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Review Article

The Binding of Drugs by Plasma Proteins

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GOLDSTEIN'S CLASSIC REVIEW (1) of interactions between drugs and proteins, which was published in 1949, surveyed and summarized a rather large literature and, in addition, clearly and lucidly emphasized the potential importance of protein binding with respect to the behavior of drugs. His review undoubtedly contributed to an increased awareness of the significance of protein binding and stimulated many investigators to study various aspects of the phenomenon. A large number of publications, describing such studies, has appeared in the 18 years since his review. All, either directly or indirectly, resulted from the desire to answer one or more of the five fundamental guideline questions posed by Scatchard *et al.* (2): "How many? How tightly? Where? Why? What of it?" Thus, many of the reported studies were physical-chemical in nature, utilized synthetic *in vitro* systems, and were oriented to the determination of the maximum number of: (*a*) small molecules which could be reversibly bound by a protein molecule, (*b*) the association constant or constants and other thermodynamic parameters which characterized the reversible association, (*c*) the chemical and conformational nature of the binding sites on the protein molecule, and (*d*) the nature of the intermolecular forces which were responsible for interaction. Other studies, by necessity of a more qualitative nature, attempted to experimentally and/or theoretically assess the importance of protein binding as it relates to the actions and uses of specific drugs.

Excellent general discussions of such studies have appeared. For example, principles and concepts fundamental to an understanding of the phenomenon were discussed by Foster (3), Weber (4), Klotz (5), Edsall and Wyman (6). Brodie and Hogben (7), in a review of physicochemical factors in drug action, discussed reversibility of binding, binding forces, and possible therapeutic consequences resulting from drug-protein interactions. Van Os *et al.* (8) briefly considered the influence of protein binding on drug excretion, drug concentration in tissue fluids, therapeutic activity, and toxicity of drugs. Several chapters, which were devoted to this topic, appeared in a book edited by Binns (9). Brodie (10), for example, cited the importance of binding on the kinetics of drug penetration through biological membranes. He also reviewed the concept of protein-drug complexes functioning as "storehouses" for drug in the body, effectively buffering the level of free drug in the biological fluids. Thorp (11) considered some general aspects of the binding phenomenon and noted that the majority of drug interactions with albumin appear to involve a single binding site on the protein. He speculated that rates of association and dissociations of drug-protein complexes are probably sufficiently great so as to not hinder drug transport from the plasma. The proceedings of a symposium entitled "The Transport Function of Proteins" edited by Desgrez and De Traverse (12) contains a number of chapters

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which are directly related to important aspects of protein–small molecule interactions. Bennhold (13), for example, summarized studies pertaining to the transport of dyes. Guillot (14) reviewed the nature of intermolecular forces responsible for binding. Presentations by Vannotti *et al.* (15) and by Salvatore *et al.* (16) dealt with the binding of thyroid hormones. Polonovski (17), Desgrez (18), Raoul (19), and Lathe (20) discussed the role of plasma proteins in the transport of lipids, corticosteroids, vitamins, and bilirubin. Brodie (21) reviewed possible therapeutic implications of drug–protein interactions and considered "drug buffering," the absorption, distribution, and excretion of highly bound drugs, saturation of binding sites, and the displacement of drugs and endogenous substances by the concomitant administration of certain drugs. Truhaut (22) contributed a review of the interactions between a variety of toxic compounds and plasma proteins. Serum inactivation of penicillins due to protein binding has been recognized for many years and has been the subject of many investigations. Warren (23) has summarized much of the current literature and has attempted the difficult task of relating and correlating binding behavior with the clinical performances of a number of penicillins.

Scope of the Present Review—It did seem desirable to supplement the works cited above with a current review of drug–plasma protein interactions. Because of the extensiveness of the literature in this field, the objectives of a review required limitations. It was decided, therefore, to briefly summarize, in tabular form similar to that employed by Goldstein, some important observations for many of the studies reported during the past 18 years. Supplemental to this tabulation, this report will attempt to review some of the newer experimental techniques which have been employed to investigate protein–small molecule interactions, and the methods used to present and to treat experimental data. Additional emphasis will be directed, by reference to pertinent and representative studies, to recent advances which have been made in understanding, evaluating, and predicting the extent to which drug–protein interactions can influence the distribution of drug in the body and the removal of drug from the body. Finally, attention will be drawn to the many studies which have been concerned with the use of agents which compete with a particular small molecule for binding sites on the protein and can, thus, cause an inhibition or diminution of binding.

Tabulations—Studies which have been specifically concerned with the binding, by plasma proteins, of therapeutic agents or endogenous substances of physiologic importance are summarized in Table I. The tabulation is self-explanatory and reflects the current and continuing interest in the interactions of drugs, metabolites, and naturally occurring compounds with plasma proteins. Table II refers, without commentary, to a number of studies which have employed dyes as the substrates in binding experiments. This class of compounds will not be considered in detail and the table is merely presented as a reference to current work for those interested in such systems. While the majority of dyes included in Table II are not of therapeutic importance, they serve as useful tools in general studies of the binding process and such studies have provided insights into the mechanisms and sites of interaction.

References to the many studies dealing with the binding of steroids have been omitted from Table I because of the availability of several recent and extensive reviews of this topic (24–34). The binding of steroids by proteins is the subject of considerable current interest which stems from the possible involvement of binding in events which are of physiological and pharmacological importance. For example, the apparent absence of the manifestations of Cushing's syndrome during pregnancy, when the corticosteroid level is elevated, has been attributed to an increase in plasma protein binding (35). Evidence has been presented by a number of groups to support the existence of a specialized plasma protein, transcortin, which has a high affinity but low capacity for corticosteroids. It has been speculated (21) that many of the important nonsteroidal anti-inflammatory agents may function *in vivo* by displacing bound endogenous corticosteroid. It has also been reported (36, 37) that the administration of oral contraceptives can result in a marked increase in the fractions of cortisol and aldosterone which are bound in the plasma.

In addition to the many studies which have been considered by authors of the various review articles, the results of a number of recent investigations have appeared and are of interest. Akasu *et al.* (38) demonstrated that dehydroepiandrosterone is highly bound. Several workers have studied the binding of free (39) and conjugated estrogens (40, 41) to serum proteins. Pearlman and Crepy (42) utilized gel filtration to measure the binding of testosterone. Kroneberg and Stoepel (43), using the isolated frog heart, determined that the presence of protein inhibited the digitalis-like activity of a prednisolone derivative. Davidson *et al.* (44) determined the binding characteristics of aldosterone and spirolactones. Hydrocortisone binding to rat and guinea pig pro-

TABLE I—STUDIES INVOLVING INTERACTIONS BETWEEN PROTEINS AND DRUGS OR ENDOGENOUS SUBSTANCES

Substance	Protein Studied[a]	Method[b]	Remarks[c]	Ref.[d]
		Antibiotics		
I. *Penicillins*				
A. Studies of one drug				
Dicloxacillin	S(H)	DF	B = 97%.	272
Benzylpenicillin	S(H)	B	B = 20–40%.	273
	S(B,R)	D	B = 35–40% (4°-pH 6.2).	274
	S(B,H)	D	B = 35–40% (4°-pH 6.8–7.0).	275
Methylchlorophenylisoxazolylpenicillin	S(H)	D	B = 62% (4°).	276
Penicillin	S,A(H)	D	—	277
	A(H)	D,BF	No binding at therapeutic levels. Binding at higher levels.	278
	A(H)	B	Binding inhibited biological activity.	190
Penicillin G-(piperidine salt)	S(H)	EP(^{35}S)	1° binding to albumin.	279
Penicillin G	S(H,D)	D	B = 34%(H), 14%(D). Displaced by probenecid.	210
	A(GP,H,E)	B	Antigenicity measured.	280
	S(H)	EP(^{35}S)	1° binding to albumin.	281
K-Penicillin G	S	D	0.94U bound/mg. albumin. 1° binding to albumin.	282
	S(D,R)	BF	Determined serum and CSF levels. Displaced by probenecid.	178
B. Studies of two drugs				
Na-penicillin G,V	S(D)	D	B = 25%(pen.G), 34%(pen.V). Probenecid caused no displacement.	209
Penicillin & mycerin	PF	EG	Penicillin binding greater than that of mycerin.	283
Oxacillin & methicillin	S(R)	—	B = 84–90%(oxacillin), 33–51%(methicillin).	284
Aminobenzyl & phenoxypropyl penicillins	S(E)	—	—	285
C. Studies of three or more drugs				
Syncillin, penicillins G,V	S(D)	D	B = 40%(Syn.), 28%(G), 36%(V).	286
3 Penicillin derivatives	S(H)	D,Dc,GF	Reversibility of binding observed.	287
	S,T(R),S(H)	BF	Distribution determined after i.v. administration. Orthocresotinic acid used as displacing agent.	177
	S(G,P,O,E,S)	U	—	288
	S(H)	D	Determined *in vitro* activity and studied displacement by sulfonamides, salicylate, and penicillins.	207
	S(H)	U,B	B = 87%(oxacillin), 63%(phenethicillin), 22%(methicillin).	289
4 Penicillin derivatives	S(H,R), A(H)	D	Over 250 compounds tested for displacing activity.	156, 157
	S(H)	EP	1° binding to A & G(α).	290
	S,L(D,H)	U,BF	Binding and distribution determined in blood and lymph.	174
	S(H)	BF,D	Binding studied after i.v. administration.	291
5 Penicillin derivatives	S(E)	D	n = 1 for 4 of 5 derivatives studied.	292
	S(H)	U		123
7 Penicillin derivatives	S,L(D)	U	Concentration of unbound drug similar in plasma and lymph.	175
	S(H,B)	D	1° binding to albumin, binding reversed by dilution.	293
	S(H,M)	—	—	294
	S,L(D)	BF	Lymph concentration always lower than plasma concentration.	173
8 Penicillin derivatives	S(H)	U,D	Finding decreased *in vitro* activity. Displacement by sulfonamides.	295
9 Penicillin derivatives	S(H), A(B)	B	Minimum inhibitory concentrations determined in serum and broth.	191
	S,CSF,T(R)	BF	B = 30–95% in serum. CSF and brain levels determined.	296
Series of several penicillins	S(H)	B	Binding reduced biological activity.	297
	S	—	Determined biological activity, distribution, and excretion.	298
	S(H)	U,D	Determined urinary excretion.	192
	S(H)	B	Binding increased with increasing mol. wt. of side chain.	299
	A(B)	D,S	Determined Δ F°.	300
	A,G(α)(B)	NMR	Phenyl group implicated in binding.	160
II. *Tetracycline(TC) and Derivatives*				
A. Studies of one drug				
Na *N*-methyllolchloro	S	—	Binding similar to other 7-chloro. derivatives.	301
Demethylchlor-(DMC)	S(H)	D,DF	—	302
Oxy-	S	P	—	303
	S(H)	D	B = 27%, renal clearance was determined.	304
Chlor-	S(H)	U	B = 70% at therapeutic levels. 1° binding to albumin.	262
B. Studies of two drugs				
DMC & TC	S(H)	B	Binding decreased *in vitro* activity.	305
2 TC salts	S(M)	—	—	306
Methacycline derivative & TC	S(H)	D	Half-life, volume of distribution, and clearance determined after i.v. administration.	263
DMC,TC, & derivative	S(H)	U,B	Blood levels studied after oral administration.	307
C. Studies of three or more TC's				
3 TC's	A,G(β,γ)	E	Binding due to several serum fractions.	308
	S(H,D)	D	—	309
	S,T(R)	BF	Binding reversible with dilution.	310
	S(H)	E	—	311

(Continued on next page.)

TABLE I—(*Continued.*)

Substance	Protein Studied[a]	Method[b]	Remarks[c]	Ref.[d]
	S(B,H)	EP	Derivatives ranked by degree of association.	312
	S(H,B)	D,T	—	313
4 TC derivatives	S(H)	U	—	314
5 TC derivatives	S(P)	D	1° binding to albumin.	315
6 TC derivatives	S(D,H)	D,U	B = 22–95%, excretion rate highest for least-bound derivative.	316
III. *Miscellaneous Antibiotics*				
Novobiocin	S	D,E	1° binding to albumin.	317
	S(H,B,R)	D	B = 75%(R), 64%(H), 62%(B) at drug concentration of 3 units/ml.	318
	S	B	Serum decreased biological activity *in vitro*.	319
	S(D,B)	D	B = 10%.	320
Streptomycin	A,G(γ)(B)	D	Also studied binding of large organic cations.	321
Spiramycin	A,G(γ)(B,R,M)	E	Tissue distribution determined.	322
Puromycin	A(B),S,G(γ)(H)	D	B = 5–10%, 1° binding to albumin.	323
Antimycin A	A(H,B,E)	B	Succinoxidase bioassay used to detect binding.	324
d,l-Chloramphenicol	S(H)	EP,D	1° binding to albumin. *d* form bound more extensively than *l* form.	325
IV. *Studies with More Than One Class of Antibiotics*				
3 Antibiotics	S	—	—	326
5 Antibiotics	PF(H)	U	—	327
8 Antibiotics	S	D	—	328
	S(H,R,Rt)	E	—	329
10 Antibiotics	PF(H,R)	E,P	Determined distribution into pleural cavity.	179, 180
12 Antibiotics	S(H)	D,GF,B	Also determined sulfonamide binding.	141
14 Antibiotics	S(H)	D,B	Used Freundlich isotherm in treating data.	330
17 Antibiotics	S(E,S,R,B,H)	U	Competition with sulfonamides, phenylbutazone, and salicylate.	163
	S(H)	E	Some association with α-globulin.	331
	S(B,D,Rt,M)	U	Reported % binding and concentration in liver.	332
	S,A(H)	D,B	Determined structure-binding relationships.	333
Sulfonamides				
I. *Studies of One Sulfa*				
Sulfadiazine, 5-ethyl	A(H)	U	*n* and *k* decreased in presence of tolbutamide.	334
Sulfamethoxypyridazines	A,G(γ)(B)	ST,D	B = 60%(3% alb.), determined *in vitro* acetylation rate.	250
	S(R)	BF	Binding decreased activity. Excretion rate not related to binding.	335
	S	—	B = 34%.	258
	S(H)	E,GF	$n = 70$, $k = 6.85 \times 10^3$. Also determined $\Delta F°$, $\Delta H°$, $\Delta S°$.	140
Sulfaethoxypyridazine	S(B)	D	B = 74–98%.	336
5-Methylsulfadiazine	S(R)	E,D(^{35}S)	B = 80%, 1° binding to albumin.	337
2-Sulfanilamido-5-ethylpyrimidine	S(H)	BF	B = 81%; clearance 2.1 ml./min.	338
Dimethoxysulfanilamidodiazine	S	BF	Determined plasma levels.	339
Sulfaethidole	A(H)	D	Variety of drugs displaced bound sulfa.	206
Sulfaethylthiadiazole	A(B)	D	Determined binding inhibition by tetracyclines.	205
	S(H)	D,U	—	340
	S,A(B,R)	BF,D	Studied displacement by 30 drugs.	204
Sulfathiazole	A(B)	ST	Studied stabilization of proteins to X-rays.	341
Sulfamethoxydiazine	S,T	BF	Determined distribution between blood and other body fluids.	342
II. *Studies of Two Sulfas*				
Sulfamethoxypyrazine, sulfamethazine	S(O,P)	BF	Blood levels determined.	343
Sulfathiazole, sulfamethazine	S(D)	EP	No clear association noted.	344
Sulfaproxyline, sulfamerazine	S(R)	E,D	B = 38–48%(Sp), 0–3%(Sm).	345
Sulfamoxole, sulfamethoxine	S(H)	D	Both bound similarly.	346
Sulfametopyrazine, sulfamethoxypyridazine	S(H)	BF	—	259
2 Sulfapyrazine derivatives	S	BF	Determined plasma and urine levels.	347
2 Sulfanilamido derivatives	S(H)	BF	Compared normal and renal patients.	348
III. *Studies of Three or More Sulfas*				
3 Long-acting sulfonamides	S(H)	D,BF	Activity not related to binding.	196
3 Sulfonamides	S(H)	U	Only unbound fraction biologically active.	194
	S(H)	BF	Binding had no effect on activity, but authors used large serum dilutions.	195
	S(H)	BF	Binding high, excretion rate low, acetylation low.	256
	S	—	Concluded that binding important in drug distribution.	349
3 Sulfonamides & N[4] glycosides	S(R)	D	Glycosides binding similar to that of bases.	350
3 Sulfonamides & 3 derivatives	S(H)	D,BF	B = 19–57%, blood levels determined.	351
4 Sulfonamides	S(H)	D	Biological activity and acetylation dependent on unbound drug.	249
	S(H)	—	Determined protein & RBC binding.	352
	S(10 species)	D,B	Determined species differences in binding. Phenylbutazone displaced bound drug.	197
	S(H)	E,D	Determined excretion rate and displacement by diethylbarbiturate.	253
4 S-containing compounds	A(B)	D	—	353
4 Sulfonamides & acetylated	S(R,Rt,D)	D,BF	Acetylated derivatives bound similarly to	354

(*Continued on next page.*)

TABLE I—(*Continued.*)

Substance	Protein Studied[a]	Method[b]	Remarks[c]	Ref.[d]
derivatives			parent compounds.	
5 Sulfonamides	S(H,O,S,P,E,T)	D	Human serum possessed highest affinity, turkey serum had least.	355
	S(R)	BF,U	Determined blood and tissue levels, excretion rate and volume of distribution after injection.	356
	A(B)	D	Determined n & k at pH 4.9–8.	357
	PF(H)	EP	1° binding to γ-globulin.	358
6 Sulfonamides	S(H)	U,UC	B = 62–96%.	359
	A(B)	D	Data fit Langmuir isotherm.	360
	S(H)	U	—	361
7 Sulfonamides	S	U,D,DF	B = 63% to over 90%.	362
	S(Rt)	D,BF	Data fit Langmuir isotherm; *in vivo* and *in vitro* studies reported.	184
8 Sulfonamides	S(H)	D	n = 1.56 to 3.26; kd = 6.1 to 848.6 μmoles/L.	363
14 Sulfonamides	S,A	D,U	Discusses types of binding and pharmacodynamic effects.	193
19 Sulfonamides	S(R)	U	Discusses binding, distribution, and excretion.	183
21 Sulfonamides & 6 metabolites	S(H)	U	Binding resulted in decreased glucuronidation, increased acetylation.	364
35 Sulfonamides	S(H)	U	Only unbound drug found to be active.	194
Series of several sulfonamides	S(H)	D	Discusses structure-binding correlation.	365
	A(H)	OR	Tertiary structure of albumin altered by binding.	366
	A,S(H)	D	Determined temperature and concentration effects.	367
	S(H)	U	Correlated binding, ionization, and metabolism.	251
	S,A(H)	UC,GF,D	Good agreement between experimental methods.	368
	S,A,G(γ)(H)	D	Influence of pH and sulfa structure studied.	369
	A(B)	NMR	—	159
	S(B)	U	Ultrafiltration discussed.	370
	S(H)	UC	Compared results with dialysis and ultrafiltration.	371
	S	—	Tripiraphen displaced bound sulfa.	372
Several pyridazine sulfonamides	S(H,M,Rt)	U	B = 60–90%.	373
Series of long-acting sulfonamides	S(H,R,Rt,B,D,M)	D,U,E,	Data fit Freundlich isotherm; determined activity, tissue distribution, and excretion.	182
	S(M,D,B,R,Rt,H)	D	Elimination rate independent of binding, activity, and tissue distribution dependent on binding.	181
	S(H)	U	Noted dependency of kidney elimination on binding.	255
	S	BF	Slow elimination due to protein binding.	254
	S(H)	BF	Factors affecting renal excretion discussed.	374
	S,A,G(γ)(H)	D,U	—	375
	S(M)	D,BF	Determined blood, tissue, and CSF levels.	376
	A(H,B)	—	n's reported.	
	G(γ)(H)	D	—	377
		Barbiturates		
Phenobarbital	A(B,H)	D	B = 0%(4% alb.), pH independent 6.1–8.6.	378
Pentobarbital	S(D)A(H)	(^{14}C)	Studied influence of binding on peritoneal dialysis in dogs.	186
	S(Rt)	B	Radiocontrast media and dextran potentiated barbiturate activity.	212
Thiopental	A(B)	D	n = 5, k = 12,000 (8° C., pH 7.4). EDTA decreased n and k.	379
	S(R)A(H,B)	U	Correlated sleeping time and binding.	380
Thiopentone & buthalitone	S(H)	D,B	Determined binding, partition coefficients, and plasma levels.	381
3 Barbituric acids	S(H)	U	Determined % bound and clearance.	382
4 Barbiturates	S(H)	GF(^{14}C)	Studied effect of urea and SO_4.	145
7 Barbiturates	S(H)	D	B = 46–81%.	383
Series of barbiturates	S(R)A(B)	U	B = 5–65%. Determined effects of pH, salts, and temperature. Reported n and k.	384
		Miscellaneous Drugs		
I. *Anti-inflammatory Agents, Analgesics, and Antipyretics*				
A. Salicylates	S(H)	D,EP	Compared binding behavior *in vitro* and *in vivo*. Studied effects of heparin & thyroid hormone.	237
	A(H)	D	B = 66–82%, n = 2.7–3.4(5°, pH 7.4).	385
	S(H,R)	D,B	Measured febrile response in rabbit.	198
	S,PF(B)	D	—	386
	S(R,B,H)	U	Binding and excretion rate compared.	387
	A(B)	D	Determined structure-binding correlations.	388
	A(B)	D	Measured competition by methyl orange.	389
	S	F	1° binding to albumin.	390
	A(B)	D	Studied 27 benzoate derivatives. Reported n, k, % binding, and pH effects.	211
B. *p*-Aminosalicylic acid	A(B)	D	Varied pH and ionic strength.	391
	S(H)	U	B = 58–73%	392
C. Na salicylate & morphiline sal.	S(H)	BF	Determined blood level and excretion rates with and without PABA.	393
D. Several nonsteroidal drugs	PF(B,H)	ST	Anti-inflammatory activity related to binding.	213

(*Continued on next page.*

TABLE I—(*Continued.*)

Substance	Protein Studied[a]	Method[b]	Remarks[c]	Ref.[d]
E. Other misc. drugs				
Morphine	S(H,D)	U	Suggested tolerance and prolonged blood levels related to binding.	264
Phenylbutazone	S	D	B = 99%.	394
	S(H)	D,U	n = 1.	395
	S		Measured association *in vivo* and *in vitro*.	396
	S(H)	D,SP	Loose binding, not displaced by salicylamide.	397
	S(H)	D		398
p-Hydroxyphenylbutazone	S(H)	D	Binding behavior similar to phenylbutazone.	399
Colchicine	S	SP	—	400
Probenecid	PF(H)	D	Reported % binding and half-lives after oral and i.v. doses.	401
	S(D)	U	Data fit Freundlich isotherm. B = 70–80%. Determined blood levels after injection.	402
Several anti-inflamm. drugs	A(B)	SP	Determined displacement of pyridoxal PO_4 and trinitrobenzaldehyde.	216
	S(B)G(H)	ST	Measured stability of protein to UV, heat, urea, and formalin denaturation.	403
II. *Tranquilizers and Antidepressants*				
A. Phenothiazines				
Chlorpromazine	S(R)	E	1° binding to albumin and globulins.	404
Phenothiazone & thional	A(B)	SP,D	Detn. n's and k's for oxidation products of phenothiazine.	405
Chlorpromazine & imipramine	S(D)	D,E	B = 31–36%, pH 8.6.	406
4 Phenothiazines	A	GF	Correlated antihistaminic activity with degree of binding.	200
B. Dicarbamates				
Meprobamate & 3 other derivatives	S(H)	D(^{14}C)	Correlated % binding with partition coefficients.	407
III. *Diuretics*				
Chlorthiazide	S(H)A(B)	D	Caused increased binding of pempidine.	247
Meralluride Na	S(H)	D	B = 90%(1:1000 concn.) and 50%(1:100 concn.).	408
Sulfamoyl-anthranilic acid derivative	S(E)	D	—	409
Ethacrynic acid	A(B)	D,EP(^{14}C)	n_1 = 4, n_2 = 12 (26°, pH 7.4.).	410
Acetazolamide	S(R)	D	B = 10–35%.	411
IV. *Antimalarials*				
Chloroquine	S(H)	E	1° binding to albumin.	412
	A(B)	SP	B = 33%, determined pH effect.	413
Acridine & quinoline derivative	S(B)	SP	Derived equations for spectral analysis.	414
V. *Antineoplastics*				
Methotrexate	S(H,D,M)	U(^{3}H)	B = 50%, determined excretion rate after i.v. dose.	415
	S,A(B)	U(^{3}H)	B = 50% BSA, displaced by PABA, sulfas, salicylates.	226
Melphalan	S(H)	U,D	B = 50–60%.	416
Pluramycin	PF(H)	C,E,UC	Bound drug less toxic.	202
2 Nitrogen mustards	S(H)	U	Data fit Freundlich isotherm.	417
3 Nitrogen mustards	S(H)	U,ST	Hydrolysis rate decreased in serum. Strong binding of 2 derivatives.	418
VI. *Anticoagulants*				
Heparin	A	E,T,B	Also studied suramin binding.	419
	A(B)	E	Also studied suramin binding.	420
	A(B)	E	1:2 heparin–alb. complex.	421
Bishydroxycoumarin	PF	D	B = 99%, half-life is dose-dependent.	261
Ethyl biscoumacetate	S(H)A(H)	D	B = 90%.	422
Warfarin Na	S,A(H)	B,BF	Displacement by phenylbutazone caused increase in activity.	222
	S(H)	B,BF	Hemorrhagic episodes with co-administration of phenylbutazone.	223
	S(H)		Clotting time increased with co-administration of oxyphenbutazone.	224
	S(H)	E,D	n_1 = 1, k = 1.54 × 10^5, also calculated $\Delta F°$, $\Delta H°$, $\Delta S°$. Bound to albumin.	423
VII. *Hypoglycemic Agents*				
Glycodiazine	S(R,Rt,D)	B,BF(^{3}H)	B = 81–98%.	424
Tolbutamide	S,A(H,B)	U,B	Determined n and k, 5-ethyl sulfadiazine increased activity.	217
	S(H)	D,B,BF	Determined displacement by sulfa and phenylbutazone.	219
Chlorpropamide	S(H)	B	ASA increased hypoglycemic effect.	425
	S(H)	D(^{35}S)	% bound increased with increasing concentration.	426
	S(H)	D	B = 80%.	151
	S(H)	D(^{35}S)	B = 80%.	150
Phenethylbiguanide	A,G(B)	D	No significant interaction.	427
4 Sulfonylureas	A(B,H,D,E,P,R)	D,U	Noted species differences in binding. Salicylate displaced bound drug.	218
VIII. *Cardiovascular Drugs*				
Digitoxin	S(Rt)A(B)	D,U	Also determined binding of metabolites.	428
	S(Rt)	E(^{3}H)	Binding capacity = 0.01 Gm./mg. protein.	148
	PF(H,Rt)	EG(^{14}C)	1° binding to albumin fraction.	149
Several digitalis derivatives	A,G(H)	UC	Determined k and $\Delta F°$. Discussed binding forces.	429
Quinidine	A(H)	D	n = 3 (pH 10); ε-amino and carboxyl groups involved. Determined pH effects.	430
	A,G(H)	D	n = 1 (pH 7.4) k = 7.7 × 10^3. Displaced by other quinoline drugs.	431

(*Continued on next page.*)

TABLE I—(*Continued.*)

Substance	Protein Studied[a]	Method[b]	Remarks[c]	Ref.[d]
	A(H)	D	n = 1, k = 0.2 to 0.8 × 10^4 at pH 5.5–8. n = 3, k = 1.1 × 10^4 at pH 10.	432
Procainamide	S(D)	D	B = 15%, calculated that most of drug localized in tissue.	433
IX. *Anesthetics*				
Procaine	S(D,R)	U	Data fit Freundlich isotherm. Determined effects of citrate, NaF, and PABA.	434
Procaine & 3 other local anesthetics	A,G(H)	D	n = 2, k varies with drug. 1° binding to albumin.	435
3 Anesthetic gases	A(H)	S	Albumin increases solubilities of gases.	436
14 Anesthetic gases	A(B)	OR	Correlated high activity with high degree of binding.	437
X. *Radiopaque Agents*				
	S(H)	E,A(^{131}I)	Used normal and diseased plasma.	438
	S(Rt)		Studied 4 compounds ranked in order of binding.	439
XI. *Plasma Extenders*				
Dextran	A(H)	E	Determined effect on binding of age and mol. wt. of dextran.	440
	PF(H)	E	1° binding to albumin.	441
PVP	S	E	Mol. wt. 33,000 bound; mol. wt. 12,500 not bound.	442
XII. *Parasympatholytics*				
Atropine	S(H)	U,UC	Calcd. 50% of therapeutic dose bound *in vivo*.	443
	S,G(H,R)	EP,D	—	444
	A(B)	B,U,SP	N = 20 (pH 6), n = 100 (pH 8). Albumin decreased anticholinergic activity.	201
	A(B)	U,D	Determined effect of pH and buffers. Displaced by choline and acetylcholine.	248
XIII. *Anti-infectives*				
Furazolidone & nitrofurantoin	S(B,H)	E	1° binding to albumin.	445
5 Nitrofurans	PF(B)	—	—	446
20 Nitrofurans	S(Rt)	U	No correlation between binding and pharmacological effects.	447
XIV. *Tuberculostatics*				
Isoniazid	S(H)	(^{14}C)E	No apparent binding noted.	147
	S	PG	—	448
	PF(B,H)	U,E	PAS, streptomycin bound, but not isoniazid.	449
Sulzolin	S(H)	U	B = 16%.	450
XV. *Misc. Studies and Studies with Drugs of Various Classes*				
Rivanol	A(B)	E,D,P	n = 17, k = 150.	451
Disulfuram & diethyldithiocarbamate	S(H)	GF	Suggested formation of disulfide bond with disulfuram.	452
d-Tubocurarine	S(H)	BF,D,E	Used plasma from normal and refractory patients.	199
Epinephrine & norepinephrine	S(H)	BF	Epinephrine highly bound.	453
Several basic amines	S	SP	Studied ephedrine, amphetamine, and adrenalin.	454
Xanthine derivatives	A(B)	D	Determined % bound, n, k, and structural requirements for binding.	455
	A(B,H)	SP	Determined effects of temperatures, pH, and modified protein structure.	456
Several alkaloids & mercurials	A,G	E	—	457
3 Drugs	S(Rt)	Dc	37°. Studied thiopental, acetazolamide, and iproniazid.	458
5 Drugs	S	D	Studied sulfadimethoxine, chlorpropamide, phenbutamide, acepromaxine, and dimethylbebeerine.	459
Vitamins				
Vitamin B_{12}	S(H)	E,B	B = 93%, 1° binding to α_1-globulin.	460
	S(H,B)	E	Determined effect of pH. 1° binding to α-globulin.	461, 462
	S(var.)	C(^{60}Co)	Used 11 animal species. 1° binding to albumin.	154
	S(H)	D,C(^{57}Co)	Noted several binding sites.	155
	S(H)	—	Binding by transcobalamin studied.	463
	S(H)	C	Hydroxycobalamin and cyano derivative bound by transcobalamin.	464
	S(H)	C	Normal serum saturated *in vivo*.	465
	S(H)	Ad	Bound vitamin resists charcoal adsorption.	466
	S(H,D,R,F,C)	D	n = greater than one.	467
	S(var.)	U,E	Studied binding in 7 animal species.	468
	S(H)	C	Noted two classes of binding sites.	469
Vitamin B_{12}	S(H)	EP,C	Studied normal and leukemic serum.	470
	S(H)	Dc	Endogenous B_{12} bound greater than exogenous B_{12}.	471
	S(H)	E	1° binding to α-globulin.	472
	S(H)	B	Noted a limited binding capacity.	473
	S(H)	D	Used normal and leukemic serum. 1° binding to β-globulin.	474
	S(H)	EG	Used normal and leukemic serum.	475
	S	E,A	1° binding to γ-globulin.	476
	S(H)	E	1° binding to α-globulin.	477
	S(H)	D	1° binding to γ-globulin, trypsin, and pepsin displaced bound vitamin.	478
	S(H)	EP	1° bound to α_2-globulin.	479
	S(H,C)	E	Measured effect of sialidase on binding.	480
	S(H)	E	*In vivo* 1° binding to α_1-globulin. *In vitro* 1° binding to α_1 and α_2-globulin.	481

(*Continued on next page.*)

TABLE I—(*Continued.*)

Substance	Protein Studied[a]	Method[b]	Remarks[c]	Ref.[d]
	S,CSF	E,A(^{58}Co)	Also measured binding of ^{45}Ca.	153
	S(H)	D(^{60}Co)	Compared normal and leukemic serum.	482
	S(H)	GF	Compared normal and leukemic serum.	483
Hydroxocobalamin	ST	—	Binding to serum protein greater than to liver tissue.	484
Vitamin A	S(R)	BF,E	1° binding to albumin.	485
Vitamin D_2	S(H)	E(^{14}C)	1° binding to α-globulin and albumin.	486
Biotin	S(H)	EP(^{14}C)	1° binding to albumin.	152
Vitamin D_3	S(D)	P,UC,BF(^{3}H)	Noted strong binding *in vivo* and *in vitro*.	487
Tocopherol	A(B)	Pt	—	125
	S	B	Bound vitamin apparently had same activity as unbound.	488
Several fat-soluble vitamins	S(H)	E,D	Vitamin A bound 90%, other vitamins tested bound 10%.	489
13 Vitamins	PF(H)		Determined distribution of vitamins between globulin, albumin, and other proteins.	490
			Endogenous Substances	
Insulin	S(H,Rt,GP)	—	Tolbutamide displaced bound insulin.	246
	S(H)	E(^{131}I)	Binding to a specific insulin antibody reported.	491
	S(H)	E(^{131}I)	Used serum from normal and diseased patients. 1° binding to albumin and globulin.	492
	S(H)	E	Bound insulin found not to be active.	493
	S	Ad	Measured differential adsorption to Cellulose.	494
Glucagon	S(H)	(^{131}I)	Measured effect of pH and growth hormone.	495
Growth hormone	S(H)	E	1° bound to α_2-globulin.	496
Vasopressin	S(H)	B	Pressor activity decreased in presence of protein.	497
Serotonin	S(H)	B	$k = 6.5 \times 10^2$. Activity decreased by albumin. Measured effects of pH, Cu, and Zn.	498
Hyaluronic acid	A(B)	E,UC	—	499
Urate	S(H)	GF	Saturation of protein observed in hyperuricemic patients.	500
	S(H)	GF	Saturation at 10 mg.% urate with 50 mg.% HSA.	138
Bile acids	PF(H)	D	Detn. k and n. Studied effect of pH and structure.	501
Palmitic acid	A(B)	(^{14}C)	Measured uptake of acid by rat epididymal fat pads.	502
Pyridoxal-5-PO_4	A(B)	SP	$n_1 = 1$, $n_2 = 1.9$, $n_3 = 2.5$, $k_1 = 10^6$, $k_2 = 10^5$, $k_3 = 7.8 \times 10^3$.	503
18 Amino acids	S(H)	—	—	504
Indole analogs	A(H)	D	Determined effect of fatty acids, $\Delta F°$, $\Delta H°$, $\Delta S°$.	505
	A(H)	D	Determined effect of pH, salts, and fatty acids.	119
	A(H)	D	Determined effect of pH, salts, and fatty acids.	506
Tryptophan	A(H,B)	D	Reported n and k, effect of pH, fatty acids, and competing compounds.	507
	S(H)	U,UC	$n = 1$, B = 75%, k reported with and without competitors.	508
	S(H)	U,D	Calculated $\Delta F°$, $\Delta H°$, $\Delta S°$.	509
	S(H)	D	Used D and L isomers.	510
Acetyl tryptophan and skatol	A(B)	D	Studied effect of KCl, dioxane, temperature, pH, urea, and glycine.	511
Phosphatidylserine	A(H)	T	$n_1 = 2$, $n_2 = 30$; $k_1 = 2 \times 10^5$, $k_2 = 1.3 \times 10^3$.	512
Bilirubin	A(H)	B	Measured oxidative phosphorylation in brain and liver mitochondria.	513
	S		pH effect determined, 1° association with albumin.	514
	A,G(H)	SP,EP	1° association with albumin, none with globulin.	515
	A(B,H)	SP	Bound to extent of 20 mg./Gm. protein.	516
	PF	E	1° association with albumin. Association with globulins occurred at high concentration.	517
	S(H)	EG	Only albumin binding found in hyperbilirubinemic serum.	518
	A(H)		Studied peritoneal dialysis.	187
	S(H)	D,UC	B = 98%, $n = 10$, also studied metabolites.	519
	S(H)	SP	Displaced by sulfa, salicylates, pH, caffeine Na benzoate.	229
	S(H)A(B)	SP,U,D	Bound form displaced by salicylates and sulfas.	228
	S(H)	BF	Studied 40 infants with neonatal jaundice. Bilirubin displaced by heme, salicylates, and sulfonamides.	227
	S(H)	BF	Compared ability of drug to displace bilirubin in infants.	232
	S(H)	BF	Studied effect of sulfisoxazole on bilirubin level in infants.	230
Bilirubin	A,S(H)	U,GF	$n = 2$ for HSA, displaced by sulfonamides.	231
	S(D,H)	U,D,UC,E	1° bound to albumin, measured excretion rate.	520

[a] Type of protein: A, albumin; CSF, cerebral spinal fluid; G, globulin; L, lymph; PF, plasma fractions; S, serum or whole plasma; T, tissues. Animal species in parentheses: B, bovine; C, chicken; D, dog; E, horse; F, frog; G, goat; GP,

(*Continued on next page.*)

TABLE I—(*Continued.*)

guinea pig; H, human; M, mouse; O, ox; P, pig; R, rabbit; Rt, rat; S, sheep; T, turkey. [b] A, autoradiography; Ad, adsorption; B, assay for biological activity; BF, assay of biological fluids for drug; C, column chromatography; D, dialysis; Dc, continuous flow dialysis; DF, diffusion; E, electrophoresis; EG, gel electrophoresis; EP, paper electrophoresis; F, fluorescence; GF, gel filtration; NMR, nuclear magnetic resonance spectroscopy; OR, optical rotation; P, precipitation; PG, polarography; Pt, partitioning; S, solubility; SP, spectrophotometric; ST, stabilization of protein or drug; T, turbidimetric analysis; U, ultrafiltration; UC, ultracentrifugation. [c] Includes experimental conditions, observations, and conclusions derived from the various studies. B = percentage of drug which was bound; 1° = protein involved in primary interaction; n = number of binding sites on the protein; k = binding association constant; k_{dp} = binding dissociation constant; U = drug concentration in units. [d] Grouped by class of compound investigated.

TABLE II—SUMMARY OF STUDIES INVOLVING PROTEIN BINDING OF VARIOUS DYES

Dyes	Ref.
Azo Dyes	
Methyl orange	(117, 122, 521–534)
Methyl red	(535)
Trypan blue	(536–538)
Trypan red	
Congo red	(534, 539–543)
Evans blue	(124, 544, 545)
Miscellaneous azo dyes	(91, 116, 265, 546–556)
Phthalein Dyes	
Phenol red	(557–562)
Bromophenol blue	(563–567)
Bromocresol green	(565, 568–570)
Bromothymol blue	(571)
Sulfobromophthalein	(137, 572–574)
Fluorescein	(575)
Miscellaneous dyes and studies involving several classes of dyes	(576–595)

tein was measured by several investigators (45, 46). Kripalani and Sorby (47) measured albumin binding of cortisol and its degradation products and have emphasized the necessity of determining the extent of steroid degradation when equilibrium dialysis and other methods requiring extended periods of time are employed to detect and quantitate binding behavior. Blair *et al.* (48) and Beisel *et al.* (49) studied the binding and renal excretion of cortisol and the glucuronides of 17-hydroxy-corticosteroids. A number of other investigations employing electrophoresis (50–52), ultracentrifugation (53), ultrafiltration (54–56), and biochemical assays (54, 57) have dealt with the binding of corticosteroids to plasma proteins of man, rat, cow, and a variety of other animal species.

Similarly, the binding of thyroid hormones by proteins has been the subject of comprehensive reviews (58–66) and references to this topic were also omitted from Table I. Such interactions are thought to be of consequence in the distribution, biotransformation, elimination, and activity of the hormones. Specialized plasma proteins with high affinities for thyroxine have been identified and classified. Of possible therapeutic importance is the observation that 2,4-dinitrophenol, salicylate (58, 59), trypan blue (58), organic dyes, and diphenylhydantoin (67) can inhibit the binding of thyroxine by some proteins. An interesting study (68) showed that ethylchlorophenoxyisobutyrate can apparently increase the binding capacity of serum proteins for triiodothyronine. More recent studies, which were not included in the cited review articles, utilized a variety of techniques including fluorescence (69), dialysis (69, 70), and electrophoresis (71–77) to demonstrate binding. Other studies were concerned with the effect of subject age (78), sex (79), thyroidectomy (80), pH, and buffers (81) on the plasma binding of thyroid hormones.

A number of studies dealt with the interactions of plasma proteins with compounds which are not of therapeutic significance, and illustrate the ability of albumin, in particular, to interact with a seemingly limitless variety of structures. Many surfactants (82–92, 3), for example, have been studied in this respect. Ethanol (93), various polyelectrolytes (94), polymers (95), fatty acids (96–98), buffer components (99–101), thio acids (102), quinone imides (103), acetamide (104, 105), octane and dodecane derivatives (106), *p*-aminobenzene (107), phosphoric esters (108), aromatic acids (109), benzpyrene (110), and a variety of other compounds have been shown to reversibly combine with plasma proteins. In addition, many inorganic anions and cations were observed to interact with albumin (111–113, 2). It has been demonstrated that the presence of inorganic ions can influence drug protein interactions by forming a bridge between protein and small molecule (114, 115), by competing for available binding sites (116–119), by altering the configuration of the macromolecule or inducing changes in ionization characteristics of the protein (2, 120).

New Experimental Methods — Classical methods, employed to detect, determine, and study binding characteristics, such as equilibrium dialysis, ultrafiltration, ultracentrifugation, and electrophoresis have been discussed by a number of authors (1, 6, 121). Although most of the studies tabulated in Tables I and II utilized these methods, modifications and some relatively new approaches were also described. Stein (122) described a rapid, nonequilibrium dialytic method which was shown to yield, for the protein binding of methyl orange, results which were comparable to those obtained by equilibrium methods. Bennett and Kirby (123) developed a vacuum ultrafiltration cell which was used to investigate the

binding of penicillins. Differences in the polarographic behavior of bound and free substrate have been demonstrated and applied to the determination of binding parameters as illustrated by the studies of Markus and Baumberger (124) with Evans blue. The interactions of protein with methyl orange (117), fatty acids (97), tocopherol (125), testosterone (118, 126–128), and other steroids (129) were examined by determining the influence of protein concentration on the apparent partition coefficient of the small molecule. Gel filtration has, in recent years, been extensively used to detect the binding of small molecules. Techniques and theory applicable to this method have been presented by several authors (130–134). Such procedures employ beads of cross-linked polysaccharides which are water insoluble but which undergo extensive swelling when in contact with water. Macromolecules such as proteins and protein complexes are unable, because of size, to penetrate into the internal volume of the gel matrix while small molecules do penetrate. The method is capable of yielding data similar to that obtainable from dialytic techniques without concomitant problems of membrane binding, Donnan effects, and time considerations. However, difficulties can be encountered. For example, many small molecules can interact with the gel material resulting, from mass law considerations, in dissociation of the protein complex (135, 136). In addition, when gel-filtration chromatography is used to demonstrate binding, the elution step results in dilution of the binding system and possible significant dissociation of the complex. The technique has been employed to study the binding by plasma proteins of a variety of compounds such as dyes (137), uric acid (138), antibiotics and sulfonamides (139–141), corticosteroids (142–144), and barbiturates (145).

Improved sensitivities have been realized with many of the classical techniques through the use of isotopically labeled small molecules. The ease of analysis afforded by this approach has permitted *in vitro* studies at concentration levels comparable to those encountered *in vivo*. This subject was recently reviewed by Cohen *et al.* (146). Illustrative of the utility of this approach are studies which employed isoniazid-^{14}C (147), ^{3}H or digitoxin-^{14}C (148, 149), chlorpropamide-^{35}S (150), testosterone-^{14}C (151), biotin-^{14}C (152), vitamin B_{12} ^{57}Co-, ^{58}Co-, ^{60}Co- (153–155), penicillins-^{14}C (156, 157), and progesterone-^{14}C (158).

Recent studies have established the value of nuclear magnetic resonance spectroscopy as a tool for studying protein–small molecule interactions and for assessing the extent to which various functional groups on the small molecule participate in the interaction. Jardetsky and Wade-Jardetsky (159) and Fisher and Jardetzky (160), for example, studied the binding of sulfonamides and penicillins by determining NMR spectra of the compounds in the presence and absence of albumin. They observed that relaxation rates for certain protons changed in the presence of protein and were able to conclude that the *p*-aminobenzene sulfonamide moiety of sulfonamides and the phenyl ring of penicillin were the functional groups which participated in the interaction.

The majority of reported studies were concerned with a characterization of binding behavior at equilibrium. A limited number of studies were also conducted to assess the kinetics of binding. Robbins *et al.* (161) investigated the rate of interaction of thyroxine with serum protein. They employed a rapid dialysis technique which consisted of placing a thin piece of lens paper, which was saturated with a solution of drug or drug and protein, directly on a dialysis membrane. It was shown that the association and dissociation reactions were complete within 2 min. A more sophisticated method was also employed and consisted of determining the rate of quenching of the fluorescence of albumin caused by the interaction with thyroxine. Association was determined to be complete within 150 msec. The dissociation process was resolved into two steps with half-lives of 0.1 sec. and 7 sec. Froese *et al.* (162) employed a temperature-jump technique to investigate the kinetics of interaction between albumin and two azo dyes. The reaction system was perturbed by about 10° within 1 μsec. and the readjustment of the system to equilibrium conditions at the higher temperature was followed spectrophotometrically. Rate constants for association were found to be 0.36×10^6 and 2.1×10^6 mole^{-1} sec.$^{-1}$. Rolinson and Sutherland (163) attempted to investigate the kinetics of penicillin binding using dialysis and ultrafiltration. They found that equilibrium was attained rapidly and that the rate of attainment was too fast to investigate by these methods. Although there is general agreement that, in most systems, rates of association and dissociation are very rapid, there is a paucity of quantitative information and a need for additional studies in this area.

Treatment and Presentation of Experimental Data—Most investigators have been concerned with protein–small molecule systems at equilibrium and with the determination of degree of binding as a function of compositional and environmental variables. A number of methods for treating and presenting the results of such investi-

gations are commonly used. The reciprocal plot and the Scatchard plot of experimental data are employed frequently and are directly derived from mass-law considerations. It can be shown (6) from such considerations that reversible binding is described by the familiar equation:

$$r = \sum_{i=1}^{i} \frac{n_i k_i D_f}{1 + k_i D_f} \qquad \text{(Eq. 1)}$$

where

- r = moles of small molecule bound to total moles of protein in the system.
- n_i = number of binding sites in the i'th class of sites.
- k_i = intrinsic association constant for the binding of small molecule by sites in the i'th class.
- D_f = concentration of unbound small molecule.

Equation 1 assumes that activities can be represented by concentrations, that all sites within a class are equivalent in binding affinity, and that all sites are mutually independent. For a single class of sites:

$$r = \frac{nkD_f}{1 + kD_f} \qquad \text{(Eq. 2)}$$

Equation 2 can be rearranged to yield:

$$1/r = 1/n + 1/nk\, D_f \qquad \text{(Eq. 3)}$$

which is the basis for the reciprocal plot in which $1/r$ is plotted as a function of $1/D_f$. Estimations of n and k are made from the slope and intercept values of such a plot. The Scatchard plot is based on a different rearrangement of Eq. 2;

$$\frac{r}{D_f} = nk - rk \qquad \text{(Eq. 4)}$$

A plot is made of r/D_f as a function of r; extrapolation to abscissa and ordinate allows estimation of n and nk. Curvature of such plots is usually indicative of the existence of more than one class of sites, *i.e.*,

$$r = \frac{n_1 k_1 D_f}{1 + k_1 D_f} + \frac{n_2 k_2 D_f}{1 + k_2 D_f} + \cdots + \frac{n_i k_i D_f}{1 + k_i D_f} \qquad \text{(Eq. 5)}$$

Graphical treatment of data by means of the reciprocal plot heavily weights those experimental points which are obtained at low D_f and can, therefore, lead to misinterpretations concerning behaviors at high D_f. The Scatchard plot does not suffer from this disadvantage and is the graphical method of choice. Extrapolation of a Scatchard plot curve, obtained when more than one class of sites are responsible for binding, yields $\Sigma n_i k_i$ and Σn_i. Various curve-fitting techniques can be employed to estimate individual n and k values. Hart (164) has recently discussed the analysis of such curves and showed that n and k values for each class of site can be evaluated by solving a set of simultaneous linear equations. For a system containing i classes of sites, $2i$ experimental points are required to generate $2i$ linear equations which can be solved for the i sets of n and k.

Reciprocal and Scatchard plots cannot be constructed if the nature and amount of protein in the experimental system is not known, as would be the case, for example, in determining the binding characteristics of plasma. Many authors have presented the results of such determinations in terms of "percent bound" or "fraction bound." This way of describing binding behavior is so commonly used that it is pertinent to repeat Goldstein's criticism (1): "The statement that a given fraction is bound in plasma is meaningless, unless qualified by an indication of the unbound drug concentration at equilibrium." The relationship between "fraction bound," β, and the fundamental parameters of binding, n and k, for the case of a single class of binding sites, is:

$$\beta = \frac{1}{1 + D_f/n\,P_t + 1/nk\,P_t} \qquad \text{(Eq. 6)}$$

where, P_t = total molar concentration of protein.

It is apparent that "fraction bound" exhibits a dependency on the concentration of free drug and the concentration of protein. Thus, "fraction bound" is a useful characterization of intrinsic binding strength only when D_f/nP_t approaches zero. Under such conditions, β approaches a maximum value and

$$nk\,P_t = \frac{\beta}{1 - \beta} \qquad \text{(Eq. 7)}$$

It should also be noted in this respect that experimental techniques which perturb the protein–small molecule system by dilution prior to the determination of the fraction of drug which is bound can grossly underestimate the true extent of binding. Saris (165) has presented data related to sulfonamide binding which illustrates this potential source of error. Similarly, Oppenheimer and Surks (166) have shown that when the association constant is large, such as in thyroxine-plasma systems, "fraction bound" is unaffected by reasonable dilution.

Krüger-Thiemer *et al.* (167) have discussed mathematical problems associated with studies on the binding of drugs in plasma samples. They showed that plasma binding, if due to a single plasma protein with a single set of sites, can be characterized by two parameters, according to the equation:

$$D_t = D_f\left(w + \frac{\beta' p}{D_f + k_{dp}}\right) \qquad \text{(Eq. 8)}$$

where

D_t = total molar concentration of drug in the plasma
D_f = molar concentration of unbound drug in the plasma
w = volume fraction of water in the plasma
β' = maximum binding capacity of plasma for drug in μmoles/Gm.
k_{dp} = intrinsic dissociation constant for the drug-protein complex
p = protein concentration in Gm./L.

They demonstrated the application of three equations, first published by Woolf (168), for linearizing data and for the graphical determination of β' and k_{dp}:

$$\frac{\beta' p}{D_b} - \frac{k_{dp}}{D_f} = 1, \qquad \text{(Eq. 9)}$$

$$D_b = \beta' p - k_{dp} \frac{D_b}{D_f}, \qquad \text{(Eq. 10)}$$

$$D_f = \beta' p \frac{D_f}{D_b} - k_{dp} \qquad \text{(Eq. 11)}$$

where D_b = concentration of bound drug. They also described the application of a digital computer to the estimation of β' and k_{dp} from experimental data and illustrated their approach with studies on three sulfa drugs. The binding behaviors of two of these could be adequately described by Eq. 8. The third drug exhibited a more complex behavior which could be explained by expanding Eq. 8 to include a term for a second binding process.

Sandberg *et al.* (169) and Rosenthal (170) have also discussed treatment of binding results when the nature and concentration of the protein is not known. They have recommended a Scatchard-type plot based on a rearrangement of Eq. 2:

$$\frac{D_b}{D_f} = nkP_t - kD_b, \qquad \text{(Eq. 12)}$$

which will also be recognized as a slightly modified form of Eq. 10. A plot of D_b/D_f as a function of D_b is independent of protein concentration and allows estimation of nkP_t, nP_t, and k from ordinate intercept, abscissa intercept, and slope. If more than one protein or more than one class of binding site on a single protein are involved in the binding, curvature of the plot results. This plot can also be used to provide a useful display of data from which can be determined the concentrations of all drug-containing species at any total or free drug concentration.

Hansch *et al.* (171) discussed a novel type of data treatment which was designed to provide an insight to the nature of intermolecular forces responsible for binding. They studied the binding by albumin of phenol and 18 substituted phenols and determined in each case the concentration, C, required to yield a 1:1 complex with the protein. They also determined the partition coefficient of each compound between octanol and water and calculated a substituent constant, π, which was defined as:

$$\pi = \log P_X/P_H, \qquad \text{(Eq. 13)}$$

where

P_X = partition coefficient of a phenol derivative
P_H = partition coefficient of phenol.

A semi-log plot of $1/C$, which is proportional to the complex association constant, *versus* π was found to be linear. The authors concluded that, mechanistically, binding closely paralleled the transfer of phenol from water to octanol, that binding was rather nonspecific, and was best described as being due to hydrophobic bonding.

Influence of Protein Binding on Drug Distribution—There is general consensus that, because macromolecules and macromolecular complexes pass across membranes only with difficulty, protein binding in plasma can influence the distributional pattern of a drug in the body. The extent to which this influence is manifest and significant has been the subject of a number of studies. Martin (172) has provided, by means of a simple model, a quantitative visualization of the potential effects of plasma binding on drug distribution. His treatment admirably serves as a basis for a discussion of this topic. His approach to this evaluation was based on the following assumptions: (*a*) drug in the body is distributed into two aqueous compartments—plasma and a compartment composed of the remaining body water, (*b*) unbound drug is in equilibrium in the two compartments, (*c*) binding of drug occurs only in the plasma and results from the formation of 1:1 complexes with albumin, (*d*) for a 70-Kg. man, the plasma volume is 3 L. and the total volume of water is 42 L., (*e*) the molar concentration of albumin in the plasma is 5×10^{-4}. He then conducted calculations related to the binding of four hypothetical drugs whose interactions with albumin could be characterized by association constants (k) of 10^4, 10^5, 10^6, and 10^7 L. mole^{-1}, respectively. For each drug he assumed a concentration of unbound drug, D_f, and calculated, first, the total concentration of drug in plasma, D_t, by an equation directly derivable from Eq. 6:

$$D_t = D_f \left(1 + \frac{P_t}{1/k + D_f}\right) \qquad \text{(Eq. 14)}$$

He then calculated for each value of D_f: (*a*) amount of drug in body = $39\,D_f + 3\,D_t$; (*b*) percent of drug, free in body = $42\,D_f \times 100/(39\,D_f + 3\,D_t)$; and (*c*) percent of drug in plasma = $3\,D_t \times 100/(39\,D_f + 3\,D_t)$.

The results of such calculations were presented in graphical form such as illustrated by Figs. 1 and 2. It is apparent from these representative theoretical curves that binding can influence drug distribution in the body and that the magnitude of the effect will depend both on the strength of association and the dosage of drug. For example, a strongly bound drug such as that with a k of 1×10^7 will, at low dosage levels, be concentrated primarily in the plasma compartment. However, at higher dosage levels, the fraction of drug in the plasma will be markedly reduced. Martin also emphasized another characteristic attributable to the plasma binding of drugs with a high affinity for proteins; that there is a dosage range within which small increases in dose result in relatively large increases in the amount of drug in the body which is not bound. He noted that this behavior can have interesting manifestations on dose-response characteristics and pharmacokinetic properties of such drugs. The treatment additionally emphasizes that binding to the plasma proteins will have an appreciable effect on drug distribution only if the strength of binding is quite large ($k > 10^4$). Martin (172) showed, to illustrate this point, that with a drug having a k of 10^4, the "fraction bound" in plasma can be 83.4% but, nevertheless, 73% of the total dose is present in the body in the unbound form. Martin's model is a highly simplified representation of what might indeed be a highly complex distributional pattern involving binding to tissue proteins, partitioning into fatty compartments, the unavailability of certain aqueous compartments, *etc.* Nevertheless, he clearly demonstrates that plasma-protein binding can be an important determinant in drug distribution for some drugs but that for many others, it might not be of significance in spite of *in vitro* demonstrations that interaction occurs to produce a seemingly high "fraction bound."

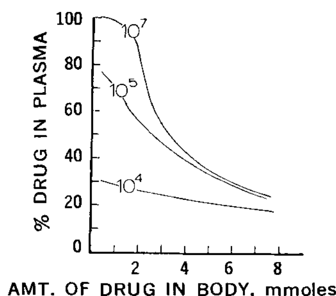

Fig. 1—A plot, based on theoretical considerations, illustrating the potential influence of plasma binding on the distribution of drug between plasma and other aqueous compartments in the body. Each curve represents a different value of k. *After Martin (172).*

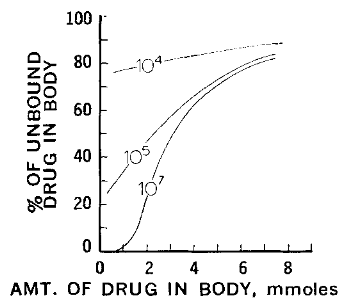

Fig. 2—A plot, based on theoretical considerations, illustrating the potential influence of plasma binding on the distribution of drug between bound and unbound forms in the body. Each curve represents a different value of k. *After Martin (172).*

A number of recent experimental studies have demonstrated, in a qualitative sense, that plasma binding can influence drug distribution. Verwey and co-workers (173–175) developed a method for sampling lymph by cannulation of the peripheral lymphatics of dogs. They noted that examination of peripheral lymph is useful in drug distribution studies since it is derived directly from interstitial fluid. They studied, in dogs, the distribution of seven penicillins between plasma and lymph and showed that for six of the seven analogs, plasma concentrations were significantly higher than those found in the lymph. The degree of binding in plasma and in lymph was determined and it was demonstrated that unbound drug was in equilibrium between the two fluids. Their results supported the conclusions of Rolinson (176) who considered theoretical aspects of protein binding as it relates to the distribution of penicillins and emphasized that only unbound drug is free to diffuse from plasma. Similar conclusions were made by Kunin (177) who showed with a series of penicillins that as the degree of protein binding decreased, localization of antibiotic in brain, muscle, lung, and heart of rabbits increased. He also showed that the co-administration of an agent capable of displacing protein-bound drug increased the amount of antibiotic which localized in the tissues.

Fishman (178) found that probenicid, when co-administered with penicillin, resulted in a higher level of antibiotic in the cerebral spinal fluid of the dog. He postulated that this effect was due, in part, to the displacement of protein-bound antibiotic by probenicid. Additional studies of this nature will be discussed in a subsequent section. Kharchenko (179, 180) also conducted studies on the distribution of antibiotics in the rabbit. He showed that when human serum was injected into the pleural cavity, distribution into this compartment occurred to a much greater extent than when saline controls were injected. Johnson *et*

al. (150) reported on the distribution of chlorpropamide-^{35}S in a patient with edema. They determined that the plasma concentration of drug was two to six times higher than that in the edematous fluid. Scholtan (181, 182) showed that a relationship existed between the degree of binding of sulfonamides and tissue distribution, in several species of animals. Rieder (183) also found a correlation between binding parameters and sulfonamide distribution in the rabbit and reported that the concentration of unbound drug in the plasma provides a reasonable estimate of the concentration of unbound drug in extravascular fluids. Ruiz-Torres and Meinig (184) reported, however, that no such correlation existed in their studies on the distribution of sulfonamides in the rat.

An interesting application of the influence of drug–protein interactions on drug distribution is illustrated by studies in which albumin was employed as an additive to the fluid used for reducing, by peritoneal dialysis, body levels of toxic substances. Campion and North (185) treated, in this way, dogs that were intoxicated with phenobarbital, amobarbital, and secobarbital and reported that the dialysis fluid containing albumin was more efficient than a control fluid. Bourne *et al.* (186) utilizing pentobarbital-intoxicated dogs, found that only a slightly greater efficiency was achieved with the technique. Grollman and Odell (187) observed that inclusion of albumin in dialysis fluid increased the amount of bilirubin which could be removed by a factor of 25. Other authors have noted enhanced removal, by this method, of salicylates from dogs (188) and barbiturates from humans (189).

Competitive Inhibition of Plasma Binding—A number of investigations have clearly demonstrated that the pharmacological response to a drug can be diminished as a result of interaction to form nondiffusable protein complexes. It is pertinent to briefly review some of these studies and to then examine the possibility of eliciting, either intentionally or unintentionally, a more pronounced response through the administration of agents which competitively inhibit binding.

Antibiotics and anti-infective agents have been intensely investigated in this respect. Plasma inactivation of penicillins, cephalosporins, and other antibiotics is a well-known phenomenon (190–192). Rolinson and Sutherland (163), for example, studied the plasma binding of 17 antibiotics and found that *in vitro* activity was inversely proportional to the observed extent of binding. Evidence supporting the premise that only unbound sulfonamides are antibacterially active was accumulated by Witzgall and Boyens (193) and Krüger-Thiemer *et al.* (194). Other workers (195, 196) failed to observe this effect, but it should be noted that their experimental methods employed rather large dilutions of plasma samples with concomitant pertubation of binding equilibria. Anton (197) also demonstrated an inverse correlation between bacteriostatic activity and extent of binding of sulfonamides.

Reynolds and Cluff (198) have shown that the antipyretic activity of sodium salicylate in rabbits was decreased when albumin was coadministered. Aladjenoff *et al.* (199) observed that plasma levels of *d*-tubocurarine were unusually high in patients who were refractory to the drug. They also found that the drug, when preincubated with albumin, lacked a curarizing effect in dogs. They suggested that the highly bound drug was unable to diffuse from the plasma to the neuromuscular junction. Jindrova *et al.* (200) evaluated four phenothiazine derivatives and determined that those which were least bound exhibited the greatest antihistaminic activity. Oroszlan and Maengwyn-Davies (201) found that the cholinolytic activity of atropine, on the isolated rat colon, was inhibited by the presence of albumin. Ogawara *et al.* (202) showed that a pluromycin A–albumin complex was less toxic than pluromycin A, and that the complex had a greater antitumor activity. Several investigators (54, 57, 203) reported that the presence of protein interfered with the participation of various steroids in biochemical processes.

Reversal of this type of drug "inactivation" by the addition of a competitive inhibitor to the binding system has been considered by a number of workers and attempts to evaluate such a possibility, *in vitro* and *in vivo*, have been reported. The theoretical foundations for this approach can be readily demonstrated. If drug, D, and competitor, C, for example, form 1:1 protein complexes and compete for the same binding site on the macromolecule, it can be shown that:

$$\frac{D_f}{D_t} = \frac{1 + k_D D_f + k_c C_f}{1 + k_D P_t + k_D D_f + k_c C_f}, \quad \text{(Eq. 15)}$$

where

k_D = association constant for the drug–protein complex
k_c = association constant for the competitor–protein complex
C_f = concentration of unbound inhibitor.

It is readily apparent, from Eq. 15, that the effectiveness of an inhibitor in "freeing" drug from combination with protein will depend on its concentration and affinity for the protein. It is il-

lustrative to assess quantitative aspects of such drug "displacement" by the construction of drug distribution curves in the same manner as was previously discussed. The results of such a treatment for a strongly bound drug ($k_D = 10^7$) is shown in Fig. 3. These theoretical curves were generated from Eq. 15 by the calculations and assumptions that were employed by Martin (172) and which were discussed previously. Various values of k_cC_f were assumed in making these calculations. It is obvious from examination of the curves that the influence of a competitive inhibitor on the fraction of drug which is free in the body can be quite pronounced and that this influence will be especially significant at lower dosage levels of the drug. It is also apparent that, in order to displace a strongly bound drug by this mechanism, an inhibitor must be present in the system at a relatively high concentration or have an affinity for the protein which is significantly greater than that exhibited by the drug.

Anton's studies (197, 204) have been frequently cited as an example of this approach to the possible potentiation of the pharmacological activity of a strongly bound drug. He was concerned with the inhibition, *in vitro* and *in vivo,* of sulfonamide binding. He demonstrated that phenylbutazone, sulfinpyrazine, ethyl biscoumarate, and iophenoxic acid were, among many compounds evaluated, effective in displacing protein-bound sulfonamides and that the antibacterial activity of a sulfonamide in the presence of albumin was markedly increased by the presence of a displacing agent. He showed quite dramatically that administration of sulfinpyrazone to rats which were dosed with sulfaethylthiadiazole or sulfamethoxypyridazine resulted in a precipitous decline in the total plasma concentration of sulfonamide but an increase in the concentration of unbound drug. Concomitant with this was an increase in the concentration of sulfonamide in tissues. Studies with other sulfonamides showed that displacing agents altered tissue and plasma concentrations of only those which were reasonably strongly bound. Genazzani *et al.* (205) determined that various tetracycline derivatives competed with sulfaethylthiazole for binding sites on albumin. Bahal (206) found that the binding of sulfaethylthiazole was modified by the presence of salicylate. His attempts to evaluate *in vivo* displacement of bound sulfonamide and the resulting effect on excretion rate was complicated by the observation that the agents which were effective in displacing the sulfonamide also retarded tubular secretion of the drug.

Kunin (156, 157, 207, 208) has published the results of exhaustive studies in which over 250

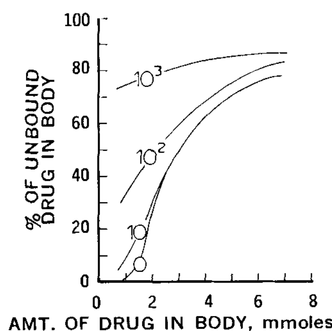

Fig. 3—A plot, derived from theoretical considerations, illustrating the potential influence of competitive inhibitors of plasma binding on the distribution of drug between unbound and bound forms in the body. See text for the assumptions made in deriving the relationship. Each curve represents a different value of k_cC_f.

compounds, representing a wide variety of structures, were evaluated for their ability to displace protein-bound penicillin G, penicillin V, and other derivatives. Three compounds—sulfamethoxypyridazine, sulfaethylthiadiazole, and acetylsalicylic acid—were found to be effective and were employed in studies with human volunteers to determine their effects *in vivo.* The results indicated that it was possible to reduce serum binding of penicillins in man by this approach. The administration of binding inhibitors tended to lower the total serum concentration and to increase the concentration of unbound antibiotic in the serum. His results demonstrated that the relative effectiveness of an inhibitor is defined by both the relative concentrations and relative affinities for the protein of drug and inhibitor. Thus, administration of sulfaethylthiadiazole was effective in reducing the binding of the moderately bound penicillin V from approximately 81 to 65%. However, the binding of strongly bound dicloxacillin was reduced only slightly from 98.3 to 97.7%. Kunin discussed the possible clinical implications of reversing, by even a small amount, the binding of a strongly bound drug. He also noted that attainment of enhanced clinical activity by displacement might be realized only with difficulty because of the large doses of inhibitors required and the often limited degree of displacement which may be achieved in man. Rolinson and Sutherland (163), Anderson *et al.* (209), and Fishman (210) have also reported the results of *in vitro* investigations on the competitive inhibition of penicillin binding.

Other studies in this general area include those of Davison and Smith (211) who observed that a variety of benzoates and salicylates are bound to the same sites on albumin. Lasser *et al.* (212) found that the administration of a highly bound radiopaque agent caused potentiation of pento-

barbital hypnosis which they attributed to the displacement of the barbiturate from protein complexes. Brodie (21) reported a correlation between the activity of nonsteroidal anti-inflammatory agents and their ability to displace bound corticosterone. Mizushima and Suzuki (213) similarly noted some correlation between the antirheumatic activity of nonsteroidal drugs and their ability to interact with various serum protein fractions. Florini and Buyske (214), however, concluded from their investigations that such compounds did not appear to function *via* displacement of bound hydrocortisone. Other studies have been concerned with the displacement of aldosterone by spirolactones and other steroids (44), and the mutual competition between cortisol, corticosterone, and progesterone (55), as well as estrone (215) for plasma protein binding sites. Skidmore and Whitehouse (216) have suggested that displacement of protein-bound pyridoxal phosphate or 2,4,6,-trinitrobenzaldehyde might be useful as an approach to the screening of anti-inflammatory activity. Buttner and Portwich (217) observed that 5-ethylsulfadiazine and tolbutamide mutually displaced each other from albumin binding sites. Concomitant administration of the two drugs resulted in enhanced clinical activity. Wishinsky *et al.* (218) measured the binding of four sulfonylurea drugs and found that addition of salicylate to the binding system resulted in a substantial increase of unbound drug. The occurrence of hypoglycemic episodes was reported with a patient who was receiving both tolbutamide and sulfaphenazole (219). It was explained, however, as resulting not from drug displacement but rather from an increased half-life for tolbutamide due to inhibition of a carboxylation process.

A general discussion of possible therapeutic difficulties which might be encountered in the concomitant administration of drugs has been presented by MacGregor (220) and Brodie (221). Several reports have been concerned with possible hazards resulting from the displacement of bound anticoagulants. Aggeler *et al.* (222) observed that phenylbutazone can decrease warfarin plasma concentrations 35 to 60%, decrease biological half-time 20 to 60%, and increase anticoagulant effects 40 to 80% in normal patients and 250% in warfarin-resistant patients. Other clinical reports (223, 224) noted a marked increase in prothrombin time as a result of the co-administration of sodium warfarin and phenylbutazone or oxyphenbutazone. Solomon and Schrogie (225) demonstrated by *in vitro* studies that a variety of agents were capable of displacing protein-bound warfarin. Reference has been made to the fact that methotrexate (226), which is administered in near toxic doses, can be displaced by salicylates and certain sulfonamides.

Consequences resulting from the displacement by drugs of protein-bound endogenous compounds have also been considered and studied. Bilirubin is an important example since its displacement can result in toxic manifestations in infants and in individuals with impaired bilirubin conjugating mechanisms. Odell (227, 228) showed that salicylates and sulfonamides, in concentrations which are encountered clinically, could displace bilirubin. He reviewed (229) the clinical implications of such an event. Kantor *et al.* (230) and Josephson and Furst (231) also considered displacement of this compound by the administration of sulfonamides to premature infants with kernicterus or to mothers before and during labor. Silverman *et al.* (232) compared the mortality rate of premature infants who received either penicillin and sulfisoxazole, or tetracycline. Their data suggested that kernicterus was more prevalent in the population which received the sulfonamide.

Christensen (233) has studied the effect of several benzoates and salicylates on thyroxine binding and has attempted to relate physiological and toxicological effects to the displacement of bound hormone. Yamada *et al.* (234) have concluded that a prime effect of diazo dyes, such as trypan blue, on the thyroid occurs due to displacement of thyroxine from plasma proteins and subsequent inhibition of TSH release. Numerous studies have shown that salicylate (235–237), hydantoin derivatives (238, 239), penicillins (239, 240), thyroxine analogs (241), dinitrophenol (242–244), and novobiocin (245) were capable of displacing bound thyroxine. In addition, studies on the binding of insulin (246) indicate that tolbutamide was able to liberate protein-bound insulin in guinea pig serum.

Of interest are two studies which indicate binding enhancement induced by the presence of a second compound. Thus, Dollery *et al.* (247) noted that pempidine, a ganglionic blocking agent, did not interact with bovine serum albumin. However, when chlorothiazide was added to the system, considerable binding was apparent. It was speculated that chlorothiazide induced a structural alteration in the protein which resulted in a configuration capable of binding pempidine. Similarly, acetylcholine was found to increase binding of atropine to albumin in systems buffered at pH values from 5 to 6 (248).

Protein Binding and Pharmacokinetic Behavior—The influence of protein binding on the time course of drug in the body has been con-

sidered by a number of authors. Many qualitative and semiquantitative observations have indicated that binding can be an important parameter in the pharmacokinetic characterization of a drug. *In vitro* studies have, for example, established that the addition of protein to isolated biochemical and physiological preparations can result in a decreased rate of substrate disappearance. Thus, Newbould and Kilpatrick (249) showed that the rate of acetylation of two long-acting sulfonamides in a perfused rabbit liver preparation was decreased when plasma was added to the perfusion fluid. They determined that the observed rate depended on the concentration of unbound drug. Similarly, Anton and Boyle (250) showed that, in an *in vitro* enzyme system, albumin interfered with the acetylation of sulfamethoxypyridazine. Wiseman and Nelson (251) reported a rank-order correlation between the rates of *in vivo* metabolism of a number of sulfonamides and their degree of protein binding. A number of other studies (252–256) have suggested that an interdependence does exist between the rates of elimination of sulfonamides and degree of plasma binding. Rieder (183), however, did not observe a correlation between binding parameters and rate of disappearance from plasma or rate of renal excretion for the various sulfonamides which he studied. Similar results were obtained by Scholtan (181, 182) and by others (257–259). Rieder, in his discussion, points out that only when elimination proceeds primarily *via* glomerular filtration and without significant tubular secretion and reabsorption should binding be anticipated to exert an appreciable effect on the rate of elimination of a drug.

Other observations pertinent to this topic include those of Bennhold (260). He noted that the rate of elimination of congo red from patients with depressed albumin blood levels (0.1 to 0.2% of normal) was approximately 60% more rapid than with normal subjects. Upon infusion of human albumin, the elimination rate of the dye was depressed to near normal levels. Weiner *et al.* (261) have suggested that the slow biotransformation and negligible kidney elimination of dicoumarol is a result of the high degree of protein binding of this drug. The prolonged blood levels of chlortetracycline[1] were explained by Sirota and Saltzman (262) as being due to protein binding since the renal excretion of the drug was found to be solely a process of glomerular filtration. Kunin (263) has similarly related the prolonged plasma levels, slow kidney clearance, and low apparent volume of distribution of methacycline to its degree of binding. Nadeau and Subolewski (264) have suggested that extended blood levels and tolerance to morphine in dogs may be related to binding. Priestly and O'Reilly (265) reported a qualitative correlation between the biliary excretion rates of four azo dyes and their relative degrees of binding to liver homogenates and blood proteins. Beisel *et al.* (266), from their studies on cortisol elimination, concluded that plasma binding provides a reservoir of readily available steroid which is protected from excretion and metabolism. Other workers (267–269) studying different steroidal compounds have made similar conclusions.

Krüger-Thiemer and his associates, who have made outstanding contributions to the quantitative understanding of the influence of protein binding on the behavior of drugs, recently (270) discussed pharmacokinetic models which focus attention on the involvement of the binding phenomenon. One model, which was discussed in detail, depicted a drug as being distributed and in rapid equilibrium between two compartments, plasma water of volume V_1, and residual body water of volume V_3. Protein binding occurred only in the plasma compartment and the rate of renal excretion or elimination, characterized by the rate constant k', was proportional to the concentration of unbound drug in the plasma water. A rate equation analogous to the following was derived:

$$-\frac{d \ln Dt}{dt} = \frac{k'}{\left(1 + \frac{nP_t}{k_{dp} + D_f}\right)\left(V_1 + \frac{V_3}{1 + \frac{k_{dp}nP_t}{(k_{dp} + D_f)^2}}\right)} \qquad \text{(Eq. 16)}$$

It should be noted that for the purposes of consistency in this paper, Eq. 16 was derived on the assumption that drug binding resulted from the interaction of drug with a specific plasma protein having n equivalent sites, and is, therefore, slightly different from that presented by Krüger-Thiemer *et al.* (270). Examination of the equation leads to the conclusion that, in such a case, a semi-log plot of D_1 (plasma concentration, determined as the sum of bound and unbound drug) *versus* time will not be linear but will be characterized by a diminishing steepness of slope as time proceeds. They similarly showed that:

$$-\frac{d \ln D_f}{dt} = \frac{k'}{V_3 + V_1\left(1 + \frac{k_{dp}\, nP_t}{(k_{dp} + D_f)^2}\right)} \qquad \text{(Eq. 17)}$$

Equation 17 predicts nonlinearity of a semi-log

[1] Trademarked as Aureomycin, Lederle Labs., Pearl River, N. Y.

plot of D_f (the concentration of unbound drug in the plasma) as a function of time. Moreover, the slope of such a curve will be initially greater than the corresponding D_t curve and will change more rapidly with time.

The model was examined by Krüger-Thiemer *et al.* (270) using computer generated data for various values of k_{dp}. In addition, blood level data obtained from a patient who was administered a long-acting sulfonamide, sulfaorthodimethoxine, were evaluated and found to be consistent with the behavior predicted by the model.

Martin (271) also considered theoretical aspects of the kinetics of elimination of drugs possessing high affinities for plasma proteins. He discussed a model in which drug was distributed between residual water and plasma. Binding occurred to form a 1:1 complex and drug disappearance proceeded at a rate which was directly proportional to the concentration of free drug. He did not present a rigorous mathematical analysis of the model but by approximate graphical methods was able to show that the rate of decline of drug concentration in the plasma underestimates the rate of drug elimination from the body. His calculations led to a conclusion that, because of protein binding, it could be possible to obtain a nonlinear semi-logarithmic plot of plasma concentration *versus* time in which slope values increase as time proceeds. It would seem, however, that this could not be the case and that this result was artifactually generated by his method of estimating plasma levels of drug. This model is similar to that discussed by Krüger-Thiemer *et al.* (270) and should exhibit the same type of behavior as they described.

Conclusions—The binding of drugs by plasma proteins continues to be a subject of active research interest and has attracted the attention of investigators from diverse disciplines. Research in this field is dipolar in character, consisting of, on the one hand, studies oriented to a fundamental physical-chemical understanding of the phenomenon and, on the other, investigations directed toward evaluation and quantitation of possible clinical manifestations and implications. Substantial progress has been made in both general areas.

While the majority of the reported *in vitro* studies have been semi-quantitative in nature, in recent years an increasing number have been conducted to yield a quantitative characterization of fundamental binding parameters. Analytically, the newer techniques of gel filtration, nuclear magnetic resonance spectroscopy, and fluorescence quenching provide the means of rapidly detecting binding, elucidating active sites involved in binding, and estimating the rates of interaction.

Considerable *in vitro* and *in vivo* evidence has been accumulated to demonstrate that plasma-protein binding can influence the distributional, pharmacological, and pharmacokinetic properties of certain drugs. In addition, theoretical contributions have been made which are of value in assessing the possible magnitudes of such influences. An impression, gained from the literature, is that there appears to be a tendency to overemphasize the general importance of the binding phenomenon in the behavior of drugs in the body. Evidence exists that only in the case of highly bound agents will binding be important in a practical sense. Many workers, in attempting to extrapolate *in vitro* data to *in vivo* expectations, tend to lose sight of the fact that the plasma comprises a relatively small fraction of the total volume available for drug distribution and that protein–drug complexes of rather extraordinary stability must be formed to substantially reduce the amount of drug that exists in the body in the active, diffusible, unbound form. A number of important drugs do, however, fall in the category of "strongly bound" and these serve as examples which emphasize the need to at least consider protein binding as a necessary parameter in the characterization of drug behavior.

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Keyphrases

Drug binding by plasma proteins—review
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Research Articles

New Method for Calculating the Intrinsic Absorption Rate of Drugs

By J. C. K. LOO and S. RIEGELMAN

If conceiving the body to be a single compartment is correct, calculations based on this presumption should result in an exact estimate of the rate of appearance of a drug into the blood when administered intravenously at a precisely known rate. In order to test this hypothesis selected drugs were administered intravenously at known logarithmic and linear rates of infusion, thereby mimicking first- and zero-order absorption conditions. It is shown that methods based on the single-compartment concept do not result in acceptable estimates of the absorption rates. Not only do these methods lead to an incorrect rate constant but occasionally allow incorrect assignment of the order of the process. A new equation is presented, presuming the drug distributes between a central and one peripheral compartment, which allows one to calculate the rate of absorption. The equation results in an accurate estimate of the known rates of infusion (absorption) for the drugs studied to date.

PROBABLY THE oldest published method for estimation of the rate of absorption of a drug into the blood was published by Dominguez and Pomerene (1). Their method was based on a presumption that the body may be treated as a single-compartmental reservoir from which the drug is eliminated by first-order processes. The calculation of the absorption by their method required estimation of the apparent volume of distribution of the drug in this single-compart-

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