III. HYDROXYPYRUVIC ALDEHYDE: ITS PREPARATION AND PHYSIOLOGICAL BEHAVIOUR.

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PREPARATION OF HYDROXYPYRUVIC ALDEHYDE.

THE general methods for the preparation of α -keto-aldehydes, devised by Wohl and Lange [1908] and Meisenheimer [1912] and developed so successfully by Dakin and Dudley [1914], being inapplicable to the case of hydroxypyruvic aldehyde, it is not surprising that this compound remained unknown until 1926, when its preparation was described by Evans and Waring [1926].

The method adopted by these workers consists essentially in the oxidation of dihydroxyacetone by an excess of finely powdered copper acetate suspended in water. Oxidation is allowed to proceed for 7–10 days at room temperature, when the precipitated cuprous oxide is removed by filtration and the filtrate is freed from the excess of copper by hydrogen sulphide. The resulting "golden yellow" solution is then concentrated under diminished pressure to an oil, which when the pressure is lowered to 9 mm. forms white crystals (M.P. 99°). The yield is 80 % of the theoretical, and the results of qualitative tests and molecular weight determinations by the cryoscopic method, accord with the supposition that the white crystalline material is a trimeride of hydroxypyruvic aldehyde.

Following the details for the oxidation, as described by Evans and Waring, we have confirmed that the weight of cuprous oxide produced after a period of 8 days corresponds with 97.2 % of the theoretical amount required for the usage of one equivalent of oxygen. We doubt, however, whether it is correct to conclude from this result that hydroxypyruvic aldehyde is the sole product of the reaction, and we have failed so far to obtain the crystalline trimeride. Kermack, Lambie and Slater [1929] were also unsuccessful in obtaining a crystalline product under the conditions indicated by the American workers, but claimed that the syrup produced rapidly crystallised if the oxidation had been allowed to continue for 30 days at room temperature. With this modification, we found that the cuprous oxide was contaminated with a greyish white crystalline precipitate of copper oxalate, and, moreover, the total weight of cuprous oxide was then greater (by 19–20 %) than that required for one oxygen equivalent. Hence the resulting product was less pure and was in

fact shown to contain other oxidation products including oxalic acid. The syrupy product when the pressure was diminished to 9 mm. soon passed into a pseudocrystalline mass, which was readily detached from the distilling flask and melted indefinitely just under 100° to a clear yellow liquid. It showed no crystalline structure, and no crystallising medium could be discovered. When the dry material was warmed at 4 mm. pressure, a sublimate of anhydrous oxalic acid formed, and on further heating decomposition of the bulk of the material took place. The product obtained by Evans and Waring's method showed similar behaviour, but yielded no sublimate and was thus probably free from oxalic acid. It may be mentioned that Friedemann [1927] had employed the same method for the preparation of a solution of hydroxypyruvic aldehyde, but made no attempt to isolate the product in the solid state. He considered the oxidation complete after 7 days at room temperature. We have formed the opinion that oxalate formation begins soon after this period, and at any rate is evident after 10 days, when separation of copper oxalate occurs.

The pseudocrystalline solid, obtained as indicated above, was found to be insoluble in all the ordinary solvents except water and alcohol. In the dry state it was practically odourless, but its alcoholic or aqueous solution soon developed an offensive smell characteristic of sulphur compounds, and later sulphur was gradually and continuously deposited. Evans and his co-workers [1928], in their experiments on the oxidation of sugars with copper acetate, add sodium chloride to prevent the formation of colloidal cupric sulphide, but make no mention either of this difficulty or of the presence of sulphur compounds in their preparation of hydroxypyruvic aldehyde. Kermack, Lambie and Slater, however, state that it is difficult to free the product from hydrogen sulphide and colloidal sulphur; in our material the presence of a sulphur derivative was further indicated by the fact that the solutions gave a very marked reaction with sodium nitroprusside. In other respects our product exhibited all the reactions of hydroxypyruvic aldehyde. Both the phenylosazone and the *p*-nitrophenylosazone were obtained in good yield, although during their formation hydrogen sulphide was evolved from the reaction mixtures. The phenylosazone after crystallisation from benzene melted at $132-3^{\circ}$ and decomposed about 170° : while the crude *p*-nitrophenylosazone melted with decomposition at 234° , and after recrystallisation from nitrobenzene decomposed sharply at 292° , and had N 23.02 % ($C_{15}H_{14}O_5N_6$ requires N 23.46 %). Evans and Waring quote $230-2^{\circ}$ as the melting-point of this compound, but this value is evidently too low, as Dakin and Dudley [1913] describe the compound as decomposing with the evolution of much gas at 315° . Further, it is to be noted that the formation of these osazones is no proof of the presence of the keto-aldehyde in the product, since the same osazones would arise from dihydroxyacetone. However, we found (1) that the product from the oxidation forms the osazones much more readily than does dihydroxyacetone, and (2) that the crop which separates after 4 hours'

interaction between *p*-nitrophenylhydrazine and dihydroxyacetone has a lower melting-point (243°) than the second crop separating after 36 hours (257°). This is probably due to the presence of varying amounts of hydrazone along with the osazone, as the crop melting at 243° had N 21.04 %, a value intermediate between those of the hydrazone (18.67 %) and osazone (23.46 %).

As a freshly prepared aqueous solution of the pseudocrystalline material gave no immediate Schiff reaction, whereas a solution previously boiled did so at once, the product was evidently present in a polymerised form as suggested by Evans and Waring. The molecular weight determinations quoted by these workers, however, are rendered somewhat unreliable owing to the presence of by-products containing sulphur. The exact nature of these by-products has not been determined so far, but it may be recalled that aldehydes in acid solution on treatment with hydrogen sulphide give rise to complexes containing the corresponding thioaldehydes, which on oxidation yield sulphones, a class of compounds with well recognised hypnotic properties. Accordingly, compounds of this character may here be involved, and we have invariably found it impossible, with the hydrogen sulphide method, to obtain a product, which gives no nitroprusside reaction. The main product of the action of copper acetate on dihydroxyacetone is probably hydroxypyruvic aldehyde, but while the excess of copper is most conveniently removed by means of hydrogen sulphide, the use of the latter introduces a complication.

Being unsuccessful with Evans and Waring's method and being in need of a reliably pure specimen of hydroxypyruvic aldehyde in order to test its physiological action, we have tried other methods of preparation. Thus the phenylosazone was prepared from dihydroxyacetone and experiments were carried out to transform it into the osone (1) by Fischer's method with fuming hydrochloric acid, and (2) by the benzaldehyde method. Unfortunately neither process proved successful; in the former extensive decomposition occurred, and the osazone proved too insoluble in water for the latter method to be applicable.

An experiment in which copper acetate was replaced by the corresponding silver salt led to a curious result. After allowing the oxidation to proceed, and subsequently removing the excess of silver by addition of dilute hydrochloric acid, there was obtained on evaporating the solution *in vacuo* a white amorphous product, resembling starch in appearance. It was free from inorganic material, but had no definite melting-point. Once isolated it was sparingly soluble in all solvents, but an aqueous suspension of the material reduced Fehling's solution vigorously in the cold. The sparing solubility of the product precluded the testing of its physiological action, but it was considered to be either a highly polymerised form of hydroxypyruvic aldehyde, or a condensation product analogous to that described by Levene and Walti [1928]. The material has so far not been further examined, but, as a second experiment yielded a somewhat similar result, its preparation is being repeated on a larger scale.

Attempts were then made to modify the copper acetate method of Evans and Waring, avoiding the use of hydrogen sulphide. The oxidation was carried out exactly as described by the American workers, and various methods were then tried for the removal of the excess of copper from the green-coloured filtrate. The only process which proved successful was the careful addition, with cooling and stirring, of saturated baryta until the acid reaction to litmus disappeared. The bulky precipitate containing the cupric hydroxide settled on standing overnight, and the colourless filtrate was freed from barium by means of sulphuric acid, excess of acid being carefully avoided. The filtered solution was then concentrated *in vacuo* to a syrup. This was extracted with alcohol and the filtered alcoholic extract was again evaporated under diminished pressure to a syrup, which under 6–9 mm. passed into the pseudo-crystalline state. The product obtained in this way had all the properties of a polymeride of hydroxypyruvic aldehyde. An unheated solution gave no immediate reaction with Schiff's reagent, but a solution which had been heated for a few minutes and subsequently cooled gave a marked reaction at once: it also reduced Fehling's solution in the cold and gave an intense coloration with phosphotungstate, but a negative test with sodium nitroprusside. It gave a purplish-red coloration with the phenol reagent used by Kermack, Lambie and Slater [1926] for the detection and estimation of dihydroxyacetone, and readily formed a *p*-nitrophenylosazone, m.p. 285°. No attempt was made to estimate by molecular weight determinations the degree of polymerisation, as this will probably vary in different experiments. The material was not definitely crystalline, but was undoubtedly hydroxypyruvic aldehyde, and not being contaminated with sulphur derivatives was purer than that previously described. In view of the ease with which keto-aldehydes are affected by alkali, a control experiment was carried out, in which a mixture of glyoxal and copper acetate was subjected to the same baryta treatment as outlined above. The glyoxal was quite unaffected by the process.

PHYSIOLOGICAL ACTION OF HYDROXYPYRUVIC ALDEHYDE.

As already reported [Hynd, 1930], the toxic action on mice of hydroxypyruvic aldehyde prepared by the method of Evans and Waring was observed by me 3 years ago, but the matter was not further investigated until Kermack, Lambie and Slater [1929] published their paper claiming that the dimeric form of hydroxypyruvic aldehyde produced symptoms similar to those following the administration of glucosone.

The material obtained by the methods described above has been injected subcutaneously into normal mice, and the effects produced have been carefully studied in a large number of experiments. Some typical cases are detailed in Table I, in which are also included experiments in which charcoal was employed to remove colloidal copper sulphide from the filtrate after the treatment with hydrogen sulphide. As we were concerned mainly with the effect of hydroxypyruvic aldehyde prepared by different processes, the con-

centration of the solutions used for injection was in all cases 15 %, and a uniform dose of 0.05 g. per 20 g. mouse was employed.

Table I. *Effect of subcutaneous injections into normal mice of hydroxypyruvic aldehyde prepared by different methods.*

Method of preparation	Test with nitro-prusside	Behaviour of animal	General inference
I. Copper removed by hydrogen sulphide	+	1. Became inactive almost at once. After 1 minute legs sprawling: then began to gasp. Rolled over on its side, and lay comatose. Two minutes after injection, animal suddenly righted itself: then followed general convulsive movements. Dead in 3 minutes after injection	The material has a very rapid effect, and is highly toxic
Do.	+	2. Breathing difficult after 1 minute. After 2 minutes convulsion, followed by slight recovery, when animal walked about in an excited condition. A second convulsion after 4 minutes, then coma and death in 6 minutes	Do.
Do.	+	3. After 2 minutes very irritable, sprawling limbs, rapid breathing. Jumps if handled. Convulsion in 4 minutes: dead 5 minutes after injection	Do.
II. Copper removed by hydrogen sulphide and the filtrate shaken with charcoal	+	1. Slight sprawling in 2 minutes. Rapid respiration. Disinclined to walk unless disturbed. Then became very drowsy and remained in that state all day. Found dead next morning	Effect is slow: drowsiness the main symptom
Do.	+	2. Tail erect: very irritable for 5 to 10 minutes after injection. Then gradually became lethargic, and died in 6½ hours	Do.
Do.	+	3. Active for 2 minutes after injection. Then sat quietly. Irritable when disturbed. Very drowsy after 20 minutes. Slight convulsion after 1 hour. Then moribund: died after 3 hours	Do.
III. Copper removed by means of baryta	-	1. No effect was noted during first hour after injection. Then gradually became drowsy and remained so all day. Refused to take food. Normal next day	Slow effect. Drowsiness the main symptom
Do.	-	2. No symptoms except drowsiness, which developed gradually during forenoon. Continued very drowsy all day. Found moribund next morning. Died 26 hours after injection	Do.
Do.	-	3. Slight sprawling 2 minutes after injection. Rapid respirations. Then became drowsy. When disturbed moved about with difficulty. Remained in this condition all day. Normal next morning	Do.

In the above experiments, apart from the concentration of the solution at 30° under diminished pressure, no heating was employed in the course of the preparation of the material used for the injections. All the solutions used gave negative tests to Schiff's reagent. Boiling for 5 to 10 minutes gave solutions, which when cold responded to Schiff's test readily, and the effects of the injection of these solutions are shown in Table II.

Table II. *Effect of subcutaneous injections into normal mice of monomeric hydroxypyruvic aldehyde obtained by different methods.*

Method of preparation	Test with nitro-prusside	Behaviour of animal	General inference
I. Copper removed by hydrogen sulphide	+	1. Walking about 2 minutes after injection. Sprawling after $\frac{1}{4}$ hour. Then very drowsy and remained so until death in $5\frac{1}{4}$ hours	Main symptom is drowsiness
Do.	+	2. No definite symptoms developed. Animal remained quiet and drowsy all day. Found dead next morning	Do.
II. Copper removed by baryta	-	1. Slight sprawling after $\frac{1}{4}$ hour. Then very drowsy: but jumps when handled. Found dead next morning	Drowsiness the main symptom
Do.	-	2. Very little effect: tail stiff: then slight sprawling after $\frac{1}{4}$ hour. Drowsy but irritable if disturbed. Normal next morning	Do.

It should also be noted that 30 animals have been injected with hydroxypyruvic aldehyde, prepared by a method involving the use of hydrogen sulphide, and death, within 12 hours, has resulted in every case. On the other hand, of the 15 mice injected with hydroxypyruvic aldehyde prepared by the "baryta" method, 10 recovered completely, and the remainder died after periods ranging from 6 to 48 hours.

DISCUSSION.

Kermack, Lambie and Slater [1929] reported that the monomeric form of hydroxypyruvic aldehyde, although holding the aldehyde group in a more reactive condition, was much less toxic than the "dimeric" form, but furnished no explanation of this somewhat unexpected finding. From the data tabulated above, our results are seen to be in agreement with their observation that the monomeric form of hydroxypyruvic aldehyde does not produce a train of symptoms suggestive of insulin hypoglycaemia. It is also clear that the hydroxypyruvic aldehyde prepared in absence of hydrogen sulphide shows little if any difference when used in the monomeric or polymeric state. Similarly it was found that both monomeric and polymeric glyoxal exert practically the same effect when injected subcutaneously into mice. Moreover, the symptoms produced by glyoxal resemble in every detail those described for sulphur-free hydroxypyruvic aldehyde. Thus, even when a lethal dose is

administered, the onset of symptoms is not rapid: but the animal gradually becomes inactive and drowsy, and when disturbed moves about with difficulty owing to weakness of the limbs. Breathing gradually becomes more shallow and death follows, without convulsions, in 2-6 hours. This is typical of five experiments we have carried out using glyoxal purchased from the British Drug Houses.

We conclude therefore that the very rapid and highly toxic effect observed by Kermack, Lambie and Slater, which we also find to ensue as the result of injecting polymeric hydroxypyruvic aldehyde prepared by Evans and Waring's method, is not due to a difference in molecular complexity of the material used, but rather to the presence of an unstable by-product. This is supported by the fact that treatment with charcoal reduces the toxicity of the product in the same way as heating the solution. Yet, it is to be noticed, the charcoal-treated material gives no Schiff reaction, and hence contains the hydroxypyruvic aldehyde in a polymeric form.

As already indicated, we have failed so far to elucidate the precise nature of the by-product. The impurity which gives rise to the reaction with sodium nitroprusside is not alone responsible for the rapid toxic effect, as this reaction is still marked after charcoal treatment and also after heating. Yet the toxicity is greatly diminished by these procedures. Neither is the toxicity to be explained by the presence of oxalic acid, as we have shown by control experiments that rats withstand much larger doses of this acid than could possibly have been present in the solution of hydroxypyruvic aldehyde injected. Moreover, heating the solution would not materially influence the effect of an injection of oxalic acid.

Whatever the toxic material may prove to be, it is certainly highly unstable. The symptoms which follow on a subcutaneous injection are quite different from those observed after an injection of glucosone and are more suggestive of the effects produced by hydrogen sulphide or colloidal sulphur. Kermack, Lambie and Slater admit that in the preparation of hydroxypyruvic aldehyde it is very difficult to eliminate the last traces of hydrogen sulphide and colloidal sulphur, but conclude that hydrogen sulphide plays no part in the production of the toxic symptoms. From the results of several control experiments with various solutions containing hydrogen sulphide, we are quite satisfied that the amount of preformed hydrogen sulphide in the unheated solution of hydroxypyruvic aldehyde injected would give rise to no toxic symptoms. It is to be remembered, however, that an aqueous solution of the material at laboratory temperature continuously evolves hydrogen sulphide or some similar sulphur-containing product, and at the same time sulphur is deposited. It is conceivable that this process may be considerably accelerated by contact at 37° with the body tissues and fluids.

Again, although the material (*i.e.* sulphur) precipitated on heating the solution had, as shown by Kermack and his co-workers, no toxic effects, it does not follow that the substance from which this precipitate was produced

by heat had no toxic effect. Thus it is usually accepted that the toxicity of sulphur is due to its reduction in the organism to hydrogen sulphide, and that the extent to which the reduction occurs, and hence the toxicity, depends on the physical condition, being most intense in colloidal, less in amorphous, and least in crystalline sulphur. The degree of dispersion is thus an important factor in the effect of a preparation of colloidal sulphur [Messini, 1928]. Further, as is well known, the injection of colloidal sulphur gives rise to a hypoglycaemia and hence to symptoms resembling those following the administration of insulin. It has been pointed out, however, that there is a marked difference between the action of insulin and that of colloidal sulphur, for in the latter case there is no strict proportionality between the amount of colloidal sulphur injected and the intensity of the resulting hypoglycaemia [Pennetti, 1928]. Moreover, the hypoglycaemia is more prolonged and is not relieved by glucose, as it is accompanied by a toxic effect comparable with that caused by asphyxiating substances, such as hydrogen sulphide, or cyanides, on the respiratory centre.

While it seems improbable that colloidal sulphur would withstand the process of drying under a pressure of 9 mm. employed in the preparation of hydroxypyruvic aldehyde, a sulphur-containing by-product having an action similar to that indicated above for colloidal sulphur might conceivably still be present. This view seems to fit in best with the results of further experiments which we have carried out. Thus we have found that similar toxic compounds arise when solutions of dihydroxyacetone and glyoxal are subjected to the action of hydrogen sulphide, but apparently no such compounds form with either the sugars, glucose and fructose, or with glucosone.

The toxic product obtained from glyoxal and hydrogen sulphide differs from that encountered in the preparation of hydroxypyruvic aldehyde in that it is more stable to heat. Boiling a solution of glyoxal, which has been subjected to the action of hydrogen sulphide, gives rise to no turbidity, and death with convulsions results in 3–8 minutes after the injection of an amount of the heated solution containing the equivalent of a dose of glyoxal, which causes death in 2–6 hours. The same dose of the unheated solution of glyoxal treated with hydrogen sulphide causes death in 2–5 minutes.

As the sugars do not give rise to toxic products with hydrogen sulphide, the positive result with dihydroxyacetone may be due to the presence of traces of pyruvic aldehyde in the sample used. The negative result with glucosone is surprising, but it may be that the unchanged glucosone exerts a protective action against the toxic product, just as it antagonises the action of cyanides [Hynd, 1927], which it may be added, give rise to symptoms remarkably similar to those ascribed by Kermack, Lambie and Slater to the "dimeric" form of hydroxypyruvic aldehyde.

The contention of the above-named authors that hydroxypyruvic aldehyde produces symptoms similar to those caused by glucosone is absolutely refuted by the results recorded in the present communication.

SUMMARY.

1. When hydrogen sulphide is used in the preparation of hydroxypyruvic aldehyde, the resulting material is contaminated with sulphur-containing by-products. Some of these are stable to heat, others unstable, and the latter are highly poisonous, producing symptoms similar to those following the administration of cyanides.

2. Somewhat similar toxic compounds arise when dihydroxyacetone and glyoxal are treated with hydrogen sulphide, but apparently not when the sugars and glucosone are so treated.

3. A modification of the method of preparing hydroxypyruvic aldehyde is described whereby a product is obtained free from sulphur-containing compounds.

4. It is also clearly shown that no material difference exists between the effects produced by monomeric and polymeric hydroxypyruvic aldehyde, when injected subcutaneously into mice. The effect of either is practically identical with that which ensues after the injection of glyoxal, the main symptom being drowsiness.

5. The presence of an unstable, highly toxic by-product, which arises through the use of hydrogen sulphide, adequately explains the reported greater toxicity of the "dimeric" form of hydroxypyruvic aldehyde.

6. The glucosone effect is quite distinct from that produced by any of the compounds dealt with in this paper.

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