INHIBITION OF THE RATE OF ALKALINE HYDROLYSIS OF METHYL *trans*-CINNAMATE BY SOME HETEROCYCLIC COMPOUNDS

BY

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A Thesis Submitted in Partial Fulfillment

of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

at the

University of Wisconsin

1966

ACKNOWLEDGEMENT

I wish to express my sincere appreciation to Dr. Kenneth A. Connors, not only for his suggestions and guidance connected with this investigation, but also for his help and understanding with other facets of my graduate career.

I wish to thank the National Institutes of Health, Public Health Service whose financial assistance (PHS Research Grant GM 11136) made this study possible.

Joseph A. Sullivan

To MJ

TABLE OF CONTENTS

I. INTRODUCTION

A. General Background - The term "complex" appears in many branches of chemistry. The forces present in these entities, the types of bonding involved, and the structures of the various kinds of complexes are not yet understood for all types of complexes (1-4). In inorganic chemistry are encountered complexes of transition metals with inorganic and organic ligands; the bonding in these complexes is fairly well understood in terms of conventional orbital concepts, and many structures have been determined (5). Metal ion complexes with various olefins and aromatic hydrocarbons, although not as well characterized as the complexes with simpler ligands, are fairly well understood. Complexes of silver ion, for example, are believed to involve π bonding between the d orbital in the silver and a π^* antibonding orbital of an alkene.

In the realm of organic chemistry, one encounters the so-called σ complexes, which occur, for example, in the Friedel-Crafts reaction when a proton (or other positively charged residue) becomes tetrahedrally bonded to one of the ring carbon atoms and forms a carbonium ion intermediate. Much organic chemistry of importance in biological and pharmaceutical systems appears to involve other types of complexes. The forces involved in these molecular complexes are not well defined, except in such

cases as pure hydrogen-bonding (6).

Electron donor-acceptor interactions are believed to play a significant role in many complexes that are commonly called molecular complexes. For simple systems such as iodine in various donor solvents, relationships between the frequency of the charge transfer interaction, the donor ionization potential, and the electron affinity of the acceptor have been proposed, and some good correlations have been obtained. However, with more complicated molecules simple relationships between ionization potential and election affinity are not so easy to establish, partly because of the difficulty in determining these parameters, but also because forces such as localized dipole-dipole interactions may be brought into play (7). It is, however, such complicated polyfunctional compounds that are of greatest theoretical and practical interest in living systems. Investigations into the interactions of various purines and pyrimidines have been conducted (8-11), as it is believed that the formation of helices in the nucleic acid strands occurs through the interaction of the nitrogenous bases and nucleotides on the polynucleotide chains. Another widely encountered type of complex, perhaps the least understood, is the enzyme-substrate complex. The forces involved may be similar to those involved in molecular complexes between smaller species than proteins. Current thinking as advanced by Woolley, et. al. (12) is that enzyme specificity may be due to non-

covalent bonding between the enzyme and substrate to form the enzyme-substrate complex. That sites for interaction on enzyme and substrate do not necessarily have to be identical or even similar (as suggested by Woolley) may be seen from the hundreds of demonstrated complexes between unlike, but relatively simple, low molecular weight organic compounds.[1] So many biological substances undergo some type of charge-transfer interaction that Szent-Györgi (13) places this phenomenon in a "basic triad" of processes that includes the broader acid-base concept and oxidation-reductions. References 2, 3, 4, 14, on the current status of complexes and methods for their detection have treated these concepts in detail.

Of particular interest to the pharmacist and biochemist is the effect that molecular complexes have on reaction rates. A generalized schematic for a three-component system containing reagent (R), substrate (S), and ligand (L) is shown in Fig. 1. Rate constants are designated $\underline{k}$ and equilibrium constants as K; α, β, γ are dimensionless proportionality constants, and P represents the products of the reaction.

The pathway outlined in the upper part of the figure is the one of primary interest. This indicates that R + S can react to form products P, while S + L can interact to form a complex SL, which may also react with reagent to

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1. A compilation of many of the systems investigated by Higuchi and co-workers is given in Ref. 4.

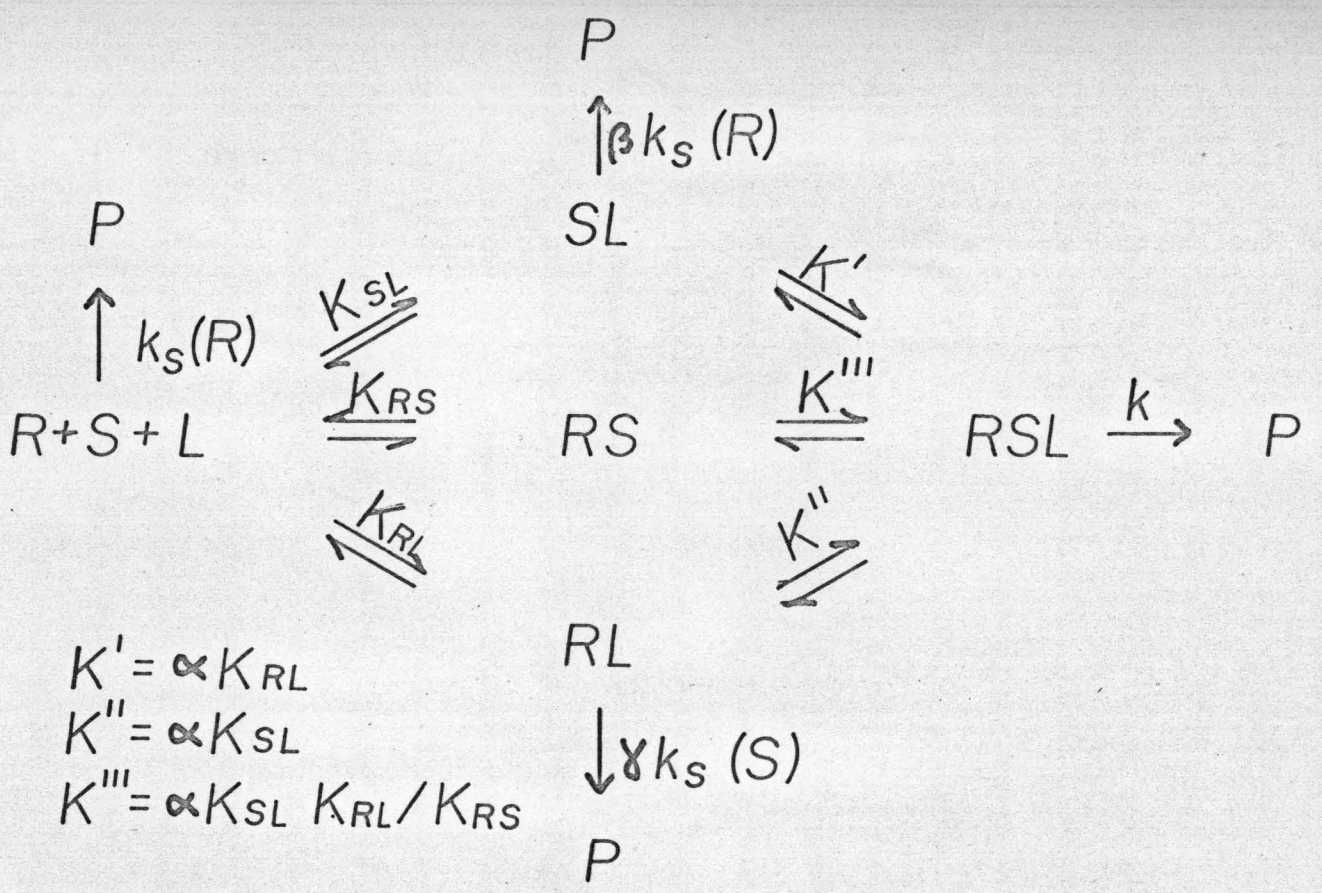

Fig. 1. Schematic diagram for a three component interacting system.

form products. If $\beta < 1$, an apparent inhibition of reaction rate will appear to result from addition of L; if $\beta > 1$, catalysis is said to occur. The lower pathway is practically identical to the upper as the designation of substrate and reagent is usually arbitrary. The middle path is similar to Michaelis-Menten kinetics, in which a reagent-substrate complex is formed, and this undergoes reaction.

Table I lists some previous (non-enzymatic, homogeneous) studies that have been conducted in which modification of reaction rates has been attributed to some type of complex formation. In most of these cases inhibition was observed, although catalysis has also been seen. The nature of these rate inhibitions or catalyses is not completely understood. Some of the examples may be readily rationalized. Thus, benzocaine and β-cyclodextrin probably form an inclusion compound in which the benzocaine is adsorbed into the non-polar region of the sugar residues and the hydrolytic rate is reduced just as it would be if a non-polar solvent were added to the aqueous solution. The catalysis of the decarboxylation of various acids with the α and β forms of cyclodextrin again indicates that the acid is included in the cavities as the β form, with larger cavities, was much more effective than the α form. The catalysis of at least some of the acids could be accounted for by postulating that after inclusion in the cavity, the carboxyl carbon is activated by nucleophilic attack by ether and hydroxyl oxygens. The inhibition observed by

Table I. Reported Instances of Rate Modification Attributed to Complex Formation[a]

	Substrate	Reagent or Reaction	Ligand	i_{50}[b]	Ref.
1.	Picryl Chloride	Triethylamine	Hexamethylbenzene	1.0	15
2.	Aniline	2,4-Dinitrochloro-benzene	Aniline	c	16
3.	β-Naphthol	p-Diazobenzene-sulphonic acid	Caffeine	0.015	17
4.	Benzocaine	Alkaline hydrolysis	Caffeine	0.039	18
5.	Procaine	Alkaline hydrolysis	Caffeine	0.10	19
6.	Tetracaine	Alkaline hydrolysis	Caffeine	0.052	20
7.	Benzocaine	Alkaline hydrolysis	1-Ethyltheobromine	0.041	21
8.	Benzocaine	Alkaline hydrolysis	Polyvinylpyrrolidone	6.9%[d]	22
9.	Benzocaine	Alkaline hydrolysis	N-Methyl-2-pyrrolidone	0.41	22
10.	Benzocaine	Alkaline hydrolysis	N,N-Dimethylacetamide	0.98	22
11.	Benzocaine	Alkaline hydrolysis	Dimethyl urea	e	22
12.	Benzocaine	Alkaline hydrolysis	Thiourea	1.0	22
13.	Benzocaine	Alkaline hydrolysis	Desoxycholic acid	2%[d]	22

	Substrate	Reagent or Reaction	Ligand	i_{50}	Ref.
14.	Benzocaine	Alkaline hydrolysis	Cholic acid	2.5%[d]	22
15.	Benzocaine	Alkaline hydrolysis	Polyethylene glycol 4000	f	22
16.	Benzocaine	Alkaline hydrolysis	Urea	g	22
17.	Ethyl benzimidate	Alkaline hydrolysis	Morpholine	3.5[h]	23
18.	Ethyl benzimidate	Alkaline hydrolysis	Trimethylamine	1.0[h]	23
19.	Ethyl benzimidate	Alkaline hydrolysis	t-Butyl amine	0.75[h]	23
20.	Ethyl benzimidate	Alkaline hydrolysis	n-Butyl amine	0.40[h]	23
21.	Riboflavin	Alkaline hydrolysis	Caffeine	0.035[h]	24
22.	Pyridoxal	α-Aminophenyl-acetic acid	Imidazole	i	25
23.	Ethylenebis (N,N-dimethyl N-(phenyl) carboxy methyl ammonium) bromide	Alkaline hydrolysis	Maleic acid-Acrylic acid copolymer	j	26
24.	2,4,7-Trinitro-9-fluorenyl p-toluene sulfonate	Acetolysis	Phenanthrene	i	27
25.	Benzocaine	Alkaline hydrolysis	β-cyclodextrin	0.25%[d]	28
26.	Riboflavin	Alkaline hydrolysis	Borate	0.15	29

Substrate	Reagent or Reaction	Ligand	i_{50}	Ref.
27. Methyl cinnamate	Alkaline hydrolysis	Imidazole	0.80	30
28. 9-Methyliso-alloxazine	Alkaline hydrolysis	8-Chlorotheophylline	0.014	31
29. 3,9-Dimethyliso-alloxazine	Alkaline hydrolysis	8-Chlorotheophylline	0.010	31
30. α-Methyl-α-benzyl-acetoacetic acid	Decarboxylation	α and β -cyclodextrin	i	32
31. α-Benzylacetoacetic acid	Decarboxylation	α and β -cyclodextrin	i	32
32. α-Methylacetoacetic acid	Decarboxylation	α and β -cyclodextrin	i	32
33. Acetoacetic acid	Decarboxylation	α and β -cyclodextrin	i	32
34. Tribromoacetic acid	Decarboxylation	α and β -cyclodextrin	i	32
35. Trichloroacetic acid	Decarboxylation	α and β -cyclodextrin	i	32
36. Methyl (4-chlorophen-yl)-cyanoacetic acid	Decarboxylation	α and β -cyclodextrin	i	32
37. Methyl (2-chloro-phenyl)-cyano-acetic acid	Decarboxylation	α and β -cyclodextrin	i	32
38. Methyl phenyl-cyanoacetic acid	Decarboxylation	α and β -cyclodextrin	i	32

	Substrate	Reagent or Reaction	Ligand	i_{50}	Ref.
39.	N-(Indole-3-acroyl) imidazole	Alkaline hydrolysis	3,5-Dinitrobenzoate	0.05	33
40.	p-Nitrophenyl 3-Indoleacetate	Alkaline hydrolysis	3,5-Dinitrobenzoate	0.15	33
41.	p-Nitrophenyl 3-Indoleacrylate	Alkaline hydrolysis	3,5-Dinitrobenzoate	0.045	33

a. Inhibitions except as noted. Systems listed are limited to homogeneous, non-enzymatic, non-free radical systems.

b. i_{50} = Molar concentration of ligand which caused a 50% decrease in reaction rate.

c. Not readily expressed, since aniline is both substrate and ligand.

d. Percent concentration of ligand which caused 50% decrease in reaction rate.

e. 25% decrease at 0.68M.

f. 28% decrease at 6%.

g. 8% decrease at 1.0M.

h. No spectral evidence of complexing observed. In a later paper Hand (34) attributed this inhibition to hydrophobic bonding.

i. Catalysis observed.

j. Rate of completely bound ester was about 5% of the free ester.

Morawetz with a cationic ester adsorbed to the surface of a polyanionic polymer could be interpreted as caused by electrostatic repulsion of the catalytic hydroxide ion. With inhibitions involving caffeine as a ligand arguments may be presented implicating steric hindrance, or electronic changes in the complex, or both of these. A more complete discussion concerning caffeine and similar ligands will be given later.

B. Research Plan - This study is concerned with the inhibition of the rate of alkaline hydrolysis of methyl trans-cinnamate with various imidazole, purine, and pyrimidine derivatives. This inhibition has been ascribed to complex formation between the substrate (methyl trans-cinnamate) and the ligands. It was possible to evaluate an equilibrium stability constant from the kinetic measurements as shown in Appendix A; and independent information bearing on the hypothesis was obtained by means of two other non-kinetic techniques for evaluating stability constants: namely, those based on absorption spectroscopy and solubility measurements. Although these are all well known techniques for studying complexation, few investigators have systematically applied more than one of them to a single system.

If the hypothesis is valid that the ester and ligand exist in rapid equilibrium with a complex composed of one

molecule[2] of each species, then all three methods should yield the same numerical value for the equilibrium constant (taking into account the expected experimental uncertainty). This was not always the case. One reason, and probably the most important one, for these descrepancies is treated in detail in Appendix A.

Methyl cinnamate (the trans isomer is to be understood) was selected as the substrate because (a) it absorbs radiation strongly in the ultraviolet region and the hydrolysis product, cinnamate ion, has an ultraviolet spectrum that is shifted sufficiently from that of the ester, so that the reaction can be conveniently followed directly; (b) it hydrolyzes at a convenient rate at pH 12 but the hydrolytic rate at lower pH's is sufficiently slow so that solubility analyses could be performed; (c) the aromatic ring is conjugated with the carbonyl group.

The reasons given in (b) offset each other in that there is great experimental difficulty in maintaining and measuring accurately high alkaline pH values, yet the ester must be sufficiently resistant to hydroxide attack to enable one to equilibrate its solutions during the solubility method for determining interactions.

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2. As pointed out by Mulliken (35), whether one considers the system as consisting of a single 1:1 complex with its many possible configurations due to vibrations and rotations, or to a statistical distribution of molecules in a continuum is a matter of taste, since the observable properties of molecules are statistical averages over all possible configurations in thermal equilibrium.

Imidazole was observed to exhibit an inhibitory effect on the alkaline hydrolysis of various cinnamate esters (36). This was unusual in that many other esters, including nonlabile aliphatic esters, had earlier shown catalytic effects with imidazole. Although the inhibitory effect was small, it was believed to be larger than could be accounted for by a general solvent effect.

In the present investigation other imidazole derivatives were then studied to see if the effect was peculiar to imidazole itself or if the imidazole nucleus was responsible. The study was expanded to include purine derivatives, since many purines have been shown to interact with a wide variety of organic compounds; moreover, they contain the imidazole nucleus. It was anticipated that some purine derivatives might interact more strongly with methyl cinnamate, which would be desirable in attempting to demonstrate the worth of the hypothesis that inhibition is due to complex formation.

Other heterocyclic compounds were selected to evaluate the effects of N-methylation, localized negative charge, distributed charge on the ring, electronegative substitution and alkyl substitution on the interaction with methyl cinnamate.

II. EXPERIMENTAL

A. Materials.- Methyl trans-cinnamate (Matheson Coleman & Bell) was distilled under reduced pressure; b.p. 91-94° (2-3 mm.); m.p. 33-34° (lit. (37) 33.5-34.5°). The molar absorptivity, ϵ_λ , at 279 mμ (the absorption maximum) was $\epsilon_{279} = 2.19 \times 10^4$ (lit. (38) 2.21×10^4) in water. The purity was checked by spectrophotometric determination of the cinnamate ion liberated upon alkaline hydrolysis; the ester was found to be at least 99% pure by this criterion. The second order rate constant for alkaline hydrolysis of methyl cinnamate, based on hydroxide ion concentration, is $6.18 \pm 0.15 \times 10^{-2}$ M^{-1} $sec.^{-1}$ in 0.4% acetonitrile, which is in excellent agreement with the value; $6.04 \pm 0.10 \times 10^{-2}$ M^{-1} $sec.^{-1}$, reported by Bender and Zerner (37), which also is based on hydroxide ion concentration. The second order constant based on hydroxide ion activity (from pH measurements) was found to be $8.26 \pm 0.20 \times 10^{-2}$ M^{-1} $sec.^{-1}$, giving an apparent activity coefficient for hydroxide ion of 0.75; this value is reasonable at the ionic strengths employed (39). Table II lists the rate constants obtained. In these calculations K_w was taken to be 1.00×10^{-14} at 25.0°.

Trans-cinnamic acid (Matheson) was recrystallized from ethanol-water; m.p. 133-134° (lit. (38) 134-135°). $\epsilon_{269} = 2.02 \times 10^4$ (lit. (38) 2.03×10^4) for cinnamate

Table II

Alkaline Rate Constants for Methyl Cinnamate.[a]

pH	$10^3 k_{obs}$ (sec^{-1})	$10^2 k_{OH}$ (M^{-1} sec^{-1})	Buffer	μ
9.19	0.00123	8.00	Borate	0.01
10.04	0.00903	8.43	Borate	0.10
11.28	0.159	8.31	Phosphate	0.10
11.34	0.180	8.27	Phosphate	0.10
12.58	3.06[b]	8.05	Hydroxide/Chloride	0.20
12.58	3.15[b]	8.29	Hydroxide/Chloride	0.20
12.62	3.42[b]	8.21	Hydroxide/Chloride	0.10
12.77	4.98[b]	8.49	Hydroxide/Chloride	0.30
12.77	4.95[b]	8.40	Hydroxide/Chloride	0.30
12.89	6.30	8.13	Hydroxide	0.10

[a] At 25.0° in 1% acetonitrile.

[b] 0.83% acetonitrile.

ion in water.

Imidazole (Aldrich Chemical Co.) was recrystallized three times from benzene using Norit as a decolorizing adsorbent; m.p. 89-90° (lit. (40) 89-90°). 2-Methylimidazole (Aldrich Chemical Co.) was recrystallized twice from benzene with Norit as a decolorizing adsorbent; m.p. 144-145° (lit. (40, 41) 144°, 140-141°). N-Methylimidazole (K & K Laboratories, Inc., Aldrich Chemical Co.) was distilled through a packed column; b.p. 194° (748 mm.) (lit. (42) 195-197° at 760 mm.). Benzimidazole (Eastman Kodak Co., white label) was recrystallized three times from water with Norit; m.p. 172-173° (lit. (40) 171-173°).

Mann Research Laboratories, Inc. "Mann Assayed" guanine and purine were employed without further purification. Guanine did not melt up to 360° (lit. (43) dec. above 360°), and purine gave m.p. 216-217° (lit. (44) 215-216°). Uracil (National Biochemicals Corporation) was recrystallized from water; m.p. 334°, turning brown around 300° (lit. (45) m.p. 335°, turns brown around 280°).

Caffeine, USP was recrystallized from water; m.p. 235-236°. The solubility of caffeine in pH 8.5 borate buffer was 0.108 M at 25°, in excellent agreement with the value 0.107 M in water reported by Emery and Wright (46). Its solubility in our experimental solvent, pH 8.5 borate buffer containing 1% acetonitrile, was found to be $1.13 \pm 0.01 \times 10^{-1}$ M. The solubility was determined by measuring the absorbance of a saturated solution appropriately

diluted; $\epsilon_{273} = 1.00 \times 10^4$.

8-Chlorotheophylline (Aldrich Chemical Co.) was recrystallized from alcohol-water; m.p. 290-292°d. (lit. (47)[3] 290°, 291°). Its purity was checked by potentiometric titration with standard NaOH; the sample assayed 100.0%. The pK_a determined potentiometrically in 15% acetonitrile at 25° was 5.38 (lit. (31) 5.28). 8-Chlorotheophylline appeared to be stable for at least one week in 0.07 NaOH and for at least two months in pH 9.0 borate buffer, as determined spectrophotometrically.

Theophylline-7-acetic acid (Purine-7-acetic acid, 1,2,3,6-tetrahydro-1,3-dimethyl-2,6 dioxo) (Aldrich Chemical Co.) was recrystallized from 95% ethanol; m.p. 269-270° (lit. (49) 269-270°). The purity was determined by titration with standard NaOH and was found to be at least 99.5%, based on the monohydrate. The pK_a determined potentiometrically in 10% acetonitrile at 25° was 3.20. That the compound recrystallized from ethanol was the monohydrate was determined by loss of weight on drying at 150°. It has been reported that degradation of the pyrimidine ring occurs in strong alkali (50). This was investigated spectrophotometrically, as a marked spectral change is expected upon ring cleavage. The half-life was estimated to be approximately two days in 0.16 N NaOH.

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3. Donbrow (48) has reported the value 311°, which is in wide disagreement with our value and the other reported values.

This cleavage was assumed to be negligible in our studies, where the maximum hydroxide ion concentration was 0.04 M and the time involved was a matter of minutes.

Theophylline (Merck & Co., Inc., USP) was recrystallized from water, m.p. 270-271°. That the compound recrystallized from water was the monohydrate was determined by drying at 150° and by titration in N,N-dimethylformamide solution with standard sodium methoxide in benzene-methanol, thymol blue being the indicator. The solubility of theophylline in our experimental solvent, pH 6.75 phosphate buffer containing 1% acetonitrile, was determined spectrophotometrically by appropriate dilution of a saturated solution to be $3.58 \pm 0.03 \times 10^{-2}$ M; $\epsilon_{272} = 1.00 \times 10^{4}$.

Acetonitrile (Eastman Kodak Co., technical grade;[4] Matheson Coleman & Bell, practical grade) was refluxed over phosphorous pentoxide (51) and distilled twice from phosphorous pentoxide; the final distillation was through a packed column; b.p. 80-81°. Isooctane (2,2,4-Trimethylpentane) (Matheson Coleman & Bell, practical grade) was shaken with concentrated H_2SO_4 intermittently for at least two days, filtered through anhydrous potassium carbonate and distilled. The middle fraction, b.p. 98-99°, was

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4. Some batches of Eastman acetonitrile when purified by the above procedure did not yield a spectrally clear product. Further attempts at purification by the method of O'Donnell, et. al. (52) gave no significant improvement. Acetonitrile supplied by Matheson Coleman & Bell gave a spectrally pure solvent when treated in the above fashion.

collected. This procedure yielded a product that was spectrally clear in the region studied. Silicic acid (Mallinckrodt AR) was used without further purification. All water was redistilled from alkaline permanganate in an all glass system. Buffer chemicals were of reagent grade quality. Standard buffers were prepared with freshly boiled double distilled water according to Bates (53). Other buffers were prepared according to Bates and Bower (54) or Koltoff and Rosenblum (55).

B. Apparatus. pH was measured with a Radiometer pH meter model 25 with scale expander, equipped with a wide pH range glass electrode G 202 B.

Most of the spectra and rate constants reported here were determined with a Cary Recording Spectrophotometer model 14; some measurements were made on a Cary Recording Spectrophotometer model 15 or on a Beckman DU equipped with a Gilford recorder and a digital readout. Each instrument was fitted with a thermostated cell compartment that maintained temperature to $\pm$ 0.1°.

Water bath temperatures were maintained to $\pm$ 0.05° with a Sargent Thermonitor Electronic Relay. Thermometers were checked against a thermometer carrying a National Bureau of Standards calibration certificate.

Solubility determinations were made with an apparatus capable of rotating ten 15 ml screw-capped vials in a constant temperature water bath. This apparatus was

composed essentially of a vertical shaft rotated from the top by an electric motor (whose speed could be varied), and connected through gears at its lower end to a horizontal axle, which was rotated at about 70 RPM. The vials were fastened radially (in holders constructed from ointment jar covers), so that they tumbled end-over-end.[5]

C. Kinetic Measurements. Kinetic measurements were performed in one of three ways depending upon the half-life of the reaction.

(i) Reactions with half-lives in the range 2 to 8 minutes. In a typical run, cuvettes with 3.0 ml of buffer containing the ligand were placed in the reference and sample compartments of the spectrophotometer and were allowed to stand for about 15 minutes to reach temperature equilibrium. The spectrophotometer signal was balanced and then 25 microliters of acetonitrile was added to the reference cell by means of a small glass stirring rod and 25 µl of ester in acetonitrile solution was added to the sample cell, which was covered with a Teflon stopper. Recording was usually started within 10 seconds after initiation. Reactions were generally followed for at least two half-lives. However at least one of the samples containing the ligand was followed to about 95% of completion to insure that the reaction adhered to apparent

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5. I wish to thank Mr. Herbert E. Bird for constructing this apparatus.

first-order kinetics throughout its course.

(ii) Reactions with half-lives in the range 8 to 25 minutes. Twenty-five ml of the buffer solution containing ligand was equilibrated in a water bath. Then 0.2 ml of ester in acetonitrile solution was added to initiate the reaction. Three ml of this solution was rapidly transferred to a cell that had been equilibrating in the cell compartment. Recording was usually started within 50 seconds of zero time.

(iii) Reactions with half-lives greater than one hour. The appropriate solution was prepared in a 100 ml volumetric flask. 1.0 ml of ester in acetonitrile was added and the solution was brought to volume. Aliquots were taken at appropriate time intervals for analysis.

The reactions were followed either at 295 mμ or 320 mμ. For analyses at 295 mμ the initial ester concentration was about 6×10^{-5} M and for studies at 320 mμ the initial ester concentration was 7×10^{-4} M. The spectral absorption of each of the ligands employed could be blanked out satisfactorily at the wavelengths chosen.

In an additional experiment a set of determinations was performed using caffeine as the ligand in the following manner: Solutions were prepared in which the initial ester concentration was 5×10^{-4} M. Aliquots (5 ml) were withdrawn at appropriate time intervals and extracted with 5.0 ml of isooctane. Three ml of the isooctane extract was placed on a partition column (internal phase: 20 g.

silicic acid, 20.0 ml pH 7.4 phosphate buffer; external phase: isooctane). Fifty ml of eluate was collected and the absorbance of the unreacted methyl cinnamate was measured at 271 mμ. The relative rate constants were in good agreement with those obtained by direct determination at 320 mμ.

Stock solutions of the ligand were prepared by adding a weighed amount of agent to a volumetric flask. If the ligand was ionizable at the pH of the studies, an equimolar amount of 1 M NaOH was added. A buffer solution and sufficient KCl to adjust the ionic strength were added. This solution was brought to temperature and its pH was determined. The buffer stock solution was adjusted to the same pH by adding small amounts of hydrochloric acid or sodium hydroxide solution. Then solutions with different concentrations of ligand, but identical pH, could be prepared by serial dilution of the ligand stock solution with the buffer stock solution.

Rate constants were obtained by plotting log (A_t - A_∞) vs time or by the method of Guggenheim (56).

D. Solubility Measurements. Methyl cinnamate in excess of its solubility was added (in 0.1 ml of acetonitrile solution) to 10.0 ml of buffer solution containing varying amounts of ligand.[6] The ligand concentrations were obtained by serial dilution of a stock solution of the agent for

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6. For a review of the solubility method see reference ~~5~~ 4

those concentrations below its solubility. For concentrations of ligand above its solubility, accurately weighed amounts were placed in each vial and 10.0 ml of the buffer solution added. The vials were then closed, placed in the apparatus described earlier, and rotated for 24 hours. (This time was sufficient as identical values were obtained at 16, 24 and 48 hours.) An aliquot of the supernatant liquid ligand was then withdrawn by pipette, around the tip of which glass wool had been wrapped to act as a filter. Depending on the properties of the ligand, the analysis was conducted in one of three ways. For those ligands that did not have an absorption band in the analytical wavelength region, the aliquot was diluted with water and the concentration of methyl cinnamate determined spectrophotometrically. Ligands that did absorb but were not appreciably soluble in isooctane were treated as follows: The aliquot (usually 1.0 ml) was extracted with 5.0 ml of isooctane, then 1.0 or 2.0 ml of the isooctane extract was diluted to 50.0 ml with isooctane, and the absorption by methyl cinnamate measured at 271 mμ.

When caffeine was the ligand, the procedure was modified to include a chromatographic separation. After the aliquot was extracted with isooctane, 2.0 ml of the isooctane extract was placed on the partition column described earlier and the ester was eluted with isooctane.

The absorption spectra after equilibration and dilution were all identical with that of pure methyl

cinnamate, indicating that any changes in solubility were not caused by irreversible chemical reaction between the ester and ligands.

Apparent equilibrium constants were determined from plots of total apparent molar solubility of ester against total concentration of ligand. (See Appendix A.)

E. Spectral Studies. Stability constants were also evaluated from spectroscopic measurements. A typical run was conducted as follows: two 25 ml flasks each containing the same amount of ligand in an appropriate buffer were equilibrated at 25.0°. To one flask was added 0.2 ml of pure acetonitrile, to the other 0.2 ml of ester in acetonitrile. Three ml of the former solution was placed in the reference cell and 3 ml of the latter solution was placed in the sample cell; these were brought to temperature in the spectrophotometer. The absorption was then measured, usually at 320 mμ. The solutions containing buffer and ligand were usually filtered through a sintered glass funnel to remove any dust or lint.

Alternative procedures were also used. The appropriate solution (3.0 ml) was placed in each of two 1 cm cuvettes and equilibrated in the sample and reference compartments of the spectrophotometer. After balancing the signals instrumentally, 25 μl of pure acetonitrile was added to the reference cell and 25 μl of ester in acetonitrile was added to the sample cell by means of a small glass stirrer and the absorbance was measured. It was also

possible to obtain the same data by extrapolation to zero time on plots of log $(A_t - A_\infty)$ *vs* time. This method was not as accurate as the former due to the difficulty in reading the graphical extrapolation. More variation was found when using a 25 µl pipette than with one of larger volume.

For some systems we were able to utilize a new spectrophotometric method. The equations involved for the application of this technique are given in Appendix B. An aliquot of a saturated solution of methyl cinnamate was placed in the reference cuvette and an aliquot of a saturated solution of the ester, containing ligand (obtained from the solubility studies) was placed in the sample cuvette, then the absorbance was determined. Spectra were obtained over a narrow wavelength range in the region where there was no appreciable ligand absorbance. Because our reference cell contained a solution saturated with respect to free ester, the difference spectra obtained are due to the complex or complexes present.

F. *Partition Studies*. In order to determine the amount of monomeric caffeine present at a given total caffeine concentration, a method similar to that utilized by Guttman and Higuchi (57) was employed.

Various amounts of a 0.09 M caffeine solution in pH 9.2 borate buffer were measured by buret into 15 ml vials; buffer solution was added to adjust the volume to 5.0 ml. Isooctane (5.0 ml) was added and the vials were

stoppered with screwcaps fitted with Teflon liners. The vials were equilibrated for about 10 hours at 25.0° with the solubility apparatus described earlier.

The organic layer was removed and assayed spectrophotometrically. The limiting partition coefficient (concentration in aqueous phase/concentration in organic phase, at infinite dilution) was 215. The limiting value is given since the partition coefficient is nonlinear.

G. Treatment of Data. The data were analyzed in a fashion that would enable us to evaluate an apparent 1:1 stability constant (K'_{11}). The manner in which the experimental points obtained by the three methods were plotted are given below. Derivations, assumptions and approximations involved in each of the basic equations are treated in Appendix A.

From the rate constants obtained in the presence and absence of ligand it is possible to evaluate K'_{11} from the following equation:

$$\frac{1}{k_s - k'_s} = \frac{1}{q_{11} k_s K_{11} [L]} + \frac{1}{q_{11} k_s} \qquad (1)$$

where k_s = rate constant for the hydrolysis of methyl cinnamate in the absence of ligand; k'_s = rate constant in presence of ligand concentration, [L]; $q_{11} = 1 - k_{11}/k_s$, in which k_{11} is the rate constant for the hydrolysis of the complex, and K_{11} = complex stability constant. K'_{11} is equal to the intercept divided by the slope in a plot of

the left-hand member of eq. (1) *vs* $1/L_t$.

The solubility data are treated by plotting the apparent total molar solubility (S_t) of methyl cinnamate *vs* total ligand concentration (L_t). If a 1:1 complex is formed one should obtain a linear plot. The equation governing this relationship is:

$$S_t = \frac{K_{11} S_o L_t}{1 + K S_o} + S_o \qquad (2)$$

and

$$K'_{11} = \frac{\text{Slope}}{S_o(1 - \text{Slope})} \qquad (3)$$

The conventional spectroscopic evaluation of a stability constant utilizing data obtained at constant total substrate concentrations can be described by the equation:

$$\frac{b}{\Delta A} = \frac{1}{K'_{11} S_t \Delta \epsilon [L]} + \frac{1}{S_t \Delta \epsilon} \qquad (4)$$

where b = cell path length; $\Delta A = A - A_o$, where A is the absorbance of methyl cinnamate in the presence of ligand at concentration $[L]$ and A_o represents the absorbance of methyl cinnamate in the absence of ligand; S_t is the total molar concentration of methyl cinnamate present; $\Delta \epsilon = \epsilon_{11} - \epsilon_s - \epsilon_L$, where ϵ_{11} is the molar absorptivity of the complex and ϵ_s and ϵ_L are the molar absorptivities of methyl cinnamate and ligand, respectively.

Spectroscopic evaluation of the stability constant at varying S_t (the data obtained by measuring the absorbance

of saturated solutions in the presence of ligand vs a saturated solution of methyl cinnamate) is made as follows: a plot of absorbance vs L_t should be linear passing through the origin with a slope equal to $\epsilon_{11}K_{11}S_o$. From the solubility isotherm the concentration of complex present can be determined; then a plot of absorbance vs [SL] should give a linear relationship going through the origin with a slope equal to ϵ_{11}. These two plots now permit the evaluation of K'_{11}. (See also Appendix B.)

As stated in the opening of this section, the data were generally treated to evaluate K'_{11}. The data were, however, not always amenable to such treatment, and the K'_{11} evaluated by the different techniques for the same system were not always in agreement. A more complete treatment of these discrepancies is given in Appendix A.

III. RESULTS

A. Imidazole - Since the reports of Bender ('58) and Bruice (40) and their co-workers on the catalytic properties of imidazole toward p-nitrophenyl acetate, many studies into the catalytic activity of imidazole have been conducted (59-62). These investigations have shown that imidazole catalysis may proceed by one or more pathways depending upon the substrate and the reaction conditions. An overall rate expression for the hydrolysis of an ester in the presence of imidazole may be written (63):

$$V = k_w[RCOOR'] + k_{OH}[RCOOR'][OH] + k_1[RCOOR'][Im] + k_2[RCOOR'][Im]^2 + k_3[RCOOR'][Im][OH].$$

The first rate term in this equation represents "uncatalyzed" hydrolysis, and the k_{OH} term accounts for hydroxide ion catalysis. The k_1 term represents the simple catalysis by imidazole through either nucleophilic attack or general base catalysis; the particular ester determines the mechanism, and for most esters it is not known which pathway is followed as they are kinetically indistinguishable. The k_2 term refers to the general base catalysis by imidazole of the nucleophilic reaction of imidazole and the k_3 term refers to hydroxide ion catalysis of the nucleophilic attack by imidazole.

This overall rate equation, however, considers only

the catalytic (i.e. rate enhancing) properties of imidazole. In this study, evidence is presented to show that imidazole is capable of rate inhibition in the hydrolysis of methyl cinnamate. (Table III, Fig. 2)

It is postulated that an unreactive complex is formed between the methyl cinnamate and imidazole, which leads to an overall decrease in the observed hydrolytic rate. Complex formation between imidazole and α-amino-phenylacetic acid has been demonstrated by Bruice and Topping (25). A stability constant for the methyl cinnamate-imidazole complex was evaluated from the solubility analysis according to the method described by Higuchi and Connors (4). The stability constant was calculated on the assumption (which is consistent with the solubility data) that the interaction product contains one molecule each of the imidazole and ester. The constant evaluated was 1.0 ± 0.1 M^{-1}.

Although this effect is small, we believe it is greater than can be accounted for by an activity coefficient effect. For example, Kirsch and Jencks (64) have determined approximate activity coefficients for trifluoroethyl acetate and ethyl acetate in 1M imidazole (95% free base). They found that the ratio of the solubility in 1M imidazole to the solubility in water was 1.12 and 1.37 for the trifluoroethyl and ethyl esters, respectively. We have found that at this concentration the solubility ratio in

TABLE III

Rate Constants for Alkaline hydrolysis of methyl cinnamate in the Presence of Imidazole at 25°.[a]

Total Imidazole (M)	$10^2 k_s'$ (M^{-1} sec^{-1})[b]
0.000	6.17 6.19
0.080	5.80[c]
0.158	5.49 5.43
0.160	5.39[c]
0.316	4.87 4.96
0.320	4.76[c]
0.400	4.51[c]
0.632	4.02 3.95
0.790	3.50 3.58 3.53 3.53

[a] OH = 0.0181 or 0.0213 M; μ = 0.02; Acetonitrile, 0.4%.

[b] k_s' is the apparent second-order rate constant (concentration basis).

[c] μ = 1.0

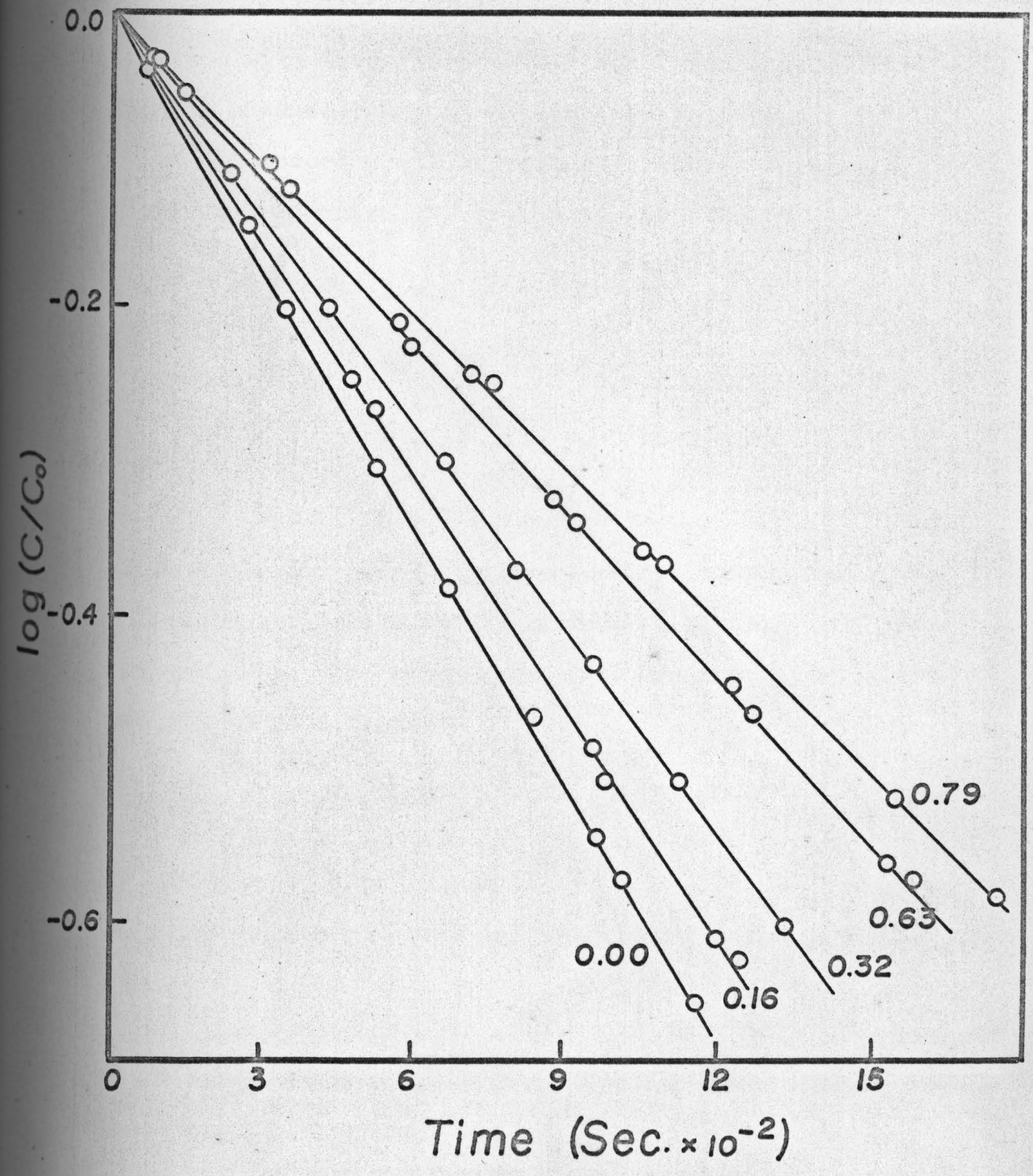


Fig. 2. Pseudo-first order plots for the hydrolysis of methyl cinnamate in the presence of imidazole; molar concentrations of imidazole are given.

our system was 2.50.[7]

Moreover, the non-specific solvent effect could be independently estimated with a compound less apt to interact with the ester; and it was found that 1M acetonitrile produced only an 8% decrease in rate.

In accordance with the experimental data, the following mechanism has been proposed (30).

$$S + L \underset{}{\overset{K_{11}}{\rightleftharpoons}} SL \qquad (1)$$

$$SL + OH \xrightarrow{k_{11}} P \qquad (2)$$

$$S + OH \xrightarrow{k_s} P \qquad (3)$$

$$S + L + OH \xrightarrow{k_c} P \qquad (4)$$

where S, L, SL, and P represent methyl cinnamate, imidazole, imidazole-methyl cinnamate complex, and products, respectively. The rate expression for the formation of products may be written

$$\frac{d[P]}{dt} = k_{11}[SL][OH] + k_s[S][OH] + k_c[S][L][OH] \qquad (5)$$

At any time t, $S_i = [S] + [SL] + [P]$ (6)

where S_i = initial ester concentration. We also write

$$K_{11} = \frac{[SL]}{[S][L]} \qquad (7)$$

which defines the complex formation constant. Combining equations (5-7) yields

---

7. Concentrations greater than 0.79M imidazole were not used in our calculations as the solubility isotherm showed some positive curvature above this concentration.

$$\frac{d[P]}{dt} = \frac{[S_i - P]}{1+K_{11}[L]}\left(k_{11}K_{11}[L] + k_s + k_c[L]\right)[OH] \qquad (8)$$

or, since

$$\frac{d[P]}{dt} = k_{obs}\left[S_i - P\right] \qquad (9)$$

we obtain the observed rate constant

$$k_{obs} = \frac{[OH]}{1+K_{11}[L]}\left(k_{11}K_{11}[L] + k_s + k_c[L]\right) \qquad (10)$$

Jencks (63) has demonstrated hydroxide ion catalysis of the nucleophilic reaction of imidazole with esters; this effect has been observed, however, only with aromatic esters. The only aliphatic ester studied by Jencks was ethyl acetate, and, in the presence of 1.0M imidazole, no catalysis was observed; it was therefore estimated that the third order rate constant was less than 10 M^{-2} min^{-1}. Assuming that the result of that is reasonably applicable in the present case, equation (10) reduces to

$$k_{obs} = \frac{[OH]}{1+K_{11}[L]}\left(k_{11}K_{11}[L] + k_s\right) \qquad (11)$$

This equation may be rearranged into a convenient form to determine K_{11} (See eq. A-23, Appendix A). A plot for the imidazole system is shown in Fig. 3. K_{11} evaluated from this graph is 0.9 ± 0.1 M^{-1}. The y - intercept agrees, within our experimental error, with k_s (6.18×10^{-2} M^{-1} sec^{-1}), which means that the complex hydrolyzes at a rate less than 3% of the free ester.

Some additional evidence for the support of this

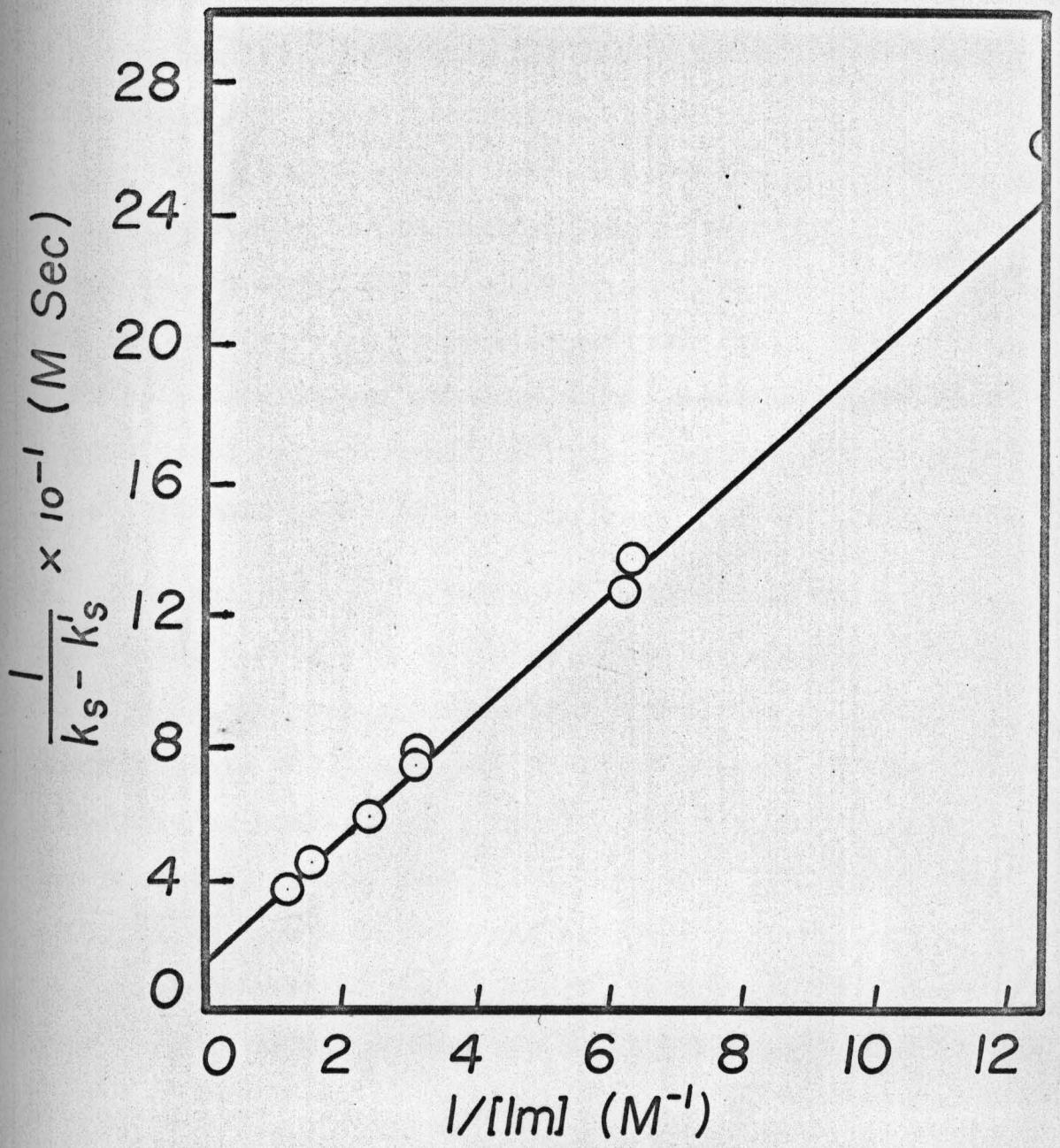


Fig. 3. Plot of kinetic data for the methyl cinnamate-imidazole system (data are given in Table III).

mechanism is a slight shift in the spectrum of the ester, to longer wavelengths, in the presence of imidazole. A non-polar solvent, isooctane, on the other hand causes a hypsochromic shift. The bathochromic shift with imidazole would be expected if charge transfer forces were responsible for the complex. Imidazole has been postulated to possess moderately good donor properties (65).

The spectral shift cannot be due to the formation of an acylimidazole as the acylimidazole formed in this case would be cinnamoylimidazole, which absorbs powerfully at 310 mμ; moreover, if it were formed one would observe catalysis because the alkaline rate constant for cinnamoylimidazole is more than 10^3 times larger than is that of methyl cinnamate (66).

B. 2-Methylimidazole - Imidazole substituted on the number two carbon with a methyl group has also been shown to have a catalytic effect on the hydrolysis of p-nitrophenylacetate (67), although it is only about 13% as effective as imidazole. A kinetic and solubility study was conducted in the same fashion as with imidazole. The equilibrium constants obtained were not significantly different from those obtained with imidazole. K_{11} was evaluated to be 1.2 M^{-1} by the solubility method and to be 1.0 M^{-1} from the kinetic data.

C. N-Methylimidazole has also been shown to be effective

as a general basic catalyst toward certain esters (60). In our studies an inhibition was exhibited, but the nature of the effect suggested that there is some mechanism operative other than an equilibrium process (Fig. 4), since a plot of k_s' vs [L] should not be linear but should be hyperbolic if a complex is reversibly formed. This was not investigated further, but we will later consider some possible mechanisms.

D. Benzimidazole - In concentrations up to 0.03 M, benzimidazole had no apparent effect on the solubility of methyl cinnamate. Benzimidazole up to 0.06 M had a small inhibitory effect on the hydrolysis of methyl cinnamate (Table IV). The kinetics were conducted at pH 12.8, therefore part of the benzimidazole was in the anion form (pK_a (68) 12.57). Also, benzimidazole had no detectable effect on the spectrum of methyl cinnamate.

E. 8-Chlorotheophylline - After the imidazole series was investigated, it was decided to study some compounds containing the purine nucleus. These compounds were chosen for several reasons: (1) they have been shown to interact with many types of compounds (4, 69, 70, 71); (2) when benzimidazole did not show any marked effect over that of the simple imidazoles it was decided to see how the purine ring system compared (purine based compounds contain the imidazole nucleus); (3) xanthines and purines are closely related to many compounds of biological and pharmaceutical

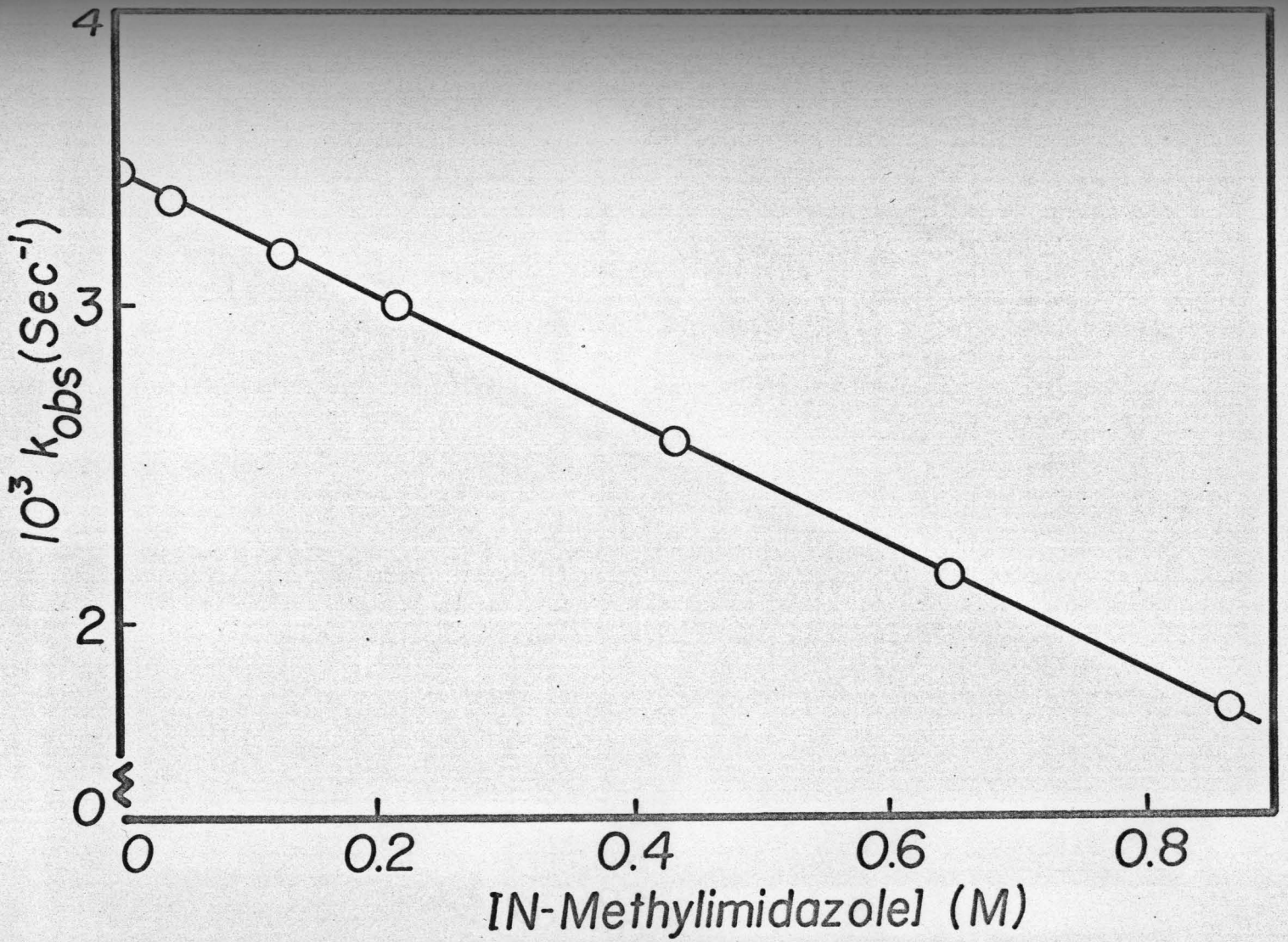


Fig. 4. The observed hydrolytic rate constant as a function of N-methylimidazole concentration

TABLE IV

Effect of Benzimidazole on the Alkaline Hydrolysis of Methyl Cinnamate at 25°.[a]

Benzimidazole ($M \times 10^2$)	$10^3 k_{obs}$ (sec^{-1})
2.38	5.71
3.56	5.45
4.75	5.37
5.94	5.35

[a] 0.83% acetonitrile; hydroxide-chloride buffer; $\mu = 0.2$.

importance.

8-Chlorotheophylline itself has been shown to complex with 9-methylisoalloxazine and 3,9-dimethylisoalloxazine (31) and the related compounds 8-chlorocaffeine and 8-chlorotheobromine have been shown to interact with 3,4-benzpyrene (72).

The interaction of methyl cinnamate and 8-chlorotheophylline (8-chlorotheophylline has a pKa of 5.3 and therefore was ionized under the conditions of our experiments, so whenever 8-chlorotheophylline is specified the anionic form is meant) was studied by three techniques. There was a marked decrease in the rate of hydrolysis with increasing amounts of 8-chlorotheophylline (Fig. 5). The shape of the curve indicates that an equilibrium process is responsible for the rate decrease, with a "saturation" effect being apparent at high ligand concentrations.

If the hypothesis presented for imidazole is valid here also, we should be able to evaluate a complexing constant. It was also deemed necessary to check this hypothesis by evaluating the stability constant by other, non-kinetic, methods. If the hypothesis is correct and only a single 1:1 complex is present then all three methods should yield the same result. A survey of the literature has not revealed any instance where an interaction system has been studied by three methods to give three essentially identical constants. Many reasons have been given for this disagreement among constants, but our

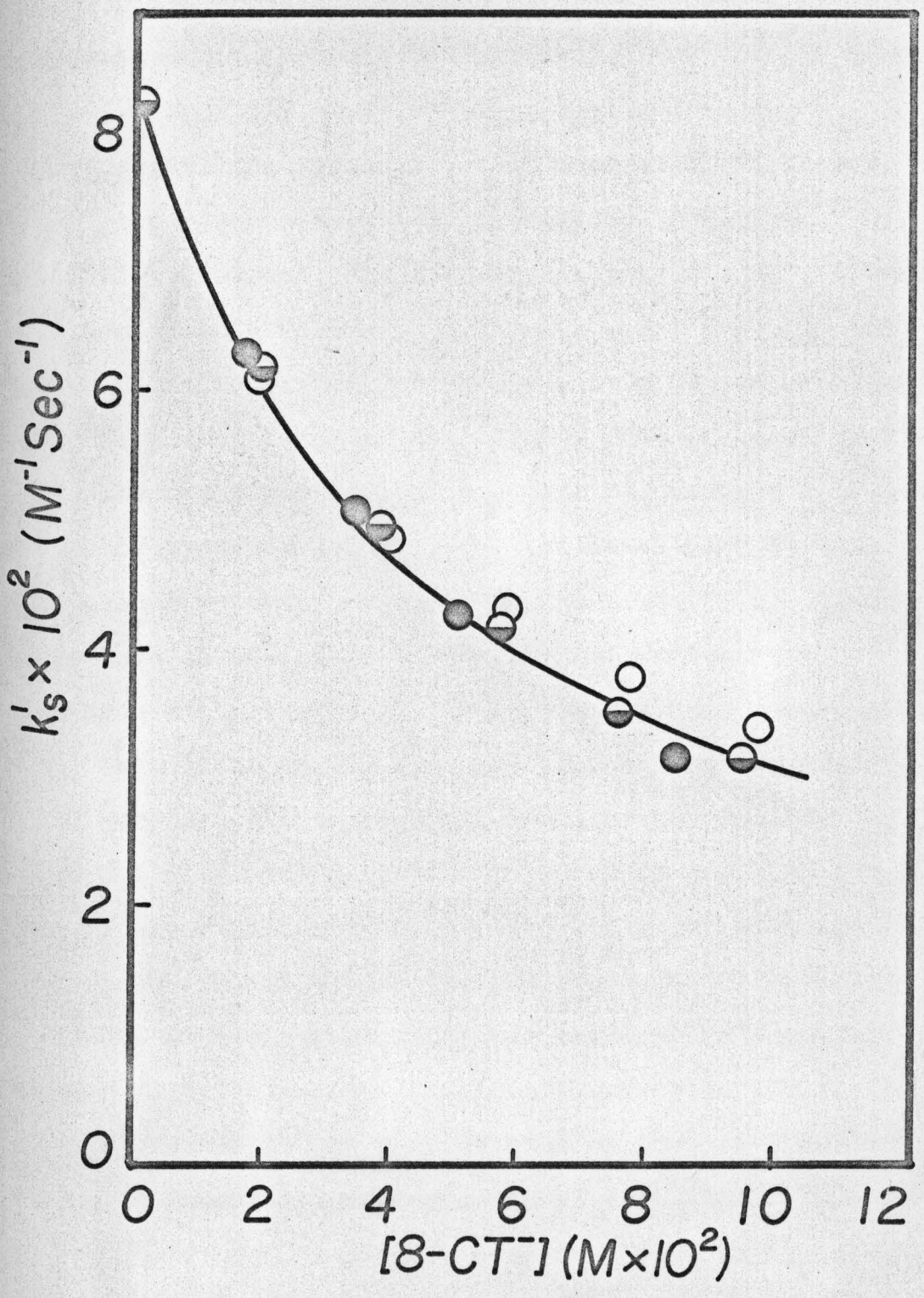


Fig. 5. Second order rate constants for alkaline hydrolysis of methyl cinnamate in the presence of 8-chlorotheophylline; ⊖-pH 10.04 borate buffer; ○-0.1 N NaOH; ●-pH 12.57 hydroxide/chloride buffer; 1% CH_3CN; 25.0°.

analysis indicates that many such instances may be explicable if multiple complexes, or stoichiometries other than 1:1, are present. For a complete discussion of this problem Appendix A should be consulted.

A solubility study of the methyl cinnamate-8-chlorotheophylline system was conducted at three temperatures; the results are shown in Fig. 6. From the equilibrium constants obtained it was possible to evaluate the thermodynamic values of ΔG°, ΔH°, and ΔS° for the complex formation reactions (Table V). ΔH° was obtained by the method of least squares from a plot of log K vs 1/T.

The xanthines have been postulated to have relatively good donor properties (65) and if at least part of the intermolecular force is due to charge transfer it should be possible to evaluate the equilibrium constant by spectrophotometric methods. Fig. 7 shows that a spectral change does occur in the presence of 8-chlorotheophylline. This figure shows the effect of 8-chlorotheophylline on the methyl cinnamate spectrum at 320 mμ at two concentrations of ester differing by a factor of 2.5. That the relative absorbance change for the two cases is constant throughout at least indicates that the complex contains only one methyl cinnamate molecule (Table VI). If these data are plotted in the conventional manner, as shown in Fig. 8, the stability constant can be evaluated. Both constants are 26 M^{-1}. This agreement at two values of the substrate concentration is further evidence that a 1:1

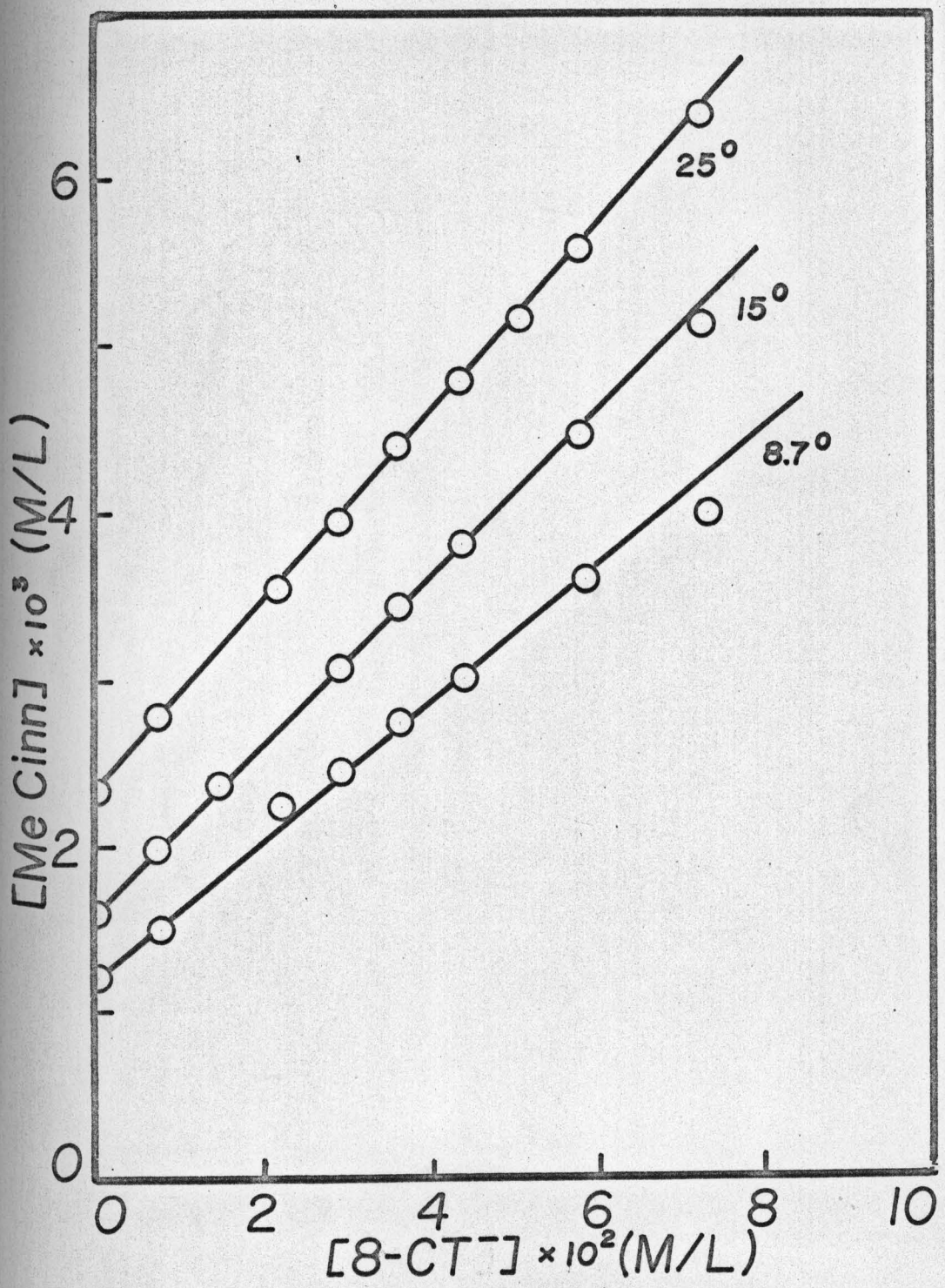


Fig. 6. The apparent solubility of methyl cinnamate as a function of 8-chlorotheophylline concentration in borate buffer.

TABLE V

Thermodynamic Values Obtained from Equilibrium Constants Determined by the Solubility Method for Methyl Cinnamate-8-Chlorotheophylline Complex.

T(°C)	$1/T$	K(M^{-1})	ΔG°(cal/mole)	ΔS°(cal/mole deg)	ΔH°(cal/mole)
25.0°	3.36×10^{-3}	26	-1900(-1930)	-4.8 (-4.76)	
15.0°	3.47	32	-2000(-1980)	-4.7 (-4.76)	-3350
8.7°	3.55	36	-2000 (-2000)	-4.7 (-4.80)	

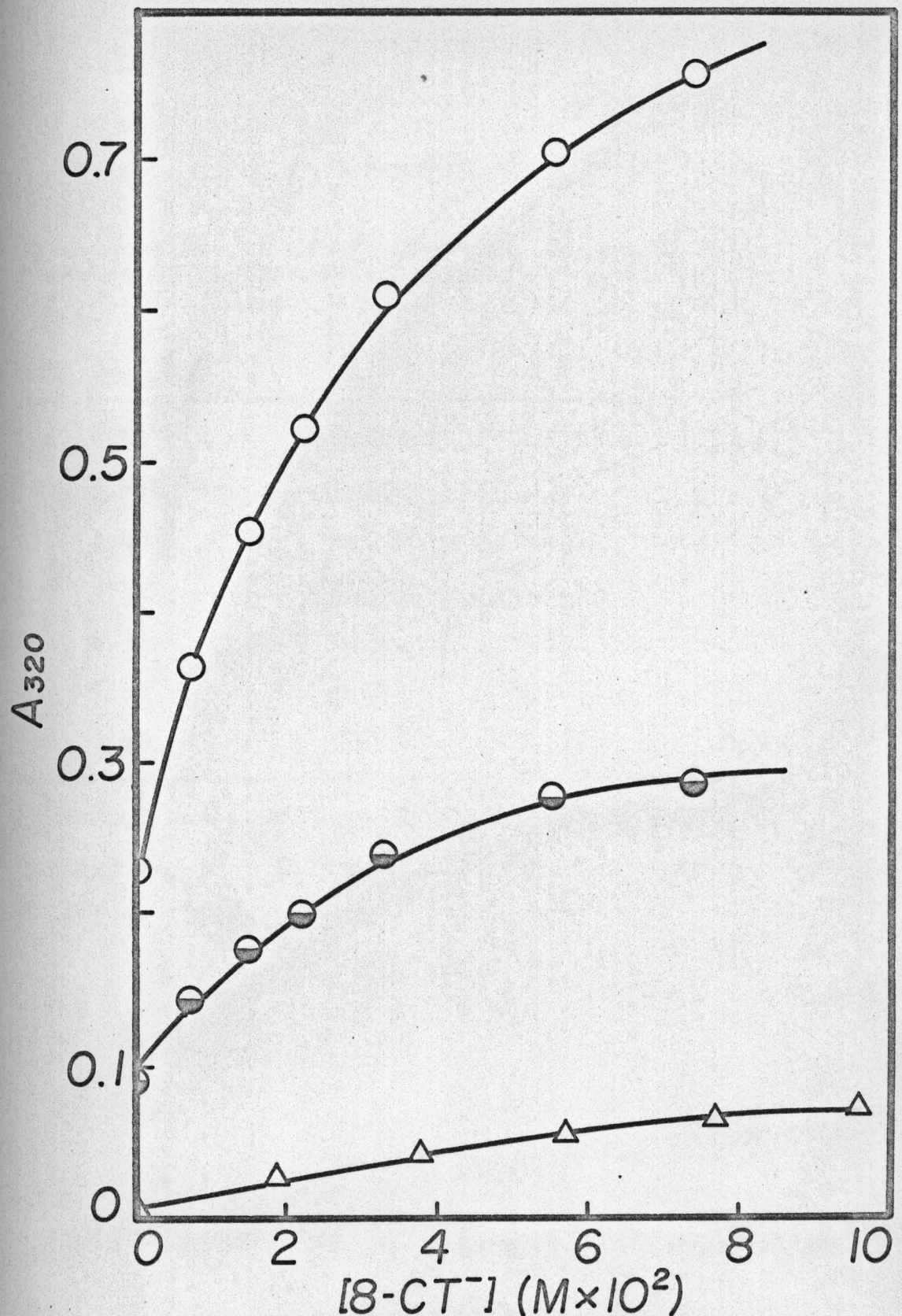


Fig. 7. Upper curves--Absorbance of methyl cinnamate solutions at two concentrations as a function of the 8-chlorotheophylline concentration (data from Table VI); bottom curve--absorbance of cinnamate ion (4.96×10^{-4} M) in the presence of the same ligand.

TABLE VI

The Absorbance of Methyl Cinnamate at 320 mμ in the Presence of 8-Chlorotheophylline.[a]

8-CT ($M \times 10^2$)	A_1[b]	A_2[c]	A_2/A_1
0.00	0.091	0.233	2.56
0.740	0.144	0.367	2.55
1.48	0.178	0.455	2.56
2.22	0.200	0.523	2.62
3.31	0.241	0.611	2.54
5.55	0.280	0.707	2.53
7.40	0.288	0.759	2.64

[a] pH 8.47 borate buffer; 1% CH_3CN; 25°; $\mu = 0.1$.

[b] ester = 2.38×10^{-4} M.

[c] ester = 6.16×10^{-4} M.

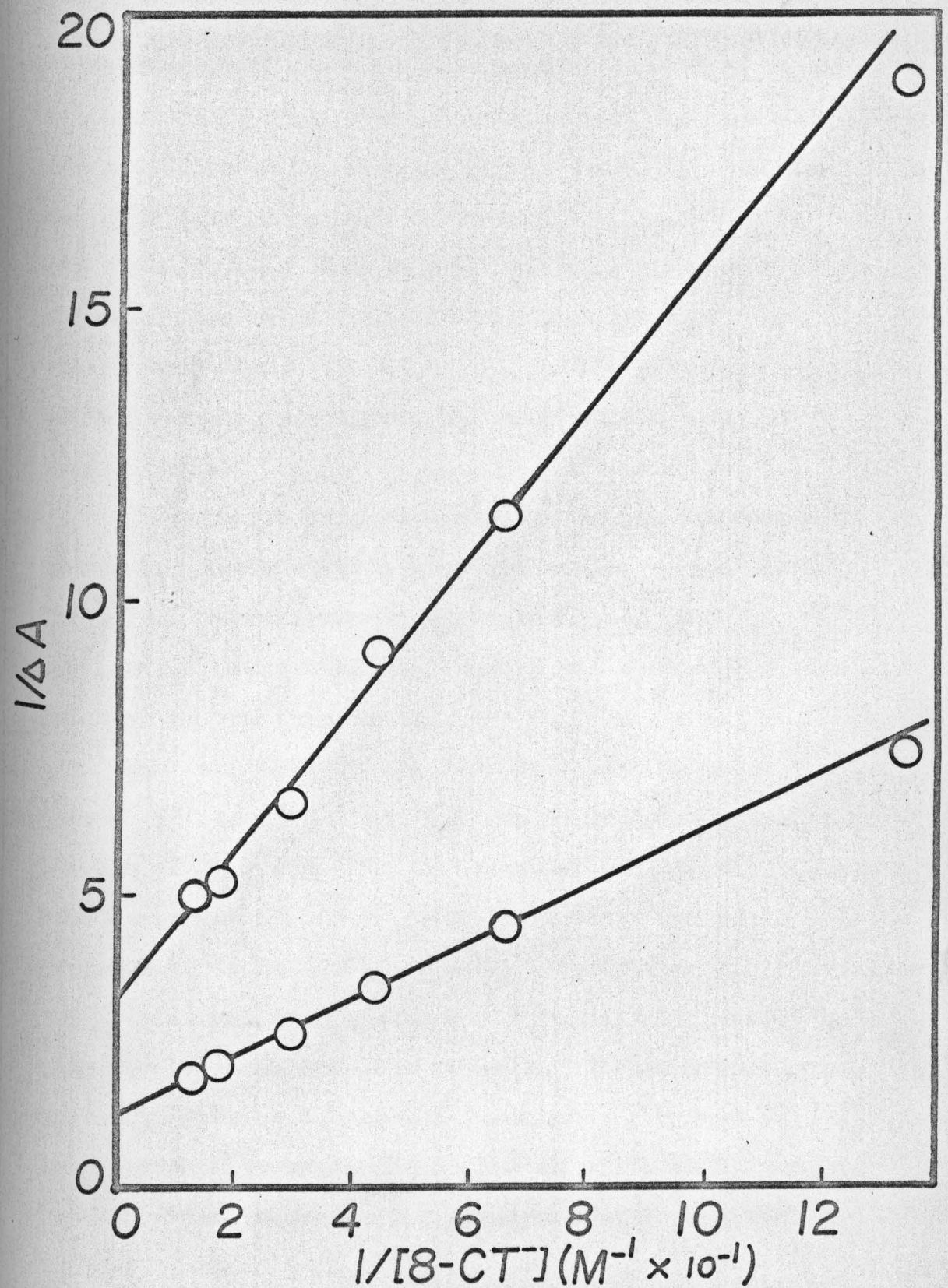


Fig. 8. Plots of spectral data from Table VI and Fig. 7 for the methyl cinnamate-8-chlorotheophylline system.

complex is responsible for the effect.

Spectrophotometric measurements were made at three temperatures. Fig. 9 shows studies for the same ester concentration at two temperatures (the values obtained are 23 M^{-1} at 25° and 18 M^{-1} at 35°). The two plots are shown as they were done at the same concentration, and over this small temperature range one would not expect much change in $\Delta\epsilon$ or S_t, so the same intercept, which is equal to $1/S_t\Delta\epsilon$, is obtained for both temperatures.

The kinetic data were plotted in the conventional manner and the stability constant evaluated was 22 M^{-1} (Fig. 10). The intercept value indicated that q_{11} = 0.91, which means that the complex was about 9% as reactive as the free ester.

The constants obtained by the three methods range from 22 M^{-1} to 26 M^{-1} at 25°. We could therefore assign a value of 24 $\pm$ 2 M^{-1} to the constant at this temperature. This agreement of the constants evaluated by three independent techniques is strong support for the hypothesis that only a single 1:1 complex is present or isomeric 1:1 complexes are present. The uncertainty of about 10% must be considered reasonable for data of this type, especially considering that in the kinetic methods the individual second order rate constants are reproducible to about $\pm$ 5%.

Fig. 7 also shows that there is some interaction

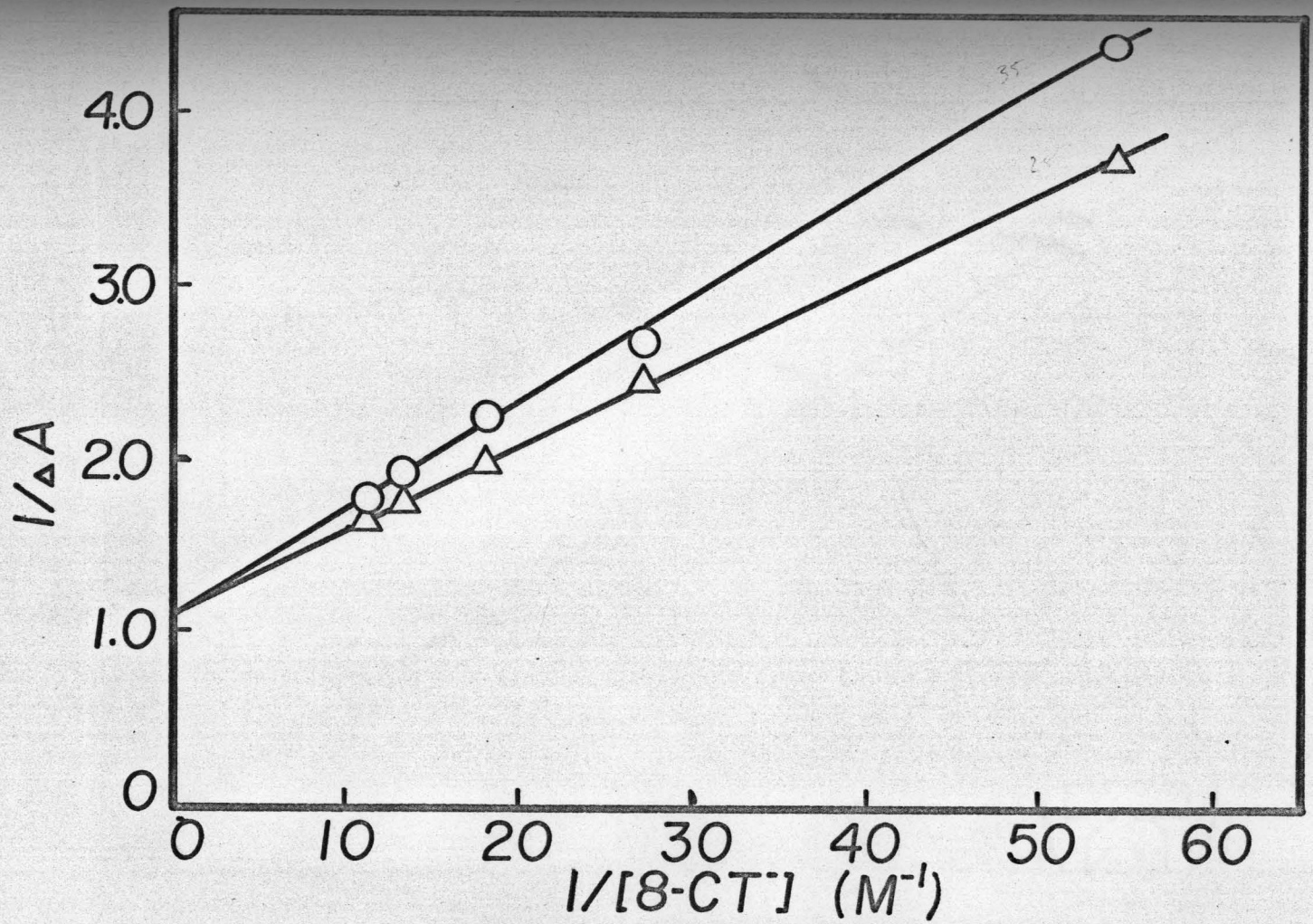


Fig. 9. Plots of spectral data at 25.0° (triangles) and 35.0° (circles) for methyl cinnamate-8-chlorotheophylline system; wavelength 320 mμ, ester concentration 7.3×10^{-4} M, in 1.2% CH_3CN.

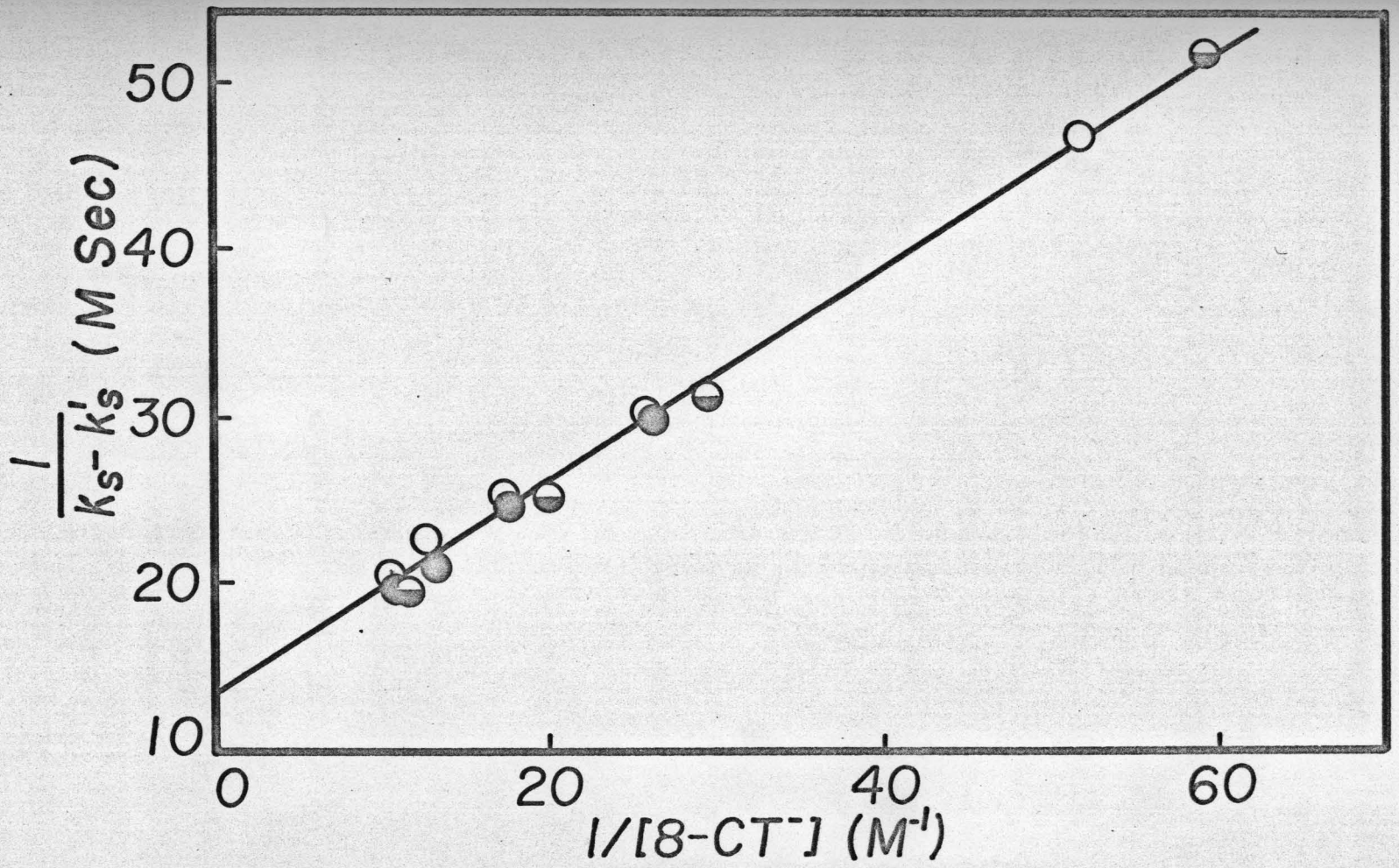


Fig. 10. Plot of kinetic data from Fig. 5.

between 8-chlorotheophylline and cinnamate ion. This was first detected when infinity absorbance values in the kinetic studies did not approach the same value as had been expected. This is a somewhat surprising result, since it shows that two negatively charged ions can interact. The stability constant for this interaction was estimated to be 6.8 M^{-1} at 25° from the spectral data. This evidence is interesting because it indicates one cannot assume a priori that there will be no interaction between two similarly charged ions. Thus it seems entirely possible that dimerization of a negatively charged species could occur to a significant extent.

F. Theophylline-7-acetic acid - This compound was chosen partly because it was supposed that, like other members of the xanthine family, it should be a good complexing agent. Because this compound has an ionizable proton (pK_a 3.20) it was anticipated that the negative charge would decrease the tendency prevalent among purines to dimerize and form even larger aggregates. The data suggested, however, that self-association occurs. The negative charge on this molecule would be localized at the carboxyl group, rather than distributed into the ring systems, which might not prohibit stacking of the planar molecules, the type of interaction believed to occur between purines (8).

The three methods of study were applied to this compound. The solubility analysis yielded a linear

isotherm (Fig. 11). The constant evaluated was 19 M^{-1}. There was also an inhibitory effect on the alkaline hydrolysis rate (Table VII). The kinetic K'_{11} was estimated to be 14 ± 2 M^{-1}. The spectral data obtained at the two ester concentrations 2.86 x 10^{-4} M and 6.40 x 10^{-4} M gave apparently linear reciprocal plots and K'_{11} values of 12 ± 2 M^{-1} and 10 ± 2 M^{-1} respectively.

These differences among the apparent stability constants would be expected if stoichiometries other than 1:1 were involved, as is discussed in detail in Appendix A. It is entirely possible that we have a system of 1:1, 1:2 complexes and a dimer of the ligand. The postulate of a ligand dimer as well as a 1:2 complex is consistent with the essentially linear solubility diagram, for if just 1:1 and 1:2 complexes were present this plot should have a positive curvature, while if only a 1:1 complex and ligand dimer were present negative curvature would be observed. A combination of the two is entirely possible. 7-Propyl-theophylline has been shown to interact with itself almost as strongly as does caffeine (57). Also, it seems that ligands capable of self-association also tend to form higher order complexes.

G. Theophylline - As with other members of the purine family, theophylline has been shown to form complexes in aqueous solution with a wide variety of compounds (4). Theophylline ionizes in a convenient pH range (pK_a (43) 8.73), which allows study of its interaction in both forms.

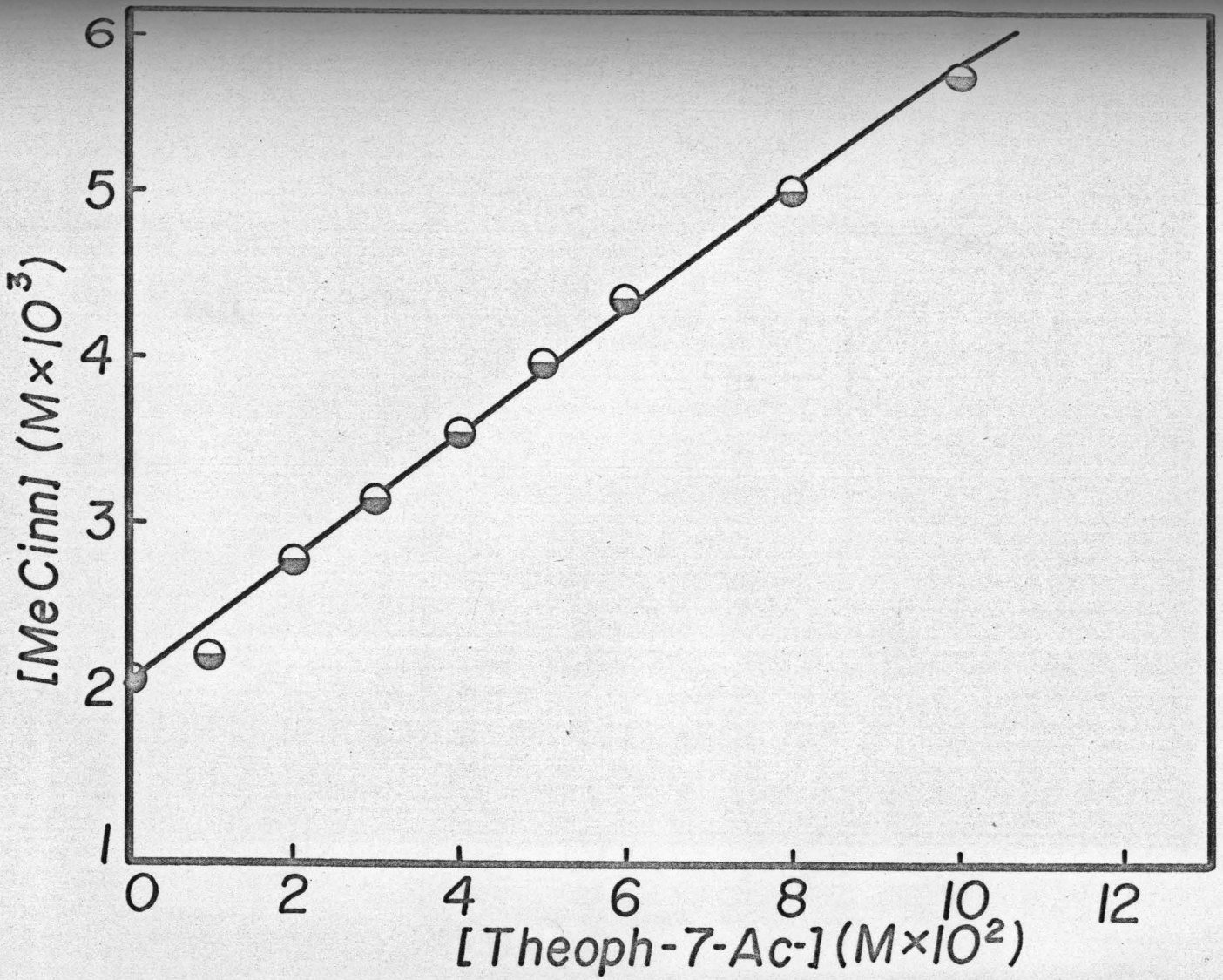


Fig. 11. The apparent solubility of methyl cinnamate as a function of the theophylline-7-acetic acid concentration in pH 8.25 borate buffer; μ = 0.3; 25.0°.

Table VII

The Observed Rate Constant for the Hydrolysis of Methyl Cinnamate in the Presence of Theophylline-7-Acetic Acid[a]

Ligand ($M \times 10^2$)	$10^3 k_{obs}$ (sec^{-1})
0.00	1.46
0.940	1.34
1.56	1.26
3.13	1.12
4.70	1.02
6.26	0.948
7.82	0.906

[a] 25.0°; pH 12.26; 0.83% acetonitrile; $\mu = 0.30$; ester = 5.66×10^{-4} M.

(Hereafter the ionic form will be referred to as theophyllinate.)

A stability constant of 25 M^{-1} was evaluated by the solubility method at pH 6.75 in phosphate buffer; the data are given in Table VIII and are plotted in Fig. 12. K'_{11} evaluated under the same conditions by the spectral method gave a value of 22 M^{-1} (Fig. 13).

The plateau region shown on the solubility isotherm was attributed to the appearance of solid theophylline. It was possible to estimate a stoichiometric ratio for the complex. Point A represents the normal solubility of theophylline (3.58×10^{-2} M). The discontinuity is estimated to occur at a total theophylline content of 3.76×10^{-2} M, representing an increase of 0.18×10^{-2} M. The solubility increase of methyl cinnamate is given by the vertical distance from the intercept to the solubility at the break-point, or 0.19×10^{-2} M. This gives a stoichiometric ratio of methyl cinnamate-to-theophylline of 1.05:1 in the complex.

Unlike many other purines and xanthines, theophylline does not appear to dimerize in aqueous solution (57). No explanation has been given for this. One explanation may be that theophylline is able to form a strong hydrogen bond with water, and this limits its ability to form aggregates with other theophylline molecules. This is suggested by the fact that theophylline crystallizes from water as the monohydrate.

Table VIII

Solubility of Methyl Cinnamate as a Function of the Theophylline Concentration at 25°.[a]

Theophylline ($M \times 10^2$)	Me Cinnamate ($M \times 10^3$)
0.000	2.15
0.368	2.37
0.736	2.54
0.784	2.59
1.10	2.76
1.31	2.87
1.84	3.09
2.57	3.51
2.61	3.56
2.94	3.65
3.41	3.86
3.68	4.06
3.91	4.09
5.42	4.07

[a] In pH 6.75 phosphate buffer containing 1% CH_3CN; $\mu = 0.3$.

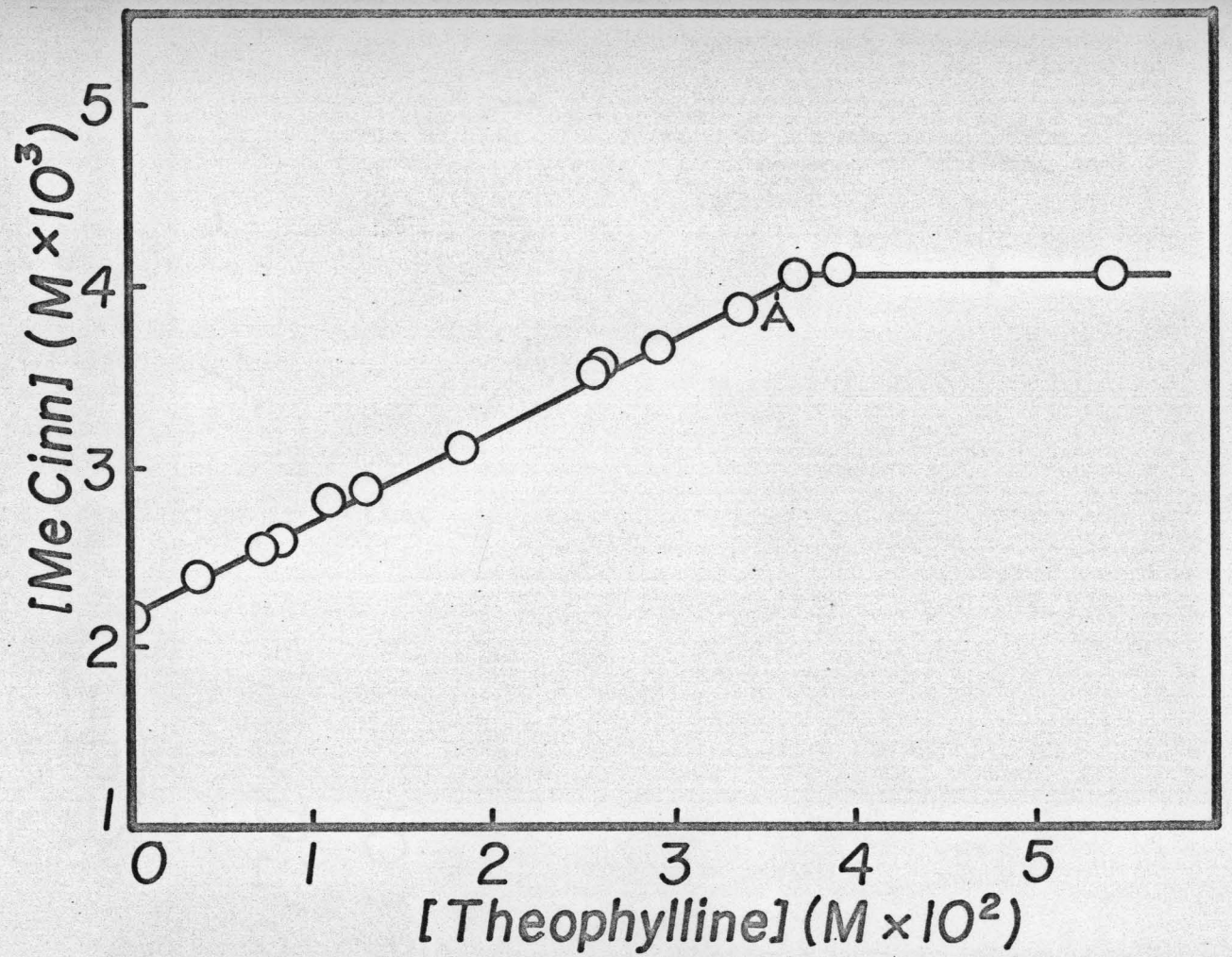


Fig. 12. The apparent solubility of methyl cinnamate as a function of the theophylline concentration (data are given in Table VIII).

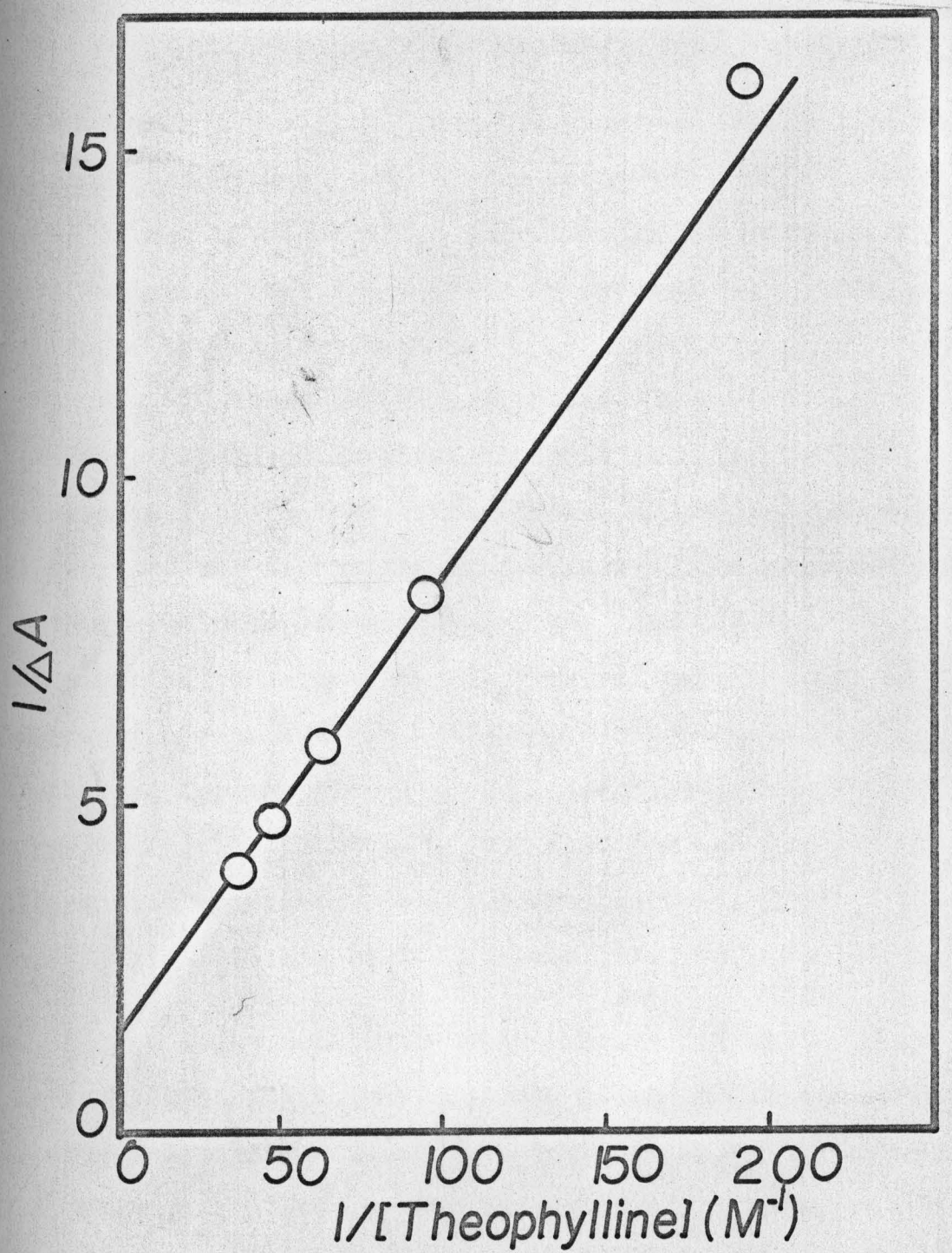


Fig. 13. Plot of spectral data for the methyl cinnamate-theophylline system; pH 6.75 phosphate buffer; μ = 0.3; 1% CH_3CN; 25.0°.

Theophyllinate was also investigated by two techniques, spectral and kinetic, and there was good agreement between the two stability constants. The constant evaluated by the kinetic method was 11 M^{-1} and the constant obtained by the spectral method was 13 M^{-1}. The estimated uncertainty of these values is about 10%. The intercept on the kinetic plot indicates that the complex is unreactive, within the limits of the experimental error (Fig. 14).

Fig. 15 shows the kinetic plots, while the rate constants obtained are given in Table IX. The initial absorbance (obtained by extrapolation of the log $(A_t - A_\infty)$ plots), and the absorbance at "infinity" time as a function of theophyllinate concentration, are shown in Fig. 16. The graphical treatment of the spectral data to give an interaction constant for the methyl cinnamate-theophyllinate system and for the cinnamate ion-theophyllinate system are given in Fig. 17. The value obtained for the apparent 1:1 interaction constant with cinnamate ion was 4.5 M^{-1}. This value has a probable error of about 25%.

H. Purine - The methyl cinnamate-purine interaction was studied kinetically. The data are given in Table X. The apparent stability constant was found to be 2.3 M^{-1} from the reciprocal plot, and the intercept value gave, to within experimental error, the known value of k_s, indicating that the reactivity of the complex is negligible.

It is seen that this stability constant is larger than that obtained with the imidazoles, but is much smaller

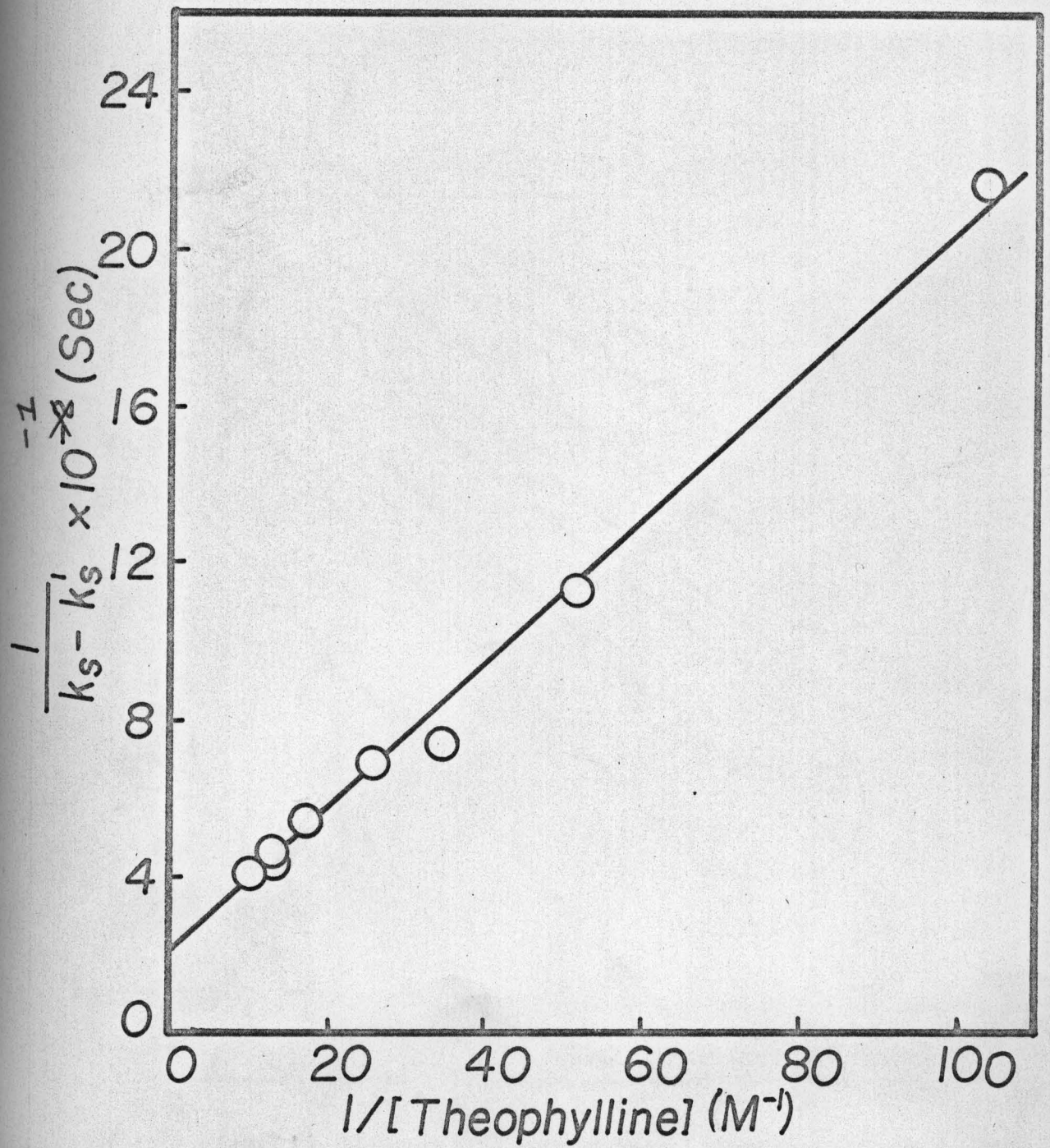


Fig. 14. Plot of kinetic data for the methyl cinnamate theophylline system (data are given in Table IX).

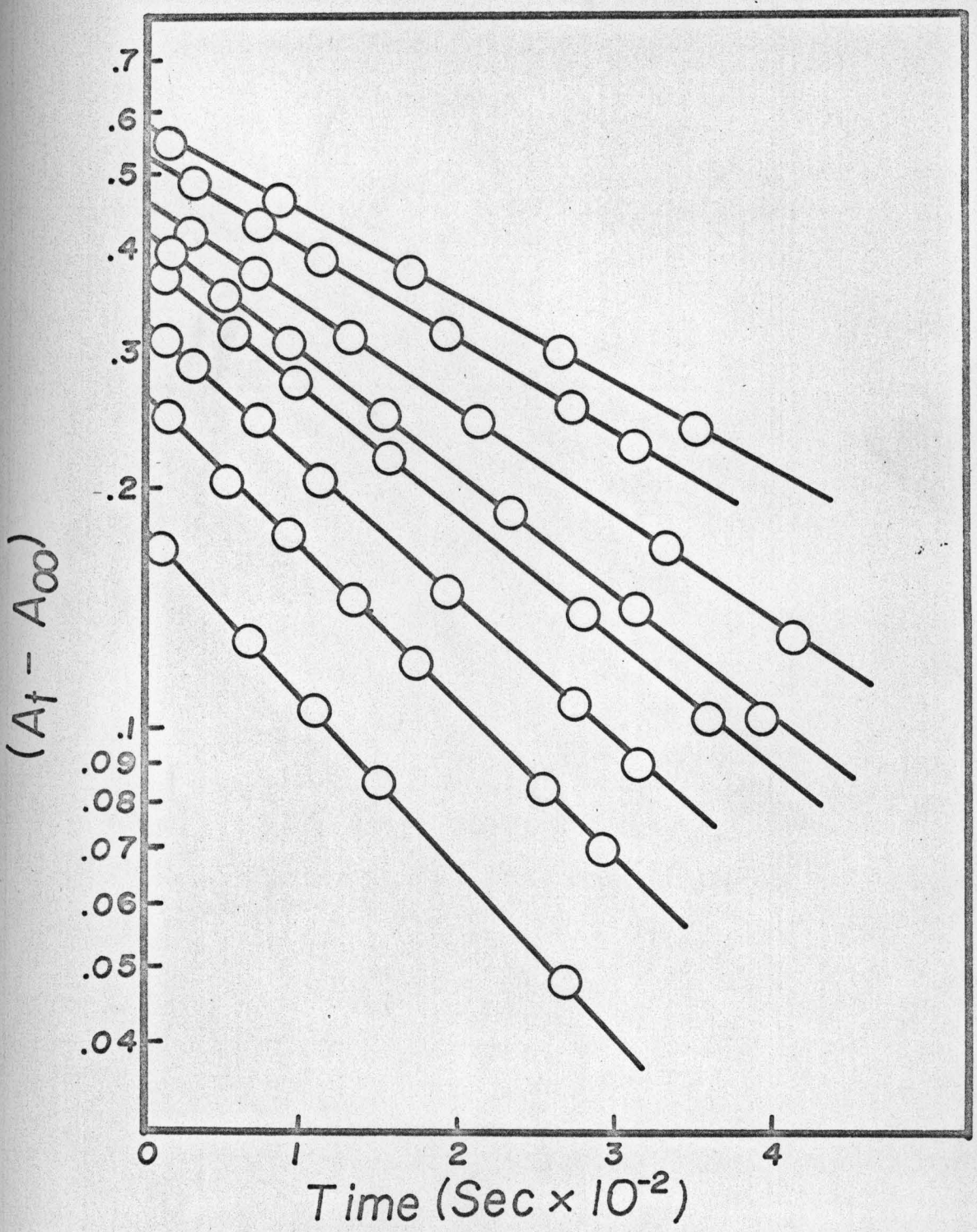


Fig. 15. Pseudo-first order plots for the alkaline hydrolysis of methyl cinnamate in the presence of theophyllinate. (See Table IX for conditions).

Table IX

Apparent First-Order Rate Constants for the Alkaline Hydrolysis of Methyl Cinnamate in the Presence of Theophyllinate.[a,b]

Theophyllinate ($M \times 10^2$)	$10^3 k_{obs}$ (sec^{-1})
0.00	5.00
0.962	4.54
1.92	4.12
2.88	3.70
3.85	3.55
5.77	3.17
7.70	2.77
7.70	2.73
9.62	2.52

[a] pH = 12.77, 0.83% CH_3CN, μ = 0.3

[b] Rate constants calculated from slopes of lines shown in Fig. 15.

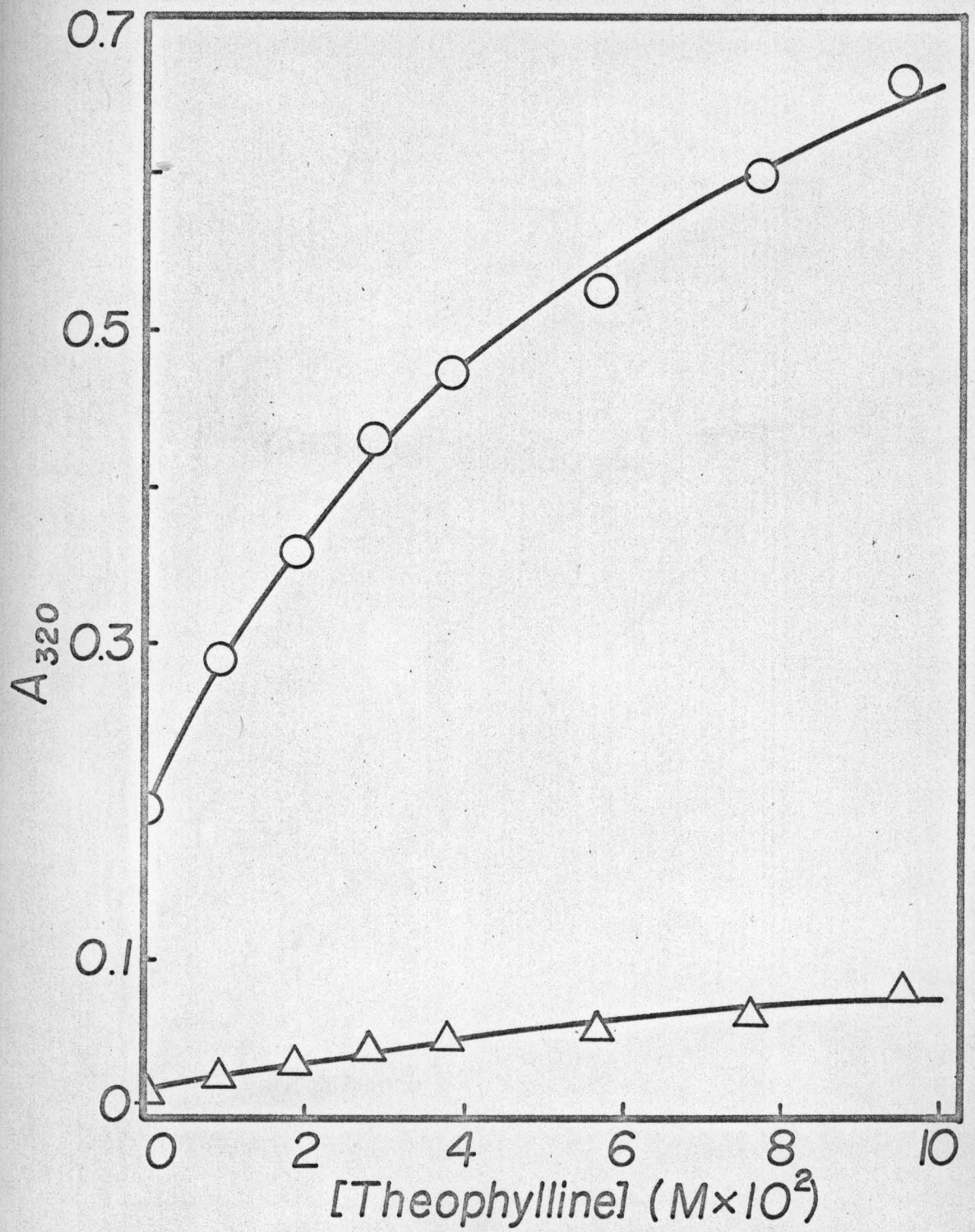


Fig. 16. Absorbance (at 320 mμ) at t = 0 (circles) and t = ∞ (triangles) from the kinetic data shown in Fig. 15.

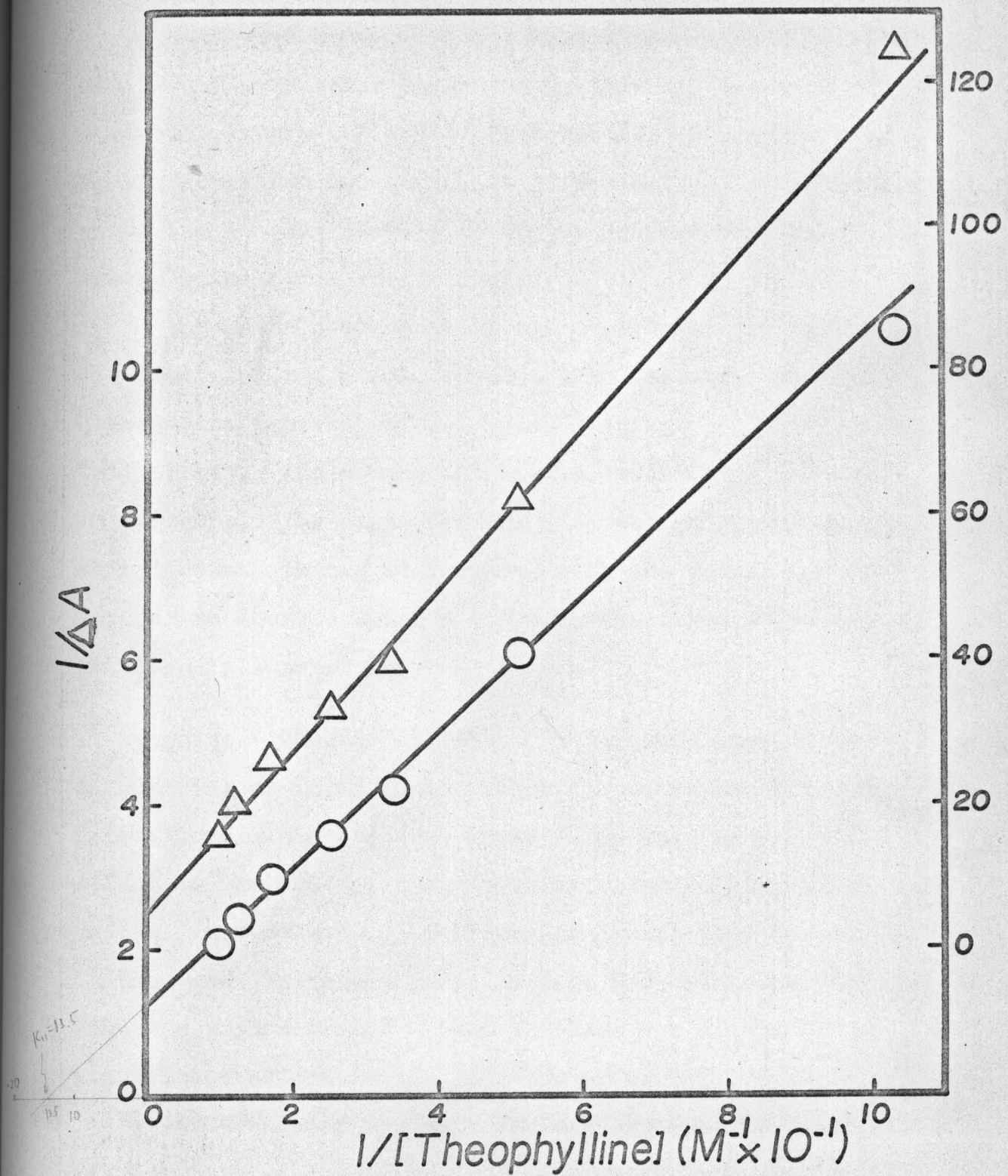


Fig. 17. Plot of spectral data shown in Fig. 16; right axis; triangles; left axis, circles.

than values obtained for the xanthines.

Much work concerning the interaction of purine with itself and with other bases and nucleosides has been conducted, since it is felt that the forces contributing to the formation and stability of the helices in nucleic acids lie in the interaction of the nitrogeneous bases on the polynucleotide chains (8-11).

Under the conditions of our experiment purine was in the ionized form, which, coupled with the apparent self-association constant of 2.1 molal^{-1} in water (8), indicates that probably the monomeric form is essentially the only one present. The value for the interaction of purine-methyl cinnamate may be compared with the values of 9 M^{-1} for purine-adenine and 2 M^{-1} for purine-thymine complexes obtained in water.

I. Guanine - Guanine differs from the xanthines in that it contains an imino group rather than a carbonyl in the 2-position. This compound appeared to have no greater effect on the rate of hydrolysis of methyl cinnamate than did the imidazole. (0.06 M guanine caused a 6% decrease in the reaction rate whereas in 0.06 M 8-chlorotheophylline the rate was decreased by 60%.) There was also no significant effect on the spectrum of methyl cinnamate. The anionic form of the molecule was employed in these studies.

J. Uracil - To be able to separate the effect of the

Table X

Observed Rate Constant of Methyl Cinnamate Hydrolysis With Varying Purine Concentration.[a]

Purine ($M \times 10^2$)	$10^3 k_{obs}$ (sec^{-1})
0.00	4.85
1.52	4.67
3.04	4.50
6.08	4.20
7.61	4.02

[a] 25.0°; pH = 12.76; 0.83% CH_3CN; μ = 0.3.

pyrimide ring from that of the imidazole ring it was decided to study uracil. This compound caused no significant change in the spectrum of methyl cinnamate and had about the same effect on the hydrolytic rate as did imidazole and guanine; an approximately 6% decrease in rate was observed in the presence of 0.066 M uracil.

In the kinetic studies, the molecule was in the anionic form (pK = 9.45) and this may account in part for the small effect. However, the pyrimidine ring itself does not appear to interact strongly; for example the analogous compounds uridine and pyrimidine have formation constants with thymine of only 1.2 M^{-1} and 0.8 M^{-1}, respectively, in water (8).

K. Caffeine - Since 1877, when caffeine was postulated by Daudt to form "molecular compounds" with salicylate and benzoate (73), many workers have studied the interactions of caffeine with a wide variety of compounds.

Caffeine is 1,3,7-trimethylxanthine; it may be considered as theophylline substituted in the 7-position with methyl. Its choice in the present study was based partly upon the great amount of data already available in the literature relating to its behavior, but also upon the interesting contrast with theophylline, since caffeine can neither ionize nor hydrogen-bond. It was recognized that caffeine is capable of self-aggregation (46, 57), and therefore might not yield a simple system for analysis.

The solubility isotherm (Fig. 18), which was plotted

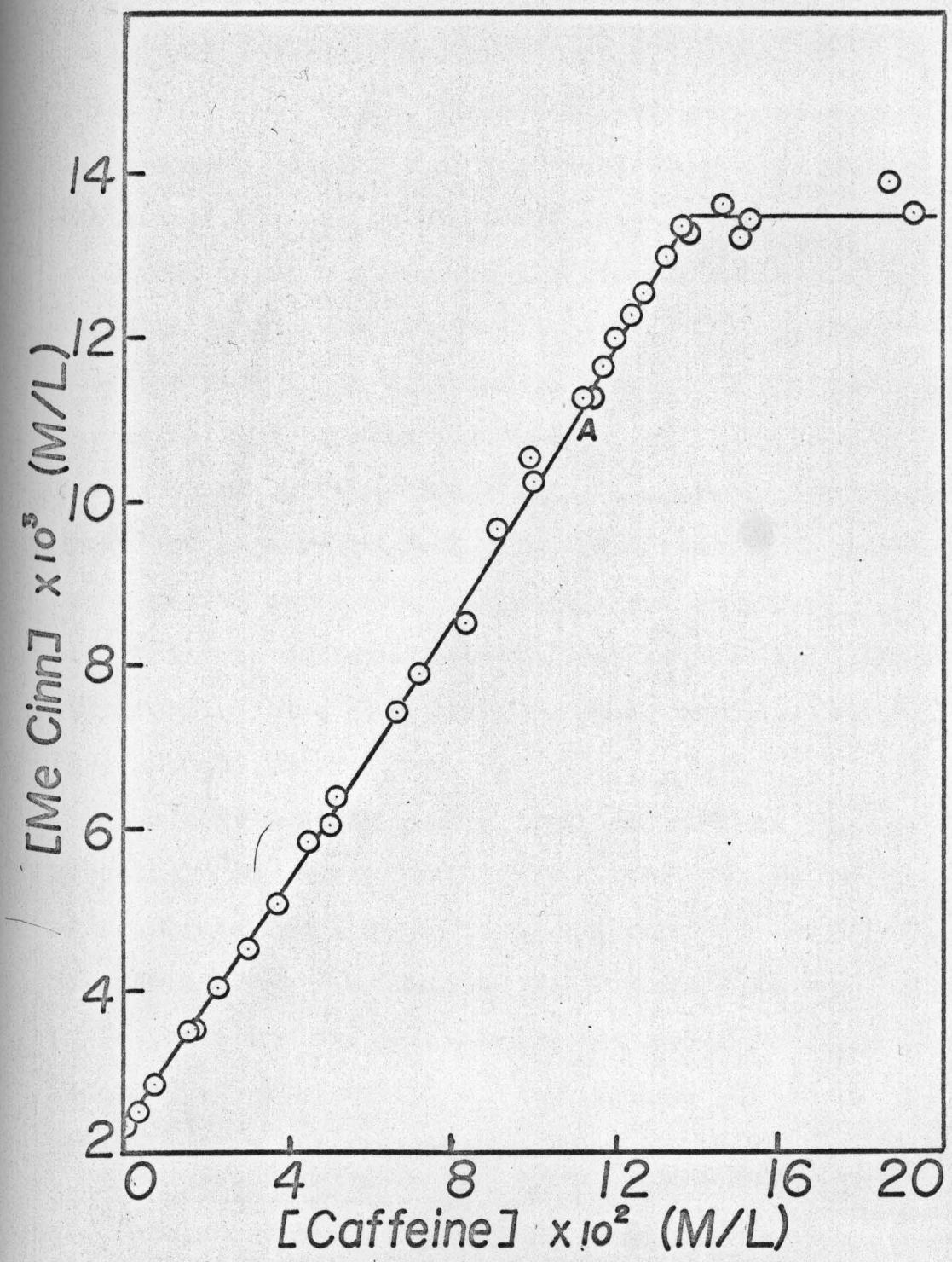


Fig. 18. The apparent solubility of methyl cinnamate as a function of caffeine concentration (data are given in Table XI.)

from the data in Table XI, appears to be reasonably linear and gives a K'_{11} of 36 M^{-1}, but careful inspection reveals that it is essentially linear only up to about 0.08 M total caffeine, beyond which a slight positive curvature is observed. Analysis of the discontinuity indicates that the total increase in the solubility of caffeine is 27.0 x 10^{-3} M; point A gives the equilibrium solubility of caffeine in the experimental solvent. The vertical distance from S_o to the plateau represents the increase in the solubility of methyl cinnamate, which is 11.2 x 10^{-3} M. The ratio of caffeine-to-methyl cinnamate in the complex therefore appears to be 2.4:1. This result, together with the positive curvature, indicates that complexes containing more than one caffeine molecule may be present. Similar observations have been made for other caffeine systems (19, 20, 48, 74).

A complicating factor, one that must be taken into account in any analysis of this system, is the presence of aggregate forms of caffeine. A partition study similar to that conducted by Guttman and Higuchi (57) was performed to estimate the concentration of monomeric caffeine as a function of total caffeine. Our aqueous phase was pH 9.2 borate buffer and the organic layer was isooctane.[8]

---

8. It was assumed that only the monomer partitions into the organic phase. This assumption will be valid if the association constant in the organic phase is less than 10^3, as the concentration in the organic phase is in the range 10^{-5} - 10^{-4} M.

Table XI

Solubility of Methyl Cinnamate in the Presence of Varying Amounts of Caffeine.[a]

Caffeine ($M \times 10^2$)	10^3 St (M)
0.000	2.35
0.367	2.56
0.735	2.84
1.47	3.50
1.63	3.56
2.20	4.02
2.87	4.51
3.67	5.07
4.41	5.82
4.89	6.02
5.14	6.37
6.52	7.41
7.18	7.86
8.15	8.50
9.01	9.67
9.82	10.5
9.91	10.3
11.1	11.3
11.3	11.3
11.4	11.6
11.9	12.0
12.3	12.3
12.6	12.6
13.0	13.0
13.5	13.4
13.8	13.3
14.4	13.7
14.7	13.2
15.2	13.5
18.6	13.9
19.2	13.6

[a] 25.0°; 1% CH_3CN; pH 8.5 borate buffer; $\mu = 0.025$.

Fig. 19 shows the results of the partition study. Our results were in good agreement with the values extrapolated from the data in ref. 57.

From these data it was possible to construct a calibration curve from which the monomer present at any total caffeine concentration could be read (Fig. 20).

The solubility data in Table XI were then replotted against caffeine monomer concentration, and the isotherm obtained was parabolic (Fig. 21). This curve was now analyzed for the presence of 1:1 and 1:2 complexes according to the method given in ref. 4 (Fig. 22). This plot shows some positive deviation at higher monomer concentrations, indicating the presence of complex species containing more than two caffeine molecules. This is not unreasonable; if caffeine can associate to form aggregates higher than the dimer, it is entirely feasible that these species could interact with the substrate.

The formation of the methyl cinnamate-caffeine complexes could occur from a stepwise build-up of simpler complexes, which react with monomer: $S + L = SL$, $SL + L = SL_2$,...$SL_{n-1} + L = SL_n$; or by combination of substrate with the appropriate aggregate form: $S + L = SL$, $S + L_2 = SL_2$,...$S + L_n = SL_n$. The two processes are mathematically equivalent, though the constants obtained will depend upon which formulation is chosen. The values obtained in this study were $K_{11} = 28\ M^{-1}$ and $K_{(12)} = 55\ M^{-1}$, following the first, stepwise, approach. These values

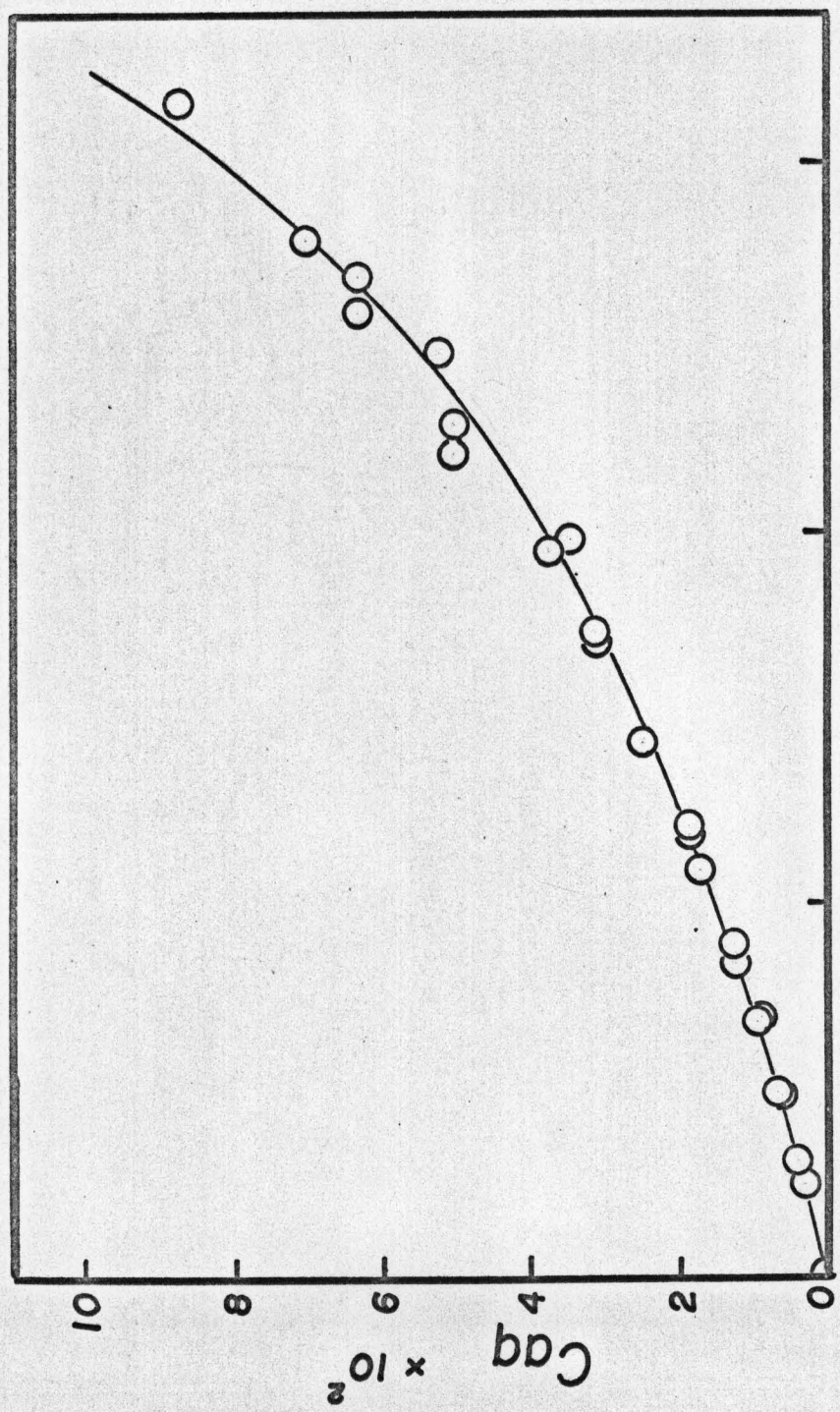


Fig. 19. Distribution isotherm of caffeine between isooctane and pH 8.5 borate buffer at 25.0°.

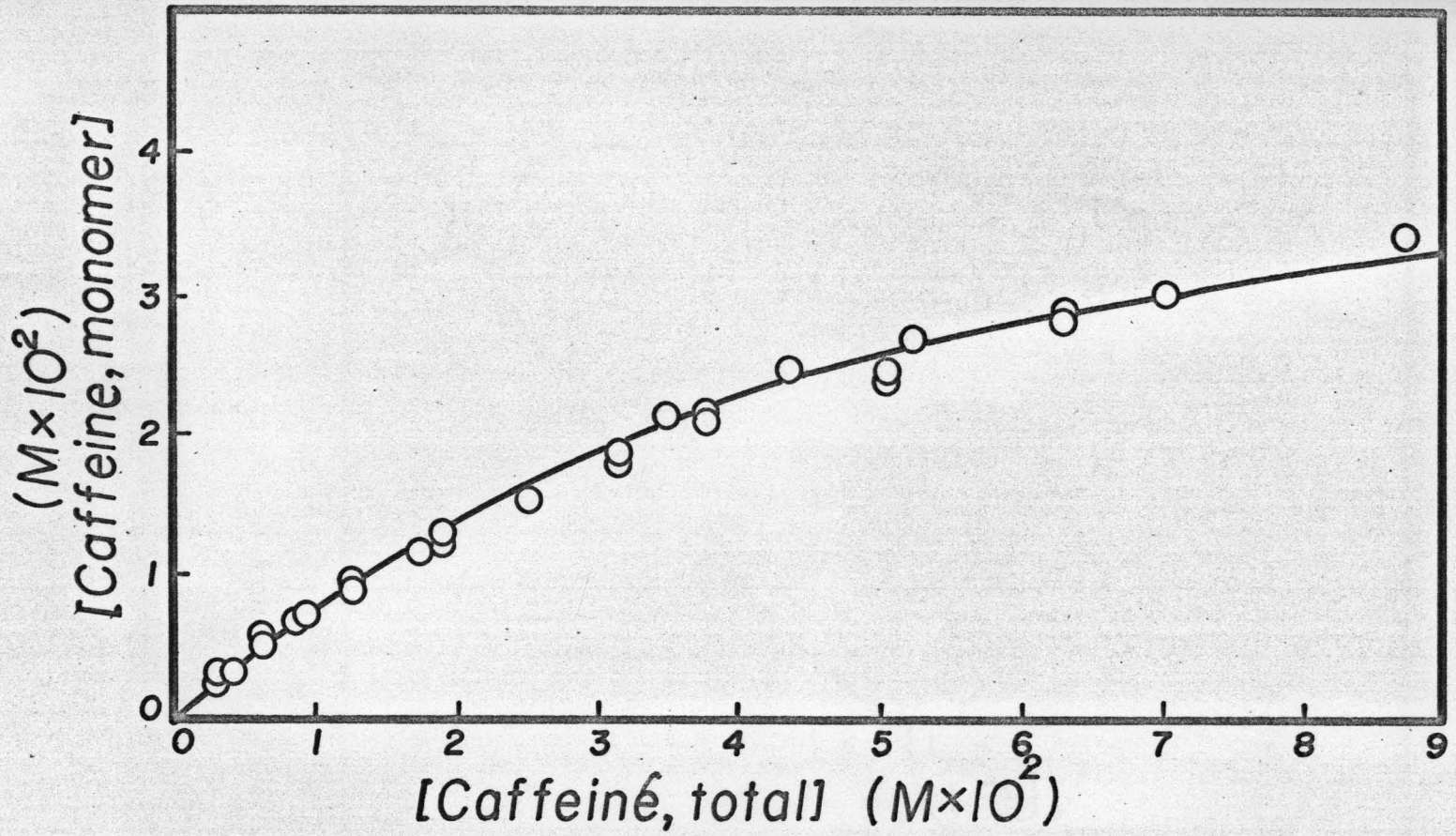


Fig. 20. Concentration of monomeric caffeine as a function of total caffeine concentration in aqueous solution at 25.0°.

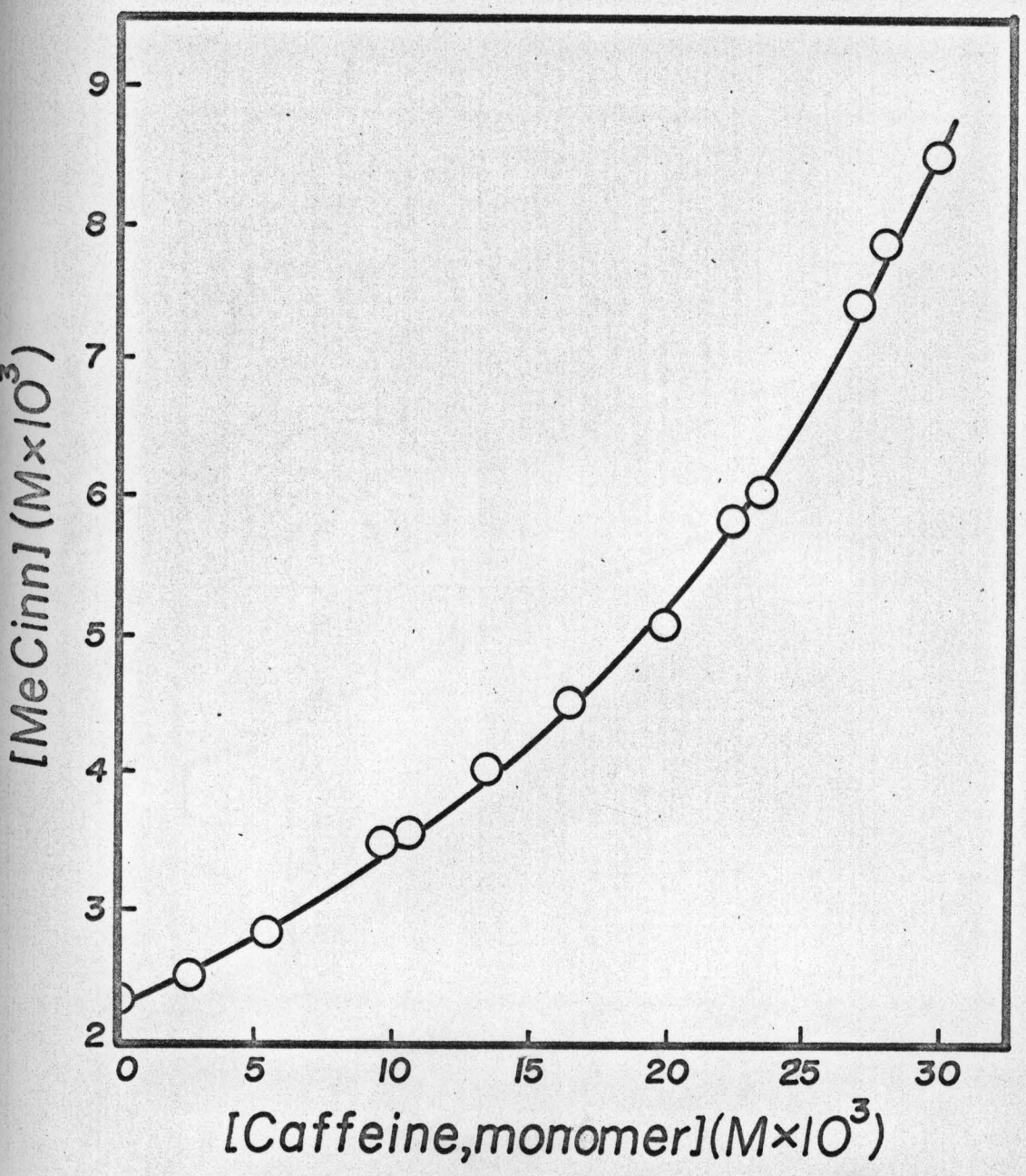


Fig. 21. The apparent solubility of methyl cinnamate as a function of monomeric caffeine (from the data in Table XI and Fig. 20).

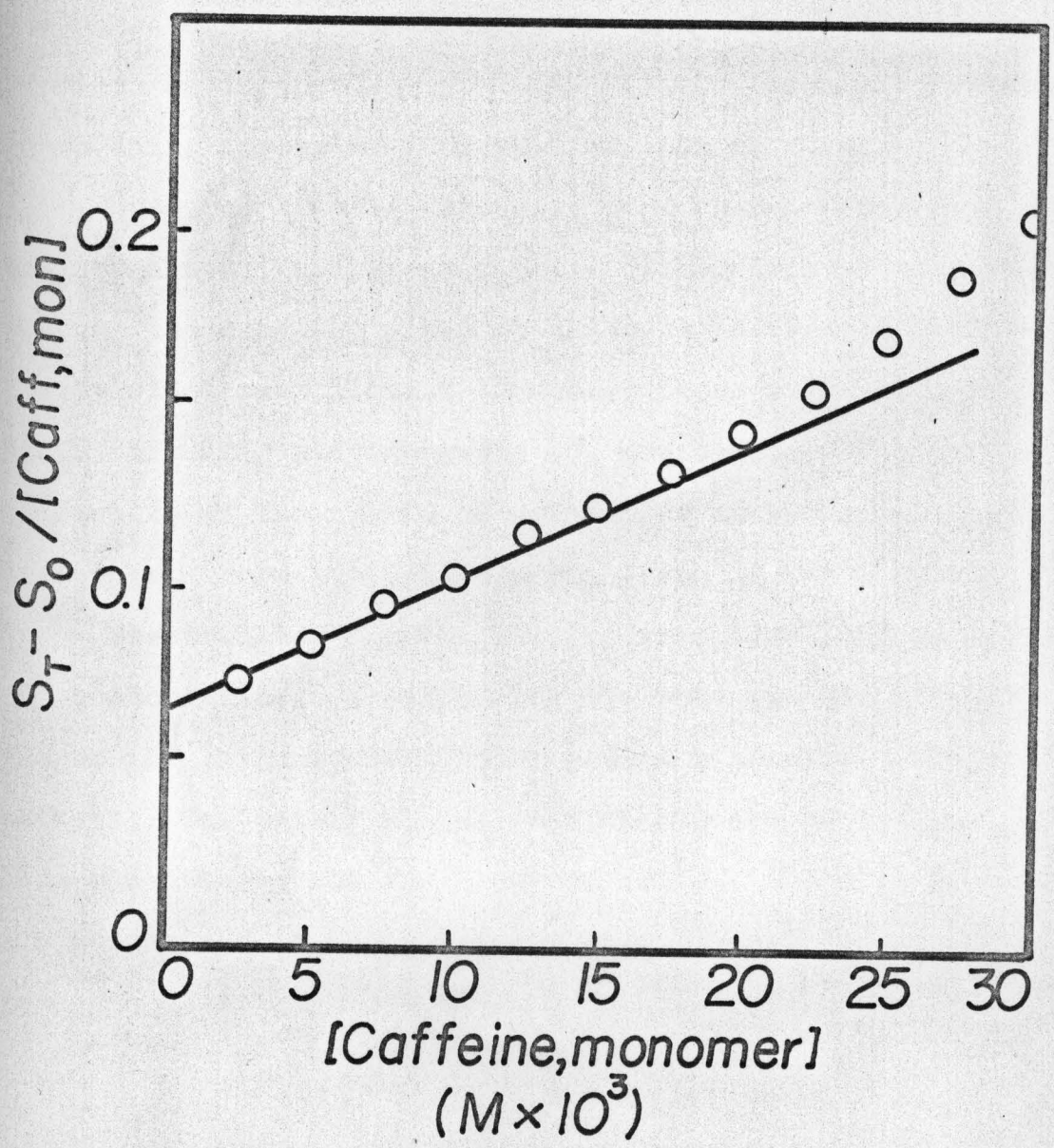


Fig. 22. Graphical estimation of K_{11} and $K_{(12)}$ based on the solubility data (the points are from the smooth curve in Fig. 21).

are probably accurate to within ± 15%.

Caffeine had a marked inhibitory effect on the rate of alkaline hydrolysis, as expected (Table XII; Fig. 23). The data suggested, however, that the complex (complexes) had some degree of reactivity. It would be difficult to generate an independent estimate of this reactivity without introducing approximations that may not be valid.

Spectral studies were also conducted and the data yielded linear reciprocal plots. (Other forms of analysis such as the equation given by Rossotti and Rossotti (14) also yield linear plots.) However, these plots have no useful significance (Appendix A), and because of the complexity of the system no convenient analysis could be performed. Some typical data are given in Table XIII.

Spectral and kinetic studies were also conducted in 10% acetonitrile. A solubility analysis was not feasible due to the large solubility of methyl cinnamate in this solvent. The extent of interaction appears to be less than the interaction in 1% acetonitrile. The kinetic data are summarized in Table XII and Fig. 23, and the spectral data are given in Table XIV.

Table XII

Relative Hydrolytic Constants of Methyl Cinnamate in the Presence of Varying Amounts of Caffeine.[a]

Total Caffeine ($M \times 10^2$)	k_s'/k_s
0.00	1.00
1.30[b]	0.833
1.65	0.791
1.97	0.774
2.59[b]	0.718
3.30	0.674
3.89[b]	0.641
3.94	0.644
4.95	0.603
5.18[b]	0.590
7.79	0.518
8.16	0.505
1.57[c]	0.882
2.15	0.824
3.41	0.777
4.29	0.732
5.12	0.719
6.82	0.673
7.73	0.623
2.57[d]	0.760
5.15	0.650
7.62	0.558
10.2	0.504

[a] 25.0°; 1% acetonitrile; pH 9.20 borate buffer.

[b] pH 12.56 hydroxide/chloride.

[c] 10% acetonitrile; 11.60 phosphate buffer.

[d] 35.0°; 1% acetonitrile; pH 9.10 borate buffer.

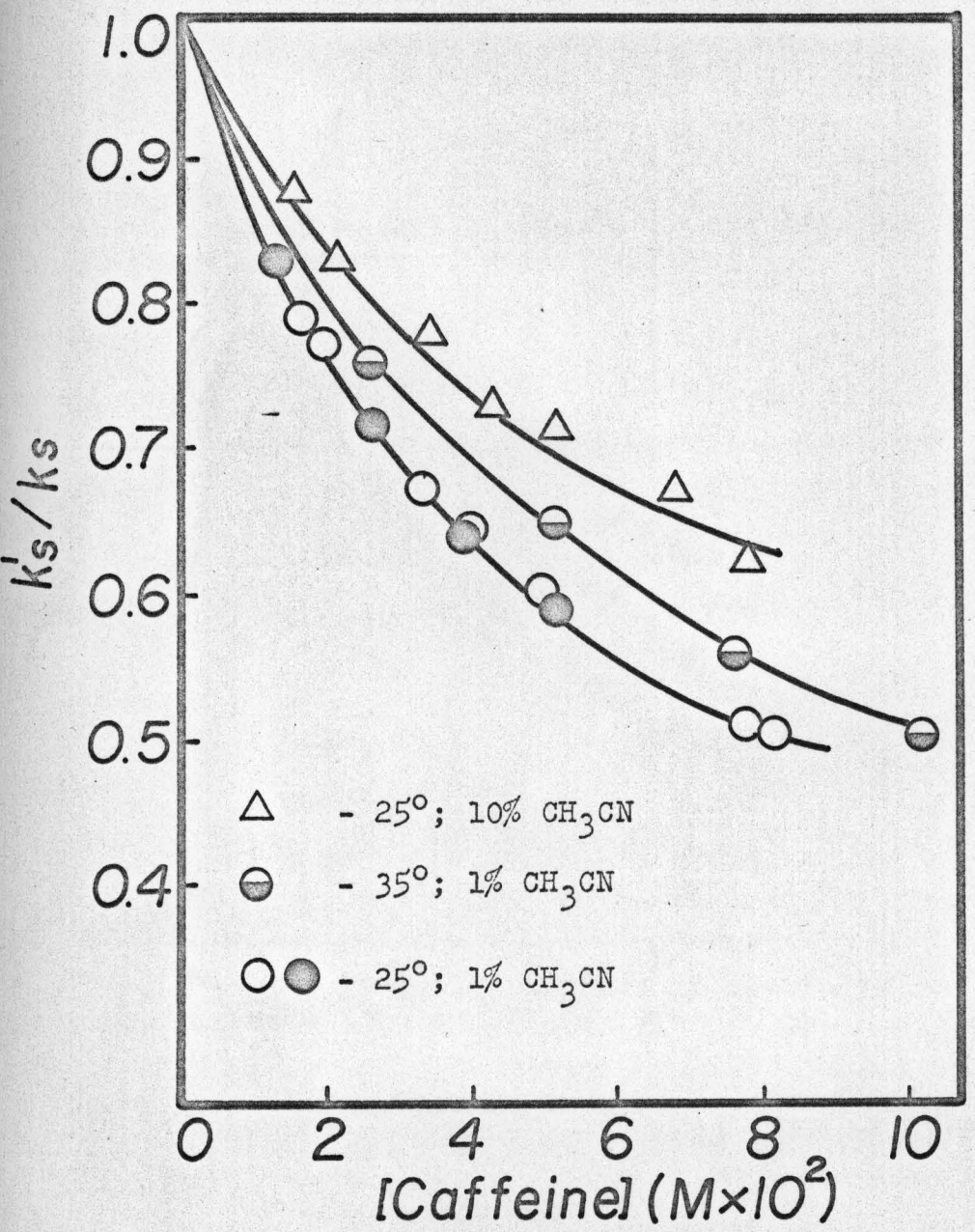


Fig. 23. Relative rates of hydrolysis of methyl cinnamate with varying concentration of caffeine (data are given in Table XII).

Table XIII

Absorbance of Methyl Cinnamate in the Presence of Varying Amounts of Caffeine.[a]

Total Caffeine ($M \times 10^2$)	$A_{320\ m\mu}$
0.00	0.284
1.72	0.537
3.44	0.679
5.17	0.781
6.89	0.861
8.27	0.910

[a] 25.0°; pH 9.20 borate buffer; 1% acetonitrile. Substrate = 7.80×10^{-4} M; λ = 320 mμ.

Table XIV

Absorbance of Methyl Cinnamate in the Presence of Varying Amounts of Caffeine.[a]

Total Caffeine ($M \times 10^2$)	$A_{325\ m\mu}$
0.000	0.132
0.000	0.134
0.911	0.209
1.82	0.271
2.73	0.329
3.64	0.377
4.55	0.404
6.38	0.472
8.20	0.510

[a] 25.0°; acetonitrile-water 10% (v/v). Substrate = 1.28×10^{-3} M; λ = 325 mμ.

IV. DISCUSSION

A. Validity of the Complexation Hypothesis

One of the objectives of the research was to establish, as far as possible, the general mechanism of the rate inhibition observed for the alkaline hydrolysis of methyl trans-cinnamate in the presence of certain heterocyclic compounds. Two hypotheses can be suggested to account for the general effect:

(1) The effect may be a general solvent effect; that is, the inhibition is ascribed to a change in the activity of the substrate by an alteration in the solvent properties upon addition of ligand to the system. This hypothesis has been referred to as the activity coefficient effect.

(2) A specific phenomenon may be invoked, the suggestion being made that the observed effects are markedly dependent upon the molecular and electronic structures of substrate and ligand, and that interaction between these species to form one or more additional species, with altered properties, is responsible for the inhibition. These new species are called complexes.

A distinction between the two hypotheses may seem somewhat arbitrary since all deviation from expected behavior could, in principle at least, be described in terms of changes in activity coefficients. The deciding factor is usually the magnitude of the effects observed

and the ligand concentrations employed, as many of the techniques used to detect the presence of complexes are similar to those used to measure activity coefficients. For example, the solubility method of analysis is identical to a classical method for determining activity coefficients. The relationship of the activity coefficient ratio of the substrate in the presence and absence of ligand to the respective solubility ratio is given by

$$S_o/S = \gamma/\gamma_o$$

The effect on reaction velocity could be explained in an analogous manner. However, the magnitude of the effects observed at the ligand concentrations employed in this study justifies the assumption that a specific interaction occurs, i.e. a water soluble complex is formed between the substrate and ligand, rather than ascribing the effects to bulk changes in the solvent (8, 79, 80). Also, the kinetic analysis and the non-kinetic analyses - solubility and spectral methods - gave consistent results, whereas in contrast the effect of acetonitrile on methyl cinnamate produces effects which cannot be correlated. Small amounts of acetonitrile increase the solubility of methyl cinnamate but have insignificant effects on the rate of alkaline hydrolysis or on its absorption spectrum. Thus the acetonitrile effect is considered characteristic of a general solvent effect, while the other ligands exemplify complex formation.

Moreover this hypothesis suggests that small alter-

ations in molecular structure of the ligands may lead to profound differences in substrate properties, and this is precisely the behavior that is observed.

The data reported in this thesis therefore provide further evidence for the validity of the complexation hypothesis, and illustrate some new results obtained with its application.

B. Structural Implications of Rate Inhibitions

The structures of the complexes discovered in this study are not known. However, from work that has been done on the structure of and electron distribution in purines, imidazoles, and pyrimidines (7, 65, 75, 76), and from molecular models, some possibilities can be considered. For example, the imidazole nucleus could interact with the double bond and carbonyl group in the cinnamate, which would provide π overlap and localized interaction with the carbonyl group. There could also be ring-ring interactions involving the phenyl ring of the ester and the planar pyrimado ring, which might lead to optimal π overlap.

These possible configurations could account for the observed inhibition in rate, which may be due to steric effects, electrostatic repulsion (in those cases where the complexing agent is an anion), or electronic changes in the substrate upon complexation.

Steric hindrance could play a significant role if the ligand is in close proximity to the labile ester group.

For the benzocaine-caffeine system (18), Martin (7) suggests such interactions. However, in that instance the carbonyl group is conjugated directly with the ring, so there is also π overlap between the pyrimado ring and phenyl ring. The possibility for both interactions to occur in that system may be why there is such strong interaction. With methyl cinnamate, however, if the overlap is with the aromatic ring, which is quite distant from the ester group, one would not expect steric blocking of the attacking reagent to be important. Bender (33) postulated an overlap between the indole ring of N-(indole-3-acroyl) imidazole and 3,5-dinitrobenzoate in his studies and discounted steric effects. When the complexing agent is a negatively charged species, electrostatic repulsion of hydroxide must be considered. This probably can be discounted because of the large effect demonstrated with caffeine, a neutral molecule; also, anionic complexing agents have been as effective against n-butylamine attack as against hydroxide ion (33). An explanation that would explain the inhibition is that upon complex formation the electronic distribution is distorted in such a fashion that the ground state is stabilized to a greater extent than the transition state and hence the activation energy is raised. The carbonyl group of the ester is in complete conjugation with the ring system, and the agents studied are postulated to possess relatively good donor properties, so whether the interaction is localized near the carbonyl or on the aromatic

ring, the trigonal ground state carbonyl would be expected to aid complex formation relative to the tetrahedral intermediate that the transition state may be expected to resemble.

In the above discussion, solvent effects have been neglected. Murrell (77) has suggested they could be handled by assuming a well defined solvent shell and writing the equilibrium:

$$S(Q_n) + L(Q_m) = SL(Q_p) + qQ$$

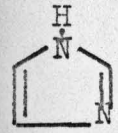
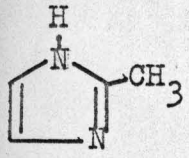
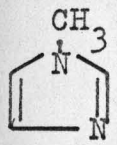
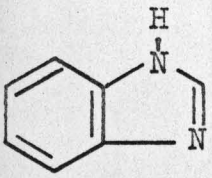
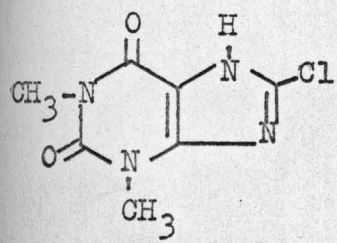
where n, m, p and q are the number of solvent molecules (Q); and $n + m = q + p$.

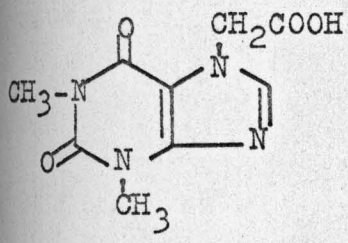
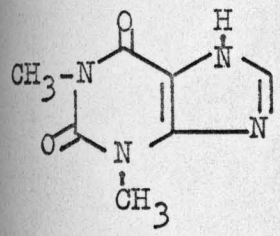
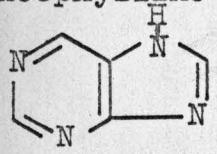
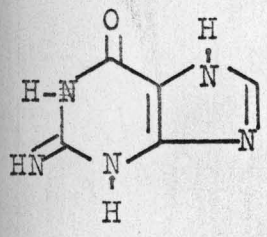
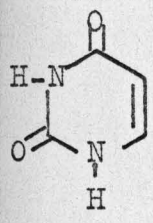
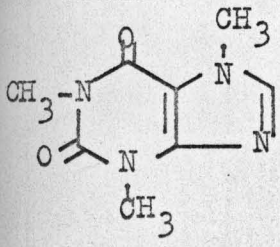
This is of importance here in that the hydrophobic rings may orient the solvent (see Higuchi's "squeezing out" effect (78)) in the neighborhood of the carbonyl group and the inhibition could be ascribed, in part, to the inability of water molecules to properly solvate the transition state.

C. Structural Effects on Complex Formation

The stability constants obtained in this study are presented in Table XV. Some qualitative interpretations may be made from these data. Imidazoles gave values of approximately 1 M^{-1}, which may be compared with the values observed for oxytetracycline with creatinine and histidine hydrochloride - 2.6 and 1.4 M^{-1}, respectively (4). N-methylimidazole also caused a rate retardation, but the plot of k_{obs} vs. N-methylimidazole concentration showed a linear decrease, which indicates that some effect other

Table XV. Apparent Stability Constants with Methyl trans-Cinnamate[a]

Ligand	K'_{11} Solubility	Kinetic	Spectral
 Imidazole	1.0	0.9	-
 2-Methylimidazole	1.2	1.0	-
 N-Methylimidazole	-	b	-
 Benzimidazole	≤ 3	≤ 1[c]	≤ 3
 8-Chlorotheophylline[c]	26 32[d] 36[e]	22 17[f] -	24 18[f] 33[g]

Ligand	K'_{11} Solubility	Kinetic	Spectral
 Theophylline-7-acetic acid[c]	19[b]	14	11
 Theophylline	25 -	- 11[c]	22 13[c]
 Purine	-	2.3[c]	-
 Guanine	-	≤ 1[c]	-
 Uracil	-	≤ 1[c]	-
 Caffeine	36[b,h]	18	18

a. Determined at 25.0° and in 1% or 0.83% acetonitrile.

b. See text.

c. Anionic form employed.

d. 15.0°.

e. 8.7°.

f. 35.0°.

g. 11.0°.

h. $K_{11} = 28\ M^{-1}$; $K_{(12)} = 55\ M^{-2}$.

than or in addition to an equilibrium process is operative. With imidazole the solubility isotherm was linear up to 0.7 M and with 2-methylimidazole up to 0.4 M ligand concentrations, a dependence that can be explained in terms of 1:1 complex formation, which predicts a linear increase in solubility, while a non-specific solvent effect usually follows the Setschenow equation (79, 80). This equation predicts a logarithmic increase of solubility with increasing concentration of added agent. With imidazole and 2-methylimidazole our kinetic studies were conducted at ligand concentrations that were in the linear region. With N-methylimidazole our kinetic study extended to greater than 0.8 M, and the system may be in a region where bulk solvent effects are also operative. The imidazole and 2-methylimidazole have the ability to form hydrogen bonds with the ester, which may contribute to their specificity.

That benzimidazole had little effect is not highly surprising. The low solubility of benzimidazole placed an upper limit of 0.03 M on the ligand concentration in the solubility and spectral studies; if the stability constant is $\leq$ 3 significant effects would be difficult to detect. In the kinetic studies higher concentrations were achieved, but about half of the molecules were in the ionic form. So even if the neutral molecule had a stability constant of 3 M^{-1}, interaction appears to be diminished with ionic forms (see below) and this may be the reason why a greater effect was not observed at higher concentrations.

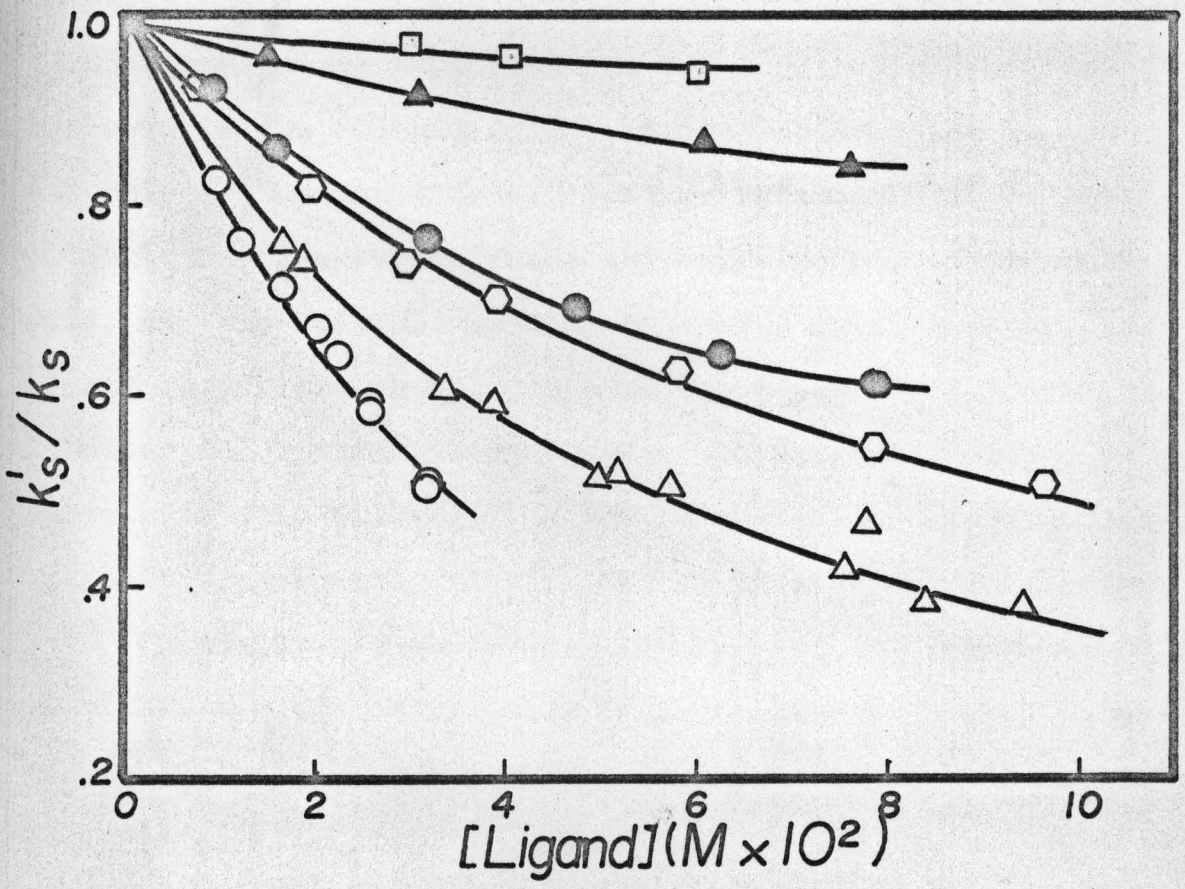
The purine derivatives offer some interesting comparisons. Fig. 24 summarizes the effect of various purines on the rate constant. The following order for interaction (expressed in terms of K'_{11}) with methyl cinnamate was observed: Caffeine > 8-chlorotheophylline ≅ theophylline > theophylline-7-acetic acid > theophyllinate > purine > guanine. As these compounds all contain the same basic ring structure, the various substituents appear to affect the interaction. Several factors can be considered:

a) N-methylation: Caffeine, which differs from theophylline in that it is methylated at the 7-N position, interacts more strongly. Similar relative results have been observed with a wide variety of substrates, and N-methylation has been postulated by the Pullmans to increase the donor properties of purines. Another possibility is that the methyl group aids interaction through London dispersion forces.

b) Distributed negative charge: The interaction with theophylline was decreased by two-fold when the molecule was ionized. The anion is solvated to a greater extent than is the free acid, which probably reduces the ability of the ligand to achieve close contact with the substrate.

c) Localized negative charge: The decreased interaction with theophylline-7-acetic acid with respect to caffeine may be attributed partly to the negative charge and also to the steric effect due to the increased bulk of the acetate over the methyl. As has been shown in self-

Fig. 24. Relative rates of hydrolysis of methyl cinnamate in the presence of various ligands at 25.0°.



□ - guanine; ▲ -purine; ● -theophylline-7-acetic acid;[a] ⬡ -theophyllinate; △ -8-chlorotheophylline; ○ -caffeine (monomer)[a]. [a]Consult text.

association studies (57), 7-propyltheophylline interacts less strongly than does caffeine. The negative charge on theophylline-7-acetate cannot be delocalized by resonance and a complete solvation as is the case with theophyllinate would not be expected to occur. The acetate group would be solvated more than the methyl group but the ring system probably is not solvated to any greater extent than it is in caffeine.

d) Electronegative substitution: 8-Chlorotheophylline was employed in the anionic form. It interacted twice as strongly as theophyllinate. Similar relative results were obtained for 8-chlorocaffeine and caffeine with benzpyrene (72), and the Pullmans predict that uric acid, with an electronegative carbonyl group in the 8-position, should be a better donor than the xanthines. It was not possible to study uric acid and xanthine due to their limited solubility, although they would have afforded an interesting comparison.

There is a rough correlation between the interaction constants and the donor ability of the ligands as predicted by the Pullmans (65) and others (13) for the neutral molecules.

In some reported systems an actual charge transfer, as defined by Mulliken (81-84), occurs. For example, caffeine, like tryptophan, appears to give up an electron in complexing with riboflavin (85, 86). Eckert (87) reported the existence of a charge transfer spectral band with caffeine and procaine (though the reported spectrum

seems questionable). In our spectral studies pertubation to longer wavelengths, which may be indicative of charge transfer, was observed.

Unfortunately, many instances appear to contradict the hypothesis. For example, complexes with xanthines and substituted benzoic acid derivatives show greater interaction with hydroxy and amino substituted benzoates than with the nitro or unsubstituted. If the interaction was due solely to donor-acceptor forces involving π electrons then one would expect greater interaction with a benzoate with electron donating groups (4). Also, in Bender's study (33) with N-(indole-3-acroyl) imidazole (donor) and 3,5-dinitrobenzoate (acceptor), he had anticipated catalysis based on the presumed interaction forces, yet inhibition was observed.

In the present study and in other similar studies involving heterocyclic polyfunctional substrates and ligands, charge transfer involving mobile π electrons undoubtedly plays a major role. But with the systems employed here hydrogen bonding is possible with many of the substrate-ligand interactions, as is localized charge-transfer (7) and other interactions between polar groups. All of these forces appear to be necessary to account for the existence of the complexes reported here.

D. Further Studies Suggested by this Investigation

In this study methyl trans-cinnamate was employed as

the substrate. Variation of the substrate structure by substitution on the cinnamate ring may be expected to influence the interaction. The influence of the double bond can be studied with the aid of hydrocinnamic acid esters; and the effect of the distance between the ring and the carbonyl group may be investigated to give further insight into the steric effects. The complexing tendencies of the cis isomer, which models show is non-planar, may be instructive. With a substrate that interacts to a greater extent, it would be possible to study ligands such as uric acid and xanthine, which are only slightly water soluble. The electron donor abilities of the purine and pyrimidine based compounds is well documented, and with a substrate that is a good acceptor it would be possible to investigate these postulates experimentally and systematically.

The ΔH and ΔS values determined for the 8-chloro-theophylline-methyl cinnamate system were not too informative in themselves as most weak intermolecular or intramolecular interactions, whether charge transfer or hydrogen bonding in nature, show thermodynamic functions of approximately the same magnitude (88-91). An important comparison would be of $\Delta\Delta H$ and $\Delta\Delta S$ values in a related series of ligands (92). This would be a more valid comparison than would one utilizing stability constants (free energy changes), because it separates enthalpy and entropy effects, which may vary independently within a series. These comparisons would yield a greater insight into the possible forces and structures involved in complexes of this type.

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APPENDIX A

A Theoretical Analysis of Comparative Studies of Complex Formation. The Solubility, Spectral, and Kinetic Techniques

INTRODUCTION

The concept of complex formation has been adopted as a simple hypothesis that can account for non-additive behavior in the physical and chemical properties of solutions of two or more species. With this hypothesis it becomes possible to utilize quantitative measures of these properties to describe the extent of interaction between the species and to investigate the nature of the interaction product, or complex. Many definitions of "a complex" have been proposed, but for the purpose of this discussion it will not be necessary to limit sharply the chemical nature of the species, and the techniques to be discussed may be applicable to the study of reactions that may not be accepted as complexation reactions. Throughout this discussion we consider complex formation to be a reversible chemical reaction in which the rate of attainment of equilibrium is much greater than any rates involved in the measuring processes. The system is therefore considered to be at equilibrium.

The basic purpose of studies of complex formation is to provide a comprehension of the properties of complexes, including their structure and reactivity. Since the reversibility of complex formation is the fundamental aspect relating all of these processes, the general reaction may be written

$$mS + nL \rightleftharpoons S_mL_n$$

where S represents the substrate and L is the ligand. (The substrate is the compound whose apparent properties are measured). Given adequate evidence that a complex is present in a system, the first information to be sought is its stoichiometry, that is, the values of m and n. It is probable that in many (perhaps most) systems more than one complex is formed, and the stoichiometries of all species are desired. Note also that it is entirely conceivable that two or more complexes may co-exist with the same stoichiometry but different structures (1). (A single complex species will possess a unique average molecular and electronic configuration).

The strength or stability of a complex is specified in terms of its stability (association, formation) constant. The overall stability constant K_{mn} for the complex formation reaction is written

$$K_{mn} = \frac{[S_mL_n]}{[S]^m[L]^n} \qquad \text{(A-1)}$$

where brackets signify molar concentrations and K_{mn} is the constant applicable to the solvent system and temperature employed. The ~~standard~~ reference state of the solute is taken to be the infinitely dilute solution in the experimental solvent. Often the concentrations of S and L are sufficiently low that they do not affect the value of the stability constant. An alternative description of

the stability of S_mL_n is available in the step stability constant; the assumption is that S_mL_n is formed from $S_mL_{(n-1)}$ by reaction with one L, or from $S_{(m-1)}L_n$ by reaction with one S. The monograph of Rossotti and Rossotti (2) reviews methods for the determination of stability constants.

Most of the molecules of pharmaceutical and biological interest are of complicated structure and contain numerous functional groupings. Such molecules will therefore possess multiple interaction sites for complex formation. A given system of substrate, ligand and solvent can be described as a member of one of these two classes:

I. Only one complex species is formed. This complex may be a resultant of multiple interactions. The possibility is admitted that the complex contains only substrate or only ligand molecules.

II. Two or more complex species are formed. The several complexes may be formed by means of different types of interaction forces or by the same interactions differently oriented.

In our studies of complexation equilibria and their relation to enzyme specificity behavior we have employed solubility measurements, absorption spectroscopy, and rate measurements for the determination of complex stability. These are all well-known techniques, but few investigators have systematically applied more than one

of them to a complexation system (3, 4, 5). The three methods do not always yield the same numerical result (taking into account the expected experimental uncertainty), and the analysis of these differences may be of value to others.

The usual procedure is to assume that a single complex of one-to-one stoichiometry is responsible for the observed effects. If the data suggest that this simple assumption is untenable, another will of course be made in its place, but the observations are not always susceptible to such an interpretation. The problem, therefore, is to find the relationship between the apparent 1:1 stability constant (as evaluated by each of the experimental methods) and the actual parameters of the system. This analysis will be carried out for each technique as applied to the systems most likely to be encountered. A comparison of the three methods will then be given.

ONE COMPLEX PRESENT

Solubility Method.- (1:1 Complex)- The theory and practice of the solubility method have recently been reviewed in detail (6), and only a brief outline will be given here. The experimental operation entails the addition of an equal weight (in excess of its normal solubility) of the slightly soluble substrate into each of several vials. A constant amount of solvent is added to each container. Then successively increasing portions of the relatively

soluble ligand are added to these vessels, which are closed and brought to solubility equilibrium at constant temperature. The solution phases are analyzed for their total concentration of S, no matter what its molecular state may be. A phase diagram is constructed by plotting, on the vertical axis, the total molar concentration of S found in the solution, S_t, against the total molar concentration of L added, L_t. We consider here only the formation of soluble complexes; these produce a phase diagram consisting of a smooth curve with a positive slope. In general, if the solution contains but one complex S_mL_n, the concentrations at any point on the curve can be expressed (see Appendix for an explanation of the symbols):

$$\begin{aligned} [S] &= S_o \\ [S_mL_n] &= (S_t - S_o)/m \\ [L] &= L_t - n[S_mL_n] \end{aligned} \qquad \text{(A-2)}$$

since the concentration of free S is maintained constant by the presence of solid substrate. Let us consider the case in which $\underline{m} = \underline{n} = 1$. Then Eq. 1 for K_{11} is combined with Eqs. A-2 to give

$$S_t = \frac{K_{11}S_oL_t}{1 + K_{11}S_o} + S_o \qquad \text{(A-3)}$$

showing that the plot of S_t $\underline{vs}$ L_t is a straight line with intercept S_o on the vertical axis; the slope of this line is $K_{11}S_o/(1 + K_{11}S_o)$, leading to

$$K_{11} = \frac{\text{Slope}}{S_o(1 - \text{Slope})}$$

In general, the apparent 1:1 stability constant is evaluated from solubility measurements by means of Eq. A-4; if a single 1:1 complex is present, the apparent K_{11} (symbolized K'_{11}) determined in this manner is equal[1] to the actual K_{11}.

$$K'_{11} = \frac{\text{Slope}}{\text{Intercept}(1 - \text{Slope})} \qquad \text{(A-4)}$$

(2:1 Complex)- If we more generally let $n = 1$ but let m assume any value, Eqs. A-1 and A-2 give

$$S_t = \frac{mK_{m1}S_o^m L_t}{1 + K_{m1}S_o^m} + S_o \qquad \text{(A-5)}$$

The phase diagram is linear as long as the complex contains only one molecule of L. If the slope is greater than unity, then at least one species must be present in which m is greater than one, for it is clearly impossible for one mole of L to take more than one mole of S into solution if the complex is of the 1:1 type. On the other hand, a slope smaller than one does not necessarily mean that a 1:1 complex is formed, though this assumption is usually made. More definite statements concerning the

---

1. This is not exactly true, of course, for we have neglected the general solvent effect of S and L on the constant; these relatively minor effects may be responsible for small differences, but can be ignored as long as S_t and L_t remain fairly small. We also neglect the "statistical" or "contact" complexes formed as a result of random distribution of the molecules (7, 8).

order with respect to S cannot usually be made since the presence of solid substrate is responsible for maintaining a constant activity of S in the system.

If a single 2:1 complex is present, the slope is given by Eq. A-6.

$$\text{Slope} = \frac{2K_{21}S_o^2}{1 + K_{21}S_o^2} \qquad \text{(A-6)}$$

If this quantity is less than one, the system will be interpreted as a probable 1:1 complex, and an apparent K'_{11} will be calculated from Eq. A-4. The actual nature of this quantity is found by combining Eqs. A-4 and A-6,

$$K'_{11} = \frac{2K_{21}S_o}{1 - K_{21}S_o^2} \qquad \text{(A-7)}$$

where K'_{11} is the apparent 1:1 stability constant. (If the true stoichiometry were known, it would be a simple matter to evaluate the correct constant, K_{21}, but this information will seldom be available).

This discussion has ignored the route of formation of S_2L. This can conceivably occur in three ways: $2S + L = S_2L$; $S_2 + L = S_2L$; $SL + S = S_2L$. The first of these has been employed in the preceding discussion. The other possibilities require the presence of another complex, and can be treated with methods developed later for these more complicated systems.

(1:2 Complex)- When a complex is present that is second-order in L, the solubility diagram will not be linear but

will show a positive curvature (6). Such a curvature would be recognized and would prevent evaluation of an apparent K'_{11}. If, however, the system contains both 1:1 and 1:2 complexes, the deviation from linearity may be unnoticed and misinterpretation may result. This system will be analyzed in a later section.

(Substrate Dimer)- Suppose S undergoes reaction to form the dimer S_2 with dimerization constant K_{SS}. Then the total concentration $S_t = [S] + 2[S_2]$, or $S_t = S_o + 2K_{SS}S_o^2$. Obviously the apparent complexation constant evaluated from the phase diagram will be zero, but the intercept will give $S_o + 2K_{SS}S_o^2$ rather than S_o. No solubility experiment will reveal this anomaly, however.

Spectral Method.-(1:1 Complex)- If the molar absorptivities of the complex and the substrate differ at the same wavelength, it may be possible to determine the stability constant spectrophotometrically. We assume that Beer's law is followed by all species. Then at a concentration S_t of substrate, in the absence of ligand, the solution absorbance is

$$A_o = a_S b S_t \qquad \text{(A-8)}$$

In the presence of ligand at total concentration L_t, the absorbance of the solution containing the same total substrate concentration is

$$A_L = a_S b[S] + a_L b[L] + a_{11} b[SL]$$

which, combined with the material balance on S, gives

$$A_L = a_S b S_t + a_L b L_t + \Delta a b [SL]$$

where $\Delta a = a_{11} - a_S - a_L$. By measuring the solution absorbance against a reference containing ligand at concentration L_t, the measured absorbance becomes

$$A_L' = a_S b S_t + \Delta a b [SL] \qquad \text{(A-9)}$$

Combining Eqs. A-8 and A-9 with the stability constant definition leads to

$$\Delta A/b = K_{11} \Delta a [S][L]$$

where $\Delta A = A_L' - A_o$. Utilizing the expression $[S] = S_t/(1 + K_{11}[L])$, this becomes

$$\Delta A/b = \frac{K_{11} S_t \Delta a [L]}{1 + K_{11}[L]} \qquad \text{(A-10)}$$

This equation can be put into several linear forms, one of these being Eq. 11, which is similar to the equation used by Benesi and Hildebrand (9, 10) to determine stability constants spectrophotometrically.

$$b/\Delta A = 1/K_{11} S_t \Delta a [L] + 1/S_t \Delta a \qquad \text{(A-11)}$$

If $[L]$ can be approximated by L_t, a plot of $b/\Delta A$ <u>vs</u> $1/L_t$ will be linear. The stability constant K_{11} is taken as the ratio <u>Intercept/Slope</u> of this plot. This is the operational definition of the spectrally measured 1:1 stability constant. Note that the types of interaction or their distribution in the complex are irrelevant, the only requirement in the application of the method being that Δa is not equal to zero.

No approximations have been introduced in the derivation of Eq. A-11, but it is necessary to assume $[L] = L_t$ in its application. This assumption is equivalent to supposing that $1 + K_{11}[S] = 1$, because of the relationship $L_t = [L](1 + K_{11}[S])$. The assumed equality $[L] = L_t$ is therefore sensitive to the magnitude of the stability constant and to the substrate concentration. If K_{11} is quite large it is essential to hold S_t to a small value if Eq. A-11 is to be applied.

As noted above, Eq. A-11 is exact, but its use requires an approximation. It is possible to introduce the approximation during the derivation (2), leading to the equation

$$bL_t/\Delta A = (S_t + L_t)/\Delta a S_t + 1/\Delta a K_{11} S_t$$

It can be shown that the application of this equation requires conditions that are similar to those adopted in the use of Eq. A-11. Throughout this paper the spectral method will be discussed in terms of Eq. A-11.

(2:1 Complex)- In this system the equation corresponding to Eq. A-9 is written

$$A_L' = a_S b S_t + \Delta ab [S_2 L]$$

where $\Delta a = a_{21} - 2a_S - a_L$. The concentration of free substrate is given by

$$[S] = \frac{S_t}{1 + 2K_{21}[S][L]}$$

The resulting equation in its reciprocal form is

$$b/\Delta A = 1/K_{21}S_t\,\Delta a[S][L] + 2/S_t\,\Delta a \qquad (A\text{-}12)$$

so the apparent spectral constant is

$$K_{11} = 2K_{21}[S] \qquad (A\text{-}13)$$

Note, however, that the plot should not theoretically be linear, since the slope is a function of [S]. This complication will be treated in more detail in later sections dealing with multiple complexes.

(Substrate Dimer)- If S dimerizes with a change in spectrum, the quantity ΔA will be independent of L_t. The apparent stability constant will be zero. Beer's law will not be followed by the substrate if the dimer's molar absorptivity is not twice that of the monomer.

Kinetic Method.-(1:1 Complex)- The kinetic method, as it has been most frequently applied, utilizes a reduction in rate of a reaction of S when L is present to obtain information about the nature of the complex; the basic assumption is that the decreased reactivity is the result of complexation, the complexed S being less reactive than free S. The kinetic scheme can be represented

$$S + L \underset{}{\overset{K_{11}}{\rightleftharpoons}} SL$$

$$S + R \xrightarrow{k_S} \text{Products}$$

$$SL + R \xrightarrow{k_{11}} \text{Products}$$

If, as is usually the case, a reagent R is involved in the reaction, k_S is the second-order rate constant (often

determined under pseudo first-order conditions with reagent in excess). It is assumed that R does not form complexes with S or L. The theoretical rate equation is

$$v = k_S [S][R] + k_{11}[SL][R] \qquad (A\text{-}14)$$

and the experimental rate equation is

$$v = k_{obs} S_t \qquad (A\text{-}15)$$

where k_{obs} is the pseudo first-order rate constant. Setting Eqs. A-14 and A-15 equal and dividing through by $[R]$ and S_t,

$$k_S' = k_S f_S + k_{11} f_{11} \qquad (A\text{-}16)$$

where k_S' is the apparent second-order rate constant, f_S is the fraction of S in the uncomplexed form, and f_{11} is the fraction present as SL. The stability constant K_{11} is combined with the definitions of these fractions, giving Eq. A-17.

$$f_S = \frac{1}{1 + K_{11}[L]} \quad ; \quad f_{11} = \frac{K_{11}[L]}{1 + K_{11}[L]} \qquad (A\text{-}17)$$

In the special case that $k_{11} = 0$, Eq. A-16 can be expressed in the forms Eqs. A-18 and A-19.

$$k_S' = k_S f_S \qquad (A\text{-}18)$$

$$k_S / k_S' = K_{11}[L] + 1 \qquad (A\text{-}19)$$

According to Eq. 18, a plot of k_S' vs f_S is linear, passing through the origin, with slope k_S. Prior knowledge of K_{11}

is required to calculate f_S. Eq. A-19, however, can be plotted without this knowledge if the equality $[L] = L_t$ may be made. Then the slope of the plot of k_S/k_S' vs L_t gives K_{11}.

If $k_{11} \neq 0$, the general equation A-16 must be used. Since $f_S + f_{11} = 1$, this can be written

$$k_S - k_S' = f_{11}(k_S - k_{11}) \qquad \text{(A-20)}$$

Introducing the definitions $r_{11} = k_{11}/k_S$ and $q_{11} = 1 - r_{11}$ permits Eq. 20 to be transformed to Eq. 21.

$$k_s - k_S' = q_{11}k_S f_{11} \qquad \text{(A-21)}$$

or

$$k_S - k_S' = \frac{q_{11}k_S K_{11}[L]}{1 + K_{11}[L]} \qquad \text{(A-22)}$$

Eq. A-22 can be placed in the following three forms amenable to linear graphing:

$$\frac{1}{k_S - k_S'} = \frac{1}{q_{11}k_S K_{11}[L]} + \frac{1}{q_{11}k_S} \qquad \text{(A-23)}$$

$$\frac{[L]}{k_S - k_S'} = \frac{[L]}{q_{11}k_S} + \frac{1}{q_{11}k_S K_{11}} \qquad \text{(A-24)}$$

$$\frac{k_S - k_S'}{[L]} = -K_{11}(k_S - k_S') + q_{11}k_S K_{11} \qquad \text{(A-25)}$$

Throughout this paper the kinetic method will be treated in terms of Eq. A-23, which predicts that a plot of $1/(k_S - k_S')$, or of $k_S/(k_S - k_S')$, vs $1/[L]$ should be linear. The kinetically determined 1:1 stability constant is then

defined as the ratio Intercept/Slope of this plot. From the intercept value the quantity q_{11}, and ultimately k_{11}, can be evaluated.

Eq. A-19 has frequently been employed for the estimation of stability constants from rate measurements. This procedure is not recommended, however, for the reason made evident in Fig. 1-A. In this figure Eq. A-19 is plotted for three hypothetical systems having q_{11} values of 0.5, 0.9, and 1.0; in each instance the true K_{11} = 25.0 M^{-1}. Only the topmost line should be straight since Eq. A-19 is valid only when $q_{11} = 1.0$, and in fact the other lines do exhibit a slight negative curvature. But if these were experimental points based on ordinary rate data, rather than calculated theoretical points, it is probable that these curves would be interpreted as straight lines.[2] The slopes of these lines, which are the apparent 1:1 stability constants according to Eq. A-19, are 25 (for $q_{11} = 1.0$), 18 (for $q_{11} = 0.9$), and 5.4 (for $q_{11} = 0.5$). We suggest that Eq. A-23, A-24, or A-25 be used in analyzing kinetic data. The same data plotted in Fig. 1 have been replotted in Fig. 2-A according to Eq. A-23; the apparent 1:1 stability constant is 25 M^{-1} in each case.

(2:1 Complex)- In dealing with this system it becomes necessary to take into account various possible fates of

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2. As K_{11} is made larger, the curvature in these plots becomes more evident.

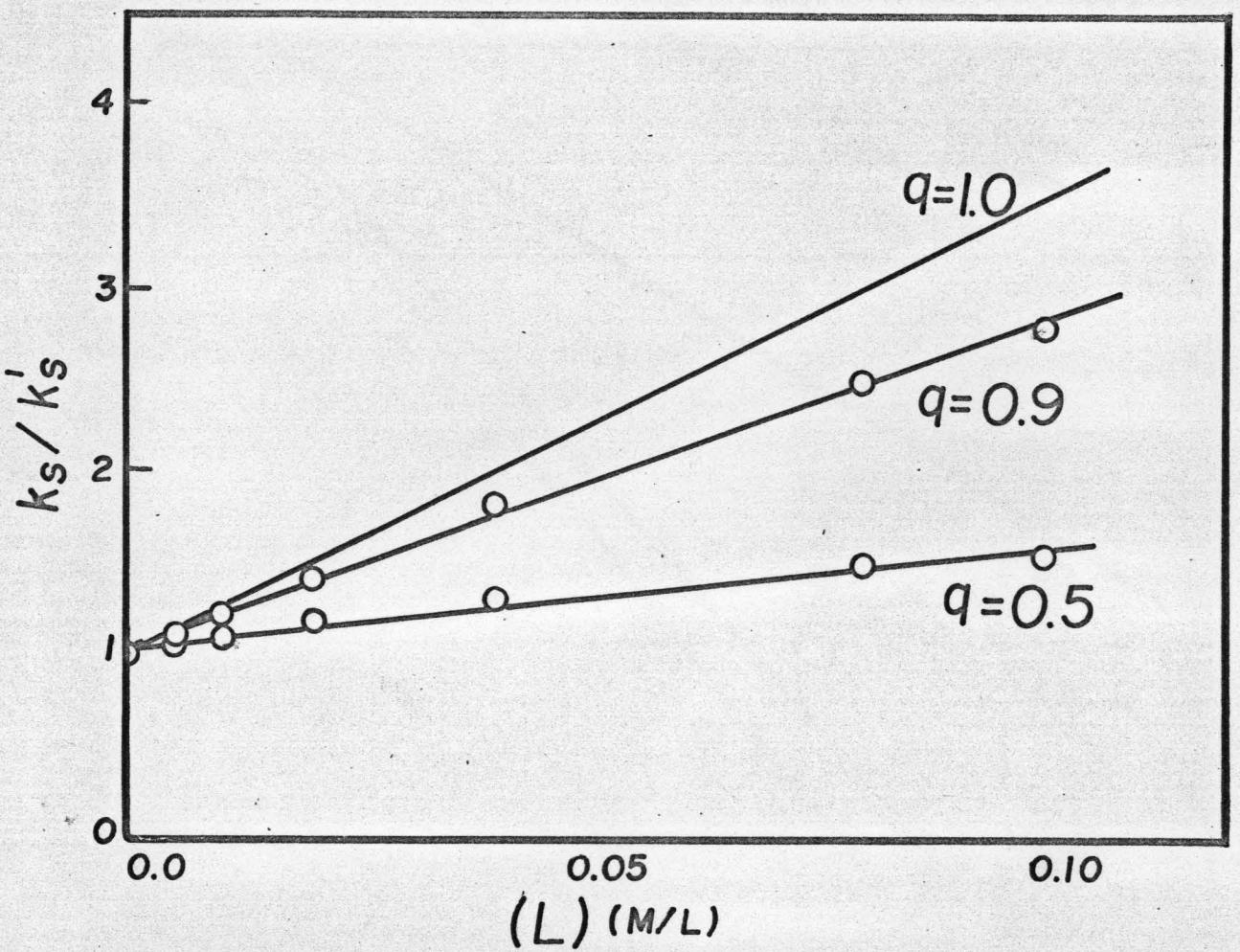

Fig. 1A. Plots of Eq. A-19 for systems containing a single 1:1 complex with stability constant $K_{11} = 25.0 M^{-1}$ and the q values shown.

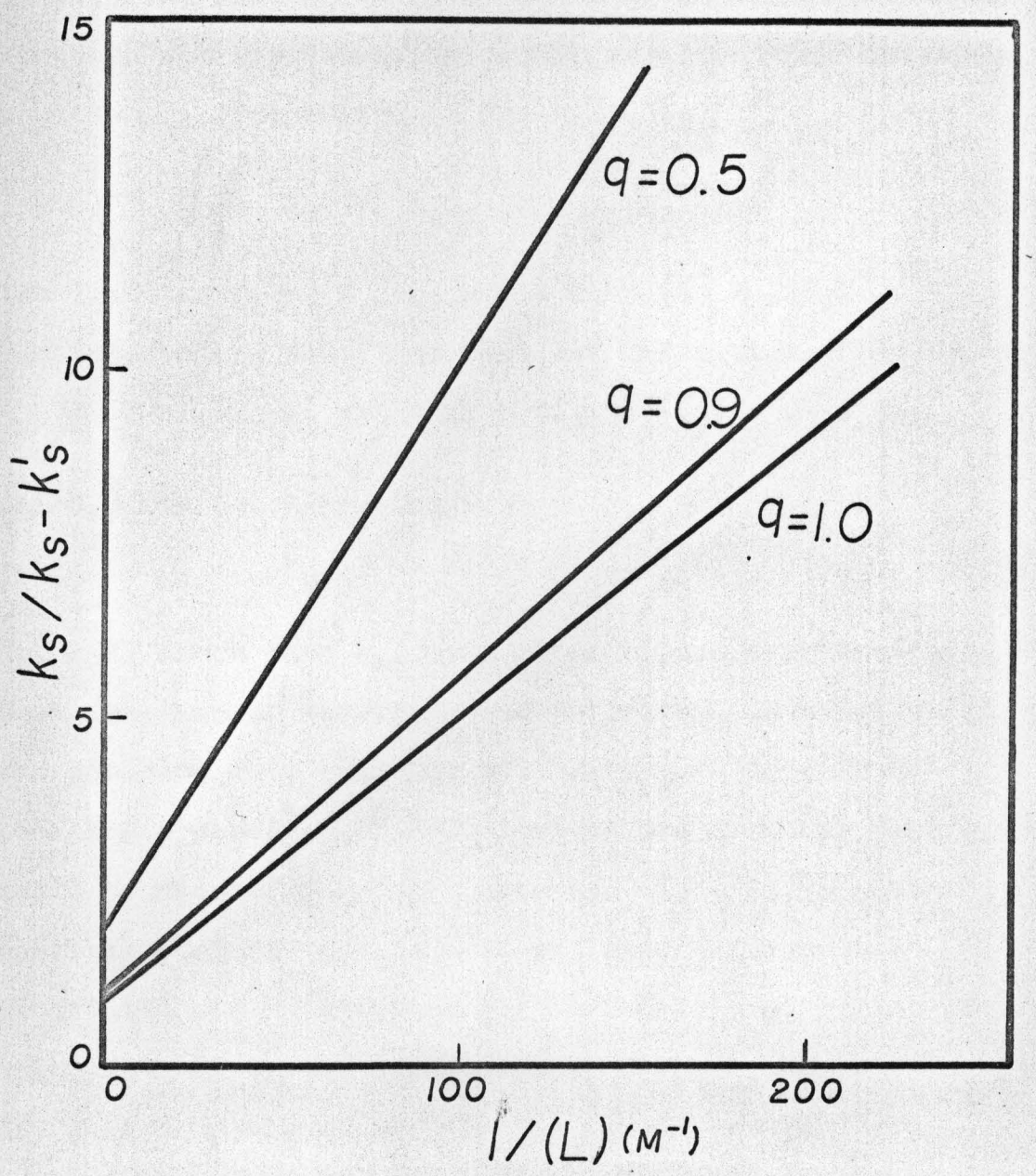

Fig. 2A. Plots of the data shown in Fig. 1A according to Eq. A-23.

the complex S_2L. It may undergo reaction with R to give products from one S molecule, releasing the other unreacted, or both S molecules may react, or two molecules of R may be required, etc. We shall adopt the simplest assumption, that $k_{21} = 0$, recognizing that this places a limit on the applicability of the result. Then the basic equation, corresponding to Eq. A-16, is

$$k_s' = k_S f_S \qquad \text{(A-26)}$$

The fraction $f_S = [S]/S_t$, while $S_t = [S] + 2[S_2L]$. Combining these equations with the definition of K_{21} gives

$$f_S = 1/(1 + 2K_{21}[S][L])$$

which, with Eq. A-26, leads to

$$k_S' = k_S/(1 + 2K_{21}[S][L]) \qquad \text{(A-27)}$$

Eq. A-27 shows that k_S', at a given ligand concentration, is a function of substrate concentration; in other words, the apparent rate constant will vary with time as the reaction proceeds. If measurements are made for only a small portion of the total reaction time, it is quite possible (taking into account ordinary experimental uncertainties) to overlook the variability of k_S' and to interpret the system as belonging to the 1:1 class. Eq. A-27 can be converted to the usual plotting form:

$$1/(k_S - k_S') = 1/2k_S K_{21}[S][L] + 1/k_S \qquad \text{(A-28)}$$

The kinetically evaluated K_{11}' is equal to the ratio

Intercept/Slope, or

$$K'_{11} = 2K_{21}[S] \qquad \text{(A-29)}$$

(Substrate Dimer)- If S dimerizes, and the dimeric form is essentially unreactive, the apparent constant k'_S will be related to the substrate concentration by the equation $k'_S = k_S(1 + 2K_{SS}[S])$. The apparent constant will therefore vary with time during a reaction. Since k'_S is not dependent upon the ligand concentration, however, the apparent 1:1 stability constant will be zero.

TWO COMPLEXES PRESENT

Solubility Method-(Two 1:1 Complexes)- It is possible that two complexes of 1:1 stoichiometry but different structures may co-exist. If we distinguish between these by representing them as SL and LS, the solubility conservation equations may be written

$$S_o = [S]$$
$$S_t = [S] + [SL] + [LS]$$
$$L_t = [L] + [SL] + [LS]$$

These are combined with the stability constants to give

$$S_t = \frac{(K_{SL} + K_{LS})S_oL_t}{1 + (K_{SL} + K_{LS})S_o} + S_o \qquad \text{(A-30)}$$

which has the same form as Eq. A-3 for a single 1:1 complex. Applying Eq. A-4 shows that

$$K'_{11} = K_{SL} + K_{LS} \qquad \text{(A-31)}$$

Thus the apparent 1:1 stability constant evaluated by solubility measurements gives the sum of the individual constants. This can be generalized to any number of 1:1 complexes. Note that the slope of the phase diagram cannot exceed unity as long as only 1:1 complexes are present.

(1:1 and 2:1 Complexes)- We define the step stability constants

$$K_{11} = [SL]/[S][L]$$

$$K_{(21)} = [S_2L]/[S][SL]$$

The development follows the lines already indicated. The equation of the phase diagram is

$$S_t = \left[\frac{K_{11}S_o + 2K_{11}K_{(21)}S_o^2}{1 + K_{11}S_o + K_{11}K_{(21)}S_o^2}\right] L_t + S_o \qquad (A\text{-}32)$$

Combining the slope of this plot with Eq. A-4:

$$K'_{11} = \frac{K_{11} + 2K_{11}K_{(21)}S_o}{1 - K_{11}K_{(21)}S_o^2} \qquad (A\text{-}33)$$

That a mathematically equivalent expression would be obtained if the overall stability constant had been employed can be seen from the equality $K_{21} = K_{11}K_{(21)}$.

(1:1 and 1:2 Complexes)- Combining the step constants K_{11} and $K_{(12)}$ with the material balance equations gives

$$S_t = \left[\frac{K_{11}S_o + K_{11}K_{(12)}S_o[L]}{1 + K_{11}S_o + 2K_{11}K_{(12)}S_o[L]} \right] L_t + S_o \qquad (A\text{-}34)$$

which, with Eq. A-4 leads to

$$K'_{11} = \frac{K_{11} + K_{11}K_{(12)}[L]}{1 + K_{11}K_{(12)}S_o[L]} \qquad (A\text{-}35)$$

According to Eq. A-34 the phase diagram should show a positive curvature, but if the 1:1 complexing is much more extensive than the 1:2 type this non-linearity may not be noticed. Methods are available to analyze this system, when it is recognized, to obtain the individual stability constants (6).

(1:1 Complex and Ligand Dimer)- The equation of the phase diagram is easily developed as before:

$$S_t = \frac{K_{11}S_oL_t}{1 + K_{11}S_o + 2K_{LL}[L]} + S_o \qquad (A\text{-}36)$$

where $K_{LL} = [L_2]/[L]^2$. The phase diagram will exhibit a negative curvature, but if the curve is mistaken for a straight line the apparent 1:1 stability constant that will be evaluated is given by Eq. A-37.

$$K'_{11} = \frac{K_{11}}{1 + 2K_{LL}[L]} \qquad (A\text{-}37)$$

(1:1 Complex and Substrate Dimer)- In this case the equation of the phase solubility diagram is

$$S_t = \frac{K_{11}S_oL_t}{1 + K_{11}S_o} + S_o + 2K_{SS}S_o^2$$

The slope of the straight line is the same as that that would be observed in the absence of dimer formation, but the intercept is different. The apparent constant is

$$K'_{11} = \frac{K_{11}}{1 + 2K_{SS}S_o} \qquad (A\text{-}38)$$

Spectral Method-(Two 1:1 Complexes)- If the two complexes SL and LS are formed, and at least one of them possesses a molar absorptivity different from free S, a spectral change will be observed. The analysis follows that given for a single 1:1 complex. The concentration of free substrate is related to the other system variables by Eq. A-39.

$$[S] = \frac{S_t}{1 + (K_{SL} + K_{LS})[L]} \qquad (A\text{-}39)$$

The reciprocal form of the equation for this system is

$$b/\Delta A = \frac{1}{S_t(K_{SL}\Delta a_{SL} + K_{LS}\Delta a_{LS})\,L} + \frac{K_{SL} + K_{LS}}{S_t(K_{SL}\Delta a_{SL} + K_{LS}\Delta a_{LS})} \qquad (A\text{-}40)$$

(where $\Delta a_{SL} = a_{SL} - a_S - a_L$ and $\Delta a_{LS} = a_{LS} - a_S - a_L$), showing that the apparent 1:1 stability constant is given by

$$K'_{11} = K_{SL} + K_{LS} \qquad (A\text{-}41)$$

This result has been pointed out by several authors (1).

Even if one of the complexes has an absorption spectrum identical with that of the free substrate, K'_{11} will be given by Eq. A-41. This may be intuitively pictured as the result of a depletion of free S by formation of this second complex, even though it is not spectrally distinctive.

(1:1 and 2:1 Complexes)- This system should not, theoretically, yield a linear reciprocal plot, yet, as Johnson and Bowen have shown, the experimental plots may well appear to be linear (11). The analysis may be conducted as in earlier examples, leading to

$$\Delta A/b = K_{11}[S][L](\Delta a + \Delta a' K_{(21)}[S])$$

where $\Delta a = a_{11} - a_S - a_L$ and $\Delta a' = a_{21} - 2a_S - a_L$. For the present purpose the substrate concentration is written as

$$[S] = \frac{S_t}{1 + K_{11}[L](1 + 2K_{(21)}[S])}$$

These equations are combined to give

$$b/\Delta A = \frac{1}{K_{11}S_t(\Delta a + \Delta a' K_{(21)}[S])\ L} + \frac{1 + 2K_{(21)}[S]}{S_t(\Delta a + \Delta a' K_{(21)}[S])} \qquad (A\text{-}42)$$

from which it is seen that the apparent spectrally measured constant is

$$K'_{11} = K_{11} + 2K_{11}K_{(21)}[S] \qquad (A\text{-}43)$$

This conclusion is not always valid, however; a fuller discussion is given under the next system.

(1:1 and 1:2 Complexes)- Again it is evident that the plot should be non-linear, but Johnson and Bowen (11) have found that the curvature may be overlooked. The development of the appropriate equation is similar to that in the preceding example; the equation is

$$b/\Delta A = \frac{1}{K_{11}S_t(\Delta a_{11} + \Delta a_{12}K_{(12)}[L])\,L} + \frac{1 + K_{(12)}[L]}{S_t(\Delta a_{11} + \Delta a_{12}K_{(12)}[L])} \qquad (A\text{-}44)$$

where $\Delta a_{11} = a_{11}-a_S-a_L$ and $\Delta a_{12} = a_{12}-a_S-a_L$. The apparent stability constant is

$$K'_{11} = K_{11} + K_{11}K_{(12)}[L] \qquad (A\text{-}45)$$

That Eq. A-44 is not the equation of a straight line must be kept in mind, however, and it may be expected that the range of ligand concentration over which the system is studied may affect the results. Suppose the ligand concentration is made very large, so that $\Delta a_{12}K_{(12)}[L] \ggg \Delta a_{11}$; then Eq. A-44 becomes

$$b/\Delta A = 1/\Delta a_{12}K_{11}K_{(12)}[L]^2 + 1/\Delta a_{12}S_t$$

and the apparent constant is

$$K'_{11} = K_{11}K_{(12)}[L] \qquad (A\text{-}46)$$

The necessary condition for Eq. A-46 to be approached is

a function not only of [L], but also of the quantities Δa_{11}, Δa_{12} and $K_{(12)}$. This conclusion agrees with the calculations of Johnson and Bowen (11), who designed hypothetical systems to demonstrate these effects. (These remarks apply also to the system described by Eq. A-42, which is, however, not as sensitive to these effects because [S] is usually much smaller than [L]). Thus the apparent stability constant may vary with the wavelength at which the absorbance measurements are made.

(1:1 Complex and Ligand Dimer)- This situation is made rather complicated because the assumption that $L_t = [L]$, made hitherto in the spectral analysis, is not valid; a very appreciable fraction of the uncomplexed ligand may exist as the dimer. This system does not appear to be amenable to a useful treatment according to the manner of the earlier examples.

(1:1 Complex and Substrate Dimer)- This system does not lead to a useful analysis. The extent of dimerization will depend upon the ligand concentration (unlike the case, discussed earlier, where S_2 was the only complex present) because the free substrate concentration is a function of ligand concentration.

Kinetic Method-(Two 1:1 Complexes)- Proceeding as for a single complex, this basic equation is obtained

$$k'_S = k_S f_S + k_{SL} f_{SL} + k_{LS} f_{LS} \qquad \text{(A-47)}$$

which, since $f_S + f_{SL} + f_{LS} = 1$, leads to

$$k_S - k_S' = q_{SL}k_S f_{SL} + q_{LS}k_S f_{LS} \qquad \text{(A-48)}$$

where the q's are defined as before. Expressions for the fractional compositions are found by combination of the material balance and stability constant equations,

$$f_{SL} = \frac{K_{SL}[L]}{1 + K[L]} \; ; \quad f_{LS} = \frac{K_{LS}[L]}{1 + K[L]}$$

where $K = K_{SL} + K_{LS}$. Substituting these into Eq. A-48 and rearranging gives the linear form

$$\frac{1}{k_S - k_S'} = \frac{1}{(q_{SL}K_{SL} + q_{LS}K_{LS})[L]} + \frac{K_{SL} + K_{LS}}{(q_{SL}K_{SL} + q_{LS}K_{LS})} \qquad \text{(A-49)}$$

showing that K_{11}' is given by Eq. A-50.

$$K_{11}' = K_{SL} + K_{LS} \qquad \text{(A-50)}$$

This result will be obtained even if one of the complexes has a reactivity equal to that of the free substrate (i.e., if one of the q's equals zero).

(1:1 and 2:1 Complexes)- We suppose that the S_2L complex is unreactive. Then the basic equation of the system is

$$k_S' = k_S f_S + k_{11} f_{11}$$

But the fractions f_S and f_{11} are functions of [S], so k_S' will vary during the course of the reaction, as pointed out in connection with Eq. A-27. If this variability should not be evident, the usual kinetic treatment will be made. The above equation is transformed into

$$k_S - k'_S = k_S(q_{11}f_{11} + f_{21})$$

where $f_{11} = [SL]/S_t$ and $f_{21} = 2[S_2L]/S_t$. This leads finally to Eq. A-51.

$$\frac{1}{k_S - k'_S} = \frac{1}{k_S K_{11}[L](q_{11} + 2K_{(21)}[S])} + \frac{1 + 2K_{(21)}[S]}{(q_{11} + 2K_{(21)}[S])k_S} \qquad (A\text{-}51)$$

This is not the equation of a straight line, but, if k_s appears to be constant during a reaction (perhaps because only a few percent of total substrate is allowed to react during the observation period), then Eq. A-51 will be essentially linear. The apparent stability constant will be

$$K'_{11} = K_{11} + 2K_{11}K_{(21)}[S] \qquad (A\text{-}52)$$

if, however, $2K_{(21)}[S] \ggg 1$, the apparent constant will be $K'_{11} = 2K_{11}K_{(21)}[S]$.

(1:1 and 1:2 Complexes)- The basic equation is

$$k'_S = k_S f_S + k_{11}f_{11} + k_{12}f_{12} \qquad (A\text{-}53)$$

which can be transformed to

$$k_S - k'_S = q_{11}k_S f_{11} + q_{12}k_S f_{12}$$

By means of the stability constant definitions and the material balance on S_t, this is converted to

$$\frac{1}{k_S - k'_S} = \frac{1}{k_S K_{11}[L](q_{11} + q_{12}K_{(12)}[L])} + \frac{1 + K_{(12)}[L]}{(q_{11} + q_{12}K_{(12)}[L])k_S} \qquad (A\text{-}54)$$

This is not the equation of a straight line, but under many circumstances it will probably yield an essentially linear plot. Eq. A-54 has the same form as Eq. A-44 for the spectral treatment of this system, and the earlier comments apply. The apparent stability constant can range from

$$K'_{11} = K_{11} + K_{11}K_{(12)}[L] \qquad \text{(A-55)}$$

to

$$K'_{11} = K_{11}K_{(12)}[L] \qquad \text{(A-56)}$$

depending on the relative magnitudes of q_{11} and $q_{12}K_{(12)}[L]$; thus the value of K'_{11} may be dependent upon the quantities q_{11} and q_{12}.

(1:1 Complex and Ligand Dimer)- As in the spectral method, this system does not give a simple analytical solution. If such a system is detected, perhaps the best way to treat it would be to determine by an independent method the ligand dimerization constant, then to calculate [L] as a function of L_t, and finally to treat the system as containing the single 1:1 complex (thus using Eq. A-23), with the calculated monomer concentration taking the place of total concentration in constructing the graph. This procedure uses the approximation $L_t = [L] + 2[L_2]$; that is, consumption of ligand by formation of SL is ignored.

(1:1 Complex and Substrate Dimer)- The experimental rate constant is a function of [S] and therefore varies during the reaction. This system cannot be conveniently analyzed.

DISCUSSION

Criteria for System Classification.- The operational definitions of the apparent 1:1 stability constants may be summarized as follows:

Solubility: Plot S_t vs L_t; then

$$K'_{11} = \frac{\text{Slope}}{\text{Intercept}(1 - \text{Slope})}$$

Spectral: Plot $b/\Delta A$ vs $1/L_t$; then

$$K'_{11} = \text{Intercept}/\text{Slope}$$

Kinetic: Plot $1/(k_S - k'_S)$ vs $1/L_t$, then

$$K'_{11} = \text{Intercept}/\text{Slope}$$

The results of the preceding analyses, giving K'_{11} in terms of stability constants and concentrations, are gathered in Table IA. The earlier discussion should be consulted for details concerning assumptions, approximations, and limits of applicability of these relationships. We shall, with their aid, consider how the comparative study of complexation systems with several techniques may yield information inaccessible with a single probe.

The usual order of investigation of a complex system will be: (1) the determination of the stoichiometries of all complexes present in significant concentrations or proportions; (2) the evaluation of stability constants for

these complexes; (3) ultimately the determination of the structure and chemical and physical properties of each complex. Several criteria can be suggested to help in establishing stoichiometries and stability constants.

(a) Relative values of K'_{11} by the solubility, spectral, and kinetic techniques. Table I-A shows the rationale for this criterion. If a finite value of K'_{11} is obtained (concerning this point see the later discussion), its relative value by the three methods may allow a partial assignment of stoichiometric types. Thus if all three methods yield the same numerical value, the system probably contains only 1:1 complexes. The possibility exists, however, that identical values can be observed with two methods by a coincidental combination of constants and concentrations. This can easily be detected as pointed out below.

(b) Dependence of K'_{11} on initial total substrate concentration by the spectral and kinetic techniques. When a complex S_mL_n is present for which m is greater than one, K'_{11} by the spectral and kinetic methods will be a function of substrate concentration. K'_{11} should be determined with at least two appreciably different initial substrate concentrations. A significant dependence of K'_{11} on substrate concentration means that at least one complex is present with m larger than one. The functional form of this dependence may yield further information. Because of this dependence, the substrate concentration should be

Table 1-A

Theoretical Equivalents of Apparent Stability Constants Determined Assuming 1:1 Complexation

Complexes Present	K'_{11} as found from Solubility	Spectra	Kinetics
None	0	0	0
L_2	0	0	0
S_2	0	0	0
SL	K_{11}	K_{11}	K_{11}
S_2L	$\frac{2K_{21}S_o}{1 - K_{21}S_o^2}$	$2K_{21}[S]$	$2K_{21}[S]$
SL + LS	$K_{SL} + K_{LS}$	$K_{SL} + K_{LS}$	$K_{SL} + K_{LS}$
$SL + S_2L$	$\frac{K_{11} + 2K_{11}K_{(21)}S_o}{1 - K_{11}K_{(21)}S_o^2}$	a	a
$SL + SL_2$	$\frac{K_{11} + K_{11}K_{(12)}[L]}{1 + K_{11}K_{(12)}S_o[L]}$	a	a
$SL + L_2$	$\frac{K_{11}}{1 + 2K_{LL}[L]}$	b	b
$SL + S_2$	$\frac{K_{11}}{1 + 2K_{SS}S_o}$	b	b

[a] Variable; see text for discussion.

[b] See text.

specified when complex stability constants are reported.

(c) Dependence of K'_{11} on ligand concentration by the solubility technique. In each of the three techniques the ligand concentration is the independent variable. As noted earlier, linear spectral plots may be observed even though a curve is theoretically to be expected, and similar results will apply in the kinetic method. The solubility method offers the best chance to detect a dependence of K'_{11} on ligand concentration. If a positive curvature is noted in the phase diagram at least one complex is present of the form SL_n where n is greater than one. Negative curvature may indicate dimerization (or higher aggregate formation) of the ligand, as in the system $SL + L_2$. A linear phase diagram does not prove that there are no complexes of these types, for certain combinations, as for example the system $SL + SL_2 + L_2$, may give rise to an essentially linear curve over wide ranges of ligand concentration (12).

(d) Dependence of k'_S on time. When a complex is present with two or more S molecules per complex molecule, the apparent rate constant should vary with time. In order to detect this variation it may be necessary to follow the reaction for at least two half-lives. If variability of k'_S is not observed the conclusion cannot be positive that all complexes contain only one S molecule, because of the assumptions made concerning the fate of the higher order complex, but this is a reasonable tentative inference.

(e) Dependence of K'_{11} on wavelength in the spectral technique. This criterion has been emphasized by Johnson and Bowen (11). If K'_{11} varies with the wavelength, at least one higher order complex is present. The theoretical reason for this dependence was pointed out in connection with Eq. A-46.

(f) Independent evidence relating to stoichiometry and stability. Some of these additional sources are: estimate of stoichiometry from isolable complexes or from the solubility phase diagram (6); Beer's law behavior of pure substrate and ligand; liquid-liquid partition studies of substrate and ligand to detect and measure the extent of self-aggregation processes; and spectral studies leading to stoichiometric ratios (e.g., the method of continuous variations).

These criteria will obviously not be capable of defining the nature of all complexation systems, but they should help considerably in this problem. The possibility that systems may be encountered that are more complicated than those in Table I-A is very real and must be kept in mind.

It is most important to realize that when the spectral K'_{11} is smaller than either the solubility or the kinetic constant this does not constitute evidence that only the charge-transfer portion of the complex interactions is being measured. If only 1:1 complexes are present, the three methods will yield the same apparent stability

constant no matter what the distribution of forces responsible for maintaining the complexes. As long as one of these complexes possesses a changed absorption spectrum this will be true, even if the other complexes cause no spectral change. The same kind of argument applies to the kinetically determined K'_{11}; if only 1:1 complexes exist, and at least one of these has an altered reactivity, the apparent K'_{11} will be equal to the sum of all the true 1:1 constants. The general statement may be made that if reliable K'_{11} values for a system differ when determined by the three methods, some complexes are present other than 1:1 combinations of substrate and ligand.

The reliability of stability constants evaluated spectrophotometrically as evidence for the existence of complexes has been explored by Person (13), who suggests that as a practical guide a 1:1 stability constant must be equal to or greater than $0.1/L_{max}$ where L_{max} is the highest ligand concentration used in the study, in order for the constant to be considered significantly different from zero. Suppose, for example, that the upper limit of ligand concentration in a spectral study is 0.2M; then the borderline value of K'_{11} is $0.5\underline{M}^{-1}$. A value smaller than this cannot be taken as evidence for complexing. Similar guides could be formulated for other techniques. Throughout this paper we have supposed that non-zero values of stability constants can be demonstrated.

Capabilities of the Solubility, Spectral, and Kinetic

Methods. The solubility method is considered by many to possess the disadvantage of non-selectivity, in that it measures the results of all types of interactions. But the foregoing analysis shows that the spectral and kinetic methods are also subject to this type of non-selectivity, and, in the mathematical terms of the analysis as represented in Table I-A it may be held that the solubility method is actually more selective than the other techniques. The solubility method possesses two real drawbacks: it is primarily limited to slightly soluble solid substrates, and the substrate concentration cannot be varied. In those systems where the ligand is not too soluble the second disadvantage may be eliminated by reversing the system, treating S as L and vice versa. It is of course not possible to extrapolate a solubility K'_{11} to zero concentration of substrate.

Solubility studies are carried out at constant [S], and spectral studies are at constant S_t. Part of the difficulty in analyzing spectra data follows from this difference, but the capability of varying S_t when desired is an advantage of the spectral method. (It is possible to perform some spectral complexation studies at solubility equilibrium, thus setting $[S] = S_o$ and letting S_t vary throughout the run; this may simplify some analyses). The great disadvantage of the spectrophotometric method is of course that a spectral change must occur upon complexation, but when a change is observed the method is very convenient,

especially since it provides wavelength as an additional variable. In a general sense, the spectral technique is neither more nor less selective, when applicable, than are other methods. The mathematics developed for spectral studies can be applied to any other physical property that is directly proportional to a species concentration; examples are refractive index (14), optical rotation (15), and fluorescence intensity.

The kinetic method is carried out with neither [S] nor S_t held constant (though if initial rates were measured S_t could be considered the constant factor). Mathematically it is similar to the spectral method; but it possesses the advantage that it is applicable even if no spectral change occurs, and the disadvantage that it does not include a convenient variable corresponding to wavelength. (The parallel to wavelength is complex reactivity, but this cannot easily be altered without changing the system). Throughout this paper the inhibition of rates by complex formation has been taken as the basis for the analysis, but the complex may in some systems exhibit an enhanced reactivity, and this phenomenon also can serve for study of the complex equilibrium (16, 17). The outstanding potential advantage of the kinetic technique is its capability for providing information about the reactivity, and thus the structure, of the complex. This capability has not yet been exploited, though some attempts have been made to utilize it (5), and further studies in the chemistry

of organic complexes may find its application valuable.

Conclusions. The application of more than one experimental technique is advisable in the study of complexation systems. By comparing the apparent stability constants evaluated by the several methods on the basis of an assumed 1:1 stoichiometry between substrate and ligand, it may be possible to establish, in part, the stoichiometries of the complexes present. If the solubility, spectral, and kinetic techniques yield essentially identical values for the apparent 1:1 stability constant (and if certain other criteria suggested in this paper are satisfied), it may be concluded that only 1:1 complexes between substrate and ligand are present. If the three techniques do not give the same value, the manner in which they differ and their dependence on variables of the system may permit a further classification. On the basis of the mathematical analysis it is concluded that the solubility, spectral, and kinetic methods for studying complex formation are about equally non-selective in their responses to multiple complexes, with the solubility method perhaps possessing a slight advantage in specificity.

APPENDIX - LIST OF SYMBOLS

S	Substrate molecule
L	Ligand molecule
S_mL_n	General formula for complex
S_t	Total (formal) concentration of S
L_t	Total (formal) concentration of L
S_o	Equilibrium molar solubility of substrate monomer in absence of L
$[i]$	Molar concentration of species i
$f_S=[S]/S_t$	Fraction of S in uncomplexed form
$f_{mn}=m[S_mL_n]/S_t$	Fraction of S in form of S_mL_n
$K_{mn}=[S_mL_n]/[S]^m[L]^n$	Overall stability constant for the complex S_mL_n
$K_{(mn)}=[S_mL_n]/[S][S_{m-1}L_n]$ or $[S_mL_n]/[S_mL_{n-1}][L]$	Step stability constant for S_mL_n
K'_{11}	Apparent stability constant assuming 1:1 stoichiometry
k_S	Specific rate constant for a reaction of S
k'_S	Apparent rate constant for S in presence of L
k_{mn}	Specific rate constant for a reaction of S_mL_n
$r_{mn} = k_{mn}/k_S$	Relative reactivity of S_mL_n
$q_{mn} = 1 - r_{mn}$	
b	Cell path length
a_S	Molar absorptivity of S
a_{mn}	Molar absorptivity of S_mL_n
A	Absorbance

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APPENDIX B

A Combined Solubility-Spectral Method for Studying Complex Equilibria

All of the commonly employed spectrophotometric methods for evaluating stability constants are based on the original Benesi-Hildebrand treatment (1), but they have been modified in methods of plotting and expressing the concentrations to suit particular purposes (2-4). These methods generally hold one total species concentration constant (substrate) and vary the other (ligand) and measure the absorbance as a function of ligand concentration. We present a new method based on a combination of the solubility method and spectrophotometric method in which the free (not total) substrate concentration is held constant. At solubility equilibrium, a system containing a 1:1 complex may be described by (see Appendix A for list of symbols):

$$S_t = S_o + K_{11}S_o[L] \qquad \text{(B-1)}$$

The absorbance of this solution will be given by

$$A = a_s bS_o + a_{11}bK_{11}S_o[L] + a_L b[L] \qquad \text{(B-2)}$$

If the absorbance is measured at a wavelength where $a_s = a_L = o$, then

$$A = a_{11}bK_{11}S_o[L] \qquad \text{(B-3)}$$

which predicts that a plot of A vs. [L] will be linear with a slope equal to $a_{11}bK_{11}S_o$. (Usually $[L] >> S_o$ and the approximation $[L] \cong L_T$ will hold).

From the solubility isotherm the concentration of complex present at any given ligand concentration can be

determined (as the substrate concentration in excess of S_o), so we can also write

$$A = a_{11}b[SL] \qquad (B\text{-}4)$$

A plot of absorbance vs [SL] ([SL] = $S_t - S_o$) should give a linear relationship passing through the origin with a slope equal to $a_{11}b$. Equations (B-3) and (B-4) will then yield a_{11} and K_{11}.

In this investigation, at the wavelengths suitable for measurement, $a_L = o$, but $a_S \neq o$, so spectral measurements were made against a saturated solution of substrate as the reference solution. Although fairly good correlations were obtained with this analysis, the data obtained in this fashion were not used for comparative purposes in this study. An obvious source of error is that although a_S was smaller than a_{11} and its contribution was blanked out, slight differences in the concentration of free substrate would introduce error.

The method would be of some use in studies where the substrate solubility is very low and suitable wavelengths could be chosen such that $a_S = a_L = o$. It serves as a means of obtaining an extra piece of information, the molar absorptivity of the complex, from a solubility study.

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APPENDIX C

Inhibition of an Ester Hydrolysis by Imidazole