XXVI. EXPERIMENTS ON THE INTERACTION OF HYDROXY- AND AMINO-ACIDS WITH NITROPHENYLHYDRAZINE WITH SPECIAL REFERENCE TO THE FORMATION OF GLYOXAL DERIVATIVES.

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Dudley and the writer [1913, 1914] have shown that when α -hydroxy-acids, such as glycollic, lactic, glyceric or mandelic acid, are digested in aqueous solution with *p*-nitrophenylhydrazine, small amounts of substances identical in properties with the bis-nitrophenylhydrazones of glyoxals are formed. Amino-acids, such as glycine, alanine, valine, leucine, aspartic acid and phenylalanine, in feebly acid solution under similar circumstances gave the same products. The course of the reaction was crudely represented as follows:

But it should be recalled that the direct synthesis, outside the body, of amino-acids from glyoxals by the action of ammonia, or similar substances was never observed.

The qualitative demonstration of the reactions upon which these conclusions are based is, in some respects, easy to carry out, but the yield of glyoxal derivatives is extremely small and their bis-nitrophenylhydrazones are not readily converted into other derivatives and moreover the reactions are complicated by numerous side changes.

The object of the following paper is to record a few additional experiments which throw some light on the reaction. These experiments are manifestly incomplete but as the opportunity to carry them further is at the moment lacking, it appeared worth while to describe them now.

(I) *Formation of p-nitrophenylhydrazides from α-hydroxy-acids.*

It has already been shown that when dilute lactic acid is digested with *p*-nitrophenylhydrazine, the following products are formed—(i) methylglyoxal bis-nitrophenylhydrazone, (ii) α-nitrophenylhydrazinopropionic acid, (iii) pyruvic acid nitrophenylhydrazone. This last substance was derived from the hydrazino-acid (ii), from which it is readily obtained by oxidation.

The amount of these three products was however not nearly enough to account for the amount of nitrophenylhydrazine which disappeared. It has now been found that a fourth product, namely, the *p*-nitrophenylhydrazone of lactic acid, $\text{CH}_3 \cdot \text{CHOH} \cdot \text{CO} \cdot \text{NH} \cdot \text{NH} \cdot \text{C}_6\text{H}_4 \cdot \text{NO}_2$, is formed in relatively large amounts. The substance possesses attractive properties and is easily prepared. A similar crystalline derivative was prepared from *d*-lactic acid and from glycollic acid, while α-hydroxybutyric acid and β-hydroxybutyric acid gave only oily products.

Glycollyl-p-nitrophenylhydrazone, $\text{CH}_2\text{OH} \cdot \text{CO} \cdot \text{NH} \cdot \text{NH} \cdot \text{C}_6\text{H}_4\text{NO}_2$. Glycollic acid (2 g.), nitrophenylhydrazine (4 g.), water (20 cc.) and alcohol (20 cc.) were warmed on a water bath, then diluted with 100 cc. of water and allowed to stand overnight. Most of the unchanged nitrophenylhydrazine was precipitated by adding 2 cc. of saturated sodium acetate solution and the remainder was extracted from the filtrate by shaking with three successive portions of chloroform. The hydrazone was next separated by repeated extraction with amyl alcohol. The latter solvent was removed by evaporation at a low temperature under diminished pressure and the product (3.1 g.) was recrystallised from boiling alcohol in which it is only moderately soluble. The substance crystallises from alcohol in lemon yellow needles melting at 192°–194°. It is readily soluble in water, very sparingly soluble in chloroform or benzene and is easily hydrolysed on boiling with acids. On analysis the

substance gave 20.1 per cent. nitrogen compared with 19.9 for the calculated amount.

i-Lactyl-p-nitrophenylhydrazide. Lactic acid (8.5 g.), *p*-nitrophenylhydrazine (17 g.) and 95 per cent. alcohol (100 cc.) were heated on the water bath in a loosely corked flask for a couple of hours, a little more alcohol was then added and the mixture allowed to stand overnight in an incubator at 37°. Most of the alcohol had then evaporated and the mixture was diluted with half a litre of water. A small precipitate of unchanged base was filtered off and the rest was removed by three extractions with chloroform. The hydrazide was then extracted by repeated shaking with amyl alcohol. The extract was evaporated under diminished pressure and the solid residue of crude hydrazide (15 g.) was recrystallised from a mixture of alcohol and toluene. The yield was 12 g.

In another experiment the reaction was carried out in aqueous solution with excess of lactic acid. Nitrophenylhydrazine (2 g.) was heated for two hours on the water bath with lactic acid (5 cc.) and water (45 cc.). After standing overnight the solution was partly neutralised by adding sodium carbonate until a pink colour transitorily appeared and then successively extracted with chloroform and amyl alcohol as before. The solid crystalline residue was dissolved in alcohol (5 cc.) and toluene added to the warm solution to a point just short of the appearance of turbidity. A yield of 2.1 g. of the hydrazide was obtained.

Lactyl-*p*-nitrophenylhydrazide is an almost colourless substance when pure and crystallises from alcohol in prisms and plates which sinter at 162° and melt at 165°–167°. It is readily soluble in water, alcohol and acetone, sparingly soluble in ether, chloroform and toluene, insoluble in light petroleum. On analysis the substance gave 18.6 per cent. nitrogen compared with a calculated value of 18.7 per cent.

d-Lactyl-nitrophenylhydrazide. This substance was prepared from *d*-lactic acid in the same way as the inactive variety. Two g. of the acid and 3 g. of the hydrazine gave 2.1 g. of product crystallising from alcohol and toluene in fine prisms melting at 161°–163° with slight previous sintering. On analysis the substance gave 18.9 per cent. nitrogen. The substance is strongly laevorotatory. Its rotation was observed in absolute alcohol solution.

$$c = 2.35, \quad l = 2 \text{ dm}, \quad \alpha = 1.37^\circ,$$

$$[\alpha]_D^{17} = -34.5^\circ.$$

Another preparation had $[\alpha]_D^{17} = -33.6^\circ$.

(II) *Conditions affecting the formation of methylglyoxal derivatives and other substances from lactic acid and nitrophenylhydrazine.*

It has been shown that when aqueous lactic acid is digested with *p*-nitrophenylhydrazine at least four well characterised substances are found in addition to indefinite decomposition products arising from the hydrazine. The results of some preliminary experiments relating to the formation of these compounds may be referred to. The products of the reaction were separated in a roughly quantitative fashion as follows. The mixture of aqueous lactic acid and nitrophenylhydrazine prepared as described in previous papers [1913, p. 130], after digestion at 37° for a determined period, was filtered through a weighed Gooch crucible. The insoluble part consists of methylglyoxal-bis-nitrophenylhydrazine with α -nitrophenylhydrazinopropionic acid and the nitrophenylhydrazone of pyruvic acid. The last two acid substances were separated by washing the precipitate with hot sodium carbonate solution and recovering them from the alkaline solution by acidifying with acetic acid. The very sparingly soluble methylglyoxal-bis-nitrophenylhydrazone was then washed with water and alcohol. The original aqueous solution after filtering off the precipitate was examined for unchanged hydrazine by acetone while lactyl-nitrophenylhydrazide was separated by partial neutralisation with sodium carbonate, half saturation with ammonium sulphate and extraction with amyl alcohol.

It does not seem of value to record a number of these experiments in detail but the following definite points were established.

(a) If the lactic acid is completely neutralised with sodium hydroxide before digestion with nitrophenylhydrazine no formation of methylglyoxal-bis-nitrophenylhydrazone is observed. A free carboxyl group is apparently necessary for methylglyoxal formation.

(b) Mineral acids added in small amounts (e.g. 2 per cent. sulphuric acid) to lactic acid solutions (10 per cent.) with nitrophenylhydrazine (0.5 per cent.) inhibit phenylhydrazide formation and slightly diminish the yield of methylglyoxal derivative. The yield of hydrazino-acid is not materially influenced.

(c) When aqueous lactic acid and nitrophenylhydrazine are digested together at 37°, a low concentration of lactic acid (e.g. 2 per cent.) favours both the formation of methylglyoxal-bis-nitrophenylhydrazone and of the hydrazino-acid but is unfavourable to the formation of lactyl-nitrophenylhydrazide. Concentrated solutions (e.g. 10 per cent. and over) favour hydrazide formation but are prejudicial to the production of the other products.

(d) Formation of methylglyoxal-bis-nitrophenylhydrazone is not conditioned by the prior formation of lactyl-nitrophenylhydrazide since the latter substance could not be converted into the former.

As an example of the relative yields of products the following experiment may be cited. Lactic acid (300 cc.), water (300 cc.) and *p*-nitrophenylhydrazine (30 g.) were rapidly heated to boiling, cooled and filtered. The mixture was divided into three parts; (a) was digested undiluted at 37°, the others, (b) and (c), were diluted with one and three litres of water respectively and digested for ten days. After three days practically all unchanged hydrazine had disappeared from (a) while a little more remained in (b) and (c). The yields of methylglyoxal-bis-nitrophenylhydrazine from (a), (b) and (c) were respectively 31, 77 and 203 milligrams. The yields of α -nitrophenylhydrazino-propionic acid were 116, 482 and 808 milligrams respectively. Almost the whole of the remaining nitrophenylhydrazine was converted into lactyl-nitrophenylhydrazide.

(III) *Glyoxal derivatives from other acids.*

As stated in the introduction, evidence of the formation of glyoxal derivatives has been observed in the case of a number of α -hydroxy- and α -amino-acids when digested with nitrophenylhydrazine. Since the yield of these glyoxal derivatives is very small, it appeared desirable to make comparative experiments with other acids, in order to see if confirmatory evidence could be obtained as to the character of the reaction under investigation.

The following experiments were simply of a qualitative kind. In order to detect glyoxal formation the precipitates obtained on digestion with nitrophenylhydrazine were filtered off and washed first with hot sodium carbonate solution and then with alcohol. The residue was then tested with caustic soda solution and alcohol. A clear blue colour resembling that of Fehling's solution is taken as a probable indication of glyoxal formation since all bis-nitrophenylhydrazones yield this extremely sensitive reaction. Brown, reddish or violet colourations such as many nitrophenylhydrazine derivatives yield are not obtained if the glyoxal derivatives are properly purified with sodium carbonate and alcohol.

The solutions were usually prepared by warming together 4 g. of the acid, 40 cc. of water and 0.2 g. nitrophenylhydrazine. The solutions were rapidly cooled, filtered and digested at 37°. After two or three days any precipitate was filtered off, and examined as described. The results were as follows:

Acid		Reaction of product with sodium hydroxide
Butyric acid	$\text{CH}_3 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{COOH}$	—
α -Hydroxybutyric acid	$\text{CH}_3 \cdot \text{CH}_2 \cdot \text{CHOH} \cdot \text{COOH}$	+
β -Hydroxybutyric acid	$\text{CH}_3 \cdot \text{CHOH} \cdot \text{CH}_2 \cdot \text{COOH}$	—
Crotonic acid	$\text{CH}_3 \cdot \text{CH}=\text{CH} \cdot \text{COOH}$	?
Isobutyric acid	$(\text{CH}_3)_2 \cdot \text{CH} \cdot \text{COOH}$	—
α -Hydroxy-isobutyric acid	$(\text{CH}_3)_2 \cdot \text{COH} \cdot \text{COOH}$	+
α -Methoxy-isobutyric acid	$(\text{CH}_3)_2 \cdot \text{C}(\text{OCH}_3) \cdot \text{COOH}$	—
Diethylglycollic acid	$(\text{C}_2\text{H}_5)_2 \cdot \text{COH} \cdot \text{COOH}$	+
Tartaric acid	$\text{COOH} \cdot \text{CHOH} \cdot \text{CHOH} \cdot \text{COOH}$	+
Gluconic acid	$\text{CH}_2\text{OH} \cdot (\text{CHOH})_4 \cdot \text{COOH}$	+

The product from crotonic acid gave a blue colour with caustic soda typical of glyoxal derivatives but the substance differed from the latter in being readily decomposed by hydrochloric acid. It appears likely that some substance with two adjacent nitrophenylhydrazine groups was formed but its structure is quite obscure.

(IV) *Action of semicarbazide on leucine.*

In all the experiments hitherto recorded on the formation of glyoxals from α -hydroxy- and α -amino-acids use has been made of nitrophenylhydrazine as being peculiarly suitable on account of the extraordinarily low solubility of its glyoxal derivatives. Dudley and the writer have shown that isobutylglyoxal gives a fairly insoluble compound with semicarbazide [1914, p. 38]. Accordingly an attempt was made to isolate isobutylglyoxal semicarbazone on long digestion of leucine with semicarbazide sulphate. Unfortunately the experiment gave a negative result. Whether this is due to a too great solubility of the semicarbazone or whether glyoxal formation is in some way conditioned by the presence of nitrophenylhydrazine is not clear. Further work is needed to determine these points. In any case it should be recalled that the simple equation given at the commencement of this paper to represent the dissociation of α -hydroxy- and α -amino-acids is adopted for convenience in explaining certain experimental results rather than on account of a belief in its precise accuracy. Many other alternative forms of dissociation are conceivable.

REFERENCES.

- Dakin and Dudley (1913). *J. Biol. Chem.*, **15**, 127.
 ——— (1914). *J. Biol. Chem.*, **18**, 29.