2. A calibration graph is constructed relating the initial velocity of angiotensin formation to renin concentration when a range of dilutions of standard rabbit renin are incubated with a prepared substrate.

3. The initial velocity of angiotensin formation is used to determine the renin concentration of plasma.

4. Renin is extracted from plasma by adsorption on diethylaminoethylcellulose, eluted, and acidified to remove traces of angiotensinase.

5. Recoveries, though low (40 %), are consistent, and the method can be used to measure renin in the peripheral plasma of normal rabbits.

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REFERENCES

Braun-Menendez, E. (1956). *Pharmacol. Rev.* **8**, 25.
Brown, J. J., Davies, D. L., Lever, A. F., Parker, R. A. & Robertson, J. I. S. (1963). *Lancet*, ii, 668.
Brown, J. J., Davies, D. L., Lever, A. F., Robertson, J. I. S. & Tree, M. (1963*a*). *Proc. 2nd int. Congr. Nephrol., Prague* (in the Press).
Brown, J. J., Davies, D. L., Lever, A. F., Robertson, J. I. S. & Tree, M. (1963*b*). *Proc. Boerhaave Symp. Hypertension, Leiden*, p. 40. Ed. by de Graeff, J.
Dexter, L., Haynes, F. W. & Bridges, W. C. (1945). *J. clin. Invest.* **24**, 62.
Goldblatt, H., Katz, Y. J., Lewis, H. A. & Richardson, E. (1943). *J. exp. Med.* **77**, 309.
Helmer, O. M. & Judson, W. E. (1963). *Circulation*, **6**, 1050.
Lever, A. F. & Peart, W. S. (1962). *J. Physiol.* **160**, 548.
Lever, A. F. & Robertson, J. I. S. (1964). *J. Physiol.* **70**, 212.
Lever, A. F., Robertson, J. I. S. & Tree, M. (1963). *Mem. Soc. Endocrin.* **13**, 285.
Lever, A. F. & Tree, M. (1963). *J. Physiol.* **166**, 26*P*.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Page, I. H. & Bumpus, F. M. (1961). *Physiol. Rev.* **41**, 331.
Peart, W. S. (1955). *Biochem. J.* **59**, 300.
Peart, W. S. (1959). *Ergebn. Physiol.* **50**, 410.
Peart, W. S., Robertson, J. I. S. & Grahame-Smith, D. G. (1961). *Circulation Res.* **9**, 1171.
Robertson, J. I. S. (1963). *J. Physiol.* **166**, 27*P*.

Biochem. J. (1964) **91**, 352

The Non-enzymic Condensation of Acetoacetate and Glyoxylate: an Explanation for the Antiketogenic Effect of Glycolaldehyde and Related Compounds

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Certain oxidizable substrates, for example, glycerol and sorbitol, decrease the formation of ketone bodies (Edson, 1936; Blakley, 1951; Haydon, 1961). During a survey of other potential antiketogenic compounds it was found that glycolaldehyde decreased the amount of acetoacetate formed by rat-liver slices. Evidence is presented in this paper which suggests that this effect is due to a non-enzymic condensation of acetoacetate and glyoxylate; the latter is an intermediate on the pathway of glycolaldehyde metabolism in animal tissues (Kun, Dechary & Pitot, 1954; Friedmann, Levin & Weinhouse, 1956; Nakada & Sund, 1958). A product of the condensation reaction has been isolated and characterized as β-acetylacrylic acid (4-oxopent-2-enoic acid).

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MATERIALS AND METHODS

Radioactive chemicals. [1-^{14}C]Octanoate and [1-^{14}C]-glyoxylate were obtained from The Radiochemical Centre, Amersham, Bucks. A solution containing [1,3-$^{14}C_2$]acetoacetate was prepared as follows: 5 μmoles of [1-^{14}C]-octanoate (2 μc) were incubated with washed rat-liver particles (Lehninger & Kennedy, 1948). The incubation mixture was deproteinized with perchloric acid (final concn. 3%, w/v), the coagulated protein removed by centrifugation and the supernatant fluid neutralized with 20% (w/v) KOH. After removing the precipitate of

Table 1. *Effect of glycolaldehyde on production of ketone bodies by rat-liver slices*

Liver slices (about 80 mg.) from a well-fed rat were incubated in 4·0 ml. of phosphate–saline for 1 hr. at 37° in O_2. Final concentration of the substrates was 2·5 mM. Results are expressed as μmoles/20 mg. dry wt. of tissue/hr.

Substrate added	Glycolaldehyde removed	Glycollate formed	Glyoxylate formed	Acetoacetate formed	D(−)-β-Hydroxybutyrate formed	Total ketone bodies formed
None	—	—	—	0·78	0·10	0·88
Glycolaldehyde	5·50	1·09	0·35	0·25	0·07	0·32
Octanoate	—	—	—	1·82	1·29	3·11
Glycolaldehyde + Octanoate	4·10	0·91	0·22	1·42	1·33	2·75

potassium perchlorate by centrifugation, the supernatant fluid (containing 7·3 μmoles of acetoacetate) was used directly for the experiments.

Other chemicals. Glycolaldehyde and sodium glyoxylate (monohydrate) were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A.; sodium glycollate was obtained from British Drug Houses Ltd., Poole, Dorset. β-Acetylacrylic acid was prepared by dehydrobromination of β-bromolaevulic acid according to the method of Overend, Turton & Wiggins (1950). After several recrystallizations from light petroleum (b.p. 60–90°) the material melted at 121° and analysed as follows: C 51·9 and H 5·6% (calc. for $C_5H_6O_3$, C 52·6 and H 5·3%).

Enzymes. Glyoxylate reductase was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. D(−)-β-Hydroxybutyrate dehydrogenase was prepared as described by Williamson, Mellanby & Krebs (1962).

Determination of metabolites. Glycollate was determined with 2,7-dihydroxynaphthalene by the colorimetric procedure of Calkins (1943). Glycolaldehyde was determined by reduction to ethylene glycol with $NADH_2$ and alcohol dehydrogenase (Holzer & Goedde, 1962). Glyoxylate was determined by reduction to glycollate with $NADH_2$ and glyoxylate reductase (Klotsch & Bergmeyer, 1962). Hydroxypyruvate, which is the only other known substrate for this enzyme, was unlikely to be present in these experiments. D(−)-β-Hydroxybutyrate dehydrogenase was used for the determination of acetoacetate and D(−)-β-hydroxybutyrate (Williamson *et al.* 1962).

Measurement of radioactivity. An Ecko scintillation counter (N612A) connected to an Ekco decatron scaler (N530F) were used for the measurements of radioactivity. The scintillator used was diphenyloxazole (0·4%, w/v) in toluene. A molar solution of Hyamine 10-X [2-methyl-4-(1,1,3,3 - tetramethylbutyl)phenoxyethoxyethyldimethyl - benzylammonium chloride monohydrate; C. Lennig and Co. (G.B.) Ltd., London, W.C. 1] was used to blend the samples with the scintillator (Brown & Badman, 1961). The vessels used for the counting were Ekco (N671A; capacity 16 ml.). The sample (0·1 ml. in 0·2N-KOH) was mixed with Hyamine solution (1·5 ml.) and this was blended with the scintillator (6 ml.).

Experiments with liver slices. The incubation technique and the method of deproteinizing the medium after incubation were as described by Mellanby & Williamson (1963). In certain experiments in which radioactive compounds were used measurements of both the 'free CO_2' (absorbed by KOH during the experiment) and the 'bound CO_2' (liberated from the incubation medium by addition of acid) were required. In this case, the Warburg vessels were cooled in ice–water after the incubation and the KOH contained in the centre well (0·2 ml. of 2N) was removed quantitatively ('free CO_2'). A further 0·2 ml. of 2N-KOH was added to the centre well of each vessel and, after equilibration at 25°, 0·5 ml. of N-HCl was added from the side arm and the incubation continued for a further 30 min. The KOH collected from the centre well at the end of this second incubation represented the 'bound CO_2'. The radioactivity in the carboxyl carbon atom of the acetoacetate formed during the experiments was determined by incubation of the medium (after removal of slices) with aniline citrate and absorption of the CO_2 liberated in KOH.

RESULTS

Antiketogenic action of glycolaldehyde and related compounds in rat-liver slices. When rat-liver slices were incubated with glycolaldehyde (2·5 mM) the amount of acetoacetate found in the medium at the end of the incubation was much lower than that in controls without glycolaldehyde; the amount of D(−)-β-hydroxybutyrate was not greatly altered (Table 1). Glycolaldehyde had a similar, though quantitatively less marked, effect when a ketone-body precursor, such as octanoate, was also added (Table 1). About 50% of the glycolaldehyde was removed and, of this, 30% was accounted for by the accumulation of glycollate and glyoxylate in the incubation medium (Table 1). Glycollate or glyoxylate, but not formate or glycine, also reduced the amount of acetoacetate found (Table 2). This suggested that glyoxylate might be the compound responsible for the antiketogenic effect of glycolaldehyde and glycollate.

Glyoxylate decreased the amount of acetoacetate formed (as measured by the radioactivity in the carboxyl carbon atom and by enzymic assay) from [1-^{14}C]octanoate and this decrease was accompanied by an increase in the radioactivity of the free and bound carbon dioxide (Table 3).

Non-enzymic condensation of acetoacetate and glyoxylate. Since the results of the slice experiment with [1-^{14}C]octanoate could not be accounted for by glyoxylate having a sparing effect on fatty acid oxidation, the possibility of a non-enzymic reaction occurring between acetoacetate and glyoxylate was tested in view of the analogous reactions

described by Henze (1930) and Ruffo, Testa, Adinolfi & Pelizza (1962). When equimolar concentrations of acetoacetate and glyoxylate were incubated together at 37° in neutral solution there was a stoicheiometric removal of the two acids and the reaction was accelerated by Mg^{2+} ions (Table 4). The reaction was faster at pH 7·4 (the pH employed for the rat-liver slice experiments) than at pH 5·0.

Chromatography of a mixture containing [1-^{14}C]-glyoxylate (10 μmoles), unlabelled acetoacetate (12 μmoles) and magnesium chloride (10 μmoles) in a final volume of 1 ml., which had been incubated for 4 hr. at 37°, showed the formation of a new compound. Small amounts (0·05 ml.) of the mixture and of an unincubated control were chromatographed on Whatman no. 4 paper in two

Table 2. *Comparison of the antiketogenic effect of glycolaldehyde and its metabolic derivatives in rat-liver slices*

Experimental conditions are as given in Table 1. Final concentration of the substrates was 2·5 mM. Results are expressed as μmoles/20 mg. dry wt. of tissue/hr.

Expt. no.	Substrate added	Acetoacetate formed	D(−)-β-Hydroxybutyrate formed	Total ketone bodies formed
1	None	0·70	0·04	0·74
	Glycolaldehyde	0·23	0·03	0·26
	Glycollate	0·31	0·01	0·32
	Glyoxylate	0·22	0·01	0·23
2	None	0·79	0·03	0·82
	Formate	0·78	0·01	0·79
	Glycollate	0·32	0·01	0·33
	Glyoxylate	0·26	0·02	0·28
3	None	0·88	0·01	0·89
	Formate	0·94	0·02	0·96
	Glycollate	0·59	0·02	0·61
	Glyoxylate	0·46	0·03	0·49
	Glycine	0·90	0·02	0·92

Table 3. *Effect of glyoxylate on the distribution of* ^{14}C *between free and bound carbon dioxide, and acetoacetate when rat-liver slices were incubated with* [1-^{14}C]*octanoate*

Rat-liver slices (100 mg. wet wt.) were incubated with [1-^{14}C]octanoate (2·5 mM) in the presence and absence of unlabelled glyoxylate (2·5 mM). The free and bound CO_2, and the CO_2 from the carboxyl group of acetoacetate, were collected separately. Acetoacetate was also determined enzymically. Radioactivity is expressed as total counts/100 sec. The changes in parentheses refer to the difference between the results with octanoate alone and those with octanoate plus glyoxylate. Experimental details are given in the text.

Substrate added	Free CO_2 (counts/100 sec.)	Bound CO_2 (counts/100 sec.)	Total CO_2 (counts/100 sec.)	Acetoacetate CO_2 (counts/100 sec.)	Acetoacetate (μmoles)
Octanoate	53000	13000	66000	77000	1·00
Octanoate + Glyoxylate	91000	19000	110000 (+44000)	47000 (−30000)	0·52 (−0·48)

Table 4. *Non-enzymic condensation of acetoacetate and glyoxylate*

Acetoacetate and glyoxylate (10 μmoles) were incubated both separately and together in a phosphate buffer (100 μmoles; pH 7·4) or an acetate buffer (100 μmoles; pH 5·0) in a total volume of 5 ml. After incubation for 90 min. at 37°, the solutions were placed in an ice bath and the acetoacetate and glyoxylate were determined enzymically. Inorganic salts (50 μmoles) were added as indicated. Results are expressed as μmoles of keto acid removed/5 ml.

			Acetoacetate removed		Glyoxylate removed	
Buffer	pH	Salts added	Incubated alone	Incubated with glyoxylate	Incubated alone	Incubated with acetoacetate
Phosphate	7·4	None	0·11	0·93	0·0	0·94
Phosphate	7·4	$MgCl_2$	0·12	3·14	0·0	3·44
Phosphate	7·4	NaCl	0·12	1·02	0·0	1·06
Acetate	5·0	None	0·0	0·10	0·0	0·13
Acetate	5·0	$MgCl_2$	0·0	2·50	0·0	2·55

Table 5. *Incubation of glyoxylate with* [1,3-$^{14}C_2$]*acetoacetate*

Glyoxylate (10 μmoles) and [1,3-$^{14}C_2$]acetoacetate (10 μmoles) were incubated with $MgCl_2$ (10 μmoles) and phosphate buffer (100 μmoles; pH 7·4) in a final volume of 2·0 ml. in a Warburg vessel for 1 hr. at 37°. A control was incubated at the same time, with the omission of glyoxylate. Free and bound CO_2, and the CO_2 from the carboxyl group of acetoacetate, were collected separately. Experimental details are given in the text. Radioactivity is expressed as total counts/100 sec. Values in parentheses refer to the difference between the vessel containing acetoacetate alone and that containing acetoacetate plus glyoxylate.

Additions	Free CO_2 (counts/100 sec.)	Bound CO_2 (counts/100 sec.)	Total CO_2 (counts/100 sec.)	Acetoacetate CO_2 (counts/100 sec.)
Acetoacetate	4000	9000	13000	118000
Acetoacetate + Glyoxylate	42000	33000	75000 (+62000)	67000 (−51000)

solvents [ethanol–ammonia–water, 8:1:1 (solvent A) and isobutyric acid–aq-N-ammonia soln., 10:6 (solvent B)]. After the papers had been dried in air, the free acids were located by spraying with 0·05% (w/v) bromophenol blue in ethanol and the keto acids by spraying with 0·4% (w/v) 2,4-dinitrophenylhydrazine in 2N-hydrochloric acid. A new keto acid area was detected (R_F 0·65 in solvent A; R_F 0·66 in solvent B) and this area contained about 75% of the radioactivity found in the glyoxylate area of the unincubated control. Enzymic determination of glyoxylate indicated that 70% had reacted during the incubation.

In view of this non-enzymic condensation it appeared likely that the apparent increase in $^{14}CO_2$ production from [1-^{14}C]octanoate in the presence of glyoxylate was due to decarboxylation of the condensation product. To test this possibility unlabelled glyoxylate and [1,3-$^{14}C_2$]acetoacetate were incubated in Warburg vessels and the free carbon dioxide, bound carbon dioxide and carbon dioxide from the carboxyl group of acetoacetate were collected as described (see the Materials and Methods section). The results indicated that the initial product of the condensation reaction was unstable and slowly decarboxylated during the incubation (Table 5). After addition of acid, the total yield of $^{14}CO_2$ liberated in the vessel containing glyoxylate and [1,3-$^{14}C_2$]acetoacetate practically balanced the decrease in the carbon dioxide from the carboxyl group of the acetoacetate (as compared with the vessel containing [1,3-$^{14}C_2$]acetoacetate alone).

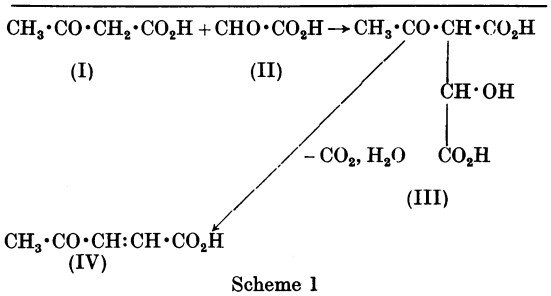
To obtain sufficient material to characterize the condensation product the reaction was carried out on a larger scale. The sodium salts of the acids (5 m-moles of each) were incubated with magnesium acetate (100 μmoles) in a final volume of 10 ml. for 16 hr. at 37°. At the end of this incubation period at least 95% of the glyoxylate and acetoacetate had reacted. The solution was diluted to 50 ml. with water and shaken with 10 g. of Amberlite IR 120 (H^+ form) for 20 min.; there was a considerable evolution of carbon dioxide. The resin was then filtered off and washed with 20 ml. of water. The filtrate and washings were concentrated *in vacuo* at 35–40° and the resulting syrup was dried over phosphorus pentoxide. After a few days it solidified to a yellow mass (yield 520 mg.), which on recrystallization from light petroleum (b.p. 60–90°) yielded white crystals (m.p. 121°, uncorr.).

The compound readily gave the iodoform reaction, indicating the presence of a $CH_3 \cdot CO$ grouping. The infrared-absorption spectrum showed a stretching frequency at 1670 cm.$^{-1}$ consistent with an $\alpha\beta$ unsaturated ketone and CH : CH stretching frequencies at 3000 cm.$^{-1}$ and 980 cm.$^{-1}$, and was identical with the spectrum of an authentic specimen of β-acetylacrylic acid. The twice-recrystallized condensation product analysed as follows: C 52·09 and H 5·4% (calc. for $C_5H_6O_3$ C 52·6 and H 5·3%).

DISCUSSION

The evidence presented in this paper indicates that acetoacetate (I) and glyoxylate (II) react to form a relatively unstable product (III) which readily decarboxylates to give an unsaturated keto acid (IV). By analogy with the condensation between methylglyoxal and acetoacetate, described by Henze (1930), the reaction probably proceeds as shown in Scheme 1.

It is not at present certain whether there are any intermediates in the conversion of the initial condensation product (III) into β-acetylacrylic acid (IV) or whether an acid medium is essential for the



Scheme 1

dehydration. The liberation of $^{14}CO_2$ when unlabelled glyoxylate and [1,3-$^{14}C_2$]acetoacetate were incubated together (Table 5) is proof that the decarboxylation of the initial product can occur at neutral pH, and explains the increase in $^{14}CO_2$ production by rat-liver slices in the presence of glyoxylate and [1-^{14}C]octanoate (Table 3).

The reaction between acetoacetate and glyoxylate probably has no physiological importance since the normal concentration of glyoxylate occurring *in vivo* is very low (e.g. in rat liver: 0·05 μmole/100 mg. dry wt.; Liang, 1962). However, the condensation reaction reported here is of interest as an explanation of the apparent antiketogenic action of glyoxylate and its metabolic precursors glycollate and glycolaldehyde.

SUMMARY

1. Glycolaldehyde, glycollate and glyoxylate, but not formate or glycine, decrease the amount of acetoacetate formed by rat-liver slices.

2. Incubation of glyoxylate and acetoacetate in neutral solution at 37° results in a stoicheiometric removal of the two acids and the reaction is accelerated by Mg^{2+} ions.

3. A product of this condensation reaction has been isolated and characterized as β-acetylacrylic acid (4-oxopent-2-enoic acid).

4. It is suggested that the apparent antiketogenic action of glyoxylate (and also of glycolaldehyde and glycollate) is due to its non-enzymic condensation with acetoacetate.

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REFERENCES

Blakley, R. L. (1951). *Biochem. J.* **49**, 257.
Brown, W. O. & Badman, H. G. (1961). *Biochem. J.* **78**, 571.
Calkins, V. P. (1943). *Analyt. Chem.* **15**, 762.
Edson, N. L. (1936). *Biochem. J.* **30**, 1862.
Friedmann, B., Levin, H. W. & Weinhouse, S. (1956). *J. biol. Chem.* **221**, 665.
Haydon, R. K. (1961). *Biochim. biophys. Acta*, **46**, 598.
Henze, M. (1930). *Hoppe-Seyl. Z.* **189**, 121.
Holzer, H. & Goedde, H. W. (1962). In *Methoden der enzymatischen Analyse*, p. 297. Ed. by Bergmeyer, H. U. Weinheim: Verlag Chemie.
Klotsch, H. & Bergmeyer, H. U. (1962). In *Methoden der enzymatischen Analyse*, p. 300. Ed. by Bergmeyer, H. U. Weinheim: Verlag Chemie.
Kun, E., Dechary, J. M. & Pitot, H. C. (1954). *J. biol. Chem.* **210**, 269.
Lehninger, A. L. & Kennedy, E. P. (1948). *J. biol. Chem.* **173**, 753.
Liang, C.-C. (1962). *Biochem. J.* **82**, 429.
Mellanby, J. & Williamson, D. H. (1963). *Biochem. J.* **88**, 440.
Nakada, H. I. & Sund, L. P. (1958). *J. biol. Chem.* **233**, 8.
Overend, W. G., Turton, L. M. & Wiggins, L. F. (1950). *J. chem. Soc.* p. 3500.
Ruffo, A., Testa, E., Adinolfi, A. & Pelizza, G. (1962). *Biochem. J.* **85**, 588.
Williamson, D. H., Mellanby, J. & Krebs, H. A. (1962). *Biochem. J.* **82**, 90.

Biochem. J. (1964) **91**, 356

The Isolation of γ-Hydroxyarginine, as its Lactone, from Seeds of *Vicia sativa*, and the Identification of γ-Hydroxyornithine as a Naturally Occurring Amino Acid

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The natural amino acid γ-hydroxyarginine was first isolated from the sea cucumber *Polycheira rufescens* by Fujita (1959) and subsequently from the sea anemone *Anthopleura japonica* Verrill by Makisumi (1961). Its occurrence in plants too has been reported by Bell & Tirimanna (1963*a*), who identified it in the seeds of 17 species of *Vicia*.

The present paper describes its isolation, as the lactone, from seeds of *Vicia sativa*, and the identification of γ-hydroxyornithine in other species.

EXPERIMENTAL

Methods

Chromatography. One-dimensional chromatograms were prepared by the descending technique on Whatman no. 1 paper. The chromatograms were developed with ethyl methyl ketone–propionic acid–water (2:1:2, by vol.), butan-1-ol–acetic acid–water (12:3:5, by vol.), butan-1-ol–pyridine–water (1:1:1, by vol.), lutidine (mixed 2,4- and 2,5-isomers)–water (11:5, v/v), and phenol–water