of transport the membrane can be considered to behave as an inert lipid solvent or system of aqueous channels through which a drug passes. According to O'Reilly (24) the following points should be noted concerning passive transfer:

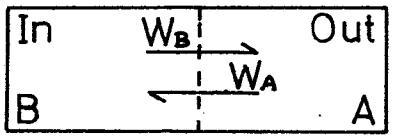
a. The rate of transfer is maximal when the solute concentration on one side of the membrane is zero, and decreases till equilibrium is reached.

b. The rate of transfer is increased proportionally as the concentration of solute in the outer side is increased.

c. The rate of transfer depends on the concentration gradient and on the lipid solubility of the drug.

d. Other factors influencing passive transport are Donnan equilibrium, pH partition, and protein binding effects.

The following is a mathematical derivation of a passive transport process across a membrane (25).



Symbols and Abbreviations

C_o = Concentration of solute in A at t = o.

t = time

C_A = Concentration of solute in A at any time t.

C_B = Concentration of solute in B at any time t.

W_A = Unidirectional flow rate of solute from A to B.

W_B = Unidirectional flow rate of solute from B to A.

k = Proportionality constant characteristic of solute and membrane.

$\frac{dC_A}{dt}$ = Rate of change in solute concentration in A at any time.

$\frac{dC_B}{dt}$ = Rate of change in solute concentration in B at any time.

V_A and V_B are volumes of chambers A and B respectively.

Conditions:

The following conditions must be satisfied prior to the derivation of equations iv and v.

1. $V_A = V_B$
2. Perfect stirring. The time of mixing by bubbling the gas is much less than the time of the experiment.
3. The thickness of the membrane must remain constant.
4. The area of the membrane exposure must remain constant.

Derivation:

Assuming that the solute is not altered or metabolized, then the rate of solute disappearance from chamber A, equals the rate of solute appearance in chamber B at any time.

Therefore, $$-\frac{dC_A}{dt} = \frac{dC_B}{dt} = W_A - W_B$$

and $W_A = k\,C_A$, $W_B = k\,C_B$

Substituting in (i), $\frac{dC_B}{dt} = k\,(C_A - C_B)$ (ii)

At any time $C_o = C_A + C_B$, therefore $C_A = C_o - C_B$.
Substituting in (ii), and arranging terms,

$\frac{dC_B}{dt} = k(C_o - 2C_B)$. Therefore $\frac{dC_B}{(C_o - 2C_B)} = kdt$

Integrate, $\int \frac{d\,C_B}{C_o - 2C_B} = k \int dt$

Therefore $-\frac{1}{2} \ln (C_o - 2C_B) = kt + \text{constant}$ (iii)

When $t = 0$ then $C_B = 0$, therefore the constant in (iii) $= -\frac{1}{2} \ln C_o$.

Substituting in (iii), and rearranging terms, and changing to $\log_{10}$,

$$- \log \frac{(Co - 2C_B)}{Co} = \frac{2}{2.303} kt \qquad \text{(iv)}$$

$$\text{or } - \log \frac{(2\,C_A - Co)}{Co} = \frac{2}{2.303} kt \qquad \text{(v)}$$

A linear relationship will result when the logarithmic terms of equations (iv) and (v) are plotted versus time.

2. Carrier Transport

In this kind of transport, the membrane can be thought of being actively participating in the process. In the diffusion process a chemical is beleived to exist in the membrane which combines with the solute and carries it across to be discharged free on the other side. The process could either

be:

a. Facilitated diffusion, a process that can attain equilibrium with respect to solute concentration across both sides of a membrane.

b. Active diffusion, a process resulting in a movement across a concentration gradient even after reaching an equilibrium state (24).

Although a carrier transport system could be shown to follow a complicated mathematical equation (26), this does not fully include all the physico-chemical factors of membrane solute and solvent relationship pertinent to membrane permeability.

In this report, we will be satisfied to take into account that a system not obeying the passive transport mathematical relationship (equations iv and v) will probably be considered a carrier transport system. Kedem and Katchalsky (27) tried to derive permeability equations on the basis of irreversible thermodynamics taking into account the following three coefficients required to characterize permeability for a solute-solvent system.

a. Friction between solute and solvent.

b. Friction between solvent and membrane.

c. Friction between solute and membrane.

The latter points, b and c, were inclusive in the permeability constant of passive transport conditions. It is of importance to note that not in all processes will the

three coefficients be equally important.

Carrier Transport Inhibitors

Carrier transport involves expenditure of energy, and consequently substances which poison energy production inhibit the process. Such inhibitors include 2,4 dinitrophenol, phlorizine and its derivatives, potassium cyanide, mercury and various mercury compounds, lachrymators, narcotics, tannic acid, formaldehyde, Di-isopropylfluorophosphate (DFP) and a number of other compounds. The inhibitors might act by blocking the transfer of the solutes or by transforming their mode of diffusion from an active to a passive (24, 26). Crawford and McCane (21) while experimenting on the chorioallantoic membrane of the pig, found that when 95% oxygen mixed with 5% carbon dioxide was bubbled in the buffer solution bathing the membrane, the active transport of sodium ions was inhibited as shown by a drop in the short-circuit current employed to indicate unequal distribution of electrolytes across the membrane. Similarly when oxygen was substituted with nitrogen an inhibition of the active transport of sodium ions occured proving that gases such as nitrogen and carbon dioxide, may act as inhibitors on active transfer.

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Results and Discussion

The validity of *in vitro* studies with biological membranes depends on several conditions under which the experiments are carried. Before any conclusions can be made with experimental findings, one has to show that the metabolic activity of the membrane remains reasonably constant. The rate of oxygen consumption of a biological membrane can be considered as a good measure of its metabolic activity. Friedman and Sachtleben (22) showed that the average oxygen quotient (+) (QO_2) of normal placental lobes dropped 42.5% when the representitive pieces from the lobes were bathed in a Kreb-Ringer's Phosphate buffer solution (pH 7.4) for a period of 7 hours.

Fig. 3 shows a plot of the average QO_2 in µl/mg dry weight/hour of the chorioamnion membrane from eight different placentas. The duration time of the experiment was five hours after which a drop of 4.16% from the initial metabolic activity was observed.

It was also found by Friedman and Sachtleben (22) that the average metabolic activity decay rate of the placental tissue lobes over the 7 hours period ranged from 2.1 µl/mg/hr. to 1.25 µl/mg/hr., while in our hands, that of the chorioamnion (over a 5 hours interval) ranged from 0.72 µl/mg/hr.

(+) Oxygen quotient is defined as the number of microliters of oxygen taken up per milligram of dry weight of tissue per hour and will be abbreviated as QO_2 throughout the text.

to 0.69 µl/mg/hr. It is also interesting to note that the QO_2 of the placental lobes was found to be higher than that of the chorioamnion membrane and furthermore that the latter was metabolically active under the experimental conditions used during the diffusion studies.

The choice of the drug studied depended primarily on their stability at the experimental conditions as well as on their gynecological applicability. The results, described below, have been obtained by experimental studies that required about one hundred full term normal delivery human placentas obtained from the Hospital of the American University of Beirut.

Diffusion Studies with Procaine Hydrochloride.

Tables I, II, III and IV as well as Fig. 4, 5, 6 and 7 describe the results obtained from the diffusion studies of 0.04% $W/_V$ procaine hydrochloride with human chorioamnion membranes in a Kreb Ringer's Phosphate buffer (pH 7.4), at the conditions described under the section of materials and methods. The addition of 10^{-3}M potassium cyanide in the buffer caused a considerable acceleration on the diffusion rate of procaine hydrochloride as compared to the diffusion of the drug through a neighbouring piece of the chorioamnion in the absence of the inhibitor (Table I, Fig. 4). It is interesting to note that the diffusion of the drug, at a concentration of 0.04% $W/_V$, did not follow simple diffusion kinetics (equations iv and v). However in the presence of

potassium cyanide the diffusion followed simple diffusion kinetics, as can be seen from the straight line plot in Fig. 4. One possible interpretation of the above results is that the membrane normally provides a "resistance" (20), towards the passage of such molecules as procaine hydrochloride, which could be enzymatically controlled. This resistance is abolished by the inhibiting effect of potassium cyanide (26) on the enzymes. Consequently, the passage of procaine hydrochloride becomes effectively passive.

Since the above experiment suggested that enzymes might play a role (among other factors) in the transfer process of procaine hydrochloride, the effect of an energy providing substance such as D-glucose was studied (Table II, Fig. 5). It was found that the addition of 0.1% $W/_V$ of D-glucose in the buffer caused an increase in the diffusion rate of procaine. It might be added here that Battaglia and coworkers (18) have recently shown a considerable uptake of D-glucose by the chorion membrane. If the effect of D-glucose is associated with enzymes, then one would expect that the potassium cyanide be opposing D-glucose activity when both substances are present near the membrane site. Table III and Fig. 6 show that the cyanide abolished the D-glucose accelerating action and surprisingly enough it caused a decrease in the diffusion rate of procaine. A possible explanation to the above observation is that in the absence of enzymatic activity (caused by potassium cyanide) the D-glucose

molecules provide a mechanical interference to the passage of procaine.

An increase in the temperature of the experiment (5°C interval range between constant temperature bath control) caused a rise in the diffusion rate of procaine (Table IV, Fig. 7) substantiating the participation of enzymes in the transfer of this drug.

Diffusion studies with Sodium Salicylate.

Table V and Fig. 8 describe the results obtained from diffusion studies of 0.04% sodium salicylate through the human chorioamnion membrane. The addition of 10^{-3}M potassium cyanide in the buffer had little or no effect on the diffusion rate as compared to the diffusion of the drug through a neighbouring piece of the chorioamnion in the absence of the inhibitor. Moreover, it is interesting to note that the addition of 0.1% $W/_V$ D-glucose in the buffer solution did not cause an acceleration in the drug transfer, as was the case with procaine hydrochloride, but a decrease in the drug diffusion (Table VI, Fig. 9) indicating an interference by D-glucose to the passage of salicylate. Since the above experiments suggested no enzymatic role in the transfer process, one could propose that the latter followed a simple diffusion kinetic rate.

It is worth noting here that at the pH of the experiment the drug was mainly as the salicylate ion (Salicylic

Acid pKa 3.0) as compared to procaine hydrochloride which at pH 7.4 was in its half salt form (28). Possible explanations for the transfer rate differences between the procaine hydrochloride and the salicylate ion is that either the chorioamnion membrane is selectively permeable to water soluble substances (such highly ionic substances) or that an active resistance exists opposing the penetration of procaine.

The nature of transfer of the salicylate ion in the above experiments appears to be similar to that observed by Battaglia and coworkers for D-glucose, D-arabinose and urea (15, 16, 18). These workers have shown that the above water soluble compounds pass through mounted chorion, amnion and chorioamnion membranes by a simple diffusion process.

Diffusion Studies with Sodium Ampicillin, Tetracycline Hydrochloride, and Dexamethasone-21-Phosphate.

The diffusion studies on these drugs were simply performed under usual experimental conditions with the purpose of measuring the extent of their permeability. The membrane was exposed to the drug solution with no other ingredient present other than the buffer.

Felton and Williams (29) have recently shown that sodium ampicillin (Sodium-D- α -Aminobenzylpenicillin) passes from the maternal blood to the liquor amnii. The in vitro experimental results shown in Table VII and Fig. 10 demonstrated the drug passage.

Charles (17) while performing *in vivo* experiments on the passage of tetracycline hydrochloride from the maternal blood into the amniotic fluid, discovered little or no diffusion of the drug. The *in vitro* experimental results (Table VII, Fig. 10) with tetracycline hydrochloride can be paralleled to the *in vivo* results in that little or no passage of the drug occured.

Dexamethasone-21-Phosphate (9α-Fluoro-16α-methyl-prednisolone-21-phosphate) was shown to penetrate also through the chorioamnion membrane as can be seen from Table VII and Fig. 10.

All the above five drugs studied were found (by thin layer chromatography) to diffuse chemically unaltered through the human chorioamnion membrane. The conditions used for the thin layer chromotography are summerized in Table X.

Diffusion Studies with 4-C^{14}-Cholesterol.

When the diffusion of 4-C^{14}-Cholesterol was studied, the substance was found to disappear in a considerably fast rate (Table VIII, Fig. 11) from the chamber facing the maternal side of the chorioamnion membrane, while very little of it appeared in the opposite side. It is suspected that the substance is absorbed by the membrane and metabolized. These results are concomitant to the findings of Katz and coworkers (19) who demonstrated that enzymes are present in the chorion membrane capable of causing biotransformations on steroids

such as estriol-3-sulfate. Whatever the exact fate of 4-C^{14}-cholesterol may be, it is noteworthy to point that the rate of disappearance of 4-C^{14}-cholesterol in our experiments followed zero order kinetics as it is depicted by Fig. 11. Moreover, when the homogenate of the membrane at the end of the experiment was extracted with chloroform, filtered and counted in the scintillation "cocktail"; the counts seemed to be nearly equivalent to the total amount lost from the chamber facing the maternal site of the membrane (Table VIII).

Fig. 10 shows the rate of diffusion across the human chorioamnion membrane of all the drugs studied. From the figure and Table IX, it can be seen that sodium salicylate had the highest diffusion rate followed by sodium ampicillin and dexamethasone-21-phosphate and tetracycline hydrochloride. Table IX shows the percentage of the drug diffused at the end of a three hours interval.

The evaluation of the <u>in vitro</u> results with the chorioamnion membrane is complicated by the fact that it is impossible to reproduce exactly the physiological conditions of the three compartment system involving the mother, the fetus and the amniotic fluid. Furthermore, a drug which is shown <u>in vivo</u> to pass at a certain rate into the amniotic fluid might follow one or several pathways as depicted in Scheme I of the introduction. Thus great care must be exercised in interpreting the <u>in vitro</u> results. It can therefore

be said, that the results presented in this account can only be used at present to throw more light on the mechanism of transfer of organic compounds across the human chorioamnion membrane.

Conclusion

From the above studies, the following conclusions can be made:

1. The human chorioamnion membrane was shown to be metabolically active for a long period of time in a simulated buffer solution.

2. Different drugs penetrate through the chorioamnion membrane at different rates and probably with different modes of transfer.

3. The present study suggests that the molecular weight is not a major factor in the drug's diffusion as was with dexamethasone-21-phosphate (molecular weight of dexamethasone 392.45) and tetracycline hydrochloride (molecular weight of tetracycline 444.43).

4. The membrane was found to contain enzyme systems as could be seen from the diffusion and the uptake of some drugs.

5. The membrane can be considered suitable for diffusion studies due to its flexibility, elasticity and durability.

Table I.

Effect of 10^{-3}M Potassium Cyanide Solution on the Diffusion Rate of 0.04% $W/_V$ Procaine Hydrochloride.

Experiment	Time in Minutes	Absorbance (C_B) at 292 mμ	Co	$-\log\left(1-\frac{2C_B}{Co}\right)$
	0	0.00	0.96	0.00
10^{-3}M KCN & 0.04% Procaine HCl	60	0.08	0.96	0.08
	180	0.21	0.96	0.25
	300	0.285	0.96	0.39
	0	0.00	1.05	0.00
0.04% Procaine HCl	60	0.02	1.05	0.017
	180	0.125	1.05	0.12
	300	0.255	1.05	0.29

Table II

Effect of 0.1% $W/_V$ D-Glucose Solution on the Diffusion Rate of 0.04% $W/_V$ Procaine Hydrochloride

Experiment	Time in Minutes	Absorbance (C_B) at 292 mμ	Co	$-\log\left(1-\frac{2C_B}{Co}\right)$
0.1% D-Glucose & 0.04% Procaine HCl	0	0.00	1.02	0.00
	60	0.12	1.02	0.11
	180	0.24	1.02	0.28
	360	0.40	1.02	0.67
0.04% Procaine HCl	0	0.00	0.95	0.00
	60	0.055	0.95	0.053
	180	0.160	0.95	0.18
	360	0.285	0.95	0.40

Table III

Effect of 0.1% $W/_V$ D-Glucose Solution in the Presence of 10^{-3}M Potassium Cyanide on the Diffusion Rate of 0.04% $W/_V$ Procaine Hydrochloride.

Experiment	Time in Minutes	Absorbance (C_B) at 292 mμ	Co	$-\log \left(1 - \frac{2C_B}{Co}\right)$
0.1% D-Glucose & 10^{-3}M KCN & 0.04% Procaine HCl	0	0.00	1.06	0.00
	30	0.08	1.06	0.07
	60	0.11	1.06	0.10
	90	0.125	1.06	0.12
	150	0.22	1.06	0.23
	210	0.25	1.06	0.27
10^{-3}M KCN & 0.04% Procaine HCl	0	0.00	1.02	0.00
	30	0.05	1.02	0.045
	60	-	-	-
	90	0.175	1.02	0.18
	150	0.23	1.02	0.26
	210	0.345	1.02	0.49

Table IV

Effect of Temperature on the Diffusion Rate of 1% $W/_V$ Procaine Hydrochloride

Temperature	Time in Minutes	Absorbance (C_B) at 292 mμ	Co	$-\log \left(1 - \frac{2C_B}{Co}\right)$
	0	0.00	1.43	0.00
	60	0.18	1.43	0.13
40° C	180	0.28	1.43	0.22
	240	0.38	1.43	0.33
	300	0.44	1.43	0.41
	0	0.00	1.41	0.00
	60	0.15	1.41	0.10
35° C	180	0.24	1.41	0.24
	240	0.32	1.41	0.32
	300	0.34	1.41	0.34

Table V.

Effect of 10^{-3}M Potassium Cyanide on the Diffusion Rate of 0.04% $W/_V$ Sodium Salicylate

Experiment	Time in Minutes	Absorbance (C_B) at 297.5 mμ	Co	$-\log \left(1 - \frac{2C_B}{Co}\right)$
10^{-3}M KCN & 0.04% Na Salicylate	0	0.00	0.685	0.00
	30	0.035	0.685	0.047
	60	0.085	0.685	0.120
	150	0.210	0.685	0.41
0.04% Na Salicylate	0	0.00	0.84	0.00
	30	0.045	0.84	0.051
	60	0.105	0.84	0.125
	150	0.235	0.84	0.235

Table VI.

Effect of 0.1% $W/_V$ D-Glucose Solution on the Diffusion Rate of 0.04% $W/_V$ Sodium Salicylate

Experiment	Time in Minutes	Absorbance (C_B) at 297.5 mμ	Co	$-\log \left(1 - \frac{2C_B}{Co}\right)$
0.1% D-Glucose & 0.04% Na Salicylate	0	0.00	0.76	0.00
	60	0.05	0.76	0.06
	180	0.14	0.76	0.20
	240	0.19	0.76	0.30
0.04% Na Salicylate	0	0.00	0.72	0.00
	60	0.055	0.72	0.072
	180	0.18	0.72	0.29
	240	0.22	0.72	0.39

Table VII

Diffusion Study Data of Sodium Ampicillin, Tetracycline Hydrochloride and Dexamethasone-21-Phosphate.

Name of Drug	Time in Minutes	Absorbance (C_B) at λ Max.	Co	$-\log \left(1-\frac{2C_B}{Co}\right)$
1% $W/_V$ Sodium Ampicillin λ max = 258 mμ	0	0.00	0.99	0.00
	30	0.03	0.99	0.027
	90	0.095	0.99	0.09
	150	0.160	0.99	0.17
	210	0.230	0.99	0.27
0.05% $W/_V$ Dexamethasone-21-Phosphate λ max = 243 mμ	0	0.00	1.255	0.00
	30	0.065	1.255	0.048
	60	0.14	1.255	0.11
	150	0.21	1.255	0.18
	210	0.29	1.255	0.27
0.036% $W/_V$ Tetracycline HCl λ max = 380 mμ	0	0.00	0.76	0.00
	30	0.00	0.76	0.00
	90	0.02	0.76	0.024
	150	0.04	0.76	0.048

Table VIII

Uptake of 4-C^{14}-Cholesterol by the

Human Chorioamnion Membrane.

Time in Min.	Counts/5 min. in Chamber A	Counts/5 min. in Chamber B	Counts/5 min. from the membrane homogenate
0	16032	0	
30	14996	71	
120	11840	6	
210	9756	14	
310	7384	5	
370			10400

Table IX.

Percent Permeability of the Studied Drugs

During a 3 hours Interval

Procaine HCl	Na Salicylate	Na Ampicillin	Dexametha-sone-21-Phosphate	Tetracycline Hydrochlo-ride
29%	48%	40%	40%	11%

Table X.

Thin Layer Chromotography Data for the Analysis of the Drugs Studied

Name of Drug	Adsorbent Used	Eluent	Visualizing Agent
Procaine HCl	Silica Gel G	Chloroform-Methanol (80 + 10)	Dragendorffs-reagent.
Tetracycline HCl	Kiesel-guhr G	Chloroform-Acetone-Phosphate-Citrate Buffer (50+50+20)	U-V light after plate exposure to ammonia Vapor
Dexamethasone-21-Phosphate	Silica Gel G	Methylene Chloride	Acetic Anhydride-Sulfuric Acid Mixture.
Sodium Salicylate	Silica Gel G	Methanol-Acetic Acid-Ether-Benzene - (1+18+60+120)	Ferric Chloride for hydroxamic acids and phenols.
Sodium Ampicillin	Silica Gel G	Acetone-Methanol (50+80)	Iodine-Azide solution.

Figure Legends

Fig. 1. Photograph of apparatus used in the diffusion studies.

Fig. 2. A schematic representation of the apparatus pictured in Fig. 1.

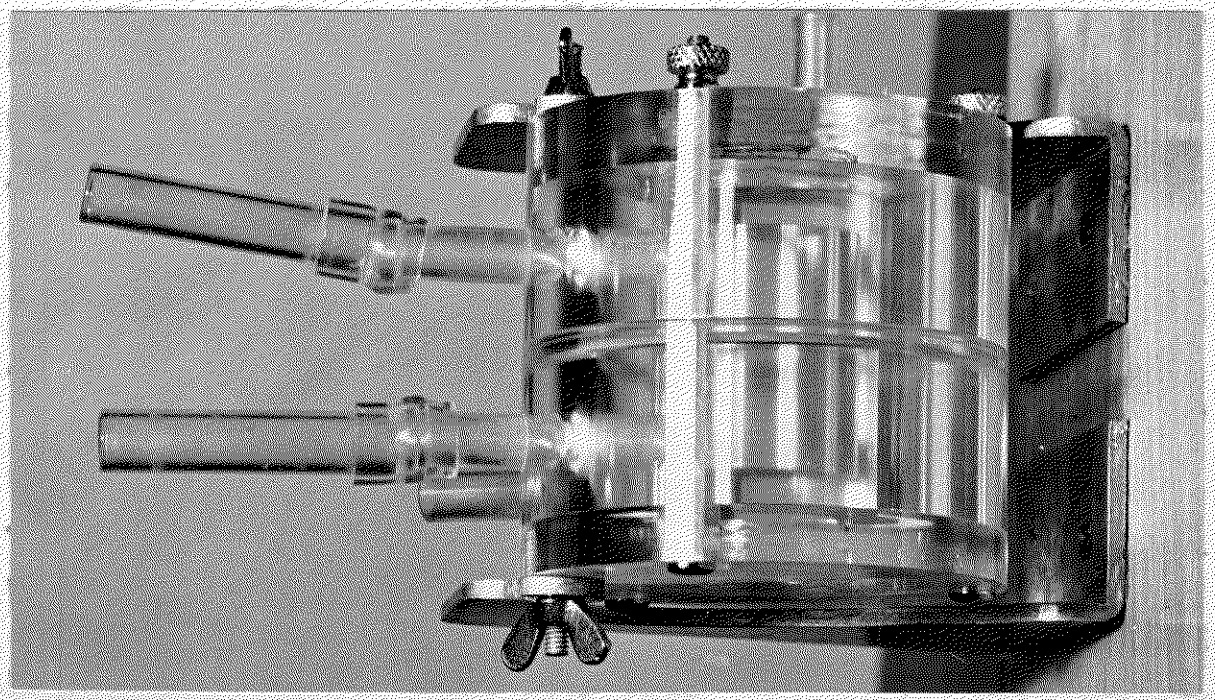

Fig. 1.

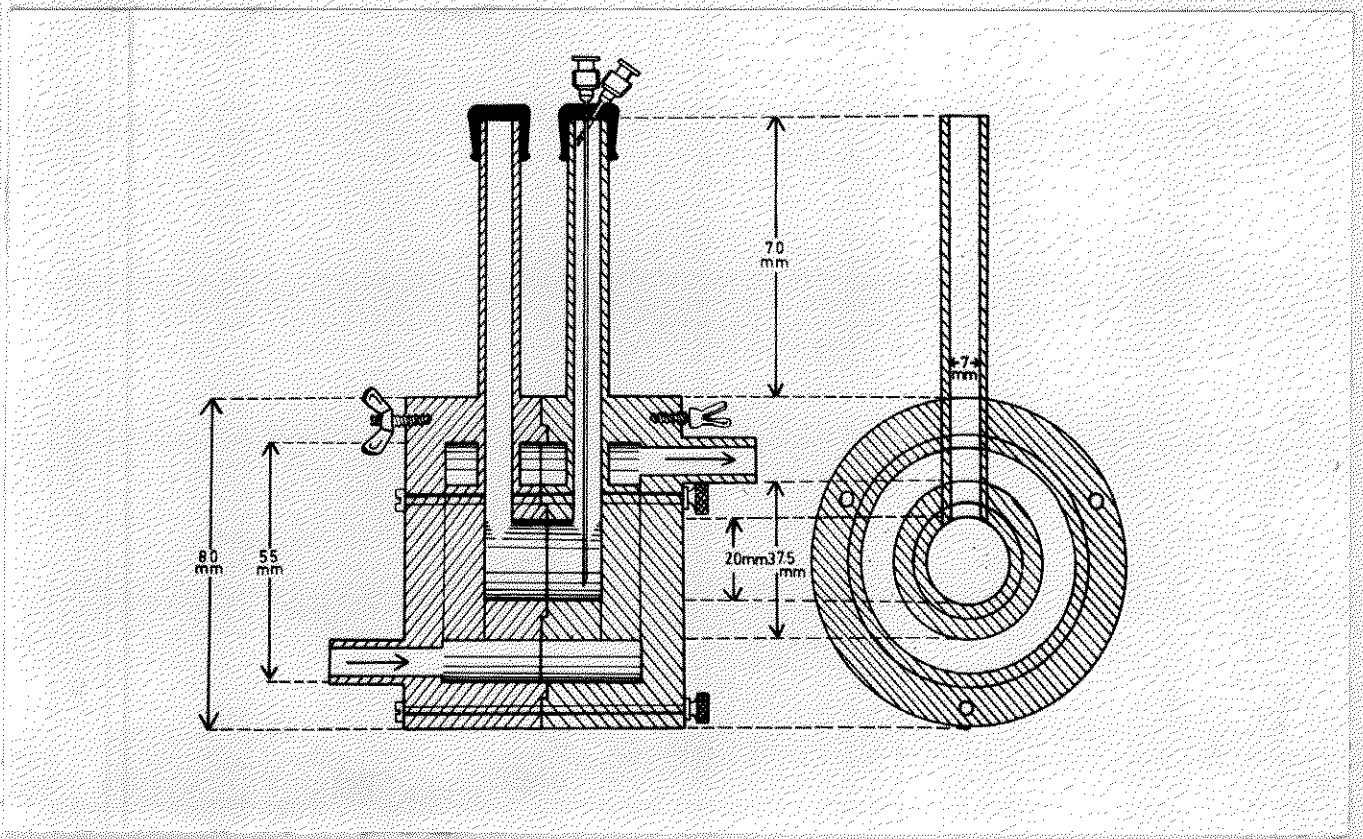

Fig. 2.

Figure Legends

Fig. 3. The scatter of the QO_2 determinations of the human Chorioamnion membrane. The line represents the mean linear regression curve of all the data.

Fig. 4. The effect of 10^{-3}M Potassium cyanide solution on the diffusion rate of 0.04% $W/_V$ procaine hydrochloride.

o—o 0.04% $W/_V$ Procaine HCl and 10^{-3}M KCN.

Δ—Δ 0.04% $W/_V$ Procaine HCl.

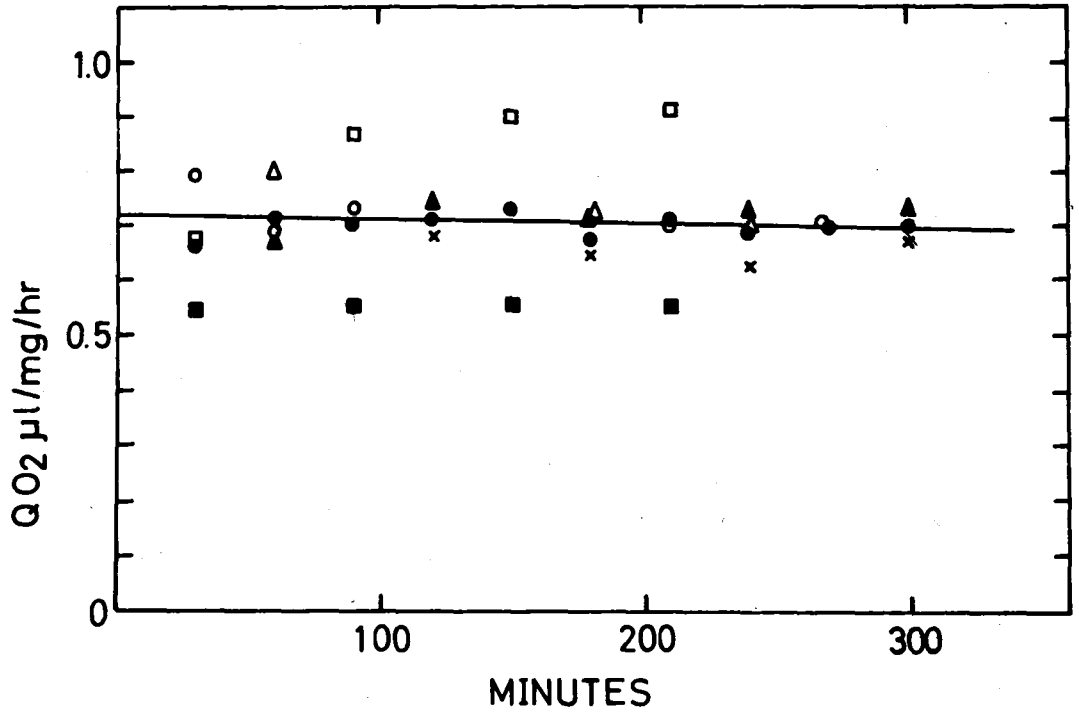
1.0
0.5
0
$Q O_2$ µl/mg/hr
100
200
300
MINUTES

Fig. 3.

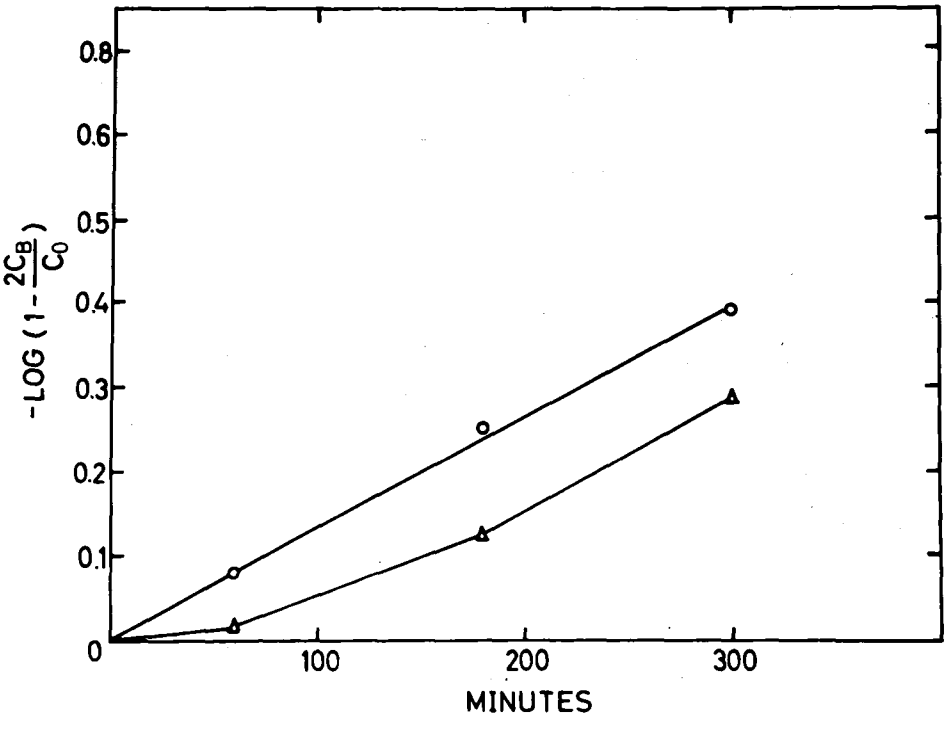
0.8
0.6
0.5
0.4
0.3
0.2
0.1
0
$-LOG\left(1-\frac{2C_B}{C_0}\right)$
100
200
300
MINUTES

Fig. 4.

Figure Legends

Fig. 5. The effect of 0.1% $W/_V$ D-glucose solution on the diffusion rate of 0.04% $W/_V$ procaine hydrochloride.

Δ—Δ 0.04% $W/_V$ Procaine HCl + 0.1% $W/_V$ D-Glucose

o—o 0.04% W/V Procaine HCl.

Fig. 6. The effect of 0.1% $W/_V$ D-glucose solution in the presence of 10^{-3}M potassium cyanide solution on the diffusion rate of 0.04% $W/_V$ procaine hydrochloride solution.

Δ—Δ 0.04% $W/_V$ procaine HCl and 10^{-3}M KCN.

o—o 0.04% $W/_V$ procaine HCl, 10^{-3}M KCN and 0.1% $W/_V$ D-glucose.

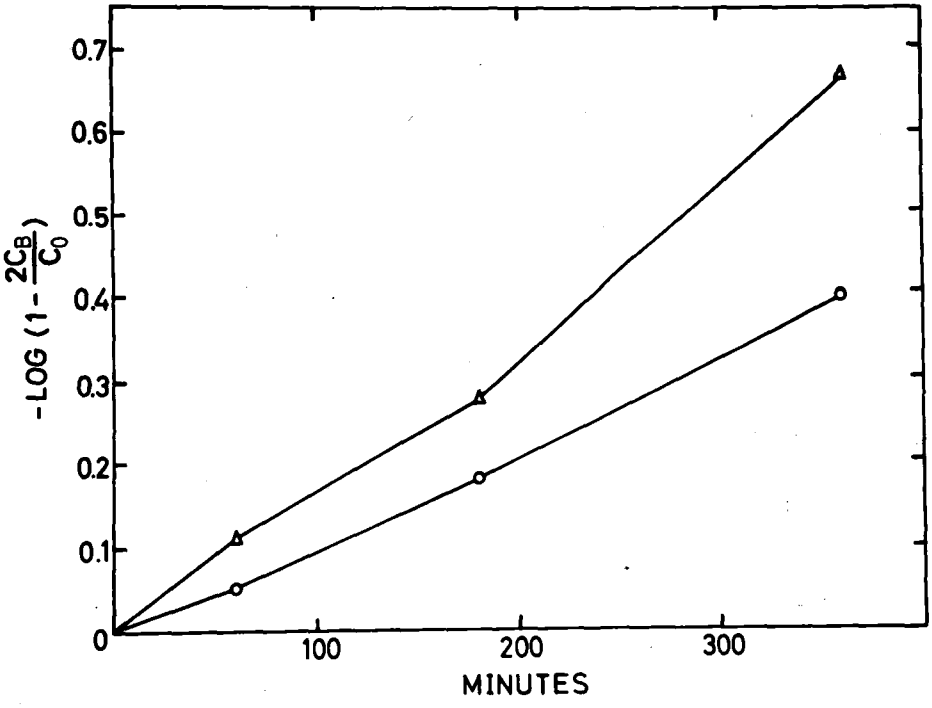
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
100
200
300
MINUTES
$-LOG\ (1-\frac{2C_B}{C_0})$

Fig. 5.

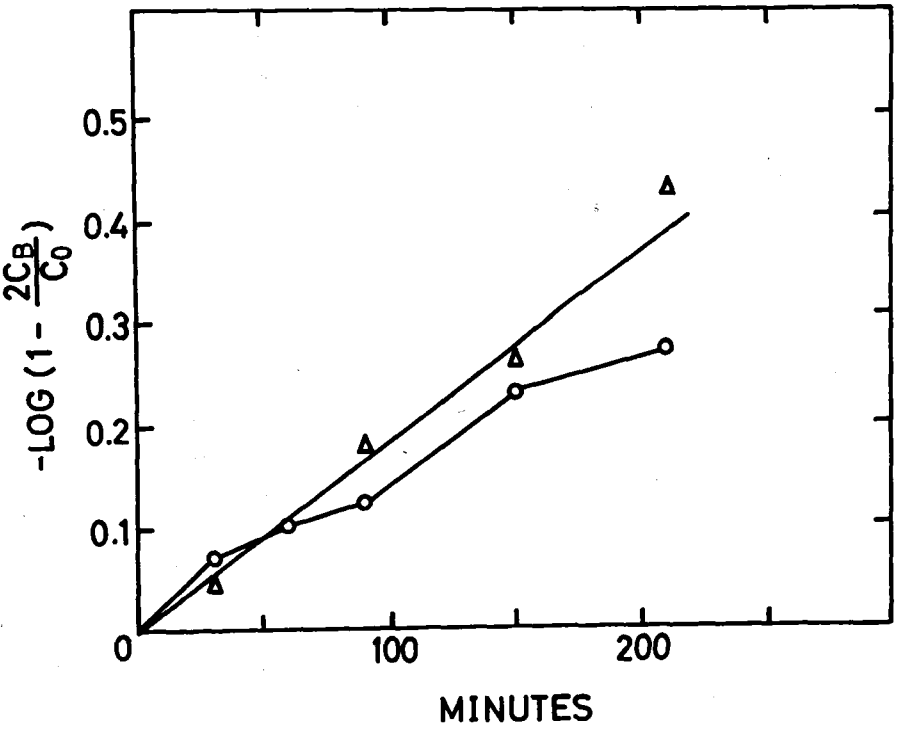
0.5
0.4
0.3
0.2
0.1
0
100
200
MINUTES
$-LOG\ (1-\frac{2C_B}{C_0})$

Fig. 6.

Figure Legends

Fig. 7. The effect of temperature changes on the diffusion of 1% $W/_V$ procaine hydrochloride solution.

Δ—Δ 35°C

o—o 40°C.

Fig. 8. The effect of 10^{-3}M potassium cyanide on the diffusion rate of 0.04% $W/_V$ sodium salicylate solution.

o—o 0.04% $W/_V$ Sodium Salicylate.

●-● 0.04% $W/_V$ Sodium Salicylate + 10^{-3}M KCN.

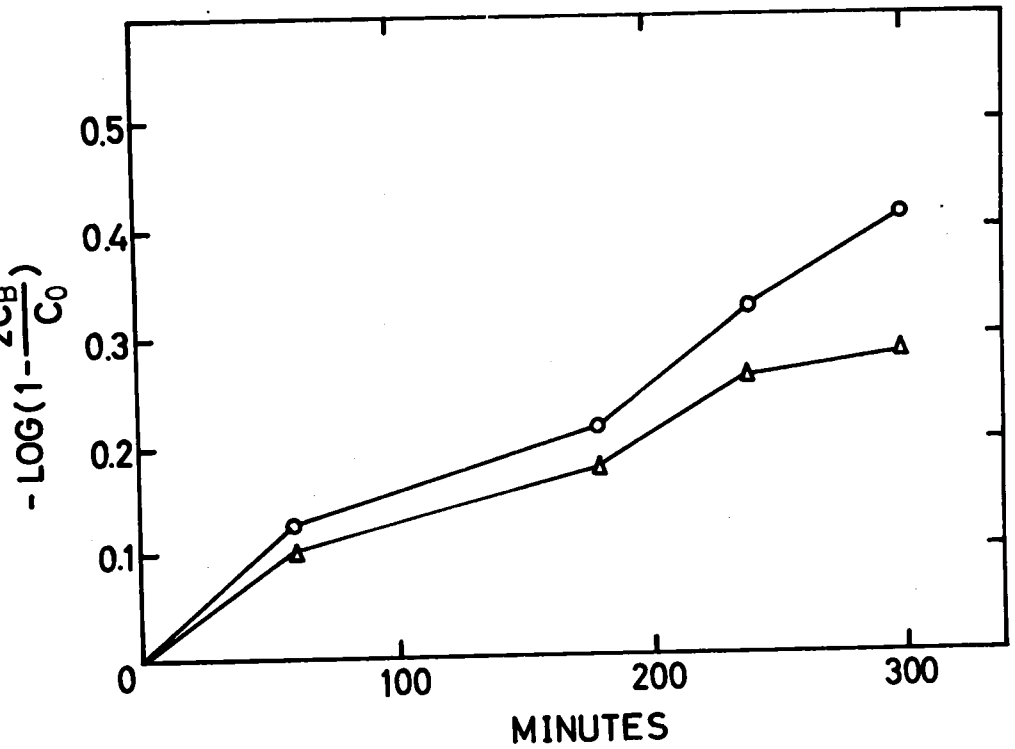
0.5
0.4
0.3
0.2
0.1
0
100
200
300
MINUTES
$-\text{LOG}(1-\frac{2C_B}{C_0})$

Fig. 7.

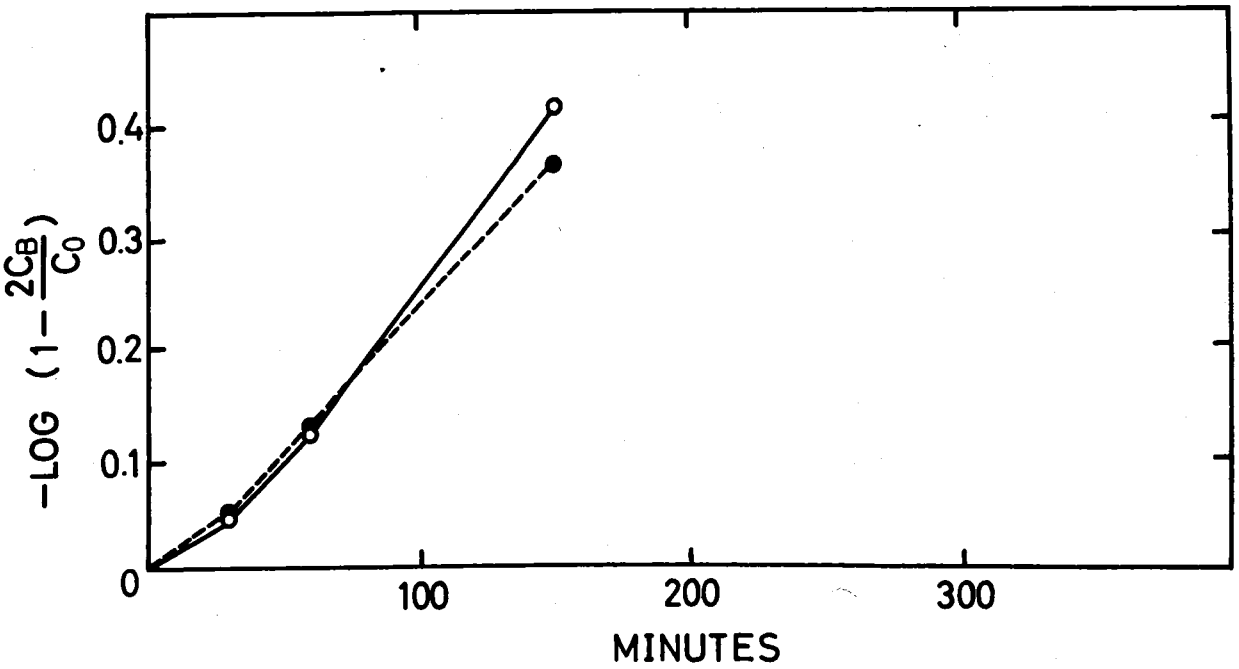
0.4
0.3
0.2
0.1
0
100
200
300
MINUTES
$-\text{LOG}\ (1-\frac{2C_B}{C_0})$

Fig. 8.

Figure Legends

Fig. 9. The effect of 0.1% $W/_V$ D-glucose solution on the diffusion of 0.04% $W/_V$ Sodium salicylate solution.

●—● 0.04% $W/_V$ sodium salicylate + 0.1% $W/_V$ D-glucose.

o—o 0.04% $W/_V$ sodium salicylate.

Fig. 10. The diffusion rate of sodium ampicillin, tetracycline hydrochloride, dexamethasone-21-phosphate, procaine hydrochloride and sodium salicylate.

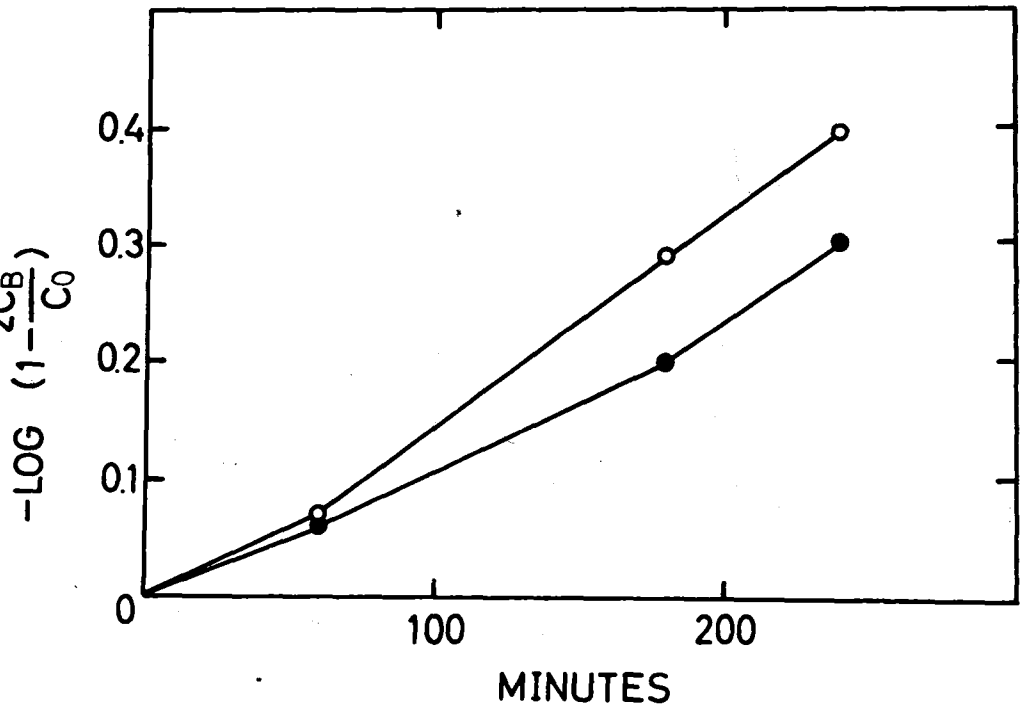
0.4
0.3
0.2
0.1
0
100
200
MINUTES
$-\text{LOG}\ (1-\frac{2C_B}{C_0})$

Fig. 9.

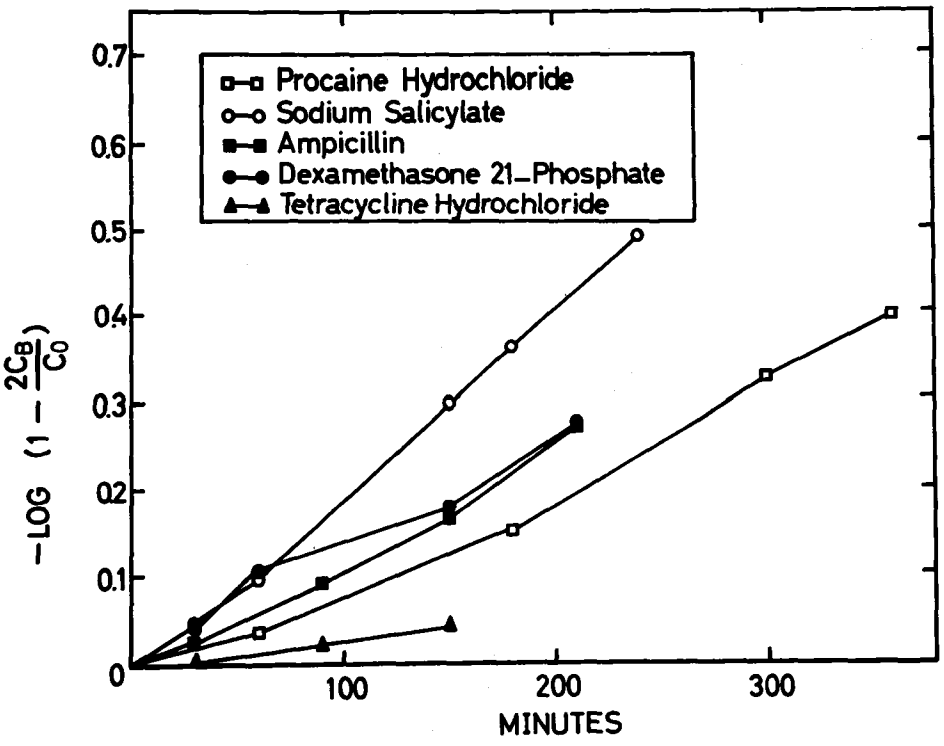
Procaine Hydrochloride
Sodium Salicylate
Ampicillin
Dexamethasone 21-Phosphate
Tetracycline Hydrochloride
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
100
200
300
MINUTES
$-\text{LOG}\ (1-\frac{2C_B}{C_0})$

Fig. 10.

Figure Legend

Fig. 11. The uptake of 4-C^{14}-cholesterol by the human chorioamnion membrane.

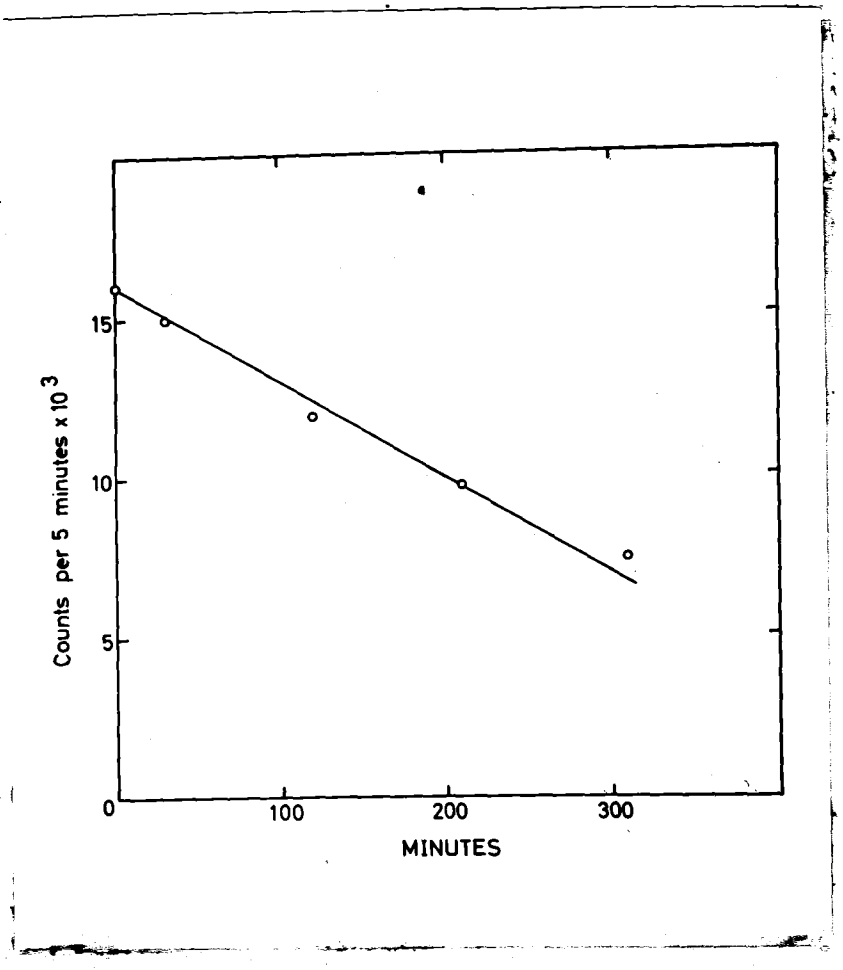

Fig. 11.

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CURRICULUM VITAE

Anwar B. Bikhazi, a Lebanese citizen, was born in Accra, Ghana, of Lebanese parents, on November 29, 1942. He received his elementary and high school education at International College, Beirut, Lebanon. In 1965, he received his Bachelor of Science degree in Pharmacy with Distinction from the School of Pharmacy, American University of Beirut. He then enrolled as a graduate student at the same School where he also served as a teaching assistant for two years.

During his undergraduate study, he was a member of the Pharmacy Students Society at the American University of Beirut, and served on its cabinet first as a secretary, then as president of the Society. He is a registered member of the Lebanese Order of Pharmacists, and is licensed to practice pharmacy in Lebanon.

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