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The Occurrence of Guanidinoacetic Acid and Other Substituted Guanidines in Mammalian Liver

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The synthesis of creatine from guanidinoacetic acid in liver was originally demonstrated by Borsook & Dubnoff (1940*a, b*), and these authors later concluded that guanidinoacetic acid was not a normal constituent of that organ (Borsook & Dubnoff, 1941). This conclusion was confirmed by Bodansky, Duff & McKinney (1941), who reported that no guanidinoacetic acid could be detected in normal animals. However, Melville & Hummel (1951) reported 3.3–11.0 mg. of this acid/100 g. of rabbit liver, compared with a creatine content of 4–26 mg./100 g. These authors estimated guanidinoacetic acid by a modification (Macpherson, 1942) of the Sakaguchi reaction, after destruction of the arginine present with arginase. Eden, Harrison & Linnane (1954*a, b*), using a method similar to that of Melville & Hummel (1951), reported appreciable amounts of guanidinoacetic acid in normal rat liver. The last-mentioned findings were at variance with the early reports as well as with some observations made in this Laboratory. In view of this, and because of the considerable error which would be introduced into the estimation of liver creatine (Ennor & Stocken, 1948) if large amounts of guanidinoacetic acid were present, the problem has been reinvestigated.

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EXPERIMENTAL

All guanidine compounds used were as previously described (Rosenberg, Ennor & Morrison, 1956). All organic solvents used in chromatography were purified by fractional distillation.

Preparation of tissue extracts. Rats, starved for 16 hr., were killed by cervical dislocation and the livers removed, rinsed in water and weighed. Single or pooled livers were dispersed in a Potter-Elvehjem type of homogenizer with 5 vol. of 1.5*N*-HClO₄. The suspension was centrifuged and the supernatant filtered. The residue was re-extracted with 3 vol. of 1.5*N*-HClO₄ and the supernatant, after filtration, combined with the original extract. The final volume of the pooled extracts did not exceed ten times that of the original tissue. A small sample (5–10 ml.) was removed and adjusted to pH 7.2 by the addition of 10*N*-KOH. The mixture was cooled to 0° and filtered to remove KClO₄. This neutralized extract was used for the colorimetric estimation of total guanidine.

The remainder of the extract was then passed through a column containing Zeo-Karb 225 ion-exchange resin, H⁺ form. The volume of the resin bed was about 20 ml./250 ml. of extract, and with a flow rate of 3 ml./min. the effluent was free from guanidines. The column was then washed with water and eluted with aq. 2.5*N*-NH₃ soln. (flow rate, 1 ml./min.). The effluent was collected in 2 ml. fractions and the first guanidine-positive fraction appeared when the pH of the effluent rose above 7. All guanidine-positive fractions were pooled and the excess of ammonia was removed by vigorous boiling under reduced pressure. The solution was then adjusted to pH 6.8 with 5*N*-HCl and freeze-dried.

The dry residue was dissolved in the minimum volume of water (about 1 ml./20 g. of liver), cooled to 0° and a small amount of insoluble material removed by centrifuging. This was washed with 0.2 ml. of cold water, centrifuged and the supernatant combined with the first extract. The residue was discarded and the combined extract evaporated under reduced pressure to near dryness. Water was added so that the solution contained the equivalent of 10 g. of liver/0.2 ml. This concentrated extract was used for chromatography.

Apart from the extracts described above, 'fortified' extracts were also prepared when a known amount of guanidinoacetic acid was added to the liver before extraction. The amount added was 1 mg. of the acid/100 g. of liver, i.e. about one-third of the minimum amount of guanidinoacetic acid claimed to be present in liver (cf. Melville & Hummel, 1951; Eden *et al.* 1954*b*).

Chromatography. The solvent systems used were: phenol-water (4:1, v/v) in an SO₂ atmosphere (Makisumi, 1952); pyridine-3-methylbutan-1-ol-water (8:4:7, by vol.) (Thoai, Olomucki, Robin, Pradel & Roche, 1956); propan-1-ol-2*M*-sodium acetate-acetic acid buffer, pH 4.0 (7:3, v/v) (Caldwell, 1955). All chromatograms were run in the ascending direction, on Whatman no. 3 MM papers (28 cm. x 28 cm.). Unless otherwise stated, 10 μ l. of the concentrated extract (equivalent to 0.5 g. of tissue) or 10 μ l. of a solution of reference compound (1 mg./ml.) was applied to each spot.

Estimation and detection of guanidines. Quantitative estimation of guanidines was carried out as described by Rosenberg *et al.* (1956) with aqueous reagent or the reagent containing 20% of propanol. For detection of compounds on paper the propanol-containing reagent (2 vol.) was mixed with 3*N*-NaOH (1 vol.) and water (1 vol.) immediately before spraying. Pink spots on a white background were produced by all guanidines within 10 min. of spraying and maximum development took place in about 25 min. Specific detection of monosubstituted guanidines was achieved by the use of a modified Sakaguchi reagent as described by Acher & Crocker (1952). With this reagent it was necessary, with chromatograms run in phenol, to dry the papers overnight in a current of warm air, and then to wash in ether to reduce the dark background which otherwise resulted.

RESULTS

Eggleton, Elsdon & Gough (1943) observed that the molecular extinction produced by creatine (and other asymmetrically disubstituted guanidines) in the Barrit reaction was approximately nine times that produced by monosubstituted guanidines.

Rosenberg *et al.* (1956) described a modified reagent, containing α -naphthol, diacetyl and propanol, which increased the molecular extinctions of monosubstituted guanidines five- to nine-fold, without having any appreciable effect on the extinctions of the asymmetrically disubstituted guanidines. It was therefore thought that the ratio of extinctions obtained with any tissue extract in the presence and absence of propanol should provide some indication of the nature of the guanidines present.

Total guanidine was estimated by the two methods on normal rat-liver extracts, on such extracts to which guanidinoacetic acid had been added and on 'fortified' extracts. The extinctions given by the aqueous and propanol-containing reagents were compared and the ratio of the two extinctions was calculated for each type of extract. Similar comparisons were made with model systems, containing either creatine or guanidinoacetic acid, or a mixture of the two compounds. The results are presented in Table 1.

With creatine the ratio of the extinction in the presence of propanol to that in the aqueous reagent was slightly greater than unity. A similar ratio was found in the normal rat-liver extract. The corresponding ratio for guanidinoacetic acid was 5.3, and for a mixture of the two guanidines an intermediate value was obtained. A similar intermediate value was obtained when guanidinoacetic acid was added either to the normal extract or to the liver. There was thus no apparent loss of guanidinoacetic acid during the preparation of the liver extract. Similar results were obtained in six experiments.

Chromatography of liver extracts. Although the method described above established the absence of guanidinoacetic acid in liver in an amount comparable with that of creatine, it was not accurate enough to exclude the presence of small amounts of the compound. For the paper-chromatographic separation of the guanidines the phenol-water-SO₂ solvent gave excellent resolution of creatine, guanidinoacetic acid and arginine with *R_f* 0.75, 0.58 and 0.36 respectively. Extracts, prepared as described above, were chromatographed in this system. No guanidinoacetic acid was detected in

Table 1. *Extinction values of various compounds and mixtures in the Barrit reaction, with and without propan-1-ol*

	Extinction in aqueous reagent (A)	Extinction with 20% propan-1-ol (B)	B/A
Creatine (20 μ g.)	0.250	0.290	1.16
Guanidinoacetic acid (20 μ g.)	0.041	0.217	5.3
Creatine (20 μ g.) + guanidinoacetic acid (20 μ g.)	0.277	0.499	1.8
Normal liver extract (1 ml.)	0.060	0.069	1.15
Normal liver extract (1 ml.) + guanidinoacetic acid (20 μ g.)	0.095	0.275	2.9
Fortified extract (1 ml.) (see text)	0.092	0.251	2.7

the normal extract, although excellent resolution was obtained when a guanidinoacetic acid marker was spotted together with the liver extract. A guanidinoacetic acid spot comparable in strength with that of creatine was also given by the fortified extract, indicating that, if present originally, this compound would not have been lost in the extraction procedure and subsequent treatment. In the chromatogram shown in Fig. 1 (A) the amount of extract applied was equivalent to 1.8 g. of liver. An extremely faint spot in the position of guanidinoacetic acid was then apparent. By visual comparison with the standard reference spot it was concluded that the amount in rat liver did not exceed 2 $\mu\text{g./g.}$

As the claims (Melville & Hummel, 1951; Eden *et al.* 1954*a, b*) for the presence of guanidinoacetic acid in liver were based on a positive Sakaguchi reaction after treatment with arginase, attempts were made to demonstrate chromatographically the presence of such compounds. The result is shown in Fig. 1 (B). Three faintly positive spots were apparent, but none corresponded with guanidinoacetic acid. The slowest spot (c) was identified as arginine and the fast-running one (a) had an R_f similar to that of γ -guanidinobutyric acid or methylguanidine. Spot (b) corresponded in position to β -guanidinopropionic acid. Since detection of the spots with the Sakaguchi reagent in the presence of phenol was not satisfactory, other solvents were employed. The propan-1-ol-sodium acetate buffer solvent afforded good separation of the three weak components from creatine, and the α -naphthol reagent could thus be used successfully for detection. In this solvent the slowest-moving

component of the three again corresponded to arginine, the fastest component behaving as γ -guanidinobutyric acid, although the possibility of methylguanidine was not excluded. The intermediate component was identified by R_f and by reinforcement with a reference compound as β -guanidinopropionic acid (Fig. 2). To check the possibility of the fast component's being methylguanidine, the extract was chromatographed in pyridine-3-methylbutan-1-ol-water, where satisfactory separation of γ -guanidinobutyric acid (R_f 0.31) and methylguanidine (R_f 0.52) was obtained. An extremely faint spot in the position of methylguanidine was detected and was reinforced by the methylguanidine reference standard. A rough estimate, from spot size, suggested that the contribution of methylguanidine to the unknown fast spot [a, Fig. 1 (B)] was less than one-tenth.

DISCUSSION

The results leave no doubt that creatine is the only major substituted guanidine present in rat liver. Of the five additional guanidines which have been detected, three are present in readily detectable amounts: one has been identified as arginine and the other two, tentatively, as β -guanidinopropionic acid and γ -guanidinobutyric acid. The two remaining compounds were detected only with difficulty because of the extremely small amounts present, but from their chromatographic behaviour

Fig. 1. Paper chromatography of liver extracts in phenol-water (4:1, v/v) in SO_2 atmosphere. The spots were developed with the diacetyl- α -naphthol reagent (A) or with the Sakaguchi reaction (B). 1, Arginine; 2, guanidinoacetic acid; 3, creatine; 4, liver extract; 5, 'fortified' liver extract. (Broken outlines denote faint spots.) Spots a, b and c are referred to in the text.

Fig. 2. Paper chromatography of liver extracts in propan-1-ol-2M-sodium acetate-acetic acid buffer, pH 4 (7:3, v/v). The spots were developed with the diacetyl- α -naphthol reagent. 1, Arginine; 2, β -guanidinopropionic acid; 3, γ -guanidinobutyric acid; 4, methylguanidine; 5, liver extract. (Broken outlines denote faint spots.)

there seems little doubt that they are methylguanidine and guanidinoacetic acid. Control experiments in which the acid was added either to the liver before extraction or to the extract after preparation indicate clearly that guanidinoacetic acid, if present in larger amounts than detected, would have been recovered after column treatment. It must be concluded therefore that guanidinoacetic acid is present in rat liver in only minute amounts, as indeed would be anticipated with an intermediate which is completely methylated in the presence of adequate amounts of methyl donor (cf. Cantoni & Vignos, 1954). The result, however, is in contrast with those obtained by Melville & Hummel (1951) and Eden *et al.* (1954*a*). The former authors based their conclusions on the detection of Sakaguchi-positive material in extracts treated with arginase to remove arginine. However, even if the arginine were not so removed, the amount of Sakaguchi-positive material present would not be sufficient to account for the result claimed and it is thus difficult to offer an explanation of their results. It is equally difficult to explain the results of Eden *et al.* (1954*a*), for, whereas the chromatographic solvent system which they used does not separate creatine and guanidinoacetic acid satisfactorily, the latter was detected by spraying with the Sakaguchi reagent, which does not react with creatine.

Although the evidence presented for the occurrence of γ -guanidinobutyric acid and β -guanidinopropionic acid is not conclusive, the presence of the former was to be expected in view of the results of Irreverre, Evans, Hayden & Silber (1957) and Pisano, Mitoma & Underfield (1957). The amounts present have been calculated to be about 3 $\mu\text{g./g.}$, which is of the same order of magnitude claimed by Irreverre *et al.* (1957) for γ -guanidinobutyric acid in dog liver. In view of the small amounts of these guanidines (other than creatine and arginine) present in rat liver, it seems unlikely that a more positive and satisfactory identification will be achieved. Some experiments were carried out with large amounts of ox liver, but no guanidines other than creatine and arginine could be detected.

SUMMARY

1. The claim that guanidinoacetic acid is present in the liver in amounts comparable with those of

creatine could not be confirmed. Chromatographic evidence indicated that the concentration of this compound in rat liver was below 2 $\mu\text{g./g.}$

2. The major substituted guanidine found in rat liver is creatine. Small amounts of arginine were also found. Two other compounds, tentatively identified as β -guanidinopropionic acid and γ -guanidinobutyric acid, were present in amounts not exceeding 3 $\mu\text{g./g.}$ of tissue.

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