# STUDIES IN THE SYNTHESIS AND HYDROLYSIS OF 2,5-DIKETOPIPERAZINES.

by

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A thesis submitted to the Faculty of Graduate Studies and Research, University of Manitoba, in partial fulfilment of the requirements for the degree of Doctor of Philosophy (Ph.D.).

## Abstract.

Five simple diketopiperazines have been prepared in good yield in extending a similar investigation made by Sannié. They were obtained in a satisfactory state of purity, indicating the suitability of the method for such purposes.

Fifteen mixed diketopiperazines have been prepared by a similar reaction, using different methods in general for isolation of final product. The yields obtainable are reasonably satisfactory. Purity of products in some cases, as judged by colour and melting point, is open to improvement by much more exhaustive investigation, but generally has been of a reasonable order. It has certainly been high enough to demonstrate that the method of carrying out the reaction is suitable for the preparation of mixed diketopiperazines.

Dielectric constants of acetic acid solutions of three simple diketopiperazines have been measured. The data obtained show that the compounds studied are sufficiently polar to form solutions of higher dielectric constant than the solvent, which itself had a fairly high value. It has also been shown that with increasing molecular weight, the effect diminishes due to the increased influence of the larger alkyl groups.

Investigations have been made of the hydrolysis of a number of mixed diketopiperazines by N/HCl and by N/NaOH. In some

cases hydrolysis was not accomplished, and in one other case success in accomplishing the reaction was variable. In other cases, hydrolysis either gave much more of one dipeptide than the other, or both possible products were formed in more nearly equal quantities.

Acknowledgments.

The author wishes to acknowledge the parts played by each of the following in making the prosecution of the research possible:-

Dr. W. M. Sandstrom, Division of Agricultural Biochemistry, University of Minnesota, for suggesting the problem and outlining the phases for attack.

Also, to the following members of the Staff of the Department of Chemistry, University of Manitoba:-

Dr. A. D. Robinson, for guidance and supervision of the author in his capacity as a candidate in the Faculty of Graduate Studies, and for making possible the provision of the facilities necessary for the performance of the work.

Dr. W. J. Biermann, for his valuable guidance and advice in the construction of the instrument for dielectric constant measurements. In this latter connection, acknowledgement must also be made of the time given by Dr. B. L. Funt.

Dr. G. E. Dunn, for suggesting the treatment of the dielectric constant cell to make measurements possible on polar liquids, and for much valuable advice on the various parts of the research.

The apparatus and chemicals used were purchased out of grants made from time to time from the President's Research Fund.

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# INTRODUCTION

2,5-diketopiperazines have attracted considerable attention in past years as derivatives of amino acids and dipetides, being capable of synthesis from both types of compound. Amino acids and peptides generally are known to contribute to the building up of protein molecules. The question was investigated by many workers as to whether diketopiperazines also played such a part, since various members of the series have been isolated along with amino acids and peptides in the degradation of proteins by acids and alkalis.

It is possible to use many simple diketopiperazines to prepare the corresponding dipeptides by hydrolysis. In certain instances workers in the field have published accounts of the hydrolysis of mixed diketopiperazines to dipeptides. The theory has generally been held that a mixture of two possible dipeptides is inevitably formed. It was with the object of determining whether hydrolysis of mixed diketopiperazines derived from aliphatic monamino-monocarboxylic acids was in any way selective that this part of the work was undertaken.

It has long been known that amino acids, peptides and proteins form solutions in aqueous solvents in which the dielectric constant is not only higher than that of water, but also that the increase follows a linear relation to concentration of solution.

Amino acids and peptides are known to give solutions of lower dielectric constant than solvent when the latter has a dielectric constant less than 20. Although contemporary opinion does not seem to regard diketopiperazine rings as such to be present in the structure of protein molecules, it was considered worth while to determine what information might be derived from measurement of dielectric constants of solutions of some members of the series.

# REVIEW OF THE LITERATURE

## THE CHEMISTRY OF 2,5-DIKETOPIPERAZINES.

2,5-Diketopiperazines are mentioned throughout the literature with respect to their methods of preparation, their possible relationship to proteins, their chemical reactions (most particularly hydrolysis), and instances where they have been formed, either as by-products or as intermediates in some reaction not necessarily designed for their preparation. In this review, attention will be directed mainly to:-

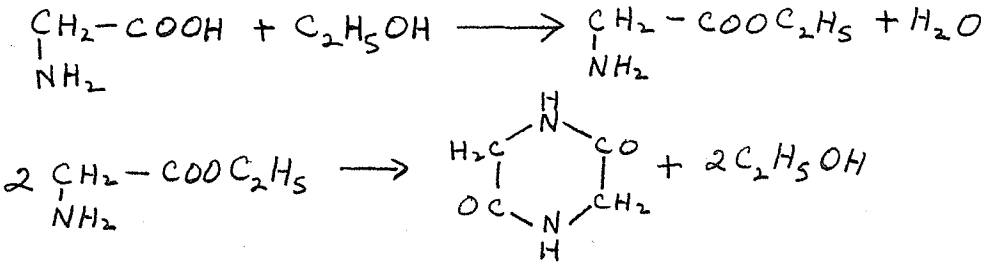
I - Their methods of preparation, with some mention of other cases where they have reported as being formed as intermediates or as by-products in the preparation of other substances.

II - Their hydrolysis.

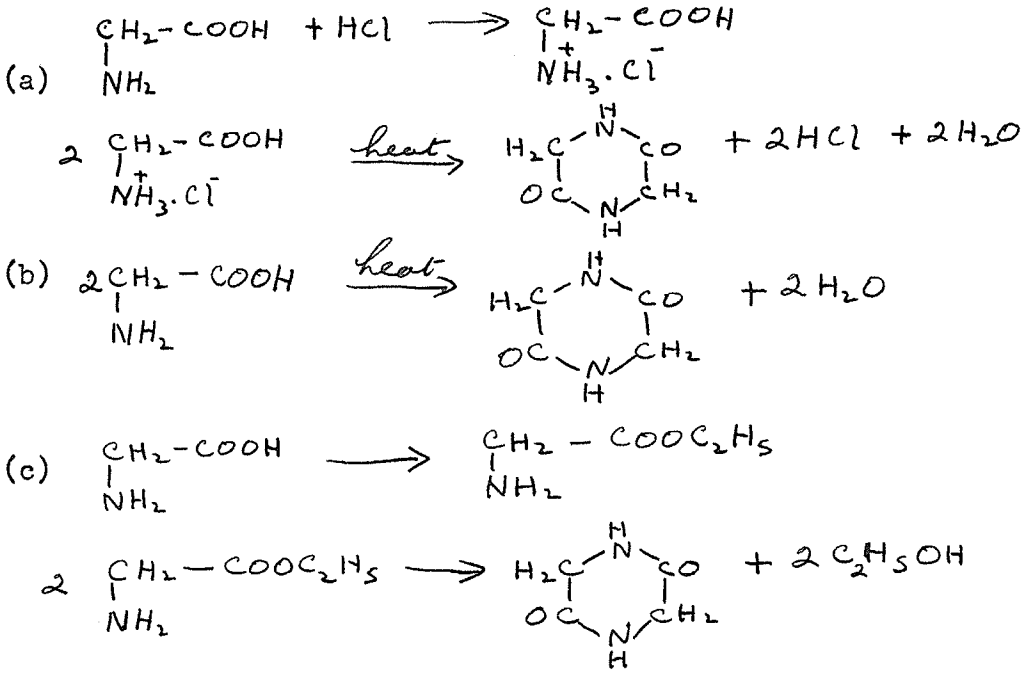
III- Their possible importance to the study of proteins.

### I. Preparation of 2,5-Diketopiperazines.

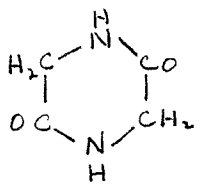
The simplest substance in this class is 2,5-diketopiperazine, frequently called glycine anhydride. The first report of preparation of this substance was published by Curtius and Goebel, who reported that it was formed by the treatment of glycine with ethyl alcohol and sulphuric acid (1).



Other methods reported by Curtius and Goebel for conversion of glycine were (a) heating glycine in a current of hydrogen chloride, (b) by heating glycine at 150°-170° with glycerine, (c) converting glycine to its ethyl ester and leaving in contact with water.

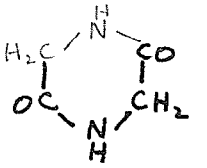


Emil Fischer and Fourneau also synthesised the substance by method (c) above (2), and Fischer with his associates carried out a long series of researches into methods of synthesising it and other members of the series. They found that 2,5-diketopiperazine could also be formed from glycylglycine ethyl ester by (a) heating to 190° (b) standing in contact with water (c) treating with a saturated alcoholic solution of ammonia, or (d) treating with sodium ethoxide solution (2).

(a) $CH_2(NH_2)-CO-NH-CH_2-COOC_2H_5 \xrightarrow[\text{or } H_2O]{\text{heat}}$  $+ C_2H_5OH$

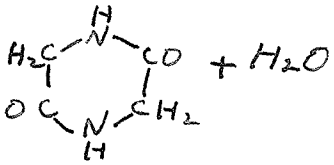
(b) $NH_2$

(c) $CH_2(NH_2)-CO-NH-CH_2-COOC_2H_5 + NH_3 \longrightarrow (CH_2(NH_2)-CO-NH-CH_2-CO-NH_2) + C_2H_5OH$

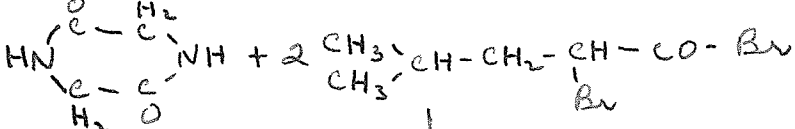
$(CH_2(NH_2)-CO-NH-CH_2-CO-NH_2) \longrightarrow$  $+ NH_3$

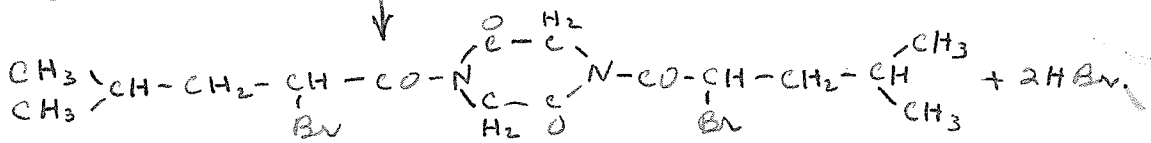
Fischer also obtained the substance from chloracetylglycine or chloracetylglycylglycylglycine by warming with concentrated ammonia solution (3).

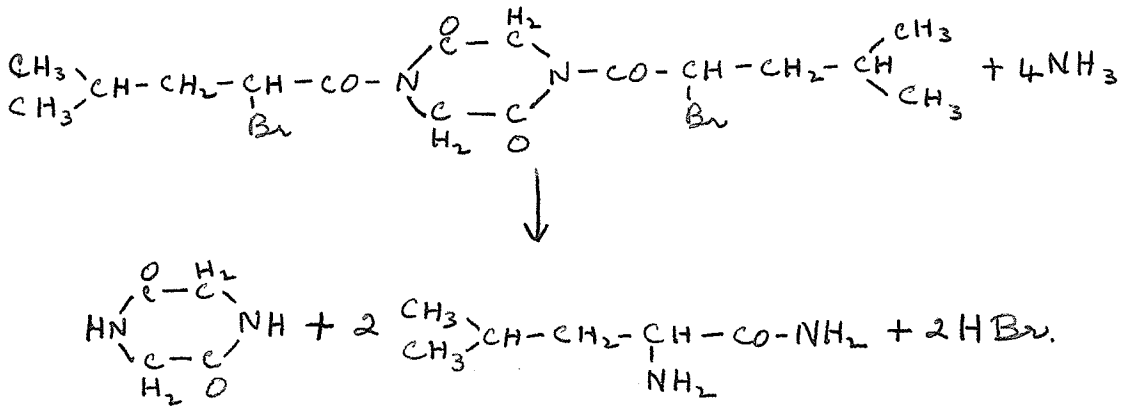
$CH_2(Cl)-CO-NH-CH_2-COOH + NH_3 \longrightarrow CH_2(NH_2)-CO-NH-CH_2-COOH + HCl$

$CH_2(NH_2)-CO-NH-CH_2-COOH \longrightarrow$  $+ H_2O$

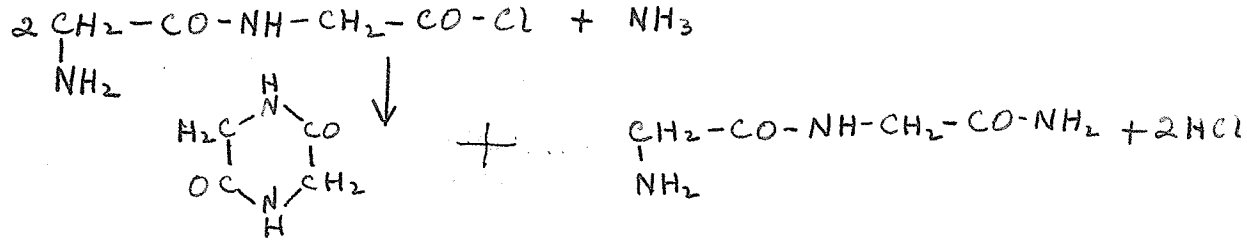
In a later investigation, Abderhalden and Klarmann condensed the substance with $\alpha$-bromoisocaproyl chloride, by heating with thionyl chloride, possibly in an attempt to build up more complex ring systems. The product, 1,4-di ($\alpha$-bromoisocaproyl)-2,5-diketopiperazine, on treatment with alcoholic ammonia, merely reverted to a mixture of the original glycine anhydride and leucinamide (4).



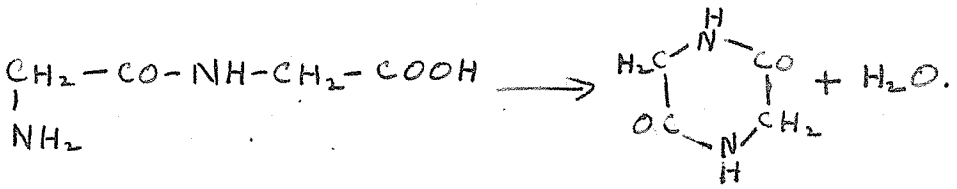




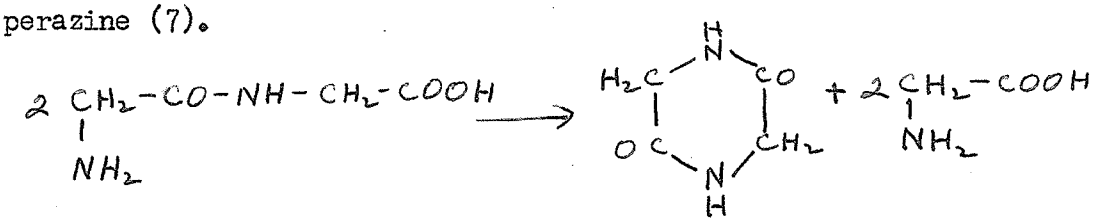
Bergell, in an attempt to convert glycylglycine chloride to glycylglycinamide by the action of aqueous ammonia, obtained instead 2,5-diketopiperazine as main product and only traces of the desired amide in a state of impurity (5).



Abderhalden and Komm also succeeded in preparing the substance from glycylglycine by each of the following procedures (a) heating to 150-160° for several hours with water in a sealed tube (b) heating with dilute hydrochloric acid or sulphuric acid or (c) by refluxing for several days with water (6).



Levene and his associates carried out extensive investigations in the chemistry of 2,5-diketopiperazines, and reported that when studying the catalytic effect of the enzyme erepsin (nowadays regarded as a mixture of dipeptidases) on the hydrolysis of glycylglycine, this dipeptide formed glycine and 2,5-diketopiperazine (7).



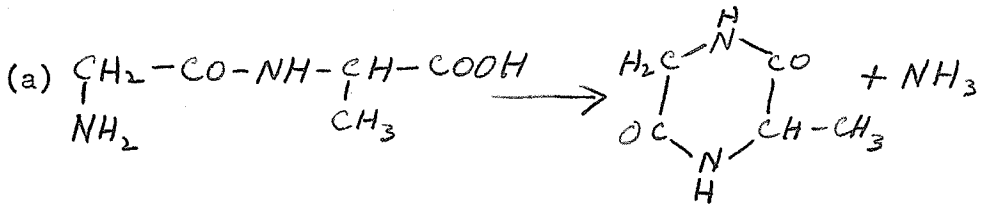
Lichtenstein in a study of conversion of amino acids and dipeptides to diketopiperazines by heating in α-naphthol to 135°-140°, found that glycylglycine could not be converted to the anhydride (8).

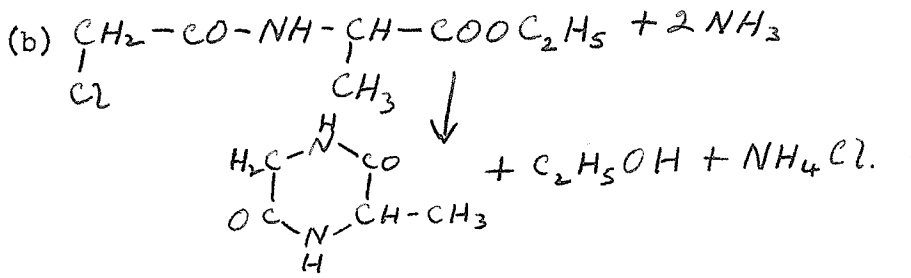
A German patent describes the preparation of the substance by passing ammonia into a benzine-benzene solution of glycine ethyl ester hydrochloride at low temperature (9).

Sannié succeeded in preparing the substance by heating glycine with ethylene glycol (10). Schott, Larkin, Rockland and Dunn (84) describe a procedure similar to that of Sannié, in which they stirred glycine with hot ethylene glycol for about one hour, but the yield of product is much lower than that obtained by Sannié's method (less than 40%, compared to 45-67%).

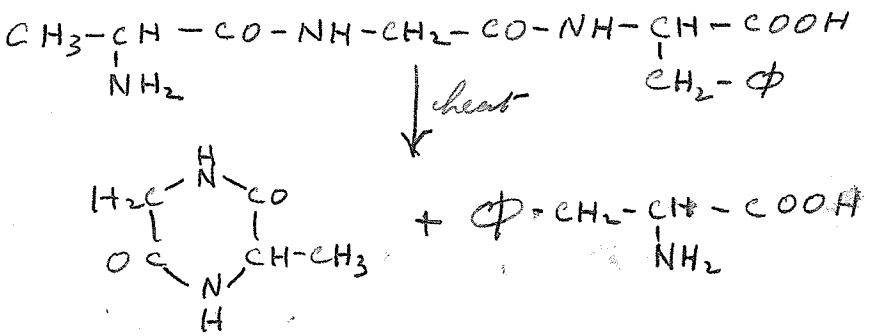
3-Methyl-2,5-diketopiperazine, or glycylalanine anhydride.

Fischer and his co-workers prepared this member of the series by an extension of the methods established earlier for the synthesis of the first member. The reactions were (a) treatment of glycyl-D-alanine or its hydrochloride with saturated alcoholic ammonia solution at low temperature, and (b) heating chloracetyl-DL-alanine ethyl ester with a saturated alcoholic ammonia solution to 100° (11,12,13).

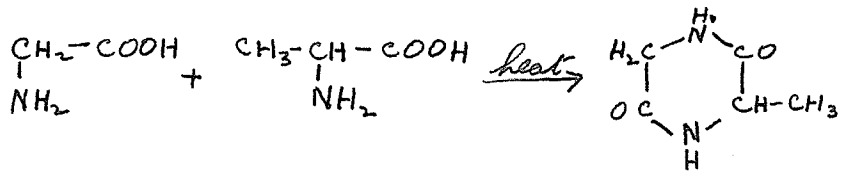




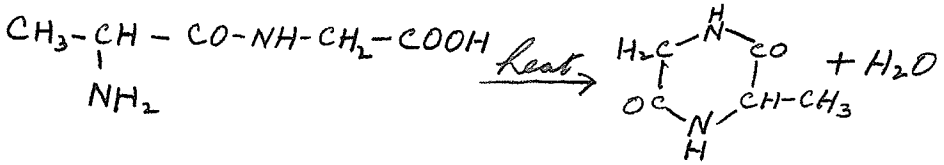
Lichtenstein, in his study of the effect of heating amino acids with α-naphthol found that DL-alanylglycyl-DL-phenylalanine formed 3-methyl-2,5-diketopiperazine along with DL-phenylalanine (8).



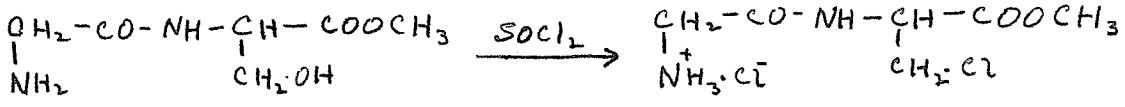
Sannié's method of heating amino acids in ethylene glycol (10) resulted in formation of the compound when a mixture of glycine and DL-alanine was so treated.

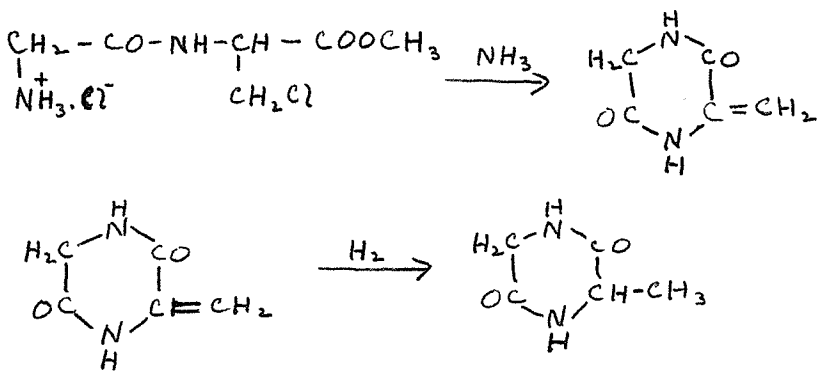


Abderhalden and Komm also prepared it by several methods, for example, they isolated it from the hydrolysates when dog hair had been heated for several hours at 150°-160° with 1% hydrochloric acid (14), and when silk fibroin had been subjected to prolonged action of concentrated hydrochloric or 70% sulphuric acid below 25° (15). He also prepared it from alanylglycine by heating to high temperature with (a) water, (b) dilute acids (6).



Bergmann described an indirect preparation of the compound from glycylserine methyl ester. The latter, with thionyl chloride, formed ∝ -glycylamino-β -chloropropionyl chloride hydrochloride methyl ester, which in turn was converted to 3-methylene-2,5-diketopiperazine by the action of concentrated aqueous ammonia. Catalytic reduction of this anhydride gave the 3-methyl compound (16).



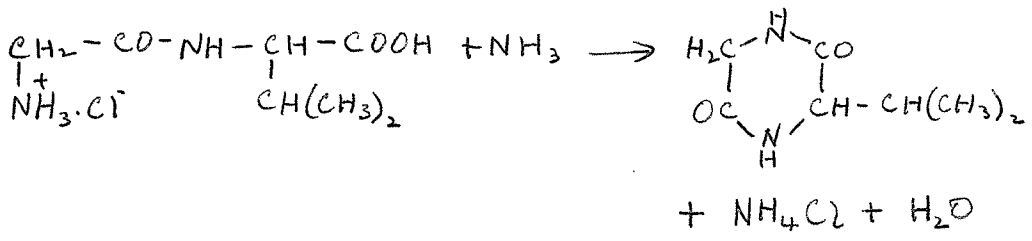


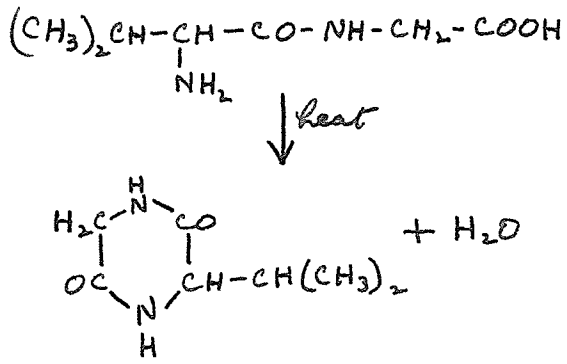
Bergell attempted to synthesise alanylglycinamide by the action of aqueous ammonia solution on $\alpha$ -bromopropionylglycine. The amide, if formed, was not sufficiently stable to be isolated, and the only product of the reaction was a small yield of 3-methyl-2,5-diketopiperazine (5).

Levene and co-workers reported its formation as a result of the catalytic effect of erepsin on the hydrolysis of alanylglycine and of glycylalanine (7).

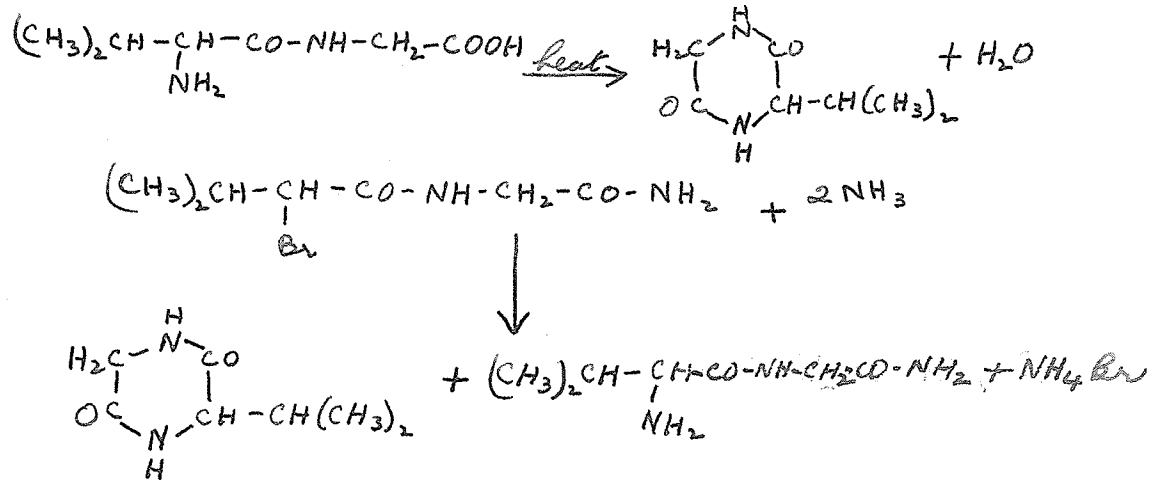
3-Isopropyl-2,5-diketopiperazine, or glycylvaline anhydride.

Preparations of this compound have been made by Fischer and his associates, from appropriatedipeptides and on protein hydrolysis, by Bergell and by Lichtenstein. Fischer's preparations were carried out by treating glycyl-D-valine hydrochloride with saturated alcoholic ammonia at low temperature (17), by heating DL-valylglycine to its melting point, (18), and by hydrolysing elastin with strong hydrochloric acid, and isolating the compound from other products in the hydrolysate (19).



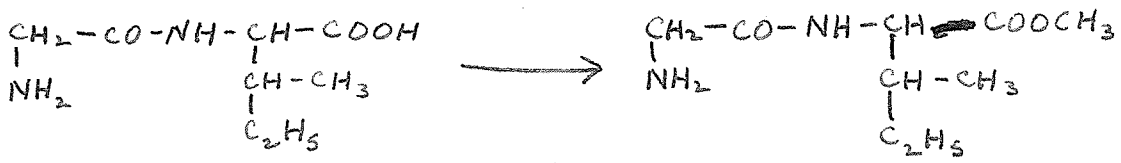


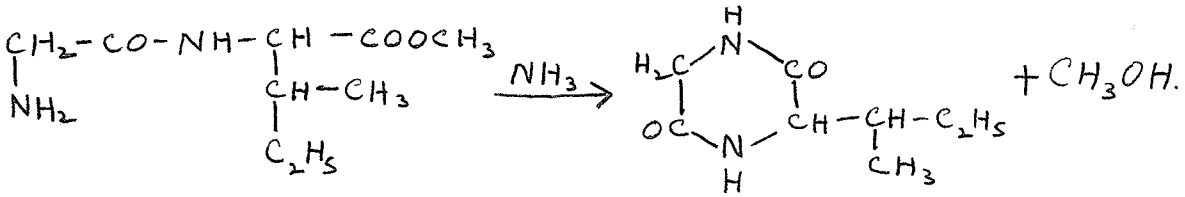

Lichtenstein prepared it by heating DL-valylglycine to 135-140° in ∝-naphthol (8). Bergell was studying the action of ammonia on (∝-bromovaleryl) glycine amide (5). Aqueous ammonia had hardly any action, but on heating to 120° in a sealed tube with alcoholic ammonia the diketopiperazine was formed along with the dipeptide amide.




3-Sec. butyl-2,5-diketopiperazine, or glycylisoleucine anhydride.

Abderhalden, Hirsch and Schuler effected its synthesis from glycyl-D-isoleucine, by first converting it to the methyl ester, and treating the latter with concentrated ammonia solution (20).

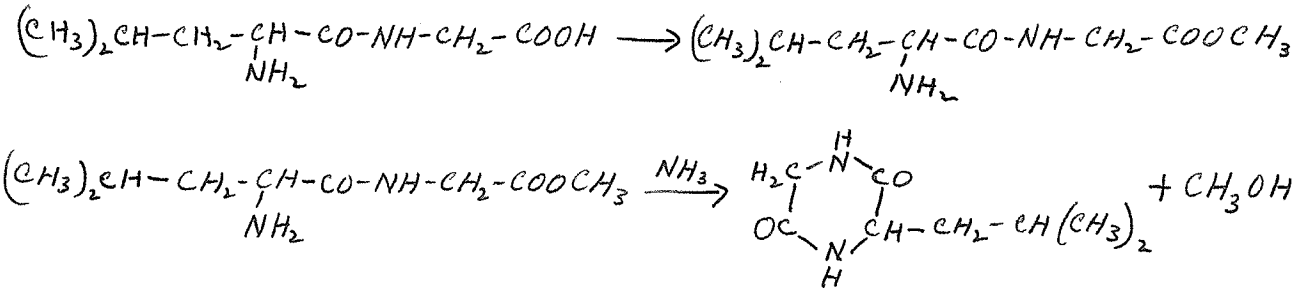




3-Isobutyl-2,5-diketopiperazine or glycylleucine anhydride.

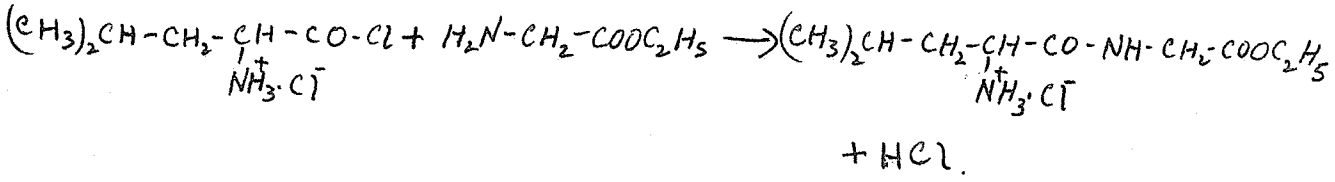
Numerous preparations of this compound have been carried out in the laboratories of Fischer, Abderhalden, and Lichtenstein. Fischer's methods were:-

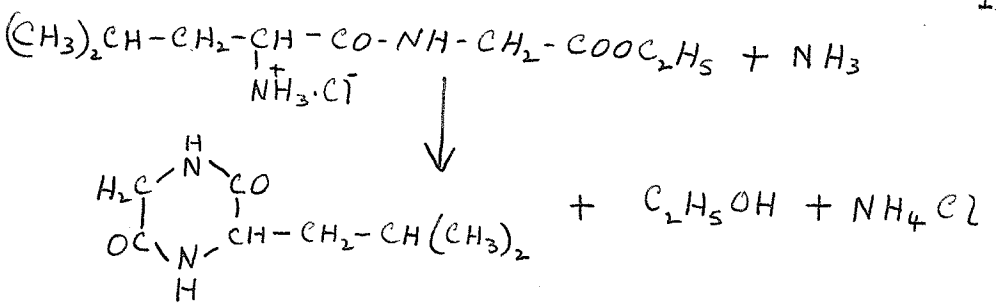
(i) Conversion of L-leucylglycine to its methyl ester and treating the latter with concentrated methyl alcoholic ammonia solution (21).



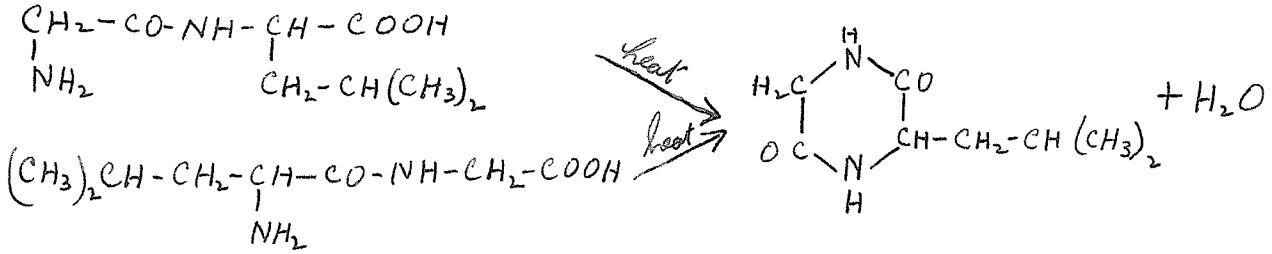
(ii) Hydrolysis of elastin with 70% sulphuric acid, esterifying the hydrolysate with ethyl alcohol in presence of hydrogen chloride, and treating the ester with ammonia in alcohol (22).

(iii) Shaking DL-leucyl chloride hydrochloride with glycine ethyl ester, and evaporating the solution with ammonia (23).





(iv) Heating glycyl-DL-leucine or DL-leucylglycine to the melting point (24, 25).

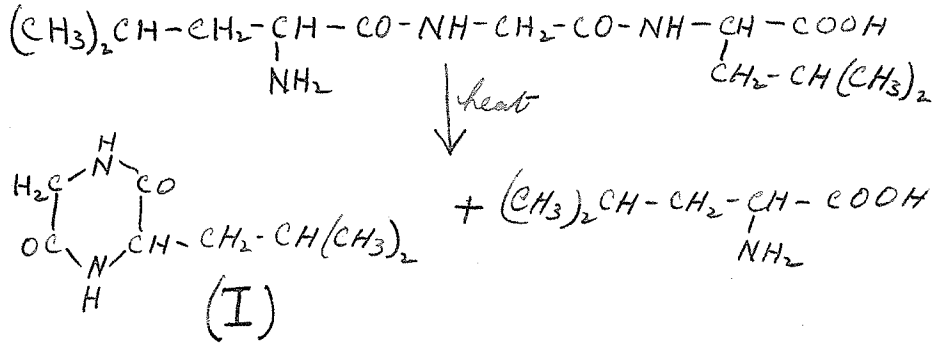


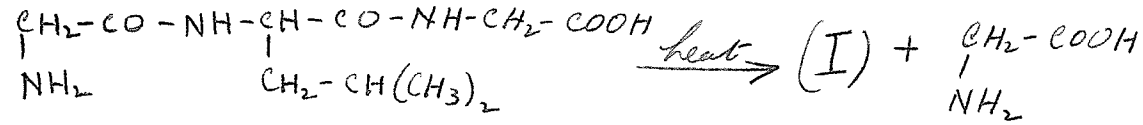
(v) Refluxing L-leucylglycine with quinoline (the DL-compound was formed) (24).

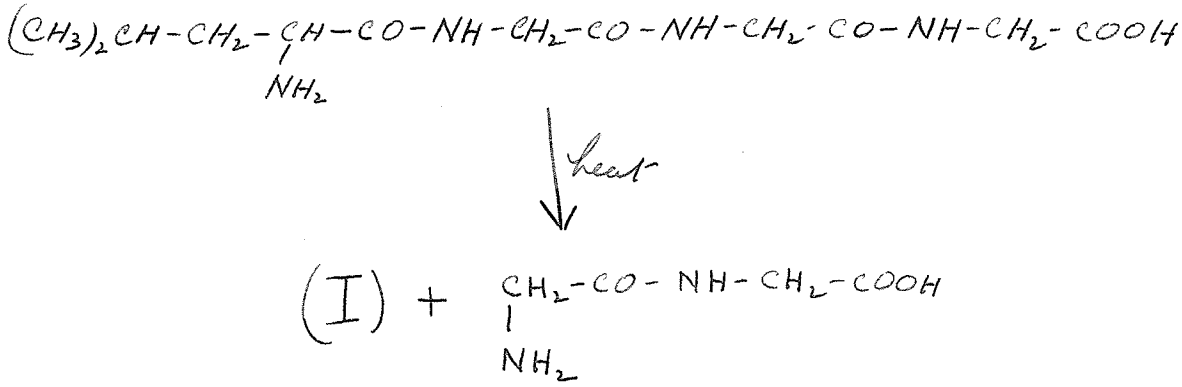
Abderhalden and Komm also obtained the compound by heating various dipeptides, polypeptides, and blood protein in an autoclave, thus:-

(i) Glycylleucine and leucylglycine each formed the substance when heated with water to 150-160° in a sealed tube (6).

(ii) L-leucylglycyl-L-leucine, glycyl-DL-leucylglycine and DL-leucylglycylglycylglycine were each heated in an autoclave (26).







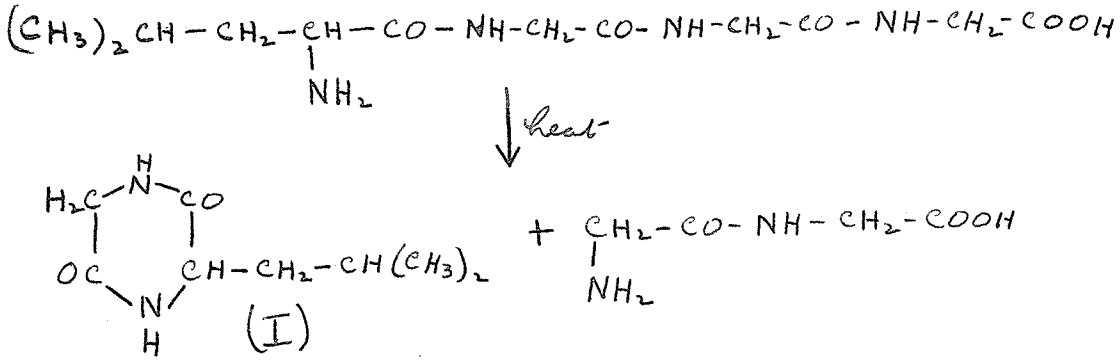
(iii) Isolation from the hydrolysate after blood protein had been heated with water at 180° for seven hours (26).

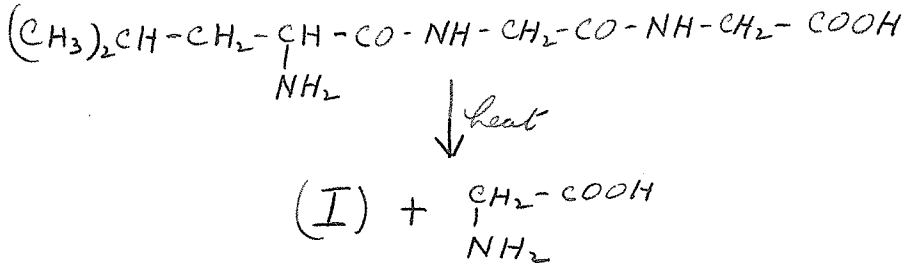
Lichtenstein converted each of the following peptides (8) to the anhydride by heating to 135-140° in $\alpha$-naphthol:-

DL-leucylglycine; DL-leucylglycylglycylglycine;

glycyl-DL-leucine; DL-leucylglycylglycine;

The overall equations for the conversion of the two dipeptides are the same as with Fischer's method no. (iv) above. The conversion of the other peptides is as shown below:-




3,6-Dimethyl-2,5-diketopiperazine or alanine anhydride, was prepared by Fischer, Preu, Pellizzari, Bergmann, Lichtenstein, and Sannié. Fischer's preparations were carried out by the following reactions:-

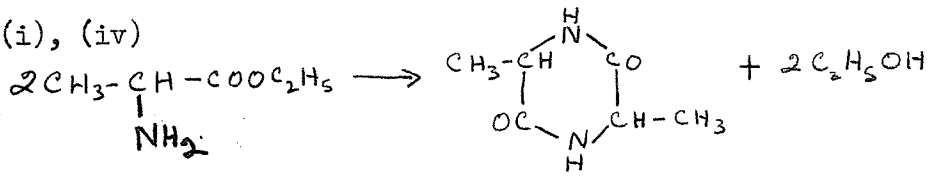
(i) Heating D-alanine ethyl (or methyl) ester for 12 hours or several days at 100° (27).

(ii) Condensing D-alanyl chloride hydrochloride with D-alanine ethyl ester, neutralising with sodium ethoxide and saturating the free ester with ammonia at 0° (27).

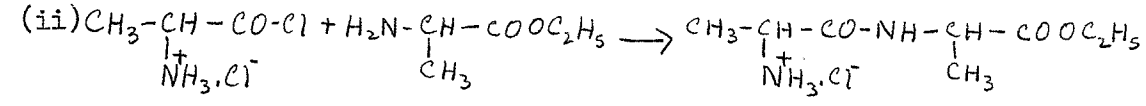
(iii) Esterifying alanylalanine and treating with alcoholic ammonia in the cold (27) (28).

(iv) Allowing DL-alanine ethyl ester to stand for several weeks, or, better, by heating to 180° in a sealed tube for 24 hours. (29). The reactions for those procedures are as represented by the equations below.

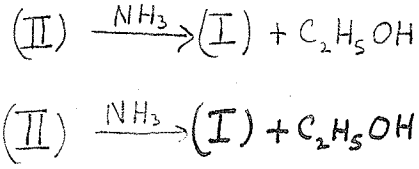
(i), (iv)



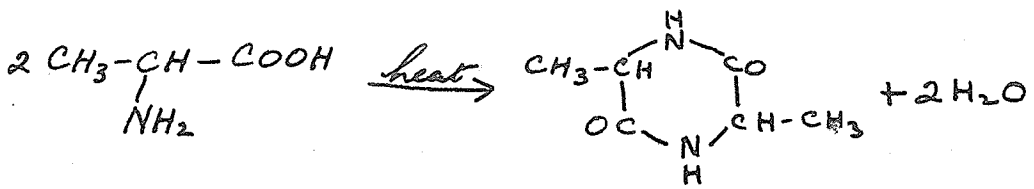
(ii)



(iii)

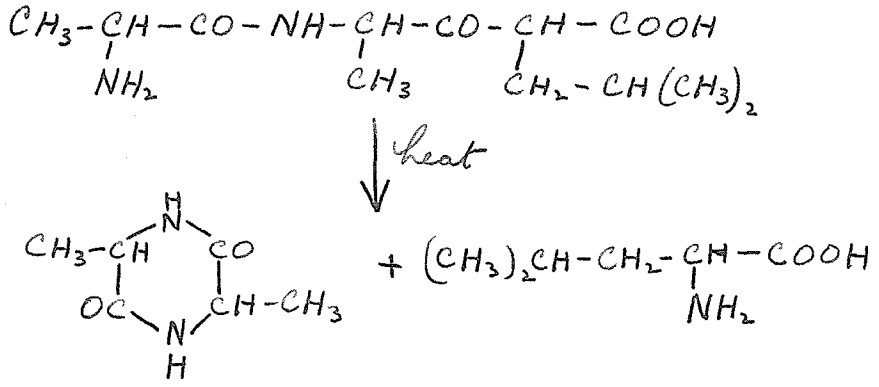


Preu's method was to heat DL-alanine to 180° in a stream of hydrogen chloride (30), while Pellizzari effected the synthesis by heating DL-alanine hydrochloride with ethyl benzoate (34).

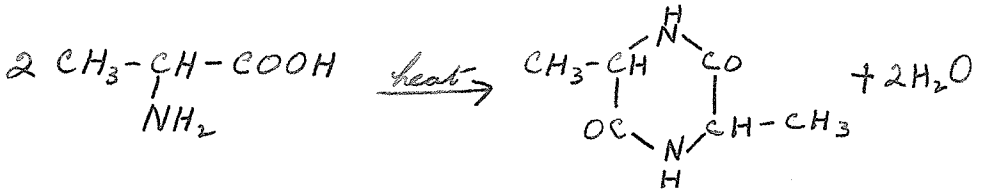


Bergmann carried out an indirect synthesis of the compound from alanine and serine. These two amino acids were condensed to form a compound $C_6H_{11}O_3N_2Cl.HCl$; the product was converted to 3-methylene-2,5-diketopiperazine by ammonia. Reduction of this unsaturated material by catalytic hydrogenation gave alanine anhydride (16).

When Lichtenstein heated DL-alanyl-DL-alanyl-DL-leucine to 135°-140° with ∝-naphthol (8), DL-alanine anhydride and DL-leucine were formed.

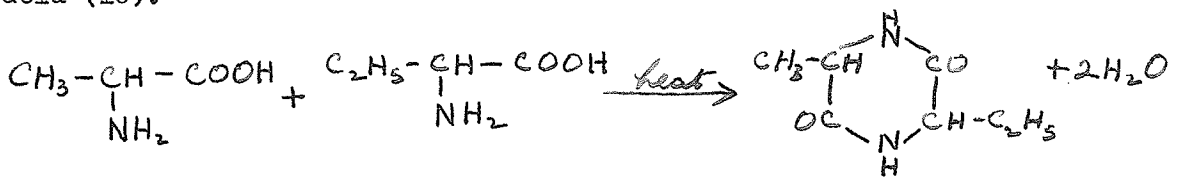



DL-Alanine was one of the amino acids whose behaviour Sannié studied; on heating with glycol DL-alanine anhydride was formed (10).



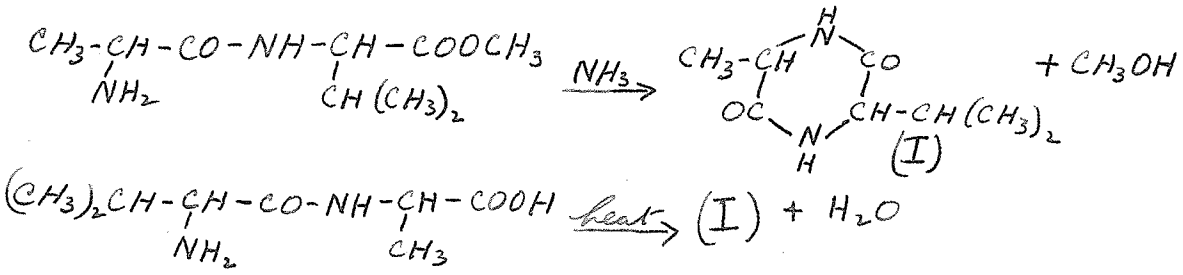
3-Methyl-6-ethyl-2,5-diketopiperazine or alanylbutyric anhydride.

Sannié describes the preparation of this compound in his paper on the heating of amino acids in glycol, by carrying out the reaction on a mixture of DL-alanine and DL-∝-aminobutyric acid (10).

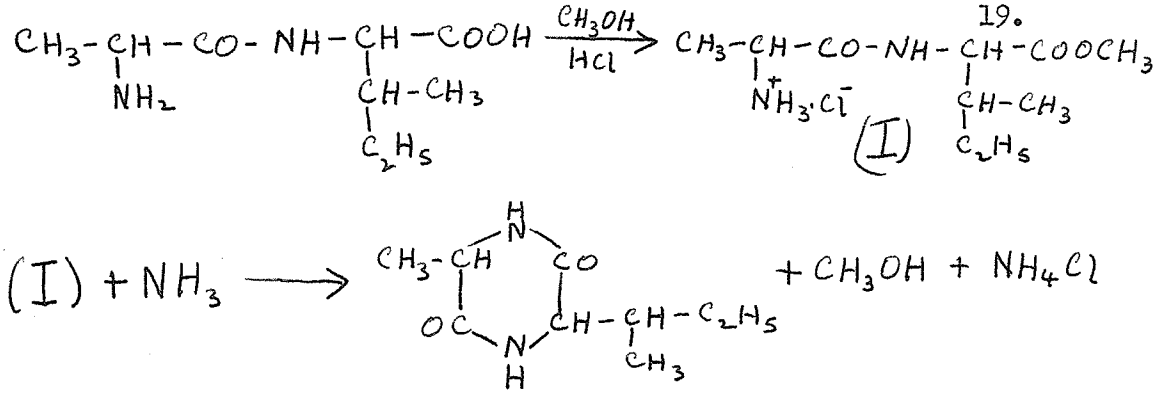


3-Methyl-6-isopropyl-2,5-diketopiperazine or alanylvaline anhydride.

By converting D-alanyl-D-valine to its methyl ester and subsequently treating the ester with ammonia, or by heating DL-valyl-DL-alanine to its melting point, Fischer and Scheibler obtained this diketopiperazine (17).



3-Methyl-6-sec.butyl-2,5-diketopiperazine or alanylisoleucine anhydride, was prepared by Fischer, Hirsch and Schuler. Hydrogen chloride was passed into a mixture of D-alanyl-D-isoleucine and methyl alcohol, followed by the action of methyl alcoholic ammonia at low temperature (20).



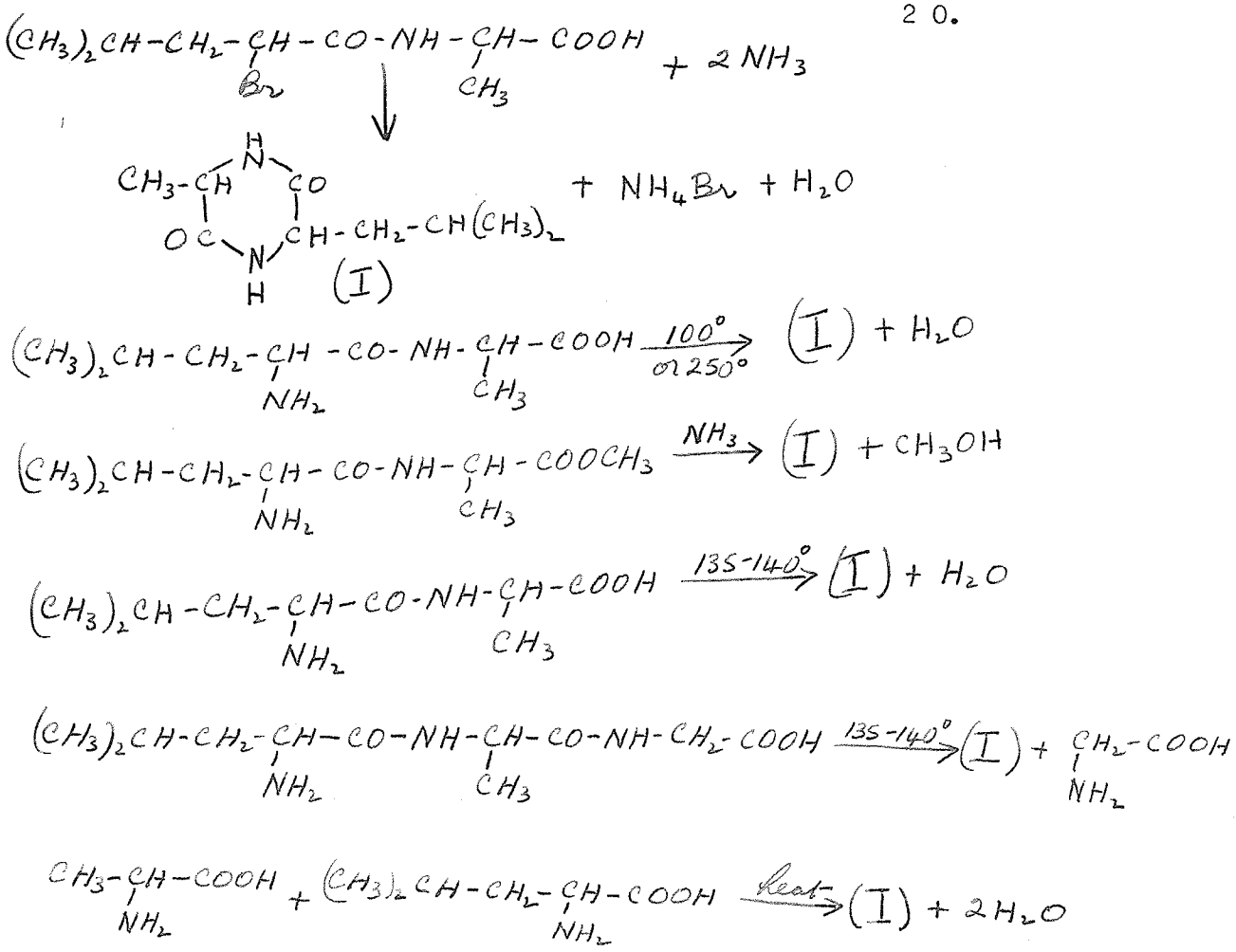


3-Methyl-6-isobutyl-2,5-diketopiperazine or alanylleucine anhydride.

Abderhalden obtained it from the hydrolysis of hog bristles by 1% hydrochloric acid.

Fischer obtained it in low yield by the prolonged action of concentrated ammonia solution at 25° on D-bromoisocaproyl-D-alanine, and by warming L-leucyl-D-alanine to 100°. A better yield was obtained by esterifying the latter dipeptide, followed by the action of methyl alcoholic ammonia (21). He also effected its preparation by heating DL-leucyl-DL-alanine to about 250° (25).

Lichtenstein's conversion of peptides to diketopiperazines by heating in ∝ -naphthol was effective when applied to DL-alanyl-DL-leucine and DL-alanyl-DL-leucylglycine (8). The method of Sannié when applied to a mixture of alanine and leucine also produced the anhydride (10).



3,6-Diethyl-2,5-diketopiperazine or ∝ -aminobutyric anhydride.

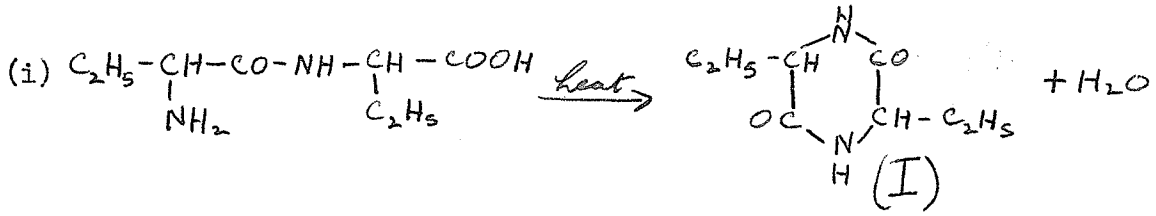
Preparation of this derivative has been carried out mainly by Fischer and Raske (28, 32) and by Sannié (10). Fischer's syntheses were mainly applications of the usual reactions on the corresponding dipeptide;-

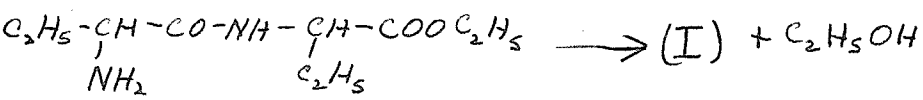
(i) Heating the dipeptide to its melting point.

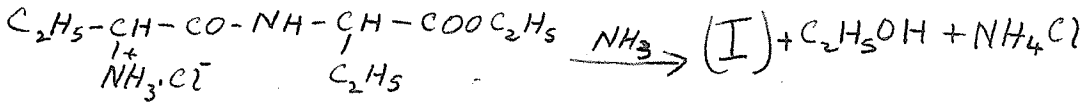
(ii) Converting the dipeptide to the ester hydrochloride, neutralising and allowing the ester to stand for a prolonged period of time.

(iii) Better than (2) above--by the action of alcoholic ammonia at a low temperature on the dipeptide ester hydrochloride.

(iv) Sannié obtained it by heating ∝ -amino butyric acid in ethylene glycol (10).

(i) 

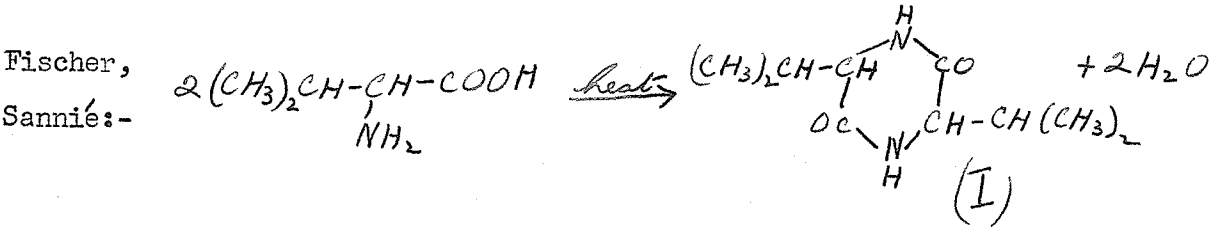
(ii) 

(iii) 

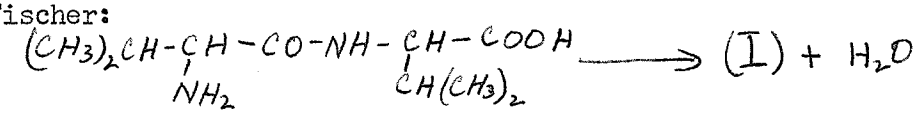
(iv) 

3,6,Diisopropyl-2,5-diketopiperazine or valine anhydride.

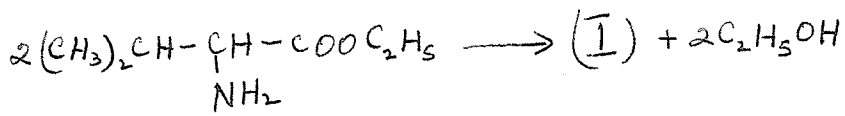
Syntheses were performed by Fischer and associates from DL-valine and from L-valyl-D-valine (17, 18). Krause obtained it by allowing DL-valine ethyl ester to stand or by heating the ester in a sealed tube to 180-190° (33), and Slimmer by allowing the ester to stand (35). Sannié also obtained it from valine.

Fischer, Sannié:- 

Fischer:



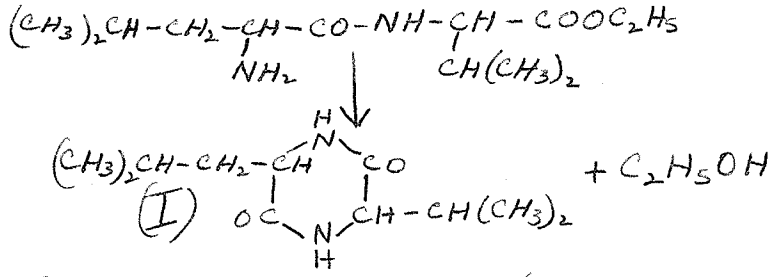
Krause, Slimmer: -



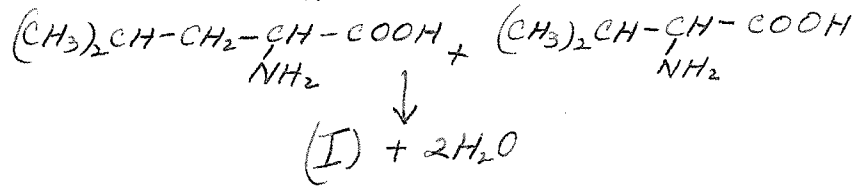
3-Isopropyl-6-isobutyl-2,5-diketopiperazine or leucylvaline anhydride.

Fischer and Scheibler prepared this compound by the frequently mentioned method of esterification and ring closure from the dipeptide (17). Krause obtained the diketopiperazine by raising a mixture of DL-valine and DL-leucine to 340° in an evacuated tube, and also by heating a mixture of the two amino acid esters at 180°-190° in a sealed tube (33). Maillard also prepared it from DL-valine and DL-leucine (36).

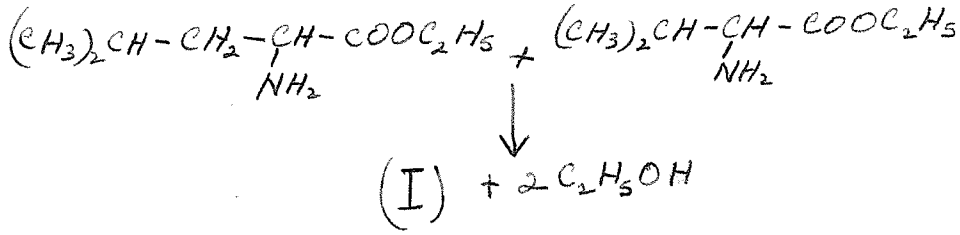
Fischer:



Krause, Maillard:

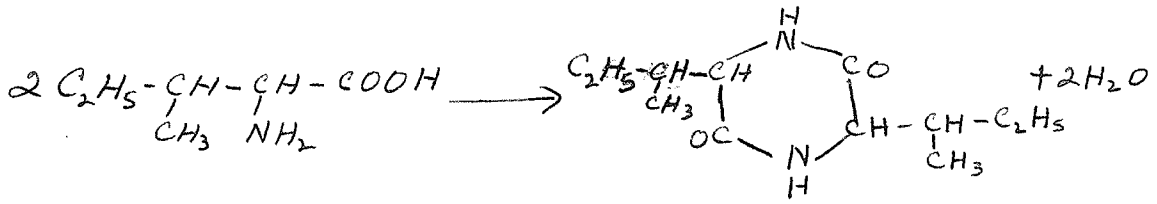


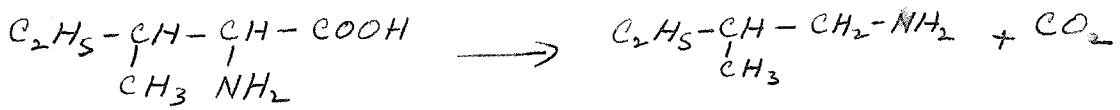
Krause:



In a study of the catalytic hydrolysis of goose feathers, Sadikov and Zelinskii reported the hydrolysate to contain this diketopiperazine, along with those derived from phenylglycylglycine, prolylleucine, and methylprolylproline (37).

3,6-Disec.butyl-2,5-diketopiperazine or isoleucine anhydride, was prepared by Ehrlich. He heated isoleucine above its melting point, and found that a certain amount of decarboxylation to the corresponding amylamine occurred, although the diketopiperazine was the main product (38).





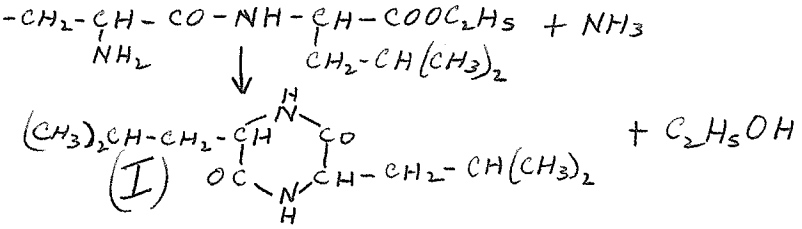
3-isobutyl-6-sec.butyl-2,5-diketopiperazine or leucylisoleucine anhydride was isolated by Abderhalden and Komm from the hog bristle hydrolysate (26).

3-Methyl-3-ethyl-6-isobutyl-2,5-diketopiperazine or leucylisovaline anhydride was isolated by Sadikov after hydrolysing blood albumin with 4% sulphuric acid at 220° in an autoclave (39, 40).

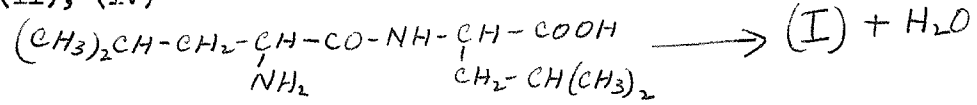
3,6-Diisobutyl-2,5-diketopiperazine or leucine anhydride.

A considerable amount of attention has been paid to the preparation of this homologue, particularly in the early years of the present century, and more especially by Fischer. His methods were: (i) esterification of the dipeptide followed by action of ammonia (21), (ii) (with Koelker) from L-leucyl-D-leucine or D-leucyl-L-leucine (41), (iii) allowing DL-leucine ethyl ester to stand for a month, or by warming this ester with sodium ethoxide on a water-bath. He found it better to heat either the DL- or the L-ester in a sealed tube to 180-190° (29), and (iv) by raising the DL-dipeptide to its melting point (42).

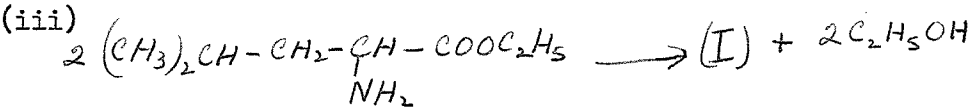
(i)



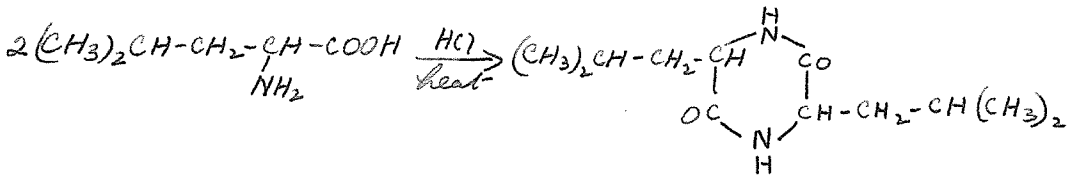
(ii), (iv)



(iii)



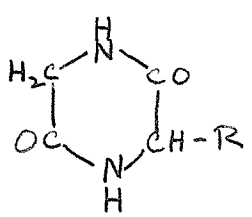
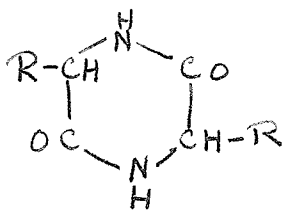
Cohn also prepared this diketopiperazine by prolonged action of hydrochloric acid on casein (43, 44, 45), and by heating L-leucine at 235-240° in a current of dry hydrogen chloride (the DL-anhydride was obtained) (45).



$$+ 2H_2O$$

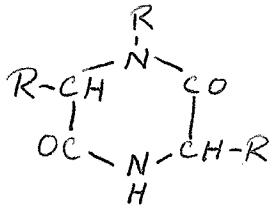
Maillard prepared the substance synthetically by Cohn's method above, and by hydrolysing plant and animal proteins with boiling sulphuric or boiling concentrated hydrochloric acid (45,46). Other reports of its preparation by protein hydrolysis have been made by various workers. For example, Ritthausen obtained it from grain protein hydrolysate (48), Abderhalden by hydrolysing casein with hydrochloric or 25% sulphuric acid and by heating horse haemoglobin with hydrochloric acid.

Salaskin prepared the compound by prolonged tryptic digestion of horse haemoglobin (49). Hydrolysis of plant and animal proteins to give the anhydride was reported by Hlasiewitz and Habermann (51). Syntheses were reported by Kohler (34), Krause (33), and Destrem (50). Sannié's technique of heating amino acids in glycol was successful in the case of leucine, as were the other applications mentioned in this review (10).

All of the diketopiperazines reviewed up to this point, other than the simplest member of the series, have been of the type  (I) or  (II).

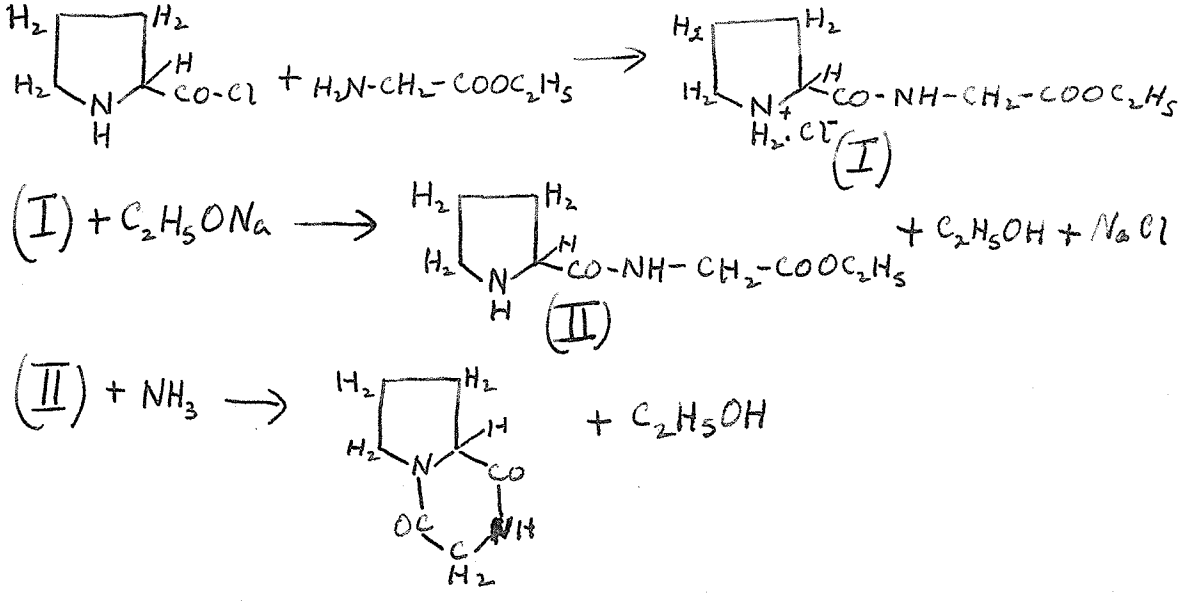
R in the compounds so far reviewed is an alkyl group, and in type II the R groups may or may not be identical. Numerous cases have been reported in the literature in which the R group is some other type of organic radical, and some instances have also been reported of compounds having R groups in the 1,4-position. Most of the diketopiperazines whose preparations are reviewed in the following pages are of types (I) and (II), where R represents any organic radical other than alkyl, and some reference is made to a few isolated examples of type (III) (R denoting various types of organic radical).

(III)



Glycylproline anhydride.

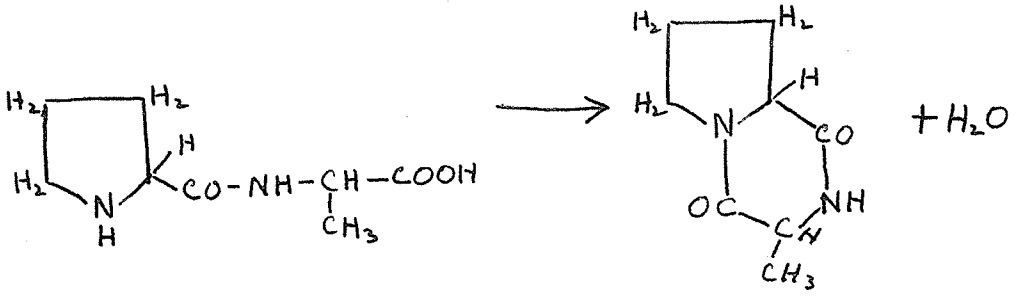
Fischer condensed L-prolyl chloride with glycine ethyl ester. He converted the resulting prolylglycine ester hydrochloride to the free ester with sodium ethoxide; the product was then made to undergo ring closure by the action of alcoholic ammonia (52).



Levene and his co-workers obtained the compound by digesting gelatin with trypsin continually for a period of 15 months (53,54). Abderhalden and Komm (14) also reported its isolation from the hydrolysate obtained by digesting edestin with pancreatin for three weeks; they also obtained dihydroxy-prolylglycine anhydride when hog bristles were heated with 2% hydrochloric acid in an autoclave for 10 hours (26).

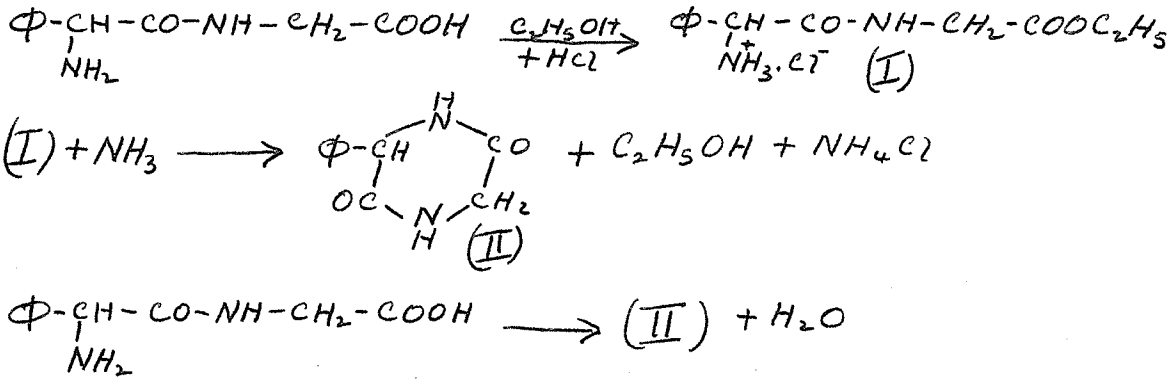
Alanylproline anhydride.

Fischer and Suzucki carried out its preparation by heating DL-prolylalanine to 225° (55).



3-Phenyl-2,5-diketopiperazine or glycylphenylglycine anhydride.

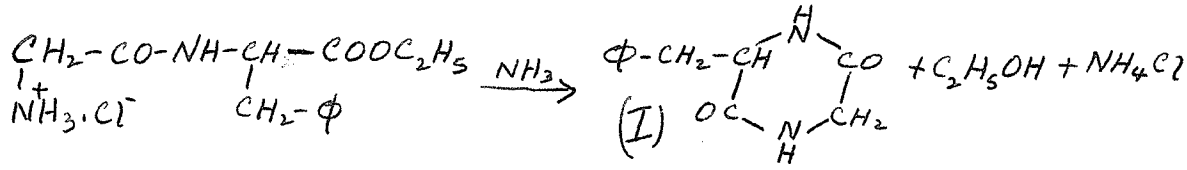
Fischer and his co-workers obtained this compound by warming DL-phenylglycylglycine with alcoholic HCl and treating the resulting ester with alcoholic ammonia (32), and also in small quantities when phenylglycylglycine was melted (32).

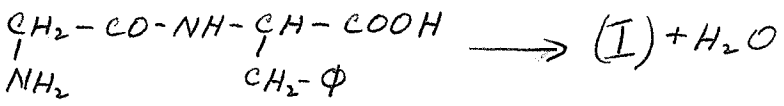


Its formation was also reported by Sadikov and Zelinskii in the catalytic hydrolysis of goose feathers (37).

3-Benzyl-2,5-diketopiperazine or glycylphenylalanine anhydride.

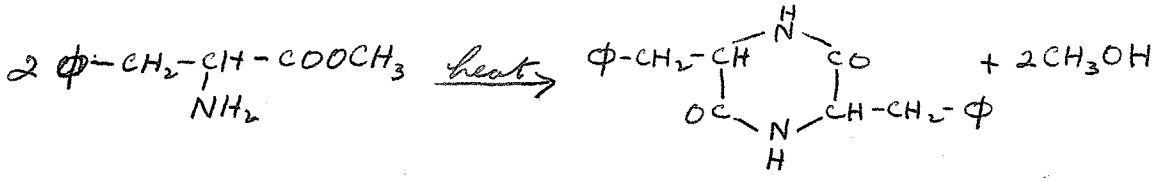
Fischer's school esterified glycyl-l-phenylalanine, and obtained the diketopiperazine by treating the ester hydrochloride with alcoholic ammonia (56,57). Abderhalden and Komm obtained the compound when glycyl-DL-phenyl-alanine was heated with water at 150-160° for six hours in a sealed tube (6), and Lichtenstein obtained it when he heated glycyl-DL-phenylalanine to 135-140° in $\alpha$-naphthol (8).



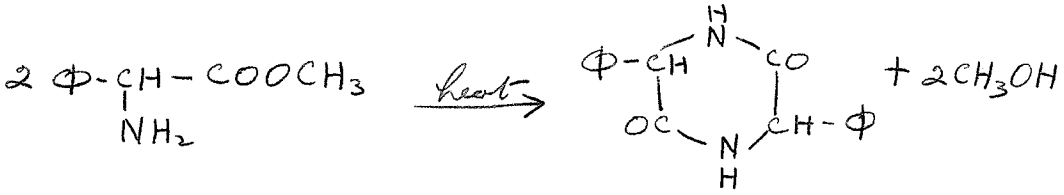


3,6-Dibenzyl-2,5-diketopiperazine or phenylalanine anhydride.

Levene and his associates prepared this compound by heating DL-phenylalanine methyl ester to 170° (58). It was also prepared by Sasaki's method, mentioned and discussed more fully on page 35 of this review.



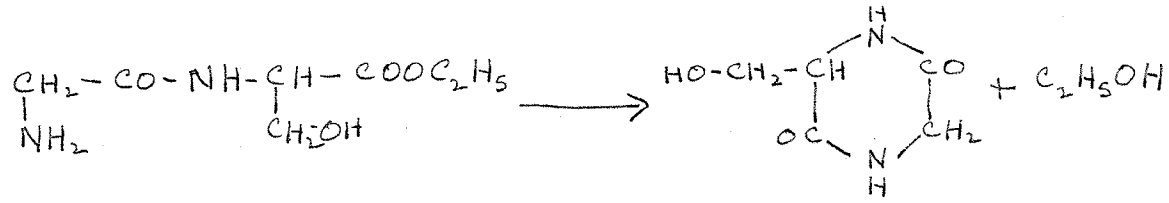
They used a similar method to prepare 3,6-diphenyl-2,5-diketopiperazine, or phenylglycine anhydride, when they heated the methyl ester of phenylglycine in a sealed tube at 160° for nine hours (58).



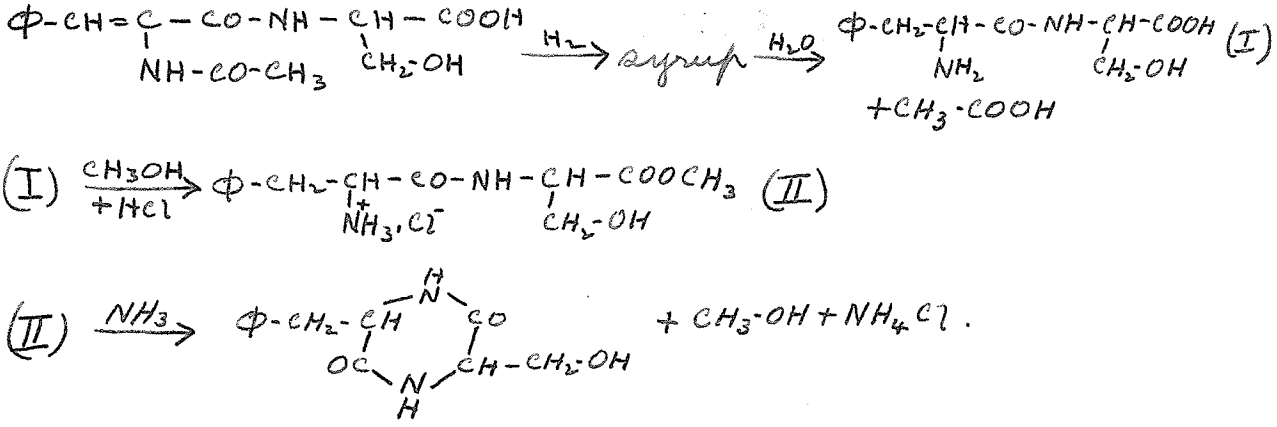
3-Benzyl-6-methyl-2,5-diketopiperazine or alanylphenylalanine anhydride.

This compound was isolated by Abderhalden from the hydrolysate after hog bristles had been hydrolysed with 1% hydrochloric acid (26).

Several diketopiperazines derived from serine have been prepared, mainly by Abderhalden and by Bergmann. Abderhalden and Bahn prepared 3-(2-hydroxymethyl)-2,5-diketopiperazine (glycyl-DL-serine anhydride) by esterifying glycyl-DL-serine and bringing about ring closure of the ester to the diketopiperazine (59).



3-Hydroxymethyl-6-benzyl-2,5-diketopiperazine (phenylalanylserine anhydride) was prepared by Bergmann as follows: - N-(∝-acetaminocinnamyl)-DL-serine on reduction gave a syrupy material. The syrup was hydrolysed to the dipeptide phenylalanylserine by heating for five minutes with 5N HCl. The dipeptide reacted with methyl alcohol in presence of hydrogen chloride to form the methyl ester hydrochloride, which in turn was converted to the diketopiperazine by the action of a methyl alcoholic solution of ammonia (60).




Mention has been made on page 26 of a few diketopiperazines derived from proline. Three other such compounds derived from this amino acid are: -

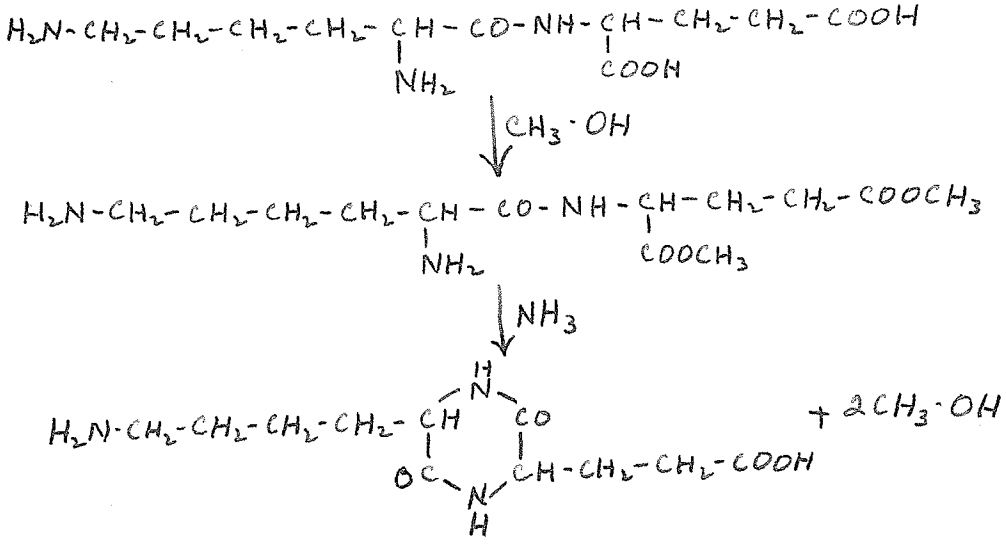
(i) DL-prolylvaline anhydride, isolated by Abderhalden and Komm following upon the hydrolysis of blood protein by heating with water at 180° for seven hours in an autoclave (26).

(ii) Proleucylleucine anhydride
(iii) Methylproline anhydride } both isolated by Sadikov and Zelinskii, following the catalytic hydrolysis of goose feathers (37).

(iv) L-Phenylalanyl-L-proline anhydride and (v) dehydrophenylalanyl-L-proline anhydride, both prepared by Bergmann (61).

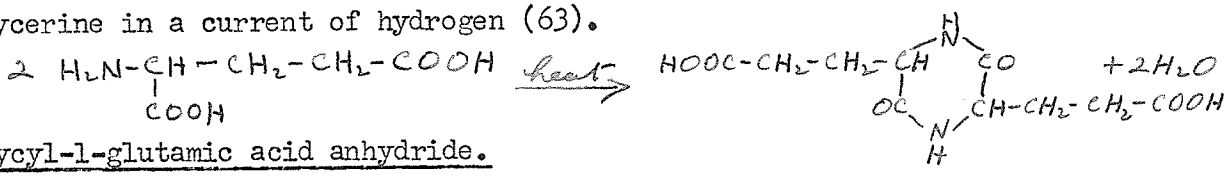
Lysylglutamic acid anhydride.

Greenstein adapted the method so often used by Fischer, of dipeptide esterification followed by ring closure. Lysylglutamic acid was allowed to stand for five days at 0° in a solution of ammonia in absolute methyl alcohol (62).



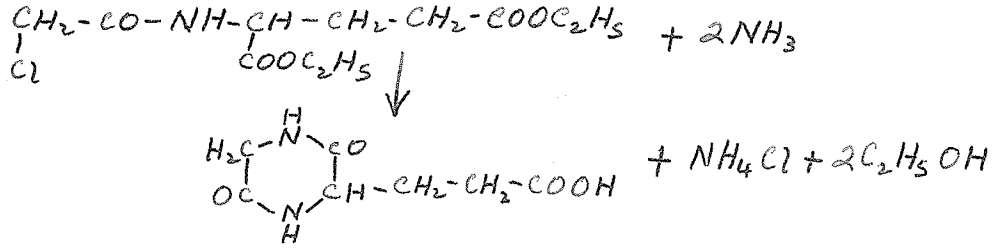
Glutamic acid anhydride.

This compound was obtained along with other products by Blanchetière from glutamic acid by heating the latter with glycerine in a current of hydrogen (63).



Glycyl-l-glutamic acid anhydride.

Abderhalden, Weichert and Haase treated the diethyl ester of chloracetyl-L-glutamic acid with ammonia (64).

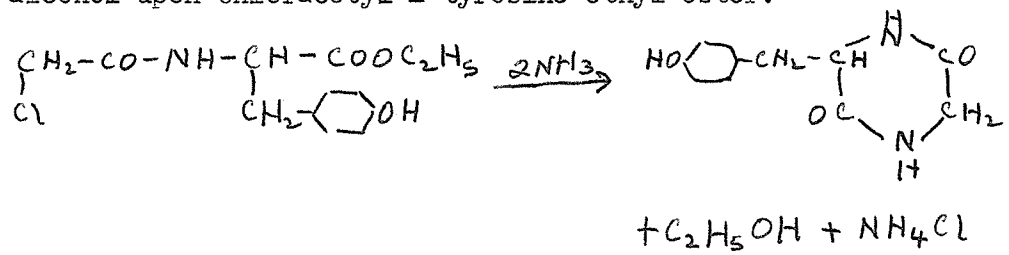


Glycyl-l-asparagine anhydride.

Levene and Steiger prepared this compound by leaving chloracetyl-L-asparagine (65) in contact with concentrated ammonia solution for several days.

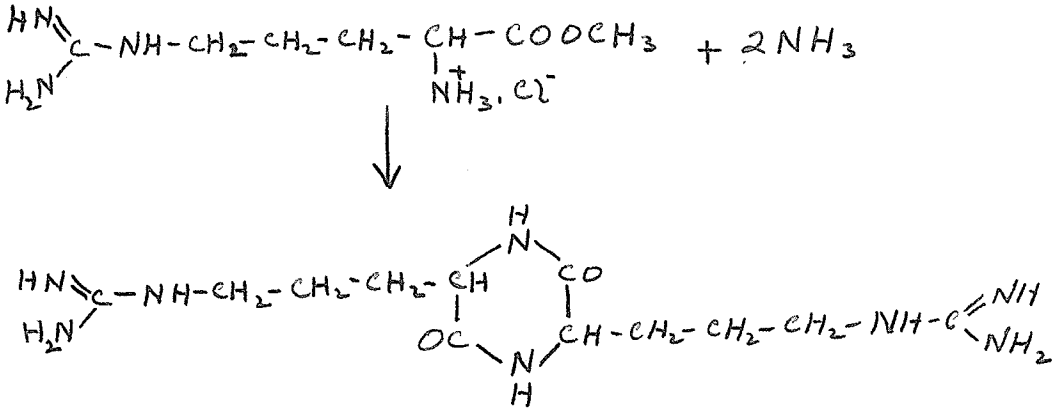
3-(p-hydroxyphenyl)-2,5-diketopiperazine or glycyl-L-tyrosine anhydride, was isolated from the hydrolysate which Abderhalden and Komm obtained when they left silk fibroin in contact with concentrated hydrochloric or 70% sulphuric acid at a temperature below 25° (15). The same compound was made synthetically

by Levene and Steiger by the action of ammonia in ethyl alcohol upon chloracetyl-L-tyrosine ethyl ester.

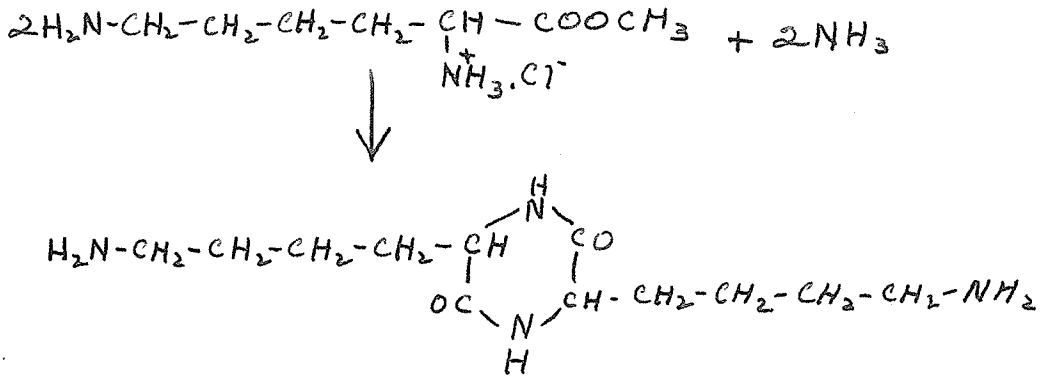


$+C_2H_5OH + NH_4Cl$

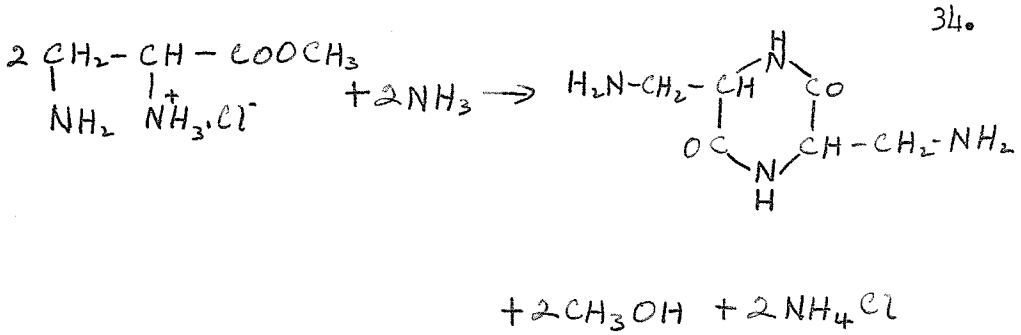
<u>D-Arginine anhydride, D-lysine anhydride,</u> and <u>DL-diamino-propionic anhydride</u> were prepared by Tazawa by the action of methyl alcoholic ammonia on the hydrochlorides of the amino acid esters. (66).



$+2CH_3OH + 2NH_4Cl$



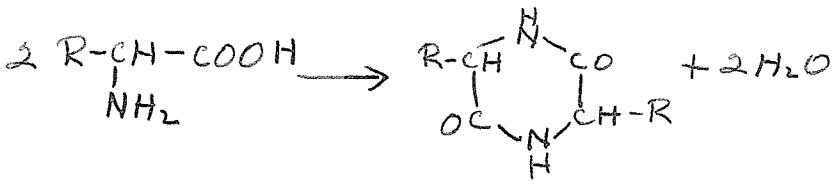
$+2CH_3OH + 2NH_4Cl$



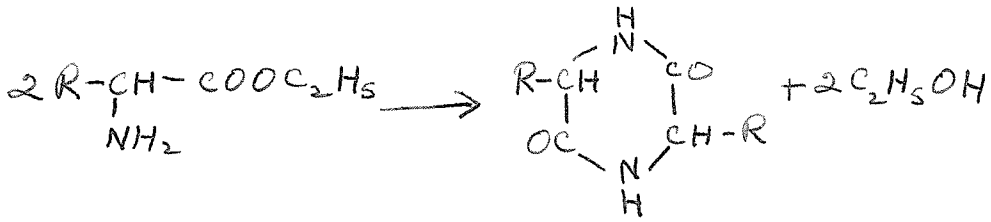
$$+2CH_3OH \quad +2NH_4Cl$$

Most of the 2,5-diketopiperazines reviewed so far have been made by application of some of the following synthetic methods:-

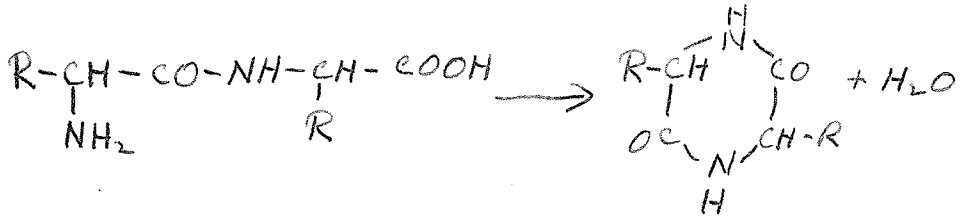
(i) Dehydration of an $\alpha$-amino acid.



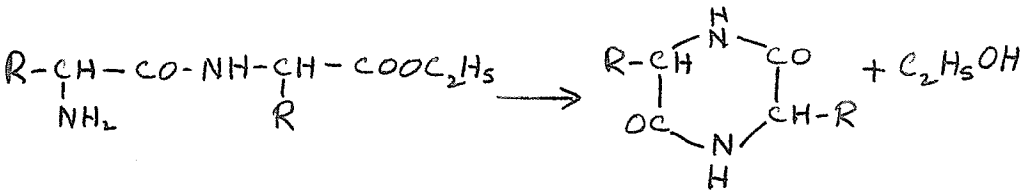
(ii) Condensation of two molecules of an amino acid ester.

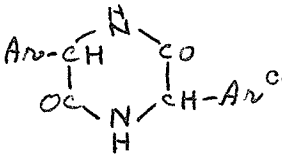
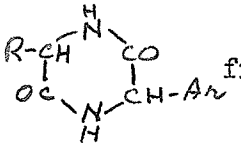
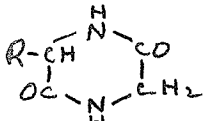


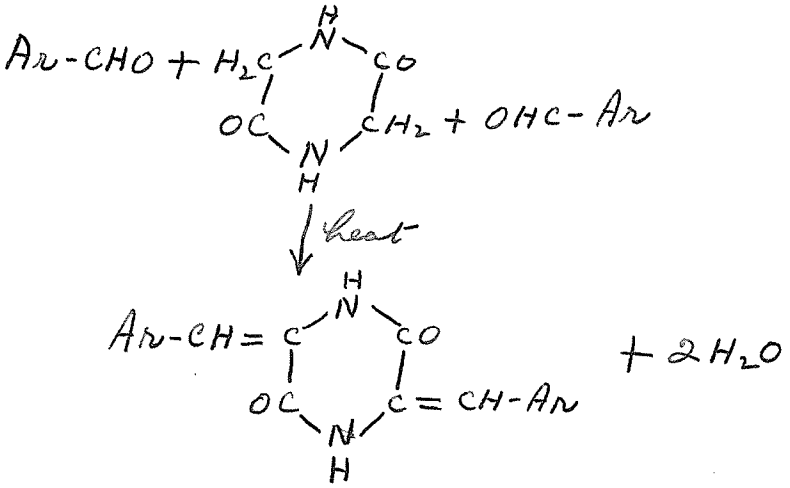
(iii) Dehydration of a dipeptide.



(iv) Elimination of an alcohol molecule from a dipeptide ester.



A method was developed by Sasaki in which anhydrides of the type  can be synthesised from 2,5-diketopiperazine, and compounds of the type  from the type  . Ar represents an aromatic radical. The method consists in heating 2,5-diketopiperazine or a monosubstituted homologue with an aromatic aldehyde, using a mixture of anhydrous sodium acetate and acetic anhydride as condensing agent; the derivative formed is unsaturated.



The unsaturated linkage can then be reduced, if desired, by treating the condensation product with zinc and acetic acid or other suitable reducing agent. Other workers have found it convenient to apply Sasaki's method. Using these procedures, the following 2,5-diketopiperazines have been prepared:

(i) 3,6-Dibenzal-2,5-diketopiperazine and 3,6-dibenzyl-2,5-diketopiperazine.

Sasaki made the first named product by condensing benzaldehyde with glycine anhydride; reduction then gave the second compound (67). This latter was also prepared by Levene and co-workers on heating dl-phenylalanine methyl ester to 170° (58).

(ii) 3-sec.butyl-6-benzyl-2,5-diketopiperazine (leucylphenylalanine anhydride).

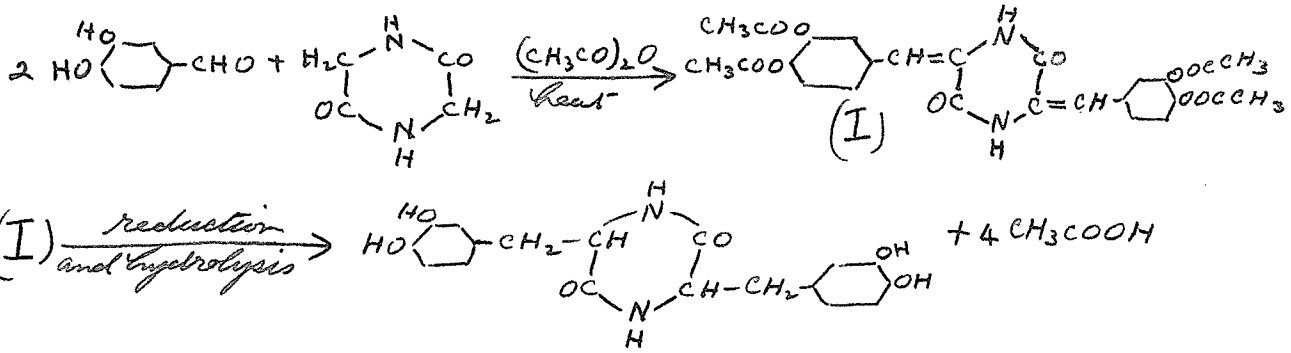
Sasaki condensed benzaldehyde with 3-sec.butyl-2,5-diketopiperazine. Reduction of the condensation product yielded the phenylalanine derivative (68).

(iii) 3,6-difuryl-2,5-diketopiperazine (furylalanine anhydride).

Sasaki applied his method to the synthesis of this substance by condensing 2,5-diketopiperazine with furfural. The 3,6-difural compound thus formed was reduced to the furyl derivative (69).

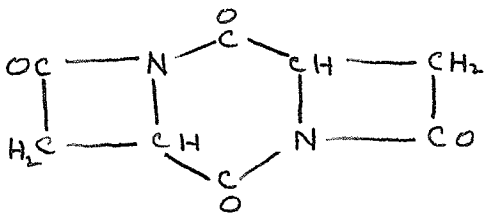
(iv) Kuster and Koppenhöfer utilised Sasaki's procedure in preparing 3,6-(dipyrrylmethyl)-2,5-diketopiperazine. These investigators carried out the condensation between pyrrole-∝-aldehyde and glycine anhydride, followed by reduction of the product (70).

(v) Deulofeu utilised Sasaki's technique to synthesise dihydroxyphenylalanine anhydride. 2,5-Diketopiperazine was made to react with 3,4-dihydroxybenzaldehyde, forming the diacetyl derivative of the dihydroxybenzal compound. Reduction of the latter gave the anhydride of diacetyldihydroxyphenylalanine, which was hydrolysed with hydriodic acid to give the required anhydride (71).

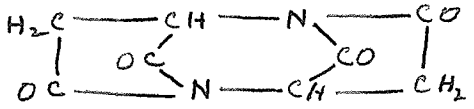



A more complex type of structure which contained the diketopiperazine ring was prepared by Ravenna and Bosinelli (72). On applying the procedure so often used by Fischer and Abderhalden to synthesise a diketopiperazine by heating a dipeptide, they obtained, on heating asparagylaspartic acid for several hours at 210° in an open vessel, a substance which they chose to call 2,5-diketopiperazinediacetic anhydride.

The product was found neither to melt nor decompose on heating to as high a temperature as 320°. They believed that its structure might be either A or B below, and that B was more likely to be the correct structure.

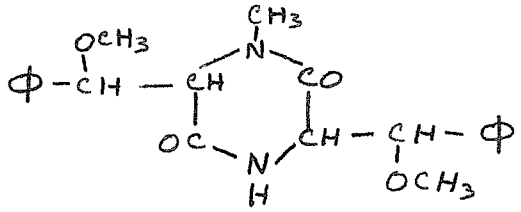


A



B

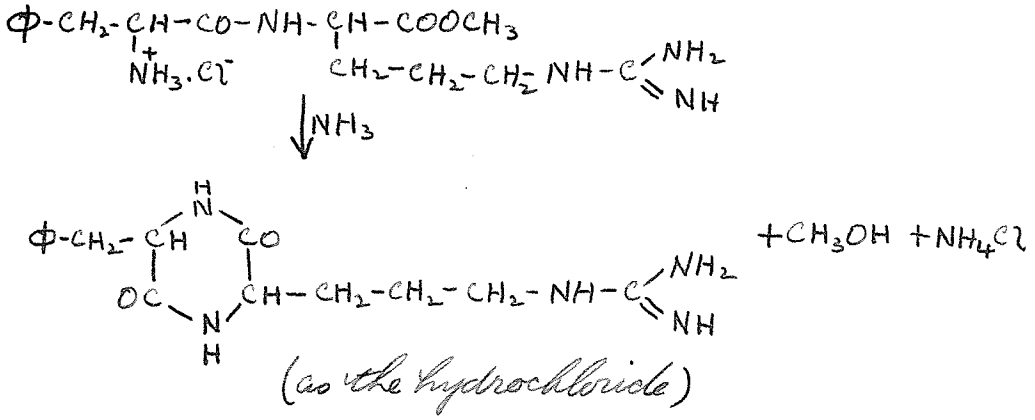
One instance has been reported of the occurrence of a 3,6-disubstituted 2,5-diketopiperazine in nature. Saville and Forster (73) isolated from a species of lichens a substance which they named picrorocelline. From an investigation of its properties they concluded that its structure was



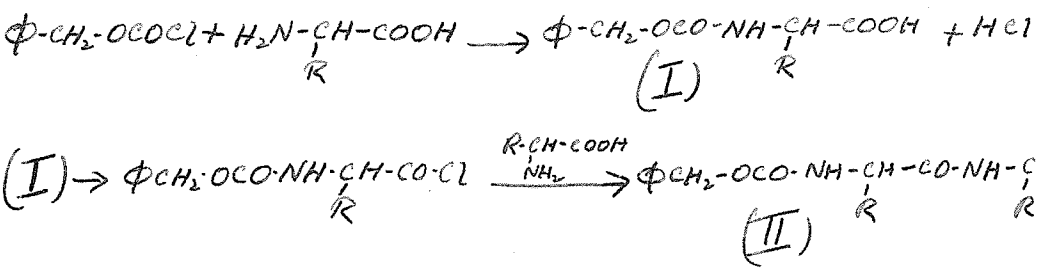
D-Phenylalanyl-D-arginine anhydride.

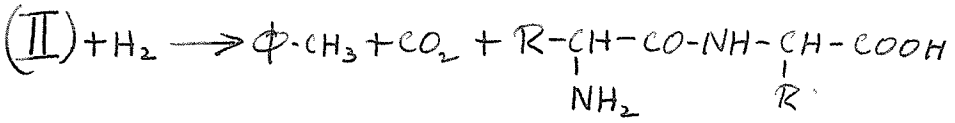
Bergmann and Köster used Fischer and Abderhalden's method of acting on a dipeptide ester with ammonia to prepare this diketopiperazine. The hydrochloride of D-phenylalanyl-D-arginine methyl ester was allowed to react with ammonia gas for several days at room temperature. The hydrochloride of the anhydride

was formed, but only in 25% yield (74).

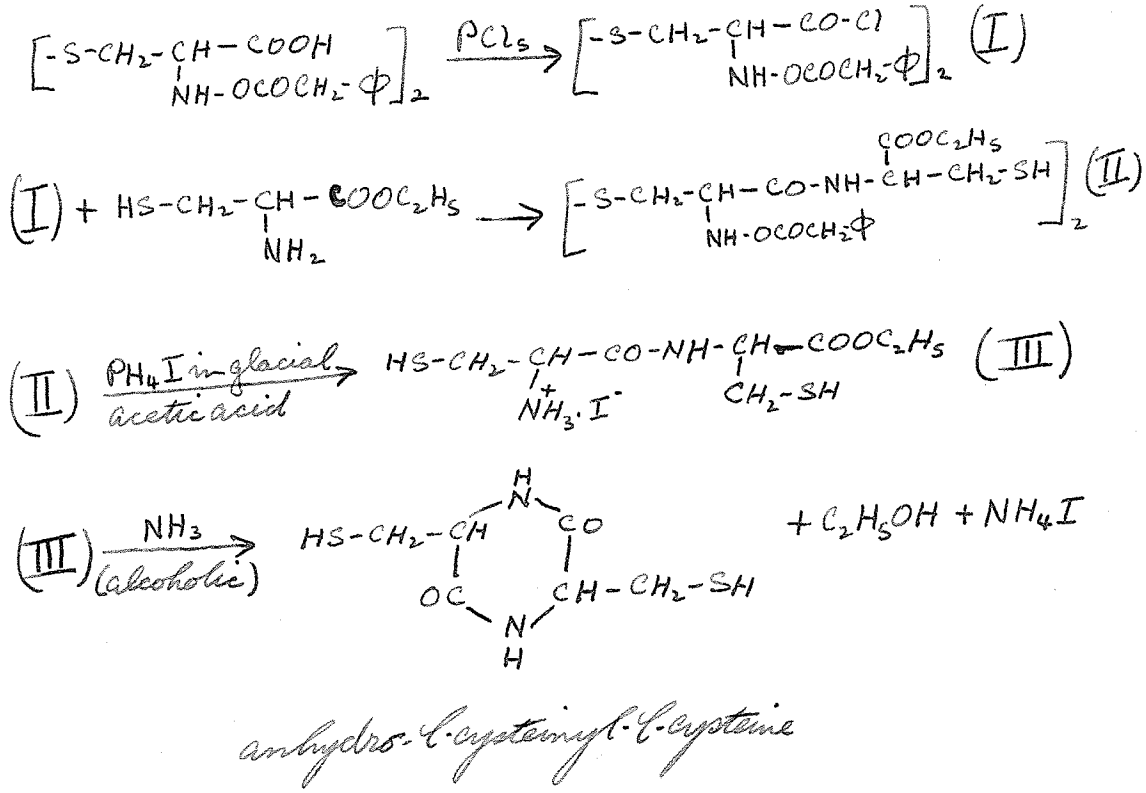



Bergmann and Zervas developed a procedure for the synthesis of dipeptides which has since come to be referred to as the "carbobenzoxy" method. Carbobenzoxy chloride is made to react with an amino acid. The carbobenzoxy derivative can be converted to its acid chloride, and this can be condensed with another amino acid to form a carbobenzoxy dipeptide. If desired, the latter can be chlorinated, and the resulting chloride condensed with still another amino acid; the process can be repeated until a chain consisting of the required number of amino acid residues is obtained. The product is the carbobenzoxy derivative of the polypeptide. Catalytic hydrogenation removes the carbobenzoxy group as toluene and carbon dioxide, thus liberating the free polypeptide (75).



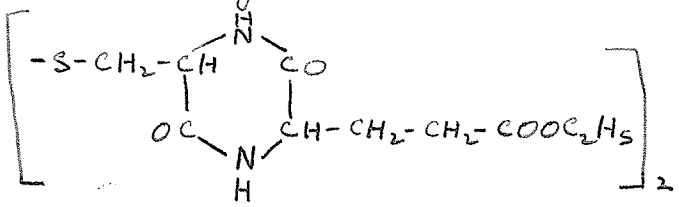


Greenstein made use of Bergmann's carbobenzoxy reaction to synthesise some diketopiperazines derived from cysteine, cysteine and aspartic acid, and cysteine and DL-tyrosine. The equations for the reactions are as shown below, and the names assigned to the diketopiperazines are the names given by Greenstein (76).

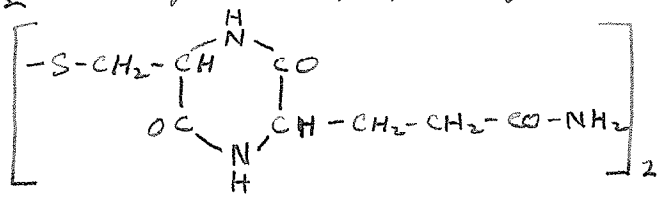



anhydro-L-cysteinyl-L-cysteine

(I) + diethyl glutamate → product $\xrightarrow{PH_4I}$ anhydro-l-cystyl-l-γ-ethyl glutamate (IV)

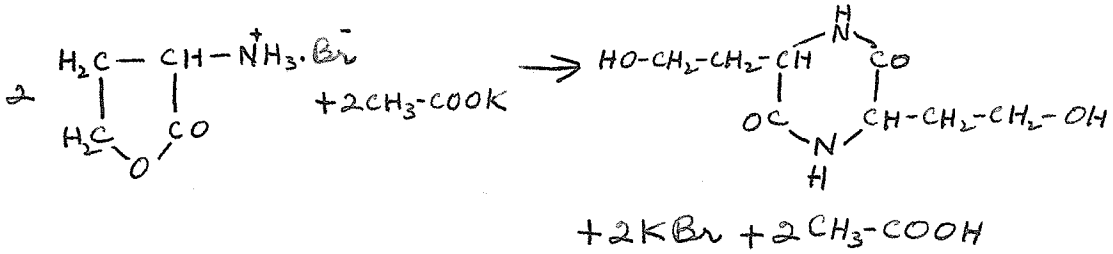


(IV) $\xrightarrow[\text{(alcoholic)}]{NH_3}$ product $\xrightarrow[\text{aeration}]{FeCl_3 \text{ with}}$ anhydro-l-cystyl-l-glutamine

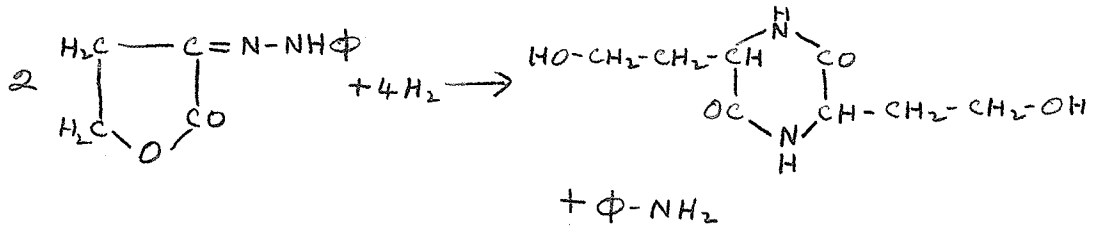


3,6-bis(2-hydroxyethyl)-2,5-diketopiperazine.

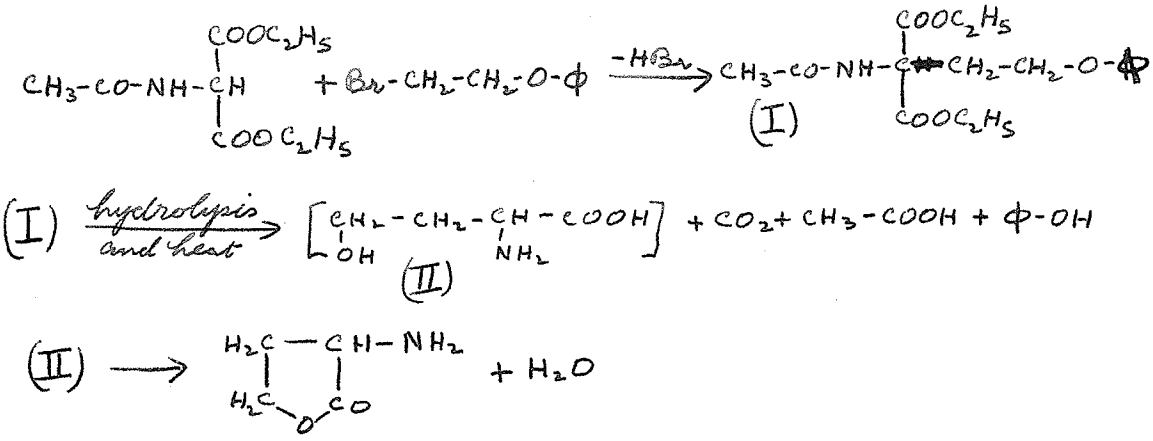
Several accounts of the preparation of this compound have been published. Britton and Livak prepared it by refluxing the hydrobomide of ∝-amino-γ-butyrolactone at 75° with potassium acetate in absolute alcohol (77).



Snyder and his co-workers obtained the compound by carrying out a catalytic hydrogenation of the phenylhydrazone of $\alpha$-keto-$\gamma$-butyrolactone at 100° under high pressure (78).



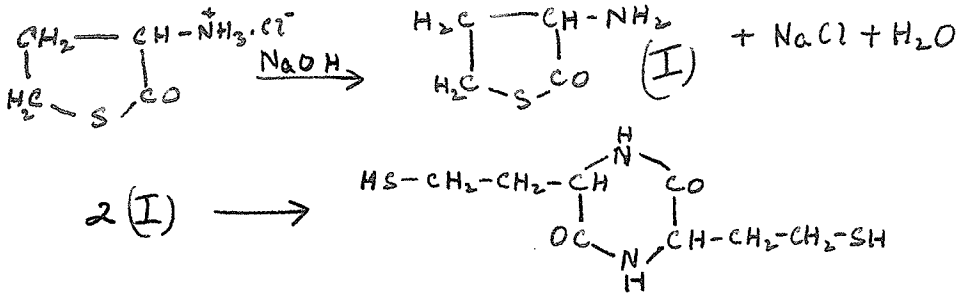
Armstrong also prepared the compound from $\alpha$-amino-$\gamma$-butyrolactone, but the latter was only an intermediate formed in a sequence of reactions. Initially he condensed acetamidomalonic ester with the phenyl ether of ethylene bromhydrin, and converted the product to the lactone (79).



Mention has been made on pages 40 and 41 of some sulphur-containing diketopiperazines derived from cysteine. Other diketopiperazines, derived from the amino acids homocysteine and methionine, were described in papers published by Du Vigneaud (80) and Snyder (81).

Du Vigneaud and his co-workers prepared DL-homocysteine anhydride, the L-compound and the D-compound, and converted each of these to the S-benzyl derivative. Details of the synthesis were as follows:-

DL-Homocysteine anhydride -- The thiolactone hydrochloride of DL-homocysteine was treated with dilute alkali at room temperature.

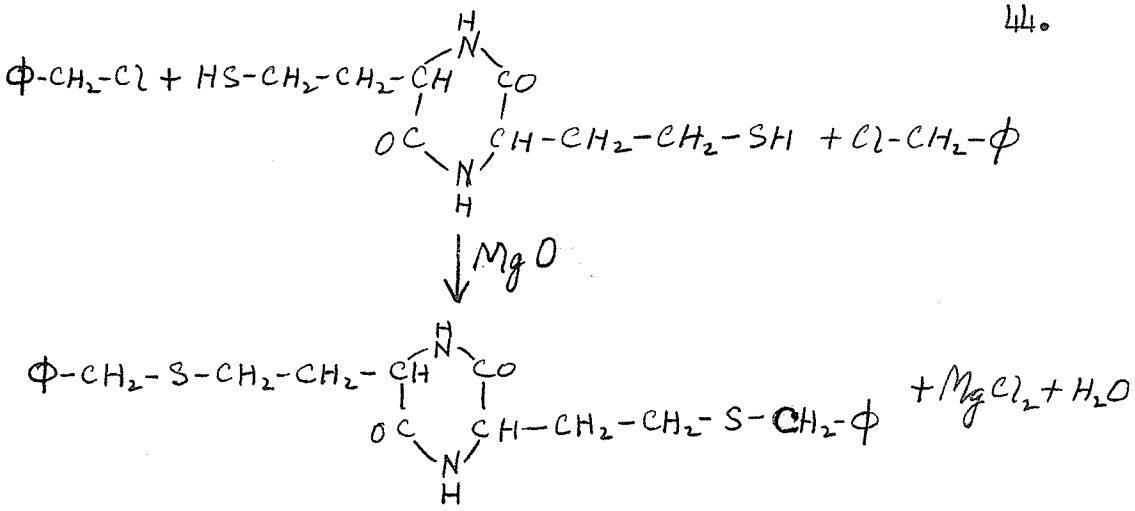


D-Homocysteine anhydride -- D-Homocysteine thiolactone hydrochloride was allowed to stand with sodium bicarbonate solution. The free lactone thus liberated would then react similarly to that of the DL-compound as shown above.

L-Homocysteine anhydride -- The same procedure was followed as for preparing the D-compound.

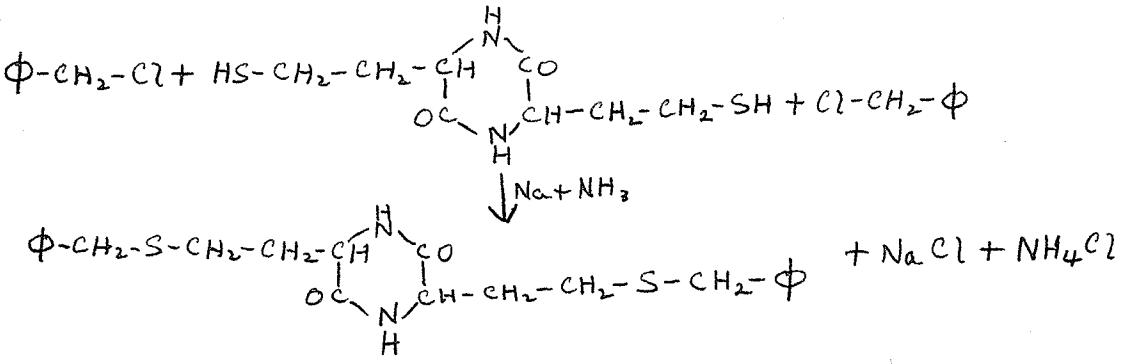
S-Benzyl-DL-homocysteine anhydride.

DL-Homocysteine anhydride was condensed with benzyl chloride in presence of magnesium oxide.

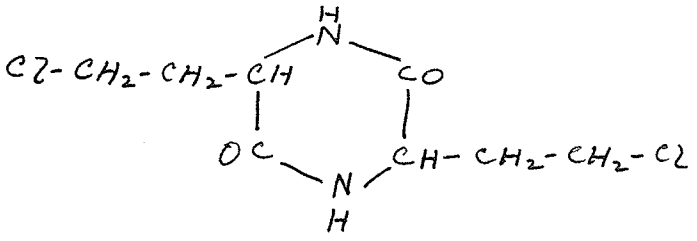


S-Benzyl derivatives of the D- and of the L- compounds.

Sodium, benzyl chloride and ammonia acted on the respective D- and L-anhydrides.



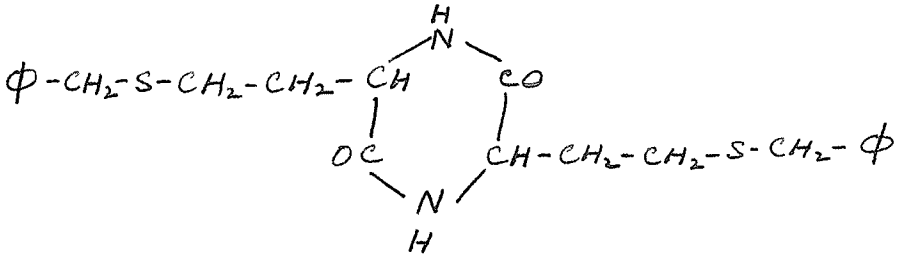
Snyder and Chiddix effected the conversion of 3,6-bis (chloroethyl)-2,5-diketopiperazine to S-benzylhomocysteine anhydride (81), and to 3,6-bis 2-(4-morpholinyl)ethyl - 2,5-diketopiperazine (82), and of 3,6-bis(bromoethyl)-2,5-diketopiperazine to methionine anhydride (81); they also brought about the interconversion of 3,6-bis(bromoethyl)-2,5-diketopiperazine and 3,6-divinyl-2,5-diketopiperazine (81).



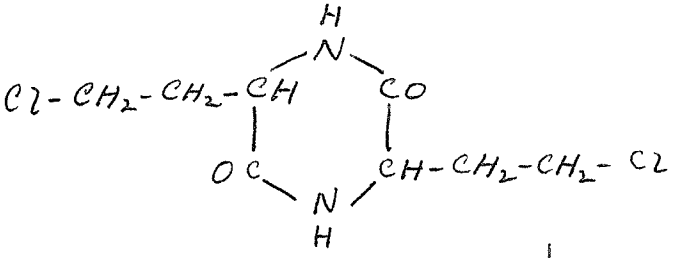
+ 2 $\phi-CH_2-SH$

I

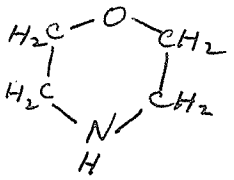
$\downarrow 2\,C_2H_5ONa$



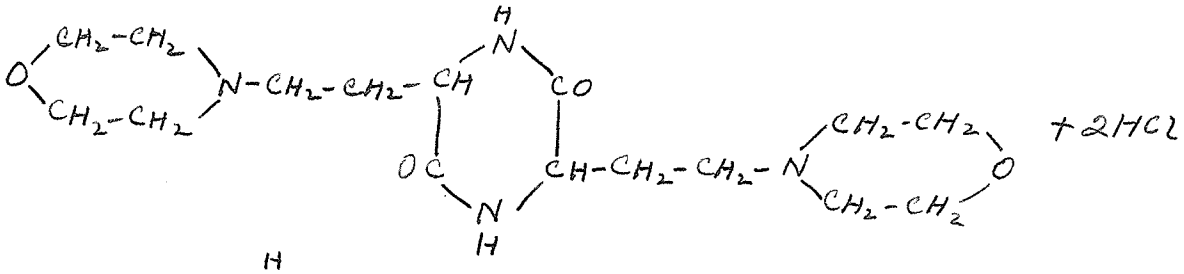
$+2NaCl + 2\,C_2H_5OH.$



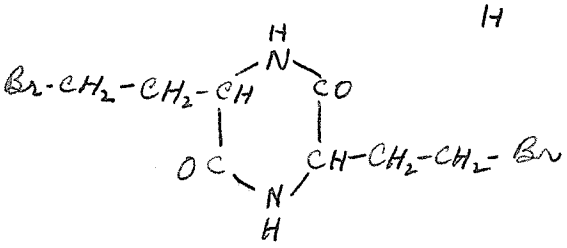
+ 2



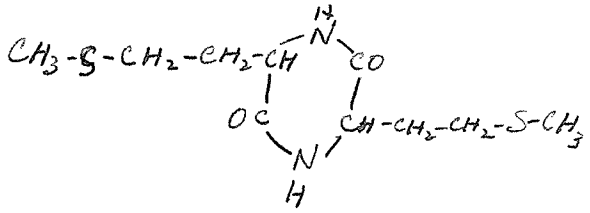
$\downarrow$



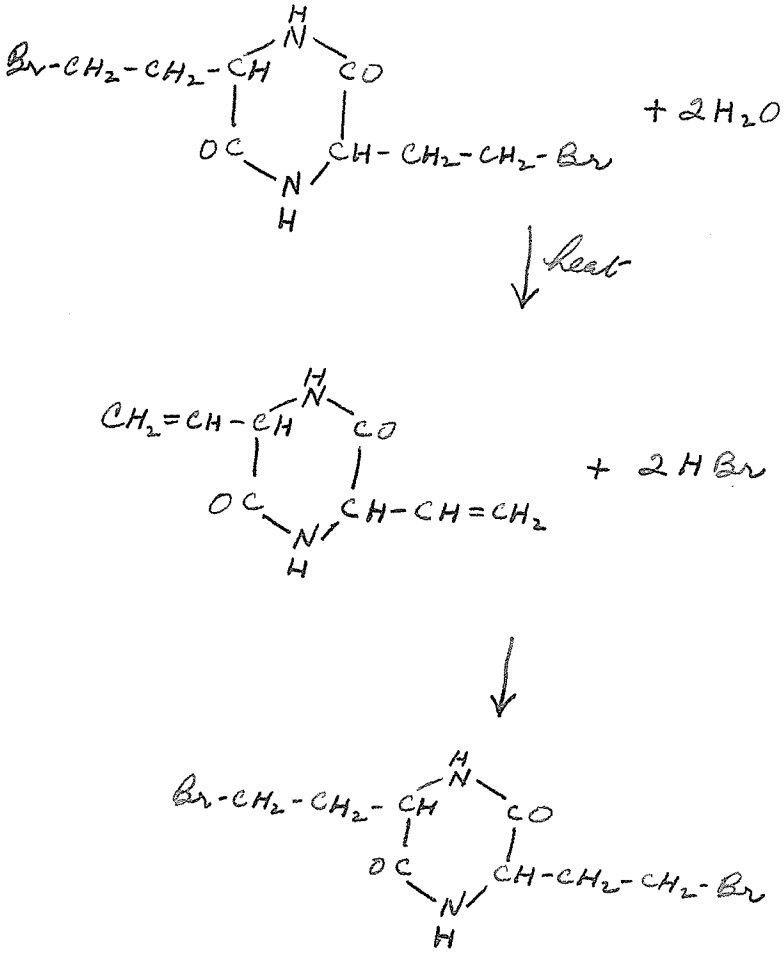
$+2HCl$



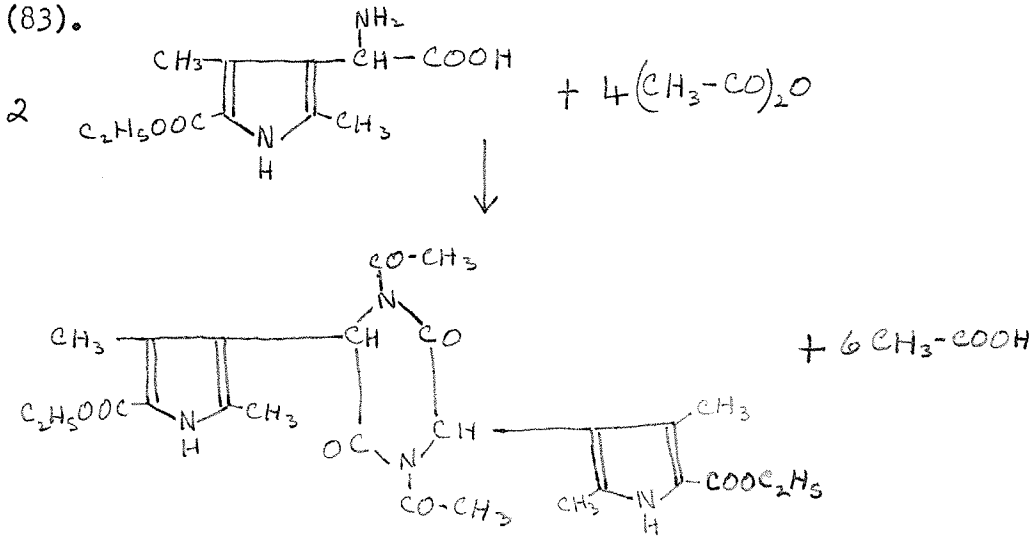
$\xrightarrow{2\,CH_3SNa}$



$+ 2NaBr$


+ 2 H2O
heat
+ 2 HBr

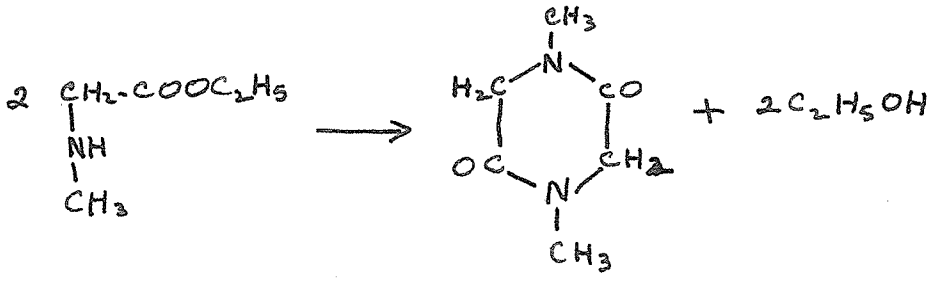
H. Fischer, Neumann and Hirschbleck heated <u>α-amino-2,4-dimethyl-5-carbethoxy-3-pyrroleacetic acid</u> with acetic anhydride, obtaining the corresponding 1,4-diacetyl anhydride (83).



<u>Sarcosine anhydride.</u>

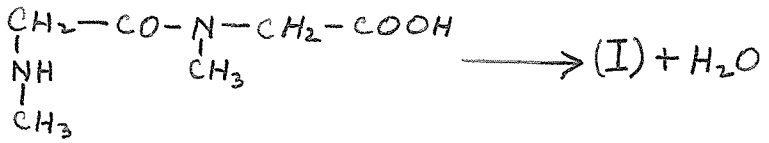
Siegmund and Liedl prepared this compound by adaptation of the methods of Fischer and Abderhalden (85):-

(i) By heating sarcosine ethyl ester in a bomb tube at 170° for 20-24 hours.

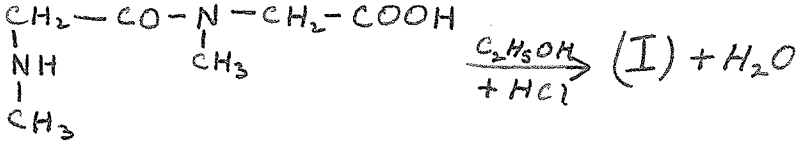


(I)

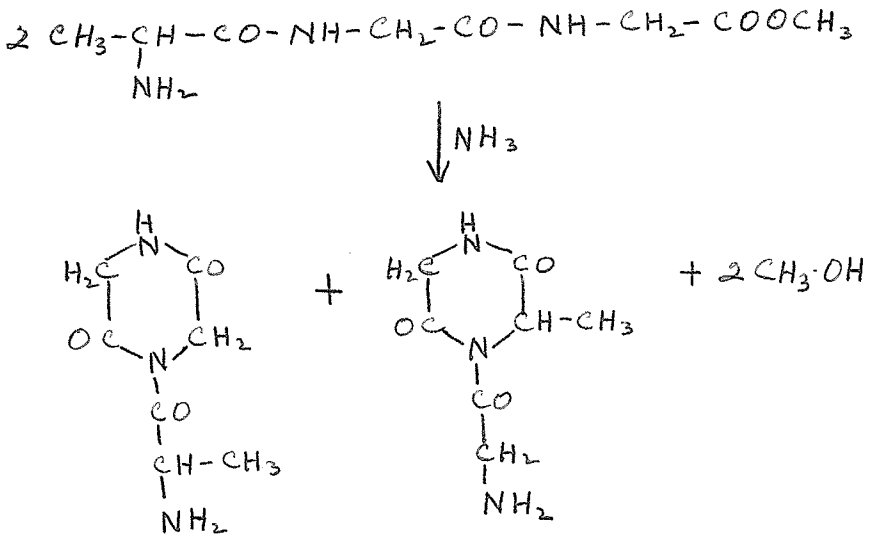
(ii) By subliming sarcosylsarcosine under vacuum at a temperature below its melting point.

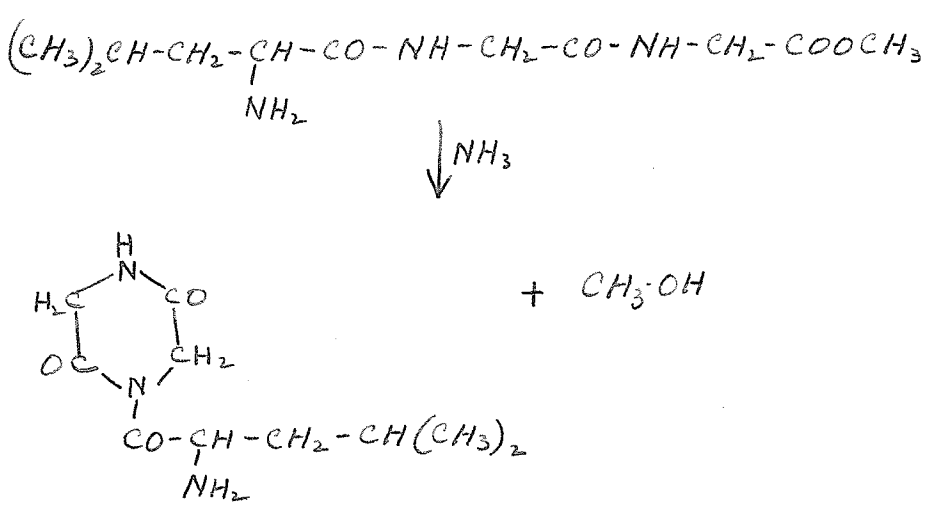


(iii) Attempts to esterify sarcosylsarcosine by means of ethyl alcohol and hydrogen chloride were unsuccessful, the anhydride being obtained instead.



Abderhalden and Schwab reported preparations of diketopiperazines having an amino acid residue linked to one of the nitrogen atoms of the ring (86). Conversion of DL-alanylglycylglycine to its methyl ester, followed by the action of ammonia in methyl alcohol, was reported as forming DL-alanyl(glycylglycine) anhydride and glycyl(glycylalanine) anhydride; similarly, DL-leucylglycylglycine was reported to form DL-leucyl(glycylglycine) anhydride.

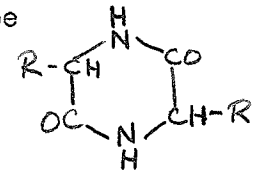




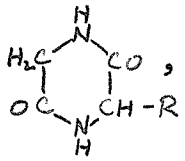
## II. Hydrolysis of 2,5-Diketopiperazines.

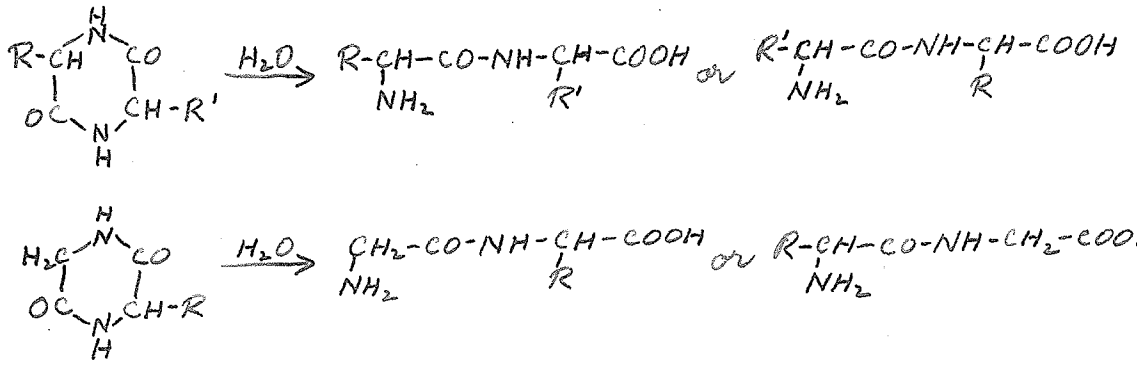
Emil Fischer, Abderhalden, Bergmann, and Levene have each contributed to information derived from studies on the hydrolysis of diketopiperazines by the action of acids and of alkalis. Other investigators who have either made a study of the effect of such reagents on specific diketopiperazines, or made use of their reaction, are (i) Euler and Petterson, (ii) Dakin, (iii) Ravenna and Bosinelli, (iv) Sasaki, (v) Kurt Meyer, (vi) Siegmund and Liedl, (vii) Kawai, (viii) Sreenivasaya, (ix) Yaichnikov and Spiridinova, (x) Snyder (xi) Fraenkel-Conrat, Cooper and Olcott, (xii) Parskin and Nikolaeva, and (xiii) Lichtenstein.

The hydrolysis of 2,5-diketopiperazine might yield the dipeptide glycylglycine or the amino acid glycine. Similarly a substituted diketopiperazine of the type



where the R-groups are identical, might yield the corresponding dipeptide $R\text{-}\underset{\underset{NH_2}{|}}{CH}\text{-}CO\text{-}NH\text{-}\underset{\underset{R}{|}}{CH}\text{-}COOH$ or the amino acid $R\text{-}\underset{\underset{NH_2}{|}}{CH}\text{-}COOH$. Where the R-groups are not identical, one dipeptide might be formed, or a mixture of two dipeptides, or a mixture

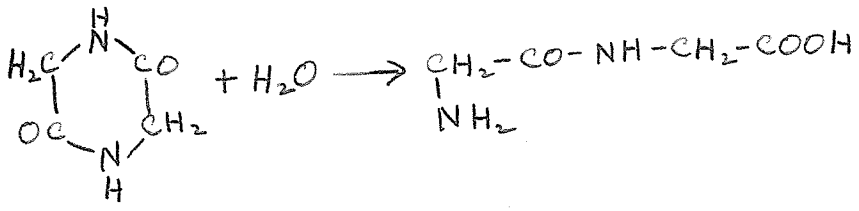
of the two amino acids. A similar possibility might result from the hydrolysis of diketopiperazines of the type  , and equations for such possible reactions are given below.



Some information on these possible reactions has been submitted in the published work of the above mentioned investigators, although in most cases they merely report the actual products obtained when hydrolysis of a specific diketopiperazine does occur under the conditions used. In this review a summary of work carried out by these workers is made.

The work of Emil Fischer.

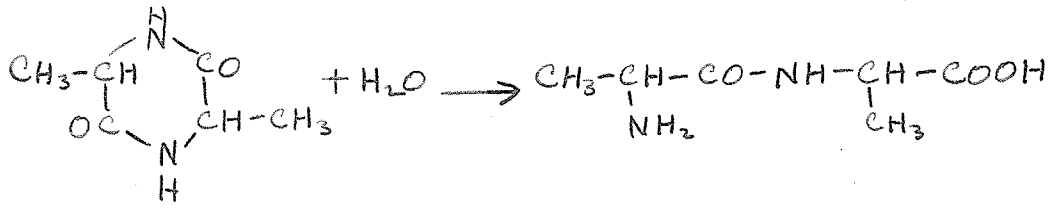
Fischer and Fourneau found that 2,5-diketopiperazine was hydrolysed to glycylglycine by concentrated hydrochloric acid, either by allowing the anhydride to stand in the acid, or by heating with the acid (2). Fischer found that hydrolysis occurred more readily with N. NaOH at ordinary temperature (23).



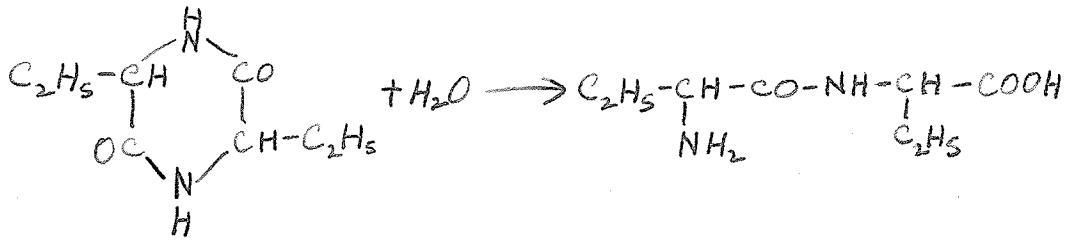
In submitting 3,6-dimethyl-2,5-diketopiperazine to the action of alkali, alanylalanine was obtained, details being as under:-

The anhydride derived from D-alanine was hydrolysed to a mixture of D-alanyl-D-alanine and DL-alanyl-DL-alanine (27). The anhydride derived from DL-alanine was hydrolysed by NNaOH to DL-alanyl-DL-alanine (28).

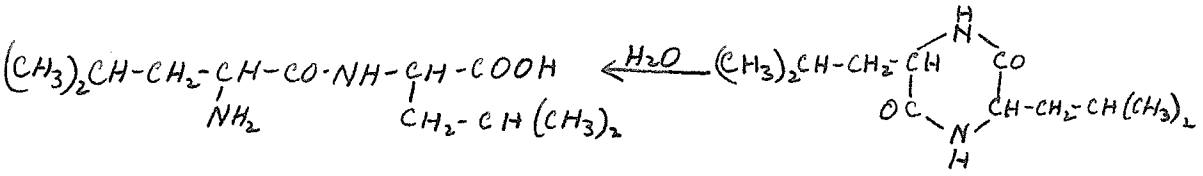
The anhydride derived from DL-alanine hydrolysed at room temperature to DL-alanyl-DL-alanine by the action of 0.4NNaOH (23).



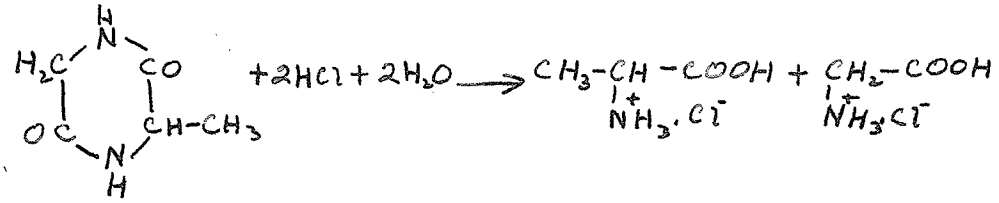
3,6-Diethyl-2,5-diketopiperazine yielded ∝-aminobutyryl-∝-aminobutyric acid when warmed with dilute alkali at 37° C (28).



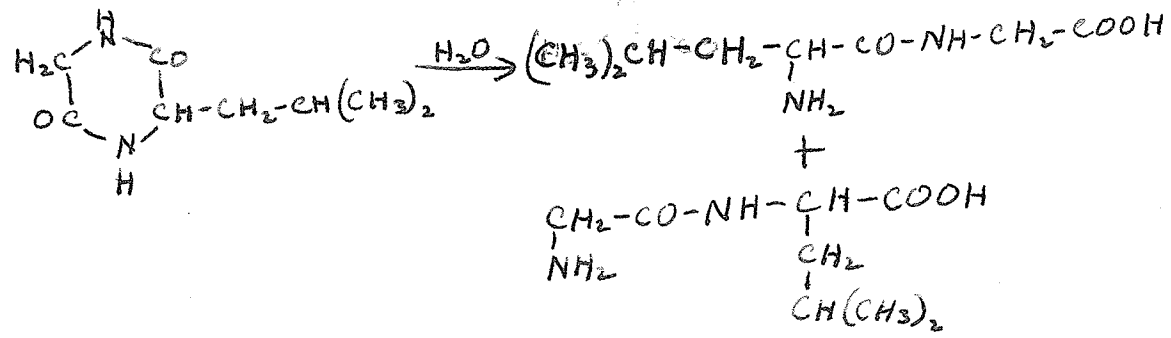
Fischer found 3,6-diisobutyl-2,5-diketopiperazine, derived from DL-leucine, very resistant to the action of alkali (23), and in order to effect hydrolysis he found it necessary to heat the anhydride with hydrobromic acid at 100° in a sealed tube; the product was DL-leucyl-DL-leucine (42).



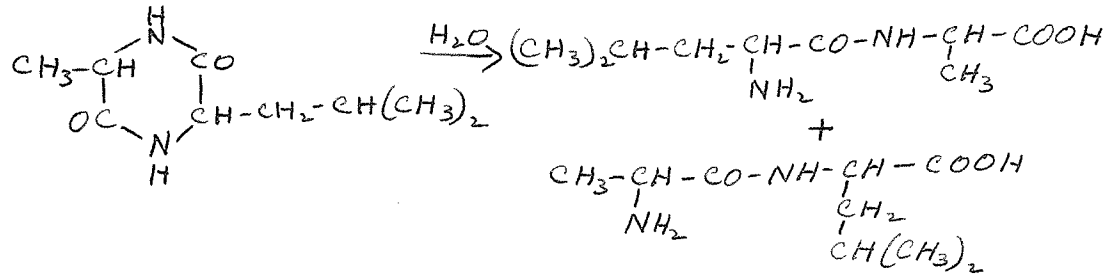
3-Methyl-2,5-diketopiperazine was hydrolysed to alanine hydrochloride and glycine hydrochloride when Fischer and Abderhalden heated it at 100° in a sealed tube with hydrochloric acid (11).



3-Isobutyl-2,5-diketopiperazine, derived from DL-leucylglycine, was quite difficult to hydrolyse with alkali. Fischer and Schrauth found that prolonged shaking with dilute alkali over a long period of time was necessary to effect opening of the ring, and both dipeptides were obtained (87).

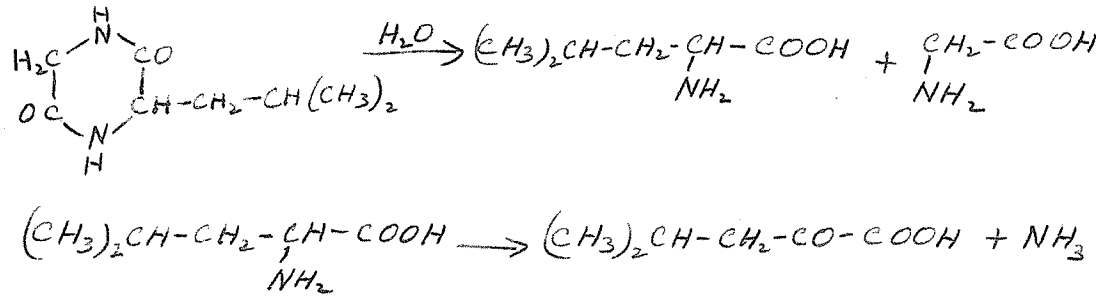


3-Methyl-6-isobutyl-2,5-diketopiperazine was also difficult to hydrolyse with alkali, and when warmed with dilute sodium hydroxide at 37° C, several days were required for the reaction to take place. Fischer and Schrauth reported the products to be a mixture of DL-leucyl-DL-alanine and DL-alanyl-DL-leucine (87).



The Work of Abderhalden.

Abderhalden and Rossner described 3-isobutyl-2,5-diketopiperazine as being resistant to hydrolysis by NNaOH (88), (cf. Fischer, p. 53), while treatment with 25% sulphuric acid gave not dipeptides, but a mixture of glycine, ammonia, and $\alpha$-ketoisocaproic acid. Presumably DL-leucine was formed initially, and then underwent oxidative deamination to the $\alpha$-ketonic acid.



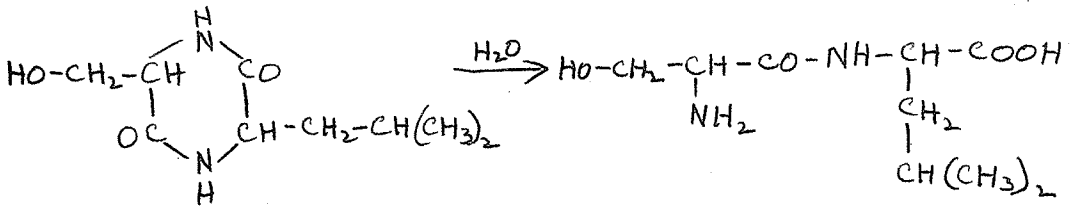
An investigation into the problem of possible hydrolysis of dipeptides into amino acids occurring, as soon as the dipeptide was formed on opening of the ring, was made (89). Conclusions were drawn that in individual cases, opening of the diketopiperazine ring took place much more readily than the further hydrolysis of the dipeptide, and that opening of the ring could take place at pH values of the same order as those found in biological fluids. In the case of 2,5-diketopiperazine, the 3-methyl-compound, and the 3,6-dimethyl-compound, partial hydrolysis occurred at room temperature when these anhydrides were allowed to stand in a solution with an initial pH of 11.40. Equilibrium was reached after 160 hours, when the pH was found to have dropped to a value of 8.50 (cf. Yaichnikov and Spiridonova, p. 62 ).

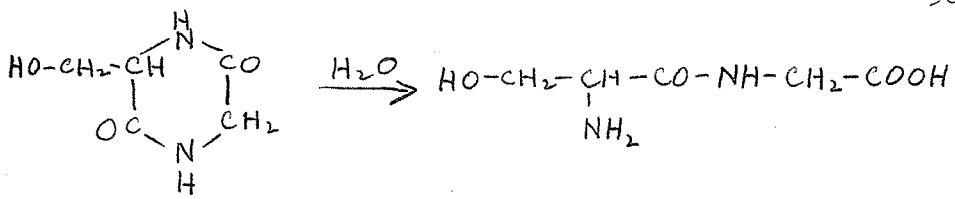
Abderhalden and Bahn found results of considerable interest from a study of the hydrolysis of some mixed diketopiperazines derived from serine (59). (cf. Bergmann, p. 56 ). Three such diketopiperazines were hydrolysed with 10% sulphuric acid solution:-

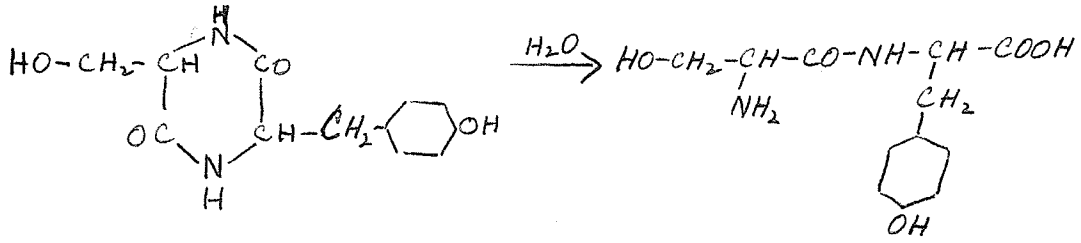
DL-leucyl-DL-serine anhydride formed DL-seryl-DL-leucine.

glycyl-DL-serine anhydride formed DL-serylglycine.

L-Tyrosyl-L-serine formed L-seryl-L-tyrosine.

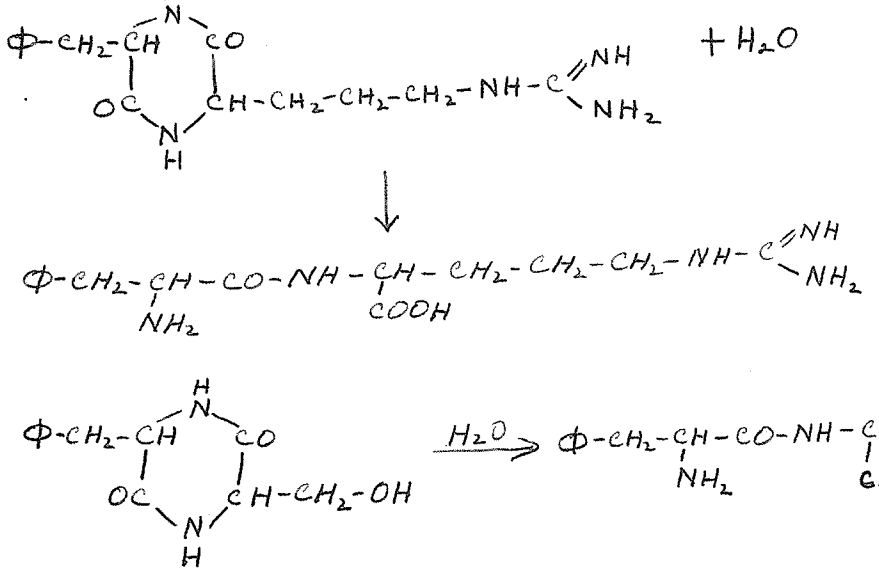






The Work of Bergmann.

Bergmann and Mickely studied the effect of hydrolysis on two diketopiperazines derived from phenylalanine, namely phenylalanylserine anhydride (60) and phenylalanylarginine anhydride. The latter, on standing in water for five days gave a 90% yield of phenylalanylarginine, and the former was hydrolysed to phenylalanylserine by shaking with barium hydroxide solution for 24 hours (cf. Abderhalden above).

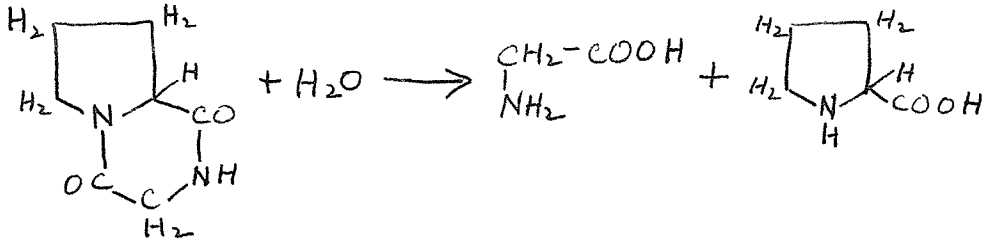


The work of Levene.

When protein hydrolysis is carried to completion, a mixture of amino acids is obtained. Most of the amino acids have at least one asymmetric carbon in the molecule, namely the $\alpha$-carbon atom; a notable exception is glycine. All the other amino acids have the L-configuration on the $\alpha$-carbon atom when the hydrolysis is effected by means of acids or enzymes. Alkali hydrolysis, however, produces the DL-amino acids. This is attributed to racemisation of the $\alpha$-carbon atom of the respective amino acids, during the hydrolysis, by the alkali.

A considerable portion of Levene's career was devoted to racemisation studies, some of which were made on 2,5-diketopiperazines, not necessarily for purposes of hydrolysis, but he and his co-workers did carry out a number of such hydrolyses as summarised below.

In a study of the diketopiperazine derived from glycyl-L-proline, Levene and Beatty found that glycine and L-proline were produced when the anhydride was heated at 150° with concentrated hydrochloric acid (54).



Reference has already been made to the experience of Fischer and of Abderhalden, in so far as finding that not all diketopiperazines are hydrolysed with equal facility, particularly with alkali (23,42,87,88). Levene, Bass and Steiger sought to find what conclusions could be drawn from the effect of structure of the substituted diketopiperazines on rate of hydrolysis (90). In this work they compared the rates of hydrolysis of 2,5-diketopiperazine (glycine anhydride), 1-methyl-2,5-diketopiperazine (glycylsarcosine anhydride), 1,4-dimethyl-2,5-diketopiperazine (sarcosine anhydride), 1,4-dimethyl-3-methyl-2,5-diketopiperazine (N-methylalanylsarcosine anhydride), and 1,4-dimethyl-3-isopropyl-2,5-ketopiperazine (N-methylvalylsarcosine anhydride), in a medium of pH 13.4.

The first three of the compounds listed were hydrolysed rapidly, glycine anhydride more rapidly than glycylsarcosine anhydride and sarcosine anhydride. The N-methyl groups of the latter two seemed to have the effect of lowering the rate of hydrolysis, a conclusion which seemed well supported by the observation that N-methylalanylsarcosine anhydride and N-methylvalyl sarcosine anhydride were hydrolysed much more slowly.

In another investigation, Levene and associates carried out studies on the rate of hydrolysis of the following diketopiperazines in 0.5N NaOH at 25° (91):-

(i) glycyl-D-alanine anhydride

(ii) glycyl-L-asparagine anhydride

(iii) glycyl-DL-valine anhydride

(iv) glycyl-D-isovaline anhydride

(v) glycyl-L-leucine anhydride

(vi) glycyl-L-phenylalanyl anhydride

(vii) glycyl-D-phenylalanine anhydride

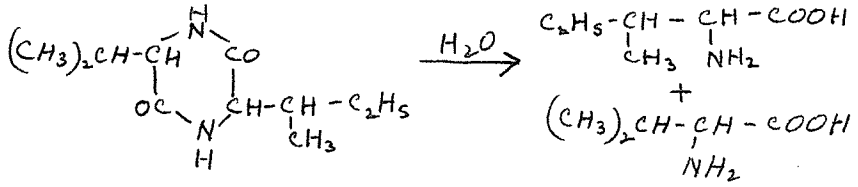
(viii) glycyl-D-tyrosine anhydride

No attempt was made to identify hydrolysis products, and they assumed that the reaction did not proceed beyond the stage of dipeptide formation. They concluded that (i) was much more easily hydrolysed than the remainder, and of the remainder, (ii) and (v) were the most rapidly hydrolysed, (vii) seemed less stable than (viii), and (iv) was the least readily hydrolysed of all.

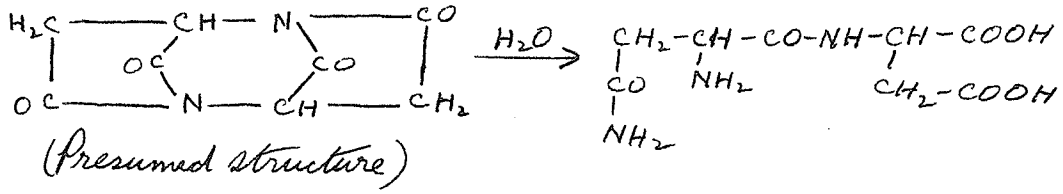
Other Workers.

Euler and Petterson (92) carried out a study of the susceptibility of glycine anhydride to acid and alkali, using solutions of pH range 0.10-9.96 at 60°. They found that the rate of hydrolysis was least at pH 4.9, and increased more rapidly with increase of pH than with decrease of pH from this value.

Dakin found 3-isopropyl-6-sec.butyl-2,5-diketopiperazine resistant to hydrolysis by dilute acids and alkalis, due to inability to effect solution. Hydrobromic acid (specific gravity 1.48) however hydrolysed the compound completely to D-isoleucine and D-valine (93).

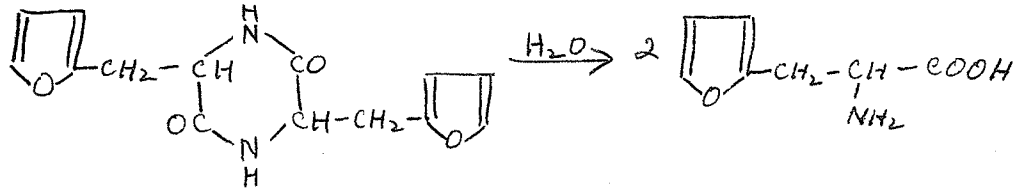


Ravenna and Bosinelli (72) obtained asparagylaspartic acid when the compound they named 2,5-diketopiperazinediacetic anhydride was hydrolysed in the cold with barium hydroxide solution.

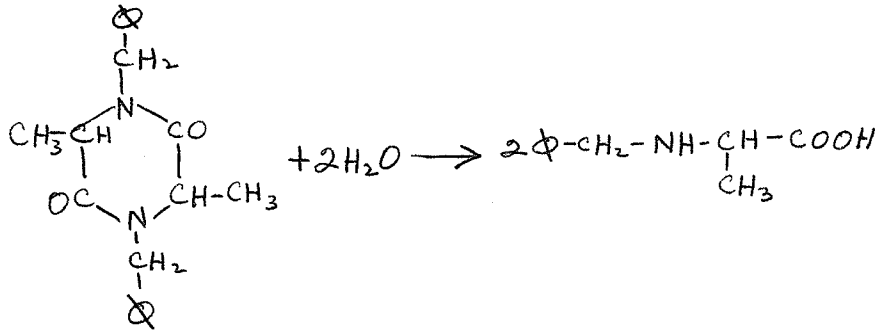


(Presumed structure)

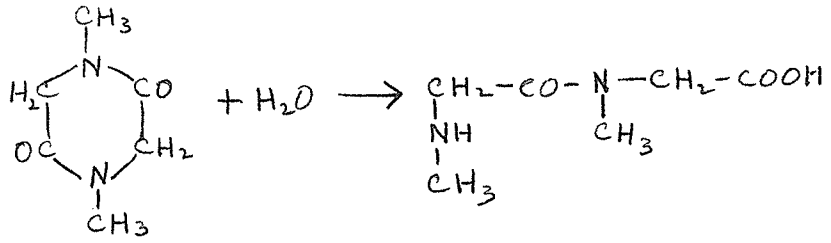
Sasaki hydrolysed furylalanine anhydride to furylalanine by boiling with barium hydroxide solution (69).



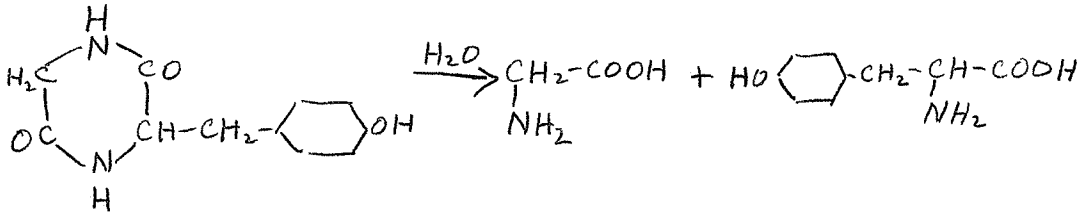
Kurt Meyer and Mark obtained N-benzylalanine when 1,4-dibenzylalanine anhydride was boiled with concentrated hydrochloric acid for 12 hours (94).



Siegmund and Liedl found that the best procedure for hydrolysing sarcosine anhydride to sarcosylsarcosine was to warm with barium hydroxide at 30°. A yield of 70% was obtained (85).



Kawai accomplished hydrolysis of glycyl-DL-tyrosine anhydride by refluxing with 25% sulphuric acid solution for 14 hours (95).



Srinivasan and Sreenivasaya

Like Abderhalden, these investigators made a study of the effect of alkali concentration and of the possibility of the resulting dipeptide being hydrolysed to amino acids (96). Their findings were as follows:-

Glycine anhydride was hydrolysed to glycylglycine by dilute alkali; if the alkali was stronger than 0.01N, the dipeptide was hydrolysed to glycine.

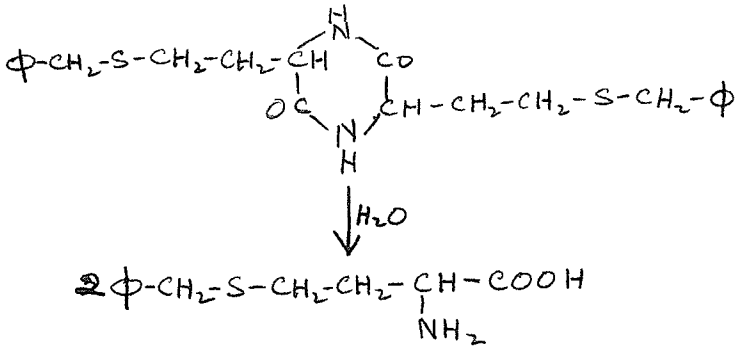
Alanine anhydride was likewise hydrolysed to alanylalanine, but the latter was not hydrolysed under the same conditions.

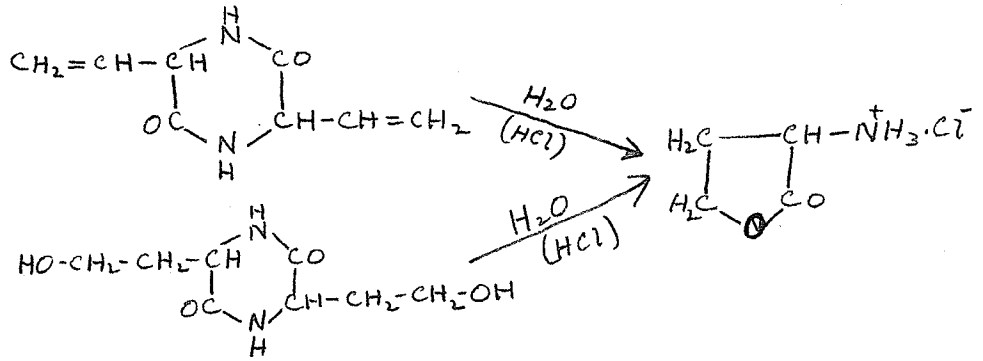
Yaichnikov and Spiridonova studied the hydrolysis of glycine anhydride by N HCl at 15° and 95°. The progress of the reaction was followed by taking samples at intervals of hours and days, keeping the volume constant by adding water. The samples were neutralised with 0.2N NaOH and titrated by Sorensen's formol method (97). They concluded that:-

At 15°-- hydrolysis to the dipeptide was rapid during the first 15 days, reaching completion in 25 days.

At 95°-- Hydrolysis to the dipeptide was complete in one hour. The dipeptide was hydrolysed to the amino acid, but more slowly, and incompletely. At advanced stages, the volume of NaOH necessary to neutralise the samples began to decrease, seemingly an indication that the reaction was being reversed.

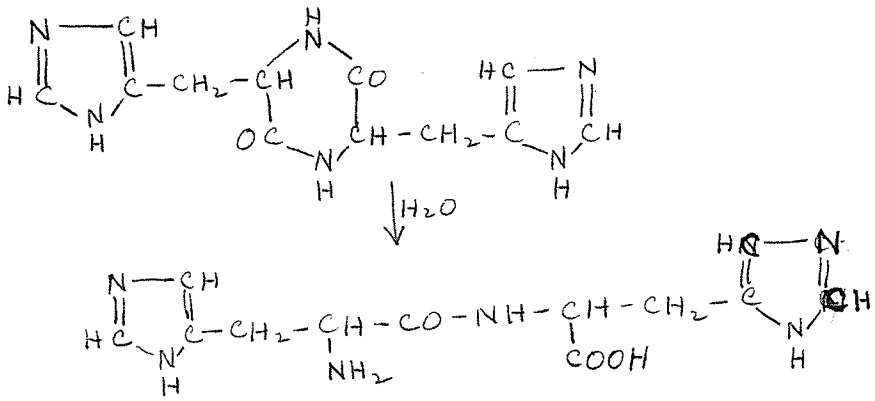
Snyder and Chiddix obtained DL-S-benzylhomocysteine by refluxing the corresponding diketopiperazine with hydrochloric acid for 7 hours (81). They also prepared ∝-amino- ɣ - butyrolactone hydrochloride by heating 3,6-divinyl-2,5-diketopiperazine on a steam cone with 20% hydrochloric acid, and by refluxing 3,6-bis(2-hydroxyethyl)-2,5-diketopiperazine with concentrated hydrochloric acid for $2\frac{1}{2}$ hours (82).



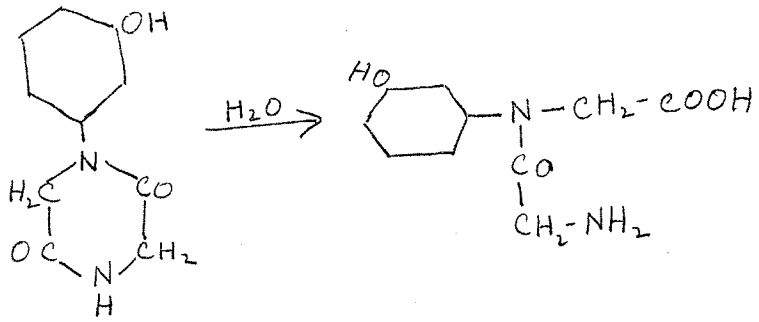


Fraenkel-Conrat, Cooper and Olcott studied the effect of 5NHCl on 3,6-dicarbanilyl-2,5-diketopiperazine. They found the anhydride quite resistant to the reagent at 120°, but hydrolysis did occur at 150° (98). They did not report identification of products beyond stating that aniline was formed in the hydrolysis.

Parshin and Nikolaeva studied the hydrolysis of the diketopiperazine derived from L-histidine to the dipeptide by acids and alkalis. They found $\frac{N}{2}H_2SO_4$ at 37° satisfactory, but $\frac{N}{2}$NaOH was less convenient, because it gave free histidine and unchanged anhydride (99).



Lichtenstein and his associates obtained N-glycyl-N-(m-hydroxy)phenylglycine when L-(m-hydroxyphenyl)-2,5-diketopiperazine was kept at 30° for 3 days in $\frac{N}{2}$NaOH (100).



## III. Possible Relation of 2,5-Diketopiperazines to the Study of Proteins.

Much controversy has raged as to whether 2,5-diketopiperazine rings (as such, or in some modified form) constitute part of the molecular structure of proteins. Many experimental findings have been presented which were claimed as evidence in favour of the theory, and many which were claimed as evidence to the contrary. It can be truthfully stated that the net result of all such investigations is such that no conclusive proof, either in favour or to the contrary, is available. In more recent years, however, it might be true to say that the general consensus of opinion disregards the theory that the ring forms part of protein molecules. This disposition is based on the fact that, while the presence of the ring in proteins may not have been conclusively disproved, no convincing proof of its presence has as yet been demonstrated.

The first suggestion that the diketopiperazine ring was an integral part of the protein molecule was made by Emil Fischer. In hydrolysing horse haemoglobin with trypsin and pepsin, 3,6-diisobutyl-2,5-diketopiperazine was one of the products obtained. Fischer concluded that the diketopiperazine ring must therefore be present in the original protein molecule (42).

Much later, Abderhalden commenced a long series of researches to determine whether definite proof could be obtained for this theory.

The Work of Abderhalden.

Initially he sought means of distinguishing between polypeptides and diketopiperazines in protein hydrolysates (102). He found that 2,5-diketopiperazines could be reduced to the corresponding piperazines, whereas polypeptides were unaffected under the same experimental conditions. When Abderhalden applied this fact to silk fibroin partial hydrolysate, he was able to isolate 2-methylpiperazine. From this observation he concluded that 3-methyl-2,5-diketopiperazine was present in the fibroin molecule.

Whatever doubt may exist about the latter diketopiperazine being part of the fibroin molecule, no doubt could exist about its presence in the hydrolysate. Abderhalden isolated the anhydride after silk fibroin had been left standing in contact with 70% sulphuric acid at 37° for three days (103). Having isolated the compound, Abderhalden made a study of its behaviour towards a hydrochloric acid solution of pepsin, and a sodium carbonate solution of trypsin. At any given time, the extent of hydrolysis was no greater than when hydrochloric acid alone or sodium carbonate alone had been used.

Reference has already been made in the literature review to the isolation of some 2,5-diketopiperazines when hog bristles were heated in an autoclave with 2% hydrochloric acid, and when blood albumin was heated with water in an autoclave (14).

Abderhalden did express the opinion in this work that the ordinary anhydrides, comprising two amino acid residues, were formed during the hydrolysis by secondary reaction from intermediate polypeptides. As supporting evidence for this belief, he cited the conversion of polypeptides to anhydrides by heating in an autoclave.

When a number of 2,5-diketopiperazines were heated with a solution of picric acid in sodium hydroxide, a mahogany-red colour developed; peptides and amino acids did not give this reaction (104,105). The reaction was positive when applied to peptones and to most proteins. Abderhalden concluded that these results were a clear demonstration of the presence of 2,5-diketopiperazine rings in the protein molecules, the anhydrides being in the keto form.

A further attempt was made to support the theory in a series of oxidation studies performed on silk peptone, some 2,5-diketopiperazines, and some dipeptides (106). These were all treated with zinc permanganate solution. The results are summarised below:-

2,5-diketopiperazines $\xrightarrow{O}$ oxamide.

Dipeptides $\xrightarrow{O}$ an unidentified product (I), but no oxamide.

Silk peptone $\xrightarrow{O}$ oxamide + (I).

Abderhalden regarded these results as good evidence for the theory, certainly for the structure of silk peptone. The fact that egg albumin and casein both gave oxamide and (I), while gelatin and serum globulin each formed oxamide only, appeared to be still further supporting evidence. It should be stated, however, that in a later paper (107) in which several dipeptides were shown as giving no oxamide, three polypeptides containing glycylglycine residues did produce some oxamide along with (I).

On page 55 , in the review of the literature on diketopiperazine hydrolysis, mention was made of Abderhalden's study of ring opening and dipeptide splitting when the anhydrides were placed in a solution of pH 11.40 at room temperature. He found similar behaviour for silk peptone and casein (88). The picric acid test was positive at first, gradually diminishing in intensity (104,105). While adducing this behaviour as one more supporting point for the theory, he did say that in some proteins the ring might revert to a more labile modification. Furthermore, stability of a protein would be dependent on the nature of the amino acid residues comprising the ring. Such opinions were expressed to account for the fact that albuminoids, such as keratin and fibroin, were less susceptible to hydrolytic action by enzymes than were other types of protein.

In an attempt to elucidate to some extent the steps involved in protein hydrolysis, Abderhalden studied the action of NHCl

and of N/NaOH on elastin and gelatin at various temperatures. He formed the opinion that polypeptides were produced first, and the amino acids later. This investigation did not give any indication of diketopiperazine formation. Nevertheless, still adhering to the belief that the rings were part of the structure, Abderhalden concluded that the study of the action of acid and alkali gave no insight into the behaviour of such rings (101).

It was inevitable that the behaviour of diketopiperazines in presence of proteolytic enzymes should be investigated, in view of the problem they presented concerning protein studies. As a first step in this direction, Abderhalden and Schwab subjected the product which was believed to be DL-leucyl(glycine anhydride) to the action of trypsin and erepsin. Hydrolysis was reported to occur, leucine being split off by hydrolysis (108).

Attempts were also made to ascertain if the behaviour of diketopiperazines in the animal body could give more tangible evidence of their significance in relation to proteins. Abderhalden fed DL-leucylglycine anhydride and glycyl-D-alanine anhydride to dogs. The former appeared in the blood stream after 80 minutes, showing that no hydrolysis had occurred in the digestive tract. The maximum concentration was reached in the blood stream after 5 hours, and the substance had disappeared after a total of eight hours after feeding. Eighty per cent

of the anhydride was recovered from the urine, and none was found in the faeces.

The latter of the two anhydrides, however, gave the impression of having been utilised to a much greater extent (it must be borne in mind, as Abderhalden pointed out, that it is more sensitive to the action of alkali). The urine contained glycylalanine, but no anhydride (109).

Despite these results, Abderhalden still upheld the possibility--if not, in his view, probability--of the anhydride ring system being present in food protein. To justify the theory against the background of the results, he suggested that linkage of the rings to other amino acids might modify the ring system so much, that on splitting off side chains, the ring would be ruptured to form dipeptides.

Further work was instituted to determine whether the presence of amino acid residues on the imido nitrogen atom of the ring would confer greater susceptibility to enzyme action (110). 0.1N NaOH, 5N NaOH trypsinkinase, and erepsin were all used on the anhydrides examined. In each case a tripeptide was formed. Abderhalden submitted these results as strong evidence in support of the theory. The results appeared to him to meet the objection that ordinary diketopiperazines were resistant to enzyme action.

Shibata and Tazawa had stated that pepsin should be capable of bringing about hydrolysis of diketopiperazines derived from basic amino acids (lysine, arginine, histidine). Those derived from acidic amino acids (aspartic, glutamic) on the other hand, should be hydrolysed by the enzymes trypsin, papain, cathepsin. Abderhalden, Weichert and Haase repeated this work of Shibata, and extended it to a wider range of anhydrides. The enzymes used were dipeptidase, polypeptidase, trypsinkinases and pepsin (64).

Despite a wide variation of experimental conditions, Abderhalden obtained nothing but negative results. Repetition seemed to indicate hydrolysis of glycyl-L-glutamic anhydride by trypsinkinase. The extent of hydrolysis, however, only seemed to proceed as far as 15-20% of original substrate. Further repetition on carefully purified materials gave completely negative results. The decomposition previously observed must have been due to the presence of impurities. From these experiments, Abderhalden concluded that no very definite evidence existed to show that anhydrides derived from acidic amino acids could be split by pepsin preparations from different sources.

Finally, in experiments where a wide variety of diketopiperazines were fed to rats and rabbits, all the anhydrides were recovered unchanged from the urine (111).

The Work of Bergmann.

Bergmann proposed a theory that the diketopiperazine ring underwent some changes, and that proteins were built up of complex cyclic structures involving secondary as well as primary valences. Mixed crystals were also postulated as being involved (112).

In an effort to gain an insight into the structure of the fish protein clupeine, Bergmann, Zervas and Köster sought to determine whether the arginine component was in diketopiperazine form (113). They made a separate study of D-phenylalanyl-D-arginine anhydride. This was found to racemise very rapidly in solution, and to undergo autohydrolysis to the corresponding anhydride, which itself did not racemise in solution.

Clupeine, under the same conditions, was found to show a much lesser degree of racemisation. Bergmann felt certain, therefore, that the arginine in clupeine was not in the form of an anhydride. Some significance was attributed, however, to the fact that some racemisation of the protein did take place, whereas the dipeptide mentioned above was not racemised.

The Work of Gavrilov.

This investigator has shown much interest in the possibility of diketopiperazines being part of the protein molecule.

From a standpoint favouring the theory, he stated that the extent to which they would be present in any given instance varied according to the type of protein, being greatest in gelatin, least in sturine (114). He stated that hydrolysis of gelatin by autoclaving at 180° required at least 24 hours, whereas the amino nitrogen content of the protein hydrolysates was the same at the end of 9 hours as at the end of 3 hours. Diketopiperazines appear to be more difficult to hydrolyse as the molecular weight increases; he attributed the longer reaction time for gelatin therefore, as being due to slower attack on the diketopiperazine rings which he thought would inevitably be part of the gelatin molecule.

He went on further to state that dipeptides formed in hydrolysis did not originate from opening of the anhydride ring. The latter, he claimed, did not form dipeptides, but in a neutral solution any dipeptide produced from breakdown of longer chains (or other structures) would form systems of type dipeptide: anhydride.

In a later report, Gavrilov stated that when hydrolysates produced by enzyme action (pepsin, trypsin) are heated in an autoclave, diketopiperazines are not formed. From such observations, he suggested enzyme hydrolysis breaks down both polypeptide and diketopiperazine structures (115).

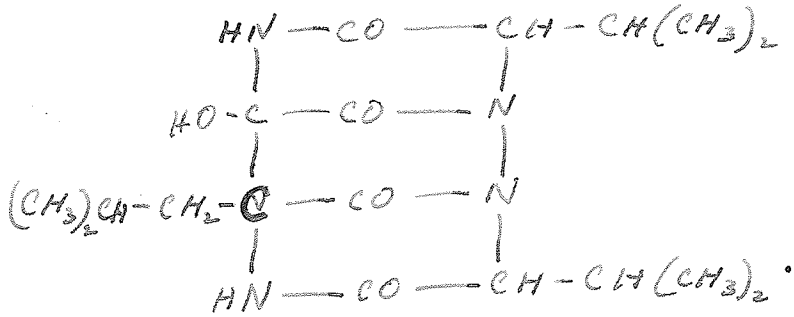
Experiments were also carried out on collagen, in which this

protein was heated at 180° with 1.5% sulphuric acid under a pressure of 10 atmospheres (116). From examination of the hydrolysate, Gavrilov drew two conclusions, that collagen had more diketopiperazine rings in its structure than gelatin has (117), and that two types of such rings were derived from phenylalanylproline and phenylalanylglycine.

Gavrilov took issue with the claims made by Abderhalden (111) and Waldschmidt-Leitz (133) that ordinary diketopiperazines were not attacked by proteolytic enzymes. He maintained that after heating with aniline, the anhydrides became more susceptible to the action of trypsinkinase, but not to trypsin or erepsin (118).

Other Workers.

Sadikov and Zelinskii isolated diketopiperazines from goose feathers (37). They reported that diketopiperazines could be converted by cold concentrated hydrochloric acid to a more complex ring structure which they called dipeptines. For example, valylleucine anhydride formed a product which they judged to have the structure



Some of the anhydrides which they isolated from the hydrolysate were related to proline. They concluded that the protein of goose feathers consisted of peptines and proline rings, together with other complex cyclic structures of unknown character.

Goldschmidt and Steigerwald tried to obtain a more intimate knowledge of protein structure by treating various proteins with sodium hypobromite solution at $0^{\circ}$ (119). The reagent was reported as reacting primarily with protein, and secondary reaction with products of alkaline hydrolysis was discounted as unlikely. They felt confident that few amino groups were present in whole protein, and that the conditions were so mild that any possibility of oxidation or nuclear halogenation was not likely to be significant. The peptide linkages were known not to be affected, but diketopiperazines reacted rapidly. The proteins underwent degradation. The degradation was interpreted as being due to diketopiperazine rings.

Levene, like Abderhalden, favoured the anhydride theory of protein structure, and tried to prove it (120,7). Reference has already been made to the reported conversion of dipeptides to amino acids and anhydrides by erepsin.

Pfeiffer and Angern also tried to uphold the theory. They reported that amino acids, polypeptides, and diketopiperazines formed complexes under the influence of neutral salts. Such findings indicated to them that these compounds must possess

residual valences; they expressed the opinion that the same residual valences might play an important part in the building up of proteins (121).

Dakin sought without success to demonstrate the presence of diketopiperazine rings in gelatin (122). He tried to make use of Sasaki's condensation of aromatic aldehydes with glycine anhydride (68). He found, however, that not only gelatin (rich in glycine) but other proteins containing no glycine reacted with benzaldehyde. The condensation products failed to give the biuret reaction, and benzaldehyde could not be liberated from them.

Wrede and Brusch departed from the usual hydrolytic method of protein degradation. They acetylated proteins first, then reduced the products (123,124). Among reduction products they identified 2-methylpiperazine and 2-isopropylpiperazine. These were presumed to have been formed from glycylalanine anhydride and glycylvaline anhydride, which they felt must be present in the original structure.

In a discussion of what might be a possible structure of silk fibroin, Kurt Meyer and Mark (94) visualised the molecule as consisting of four principal valence chains of glycylalanine residues; these four chains were visualised as having a spiral shape. They speculated that glycylalanine anhydride could assume a form whose shape and size would be in close agreement with the data which seem to be pertinent to the silk fibroin.

Kuzin and Polyakova studied the effect of formaldehyde, acetaldehyde and acetone on the conversion of glycine ethyl ester to 2,5-diketopiperazine (125). These did not appear to have any effect. However, the yield was almost doubled when, in place of these carbonyl compounds, glucose, fructose or galactose was used, all of which have a potential carbonyl group in their structures. Compounds closely related to these simple sugars but having no potential carbonyl group (e.g. mannitol), were found to have no effect.

They concluded that the potential carbonyl group of the simple sugars activated the hydrogen of the amino group in the ester, forming an unstable intermediate. Such formation, they argued, would accelerate condensation of the ester. They proposed the theory that in living matter similar conditions would bring about formation of peptides and diketopiperazines.

The remainder of this section of the literature review embraces work carried out by various investigators in which the effect of enzymes on diketopiperazines has been studied. Studies in this category can be classified in two main groups;-

(i) Experiments in which positive results were claimed. Such results were either presented by the investigator as evidence in support of the diketopiperazine theory of protein structure, or could be cited as such by anyone favouring the theory.

(ii) Experiments producing negative results. These results were either submitted as disproving the theory, or could be used for this purpose. In one such study, however, the author of the paper did not regard his negative results as necessarily disproving the theory; he merely stated that extreme caution must be exercised before applying the anhydride theory to protein structure.

(i) Experiments in which 2,5-diketopiperazines were claimed to have been hydrolysed by proteolytic enzymes.

Blagowestschenski reported that enzymes prepared from beans were capable of synthesising diketopiperazine derivatives from amino acids. Powdered seeds of Phaseolus Mungo, L. were allowed to stand in glycerol solutions of leucine or glycine in presence of toluene. The amino nitrogen content decreased over a four week period, and in the case of leucine a crystalline material reported as resembling 3,6-diisobutyl-2,5-diketopiperazine was extracted from the solution (126).

Blanchetière published an account of formation of diketopiperazines when natural and denatured ovalbumin were subjected to hydrolysis by trypsin. They were not formed when hydrolysis was effected by pepsin (127). He also studied the action of pepsin and of trypsin on the gliadin of wheat; his conclusions were that diketopiperazine formation occurred to a much greater

extent from gliadin than from albumin (128).

Shibata and Tazawa (129) claimed that hydrolysis of diketopiperazines by proteolytic enzymes was dependent on enzyme concentration, Abderhalden repeated and extended this work and obtained completely negative results. Fuller details of Abderhalden's contradiction of Shibata's work were outlined on p. 71 of this review.

Tazawa extended this work to a study of the effect of crystalline trypsin on glycyl-D-glutamic anhydride. He reported that this compound was hydrolysed by trypsin, and that the hydrolysis was not affected by the presence of other amino acid derivatives (130). In another publication he contradicted Abderhalden's negative results on the action of enzymes on L-histidine anhydride (131). Tazawa said that the compound was hydrolysed by pepsin at 37°, but not by trypsin or papain. As a check to ensure that the hydrolysis by pepsin was not caused solely by the hydrochloric acid but by the enzyme, he made a separate study of the action of the acid on the compound at 37°. The anhydride was not hydrolysed by the acid, so the hydrolysis appeared to be due to the pepsin.

Ishiyama exposed three acidic anhydrides (glycylaspartic, glycylglutamic, and aspartic) to the action of trypsin (132). All three were reported to be hydrolysed, especially when the trypsin had been activated, but other proteolytic enzymes had no effect. Glycylglutamine and pyrrolidone anhydrides he found very resistant to enzyme action. The validity of these results

was rejected by Waldschmidt-Leitz (133).

(ii) Experiments in which proteolytic enzymes were without effect on 2,5-diketopiperazines.

Waldschmidt-Leitz and Schäffner failed to detect any hydrolysis of glycine anhydride by pepsin, trypsin, erepsin, papain, or yeast autolysate (133). The experiments were performed under the conditions regarded as the most favorable for the respective enzymes. They maintained that, in those experiments of Abderhalden where enzyme action was stated to be dependent on acid or alkali concentration, the results were inconclusive. Abderhalden's experiments, as Waldschmidt-Leitz pointed out, were carried out at a pH outside the range of activity of body fluids.

Waldschmidt-Leitz proceeds further to cast doubt on the possibility of diketopiperazines constituting a building unit for proteins in view of their resistance to enzyme hydrolysis. Since silk fibroin and keratin are remarkably resistant to enzyme action, however, he acknowledged that several types of structure must exist, depending on the type of protein. With the knowledge available, he believed that protein material resistant to enzyme action may be rich in diketopiperazines.

The validity of Ishiyama's results was rejected by Waldschmidt-Leitz and Görtner on the claim that they were erroneous. Support was offered for their claim by their failure to

effect hydrolysis of aspartic anhydride and glycylglutamic anhydride by trypsin, carboxypeptidase, protaminase, chymotrypsin, whole pancreas extract, erepsin, or papain (134).

Kawai failed to hydrolyse glycyl-DL-tyrosine with pepsin, erepsin, glycerol extract of kidneys, yeast press juice, takadiastase (95).

Morel and Bay were unable to culture microorganisms in media where diketopiperazines served as the only source of nitrogen. As examples, Sterigmatocystis nigra and Rhizopus nigricans were unable to utilise the compounds (135). Also, four species of saccharomyces only showed negligible growth compared to growth in presence of glycine.

Akabori and Maeda failed to hydrolyse diketopiperazineacetic acid with trypsin or papain (136). In view of Shibata's statement that opening of the ring was dependent upon enzyme concentration, they repeated the work with larger quantities of enzyme; no hydrolysis occurred.

London and Kotchneva (137) sought to throw further light on the problem by determining whether glycine anhydride could be utilised in the body; if it could, they hoped to determine the manner of utilisation. Glycine anhydride was injected intravenously into dogs. No hydrolysis occurred as the substance passed through the organs; the only result produced seemed to be an increase in the polypeptide content of the blood.

Parskin and Nikolaeva found that L-histidine anhydride was hydrolysed to DL-histidyl-DL-histidine by acids and alkalis, and that the rate of hydrolysis was unchanged on addition of pepsin (99). They concluded that any diketopiperazines formed in protein hydrolysis was purely a side effect. They fed a number of diketopiperazines subcutaneously to rats and rabbits, but all were recovered unaltered.

A publication by Greenstein is worthy of note (138). The diketopiperazine derived from L-lysyl-L-glutamic amide was completely resistant to pepsin, trypsin and papain. Greenstein stated that resistance of anhydrides to enzyme hydrolysis did not necessarily discredit the diketopiperazine theory of protein structure, but that caution was necessary before applying the structure to proteins.

## Dielectric Constants of Solutions of Amino Acids, Peptides, and Proteins.

A considerable number of measurements were made by Devoto and his co-workers, and by Wyman and his co-workers, of dielectric constants of amino acids and peptides (139-149). Within the ranges of concentration studied, a linear relationship was found to connect increase of dielectric constant with concentration, and can be expressed by equation (i) below.

$$D = D_o + \delta C \quad \text{----------(i)}$$

$D$ = dielectric constant of solution

$D_o$ = dielectric constant of pure solvent

$C$ = concentration of solution

$\delta$ = increment of dielectric constant with concentration.

The value of $\delta$ is characteristic of the amino acid or peptide in question, and is usually close to 23 for monaminomonocarboxylic acids. It can be much higher for the acidic and the basic amino acids, and still higher for peptides. Furthermore, the value of $\delta$ is more or less constant for a given substance in these classes, irrespective of the solvent; the solvents used were mainly water, 20%, 40%, 60% and 80% aqueous ethanol solutions, and 2.58 M solutions of urea in water.

Equation (i) was valid irrespective of the frequency at which the measurements were made in the case of the amino acids and peptides. 2,5-diketopiperazine showed a negative value (-10)

for $\delta$ in water; this was interpreted as being consistent with its amide character, the cyclic structure of its molecule, and the absence of the zwitterion structure. The observation was also made that, in solvent media having dielectric constant of 20 or less, the value of D was apt to decrease with amino acid concentration; the decrease was attributed to a tendency for zwitterion structure to be depressed in solvents of low dielectric constant.

The linear relationship was found to be valid in the case of protein "solutions" provided the frequency at which the measurements were made did not exceed $2.4 \times 10^5$ cycles. Above this frequency, dielectric constant was found to decrease linearly with concentration.

Wyman found a rather interesting relation between the values of $\delta$ for glycine and peptides of glycine residues; the value of $\delta$ was related linearly to the number of glycyl residues, from glycine up to the heptapeptide. A copy of his graph is appended in Fig. 1.

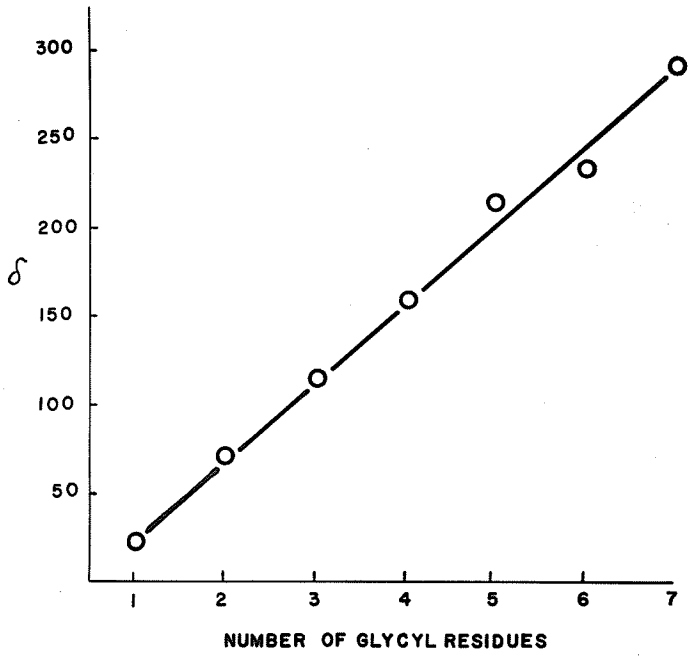



FIGURE 1.

RELATION OF δ TO NUMBER OF GLYCYL RESIDUES IN GLYCYL PEPTIDES.

From Wymann (144).

## Hydrolysis of Acid Amides.

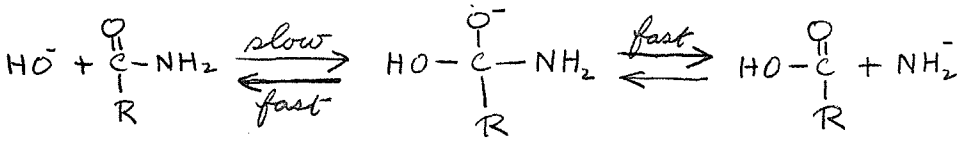
Amides of the type $R\text{-}CO\text{-}NH_2$ can be hydrolysed to the corresponding carboxylic acid in either acid or alkaline medium.

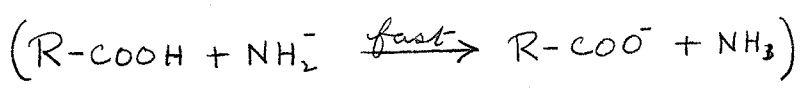
$$R\text{-}CO\text{-}NH_2 + H_2O \longrightarrow R\text{-}COOH + NH_3.$$

Very little information is available on the mechanism of the reaction, but Ingold in his text on organic reaction mechanisms (150) has proposed a mechanism, based on studies made by Reid (151). It can be seen by inspection of the above equation that the $-NH_2$ group attached to the acyl radical is replaced; Ingold regards such a reaction as analogous to what he calls acyl oxygen fission.

When any type of fission reaction occurs in basic or neutral medium, Ingold symbolises the mechanism as "B"; when fission occurs in acid medium, the mechanism is symbolised by "A". An acyl oxygen fission taking place by the B mechanism is described by $B_{AC}$, and similarly when the fission proceeds by the A mechanism, the symbol $A_{AC}$ is used. Such reactions, however, might be unimolecular or bimolecular; complete representation of the mechanism is achieved by such symbols as $B_{AC}1$, $B_{AC}2$, $A_{AC}1$, $A_{AC}2$; unimolecular reactions are designated by "1", bimolecular reactions by "2".

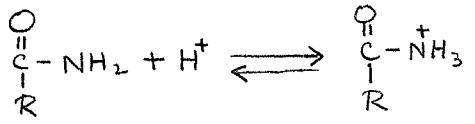
Ingold describes alkaline hydrolysis of amides as $B_{AC}2$, and on this basis the detailed mechanism which he proposes is as follows:-

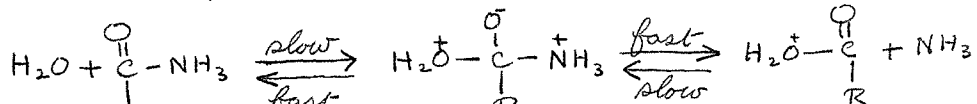


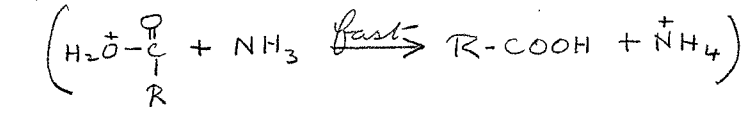


If the R group contains any electron attracting groups, the reaction is accelerated, whereas electron releasing groups retard the reaction. If any part of the R group is capable of exerting a steric effect, the reaction is retarded regardless of the polarity of the group.

Acid hydrolysis is generally quite slow, and the mechanism is stated by Ingold to be $A_{AC}2$; the detailed mechanism proposed is thus:-







Substituents have little polar effect, but when suitably situated have a strong steric effect, which retards the reaction independently of their polarity.

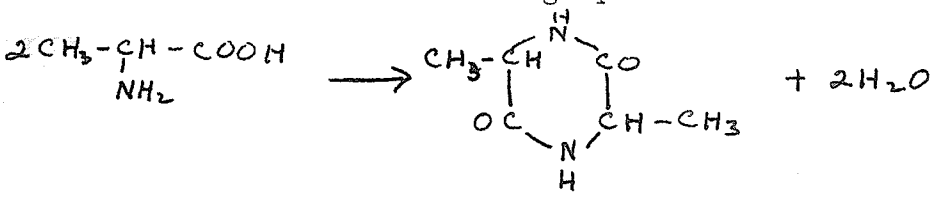
## Experimental.

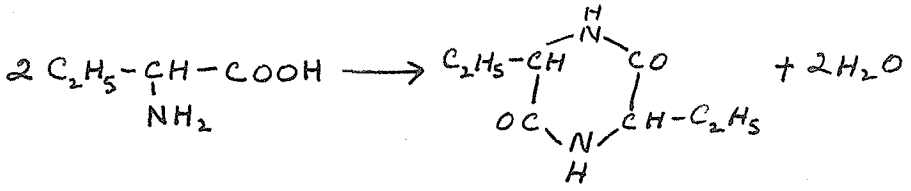
### I - Synthesis of 2,5-Diketopiperazines.

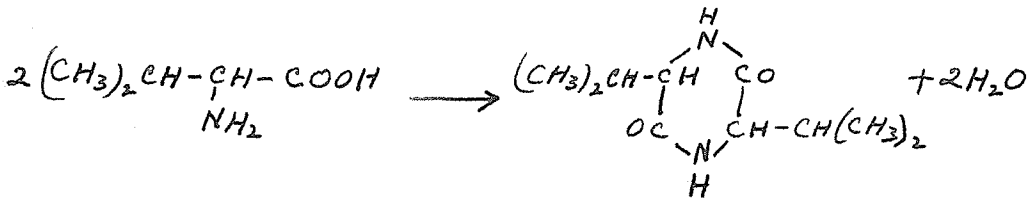
All the diketopiperazines prepared were those derived from monaminomonocarboxylic acids. Two types were made, simple -- made by condensation of two molecules of a single amino acid, -- and mixed --obtained by the condensation of a molecule of one amino acid with a molecule of another amino acid. The method was essentially an extension of Sannié's technique (10).

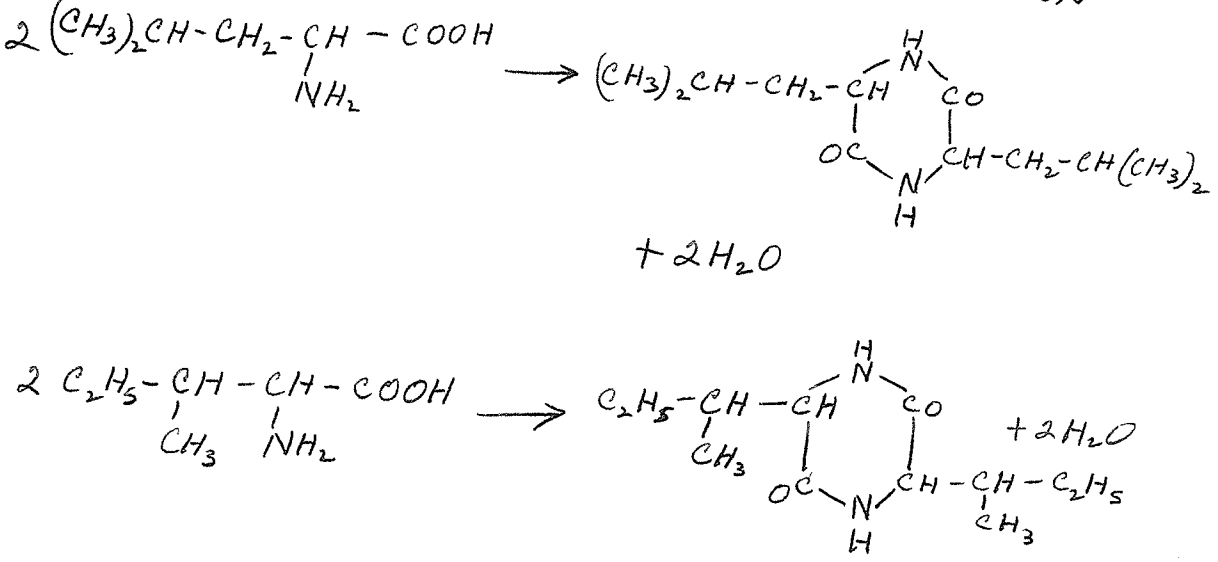
#### Simple diketopiperazines.

Those prepared were 3,6-dimethyl-2,5-diketopiperazine from DL-alanine; 3,6-diethyl-2,5-diketopiperazine from DL-$\alpha$- amino butyric acid; 3,6-diisopropyl-2,5-diketopiperazine from DL-valine; 3,6-diisobutyl-2,5-diketopiperazine from DL-leucine; 3,6-disec. butyl-2,5-diketopiperazine from DL-isoleucine. The overall reactions are as shown in the following equations:-











Preparation of 3,6-dimethyl-2,5-diketopiperazine.

Materials:-

| | |
|---|---|
| DL-Alanine | 12 grams (0.135 mole). |
| ethylene glycol | 45 c.c. |
| water | 30 c.c. |

The materials were mixed in a flask fitted with a tube to serve partly as a rough fractionating column as well as a means of removing the water originally present in the mixture and given off during the reaction. Although no effort was made to fractionate the distillate, its reaction was tested with litmus and found to be basic. The mixture was heated to 180° - 185°.

Solution was complete when the reaction temperature was

reached. The solution was kept at 180°-185° for 3½ hours, during which period the solution became straw coloured and somewhat fluorescent. At the end of the reaction period the reaction mixture was allowed to cool to room temperature and then refrigerated for 48 hours. The straw coloured, fluorescent, supernatant liquid was filtered off by suction from the white solid which had separated, and the solid subsequently washed with small quantities of ethyl alcohol, followed with small quantities of ether.

The yield of product was 6.4 grams - 7.2 grams, or 66.8-75% of theory.

Preparation of 3,6-diethyl-2,5-diketopiperazine.

Materials:-

| | |
|---|---|
| DL-α-aminobutyric acid | 11 grams (0.107 mole). |
| ethylene glycol | 36 c.c. |
| water | 24 c.c. |

The materials were heated to 180° - 185°, the temperature maintained for 3½ hours, and the product isolated in exactly the same manner as described in the previous preparation.

In each of two consecutive experiments the yield was 6.5 grams or 71.6% of theory.

Preparation of 3,6-Diisopropyl-2,5-diketopiperazine.

Materials:-

| | |
|---|---|
| DL-valine | 8 grams (0.069 mole) |
| ethylene glycol | 25 c.c. |
| water | 15 c.c. |

The materials were heated together under the same experimental conditions and the same procedure followed for isolation of the product as outlined for the two previous members of the series.

The yield of product was 4.1-4.9 grams, or 60.6-72.2% of theory.

Preparation of 3,6-diisobutyl-2,5-diketopiperazine.

Materials:-

| | |
|---|---|
| DL-leucine | 7 grams (0.053 mole) |
| ethylene glycol | 18 c.c. |
| water | 12.c.c. |

The same experimental procedures for reaction and isolation of product as described above were used.

Yield of product was 3.5-4 grams, or 70-80% of theory.

Preparation of 3,6-diisecbutyl-2,5-diketopiperazine.

Materials:-

| | |
|---|---|
| DL-isoleucine | 7 grams (0.053 mole). |
| ethylene glycol | 18 c.c. |
| water | 12 c.c. |

Using the same conditions and isolation methods, a yield of 2-3 grams, or 40-60% of theory was obtained.

Attempts to synthesise the above diketopiperazines, using the monethyl ether of ethylene glycol (ethyl cellosolve) or the monomethyl ether (methyl cellosolve) as solvent instead of the free glycol were given a cursory preliminary trial and abandoned. The amino acids were recovered unchanged; presumably the boiling points of these liquids are too low to enable a high enough temperature to be reached for the reaction to take place. In those instances reported in the literature where the syntheses have been carried out directly from amino acids, temperature has evidently been an important factor. Lichtenstein's use of $\alpha$ - naphthol at 135-140° appears to be the lowest practicable temperature for the conversion; in other cases a temperature of at least 150° has been necessary when carried out in presence of a liquid; otherwise, much higher temperatures have been required, e.g. the melting point of the amino acid.

The question might arise with respect to the possibility of using other liquids, e.g. a high boiling petroleum fraction

or a high boiling coal tar fraction. No doubt this would be feasible, but consideration has to be given to the insolubility both of the amino acids and of the anhydrides in those liquids; this factor would necessitate the development of a stirring device of such a nature as to prevent any settling of solid during the heating period. Achievement of such an object at any time would present a little difficulty, and would not in any event surmount the problem of insoluble product coating the surface of unreacted amino acid, a phenomenon which would introduce uncontrollable variables into the reaction, with possibly wide variation in yields.

With these considerations in mind, and since solution did occur at higher temperatures in the glycol, the possible use of other liquids was not investigated further.

Purity of products.

The diketopiperazines derived from the amino acids used usually have very high melting points and sometimes melt with decomposition. The temperatures observed for the products were all found to correspond fairly closely, but not always exactly to the literature values. As these temperatures are very high, it was decided to have analyses made on the compounds in order to determine their degree of purity before embarking on the preparation of other members of the series. The analyses were carried out by an analyst in the Department of Chemistry, University of Minnesota; details of the results are recorded in Table 1.

TABLE 1.

Analytical Data for Simple Diketopiperazines.

| Substance | C% theor. | C% found | H% theor. | H% found | N% theor. | N% found |
|---|---|---|---|---|---|---|
| alanine anhydride | 50.7 | 50.89 | 7.0 | 7.22 | 19.7 | 19.25 |
| ∝-aminobutyric anhydride | 56.4 | 56.77 | 8.2 | 8.46 | 16.5 | 16.59 |
| valine anhydride | 60.6 | 60.9 | 9.1 | 9.19 | 14.1 | 13.96 |
| leucine anhydride | 63.7 | 64.07 | 9.7 | 9.95 | 12.4 | 12.57 |
| isoleucine anhydride | 63.7 | 63.96 | 9.7 | 9.90 | 12.4 | 12.86 |

The agreement between the theoretical values for carbon, hydrogen and nitrogen content and the values actually found is comparable with that encountered in most organic compounds, indicating that the products were in a sufficiently high degree of purity for the observed temperatures to be used as a reasonable criterion of identity in subsequent preparations of members of the diketopiperazine series.

Mixed Diketopiperazines.

The ∝-amino acids used for preparing this series were glycine, DL-alanine, DL-∝-aminobutyric acid, DL-valine, DL-leucine, and DL-isoleucine. The diketopiperazines made were:-

(1) 3-methyl-2,5-diketopiperazine

(2) 3-ethyl-2,5-diketopiperazine

(3) 3-isopropyl-2,5-diketopiperazine

(4) 3-isobutyl-2,5-diketopiperazines

(5) 3-sec.butyl-2,5-diketopiperazine

(6) 3-methyl-6-ethyl-2,5-diketopiperazine

(7) 3-methyl-6-isopropyl-2,5-diketopiperazine

(8) 3-methyl-6-isobutyl-2,5-diketopiperazine

(9) 3-methyl-6-sec.butyl-2,5-diketopiperazine

(10) 3-ethyl-6-isopropyl-2,5-diketopiperazine

(11) 3-ethyl-6-isobutyl-2,5-diketopiperazine

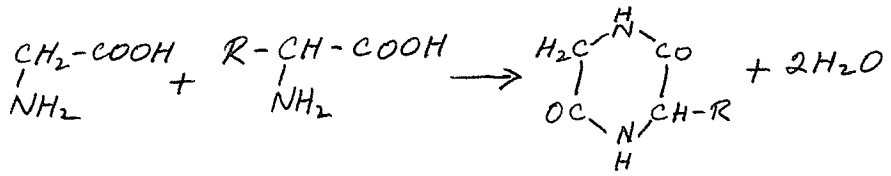
(12) 3-ethyl-6-sec.butyl-2,5-diketopiperazine

(13) 3-isopropyl-6-isobutyl-2,5-diketopiperazine

(14) 3-isopropyl-6-sec.butyl-2,5-diketopiperazine

(15) 3-isobutyl-6-sec.butyl-2,5-diketopiperazine

The equations for the respective reactions are as follows;-

(1) - (5) 

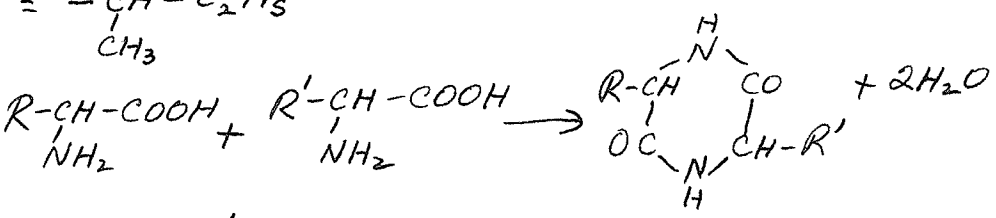
(1) $R = -CH_3$

(2) $R = -C_2H_5$

(3) $R = -CH(CH_3)_2$

(4) $R = -CH_2-CH(CH_3)_2$

(5) $R = -\underset{CH_3}{CH}-C_2H_5$

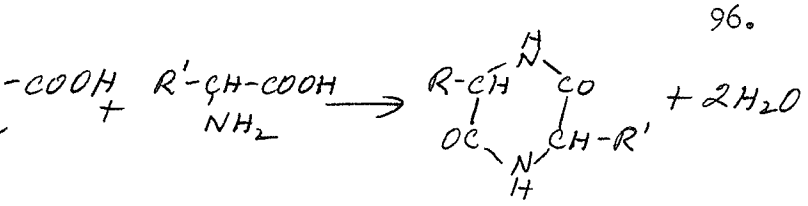
(6) - (9) 

(6) $R = -CH_3$ ; $R' = -C_2H_5$

(7) $R = -CH_3$ ; $R' = -CH(CH_3)_2$

(8) $R = -CH_3$ ; $R' = -CH_2-CH(CH_3)_2$

(9) $R = -CH_3$ ; $R' = -\underset{CH_3}{CH}-C_2H_5$

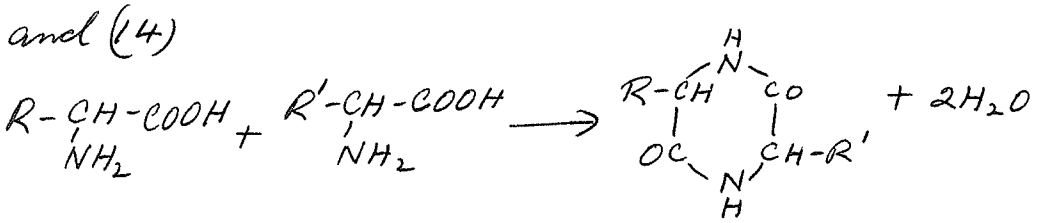
(10) – (12) 

(10) $R = -C_2H_5$ ; $R' = -CH(CH_3)_2$

(11) $R = -C_2H_5$ ; $R' = -CH_2-CH(CH_3)_2$

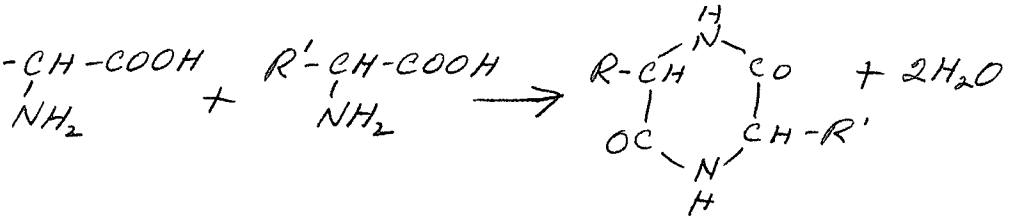
(12) $R = -C_2H_5$ ; $R' = -\underset{CH_3}{\underset{|}{CH}}-C_2H_5$

(13) and (14)



(13) $R = -CH(CH_3)_2$ ; $R' = -CH_2-CH(CH_3)_2$

(14) $R = -CH(CH_3)_2$ ; $R' = -\underset{CH_3}{\underset{|}{CH}}-C_2H_5$

(15) 

$R = -\underset{CH_3}{\underset{|}{CH}}-C_2H_5$ ; $R' = -CH_2-CH(CH_3)_2$

The reaction was superficially different in this series from what obtained in preparing the simple diketopiperazines. During the reaction period, effervescence was noticed, presumably due in some measure at least to the water formed as by-product of the reaction boiling off; this effervescence was not observed to any extent in the previous preparations. In the previous series the reaction time was set at $3\frac{1}{2}$ hours arbitrarily; in this series, the end of the reaction time was judged by the failure of a sample from the reaction mixture to give a blue colour with copper hydroxide. Sannié used this test in his preparations. As long as a blue colour was obtained with the copper hydroxide, free amino acid was judged to be still present. On the basis of this test, the period during which the system was kept on temperature was in most cases less than $3\frac{1}{2}$ hours.

It was also necessary to alter the procedure for isolating the final products, except in the case of (13) and (14) above. When the same method of isolating the compounds was tried as used for the simple anhydrides, losses were very serious, especially in (1)-(5) inclusive. No doubt the losses can be attributed in part to greater solubility of the mixed diketopiperazines in ethyl alcohol, but this in itself need not always be a serious deterrent, because careful washing of a filter cake with small quantities might be performed without too great a loss in yield. A much more serious factor seemed to be associated with the formation of an oily material as a by-product of

the reaction, especially in reactions (1)- (8) inclusive.

Since diketopiperazines are not generally soluble in ether or in hydrocarbon solvents, trials were made in which these solvents were poured on the filter cake to see if any one of them could be of any avail in ridding the filter cake of adhering oil, before or after draining off as much glycol as possible by suction filtration. These trials were unsuccessful; the only result was formation of a pasty mass which could not be filtered, and in some instances the oil seemed to form a separate layer when these solvents were added to the cake.

The oil presumably exerted a solvent effect on the products, and when the filter cake was washed with alcohol as in isolating the simple anhydrides, it is possible that a solution of the oil was formed having a still greater solvent effect on the product.

In the account of the heating of the reaction mixture for simple diketopiperazines, mention was made of the fitting of a distillation tube to the reaction flask. When this was used in the mixed series, the reaction took a longer time than when carried out in an open flask. Not only was it necessary to maintain the reaction temperature for a longer period, but a longer time was needed for the reaction temperature to be reached. The exact reason for this was not investigated, but it may quite well have been due to a partial refluxing action slowing down the escape of the water from the system. In view of these circumstances, it was decided to dispense with the tube and use an open flask.

Temperature control was more difficult for this series. In making the simple compounds, a small flame was all that was needed to keep the system at 180° - 185° C once the temperature had been reached. No such ease of control was encountered when similar methods were adopted for the mixed compounds considerable fluctuation of temperature occurring at all times. This experimental detail was resolved by carrying out the heating in a wax bath.

In certain instances Sannié was able to perform his conversions at lower temperatures than were used in this work. In the project of this problem, the control test with copper hydroxide was more definite at the higher reaction temperature, i.e. definitely positive or definitely negative. In some small scale trials at lower temperature, tests over a period of time were often rather vague and indefinite.

Preparation of 3-methyl-2,5-diketopiperazine.

Materials:-

| | |
|---|---|
| glycine | 5 grams (0.67 mole) |
| DL-alanine | 5.94 grams (0.67 mole) |
| ethylene glycol | 54 c.c. |
| water | 30 c.c. |

On heating the mixture, solution was complete at 110° C, and no solid separated out again during the heating period.

Boiling of the water occurred shortly after solution was complete and continued until it was all driven off. On reaching 180° - 185°, most of the original water seemed to have boiled out of the solution, but effervescence commenced in this range and persisted for more than an hour. Evolution of the water eliminated in the chemical reaction may have been a contributing factor to the effervescence.

A reddish-brown colour began to develop between 170° and 180°, becoming progressively deeper with continued heating. On testing samples of the solution with copper hydroxide, the test was positive as long as the effervescence persisted. The test was negative after the solution had been at 180° for 1½-2 hours.

At the end of this time, as much of the glycol was distilled off as possible under reduced pressure. The residual solid and dark red oil were refluxed with 50 c.c. ethyl alcohol; a varying amount of white solid did not dissolve, and was removed from the dark coloured solution by suction filtration and washed with further small quantities of hot alcohol. The undissolved residue might quite well have been glycine anhydride, but was not investigated further.

About one half to two thirds of the alcohol was distilled off and the residue in the distilling flask refrigerated 24 hours. A pasty solid separated which was filtered and drained as much

as possible without washing. Although the filter cake was pressed in an endeavour to assist the draining process, some dark oily matter remained with the solid. The solid was spread in a thin layer on a large piece of filter paper and after 24 hours had dried to a hard, brittle powder of a slight brown colour. Storage in a vacuum dessicator did not accomplish any noticeable change in colour, although some lightening did seem to take place when it had been exposed to the atmosphere for several weeks.

Further small quantities of solid separated from the filtrate when the latter was refrigerated 24-48 hours, but the amount was small and the purity questionable, and the material was rejected.

Yield of main product was 5.34 grams, or 62.5% of theory. This was the yield ultimately obtained in each of two consecutive runs.

Preparation of 3-ethyl-2,5-diketopiperazine.

Materials:-

| | |
|---|---|
| glycine | 5 grams (0.67 mole) |
| DL- $\alpha$ -aminobutyric acid | 6.85 grams (0.67 mole) |
| ethylene glycol | 45 c.c. |
| water | 30 c.c. |

The materials were heated together in the usual manner.

At 170°C the solution turned the usual dark reddish brown colour, and the effervescence persisted for about 40 minutes at 180°C. The reaction time was 2 hours. The ethylene glycol was distilled off under reduced pressure, and the dark residue refluxed ½ hour with ethyl alcohol. The undissolved solid was removed by filtering, and the filtrate refrigerated 48 hours.

The weight of solid which separated during the refrigeration was 5.04-5.59 grams, or 53.5-59.15% of theory.

Preparation of 3-isopropyl-2,5-diketopiperazine.

Materials:-

| | |
|---|---|
| glycine | 5 grams (0.067 mole) |
| DL-valine | 7.8 grams (0.067 mole) |
| ethylene glycol | 45 c.c. |
| water | 30 c.c. |

Solution was complete during the initial stages of the heating, but at 130°C a white solid appeared over the entire surface of the bubbling liquid and also round the wall of the flask, just above the edge of the reaction mixture. This solid was completely redissolved when the temperature had reached 160°C; at this temperature the solution began to acquire the customary darkening in colour, and the effervescence was very slow.

On reaching 180°C, the effervescence became much more

vigorous; after one hour at 180° - 185°, the effervescence seemed to have ceased, and the copper hydroxide test was negative. At this stage the glycol was distilled off under reduced pressure, leaving a dark brown coloured residue which was now refluxed ½ hour with 50 c.c. ethyl alcohol. The undissolved solid was filtered off and washed with two 25 c.c. portions of hot ethyl alcohol. The volume of the combined filtrate and washings, dark brown in colour, was reduced by about 1/3 to ½ by distilling off some of the alcohol.

Refrigeration of the alcoholic solution for 24 hours resulted in separation of a white solid. The solid did not settle to the bottom of the flask, but formed a mass in which the mother liquor was occluded, and filtration was extremely protracted. The attempt to filter as it stood was abandoned. The mass was detached from the filter, mixed with a further 25 C.C. of ethyl alcohol and the whole refrigerated a further 24 hours. The consistency of the mixture was no different from before, and filtration was no more rapid than in the first attempt. Some ether was added to the filter cake in an effort to speed up the filtration, but without success.

The ultimate yield of product was 5.8 grams, or 55.8% of theory. The product was a white solid, slightly tinged with yellow.

In another attempt to prepare this compound the same difficulty of slow filtration was encountered. The cake was re-

moved and spread out on a large piece of filter paper, but over a period of 48 hours very little loss in weight had occurred, indicating that separation of alcohol from the solid was extremely difficult. The material was mixed with some ether in a beaker; the reddish brown oil separated in considerable amount from the solid, but did not dissolve completely, if at all, in the ether. Despite this heterogeneous condition, the mixture was placed on the filter and drained by suction. The filtration was much more rapid than in the previous preparation, and the solid residue on the filter was much whiter than the previous product. The weight and yield were the same.

Preparation of 3-isobutyl-2,5-diketopiperazine.

Materials:-

| | |
|---|---|
| Glycine | 5 grams (0.067 mole) |
| DL-leucine | 8.73 grams (0.067 mole) |
| ethylene glycol | 45 c.c. |
| water | 30 c.c. |

The mixture was heated to 180° - 185° as usual, and solution was complete throughout the entire heating period. The effervescence persisted for 1¼ hours after reaching the reaction temperature. When the reaction temperature had been maintained a total of 2 hours, the copper hydroxide test was finally judged to be negative; the solution possessed the usual dark colour.

In distilling off the glycol under reduced pressure, care had to be exercised to prevent frothing of the solution with consequent loss of yield. In one experiment, the dark coloured residue was refluxed with 75 c.c. ethyl alcohol, the dark solution filtered to remove the small amount of undissolved solid and the latter washed with small quantities of hot alcohol. The combined filtrate and washings were distilled until about 70 c.c. of the alcohol had been removed, and the residual solution refrigerated 48 hours.

A solid separated out which was filtered and washed with ether. As was the case with the 3-methyl compound, some dark coloured oil separated from the solid on the filter. Addition of a little acetone to the filter cake seemed to dissolve the oil and wash it into the filtrate. The product left on the filter was slightly brown in colour.

Yield of product was 5.364 grams, or 47.34% of theory. In another trial using the same procedure up to and including the refluxing with alcohol and filtering off the undissolved solid, the isolation procedure was altered beyond this point. Distillation of alcohol was continued until no more could be driven off. Fifty c.c. acetone was added to the dark coloured residue in the distilling flask, and the whole refrigerated 48 hours. The resulting suspension was filtered and washed with cold acetone. The solid product remaining on the filter was much whiter than in the previous trial.

The yield was 5.354 grams, or 47.26% of theory.

Preparation of 3-sec.butyl-2,5-diketopiperazine.

Materials:-

| | |
|---|---|
| glycine | 5 grams (0.067 mole) |
| DL-isoleucine | 8.73 grams (0.067 mole) |
| ethylene glycol | 45 c.c. |
| water | 30 c.c. |

The effervescence persisted for 2 hours after the reaction temperature (180°-185°) was reached. The copper hydroxide test was judged to be definitely negative ½ hour later, and the solution possessed the usual dark colour. Frothing was particularly serious when vacuum distillation of the glycol was attempted. In order to minimise the possibility of losses due to frothing, it was necessary in one of the trials to distil some of the glycol at atmospheric pressure before resorting to vacuum distillation. The dark coloured residue was refluxed with 50 c.c. ethyl alcohol. As in the previous preparations where glycine was one of the amino acids used, a small amount of solid remained undissolved, was removed in the usual way and washed with two 25 c.c. portions of hot ethyl alcohol.

The combined filtrate and washings were reduced about 1/3 to½ in volume by distilling off some of the alcohol. The dark coloured solution was refrigerated 24-36 hours and filtered by suction. In one trial, the solid on the filter was light yellow in colour, the colour being lightened somewhat by washing with a

few drops of acetone, and the yield was 5 grams or 45.3% of theory. In another trial a few drops of ether were used in an effort to remove the coloured matter adhering to the solid, but without success. The cake was drained at the suction pump as much as possible and then spread out on filter paper. At this stage, the solid was dark brown in colour, but after standing overnight had changed to a light brown or yellowish brown colour, and was rather brittle.

The weight of product was 6.3 grams or 55.6 % of theory.

Preparation of 3-methyl-6-ethyl-2,5-diketopiperazine.

Materials:-

| | |
|---|---|
| DL-alanine | 5.94 grams (0.067 mole) |
| DL-$\alpha$-aminobutyric acid | 6.85 grams (0.067 mole) |
| ethylene glycol | 45 c.c. |
| water | 30 c.c. |

Solution was complete when the temperature had reached 120°C. At 180°C the effervescence had ceased and the copper hydroxide test was negative 50 minutes later; the solution at this point had become a light, golden yellow colour. Removal of the ethylene glycol by vacuum distillation left a light yellow solid residue in the distilling flask. Warming the solid slightly with 30 c.c. ethyl alcohol dissolved out the colour. Partial solution of the solid occurred in this treatment, but some separated out again on cooling, and addition of 80 c.c.

of ether caused further precipitation. The suspension was allowed to stand overnight and then filtered by suction, leaving a white crystalline solid on the filter.

The yield of product was 6.06-6.68 grams, or 58.3-64.3% of theory.

Preparation of 3-methyl-6-isopropyl-2,5-diketopiperazine.

Materials:-

| | |
|---|---|
| DL-alanine | 5.94 grams (0.067 mole) |
| DL-valine | 7.8 grams (0.067 mole) |
| ethylene glycol | 45 c.c. |
| water | 30 c.c. |

Solution was complete at 110°C, but a white solid separated out on the surface of the solution at 130°; at 170°C this had almost disappeared except for a small fragment which, however, had completely dissolved on reaching the reaction temperature of 180°C. After 80 minutes at 180°-185°C, the reaction was complete, and the solution had turned to a reddish brown colour, although it was still transparent. As much of the glycol was distilled off as possible under reduced pressure.

The residue was heated with 30 c.c. ethyl alcohol for 30 minutes, and, after cooling, 70 c.c. ether was added and the whole allowed to stand overnight. The dark colour had been dissolved out of the solid by the alcohol, and remained dis-

solved in the ether-alcohol mixture. The suspension was filtered and the white solid washed with ether.

The yield of product was 5.25 grams or 46.3% of theory.

In another preparation the effervescence had ceased and the copper hydroxide test was negative after 50 minutes at 180°-185°C. The solution at the end of this time was of a light straw colour. The glycol was evaporated under vacuum and the residual solid refluxed 30 minutes with 30 c.c. ethyl alcohol. Solution was complete, and the alcoholic solution was of a reddish colour similar to that observed in the glycine series. On cooling, 70 c.c. ether was added and the mixture allowed to stand overnight. The white solid was filtered off and drained at the suction pump. A further quantity of solid separated from the filtrate on standing, and was recovered by filtering and draining at the suction pump.

The yield of product in this run was 7.33 grams or 64.68% of theory.

The yield of second crop recovery was 0.4 grams or 3.53% of theory.

The total yield was 68.21% of theory.

Preparation of 3-methyl-6-isobutyl-2,5-diketopiperazine.

Materials:-

| | |
|---|---|
| DL-alanine | 5.94 grams (0.067 mole) |
| DL-leucine | 8.5 grams (0.067 mole) |
| ethylene glycol | 45 c.c. |
| water | 30 c.c. |

Solution was not complete until a temperature of 177° had been reached. After 1 hour at 180°-185°C effervescence was observed to have ceased and the copper hydroxide test found to be negative. During removal of the glycol under reduced pressure the mass in the distilling flask turned to a darker colour. The residue dissolved completely on refluxing with 30 c.c. ethyl alcohol, giving a dark coloured solution reminiscent of the glycine series. Seventy c.c. ether was added, causing a bulky white precipitate to separate out, and the mixture left standing overnight. The precipitate did not settle as had the previous two compounds, but formed a mass with solvents presumably adhering to the particles. Suction filtration was very difficult, and the cake was spread out on a large sheet of filter paper to allow adhering solvents to evaporate. Evaporation was slow, the time taken for the solid to decrease to constant weight being 6-8 weeks.

Weight of product obtained was 7.82 grams, 64.27% of theory.

Preparation of 3-methyl-6-sec.butyl-2,5-diketopiperazine.

Materials:-

| | |
|---|---|
| DL-alanine | 5.94 grams (0.067 mole) |
| DL-isoleucine | 8.5 grams |
| ethylene glycol | 45 c.c. |
| water | 30 c.c. |

Most solid matter had dissolved by 120°C, but a certain amount remained undissolved until 160°C. Effervescence had ceased and the copper hydroxide test was negative after about one hour at 180°-185°C. The solution was a clear golden syrup colour, and nothing precipitated out even on cooling to room temperature. The glycol was removed as completely as possible under reduced pressure, leaving a pale yellow solid.

Only partial solution of the solid occurred on refluxing with 30 c.c. ethyl alcohol, but the colour was dissolved out completely. Seventy c.c. ether was added, causing further precipitation. After standing overnight, the precipitate had settled. It was filtered and drained at the suction pump and recovered as a white solid.

Yield of product was 7.1 grams or 58.34% of theory.

Second crop recovery (white solid) from filtrate was 1 gram, or 8.22% of theory.

Total yield was 66.56%.

Preparation of 3-ethyl-6-isopropyl-2,5-diketopiperazine.

Materials:-

| | |
|---|---|
| DL-$\alpha$-aminobutyric acid | 6.85 grams |
| DL-valine | 7.8 grams |
| ethylene glycol | 45 c.c. |
| water | 30 c.c. |

All materials were completely dissolved by 180°C, and the time necessary to keep the solution on temperature was 75-110 minutes. At the end of the reaction time the solution was only of a very pale yellow colour, and a precipitate separated on cooling. The glycol was removed in the usual manner, leaving a light brownish yellow residue in the flask. Refluxing $\frac{1}{2}$ hour with 30 c.c. ethyl alcohol only effected partial solution, but all the colour was dissolved out. Eighty c.c. ether was added and the mixture allowed to stand overnight. On filtering, a white solid was obtained.

Yield of product was 7.38 grams, 60.64% of theory.

3-ethyl-6-isobutyl-2,5-diketopiperazine.

Materials:-

| | |
|---|---|
| DL-$\alpha$-aminobutyric acid | 6.85 grams |
| DL-leucine | 8.5 grams |
| ethylene glycol | 45 c.c. |
| water | 30 c.c. |

The reaction mixture was heated to 180°-185°C and maintained for one hour; solution had been complete at 170°C. The solution was slightly golden yellow in colour, and the solid remaining after the glycol was evaporated under vacuum had a slight yellow tinge. On refluxing ½ hour with 30 c.c. ethyl alcohol practically everything dissolved, some white solid separating out on cooling. Ether (80 c.c.) was added, the mixture left standing overnight and then filtered. Evaporation of solvent in the Buchner flask due to the vacuum caused more solid to precipitate out.

Yield of main product was 9.706 grams, 73.53% of theory.

Weight of second crop recovery was 1.754 grams, 13.3% of theory.

The total yield was 86.83% of theory.

Preparation of 3-ethyl-6-sec.butyl-2,5-diketopiperazine.

Materials:-

| | |
|---|---|
| DL-∝-aminobutyric acid | 6.85 grams |
| DL-isoleucine | 8.5 grams |
| ethylene glycol | 45 c.c. |
| water | 30 c.c. |

The materials were completely dissolved at 175°C, having a sort of brownish yellow colour; it was found necessary to keep the temperature at 180°-185°C for about 60 minutes.

Evaporation of the glycol under reduced pressure left a creamy-yellow solid. Refluxing with 30 c.c. ethyl alcohol gave complete solution, to which was added 80 c.c. ether. On standing overnight, the solid was obtained white on filtering. Some filtrate evaporated due to the vacuum and some more solid precipitated. This was filtered separately and found to be more yellow than the main product. On standing, however, it became white.

Weight of main product was 7.607 grams, 57.88% of theory.

Weight of second crop was 1.245 grams, 9.45% of theory.

Total yield was 67.33% of theory.

3-isopropyl-6-isobutyl-2,5-diketopiperazine )
( - These were made
3-isopropyl-6-sec.butyl-2,5-diketopiperazine )

by the same procedure as outlined for the simple diketopiperazines, in yields of 45.7-80% and 48-64% of theory respectively.

Preparation of 3-isobutyl-6-sec.butyl-2,5-diketopiperazine.

Materials:

| | |
|---|---|
| DL-leucine | 8.5 grams |
| DL-isoleucine | 8.5 grams |
| ethylene glycol | 45 c.c. |
| water | 30 c.c. |

The reaction mixture had to be kept on temperature for 70 minutes, and solution was not complete until the mixture had been on temperature for fully 30 minutes; the colour of the solution was asort of golden brown. When as much glycol as possible had been removed after completion of reaction, a yellow solid remained. On refluxing with 30 c.c. ethyl alcohol for 30 minutes the colour was dissolved out, but solution of the solid was nowhere near complete. To the suspension was added 80 c.c. ether, and the mixture left standing overnight and then filtered. A white solid was collected on the filter, and the filtrate was observed to revert to a reddish brown colour on standing. Some yellow solid separated from the filtrate as solvent evaporated in the Buchner flask, but on filtering off it became white.

Weight of main product was 10.31 grams, or 70.38% of theory.

Weight of second crop was 1.98 grams, or 13.53% of theory.

II- Measurement of Dielectric Constants.

The apparatus used was constructed according to the design proposed by Bender (152). The diagram of the electrical circuit is shown in Fig. 2. The instrument actually comprises two portions, a resonance circuit and a power supply circuit, and details of the parts are listed below:-

Resonance circuit.

$R_1$ - resistance, 150,000 ohms

$R_2$ - resistance, 40,000 ohms

$L_1$ - RF choke, 2.5 millihenris.

$L_2$ - inductance consisting of 5 turns of no.26 enamelled wire closely wound on a $1\frac{1}{4}$ inch diameter 4-prong moulded coil form.

$C_1$ - condenser, capacitance 0.001 mf, 450 wv.

$C_2$ - condenser, capacitance 0.01 mf, 450 w.v.

$C_A$ - condenser, precision variable type, consisting of 3 gangs, each of 150 mmf capacitance.

$C_x$ - the dielectric constant cell, described in more detail on p.119.

K - a Bliley crystal, type MC-7, frequency 2000 kilocycles, the whole mounted in a crystal socket and adapter.

B - 6E5 tuning eye, mounted on a 1 inch 8-prong socket. The tube functions both as an oscillator tube and as a resonance detector.

Power supply circuit.

T - power transformer

$C_3$ - oil filled condensers, each of capacitance 6 mf, 600 volts.

$L_3$ - chokes, each 10 HY, 80 MA, 240 ohms.

D - 5Y3GT power tube, mounted on a 1 inch 8-prong socket.

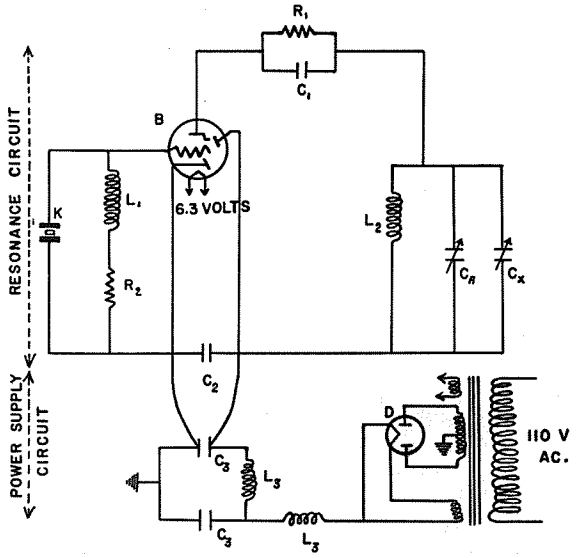



FIGURE 2.

ELECTRICAL CIRCUIT FOR DIELECTRIC CONSTANT MEASUREMENTS (As proposed by Bender).

All connections were made by means of 20-solid thermoplastic hook-up wire.

Variation of $C_A$.

One end of the rotor was fitted with a gear to one end of a shaft at right angles to the axis of the rotor, and protruding to the outside of the enclosing cabinet. The protruding portion of the shaft was fitted with a knob, and just inside the cabinet the shaft was connected by a gear to a Veeder counter. The knob enabled variation of capacitance to be made by manual rotation of the shaft, and the counter showed the number of complete turns made. The knob was fitted with a circular scale graduated to 1/100 turn.

The circuit was enclosed in a sloping panel cabinet of iron, 8" x 14" x 8".

The Dielectric Constant Cell.

The cell used was similar to that used by Funt and Mason, (153), and is shown in Fig. 3.

A,B- two concentric brass cylinders, silver plated on the inside and outside.

C - a bakelite block machined so that A,B were held firmly in position when closed by the block.

D - the leads connecting the cell to the instrument.

A was cemented firmly to C by means of a cement made by mixing calcium fluoride with water glass to a stiff paste. The liquid whose dielectric constant is to be measured is introduced into the annular space between A and B.

Preparation of the Cell.

Resonance was established when liquids such as benzene, toluene, petroleum spirit, ether and chloroform were used. 2,5-diketopiperazines are not generally soluble in such solvents, however, recourse to liquids such as ethyl alcohol or glacial acetic being necessary.

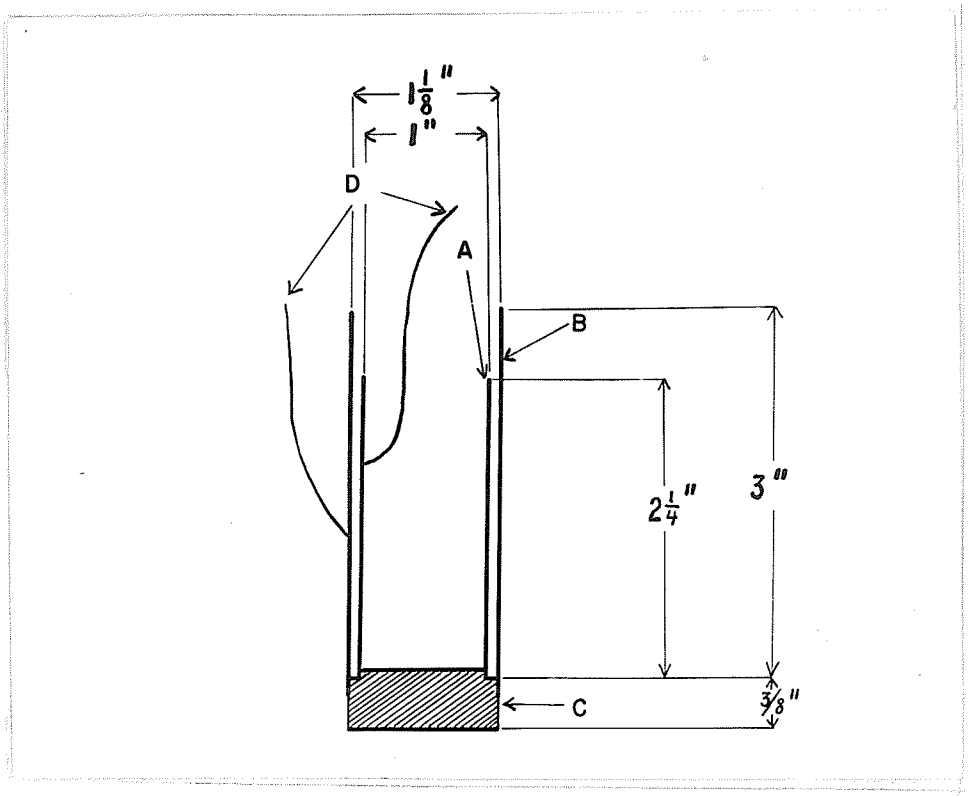



FIGURE 3.

DIELECTRIC CONSTANT CELL, ADAPTED FROM THE DESIGN BY FUNT AND MASON (153).

In addition, it was desirable to seek a solvent of fairly high dielectric constant to ascertain whether diketopiperazines were sufficiently polar to raise the dielectric constant of a liquid for which the value was alread fairly high.

No readings could be obtained when acetic acid, ethyl alcohol, or water was placed in the cell, a fact which is generally attributed to conductance. Nitrobenzene has a high dielectric constant but is not regarded as possessing conductance; no reading was obtained with this liquid.

The problem of conductance was resolved by immersing B in molten paraffin wax, and the same treatment was applied to the permanent A-C structure. B was refitted to the A-C assembly after all the paraffin coatings had hardened, and the join at B and C made water-tight by applying another coating to the outside. To provide further support and protection for the lead on the outside of B, the outside of the cylinder was wrapped with rubber insulation tape.

Before use it was found necessary to let the cell stand several days, usually about three. Readings were now obtainable with acetic acid, ethyl alcohol, nitrobenzene, acetone, and water. Reproducibility could only be attained with the acetic acid and the alcohol, however. Although for any one preparation of the cell the volume of acetone needed to fill the annular space was constant, the readings on the instrument varied.

When the same sample was removed from the cell, stored in a tightly stoppered container and later replaced in the space, the same variations were found as before. Fresh samples from the same stock bottle and from different containers behaved similarly. Distillation with or without a fractionating column produced no change in behaviour.

In the case of nitrobenzene, the volume of sample filling the annular space was never constant, possibly due to inability of the liquid to cover the inside surface completely, and no improvement was achieved on redistillation, the reading varying for any single filling of the cell. A similar situation was experienced with distilled water.

For acetic acid and for ethyl alcohol the volume for one filling of a given assembly was constant, and the reading was constant and reproducible for any given assembly. The acetic acid used was glacial, C.P., Grasselli brand as supplied by Canadian Industries Ltd., and no change in reading occurred when repeatedly redistilled or when samples were taken from different containers. The alcohol was absolute as supplied by Canadian Industrial Solvents Ltd., and showed no change in behaviour when redistilled from quicklime, and no variation was met when samples were taken from another bottle.

Calibration of the Instrument.

The amount of capacitance from $C_A$ for any given setting

must be related to the number of turns of the manually rotated shaft out from the fully closed position. In order to determine capacitance change between different settings of $C_A$ a precision standard variable condenser was connected in parallel with $C_A$. For any given setting of $C_A$ a certain amount of capacitance had to be removed from the standard to establish resonance. If for a certain setting $C_{A1}$ the capacitance taken out of the standard was $C_X$, while for another setting the capacitance taken out of the standard was $C_Y$, then the difference in capacitance in changing the settings is $C_{A2} - C_{A1} = C_Y - C_X$

The setting $C_{A0}$, corresponding to that obtained when the cell is not included in the circuit, was the lowest at which resonance could be established, and was taken as the reference setting for all calculations.

In Table 2, column I shows the changes in settings relative to $C_{A0}$, column II the difference ($\triangle T$) in settings from the initial one, column III the actual capacitance in the standard corresponding to the setting (A is unknown), column IV the total change in capacitance $\triangle C$ corresponding to a given value of $\triangle T$.

Table 2.

Data relevant to calibration of dielectric constant instrument.

| I | II($\triangle T$). | III | IV($\triangle C$) |
|---|---|---|---|
| 5.8 | 0 | A- 47.7 | 0 |
| 6.8 | 1 | A- 55.9 | 8.2 |
| 12.95 | 7.15 | A-109.2 | 61.5 |
| 21.0 | 15.2 | A-168 | 120.3 |

Figure 4 shows the relationship of $\triangle T$ to $\triangle C$. The equation of the curve may be obtained from the values of the logarithms of corresponding values of $\triangle T$ and $\triangle C$ as listed in Table 3.

Table 3.

Values of $\triangle T$, Log $\triangle T$, $\triangle C$, log $\triangle C$.

| $\triangle T$ | log. $\triangle T$ | $\triangle C$ | log. $\triangle C$ |
|---|---|---|---|
| 1 | 0 | 8.2 | 0.9138 |
| 7.15 | 0.8453 | 61.5 | 1.7889 |
| 15.2 | 1.1818 | 120.3 | 2.0897 |

Log. $\triangle T$ is plotted against log $\triangle C$ in Figure 5. The three points plotted on the curve seem to lie on a straight line making an intercept on the vertical axis at 0.9138. Measurement of the angle made by the curve with the horizontal axis gives a value of $45^{\circ}$, for which the gradient is 1, and the equation for the curve is given by

$$\log \triangle C = \log \triangle T + 0.9138 \quad \text{---------- eqn. (1)}$$

To check the validity of the equation, the gradient may be calculated from the coordinates of the points plotted. Thus, the gradient between the points ( 0.8543,1.7889) and (0, 0.9138) is

$$\frac{1.7889-0.9138}{0.8543-0.00}$$

$$= \frac{0.8751}{0.8543}$$

$$= 1.0243$$

The gradient between the points (1.1818, 2,0897) and (0,0.9138)

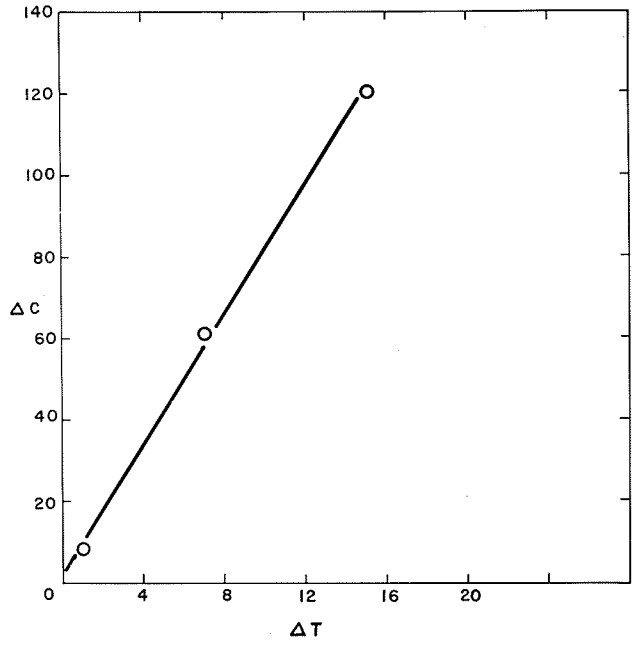



FIGURE 4.

RELATION OF $\triangle C$ TO $\triangle T$.

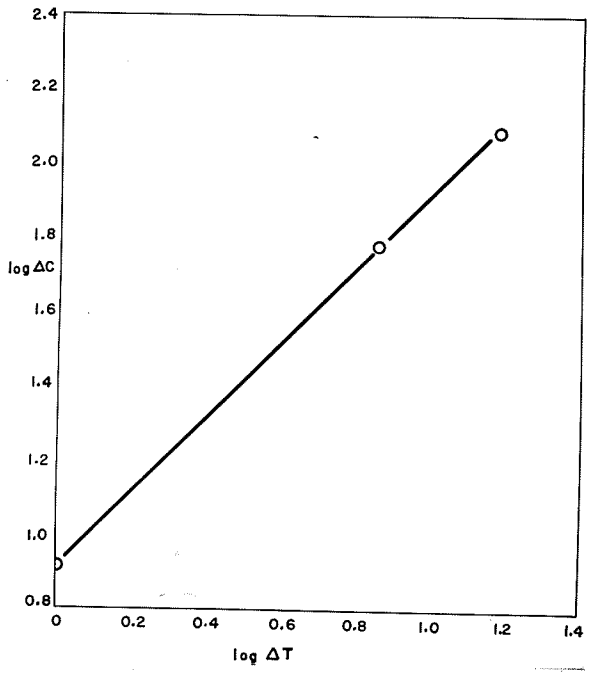



FIGURE 5.

RELATION OF LOG △C TO LOG △T.

is $\frac{2.0897-0.9138}{1.1818-0.00} = \frac{1.1759}{1.1818}$

$= 0.9950.$

The average of these two values is 1.0097, indicating that assumption of the validity of the equation is justified.

Principle of the Apparatus.

The power supply circuit provides the power necessary for the operation of the resonance circuit. The resonance circuit is the one in which the dielectric constant is measured, and in this section it is solely the principle of the resonance circuit which will be discussed.

The frequency f in the part of the circuit comprising $L_2$, $C_A$, and $C_X$ is given by the relationship

$$f = \frac{1}{2\pi\sqrt{LC}}$$

where L= inductance, C= capacitance.

When this part of the circuit is in resonance with the rest of the circuit, the shadow angle of the tuning eye closes to a minimum, and a slight increase in the value of C will cause the angle to open suddenly.

$$C = C_A + C_X .$$

When $C_X$ is removed from the circuit, $C = C_A$, and resonance is possible for only one particular setting of the instrument. When the empty cell is included in the circuit, the capacitance

in $C_A$ must be reduced in order to reestablish resonance, and the setting must be altered accordingly. When the cell is filled with some liquid or solution, its capacitance is increased, and a still greater reduction must be made in $C_A$, and the setting must be further altered to attain resonance.

For purposes of dielectric constant determinations, the following relationships apply;-

If $C_o$ = capacitance in $C_A$ to establish resonance without the cell,

$C_g$ = capacitance of $C_x$ when empty,

$C_l$ = capacitance in $C_x$ when filled with liquid,

the change in capacitance $\triangle C$ when the empty cell is connected

$$= C_o - C_g$$

and when the cell contains liquid $= C_o - C_l$.

The dielectric constant of the liquid is given by the equation

$$\frac{C_o - C_l}{C_o - C_g} = \epsilon \qquad \text{---------- eqn. (2)}$$

Calculation of $C_g$.

The value of $\triangle T$ when the empty cell is connected is 4.2; substituting this value in eqn. (1),

$$\log \triangle C = \log 4.2 + 0.9138$$
$$= 0.6232 + 0.9138$$
$$= 1.5370$$
$$= \log 34.43$$
$$\therefore \triangle C = 34.43 \text{ mmf}$$

Calculation of $C_o$.

Various values of $\epsilon$ for ethyl alcohol have been reported in the literature, and values are quoted for various alcohol-water mixtures. The valuesfor absolute alcohol are usually within the range 24-26 at 25°C, while for 90% alcohol Harned and Owen (154) quote values within this range. The possibility of the absolute alcohol used taking up moisture rapidly enough for $\epsilon$ to increase had to be considered. The reading was constant, and the laboratory supplies of 95% alcohol gave a different but nonetheless constant, reading when samples were taken from different containers. From these observations it was concluded that the extent to which the absolute alcohol would take up moisture was not sufficient to introduce a large error over the duration of the measurement. The material was therefore regarded as practically pure and the value for $\epsilon$ at 25° quoted in N.B.S. circular 514 (154) as 24.3 was taken.

The value of $C_1$ for the alcohol was obtained from equation (1):-

$$\triangle T = 9.3$$

$$\log. \triangle C = \log. 9.3 + 0.9138$$

$$= 0.9685 + 0.9138$$

$$= 1.8823$$

$$= \log. 76.26$$

$$\triangle C = 76.26 \text{ mmf.}$$

$$\therefore C_1 = 76.26 \text{ mmf.}$$

Substituting these values for $C_l$, $C_g$, and $\epsilon$ in equation (2),

$$\frac{C_o - 76.26}{C_o - 34.43} = 24.3,$$

giving a value of 32.63 mmF for $C_l$, and -1.80 for $C_o - C_g$.

Calculation of $\epsilon$ for the acetic acid used.

When the cell $C_x$ is filled with glacial acetic acid, $\triangle T$ = 7.5. Substituting once more in equation (1).

$$\log \triangle C = \log.7.5 + 0.9138$$
$$= 0.8751 + 0.9138$$
$$= \log.61.51$$
$$\triangle C = 61.51 \text{ mmf}$$
$$\therefore C_l = 61.51 \text{ mmf}$$

Putting this value for $C_l$ and -1.8 for $C_o - C_g$ in equation (2),

$$\epsilon = \frac{32.63 - 61.51}{-1.8} = 16.04.$$

The value of $\epsilon$ quoted for acetic acid in N.B.S. circular 514 (155) is given as 6.19 at 25°. Glacial acetic acid is an associated compound, however, and an extremely minute trace of moisture is quite capable of causing a considerable measure of dissociation into single molecules. The formation of single molecules of acetic acid would produce a more highly polar system with a higher dielectric constant. The material is also reported to contain glyoxylic acid, $\begin{matrix} CHO \\ | \\ COOH \end{matrix}$, which can be

expected from its structure to make the system still more polar and raise the dielectric constant appreciably. As the readings were constant, it was apparent that the hazards due to the possible presence and uptake of moisture and the possible presence of other polar impurities, did not constitute a variable under the conditions of the experiment. Different samples of acetic acid from different containers gave the same reading. The value of 16.04 for $\epsilon$ was therefore taken as the value for the acetic acid used. If, as turned out to be true in some measure, the diketopiperazines gave solutions of higher dielectric constant than the liquid itself, they would be shown to possess a highly polar character even if no other significant conclusions could be drawn.

Dielectric Constants of Diketopiperazine Solutions.

Only simple diketopiperazines were studied. Mixed diketopiperazines would be polar in any case due to their unsymmetrical structure. An indication of polarity by the simple diketopiperazines of symmetrical structure, however, might be informative in showing whether they had some property which could be studied analogous to amino acids, peptides, and proteins. 2,5-Diketopiperazine and the 3,6-dimethyl compound did not dissolve in significant amount in ethyl alcohol, and neither appeared to give a stable solution in acetic acid, so no measurements were made with them. The 3,6-diethyl compound gave every indication of dissolving more readily in acetic acid than in the alcohol and measurements were made on this solution.

To carry out the measurements, the setting of the instrument for acetic acid was first determined. The cell was next emptied and allowed to dry out by exposure to the atmosphere until the reading for the cell had returned to the original value for the empty cell. The time required for this varied from 24 hours to 4 days. Solution of a given strength was then introduced, the reading taken after time had been allowed for the system to come to equilibrium with the constant temperature bath, the cell emptied once more and washed several times with acetic acid. When the acetic acid gave a constant reading identical with previous measurements, the cell was dried out again as before and filled with solution of different strength.

In calculating the dielectric constants of the solutions, the value of $\epsilon$ for acetic acid was arbitrarily taken for simplicity as 16, rather than 16.04 as calculated. An alternative method has been chosen for calculating $\epsilon$ for the solutions on the following grounds:-

$\Delta$ T from setting at $C_g$ to setting at $C_l$ for alcohol = 5.1

$\Delta$ T from setting at $C_g$ to setting at $C_l$ for alcohol = 3.3

The ratio of $\Delta$ T for ethyl alcohol to $\Delta$ T for acetic acid should be the same as the ratio of their dielectric constants,

$$\frac{\Delta \text{ T for ethyl alcohol}}{\Delta \text{ T for acetic acid}} = \frac{1.7}{1.1} = 1.55$$

If $\epsilon$ = 16 for acetic acid, then

for ethyl alcohol $\epsilon$ = 16 x 1.55

= 24.8

This is a little higher than the value originally taken for the alcohol, but the differences between the acetic acid solutions and the acetic acid alone are within a much more restricted range than between the acid and the alcohol. Because of this greater restriction in range, less inaccuracy might be involved by finding the ratios of $\frac{\triangle T \text{ from } C_g \text{ to solution}}{\triangle T \text{ from } C_g \text{ to acetic acid}}$ instead of calculating the different values of C within this range. Table 4 lists the values of the dielectric constants calculated from measurements made on acetic acid solutions of 3,6-diethyl-2,5-diketopiperazine, of various concentrations, and the graph showing the relation between $\mathfrak{E}$ and concentration is shown in Fig. 6. Attention is drawn to the variation in the setting for the 0.222 M solution. Using the lower value, $\mathfrak{E}$ at this concentration is seen to lie close to the linear curve which seems to connect the two functions up to 0.445 M. Up to this concentration, the dielectric constant of the solutions appears to increase linearly relative to that of acetic acid and is given by the equation

$\mathfrak{E} = 16.0 + 6.02C$, where C is the concentration.

The value for the 0.557 M solution does not lie on the curve. From experience gained in preparing solutions of the anhydride, this appeared to be approaching the limit of solubility; this may have bearing on the marked drop in dielectric constant.

Table 4.

Dielectric Constants of Acetic Acid Solutions of

3,5-Diethyl-2,5-Diketopiperazine.

| Strength (moles / litre) | $\Delta$T from Cg setting to solution setting | $\frac{\Delta T \text{ solution}}{\Delta T \text{ acetic acid}}$ | $\epsilon$ |
|---|---|---|---|
| 0.111 | 3.44 | 1.042 | 16.67 |
| 0.222 | 3.6--3.68 | 1.091--1.112 | 17.46--17.78 |
| 0.278 | 3.65 | 1.106 | 17.7 |
| 0.371 | 3.75 | 1.136 | 18.18 |
| 0.445 | 3.80 | 1.152 | 18.43 |

After all the measurements had been made, the cell was washed and dried out, disassembled and recoated with fresh paraffin wax, and the cell reassembled. A few days were necessary for the coating to harden sufficiently for continued handling. The settings for the empty cell, for acetic acid, and for the alcohol were found to be the same as before, as was also the volume required to fill the annular space. This duplication is regarded as fortuitous.

3,6-diisopropyl-2,5-diketopiperazine.

This compound was found to be more readily soluble in acetic acid than in ethyl alcohol, but even in acetic acid its solubility was much more restricted than was that of the 3,6-diethyl compound. The maximum strength of solution that could be prepared for purposes of measurement was one containing 0.251 mole per litre. This solution and one of half that strength were the only ones that could be studied within the range of sensitivity of the instrument with the kind of cell being used. Details of strength of solution, change of settings, and calculated dielectric constants are shown in Table 5, and the graph drawn from these data is reproduced in Figure 7.

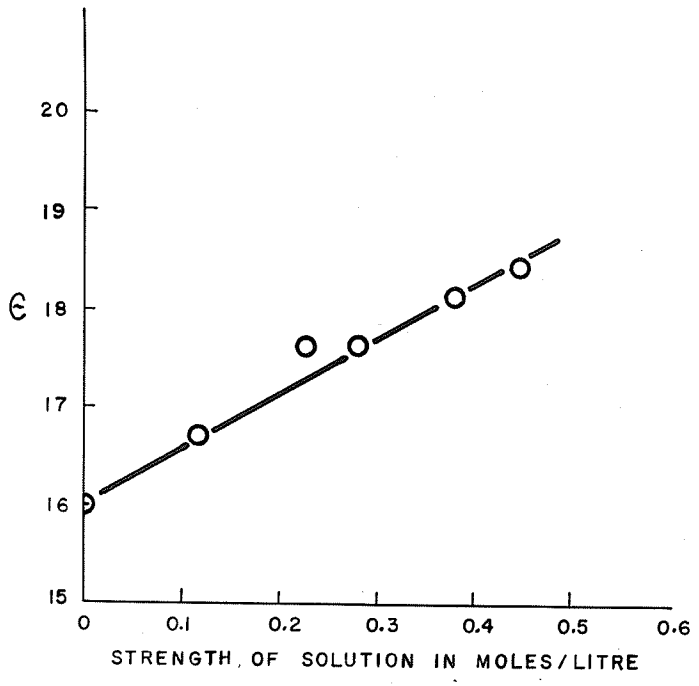



FIGURE 6.

RELATION OF DIELECTRIC CONSTANTS TO CONCENTRATION FOR SOLUTIONS OF 3,6-DIETHYL-2,5-DIKETOPIPERAZINE IN GLACIAL ACETIC ACID AT 25°.

Table 5.

Dielectric Constants of Acetic Acid Solutions of

3,6-Diisopropyl-2,5-diketopiperazine.

| Strength (moles / litre) | $\Delta$ T from $C_6$ setting to solution setting | $\Delta$ T solution / $\Delta$T acetic acid | $\epsilon$ |
|---|---|---|---|
| 0.126 | 3.55 | 1.076 | 17.22 |
| 0.251 | 3.52 | 1.067 | 17.07 |

As with the 3,6-diethyl homologue, the drop in the value of $\epsilon$ at the highest concentration may be associated with the fact that the limit of solubility is being reached.

3,6-Diisobutyl-2,5-diketopiperazine.

This member of the series, as far as studied, formed solutions in acetic acid which had generally higher values for the dielectric constant than the solvent alone, but the value decreased with increase of concentration, in a more or less linear fashion. The greatest concentration made was 0.426 M. No attempt was made to make a more concentrated solution, because the solid was already taking a longer time to dissolve in the liquid and the limit of solubility was not known. With the previous two compounds, too close an approach to the limit resulted in the formation of an apparently crystalline precipitate which separated from solution; the separation in those cases was only partial, and on filtering, the filtrates gave the same reading as acetic acid alone, and further separation from the

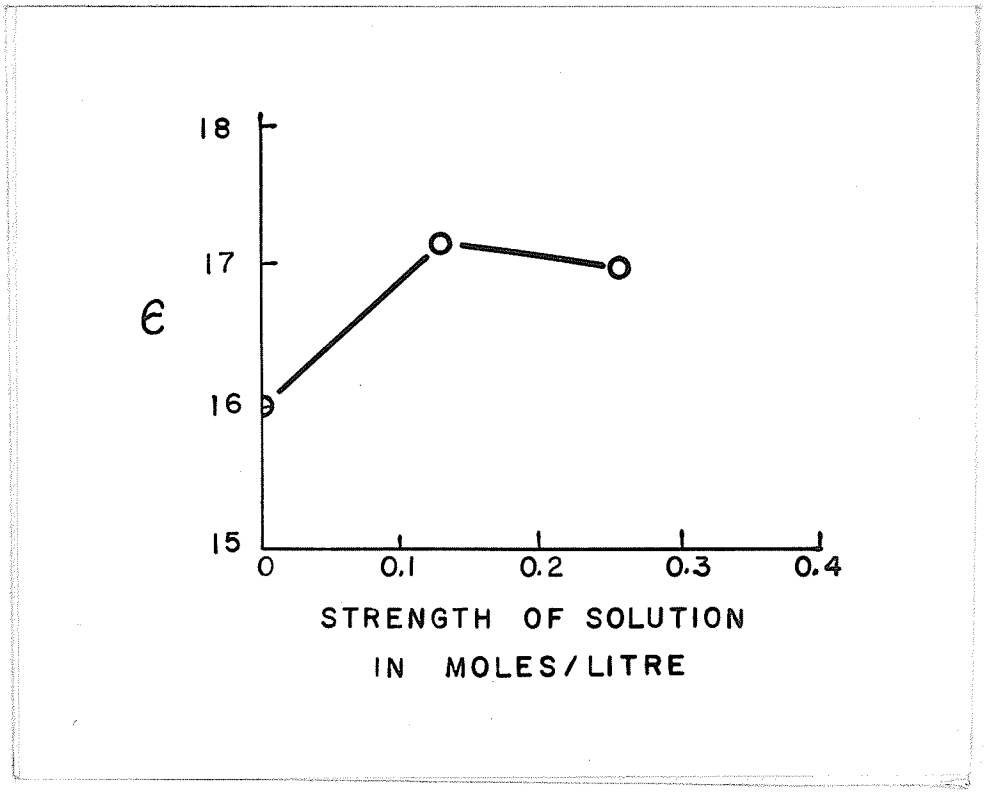



FIGURE 7.

RELATION OF DIELECTRIC CONSTANT TO CONCENTRATION

FOR SOLUTIONS OF 3,6-DIISOPROPYL-2,5-DIKETOPIPERAZINE

IN GLACIAL ACETIC ACID AT 25°.

filtrates took place on standing. The addition of further amounts of acetic acid to the suspensions never effected solution. From these previous encounters and since solution was becoming slower with the compound in question, it was deemed more prudent to avoid the risk which might be inherent in trying to attain higher concentrations. Data pertinent to concentration, change of instrument setting, and calculated values of $\epsilon$ are recorded in Table 6, and the graph from these data is shown in Figure 8. This time the settings for the empty cell, and for acetic acid, were different from those obtained before; this is not unexpected, for it is not to be expected that successive preparations of the cell would give an assembly alike in all respects. The volume of liquid to fill the annular space was also greater.

Table 6.

Dielectric Constants of Acetic Acid Solutions of 3,6-Diisobutyl-2,5-diketopiperazine.

| Strength (moles / litre) | $\Delta$T from $C_g$ setting to solution setting | $\frac{\Delta T \text{ solution}}{\Delta T \text{ acetic acid}}$ | $\epsilon$ |
|---|---|---|---|
| 0.107 | 6.2 | 1.03 | 16.48 |
| 0.213 | 5.85 | 0.98 | 15.68 |
| 0.284 | 6.1 | 1.02 | 16.32 |
| 0.426 | 6.06 | 1.01 | 16.16 |

The gradient of the linear portion of the curve (5) is calculated from the co-ordinates (0.107, 1648) and 0.426,1616) to be 1.003. If the linear part is produced back to the axis of ordinates, it is found to make an intercept of 16.56.

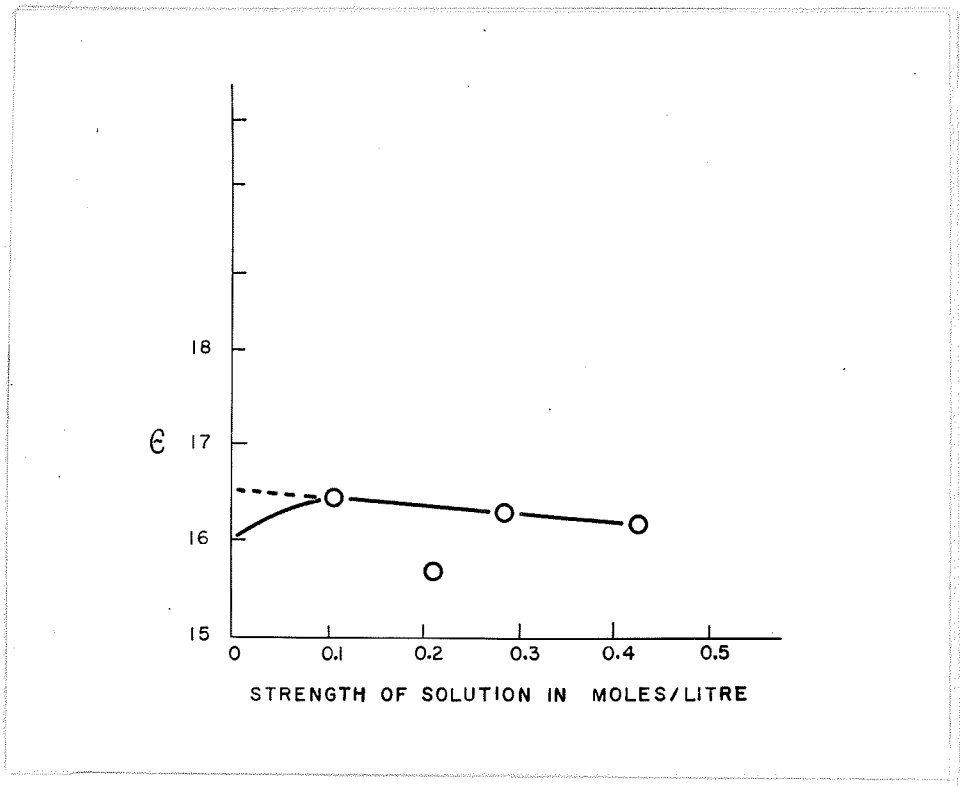
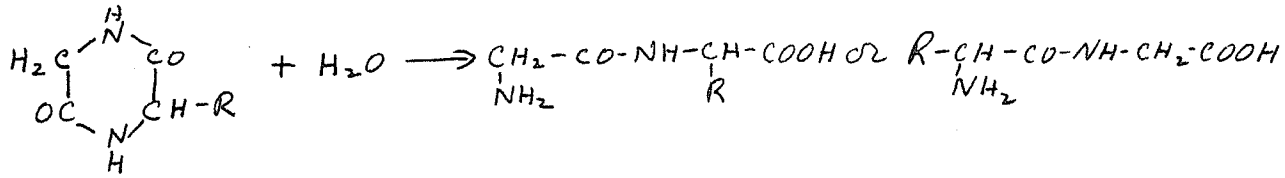


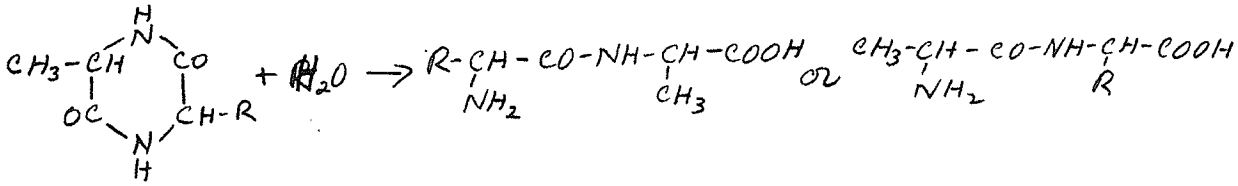

FIGURE 8.

RELATION OF DIELECTRIC CONSTANT TO CONCENTRATION FOR SOLUTIONS OF 3,6-DIISOBUTYL-2,5-DIKETOPIPERAZINE IN GLACIAL ACETIC ACID AT 25°.

III - Hydrolysis of Diketopiperazines.

The hydrolysis of mixed diketopiperazines of the 3-alkyl substituted group and of the 3-methyl-6-alkyl group was studied. The dipeptides formed from members of each class, by acid or alkali, might be as follows:-





In each class, two dipeptides might be formed, or perhaps more of one than the other might be formed; in the latter event some influence due to the substituents in the ring may be exerted. This part of the project was undertaken to determine what answer might be revealed to such problems. The action of 1.0828 N/HCl or of 0.9843 N/NaOH was studied on the following members of the series:-

3-isopropyl-2,5-diketopiperazine

3-sec.butyl-2,5-diketopiperazine

3-isobutyl-2,5-diketopiperazine

3-methyl-6-isopropyl-2,5-diketopiperazine

3-methyl-6-isobutyl-2,5-diketopiperazine

3-methyl-6-sec.butyl-2,5-diketopiperazine.

The end-point of the reaction was always judged in the case of the monosubstitued anhydrides (i.e. the 3-alkyl members) by the absence

of a red colour when a trace of the reaction mixture was heated with a sodium carbonate solution of picric acid, and in the other cases by the absence of a red colour when a trace of the reaction mixture was heated with a sodium hydroxide solution of picric acid. The time and temperature necessary for completion of hydrolysis varied according to the anhydride.

Identification of the products of hydrolysis.

The possible dipeptides to be sought in the products of the reaction from the respective diketopiperazines are listed in the summary below:-

| Substitued diketopiperazine | Possible dipeptides. |
|---|---|
| 3-isopropyl | glycyl-DL-valine, DL-valylglycine. |
| 3-isobutyl | glycyl-DL-leucine, DL-leucylglycine. |
| 3-sec.butyl | glycyl-DL-isoleucine, DL-isoleucylglycine. |
| 3-methyl-6-isopropyl | DL-alanyl-DL-valine, DL-valyl-DL-alanine. |
| 3-methyl-6-isobutyl | DL-alanyl-DL-leucine, DL-leucyl-DL-alanine. |
| 3-methyl-6-sec.butyl | DL-alanyl-DL-isoleucine, DL-isoleucyl-DL-alanine. |

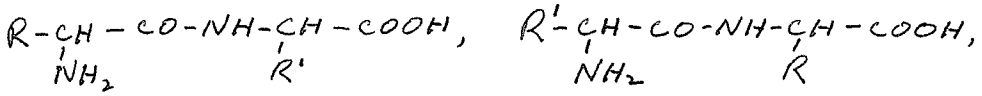
For purposes of identification, it was necessary to have available as many authentic samples of the dipeptides as pos-

sible to enable a comparison to be made of some suitable property of reaction product with the same property of the two possible known dipeptides. Glycyl-DL-valine and DL-valyl-glycine, glycyl-DL-isoleucine and DL-isoleucylglycine, glycyl-DL-leucine and DL-leucylglycine, and DL-alanyl-DL-valine were all purchased commercially, while DL-alanyl-DL-leucine, DL-leucyl-DL-alanine and DL-valyl-DL-alanine were made synthetically by the method of Fischer and associates (157, 158). This method consists of condensing a bromacid bromide with an amino acid in alkaline solution. Acidification liberates the free halogenacylamino acid, which is subsequently converted to the dipeptide by the action of ammonia.

Several approaches to the problem of identification suggest themselves to determine which dipeptides are formed in the hydrolysis of a given diketopiperazine. X-ray powder pictures could be taken of authentic samples of known dipeptides, and the pictures compared with that given by the unknown product. The powder pictures taken of the known dipeptides were unsatisfactory; this was attributed to the lack of a good crystalline form in the samples, possibly arising from the size of the dipeptide molecules. Powder photographs of the unknowns were equally unsatisfactory, so no helpful comparisons could be made for identification. The procedure was therefore suspended pending an investigation of other approaches to the problem.

If the known dipeptides were found to have characteristic

absorption maxima at some definite wavelengths, and if any characteristics were found in the absorption curves which would distinguish a pair of isomers of the type



a study of the absorption characteristics of an unknown would show which of the two isomers were present, thereby serving to identify the products of hydrolysis. With these considerations in mind, dilute aqueous solutions of glycyl-DL-leucine and DL-leucylglycine, were scanned in the near infra-red and in the ultraviolet regions. No characteristic absorption was found, and the use of this technique for identification purposes was discontinued.

The authentic dipeptides could be converted to suitable derivatives and the behaviour of individual dipeptides and of a mixture of two isomeric dipeptides on a chromatographic column could be made. Comparison of the behaviour of such a mixture could then be made with the behaviour of an unknown suspected of containing the isomers under similar conditions and the appropriate conclusions drawn. Sanger developed such a procedure in identifying amino acid mixtures (156). He reacted amino acids with 2,4-dinitrofluorobenzene in presence of sodium bicarbonate solution, liberated the free dinitrophenyl derivatives by acidifying, extracted with suitable organic solvents and studied the behaviour of the solutions when passed through a suitable adsorption column.

An attempt was made to adapt Sanger's technique to the dipeptides available. Glycyl-DL-leucine and DL-leucylglycine were each separately stirred with an alcoholic solution of 2,4-dinitrofluorobenzene in presence of aqueous sodium bicarbonate solution for 24 hours. The solution was acidified and the alcohol evaporated of as much as possible under reduced pressure. No solid separated out (contrary to the description of isolation of such derivatives recorded later on page 147). The residue (essentially aqueous) was shaken repeatedly with chloroform to extract the derivative presumed to have been formed, and the chloroform layer removed.

The chloroform solution (estimated about 0.1 gm. product in 100 c.c. solvent) was placed in a chromatographic tube packed with activated magnesia. Passage of solvent through the column was extremely slow, and all colour was situated at the top of the column. This might indicate that complete adsorption had taken place at the top of the column, or else the chemical reaction had occurred between the magnesia and the acidic derivative. Further quantities of chloroform failed to shift the band, as was also the case when other liquids such as petroleum ether, benzene, or toluene were added. Similarly, when a solution of the two dipeptides in chloroform was placed in a like column, the yellow band at the top did not separate.

The use of chromatographic cellulose instead of magnesia resulted in initial adsorption of the coloured matter over the whole length of the adsorbent, colourless solvent issuing from

the bottom of the column. As more solution was introduced to the top of the column, however, the adsorbed colouring matter was rapidly eluted. Sanger reports that the 2,4-dinitrophenyl derivatives of the amino acids were only weakly adsorbed by cellulose, so the findings in this instance are perhaps not surprising.

The use of filtercel as an adsorbent did seem to effect much stronger adsorption than the cellulose, but elution was very rapid, and no separation of bands occurred when a solution of the two dipeptides together was passed through the column.

A dry silica gel column was tried, but no adsorption from the solution took place. Another was prepared by mixing silica gel with chloroform. On introducing the solution, a concentration of colour at the top of the column was all that was observed, and the solution above the column was not decolourised. Similar negative results were obtained when a column was made by mixing silica gel with water.

Preliminary attempts to make use of paper chromatography were equally unsuccessful. The problem of evaporation proved a major one, and when it was surmounted, the distances travelled by the bands from two different solutions, one isomer in each, were equal. No resolution from a solution of the two was achieved, which would seem to be consistent with the observation on the two separate solutions. Sanger reported failure in the use of paper chromatography with the dinitrophenyl derivatives of amino acids.

Before pursuing these preliminary attempts to devise suitable means of utilising chromatographic techniques, i.e. seeking the right solvent or combination of solvents, the right adsorbent or combination of such materials, and the right liquid or combination of liquids for developing and eluting the products, it was decided to investigate the possibility of preparing and isolating the 2,4-dinitrophenyl derivatives or other suitable derivatives, and seeking solution of the identification problem by melting point determination.

This latter approach turned out to give much more indication of being fruitful, so all the previous approaches, viz. X-ray powder photographs, absorption spectra, and chromatographic methods, were abandoned in its favour.

Preparation of the 2,4-dinitrophenyl derivatives of the dipeptides.

A solution of 2,4-dinitrofluorobenzene was made by dissolving 1 gram of the reagent in 50 c.c. ethyl alcohol. 0.1 gram of dipeptide was dissolved in a solution of 0.1 gram sodium bicarbonate in 5-7 c.c. water. Five c.c. fluorodinitrobenzene solution was added to the dipeptide solution, and the resulting yellow turbidity redissolved by adding an additional 5-10 c.c. ethyl alcohol. The solution was allowed to stand 24 hours at room temperature, and at the end of this time the volume of the solution was reduced by evaporating off as much of the alcohol as possible under reduced pressure from a water bath at 35°-40° C. A brownish yellow solid was formed and was

converted to the required derivative by the addition of dilute hydrochloric acid. This treatment produced an intensely yellow solid which was filtered off by suction and washed several times with water. The solid was detached from the filter, pressed on a piece of porous plate and kept in a vacuum desiccator for 48 hours. The procedure is more or less an exact duplication of Sanger's preparation of the amino acid derivatives.

The author wishes at this stage to draw attention to certain incidentals which evidently have to be borne in mind in carrying out the preparations. The observations made are merely an aside with respect to detail. At the time it seemed surprising that they should play any part in the success or failure of the experiment, but failure was always inevitable when they were ignored.

(i) Turbidity on adding the alcoholic FDNB solution.

If further quantities of alcohol were not added to regain complete solution, the only product obtained on acidifying was a brown oily mass. This happened even when the system had been stirred from the outset. Attempts to purify were unsuccessful. Either the reaction had not taken place, or was incomplete and the presence of unreacted reagent prevented solidification of any derivative formed.

(ii) Even if enough alcohol had been added to effect complete solution, it was necessary to perform the reaction in a flask rather than in a tube. In the latter case, no matter whether stirring had been used or not, a brown solid was liable to separate after several hours, and could not be redissolved on stirring or shaking. The addition of further quantities of alcohol failed to bring about solution. Acidification after evaporation only produced a yellow turbidity turning to a red sticky mass which could not be recrystallised. It was decided to carry out the reaction in a 125 ml. Erlenmeyer flask or a 100 ml. round flask. In the event of this intermediate precipitation recurring, it was possible that stirring might be more effective in redissolving the material. When this change in detail was made, precipitation never occurred, and stirring of the reaction mixture was found unnecessary.

The derivatives and their melting points <u>as prepared</u> are tabulated in Table 7. The general type equations for the reaction are as shown below.

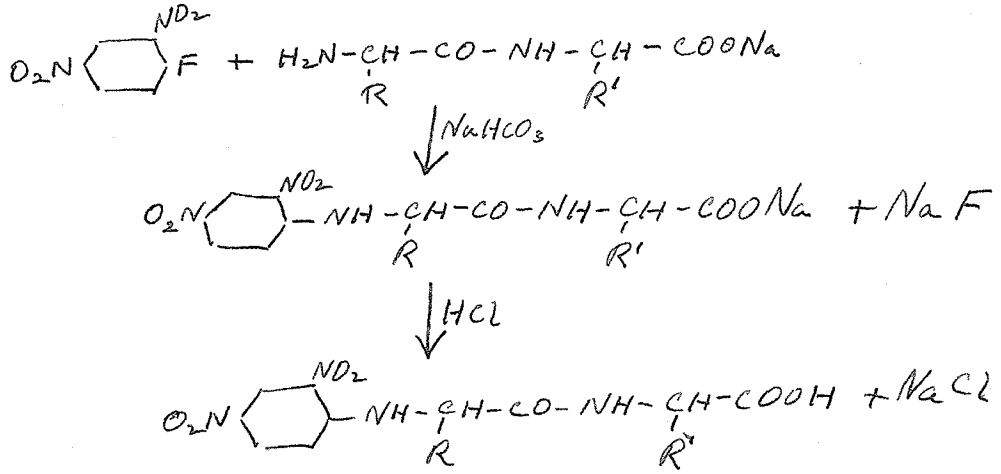


Table 7.

Melting points of 2,4-dinitrophenyl derivatives of dipeptides as prepared.

| Name of derivative. | Melting point. (uncorrected) |
|---|---|
| 2,4-dinitrophenylglycyl-DL-valine | 183°-190° |
| 2,4-dinitrophenyl-DL-valylglycine | 189°-194° |
| 2,4-dinitrophenylglycyl-DL-isoleucine | 125°-128° |
| 2,4-dinitrophenyl-DL-isoleucylglycine | 186°-189° |
| 2,4-dinitrophenylglycyl-DL-leucine | 155°-157° |
| 2,4-dinitrophenyl-DL-leucylglycine | 134°-137° |
| 2,4-dinitrophenyl-DL-alanyl-DL-valine | 188°-191° |
| 2,4-dinitrophenyl-DL-valyl-DL-alanine | 214°-218° |
| 2,4-dinitrophenyl-DL-alanyl-DL-leucine | 125°-130° |
| 2,4-dinitrophenyl-DL-leucyl-DL-alanine | 188°-191° |

Acid Hydrolysis of Diketopiperazines.

The anhydrides were shaken with 1.0828N HCl at room temperature and tested for completion of reaction by means of the picric acid test. At the end of thirty days the reaction, if any, was most certainly incomplete. Resort was made to immersion of the reaction mixtures in a hot water bath, and the hydrolysis generally was found to be considerably accelerated. Details of time and temperature for any given reaction are given in the appropriate heading. When the end point had been deter-

mined for hydrolysis of a particular diketopiperazine, a repeat run was made for the same length of time at the same temperature; solution was always complete and the picric acid test found negative at the end of the period. The hydrochloric acid was neutralised by addition of the theoretical amount of 0.9843 N NaOH as determined by previous titration. The water was now removed by evaporation under reduced pressure from a water bath at 35°C. The residual solid was rendered as dry as possible by covering with alcohol and evaporating from a boiling water bath, repeating this operation a further two times. The residual solid was collected, weighed, and placed in a stoppered container. No attempt was made to separate the product from the sodium chloride formed on adding the alkali.

Attempts were made to identify the dipeptide constituents of the residues by conversion to the 2,4-dinitrophenyl derivatives. It was necessary to make allowance for the amount of sodium chloride present in the solid when calculating the amount of fluorodinitrobenzene solution required. Failure to observe this precaution resulted in failure to obtain a satisfactory derivative; instead of a crystalline precipitate on acidifying, a brown or yellow resinous mass was formed which defied efforts to recrystallise. Presumably the presence of excess reagent prevented proper solidification of the product.

To meet such exigencies, a stock solution of fluorodinitrobenzene was made by dissolving 1 gram of the reagent in 100 c.c.

of ethyl alcohol. Five c.c. of the solution was added to an aqueous solution of 0.1 gram of hydrolysis product in 7-10 c.c. water and 0.1 gram sodium bicarbonate. The remainder of the procedure was the same as described previously for the known dipeptides. The melting points (uncorrected) of the dinitrophenyl derivatives of the unknowns as prepared were determined and mixed melting points with the derivatives of the known dipeptides studied.

## Acid Hydrolysis of 2,5-Diketopiperazines.

### 3-isopropyl-2,5-diketopiperazine.

Four hours was required for the hydrolysis of 0.5 gm. anhydride by 6.3 ml. 1.0828 NHCl. The melting point of the dinitrophenyl derivative was 149-156°C. A mixture of 50% unknown derivative and 50% dinitrophenylglycyl-DL-valine melted at 162°-168°C, and a mixture of 50% unknown derivative with 50% DL-valylglycine melted at 172-175°.

Conclusions:- Hydrolysis of 3-isopropyl-2,5-diketopiperazine under the conditions described gives glycyl-DL-valine and DL-valylglycine, both in considerable proportion.

### 3-sec.butyl-2,5-diketopiperazine.

Time required for complete hydrolysis of 0.5 gm. compound by 6.3 ml. 1.0828 NHCl - 3½ hours at 90-100°.

Melting point of 2,4-dinitrophenyl derivative as prepared 180-192°C.

Melting point of 50% DNP unknown and 50% DNP -glycyl-DL-isoleucine was 125°-172°C.

Melting point of 50% DNP unknown and 50% DNP-DL-isoleucylglycine was 172-184°C.

Conclusions: - Hydrolysis of 3-sec. butyl-2,5-diketopiperazine under the conditions described gives glycyl-DL-isoleucine and DL-isoleucylglycine, rather more of the latter being formed than of the former.

3-isobutyl-2,5-diketopiperazine.

Time required for complete hydrolysis of 0.5 gm. compound with 6.3 ml. 1.0828 NHCl-4 hours at 90°- 100°C.
No satisfactory derivative could be obtained. As this may have been due to some inherent factor in the character of the products, a further attempt was made to effect the hydrolysis. This time the reaction required nine hours for completion of hydrolysis by heating 0.5 gm. anhydride in a boiling water bath with 12.4 ml. 1.0828 NHCl. A considerable amount of solid was still undissolved after 8 hours, at which time solution was observed to commence. Solution was very slow, and was only complete at the end of the ninth hour. The solid was recovered in the usual way.

Condensation with fluorodinitrobenzene gave a yellow crystalline solid melting sharply at 84°.

When a small amount of DNP-glycyl-DL-leucine was mixed with a sample of the unknown, the melting point was depressed $10^{\circ}$, and when a small amount of DNP-DL-leucylglycine was mixed with a sample of the unknown, the melting point was depressed $5^{\circ}$. It is concluded that the DNP derivative of the unknown is not identical with that of either dipeptide, showing that neither dipeptide was present in the hydrolysis product.

3-Methyl-6-isopropyl-2,5-diketopiperazine.

Time for complete hydrolysis of 0.5 gm. compound with 6.3 ml. 1.0828 N/HCl -- 4 hours at $90^{\circ}$-$100^{\circ}$C.

No satisfactory derivative could be obtained. Another attempt at hydrolysis of 0.5 gm. anhydride with 12.4 ml. 1.0828 N/HCl on a boiling water bath was carried out. Hydrolysis, if any, was incomplete after 17 hours, no evidence of any solution being apparent, and the reaction was not examined further.

3-Methyl-6-isobutyl-2,5-diketopiperazine.

Time for complete hydrolysis of 0.5 gm. compound with 5.8 ml. 1.0828 N/HCl -- 3 hours at $90^{\circ}$-$100^{\circ}$.

No satisfactory derivative could be obtained. A further trial was made by heating 0.5 gm. anhydride with 11.6 ml. 1.0828 N/HCl on a boiling water bath. Complete hydrolysis and solution required 11 hours. The required volume of 1.006 N/NaOH was added

and the solid recovered in the usual manner. A dinitrophenyl derivative was successfully prepared from the residue, and melted at 82-84°. When mixed with a small amount of DNP-alanyl-leucine, the melting point was 77-80°C. When DNP-alanylleucine was mixed with a small amount of the unknown, the melting point was 110°-120°.

When a small amount of DNP-leucylalanine was mixed with the unknown derivative, the melting point was 90-130°, remelt 84°, and when a small amount of unknown derivative was added to DNP-leucylalanine, the melting point was 180-185°, remelt 177-179°.

Conclusion:- 3-methyl-6-isobutyl-2,5-diketopiperazine under the conditions described gives mainly DL-leucyl-DL-alanine.

Alkaline Hydrolysis of 2,5-diketopiperazines.

The hydrolysis was carried out at room temperature or at 37° as required. The time was usually quite protracted. Clear solutions such as resulted from acid hydrolysis were not obtained. At the end of the reaction period, as judged by the picric acid test, the liquid was milky but no gross particles remained. The alkali was neutralised by adding the theoretical amount of 1.0828 NHCl, and the milkiness persisted. The solid matter was recovered in a similar manner to that outlined under acid hydrolysis, and rendered as dry as possible by a similar alcohol treatment. Identification was sought through the medium of the dinitrophenyl derivative, prepared as described before and com-

pared with the corresponding derivatives of the standards. As with the products of acid hydrolysis, allowance had to be made for the sodium chloride present in the unknown before carrying out the condensation.

3-isopropyl-2,5-diketopiperazine.

Time for complete hydrolysis of 0.5 gm. with 7.42 ml. 0.9843 N/NaOH -- 4 weeks at room temperature.

Melting point of DNP derivative as prepared. 165°-172°.

Melting point of 50% DNP unknown with 50% DNP-glycyl-DL-valine 160°-168°.

Melting point of 50% DNP unknown with 50% DNP-DL-valylglycine 169°-181°.

Conclusions:- 3-isopropyl-2,5-diketopiperazine under the conditions described gives some glycyl-DL-valine, but rather more DL-valylglycine.

3-sec.butyl-2,5-diketopiperazine.

Time for complete hydrolysis of 0.5 gm. compound with 6.81 ml. 0.9843 N/NaOH -- about one week at room temperature.

Melting point of DNP derivative as prepared 129°-133°C.

Melting point of 50% DNP unknown with 50% DNP-glycyl-DL-isoleucine 123°-127°.

Melting point of 50% DNP unknown with 50% DNP-DL-isoleucylglycine 169 - 173°.

Conclusion: - 3-sec.butyl-2,5-diketopiperazine under the conditions described gives mainly glycyl-DL-isoleucine.

3-isobutyl-2,5-diketopiperazine

Time for complete hydrolysis of 0.5 gm. compound with 6.81 ml. 0.9843 N NaOH--one week at 37°, somewhat longer at room temperature.

Melting point of DNP derivative as prepared 143° - 146°.

Melting point of 50% DNP derivative and 50% DNP-glycyl-DL-leucine 140°-145°.

Melting point of 50% DNP derivative and 50% DNP-glycyl-DL-leucylglycine 110°-117°.

Conclusion:- 3-isobutyl-2,5-diketopiperazine under the conditions described gives glycyl-DL-leucine as main product.

3-methyl-6-isopropyl-2,5-diketopiperazine

When 0.5 gm. of the anhydride was shaken with 6.81 ml. 0.9843 N NaOH, hydrolysis, if any, was incomplete after 8 weeks, and little if any solution occurred. The residue was not examined further.

3-methyl-6-sec.butyl-2,5-diketopiperazine.

Time for complete hydrolysis of 0.5 gm. compound with 6.22 ml. 0.9843 N NaOH--one to six weeks at room temperature. No DL-alanyl-DL-isoleucine or DL-isoleucyl-DL-alanine was available for making derivatives for comparison, so the residue was not examined further.

3-methyl-6-isobutyl-2,5-diketopiperazine.

Attempts to hydrolyse this compound were no more successful than the previous two cases.

It is convenient to summarise the results of the acid and of the alkaline hydrolyses respectively as in Table 8.

Table 8.

Summary of results of acid and alkaline hydrolysis of diketopiperazines.

| 2,5-diketopiperazine studied | Main product of acid hydrolysis. | Main product of alkaline hydrolysis. |
|---|---|---|
| 3-isopropyl | glycyl-DL-valine and DL-valylglycine, both in considerable proportion. | rather more DL-valyl-glycine than glycyl-DL-valine. |
| 3-isobutyl | no evidence of presence of dipeptides. | mainly glycyl-DL-leucine. |
| 3-sec.butyl | rather more DL-iso-leucylglycine | mainly glycyl-DL-isoleucine |
| 3-methyl-6-isopropyl | not identified when hydrolysis effected, and in repeat trial anhydride was not hydrolysed. | hydrolysis not effected. |
| 3-methyl-6-sec.butyl | ---------- | product not identified. |
| 3-methyl-6-isobutyl | mainly DL-leucyl-DL-alanine | hydrolysis not effected. |

## Discussion of Results.

### I. Synthesis of Diketopiperazines.

The procedures described for the syntheses are essentially a variation and extension of those used by Sannié. In his synthesis of alanine anhydride by heating alanine with water and glycol, he first dissolved the amino acid in boiling water, then added the glycol, and used a reaction temperature of 170-175° for a total of 6 hours. He reports considerable precipitation of amino acid after 1½ hours on temperature, redissolving after 2 hours. In the work of this thesis no precipitation occurred in the preparation of any of the simple diketopiperazines. He also reports evolution of a dense white vapour from the reaction flask during the heating period, a phenomenon never encountered in any preparation in this project.

In this work, no effort was made to recover further quantities of simple diketopiperazines from the glycol and alcohol-ether filtrates as Sannié did. He obtained a 70% yield of pure product in the first instance, and 6.27% recovered from the filtrates, but less nearly pure. The yield of 66.8%-75% of pure product described in the experimental section of this thesis seemed to warrant dispensing with attempted recovery of further product from washings. A comparison is appended in Table 9 of the yields obtained by Sannié with those described in the experimental section, using the methods described.

Table 9.

Comparison of yields of simple diketopiperazines with these of Sannié. %

| 2,5-diketopiperazine | main crop | less pure product from washings. | Yield in this work. |
|---|---|---|---|
| 3,6-dimethyl | 70 | 6.27 | 66.8-75 |
| 3,6-diethyl | 52.6 | 4 | 71.6 |
| 3,6-di-n-propyl | 55.5 | 9.5 | ------- |
| 3,6-diisopropyl | 47.3 | 10 | 60.6-72.2 |
| 3,6-diisobutyl | ---- | --- | 70 -80 |
| 3,6-disec-butyl | ---- | --- | 40 -60 |

As the water initially used evaporates during the heating, the possibility of dispensing with its use is a natural consideration, and Sannié did so claiming some success. Table 10 shows the comparison of his yields without water with those of this project using water.

Table 10.

Comparison of yields of simple diketopiperazines obtained by Sannié (using anhydrous glycol) and in this work (using aqueous glycol initially). %

| 2,5-diketopiperazine | Sannié main crop | less nearly pure product from washings. | Yield in this work. |
|---|---|---|---|
| 3,6-dimethyl | 50.2 | 20 | 66.8-75 |
| 3,6-diethyl- | 55.8 | 17 | 71.6 |
| 3,6-di-n-propyl | 52 | 11.8 | ------- |
| 3,6-diisopropyl | 47.3 | 9.5 | 60.6-72.2 |
| 3,6-diisobutyl | 40 | ---- | 70 -80 |
| 3,6-disec.butyl | ---- | ---- | 40 -60 |

In all these preparations, his heating period was 30-60 minutes.

A preliminary procedure had been developed by another worker at the University of Minnesota for the preparation of glycine anhydride, and it had appeared that it was desirable to use water in the beginning. It was decided, therefore, that in all the preparations carried out in this project, the water should be used. Furthermore, Sannié in this phase of his investigations carried out the reaction at a higher temperature--the boiling point of glycol. In the work of this thesis the experience seemed to indicate that it was desirable to adhere to the range 180-185°.

It is not absolutely clear whether Sannié used the glycol alone in preparing mixed diketopiperazines, but it appears that such was the case. The compounds which he prepared in this series were the 3-methyl, the 3-methyl-6-ethyl, the 3-methyl-6-n-propyl, and the 3-methyl-6-isobutyl homologues. In his first attempt to prepare the 3-methyl compound he used a mixture of glycine and alanine, but evidently found difficulty in obtaining the desired product. He modified his procedure dissolving alanine in the glycol by heating, then adding a slight deficiency of glycine to the solution, and performed the reaction at the boiling point for 25 minutes. He removed the glycol by vacuum distillation, added ethyl alcohol to the residue, and allowed to refrigerate 24 hours. A quotation of the translation of his procedure from that point follows:-

"----4.8gm. of a slightly coloured product is obtained. By crystallisation from alcohol, two fractions are easily separated: the first ---- consists of a mixture of glycine anhydride and glycylalanine anhydride, and a second fraction of 8.8 gm. consisting of 3-methyl-2,5-diketopiperazine".

As he used 5 gm. alanine and 4.8 gm., the theoretical yield of product would be 6.83 gm.

To what extent his original difficulty in effecting the condensation may have been caused by solubility problems is hard to say. As stated in the experimental section, solution of both amino acids was complete before boiling commenced, due to the use of water; the product obtained was the correct one.

3-Methyl-6-ethyl-2,5-diketopiperazine.

In this preparation Sannié does not state whether he mixed the alanine and ∝-aminobutyric acid initially or added one to the solution of the other in hot glycol, nor does he state whether this is necessary in order to secure the right product. Such a problem did not arise in the author's preparation because solution was complete at a temperature well below that necessary for reaction; the method of isolation, while different from Sannié's (ether instead of acetone) gave a yield quite comparable to his yield of 62%. (Sannié does not give details of his isolation procedure).

3-methyl-6-isobutyl-2,5-diketopiperazine.

In this work the details of reaction time, temperature, and isolation procedure were different from those used by Sannié. Briefly, he boiled the glycol solution for 1½ hours, to about one-half the original volume. To isolate the product, he diluted the mixture, after cooling, with three times its volume of acetone, obtaining an impure product in 60.5% yield. The yield of 64.27% quoted in the experimental section is comparable with his, and while a greater degree of purity would be desirable, it was adequate for later requirements. The author was fortunate enough to escape the problem of frothing which Sannié encountered when he endeavoured to remove the glycol from the reaction mixture under vacuum (Sannié met the same problem in the preparation of alanylaminobutyric anhydride). He (Sannié) recommends preliminary evaporation of the glycol at ordinary pressure to small bulk, followed by addition of acetone and ether, and refrigerating. The solid which separates is filtered off, the acetone and ether evaporated from the filtrate by heating on a water bath, and the residual glycol distilled under vacuum.

The purpose of changing the isolation procedure in this work was to avoid using these various steps, since in any case the frothing did not occur, and to determine whether improvement in yield and purity could be accomplished. While the yield of crude product is somewhat higher, improvement in quality has evidently not been achieved to a marked degree, and the time

taken for escape of solvents from product is a marked disadvantage.

3-ethyl-2,5-diketopiperazine

A yield of 55.8% is lower than might be hoped for, but the reddish-brown oil passing into the filtrate could have been responsible for some losses.

3-isobutyl-2,5-diketopiperazine.

Yields of 47.26-47.34% are disappointingly low, but the problem of the oily by-product seems to be a contributing factor. It will be noted that the problem of frothing was encountered during vacuum distillation of the ethylene glycol.

3-sec.butyl-2,5-diketopiperazine.

The yield of 55.6% is lower than the yields in most of the other preparations, and the product was impure as judged by colour. The coloured oily by-product could be responsible for losses as postulated in other instances.

3-methyl-6-isopropyl-2,5-diketopiperazine.

The yield of 64.68% of theory for the main crop is more in keeping with the yields obtained in most of the other preparations in this work and in Sannié's experiments. The variation in reaction time is not understood. No further trials

were carried out as supplies of chemicals were not adequate to permit of a more extensive investigation into the synthesis

3-methyl-6-sec.butyl-2,5-diketopiperazine

The yield of main crop, though slightly lower than might be wished, is compensated by the high recovery in the second crop from the filtrate.

3-ethyl-6-isopropyl diketopiperazine

The yield of 60.64% is as satisfactory as that of other products prepared in this work, and purity as judged by colour is satisfactory. The reaction time is variable, but the cause was not sought since the yield was consistent in two consecutive preparations.

3-ethyl-6-isobutyl-2,5-diketopiperazine

Quality as judged by colour was satisfactory, and the yield was the highest obtained for any mixed anhydride in this work.

3-ethyl-6-sec.butyl-2,5-diketopiperazine

Quality as judged by colour was satisfactory, and while the total yield was below 70%, it was higher than the usual for the mixed series.

3-isopropyl-6-isobutyl-and 3-isopropyl-6-sec.butyl-2,5-diketopiperazines.

The lower yields are attributed to greater solubility in ethyl alcohol than the simple diketopiperazines.

Identification of the mixed diketopiperazines.

It was necessary to ensure that in each case the product was the mixed anhydride and not a mixture of the two simple anhydrides. This objective was determined in each case by determining the melting point of the product, and of mixtures of the product with each of the two simple anhydrides in turn. As the melting points are generally very high, little regard was paid to differences between observed temperature for product alone and that recorded in the references in Beilstein, attention being focussed solely on the qualitative observation of the behaviour of mixtures.

In no single case of compounds in the following list was product found identical with a simple anhydride, indicating that the products were the desired mixed anhydrides. Addition of a small amount of simple anhydride usually depressed the melting point of the product; in any instance where this was not so, a small amount of product depressed the melting point of a simple anhydride.

Purity of the Mixed Diketopiperazines.

Melting-points are recorded in the literature references in Beilstein for each of the following 2,5-diketopiperazines:-

3-methyl; (i)

3-ethyl; (ii)

| | |
|---|---|
| 3-sec.butyl; | (iii) |
| 3-isopropyl; | (iv) |
| 3-isobutyl; | (v) |
| 3-sec.butyl; | (vi) |
| 3-methyl-6-isopropyl | (vii) |
| 3-methyl-6-isobutyl | (viii) |
| 3-methyl-6-sec.butyl | (ix) |
| 3-isopropyl-6-sec.butyl | (x) |
| 3-isopropyl-6-isobutyl | (xi) |
| 3-isobutyl-6-sec.butyl | (xii) |

In all cases, except (vii) and (ix), the melting points obtained were of an order of magnitude comparable to the values given in the Beilstein references, and as already stated, mixed melting points with the corresponding simple anhydrides usually showed complete lack of identity. The melting points of (vii) and (ix), however, were below 200°, indicating presence of impurity. The impurity possibly consisted of adhering solvents.

As an additional check on the identity of (x) and (xi), samples of these were analysed at the Microanalytical Laboratory of the National Research Council of Canada through the courtesy of Dr. L. Marion and Dr. J. F. Eagen. The data obtained were:-

| | (X) | (XI) |
|---|---|---|
| C% - | 62.43, 62,33 (theor. 62.26%) | 62.10, 62.18 |
| H% - | 9.29, 9.28 (theor. 9.43%) | 9.24, 9.38 |
| N% - | 13.36, 13.20 (theor. 13.21%) | 12.94, 13.04 |

These results are quite different from the analytical data recorded in Table 1 for valine anhydride, isoleucine anhydride and leucine anhydride.

3-ethyl-6-isopropyl-2,5-diketopiperazine, the 3-ethyl-6sec.butyl, and the 3-ethyl-6-isobutyl compounds are less well known than the others mentioned, as there seems to be little if any reference to them in the literature. The first showed a slight increase in melting point when mixed with small amounts of aminobutyric anhydride or valine anhydride, but each of these simple anhydrides had their melting points decreased by addition of small amounts of product. Similar behaviour was observed when mixed melting points of the second with aminobutyric anhydride and isoleucine anhydride were determined. The melting point of the third was lowered when a small amount of aminobutyric anhydride was added, but was raised slightly when a small amount of leucine anhydride was added; the melting point of leucine anhydride was lowered when a small amount of product was added to it.

Identification of (xii) was a little difficult. A mixed melting point of product with a little leucine anhydride showed a slight lowering of melting point of product, but when a little isoleucine anhydride was added to product, a melting range was observed commencing slightly below the melting point of product and ending at a slightly higher temperature than the melting point of the simple anhydride. The close similarity in the structures of the three isomers may lead to the formation of solid

solutions of fairly similar melting points, as their separate melting points were all fairly similar.

II. Dielectric Constants.

The diketopiperazines and the apparatus used were available, so the dielectric constants were measured, using the one solvent which seemed to serve equally well for all three compounds. The value of 6.02 for $\epsilon$ in the case of the 3,6-diethyl compound seems to indicate an appreciable polar character due to the imide character of the ring, even though, as De Voto points out, the ring does not have a zwitterion structure. De Voto also points out the amino acids have a negative value for $\delta$ in solutions where the solvent has a dielectric constant less than 20, so that the ring would appear to have a polar character due to its imide structure and independent of the lack of zwitterion structure.

The data obtained for 3,6-diisopropyl-2,5-diketopiperazine may not be spread over a sufficiently wide range of concentrations to make possible the deduction of a relationship showing the main trend of the effect of concentration on the value of $\epsilon$. Due to the cell, the instrument was not sufficiently sensitive to give a reliable reading for a concentration intermediate between the two for which measurements were made. An attempt to study a solution of half the strength of the lower of the two concentrations gave readings very little different from the setting for acetic acid. Attention would have to be focussed on the

two sole readings obtained in order to make any deductions regarding the effect of concentration on $\epsilon$, if the two readings were adequate for such a purpose. In the absence of further data, the only positive statement that could be made is that the compound does appear sufficiently polar to form solutions having a higher value for $\epsilon$ than the solvent, and that increasing concentration tends to lower the value of $\epsilon$. From the coordinates of these two points the value for $\delta$ would be -1.20.

The change from a value of + 6.02 for the 3,6-diethyl compound to - 1.20 for the 3,6-diisopropyl compound could then be explained perhaps by the increase in the size of the alkyl side chains, if not in their length, causing the dielectric constant of the 3,6-diisopropyl compound to have a lower value than the lower homologue.

The data obtained for 3,6-diisobutyl-2,5-diketopiperazine are perhaps more adaptable for discussion in view of their slightly greater number. In this case the effect is an increase in the value of $\epsilon$ (except at 0.213 M), but much less than with the two lower homologues, and a lower (numerically speaking) value for $\delta$, namely 1.003. Such results could be explained on the basis of the increase in length of the alkyl side chains, causing a diminution in the polar character of the molecule.

III. Hydrolysis of Diketopiperazines.

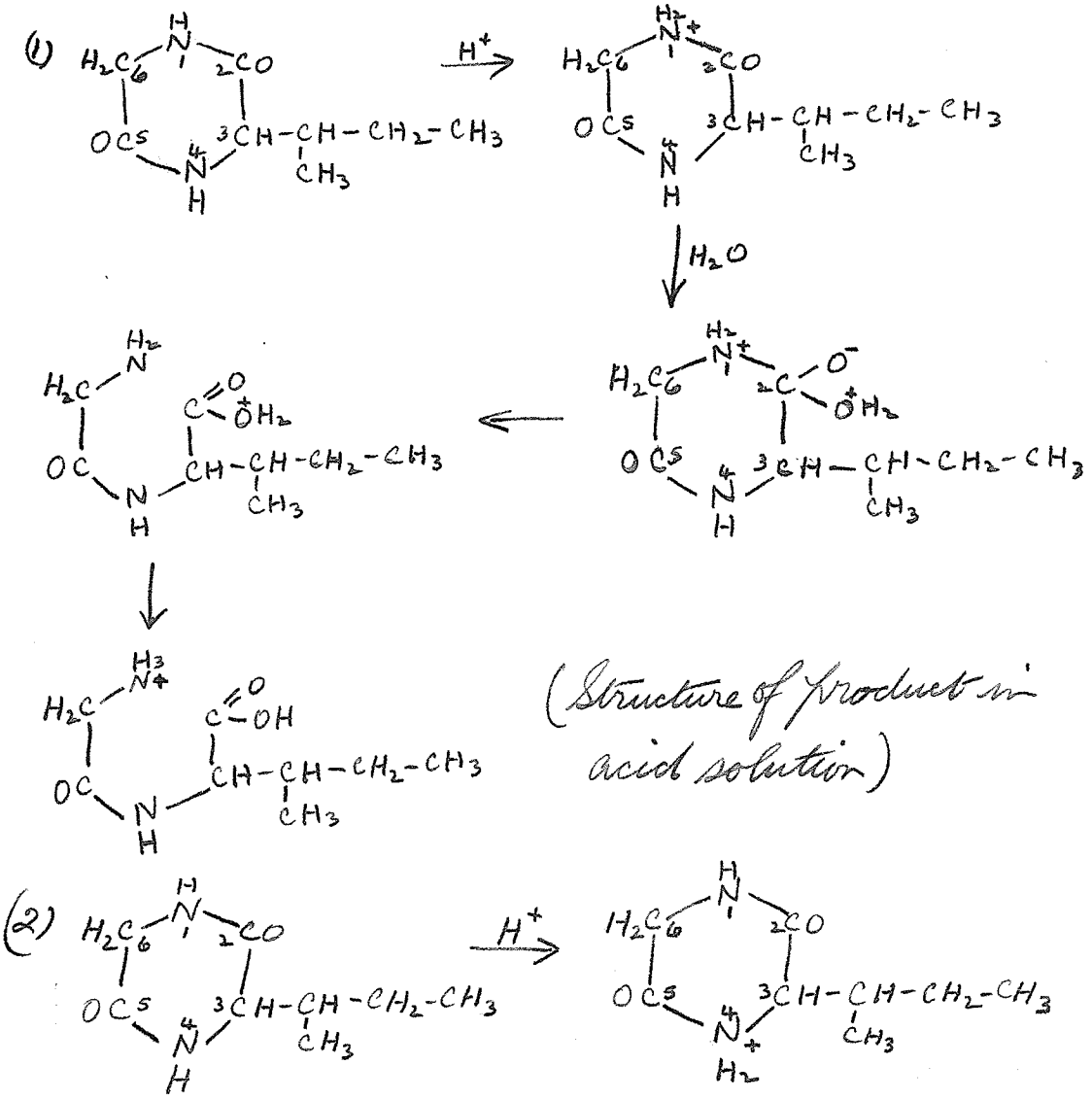
Acid Hydrolysis.

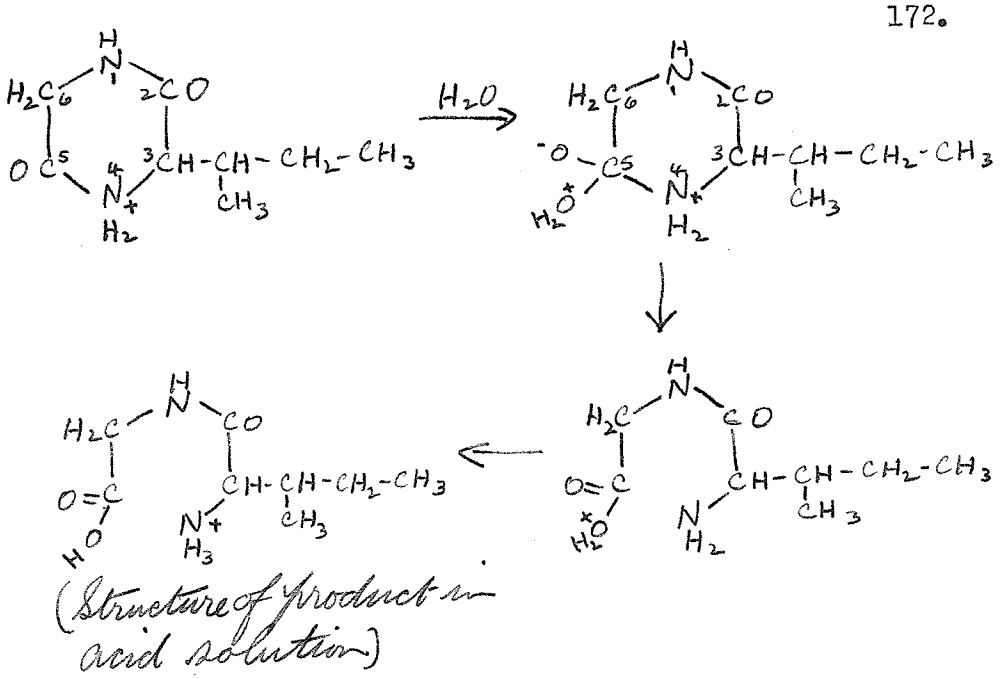
3-isopropyl-2,5-diketopiperazine.

Evidently no steric or other effects arise from the presence of the isopropyl group in position no.3 of the ring.

3-sec.butyl-2,5-diketopiperazine.

In attempting to apply Ingold's (150) proposed mechanism for amide hydrolysis, two possibilities are suggested:-





(Structure of product in acid solution)

That both possibilities are realized is evident from the fact that both dipeptides were obtained. Since DL-isoleucyl-glycine appeared to be formed in somewhat greater amount than glycyl-DL-isoleucine, the second possibility seems to be favoured to a somewhat greater extent than the first. This might suggest that the presence of the sec.butyl group at position 3 of the ring would favour the capture of hydrogen ion by the imido nitrogen atom in position 4. The converse might actually be expected from purely steric considerations, since position 1 is further removed from position 3, but repulsion of electrons by the alkyl group might conceivably increase the electron density at position 4 sufficiently to make capture of proton by this nitrogen atom a little easier than by the other more distant one.

3-isobutyl-2,5-diketopiperazine.

The variation in reaction time and in the amount of acid required may be due to difficulty in effecting solution. Evid-

ently a considerable amount of hydrolysis to glycine and DL-leucine occurred, since little if any of either dipeptide seems to have been present in the products of hydrolysis.

3-methyl-6-isopropyl-2,5-diketopiperazine.

The variation in time and amount of acid used may be due to variable ease or difficulty in effecting solution. When hydrolysis was effected under the conditions where the shorter time prevailed, DL-alanine and DL-valine must have been formed to an appreciable extent.

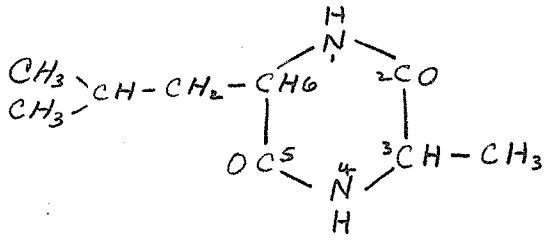
3-methyl-6-isobutyl-2,5-diketopiperazine.

Variable reaction conditions as reported are possibly the result of solubility problems. Mixtures of the 2,4-dinitrophenyl derivatives of the hydrolysis product and of DL-alanyl-DL-leucine clearly indicate the absence of the latter in the former, so absence of DL-alanyl-DL-leucine in the hydrolysis product seems established.

Mixtures of the DNP derivatives of the unknown and of DL-leucyl-DL-alanine, however, have a less profound effect on their respective melting points. When more of the latter was added to a mixture consisting largely of the former, no further change in melting point was observed. Also, when further quantities of the DNP derivative of the unknown were added to a mixture consisting largely of the DL-leucyl-DL-alanine derivative, no further lowering

of the melting point occurred. It is on the basis of such observations that the conclusion is drawn that the unknown derivative contains DNP - DL-leucyl-DL-alanine, despite the vast difference in the melting points, and that DL-leucyl-DL-alanine was present in the hydrolysis product.

It is quite conceivable that the only dipeptide present was DL-leucyl-DL-alanine, possibly containing also the two amino acids, DL-alanine and DL-leucine. In this case the presence of their DNP derivatives might produce quite a profound lowering of the melting point of the dipeptide derivative, even if they were only present in small amounts. It is not possible to say to what extent they might have been present, however. It is therefore not inconceivable that DL-leucyl-DL-alanine could be the main component of the hydrolysis products.



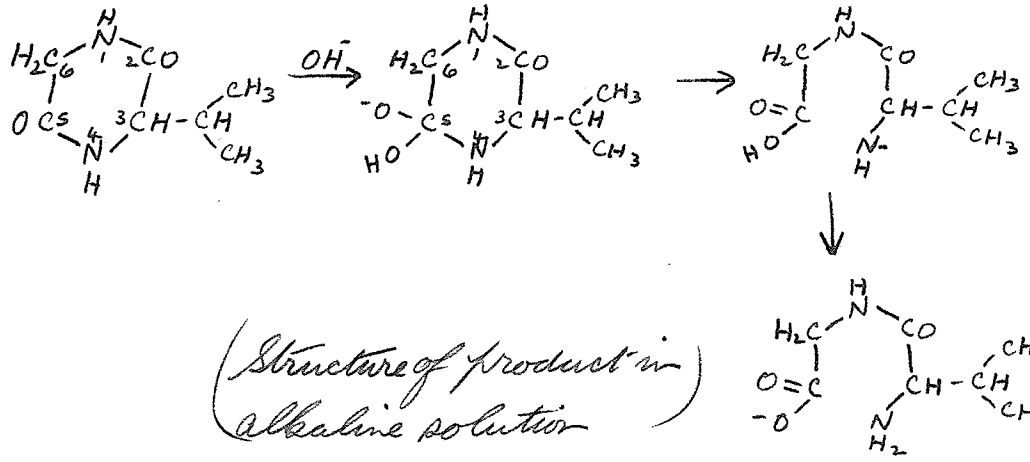
The isobutyl group in position 6 could increase the electron density at position 1 more than the methyl group at 3 would increase the electron density at position 4. Proton capture at position 1 would then be easier than at position 4.

Alkaline Hydrolysis.

3-isopropyl-2,5-diketopiperazine.

An attempt to apply Ingold's proposed mechanism for alkaline hydrolysis of amides suggests two possibilities:-

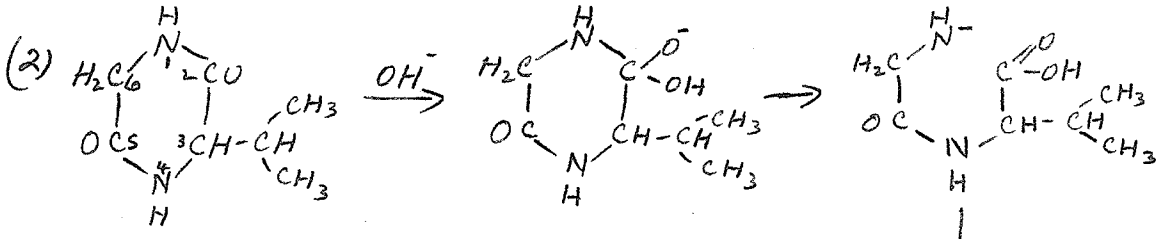
(1)



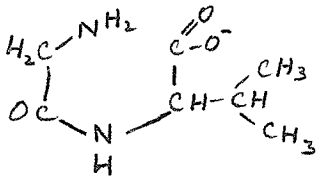
(Structure of product in alkaline solution)



(2)



(Structure of product in alkaline solution)

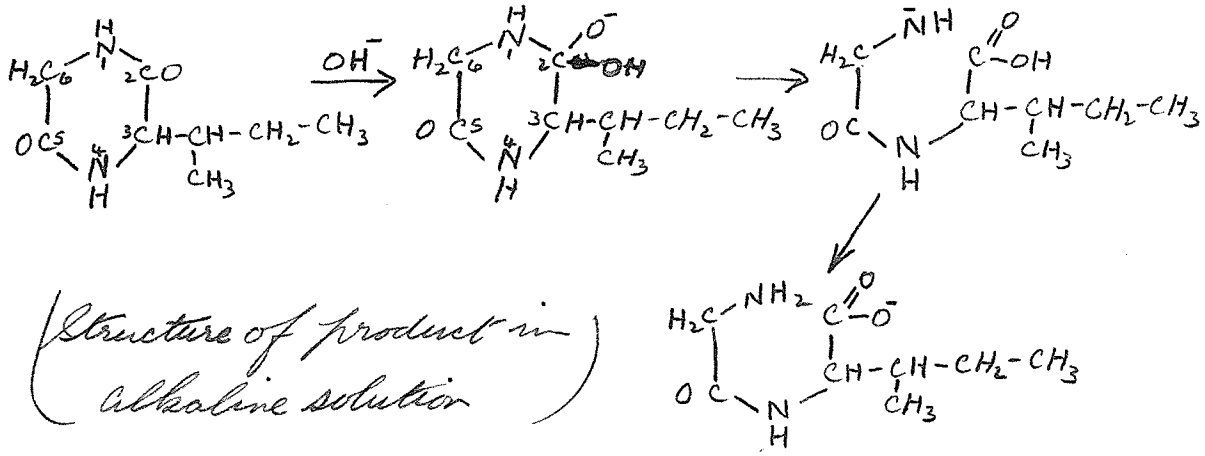


Since both DL-valylglycine and glycyl-DL-valine appear to have been formed, and rather more of the former than the latter, it would appear that both mechanisms obtain, with the first favoured somewhat more than the second. Such an effect would be due to the presence of the isopropyl group at position 3 of the ring.

It is conceivable that the isopropyl group might increase the electron density at positions 2 and 4, making addition of $OH^-$ to position 5 easier and thereby favouring the first mechanism. With this reasoning, however, it might be expected that acid hydrolysis would likewise give rather more DL-valylglycine than glycyl-DL-valine. Since preliminary proton capture by imido nitrogen is a rapid reaction on Ingold's reasoning, and addition of $OH^-$ to the $>C=O$ group is slow, it might be reasonable to conclude the isopropyl group is perhaps not large enough to make preferential proton capture significant enough to affect the course of acid hydrolysis, but yet is enough to make $OH^-$ addition to position 2 less easy than to position 5.

3-sec.butyl-2,5-diketopiperazine.

The fact that glycyl-DL-isoleucine appears to be the main product of hydrolysis seems to indicate the following path of reaction:-



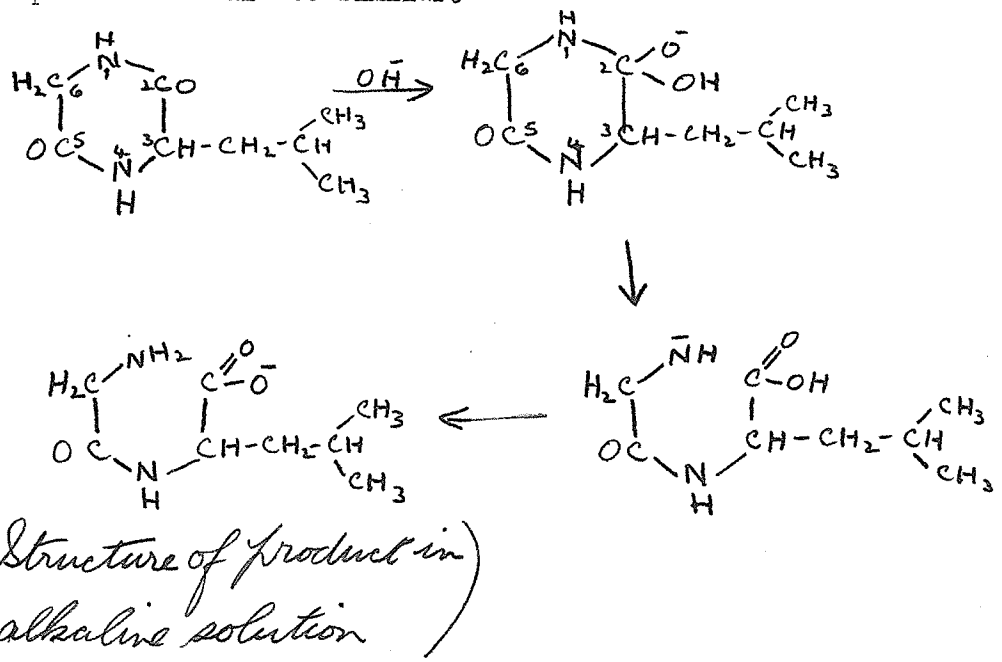
(Structure of product in alkaline solution)

Such a conclusion would imply that the slow reaction, addition of $OH^-$ to position 2, is favoured more than to position 5.

Since the sec.butyl group might be expected to increase the electron density at position 4, addition of $O\bar{H}$ to position 5 might be expected to predominate, giving DL-isoleucylglycine as was the case in acid hydrolysis. Evidently the electronic effect of the sec.butyl radical is not the only factor determining the mechanism of hydrolysis in this case, if Ingold's theory can be applied.

3-isobutyl-2,5-diketopiperazine.

That glycyl-DL-leucine appears to be formed in larger amount than DL-leucylglycine would indicate that addition of $O\bar{H}$ to position 2 is favoured, following a similar path to the alkaline hydrolysis of the 3-sec.butyl compound. The theoretical explanation would be similar.



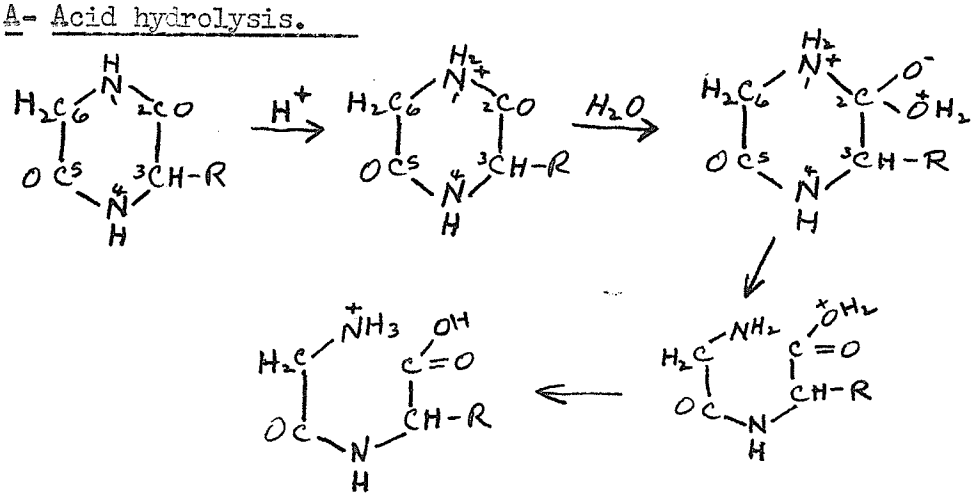
(Structure of product in alkaline solution)

3-methyl-6-isopropyl-2,5-diketopiperazine and 3-methyl-6-isobutyl-2,5-diketopiperazine were not hydrolysed, possibly due to difficulty in effecting solution. Fischer and Schrauth (87) found some difficulty in hydrolysing the latter with alkali. It would seem that the tendency for the alkyl substituted anhydrides to be somewhat resistant to alkaline hydrolysis, as reported by Emil Fischer and Abderhalden and their collaborators, could lead to variable results, at least with respect to success or failure in effecting hydrolysis.

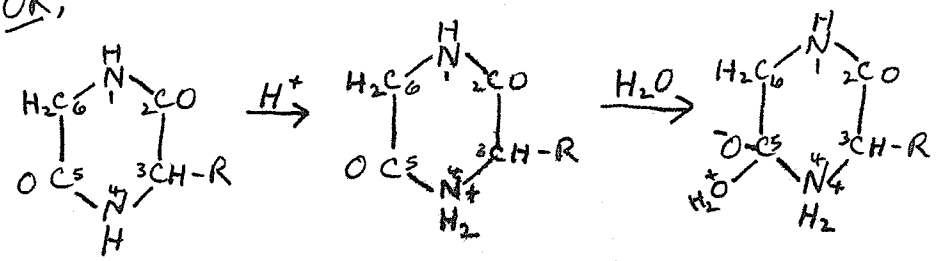
Summary of discussion of results of acid and alkaline hydrolysis studies.

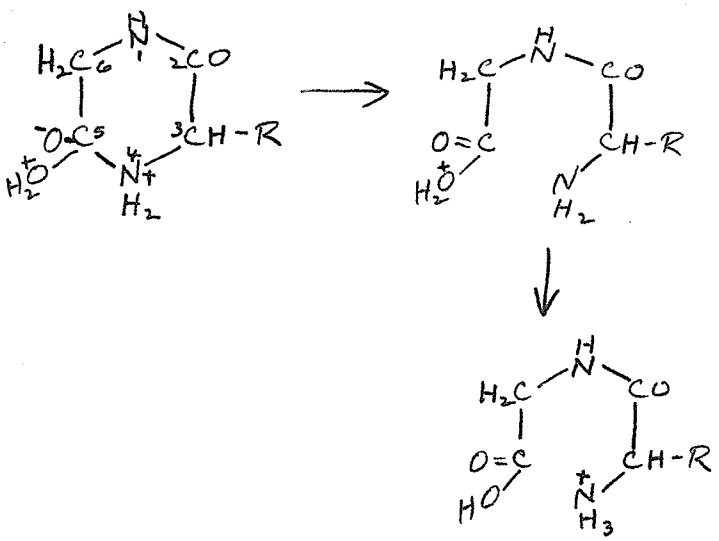
If Ingold's theory of amide hydrolysis can be applied to the 2,5-diketopiperazines, the steps involved would be:-

A- Acid hydrolysis.



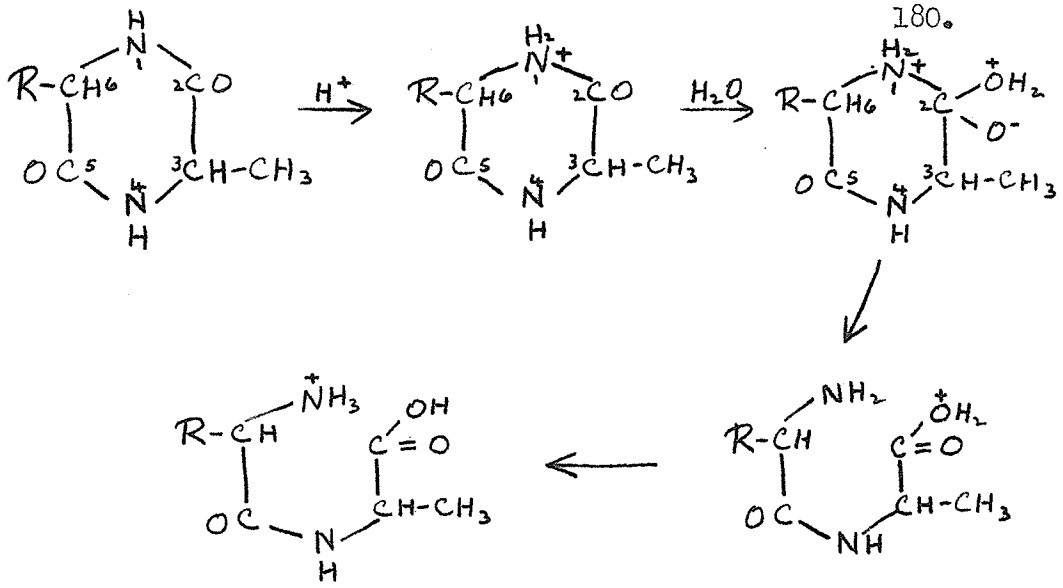
OR,





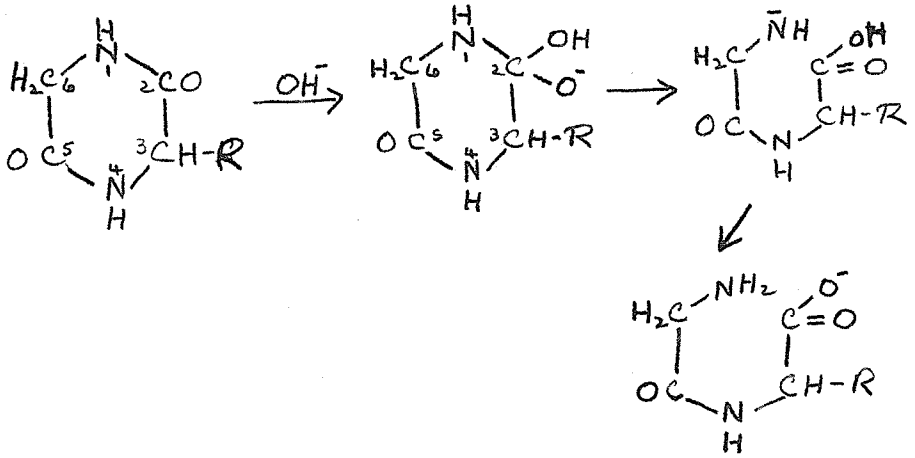
From the findings of this investigation, it would appear that when R is $CH_3>CH-$ $CH_3$ the group does not exert sufficient influence to cause either course to be followed more than the other, at least not to a significant extent. The sec.butyl radical, $CH_3-CH_2-\underset{\displaystyle CH_3}{CH}-$, however, does seem to favour the second course rather more than the first. The effect would be electronic rather than steric, and would require that the radical should increase the electron density of the nitrogen atom at position 4. If this did occur, addition of proton to this atom would be aided, accounting for the second course being followed.

Sufficient increase of electron density for this purpose at position 4 by the sec.butyl group but not by the isopropyl group would be due to the larger size of the sec.butyl radical.

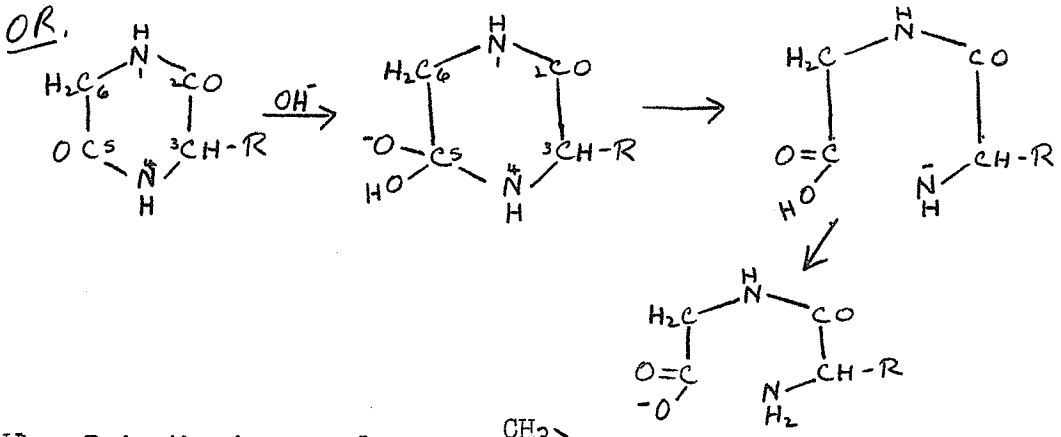


With this class of homologues, results could only be identified in the case where R- is the isobutyl radical, and the course outlined above seems to have been the one favoured in this particular example. The methyl radical might be expected to have some effect in increasing electron density at position 4, and the isobutyl radical to have a similar effect at position 1. The isobutyl radical is much larger than the methyl radical, and therefore would be expected to have a greater effect on position 1 than the methyl radical would have on position 4, thus accounting for the course apparently followed.

B - Alkaline Hydrolysis.



OR.



When R is the isopropyl group, $\begin{matrix}CH_3\\CH_3\end{matrix}\!\!>\!CH-$ , the second course appears to be favoured, although the first course is followed to some extent. The isopropyl group might be expected to increase the electron density at position 2 and position 4. The increase of electron density at position 2 would make addition of $O\bar{H}$ to position 5 a little easier.

When R is sec.butyl $CH_3-CH_2-\underset{CH_3}{CH}-$ or isobutyl $\begin{matrix}CH_3\\CH_3\end{matrix}\!\!>\!CH-CH_2-$ ,

the products are the converse of what would be expected in terms of the electronic effect of the alkyl groups. Some other factors additional to or independent of the latter effect must be involved if Ingold's theory of amide hydrolysis can be applied in these instances.

The speculative interpretations made regarding possible electronic effects in the diketopiperazine ring due to the presence of alkyl groups at positions 3 and/or 6 require the exercise of considerable caution. Some variation has been noted regarding time of reaction for hydrolysis, and in the case of acid hydrolysis, variation in the amount of acid required. Without paying any regard whatever to the solubility of any given

anhydride in aqueous medium, particularly acid or alkali, and more particularly at temperatures higher than room temperature, it is not absolutely certain whether reaction can occur without initial solution of the anhydride.

If the anhydride has to be in solution before hydrolysis can occur, apparently long reaction times may actually be long periods required to dissolve the anhydride. Following upon solution, hydrolysis might be fairly rapid. On the other hand, if solution of anhydride is not a prerequisite for hydrolysis, factors at present unknown may introduce variables into the reaction. Of these variables, reaction time, quantities of reagent, and temperature required, may only be outward manifestations which cannot be treated as being informative without some other correlating information.

If it were known that preliminary solution of anhydride was a prerequisite for hydrolysis, it might be possible to devise experimental conditions in which complete solution of anhydride was obtained. The course of the hydrolysis would then be easier to follow, and much more definite information could be obtained with respect to the optimum conditions and most probable mechanism of the reaction. Unfortunately excessive dilution is very apt to be the consequences of attempting to achieve these ends, with consequent obscuring of results, certainly concerning the anhydrides derived from monaminomonocarboxylic acids.

It would appear therefore, that the problem of solubility, to which reference has so frequently been made in this discussion,

is capable of introducing complexity into the study of the hydrolysis reaction, the nature of which complexity cannot as yet be deduced.

## SUMMARY.

The anhydrides of DL-alanine, DL-$\alpha$-aminobutyric acid, DL-valine, DL-isoleucine, and DL-leucine have been prepared in good yield by heating the respective amino acids at 180°-185° in ethylene glycol.

Fifteen mixed diketopiperazines have been synthesised by heating pairs of glycine, DL-alanine, DL-$\alpha$-aminobutyric acid, DL-valine, DL-isoleucine and DL-leucine in ethylene glycol at 180°-185°. Yields and purity have been such as to indicate that the method could become a reasonably satisfactory one for the preparation of such compounds.

Dielectric constants of acetic acid solutions of $\alpha$-aminobutyric anhydride, valine anhydride and leucine anhydride have been measured at 25°. The solutions had a higher dielectric constant than the acetic acid used. Solutions of $\alpha$-aminobutyric anhydride showed a more or less linear increase of dielectric constant with increasing concentration until the limit of solubility was being approached. Valine anhydride, within the range of concentrations studied and the sensitivity of the instrument, gave an increase in dielectric constant for two increasing concentrations, followed by a decrease at a concentration approaching the limit of solubility. For leucine anhydride, the solutions which could be studied with the apparatus available had a higher dielectric constant than the acetic acid, but the value decreased linearly with increasing concentration.

The hydrolysis of glycyl-DL-valine anhydride, glycyl-DL-leucine anhydride, glycyl-DL-isoleucine anhydride, DL-alanyl-DL-valine anhydride, DL-alanyl-DL-leucine anhydride and DL-alanyl-DL-isoleucine anhydride by NHCl and by NNaOH have been studied. Hydrolysis did not always occur, and in cases where hydrolysis to identifiable dipeptides took place, possible interpretations of the results have been advanced. These interpretations are subject to reservation because of variable solubility behaviour of anhydrides in aqueous media, on the grounds that such behaviour is capable of introducing unknown factors into the reaction.

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