Biochemical Pharmacology, Vol. 28, pp. 1977–1981.

0006-2952/79/0615–1977 $02.00/0

HEPATIC AND BILIARY TRANSPORT OF OROTATE AND ITS METABOLIC CONSEQUENCES *

ROBERT E. HANDSCHUMACHER and JOHN COLERIDGE

Department of Pharmacology, Yale University School of Medicine, New Haven, CT 06510, U.S.A.

(*Received* 15 *November* 1978)

Abstract—Entry of ratioactive orotic acid ([^{14}C]OOH-orotate) into tissues of rats treated with pyrazofurin to inhibit metabolism of this precursor could be shown to be limited primarily to liver and kidney, both of which actively concentrate this precursor. A saturable active transport system responsible for excretion into the bile at concentrations up to 16 times that found in plasma was shown to be inhibited by probenecid and *p*-aminohippurate. Evidence for active transport between the plasma and the cytoplasma of hepatic cells *in vivo* and in liver slices is also presented.

Orotate, administered by parenteral routes to rats, is utilized effectively as a precursor of pyrimidine nucleotides in the liver and kidney. However, other tissues that have rapid rates of nucleic acid synthesis, such as the intestinal mucosa, thymus and a variety of neoplasms, incorporate only small amounts of this precursor during short term *in vivo* experiments [1–4]. It has been suggested that this reflects a lack of permeability of this precursor into most mammalian cells [3].

In an earlier report from this laboratory, active transport of orotate in the kidney of the chicken via the organic acid transport system was demonstrated [5]. Preliminary studies extended this to transport of orotate through the liver into the bile [6]. This finding was also the basis for studies of the pyrimidine analog 5-azaorotate as a potentially selective therapeutic agent for differentiated neoplasms of the liver and kidney that might retain this permeability and transport system [7, 8].

The current report extends earlier studies on the distribution of orotate and documents the transport of orotate by the organic acid transport system through the parenchymal cells of the liver into the bile. The use of radioactive orotic acid ([^{14}C]OOH-orotate) and an inhibitor of its metabolism, pyrazofurin, permits a more direct analysis of entry into cells, as distinguished from subsequent metabolism to pyrimidine nucleotides.

MATERIALS AND METHODS

In vivo *studies*. Male Sprague–Dawley rats (250–450 g) were lightly etherized prior to complete anethetization with Diabutal (pentobarbital, 50 mg/kg, intraperitoneally). The carotid artery, femoral vein, bile duct and trachea were cannulated. Body temperature was monitored, using a rectal thermometer, and maintained at 36–38° by an overhead heating lamp. Since renal clearance of orotic acid is rapid, the renal pedicles were ligated in studies of the saturation of the biliary orotate secretory mechanism. [^{14}C]OOH-orotate (41.25 mCi/m-mole) was obtained from the New England Nuclear Corp (Boston, MA), and diluted with a 10 mM solution of orotic acid in physiological saline for infusion (final specific activity of 0.1 or 0.4 mCi/m-mole). Variable infusion rates were achieved using a Harvard infusion pump, Harvard Apparatus Co., Dover, MA (model 600–910); the total volume of infused solutions was controlled so that the hematocrit was not reduced by more than 7 per cent. Pyrazofurin, kindly supplied by E. Lilly & Co. (Indianapolis, IN), was administered intravenously (70 mg/kg) 10 min prior to orotate infusion. A loading dose of probenecid (20 mg/kg); Merck, Sharpe & Dohme, West Point, PA) was also given intravenously 10 min before orotate infusion, and plasma levels were maintained by infusion of 1 mg probenecid/min throughout the biliary excretion experiments. All rats were heparinized with 200 units/animal. Carotid blood samples were taken every 10 min, and the corresponding bile sample was taken 5 min later to allow for the time required for bile to traverse the biliary tree and appear at the tip of the cannula. Both blood and tissue samples were homogenized in 5% trichloroacetic acid and centrifuged, and the supernatant fluid was counted in an aqueous scintillation mixture. The hematocrit was determined on all blood samples using a Microfuge. Bile samples were counted directly in scintillant. All samples were adjusted for quench by internal standardization.

In vitro *studies*. Rats were stunned and decapitated, and the liver was removed quickly and placed in ice-cold Eagle's Minimal Essential Medium containing a 0.05 M HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) buffer adjusted to pH 7.4. When thoroughly chilled, the liver was sliced with a Stadie–Riggs microtome. Care was taken to ensure uniformity of slice mass (100 mg ± 10 per cent) and surface area. All reactions were performed in 15-ml plastic scintillation vials containing 3 ml medium. One liver slice (~100 mg) was added to each vial. Carbon dioxide was trapped on a filter paper soaked in 0.1 ml of NCS Tissue Solubilizer (New England Nuclear Corp.) and impaled on a needle pushed through the centre of each vial cap. After a 10-min incubation in a shaking water bath at 37°, 1.0 μmole [^{14}C]OOH-orotate (0.4 mCi/m-mole) was added and incubation continued for the

* Supported by grants from the USPHS (CA10748 and CA16359) and the American Cancer Society (CH67R).

desired time. To stop the reaction, concentrated perchloric acid (0.3 ml) was injected through the needle in the cap and incubation continued for an additional hour to ensure release of carbon dioxide and absorption onto the filter paper. The filter paper was counted directly in an aqueous scintillation mixture. All counts were corrected for release of carbon dioxide in the absence of liver slices, and for quench by internal standardization.

RESULTS

Although several previous studies have indicated that the liver and kidneys incorporate the majority of a dose of orotate given by intravenous injection, they have all involved single injections of orotate labeled with ^{14}C or ^{3}H in the heterocyclic ring and measured total radioactivity. Under these conditions, differences in tissue concentration can reflect competition for uptake from the plasma or the sum of entry into tissues and subsequent conversion to uridine and cytidine nucleotides. A more direct measurement of permeation has been obtained in the current study using [^{14}C]OOH-orotate given as a continuous intravenous infusion after a priming dose to achieve a steady state concentration in the plasma (Fig. 1). Under these conditions, only orotate is determined, since $^{14}CO_2$ released by decarboxylation in the formation of uridine nucleotides is not counted. The concentration of orotate in erythrocytes is less than 15% of that in the plasma during the 2 hr of these experiments and plasma concentrations can be calculated by dividing the total blood concentration by the hematocrit. In some experiments, pyrazofurin (70 mg/kg) was given to inhibit orotate metabolism since entry might be rate limiting and the pool or orotate would be negligible if it was rapidly converted to uridine nucleotides. Little difference was seen, however, in tissue levels and the data presented in Fig. 1 are nearly identical to the distribution seen in tissues in the absence of pyrazofurin.

The concentration of orotate in most tissues after attainment of plasma equilibrium was that expected for entry only into the extracellular space or less. Orotate is essentially excluded from the brain and cerebral spinal fluid and that which enters is probably secreted by the organic acid secretory system of the choroid plexus [9]. The extremely high concentration in the kidney undoubtedly reflects the high concentration of orotate in the urine as a consequence of the organic acid secretory system. The concentration in the liver was consistently above that seen in plasma.

Orotate is actively secreted into the bile of rats by a transport system of limited capacity (Fig. 2). As increasing amounts of orotate are infused, the bile/plasma concentration ratio increases until a maximum value of approximately 15 is attained at plasma orotate concentrations of 0.05 to 0.15 mM. At high plasma concentrations the ratio declines, presumably as a consequence of saturation of the biliary secretory mechanism. Despite the fall in the bile/plasma ratio, the absolute amount of orotate transported into the bile continues to increase, in part due to the contribution of passive diffusion to orotate excretion at plasma levels which saturate the active transport system. At low infusion rates the plasma concentrations of orotate were less than those which might have been predicted as a consequence of infusion. Since biliary secretion can only clear 1 ml of plasma every 3 min and the renal pedicles were ligated to minimize loss to the urine, hepatic metabolism must be responsible for these initially low plasma concentrations.

In other experiments not shown, a constant infusion of orotate at rates of 0.1 and 0.5 μmole/min for a total

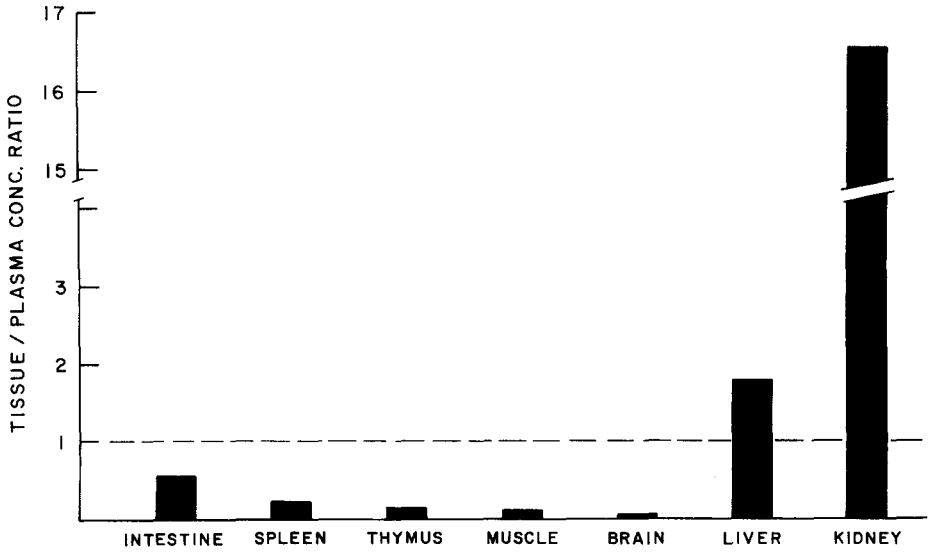

Fig. 1. Entrance of orotate into various tissues after steady state infusion. Rats (250 g males) were prepared, as described in Materials and Methods, and given 30 mg pyrazofurin 4 min prior to infusion of [^{14}C]OOH-orotate (10 mM, 2.5 μCi/ml). Infusion was started with a 0.1 ml priming dose and continued at 0.01 ml/min for 30 min. Bile was collected to eliminate this contribution to intestinal orotate concentration. Plasma concentrations of orotate had achieved an average plateau of 4.5×10^{-6} M after 30 min. Data are the average of three rats each in two different experiments.

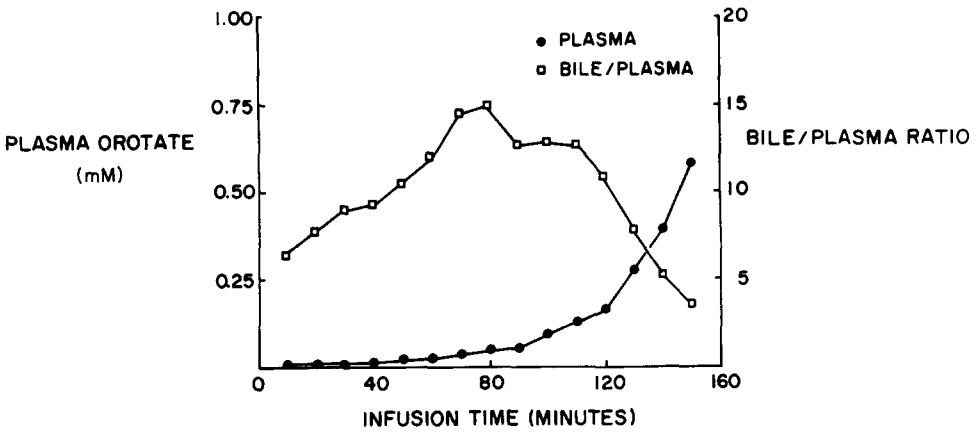

Fig. 2. Biliary secretion of orotate in the rat. A solution of [^{14}C]OOH-orotate (10 mM, 2.5 μCi/ml) was infused intravenously for five successive 30-min periods. The animal was primed with a dose of 0.5 μmole [^{14}C]OOH-orotate immediately prior to infusion at rates of 0.05, 0.1, 0.2, 0.5 and 1.0 μmoles/min. The animal was prepared and samples were processed as described in Materials and Methods.

of 120 min was established. The bile/plasma ratios remained between 10 and 12 throughout the entire 2 hr, indicating that the decreasing ratios seen in Figs. 2 and 3 were not the consequence of a decrease in the functional state of the biliary transport system.

To minimize the effect of orotate metabolism, rats were pretreated with pyrazofurin, a potent inhibitor of orotate metabolism [10, 11]. A dose of 70 mg/kg is sufficient to inhibit by at least 95 per cent the conversion of orotate to uridine nucleotides in most tissues. Under these conditions, the concentration of orotate rose more rapidly in the plasma, and high bile/plasma ratios were observed much earlier in the experiment (Fig. 3). These values correspond closely to the descending portion of the bile/plasma ratio curve in Fig. 2 for each level of plasma orotate. When metabolism is inhibited, a peak in the biliary transport is not seen, presumably because intracellular conversion to uridine nucleotides no longer reduces the concentration of orotate within the hepatocyte available for transport. The plasma levels of orotate in this experiment correspond to values anticipated, if orotate was distributed essentially in the extracellular space, and increase in step-wise fashion in proportion to the increase in the infusion rate. Only during the final 30 min of the experiment are the plasma concentrations similar in both the control and the pyrazofurin-treated rats, a result that suggests that metabolism is saturated during the latter portion of the control experiment. The bile/plasma ratios as presented are minimal values in the pyrazofurin-treated animals since the orotate formed endogenously within the hepatocyte dilutes the infused orotate up to 30 per cent.

To assess the role of the hepatic organic acid transport system [12] in the biliary secretion of orotate, inhibition of the transport by simultaneous infusion of *p*-aminohippurate was tested. When both organic acids were infused at a rate of 0.5 μmole/min in rats not treated with pyrazofurin, the biliary secretion ratios decreased 13 and 20 per cent in two different experiments. When the rate of infusion of *p*-aminohippurate was 5 μmoles/min and orotate 0.5 μmole/min, the bile/plasma ratio for [^{14}C]OOH-orotate was decreased 40 per cent. More effective inhibition of biliary secretion

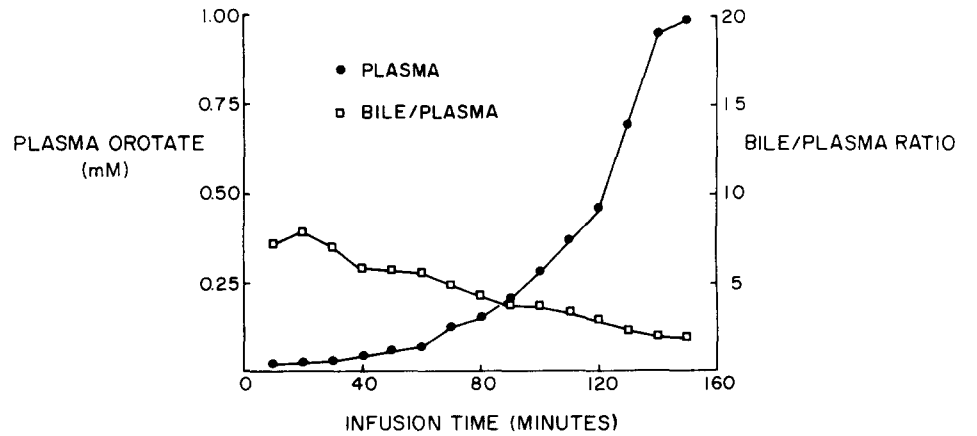

Fig. 3. Effect of pyrazofurin upon biliary secretion of orotate. The treatment was identical to that in Fig. 1, except that pyrazofurin (70 mg/kg) was administered intravenously 10 min prior to the orotate infusion.

could be observed with probenecid. Rats were pretreated with probenecid (20 mg/kg) and infused at a rate of 0.4 mg/kg/min during the experiment. Since probenecid impairs the renal excretion of orotate, a comparable range of plasma orotate concentrations was attained in rats not receiving probenecid only by prolonging the infusion period or by ligating the renal pedicles. In the control experiments, the decline of bile/plasma ratios as a function of increasing plasma orotate concentrations represents the saturation phase of biliary secretion, as seen in Fig. 2. The inhibition of secretion created by probenecid treatment is almost complete at lower plasma concentrations of orotate. As the concentrations are increased, the per cent inhibition is decreased since the ability of orotate to compete for the transport system becomes greater and passive diffusion may make a more significant contribution to secretion.

Since transport of orotate into the hepatic cell as well as secretion into bile might be occurring, the steady state concentration of orotate in rat liver *in vivo* was determined after a 30-min orotate infusion (Table 1). Although the bile/plasma ratio was 7.2 in the control animals, there was only minimal evidence of concentration of the compound within the hepatocyte. In rats pretreated with pyrazofurin to inhibit metabolism of orotate within the hepatocyte, the liver/plasma ratio was increased to 1.4. Treatment with probenecid, however, decreased the concentration of orotate in the liver to less than that of plasma. Under these conditions, the bile/plasma ratios was also reduced, as noted in Fig. 4. In the presence of both pyrazofurin and probenecid, values approximating those seen in the control rats were obtained, possibly the result of competitive reduction of probenecid blockade by the increased levels of orotate within the hepatocyte.

Since orotate is metabolized very rapidly to uridine nucleotides by cell-free extracts of liver [7, 8], the limiting factor for its metabolism by slices of liver is probably the rate of entry into the cell. This affords an alternative measure of transport across the plasma membrane into the cytoplasm by measurement of $^{14}CO_2$ formation in slices incubated in medium containing [^{14}C]OOH-orotate (Fig. 5). The release of $^{14}CO_2$ was linear with time for more than 10 min under these conditions, and probenecid caused a dose-dependent inhibition of orotate metabolism. A 40 per cent reduction of $^{14}CO_2$ release was noted at a probenecid concentration of 1.1 mM. This same concentration of probene-

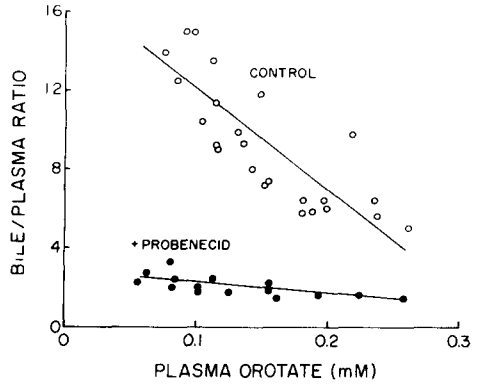

Fig. 4. Effect of probenecid upon biliary secretion of orotate. Both control and probenecid-treated rats were primed with 5 μmoles [^{14}C]OOH-orotate (0.4 mCi/m-mole) and then infused at a constant rate of 0.5 μmole/min. Probenecid was injected intravenously (20 mg/kg) 10 min before orotate and infused at a rate of 1 mg/min during the experiment. Data were obtained from five rats over a period of 2 hr for each treatment group, pooled, and lines fitted by linear regression.

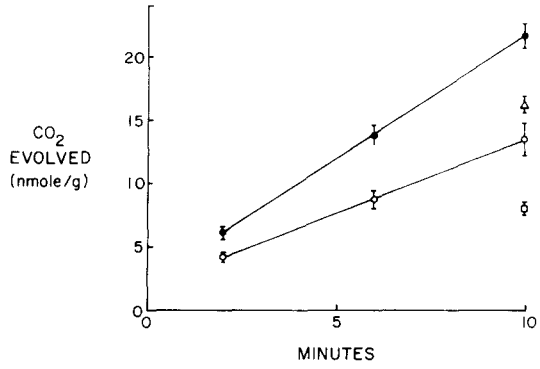

Fig. 5. Effect of probenecid upon evolution of $^{14}CO_2$ from [^{14}C]OOH-orotate by slices of rat liver. Slices (~100 mg) were incubated at 37° in medium containing 0.32 mM [^{14}C]OOH-orotic acid (0.4 mCi/m-mole). $^{14}CO_2$ evolved in 10 min/g of liver was measured as described in Materials and Methods and is presented as a function of probenecid concentration: (●) control; (△) 0.11 mM; (○) 1.1 mM; and (□) 11 mM probenecid. All data points are the means ± S.E.M. of six determinations.

Table 1. *In vivo* uptake of orotate by rat liver*

Treatment group	Plasma orotate concentration (mM)	Liver orotate concentration (mM)	Bile/plasma ratio	Liver/plasma ratio
Control (4)	0.22 ± 0.03	0.23 ± 0.02	7.2 ± 1.0	1.1 ± 0.1
Pyrazofurin (4)	0.21 ± 0.03	0.28 ± 0.04	9.8 ± 1.1	1.4 ± 0.1
Probenecid (3)	0.21 ± 0.03	0.10 ± 0.02	1.5 ± 0.1	0.5 ± 0.1

* All rats were given an initial injection of 5 μmoles orotate and infused at a rate of 0.5 μmole/min for 30 min. Both pyrazofurin (70 mg/kg) and probenecid (20 mg/kg) were given 10 min prior to orotate infusion; probenecid was also infused at a rate of 1 mg/min throughout the experiment. The number of rats in each treatment group is shown in parentheses. The values are means ± S.E.M.

cid had no effect upon the metabolism of orotate in cell-free extracts prepared as described previously [7]. Thus, the reduced rate of CO_2 release in the presence of probenecid probably reflects an inhibition of orotate entry into the hepatocyte.

DISCUSSION

The selective utilization of orotate as a pyrimidine precursor by liver and kidney tissue can be attributed to the organic acid transport systems responsible for its entrance into hepatic parenchymal and tubular epithelial cells. This is of some interest since these systems are generally associated with xenobiotics, sulfonic acid dyes, selected acidic drugs and metabolic end products, such as bilirubin and *p*-aminohippurate [12]. An exception is the clearance of folic acid [13] and its analog methotrexate [14] by biliary excretion. Furthermore, an increase in the toxicity of the folate analog is observed after treatment with probenecid [15]. That a normal precursor of nucleic acid pyrimidines is also handled by the same transport system adds another dimension to our understanding of its role. It has been suggested that active biliary secretion of compounds in general must be associated with a process of active transport from the plasma into the cellular cytoplasm. Our determinations of hepatic concentration of orotate *in vivo* and its metabolism by liver slices are consonant with this view. The fact that the concentration of orotate in the liver was less than that in both plasma and bile when probenecid was infused suggests that entry from the plasma can be made rate limiting. Earlier studies had shown that the intrinsic capability of suitably supplemented liver extracts to metabolize orotate exceeded almost 10-fold the rate observed with slices [7]. The current report extends these findings by demonstrating a probenecid blockade of orotate entry and metabolism in slices at concentrations that do not affect the same reactions in cell-free extracts.

The use of pyrazofurin to inhibit metabolism of orotate while studying secretion must be recognized as having several modifying effects on the system. Within 3 hr, intracellular concentrations of orotate and orotidine as high as 0.8 mM could be detected in spleen and Lewis lung tumors [16]; however, orotate could not be detected in the liver. This is similar to results reported previously for 6-azauridine which accumulates orotate and orotidine in tumor cells [17, 18] but not in liver [18]. This could be consequent to rapid secretion into the bile of any orotate that accumulates behind the site of inhibition; in fact, biliary concentrations as high as 0.6 mM have been observed in rats treated with a single injection of pyrazofurin.* Orotidine, formed from the action of a phosphatase on orotidylate, also accumulates in many tissues but not in the liver. Since this nucleoside is not secreted actively into bile, it must diffuse into the plasma and be eliminated in the urine, as reported in patients treated with pyrazofurin [10, 19].

The delineation of this tranport system suggests means by which not only the natural precursor, orotate, but also analogs of this precursor might be affected in their enterohepatic circulation, thereby altering toxicity to other tissues. Less well understood, however, are the factors that control uptake of orotate from the plasma across the sinusoidal spaces, endogenous synthesis of this precursor, and subsequent secretion into biliary cannuliculi to establish a steady state in the liver. It would appear that under normal circumstances the balance favors biliary excretion and, consequently, orotate is not detectable in the liver parenchyma.

Acknowledgements—These experimental studies are an outgrowth of discussions held many years ago with Arnold D. Welch. His introduction to concepts of organic acid and base transport and assistance in establishing collaborative studies with Dr. L. Peters and Dr. R. L. Volle were the model for a preceptor-fledgling relationship—facilitatory but not intrusive. Thanks must also go to Dr. J. R. Vane for guidance in some of the methods subsequently used in these studies during a sabbatical leave at the Royal College of Surgeons. Able assistance in this work was provided by Mrs. Gisella Johns and Mr. Louis Pacilio.

REFERENCES

1. R. B. Hurlbert and V. R. Potter, *J. biol. Chem.* **195**, 257 (1952).
2. M. G. Ord and L. A. Stocken, *Biochem. J.* **132**, 47 (1973).
3. M. A. Lea, J. Bullock, F. L. Khalil and H. P. Morris, *Cancer Res.* **34**, 3414 (1974).
4. G. Weber, R. L. Signhal and S. K. Srivastave, *Adv. Enzyme Regulat.* **3**, 369 (1965).
5. R. L. Volle, R. E. Green, L. Peters, R. E. Handschumacher and A. D. Welch, *J. Pharmac. exp. Ther.* **136**, 353 (1962).
6. R. E. Handschumacher and J. R. Vane, *Pharmacologist* **5**, 252 (1963).
7. R. E. Handschumacher, *Cancer Res.* **23**, 634 (1963).
8. P. Granat, W. A. Creasey, P. Calabresi and R. E. Handschumacher, *Clin. Pharmac. Ther.* **6**, 436 (1965).
9. J. R. Pappenheimer, S. R. Heisey and E. F. Jordan, *Am. J. Physiol.* **200**, 1 (1961).
10. G. E. Gutowski, M. J. Sweeney, D. C. DeLong, R. L. Hamill and K. Gerzon, *Ann. N.Y. Acad. Sci.* **255**, 544 (1975).
11. E. C. Cadman, D. E. Dix and R. E. Handschumacher, *Cancer Res.* **38**, 682 (1978).
12. L. S. Schanker, in *Handbook of Physiology* (Ed. Werner Heidel), Vol. V, Chap. 114. American Physiology Society, Washington, DC (1968).
13. R. S. Hillman, R. McGuffin and C. Campbell, *Trans. Ass. Am. Physns* **90**, 145 (1977).
14. E. S. Henderson, R. H. Adamson, C. Denham and V. T. Oliverio, *Cancer Res.* **25**, 1008 (1965).
15. R. E. Kates, T. N. Tozer and D. L. Sorby, *Biochem. Pharmac.* **25**, 1485 (1976).
16. J. D. Moyer and R. E. Handschumacher, *Fedn Proc.* **37**, 243 (1978).
17. C. M. Janeway and S. Cha, *Cancer Res.* **37**, 4382 (1977).
18. C. A. Pasternak and R. E. Handschumacher, *J. biol. Chem.* **234**, 2992 (1959).
19. T. Ohnuma, J. Roboz, M. L. Shapiro and J. F. Holland, *Cancer Res.* **37**, 2043 (1977).

* J. Moyer, unpublished results.