THE ROLE OF LYSINE
IN
NONENZYMIC BROWNING

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ABSTRACT

Quantitative and qualitative studies were carried out on sulfited lysine-sugar and casein-sugar systems incubated at 55°C and pH 5.5 or 7.6. Measurements of "free", "combined" and "total" SO_2 were carried out in addition to absorbance at 490 mμ to follow the color intensity of the solutions as the browning reaction progressed. Browning was observed concomitant with a fall of the "free" SO_2 level under acid conditions while under alkaline conditions browning developed much earlier and did not appear dependent upon the level of "free" SO_2 present. An attempt to isolate intermediates from sulfited lysine-glucose and lysine-sucrose systems was also reported. Quantitative enzymic studies employing trypsin were also conducted on casein and casein-sugar systems at pH 7.6 incubated at 37°C and 55°C. Statistical analysis of the individual slopes indicated significant decreases in the rate of hydrolysis for the casein-sugar systems when compared to the casein control system.

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INTRODUCTION

Proteins provide a major part of man's diet with many of the traditional food sources also being rich in carbohydrates. In the course of processing or storage many of these food products undergo chemical changes which inevitably have a deleterious effect on their nutritive value. Among the principal reactions involved are protein-sugar interactions, which form part of a group of reactions referred to as nonenzymic browning.

The decrease in nutritive value is related to the availability of amino acids in the protein, particularly the essential ones. The basic amino acid, lysine, an essential substance for man is rendered unavailable during the course of processing for many foods. Initial studies were therefore carried out on the inhibition of reactions involving lysine using bisulfite. Lysine-sugar sulfited systems were investigated under acid and alkaline conditions in order to observe the effects of the bisulfite inhibitor under the different conditions of pH. The implication of lysine in casein-sugar interactions by many workers led to similar inhibition studies on several casein-sugar systems under alkaline conditions.

In order to provide more effective control of these reactions a deeper understanding of the mechanisms involved is mandatory. An attempt was made to isolate and identify

some of the carbonyl intermediates formed in the sulfited lysine-sugar systems incubated at pH 7.6.

The losses in available lysine due to interaction with sugars were related to a decrease in biological or microbiological growth. This was thought to be a consequence of the inability of the amino acid in the sugar complex to be metabolically utilized. A possible theory is proposed concerning the unavailability of amino acids in terms of the inaccessibility of the peptide bond. Since the epsilon amino group of lysine has been widely implicated as it lies out of the main protein chain, possible interaction with sugar might render the adjacent peptide bond inaccessible for attachment by the enzyme trypsin. An experiment was designed to measure the effect of a series of mono- and disaccharides on the hydrolysis of casein by trypsin, the results being compared to the carbohydrate-free casein controls. These experiments were conducted at pH 7.6 and at two different temperatures 37°C and 55°C.

REVIEW OF LITERATURE

Nonenzymic Browning

Browning phenomena are well recognized in food systems. In addition to those involving enzymes many are of a purely chemical or nonenzymic nature. These nonenzymic browning reactions occur during the processing and storage of foods and have proved to be extremely beneficial in the production of a whole variety of food products e.g. coffee, bread, maple syrup, potato chips and roasted nuts. If these reactions are not carefully controlled, however, undesirable changes can occur including off-flavors and poor appearance generally accompanied by a decrease in the nutritional value of the food products concerned.

Three distinct nonenzymic browning reactions are generally recognized; of these the Maillard reaction appears to be the most widely occurring one. The reaction involves interaction between reducing sugars, aldehydes or ketones with amino acids, peptides or proteins. At one time it was referred to as melanoidin browning until Stadtman (1948) proposed the term "Maillard reaction" in recognition of the original investigations carried out by Maillard (1912).

During the 40 years following Maillard's original experiments extensive studies have been carried out on nonenzymic browning reactions. Hodge (1953) integrated all the information reported up to that time on all aspects

of nonenzymic browning which provided a useful foundation for further investigations. In the nonenzymic browning process reviewed by Hodge (1953) amino compounds, which may be amino acids or proteins, and reducing sugars react to form N-substituted glycosylamine which then undergoes the Amadori rearrangement to form the deoxyaminoketose. Evidence for the formation of the latter compound was presented by Brueggemann and Erbersdobler (1968) who isolated fructose-lysine as a major reaction product of lysine with glucose in the heat degradation of foods and feeds.

The reactions leading to the formation of the final products are still incompletely understood, although the present state of knowledge is well documented in a number of comprehensive reviews on the subject (Reynolds, 1963; 1965; Hodge, 1967). The final products of this reaction, however, are still chemically unknown, but they are believed to consist of high molecular weight polymers.

Several studies have attempted to determine which of the amino acids are more rapidly utilized in these reactions. Underwood et al. (1959) showed that the basic amino acid lysine appeared to produce the darkest coloration when heated in the presence of sugars. The color was found to develop with greater intensity as the solution was made more alkaline. Spark (1969) followed the rate of browning for a series of glycine-sugar model systems reporting that the

rate of reaction varied with the different sugars used. He further reported the order of reactivity to be pentoses > hexoses > reducing disaccharides > nonreducing disaccharides. In the case of the nonreducing disaccharides breakdown to reducing monosaccharides was thought necessary before the reaction could proceed.

Nutritional Effects of Protein-Sugar Interactions

The deleterious effects of heating or autoclaving on the nutritive value of protein in foods was first reported by Morgan (1931) and later in studies by Greaves et al. (1938) and Block et al. (1946). This decrease in nutritive value was soon correlated with protein damage caused as a direct result of amino acid destruction or from combination of one or more amino acids in the protein with other components of the food. In the former case the high temperatures necessary to destroy amino acids would probably only affect the surface layers of the food resulting in relatively insignificant losses (Bender, 1966). The latter case, however, has been shown to produce much more significant losses. The importance of sugars in enhancing protein damage was reported by several workers over twenty years ago (Patton et al., 1948; McInroy et al., 1949). Such evidence subsequently implicated the Maillard reaction as a major cause, since it involved interactions between amino acids and sugars. The decrease in nutritive value associated with

processed or stored products is related to the unavailability of the amino acids, due to combination between the amino acids and sugar through their α-amino and carbonyl groups respectively. The resulting linkage formed cannot be readily broken down by digestive enzyme action and results in the amino acid being rendered unavailable for metabolic utilization. To study the mechanism whereby amino acids were rendered unavailable in carbohydrate foods Horn et al. (1968) prepared a methionine-fructose complex and more recently a fructose-glycine complex (Hagan et al., 1970). In both cases these complexes were assayed microbiologically and biologically and were found to exhibit a 20-30% decrease in their growth stimulating ability with respect to the free methionine and glycine as well as rendering the respective amino acids in the complex unavailable. Later workers showed that the products of fat oxidation can also provide carbonyl compounds for similar interactions (Lea, 1958; Ellis, 1959). If the protein is allowed to undergo acid hydrolysis as is normally required prior to amino acid analysis, this linkage can be broken thus giving misleading results from the chemical analysis. Consequently biological methods appear to be the only reliable method for determining the availability of amino acids at the present time.

Changes in Lysine Availability

Particular attention has been directed to lysine,

since it appears to be the most heat-labile of the amino acids and provides a useful index to determine the extent of protein damage for many protein foods (Eskin and Shenai, 1970). The loss of nutritive value for many foods has been correlated with a change in lysine availability. The reaction between the lysine residues in the protein chain appears to involve the ϵ-amino groups since the -amino groups are thought to be chemically bound in the peptide bonds. The effect of reducing sugars on lysine availability was clearly demonstrated by Halevy and Guggenheim (1953) in studies on wheat gluten. When heated alone the gluten was undamaged with a biological value of 55, but in the presence of glucose the value fell to 18. When lysine was added to the undamaged gluten-glucose heated system the biological value rose to 63 thus suggesting that lysine was the main amino acid affected.

Studies on milk protein have also implicated substantial losses in lysine as a result of interaction with sugars (Henry et al., 1948; Mauron and Mottu, 1958). The browning of milk has been associated with losses in lysine, and was observed for many years as a feature of milk deterioration brought about by a Maillard type of reaction involving lactose and the milk protein casein (Coulter et al., 1951; Patton, 1955). The -amino group of lysine, which represents approximately 90%

of the total free amino groups of casein was shown to be the main factor in the decrease of the biological value of casein in dry milk (Henry et al., 1948; Lea and Hannan, 1950). In more recent studies by Rao et al. (1963) on the effects of different heat treatments on buffalo casein and glucose mixtures they reported a depression in the growth of rats. This depression in growth was attributed to a major loss of lysine accompanied by smaller losses of methionine and cysteine. Recent studies carried out by Brueggemann and Erbersdobler (1968) reported a 50% loss of lysine when casein mixtures were dried with 20% glucose at 100-105°C accompanied by a reduction in the apparent digestibility of the protein.

Recent interest in oil seed meals as a potential food source have also been concerned with these reactions since they are generally heated prior to their utilization in order to eliminate any toxic factors present. Stevens and McGinnis (1947) in studies on soybean oil meal implicated a loss in lysine availability as a result of the extensive heating carried out. Later work by Hsu et al. (1948) reported a decrease in the nutritive value of soybean protein autoclaved in the presence of glucose when fed to chicks. Further work on soybean protein by Evans et al. (1951) in the presence of sucrose also indicated substantial losses in lysine. Del Cueto et al. (1960) carried out

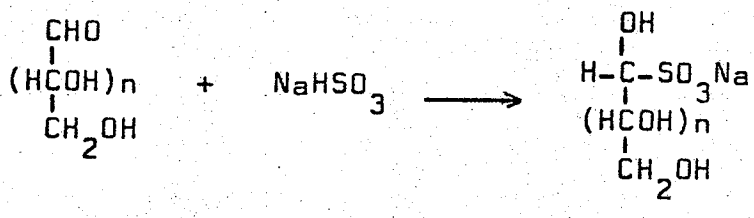
investigations on chick pea, a starchy seed rich in lysine, and reported substantial losses of lysine when autoclaved attributing this effect to interaction with sugars.

In addition to studies involving proteins and reducing sugars in which lysine-sugar interactions have been implicated, other investigations have reported lysine interaction with nonreducing sugars. Martinez et al. (1961) reported a reduction in lysine availability in glandless cottonseed meals and attributed this to the interaction of the epsilon amino groups of lysine with raffinose, a non-reducing trisaccharide normally present. Further experiments by El-Nockrashy and Frampton (1967) on a number of model systems including lysine-sucrose led to a report that the epsilon amino group of lysine interacted with the 1 : 2 glycosidic bond of the nonreducing disaccharide, sucrose, under the mild conditions in which they were working. They referred to this mechanism as "aminolysis", and suggested that lysine may accelerate the breakdown of the reducing disaccharide to its reducing monosaccharide constituents by this process.

Sulfite Inhibition of Nonenzymic Browning

A great deal of research has been carried out to find suitable methods for controlling nonenzymic browning. One of the more successful methods involves the use of sulfites. Bisulfite was found to interact with the reducing

group of sugars theoretically preventing interaction with amino acids, as shown in the following equation:



Pill-Soon Song and Chichester (1967) in their studies on sulfited model systems containing D-glucose—glycine, refuted the formation of sugar sulfates, and suggested the inhibition step occurred much later by a free radical process. McWeeny et al. (1969), however, disagreed with these findings which reported that 64-77% of the original bisulfite was still present at the end of the inhibition period. They investigated the sugar-bisulfite complex and reported that it tended to undergo hydrolysis during chromatographic analysis. Since the early experiments of Pill-Soon Song and Chichester (1967) involved electrophoretic and chromatographic techniques, the high level of original bisulfite reported probably resulted from hydrolysis of the bisulfite complex during the course of the experiment. McWeeny et al. (1969) determined the "free" (uncombined) SO_2 and "total" SO_2 by titrametric analysis for glycine-glucose sulfited systems and demonstrated a dramatic decrease in original bisulfite at the termination of the inhibition period.

A further and somewhat simpler explanation of sulfite

inhibition was proposed by Burton et al. (1963). This involved interaction of the bisulfite with the olefinic bond of α,β-unsaturated carbonylic compounds to form stable products from which the bisulfite could not be regenerated. This reaction is probably responsible for the irreversible removal of measurable sulfite by the nonenzymic browning system.

EXPERIMENTAL

Introduction

Many model systems have been studied containing the amino acid glycine, even though lysine has been shown to be the nutritionally limiting amino acid for many proteins. Studies were therefore carried out on lysine model systems with an attempt to identify some of the intermediates formed. Further studies were also carried out on a number of casein-sugar systems, since earlier work on casein had implicated the epsilon amino group to be primarily involved in casein-sugar interactions. Enzymic assays using trypsin were conducted on casein-sugar systems to observe any possible effects of these reactions on the accessibility of the peptide bonds to proteolytic attack.

Experiment I

Studies on sulfited lysine-sugar and casein-sugar model systems.

Materials and Method

The first experiment was designed to investigate the inhibition of nonenzymic browning in sulfited lysine-glucose and lysine-sucrose model systems under acid (pH 5.5) and alkaline (pH 7.6) conditions. A similar experiment was also conducted on casein-glucose, casein-lactose and casein-sucrose systems under alkaline (pH 7.6) conditions only.

The model systems were set up as in Table I and II.

The solutions were adjusted to pH 5.5 or 7.6 by the addition of 1N hydrochloric acid or 1N sodium hydroxide. Aliquots (6 ml) of each mixture were placed in a series of small vials which were sealed and incubated in an oven at 55°C. Duplicate samples were removed at intervals of 1-3 days and examined for measurable "SO_2" and rate of browning using the following methods:

1. "Free" SO_2 (the uncombined inorganic sulfite). This was determined by titrametric analysis. 1.0 ml aliquots of each system were pipetted into a 250 ml conical flask containing distilled water (50 ml), 5N sulfuric acid (2 ml) and freshly prepared 1% starch solution (1 ml). The solution was titrated against 1.0×10^{-2}N

TABLE I

Sulfited lysine-sugar model systems

Amino acid (0.5M)	Sugar (1.0M)	Sulfite inhibitor (0.043M)
lysine	glucose	sodium metabisulfite
lysine	sucrose	sodium metabisulfite

TABLE II

Sulfited casein-sugar model systems

Protein (1%)	Sugar (0.55M)	Sulfite inhibitor (0.043M)
casein	glucose	sodium metabisulfite
casein	lactose	sodium metabisulfite
casein	sucrose	sodium metabisulfite

iodine solution until a blue end point was observed. The volume of titrant was converted to mg of SO_2 since 1 ml $1.0 \times 10^{-2}N$ iodine = 0.32 mg SO_2. (McWeeny et al., 1969).

2. "Combined" SO_2 (reversibly bound organic sulfur). This was determined by taking the solution previously titrated, adding 1N sodium hydroxide (40 ml) and allowing to stand for 10 minutes. The "combined" SO_2 is released during this period and then acidified with 5N sulfuric acid (15 ml) and titrated with $1.0 \times 10^{-2}N$ iodine solution. The final titrant volume was converted to mg SO_2 as previously described.
3. "Total" SO_2 was determined by taking the sum of the "free" and "combined" SO_2 previously determined.
4. Rate of browning was measured by the increase in absorbance at 490 mμ using the Unicam SP 600 spectrophotometer against a distilled water blank or freshly prepared casein/casein sugar solutions. This permitted comparison between the present work and that of McWeeny et al. (1969).

Results and Discussion

The results from studies on the sulfited lysine-sugar and casein-sugar systems are shown in Figures 1 to 7

respectively.

In Figure 1 for the sulfited lysine-glucose system at pH 5.5 the development of browning did not occur until the "free" SO_2 level had almost disappeared. The level of "free" SO_2 at the end of the inhibition period corresponded to approximately 5% of that reported initially after incubation for an hour. The relative amounts of "free" and "combined" SO_2 appeared to change appreciably, in a manner similar to that reported by McWeeny et al. (1969) working with sulfited glycine-glucose systems under similar conditions. McWeeny et al. (1969) related these changes to the equilibrium between the bisulfite and glucose:

$$\underset{\text{(free)}}{\text{bisulfite}} + \text{glucose} \longleftrightarrow \underset{\text{(combined)}}{\text{glucose hydroxy-sulfonate}}$$

During the course of the incubation studies some of the bisulfite was thought to be irreversibly bound by compounds formed in the reaction while other compounds were thought to be responsible for combining reversibly with bisulfite. This resulted in a larger proportion of the total measurable bisulfite being in the "combined form" which was also observed in the present studies. Burton et al. (1963) suggested that the bisulfite also reacted with the olefinic bond of $\alpha\beta$ - unsaturated carbonylic compounds forming stable sulfonated products from which bisulfite could not be regenerated and may be the factor responsible for the loss in "total" SO_2

Figure 1.

Color production and loss of measurable SO_2 from sulfited lysine-glucose system incubated at pH 5.5 and 55°C.

- ○ "Total" SO_2
- △ "Combined" SO_2
- ● "Free" SO_2
- ▲ Absorbance at 490mμ

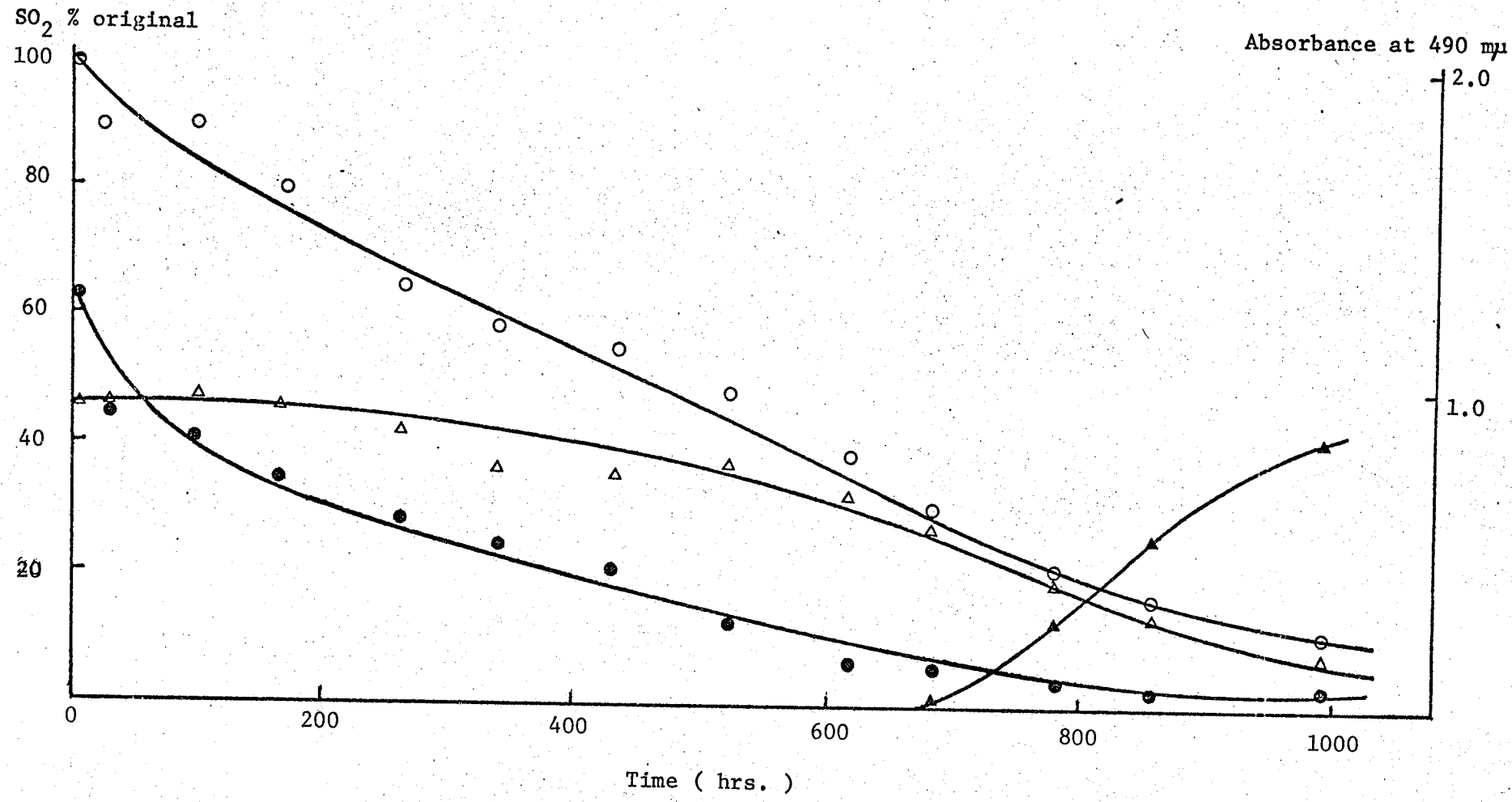
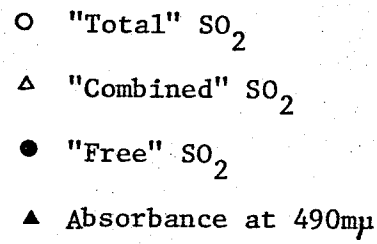
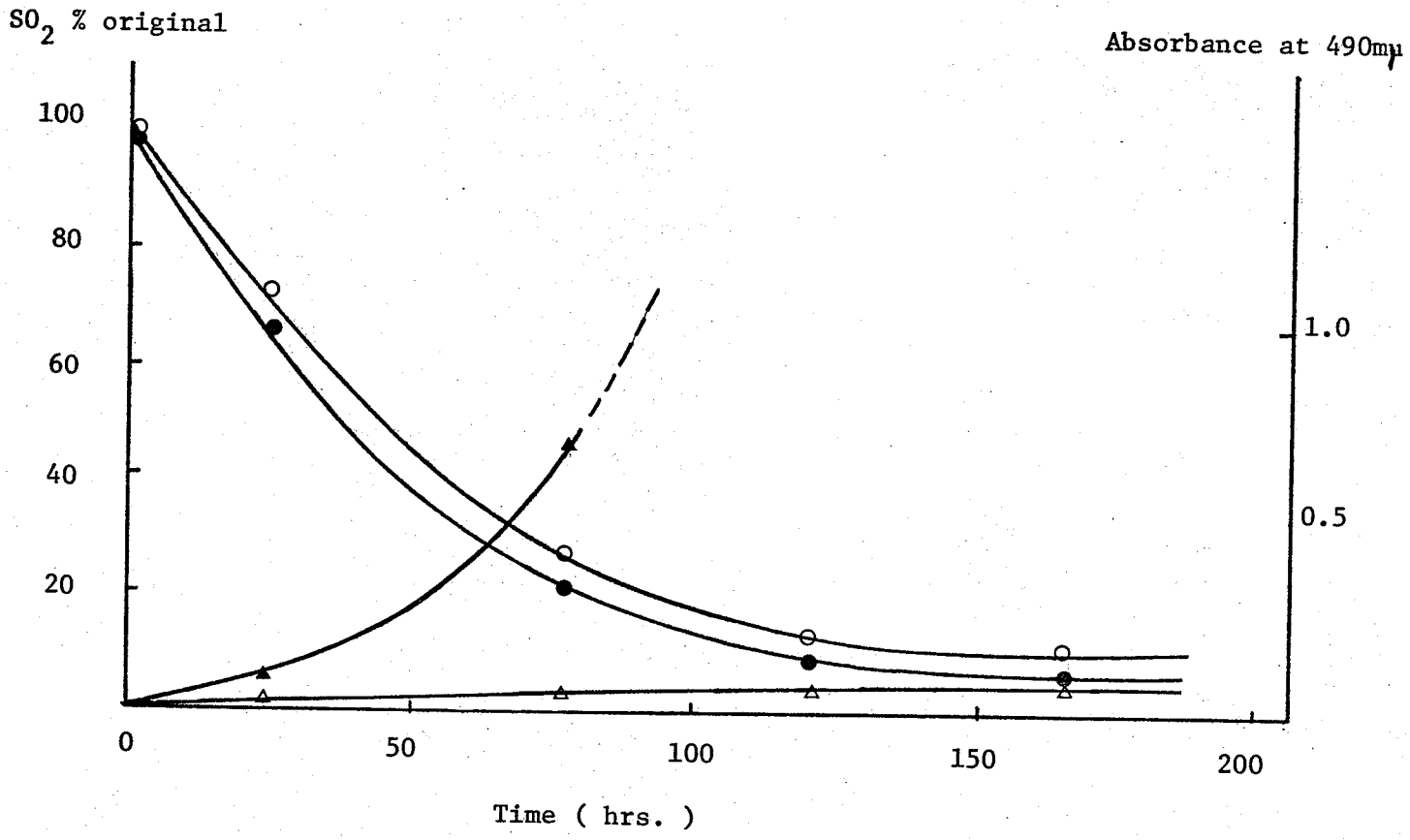

SO_2 % original
100
80
60
40
20
Absorbance at 490 mμ
2.0
1.0
0
200
400
600
800
1000
Time (hrs.)

Figure 2.

Color production and loss of measurable SO_2 from sulfited lysine-glucose system incubated at pH 7.6 and 55°C.




SO_2 % original
Absorbance at 490mμ
100
80
60
40
20
1.0
0.5
0
50
100
150
200
Time (hrs.)

during the course of the reaction.

In the present studies the inhibition period appeared to terminate after 24 days which is considerably shorter than the 41 days McWeeny *et al.* (1969) reported for their sulfited glycine-glucose system in spite of the higher bisulfite level used in the present experiments. The comparatively shorter inhibition period may probably be related to the amino acid lysine used in these experiments. A number of studies have shown that lysine browns far more rapidly than glycine in the presence of the same sugars (Lento *et al.*, 1958; Underwood *et al.*, 1959).

In Figure 2 the effect of bisulfite on the lysine-glucose system is shown to be somewhat different. During the course of the incubation period the "combined" SO_2 remained at a constant low level under the alkaline conditions. While the level of "free" SO_2 appeared to decrease rapidly concomitant with a rapid rise in the browning level. The formation of "combined" SO_2 is not favored under these alkaline conditions since 1N sodium hydroxide is used to break down the complex, so that the loss in measurable SO_2 must be due to irreversible combination with some of the intermediates formed during the nonenzymic browning process.

The progress of the sulfited lysine-sucrose system at pH 5.5 is illustrated in Figure 3. At the beginning of the incubation period all the "measurable" SO_2 appeared to

Figure 3

Color production and loss of measurable SO_2 from sulfited lysine-sucrose system incubated at pH 5.5 and 55°C.

- ○ "Total" SO_2
- △ "Combined" SO_2
- ● "Free" SO_2
- ▲ Absorbance at 490mμ

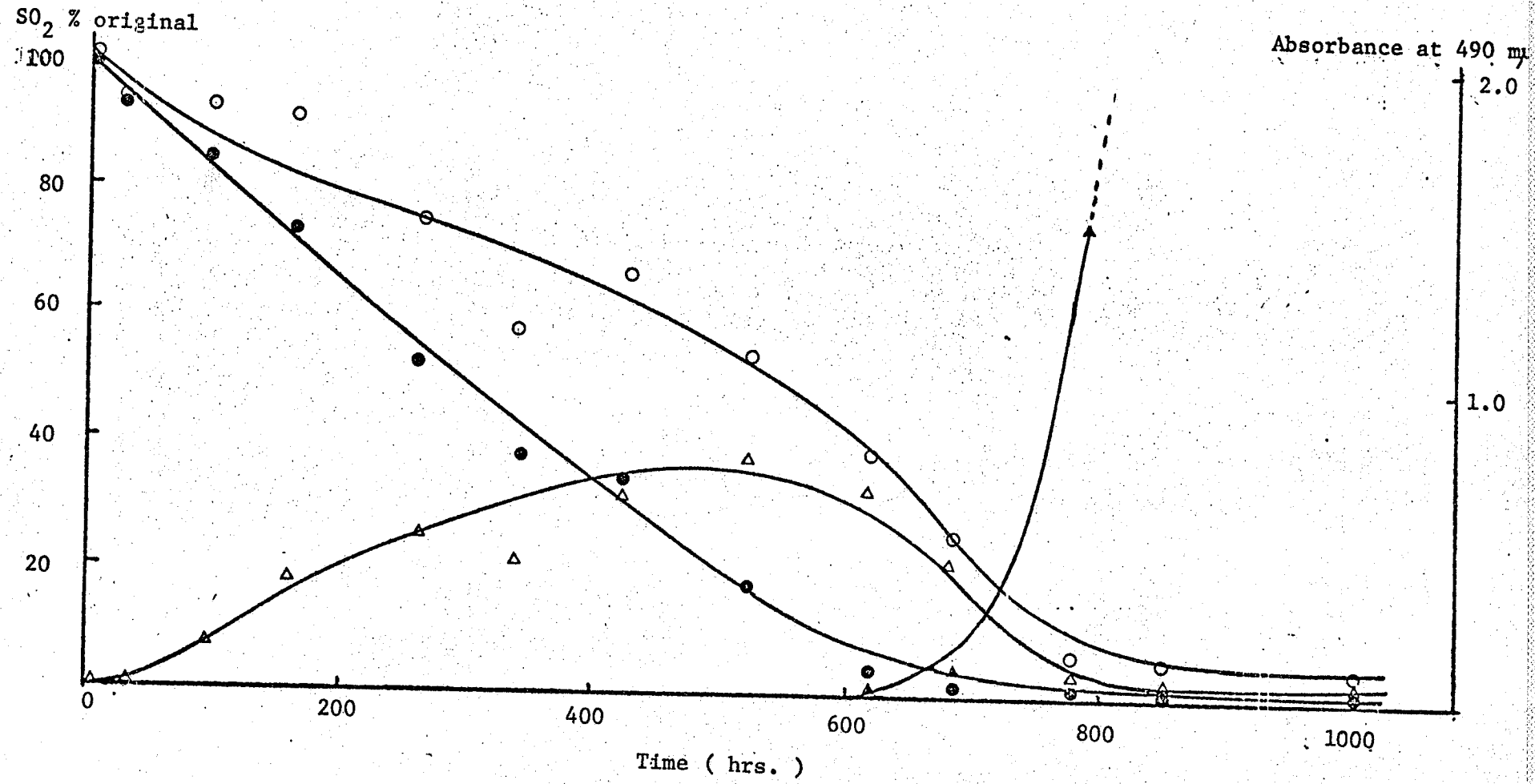
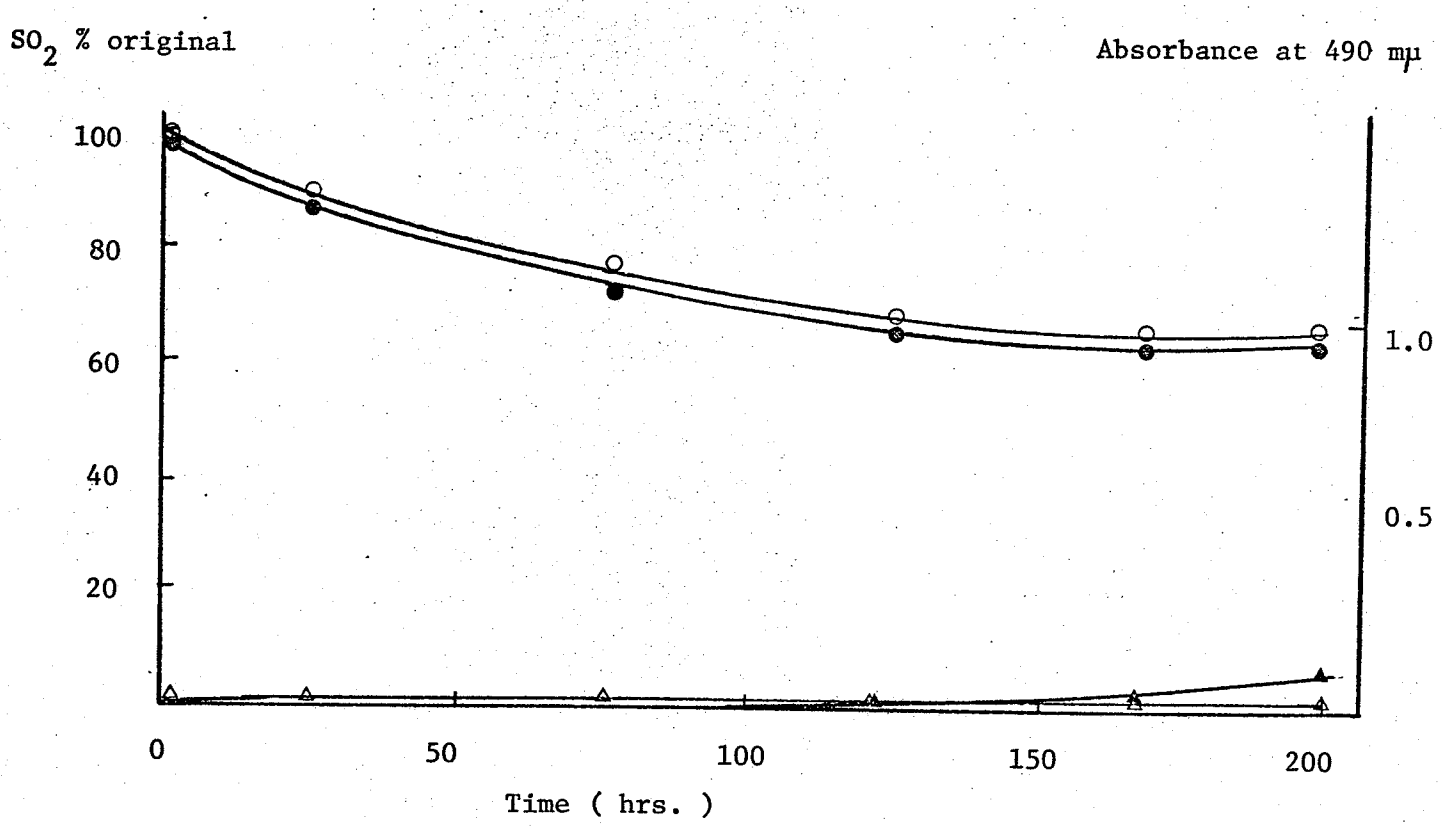

SO_2 % original
100
80
60
40
20
0
200
400
600
800
1000
Time (hrs.)
Absorbance at 490 mμ
2.0
1.0

Figure 4.

Color production and loss of measurable SO_2 from sulfited lysine-sucrose system incubated at pH 7.6 and 55°C.

- ○ "Total" SO_2
- △ "Combined" SO_2
- ● "Free" SO_2
- ▲ Absorbance at 490mμ


SO_2 % original
Absorbance at 490 mμ
100
80
60
40
20
1.0
0.5
0
50
100
150
200
Time (hrs.)

be in the "free" form which is probably due to the sucrose molecule being unable to interact with the bisulfite at this stage. During the course of the incubation period, however, breakdown to reducing monosaccharides probably occurred as was detected by positive Fehlings test. This was accompanied by an increase in the "combined" SO_2 which reached a maximum after 500 hours, corresponding to 35% of the original "total" measurable SO_2. The development of browning appeared after 25 days at a very much faster rate than that reported for the sulfited lysine-glucose system. This effect could be due to the rapid utilization of fructose released during the course of the reaction. The corresponding effect under alkaline conditions is illustrated in Figure 4. While the incubation period was investigated over a 200 hour period, since the browning reaction was extremely rapid, it nevertheless illustrates a significant difference in effect of bisulfite. The level of "combined" SO_2 appears negligible as in the case of sulfited lysine-glucose under alkaline conditions while the "free" SO_2 level fell around 20% during the 7 day incubation period. The development of browning was not quite as dramatic although color development appeared after 120 hours. The slow rate of browning was probably determined by the rate of hydrolysis of the sucrose molecule to produce reducing compounds. These were then responsible for

Figure 5

Color production and loss of measurable SO_2 from sulfited casein-glucose system incubated at pH 7.6 and 55°C.

○ "Total" SO_2

△ "Combined" SO_2

● "Free" SO_2

▲ Absorbance at 490mμ

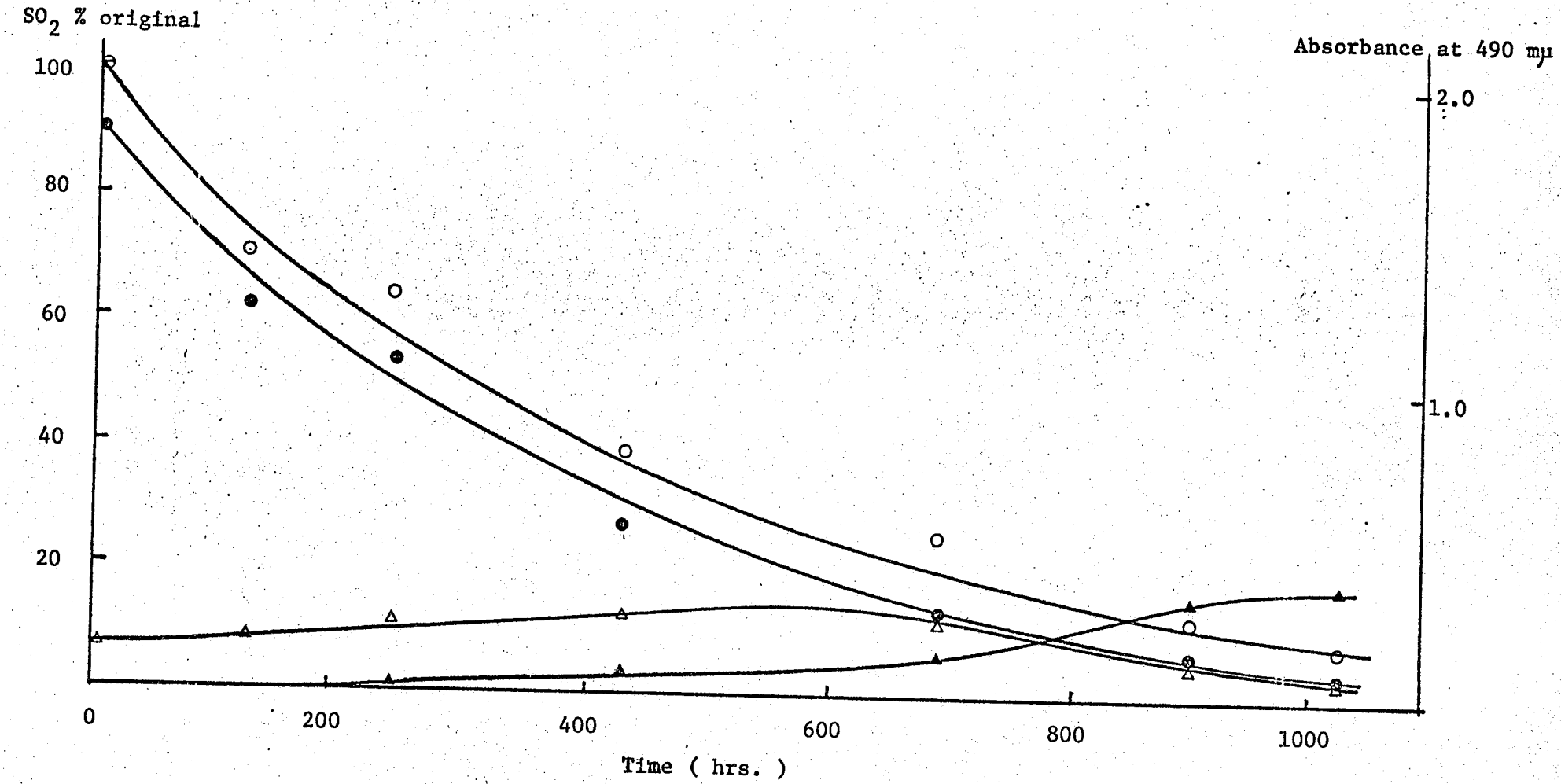

SO_2 % original
100
80
60
40
20
0
200
400
600
800
1000
Time (hrs.)
Absorbance at 490 mμ
2.0
1.0

Figure 6

Color production and loss of measurable SO_2 from sulfited casein-lactose system incubated at pH 7.6 and 55°C.

○ "Total" SO_2

△ "Combined" SO_2

● "Free" SO_2

▲ Absorbance at 490mμ

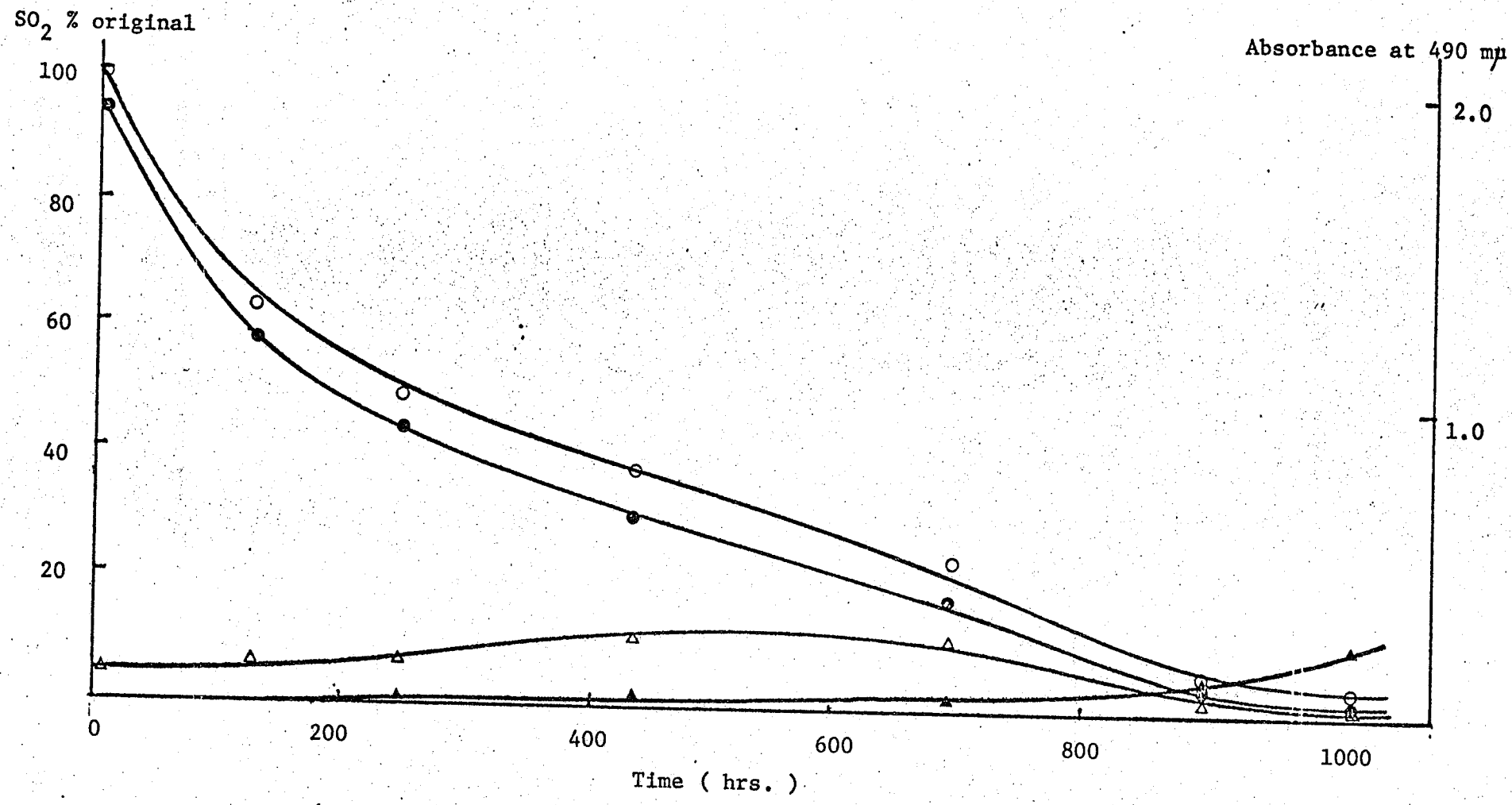
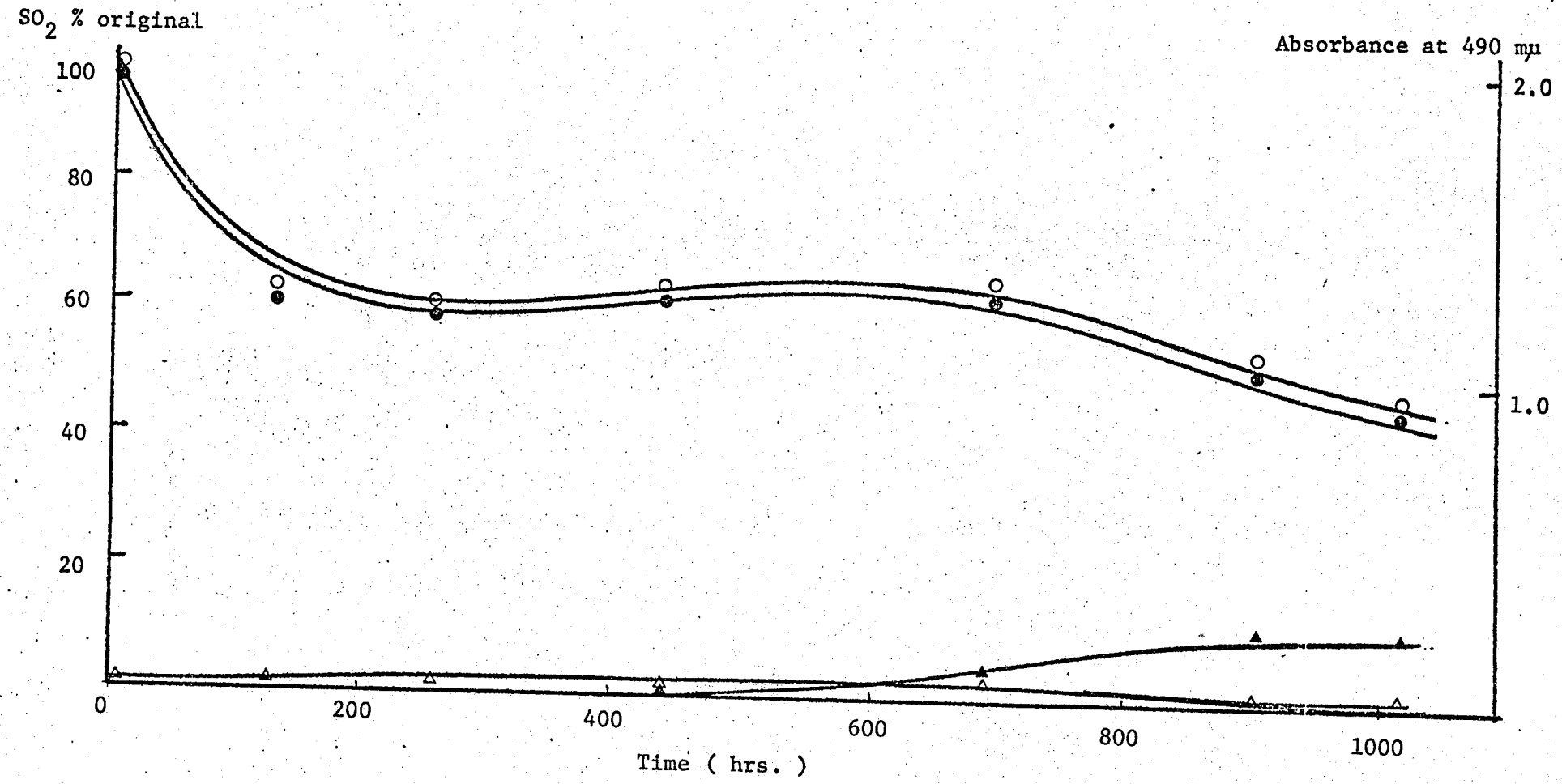

SO_2 % original
100
80
60
40
20
Absorbance at 490 mμ
2.0
1.0
0
200
400
600
800
1000
Time (hrs.)

Figure 7

Color production and loss of measurable SO_2 from sulfited casein-sucrose system incubated at pH 7.6 and 55°C.

- ○ "Total" SO_2
- △ "Combined" SO_2
- ● "Free" SO_2
- ▲ Absorbance at 490mμ


SO_2 % original
Absorbance at 490 mμ
100
80
60
40
20
2.0
1.0
0
200
400
600
800
1000
Time (hrs.)

interacting with lysine resulting in the formation of the various intermediate carbonylic compounds which combined irreversibly with bisulfite as indicated by a decrease in the "free" SO_2.

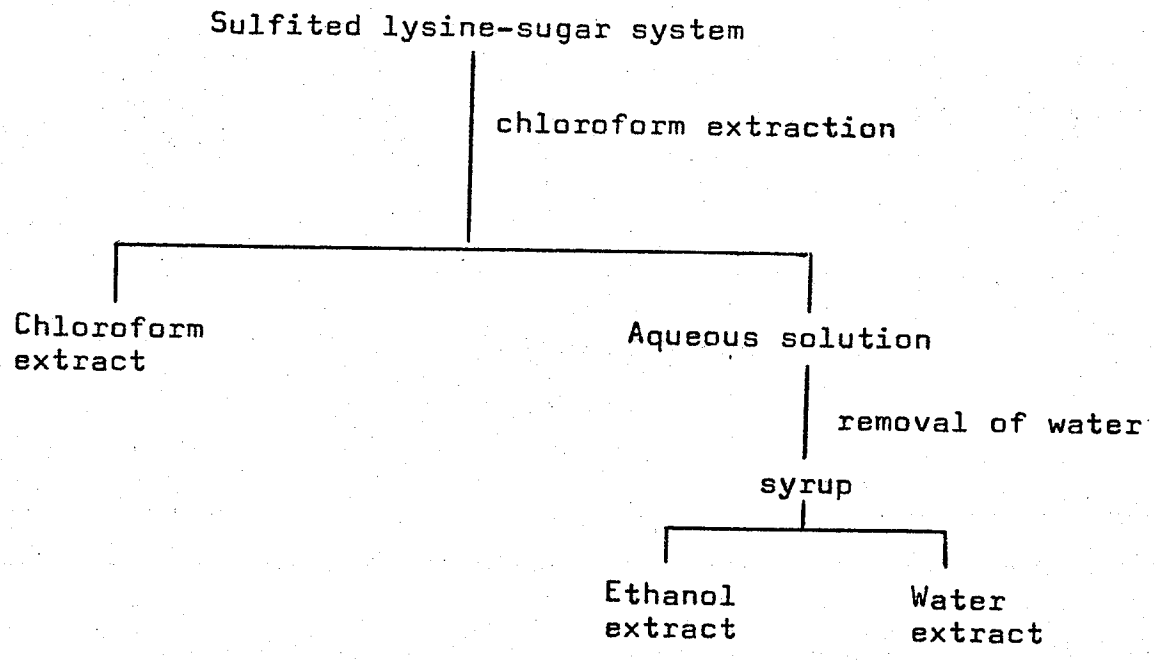
The changes in measurable SO_2 in sulfited casein-sugar systems are shown in Figure 5, 6 and 7. While the systems are somewhat more complex as the protein casein is involved, the graphs nevertheless appear to have some similarities with those observed for sulfited lysine-glucose and lysine-sucrose systems at pH 7.6. In all cases the level of "combined" SO_2 remains at a constant low value although there appears to be a slight increase for casein-lactose and casein-glucose systems, however these still appear insignificant when compared to the "total" measurable SO_2. The "free" SO_2 appears to decrease rapidly for the sulfited casein-glucose and casein-lactose systems but decreased only 40% in the sulfited casein-sucrose system. The development of browning appeared after a considerable decrease in the "free" SO_2 levels for casein-glucose and casein-lactose in contrast to casein-sucrose where browning starts to develop even though 50% of the "total" measurable SO_2 was still present in the "free" form.

Experiment II

Identification and isolation of the intermediate carbonyl compounds formed during the browning of sulfited lysine-glucose and lysine-sucrose systems at pH 7.6 and 55°C.

Materials and Method

Samples from the sulfited lysine-glucose and lysine-sucrose solutions were removed after incubating for 1, 24, 120 and 168 hours and their absorption spectra observed over the wavelength range 220-350 mμ characteristic for carbonyl intermediates. A 40 ml aliquot of each system was extracted by the following procedure in an attempt to characterize the intermediates formed.



The 2,4-Dinitrophenylhydrazone derivatives were prepared by reacting the carbonyl compounds with 2,4-dinitrophenylhydrazine at room temperature for 2 hours. (Shriner et al., 1967).

The carbonyl intermediates for each fraction were precipitated as 2,4-DNPH, collected by filtration and washed with water. Recrystallization was carried out using hot ethanol and water. The purified 2,4-DNPH (3 mg) derivatives were dissolved in chloroform (3 ml) and an aliquot spotted on Whatman No. 1 chromatographic paper. The paper was dipped in a mixture of N,N-dimethylformamide and ethyl ether (1 : 1 ratio) and dried in air to provide the stationary phase and then developed with cyclohexane by the descending method of paper chromatography. The chromatograms were removed after several hours and dried in air. Further analysis was carried out using thin layer chromatography. The thin layer plates were coated with Silica Gel G and developed with benzene-ethyl acetate (19 : 1 v/v).

Results and Discussion

A rapid increase in absorption was observed for the sulfited lysine-glucose over 220-350 mμ region exhibiting a number of rather broad peaks. In the case of the sulfited lysine-sucrose system, however, a broad peak at 300-350 mμ was found to increase as illustrated in Figure 8, exhibiting a maximum at 310-314 mμ.

Figure 8

Absorbance spectra for sulfited lysine-sucrose system incubated at pH 7.6 and 55°C.

I. After 1 hour incubation.

II. After 24 hours incubation.

III. After 120 hours incubation.

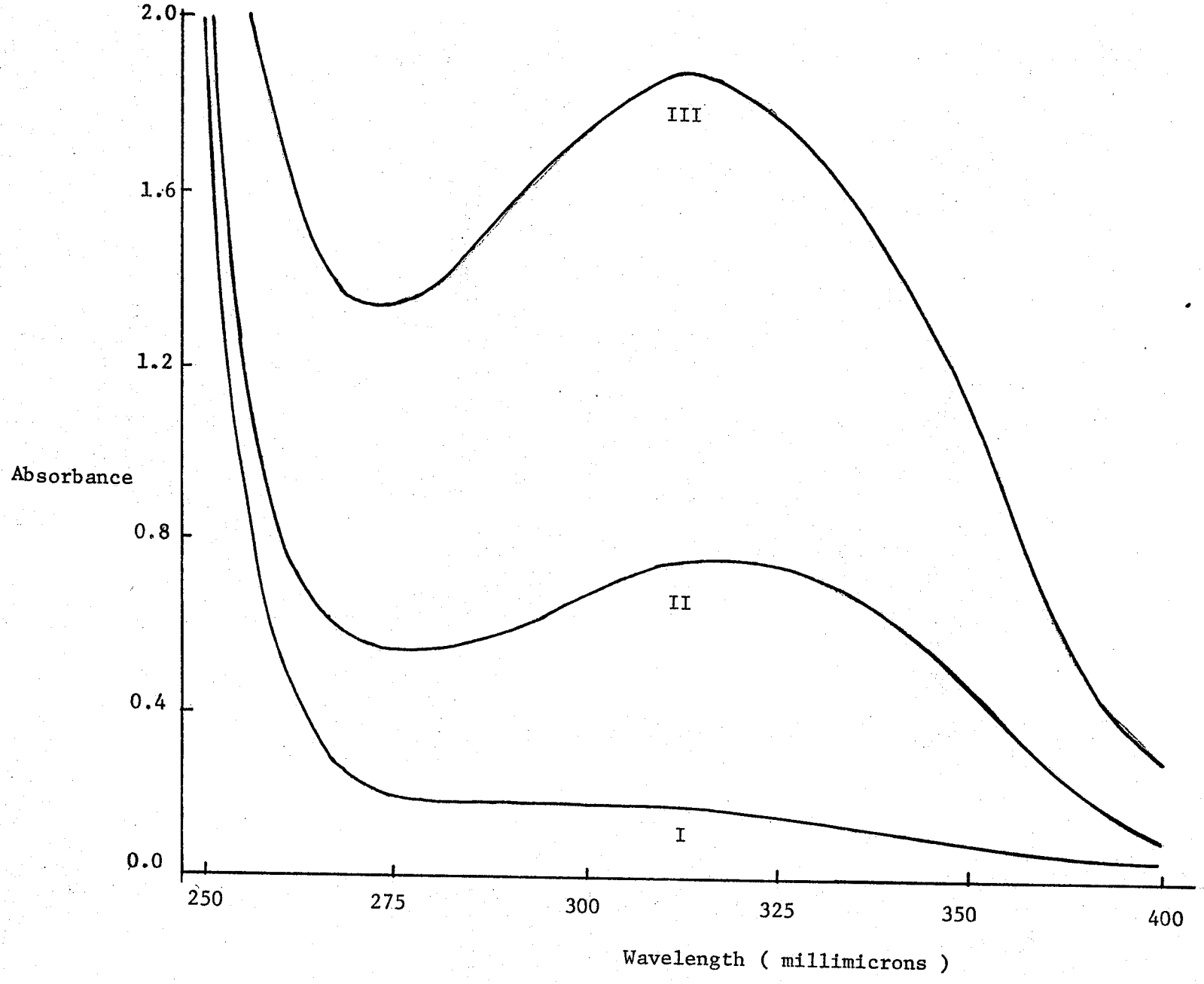
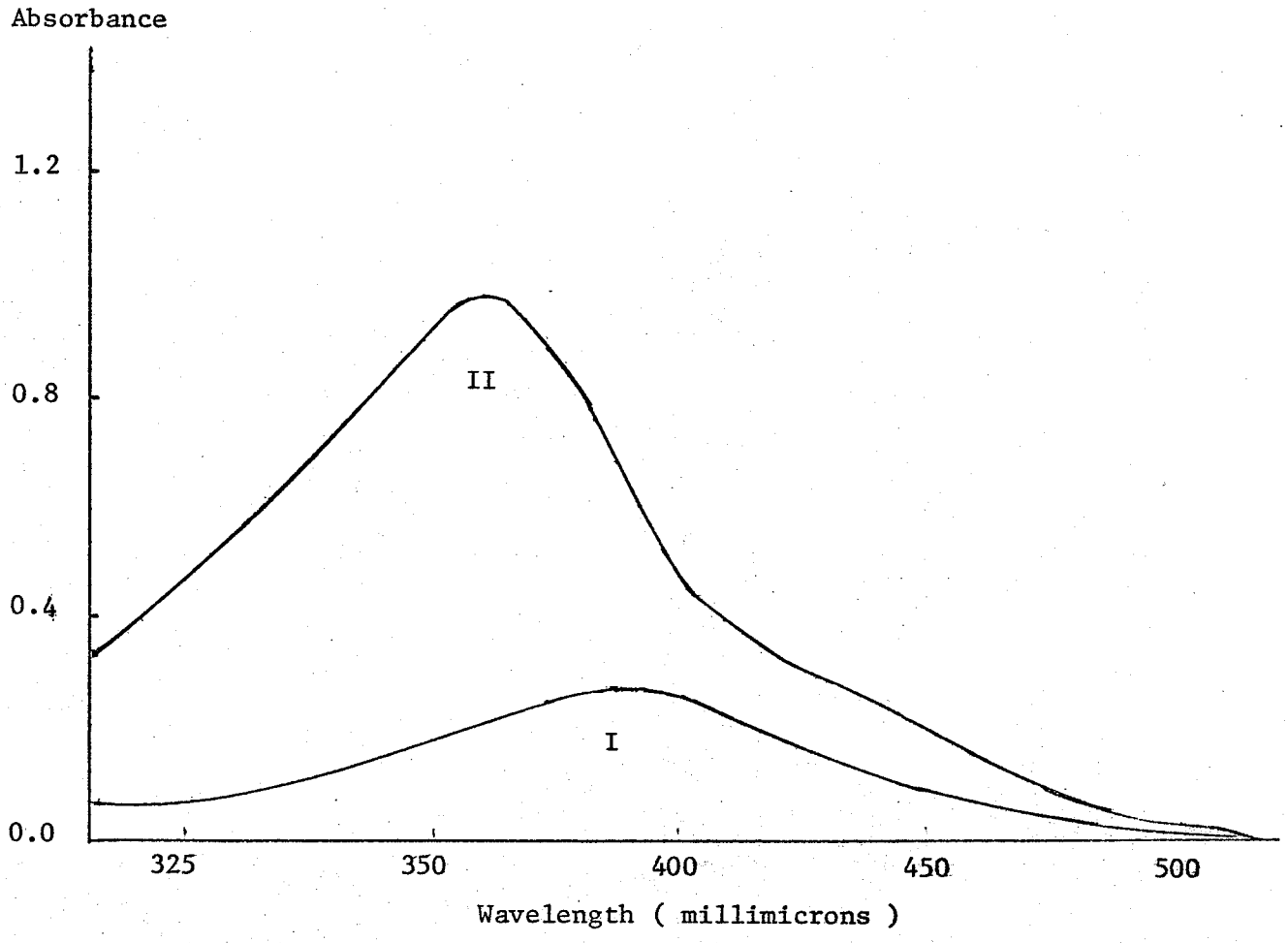

2.0
1.6
1.2
0.8
0.4
0.0
Absorbance
III
II
I
250
275
300
325
350
400
Wavelength (millimicrons)

Figure 9

Absorbance spectra for 2,4-DNPH derivatives isolated by paper chromatography.

I. Reference 2,4-DNPH fufural derivative.

II. Isolated 2,4-DNPH carbonyl derivatives.


Absorbance
1.2
0.8
0.4
0.0
II
I
325
350
400
450
500
Wavelength (millimicrons)

The 2,4-dinitrophenylhydrazones were prepared from the extracts isolated and the spots observed by their orange-yellow color. The migration of the spots on the paper chromatogram were measured relative to the Rf of methyl ethyl ketone which was expressed as 1.00 (R_{MEK}). The results are tabulated in Table III.

The individual spots from thin layer chromatographic analysis of the 2,4-DNPH derivatives were recorded as Rf values as indicated in Table IV.

Two spots were recorded for most extracts although the slower running spot was not as well defined. In the lysine-glucose extracts two spots were observed in the chloroform extract when separated by paper chromatography, while only a single spot was observed when examined by thin layer chromatography, the reverse being true for the corresponding ethanol extract.

The individual spots recorded by paper chromatography were cut out and eluted individually with spectrosopically pure methanol. The ultraviolet and visible absorption spectra of the eluted 2,4-DNPH spots were determined. All the derivatives showed a major absorption peak at 364-366 mu and a smaller and broader peak at 420-430 mu, indicating the derivatives were probably very similar as illustrated in Figure 9. Further investigations are still required, however, to identify these derivatives more completely.

TABLE III

R_{MEK} values for 2,4-DNPH derivatives

Sulfited system	R_{MEK}		
	Chloroform extract	Ethanol extract	Aqueous extract
lysine-glucose	0.82		-
	1.00	1.02	
lysine-sucrose	0.80	0.80	unresolved
	0.94	0.93	

Reference compound: furfural 0.18

TABLE IV

Rf values for 2, 4-DNPH derivatives

Sulfited system	Rf values		
	Chloroform extract	Ethanol extract	Aqueous extract
lysine-glucose		0.07	-
	0.12	0.12	
lysine-sucrose	0.08	0.08	unresolved
	0.12	0.13	

Reference compounds: methylethyl ketone 0.12
furfural 0.04

Experiment III

The enzymic hydrolysis of casein and casein-sugar systems using trypsin.

Materials and Method

A study of the rates of proteolytic hydrolysis of casein and a series of casein-sugar systems incubated at 37° and 55°C was carried out using trypsin.

A stock casein solution (1%) was prepared by dissolving (1 g) casein in 100 ml phosphate buffer. (0.1M; pH 7.6). The model systems set up for enzymic assay with trypsin are shown in Table V and VI.

A series of duplicate solutions for each system (6 ml) were sealed in small vials and incubated in an oven at 37°C and 55°C respectively. Merthiolate was added to those samples incubated at 37°C to prevent microbial growth. The procedures followed included:

1. Enzyme assays using trypsin carried out according to the method described by Rick (1963).

2. A curve indicating the relationship between enzyme concentration and reaction velocity for the hydrolysis of casein by trypsin was prepared as follows:

   Trypsin (10 mg) was dissolved in 0.001N

TABLE V

Casein-monosaccharide systems

Protein (1%)	Sugar (0.55M)
casein	--
casein	glucose
casein	fructose
casein	galactose

TABLE VI

Casein-disaccharide systems

Protein (1%)	Sugar (0.35M)
casein	--
casein	lactose
casein	sucrose

hydrochloric acid (HCl) and diluted to 100 ml with phosphate buffer (0.1M; pH 7.6). The incubation mixture consisted of trypsin (1 ml) (0.5 - 49.5 µg) and casein (1 ml). After allowing the mixture to incubate at 37°C for 1 hour, 3.0 ml of 5% trichloroacetic acid was added and the solution mixed thoroughly. The mixture was permitted to stand at room temperature for 30 minutes and the precipitated protein removed by centrifugation at 3,000 g for 20 minutes at room temperature. The filtrate containing the products of hydrolysis was determined by measuring their absorbance at 280 mµ.

3. Rate studies on the hydrolysis of casein and casein-sugar systems using trypsin were carried out on the following incubation mixtures:

Enzyme (4.0 ml)

Casein system (4.0 ml)

1.0 ml aliquots were removed at 0, 30, 60, 70, 80 and 90 minutes, the reaction was terminated by the addition of 5% trichloroacetic acid (3.0 ml). For each set of duplicates the change in absorbance at 280 mµ was determined

as described earlier. The blank was taken as the incubation mixture at zero time. The slope of the graph for the casein control and casein mixtures were compared statistically by the method of analysis involving linear regression discussed in the results.

Results and Discussion

The curve indicating the relationship between the enzyme concentration and reaction velocity for the hydrolysis of casein by trypsin is illustrated in Figure 10. The enzyme concentration used in subsequent assays was 12.5 µg/ml.

The hydrolysis curves for the casein and casein-sugar systems were prepared as illustrated for casein-glucose incubated at 55°C (Fig. 11). Increase in absorbance was plotted against time in minutes for all systems. Trypsin assays were carried out on all the systems and the slopes of the graphs compared statistically as follows:

$$y = a + bx$$

Where x = square root of time

y = optical density

a = the y intercept

b = a measure of the slope of the line.

The absorbance was found to be a linear function of

Figure 10

The relationship between enzyme concentration and reaction velocity for the hydrolysis of casein by trypsin.

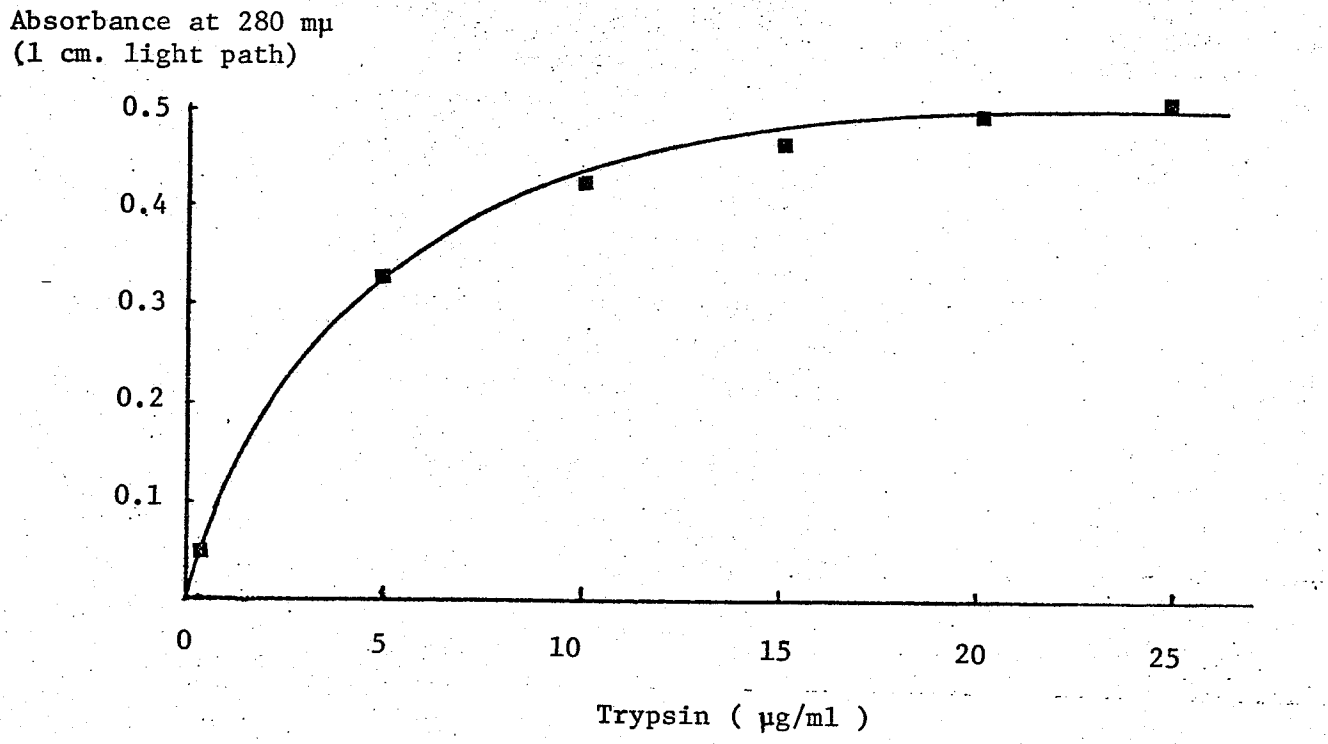

Absorbance at 280 mμ
(1 cm. light path)
0.5
0.4
0.3
0.2
0.1
0
5
10
15
20
25
Trypsin (μg/ml)

Figure 11

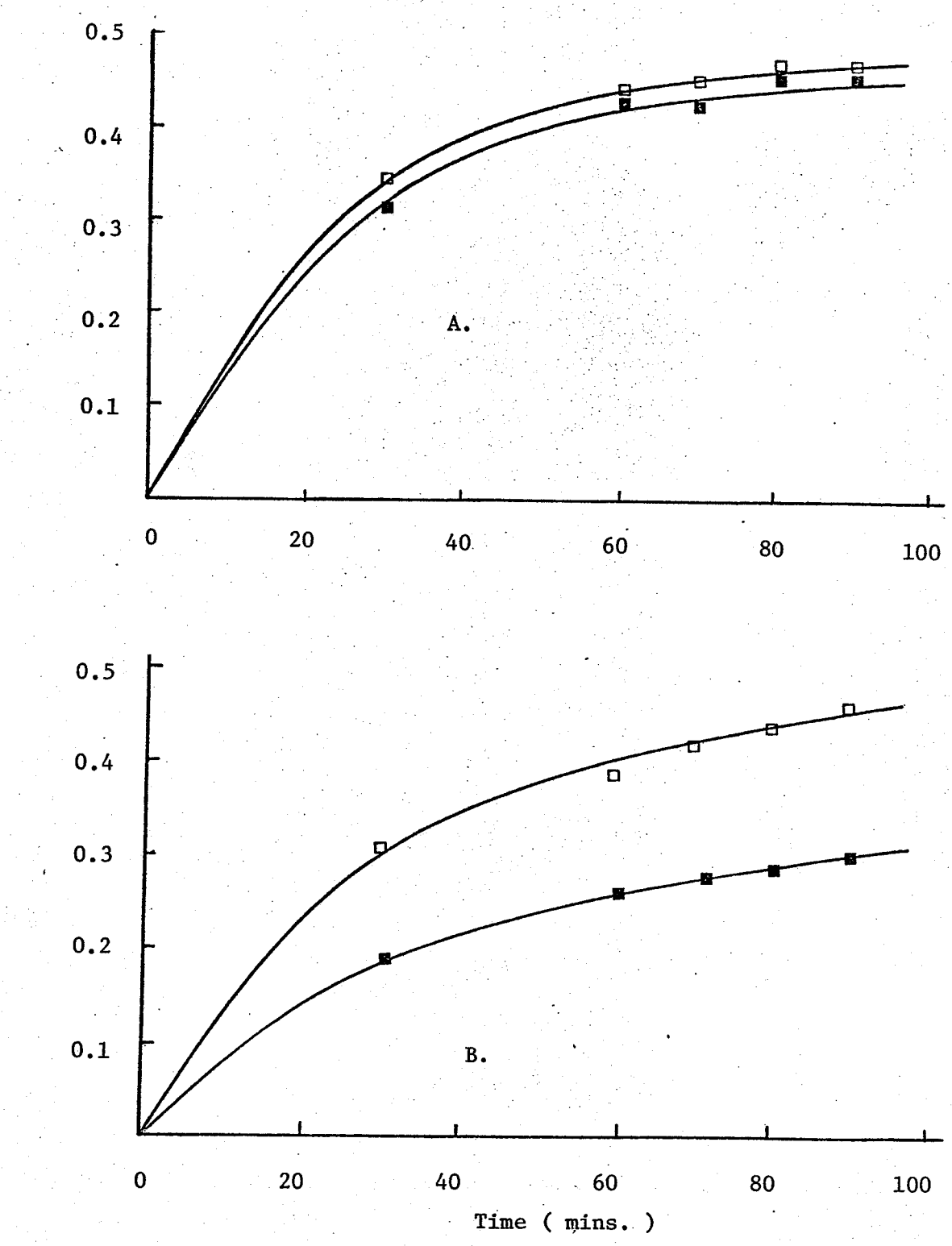
Hydrolysis of casein and casein-glucose systems incubated at 55°C by trypsin.

A. Initially

B. After 4 days incubation.

□ Casein control systems.

■ Casein-glucose systems


Absorbance at 280 mμ
(1 cm. light path)
0.5
0.4
0.3
0.2
0.1
A.
0
20
40
60
80
100
0.5
0.4
0.3
0.2
0.1
B.
0
20
40
60
80
100
Time (mins.)

the square root of time. The regression line between absorbance and square root of time was estimated by the method of linear regression. Two slopes were obtained from the two regression lines and calculated for the control casein and casein-sugar systems. Student's t values were determined using the method of Steel and Torrie (1960) to ascertain whether or not these values could be considered estimates of a common slope. A 5% probability level was used.

$$t = \frac{b_1 - b_2}{\sqrt{S_p^2 \left(\frac{1}{\sum_j X_{1j}^2} + \frac{1}{\sum_j X_{2j}^2} \right)}}$$

since $X_1 = X_2$

$$t = \frac{b_1 - b_2}{\sqrt{S_p^2 \left(\frac{2}{\sum_j X_j^2} \right)}}$$

Where: t = student's t test

b_1 = the slopes of casein control line

b_2 = the slope of casein-sugar systems line

S_p^2 = variation about regression

$$S_p^2 = \frac{\left\{ \sum Y_{1j}^2 - \left[(\Sigma X_j Y_{1j})^2 / \Sigma X_j^2 \right] \right\} + \left\{ \sum Y_{2j}^2 - \left[(\Sigma X_j Y_{2j})^2 / \Sigma X_j^2 \right] \right\}}{n_1 - 2 + n_2 - 2}$$

The difference between the slopes obtained from the hydrolysis of casein and casein-sugar systems at initial (0) time and after several days at 37°C and 55°C incubation was determined. The data was processed by a computer programme

given in the Appendix (I). The results obtained from statistical analysis are tabulated in Table VII and VIII. Significant decrease in the rates of hydrolysis were observed for the majority of the casein-sugar systems studied with the exception of the casein-sucrose system incubated at 37°C. This could possibly be related to the rate of breakdown of the sucrose molecule since at the higher temperature (55°C) a significant change was observed in the later incubation period.

One explanation for the decrease in the rate of proteolysis observed might be related to the activity of trypsin. The enzyme is highly specific for peptide bonds involving the carboxyl group of lysine or arginine, so that there is always the possibility that interaction of the sugars with one or other of these two amino acids in the protein chain may render the adjoining peptide bond less accessible to proteolytic attack. Interaction of the side chain of arginine has been postulated in several reviews but that involving the epsilon amino group of lysine has been confirmed in a number of investigations including that by Waterworth (1964). The binding of lysine in the protein chain is known to involve interaction between the epsilon amino group projecting from the protein chain with reducing compounds such as carbohydrates. In the absence of carbohydrates proteins heated to high temperatures have also been

TABLE VII

Casein-monosaccharide systems (Statistical Analyses)

System	Time (incubation)	b_1 (slope of casein control)	b_2 (slope of casein-sugar)	T
casein-glucose (37°C)	initial	0.03190	0.03182	N.S.
	6 days	0.03586	0.02739	N.S.
	14 days	0.04185	0.02700	7.54645
casein-glucose (55°C)	initial	0.03406	0.03630	N.S.
	4 days	0.03766	0.02812	6.94791
casein-glucose (55°C) (double enzyme)	initial	0.03708	0.03751	N.S.
	5 days	0.04002	0.02858	5.02197
casein-fructose (37°C)	initial	0.03370	0.03584	N.S.
	3 days	0.03228	0.02928	N.S.
	6 days	0.03605	0.02940	3.87728
casein-fructose (55°C)	initial	0.03452	0.03025	N.S.
	1 day	0.03217	0.02801	N.S.
	2 days	0.03452	0.02236	3.22809
casein-galactose (37°C)	initial	0.03370	0.03584	N.S.
	6 days	0.03190	0.03047	N.S.
	12 days	0.03452	0.02659	3.79103
casein-galactose (55°C)	initial	0.03370	0.03584	N.S.
	1 day	0.03778	0.03093	3.12083
	2 days	0.03605	0.02775	4.42333

$t_{0.025} = 2.447$ $t_{0.005} = 3.707$ N.S. not significant

TABLE VIII

Casein-disaccharide systems (Statistical Analyses)

System	Time (incubation)	b_1 (slope of casein control)	b_2 (slope of casein-sugar)	T
casein-lactose (37°C)	initial	0.04290	0.04155	N.S.
	10 days	0.04002	0.03650	2.55891
	20 days	0.04058	0.02843	6.06841
casein-lactose (55°C)	initial	0.03190	0.03287	N.S.
	3 days	0.03725	0.03408	N.S.
	13 days	0.03478	0.01931	8.62055
casein-sucrose (37°C)	initial	0.03364	0.03395	N.S.
	6 days	0.03362	0.03313	N.S.
	12 days	0.04208	0.03548	N.S.
casein-sucrose (55°C)	initial	0.03863	0.03766	N.S.
	4 days	0.03837	0.03478	N.S.
	10 days	0.04147	0.02647	12.64974

$t_{0.025} = 2.447$ $t_{0.005} = 3.707$ N.S. not significant

associated with a decrease in lysine availability. Bjarnason and Carpenter (1970) in studies on heat damage in protein postulated that the loss in available lysine was the result of interaction between the epsilon amino group of lysine and the amide group of asparagine or glutamine.

Recent work by Finot et al. (1968) on the acid hydrolysis of heated milk led to the identification of a new lysine derivative. This was identified as a new basic amino acid referred to as furosine and consisted of ϵ-N-(2-Furoylmethyl)-L-lysine. The derivative is presumably derived from a reaction involving lysine and sugars and is the first example of a stable complex formed. Previous studies concerned with a decrease in available lysine had indicated the lysine was released when the system was acidified prior to amino acid analysis. The important factor to be considered, however, is whether such a complex might affect the accessibility of the peptide bond. While the present studies indicate significant decreases in the ability of trypsin to hydrolyze the casein systems, the importance of lysine-sugar interactions cannot be overlooked. An amino acid analysis of the protein showed a relatively high content of arginine and lysine. The possibility of intermediates produced during the nonenzymic browning process having an inhibitory effect on trypsin must also be considered. Further investigations on lysine availability

of these systems in addition to studies on peptide-sugar systems are required to clarify the situation more fully.

GENERAL DISCUSSION

The results reported for Experiment I showed a distinct difference in the effect of the bisulfite inhibitor under acid and alkaline conditions. This investigation extended the earlier work with glycine reported by McWeeny *et al.* (1969), by studying lysine-sucrose and lysine-glucose sulfited systems in addition to several sulfited casein-sugar systems. The importance of sulfites as chemical preservatives for a large number of food products is well established although the precise mechanism of inhibition is still incompletely understood. These studies were designed to determine the mechanism of sulfite inhibition in the hope of improving the efficiency of nonenzymic control in food products. An interesting feature of these studies was the dramatic decrease in "free" SO_2 under alkaline conditions, which could not be accounted for by a corresponding rise in the "combined" SO_2 level. The loss in "free" SO_2 could only be accounted for by the possible irreversible combination with carbonylic intermediates at the higher pH which appeared to have a negligible effect on the inhibition of the browning process. Consequently there may be several classes of carbonyl intermediates, not all of which effect the rate of the browning process as suggested in earlier studies by Burton *et al.* (1963).

In Experiment II an attempt to isolate and identify

some of the carbonyl intermediates from the sulfited lysine-sugar systems incubated at pH 7.6 was discussed. While the results were not conclusive the products obtained appeared to be common for both the lysine-sucrose and lysine-glucose systems.

The results from Experiment III appeared to indicate an inhibitory effect on the activity of trypsin for the casein systems incubated with carbohydrates, which was not observed with the casein controls incubated under similar conditions of temperature and pH. This could be interpreted as support for the proposed theory that if the epsilon amino group of lysine combined with the reducing sugar an adverse stereochemically effect might be produced limiting the accessibility of the adjoining peptide bond to trypsin attack. While this may be the case, the possibility of other factors such as intermediates formed during the incubation period having an inhibitory effect on the enzyme itself cannot be overlooked. The implications of this in foods could be one of the important factors responsible for the lowering of the nutritional value of the food products.

SUMMARY AND CONCLUSIONS

The results obtained from these investigations are summarized as follows:

1. The efficiency of bisulfite as an inhibitor of browning in the systems studied appeared to depend on the pH. The more acid the environment the more effective was the inhibition.

2. Isolation of some carbonyls were reported although their exact chemical nature was not defined.

3. Significant decreases were reported in the activity of trypsin in a number of casein systems incubated with a series of carbohydrates at 37°C and 55°C.

In order to understand some of the reaction mechanisms involved, further identification of the carbonyls isolated in this study should be carried out. The use of gas chromatography to identify some of the more volatile carbonyls would also provide a useful method for future work. In order to substantiate the theory proposed to explain the reduction of trypsin activity in casein-sugar systems, comparison of these results with lysine availability measurements would be desirable. Further enzyme studies on

peptide-sugar systems could also provide useful information in future studies.

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APPENDIX

APPENDIX I

The following program computes the regression coefficient b, Student's t and variation about regression s_p^2. Two sets of calculations are performed with Y and Y1 respectively as the dependent variables and X the independent variable. Y or Y1 corresponds to optical density and X the square root of time in the experiment. The program represents b by B or B1 depending whether Y or Y1 is the dependent variable, t by T and s_p^2 by SPSQR. Five pairs of values, X and Y or X and Y1, are used in each set of calculations.

```
      DIMENSION X(5),Y(5),Y1(5)
      DATA X/5.48,7.75,8.37,8.94,9.49/
      SUMX=0.0
      SXX=0.0
      XX=0.0
      DO 18 I=1,5
      SUMX=SUMX + X(I)
      XX=XX + X(I) * X(I)
18    CONTINUE
      BARX=SUMX/5.0
      DO 31 J=1,5
31    SXX=SXX + (X(J)-BARX) * (X(J)-BARX)
C
28    READ(5,23) Y,Y1
C
      IF (Y(1) .EQ. 0.0) GO TO 98
23    FORMAT(10F4.2)
      YX=0.0
      Y1X=0.0
      SYY=0.0
      SUMY1=0.0
      SY1Y1=0.0
      SXY1B=0.0
```

```
      SUMY=0.0
      SXYB=0.0
      WRITE(6,34) Y,Y1
      DO 10 I=1,5
      SUMY=SUMY + Y(I)
      SUMY1=SUMY1 + Y1(I)
      YX=YX + (X(I)*Y(I))
      Y1X=Y1X + (X(I)*Y1(I))
      SYY=SYY + Y(I)**2
      SY1Y1=SY1Y1 + Y1(I)**2
10    CONTINUE
      BARY=SUMY/5.0
      BARY1=SUMY1/5.0
      DO 30 I=1,5
      SXY1B=SXY1B + (X(I)-BARX) * (Y1(I)-BARY1)
      SXYB=SXYB + (X(I)-BARX) * (Y(I)-BARY)
30    CONTINUE
      B=SXYB/SXX
      B1=SXY1B/SXX
      SPSQR=((SYY-YX**2/XX) + (SY1Y1-Y1X**2/XX))/6.0
      T=(B-B1)/SQRT(SPSQR*2.0/XX)
      WRITE(6,33) BARY,BARY1,B,B1,SPSQR,T
34    FORMAT(///15X,10F8.2)
33    FORMAT(' ',20X,6F15.5)
      GOTO 28
98    CALL EXIT
      END
```