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Net Biosynthesis of Antithrombin III by the Isolated Rat Liver Perfused for 12-24 Hours. Compared with Rat Fibrinogen and α-2 (acute Phase) Globulin, Antithrombin III is Not an Acute Phase Protein

by

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DISCLAIMER

SUMMARY

Antithrombin III-heparin cofactor has been isolated from normal rat plasma, purified to homogeniety on acrylamide gel electrophoresis and used to prepare a monospecific antiserum in rabbits. Measurements of rat AT-III were made by a single radial immunodiffusion assay.

Net synthesis of AT-III was investigated during 12 or 24 hour perfusions of the isolated rat liver. In perfusions performed under basal conditions cumulative synthesis of AT-III was observed to occur at a rate sufficient to replace the total circulating plasma AT-III in about 6 hours. In perfusions performed under full supplementation conditions which greatly enhanced synthesis of fibrinogen and α-2 (acute phase) globulin (known acute phase reactant proteins) net synthesis of AT-III was not significantly greater than that observed in control perfusions. Although these prolonged perfusion studies conclusively demonstrate net synthesis of AT-III by the isolated rat liver, they afford no evidence that this protein is an acute phase reactant.

I. Introduction

Antithrombin III - heparin cofactor, an α-2 globulin, is believed to be the chief inhibitor of activated thrombin and Factor X-a in plasma. [1-3] The possible significance of AT-III in regulation of the coagulation system has been emphasized in studies of families where an inherited deficiency of AT-III has been found to occur along with a strong predisposition toward thrombotic disorders. [4-6] Clinical studies in which decreased plasma levels of AT-III have been observed in states of advanced liver failure have implicated the liver as a probable site of synthesis of AT-III. [7]

Perfusion of the isolated rat liver has been utilized extensively both in studies of synthesis of blood coagulation factors [8-12] and in defining effects of hormones in modulating plasma protein synthesis. [13,16] Synthesis of AT-III by the perfused rat liver has been looked for in two previous studies. [17,18] The first failed to demonstrate net synthesis over a four hour perfusion period. [17] In the second, Koj et al [18] demonstrated hepatic synthesis of AT-III over a three hour period, an interval we believe too brief to permit eliciting hormonal effects on AT-III synthesis, since a minimum interval of four to eight hours has been found necessary to demonstrate an acute phase response both in vivo and in vitro. [15,19] Our study was undertaken to clarify the role of the liver as the site of synthesis of AT-III and to determine whether the synthesis of AT-III is enhanced by those hormones which are known to induce increased synthesis of the acute phase proteins: fibrinogen, α-1 acid glycoprotein, α-2 (acute phase) globulin, and haptoglobin. [13-16] This study involved the

isolation of rat AT-III, the preparation of potent rabbit antiserum to rat AT-III, and the demonstration of substantial net synthesis of AT-III by the isolated rat liver perfused for 12 or 24 hours.

II. Methods

A. Liver Perfusion Technique

The technique of prolonged perfusion of the isolated rat liver, previously described by our laboratory, was utilized in this study without modification. [20] The perfusate consisted of 36 ml of washed bovine red cells suspended in 50 ml of Krebs-Ringer bicarbonate buffer containing 3 gm% of bovine serum albumin. In addition each perfusate contained glucose 100 mg, heparin 10,000 u, penicillin 3,000 u and streptomycin-HCl 3 mg. The final volume of the perfusate was brought to 100 ml by the addition of Ringer solution.

In control experiments, a constant infusion consisting of 18 ml of Ringer solution, containing glucose 500 mg, penicillin 3,000 u, streptomycin-HCl 3 mg and amino acids 320 mg was added to the perfusate at a rate of 1.5 ml per hour for twelve hours. In "full supplementation" experiments, in addition to 5 mg cortisol and 5.1 u insulin (generously supplied by The Eli Lilly Co.) added to the perfusate at the onset, the constant infusion also contained cortisol 5 mg and insulin 6.8 u, hormones which are known to enhance synthesis of the acute phase reactant proteins. [13,15] In selected experiments to block protein synthesis, puromycin 15 mg was added directly to the perfusate at the outset and 7.5 mg was added to the constant infusion.

Throughout each experiment, the pH of the perfusate was maintained constantly at either 7.10 or 7.40 by an infusion of 1.0 M $NaHCO_3$

from a Radiometer Autoburette (ABU-12) and Titrator (TTT-10) equipped with a combined glass calomel electrode (GK202LC). At the start of each perfusion, to wash out preformed rat plasma proteins, the first 10 ml of perfusate to pass through the liver was collected and discarded. Four control experiments were run at pH 7.4 and five were run at pH 7.1. A total of four full supplementation experiments were run at pH 7.4 and seven at pH 7.1.

B. Liver Donors

Liver donors were adult male Sprague-Dawley rats from the Holtzman Company of Madison, Wisconsin. Liver donor weights ranged from 350 to 450 g.

C. Purification and Characterization of Rat Antithrombin III

Rat AT-III heparin cofactor was prepared from pooled defibrinated rat plasma by chromatography on heparin-sepharose and Sephadex G-100. Heparin-sepharose chromatography was performed essentially as described by Damus for isolation of canine antithrombin. [21] In brief, blood was collected from normally fed rats under ether anesthesia by means of aortic puncture into syringes which contained 0.1 M sodium oxalate. The ratio of anticoagulant to whole rat blood was 1:9. The plasma component was separated by centrifugation and was then defibrinated by heating at 56^{o} C for four minutes. The heat-denatured fibrinogen comes down as a flocculent precipitate which is removed by centrifugation. The defibrinated rat plasma sample was then dialyzed for 24 hours against 0.145 M NaCl - 0.01 M tris buffer pH 7.5. Crude heparin was obtained from the Wilson Chemical Corporation of Chicago. The crude material was purified by three precipitations with cetylpyridinium

chloride as described by Iverius [22] and modified by Damus. 50 mg of the purified heparin was coupled to 100 ml of Sepharose 4B utilizing cyanogen bromide. A 10 ml column of the heparin-sepharose was prepared and extensively washed with 0.145 M NaCl - 0.01 tris buffer, pH 7.5. A 30 ml aliquot of the dialyzed defibrinated rat plasma was applied to the column and it was then washed with 40 ml of the tris buffer. A progressive step-wise saline gradient ranging from 0.3 M to 0.8 M NaCl (see Fig. 1) was utilized to elute AT-III. The flow rate was 3 ml per hour and 3 ml fractions were collected. The fractions were initially assayed for thrombin inhibitory capacity. Those fractions containing antithrombin activity were combined and further purified by gel filtration chromatography on Sephadex G-100.

To assess the antithrombin activity of the purified AT-III, a dilute solution of bovine thrombin (15 u/ml) was incubated at 37^{o}C in veronal buffer with the purified protein; at progressive intervals, 0.2 ml aliquots of the reaction mixture were withdrawn, added to 0.2 ml of a 1% bovine fibrinogen solution, and clotting times were recorded. A second identical incubation was done with the addition of 5 u of heparin (Abbott Labs) to the mixture of thrombin and AT-III. The stability of the thrombin solution in the absence of AT-III was assessed by substituting buffer for AT-III in a third incubation.

D. The molecular weight of the purified rat AT-III was estimated by the method of Weber and Osborn using SDS acrylamide gel electrophoresis. [23] The electrophoretic mobility of the rat AT-III was compared to that of a series of standards with molecular weights ranging from 10,000 to 100,000 (Lysosyme, Carbonic anhydrase, Ovalbumin, BSA

and Phosphorylase B).

E. Antiserum Preparation

For preparation of antiserum to rat AT-III, 0.75 ml (0.2 mg) of the purified protein was emulsified with 0.75 ml of complete Freund's adjuvant and injected subcutaneously at multiple (12-15) sites on the backs of the rabbits. Each rabbit received 0.2 mg weekly for four weeks and antiserum titer was measured at that time. The preparation of rabbit antiserum to rat fibrinogen and α-2 (acute phase) globulin were carried out as previously described. [24,25]

Protein concentrations in purified preparations of rat AT-III and fibrinogen were determined by a modification of the method of Lowry [26] with a commercial protein assay reagent (Bio-Rad). Dilutions of the purified protein preparations were then used as standards in performing quantitative immunologic assays of the proteins in the liver perfusate and in pooled rat plasma. Concentrations of rat α-2 (acute phase) globulin were assigned values in units as previously described. [15] Quantitative immunologic assays of the three proteins, rat AT-III, fibrinogen, and α-2 (acute phase) globulin were performed by the single radial immunodiffusion assay of Mancini as modified by Fahey and McKelvey. [27,28] Dimensions of the precipitant rings were read by means of a calibrating viewer (Transidyne General Corporation).

III. Results

A. Purification of Rat AT-III Heparin Cofactor

A representative isolation of rat AT-III by affinity chromatography on a heparin-sepharose column is depicted in Figure 1. AT-III was eluted from the column at 0.55 M NaCl. The fractions containing

AT-III activity (16-22) were pooled and examined by SDS acrylamide gel electrophoresis. Two distinct bands were formed by the pooled fractions from the heparin-sepharose column (see Figure 2). The partially purified AT-III sample (18 ml) was concentrated to a volume of 4 ml using an immersible vacuum filtration device (Millipore Corporation) and was then applied to a 2.5 x 25 cm Sephadex G-100 column which had been washed extensively with 0.145 M NaCl - 0.1 M tris, pH 7.5. Fractions of 3 ml were collected and assayed for AT-III activity. AT-III was recovered in the void volume of the column, and the pooled active fractions yielded a single heavy band on SDS acrylamide gel electrophoresis (see Figure 2). The substance corresponding to the second band seen in the samples obtained from the heparin-sepharose column was not recovered during Sephadex G-100 filtration.

B. Activity of Purified AT-III

The thrombin-inhibitory capacity of the purified AT-III was measured in the presence and absence of heparin. As seen in Figure 3, AT-III in the absence of heparin destroys thrombin activity in a slow progressive fashion. The addition of heparin to the mixture dramatically accelerates the destruction of thrombin by AT-III.

C. Molecular Weight of Rat AT-III

The molecular weight of purified rat AT-III estimated by the method of Weber and Osborn [23] in comparison to molecular weight standards ranging from 10,000 to 100,000 was 64,000 as revealed in Figure 4.

D. Net Biosynthesis of AT-III by the Isolated Perfused Rat Liver

Multiple injections of rat AT-III into rabbits raised a mono-

specific antiserum which was utilized in a single radial immunodiffusion assay to estimate the synthesis of rat AT-III by the perfused rat liver. Synthesis of AT-III was studied under control and "full supplementation" conditions at both pH 7.4 and 7.1. The two pH levels were chosen because the synthesis of some acute phase proteins is known to be enhanced at the lower pH level. [14] The mean values for cumulative synthesis of AT-III in both control and full supplementation experiments at pH 7.4 are shown in Figure 5. As can be seen from the Figure the mean values for AT-III synthesis are slightly higher in the full supplementation experiments than for those of control perfusions. The difference is significant only at 12 hours but does not approach those proportions seen with the induction of known acute phase reactant proteins such as fibrinogen or haptoglobin. [15]

To determine whether the observed levels of AT-III seen in perfusate samples represented newly synthesized protein or preformed protein, puromycin was added to the initial perfusate and as a constant infusion. The dose utilized (15 mg at the start plus 7.5 mg infused constantly) has been shown to effectively inhibit protein synthesis by the perfused rat liver. [24] The effects of puromycin on AT-III synthesis are shown in Figure 5 along with the values for control and full supplementation experiments. A small amount of AT-III appeared in the perfusate during the first two hours of the experiment but no significant increase was observed thereafter. There was neither net gain nor loss of AT-III in the liver perfusate when protein synthesis was inhibited by puromycin. The small quantity of AT-III noted during the first two hours of perfusion is most likely preformed AT-III.

The synthesis of AT-III under control and "full supplementation" conditions in 24 hour perfusions was also investigated at pH 7.1. As demonstrated in Figure 6, the mean values for net synthesis in four control and seven full supplementation experiments were not significantly different from each other at this pH level. As would be expected, the net synthesis of AT-III in these 24 hour experiments was substantially greater than that found in the 12 hour perfusions.

E. Synthesis of Known Acute Phase Reactant Proteins by the Perfused Rat Liver

The above results suggest that AT-III is not influenced by the hormones cortisol and insulin or by pH conditions which enhance synthesis of some acute phase proteins. To compare the effectiveness of the full supplementation regimen on synthesis of AT-III with the induction of increased synthesis of acute phase proteins, the synthesis of fibrinogen and of α-2 (acute phase) globulin were assayed in the perfusates from the same experiments. Fibrinogen synthesis under control and "full supplementation" conditions is shown in Figure 7. The characteristic induction pattern with dramatic increase in synthesis under full supplementation conditions is obvious. Alpha-2 (acute phase) globulin, a protein that in the rat appears to be synthesized only under acute phase conditions was also assayed. The synthesis of this protein, shown in Figure 8, was manifested only under full supplementation conditions and became apparent only after eight hours of perfusion. Our failure to demonstrate effects of cortisol and insulin on the production of AT-III cannot reasonably be ascribed to a failure of the induction regimen.

IV. Discussion

The technique of perfusion of the isolated rat liver has added greatly to our knowledge of the dominant role of the liver in plasma protein synthesis. [20,29] Prolonged perfusion studies have helped define a certain group of "acute phase reactant proteins": fibrinogen, haptoglobin, α-2 (acute phase) globulin and α-1 acid glycoprotein. [13,16] Perfusion studies have also provided insight into the nature of hepatic production of proteins involved in blood coagulation. Coagulation factors II, VII, IX, X, V, XI, XII and fibrinogen have been shown to be produced by the perfused rat liver. [8-12] Of these coagulation factors, fibrinogen has been shown both in vivo and in vitro to behave as an acute phase reactant protein. [15,19]

This study was undertaken to evaluate the role of the liver in synthesis of AT-III under basal conditions and under conditions known unequivocally to induce enhanced synthesis of acute phase reactant proteins. Two studies have recently been reported in which the synthesis of AT-III was examined during rat liver perfusion. Losito et al [17], using whole rat blood as perfusate, failed to show net synthesis of AT-III during five hour perfusions; presumably AT-III levels present in normal rat plasma of the perfusate obscured immunologically detectable net change in AT-III levels during his experiments. More recently, Koj et al [18] presented evidence for hepatic synthesis of AT-III during three hour perfusions, too short an interval to elicit an acute phase response either in vivo or in vitro. [15,19] Their results based on [^{3}H] leucine incorporation were inexplicably higher than their serologic values for AT-III synthesis. They concluded that AT-III is

not an acute phase protein in the rat.

We have isolated rat AT-III by chromatography on heparin-sepharose and Sephadex G-100; the purified protein yielded a single band on SDS acrylamide gel. As has been shown for AT-III of other species, rat AT-III possesses both progressive antithrombin and heparin cofactor activities. The relative mobility of the protein in SDS acrylamide gels corresponds to a molecular weight of 64,000, which is slightly higher than that reported for human AT-III (62,300) and slightly lower than that of rabbit AT-III (67,000). [1,30]

A monospecific antiserum to rat AT-III was prepared and utilized to measure levels of AT-III produced during prolonged perfusion of the isolated rat liver. Steady increases in perfusate levels of AT-III were observed throughout perfusion periods of 12 or 24 hours. Perfusions with puromycin support the view that the observed increasing levels seen in the absence of puromycin represent newly synthesized protein rather than preformed protein. In the puromycin experiments only a small amount of AT-III was noted in the samples drawn during the early hours of perfusion without further increases.

The observed average net synthesis of AT-III during 12 hour perfusion periods was 4.94 ± 0.53 mg per 300 cm^2 body surface area of the rat liver donor, which corresponds to a body weight of 195 g. Our immunodiffusion assay for AT-III yielded values of 26 mg per 100 ml pooled normal rat plasma; this value is comparable to that reported for human plasma, normally about 20 mg per 100 ml. [31] If the plasma volume of the rat is expressed as 4.5% of body weight, a 195 gm rat would have a plasma volume of 8.8 ml containing approximately 2.3 mg of AT-III.

The production of 4.94 ± 0.53 mg of AT-III in a 12 hour perfusion indicates that the rat liver can replace the circulating plasma pool of AT-III in a relatively short period of time. There is no data available on the biological half life of AT-III in the rat. Data available from human studies indicate a half life of 2.69 days for labelled AT-III. [31] The values for normal rat turnover of AT-III may be briefer than this, however, since other proteins such as fibrinogen have a substantially shorter half life in rats than in humans. [32,33]

In previous studies we have demonstrated direct effects of certain hormones, in particular glucocorticoids, on the induction of synthesis of acute phase reactant proteins by the perfused rat liver. [15] The experiments reported here examined the effects of the "full supplementation" regimen consisting of glucose, amino acids, insulin and cortisol, which is known to enhance synthesis of the acute phase proteins, fibrinogen, and α-2 (acute phase) globulin. [15,13] We did indeed observe a typical acute phase response in the synthesis of fibrinogen and α-2 (acute phase) globulin. The fact that synthesis of AT-III in both 12 and 24 hour perfusion periods was not significantly greater than normal is strong presumptive evidence that synthesis of AT-III is not like that of typical acute phase proteins. The current study demonstrates cumulative net synthesis of AT-III by the perfused rat liver during 12 or 24 hour perfusions but fails to support the view that AT-III is an acute phase reactant protein in the sense manifested by fibrinogen, α-1 acid glycoprotein, α-2 (acute phase) globulin and haptoglobin.

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Figure 1. Chromatography of rat AT III on heparin-sepharose.

Thirty ml of heat-defibrinated rat plasma was dialyzed for 24 hours vs. 0.145 M NaCl/0.01 M Tris, pH 7.4. The plasma was then applied to a heparin-sepharose column which had been washed extensively with the same buffer. AT III activity is expressed in the figure as % normal rat plasma activity. AT III was eluted from the column with a progressive NaCl gradient from 0.3 to 0.8 M in 0.01 M Tris, pH 7.4. The AT III activity peaked at 0.55 M NaCl. The activity in fractions 16-22 was greater than that of normal plasma and is expressed arbitrarily as 100%.

Figure 2. SDS acrylamide gel electrophoresis of rat AT III.

Six ug of protein were applied to each of the gels (see Methods). The gel on the left contains protein obtained from the heparin-sepharose column eluted at 0.55 M NaCl. The fractions containing AT III activity were pooled and further purified by gel chromatography on Sephadex G-100. The gel on the right contains 6 ug of AT III from the Sephadex column.

Figure 3. Inactivation of thrombin by purified rat AT III.

A dilute thrombin solution (15u/ml) was incubated at 37° C with purified AT III in veronal buffer. Samples of 0.2 ml were withdrawn from the mixture at specified time intervals and admixed with 0.2 ml aliquots of a 1% bovine fibrinogen solution. The remaining thrombin activity at each time interval is expressed as a % of the activity in the starting thrombin mixture. In the uppermost curve, (open circles) buffer was substituted for AT III and very little decrease in thrombin activity occurred. The second curve (closed circles) shows the progressive destruction of thrombin activity by AT III. In the third curve,(squares) heparin was first added to the AT III 30 seconds before the addition of thrombin and the characteristic rapid destruction of thrombin activity by AT III is seen.

Figure 4. Molecular weight of rat AT III.

The relative mobility of purified AT III in SDS acrylamide gel was compared to that of known standards and the molecular weight was calculated by the method of Weber and Osborn (23).

Figure 5. Net synthesis of AT III at pH 7.4.

Perfusion of the isolated rat liver was carried out for 12 hours under control (open circles) and full supplementation conditions (closed circles) at pH 7.4. The numbers in brackets here and in Figs. 5 and 6 indicate the number of experiments in each group. The cumulative production of AT III is expressed in mg/300 cm^2 body surface area of the rat liver donor. Included in the figure is data from two experiments in which, to inhibit protein synthesis, puromycin, 15 mg was added to the perfusate at the start and also 7.5 mg as part of the constant infusion (squares).

Figure 6. Net synthesis of AT III at pH 7.1.

Cumulative production of AT III by the perfused rat liver is shown under control (open circles) and full supplementation (closed circles) conditions during 24 hour perfusions.

Figure 7. Net synthesis of fibrinogen at pH 7.4.

The net synthesis of fibrinogen is shown under control (open circles) and full supplementation (closed circles) conditions at pH 7.4. The typical acute phase response is apparent in the greatly increased rate of synthesis under full supplementation conditions.

Figure 8. Net synthesis of alpha-2-acute phase globulin.

Alpha-2-acute-phase globulin was assayed in perfusions under control (open circles) and full suplementation (closed circles) conditions at pH 7.4. In control perfusions no detectable amounts of this protein appeared. In full supplementation experiments, the induction of synthesis of this protein is apparent.

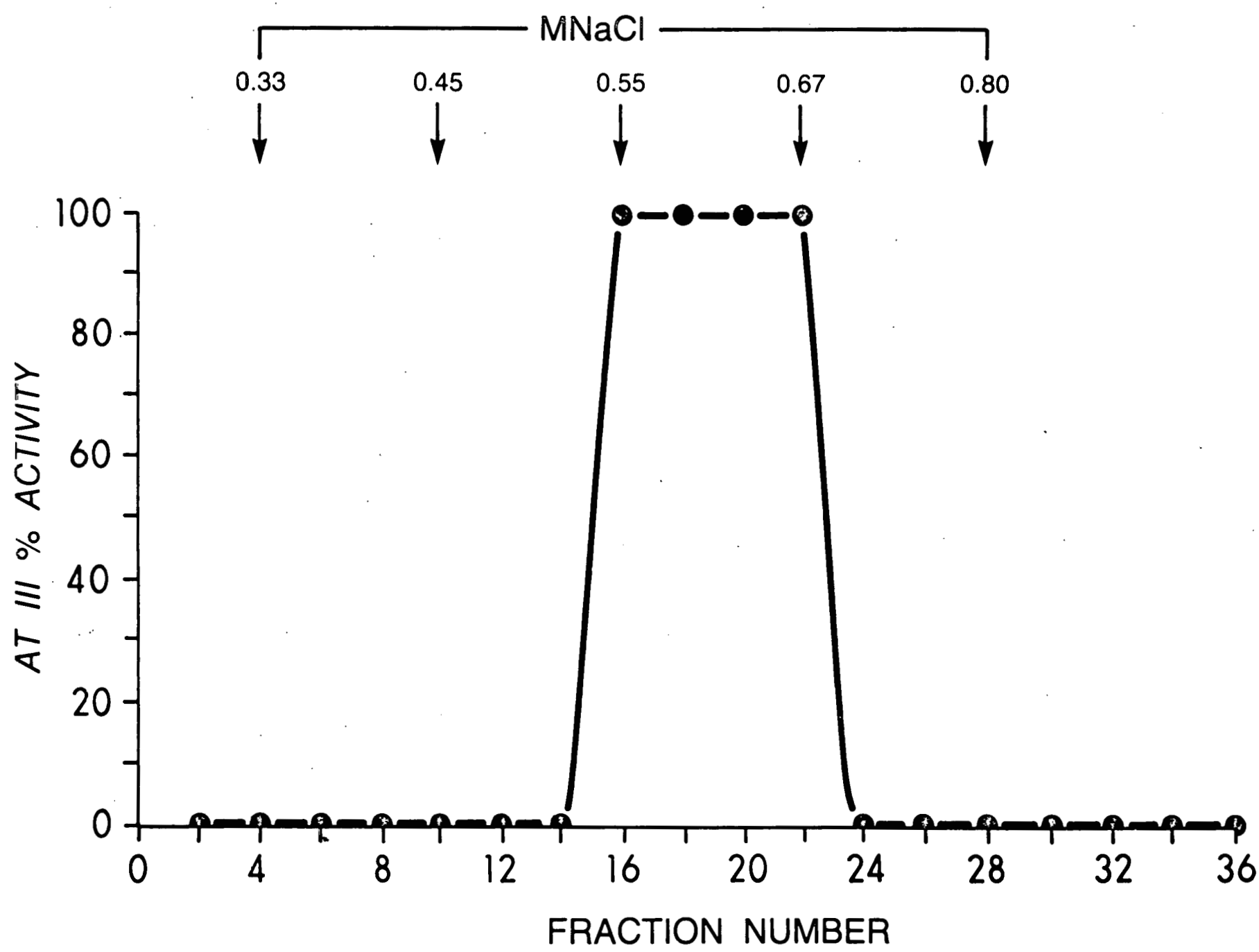
MNaCl
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Figure 1

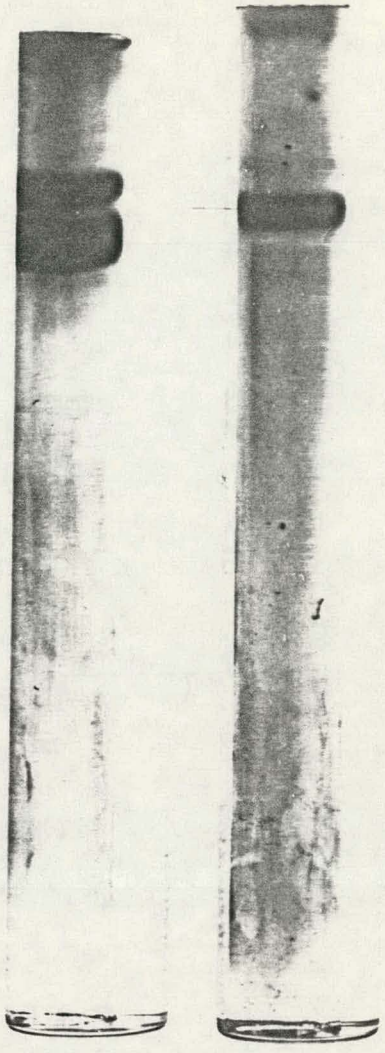

Figure 2

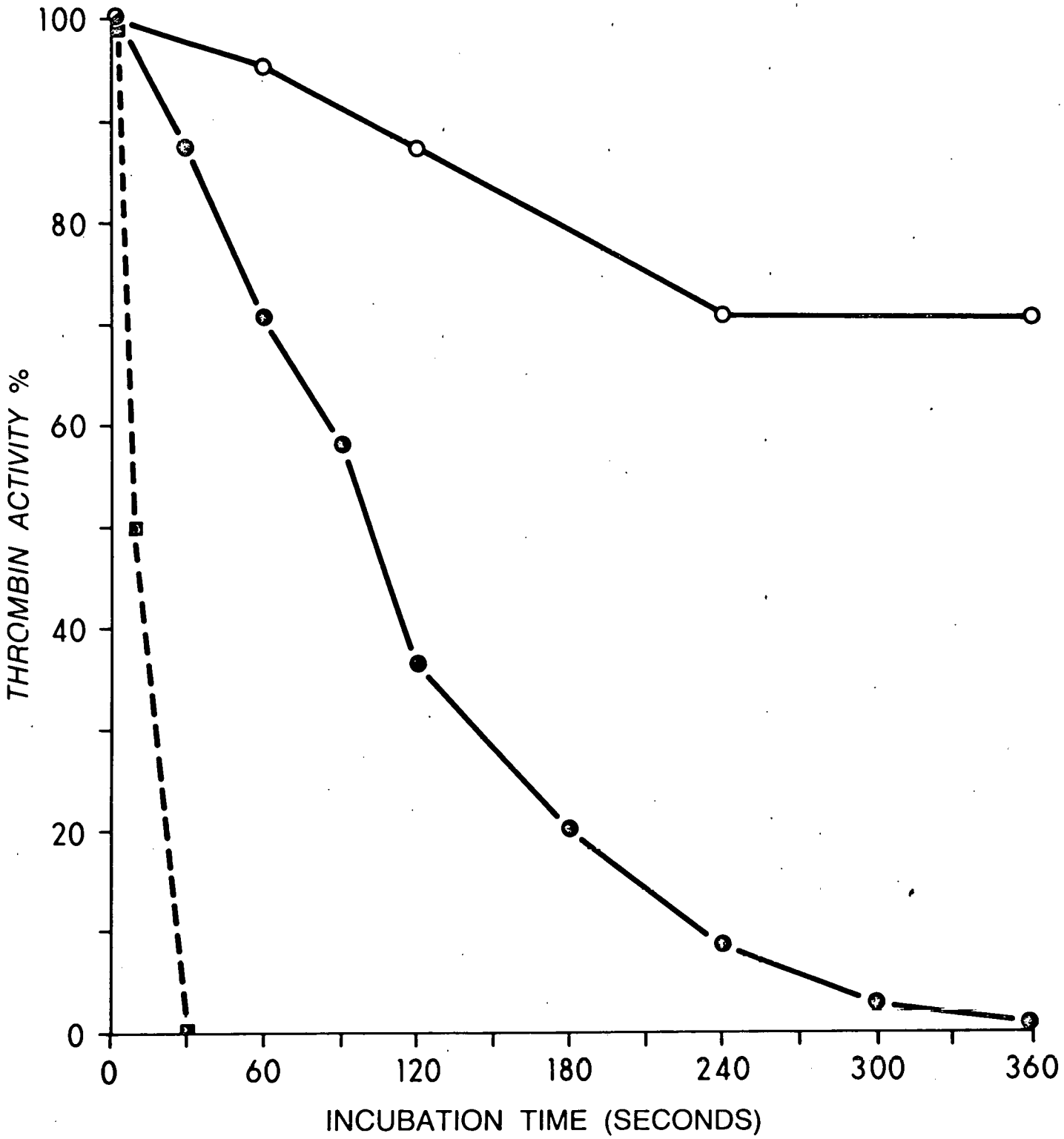
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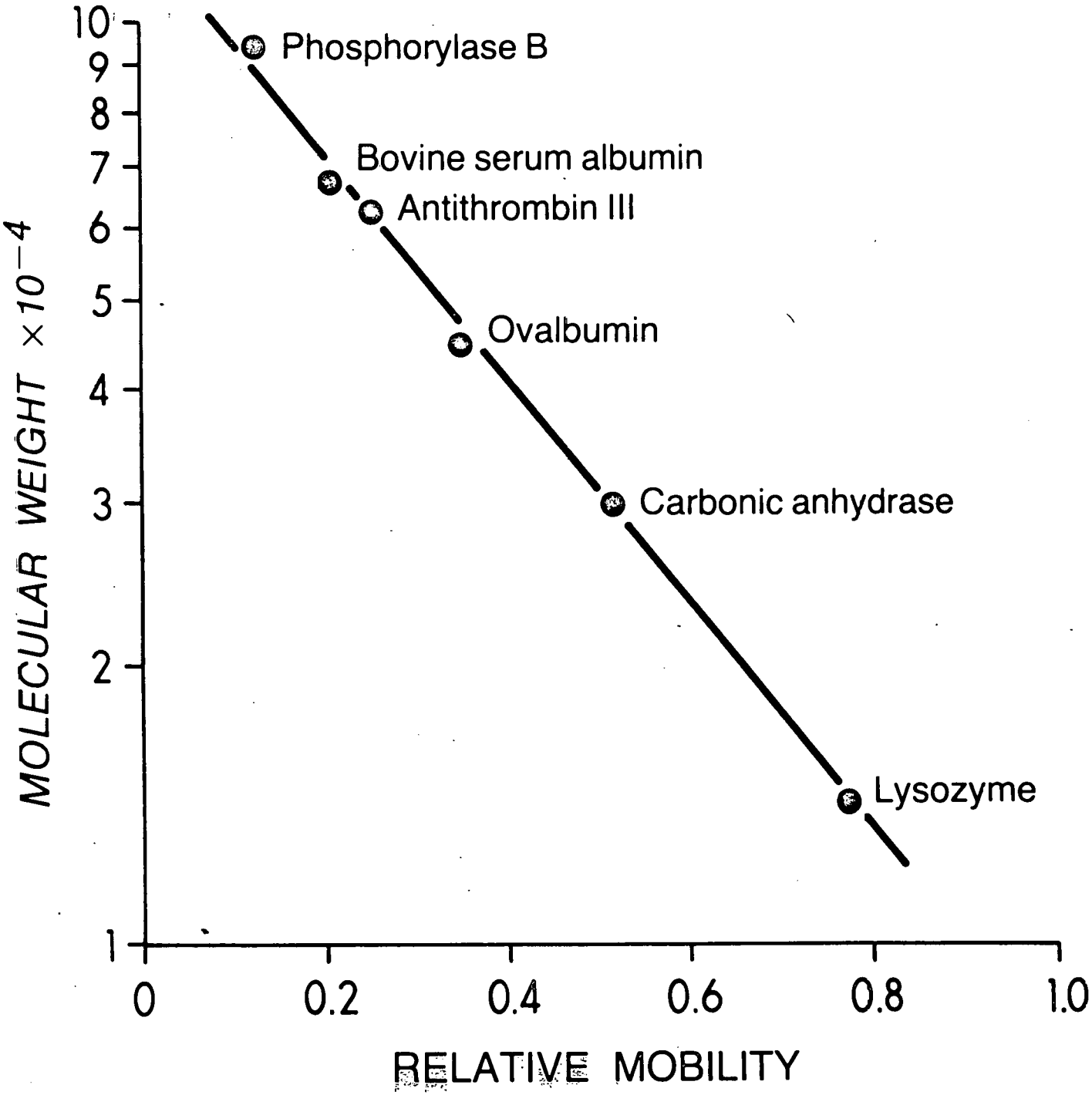
Figure 3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RELATIVE MOBILITY

Figure 4

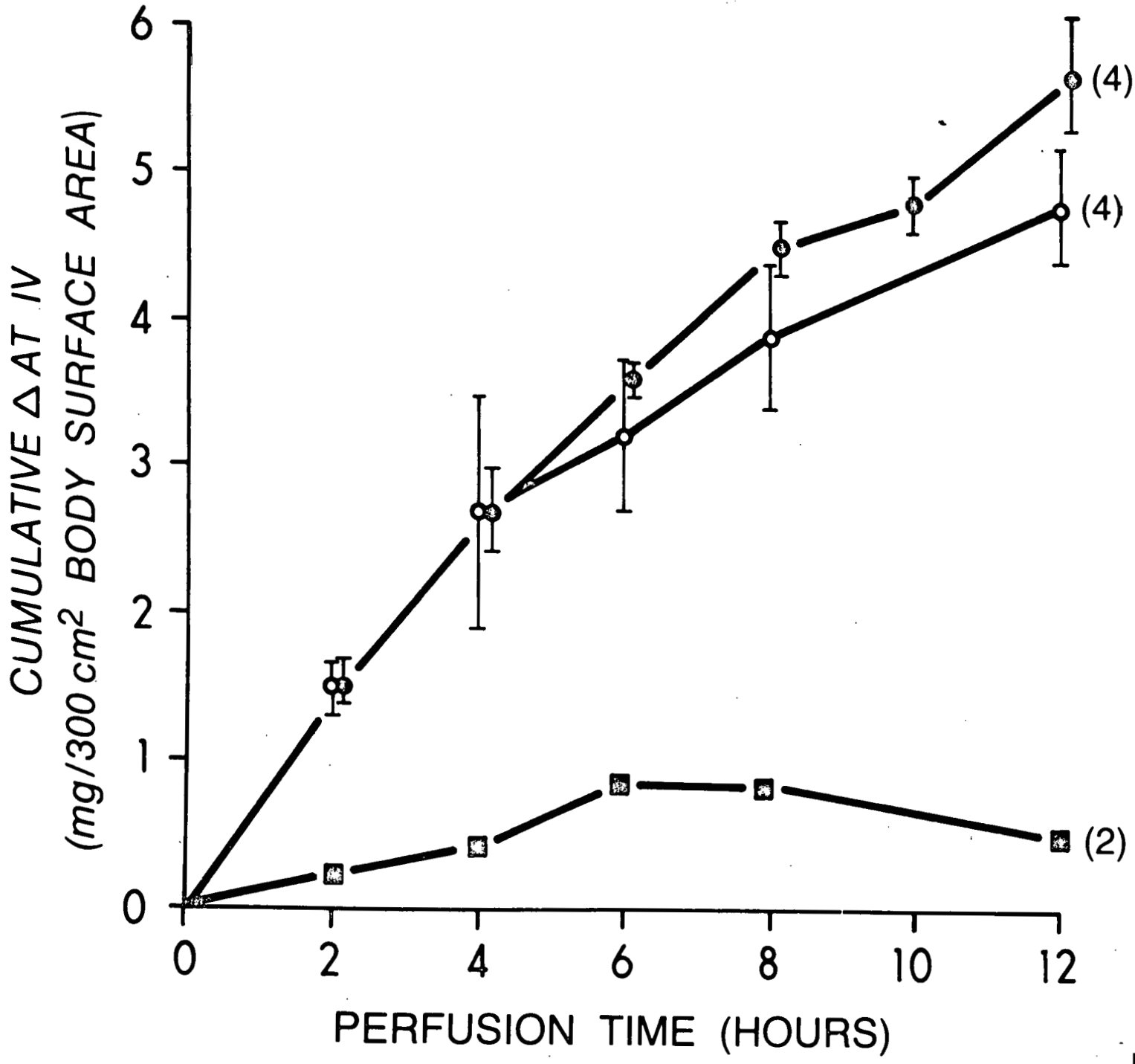
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Figure 5

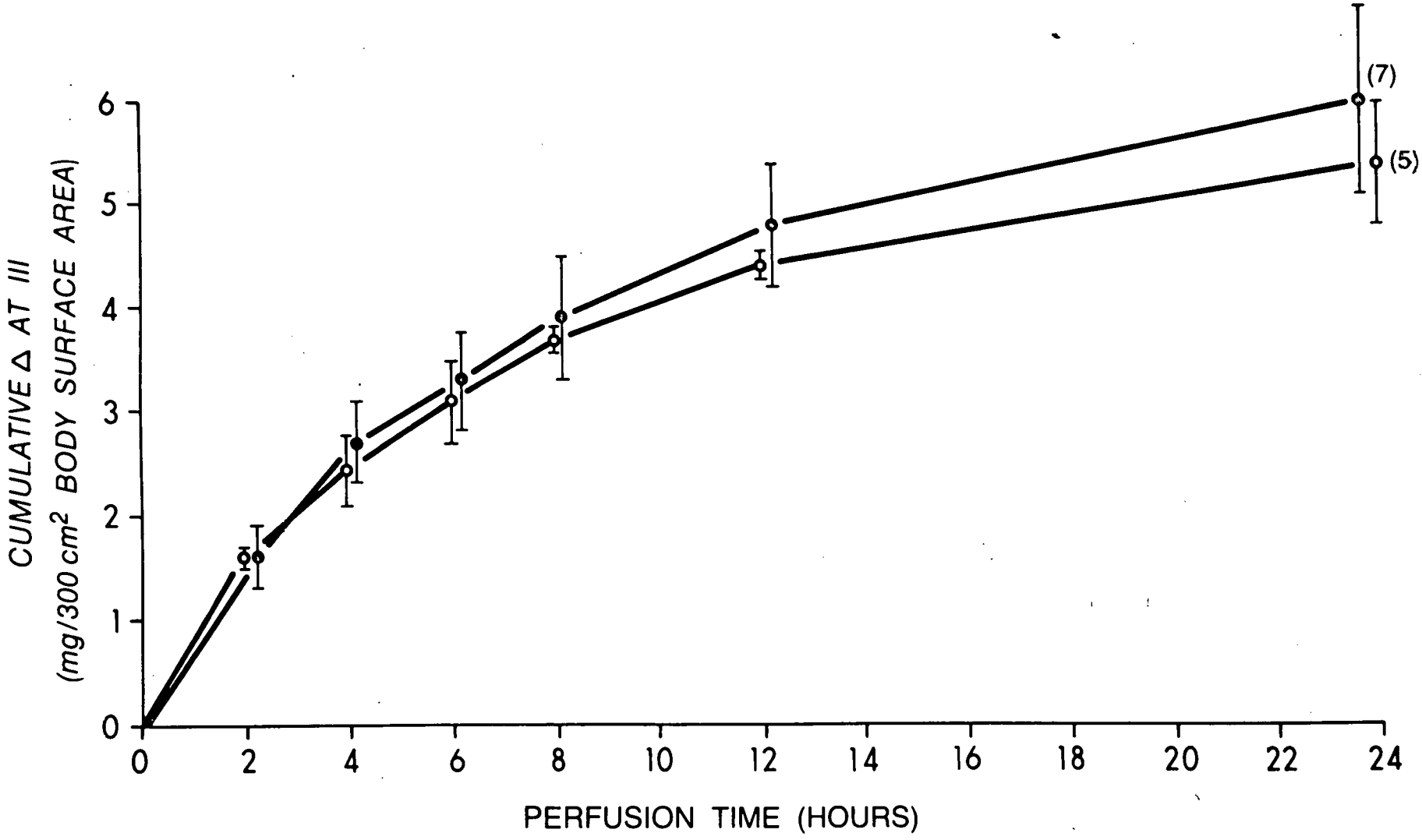
CUMULATIVE Δ AT III
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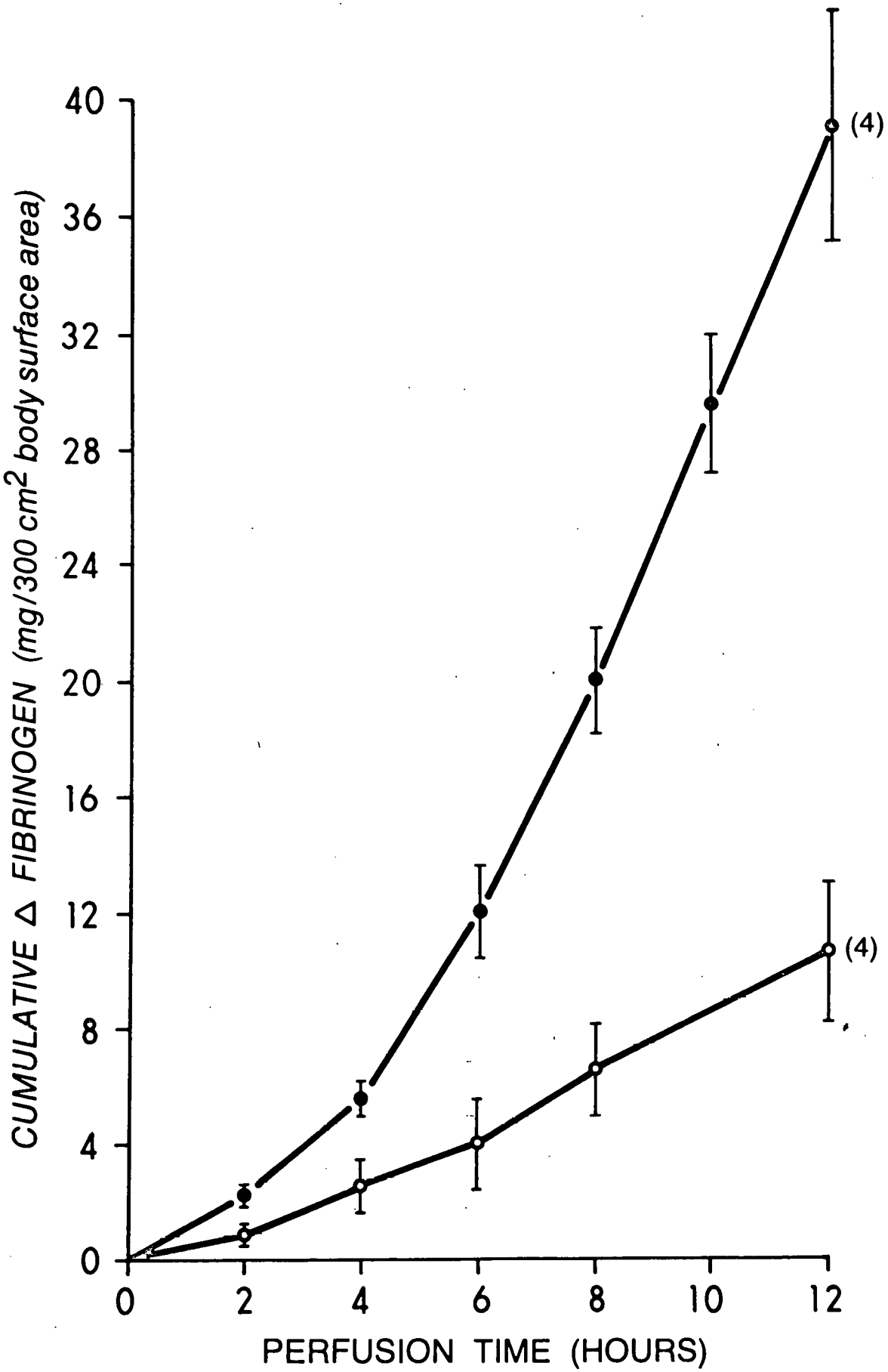
Figure 6


CUMULATIVE Δ FIBRINOGEN (mg/300 cm² body surface area)
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24
20
16
12
8
4
0
0
2
4
6
8
10
12
PERFUSION TIME (HOURS)
(4)
(4)

Figure 7

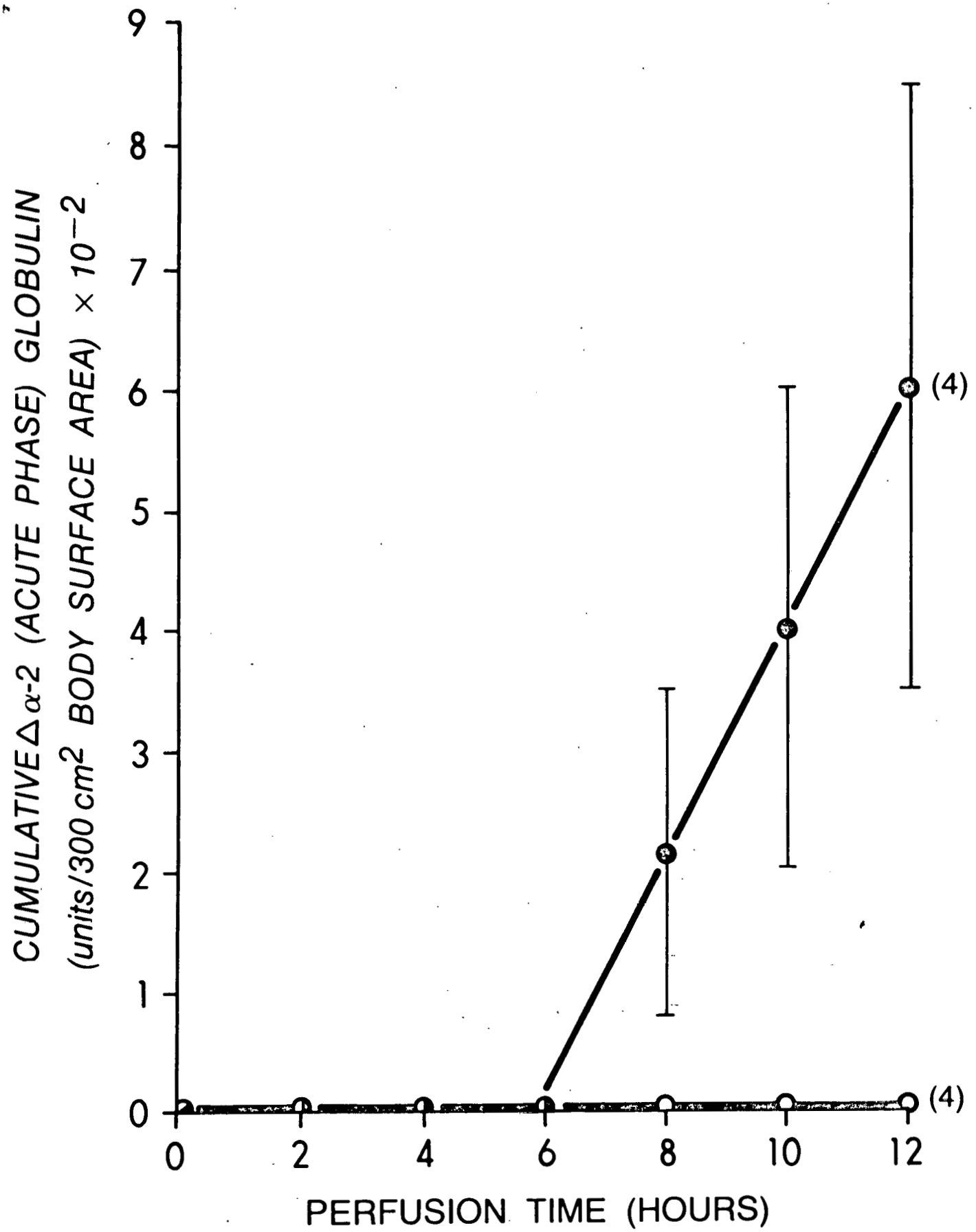
CUMULATIVE Δ α-2 (ACUTE PHASE) GLOBULIN
(units/300 cm² BODY SURFACE AREA) × 10⁻²
9
8
7
6
5
4
3
2
1
0
0
2
4
6
8
10
12
PERFUSION TIME (HOURS)
(4)
(4)

Figure 8