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AMINO CARBOXYLIC INHIBITION OF LIPID OXIDATION IN DEHYDRATED FOODS

By

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Massachusetts Institute Of Technology
Division Of Sponsored Research
Cambridge, Massachusetts

Contract No. DA 19-129-AMC-252(N)

September 1966

UNITED STATES ARMY
NATICK LABORATORIES
Natick, Massachusetts 01760

Food Division
FD-53

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FOREWORD

Dehydrated foods are particularly susceptible to autoxidation; oxidative deterioration of the lipids and secondary reactions proceeding therefrom have serious consequences on quality. In order to improve the stability of dehydrated foods for military use, it is important to understand both stabilizing and destabilizing subsystems. Amino acids have been known to affect the course of autoxidation of food lipids, but have not been studied systematically in dehydrated systems.

The work covered in this report, performed in the Division of Sponsored Research, Massachusetts Institute of Technology under Contract Number DA 19-129-AMC-254(N) (April 1965 - April 1966) represents the final phase of an investigation into the determination of the anti-oxidant and/or prooxidant effects of various amino acids in model systems simulating dehydrated foods and of the nature of the observed anti-oxidant activity with a view toward the elucidation of its mechanism. The investigator was Dr. Marcus Karel; his collaborator was Dr. S. R. Tannenbaum.

The U. S. Army Natick Laboratories Project Officer was A. S. Henick, and the Alternate Project Officer was S. J. Bishov both of Food Chemistry Branch, Food Division.

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SUMMARY

Oxidation of methyl linoleate was studied in a freeze-dried model system based on microcrystalline cellulose in the presence and in the absence of amino acids. The antioxidant activity of some of the amino acids, which was quite substantial in several runs, was found to be highly variable from run to run in spite of good replication between different samples of a given treatment in a given run. Attempts to eliminate the variability through purification of the methyl linoleate, through addition of cobalt nitrate, washing of the cellulose, changing the pH to 9.5, and through variations in model system preparation procedures were not successful.

In parallel work on conversion products of histidine in the model system, it was demonstrated that a major conversion product of histidine did not contain any substantial amounts of carbon derived from linoleate. In subsequent work it was determined that the conversion of histidine could occur as a result of mixing of the amino acid with the cellulose used in preparation of the model system, or with other types of cellulose including filter paper and cotton. The extent of conversion, however, was quite variable being similar in this respect to the antioxidant activity of the amino acid. Data were inadequate to correlate the conversion of the histidine with its antioxidant activity. However, in two runs in which analysis for the conversion product and oxidation studies were run concurrently it was observed that histidine showed an antioxidant effect when the conversion product was absent, but showed no effect in early oxidation stages, and some pro-oxidant activity in later oxidation stages. In these two runs analysis for the conversion product was carried out after freeze-drying but prior to incubation in air.

I. INTRODUCTION

The present investigation has as its aim the elucidation of the mechanism of antioxidant activity of amino carboxylic compounds in lipid-containing, dehydrated systems. In previous work (Karel and Tannenbaum, 1965) it was observed that several amino carboxylic compounds had substantial antioxidant activity. Compounds for which such activity was observed included histidine, cysteine, alanine, lysine, β-amino butyric acid, γ-amino butyric acid, and ε-amino caproic acid; whereas no antioxidant activity was observed with methionine, arginine, phenylalanine and isoleucine.

In the initial phase of this investigation work was also undertaken on the separation and characterization of reaction products from model systems containing linoleate and amino compounds. Reaction products in the model system oxidized in the presence of linoleate and histidine were studied using radioactive tracer compounds. Reaction products derived from histidine were located through the use of ring labelled histidine (C^{14}) and those derived from linoleate through the use of uniformly labelled methyl linoleate (C^{14}). Fractions of the reaction products were obtained by chromatographic and electrophoretic techniques. The fractions were characterized by the source of radioactivity, the relative mobility in different solvent systems, and the presence of specific functional groups as determined with suitable reagents.

The present phase of the investigation is devoted in particular to the following problems:

1. Further study of conditions under which amino carboxylic compounds act as antioxidants.

2. Study of reaction products of histidine, including investigation of conditions under which histidine is converted and the possible significance of this conversion to the mechanism of antioxidant activity of amino acids.

II. OXIDATION EXPERIMENTS

A. EXPERIMENTAL

1. Materials

a. Methyl linoleate. Purified methyl linoleate was obtained from the following sources: Mann Research Laboratories, New York, N.Y.; Hormel Institute, Minneapolis, Minn.; Applied Science Laboratories, Inc., State College, Pa.; and Sigma Chemical Company, St. Louis, Mo.

The methyl linoleate was further purified by urea adduct formation in methanol and thorough washing with urea saturated methanol. The adduct was then dissolved in water and solvent extracted with benzene to remove the methyl ester. As a further precaution the benzene layer was washed with 0.1 M citric acid and then rewashed with water. The benzene was then evaporated under vacuum and the methyl linoleate was vacuum distilled. Only the center cut of the distillate was used for subsequent oxidation studies. The use of n-hexane rather than benzene to extract the methyl linoleate from the urea adduct has been mentioned in a previous report; this procedure was used for runs 19 through 24.

In run 15 the following additional purification procedure was used before formation of the urea adduct:

Column chromatography of methyl linoleate was carried out on a silicic acid column using n-hexane:methanol (95:5 by volume).

In runs19 to 24 the purification procedure was modified as follows:

Methyl linoleate was distilled in a simple vacuum still of our own design constructed from standard laboratory glassware. Standard purification procedures, including urea adduct formation and redistillation, followed. Column chromatography was not used since the results obtained using the initial distillation step were more satisfactory. The impurity described previously as giving rise to emulsification of the preparation was removed by the distillation, as evidenced by TLC analysis. The initial distillation was effective in reducing the degree of oxidation of linoleate from 10-1 mM oxygen/M linoleate.

The purification was checked by procedures described previously (Karel and Tannenbaum, 1965).

b. Carbohydrate matrix. Microcrystalline cellulose (Avicel, American Viscose Co.) was used without further purification, except in run 22 in which the cellulose was washed by following the sequence of solvent elution used previously for elution of oxidation products as described fully in a previous report (Karel and Tannenbaum, 1965)

c. Amino carboxylic compounds. Amino carboxylic compounds used were as follows:

Compound	Source	Purity (Grade)
DL-alanine	Calbiochem	A
DL-β-amino-n-butyric acid	"	C
γ-amino butyric acid	"	A
ε-amino-n-caproic acid	"	C
L-cysteine hydrochloride hydrate	"	A

Compound	Source	Purity (Grade)
L-histidine	Calbiochem	A
DL-lysine·HCl	"	A

c. Other materials used. Other materials used included Cobalt nitrate, Malinckrodt, Analytical Reagent; n-hexane, Fisher Certified Reagent, redistilled; Methanol, Fisher Certified Reagent; Propyl gallate, Tenox, Food Grade Antioxidant, Eastman Chemical Products, Inc.; Silicic acid, 100 mesh, (chromatographic method of Ramsey and Patterson) Mallinckrodt; and water, deionized, redistilled, laboratory distilled water.

2. Procedures

Preparation of the model system was identical to that described in a previous report (Karel and Tannenbaum, 1965) with the exception of run 22 in which water was added to the cellulose before the addition of linoleate. The samples for which this modified procedure was used are referred to as "reverse mix".

Oxidation was followed by procedures described previously, except that in most of the runs a Gilson Microrespirometer was used instead of a Warburg Microrespirometer.

The pH adjustment was carried out by adding an aliquot of 0.1 N NaOH to the aqueous phase. The size of the aliquot was previously determined by potentiometric titration.

B. RESULTS AND DISCUSSION

1. Results

The effect of addition of amino carboxylic compounds on rate of oxidation in the model system was studied in 11 separate runs in which oxygen absorption was followed manometrically and the initial degree of oxidation was estimated by spectrophotometric determination of diene conjugation (Privett and Blank, 1962).

The composition of the model systems used in each of the runs is presented in Table 1, and the initial degree of oxidation for each treatment for each of the runs is presented in Table 2.

The oxygen absorption data during the course of oxidation are presented as follows: run 12 in Figure 1; run 13 in Figure 2; run 15 in Figure 3; run 16 in Figure 4; run 17 in Figure 5; run 19 in Figures 6 and 7; run 20 in Figures 8, 9 and 10; run 21 in Figures 11 and 12 and in Table 3; run 22 in Figures 13 and 14; run 23 in Figure 15 and in Table 4; and run 24 in Figure 16 and in Table 5.

In addition in order to compare the effects of various treatments with respect to control samples used in the same run, the following values were calculated and are presented in Table 6:

1. The times required by the control to reach oxidation levels of 10 mM oxygen/M linoleate and of 30 mM oxygen/M linoleate and the corresponding times required by the treated sample in the same run under identical conditions.

2. Relative degrees of oxygen absorption for each treatment with respect to its own control at two stages in the oxidation. This is calculated as the amount of oxygen absorbed by the

treated sample divided by the amount absorbed by the control at two different times; namely, when the control oxidation level is 10 mM/M and when it is 30 mM/M.

2. Discussion

The aim of the present investigation is the elucidation of the mechanism of antioxidant activity of amino carboxylic compounds in lipid-containing dehydrated systems. In previous work (Karel and Tannenbaum, 1965) it was observed that several amino carboxylic compounds had substantial antioxidant activity. Compounds for which such activity was observed included histidine, cysteine, alanine, lysine, β-amino butyric acid, γ-amino butyric acid, and ε-amino caproic acid; whereas no antioxidant activity was observed with methionine, arginine, phenylalanine and isoleucine.

The nature of the antioxidant activity of the amino compounds was found to be different from that observed in the identical system with a phenolic antioxidant since the main effect of the amino compound was to prolong the induction period. However, the phenolic compound prolonged the induction period; and in addition, reduced the rate of oxidation throughout the course of the oxidation.

In subsequent work it was determined that the antioxidant effects of the amino acids were extremely variable depending on the initial degree of oxidation of the linoleate, on the presence of impurities, as well as on some factors which were unknown but which apparently varied from run to run.

A survey of the literature in the field indicated that similar variations in the effectiveness of amino acids as antioxidants have been observed by almost all investigators studying this problem. A concise statement of the extent of this variation is given by Castell et al. (1966) who, in reviewing previous work, state:

"....many of the amino acids have antioxidant activity. The results are not always clearcut. Cysteine, for example, has been found more often to be pro-oxidant than antioxidant; certain others have been reported as being pro-oxidant in one experiment and antioxidant in another."

Among the variables which may cause this variation, and which are difficult to control in experimentation, are the following:

1. Nature of the fatty substrate and its degree of oxidation (Karel and Tannenbaum, 1965; Harris and Olcott, 1966; Castell et al., 1966)

2. Concentration of the amino acid (Marcuse, 1961; 1966)

3. pH (Marcuse, 1966; Castell et al., 1966).

The effect of pH was also shown to be important in the case of porphyrins which may act either as pro-oxidants or antioxidants (Matsushita and Iwami, 1965).

4. Presence of metals in trace concentrations, especially of cobalt, manganese, and copper which are apparently capable of causing complete inversion of amino acid antioxidant activity. However, the effects of these metals reported by different authors are quite contradictory. Marcuse (1966) reported that histidine

was strongly antioxidative in presence of low concentrations of copper, cobalt and manganese, but became pro-oxidative at high concentrations of metals. Saunders et al. (1962) found that histidine was pro-oxidative in their system, and that manganese increased this pro-oxidant effect; but that copper, cobalt, chromium and nickel had no effect. Castell et al. (1966), on the other hand, found that histidine as well as other amino acids were pro-oxidative when the copper concentration was low but became strongly antioxidative as the concentration of the metal increased.

In addition to these general effects creating problems in studies in solutions, solid model systems and in foods, there are specific problems arising from the use of a dehydrated model system based on a carbohydrate matrix of cellulose. These include:

1. The effect of the relative position and orientation of model system components on the rate of oxidation. Such effects were observed specifically in solid model systems by Togashi et al. (1961), Bishov et al. (1961), Trice (1965) and are implied in the work on emulsions by Coleman et al. (1964).

2. The effect of carbonyl groups and other reactive groups present in the cellulose which may vary considerably within a lot of the cellulose. The fact that such groups exist in cellulose has been shown by Lewin (1965) and a specific effect of cotton cellulose on rates of oxidation was shown by Frank and Roberts (1965). Pro-oxidant effects of soluble carbonyl compounds were shown by Anderson and Huntley (1964).

3. The possible effects of conversion of amino acids to new compounds with different antioxidant or pro-oxidant properties

from the parent compounds, either during preparation of the model system or during the autoxidation. As is shown in subsequent sections of this report we do, in fact, find that histidine may be converted to a new compound under our experimental conditions.

A conversion of aliphatic tertiary amines to strongly antioxidant compounds as a result of reaction with hydroperoxides was reported recently by Harris and Olcott (1966).

The experiments discussed below were undertaken in the hope of unraveling some of the peculiarities of the amino acid activity.

a. Comparison of activities of different amino acids within a given run. The results of run 12 indicated that β-amino butyric acid and γ-amino butyric acid, which have previously been shown to give antioxidant activity, gave a very definite pro-oxidant activity in this run.

The methyl linoleate used in these model systems, however, was not typical of the lipid normally obtained from our source of supply. This sample of lipid contained an impurity which caused emulsification at the benzene-water interface during extraction of the lipid from the urea adduct. This impurity did not allow adequate purification of the lipid, and its presence may have been responsible, in part, for the resultant pro-oxidant effect of the two test compounds.

This problem had occasionally occurred before, as noted in Report #3 (Karel and Tannenbaum, 1965). However, since this

run was completed, all of the methyl linoleate purchased from Mann Research Laboratories has contained this impurity and could not be purified properly.

Run 13 was undertaken with linoleate obtained from the Hormel Foundation. No difficulties were encountered in the purification of this batch; and the run was carried out on the seven amino acids which had previously given antioxidant activity, namely histidine, β-amino-n-butyric acid, alanine, γ-amino-n-butyric acid, ε-amino-n-caproic acid, cysteine, and lysine. A control sample and a sample containing propyl gallate were included in this run. All of the additives were tested in concentrations of 10^{-3} M/M linoleate.

The run gave a rather unexpected result. Four of the additives tested, ε-amino caproic acid, cysteine, lysine, and propyl gallate, gave good antioxidant activity. The other additives, however, acted as pro-oxidants. A detailed review of the experimental procedure showed that during the preparation of the model systems, two omni-mixer cups were used alternately to allow for washing of the cups after use. The four test compounds which acted as pro-oxidants were mixed in the first cup; the four which acted as antioxidants were mixed in the second cup. Examination of the first cup revealed a small hole in its stainless steel wall; this hole probably developed due to an impurity in the stainless steel sheet which was eventually completely dissolved out by the acid in our washing procedure. The presence of this hole probably allowed cooling water to enter the cup during mixing, thereby contaminating the samples.

The samples from the different batches were analyzed for cobalt and for iron, but no significant differences were found. (Cobalt analyses were included because the cups were used occasionally for preparation of model systems containing this metal.) Analyses for other metals were not undertaken.

Results of runs 12 and 13 have shown that, as suspected previously, the presence of impurities in the model system either as contaminants in the original linoleate preparation or as contaminants from preparation procedures, is capable of inverting the antioxidant activity of the amino acids.

Further work was directed to the study of conditions under which amino acids would show antioxidant activity and to try to isolate the factors negating such activity. All of the subsequent runs were conducted with either ε-amino caproic acid or histidine.

b. Effect of linoleate purification. One of the aims of the investigation was to improve the purification procedures for the linoleate. In run 15 which was conducted using histidine, a comparison was made between linoleate purified by column chromatography and that purified by regular procedures. In neither case was there a significant effect of histidine on oxidation rates.

In run 16 we used Applied Science Laboratories as the new source of methyl linoleate. The linoleate appeared to be of good quality, the initial degree of oxidation was low (Table 2), and no emulsifying impurity could be detected. However, histidine (10^{-3} M/M linoleate) did not show any significant antioxidant activity.

In run 17 another new source of methyl linoleate was explored. The linoleate was obtained from Sigma Chemical Company and was purified in the normal manner. The results of run 17 indicated that while propyl gallate exhibited antioxidant activity in later stages of oxidation, histidine was not an effective antioxidant.

Beginning with run 19 a new purification procedure was introduced based on two distillation steps as described in the section entitled "Experimental". This procedure was found to be very effective in reducing the extent of dieneconjugation after purification, and also (in runs 20-24) resulted in linoleate having considerable resistance to oxidation as evidenced by the very long time required to reach a given extent of oxidation. In spite of this standardization of linoleate preparation, the effect of histidine remained variable. In run 23 histidine showed a definite antioxidant effect but showed none in run 24 in spite of identical linoleate preparations.

c. Effect of added cobalt nitrate and of increase in pH.

Literature reports indicated that amino acid activity was strongly affected by metals. Castell et al. (1966), for instance, showed that at a concentration of 5 ppm of copper histidine was a pro-oxidant; but at a concentration of 15 ppm it was strongly antioxidant. Marcuse (1966) showed that addition of cobalt enhanced the antioxidant activity of amino acids.

In our system it was impossible to assure that the model system would always remain at the same trace metal content. It was decided, therefore, to conduct some experiments at a high

level of added metal in the hope that at this level variations in unintentional trace contaminants would be of little consequence. Ten ppm of cobalt nitrate were used in runs 16, 19 and 20. In run 16 histidine was the amino acid used; by itself it had little effect on autoxidation, but in combination with added cobalt it showed a pro-oxidant effect greater than cobalt alone.

In run 19 three different concentrations of ε-amino caproic acid were tested, in both the presence and absence of cobalt nitrate. The results of the run appear quite abnormal since, with one exception, all treated samples including those containing cobalt alone showed oxidation rates slower than those for the control. The one exception was the treatment containing cobalt and the highest concentration of the amino acid which showed a rate somewhat higher than that for the control.

The run was repeated with a fresh preparation of cobalt nitrate and at two different pH's (run 20). The results of that run showed the following:

1. When the pH was in the normal range (∿4.5), cobalt alone or cobalt in combination with ε-amino caproic acid gave a very strong pro-oxidant activity. A high concentration of the amino acid (10^{-2} M/M) gave some pro-oxidant activity. The lower concentration (10^{-3}) had a slight pro-oxidant effect.

2. At pH 9.5 cobalt alone or cobalt in combination with the amino acid showed extremely strong pro-oxidant activity. The amino acid alone had little effect.

It appears therefore, that addition of 10 ppm of cobalt fails to produce a consistent antioxidant effect of either histidine

or ε-amino caproic acid. The increase of pH to 9.5 fails to produce a consistent antioxidant effect of ε-amino caproic acid.

These results were disappointing, since the above measures might have been expected to produce drastic changes in the activity of amino acids, if the findings of Marcuse (1966) and Castell et al. (1966) could be applied to our system.

d. Effects of mixing procedure and of cellulose pre-washing.

Since the type of mixing achieved during the preparation of the model system might affect the relative position of the model system components, consideration was given to the possibility that mixing procedures affect the rate of oxidation. This was of importance also because of the fact that controls and treatment had to be prepared in separate mixes. In runs 21, 22, 23 and 24, therefore, several mixes were used for each of the treatments (control and amino acid-containing systems). The results, which are shown in detail for runs 21, 23 and 24 in Tables 3, 4 and 5, showed that the variation between the replicates of a given mix is of the same order of magnitude as the variation between mixes. While this confirms the reality of the antioxidant effects when observed in past runs, it sheds no light on the causes of lack of such effects in other runs.

A variation of the mixing procedure was tried in run 21 in which some of both the control and the treated samples were prepared by adding to the cellulose the aqueous phase before the addition of the linoleate. This change in procedure did not change the general effect of the amino acid (which was antioxidant to some extent).

Another procedure tried in run 22 was the washing of the cellulose with a series of solvents of increasing polarity prior to the preparation of the system. The results, which are shown in figures 11 and 12, indicate no substantial differences in oxidation rates or in amino acid effect between washed and unwashed cellulose. The results discussed above indicate that the variability in the antioxidant effect of amino acids could not be eliminated by purifying linoleate, by increasing the pH, by varying the mixing procedure, or by prewashing the cellulose. Furthermore, it appears that the variability occurs between runs, since even when several different mixes are used in a given run, the replicates of a given treatment tend to be comparable.

A similar unexplained variability was observed in work on conversion of histidine to an unknown compound ("slow component"; see Section III of this report). This conversion was found to occur even in absence of a lipid phase provided that cellulose was present. It seemed of interest, therefore, to study the effect of conversion of histidine on its antioxidant activity. This was done in runs 23 and 24. In run 23 two separate mixes were prepared for each of the two treatments (control and histidine); each mix was divided into four replicate samples. The results show a definite antioxidant effect of histidine. Analysis of the histidine-containing samples prior to oxidation showed no conversion of histidine to the "slow component".

In run 24 the experiment was repeated under similar conditions. Again there was good replication between mixes for the same treatment, but the amino acid showed no antioxidant activity, and

some pro-oxidant effect in later oxidation stages. Analysis of the histidine-containing samples showed the presence of the "slow component" in addition to histidine. The slow component was shown to be present prior to oxidation, and appeared to increase in concentration after oxidation.

Unfortunately, further experiments could not be undertaken because of completion of the contract period. The possibility that histidine, and possibly other amino acids may be converted to compounds with pro- or antioxidant properties vastly different from the parent compound seems worthy of further investigation.

III. REACTION PRODUCTS OF AMINO ACIDS IN OXIDIZING LINOLEATE SYSTEMS

As has been indicated in previous reports (Karel and Tannenbaum, 1965) it is necessary to understand the mechanism by means of which an amino acid may act as an oxidation inhibitor in order to control and predict its effectiveness. Investigations by numerous workers in this field have delineated four main mechanisms of antioxidant activity. These include:

1. Reaction of an inhibitor molecule (InH) with a peroxide to give a compound of the type InOOR (Campbell and Coppinger, 1952; Bickel and Kooyman, 1953; Bailey, 1962).

2. Reaction of InH with a peroxide radical to give a hydroperoxide and a stable inhibitor radical (In·)(Boozer et al., 1959; Ingold, 1961).

3. Reaction of InH with a hydroperoxide giving a ketone, a molecule of water, and the unchanged inhibitor (Shelton and Cox, 1954).

4. Chelation of pro-oxidative metal ions to inactive forms.

Experiments have been conducted with the freeze-dried histidine-methyl linoleate-cellulose system to determine which of the mechanisms above might be effective under the conditions of our investigations.

A. STUDIES WITH METHYL LINOLEATE-U-C^{14}

1. Experimental

The system (with or without 10^{-3} M histidine/M linoleate) was prepared and subjected to the standard column chromatographic procedure as previously reported (Karel and Tannenbaum, 1965). The major Fractions designated as I, II, and III were examined with thin layer chromatography on silica gel or cellulose powder using a variety of solvent systems.

A critical experiment to determine whether the major reaction products of histidine found in Fraction III are also reaction products of methyl linoleate was performed in the following manner. Two freeze-dried model systems were prepared in the conventional manner; only one of them contained histidine. Each system contained one gram methyl linoleate-U-C^{14} (29 uC) and was oxidized to a level of 0.075 M O_2/M linoleate. The systems were then packed into columns and eluted with the standard series of solvents to yield Fraction III which contains approximately 95% of the histidine in the original system. Fraction III from each system was concentrated into a small volume. Fifty per cent of each concentrate was spotted onto a cellulose thin layer plate and the plate eluted with n-butanol-acetic acid-water (25:6:25,

upper phase). When the chromatography was complete, the histidine reaction components were located on a guide strip of the plate with ninhydrin and diazotized sulfanilic acid, and the plate was subdivided into 1 cm sections which were scraped into a liquid scintillation counting vial for measurement of radioactivity.

2. Results and Discussion

There was no significant radioactivity above background radiation at the positions on the thin layer chromatogram of Fraction III which correspond to reaction products of histidine. The calculation of the sensitivity of the method for the detection of linoleate carbon in any histidine reaction product is given in Table 7.

Since the amount of radioactivity found on the thin layer plate is significantly below the level which would have been expected in either Case I, II, or III (Table 7), it can be concluded that none of the reaction products of histidine in Fraction III are also reaction products of methyl linoleate. This would apparently eliminate mechanism (a) from consideration as a major pathway of histidine antioxidant activity. A conclusion concerning other possible mechanisms cannot be reached on the basis of this experiment.

B. ORIGIN OF HISTIDINE REACTION PRODUCTS

Since the major histidine reaction product was clearly shown not to be also a reaction product of methyl linoleate, it was decided to further explore the possibility that another component of the system might be involved. Accordingly, studies were

conducted with histidine and water, and histidine, water and cellulose to determine whether the reaction product would be formed during any of the various stages of sample preparation. These include the stages of mixing, freezing, dehydration, and incubation. Each of these operations was conducted both in the absence and presence of oxygen, and separation and analysis of the reaction products were conducted through the column and thin layer chromatographic procedures which have been previously described.

1. Results and Discussion

The results of these studies indicate that the major histidine reaction product is not formed under a variety of conditions in the system histidine-water. The reaction product is, however, formed under a variety of conditions in the system histidine-water-cellulose as shown in Tables 8 and 9. In the studies conducted thus far the extent of product formation has been so variable (0-100%) as to be unpredictable. The product can, however, be formed simply by mixing the system; and oxygen does not seem to be a necessary component of the system.

An obvious question to ask is whether the reaction product has anti or pro-oxidative properties when linoleate is incorporated into the system. An attempt has been made to answer this question by chromatographic examination of oxidized model systems where data have been available on oxidation. Unfortunately present evidence is not sufficient to draw any conclusions. The answer

to this question would, however, appear sufficiently important to be considered for further research at some future date.

C. STRUCTURE OF HISTIDINE REACTION PRODUCT

Although a great deal of effort over the past year has gone into the elucidation of the structure of the histidine reaction product, this goal has not been accomplished. The major problems hindering this work have been the relatively low concentration of histidine in the system, and the difficulty in obtaining uncontaminated products from a cellulosic chromatographic system. The available evidence is summarized in the following sections.

1. Separation of Histidine from the Reaction Product

This topic has been thoroughly covered in a previous report (Karel and Tannenbaum, 1965). The two compounds are not separable by ion-exchange chromatography or by electrophoresis in alkaline systems. Separation has been achieved by chromatography and by electrophoresis in acidic systems. The best separations have been achieved by chromatography with butanol-acetic acid-water systems on thin layers of cellulose powder. Recovery from this system has been complicated by co-extraction of extraneous material from the cellulose.

2. Chemical Evidence

Both histidine and the reaction product give a positive test with ninhydrin and with diazotized sulfanilic acid. The response of the reaction product to ninhydrin appears to be somewhat slower than that of histidine. The visible absorption

spectra of the reaction product of diazotized sulfanilic acid with the histidine reaction product and with histidine are similar.

Prolonged refluxing of the histidine reaction product with constant boiling hydrochloric acid has no effect. Cold hydriodic acid, however, which does not react with histidine, does react with the histidine reaction product to give a partial regeneration of histidine. Reconsideration of this information in the light of the evidence that the reaction product is derived through a cellulose interaction leads to the inference that HI may cleave a bond between histidine and some cellulose-derived product. A possible route to the reaction product may, therefore, be a histidine catalyzed depolymerization of cellulose. Recent evidence indicates substantial reactivity of various groups in cellulose under a variety of conditions (Lewin, 1965).

3. Spectroscopic Evidence

Numerous attempts have been made to obtain spectroscopic evidence toward the interpretation of the structure of the reaction product. The ultraviolet absorption spectrum shows only end-absorption, similar to that of histidine.

Infra-red spectral analysis has been attempted via potassium bromide pellets prepared by a freeze-dehydration technique. Application of this technique has been found necessary because of the very small amounts of material available and the limited solubility in any solvent other than water. Good spectra of authentic samples of histidine have been obtained with this technique, although occasional difficulties arise due to unexplained

causes. The spectrum of the histidine reaction product invariably shows broad, poorly defined bands, which have thus been uninterpretable. A very broad, strong band is seen in the region of 9 microns. An attempt has been made to reduce possible cellulosic contamination by rechromatography of the reaction product recovered from a cellulose thin layer plate on a gel filtration column (Biogel, P-2). This resulted in a reduction of the intensity of the 9 micron band but not its disappearance. The evidence only tends to substantiate a histidine-cellulose reaction product.

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Table 1. Description of Run Conditions

Run #	Lot # of Methyl Linoleate	Additives*	Remarks
12	Mann - N1910	β-amino butyric acid γ-amino butyric acid	
13	Hormel - 2M	Histidine, lysine, cysteine, alanine, β-amino butyric acid, γ-amino butyric acid, ε-amino caproic acid, propyl gallate	
15	Hormel - 2M	Histidine	Column chromatography of linoleate for half of samples
16	Applied Science 580 - 65	Histidine, cobalt, cobalt & histidine	Cobalt concentration 10 ppm basis linoleate
17	Sigma 55B - 1660	Histidine, propyl gallate	
19	Laboratory preparation from pooled Hormel and Mann Lots	Cobalt, ε-amino caproic acid (10^{-2}, 10^{-3}, 10^{-4}), cobalt & ε-amino caproic acid (10^{-2}, 10^{-3}, 10^{-4})	Cobalt concentration 10 ppm basis linoleate
20	"	Cobalt, ε-amino caproic acid (10^{-2}, 10^{-3}), cobalt & ε-amino caproic acid (10^{-2})	2 sets - one at pH 4.8, one at pH 9.6, cobalt concentration 10 ppm basis linoleate
21	"	ε-amino caproic acid	3 mixes + "reverse mix"
22	"	ε-amino caproic acid	2 mixes each, washed and untreated cellulose

Table 1. (continued)

Run #	Lot # of Methyl Linoleate	Additives*	Remarks
23	Laboratory preparation from pooled Hormel and Mann Lots	Histidine	4 replicate samples for each mixing 2 mixings
24	"	Histidine, propyl gallate	3 replicate samples for each mixing 2 mixings

*The concentration of each of the additives is 10^{-3} M/M linoleate unless otherwise indicated.

Table 2. Initial Degree of Oxidation of Model Systems

Run #	Degree of oxidation (M O_2/ M linoleate) x 10^3		
	Control		Treated	
12		2.45	β-amino butyric acid*	3.08
			γ-amino butyric acid	3.55
13		1.20	Histidine	2.17
			β-amino butyric acid	4.74
			alanine	2.02
			γ-amino butyric acid	2.18
			ε-amino caproic acid	1.34
			cysteine	1.30
			lysine	1.08
			propyl gallate	1.65
15		6.50	Histidine	6.40
	Chromatographed	6.20	Histidine(chromatographed)	5.20
16		2.10	Histidine	2.40
			cobalt**	2.40
			cobalt & histidine	2.20
17		4.22	Histidine	2.16
			propyl gallate	3.35
19		4.58	Cobalt	4.37
			ε-amino caproic acid (10^{-2})	3.20
			(10^{-3})	2.69
			(10^{-4})	2.61
			cobalt & ε-amino caproic acid (10^{-2})	3.25
			(10^{-3})	2.64
			(10^{-4})	2.74
20		1.34	Cobalt	2.30
	pH 9.6	1.86	ε-amino caproic acid (10^{-2})	1.56
			(10^{-3})	0.86
			cobalt &ε-amino caproic acid (10^{-2})	1.40
			cobalt, pH 9.6	2.82
			ε-amino caproic acid (10^{-2}), pH 9.6	1.47
			(10^{-3}) pH 9.6	1.09
			cobalt & ε-amino caproic acid (10^{-2}), pH 9.6	2.03

Table 2. (continued)

Run #	Degree of oxidation (M O_2/M linoleate) x 10^3)			
	Control			Treated
21			ε-amino caproic acid	
	Mix #1	2.34	Mix #1	1.46
	Mix #2	2.30	Mix #2	1.51
	Mix #3	2.17	Mix #3	1.54
	"reverse mix"	2.21	"reverse mix"	1.58
22			ε-amino caproic acid	
	Mix #1	3.06	Mix #1	1.79
	Mix #2***	2.09	Mix #2***	1.41
	Mix #3	2.53	Mix #3	1.58
	Mix #4***	2.12	Mix #4***	1.76
23			Histidine	
	Mix #1	1.70	Mix #1	0.98
	Mix #2	1.76	Mix #2	1.06
24			Histidine	
	Mix #1	1.96	Mix #1	1.38
	Mix #2	2.04	Mix #2	1.65
			propyl gallate	1.28

* Amino carboxylic compounds or propyl gallate, where used, are present in concentration of 10^{-3} M/M linoleate unless otherwise specified.

**Cobalt, where used, is present in a concentration of 10 ppm - (basis:linoleate).

***"Washed avicel used".

Table 3. Oxygen Absorbed by Model Systems (Run 21)

Time days	Oxygen Absorption - (M O_2/M linoleate) x 10^3											
	Control						ε-Amino caproic					
	Mix 1		Mix 2		Mix 3		Mix 1		Mix 2		Mix 3	
	a	b	a	b	a	b	a	b	a	b	a	b
0	0	0	0	0	0	0	0	0	0	0	0	0
1	5.01	3.96	4.30	4.19	4.00	4.12	4.06	4.76	3.67	3.87	4.18	3.84
2	10.2	8.81	9.01	8.67	8.36	8.69	8.52	9.95	7.69	8.09	8.80	8.05
3	12.9	11.3	11.5	11.3	10.8	11.2	11.0	12.6	9.96	10.5	11.4	10.3
4	18.0	15.8	15.5	15.5	14.3	15.4	14.7	17.6	13.1	14.7	15.0	14.4
5	20.2	17.8	17.2	17.3	15.8	17.4	16.4	19.8	14.4	16.7	16.6	16.1
6	21.0	18.6	17.5	17.9	15.9	17.8	16.6	20.4	14.5	17.5	16.6	16.5
7	21.9	19.3	18.1	18.5	16.5	18.3	17.3	20.5	15.0	18.4	17.1	16.7
8	24.9	22.1	20.9	21.0		20.8	20.0	23.5	17.2	21.4	19.5	19.1
9	28.8	25.6	24.3	23.9		23.7	22.8	27.0	19.5	24.8	22.3	21.9
10	33.0	29.2	27.9	26.9		27.1	25.9	31.0	22.1	28.4	25.2	24.7
11	38.3	34.3	33.3	31.0		31.8	30.6	36.4	25.9	33.6	29.6	28.7
12	43.0	38.3	37.1	33.9		36.2	33.6	40.9	28.4	38.3	32.5	31.7
13	48.4	43.2	41.6	37.1		41.8	36.2	46.3	31.1	44.5	35.4	35.2
14	54.1	48.1	45.1	39.2		48.7	39.0	51.3	32.3	51.4	37.0	37.8
15	65.4	56.4	51.8	42.7			42.8	59.9	34.8	63.0	39.9	41.9
16	85.6	68.1	61.3	46.4			46.1	71.3	36.7	80.1	41.5	46.2
17	122	85.4	76.8	51.5			50.1	89.9	38.5	105	43.8	52.2

Continued/

Table 3. (continued)

Time days	Reverse mix Control a	Control b	ε-amino caproic acid a	ε-amino caproic acid b
0	0	0	0	0
1	3.84	3.37	3.17	3.63
2	7.93	7.30	6.02	8.11
3	10.5	9.55	8.00	10.6
4	14.1	13.4	10.7	14.8
5	16.0	15.2	12.2	16.5
6	16.6	16.0	12.9	16.8
7	17.4	16.9	13.6	17.6
8	20.6	19.6	15.6	20.1
9	24.3	22.8	18.1	22.8
10	28.8	26.5	20.6	25.8
11	35.7	31.9	24.0	29.6
12	42.2	38.5	26.7	32.6
13	50.6	48.9	29.8	35.7
14	60.3	64.0	32.6	37.5
15	76.9	87.0	36.9	40.4
16	103	120	42.9	42.1
17			53.1	44.8

Table 4. Oxygen Absorption by Model System (Run 23)

Time days	Oxygen absorption - (M O_2/M linoleate) x 10^3															
	Control								Histidine							
	Mix 1				Mix 2				Mix 1				Mix 2			
	1	2	3	4	1	2	3	4	1	2	3	1	2	3	4
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	2.87	2.05	3.08	2.47	2.39	2.22	2.29	2.42	2.80	2.54	2.20	2.14	2.16	2.01	2.76
2	6.86	5.46	7.00	6.39	5.79	5.46	5.12	5.36	6.15	5.89	4.79	4.77	4.92	4.72	6.31
3	10.6	8.70	11.0	9.93	9.10	8.72	7.72	8.24	8.94	9.07	7.31	7.34	7.40	7.19	9.59
4	16.2	13.9	17.3	15.4	14.6	14.0	11.6	12.7	13.2	13.3	11.1	11.0	11.1	10.5	14.2
5	25.8	24.3	28.8	25.0	24.6	24.1	16.8	19.7	18.9	20.7	17.2	16.5	17.6	15.4	22.1
6	36.7	38.2	46.7	37.5	39.1	36.7	20.2	27.1	23.3	28.1	23.4	21.7	24.8	19.2	29.8
7	84.3	96.2		92.1	100	84.8	29.0	55.6	35.1	53.4	45.8	40.0	53.2	29.3	58.3
8							55.2		70.8					62.9	

Table 5. Oxygen Absorption by Model System (Run 24)

Time days	Oxygen Absorbed - (M O_2/M linoleate) x 10^3													
	Control						Histidine						Propyl gallate	
	Mix 1			Mix 2			Mix 1			Mix 2				
	1	2	3	1	2	3	1	2	3	1	2	3	1	2
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	2.25	2.60	2.83	3.23	2.89	3.20	2.92	2.62	2.95	2.79	2.99	2.93	2.66	3.58
2	5.42	5.81	6.22	7.27	6.22	6.88	6.38	5.44	6.46	5.84	6.43	6.05	5.90	8.52
3	8.52	8.89	9.68	11.3	9.60	10.6	9.78	8.44	9.85	9.06	9.68	9.15	9.43	14.0
4	12.1	12.5	14.0	16.2	13.5	14.7	14.3	12.5	14.1	13.1	13.5	13.1	13.8	20.6
5	17.4	17.4	20.6	24.1	18.9	20.7	22.0	19.9	21.0	19.5	19.0	19.3	20.3	30.4
6	25.2	24.3	32.2	38.3	26.3	29.0	42.8	37.9	35.6	34.0	27.5	32.4	28.3	44.5
7	50.2	44.6	72.9	77.8	44.6	54.4		99.9	90.7	97.2	52.7	80.8	43.2	74.1
8													81.1	

Table 6. Summary of Effects of Amino Acids on Oxidation

Run #	Additive (A.A. concentrations in M/M linoleate)	Average time to reach the specified oxidation level (hrs) to 10 mM O_2/M Control	to 10 mM O_2/M Treated	to 30 mM O_2/M Control	to 30 mM O_2/M Treated	Oxygen absorption relative to control: Control level of 10 mM/M	Control level of 30 mM/M
13	Histidine, 10^{-3}	18.4	10.7	27.4	16.4	3.95	3.2
15	" 10^{-3}	25	25	36.8	36.8	1.00	1.0
16	" 10^{-3}	15	15	24.8	24.8	1.00	1.0
17	" 10^{-3}	32.5	32.5	57.5	62	1.00	0.82
23	" 10^{-3}	67	78	130	154	0.86	0.66
24	" 10^{-3}	75	75	139	135	1.00	1.18
16	Histidine, 10^{-3} + Co 10 ppm	15	10.6	24.8	17.3	2.04	2.4
13	ε-amino caproic acid, 10^{-3}	18.4	22.6	27.4	32.3	0.61	0.6
19	" 10^{-2}	23.5	33	52.5	68	0.66	0.61
19	" 10^{-3}	23.5	41	52.5	77.5	0.66	0.44
19	" 10^{-4}	23.5	33	52.5	67.5	0.74	0.64
20	" 10^{-2}	40	29	60.5	43	2.42	3.66
20	" 10^{-3}	40	38	60.5	55.5	1.16	1.65
21	" 10^{-3}	58	58	238	290	1.00	0.77
22	" 10^{-3}	65	65	175	180	1.00	0.88
20	" 10^{-2}, pH 9.5	20	20	28.5	28.5	0.76	0.94
20	" 10^{-3}, pH 9.5	20	20	28.5	27.5	1.10	1.10
19	ε-amino caproic acid, 10^{-2}, + Co 10 ppm	23.5	23.5	52.5	43.5	1.0	1.66
19	10^{-3}, + Co 10 ppm	23.5	28.5	52.5	60	0.82	0.79
19	10^{-4}, + Co 10 ppm	23.5	32	52.5	59.5	0.66	0.89
20	10^{-2}, + Co 10 ppm	40	20	60.5	28.5	8.75	--
20	10^{-2}, + Co 10 ppm, pH 9.5	20	0.5	28.5	1.5	>>10	>>10
16	Co 10 ppm	15	11.4	24.8	18.8	1.65	1.95
19	" 10 ppm	23.5	33	52.5	62.5	0.74	0.75
20	" 10 ppm	40	20	60.5	28.5	10	--
20	" 10 ppm, pH 9.5	20	0.5	28.5	1.5	>>10	>>10

Table 6. (continued)

Run #	Additive (A.A. concentrations in M/M linoleate)	Average time to reach the specified oxidation level (hrs) to 10 mM O_2/M Control	to 10 mM O_2/M Treated	to 30 mM O_2/M Control	to 30 mM O_2/M Treated	Oxygen absorption relative to control: Control level of 10 mM/M	Control level of 30 mM/M
12	β-amino butyric acid, 10^{-3}	23.2	17.8	33.2	26.8	2.03	1.87
13	" 10^{-3}	18.4	11	27.4	17.9	3.2	2.6
12	γ-amino butyric acid, 10^{-3}	23.2	18.5	33.2	28	1.76	1.67
13	" 10^{-3}	18.4	15.6	27.4	24	1.51	1.45
13	Alanine, 10^{-3}	18.4	16.4	27.4	25	1.34	1.27
13	Cysteine, 10^{-3}	18.4	25.2	27.4	35.7	0.51	0.44
13	Lysine, 10^{-3}	18.4	28.4	27.4	38.4	0.32	0.30
13	Propyl gallate, 10^{-3}	18.4	36	27.4	--	0.26	0.22
17	" 10^{-3}	32.5	32.5	57	76	1.00	0.66

Table 7. Calculation of the Sensitivity of Detection of Methyl Linoleate Carbon in the Histidine Reaction Products

1. specific activity of methyl linoleate = 29 uC/g
   = 8.7 uC/mM
2. concentration of histidine = 10^{-3} M/M linoleate
3. size of experimental sample = 3 1/3 mM linoleate
   + 3 1/3 x 10^{-3} mM histidine
4. amount of sample placed on TLC plate is 50% of Fraction III
5. on the basis of previous work with radioactive histidine, 2/3 of the histidine has been converted to the major reaction product
6. assume 25% overall extraction and counting efficiency for radioactive material on TLC plate

Case I. assume a 1:1 molecular reaction product of histidine and methyl linoleate (or derived product); then amount of radioactivity on TLC plate corresponding to the reaction product would be (8.7 uC/mM)(10/3 x 10^{-3} mM)(1/2 of sample) (1/4 efficiency)(2/3 of histidine in product) = 5400 cpm

Case II. assume one molecule of histidine reacts with a one carbon atom product derived from linoleate; then amount of radioactivity on TLC plate corresponding to the reaction product would be 5400 cpm/18 carbon atoms per molecule = 300 cpm

Case III. assume a 1.1 molecular reaction between histidine and linoleate (or derived product) in which only 1% of the histidine has reacted: then amount of radioactivity on TLC plate associated with any histidine derived product would be (5400 cpm)(1/60 of amount of histidine assumed in Case I) = 90 cpm

Table 8. Conversion of Histidine in Different Experiments Using Similar Conditions

Experiment number	% Conversion of histidine			
	Wet system	Freeze-dried system	Freeze-dried system incubated in air (48 hrs, 37°C)	
	HA*	HA*	HA*	HLA**
1	-	-	-	40
2	-	-	-	30
3	-	20	35	-
4	0	0	-	-
5	-	-	35	-
6	-	-	97	-
7 (pH 9.5)	-	-	62	-
8	83	20	30	-

*Histidine + Avicel = HA

**Histidine + Avicel + Methyl Linoleate = HLA

Table 9. Conversion of Histidine in an Experiment Using Lipid-Free Systems with Different Types of Cellulose

Type of cellulose	% conversion		
	After mixing & incubation in wet state	After freeze-drying	After freeze-drying & incubation in air. (37°C)
Avicel	30	n.a.	n.a.
Washed Avicel	35	20	20
Chromatographic cellulose	35	20	20
Filter paper	30	n.a.	n.a.
Cotton	70	n.a.	n.a.

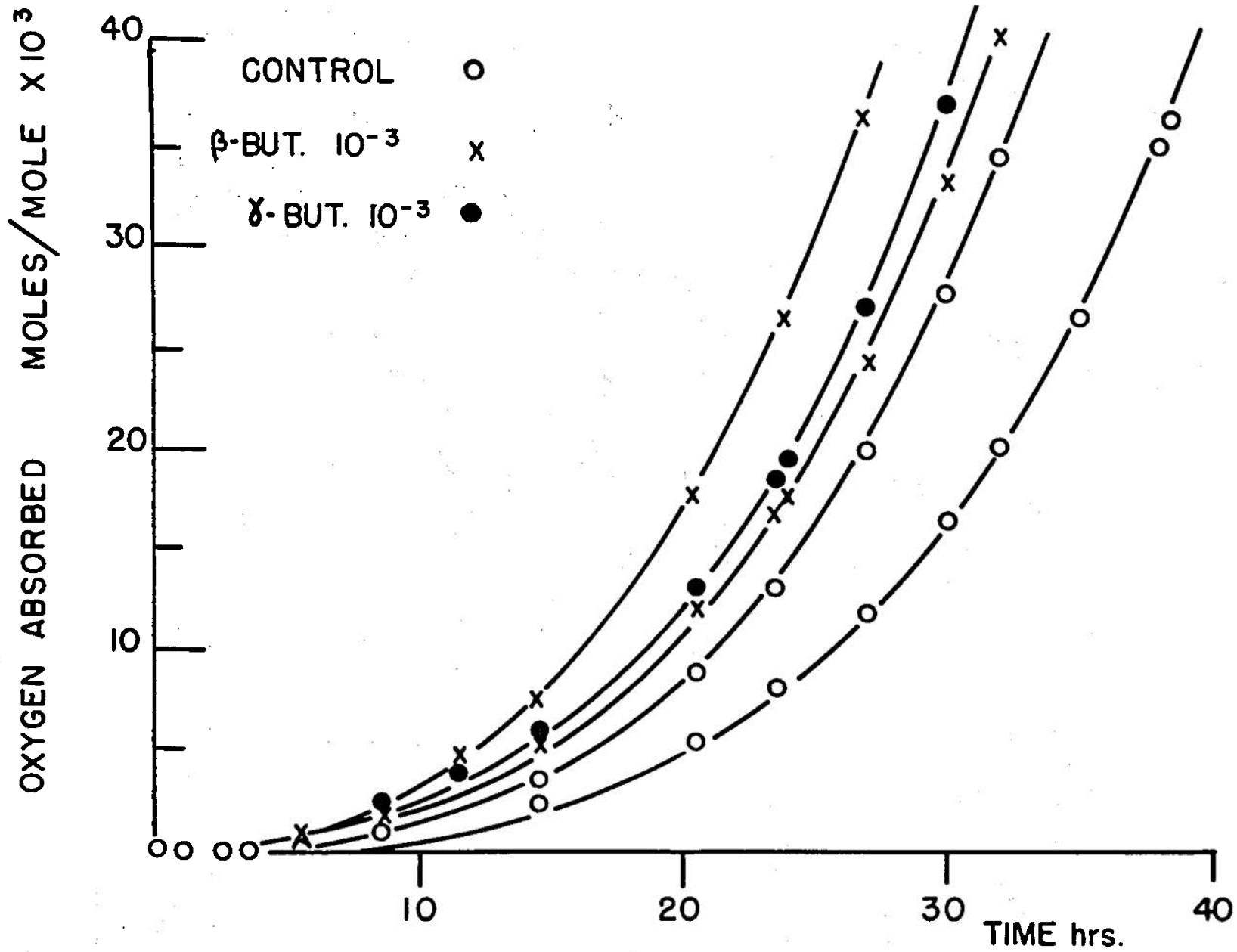

Figure 1. Oxygen absorbed by model system. Run 12
(Additives: β-amino butyric acid, γ-amino butyric acid)

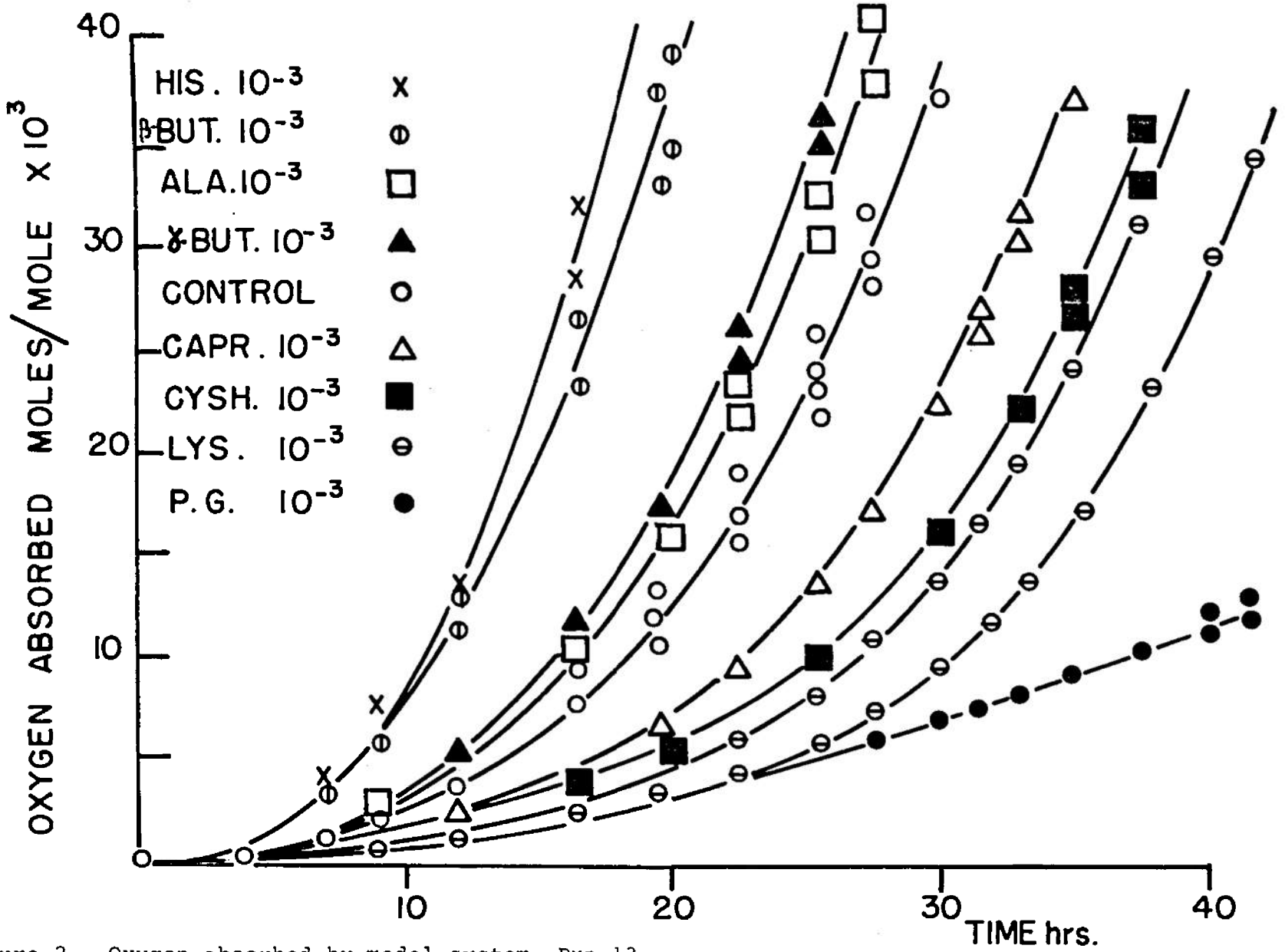

Figure 2. Oxygen absorbed by model system. Run 13
(Additives: histidine, β-amino butyric acid, γ-amino butyric acid, alanine, ε-amino-n-caproic acid, cysteine, lysine, and propyl gallate)

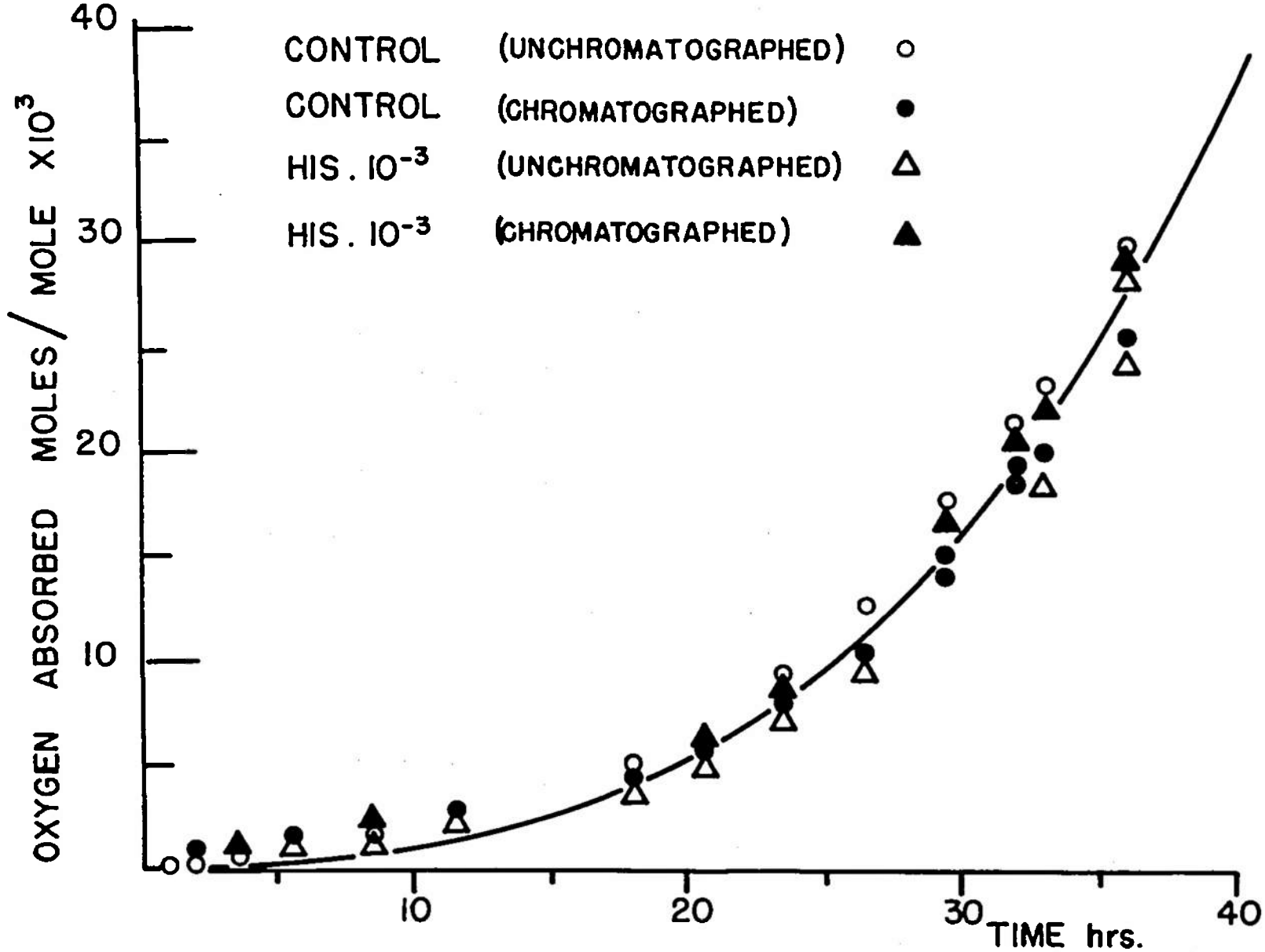

Figure 3. Oxygen absorbed by model system. Run 15
(Additive: histidine)

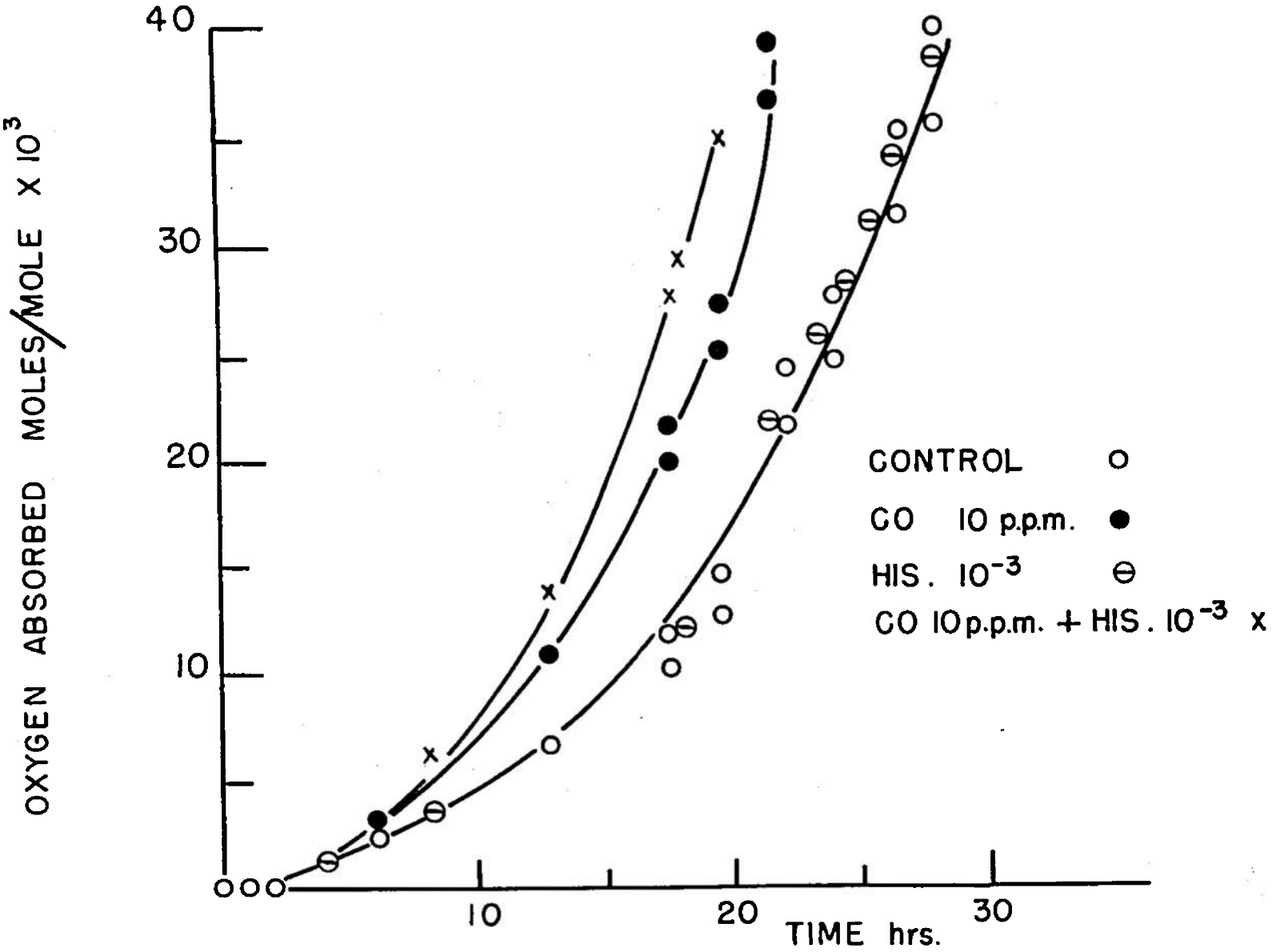

Figure 4. Oxygen absorbed by model system. Run 16

(Additives: histidine and cobalt nitrate)

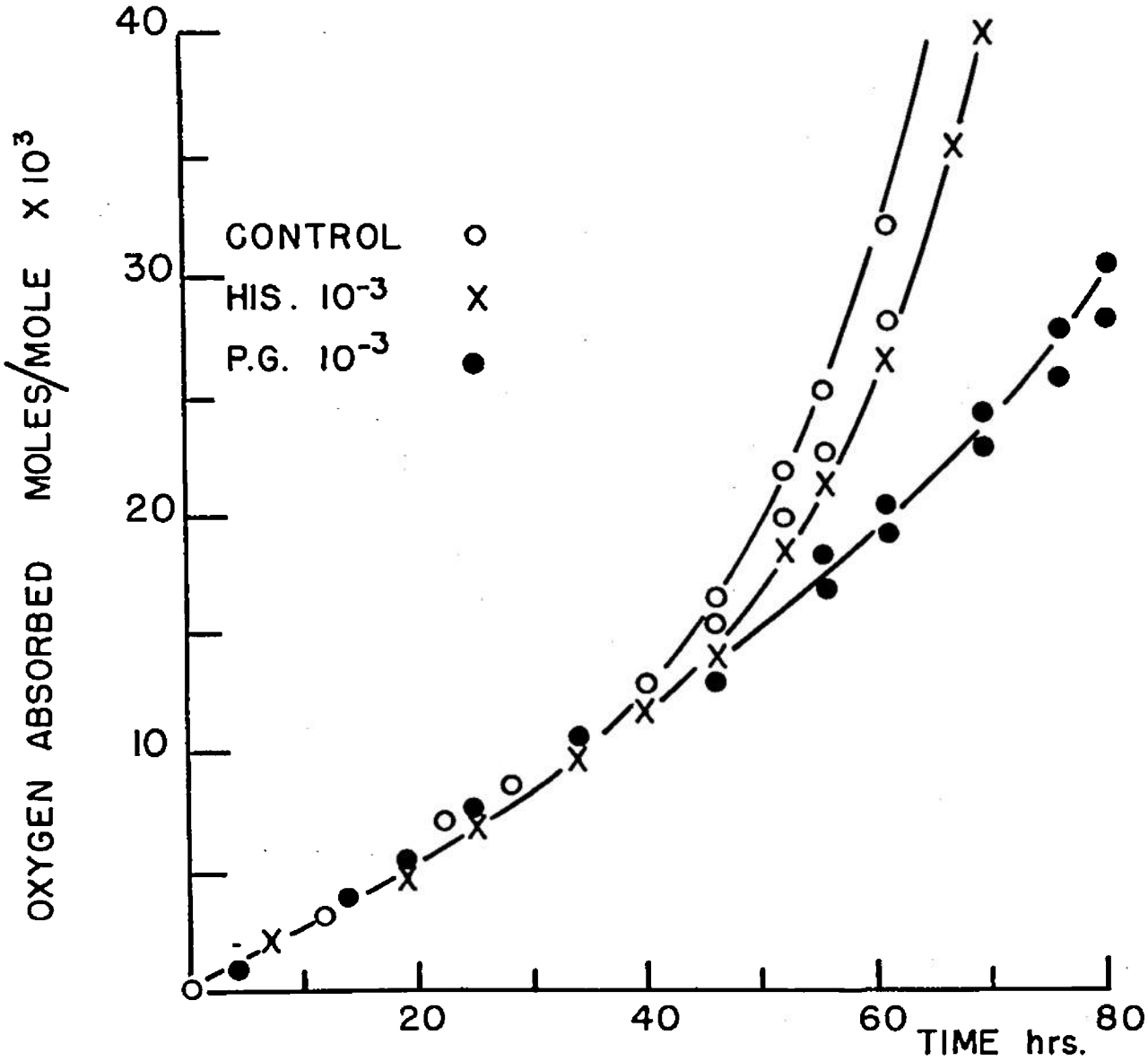

Figure 5. Oxygen absorbed by model system. Run 17

(Additives: histidine and propyl gallate)

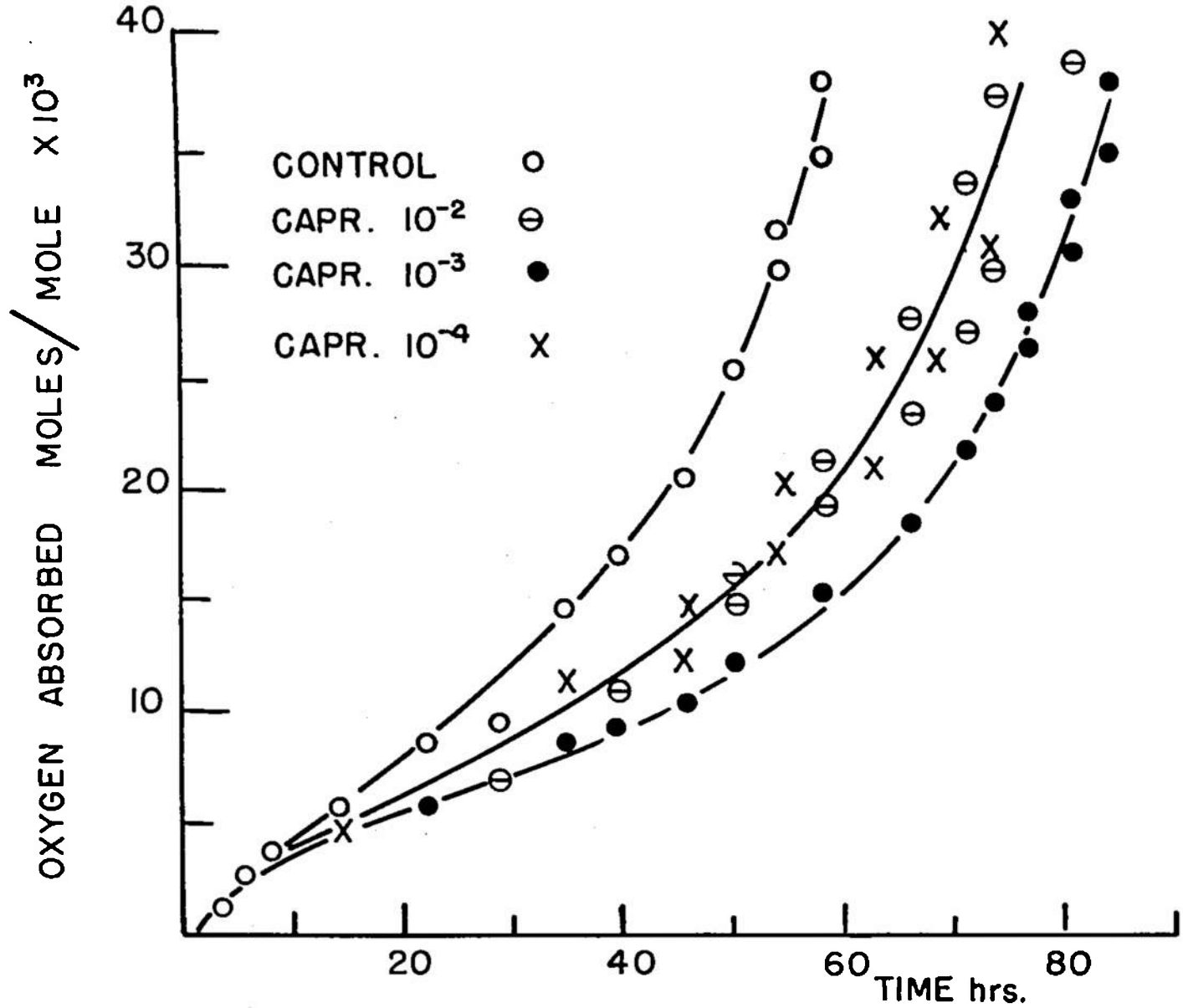

Figure 6. Oxygen absorbed by model system. Run 19

(Additives: ε-amino-n-caproic acid)

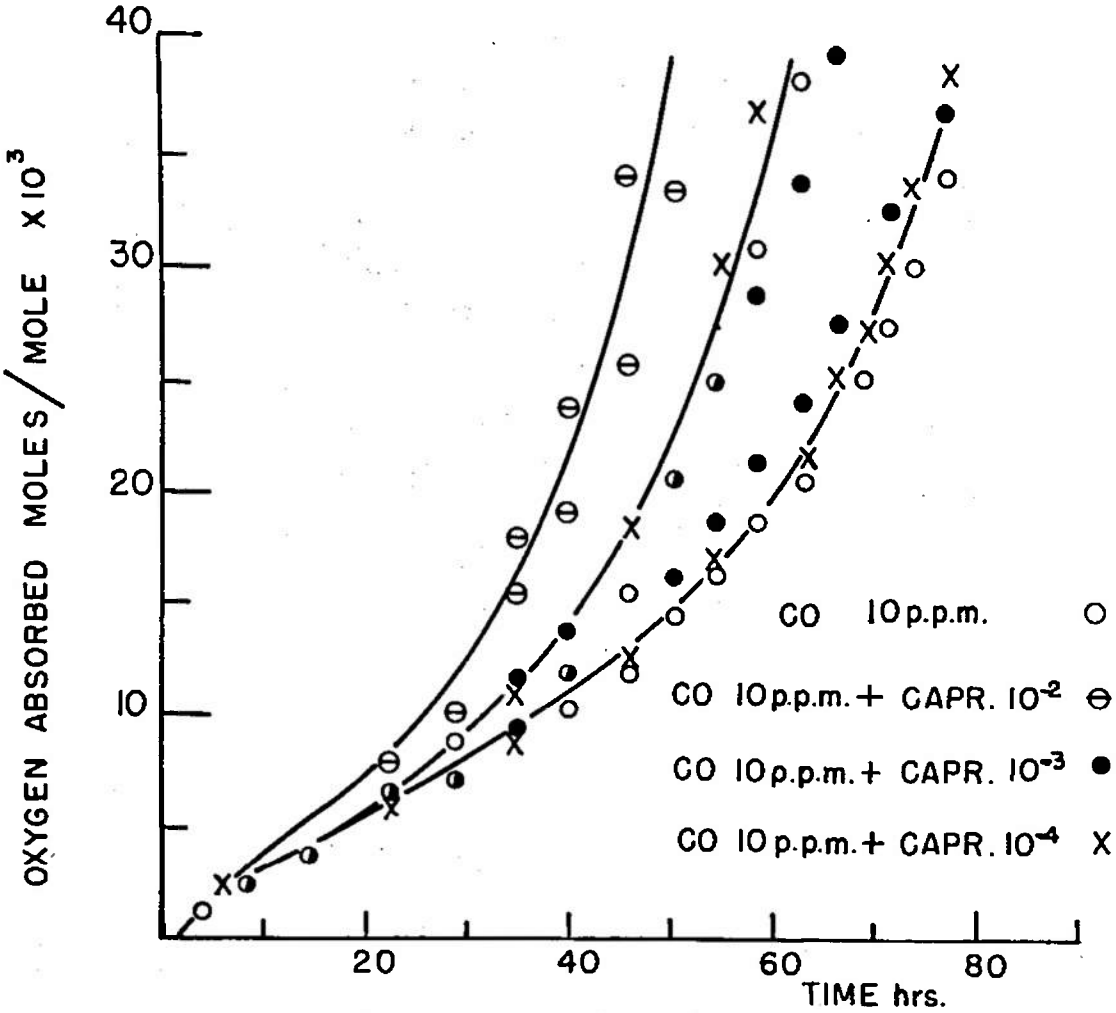

Figure 7. Oxygen absorbed by model system. Run 19
(Additives: ε-amino-n-caproic acid and
cobalt nitrate)

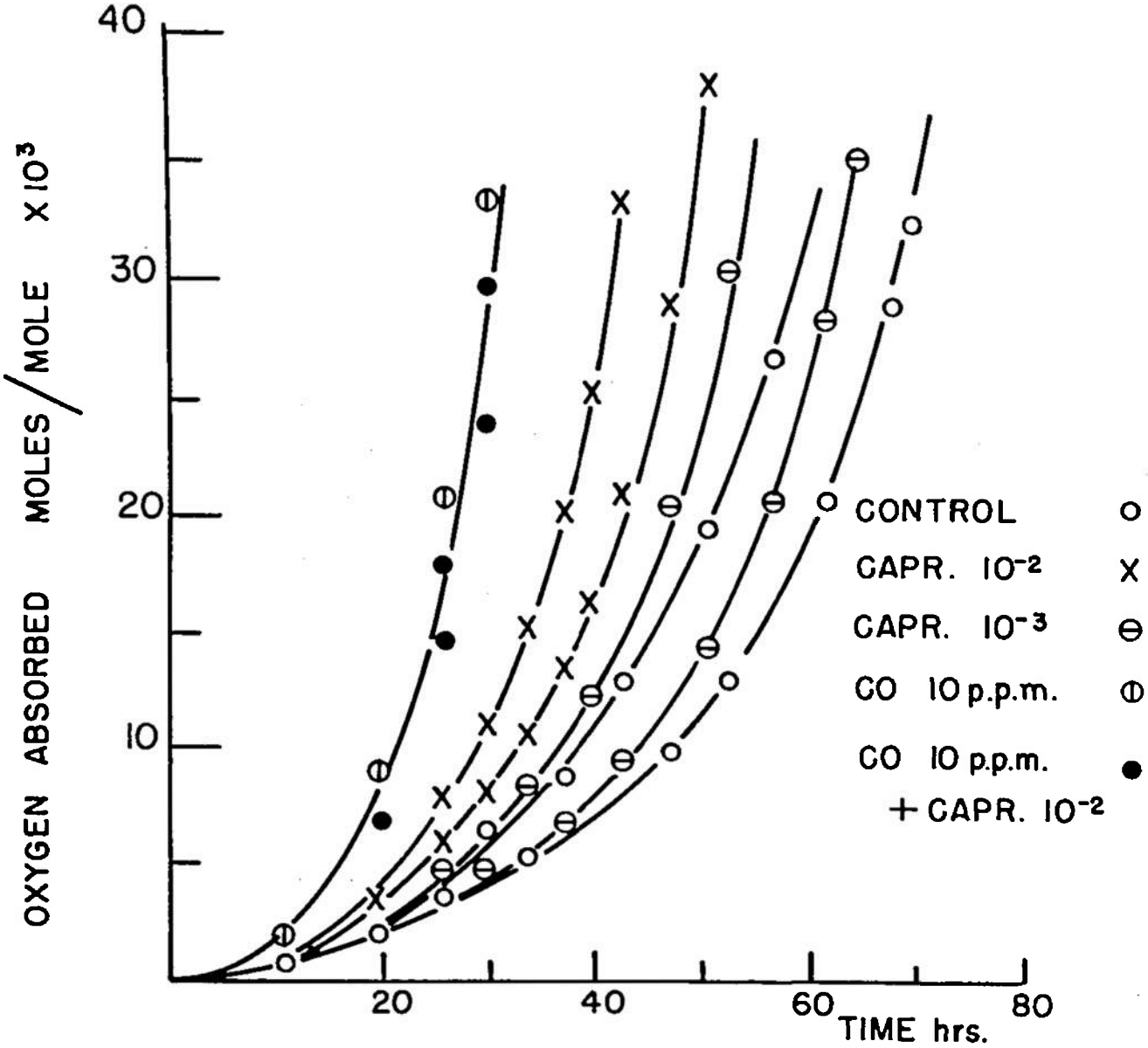

Figure 8. Oxygen absorbed by model system. Run 20 (Additives: ε-amino-n-caproic acid and cobalt nitrate)

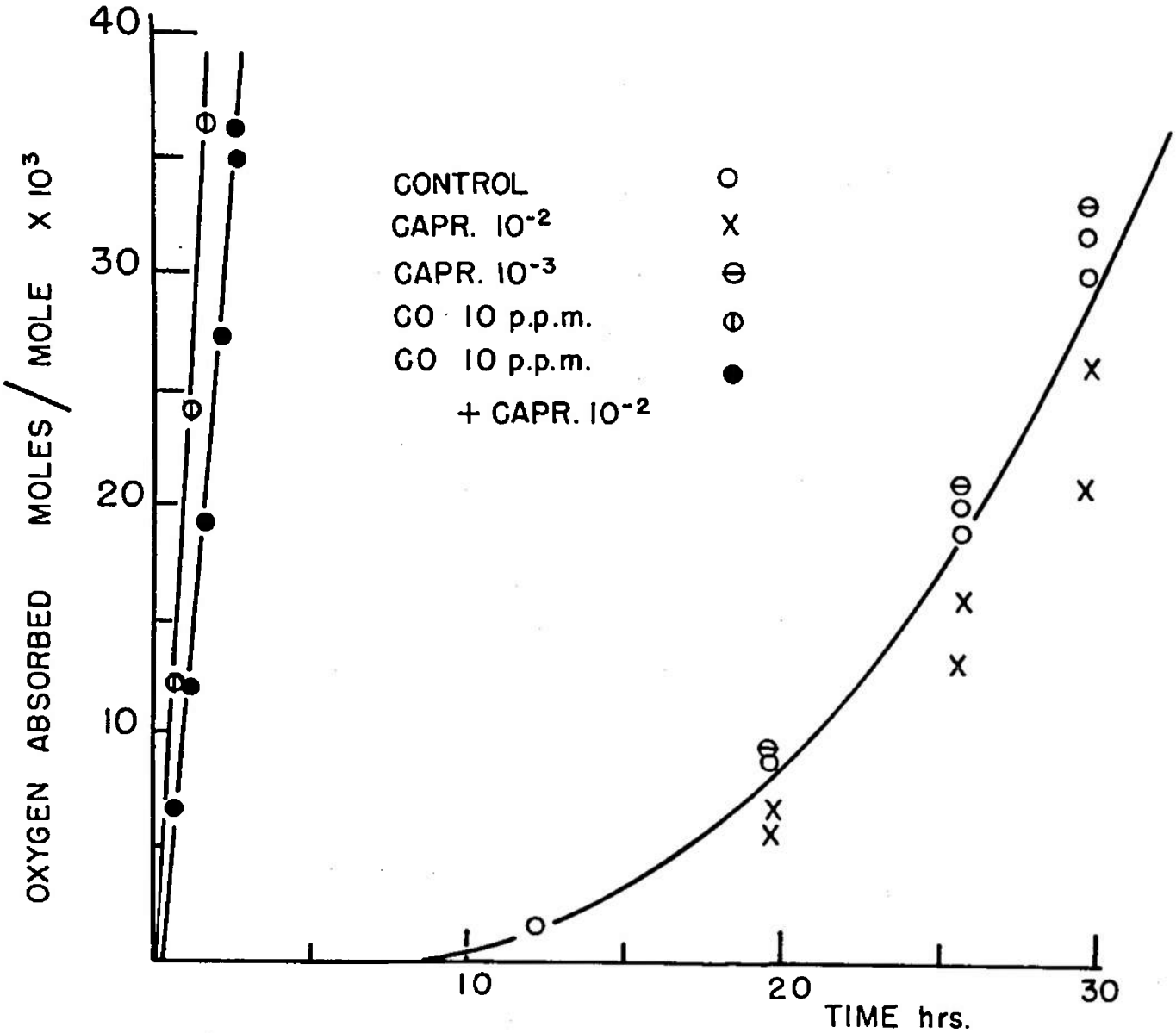

Figure 9. Oxygen absorbed by model system. Run 20
(System adjusted to pH 9.5. Additives:
ε-amino-n-caproic acid and cobalt nitrate)

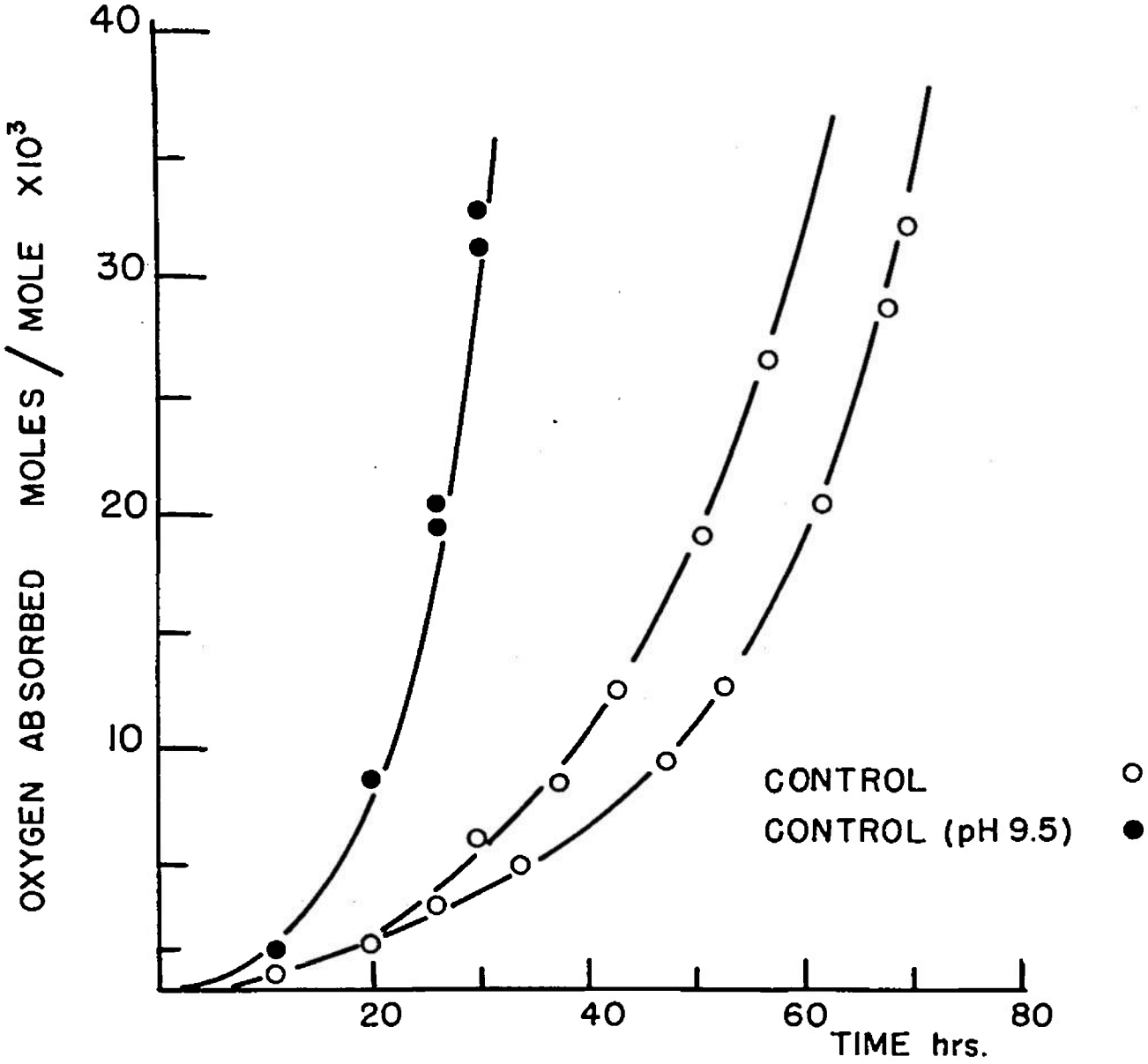

Figure 10. Oxygen absorbed by model system. Run 20
(Comparison of controls for the normal system,
and the system adjusted to pH 9.5)

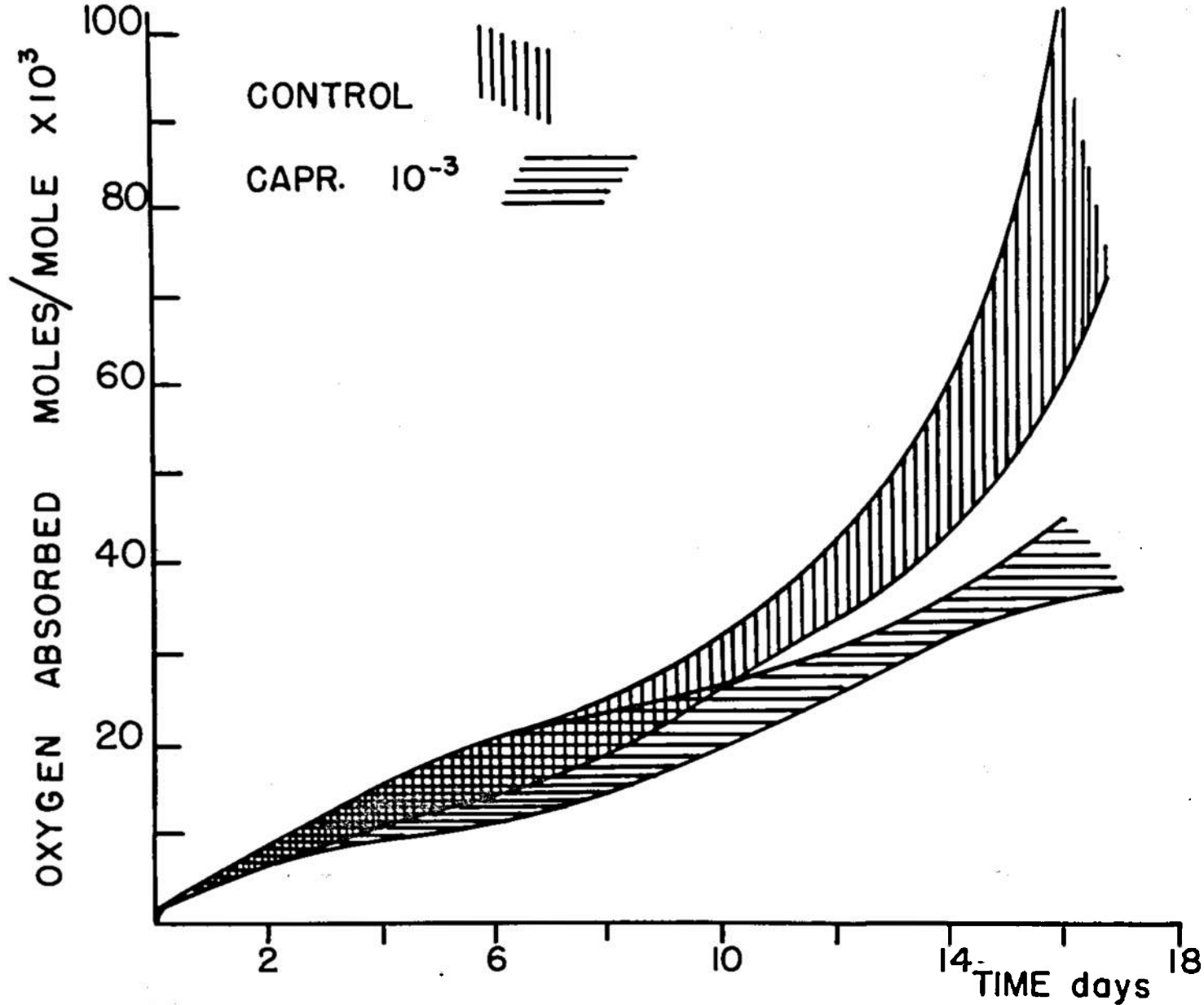

Figure 11. Oxygen absorbed by model system. Run 21
(Additive: ε-amino caproic acid)

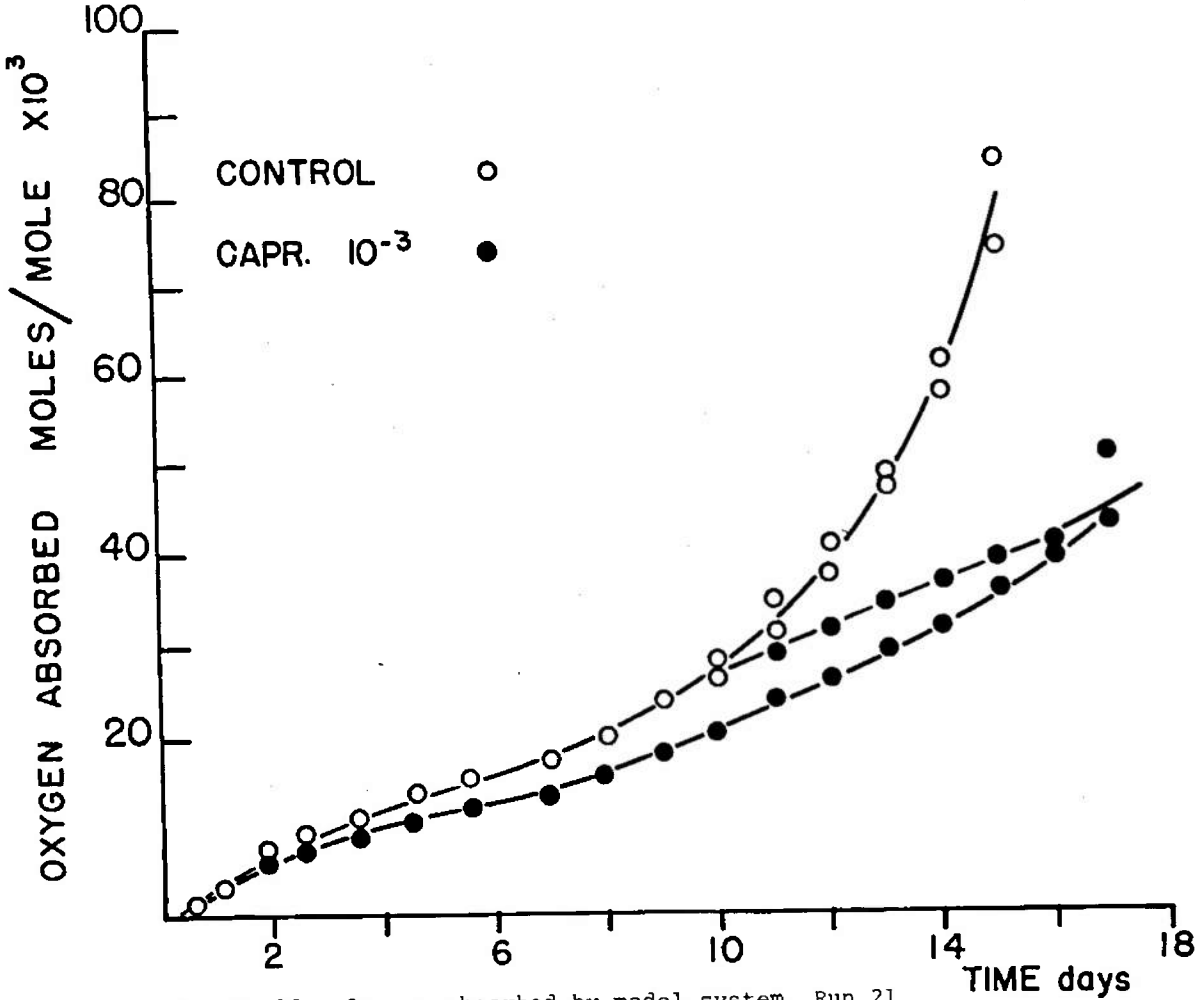

Figure 12. Oxygen absorbed by model system. Run 21
(Additive: ε-amino caproic acid; reverse mix samples only)

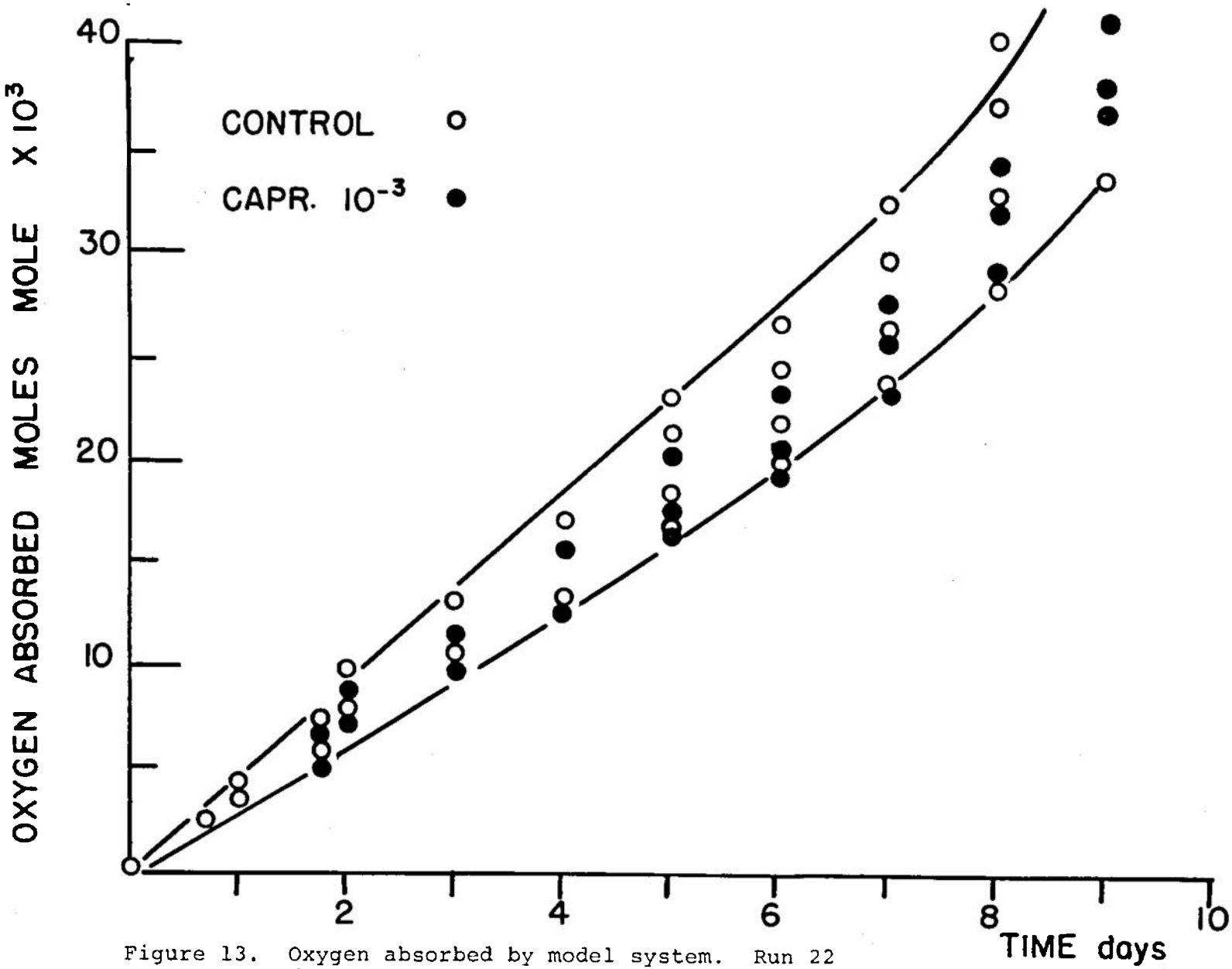

Figure 13. Oxygen absorbed by model system. Run 22
(Additive: ε-amino caproic acid; unwashed cellulose)

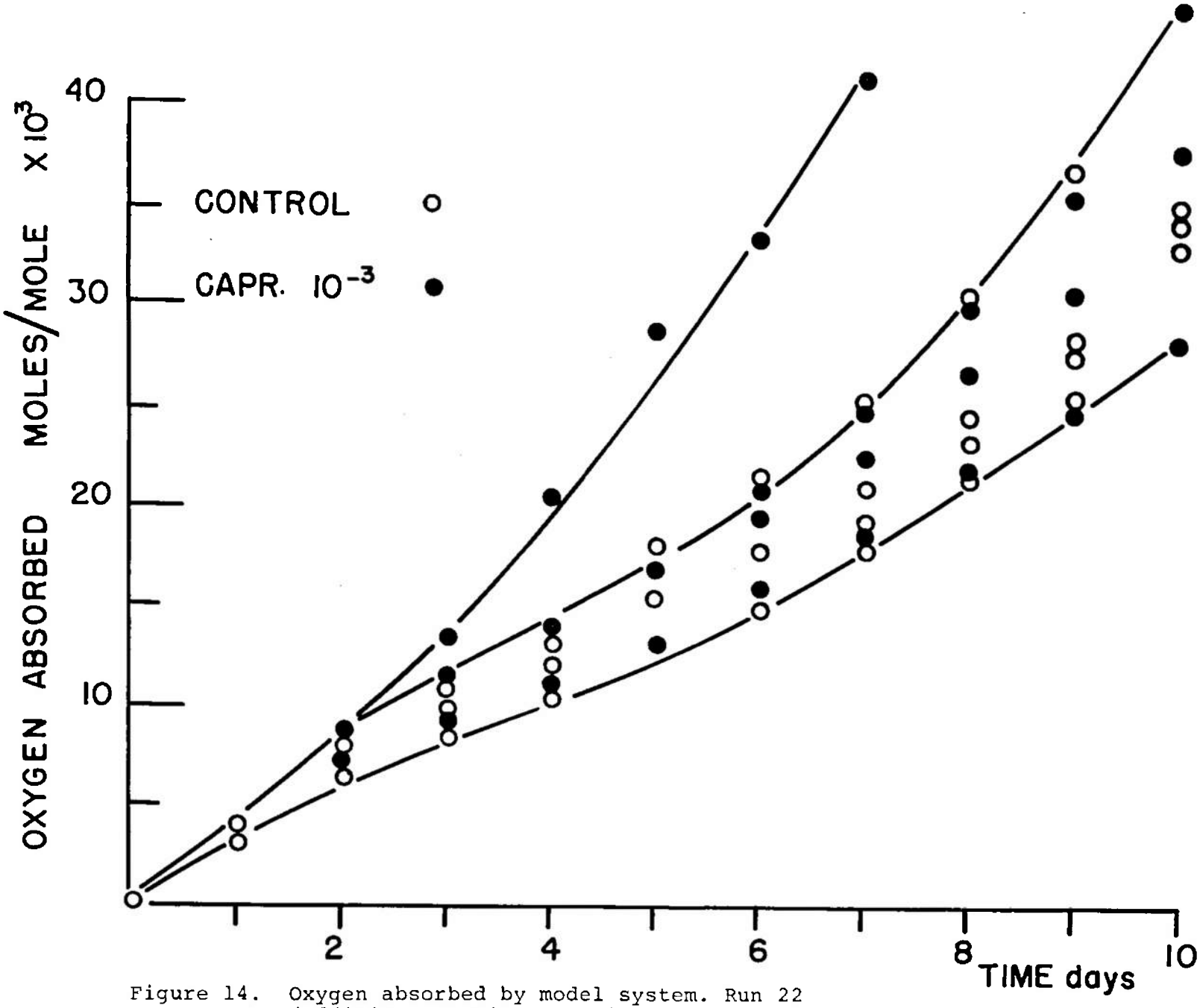

Figure 14. Oxygen absorbed by model system. Run 22
(Additive: ε-amino caproic acid; washed cellulose)

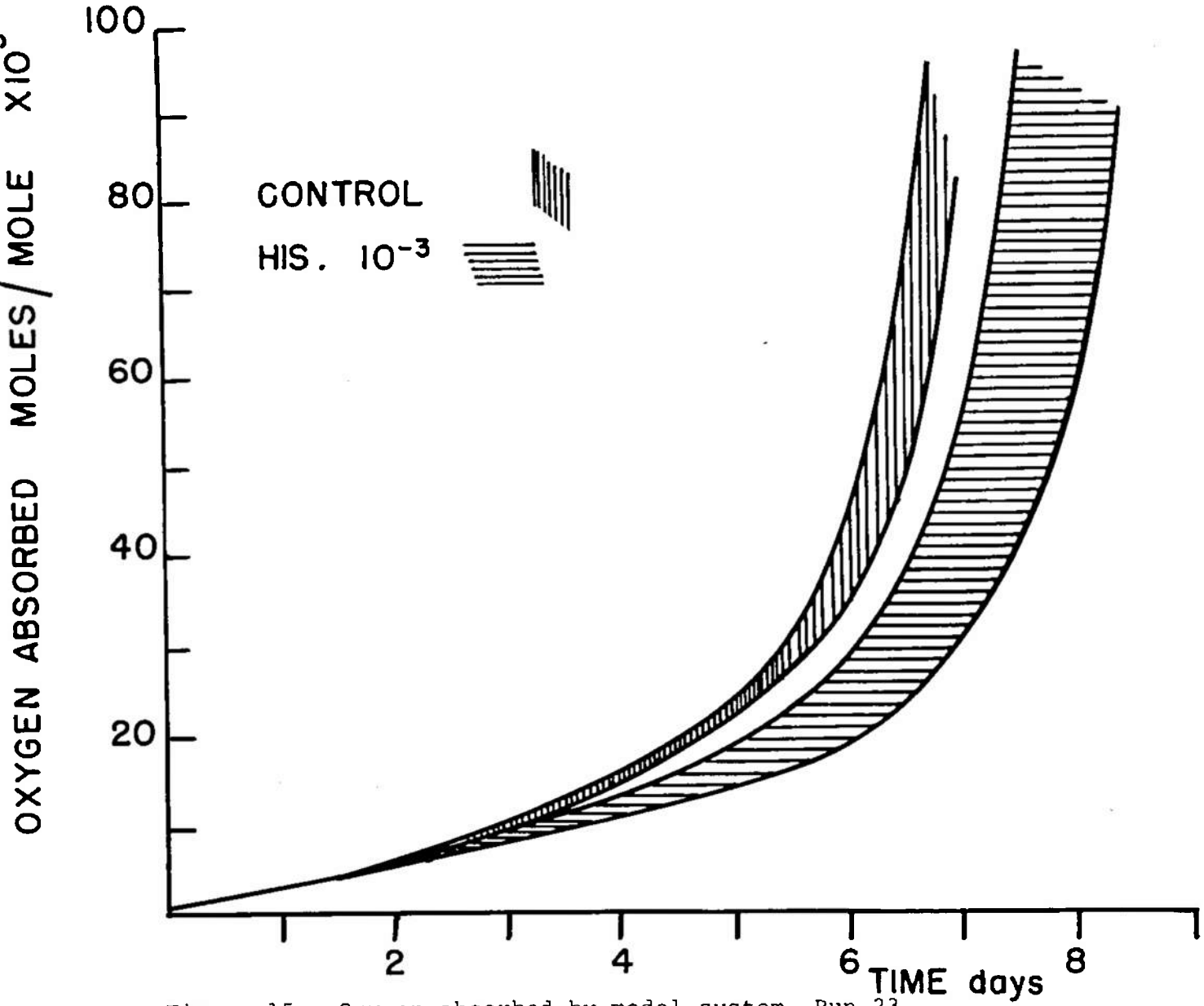

Figure 15. Oxygen absorbed by model system. Run 23 (Additive: histidine)

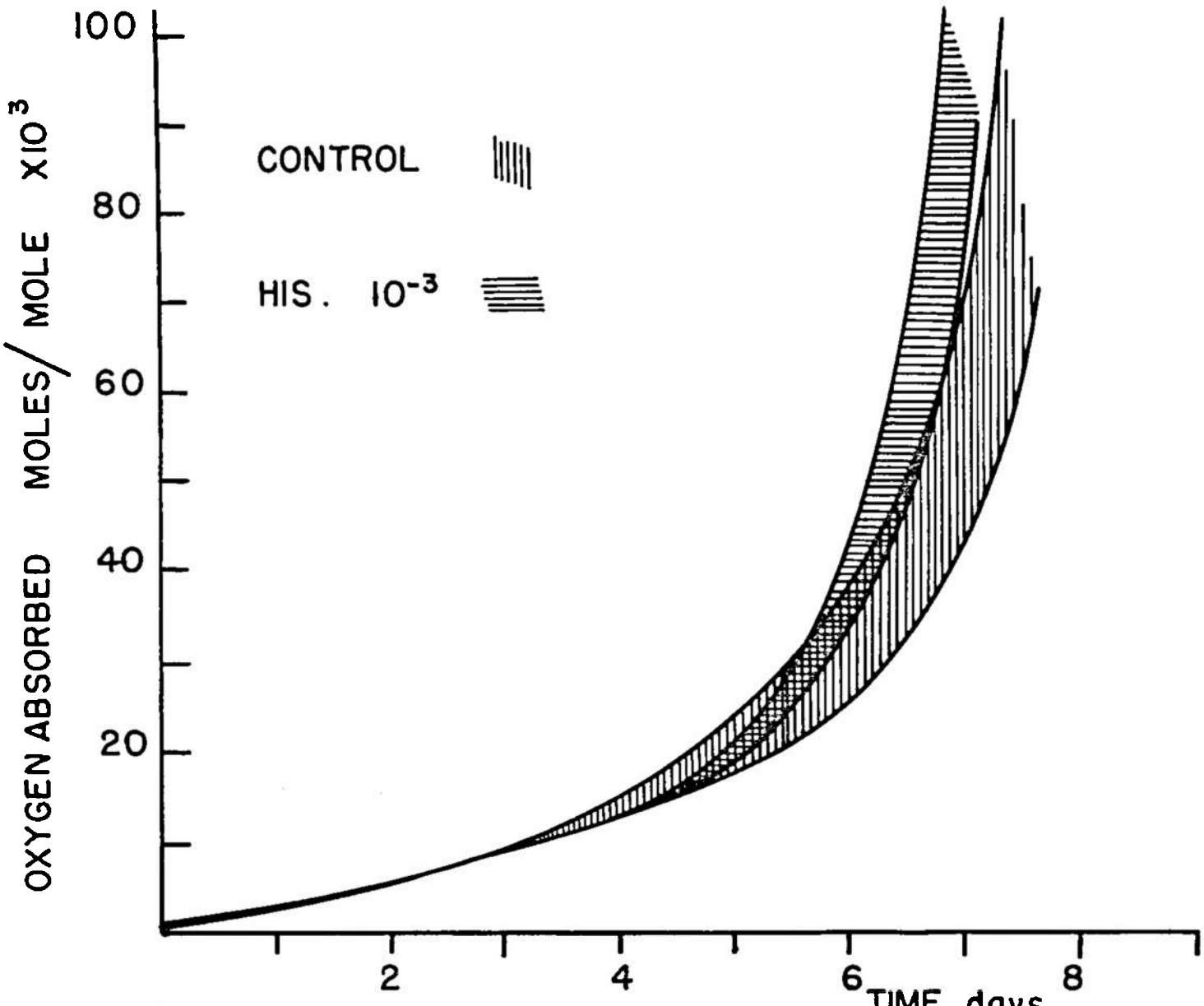

Figure 16. Oxygen absorbed by model system. Run24 (Additive: histidine)

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13. ABSTRACT Oxidation of methyl linoleate was studied in a freeze-dried model system based on microcrystalline cellulose in the presence and in the absence of amino acids. The antioxidant activity of some of the amino acids, which was quite substantial in several runs, was found to be highly variable from run to run in spite of good replication between different samples of a given treatment in a given run. Attempts to eliminate the variability through purification of the methyl linoleate, through addition of cobalt nitrate, washing of the cellulose, changing the pH to 9.5, and through variations in model system preparation procedures were not successful.

In parallel work on conversion products of histidine in the model system, it was demonstrated that a major conversion product of histidine did not contain any substantial amounts of carbon derived from linoleate. In subsequent work it was determined that the conversion of histidine could occur as a result of mixing of the amino acid with the cellulose used in preparation of the model system, or with other types of cellulose including filter paper and cotton. The extent of conversion, however, was quite variable being similar in this respect to the antioxidant activity of the amino acid. Data were inadequate to correlate the conversion of the histidine with its antioxidant activity. However, in two runs in which analysis for the conversion product and oxidation studies were run concurrently it was observed that histidine showed an antioxidant effect when the conversion product was absent, but showed no effect in early oxidation stages, and some pro-oxidant activity in later oxidation stages. In these two runs analysis for the conversion product was carried out after freeze-drying but prior to incubation in air.

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