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MOLECULAR SIEVING OF RED CELL MEMBRANES DURING GRADUAL OSMOTIC HEMOLYSIS*

by

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RUNNING TITLE: MOLECULAR SIEVING OF RED CELL MEMBRANES

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SUMMARY

Rat red blood corpuscles were held stationary with respect to a continuously flowing solution in either a specially constructed centrifuge or in glass filters. The concentration of the solution was gradually decreased to cause the swelling and subsequent gradual osmotic hemolysis of the cells. The passage of the intracellular molecules--potassium, adenylate kinase, and hemoglobin--across the cell membranes and into the flowing solution was determined as a function of time. Ions and molecules begin passage across the membranes in the order of increasing molecular size. The initial flow of potassium is followed by the initial flows of hemoglobin and adenylate kinase. The flow of hemoglobin has been interpreted as the flows of hemoglobin monomers, dimers, and tetramers such that the time sequence is: potassium; hemoglobin monomer;(adenylate kinase/ hemoglobin dimer); and finally, hemoglobin tetramer. It is concluded that the stressed cell membrane has molecular sieving properties and that the exclusion limit (effective hole size) increases as a function of time during the initial stages of gradual osmotic hemolysis. The process of gradual osmotic hemolysis is discussed in terms of molecular sieving through stress-induced effective membrane holes. It is suggested that a portion of the membrane protein might form an elastic network which would account for the gradual increase in size and apparent homogeneity of the effective holes.

INTRODUCTION

A red blood cell can be considered an assembly of intracellular molecules of various sizes separated from an extracellular fluid by a membrane. When the membrane is sufficiently stressed by either mechanical (Rand, 1964) or osmotic (Ponder, 1948) means, it becomes permeable to hemoglobin, and the cell hemolyzes. A detailed study of the process of osmotic hemolysis, that is, the yielding of the cell membrane to applied stress, would be expected to provide information concerning the structure of the cell membrane.

Marsden and Östling (1959) found that during drastic hemolysis low molecular weight dextran entered the cells to a greater extent than high molecular weight dextran. Hjelm, Östling and Persson (1966) found a greater percentage of small molecular weight molecules released from the cells during hemolysis than high molecular weight molecules. Both observations suggest molecular sieving by the cell membrane. Katchalsky, Kedem, Klibansky, and de Vries (1960) employed a molecular sieving pore model of the cell membrane in discussing the process of osmotic hemolysis. In our work, methods were developed which allowed the flow of intracellular molecules through the cell membrane to be followed as a function of time during gradual osmotic hemolysis. This permitted a direct evaluation of the hypothesis that the stressed cell membrane has molecular sieving properties.

METHODS

General Experimental Procedure

Red cells were held stationary with respect to a flowing solution, the concentration of which was gradually decreased with time in order to slowly swell and then osmotically stress the cell membranes. The

solution was collected in serial fractions after it passed the cells and was analyzed for intracellular molecules of various sizes.

Cell Preparation

Blood was obtained from the posterior vena cava of ether anesthetized female Sprague-Dawley rats weighing in excess of 250g. The blood was withdrawn, without the use of an anticoagulant, through a number 19 needle into a glass syringe which had been previously wetted with isotonic solution. The needle was removed and the blood immediately added to at least five volumes of isotonic solution and centrifuged at 1000G for three minutes in a fixed angle rotor (Servall SS-1). The supernatant and buffy coat were removed by aspiration and the cells washed an additional four times with 30-40 ml portions of isotonic solution. The cells were resuspended at approximately 30% hematocrit and immediately placed in the experimental apparatus.

$^{42}K^{+}$Labeled Red Cells

Approximately 1 mc $^{42}K^{+}$(specific activity 170 mc/g K^{+}; ISO/SERVE) was administered to a rat by stomach tube. Ten hours later the blood was withdrawn from the posterior vena cava and washed as described above.

Flow Solutions

Two solutions, which differed in the concentration of the major solute, were prepared for each experiment and were termed "isotonic" and "dilute". The major solutes employed in different experiments were NaCl, LiCl, and dextrose. The composition of the solutions and other experimental parameters are listed in Table 1. Prior to the start of an experiment, a fixed volume

mixing chamber containing a magnetic stir bar was completely filled with the isotonic solution. The mixing chamber was connected to a cell-holding device (described below) and isotonic solution was pumped into and through the mixing chamber and past the cells in order to further wash them. The experiment was then initiated by switching the solution pumped into the mixing chamber from isotonic to dilute. This resulted in an exponential decrease in solution concentration such that the concentration flowing from the mixing chamber to the cells, C, was found experimentally to follow the theoretical equation: $C = C_d + (C_i - C_d)e^{-v/V}$

where C_d = concentration of dilute solution;

C_i = concentration of isotonic solution;

v = total volume flow since start of dilute flow;

V = volume of mixing chamber.

After passing the cells, the solution was collected in serial fractions. The volume and collection times were constant within each experiment but varied between experiments (Table 1). The approximate milliosmolar concentration of the flowing solution was calculated by assuming an osmotic coefficient of 1.00 for dextrose and of 0.94 for NaCl and LiCl. Plug flow was assumed through the experimental apparatus in order to calculate the concentrations in the collected fractions.

Cell-holding Devices

The red cells were restrained against the flowing solution by the use of either a continuous flow centrifuge or a glass wool filter.

Centrifuge

The cells were sedimented with approximately 1500G against the inside wall of Intramedic PE-260 polyethylene tubing which ran in the circumferential rotor grove of an especially constructed continuous flow centrifuge (details to be published). The ends of the tubing were brought to the center of the rotor and connected to fittings which allowed the flow solutions to pass the cells.

Filters

Glass wool filters were constructed by packing approximately 3/4 cm^3 chambers with glass wool. When the red cells were allowed to sediment into intimate contact with the glass fibers, they became attached to the glass and were restrained against the fluid flow.

Chemical analyses

The intracellular molecules chosen for analysis were potassium (MW 39), adenylate kinase (MW 22028), and hemoglobin (tetramer MW 64459).

Potassium

Total K^{+1} was determined by flame emission with a Beckman DU spectrophotometer with flame attachment and oxy-acetylene flame at 766 mμ or by atomic absorption with a modified Jarrell-Ash 82-360 spectrophotometer with hydrogen-air flame at 7665Å. The atomic absorption data was corrected for drift by a computerized comparison to a reference solution.

$^{42}K^{+}$ was counted in a Nuclear Chicago C120-1 well counter and the counts obtained corrected for background and decay.

Hemoglobin

Total Hb was estimated as oxy-Hb at 540 or 414 mμ (MacFate 1964, Martinek 1966) or as cyano-met-Hb at 540 mμ (Austin and Drabkin, 1935).

The concentrations of deoxy-, oxy-, and met-Hb were determined simultaneously by measuring the optical density at 401, 417, and 433 mμ with a Cary 14 spectrophotometer and flow cuvette and then solving a set of three simultaneous equations, one for each wavelength, using the micromolar extinction coefficients listed in Table 2 (details to be published).

Adenylate kinase

Adenylate kinase was determined by the method of Noda and Kuby (Noda and Kuby, 1957; Kuba, Noda and Lardy, 1954) after one hour incubation.

RESULTS

In these experiments higher resolution data were obtained with the centrifuge than with glass filters.[2]

Figure 1 indicates the concentrations of K^+ and Hb in the collected fractions when a LiCl solution of continuously decreasing concentration was flowed past cells restrained with the centrifuge. Since the collection times and fraction volumes were constant, the concentrations are a measure of the average flow through the cell membranes during the experiment. A roughly constant K^+ flow occurred during the isotonic wash and through the first ten tubes after the start of the dilute flow into the mixing chamber. The major K^+ flow started in tube 11 when the external solute concentration was approximately 175 mOsm. There was virtually no Hb flow during the iotonic wash and through the first twelve tubes after the start of the dilute flow. The flow started in tube 13 and increased rapidly in tube 15. Thus, the initial rapid flow of K^+ at tube 11 occurred before the initial flow of Hb at tube 13. The insert of Fig. 1 shows, on an expanded scale, the losses of K^+, AK, and Hb. The rapid rise in the AK flow occurred after the start of

the initial Hb flow but before the rapid Hb flow.

The flows of K^+ and Hb from cells restrained in a glass filter into a NaCl solution of continuously decreasing concentration are indicated in Fig. 2. As in the previous experiment, the major flows of both K^+ and Hb occurred after the start of the dilute flow. However, due to the variable hemolysis caused by the flow of any solution past cells restrained with a glass filter, it is difficult to pinpoint the start of these major flows. Within the resolution of the data, the major K^+ and Hb flows both started about 60 minutes after the start of the dilute flow when the external solute concentration was approximately 172 mOsm.

The flows of K^+ and Hb from cells restrained in a filter into a dextrose solution of approximately constant ionic strength are indicated in Fig. 3. The major K^+ flow started approximately 96 minutes after the start of the dilute flow when the external concentration was approximately 159 mOsm. The major Hb flow started approximately 24 minutes after the start of major K^+ flow, a much greater time interval than with LiCl or NaCl flow solutions.

The concentrations of deoxy-, met-, and oxy-Hb in the collected fractions of a centrifuge experiment with NaCl flow solutions are indicated in Fig. 4. The deoxy-Hb flow rises dramatically in tube 11, met-Hb flow rises in tube 11 but more steeply in tube 12, and oxy-Hb rises steeply in tube 13. The deoxy-Hb flow at tube 11 and sharp rise in oxy-Hb in tube 13 of Fig. 4 correspond to the initial Hb flow at tube 13 and its sharp rise in tube 15 of Fig. 1.

INTERPRETATION OF DATA

The sharp increases in flow of the various solutes through the cell membrane can be interpreted in terms of normal membrane flow

followed by a transition to a state which includes flow through a sress-induced pathway. The molecular sieving properties of this stress-induced pathway are best illustrated with reference to Figs. 1 and 4. At tube 11 of Fig. 4 deoxy-Hb is rapidly flowing from the cells, met-Hb has begun to flow, but oxy-Hb is retained by the cells. By tube 13, however, deoxy-, met-, and oxy-Hb can all flow through the membrane. That the different Hb species correspond to different Hb size units can be seen in Fig. 1. The insert to this figure indicates that the initial flow of AK occurs after the initial flow of Hb, which corresponds to the deoxy-Hb flow of Fig. 4, but before the rapid increase in Hb flow, which corresponds to the oxy-Hb flow of Fig. 4. If it is assumed that, to first order, the time of the initial flow of a molecule is determined by its molecular weight, molecular shape and charge being neglected, the flow of AK with a molecular weight of about 22028 indicates that the flows of deoxy-, met-, and oxy-Hb probably represent the flows of Hb monomers, dimers, and tetramers, with molecular weights of about 16115, 32230 and 64459, respectively.

Potassium loss from red cells increases at low ionic strength (Davson, 1939). However, in these experiments the initial rise in the major K^+ flow occurred at approximately the same milliosmolar

concentration regardless of whether the major external solute was a salt (NaCl or LiCl) or a nonelectrolyte (dextrose), and was independent of the method employed to restrain the cells. Thus, the primary cause of the increased K^+ flow must be the osmotic stress of the cell membrane, and it would appear reasonable that the increased K^+ flow occurred through a similiar or identical stress-induced pathway by which the larger molecules were lost from the cells.

Thus, it is suggested that during the process of gradual osmotic hemolysis the gradual stress of the cell membrane causes the opening of effective holes of an undefined nature in or through the membrane. Solute molecules can flow, or be transported, across the cell membrane via these effective holes in both directions provided the dimensions of the solute molecules are less than the dimensions of the effective holes. The dimensions of these effective holes increase during the initial stages of gradual osmotic hemolysis.

DISCUSSION

We believe that in these experiments the overall structure of the cell membrane remained relatively intact. Had appreciable numbers of large tears occurred, we would expect the simultaneous exchange of large and small molecules.

Since there are times during the process of gradual osmotic hemolysis when molecules larger than a certain size can not pass through the membrane, the stressed membrane has molecular sieving properties. However, the molecular weight exclusion limit, which is discussed here in terms of an effective hole size, is a function of time and increases during the initial stages of gradual osmotic hemolysis.

The delay between the initial K^+ and Hb flows in dextrose solutions as compared to the flows in NaCl or LiCl solutions can be explained in terms of the proposed molecular sieving mechanism of gradual osmotic hemolysis. When the experiment is conducted in a salt solution, the continuously decreasing external solute concentration eventually osmotically stresses the membrane enough to open the effective holes sufficiently to allow K^+ to flow from the cells. Because Na^+ and Li^+, whether hydrated or not, are, for the purposes of these experiments, approximately the same size as K^+, the external cation can begin to flow into the cell at approximately the same time as K^+ can begin to flow out of the cell. Since the external cation concentration is controlled at a slowly changing but relatively high concentration, there is a net gain of cation within the cell as

the external cation approaches a Donnan equilibrium. If it is assumed that the membrane is freely permeable to anions and water in comparison to cations, and that the volume of the swollen cell is approximately constant, the gain of cation causes an increase in osmotic pressure within the cell, a rapid enlarging of the effective hole size, and the loss of Hb shortly after the initial flow of K^+ from the cells.

In contrast, when the experiment is conducted in a dextrose solution, the initial flow of K^+ from the cells is not accompanied by a gain of the major external solute since the larger dextrose molecule is excluded from the effective holes. Thus, the gradually decreasing external solute concentration can be balanced to some extent by the loss of cellular K^+ and the effective hole size does not rapidly increase. Eventually the membrane is stressed enough to open the effective holes sufficiently to allow dextrose to enter the cell. The self-propagating entrance of dextrose leads to a rapid increase in effective hole size and to the loss of Hb.

Thus, the proposed molecular sieving mechanism of gradual osmotic hemolysis predicts that, to first order, the initial flow of K^+ through the cell membrane would start at the same milliosmolar concentration regardless of the external solute, provided that normal membrane permeability can be neglected. The flow of Hb, however, would occur shortly after the entrance of the external solute, provided that the external solute were smaller than Hb. The flow of Hb would be delayed in solutions of molecules larger than K^+. These predictions are confirmed in the data presented.

The amount of K^+ and other small solutes lost from the cell

before the loss of Hb depends on the composition of the external solute with primary emphasis on the size of the solute molecules and would be expected to depend upon the rate of stress of the membrane. The permeability of the red cell membrane to solutes is often estimated by determining the time required for hemolysis in a solution of the permeant molecule (Hŭber and Ørskov, 1933). The entrance of the molecule into the cell, accompanied by water molecules, causes the gradual swelling and subsequent stressing of the membrane in an analogous manner that reducing the external solute concentration does in these experiments. Thus, it would be expected that there would be a loss of small cellular molecules prior to the flow of Hb, and that the amount of these losses would depend on the size of the external solute molecules and the rate of entrance of the permeant molecules. it is suggested that the undetected loss of small molecules introduces undesirable variables into this traditional permeability data which could be avoided by observing the flow of a molecule much smaller than Hb, such as K^+.

The colloid-osmotic theory of hemolysis (Wilbrandt, 1941) is extended in the present work by suggesting that osmotic hemolysis proceeds by a molecular sieving mechanism which reduces the cation permeability barrier and leads to hemolysis. Further work is required to assess the effects of cation size. The all-or-none hemolysis of individual red cells (Hendry, 1947; Hoffman, 1958) can be explained in terms of the self-propagating increase in effective hole size once the enxternal solute can enter the cell.

It is not known if the mechanism by which solutes pass through the stressed cell membrane is operative in the unstressed membrane.

It is tempting to speculate that the approximately one-for-one exchange of K^+ for Na^+ (Ponder, 1948) might occur by this mechanism, since both ions are approximately the same size and are at roughly the inverse concentrations on opposite sides of the membrane.

We believe that gentle stress does not produce rips or tears of the membrane, but, rather, small effective holes which gradually increase in size. This gradual increase in size suggests that they do not arise from a stress-induced phase change in the membrane, but more likely from a stretching of the membrane. The effective hole size appears homogeneous as evidenced by the separation of the molecular flows and might result from an underlying structural membrane architecture. Thus, it is suggested that a portion of the membrane protein forms an elastic network which is evidenced in this work by the molecular sieving properties of the stressed membrane. Such a network might be similar to the molecular fabric envisioned by Katchalsky et al (1960).

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References

Austin, J.H., Drabkin, D.L. 1935. Spectrophotometric studies. III. Methemoglobin. J. Biol. Chem. 112:67.

Connerty, H.V., Briggs, A.R. 1962. New method for the determination of whole-blood iron and hemoglobin. Clin. Chem. 8:151.

Davson, H. 1939. Studies on the permeability of erythrocytes. VI. The effect of reducing the salt content of the medium surrounding the cell. Biochem. J. 33:389.

Hendry, E.B. 1947. Physical and chemical factors concerned in haemolysis. Edinburgh Medical J. 54:476.

Hjelm, M., Östling,S.G., Persson, A.E.G. 1966. The loss of certain cellular components from human erythrocytes during hypotonic hemolysis in the presence of dextran. Acta Physiol. Scand. 67:43.

Hober, R., Ørskov, S.L. 1933. Untersuchungen über die Permeiergeschwindigkeit von Anelektrolyten bei den roten Blutkörperchen verschiedener Tierarten. Pfluegers Arch. Ges. Physiol. 231:599.

Hoffman, J.F. 1958. Physiological characteristics of human red blood cell ghosts. J. Gen. Physiol. 42:9.

Katchalsky, A., Kedem, O., Klibansky, C., deVries, A. 1960. Rheological considerations of the haemolysing red blood cell. In: Flow Properties of Blood and other Biological Systems. A.L. Copley & G. Stainsby, editors. p. 155. Pergamon, New York.

Kuby, S.A., Noda, L., Lardy, H.A. 1954. Adenosinetriphosphate-creatine transphosphorylase. I. Isolation of the crystalline enzyme from rabbit muscle. J. Biol. Chem. 209:191.

MacFate, R.P. 1964. Measurement of hemoglobin in blood. In: Hemoglobin. Its Precursors and Metabol tes. F.W. Sunderman & F.W. Sunderman, Jr., editors. Lippincott, Philadelphia, p. 31.

Marsden, N.V.B., Östling, S.G. 1959. Accumulation of dextran in human red cells after hemolysis. Nature 184:723.

Martinek, R.G. 1966. A simple spectrophotometric method for quantitating hemoglobin in serum or plasma. J. Am. Med. Technologists 28:42.

Noda, L., Kuby, S.A. 1957. Adenosine triphosphate-adenosine monophosphate transphosphorylase (myokinase). I. Isolation of the crystalline enzyme from rabbit skeletal muscle. J. Biol. Chem. 226:541.

Ponder, E. 1948. Hemolysis and Related Phenomena. Grune and Stratton, New York.

Rand, R.P. 1964. Mechanical properties of the red cell membrane. II. Viscoelastic breakdown of the membrane. Biophysical J. 4:303.

Wilbrandt, W. 1941. Osmotische Natur sogenannter nicht-osmotischer Hämolysen (Kolloidosmotische Hämolyse). I. Mitteilung. Pfluegers Arch. Ges. Physiol. 245:22.

Williams, L.A., Zak, B. 1957. Standardization of hemoglobin. Amer. J. Clin. Path. 28:195.

FOOTNOTES

[1]Abbreviations used: AK = adenylate kinase, K^+ = potassium ion, Hb = hemoglobin.

[2]The problem will be discussed in more detail by R. MacGregor (Thesis, University of California, Berkeley, in preparation).

Figure legends.

Fig. 1. Concentrations of K^+ and Hb in a LiCl solution of gradually decreasing concentration which was flowed past rat red cells restrained in a centrifuge. Insert. Concentrations of K^+, AK and Hb on an expanded scale. The concentrations of intracellular molecules in this and following figures have been normalized in order to facilitate comparisons.

X Potassium (full scale = 82.8μM),

□ Hemoglobin (full scale = 0.517 OD units),

O Adenylate kinase (full scale = 0.021 OD units).

The external solute concentration is indicated by the line and the scale on the left ordinate.

Fig. 2. Concentrations of K^+ and Hb in a NaCl solution of gradually decreasing concentration which was flowed past rat red cells restrained in a glass filter.

X Potassium (full scale = 5600 counts/5 min.),

□ Hemoglobin (full scale = 0.369 OD units).

The external solute concentration is indicated by the line and the scale on the left ordinate.

Fig. 3. Concentrations of K^+ and Hb in a dextrose solution of gradually decreasing concentration which was flowed past rat red cells restrained in a glass filter.

X Potassium (full scale = 43.9μM),

□ Hemoglobin (full scale = 0.128 OD units).

The external solute concentration is indicated by the line and

Fig. 3. (cont.) the scale on the left ordinate.

Fig. 4. Concentrations of oxy-, deoxy-, and met-Hb in a NaCl solution of gradually decreasing concentration which was flowed past rat red cells restrained in a centrifuge.

Table 1. Experimental parameters and assays

Figure	Solution composition		Cell-holding device	Temp. (°C)	Fraction collection		Mixing chamber volume (ml)	Fluid delay[b] (ml)	Assay	Method
	Isotonic	Dilute			Interval (minutes)	Volume (ml)				
1	150mMLiCl 1mMTris[a] pH 7.4	30mMLiCl 1mMTris pH 7.4	centrifuge	29	1.0	8.5	129.6	5.1	K AK Hb	Atomic absorption Conversion of ADP to ATP coupled to formation of creatine ~ P OD at 414mμ
2	150mMNaCl 5mMdextrose 1mMTris pH 7.4	30mMNaCl 5mMdextr. 1mMTris pH 7.4	filter	22	5.0	7.5	108.0	8.8	K Hb	^{42}K OD of cyanomet-Hb at 540 mμ
3	240mMdextr. 38mMNaCl 1mMTris pH 7.4	35 mMNaCl 1mMTris pH 7.4	filter	22	6.0	9.0	108.0	35.2	K Hb	Flame emission OD at 540 mμ
4	150mMNaCl 1mMTris pH 7.4	30mMNaCl 1mMTris pH 7.4	centrifuge	29	1.0	8.5	129.6	5.1	deoxy-Hb oxy- Hb met-Hb	OD at 401, 417, and 433mμ

[a]Tris = Tris(hydroxymethyl)aminomethane.

[b]Volume of experimental apparatus between inlet valve of mixing chamber and outlet of fraction collector, less volume of mixing chamber.

Table 2. Approximate micromolar extinction coefficients for hemoglobin derivatives[a] at pH 7.2[b]

Hemoglobin derivative[c]	Wavelength in mμ 401	417	433
deoxy	.4014	.3560	.5247
oxy	.2986	.4927	.1691
met	.5151	.3300	.0825

[a]Based upon Fe determination and assuming 4 Fe/Hb (Williams and Zak, 1957; Connerty and Briggs, 1962).

[b]The met-Hb spectrum is a strong function of pH at pH 7.2.

[c]Derivatives of unpurified rat hemoglobin were prepared as follows:

deoxy-Hb — 25 ml 50 mM phosphate buffer was added to a tube containing 0.125g dry sodium dithionite. The dithionite was dissolved and this solution was immediately used to dilute 1 ml of hemoglobin stock solution to 10 ml.

oxy-Hb — 1 ml hemoglobin stock solution was diluted with air or O_2 saturated buffer to 10 ml. Some samples were additionally shaken under an O_2 atmosphere.

met-Hb — 1 ml hemoglobin stock solution was diluted to 10 ml with 50 mM phosphate buffer containing $K_3Fe(CN)_6$ such that a 10-fold excess of $K_3Fe(CN)_6$ was present.

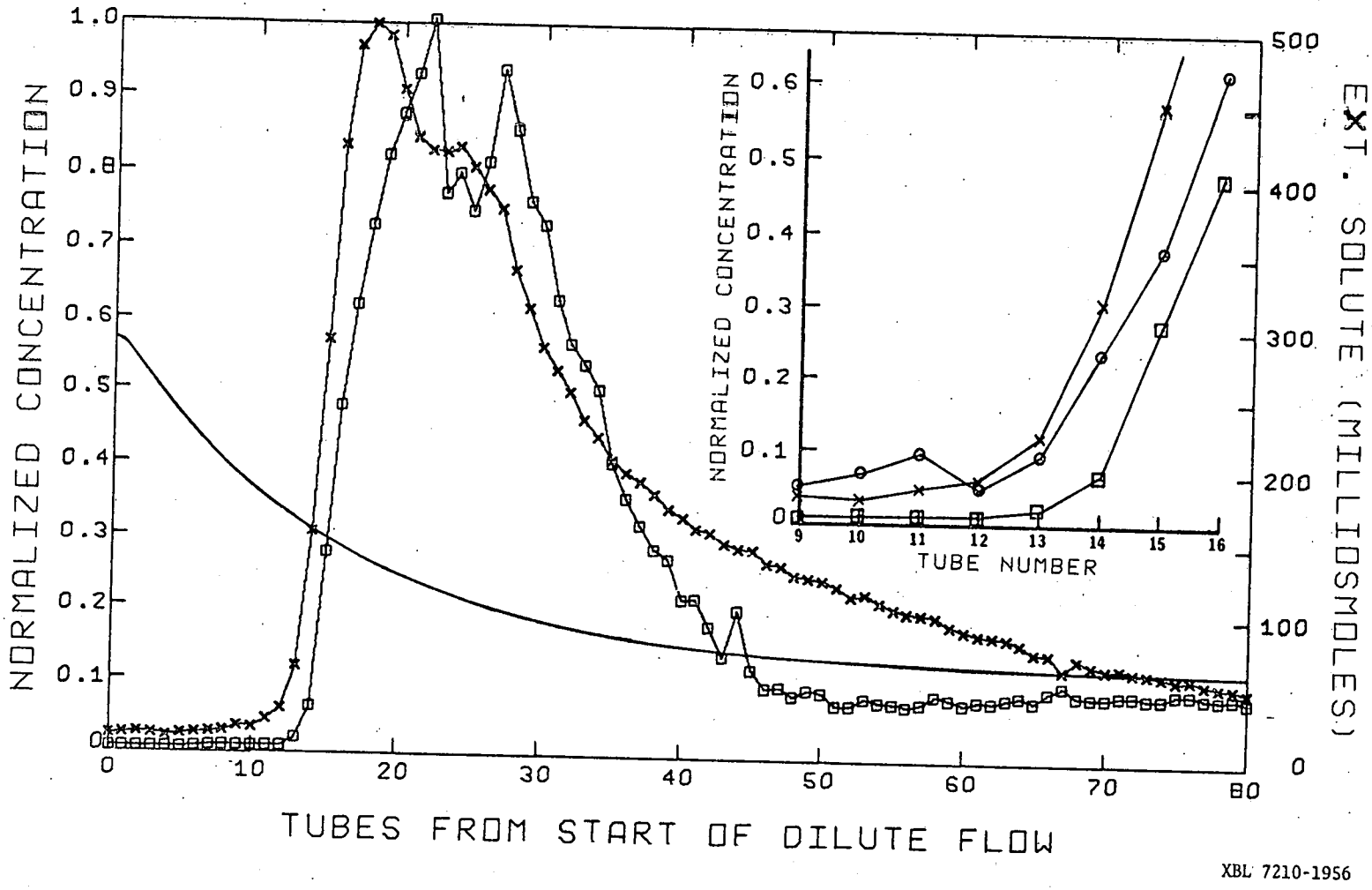

NORMALIZED CONCENTRATION
EXT. SOLUTE (MILLIOSMOLES)
TUBES FROM START OF DILUTE FLOW
NORMALIZED CONCENTRATION
TUBE NUMBER
XBL 7210-1956

Figure 1.

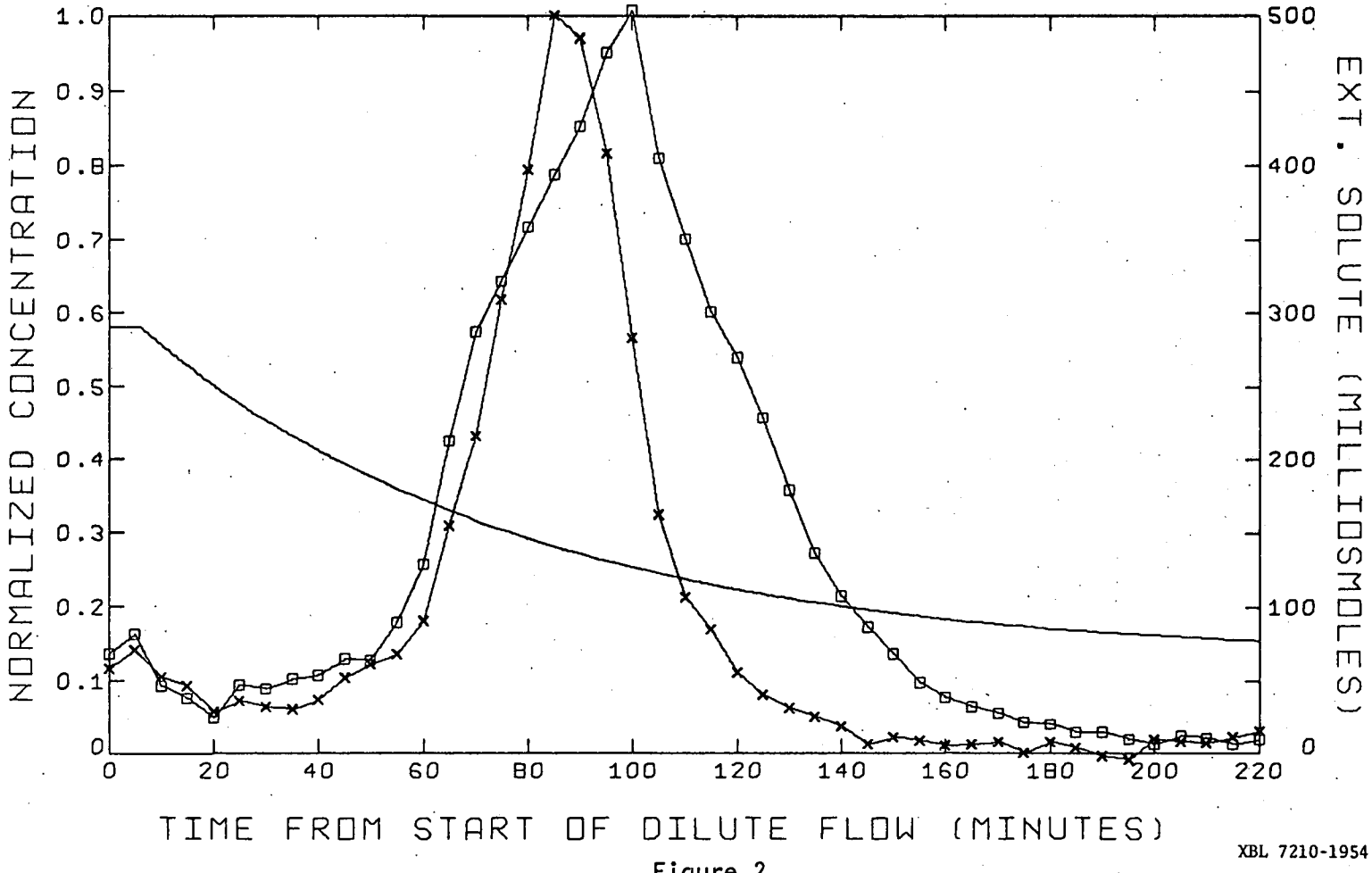

NORMALIZED CONCENTRATION
EXT. SOLUTE (MILLIOSMOLES)
TIME FROM START OF DILUTE FLOW (MINUTES)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
0
100
200
300
400
500
0
20
40
60
80
100
120
140
160
180
200
220
XBL 7210-1954

Figure 2.

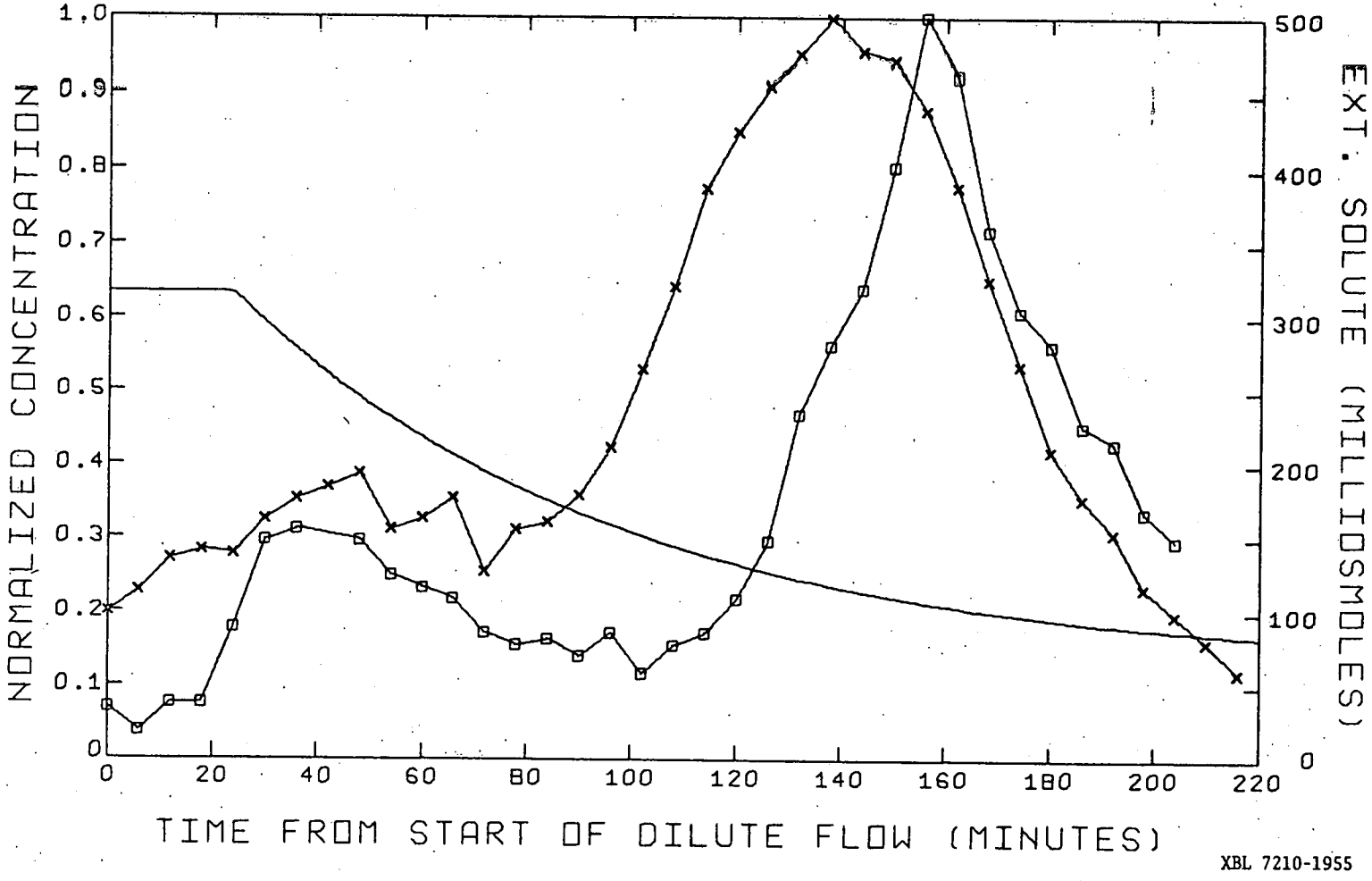
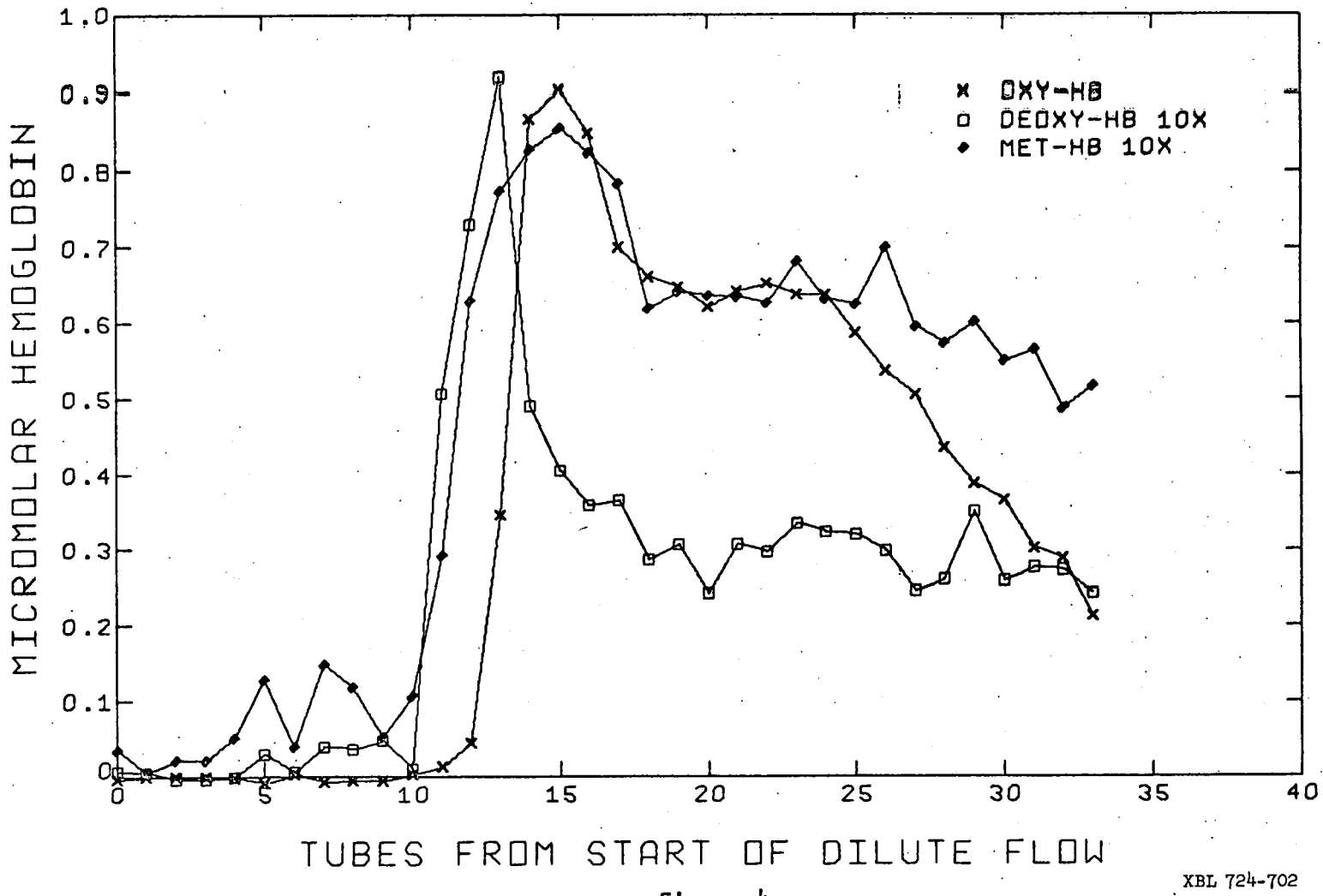

NORMALIZED CONCENTRATION
EXT. SOLUTE (MILLIOSMOLES)
TIME FROM START OF DILUTE FLOW (MINUTES)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
0
100
200
300
400
500
0
20
40
60
80
100
120
140
160
180
200
220
XBL 7210-1955

Figure 3.



XBL 724-702

Figure 4.