tantly, there is insufficient methotrexate entering the cell to saturate all the dihydrofolate reductase present and, therefore, little free methotrexate exists inside the cell at any time. It has been demonstrated (24, 25), however, that the persistence of the free intracellular methotrexate accounts for its cytotoxic action. Thus, through model simulations, the measured concentrations in tumors are shown to consist almost entirely of extracellular and bound intracellular concentrations in various proportions, depending on the time and the dose. Since the transport rate has already reached its maximal value at this high dose (Fig. 6), a further increase in the dose would only shift the extracellular curve and, correspondingly, the tissue average curve upwards without a large concomitant increase in the intracellular concentration during the first 2–3 hr. The transport rate would be near the maximum level, only dropping at a later time.

These predictions will be altered, however, if a passive process plays an increasingly significant role as the dose is increased further. This role is still largely unknown at the present time. Nevertheless, what happens at even higher doses is probably of theoretical interest only. In practice, there is a limit to which the dose can be further increased; the LD_{50} of the drug in mice is 350 mg/kg (26).

REFERENCES

(1) D. S. Zaharko, R. L. Dedrick, A. L. Peale, J. C. Drake, and R. J. Lutz, *J. Pharmacol. Exp. Ther.*, **189**, 585 (1974).

(2) D. S. Zaharko, "Pharmacokinetics and Cancer Chemotherapy," Proceedings of the 7th International Congress of Pharmacology, Paris, France, July 1978.

(3) G. Zubrod, *Cancer*, **30**, 1474 (1972).

(4) R. J. Lutz, R. L. Dedrick, J. A. Straw, M. M. Hart, P. Klubes, and D. S. Zaharko, *J. Pharmacokinet. Biopharm.*, **3**, 77 (1975).

(5) J. G. Mayo, *Cancer Chemother. Rep., Part 2*, **3**, 325 (1972).

(6) J. M. Venditti, in "Pharmacological Basis of Cancer Chemotherapy, 27th Annual Symposium on Fundamental Cancer Research," Williams & Wilkins, Baltimore, Md., 1975, p. 245.

(7) T. Giraldi, P. J. Houghton, D. M. Taylor, and L. Nisi, *Cancer Treat. Rep.*, **62**, 721 (1978).

(8) R. L. Dedrick, D. S. Zaharko, and R. J. Lutz, *J. Pharm. Sci.*, **62**, 882 (1973).

(9) I. D. Goldman, *Ann. N.Y. Acad. Sci.*, **186**, 400 (1971).

(10) M. T. Hakala, in "Antineoplastic and Immunosuppressive Agents, Part 1," A. C. Sartorelli and D. G. Johns, Eds., Springer-Verlag, Berlin, Germany, 1974, p. 240.

(11) D. Kessel, T. C. Hall, and D. Roberts, *Cancer Res.*, **28**, 564 (1968).

(12) V. T. Oliverio, *Anal. Chem.*, **33**, 263 (1961).

(13) D. S. Zaharko and V. T. Oliverio, *Biochem. Pharmacol.*, **19**, 2923 (1970).

(14) D. M. Valerino, D. G. Johns, D. S. Zaharko, and V. T. Oliverio, *ibid.*, **21**, 821 (1972).

(15) R. E. Kates and T. N. Tozer, *J. Pharm. Sci.*, **62**, 2056 (1973).

(16) J. E. Fletcher and R. I. Shrager, "Users Guide to Least Squares Model Fitting," Technical Report No. 1, Division of Computer Research and Technology, National Institutes of Health, Washington, D.C., 1968.

(17) K. B. Bischoff, R. L. Dedrick, D. S. Zaharko, and J. A. Longstreth, *J. Pharm. Sci.*, **60**, 1128 (1971).

(18) W. D. Stein, "The Movement of Molecules Across Cell Membranes," Academic, New York, N.Y., 1967, p. 148.

(19) W. C. Werkheiser, *J. Biol. Chem.*, **236**, 888 (1961).

(20) D. S. Saperstein, A. J. Rein, M. Poe, and M. F. Leahy, *J. Am. Chem. Soc.*, **100**, 4296 (1978).

(21) I. D. Goldman, N. S. Lichenstein, and V. T. Oliverio, *J. Biol. Chem.*, **243**, 5007 (1968).

(22) M. Dembo and F. M. Sirotnak, *Biochim. Biophys. Acta*, **448**, 505 (1976).

(23) F. M. Sirotnak and R. C. Donsbach, *Cancer Res.*, **36**, 1151 (1976).

(24) I. D. Goldman, *Mol. Pharmacol.*, **10**, 257 (1974).

(25) J. C. White and I. D. Goldman, *ibid.*, **12**, 711 (1976).

(26) D. S. Zaharko, in "Pharmacological Basis of Cancer Chemotherapy, 27th Annual Symposium on Fundamental Cancer Research," Williams & Wilkins, Baltimore, Md., 1975, p. 69.

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Effect of Enzyme-Inducing and Enzyme-Inhibiting Agents on Drug Absorption II: Influence of Proadifen on 3-*O*-Methylglucose Transport in Rats

WILLIAM R. RAVIS * and STUART FELDMAN ‡×

Received September 5, 1978, from the **School of Pharmacy, Auburn University, Auburn, AL 36830*, and the ‡*Department of Pharmaceutics, College of Pharmacy, University of Houston, Houston, TX 77004.* Accepted for publication January 22, 1979.

Abstract □ Studies utilizing the *in vitro* everted rat jejunum were performed to investigate the effects of proadifen pretreatment and fasting on active and passive intestinal transfer of 3-*O*-methylglucose. Animals were pretreated 24 hr prior to the experiments with 100 mg of proadifen/kg ip. With mucosal concentrations of 0.1 m*M* 3-*O*-methylglucose and ^{14}C-3-*O*-methylglucose, transfer to the sugar-free serosal buffer after pretreatment with proadifen was 50% greater than controls after a 24-hr fast and 120% greater than controls after a 48-hr fast. Everted intestinal segments obtained from unfasted control animals showed diminished ability to transfer the sugar derivative, with transfer rates approximately 50% less than those of segments obtained from 24-hr fasted control animals. Wet and dry tissue weights for 24- and 48 hr-fasted groups increased following proadifen pretreatment. The results suggest that proadifen enhances 3-*O*-methylglucose transport across the rat jejunum and also increases intestinal tissue weight.

Keyphrases □ Enzyme induction—effect of proadifen on intestinal methylglucose transport, rats, fasting □ Enzyme inhibition—effect of proadifen on intestinal methylglucose transport, rats, fasting □ Methylglucose—effect of proadifen on intestinal transport, rats, fasting □ Proadifen—effect on intestinal methylglucose transport, rats, fasting

Several monosaccharides, amino acids, vitamins, and clinically employed drugs are absorbed from the intestinal lumen by carrier-mediated processes. A 3-*O*-methyl derivative of *d*-glucose has been shown to be absorbed across the mucosal epithelia by an active transport mechanism (1, 2). Since this derivative is not utilized by animal tissue (1), as is *d*-glucose, it is a useful agent for specific examination of active sugar transport by intestinal tissue.

Chemical agents capable of altering cell metabolism influence active absorption processes in the intestine (3–8).

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Dicumarol in mucosal solutions inhibited galactose active uptake by the rat small intestine (3). Cardiac glycosides such as strophantidin (4) and ouabain (5) reduced active sugar absorption, possibly due to influence on Na^+-K^+ ATPase, an apparently important component of the translocation mechanism. On the other hand, pretreatment of rats with parathion, a widely used insecticide, elevated intestinal glucose transport, as shown by increases in serosal–mucosal gradients (6). Subcutaneous phenobarbital in rats increased active bile salt ileal absorption (7) and enhanced the ability of duodenal tissue to take up ^{59}Fe-iron sulfate (8).

Previous studies demonstrated that 24-hr pretreatment of rats with 100 mg of proadifen/kg ip increased intestinal transport capabilities for 3-*O*-methylglucose (2). Phenobarbital and 3,4-benzo[*a*]pyrene treatment schedules had no significant influence on 3-*O*-methylglucose transport. In addition, proadifen possessed spasmolytic activity on the small intestine since 24-hr fasting periods prior to the experiments were insufficient to render the lumen free of food and fecal materials.

The present investigation was undertaken to distinguish the nonfasting effects on active 3-*O*-methylglucose absorption from the proadifen pretreatment effects.

EXPERIMENTAL

Animals and Treatment—Male Sprague-Dawley rats[1], 176–255 g, were maintained on regular rat chow and water *ad libitum*. Prior to preparation of *in vitro* everted sacs, the rats were fasted for 24 or 48 hr, depending on the experiment. Water was allowed *ad libitum*.

Proadifen hydrochloride[2]-pretreated animals received 100 mg of drug/kg ip either 24 or 48 hr prior to the experiment. The necessary proadifen hydrochloride dose was administered in <0.5 ml of water, with control animals receiving 0.4 ml of water.

Preparation of Everted Jejunal Sacs—While anesthesia was maintained with ether, a midline incision was made; the small intestine was removed carefully and then rinsed several times with cool saline. The first 15 cm distal to the pylorus was discarded, and the remaining intestine was everted over a glass rod.

Ten-centimeter segments of everted intestine were prepared as everted ligated sacs as described previously (9) or were cannulated (10). Both the serosal and mucosal bathing buffers consisted of pH 7.4 Krebs–Henseleit buffer with the pH determined before and after incubation in the tissue preparation. The serosal buffers were bubbled with 95% oxygen–5% carbon dioxide before installation into the sacs; the mucosal buffers were bubbled prior to and during the experiment. Temperature over the incubation period was maintained at 37 ± 0.2° by a circulating water bath[3].

Uptake and Transfer of ^{14}C-3-*O*-Methylglucose from 0.1 m*M* Mucosal Solutions—Everted ligated sacs from 24- and 48-hr fasted and unfasted animals were filled with 1.0 ml of buffer free of 3-*O*-methylglucose and incubated in 0.1 m*M* methylglucose in 10 ml of mucosal buffer. Sugar transfer and uptake were examined for sacs from both control and proadifen-pretreated animals under various fasting conditions, with one-half of all sacs incubated in 0.08 m*M* phlorizin[4] as well. All mucosal solutions contained initially 0.3 μCi of ^{14}C-3-*O*-methylglucose[5] with appropriate addition of unlabeled 3-*O*-methylglucose[6]. In separate studies, 10^{-3} *M* proadifen hydrochloride was incorporated into mucosal and serosal buffers of segments from control animals.

After 30 min of incubation, the sacs were emptied and the volumes were recorded with sequential dilution of 2 ml with water. One-tenth-milliliter serosal and mucosal samples were saved for liquid scintillation counting. Following rinsing and weighing of the sac tissue, the tissue was homogenized[7] with 2 ml of methanol and centrifuged. A portion of the methanol supernate was counted. Approximately 90% of the total initial label could be accounted for by the buffer and tissue samples.

Table I—Effects of Fasting and Proadifen Pretreatment on ^{14}C-3-*O*-Methylglucose[a] Transfer and Tissue Uptake across the Everted Rat Small Intestine

Condition	*n*	Amount Transferred to Serosal Fluid in 30 min[b], m*M* × 10^{-6}	30-min Tissue Uptake[b], m*M* × 10^{-6}
1-Day fast			
Untreated	19	21.82 ± 4.56	26.60 ± 3.74
Proadifen	8	33.09 ± 7.84[c]	33.64 ± 7.11[c]
2-Day fast			
Untreated	4	17.73 ± 2.64	26.68 ± 4.71
Proadifen	4	39.04 ± 8.40[c]	32.82 ± 3.29[c]
Unfasted			
Untreated	6	9.95 ± 1.25[d]	20.45 ± 0.99[d]

[a] Initial mucosal concentration = 0.1 m*M*. [b] Mean ± *SD*. [c] $p < 0.05$ compared to untreated of same fast period. [d] $p < 0.05$ compared to 1-day untreated.

Serosal to Mucosal Concentration Gradients—Cannulated sacs from 24-hr fasted treated and control animals as well as untreated unfasted animals were incubated with 1.0 m*M* 3-*O*-methylglucose in both mucosal and serosal buffers. The mucosal volume was 60 ml; the serosal volume was 2 ml. After 30 min, the concentration and amount of ^{14}C-3-*O*-methylglucose in the mucosal solution, the serosal solution, and the intestinal tissue were determined. Before the segments were homogenized with methanol, they were rinsed with 1 ml of water and their wet weights were recorded.

Transfer Rates of ^{14}C-3-*O*-Methylglucose as a Function of Incubation Time—Ten-centimeter everted segments from 24-hr fasted control and proadifen-pretreated animals were suspended in 60 ml of 10 m*M* 3-*O*-methylglucose buffer. Of the two segments utilized from each animal, one was randomly chosen for incubation with either 0.08 or 0.8 m*M* phlorizin. Two milliliters of sugar-free buffer was placed into the serosal compartment. Every 10 min over the 2-hr experiment, the 2 ml of buffer was withdrawn followed by a 2-ml serosal rinse and instillation of another 2 ml for the subsequent time interval. The rinse was combined with the initial sample for that 10-min period, and its radioactivity was determined.

Determination of Tissue Weights and Extracellular Space—In separate experiments, wet and dry intestinal tissue weights were recorded for 24- and 48-hr fasted control and pretreated animals. Wet weights were measured after segments were thoroughly rinsed and drained. The wet tissue was placed on tared slides, dried for 2 hr at 120°, and reweighed.

Extracellular space values were obtained for whole sac preparations. Five-centimeter ligated everted segments were incubated with serosal and mucosal buffers containing 0.625 μCi/ml of ^{14}C-carboxyinulin[5] (0.05 mCi/19.6 mg). The mucosal volume was 10 ml; the serosal volume was 0.5 ml. All buffers were gassed with the oxygen carbon dioxide mixture, and the preparations were incubated at 37° for 60 min.

Upon completion of the incubation, the sacs were rinsed, drained, and halved for weighing in tared vials. The tissue contents of the vials were then digested with 1.5 ml of alkaline digestant[8] at 45° until digestion was complete. Before neutralization with acetic acid, the vials were allowed to cool. Five milliliters of liquid scintillation cocktail[9] was added to each vial, and the radioactivity was determined. Values of extracellular space were calculated from the following relationship (11):

$$\text{extracellular space (ml/g)} = \frac{\text{cpm/g of tissue}}{\text{cpm/ml of mucosal inulin solution}} \quad \text{(Eq. 1)}$$

Assay—Samples of serosal buffers, mucosal buffers, and tissue extracts were placed in vials containing 7 ml of scintillant, and their radioactivity was determined with a liquid scintillation spectrometer[10]. Counts per minute were corrected for quenching by standardized quench curves.

RESULTS

These studies were designed to examine the effect of proadifen pretreatment and length of fast on active and passive 3-*O*-methylglucose

[1] Texas Inbred Mice Co., Houston, Tex.
[2] Supplied by Smith Kline and French Laboratories, Philadelphia, Pa.
[3] Blue M, model 1152.
[4] Sigma Chemical Co., St. Louis, Mo.
[5] New England Nuclear, Boston, Mass.
[6] Calbiochem, Los Angeles, Calif.
[7] Polytron model 125-C, Brinkmann Instruments.
[8] NCS, Amersham/Searle Corp., Arlington Heights, Ill.
[9] PCS, Amersham/Searle Corp., Arlington Heights, Ill.
[10] Packard Tri-Carb model 3326.

Table II—Effects of Fasting and Proadifen Pretreatment on ^{14}C-3-*O*-Methylglucose [a] Transfer and Tissue Uptake across Phlorizin [b]-Incubated Everted Rat Intestinal Sacs

Condition	n	Amount Transferred to Serosal Fluid in 30 min[c], mM × 10^{-6}	30-min Tissue Uptake[c], mM × 10^{-6}
1-Day fast			
Untreated	19	4.87 ± 1.22	7.85 ± 1.68
Proadifen	8	2.64 ± 0.81[d]	5.32 ± 1.53
2-Day fast			
Untreated	4	4.10 ± 0.52	7.93 ± 0.46
Proadifen	4	4.50 ± 1.24	7.88 ± 0.90
Unfasted			
Untreated	6	2.50 ± 0.62[e]	7.41 ± 0.64

[a] Initial mucosal concentration = 0.1 mM. [b] Concentration of phlorizin in mucosal and serosal buffer = 0.08 mM. [c] Mean ± SD. [d] $p < 0.05$ compared to untreated of same fast period. [e] $p < 0.05$ compared to 1-day fasted untreated.

transfer across everted rat intestinal segments. Following 24 hr pretreatment with proadifen, the lumens of the 24-hr fasted group were distended with fecal matter; 48-hr fasting provided lumens free of matter. Variation of fasting periods allowed an assessment of fasting and proadifen effects on the mucosal to serosal sugar transfer. By including phlorizin, a glucose transport inhibitor (12), in the incubation medium, passive 3-*O*-methylglucose transfer could be examined separately. Further studies were conducted to determine alterations in wet and dry intestinal tissue weight and whole tissue extracellular space as a result of fasting and proadifen pretreatment.

Study A: Uptake and Transfer of ^{14}C-3-*O*-Methylglucose from 0.1 m*M* Mucosal Solutions—To examine the effects of fasting and proadifin pretreatment on mucosal to serosal transfer and tissue uptake of the actively transported sugar, animals were divided into five groups: unfasted animals, 24- and 48-hr fasted control animals, and 24- and 48-hr fasted animals that were pretreated with proadifen 24 hr prior to segment preparation. Table I gives values for the amount of 3-*O*-methylglucose transferred to the initially sugar-free serosal buffer in 30 min. Everted segments from 24- and 48-hr fasted proadifen-pretreated animals transferred significantly greater amounts of the labeled sugar than the corresponding fasted controls, with increases approximating 52 and 120%, respectively. Segments obtained from unfasted animals transferred 54% less labeled 3-*O*-methylglucose in 30 min than the 24-hr fasted controls.

Tissue sugar levels were elevated in the proadifen-treated group while the sugar levels in the sacs from unfasted animals were significantly ($p < 0.05$) lower than the fasted controls. Due to measured increases in wet tissue weight for the pretreated group, no differences were noted between treated and control fasted groups when comparisons were made based on counts per gram of tissue. Counts per gram of tissue determined from sacs from unfasted animals were significantly ($p < 0.05$) less than the other experimental groups.

Of the two 10-cm intestinal segments utilized from each animal, one was chosen in an alternating pattern for incubation in a similar 3-*O*-methylglucose buffer containing 0.08 mM phlorizin. In experiments where the phlorizin concentration was varied, this inhibitor level provided maximum 3-*O*-methylglucose transport inhibition. Phlorizin dramatically reduced sugar mucosal to serosal transfer and tissue uptake in all experimental conditions, and this transfer was assumed to represent the

Table III—Effects of Unfasting and 1-Day Fasting of Controls and Proadifen-Pretreated Animals on Final ^{14}C-3-*O*-Methylglucose [a] Concentration Ratios and Tissue Levels (n = 6)

Parameter	Fasted[b]	Proadifen Treated[b]	Unfasted[b]
Final ratio, serosal-mucosal	1.152 ± 0.057	1.426 ± 0.094[c]	1.091 ± 0.173
Serosal gain[d], mM × 10^{-3}	0.184 ± 0.092	0.706 ± 0.190[c]	0.082 ± 0.340
Wet tissue weight	0.701 ± 0.102	0.859 ± 0.080[c]	0.897 ± 0.091[c]
Tissue concentration, mM × 10^{-3}/g	1.504 ± 0.307	1.729 ± 0.271	1.238 ± 0.463

[a] Initial mucosal and serosal concentration = 1.0 mM. [b] Mean ± SD. [c] $p < 0.05$ compared to 1-day fasted control. [d] Obtained by subtracting initial amount from final amount.

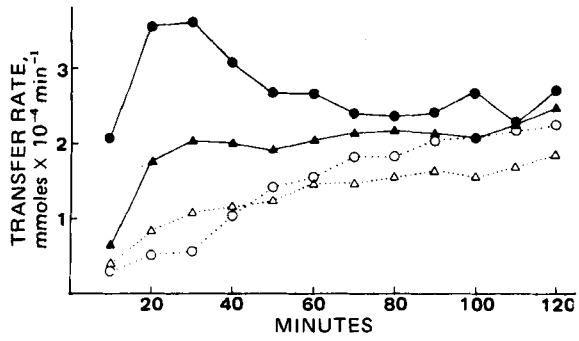

Figure 1—*Mean 10-min transfer rates of ^{14}C-3-O-methylglucose across the everted rat intestine of control and proadifen-pretreated animals* versus *incubation time in a 10 m*M *mucosal buffer. Key:* ●, *proadifen pretreated alone;* ▲, *control alone;* ○, *proadifen pretreated in the presence of phlorizin (0.08 m*M*); and* △, *control in the presence of phlorizin (0.08 m*M*).*

passive component of the sugar transfer. As can be seen in Table II, significantly less 3-*O*-methylglucose was transferred to the serosal compartment for unfasted and 24-hr fasted treated animals.

Study B: Serosal to Mucosal Concentration Gradients across Intestinal Segments from 1-Day Fasted Control, 1-Day Fasted Proadifen-Treated, and Unfasted Control Animals—The ability of everted segments from unfasted control, 24-hr fasted control, and 24-hr fasted proadifen-treated animals to generate concentration gradients for the sugar between mucosal and serosal buffers was examined. 3-*O*-Methylglucose, 1 mM, was incorporated into both the 60-ml mucosal buffers and the 2-ml serosal buffers followed by a 30-min incubation. Final concentration ratios, net serosal gains in 3-*O*-methylglucose, and wet tissue weights are presented in Table III. Segments from pretreated animals exhibited greater final gradients than those of the control and unfasted groups. Intestinal tissue from treated and unfasted animals was greater in wet tissue weight per length than that of 24-hr fasted controls.

Study C: ^{14}C-3-*O*-Methylglucose Transfer as a Function of Incubation Time—Cannulated everted intestinal sacs, prepared from 24-hr fasted control and pretreated animals, were utilized to examine active and passive transfer of 3-*O*-methylglucose across isolated segments over extended incubation periods. The sugar transfer rates at 10-min intervals as a function of incubation time appear in Fig. 1. With phlorizin excluded from the bathing solutions, marked increases in 3-*O*-methylglucose transfer to the serosal buffers were noted at 10, 20, and 30 min for segments obtained from pretreated animals, with percentage increases over controls of 120, 100, and 75% for the three time periods, respectively.

After 30 min, the average rate for the proadifen group progressively fell; rate values from 70 to 120 min were similar to those of the control sacs and phlorizin-incubated sacs of treated and untreated animals for the same time period. The fall in transfer rates and the similarity to passive rates may represent transport system deterioration in isolated segments with long incubation times.

For phlorizin-incubated sacs, the amount of labeled sugar appearing in the serosal buffers up to 30 min was reduced in the proadifen group compared to controls. Passive transfer rates in both control and treated groups progressively increased with incubation, possibly suggesting mucosal membrane barrier deterioration with extended incubation periods (13).

Study D: Uptake and Transfer of ^{14}C-3-*O*-Methylglucose by Intestinal Tissue following 40-hr Proadifen Pretreatment—Preliminary studies examining concentration gradients across ligated sacs from 24-hr fasted animals representing control, 12-hr proadifen pretreatment, and 24-hr proadifen pretreatment groups demonstrated only moderate increases in final concentration gradients following 12-hr pretreatment. To determine whether the effects noted after 24 hr were temporary or became more evident when longer intervals separated the time of injection and transfer measurements, a 40-hr interval between dosing and sacrifice was chosen.

The animals were fasted 24 hr prior to the experiment. Thirty-minute transfer and uptake were determined for ligated sacs in 0.1 mM 3-*O*-methylglucose buffers. No differences were observed between control and 40-hr treated animals, with serosal transfer values of 2.26 ± 0.50 × 10^{-5} and 2.75 ± 0.24 × 10^{-5} mM/10 cm/30 min, respectively. Likewise, tissue levels of the labeled sugar suggested no enhanced sugar uptake by

Table IV—Effects of *In Vitro* Proadifen on 10-min Serosal Transfer and Tissue Uptake of ^{14}C-3-*O*-Methylglucose [a] across the Everted Rat Small Intestine (n = 4)

Treatment	Amount Transferred to Serosal Fluid in 10 min [b], $mM \times 10^{-6}$		10-min Tissue Uptake [b], $mM \times 10^{-6}$	
	Phlorizin [c]	Control	Phlorizin [c]	Control
Untreated	1.454 ± 0.255	4.534 ± 0.220	6.302 ± 0.860	17.910 ± 6.259
Proadifen incubated	1.594 ± 0.333	5.133 ± 0.576	7.082 ± 0.933	17.838 ± 4.449

[a] Initial mucosal concentration = 0.1 m*M*. [b] Mean ± *SD*. [c] Phlorizin concentration in serosal and mucosal buffer = 0.08 m*M*.

the treated group, with a value of $2.68 \pm 0.24 \times 10^{-5}$ m*M*/10 cm/30 min for the 40-hr pretreated group.

Study E: Uptake and Transfer of ^{14}C-3-*O*-Methylglucose for Ligated Segments Incubated in 10^{-3} *M* Proadifen—Possible direct alterations of sugar transport by proadifen were investigated by dissolving 10^{-3} *M* proadifen in the incubation buffers. This concentration was based on reported alterations in the permeability of human erythrocytes (14) and rat hepatocytes (15) in the presence of 10^{-9}–10^{-4} *M* proadifen. *In vitro* drug incubation did not influence mucosal to serosal passive transfer of the sugar in the sac preparations (Table IV). Wet tissue weights were also similar.

Study F: Determination of Wet and Dry Intestinal Tissue Weights—To determine whether the larger values for wet tissue weight were the result of fluid accumulation or tissue proliferation, wet and dry intestinal tissue weights of 24-hr fasted control and treated animals as well as 48-hr fasted groups were recorded. While the lumens of 24-hr fasted proadifen-treated animals were filled with fecal matter, the lumens of the remaining three groups were clear. In both treated groups, the wet and dry tissue weight increased significantly over controls (Table V). Wet tissue weight determinations were greater by 23 and 32% and dry tissue weight determinations were greater by 30 and 27% for 24- and 48-hr fasts, respectively.

Study G: Determination of Whole Tissue Extracellular Space—Measurements of the intestinal tissue extracellular space of 24- and 48-hr fasted, control and treated groups were conducted with ^{14}C-carboxyinulin. No significant differences in whole tissue extracellular space were demonstrated (Table V).

DISCUSSION

As noted in Studies A and C, intestinal segments from unfasted and 24-hr fasted proadifen-treated animals displayed reduced mucosal to serosal passive transfer compared to fasted controls. These two groups coincided with those found to have fecal material in their lumens at the time of sac preparation. Literature reports suggest that differences exist between fasted and unfasted animals with respect to intestinal absorption (16, 17). Alterations in extracellular space as a result of fasting or drug treatment could influence the passive absorption of water-soluble drugs that transverse the mucosal epithelia by aqueous channels. Similar increases in passive transfer rates with incubation time, as seen with 3-*O*-methylglucose in Study C, were reported for water-soluble drugs that are believed to pass the mucosal epithelia by aqueous pathways (13). However, in the present study, no differences were observed in the estracellular space volume per gram of tissue. On the other hand, such measured values are based on whole tissue space and may not be useful in evaluating aqueous channel volume in the absorption-limiting mucosal epithelia (11).

In the present study, segments from unfasted animals displayed diminished serosal transfer when the active transport pathway was not inhibited. If active and passive transfer and uptake are additive, the lower transfer in the absence of phlorizin for unfasted segments cannot be accounted for solely by lower passive transfer rates but must represent the reduced ability of unfasted tissue to transport sugar actively. In contrast, Newey *et al.* (18) found that fasting for 72 hr reduced active D-glucose transport in hamsters. However, other investigators (19) reported elevations in glucose and histidine transport with fasting.

Transfer and tissue weight data indicate that an intestinal segment's transfer abilities are not correlated with segment weight. Proadifen elevated both the amount of 3-*O*-methylglucose transferred and the tissue weight. However, increased intestinal weights noted for the unfasted groups did not coincide with elevations in transfer rates but with reduced rates. Newey *et al.* (18) also showed the inappropriateness of direct comparisons of the amount of sugar transported per gram of tissue in hamsters.

Table V—Effects of 24-hr Proadifen Pretreatment on Rat Intestinal Tissue after 1- and 2-Day Fast [a]

Tissue	Control [b,c]	Treated [b,c]
1-Day fast		
Wet tissue weight, mg/cm	62.14 ± 7.58	76.30 ± 13.70 [d]
Dry tissue weight, mg/cm	7.04 ± 0.82	9.16 ± 1.84 [d]
Extracellular space, ml/g	44.15 ± 5.30	42.27 ± 2.56
2-Day fast		
Wet tissue weight, mg/cm	62.12 ± 1.94	82.10 ± 4.98 [d]
Dry tissue weight, mg/cm	7.30 ± 0.26	9.30 ± 0.48 [d]
Extracellular space, ml/g	41.58 ± 3.43	41.59 ± 3.84

[a] One- and 2-day fast studies performed on separate days. [b] Number of sacs = 8. [c] Mean ± *SD*. [d] $p < 0.05$ compared to control.

Despite the presence or absence of fecal material in the intestinal lumen as noted in Study A, proadifen pretreatment elevated mucosal to serosal transfer of the sugar derivative. Likewise, wet and dry tissue weights were greater in all pretreated groups than in controls of the same fast period. Both effects appeared to be caused by an indirect mechanism and/or *via* a proadifen metabolite since direct drug incubation did not alter sugar transfer (Study E). Of the three pretreatment periods in Study D, 24-hr pretreatment produced optimum enhancement.

Possible indirect mechanisms include the prolongation of the half-life of endogenous steroids (20) or stimulated pituitary hormone release (20) by proadifen or its metabolites. The hepatic microsomal enzyme inducer phenobarbital increased intestinal tissue weight following several days of administration (8). Further studies are necessary to clarify the mechanism of proadifen's effects on intestinal tissue and to evaluate possible alterations in drug absorption following exposure to enzyme-inducing and inhibiting agents.

In summary, enhanced 3-*O*-methylglucose transfer across *in vitro* everted intestinal sacs and increased wet and dry intestinal tissue weights were observed in 24-hr proadifen-pretreated rats. Both effects apparently were unrelated to the unfasted state of the lumen, resulting from proadifen's spasmolytic effects, since 48-hr fasting of treated animals provided material-free lumens with the same effects. Proadifen enhancement of sugar transport appeared to be indirect since drug incubation of segments produced no alterations. In control animals, 24- and 48-hr fasting decreased intestinal weight and increased mucosal to serosal sugar transport as determined by everted intestine techniques.

REFERENCES

(1) T. Z. Csaky, *Fed. Proc.*, **22**, 3 (1963).

(2) W. R. Ravis and S. Feldman, *J. Pharm. Sci.*, **67**, 245 (1978).

(3) E. Anselmi, R. Jordana, and J. Larralde, *Rev. Esp. Fisiol.*, **26**, 325 (1970).

(4) R. K. Crane, D. Miller, and I. Bihler, in "Membrane Transport and Metabolism," A. Kleinzeller and A. Kotyk, Eds., Academic, New York, N.Y., 1961, p. 439.

(5) T. S. Csaky, H. G. Hortzog, III, and G. W. Fernald, *Am. J. Physiol.*, **200**, 459 (1961).

(6) C. R. Robinson, J. K. Chai, and J. T. Pinto, *J. Pharm. Sci.*, **66**, 879 (1977).

(7) V. S. Ostrower, P. Coan, and F. Kern, *Gastroenterology*, **67**, 1162 (1974).

(8) F. B. Thomas, F. S. McCullough, and J. J. Greenberger, *ibid.*, **62**, 590 (1972).

(9) T. H. Wilson and G. Wiseman, *J. Physiol.*, **123**, 116 (1954).

(10) S. Feldman and M. Gibaldi, *J. Pharm. Sci.*, **58**, 425 (1969).

(11) M. Mayersohn, M. Gibaldi, and B. Grundhofer, *ibid.*, **60**, 1813 (1971).

(12) H. Newey, B. J. Parsons, and D. H. Smyth, *J. Physiol.*, **148**, 83 (1959).

(13) M. Gibaldi and B. Grundhofer, *Proc. Soc. Exp. Biol. Med.*, **141**, 564 (1972).

(14) I. P. Lee, H. I. Yamamura, and R. L. Dixon, *Biochem. Pharmacol.*, **17**, 1671 (1968).

(15) P. T. Henderson and J. H. Dewade, *ibid.*, **18**, 2087 (1969).

(16) J. M. Orr and L. Z. Benet, *Digest. Dis.*, **20**, 858 (1975).

(17) J. T. Doluisio, G. H. Tan, N. F. Billups, and L. Diamond, *J. Pharm. Sci.*, **58**, 1200 (1969).
(18) H. Newey, P. A. Sanford, and D. H. Smyth, *J. Physiol.*, **208**, 705 (1970).
(19) J. T. Hindmarsh, D. Kolby, B. Ross, and G. Wiseman, *ibid.*, **188**, 207 (1967).
(20) R. D. Magus, D. E. Rickert, and J. R. Fouts, *Biochem. Pharmacol.*, **17**, 2071 (1968).

Prostaglandin Prodrugs III: Synthesis and Biological Properties of C_9- and C_{15}-Monoesters of Dinoprost (Prostaglandin $F_{2\alpha}$)

W. MOROZOWICH ˣ, T. O. OESTERLING, W. L. MILLER, C. F. LAWSON, J. C. CORNETTE, J. R. WEEKS, and S. L. DOUGLAS

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Abstract □ Methods are described for the synthesis of dinoprost C_9- and C_{15}-monoesters using protective groups. Esters at C_9 were synthesized by acylation of dinoprost 11,15-bis(tetrahydropyran-2-yl)ether followed by acid-catalyzed protective group removal. Esters at C_{15} were synthesized by initial formation of the protected intermediate, dinoprost 9,11-*n*-butylboronate, followed by acylation and hydrolytic protective group removal. Many esters were active *in vivo* in the hamster antifertility screen. Plasma hydrolysis studies showed that the C_{15}-esters were more readily cleaved than the C_9-esters. *In vivo* studies in the rat showed that both the C_9- and C_{15}-esters resulted in urinary excretion of 5α,7α-dihydroxy-11-ketotetranorprosta-1,16-dioic acid in amounts comparable to those obtained after dosing with dinoprost, indicating that ester hydrolysis occurred *in vivo*.

Keyphrases □ Dinoprost—prodrugs, C_9- and C_{15}-monoesters, synthesis, bioactivity □ Prostaglandins—dinoprost, prodrugs, C_9- and C_{15}-monoesters, synthesis, bioactivity □ Prodrugs—dinoprost, C_9- and C_{15}-monoesters, synthesis, bioactivity

Previously, methods were described for the synthesis of aromatic and aliphatic prostaglandins C_1-esters, and the utility of these prodrugs in improving solid-state stability and oral absorption was reported (1, 2). This report describes synthetic routes for dinoprost C_9- and C_{15}-monoesters and an evaluation of ester bioactivity.

EXPERIMENTAL

Materials and Methods—Dinoprost and dinoprost 11,15-bis(tetrahydropyran-2-yl)ether (I)[1] (3) were pure by silica gel TLC. The acid chlorides were obtained commercially[2]. Pyridine (analytical reagent) was dried over molecular sieves[3] for 1 week prior to use. All other solvents were glass-distilled quality[4].

Column chromatography was conducted on 0.063–0.2-mm silica gel[5]. Silica gel TLC was conducted on 250-μm layer plates[6], and visualization was by spraying the developed plates with aqueous 15% ammonium sulfate followed by charring on a hot plate. Prostaglandin ester mass spectra were obtained[7] for (*a*) the trimethylsilyl derivative, (*b*) the C_1-methyl ester of the trimethylsilyl derivative, and (*c*) the nonderivatized compound. The methyl esters were obtained using diazomethane, and the trimethylsilyl derivatives were obtained with a mixture of hexamethyldisilazane – trimethylchlorosilane – bis(trimethylsilyl)acetamide–pyridine (2:2:1:5).

Synthesis—Representative ester syntheses are described. The other esters were synthesized similarly.

Dinoprost 9-n-Butyrate (*III*b)—A solution of 100 mg of I (3) in a mixture of 0.5 ml of butyric anhydride and 1 ml of pyridine was allowed to stand at room temperature for 24 hr. Upon solvent removal at 45° under vacuum, the residue was dissolved in 50 ml of acetone–acetic acid–water (1:1:1). After 24 hr at 40°, the solvent was removed and the residue was subjected to column chromatography on 75 g of silica gel. Elution was achieved with ethyl acetate–acetic acid (97:3), and the product fractions were identified by silica gel TLC with the same solvent system. Evaporation of the pooled product fractions gave 51 mg of a colorless oil, which was pure by TLC.

Dinoprost 15-Acetate (*VIII*a)—A solution of 48.4 mg (0.137 mmole) of IV and 24 mg (0.236 mmole) of *n*-butylboronic acid in 2 ml of dry pyridine was allowed to stand at room temperature for 1 hr. Acetic anhydride, 2 ml, was added; after 24 hr at room temperature, 3 ml of water was added and the solvent was removed at 45° under vacuum. The residual oil was dissolved in 50 ml of ethyl acetate and extracted with 50 ml of 0.2 *M* citrate buffer, pH 3.0.

The organic phase was dried over sodium sulfate; upon solvent removal, the residue was subjected to column chromatography on 50 g of silica gel. Elution was achieved with ethyl acetate–acetic acid (97:3). The product fractions were identified by TLC; upon solvent removal, 25.3 mg of a colorless oil was isolated.

Biological Activity—The ester activity was studied in the gerbil colon assay, the rat pressor blood pressure assay, and the hamster antifertility assay (subcutaneous administration) as described previously (1, 4).

Plasma Hydrolysis—Standard aqueous solutions (0.1 ml) of dinoprost, the 9-acetate (IIIa), the 15-acetate (VIIIa), the 9-*n*-butyrate (IIIb), and the 15-*n*-butyrate (VIIIc) containing quantities in terms of IV ranging from 5 to 20×10^3 ng/ml were incubated separately with 0.05 ml of each of the following: (*a*) saline solution, (*b*) heparinized rat plasma, (*c*) heparinized monkey plasma, and (*d*) heparinized human plasma. After 2 hr at 37°, the affinity of each incubated sample for anti-dinoprost serum was determined using a double antibody radioimmunoassay (5). Relative cross-reactivity was calculated by comparing the amount of the compound required to displace 50% of the label with the amount of IV required. Increased antibody affinity occurred as ester hydrolysis proceeded to yield IV.

***In Vivo* Metabolism**—Mature Sprague–Dawley female rats were injected subcutaneously with one of the following: I, IIIa, VIIIa, IIIb, VIIIc, and the vehicle as the control. The compounds were administered in amounts equivalent to 1 mg of IV dissolved in 0.5 ml of vehicle consisting of polyethoxylated vegetable oil[8]–ethanol–5% aqueous dextrose (1:1:8).

The animals were kept in metabolism cages, and total urine volumes

[1] Supplied by the Research Division, The Upjohn Co.
[2] Aldrich Chemical Co., Milwaukee, Wis., and Eastman Kodak Co., Rochester, N.Y.
[3] Beads, 10–16 mesh, 4 Å, Davison Chemical Co., Baltimore, Md.
[4] Burdick & Jackson Co., Muskegon, Mich.
[5] Silica gel 60, EM Laboratories, Elmsford, N.Y.
[6] Uniplate, Analtech Inc., Newark, Del.
[7] LKB 9000 mass spectrometer.
[8] Emulphor EL-620, GAF Corp., New York, N.Y.

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