A Study of the Effect of Particle-Bound γ -Glutamyltranspeptidase on the Product of Interaction of Fluoropyruvate with Glutathione

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Fluoropyruvate has been shown to interact with thiol compounds (Avi-Dor & Mager, 1956*a*; Peters & Hall, 1957). Its inhibitory action on the respiration of mitochondria (Avi-Dor & Mager, 1956*b*; Chari-Bitron & Avi-Dor, 1958) and of tissue cells (Traub & Ginzburg, 1959) is presumably due to combination with thiol groups. We have observed that, during an incubation of the product formed between glutathione and fluoropyruvate with a rat-kidney homogenate, glutamate is liberated. Concomitantly the extinction in the ultraviolet region increases greatly. The change in the spectrum allows the kinetics of the reaction to be followed in the spectrophotometer. It can be demonstrated that the enzyme responsible for the reaction is in the microsomal fraction. The pattern of the reaction of the microsome-bound enzyme suggests that it is identical with γ -glutamyltranspeptidase, which is known to transfer the γ -glutamyl moiety of glutathione to L-amino acids, or, in their absence, to water (for references see Revel & Ball, 1959).

EXPERIMENTAL AND METHODS

Materials. Fluoropyruvic acid was synthesized as described by Blank, Mager & Bergmann (1955). The amino acids and peptides used were the products of the following firms: Glycylglycine, glycylglycylglycine and L-S-ethylcysteine of Mann Research Lab.; DL- α -amino-n-butyric acid, DL-p-fluorophenylalanine, L-ethionine and D-methionine of the California Foundation; the other amino acids of Schwarz Lab. Inc. All other chemicals used were of A. R. grade.

Preparation of rat-kidney microsomes. The cortex of the kidneys from rats (male and female) of 200–230 g. weight was homogenized with 10 (v/w) parts of an ice-cold 0.25 M-sucrose solution and the microsomal fraction separated as described by Schneider (1948). The microsomes were washed twice with water and resuspended in water. The final suspension, which contained approx. 20 mg. of protein/ml., was frozen and kept at -15° . No loss in the enzymic activity was noted during a storage of 6 weeks.

Preparation of the product of the reaction of thiols and fluoropyruvic acid. A mixture containing 50 mM-2-amino-2-hydroxymethylpropane-1:3-diol (tris buffer), pH 8.2, the respective thiol compound (3 mM) and 10 mM-fluoropyruvic acid was incubated at 37° for 20 min. Analysis by methods described previously (Avi-Dor & Mager, 1956*a*) has shown

that the free SH groups of all the thiol compounds used disappeared at the end of incubation, with the liberation of an equivalent amount of hydrofluoric acid. While the structures of the products of interaction are not elucidated they are designated below as glutathione pyruvate, cysteinylglycine pyruvate and cysteine pyruvate respectively.

Determination of enzyme activity. During the action of the enzyme on glutathione pyruvate the largest increase in the extinction, E , occurred at 300 m μ . Thus, by recording the change in the extinction at this wavelength, the time curve of the reaction could be followed. Four Beckman silica cells of 1 cm. light-path were used; all cells (1, 2, 3 and 4) contained 50 mM-tris buffer (pH 8.2), the acceptor amino acid at the concentration required and water to 3.0 ml. Cells 1 and 2 also contained glutathione pyruvate (as a rule 0.3 mM). Before starting the experiment, the microsomes or other cell fractions were added to cell 3 in a volume of 0.02–0.03 ml. and the same volume of water was added to cells 2 and 4. The reaction was initiated by adding the cell fraction to be tested to cell 1. The amount of the particles tested for enzymic activity was chosen in such a way that the sample added to cell 1 could cause an increase in $E_{300\text{ m}\mu}$ of 0.05–0.10 unit/min. when measured against cell 3 as the control. Measurements were carried out in the Beckman DU spectrophotometer at time-intervals of 15 or 30 sec. If not stated otherwise, measurements were carried out at room temp. (26 – 28°).

Calculation. When the reaction continued until no further change in $E_{300\text{ m}\mu}$ was noted, the final level of

Fig. 1. Final extent of hydrolysis of glutathione pyruvate in the hydrolytic reaction (O) and in the transpeptidase reaction (●). Microsomes in amount equivalent to 120 $\mu\text{g.}$ of protein/ml. were present. In the transpeptidase assay, a concentration of 10 mM-glycylglycine was present. Incubation was carried out for 30 min. at 37° . The microsomes were removed after the incubation by centrifuging and the extent of the hydrolysis was determined as described in the Experimental section.

absorbancy increased proportionally with the glutathione concentration in a wide concentration range. The same final state was reached whether the enzymic reaction had been carried out in the presence or in the absence of glycylglycine (Fig. 1). The increment in $E_{300\text{ m}\mu}$ was 5.2 for a mM-glutathione pyruvate solution. Hence, without making any assumptions on the nature of the chromogenic hydrolysed product, the amount of glutathione pyruvate disappearing can be calculated from the expression:

$$(E_{300\text{ m}\mu} \text{ cell 1/cell 3} - E_{300\text{ m}\mu} \text{ cell 2/cell 4})/5.2.$$

It is, however, shown below that at least 90% of the increase in $E_{300\text{ m}\mu}$ is due to formation of cysteinylglycine pyruvate.

Interference. The effects of potassium cyanide, reducing agents (Avi-Dor & Mager, 1956a), borate (Avi-Dor & Lipkin, 1958) and bivalent metals (Avi-Dor, 1959) have been described before. During the present investigation 0.6 mM-chloramphenicol or 0.06 mM-tetracyclin (achromycin) depressed the extinction of cysteinylglycine pyruvate or cysteine pyruvate at the peak by 50%.

Definitions. Hydrolytic activity (V_H) is the number of μ moles of glutathione pyruvate hydrolysed/min./mg. of protein, in the absence of added acceptor amino acid. Transpeptidase activity (V_{TP}) is defined similarly, but in the presence of an acceptor amino acid.

The acceleration (Acc) caused by an acceptor is given by V_{TP}/V_H . The maximum acceleration (Acc_{max}) is that caused by an infinitely high concentration of the acceptor.

Rates were calculated always from the linear part of the activity-time curve.

Protein. This was estimated by the method of Lowry, Rosenbrough, Farr & Randall (1951).

RESULTS

Identification of the products formed during the action of microsomes on glutathione pyruvate

Paper chromatography. With water-saturated phenol containing 0.002% of 8-hydroxyquinoline as the solvent in descending chromatograms run for 8 hr. at 25° untreated glutathione pyruvate remained near the starting line. The spot showed no fluorescence on illumination with an ultraviolet-light source and gave a positive reaction with ninhydrin. When the glutathione pyruvate had been incubated with microsomes until the increase in E reached its maximum, a fluorescent, ninhydrin-negative spot remained near the starting line and a strongly ninhydrin-positive spot appeared, possessing the same R_F (approx. 0.22) as glutamate run simultaneously. After prolonged incubation with the microsomes a weak spot corresponding to glycine could be detected. When L-methionine in an equivalent concentration to glutathione pyruvate was included in the incubation mixture the chromatogram revealed, besides the glutamate spot and the methionine spot (R_F 0.80), an additional spot (R_F 0.50). When this compound was extracted from the paper with water, hydrolysed in 6N-HCl for 14 hr. in a closed

vessel at 110°, evaporated to dryness, redissolved in water and rechromatographed, it yielded two spots identified as methionine and glutamate. Thus, in the presence of the acceptor amino acid, the microsomal enzyme transferred the γ -glutamyl moiety to L-methionine and a dipeptide, presumably γ -glutamylmethionine, was formed.

Paper electrophoresis. On electrophoretic fractionation, glutathione pyruvate (Whatman no. 1 paper, 0.2M-acetate buffer of pH 4.6, 26V/cm., 2.5 hr., 20°) produced a single line (non-fluorescent, ninhydrin-positive, approx. 7.5 cm. from the starting line in the direction of the cathode). Cysteinylglycine pyruvate separated clearly from this and was fluorescent, ninhydrin-negative, approx. 9.5 cm. from the starting line. Cysteine pyruvate produced a yellow, visible line (weakly ninhydrin-positive, yellow fluorescent, approx. distance from starting line 4 cm.) and a second line which ran only slightly faster than cysteinylglycine pyruvate (fluorescent, ninhydrin-negative, approx. distance from starting line 10.0 cm.). The product of incubation of glutathione pyruvate with microsomes showed a fluorescent, ninhydrin-negative line corresponding exactly to that of cysteinylglycine pyruvate and a ninhydrin-positive line running just behind the glutathione pyruvate reference and corresponding to glutamate (identified also after elution, by paper chromatography). When the line given by the product of the enzyme action, corresponding to cysteinylglycine pyruvate, was eluted, hydrolysed and rechromatographed (with water-saturated phenol as solvent above), the liberation of glycine could be demonstrated.

Spectrophotometry. Further support for the assumption that the chromogenic compound formed during the action of microsomes on glutathione pyruvate is cysteinylglycine pyruvate came from an investigation of the spectra of the thiol-pyruvate compounds. Avi-Dor & Mager (1956a) reported that only those β -aminothiols which possess an unsubstituted amino group show high light-absorption in the ultraviolet. An increase in the extinction is, therefore, expected to occur when glutathione pyruvate is hydrolysed either to glutamate and cysteinylglycine pyruvate or to glutamate, glycine and cysteine pyruvate. Cysteine pyruvate and cysteinylglycine pyruvate have very similar spectra (Table 1) and the millimolar extinction of both compounds is 5.2 at 300 m μ . On the addition of 4 mM-zinc sulphate, however, the peak of cysteine pyruvate is strongly depressed while that of cysteinylglycine pyruvate remains unchanged. When the two compounds were mixed in various proportions each of them retained its properties in relation to the effect of the addition of Zn^{2+} ions. Even the presence of 10% cysteine pyruvate in cysteinylglycine pyruvate could be

Table 1. *Peaks of light extinction of thiol pyruvate compounds*

Incubation of glutathione pyruvate with microsomes was carried out as described for Fig. 1. When indicated 4 mM-zinc sulphate was added to the reaction mixture and the light absorption was measured 5 min. after the addition of the metal ions.

Compounds	$\lambda_{\text{max.}}$ (m μ)		$\epsilon_{\text{max.}}$ mM	
	No addition	Zn ²⁺	No addition	Zn ²⁺
Glutathione pyruvate				
Untreated	270	295	0.54	5.38
Incubated with microsomes	297	297	5.30	5.26
Cysteinylglycine pyruvate	297	297	5.35	5.39
Cysteine pyruvate	300	300	5.20	0.70

Table 2. *Distribution of the γ -glutamyltranspeptidase activity of rat-kidney cortex between various fractions of the cytoplasmic extract*

Temp. 28°. For the method of assay and the definition of the units used see Experimental section. The sum of the activity of the separated fractions was higher than that of the original cytoplasmic extract, namely for the hydrolytic reaction 160% and for the transpeptidase reaction 145%.

Fraction	Protein (% of total)	Hydrolytic activity (μ mole/mg. of protein/min.)	Transpeptidase activity (μ mole/mg. of protein/min.)
Cytoplasmic extract	100	0.03	0.45
Mitochondria	18	0.015	0.19
Microsomes	25	0.16	2.15
Supernatant	53	0.01	0.15
Microsomes (1 part) + mitochondria (1 part)	—	0.10	1.42
Microsomes (1 part) + supernatant	—	0.09	1.28

detected by the depression of *E*. The product of incubation of glutathione pyruvate with microsomes showed the same spectrum as cysteinylglycine pyruvate and was almost unaffected by the addition of zinc sulphate.

Distribution of γ -glutamyltranspeptidase activity in kidney homogenates

Transpeptidase and hydrolytic activity of various cell fractions isolated according to the method of Schneider (1948) were assayed by the standard procedure described under 'Methods'. The so-called nuclear fraction, which sedimented by using a centrifugal force of 700 *g* for 10 min., showed an activity varying from preparation to preparation. Since this fraction contains, besides nuclei, some damaged cells, it was suspected that the activity was due to this contamination. When a more homogeneous nuclear fraction was prepared from a sucrose-CaCl₂ homogenate (Hogeboom, Schneider & Striebich, 1952), the activity of the nuclear fraction was negligible. In the following experiments the sucrose homogenate, after the removal of the nuclear fraction (cytoplasmic extract), served as the starting material for the differential centrifuging. The data presented in Table 2 show average values from three experiments. In each experiment the homogenate was prepared from the kidneys of twelve rats. It can be seen that the highest activity is connected with the

microsomal fraction. The small activity of the other fraction may be due to microsomal contamination. When microsomes were diluted with an equal amount of mitochondria or supernatant (based on the protein contents) and the dilution caused by the inert protein was taken into account, no significant change in the activity was observed.

Properties of the enzyme

Time course of the enzymic reaction. The time course of the reaction is shown in Fig. 2. In the absence of an added acceptor, a lag period is apparent. Glycylglycine, which accelerated the hydrolysis nearly 20 times, abolished the lag. Tests on other amino acids showed that their effect on the lag is related to their effectiveness as accelerators (acceptors). L-Methionine and L-glutamine shortened the lag, while glycine and L-glutamic acid had no effect.

Effect of the concentration of glutathione pyruvate. A double reciprocal plot of the glutathione pyruvate concentration against the velocity of the reaction (Fig. 3) shows that the affinity of the substrate to the enzyme is the same in the hydrolytic reaction as in the transpeptidation. The presence of the acceptor alters the maximum velocity but not Michaelis's constant (K_m 0.24 mM). When free glutathione (GSH) was used as the substrate instead of glutathione pyruvate, and the rate of hydrolysis was followed by determining the

amount of cysteinylglycine liberated (Avi-Dor & Mager, 1956a) K_m calculated from a Lineweaver-Burk graph was 0.5 mM.

Effect of the concentration of the enzyme and of pH. In the range 10–100 μ g. of protein/ml. for the hydrolytic reaction and 0.8–8.0 μ g. of protein/ml. for the transpeptidase activity, the velocity of the reaction was proportional to the amount of microsomes added. The optimum pH, in the presence as well as in the absence of acceptor, was about 8.15–8.30. The slope of the pH-activity curves on the acid side of the optimum depends on the pK of the amino group of the acceptor. Half-

maximum activity was found with glycine (pK 9.60) at pH 7.75 and with glycylglycine (pK 8.15) at pH 6.90. These results closely resemble the findings of Kinoshita & Ball (1953) and Ball, Revel & Cooper (1956).

Comparative activity of various acceptors. The comparative reactivity of various amino acids with GSH has been investigated in the past mostly by using an arbitrarily fixed concentration of the acceptor. However, the slope of the concentration-activity curves differs for each substance, and at higher concentrations a decline in the activity has been noted in some cases (Fodor, Miller & Waelsch, 1953; Hird & Springell, 1954; Revel & Ball, 1959). A comparison of the activities at an arbitrarily fixed level is therefore not strictly valid. The simplicity of the spectrophotometric method made it feasible to run concentration-activity curves for each acceptor and (from a double reciprocal plot, as exemplified in Fig. 4) to calculate the concentration causing half-maximum acceleration ($K_{Acc.}$) and maximum acceleration ($Acc_{max.}$) attainable at an infinitely high concentration of the substance. $K_{Acc.}$ and $Acc_{max.}$ values for a number of acceptors are listed in Table 3. D-Alanine, D-glutamic acid and D-methionine neither accelerated the hydrolytic reaction nor inhibited the transpeptidase activity with the respective L-isomer as the acceptor.

Fig. 2. Time course of the hydrolytic reaction (O) and of the transpeptidase activity (●). In the transpeptidase assay, 10 mM-glycylglycine was present and microsomes in an amount equivalent to 1.5 μ g. of protein/ml. were used. To make the rate of the reaction of comparable magnitude microsomes equivalent to 30 μ g. of protein/ml. were added in the hydrolytic reaction. In other respects the conditions of the reaction were as described in the Experimental section.

Fig. 3. Lineweaver-Burk plot for the hydrolytic reaction (O) and for the transpeptidation (●). The concentration of glutathione pyruvate was varied as indicated. In other respects conditions were as in Fig. 2.

DISCUSSION

The pattern of the reaction of the microsome-bound enzyme obtained from rat-kidney homogenates suggests that it is identical with the so-

Fig. 4. Effect of the concentration of the acceptor on the extent of acceleration of the hydrolytic reaction: double reciprocal graph of the acceleration (V_{TP}/V_H) against the concentration of the acceptor (S). O, Glycine; ●, ethylglycine; Δ, glycylglycylglycine; ▲, glycylglycine. Standard conditions of assay. The negative reciprocal value of the point of intersection of the graphs with the abscissa gives $K_{Acc.}$; the reciprocal value of the intersection with the ordinate gives $Acc_{max.}$.

Table 3. *Comparative reactivity of various acceptors*

The condition of the experiment and the method of the calculation of K_{Acc} and Acc_{max} were as described for Fig. 4. When DL-amino acids were used, the figure given is related to the concentration of the L-isomer.

Acceptor	$10^3 K_{Acc}$	Acc_{max}	Acceptor	$10^3 K_{Acc}$	Acc_{max}
Glycine	12.0	10.0	DL- α -Amino- n -butyric acid	4.0	9.0
Glycine ethyl ester	10.0	10.0	DL-Norvaline	5.0	9.0
Glycylglycine	0.7	18.0	DL-Norleucine	5.5	9.0
Glycylglycylglycine	1.4	13.0	L-Leucine	6.5	5.0
L-Alanine	2.4	9.0	DL-Isoleucine	10.0	2.5
L-Serine	5.0	7.0	L-Glutamic acid	4.3	4.5
Azaserine	—	1.0	L-Aspartic acid	—	1.0
L-S-Ethylcysteine	2.5	12.5	L-Glutamine	1.2	10.0
DL-Phenylalanine	5.0	6.0	L-Asparagine	2.2	5.0
DL- p -Fluorophenylalanine	5.0	4.0	L-Methionine	2.0	10.0
			L-Ethionine	2.0	10.0

called γ -glutamyltranspeptidase. Glutathione pyruvate as a substrate for the enzyme has a K_m of about the same magnitude as free glutathione. The products of hydrolysis are the same, except that for the former, instead of the free thiol compound, the thiol pyruvate derivative of high extinction is formed. The possibility of following the reaction spectrophotometrically greatly facilitates its quantitative study. There is no close correlation between the concentration of the acceptor causing half-maximum acceleration and the extent of the activation induced. This is especially striking if one compares the respective values for glycine (K_{Acc} , 12; Acc_{max} , 10) on the one hand and for its next homologue L-alanine (K_{Acc} , 2.4, Acc_{max} , 9) on the other. The effects due to branching of the carbon chain, length of the peptide chain, presence of an additional carboxyl group and stereochemistry were the same as those found by other investigators and will not be discussed.

The initial acceleration of the hydrolytic reaction could be the result of the accumulation of a substance derived from glutathione. This substance could serve, instead of the water, as the primary acceptor for the glutamyl moiety in the subsequent quicker steady phase of the reaction. The abolition of the lag by glycylglycine is in accordance with such a view. However, the addition of possible products of hydrolysis of glutathione, e.g. glutamic acid or glycine, did not affect the duration of the lag. The effect of other cleavage products containing a thiol group (cysteine, cysteinylglycine) could not be tested by the present method. Hence the identity of the acceptor which is assumed to accumulate during the lag is still obscure. The physiological role of γ -glutamyltranspeptidase is still unknown. Hanes, Hird & Isherwood (1950) made the suggestion that it may be involved in some way in the synthesis of proteins. The finding that the enzymic activity is bound to the microsomal fraction is therefore of interest, since this fraction is considered to be the main site of protein synthesis.

SUMMARY

1. It is shown that a microsome-bound enzyme, which can be separated from rat-kidney homogenates, splits the compound formed by the interaction of glutathione and fluoropyruvic acid with a concomitant increase in the ultraviolet light absorption.

2. An analysis of the products and the pattern of the reaction suggest that the microsomal enzyme is identical with γ -glutamyltranspeptidase.

3. By a spectrophotometric method some quantitative aspects of this enzyme activity have been studied.

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