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REACTION OF UREA WITH DIACETYL MONOXIME AND DIACETYL

BY

ROSITA (YU SI TAN) LUGOSI

A Thesis
Submitted to the Faculty of Graduate Studies through the
Department of Chemistry in Partial Fulfillment
of the Requirements for the Degree of
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Windsor, Ontario

1971

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ABSTRACT

The different methods used for the determination of urea in clinical laboratories as well as in industry have been reviewed. An attempt to synthesize 3-hydroxy-5,6-dimethyl-1,2,4-triazine as a potential pigment precursor for the urea color reaction was unsuccessful. While preparing diacetyl monosemicarbazone as a reactant for the above synthesis, a compound was obtained which was shown to be the isomer of diacetyl monosemicarbazone.

From the similarities in the absorption maximum peak (478 nm) and the superimposability of the curves ($\log_{10}$ A vs. wavelength) of the urea-alpha diketone reaction product and that of the compound synthesized, it was proven that this compound is the potential pigment precursor. The evidence obtained from the i. r. spectra and the $\log_{10}$ A vs. wavelength curves showed that, irrespective of whether diacetyl or its monoxime was used in the reaction, the same compound was obtained. This thesis favours the concept that the protochromogen is a straight chain condensation product, diacetyl diureide, rather than a cyclized product, 3a,6a-dimethylglycoluril, which has been suggested as the protochromogen.

A Beer's law study was performed using the synthesized compound as the protochromogen and compared to the urea-diacetyl and urea-diacetyl monoxime color reactions.

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Acknowledgement is also extended to the National Research Council of Canada who sponsored this study in part.

Last but not least I thank my husband for his unending patience and encouragement.

DEDICATION

TO MY HUSBAND

AND

TO OUR DAUGHTER

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ABBREVIATIONS

°C	degree centigrade
$E_{1/2}$	half-wave potential
μg	microgram
g	gram
ml	milliliter
μl	microliter
α	alpha
DAM	diacetyl monoxime
DA	diacetyl
mp	melting point
min	minute
N	normal
M	molar
(d)	decomposition
DMS	diacetyl monosemicarbazone
λ_E	excitation wavelength
% I	percent fluorescent intensity
nm	nanometer
mg	milligram
Pd/C	palladium on charcoal
A	absorbance

w/w	weight by weight
w/v	weight by volume
BUN	blood urea nitrogen
i. r.	infrared

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CHAPTER I

INTRODUCTION

In man the end-product of nitrogen metabolism is urea. When amino acids (in the form of proteins) are provided in excess of requirements for synthesis of new protein molecules and other nitrogenous substances, the excess nitrogen enters into the formation of urea, which is the chief end-product of amino acid metabolism. Ammonia and carbon dioxide are carried by a derivative of glutamic acid (carbamyl-glutamic acid) and are transferred to another amino acid, ornithine. This undergoes amination (transfer of NH_2 from aspartic acid) to form arginine, which is split by the enzyme, arginase, into urea and a molecule of ornithine, which can then begin again the so-called ornithine-citrulline-arginine or urea cycle (1). The principal sources of ammonia for biosynthesis of urea include the oxidative (2) and non-oxidative deaminations of amino acids and the hydrolysis of the amides of glutamic and aspartic acids. Carbon dioxide, on the other hand, is derived from the oxidation of carbohydrates and fats in the Krebs or citric acid or tricarboxylic acid cycle (1). Formation of urea (i.e., starting with ammonia) takes place practically exclusively in the liver. Although other tissues may contain and form small amounts of urea, this apparently results only from the hydrolysis of the arginine which happens to be present. The indication at present is that the formation of urea is practically irreversible in the body and that once urea is formed it enters

the systemic circulation and is excreted from the body, mainly in the urine. While nitrogen containing substances other than urea certainly occur in the urine of man, urea is quantitatively the most important.

Urea is an extremely diffusible substance and, as such, exists in all body fluids in practically the same concentration. Thus, it is present in the spinal fluid, saliva, exudates, and transudates in approximately the same amount as in blood. Considerable amounts may be lost also through the skin if perspiration is active. The blood urea normally ranges from 20 to 35 mg per 100 ml, the urea nitrogen being 9 to 17 mg (46.6 per cent of the total urea molecule). The extreme normal limits under conditions of very low to very high protein intake are 5 to 23 mg urea nitrogen per 100 ml. Values of 5 to 12 mg are common in the last months of normal pregnancy. The blood urea nitrogen (BUN), as well as the quantity of urea excreted in the urine, depends largely upon the protein intake. With an average diet, approximately 30 g of urea are eliminated in twenty-four hours, constituting about 50 per cent of the total urinary solids.

Abnormal levels of urea are usually an indication of certain diseases (3-4). The most common cause for increased BUN is inadequate excretion due usually to kidney diseases, urinary tract obstruction, hepatic and biliary tract disease, shock and hemoconcentration, excessive protein catabolism, renal failure in hyperparathyroidism, and after hemorrhage. Decreased BUN is observed in acute hepatic insufficiency and in normal pregnancy.

Clinically urea is routinely determined mostly in blood and urine, although sometimes it is also estimated in other biological fluids such as spinal fluid, saliva, and sweat. In industry, urea is determined in fertilizers, feeds, milk, natural and swimming-pool waters, cotton filling materials, agricultural nitrogen solutions, volatile corrosion inhibitor materials, and in synthetic resins. Since urea is one of the most important of the simple nitrogenous compounds from the point of view of physiology and industry, a review of the different methods used for its determination will be presented.

In soda production, urea is determined in mother liquors by the diacetyl monoxime (DAM) method (5). Urea, in aqueous solution containing ammonium nitrate and ammonia, is determined rapidly and reliably by neutralizing to methyl purple, incubating with urease (an enzyme specific only for urea), and then titrating with standard acid to the methyl purple end-point (6). Gullstrom and Demkovich (7),however, determined the urea content by decomposing it with nitrous acid, absorbing the carbon dioxide evolved in a solution of $Ba(OH)_2$ and $BaCl_2$, and titrating the excess of base. By developing a chemical and fluorescence (violet) test, Jephcott and Bishop (8) were able to detect second-hand white cotton filling materials used in articles of bedding and upholstered furniture, that is, if the concentration of urea exceeds 0.0105 %. Since urea is the best nitrogen source for the production of yeast, Nase (9) found among the several methods he examined that the phenol-hypochlorite procedure is the best for determining urea added in

molasses and synthetic media. The method is based on measuring colorimetrically the bluish green color developed. Urea, normally present in calcium cyanamide, can be estimated by means of urease (10), by decomposition with nitric acid (11), and by precipitating as dixanthylurea with xanthydrol reagent (12). This latter method is also found to be useful in determining urea in a bath for black chromium plating (13). The classical method for the determination of urea in fertilizers makes use of the fact that urea can be estimated as secondary oxalate with oxalic acid (14). The rapid volumetric urease method (10) is applicable with satisfactory precision and accuracy to phosphatic fertilizer mixtures containing 1-10% of urea (15). The recent and modern method is based on the yellow-green color developed by urea with p-dimethylaminobenzaldehyde and is measured spectrophotometrically at 420 or 440 nm (16-17). This same method is also widely used in the determination of the urea content of urea-formaldehyde resin solution, alone or in mixtures with melamine resins (18), and in feeds (16, 19-20). The resins are first hydrolyzed in methanol with hydrochloric acid. Slater, Hamm, and Sesso (21) measured the content of urea in fertilizers and animal feed mixture by titrating the ammonia, liberated after hydrolysis with urease, with sulfuric acid to a potentiometric end-point at pH 4.1 $\pm$ 0.05. The determination of urea in water proves to be useful as an indicator of the presence of sewage or the degree of contamination of chlorinated water. The urease method (22-23), the xanthydrol method (24), and the phenol-hypochlorite method (25) are used for the detection and determination of urea in natural, sea, or swimming-pool water. In milk,

the only method used so far for urea determination is by urease hydrolysis (26–27). Urea is also estimated in soils by the DAM-thiosemicarbazide method (28), and in soils and vegetables by its reaction with p-dimethylaminobenzaldehyde in acid solution (29). LeMar and Bootzin (30) used diacetyl (DA) to determine very small quantities of urea in volatile corrosion inhibitor materials.

One of the most common analyses performed in the clinical laboratory is the determination of urea nitrogen in blood – second only in importance to glucose. Methods for its determination in blood and urine may be grouped into miscellaneous methods, methods employing urease action, and methods based on color reaction with diacetyl monoxime, diacetyl or similar compounds.

Urea at temperatures of about 125°C and above is hydrolyzed to NH_3 and CO_2. Quantitation of the NH_3 thus formed as a basis of analysis (31–32) goes back to the nineteenth century. This technique is not very popular due to the inconvenience of autoclaving, but from time to time it is revived (32–34). Lindsay (35) based his method on the decomposition of urea with $MgCl_2$ and the NH_3 distilled in vacuo. Several other chemicals, such as HNO_2 (36), LiCl (37), $Hg(NO_3)_2$ (38), and Millon's reagent (39) are also used to decompose urea and the CO_2 or N_2 set free is then measured. By the use of $HgCl_2$, urea can be measured in blood gravimetrically (40) or volumetrically (41). Titration with acid (42–43) is one of the early methods for the determination of urea in blood and urine. Recently, this method has been reintroduced with the use of a Spinco Microtitrator (44). Gasometric measurement of the nitrogen or ammonia

formed by reaction of urea with alkaline hypobromite (45–47) is also an early method. A manometric technique (48–50) based on the hypobromite reaction has also been developed for estimating urea. Jolles (51) and Haesler (52) measured gravimetrically the CO_2 produced by reaction of urea with sodium hypobromite instead of the nitrogen formed. These classical methods are apparently seldom used today, although the hypobromite method has been recently (53) proposed as an ultramicro technique with the Scholander-Roughton Syringe. Vinogradova (54) modified the method for his determination of urea in blood and urine. The hypobromite reaction is not strictly stoichiometric (55) and not entirely specific (56). The use of N-bromosuccinimide has been introduced as a substitute for hypobromite in the determination of urea in urine (57).

In 1914, Fosse (58) introduced a gravimetric method in which urea was precipitated as dixanthylurea by the addition of an alcoholic solution of xanthydrol. Several workers (59–61) afterwards used Fosse's reagent (xanthydrol) for the estimation of urea in blood and urine. This technique was converted to a photometric procedure (62–67) by reaction of the precipitate to form a yellow color in an acidic medium. There is, however, a six-hour waiting period for a refrigeration step and some slight interference from thiourea and allantoin. Allen and Luck (68) precipitated urea as dixanthylurea and the latter was estimated by oxidation with $K_2Cr_2O_7$. A radiochemical method for the submicro determination of urea (15–75 μg) was developed by Herbain and Bertin (69). Urea was treated with labelled xanthydrol-9-C^{14} and the specific activity of the dixanthyl-

urea precipitate was measured. The xanthydrol method has also been applied to the estimation of urea in biological fluids other than blood and urine (70-71), and in organs and tissues (72).

The yellow-green color produced when p-dimethylaminobenzaldehyde is added to urea in dilute HCl has also been proposed as the basis of a photometric method (73-82), but it cannot be used in the presence of p-aminosalicylic acid or sulfa drugs (75). Recently, Nakagawa, Heirwegh, and De Groote (83) based their urea determination on this method in combination with the use of the specificity of urease to prepare a blank containing the color formed by components of the sample (urine and serum) except the urea. A test for the detection of urea in body fluids has been developed by Okuda (84). This device is comprised of a permeable-base material containing a strong cation exchange resin (Amberlite SA-2) and coated with an acidic solution of p-dimethylaminocinnamaldehyde. This treated paper is dipped into the serum sample and the resulting red color varies with the amount of urea present. A quick and reasonably accurate polarographic method for estimation of urea both as a solid and in solution was developed by Bhatnagar, Singh, and Roy (85). The method is based on the effect of urea on the limiting current as well as $E_{1/2}$ of Ti(IV) in a solution containing 0.005M Ti(IV), 0.4N H_2SO_4, and 0.3M $(NH_4)_2SO_4$.

The extreme specificity of the enzyme urease is the chief attraction of any method based on the hydrolysis of urea by the enzyme. No other substances has ever been found yet that is acted upon by urease. Most of the urease techniques measure the ammonia formed,

although the quantity of urea originally present can be determined also by gasometric measurement of the CO_2 formed (86-87). In 1913, Marshall (88) was the first to utilize urease as a tool for the determination of urea in blood, measuring the ammonia formed by titration after its isolation by aeration. Lisle (89) makes use of this principle in developing a rapid and accurate procedure.

Conway (90) devised an absorption apparatus for the micro-determination of certain volatile substances. Abelin (91) and other workers (92-93) applied this device to the estimation of urea whereby ammonia is isothermally distilled and titrated after urease treatment. The Conway microdiffusion method has been modified recently for use on chromatographic paper (94). The method depends upon the capillary migration of serum or plasma along strips of chromatographic paper impregnated with successive bands of different reagents separated by plastic screen. The Conway microdiffusion method has also been used for the determination of urea in saliva (95). The Conway technique suffers a few drawbacks : extra labor is involved; complete recovery of the ammonia by aeration has been claimed to be extremely difficult; and when performed at room temperature, recovery may be only 85% (96). Other workers, however, have obtained complete recovery (97). To avoid this possible difficulty and to shorten the procedure, nesslerization is applied to a protein-free filtrate (98-106). This direct procedure can be carried out either by adding urease to a protein-free filtrate or by adding it to the whole blood or serum. An example of the first case is that of Karr (100) in which he added the urease to a tungstic acid filtrate.

This technique requires buffering of the filtrate at which the urease is active; for phosphate or acetate buffers the optimal pH is about 7.1 (107). The second procedure can be either with or without buffer, and a protein-free filtrate is prepared after the urea is converted to ammonia (108). Filtrates from trichloroacetic or tungstic acid have been stated to be inferior to Somogyi's zinc hydroxide filtrate since they contain substances other than ammonia which also give color after nesslerization. Henry and Chiamori (109), however, showed that both types of filtrate behave the same : that is, substances are present other than NH_4^+ which slowly develop a yellow color.

Another urease method is based on the difference in electrical conductivity of urea and of the ammonium carbonate produced from urea by urease (110). Nielsen (111) based his method on the change in pH caused by the enzymatic hydrolysis of urea. The NH_4^+ liberated by urease-catalyzed hydrolysis of urea can be determined potentiometrically (112). Ammonia resulting from urease hydrolysis of urea can be titrated with coulometrically-generated OBr^- using a direct amperometric end-point detection (113). The reaction between ammonia (from urea by urease), reduced NAD and 2-oxo-glutarate to form L-glutamate, NAD and H_2O (which takes place in the presence of glutamate dehydrogenase) is the basis of a method (114-115) for determining urea in blood and serum. The ammonia is measured by the decreased in the extinction of the NADH at 366 nm. This reaction was modified into a photofluorimetric technique (116) for estimating urea concentrations in nanolitre specimens. The concentration of

NAD formed is determined fluorimetrically after treatment with strongly alkaline solution or by condensation with ethyl methyl ketone. The fluorescence is measured at 460 nm with excitation at 340 nm.

Silver manganese nitrate, a reagent more sensitive to ammonia than Nessler's reagent, has been used in a urease method for urea determination (117). The test is particularly useful as a screening test for uremia.

A semi-quantitative procedure called Urograph or Urastrat has been offered by Warner-Chilcott Laboratories. A strip of test paper comprising four zones (urease, K_2CO_3, a barrier, and bromocresol green or purple in tartaric acid) is placed in a stoppered tube containing 0.1 ml of serum or plasma. The liberated ammonia causes a blue color change in the indicator zone, which is compared with a standard chart. The claims made by the company have been confirmed (118) and it was applied by other workers (119-120) as a screening test.

In 1859, Berthelot (121) described the reaction between ammonia and phenol in the presence of hypochlorite to yield a blue color. Early application of the Berthelot reaction to the analysis of preformed NH_3 (122-124) or for NH_3 derived from urea by urease action (125-126) did not employ a catalyst for the reaction. The final color, however, was not stable, and reproducibility was a problem. Lubochinsky and Zalta (127) introduced the next technical advance with the discovery that sodium nitroprusside catalyzed the Berthelot reaction, improving the intensity, reproducibility, and stability.

of the blue color. This very sensitive colorimetric procedure for the determination of ammonia in combination with the specificity of the urease reaction has been applied to the determination of urea in serum plasma, whole blood, and urine (128-132). Acetone (133) and acetone in combination with nitroprusside (134) have also been used as catalysts. Chaney and Marbach (135) simplified the procedure of Fawcett and Scott (128) by reducing the number of reagents from three to two and increasing their stability. La Rosa (136) makes use of the emerald green color produced by urea with phenol-sodium hypobromite after urease treatment for his colorimetric method. The procedure as described by Fawcett and Scott (128) and modified by Chaney and Marbach (135) was adapted for use with the AutoAnalyzer (137-139).

Recently, a specific enzyme electrode has been devised for the determination of urea in blood and urine (140-145). The electrode is prepared by immobilizing a layer of urease in polyacrylamide over the surface of a Beckman cation electrode responsive to NH_4^+ and is used in an ion-exchange system with an uncoated cation electrode as a reference. The immobilized enzyme catalyzes the decomposition of urea to NH_4^+ at the surface of the cationic electrode. The NH_4^+ is sensed by the cation electrode; the steady-state potential developed is proportional to the logarithm of the urea concentration.

In 1939, Fearon (146) found that reaction with diacetyl monoxime (DAM) followed by oxidation gives colors with $R_1NH-CO-NHR_2$ when R_1 is H or a single aliphatic radical and R_2 is not an acyl radical. Thus, colors were produced with urea, citrulline, methyl-

urea and urea derivatives, allantoin, and proteins. These various compounds give varying colors but any substance with a ureide grouping will generally give a yellow color (147). Many substituted ureas give a red color, but only urea yields a yellow pigment. Abelin (148) first applied the reaction to the determination of urea in serum, but, since the reaction was carried out in neutral solution and no oxidizing agent was added, only a very rough approximation of the amount of urea present was given. In 1942, Ormsby (149) applied this reaction to the determination of urea. The sample is heated with DAM in strongly acid solution and the resultant yellow color is intensified by oxidation, with potassium persulfate, of the hydroxylamine formed in the reaction (150-151). Thus, the determination of urea has turned away from enzymatic methods and back to more strictly chemical ones. This change certainly involves a decrease in specificity, but at the same time a tremendous increase in convenience. Urease methods will remain the standards of reference, but need not be required for many ordinary purposes. Kawerau (151) and Rosenthal (152) used an arsenic pentoxide-hydrochloric acid mixture for simultaneous color development with DAM in dilute acetic acid solution, the hydroxylamine being oxidized as it is liberated during the heating period. Friedman (153) and other workers (154-159) modified the procedure by using sulfuric acid in place of the concentrated hydrochloric acid. This eliminates the objectionable odors of fuming hydrochloric acid. Richter and Lapointe (160) used a ferric alum reagent in an automated analysis for urea to eliminate the turbidity in the test solution.

Natelson and his associates (161) have shown that the potassium persulfate serves to destroy the hydroxylamine formed, which inhibits color formation. Natelson proposed the use of free diacetyl (DA), since this appears to be the active reagent. This, however, has been reported and confirmed (153, 162) to be capricious, indicating that certain as yet unknown variables are operative. Dickenman, Crafts, and Zak (163) modified the method by using a DA-phosphoric acid mixture as the color reagent.

Condensation of urea with DAM in the presence of N-phenylanthranilic acid (164-167) has also been proposed to enhance the color formation. The reaction of urea with DAM, as modified by the addition of phenazone or antipyrine (168-172) fulfills many of the requirements for a good microquantitative method : it is very sensitive, simple and reproducible, gives a linear response over a wide range of concentration, and the color produced is stable. However, the development of color is rather slow which hinders its application to automated analysis. To overcome this difficulty, the reaction rate was increased by catalysis with various cations, such as Fe^{3+} and Ce^{4+}, which makes it possible to automate the method (173-174). Recently, a single reagent mixture of DAM, antipyrine, and arsenic pentoxide in acid solution was used for the direct determination of urea nitrogen in serum (175). In acid medium, DAM and thiosemicarbazide form a red complex (max. 530 nm) with urea which is stable at room temperature. This was the basis used by several workers (176-178) for their manual as well as automated colorimetric determination of urea. Thiosemicarbazide minimizes the photosensitivity of the reaction.

Laboratories of clinical chemistry are confronted with an ever-increasing number and variety of determinations. It seemed worthwhile, therefore, to explore the possibility of automated methods for analyses in clinical laboratories. In 1957, Skeggs (179) discovered that colorimetric analysis could be performed in continuously flowing streams, thus eliminating the need for stepwise measurement, addition, and processing of samples and reagents. An automatic device was then designed for blood analysis in which the removal of blood proteins is made possible by the use of a dialyzer that was designed to process continuously flowing streams of solutions. Marsh, Pingerhut, and Kirsch (180) adapted this device in the determination of urea. The urea, which is dialyzed from the blood, is then permitted to react with DAM in order to produce a colored product, the intensity of which is automatically measured and recorded on a chart as percentage transmission. Technicon Corporation adapted Skeggs' continuous flow system and manufactured the "AutoAnalyzer", which is designed primarily for automated analyses in clinical laboratories as well as in industry. The method of Marsh, Pingerhut, and Kirsch (180) was the basis of the urea determination adapted by the Technicon Corporation for use with the AutoAnalyzer (181). Pellerin (182), Moore and Sax (183), and Mather and Roland (184) used strong acids in their automated system. Marsh, Pingerhut, and Miller (185) take advantage of the increased sensitivity gained by combined use of ferric ion and thiosemicarbazide to reduce the acid requirement to about one-tenth that originally needed in their automated and manual methods for the direct determination of urea. This was

modified and adapted for use with the AutoAnalyzer (186) by the Technicon Corporation. Evans (187) made some modifications of the procedure so that it can be used manually in case of emergency. Simultaneous determination of blood glucose and urea using the Auto-Analyzer has also been developed (188-190). Glucose analysis is based upon its reduction of ferricyanide to ferrocyanide, and determination of the ferrocyanide formed with molybdate (191). The urea nitrogen technique is based on the method of Marsh, Fingerhut, and Miller (185). Girardet and Cornillot (192), however, adapted the procedure of Ceriotti and Spandrio (168) in which antipyrine is used instead of thiosemicarbazide. Vigneron and Siest (193) used a phosphoferric reagent in their DAM procedure since it is less toxic and corrosive, easier to prepare, and has a range of sensitivity to cover high urea concentrations. They later modified the method by suppression of dialysis, that is, an automated determination of urea in a protein medium (194).

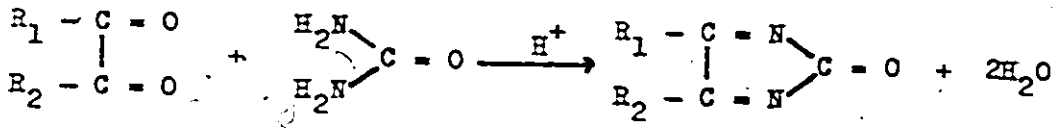
The use of semidine (195) as a sensitizing agent in direct assay method for determination of urea, citrulline, and carbamyl derivatives allows elimination of the deproteinization of serum and provides a simple, sensitive, and rapid procedure. Girard and Dreux (196) used chloramine-T solution to intensify the color development. D-Glucurono-lactone or D-glucuronic acid amide was found to produce an intense orange and photostable color when added to the reaction mixture of DAM and urea in moderately concentrated phosphoric acid. A simple, accurate, and reproducible method for determination of urea in blood and urine was then developed utilizing this reaction (197-198). This was later adapted in automated procedure (199). A fluorescent

property of the urea-alpha diketone compound was discovered by McClesky (200). The fluorescent peak was observed at a wavelength of 415 nm when the compound was activated at an optimal wavelength of 380 nm.

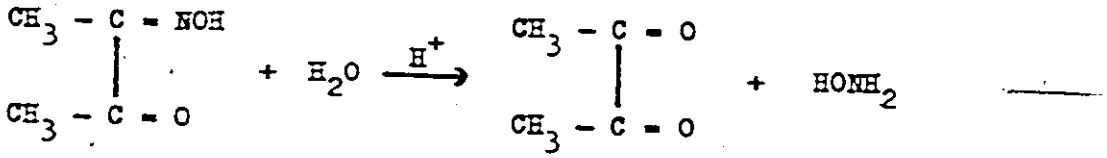
Dudek (201) and others (202-203) described a method for the direct colorimetric determination of urea in blood. This is based on the reaction between dimethylglyoxime and urea in the presence of sulfuric acid. Khramov and Galaev (204) modified the procedure by adding thiosemicarbazide to the reaction which is sensitive to 1 µg of urea per ml. The colorimetric method developed by Archibald (205-206) which requires a 2-ml sample permits direct determination of urea using α-isonitrosopropiophenone, the monoxime of benzoyl acetyl, as the reagent for color development. The adaptation of this procedure used with a 5-µl sample of plasma (207) gives reproducible results and quantitative recovery of urea and checks with analysis of synthetic control serum analyzed by a macromethod. Siest (208) studied the influence of proteins on the color reaction of urea with α-isonitrosopropiophenone. The results indicate that the presence of some proteins is necessary for the maximum development of the violet color. Timmermans (209) and Pantini (210) also based their procedure on Archibald's color reaction. Several α-diketones (211), such as 1,2-cyclo-heptanedione dioxime (heptoxime) (212), 1,2-cyclo-hexanedione (213), and 1,2-cyclo-hexane dioxime (nioxime) (214), are also found to produce a photostable color with urea in an acidic medium at 37°C.

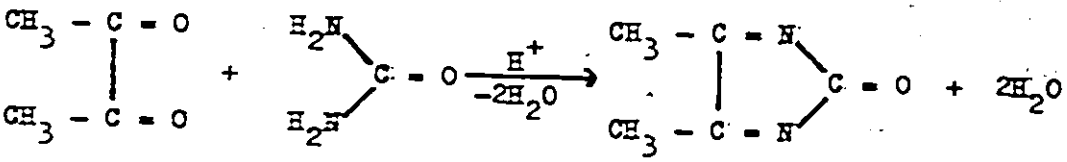
The mechanism of the reactions between alpha-diketones and urea

or urea derivatives is not known (215); however, analogous reactions such as those involving o-phenylenediamine and benzil have been described (216-217). These involve ring formation on condensation under given conditions. Based on this Dickenman, Crafts, and Zak (163) postulated a mechanism for urea-diacetyl reaction in which a 5-membered heterocyclic ring is formed,

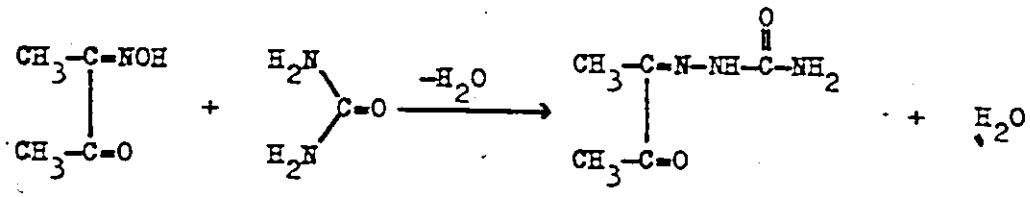


where R_1 and R_2 is a methyl group or R_2, a phenyl. Tietz (218) presents a reaction mechanism scheme for urea-DAM reaction,

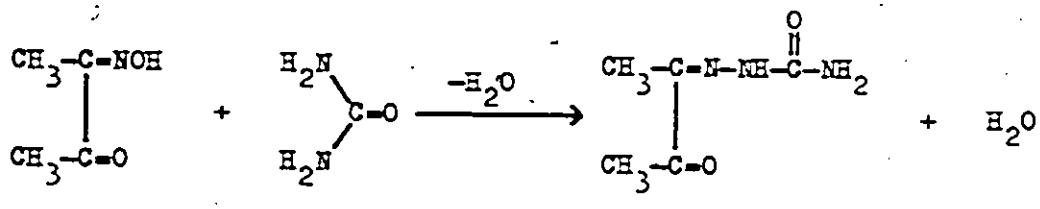




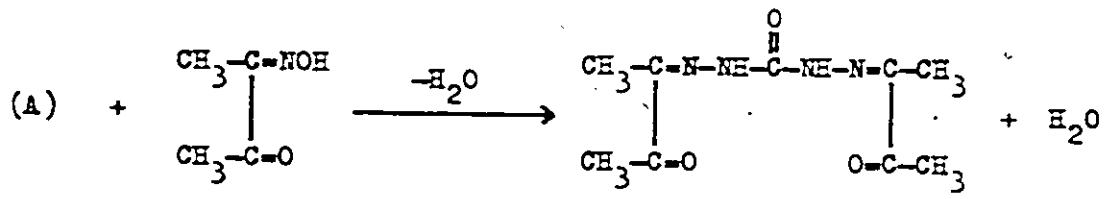
in which the DAM is first hydrolyzed to the free diacetyl and hydroxylamine; the liberated diacetyl then react with urea to form a 5-membered heterocyclic ring compound. Beale and Croft (165), however, gave three possibilities as to the nature of the reaction between urea and DAM. The first possibility is a straight chain condensation between one molecule of DAM and one molecule of urea;



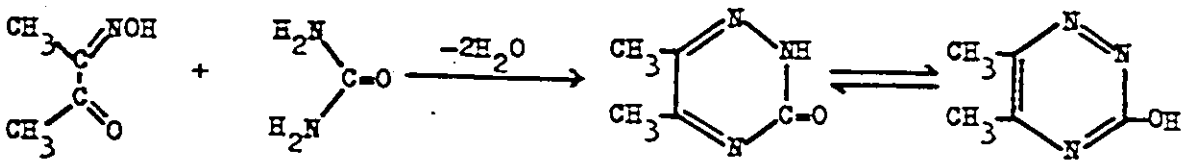
the second is similar to the first one except that the ratio of DAM to urea is 2 to 1;



(A)

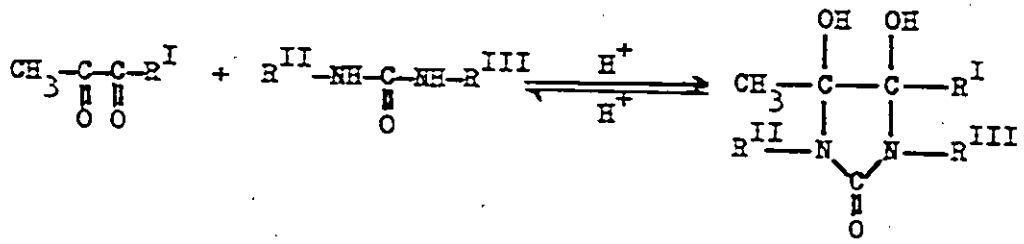


and the last one is a 1:1 ring condensation between urea and DAM to form a substituted 1,2,4-triazine:



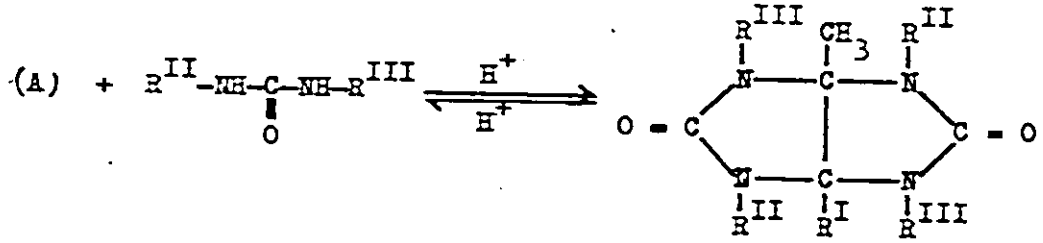
The condensation product is 3-hydroxy-5,6-dimethyl-1,2,4-triazine. Khramov and Galaev (204) modified Pearon's reaction by using dimethyl-

glyoxime and thiosemicarbazide as the color reagent. According to them, the probable variant appears to be a cyclic condensation with formation of a 1,2,4-triazine. Veniamin and Vakirtzi-Lemonias (219) recently investigated the carbamido-diacetyl colorimetric assay and they presented evidence establishing the involvement of either 7- or 8-methyl, or 7,8-dimethyltetrahydroimidazo(4,5-d)imidazole-2,5-diones as chromogens, all three being equally acceptable. Their experimentation has led to the proposal of an overall reaction scheme,



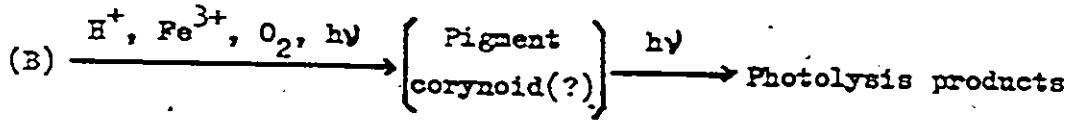
(A)

4,5-dihydroxy-2-imidazolidinone



(B)

glycoluril derivatives



which essentially involves either of the above-mentioned glycolurils (7- or 8-methyl or 7,8-dimethyltetrahydroimidazo(4,5-d)imidazole-2,5-

diones) as the potential pigment precursor.

Up to the time that this project was undertaken, the exact nature of the substance formed between the reaction of urea with DAM and DA was not known, except that it is yellow and yields maximum absorption at 480 nm. Unfortunately the color is unstable because it is reduced by light. It is therefore the main object of this research study to try and find out, if possible, the nature of the pigment formed, or at least the protochromogen to the yellow pigment.

CHAPTER II

EXPERIMENTAL

A. Preparation of Diacetyl Monosemicarbazone (DMS)

Materials and Methods

1) Apparatus

Corrected melting point measurements were taken with a Fisher-Johns Melting Point Apparatus. Infrared spectra were obtained with a Beckman IR-12 infrared spectrophotometer using 0.25% w/w potassium bromide discs. Ultraviolet spectra were obtained in ethanol with a Beckman Model DB spectrophotometer equipped with a Wavelength Drive Unit and a Sargent SRL recorder. The fluorimetric spectra were made in ethanol with a Perkin-Elmer Model 203 fluorescence spectrophotometer.

2) Reagents

2,3-Butanedione (or diacetyl) and semicarbazide hydrochloride (Fisher Certified Reagent) were obtained from Fisher Scientific Company, Canada. All solutions were made up in deionized, distilled water.

3) Synthesis

Diacetyl monosemicarbazone was prepared for the synthesis of 3-hydroxy-5,6-dimethyl-1,2,4-triazine. The procedure followed was basically that of the first method given by Diels (220). The first method was chosen simply because it is simple and gave a better yield. Diacetyl (DA), 5 g, was dissolved in 25 ml of deionized, distilled water and to this was added a solution of 10 g semicarbazide

hydrochloride in 25 ml of deionized, distilled water. The reaction was carried out in an ice bath with constant stirring. A thick slurry of white precipitate was formed immediately. The reaction mixture was allowed to stand for 30 min to insure completion of reaction. The precipitate was then filtered, washed with cold water, and dried under vacuum. The yield was 7.4 g (57.7%). The precipitate was boiled in about 200 ml of hot water for 5 to 10 min and filtered through a Büchner funnel which has two filter papers covered with a thin layer of moistened activated charcoal (Norit A). The colorless filtrate was cooled to room temperature, at which time fine needle-like crystals started to separate out from the solution. This was allowed to stand overnight in the cold (refrigerator) to insure complete crystallization. Fine white needle-shaped crystals in leaflet aggregates were obtained. The crystals were filtered, washed with cold water, and dried in an oven at 100°C for at least two hours. Recrystallization from boiling water was repeated until a constant melting point was obtained. There were obtained 2.1 g of white crystals, mp 234°-5°C (d).

B. Attempted Synthesis of 3-Hydroxy-5,6-Dimethyl-1,2,4-Triazine

Materials and Methods

1) Reagents

Diacetyl monosemicarbazone was prepared in Part A. Sodium hydroxide and concentrated hydrochloric acid were of analytical grade.

These were used for the preparation of 2N sodium hydroxide and 6N hydrochloric acid solutions, respectively. Both were prepared in deionized, distilled water.

2) Synthesis

The method followed was that of Seibert (221). To a 250-ml, round-bottomed flask, equipped with a reflux-distillation condenser, were added 6.9 g of the diacetyl monosemicarbazone and 60 ml of 2N sodium hydroxide. The mixture was stirred thoroughly and then heated at reflux temperature with a heating mantle. Soon after the semicarbazone began to melt, evolution of gas and formation of a yellow-orange solution were observed. All the semicarbazone dissolved in 15 min. Reflux and subsequent distillation was carried out at 100°C for one hour, at which time the liquid portion was distilled off completely leaving a dark-brown residue in the flask. The distillate was pale yellow in color. The residue was allowed to cool and then acidified with 6N hydrochloric acid to pH 4.0. A few drops of concentrated hydrochloric acid were added as the pH approached 4.0 to keep the volume at a minimum. The mixture was allowed to stand overnight in the refrigerator. The dark-brown precipitate was then filtered, washed with cold water, and recrystallized from hot water after purifying with activated charcoal (Norit A) as in Part A. The colorless filtrate was allowed to stand overnight again in the refrigerator because the crystallization process is very slow. The solvent was removed on a rotary evaporator at a temperature of 50°C. A white precipitate was formed. This did not melt even when a temperature of 300°C was reached.

C. Synthesis of Diacetyl Monosemicarbazone Isomer

Materials and Methods

1) Apparatus

The apparati used were similar to those used in Part A. The elementary analysis was performed by Schwarzkopf Microanalytical Laboratory, 56-19 37th Avenue, Woodside, New York 11377.

2) Reagents

Diacetyl monoxime (2,3-butanedione monoxime) and urea (A.C.S. grade) were obtained from Fisher Scientific Company, Canada. The diacetyl monoxime (DAM) was Fisher reagent grade for urea nitrogen. The DAM and urea solutions were prepared with deionized, distilled water. Concentrated sulfuric acid and phosphoric acid (85% w/w) were of analytical grade.

3) Synthesis

The isomer of DMS was obtained from the filtrate in Part A. This filtrate, which was colorless when the semicarbazone was filtered, turned pink after allowing to stand in the ice bath for half an hour. Some white precipitate was observed to appear during this period. Then the color turned lavender and subsequently purple upon standing overnight at room temperature. The precipitate was filtered, washed with cold water, and dried under vacuum. A first recrystallization was carried out with hot water and Norit A in the same manner as in Part A. Subsequent recrystallization from alcohol gave 0.4 g of white, glossy, flake-like crystals, mp 222°-223°C (d).

Anal. Calc. for $C_5H_9N_3O_2$: C, 42.0; H, 6.33; N, 29.7.

Found: C, 42.03; H, 6.24; N, 28.76.

4) Spectral Study of the DMS Isomer

The spectrum of the reaction product between urea and DAM and that of the DMS isomer in acid medium were compared. The basic experimental procedure was essentially that of Natelson (222). The DMS isomer, 30 mg, was dissolved in 50 ml of hot water. Urea solution was prepared in the same manner and concentration except that no heating is necessary. A sulfuric acid-phosphoric acid reagent was prepared by mixing thoroughly 150 ml of 85% (w/w) phosphoric acid with 140 ml of water. To this solution concentrated sulfuric acid (50 ml) was added slowly while mixing. DAM solution (2%) was made by adding 2 g of DAM to about 60 ml of water in a 100-ml volumetric flask, followed by 2 ml of glacial acetic acid. The mixture was shaken to effect dissolution, with slight warming if necessary, and then made up to the mark.

To 1 ml of the urea solution in a test tube were added 1 ml of water, 0.4 ml DAM, and 1.6 ml of the sulfuric acid-phosphoric acid mixture. The test tube was then stirred thoroughly and placed in a boiling water bath for 30 min, cooled, and the absorbance read against a water blank. At the same time the color from the DMS isomer was developed in the same way, except that 2.4 ml of the sample were nsed in place of the 1 ml urea solution, 1 ml water, and 0.4 ml DAM.

D. Synthesis of the Protochromogen in the Urea-Alpha Diketone Reaction

Materials and Methods

1) Apparatus

Absorbance measurements were made with a Beckman DU monochromator with Gilford Model 2000 Multiple Sample Recorder and a Hitachi Perkin-Elmer Model 139 spectrophotometer. Uncorrected melting point measurements were obtained with a Cenco Electrothermal Melting Point Apparatus. Class A volumetric glassware was used. Infrared spectra were obtained with a Beckman IR-12 infrared spectrophotometer using 0.25% w/w potassium bromide discs. The hydrogenation experiment was run on a sloping-manifold atmospheric pressure hydrogenator designed and assembled by Professor J. M. McIntosh, Ph. D., Chemistry Department, University of Windsor, Windsor, Ontario, Canada.

2) Reagents

Diacetyl monoxime (2,3-butanedione monoxime), diacetyl (2,3-butanedione), sodium nitrite, palladium on charcoal, and urea were obtained from Fisher Scientific Company, Canada. The diacetyl monoxime (DAM) was Fisher reagent grade for urea nitrogen. Urea, sodium nitrite, 85% (w/w) phosphoric acid, concentrated sulfuric and hydrochloric acids were of A. C. S. analytical grade. Glycoluril (practical grade) was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wisconsin. 1,3-Dimethylurea was supplied by Eastman Organic Chemicals, Rochester, New York. Solutions of all of the above reagents were prepared with deionized, distilled water.

3) Synthesis

The potential pigment precursor was synthesized by three means. In the first method, (a), a solution of 10 g 2,3-butanedione (diacetyl) in 50 ml water was mixed thoroughly with a solution of

20 g urea in 50 ml water. To this mixture was added 20 ml of concentrated hydrochloric acid, dropwise, with constant stirring. A pure white precipitate was slowly formed. The reaction mixture was then allowed to stand for two hours at room temperature, after which the precipitate was filtered, washed with cold water thoroughly, and dried under vacuum. The yield was 10 g (A). The same procedure was repeated using a solution of DAM of the same concentration as diacetyl (DA), in place of DA, and 38 ml of concentrated hydrochloric acid were used to effect precipitation. The reaction mixture was allowed to stand overnight in the cold before filtration. The yield was 17.5 g (B). Crude products (A) and (B) were recrystallized first from hot water after treatment of their solutions with Norit A, and subsequently two or three times from hot water alone. White, glossy, flaky crystals were obtained in minute quantity. The crystals were filtered, washed thoroughly with cold water, and dried in the oven at 100°C for at least two hours.

The second method, (b), was essentially that of Williams (223). In a 2-litre, round-bottomed flask, equipped with reflux condenser, were added 50.5 g DAM, 30 g urea, 1 litre 95% alcohol, and 25 ml concentrated hydrochloric acid. The mixture was refluxed for 9 hours; 30 g urea was added every 3 hours until 120 g was present. Alcohol (250 ml of 95%) and 25 ml of concentrated hydrochloric acid were added after the last addition of urea. The mixture was refluxed for six additional hours, cooled, and filtered. The precipitate was washed with 250 ml alcohol and dried in the oven at 100°C for one hour. The sample was then slurried with 750 ml boiling water, filtered, washed with 250 ml alcohol, and dried in the oven at 100°C

for two hours. The yield was 18.4 g (C). This product was recrystallized in the same manner as in the first method.

Anal. Calc. for $C_6H_{10}N_4O_2$: C, 42.36; H, 5.93; N, 32.94.

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

The elementary analysis was done by the same laboratory as in Part C.

The method of Franchmont and Klobbie (224) was followed in the third procedure. In a 250-ml beaker were mixed 20 g of DA, 50 g of water, and 35 g of urea; all dissolved completely. This reaction mixture was allowed to stand overnight. The following day a white precipitate was formed. The precipitate was then filtered, washed with hot water, and dried under vacuum. The yield was 19.44 g (D). Recrystallization was also accomplished in the same way as the other two methods.

All the four compounds (A,B,C,D) obtained from the three methods above exhibit the same appearance and melting behaviour. The crystals were white, glossy, and flaky in shape. The compound turned light brown at 340°C, dark brown at 345°C, and foamed above 354°C without melting.

4) Spectral Study of the Protochromogen

The spectra of the four compounds (A,B,C,D) prepared above and that of urea-DAM in acid medium were examined and compared. The method used in obtaining the absorption curves and the standard curves was essentially that of Natelson (222) as in Part C, except for that involving DA as the color reagent in which the procedure of Dickenman, Crafts, and Zak (163) was followed.

Solutions of the four compounds (A,B,C,D) were prepared by

dissolving each of the samples (A, 10 mg; B, 11 mg; C, 10 mg; D, 10.5 mg) in about 80 ml of hot boiling water and then made up to the mark in a 100-ml volumetric flask. A urea solution of 11.5 mg per 100 ml of water was prepared. The DAM solution was made up in the same manner as that used in Part C. These were used in obtaining the various spectral absorption curves.

For the Beer's law study, a solution of the sample (A, 40.8 mg per 100 ml; B, 40.0 mg per 100 ml; C, 40.3 mg per 100 ml; D, 40.9 mg per 100 ml) was prepared in hot water and a 1:10 dilution was made as the working solution. For a urea stock standard 250 mg per 100 ml was used and the working standard used was a 1:100 dilution of the urea stock standard (0.0250 mg per ml). The urea stock standard used in the urea-DA reaction was 214.4 mg per 100 ml and a 1:100 dilution was used as the working standard. The absorbance was measured at 480 nm. The diacetyl color reagent was prepared by adding a phosphoric acid solution (400 ml 85% phosphoric acid in 100 ml water) to 0.2 ml DA until the mark of a 100-ml volumetric flask was reached. Two milliliters of the sample (A,B,C,D) or urea working standard and 2 ml of the color reagent were mixed in a test tube and placed in a boiling water bath for 10 minutes. The tubes were then removed and cooled in an ice bath for 10 min, and the absorbance of the solutions were read against a water blank at 475 nm. The Beer's law studies were carried in triplicate.

5) Nitrous Acid Test

The procedure is based on that of Bouveault's method (225). The solutions of the synthesized compounds (A,B,C,D) prepared in experiment 4 were treated with an equal volume of 6N hydrochloric

acid and heated in a boiling water bath for 10 minutes. The test tubes were cooled in an ice bath and a 20% solution of sodium nitrite was added below the surface of each of the reaction mixtures. The amount of sodium nitrite used was one-tenth of the volume of the reaction mixture. The procedure was repeated using solutions of glycoluril and 1,3-dimethylurea. The results of the reactions of the three compounds were compared. Glycoluril was recrystallized first from hot water after purifying with Norit A, and subsequently two times from hot water alone. 1,3-Dimethylurea was recrystallized from water:ether (1:2) mixture until a constant melting point (107°C) was reached.

6) Hydrogenation Study

The hydrogenation technique followed was basically that given by Augustine (226). To a 1-litre, round-bottomed flask 80 mg of the synthesized sample were dissolved in 510 ml of hot water. The solution was cooled to room temperature and 4 drops of concentrated hydrochloric acid were added. Then 0.4146 g of 10% Pd/C catalyst was suspended in the solution. A magnetic stirring bar was also placed in the flask. The flask was connected to the hydrogenator and a magnetic stirrer was placed under it. The gas burette was filled with mercury and the stopcock at the top closed. The system was alternately evacuated with a water aspirator and filled with hydrogen three times. After the last hydrogen intake, the stopcock on the burette was opened and the burette was filled with hydrogen. The three-way stopcock was closed and the volume of the hydrogen in the burette was recorded after the pressure has been equalized with the atmosphere by adjusting the leveling bulb. The stirrer was

turned on and hydrogen uptake was allowed to proceed with only occasional adjustment of the leveling bulb.

The hydrogenation was run continuously, with constant stirring, for approximately 17 hours, at which time hydrogen absorption ceased. The pressure of the system was readjusted to atmospheric pressure, the hydrogen volume recorded, and the flask removed. The catalyst was filtered through a Büchner funnel coated with a cake of hyflo. The solvent was removed under vacuum with slight heating (40°-60°C). A white precipitate separated out when the volume of the solvent was reduced to about 100 ml. The precipitate was then filtered, washed with cold water, and dried in the oven at 60°C overnight. The reduced substance was then used to run an i. r. spectrum. The procedure was repeated on a control containing the same amount of water, acid, and catalyst. The difference in the hydrogen uptake between the sample and the control is the amount of hydrogen absorbed by the sample.

CHAPTER III

RESULTS AND DISCUSSION

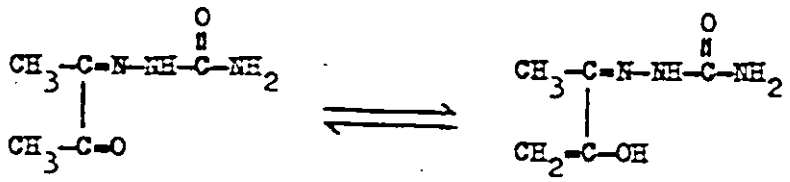
A. Preparation of Diacetyl Monosemicarbazone

The results of the infrared, ultraviolet, and fluorimetric spectra on the compound, diacetyl monosemicarbazone, prepared are reported in Table I. The corrected melting point obtained for the diacetyl monosemicarbazone, 234-5°C, agrees with that of Diels (220). It melts with accompanying active gas evolution and decomposition. With most organic solvents, such as alcohol, methanol, acetone, chloroform, ethyl ether, benzene, toluene, dimethyl sulfoxide, etc., the compound is very difficult to solubilize, even by boiling. Its solubility in boiling water is approximately 1:30 (w/v), and thus its recrystallization was carried out in boiling water.

As Diels had observed, the substance exhibited a weak acid character and forms a yellow solution when dilute sodium hydroxide was added, and when concentrated sodium hydroxide was used, yellow needle-like crystals were obtained. The same observations were noted in the present study. It is soluble in the cold in glacial acetic acid and trifluoroacetic acid. The semicarbazone is not dissolved in most acids in the cold, but it dissolves when heated to boiling. Thus, when the solution of semicarbazone was subjected to the standard conditions for the determination of urea in clinical laboratories, a pinkish solution was obtained.

Figure 1 and Table I show that DMS has an ultraviolet maximum at 267 nm with a molar absorbancy index of 7.4×10^6. A fluorimetric study of the compound was undertaken using the excitation wavelengths (λ_E), 260 nm and 315 nm. The results are shown in Table I and in figures 2 and 3. When the compound was activated at 260 nm, two fluorescent peaks were observed at 312 nm and 395 nm;at an excitation wavelength of 315 nm, the fluorescent peaks appeared at 345 nm and 465 nm. The fluorescence intensity at the lower excitation wavelength (260 nm) was observed to be lower than that activated at the higher excitation wavelength (315 nm).

The i. r. spectrum shows that the compound has the functional groups characteristic of a primary amide (227). It has two moderately intense N-H stretching frequencies, 3350 cm^{-1} and 3200 cm^{-1}, corresponding to the asymmetrical and symmetrical N-H stretching vibrations typical of a primary amide in the solid phase. A moderately intense O-H stretching vibration occurs at a higher frequency, 3500 cm^{-1}. The spectrum shows this band due to the fact that the semicarbazone can also exist in its tautomeric form:



A strong carbonyl absorption band (amide I band) occurs at 1685 cm^{-1} (227). The increase in the frequency of absorption may be attributed to the inductive effect of the NH_2 group adjacent to the carbonyl grouping. An amide II band, which is less intense than the amide I

TABLE I

COMPARISON OF SPECTRAL DATA FOR DMS AND ITS ISOMER

	DMS (mp 234-5°C)	DMS isomer (mp 222-3°C)
Ultraviolet maxima and molar absorbancy index	267 nm and 7.4×10^6	285 nm and 8.0×10^6 210 nm and 5.2×10^6
Fluorescent peaks		
λ_E = 260 nm	312 nm and 395 nm	323 nm and 510 nm
λ_E = 315 nm	345 nm and 465 nm	345 nm and 460 nm
Characteristic infrared bands, cm^{-1}	3500	3490
	3350	3380
	3200	3220
	1685 sh	1680 sh
	1600	1615
	1580	1585
	1450 w	1565
	1425 w	1430 b
	1390	1380
	1360 sh	1300
	1300 sh	1100
	1170	980
	1130	960
	1000	940
	945	880
	765	755 sh
	745	

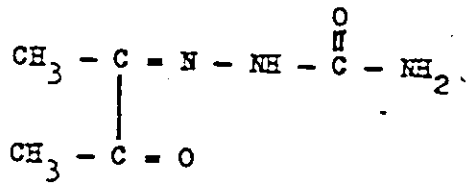
sh sharp

w weak

b broad

band, appears as a doublet at 1600 cm^{-1} and 1580 cm^{-1}. These bands correspond to the N-H bending vibration. The C-N stretching band is found at 1450-1425 cm^{-1} and a broad, medium band in the region of 765-745 cm^{-1} results from the out-of-plane N-H wagging. The spectrum also shows two bands at 1390 cm^{-1} and 1360 cm^{-1}, respectively, corresponding to the asymmetrical and symmetrical methyl bending. The methyl deformation and the C-C stretching vibrations occur at 1170 cm^{-1}.

From the melting point and the different functional groups shown in the i. r. spectrum, the compound prepared is confirmed to be diacetyl monosemicarbazone, which has the structure :



This is the same structure that had been assigned by Diels (220) for this compound.

B. Attempted Synthesis of 3-Hydroxy-5,6-Dimethyl-1,2,4-Triazine

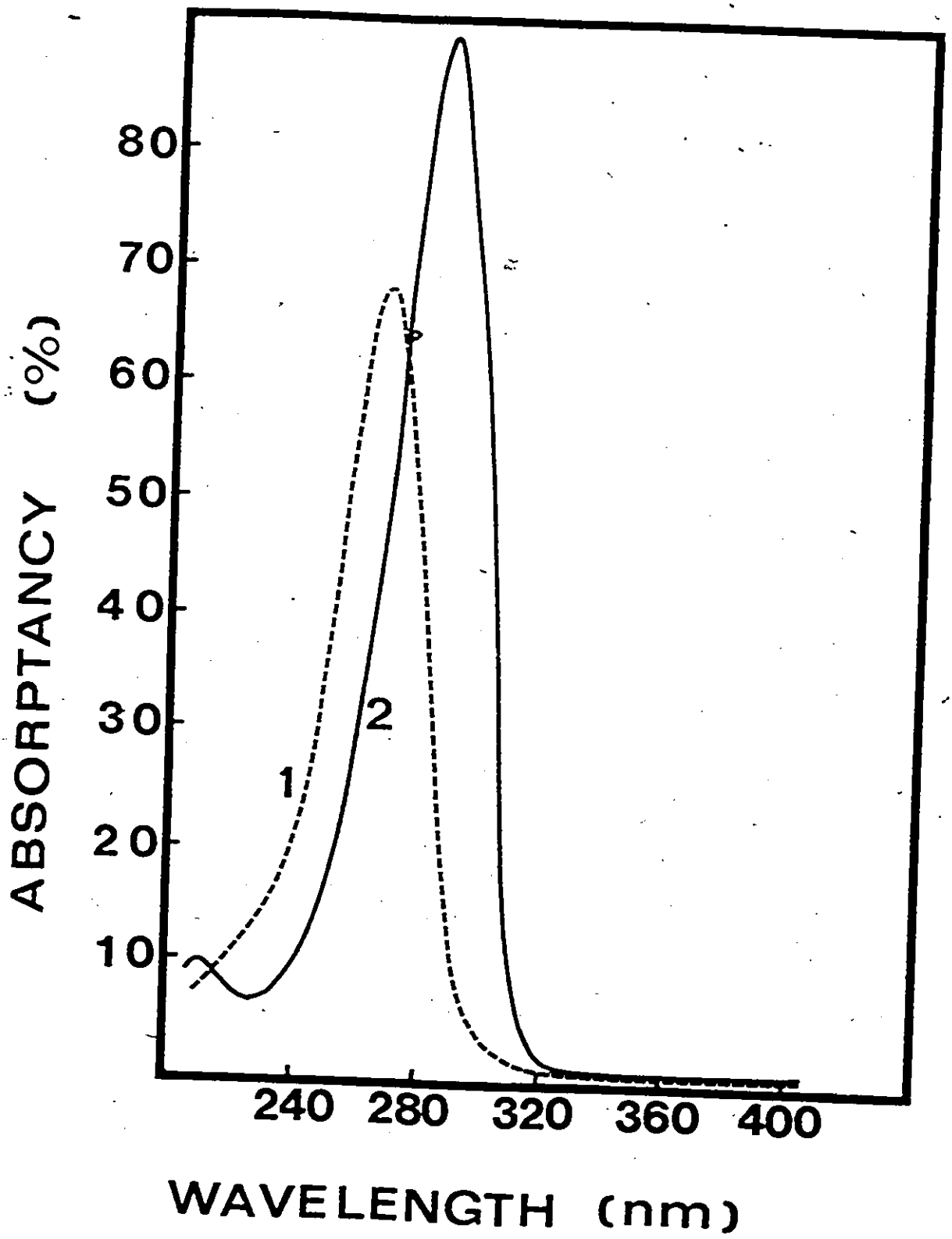
The procedure of Seibert (221) was followed closely for the synthesis of 3-hydroxy-5,6-dimethyl-1,2,4-triazine, the suspected chromogen in the urea-alpha diketone reaction as postulated by Beale and Croft (165). The synthesis could not be accomplished in this laboratory. A dark-brown residue was left in the bottom of

Legend to Figure 1

Fig. 1 Ultraviolet spectra of DMS and its isomer. Each curve represents the following: Curve 1, 3.62×10^{-5} M diacetyl monosemicarbazone (DMS) read against an alcohol blank; Curve 2, 3.55×10^{-5} M DMS isomer read against an alcohol blank.

FIGURE 1

ULTRAVIOLET SPECTRA OF DMS AND ITS ISOMER

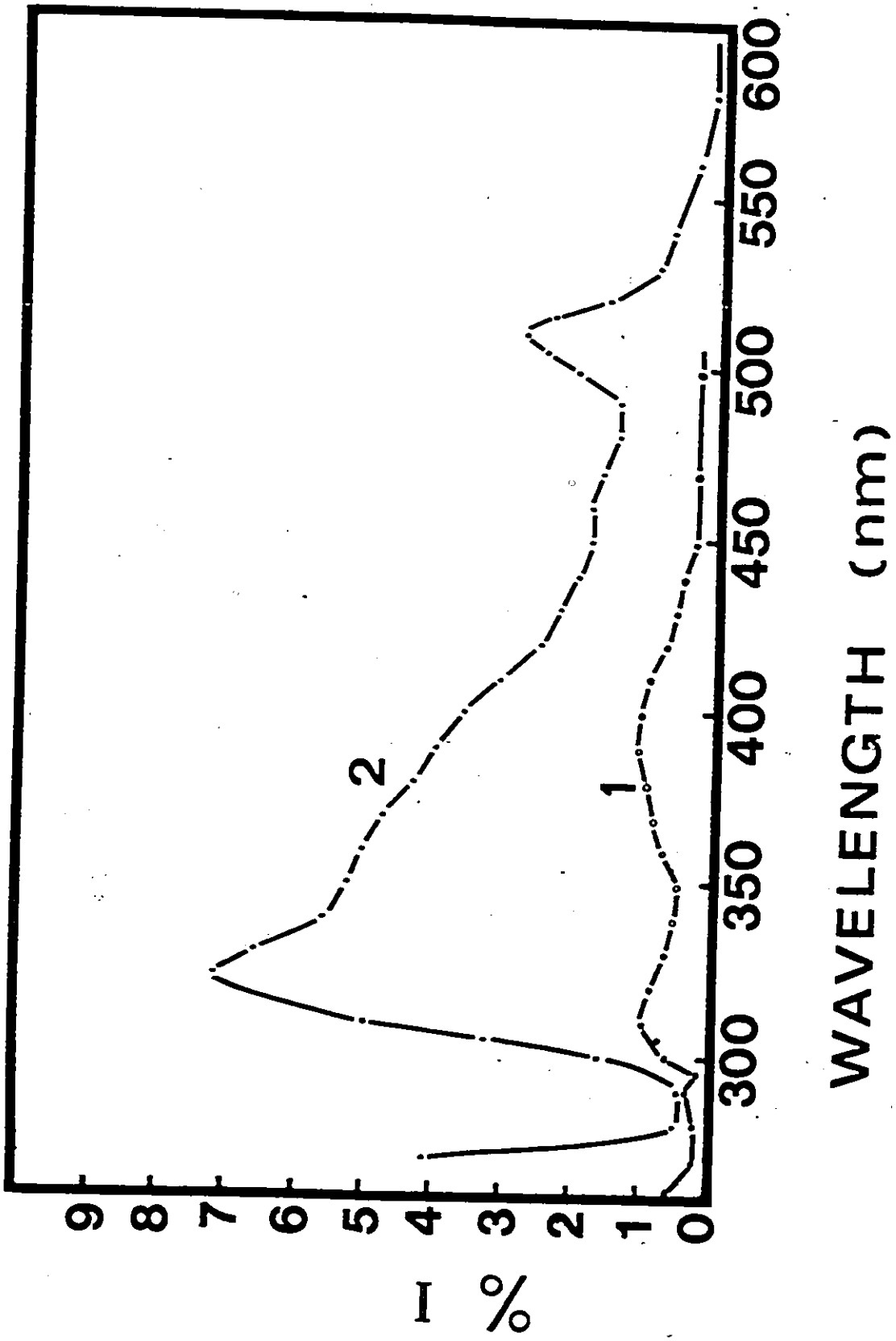


Legend to Figure 2

Fig. 2 Fluorimetric spectra of DMS and its isomer - λ_E, 260 nm. Curve 1, 2.1×10^{-4}M DMS in alcohol; Curve 2, 1.7×10^{-4}M DMS isomer in alcohol.

FIGURE 2

FLUORIMETRIC SPECTRA OF DMS AND ITS ISOMER - λ_E, 260 nm

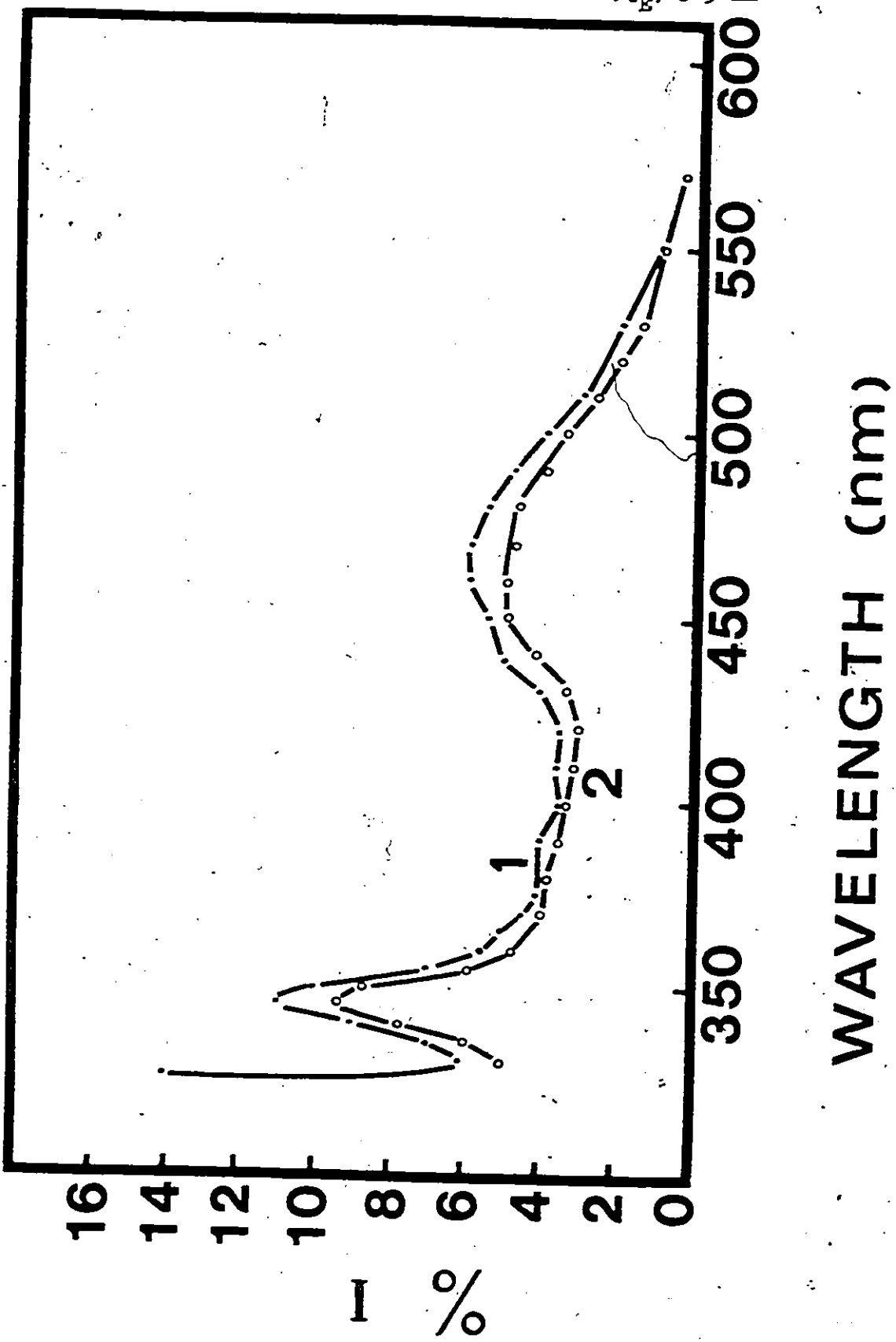


Legend to Figure 3

Fig. 3 Fluorimetric spectra of DMS and its isomer - λ_E, 315 nm. Curve 1, 2.1 x 10^{-4}M DMS in alcohol; Curve 2, 1.7 x 10^{-4}M DMS isomer in alcohol.

FIGURE 3

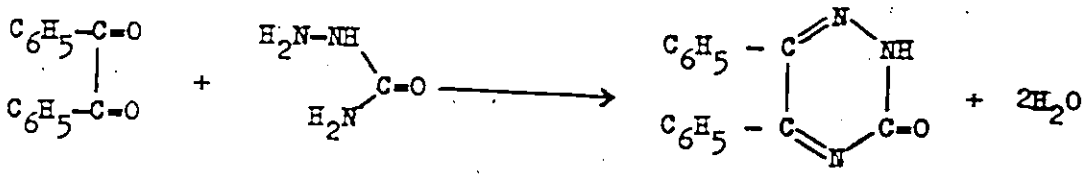
FLUORIMETRIC SPECTRA OF DMS AND ITS ISOMER - λ_E, 315 nm



the flask after the reflux and subsequent distillation steps. Acidification of this residue was carried out with either 6N HCl or 1N H_2SO_4 to pH 4.0. Frothing and the appearance of a grayish-yellow precipitate were observed upon addition of the acid. When this precipitate was boiled with hot water and Norit A, no crystals were observed even after standing overnight or longer. A solution of 40% KOH was tried to effect crystallization, but to no avail - only a very minute quantity of gelatinous substance was obtained.

The colorless filtrate from hot water and Norit A treatment was then transfered to a rotary evaporator at 45°-50°C in order to remove the solvent. White crystals were obtained; however, when subjected to heat only a few crystals melted, appearing as orange spots or droplets, at about 228°C. The only possible explanation for this is that sodium salts are coprecipitated. To overcome this possible difficulty, extractions into n-amyl alcohol or iso-amyl alcohol were tried. No colorless crystals were obtained after removing the solvent on a rotary evaporator. An orange and white residue was left in the flask. The orange portion of the mixture melted at a range of 190°-202°C. The amount of orange residue is so minute in quantity as compared to the white solid that its separation could not be accomplished. No significant results can then be deduced from the synthesis attempted.

Biltz (228) obtained with ease 5,6-diphenyl-3-oxy-1,2,4-triazine by boiling benzil and semicarbazide hydrochloride in acetic acid solution for several hours:



Based on this principle he tried to synthesize 5,6-dimethyl-3-oxy-1,2,4-triazine using diacetyl in sodium ethoxide, but did not succeed. Several unsuccessful attempts (220, 229) were made to prepare 3-hydroxy-5,6-dimethyl-1,2,4-triazine from the monosemicarbazone of diacetyl. Diels (220) found that cold dilute alkali merely dissolved the semicarbazone without causing cyclization. The cyclization of diacetyl monosemicarbazone was finally achieved in 1947 by Seibert (221). Unfortunately, no reaction time and acidification conditions were given. Following his procedure, the synthesis undertaken here did not achieve the expected results (i.e., cyclization to a triazine did not occur). According to him, crystallization of the triazine requires weeks for completion. It is clear then, that the cyclization of diacetyl monosemicarbazone is quite difficult.

Thiele and Dralle (230) studied the reaction of diacetyl with aminoguanidine hydrochloride or nitrate in aqueous solution. They did not obtain ring compounds, only osazone-like compounds. Erickson (231) was able to obtain 3-amino-5,6-dimethyl-1,2,4-triazine by reacting diacetyl with aminoguanidine bicarbonate in an aqueous medium at room temperature. The ring closure does not proceed nearly as readily with semicarbazide as with aminoguanidine. The ease of reaction thus appears to be greatly affected by the nature of

substituent groups, for aromatic diketones give much more satisfactory results than do aliphatic diketones.

C. Synthesis of Diacetyl Monosemicarbazone Isomer

During the preparation of diacetyl monosemicarbazone, a new compound was accidentally found in the mother liquor (See EXPERIMENTAL, Part A). This was assumed to be the isomer of DMS based on the comparison of its spectral data, elementary analysis, and melting point with those of DMS. The spectral data are reported in Table I. The elementary analyses are very close to those of DMS, but the melting points are different. The ultraviolet spectra (Fig. 1) show that the assumed isomer has two maxima : one at 285 nm with a molar absorbancy index of 8.0×10^6, and a shoulder at 210 nm with a molar absorbancy index of 5.2×10^6. This differs from DMS in that it has a maximum at 267 nm.

In the fluorimetric study, at an excitation wavelength of 260 nm (Fig. 2), the compound has a greater fluorescent intensity than DMS and has two distinct fluorescent peaks at 323 and 510 nm, respectively. At an excitation wavelength of 315 nm (Fig. 3), however, the isomer's fluorescent intensity is less than that of DMS, but the curve has a similar pattern and is superimposable on that of DMS.

The functional group portion of the i. r. spectrum shows similar characteristic bands to DMS : O-H stretching vibration at 3490 cm^{-1}; asymmetrical and symmetrical N-H stretching vibration at 3380 cm^{-1} and 3220 cm^{-1}, respectively; a strong C=O and C=N stretching vibration at 1680 cm^{-1}. The fingerprint portion shows differences in the characteristic bands which are summarized in Table I.

Based on the above similarities and differences, the compound obtained was assumed to be the isomer of DMS.

A solution of this compound was treated with a $H_2SO_4 - H_3PO_4$ acid mixture and heated in a boiling water bath, the standard clinical conditions for the determination of urea according to the method of Natelson (222). The resulting reaction mixture was compared with that of urea-alpha diketone reaction mixture with respect to its color. The color developed by the isomer was pink instead of the yellow color produced by urea-DAM. Further study of its absorption curve was not undertaken for the simple reason that this compound could not have been the protochromogen in the urea-DAM or urea-DA reaction because the expected yellow color was not obtained.

D. Synthesis of the Protochromogen in the Urea-Alpha Diketone Reaction

The i. r. frequencies of characteristic groups are listed in Table II. All four compounds (A,B,C,D) prepared, using DA or DAM by different methods, were found to show similar and almost superimposable i. r. spectra (Fig. 4). The characteristic group frequencies as shown by the spectrum favours a diacetyl diureide structure,

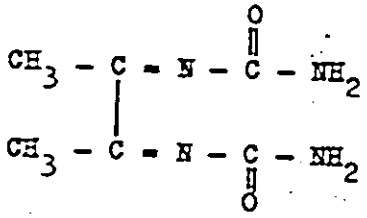


TABLE II

CHARACTERISTIC INFRARED BANDS OF THE PROTOCHROMOGEN

Functional groups	Frequencies (cm^{-1})
N–H stretching vibrations (primary amide, solid phase)	3400 3240
C–H stretching vibration (asymmetrical methyl)	2920
C–H stretching vibration (symmetrical methyl)	2840
C=O stretching vibration (non-bonded)	1720[a]
C=O stretching vibration (hydrogen bonded)	1680[a]
N–H deformation (hydrogen bonded) and C–N	1640[b]
C=N (conjugated)	1515
C–H bending vibration (asymmetrical methyl)	1440
C–H bending vibration (symmetrical methyl)	1385
C–H deformation and C–C stretching vibration	1160
N–H out-of-plane bending or wagging	740–720

[a] Amide I Band

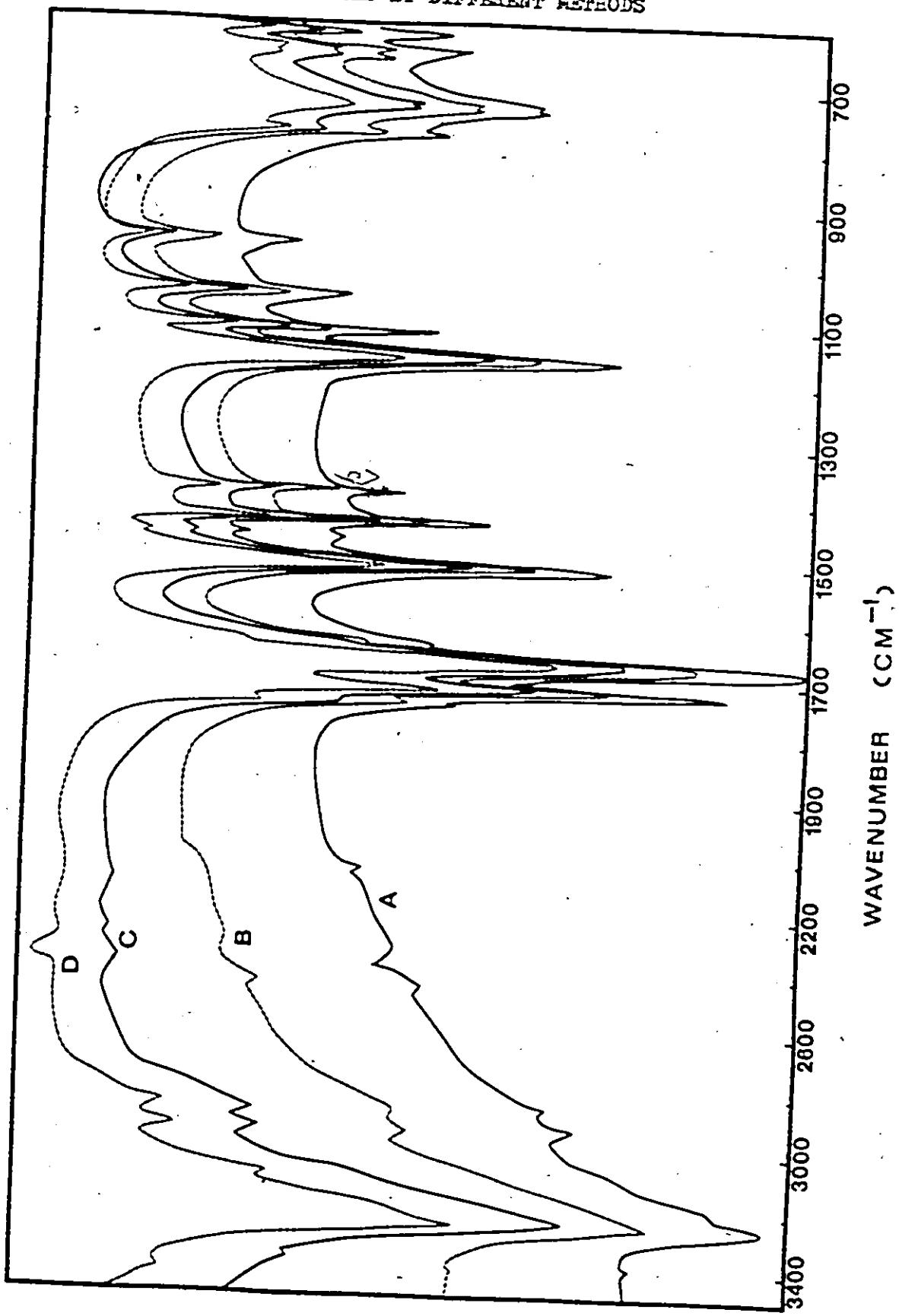
[b] Amide II Band

Legend to Figure 4

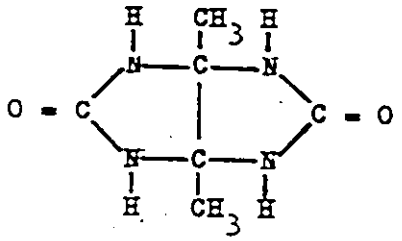
Fig. 4 Comparison of infrared spectra for the protochromogen synthesized by different methods. Curves A and D, compound synthesized from urea and DA by different methods; Curves B and C, compound synthesized from urea and DAM by different procedures.

FIGURE 4

COMPARISON OF INFRARED SPECTRA FOR THE PROTOCHROMOGEN SYNTHESIZED BY DIFFERENT METHODS



rather than 3a,6a-dimethylglycoluril (3a,6a-dimethyl-tetrahydro-imidazo[4,5-d]imidazole-2,5-dione),



as assigned by Franchimont and Klobbie (224). These absorption bands are characteristic of an open-chain primary amide in the solid phase.

Regardless of whether DAM or DA was used in the reaction, the same final product was obtained. All four products show a similarity in their melting behaviour when subjected to heat. They all turned light brown at 340°C, darker at 345°C, and above 350°C they evolved gas without melting. Compound A was obtained from the reaction of urea and DA in the presence of concentrated hydrochloric acid; compound B from urea and DAM under the same conditions. Compound C was prepared following Williams' method (223) using DAM to react with urea; while compound D was that obtained by the method of Franchimont and Klobbie (224). Whether the reaction was carried out under reflux or not, the same substance resulted. Hatelson and co-workers (161) also found that the active reagent in the urea-alpha diketone reaction was diacetyl not DAM. When diacetyl was used, the reaction time in a boiling water-bath was markedly decreased and maximum color developed in a few minutes. This color is not enhanced by potassium persulfate, and it became apparent

that the function of the persulfate was to destroy the hydroxylamine which tended to reduce the intensity of the color. Beale and Croft (165), on the other hand, claimed that DAM, and not diacetyl, reacts with the urea. In investigations of the Fearon reaction as applied to urea, many workers have included, as did Fearon, an oxidant, the purpose of which usually has been stated to be to destroy the hydroxylamine produced by hydrolysis of DAM. Recently, Tietz (218) presented a reaction scheme for urea-DAM showing that DAM is first hydrolyzed to DA and hydroxylamine. The free diacetyl was postulated to react with urea to produce the yellow color. The fact that the same compound was obtained either from DA or its monoxime supports the above hypothesis.

The above compounds (A,B,C,D) were studied with respect to absorption maxima employing the standard clinical conditions for determination of urea in blood and urine. The method followed was that of Natelson (222). The absorption maximum for the reaction solution using the above compounds (A,B,C,D) as well as that for the urea-DAM reaction is similar and occurs at 478 nm (Fig. 5). The heating step in a boiling water-bath (30 min) was carried out in the dark since the reaction is photosensitive. The sample and blank were both stable for the length of the study (1-1/2 hours). The compounds produced the same yellow color as urea does under the same standard conditions. The absorption curves obtained for the solutions of the four compounds are superimposable on each other and on that of a urea-DAM reaction mixture.

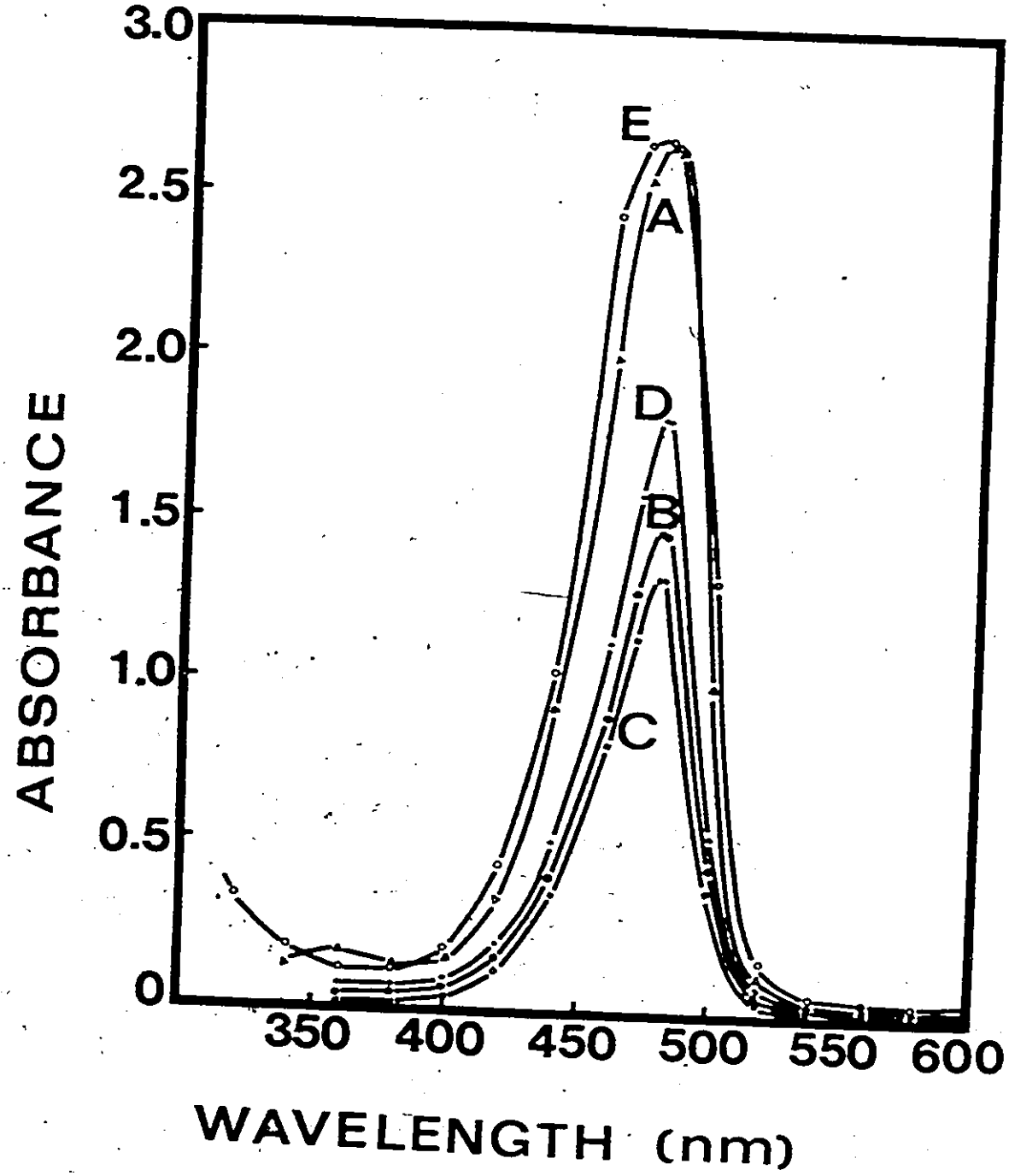
The logarithm of the absorbance was plotted against wavelength for the above five samples (Fig. 6). The portions of the curves

Legend to Figure 5

Fig. 5 Absorption spectra of the protochromogen and the urea-alpha diketone reaction product. Curves A and D, compound synthesized from urea and DA by different methods; Curves B and C, compound synthesized from urea and DAM by different procedures; Curve E, urea-DAM reaction product by the standard clinical method (Natelson (222)).

FIGURE 5

ABSORPTION SPECTRA OF THE PROTOCHROMOGEN AND THE UREA-ALPHA DIKETONE REACTION PRODUCT

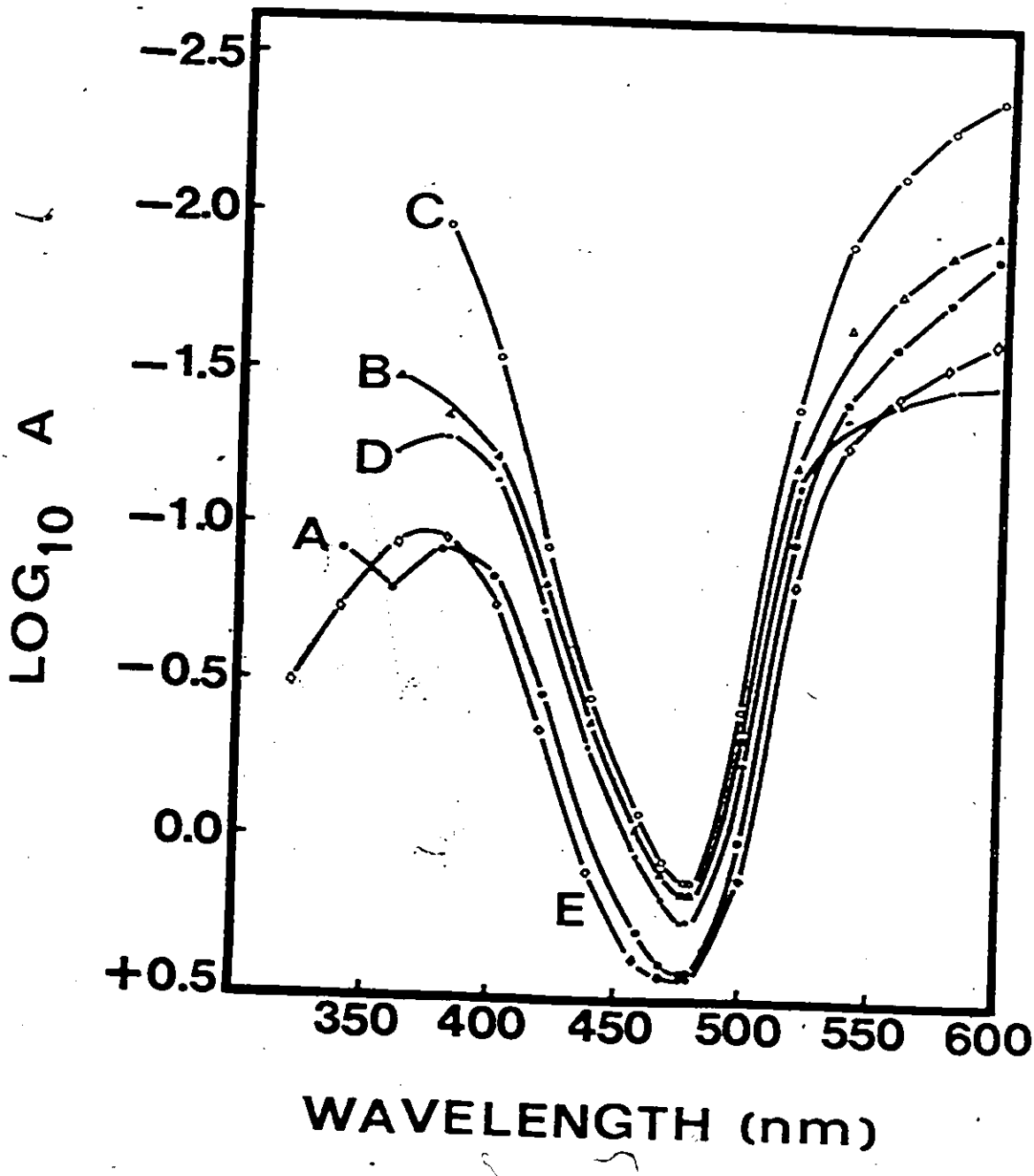


Legend to Figure 6

Fig. 6 Chemical identity of the protochromogen and the urea-alpha diketone reaction product. Curves A and D, compound from urea-DA reaction by different methods; Curves B and C, compound from urea-DAK reaction by different procedures; Curve E, urea-DAK reaction product by standard clinical method (Natelson (222)).

FIGURE 6

CHEMICAL IDENTITY OF THE PROTOCHROMOGEN AND THE UREA-ALPHA DIKETONE REACTION PRODUCT



between 400 and 520 nm are exactly parallel and superimposable regardless of concentration. According to Siggia and Stolten (232), for a given substance in a given solvent the curves resulting from this type of plot are exactly parallel and superimposable even if the concentrations of the different samples are not the same. Such a coincidence of curves is an indication that these compounds are identical. From Fig. 6, it is clear that the curves as compared to each other and with that of the standard urea-DAM curve are exactly parallel and superimposable. This is an additional piece of evidence, besides the similarity in the i. r. spectra (Fig. 4) and absorption maximum (478 nm), that compounds A, B, C, and D are chemically identical.

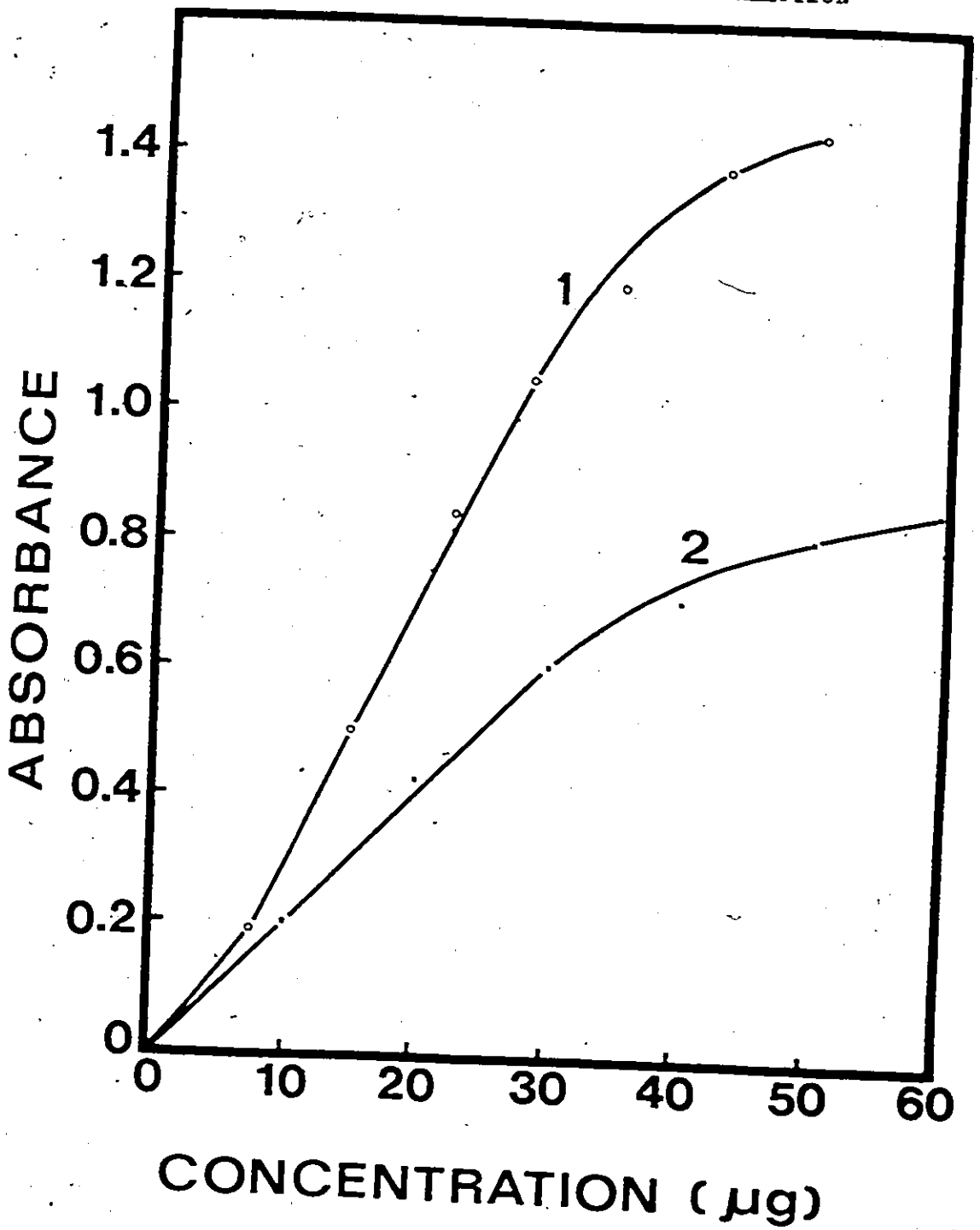
Beer's law studies were carried on both urea-DAM and urea-DA reactions. Both color reagents appear to follow Beer's law over limited ranges under given conditions of time and temperature control. The absorbancy-concentration curves are shown in Fig. 7. Both the urea-DAM and urea-DA curves are linear to 30 μg per 4 ml except that the urea-DAM curve has a break from 0 to 7.5 μg per 4 ml. Using Natelson's reaction conditions (222), Beer's law studies of compounds A, B, C, and D were carried out at the same concentrations. The results of the study are shown in Fig. 8. All four curves exhibit the same relationship with respect to absorbance and concentration. In each case, there is a break in the curve at about 35 μg per 4 ml, creating two linear portions : one from 0 to 35 μg per 4 ml; the other one from 35 to 95 μg per 4 ml. The curves almost coincide with each other except for curve B, which has a higher absorbance than the

Legend to Figure 7

Fig. 7 Beer's law study of the urea-alpha diketone reaction. Curve 1, urea-DAM reaction; Curve 2, urea-DA reaction. Each analysis was performed in triplicate.

FIGURE 7

BEER'S LAW STUDY OF THE UREA-ALPHA DIKETONE REACTION

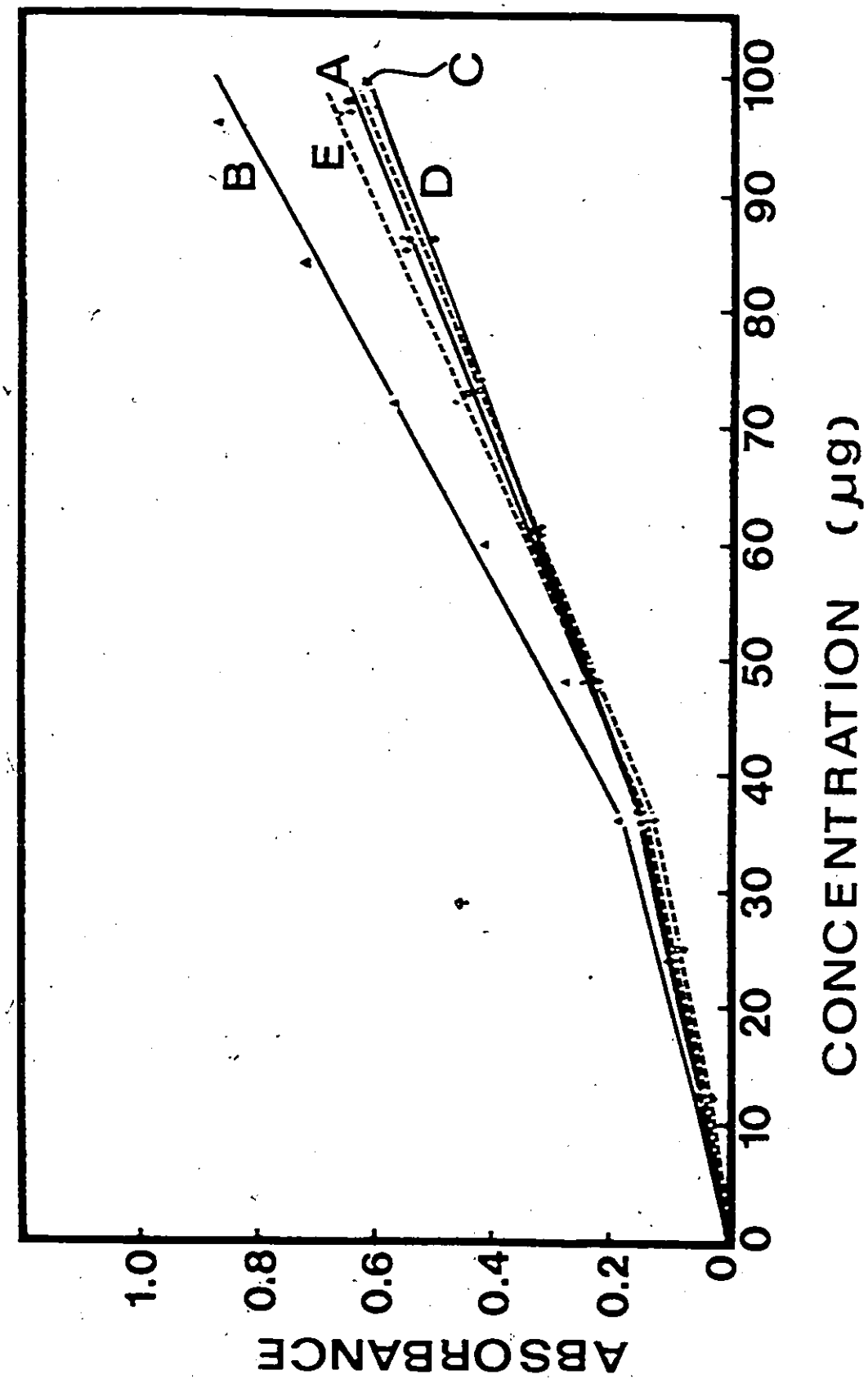


Legend to Figure 8

Fig. 8 Beer's law study of the protochromogen. Curves A and D, compound from urea-DA reaction by different methods; Curves B and C, compound from urea-DAM reaction by different methods; Curve E, combined sample of the four compounds from different methods. Each analysis was performed in triplicate.

FIGURE 8

BEER'S LAW STUDY OF THE PROTOCHROMOGEN



rest. It was shown above (Fig. 6) that the four compounds are chemically identical and therefore, one would expect their standard curves should coincide with each other. The deviation of curve B from the rest may be attributed to the higher concentration of B present in solution. During the preparation of the sample there is difficulty in keeping the final total volume exact although careful precautions were taken. In boiling the sample to bring about its solution, there is always the possibility of losing a certain portion of the solvent through evaporation during and after heating. Although hot water may be added for the portion of solvent evaporated, evaporation can still occur during the cooling step. This might be the reason, in the case of curve B, which accounts for its higher concentration and consequently higher absorbance. Comparing these results with those of the urea in Fig. 7, there is similarity observed in that all the curves exhibit linearity from 0 to 35 μg per 4 ml. Above 30 μg per 4 ml both urea-DAH and urea-DA curves (Fig. 7) level off and a plateau is reached. The four curves resulting from compounds A, B, C, and D, however, were linear up to a concentration of 95 μg per 4 ml. A Beer's law study was also run on a combined sample of the four compounds and the curve (curve E, Fig. 8) obtained is similar to those of the separate samples.

The nitrous acid test was based on Bouveault's method (225). The test was performed on the synthesized compound, glycoluril, and on 1,3-dimethylurea. In the case of the synthesized compound and the glycoluril solutions, both gave a colorless, odorless gas which does not support combustion. This gas is nitrogen, which is liberat-

ed by the action of nitrous acid on a primary amide. Amides which resist hydrolysis with alkalies or acids can be converted to the corresponding acids, with a few exceptions, by reaction with nitrous acid (233) :

$$RCONH_2 + HNO_2 \longrightarrow RCOOH + N_2 + H_2O$$

This only applys to primary amides. The reaction is formally analogous to that given by primary aliphatic amines. N-Substituted amides of type R-CONH-R' react with nitrous acid to give nitroso derivatives of the general formula R-CON(NO)-R'. The solution of 1,3-dimethylurea when reacted with nitrous acid gave a brown gas instead of a colorless, odorless gas.

Hydrogenation was carried out on the synthesized compound using a sloping-manifold atmospheric pressure hydrogenator. The reduced substance was used to run an infrared spectrum. The resulting spectrum did not have any significant changes from the starting compound even though it had taken up so large an amount of hydrogen (60 ml). At present, we cannot explain this finding. The hydrogenation was repeated and the same result was obtained.

The result of the elementary analysis on the synthesized compound agrees with that obtained by Franchimont and Klobbie (224).

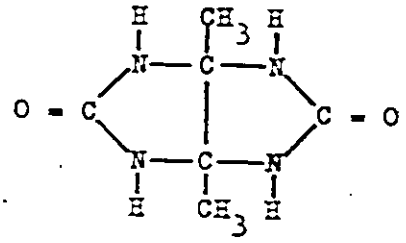
CHAPTER IV

SUMMARY AND CONCLUSIONS

The diacetyl monosemicarbazone prepared in this laboratory is identical with that obtained by Diels (220). The synthesis of 3-hydroxy-5,6-dimethyl-1,2,4-triazine was unsuccessful. Due to failure to cyclize the diacetyl monosemicarbazone to the ring condensation product, a triazine, and the results obtained from using either DAM or DA producing the same compound, it is concluded that the proto-chromogen in the urea-alpha diketone reaction could not be a 1,2,4-triazine. During the preparation of diacetyl monosemicarbazone a new compound was found. It was shown to be an isomer of the original diacetyl monosemicarbazone since the elementary analysis and the functional group portion of the i. r. spectrum were similar; however, the fingerprint portion of the i. r. spectrum, the ultraviolet absorption peak, and the melting point were different.

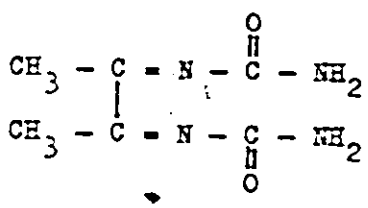
Four compounds were synthesized using either DAM or DA as a reagent in the reaction. From the absorption spectra and the $\log_{10} A$ vs wavelength curves, the four compounds were shown to be the same substance. The similarity in their i. r. spectra confirmed this finding. The compounds obey Beer's law in the range of 0 to 35 μg per 4 ml and from 35 to 95 μg per 4 ml. Under the standard clinical conditions for urea determination, the compound produced the same yellow solution as urea (absorption maximum, 478 nm). The reaction mixture is photosensitive and is not stable.

Pranchimont and Klobbie (224) first synthesized a compound to which they assigned the following structure :



Kouno and Ueda (234) and Ueda and co-workers (235) investigated the urea-alpha diketone reactions using butyl urea, DA and its monoxime in the presence of D-glucuronolactone. Their results showed that the reactions of diacetyl and its monoxime gave tetrahydroimidazo[4,5-d]-imidazole-2,5-dione derivatives as common products. Their assignment of the structure is similar to that given by Pranchimont and Klobbie (224) and by Biltz (228).

The findings presented in this thesis are that the same compound is obtained regardless of whether DA or its monoxime was used. This agrees with the observations of Kouno and Ueda (234) and Ueda and co-workers (235). Another chemical structure was found to have the exact elementary analysis as the glycoluril derivatives, i.e., a di-acetyl diureide :



This is in agreement with the observations of Thiele and Dralle (230) for the case of the reaction of diacetyl and aminoguanidine as the

hydrochloride or nitrate, where osazone-like compounds were isolated instead of the expected amino triazines.

From the results obtained in the nitrous acid test, hydrogenation experiment, and the characteristic i. r. bands of a primary amide compound shown by the spectrum of the synthesized substance, we do firmly believe that the protochromogen in the urea-alpha diketone reaction is a diacetyl diureide type of compound, and not a derivative of tetrahydroimidazo[4,5-d]imidazole-2,5-dione (3a,6a-dimethylglycoluril) as has been proposed by several authors (219, 224, 228, 234,235).

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