

METABOLISM OF γ -GLUTAMYL DOPAMIDE AND ITS CARBOXYLIC ACID ESTERS*

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Abstract—The release of dopamine (DA) from L- γ -glutamyl dopamide (GDA) during incubation with renal homogenates from the dog was catalyzed by γ -glutamyltranspeptidase. This was shown by the increased release in the presence of glycylglycine, by the formation and isolation of glutamylglycylglycine, and by the complete inhibition of release by a combination of serine and tetraborate. Cleavage was localized predominantly in the renal cortex. Carboxylic acid esters of GDA were extremely effective prodrugs for DA, as measured by the increased renal DA level following their intragastric or intraperitoneal administration to rats. There was a close correlation between the *in vitro* susceptibility of the esters to hydrolysis by hepatic esterase and the renal level of DA following their intragastric administration; the *n*-amyl, *n*-hexyl and benzyl esters were very active in both tests. Hydrolysis of the ester bond appeared to be the most decisive step among those that occur during the transport of DA in the form of a GDA ester in the intestine to unbound DA in the kidney. When esters were administered intraperitoneally, renal DA levels were approximately 100 times higher than after intragastric administration, and the largest increases were produced by the *n*-heptyl and *n*-octyl esters; little correlation existed between the rate of ester hydrolysis and renal DA level. Because of the intensive renal activity of γ -glutamyltranspeptidase, it is likely that most of the DA appearing in kidney from GDA and its carboxylic acid esters is released within the kidney itself and does not originate in other body organs.

A specific receptor for dopamine (DA) that causes both a dilatation of the renal vasculature and an increased renal blood flow was demonstrated in the kidney by Goldberg *et al.* [1]. Because circulation through the kidney is frequently decreased in cardiovascular disorders such as congestive failure and hypertension, a derivative of DA that remains intact during intestinal absorption and subsequently releases unbound DA to the kidney could be of value in the therapy of such conditions. It is for this purpose that many amides of DA with naturally occurring amino acids have been synthesized in these laboratories and their metabolism and pharmacology investigated.

Mammalian kidney is characterized by two enzymes having high activities relative to other body organs, aminoacylarylamidase (EC 3. 4. 1. 2) and γ -glutamyl transpeptidase (GTase). It has now been demonstrated that each of these enzymes can release DA from amides with specific amino acids. Details of the hydrolysis by arylamidase of the amides of DA with neutral α -amino acids were reported previously [2]. The present investigation is concerned with the metabolism of L- γ -glutamyl-DA (GDA), a dopamide that is shown here to be cleaved by GTase. It was found, following their intragastric administration, that this particular amide and its carboxylic esters transport DA into the animal body more effectively than the dopamides of the neutral α -amino acids.

MATERIALS AND METHODS

To establish the formation of γ -glutamyl peptides during enzymatic cleavage of GDA, a partially purified preparation of GTase was prepared from canine renal cortex according to the method of Orłowski and Meister [3]. Purification was carried through Step 2 of their procedure, and the resulting material was stored as a frozen suspension in 0.9 per cent saline. The ratios of arylamidase activity [2] to GTase activity [3] (respective substrates were L-alanyl-DA and L- γ -glutamyl-*p*-nitranilide) for the initial and final preparations were 18.2 and 0.08, respectively, showing the relative loss of arylamidase activity during the purification procedure. This suspension of GTase contained 2.4 units of activity/mg of protein when measured as described [3].

The final concentrations in the 2.0ml incubation mixture were: GDA, 12mM; amino acid or peptide acceptor, 120mM; Tris, 100mM; magnesium chloride, 10 mM; above suspension of GTase, 2.4 units. After 3 hr of incubation at pH 9.0 and 37°, the mixture was centrifuged at 10,000g for 5 min at 0° to sediment the particulate material. The resulting supernatant fraction was passed over a 0.65 cm diameter \times 25 cm length column of cation exchange resin Dowex 1 \times 8 (200-400 mesh, acetate form), and after washing the neutral amino acids and peptides through the column with 40 ml of water, glutamic acid and glutamyl peptides were sequentially eluted with an increasing gradient of acetic acid (100 ml of water in a mixer and 1.0 N acetic acid in the reservoir). Eluates were collected as 3.0 ml fractions and analyzed by the ninhydrin reaction adapted to the AutoAnalyzer. Peptides were hydrolyzed for

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15 hr at 110° in constant boiling hydrochloric acid, and the resulting amino acids were separated by sequential elution from a standardized cation exchange resin column and also were measured by the ninhydrin reaction.

The effects of glycylglycine and tetraborate-serine upon DA release from GDA were demonstrated by the addition of 0.5 ml of a homogenate of canine renal cortex to 10.0 ml of a solution containing reactants and buffer in a water bath at 37°. Each mixture was gassed with nitrogen before and during incubation to prevent the oxidation of DA by monoamine oxidase. Final concentrations were: GDA, 2 mM; Tris, 100 mM; and magnesium chloride, 10 mM; pH 9.0; other conditions were as indicated. Two-ml aliquots were removed at 5, 40, 80 and 120 min and passed directly over columns (0.8 cm diameter \times 3.0 cm length) of BR-70 resin (100–200 mesh, Bio-Rad Laboratories, Richmond, CA) previously equilibrated to pH 6.0. Each column was washed with 2 \times 10 ml of water, and DA was eluted with 2 \times 5 ml of 2% boric acid solution. Aliquots (0.5 ml) of each 10.0 ml eluate were used for the measurement of DA by the I₂-trihydroxyindole method described by Welch and Welch [4]. Details of the isolation method for DA have been described previously [5].

In order to demonstrate the relative release of DA from GDA by homogenates and cellular fractions of canine renal cortex and medulla, 0.25 ml of tissue preparation was added to 5.0 ml of a solution containing the reactants and buffer in a water bath at 37°. The final mixture contained 2 mM GDA and 10 mM magnesium chloride in 100 mM Tris buffer (pH 9.0) and, when included, 20 mM glycylglycine. A nitrogen atmosphere was used to prevent oxidation of the released DA. Aliquots of 2.0 ml were removed at 1 and 61 min and were chromatographed and analyzed for DA according to the preceding methods.

The ability of rat plasma and tissue homogenates to release DA from GDA was determined, in the absence of added glycylglycine, by the incubation of 1.0 ml of tissue sample with 20 ml of a solution containing 2.0 mM GDA in Krebs–Ringer phosphate buffer (pH 6.9). Pargyline (1.0 mM) was included to inhibit monoamine oxidase. Aliquots of 10 ml were removed at 1 and 61 min, treated with 0.72 ml of 60% perchloric acid in ice, centrifuged, and the supernatant fractions were adjusted to pH 6.0 with cold potassium hydroxide. The entire extracts were passed over columns of BR-70 as described above, and DA was measured in the boric acid eluates.

To measure the hydrolysis of GDA esters to GDA itself, 0.5 ml of a rat liver homogenate was added to 10.0 ml of a solution of an ester in sodium phosphate buffer (pH 6.9). The final concentrations were: ester, 2.0 mM; and phosphate, 50 mM. Two-ml aliquots were removed at 2, 20, 40 and 60 min and were passed directly over 0.8 \times 3.0 cm columns of BR-70 resin. Dopamine and the esters of GDA were retained on the column while GDA was in the

effluent; traces of GDA were washed through with 2 \times 2 ml of water. Aliquots of each 6.0 ml effluent were analyzed for GDA by the ninhydrin assay adapted to the AutoAnalyzer. Initial hydrolysis rates were obtained graphically.

Homogenates of canine renal cortex and medulla and of rat organs were prepared with a motor-driven glass–Teflon homogenizer in a proportion of 1.0 g tissue/4 ml of 0.9% saline, and they were centrifuged at 33 g for 5 min to remove gross particulate matter. Where indicated in Table 1, a canine cortical homogenate in saline was also centrifuged at 100,000 g for 1 hr to separate the particulate and supernatant fractions; the sediment of particulate matter was resuspended in fresh saline equal to the original volume prior to incubation.

For the measurement of DA in rat kidney, non-fasted animals were decapitated and pairs of kidneys were homogenized in 7.0 ml of 0.4 M perchloric acid. The centrifuged extracts were neutralized to pH 6.0 with KOH, again centrifuged, and then passed over 0.65 \times 3.0 cm length columns of BR-70 (100–200 mesh). DA was eluted with 7.0 ml of 2% boric acid solution as described previously [5] and was measured with the I₂-trihydroxyindole method. GDA and its esters do not fluoresce by this method.

All of the dopamides were synthesized at Abbott Laboratories, North Chicago, IL.*. They were administered to animals as solutions of their hydrochloride salts in 0.9% saline. Male Long–Evans rats (150–200 g, Simonsen Laboratories) and male beagle dogs served as test animals and sources of tissue. Crystalline porcine renal aminoacylarylamidase was purchased from the Boehringer–Mannheim Corp., New York, N.Y.

RESULTS

Cleavage of GDA by renal GTase. The involvement of GTase in the cleavage of GDA was established by the following experiments.

During the incubation of GDA with a homogenate of canine renal cortex, DA release was stimulated 250 per cent by the inclusion of 20 mM glycylglycine (Fig. 1A), a known acceptor for the γ -glutamyl moiety in reactions mediated by GTase [6].

DA release was similarly stimulated by glycylglycine during an incubation of GDA with a purified preparation of canine renal cortical GTase under conditions identical to the above (data not included). Furthermore, glutamyl glycylglycine was isolated from a 3-hr incubation (see Materials and Methods) of GDA and glycylglycine with this purified GTase; a preparation such as this was needed to produce a sufficient quantity of peptide for characterization. After isolation of the tripeptide by ion exchange chromatography and its acid hydrolysis, the ratio of constituent amino acids was found to be the nearly theoretical value of 1.98 moles glycine/mole of glutamic acid. The assumption was made that this newly synthesized tripeptide contained a γ -glutamyl linkage and not an α -linkage. A significant quantity of glutamic acid was released simultaneously with the formation of glutamyl glycylglycine during the incubation, and the molar ratio of tripeptide

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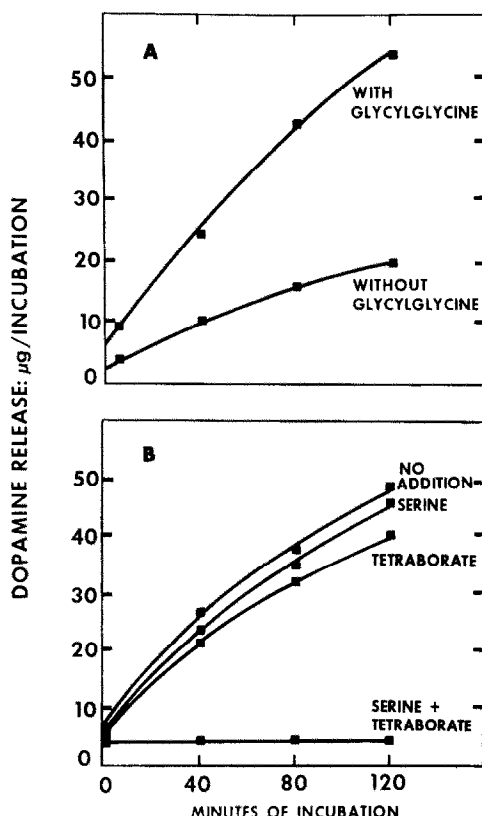


Fig. 1. Effects of glycylglycine (20 mM), sodium tetraborate (50 mM) and DL-serine (50 mM) upon DA release from GDA (2 mM) by a homogenate of canine renal cortex in a Tris (100mM)–magnesium chloride (10 mM) buffer (pH 9.0). In panel B, all incubations contained glycylglycine (20 ml).

formed/glutamic acid released was 2/1. Glutamyl peptides were also isolated and characterized from incubations in which glycine and leucine were acceptors.

Further evidence that GDA is cleaved by the GTase in renal homogenates was provided by an

experiment in which sodium tetraborate and DL-serine (each at 50mM) were simultaneously included in an incubation; a complete inhibition of DA release resulted (Fig 1B). In contrast, when they were added individually, there was little inhibition. A combination of these two substances was shown previously to exert an inhibition of GTase that is competitive to the γ -glutamyl substrate [7].

Subsequent experiments with homogenates of dissected areas of canine kidney showed that the cleavage of GDA occurred mainly within the cortex; renal medulla exhibited only 1 per cent of the cortical activity when compared on the basis of DA released per g of tissue (Table 1). Moreover, the releasing activity was entirely in the material sedimented by high-speed centrifugation of cortical homogenates (Table 1). This distribution of releasing activity for DA is consistent with the observed localization of renal GTase in the proximal tubules and the loop of Henle [8].

Null hydrolysis of GDA by crystalline arylamidase. Amides of DA with the carboxyl group of α -amino acids were shown previously to be hydrolyzed at varying rates by crystalline porcine arylamidase [2]. L-Alanyl-DA was the dopamide most rapidly hydrolyzed in this series of amides, and it served as the reference substrate for expression of hydrolysis rates at pH 6.9 under standardized incubation conditions. When GDA was incubated under these same conditions with the crystalline enzyme from hog kidney, the release of less than 0.1 μ g DA could be detected during 60 min. Because 85 μ g DA were released from L-alanyl-DA during this time period by the same solution of enzyme, GDA was considered to be non-hydrolyzed by arylamidase.

Relative rates of DA release from GDA and alanyl-DA by a homogenate of canine renal cortex. To illustrate the relative release of DA from these two types of dopamide, each amide was incubated without added glycylglycine under the previously described conditions with a cortical homogenate from dog kidney. The arylamidase activity in this particular homogenate hydrolyzed L-alanyl-DA at a rate of 0.69 μ mole/min, while the GTase activity in this same homogenate cleaved GDA at only 0.00012 μ mole/min.

Table 1. Release of DA from GDA by a renal cortical homogenate from dog*

	DA released (μ g DA/60 min/g tissue)	
	No addition	Plus glycylglycine
Renal homogenate		
Cortex		488
Medulla		6
Renal cortical homogenate		
particulate fraction	104	450
supernatant fraction	17	7

* Tissue preparations were incubated at 37° under a nitrogen atmosphere with 2 mM GDA and 10 mM magnesium chloride in 100 mM Tris buffer at pH 9.0 and in the presence and absence of 20 mM glycylglycine. The particulate and supernatant fractions were prepared by centrifugation at 100,000 g for 1 hr. Data are from single experiments.

In vitro cleavage of GDA by body organs of the rat. The relative cleavage of GDA by homogenates of various body organs and plasma of the rat was measured by the total DA released during incubation in Krebs-Ringer phosphate buffer for 1 hr in the absence of added glycylglycine acceptor. Without this addition, the rate of cleavage depends upon the activity of endogenous glutamyl acceptors in addition to that of GTase. Pargyline (1.0mM) was present during each incubation to protect the newly released DA from degradation by monoamine oxidase.

The data in Table 2 show that the kidney was by far the most active of the seven tissues tested, although each of the other organs exhibited some activity. Kidney was also the most active organ when slices were incubated with GDA in Krebs-Ringer phosphate under 100% oxygen (data not presented).

Hydrolysis of esters of GDA by rat liver homogenate. The initial rates of hydrolysis of GDA esters by rat liver homogenate were determined by their incubation as described in Materials and Methods. These esters ranged in chain length from the methyl to the hexadecyl and included the isopropyl, isobutyl, and benzyl esters. Liver was chosen as the source of tissue in preference to kidney or duodenum mainly because its homogenate produced a much lower blank in the ninhydrin assay; the specificities of hepatic, duodenal, and renal esterases are probably similar [9].

As may be seen in Fig. 2, a plot of the enzymatic hydrolysis rate of each aliphatic ester versus the number of carbon atoms in the alcohol moiety of the ester revealed a parabolic relationship with a maximum rate occurring with C-4, C-5, C-6. The isopropyl and isobutyl esters were hydrolyzed more slowly than the corresponding straight-chain esters, while the benzyl ester was hydrolyzed at a faster rate than any of the aliphatic esters (6.3 times that of the ethyl ester).

Renal levels of DA after administration of GDA and its esters to rats. Rats received GDA and its esters by either the intragastric or the intraperitoneal route, and each substance was given as a solution in 0.9% saline at a dose of 0.3 mmoles/kg body weight corresponding to 96 mg/kg of GDA itself.

Table 2. Release of DA from GDA by plasma and homogenates of rat organs*

Tissue	DA released ($\mu\text{g DA}/60 \text{ min/g organ or /ml plasma}$)	
Kidney	1860 \pm 180.0	(4)
Brain	61 \pm 1.8	(2)
Heart	37 \pm 6.4	(2)
Liver	122 \pm 31.0	(2)
Small intestine	71 \pm 21.3	(2)
Stomach	91 \pm 5.1	(2)
Plasma	0.9 \pm 0.2	(2)

* Tissues were incubated at 37° with 2.0 mM GDA and 1.0 mM pargyline in Krebs-Ringer phosphate buffer at pH 6.9 and in the absence of added glycylglycine. Values are means \pm S.E.M. with the number of determinations in parentheses.

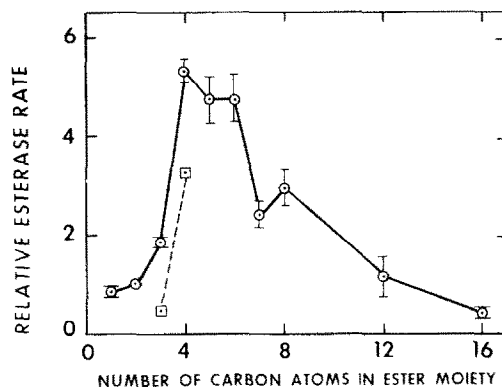


Fig. 2. Variation of initial hydrolysis rates of GDA esters with chain length of ester moiety (GDA ester, 2 mM; phosphate buffer, 50mM; pH 6.9; homogenate of rat liver). The hydrolytic rate of GDA ethyl ester was assigned a value of unity and other rates were expressed as ratios to it. The broken line connects values for isopropyl and isobutyl esters; other esters are with *n*-alcohols. Replicate values are means \pm S.E.M. of two to three determinations.

Renal levels of DA were determined at 0.5 hr following intraperitoneal administration and at 1.0 hr following intragastric administration, because maximum levels of DA occurred at these times (data not included) following the administration of GDA (intraperitoneal) and its ethyl ester (intragastric).

Esters of intermediate chain length produced the highest levels of renal DA when given by either route. After their intragastric administration, the peak level of DA occurred with esters having chain lengths of 5 and 6 (Fig. 3A), while after intraperitoneal administration the peak level occurred with chain lengths of 7 and 8 (Fig. 3B). This latter level was two orders of magnitude greater than that produced by intragastric administration. Furthermore, a comparison of panels A and B of Fig. 3 shows that there is considerable loss of these esters during gastrointestinal absorption because they then produced only 1 per cent of the DA level found after their intraperitoneal administration. Levels of DA from GDA itself were relatively low and were plotted in Fig. 3, panels A and B, corresponding to "0" number of carbon atoms. The elevated DA levels found after the intraperitoneal administration of GDA octyl ester, as measured by the trihydroxyindole fluorescence assay, were confirmed by a previously published chromatographic-native fluorescence method [5].

A scatter plot of the renal levels of DA after intragastric administration of the various esters versus their rates of hydrolysis by hepatic esterase revealed a distinct correlation ($r = 0.825$; $P < 0.001$); higher levels were produced by esters having faster hydrolysis rates (Fig. 4A). However, a comparable plot of renal levels of DA resulting from intraperitoneal administration of the esters versus their rates of hydrolysis by hepatic esterase (Fig. 4B) clearly shows that, in this situation, factors other than the relative ease of ester hydrolysis determine the level of renal DA.

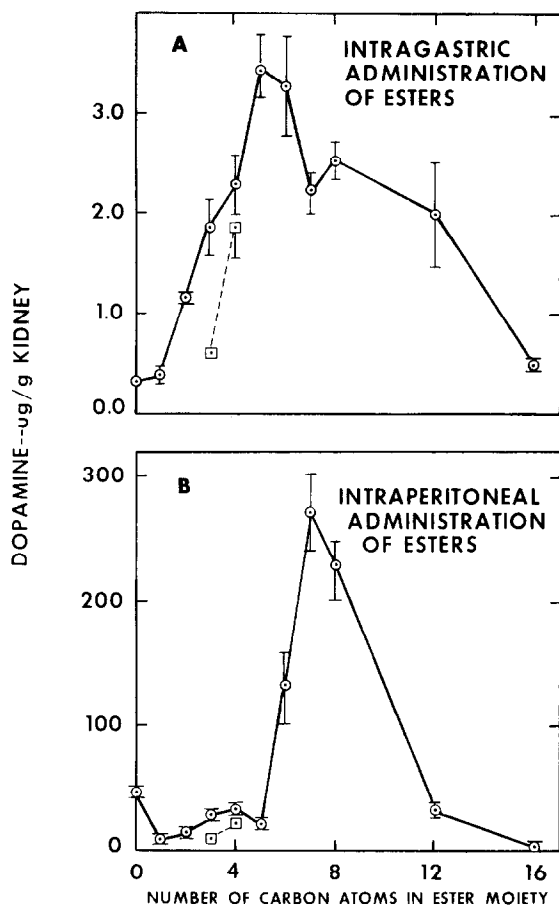


Fig. 3. Variation of the level of renal DA with chain length of GDA ester moiety after administration of esters (0.3 mmoles/kg) to rats. Panel A: intra gastric administration; kidneys removed 1.0 hr later. Panel B: intra peritoneal administration; kidneys removed 0.5 hr later. The value for GDA is at "0" chain length. The broken line connects values for isopropyl and isobutyl esters; other esters are with *n*-alcohols. Values are expressed as means \pm S.E.M. for groups of four animals.

In order to demonstrate the relative effectiveness of these esters as DA precursors, renal levels of DA following the intra gastric and intra peritoneal administration of a variety of precursors to rats were

determined (Table 3). The greater activity of GDA octyl ester as a source of renal DA in comparison with that of L-alanyl-DA and with DA itself is clearly seen. L-DOPA and GDA octyl ester were similar in potency upon intra gastric administration, but the ester was considerably more active in raising the renal level following intra peritoneal administration.

DISCUSSION

It is clear from the data presented that DA can be released from GDA by the action of GTase and that GTase is apparently the only enzyme in kidney which cleaves the amide. This unexpected action of GTase was demonstrated by the stimulation of cleavage by glycylglycine, by the isolation of glutamyl glycylglycine from the incubation mixture, and by the complete inhibition of both the non-stimulated and the glycylglycine-stimulated cleavages with a mixture of tetraborate and serine. The absence of cleavage activity from the supernatant fraction of a renal homogenate indicates that GDA is not cleaved by γ -glutamyl cyclotransferase, a soluble enzyme that cleaves γ -glutamyl peptides to pyrrolidone carboxylic acid and amino acids [10]. In addition, crystalline arylamidase did not attack GDA even though it rapidly hydrolyzed many amides of DA with neutral α -amino acids [2].

The observation that the kidney is able to entrap DA, whether it is derived from perfusing blood (as after administration of DA itself) or after release within the kidney (as after GDA and its esters), offered a convenient method for quantifying DA release from GDA esters and a variety of other precursors. The times required to produce maximum DA levels were undoubtedly different for these agents. However, as noted previously, they were assumed to be sufficiently near 0.5 and 1.0 hr, when maximum levels occurred after treatment with GDA (intra peritoneal) and GDA ethyl ester (intra gastric), to permit valid comparisons of efficacy.

All of the esters of GDA were more effective than GDA itself in their ability to increase renal DA after intra gastric administration. In addition, their relative effectiveness was almost a linear function of their rate of hydrolysis by esterase as shown by the continuous-line relationship between these rates and renal DA, i.e. hydrolysis of the ester bond is the

Table 3. Ability of substances to increase the level of renal DA in the rat*

Substance	Renal DA (μ g/g kidney)	
	Intra gastric administration	Intra peritoneal administration
Saline (24)		0.027 \pm 0.008
DA (4)	0.035 \pm 0.003	1.61 \pm 0.31
L-DOPA (4)	2.86 \pm 0.89	16.7 \pm 2.1
L-Alanyldopamide (4)	0.24 \pm 0.03	6.02 \pm 0.25
L-Isoleucyldopamide (4)	0.38 \pm 0.05	2.12 \pm 0.27
GDA (4)	0.31 \pm 0.06	41.2 \pm 2.89
GDA- <i>n</i> -octyl ester (12)	2.55 \pm 0.19	225.8 \pm 23.0

* Animals received the test substances (0.3 mmoles/kg) as solutions of hydrochloride salts in 0.9 per cent saline; kidneys were removed at 1.0 and 0.5 hr, respectively, after intra gastric and intra peritoneal administrations. Values are expressed as means \pm S.E.M.; the number of animals is given in parentheses. Values for intra gastric and intra peritoneal administrations of saline were treated as one group.

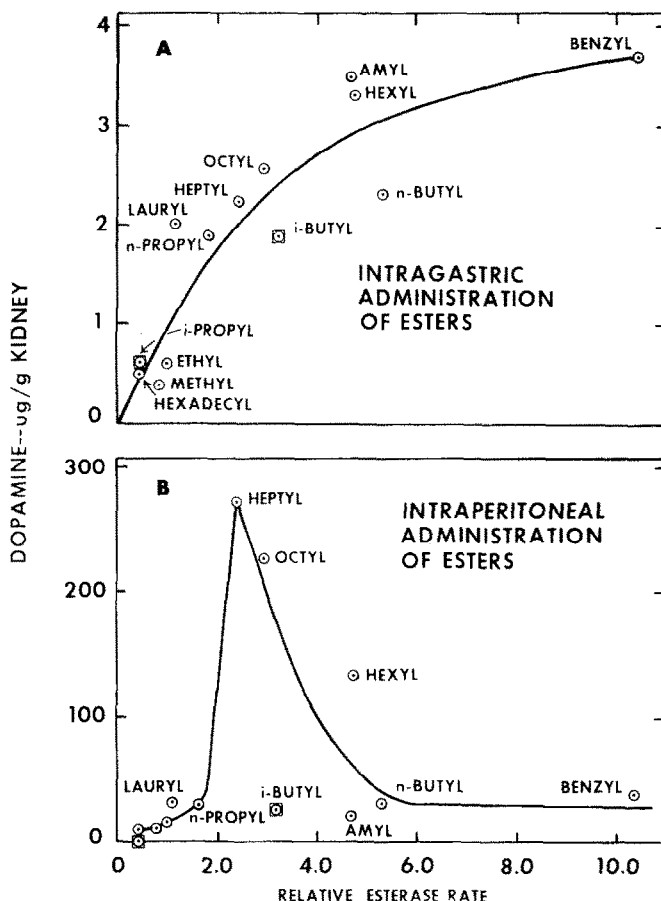


Fig. 4. Variation of level of renal DA after administration of GDA esters to rats with the relative rate of hydrolysis by rat liver esterase. Panel A: intragastric administration. Panel B: intraperitoneal administration. Values are from Figs. 2 and 3.

most important step among those that occur between gastrointestinal administration and the appearance of DA within the kidney. Moreover, there was a pronounced lack of correlation between renal DA levels and the lipophilicity of the esters because those producing the highest levels were in the middle of the range from low to high lipophilicity, and the longer chain esters produced lower levels of DA.

Similar comparisons following intraperitoneal administration of the esters show that a factor(s) other than the rate of esterolytic hydrolysis determines the renal level of DA, and that again there is a lack of correlation between DA levels and lipophilicity.

These esters obviously provide an effective vehicle for transporting DA from the gastrointestinal tract into the animal body and the kidney. Even so, there was a considerable loss of both parent amide and ester during the transport process because the levels of renal DA following the intraperitoneal administration of GDA and its octyl ester were, respectively, 133 and 88 times greater than those following their intragastric administration. Furthermore, it appears likely that most of the DA appearing in the kidney from GDA and its esters is released within the kidney

itself and does not originate in other organs. This is indicated by the findings that GDA was considerably more effective than DA and L-DOPA in its elevation of renal DA following intraperitoneal administration, and that it stimulated the canine heart to a lesser extent than did an equimolar quantity of DA when administered intravenously [11]. Moreover, when given orally to the dog, a significant level of GDA, together with an insignificant level of DA, was found in plasma, whereas a large amount of DA was recovered from urine [11]. Such findings are consistent with a kidney-specific release of DA from γ -glutamyl amides as a consequence of the high renal activity of GTase.

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