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Effect on Glutamate on the Incorporation of Cystine Into the Plasma Proteins

Robert Orman
Loyola University Chicago

EFFECT OF GLUTAMATE ON THE INCORPORATION OF CYSTINE INTO THE PLASMA PROTEINS

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ROBERT ORMAN

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LIFE

Robert Orman was born in Chicago, Illinois, July 20, 1944.

In June of 1966, he received the B. Sc. degree in mathematics from the University of Illinois in Champaign.

He began further studies in the Department of Biochemistry and Biophysics, Loyola University in September of 1967.

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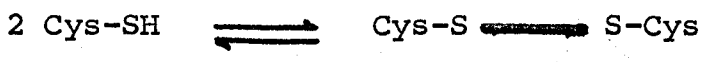
CHAPTER I

INTRODUCTION

Protein chemistry is becoming increasingly involved with the critical roles of cystine and cysteine in protein structure and function. The amino and carboxyl groups of these amino acids enter into the amide linkage in a similar fashion as do the other naturally occurring amino acids. However, it is the disulfide bond, which reversibly gives rise to sulfhydryl groups which gives cystine its unique function in proteins. The conversion of sulfhydryl groups to the disulfide bond proceeds according to the following reaction:



(1)

Thus these sulfur amino acids can be incorporated into proteins not only by biochemical mechanisms but also by direct chemical reaction. The position and state of oxidation of cystine and cysteine are essential to the function of many proteins.

Considerable work has been carried out on the reactions of cystine and cysteine *in vitro*. Fundamentally, this work seems to indicate that the reactions of these amino acids involve oxidation and reduction as illustrated in Reaction 1 above. However, additional factors seem to be operating *in vivo* which add complexities to the *in vitro* studies. The work described herein is a first attempt at unraveling some of these complexities.

Cystine and Cysteine in Protein Structure and Function

Cystine and cysteine enter into the primary and secondary structures of proteins in a similar fashion as that displayed by the other amino acids. Two codons direct the incorporation of cysteine into proteins. These codons are uracil-guanine-uracil and uracil-guanine-cytosine (32). Only cysteine may be incorporated into peptidechains by the RNA mechanism. Cystine, in proteins, arises from the oxidation of peptide-bound cysteine sulfhydryl groups. Thus the presence of cysteine in proteins is under genetic control whereas that of cystine is a resultant of metabolic reactions.

Protein sulfhydryl groups may be classified as rapidly reacting or slowly reacting when treated with metals and organometallic reagents. Rapidly reacting sulfhydryl

groups have been considered functional in nature in that they directly participate in the biochemical reaction of the protein. The cysteine residues at the active site of enzymes have classically been considered of this type.

The sulfhydryl groups which react slowly with metals and organometallic reagents have been considered structural in nature. These sulfhydryl groups organize the protein and help it assume a preferred configuration so that other groups may react. The precise nature of this property of the sulfhydryl group is not as yet understood although many contemporary theories are currently under consideration (22). Cori and Green have demonstrated the structural requirements of the sulfhydryl group in muscle phosphorylase A (7). Treatment of phosphorylase A with p-mercuribenzoate resulted in dissociation of the molecule into four subunits and concommitant loss of activity. Further studies indicated that it was indeed the sulfhydryl group and not the disulfide linkage that provided the cohesive organization of the molecule. Cecil and Snow have demonstrated that sulfhydryl groups are involved in "intramolecular linkages" in the structure of hemoglobin (5). Similarly, it has been determined that the heme in cytochrome C is bound to the protein through two cysteine residues (22). It should be

noted that the sulfhydryl group functions differently in hemoglobin structure than it does in cytochrome C. However, the exact role of cysteine in these proteins is only vaguely understood (21).

The disulfide bond has classically been assigned a structural role in the architecture of proteins. Disulfide linkages hold the protein in the preferred functional configuration. This premise has been expanded to recognize different kinds of disulfide linkages in proteins. Polarographic studies indicate that disulfide bonds linking two different peptide chains appear to be easier to reduce than those between half-cystine residues in the same peptide chain (20, 21, 30).

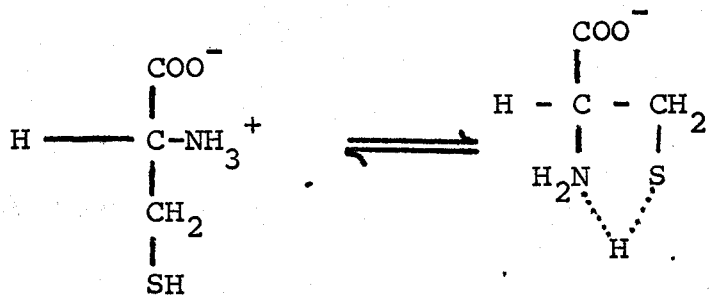
The Sulfhydryl-Disulfide Exchange Reaction

Compounds containing sulfhydryl or disulfide groups such as cysteine and cystine have been shown to react with each other according to the following scheme:

$$\text{RSH} + \text{R'-S-S-R"} \rightleftharpoons \begin{matrix} \text{R'SH} + \text{R-S-S-R"} \\ \text{or} \\ \text{R"SH} + \text{R-S-S-R} \end{matrix} \qquad (2)$$

Fava (11) and Eldjarn and Pihl (9, 10) have determined that the reaction between thiols and disulfides is

proportional to the concentration of mercaptide ion. Thus the degree of ionization of thiol groups limits the rate of the exchange reaction. The nature of the compound to which the sulfhydryl group is attached appears to effect the degree of ionization (11). When the group attached to the sulfhydryl group is electrophilic, the rate of reaction is more rapid than when an electrophobic substituent is involved. Increasing the pH of the medium tends to increase the rate of reaction because ionization increases with basicity (11). As would be expected, ionization is also a function of the nature of the medium since some solvents promote ionization to a greater extent than others (12). In the case of the free amino acid, cysteine, it has been suggested that hydrogen bonding between the sulfhydryl and amino groups retards ionization according to the following scheme:



(3)

This bonding makes for a slower reaction rate than that observed when the amino group cannot react. Equilibrium

constants have been calculated for exchange reactions involving many different thiols and disulfides (11).

The exchange reaction may be considered to be an oxidation-reduction reaction with special features. The oxidation reduction indicated in Reaction 1 may be brought about by molecular oxygen, alkaline pH, or by intermediary enzymes in the presence of NAD or NADP (6, 19, 25). On the other hand, the exchange reaction (Reaction 2) involves oxidation of one reactant by the oxidized form of the same reactant.

Katchalski used thioglycolic acid to determine the number of disulfide bonds present in serum albumin (18). It had already been demonstrated that both bovine and human serum albumin dimerize in acid solution and that this behavior was the result of an exchange reaction (2). Only one disulfide bond was shown accessible to the thiol reagent at physiological pH (18). Thus albumin in physiological solution has only one reducible disulfide bond capable of exchanging with free sulfhydryls.

Thiol reagents similar to thioglycolic acid have been used in studies on the various components of the γ-globulin fraction of serum (17). It has been reported that the 7-S species of human γ-globulin contained twelve

disulfide bonds which, if reduced, result in a loss of antibody activity (14). Karush has postulated that the intricate folding of γ-globulin fractions, stabilized by the disulfide linkages are responsible for antibody activity and that the exchange reactions with free cystine reduces antibody activity in vitro (17).

Glutathione (α-glutamyl-cysteinyl-glycine), by virtue of its cysteine residues enters into reactions similar to Reaction (1) and Reaction (2) (15). Glutathione has been found in all tissues thus far studied, with the possible exception of bone (2). Oxidized glutathione is found in considerable amounts in the blood but is negligible in other tissues during normal metabolism (15). It has been suggested that oxidized glutathione is constantly interacting with the plasma proteins by means of the exchange reaction. Supposedly, this permits the plasma proteins to carry out their functional role in metabolism (14). However, the precise role of glutathione and its relation to plasma proteins is still unknown.

Glutathione has been found as a mixed disulfide with protein in different tissues such as spleen, heart, liver, kidney, and muscle (2). The concentration of glutathione-protein mixed disulfides has been found to be essentially

the same in all the above tissues (14). This has been interpreted to mean that glutathione plays a similar role in all tissues mentioned (24). The requirement for glutathione in protein synthesis, a process common to all cells, has been well documented (32).

Enzymes have been isolated from rat stomach that catalyze the exchange reaction between glutathione and protein in the formation of mixed disulfides (6, 19). These enzymes require NADP and appear relatively specific for glutathione (25). Whatever the function of glutathione, the available evidence indicates, as was suggested by Jocelyn (15), that glutathione functions largely "as a carrier of sulfhydryl groups" and not "by virtue of its peptide nature."

The cysteine in proteins may be involved in peptide bonds, disulfide bonds, or both. The half-cystine residues involved in the peptide bond, whether in the disulfide or sulfhydryl form will be designated as "peptide-bound half-cystine"; the half-cystine bound to proteins through the disulfide linkage only will be designated as "disulfide-bound half-cystine." Disulfide bound cystine arises from the reaction of free cystine with the sulfhydryl group in proteins.

It has been demonstrated that the disulfide-bound half-cystine in proteins turns over more rapidly than peptide-bound half-cystine. Disulphide bound half-cystine represents less than ten percent of the total cystine in proteins. The possibility exists that oxidized glutathione may act in a manner similar to cystine to form mixed disulfides with tissue proteins. In the case of regenerating wound tissue, most if not all mixed disulfides involve half-cystine residues (36).

The disulfide-sulfhydryl exchange reaction appears to occur in many tissues (2). Evidently both glutathione and cystine may participate. Several studies on this reaction in regenerating wound tissue have appeared (34, 36, 37, 38). The highest concentration of protein-bound mixed disulfides appears to be in the nuclei of wound tissue cells (33). However, the greatest turnover of disulfide bound cystine appears to take place in the microsomes (33). Preliminary evidence indicates that the principle form of protein mixed disulfide involves half-cystine residues (35). Modig has reported both half-cystine and glutathione mixed disulfides in protein of ascites tumor cells (24). Mixed disulfides involving glutathione and half-cystine have also been observed in other tissues (2).

Unpublished data is available indicating that glutamate inhibits the exchange reaction (35). This inhibition occurs when either tissue proteins or purified proteins are allowed to react with cystine in vitro. Furthermore the effect of glutamic acid is greatest at physiological pH. Significantly, glutamic acid can reduce but not totally inhibit the rate of exchange. The structure of glutamic acid is relatively unique with regard to the ability to slow down the rate of the exchange reaction. In an attempt to determine the minimal structural requirements for inhibition of the exchange reaction, analogs of glutamic acid were incubated with a liver homogenate and ^{35}S-cystine. The rate of incorporation of the disulfide-bound half-cystine was determined. The data from these experiments indicates that the inhibitory activity of glutamic acid disappears if either or both of the carboxylate groups are bound by ester, amide or peptide bonds (35). Five carbon dicarboxylic acids such as glutaric or α-ketoglutaric acid have no effect on the sulfhydryl-disulfide exchange reaction. However, the inhibition is apparently not affected if the amino group of glutamic acid is acetylated or bound to a dinitrophenyl substituent. Aspartate, a four carbon analog of glutamic acid, shows no inhibitory activity.

The question now arises as to whether glutamic acid has the same effect *in vivo* as it has been shown to have *in vitro*. The approach to answering this question has been undertaken by following the rates of binding of labeled cystine to the plasma proteins as affected by different situations. The plasma proteins were chosen as a test system because (1) they are relatively easy to isolate, (2) they represent a relatively simple protein system when compared to other cellular protein systems and (3) their environment is relatively easy to control.

CHAPTER II

EXPERIMENTAL PROCEDURE

Previous work has shown that the metabolism of the sulfur amino acids is greatly increased during wound healing (37). Since the present experiment is specifically structured to study the rate of cystine incorporation into the plasma proteins, it was felt that a study of the plasma proteins of wounded animals, as well as unwounded animals, would provide enough diverse experimental situations to give several areas of insight into the problem.

Design of the Experiment

Twenty mature female rats of the Sprague-Dawley strain weighing 200-250 grams were housed in individual cages, maintained on a diet of standard laboratory chow (Appendix I) and tap water for seven days (23°-25°C.). The rats were closely observed during these seven days to insure that they were healthy and fully acclimated to their environment.

At the end of seven days, the twenty rats were randomly divided into four groups of five rats each designated as follows:

Group 1 - Unwounded Rats

Group 2 - Wounded Rats

Group 3 - Wounded Rats Treated with Aspartate

Group 4 - Wounded Rats Treated with Glutamate

Twelve hours prior to wounding, food was removed from the rats. Rats in Groups 2, 3 and 4 were injected subcutaneously with pentabarbital at a dose level of 30 mg./kg. body weight. When the animals were quiescent (as determined by pinching the tail with forceps), the outline of a coin four centimeters in diameter was traced in the scapular region and the skin was excised. No underlying fascia or muscle was taken. The wound was blotted with a cotton swab wetted with 95 per cent ethanol and the rats returned to their cages to regain consciousness. For the next seven days, the rats were maintained on a protein free diet (10 gms/day--see Appendix I) and tap water.

On the seventh day after wounding, the animals were again anesthetized with pentabarbital as above. Each animal in each group was given three 5.0 ml. injections of experimental solution subcutaneously at fifteen minute intervals. The experimental solution for Groups 1 and 2 was 0.2 M NaCl in $NaHCO_3$ buffer (pH 7.4). Group 3 was given 0.2 M aspartate in $NaHCO_3$ buffer (pH 7.4). Group 4 was given 0.2 M glutamate in $NaHCO_3$ buffer (pH 7.4). Fifteen minutes after the third

injection of experimental solution, the rats were given a subcutaneous injection of one percent sodium heparin (10 mg./kg. body weight). Fifteen minutes after the injection of heparin, the rats were subcutaneously injected with 1.0 ml. of 35 S-L-cystine in bicarbonate buffer, pH = 7.4. This solution contained 197 uCuries/ml. The 35 S-L-cystine had a specific activity of 47 mCuries/mM.

Thirty minutes after the administration of the cystine, blood was taken from the tail. Exactly 1.0 ml. of blood from each rat in the group was pooled in a heparinized centrifuge tube. The freshly removed blood was centrifuged at 3000 r.p.m., at 4°C., for 20 minutes. The plasma was separated from the packed cells and lyophilized. Subsequently three more samples were taken at 60, 90 and 120 minutes after the administration of the labeled cystine. Forty-five minutes after the injection of the labeled cystine, the rats were given a fourth 5.0 ml. injection of experimental solution.

It was decided to administer the greatest possible dose of glutamate to the animals to insure maximal effectiveness, if any. Glutamate could not be given too far in advance of the labeled cystine because of its effect on the nervous system (29). The total maximum volume considered feasible to administer was 20 ml. (approximately twice the rat's blood volume). A volume greater than 20 ml. tended

to leak out of the rats subcutaneous pockets.

It was also observed that 20 ml. of solution greater than 0.3 M resulted in loss of water from the circulatory system making bleeding virtually impossible. Death occurred in about 30 minutes. Consequently, 20 ml. of 0.2 M glutamate was used. Twenty ml. of 0.2 M NaCl were injected into the control animals.

Techniques have been devised to distinguish between cystine as the free, disulfide bound, and peptide bound amino acid. Free cystine may be separated from the protein bound by dialysis against distilled water (36). The disulfide bound half-cystine residues may be further distinguished from those bound into the peptide chains by peptide bounds by dialyzing the water dialyzed samples against dilute Na_2SO_3 (27, 31). Sulfite ion reacts with the disulfide bond according to the following reactions:

$$R\text{-}S\text{-}S\text{-}R + SO_3^{=} \rightleftharpoons R\text{-}S\text{-}SO_3^{-} + R\text{-}S^{-} \qquad (4)$$

$$R\text{-}S^{-} + SO_3^{=} + 2\ Cu^{++} \rightleftharpoons R\text{-}S\text{-}SO_3^{-} + 2\ Cu^{+} \qquad (4A)$$

After the disulfide bond has been ruptured, the smaller fragments can be removed by dialysis against distilled water leaving only the peptide-bound half-cystine residues in the dialysand.

Employing these techniques, approximately 500 mg. of each lyophilized plasma protein sample was dissolved in 30 ml. of 0.1 M Na_2CO_3. Duplicate 7.5 ml. samples were dialyzed for four hours against three consecutive one liter portions of distilled water with shaking at 4°C. The theoretical dilution resulting from such treatment is 64,000:1. The samples were then lyophilized and stored.

Other 7.5 ml. aliquots of the remaining dissolved plasma protein solution were then dialyzed in duplicate against one liter of 0.03 M Na_2SO_3 for eight hours at 4°C. with shaking. These solutions were then dialyzed against distilled water as above and lyophilized.

When plasma protein solutions are dialyzed against distilled water, the globulins may precipitate. For this reason, the dialysis tubes were extensively rinsed with distilled water after dialysis to insure complete removal of all the plasma proteins. In this way, two derivatives of the plasma proteins were prepared. The first contained both disulfide-bound and peptide bound half-cystine residues. The second contained peptide bound half-cystine residues only.

Fractionation of plasma proteins.--The samples of plasma proteins were fractionated in order to determine the activity of each fraction.

The lyophilized plasma protein dialyzed either against distilled water or Na_2SO_3 as described above, were dissolved in 0.1 M Na_2CO_3 to give a 2.8 percent solution (W:V). These solutions were fractionated with Na_2SO_4.

An equal volume of Na_2SO_4 (saturated at 25°C.) was added to the 2.0 percent solution of plasma proteins. The resulting mixture was allowed to stand for twenty minutes at room temperature. The solution was then centrifuged at 3000 r.p.m. for thirty minutes. The supernatants solution was removed and labeled Supernatant 1. The precipitates were redissolved in the original volume of 0.1 M Na_2CO_3 and reprecipitated with the same volume of saturated Na_2SO_4 used above. The precipitate was labeled Fraction 1. The reprecipitation was carried out to remove proteins which were physically entrapped during the precipitation process.

Supernatant 1 was combined with an equal volume of saturated Na_2SO_4 and treated in the above manner. The precipitate obtained was redissolved, reprecipitated, and labelled Fraction 2. Supernatant 2 (obtained after removal of Fraction 1) was then combined with an equal volume of saturated Na_2SO_4 and again treated as described above. The precipitate reprecipitated, and labeled Fraction 3. The supernatant obtained was labeled Fraction 4.

Fractions 1-3 were dissolved in 0.1 M $NaHCO_3$ buffer, pH 8.6. Fraction 4 was desalted by passing through a Bio-Gel (P-10 mesh 150) column. The pH of Fraction 4 was adjusted to 8.6 using 0.5 M Na_2CO_3. An electrophoretic separation was run on each plasma fraction using the Beckman microzone apparatus and following the procedure described in the instructions for that apparatus (41). The Beckman densitometer and integrator for use with the microzone apparatus was used to scan the electrophoretograms obtained. The fractions considered were the albumins, α, β, and γ-globulins. Figure 1 shows the electrophoretic pattern obtained from a typical plasma sample. From such patterns, the relative proportions of the proteins present in each fraction may be determined.

Measurement of Tyrosine in the Plasma Protein Fractions

The protein content of the fractions obtained by Na_2SO_4 fractionation was measured in terms of tyrosine content. A modification of the Folin-Ciocalteau method was used. In this procedure, 5.0 ml of 0.5 M Na_2CO_3 were added to 1.0 ml. of the protein solution to be studied. After mixing, 1.0 ml. of dilute Folin-Ciocalteau reagent was added. After mixing, it was allowed to stand at room temperature for thirty minutes. The color was found stable for more than

Fig. 1. Densitometric patterns of electrophoretograms of rat plasma proteins. The samples were prepared as described on page 16. Electrophoresis was carried out on cell acetate for twenty minutes at pH 8.6. Pattern A represents whole plasma; B = Fraction I; C = Fraction II, D = Fraction III, E = Fraction IV. Peak 1 represents albumin; peak 2 = α-globulins, peak 3 = β-globulins, peak 4 = γ-globulins.

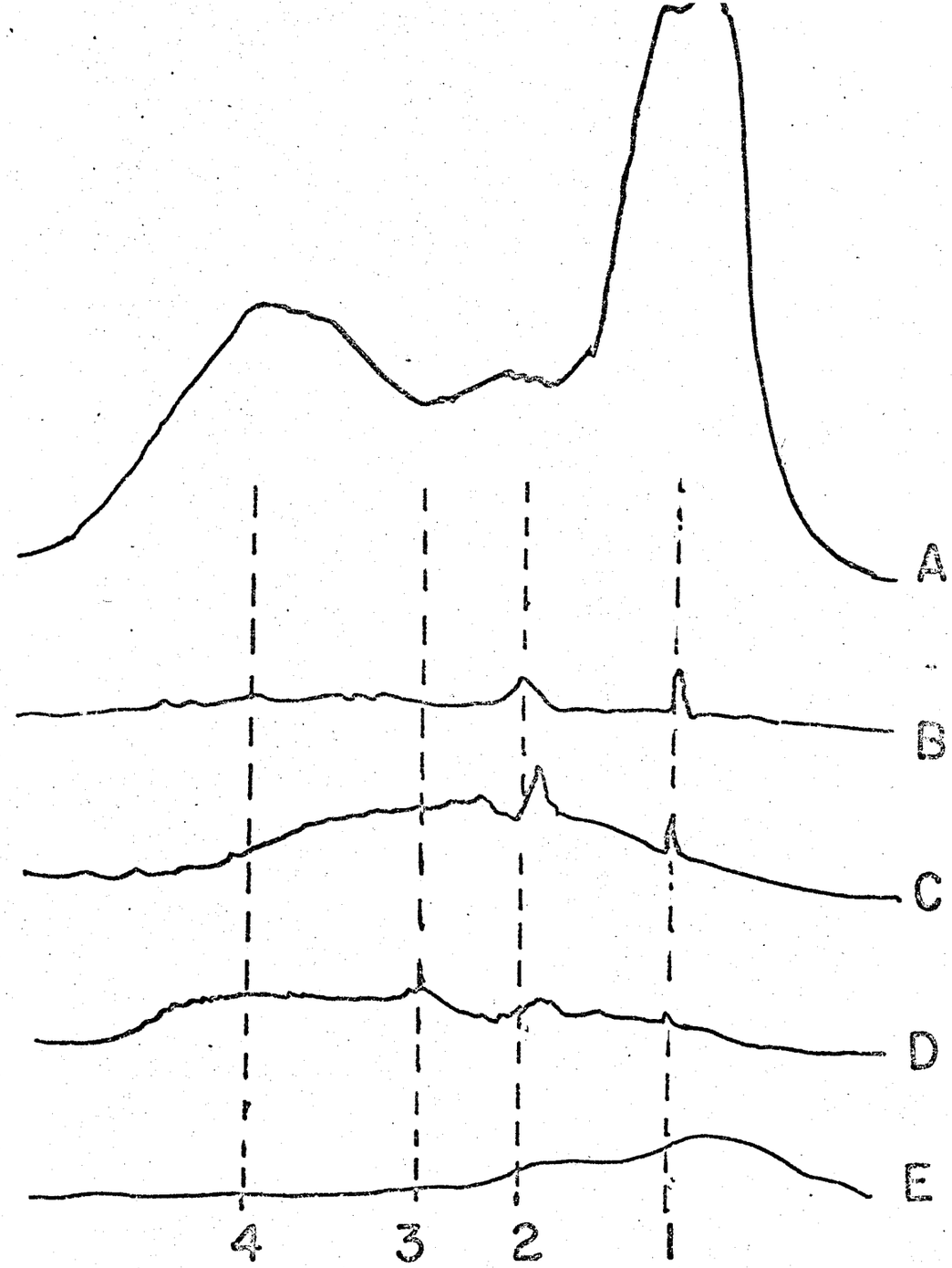


FIG. 1

three hours. The optical density was measured at 540 mu.

A stock solution of tyrosine was prepared and diluted to various concentrations. Using the above procedure, a standard curve was constructed for tyrosine determination. The standard curve was a plot of optical density recorded against tyrosine concentration and is given in Figure 2.

To determine the validity of the above technique and the range of optical densities over which the procedure remained sensitive, the following recovery procedure was performed. To a known amount of bovine serum albumin, various known concentrations of bovine serum albumin were added, total tyrosine determined, and the percent recovery calculated. The results of this experiment are given in Table I. From these results, it appears that (1) the method is valid for determining protein tyrosine and (2) the validity holds over a range of 0.020 to 0.068 mg. protein.

Measurement of ^{35}S Activity

A liquid scintillation counter was employed to determine ^{35}S activity. The model counter used was the Beckman, 154. The fluor used was prepared by dissolving four grams of 2,5 biphenyl oxazole, 100 grams of naphthalene, and 25 grams of Cab-o-sil, in one liter of dioxane. All materials used were of scintillation grade. Since the plasma proteins

Fig. 2. Standard curve for the determination of tyrosine in proteins measured in terms of concentration of tyrosine (mg./ml.) against absorbence at 540 mu.

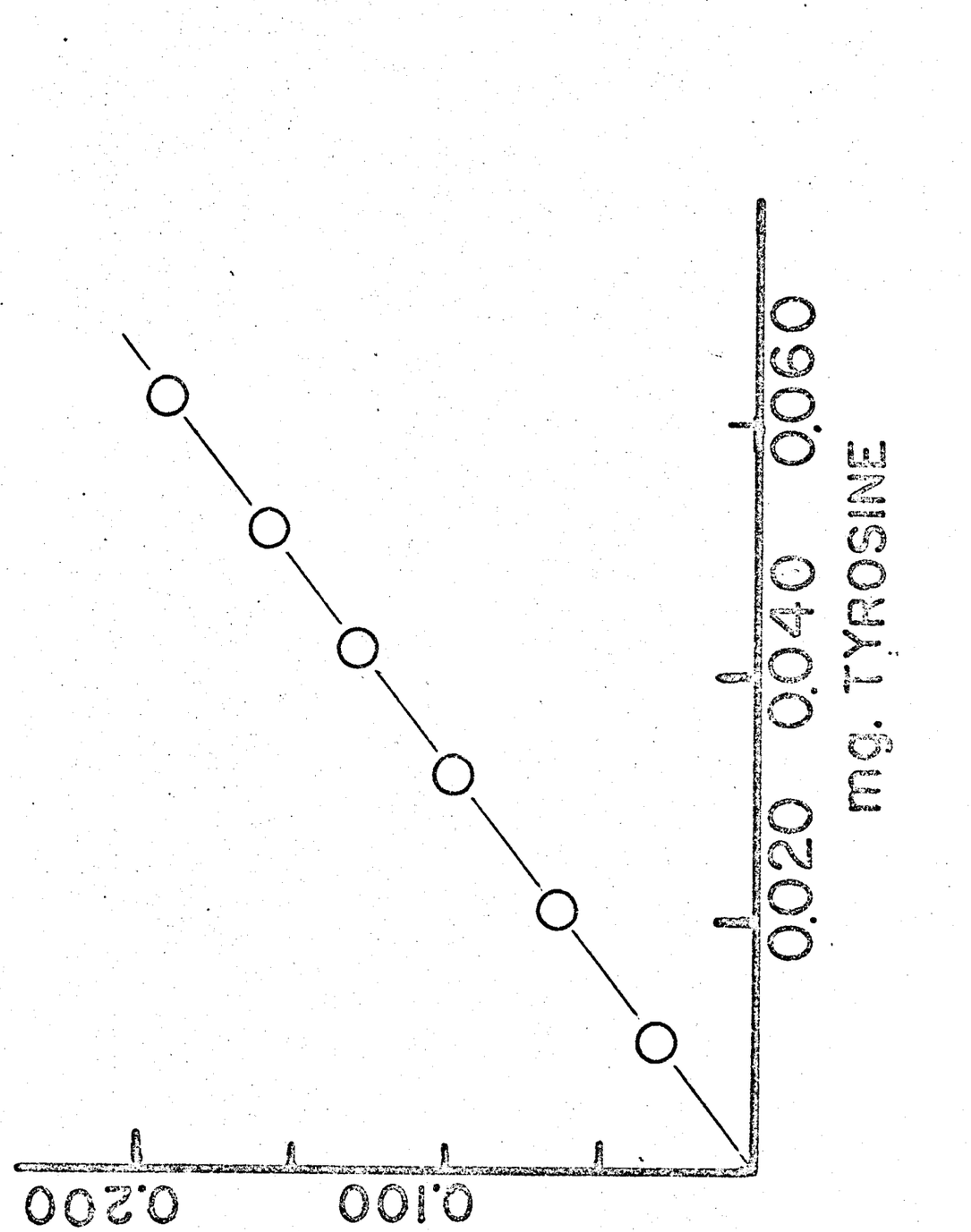


FIG. 2

TABLE I

RECOVERY OF SERUM ALBUMIN*

Sample Number	Serum Albumin mg.	Serum Albumin Added mg.	Total Serum Albumin mg.	Percent Recovery
1	0.400	0.000	0.380	95.0%
2	0.400	0.200	0.600	100.0%
3	0.400	0.400	0.780	97.5%
4	0.400	0.600	0.096	96.0%
5	0.400	0.800	1.180	98.4%
6	0.400	1.000	1.360	97.3%
7	0.400	1.200	1.460	91.2%
8	0.400	1.600	1.500	75.0%
9	0.400	1.800	1.520	69.0%
10	0.400	2.000	1.600	66.3%

*Measured in terms of protein tyrosine.

were dissolved in Na_2CO_3, the salt would precipitate when added to the dioxane-based fluor. Cab-o-sil is a gelling agent which suspends insoluble materials in a homogeneous gel for scintillation counting.

One ml. of each plasma sample was pipetted in duplicate into scintillation vials containing 10.0 ml. of fluor. The samples were allowed to equilibrate in the dark for thirty minutes prior to counting. Each sample was counted for ten minutes.

Data Analysis

From the data on the activity of the four fractions obtained from each plasma protein sample and the relative proportions of the four principle plasma proteins present in each, four simultaneous equations with four unknowns may be written. The unknowns are the activity of albumin, α -, β -, and γ -globulin in terms of counts/min/mg. protein tyrosine.

Since these equations were derived from experimental data, each had an intrinsic experimental error. Consequently, no exact solution could be found. Thus, the aid of a computer was enlisted to determine a statistical solution to the equations. A statistical solution is the solution which best fits the conditions described by the equations. In this

case, all statistical solutions obtained approximated the exact solutions with an error no greater than 3.6 percent. The program used was that developed by the Controls Data Corporation and was provided by Robert A. Pekar of the University of Illinois at the Chicago Circle Campus.

CHAPTER III

RESULTS AND DISCUSSION

As mentioned previously, dialysis of plasma proteins against sulfite ion provides a method for distinguishing peptide bound ^{35}S from disulfide bound ^{35}S. In this experiment, a plot of the residual activity of the plasma proteins after dialysis against sulfite ion reflects the rate of protein synthesis. Figures 3-6 compare the rate of synthesis of the four principle plasma protein fractions for wounded and unwounded animals. From the data in Figure 3, it appears that the rate of albumin synthesis is markedly depressed in wounded animals as compared with that of unwounded animals. However, the rate of synthesis of the three major plasma globulin fractions seems to be faster in the wounded than in normal rats. In particular, after two hours, the ^{35}S activity of the α-globulin fraction of the wounded animals is more than twice that of the unwounded animals. A summary of the data is given in Table II.

These results seem consistent with previous studies concerning the effect of wounding on the level of plasma protein fractions. In those studies (39), it was similarly shown that wounding lowers serum albumin levels with a concommitant increase in α-globulin fraction (30).

The rate of incorporation of labeled cysteine into peptide bonds may be taken as a measure of the rate of protein synthesis. Aspartate and glutamate appear to have little, if any, effect on the four plasma functions.

It was found that very little ^{35}S is bound into serum albumin through the disulfide linkage. Furthermore, the variation in ^{35}S activity was as great as the amount bound. Consequently, no consistent or reliable patterns of finding could be established.

The limited binding of ^{35}S to serum albumin through the disulfide bond may be attributed to the fact that serum albumin contains only one sulfhydryl group per molecule. With only one available site per molecule for the disulfide attachment of ^{35}S, it is impossible to distinguish any effect exerted by glutamate on disulfide

half-cystine incorporation. Even data from *in vitro* studies measuring the effect of glutamate on disulfide ^{35}S incorporation into serum albumin is difficult to interpret because of this sparcity of sulfhydryl groups.

The globulin fractions however have ample sulfhydryl groups and lend themselves to a more complete analysis. The rate of disulfide incorporation of ^{35}S into each of the three principal globulin fractions of unwounded animals is shown in Figures 7-9. For each of these three globulin fractions, the ^{35}S activity reaches at a maximum one hour after administration of the tracer. Two hours after the tracer dose was given, the disulfide bound ^{35}S activity was essentially gone in all three globulins. The activity falls to zero because the disulfide bound labeled half-cystine residues are apparently being replaced by unlabelled half-cystine residues. Thus it appears that in unwounded animals, the half-life of disulfide bound half-cystine is about thirty minutes in the globulin fractions.

The disulfide incorporation of ^{35}S into the globulin fractions of wounded and wounded animals treated with aspartate appear to be similar. In both cases, disulfide bound activity reaches a maximum thirty minutes after administration of the tracer. Two hours after the tracer

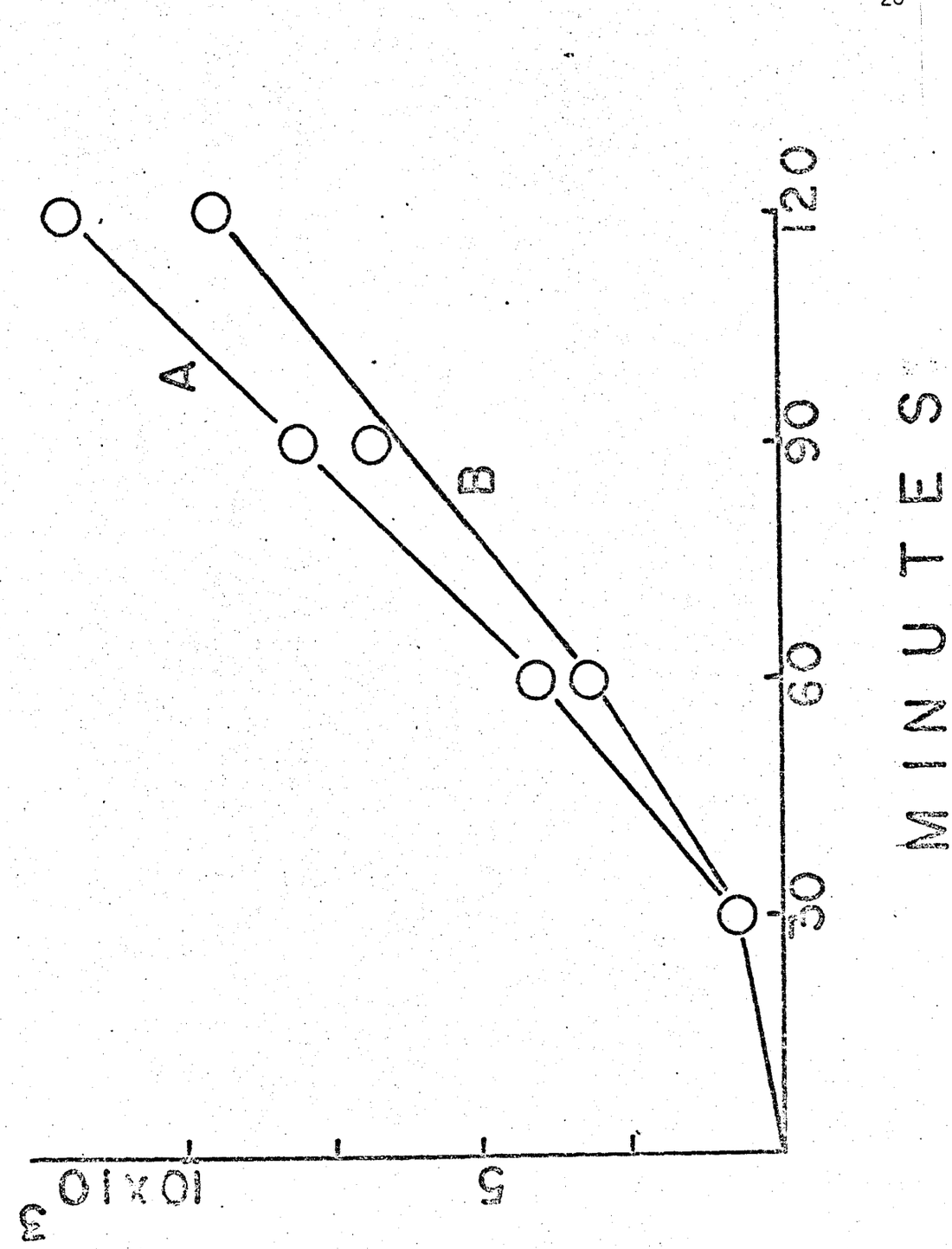


Fig. 3 The rate of incorporation of peptide-bound ^{35}S into the albumin fraction of the plasma proteins. Data is presented in terms of specific activity (cpm/mg. Tyr.) versus minutes after the injection of ^{35}S-L-cystine. A represents wounded animals. B = unwounded.

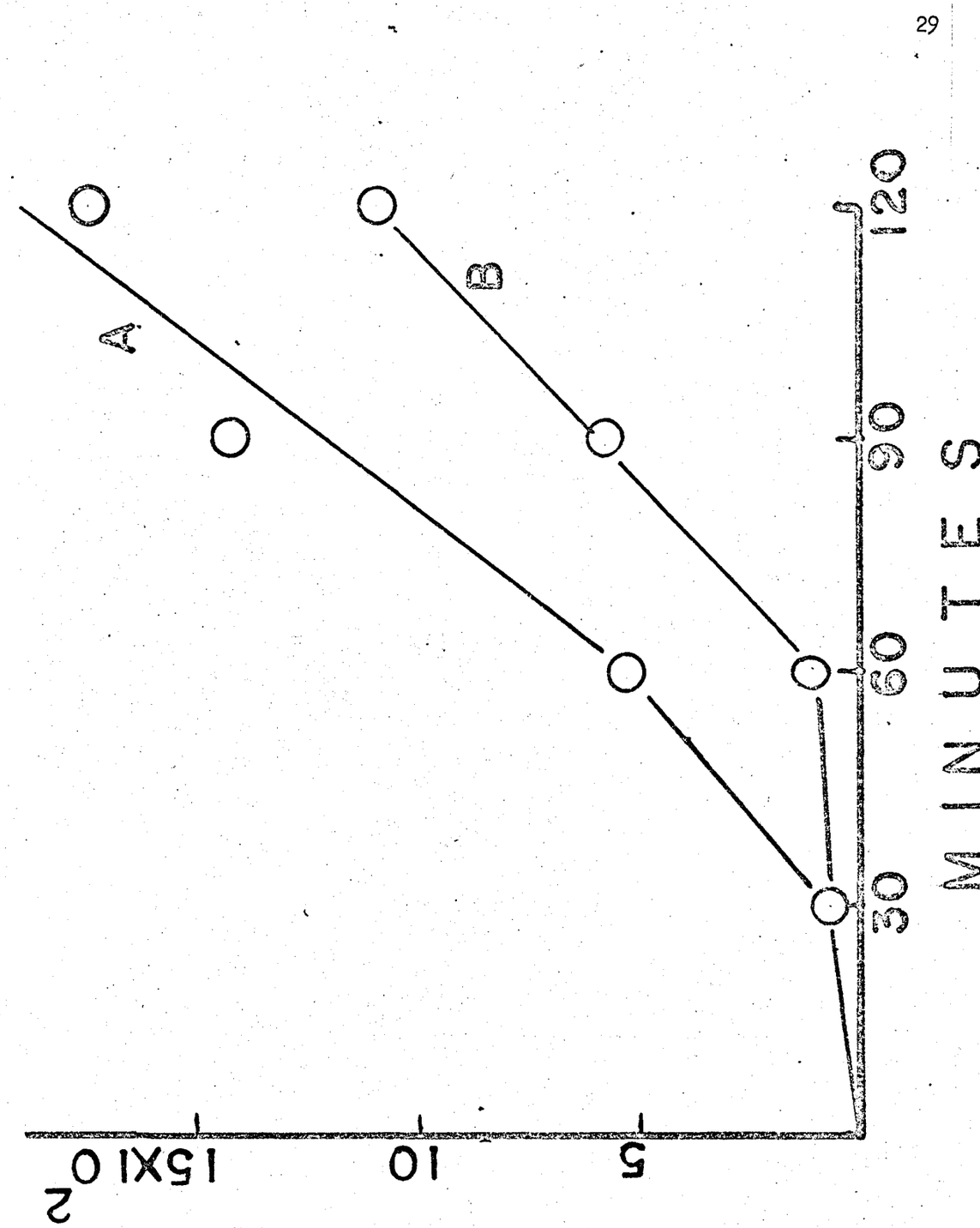


Fig. 4 The rate of incorporation of peptide-bound ^{35}S into the α-globulin fraction of the plasma proteins. Data is presented in terms of specific activity (cpm/mg. Tyr.) versus minutes after the injection of ^{35}S-L-cystine. A represents wounded animals. B = unwounded.

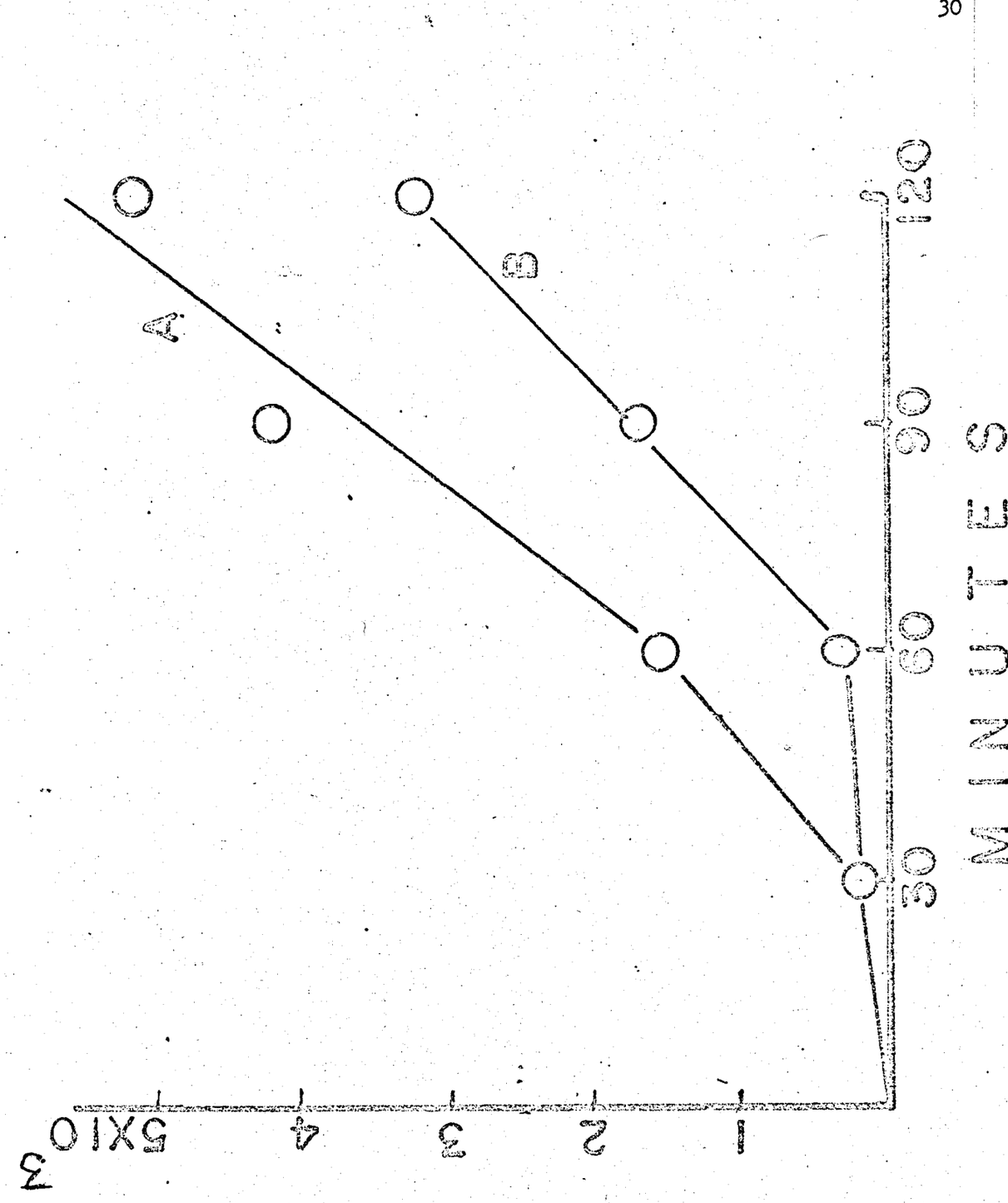


Fig. 5 The rate of incorporation of peptide-bound ^{35}S into the β-globulin fraction of the plasma proteins. Data is presented in terms of specific activity (cpm/mg. Tyr.) versus minutes after the injection of ^{35}S-L-cystine. A represents wounded animals. B = unwounded.

Fig. 6 The rate of incorporation of peptide-bound ^{35}S into the γ -globulin fraction of the plasma proteins. Data is presented in terms of specific activity (cpm/mg. Tyr.) versus minutes after the injection of ^{35}S-L-cystine. A represents wounded animals. B = unwounded.

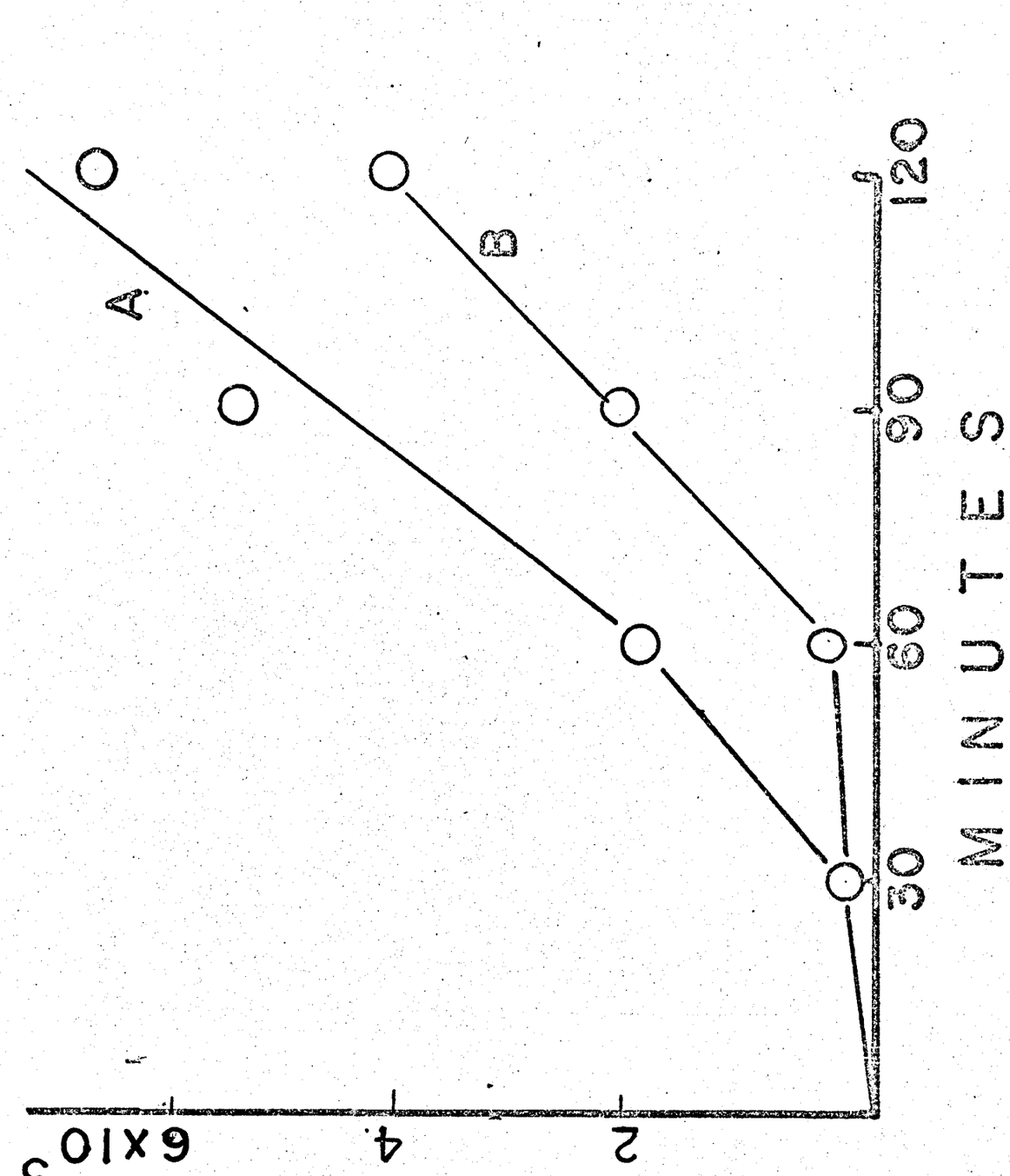


FIG 6

TABLE II

EFFECT OF WOUNDING ON THE INCORPORATION OF PEPTIDE-BOUND ^{35}S CYSTINE INTO PLASMA PROTEINS*

Fraction	Unwounded Rats counts/min/mg. Tyr	Wounded Rats counts/min/mg. Tyr
Albumin	12,000	9,400
α-globulin	8,000	18,000
β-globulin	2,000	3,800
γ-globulin	4,200	6,700

*Data obtained 120 minutes after administration of 197 Curies of ^{35}S-L-cystine.

was given, disulfide bound ^{35}S activity essentially disappears. It appears that the turnover of disulfide bound half-cystine is greater in the wounded than in the unwounded.

The disulfide incorporation of ^{35}S into the globulin fractions of wounded animals treated with glutamate is characterized by a much slower rate of incorporation than that observed with the wounded animals discussed above. The disulfide bound ^{35}S activity in these animals is further differentiated from previous cases in that the activity remains relatively high after two hours. In both wounded and unwounded animals, the disulfide bound ^{35}S activity was found to turn over completely. Glutamate apparently retards this turnover.

The binding and removal of disulfide bound labeled half-cystine may be represented by the following equations:

$$R\text{-}SH + Cys\text{-}{}^{35}S \longrightarrow {}^{35}S\text{-}Cys \rightleftharpoons R\text{-}S\text{-}{}^{35}S\text{-}Cys + H{}^{35}S\text{-}Cys \quad (5)$$

$$R\text{-}S\text{-}{}^{35}S\text{-}Cys + Cys\text{-}{}^{32}SH \rightleftharpoons R\text{-}S\text{-}{}^{32}S\text{-}Cys + Cys\text{-}{}^{35}SH \quad (5A)$$

By treating the animals in the various ways discussed above, changes were brought about in the rates of reactions. And all the observed phenomena previously discussed can be explained on the basis of change in reaction rates.

In general, two factors control reaction rates in an *in vivo* system. One is the concentration of reactants and the other is the presence or absence of a catalyst. The two reactants of concern here are the sulfhydryl groups of the plasma proteins and the labeled cystine. Since all data reported here is on the basis of activity per mg. protein tyrosine, variations in protein concentration cannot affect the reaction rate.

The probability that a labeled cystine molecule will react with a protein sulphydryl group is given by the ratio of the concentration labeled cystine to the concentration of total cystine present (specific activity).

$$\text{Prob.} = \frac{(^{35}\text{S Cystine})}{(^{35}\text{S Cystine} + ^{32}\text{S Cystine})} \qquad (6)$$

By lowering the concentration of ^{32}S-cystine, the probability of reaction between protein and ^{35}S cystine is increased. Wounding has been shown to affect plasma cystine levels in three ways. First, wounding increases the output of cystine by the liver (36). Secondly, wounding promotes the conversion of cysteine to taurine (38). Thirdly, the wound tissue itself accumulates cystine (37). Thus one tendency is to increase plasma cystine as shown by the liver while the

other two lower cystine levels. It is probable that the overall effect of wounding however is to lower the total plasma concentration of free cystine. Consequently the probability of reaction of protein with labeled cystine is increased. Therefore the wounded animals exhibit a faster turnover of labeled cystine than the unwounded animals.

Other factors are probably operating in the case of the wounded animals treated with glutamate. These factors account for the radical deviation in the disulfide incorporation of ^{35}S from that exhibited in wounded animals or the wounded animals treated with aspartate. This deviation can be accounted for by postulating the presence of a catalyst which helps form and break disulfide bonds. This catalyst may be enzymic in nature. As mentioned previously, enzymes catalyzing the exchange reaction have been found in various tissues. Glutamate somehow reduces the efficiency of this catalyst for the rate of disulfide half-cystine is markedly reduced and turnover is essentially inhibited. If the catalyst is an enzyme, glutamate may react directly with it in one of the classically described types of inhibition. The possibility also exists that glutamate reacts with a cofactor required for proper enzyme functioning, consequently impairing enzyme efficiency. Many enzymes have been shown

to require a divalent metal ion as a cofactor. It is possible that glutamate complexes some divalent metal ions thus making them less accessible to the enzyme. Whatever the case, it is clear that aspartate does not exert the same effect as exhibited by glutamate. This is in accord with the *in vitro* studies previously discussed.

Fig. 7 The rate of incorporation of disulfide-bound ^{35}S into the γ-globulin fraction of the plasma proteins. Data is presented in terms of specific activity (cpm/mg. Tyr.) versus minutes. Empty circles represent unwounded animals; split circles represent wounded animals given either aspartate or saline; darkened circles represent animals given glutamate.

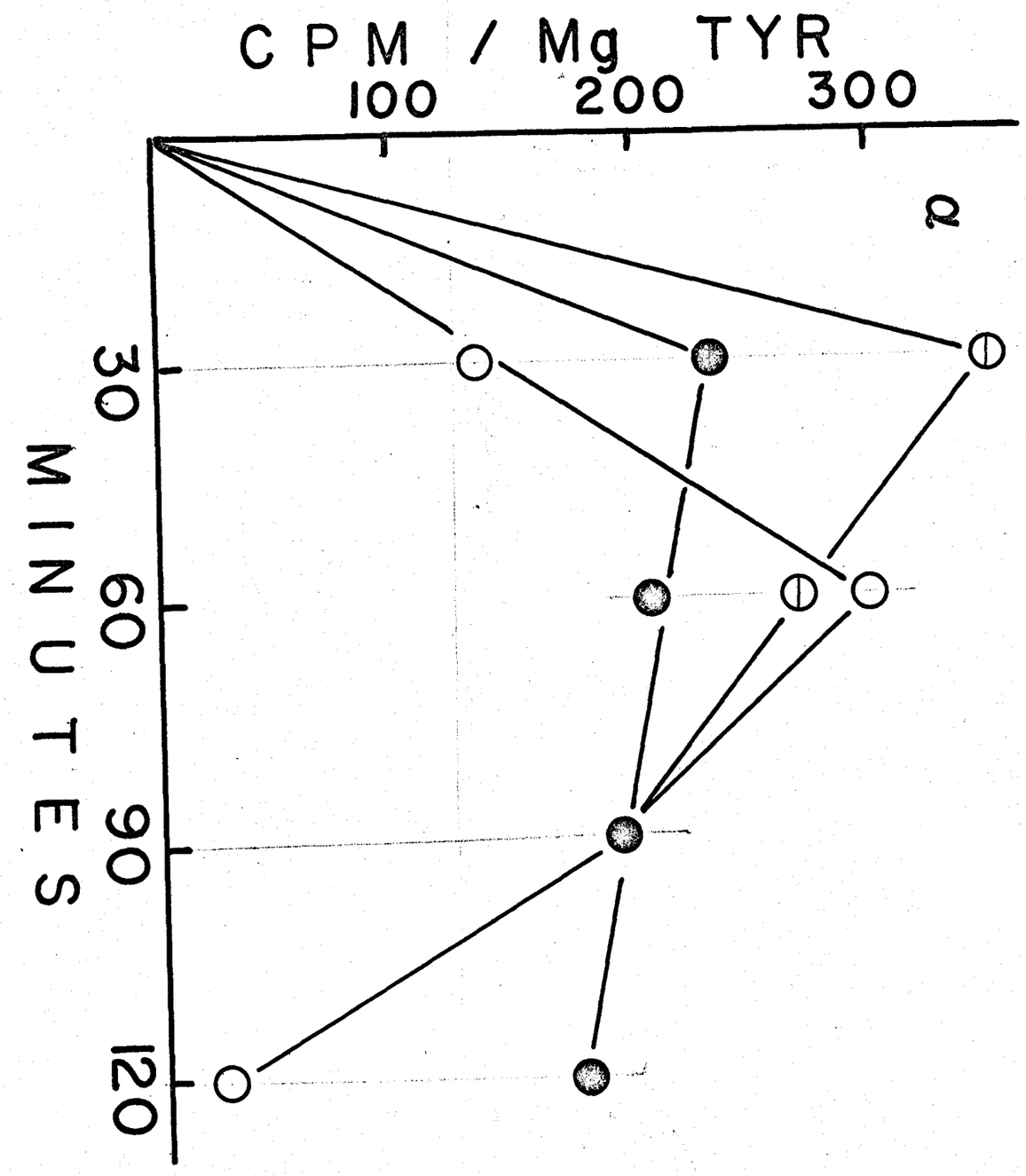


Fig. 8 The rate of incorporation of disulfide-bound ^{35}S into the β-globulin fraction of the plasma proteins. Data is presented in terms of specific activity (cpm/mg. Tyr.) versus minutes. Empty circles represent unwounded animals; split circles represent wounded animals given either aspartate or saline; darkened circles represent animals given glutamate.

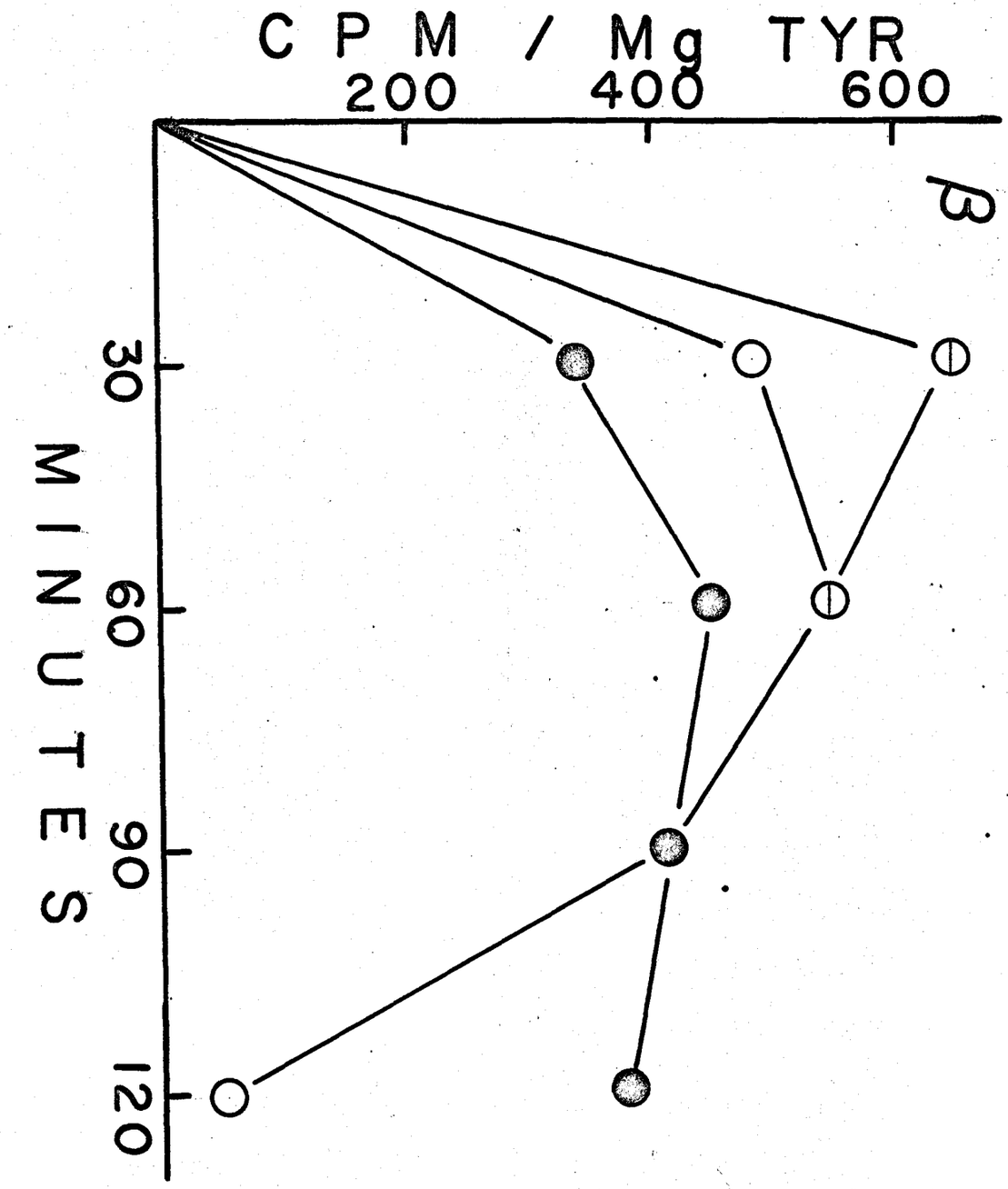
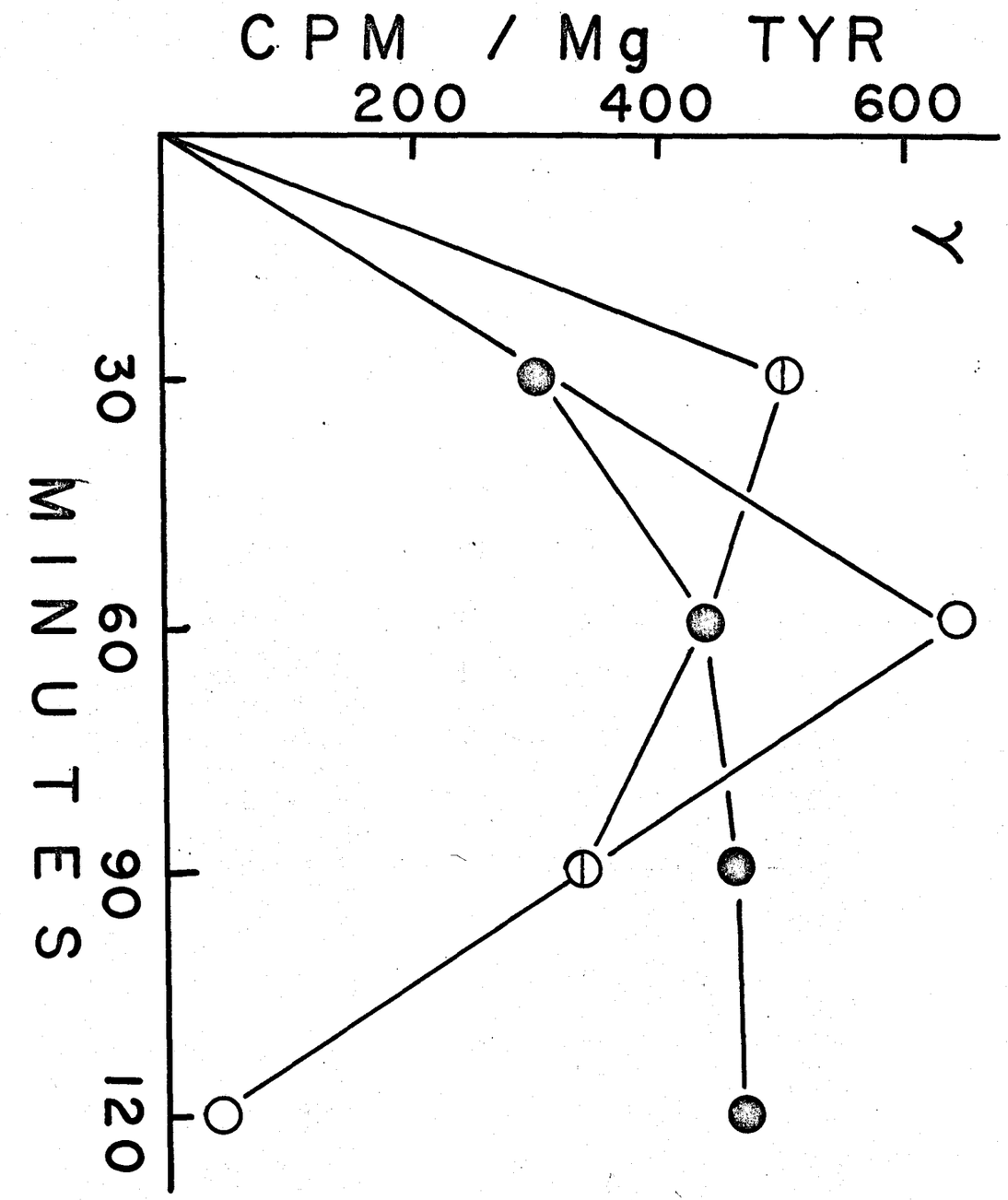


Fig. 9 The rate of incorporation of disulfide-bound ^{35}S into the γ -globulin fraction of the plasma proteins. Data is presented in terms of specific activity (cpm/mg. Tyr.) versus minutes. Empty circles represent unwounded animals; split circles represent wounded animals given either aspartate or saline; darkened circles represent animals given glutamate.



CHAPTER IV

SUMMARY AND CONCLUSIONS

1) Wounding has the effect of decreasing the rate of synthesis of serum albumin. It also has the effect of increasing the rate of synthesis of all three globulin fractions. The rate of synthesis of the α-globulins was found to be twice as great in wounded animals when compared with unwounded animals. These results are consistent with previous by published data on plasma protein levels during wound tissue formation.

2) In the wounded animals which received the saline solution, the rate of incorporation of half-cystine into the disulfide linkages of all three globulin fractions has been found to be greater than that observed in unwounded animals. The same effect was observed in the rats given aspartate.

3) The half-life of disulfide-bound half-cystine in the plasma globulins of unwounded animals was estimated to be less than thirty minutes.

4) The rate of incorporation of disulfide-bound half-cystine into the globulins of wounded animals which received

glutamate is lower than that observed in wounded animals which were given either saline or asparate. The rate of removal of the labeled disulfide-bound-half-cystine from the plasma globulins also is slower. These findings are consistent with the hypothesis that some catalyst is responsible for the attachment and removal of disulfide-bound half-cystine to proteins. Glutamate may reduce the efficiency of the catalyst by any of the classical types of inhibition or by reaction with a required cofactor. A loss in catalytic efficiency may then result in the observed decline in the rates of binding and removal.

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APPENDIX I

The diet was purchased from Nutritional Biochemical Corporation, Cleveland, Ohio, and had the following composition:

Corn Starch	70%
Alphacel (cellulose)	15%
Vegetable Oil	10%
Salt Mixture U.S.P. XIV	4%
Cod Liver Oil	1%

The Salt Mixture U.S.P. XIV contained the following ingredients:

Cupric Sulfate	0.48 gm.
Ferric Ammouium Citrate	94.33 gm.
Manganese Sulfate	1.24 gm.
Ammonium Alum	0.57 gm
	1.62%
Potassium Iodide	1.25 gm.
Sodium Fluoride	3.13 gm.
Calcium Carbonate	6.86%
Calcium Citrate	30.83%

Calcium Biphosphate	11.28%
Magnesium Carbonate	3.52%
Magnesium Sulfate	3.83%
Potassium Chloride	12.47%
Dibasic Potassium Phosphate	21.88%
Sodium Chloride	7.71%

The diet also contained the fat soluble Vitamins A and D and it was supplemented with E Complex Vitamins.

APPROVAL SHEET

The thesis submitted by Robert Orman has been read and approved by the director of the thesis.

Furthermore, the final copies have been examined by the director and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

6/13/69
Date

M B Williamson
Signature of Advisor