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The Effect of Tetrathionate on the Stability and Immunological Properties of Muscle Triosephosphate Dehydrogenases*

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Abstract

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When three sulfhydryl groups of rabbit, turkey, sturgeon, and lobster triosephosphate dehydrogenases (TPD's), are modified by tetrathionate, the enzymes are completely inhibited. Depending on the temperature and length of incubation with tetrathionate, this inhibition can be reversed by the addition of thiols. The muscle TPD's become irreversibly inactivated in the presence of tetrathionate at 37°. The rate of this irreversible inactivation varies with the different muscle TPD's. The sturgeon TPD is significantly more stable than the turkey and lobster TPD's at 37° in the presence of tetrathionate. Losses in immunological activity with specific rabbit antisera prepared against the active forms of the enzymes are observed when the turkey, sturgeon, and lobster TPD's are reversibly and irreversibly inactivated with tetrathionate. The immunological properties of the sturgeon enzyme are only slightly altered when it is inactivated with tetrathionate, while the immunological properties of the tetrathionate inactivated turkey and lobster TPD's differ significantly from the native enzymes. The sulfhydryl groups in the TPD's show a differential reactivity with p-hydroxymercurobenzoate (PCMB) in the presence and absence of 8.0 M urea. Only 2.5 out of 10 sulfhydryl groups in the sturgeon enzyme react with PCMB in the absence of urea; the sulfhydryl groups of this enzyme react sluggishly with PCMB in the presence of 8.0 M urea. The "buried" sulfhydryl groups of the lobster and turkey TPD's are much more accessible to PCMB. All of the TPD's studied appear to have no disulfide linkages, since all of the non-methionine sulfur exists as sulfhydryl groups. It is concluded that the active conformations of the different TPD's are partly stabilized by the three active sulfhydryl groups which react with tetrathionate, but the degree of stabilization varies with the different enzymes.

Author

Introduction

It is well established that the enzymatic activity of yeast and rabbit muscle triosephosphate dehydrogenases (TPD) depends on the state of the active sulfhydryl groups in the enzymes (Cori et al., 1948; Krebs, 1955). The crystalline rabbit muscle enzyme prepared in the absence of reducing thiols or heavy metal chelating agents such as EDTA becomes reversibly inactivated, but it may be reactivated by incubation with thiols such as cysteine or mercaptoethanol (Velick, 1955). When the crystalline rabbit muscle enzyme is prepared in the presence of EDTA, it does not require activation with thiols and its thermal and pH stability are increased (Velick and Furfine, 1963). The work of Krimsky and Racker (1955), Velick and Hayes (1953) and Segal and Boyer (1953) has shown that the oxidative phosphorylation of D-glyceraldehyde-3-phosphate proceeds in two steps, the first of which is the oxidation of the substrate by bound DPN to form an S-acyl enzyme intermediate. The second step is the phosphorolysis of the S-acyl enzyme to form 1,3-diphosphoglyceric acid. Evidence indicates that there are at least three active -SH groups per 120,000 g of the rabbit muscle enzyme that are involved in the catalytic process (Velick, 1953; Perham and Harris, 1963). This evidence has been obtained by the use of -SH reagents such as p-hydroxymercuribenzoate (PCMB) (Velick, 1953), iodoacetate (Krimsky and Racker, 1952), and tetrathionate (Pihl and Lange, 1962).

Crystalline turkey, sturgeon, and lobster TPD's are quite similar to the rabbit muscle enzyme in their sensitivity to -SH reagents (Allison and Kaplan, 1964). Like the rabbit muscle enzyme, they are completely inhibited by three moles of tetrathionate per 120,000 g of enzyme at 0°, and this inhibition can be completely reversed by

the addition of thiols under proper conditions. However, changes in the heat stability, susceptibility to proteolytic digestion, and immunological properties accompany inactivation of these dehydrogenases with tetrathionate. These changes are presented and discussed in this publication.

Experimental Procedure

Materials.--The enzymes used in this study were prepared by the methods described elsewhere (Allison and Kaplan, 1964). Bound DPN was removed from the lobster TPD with acid activated Norite A which had been water washed to pH 4.5 and dried before use. 0.2-0.5% solutions of lobster TPD in 0.1 M sodium phosphate, pH 7.0, containing 10^{-3} M EDTA were stirred with the treated Norite A (100 mg/ml of enzyme solution) at 0° for 30 seconds. The Norite A was then removed by suction filtration through a sintered glass funnel previously chilled to 5°. At least 85% of the bound DPN was removed from the enzyme by this procedure as estimated by the increase in extinction at 340 mμ when substrate and arsenate were added to solutions of the enzyme under conditions which reduce bound DPN to DPNH.

DPN and PCMB were purchased from the Sigma Chemical Company. The dihydrate of sodium tetrathionate was prepared from thiosulfate by oxidation with iodine. Solutions of tetrathionate were prepared by weight and were stored at 5° for no longer than 3 weeks. The lithium salt of acetyl phosphate was prepared as described by Stadtman (1957). Solutions of acetyl phosphate were assayed with hydroxylamine by the procedure of Lipmann and Tuttle (1945).

Methods.--Immunological Techniques.--The crystalline enzymes were dissolved in 10^{-3} M EDTA, pH 7.0, and dialyzed against the same

solution before injection into rabbits. During the first course of immunization 20 mg of antigen in 1.0 ml of 10^{-3} M EDTA, pH 7.0, mixed with an equal volume of complete Freund's adjuvant, was injected into the toepads and intramuscularly. Three weeks after the initial injection rabbits were boosted by the intravenous injection of 2 mg of antigen a day for 5 days. Seven days after the last intravenous injection, 40 ml were bled from the ear. The anti-lobster TPD used in this study was obtained after a single course of intravenous boosting, while the anti-turkey and anti-sturgeon TPD's were obtained after three courses of intravenous boosting administered at four-week intervals. All sera were tested for the presence of precipitating antibody by the gel diffusion method of Ouchterlony (1949). Quantitative micro-complement (C') fixation was performed as described by Wasserman and Levine (1961).

Trypsin Digestion.--Proteolytic digestion was carried out at 35° with twice recrystallized, salt-free trypsin purchased from Worthington Biochemical Corporation with a weight ratio of TPD to trypsin of 200:1. The rate of digestion was determined by measuring the increase in extinction at 280 mμ with time of samples of the digestion mixtures deproteinized with 5% trichloro-acetic acid.

Cysteine Determinations.--Cysteine was determined spectrophotometrically with PCMB in the presence of 8.0 M urea (Boyer, 1956). 0.10 ml of the enzymes (5-10 mμ moles) were added to 2.9 ml of 0.10 M sodium phosphate, pH 7.0, containing 8.0 M urea and 10^{-4} M PCMB. The increase in extinction at 250 mμ due to mercaptide formation was then measured with time in a Zeiss model PMQ II spectrophotometer. A correction factor for the effect of urea on mercaptide absorption at 250 mμ was determined with reduced glutathione.

This correction factor was applied to the extinction coefficient of 7.6×10^3 reported by Boyer (1954) for mercaptide formation in 0.10 M phosphate, pH 7.0. The amount of non-methionine sulfur in the enzymes was determined as cysteic acid following performic acid oxidation at room temperature by the procedure of Schram et al. (1954). Cysteic acid was determined by automatic amino acid analyses in a Spinco amino acid analyzer by the procedure of Moore et al. (1958).

Results

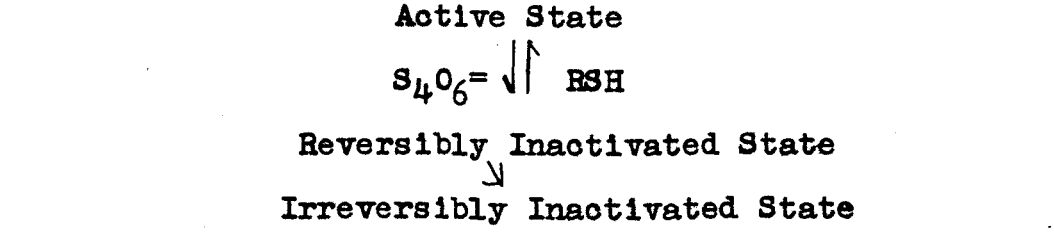
The Effect of Tetrathionate Inactivation on the Heat Stability of Muscle TPD's.--Pihl and Lange (1962) have shown that rabbit muscle TPD is inactivated by the addition of three moles of tetrathionate per mole of enzyme. The evidence of Pihl and Lange (1962) suggests that tetrathionate reacts specifically with three thiol groups in the enzyme that appear to be involved in thioester formation at the active site of TPD during the catalytic oxidation of D-glyceraldehyde-3-phosphate. Similarly, turkey, sturgeon and lobster TPD's are completely inactivated by tetrathionate within 2 minutes at 0° when three moles of inhibitor are added per mole of enzyme. As was observed with the rabbit muscle enzyme, this inhibition can be completely reversed by reducing thiols such as cysteine and mercaptoethanol if the inhibited enzymes are incubated at low temperatures and the activating thiol is added within an hour after the introduction of the inhibitor. After prolonged incubation at 0°, the tetrathionate-inactivated enzymes can be only partly reactivated with thiols, as shown in Table I for the lobster muscle TPD. The rate of irreversible inactivation of the enzymes by tetrathionate is much faster at higher temperatures and varies for different muscle TPD's. Figures 1-3 show the effect

Table I

Figs 1-3

of tetrathionate inactivation on the stability of turkey, sturgeon, and lobster TPD's at 37°. Each of the tetrathionate inactivated enzymes is much less stable at 37° than it is in the native state, although there are some significant differences in the stability of the three TPD's. The tetrathionate inactivated lobster TPD is the most labile. It is irreversibly inactivated within 10 minutes at 37°. The treated turkey enzyme is somewhat more stable than the treated lobster enzyme; the sturgeon dehydrogenase is much less sensitive to irreversible inactivation following treatment with tetrathionate. Figures 1-3 also show that the heat stability of both the native and tetrathionate inactivated TPD's depends on the ionic composition of the buffer. The presence of 0.4 M ammonium sulfate prevents the spontaneous denaturation of the native TPD's and increases the stability of the tetrathionate inactivated TPD's at 37°. Preliminary experiments have indicated that the salt effect is due to the sulfate anion, since sodium sulfate produces the same effect as ammonium sulfate, whereas soidum chloride has little effect on the lability of the enzymes.

The decrease in stability of the tetrathionate inactivated enzymes over the native enzymes in the absence and presence of sulfate suggests that the chemical modification of the active -SH groups of TPD by tetrathionate, which eventually leads to irreversible inactivation of the enzyme, proceeds in two steps as shown in the following scheme:



The first step occurs rapidly even at 0°, while the second occurs slowly at low temperatures and at a much faster rate at moderate temperatures.

The Effect of Tetrathionate on the Antigenic Activity of Lobster Muscle TPD.--The strength of precipitin bands in two dimensional gel diffusion experiments between lobster TPD and a rabbit antiserum prepared against fully active TPD depends on the state of the catalytically active thiol groups of the enzyme. During gel diffusion experiments conducted at room temperature, precipitin bands between wells containing a solution of the enzyme, which had lost 70% of its original activity after standing for 3 days at 5° in 0.01 M sodium phosphate, pH 8.0, in the absence of EDTA or thiols, were barely visible; while strong precipitin bands between wells containing the same antiserum and solutions of the aged enzyme which had been partially reactivated with mercaptoethanol (85% of the original activity was recovered) were observed. A precipitin band between a well containing the tetrathionate inhibited enzyme and a well containing the antiserum was not observed even after 48 hours of incubation at room temperature, while a strong precipitin band appeared within 20 hours between a well containing the tetrathionate inhibited enzyme, which was reactivated with mercaptoethanol, and a well containing the antiserum. Drawings of these observations are shown in Figure 4a. When agar gel diffusion experiments were conducted at 5°, the rate of formation of precipitin bands was decreased when the enzyme was partially or completely inactivated as shown in Figures 4b and 4c. Precipitin bands between wells containing the tetrathionate inhibited enzyme or solutions of aged enzyme, which possessed 30% of maximal activity in the absence of thiols, and wells containing the anti-

Fig. 4

serum appeared after 16 hours. However, precipitin bands between the fully-reduced enzyme were observed within 7 hours under the same conditions as shown in Figure 4b. Losses in antigenic activity with anti-lobster TPD were observed when the lobster muscle enzyme was inactivated, as determined by quantitative C' fixation analysis. Complete inactivation of the lobster muscle enzyme by tetrathionate resulted in a loss in C' fixation activity, as shown in Figure 5. Compared to the C' fixation curve for the fully activated enzyme in the presence of cysteine, the C' fixation curve for the enzyme, which was completely inactivated with tetrathionate, is shifted toward higher antigen concentrations and has a lower peak height. The C' fixation curve for the "aged" enzyme, which in this case possessed 70% of maximal activity in the absence of thiols, is slightly shifted toward higher antigen concentrations and has a lower peak height when compared to the curve for the fully activated enzyme. When the lobster muscle enzyme was irreversibly inactivated by incubation with tetrathionate for 30 minutes at 30°, antigenic activity was completely
Fig. 5 lost, as shown by curve 5 in Figure 5. The addition of cysteine to the irreversibly inactivated enzyme led to partial restoration of antigenic activity. However, after this treatment with cysteine, no enzymatic activity was recovered.

The results of both the gel diffusion experiments at 5° and the C' fixation experiments indicate that the reversibly inactivated enzyme (enzyme activity is restored by the addition of thiols) is more antigenic than the irreversibly inactivated enzyme (enzyme activity is not restored by thiols). However, a slow transition of the reversibly inactivated state to the irreversibly inactivated state occurred when the lobster muscle TPD was incubated with tetrathionate

at 0° as shown in Table I. Therefore, the losses in antigenic activity observed in the C' fixation experiments and the gel diffusion experiments that were conducted at 5° may be directly related to the blocking of the active sulfhydryl groups, or they may be due to changes in antigenic determinants that accompany the irreversible inactivation of the enzyme that occurs slowly at 5°. To test between these two possibilities the antigenic activity of the fully active, the reversibly inactivated, and the irreversibly inactivated states of the enzyme was determined by measuring turbidity development due to formation of an insoluble antigen-antibody complex at 600 mμ with a Zeiss Model PMQ II spectrophotometer equipped with a cell holder jacketed at 7°. This method has the advantage of measuring the antigenic activity of the reversibly inactivated state of the enzyme immediately after inactivation with tetrathionate. Under these conditions all of the enzyme will be in the reversibly inactivated state as shown in Table I. Figure 6 shows that the rate of formation of turbidity was decreased when the lobster enzyme was reversibly inactivated with tetrathionate before incubation with the antiserum. When the enzyme was irreversibly inactivated by heating in the presence of tetrathionate, the rate of formation of turbidity was decreased even further. This experiment indicates that the reversible inactivation of the lobster muscle enzyme leads to changes in antigenic determinants on the enzyme. Furthermore, the rate at which turbidity developed at 7° indicates that the formation of a three dimensional complex between the anti-enzyme and the reversibly inactivated enzyme occurs long before an appreciable amount of the modified enzyme is irreversibly denatured in the C' fixation experiments.

Fig. 6

The Effect of Tetrathionate on the Antigenic Activity of Other Triosephosphate Dehydrogenases.--Inactivation of chicken and turkey TPD's with tetrathionate at 5° was accompanied by decreases in antigenic activity similar to those described for the lobster muscle enzyme. Addition of cysteine or mercaptoethanol to the enzymes that were inactivated with tetrathionate at 5° completely restored both antigenic and enzymatic activity. The changes in the C' fixation reaction, which accompany both the reversible and irreversible
Fig. 7 inactivation of the turkey enzyme, are shown in Figure 7. Inactivation of the enzyme leads to a complete loss in the C' fixation reaction at an antibody dilution of 1/8000, whether the inactivation was carried out under reversible or irreversible conditions. When mercaptoethanol was added to the irreversibly inactivated turkey TPD, no antigenic activity was recovered at an antiserum dilution of 1/8000.

When sturgeon muscle TPD was inactivated with tetrathionate, the reaction between the modified enzyme and an antiserum prepared against the active form of the sturgeon enzyme was only slightly
Fig 8 altered as shown in Figure 8. The C' fixation curve for the reversibly inactivated enzyme was slightly shifted toward higher antigen concentrations and has nearly the same peak height when compared to the C' fixation curve for the active enzyme. Moreover, irreversible inactivation of the sturgeon enzyme failed to produce further changes in the C' fixation reaction. The differences between the changes in the antigenic activity, which accompany tetrathionate inactivation of the lobster and turkey TPD's, may be due to a structural difference in the sturgeon enzyme or to a difference in the specificity of the antibody prepared against the sturgeon enzyme. To test

between these possibilities advantage was taken of the ability of the sturgeon enzyme to cross react with the anti-turkey TPD. The Fig. 9 C' fixation curve in figure 9 between the tetrathionate inactivated sturgeon TPD and the anti-turkey enzyme is only slightly shifted toward higher antigen concentrations when compared to the curve for the reaction between active sturgeon enzyme and the anti-turkey serum.

The Effect of Bound DPN on the Antigenic Activity of Muscle Triosephosphate Dehydrogenases.--Like the rabbit muscle enzyme (Cori et al., 1948) lobster muscle TPD crystallized with approximately 2 moles of firmly bound DPN which cannot be removed by dialysis. On the other hand, turkey muscle TPD crystallizes with less than 0.2 moles of bound DPN per mole of enzyme and the sturgeon muscle enzyme crystallizes free of coenzyme (Allison and Kaplan, 1964). Solutions of the active lobster muscle TPD-DPN complex have the same broad absorption band between 320 and 400 mμ, as has been observed for the active rabbit muscle TPD-DPN complex. This absorption band has been attributed to an interaction between the coenzyme and the active -SH groups of the enzyme, since it is destroyed by -SH reagents such as iodoacetate (Krimsky and Racker, 1952), PCMB (Velick, 1953) and tetrathionate (Pihl and Lange, 1962). When exogenous DPN was added to solutions of the turkey and sturgeon muscle TPD's, this absorption band appeared.

When the bound DPN was removed from the lobster muscle enzyme with charcoal under conditions described in Experimental Procedure, both enzymatic and antigenic activity were retained. Moreover, the addition of exogenous DPN to solutions of the charcoal-treated apoenzyme did not increase either enzymatic or C' fixation acti-

Fig. 10 vity, as shown in Figure 10. Reversible inactivation of the apoenzyme with tetrathionate at 5° resulted in losses in C' fixation activity similar to those observed when the native enzyme was reversibly inactivated. Figure 10 also shows that the addition of mercaptoethanol to the reversibly inactivated apoenzyme led to complete restoration of C' fixation activity which was accompanied by complete restoration of enzymatic activity. The addition of DPN to the tetrathionate inactivated apoenzyme did not increase either C' fixation activity (Figure 10) nor did it restore enzymatic activity. These observations indicate that the native lobster enzyme (containing bound DPN) and the lobster apoenzyme are immunologically indistinguishable. Similarly, when DPN was added to solutions of turkey and sturgeon muscle TPD's, no changes in the C' fixation activity between the enzymes and their specific anti-enzymes were observed.

The Effect of Acetyl Phosphate on the Antigenic Activity of Lobster Muscle Triosephosphate Dehydrogenase.--Krimsky and Racker (1955) have shown that the catalytically active -SH groups of rabbit muscle TPD are acetylated when the charcoal-treated apoenzyme is treated with excess acetyl phosphate in the cold and that the addition of DPN and arsenate to the resulting acetyl enzyme leads to its arsenolysis. When a 1,000 molar excess of acetyl phosphate was added to the lobster muscle apoenzyme at 5°, approximately 90% of antigenic activity was lost, as estimated by the lateral displace-

Fig. 11 ment of the C' fixation curve shown in Figure 11. When the apoenzyme was incubated with a 100 molar excess of acetyl phosphate at 5°, the loss of C' fixation activity was less marked, as shown in Figure 11. At both acetyl phosphate concentrations, incubation of the acetyl phosphate treated apoenzyme with DPN and arsenate led to some

recovery in antigenic activity, as shown in Figure 11.

The arsenolysis of acetyl phosphate by lobster muscle TPD has been studied at 37°. Table II shows that tetrathionate completely inhibits the arsenolysis reaction. The addition of DPN to the native enzyme enhances the arsenolysis reaction, while it has no effect on the tetrathionate inhibited enzyme. The arsenolysis reaction of the native enzyme was also enhanced by the addition of cysteine. These data suggest that the addition of acetyl phosphate to lobster muscle TPD leads to the formation of an acetyl-enzyme intermediate. However, since nothing is known about the kinetics and equilibria involved during acetyl-enzyme formation and arsenolysis at 5° (the average temperature at which the previously described C' fixation experiments were conducted), a more quantitative study must be completed in which the extent of acetyl-enzyme formation can be related to the observed losses in antigenic activity.

Table II

The Effect of Irreversible Tetrathionate Inactivation on the Proteolytic Digestion of Muscle TPD's.--Irreversible inactivation of lobster muscle TPD with tetrathionate leads to an increased susceptibility of the enzyme to proteolysis by trypsin. When the lobster muscle enzyme was irreversibly inactivated with tetrathionate and then incubated with trypsin at 37°, the rate of proteolysis, compared to that observed for the native enzyme, was increased as shown in Table III. The rate of proteolysis of the native enzyme was decreased in the presence of excess DPN, as was observed by Racker and Krimsky (1958) for the rabbit muscle enzyme and by Elödi and Szabolcsi (1959) for the swine muscle enzyme. Table III also shows that DPN protects the tetrathionate inactivated enzyme against

Table III

tryptic digestion. This indicates that the DPN protection is unrelated to the state of the active -SH groups of the enzyme. Further evidence of the protection of TPD from tryptic digestion by DPN is also shown in Table III. The pyridine-3-aldehyde analogue of DPN is a strong competitive inhibitor of lobster muscle TPD which, when bound to the enzyme, abolishes the 320-400 mμ absorption band associated with the active TPD-DPN complex (Kaplan et al., 1957; Allison and Kaplan, 1964). However, when excess pyridine-3-aldehyde DPN was added to lobster muscle TPD, the rate of trypsin digestion was decreased. Furthermore, TPN, which is completely inactive as coenzyme for the TPD, partly inhibits the rate of proteolysis of the lobster muscle enzyme. When bound DPN was removed from lobster TPD with charcoal, the enzyme became more susceptible to tryptic digestion, as observed by Racker and Krimsky (1958) and by Elödi and Szabolcsi (1959) for mammalian TPD's. Addition of DPN to the apoenzyme decreased the rate of digestion, while inactivation of the apoenzyme by tetrathionate increased the rate of digestion. These data also suggest that the inhibition of TPD by DPN is unrelated to the state of the active -SH groups but may be due to a polyanionic stabilization effect similar to that observed for the sulfate anion in the heat stability studies.

The Number of Free -SH Groups in Turkey, Sturgeon, and Lobster Muscle TPD's.--Spectrophotometric analysis with PCMB (Boyer and Schulz, 1959), amperometric titration with Ag^+ (Benesch et al., 1955), and oxidation with iodosobenzoate (Rafter, 1957) have shown that there are 11 ±2 free -SH groups per rabbit muscle TPD if one assumes a molecular weight of 120,000. Amino acid analysis for total cystine plus cysteine indicates that all of the non-methionine sulfur of the rabbit muscle enzyme is equal to the number of free -SH groups

(Velick, 1954). Therefore, the enzyme lacks stabilizing disulfide bonds. The -SH groups of the rabbit, turkey, sturgeon, and lobster muscle TPD's were determined in 0.10 M sodium phosphate, pH 7.0, both in the presence and absence of 8.0 M urea. A differential reactivity of the -SH groups in each of the TPD's was found in the experiments
Table IV carried out in the absence of urea, as shown in Table IV. During experiments with the rabbit, turkey, and lobster TPD's there was a slow, steady increase in the extinction at 250 mμ after the initial burst of mercaptide formation between the reactive -SH groups and PCMB. Turbidity due to protein precipitation occurred after 8 to 10 -SH groups in the turkey muscle TPD had reacted with PCMB. However, during experiments in the absence of urea with the rabbit and lobster muscle TPD's, the number of -SH groups that reacted with PCMB reached a maximum within an hour and no turbidity developed long after this maximum value was reached. No increase in extinction at 250 mμ over the initial burst of mercaptide formation was observed during experiments with the sturgeon muscle enzyme in the absence of urea. The results of -SH determinations in the presence of 8.0 M urea are also shown in Table IV. The number of -SH groups in the rabbit enzyme which reacted with PCMB in 8.0 M urea reached a maximum value of 12.7 within 10 minutes, while the number of -SH groups that reacted with PCMB in the turkey and lobster enzymes reached maximum values of 13.2 and 11.8, respectively, within 25 minutes in
Fig. 12 8.0 M urea as shown in Figure 12. Some of the -SH groups in the sturgeon muscle TPD reacted very slowly with PCMB even in the presence of 8.0 M urea. The number of -SH groups in the sturgeon enzyme that reacted with PCMB in the presence of 8.0 M urea did not reach a maximum value until 90 minutes after the enzyme was mixed

with the reagent. All of these experiments were carried out at 23° under nearly identical conditions of enzyme and PCMB concentrations. Therefore, the differences in the reactivity of the -SH groups in the various muscle TPD's with PCMB are not due to the effects of concentration.

Automatic amino acid analyses of the rabbit, turkey, sturgeon, and lobster TPD's for cysteic acid following performic acid oxidation of the enzymes revealed that each of the TPD's lacks stabilizing disulfide bonds. Table IV shows that the number of cysteic acid residues determined by automatic amino acid analyses for each of the performic acid oxidized enzymes is equal to the number of free -SH groups determined with PCMB in the presence of 8.0 M urea.

Discussion

The heat stability studies show that the active -SH groups of muscle TPD's are involved in maintaining the tertiary structure of the molecule in an active configuration. Modification of the active -SH groups of the turkey, sturgeon, and lobster TPD's with tetrathionate decreased the heat stability of all of the enzymes, but to varying degrees with the different enzymes. There is a strong correlation between changes in the immunological properties of the enzymes and the decrease in heat stability after inactivation with tetrathionate. The turkey and lobster TPD's, when inactivated with tetrathionate, are quite susceptible to heat denaturation at 37°, while the tetrathionate inactivated sturgeon TPD is much more stable. The immunological reactivity of the lobster and turkey TPD's with specific antisera prepared against the active form of the enzymes is decreased to a great extent when the enzymes are reversibly

inactivated with tetrathionate. On the other hand, the immunological reactivity of the sturgeon enzyme with a specific antiserum prepared against the active form of the enzyme is only slightly decreased when the enzyme is reversibly inactivated with tetrathionate. These results indicate that the forces holding the tertiary structure of the lobster and turkey enzymes in a specific, active conformation are dependent on the state of the three active -SH groups of the two dehydrogenases. This is true to a lesser degree for the sturgeon enzyme. The fact that only the three active -SH groups of the sturgeon enzyme react with PCMB in the absence of urea supports the hypothesis that this dehydrogenase does not unfold to a great extent, even after modification of these three -SH groups. Furthermore, some of the -SH groups in the sturgeon enzyme react sluggishly with PCMB even in the presence of 8.0 M urea indicating that part of the structure of the enzyme is very stable.

The losses observed in antigenic activity accompanying chemical modification of the active -SH groups of the TPD's are subject to two interpretations. They may indicate that a specific antigenic determinant is modified by steric or charge effects introduced by covalent bonding of the -SH groups, or alternatively, they may indicate that the positions of several antigenic determinants in three-dimensional space are modified by a conformational change following chemical modification of the active -SH groups.

There is evidence that antisera prepared against protein antigens are specific for a specific three-dimensional conformation. Mills and Haber (1962) have shown that a rabbit antiserum prepared against bovine ribonuclease is specific for a certain spatial configuration of the protein. When ribonuclease is reduced and then

re-oxidized under conditions where protein molecules are formed that differ from the native protein only in the arrangement of disulfide linkages, enzymatic activity is lost. These inactive ribonuclease derivatives failed to cross react with an antiserum prepared against the native enzyme as determined by agar gel diffusion analysis. Further evidence that antibodies are specific for a given tertiary structure of an enzyme has been obtained from immunologic studies of the pepsinogen system. Using the quantitative C' fixation technique, Van Vunakis et al. (1963) and Gerstein et al. (1963) have shown that rabbit antisera prepared against native pepsinogen and denatured pepsin (both isolated from swine) are specific for the conformation of their homologous antigens. When pepsinogen is treated with alkali, urea, or heat, or is photooxidized with methylene blue, the pepsin moiety of the zymogen is unmasked. This conformational change is detected immunologically. Treatment of pepsinogen with alkali, urea, heat, or methylene blue, leads to increased reactivity with the anti-pepsinogen serum. When pepsinogen was treated with these agents, the C' fixation curve in experiments with the anti-pepsinogen serum was shifted to higher antigen concentrations, and decreases in peak height were observed after prolonged treatment. When the TPD's investigated in this study were inactivated with -SH reagents, both a lateral shift toward higher antigen concentrations and a decrease in the height of the C' fixation curve were observed. Modification of the active -SH groups of the sturgeon TPD with tetrathionate leads to only a slight change in antigenic activity with both the anti-sturgeon and anti-turkey TPD's. On the other hand, such modification of the turkey and lobster TPD's results in extensive losses in antigenic activity with the antisera prepared against the active form of

the enzyme. The heat stability studies and PCMB titrations indicate that the sturgeon TPD does not unfold to a great extent in the absence of urea after modification of its active -SH groups, while the same studies indicate that the turkey and lobster TPD's are altered significantly after chemical modification of their active -SH groups. Therefore, it appears that the changes in antigenic activity accompanying chemical modification of the active -SH groups of the TPD's are due to changes in the tertiary structure of the enzymes and are not primarily due to the specific blocking of an antigenic determinant.

The possibility arises that tetrathionate inactivation of the TPD might be a result of a conformational change rather than a blocking of essential -SH groups. The fact that the sturgeon TPD apparently does not undergo structural change upon addition of tetrathionate appears to rule out this possibility.

Elödi and Szabolcsi (1959) have hypothesized that bound DPN stabilizes muscle TPD's in their native conformations, since removal of bound DPN from mammalian muscle TPD's with charcoal resulted in changes in optical rotation, intrinsic viscosity, and trypsin digestibility, all of which were reversed by the addition of coenzyme. They have noted, however, that the addition of more than three moles of DPN to the swine muscle apoenzyme increased the intrinsic viscosity over that of the native enzyme (2 moles of DPN/mole of enzyme) and reduced trypsin digestibility to very low values. During their original studies with the rabbit muscle enzyme, Taylor et al. (1948) observed that the charcoal-treated apoenzyme could not be crystallized, while it was easily crystallized after the readdition of DPN. Velick and Furfine (1963) have shown that excess

DPN stabilizes rabbit muscle TPD against spontaneous inactivation at 39°. They found that the enzyme in the presence of a 5,000 molar excess of DPN was completely stabilized at 39°. The results of the immunologic experiments of this study indicate that bound DPN does not play a prominent role in the stabilization of the native conformation of the TPD's. The addition of DPN to the charcoal-treated lobster muscle enzyme or to the turkey and sturgeon enzymes does not alter their antigenicity with antisera prepared against the native enzyme. The observation that the rate of proteolytic digestion of mammalian TPD's is decreased by bound DPN (Racker and Krimsky, 1958; Elödi and Szabolcsi, 1959) has been confirmed for the lobster enzyme. However, it appears that the effect of DPN on the proteolysis of the dehydrogenase is independent of the state of the active -SH groups, since the rate of trypsin digestion of the irreversibly inactivated enzyme is decreased by DPN. Analyses of several crystalline muscle TPD's for bound DPN has revealed that the turkey, pheasant and sturgeon dehydrogenases are virtually free of coenzyme, while the halibut and chicken enzymes crystallize with less than one mole of DPN bound per mole of the enzyme (Allison and Kaplan, 1964). All of these enzymes are as stable as the rabbit muscle enzyme when stored at 5° as suspensions in ammonium sulfate. The observed stabilization of the mammalian muscle TPD's by excess DPN against tryptic digestion and spontaneous inactivation at 39° may be due to a polyanionic effect similar to stabilization of the native and reversibly inactivated TPD's by sulfate discussed in this study. Both the sulfate stabilization and the DPN stabilization are unrelated to the state of the active -SH groups of the enzyme, since DPN partly protects the tetrathionate inactivated lobster enzyme against

tryptic digestion and sulfate protects the tetrathionate inactivated sturgeon, turkey, and lobster muscle enzymes against heat denaturation.

With different methods of isolation, muscle TPD-DPN complexes can be crystallized in an active or an inactive form, depending on the state of the active -SH groups (Astrachan et al., 1957; Velick et al., 1953). When the rabbit muscle enzyme is crystallized in the absence of EDTA or activating thiols, 2 moles of DPN are firmly bound per mole of enzyme, but the enzyme must be activated with thiols before maximal enzyme activity can be measured (Velick, 1955). When DPN is bound to the active form of TPD (-SH groups reduced) the characteristic 360 mμ absorption band is observed, while the inactive TPD-DPN complex does not have the 360 mμ chromophore (Astrachan et al., 1953). When an aged preparation of lobster TPD (stored for six months as a crystalline suspension in ammonium sulfate) is fully activated with cysteine, both antigenic activity and absorption at 360 mμ are increased. Astrachan et al. (1957) observed that DPN bound to inactive TPD is more susceptible to other enzymes than is the DPN bound to the active enzyme. Moreover, Nygaard and Rutter (1956) found that DPN bound to rabbit muscle TPD was reduced more slowly than free DPN by beef heart lactic dehydrogenase in the presence of lactate, but the bound DPN was reduced at the same rate as free DPN when the TPD was inactivated by mercuribenzoate. From the evidence presented in the present study, it appears that the increased susceptibility of DPN to enzymes in the inactive TPD-DPN complex over that in the active TPD-DPN complex to enzymatic attack is due to a change in enzyme conformation following chemical modification of the active -SH groups.

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Figure Legends

Figure 1. The effect of tetrathionate inactivation on the heat stability of turkey muscle TPD. 10^{-6} M solutions of the enzyme in 0.05 M sodium pyrophosphate, pH 8.5, were incubated at 37° with the following reagents: ●, none; o, 0.4 M $(NH_4)_2SO_4$, pH 8.5; Δ, 3 x 10^{-6} M $S_4O_6^=$; ▲, 3 x 10^{-6} M $S_4O_6^=$ and 0.4 M $(NH_4)_2SO_4$, pH 8.5. 5 μg samples were withdrawn at the times indicated and were incubated with 10^{-3} M mercaptoethanol for 5 minutes at which time they were assayed.

Figure 2. The effect of tetrathionate inactivation on the heat stability of sturgeon muscle TPD. 10^{-6} M solutions of the enzyme in 0.05 M sodium pyrophosphate, pH 8.5, were incubated at 37° with the following reagents: ●, none; o, 0.4 M $(NH_4)_2SO_4$, pH 8.5; Δ, 3 x 10^{-6} M $S_4O_6^=$; ▲, 3 x 10^{-6} M $S_4O_6^=$ and 0.4 M $(NH_4)_2SO_4$, pH 8.5. 5 μg samples were withdrawn at the times indicated and were incubated with 10^{-3} M mercaptoethanol for 5 minutes at which time they were assayed.

Figure 3. The effect of tetrathionate inactivation on the heat stability of lobster muscle TPD. 10^{-6} M solutions of the enzyme in 0.05 M sodium pyrophosphate, pH 8.5, were incubated at 37° with the following reagents: ●, none; o, 0.4 M $(NH_4)_2SO_4$, pH 8.5; Δ, 3 x 10^{-6} M $S_4O_6^=$; ▲, 3 x 10^{-6} M $S_4O_6^=$ and 0.4 M $(NH_4)_2SO_4$, pH 8.5. 5 μg samples were withdrawn at the times indicated and were incubated with 10^{-3} M mercaptoethanol for 5 minutes at which time they were assayed.

Figure 4. The effect of tetrathionate inactivation of lobster TPD on precipitin band formation. Each enzyme well contained 0.10 ml of 3×10^{-6} M lobster muscle TPD in 0.05 M sodium pyrophosphate, pH 8.5, after the treatments indicated. Well 1, 3 days aging in 0.01 M sodium phosphate, pH 8.0; Well 2, 10^{-5} M $S_4O_6^=$; Well 3, same as well 1 with the addition of 10^{-4} M mercaptoethanol; Well 4, 10^{-5} M $S_4O_6^=$ followed by the addition of 10^{-4} M mercaptoethanol. The center well contained 0.10 ml of a rabbit serum prepared against the active form of lobster muscle TPD. The time represents the period after the wells were filled.

Figure 5. The effect of tetrathionate inactivation on the complement fixation reaction of lobster muscle TPD with anti-lobster TPD. The following additions were made to 3×10^{-6} M solutions of the "aged" enzyme in 0.05 M sodium pyrophosphate, pH 8.5: curve 1, none; curve 2, 10^{-3} M cysteine; curve 3, 10^{-5} M $S_4O_6^=$ at 0°; curve 4, 10^{-5} M $S_4O_6^=$ at 0° then 10^{-3} M cysteine; curve 5, 10^{-5} M $S_4O_6^=$ at 30°; curve 6, 10^{-5} M $S_4O_6^=$ at 30° and then 10^{-3} M cysteine. The mixtures were incubated at 0° for 20 minutes and at 30° for 30 minutes where indicated. Cysteine was added at the end of these incubations where indicated. A sample of each mixture was diluted to a final enzyme concentration of 0.44 µg per ml with isotonic veronal buffer for the C' fixation assay.

Figure 6. The effect of tetrathionate inactivation on precipitate formation between lobster TPD and anti-lobster TPD. 3×10^{-6} M enzyme solutions in 0.05 M sodium pyrophosphate, pH 8.5, contained the following additions: o, 10^{-4} M mercaptoethanol; •, 10^{-5} M

$S_4O_6^=$ at 0°; Δ, 10^{-5} M $S_4O_6^=$ at 0°, then 10^{-4} M mercaptoethanol; ▲, 10^{-5} M $S_4O_6^=$ at 35°; □, 10^{-5} M $S_4O_6^=$ at 35° then 10^{-4} M mercaptoethanol. The mixtures were incubated with tetrathionate for 20 min. at 0° or 35° where indicated. Mercaptoethanol was added to the mixtures at the end of the incubation period where indicated. After the incubation period, 0.5 ml of the enzyme solutions were mixed with a 2.5 ml solution of the antiserum (0.20 ml of the antiserum, 2.3 ml of buffer) in 0.05 M sodium pyrophosphate, pH 8.5, in a cuvette at 7°. Immediately after mixing, the optical density of the reaction mixtures was determined at 600 mμ at 30-second intervals.

Figure 7. The effect of tetrathionate inactivation on the C' fixation reaction of turkey muscle TPD with anti-turkey TPD. The following additions were made to 3 x 10^{-6} M solutions of the enzyme in 0.05 M sodium pyrophosphate, pH 8.5; o, none; ■, 10^{-5} M $S_4O_6^=$ at 0°; •, 10^{-5} M $S_4O_6^=$ at 0° then 10^{-3} M mercaptoethanol; □, 10^{-5} M $S_4O_6^=$ at 37°; x, 10^{-5} M $S_4O_6^=$ at 37° and then 10^{-3} M mercaptoethanol. The mixtures were incubated at 0° for 30 min. where indicated and at 37° for 15 min. where indicated. Mercaptoethanol was added at the end of these incubations where indicated. A sample of each mixture was diluted to a final enzyme concentration of 0.80 μg per ml with isotonic veronal buffer for the C' fixation assay.

Figure 8. The effect of tetrathionate inactivation on the C' fixation reaction of sturgeon muscle TPD with anti-sturgeon TPD. The following additions were made to 3 x 10^{-6} M solutions of the enzyme in 0.05 M sodium pyrophosphate, pH 8.5; o, none; •, 10^{-5} M $S_4O_6^=$ at 0°; Δ, 10^{-5} M $S_4O_6^=$ at 37°; ▲, 10^{-5} M $S_4O_6^=$ at 37° and then 10^{-3} M mercaptoethanol. The mixtures were incubated at 0° for 30 min. where

indicated and at 37° for 120 min. where indicated. Mercaptoethanol was added at the end of the incubation period where indicated. A sample of each mixture was diluted to a final enzyme concentration of 1.0 μg per ml with isotonic veronal buffer for the C' fixation assay.

Figure 9. The effect of tetrathionate inactivation on the C' fixation reaction of sturgeon muscle TPD with anti-turkey TPD. The following additions were made to 3 x 10^{-6} M solutions of the enzyme in 0.05 M sodium pyrophosphate, pH 8.5: o, none; •, 10^{-5} M $S_4O_6^=$ at 0°; Δ, 10^{-5} M $S_4O_6^=$ at 37°; ▲, 10^{-5} M $S_4O_6^=$ at 37° and then 10^{-3} M mercaptoethanol. The mixtures were incubated at 0° for 30 min. where indicated and at 37° for 120 min. where indicated. Mercaptoethanol was added at the end of the incubation period where indicated. A sample of each mixture was diluted to a final enzyme concentration of 1.0 μg per ml with isotonic veronal buffer for the C' fixation assay.

Figure 10. The effect of DPN and $S_4O_6^=$ on the C' fixation reaction of charcoal-treated lobster muscle TPD with anti-lobster TPD. The following additions were made to 3 x 10^{-6} M solutions of the charcoal treated apoenzyme in 0.05 M sodium pyrophosphate, pH 8.5: •, none; o, 10^{-5} M DPN; ▲, 10^{-5} M $S_4O_6^=$ and Δ, 10^{-5} M DPN and 10^{-5} M $S_4O_6^=$. The mixtures were incubated for 20 min. at 0° at which time a sample of each was diluted to a final enzyme concentration of 0.50 μg per ml with isotonic veronal buffer for the C' fixation assay.

Figure 11. The effect of acetyl phosphate on the C' fixation reaction of charcoal-treated lobster muscle TPD with anti-lobster TPD. The following additions were made to 10^{-5} M solutions of the apoenzyme in 0.05 M sodium pyrophosphate, pH 8.5; curve 1, 0.75 µmoles DPN and 3 µmoles Na_2HAsO_4; curve 2, 0.01 M acetyl phosphate; curve 3, 0.01 M acetyl phosphate, 0.75 µmoles DPN and 3 µmoles Na_2HAsO_4; curve 4, 0.001 M acetyl phosphate; curve 5, 0.001 M acetyl phosphate, 0.75 µmoles DPN, and 3 µmoles Na_2HAsO_4. The apoenzyme was incubated with acetyl phosphate for 30 min. at 5° before DPN and arsenate were added. After DPN and arsenate were added, the reaction mixtures were inactivated at 5° for an additional 30 min., at which time samples were diluted with isotonic veronal buffer to a final enzyme concentration of 0.50 µg per ml for the C' fixation assay.

Figure 12. The rate of reaction of the -SH groups of various TPD's with PCMB in 8.0 M urea.

Table I

The Stability of Lobster Muscle Triosephosphate in the Presence of Tetrathionate at 5°

The enzyme (10^{-6} M) was inactivated by the addition of 3×10^{-6} M $S_4O_6^{=}$ in 0.05 M sodium pyrophosphate, pH 8.5, and was incubated at 5°. Five µg samples were withdrawn in the times indicated and were incubated with 10^{-3} M mercaptoethanol for 5 minutes at room temperature and then assayed.

Time at 5°	% Activity recovered after addition of mercaptoethanol
0	100
1 hr	96
4 hrs	79
8 hrs	70
12 hrs	60
24 hrs	51

Table II

The Arsenolysis of Acetyl Phosphate by Lobster Muscle Triosephosphate Dehydrogenase

In a volume of 1 ml, reaction mixtures contained 100 μmoles of $KHCO_3$, 3 μmoles of dibasic sodium arsenate, 10 μmoles of acetyl phosphate, 3 mg of the lobster muscle enzyme, and the additions indicated in the Table. Acetyl phosphate was assayed by the method of Lipmann and Tuttle (1945).

Additions			Acetyl phosphate destroyed in 30 min.
10^{-3} M $S_4O_6^=$	0.75 moles DPN	5×10^{-3} M cysteine	
-	-	-	4.0 moles
-	-	+	5.6
-	+	+	7.4
+	-	-	0
+	-	+	4.5
+	+	+	6.7
+	+	-	0

Table III

The Effect of Coenzyme on the Tryptic Digestion of Lobster Muscle Triosephosphate Dehydrogenase

Experiments were carried out in 0.05 M $NaPO_4$, pH 8.0 at 37°. Reaction mixtures contained 1 x 10^{-5} M enzyme or apoenzyme, the compounds listed below in the concentrations indicated, and 36 µg of trypsin. The rate of proteolysis was estimated by the increase in optical density of aliquots deproteinized with 5% TCA. The values shown were recorded after 60 min. of digestion.

Additions	Rate of proteolysis relative to the native enzymes	Rate of proteolysis relative to the apoenzymes
Experiment 1		
None	1.0	-
5 x 10^{-5} M DPN	0.50	-
10 x 10^{-5} M DPN	0.50	-
15 x 10^{-5} M DPN	0.35	-
15 x 10^{-5} M Pyr-3-ald-DPN	0.65	-
15 x 10^{-5} M TPN	0.70	-
3 x 10^{-5} $S_4O_6^=$	2.70	-
3 x 10^{-5} $S_4O_6^=$ + 15 x 10^{-5} M DPN	1.50	-
Experiment 2		
Apoenzyme	-	1.0
Apoenzyme + 3 x 10^{-5} M DPN	-	0.39
Apoenzyme + 6 x 10^{-5} M DPN	-	0.23
Apoenzyme + 3 x 10^{-5} M $S_4O_6^=$	-	1.4

Table IV

The Number of Sulfhydryl Groups in Various Muscle Triosephosphate Dehydrogenases

Enzyme	-SH's reacting with PCMB within 2 mins. in 0.1 M Na-PO_4, pH 7.0	Total -SH's reacting with PCMB in 0.10 M Na-PO_4, pH 7.0	Total -SH's reacting with PCMB in the presence of 8.0 M urea	Half cystines determined as cysteic acid
Rabbit	7.2	13.4	12.7	12
Turkey	6.2	8-10*	13.2	13
Sturgeon	2.5	2.5	9.6	10
Lobster	5.8	11.6	11.8	12

*Turbidity due to the formation of a precipitate developed before titration was complete; 8 to 10 -SH groups were titrated before the detectable turbidity was observed.

Footnotes

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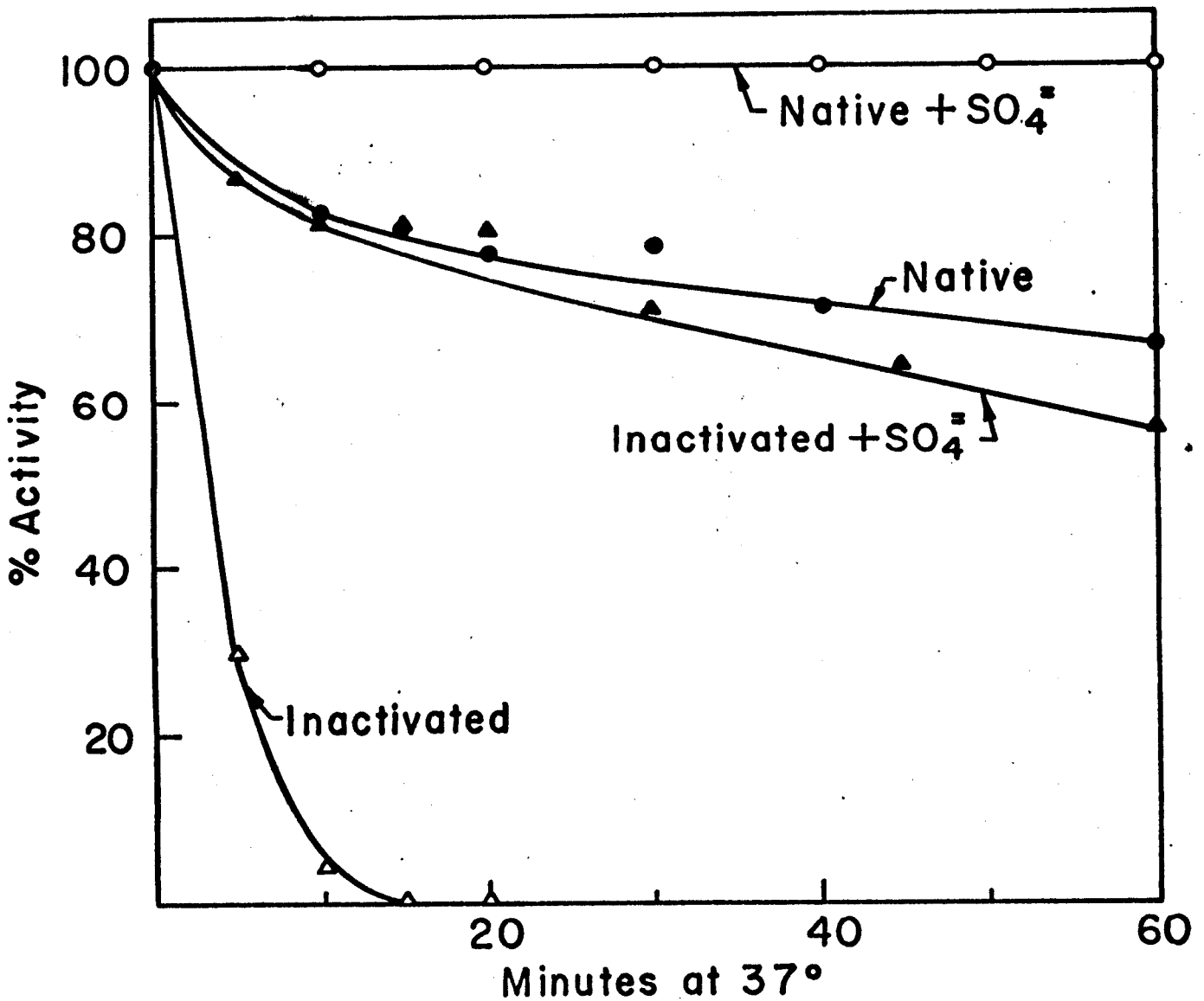
** Public Health Service Predoctoral Fellow (GM-12,638-3). Present address: Laboratory of Molecular Biology, Cambridge, England.

† Publication No. 000.


100
80
60
40
20
% Activity
Native + $SO_4^=$
Native
Inactivated + $SO_4^=$
Inactivated
20
40
60
Minutes at 37°

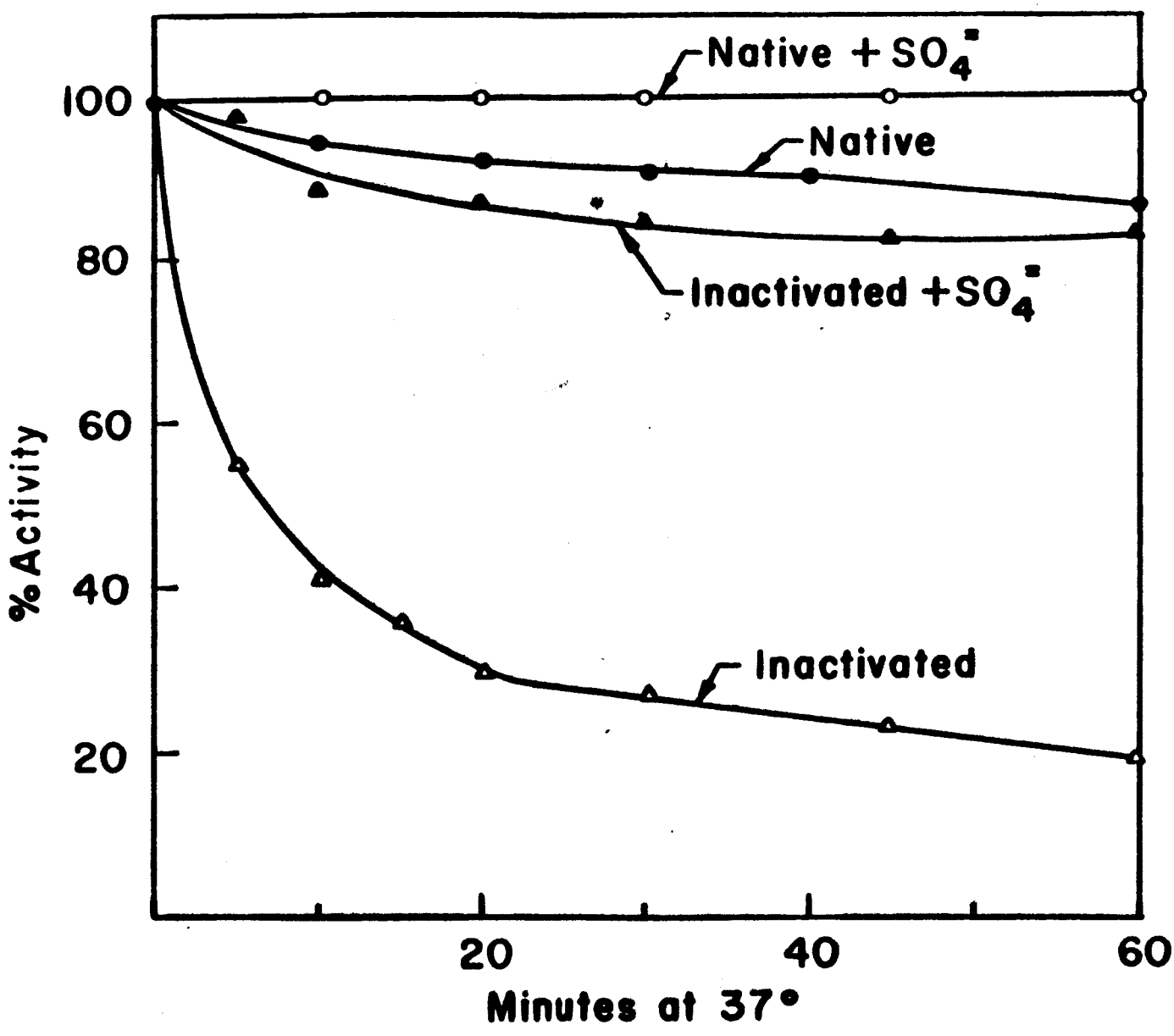


Fig 2 Allison-Kaplan

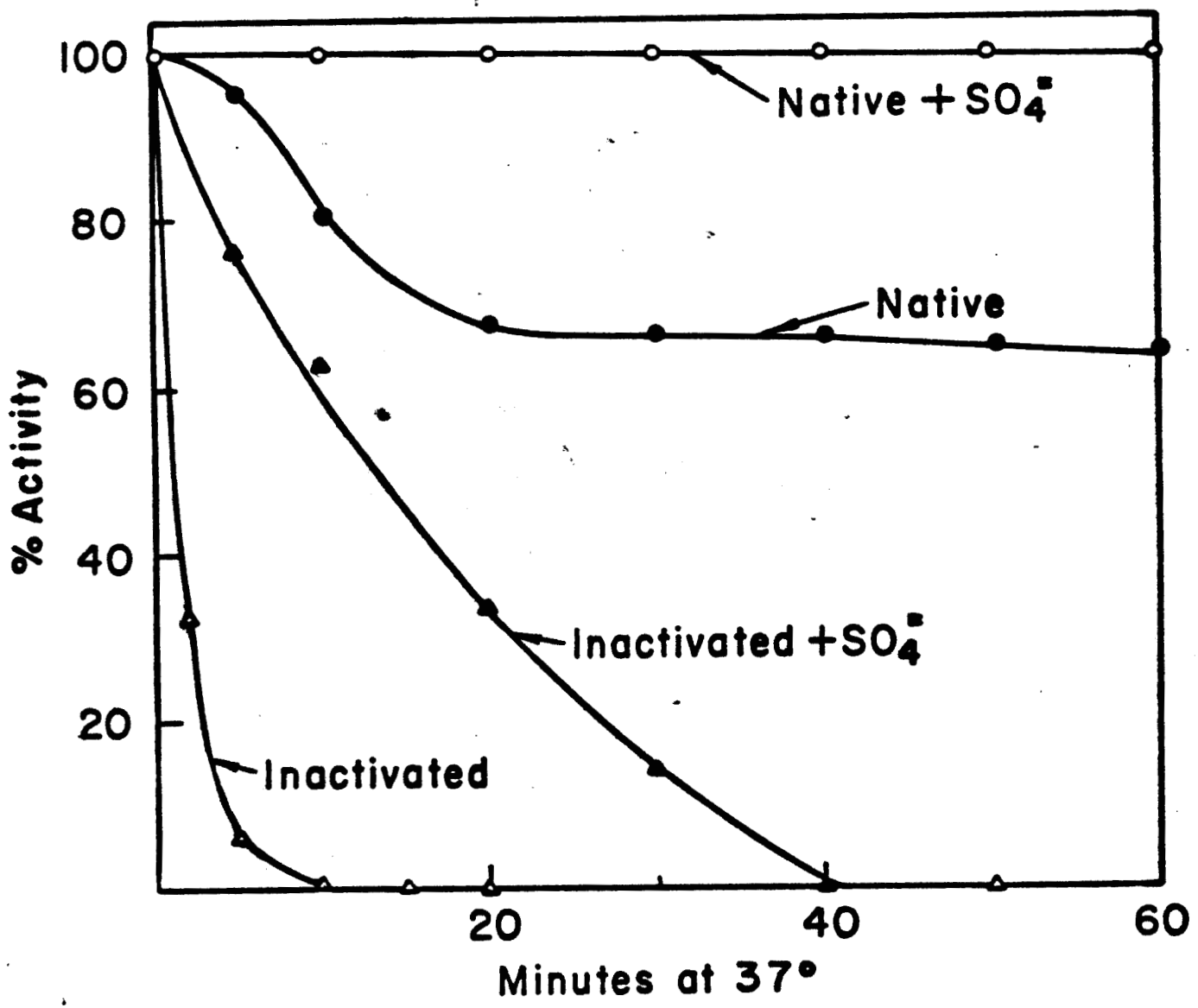
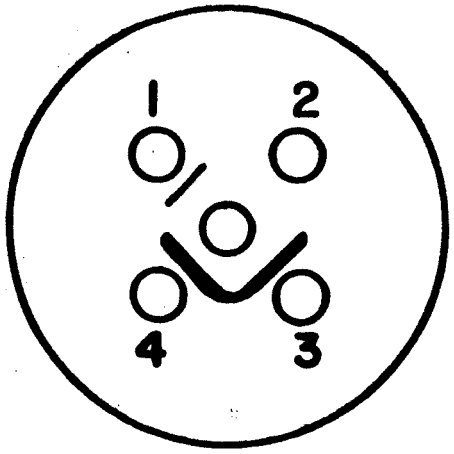
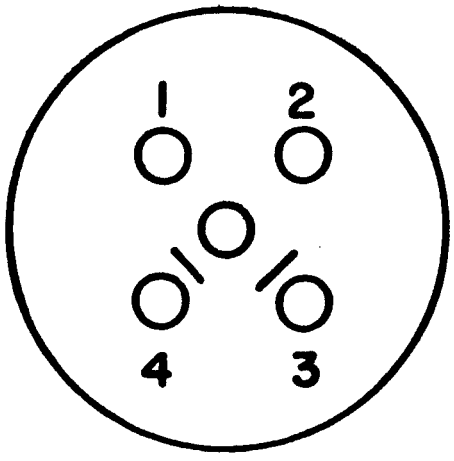


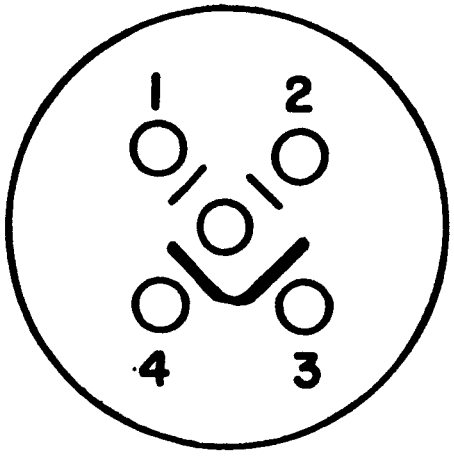
Fig 3 Alk --Kaplan



A. After 48 hours at 23°



B. After 7 hours at 5°



C. After 16 hours at 5°

F.194 Allison/Kaplan

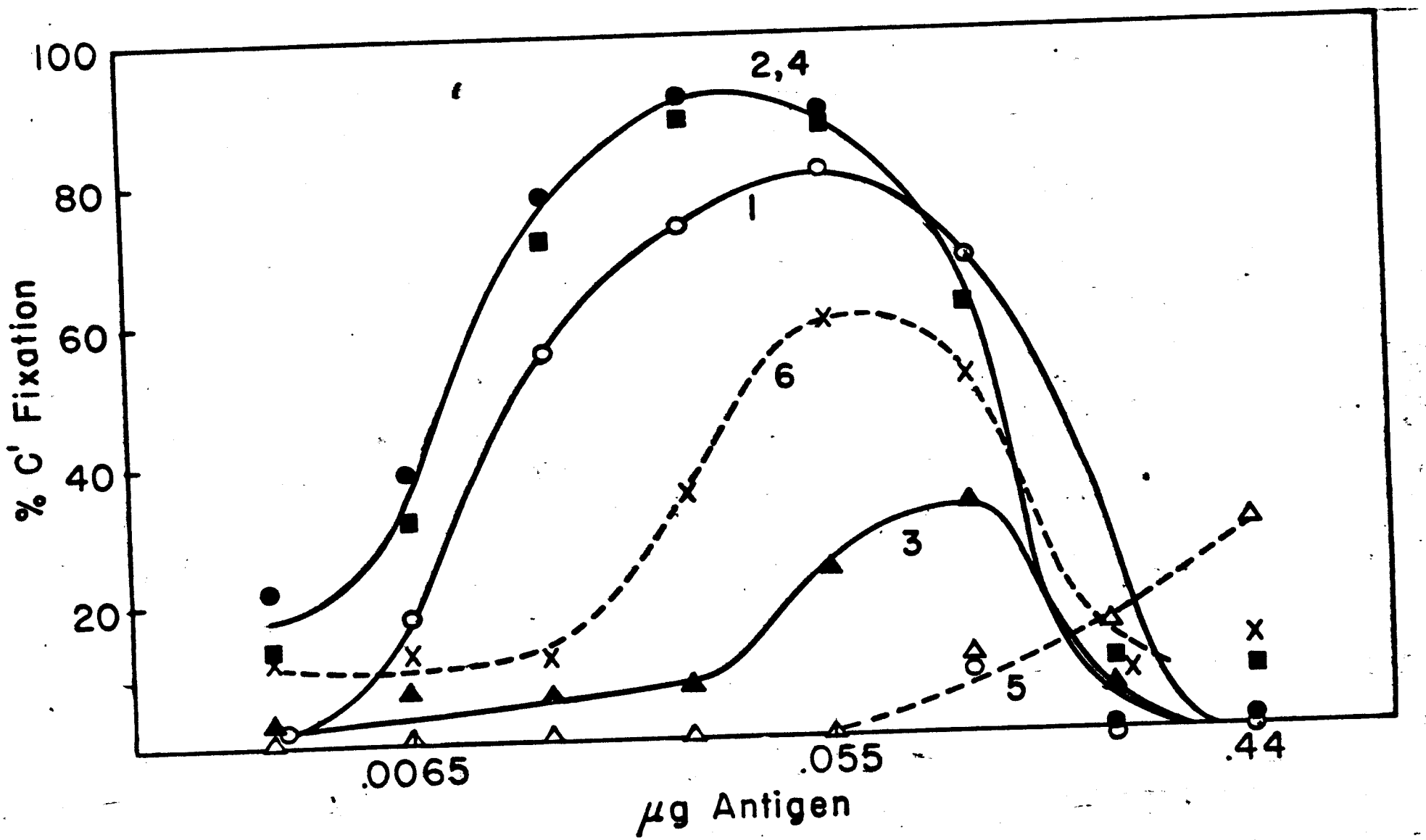


Fig 5 Allison-Kabat

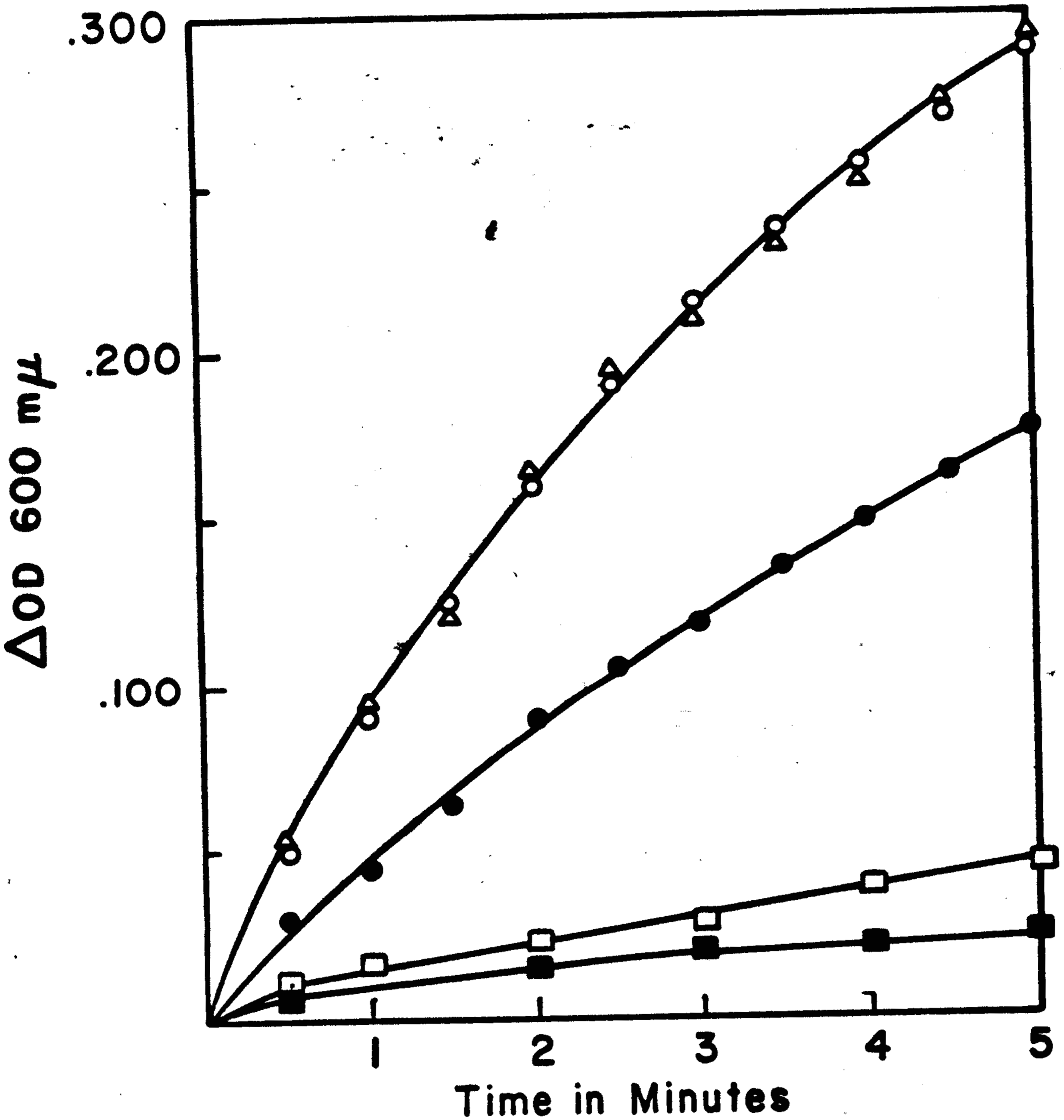


Fig. Allison - Kaplan

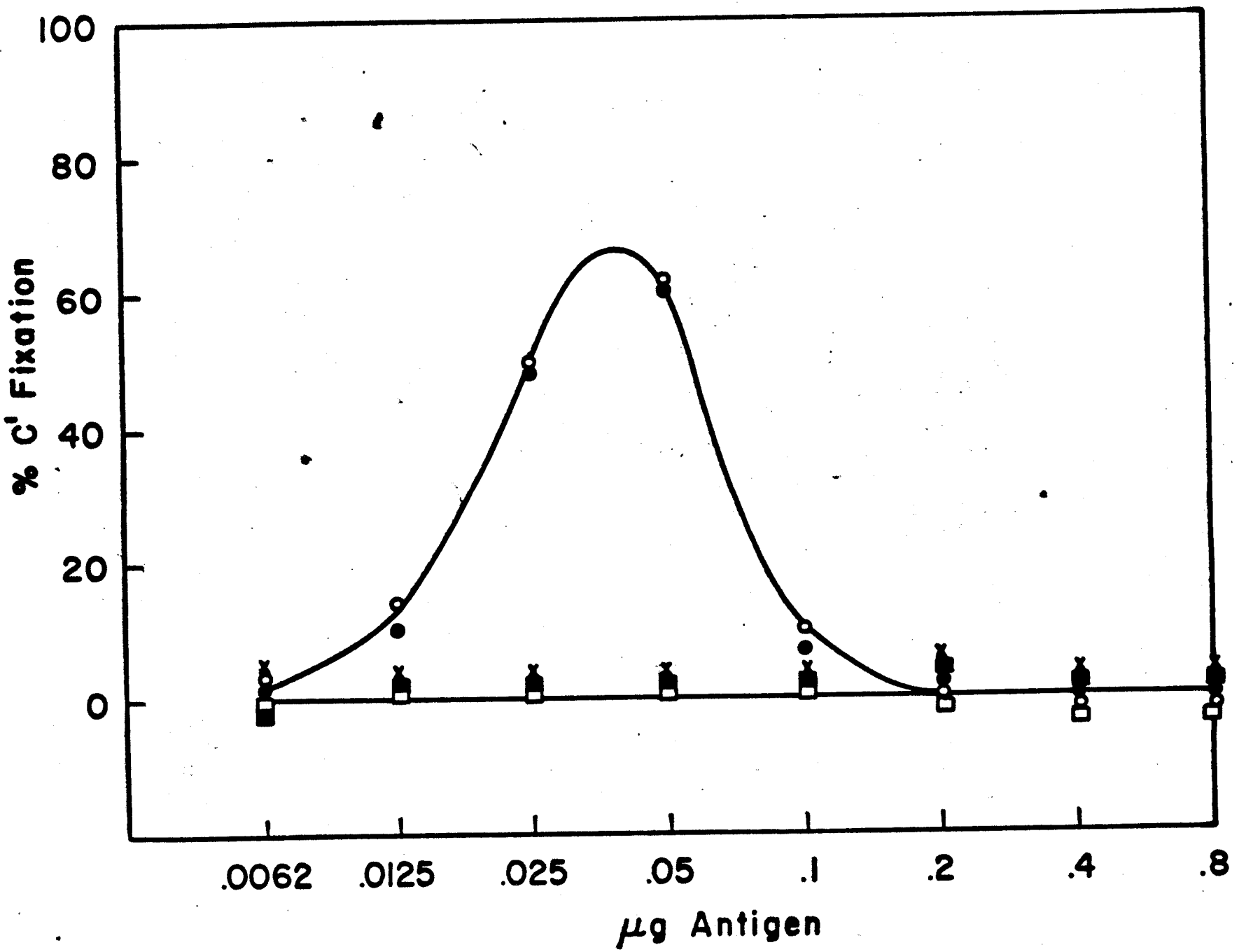


Fig 7 Allison-Kaplan

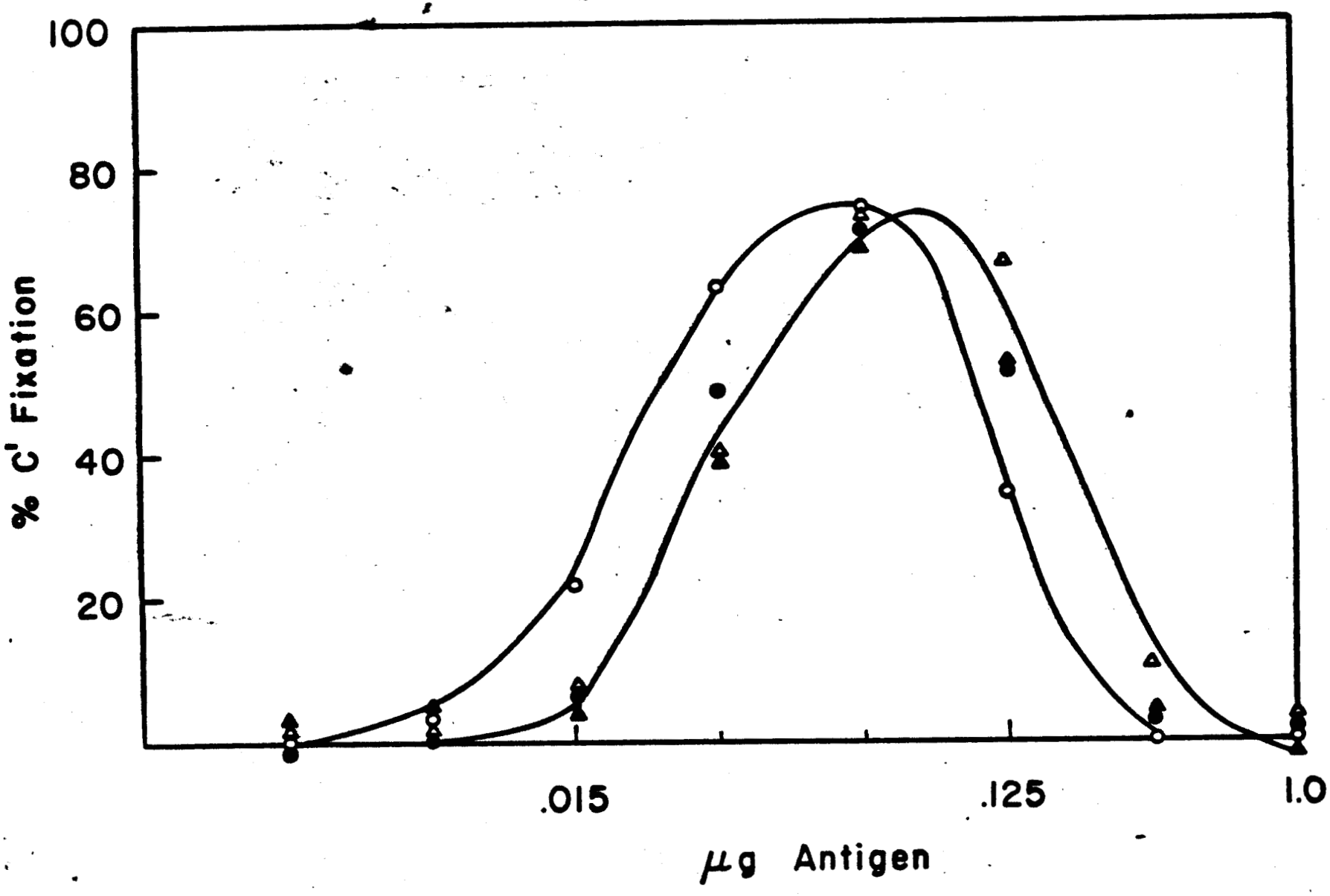


Fig 8. Nelson-Kaplan

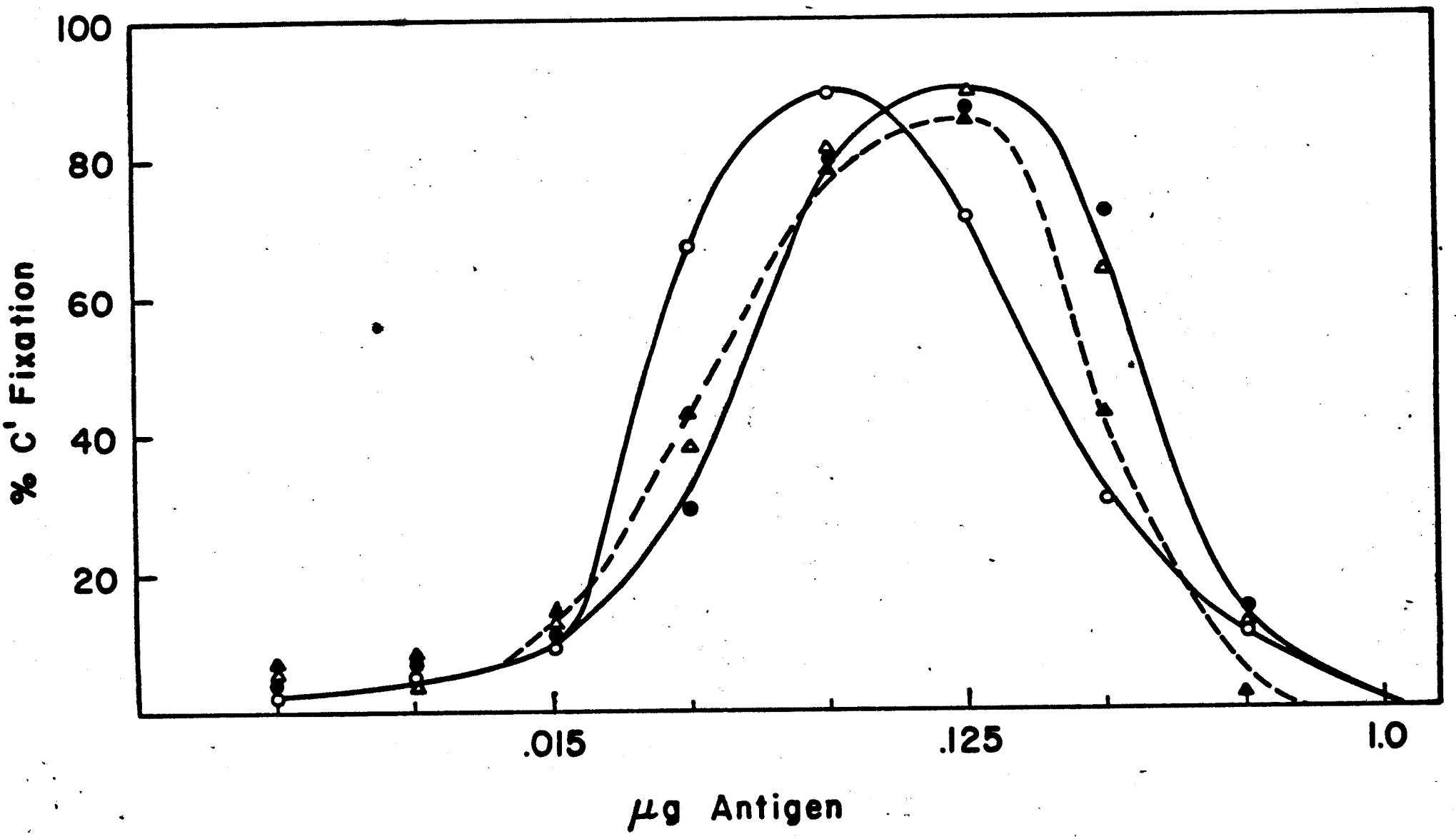


Fig 9 Oudin-Kabat

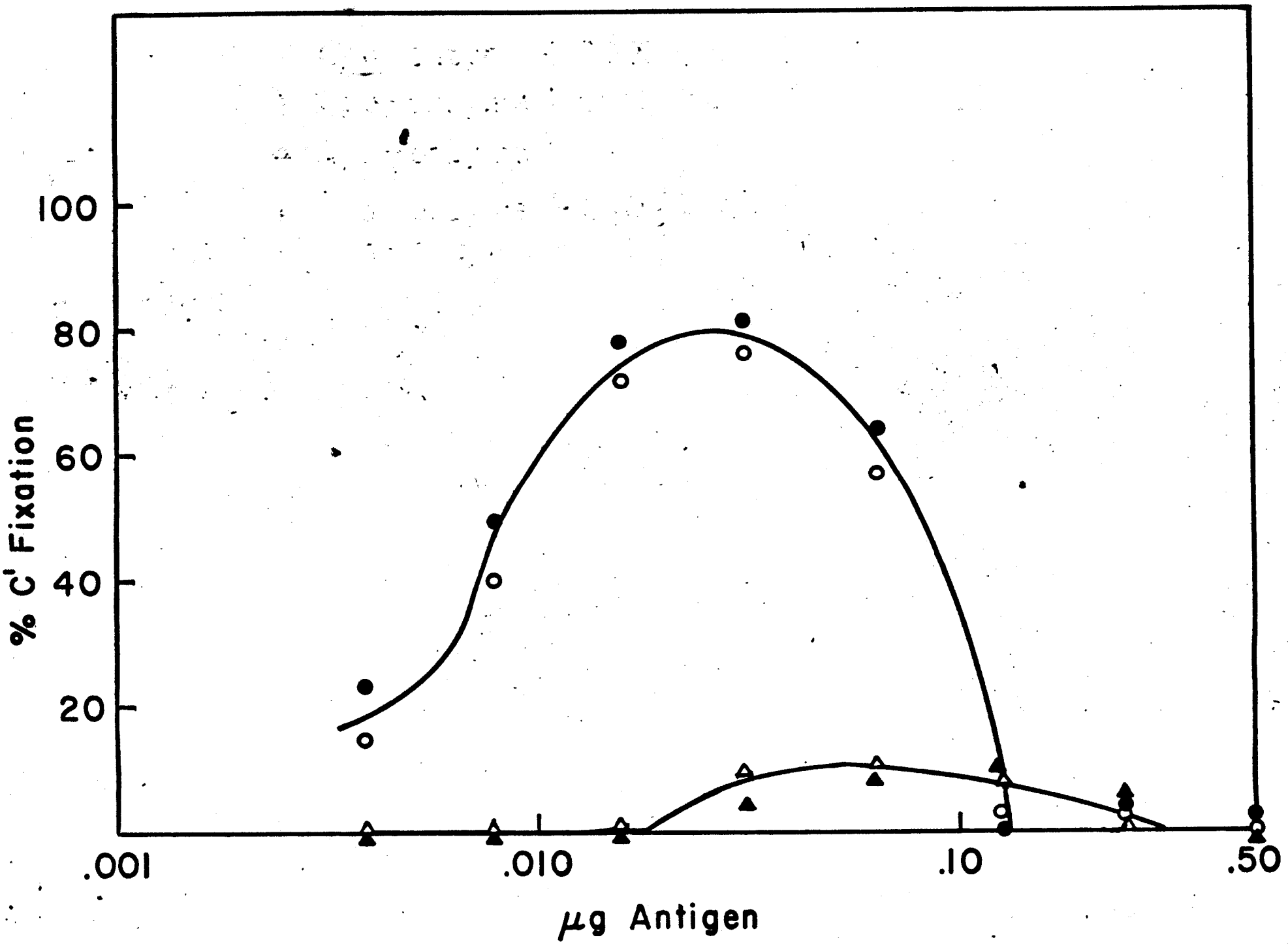


Fig 10 Albumin-Kaplan

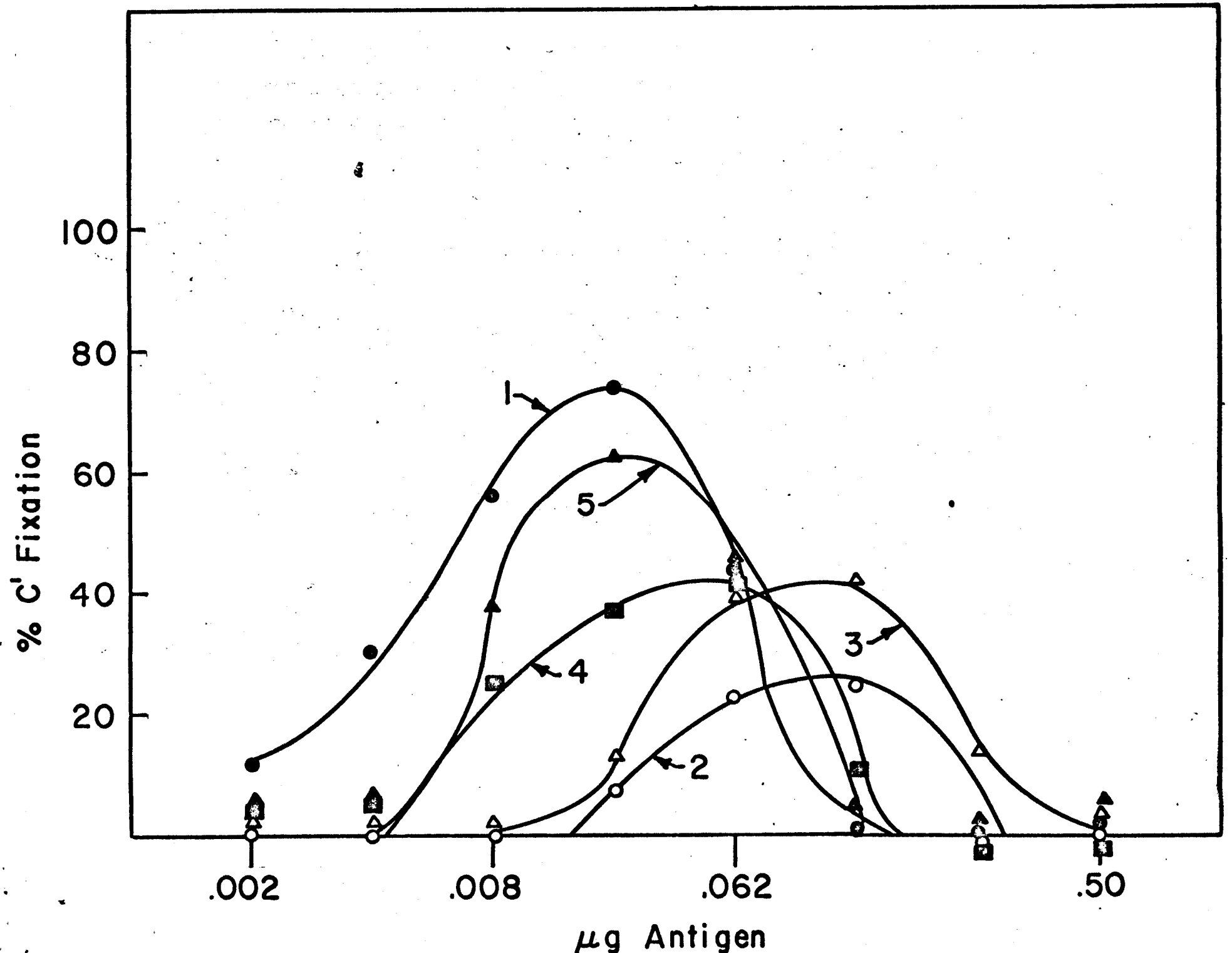


Fig II Adler - Kabat

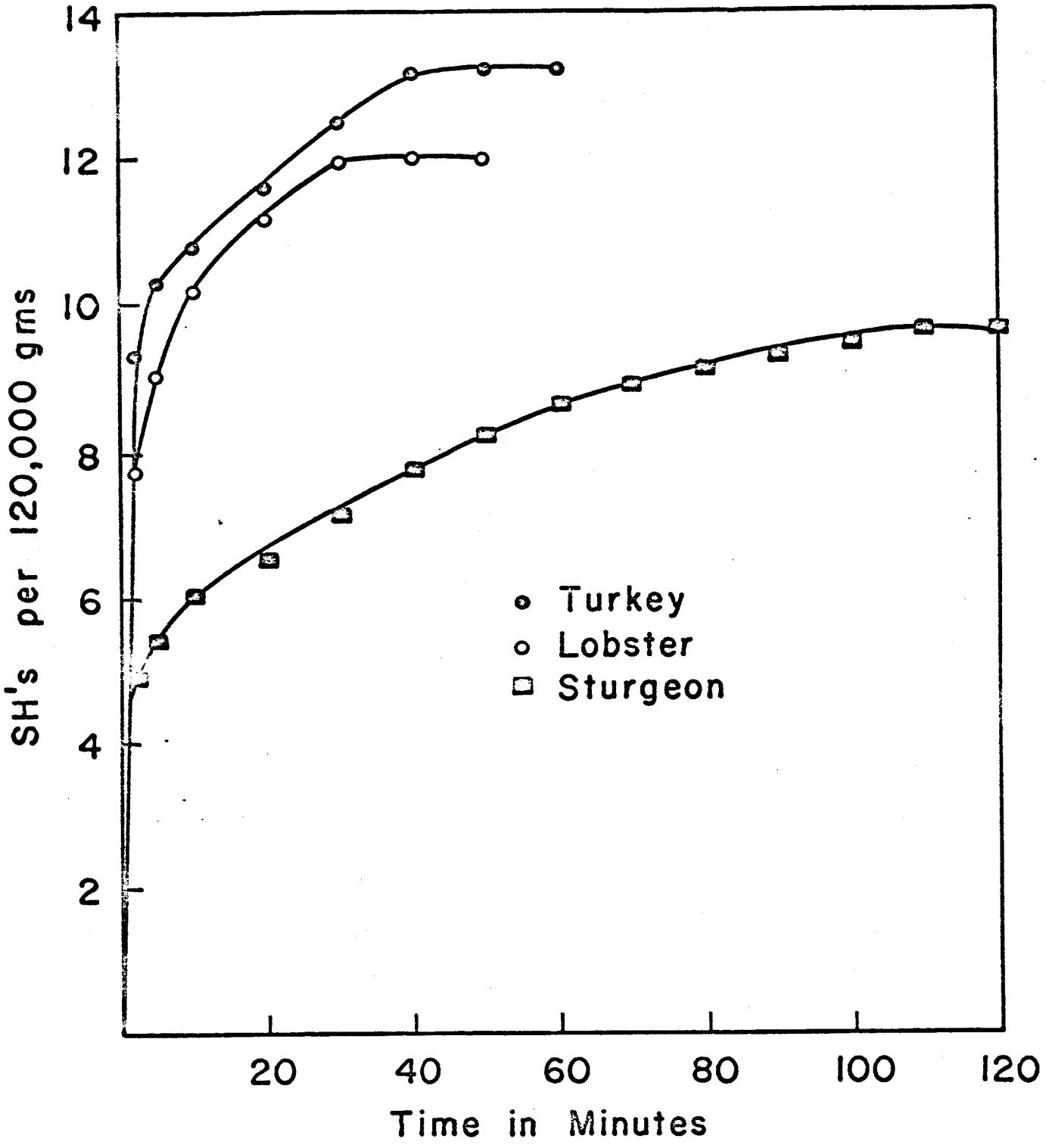


Fig 12 Allison-Kaplan