Enzymes and Cancer

PREPARATION AND SOME PROPERTIES OF GUANASE FROM RABBIT LIVER

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1. Guanase has been purified 200-fold in 20% yield from the supernatant fraction of rabbit-liver homogenates, by using ammonium sulphate fractionation, calcium phosphate-gel adsorption and chromatography on DEAE-cellulose and Sephadex G-200. 2. K_m with guanine as substrate at the optimum pH of 7.7 was found to be $1.05 \times 10^{-5} M$. Q_{10} was 1.4 between 23° and 48°. 3. Substrate activity and pH optima of compounds related to guanine have been studied. 8-Azaguanine, 1-methylguanine, thioguanine and 1-methylthioguanine are all substrates.

During our investigations of enzymes that might control metabolic events, particularly in neoplastic tissues, we carried out an extensive study of xanthine oxidase (see Bray, 1963). It appeared logical to extend such investigations to include other enzymes of purine metabolism. Of these, guanase (guanine aminohydrolase, EC 3.5.4.3) was of special interest as guanine is metabolically a precursor of xanthine and is thus indirectly a source of uric acid.

Guanase was discovered by Schmidt (Schmidt, 1932; Schmidt & Engel, 1932) in rabbit liver and is present in tissues of other animals (Greenstein, Carter, Chalkley & Leuthardt, 1946; Block & Johnson, 1955; Hall, Levine & Harris, 1961; Roy, 1966), in certain bacteria (Rakosky & Beck, 1955; Rashba & Tsinskalovska, 1956) and in the adapted yeast *Torula utilis* (Roush, 1954). Partial purification of the enzyme has been reported from rabbit liver (Hitchings, & Falco, 1944), rat liver (Kalekar, 1947) and *Clostridium acidivoracis* (Rakosky, Zimmerman & Beck, 1955), with few indications of the degree of purification and yield. Mansoor, Kalyan- kar & Talwar (1963) have published a procedure for purification of the enzyme from rat brain and, since this work (Currie, 1965) was completed, Roy (1966) has purified the enzyme from ling-cod muscle. Both of these more recent preparations appear to differ in some kinetic properties from those of liver extracts.

A comparison (R. Yasin, unpublished work) of the activities of liver extracts from mouse, rat, calf, pig, ox, chicken, sheep, horse, dog, rabbit and man indicated that rabbit liver was the best available

source. The present paper describes a convenient partial purification of the enzyme from this source and some of its properties.

MATERIALS AND METHODS

Materials. Analytical-reagent grades were generally employed where these were available. Special materials were obtained from the following sources: ribonuclease (bovine, crystalline, was from Armour Pharmaceutical Co. Ltd. (Eastbourne, Sussex); deoxyribonuclease (crystalline) was from L. Light and Co. Ltd. (Colnbrook, Bucks); lactate dehydrogenase [$(NH_4)_2SO_4$ suspension] was from British Drug Houses Ltd. (Poole, Dorset); xanthine oxidase was obtained from cow's milk by the method of Palmer, Bray & Beinert (1964); Amido Black (Naphthalene Black 10B) and nitro-blue tetrazolium were from E. Gurr Ltd. (London, S.W. 14); starch for electrophoresis was from Connaught Medical Laboratories (Toronto, Canada); guanine was from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany); 8-azaguanine was from Sigma Chemical Co. (St Louis, Mo., U.S.A.); 1-methylguanine was from Dr C. Reese (University of Cambridge); 8-bromoguanine (C grade) was from Calbiochem (Los Angeles, Calif., U.S.A.); 2,6-diaminopurine, thioguanine, thioazaguanine disulphide and 4-hydroxy-6-aminopyrazolo-(3,4d)-pyrimidine were kindly supplied by Dr G. Hitchings (Wellcome Research Laboratories, Tuckahoe, N.Y., U.S.A.); DEAE-cellulose was from Calbiochem; calcium phosphate gel was prepared by the method of Tiselius, Hjerten & Levin (1956); Sephadex G-200 was from Pharmacia (Uppsala, Sweden). Saturated $(NH_4)_2SO_4$ solutions were prepared as follows. Glass-distilled water was saturated with $(NH_4)_2SO_4$ at room temperature and the pH was adjusted to 9.0 with aq. NH_3 . The solution was kept overnight to allow for precipitation of any $Fe(OH)_3$. The solution was filtered and then adjusted to the required pH with H_2SO_4 . The $(NH_4)_2SO_4$ solution was placed at 3° for at least 24 hr. before use.

Spectrophotometric and ammonia production assay of guanase activity. Measurements were carried out with a

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Unicam SP. 500 spectrophotometer with a Gilford recording attachment at 37° and with a mixture containing sodium pyrophosphate buffer, pH 8.2 (50 mm), guanine (87 μ M) and enzyme in a final volume of 3.0 ml. The reaction was started by adding the enzyme at a suitable dilution and continuous recording of the rate of E_{250} decrease was used to obtain the initial rate. The 'unit' of enzyme activity as mentioned in this paper is defined as that amount of enzyme which is required to produce an E_{250} change of 0.01 unit/min. under the conditions of assay; the specific activities of the enzyme samples were expressed as units/mg. of protein.

When guanase activity was measured by NH_3 production the assay mixture consisted of: 0.1 M-sodium pyrophosphate (2.8 ml.) adjusted to the pH being used, 1 mM-guanine (0.2 ml.) and enzyme solution of a suitable dilution (0.1 ml.). A blank solution without enzyme was also set up. The mixtures were incubated at 37° for suitable time-intervals, then the reaction was stopped by the addition of 20% (w/v) trichloroacetic acid (1.0 ml.). The NH_3 produced in 0.1 ml. of each of the samples was assayed by the method of Konitzer & Voigt (1963). The quantities of NH_3 were plotted against the time of incubation and the initial rate obtained was used as a measure of enzyme activity.

Assay of lactate-dehydrogenase activity. Lactate-dehydrogenase activity was assayed spectrophotometrically at 340 μ by the method of Wu & Racker (1959).

Preparation of columns. DEAE-cellulose was treated with approx. N-NaOH for at least 30 min. and then washed free of most of the alkali and poured into columns as a slightly alkaline suspension. When the DEAE-cellulose was packed to the desired height it was washed with distilled water until the effluent was neutral. Equilibrating buffer was allowed to flow slowly through the column at about 3°. When the effluent attained the same pH as that of the ingoing buffer the column was ready for the protein solution to be applied. Exponential gradients were set up according to the method of Bock & Ling (1954).

Dry Sephadex G-200 was stirred into ammonium phosphate buffer, pH 7.3 and 1.0 M, and kept at room temperature for 5–6 days. Buffer and suspension were deaerated by evacuation. The column, with an extension tube of at least equal length, filled with buffer at room temperature and the gel suspension was then run into the top of the column at the same rate as buffer was run out. The buffer in the top of the column was slowly stirred (Andrews, 1964). When the gel grains had settled to the required height, the extension tube was removed and the column was washed in the cold with buffer.

Starch-gel electrophoresis. Special starch (12.2 g./100 ml.) was suspended in 8.6 mM- Na_2HPO_4 –0.14 M-citric acid buffer, pH 7.0, and heated with continuous swirling in a Buchner flask over a naked flame, until the starch solution was just boiling. The stoppered flask was then

attached to a pump to reduce the air pressure and the solution was swirled until boiling ceased. The hot liquid starch solution was quickly poured into a Perspex container (18 cm. $\times$ 9 cm. $\times$ 2 cm.). The top of the starch gel was flattened with a Perspex sheet and then left at 3° for at least 1 hr. to set.

The enzyme solution (50 μ l. containing 3–4 mg.) on Whatman no. 42 filter paper was placed in a cut in the gel. A potential of 6 v/cm. was applied to the gel at 3° for 4 hr. The electrode buffer used was 89 mM- Na_2HPO_4 –11 mM-citric acid buffer, pH 7.0.

Transverse sections of the gel were cut and the protein in one section was stained with saturated Amido Black in methanol–water–acetic acid (5:5:1, by vol.) for 1 min. The free Amido Black was then washed away with methanol–acetic acid–water (5:5:1, by vol.) until the background gel was colourless. The other transverse sections were stained for guanase activity as follows: nitro-blue tetrazolium (10 mg.) was dissolved in 0.1 M-tris buffer, pH 8.0 (52 ml.), and added to 1 mM-guanine (8.0 ml.). Xanthine oxidase (2 ml., 0.5% solution) was added and a slice of the starch gel incubated in this solution at 37° for 30–60 min. The position of the guanase activity, via the reduction of the tetrazolium reagent by xanthine oxidase, could be located by the appearance of a mauve–blue area.

Purification. R. Yasin (unpublished work) carried out a survey of guanase concentration in livers of a number of animals. From this it was concluded that the enzyme activity in the livers of dog, mouse, rat, man and rabbit was distinctly higher than that in the livers of calf, pig, ox, chicken, sheep and horse. The highest activity was found in rabbit liver. This was therefore selected as starting material. The final purification procedure is summarized below and in Table 1: all operations were carried out at 0–5°.

Stage 1. Fresh rabbit liver from a commercial slaughterhouse was cut into dices and then homogenized in a Waring Blendor in 2 vol. of 49 mM-ammonium phosphate buffer, pH 5.0 and 1.0 M. The pH of the homogenate was carefully adjusted to 5.0 with approx. N-acetic acid. The homogenate was then left for 2–4 hr. before centrifugation at 41000g for 20 min. The red–brown supernatant was decanted and filtered through muslin to remove any floating lipid or protein.

Stage 2. The supernatant was brought to 40% saturation by dropwise addition of saturated $(\text{NH}_4)_2\text{SO}_4$ solution, pH 6.6, and left for at least 1 hr. before centrifugation at 1000g for 30 min. The supernatant obtained was then brought to 75% saturation with respect to $(\text{NH}_4)_2\text{SO}_4$ and left for 2–3 hr. before centrifugation at 1000g for 30 min. The 40–75%-saturated $(\text{NH}_4)_2\text{SO}_4$ precipitate was dissolved in a minimum volume of 50 mM-ammonium acetate buffer, pH 5.8 and 1.0 M.

Table 1. *Preparation of guanase from rabbit liver*

Fraction	Stage	Total enzyme (units/kg. of liver)	Specific activity	Purification	Yield (%)
pH 5.0 tissue extract	1	52000	0.25	—	100
40–75% satd. $(\text{NH}_4)_2\text{SO}_4$ fraction	2	52000	1.8	7.2 $\times$	100
Calcium phosphate fraction	3	28600	7.4	32 $\times$	55
DEAE-cellulose chromatography	4	13500	31	124 $\times$	26
Sephadex-G-200-gel filtration	5	11400	51	202 $\times$	20

Stage 3. The solution was dialysed against 50mm-ammonium acetate buffer, pH 5.8 and *I*0.05 (two changes each of 20–30 vol.), and then mixed with calcium phosphate gel equilibrated with the same acetate buffer: 20g. of calcium phosphate gel was used for the extract from 200g. of liver. The pH of the suspension was adjusted to 5.0 with *N*-acetic acid. The suspension was stirred slowly for 2 hr. and then centrifuged at 1000*g* for 10 min. The supernatant, which still contained a small percentage of the enzyme activity, was discarded and the gel was washed with a small amount of 80mm-ammonium phosphate buffer, pH 7.5 and *I*0.2 (30ml. of buffer/20g. of gel), and this washing discarded. The gel was then eluted five times with 80mm-ammonium phosphate buffer, pH 7.5 and *I*0.2 (50ml. of buffer/20g. of gel), and the eluate retained.

Stage 4. Saturated (NH₄)₂SO₄ solution, pH 6.6 (3 vol.), was added to bring the eluate to 75% saturation. The mixture was left for 2 hr. before centrifugation at 1000*g* for 45 min. The precipitate was dissolved in the minimum volume of 19mm-ammonium phosphate buffer, pH 7.3 and *I*0.04, to which had been added 1mg. each of ribonuclease and deoxyribonuclease. The solution was then dialysed overnight (against 19mm-ammonium phosphate buffer, pH 7.3) to allow the enzymes time to hydrolyse any nucleic acids present, with two changes, each of 20 vol. (If the enzymic treatment was omitted, the purification achieved in the subsequent chromatography was less complete.) The protein solution was run on to a DEAE-cellulose column (770ml. packed volume for 1g. of protein) that had previously been equilibrated with 19mm-ammonium phosphate buffer, pH 7.3. The column was then washed with 19mm-ammonium phosphate buffer, pH 7.3, until enzyme activity was just beginning to be eluted (0.5 column vol. of buffer). The eluting buffer was changed to 120mm-ammonium phosphate buffer, pH 7.3 and *I*0.25, and 3ml. fractions were collected until no further protein was eluted from the column. The fractions were assayed for both protein concentration and enzyme activity and those fractions of specific activity greater than 25 were pooled.

Stage 5. The pooled fractions were concentrated to approx. 50mg./ml. on a vacuum dialyser (Membranfiltergesellschaft, Göttingen, Germany) and run through a column of Sephadex G-200 previously equilibrated with 19mm-ammonium phosphate buffer, pH 7.3 and *I*0.04 (2.5cm. × 45cm. column for up to 50mg. of protein). The protein was washed through the column with 19mm-

ammonium phosphate buffer, pH 7.3 and *I*0.04. Fractions (2ml.) were collected and assayed for enzyme activity and protein concentration (Fig. 1). Fractions of specific activity greater than 40 were retained and pooled. The width and asymmetry of the activity band suggested that aggregation of the enzyme may have occurred. (If the gel filtration was carried out at pH 5.7 this aggregation was much more marked.)

At this stage the specific activity was approx. 50, corresponding to a 200-fold purification of the initial tissue extract, and the yield was 20%. However, the enzyme was not completely pure, since it showed guanosine-phosphorylase activity and since, after starch-gel electrophoresis, staining indicated that protein and activity were not fully coincident.

RESULTS

The enzyme purified by the procedure described above had the following properties.

Storage. Enzyme solution could be stored for long periods (tested up to 6 months) in 40%-saturated ammonium sulphate at pH 6.6 and -13° . If the solutions were cooled down to -35° then a large proportion of the activity was lost; the same loss occurred after repeated freezing and thawing. The enzyme solutions are also much more stable to dialysis in ammonium than in sodium or potassium phosphate buffers.

Molecular weight. Gel-filtration behaviour has been used to determine molecular weights of proteins (see Andrews, 1964) in that log (molecular weight) was shown to be directly proportional to the volume of solution necessary to elute the compound from a standardized column.

A mixture of the proteins xanthine oxidase, lactate dehydrogenase and cytochrome *c* was placed on to a column of Sephadex G-200 (see the Materials and Methods section), washed through with phosphate buffer, pH 7.3, and the fractions were assayed for enzyme activity and protein concentration. Purified guanase (stage 5) gave a skewed peak of enzyme activity, suggesting multimolecular forms of the enzyme. The main peak indicated a molecular weight of 170 000, and a shoulder corresponded to a molecular weight of about 525 000.

Effect of pH on enzyme activity. Liver guanase has been reported to have a broad pH optimum with a maximum at about pH 9.0 with guanine as substrate (Schmidt, 1932; Roush & Norris, 1950; Takahashi, 1954), but a sharp pH-activity curve reaching its peak at pH 6.3 when 8-azaguanine was used as substrate (Roush & Norris, 1950). The purified guanase described in this paper was tested with three substrates: guanine, 8-azaguanine and 1-methylguanine in the range pH 4.5–11.5 at 37°. The pH-activity curves showed a relatively sharp maximum at pH 5.9 for 8-azaguanine, and slightly broader maxima at pH 7.1 for 1-methylguanine and at pH 7.7 for guanine (Fig. 2).

Fig. 1. Gel filtration of guanase (stage 4) on Sephadex G-200 at pH 7.3. —, Protein concentration; ----, guanase activity. The shaded section indicates the range of fractions pooled.

Fig. 2. pH-activity curves for purified guanase with the following substrates: guanase (—); 1-methylguanase (---); 8-azaguanine (—). Δ , $\circ$ and $\bullet$, Ammonia determination method; $\blacktriangle$, spectrophotometric method.

Table 2. K_m values of rabbit-liver guanase at the optimum pH values for each of the three substrates

Incubations were at 37° in 0.1 M-sodium pyrophosphate-citrate buffer.

Substrate	Optimum pH	$10^5 K_m$ (M)
Guanine	7.7	1.05
8-Azaguanine	5.9	10.2
1-Methylguanine	7.1	270

Substrate concentration. Values for K_m were obtained for each substrate at its pH optimum in 0.1 M-pyrophosphate-citrate buffer by drawing the usual Lineweaver-Burk graphs. Results are given in Table 2.

Temperature effect. Guanase activity in 0.1 M-sodium pyrophosphate buffer, pH 7.7, with 67 μ M-guanine was measured between 23° and 48°. A linear plot was obtained and Q_{10} was calculated to be 1.4.

Deamination of guanine analogues. Solutions (10 mM) of 2,6-diaminopurine, 1-methylthioguanine, thioguanine, thioazaguanine disulphide and 4-hydroxy-6-aminopyrazolo-(3,4d)-pyrimidine were prepared and mixed with the enzyme in 0.1 M-sodium pyrophosphate buffer, pH 6.5. After incubation at 37° for 3 hr. the reaction was stopped by the addition of perchloric acid. The only substrates that showed any measurable ammonia liberation were thioguanine and 1-methylguanine with respectively 0.75% and 0.36% compared with the guanine 100% degradation.

DISCUSSION

There is no doubt that the present preparation of guanase could be further improved. However, the

purity and yield compare with the preparation by Mansoor *et al.* (1963) from rat brain and with that of Roy (1966) from ling-cod muscle. In the present case and also in that of Mansoor *et al.* (1963) five major steps are required to produce an enzyme concentrate of about the same specific activity. The method of Roy (1966) has three major steps. The overall yield obtained in the present method was 20%, whereas that of other investigators was about 5%.

Guanases from different sources do not appear to be identical. The enzymes from both rat and sheep brain exhibit a double peak of optimum activity at pH 6.5 and pH 8.5–9.0 in tris-acetate buffers with guanine as substrate (Mansoor *et al.* 1963), whereas the crude extract of ling-cod muscle has double peaks of activity at pH 5.6 and pH 8.5 (Roy, 1966). On heat treatment, however, this enzyme only exhibits a single optimum at pH 6.0. The optimum activity of the enzyme from rabbit liver with guanine as substrate was found to be at pH 7.7 in pyrophosphate-citrate buffers. As stated above, previous workers have reported a broad pH optimum for the crude rabbit-liver enzyme at approx. pH 9.0. The purified rabbit-liver enzyme appears to manifest a much sharper pH-activity curve than that exhibited by the crude enzyme. Optimum activity with 8-azaguanine as substrate is at pH 5.9 in pyrophosphate-citrate buffers. This value is again somewhat lower than that obtained by other workers with a crude enzyme preparation, i.e. pH 6.5 (Roush & Norris, 1950).

The present K_m value with guanine as substrate also differs from that reported for the rat-brain enzyme, but, since measurements were carried out at different pH values, no definite comparison can be made.

Roy (1966) could only demonstrate the deamination by the fish guanase of guanine and 8-azaguanine, whereas Mansoor *et al.* (1963) found that guanase from rat brain would deaminate thioguanine as well. Purified rabbit-liver guanase will deaminate guanine, 8-azaguanine, 1-methylguanine, thioguanine and 1-methylthioguanine. Hitchings & Falco (1944) demonstrated that the enzyme from rabbit liver did not deaminate adenine, 7-methylguanine or 1,7-dimethylguanine. Thus the nature of the group at position 6 of the purine ring is very important in relation to enzyme activity. Deamination of the 2-amino group occurs with a hydroxyl or thiol group in position 6 (though in the latter case the substrate affinity is lowered), but not with an amino group, i.e. 2,6-diaminopurine is not deaminated. Methylation at position 1 of the ring allows a much diminished rate of deamination to occur, and on methylation at position 7 no deamination occurs at all. The replacement of the $-\text{CH}=\text{}$ group at position 8 of the ring by an $-\text{N}=\text{}$ group

also lowers this affinity. Thus the groups at positions 2, 6, 7 and 8 are all critical for activity.

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