Table V—Analysis of Standard Mixtures of Isosorbide Dinitrate and Alprenolol Hydrochloride

Standard Mixture	Internal Standard, mg	Alprenolol Hydrochloride			Isosorbide Dinitrate		
		Added, mg	Found, mg	Recovery, %	Added, mg	Found, mg	Recovery, %
1	101.7	253.0	252.5	99.7	53.2	53.5	100.6
2	102.7	250.7	261.0	104.1	53.5	53.5	100.0
3	114.0	258.2	252.2	101.5	52.5	54.2	103.3
4	97.7	260.5	256.5	98.5	57.5	56.5	98.3
5	94.5	251.0	256.5	102.2	57.0	56.0	98.2
6	94.8	252.0	258.0	102.4	57.0	58.5	102.6
7	91.2	249.5	254.0	101.8	52.5	49.0	93.3
8	91.5	258.5	266.0	102.9	62.0	63.0	101.6
				Mean = 101.6			Mean = 99.7
				%SD = ±1.78			%SD = ±3.18

The signal chosen for the analysis of I was the multiplet at 5.5 ppm due to the protons on C_2 and C_5. The presence of this signal at low fields is due to the electronegativity of the nitrate ester group as in 1,4:3,6-dianhydro-D-glucital. In this compound both diacetoxy and dimesyloxy derivatives have their C_2 and C_5 protons shifted to lower fields (9).

The doublet at 1.25 ppm due to both methyl groups was chosen for II. The signal given by the same group in III, a doublet at 1.30 ppm, was selected for III. The singlets at 6.2 ppm for IV and 8 ppm for V were used for the internal standards.

The results of the analysis of a group of known standard mixtures of I and II and I and III are summarized in Tables IV and V (Figs. 1 and 2). The method is accurate and precise, with an $SD \pm 3.06$ for I. The standard deviation is ±1.80 and ±1.78 for II and III, respectively, for the standard mixtures.

This procedure was also applied to commercial lots of tablets from three companies containing I and its mixtures with II and III.

The results obtained are in agreement with those obtained using the official USP procedure for I (4) and the BP procedures for II (6) and III (7).

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Ion-Specific Electrode Study of Copper Binding to Serum Albumins

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Abstract □ The binding of copper to bovine, human, rabbit, rat, and porcine albumin has been studied using a cupric ion-specific electrode. The results were analyzed in terms of Scatchard expression assuming two classes of independent binding sites. The high-affinity constants for copper binding to the albumin show the same trend as the first association constants for nickel binding, namely, rabbit > human > rat > pig. Despite the similarity in the primary amino acid sequence for human and bovine serum albumin, the former has only one high-affinity site for copper, while the latter has more than three sites.

Keyphrases □ Binding—copper to serum albumin, ion-specific electrode study, bovine, human, rabbit, rat, porcine □ Serum albumin—bovine, human, rabbit, rat, porcine, ion-specific electrode study, copper binding □ Ion-specific electrode—copper binding to serum albumin, bovine, rabbit, human, rat, porcine

Serum copper levels play a dual role in several pathological conditions, both in humans and experimental animals (1, 2). Anti-inflammatory properties are associated with several salts and chelates of copper (3–9). On the other hand, increased copper levels have been observed in several diseases such as tuberculosis and pneumonia (10), rheumatoid arthritis (11, 12), and ankylosing spondilitis (13). In serum the weakly bound form of copper is associated with the transport protein, albumin. Sequence analysis studies have shown that the first three amino acids at the *N*-terminal constitute a high-affinity copper binding site in bovine (14–16), human, and rat albumin (17). Nickel, which has been implicated as a carcinogen in humans and experimental animals (18), also binds to this *N*-terminal site.

There have been several previous attempts to measure the binding of copper to serum albumins from different animal species. In an early study (19) the thermodynamics of copper binding to bovine serum albumin using absorption spectroscopy were examined. However, the insensitivity of the spectroscopic method necessitated the use of high protein concentrations, which precluded the determination of the high-affinity binding constants. A later study (20) estimated the number of copper binding sites on bovine and human albumin but the relative affinity constants were not determined. A more recent study (21)

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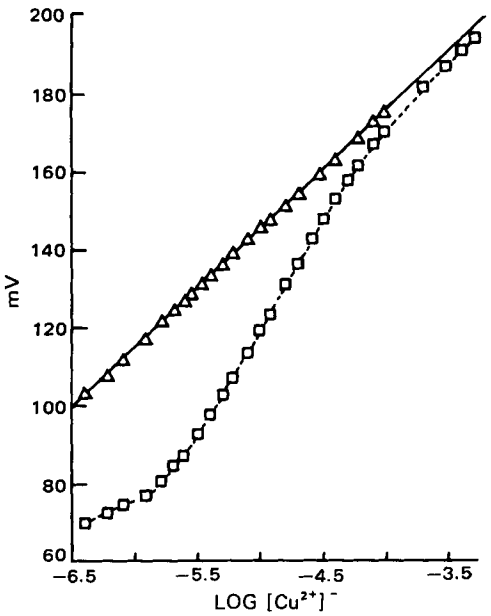

Figure 1—*Response of the electrode to the addition of Cu^{2+} Cu^{2+} was added in microliter quantities to a stirred solution of 0.1 M KNO_3 (50 ml) in the absence (△) and in the presence (□) of human serum albumin, and the change in potential was measured after each addition.*

employed a cupric ion-specific electrode to determine the copper binding parameters of bovine serum albumin. In this report the copper binding parameters for bovine, human, rat, porcine, and rabbit albumins have been estimated using a cupric ion-specific electrode. The sensitivity of this technique is such that it permits the determination of the association constant of copper for the high-affinity primary albumin binding site(s).

EXPERIMENTAL

Materials—Crystallized bovine, human, and rabbit serum albumins[1] were used without further purification. Rat serum albumin[1] (Fraction V) was extensively dialyzed against large volumes of 5 m*M* edetic acid and deionized water, freeze-dried, and stored at 4° until use. Porcine Fraction V[2] was decolorized with charcoal at pH 3 and eluted with 0.2 *M* NaCl over a cross-linked dextran gel column (100 × 3.5 cm). The albumin fractions from the column were concentrated by ultrafiltration[3], dialyzed against large volumes of 5 m*M* edetic acid, lyophilized after dialysis against large volumes of deionized water, and stored cold (4°). The monomer and higher oligomers were pooled together before use. Human and bovine albumin concentrations in the solutions were estimated from their respective absorbances at 279 nm (22, 23). Other albumin solutions were prepared by dissolving a known amount of protein in a desired volume of 0.1 *M* KNO_3 solution. Typically, the titration solutions contained 10–40 μ*M* albumin. Stock solutions of 2 and 100 m*M* $CuSO_4$ were prepared and their concentrations were estimated by atomic absorption spectroscopy.

Titration Experiments—The titrations were carried out with a cupric ion-specific electrode[4] and a single-junction electrode[5] both connected to a digital pH/mV meter and dipped in a 0.1 *M* KNO_3 solution (with or without albumin). Aliquots of the copper sulfate solution (first 2 and later 100 m*M*) were added to the albumin solutions (20–50 ml) using a microsyringe such that the logarithm of the cupric ion concentration in the medium increased linearly with each addition. The corresponding potential as shown by the pH/mV meter was noted after each addition. The parameters of the electrode (the slope and the intercept) were determined from control (no protein) titrations in each case. All titrations were performed at room temperature (23–25°). The pH of the solution at the beginning and end of each titration was 5.2–5.4.

[1] Miles Laboratories, Elkhart, Ind.
[2] Sephadex G-150 Sigma Chemical Co., St. Louis, Mo.
[3] UM-10 membrane, Amicon, Danvers, Mass.
[4] Model 94-29, Orion Res. Inc., Cambridge, Mass.
[5] Model 90-01, Orion Res. Inc., Cambridge, Mass.

Table I—Parameters of Cu^{2+} Binding to Serum Albumins [a]

Source	n_1	$K_1 \times 10^{-6}, M^{-1}$	n_2	$K_2 \times 10^{-3}, M^{-1}$
Bovine	3.68	3.14	12.73	20.15
	3.0[b]	3.0[b]	16.0[b]	20.0[a]
Human	1.13	2.33	4.5	67.54
Rabbit	3.9	2.83	15.14	7.52
Rat	2.62	1.85	26.4	4.34
Porcine	1.86	0.41	18.01	5.76

[a] The values represent the computer analysis of the results of three or more titration experiments. The values are the best fit for both *R versus C* and *(R/C) versus R* Scatchard analysis. The standard deviation was <8% for the computer fittings. [b] Taken from Reference 21.

It is known that above pH 4 at the concentrations employed in this study, the Cu^{2+} ion begins to hydrolyze and precipitates as the oxide or hydroxide soon thereafter (24). Although a more physiological pH of 7.4 would have been the ideal choice, the sensitivity of the electrode may decrease above pH 6 because of the formation of both soluble and insoluble hydroxo–oxo complexes under experimental conditions. Hence, no pH adjustments were made before each titration.

Data Analysis—The titration data were analyzed in terms of the Nernst equation to find the concentration of free and bound copper in solution. Since the resultant Scatchard plots exhibited considerable curvature, the assumption was made that there were two sets of independent binding sites and the data were fitted to the expression (25):

$$r = \frac{n_1 K_1 C}{1 + K_1 C} + \frac{n_2 K_2 C}{1 + K_2 C}$$

where r is the number of Cu^{2+} bound per mole of protein, n_1 and n_2 are the number of binding sites, K_1 and K_2 are the corresponding constants[6], and C the molar concentration of free Cu^{2+}. The curve fitting was performed using a modeling program[7] (26) running on a computer[8,9].

RESULTS AND DISCUSSION

A typical titration curve is shown in Fig. 1. Figure 2 is a computer-fitted Scatchard plot for human serum albumin. The binding parameters determined in this study for albumins from different species are listed in Table I. Also listed in Table I are the binding parameters for bovine serum albumin reported previously (21) for comparison. It can be seen that the results of the present study are in reasonable agreement with previous results (21).

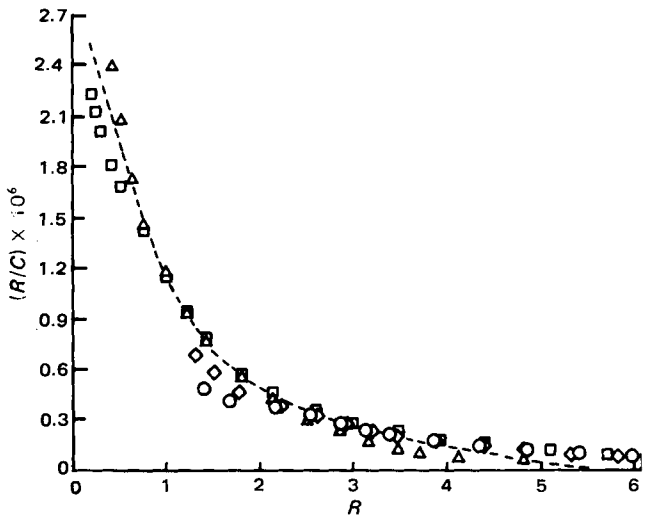

Figure 2—*Scatchard analysis of Cu^{2+} binding to human serum albumin. R and C are the number of Cu^{2+} ions bound per mole of albumin and the molar concentration of free Cu^{2+}, respectively. The figure is the result of a computer fit of the results from four different (○,△,□,◇) titration experiments.*

[6] K_1 and K_2 above are defined for the equilibrium: albumin + n Cu^{2+} ⇌ (albumin-n Cu^{2+}).
[7] Modeling Laboratory, MLAB.
[8] DEC-10, Digital Equipment Company, Marlborough, Mass.
[9] Computer at the Division of Computer Research & Technology, the National Institutes of Health, Bethesda, Md.

The trend in the high-affinity association constants, namely, rabbit > human > rat > pig, parallels that observed for nickel binding to the same albumins as determined previously (27). While it was not possible to obtain consistent values for copper binding to dog serum albumin, the results seemed to indicate that the primary binding sites for canine albumin had a lower affinity than those found in porcine albumin. Again, this is the case for nickel binding (27). If both Cu^{2+} and Ni^{2+} occupy the same albumin binding sites and form complexes of the same geometry type, then one would expect the same trend for both copper and nickel binding. This suggests that the *N*-terminal Cu^{2+}-binding site may also constitute the primary binding site for nickel. The relatively lower binding constants for dog and pig albumins are probably due to the absence of a histidine at the third amino acid position from the *N*-terminal end of these proteins (26).

The number of high-affinity sites for human albumin is less than that for bovine (Table I). From a comparison of the proposed primary structures of these proteins (28, 29), and based on the assumption that the geometry at the additional sites should be comparable to that at the *N*-terminal site, two extra high-affinity sites in bovine albumin may be provided by the amino acid sequences of Glu^{16}–Glu–His^{18} near the *N*-terminal and Asp^{373}–Lys–Leu–Lys–His–Leu–Val–Asp^{380} between the second and the third domains. However, it should be pointed out that this assignment is only tentative and needs to be further substantiated experimentally. It is our experience that the extrinsic circular dichroism spectra of bound copper are not the same for human and bovine albumins[10]. Obviously, there must be some significant differences in both the number and the nature of the copper binding sites for these two types of albumin.

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Effect of Dose Size on the Pharmacokinetics of Oral Hydrocortisone Suspension

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Abstract □ The pharmacokinetics of hydrocortisone were examined following single doses of 5-, 10-, 20-, and 40-mg hydrocortisone suspensions to healthy male volunteers. Endogenous hydrocortisone was suppressed by giving 2 mg of dexamethasone the night before hydrocortisone administration. Plasma samples obtained serially for 12 hr after hydrocortisone administration were assayed by reversed-phase high-pressure liquid chromatography using a fixed-wavelength (254 nm) UV absorbance detector. Drug absorption was rapid, with mean maximum plasma hydrocortisone concentrations occurring within 60 min of dosing. Subsequent drug elimination was monophasic with mean elimination half-lives increasing from 1.2 hr for the 5-mg dose to 1.7 hr for the 40-mg dose. Increases in *AUC* and C_{max} with increasing dose were linear but not directly proportional to dose size. This was attributed to dose-dependent absorption or to loss of drug during the first-pass through the liver.

Keyphrases □ Hydrocortisone—effect of dose size on pharmacokinetics of oral suspension, absorption, elimination □ Pharmacokinetics—oral hydrocortisone suspension, absorption, elimination, effect of dose size □ Absorption—oral hydrocortisone suspension, elimination, effect of dose size on pharmacokinetics □ Suspension—oral hydrocortisone, absorption, elimination, effect of dose size on pharmacokinetics

Hydrocortisone was designated by the U.S. Food and Drug Administration as a drug whose different brands and dosage forms should be examined for bioequivalence (1). A series of studies was initiated to examine the pharmacokinetics of hydrocortisone and to assess the feasibility of conducting bioequivalence studies on commercial products.

In a previous study (2), dexamethasone was shown to

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