Comparative Detoxication

10. THE ENZYMIC CONJUGATION OF CHLORO COMPOUNDS WITH GLUTATHIONE IN LOCUSTS AND OTHER INSECTS

BY A. J. COHEN, J. N. SMITH AND HELEN TURBERT
Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2

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It was shown by Cohen & Smith (1964) that some halogenated organic compounds were metabolized in the locust to *S*-substituted glutathione derivatives and that this was an effective detoxication of the administered compound. It is probable that the insecticide Gammexane (γ-hexachlorocyclohexane) is inactivated in insects by a similar glutathione conjugation (Smith, 1962; Bradbury & Standen, 1959), and so it was decided to study the enzymes catalysing this glutathione detoxication and also the action of possible inhibitors.

An enzyme catalysing similar reactions in rat liver has been studied by Booth, Boyland & Sims (1961) and by Al-Kassab, Boyland & Williams (1963). In man and other vertebrates, the formation of a conjugate from phenoltetrabromophthaleindisulphonate, used in liver-function tests, is also attributable to a reaction catalysed by 'glutathiokinase' (Combes & Stakelum, 1961).

It is shown below that marked differences in response to inhibitors are found between the analogous enzymes in vertebrates and insects.

EXPERIMENTAL

Materials

Reference compounds. Substrates and glutathione conjugates were the same as the samples used by Cohen & Smith (1964). Inhibitors were reagent-grade commercial products and were used without further purification.

Preparation of enzyme extracts. Tissues or whole insects were ground with water in a glass Potter–Elvehjem homogenizer, 1 ml. of water being normally used for each organ and 3–5 ml./g. for whole insects. Except for haemolymph and crop fluid, where 0·1 ml. was diluted to 1 ml. with water, all the designated tissue from each locust was used for distribution studies and results of locust-tissue assays were expressed in activities/insect (Table 1). Extracts of whole insects were centrifuged and the supernatants diluted to suitable volumes for assay.

Acetone-dried powders of enzymes were prepared by homogenizing tissues or chopped insects in the minimum quantity of water. Acetone at $-10°$ was then added until no more precipitation occurred. The precipitate was filtered off and dried over $CaCl_2$ *in vacuo*.

Methods

Determination of enzyme activity: (*a*) With *p*-nitrobenzyl chloride. Incubation mixtures contained 0·5 ml. of enzyme in a total volume of 1·0 ml. of 0·1 M-citrate–phosphate buffer (McIlvaine, 1921) at the required pH containing glutathione and 0·5% of bovine blood albumin. *p*-Nitrobenzyl chloride was added at the start of incubation in 0·01 ml. of acetone to give the required concentration in the incubation mixture. In routine assays, 5 mM-glutathione and 5 mM-*p*-nitrobenzyl chloride were used at pH 6. Controls were prepared similarly except that the enzyme was replaced by an equivalent volume of water.

After incubation at 37°, 0·5 ml. of enzyme was added to the controls and 0·5 ml. of water to the test solutions, followed by 0·1 ml. of N-HCl to each tube. After shaking with 8 ml. of $CHCl_3$ to remove excess of substrate, 1 ml. of the aqueous layer was removed and the *S*-(*p*-nitrobenzyl)-glutathione present measured after reduction with zinc amalgam as described by Cohen & Smith (1964).

(*b*) With 1-chloro-2,4-dinitrobenzene. Incubation mixtures contained 1·0 ml. of enzyme in a total volume of 2·5 ml. of 0·05 M-acetate buffer containing the desired concentrations of glutathione and 1-chloro-2,4-dinitrobenzene. Stock solutions of 1-chloro-2,4-dinitrobenzene were made by adding the required amount of an acetone solution to buffer. The acetone concentration in the incubation mixtures did not exceed 0·5%. Control mixtures were prepared similarly except that the enzyme was replaced by water. In routine assays, 5 mM-glutathione and 1 mM-1-chloro-2,4-dinitrobenzene were used at pH 5·4. After 20 min. at 25°, 0·5 ml. of 2 N-HCl was added to each tube, 0·5 ml. of enzyme to the controls and 0·5 ml. of water to the test solution. Solutions were measured in a Unicam SP. 500 spectrophotometer at 350 mμ, with the control solution as the instrument blank. The amount of *S*-(2,4-dinitrophenyl)glutathione was calculated by reference to a calibration curve prepared from solutions of the pure compound in 0·2 N-HCl.

(*c*) With 1,2-dichloro-4-nitrobenzene. The direct spectrophotometric method of Booth *et al.* (1961) was used.

(*d*) With phenoltetrabromophthaleindisulphonate. Incubation mixtures contained 0·1 ml. of enzyme in a total volume of 0·3 ml. of citrate–phosphate buffer, pH 6 (McIlvaine, 1921), containing 5 mM-glutathione (reduced) and the required concentration of phenoltetrabromophthaleindisulphonate. Control tubes were prepared similarly with water in place of the enzyme. After 1 hr., 0·1 ml. of reaction mixtures was applied to 2 cm. strips of Whatman no. 1 paper and developed in the upper layer of propan-1-ol–acetic acid–water (10:1:5, by vol.).

When the solvent front had moved 14–16 in., the zones corresponding to the phenoltetrabromophthaleindisulphonate conjugate were eluted in 0·1 N-KOH and the extinctions measured at 580 mμ in cells of 3 cm. light-path (Combes & Stakelum, 1961). An estimate of the amount of

conjugate was made by reference to a calibration curve prepared from the synthetic reference material.

Inhibition experiments. Water-soluble inhibitors were added to the incubation mixtures and controls to give the desired concentration; water-insoluble compounds were added in 0·01 ml. of acetone.

RESULTS

Distribution of the enzyme in locusts. Extracts of *Schistocerca gregaria* tissues were assayed with *p*-nitrobenzyl chloride as described above (Table 1). Paper chromatography (Cohen & Smith, 1964) of the aqueous layer of the chloroform–hydrochloric acid partition system showed that only *S*-(*p*-nitrobenzyl)glutathione was present when the enzyme was prepared from gut, fat body or malpighian tubes. No nitro compounds were detectable when the aqueous layer from assays of integument, haemolymph, gut contents or crop fluid were chromatographed.

Properties of the locust fat-body enzyme. Except for the tissue and species-distribution studies (Tables 1 and 3) all experiments were carried out with locust fat-body homogenates, since these were relatively free of enzymes that degrade the glutathione conjugates (Cohen & Smith, 1964). Homogenates were centrifuged in a bench centrifuge to remove a layer of fat and some large debris.

With all the substrates tested, the relation between amount of glutathione conjugate formed and time was linear for at least 3 hr. under the experimental conditions used. The optimum pH for the 'glutathiokinase' of fat-body was at pH 7 with *p*-nitrobenzyl chloride as substrate and at pH 8 when 3,4-dichloronitrobenzene was used. No significant differences in pH optima were found when phosphate (Sorensen, 1909) or universal buffer (Ellis, 1961) was used in place of McIlvaine's buffer. The chemical reaction between glutathione and *p*-nitrobenzyl chloride became increasingly important at pH values greater than 6, and this pH was therefore used in routine assays with this substance to minimize the colour in control tubes.

The non-enzymic reaction between 1-chloro-2,4-dinitrobenzene and glutathione was so rapid at pH values greater than 6 that no reliable value could be obtained for the optimum pH of the enzyme reaction. Routine assays were carried out at pH 5·4, where the chemical reaction was negligible under the conditions used.

High concentrations of glutathione were needed for optimum activity of the enzyme, and, in the presence of 5 mM-*p*-nitrobenzyl chloride, K_s for glutathione, determined from Lineweaver & Burk (1934) plots, was 2 mM. Values for phenoltetrabromophthaleindisulphonate (K_s = 2 mM at pH 6), *p*-nitrobenzyl chloride (K_s = 3 mM at pH 6) and 1,2-dichloro-4-nitrobenzene (K_s = 5 mM at pH 8) were also determined graphically from plots of $1/v$ against $1/s$ in 5 mM-glutathione. Inhibition by substrate occurred in all cases at concentrations greater than 2 mM. The K_s value for 1-chloro-2,4-dinitrobenzene could not be determined in this way as plots of $1/v$ against $1/s$ were non-linear (Fig. 5).

The enzyme-catalysed reaction between phenoltetrabromophthaleindisulphonate and glutathione was very slow and of the same order as the non-enzymic reaction in the enzyme extracts used. The relative velocities of the reactions of *p*-nitrobenzyl chloride, 1,2-dichloro-4-nitrobenzene and phenoltetrabromophthaleindisulphonate at pH 7 and 37° with 5 mM-glutathione were 29:14:1 when 1 mM substrates were used.

Enzyme activity was lost on heating the enzyme extract to 100° for 2 min. About half the activity was lost at room temperature in 24 hr. or at 0° in a week. No activity towards *p*-nitrobenzyl chloride was lost on dialysis at 0° against water overnight

Table 1. *Distribution of 'glutathiokinase' activity in locust tissues*

Incubation mixtures contained glutathione (5 mM) and *p*-nitrobenzyl chloride (5 mM) at pH 6 and 37°.

	S-(*p*-Nitrobenzyl)glutathione formed (mg./locust/hr.)					
Expt. no.	1	2	3	4	5	6
Blood*	0·14	0·11	0·01	0·04	0·04	0·02
Crop fluid*	0	0·01	0·04	0·02	0	0
Fat body	1·10	0·75	2·00	1·50	1·60	1·80
Malpighian tubes	0·76	1·50	2·30	1·40	1·40	1·60
Integument	0·09	0·01	0	0·1	0·06	0·14
Fore-gut	0·20	0·24	0·11	0·08	0·11	0·30
Mid-gut	0·21	0·52	0·24	0·40	1·40	0·60
Hind-gut	0·20	0·38	0·14	0·25	0·17	0·50
Gastric caecae	0·60	0·33	2·0	0·48	0·21	0·38
Gut contents	0	0	0·57	0	0·09	0·11
Totals	3·30	3·85	7·41	4·27	5·12	5·45

* 0·1 ml. used from each locust.

or on gel-filtration through Sephadex G-25. The presence of 1 mM-CoA or 1 mM-2-mercaptoethylamine had no effect on the activity of the enzyme.

Effect of inhibitors on locust fat-body enzyme. The effect of benzyl chloride, and of some other potential competitive substrates for the enzyme, was measured over a range of inhibitor concentrations from 0·5 to 5 mM. *p*-Nitrobenzyl chloride was used as substrate at 0·5, 1·25, 2·5 and 5 mM in the presence of 5 mM-glutathione at pH 6. When the results were plotted graphically according to the method of Dixon (1953), a reasonable fit for a case of competitive inhibition was found with experimental value of 2 mM for the inhibitor constant, K_i (Fig. 1). The same results plotted according to the method of Lineweaver & Burk (1934), however, suggested a mixed inhibition (Fig. 2). Similar experiments with 1-bromobutane or phenoltetrabromophthaleindisulphonate as inhibitor suggested that the inhibition was non-competitive. A form of partial inhibition was exhibited by 1-chloromethylnaphthalene (Fig. 3), but no inhibition by bromoethane, 1,2-epoxypropane, EDTA, cysteine or Gammexane was observed over the range 0·5–5 mM.

In the reaction between 1-chloro-2,4-dinitrobenzene and glutathione, non-linear plots of $1/v$ against $1/s$ were obtained when concentrations of either of the two substrates were varied at a constant concentration of the other (Figs. 4 and 5). This was more noticeable in reactions inhibited with phenoltetrabromophthaleindisulphonate. When the inhibitor concentration was altered at fixed concentrations of glutathione and 1-chloro-2,4-dinitrobenzene, plots of $1/v$ against i (Dixon, 1953) indicated partial inhibition of the reaction (Fig. 6).

A number of compounds were tested as inhibitors at concentrations of 0·1 mM or 0·01 mM by using either the *p*-nitrobenzyl chloride or the 1,2-dichloro-4-nitrobenzene assay method. With the exception of the compounds listed in Table 2, 0·1 mM concentrations of substances tested exerted only weak (less than 20 %) or no inhibition. These included piperonyl butoxide, 2,4-dinitro-*o*-cresol, *p*-iodophenol, bromoquinol, 2,4-dichlorophenol, 2,4,5-trichlorophenol, chlorotriphenylmethane, salicylic acid, cresotic acid, gentisic acid, benzoquinone, rhodizonic acid, penicillin G, semi-carbazide, sodium arsenite, chloroacetamide, phenolphthalein, phenolphthaleindisulphonate, tetrabromophenolphthalein, thymolphthalein, *o*-cresolphthalein, thymol blue, phenol red, saccharin, fluorescein, rhodamine, phthalic acid, phthalide,

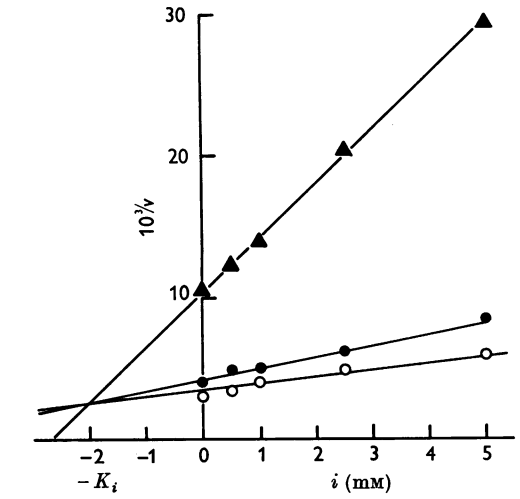

Fig. 1. Inhibition of reaction between glutathione and *p*-nitrobenzyl chloride by benzyl chloride. v, μg. of *S*-(*p*-nitrobenzyl)glutathione formed/hr.; i, Concn. of benzyl chloride. Incubation mixtures contained enzyme from 0·3 locust in a total volume of 1 ml. of pH 6 buffer containing glutathione (5 mM) and *p*-nitrobenzyl chloride. Concn. of *p*-nitrobenzyl chloride: ○, 5 mM; ●, 2·5 mM; ▲, 0·5 mM.

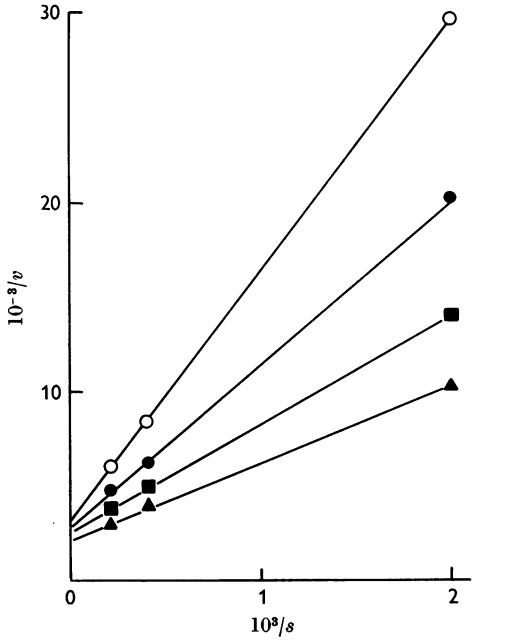

Fig. 2. Inhibition of reaction between glutathione and *p*-nitrobenzyl chloride by benzyl chloride. v, μg. of *S*-(*p*-nitrobenzyl)glutathione formed/hr.; s, concn. (M) of *p*-nitrobenzyl chloride. Incubation mixtures contained enzyme from 0·3 locust in a total volume of 1 ml. of pH 6 buffer containing glutathione (5 mM) and benzyl chloride. Concn. of benzyl chloride: ○, 5 mM; ●, 2·5 mM; ■, 1 mM; ▲, inhibitor absent.

phthalimide and tetrachlorophthalic anhydride. The chlorinated insecticides aldrin, dieldrin and DDT were also tested in 5 mM solution but had no effect on the reaction between *p*-nitrobenzyl chloride and glutathione under the standard assay conditions.

No enzymic reaction was found when glutathione was replaced in the routine assay mixture by 2·5–10 mM-L-cysteine.

Behaviour of the enzyme on Sephadex columns. Sephadex G-25, G-50, G-75, G-100 and G-200 gels were prepared as recommended by the makers (Pharmacia A.B., Uppsala, Sweden; see Flodin, 1962) and packed into 20 cm. × 1 cm. columns in 0·05M-acetate buffer, pH 5·4. Acetone-dried enzyme powders were shaken with suitable volumes of the acetate buffer (usually 20 mg. of powder and 2–5 ml. of water) and centrifuged to remove insoluble proteins. The supernatant solution (0·5 ml.) was applied to the column and washed through with 0·05M-acetate buffer, pH 5·4. Fractions (1 ml.) were collected until a volume of buffer equal to the total column volume had passed through. The extinctions of the fractions at 280 mμ were measured, the enzyme activities were assayed by the 1-chloro-2,4-dinitrobenzene procedure and the results were plotted graphically. With enzymes from all species, the peak enzyme activity appeared with the void volume on Sephadex G-25, G-50 and G-75, and at intermediate volumes on Sephadex G-100 and G-200 columns. Small species differences, possibly indicative of differences in the molecular weights of the enzymes, were noted in the elution volumes of enzymes from different sources (Table 3), but these were all close to the elution volume of haemoglobin.

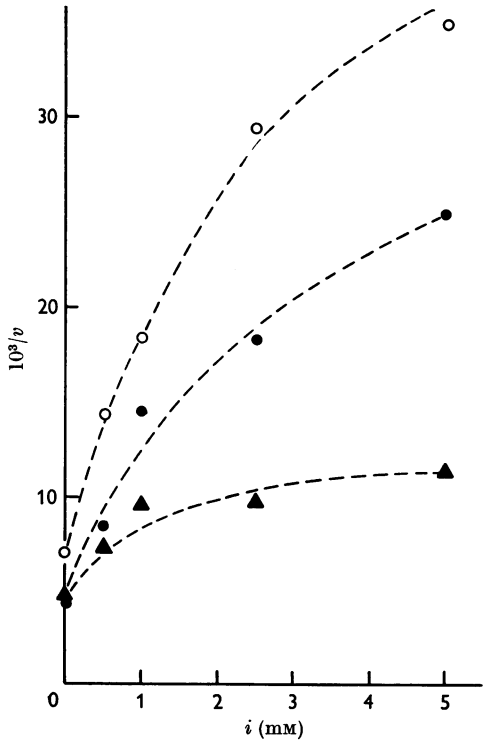

Fig. 3. Inhibition of reaction between glutathione and *p*-nitrobenzyl chloride by 1-chloromethylnaphthalene. Concn. of *p*-nitrobenzyl chloride: ▲, 5 mM; ●, 1·25 mM; ○, 0·5 mM. Other values and conditions were as given in Fig. 1.

DISCUSSION

The insect enzyme catalysing the condensation between reactive halogen compounds and glutathione appears to be generally similar to the rat-liver 'glutathiokinase' described by Booth *et al.* (1961). Experiments with Sephadex columns show that it is found in the soluble part of the cell homogenate and may have a molecular weight about 65000 similar to that of haemoglobin, since it behaves similarly in Sephadex columns. No coenzyme requirements could be demonstrated. Glutathione could not be replaced by L-cysteine, but specificity to the second substrate was low and a variety of halogen compounds reacted. Among

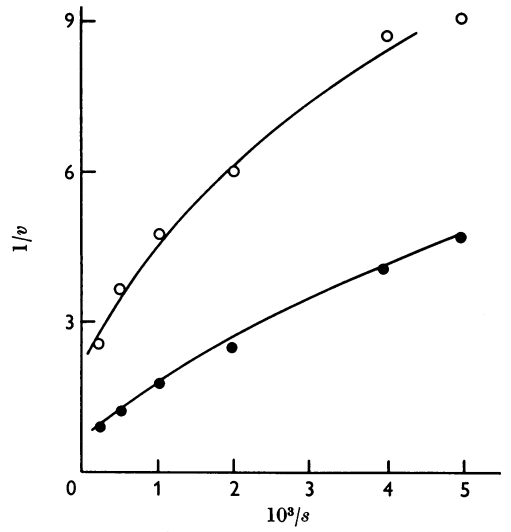

Fig. 4. Inhibition of reaction between glutathione and 1-chloro-2,4-dinitrobenzene by 2 μM-phenoltetrabromophthaleindisulphonate. v, μmoles of *S*-(2,4-dinitrophenyl)-glutathione formed/hr.; s, concn. (M) of glutathione. ○, Inhibitor present; ●, inhibitor absent. Each tube contained 1-chloro-2,4-dinitrobenzene (1·0 mM) and enzyme from 0·25 locust at pH 5·4.

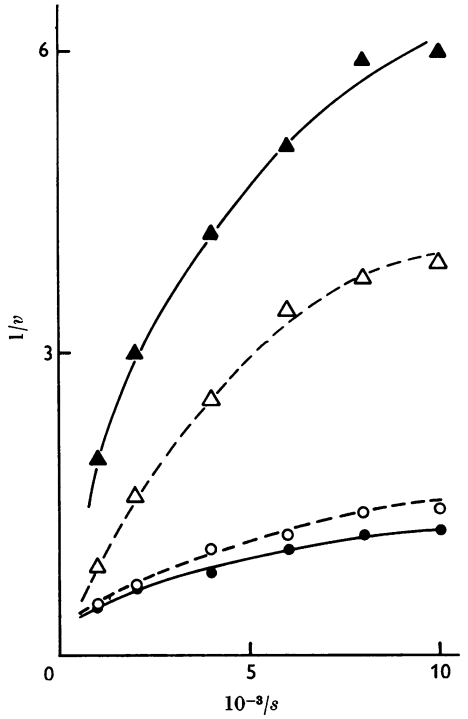

Fig. 5. Inhibition of enzymic reaction between glutathione and 1-chloro-2,4-dinitrobenzene by 2 μM-phenoltetrabromophthaleindisulphonate. v, μmoles of S-(2,4-dinitrophenyl)glutathione formed/hr.; s, concn. (M) of 1-chloro-2,4-dinitrobenzene. With locust enzyme: ▲, inhibitor present; ●, inhibitor absent. With housefly enzyme: △, inhibitor present; ○, inhibitor absent. Each tube contained glutathione (5 mM) and enzyme from two flies or 0·25 locust in 0·1 M-acetate buffer, pH 5·4.

the compounds studied, those that reacted most readily with glutathione non-enzymically also had the fastest enzyme-catalysed rate of reaction.

1-Chloro-2,4-dinitrobenzene was particularly convenient as a substrate for routine assays because of its high enzyme-catalysed rate of reaction at 25° even at pH 5·4, away from its optimum pH. Since

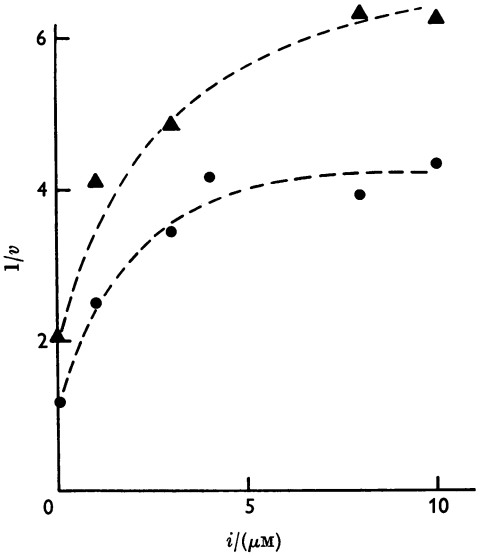

Fig. 6. Inhibition of reaction between glutathione and 1-chloro-2,4-dinitrobenzene by phenoltetrabromophthaleindisulphonate. i, concn. of phenoltetrabromophthaleindisulphonate. Concn. of 1-chloro-2,4-dinitrochlorobenzene: ●, 1 mM; ▲, 0·25 mM. Other values and conditions were as given in Fig. 5.

Table 2. *Inhibitors of locust fat-body 'glutathiokinase'*

Incubation mixtures contained glutathione (5 mM) and either *p*-nitrobenzyl chloride (5 mM) at pH 6 or 1,2-dichloro-4-nitrobenzene (1 mM) at pH 8.

	Inhibition of reaction (%)					
	With *p*-nitrobenzyl chloride			With 1,2-dichloro-4-nitrobenzene		
Concn. of inhibitor (μM)	5000	100	10	100	10	1·0
2,4,6-Tribromophenol	—	—	—	68	—	—
2,3,4,6-Tetrachlorophenol	—	—	—	81	—	—
Pentachlorophenol	80	—	—	95	44	—
3,5-Di-iodosalicylic acid	—	—	—	89	72	—
2,4-Dinitro-1-naphthol-7-sulphonic acid (flavianic acid)	—	—	—	50	—	—
Phenoltetrabromophthaleindisulphonate	—	—	80	95	83	25
Tetrabromo-*m*-cresolsulphonphthalein (bromocresol green)	—	—	60	80	40	—
Tetraiodophenolsulphonphthalein (iodophenol blue)	—	50	35	95	49	—
Tetrabromophenolsulphonphthalein (bromophenol blue)	—	—	50	76	37	—
Tetrabromo(R)fluorescein (eosin)	—	—	—	60	—	—
Tetrachloro(P)fluorescein	—	65	23	50	—	—
Tetrachloro(P)tetraiodo(R)fluorescein (rose Bengal)	—	60	—	50	—	—
Tetrabromo(R)tetrachloro(P)fluorescein (phloxin)	—	65	37	66	—	—

Table 3. *'Glutathiokinase' activity in different species*

The enzyme was assayed with 1-chloro-2,4-dinitrobenzene (1 mM) and glutathione (5 mM) at 25° and pH 5·4. Fresh homogenates of whole insects or of vertebrate liver were used as the enzyme source except that reconstituted acetone-dried powders were used for elution and inhibition experiments. Enzyme activities are given as the means of six experiments each with locusts, ticks and periplaneta, or from 1 g. of the smaller insects.

	Enzyme activity (μmoles/hr./g. of animal or organ)	Elution volume from Sephadex G-100* (ml.)	Inhibition by 2 μM-phenoltetrabromophthalein-disulphonate (%)
Locust (*Schistocerca*)	19	9·0	65
Housefly (*Musca*)	200	7·0	50
Flourbeetle (*Tenebrio*)	70	8·5	43
Cockroach (*Blatta*)	215	—	—
Cockroach (*Periplaneta*)	70	—	—
Cockroach (*Blattella*)	120	9·0	50
Tick (*Boophilus*)	100	7·0	5
Cotton stainer (*Dystercus*)	7	—	—
Turnip beetle (*Phaedon*)	6000	7·0–9·0	85
Rat liver	20	10·0	5
Rabbit liver	80	9·0	5

* Void volume of column = 5 ml., elution volume of haemoglobin = 8 ml.; fresh homogenate was used as the enzyme.

its conjugate, *S*-(2,4-dinitrophenyl)glutathione, had a very high extinction at 350 mμ, where 2,4-dinitrochlorobenzene had almost none, it was also suitable for a direct photometric assay as described by Booth *et al.* (1961) with 1,2-dichloro-4-nitrobenzene.

The 'glutathiokinases' appear to catalyse reactions that either take place spontaneously or are energetically favoured, and for the vertebrate enzymes a variety of alkylating agents act as substrates (Booth *et al.* 1961; Bray & Garrett, 1961; Al-Kassab *et al.* 1963). These substrates are already 'high-energy' compounds and the glutathione detoxication does not seem to require the formation of activated intermediates such as the benzoyl-CoA or UDP-glucose that are necessary in other detoxication conjugations.

High concentrations of glutathione were required for optimum activity of the locust enzyme. The initial rate of conjugation of *p*-nitrobenzyl chloride in intact locusts was about 100 μg./hr. (Cohen & Smith, 1964), but enzyme assays showed that sufficient enzyme was frequently available in the insect to catalyse reaction rates 50 times as great as this (Table 1) when optimum concentrations of glutathione were provided. It might be expected from this that strains of organisms that, through exposure to arsenical insecticides, have developed higher glutathione contents than normal (see, for example, Whitehead, 1961) would be able to dispose of foreign organic chloro compounds more rapidly. Whether this has any relevance to the development of resistance to chlorinated insecticides in these organisms must also depend also on the presence of the appropriate 'glutathiokinase'.

The insect enzymes were clearly distinguished from vertebrate-liver 'glutathiokinase' by their response to the phthalein group of inhibitors, particularly phenoltetrabromophthaleindisulphonate which produced measurable inhibition at a concentration of 0·1 μM. The enzyme in the cattle tick, a non-insect arthropod, was similar to the vertebrate enzymes and was little affected by phenoltetrabromophthaleindisulphonate. Among the insects, small differences in the enzymes were found in the degree of their inhibition by phenoltetrabromophthaleindisulphonate also in their behaviour on Sephadex columns. From most sources (Table 3) the enzyme elution peaks were symmetrical, but the turnip-beetle enzyme was eluted as an asymmetrical peak, suggesting that it was made up of components with elution volumes of about 7 and 9 ml. respectively.

A detailed study of the enzyme from locusts with a range of possible inhibitors showed that inhibition was usually complex. Lineweaver–Burk plots indicating a simple non-competitive inhibition were found only with *p*-nitrobenzyl chloride inhibited by 0·01 mM-phenoltetrabromophthaleindisulphonate. In other cases, even with closely related compounds such as benzyl chloride and 1-chloromethylnaphthalene acting as inhibitors of *p*-nitrobenzyl chloride, the plots of the experimental values suggest mixed or partial inhibitions. It is possible that these results indicate the presence of more than one 'glutathiokinase' with different responses to the inhibitor and substrates. This interpretation is supported by the observation that the enzymes from vertebrates and insects show just such a difference in their response

to inhibition by phenoltetrabromophthaleindisulphonate. Evidence has also been presented by Johnson (1963) that the rat enzyme has two components that differ in their substrate specificities.

The primary detoxication product of Gammexane in flies (Bradbury & Standen, 1959) and locusts (J. N. Smith, unpublished work) is probably a glutathione conjugate, and if several 'glutathiokinases' are present in the insects one of these may be more affective in metabolizing the insecticide. The results quoted above give no indication of any competition by the insecticide for the enzyme metabolizing *p*-nitrobenzyl chloride, and preliminary results (A. J. Cohen, J. N. Smith & H. Turbert, unpublished work) suggest that the 'glutathiokinase' in locusts which act on Gammexane is insensitive to inhibition by phenoltetrabromophthaleindisulphonate.

The large amount of enzyme activity present in most of the organisms studied raises the question of a possible normal role for this enzyme which is separate from its detoxication function. Since it catalyses condensations with reactive compounds capable of non-enzymic reaction the postulated normal substrate might be a reactive molecule such as an acyl-CoA derivative (cf. Drummond & Stern, 1961) or a quinone (cf. Bray & Garrett, 1961).

Another less likely possibility is that 'glutathiokinase' activity is a subsidiary function of another enzyme. The unusual and sensitive inhibition by phthaleins and flavianic acid is reminiscent of the γ-glutamyltransferase activity of the glutathionase complex (Binkley, 1961) and of the phosphate-activated glutaminase I (Sayre & Roberts, 1958). Both these enzymes, however, are associated, in vertebrates, with particulate fractions (de Duve, Wattiaux & Baudhuin, 1962), whereas 'glutathiokinase' is found in the soluble part of cell homogenates.

The number of enzymes having a specific requirement for glutathione as coenzyme or substrate is small, and two of those which have this requirement are concerned with detoxication. The DDT dehydrochlorinase of flies needs a high concentration of glutathione for optimum activity, and it has been suggested (Gessner & Smith, 1960; Bradbury, 1960) that a DDT–cysteine or DDT–glutathione conjugate might be an intermediate in the dehydrochlorination reaction. Lipke & Kearns (1960) found no evidence of this, and from the present work it also seems unlikely that 'glutathiokinase' and DDT dehydrochlorinase are the same since the probable molecular weight of the former, about 65000, differs greatly from the 36000 quoted by Lipke & Kearns (1960) for the molecular weight of fly DDT dehydrochlorinase.

That 'glutathiokinase' has a normal role separate from its function in detoxication is also suggested by its distribution in Nature. In addition to its occurrence in ticks (Hitchcock & Smith, 1963), vertebrates and insects, it may also be present in plants. A variety of *S*-alkylcysteines have been found in plants, and *S*-(carboxypropyl)-glutathione and related γ-glutamylcysteine derivatives have been isolated from onion and garlic bulbs (Virtanen, 1962*a*, *b*). A relation to protein metabolism has been suggested for these compounds by Virtanen & Matikkala (1960), and it has also been shown that the thiol-substituting group is probably derived from an amino acyl-CoA derivative (Suzuki, Sugii & Kakimoto, 1962).

SUMMARY

1. 'Glutathiokinase' activity in insects has been assayed by using the reaction between reduced glutathione and *p*-nitrobenzyl chloride, 1-chloro-2,4-dinitrobenzene, 1,2-dichloro-4-nitrobenzene or phenoltetrabromophthaleindisulphonate.

2. In locusts, the highest activity was found in fat body, malpighian tubes and gut, with small amounts in other tissues.

3. 'Glutathiokinase' activity has been found in seven other insect species and in the cattle tick.

4. The insect enzymes were distinguished from the analogous enzymes in rat, rabbit and cattle tick by their marked sensitivity to inhibition by phthaleins.

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REFERENCES

Al-Kassab, S., Boyland, E. & Williams, K. (1963). *Biochem. J.* **87**, 4.

Binkley, F. (1961). *J. biol. Chem.* **236**, 1075.

Booth, J., Boyland, E. & Sims, P. (1961). *Biochem. J.* **79**, 516.

Bradbury, F. R. (1960). *J. Sci. Fd Agric.* **11**, 92.

Bradbury, F. R. & Standen, H. (1959). *Nature, Lond.*, **183**, 983.

Bray, H. G. & Garrett, A. J. (1961). *Biochem. J.* **80**, 6P.

Cohen, A. J. & Smith, J. N. (1964). *Biochem. J.* **90**, 449.

Combes, B. & Stakelum, G. S. (1961). *J. clin. Invest.* **40**, 981.

de Duve, C., Wattiaux, R. T. & Baudhuin, P. (1962). *Advanc. Enzymol.* **24**, 291.

Dixon, M. (1953). *Biochem. J.* **55**, 170.

Drummond, I. & Stern, J. R. (1961). *Arch. Biochem. Biophys.* **95**, 323.

Ellis, D. A. (1961). *Nature, Lond.*, **191**, 1099.

Flodin, P. (1962). *Dextran Gels and their Applications in Gel Filtration.* Uppsala: Pharmacia A.B.

Gessner, T. & Smith, J. N. (1960). *Biochem. J.* **75**, 165.

Hitchcock, M. & Smith, J. N. (1963). *Biochem. J.* **87**, 34P.
Johnson, M. K. (1963). *Biochem. J.* **87**, 9P.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Lipke, H. & Kearns, C. W. (1960). *Advanc. Pest Control Res.* **3**, 253.
McIlvaine, T. C. (1921). *J. biol. Chem.* **49**, 183.
Sayre, F. W. & Roberts, E. (1958). *J. biol. Chem.* **233**, 1128.
Smith, J. N. (1962). *Annu. Rev. Entom.* **7**, 465.
Sorensen, S. P. L. (1909). *Biochem. Z.* **21**, 131.
Suzuki, T., Sugii, M. & Kakimoto, T. (1962). *Chem. Pharm. Bull.* **10**, 328.
Virtanen, A. I. (1962*a*). *Angew. Chem.* (*Int. Ed.*), **1**, 229.
Virtanen, A. I. (1962*b*). *Arch. Biochem. Biophys.* suppl. **1**, 200.
Virtanen, A. I. & Matikkala, E. J. (1960). *Hoppe-Seyl. Z.* **322**, 8.
Whitehead, G. B. (1961). *J. Insect Physiol.* **7**, 177.

Biochem. J. (1964) **90**, 464

The Metabolism of D(—)-β-Hydroxybutyrate in Sheep

BY R. A. LENG AND E. F. ANNISON
Department of Biochemistry and Nutrition, University of New England, Armidale, New South Wales, Australia

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The association of raised blood concentrations of ketone bodies with certain pathological conditions, e.g. diabetes mellitus in man and bovine ketosis, has resulted in the intensive study of occurrence and metabolism in mammals of acetoacetate and β-hydroxybutyrate, as reviewed by Campbell & Best (1956). It is now recognized that many tissues are capable of utilizing ketone bodies as substrates for oxidative metabolism (Krebs, 1961), but much work on β-hydroxybutyrate based on the racemic mixture of the acid is difficult to interpret since there is evidence that the L(+)- and D(−)-forms are utilized by tissues at different rates (Marriott, 1914; Lehninger & Greville, 1953; McCann, 1957). In the present studies the metabolism of D(−)-β-hydroxybutyrate in sheep was investigated both *in vitro* and *in vivo*. D(−)-β-Hydroxy[^{14}C]butyrate was prepared by incubating sodium [^{14}C]butyrate with liver slices, or with sheep-rumen epithelium. The capacity of sheep tissues to oxidize D(−)-β-hydroxy [^{14}C]butyrate was examined by incubating the acid with preparations of rumen epithelium, liver, kidney, brain, muscle, lung and spleen. The metabolic interrelations of β-hydroxybutyrate with glucose and short-chain fatty acids in isolated tissues were also examined. In studies on the intact conscious sheep entry rates of β-hydroxybutyrate were measured by isotope dilution by using continuous-infusion procedures. Comparison of the specific activities of blood β-hydroxybutyrate and blood carbon dioxide during the terminal stages of β-hydroxy[^{14}C]butyrate infusions allowed rough calculation of the contribution of this substrate to total carbon dioxide production.

MATERIALS AND METHODS

Experimental animals. Merino wethers (aged 2–3 years) were housed indoors and fed on lucerne chaff (800 g./day). Animals used for entry rate measurements were trained to stand quietly in stocks.

Preparation of tissues. Liver, heart, ventricle, kidney-cortex, spleen and brain (cerebrum and cerebellum) slices were cut free-hand by the procedures described by Leng & Annison (1963). Brain tissue was removed within 2–3 min. from sheep killed by exsanguination. A mechanical saw was used to open the top of the head and provide access to the brain. A tissue mince of lung was prepared by slicing and chopping the tissue with a razor blade. Individual bundles of skeletal-muscle (sartorius) fibres were teased out with fine dissecting needles. Rumen epithelium was prepared as described by Pennington (1952), and thin (1 mm.) sheets of rumen-wall muscle were stripped from rumen epithelium and cut into 1 cm. squares.

Tissues were incubated in Warburg flasks at 39° in Krebs–Ringer phosphate (gas phase: 100% O_2) or Krebs–Ringer bicarbonate [gas phase: CO_2+O_2 (5:95)] prepared as described by Umbreit, Burris & Stauffer (1957). The flasks were shaken at 100 strokes/min. Results are expressed as μmc (or μmoles or mg.)/hr./g. of tissue.

Preparation of D(−)-β-*hydroxy*[^{14}C]*butyrate.* Liver slices (3 g.) or rumen epithelium (4 g.) were incubated with sodium [1-^{14}C]butyrate or sodium [2-^{14}C]butyrate (200 μmoles; 300 μc) in 24 ml. of Krebs–Ringer bicarbonate for 4 hr. by the incubation procedures described by Leng & Annison (1963). After removal of tissues by centrifuging, the flask contents were deproteinized with an equal volume of 6% $HClO_4$. The combined filtrates from three flasks were adjusted to pH 7·0, concentrated *in vacuo* with a rotary evaporator to 3–4 ml. and mixed with 15 g. of anhydrous $CaSO_4$. The product was mixed with 2 ml. of N-H_2SO_4 and extracted with ether for 6 hr. in a Soxhlet