I. INVESTIGATION OF REACTION INTERMEDIATES OF THE UREA-DIACETYLMONOXIME REACTION. II. AUTOMATED MICROANALYSIS OF IRON USING SYNTHESIZED TEROSITE SULFONATE AS CHELATING REAGENT.

CHANDRAVADAN PATEL

University of Windsor

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I. INVESTIGATION OF REACTION INTERMEDIATES OF THE UREA-DIACETYLMONOXIME REACTION

II. AUTOMATED MICROANALYSIS OF IRON USING SYNTHESIZED TEROSITE SULFONATE AS CHELATING REAGENT

by

CHANDRAVADAN PATEL

A Dissertation
Submitted to the Faculty of Graduate Studies Through the Department of Chemistry in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy at the University of Windsor

Windsor, Ontario, Canada
1978
ABSTRACT

PART I

INVESTIGATION OF REACTION INTERMEDIATES OF THE
UREA-DIACETYLMONOXIME REACTION

by

Chandravadan Patel

An investigation of the intermediates of the urea-diacetylmonoxime reaction and the isolation of the protochromogen is described. A comparative spectral study of the protochromogen with that of the literature values suggests the structure of the protochromogen to be a diureide. Mass spectrometric (field desorption) studies gives the molecular weight of the compound to be 170 as expected. A comparative study of electron impact mass spectrometry on the fragmentation patterns of glycoluril and the diureide (ethylmethyldiureide) provides no conclusive evidence regarding the structure.

The diureides of 1-phenyl-1,2-propanedionemonoxime and 2,3-pentanedione were synthesized and their characteristic visible, U.V., I.R., NMR, and spectrometric data were analyzed. The study suggests that the structure of these compounds are that of a diureide. When these diureides were redissolved in acid media, they gave the color expected of the corresponding chromogens with the respective diketones and urea.

The 3a-methyl-6a-phenylglycoluril was synthesized
by a base-catalyzed reaction. When this glycoluril was redissoved in concentrated HCl, a pink color was obtained, similar to that of the corresponding diureide. These studies indicate that both the diureide and the glycoluril (if at all formed in acid-catalyzed reaction) are converted to the same chromogen, possibly via a rearrangement.

The reaction of urea-diacetylmonoxime with thiosemicarbazide in an acid medium results in a bathochromic shift from 480 nm to 525 nm as expected. When 2,3-butanedionemonoximethiosemicarbazone reacts with urea in acid medium, the spectral characteristics obtained are very similar to those of the urea-diacetylmonoxime-thiosemicarbazide reaction. From the comparison of the spectral characteristics of the final reaction concentrates of the preceding reactions, similar visible and U.V. spectra are obtained which suggests that the structure of the protochromogens is similar to that of the open chain diureide type.
PART II

AUTOMATED MICROANALYSIS OF IRON USING SYNTHESIZED TEROSITE SULFONATE AS CHELATING REAGENT,

by

Chandravadan Patel

The synthesis of terosite sulfonate is described. Sulfonation eliminates the water insolubility problem of the parent terosite used for the determination of iron. The absorption maxima of the sulfonated material is identical to that of the parent compound. The ferrous complex of the sulfonated terosite is found to be very stable, and the molar extinction coefficient, ($\epsilon = 30,971$), is very similar to that of the unsulfonated terosite, ($\epsilon = 30,200$). The effect of pH on the stability of ferrous-bis(terosite sulfonate) was studied and the optimum pH range for chelate formation was found to be between pH 2 - 4.5. The procedure for the determination of microquantities of iron by the use of water-solubilized terosite sulfonate has been automated on the Technicon ® AutoAnalyzer ® (AAI).
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My special thanks goes to my typist, Mrs. A. Holder,
especially for battling my handwriting, proofreading and typing the manuscript. I thank her for her patience and understanding.
DEDICATION

to

My Wife Patricia
and

My Children: Jennifer, Stephanie, Nicole

for their patience, moral support and continued encouragement and tolerance of my neglect of other duties which made the completion of my education possible.
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PART I

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ABBREVIATIONS

U = Urea
BUN = Blood Urea Nitrogen
DAM = Diacetylmonoxime
TSC = Thiosemicarbazide
DAMTSCO = 2,3-Butanedioneoximethiosemicarbazone
PPDMO = 1-Phenyl-1,2-propanedionemonoxime
PPDDU = 1-Phenyl-1,2-propanedionediureide
AAI = Technicon AutoAnalyzer System I
M = Molecular ion

Bathophenanthroline = 4,6-Diphenyl-1,10-phenanthroline
Ferrozine = 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine
DMPP = 2,6-Dipyridyl-(2)-4-p-methoxy-phenylpyridine
Terosine = 2,6-Bis(2-pyridyl)-4-phenylpyridine
Terosole = 2,6-Bis(4-methyl-2-pyridyl)-4-phenylpyridine
Terosole = 2,6-Bis(4-ethyl-2-pyridyl)-4-phenylpyridine
Terosite = 2,6-Bis(4-phenyl-2-pyridyl)-4-phenylpyridine
Terpyridine = 2,2',2''-Tripyridine
NED = N-(1-naphthyl)-ethylenediamine
RA = Relative abundance
NPN = Nonprotein nitrogen
Ts = Terosite sulfonate
DMF = Dimethylformamide
TPTZ = 2,3,6-Tripyridyl-3-triazine
TGA = Thioglycollic acid
BCS = Bathocuproine sulfonate

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DMSO = Dimethyl sulfoxide
ε = Molar extinction coefficient
FD = Field desorption
FI = Field Ionization
EI = Electron Impact
PDDU = 2,3-Pentanedioniureide
A = Absorbance
PART I

INVESTIGATION OF REACTION INTERMEDIATES OF THE
UREA-DIACETYLMONOXIME REACTION
CHAPTER I

UREA-DIACETYLMONOXIME REACTION

A. INTRODUCTION

Urea is the chief end-product of amino acid metabolism and is formed mainly in the liver by cleaving the amidino group from the side chain of arginine. The overall reaction of the urea cycle is the formation of one molecule of urea with consumption of one molecule each of \( \text{NH}_4^+ \), aspartate, and \( \text{CO}_2 \), with a cleavage of four high-energy phosphate bonds (1). The urea enters the systemic circulation and is excreted from the body, mainly in the urine. In cases of severe liver impairment, there is a decrease in the blood urea concentration, while in kidney disease the BUN levels usually increase. Therefore, studies of urinary nonprotein nitrogenous (NPN) elements from the standpoint of the investigation of renal function have been largely directed towards urea and creatinine (2). The measurement of blood urea nitrogen (BUN) is the second most frequently ordered chemical test because of its utility in detecting and managing renal disorders (3-6). Urea nitrogen constitutes about 45% of the total NPN in serum or plasma. Methods for determining urea are classified into three groups (7):
a) Direct: condensation of urea with diacetylmonoxime.

b) Indirect: determination of ammonia formed by urease.

c) Miscellaneous: methods involving other physical principles involved in measuring urea.

The carbamidodiacetyl reaction of Fearon (8) for the measurement of citrulline, is one of the most common methods used in many clinical laboratories for the determination of urea. The diacetyl reaction does not measure ammonia, the reagents are stable and the reaction can be sensitive.

In numerous modifications of the usual procedure described in the literature (9-11) little success was reported in increasing the sensitivity for urea.

The condensation of urea with diacetylmonoxime in acid and alkaline medium has been studied by many workers, in order to isolate intermediate(s) which are responsible for the color development (yellow). This is a requirement in understanding the mechanism of the reaction, which, if elucidated, could help in evaluating the specificity of the reaction. The establishment of optimum reaction conditions and the prediction of interferences from other substances present in the biological fluid under examination would also be better understood.

Several postulates regarding the structure(s) of the intermediate(s) have been proposed in the literature. Structures of the proposed intermediates are as follows:
A. Franchimont and Klobbie (12) 1888, and Biltz (13) 1907;

\[
\begin{align*}
\text{CH}_3 & \quad \text{NH} \quad \text{C} \quad \text{NH} \\
& \quad \text{O=\text{C}} \\
& \quad \text{NH} \quad \text{C} \quad \text{NH} \\
& \quad \text{CH}_3
\end{align*}
\]

B. Fearon (8) 1939;

\[
\begin{align*}
\text{CH} & \quad \text{CH} \\
& \quad \text{NH} \\
& \quad \text{C} \\
& \quad \text{C=O} \\
& \quad \text{CH}_3
\end{align*}
\]

C. Pechman (14) 1888, Dickenman, Zak. (15) 1954;

\[
\begin{align*}
\text{CH}_3 & \quad \text{C=N} \\
& \quad \text{C=O} \\
& \quad \text{C=N} \\
& \quad \text{CH}_3
\end{align*}
\]

D. Beale and Croft (16) 1961;

\[
\begin{align*}
\text{CH}_3 & \quad 0 \\
& \quad \text{C=N-NH-C-NH}_2 \\
& \quad \text{C=O} \\
& \quad \text{CH}_3
\end{align*}
\]
E. Nematollahi and Ketcham (17) 1963 (glyoxal + urea);

1. \[
\begin{align*}
\text{H} & \\
\text{N} & \text{CH}_2 \\
\text{O} & \text{C} \\
\text{N} & \text{C} = \text{N} - \text{C} - \text{NH}_2 \\
\text{H} & \\
\end{align*}
\]

2. \[
\begin{align*}
\text{H} & \\
\text{N} & \text{CH} \\
\text{O} & \text{C} \\
\text{N} & \text{C} = \text{N} - \text{C} - \text{NH}_2 \\
\text{H} & \\
\end{align*}
\]

3. \[
\begin{align*}
\text{CH} - \text{N} & \text{C} - \text{NH}_2 \\
\text{CH} - \text{N} & \text{C} - \text{NH}_2 \\
\end{align*}
\]
F. Siest (18) 1966;

\[
\begin{array}{c}
\text{CH}_2 \\
\| \\
\text{C} - \text{N} \\
\| \\
\text{C} = \text{O} \\
\| \\
\text{C} - \text{N} \\
\| \\
\text{(CH}_2\text{)_n}
\end{array}
\]

G. Ueda, et al. (19) 1968 and 1971;
(butylurea and p-tolylurea with diacetylmonoxime);

\[
\begin{array}{c}
\text{O} \\
\text{R} - \text{N} \\
\| \\
\text{CH}_3 - \text{C} = \text{NH} \\
\| \\
\text{R} - \text{N} \\
\| \\
\text{NH} \\
\| \\
\text{R} = \text{H}
\end{array}
\]

H. Lugosi and Thibert (20) 1972;

\[
\begin{array}{c}
\text{CH}_3 \\
\| \\
\text{C} = \text{N} - \text{C} - \text{NH}_2 \\
\| \\
\text{C} = \text{N} - \text{C} - \text{NH}_2 \\
\| \\
\text{CH}_3 \\
\| \\
\text{O}
\end{array}
\]

Siest (18), in his work, has mentioned that Job's method of continuous variation with urea and their substituted derivatives gave colored complexes with equimolar quantities of diacetyl. However, cyclohexanedione and its dioxime did not form colored complexes.

The formation of diureide was excluded by Siest (18), because they did not give colored complexes and secondly, that the one molecule of urea reacts with...
one molecule of diacetyl.

Fearon's proposal of oxidative condensation is to be considered as possible since the proposal requires the presence of an α-methylene group next to the functional carbonyl group. However, the reaction is not an oxidative condensation, since the reaction proceeds to a colored product even in the presence of a reducing agent (8).

The proposal of Beale and Croft was rejected simply because diacetyl and other α-diketones give a reaction equal to if not superior to that of their oximes. It has been indicated that the probable cause of their product formation could be undistilled diacetyl or use of improper experimental conditions which were applicable to diacetyl monoxime and not to diacetyl (18).

Siest (18) proposes that the formula of the chromogen was the condensation of a single urea molecule with diacetyl monoxime (see Scheme 1.).

However, no isolation or identification of the chromogen was carried out by Siest.

Lugosi, Thibert, Holland, and Lam (20) have synthesized diacetyl monosemicarbazone according to the method.

---

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of Diels (21). They could not cyclize the diacetyl mono-
semicarbazone to a triazine ring condensation product.
Therefore, they concluded that the reaction product of 
urea with diacetylmonoxime or diacetyl in acid medium 
could not be a triazine. The existence of the proposed 
structure (tetrahydroimidazo-(4,5-d)-imidazole-2,5-dione 
of Franchimont and Klobbie (12) was shown to be unlikely 
due to the positive test given by diureide obtained by 
Lugosi et al. (20), as well as the characteristic in-
frared bands which show the presence of a primary amide 
as illustrated by a diacetyl diureide type of structure 
synthesized by them. Results indicated that the follow-
ing two compounds are possible; diureide; as well as 
3a,6a-dimethyl-tetrahydroimidazo-(4,5-d)-imidazole-2,5-
dione.

\[
\begin{align*}
\text{HN} & \quad \text{CH}_3 \\
C & \quad \text{NH} \\
\text{O} = \text{C} & \quad \text{C} = \text{O} \\
\text{HN} & \quad \text{NH} \\
\text{CH}_3 & \quad \text{O}
\end{align*}
\]

3a,6a-dimethyltetrahydroimidazo-(4,5-d)-imidazole-2,5-dione

diacetyl diureide

The literature cites many examples of base-catalyzed 
condensation reactions of glyoxal, diketones, benzil 
with urea and similar compounds. In the base-catalyzed 
reactions, generally the intermediate found is 2-imidazo-
lidones or substituted 2-imidazolidones. The simplest
example of this reaction is the formation of 4,5-dihydroxy-2-imidazolidone from glyoxal and urea. (See Scheme 2).

\[ \text{H} - \text{C} = \text{O} + \text{NH}_2 \rightarrow \text{H} - \text{C} - \text{N} \]

\[ \text{OH} \]

\[ \text{SCHEME 2} \]

The formation of 5,5-disubstituted hydantoin from 4,5-disubstituted-4,5-dihydroxy-2-imidazolidones involves a pinacolic or benzilic acid shift. Dunnivant and James (22) have shown the formation of 5,5-diphenylhydantoin by condensation of benzil with urea in alkaline medium and have concluded that this is most probably a benzilic type rearrangement and not a pinacol rearrangement. In their study they have prepared many substituted phenylhydantoins and 3a,6a-di-(4-substituted phenyl)-glycolurils and have proposed the mechanism for the formation of hydantoin and glycoluril. (See Scheme 3).

\[ \delta^+ \]

\[ \text{H} - \text{C} - \text{C} - \theta + \text{NH}_2 \rightarrow \text{H} - \text{C} - \text{C} - \theta + \text{H}_2\text{O} \]

\[ \delta^- \]

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In contrast to the above pinacol-pinacolone rearrangement, is the acid-catalyzed conversions of 1,2-glycol into aldehyde or ketone, in which the reaction is facilitated by the presence of both hydroxyl groups as tertiary groups. In this rearrangement the aryl groups migrate more easily than the methyl groups. (23).

The 4,5-dihydroxy-2-imidazolidone can be transformed to hexahydro-2,5-dioximidaz-[d]-imidazoles (glycolurils). Acid hydrolysis converts a number of these imidazoles into hydantoins (24) (See Scheme 4).

\[
\begin{align*}
\text{NH}_2 & \quad \text{NH} \\
\text{C}_6\text{H}_5 & \quad \text{C}_6\text{H}_5
\end{align*}
\]

**Scheme 4**

Neville (25) has shown that the ratio of glycoluril...
to hydantoin formation depends upon the proportions of benzil to urea used. Similarly Fisher et al. (26) have reported condensation of urea with phenylglyoxal, and obtained 5-phenyl-dihydroxy-2-imidazolone which undergoes rearrangement in alkaline medium to the corresponding hydantoin.

Dunnavant and James (22) attempted a condensation of benzil with urea in neutral or acidic solution, but failed to attain the corresponding glycoluril, indicating that alkaline medium was needed for formation of glycoluril.

Veniamin et al. (27) in their work considered either 7- or 8-methyl, or 7,8-dimethyltetrahydroimidazo (4,5-d)-imidazole-2,5-diones as being the chromogens in the carbamido-diacetyl reaction; however, they did not isolate any compound.

Since hydantoin, when dissolved in hydrochloric acid solution, does not give the typical reaction color, one can rule out its role as the chromogen of the urea-diacetyl reaction.

Erickson (28) has reported the studies of Thiele and Dralle with aliphatic 1,2-dicarbonyl compounds with aminoguanidine salts. They used glyoxal, diacetyl and aminoguanidine hydrochloride and isolated monoguanyl-hydrazone and osazone-like compound (III) instead of triazine (II) (See Scheme 5). Triazines were obtained when they used aminoguanidine bicarbonate instead of
hydrochloride salts. Therefore, formation of a triazine type of structure of the chromogen can be eliminated since the reaction of diacetyl-urea is carried out in strong acid medium. The structure proposed by Siest (18) and Fearon (8) seems difficult to visualize in enol-cyclic form for the chromogen even though it does possess the required conjugated system for color formation. The condensation of diacetyl with urea may be taking place via an enol form (See Scheme 6).

Instead of forming the cyclic 4-methyl-5-methylene-2-imidazolidone, one might find condensation of a second urea molecule to the monoureide to form diureide as proposed and isolated by Lugosi et al. (20).
In acid-catalyzed additions glycoluril and hydantoin are reported products (17, 29, 30). However, Vail et al. (31) has reported that formation of the $N,N'$-dimethyl derivative of hydantoin was not obtained by acid-catalyzed addition. Glycoluril does not possess the required conjugated system for a chromogen, therefore, it is most likely that it might not be the chromogen responsible for the
formation of the color.

Smith (32) has shown that carbazides and carbanilides exhibit a color reaction when treated with diacetyl and/or diacyldioxime in hydrochloric acid and that the intensity of the color varies with the relative proportions of either diacetyl or dioxime and semicarbazide.

Lugosi et al. (20) has prepared diacetylmonocarbazone which exhibits color in acid medium thus providing more support for the monodiureide structure as a possible protochromogen (protochromogen is a substance that was isolated from the reaction mixture which may exhibit an isomeric form of the chromogen in the reaction media) in the diacetyl-urea reaction.

In order to arrive at a final conclusion regarding the structure of the protochromogen of the urea-diacetylmonoxime reaction, the following approach included the isolation of the protochromogen and comparing the infrared spectra with that of the previously isolated diureide of Lugosi et al. (20). Further studies included the synthesis of glycoluril, comparison of the infrared spectra of glycoluril with that of the isolated chromogen, and the study of the mass spectrophotometric data on both compounds. Field desorption mass spectrometric data was collected to determine the molecular weights of these compounds. Elemental analysis was also carried out. Further ultraviolet spectra of the starting materials and compounds related to the structures of
the proposed intermediates in the literature will be compared. Finally the absorption maxima of the urea-diacetylmonoxime reaction mixture will be studied together with that of the prepared diureide in the acid media.
B. EXPERIMENTAL

1. APPARATUS

Visible spectra were obtained with a Beckman Acta III recording spectrophotometer. Ultraviolet spectra were recorded in hydrochloric acid solutions with a Beckman Acta III spectrophotometer. A Beckman infrared spectrophotometer (IR-12) was used for the measurements of 1 - 2% samples of the compound mixed and dispersed in KBr powder.

The nuclear magnetic resonance (NMR) spectra were obtained where applicable with a Jeolco C60-HL NMR spectrometer at 60 MHz on an ≈20 mg sample dissolved in ≈0.40 mL solvent, with tetramethylsilane as the internal standard. A Varian MAT CH-5 double-focussing mass spectrometer with a triple source FI/EI/FD equipped with Incos 2000 data system was used to obtain EI/FI data on approximately 1 mg sample in the project.

For the field desorption mass spectrometry (EI/D and FD), the samples were introduced via the Beckey (emitter dipping) technique-(33), using acetone as the solvent on a 1 mg sample. The aim of the mass spectrometric study with FD was to determine the molecular weight of the compound.

Melting point determinations were carried out on a Fisher-Johns melting point apparatus.

The elemental analysis was performed by Schwarzkopf
2. REAGENTS

Diacetyl (diketobutane), urea, and diacetylmonoxime (Reagent grade) were purchased from Fisher Scientific Company, Chemical Manufacturing Division, Fairlawn, N.J.

Hydrochloric acid ('Baker Analytical' Reagent) was obtained from J.T. Baker Chemical Company, Philadelphia, N.J.

Concentrated sulfuric acid and phosphoric acid (85% w/w) were of analytical grade. All solutions were prepared in distilled water.

Thiosemicarbazide was purchased from Eastman Kodak Company, Rochester, N.Y.

Glyoxal (approx. 30% aqueous solution) was purchased from Fisher Laboratory Chemicals, Fisher Scientific Company, Fairlawn, N.J.

Ethanol (absolute) was obtained from Consolidated Alcohols, Toronto, Ontario.

Potassium hydroxide (Laboratory grade) was obtained from Fisher Scientific Company, 184 Railside Road, Don Mills, Toronto, Ontario.

Furil was obtained from the Aldrich Chemical Company Inc., 940 W. Saint Paul Avenue, Milwaukee, Wisconsin, 53233.

Benzil was purchased from the British Drug Houses Ltd., B.D.H. Laboratory Chemicals Division, Toronto, Ontario.
3. METHODS

(a) Synthesis of the Protochromogen in the Urea-
Diacetylmonoxime Reaction

To a mixture of 25 mL of distilled water and 38 mL of concentrated HCl, were added 5.0 g (0.05 mole) of diacetylmonoxime and 10.0 g (0.166 mole) of urea. The mixture was heated in a 250-mL Erlenmeyer flask on a steam bath with continuous agitation (=15 - 20 minutes) until a clear yellow solution was obtained. On cooling and upon overnight standing at room temperature, a white-buff-colored product was obtained. The mixture was filtered by suction and the filtrate discarded. The precipitate was washed with cold water and recrystallized from hot distilled water after treatment with Norit A. The yield was 3.5 g (41% of the theoretical yield). The melting point of the white product was over 300°C with decomposition. The reported melting point of the diacetyl-diureide and 3α,6α-dimethylglycoluril was >300°C (d).

The infrared spectra were run using KBr pellets and the field desorption mass spectra was recorded for the molecular weight determination of this compound. The visible spectrum of this compound had a $\lambda_{\text{max}}$ of 478 nm as reported in the literature. This product was identical to that of Lugosi et al. (20).

Anal. calc. for C$_{6}$H$_{10}$N$_{4}$O$_{2}$: C, 42.36; H, 5.93; N, 32.94

Found: C, 42.54; H, 5.88; N, 32.76

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(b) Synthesis of 2,3-Diacetyldithiosemicarbazone

Thiosemicarbazide (3.0 g [0.03 mole]) was dissolved in 50 mL of distilled water by application of heat, till a clear solution was obtained. To this were added, with continuous stirring, 5.0 g (0.05 mole) of DAM dissolved in 50 mL of distilled water, followed by 37 mL of concentrated hydrochloric acid. The reaction mixture was heated on a steam bath for 15 - 20 minutes. A clear solution was obtained. The solution was concentrated to approximately 25 mL by evaporating the excess water on the steam bath. A white crystalline product was obtained upon overnight standing at room temperature. The thiosemicarbazone was filtered by suction and washed with ice-cold water. The molecular weight, as determined by field desorption mass spectrometry, was found to be 232 which corresponds to the calculated molecular weight of 232 (diacetyldithiosemicarbazone). Infrared data corresponds to the structure of the compound.

(c) Synthesis of Glycoluril (Tetrahydroimidazo-(4,5-d)-imidazole-2,5-dione)

Tetrahydroimidazo-(4,5-d)-imidazole-2,5-dione was prepared from 30% glyoxal and urea according to the method of Slezak (29, 34). This product, or its derivative have been prepared by other workers (17, 30, 31).

An aqueous solution (30%) of glyoxal (17.40 g; 0.1 mole) was weighed out in a 250-mL round-bottomed flask equipped with a reflux condenser. To this was added 0.5 mL of concentrated HCl and 1.8 g (0.03 mole) of urea
in 100 mL of ethanol. The solution was refluxed for 30 minutes on a steam bath, then allowed to stand for 4 hours at room temperature. A white crystalline needle-shaped product was obtained. The crude product was recrystallized from hot distilled water (0.8 g; yield 70%). The m.p. was found to be above 300°C (d) [lit. m.p. above 300°C (d)]. The characteristic I.R. band for glycoluril in KBr, as reported in the literature, was 1690, 3200 cm⁻¹ (35). The NMR spectra was found to be δ 5.45 for the CH protons.

(d) Attempted Synthesis of 4,5-Dihydroxy-4,5-Dimethyl-2-Imidazolidinone

i) The procedure followed was basically that of Vail et al. (31) for the synthesis of 4,5-dihydroxy-2-imidazolidinone. To 12.0 g (0.2 mole) of urea were added 13.2 g (0.153 mole) of 2,3-butanedione in 50 mL ethanol which was made alkaline with 20% NaOH to a pH of 9. A brown solution was obtained on stirring. The mixture was allowed to stand at room temperature for 4 hours and then stored for a week at -20°C. No crystalline product was obtained.

ii) In the second attempt the concentration of sodium hydroxide was changed. In a 250-mL Erlenmeyer flask, 9.0 g (0.15 mole) of urea and 8.6 g (0.1 mole) of diacetyl were weighed out and dissolved in 50 mL of alcohol. The mixture was titrated to a pH of 9 with 6N NaOH. No crystalline product was obtained on cooling the reaction mixture.
iii) The procedure of Neville et al. (25) for the synthesis of 1,3-dimethyl-4,5-dihydroxy-4,5-diphenyl-2-imidazolididone was employed. To 12.75 g sodium ethoxide in 100 mL ethanol were added 12.0 g (0.2 mole) of urea and 17.2 g (0.2 mole) of diacetyl. The mixture was refluxed in a 250-mL round-bottomed flask equipped with a reflux condenser for one hour, then poured while hot into a solution of sodium acetate (40 g) dissolved in 2 L of water. After refrigeration for 24 hours no crystalline product was obtained.

(e) Attempted Synthesis of 3a, 6a-Dimethylglycoluril

The method followed was that of Dunnavant et al. (22) for the base-catalyzed condensation of benzil with urea. A mixture of 1.6 g (0.019 mole) of 2,3-butanedione, 1.99 g (0.033 mole) of urea, 1.99 g (0.0356 mole) KOH and 40 mL of ethanol was refluxed in a 250-mL round-bottomed flask equipped with a reflux condenser on a steam bath for 2 hours. The hot reaction mixture was then poured into ice water. No precipitate was recovered even after standing at room temperature for 48 hours. Addition of more distilled water did not produce any precipitate.

(f) Attempted Synthesis of 3a,6a-Diphenylglycoluril

Benzil (4.20 g [0.02 mole]) was dissolved in approximately 50 mL of ethanol, and placed in a 200-mL round-bottomed flask attached with a reflux condenser. To this was added 3.6 g (0.06 mole) urea dissolved in 10 mL
of water. To the mixture was added approximately 1.0 - 1.5 mL of concentrated HCl. The reaction mixture was refluxed on a steam bath for about one hour and cooled. The precipitate was filtered and washed with alcohol. Recrystallization of the product from alcohol gave almost a theoretical amount of the starting material, with a melting point of 95°C. Condensation of benzil with urea in a base-catalyzed reaction has been reported in the literature (22).

(g) Attempted Condensation Reaction of Furil with Urea

In a 250-mL round-bottomed flask were added 1.9 g (0.01 mole) of furil with 100 mL of alcohol. The mixture was heated on a steam bath to dissolve furil. A yellow-greenish solution was obtained with some undissolved furil. To this were added 1.8 g (0.03 mole) of urea and about 1.0 - 1.5 mL of concentrated HCl. The solution had a pH of 1.5 - 2.0. At the end of one hour of refluxing time, a green solution with a greenish crystalline product was obtained. On recrystallization of the product, a yellow needle-shaped crystalline product was produced with melting point of 163° - 165°C. Field desorption mass spectrometric data showed the molecular weight of the compound to be 190, which corresponds to that of the starting material (furil).
C. RESULTS AND DISCUSSION

The comparative infrared characteristic bands of the starting material, the isolated chromogen, and the literature value (Lugosi et al. [20]) is reported in Table I. The characteristic bands of the isolated product of the diacetylmonoxime-urea reaction is identical with the literature value. The other physical characteristics such as melting point match the literature value (>300°C [d]). The visible spectrum in HCl shows the $\lambda_{\text{max}}$ at 478 nm and 345 nm as expected. Table II lists the typical infrared bands of glycoluril and diureide. As expected, the synthesized glycoluril spectrum corresponds to the reported literature values, whereas, that of diureide shows discrepancies (33, 36). The strained ring C=O which occurs in glycoluril at 1760 cm$^{-1}$ (literature value for strained ring C=O is 1850 - 1750 cm$^{-1}$) is absent from the diureide spectrum. In amides (R-C-NH$_2$), the C=O stretching and NH$_2$ deformation band is found near 1650 cm$^{-1}$ (amide band II), which is seen in the diureide in Table I but seems to be absent from glycoluril data in Table II as expected. Secondary acyclic amides in the solid state display an amide II band in the region of 1570 - 1515 cm$^{-1}$. The 1760 cm$^{-1}$ band in glycoluril could be due to a strained ring C=O (literature value 1850 - 1750 cm$^{-1}$). This band is not
<table>
<thead>
<tr>
<th>Functional groups</th>
<th>Diacetyl monoxime</th>
<th>Protochromogen Exp. data</th>
<th>Lugosi et al. data</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-H stretching vibrations (primary amide solid phase)</td>
<td>3400</td>
<td>3240</td>
<td>3240</td>
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<tr>
<td>C-H stretching vibrations (assymmetrical methyl)</td>
<td>2920</td>
<td>2930</td>
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<tr>
<td>C-H stretching vibrations (symmetrical methyl)</td>
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<td>C=O stretching vibrations (non-bonded)</td>
<td>1720</td>
<td>1730</td>
<td>1730</td>
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<td>C=O stretching vibrations (H-bonded)</td>
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<td>1680</td>
<td>1670</td>
</tr>
<tr>
<td>N-H deformation and C=N</td>
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<td>1640</td>
<td>1640</td>
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<tr>
<td>C=N (conjugated)</td>
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<td></td>
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<tr>
<td>C-H bonding vibrations (as assymmetrical methyl)</td>
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<td>1440</td>
<td>1440</td>
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<td>C-H bonding vibrations (symmetrical methyl)</td>
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<td>1370</td>
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<tr>
<td>C-H deformation and C-C stretching vibrations</td>
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<td>1160</td>
<td>1145</td>
</tr>
<tr>
<td>N-H out-of-plane bonding or wagging</td>
<td>740-720</td>
<td>720-740</td>
<td></td>
</tr>
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*a = amide I band  
*b = amide II band
<table>
<thead>
<tr>
<th>Functional groups</th>
<th>Group frequencies</th>
<th>Lit. data</th>
<th>Exp. data</th>
<th>Diureide data</th>
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<td>N-H stretching vibrations bonded NH</td>
<td>3220-3180</td>
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<td>3200</td>
<td>3240</td>
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<td>Bonded NH (cis &amp; trans)</td>
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<td>3060</td>
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<tr>
<td>C-H stretching vibrations (symmetrical)</td>
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<td>2850</td>
<td>2830</td>
<td>2860</td>
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<tr>
<td>C=O stretching vibrations (I band)</td>
<td>1700-1665</td>
<td>1680-1630</td>
<td>1760</td>
<td>1760</td>
</tr>
<tr>
<td>Secondary amides or ring fused lactams</td>
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<td>1510</td>
<td>1510</td>
<td>1515(^b)</td>
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<tr>
<td>Combination bonds of N-H deformation and C-N stretching vibrations</td>
<td>1305-1200</td>
<td>1340</td>
<td>1340</td>
<td>1260</td>
</tr>
<tr>
<td>Combination amide III</td>
<td>1135</td>
<td>1130</td>
<td>1110</td>
<td>1145</td>
</tr>
<tr>
<td>C-H deformation and C-C stretching</td>
<td>ca700</td>
<td>720-740</td>
<td></td>
<td></td>
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<tr>
<td>NH deformation vibration</td>
<td>770-620</td>
<td>740</td>
<td>740</td>
<td></td>
</tr>
</tbody>
</table>
found in the diureide spectrum. As in the secondary amines, a fairly prominent C-N-C out-of-phase stretching band near 1130 cm\(^{-1}\) is present in glycoluril. The characteristic bands at 3200 cm\(^{-1}\), 740 - 720 cm\(^{-1}\) are due to the deformation overtone of NH\(_2\) and an NH\(_2\) wag band. The expected CH\(_2\) wag, twist and rock vibration are present at 2930 cm\(^{-1}\), 2850 cm\(^{-1}\) and 1460 cm\(^{-1}\) in diureide spectra (35 - 38). The analysis of the data in Table I and II, leads us to believe that the compound isolated is not a glycoluril but rather a diureide as reported by Lugosi et al. (20).

In order to gain further information, mass desorption and electron impact mass spectrometric data were collected on glycoluril and other diureides. The molecular weight for glycoluril was found to be 142 as calculated. Fragmentation patterns on glycoluril gave the proposed ions and molecular ion. (See Scheme 7).

![Scheme 7](image.png)

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Since the mass spectrum displays a peak m/e 28 mass units above the molecular ion peak, the three most likely candidates for a species of mass 28, the CH₂=CH₂, C=O, N₂ can be taken into consideration (39). Since glycoluril possessed a C=O grouping, the abundance of fragments with m/e 28 is easily visualized, and possibility of contamination of the sample can be eliminated. Similarly, the proposed fragmentation pattern on ethylmethyldiureide were obtained as indicated in Scheme 8.

\[
\begin{align*}
&\text{CH}_3 \\
&\text{C} = \text{N} - \text{C} - \text{NH}_2 \\
&\text{C} = \text{N} - \text{C} - \text{NH}_2 \\
&\text{C}_2\text{H}_5
\end{align*}
\]

\[\text{A} \rightarrow \text{B} \rightarrow \text{C} \rightarrow \text{D}\]

\[
\begin{align*}
&M \rightarrow \text{M-CO} \rightarrow \text{M-85} \\
&m/e 184 \quad m/e 156 \quad m/e 99
\end{align*}
\]

**SCHEME 8**

From the fragmentation patterns on glycoluril and the diureide, no conclusive evidence with regard to the structure can be deduced. (Compare Scheme 7 to Scheme 8, and see Table II).

Furthermore, 3a-methyl-6a-phenylglycoluril and the
corresponding diureide (methylphenyldiureide) were synthesized. The glycoluril was synthesized via the base-catalyzed reaction (see CHAPTER III).

Mass spectrometric (FD) data of both compounds indicated that there was a phenyl radical and a molecular ion with m/e of 232.28. The electron impact mass spectrometric data gave fragmentation patterns similar for both compounds as shown in Table III, and only relative abundance values were found to be different. Both contained peaks at m/e of 28 as shown by previous compounds.

Since 3a-methyl-6a-phenylglycoluril was synthesized via base-catalyzed reaction and because when dissolved in acidic media it gave a pink-colored solution with a similar visible spectrum to that of the corresponding diureide, one can possibly conclude that the glycoluril was being converted via rearrangement to the same protochromogen as the diureide.

Table IV lists the U.V. absorption maxima for some of the starting materials, hydantoin, triazine, glycoluril and diureide. The data proposes the structure of the protochromogen to be similar to diacetylmonosemicarbazone and not to hydantoin, triazine or glycoluril.

Therefore, a diureide as a possible structure for the protochromogen of the urea-diacetylimonoxime reaction can not be ruled out.
<table>
<thead>
<tr>
<th>Possible ions formed</th>
<th>Phenylmethylglycolauril</th>
<th>Phenylmethylbiureide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>m/e</td>
<td>% R.A.</td>
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<tr>
<td>[C=O]⁺</td>
<td>28.06</td>
<td>94.78</td>
</tr>
<tr>
<td></td>
<td>32.06</td>
<td>13.42</td>
</tr>
<tr>
<td>[CH₃-C=NH]⁺</td>
<td>42.12</td>
<td>32.65</td>
</tr>
<tr>
<td>[NH=C=O]⁺</td>
<td>43.12</td>
<td>15.07</td>
</tr>
<tr>
<td>[NH₂-C=O]⁺</td>
<td>44.09</td>
<td>100</td>
</tr>
<tr>
<td>[NH₂-C-NH]⁺</td>
<td>58.15</td>
<td>26.77</td>
</tr>
<tr>
<td></td>
<td>59.18</td>
<td>43.16</td>
</tr>
<tr>
<td>[C₆H₅]⁺</td>
<td>77.15</td>
<td>28.05</td>
</tr>
<tr>
<td>[CH₃-C=N-C-NH₂]⁺</td>
<td>85.18</td>
<td>37.94</td>
</tr>
<tr>
<td></td>
<td>105.21</td>
<td>6.79</td>
</tr>
<tr>
<td>[C₆H₅-C=NH]⁺</td>
<td>104.21</td>
<td>54.79</td>
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<tr>
<td>[C₆H₅CH=CH-CH₃]⁺</td>
<td>120.24</td>
<td>12.02</td>
</tr>
<tr>
<td>[C₆H₅-C=N-C-NH₂]⁺</td>
<td>147.24</td>
<td>99.63</td>
</tr>
<tr>
<td></td>
<td>174.24</td>
<td>25.47</td>
</tr>
<tr>
<td>[M-58]⁺</td>
<td>188.31</td>
<td>13.31</td>
</tr>
<tr>
<td>[M-44]⁺</td>
<td>189.34</td>
<td>56.09</td>
</tr>
<tr>
<td>[M-43]⁺</td>
<td>204.34</td>
<td>10.34</td>
</tr>
<tr>
<td>[M-CO]⁺</td>
<td>232.37</td>
<td>45.77</td>
</tr>
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</table>
**TABLE IV**

**CORRELATION OF ABSORPTION MAXIMA**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Absorption maxima</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>195</td>
<td>Water</td>
</tr>
<tr>
<td>2,2-Butanedione</td>
<td>228</td>
<td>Alcohol</td>
</tr>
<tr>
<td>2,3-Butanedionemonoxime</td>
<td>230</td>
<td>HCl</td>
</tr>
<tr>
<td>Hydantoin</td>
<td>223</td>
<td>NaOH</td>
</tr>
<tr>
<td>1,3,5-Triazine</td>
<td>272</td>
<td>Isooctane</td>
</tr>
<tr>
<td>Glycoluril</td>
<td>236,213</td>
<td>50% HCl</td>
</tr>
<tr>
<td>Diacetylmonosemicalbazone</td>
<td>268</td>
<td>Acid</td>
</tr>
<tr>
<td>U + DAM + HCl</td>
<td>268,226</td>
<td>HCl</td>
</tr>
<tr>
<td>Glyoxal + U</td>
<td>265,205</td>
<td>HCl</td>
</tr>
<tr>
<td>3a,6a-Dimethylglycoluril</td>
<td>256</td>
<td>Alcohol</td>
</tr>
<tr>
<td>5,5-Dimethylhydantoin</td>
<td>219</td>
<td>Alc-KOH</td>
</tr>
</tbody>
</table>

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D. CONCLUSIONS

The urea-diacetylmonoxime reaction was studied in acid medium. A product was isolated (white crystals) which melted above 300°C. Mass spectral data (desorption mass spectrometry) indicates that the molecular weight of the compound was 170 which corresponds to both diureide and 3a,6a-dimethylglycoluril. Electron impact mass spectral data indicates the presence of the base peak at mass 28 which represents the $[\text{C}=\text{O}]^{+}$ ion which is as easily fragmentable from glycoluril as from the diureide. No conclusive evidence can be derived from mass fragmentometry. Usually, the interaction between an $\alpha$-dicarbonyl compound and urea in the presence of acids or alkali does not result in the formation of a 4,5-dihydroxy-2-imidazolidone but produces a hydantoin instead. Since hydantoin (5,5-dimethylhydantoin) when dissolved in HCl did not give the typical color reaction of the urea-diacetylmonoxime, it must not be the protochromogen of the reaction. Hydantoins absorb at $\lambda_{\text{max}}$ 256 nm and, therefore, the absence of this absorption band indicates that the reaction does not produce hydantoin under the reaction conditions used.

Veniamin and Vakirtzi-Lemonias (27) have demonstrated the linearity of molar response in pigment production by the glycoluril, with and without exposure to light. The standard curves obtained for 3a,6a-dimethylglycoluril...
show some decrease in color intensity after 48 hours. They have also studied the time course conversion of 3a,6a-dimethylglycoluril ($\lambda_{\text{max}} = 478 \text{ nm}$). As the color starts developing, the absorption at $\lambda_{\text{max}} 256 \text{ nm}$ starts decreasing. This indicates that 3a,6a-dimethylglycoluril is being transformed into some other product in the acid medium which must be the actual chromogen. Therefore, it is possible that the glycoluril may be transformed into the open chain diureide, which then represents the chromogen or protochromogen in solution in the urea diacetylmonoxime reaction.

Diels (21, 40) has shown that diacetylmonosemicarbzone was apparently, unaffected by either cold dilute alkali or warm sodium ethoxide (26). Boiling acetic acid hydrolyzes it to diacetyl. However, the cyclization to a triazine of diacetylmonosemicarbzone was ultimately achieved by refluxing it in 2 N sodium hydroxide (41). Therefore, it would appear that under the urea-diacetylmonoxime reaction condition one could safely eliminate the possibility of triazines as protochromogens. (See Scheme 9).

As previously reported (20), a positive nitrous acid test also confirms the open chain structure for the isolated product.
The condensation of diacetylmonoxime with urea can then be visualized as shown in Scheme 10.

Thus one can safely predict the existence of diureide as the major protochromogen of the urea-diacetylmonoxime reaction.
CHAPTER II

INVESTIGATION OF THE FUNCTION OF THIOSEMICARBAZIDE IN THE UREA-DIACETYLMONOXIME REACTION

A. INTRODUCTION

The original carbamido reaction of Fearon (8) has been modified by various workers in order to stabilize and/or intensify the color of the diacetylmonoxime reaction with urea. The modifications involves addition of one or a combination of the following substances to the reaction:

- semidine (27)
- potassium persulfate (10, 42-44)
- arsenic acid (45 - 49)
- cations (5, 50 - 52)
- phenazone (53 - 56)
- p-dimethylaminobenzaldehyde (57)
- phenylantranilic acid (5, 58)
- glucuronolactone-glucosaccharodilactone (19, 59)
- thiosemicarbazide (5, 60 - 62)

It has been shown that when diacetyl is used instead of diacetylmonoxime, the color intensity of the chromogen seems to be one tenth of that of diacetylmonoxime. The more intense color with diacetylmonoxime has been attributed to slow production of diacetyl and continuous destruction of the generated hydroxylamine by the acid medium of the reaction. Therefore, the function of the oxidizing agent seems to be mainly concerned with the destruction of the hydroxylamine formed in the reaction. However, it is not unlikely that it might have other involvement in the reaction which is not clear at the present time.
Color reactions of carbazides and carbamides with diacetyl and diacetyldioxime in warm hydrochloric acid were carried out by Smith (32) in 1935 and used for rapid differential qualitative tests for these compounds. Semicarbazide and diacetyl in warm hydrochloric acid gave a red coloration; on subsequent addition of ammonia, bluish-violet colors developed.

Lugosi et al. (20) observed that when the solution of diacetylmonosemicarbazone was subjected to the standard conditions (dissolved in acid medium with application of heat) for the determination of urea in clinical laboratories (5), a pink solution was obtained. The data in Table V was obtained from their work on diacetylmonosemicarbazone.

Veniamin and Vakirtzi-Lemonias (27) suggested that semidine or thiosemicarbazide is the final electron acceptor from the chromogen (glycoluril) and proposed the reaction mechanism as presented in Scheme 11.

A continuous shift of the equilibrium to the right can be visualized by presuming that the formation of the color pigment is irreversible. They observed that addition of semidine or thiosemicarbazide produce a more stable pink color with $\lambda_{\text{max}} = 535$ nm and conclude that the thiosemicarbazide is the final electron acceptor in the above reaction.

From the study of the urea-diacetylmonoxime in CHAPTER I and work published by Lugosi et al. (20), the
open diureide structure for this reaction must be taken into consideration. The main object of the project was to try and find out, if possible, the functional involvement of thiosemicarbazide in the urea-diacetyl reaction, and study the nature of the protochromogen(s) in this reaction. Therefore, the spectral studies in the visible and the ultraviolet range of the above reaction under standard conditions for the determination of BUN in the clinical laboratory was undertaken. At the same time, an attempt was made to isolate the chromogen(s) of the reaction and/or synthesize the product(s) under concentrated reagent conditions, and obtain I.R., U.V. and mass spectrophotometric data on the product(s).
<table>
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<tr>
<th>Functional groups</th>
<th>Frequencies cm(^{-1})</th>
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<tr>
<td>N-H stretching vibrations</td>
<td>3490, 3380</td>
<td>268 nm; (\varepsilon = 8.0 \times 10^6)</td>
<td>(\text{CH}_3\text{-C=N-NH-C-NH}_2)</td>
</tr>
<tr>
<td>asy. &amp; sym.</td>
<td>3220</td>
<td>210 nm; (\varepsilon = 5.2 \times 10^6)</td>
<td>(\text{CH}_3\text{-C=O})</td>
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<td>Amide I band (carbonyl)</td>
<td>1680</td>
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<td>C-C stretching vibrations</td>
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<tr>
<td>C-N stretching vibrations</td>
<td>880, 755</td>
<td></td>
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In addition, a comparison study of the reaction of diacetylmonoximethiosemicarbazone with urea in acid medium, with that of the urea-diacetylmonoxime-thiosemi-carbazide, reaction was carried out.
B. EXPERIMENTAL

1. APPARATUS

The apparatus used was the same as those used in CHAPTER I section B.1. The freeze drier used was Model USM-15 (Universal sub-mobile 15) purchased from the Virtis Company Inc., Gardiner, N.Y. 12525.

2. REAGENTS

Urea and 2,3-butanedionemonoxime (diacetylmonoxime) Reagent grade were obtained from Fisher Scientific Company, Fairlawn, New Jersey.

Thiosemicarbazide was purchased from Eastman Kodak Company, Rochester, N.Y.

Stock urea solution: Urea (0.50 g) was dissolved and diluted with distilled water saturated with benzoic acid to 1000 mL.

Working urea solution: Stock urea solution (10 mL) was diluted with distilled water to 100 mL.

Diacetylmonoxime stock solution: DAM (25.0 g) was dissolved in a 1000-mL volumetric flask and diluted to the mark with distilled water.

Thiosemicarbazide stock solution: TSC (5.0 g) was dissolved in a one-liter volumetric flask and diluted to the mark with distilled water.

Color Reagent: This was made by adding 67 mL of DAM (stock) to 67 mL of TSC (stock) solution. They were
mixed together in a one-liter volumetric flask and diluted to one liter with distilled water.

Ferric chloride solution: FeCl₃·6H₂O (19.0 g) was dissolved in a one-liter volumetric flask with distilled water. Phosphoric acid (300 mL of 85%) was added to it, and the mixture was diluted to the mark with distilled water.

20% Sulfuric acid solution: This solution was prepared by adding 200 mL of concentrated sulfuric acid to 700 mL of distilled water in a one-liter volumetric flask, and diluted with sufficient quantity of distilled water to the 1000 mL mark.

Working acid solution: The ferric chloride solution (1.0 mL) was pipetted in a one-liter volumetric flask and diluted to the mark with 20% sulfuric acid.

2,3-Butanedioneoximethiosemicarbazone was purchased from Eastman Organic Chemicals, Distillation Products Industries, Rochester, N.Y.

Stock color reagent: DAMTSCO (0.3524 g [2 x 10⁻³ moles]) was dissolved in 50 mL of concentrated HCl in a 100-mL volumetric flask and diluted to the mark with distilled water. A yellow solution was obtained which changes color to orange on standing at room temperature.

Working color reagent: The stock DAMTSCO was diluted 1/10 with distilled water.

Stock urea solution: Urea (0.5 g [8.3 x 10⁻³ moles]) was dissolved in a 100-mL volumetric flask with distilled water.
Working acid solution: Concentrated HCl (50 mL) was diluted to 100 mL with distilled water (50% HCl).

Acetonitrile (Certified) was purchased from Fisher Scientific Company, Fairlawn, N.J. 07410.

3. METHODS

(a) The Measurement of Urea Nitrogen by Diacetylmonoxime Procedure

The manual procedure by Bousquet et al. (63) which was a variation of the automated procedure (5, 34, 64, 65) was used. The working urea solution (0.1 and 0.2 mL) was pipetted into the corresponding tubes marked #1 and #3. To the tube #1 was added 0.1 mL of distilled water. To the tube #2 was added 0.2 mL of distilled water. Working color reagent (4 mL) and working acid reagent (4 mL) were transferred to all the tubes. The tubes were covered with a piece of aluminum foil and heated in a boiling water bath for 15 minutes, then cooled in running water. The absorption spectra were obtained by scanning the solutions from 400 nm to 600 nm against distilled water. The absorption spectra of the developed color (absorption maximum, 525 nm) corresponded to that of the literature data (5, 63 - 65).

The ultraviolet spectral study of the developed color was performed on a 100-fold dilution of the above mixtures.
Modified Procedure for the Measurement of Urea Nitrogen

Instead of the above working acid solution, 50% HCl was substituted as the working acid. In this procedure, tube #1 was the blank, tube #2 contained 0.1 mL working urea standard with 0.1 mL of distilled water, #3 contained 0.2 mL of working urea standard, and tube #4 had 0.2 mL working urea standard and 4 mL of 20% sulfuric acid instead of 50% HCl.

For the ultraviolet spectral study, all the above solutions were diluted 100-fold.

(c) Isolation of the Protochromogen from the Urea-Diacetyl Reaction with Thiosemicarbazide

Thiosemicarbazide (3.0 g [0.03 mole]), 2.0 g (0.03 mole) of urea and 3.3 g (0.03 mole) of diacetylmonoxime were each dissolved separately in 15 mL of distilled water. The urea and DAM solutions were mixed and 25 mL of 85% phosphoric acid was added, followed by TSC solution and 30 mL of distilled water. The reaction mixture was then heated to 100°C on a hot plate for 15 minutes. A red solution was obtained containing a white precipitate. The mixture was cooled and filtered, yielding 5 g (71.8%) of nearly white solid. The red filtrate was kept for further n-butanol extraction treatment. The precipitate was dissolved in DMF and treated with Norit A, boiled and filtered. To this filtrate was added acetone, in order to obtain a white crystalline product whose field desorption mass spectrometric data indicated
the molecular weight to be 232.78, which corresponds to that of diacetyldithiosemicarbazone. Infrared spectra suggested an open chain structure for the compound. The red filtrate was extracted several times with small amounts of n-butanol. The butanol extract was dried over anhydrous magnesium sulfate and concentrated by vacuum distillation to about 3 mL. The concentrate was first treated with 1:3 mixture of methanol: ethylacetate followed by acetone to obtain a black-blue solid. Further spectroscopic work was done on this crude product.

(d) Attempted Isolation of the Protochromogen from the Urea-Diacetylmonoxime Reaction with Thiosemicarbazide under Clinical Analytical Conditions

An identical extraction to the above was carried out with n-butanol at the clinical concentration of urea. A pink-colored butanol extract was obtained, however, a negligible amount of black-blue precipitate was obtained from the butanol concentrate obviating any analytical work. Several attempts to obtain crystalline material from the concentrates were unsuccessful.

(e) Spectral Study of the Reaction Mixture of 2,3-Butanedioneoximidethiosemicarbazone with Urea

To test tube #1, was added 20 μL of stock urea solution. To tubes #2 and #3 were added 20 μL of distilled water. Color reagent (4 mL) was transferred to all the tubes followed by 4 mL of working acid. The tubes #1 and #2 were covered with aluminum foil and heated for 15 minutes in a boiling water bath. The
absorption spectra of the cooled solutions were measured against 50% HCl solution as a blank. The ultraviolet spectral study was conducted on the developed color solutions as well as on the unincubated sample after a 100-fold dilution with distilled water.

(f) Isolation of the Protochromogen from Urea-Diacetylmonoxime Reaction with Thiosemicarbazide

In a porcelain evaporating dish were weighed out 1.5 g (0.025 mole) of urea. A solution of 2.27 g (0.025 mole) thiosemicarbazide was prepared by dissolving it in a few mL of 50% HCl. This was added to urea with a solution of diacetylmonoxime (2.5 g [0.025 mole]) which was dissolved in a minimum amount of 50% HCl. The mixture was heated on a steam bath with agitation. Within about 5 minutes a pink-purple solution was formed. The solution was then concentrated on the steam bath to about 1.5 - 2.0 mL. A few milligrams of blue crystalline product were recovered. Spectral studies were carried out on the reaction concentrate.

(g) Isolation of the Protochromogen of the Urea-Diacetyl-thiosemicarbazidemonoxime Reaction

The procedure was identical to the reaction of urea-diacetylmonoxime with thiosemicarbazide as described in 3 (f). The concentrate obtained was pink-blue in color (1.5 - 2.0 mL). The procedure was further modified by running the reaction in acetonitrile instead of an aqueous medium. The concentrate produced was identical
to that of the aqueous reaction. Both of the concentrates were further used to study the visible and ultraviolet spectral characteristics of the concentrate.

Further trials involved different combinations of the molar ratios of the reactants. All of these produced pink-purple-blue solutions.

(h) Crystallization of Products from Reactions in Section B. 3 (f) and B. 3 (g).

The freeze drier model USM-15 was pre-cooled to -40°C, then the drier was loaded with the above two products (approximately 2 mL of each in their respective 50-mL porcelain evaporating dish). A vacuum was then applied until the pressure reached 100 microns. Next, the shelf heater was turned on to 30°C, and the vacuum continued until it reached a reading of 5 - 10 microns. No crystallization of the concentrates was achieved.
C. RESULTS AND DISCUSSION

The reaction of diacetylmonoxime-urea with thiosemicarbazide in acid medium produced a pink (red) color whose \( \lambda_{\text{max}} \) was 525 nm. The shift in \( \lambda_{\text{max}} \) from 480 nm to 525 nm indicates possibly a \( \pi \) electronic system which contains more conjugation than the diacetylmonoxime interaction with urea. Figures 1 and 2 represent the scans of the reaction according to the AutoAnalyzer® I procedure and the reaction in HCl, respectively. The first system contains ferric ions while the second system does not contain any oxidizing metal ions. Since the color produced in both have the same \( \lambda_{\text{max}} \), one can conclude that the ferric ion plays no significant role in the color formation of the above reaction, except that it may act as a catalyst.

Further investigation of the reaction mechanism was carried out using an ultraviolet spectroscopic procedure, since it can be useful in the determination of the structure of molecules containing conjugated systems of double bonds. The presence of conjugated unsaturation always results in one or more intense absorption maxima at wavelengths greater than 200 nm. Therefore, very useful correlations can be deduced for the positions and intensities of these peaks as a function of the structure of the conjugated system. Presence of O, N, S, or halogen atoms in the compound can absorb light in...
FIGURE 1

VISIBLE SPECTRA OF DIACETYLMONOXIME-UREA WITH THIOSEMICYCARBAZIDE IN 20% H₂SO₄ (AAI METHOD)

Legend

1. Incubated for 15 minutes at 100°C
   Conc: DAM: 1.65 x 10⁻² M
   TSC: 3.45 x 10⁻³ M
   Urea: 8.33 x 10⁻⁴ M
   Total reaction volume: 8.1 mL
   $\lambda_{\text{max}}$ 525; $\epsilon = 1.27 \times 10^4$

2. Working reagent incubated

3. Incubated for 15 minutes at 100°C
   Conc: DAM: 1.65 x 10⁻² M
   TSC: 3.45 x 10⁻³ M
   Urea: 16.6 x 10⁻⁴ M
   Total reaction volume: 8.2 mL
   $\lambda_{\text{max}}$ 525; $\epsilon = 1.32 \times 10^4$

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FIGURE 1

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FIGURE 2

VISIBLE SPECTRA OF UREA-DIACETYLMONOXIME REACTION WITH THIOSEMICARBAZIDE IN 50% HCl

Legend

Conc: DAM: $1.65 \times 10^{-2}$ M
TSC: $3.45 \times 10^{-3}$ M

Ratio: DAM/TSC = 4.8; DAM/Urea = 19.8
Incubation time: 15 minutes at 100°C

1. Reagent blank (pale yellow-green)

2. Urea conc: $8.33 \times 10^{-4}$ M
   $\lambda_{\text{max}}$ 525; $\varepsilon = 0.84 \times 10^4$; pink solution

3. Urea conc: $16.6 \times 10^{-4}$ M
   $\lambda_{\text{max}}$ 525; $\varepsilon = 0.88 \times 10^4$; pink solution

4. Urea conc: $16.6 \times 10^{-4}$ M
   $\lambda_{\text{max}}$ 525; $\varepsilon = 1.32 \times 10^4$; pink solution

This was run in 20% H$_2$SO$_4$
FIGURE 2

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the ultraviolet range because of the presence of unshared pairs of electrons (n electrons) of these atoms. The two features of an ultraviolet spectrum which can give information concerning molecular structure are:

1. the wavelength at which the maximum light is absorbed ($\lambda_{\text{max}}$).

2. the intensity of absorption at that wavelength.

Since many unsaturated compounds absorb strongly, even at a very low concentration, the reaction mixture of the diacetylmonoxime-urea with thiosemicarbazide was diluted 1/100 before taking any readings in the ultraviolet region. Figure 3 illustrates the $\lambda_{\text{max}}$ for the reaction according to the AAL procedure. When the reaction mixture was incubated, the spectrum was identical to that of diacetylmonoxime-urea with thiosemicarbazide. Measurement against working acid solution did not give very intense absorption peaks for a proper evaluation of the result. Urea, which absorbs around 198 nm, appeared nearly transparent at this concentration, because the absorption band was completely obscure and weak. It was therefore not used to study the reaction in the phosphoric-sulfuric-Fe$^{+3}$ working acid medium.

Hydrochloric acid was substituted for sulfuric-phosphoric acid and the reaction was repeated. Figure 4 shows the shape and absorption maximum of the absorption curve which has an identical $\lambda_{\text{max}}$ to that of the sulfuric acid reaction. The reaction conditions were
FIGURE 3

ULTRAVIOLET SPECTRA OF DIACYTLMONOXIME-UREA REACTION
WITH THIOSEMICARBAZIDE IN 20% H₂SO₄ (AAI METHOD)

Legend

1. Pink colored solution after incubation
   for 15 minutes at 100°C
   Conc: Urea: 8.33 x 10⁻⁴ M
   DAM: 1.65 x 10⁻² M
   TSC: 3.45 x 10⁻³ M
   Total reaction volume: 8.1 mL
   Dilution for ultraviolet: 1/100
   λ_max 268; ε = 2.05 x 10⁶
   λ_max 226; ε = 3.23 x 10⁶

2. Reagent blank (TSC + DAM), incubated
   λ_max 268; λ_max 226

3. #1 read against distilled H₂O instead
   of (4 mL 20% H₂SO₄ + 4 mL H₂O)
   λ_max 268; λ_max 226

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almost identical with that of the sulfuric acid method. The ultraviolet spectrum in Figure 4 illustrates the $\lambda_{\text{max}}$ which coincides with that of the sulfuric acid reaction. However, the results of this experiment also indicates almost an identical absorption intensity even though different concentrations of urea were used. Absorption due to urea was obscure and could not be resolved as a sharp peak even after changing the slit width. The identical absorption intensity could be explained by nonstoichiometric proportions of the working color reagent compound to urea concentration. (DAM = 1.65 x 10^{-2}M; TSC = 3.45 x 10^{-3}M; urea = 8.33 x 10^{-4}M; DAM/TSC = 4.8; DAM/urea = 19.8). Molar absorptivity of the protochromogen at these concentrations in both HCl and sulfuric acid reaction are listed. (See Figure 1 and 2).

From Figures 1 and 2, it was clear that the color formation is proportional to the urea concentration, the time of incubation, and probably to the acidity of the reaction medium. Therefore, besides the pink color, other chromogens (yellow) are formed depending upon the reaction conditions. A mass spectrometric study of the isolated material proved that there was more than one compound formed.

The n-butanol extract of diacetylmonoxime-urea reaction with thiosemicarbazide in acid medium (HCl) at relatively high concentrations of the starting materials showed an absorption at 265 nm and 202 nm. The $\lambda_{\text{max}}$ due
FIGURE 4
ULTRAVIOLET SPECTRA OF UREA-DIACETYLMONOXIME REACTION
WITH THIOSEMICARBAZIDE IN 50% HCl

Legend

Conc. DAK: \(1.65 \times 10^{-2}\) M

TSC: \(3.45 \times 10^{-3}\) M

Total reaction volume: = 8.2 mL

Incubation time: 15 minutes at 100°C

1. Urea solution: \(8.33 \times 10^{-4}\) M

\(\lambda_{\text{max}}\) 268; \(\lambda_{\text{max}}\) 226

2. Urea solution: \(16.6 \times 10^{-4}\) M

\(\lambda_{\text{max}}\) 268; \(\lambda_{\text{max}}\) 226

3. Reagent blank

\(\lambda_{\text{max}}\) are identical with #1 and #2

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to diacetyl at 228 nm and TSC at 237 nm were absent since the reagents were close to stoichiometric proportions.

In order to prove the relationship of the ultraviolet spectral data to the open chain structure of the chromogen, a compound, 2,3-butanedioneoximethiosemicarbazone (DAMTSCO) was selected as a substitute for the color reagent from the previous experiments (DAM + TSC). In the first experiment a $1.35 \times 10^{-2}$ M concentration of the above compound was used in concentrated HCl. This stock solution was yellow to start with and turned orange on standing at room temperature during the next 24 hours. The reaction mixture obtained, by use of DAMTSCO with urea, was green to green-orange. (See Scheme 12). It was clear from this experiment that the concentration of the stock solution had to be cut down.

![Scheme 12](image)

A second experiment consisted of a 1/10 dilution of the stock reagent (i.e., conc. = $1.35 \times 10^{-3}$ M). The color produced by this reagent with urea ranged from pale-green to purple. The solutions changed their color to pale-yellow to pink-red on standing at room temperature for 24 hours.
This observation indicated that the generation of and/or destruction of hydroxylamine may be slow and could be responsible for these changes. However, the visible spectrum (Figure 5) showed that the $\lambda_{\text{max}}$ of both the pink and the purple solution was 525 nm. The only part of the spectrum that changed during the 24-hour interval was the yellow portion (i.e., $\lambda = 400 \text{ nm} - 425 \text{ nm}$). The final pink color was identical to the pink color produced by the $\text{H}_2\text{SO}_4$-$\text{H}_3\text{PO}_4$ and $\text{HCl}$ reactions. It is possible that the green color could be eliminated, if the reaction was carried out at 1/100 dilution of the stock and if the acidity of the solution was reduced (dil. $\text{HCl}$ instead of conc. $\text{HCl}$). However, this would produce a decrease in color due to reduction in acidity.

A similar yellow-greenish color was produced when one incubated the color reagent ($\text{DAM} + \text{TSC}$) in 50% $\text{HCl}$ for 15 minutes at 100$^\circ$C. An additional observation was that the resulting pink color of the reaction of urea-diacetylmonoxime with thiosemicarbazide in 20% $\text{H}_2\text{SO}_4$ slowly decreased at room temperature and eventually turned orange-yellow within the next 48 hours, with the formation of a minute amount of a white precipitate. The amount of precipitate formed was too small for characterization. The $\lambda_{\text{max}}$ shown in the ultraviolet spectrum had shifted to 292 nm and 260 nm, while the visible absorption had shifted to 457 nm and 433 nm (hypsochromic shift).

A solution of hydroxylamine $\text{HCl}$ was measured in $\text{HCl}$.
FIGURE 5

VISIBLE SPECTRUM OF 2,3-BUTANEDIONEOXIMETHIOSEMICARBAZONE
WITH UREA IN 50% HCl

Legend

Conc. DAMTSCO: $2.02 \times 10^{-3}$ M
Urea: $8.33 \times 10^{-2}$ M
Total reaction volume: 8.02 mL
Incubation time: 15 minutes at 100°C
$\lambda_{\text{max}}$ 525 nm; $\varepsilon = 2 \times 10^4$

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and a $\lambda_{\text{max}}$ at 202 nm was found to be present. A similar peak at 198 nm was found with the DAMTSCO reaction with urea (Figure 6). The ultraviolet spectrum shows two sharp peaks at 272 nm and 235 nm which are similar to those of the other diacetylmonoxime-urea reaction mixture with thiosemicarbazide. The absence of a peak at 226 nm is due to the omission of diketone from the reaction mixture.

The peak at 235 nm indicated the presence of the thiosemicarbazide type structure in the reaction mixture ($2.02 \times 10^{-3} \text{M} \text{DAMTSO}; \text{urea} = 8.33 \times 10^{-2}\text{M}$). The $\lambda_{\text{max}}$ at 268 nm and 272 nm which were present in the reactions of DAM + TSC and DAMTSCO indicated the existence of the pink chromogen.

Several attempts were made to synthesize or isolate the chromogen from either dilute reaction mixtures (clinical condition of BUN reaction) or concentrated reaction solutions (stoichiometric ratio of urea to the color reagents). In the first instance the dilute reaction mixture was extracted with n-butanol. The red extract was concentrated by means of vacuum distillation and the distillate solidified by treatment with a 1:1 ethylacetate-acetone mixture. A blue-black solid was obtained. When this material was dissolved in a solution of HCl a yellow-pink colored solution resulted, which could indicate that the chromogen might have decomposed during the isolation procedure. The second attempt to obtain the chromogen
FIGURE 6

ULTRAVIOLET SPECTRA OF 2,3-BUTANEDIONEOXIME-THIOSEMICARBAZONE WITH UREA IN HCl

Legend

Conc. DAMTSCO: $2.02 \times 10^{-3}$ M
Urea: $8.33 \times 10^{-2}$ M
Total reaction volume: 8.02 mL
Incubation time: 15 minutes at 100°C
Dilution 1/100 for ultraviolet measurement

1. Purple solution
   $\lambda_{\text{max}}^{\text{268 nm}}; \lambda_{\text{max}}^{235 \text{ nm}}; \lambda_{\text{max}}^{197 \text{ nm}}$

2. Pink solution
   (#1 solution measured again after 24 hours)
   $\lambda_{\text{max}}^{268 \text{ nm}}; \lambda_{\text{max}}^{237 \text{ nm}}; \lambda_{\text{max}}^{198 \text{ nm}}$

3. Nonincubated DAMTSCO
   $\lambda_{\text{max}}^{297 \text{ nm}}; \lambda_{\text{max}}^{237 \text{ nm}}; \lambda_{\text{max}}^{197 \text{ nm}}$

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resulted in treatment of $2 \times 10^{-3} \text{M}$ urea with $1 \times 10^{-3} \text{M}$ of DAM and TSC, respectively. On heating this mixture on a steam bath for several minutes, followed by the addition of concentrated HCl, a red-blue concentrate was obtained. On changing the molar ratio of urea from $2 \times 10^{-2} \text{M}$ to $4 \times 10^{-3} \text{M}$ in an aqueous HCl medium, a minute amount of blue crystals was found suspended in the reaction mixture, the amount of precipitate being too small to allow its characterization.

A third attempt was carried out by performing the reaction in acetonitrile (with a few mL of conc. HCl). On heating, a similar red-blue concentrate was obtained with several crystals suspended in the concentrate. A freeze-drying process was applied to this concentrate in order to crystallize the product. This process was unsuccessful in yielding a crystalline product for characterization.

In order to prove that the same reaction product is formed when urea reacts with DAMTSCO or DAM + TSC, $1 \times 10^{-3} \text{M}$ DAMTSCO was treated with $2 \times 10^{-3} \text{M}$ urea in a solution of acetonitrile containing a few mL of concentrated HCl. A purple-blue colored concentrate was obtained within a few minutes with the formation of a blue precipitate. This precipitate was washed with acetone and visible-ultraviolet spectrophotometric studies were performed. The visible spectra of these concentrates are shown in Figure 7.
FIGURE 7
VISIBLE SPECTRA OF CONCENTRATE OBTAINED FROM THE REACTION OF UREA IN ACETONITRILE AND CONCENTRATED HCl

Legend

1. 2',3-Butanedionemonooximethiosemicarbazone
   Sample volume: 20 µL diluted to 100 mL with 50% HCl
   Pink solution: \( \lambda_{\text{max}} \) 525 nm (0.08)
   Concentration: \( 1 \times 10^{-3} \) mole DAMTSCO (0.174 g)
                  \( 2 \times 10^{-3} \) mole urea (0.120 g)
   Total concentrate volume: = 1.5 - 2 mL

2. DAM-TSC
   Sample volume: 20 µL diluted to 100 mL with 50% HCl
   Pink solution: \( \lambda_{\text{max}} \) 525 nm (0.07)
   Concentration: \( 1 \times 10^{-3} \) mole DAM (0.01 g)
                  \( 1 \times 10^{-3} \) mole TSC (0.097 g)
                  \( 2 \times 10^{-3} \) mole urea (0.120 g)
   Total concentrate volume: = 1.5 - 2 mL

3. 1 mg of blue precipitate dissolved in 50% HCl and diluted to 100 mL (precipitate obtained when \( 4 \times 10^{-3} \) mole urea was used).
   Heat was used to dissolve most of the precipitate.
Both the DAM + TSC as well as the DAMTSCO gave identical spectra and absorbance intensities (0.08 compared to 0.07 A). The blue precipitate obtained from the DAM + urea + TSC had a $\lambda_{max}$ of 532 nm which is very close to $\lambda_{max}$ of 525 nm of the reaction concentrates. Since only a few milligrams of the blue precipitate was at our disposal, a dilute solution was made for this spectral study (i.e., $4.97 \times 10^{-6}$ M assuming that the structure of the chromogen was 2,3-butanedionemonothiosemicarbazone-monoureide mol. wt. = 201).

The ultraviolet spectra of the above concentrates are shown in Figure 8. Both concentrates have identical $\lambda_{max}$ of 245 nm and 199 nm, respectively. The molar absorptivities were also similar, indicating the formation of the same substance as the pink chromogen. The blue precipitate demonstrated a similar $\lambda_{max}$ to the above. The product obtained from the n-butanol extract, however, does not give a $\lambda_{max}$ of 245 nm (262 nm: bathochromic shift). This could be due to the presence of various impurities (other chromogens formed between DAM + TSC), or partial decomposition of the chromogen as mentioned previously.

From the above data, one can summarize that when working at a stoichiometric ratio of the color reagent (DAM + TSC) and excess of urea, the absorbance maxima at 237 and 226 nm disappear, as predicted. The new $\lambda_{max}$ formed being due to the pink chromogen absorbing
FIGURE 8
ULTRAVIOLET SPECTRA OF CONCENTRATE OBTAINED FROM THE REACTION OF UREA IN ACETONITRILE AND CONCENTRATED HCl

Legend

1. 2,3-Butanedionemonooximethiosemicarbazone
   Sample volume: 20 μL of concentrate diluted to 100 mL with 50% HCl. Further dilution of 1/10 of the above with distilled H₂O was used.

2. DAM + TSC
   Sample Volume: 20 μL of concentrated diluted to 100 mL with 50% HCl. Further dilution of 1/10 of the above with distilled H₂O was used.

3. 1 mg of blue precipitate dissolved and diluted to 100 mL with 50% HCl. (see Legend for Figure 7).

4. Blue-black crystals obtained from butanol extract of DAM + U + TSC reaction. A few crystals were dissolved in 50% HCl. An orange-pinkish solution was obtained whose spectra is shown here.
at 245 nm in concentrated solutions (i.e., stoichiometric ratio) and 268 nm in dilute solutions (i.e., clinical analytical conditions). That the chromogen possibly had a structure similar to DAMTSCO (open chain), and that there is a strong possibility that the compound responsible for the pink color is 2,3-butanedionemonothiosemicarbazonemonoureide can not be ruled out. The color intensity of the reaction mixture depends on the acidity of the medium, temperature, time and the concentration of the urea in the reaction mixture. Other yellow chromogens are also formed depending on the stoichiometric ratios of the reagents employed.

All of the products formed in the urea-diacetyl-monoxime reaction with thiosemicarbazide in 50% HCl had not been isolated and fully elucidated. However, there is sufficient evidence that urea does condense with diacetyl to form an adduct as shown by Lugosi et al. (20) and in CHAPTER I. The isolated and/or synthesized adduct of DAM and 2SC, the protochromogen and DAMTSCO reaction product, all show similar open chain structure as indicated in Table VI by their characteristic infrared bands. The molecular weights were determined by field desorption mass spectrometry and are reported in Table VII. When DAMTSCO is allowed to react with urea under the condition of analysis, a pink color is developed, suggesting that it is the DAMTSCO derivative (monothiocarbazonemonoureide) which is formed in the reaction.
<table>
<thead>
<tr>
<th>Functional groups</th>
<th>Thiosemicarbazide</th>
<th>Adduct DAM + TSC</th>
<th>Protochromogen U + DAM + TSC</th>
<th>DAMTSCO reaction with urea</th>
<th>DAMTSCO</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-H stretching vibrations (primary amide solid phase)</td>
<td>3290, 3260</td>
<td>3420, 3240</td>
<td>3400, 3260</td>
<td>3410, 3240</td>
<td>3410, 3220</td>
</tr>
<tr>
<td>Combination bands of NH deformation &amp; C-N stretching vibrations amide II band</td>
<td>3200</td>
<td>3160</td>
<td>3160</td>
<td>3160</td>
<td>3180</td>
</tr>
<tr>
<td>Combination bands of NH deformation &amp; C-N stretching vibrations amide II band</td>
<td>1650, 1630</td>
<td>1610</td>
<td>1655, 1600</td>
<td>1605</td>
<td>1610</td>
</tr>
<tr>
<td>Combination bands of NH deformation &amp; C-N stretching vibrations amide II band</td>
<td>1540, 1490</td>
<td>1505, 1455</td>
<td>1500, 1455</td>
<td>1495, 1460</td>
<td>1510, 1465</td>
</tr>
<tr>
<td>Combination bands of NH deformation &amp; C-N stretching vibrations amide II band</td>
<td>1330, 1300</td>
<td>1370, 1340</td>
<td>1370, 1300</td>
<td>1375, 1340</td>
<td>1380, 1310</td>
</tr>
<tr>
<td>Combination bands of NH deformation &amp; C-N stretching vibrations amide II band</td>
<td>1300</td>
<td>1260</td>
<td>1290, 1255</td>
<td>1265</td>
<td></td>
</tr>
<tr>
<td>Combination bands of NH deformation &amp; C-N stretching vibrations amide II band</td>
<td>1170</td>
<td>1150, 1120</td>
<td>1160</td>
<td>1150, 1120</td>
<td>1160</td>
</tr>
<tr>
<td>Combination bands of NH deformation &amp; C-N stretching vibrations amide II band</td>
<td>1020</td>
<td>1050</td>
<td>1020</td>
<td>1100</td>
<td></td>
</tr>
<tr>
<td>Combination bands of NH deformation &amp; C-N stretching vibrations amide II band</td>
<td>1010</td>
<td>1060, 980</td>
<td>1090, 950</td>
<td>985, 940</td>
<td>960</td>
</tr>
<tr>
<td>Combination bands of NH deformation &amp; C-N stretching vibrations amide II band</td>
<td>800</td>
<td>850</td>
<td>835</td>
<td>.845</td>
<td>840</td>
</tr>
<tr>
<td>Combination bands of NH deformation &amp; C-N stretching vibrations amide II band</td>
<td>760, 720</td>
<td>725</td>
<td>780, 760</td>
<td>730</td>
<td></td>
</tr>
</tbody>
</table>
**TABLE VII**

MASS SPECTRAL DATA (FD)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Major m/e</th>
<th>R.A. %</th>
<th>Expected mol. wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diacetylmonothiosemicarbazone (adduct DAM + TSC)</td>
<td>158 (p)</td>
<td>100</td>
<td>159</td>
</tr>
<tr>
<td></td>
<td>M⁺ + 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diacetyldithiosemicarbazone (protochromogen from U + DAM + TSC reaction)</td>
<td>232.78 (p)</td>
<td>100</td>
<td>232</td>
</tr>
<tr>
<td></td>
<td>M⁺ + 2</td>
<td>5.60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>215</td>
<td>1.94</td>
<td></td>
</tr>
<tr>
<td>Product from DAMTSCO + U reaction</td>
<td>232 (p)</td>
<td>100</td>
<td>201</td>
</tr>
<tr>
<td>(diacetylmonothiosemicarbazonemonoureide)</td>
<td>M⁺ + 1</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M⁺ + 2</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>215</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Diacetylmonothiosemicarbazone oxime</td>
<td>174 (p)</td>
<td>100</td>
<td>174</td>
</tr>
<tr>
<td></td>
<td>M⁺ + 1</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M⁺ + 2</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>
This adduct would then have a conjugated system which should account for the bathochromic shift from 480 nm to 525 nm.
D. CONCLUSIONS

The function of thiosemicarbazide in the urea-diacetyl reaction was investigated. Addition of thiosemicarbazide produced a bathochromic shift with a hyperchromic effect.

To prove that thiosemicarbazide is involved in the condensation reaction to yield an open chain protocromogen, the reactions in Scheme 13 were carried out.

Products I and II were isolated. When dissolved in concentrated HCl, they produced an orange-pink color.
Product IV was presumed to be formed. It was not separated as it was identified in a previous study. Product III was most probably present in the isolated mixture. Due to a very limited quantity of the isolated blue material, further work on purification was not possible. Therefore, spectroscopic data was collected on the concentrate and extracts of the reaction mixture. The color reagent was substituted with 2,3-butanedioneoximethiosemicarbazone. The proposed condensation products formed are shown in Scheme 14 (compare to products in Scheme 13).

Product II was identified once again. The remaining reaction mixture gave pink color. The spectroscopic data on the reaction concentrate or extract gave a visible spectrum identical to I (Scheme 13). The ultraviolet study confirmed the absorption maxima to be similar. Therefore, the proposal showing the involvement of thiosemicarbazide in the condensation reaction on a 1:1 ratio with the diketone was substantiated. The observation of the presence of more than one chromogenic
compound was proved to be true, and depended on the reaction conditions and the amount of reactants.

The ultraviolet spectra of reactions (a) urea-diacetylmonoximethiosemicarbazone and (b) urea-1-phenyl-1,2-propanedionemonoxime-thiosemicarbazide gave spectra containing an absorption maximum at 237 nm corresponding to thiosemicarbazone moiety in both of the protochromogens, indicating the presence of a similar chromogenic reaction in both cases. The ultraviolet spectrum of 3a-methyl-6a-phenylglycoluril on the other hand contains the absorption peak at 230 nm, representing the presence of the diketone moiety in the molecule. (See Figure 9, A. B. and C.)

All these data suggest the formation of an ureide as a protochromogen in the urea diacetyl + thiosemicarbazide in the reaction to form the open chain monothiosemicarbazone with diacetyl. The presence of other protochromogens was also indicated.
FIGURE 9
COMPARATIVE ULTRAVIOLET SPECTRA

Legend

A. The ultraviolet spectrum of the 3α-methyl-6α-phenylglycoluril in HCl (pink colored solution)
B. The ultraviolet spectrum of 2,3-butanedione-monoxime-thiosemicarbazone reaction with urea in HCl (pink colored solution)
C. The ultraviolet spectrum of 1-phenyl-1,2-propanedionemonoxime reaction with urea and thiosemicarbazide in HCl (pink colored solution)

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FIGURE 9

ANOMETERS

ABSORBANCE

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CHAPTER III
INVESTIGATION OF THE REACTION INTERMEDIATE(S) OF OTHER DIKETONES AND THEIR OXIMES WITH UREA

A. INTRODUCTION

The carbamidodiacetyl method of Fearon (8) has been modified by substitution of diacetyl by 1-phenyl-1,2-propanedione or its oxime and by 2,3-pentanedione. Muller, Archibald and others (66 - 69) have used 1-phenyl-1,2-propanedione or its oxime in a H₂SO₄-H₃PO₄ medium to determine urea nitrogen. The solubility of the monoxime was found to be quite low in water, and therefore, Murayama, Archibald and Timmermans (67 - 71) have used alcohol as a solvent.

Dickenman and Zak (15) described the use of the 1-phenyl-1,2-propanedione in phosphoric acid instead of its monoxime. Velay (72) has used dioxime and has modified the Archibald procedure, while Siest (18, 73, 74) has substituted H₃AsO₄-H₃PO₄ as his acid medium. Timmermans (71) substituted HCl as the acid medium for his reaction. Therefore, the modification in the determination of urea nitrogen with 1-phenyl-1,2-propanedione involves a change in the acid medium, selection of a solvent for PPDMO, and addition of oxidants or color intensifiers to the reaction mixture.

The final color of the 1-phenyl-1,2-propanedione in an acid-catalyzed reaction with urea at 95°C was red-
purple, possessing an absorbance maximum at 540 nm. Siest (18) has shown that when phenazone was added to the reaction, a yellow-orange color with a $\lambda_{\text{max}}$ 480 nm was obtained. Thiosemicarbazide produced a blue-purple color with a $\lambda_{\text{max}}$ 560 nm.

Archibald and Muller (67, 68) has pointed out the advantages of the use of 1-phenyl-1,2-propanedionemonoxime over diacetylmonoxime or its diketone to be that the reagent was less volatile. A red color was obtained instead of yellow, and the reagent was less sensitive to citrulline and other urea derivatives.

The mechanism of the reaction involved in the formation of the red color seemed to be identical to that of the diacetylmonoxime reaction. The first step being the release of hydroxylamine from the monoxime by the acid medium, and the second step that of the condensation of the protonated diketone with urea to form the colored chromophore. Siest (18) obtained identical spectra with both the monoxime and the diketone, suggesting possibly the slow release and destruction of hydroxylamine from the monoxime. The usual incubation time was 60 minutes, however, 10 - 30 minutes could have been used with a decrease in color development. This loss in intensity could be used to an advantage, if interference from other reacting substances still in the lag phases could be reduced or kept to a minimum.

De Nieves and Pike (75) modified Archibald's (67)
method and adapted it to an ultramicro procedure for the
determination of urea nitrogen in blood serum. Their
calibration curves with urea standards indicate that
optical density was proportional to concentration be­
tween 100 - 400 µg urea nitrogen/mL. Above 400 µg urea
nitrogen/mL, a deviation from Beer's law was reported
(67, 68, 76).

The purpose of this study was to isolate the inter­
mediate(s) of the 1-phenyl-1,2-propanedione and 2,3-
pentanedione with urea in presence and absence of thio­
semicarbazide. The use of 2,3-pentanedione as a new
reagent for the determination of urea was determined.
Synthesis of the corresponding diureides of these two
diketones via an acid-catalyzed reaction was carried out:
Spectroscopic data on the synthesized diureide was com­
pared. 3a,6a-Phenylmethyglycoluril was synthesized via
a base-catalyzed reaction and its characteristic spectrum
was compared with that of its corresponding diureide.
B. EXPERIMENTAL

1. APPARATUS

The spectral studies were carried out on the equipment described in CHAPTER I, Section B 1. The procedures for handling and preparation of the samples were identical. The elemental analysis were performed by Microanalysis Laboratories Limited, 329 St. George St., Toronto, Ontario

2. REAGENTS

1-Phenyl-1,2-propanedionemonoxime and 1-phenyl-1,2-propanedione were purchased from Eastman Kodak Company, Rochester, N.Y.

2,3-Pentanedione was obtained from Chem. Service, West Chester, P.A. 19380.

3. METHODS

(a) Spectral Study of the Reaction of 1-Phenyl-1,2-Propanedionemonoxime Reaction with Urea

The manual procedure of Bousquet et al. (63) was modified. The color reagent used was 1-phenyl-1,2-propanedionemonoxime (1.03 x 10^{-3}M) instead of diacetylmonoxime and thiosemicarbazide. The concentration of urea in 0.02 mL of working standard solution was 8.3 x 10^{-6}M. The reaction was carried out under similar conditions to that of the urea nitrogen procedure. The absorption maxima were found to be 543 nm and 257 nm.
(b) Spectral Study of the Reaction of 1-Phenyl-1,2-Propanedionemonoxime and Urea with Thiosemicarbazide.

In this study, the color reagent used was a mixture of 1-phenyl-1,2-propanedionemonoxime \(1.03 \times 10^{-3} \text{M}\) and thiosemicarbazide \(3.68 \times 10^{-3} \text{M}\). Other reaction conditions were kept identical to that of Bousquet et al. (63). The absorption maxima were found to be at 568 nm, 284 nm and 237 nm. The reaction mixtures were diluted 1/20 for ultraviolet studies in (a) and (b).

(c) Synthesis of 1-Phenyl-1,2-Propanedionediucreide (PPDU).

In a round-bottomed flask, equipped with a reflux condenser, were placed 16.3 g (0.1 mole) of 1-phenyl-1,2-propanedionemonoxime. To this were added 1.2 mL of concentrated HCl through the condenser. The monoxime was dissolved on a steam bath. To the reaction mixture was added a solution of 15 g (0.25 mole) of urea in 100 mL of ethanol. The solution was refluxed for about 30 minutes on a steam bath, then allowed to cool at room temperature. A pale yellow crystalline product was formed. The crystalline product was separated by filtration and the filtrate further concentrated by solvent evaporation, upon which further additional crystals were obtained. The crystalline product was insoluble in water. When dissolved in concentrated HCl by application of heat, a pink-colored solution was formed. The yield obtained was about 14 g (=60%). The melting point was found to be above 300°C (d).
A buff-colored crystalline product, from the above filtrate, was separated, whose melting point was above 300°C (d). The product had the elemental analysis corresponding to phenylmethylureidemonoxime. This product gave pink-colored solution when dissolved in hot HCl.

(d) Synthesis of 3α-Phenyl-6α-Methylglycoluril

The procedure of Vail et al. (31) was employed to prepare 3α-phenyl-6α-methylglycoluril. A mixture of 1.41 g (0.0095 mole) 1-phenyl-1,2-propanedione, 0.996 g (0.0166 mole) of urea and 0.0178 M KOH in 40 mL ethanol was refluxed on a steam bath for about 2 hours. A brown-colored solution was obtained with some brown precipitate on filtration, and upon washing of the residue with glacial acetic acid and acetone, a white-colored product was obtained (0.21 g). Further dilution of the filtrate with water, gave large amounts of brownish product which was not washed. The melting point of the product was above 300°C (d). The elemental analysis was performed on the white product.

Anal. Calc. for C_{11}H_{12}N_{4}O_{2}: C, 56.90; H, 5.17; N, 24.14.
Found: C, 55.63; H, 5.35; N, 23.30.
(e) Synthesis of 2,3-Pentanedionediureide (PDDU)

In a round-bottomed flask equipped with a reflux condenser were added 2.5 g (0.025 mole) 2,3-pentanedione and 4.5 g (0.075 mole) urea in 5 mL of water and 20 mL of ethanol. To this mixture a one mL of concentrated HCl was added and the solution refluxed on a steam bath for about 20 - 30 minutes. The mixture was cooled and the crystalline product filtered. About 1 g of a white crystalline product was obtained with a melting point above 300°C.

Anal. Calc. for C₇H₁₂N₄O₂: C, 45.65; H, 6.52; N, 30.43. Found: C, 45.59; H, 6.94; N, 30.11.

(f) Spectral Studies on 2,3-Pentanedionediureide (PDDU)

2,3-Pentanedionediureide (2.5 x 10⁻³ g) was dissolved in 10 mL of 50% HCl. This was warmed for 15 minutes on a boiling water bath. On cooling the reaction mixture, a spectral scan of the blue solution gave an absorbance maxima at 577 nm, and 480 nm. A 1:1 dilution of the above mixture was used for the ultraviolet spectral study. The absorbance maxima were found to be at 304 nm and 212 nm.
C. RESULTS AND DISCUSSION

Absorption spectra in Figure 10 (curve #1) represents the reaction of 1-phenyl-1,2-propanedionemonoxime (PPDMO) with urea in 50% HCl at 100°C for 15 minutes. The concentration of urea used was $8.33 \times 10^{-6}$ M/0.02 mL, while that of PPDMO was $4.11 \times 10^{-6}$ M/4 mL. Excess of urea was used in order to facilitate the completion of the condensation reaction. The molar absorptivity was calculated using the concentration of PPDMO, assuming that the reaction product formed was 1-phenyl-1,2-propanedionediureide. This would also allow calculations and comparisons of molar absorptivities when propanedionediureide was used in HCl to produce the red color.

Curve #2, in Figure 10 represents the reaction of PPDMO with urea in the presence of thiosemicarbazide. The $\lambda_{\text{max}}$ obtained was 568 nm compared to 543 nm without thiosemicarbazide. This indicates a bathochromic shift of approximately 24 nm, which is similar to that of the urea-diacetylmonoxime reaction with thiosemicarbazide ($\approx 45$ nm). A hyperchromic effect has been observed in acidic media in the visible range by Ueda and Kouno (19, 66). From Figure 10 it is clear that addition of thiosemicarbazide produces a more intense blue color and, therefore, the sensitivity of urea detection could be improved by use of thiosemicarbazide. Veniamin, et al. (27) have reported that semidine might produce a
FIGURE 10
VISIBLE SPECTRA OF 1-PHENYL-1,2-PROPANEDIONEMONOXIME
REACTION WITH UREA IN 50% HCl

Legend

Total reaction volume: 8.02 mL

1. Represents the reaction without any thiosemicarbazide.
   Conc: Urea: $8.33 \times 10^{-6}$ mole/0.02 mL
   Thiosemicarbazide: zero mole
   PPDMO: $4.11 \times 10^{-6}$ mole/4 mL; $1.03 \times 10^{-3}$ M
   $U/PPDMO = 2.02; \ A = 0.47$
   $\lambda_{max} = 543$ nm; $\epsilon = 1.95 \times 10^{4}$
   pink color

2. Represents the reaction with thiosemicarbazide
   Conc: Urea: $8.33 \times 10^{-6}$ mole/0.2 mL
   Thiosemicarbazide: $1.38 \times 10^{-5}$ mole/4 mL
   $3.45 \times 10^{-3}$ M
   PPDMO: $4.11 \times 10^{-6}$ mole/4 mL; $1.03 \times 10^{-3}$ M
   $U/PPDMO = 2.0; \ \frac{PPDMO}{TSC} = 0.297; \ A = 0.627$
   $\lambda_{max} = 568$ nm; $\epsilon = 2.30 \times 10^{4}$
   blue color

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FIGURE 1.0

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bathochromic shift which is greater than that of thiosemicarbazide.

The same reaction mixture was diluted 1/20 (Figure 11, curve #1) and an ultraviolet spectrum was obtained. \( \lambda_{\text{max}} \) of 257 nm and 199 nm were found to be present for the reaction mixture containing no thiosemicarbazide. This finding could be similar if ultraviolet data were available for the urea-diacetylmonoxime reaction in the absence of thiosemicarbazide. Curve #2 illustrates that there has been a bathochromic shift (\( \lambda_{\text{max}} \) of 284 nm from \( \lambda_{\text{max}} \) of 257 nm) as well as hyperchromic effect in ultraviolet spectrum on addition of thiosemicarbazide. The peak at \( \lambda_{\text{max}} \) 237 nm represents the excess of the thiosemicarbazide reagent in the reaction mixture as expected (TSC/PPDMO = 3.35). An opposite shift in the ultraviolet spectra was observed when working with dilute solutions of DAM + urea + TSC (\( \lambda_{\text{max}} \) 268 nm) compared to the concentrated solution of DAM + urea + TSC (\( \lambda_{\text{max}} \) 245). The blue shift could result from hydrogen bonding which lowers the energy of the n-orbital (attributed to the N and S atoms). The peak of \( \lambda_{\text{max}} \) 199 - 202 is most likely due to hydroxylamine produced by the reaction.

As indicated in CHAPTER I, the protochromogen responsible for color in the diacetylmonoxime-urea reaction was postulated to be diacetyldiureide. Since it seemed most likely that the structure of the protochromogen for the PPDMO-urea reaction could be similar, an attempt
FIGURE 11
ULTRAVIOLET SPECTRA OF 1-PHENYL-1,2-PROPANEDIONEMONOXIME
REACTION WITH UREA IN 50% HCl

Legend

1. Represents the reaction without thiosemicarbazide.
   Conc: Urea: $8.33 \times 10^{-6} \text{M}/4 \text{mL}$
   Thiosemicarbazide: zero
   PPDMO: $4.11 \times 10^{-6} \text{M}/4 \text{mL}$
   Dilution: 1/20; $A = 0.150; 0.89$
   $\lambda_{\text{max}}$ 257 nm; $\epsilon = 1.50 \times 10^{5}$; $\lambda_{\text{max}}$ 199

2. Represents the reaction with thiosemicarbazide.
   Conc: Urea: $8.33 \times 10^{-6} \text{M}$
   Thiosemicarbazide: $1.38 \times 10^{-5} \text{M}$
   PPDMO: $4.11 \times 10^{-6} \text{M}$
   $\lambda_{\text{max}}$ 284 nm; $\epsilon = 2.97 \times 10^{5}$; 237 nm; 199 nm
   $A = 0.297; 0.934; 1.14$
   Ratio: $\frac{TSC}{PPDMO} = 3.35; \frac{\text{urea}}{PPDMO} = 2.02$

Total reaction volume: 8.02 mL
was made to synthesize 1-phenyl-1,2-propanedionediureide (PPDDU) and compare the visible and ultraviolet spectra of PPDDU with that of the reaction mixture of PPDMO with urea. PPDMO was refluxed in an acid-alcohol medium with urea at 80°C and isolated from the reaction mixture as a pale-yellow residue. The melting point was found to be over 300°C (d).

When 1-phenyl-1,2-propanedionediureide (PPDDU) was dissolved in HCl, a red-purple solution was obtained without incubation. This was an indication that the chromogen of the PPDMO reaction with urea was PPDDU. This color exhibits a hyperchromic effect when left at room temperature or incubated at 100°C. Figure 12 shows the visible range spectrum with a \( \lambda_{\text{max}} \) of 543 nm for PPDDU in HCl solution which is identical to that of the reaction of PPDMO with urea. For the ultraviolet measurements, the above solution was diluted 1/20, (See Figure 13, curve #1) and \( \lambda_{\text{max}} \) of 254 nm and 202 nm were obtained. When PPDDU was dissolved in alcohol, a similar spectrum with \( \lambda_{\text{max}} \) 254 nm and \( \lambda_{\text{max}} \) 212 nm were obtained (curve #2). However, when sample #1 was incubated at 100°C for 15 minutes, a bathochromic shift in the spectrum (\( \lambda_{\text{max}} \) 254 nm to 264 nm was obtained, as well as, a hyperchromic effect at \( \lambda_{264} \) nm.

A sample of PPDDU (1 mg) was thoroughly mixed with approximately 100 mg of dry, powdered potassium bromide. The mixture was pressed into a transparent disc under a
FIGURE 12

VISIBLE SPECTRUM OF 1-PHENYL-1,2-PROPANEONEDIUREIDE IN 50% HCl

Legend

Visible spectrum of stock solution (purple).
No incubation. \( A = 0.093 \)
Conc: \( 2.5 \times 10^{-3} M \) (0.0588 g/100 mL)
\( \lambda_{\text{max}} = 543; \epsilon = 3.7 \times 10^5 \)
**FIGURE 13**

ULTRAVIOLET SPECTRA OF 1-PHENYL-1,2-PROPANEDIONEDIUREIDE IN 50% HCl

**Legend**

1. **Unincubated sample**
   - Conc: $2.5 \times 10^{-4}$ M (58.8 mg/100 mL)
   - pink solution
   - Dilution: 1:20; $A = 0.12$; 1.2
   - $\lambda_{\text{max}}$ 254; $\epsilon = 9.6 \times 10^4$; 202; $\epsilon = 9.6 \times 10^5$

2. **In alcohol**: Unincubated sample
   - Conc: 10 mg/100 mL; $4.31 \times 10^{-5}$ M
   - $A = 0.403$; 1.24
   - $\lambda_{\text{max}}$ 254; $\epsilon = 9.35 \times 10^4$; 212; $\epsilon = 2.8 \times 10^5$

3. **Incubated sample (15 minutes at 100°C)**
   - Conc: $2.5 \times 10^{-3}$ M
   - Dilution: 1/100
   - $A = 0.168$; 0.47
   - $\lambda_{\text{max}}$ 264; $\epsilon = 6.72 \times 10^3$; 199; $\epsilon = 1.88 \times 10^3$

Absorbance from 230 - 360 nm is shown on right hand side of absorption spectrum.
Absorbance below 230 nm is shown on left hand side of absorption spectrum.

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pressure of \(=10,000 - 15,000\) pounds/sq. inch. A KBr-disc was obtained for the infrared measurement. Table VIII presents the characteristic infrared bands of 1-phenyl-1,2-propanedionediureide. The NH-stretching frequencies corresponding to the assymmetrical and symmetrical NH-stretching vibrations are observed at \(3220 - 3160\) cm\(^{-1}\) because of hydrogen bonding. The primary amide \((\text{C}=\text{O} \text{ bond})\) absorbs (amide I band) at \(1680\) cm\(^{-1}\) as illustrated in the table. An amide II band is also present at \(1515\) cm\(^{-1}\). The absorption pattern when compared to that of diacetyldiureide (Table I) indicates an open chain structure for PPDDU.

The visible, NMR, and mass spectrometric data on 1-phenyl-1,2-propanedionediureide are shown in Table IX. The calculated molecular weight for PPDDU was found to be 232. The mass spectrometric (FD) data indicates the presence of phenyl radical and the molecular ion with \(m/e\) of 232.28.

PPDDU was dissolved in dimethyl sulfoxide and an NMR spectrum was obtained. Chemical shifts are reported in parts per million, \(\delta\), downfield from tetramethylsilane. In the spectrum of PPDDU, the \(\text{NH}_2\) protons show absorption about \(\delta3.46\) (T6.54); the \(-\text{C}-\text{CH}_3\) absorption at \(\delta0.78\), (T9.22); phenyl protons are represented at \(\delta7.42\), (T2.54). The peaks due to dimethyl sulfoxide are not in the table. The mass spectrometry, I.R., and NMR data point towards an open chain structure for the 1-phenyl-1,2-propanedione-
<table>
<thead>
<tr>
<th>Functional groups</th>
<th>Frequency cm⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-H stretching vibrations</td>
<td>3220 - 3160</td>
</tr>
<tr>
<td>C-H stretching vibrations assymetrical</td>
<td>2995</td>
</tr>
<tr>
<td>C-H stretching vibrations symmetrical</td>
<td>2860</td>
</tr>
<tr>
<td>C=O stretching vibrations non-bonded (amide I band)</td>
<td>1740</td>
</tr>
<tr>
<td>C=O stretching vibrations H-bonded</td>
<td>1680</td>
</tr>
<tr>
<td>C=N (conjugated)</td>
<td>1515</td>
</tr>
<tr>
<td>C-H bending vibrations assymetrical methyl</td>
<td>1445</td>
</tr>
<tr>
<td>C-H bending vibrations symmetrical methyl</td>
<td>1380, 1320</td>
</tr>
<tr>
<td>C-H deformation and C-C stretching vibrations</td>
<td>1170, 1140</td>
</tr>
<tr>
<td>N-H out-of-plane bending or wagging</td>
<td>740 - 700</td>
</tr>
</tbody>
</table>
TABLE IX

**1-PHENYL-1,2-PROPANEDIONEDIUREIDE**

<table>
<thead>
<tr>
<th>Ultraviolet data (in 50% HCl)</th>
<th>Structural formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>λ254; 9.6 x 10⁴</td>
<td>CH₃ 0</td>
</tr>
<tr>
<td>λ202; 9.6 x 10⁵</td>
<td>C=N-C-NH₂</td>
</tr>
<tr>
<td>λ264; 6.72 x 10⁵</td>
<td>C=N-C-NH₂</td>
</tr>
<tr>
<td>λ199; 1.88 x 10⁶</td>
<td>incubated sample</td>
</tr>
</tbody>
</table>

λ_max 545 nm; (purple color) ε = 3.7 x 10³

**Mass spectrophotometric data (FD)**

<table>
<thead>
<tr>
<th>Experimental m/e</th>
<th>%R.A.</th>
<th>Calculated (mol. wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-78.31</td>
<td>100%</td>
<td>232</td>
</tr>
<tr>
<td>232.28</td>
<td>84%</td>
<td></td>
</tr>
</tbody>
</table>

**NMR data (dimethylsulfoxide was used as solvent)**

<table>
<thead>
<tr>
<th>δ</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0.78</td>
<td>CH₃</td>
<td>T 9.22</td>
<td></td>
</tr>
<tr>
<td>3.46</td>
<td>NH₂</td>
<td>T 6.54</td>
<td></td>
</tr>
<tr>
<td>7.42</td>
<td>phenyl</td>
<td>T 2.57</td>
<td></td>
</tr>
</tbody>
</table>

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diureide. The visible and ultraviolet spectra when compared to that of the reaction mixture of PPDMO with urea suggest that the chromogen responsible for the purple color is PPDDU. However, there is a possibility that structures such as glycolurils, as suggested by Veniamin, et al. (27) and 2,2'-dioxo-4,5'-diimidazolmethane might be formed in the reaction which might also contribute to the same type of color development, although these compounds were not isolated under the conditions studied in this laboratory.

The difficulty encountered in using PPDMO as a reagent for the determination of urea nitrogen, was that of solubility in an aqueous medium. This was overcome by taking up PPDMO in a few ml of concentrated HCl and heating the solution on a steam bath for a few minutes. Heating in an acid medium caused the liberation of hydroxylamine and the formation of yellow droplets of phenylpropanediketone. Alcohol was used to dissolve the diketone formed. This solution then served as the stock color reagent which was stable at room temperature. The working solution was made by diluting the stock with 50% HCl solution. Further, 1-phenyl-1,2-propanedione was sulphonated to facilitate its solubility in water. No reactions were carried out with this compound. No attempt was made to establish the sensitivity of the method, time required for optimum color development, nor inhibition by various guanidino derivatives which occur.
naturally and which may reach levels which would lead to interference with this reagent. The purpose of the study was to determine the structure of the chromogen responsible for the color development (purple), its isolation and/or synthesis, and obtain the spectral characteristics of the protochromogen for comparison purposes to that of the non-isolated products of the reaction mixture.

A mixture of urea and 2,3-pentanedione was refluxed in acid-alcohol mixture and the resultant product was isolated. The white powder had a melting point of >300°C (d). The elemental analysis matched the calculated data. Field desorption mass spectrometric data gave a molecular weight of 184.

The starting substance, 2,3-pentanedione, in alcohol, has a $\lambda_{\text{max}}$ of 270 nm and 230 nm. Figure 14 presents a scan of the visible spectrum of the 2,3-pentanedione-diureide in 50% HCl after incubation. The $\lambda_{\text{max}}$ obtained are at 577 nm (blue color) ($\epsilon = 4.7 \times 10^2$) and 480 nm ($\epsilon = 2.44 \times 10^2$). The ultraviolet spectra of the incubated sample diluted 1:1 (Figure 15) shows $\lambda_{\text{max}}$ of 304 nm ($\epsilon = 735$) and 212 nm while the non-incubated sample has an identical $\lambda_{\text{max}}$ except a lower molar absorptivity. Once again, incubation in HCl shows a hyperchromic effect possibly due to protonation of the diureide and formation of a more conjugated system. These hyperchromic effects are similar to that observed by Kouno and Ueda (66) when they dissolve the residue from the n-butanol extract of

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FIGURE 14
VISIBLE SPECTRUM OF 2,3-PENTANEDIONEDIUREIDE

Legend
The 2,3-pentanedionediureide (2.7 x 10^{-4} M) was dissolved in HCl and the spectrum was obtained after incubation of the sample (25 minutes at 100°C).
The blue-colored solution exhibited the absorption maxima at 577 nm (ε = 4.7 x 10^2) and 480 nm (ε = 2.44 x 10^2).
FIGURE 15

ULTRAVIOLET SPECTRUM OF 2,3-PENTANEDIONEDIUREIDE
IN 50% HCl

Legend

1. Incubated sample
   Conc: $1.35 \times 10^{-3} \text{M}$
   Dilution: 1:1
   $\lambda_{\text{max}}$ 340; $\varepsilon = 3 \times 10^2$; 212

2. Non-incubated sample
   Conc: $2.5 \times 10^{-3} \text{ g/10 mL}; 1.35 \times 10^{-3} \text{M}$
   No dilution
   $\lambda_{\text{max}}$ 304; $\varepsilon = 735$; 212

Absorbance from 250 - 400 nm is shown on right hand side of absorption spectrum.

Absorbance below 250 nm is shown on left hand side of absorption spectrum.
the reaction mixture of the butylurea and 1-phenyl-1-hydroxylmimo-2-propanone. The substitution of ketone for monoxime did not alter their observation of color intensification with time or temperature. However, as mentioned before, they suggest that the intermediate responsible for the color development is the 2,2'-dioxo-4,5'-dimidazolylmethane derivative.

Table X presents the characteristic I.R. bands from the spectrum of 2,3-propanedionediureide. On comparison of these bands with that of diacetyl diureide or the 1-phenyl-1,2-propanedionediureide, it is obvious that they resemble, in most part, the diureide, suggesting the open chain structure for this diureide. There is no band at 3150 cm\(^{-1}\) which is the characteristic I.R. absorption band for (NH) imidazolone moiety. The absorption bands at 1670 and 1680 cm\(^{-1}\) represent the C=O stretching vibrations.

The calculated molecular weight of the diureide (C\(_7\)H\(_{12}\)N\(_4\)O\(_2\)) was 184. The field desorption mass spectrometric spectrum gave m/e for the molecular ion to be 184 and M\(^{+}\) + 1 to be 185 as tabulated in Table XI. Electron impact mass spectrometry data is listed in Scheme 8, CHAPTER I.
<table>
<thead>
<tr>
<th>Functional groups</th>
<th>Frequencies cm(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-H stretching vibrations</td>
<td>3280, 3220</td>
</tr>
<tr>
<td>C-H stretching vibrations</td>
<td>2980, 2880</td>
</tr>
<tr>
<td>C=O stretching vibrations</td>
<td>1725</td>
</tr>
<tr>
<td>(I - band)</td>
<td>1670, 1680</td>
</tr>
<tr>
<td>secondary amide</td>
<td></td>
</tr>
<tr>
<td>Combination of bands of N-H deformation and C-N stretching vibrations</td>
<td>1515</td>
</tr>
<tr>
<td>C-H deformation</td>
<td>1160, 1100, 1055</td>
</tr>
<tr>
<td>and</td>
<td></td>
</tr>
<tr>
<td>C-C stretching</td>
<td>1015</td>
</tr>
<tr>
<td>NH deformation vibrations</td>
<td>770, 735</td>
</tr>
</tbody>
</table>
### Table XI

**Ultraviolet and Mass Spectrophotometric Data of 2,3-Pentanedionediureide**

<table>
<thead>
<tr>
<th>Ultraviolet data</th>
<th>Structural formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \lambda_{max} = 1.34 \times 10^5 )</td>
<td><img src="image" alt="structural_formula" /></td>
</tr>
<tr>
<td>( \lambda_{212} = 1.16 \times 10^5 )</td>
<td><img src="image" alt="structural_formula" /></td>
</tr>
<tr>
<td>in HCl</td>
<td><img src="image" alt="structural_formula" /></td>
</tr>
<tr>
<td>( \lambda_{304} = 1.52 \times 10^5 )</td>
<td><img src="image" alt="structural_formula" /></td>
</tr>
<tr>
<td>( \lambda_{272} = 6.3 \times 10^5 )</td>
<td><img src="image" alt="structural_formula" /></td>
</tr>
<tr>
<td>( \lambda_{max} ) 575 nm; (blue color) ( \lambda_{480} )</td>
<td><img src="image" alt="structural_formula" /></td>
</tr>
</tbody>
</table>

**Mass spectrometric data (FD), (major fragments over 1% R.A.)**

<table>
<thead>
<tr>
<th>Experimental</th>
<th>Expected (mol.wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>m/e</td>
<td>%R.A.</td>
</tr>
<tr>
<td>184</td>
<td>100</td>
</tr>
<tr>
<td>( M + 1 )</td>
<td>18.71</td>
</tr>
</tbody>
</table>

**Starting material:** 2,3-pentanedione

\( \lambda_{max} \) (alc.): 230; 270 nm
D. CONCLUSIONS

The protochromogen responsible for the pink color of the reaction between urea and 1-phenyl-1,2-propanedionemonoxime in acid medium was found to be 1-phenyl-1,2-propanedionediureide. When this diureide was dissolved in HCl it gave a pink-colored solution with an $\lambda_{\text{max}}$ 540 nm, which is identical to that of the urea-1-phenyl-1,2-propanedionemonoxime reaction. The infrared and nuclear magnetic resonance data suggest the structure of the synthesized compound to be that of the diureide. The mass spectrometric data gave the molecular weight of the compound to be 232. The ultraviolet spectrum indicated the absorption maxima at 257 nm and 199 nm.

When 1-phenyl-1,2-propanedionemonoxime was reacted with urea in presence of thiosemicarbazide a bathochromic shift and a hyperchromic effect was observed by shifts in absorption maxima from 540 nm to 568 nm, indicating formation of a more unsaturated structure in the chromogen. The ultraviolet spectrum showed the presence of an absorption maxima at 284 nm, 237 nm and 199 nm. This suggested the incorporation of thiosemicarbazide in the chromogen structure.

Veniamin, et al. (27) have synthesized 3a-methyl-6a-phenylglycoluril (whose $\lambda_{\text{max}}$ 290 nm was measured) in order to establish the time, course of acid hydrolysis at 20°C, and its conversion to 4-methyl-5-phenyl-4,5-dihydroxy-2-
imidazolone ($\lambda_{\text{max}}$ 264 nm). Several 1-alkyl and 1-aryl derivatives of 2-imidazolidinone (I) have been reported in the patent literature (77), but characterization of the products was incomplete. In acid-catalyzed additions, glycoluril (II) and/or hydantoin (III) were the reported products (17, 28, 29) (See Scheme 15).

\[ \text{(I) \rightarrow \text{acid}} \]

\[ \text{alkali} \]

The synthesized phenylmethyldiureide does not have a $\lambda_{\text{max}}$ 290 nm in non-incubated alcohol or HCl solutions, therefore, 3a-methyl-6a-phenylglycoluril as a structure for the synthesized compound can be eliminated.

If the corresponding 4,5-dihydroxy-2-imidazolidinone (I) with $\lambda_{\text{max}}$ 264 nm from glycoluril was formed on hydrolysis according to Veniamin et al. (27), then the molecular weight ($C_9H_8N_2O_2$) would be 176 and the NMR data of 2-imidazolidinones as reported by Vail et al. (31) would match the data of the synthesized diureide. However, they do not correspond to the diureide (molecular weight 232). The ultraviolet spectrum of the diureide

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has an absorption maxima at 254 nm compared to 264 nm for the imidazolone.

The 3a-phenyl-6a-methylglycoluril was synthesized via a base-catalyzed reaction. It produced an identical pink color when dissolved in hot HCl to that of the urea reaction with 1-phenyl-1,2-propanedionemonoxime, as well as to the corresponding diureide in HCl. The absorbance maxima were at 543 nm for all three reactions. The ultraviolet spectrum of glycoluril showed absorbance maxima at 274 nm, 230 nm, and 217 nm, which is quite different from that of the corresponding diureide ($\lambda_{\text{max}}$ 257 and 199 nm). The NMR data did not correspond to that of the glycoluril.

From the above experimental data, one can conclude that the synthesized compound represents a diureide (different infrared, ultraviolet, NMR, and mass spectrometric data).

No report has been made up to the present in the literature of the use of thiosemicarbazide with urea and 1-phenyl-1,2-propanedione for the determination of blood urea nitrogen. The protochromogen of this reaction has not been isolated nor reported in the literature. From the experimental data (ultraviolet spectra) on the reaction of thiosemicarbazide with urea, 1-phenyl-1,2-propanedionemonoxime gave absorbance maxima of 284 nm, 237 nm and 199 nm. Similarly, the reaction of diacetyl-thiosemicarbazonemonoxime with urea gave (ultraviolet
spectrum) absorbance maxima of 282 nm and 237 nm. These results suggest that the protochromogen in both reactions must be identical. Since both indicate the presence of absorption bands at 237 nm, which corresponds to a thiosemicarbazone moiety, the protochromogen can be expected to possess this group in the structure. Therefore, one can conclude that the involvement of thiosemicarbazide was that of imparting more unsaturation to the protochromogen as visualized by the observed bathochromic shift and hyperchromic effect.

The use of 2,3-pentanedione as a reagent for urea determination has not been cited in the literature. The reaction of 2,3-pentanedione with urea produced a blue-colored reaction mixture with absorbance maxima at 577 nm and 480 nm. The 2,3-pentanedionediureide was synthesized by the acid-catalyzed reaction. The infrared, ultraviolet, NMR, and mass spectrometric results indicate the structure of this compound to be a diureide with a molecular weight of 184. A bathochromic shift and hyperchromic effect would be observed, if urea and 2,3-pentanedione was allowed to react with thiosemicarbazide. However, no such effect was observed when the diureide was reacted with thiosemicarbazide in HCl, because the thiosemicarbazide apparently cannot displace a urea residue from the diureide.

It is highly possible that 2,3-pentanedione could also be used for determination of creatine in urine with
1-naphthol similar to the use of diacetyl as reported by Ennor, Stocken and Kurohara (78, 79).
CHAPTER IV
INVESTIGATION OF THE REACTION INTERMEDIATE(S) OF THE UREA-DIACETYLMONOXIME REACTION: A RETROSPECTIVE VIEW

Numerous variations of Fearon's (8) original carba-mido reaction have been cited in the literature. Modifications involve changes in acid medium, use of derivatives of diketones, and addition of oxidizing or color stabilizers.

Many oxidizing agents such as Fe\(^{3+}\) and glucurono-lactone (59) do not play any significant role in the urea-diketone reaction. The mechanism by which they stabilize or intensify the color is not known. When the reaction is run with or without Fe\(^{3+}\), it has been observed that there is no net effect on final color.

Reaction of urea with o-phthalaldehyde and N-(1-naphthyl)-ethylenediamine (NED) is described by Jung, et al. (80). They have not fully elucidated the intermediate or the chromogen of the reaction, but suggested the existence of the following isoindoline derivative (A) or a phthalan derivative (B) as intermediates which react further with NED to produce a compound (aromatic substitution) that undergoes rearrangement to yield a highly conjugated and intensely colored product. They did not isolate the colored compound nor did they characterize it.

Morin, et al. (57) have reported the use of
dimethylaminobenzaldehyde instead of a diketone. The authors do not suggest any mechanism or the structure of the chromogen(s).

![Chemical Structure](image)

Ueda, et al. (19, 66) have separated various spots on TLC from the butylurea reaction with diacetyl, 1-phenyl-1,2-propanedione, and their oximes and compared them with TLC of n-butanol extracts of the above reactions. They reported the structure to be a compound formed from the condensation of three moles of diacetyl with a simultaneous loss of two carbon atoms to form 2,2'-dioxo-2,5'-diimidazolylmethane type of structure.

Veniamin, et al. (27) have suggested the formation of glycoluril, which in acid medium hydrolyzes to form a pigment which is responsible for the color. However, they did not isolate the photolysis product.

The present study (CHAPTERS I - III) shows clearly, that diureides, whether synthesized from diacetyl, 1-phenyl-1,2-propanedione, or 2,3-pentanedione, or their monoxide do produce identical visible and ultraviolet spectrum to that of the urea reaction with the corresponding diketone or its oxime. The reconstitution of the color,
therefore, is a primary visible proof of the existence of diureide as the principle chromogen or protochromogen of the reaction. There might be other compounds formed in the reaction mixture as observed by Ueda, et al. (19, 66) on TLC separation which gave a yellow to blue-colored solution when dissolved in acid medium, when butylurea was used instead of urea.

The infrared and mass spectrometric data (in CHAPTER I - III) confirm the structure of the synthesized compound to be a diureide and not a glycoluril or a 4,5-dihydroxy-2-imidazolidinone derivative, or a 2,2'-dioxo-4,5'-diimidazolylmethane derivative.

The ultraviolet studies of the reaction: urea + thiosemicarbazide + 1-phenyl-1,2-propanedionemonoxime and urea + diacetylthiosemicarbazonemonoxime in 50% HCl gave absorbance maxima at 237 nm which indicated the presence of a thiosemicarbazone moiety in the protochromogen of both reactions. (See CHAPTER II; Figure 9).

The new maxima formed in the above reactions are at 284 and 282 nm, respectively, which further substantiated that the structure of the protochromogen was similar. However, glycoluril does not exhibit similar absorbance maxima when dissolved in 50% HCl ($\lambda_{\text{max}}$ 274 nm and 230 nm).

The use of 2,3-pentanedione in presence or absence of thiosemicarbazide as a new reagent for determination of urea nitrogen is being proposed. Its use in the
determination of creatine in urine with 1-naphthol (similar to the use of diacetyl as reported by Ennor and Stocken and Kurohara [78, 79]) is being proposed.
PART. II

AUTOMATED MICROANALYSIS OF IRON USING SYNTHESIZED
TEROSITE SULFONATE AS CHELATING REAGENT
AUTOMATED MICROANALYSIS OF IRON USING SYNTHESIZED TEROSITE SULFONATE AS CHELATING REAGENT

A. INTRODUCTION

Iron is one of the most abundant elements in the crust of the earth. It is widely distributed amongst minerals, plants, and the animal kingdom. It is one of the trace elements which plays a vital role in the body, bound to proteins such as hemoglobin, catalase, cytochrome, transferrin, ferritin and hemosiderin (7, 81). After being taken up by the intestinal mucosa, iron may be transported across the mucosal cell directly to the plasma. Alternatively, it may be incorporated into the iron-storage protein, ferritin. Both in bone marrow and liver, iron is chelated with protoporphyrin IXα to give haem, which is incorporated into various haem-proteins. Excess of iron is stored as ferritin or haemosiderin. Determination of iron is important in the investigation of disorders of iron metabolism. The presence of iron is objectionable in many manufactured products due to its redox properties.

When iron is present in large amounts, the usual procedure for its determination employs gravimetric or volumetric techniques. However, when trace amounts of iron have to be measured (parts per million), the method of choice is the colorimetric procedure in which 1,10-phenanthroline and syn-phenyl-2-pyridylketoxime are
the reagents of choice. When iron is present in parts per billion, bathophenanthroline is used because of its higher molecular extinction coefficient. Bathophenanthroline (82) combines with ferrous ion in the ratio of 3:1.

\[
\begin{align*}
\text{Fe} &\quad \text{N} \\
\text{N} &\quad \text{N} \\
\text{N} &\quad \text{N} \\
\text{C} &\quad \text{C} \\
\end{align*}
\]

Similarly, the 2,4,6-tripyridyl-s-triazine (TPTZ) with a molecular extinction coefficient of 22,600 has been used for the determination of iron in the parts per billion range. In this case, the ferrous ion unites in a ratio of 2:1 to form the chelate ring. The \(\text{(Fe[bathophenanthroline]_3)}^{\text{++}}\) and \(\text{(Fe[TPTZ]_3)}^{\text{++}}\) complexes are extracted in organic solvents, such as isoamyl alcohol or nitrobenzene prior to iron determination. In order to avoid the extraction step, some of these complexing agents were sulfonated. This imparts water solubility to the compound. Table XII presents data of the ferroin chromogens used for serum iron determinations within the past decade. They contain the functional group \(-\text{N=C-C=N-}\) (also known as "ferroin group"), which gives bidentate ligands with transition metals, particularly iron, copper, and cobalt (82 - 95).

Recently, compounds containing the functional group \(-\text{C-C=N=C-C=N-}\) with the trivial name of the "terroine"
<table>
<thead>
<tr>
<th>Compound</th>
<th>Wavelength (nm)</th>
<th>Molecular extinction coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridyl benzodiazepine-2-one type</td>
<td>580</td>
<td>17,400</td>
</tr>
<tr>
<td>Bathophenanthroline</td>
<td>534</td>
<td>22,150</td>
</tr>
<tr>
<td>Bathophenanthroline disulfonate</td>
<td>534</td>
<td>22,140</td>
</tr>
<tr>
<td>Tripyridyl-s-triazine</td>
<td>593</td>
<td>22,600</td>
</tr>
<tr>
<td>2,6-Di-[pyridyl-(2)-4-p-methoxy-phenyl]-pyridine</td>
<td>570</td>
<td>26,900</td>
</tr>
<tr>
<td>DMPP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMPP sulfonic acid sodium salt</td>
<td>570</td>
<td>26,900</td>
</tr>
<tr>
<td>Ferrozine</td>
<td>562</td>
<td>27,900</td>
</tr>
<tr>
<td>Terosine</td>
<td>569</td>
<td>22,000</td>
</tr>
<tr>
<td>Terosole (4-methyl)</td>
<td>575</td>
<td>26,300</td>
</tr>
<tr>
<td>Terosole (4-ethyl)</td>
<td>570</td>
<td>27,100</td>
</tr>
<tr>
<td>Terosite (4-phenyl)</td>
<td>583</td>
<td>30,200</td>
</tr>
<tr>
<td>Tripyridyl 2,2',2''-tripyridine (terpyridine)</td>
<td>552</td>
<td>12,500</td>
</tr>
</tbody>
</table>
have been synthesized (90 - 93) and investigated for the purpose of exploiting a common property; that of formation of complexes with ferrous, cobaltous, and cuprous species. The "terroine" reaction is analogous to the "ferroin" reaction in chelation to form Fe(II) complex cations. Table XII presents data for the ferroin reagents used for the serum iron determinations. Most of these compounds are expensive, due to difficult and tedious organic syntheses that are required for their preparation (96). For clinical analytical purposes, a fairly low cost, yet highly sensitive reagent (high extinction coefficient) with water solubility, would be highly desirable. Most of the iron reagents in Table XII are difficult to dissolve in aqueous media, thus requiring extraction steps with organic solvents.

The steps involved in serum iron determination can be summarized as follows:

1. Disruption of the Fe-protein complex by HCl or pH changes. The dissociation of the ferric ion from transferrin is a reversible reaction and procedural variations affect the dissociation.

2. Reduction of ferric ions to ferrous ions. The effectiveness of the various reducing agents depend upon several factors such as, redox potential, solubility, rate of reaction, coprecipitation of the Fe with protein. Among the common reducing agents used are ascorbic acid, thioglycollic acid (97), sodium metabisulfite (98)
and hydroxylamine·HCl (82).

3. Chelation reaction producing a ferrous ion complex which is non-reversible. Some of the complexes are extractable with organic solvents.

The 2,6-bis-(4-phenyl-2-pyridyl)-4-phenylpyridine (terosite) has been synthesized by Case (90) by a modified Chichibabin reaction in an overall yield of about 18 - 20%. The low solubility of the ferrous-terosite complex necessitates the use of mixed solvents (organic) for its extraction prior to the final determination. This shortcoming can be eliminated by sulfonation of terosite (99 - 100). Therefore, the synthesis of terosite sulfonate and its use as a reagent for the measurement of parts per billion of iron by the colorimetric method was undertaken.

Terosite's synthesis can be achieved by either of the methods of Case et al. (90) and Proevska et al. (101) whose schematics are represented by the following set of equations (A) and (B). (See Scheme 16 and 17).

The aim of this project was to prepare terosite sulfonate and to impart water solubility to the molecule, thus avoiding the extraction step involved in the determination of iron with the unsulfonated terosite; to determine the spectral characteristics of the sulfonated product and compare it with that of terosite; to measure the effects of pH on the stability of the ferrous-bis (terosite sulfonate); and finally to automate the analytical
procedure on a Technicon® AutoAnalyzer® AAI.

(A) Method of Case et al. (90)

1. Chichibabin reaction: The substituted 4-phenylpyridines have been synthesized by a modified Chichibabin reaction from α-methylstyrene, formaldehyde and ammonium chloride to give 6-methyl-6-phenyltetrahydro-1,3-oxazine I.

\[
\begin{align*}
\text{Cyclic ether I} & \xrightarrow{\text{NH}_4\text{Cl}, \text{H}_2\text{C}=\text{O}} \text{4-phenylpyridine}
\end{align*}
\]

Conversion of I to 4-phenylpyridine using nitrobenzene and palladium as a catalyst.

\[
\begin{align*}
\text{I} & \xrightarrow{\text{H}_2\text{O}, \text{excess HCl}} \text{4-phenylpyridine}
\end{align*}
\]

2. Conversion of II to 2-cyanopyridines.

\[
\begin{align*}
\text{II} & \xrightarrow{\text{CuCN}} \text{II} & \text{CN}
\end{align*}
\]

3. Conversion of III to 2-acetyl-4-phenylpyridine.

\[
\begin{align*}
\text{III} & \xrightarrow{\text{C}_2\text{H}_5\text{OH}, \text{HCl}} \text{IV}
\end{align*}
\]

4. Terosite from IV

\[
\begin{align*}
\text{IV} & \xrightarrow{\text{C}_6\text{H}_5\text{CHO}, \text{NH}_4\text{OH}} \text{Terosite}
\end{align*}
\]

**Scheme 16**

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(B) Method of Proevska et al. (101).

Scheme 17
EXPERIMENTAL

1. APPARATUS

Absorption spectra were recorded on a Beckman Acta III spectrophotometer. For the potentiometric titration, a Corning pH-meter Model 7 equipped with a Corning glass and calomel electrode was used. Ion-exchange experiments were carried out on a column of 1-cm diameter and 12-inch length containing a sintered glass plug. The synthesis of terosite sulfonate was performed using Quickfit glassware.

2. REAGENTS

Ammonium acetate buffer pH 4.5 ± 0.02: In a 1.5-liter beaker were dissolved 100 g ammonium acetate (certified ACS reagent) in 990 mL of distilled water. Sufficient glacial acetic acid was added to this solution to bring the pH of the solution to 4.5 ± 0.02.

Potassium biphthalate buffer pH 4.0 ± 0.02: The certified buffer concentrate solution was diluted 1:5 with distilled water.

Potassium biphthalate buffer pH 2.0 ± 0.02: The diluted buffer of pH 4.0 was treated with HCl to bring the pH to 2.0.

Potassium phosphate monobasic-NaOH buffer pH 7.0 ± 0.02: Fisher certified buffer concentrate was diluted 1:25 with distilled water.

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Boric acid-NaOH buffer pH 10 ± 0.02: This was prepared by dilution of the buffer concentrate (1:10) with distilled water.

L-Ascorbic acid 1 g%: Ascorbic acid (reagent grade) (1 g) was dissolved in 100 mL of distilled water.

Stock iron solution: Ferrous ammonium sulfate·6H₂O (analytical reagent) (0.702 g) was dissolved in distilled water (containing 0.5 mL concentrated sulfuric acid) and diluted to 1 liter.

Working iron reagent: In a corresponding 100-mL volumetric flask marked 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 μg of iron was pipetted 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 mL of the stock iron reagent, respectively, and diluted to the mark with distilled water.

TGA-NaCl-1 N HCl solution: In a 1-liter volumetric flask were dissolved 8.5 g of sodium chloride (reagent grade) in 500 mL of 1 N HCl solution. Thioglycollic acid (1 mL) was added to the flask, mixed and diluted to the mark with 1 N HCl solution.

HCl, 1 N solution: Concentrated HCl (33 mL) was diluted to 1 liter with distilled water.

Terosite sulfonate stock solution: In a 10-mL volumetric flask was dissolved 0.1006 g of terosite sulfonate and diluted to the mark with distilled water.

Working terosite sulfonate: One mL of the stock terosite sulfonate solution was diluted to the 50 mL mark with distilled water.
Amberlite IR-120: The ion-exchange resin was purified by washing it with concentrated hydrochloric acid to remove iron, followed by deionized water until the eluate was free of chloride.

Chlorosulfonic acid: Chlorosulfonic acid (98% pure), purchased from BDH Limited, was used.

Most of the above reagents were obtained from Fisher Scientific Company, Fair Lawn, New Jersey 07410, or from BDH Ltd., Toronto, Ontario. All the reagents were of analytical grade.

Terosite was obtained from the G. Fredrick Smith Chemical Co., Columbus, Ohio (courtesy of Dr. B. Zak).

3. METHOD

(a) Sulfonation of Terosite

To 200 mg of terosite was added chlorosulfonic acid (3 mL) in a round-bottomed flask equipped with a dropping funnel and a reflux condensor. The mixture was heated to 155° C for about 24 hours. At this time a clear-yellow solution resulted. The solution was cooled in an ice-bath and distilled water was added (≈ 20 mL) to destroy the excess of chlorosulfonic acid. The mixture was then heated on a steam-bath till most of the solid had dissolved. The unsulfonated material was separated by filtration. HCl was removed by aspiration. Ammonium hydroxide was added to neutralize the solution. To this slightly alkaline solution was added
about 500 mL of ethanol while stirring. Separation of precipitated ammonium sulfate was achieved by filtration. On evaporation of the ethanol filtrate on a steam-bath, a bluish-white product was obtained. The field desorption mass spectrometric data indicated that a mixture of mono- and disulfonate in a ratio of 10:7 was present in the crude product. The yield was about 80% of the theoretical yield.

The sodium salt of terosite sulfonate was prepared by passing the aqueous solution of the above ammonium salt of terosite sulfonate through an ion-exchange resin (IR-120 strong cation-exchange resin in H-form) column. The eluate was acidic to the pH paper. It was neutralized with sodium bicarbonate (powder). On evaporation of the aqueous solution on a hot-plate, a white product was obtained which reacted with ferrous ions to give a blue-colored complex identical to terosite. The sodium salt was extremely soluble in water and showed a blue fluorescence under ultraviolet light.

(b) Thin-layer Chromatography of Terosite Sulfonate

The technique of Barrera and Spotswood (102 - 104) who carried out some separations of terphenyls by chromatography on acetylated paper was used. The chromatographic separations were carried out by the ascending technique on a 10 x 4 cm strip of plastic plates, coated with cellulose. The glass chromatographic chambers used
were 400-mL beakers (11 cm high). The strips were then developed with various solvents (eluting power of solvents used was in increasing order). The solvents tried were ethylacetate, acetone, 1,2-dichloroethane, ethanol, water, pyridine, DMSO, and acetonitrile. The mixture of DMSO and acetone (1:1) as eluting solvent showed separation of the terosite sulfonate as two spots at room temperature (20°C). The separation of the mixture was visualized under ultraviolet light.

(c) Visible Absorption Characteristics of Ferrous-bis(terosite sulfonate) Complex

In a test tube was pipetted 4 μg of Fe²⁺ solution (1 mL), 1 mL of thioglycollic acid-NaCl-1 N HCl solution, ammonium acetate buffer (1 mL) of pH 4.5 ± 0.02, and 0.5 mL of terosite sulfonate (3.07 x 10⁻⁶ mole). The solution was mixed and the absorbance of the blue-colored complex recorded from 650 nm down to 320 nm against distilled water. The absorption maxima were found to be at 583, 383, and 340 nm. The molar extinction coefficient was calculated to be 30,971 at 583 nm (See Figure 16).

(d) Determination of the Second Dissociation Constant of Terosite Sulfonate

The potentiometric titration was carried out with a Corning pH-meter equipped with glass-calomel electrodes. A 400-mL beaker equipped with a magnetic stirrer was used as the titration vessel. A 5-mL microburette was
FIGURE 16
ABSORPTION SPECTRUM OF Fe(II)-BIS (TEROSITE SULFONATE)

Legend
The concentration of terosite sulfonate $1.53 \times 10^{-7}$ mole and ferrous ion $4 \mu g$ ($0.07168 \times 10^{-5}$ mole) was used for this determination.

The buffer system consisted of ammonium acetate of pH 4.5 ± 0.02.

The reductant was thioglycollic acid (TGA)-NaCl-l N HCl.
used for the titration containing a 0.01302 N NaOH solution. Terosite sulfonate \((1.527 \times 10^{-5} \text{ mole})\) in approximately 100 mL of distilled water was used for the analysis. The starting pH of the solution was found to be 4.65. Recordings of changes in pH values on addition of 0.1 mL of NaOH were obtained. A plot of pH vs. mL of NaOH added is shown in Figure 17. The \(pK_2\) value and the equivalence point were computed from the titration to be at pH 6.45 and at pH 7.57, respectively.

(e) Effect of pH on the Stability of Ferrous-bis (terosite sulfonate)

The pH range over which ferrous-bis(terosite sulfonate) was stable, was determined by preparing a series of solutions, each solution having the same amount of iron (8 \(\mu\)g), ascorbic acid, excess of terosite sulfonate and a buffer solution of different pH. The buffers used were potassium biphthalate of pH 2.0 and 4.0, potassium phosphate monobasic-NaOH of pH 7.0, boric acid-NaOH buffer of pH 9.0 and boric acid-KCl-NaOH buffer of pH 10.0. The absorbance of each solution was measured at 583 nm. The results plotted in Figure 18 show the influence of pH on chelation of iron with terosite sulfonate.

(f) Determination of the Formula of the Complex

The molar-ratio method was used for determination of the formula of the complex. The pH chosen was 4.5 and the buffer system used was ammonium acetate. The concentration of iron (5 \(\mu\)g) was kept constant, while
FIGURE 17
TITRATION CURVE OF TEROSITE SULFONATE

Legend
Terosite sulfonate 0.010 g was titrated with 0.01032 N NaOH. The pH at the start of the titration was 4.65. The pK₂ was found to be at pH 6.45. The end-point was determined to be at pH 7.6.
FIGURE 18

pH STABILITY STUDIES OF Fe(II)-BIS (TEROSITE SULFONATE)

Legend

The pH buffers were prepared from certified buffer concentrate solution from Fisher Scientific Company.

The pH 2.0 and 4.0 ± 0.02 buffers were potassium biphthalate buffers.

The pH 7.0 ± 0.02 buffer was potassium phosphate monobasic-NaOH.

The pH 9.0 ± 0.02 buffer was 0.5 M boric acid-0.2 M NaOH buffer.

The pH 10.0 ± 0.02 buffer was boric acid-KCl-NaOH buffer.

The reductant used was L-ascorbic acid.

The concentration of terosite sulfonate was 6.107 x 10^-7 mole.

The concentration of ferrous ion (8 μg) used was kept constant.
the concentration of terosite sulfonate was varied.

The molar ratio plot (Figure 19) showed a steady increase up to a concentration of $0.418 \times 10^{-6}$ mole at which point a break in the curve was noted and a constant amount of dissolved ligand was indicated.

(g) Automation of the Manual Procedure

The ratio of the mL of reagent delivered per minute was used in selection of the proper AutoAnalyzer pump tubings. The Figure 20 shows the single-channel flow schematic of the automated method at 583 nm using the basic Technicon AutoAnalyzer system. In Figures 21 and 22 are presented the AutoAnalyzer curves for the iron standards used at 583 nm and 340 nm, respectively. These curves were obtained following the procedural guidelines as described in steady state interaction by Technicon. Results were calculated from the calibration curve in the usual manner for methods obeying Beer's law. Therefore, the results obtained by the automated procedure were in excellent agreement with those obtained by the manual procedure.
FIGURE 19
SPECTROPHOTOMETRIC TITRATION OF FERROUS ION WITH TERO SITE SULFONATE

Legend

The concentration of the ferrous ion was kept at 0.896 x 10^-5 mole.
Terosite sulfonate concentration was varied.
The buffer used for the analysis was ammonium acetate of pH 4.5 ± 0.02. The reductant used was thioglycollic acid, in NaCl-1 N HCl.
The spectrophotometric measurements were carried out at 583 nm.
The molar ratio plot was carried out according to Reilly, C.N. and Sawyer, D.T., Experiments for Instrumental Methods, McGraw-Hill Book Company, Inc., Toronto, 1961.
FIGURE 20
MANIFOLD FOR IRON DETERMINATION

Legend

With the manifold in position, reagents are pumped through the system until the baseline stabilizes. Zero and 100% T are set on the recorder. The standards were placed on the sampler with serum specimens. A Technicon® basic AutoAnalyzer® system was used and the measurements were carried out at 583 nm.
FIGURE 20

Sampler

HCl-NaCl-Tg
Sample
Wash
Air

Dialyzer

Air
Buffer
Terosite
Pump

Waste

583 nm - 20 mm
PBG

Waste

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FIGURE 21
RECORDE TRACING WITH USE OF TECHNICON AAI SYSTEM

Legend

The typical recorder-tracing is illustrated using 100, 200 and 300 μg/100 mL Fe(II) standards and serum specimens. The measurements were carried out at 583 nm.

<table>
<thead>
<tr>
<th>Recorder Tracing Number</th>
<th>Concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>300 μg Fe(II) standards</td>
</tr>
<tr>
<td>2</td>
<td>200 μg Fe(II) standards</td>
</tr>
<tr>
<td>3</td>
<td>165 μg Serum samples</td>
</tr>
<tr>
<td>4</td>
<td>100 μg Fe(II) standards</td>
</tr>
<tr>
<td>5</td>
<td>64 μg Serum samples</td>
</tr>
</tbody>
</table>
FIGURE 21

Measurement at 583 nm

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FIGURE 22
CALIBRATION CURVE OF ABSORBANCE vs. CONCENTRATION OF IRON

Legend

The upper half of the diagram shows the calibration curves obtained at 583 and 340 nm, respectively. The lower half of the figure shows the recorder tracing of the standards (iron) in % transmittance at 340 nm.
C. RESULTS AND DISCUSSION

The synthesis of 2,6-bis-(4-phenyl-2-pyridyl)-pyridine (terosite) has been achieved via a Chichibabin reaction by Case et al. (90) with a yield of 21%. A white product was obtained with the melting point of 257-258°C. The mass spectrometric data indicated the presence of impurities in the crystallized product. The compound was found to be insoluble in water and alcohol. Terosite was extremely reactive with ferrous ions (a sensitive reagent) and gave a blue-colored ferrous complex of 2,6-bis-(4-phenyl-2-pyridyl)-pyridine (molecular extinction coefficient 30,200).

The disadvantage in using ferroin and terroine as reagents in the determination of iron in beer, wine, urine, serum, agricultural, industrial, and environmental samples, is their insolubility in aqueous media (e.g. 1,10-phenanthroline (105), bathophenanthroline (106, 107) and phenyl-2-pyridyl ketoxime (82, 108). Therefore, extraction of the complex with organic solvents is required before colorimetric determination. A means of overcoming the solubility limitation is to sulfonate the reagent. Trinder (109) was first to devise a method to sulfonate bathophenanthroline with chlorosulfonic acid. The product obtained was a water-soluble sulfonated derivative. Zak, Diehl and others have sulfonated bathocuproine (99, 100, 110), PDT (111), and terosite.
(100) using chlorosulfonic acid as the sulfonating reagent (See Scheme 18).

![Scheme 18](image)

**Scheme 18**

Terosite mono- and disulfonates were synthesized on neutralization of excess of chlorosulfonic acid with ammonium hydroxide and eventual separation of ammonium sulfate from the sulfonated ammonium salts of terosite, with ethanol. The free acid of the sulfonated terosite was generated by ion-exchange column chromatography using Amberlite IR-120. The sodium salt (tan color product), was obtained on neutralization of the free acid with NaHCO₃. The ammonium salts were bluish in color due to contamination of the reagent grade ammonium hydroxide (0.000010% Fe) present in the reagent. The isolation scheme did not adequately separate the free acid from the blue-colored metal derivatives. Thin-layer chromatography (of the blue product) on cellulose impregnated plastic plates, showed two distinct spots representing the mono- and disulphonated terosite. The spots showed fluorescence (blue) under ultraviolet light.

The infrared spectrum showed the typical bands due to aromatic substitution at the para position by sulfonic acid between 8.40 µ and 9.65 µ. The structure of
The terosite sulfonate was not determined precisely. It is reasonable to assume that the sulfonic acid has entered the phenyl ring at the para position. Furthermore, the two outside phenyl rings are most likely sulfonated, since further addition might involve steric hinderance.

The mass desorption spectrometric data indicated that the blue sulfonated product contained mono- and disulfonated products in a ratio of 10:7. If the terosite was sulfonated for a longer time and at a higher reaction temperature, a better yield of disulfonated material could be attained.

The problem of the solubility of terosite in aqueous media was overcome by sulfonation. The sulfonated product was found to be extremely soluble in water, alcohol, dimethylformamide and dimethylsulfoxide, and it was insoluble in chloroform, carbon tetrachloride, acetone, dioxane, petroleum ether, toluene, cyclohexane, methylacetate, dichloroethane, and n-butanol.

Terosite sulfonate reacted instantaneously with ferrous ions to produce a blue-colored Fe(II) terosite sulfonate complex which was found to be extremely soluble in water. Attempts to extract the complex from the aqueous medium with organic solvents, such as nitrobenzene, chloroform, carbon tetrachloride, isoamyl alcohol, benzene and a combination of nitrobenzene-isoamyl alcohol was not successful; therefore, the
characterization of the complex was not undertaken. Terosite does not form a Cu(I) complex which absorbs in the visual range of the spectrum, but forms Co(II) colored complex with low molecular extinction coefficient (2710 - 3120).

The studies of Zak et al. (99) have shown that none of the major cations present in serum (sodium, potassium, calcium and magnesium) interfered with the determination of iron with this reagent.

In Figure 23 are shown the absorption spectra of bathocuproine disulfonate-Cu(I) complex and terosite sulfonate-Fe(II) complex. A simultaneous determination of copper and iron in a single aliquot of sample is illustrated. The simultaneous equations are not required in calculations of the results. The interference of cobalt may be eliminated by adding ethylenediamine to complex the cobalt (II) cation, which otherwise will chelate with terosite sulfonate to form a Co(II)-terosite sulfonate colored complex (absorption maxima 466, 528 nm). It has been shown that other ions which, if present to the extent of 100 mg did not interfere in iron determinations using terosite (Mn(II), Al(III), Cr(III), Mg, PO$_4^{-3}$, P$_2$O$_4^{-5}$, C$_2$O$_4^{-2}$, Cl$^{-}$, NO$_3^{-}$, Ac$^{-}$, SO$_4^{2-}$) (See Table XIII).

The absorption spectrum of the ammonium salt of the sulfonated terosite is presented in Figure 16. The reaction was carried out in ammonium acetate buffer
FIGURE 23
ABSORPTION SPECTRA OF Fe(II)-BIS(TEROSITE SULFONATE) AND Cu(I)-BATHOCUPROINE SULFONATE COMPLEXES

Legend
Spectrophotometric determination of two metal ions (Cu and Fe) in a single aliquot is illustrated by use of successive addition of bathocuproine disulfonate and terosite sulfonate as complexing reagents. Since the absorption maxima were quite discrete, the small spectral interference can be subtracted from the final readings. The need for simultaneous equations in calculations of the final results is eliminated.

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<table>
<thead>
<tr>
<th>Ligand (^a)</th>
<th>Reported molar absorptivity</th>
<th>Sensitivity ratios (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. 4,7-Diphenyl-1,10-phenanthroline disulfonate</td>
<td>22,140</td>
<td>1.00</td>
</tr>
<tr>
<td>B. 2,4,6-Tris(2-pyridyl)-1,3,5-triazine disulfonate (Ferrozine)</td>
<td>27,900</td>
<td>1.26</td>
</tr>
<tr>
<td>C. 2,6-Bis(4-phenyl-2-pyridyl)-4-phenylpyridine (Terosite) (^c)</td>
<td>30,200</td>
<td>1.36</td>
</tr>
<tr>
<td>D. Terosite sulfonate</td>
<td>30,971</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) Costs of compound are $35.00 per gram for terosite; $16.00 per gram for bethophenanthroline sulfonate and $1.00 per gram for ferrozine.

\(^b\) Terosite: ferrozine = 108%

\(^c\) Must be sulfonated whereas 2 other compounds are obtained as sulfonates.
(pH 4.5 ± 0.02) containing thioglycollic acid as the reducing agent. The absorption maxima were found to be at 583, 383 and 340 nm.

Ascorbic acid can be substituted for thioglycollic acid and other buffer systems can be used instead of ammonium or sodium acetate with a pH of 4.5. The absorption spectra obtained with ascorbic acid and potassium biphthalate buffer (pH 2.0) showed absorption maxima at 583, 383 and 340 (A = 0.405, 0.285 and 0.980, respectively).

The molar-ratio method which is very similar to the method of continuous variations was used to determine the terosite sulfonate-Fe(II) complex formula. The concentration of iron was held constant and the concentration of the terosite sulfonate was varied. The absorbance was measured at 583 nm in an ammonium acetate buffer of pH 4.5 ± 0.02, containing thioglycollic acid as the reductant. A plot of absorbance vs. the molar concentration of the ligand was obtained (See Figure 19). The straight-line portions were extrapolated to where they cross. The ratio at this point was found to be 2:1 for terosite sulfonate: Fe(II). (The concentration of Fe: 0.89 x 10^{-5} mole/4mL terosite sulfonate ammonium salt: 0.418 x 10^{-2} M). Therefore, the interaction of ferrous ions with the ligand terosite sulfonate can be written as shown in Scheme 19.
Fe(II) + 2Ts $\xrightarrow{\text{pH 4.5}}$ Fe(Ts)$_2$

**SCHEME 19**

Each molecule of the ligand unites with one ferrous atom to form a five-membered ring with the whole molecule being arranged so that each nitrogen atom occupies one apex of a regular octahedron surrounding the iron atom. Figure 24 represents the proposed structure of the complex of ferrozine and terosite sulfonate with ferrous ion. Also shown in Figure 24 is the structure of the complex of 1,10-phenanthroline with iron.

The neutralization titration curves (112) of bathophenanthroline disulfonic acid gave $pK_1$: 2.83 and $pK_2$: 5.20. Bathocuproine disulfonic acid on the other hand gave $pK_1$: 2.65 and $pK_2$: 5.80. The second acid dissociation constant of bathocuproine disulfonic acid was smaller than that of bathophenanthroline disulfonic acid, presumably due to the basic character of the latter imparted by the presence of 2- and 9-methyl groups. The neutralization titration curve of the ammonium salt of terosite sulfonate with sodium hydroxide gave the second dissociation constant at a pH of 6.45. The end-point was found to be at pH of 7.58. The increase in $pK_2$ value could be due to the more acidic character of the terosite sulfonate.

The effect of pH on formation of the ferrous complex of terosite sulfonate was accomplished by keeping the
FIGURE 24
PROPOSED STRUCTURES OF FERROUS ION COMPLEXES
WITH VARIOUS LIGANDS

Legend

A. Illustrates the tridentate (three-toothed) chelate ring formed when 2,3,6-tripryidyl-
s-triazine unites with ferrous ion in the ratio of two to one.

B. This presents the proposed bis-complex of ferrous ions with ammonium salt of the terosite sulfonate.

C. Shows the tris-complex of 1,10-phenanthroline with ferrous ions.
amount of iron and the ligand constant in a buffer of different pH. Buffers of pH 8.0 and 4.0 were prepared from buffer concentrates of potassium phosphate mono-basic-sodium hydroxide. The buffer of pH 9.0 was composed of boric acid-sodium hydroxide. Potassium chloride-sodium hydroxide was used to make buffer of pH 10.0. The pH range over which the ferrous(II)terosite sulfonate complex was stable was found to be between pH 2.0 - 5.0 (See Figure 18). Below pH 2.0, the complex formation was very slow. Above pH 5.0, the complex formation was decreased slightly. The pH stability studies of Zak et al. (99) have shown that the crude product obtained on sulfonation had the stability between the range of pH 3 - 10.

The automated analytical system (Figures 20 - 22) was stable, trouble-free, and yielded precise results. A single dialysis of the serum specimen was adequate for the routine analysis.
D. CONCLUSIONS

For clinical analytical purposes, a fairly low cost, yet highly sensitive terosite sulfonate reagent for iron determination, was synthesized. The cost was calculated to be 0.35¢ per analysis, which is comparable to other reagents used for serum iron determination.

The ferrous-bis (terosite sulfonate) complex was found to be very stable and did not decolorize on storage. The optimum pH range for the formation of the complex was found to be between pH range of 2 - 5.0. The absorption spectrum of the sulfonated product had the same absorption characteristics as that of the unsulfonated terosite, and the Beer-Lambert law was found to be linear at submicrogram levels. The absorbance at the absorption maxima of 340 nm, was found to be much higher than at 583 nm and, therefore, could be used to improve the sensitivity of the procedure.

The ferrous-bis (terosite sulfonate) complex was very soluble in aqueous medium and could not be extracted with organic solvents. The potentiometric titration showed that the second dissociation constant was found to be at pH 6.45.

Finally, the manual analytical procedure for the determination of iron was automated using the Technicon® basic AutoAnalyzer® system.
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1973 - Served as Program-Chairman for
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1974 - Nominated Chairman of the
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          #10, London-Windsor.
1975 - Served on the Sandwich West
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Past Positions:

Union Rheinische Braunkohlen-Krafstoff-AG. Worked in the main laboratories, gained experience in routine analysis of petroleum products. 1956-58

Aktiebolaget LEO, Sweden. Worked in the pharmaceutical laboratories as a practical in the analytical laboratory. 1959

AEG Fabriken Brunnenstrasse, Berlin. Worked as a chemist. My chief duties were concerned with analytical work with polymers, paints, metals and bituminous compounds. 1960-64

Empire Laboratories Limited, 301 Lansdowne Ave., Toronto, Ontario. Employed as a Chief Chemist. Duties involved supervision of eleven technicians and technologists concerned with Quality Control of pharmaceutical products, and Organic Synthesis. 1965-66

Bell-Craig Pharmaceuticals, Toronto, Ontario. Employed as a research chemist. Activities included development of analytical methods in biological fluids of antibiotics and other products. 1966