ENZYMIC STUDIES ON MORPHINE GLUCURONIDE SYNTHESIS IN ACUTELY AND CHRONICALLY MORPHINIZED RATS

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During the past several years, metabolic changes and urinary excretion of morphine have been studied both in nontolerant and tolerant laboratory animals by many investigators. The major metabolite of morphine has been established as a "bound" morphine which has since been characterized as the morphine glucuronide (Woods, 1954a; Seibert *et al.*, 1954; Fujimoto and Way, 1954). *In vivo* studies were based on the measurement of the amount of free and conjugated morphine appearing in the urine, feces, or bile (Pierce and Plant, 1932; Gross and Thompson, 1940; Thompson and Gross, 1941; Fitchenberg, 1951; Zauder, 1952; Cochin *et al.*, 1954; Woods, 1954b). *In vitro* studies on morphine conjugation were investigated on liver slices of normal and tolerant animals (Zauder, 1952; Way *et al.*, 1954).

Although glucuronide synthesis was postulated over three decades ago, the mechanism of this biotransformation has been elucidated only in recent years. The overall scheme of glucuronide formation is now believed to be that shown in figure 1.

The following enzymes catalyze the sequential reactions: reaction (1) by uridine diphosphoglucose pyrophosphorylase (UDP-glucose pyrophosphorylase) (Munch-Petersen *et al.*, 1953), reaction (2) by uridine diphosphoglucose dehydrogenase (UDP-glucose dehydrogenase) (Strominger *et al.*, 1954), reaction (3) by transferase (Dutton and Story, 1954; Story and Dutton, 1955; Strominger *et al.*, 1954), and reaction (4) by nucleoside diphosphate kinase (NDP-kinase) (Berg and Joklik, 1954).

This paper is concerned with the activities of individual hepatic enzymes responsible for glucuronide formation in chronically morphinized rats. Study of the enzymic changes occurring in the liver indicates an increase in UDP-glucose dehydrogenase activity and a decrease in transferase activity in chronically morphinized rats as compared to those in control (saline injected) rats.

MATERIALS AND METHODS. Male Holtzman rats, 200 to 300 g, were chronically morphinized with an initial dose of 30 mg/kg intraperitoneally which was increased in 30 mg/kg increments weekly for five weeks until the rats were receiving 150 mg/kg during the fifth week (Mannering and Takemori, 1959). Control animals received isotonic saline injections throughout the course of the experiment. The rats were sacrificed and bled by decapitation 24 hours after the last dose. The livers were removed and 10% homogenates were prepared in ice-cold isotonic KCl using a teflon pestle–pyrex tube tissue grinder. Another group of rats was treated acutely with morphine at a dose of 150 mg/kg and 24 hours later the hepatic tissues were prepared in the same manner as above. The control animals were again injected with the equivalent volume of isotonic saline. The procedure for centrifugation of the homogenates is described below (*Enzymic preparations*). The activities of all four enzymes concerned with glucuronide synthesis were determined in duplicates in each animal. The data were statistically analyzed by the Student *t* test.

Enzymic preparations. The source for UDP-glucose pyrophosphorylase, UDP-glucose dehydrogenase, and NDP-kinase was prepared by isolating the soluble fraction of 10% hepatic homogenates in isotonic KCl. After centrifuging out the nuclei and mitochondria, the supernatant fraction was centrifuged at 105,000 × *g* for 60 minutes in a Spinco model L ultracentrifuge under refrigeration (0 to 2°C). The soluble fraction was then dialyzed against isotonic KCl in the cold room (2°C) for 20 to 21 hours. The dialyzed, clear, supernatant protein solution was used in the spectrophotometric assay. The residue (microsomes) from the above centrifugation was washed with KCl and recentrifuged at 105,000 × *g* for 30 minutes. One ml of the final suspension contained microsomes equivalent to that obtained

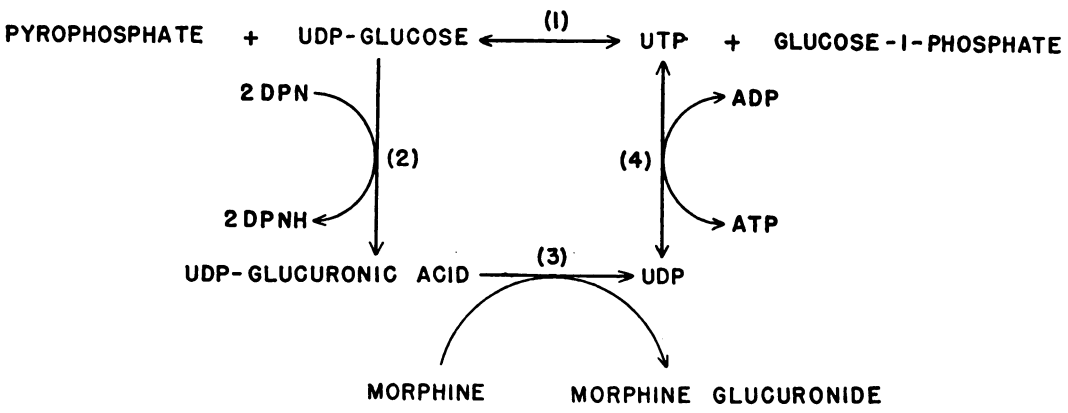

FIG. 1

from 1 g of original tissue. This suspension was used for the source of transferase.

Adenylate kinase was prepared by the method of Colowick and Kalckar (1943) and was made free of NDP-kinase according to the modification by Berg and Joklik (1954). Adenylic acid deaminase was prepared according to the "Preparation A" method of Kalckar (1947). UDP-glucose dehydrogenase, type III, was obtained from Sigma Chemical Co.

Chemicals. Uridine diphosphoglucose (UDP-glucose), uridine diphosphate (UDP), uridine diphosphoglucuronic acid (UDP-glucuronic acid), uridine triphosphate (UTP), glucose-1-phosphate, and diphosphopyridine nucleotide (DPN) were obtained from Sigma Chemical Co. UDP-glucuronic acid was received as the ammonium salt and converted to the sodium salt by employing a Dowex-50-Na^+ column. Adenosine triphosphate (ATP) was procured from Pabst Laboratories.

Enzymic measurements. The transferase assay system contained 0.4 μmol of morphine sulfate, 0.6 μmol of UDP-glucuronic acid, 25 μmol of $MgCl_2$, 250 μmol of phosphate buffer, pH 7.4, 0.5 ml of microsomal suspension, and distilled water in a total of 4.0 ml. A blank (minus morphine) was included for each determination. The reaction mixture was incubated for 20 minutes at 37°C in a Dubnoff shaker. Two-ml aliquots were taken at 0 and 20 minutes and deproteinized with 0.5 ml of 20% trichloracetic acid. After centrifugation, 2 ml of the supernatant were used for the determination of morphine content by the method of Fujimoto *et al.* (1954). When the incubated mixture was autoclaved at 15 pounds pressure for 30 minutes in 1 N HCl, all the morphine was recovered, confirming the results of Strominger *et al.* (1957b). Thus, the disappearance of morphine is the measure for glucuronide formation.

Modifications of the following established assays were employed: UDP-glucose pyrophosphorylase (Strominger *et al.*, 1957b; Kalckar and Anderson, 1957), UDP-glucose dehydrogenase (Strominger *et al.*, 1957a), NDP-kinase (Berg and Joklik, 1954).

The measurement of UDP-glucose pyrophosphorylase activity was carried out in two steps. Incubation mixture in step I contained 300 μmol of Tris[1] buffer, pH 7.4, 1 μmol of glucose-1-phosphate, 0.3 μmol of UTP, 0.5 ml of dialyzed soluble fraction, and distilled water in a total of 2 ml. After incubating the mixture for 1 minute at 37°C, the enzyme was inactivated by immersion in a boiling water bath for 1 minute. The blank contained all the ingredients above except UTP. The amount of UDP-glucose formed was determined in step II by using the UDP-glucose dehydrogenase assay method (assay is described below). One-ml aliquot from the supernatant of step I and 200 units[2] of UDP-glucose dehydrogenase were used. The reaction was followed to completion and the amount of UDP-glucose was determined (the change in optical density at 340 mμ is 12.0/μmol UDP-glucose/ml).

The reaction mixture for the UDP-glucose dehydrogenase assay contained, in a silica cuvette, 150 μmol of glycine buffer, pH 8.7, 1 μmol of DPN, 0.3 μmol of UDP-glucose, 0.5 ml of dialyzed soluble fraction, and distilled water in a total of 3.0 ml. The enzymic activity was measured by following the rate of DPN reduction spectrophotometrically at 340 mμ for 2 to 3 minutes. The blank contained all the substances above except UDP-glucose. The enzymic activity is the reaction rate minus the endogenous rate (blank).

[1] Tris = trishydroxymethylaminomethane.

[2] Units = Sigma units (amount of enzyme, in a 0.5-ml reaction mixture, required to increase the optical density 0.001/min at 340 mμ, pH 8.7, and 25°C; Strominger *et al.*, 1957a).

TABLE 1

Nitrogen contents of hepatic microsomal and soluble fractions of rats

	No. of Animals	mg N/ml ± S.E.*			P
		Control Rats	Chronically Morphinized Rats	Acutely Morphinized Rats	
Dialyzed soluble fraction	10	1.58 ± 0.06	1.73 ± 0.04		>0.05
Microsomes	10	4.03 ± 0.16	4.52 ± 0.15		>0.05
Dialyzed soluble fraction	4	1.45 ± 0.04		1.49 ± 0.02	>0.40
Microsomes	4	3.42 ± 0.15		3.52 ± 0.07	>0.50

* Mg nitrogen per ml ± standard error.

TABLE 2

Activities of various enzymes in hepatic soluble and microsomal fractions in rats

Treatment	No. of Animals	UDP-Glucose Pyrophosphorylase μmol UDP-Glucose Formed/min/mg N ± S.E.	UDP-Glucose Dehydrogenase ΔO.D. at 340 mμ/min/mg N ± S.E.	Transferase μmol Morphine Glucuronide Formed/20 min/mg N ± S.E.	NDP-kinase ΔO.D. at 265 mμ/min/mg N ± S.E.
Saline (control)	10	0.133 ± 0.019	0.034 ± 0.002	0.029 ± 0.002	0.150 ± 0.009
Chronically morphinized	10	0.131 ± 0.017	0.043 ± 0.002*	0.018 ± 0.001†	0.132 ± 0.006
Saline (control)	4	0.136 ± 0.003	0.030 ± 0.001	0.034 ± 0.001	0.186 ± 0.010
Acutely morphinized	4	0.134 ± 0.002	0.045 ± 0.002*	0.033 ± 0.001	0.178 ± 0.006

The enzymic activities of the morphinized groups are not significantly different from those of the control groups except for those comparisons that are properly indicated.

* P value between control and experimental groups < 0.01.

† P value <0.001.

The NDP-kinase assay system contained 80 μmol of succinate buffer, pH 6.0, 5 μmol of $MgCl_2$, 0.22 μmol of ATP, 0.64 μmol of UDP, 0.01 ml of a 1:10 dilution of the adenylate kinase preparation, 0.01 ml of a 1:5 dilution of adenylic acid deaminase preparation, and 0.05 ml of dialyzed soluble fraction in a total of 0.5 ml. The blank contained all the ingredients above except adenylic acid deaminase[3]. The mixture was incubated at 37°C for 15 minutes and the reaction was terminated with 5 ml of 0.1 N HCl. The optical density at 265 mμ was read against the blank.

Nitrogen content of the homogenate fractions was determined by direct nesslerization after complete acid digestion.

RESULTS. Nitrogen contents of dialyzed soluble and microsomal fractions obtained from livers of control and morphinized animals were determined to see if any significant difference was observed upon morphine administration to the rats. No significant difference in nitrogen content of either soluble fractions or microsomal fractions was seen between control and chronically morphinized rats or between control and acutely morphinized rats (table 1). In view of this, all enzymic activities were expressed on a mg nitrogen basis in order to obtain uniform comparisons.

The data in table 2 indicate that the activity of UDP-glucose pyrophosphorylase is not altered significantly upon treating the rats with single or repeated injections of morphine. Thus, reaction (1) in the scheme is not changed in either direction upon morphine administration.

There is a significant increase in the activity of UDP-glucose dehydrogenase in chronically morphinized rats as compared to that in control rats. This significant increase of enzymic activity was also noted in animals receiving only a single dose of morphine. It is apparent that reaction (2) in the scheme clearly favors UDP-glucuronic acid formation upon morphine administration.

There seems to be no significant alteration in

[3] The blank was made without adenylic acid deaminase instead of without soluble fraction since the latter had a relatively high extinction at 265 mμ.

the activity of transferase between control and acutely morphinized rats. However, upon repeated administration of increasing doses of morphine, the activity of transferase definitely decreases. This manifestation points at reaction (3) in the scheme as the possible rate-limiting step in the synthesis of morphine glucuronide in chronically morphinized rats.

Although there is a trend towards a decrease in the activity of NDP-kinase, the activity of this enzyme is not significantly changed between the control and morphinized animals. Thus, reaction (4) in the scheme remains unaltered upon morphine administration to rats.

DISCUSSION. The phenomenon of tolerance is a very perplexing one in the laboratory as well as in therapeutics. The mechanism of tolerance is still unknown despite numerous papers and theories written about this problem. Of the earlier theories on this topic, the theory of increased biotransformation of morphine in tolerant animals has had some support. However, a critical study on the fate of morphine in tolerant and nontolerant dogs by Woods (1954) illustrated that altered distribution or fate of morphine was not associated with or responsible for the development of tolerance. Way *et al.* (1954) found that no great alteration took place in the ability of liver slices to conjugate morphine as tolerance developed in rats, whereas Zauder (1952) showed an increased capacity to conjugate morphine in liver slices taken from tolerant rats. However, *in vivo*, urinary excretion of conjugated morphine decreased in rats after repeated injections of morphine (Zauder, 1952; Way *et al.*, 1954). The present study of the individual enzymes responsible for morphine conjugation supports the view that there is a lack of relationship between the conjugation of morphine and the phenomenon of tolerance.

It is apparent from the data that changes in certain enzymic activities do occur after repeated administration of morphine to rats. Of the four enzymes studied, two show altered activities, namely an increased activity of UDP-glucose dehydrogenase and a decreased activity of transferase. It is interesting to note that the UDP-glucose dehydrogenase activity increases after only a single injection of morphine, whereas the transferase activity remains at the control level. As the animal receives morphine, the first response of the animal is probably to increase the activity of UDP-glucose dehydrogenase by about 30 to 50%. This effect results in presenting the transferase reaction with an abundance of UDP-glucuronic acid; however, the transferase reaction is apparently proceeding maximally at this time. As more morphine is incorporated into the animal's body by repeated injections, the activity of transferase decreases to approximately 60% of the control level. This decrease produces a rate-limiting step in the overall scheme of morphine glucuronide production. Thus, the actual ability of the livers from tolerant rats to conjugate morphine is less than or equivalent to that of the livers from nontolerant rats. This observation, of course, assumes an accumulation of UDP-glucuronic acid in the hepatic tissue. This point has not been thoroughly investigated.

The above observations may explain the earlier results obtained *in vivo* from tolerant rats but cannot explain those obtained *in vitro* from liver slices. The results on the conjugating capacity of liver slices may have been complicated by the influence of other enzymic systems upon the transferase reaction. Also, the reaction product in Zauder's studies was not measured at a time when the transferase reaction was proceeding maximally. It should be noted, however, that the dosages used for the production of chronically morphinized rats in this report were much higher than those used by Zauder (1952) or Way *et al.* (1954).

Takemori and Mannering (1958) showed that the N-demethylating activity in the liver increases markedly when an alkyl group is substituted into the 3-hydroxyl group of morphine-type narcotics. In view of this, N-demethylation may play a more prominent role in the biotransformation of morphine once the morphine molecule is conjugated at the 3-position. On the other hand, this may not be a factor *in vivo* since excretion of conjugated morphine would be expected to be relatively rapid.

SUMMARY

The enzymes responsible for glucuronide synthesis, namely, UDP-glucose pyrophosphorylase, UDP-glucose dehydrogenase, transferase, and NDP-kinase, were studied in livers of acutely and chronically morphinized rats. The activity of UDP-glucose dehydrogenase increased in both acutely and chronically treated rats as compared to those of control animals.

The activity of transferase decreased as the animal received repeated doses of morphine. The enzymic activity of the other two enzymes was not appreciably altered. The relation of the changes in enzymic activities to the conjugating ability of the animal is discussed.

The data indicate a lack of relation between the formation of morphine glucuronide and the development of tolerance.

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