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EFFECT OF CHLOROQUINE AND PHENOBARBITAL ON MORPHINE GLUCURONIDATION AND BILIARY EXCRETION IN THE RAT*

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Abstract—The relationship between morphine glucuronidation and biliary excretion was studied in rats pretreated with saline (control), chloroquine (CQ) or phenobarbital (PB) by determining the conversion of morphine to morphine-3-glucuronide (MG) *in vitro* and the biliary excretion of intravenously administered morphine and MG *in vivo*. For the biliary excretion studies, ^{14}C-morphine or ^{14}C-MG was administered intravenously and excretion measured in anesthetized renal-ligated rats in which the common bile ducts were cannulated. The effect of PB treatment on the biliary excretion of morphine and MG was complex and it was found that: (1) PB pretreatment did not alter the proportions of morphine and MG in bile; (2) the rate of biliary excretion of morphine (as MG) was decreased in PB-pretreated rats even though MG formation was increased *in vitro*; (3) the plasma disappearance of ^{14}C after ^{14}C-morphine administration was also decreased by PB pretreatment; (4) the biliary excretion of administered MG was significantly decreased by PB pretreatment; and (5) the ^{14}C plasma disappearance after ^{14}C-MG administration was not changed by PB pretreatment. Several interpretations can be derived from these results. One possibility is that MG formation is not a rate-limiting step in the biliary excretion of morphine. An alternate interpretation would be that PB pretreatment may have an inhibitory effect on the biliary excretion of morphine and MG that is unrelated to metabolism. Chloroquine pretreatment produced only a slight effect on the biliary excretion of morphine and no effect on the biliary excretion of administered MG. Using studies *in vitro*, we could not demonstrate that CQ induced an increase in MG formation.

ONE APPROACH in studying the relationship between the metabolism of a compound and its biliary excretion is to alter metabolism with microsomal enzyme inducers and observe the effect on biliary excretion. The utility of this approach has been demonstrated by Levine,[1,2] who showed that phenobarbital (PB) pretreatment enhanced the biliary excretion of 3-methyl cholanthrene and benzpyrene. He attributed the increased biliary excretion of these compounds to the PB-induced increase in their metabolism. Because of this and other evidence, Levine[1,2] has concluded that metabolism is the rate-limiting step in the biliary excretion of 3-methyl cholanthrene and benzpyrene.

With compounds that are glucuronidated prior to being excreted into bile, the effect of PB pretreatment on biliary excretion is variable. For example, the enhanced biliary excretion of bilirubin in PB-pretreated rats may result from an increase in

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excretion of bilirubin glucuronide.[3,4] Roberts and Plaa[4] have suggested that increased bilirubin conjugation may play a role in the enhanced excretion of bilirubin after PB pretreatment. Phenol red, on the other hand, is excreted into bile as a glucuronide but its biliary excretion is not changed by PB pretreatment.[5] The increased biliary excretion of probenecid[5] and thyroxin[6] has been attributed to yet another factor, the faster rate of bile flow in PB-pretreated animals.[4]

Recently Peterson and Fujimoto[7] have shown that the biliary excretion of morphine was similar in control and PB-pretreated animals, whereas the biliary excretion of administered morphine-3-glucuronide (MG) was decreased by PB pretreatment. These experiments, however, were not concerned with the effect of PB pretreatment on MG formation. The purpose of the present study was to determine the effect of PB pretreatment on both the biliary excretion of morphine *in vivo* and the conversion of morphine to MG *in vitro*. In the studies *in vivo*, the process of biliary excretion was considered separately from morphine metabolism by administering the metabolite MG. This approach allowed us to study the biliary excretion of MG in the apparent absence of metabolism and compare it to the excretion of MG formed endogenously from morphine.

Also reported in the present investigation are results of biliary excretion experiments in which rats were pretreated with chloroquine (CQ). This compound has been shown to enhance MG formation *in vitro* and decrease the lethal dose of morphine *in vivo*.[8]

MATERIALS AND METHODS

Drugs. Morphine (N-$^{14}CH_3$) hydrochloride, 3·5 mCi/m-mole, was obtained from Mallinckrodt Chemical Company. Crystaline morphine (N-$^{14}CH_3$)-3-glucuronide (MG), 0·45 μCi/mg was prepared biosynthetically by techniques described by Fujimoto and Haarstad[9] and Fujimoto.[10] Phenobarbital sodium (PB) and chloroquine hydrochloride (CQ) were obtained fron Winthrop Laboratories.

Treatment of animals. Sprague–Dawley male rats (300–400 g) were divided into three treatment groups. The control group was injected with saline (2·0 ml/kg, i.p.) once daily for 4 days. The PB group was injected with PB (75 mg/kg, i.p.) once daily for 4 days, and the CQ group was given CQ (25 mg/kg, i.p.) on a daily basis for 5 days. All animals were used 1 day after the last injection and were fasted during this time with water available *ad lib.*

The rats were anesthetized with pentobarbital sodium (45 mg/kg, i.p.) and the femoral vein, femoral artery and common bile duct were cannulated with polyethylene tubing of the appropriate size.[7] The renal pedicles were ligated bilaterally and, in some animals, the trachea was cannulated to facilitate respiration. After the surgery was completed, a thermistor probe was inserted into the rectum and the temperature measured with a Tele-Thermometer (Yellow Springs Instrument Company). Body temperature was maintained at 37° ± 0·5° by a 40-W incandescent lamp placed near the animal. After body temperature had stabilized, approximately 1·0 μCi ^{14}C-morphine (0·097 μmole/kg) or 0·2 μCi ^{14}C-MG (0·32 μmole/kg) was administered into the femoral vein. Bile was collected in 15-min samples for 90 min and 0·3-ml blood samples were collected from the femoral artery in heparinized tubes at 2, 5, 10, 15, 20 and 30 min. Comparisons between treatment conditions were tested for statistical significance by a two-tailed *t*-test.[11]

Analytical procedures. The bile in each 15-min sample was weighed and the radioactivity in 0·05 ml was estimated by counting in a liquid scintillation counter (model 3380, Packard Instrument Company). The radioactivity in blood samples was estimated similarly in 0·05 ml plasma. The scintillation fluid consisted of 4 g of 2,5-diphenyloxazole (PPO) and 50 mg of 1,4-bis [2-(5-phenyloxazolyl)] benzene (POPOP) dissolved in 1·0 l. toluene and 0·5 l. of Triton X-100. Fifteen ml of scintillation fluid was used per vial and quenching was estimated by automatic external standardization.

The per cent ^{14}C as morphine and MG in bile was determined after morphine administration by the countercurrent distribution system of Smith *et al.*[12] The 15-, 30- and 45- min bile samples from a given rat were combined and 0·05 ml of this bile was added to the countercurrent distribution system. The countercurrent solvent system consisted of acetonitrile-*n*-butanol–1·0 M NaCl–0·02 M $NaHCO_3$ (4:25:25, v/v). Two ml of the organic solvent phase and 1·95 ml of the aqueous solvent phase (pH 10) were added to the 0·05 ml bile. After shaking, the aqueous phase was transferred with a syringe and long needle for the countercurrent distribution. After eight transfers, the radioactivity in each tube was estimated by liquid scintillation counting. The per cent radioactivity in bile as morphine and MG was determined by fitting theoretical curves to the countercurrent curve of each experiment.[7,12]

Studies in vitro. The rate of morphine glucuronidation was determined by incubating ^{14}C-morphine with rat liver microsomes and measuring the per cent of ^{14}C-morphine converted to ^{14}C-MG. Liver microsomes were obtained as a 9000 *g* supernatant from animals that had been pretreated with saline (control), CQ and PB. The rats were sacrificed by decapitation and the livers perfused with 10 ml of cold 1·15% KCl through the portal vein. The liver was homogenized with 3 vol. of cold 1·15% KCl, using four strokes of a Potter–Elvehjem homogenizer. The homogenate was centrifuged at 9000 *g* for 30 min and the pellet discarded. The supernatant was diluted with 0·05 Tris–HCl, pH 7·4, to give a protein concentration of 20 mg/ml, and was used for all assays.

The incubation mixture was similar to that of Sanchez *et al.*[8] The assay contained 0·38 μmole uridine-5′-diphosphoglucose (UDPG), 1·2 μmoles nicotinamide adenine dinucleotide (NAD), 3·0 μmoles $MgCl_2$ and 0·07 μmole morphine N-$^{14}CH_3$ hydrochloride (3·65 μCi/μmole). To each assay mixture, 10 mg protein from the 9000 *g* supernatant was added and the assay volume adjusted to 1·0 ml, using 0·05 M Tris–HCl, pH 7·4. The mixture was incubated at 37° for 30 min and the reaction was stopped by quick cooling at 0°. Countercurrent distribution, performed in the same manner as that described previously for bile, was used to estimate the relative amount of MG formed.

RESULTS

Figure 1 shows the rate of plasma disappearance (top panel), biliary excretion (middle panel) and bile flow (bottom panel) after ^{14}C-morphine administration. In these experiments, the plasma ^{14}C concentrations were similar at each time period for control (solid line) and CQ-pretreated animals (dashed line). However, with PB-pretreated rats (dotted line) there was a significant increase in the plasma ^{14}C concentration when compared to that of control rats ($P < 0·05$).

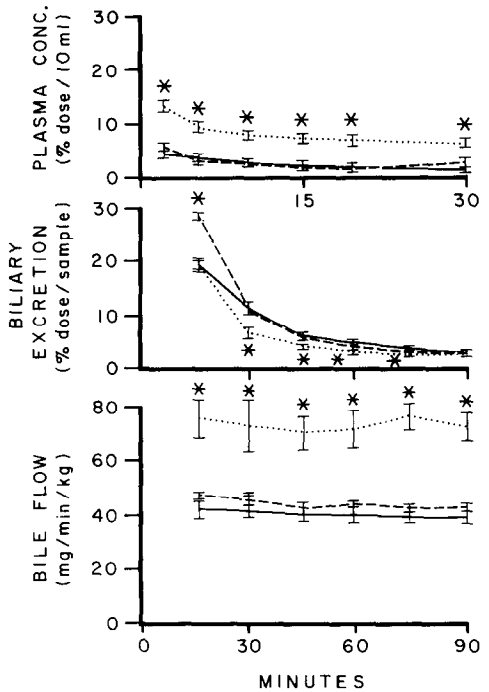

FIG. 1. Effect of saline, CQ and PB-pretreatment on the plasma concentration (top panel), biliary excretion (middle panel) and bile flow (bottom panel) for rats given ^{14}C-morphine. The ordinate in the top panel gives the plasma concentration of radioactivity in 10·0 ml plasma, represented as a percentage of the dose of ^{14}C administered. In the middle panel, the ordinate represents the biliary excretion of ^{14}C as a per cent of the dose administered. In the bottom panel, bile flow is expressed as mg bile/min/kg of body wt. Note that there is a difference in the time scale of the abscissa in the top panel as compared to the middle and bottom panels. The pretreatments are designated as: —, saline (control); – – –, CQ; and ....., PB. Each value is the mean ± S.E. of four or more animals. The asterisk (*) indicates values significantly different from control ($P < 0·05$).

administration. Chloroquine pretreatment increased the biliary excretion of ^{14}C during the first collection period (CQ 28·1 ± 0·7 per cent, mean ± S.E. vs control 20·3 ± 2·6 per cent, $P < 0·05$). This effect of CQ pretreatment would not have been predicted from the plasma disappearance data. In contrast to that observed in CQ-pretreated animals, the biliary excretion of ^{14}C in PB-pretreated rats was similar to that of controls for the first bile sample, but was significantly lower in subsequent collection periods. Furthermore, in PB-pretreated animals the lower excretion was accompanied by a higher concentration of ^{14}C in plasma.

The bottom panel of Fig. 1 shows, as has been previously reported,[3,7,13] that PB pretreatment significantly increases bile flow. Chloroquine pretreatment had no effect on bile flow.

In order to bypass the metabolic conjugation step in the biliary excretion of morphine, the metabolite, morphine-3-glucuronide was administered (Fig. 2). The top panel shows that plasma ^{14}C concentrations were similar after ^{14}C-MG administration for all pretreatment conditions. Thus, PB pretreatment, which in-

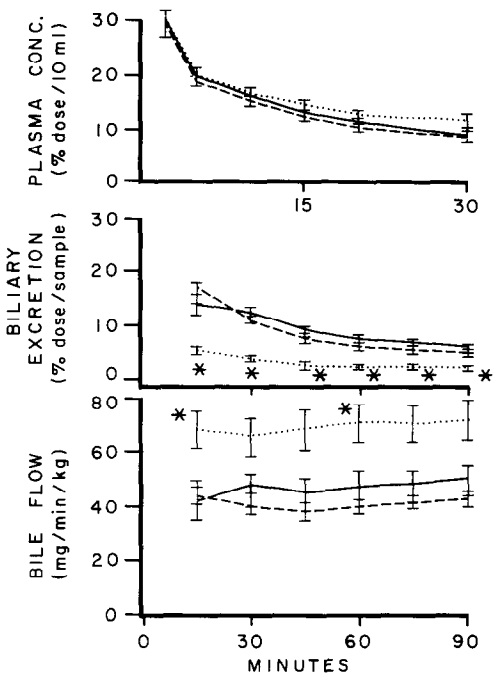

FIG. 2. Effect of CQ and PB pretreatment on the plasma concentration (top panel), biliary excretion (middle panel) and bile flow (bottom panel) for rats given MG. Other conditions as in Fig. 1.

creased plasma ^{14}C concentrations in the ^{14}C-morphine experiment (Fig. 1), had no effect in the ^{14}C-MG experiment (Fig. 2).

The middle panel of Fig. 2 shows that the biliary excretion of ^{14}C-MG in CQ-pretreated and control rats was similar. This lack of effect of CQ pretreatment is in contrast to the initial enhancement of biliary excretion obtained when ^{14}C-morphine was administered to CQ-pretreated rats (Fig. 1). Unlike CQ pretreatment, PB pretreatment caused a significant decrease in ^{14}C-MG excretion at each collection period ($P < 0.01$), as seen in Fig. 2.

The bottom panel of Fig. 2 shows that bile flow in PB-pretreated rats tended to be higher than that in control and CQ-pretreated animals. This effect of PB pretreatment was also found in the morphine experiments (Fig. 1).

An additional facet of this study was to consider the total recovery of ^{14}C in relation to the compounds excreted into bile (Fig. 3). The recovery of radioactivity after morphine administration (top panel) was similar for saline-(49·3 ± 3·6 per cent) and CQ-pretreated animals (55·3 ± 1·2 per cent), but lower in animals pretreated with PB (39·1 ± 4·7 per cent).

It should be emphasized that two radioactive compounds, morphine (stippled area) and MG (cross-hatched area), are excreted into bile after morphine administration. The data in the top panel show that the major amount of ^{14}C recovered in bile after morphine administration is MG, and that the relative proportion of morphine and MG in the total ^{14}C recovered is similar in control, CQ- and PB-pretreated rats. In addition, we have determined the relative proportions of morphine and MG in the individual 15-, 30- and 45-min bile samples and found them

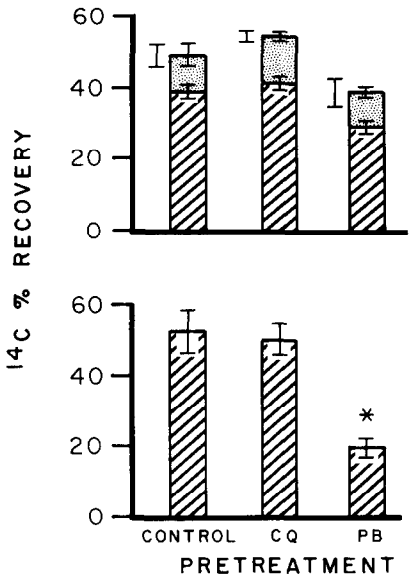

FIG. 3. Effect of pretreatment with saline (control), CQ and PB on the recovery of ^{14}C-labeled compounds in bile 90 min after i.v. administration of ^{14}C-morphine (top panel) and ^{14}C-MG (bottom panel). The ordinate gives the recovery of ^{14}C expressed as a per cent of the morphine and MG dose administered. The values are the mean ± S.E. of at least four rats. The pair of vertical lines within each bar in the top panel represents the mean ± S.E. for the countercurrent distribution analysis of the combined 15-, 30- and 45-min bile samples. The compounds found in the bile were morphine (stippled area) and MG (cross-hatched area). Countercurrent distribution analysis was not performed on the bile from rats given MG (bottom panel) because previous studies[12,14] had shown that MG was excreted unchanged.

to be similar to the relative proportions shown in Fig. 3. Individual analyses of the 60-, 75- and 90-min bile samples were not done, since the amount of ^{14}C in these samples was too low for accurate analysis by countercurrent distribution.

In the bottom panel of Fig. 3, it can be seen that the 90-min recovery of ^{14}C-MG in control (51·8 ± 6·6 per cent) and CQ-pretreated rats (49·4 ± 4·5 per cent) is similar. However, the recovery in PB-pretreated rats (19·5 ± 2·6 per cent) is significantly lower than that in the control and CQ groups ($P < 0.01$).

Since metabolic conjugation is involved in the biliary excretion of morphine but not MG, the effect of CQ and PB pretreatment on the glucuronidation reaction was studied *in vitro*. ^{14}C-morphine was incubated with the 9000 *g* supernatant from rat liver homogenate from control, CQ- and PB-pretreated animals. The results were expressed as per cent conversion of morphine to MG/30 min/10 mg of protein. We found no significant difference between the per cent conversions of morphine to MG with the 9000 *g* supernatant from control (21·0 ± 0·5 per cent) and CQ-pretreated rats (19·8 ± 0·5 per cent). This finding is in contrast to that of Sanchez *et al.*,[8] who reported that morphine glucuronidation was enhanced by CQ pretreatment. The per cent conversion using the 9000 *g* supernatant from PB-pretreated rats (51·3 ± 2·5 per cent) was significantly greater ($P < 0.01$) than the control value. This represents a 3-fold increase of MG formation *in vitro* as compared to control or CQ-pretreated rats. Most recently, Sanchez and Tephley[15] have reported as much as a 10-fold increase in the formation of MG *in vitro* after PB pretreatment.

DISCUSSION

The present study and that of Sanchez and Tephley[15] have shown that PB pretreatment increases the conversion of morphine to morphine glucuronide *in vitro*. As a result, if morphine glucuronidation were a rate-limiting step in the biliary excretion of morphine, one would expect the biliary excretion of this metabolite to be increased by PB pretreatment. Furthermore, the amount of glucuronide conjugate in bile of PB-pretreated rats, as compared to morphine, should increase. Such an expectation is supported by the work of Goldstein and Taurog,[6] who found that benzpyrene pretreatment of rats enhanced the formation of thyroxin glucuronide *in vitro* and increased the amount of glucuronide metabolite which was excreted into bile. Such a result was not obtained in the present study when morphine was administered to PB-pretreated rats.

We found that the amount of ^{14}C excreted into bile of PB-pretreated rats from 90 min after ^{14}C-morphine administration was not significantly different from that of control rats. Furthermore, the relative amounts of morphine and MG in bile after ^{14}C-morphine administration were similar in control and PB-pretreated rats. Analysis of the relative amounts of these two compounds in the 15-, 30- and 45-min bile samples showed that their proportions were constant with time and similar to proportions found in the bile collected for 90 min (Fig. 3, upper panel). These results suggest that MG formation may not be a rate-limiting step in the biliary excretion of morphine.

It is also possible that PB pretreatment is exerting an effect on biliary excretion that is not related to the metabolism of morphine. For example, PB pretreatment decreased the rate at which morphine was excreted in the bile. This effect of PB pretreatment could be due to an inhibition of the transfer of morphine from blood into liver or from liver into bile. Associated with this reduced rate at which ^{14}C-morphine is excreted into bile of PB-pretreated rats was a slower rate of ^{14}C disappearance from plasma. Since the amount of ^{14}C in plasma was too small to permit identification of the ^{14}C compound, we can only hypothesize about its identity. It is unlikely that it is $^{14}CO_2$ resulting from morphine *N*-demethylation, since Alvares and Mannering[16] and Henderson and Mazel[17] have shown that PB pretreatment does not increase the rate of morphine *N*-demethylation *in vitro*.

Another possibility is that, since hepatic morphine glucuronidation is increased, the higher plasma concentration of ^{14}C in PB-pretreated rats could be ^{14}C-MG resulting from diffusion of ^{14}C-MG from the liver into blood. Such a mechanism has been suggested by Guarino and Schanker[14] to account for the postulated presence of probenecid glucuronide in the blood of rats given probenecid. The presence of ^{14}C-MG in blood could also result from the metabolism of morphine at sites other than the liver, since lung, spleen and gastrointestinal tissues are known to make glucuronides.[18] Perhaps the simplest explanation of the increased ^{14}C plasma concentration in PB-pretreated rats after ^{14}C-morphine administration is that it is due to a decreased transfer of ^{14}C-morphine from blood into the liver. Such a situation would result in a decreased rate of biliary excretion of morphine, which was observed.

Pretreatment with PB also markedly decreased the biliary excretion of MG after MG administration, and the total MG excreted in 90 min was significantly less than that in controls ($P < 0.01$). Interestingly, plasma ^{14}C concentration after ^{14}C-MG

administration was not elevated above control values. This suggests that PB pretreatment may be inhibiting the excretion of MG from the liver into bile.

The results obtained with animals pretreated with CQ were disappointing. Although CQ pretreatment did increase the biliary excretion of morphine in the first collection period, there was no significant change in either the total ^{14}C excreted in 90 min or in the relative amounts of morphine and MG in bile when compared to controls. Furthermore, the relative proportions of morphine and MG were similar in bile at the 15-, 30- and 45-min collection periods. This may indicate that the increased biliary excretion observed in the first collection period with CQ pretreatment was not due to an increase in the metabolism of morphine. This is further supported by our finding that CQ pretreatment did not enhance the conversion of morphine to MG *in vitro* when compared to controls. This result is in contrast to the findings of Sanchez *et al.*,[8] who found that CQ pretreatment induced a 2-fold increase in MG formation. While our studies were performed using 9000 *g* supernatant of rat liver homogenate, more recent work using isolated microsomes and UDP glucuronic acid as cofactors has resulted in similar findings in that no induction of morphine glucuronidation was found with CQ-pretreated animals.*

Studies on the conversion of morphine to MG *in vitro* showed that PB pretreatment induced a 3-fold increase in MG formation. Recently, Sanchez and Tephley[15] reported a 10-fold increase in MG formation *in vitro* in PB-pretreated rats. The reasons for the large difference in induction of MG formation between this study and that of Sanchez and Tephley[15] are not known.

In conclusion, while some of the results presented suggest that metabolism is not a rate-limiting step in the biliary excretion of morphine, there is also evidence to suggest that PB pretreatment inhibits some aspect of the biliary excretion process for both morphine and MG. This is an unusual effect of PB pretreatment which has not been previously reported for any other drug. Further studies concerning this unusual phenomenon are now in progress.

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REFERENCES

1. W. G. LEVINE, *J. Pharmac. exp. Ther.* **175,** 301 (1970).
2. W. G. LEVINE, *J. Pharmac. exp. Ther.* **183,** 420 (1972).
3. C. D. KLAASSEN, *J. Pharmac. exp. Ther.* **175,** 289 (1970).
4. R. J. ROBERTS and G. L. PLAA, *Biochem. Pharmac.* **16,** 827 (1967).
5. L. G. HART, A. M. GUARINO and R. H. ADAMSON, *Am. J. Physiol.* **217,** 46 (1969).
6. J. A. GOLDSTEIN and A. TAUROG, *Biochem. Pharmac.* **17,** 1040 (1968).
7. R. E. PETERSON and J. M. FUJIMOTO, *J. Pharmac. exp. Ther.* **184,** 409 (1973).
8. E. SANCHEZ, L. TAMPIER and J. MARDONES, *Eur. J. Pharmac.* **7,** 106 (1969).
9. J. M. FUJIMOTO and V. B. HAARSTAD, *J. Pharmac. exp. Ther.* **165,** 45 (1969).
10. J. M. FUJIMOTO, *J. Pharmac. exp. Ther.* **168,** 180 (1969).
11. G. W. SNEDECOR and W. G. COCHRAN, in *Statistical Methods*, p. 104. Iowa State University Press, Ames, Iowa (1967).
12. D. S. SMITH, R. E. PETERSON and J. M. FUJIMOTO, *Biochem. Pharmac.* **22,** 485 (1973).
13. P. BERTHELOT, D. DHUMEAUX, S. ERLINGER and A. M. PREAUX, *Am. J. Physiol.* **219,** 809, (1970).
14. A.M.GUARINO and L. S. SCHANKER, *J. Pharmac. exp. Ther.* **164,** 387 (1968).

* D. L. Roerig and A. T. Hasegawa, unpublished observations.

15. E. Sanchez and T. R. Tephley, *Fedn Proc.* **32,** 763 (1973).
16. A. P. Alvares and G. J. Mannering, *Molec. Pharmac.* **6,** 206 (1970).
17. J. F. Henderson and P. Mazel, *Biochem. Pharmac.* **13,** 1471 (1964).
18. D. Schachter, D. J. Kass and T. J. Lannon, *J. biol. Chem.* **234,** 201 (1959).