Determination and Prediction of Tissue Binding of Prednisolone in the Rabbit

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Received December 27, 1982, from the [*]*Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14260* and the [‡]*Department of Pharmaceutics, Faculty of Pharmacy, University of Alexandria, Alexandria, Egypt.* Accepted for publication February 25, 1983.

Abstract □ Binding of prednisolone to rabbit tissue was determined experimentally and by computation to assess factors and proteins responsible for tissue distribution of this corticosteroid. Binding in tissue slices ranged from 36% in the lung to 83% in the liver. The results obtained were compared with steady-state tissue distribution data generated *in vivo* in the rabbit. Linear uptake with good agreement between the two sets of data was observed for the skeletal muscle and kidney, but not for the liver and lung. *In vivo* uptake of prednisolone in the liver is severely affected by metabolism and biliary excretion, while *in vitro* distribution into liver slices showed a pattern typical of saturation of a low-capacity site followed by linear nonspecific binding at higher concentrations. Specific saturable binding ($K_1 = 1.95 \times 10^7\ M^{-1}$) accounted for 74% of drug bound at a steroid concentration in the liver of 0.3×10^{-6} M, but its contribution decreased to 5% at a concentration of 20×10^{-6} M. Expected prednisolone binding in rabbit tissue was calculated based on various effective protein concentrations in tissues and binding parameters of prednisolone to rabbit plasma. The use of total Lowry protein concentrations markedly overestimates, while albumin (and transcortin) concentrations severely underestimate the tissue binding of prednisolone. The thiopental effective protein fraction best approximates the *in vivo* and tissue slice binding of prednisolone. Metabolism and saturable binding in liver complicate the otherwise relatively linear uptake of prednisolone into various tissues where macromolecules other than albumin dominate in tissue binding of the steroid.

Keyphrases □ Prednisolone—pharmacokinetics, tissue distribution, tissue binding, rabbits □ Tissue binding—prednisolone in the rabbit, liver, muscle, kidney, lung, albumin, effective protein fractions

Current interest in tissue binding stems partly from its uncertain nature and a growing recognition of the magnitude of effect on drug pharmacokinetics exerted by this process. The volume of distribution (1), biological half-life (2), and, of course, total tissue concentrations (3) are influenced by, and respond linearly in some cases to, changes in the fraction bound in tissue. Simulations and limited experimental evidence suggest a stronger influence of tissue binding on these aspects of drug pharmacokinetics compared with plasma binding (3). Occasionally, evidence of saturable tissue uptake invalidates or complicates the assumptions implied in linear pharmacokinetic models (4). Accurate estimates of individual tissue binding parameters over the therapeutic range of concentration are usually required as direct input data in mass-balance equations of physiological pharmacokinetic models (5).

From a pharmacodynamic point of view, the effect elicited by many drugs, including steroids, is a function of the amount of drug bound to receptors in tissues (6). In characterizing receptor binding as a unique type of tissue binding, it is important to be aware of the influence of methodological and pathophysiological factors on both specific and nonspecific tissue binding.

Several approaches have emerged for generating tissue binding data. *In vivo* methods involve measurement of drug uptake in mammalian tissue, with drug gaining access to the tissue while the animal is alive. Drug in tissue, in excess of that diffused into the tissue water, is considered bound to cellular constituents in the absence of time factors, metabolism, active transport, and pH effects. Steady-state conditions are necessary to be certain of the true degree of tissue binding or distribution.

A semiphysiological approach involves the use of perfused animal organs such as kidney, liver, and lung tissues (7, 8). However, the limited duration of organ viability may limit this approach, and it is best used for examining clearance processes.

In vitro determination of tissue binding requires tissue reduction to a near-liquid state manageable in equilibrium dialysis and ultrafiltration procedures (9, 10). The question is often raised, however, as to the degree that the intact tissue characteristics are retained in the homogenate. A milder alternative method is to measure drug uptake into tissue slices suspended in a physiological medium (11, 12). However, as with many other methods, the net drug uptake into slices may be the sum of several processes (transport, metabolism, and ion trapping) including binding.

Prednisolone, a synthetic corticosteroid, is used under various dosage regimens, during which body tissues are exposed to a wide range of concentrations. Specific glucocorticoid receptors have been identified in numerous tissues (6). However, little information is available in the literature concerning binding to mammalian tissues over the therapeutic plasma concentration range. We have found that this steroid is taken up by the majority of tissues of the rabbit in amounts exceeding the diffusion of free drug into tissue water (13). This species also appears to handle prednisolone pharmacokinetically in a manner similar to humans (14).

The aim of the present study was to determine prednisolone binding to rabbit tissue *in vitro* using tissue slices and to compare it with data obtained *in vivo* in rabbits (13). Further comparison will be made with predictions based on various effective (15) protein concentrations of rabbit tissues.

EXPERIMENTAL

Materials—Female New Zealand White rabbits, weighing 3.6–4.2 kg, were used. The animals were sacrificed with ether, and blood was drained from the abdominal aorta. Skeletal muscle, liver, kidney, and lung samples were obtained, washed with ice-cold saline, blotted dry, wrapped in aluminum foil, and frozen. The steroids used were prednisolone[1], prednisone[1], hydrocortisone[1], and dexamethasone[1].

Binding to Tissue Slices—The frozen tissues were cut with a razor blade into slices ~0.5 mm thick, weighing 500 mg. Slices were blotted, weighed, and placed in stoppered vials containing 3.0 mL of steroid solution in isotonic phosphate-buffered saline, pH 7.4. The vials were oscillated at 37°C for 3 h, which was predetermined to be adequate for equilibrium uptake of drug in the tissue slices. At equilibrium, slices were removed, washed quickly with 3 mL of ice-cold saline, blotted, weighed,

[1] *Sigma Chemical Co., St. Louis, Mo.*

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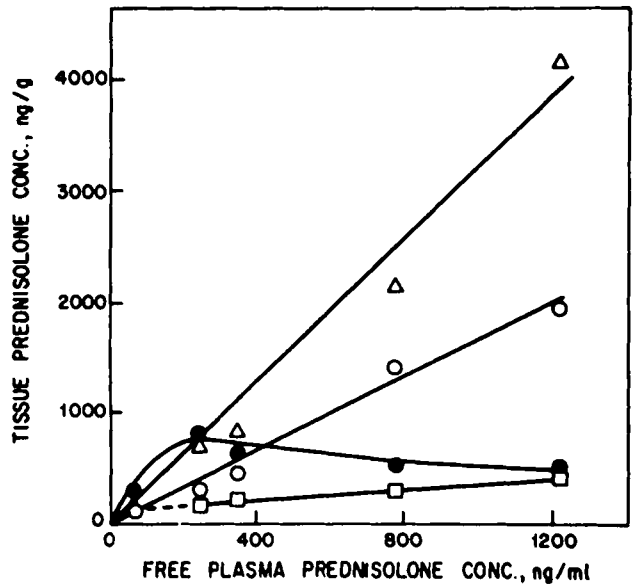

Figure 1—*Uptake of prednisolone in rabbit heart (△), skeletal muscle (○), liver (●), and fat (□) showing the relationships to free concentrations in plasma.*

and homogenized (1:5) with water in a cell disrupter. Vials containing steroid solution but devoid of tissue slices were included in all runs. Initial solutions, solutions remaining at equilibrium, and tissue homogenates were analyzed by HPLC, as described elsewhere (13). The assay yielded equilibrium drug concentrations in the slice (S) and medium (M). Percent bound in tissue (% TB) was calculated from:

$$\%TB = \frac{S - M \cdot f_w}{S \cdot 0.01} \quad \text{(Eq. 1)}$$

where f_w is the fractional tissue water, determined by drying tissue slices to a constant weight (13).

Distribution to Rabbit Tissues *In Vivo*—Distribution data were obtained in an *in vivo* study of prednisolone pharmacokinetics in the rabbit (13). Briefly, the study involved administration of a zero-order intravenous infusion of prednisolone and measurement of the steady-state uptake of the steroid in rabbit tissues and plasma. Apparent percent binding was calculated for each tissue concentration (C_{tissue}) and unbound plasma concentration (C_f) from:

$$\%TB = \frac{C_{tissue} - C_f \cdot f_w}{C_{tissue} \cdot 0.01} \quad \text{(Eq. 3)}$$

It follows that:

$$\%TB = \frac{K_p - f_w}{K_p \cdot 0.01} \quad \text{(Eq. 4)}$$

where K_p is the tissue–free plasma distribution ratio as reported previously (13).

Prediction of Binding to Rabbit Tissues—The *in vivo* tissue distribution data obtained previously in the rabbit (13), indicated linear binding of prednisolone in most rabbit tissues over the concentration range examined. Hence, bound (C_b) and free (C_f) concentrations are related by:

$$C_b = nK \cdot P \cdot C_f \quad \text{(Eq. 5)}$$

and the fraction bound (f_b) is:

$$f_b = \frac{nK \cdot P}{1 + nK \cdot P} \quad \text{(Eq. 6)}$$

where nK is the composite binding parameter (number of binding sites × association constant) and P is the effective protein concentration in tissue. The assumption was then made that the nK value obtained from binding of prednisolone to rabbit plasma can characterize its binding to other tissue proteins. The value of nK was calculated from steady-state plasma *Pn* binding, determined *in vivo* (13), using (16):

$$nK = \frac{1}{[Prot]} \cdot \frac{\beta_0}{1 - \beta_0} \quad \text{(Eq. 7)}$$

where β_0 is the extrapolated fraction bound in the linear binding range

Table I—Binding of Prednisolone to Rabbit Tissue Slices

Tissue	Medium Prednisolone Concentration at Equilibrium, ng/mL	Recovery[a] (*SD*), %	Bound[a] (*SD*), %
Lung	439	91.1 (1.3)	35.86 (10.5)
Skeletal muscle	90–8000	92.8 (7.7)	60.91 (3.1)
Skeletal muscle[b]	458	91.9 (1.8)	52.64 (4.0)
Liver	30–8000	50.8 (11.3)	82.6–54.7[c]
Kidney	399	85.1 (1.5)	61.87 (2.7)

[a] Mean of 6–16 slices. [b] Uptake of prednisolone was measured in the presence of an equal initial prednisolone and hydrocortisone concentration in the medium of 500 ng/mL. [c] Decreasing with increasing concentration.

and [Prot] is the molar concentration of binding proteins in plasma, calculated from the sum of albumin and transcortin concentrations reported in rabbit plasma (14).

Fraction bound in tissue was then predicted (Eq. 6) using P values corresponding to total protein concentrations in tissue (17), albumin concentrations in tissue (18), and effective tissue protein concentrations reported for thiopental (15). The protein values used were reported as fractions of tissue weight and were converted to molar concentration assuming a molecular weight of 69,000 and tissue density of unity. For tissue albumin content, we employed the extravascular albumin spaces determined in rats by Studer and Potchen (18), except in the case of liver where data from dogs (19) were used because of inaccuracy in the rat value.

RESULTS

***In Vivo* Distribution of Prednisolone**—Steady-state tissue-to-plasma distribution of prednisolone was measured in five rabbits. Confirmation of assay methodology, stability, recovery data, and other experimental results are provided elsewhere (13). The equilibrium tissue–unbound prednisolone in plasma ratios for four tissues is shown in Fig. 1. All tissues except the liver showed linear distribution ratios.

Binding in Tissue Slices—Steroid recoveries and percent tissue binding for the tissue slices are reported in Table I. Recovery data were generated by comparing the amount of prednisolone found in tissue slices and medium at equilibrium to the amount of drug initially added to the medium. Both liver and kidney slices generated prednisone when incubated with prednisolone. The recovery values and standard deviations indicate good reproducibility of the data, with marked loss of drug occurring only in the liver.

Binding Kinetics in Muscle and Liver Slices—Uptake of prednisolone into muscle and liver slices was examined over a wide range of

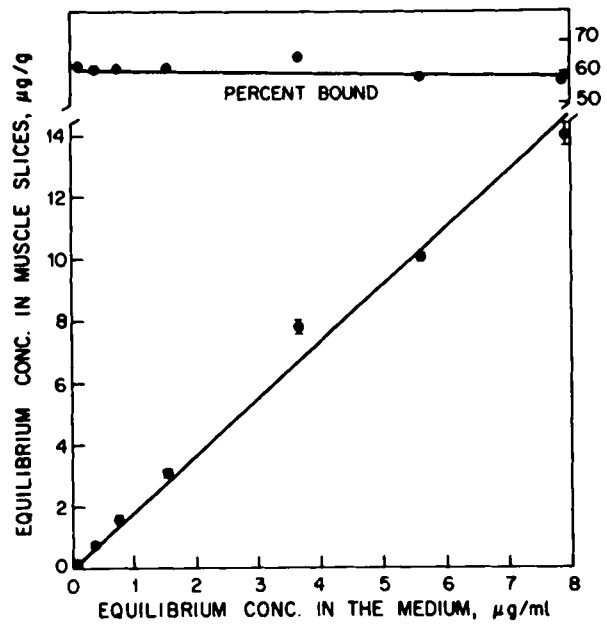

Figure 2—*Uptake of prednisolone in rabbit skeletal muscle slices at 37°C. Verticle lines denote 1* SD. *Percentage binding was calculated using Eq. 1.*

Table II—Protein Fractions Used to Predict the Binding of Prednisolone to Rabbit Tissue

Tissue	Solid (Non-water) Fraction[a]	Effective Protein Fraction		
		Total Protein Fraction[b]	Thiopental Fraction[c]	Albumin Fraction[d]
Lung	0.217	—	—	0.0040
Heart	0.210	0.144	0.0301	0.0022
Skeletal muscle	0.235	0.195	0.0175	0.0036
Brain	0.260	0.108	0.0212	0.0004
Liver	0.298	0.190	0.0362	0.0014[e]
Spleen	0.245	—	—	—
Kidney	0.207	0.152	0.0237	0.0048
Small intestine	0.177	—	—	0.0056
Skin	—	—	—	0.0072

[a] Data in the rabbit, Ref. 13. [b] Data in the rabbit, Ref. 17. [c] Data in the rabbit, Ref. 15. [d] Data in the rat, calculated from *EV* albumin space × 0.04, Ref. 18. [e] Data in the dog, uncorrected for plasma space (Ref. 19).

Table III—Binding of Prednisolone to Rabbit Tissue Determined *In Vivo*, *In Vitro*, and by Prediction

Tissue	Prednisolone Bound, %				
			Predicted Using		
	In Vivo	*In Vitro*	Total Protein Conc.	Effective Thiopental Binding Protein	Albumin Conc.
Lung	72.6	35.9	—	—	25.4
Heart	73.0	—	92.4	71.9	15.8
Skeletal muscle	50.3	60.9	94.3	59.8	23.4
Brain	59.3[a]	—	90.2	64.3	3.3
Liver	84.3– <58.7	82.6–54.7	94.2	75.5	10.6
Spleen	34.9	—	—	—	—
Kidney	72.8	61.9	92.8	66.8	29.0
Small intestine	87.6	—	—	—	32.2
Skin	—	—	—	—	38.0

[a] Based on brain to CSF ratios (Ref. 13).

concentrations (Figs. 2 and 3). Linear binding to the muscle was evident throughout the concentration range examined. Binding to liver slices was nonlinear in the low concentration range but progressed linearly beyond that. The insert in Fig. 3 is a Scatchard plot of the binding data. Drug bound in the liver (D_b) at each concentration was calculated from S − M·f_w, while M·f_w yielded free drug (D_f) in the tissue. The shape of the plot suggests both nonlinear (subscript 1) and linear (subscript 2) binding in accordance with:

$$\frac{D_b}{D_f} = \frac{n_1K_1P_1}{1 + k_1D_f} + n_2K_2P_2 \qquad \text{(Eq. 8)}$$

Individual parameters were calculated as follows. At high drug concentration the first term in Eq. 8 could be neglected and $n_2K_2P_2$ was calculated from the slope of a plot of D_b *versus* D_f. For each data point, drug bound nonspecifically (D_{bNS}) was calculated using $D_{bNS} = n_2K_2P_2 \cdot D_f$. Subtracting this term from the total drug bound yielded D_{bSP}. Plotting D_{bSP}/D_f *versus* D_{bSP} gave a straight line with an abscissa intercept of n_1P_1, and a slope of $-K_1$. The parameters generated were: $n_2K_2P_2 = 1.23$ (no units); $n_1P_1 = 0.142$ μg/g of liver; and $K_1 = 54.2$ g/μg or 1.95×10^7 M^{-1}.

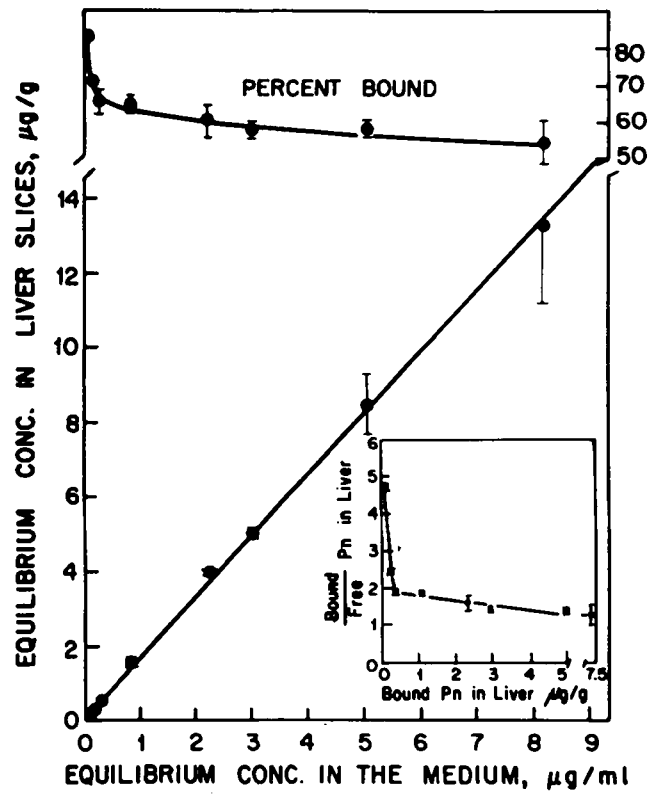

Figure 3—*Uptake of prednisolone in rabbit liver slices at 37°C. The insert is a Scatchard plot of the binding data.*

Calculated Tissue Binding—The protein fractions of the rabbit tissues, obtained from three literature sources, are listed in Table II. Of the total solids of the various rabbit tissues measured in our previous study (13), the Lowry assay-reactive proteins comprise 64–83%, except for brain (42%). The albumin fraction is extremely small and does not change perceptibly when transcortin is included. The effective protein fraction with respect to thiopental binding generally reflects 1.8–3.6% of the tissue weights.

The calculated percentage binding of prednisolone using the three protein fractions are compared with the *in vivo* and *in vitro* tissue binding values in Table III. The use of total protein concentrations markedly overestimates, while albumin concentrations severely underestimate, the tissue binding of prednisolone. The effective protein fraction best approximates both the *in vivo* and tissue slice binding of prednisolone.

DISCUSSION

The present study reports tissue binding data for prednisolone in the rabbit, obtained using different methods. The binding data were generated over a range of steroid concentrations where linear tissue uptake was evident in most tissues both *in vivo* and *in vitro*. The range of concentrations investigated in tissue slices reached an equilibrium medium concentration of 8 μg/mL, which exceeds human plasma exposure to free drug at usual therapeutic doses. However, some corticosteroids such as hydrocortisone and methylprednisolone are sometimes prescribed in large

doses, resulting in plasma levels of 1 mg/mL of total (bound and free) steroid (20, 21), and it is of interest to investigate the manner by which mammalian tissues handle high steroid concentrations.

Binding to Slices Compared with *In Vivo* Data—There was good agreement between slice uptake and *in vivo* distribution of prednisolone in the case of skeletal muscle and kidney. This permits some speculation concerning the mode of entry of the drug into rabbit tissue. The fact that *in vivo* uptake of prednisolone in tissues such as the skeletal muscle was linear and could be quantitatively reproduced in slices under the conditions described, suggests the absence of an active transport process. If the latter occurs *in vivo*, but only partly *in vitro*, drug uptake in slices may underestimate *in vivo* distribution, as reported for the tetraethylammonium ion (22).

Uptake of prednisolone into lung slices markedly differed from the *in vivo* situation. Many aspects of *in vivo* drug localization in lung tissue are not well understood, and underestimation of *in vivo* drug binding when using lung homogenates has been reported (23, 24).

Prednisolone Binding in the Liver—The liver showed a strikingly different uptake pattern *in vivo* compared with the other tissues (Fig. 1). We thus chose to further examine the uptake over a wide range of concentrations in both liver and a tissue such as skeletal muscle using the slice technique. The *in vivo* and *in vitro* data indicated a disparate type of nonlinear binding or uptake of the steroid to the liver tissue. *In vitro*, the results showed a pattern typical of saturation of a low-capacity site followed by linear, nonspecific binding at higher concentrations. The *in vivo* data showed the opposite saturation pattern in the high concentration range. However, these data were probably confounded by rapid metabolism and appreciable excretion in the bile (13) resulting in gross underestimation of the true tissue binding (5). In this respect, the slice technique offers the advantage of measuring drug uptake in the liver in the absence of total expression of its metabolic activity. However, judging by the relatively low recovery values of prednisolone from liver slices compared with other tissues (Table I), steroid metabolism in liver slices cannot be ruled out and may consequently have resulted in some underestimation of binding in the liver.

In generating binding data, part of the information sought is identification of the binding constituent, usually protein, and characterization of the ligand–protein reaction by numerical parameters. Binding data in liver slices yielded an apparent association constant (K_1) for specific nonlinear binding ($1.95 \times 10^7\ M^{-1}$), while for linear binding, only a value for percent bound (59.6%) could be calculated.

The association constant of the liver protein, exhibiting high affinity but low capacity for prednisolone, fits within the range reported for the glucocorticoid receptor, but is also compatible with steroid affinities of other liver proteins such as ligandin and transcortin. Values reported for liver receptor binding range between $5–74 \times 10^7\ M^{-1}$ for dexamethasone in humans (25), rabbits (26), and rats (27), and for hydrocortisone and corticosterone in rats (28, 29). The ligandin association constants for steroids are in the range of $10^5\ M^{-1}$ for purified ligandin, but native cytosol binding may be greater (30). Receptors have been identified in numerous rabbit tissues including the skeletal muscle (31). Prednisolone uptake in muscle slices showed no evidence of high-affinity–low-capacity binding in the low concentration range, which suggests that the corresponding binding seen in liver slices may not necessarily be to the glucocorticoid receptors.

Whatever the identity of the liver protein exhibiting high affinity for prednisolone, and whether or not it is directly involved in expression of steroid activity, it contributed significantly to drug uptake. The percent prednisolone bound specifically (related to total drug bound in liver slices) was as high as 74% at a total drug concentration of 0.3×10^{-6} M, but decreased to 5% at a corresponding concentration of 20×10^{-6} M. The activity of this protein, ($K_1 = 1.95 \times 10^7\ M^{-1}$) is higher than that of albumin in plasma ($nK = 6 \times 10^3\ M^{-1}$) and is as high as that of transcortin in plasma ($K = 2 \times 10^7\ M^{-1}$) (14). It may thus be an important factor in the mechanism of uptake and concentration of prednisolone in liver cells.

Predicted Tissue Binding Data Compared with *In Vivo* Results—The possibility of predicting prednisolone binding to rabbit tissue, based on parameters obtained for binding to rabbit plasma and an estimate of effective protein concentration, was explored in the present study (Table III). Only a portion of tissue proteins are effective in drug binding (32); hence, the observation that total tissue protein binding overestimates prednisolone binding is not unexpected. Albumin, by virtue of its high concentration, is a major binding species in plasma, but apparently assumes a less significant role in tissues. Binding of prednisolone to albumin accounts for less than half of the calculated and measured *Pn* binding in the rabbit tissues (Table III). Owing to the extremely low tissue concentrations, the addition of tissue transcortin to this calculation barely affects the binding values based on albumin alone.

It was intriguing to note that the effective protein fraction calculated for thiopental from binding data to rabbit tissue homogenates (15) yielded good estimates of prednisolone binding in the same species (Table III). Values of the effective protein fraction for prednisolone, and possibly other drugs, may be similar to those reported for thiopental. The concept of a common array of tissue binding proteins for certain types of drugs in various species is worth contemplating, bearing in mind that the binding data presented in this study correspond to a concentration range governed mainly by nonspecific binding, a likely situation for most therapeutic agents.

Recently, some criticism has been raised regarding the assumptions made when utilizing the concept of effective protein fraction in predicting tissue binding. Particularly strong are arguments against extrapolation of binding data from one species or from one protein concentration to another with the inherent assumptions that binding is independent of these factors. Experimental data and simulations have appeared which invalidate these assumptions (32, 33). Furthermore, the effective protein fraction was shown to vary with drug concentration in some of the protein media examined (33). In the present study, mingling of data from different species was minimal. The data presented, whether determined experimentally or predicted (apart from tissue albumin concentration) all pertain to the rabbit. Furthermore, the composite binding parameter, nK (Eq. 7), was determined over the same range of plasma concentration used in the determination of tissue binding *in vivo*. Hence, it is felt that the present data provided a good opportunity to test the usefulness of the effective thiopental protein fraction in predicting the tissue binding of other drugs.

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Deaza Analogues of Adenosine as Inhibitors of Blood Platelet Aggregation

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Abstract □ A number of deaza analogues of adenosine were prepared and tested as inhibitors of platelet aggregation induced by ADP and collagen to investigate the structure–activity relationships in this class of nucleoside analogues. The results showed that the presence of a 6-amino group and nitrogen atoms at positions 3 and 7 of the purine moiety are required for inhibitory activity.

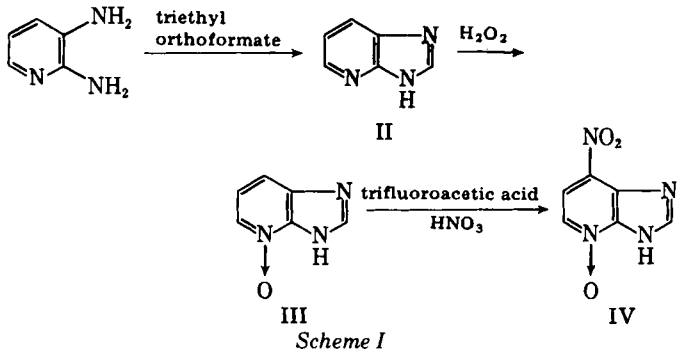
Keyphrases □ Adenosine—deaza analogues, inhibition of ADP- and collagen-induced platelet aggregation □ Platelet aggregation—induction by ADP and collagen, inhibition by deaza analogues of adenosine □ Nucleoside analogues—deazaadenosines, inhibition of ADP- and collagen-induced platelet aggregation

Blood platelets seem to play a dominant role in both the physiological and pathological events of hemostasis (1). In fact, platelet aggregation leads to the formation of either hemostatic plugs or thrombi. Several tissue constituents can give rise to platelet aggregation (2, 3). Among these substances, adenosine diphosphate (ADP) has been shown to be important as it can initiate aggregation (4) and can also mediate aggregation induced by other agents (3, 5).

Adenosine, a structural analogue of ADP, is a powerful inhibitor of platelet aggregation induced by ADP (6). A number of adenosine derivatives and analogues have been synthesized and tested in an attempt to clarify the structure–activity relationships in this class of platelet-aggregation inhibitors (6–9). The present paper investigates the influence of the purine nitrogen atoms on adenosine activity. Some deaza analogues of adenosine and purine riboside have been prepared and tested *in vitro* as inhibitors of platelet aggregation induced by ADP and collagen.

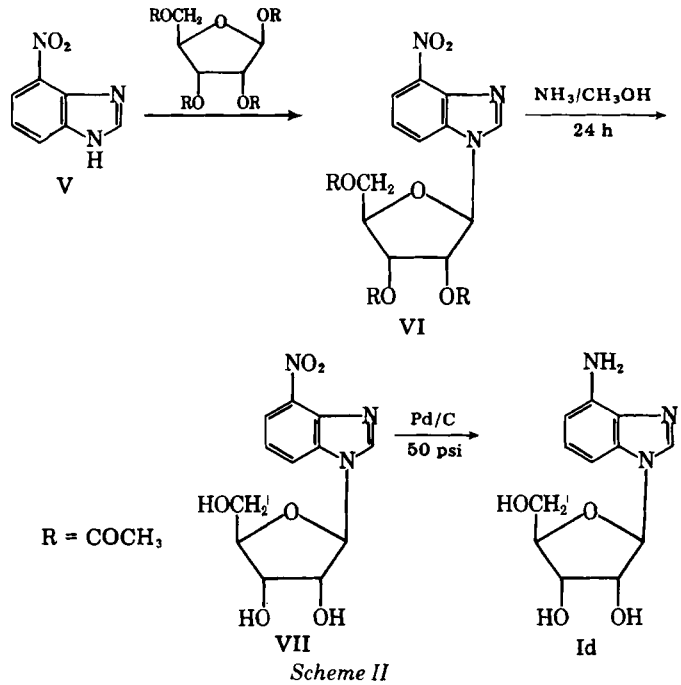
DISCUSSION AND RESULTS

Adenosine[1] (Ia), 7-deazaadenosine[2] (Ie), and purine riboside[2] (Ig) were purchased from commercial sources. 3-Deazaadenosine (Ic) was synthesized using a method previously described (10). 1-Deazaadenosine (Ib) was synthesized using a modification of previous methods (11–14) (Scheme I). Imidazo[4,5-*b*]pyridine (II) (11) was prepared in a 73% yield by the condensation of 2,3-diaminopyridine with triethyl orthoformate.



Scheme I

Nitration of *N*-oxide III, employing a nitric acid–trifluoroacetic acid mixture instead of a nitric acid–acetic acid mixture (12), gave 7-nitroimidazo[4,5-*b*]pyridine-4-oxide (IV) in good yield. The other steps involved following known procedures (13, 14). 1,3-Dideazaadenosine (Id) was synthesized as shown in Scheme II instead of the method reported



Scheme II

[1] Fluka.
[2] Sigma Chemical Co.

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