# AN INVESTIGATION OF THE DAKIN-WEST REACTION

# THE ROLE OF PEROXIDES IN MUTAGENESIS

Thesis by

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In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1951

It is a pleasure to express here my gratitude to a large number of persons.

To Dr. Carl G. Niemann I wish to extend deepest thanks for assigning to me a research problem which has held my continued interest, and for sound advice both in the prosecution of my research and in the progress of my course in general.

The acquaintanceships I have made among fellow-students and faculty members, and the conversation, discussion, debate and argument afforded by them, have been of inestimable value for both stimulus and for knowledge gained.

Finally, I want to acknowledge my grateful appreciation for the encouragement, advice and inspiration given (and for the great patience shown) by my father, Robert G. Cleland, my brother, Robert S. Cleland, and my fiancé, Norma L. Jones.

## Abstract

An investigation of the Dakin-West reaction has been made, as a result of which the scope of the reaction has been extended in a number of ways.

a. It has been found that a number of aliphatic, aromatic and heterocyclic acid anhydrides, acyl fluorides, and (with the proper choice of catalysts) acid chlorides can be used in place of acetic anhydride in the reaction. Several cyclic anhydrides of dibasic acids have been shown not to react; inconclusive results were obtained with maleic anhydride.

b. The reaction has been shown not to depend on a specific catalytic action of the pyridine structure, but to be catalyzed also by such bases as sodium acetate.

c. Several unusual $\alpha$-amino acids have been shown to undergo the reaction; conclusions have been drawn as to the failure of several other amino acids to react. It has been shown that dipeptides can be used in place of amino acids.

d. A mechanism for the reaction has been proposed, based on evidence now available.

e. Observations have been made on side reactions of importance to the reaction; a method of utility for purifying the products of the reaction has been developed.

The role of peroxides in mutagenesis has been investigated; several peroxides have proved to be effective for the induction of mutations.

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# PART I

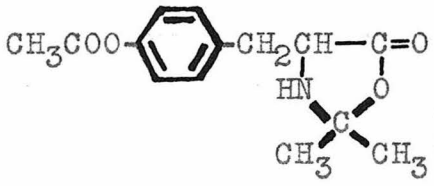
## AN INVESTIGATION OF THE DAKIN-WEST REACTION

PART I

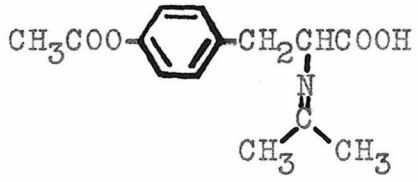
# AN INVESTIGATION OF THE DAKIN-WEST REACTION*

## Introduction.

In 1927 Levene and Steiger (1), in an attempt to acetylate tyrosine by heating the amino acid with acetic anhydride and pyridine, obtained instead of the expected product a compound with the formula $C_{14}H_{17}O_4N$. They believed that this substance resulted from a condensation of tyrosine with acetone, which was either present as an impurity in the reactants or formed during the reaction, and proposed two alternate formulas (I, II) based on this idea. Certain experiments which they



(I)



(II)

performed seemed to show that added acetone facilitated the reaction. When they heated phenylaminoacetic acid, acetone, acetic anhydride and pyridine together, a compound with the formula $C_{11}H_{13}O_2N$ resulted. They observed an evolution of

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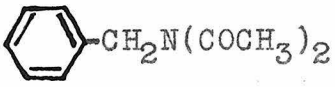
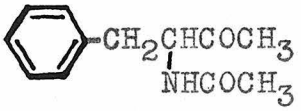
* A part of this material has appeared in an article, "Some Observations On the Dakin-West Reaction," in the Journal of the American Chemical Society. [Contribution From the Gates and Crellin Laboratories of Chemistry, California Institute of Technology, No. 1241. G. H. Cleland and C. Niemann, J.A.C.S. 71, 841 (1949).]

carbon dioxide during this experiment, and therefore suggested that the product might be (III) or (IV).

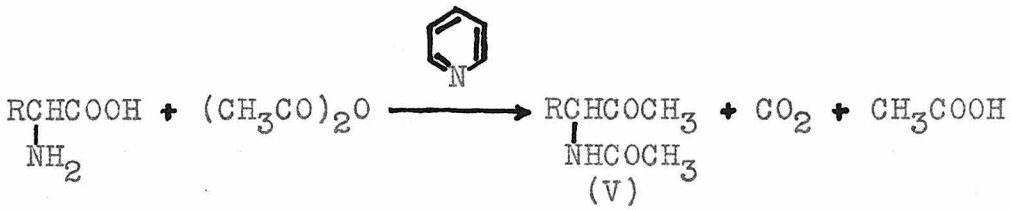


(III)



(IV)

The following year Dakin and West (2,3), attempting to acetylate cystine by heating the amino acid with acetic anhydride and pyridine, isolated instead a product which proved to be a neutral ketone with the empirical formula $C_6H_{10}O_2NS$. Further investigation showed that a large number of amino acids reacted with acetic anhydride and pyridine to yield ketones, and that carbon dioxide was another product of the reaction. Elucidation of the structures of the ketones and quantitative estimations of the carbon dioxide evolved proved the over-all reaction to be



This formulation represents the original Dakin-West reaction.*,** Dakin and West found that the greater number of α-amino acids

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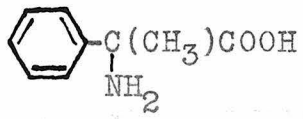
* It should be noted that one of the formulas (IV) proposed by Levene and Steiger for the product of the reaction of phenylaminoacetic acid, acetic anhydride and pyridine is the correct one.

** The reaction of course involves more than one mol of anhydride per mol of amino acid. However, in all experiments unless otherwise noted, moderate to large excesses of anhydride (or its equivalent) were used. The probable stoichiometric relationships are treated in a later section, and unbalanced equations, such as shown above, will be used until the discussion presented in that section.

tested reacted in this manner, while β-amino acids were not affected except for simple acetylation. While optically active α-amino acids were used in some experiments, the ketones isolated were invariably inactive. Complicating competitive reactions were observed in certain cases. Thus, serine and phenylserine gave little or no carbon dioxide or ketone under the conditions used, but instead were converted mainly to the azlactones of the corresponding α-acetamino-acrylic acids by intermolecular dehydration. Acids such as α-acetamidoacrylic acid did not react to give carbon dioxide or ketones. Glutamic acid gave only small amounts of carbon dioxide and ketone, most of the amino acid being changed to pyrrolidone carboxylic acid by the dehydrating action of the acetic anhydride. Proline and aminohydrotropic acid (VI)



(VI)

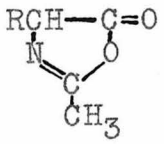
were acetylated when heated with acetic anhydride and pyridine, but gave no further reaction, and this was interpreted to mean that at least one hydrogen atom must be present on the α-carbon of the amino acid in order for the reaction to proceed. Neither glycine nor hippuric acid reacted smoothly under the conditions employed, and no ketone could be isolated in either case, although derivatives of the expected ketone (V, R=H) from the reaction of glycine were obtained from the

crude product.* Sarcosine (VII) gave little carbon dioxide

$$NH(CH_3)CH_2COOH$$

(VII)

and no detectable ketone,** and the N-methyl and N,N-dimethyl derivatives of phenylaminoacetic acid also gave negative results, which led to the conclusion that the amino group could not be alkylated if the reaction was to proceed. Dakin and West therefore suggested that azlactones (5-oxazolones) of the type of (VIII) might be intermediates in the reaction.



(VIII)

Experiments which they performed showed that such azlactones could be substituted successfully for amino acids, but that the reaction did not appear to proceed at a faster rate as a result of such substitution. A number of compounds related to pyridine, such as lutidine, nicotinic acid and

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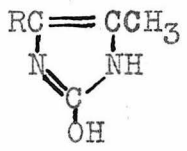
* Wiley and Borum (4) have recently prepared an acetyl derivative of acetamidoacetone, the expected ketone, in 60% yield. They used the Dakin-West reaction for this synthesis and found that much more drastic conditions were required than those employed by Dakin and West, which probably accounts for the failure of the latter authors to obtain the desired product in this case.

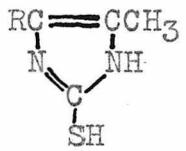
** Wiley and Borum (5) have shown recently that, under more drastic conditions than those used by Dakin and West, N-acetylsarcosine is converted by treatment with acetic anhydride and pyridine to N-methylacetamidoacetone and an acetyl derivative of this compound, in good yield. (This work is discussed further in a later section.)

isoquinoline, were found to catalyze the reaction, whereas quinoline did not, nor did dimethylaniline. No reference to the action of anhydrides or acylating agents other than acetic anhydride was made except for the statement that "the homologues of acetic anhydride are much less reactive than the latter substance. Acid chlorides such as acetyl chloride react vigorously but less smoothly than the anhydrides." Without presenting experimental evidence, Dakin and West reported that several acids other than amino acids, such as α-halo acids and phenylacetic acid, reacted to give ketones when heated with acetic anhydride and pyridine. Thus, phenylaminoacetic acid yielded methylbenzylketone.*

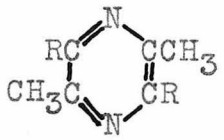
Since 1928, the Dakin-West reaction has been used to prepare a variety of ketones of the type of (V). Thus, Dakin and West (6) used the reaction to make several such ketones, and synthesized from these a number of imidazolones (IX), thioimidazolones (X) and pyrazines (XI). Wood and du Vigneaud (7) prepared one of the intermediates in a synthesis of



(IX)



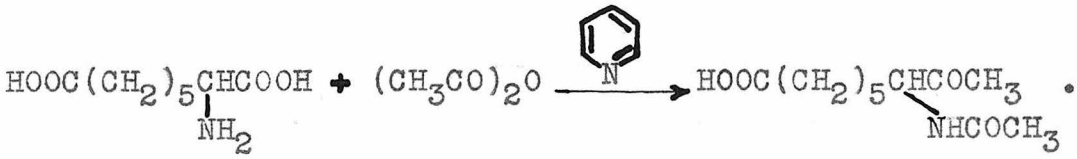
(X)



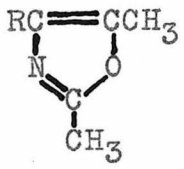
(XI)

* As will be detailed in a later section of this thesis, the mechanism of these reactions is apparently different from that operating in the case of α-amino acids. The designation of a reaction as a "Dakin-West reaction" implies that an α-amino acid or a derivative of an α-amino acid is one of the reactants.

desthiobiotin by the reaction

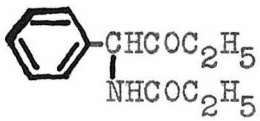


Wiley (8) used the reaction to synthesize several α-acetamido-alkyl methyl ketones, and dehydrated these with sulfuric acid to form oxazoles (XII).



(XII)

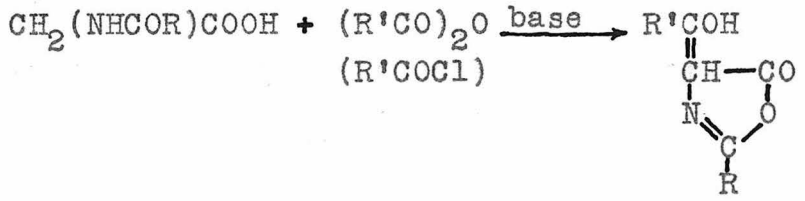
However, except for the synthesis of 1-phenyl-1-propionamido-2-butanone (XIII) by the reaction of phenylaminoacetic acid, propionic anhydride and pyridine, reported by Wiley and Borum (4) while the work in these laboratories was in



(XIII)

progress, no extensions of the nature of the original reaction were known until the work reported in this thesis. In addition, the results of research (9,10) on the base-catalyzed reaction of N-acylated glycines and acid chlorides or anhydrides to form substituted oxazolones, according to

the scheme

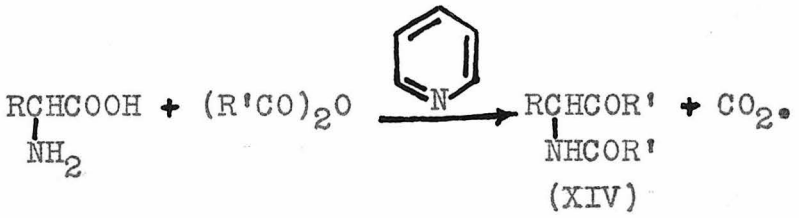


bear an intimate relationship to the Dakin-West reaction, and will be considered in a later section of this thesis.

## Discussion of results.

A. Extensions of the reaction.

The Dakin-West reaction in its original form was limited to the formation of α-acetamidoalkyl methyl ketones such as (V). It was felt that the usefulness of the reaction would be greatly increased if it could be extended to include the anhydrides of acids other than acetic acid, by the reaction




(XIV)

Experiments were therefore undertaken with this idea in view. These experiments resulted in the extension of the reaction, according to the scheme above, to include not only several aliphatic anhydrides, but aromatic and heterocyclic anhydrides as well. More strenuous conditions of reaction were usually required than when acetic anhydride was used, but the yields of ketones of the type of (XIV) were generally good.* Representative examples of the three types of ketones together with the yields obtained are given in Table 1.

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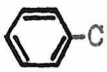
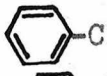
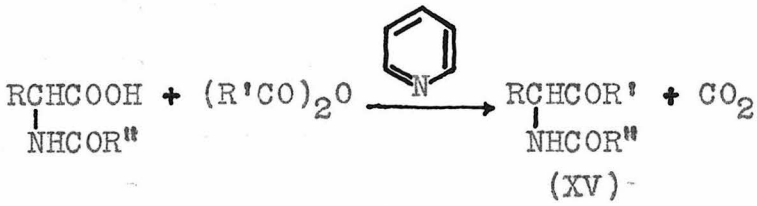
* Since the nature of this work was mainly exploratory, no attempt was made to determine the conditions for maximum yields.

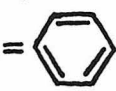
Table 1.

Acylated Aminoketones and Yields Obtained,
Using Aliphatic, Aromatic and Heterocyclic Anhydrides.

| Amino Acid Used | Anhydride Used | Product (XIV) | Yield, % |
|---|---|---|---|
| phenylalanine | butyric | R =  -$CH_2$-, R' = $C_3H_7$- | 27 |
| phenylalanine | benzoic | R =  -$CH_2$-, R' =  | 44 |
| phenylalanine | α-furoic | R =  -$CH_2$-, R' =  | 55 |

Subsequent work here and by others (11) has shown that when an N-acylated amino acid is used in place of an α-amino acid the reaction takes the course



with retention of the original N-acyl group in the product. Thus, N-acetylphenylalanine, α-furoic anhydride and pyridine gave a ketone of the type of (XV) with R=  -$CH_2$-, R' =  , R'' = $CH_3$-.

Pyridine and certain of its homologues were the only bases which were found by Dakin and West to catalyze the reaction of α-amino acids and acetic anhydride, and they felt that a catalytic effect specific to the pyridine structure was operative. However, experiments in these laboratories showed that sodium acetate was an effective catalyst for the reaction of phenylalanine and acetic anhydride to form 1-phenyl-2-acetamido-3-butanone, (V, R=  -$CH_2$-).* The yield was comparable to that obtained by using pyridine as a catalyst. Moreover, the reaction product contained much less of colored contaminants and the desired ketone was more easily isolated than in the case where pyridine was the catalyst. Subsequent experiments showed that in several syntheses where the use of pyridine or γ-picoline led to the formation of oily by-products which caused the isolation of the desired product to be troublesome, the use as catalyst of either the sodium salt of the amino acid or the sodium salt of the acid corresponding to the anhydride eliminated this difficulty in large part. It has recently been shown (11) that such bases as triethylamine and N-methyl piperidine also effectively catalyze the Dakin-West reaction.

In contrast to the successful use of a variety of acid

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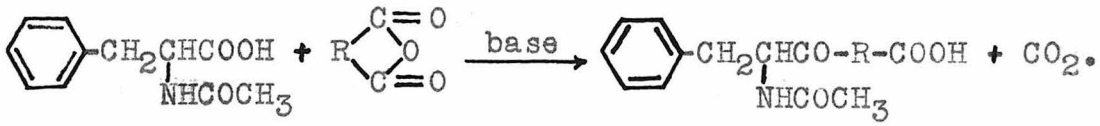
* The preparation of oxazoles by the dehydration of the crude reaction product obtained by treating an α-amino acid with acetic anhydride and sodium acetate (12,13) has been interpreted by Wiley (4,14) as proceeding through the intermediate α-acetamidoalkyl methyl ketone. The observed catalysis of the Dakin-West reaction by sodium acetate would seem to substantiate this idea.

anhydrides, the normal cyclic anhydrides of several dibasic acids failed to react as expected, in an exhaustive series of experiments under a wide variety of conditions. Thus, while adipic anhydride, a linear or cyclic polymer of two or more residues, appeared to react in a typical fashion, neither succinic nor phthalic anhydride gave appreciable amounts of carbon dioxide, or any product exhibiting typical ketonic reactions, whether the catalyst used was pyridine, γ-picoline, or any one of the sodium salts of the dibasic acids, or whether N-acetylphenylalanine* or 2-methyl-4-benzyl-5-oxazolone was used in place of the amino acid itself. The expected reaction in the case of N-acetylphenylalanine can be represented as




(XVI)

Although compounds of the type of (XVI) might react further,**

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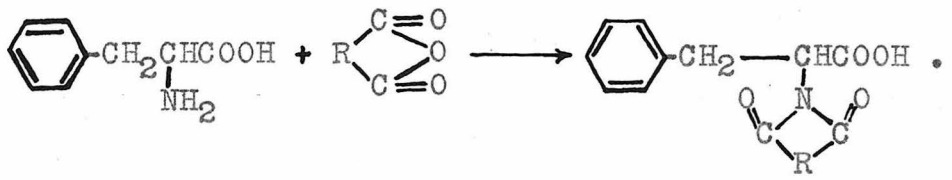
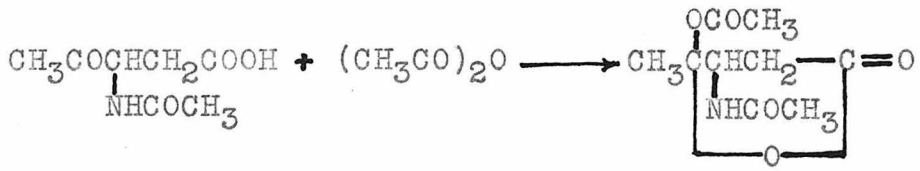
* It is probable that phenylalanine itself would react with the dibasic anhydride according to the scheme




** Thus, Dakin and West (3) found that the ketone formed from the reaction of aspartic acid, acetic anhydride and pyridine reacted further with the anhydride, according to the scheme



once formed, carbon dioxide should be evolved if the first reaction takes place. A mixture of maleic anhydride, pyridine and phenylalanine reacted vigorously, but this was soon traced to a reaction of maleic anhydride and pyridine.* A number of experiments which were then performed, using maleic anhydride, gave negative or inconclusive results. For example, the use of sodium hydrogen maleate or disodium maleate as catalysts, together with high temperatures of reaction, led to an evolution of carbon dioxide and the formation of a high-melting substance of unknown composition. This was true whether phenylalanine, N-acetylphenylalanine or 2-methyl-4-benzyl-5-oxazolone was used as the amino acid component. The substance contained no nitrogen and gave analytical results which were not in accord with the formula of fumaric acid. Further, it was found that a mixture of maleic anhydride, sodium hydrogen maleate and maleic acid evolved carbon dioxide when heated at 140°, and the same high-melting material as noted above was isolated from the resulting dark mixture. The mixture which resulted from heating together maleic anhydride, 2-methyl-4-benzyl-5-

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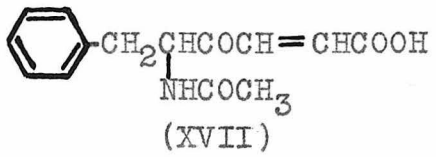
* This observation has been reported several times in the literature, but apparently the evolution of carbon dioxide as one of the products has heretofore escaped notice. The reaction has been attributed to a Diels-Alder type of condensation (15), but since a mixture of maleic anhydride and triethylamine was found to undergo the same typical changes (endothermic, then exothermic reaction, with the evolution of carbon dioxide and the production of a highly colored mixture), this cannot be a true picture of what occurs.

oxazolone and disodium maleate, followed by hydrolysis, afforded a dark oil which may have contained the desired ketone (XVII). This oil absorbed bromine and reduced

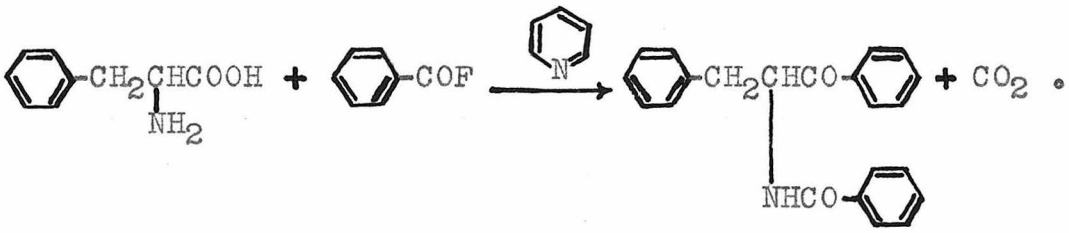


(XVII)

permanganate, gave off ammonia when heated with strong alkali, and yielded two derivatives with dinitrophenylhydrazine. However, both of these derivatives contained several per cent less nitrogen and possessed much lower melting points than would be expected for derivatives of (XVII). The results of these experiments show that, in general, the Dakin-West reaction fails to proceed in a normal fashion when the anhydrides of dibasic acids (at least, where the anhydrides are five-membered ring compounds) are used, under conditions where other anhydrides react. The case of maleic anhydride remains undecided.

The reaction of acetyl chloride with phenylalanine, using pyridine as catalyst, was found difficult to control, as Dakin and West (2) had reported. With both acetyl and benzoyl chloride the desired ketones were obtained in poor yield, although up to 50% of the calculated amounts of carbon dioxide was evolved in these reactions. However, benzoyl fluoride reacted smoothly with phenylalanine in the presence of pyridine to give the desired ketone, as shown in the

equations



It thus appears that acyl fluorides may be used in lieu of acid anhydrides in the Dakin-West reaction.

One reason for the low yields of ketones obtained when acyl chlorides were used in place of acid anhydrides was the formation of large amounts of tarry materials which made the separation of the desired products difficult. It was found that much of this tar resulted from a reaction of the acyl chloride with the heterocyclic bases used as catalysts, and that by substituting a trialkylamine such as tributylamine for the heterocyclic catalyst, the yield of ketone recovered could be much improved. Thus it appears that, with the proper choice of catalyst and control of conditions of reaction, acid chlorides may be used in place of acid anhydrides in the Dakin-West reaction. Comparative yields for several of these syntheses are shown in Table 2.*

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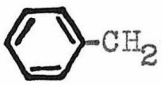
* No experiments were run using mixtures of amino acids, acyl halides and the sodium salts of acids, since there seemed to be little doubt that the reaction

$$RCOX + RCOONa \longrightarrow (RCO)_2O + NaX$$

would take precedence over a Dakin-West reaction.

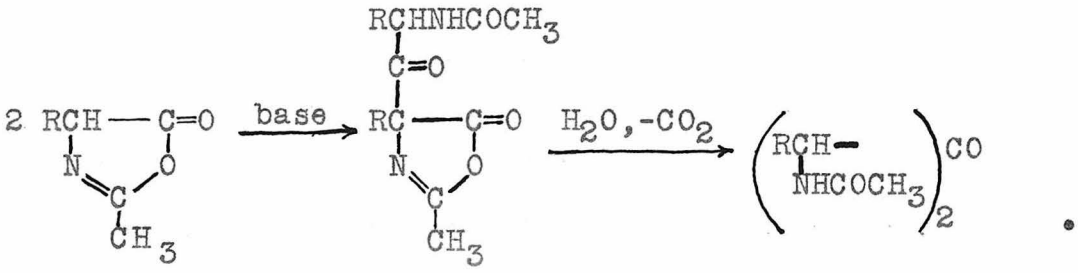
Table 2.

The Formation of α-Benzamido-β-phenyl-propiophenone (XIV), R=  , R'=  .

| Amino acid | Acylating Agent | Catalyst | Yield of ketone, % |
|---|---|---|---|
| phenylalanine | benzoic anhydride | pyridine | 44 |
| phenylalanine | benzoyl chloride | pyridine | 9 |
| phenylalanine | benzoyl chloride | tri-n-butylamine | 20 |
| phenylalanine | benzoyl fluoride | pyridine | 39 |

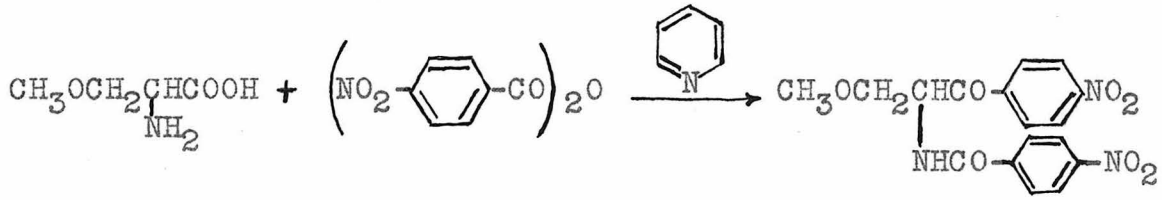
In view of the probable mechanism of the Dakin-West reaction, as discussed in another part of this thesis, it was not expected that compounds such as esters and nitriles, although having a certain relationship to acid anhydrides, would react in a manner similar to that of the latter with α-amino acids. A number of experiments showed that this was indeed the case. Such compounds as ethyl formate, amyl acetate and acetonitrile gave no carbon dioxide when heated with pyridine and either phenylalanine, N-acetylphenylalanine or 2-methyl-4-benzyl-5-oxazolone, nor was any carbon dioxide produced when the resulting reaction mixtures were treated with hydrochloric acid and again refluxed.

It was thought possible that azlactones such as (VIII), being probable intermediates in the reaction and at the same time being internal anhydrides, might condense with themselves under the influence of a basic catalyst, and yield ketones by subsequent hydrolysis, as shown in the equations



Accordingly, 2-methyl-4-benzyl-5-oxazolone was refluxed with γ-picoline, the solution acidified, and again refluxed. A small amount of carbon dioxide appeared during the first step and none was evolved during the second step.

Dakin and West (3) showed that serine is chiefly converted to α-acetamidoacrylic acid when heated with pyridine and acetic anhydride, and it was felt that O-methyl serine would be acted upon in a similar manner by anhydrides, with the production of methanol and α-acetomidoacrylic acid. However, while this was evidently the main reaction, 15% of the theoretical amount of carbon dioxide based on the reaction

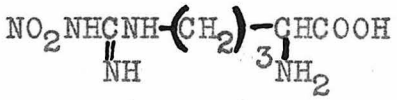


was evolved when O-methyl serine was refluxed with pyridine and *p*-nitrobenzoic anhydride.

When cysteine was refluxed with acetic anhydride and pyridine, both carbon dioxide and hydrogen sulfide were evolved, the latter probably arising from an elimination of hydrogen sulfide from cysteine with the formation of *α*-acetamidoacrylic acid.

When arginine was refluxed with acetic anhydride and either pyridine or sodium acetate, up to 50% of the theoretical amount of carbon dioxide was evolved, and a neutral residue which was isolated gave a color reaction with sodium nitroprusside, typical of methyl ketones.*

Nitroarginine, (XVIII), gave results similar to those obtained with arginine.



(XVIII)

It has been noted elsewhere that amino acids of the type of *α*-acetamidoacrylic acid, containing an aliphatic double

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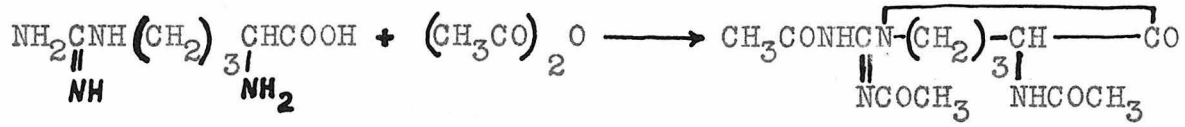
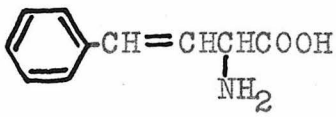
* These results are perhaps somewhat surprising since it is known (16) that on boiling arginine with acetic anhydride, the following reaction occurs



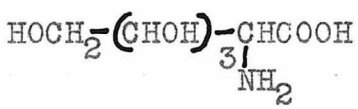
bond but no hydrogen on the α-carbon atom, do not undergo the Dakin-West reaction. On the other hand, 2-amino-4-phenyl-3-butenoic acid (XIX), which contains both an α-hydrogen atom and a neighboring aliphatic double bond,



(XIX)

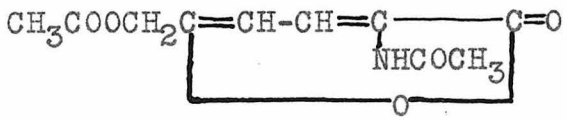
appeared to react in a normal fashion. When the amino acid was refluxed with pyridine and acetic anhydride, 70% of the theoretical amount of carbon dioxide was evolved, and the neutral residue which was isolated reduced permanganate, decolorized bromine in carbon tetrachloride, gave a precipitate with 2,4-dinitrophenylhydrazine, and showed a positive although atypical nitroprusside test.

When glucosaminic acid (XX) was refluxed with acetic anhydride and sodium acetate, the resulting mixture yielded

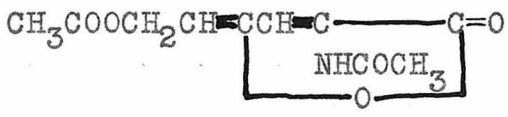


(XX)

an unsaturated lactone which is either (XXI-a) or (XXI-b).*



(XXI-a)



(XXI-b)

* The reaction of glucosaminic acid, acetic anhydride and sodium acetate to give (XXI) has been reported elsewhere (17,18). The low yield of (XXI) so obtained and discrepancies in melting points found by the several authors may have been due to a simultaneous Dakin-West reaction of glucosaminic acid.

After separation of this lactone, however, the oily red residue was found to give a positive nitroprusside test. When pyridine was substituted for sodium acetate and the mixture refluxed for several hours, traces of carbon dioxide were evolved, and the neutral residue gave a faint nitroprusside test. A very small amount of the doubly unsaturated lactone (XXI) was isolated from the mixture.

When either α-chloropropionic, α-bromostearic or dichloroacetic acid was refluxed with pyridine and acetic anhydride for one hour, ca. 50% of the theoretical amount of carbon dioxide based on the reaction

$$\underset{\underset{X}{|}}{RCHCOOH} + (CH_3CO)_2O \xrightarrow{\text{base}} \underset{\underset{X}{|}}{RCHCOCH_3} + CO_2$$

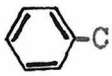
was evolved. The dark neutral residues obtained from the reaction mixtures reduced permanganate and decolorized bromine in carbon tetrachloride, but gave negative or inconclusive color reactions with sodium nitroprusside.

Dipeptides as well as polypeptides are examples of N-acylated amino acids. Since N-acylated amino acids are undoubtedly intermediates in the Dakin-West reaction, it seemed reasonable to assume that dipeptides and polypeptides would react with acid anhydrides and catalysts to yield N-acylated aminoketones according to the scheme (dipeptides):

$$\underset{\underset{NH_2}{|}\quad\underset{R}{|}}{R'CHCONHCHCOOH} + (R''CO)_2O \xrightarrow{\text{base}} \underset{\underset{NHCOR''}{|}\quad\underset{R}{|}}{R'CHCONHCHCOR''}$$

(XXII)

Experiments conducted with two dipeptides showed that the expected reaction did take place, with the evolution of up to 90% of the theoretical amounts of carbon dioxide. Alanylalanine or its sodium salt* was converted by the action of acetic anhydride and sodium acetate to the ketone (XXIII), (XXII, R=R'=R" = $CH_3$-), and phenylalanylphenylalanine reacted with acetic anhydride and sodium acetate to give

(XXIV), (XXII, R=R' =  -$CH_2$-, R" = $CH_3$-).**

The isolation of (XXIII) proved very difficult, as did the derivatization of the two ketones,*** but when finally

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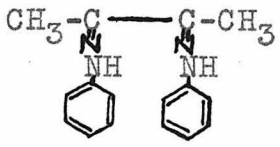
* This proved to be one of the cases where the isolation of the desired product was made difficult, when pyridine was used as catalyst, by the formation of much colored, tarry material.

** It can be seen from (XXII) that two pairs of enantiomorphs are possible for this type of ketone. The melting points of (XXIII) and (XXIV), the two ketones isolated, were quite sharp, so that a reasonable assumption is that in each case the product consisted of one or the other pair and not of all four forms. On the other hand, the melting point behavior of the oxime of (XXII) indicates that this may have been a mixture of all four forms. (This oxime was prepared from a crude reaction product.)

*** Thus, when (XXIII) was treated with phenylhydrazine in neutral or faintly acid media, no reaction occurred, while on warming with phenylhydrazine and 10% acetic acid for a few minutes, a precipitate was obtained which proved to be (XXV), the osazone of biacetyl.

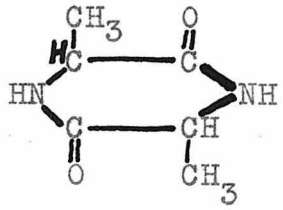


(XXV)

(XXV) was also obtained by Dakin and West (2) by treatment of 3-acetamido-2-butanone with phenylhydrazine in 2% sulfuric acid.

obtained in crystalline form, the ketones and their derivatives gave the correct analytical results. In addition, both gave positive iodoform and nitroprusside reactions, typical of methyl ketones. A mixture of alanylalanine, benzoic anhydride and pyridine also evolved carbon dioxide when refluxed. The fact that alanylalanine was C-acetylated only on the $\alpha$-carbon was shown by an experiment in which alanylalanine, acetic anhydride and pyridine were refluxed together until 92% of the anticipated amount of carbon dioxide (one equivalent) was evolved. The mixture was then acidified with 6 N hydrochloric acid and again refluxed. No carbon dioxide resulted from the last step.

Dimethyldiketopiperazine (XXVI), from which alanylalanine was prepared by alkaline hydrolysis, was refluxed with acetic anhydride and pyridine, the resulting mixture was treated with an excess of 6 N hydrochloric acid, and again refluxed. In neither step was carbon dioxide evolved.

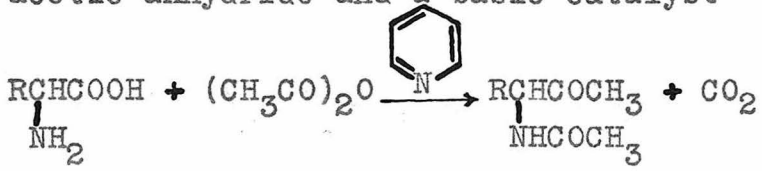


(XXVI)

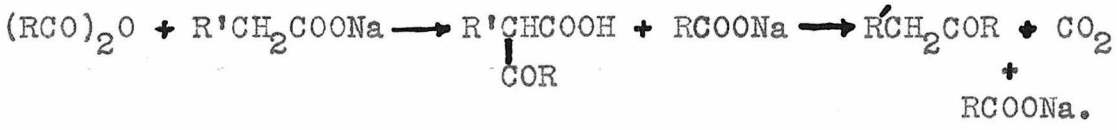
B. The Mechanism of the Reaction.

After elucidation of the nature of the over-all reaction, Dakin and West (2,3) considered, and rejected on reasonable

grounds, several possible mechanisms. They believed that an analogy existed between the reaction of α-amino acids with acetic anhydride and a basic catalyst

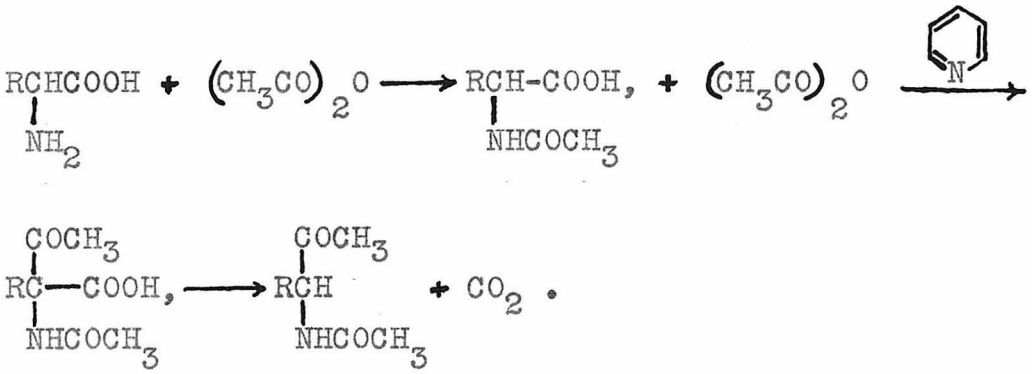


and a reaction discovered by Perkin (19) and explained by Fittig (20), whereby acid anhydrides and the sodium salts of acids react when heated together according to the scheme

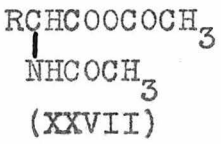


From this analogy Dakin and West felt that β-keto acids were intermediates in their reaction, and that acids lacking a hydrogen atom on the α-carbon atom could not react. They demonstrated that such an acid, α-aminohydrotropic acid (VI) did not react. Further, they believed that the function of pyridine was catalytic and, on the basis of the failure of N-alkylamino acids to undergo the reaction to any appreciable extent, concluded that alkylation of the amino group prevented α-amino acids from reacting to form α-acetamido ketones . From this they conceived the idea that azlactones (VIII) might be intermediates in the reaction; but they found that such azlactones did not react appreciably faster than the amino acids themselves, and concluded that, if formed at all, an azlactone was only one of several intermediates. They

pictured the reaction (exclusive of other intermediates) as

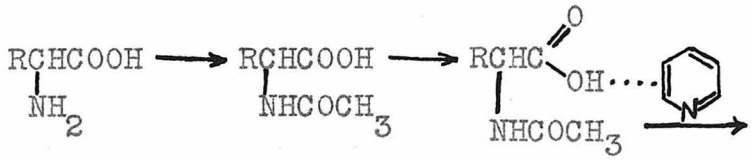


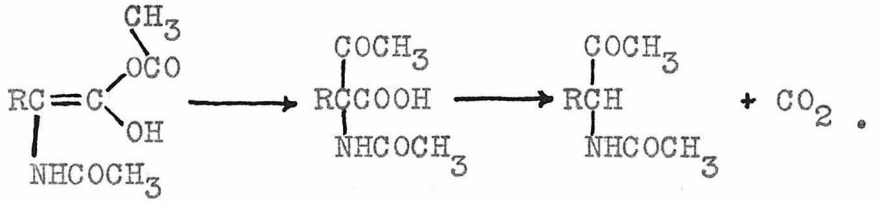
Finally, they noted without experimental evidence that a mixed anhydride such as (XXVII) might be formed and then react further to give the acetamido ketones.



(XXVII)

Levene and Steiger (21) felt that azlactones were only secondary products of the reaction and that a pyridine complex of unknown composition was an intermediate, the mechanism postulated being



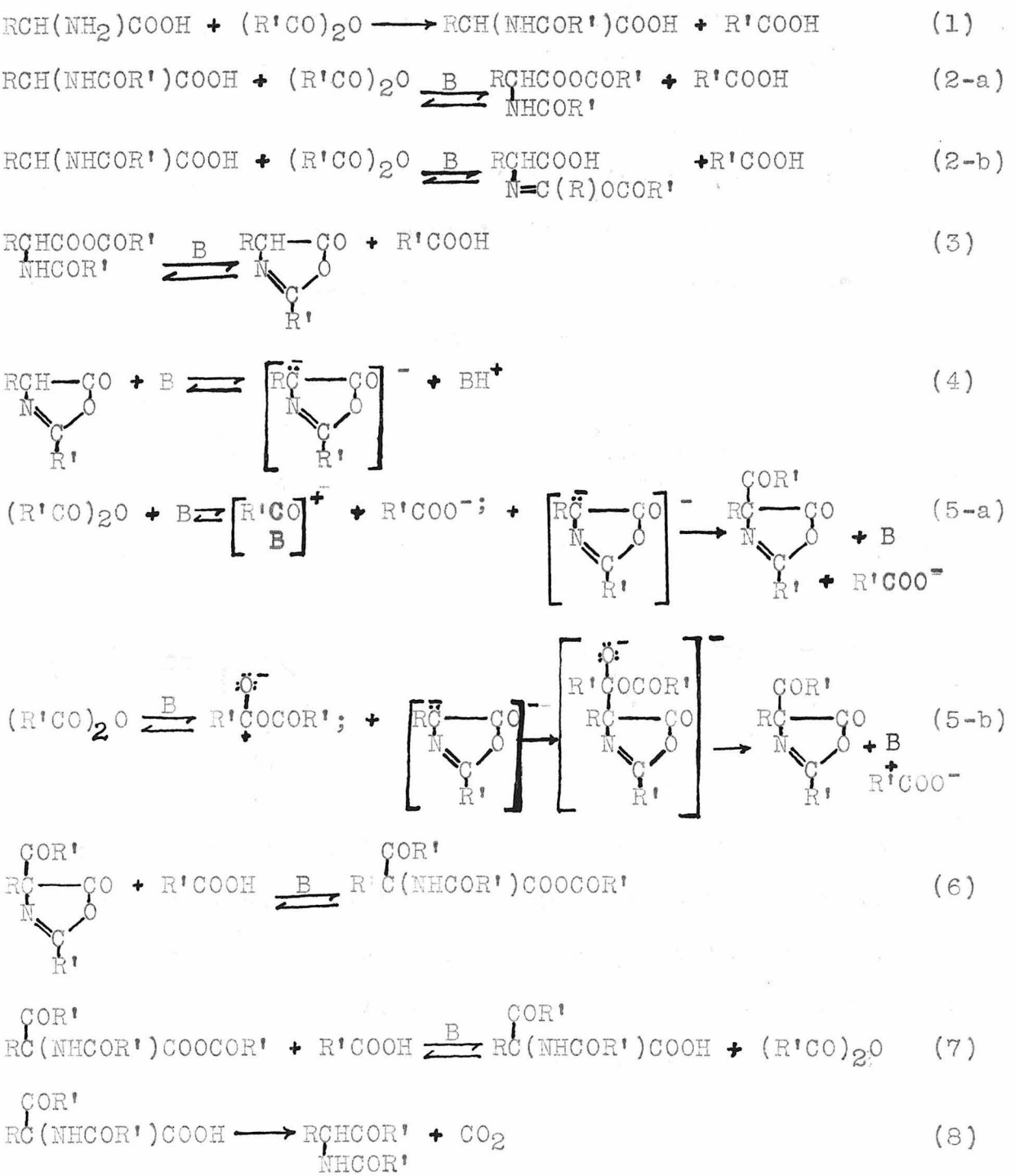


Since such bases as trialkylamines and sodium acetate catalyze the Dakin-West reaction, however, the suggested pyridine complex becomes of little interest.

Subject to certain modifications which are discussed later, Mechanism I, presented in Figure 1, is believed to represent the course of the reaction. The evidence for each of the various steps of this mechanism is analyzed in the discussion which follows.

Figure 1.

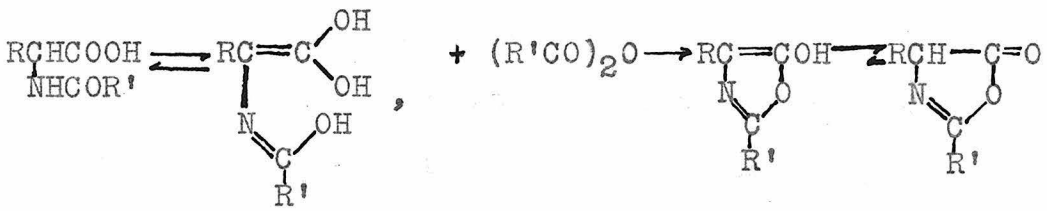
Mechanism I -- A Proposed Mechanism For the Dakin-West Reaction.



Step (1), the acylation of the amino acid, seems reasonable from all experimental evidence.

Steps (2-a), formation of a mixed anhydride as suggested by Dakin and West (2), and (2-b), involving an enolization of the acylamino acid followed by acylation of the enol hydroxyl group, cannot be evaluated on the basis of present evidence. It is probable that, if one (or both) of the intermediates is formed, one (or both) reacts only after further change to such structures as

A mechanism proposed by Wiley (14),



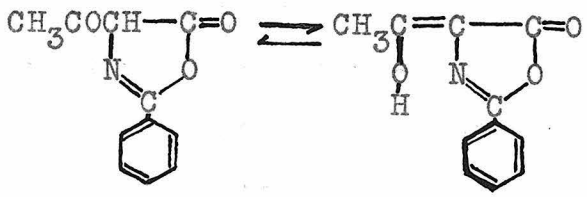
which involves steps (2) and (3), is also a possibility. Another step undoubtedly intervenes before the 5-hydroxy oxazole is formed, however, since here, as in (2-a), (2-b) and (3) of mechanism I, a picture of a simultaneous removal of the elements of water from an α-acylamino acid is quite unlikely.

Step (3), the formation of the azlactone, is consistent together with (1) and (2) with the fact that azlactones are

easily formed in good yield, in many cases, by warming or refluxing α-amino acids with acid anhydrides. It is also in accord with the fact that the ketones isolated are invariably inactive. Azlactones are racemized very easily (22).

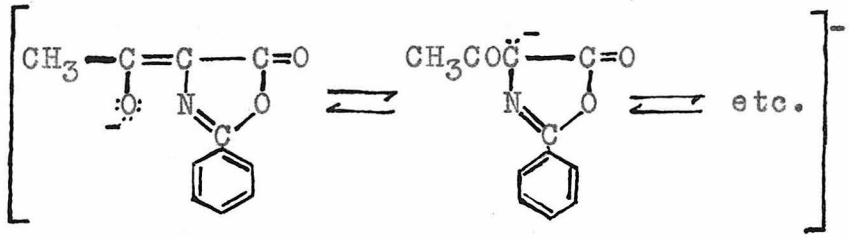
Step (4), the reaction of the azlactone with base to give a (resonance stablized) carbanion, is consistent with the fact that a great deal of evidence exists showing that the methylene group of azlactones in general is very active (22), examples of this reactivity being the Erlenmeyer condensation of aldehydes with acylated glycines and the ease of internal dehydration of the azlactones of α-amino-β-hydroxy acids. The base would be expected to facilitate the formation of the carbanion and to stablize it, when once formed.

Step (5), the reaction of the carbanion with acid anhydride to give the azlactone of an α-acylamido-β-keto acid, has been shown to be true in several cases by the isolation of such azlactones, of a particular type (9,10). Thus, a mixture of hippuric acid, acetic anhydride, γ-picoline and sodium acetate, stirred at room temperature for several hours, gave the compound (XXVIII) in good yield.



(XXVIII-a) (XXVIII-b)

When sodium acetate was omitted, the yield of (XXVIII) was poor. Presumably sodium acetate, a stronger base than γ-picoline, favored the more complete formation of the ion (XXIX) of the enol form



(XXIX)

which would be expected to be more inert to ring cleavage and subsequent decarboxylation than the keto form. Compounds such as (XXVIII) are very stable towards alkali, but are easily decomposed by acid media. In the case of the majority of amino acids, with the exception of derivatives of glycine, such C-acylated azlactones could exist only in a keto form, corresponding to (XXVIII-a), since they lack a hydrogen atom necessary for enolization. It is therefore to be expected that they would be much more susceptible to ring cleavage and subsequent decarboxylation than would (XXVIII). Glycine itself reacts when refluxed with acetic anhydride and pyridine to give acetamidoacetone. The fact that this reaction requires more drastic conditions and proceeds more slowly than in the case of other amino acids is probably due to an equilibrium between enol and keto forms. Decision between (5-a)

and (5-b) is difficult, since the base could no doubt catalyze the first step of each. However, the fact that esters do not react while anhydrides do seems to indicate that (5-a) is the better possibility. Also, the failure of succinic and phthalic anhydrides may well find its explanation in this step.

Step (6), the formation of a mixed anhydride, seems to be the most likely course for ring cleavage and the first step toward the formation of the β-keto acid. Water itself could not exist in the presence of the excess acid anhydride used, but the elements of water must be added to the C-acylated azlactone in order to form the β-keto acid, and no logical source for these elements can be found in the system except in the acid formed in previous steps. In this step as well as in steps (2), (3), and (7), pyridine (and other catalysts) probably plays a further role. It is known (23) that it hastens the establishment of equilibria in at least one system of the type

$$(RCO)_2O + 2R'COOH \rightleftharpoons RCOOCOR' + RCOOH + R'COOH \rightleftharpoons (R'CO)_2O + 2RCOOH.$$ *

Rondestvedt et. al. (24) found that when limited amounts of anhydride were used, the per cent of carbon dioxide obtained in relation to the mol ratio (R) of amino acid to anhydride

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* Azlactones are, of course, a type of internal anhydride.

was R = 1/2, 63%; R= 2/5, 82%; R= 1/3, 98%. They interpreted these results to mean that three mols of anhydride were required for the reaction instead of two, and therefore disputed the cleavage shown in steps (6) and (7). However, careful consideration of these data shows that, on the contrary, the acid-azlactone-anhydride equilibria of various steps of mechanism I are absolutely essential if an azlactone is an intermediate (as they also believed).

Step (7), in which the mixed anhydride reacts with acid to form the regular anhydride and an α-acylamido-β-keto acid, is similar to step (6).

Step (8), the decarboxylation of the β-keto acid to form the α-acylamido ketone and carbon dioxide, is typical of the behavior of β-keto acids when heated. This irreversible decarboxylation also serves to drive the reaction towards completion.*

Objections have been offered to several steps of this mechanism. Wiley and Borum (5), on the basis of the observation that N-acetylsarcosine, an amino acid incapable of

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* It should be noted that several other obvious steps, such as

$$RCOO^- + BH^+ \rightleftharpoons R'COOH + B$$

and

$$RC(NHCOR')COOH \longrightarrow \left[R\ddot{C}NHCOR'\right]^- + H^+ + CO_2 \longrightarrow RCHCOR' + CO_2$$

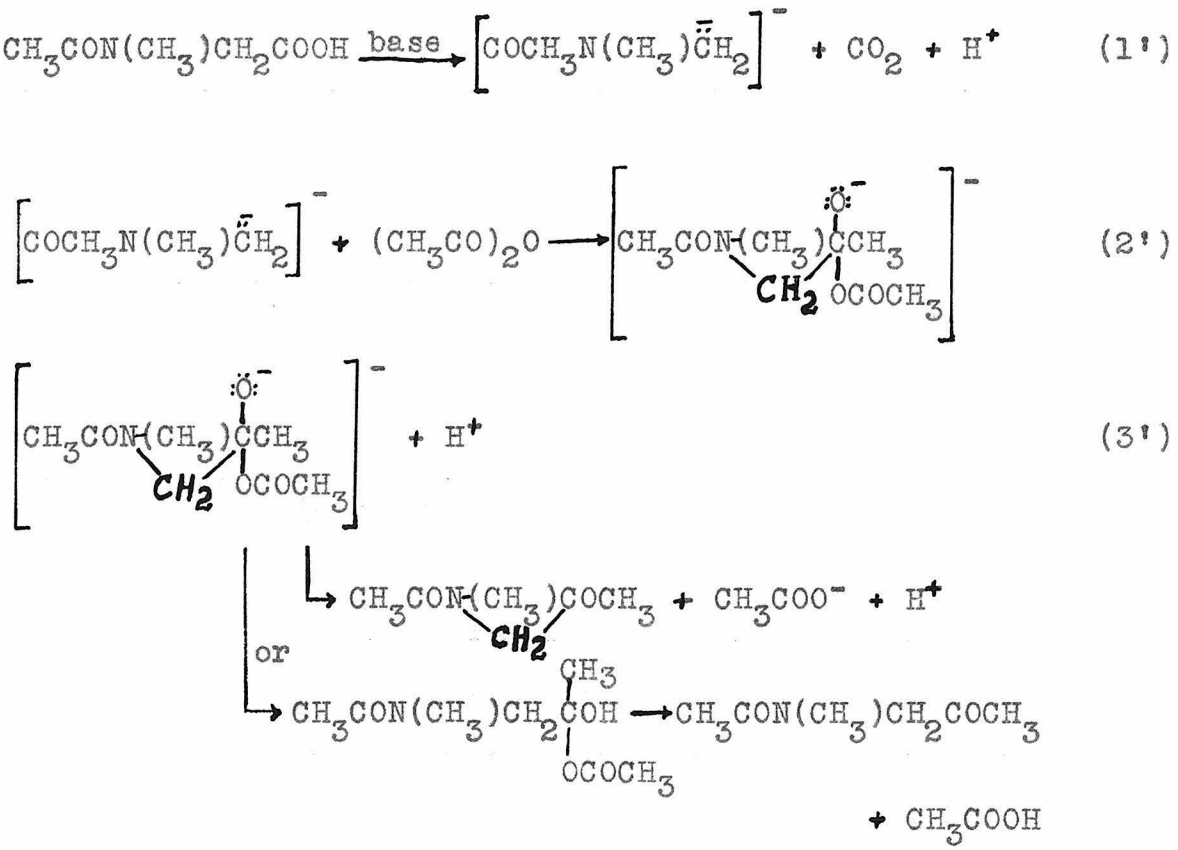
must intervene in mechanism I.

forming a typical azlactone, undergoes the Dakin-West reaction, proposed an alternate mechanism shown in Figure 2.

Figure 2.

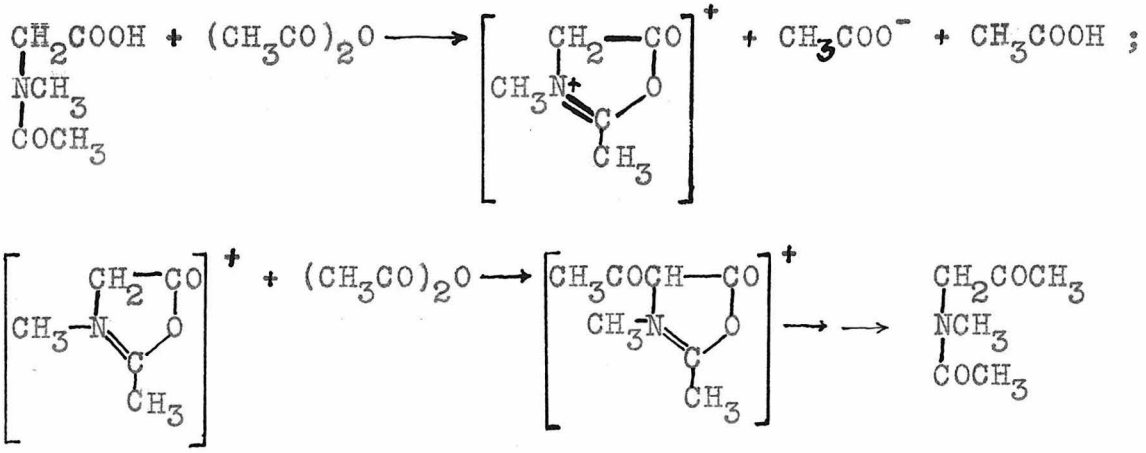
Mechanism II.

Wiley and Borum's Proposed Mechanism for the Dakin-West Reaction.



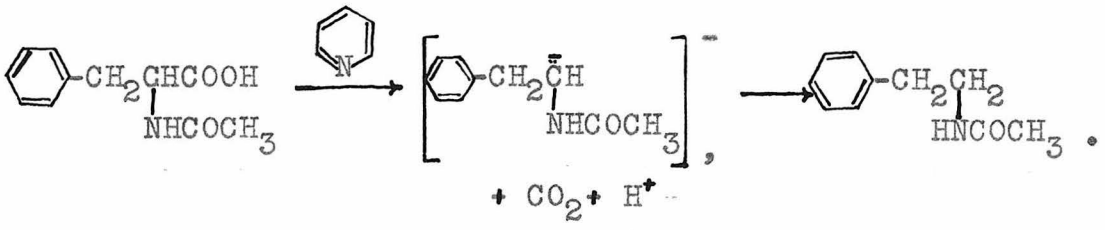

However, O'Brien and Niemann (25) have shown that under conditions similar to those used by Wiley and Borum, N-acetyl-

sarcosine can form a 5-oxazolonium (azlactonium) ion, and this could then be expected to react further, in a manner typical of azlactones themselves (acetylation, ring opening and decarboxylation) to give N-methyl-acetamidoacetone



The idea of an intermediate 5-oxazolonium ion in this case has also been suggested (26) elsewhere. Further, convincing evidence has been accumulated to show that Wiley and Borum's mechanism is not satisfactory.

A. N-acetylphenylalanine and pyridine were refluxed together for several hours. No carbon dioxide was evolved, whereas by Wiley's mechanism the following reaction might be expected to occur



Since it might have been possible that either the acid formed

in their reaction or some action of the carbonyl function of the acetic anhydride present was necessary to cause the decarboxylation, experiments were run as above in which, after refluxing, acid was added, the mixture refluxed, benzaldehyde added, and the mixture again refluxed. No carbon dioxide resulted here, nor when the addition of acid and benzaldehyde was reversed. However, as soon as acetic anhydride was added to any of these mixtures and then heated on a steamcone, carbon dioxide was evolved within three minutes and continued to appear in large amounts (35% in twenty minutes). A somewhat similar experiment has been reported elsewhere (26).

B. Dakin and West (2) found that amino acids containing no α-hydrogen atom failed to react. This is in accord with the azlactone mechanism, but is much more difficult to explain on the basis of Wiley and Borum's. The same is true for the failure of proline to react.

C. Cvejanovich and Searles (unpublished), without further elaboration, stated that "free acid was found necessary for the reaction to proceed." This is in accord with mechanism I, but not with mechanism II. However, since this statement did not appear in the published manuscript (11), it cannot be weighed heavily.

D. It is worthy of note that, since many azlactones can be prepared in near-quantitative yields by heating amino

acids with acetic anhydride for a few minutes, whereas the evolution of carbon dioxide continues in the Dakin-West reaction for several hours, mechanism II requires that constant azlactonization and de-azlactonization take place.

Evidence has been obtained in several ways to show that, in the reaction of an amino acid, acid anhydride and base catalyst, an intermediate is formed which is capable of hydrolysis by water or by reaction with acetic acid with the evolution of carbon dioxide. This is presumably the C-acylated azlactone of step (5), mechanism I. Of several methods used to demonstrate this fact, the most convincing is the following. In considering the proposed mechanism I, it was felt that, by conducting the reaction at different temperatures, the rates of the various steps could be changed relatively, and that if steps (6) and (7) could be slowed down relative to (5), it might be possible to demonstrate the presence of the C-acylated azlactone. Accordingly, a series of tests was conducted at room temperature, using 2-methyl-4-benzyl-5-oxazolone, pyridine and acetic anhydride ("95% min.") in a mol ratio of 1:10:25. The results are shown in Table III. The reactants were kept for the lengths of time as shown, concentrated at 6-8 mm. Hg and 30-35° until all of the pyridine and acetic acid and part of the acetic anhydride were eliminated, heated at 90-100° for 15-30 minutes, water added, and the mixture heated at reflux. Carbon dioxide was determined during the period of heating before water was

added, and during the period after water was added. In no case did carbon dioxide appear during the former period.

Table III.

The Formation In the Dakin-West Reaction of An Intermediate At Room Temperature Capable of Hydrolysis to Give Carbon Dioxide.

| Time, hrs. | Carbon Dioxide Evolved, % |
|---|---|
| 3 | 3 |
| 6 | 19 |
| 8 | 17 |
| 10 | 3* |
| 12 | 14 |
| 16 | 19 |
| 24 | 33** |
| 70 | 14 |
| 168 | 0 |

These results are compatible with mechanism I, wherein a base-catalyzed intermediate is formed which is then hydrolyzed, but this is not the case with mechanism II, where decarboxylation is one of the first steps, and requires base catalysis.

The steps (6, 7) leading to the cleavage of the C-acylated

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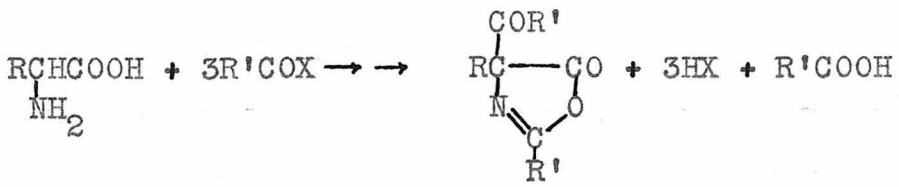
* Something went awry with this run, as judged by appearance as well as by the low per cent of carbon dioxide obtained.

** In another run of 24 hours, acetic acid was used in place of water for the cleavage. Although carbon dioxide was evolved more slowly than when water was used, the per cent of carbon dioxide evolved was essentially the same.

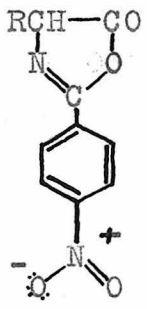
azlactone have been questioned by Rondestvedt et al. (24). They felt that the reaction of benzoyl fluoride and other acyl halides in place of anhydrides could not be explained by mechanism I, since, as inspection of the reaction



shows, only one mol of the acid R'COOH is produced, per mol of the azlactone, and this is only enough to cleave 0.5 mols of the azlactone by mechanism I. However, this is compatible with observed results, since in no case has more than 50% of the theoretical amount of carbon dioxide been observed when an acyl halide was used, and further, the acyl halides used contained several per cent of the corresponding acid, which would facilitate the latter stages of mechanism I when acyl halides were employed. Rondestvedt et al. conducted experiments, the results of which, as noted before, they interpreted to mean that three mols of anhydride are required per mol of amino acid converted to acylamidoketone, but these results, on the contrary, can be reasonably interpreted only in ways which are in accord with mechanism I. In connection with the use of acyl halides, further evidence was obtained for the intermediate formation of the C-acylated azlactone. 2-methyl-4-benzyl-5-oxazolone, benzoyl chloride and tributylamine were heated together for one hour at 90-

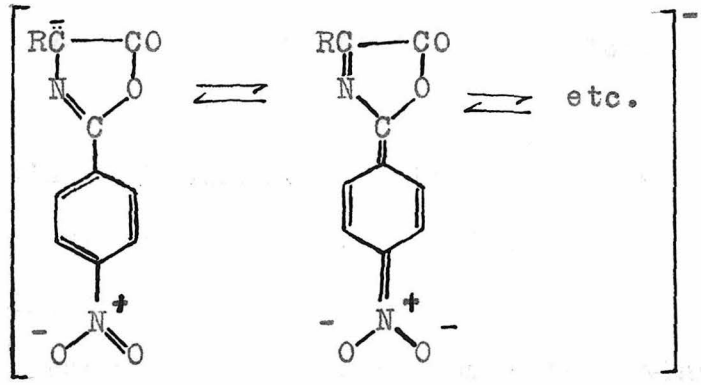
100°. A deep red color gradually developed, but only traces of carbon dioxide were evolved. However, on adding water and again heating, 38% of the theoretical amount of carbon dioxide was evolved in ten minutes, and a neutral residue which was isolated gave a yellow precipitate when treated with dinitrophenylhydrazine.

Finally, evidence that the carbanion shown in step (4) of mechanism I exists under the conditions of the Dakin-West reaction was demonstrated by the fact that a solution of any one of several amino acids in pyridine developed various colors when treated with *p*-nitrobenzoic anhydride, which changed to vivid colors on the addition of aqueous sodium hydroxide, and faded slowly over a period of several hours. These are the color sequences shown by these amino acids when treated with *p*-nitrobenzoyl chloride and any one of several bases (27), which colors have been shown (28) to be due to the formation of azlactones of the type of (XXX)



(XXX)

and from these, colored anions such as (XXXI)



(XXXI)

Thus, it appears that, based on experimental evidence (steps 1,3,4,5,8) and on reasonable and necessary assumptions (steps 6,7), the reaction picture of mechanism I is valid. The nature of step (2) remains in doubt. The rate-determining step(s) is undoubtedly one or more of (4),* (5), (6-7).

C. Miscellaneous Observations On the Reaction.

In the course of work on the Dakin-West reaction, a large number of observations were made which, for one reason or another, have found no place in previous sections. Much of this material has not been deemed of sufficient interest

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* Searles and Cvejanovich (11) found that in the reaction of acylamino acids, catalysts and acid anhydrides, the reaction was first order both with respect to the amino acid and to the catalyst. They determined that the relative catalytic effect of bases depended on basic strength, but even more on steric factors, as in the various picolines and lutidines. γ-picoline caused a much greater evolution of carbon dioxide than did pyridine, under the same conditions, but the amounts of ketone isolated were about the same and they felt this was due to a reaction of the ketone with the active methyl of the picoline. They concluded that the rate-determining step was step (4).

to merit inclusion. However, the observations recorded in the present section are felt to be of value.

1. Catalysis by impurities.

When alanylalanine and acetic anhydride were refluxed together for twenty minutes, 28% of the theoretical amount of carbon dioxide (based on the dipeptide) was evolved. After three recrystallizations from water, alanylalanine gave no carbon dioxide when refluxed for an hour with acetic anhydride. Two different commercial brands of alanine, one of which was from the same lot as that from which alanylalanine was prepared, also gave carbon dioxide when refluxed with acetic anhydride, but after three recrystallizations from water, neither brand of alanine gave carbon dioxide on refluxing with acetic anhydride. A mixture of two commercial brands of phenylalanine, which had not been recrystallized before using, heated with acetic anhydride, gave no carbon dioxide. The neutral residue from the reaction mixtures from acetic anhydride and un-recrystallized alanine gave positive nitroprusside reactions.

2. Chromatographic studies.

Chromatography of neutral fractions isolated from several Dakin-West reaction mixtures showed that from two to eight colored by-products were present. The number and amount of these by-products seemed to vary with the nature of the reactants and the temperature and length of time of heating of the reaction mixtures. When sodium acetate was

used as catalyst instead of pyridine or γ-picoline, the original solutions were much lighter in color and only one or two light yellow or yellow-orange colored zones were observed on chromatographing a portion of the neutral residues.

3. Side reactions resulting from the use of organic catalysts.

When alanylalanine, pyridine and acetic anhydride were refluxed together for an hour, a neutral residue was isolated (a dark red oil) which in aqueous solution gave precipitates with saturated aqueous solutions of picric acid and mercuric chloride. An aqueous solution of the purified ketone expected from the above reaction gave no precipitates with either of these reagants.

When γ-picoline was substituted for pyridine, and the mixture refluxed for three hours, the neutral residue was very dark, and weighed 130% of theory. An aqueous solution of this residue gave precipitates with picric acid, mercuric chloride and bromine. All of these precipitates possessed indefinite melting points. Thus, the precipitate from treatment with picric acid darkened gradually at temperatures greater than 180°, but was not melted at 300°. On boiling an aqueous solution of the neutral residue with twice its own weight of Norite for several minutes, a good deal of the colored impurities was removed, but the carbon also removed a large amount of the desired ketone. It was found

that the best means of purification was to add an excess of aqueous mercuric chloride to an aqueous solution of the residue, followed by filtration or centrifugation to give a light yellow solution, passing hydrogen sulfide into the solution until no more precipitate was formed, then by filtration, neutralization, concentration to a small volume, and extraction with chloroform. Removal of the chloroform left a nearly colorless oil, which gave the nitroprusside color reactions typical of the desired product, and no precipitates with either mercuric chloride or picric acid.

When alanylalanine, acetic anhydride and sodium acetate were refluxed together for thirty minutes, 80% of the theoretical amount of carbon dioxide was evolved. Isolation of the neutral residue gave a light orange oil. This material gave no precipitates with either picric acid or bromine, but yielded a precipitate with mercuric chloride. This was much lighter in color than the precipitates obtained by treatment of the products from the runs where pyridine or picoline were used. By boiling an aqueous solution of the product of the run where sodium acetate was used as catalyst with a small amount of Norite, followed by filtration and concentration in vacuo, an almost colorless oil was obtained. This material still gave a precipitate (white) when treated with mercuric chloride. When the reaction was run for eight hours instead of thirty minutes, the neutral residue was much darker in color, and an aqueous solution of it gave

precipitates with mercuric chloride, picric acid and bromine.

When pyridine and acetic anhydride were refluxed together for a number of hours, concentration at 3-5 mm. and 100° left a dark residue. This material gave a small precipitate with picric acid and a somewhat larger one with mercuric chloride, but exhibited no color change when treated with sodium nitroprusside.

When γ-picoline and acetic anhydride were refluxed together for several hours, concentration in vacuo left a sizeable amount of dark residue. An aqueous solution of this oil gave precipitates with picric acid and mercuric chloride, and a positive color reaction with sodium nitroprusside, typical of methyl ketones. It did not appear to react with phenylhydrazine.

From the evidence presented in the forgoing discussion, the following conclusions may be drawn:

In general, in Dakin-West reactions, side reactions occur which lead to the formation of by-products. These impurities can in large part be precipitated by mercuric chloride.

In addition, when heterocyclic organic catalysts are used, there are side-reactions between the catalyst and the anhydride which lead to large amounts of additional by-products. Some of these can also be precipitated by mercuric chloride, and where this reagent does not also react with the desired product, it affords a convenient method of purification.

Where γ-picoline is used as a catalyst, at least a part of the impurities formed seems to consist of one or several methyl ketones.*

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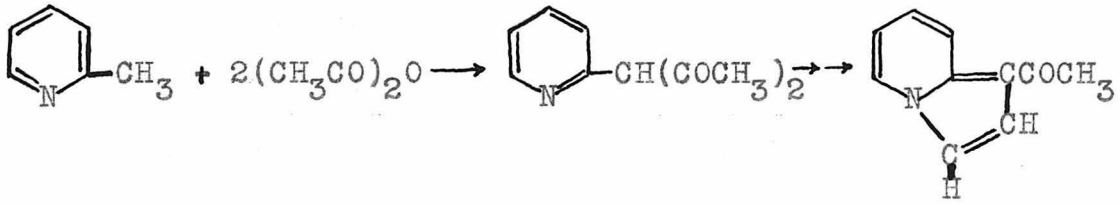
* A reaction between α-picoline and acetic anhydride at high temperatures has been recorded (29). This reaction apparently takes the course



The observed formation of methyl ketones by heating γ-picoline and acetic anhydride together may have resulted from a similar reaction, since both α- and γ-picolines contain a reactive methyl group, or it may have been that the γ-picoline used contained a certain amount of its α-isomer.

## Experimental

General.--In the following experiments no attempt has been made to determine the conditions leading to maximum yields.

All melting points are corrected.

Only optically inactive materials were used, unless otherwise noted.

The "theoretical amount of carbon dioxide," referred to in certain experiments, is the quantity of carbon dioxide that might be expected if a "normal" Dakin-West reaction took place, whereby one mol of carbon dioxide would be produced per mol of amino acid (or its equivalent) that reacted.

Apparatus.--The apparatus used for the quantitative determinations of carbon dioxide evolved during the reactions was essentially the same as that employed by Dakin and West (2). A tower (A), charged at the top with Drierite and at the bottom with Ascarite, was connected to the reaction vessel (B). (B), a three-neck, round-bottom flask, was equipped with a thermometer (C), a stirrer (D) and a condenser (E). (E) was joined to a trap (F) containing 25% sulfuric acid, and (F) was connected to a bubbler (G) containing bromophenol blue. (G) was attached to an absorption receiver (H) by a sintered glass bubbler, and (H) was joined by a controlled leak (I), with a dry-ice trap (J) interposed,

to an oil-pump (K). Heating was accomplished by means of a steambath, an oilbath or a Glascol heater.

Operation.--The reactants were introduced into (B), and the whole system, with the exception of (H), was swept rapidly by brief operation of (K). (H), containing a measured excess of standard base,* was connected into the system, (K) was started, and (I) adjusted so that a fairly rapid stream of air (40-60 ml./min.), freed of water and carbon dioxide by (A), was pulled through the system. The reactants were stirred by (D). Any carbon dioxide evolved in (B) was absorbed in (H). Basic substances entrained in the air stream were trapped in (F), while a change of the indicator in (G) warned of acid substances entering (H). The excess base in (H) was titrated to a phenolphthalein endpoint with 0.2-0.3 N hydrochloric acid at the end of an experiment.

Reagents.--The majority of the chemicals used were commercial products of a good grade, employed without further treatment unless purification seemed necessary for the purpose of the experiment. In such cases the treatment is specified in the text.

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* Barium hydroxide (0.2-0.3 N) was the preferred absorbent, since the appearance of barium carbonate furnished an indication that reaction was taking place. In certain cases, however, the carbonate produced caused stoppage of the sintered glass bubbler. In such cases sodium hydroxide was used as the absorbent, an excess of barium chloride being added before titration at the end of the experiment.

1-Phenyl-2-acetamido-3-butanone.--(A) A mixture of 12.4 g. (0.075 M) of phenylalanine, 39.3 g. (0.5 M) of pyridine and 65.2 g. (0.64 M) of acetic anhydride was heated on a steam-bath for five hours, steam distilled until free of pyridine, the residue treated with an excess of aqueous sodium bicarbonate, and extracted with six 100-ml. portions of ether. The ethereal extract was dried, the solvent removed and the waxy orange-yellow solid recrystallized twice from xylene to give 12.1 g. (79%) of 1-phenyl-2-acetamido-3-butanone, colorless needles, m.p. 98-99°.

(B) A mixture of 1.65 g. (0.01 M) of phenylalanine, 4 g. (0.05 M) of anhydrous sodium acetate and 30.6 g. (0.3 M) of acetic anhydride was heated with stirring for thirty minutes at 130-135°, and the reaction product worked up substantially as described in (A) to give 1.3 g. (62%) of the acetamidoketone, colorless needles, m.p. 98-99°.

(C) A mixture of 2 g. (0.012 M) of phenylalanine, 7.9 g. (0.1 M) of pyridine and 8.8 g. (0.11 M) of acetyl chloride was heated with stirring for one hour at 60° and the reaction mixture worked up as previously described to give 0.15 g. (8%) of 1-phenyl-2-acetamido-3-butanone, colorless needles, m.p. 97-99° after one recrystallization from xylene.

1-Phenyl-2-propionamido-3-pentanone.--A mixture of 3.3 g. (0.02 M) of phenylalanine, 16 g. (0.2 M) of pyridine and 26 g. (0.2 M) of propionic anhydride was refluxed at 140-145°

for thirty minutes, the solution concentrated *in vacuo*, treated with an excess of aqueous sodium bicarbonate, extracted successively with two 100-ml. portions of ether and of chloroform, the non-aqueous phases combined, dried, and the solvents evaporated to give approx. 5 g. of a light-orange oil. The oil was taken up in hot ligroin, b. p. 90-130°, filtered, the volume reduced to 25 ml., and the solution stored at 5° overnight. The colorless needles so obtained were recrystallized first from ligroin and then from aqueous acetone to give 1.7 g. (36%) of 1-phenyl-2-propionamido-3-pentanone, colorless needles, m. p. 67-68°. In a second experiment the reaction mixture resulting from heating 5 g. (0.03 *M*) of phenylalanine, 16 g. (0.2 *M*) of pyridine and 26 g. (0.2 *M*) of propionic anhydride at 135° for one and one-half hours was fractionally distilled to give 2.9 g. (41%) of 1-phenyl-2-propionamido-3-pentanone, colorless needles, m. p. 67-68°.

*Anal.* Calcd. for $C_{14}H_{19}O_2N$: C, 72.1; H, 8.2; N, 6.0. Found: C, 71.9; H, 8.2; N, 5.9.

The *oxime*, colorless needles, m. p. 152-153°, was recrystallized from 95% ethanol.

*Anal.* Calcd. for $C_{14}H_{20}O_2N_2$: C,67.7; H, 8.1; N, 11.3. Found: C, 67.6; H, 8.4; N, 11.1.

The *2,4-dinitrophenylhydrazone*, yellow needles, m. p. 153-154°, was recrystallized alternately from ethyl acetate and 95% ethanol.

Anal. Calcd. for $C_{20}H_{23}O_5N_5$: N, 16.9. Found: N,16.8.

1-Phenyl-2-butyramido-3-hexanone.--A mixture of 3.3 g. (0.02 M) of phenylalanine, 16 g. (0.2 M) of pyridine and 40 g. (0.25 M) of butyric anhydride was heated for three hours at 145-150°, and the reaction product worked up as described for the preceding preparation to give a light-orange oil which crystallised upon standing at 5°. This product was recrystallized successively from xylene and ligroin, 30-60°, to give 1.4 g. (27%) of 1-phenyl-2-butyramido-3-hexanone; colorless needles, m. p. 59-60°.

Anal. Calcd. for $C_{16}H_{23}O_2N$: C, 73.5; H, 8.9; N, 5.4. Found: C, 73.6; H, 8.7; N, 5.4.

The oxime, colorless needles, m. p. 145-146° was recrystallized twice from water.

Anal. Calcd. for $C_{16}H_{24}O_2N_2$: C, 69.5; H, 8.8; N, 10.1. Found: C, 69.3; H, 8.7; N, 10.1.

The 2,4-dinitrophenylhydrazone, bright yellow needles, m. p. 173-174° was obtained after successive recrystallization from ethanol and ethyl acetate.

Anal. Calcd. for $C_{22}H_{27}O_5N_5$: C, 59.9; H, 6.2; N, 15.9. Found: C, 60.1; H, 6.4; N, 16.0.

1-Methoxy-3-methoxyacetamido-4-phenyl-2-butanone.--A mixture of 5 g. (0.03 M) of phenylalanine, 12 g. (0.15 M) of pyridine and 24 g. (0.15 M) of methoxyacetic anhydride*

* Prepared by a method similar to that described by Allen, et al., "Organic Syntheses," 26, 1 (1946).

was heated with stirring at 115° for one hour, the solution evaporated *in vacuo*, the residue treated with an excess of aqueous sodium bicarbonate, extracted successively with two 100-ml. portions of ether and of chloroform, the non-aqueous phases combined, dried, the solvents removed, the residual oil taken up in 60% ethanol, the solution treated with Norite, filtered, and the solvents evaporated to give 6.2 g. (78%) of a neutral light-yellow oil which could not be induced to crystallize. A portion of the oil was treated with p-nitrophenylhydrazine to give a p-nitrophenylhydrazone, stout orange rhombs, m. p. 179-181° after two recrystallizations from 95% ethanol.

*Anal.* Calcd. for $C_{20}H_{24}O_5N_4$: C, 60.0; H, 6.0; N, 14.0. Found: C, 60.2; H, 6.1; N, 14.1.

The *2,4-dinitrophenylhydrazone* was obtained as light-yellow needles, m. p. 168-169° after two recrystallizations from chloroform.

*Anal.* Calcd. for $C_{20}H_{23}O_7N_5$: C, 53.9; H, 5.2; N, 15.7. Found: C, 53.8; H, 5.2; N, 15.8.

The *semicarbazone*, rosettes of colorless needles, m. p. 116-117°, was recrystallized twice from water.

*Anal.* Calcd. for $C_{15}H_{22}O_4N_4$: C, 55.9; H, 6.9; N, 17.4. Found: C, 55.7; H, 7.0; N, 17.5.

*α-Benzamidopropiophenone.*--A mixture of 3 g. (0.034 M) of alanine, 12 g. (0.15 M) of pyridine and 34 g. (0.15 M) of benzoic anhydride was heated with stirring at 130-135°

for two and one-half hours, and the reaction product worked up as previously described. The solid obtained was recrystallized once from ligroin and twice from aqueous ethanol to give 3.6 g. (42%) of α-benzamidopropiophenone; colorless prisms, m. p. 104-105°.

Anal. Calcd. for $C_{16}H_{15}O_2N$: C, 75.9; H, 6.0; N, 5.5. Found: C, 76.0; H, 6.0; N, 5.4.

The oxime, rosettes of colorless needles, m. p. 157-158°, was recrystallized twice from aqueous ethanol.

Anal. Calcd. for $C_{16}H_{16}O_2N_2$: C, 71.6; H, 6.0; N, 10.4. Found: C, 71.9; H, 6.2; N, 10.3.

The ketone (3 g.) in 3 ml. of cold concd. sulfuric acid was diluted with ice water, the solid collected, washed with water and recrystallized twice from ligroin to give 2,5-diphenyl-4-methyl-oxazole, prisms, m. p. 81-82°.

Anal. Calcd. for $C_{16}H_{13}ON$: C, 81.7; H, 5.6; N, 6.0. Found: C, 81.8; H, 5.7; N, 5.8.

α-Benzamido-β-phenyl-propiophenone.--(A) A stirred mixture of 2.5 g. (0.015 M) of phenylalanine, 8 g. (0.10 M) of pyridine, and 22.5 g. of benzoic anhydride (0.10 M) was heated at 140-145° for two hours. The reaction mixture was worked up as described for α-benzamidopropiophenone to give 2.2 g. (44%) of α-benzamido-β-phenylpropiophenone, long colorless needles, m. p. 146-147° after two recrystallizations from aqueous ethanol.

Anal. Calcd. for $C_{22}H_{19}O_2N$: C, 80.2; H, 5.8; N, 4.3.

Found: C, 80.3; H, 5.8; N, 4.3.

The oxime was obtained after two recrystallizations from 95% ethanol as colorless needles, m. p. 188-189°.

Anal. Calcd. for $C_{22}H_{20}O_2N_2$: C, 76.7; H, 5.8; N, 8.1. Found: C, 76.7; H, 6.0; N, 8.0.

A solution of 0.4 g. of α-benzamido-β-phenyl-propiophenone in 30 ml. of 6 N hydrochloric acid and 10 ml. of ethanol was refluxed for three hours, the solvents removed, the residue extracted with ether, and dissolved in 10 ml. of ethanol. The addition of 25 ml. of ether gave the hydrochloride of α-amino-β-phenylpropiophenone, lustrous needles, m. p. 200° with decomposition.

Anal. Calcd. for $C_{15}H_{16}ONCl$: C, 68.8; H, 6.2; N, 5.4; Cl, 13.6. Found: C, 68.6; H, 6.4; N, 5.2; Cl, 13.8.

(B) A mixture of 2.7 g. (0.01 M) of N-benzoylphenylalanine, 5.9 g. (0.075 M) of pyridine and 11.3 g. (0.05 M) of benzoic anhydride heated at 135-140° for two hours with stirring when treated as described above gave 1.2 g. (36%) of α-benzamido-β-phenylpropiophenone, colorless needles, m. p. 146-147°.

(C) When a mixture of 2 g. (0.012 M) of phenylalanine, 7.9 g. (0.1 M) of pyridine, and 17 g. (0.12 M) of benzoyl chloride was heated for one hour at 135-140°, 39-42% of the anticipated quantity of carbon dioxide was evolved, but on working up the dark tarry reaction product only 0.36 g. (9%) of α-benzamido-β-phenylpropiophenone, m. p. 146-147°,

was obtained.

(D) 1.65 g. (0.01 M) of phenylalanine, 8.4 g. (0.06 M) of benzoyl chloride and 5.6 g. (0.03 M) of tri-n-butylamine were mixed and heated at 135-140° for one hour. 48% of the theoretical amount of carbon dioxide was evolved, and from the reaction mixture, which was lighter in color and much less tarry than that from (C), was isolated 0.65 g. (20%) of α-benzamido-β-phenyl-propiophenone.

(E) A mixture of 2 g. (0.012 M) of phenylalanine, 7.9 g. (0.1 M) of pyridine and 12.5 g. (0.1 M) of benzoyl fluoride* was heated for two hours at 135-140°. 46% of the theoretical amount of carbon dioxide was evolved, and from the reaction mixture there was obtained 1.55 g. (39%) of α-benzamido-β-phenyl-propiophenone, m. p. 146-147°.

The reaction of phenylalanine, p-nitrobenzoic anhydride and γ-picoline.--A mixture of 0.5 g. (0.003 M) of phenylalanine, 6.0 g. (0.019 M) of p-nitrobenzoic anhydride** and 27 g. (0.27 M) of γ-picoline was refluxed for one hour, during which time 55% of the theoretical amount of carbon dioxide was evolved. The neutral residue obtained on working up the reaction mixture showed a positive reaction with 2,4-dinitrophenylhydrazine, presumably due to presence of the ketone

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* Prepared from benzoyl chloride and zinc fluoride in 41% yield by the method of M. Meslans and F. Girardet, Bull. soc. chim. (3) 15, 878 (1896).

** Prepared in 87% yield by the reaction of p-nitrobenzoyl chloride and sodium p-nitrobenzoate.

α-(p-nitrobenzamido)-β-phenyl-p-nitropropiophenone.

3-Phenyl-2-(α-furamido)-1-(α-furyl)-1-propanone.--1.65 g. (0.01 M) of phenylalanine, 15.5 g. (0.075 M) of α-furoic anhydride* and 59 g. (0.75 M) of pyridine were refluxed together for thirty minutes. More than 75% of the theoretical amount of carbon dioxide was evolved during this time. The dark mixture was concentrated in vacuo to 25 ml., 100 ml. of water and 17 g. (0.2 M) of sodium bicarbonate were added, and the mixture was warmed on a steamcone for two hours and then extracted with two 100-ml. portions of chloroform and one 100-ml. portion of ether. The solvents were removed from the combined non-aqueous extracts, a solution of 100 ml. of water and 50 ml. of ethanol was added, the resulting solution was boiled for two minutes with 1 g. of Nuchar and filtered. The filtrate was concentrated to 50 ml. and stored at 5° for ten hours. A white solid was filtered off, washed with cold water and dried, wt. 1.7 g. (55%) of 3-phenyl-2-(α-furamido)-1-(α-furyl)-1-propanone. Recrystallized from 60% ethanol and then from 96% ethanol, small colorless needles, m. p. 140-141°.

Anal. Calcd. for $C_{18}H_{15}O_4N$: C, 69.9; H, 4.9; N, 4.5. Found: C, 70.0; H, 5.0; N, 4.7.

3-Phenyl-2-acetamido-1-(α-furyl)-1-propanone.--2.07 g.

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* Prepared in 89% yield by the reaction of α-furoyl chloride and sodium α-furoate.

(0.01 M) of N-acetylphenylalanine, 10.3 g. (0.05 M) of α-furoic anhydride, 1.7 g. (0.015 M) of α-furoic acid and 59 g. (0.75 M) of pyridine were refluxed together for 0.75 hours. Greater than 60% of the theoretical amount of carbon dioxide was evolved in thirty minutes. The solution was concentrated in vacuo to remove pyridine, treated with an excess of aqueous sodium bicarbonate, and extracted with three 100-ml. portions of chloroform. The chloroform was removed in vacuo, a solution of 150 ml. of ethanol and 100 ml. of water was added, and the resulting solution was then decolorized by boiling for five minutes with 2 g. of Nuchar and filtering. The filtrate was concentrated in vacuo, the residue was taken up in 30 ml. of hot xylene, 20 ml. of 30-60° petroleum ether was added, and in three minutes the colorless supernate was decanted from a little yellow oil. On chilling the supernate, clumps of colorless needles separated. The solid was filtered off and dried, wt. of 3-phenyl-2-acetamido-1-(α-furyl)-1-propanone = 0.9 g. (35%), m. p. 120.5-121.5° after recrystallization from ethanol.

Anal. Calcd. for $C_{15}H_{15}O_3N_n$: C, 70.0; H, 5.9; N, 5.4. Found: C, 70.2; H, 5.9; N, 5.4.

The reaction of 2-methyl-4-benzyl-5-oxazolone, nicotinic anhydride, nicotinic acid and pyridine.--The oxazolone from 0.825 g. (0.005 M) of phenylalanine was refluxed with 3.3 g. (0.015 M) of crude nicotinic anhydride, 1 g. (0.008 M) nicotinic acid and 30 g. pyridine, and ca. 30% of the

theoretical amount of carbon dioxide was evolved in one hour. The very dark reaction mixture was not worked up.

The reaction of phenylalanine, succinic anhydride and γ-picoline.--1.65 g. (0.01 M) of phenylalanine, 4 g. (0.04 M) of succinic anhydride and 23 g. (0.25 M) of γ-picoline were refluxed together for fifteen minutes. 2% of the theoretical amount of carbon dioxide was evolved. Then 2.4 g. (0.02 M) of succinic acid was added and the mixture was refluxed one hour. 1% of the theoretical amount of carbon dioxide was evolved. 20 ml. of water was added and the mixture was again refluxed for one hour. Only traces of carbon dioxide were evolved. Similar results were observed when pyridine (20 g., 0.25 M) was substituted for γ-picoline.

The reaction of N-acetyl phenylalanine, succinic anhydride and γ-picoline.--2.1 g. (0.01 M) of N-acetylphenylalanine, 6 g. (0.06 M) of succinic anhydride and 50 g. (0.54 M) of γ-picoline were refluxed together for thirty minutes. Then 2.4 g. (0.02 M) of succinic acid was added, and the mixture was refluxed for forty-five minutes. Then 20 ml. of water was added, and the mixture was refluxed for fifteen minutes. The total amount of carbon dioxide evolved was 2% of the theoretical amount.

The reaction of 2-methyl-4-benzyl-5-oxazolone, succinic anhydride and γ-picoline.--1.65 g. (0.01 M) of phenylalanine was heated at 100° for 10 minutes with 30 ml. of acetic anhydride and the solution concentrated in vacuo. The

oxazolone was heated at 120-125° with 1.3 g. (0.013 M) of succinic anhydride and 15 g. (0.16 M) of γ-picoline. After thirty minutes only a trace of carbon dioxide was formed, nor did any more appear when 40 ml. of water was added and the mixture was refluxed for fifteen minutes. In another experiment, when the oxazolone was heated with the anhydride and catalyst (same mol ratio) at reflux (ca. 145°) for one hour, 3-4% of the theoretical amount of carbon dioxide was evolved. The reaction mixture was very dark and tarry. 20 ml. of water was added and the mixture was again refluxed for thirty minutes. Only traces of carbon dioxide were formed.

The reaction of 2-methyl-4-benzyl-5-oxazolone, phthalic anhydride, disodium phthalate and pyridine.--A mixture of 1.65 g. (0.01 M) of phenylalanine and 35 ml. of acetic anhydride was heated at 100° for 10 minutes and concentrated in vacuo. The oxazolone was refluxed together with 8.9 g. (0.06 M) of phthalic anhydride, 2.9 g. (0.014 M) of disodium phthalate and 40 g. (0.5 M) of pyridine for four hours. Only traces of carbon dioxide appeared. After concentrating to remove most of the pyridine (during which operation no carbon dioxide appeared), the orange mixture was treated with 60 ml. of water and refluxed for ten minutes. Then 4 ml. of concentrated hydrochloric acid was added and the mixture was refluxed for thirty minutes. No carbon dioxide was evolved in the last two steps.

The reaction of maleic anhydride and pyridine.--2 g. (0.02 M) of maleic anhydride was added to 5 g. (0.06 M) of pyridine. Over a three minute period the solution changed from yellow to orange to red to dark red-brown, and the temperature dropped from 26.5° to 19°. After another three minutes the temperature rose to 26°, and at the end of an additional six minutes the temperature was 64°, with carbon dioxide being evolved. The temperature dropped to 27° in thirty minutes. After one hour at room temperature the amount of carbon dioxide which had been formed amounted to 0.005 M.

In another experiment, 2 g. (0.02 M) of maleic anhydride and 5 g. (0.06 M) of pyridine were heated together at 95-100° for ninety minutes. The carbon dioxide formed amounted to 0.012 M.

The dark reaction mixture from the first run was washed several times with 10-ml. portions of benzene, leaving a dark brown, solid residue. This material was very soluble in water, ethanol and acetone, quite insoluble in benzene or ether. The aqueous solution was acidic, and gave a precipitate with 0.2 N barium hydroxide. This precipitate was redissolved on acidification with 0.2 N hydrochloric acid. Fusion of some of the material with soda lime resulted in the formation of a basic liquid with the odor of pyridine. Chromatography of an ethanolic solution on a silicic acid column, developed with 50% ethanol, revealed five colored zones.

The reaction of maleic anhydride and triethylamine.--1 g. (0.01 M) of maleic anhydride was mixed with 1 g. (0.01 M) of triethylamine. The mixture rapidly turned red. On warming at 35-40° for a few seconds a vigorous reaction began, the temperature rose rapidly, and the mixture finally foamed, producing red vapors and leaving a purple residue. A large amount (0.005 M) of carbon dioxide was evolved during the reaction.

The reaction of maleic acid with maleic anhydride and sodium hydrogen maleate.--1.5 g. (0.0015 M) of maleic acid, 2.1 g. (0.015 M) of sodium hydrogen maleate and 14.7 g. (0.15 M) of maleic anhydride were heated together at 120-125° for thirty minutes. The mixture darkened considerably, and a large amount (0.01 M) of carbon dioxide was evolved.

The reaction of 2-methyl-4-benzyl-5-oxazolone, maleic anhydride and disodium maleate.--3.3 g. (0.03 M) of phenylalanine was converted to the oxazolone by heating at 100° for fifteen minutes with 50 ml. of acetic anhydride, followed by concentration in vacuo. The oxazolone was heated at reflux with 1.9 g. (0.012 M) of disodium maleate, 2.0 g. (0.02 M) of maleic anhydride and 100 ml. of dry benzene for thirty minutes. The mixture became orange during this period (a pink color developed on mixing the oxazolone and maleic anhydride, turning to red in several minutes), and 5% of

the theoretical amount of carbon dioxide was evolved.* After cooling the mixture a solid (A) was filtered off, washed with 30 ml. of benzene and air-dried, wt. 3.3 g. The filtrate deposited a further amount of solid (B) on standing several hours. (B) was filtered off, washed with 30 ml. of benzene and air-dried, wt. 2.5 g. The filtrate from (B) was concentrated in vacuo giving 1.6 g. of red-orange oil (C).

(A) was recrystallized twice from water to give 2.8 g. of colorless plates. A concentrated aqueous solution of (A) was acidic, and on making this solution strongly acidic with 2 N hydrochloric acid, (B) was precipitated out (as shown by appearance and melting point behavior).

(B) was recrystallized once from 0.5 N hydrochloric acid [the filtrate = (D)] and twice from water to give 1.5 g. of colorless rods and needles. An aqueous solution of (B) was strongly acidic. (B) was air-dried at room temperature for seventy-two hours. It softened and then partially melted over the range 114-120°, resolidified, softened somewhat at 215°, and went from yellow through orange to very dark brown over the range 220-320°. It was not melted at 320°. A sample of (B) was analyzed:

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* In a similar experiment, when 2.3 g. (0.02 M) of maleic acid was added at this point and the mixture then refluxed, a vigorous evolution of carbon dioxide took place. After refluxing for fifteen minutes this amounted to 40% of theoretical. On working up the reaction product, however, no results were observed which differed essentially from those reported above.

loss of wt. at 110°, 7.3%; Found (after drying at 110° to constant wt.): C, 42.0; H, 4.05; N, 0.0.

(B) reduced permanganate and absorbed bromine. It did not appear to react with a saturated solution of dinitrophenylhydrazine, but treatment of the filtrate (D) in 1 N hydrochloric acid with this reagent yielded an orange-yellow precipitate. This material seemed unstable to heat. Recrystallized from warm (70-80°) 96% ethanol, it gave an indefinite melting point, softening and darkening over a considerable range. The last solid was gone at 130°.

(C) gave an acidic solution in water. On warming with 4 N NaOH a basic gas was evolved and the odor of ammonia was apparent. (C) decolorized bromine and reduced permanganate. An aqueous solution gave an immediate precipitate with a saturated solution of dinitrophenylhydrazine in 1 N HCl. The product, an unstable low-melting solid, was recrystallized from warm (50-60°) 96% ethanol. It sintered at ca. 40° and melted completely at 50°. (C) could not be induced to crystallize.

Anal. Calcd. for the 2,4-dinitrophenylhydrazone of 6-phenyl-5-acetamido-4-keto-2-hexenoic acid, $C_{20}H_{19}O_7N_5$: C, 54.4; H, 4.3; N, 15.9. Found for derivative from (D): C, 55.0; H, 5.0; N, 13.3. Found for derivative from (C): C, 54.7; H, 4.7; N, 14.1.

The reaction of phenylalanine, sodium hydrogen maleate and maleic anhydride.--1.65 g. (0.01 M) of phenylalanine,

2 g. (0.015 M) of sodium hydrogen maleate, 1.7 g. (0.015 M) of maleic acid and 9.8 g. (0.1 M) of maleic anhydride were heated together at 90-100° for ninety minutes. No carbon dioxide was formed. 4.9 g. (0.05 M) of maleic anhydride was added and the mixture was heated at 120° for fifteen minutes. The mixture foamed and darkened considerably, and 0.008 M of carbon dioxide was evolved. The residue was taken up in 50 ml. of hot water. On cooling, 1.6 g. of (B), the high-melting material described in the previous experiment, crystallized out. The filtrate gave only a faint cloudiness with a saturated solution of dinitrophenylhydrazine in 1 N hydrochloric acid.

The reaction of adipic anhydride, pyridine and phenylalanine.--0.8 g. (0.005 M) of phenylalanine, 40 g. (0.5 M) of pyridine and 10 g. of crude adipic anhydride* were refluxed together for one hour, during which time 22% of the theoretical amount of carbon dioxide was evolved. The dark reaction mixture was not worked up.

The reaction of N-acetylphenylalanine, pyridine and acetonitrile.--0.9 g. (0.004 M) of N-acetylphenylalanine, 1.8 g. (0.044 M) of acetonitrile and 3.5 g. (0.044 M) of pyridine were refluxed together for thirty minutes. No carbon dioxide was formed during this period. Then 0.4 g. (0.004 M) of acetic anhydride and 0.2 g. (.002 M) of anhy-

* Prepared by the slow distillation of a mixture of adipic acid and acetic anhydride, followed by crystallization of the residue from benzene-ligroin.

drous sodium acetate was added and the mixture refluxed for one hour. Only traces of carbon dioxide appeared.

The reaction of 2-methyl-4-benzyl-5-oxazolone, pyridine and acetonitrile.--0.7 g. (0.004 M) of the oxazolone was refluxed for one hour with 16 g. (0.2 M) of pyridine and 0.9 g. (0.02 M) of acetonitrile. Only traces of carbon dioxide were formed. 15 ml. of acetic anhydride was added and the mixture refluxed for one hour. Only traces of carbon dioxide were formed. 50 ml. of water was added and the mixture was refluxed for one hour. No carbon dioxide was evolved.

The reaction of N-acetylphenylalanine, pyridine and ethyl formate.--0.5 g. (.003 M) of N-acetylphenylalanine, 4 g. (0.005 M) of pyridine and 0.4 g. (.005 M) of ethyl formate were refluxed together for one hour. No carbon dioxide was produced, nor was any formed when 10 ml. of 3 N hydrochloric acid was added and the mixture refluxed for ten minutes.

The reaction of 2-methyl-4-benzyl-oxazolone, pyridine and ethyl formate.--0.8 g. (0.004 M) of the oxazolone was refluxed for four hours with 4 g. (0.005 M) of pyridine and 7 g. (0.1 M) of ethyl formate. No carbon dioxide appeared. Then 20 ml. of 6 N HCl was added and the mixture was refluxed for three hours. No carbon dioxide was formed during this period.

In a similar experiment using amyl acetate in place of ethyl formate, the same results were observed.

The reaction of 2-methyl-4-benzyl-5-oxazolone and γ-picoline.--1.65 g. (0.01 M) of phenylalanine was heated at 90-100° for fifteen minutes with 40 ml. of acetic anhydride. The solution was concentrated in vacuo to remove acetic acid and acetic anhydride. 15 g. (0.16 M) of dry γ-picoline was added and the solution was refluxed for thirty minutes. During the first fifteen minutes the solution became red and 0.0004 M of carbon dioxide was given off. No further carbon dioxide was formed in the second fifteen minutes, nor was any formed when 10 ml. of water was added and the solution then refluxed for ten minutes. 10 ml. of acetic acid was then added and the mixture refluxed for ten minutes, and then 10 ml. of 0.2 N hydrochloric acid was added and the mixture again was refluxed for ten minutes. In neither was carbon dioxide produced.

The reaction of O-methyl serine, p-nitrobenzoic anhydride and pyridine.--1.5 g. (0.013 M) of O-methyl serine,* 24 g. (0.079 M) of p-nitrobenzoic anhydride and 32 g. (0.4 M) of pyridine were heated together for one hour. 15% of the theoretical amount of carbon dioxide was evolved.

The reaction of cysteine, γ-picoline and acetic anhydride.--1.2 g. (0.06 M) of cysteine hydrochloride, 0.8 g.

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* Prepared in 26% over-all yield by the method of H. E. Carter and H. D. West, Organic Syntheses 20, 81 (1940).

(0.01 M) of sodium acetate, 15 g. (0.16 M) of γ-picoline and 30 g. (0.3 M) of acetic anhydride were refluxed together for thirty minutes. 32% of the theoretical amount of carbon dioxide was produced in the first twenty-five minutes (acidification of the precipitate in the receiver produced no hydrogen sulfide). After the first thirty minutes of heating, the odor of hydrogen sulfide was noted, the bromophenol blue showed a change in color, and acidification of the precipitate in the receiver resulted in the evolution of hydrogen sulfide. The solution was concentrated in vacuo to some degree, an excess of aqueous sodium bicarbonate was added, and the solution was extracted with four 100-ml. portions of ether. The combined ether extracts, extracted with 50 ml. of 4 N hydrochloric acid, washed with 50 ml. of water and dried, gave 0.6 g. of dark red oil on concentrating in vacuo. An aqueous-alcoholic solution of the oil gave a cloudy precipitate when treated with a saturated solution of dinitrophenylhydrazine in 1 N hydrochloric acid.

The reaction of arginine, pyridine and acetic anhydride.--1 g. (0.005 M) of arginine hydrochloride, 6 g. (0.08 M) of pyridine and 8 g. (0.08 M) of acetic anhydride were heated together at 120-125° for thirty minutes, and 64% of the theoretical amount of carbon dioxide was evolved during this time.

The reaction of nitroarginine, pyridine and acetic

anhydride.--1 g. (0.005 M) of nitroarginine,* 6 g. (0.08 M) of pyridine and 8 g. (0.08 M) of acetic anhydride were refluxed together for twenty minutes. 67% of the theoretical amount of carbon dioxide was evolved. The reaction mixture was concentrated in vacuo at 90°, 30 ml. of water was added and the mixture concentrated to 25 ml. An excess of sodium bicarbonate was added and the mixture was extracted with three 50-ml. portions of chloroform. On drying the combined chloroform solutions and removal of the chloroform in vacuo there remained 0.8 g. of a red oil. A dilute aqueous-alcoholic solution of this oil gave an orange-red color when treated with 2 N NaOH and sodium nitroprusside, which changed to a light violet when acidified with acetic acid.

Potassium benzalpyruvate.--A cold solution of 19 g. of "85.6% min." potassium hydroxide in 70 ml. of methanol was added with shaking and cooling to a mixture of 22 g. (0.25 M) of pyruvic acid and 26.5 g. (0.25 M) of benzaldehyde. The mixture was stirred at 0-10° for five minutes and allowed to stand at room temperature for four hours. The yellow salt was filtered off, washed with 40 ml. of methanol and then with 60 ml. of ether, and air-dried, giving 43 g. (80%) of potassium benzalpyruvate.

Benzalpyruvic acid oxime.--A cold, concentrated solution of potassium benzalpyruvate was made acid with an excess

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* Prepared in 65% yield by nitration of arginine nitrate, using the method of M. Bergmann, L. Zervas and H. Rinke, Z. physiol. Chem. 224, 40 (1934).

of 6 N hydrochloric acid. The yellow precipitate of benzalpyruvic acid was filtered off, washed with water and dried.

8.8 g. (0.05 M) of benzalpyruvic acid was dissolved in 125 ml. of ethanol, and 7 g. (0.1 M) of hydroxylamine hydrochloride and 8.5 g. (0.1 M) of sodium acetate were added. The mixture was refluxed on a steamcone for three hours, during which time the initial yellow color of the mixture disappeared. The solution was concentrated in vacuo, 100 ml. of water was added and a white solid was filtered off, washed with 50 ml. of water and recrystallized from 35% ethanol, giving 6.2 g. (65%) of benzalpyruvic acid oxime, m. p. 164°.

4-phenyl-2-amino-3-butenoic acid.--4.4 g. (0.023 M) of benzalpyruvic acid oxime was added to a solution of 9.5 g. (0.05 M) of stannous chloride in 50 ml. of 13 N hydrochloric acid. A transient yellow color appeared on mixing. The mixture was allowed to stand at room temperature for four hours and then at 5° for twenty-four hours. 3.8 g. of white solid was filtered off, dissolved in 250 ml. of hot water, the hot solution was saturated with hydrogen sulfide, filtered, and concentrated in vacuo to 50 ml. A white solid was filtered off, washed with 50 ml. of water, dissolved in 100 ml. of conc. ammonium hydroxide and the solution was concentrated to 50 ml. A white solid was filtered off and recrystallized twice from hot water to give 1.9 g. (47%) of 4-phenyl-2-amino-3-butenoic acid, m. p.

208-210° (decomp.), after sintering and discoloring in the range 190-205°.

Anal. Calcd. for $C_{10}H_{11}O_2N$: C, 67.8; H, 6.3. Found: C, 67.6; H, 6.6.

The amino acid absorbed bromine rapidly and reduced permanganate.

The reaction of 4-phenyl-2-amino-3-butenoic acid, pyridine and acetic anhydride.--0.5 g. (0.003 M) of the amino acid was heated at 130-135° for thirty minutes with 4 g. (0.05 M) of pyridine and 15 g. (0.15 M) of acetic anhydride. 74% of the theoretical amount of carbon dioxide was evolved during this period. The solution was concentrated in vacuo, treated with an excess of sodium bicarbonate in 30 ml. of water and the resulting mixture extracted with three 50-ml. portions of chloroform. Removal of the chloroform in vacuo gave 0.4 g. of red oil. An aqueous-alcoholic solution of this material gave a cloudy yellow precipitate with dinitrophenylhydrazine, and an orange-red color when treated with 2 N sodium hydroxide and sodium nitroprusside, which changed to a transient green on acidification with acetic acid and turned to a dirty orange within a few seconds.

The reaction of D-glucosaminic acid, sodium acetate and acetic anhydride.--4 g. (0.02 M) of D-glucosaminic acid,*

* Prepared in 62% yield from D-glucosamine using the procedure of H. Pringsheim and G. Ruschmann, Ber. 48, 680 (1915).

4 g. (0.05 M) of sodium acetate and 35 g. (0.35 M) of acetic anhydride were mixed and heated together with a free flame until a vigorous reaction was initiated.* The flame was removed and the reaction continued for several minutes with no external heating. The solution was cooled, treated with 100 ml. of water, extracted with four 75-ml. portions of chloroform, the chloroform extracts were dried, and acetic acid and chloroform removed in vacuo to give a brown oily residue. When this residue was rubbed with a little ether a portion of it crystallized and was filtered off, washed with three ml. of cold ether and dried, yielding 1.9 g. (42%) of the lactone of 6-acetoxy-2-acetamido-2,4-hexadienoic -4(or 5)-hydroxy- acid, m. p. 126°, after recrystallizing twice from benzene and twice from water. The oily residue left after the crystallization and separation of the lactone could not be induced to crystallize. An aqueous-alcoholic solution of this residue gave a yellow precipitate with dinitrophenylhydrazine, and a scarlet color with 2 N sodium hydroxide and sodium nitroprusside, changing to deep wine-red on acidification with acetic acid.

The reaction of D-glucosaminic acid, pyridine and acetic anhydride.--0.5 g. (0.0026 M) of D-glucosaminic acid, 2.5 g. (0.03 M) of pyridine and 7.1 g. (0.07 M) of acetic

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* The reaction conditions and isolation procedures are those used by M. Bergmann, L. Zervas and E. Silberkweit, Ber. 64B, 2428 (1931), for obtaining the lactone of 6-acetoxy-2-acetamido-2,4-hexadienoic acid (XXI).
-4(or 5)-hydroxy-

anhydride were refluxed together at 130-140° for thirty minutes. No carbon dioxide was evolved during this time. The dark solution was cooled, treated with 80 ml. of water and extracted with four 50-ml. portions of chloroform. The combined chloroform extracts were dried and the chloroform removed *in vacuo* to give 0.7 g. of a red oil. The oil was washed with three 5-ml. portions of ether and then taken up in 5 ml. of hot benzene. The benzene solution was boiled with 0.1 g. of silicic acid, filtered hot, and concentrated to 3 ml. On cooling, 0.02 g. of a white solid crystallized out, m. p. 123-124°. A mixed melting point with a sample of the doubly unsaturated lactone from the previous experiment showed no depression. An aqueous-alcoholic solution of the residue left after removal of this solid gave the same nitroprusside color reactions as those shown by the residual material in the previous experiment.

In another experiment, 0.16 g. (0.001 M) of D-glucosaminic acid was heated at 125° for thirty minutes with 2 g. (0.025 M) of pyridine and 4 g. (0.05 M) of acetic anhydride. Then half of the mixture was heated at 100° for fifteen minutes with 15 ml. of 6 N hydrochloric acid, and half was heated with 15 ml. of 6 N sodium hydroxide for fifteen minutes at 100°, treated with 30 ml. of 6 N hydrochloric acid, and again heated at 100° for fifteen minutes. Carbon dioxide was not produced in any of these operations.

4-(p-nitrophenyl)-3-acetamido-2-butanone.--A mixture of 0.63 g. (0.003 M) of p-nitrophenylalanine,* 1.6 g. (0.02 M) of pyridine and 1.5 g. (0.015 M) of acetic anhydride was heated at 95-100° for thirty minutes. 57% of the theoretical amount of carbon dioxide was evolved. The solution was cooled, an excess of aqueous sodium bicarbonate added, the mixture extracted with three 40-ml. portions of chloroform, the combined chloroform extracts dried, and the chloroform and pyridine removed in vacuo to leave a dark residue. This was taken up in 15 ml. of ethanol, 10 ml. of 30-60° petroleum ether was added and the solution was stored at 5°. After twenty hours a yellow solid was filtered off, washed with a little cold ether and recrystallized from ethanol to give 0.27 g. (36%) of 4-(p-nitrophenyl)-3-acetamido-2-butanone, small white needles, m. p. 150-151°.

Anal. Calcd. for $C_{12}H_{14}O_4N_2$: N, 11.2. Found: N, 10.9.

The reaction of α-chloropropionic acid, pyridine and acetic anhydride.--5.4 g. (0.05 M) of α-chloropropionic acid, 16 g. (0.2 M) of pyridine and 20 g. (0.2 M) of acetic anhydride were heated together at 120-125° for twenty minutes. 32% of the theoretical amount of carbon dioxide was evolved. The dark red solution was treated with an excess of aqueous sodium bicarbonate, extracted with three 75-ml. portions of ether, the combined ether extracts washed

* Prepared in 57% yield by nitration of phenylalanine, using the procedure of E. Erlenmeyer and A. Lipp, Ann. 219, 179 (1883).

with 100 ml. of 3 N hydrochloric acid and dried, and the ether removed to give 0.3 g. of light yellow oil. This material gave a fleeting red-orange color when treated with 2 N sodium hydroxide and sodium nitroprusside which faded immediately, and acidification with acetic acid caused no detectable color change. It reduced permanganate and decolorized a solution of bromine in carbon tetrachloride as did the aqueous bicarbonate phase. Neither gave a positive reaction with 2,4-dinitrophenylhydrazine.

The reaction of alanylalanine, pyridine and acetic anhydride, followed by acid hydrolysis.--A mixture of 0.7 g. (0.0044 M) of alanylalanine,* 3.6 g. (0.045 M) of pyridine and 4.6 g. (0.045 M) of acetic anhydride was heated for ninety minutes at 115°. 92% of the theoretical amount of carbon dioxide was evolved, and when the reaction mixture was refluxed with 20 ml. of 6 N hydrochloric acid, no more carbon dioxide was obtained.

N-(N-acetylalanyl)-3-amino-2-butanone.--1.6 g. (0.01 M) of alanylalanine and 50 g. (0.5 M) of acetic anhydride were heated at 100-110° for ten minutes. No carbon dioxide was evolved during this period. 4 g. (0.05 M) of anhydrous sodium acetate was then added and the mixture refluxed for thirty minutes. 83% of the theoretical amount of carbon

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* Prepared from dimethyldiketopiperazine in 78% yield by the procedure of E. Fischer and K. Kautzsch, Ber. 38, 2376 (1905).

dioxide was evolved during this time. The solution was cooled, diluted with 50 ml. of benzene, filtered, and the precipitate washed with a 30-ml. portion of benzene. The combined filtrates were concentrated *in vacuo*, 10 ml. of water was added, and again concentrated, leaving 1.8 g. (90%) of a neutral yellow-orange oil. This oil gave a positive iodoform test, and a deep red color with 2 *N* sodium hydroxide and sodium nitroprusside, which changed to a deep violet when acidified with acetic acid. The oil was taken up in 50 ml. of water, 1.5 g. of Norite was added, the mixture was heated to boiling and then warmed on the steamcone for one hour and filtered. This process was repeated twice. After standing over night, the solution was filtered from a small, light-yellow precipitate and concentrated *in vacuo*, giving 1.4 g. (70%) of a pale yellow oil. From 600 mg. of this oil, which had stood for three weeks, 51 mg. (6%) of a crystalline product was obtained. Recrystallized from benzene-ethanol and then from ether-ethanol, white needles, m. p. 130-132°.

*Anal.* Calcd. for $C_9H_{16}O_3N_2$: C, 54.0; H, 8.1; N, 14.0. Found: C, 54.1; H, 8.1; N, 13.9.

0.2 g. of the partially decolorized oil obtained from a typical run as described above was heated for 20 hours on a steamcone with 1 g. (0.014 *M*) of hydroxylamine hydrochloride, 10 ml. of pyridine and 15 ml. of ethanol. No condenser was used, and 2 ml. of pyridine was added every three hours

for the first fifteen hours. The solvents were blown off the dark solution with an air jet, leaving a mixture of dark oil and solid. This was triturated with 10 ml. of water, centrifuged, dissolved in 3 ml. of ethanol, treated with water until cloudy, heated until clear, and allowed to cool to room temperature. An amorphous solid which separated was centrifuged off, washed with 50% ethanol, and recrystallized from ethanol to give 19 mg. of a white solid. M. p. behavior: softened at 160°, last solid disappeared at 176-178°, cloudy until 183-184°.

Anal. Calcd. for the oxime of N-(N-acetylalanyl)-3-amino-2-butanone, $C_9H_{17}O_3N_3$: C, 50.2; H, 8.0; N, 19.5. Found: C, 50.4; H, 8.0; N, 19.7.

When 0.3 g. of the crude product was heated for five minutes in 10% acetic acid with 0.5 g. of 2,4-dinitrophenylhydrazine, a precipitate was obtained which melted at 238° and gave a nitrogen analysis in fair agreement with that calculated for the osazone of biacetyl.

Anal. Calcd. for $C_{16}H_{18}N_4$: N, 21.0.
Found: N, 20.5.

N-acetylphenylalanylphenylalanine.--8.25 g. (0.05 M) of phenylalanine was heated at 100-110° for ten minutes with 85 g. of acetic anhydride. The solution was concentrated in vacuo to remove acetic acid and acetic anhydride, 9.9 g. (0.06 M) of phenylalanine and 100 ml. of benzene were added, and the mixture was heated on a steamcone for five hours.

150 ml. of acetone was added, the solution was refluxed for one hour, and filtered while hot. The white precipitate was washed with 100 ml. of N hydrochloric acid and then with 30 ml. of water. The solid was air-dried, giving 4.0 g. (23%) of N-acetylphenylalanylphenylalanine, m. p. 238°. A further amount of the acetylated dipeptide could be recovered from the first filtrate.

4-phenyl-N-(N-acetylphenylalanyl)-3-amino-2-butanone.--1.3 g. (0.0037 M) of N-acetylphenylalanylphenylalanine was heated at 100° for two minutes with 45 g. of acetic anhydride. 3 g. (0.037 M) of anhydrous sodium acetate was added to the resulting colorless solution, and the mixture was refluxed for twenty minutes. 80% of the theoretical amount of carbon dioxide was evolved during this time. After standing one hour the yellow solution was filtered, the precipitate washed with 10 ml. of acetic anhydride, and the combined filtrates concentrated in vacuo. 40 ml. of water and 3.0 g. (0.036 M) of sodium bicarbonate were added, the mixture was warmed on a steamcone for 10 minutes, and extracted with three 50-ml. portions of ether. The combined ether extracts were dried, filtered, and the ether removed to give 1.1 g. (85%) of a yellow oil giving strongly positive iodoform and nitroprusside reactions. The oil was taken up in 10 ml. of ether, 10 ml. of 60-70° petroleum ether was added, and the solution set aside. Over a period of nine days a total of 0.45 g. (35%) of white needles

separated. Recrystallized twice from benzene-petroleum ether, white needles, m. p. 186-187°, with previous softening.

Anal. Calcd. for $C_{21}H_{24}O_3N_2$: C, 71.6; H, 6.9; N, 8.0. Found: C, 71.7; H, 7.0; N, 8.2.

0.3 g. of the crude product ( a yellow oil) obtained above was dissolved in 10 ml. of 50% ethanol. A warm solution of 0.15 g. of dinitrophenylhydrazine in 5 ml. of 96% ethanol and 2 ml. of acetic acid was added, the solution was warmed for five minutes on a steamcone, cooled, 5 ml. of water was added, and after ten minutes a yellow precipitate (A) was filtered off, washed with 1 ml. of acetic acid, and recrystallized from acetic acid and then from ethanol, giving clusters of small yellow needles, m. p. 246-247°.

Anal. Calcd. for the dinitrophenylhydrazone of 4-phenyl-N-(N-acetylphenylalanyl)-3-amino-2-butanone, $C_{27}H_{28}O_6N_6$: C, 60.9; H, 5.3; N, 15.8. Found: C, 61.1; H, 5.4; N, 16.1.

The supernate remaining after removal of the first precipitate was treated with 5 ml. of water, and a yellow-orange precipitate (B) was centrifuged off, washed with 1 ml. of acetic acid, recrystallized from ethanol and then from ethyl acetate, giving yellow-orange flat plates, m. p. 221-222°, with previous sintering. The amount of material was small, and the analytical results were poor.

Anal. Calcd. for C, 60.9; H, 5.3; N, 15.8. Found: C, 58.7; H, 6.0; N, 19.5.

A sample of the crystalline ketone was dissolved in 2 ml. of ethanol and treated with 10 ml. of a saturated solution of 2,4-dinitrophenylhydrazine in N hydrochloric acid, yielding a yellow precipitate which, after recrystallization from ethanol, gave a melting point of 245-247°, and showed no depression in a mixed melting point with (A).

The reaction of dimethyldiketopiperazine, pyridine and acetic anhydride.--A mixture of 0.44 g. (0.004 M) of dimethyldiketopiperazine,* 2 g. (0.025 M) of pyridine and 3.6 g. (0.035 M) of acetic anhydride was heated at 120° for two hours. No carbon dioxide was evolved during this period nor after refluxing with 20 ml. of 6 N hydrochloric acid for one hour.

The reaction of N-acetylphenylalanine with pyridine, benzaldehyde, acetic acid and acetic anhydride, added in sequence.--1 g. (0.005 M) of N-acetylphenylalanine was refluxed with 12 g. (0.15 M) of pyridine for two hours. No carbon dioxide was evolved. Then 0.6 g. (0.01 M) of dry acetic acid was added and the mixture was refluxed for two hours. No carbon dioxide was formed. 3 g. (0.03 M) of benzaldehyde was then added and refluxing continued for one hour. No carbon dioxide appeared. Finally, 4 g. (0.05 M)

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* Prepared in 66% yield from alanine by the method of C. Sannie, Bull. soc. chim. [5], 9, 487 (1942).

of acetic anhydride was added and the mixture refluxed. Carbon dioxide was evolved within five minutes, and 35% of the theoretical amount was formed in twenty minutes.

The reaction of 2-methyl-4-benzyl-5-oxazolone, pyridine and acetic anhydride at room temperature.--These experiments are summarized in Table III, page 34.

The color reactions exhibited by mixtures of α-amino acids, pyridine and p-nitrobenzoic anhydride.--0.1 g. samples of various amino acids were mixed with 0.1 g. samples of p-nitrobenzoic anhydride and 2-ml. portions of pyridine. Colors developed fairly rapidly at room temperature, and much more rapidly when the mixtures were warmed for a few seconds. The addition of 2-5 drops of 2 N sodium hydroxide caused a change to much more brilliant colors. Dilution with water caused these colors to fade over a period of several hours, leaving almost colorless solutions. However, some color persisted after several days.

The reaction of impure alanine and acetic anhydride.--When a 1.2 g. (0.013 M) sample of alanine (Dow Chemical Co.) was refluxed for two hours with 50 ml. of acetic anhydride, 31-32% of the theoretical amount of carbon dioxide was formed. After concentrating in vacuo the residue was taken up in thirty ml. of water, giving a red-orange solution which gave a deep red color when treated with 2 N sodium hydroxide and sodium nitroprusside, changing to violet when acidified with acetic acid.

After three recrystallizations from water, a 0.5 g. (0.006 M) sample of the amino acid gave no carbon dioxide when refluxed for two hours with thirty ml. of acetic anhydride.

Alanine from another commercial source gave the same results.

Samples of phenylalanine from two commercial sources were mixed, and 1 g. (0.006 M) of the mixture was refluxed for two hours with 50 ml. of acetic anhydride. No carbon dioxide was evolved during this time.

Chromatographic studies.--When 0.1 g. of the red oil obtained in the isolation of the neutral fraction from the reaction of phenylalanine, methoxyacetic anhydride and pyridine was dissolved in 10 ml. of ethanol, placed on a silicic acid column and developed with ethanol, five colored bands were observed. A neutral fraction from the reaction of alanine, acetic anhydride and pyridine at 100° for one hour gave four colored zones; while if the reaction was conducted at reflux for two hours, seven colored zones were observed.

When alanine, acetic anhydride and sodium acetate were refluxed together for two hours, the neutral fraction was much lighter in color than when pyridine was used as catalyst, and showed only two faint colored zones when chromatographed.

The action of certain reagents on the reaction products of alanylalanine, acetic anhydride and several catalysts.--3.3 g. (0.02 M) of alanylalanine was refluxed for two hours with 48 g. (0.6 M) of pyridine and 60 g. (0.6 M) of acetic anhydride. The solution was concentrated in vacuo, treated with an excess of aqueous sodium bicarbonate, concentrated to 30 ml. and extracted with 100 ml. of 1:1 ethanol-chloroform and then with 50 ml. of chloroform. The combined non-aqueous phases were dried and the solvents removed to give 4 g. of red oil. An aqueous solution of this oil gave precipitates when treated with aqueous solutions of picric acid or mercuric chloride, while a sample of the desired ketone, N-(N-acetylalanyl)-3-amino-2-butanone, showed no reaction with either reagent.

2 g. (0.01 M) of alanylalanine was refluxed for three and one-half hours with 19 g. (0.2 M) of γ-picoline and 40 g. (0.4 M) of acetic anhydride. Isolation of the neutral residue as above gave 130% of the theoretical amount of product, and this material was very dark. The aqueous bicarbonate solution contained some colored material which could not be removed by a number of extractions with chloroform or ether. After acidifying the aqueous solution, a part of the color could be removed by extraction with organic solvents, but the greater part remained in the aqueous phase. This aqueous solution showed a faint orange-red color when treated with 2 N sodium hydroxide and then

with sodium nitroprusside, which changed to dirty brown and then to green upon acidification with acetic acid. An aqueous solution of the dark, neutral residue gave a large amount of dark yellow precipitate when treated with an aqueous solution of picric acid. This precipitate darkened at temperatures greater than 180° but was not melted at 300°. The neutral residue also gave a large precipitate with 0.1 N aqueous or alcoholic solutions of mercuric chloride, and with bromine. Neither of the precipitates so obtained possessed a definite melting point. 1 g. of the dark oil was dissolved in 15 ml. of water, treated with a solution of 0.1 N mercuric chloride until no more precipitate formed, centrifuged, the supernate saturated with hydrogen sulfide, concentrated to 5 ml. and extracted with five 10-ml. portions of chloroform. The chloroform solutions were dried and the solvent removed in vacuo to leave 0.42 g. of a pale yellow oil which exhibited a positive iodoform reaction and a nitroprusside test characteristic of N-(N-acetylalanyl)-3-amino-2-butanone.

1 g. (0.006 M) of alanylalanine was refluxed for thirty minutes with 5 g. (0.006 M) of sodium acetate and 35 g. (0.35 M) of acetic anhydride. Isolation of a neutral residue gave 88% of a light yellow oil. An aqueous solution of this oil gave no precipitates with picric acid or bromine, but gave a light yellow precipitate with mercuric chloride, leaving an apparently colorless supernate. Removal of excess

mercuric chloride from the supernate, followed by concentration in vacuo, left 53% of an almost colorless oil which exhibited the nitroprusside color reaction characteristic of the ketone N-(N-acetylalanyl)-3-amino-2-butanone.

The reaction of pyridine and acetic anhydride.--2 g. (0.025 M) of pyridine and 4 g. (0.04 M) of acetic anhydride were refluxed together for four hours. On concentrating at 100° and 3-5 mm. Hg. there remained 0.08 g. of a dark residue. A part of this material was much more soluble in water than the rest. The less soluble fraction gave a small precipitate with picric acid and a somewhat larger one with mercuric chloride. Neither fraction gave a positive nitroprusside test.

The reaction of γ-picoline and acetic anhydride.--2 g. (0.02 M) of γ-picoline and 4 g. (0.04 M) of acetic anhydride were refluxed together for four hours. On concentration of the dark red solution at 110° and 3-5 mm. Hg., there was obtained 0.15 g. of a dark oil. An aqueous solution of this oil gave precipitates with picric acid and mercuric chloride, and an orange-red color when treated with 2 N sodium hydroxide and sodium nitroprusside which changed to a deeper red on acidification with acetic acid. The oil did not appear to react with phenylhydrazine.

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# PART II

# THE ROLE OF PEROXIDES IN MUTAGENESIS

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## THE ROLE OF PEROXIDES IN MUTAGENESIS

As a result of a course of study in genetics at the Kerckhoff Laboratories of Biology of this Institute, Frank H. Dickey and I became interested in the problems of induced mutations. Both of us conceived schemes of attack based on reasonable hypotheses, but Dr. Dickey's suggestions seemed much more promising. Accordingly, research was begun with the idea of testing his hypothesis--that organic peroxides would be found to cause mutations, and that such peroxides were intermediates in the induction of mutations by such other agents as ultraviolet light and the various "mustard gases." Soon after this research was begun, we were joined by Miss Carol Lotz, who made possible the success of the investigation, both by guiding us safely through the pitfalls which beset any chemist-turned-biologist and by numerous timely and valuable suggestions as to the course of research. Dr. Dickey's proposal--that organic peroxides would induce mutations--has been fully verified, and a further amount of significant evidence has been uncovered relating to other phases of mutagenesis. The research up until June, 1949 has been fully treated in Dr. Dickey's thesis (Ph. D. Thesis, California Institute of Technology, June, 1949), and the more important results appeared in the

Proceedings of the National Academy of Sciences in the article which follows. Dr. Dickey and Miss Lotz continued research on the problem from June, 1949 to September, 1950.

Reprinted from the Proceedings of the National Academy of Sciences,
Vol. 35, No. 10, pp. 581–586. October, 1949

# *THE RÔLE OF ORGANIC PEROXIDES IN THE INDUCTION OF MUTATIONS**

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Communicated by G. W. Beadle, August 15, 1949

The discovery by Wyss, Stone, and Clark[1] that bacteria grown on a substrate recently exposed to ultra-violet light are subject to high mutation rates shows clearly that some meta-stable chemical substance, probably of no great complexity, is an intermediate in at least a part of the mutagenic action of ultra-violet light. It was supposed that hydrogen peroxide might be responsible for these results, but subsequent work has shown that this cannot be the whole explanation.[2] However, organic peroxides are known to be formed by the action of ultra-violet light on many compounds and such peroxides might very well be the intermediate agents producing the substrate irradiation effect.

The process by which organic compounds, especially ethers, olefins and aldehydes, form peroxides simply on contact with molecular oxygen is not wholly understood. In many simple cases, however, a chain reaction of the sort pictured below appears to be involved.

$$\begin{aligned} \text{R—H} &\longrightarrow \text{R—} + \text{H} \\ \text{R—} + \text{O}_2 &\longrightarrow \text{R—OO—} \\ \text{R—OO—} + \text{R—H} &\longrightarrow \text{R—OO—H} + \text{R—} \end{aligned}$$

The peroxide-forming process is catalyzed by ultra-violet light[3] which, presumably, supplies the energy for breaking a carbon-hydrogen bond in the first step.

The hypothesis that organic peroxides play an essential rôle in the mutagenic action of ultra-violet light has been under investigation in this laboratory for some time. One result of this work, and the subject of the

present writing, is the discovery that many simple organic peroxides increase mutation rates.

*Testing Procedure.*—The organism used for detecting and comparing mutagenic agents has been an adenineless, colonial strain of *Neurospora crassa*, the double mutant 70007-38701. The adenineless character in this strain is subject to a low spontaneous rate of reversion to adenine independence. These occurrences are probably back mutations at the adenineless locus, but this has not been demonstrated in the present study. The colonial character, introduced into the strain to permit plate counts, appears to be quite stable. Following essentially a method described by Giles and Lederberg[4] and by Westergaard and Mitchell,[5] conidial suspensions were exposed to various peroxides and the effects calculated from the fraction of these spores that gave rise to colonies on adenine-free medium. Since as many as $2 \times 10^8$ spores may be conveniently treated in a single experiment, this method is capable of detecting very weak mutagenic activity.

In a typical experiment a thoroughly mixed suspension of two-day old spores was divided into four portions, centrifuged and decanted. Two portions were retained as controls and the other two were re-suspended in an aqueous solution of the peroxide and allowed to stand at room temperature for 30 minutes. After washing to remove the treating solution, the concentration of conidia in each centrifuge tube was determined with a hemocytometer, and dilution platings on adenine-supplemented medium were made from each sample to indicate conidial viability and percentage mortality. Finally, the suspensions were spread on a series of adenine-free plates and incubated at 25°C. Mutants formed distinct colonies that could be counted during the third or fourth day after treatment.

Mutation rates were calculated by dividing the number of mutants counted by the number of spores plated. By subtracting from the mutation rate shown by spores subjected to a particular treatment the spontaneous rate shown by untreated spores from the same spore batch there was obtained the quantity termed the "induced mutation rate." It is not practical to determine an average value for the fraction of untreated spores that produce adenine-independent colonies and to use such a fixed figure for correcting the observed mutation rates. Although the spontaneous rate is lower than $0.8 \times 10^{-7}$ in more than 75% of the spore batches, occasionally this rate is very high (e.g., $13 \times 10^{-7}$), presumably as a result of an early mutation in the culture from which the spores were obtained. The same problem has been described by Delbrück[6] in connection with mutations in bacteria.

It appears that the largest numbers of mutants are obtained with treatments that kill 60 to 80% of the spores. Mutation rates based on the numbers of spores surviving the treatment might increase up to very high

mortalities but in the present investigation more consistent values have been obtained for mutation rates based on the number of spores treated. Accordingly, treatments producing the greatest actual numbers of viable mutants have been sought.

A danger in working with very high mortalities is the occasional appearance of numbers of adenineless colonies, apparently sustained by adenine released to the medium from dead spores. Such false mutants can usually be distinguished by their frail appearance and their failure to develop beyond an early stage, and always by their inability to grow when transferred to minimal medium.

*Demonstration of the Mutagenic Action of Peroxides.*—Table 1 illustrates the mutagenic action of *tert*-butyl hydroperoxide at various concentrations. The data are taken from typical experiments selected from an extensive study of this material. The table is intended to show the effect of concentration of the agent and to give a rough idea of the reproducibility of the results at a particular concentration. The first objective of this work has been to show that peroxides induce mutations and the values obtained for induced mutation rates are only approximate. Variations in the conditions of treatment, especially temperature, may account in part for the irregularities in the results.

TABLE 1

MUTAGENIC ACTION OF *tert*-BUTYL HYDROPEROXIDE

(Treatment: 30 minutes exposure to an aqueous solution of *tert*-butyl hydroperoxide at the indicated concentration)

| CONCENTRATION, MOLES PER LITER | SPORES TREATED, MILLIONS | MUTANTS | MUTATION RATES $\times 10^7$ OBSERVED | INDUCED | MORTALITY, % |
|---|---|---|---|---|---|
| 0.004 | 48 | 6 | 1.2 | 0.5 | 5 |
| 0.010 | 96 | 51 | 5.3 | 2.5 | 42 |
| 0.089 | 70 | 106 | 15.1 | 15.0 | 29 |
| 0.089 | 72 | 140 | 19.3 | 19.2 | 38 |
| 0.089 | 40 | 51 | 12.8 | 10.5 | 80 |
| 0.089 | 29 | 21 | 7.2 | 7.1 | 91 |
| 0.11 | 32 | 6 | 1.9 | 1.8 | 85 |
| 0.27 | 38 | 0 | 0.0 | 0.0 | 100 |

In Table 2 averages of the results of many experiments with six different peroxides are presented. The concentrations of the respective agents, shown in this table, are the ones which have given the highest mutation rates. For comparison the effects of four established mutagenic agents have been included and also the results of experiments with four mildly toxic substances which had no definite effect on mutation rates. It might be noted that two of the last named group, phenol and formaldehyde, have been reported to have a weak mutagenic action in experiments with Drosophila.[7, 8]

The active principles in the mixtures of hydrogen peroxide with formaldehyde and acetone may be, respectively, HO—$CH_2$—OO—$CH_2$—OH and HO—$C(CH_3)_2$—OO—H. Hydroxymethyl *tert*-butyl peroxide, derived similarly from the interaction of *tert*-butyl hydroperoxide and formaldehyde, is a well-defined compound.[9] The peroxide derived from diisopropyl ether was obtained by extracting old samples of the ether with water and freeing the extract of volatile material by passing an air stream through it.

TABLE 2

MUTAGENIC ACTION OF VARIOUS AGENTS

(Treatment: 30 minutes exposure to indicated aqueous solutions)

| AGENT | CONCENTRATION, MOLES PER LITER | TOTAL SPORES, MILLIONS | INDUCED MUTATION RATE × $10^7$ | MORTALITY, % |
|---|---|---|---|---|
| Hydrogen peroxide | 0.21 | 1200 | 1.8 | 40 |
| *tert*-Butyl hydroperoxide | 0.089 | 211 | 14.5 | 50 |
| Hydroxymethyl *tert*-butyl peroxide | 0.089 | 270 | 16.3 | 68 |
| Peroxide derived from diisopropyl ether | 0.15 | 146 | 23.0 | 61 |
| Hydrogen peroxide and formaldehyde | 0.022 (0.033 in $CH_2O$) | 464 | 8.8 | 77 |
| Hydrogen peroxide and acetone | 0.21 (1.36 in $(CH_3)_2CO$) | 96 | 10.4 | 32 |
| X-rays | * | 61 | 15.8 | 37 |
| Ultra-violet light | † | 418 | 46.0 | 32 |
| *Bis*(β-chloroethyl)sulfide | 0.0002 | 103 | 4.0 | 70 |
| *Bis*(β-chloroethyl)methylamine | 0.004 | 279 | 3.6 | 85 |
| Phenol | 0.077 | 465 | −0.4 | 87 |
| Formaldehyde | 0.0244 | 251 | 0.5 | 73 |
| Potassium permanganate | 0.00052 | 383 | 0.0 | 35 |
| *tert*-Butyl alcohol | 1.75 | 275 | −0.3 | 84 |

* Approximately 30,000 r units.

† Exposure of an aqueous suspension with agitation for 75 seconds to 30-watt General Electric germicidal lamp at a distance of 10 cm. The incident ultra-violet energy, nearly all in the vicinity of 2537 A. U., was 6000 ergs/mm.²/min.

The peroxide concentration in the resulting solution was determined iodometrically.

Mustard gas, *bis*(β-chloroethyl)sulfide, and the nitrogen mustard, *bis*(β-chloroethyl)methylamine, were used in borate (pH 8) and acetate (pH 5) buffers, respectively. The solution of the sulfur compound contained, in addition, 1.36 moles per liter of acetone to increase its solubility.

In presenting this demonstration of the mutagenic action of peroxides it is assumed that the adenine-independent colonies appear as the result of true mutations. The action of the peroxides certainly produces a heritable change since adenine independence of the reverted strains persists through repeated subcultures on an adenine-free medium. It has been the general experience of other workers that non-genetic reversions of Neurospora mutants, which occur spontaneously in some strains, do not persist through subcultures. This, together with the observation that known mutagenic agents (radiations, mustard gas) produce effects similar to those of the peroxides, constitutes presumptive evidence for the view that the action of the peroxides is mutational. It is recognized, however, that the results of genetic crosses will constitute the only proof that gene mutations are involved. A genetic study directed toward this end is now in progress and will be reported in a later communication.

There can be little doubt that the peroxide treatment *induces* the presumed mutations. Selection cannot account for the results since the spores are not growing during the treatment and since it is an increase in the actual number of adenine-independent colonies that is observed and not merely a higher ratio of the number of such colonies to the number of surviving spores.

Conceivably, certain nuclei (the conidiospores are multinucleate) possess a latent capacity for adenine synthesis that is only made manifest by the peroxide treatment. However, there is no precedent for such a phenomenon. This question as well as the question of the mutational nature of the peroxide-induced changes will be resolved by the current genetic studies mentioned above.

Finally, it should be noted that the experimental method used here is of a rather special sort. In all probability, only the back mutation of a single gene is involved. It will be important to confirm these findings by different techniques and in different organisms.

*Conclusions.*—The foregoing evidence that organic peroxides are capable of producing mutations lends substantial support to the idea that ultraviolet light produces mutations by forming such compounds in irradiated media and in cells. A demonstration that this is the mechanism of the substrate-irradiation effect may eventually be obtained by correlating the mutagenic action of irradiated material with peroxide content.

The facts that so few mutation-inducing agents are known and that the effects of those that are known are in large measure similar suggest that they have a common mode of action. Accordingly, the possibility that peroxides are in some way involved in the action of x-rays and of the mustards is currently under investigation. Possibly compounds of the mustard group or certain products of their interaction with organic compounds are especially liable to peroxide formation.

By determining just what feature of the chemistry of peroxides is responsible for their mutagenic action one might hope to shed light on the nature of the mutation process. It seems unlikely that this action is simply related to oxidizing power, since oxidizing agents are common and organic peroxides are not especially effective ones. Of more interest is the characteristic decomposition of peroxides by which free radicals are produced. If this is the essence of peroxide action non-peroxidic free radical sources (e.g., diazomethane) should show similar effects. It should be noted that irradiation of a cell could produce free radicals directly as well as by peroxide formation.

Besides affording a basis for speculation on the nature of the mutation process, the discovery of the mutation-inducing power of organic peroxides substantially increases the number of known mutagenic agents. Organic peroxides of widely varied structure can be prepared. It will be of interest to compare the action of these various agents on different genes and to search for agents having pronounced effects on particular genes.

* This investigation was supported in part by a research grant from Merck and Co.

† DuPont Predoctoral Fellow in Chemistry 1948–1949.

‡ The authors wish to thank Professor Norman H. Horowitz for valuable assistance and advice.

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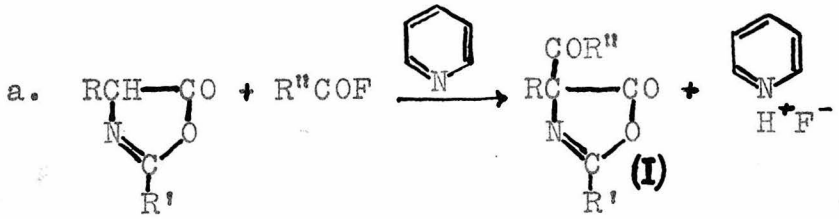
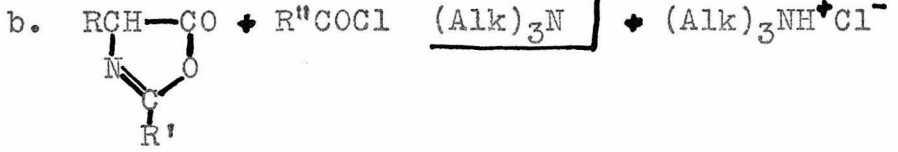
PROPOSITIONS

1. It is proposed that C-acylated azlactones such as (I), which have been postulated as being intermediates in the Dakin-West reaction, could be synthesized by either of the following reactions:

a. 

b. 

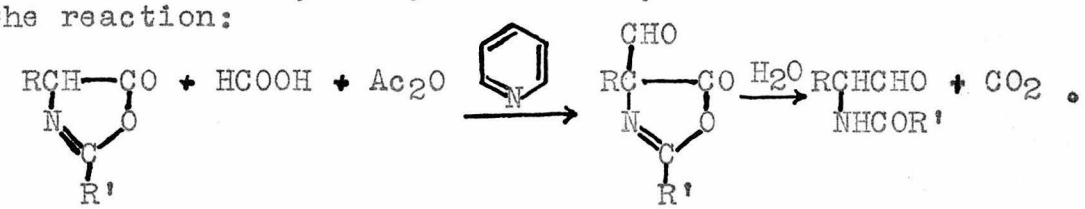
(1) Thesis, pp. 25, 27.
(2) Attenburrow, Elliott and Penny, J. C. S. 310 (1948).

2. It is proposed that the Dakin-West reaction could be used:

a. to determine the minimum molecular weights of peptides (1 M peptide $\xrightarrow{\text{D-W}}$ 1,2,3,etc. M $CO_2$).

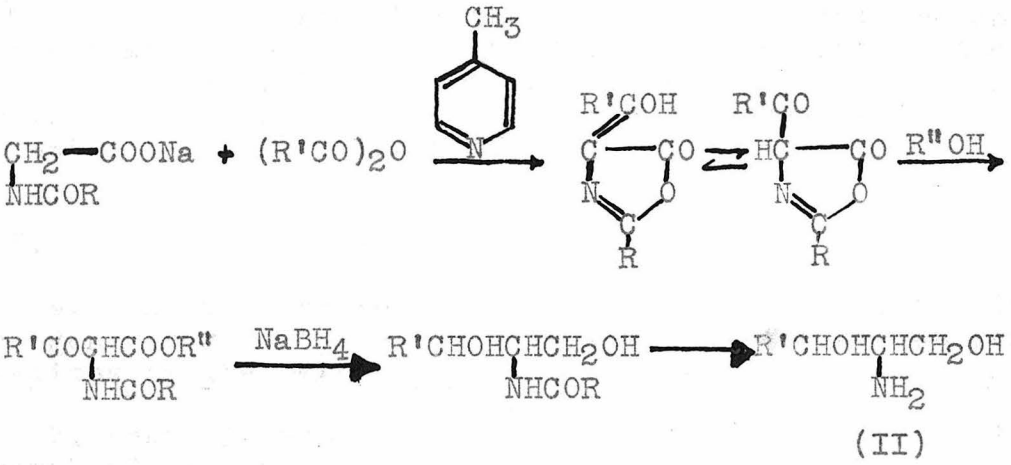
b. to determine the nature of initial amino acids of peptides (peptide $\xrightarrow{\text{D-W}}$ $\xrightarrow{\text{hydrolysis}}$ $\xrightarrow{OH^-}$ $\xrightarrow{Fe^{+++}}$ pyrazines, with the nature of initial amino acids determined by the nature of the pyrazines formed).

3. It is proposed that, by suitable modifications of reaction conditions, α-acylamidoaldehydes could be obtained by the reaction:



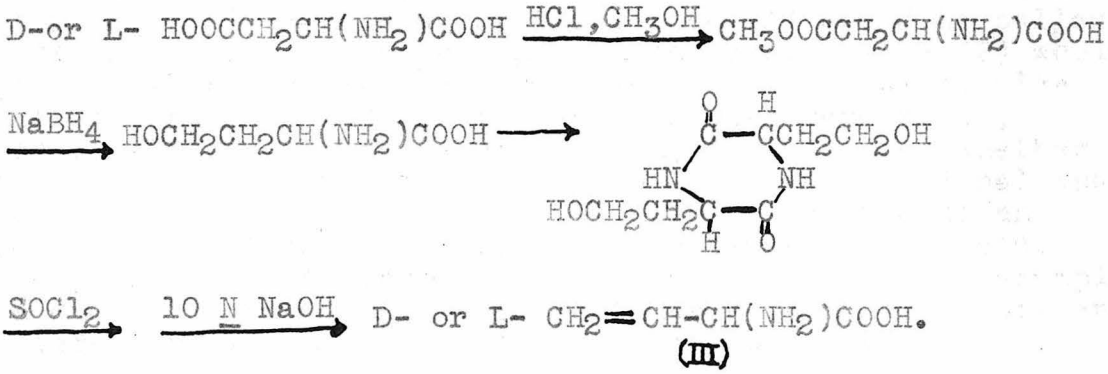
4. Compounds with the structure (II) are of considerable interest (chloromycetin, sphingosine, etc.). It is proposed

that a path leading to the synthesis of such compounds, which offers several advantages over those now in use, is the following:

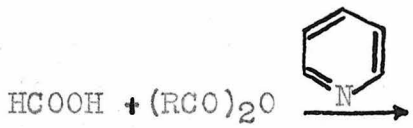


(1) Attenburrow, Elliott and Penny, J. C. S. 310 (1948).

5. It is proposed that (III), 2-amino-3-butenoic acid (C-vinyl glycine), could be synthesized in optically active form by the following path:



6. It is proposed that the reaction



could be used

a. as a much more convenient source for reasonable quantities of pure carbon monoxide than the methods usually suggested,

b. as a method for the quantitative estimation of small amounts of either formic acid or of acid anhydrides.

7. It is proposed that the production of intense colors by the reaction of maleic anhydride with tertiary amines affords a more rapid and convenient method for differentiating tertiary amines from primary and secondary amines than the methods usually recommended.

8. It is proposed:

a. that a major benefit derived from the destruction of hydrogen peroxide by catalase in vivo is the prevention of mutations by the peroxide;

b. that glutathione serves a similar function by the removal of organic peroxides;

c. that glutathione would prove by laboratory experiments to be a mutagen inhibitor.

9. It is proposed that, in view of the increasing interest in the field of mutagenesis, a system of terminology for the subject should be developed. As an example, if an agent (A) causes the formation of another agent (B), which in turn directly causes a mutation, then (A) could be called a mutagen initiator, secondary mutagen, etc., while (B) would be termed a primary mutagen or direct mutagen. Also, distinction should be made between agents with reversible, irreversible or dual mutagenic action, depending on whether they can change a gene from functional to non-functional and also cause the reverse change. An extreme example of an irreversible mutagen would be concentrated sulfuric acid. It is proposed that diazomethane would prove to be an example of a dualistic mutagen, serving both as a reversible mutagen-initiator and as an irreversible direct mutagen.

10. It is proposed that, together with the thesis and propositions, another section be submitted by a candidate if he so desires. This section would have a title such as "Miscellaneous Observations and Ideas." In this section would be included ideas on apparatus, variations on syntheses, manipulative techniques, etc., which the candidate has used to advantage in his work, which might be of value to others, but which to his knowledge have not been employed elsewhere.