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A study of the mechanism of the Jaffe reaction ; Inverse polarographic determination of creatinine with alkaline picrate and 3,5-dinitrosalicylic acid.

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I A STUDY OF THE MECHANISM OF THE JAFFE REACTION

II INVERSE POLAROGRAPHIC DETERMINATION OF CREATININE WITH ALKALINE PICRATE AND 3,5-DINITROSALICYLIC ACID

By

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ABSTRACT

PART I

The red Jaffé reaction product was isolated. A yellow creatinine picrate salt and an orange compound containing two molecules of creatinine per picric acid molecule were also isolated. Spectrophotometric, pH titration, and polarographic investigation of the Jaffé reaction was undertaken. Hydantoin was shown to react similar to creatinine, while the 5,5-dimethylhydantoin was inactive. The methylene anion of creatinine is proposed to attack the meta position of picric acid, resulting in the formation of a nitro anion. A reaction structure for the Jaffé chromogen is proposed.

PART II

A selective and highly sensitive inverse polarographic method for the determination of creatinine has been developed, involving the measurement of disappearance of diffusion current for the picric acid wave ($E_{1/2}$ = -0.501V) when creatinine is added. Creatinine levels of 0-5 µg/ml can be measured accurately.

Investigation of 3,5-dinitrobenzoic acid and 3,5-dinitrosalicylic acid revealed the latter to be more reactive and useful for the inverse polarographic determination of creatinine at levels of 0 - 30 µg/ml.

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TO MY PARENTS

TABLE OF CONTENTS

PART II

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

^{o}C	degree centigrade
cm	centimeter
cm^2	square-centimeter
g	gram
mg	milligram
µg	microgram
mg%	milligram percent (mg/100 ml)
mm	millimeter
ml	milliliter
V	volt
i_D	diffusion current
$E_{1/2}$	half-wave potential
s	second
DNSA	3,5-dinitrosalicylic acid

PART I

A STUDY OF THE MECHANISM OF THE JAFFÉ REACTION

CHAPTER I

INTRODUCTION

The reaction of creatinine with alkaline picrate, commonly known as the Jaffe reaction (1), is widely employed in clinical chemistry laboratories as a routine screening for the evaluation of kidney function.

The metabolic origin of creatinine is shown in Fig. 1.

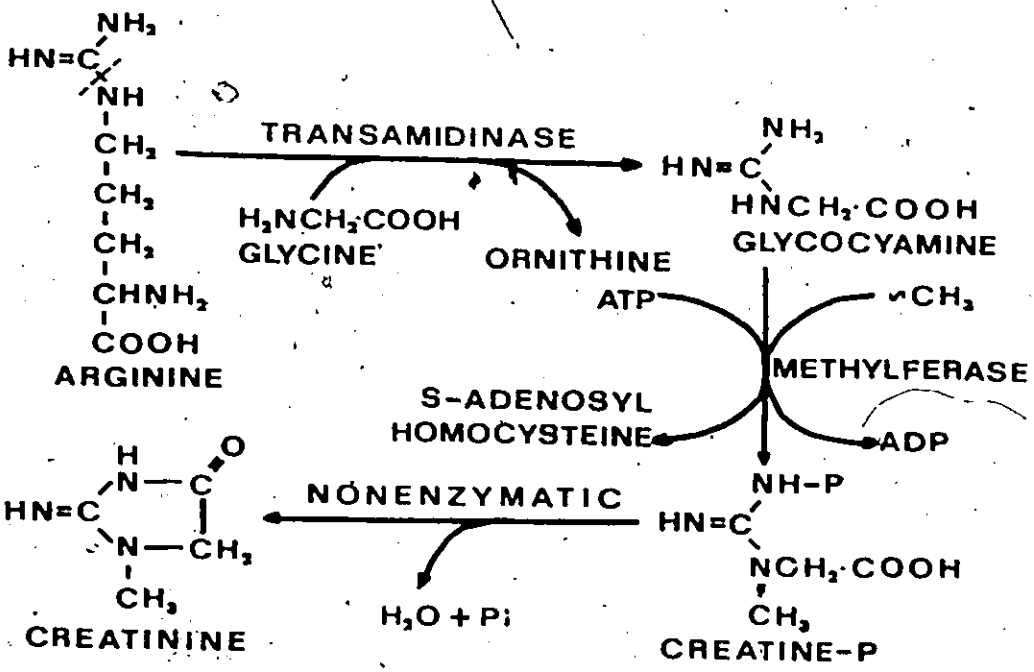


Fig. 1. The Metabolic Origin of Creatinine.

Isotope techniques have confirmed that glycine, arginine, and methionine are directly involved in the formation of creatinine. A transamidination from arginine to glycine forms guanidoacetic acid (glycocyamine). In vitro experiments have shown this to occur in the kidney, but not in the liver or in heart muscle. Methylation of the glycocyamine in the liver is accomplished with "active" methionine as the methyl

donor. The methylation of glycocyamine is not reversible. Cantoni and Vignos (2) studied the enzymatic mechanism for the methylation of glycocyamine to form creatine. The formation of active methionine was found to require ATP, magnesium ions, glutathione (GSH), and a methionine-activating enzyme. The methylation of guanidoacetic acid is catalyzed by guanidoacetate methyltransferase. The glutathione is required for optimal activity of this enzyme. The creatine is present in muscle, brain, and blood as both phosphocreatine and in the free state. Creatinine is formed by an irreversible and nonenzymatic removal of water from creatine phosphate. The creatinine is eliminated via the kidneys and increased levels of creatinine in the bloodstream generally indicate kidney malfunction. The determination of creatinine by the Jaffé reaction has gained wide acceptance since Folin (3-6) introduced the colorimetric method for quantification of creatinine. Modified methods based on the Jaffé reaction have been and are still widely employed for the determination of creatinine (Table I).

The Jaffé reaction is plagued by numerous interfering substances which react with the alkaline picrate reagent. The following substances have been reported to interfere in the Jaffé reaction: acetoacetic acid (29,57-62), acetone

TABLE 1. Modifications of the Jaffé Reaction

Modifications	Measured Wavelength (nm)	Applicable Conc.	Ref.
Original Jaffé reaction			1
Colorimetric determination of creatinine			7
Determination of small quantities of creatinine	Duboscq colorimeter	Standards 0.5 1 and 2 mg %	8
Kaolin to adsorb creatinine	Duboscq colorimeter	0.200 and 1.00 mg standards	9
Adsorption of creatinine on Lloyd's reagent			10
Micromethod with Lloyd's reagent	525	0 - 2.0 mg %	11
Pulfrich step-photometer	530	2 - 40 mg %	12,13
$Al(OH_3)$ to remove interfering substances by adsorption			14
Micromethod using Fuller's earth for adsorption of creatinine	530	0.005-0.080 mg	15
Al_2O_3, medicinal charcoal, and talc to absorb arginine and creatinine. Elute creatinine with pyridine solution			16
Adsorption of creatinine on acid clay	filter S_{53}	0 - 3.2 mg %	17
Temperature studies 15-28°C			18
CCl_3COOH for protein precipitation			19

Time, temperature, pH, and optimal wavelength	520		20
Montomorillonite to adsorb creatinine			21
Improved deproteinization procedure			22
Optimal reaction time and color stability (25-30 min.)	475-90		23
Adaption to AutoAnalyser	505	0-10 mg %	24, 25-30
Dowex 50W ion-exchange resin			31
Glass capillary micromethod	500		32
Adsorption of creatinine on bentonite			33
Adsorption of creatinine on montomorillonite			34
Ultramicromethod			35
Automethod microchemical method	Selenphotoelement with a Schott OG filter		36
Addition of NaH_2PO_4 to increase absorbance of the creatinine-picrate complex	500		37
Creatinine adsorption on Montana A2 bleaching earth	480	05-3.0 mg/100 ml	38
10% Cl_3CCO_2H washing	546	20 mg %	39
Cation-exchange resin	510		40
Extraction of creatinine with tetraphenylborate in ether			41

Shift to higher wavelength to avoid interference	540		42
Deproteinizing serum and using LiOH as base	509		43
LiOH as base	520		44,51
Improved continuous flow system	505		45
Direct method without deproteinization		0.8-6 mg/100 ml	46
Microdetermination	492		47
AutoAnalyser	505	0-15 mg %	48
Automated microanalysis			49
Centrifugal fast analyzer	520	0-10 mg %	50
Picric acid concentration of 5.7 mM	492	0.5-5.0 mg %	52
Micromethod at optimal temp., alkali conc. and time reaction	509	0-10 mg %	53
Kinetic method	515	to 25 mg %	54
Kinetic microdetermination	510	to 11.3 mg %	55
Addition of Na-dodecyl-sulfate to form stable complexes with proteins	510	to 8 mg %	56

(1,29,57,63), acetylacetone (64), adrenalin (65), amino-hippurate (66), ascorbic acid (67), diacetic acid (68,69), ethyl acetoacetate (59,64,70), furfurol (64), glucose (1,71-78), glutathione (65), glycocyamidine (59,60,77-81), glycocyamine (77), histidine (65), hydantoin (59,60,81,82), hydroxylamine (64), levulinic acid (60,64), methylhydantoin (59,81,83), protein (66,76,84-86), pyruvic acid (62,64,84,87,88), resorcinol (89), tryptophan (65). Ferro-Luzzi and researchers (65) have reported tryptophan and histidine to interfere in the Jaffé reaction, while other authors have concluded that tryptophan (64,90,91) and histidine (64,90,92) do not interfere. Photometric estimations by Schormuller and Mohr (64) have concluded that histidine is practically Jaffé negative. Some authors have concluded that temperature and time effects Jaffé positive or negative results (1,59,60,64,82,93,94). Substances like adrenalin, ascorbic acid, diketopiperazine, dimethylguanidine hydrochloride, glucose, guanidine carbonate, and histidine have been shown to influence the kinetics of the reaction (95-98). Histidine and guanine have been shown to inhibit the Jaffé reaction (89,95) while glycylglycine anhydride was shown to enhance the reaction (89). The activating effect of adrenalin and ascorbic acid and the inhibiting effect of histidine and glucose were thoroughly

investigated by Richter and researchers (96-102).

In 1936, three groups of researchers (81,103,104) published methods on the determination of creatinine with alkaline 3,5-dinitrobenzoate. Modifications and improvements of this colorimetric determination were made by Andes (105), Franceschi (106), and by Jansen and researchers (107). Many researchers claimed that 3,5-dinitrobenzoic acid was more specific for creatinine. The work of Langley and Evans (104) showed a close correlation between the results of the alkaline 3,5-dinitrobenzoate reaction and the Jaffé reaction after adsorption of creatinine upon Lloyd's reagent. Although some researchers found the alkaline 3,5-dinitrobenzoate useful (108), others found it less suitable than picric acid. Low reactivity and color instability were disadvantages of the alkaline 3,5-dinitrobenzoate reaction with creatinine (64).

The following nitro substituted aromatic compounds have been shown to give a color reaction with creatinine: 1,3,5-trinitrobenzene (109), 2,4,6-trinitrotoluene (109), 2,4,6-trinitrobenzoic acid (109), 3,5-dinitrobenzene-sulfonate (110), and 2,2',4,4'-tetranitrobiphenyl (111). Polarographic evidence reveals that creatinine also reacts with 3,5-dinitrosalicylic acid (See PART II, this Dissertation) and with m-dinitrobenzene.

Alternate methods have been developed for the determination of creatinine. One such method is the conversion of creatinine to methylguanidine by o-nitrobenzaldehyde, followed by the Sakaguchi reaction (112-115). The method is highly specific, but too complicated for routine work (113). Sullivan and Irreverre (116) introduced a method which uses 1,4-naphthoquinone-2-potassium sulfonate to determine creatinine. Cooper and Biggs (113) concluded that this method gave spuriously high results. Stelgens and researchers (117) presented a method based on the reaction of creatinine with mercury thiocyanate, followed by mercury quantification with dithizone. Normal values of 0.7 mg % were reported using the mercury thiocyanate method (118). A turbidimetric method, by Barclay and Kenney (71), claimed high specificity employing a modified Nessler's reagent. A quantitative paper chromatography method has been reported by Paumgartner and researchers (119).

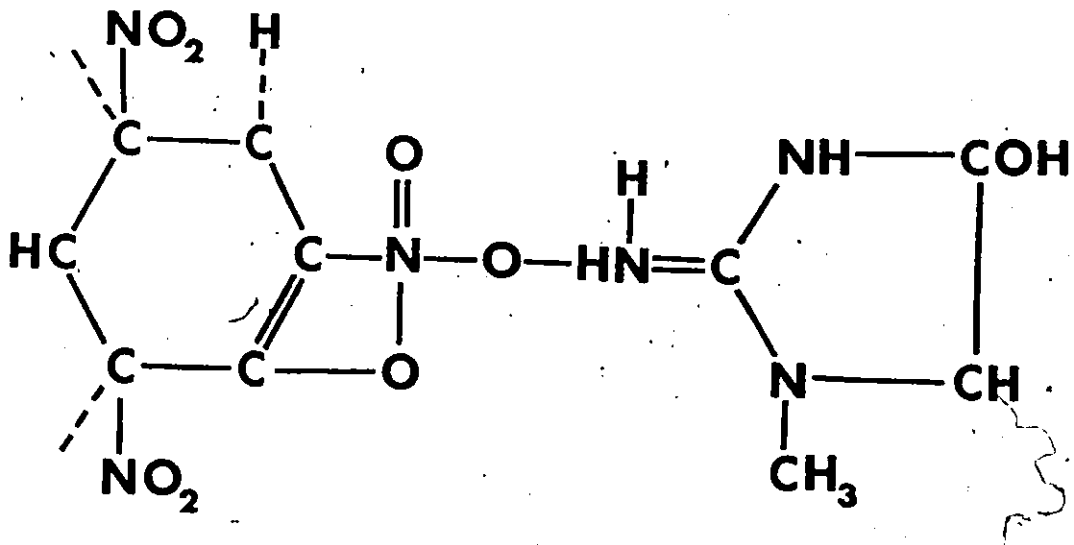
Fermentative destruction of creatinine was reported by Linneweh (120) in 1930. He concluded that putrefactive bacteria decompose creatinine to methylhydantoin with the elimination of NH_3 and then, by prolonged putrefaction to sarcosine. Simola (121) found that liver and kidney extracts of dogs and cattle did not contain creatinase. Miller and

Dubos (122) isolated several species of bacteria capable of decomposing creatinine. Their further work on determining creatinine by a specific enzymatic method (123,124) put an end to the controversy over the existence of creatinine in human blood. Other methods for determining creatinine using cultured soil bacteria have been published (125,126). Pseudomonas stutzeri (127), Pseudomonas roqueforti (128), Escherichia sarcosinogenum (129), and Pseudomonas aeruginosa (130) have been shown to assimilate creatinine. Recently, Wahlefeld and researchers (131) used the enzyme creatinine amidohydrolase in the determination of creatinine by difference of the Jaffe color (measured at 546 nm) produced before and after enzyme action at pH 7.5-9 and 37°C.

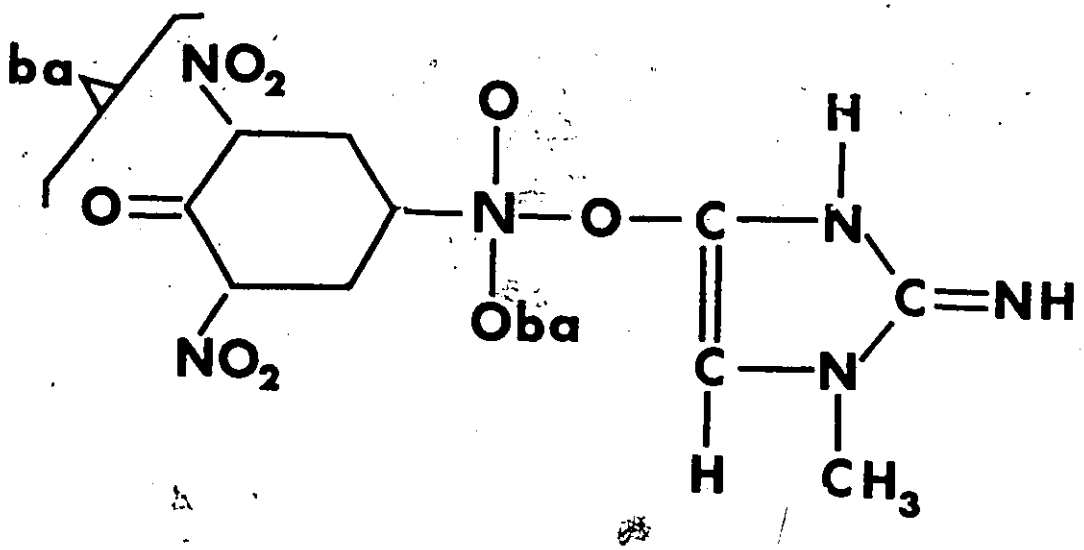
Amniotic fluid creatinine levels have been used to evaluate fetal maturity. Mischel (132) found a mean creatinine value of 5.49 mg % for over 200 human amniotic fluid samples which were examined after rupture of the membranes. Pitkin and Zwierek (133) examined both single tests and serial cases of amniotic fluid creatinine concentrations during the last half of pregnancy. In 94% of the cases, an abrupt rise of more than 2 mg % was observed after 37 weeks. They suggested that creatinine determinations might be useful for estimating fetal maturity. Some researchers have confirmed these

findings, (134-136) while others have considered amniotic fluid creatinine an unreliable index of fetal maturity (137).

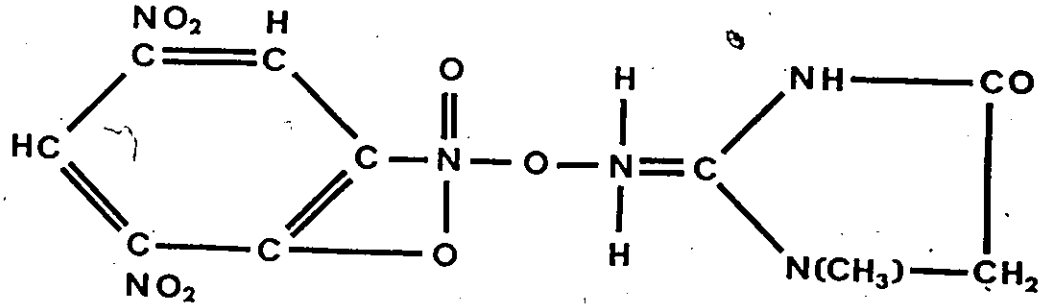
Chapman (138) reported that the red color formed during the Jaffe reaction is due to the sodium salts of picramic acid (monoamino-dinitrophenol) and diamino-nitrophenol. Greenwald and Gross (139) concluded that such an explanation was "inherently improbable". They in turn reported a procedure for isolating the red product as well as noting that when heated to 139°C, it was transformed into ordinary creatinine picrate. They concluded that only one mole of picric acid appears to be required for each mole of creatinine, although the reaction was not complete unless a considerable excess was present. The color was believed to be due to a tautomeric form of creatinine picrate (140). The following structure was proposed by Greenwald (140).



Greenwald (141) went on to hypothesize that all three nitro groups of picric acid undergo a change in the formation of the red tautomer of creatinine picrate. Greenwald (142) isolated an orange compound containing 2 molecules of creatinine, 1 of picric acid, 3 of sodium hydroxide and 3 of water. In another publication (143), he isolated the red compound which he found to be composed of 1 molecule of creatinine, 1 of picric acid, and 2 of sodium hydroxide. Weise and Tropp (82) studied numerous other substances which react with alkaline picrate. They concluded that Jaffe's reaction for creatinine is a special case of a general reaction for active methylene or methine groups. Anslow and King (144) isolated the barium salt of the Jaffé reaction product. They proposed the following structure for the red barium creatinine picrate:



In 1930, Greenwald (145) proposed the following structure for the yellow creatinine picrate:

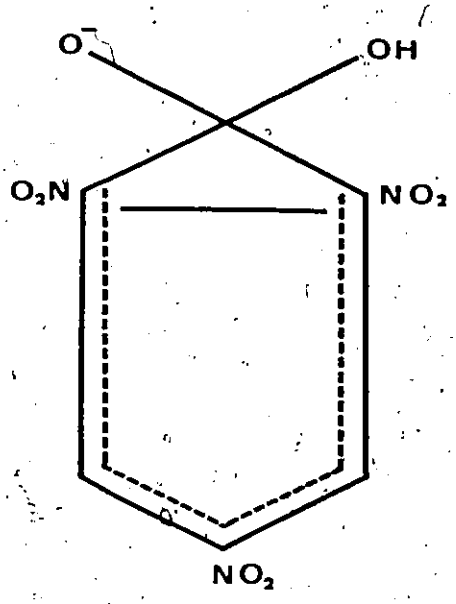


The red tautomer, or isomer was explained as a result of formation of the enolic form of creatinine. Bollinger (146) also concluded that enolization takes place in the creatinine molecule due to the presence of alkali. He isolated from an alcoholic medium, an orange-red crystalline compound which consisted of one molecule of creatinine, one of picric acid and two of sodium hydroxide. Acidification of the orange-red compound resulted in a red crystalline compound which turned orange upon heating to 130°C, yellow at 140°C, and melted at 206°C. Greenwald and Gross (139) noted similar properties of the red product. Bollinger (147) isolated four compounds which formed by varying the amounts of sodium hydroxide in an alcoholic medium of picric acid and creatinine. The compounds consisted of one molecule of picric acid, one molecule of creatinine and from two to three and a half molecules of

sodium hydroxide. The compound with the highest sodium hydroxide concentration was considered the most likely to represent the Jaffe reaction product.

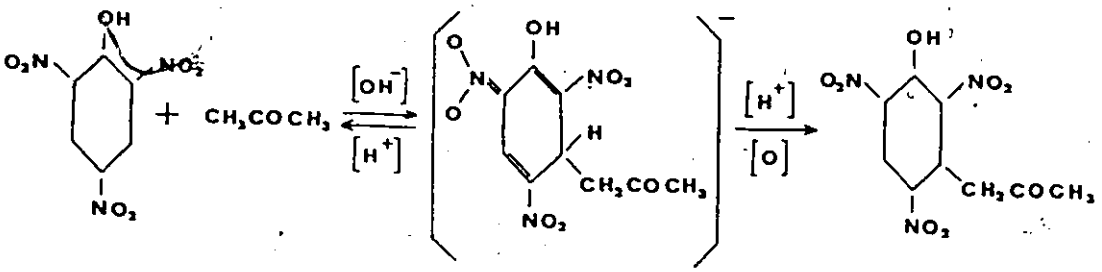
Greenwald and Gross (139) had noticed that if the Jaffe reaction was allowed to stand for 30 or more minutes, the unchanged picric acid could no longer be recovered quantitatively. Van Pilsum and researchers (148) examined the Jaffe reaction at longer reaction times and higher temperatures (30-50°C). They noted the formation of methylguanidine and a reduction product of picrate. Further evidence was presented by Archibald (149), in a study of changing optimal density with time at different temperatures.

Abe (150-152) investigated color formation of picric acid and 1,3,5-trinitrobenzene in the presence of sodium hydroxide. He attributes the red color to be due to the formation of complexes such as [trinitrobenzene $OH]^-$. The structure of the complex picrate anion OH^- was reported to be a Meisenheimer type (153). The following structure was attributed to the red chromogen formed by the interaction of picric acid with sodium hydroxide in water (152).

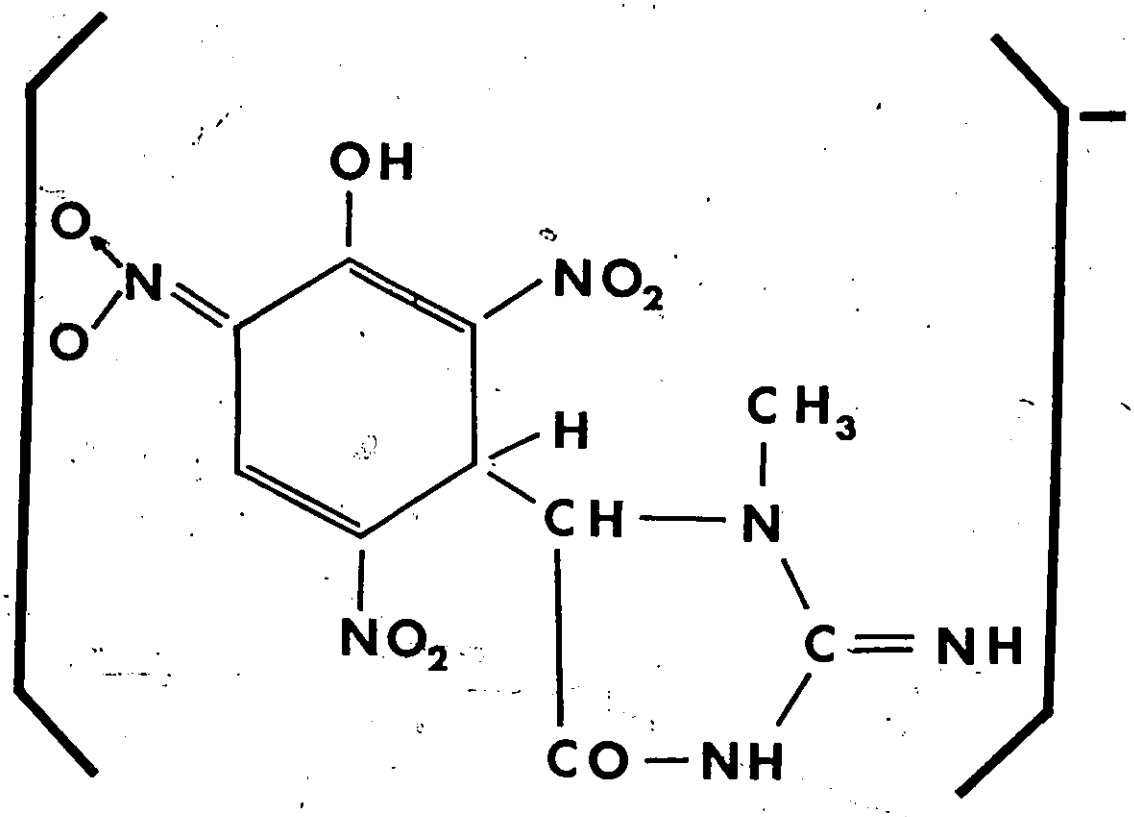


Since the creatinine molecule contains amine groups, examination of possible reactions of amine groups with nitro benzene compounds was reviewed in the literature. A number of theories on their interaction has arisen; a zwitterion was postulated by Lewis and Seaborg (154); weak-electron interaction was proposed by Miller and Wynne-Jones (155); complete electron transfer was also postulated (155); and, a less likely possibility of transfer of a ring-proton has also been proposed (156,157). Reactions of 2,4,6-trinitrobenzenesulfonate with amines and hydroxide ion were investigated by Means and coworkers (158). Charged Meisenheimer-type complexes have also been proposed by Buncel and Webb (159).

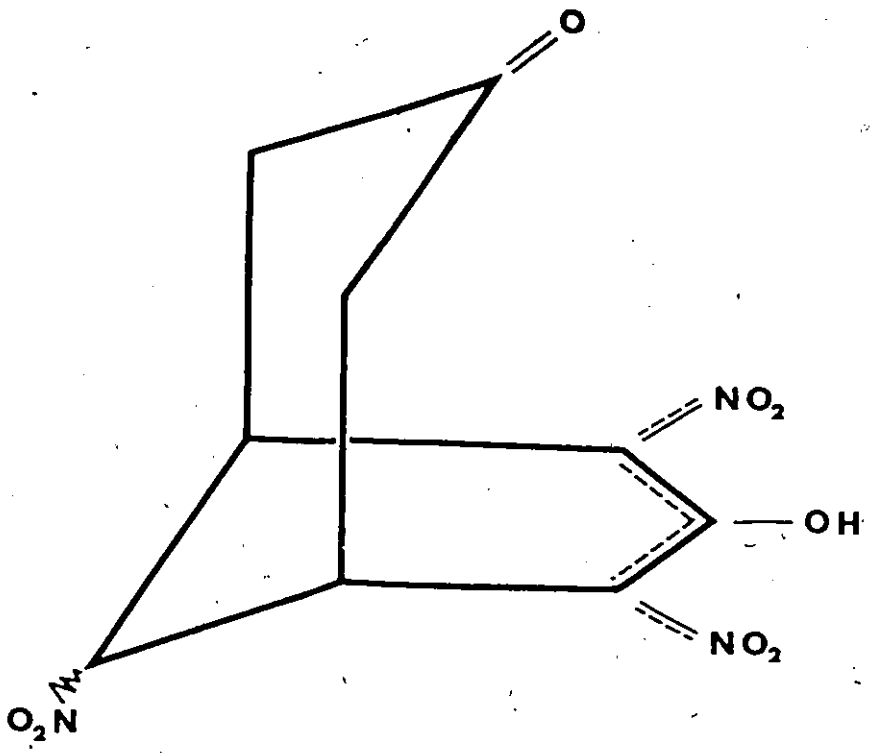
In 1955, Kimura (160) investigated the reaction between picric acid and active methylene compounds in the presence of alkali. Comparison of the chromogens observed for the Jaffé reaction, the Baljet reaction (picric acid with the cardiac glycoside digitoxigenin), and the reaction of picric acid with acetone revealed absorption spectra which closely resembled one another. For the latter reaction between picric acid and acetone in an alkaline medium, Kimura (160) proposed the principal reaction to be as follows:



He then went on to propose "probable structures of the colored complex anions" for the Jaffé reaction and the Baljet reaction. Kimura proposed the following chemical structure for the red chromogen of the Jaffé reaction:

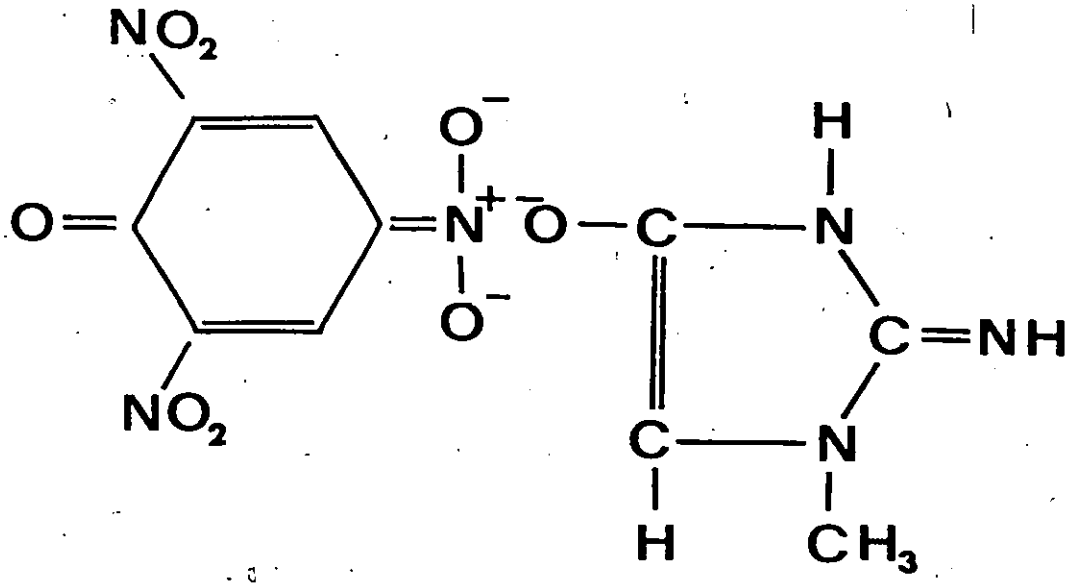


Further investigation of acetone with alkaline 1,3,5-trinitrobenzene by Kohashi et al. (161,162) revealed the formation of three types of compounds. The three major coloring matters were shown to be: a Meisenheimer type; a bicyclic type; and a tetracyclic type. The following structure was attributed to be the bicyclic compound.



Investigation of the product of the reaction between acetone and alkaline picrate revealed a similar bicyclic type of compound (163).

In 1969, Seelig (164) showed that spectrophotometric and chromatographic studies of the Jaffé reaction product do not compare with the frequently suggested picramic acid. He believed it to be an addition complex of one molecule of creatinine and one molecule of picric acid. Seelig and Wüst (165) proposed the following structure:



This structure was based on spectrophotometric evidence and takes into account the labile nature of the red Jaffé product, which was also observed by previous researchers.

Creatinine values generally determined by the Jaffé reaction, have been useful in determining kidney function, as a routine screening and as an index of renal homograft rejection (166). Further indication of renal function has been obtained by measuring creatinine clearance values (167). Measurement of creatinine in amniotic fluid has been used as an index of fetal maturity. Although the body fluid examined in the aforementioned tests changes from serum, to urine, and finally to amniotic fluid, the test for creatinine

which is generally employed is a modification of the original Jaffé reaction. Many researchers have investigated the mechanism of the Jaffé reaction and a number of structures have been proposed, but much controversy still exists as to the structure of the red Jaffé chromogen. Investigation of the mechanism of this reaction was undertaken because of the wide use and importance of the Jaffé reaction in clinical chemistry. It is hoped that solving the mechanism will aid in finding a more specific reagent for the determination of creatinine.

CHAPTER II

EXPERIMENTAL

A. Isolation of Jaffé Reaction Products

Materials and Methods

Reagent grade creatinine was purchased from Fisher Scientific Company, Don Mills, Ontario, Canada. The sodium hydroxide was "Baker Analyzed" Reagent grade from J. T. Baker Chemical Co., Phillipsburg, N. J. Reagent picric acid crystals (under water) were purchased from Allied Chemical of Morristown, New Jersey. A Fisher-Johns Melting Point Apparatus from Fisher Scientific Company, Don Mills, Ontario, Canada, was used to determine the melting point of the red Jaffé chromogen.

The red creatinine picrate was prepared by the procedure of Anslow and King (144). Creatinine, 5 g, was dissolved in 50 ml of distilled water. Picric acid, 10 g was dissolved in 200 ml of boiling distilled water. The picric acid solution was added to the dissolved creatinine and the solution was mixed. At this point a yellow precipitate was observed. To this viscous mixture, 50 ml of a cold solution of sodium hydroxide were added with mixing. The yellow precipitate dissolved with the appearance of a deep-red solution. The red solution was cooled in an ice bath and

50 ml of cold concentrated hydrochloric acid were added. The red chromogen of the Jaffé reaction precipitated and was collected by filtration and dried by rotary evaporation.

The yellow precipitate of creatinine and picric acid was prepared by mixing the aforementioned creatinine and picric acid solutions. The resulting yellow precipitate was cooled, filtered, and dried using a rotary evaporator.

Preparation of the orange precipitate was similar to the red, except that 15 g of creatinine were dissolved. During the acidification step, the concentrated acid was added very slowly. Crystals were separated by filtration of the cold solution and dried in the rotary evaporator.

B. Spectrophotometric Examination of the Jaffé Reaction Products

Materials and Methods

A Beckman DB-GT Grating Spectrophotometer with a Beckman 10" recorder (Beckman Instruments Inc., Toronto, Ontario) was employed in the spectrophotometric studies. Spectrophotometer cells with a 2 mm light path were purchased from Hellma Cells Inc., Toronto, Ontario.

A solution containing the red Jaffé color was prepared for spectrophotometric examination. Creatinine, 0.0148 g, was dissolved in 5.0 ml of distilled water. This solution

was mixed with 25 ml of aqueous picric acid, which was prepared by adding 0.030 g of picric acid to 25.0 ml of distilled water. Thirty milliliters of 0.5 N sodium hydroxide were added resulting in a reddish orange colored solution. The resulting pH of this solution was 12.94. Spectra were recorded on a Beckman DB-GT recording spectrophotometer. The pH was altered by titrating with 0.50 N hydrochloric acid. Spectra were recorded at pH values of 12.94, 9.52, 7.2, and 5.35. The solution was then backtitrated with 0.50 N sodium hydroxide. Spectra of the backtitration were recorded at pH values of 9.48, 10.78, 11.9, and 12.0.

C. Polarographic Examination of the Jaffé Products

Materials and Methods

Nitrogen 99.996% pure was purchased from Liquid Carbonic of Canada Ltd. Triple distilled mercury was obtained from Engelhard Industries of Canada Ltd.

A Sargent (Sargent-Welch Scientific Company) Model XVI Polarograph was employed for this investigation. Polarographic reduction was carried out in a ten-milliliter Heyrovsky cell. The characteristics of the capillary used were: $m = 1.767$ mg s^{-1}; $t = 4.68$ sec; $m^{2/3}t^{1/6} = 1.890$ $mg^{2/3}s^{-1/2}$. The height of the mercury column was 7.15 cm.

The cell was placed in a water bath maintained at 25 ± 0.1°C with a Haake Model ED "UNITHERM" constant temperature circulator.

Polarographic examination of the yellow creatinine-picric precipitate (isolated as described under EXPERIMENTAL Part A) was undertaken by placing 4.0 mg of the yellow precipitate into a 10-ml volumetric flask. To the flask, 2.0 ml of a 5 g/100 ml aqueous solution of sodium chloride were added to act as a supporting electrolyte during the polarographic reduction of the yellow precipitate. The volumetric flask was brought to volume with deionized-distilled water and the resulting yellow solution was mixed. A volume of 5.0 ml of this solution was placed into a Heyrovsky cell which was in a 25°C water bath. The cell was deaerated for a period of 20 min with nitrogen prior to polarography.

Polarographic analysis of the red Jaffé precipitate (isolated as described under EXPERIMENTAL Part A) was undertaken by weighing out 0.0050 g of the red precipitate into a 10-ml volumetric flask. A volume of 4.0 ml of 0.5 N sodium hydroxide was added to the flask and the volumetric flask was then filled to volume with deionized-distilled water. The resulting solution was mixed and 5.0 ml were transferred by pipet to a 5-ml Heyrovsky polarographic cell. The cell

was placed into the 25°C water bath and deaerated for a period of 15 min with nitrogen prior to polarography.

D. Polarographic and Spectrophotometric Studies of the Reaction of Creatinine with m-Dinitrobenzene

Materials and Methods

Polarographic examinations were performed using a Sargent Model XVI Polarograph with the previously described capillary. Polarography was carried out in a 10-ml Heyrovsky cell which was placed in a water bath maintained at 25 ± 0.1°C by a Haake Model ED "UNITHERM" constant temperature circulator.

An 8N sodium hydroxide solution was prepared by adding 32.0 g of sodium hydroxide pellets to a 100-ml volumetric flask which is filled to volume with distilled water. A standard solution of m-dinitrobenzene was prepared adding 0.500 g of m-dinitrobenzene into a 500-ml volumetric flask which is filled to volume with absolute ethanol. A volume of 0.2 ml of the sodium hydroxide solution is added to a 10-ml volumetric flask. To this flask, 0.1 ml of standard m-dinitrobenzene was added. The flask was then filled to volume with distilled water and mixed well. A 5-ml aliquot was pipetted into the Heyrovsky cell, which was placed into the 25°C water bath. A 15-min deoxygenation was carried out

using 99.996% nitrogen (Liquid Carbonic), which was passed through one wash bottle containing distilled water. Blank polarograms were determined on this solution. Solid creatinine, 0.020 g, was added to the Heyrovsky cell. The resulting solution was mixed by deoxygenating with nitrogen for a period of 5 min. Polarograms were run over a 3-hour period, during which a gradual decrease was observed for the diffusion current of the two reduction waves of the m-dinitrobenzene. Spectrophotometric spectra of the yellow solution were run against its blank on a Beckman DB-GT recording spectrophotometer.

E. pH Titration Studies of the Jaffe Reaction Products and Related Compounds

Materials and Methods

Picric acid, 0.0300 g, was dissolved in 60 ml of distilled water in a 150-ml beaker. Titration of picric acid was with 0.1016 N sodium hydroxide.

Creatinine, 0.01481 g, was dissolved in 40 ml of distilled water and pH titration was with 0.1016 N sodium hydroxide.

Creatinine, 0.01481 g, was dissolved in 40 ml of distilled water and acidified with 0.10 ml of concentrated hydrochloric acid. A pH titration was carried out by titration with

0.1016 N sodium hydroxide.

The yellow precipitate of creatinine-picric acid, 0.0448 g, was dissolved in 40 ml of distilled water and the resulting solution was titrated with 0.1016 N sodium hydroxide.

The red precipitate of creatinine-picrate, 0.0448g, was dissolved in 40 ml of distilled water. A volume of 14 ml of 0.5 N sodium hydroxide was added with mixing and the resulting solution was titrated with 0.1169 N hydrochloric acid.

F. Reactivity of Alkaline Picrate with Hydantoin and 5,5-Dimethylhydantoin

Materials and Methods

Hydantoin and 5,5-dimethylhydantoin were purchased from Sigma Chemical Company, St. Louis, Missouri.

A standard hydantoin solution was prepared by adding 0.150 g of hydantoin to a 10-ml volumetric flask and diluting to volume with distilled water. A standard 5,5-dimethylhydantoin solution was prepared by adding 0.150 g of 5,5-dimethylhydantoin to a 10-ml volumetric flask which was filled to volume with distilled water. A standard solution of picric acid was prepared by adding 0.3238 g of dry picric acid to a 50-ml volumetric flask which was filled to volume

with distilled water. A spectrophotometer blanking solution was made by adding 2.0 ml of the picric acid standard solution to 2.0 ml of 0.50 N sodium hydroxide and finally, adding 0.1 ml of distilled water. Test solutions of hydantoin and 5,5-dimethylhydantoin were prepared by adding 0.1 ml of the respective standard solution in place of the 0.1 ml of the water used in the blank. Spectra of hydantoin and 5,5-dimethylhydantoin were prepared using a Beckman DB-GT recording spectrophotometer.

CHAPTER III

RESULTS

A. Isolation of Jaffé Reaction Products

Determination of the Melting Point of the Jaffé Product

The yellow product of creatinine-picric acid, the red product of creatinine-alkaline picrate, and the orange creatinine-alkaline picrate were isolated in crystalline form.

The red product was examined by heating on a Fisher-Johns Melting Point Apparatus. The red compound started to lose its brilliant red color above 160°C. It turned orange at about 170°C, then yellow at about 190°C, a very pale yellow resulted above 195°C with a melting point just below 210°C.

The red product was highly insoluble in most solvents. Other solvents like acetone and isobutylamine could not be used because of further chemical reactions. When small amounts of the red precipitate were dissolved in solvents like water and nitrobenzene, a yellow color resulted. The orange product also turned yellow when dissolved in water.

B. Spectrophotometric Examination of the Jaffé Reaction Products

Spectra of the red Jaffé reaction product can be seen in Fig. 2 and 3. The plateau in the spectrum of the Jaffé-

FIGURE 2

SPECTRA OF THE JAFFÉ REACTION DURING ACID TITRATION

Legend

Spectra of the Jaffé reaction during titration with dilute hydrochloric acid are depicted. Refer to text for complete details.

FIGURE 2

SPECTRA OF THE JAFFÉ REACTION DURING ACID TITRATION

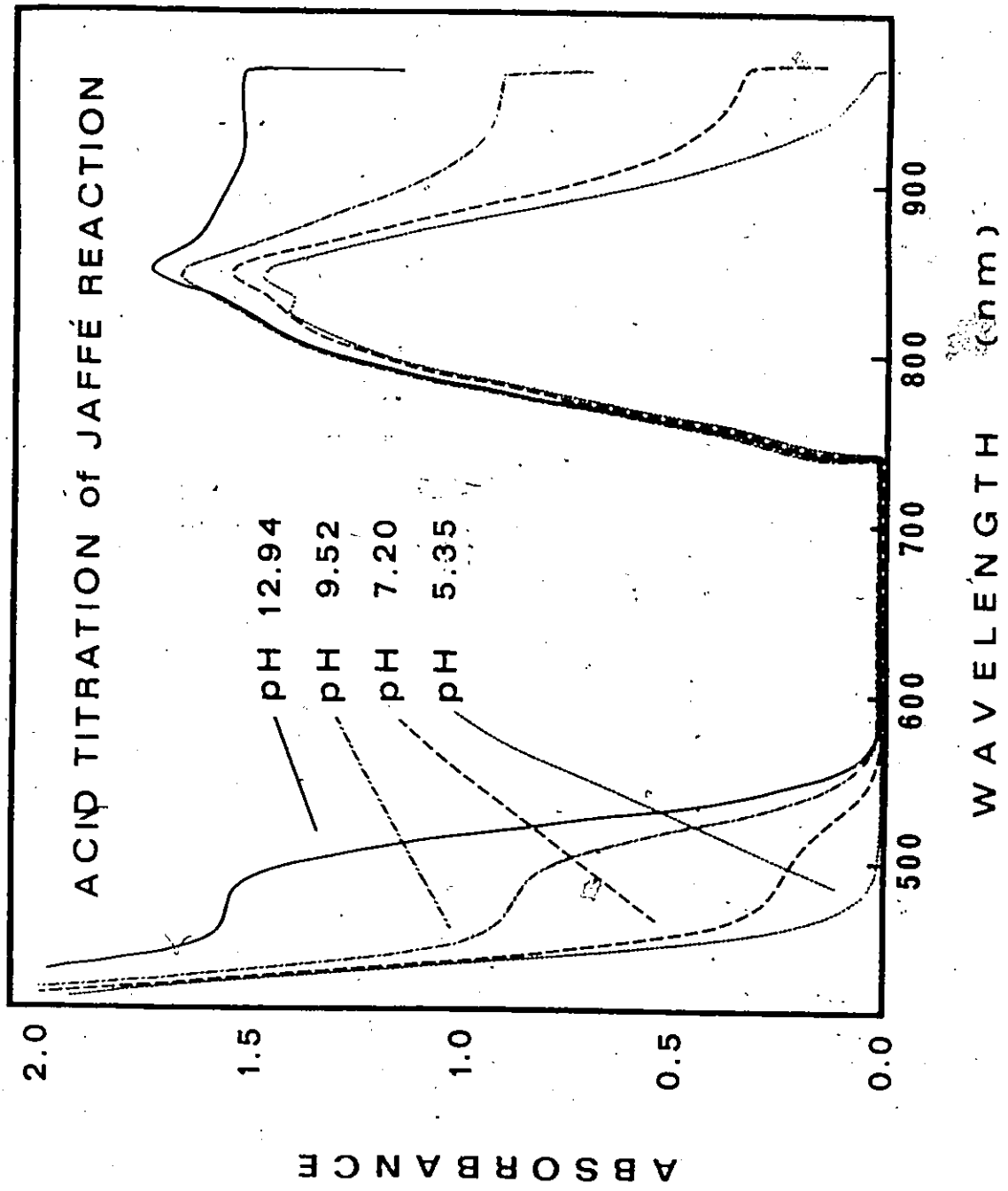


FIGURE 3

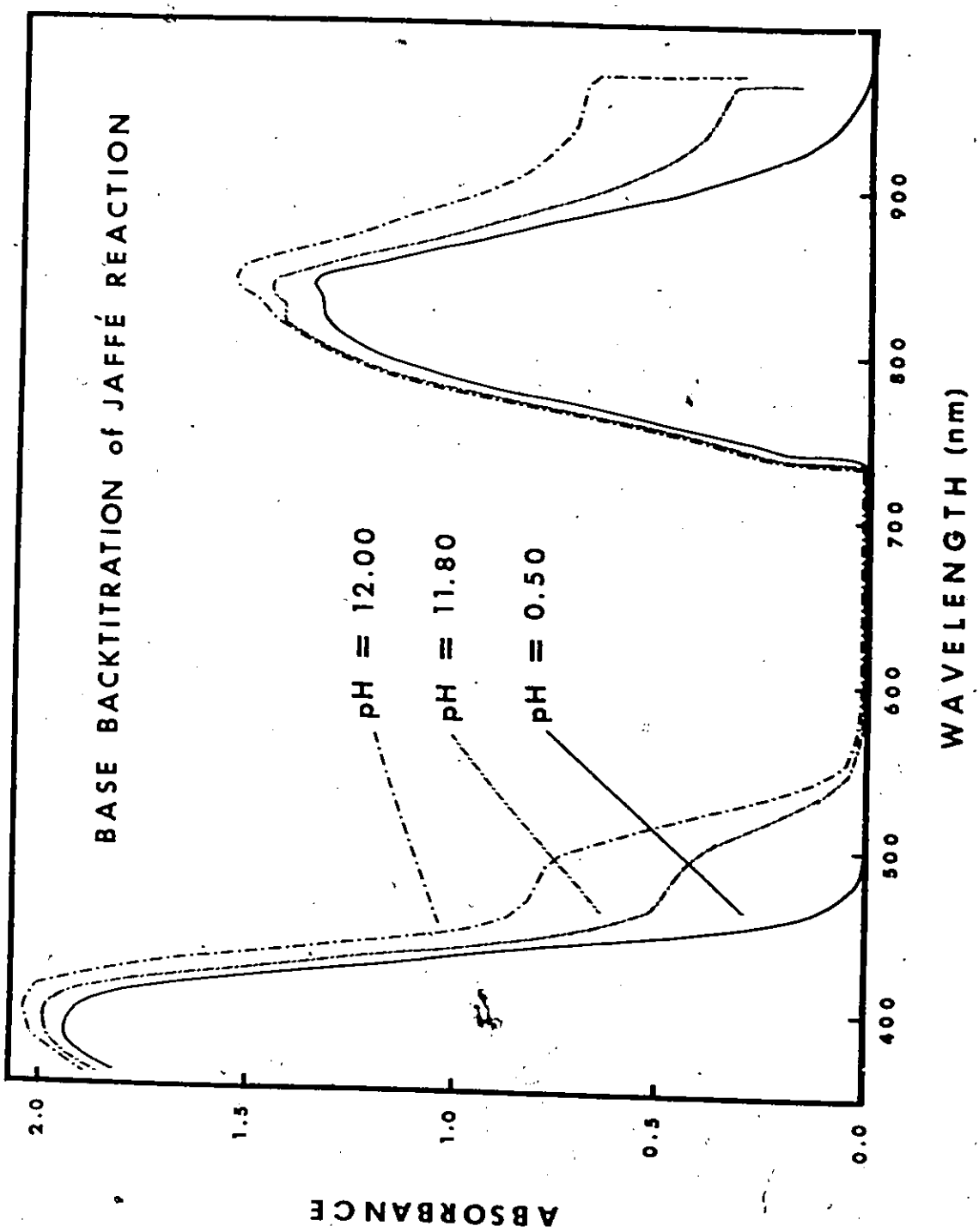
SPECTRA OF THE ACIDIFIED JAFFÉ REACTION DURING BASE BACKTITRATION

Legend

Spectra of the acidified Jaffé reaction during back-titration with dilute sodium hydroxide are depicted. Refer to text for complete details.

FIGURE 3

SPECTRA OF THE ACIDIFIED JAFFE REACTION DURING BASE BACKTITRATION



chromogen, between 470-525 nm, disappeared during a dilute hydrochloric acid titration. At a pH of 5.35, the wave completely disappeared. A base titration of an acidified solution results in the reappearance of the Jaffé absorption wave (Fig. 3).

C. Polarographic Examination of the Jaffé Products

Polarographic examination of the yellow precipitate of creatinine and picric acid revealed a large decrease in the first reduction wave of picric acid (Fig. 4).

Polarography of the red precipitate of the Jaffé reaction resulted in a three-step reduction wave which was almost identical to that obtained for the polarographic reduction of picric acid.

D. Polarographic and Spectrophotometric Studies of the Reaction of Creatinine with m-Dinitrobenzene

After the addition of creatinine to the alkaline m-dinitrobenzene, a gradual decrease was observed in the diffusion current of both nitro reduction waves of m-dinitrobenzene. The solution which remained in the 10-ml volumetric flask was used as a blank. The blank solution was clear and colorless while the solution containing the creatinine turned yellow with time. Over a 3-hour period,

FIGURE 4

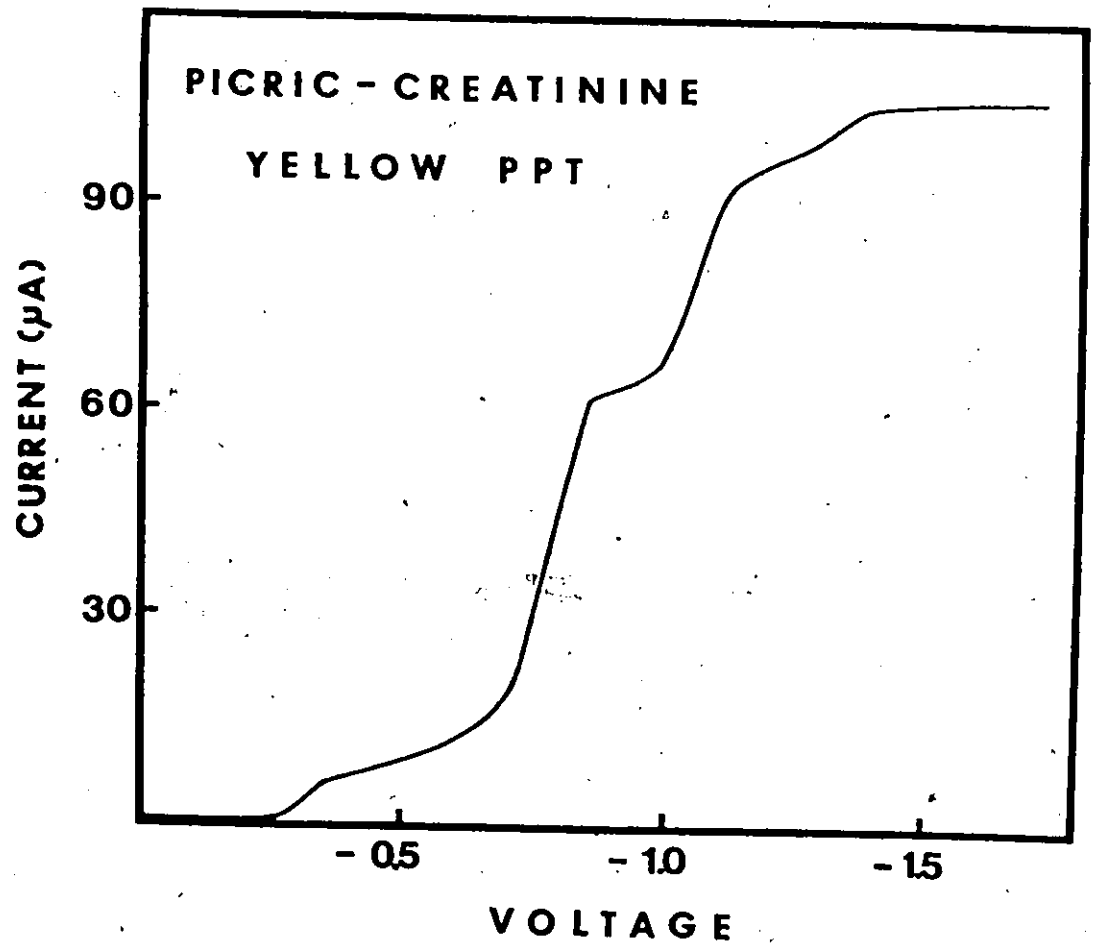
POLAROGRAM OF THE YELLOW PRECIPITATE OF PICRIC ACID AND CREATININE

Legend

This is a typical polarogram of the yellow precipitate of picric acid and creatinine. Voltage is expressed versus mercury pool.

FIGURE 4

POLAROGRAM OF THE YELLOW PRECIPITATE OF PICRIC ACID AND CREATININE



during which a gradual decrease was observed for the diffusion current of the two reduction waves of the m-dinitrobenzene. Spectrophotometric spectra of the yellow solution were run against its blank on a Beckman DB-GT recording spectrophotometer.

E. pH Titration Studies of the Jaffé Reaction Products and Related Products

The pH titration curves for picric acid, creatinine, an acidified sample of creatinine, the yellow precipitate of creatinine-picric acid, and the red Jaffé reaction product, can be viewed in Figs. 5 - 9, respectively.

F. Reactivity of Alkaline Picrate with Hydantoin and 5,5-Dimethylhydantoin

The spectra of hydantoin reacting with alkaline picrate can be seen in Fig. 10. Under identical conditions, 5,5-dimethylhydantoin does not react with alkaline picrate (Fig. 10). The spectra of hydantoin are very similar to the spectra of the red Jaffé chromogen (Fig. 2).

FIGURE 5

pH TITRATION CURVE OF PICRIC ACID

Legend

Depicted is a pH titration curve for picric acid when titrated with dilute NaOH. Refer to text for details.

FIGURE 5

pH TITRATION CURVE OF PICRIC ACID

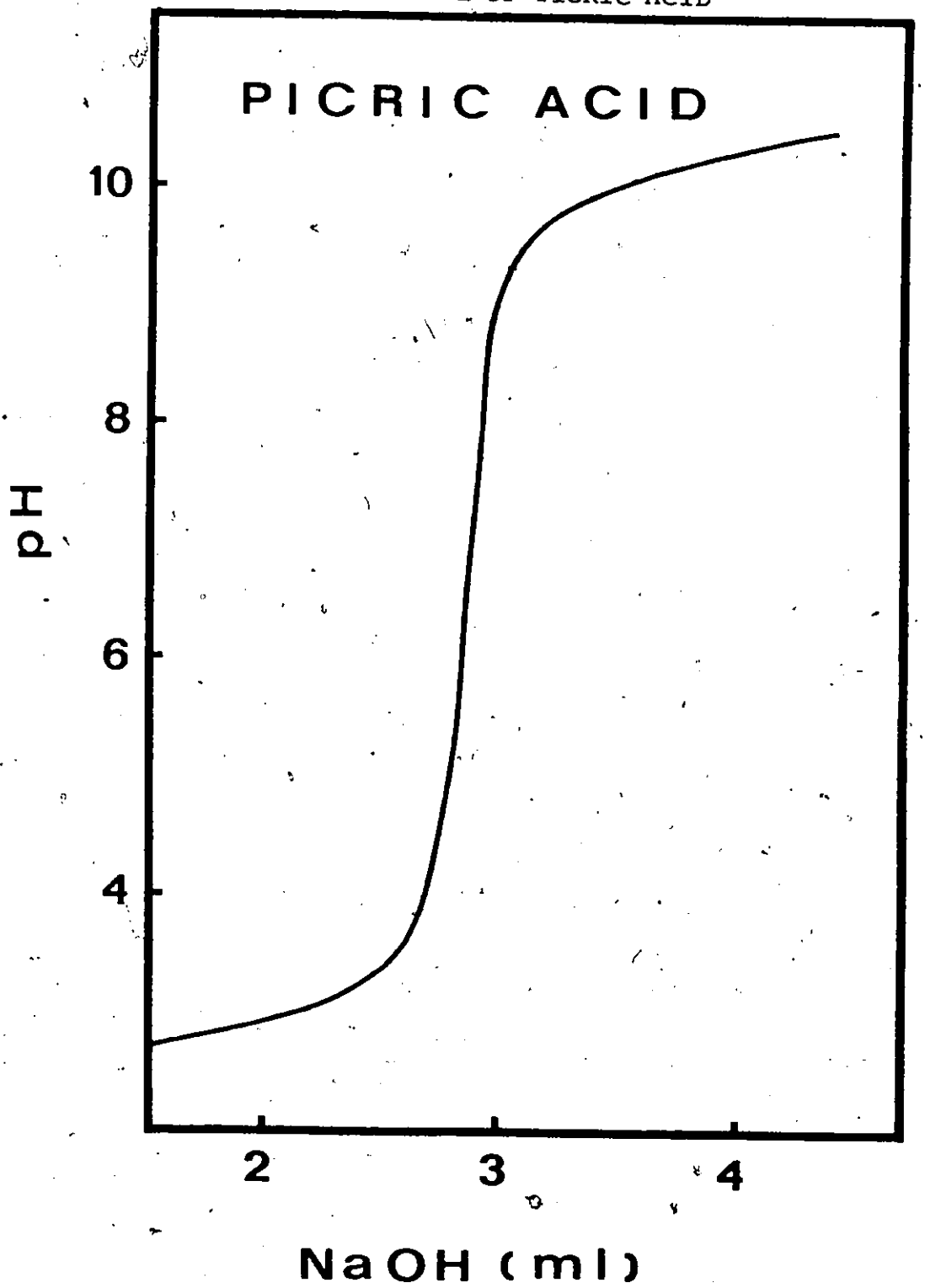


FIGURE 6

BASE TITRATION OF CREATININE

Legend

Creatinine was titrated with 0.102N NaOH. Refer to text for titration details.

FIGURE 6

BASE TITRATION OF CREATININE

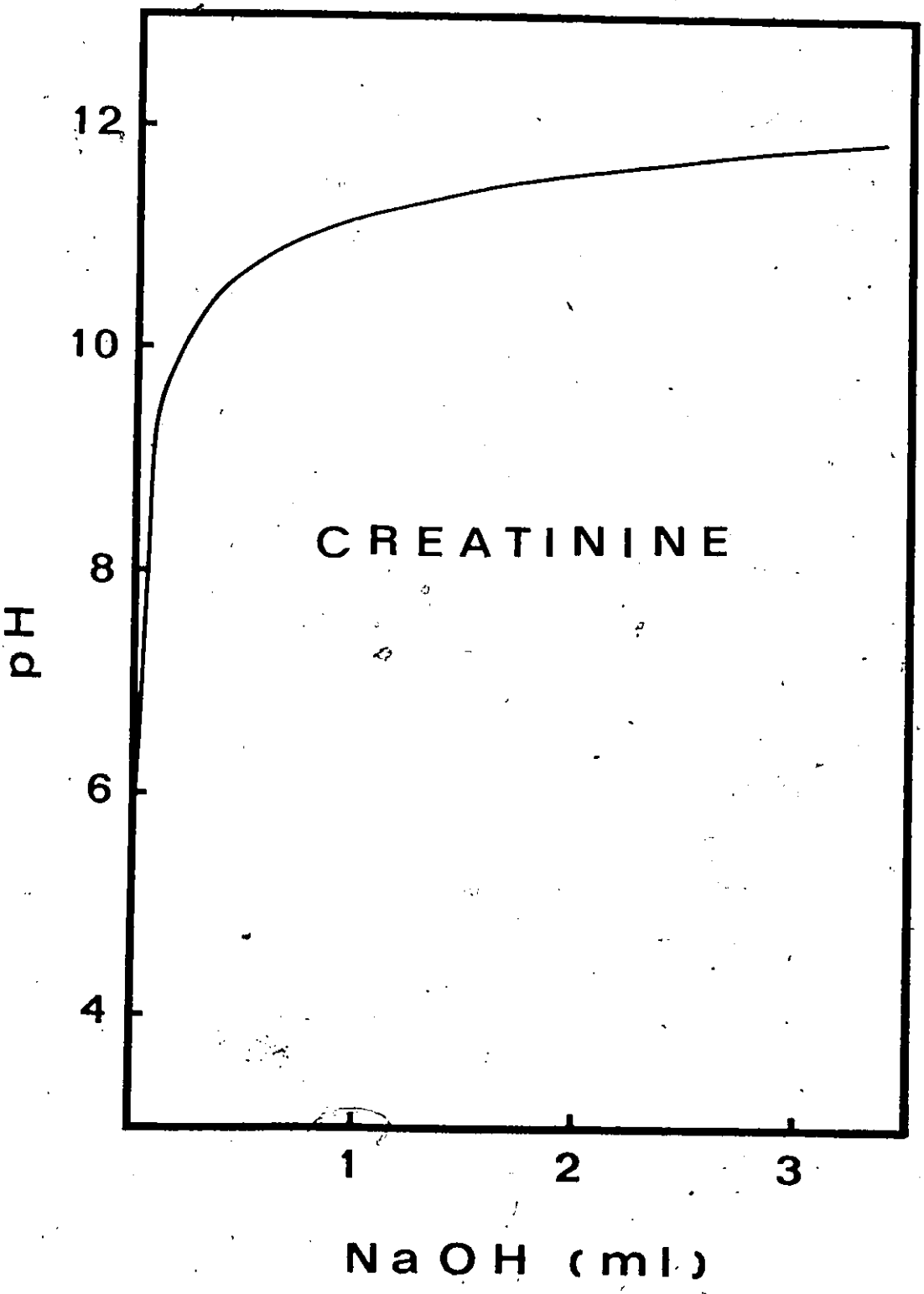


FIGURE 7

BASE TITRATION OF CREATININE IN DILUTE HCl

Legend

Depicted is a pH titration curve for a weakly acidified creatinine solution which is titrated with 0.1016N NaOH. Refer to text for titration details.

FIGURE 7

BASE TITRATION OF CREATININE IN DILUTE HCl

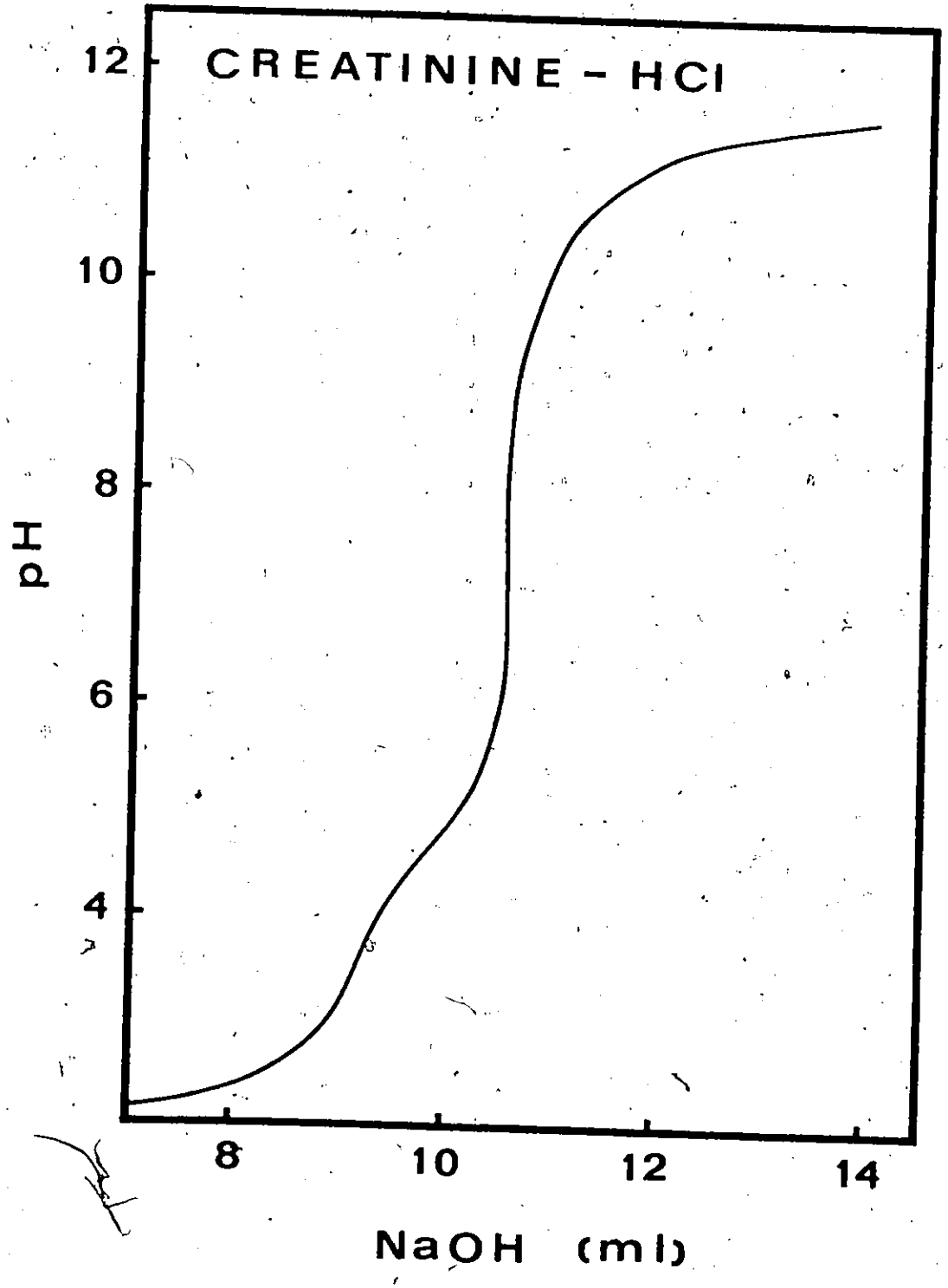


FIGURE 8

pH TITRATION CURVE FOR THE YELLOW PRECIPITATE OF PICRIC ACID AND CREATININE

Legend

Depicted is a pH titration curve for the yellow precipitate of picric acid and creatinine when titrated with dilute NaOH. Refer to text for details.

FIGURE 8

pH TITRATION CURVE FOR THE YELLOW PRECIPITATE OF PICRIC ACID AND CREATININE

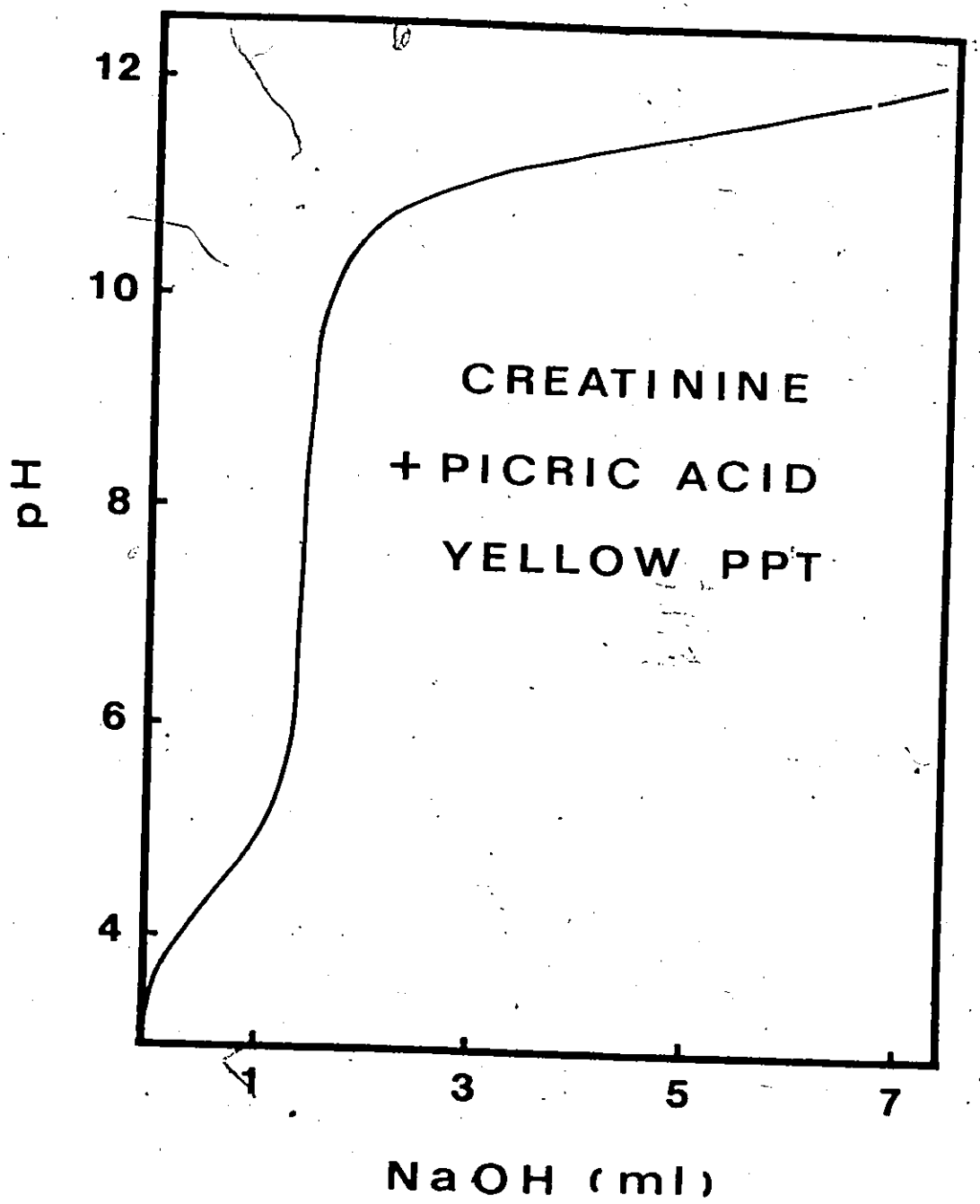


FIGURE 9

ACID pH TITRATION OF THE RED JAFFE PRODUCT

Legend

Depicted is a pH titration curve of the red Jaffe product. The red precipitate of the Jaffe reaction was dissolved in an aqueous solution of dilute sodium hydroxide and back-titrated with dilute hydrochloric acid. Refer to text for complete details.

FIGURE 9

ACID pH TITRATION OF THE RED JAFFE PRODUCT

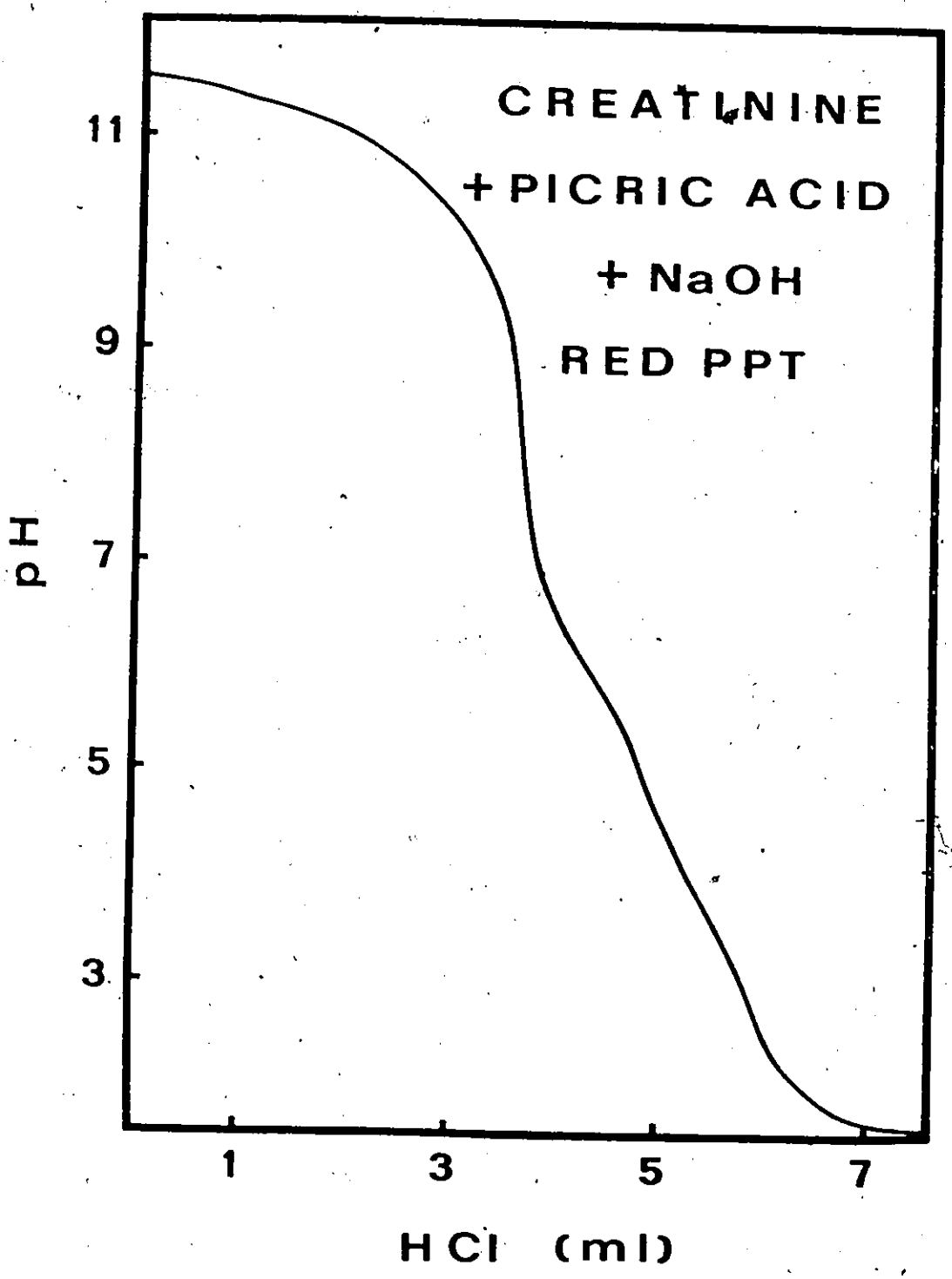


FIGURE 10

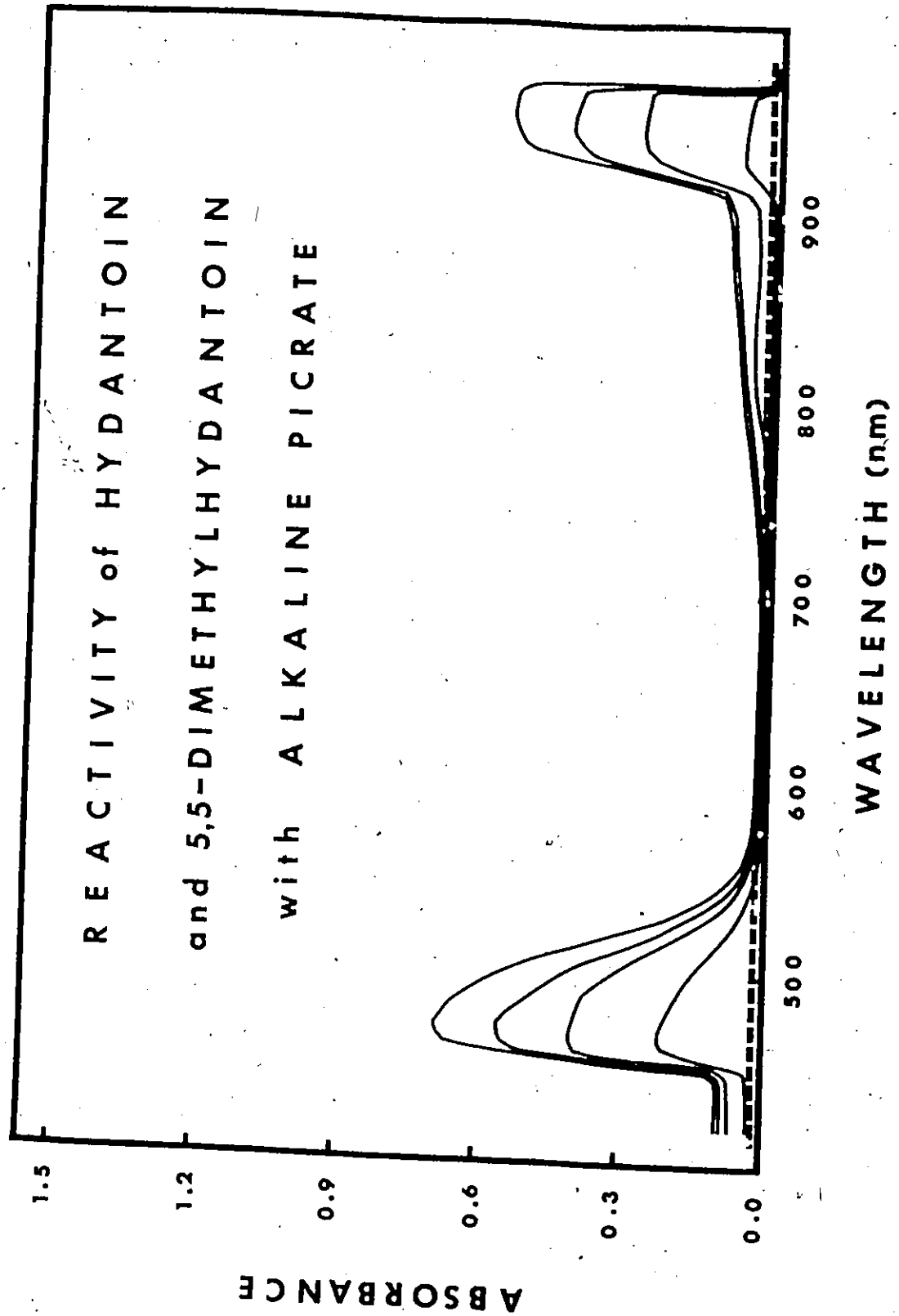
REACTIVITY OF HYDANTOIN AND 5,5-DIMETHYLHYDANTOIN WITH ALKALINE PICRATE

Legend

Spectra of hydantoin reacting with alkaline picrate are depicted. A spectrum of 5,5-dimethylhydantoin in the presence of alkaline picrate is also shown. Refer to text for details.

FIGURE 10

REACTIVITY OF HYDANTOIN AND 5,5-DIMETHYLHYDANTOIN WITH ALKALINE PICRATE



CHAPTER IV

DISCUSSION

A. Isolation of Jaffé Reaction Products

Unlike other procedures examined, the modified Anslow and King (144) procedure for isolating the red Jaffé reaction product was very useful. The rotary evaporator which was used to remove the moisture after the filtering step, proved very efficient. Fast elimination of moisture was necessary because of continued reaction. When properly carried out, a brilliant red precipitate was obtained. If allowed to stand moist and exposed to air, a dull dark red to brownish colored precipitate resulted.

B. Spectrophotometric Examination of the Jaffé Reaction Products

The superimposed spectra (Fig. 2) of the acid titration of the Jaffé chromogen at various pH levels, clearly shows the disappearance of the 470-525 nm wave. Similarly, the reappearance of the wave upon adding base is distinctly shown in Fig. 3. This difference easily lends itself to the keto and enol explanation proposed by Greenwald (145) and supported by Bollinger (146).

C. Polarographic Examination of the Jaffé Products

Polarographic examination of the yellow precipitate of creatinine and picric acid revealed that the first nitro reduction wave of picric acid had disappeared. Examination of the red product, alkaline picrate-creatinine, revealed a three-wave pattern normally found for picric acid in an alkaline medium. Polarographic investigation of the formation of the red Jaffé chromogen shows the disappearance of the first reduction wave of picric acid (See PART II, this Dissertation). The initial disappearance of the nitro reduction wave and its reappearance after base treatment of the acid precipitated compound, is an indication of a permanent bond between alkaline picrate and creatinine. The regeneration of the nitro reduction wave and reappearance of the red Jaffé color, indicate that the nitro group of picric acid is not bound as the theories of Greenwald (140) and Anslow and King (144) suggest.

D. Polarographic and Spectrophotometric Studies of the Reaction of Creatinine with m-Dinitrobenzene

Both polarographic and spectrophotometric evidence indicate a reaction between creatinine and m-dinitrobenzene under alkaline conditions. A decrease in the diffusion current

of the nitro reduction waves of m-dinitrobenzene was observed as the reaction time progressed. After about 3 hours, a decrease of over 50% in the diffusion current was observed for both reduction waves. The blank solution remained clear and colorless, while the test solution turned yellow.

E. pH Titration Studies of the Jaffé Reaction Products and Related Products

The upper portions of the pH titration curves of creatinine, the yellow product of creatinine and picric acid, and the red Jaffe product , proved almost identical. This is an indication that the guanidino group of creatinine is not bound in the red Jaffé product, nor is it bound in the yellow product of creatinine and picric acid.

F. Reactivity of Alkaline Picrate with Hydantoin and 5,5-Dimethylhydantoin

Hydantoin, a molecule which is similar to creatinine, reacts with picrid acid under alkaline conditions. Spectra of the hydantoin alkaline picrate reaction (Fig. 10) are very similar to the Jaffé reaction spectra (Fig. 2). The 5,5-dimethylhydantoin does not form a colored reaction product with alkaline picrate. This clearly implicates

involvement of the methylene group of hydantoin in the reaction with alkaline picrate. The similarity in spectra as well as the structural similarities between hydantoin and creatinine indicate that the reactive site of creatinine involves the methylene group.

CHAPTER V

SUMMARY AND CONCLUSIONS

Creatinine has been shown to react with 3,5-dinitrobenzoic acid (81, 103, 104), 1,3,5-trinitrobenzene (109), 2,4,6-trinitrotoluene (109), and 2,4,6-trinitrobenzoic acid (109). The polarographic investigation reported in this dissertation confirms the reaction of creatinine with picric acid. Further polarographic examination showed that creatinine can also react with 3,5-dinitrosalicylic acid and with m-dinitrobenzene. Reaction of acetone with picric acid and 1,3,5-trinitrobenzene has been widely investigated (160-163). Kimura (160) proposed attack of the acetone anion at the meta position of picric acid. Under special conditions bicyclic and tetracyclic reaction products of 1,3,5-trinitrobenzene and acetone have been formed (163). These reactions, in particular, the spectrophotometric and polarographic results reported in the present study of creatinine with m-dinitrobenzene indicate that the attack of creatinine is at the meta position of the picric acid molecule.

Greenwald and Gross (139) were the first to isolate a compound composed of two molecules of creatinine with one

of picric acid. Polarographic analysis of the 2:1 reaction product reported here, shows the disappearance of the first two nitro reduction waves of picric acid. This can be explained by attack at each of the meta positions of picric acid.

Although Meisenheimer-complex formation has been shown to occur in the presence of excess hydroxide concentration (152), this does not take place at the base concentrations widely employed in the determination of creatinine by the Jaffe procedures commonly used in clinical chemistry.

Identification of the reactive group of creatinine was done by two methods. First, titration curves of the yellow and red products of picric acid and creatinine were examined. These curves were superimposable on the titration curve of creatinine. This was an indication that the guanidino group of creatinine was not involved in the reaction with picric acid. If the guanidino group were involved in the reaction, a shift of about two pH units should have been observed (168). Polarographic examination shows that the nitro reduction waves of picric acid are not decreased in the presence of creatinine. Preliminary results in this laboratory indicate that guanine does not produce a color reaction with alkaline picrate and the polarographic reduction waves of picric acid

are not decreased in the presence of guanine. The second piece of evidence is that hydantoin reacts with picric acid, while 5,5-dimethylhydantoin does not react.

In 1928, Weise and Tropp (82) visually observed red color formation for hydantoin and 5-methylhydantoin alkaline picrate. They also discovered that substitution at the 1,2, or 3 positions of hydantoin gave a positive reaction, while 5,5-ethyl-phenylhydantoin gave a negative reaction. They concluded that an active methylene group is required to react with the alkaline picrate, but they did not mention the type of attachment, nor did they propose a structure for the red Jaffé chromogen. Spectrophotometric results (depicted in the RESULTS section of this Dissertation) showed very similar spectra for the hydantoin and creatinine reactions with alkaline picrate. Further spectrophotometric examination of 5,5-dimethylhydantoin proved that substitution of both methylene protons of hydantoin prevents formation of the red chromogen in the presence of alkaline picrate. Greenwald (139) found that red color was produced with methyl substituted creatinine compounds, when the methyl groups were attached to the guanidino group of creatinine. From the evidence presented, it is concluded that the binding site of the red Jaffé chromogen is between the meta position of the picric

acid and the methylene group of creatinine. Polarographic evidence further shows that the first nitro reduction wave of picric acid is decreased in the reaction. This indicates that the red chromogen has a nitro anion. Stability of the Jaffé product has been shown by titrating the red alkaline solution with dilute hydrochloric acid until a yellow color was obtained. The yellow solution was backtitrated with 0.5 N sodium hydroxide until a red color was obtained. This red solution was then examined on the polarograph. The red Jaffé color had reappeared, but the first nitro reduction wave had also reappeared. The reappearance of the first nitro reduction wave of picric acid is attributed to the loss of the negative charge which resided on the nitro group. Furthermore, this is an indication that the binding of picric acid with creatinine forms a permanent bond. Greenwald (139) found picric acid to be quantitatively inseparable from creatinine when acetic acid was added after 30 min of reaction time. The polarographic evidence also shows that the nitro group is not bound, as was proposed by some researchers (140,144).

The proposed structure for the red Jaffé chromogen is presented in Fig. 11.

FIGURE 11

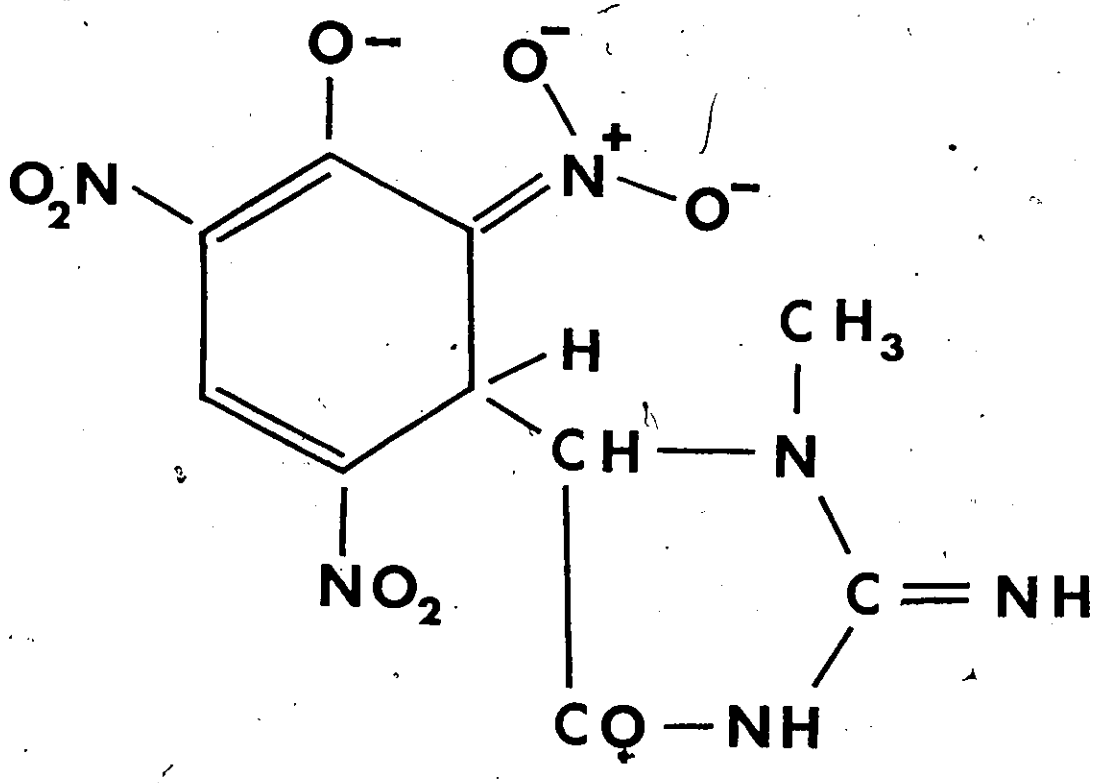
STRUCTURE OF THE RED JAFFÉ CHROMOGEN

Legend

A proposed structure of the red Jaffé chromogen is presented. Refer to text for complete details.

FIGURE 11

STRUCTURE OF THE RED JAFFÉ CHROMOGEN



PART II

INVERSE POLAROGRAPHIC DETERMINATION OF CREATININE WITH ALKALINE PICRATE AND 3,5-DINITROSALICYLIC ACID.

CHAPTER I

INTRODUCTION

The Jaffé (1) reaction of creatinine with alkaline picrate is not specific and numerous interferences which produce similar chromogens exist (59,169). Picric acid is a very reactive molecule with various types of reactions that it can undergo (59,160,170). Reactivity at the meta position of picric acid by acetone (160,161,163,171) and probably other molecules produces chromogens which interfere in the spectrophotometric determination of creatinine. Meisenheimer (151) complex formation also produces interfering chromogens.

Since the specificity of the colorimetric determination is decreased by many types of interfering chromogens, we proposed a new indirect polarographic determination. The polarographic reduction of picric acid has been investigated by a number of researchers (172-174). Three well-defined polarographic waves can be seen in an alkaline medium. After reacting the alkaline picrate with an aqueous solution of creatinine, a shift in half-wave potential and a lowering of the picric acid diffusion current are observed. This decrease in diffusion current is directly proportional to the concentration of creatinine. Meisenheimer complex

formation and the effect of the interferences that attack at the meta position are minimized in this new indirect polarographic determination of creatinine. In 1936, three different reports appeared on the reaction of alkaline 3,5-dinitrobenzoic acid with creatinine (81,103,104). The investigators described 3,5-dinitrobenzoic acid as a more specific reagent than picric acid for the determination of creatinine. However, limitations of low reactivity and instability of the purple color formed by the reaction were major disadvantages noted in the literature. Bollinger (103) attributed greater color stability to the alcoholic 3,5-dinitrobenzoic acid reaction of Benedict and Behre (81).

Since color stability is not a necessary requirement for polarographic investigation of the effect of creatinine upon the reduction waves of the nitro groups, an examination was undertaken to find a reagent which is more specific than picric acid for the polarographic determination of creatinine. An investigation of 3,5-dinitrosalicylic acid (DNSA) was also undertaken to improve reactivity and selectivity of the new inverse polarographic determination of creatinine.

CHAPTER II

EXPERIMENTAL

Materials and Methods

Reagent grade creatinine was purchased from Fisher Scientific Company, Don Mills, Ontario, Canada. The sodium hydroxide was "Baker Analyzed" Reagent grade from J. T. Baker Chemical Co., Phillipsburg, N. J. Reagent picric acid crystals (under water) were purchased from Allied Chemical of Morristown, New Jersey. Laboratory Reagent 3,5-dinitrosalicylic acid was from the British Drug Houses Ltd., Toronto, Ontario, Canada. The sodium chloride was obtained from Fisher Scientific Company, Don Mills, Ontario, Canada. Nitrogen 99.996% pure was from Liquid Carbonic of Canada Ltd. Triple distilled mercury was obtained from Englehard Industries of Canada Ltd.

A Sargent (Sargent-Welch Scientific Company) Model XVI Polarograph was employed for this investigation. The concentration study was carried out in a five-milliliter Heyrovsky cell. The characteristics of the capillary used were: $m = 1.767 mg\ s^{-1}$; $t = 4.68$ sec; $m^{2/3}t^{1/6} = 1.890\ mg^{2/3}\ s^{-1/2}$. The height of the mercury column was 71.5 cm. The cell was placed in a water bath maintained at $25 \pm 0.1^{\circ}C$ with a Haake Model ED "UNITHERM" constant temperature circulator.

A flask of stock saturated picric acid solution was prepared by adding distilled water to an excess amount of wet picric acid. The flask was immersed in the 25°C water bath until the temperature had equilibrated. Eight milliliters of this solution were added to a liter flask and brought up to volume with distilled water to form a picric acid working standard solution. To prepare the first blank solution, three milliliters of picric acid working standard and four ml of 0.5N sodium hydroxide solution were added to a 10-ml volumetric flask which was filled to the mark with distilled water. This solution (5 ml) was pipetted into the Heyrovsky cell for polarographic analysis. Creatinine standard was prepared by adding 0.025 g of creatinine to a 250-ml volumetric flask and made up to volume with distilled water. Solutions of various concentrations of creatinine (1 to 5 µg/ml) were prepared by adding appropriate alliquots of the standard creatinine to a 10-ml volumetric flask containing the picric acid working standard and the sodium hydroxide supporting electrolyte as in the blank. All solutions were deaerated for a period of 15 min with nitrogen prior to polarography.

A stock 3,5-dinitrosalicylic acid solution was prepared just prior to use by adding 0.050g of DNSA to a 500-ml

volumetric flask and diluting to volume with distilled water. The sodium chloride supporting electrolyte was prepared by adding 2.922g of sodium chloride to a 100-ml volumetric flask and filling the flask to volume with distilled water. Similarly, 0.05g of creatinine was weighed and made up to volume just prior to use in a 100-ml volumetric flask with distilled water. In the concentration study, 1 ml of sodium chloride solution, 5 ml of DNSA solution, and from 0.0 to 0.5 ml in 0.1 ml increments of creatinine solution (equivalent to 0.50 µg/ml) were added to a 10-ml volumetric flask and made up to volume with distilled water. The solution was mixed, and a 5-ml aliquot was pipetted into the Heyrovsky cell for polarographic analysis. A 15-min deaeration using nitrogen gas was accomplished by passing the gas through a water tower at room temperature, and then through the Heyrovsky cell which was kept at 25°C in a water bath. Duplicate analyses were performed for each concentration studied, with three polarograms obtained for each run.

CHAPTER III

RESULTS

A linear inverse relationship was observed between the diffusion current of the picric acid nitro group reduction wave at $E_{1/2}$ = -0.501V vs. Hg pool with increased creatinine concentration. The results of this concentration study are recorded in Table 2 and in Fig. 12. A slight change to a less negative $E_{1/2}$ was observed upon reacting the alkaline picric acid with creatinine. The $E_{1/2}$ for the reduction of the first nitro group of the alkaline picric acid is -0.501V, whereas the creatinine bound product has an $E_{1/2}$ = -0.496V.

The diffusion current was calculated from the following equations:

$$i_D = \text{Sens.} \times h$$

where i_D = the diffusion current in μA

Sens. = the sensitivity in μA/mm

h = the wave height in mm.

The wave height, h, was measured in mm. A typical polarogram for the first reduction wave of picric acid can be seen in Fig. 13. A sample calculation for a blank picric acid concentration can be calculated as follows:

TABLE 2. Effect of Creatinine Concentration on the Diffusion Current of the First Reduction Wave for Picric Acid

Creatinine concentration µg/ml	Discusion current (µA)* of the picric acid wave $E_{1/2}$= -0.501V vs Hg Pool
0	2.381
1	2.30
2	2.221
3	2.118
4	2.031
5	1.93

*The values reported represent an average of two samples at each concentration studied, with three polarograms run for each sample.

FIGURE 12

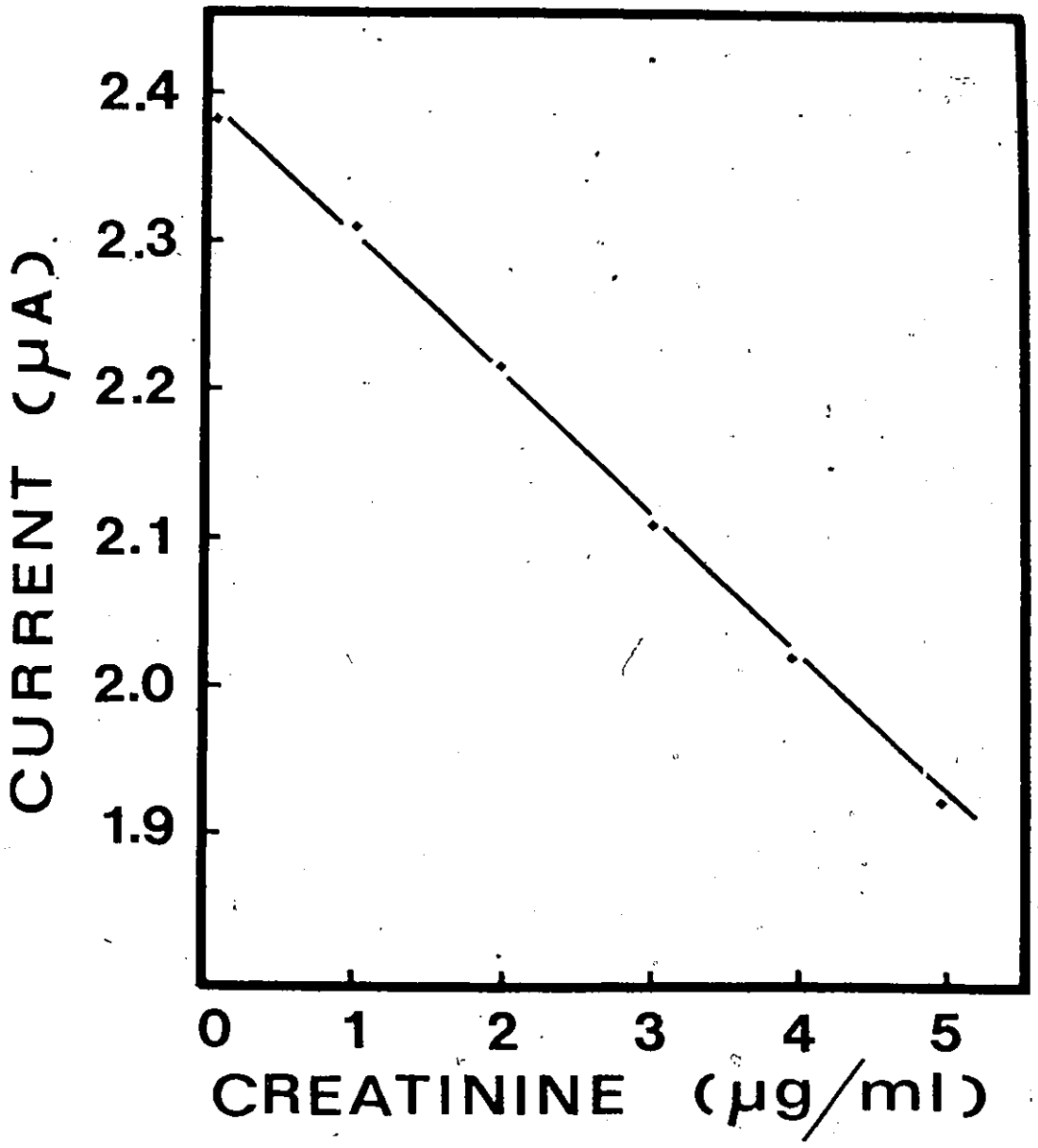
EFFECT OF CREATININE ON DIFFUSION CURRENT OF PICRIC ACID

Legend

The data for this plot was calculated from the diffusion current of the first reduction wave of picric acid. The results are an average of two samples at each concentration studied, with three polarograms run for each sample.

FIGURE 12

EFFECT OF CREATININE ON DIFFUSION CURRENT OF PICRIC ACID



Sens. = 0.020 μA/mm

h = .119 mm

i_D = 0.020 X 119 - 2.38 μA

The apparent half-wave potentials were calculated by measuring the distance in inches from the beginning of the polarogram to the midpoint of the diffusion current wave (175) as shown in Fig. 13.

These distances are substituted into the following equations:

$$\text{apparent } E_{1/2} = (-3.0/25.0) \text{ X } d$$

where apparent $E_{1/2}$ = the apparent half-wave potential

d = distance in inches

-3.0/25.0 = a constant for the polarograph.

The constant, -3.0/25.0, varies with the settings used on the Sargent Model XVI Polarograph. A polarogram is 25.0 inches long when the settings are medium and fast for the voltage drive and chart paper drive, respectively. The -3.0 volts with the negative sign indicating a negative increase in voltage. Thus every inch of the polarogram is a negative increase of 0.2 volts.

FIGURE 13

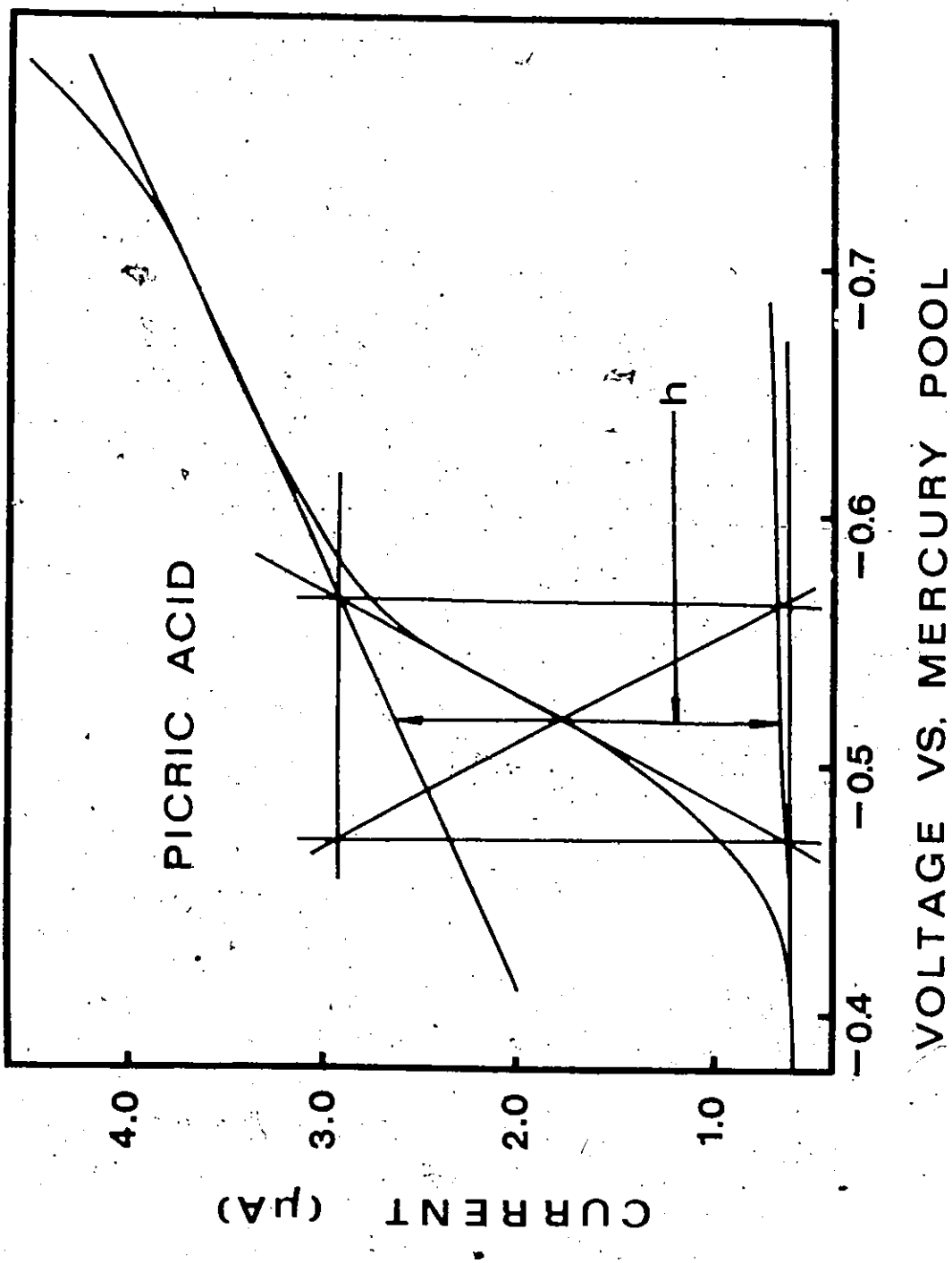
TYPICAL POLAROGRAM OF THE FIRST REDUCTION WAVE OF PICRIC ACID

Legend

This is a typical polarogram depicting only the first reduction wave of picric acid. Methods for measuring the diffusion current (h) and the apparent half-wave potential are depicted.

FIGURE 13

TYPICAL POLAROGRAM OF THE FIRST REDUCTION WAVE OF PICRIC ACID



A polarogram for the first wave of picric acid which was started at 0.0 volts and with d = 4.18 inches would yield:

$$E_{1/2} = 0.12 \times 4.18$$

$$= -0.502 \text{ volt}$$

The effect of creatinine concentration on the diffusion current of the DNSA wave at $E_{1/2}$ = -0.674 vs. Hg pool is shown in Table 3 and in Fig. 14. This study shows that the diffusion current is inversely proportional to concentration in the range of 0 to 30 µg/ml of creatinine. The half-wave potentials of the two nitro group reduction waves for DNSA were found to be -0.674V and -1.75V vs. Hg pool. A shift of $E_{1/2}$ was observed upon reacting the DNSA with creatinine. The shift in $E_{1/2}$ values for the first nitro group reduction wave of the DNSA-creatinine study was from -0.674 to -0.700V vs Hg pool.

TABLE 3. Effect of Creatinine Concentration on the Diffusion Current of the DNSA Wave at $E_{1/2} = -0.674v$

Creatinine concentration µg/ml	Diffusion current* (µA) of the DNSA wave at $E_{1/2}$=- 0.674 vs Hg pool
0	2.785
10	2.265
20	1.884
30	1.435
40	1.263
50	1.070

* The values reported represent an average of two samples at each concentration studied, with three polarograms run for each sample.

FIGURE 14

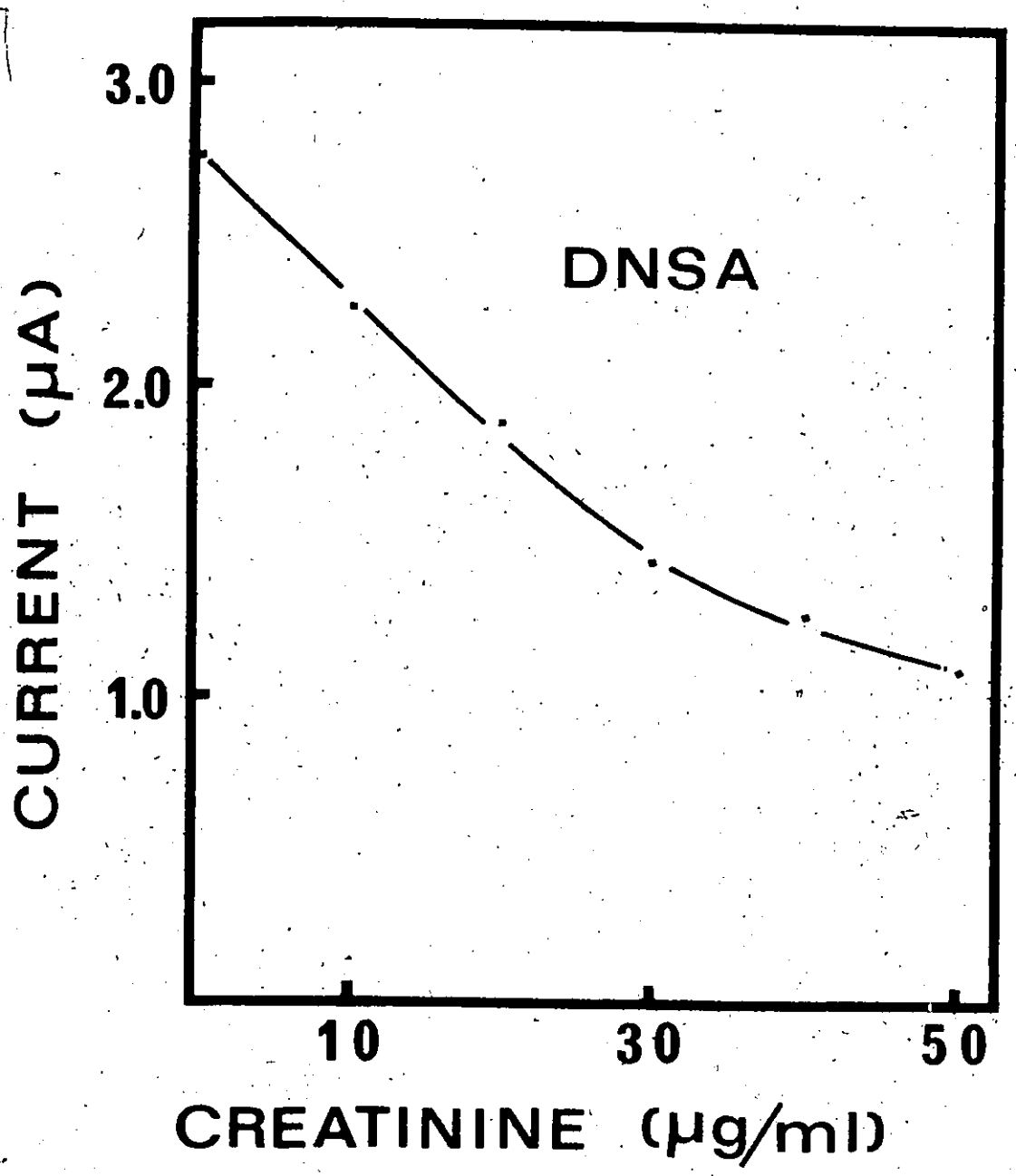
EFFECT OF CREATININE ON DIFFUSION CURRENT OF DNSA

Legend

The data for this plot was calculated from the diffusion current of the first reduction wave of DNSA. The results are an average of two samples at each concentration studied, with three polarograms run for each sample.

FIGURE 14

EFFECT OF CREATININE ON DIFFUSION CURRENT OF DNSA



CHAPTER IV

DISCUSSION

The polarographic reduction of the nitro groups of picric acid to hydroxylamine groups has been shown to involve the transfer of four electrons per nitro group (176). Inhibition of this reduction by creatinine causes a decrease in diffusion current of four electrons per molecule of creatinine. Because of this sharp decrease in the diffusion current, along with the fact that only the first nitro wave is affected by creatinine at low concentrations (1:4 C/P) accounts for the sensitivity of this new indirect polarographic determination.

Polarographic investigation is able to clarify whether the reaction between creatinine and picric acid forms a 1:1 or a 2:1 product. The difficulty in determining whether it is a 1:1 or a 2:1 product lies in the fact that creatinine can form both depending on the relative amounts of each of the chemicals present. At a low creatinine to picric acid ratio, a red 1:1 product is formed. At ratios of 1:1, a mixture consisting mainly of the 1:1 product is present, but approximately 10% is in the 2:1 form. This is easily observed by polarography of picric acid-creatinine solutions

and comparison of the reduction waves of picric acid. Spectrophotometric determination did not show clearly that the reaction product was a 1:1 or 2:1 adduct because the red product is unstable and changes to a yellow product upon dilution. Similarly, it was observed that the orange 2:1 product, which was first isolated by Greenwald (142), is also converted to a yellow color when it is dissolved in water and other solvents. Indeed, a solvent could not be found in which the red and orange products were soluble and at the same time retained their color in dilute solutions.

In 1936, three independent investigators (81,103,104) found that 3,5-dinitrobenzoic acid will produce a purple color with creatinine in alkaline solution. This method was shown to be more specific toward creatinine, but it was less sensitive. Polarographic examination of this reaction revealed, that both reduction waves corresponding to each of the nitro groups, would decrease at the same time when creatinine was added. This suggested that the reaction of 3,5-dinitrobenzoic acid was similar to that of picric acid. Since creatinine addition to 3,5-dinitrobenzoic acid affected both reduction waves at the same time, and also because of its lowered reactivity, the 3,5-dinitrobenzoic acid proved unsuitable compared to picric acid as a reagent

for the indirect polarographic determination of creatinine.

To examine a similar molecule with greater reactivity 3,5-dinitrosalicylic acid was tried. This reagent was much more reactive, in that it gave a greater decrease in the diffusion current with creatinine than did 3,5-dinitrobenzoic acid. DNSA did not produce the purple color which occurs with 3,5-dinitrobenzoic acid and creatinine in the presence of base. It was found advantageous to eliminate the addition of base and to substitute NaCl as the supporting electrolyte for the polarographic determination of creatinine with DNSA.

An experimentally insignificant change in $E_{1/2}$ is observed for the first nitro group reduction wave of picric acid when creatinine is added under the conditions described in this paper. However, in the DNSA study, a shift of 0.26V (to a more negative potential) was observed between the $E_{1/2}$ of the first nitro group reduction wave and the corresponding wave in the presence of creatinine. Although correlation between shifts in $E_{1/2}$ and the extent of conjugation have been well documented in the literature (177-179), such a conclusion cannot be drawn from the data presented in this paper. The shift in $E_{1/2}$ in the DNSA-creatinine study is believed to be due to a change in pH.

An increase in pH would decrease the availability of protons for the reduction of the nitro group to the hydroxylamine (176), and therefore a change to a more negative potential would be expected and was observed. The effect of changes in pH versus $E_{1/2}$ for nitro group reductions have been tabulated (180-182).

CHAPTER V

SUMMARY

A selective and highly sensitive inverse polarographic method for the determination of creatinine has been developed, involving the measurement of disappearance of diffusion current for the picric acid wave ($E_{1/2}$ = -0.501V) when creatinine is added. Creatinine levels of 0-5 µg/ml can be measured accurately.

Investigation of 3,5-dinitrobenzoic acid and 3,5-dinitrosalicylic acid revealed the latter to be more reactive and useful for the inverse polarographic determination of creatinine at levels of 0 - 30 µg/ml.

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