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Drug Interactions. III.[1] Binding to and Displacement from Bovine Serum Albumin of Barbiturates[2]

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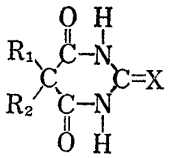
Binding to and displacement from bovine serum albumin of five barbiturates, barbital, phenobarbital, pentobarbital, secobarbital, and thiopental, were investigated by the dynamic dialysis method. The analysis of Scatchard plots indicated that there are marked differences in binding affinity and a parallelism in the relationship between chemical structure and their affinity. Furthermore, when these drugs are arranged in the order of their affinity for albumin molecule, there is a close correlation with pharmacological properties. Thus, the more highly protein-binding compounds are short-acting compounds with a rapid onset of action. They tend to be more rapidly degraded metabolically and are more potent hypnotics. For the study of competitive inhibition on the binding between barbiturate and albumin, thiopental was used as the test drug, and chlorpropamide and lauric acid as the competitors. Substantial inhibition of binding of thiopental to albnmin was found in both competitors with molar concentration of 0.7—1.0 times that of thiopental, at a ratio of 7 mol thiopental/mol of albumin. These results indicate that the competitive effect of lauric acid to inhibit the binding of thiopental to serum albumin appears to be greater than that of chlorproamide.

Keywords——drug interactions; binding and displacement; bovine serum albumin; barbiturates; dynamic dialysis; chlorpropamide; lauric acid

Two previous publications[1,4] in this series have presented the binding data of several drugs to bovine serum albumin (BSA) examined by a dynamic dialysis technique. The binding of drugs to plasma proteins, especially albumin, has been recognized as an important factor in availability, efficacy, and transport of drugs. When highly bound drugs compete for the same binding sites, weakly bound drugs may be displaced. Clinically significant responses may result when the bound fraction of one drug is displaced from its binding site by a second drug of greater affinity.[5]

In the present investigation, a series of substituted barbituric acids was selected for such binding studies. It is well known that slight changes in the structure of barbiturates signifi-

Table I. Chemical Structure of Barbiturates used in This Study

General formula	Barbiturate	R_1	R_2	X
	Barbital[a]	Ethyl	Ethyl	O
	Phenobarbital[a]	Ethyl	Phenyl	O
	Pentobarbital[b]	Ethyl	1-Methylbutyl	O
	Secobarbital[b]	Allyl	1-Methylbutyl	O
	Thiopental[c]	Ethyl	1-Methylbutyl	S

a) Long-acting.
b) Short-to intermediate-acting.
c) Ultrashort-acting.

1) Part II: S. Ozeki and K. Tejima, *Chem. Pharm. Bull.* (Tokyo), **22**, 1297 (1974).
2) Presented at the 94th Annual Meeting of the Pharmaceutical Society of Japan, Sendai, April, 1974.
3) Location: *3-1, Tanabe-dori, Mizuho-ku, Nagoya, 467, Japan.*
4) T. Tukamoto, S. Ozeki, F. Hattori, and T. Ishida, *Chem. Pharm. Bull.* (Tokyo), **22**, 385 (1974).
5) L.K. Christensen, L.M. Hansen, and M. Kristensen, *Lancet*, **2**, 1298 (1963); J.B. Field, M. Ohta, C. Boyle, and A. Remer, *N. Engl. J. Med.*, **277**, 889 (1967); J.A. Udal, *Clin. Med.*, **77**, 20 (1970).

cantly affect the activity, duration of action, fate, and excretion of these drugs. This makes them particularly suitable for investigations correlating chemical structure and physicochemical properties with pharmacological activity. Chemical structure of barbiturates used in this study are listed in Table I.

Investigations on the percentage of binding of barbiturates to serum albumin have been reported in a number of papers,[6,7] but it should be emphasized that the percentage value is not constant but changes depending on both the concentration of serum albumin and barbiturate in the system under study and that the value does not provide a reflection of the intrinsic affinity of barbiturates for serum albumin. On the other hand, only a few of fundamental binding parameters, n and k, for various barbiturates to serum albumin are known.[8] The present investigation is an attempt to clarify the relationship between the binding parameters and some pharmacological activities of these drugs. In addition, the competitive binding effect on the addition of a competitor to the binding system was examined.

Experimental

Materials——Bovine serum albumin, Fraction V (Armour Co., U.S.A.), was used in this study, and its molecular weight was assumed to be 69000. Barbital, phenobarbital sodium, pentobarbital sodium, secobarbital sodium, thiopental sodium, chlorpropamide, lauric acid, and other chemicals of analytical grade were obtained from commercial sources, and used without further purification. The bags for dialysis were prepared by the previously reported method.[4] The same phosphate buffer (pH 7.4, ionic strength 0.16) was used throughout this study.

Method——A dynamic dialysis method, similar to that described in the previous paper,[4] was used to determine the binding parameters, n (the number of binding sites per molecule of protein) and k (the association constant characterizing the interaction). The method was also applied to the experiment of competitive displacement of bound barbiturates from the binding site. Ten milliliters of a drug or drug-BSA solution, was placed in the dialysis bag, the bag was immersed in 200 ml of a buffer solution, and the whole thermostatted; runs were made at 37°, under continuous stirring of both solutions. The initial concentration of barbital was 5.43 mM, phenobarbital 3.93 mM, pentobarbital 4.03 mM, secobarbital 3.84 mM, thiopental 1.89 mM, and BSA 0.284 mM. Every 30 min, 100 ml of the external solution was removed and immediately replaced with 100 ml of a fresh buffer. Concentration of the drug in the collected sample was determined by spectrophotometry and the total concentration of the drug in the protein compartment was calculated. For the study of competitive inhibition on the binding between barbiturate and BSA, thiopental was used as the test species, and chlorpropamide (oral antiabetic agents) or lauric acid (endogeneous substance) as the competitors. A control run with thiopental was first conducted and then, using the same membrane sac, dialysis of thiopental was followed in the presence of the competitor. Finally, still using the same membrane, thiopental, BSA, and competitor were dialyzed. In this competition experiments, the initial concentration of thiopental was 1.98 mM, chlorpropamide 1.98 or 3.96 mM, lauric acid 0.28 to 1.98 mM, and BSA 0.284 mM.

Oil/water partition coefficients were found by shaking the barbiturate solution in the phosphate buffer of pH 7.4 with ethylene dichloride at 20° for 6 hr, and estimating the amounts of barbiturate in the aqueous layer before and after shaking.

Result and Discussion

Binding Parameters

The kinetic curves for each system obtained by the dynamic dialysis experiments were analyzed according to the previously reported method.[4] The treatment of data yielded values

6) L.R. Goldbaum, and P.K. Smith, *J. Pharmacol. Exp. Ther.*, **111**, 197 (1954).
7) J. Asher, *J. Pharmacol. Exp. Ther.*, **112**, 40 (1954); W.J. Waddel and T.C. Butler, *J. Clin. Invest.*, **36**, 1217 (1957); P.O. Kane and S.E. Smith, *Brit. J. Pharmacol. Exp.*, **11**, 134 (1959) [*Chem. Abstr.*, **59**, 23798 (1961)]; B.B. Brodie, H. Kurz, and L.S. Schanker, *J. Pharmacol. Exp. Ther.*, **130**, 20 (1960); P.G. Dayton, J.M. Perel, M.A. Landran, L. Brand, and L.C. Mark, *Biochem. Pharmacol.*, **16**, 2321 (1967); M. Ehrnebo, S.A. Gurell, B. Jalling, and L.O. Boreus, *Eur. J. Clin. Pharmacol.*, **3**, 189 (1971) [*Chem. Abstr.*, **76**, 68031 (1972)].
8) K. Yoshikawa and R.W. Loehning, *Experientia*, **21**, 377 (1965); S. Goto, T. Ohki, S. Kiryu, and S. Iguchi, *Yakuzaigaku*, **31**, 247 (1971); W. Mikits, A. Skinner, R.G. Spector, and D.C. Watts, *Brit. J. Pharmacol.*, **41**, 76 (1971); J. Brånsted, U. Meresar, and A. Ågren, *Acta. Pharm. Suecica*, **9**, 129 (1972).

for the concentration of unbound (Df) and bound (Db) species, for a number of different total drug concentration (Dt). The results obtained were used to construct the well-known Scatchard plots[9] where $\bar{v}/Df$ ($\bar{v}$=mol of Db per mol of protein) was plotted as a function of $\bar{V}$. In the simple binding theory, if it is assumed that BSA has n sites with constant k, then the following equation holds:

$$\bar{v}/Df = kn - k\bar{v} \qquad (1)$$

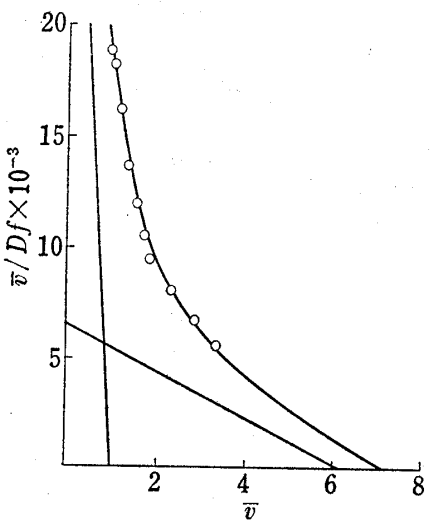


Fig. 1. Scatchard Plots the Binding of Thiopental to BSA in Phosphate Buffer at pH 7.4 and 37°

A plot of $\bar{v}/Df$ *vs.* $\bar{v}$ yields a straight line whose ordinate intercept is kn and whose abscissa intercept is n. Thus k can be explicitly calculated. If BSA has two types of sites, n_1 and n_2, then Eq. (2) may be applied.

$$\frac{\bar{v}}{Df} = \frac{k_1 n_1}{1+k_1 Df} + \frac{k_2 n_2}{1+k_2 Df} \qquad (2)$$

Where $n_1+n_2=n$, the total number of sites (per mol of BSA). The four unknowns can be evaluated in the manner previously described.[4] The typical Scatchard plots for thiopental to BSA at 37° in a phosphate buffer of pH 7.4 are shown in Fig. 1.

Thus, the analyses of Scatchard plots revealed that barbital and phenobarbital occupy one type of binding site in BSA, whereas two types of binding site was detected for pentobarbital, secobarbital, and thiopental. The binding parameters of barbiturates are summarized in Table II.

Table II. Binding Parameters and Partition Coefficient (P)

Barbiturate	n_1	n_2	$k_1\times10^{-2}$	$k_2\times10^{-2}$	$P^{a)}$	Goldbaum and Smith[6]		
						Bound[b] (%)	n_1	$k_1\times10^{-2}$
Barbital	1.0		7		1	5	0.1	87.3
Phenobarbital	1.0		25		14	20	0.3	28.7
Pentobarbital	1.0	3.4	52	3.5	39	35	0.4	21.1
Secobarbital	1.0	6.0	100	4.3	73	44	0.6	13.9
Thiopental	1.0	6.0	420	11.0	248	65	1.25	6.0

a) True partition coefficient. The pK_a values of the drugs are as follows:[c] Barbital 7.8; phenobarbital 7.3; pentobarbital 8.0; secobarbital 7.9; thiopental 7.4.
b) Binding of 0.001M barbiturate by 1% BSA in 1/15M phosphate buffer at 7.4; fraction bound.
c) M.T. Bush, "Physiological Pharmacology," vol. 1, ed. by W.S. Root, and F.G. Hofmann, Academic Press, Inc., New York, 1963, p. 185.

These results indicate that there are marked differences in the binding strength expressed as k_1 and a parallelism in the relationship between chemical structure and k_1. For example, k_1 for thiopental was 60 times greater than k_1 for barbital. The k_1 values for the barbiturates increased in the order of barbital, phenobarbital, pentobarbital, secobarbital, and thiopental. It is well established that increase in the length of one or both alkyl side-chains in barbiturates increased the binding strength. The fairly good agreement between the rank order in columns k_1 and oil/water partition coefficient is noteworthy. Thus, it indicates that there is some contribution of hydrophobic forces to the BSA binding, at the primary and/or secondary binding sites. Goldbaum and Smith[6] studied the binding of various barbiturates to BSA,

9) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).

and they showed the percentage of binding of barbiturates in phosphate buffer (pH 7.4) at room temperature (Table II). The values from Goldbaum and Smith are inreasonable agreement with those reported by other investigators,[7] and a correlation is also found between the values and the partition coefficient. In addition, they had estimated the binding parameters from Scatchard plots, using a limited number of data points. Their estimated results are shown in Table II. It should be noted that the latter results are not only different from our results, but also in the reverse order to what would be expected from percentage values for the binding of barbiturates to BSA. Simple extrapolation of the slope of the curved Scatchard plots ordinarily may give inaccurate estimates of the associate constants for all classes of sites, unless sufficient data are available. Therefore, it is likely that the results led to overestimation or underestimation of binding parameters. Disagreement between the rank order of percentage bound and k_1 may occur because of this reason.

TABLE III. Relationship between k_1 and Some Pharmacological Properties of Barbiturates

Barbiturate	$k_1 \times 10^{-2}$	Delay in onset of activity[a]	Biological half-life, hr[b]	Anesthetic dose, mg/kg[c]	Concn. inhibiting brain cortex respiration[d]
Barbital	7	22	120	234	480
Phenobarbital	25	12	84	134	44
Pentobarbital	52	0.1	42	35	8
Secobarbital	100	0.1	—	30	6
Thiopental	420	—	16	25	28

a) Minutes until anesthesia after intravenous injection in mice.[e]
b) J.M. Van Rossum, "Drug Design," Vol. 1, ed. by E.J. Ariëns, Academic Press, Inc., New York, N.Y., 1971, p. 473.
c) C.D. Barnes and L.G. Eltherington, "Drug Dosage in Labooratory Animals a Handbook," University of California Prees, Berkeley and Los Angeles, 1966, p. 15.
d) Concentration ($M \times 10^{-11}$) producing 50% inhibition of rat brain cortex respiration.[f]
e) T.C. Butler, *J. Pharmacol. Exp. Ther.*, **74**, 118 (1942).
f) F.A. Fuhrman and J. Field, *J. Pharmacol. Exp. Ther.*, **77**, 392 (1943).

Structure–activity relationship of the barbiturate has been observed. When these drugs are arranged in the order of their affinity for BSA molecule, there is a close correlation with pharmacological properties, as shown in Table III. Thus, the more highly protein-binding compounds are short-acting with a rapid onset of action. They tend to be more rapidly degraded metabolically and are more potent hypnotics. In general, the structual changes that increase lipid solubility decrease duration of action, decrease latency to onset of activity, accelerate metabolic degradation, and often increase hypnotic potency. On the other hand, since protein binding reduces the fraction of diffusible drug, a reduced concentration of drug usually exists at the receptor sites when a drug is extensively bound. This results in a decreased pharmacological effect of drug. However, it is interesting to note that there is a parallelism in the relationship between the binding affinity and pharmacological effect. Thus it is inferred that the physicochemical functions of the barbiturate binding sites on serum albumin are similar to that of the active receptor sites. However, we have no idea how the pharmacological effect is brought about, nor, in particular, what biochemical events might be implicated. In addition, it is possible that differences in protein binding of the barbiturates are manifest not only in the plasma but also in the liver, and that the rates of metabolism are influenced by binding to the enzymes concerned. However, other factors, such as chemical structure and polarity, probably influence the rate as well, and it is not therefore suggested that protein binding is the only one.

Differences in the drug-binding capacity of plasma protein are found between species, but in most cases that of between BSA and HSA (human serum albumin) are relatively small and within a group of substances the increase or decrease of the affinities is similar for both

albumins.[10] Therefor, it seems highly probable that studies utilizing HSA may also reflect the same correlations.

Competitive Inhibition of Protein Binding

Inhibition of protein-small molecule interactions due to the presence of a species capable of competing for the binding site is a well recognized phenomenon. For the study of competitive inhibition on the binding between barbiturate and BSA, thiopental was used as the test drug, and chlorpropamide and lauric acid as the competitor. Chlorpropamide is widely used as an oral hypoglycemic agent. It has been well established that chlorpropamide is strongly bound to serum albumin,[11] and this competes with compounds like sulfonamides, phenylbutazone, Aspirin, sodium salicylate, and 1-anilinonaphthalene-8-sufonic acid for the same binding site on serum albumin.[12,13] On the other hand, free fatty acids in the plasma is composed of a mixture of long-chain fatty acids[14] such as lauric, palmitic, oleic and linolenic acids. Free fatty acids and numerous drugs are transported in blood primarily bound to plasma albumin. The affinity of free fatty acids for albumin is stronger than most drugs and, therefore, then can effectively displace various drugs from their binding site on the albumin molecule, as already shown by many investigators and as recently reviewed.[15] Under the conditions of the present study, lauric acid is more suitable than other long-chain fatty acids which are virtually insoluble in aqueous buffer and tend to micelle formation when saturation is approached.

The rate of disappearance of total concentration of small molecule (Dt) from a protein compartment containing a competitor as a function of an apparent first-order elimination constant, k_e, may be expressed by the equation developed by Meyer and Guttman.[16]

$$\frac{-d(Dt)}{dt} = \frac{k_e(Dt)}{1+\dfrac{[n_1 k_{d1}(Pt)]}{[1+k_{d1}(Df)+k_{c1}(Cf)]}+\dfrac{[n_2 k_{d2}(Pt)]}{[1+k_{d2}(Df)+k_{c2}(Df)]}} \qquad (3)$$

Eq. (3) describing competitive binding defines the dependency of binding a species "D", in the presence of a competitor "C", on the unbound "D" concentration (Df), the number of binding sites (n_1 and n_2) on the protein (Pt) which interact with the drug and the competitor, and the corresponding association constants for "D" and "C" (k_{d_1}, k_{d_2}, k_{c_1} and k_{c_2}). It is apparent that the presence of a competitor will result in a faster decline of (Dt) as verfied by Eq. (3), which predicts that the rate of loss of (Dt) will increase with increasing (Cf). Further, Eq. (3) shows that if the values of k_{d_1} (Df), and k_{d_2} (Df) are significantly smaller than the values of k_{c_1} (Cf) and k_{c_2} (Cf), then the slope of a semilogarithmic plot of (Dt) *vs.* time will be constant, if (Cf) is constant.

Since the maximum number of binding sites ($n=n_1+n_2$) on BSA molecule for thiopental is 7, as mentioned above, thiopental was added to the dialysis bag to make 7 mol/mol albumin. Preliminary experiments demonstrated that chlorpropamide and lauric acid, in the concentration used in this study, do not significantly affect the intrinsic rate of thiopental dialysis. The range of concentrations of competitors in the bags extended from 1 to 14 mol/mol albumin. The results of the competitive experiments are shown in Fig. 2 and 3.

10) A.H. Anton, *J. Pharmacol. Exp. Ther.*, **129**, 282 (1960); H. Kurz and G. Friemel, *Naunyn-Schmiedeberg's Arch. Exp. Path. Pharmak.*, **257**, 35 (1967); W. Scholton, K. Schlossmann, and H. Rosenkranz, *Arzneim.-Forsch.*, **18**, 767 (1968).
11) J.M. Stowers, L.W. Constable, and R.B. Hunter, *Ann. N. Y. Acad. Sci.*, **74**, 689 (1959); L.K. Christensen, J.M. Hansen, and M. Kristensen, *Lancet*, **2**, 1298 (1963).
12) J. Judis, *J. Pharm. Sci.*, **61**, 89 (1972).
13) P. Hsu, J.K.H. Ma, and L.A. Luzzy, *J. Pharm. Sci.*, **63**, 570 (1974).
14) V.P. Dole, A.T. James, J.P.W. Webb, M.A. Rizack, and M.F. Sturman, *J. Clin. Invest.*, **38**, 1544 (1959); A. Saifer and L. Goldman., *J. Lipid Res.*, **2**, 268 (1961).
15) A.A. Spector, *J. Lipid Res.*, **16**, 165 (1975).
16) M.M. Meyer and D.E. Guttman, *J. Pharm. Sci.*, **59**, 39 (1970).

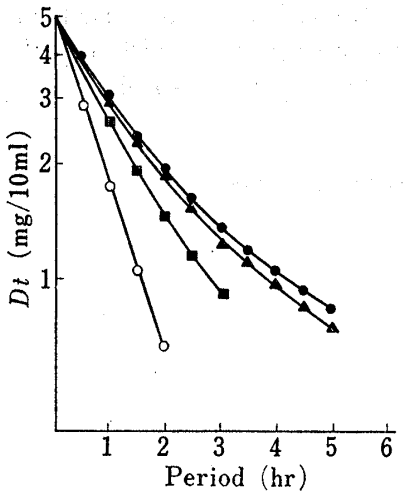


Fig. 2. Loss of Thiopental from Dialysis Sac at pH 7.4 and 37°, in the Presence of BSA, and Chlorpropamide

Initial concentration of thiopental was 1.98 mM.
—○—: in the absence of BSA.
—●—: in the presence of 0.248 mM BSA.
—▲—: in the presence of 0.284 mM BSA and 1.98 mM chlorpropamide.
—■—: in the presence of 0.284 mM BSA and 3.96 mM chlorpropamide.

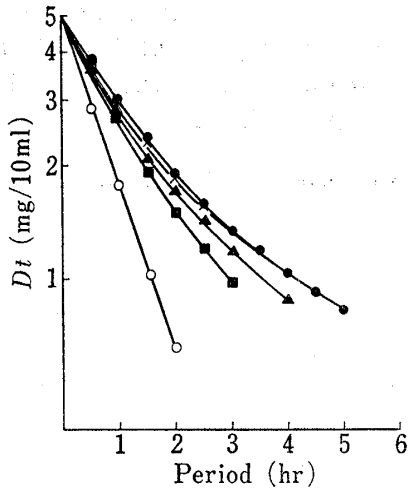


Fig. 3. Loss of Thiopental from Dialysis Sac at pH 7.4 and 37°, in the Presence and Absence of BSA, and Lauric Acid

Initial concentration of thiopental was 1.98 mM.
—○—: in the absence of BSA.
—●—: in the presence of 0.284 mM BSA.
—×—: in the presence of 0.284 mM BSA and 0.99 mM lauric acid.
—▲—: in the presence of 0.284 mM BSA and 1.42 mM lauric acid.
—■—: in the presence of 0.284 mM BSA and 1.98 mM lauric acid.

It is apparent from these graphs that the dialysis rate of thiopental was markedly increased by the presence of the competitor and with its increased concentration in the system, and thiopental must thus compete with chlorpropamide or lauric acid for the binding site on the albumin molecule. Both competitors appear to bind to the same site on the albumin, since each drug competitively inhibits the binding of the other to BSA. It is also of interest to note that when more than 5 mol of lauric acid and 7 mol of chlorpropamide to 1 mol of albumin are added, thiopental begins to displace effectively. The competitive effect of lauric acid to inhibit the binding of thiopental appears to be greater than that of chlorpropamide, because the effective concentration of the former was lower than the latter. The effect of lauric acid at a ratio of 4 was also detectable but far less marked than that seen at ratio 5, and it is inconclusive. Therefore, in the usual physiological concentration range ($\bar{v}$=0.5—2.0) [17] free fatty acids would not be able to displace effectively appreciable amount of thiopental from albumin binding site. In special situations, such as after vigorous muscular exercise,[18] an injection of heparin,[18] or in uncontrolled diabetes mellitus,[19] the molar ratio can exceed 4. It has been reported that the highest plasma level of free fatty acids in man with hyperlipidemia is about 4000 μeq/l, or about 7 mol of free fatty acids/mol of albumin.[20]

On the other hand, albumin component of the serum is either unchanged or, more usually, lowered in pathological states. Albumin levels do not rise above normal except in the presence of hemoconcentration or dehydration. A decline in serum albumin levels (hypoalbuminemia), for example, may follow chronic loss of protein (as in the nephrotic syndrome or burns), chronic liver disease (cirrhosis), or prolonged malnutrition.[21] These data indicate that the unsuitable

17) R.J. Havel, A. Nailmark, and C.F. Borchgrevink, *J. Clin. Invest.*, **42**, 1054 (1963).
18) H. Rutenberg, A.G. Lacko, and L.O. Saloff, *Biochim. Biophys. Acta.*, **326**, 419 (1973).
19) H.A. Harper, "Review of Physiological Chemistry," 13th ed., Marzen Co., Ltd., Tokyo, 1971, p. 283.
20) J.I. Gallin, D. Kaye, and W.M. Oleary, *N. Engl. J. Med.*, **281**, 1081 (1969).
21) H.A. Harper, "Review of Physiological Chemistry," 13th ed., Maruzen Co., Ltd., Tokyo, 1971, p. 197.

drug combinations have always exposed the patients to the risks. As a consequence, it has been suggested that thiopental should be given with caution to patients with hyperlipoidemia and/or hypoalbuminemia and to diabetics receiving chlorpropamide so that the potential hazards may be avoided.

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