Wittenberg, 1955). No terpene acids other than geranic acid were available for testing. However, the position of the peak for any acid may be predicted from an experimentally determined value of the distribution coefficient (α), since the chromatogram described behaves as a theoretical partition chromatogram (Martin & Synge, 1941). The pertinent values necessary for the calculation are: cross-sectional area of column (A), 255 mm.^{2}; cross-sectional area of non-mobile phase (A_s), 85 mm.^{2}; cross-sectional area of mobile phase (A_L), 113 mm.^{2}.

It is of interest that columns similar to those described here have been useful for the separation of three other groups of acidic lipids, the glycerol phosphatides (Garvin & Karnovsky, 1954), the bile acids (Bergström & Sjövall, 1951; Sjövall, 1953), and *N*-succinylsphingosine and its congenors (Wittenberg, 1955).

SUMMARY

1. The method of reversed-phase partition chromatography has been extended to the separation of the C_6–C_{12} fatty acids.

2. This type of chromatography is applicable to the separation of the terpene acids.

The author thanks Dr S. R. Korey for support and discussion, and Dr J. E. Garvin for helpful discussion of this work. I am indebted to Dr Harry Rudney for a gift of geranic acid. This investigation was supported in part by research grants from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health, U.S. Public Health Service [nos. B 242 (C2) and B 1006], and in part by grants from the National Science Foundation.

REFERENCES

Bergström, S. & Sjövall, J. (1951). *Acta chem. scand.* **5**, 1267.
Boldingh, J. (1950). *Rec. Trav. chim. Pays-Bas*, **69**, 247.
Crombie, W. M. L., Comber, R. & Boatman, S. G. (1955). *Biochem. J.* **59**, 309.
Fairbairn, D. & Harpur, R. P. (1951). *Canad. chem. J.* **29**, 633.
Garvin, J. E. & Karnovsky, M. (1954). *Fed. Proc.* **13**, 215.
Holman, R. T. & Hagdahl, L. (1950). *J. biol. Chem.* **182**, 421.
Howard, G. A. & Martin, A. J. P. (1950). *Biochem. J.* **46**, 532.
James, A. T. & Martin, A. J. P. (1952). *Biochem. J.* **50**, 679.
James, A. T. & Martin, A. J. P. (1956). *Biochem. J.* **63**, 144.
Martin, A. J. P. & Synge, R. L. M. (1941). *Biochem. J.* **35**, 1358.
Moyle, V., Baldwin, E. & Scarisbrick, R. (1948). *Biochem. J.* **43**, 308.
Nijkamp, H. J. (1951). *Analyt. chim. acta*, **5**, 325.
Peterson, M. H. & Johnson, M. J. (1948). *J. biol. Chem.* **174**, 775.
Popják, G. & Tietz, A. (1954). *Biochem. J.* **56**, 46.
Ramsey, L. L. & Patterson, W. I. (1948). *J. Ass. off. agric. Chem., Wash.*, **31**, 139.
Silk, M. H. & Hahn, H. H. (1954). *Biochem. J.* **56**, 406.
Sjövall, J. (1953). *Acta physiol. scand.* **29**, 232.
Vandenheuvel, F. A. & Hayes, E. R. (1952). *Analyt. Chem.* **24**, 960.
Wittenberg, J. (1955). *J. biol. Chem.* **216**, 379.
Zbinovsky, V. (1955). *Analyt. Chem.* **27**, 764.

The Synthesis of some Analogues of Glutathione

By W. O. KERMACK and N. A. MATHESON*
Department of Biological Chemistry, University of Aberdeen

(*Received* 8 *March* 1956)

Several analogues of glutathione were required for studies on glyoxalase described in a later paper. The preparation of these compounds was undertaken by synthesis from amino acids and by modification of glutathione itself.

King & Kidd (1949, 1951) and King, Jackson & Kidd (1951) have shown that when phthalylglutamic anhydride is condensed with amino acids or peptides γ- and not α-glutamyl derivatives are obtained, so this route was first investigated.

Phthalyl-DL-glutamic anhydride was allowed to react with glycylglycine in acetic acid, giving phthalyl-γ-DL-glutamylglycylglycine. The phthalyl group was then removed with phenylhydrazine (Boissonnas & Schumann, 1952) and, after recrystallization, the resulting γ-DL-glutamylglycylglycine behaved as a pure compound on paper chromatograms. When this compound was heated with ninhydrin, one mole of carbon dioxide was evolved/mole of peptide; since only free carboxyl groups with adjacent free amino groups react in this way, the peptide was evidently γ- not α-glutamylglycylglycine.

In a similar manner, phthalyl-γ-DL-glutamyl-DL-alanylglycine was obtained from DL-alanylglycine, and the removal of the phthalyl group gave γ-DL-glutamyl-DL-alanylglycine. To confirm the identity

* Present address: Ministry of Agriculture, Fisheries and Food, The Experimental Factory, Greyhope Road, Aberdeen.

of this peptide, an aqueous solution of glutathione was shaken with Raney nickel, which removed the sulphur. The product was shown, by hydrolysis and paper chromatography, to contain glutamic acid, alanine and glycine. It could not be distinguished chromatographically from the γ-DL-glutamyl-DL-alanylglycine prepared above, although the latter was presumably a mixture of two pairs of diastereoisomers.

The phthalyl intermediates required in these syntheses were tested for purity by paper chromatography in *n*-butanol–pyridine–water (5:2:2, by vol.). Treatment with ninhydrin then revealed any amino acids or peptides. Phthalyl compounds could be detected if the paper were sprayed with 5% (w/v) aqueous hydrazine hydrate, dried thoroughly in an oven at 100° and then examined under ultraviolet light. The phthalylhydrazide so formed showed a characteristic blue fluorescence.

The *S*-methyl derivative of glutathione was obtained by treating the peptide in liquid ammonia with metallic sodium followed by methyl iodide. *S*-Methylcysteine was prepared similarly.

It was already known that *N*-ethylmaleimide reacts with thiol groups (Friedmann, Marrian & Simon-Reuss, 1949; Hanes, Hird & Isherwood, 1950). This reaction was carried out with glutathione and with L-cysteine in aqueous solution and the resulting *S*(*N*-ethylsuccinimido)glutathione and *S*(*N*-ethylsuccinimido)-L-cysteine were isolated.

The sulphydryl group of glutathione was oxidized to a sulphonic acid group by the performic acid reagent of Toennies & Homiller (1942) to give γ-L-glutamyl-L-β-sulphoalanylglycine. This compound, though not crystalline, gave an elementary analysis in reasonable agreement with the theoretical figures.

L-Cysteinylglycine was prepared from glutathione by mild hydrolysis with hydrochloric acid, whereby the labile γ-glutamyl group was removed without fission of the other peptide bond (Olson & Binkley, 1950).

EXPERIMENTAL

All m.p.'s are uncorrected. The hydrolyses of peptides were carried out by heating in a sealed tube with 6N-HCl for 15 hr. at 100°.

Phthalyl-γ-DL-glutamylglycylglycine. Phthalyl-DL-glutamic anhydride (5·9 g.) was dissolved in hot acetic acid (50 ml.) containing glycylglycine (3·0 g.) and the solution set aside for 30 min. The acetic acid was removed under reduced pressure and the syrup dissolved in hot water (10 ml.): the white crystals of phthalyl-γ-DL-glutamylglycylglycine (5 g., 53%), which deposited as the solution cooled, had m.p. 165–167° (decomp.) after drying at 78° *in vacuo*. The product could be recrystallized from water without altering the m.p. but it did not analyse satisfactorily till treated as follows. The crystals were dissolved in ethanol and the solution was dried over anhydrous sodium sulphate. To the hot ethanolic solution, dry ethyl acetate was added till a turbidity developed. Hard masses of crystals of *phthalyl-γ-DL-glutamylglycylglycine*, m.p. 186–188° (decomp.), separated on cooling. (Found: C, 52·2; H, 4·2; N, 10·3. $C_{17}H_{17}O_8N_3$ requires C, 52·2; H, 4·4; N, 10·7%.)

When the anhydrous material was recrystallized from water, the original hydrate, m.p. 165–167°, was obtained. (Found: C, 49·6; H, 4·4; N, 10·4. $C_{17}H_{17}O_8N_3,H_2O$ requires C, 50·0; H, 4·6; N, 10·3%.)

Chromatography of the product on paper in butanol–pyridine–water gave only one spot, R_F 0·25, which reacted with the hydrazine reagent.

γ-DL-Glutamylglycylglycine. Phthalyl-γ-DL-glutamylglycylglycine (3·0 g.) was dissolved in 95% (v/v) ethanol (75 ml.) containing tri-*n*-butylamine (2·8 g.). Phenylhydrazine (1·7 g.) was added, and the solution was refluxed for 3 hr., during which a white precipitate separated. To the warm solution acetic acid (1·4 g.) was added and then ethyl methyl ketone (110 ml.) to complete precipitation. The product was washed with ethyl methyl ketone by decantation and then filtered, giving a white powder (1·1 g., 50%) which had m.p. 180–183° (decomp.). Attempts to purify the compound by precipitation from aqueous solution with ethanol were unsuccessful. However, after a concentrated aqueous solution of the peptide had been passed through a very small column (2·5 cm. × 0·5 cm.) of activated charcoal, the addition of ethanol (10 vol.) to the combined eluate and washings precipitated small white crystals which analysed satisfactorily. (Found: C, 38·3; H, 5·9; N, 15·3. Calc. for $C_9H_{15}O_6N_3,H_2O$: C, 38·7; H, 6·1; N, 15·1%.) (Cf. Le Quesne & Young, 1950.)

The compound was chromatographed in phenol–water (4:1, v/v) in an atmosphere of NH_3 [from a 1% (w/v) solution] and coal gas. On spraying with ninhydrin only one spot appeared, R_F 0·36.

After hydrolysis in 6N-HCl, the products were chromatographed similarly. Two ninhydrin-reacting spots appeared, corresponding to glutamic acid and glycine controls, the glycine spot being about twice as intense as the other.

The compound was analysed for α-amino N by Van Slyke's ninhydrin method. (Found: 4·94. Calc. for $C_9H_{15}O_6N_3,H_2O$: 5·01% α-amino N).

Phthalyl-γ-DL-glutamyl-DL-alanylglycine. Phthalyl-DL-glutamic anhydride (3·6 g.) was dissolved in hot acetic acid containing DL-alanylglycine (2·0 g.) and the solution set aside for 30 min. The acetic acid was removed under reduced pressure, leaving a syrup which was dissolved in hot ethanol. Hot ethyl acetate was added till the solution became cloudy. White crystals of *phthalyl-γ-DL-glutamyl DL-alanylglycine* (3·0 g., 54%) were filtered off next day, m.p. 188–194° (decomp.). Repeated recrystallization from ethanol–ethyl acetate gave a product with m.p. 197–198° (decomp.). (Found: C, 53·2; H, 4·6; N, 10·2. $C_{18}H_{19}O_8N_3$ requires C, 53·3; H, 4·7; N, 10·4%.) Only one spot, R_F 0·30, was obtained when the product was run on paper with butanol–pyridine–water mixture and sprayed with hydrazine hydrate. The compound was very similar to phthalyl-γ-DL-glutamylglycylglycine in its properties except that no crystalline hydrate was obtained.

γ-DL-Glutamyl-DL-alanylglycine. Phthalyl-γ-DL-glutamyl-DL-alanylglycine (1·0 g.) was dissolved in 95% ethanol (30 ml.) containing tri-*n*-butylamine (0·93 g.). Phenylhydrazine (0·54 g.) was added and the solution was refluxed for 3 hr., during which white crystals began to separate.

Acetic acid (4·5 g.) was added, then ethyl methyl ketone (45 ml.), and the solution was left overnight. The precipitate was washed with ethyl methyl ketone by decantation, filtered off and dried in air, giving *γ*-DL-*glutamyl*-DL-*alanylglycine* (0·5 g., 68%), m.p. 195–198°. The compound was purified by the addition of ethanol to its aqueous solution till cloudy. The product, which separated overnight, was dried *in vacuo* at 78° and then had m.p. 198°–200° (decomp.). (Found: C, 41·1; H, 6·4; N, 13·8. $C_{10}H_{17}O_6N_3,H_2O$ requires C, 41·0; H, 6·5; N, 14·3%.) When chromatographed in phenol–water without NH_3 it gave one spot, R_F 0·60; when 1% of NH_3 was present, the R_F fell to 0·50.

When an acid hydrolysate of the compound was chromatographed on paper in phenol–water in the presence of NH_3, three spots were obtained with R_F 0·22, 0·35 and 0·50, respectively, corresponding to glutamic acid, glycine and alanine run simultaneously.

γ-L-*Glutamyl*-L-*alanylglycine from glutathione.* Glutathione (1 g.) was dissolved in water (20 ml.) and Raney nickel C (20 ml. of 0·5 g./ml. aqueous suspension; Hurd & Rudner, 1951) was added. The mixture was shaken for 2 days at 37°, then centrifuged; the supernatant was decanted. The residue was extracted with water and the combined extracts and supernatant were evaporated to dryness *in vacuo*. Only 0·4 g. of a brown residue remained, but no more material could be extracted from the nickel with hot water or with dilute NH_3. A similar loss of product on Raney nickel has been reported by Edward & Martlew (1954).

The brown residue was dissolved in a little water and much of the pigment was removed by precipitation with methanol. The remaining solution still contained some nickel, which was removed by precipitation with H_2S in the presence of NH_3. After the solution had been freed of H_2S and the product purified by precipitation with ethanol, it was examined by paper chromatography in phenol–water with an atmosphere of NH_3. Two ninhydrin-reacting spots were found, a very faint one with R_F 0·11 and a strong one with R_F 0·50, the latter figure being increased to 0·61 when NH_3 was omitted. This behaviour is very similar to that of *γ*-DL-glutamyl-DL-alanylglycine. Confirmation of structure was obtained by hydrolysing the product in 6N-HCl and chromatographing in phenol–water with an atmosphere of NH_3. Three spots were obtained which reacted with ninhydrin and corresponded to glutamic acid, alanine and glycine run simultaneously. A very faint spot also appeared, with R_F 0·10, corresponding to the original impurity.

S-*Methylglutathione.* Glutathione (0·92 g.) was dissolved in liquid NH_3 (100 ml.) with stirring. To the faintly reddish solution, sodium (approx. 0·21 g.) was added till the resulting indigo colour persisted for at least 1 min. Methyl iodide (0·57 g.) was added dropwise over a period of 5 min. and stirring was continued for a further 15 min. The NH_3 was allowed to evaporate and the resulting solution was dried *in vacuo* over conc. H_2SO_4. The residue was taken up in water, adjusted to pH 3·0 with HI, then mixed with ethanol (3–4 vol.). Next day, amorphous, hygroscopic *S*-methylglutathione (0·67 g., 70%) was filtered off. The product was purified by solution in hot water followed by the addition of an equal vol. of hot ethanol: crystallization occurred after a few hours in the refrigerator. After recrystallization from the minimum of hot water, S-*methylglutathione* was obtained as small white crystals, m.p. 197–199° (decomp.), after drying *in vacuo* at 78°. (Found: C, 41·3; H, 6·1; N, 13·1. $C_{11}H_{19}O_6N_3S$ requires C, 41·1; H, 5·9; N, 13·1%.)

Methylation of glutathione with dimethyl sulphate gave a sticky syrup which could not be crystallized.

The products of each reaction were chromatographically identical, each showing a single spot, R_F 0·53, in phenol–water with an atmosphere of NH_3, and R_F 0·63 in the absence of the NH_3. These spots reacted with ninhydrin and with iodoplatinic acid but not with nitroprusside and cyanide (Block, LeStrange & Zweig, 1952). After hydrolysis in 6N-HCl overnight, each preparation gave three spots on chromatography in phenol–water in the presence of NH_3, corresponding to authentic glutamic acid, *S*-methylcysteine and glycine run simultaneously.

S-*Methyl*-L-*cysteine.* This compound was prepared by methylation with methyl iodide in liquid NH_3, as for *S*-methylglutathione.

S(N-*Ethylsuccinimido*)-L-*cysteine.* L-Cysteine (0·50 g.) was dissolved in water (10 ml.) with slight warming, and *N*-ethylmaleimide (0·53 g.) was added gradually with shaking. After 1 hr., acetone (3–4 vol.) was added to the solution to precipitate S(N-*ethylsuccinimido*)-L-*cysteine* (0·93 g., 92%), m.p. 185–191° (decomp.). The product, purified by repeated precipitation from aqueous solution, first by acetone then by ethanol, had m.p. 187–193° (decomp.). (Found: C, 44·1; H, 5·7; N, 11·1. $C_9H_{14}O_4N_2S$ requires C, 43·9; H, 5·7; N, 11·4%.) The compound gave only one ninhydrin-reacting spot, R_F 0·87, when run on paper in phenol–water with an atmosphere of NH_3. When the compound was treated with 6N-HCl in a sealed tube overnight, some was unchanged but this was accompanied by a larger amount of a new compound, R_F 0·05, which was presumably a succinic acid derivative of cysteine.

S(N-*Ethylsuccinimido*)*glutathione.* Glutathione (1·0 g.) was dissolved in water (10 ml.) with slight warming and *N*-ethylmaleimide (0·42 g.) was added gradually with shaking. After 1 hr., the product was precipitated with acetone, giving S(N-*ethylsuccinimido*)*glutathione* (1·3 g., 91%), m.p. 198–199° (decomp.). Purification was effected as for *S*(*N*-ethylsuccinimido)cysteine, giving fine white crystals, m.p. 202–203° (decomp.). (Found: C, 44·2; H, 5·4; N, 12·9. $C_{16}H_{24}O_8N_4S$ requires C, 44·5; H, 5·6; N, 13·0%.) When run on paper in phenol–water the compound gave only one spot, R_F 0·75, which reacted with ninhydrin and with the iodoplatinic acid reagent but not with nitroprusside and cyanide. An acid hydrolysate of the compound, run in phenol–water in the presence of NH_3, gave four ninhydrin-reacting spots, R_F values 0·05, 0·25, 0·36 and 0·87. The spots at 0·87 and 0·05 corresponded to *S*(*N*-ethylsuccinimido)cysteine and its breakdown product, referred to above, respectively.

γ-L-*Glutamyl*-L-*β*-*sulphoalanylglycine.* Glutathione (1·0 g.) was dissolved in 98% formic acid (40 ml.) and 30% (w/v) of H_2O_2 (5 ml.) was added. After 1·5 hr. the solution was placed in a vacuum desiccator over KOH and left overnight. The residual syrup was dissolved in dry methanol and a mixture of equal parts of dry ethanol and dry ether was added to give a flocculent precipitate. This could be purified by repeated solution in methanol and precipitation with ethanol–ether to yield an amorphous white powder. When heated, the peptide first shrank and then swelled to a frothy mass as the temperature rose from 120° to 180°, the froth liquefying and decomposing at 194–196°. For analysis, the product was dried *in vacuo* at room temp.

over H_2SO_4 for 3 days. (Found: C, 33·3; H, 5·5; N, 11·7. $C_{10}H_{17}O_9N_3S$ requires C, 33·8; H, 4·8; N, 11·8%.) This was not a completely satisfactory analysis, but the identity of the compound was confirmed chromatographically. When run on paper with phenol–water, the compound gave a single spot, R_F 0·13, which reacted with ninhydrin but not with nitroprusside and cyanide nor with iodoplatinic acid. After being hydrolysed in 6N-HCl, the compound run in phenol–water in presence of NH_3 gave three ninhydrin-reacting spots corresponding to glutamic acid, cysteic acid and glycine.

L-*Cysteinylglycine.* This compound was prepared by partial hydrolysis of glutathione with N-HCl (Olson & Binkley, 1950).

SUMMARY

1. The three compounds γ-DL-glutamylglycylglycine, γ-DL-glutamyl-DL-alanylglycine and *S*(*N*-ethylsuccinimido)-L-cysteine have been synthesized.

2. From glutathione the following compounds have been prepared; *S*-methylglutathione, *S*(*N*-ethylsuccinimido)glutathione, γ-L-glutamyl-L-β-sulphoalanylglycine, L-cysteinylglycine and γ-L-glutamyl-L-alanylglycine, the last being obtained only in an impure state and in low yield.

3. On a paper chromatogram, phthalimido derivatives are detected by spraying with a reagent containing hydrazine hydrate. When the paper is dried, the phthalylhydrazide formed fluoresces strongly in ultraviolet light.

We are indebted to Dr Hazel Dobbie for the α-amino nitrogen analysis carried out on γ-DL-glutamylglycylglycine. We wish to thank the Medical Research Council for a studentship to one of us (N.A.M.) and The Distillers Co. (Biochemicals) Ltd. for a generous gift of glutathione.

REFERENCES

Block, R. J., LeStrange, R. & Zweig, G. (1952). *Paper Chromatography*, p. 63. New York: Academic Press.
Boissonnas, R. A. & Schumann, I. (1952). *Nature, Lond.*, **169**, 154.
Edward, J. T. & Martlew, E. F. (1954). *Chem. & Ind.* p. 193.
Friedmann, E., Marrian, D. H. & Simon-Reuss, I. (1949). *Brit. J. Pharmacol.* **4**, 105.
Hanes, C. S., Hird, F. J. R. & Isherwood, F. A. (1950). *Nature, Lond.*, **166**, 288.
Hurd, C. D. & Rudner, B. (1951). *J. Amer. chem. Soc.* **73**, 5157.
King, F. E., Jackson, B. S. & Kidd, D. A. A. (1951). *J. chem. Soc.* p. 243.
King, F. E. & Kidd, D. A. A. (1949). *J. chem. Soc.* p. 3315.
King, F. E. & Kidd, D. A. A. (1951). *J. chem. Soc.* p. 2976.
Le Quesne, W. J. & Young, G. T. (1950). *J. chem. Soc.* p. 1959.
Olson, C. K. & Binkley, F. (1950). *J. biol. Chem.* **186**, 731.
Toennies, G. & Homiller, R. P. (1942). *J. Amer. chem. Soc.* **64**, 3054.

The Effects of some Analogues of Glutathione on the Glyoxalase System

By W. O. KERMACK and N. A. MATHESON*
Department of Biological Chemistry, University of Aberdeen

(*Received* 8 *March* 1956)

It is now established that the enzymic conversion of methylglyoxal into lactic acid takes place in two stages. First, methylglyoxal reacts with glutathione (GSH) to form *S*-lactylglutathione, the reaction being catalysed by an enzyme which Racker (1951) has called glyoxalase I. Secondly, this thioester is hydrolysed by another enzyme, glyoxalase II, giving lactic acid and regenerating GSH. The GSH therefore acts as a coenzyme for the overall reaction whereby methylglyoxal is transformed into lactic acid. Glyoxalase I and II correspond to the 'enzyme' and the 'factor' respectively of Hopkins & Morgan (1948).

Behrens (1941) has shown that, in the glyoxalase reaction, glutathione may be replaced as a coenzyme by either α-L-glutamyl-L-cysteinylglycine or β-L-aspartyl-L-cysteinylglycine, though both are less effective than GSH (γ-L-glutamyl-L-cysteinylglycine). Kögl & Akkerman (1946) found that γ-D-glutamyl-L-cysteinylglycine can also serve as a coenzyme for the glyoxalase system, but again it is less active than glutathione. Other substances tested as coenzymes but found ineffective include oxidized glutathione, cysteine, thioglycollic acid (Lohmann, 1932), γ-glutamylcysteine and cysteinylglycine (Woodward & Reinhart, 1942).

In the present work various analogues of GSH were tested as possible inhibitors of the glyoxalase reaction. Experiments with the complete glyoxalase system showed that certain of these analogues were indeed inhibitory but the simultaneous action of both glyoxalase I and II made quantitative interpretation of the results difficult. For this reason,

* Present address: Ministry of Agriculture, Fisheries and Food, The Experimental Factory, Greyhope Road, Aberdeen.