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CHEMICAL BINDING TO HUMAN DERMIS *IN VITRO* TESTOSTERONE AND BENZYL ALCOHOL

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Abstract. 1. Using techniques similar to those employed in plasma protein–drug binding studies, human skin was examined *in vitro* with testosterone and benzyl alcohol.

2. Marked dermal binding of testosterone was ascertained by dialysis from normal saline solution.

3. The partitioning of benzyl alcohol into the dermis was slight and devoid of any binding interaction. The epidermal partition coefficient of benzyl alcohol is about one hundred-fold that in the dermis.

3. The skin distribution characteristics of testosterone and benzyl alcohol were differentiated.

4. The possible clinical significance of these *in vitro* observations are discussed in relation to percutaneous penetration kinetics and allergic contact dermatitis.

Plasma protein drug binding is a well-recognized phenomenon; this study seeks to ascertain whether human dermis has similar binding properties.

The binding of steroids to serum proteins has been extensively investigated to ascertain hormonal transport and distribution in relation to metabolic pathways. Following the development of radioisotope techniques and the availability of labeled hormones, the interaction of steroid hormones with plasma proteins has been assessed (4, 8). Reviewing comprehensively the chemical and biological factors governing this activity of steroids, Bush inferred the following generalization (3): "The association with albumin is minimal for very hydrophobic steroids (e.g. monoketones and monohydroxysteroids), weak with hydrophilic steroids (e.g. cortisol) but strong with moderately hydrophobic steroids such as testosterone." Testosterone binding with bovine serum albumin at 37°C was 94% (5, 19). Sandberg et al., utilizing two alternative methods, reported that testosterone was bound to human serum albumin in the range of 95%–96% at 5°C (17). The strong binding capacity of testosterone to plasma proteins prompted comparative determinations with plasma substitutes and some animal proteins. Modified human globin and rat tail collagen have significant affinity for testosterone (5).

Lipid- and water-soluble benzyl alcohol (employed as an antiseptic and local anesthetic) has not been reported hitherto (13) to bind with proteins.

In a previous communication (12) our data suggested that the dermis functioned as a sequential barrier to that of the epidermis in percutaneous penetration of testosterone *in vitro*. Under similar experimental conditions the main barrier to benzyl alcohol resided within the epidermis. The relative affinity of the dermis to testosterone and benzyl alcohol, respectively, was evaluated in terms of lipoprotein solubilities and binding associations. A strong affinity of testosterone to human dermis paralleling that of plasma proteins was found.

MATERIALS AND METHODS

Radioisotopes

Benzyl 7-^{14}C alcohol and testosterone 4-^{14}C of the same lots used previously (12).

Skin

Human autopsy skin was used (12). Epidermis was separated from dermis by peeling after exposure to heat on an electric plate (about 50°C). Before partitioning-dialysis, each slice was weighed on a Mettler analytical balance.

Partitioning-dialysis

Increasing quantities of the chemicals were added into screw-cap vials, each containing 8 ml of N.S.S. (Normal

Table I. *N.S.S.–dermal partition dialysis of testosterone and benzyl alcohol*

Initial Amount (c.p.m. × 10^4)	Weight of Dermis (g)	Equilibrium concentrations: Dermis (c.p.m. × 10^4/g)	Equilibrium concentrations: N.S.S. (c.p.m. × 10^4/ml)	Ratio Dermis/N.S.S.
Testosterone				
52	0.8131	24.02	3.947	6.086
77	1.0320	37.09	4.711	7.873
148	1.1612	44.44	10.160	5.457
180	1.2877	65.98	11.450	5.762
Benzyl alcohol				
52	0.6596	1.66	6.137	0.270
61	0.7961	1.87	7.239	0.258
65	0.6746	1.80	7.773	0.232
112[a]	0.8494	3.43	13.393	0.256

[a] For technical reasons the upper limit was set to this amount.

Saline Solution, pH 5.5, unbuffered) at 37°C. Weighed dermis or epidermis was introduced, the caps tightly screwed and thereafter the vials were shaken on a mechanical agitator installed in a walk-in incubator, automatically controlled to 37°C ± 1°C.

Partition coefficient

Provided that a solute has the same molecular weight upon distribution into two immiscible or slightly miscible solvents at constant temperature, the partition coefficient K is the ratio of its concentrations c_I c_{II} attained at equilibrium in each of the respective phases (6). Consequently, expressed in the logarithmic form:

$$\log c_I = \log K + \log c_{II} \qquad \text{(Eq. 1)}$$

where $\log c_I$ exists in linear relationship to $\log c_{II}$ with $\log K$ as the intercept and the slope value being unity.

Freundlich adsorption isotherm

The degree of protein binding as a function of concentration can be derived by applying the Freundlich adsorption isotherm (9, 18). Transposed logarithmically this can be stated as follows:

$$\log Dp = \log K + a \log Dg \qquad \text{(Eq. 2)}$$

where Dp is the concentration of bound drug, Dg is the concentration of unbound drug and K and a are constants characteristic of the system involved. Evidently, there is a linear relationship between $\log Dp$ and $\log Dg$ with $\log K$ as the intercept and a as the slope (9). For theoretical reasons, a in Equation 2 is less than unity (6). When the value of a approximates 1, Equation 2 implicitly resembles Equation 1. Thus, the measurement of a indicates whether the drug is partitioned or associatively bound upon equilibration between the specific skin tissue and N.S.S. at constant temperature.

Sampling

At intervals, 20 μl was withdrawn from the vials and radioactivity determined as previously described (12). Equilibration was effected completely at about 60 hours and the total concentrations of the free chemicals in N.S.S. ascertained.

Chemicals retained by the tissue

Following equilibration, the tissues were subjected to digestion by 2 N potassium hydroxide–methyl alcohol solution at 60°C for 24 hours. Aliquots of the digestion fluids were prepared for scintillation counting as previously described (12) and the total calculated.

Internal standard

A correction factor was used in the computations by the addition of an internal standard (12).

RESULTS AND DISCUSSION

The data of representative partition-dialysis experiments of testosterone and benzyl alcohol with dermal tissue represent dissimilar distribution systems (Table I). Testosterone exhibits a pattern of binding association. A fairly consistent ratio was attained at equilibrium of benzyl alcohol partitioned between the aqueous solvent and the dermal tissue.

The quotients of dermal/N.S.S. equilibrium concentrations vary with testosterone from 5.457 to 7.873 (Column 6, Table I). The highest ratio differs from the lowest one by about 50%. For benzyl alcohol, these quotients are confined in the narrow range of 0.232 to 0.270 (average value, 0.254). This practically constant relationship conforms to the partition coefficient, K, of Equation 1. With the slope at unity, the value of

K calculated by regression analysis (7) is 0.254,[1] where c_I is the dermal concentration and c_{II} is the concentration of benzyl alcohol in N.S.S. at equilibrium, respectively.

The data for testosterone and benzyl alcohol was evaluated by Equation 2 (Freundlich adsorption isotherm): *Dp* and *Dg* represent the dermal and N.S.S. concentrations of the chemicals involved at equilibrium. respectively. From the equilibration data (in units of 10^4 c.p.m.) the values of K and a were computed by the method of regression (7), as follows:

	K	a
Testosterone	9.024[2]	0.81
Benzyl alcohol	0.270[3]	0.97

For benzyl alcohol, the K value ascertained by Equations 1 and 2 are of the same order of magnitude, and thus represent its dermal/N.S.S. partition coefficient K. Accordingly, the slope a of Equation 2 should have the value of about 1; the actual calculated value (0.97) deviates slightly from unity.

The K value of testosterone is high. Apparently this steroid has a great affinity toward one or more of the major constitutents of human dermis which consists, as any other biological tissue, of an interwoven mosaic of lipid, proteins and other particles (14). With higher concentrations, the testosterone dermal/N.S.S. equilibrium quotients gradually decrease (Column 5, Table I) resulting in the slope value a of less than unity (0.81) on a log scale (Fig. 1). This could be attributed to a saturation process of the binding sites characteristic of protein–testosterone association.

Further proof of binding interaction was obtained by effecting redistribution of testosterone from the dermis through an aqueous vehicle. At the conclusion of the partitioning-dialysis, the dermal slices were suspended in screw-capped vials containing 8 ml of N.S.S. at 37°C. These were shaken, sampled and assayed as described under the procedure of partitioning-dialysis. The amounts redistributed into N.S.S. fluctuated for about 36 hours which did not produce any consistent elution of testosterone. The same procedure was repeated with the dermal slices partitioned with benzyl alcohol. Within 3 hours, the benzyl alcohol was uniformly redistributed according to the established partition coefficient, K, between N.S.S. and dermis without any appreciable change up to 36 hours. These results may relate to the mechanisms involved in higher testosterone dermal retention versus benzyl alcohol (12).

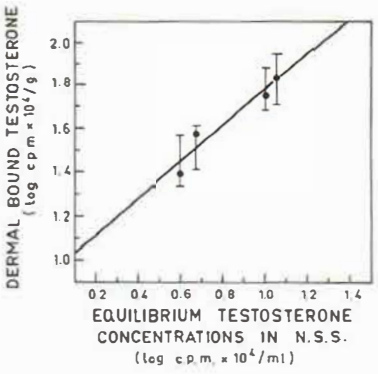

Fig. 1. Freundlich adsorption isotherm plot of dermal testosterone binding. ——, slope (+0.81) representing constant a of Equation 2. Filled circles indicate experimental data and their vertical bars denote 95% confidence limits of the regression line (12).

Since the dermal affinity for benzyl alcohol is comparatively limited and as its rate of percutaneous penetration does not differ essentially from that of testosterone (12), it follows that benzyl alcohol partitioning into the epidermis *per se* might be considerable. Apart from the partitioning dialysis of the dermis, the peeled-off epidermis was dialysed concurrently, with increasing concentrations of benzyl alcohol. The data is summarized in Table II. The ratios of epidermal/N.S.S. equilibrium concentrations are fairly constant, with an average quotient of 25.99. The K values computed by Equations 1 and 2 through regression analysis (12) are 25.98[4] and 27.22,[5] respectively. Since K remains constant, this represents the partition coefficient—epidermis/N.S.S.—of benzyl alcohol. It follows therefore that the a value of Equation 2 should

[1] $K = y$ (intercept) $= \bar{y} + 1(0\text{-}\bar{x}) = 0.3206 - 0.9162 = -0.5956$; antilog = 0.254.

[2] $K = y$ (intercept) $= \bar{y} + 0.81(0\text{-}\bar{x}) = 1.6282 - 0.6728 = 0.9554$; antilog = 9.024.

[3] $K = y$ (intercept) $= \bar{y} + 0.97(0\text{-}\bar{x}) = 0.3206 - 0.8887 = -0.5681$, antilog = 0.270.

[4] $K = y$ (intercept) $= \bar{y} + 1(0\text{-}\bar{x}) = 2.3811 - 0.9664 = 1.4147$; antilog = 25.98 (in units of 10^4 c.p.m.).

[5] $K = y$ (intercept) $= \bar{y} + 0.979(0\text{-}\bar{x}) = 2.3811 - 0.9461 = 1.4350$; antilog = 27.22 (in units of 10^4 c.p.m.).

Table II. *N.S.S.–epidermal partitioning of benzyl alcohol*

	Initial amount (c.p.m. × 10^4)			
	52	62	104	164
Partitioning data				
Weight of epidermis (g)	0.047	0.043	0.042	0.040
Equilibrium concentrations:				
Epidermis (c.p.m. × 10^4/g)	145.0	173.2	295.3	451.2
N.S.S. (c.p.m. × 10^4/ml)	5.501	6.753	11.040	17.890
Ratio: Epidermis/N.S.S.	26.359	25.048	26.748	25.221
Proportional distribution in epidermis:				
Epithelial cells (%)	34.88	32.40	27.11	34.51
Stratum corneum (%)	65.12	67.60	72.89	65.49

approach unity and was, in fact, 0.979, indicating the absence of any binding association.

For benzyl alcohol, Equation 1 restated in its non-logarithmic form (6) represents the linear partitioning relationship:

$$c_I = Kc_{II} \qquad \text{(Eq. 3)}$$

where K is the slope of the values in the skin compartments, c_I, versus that in N.S.S., c_{II}. It is seen in Fig. 2 that the epidermal slope (24.93) is about one hundred-fold that of the dermis (0.254). The significance of these results in the discernment of the epidermal barrier for benzyl alcohol is evident. The benzyl alcohol content

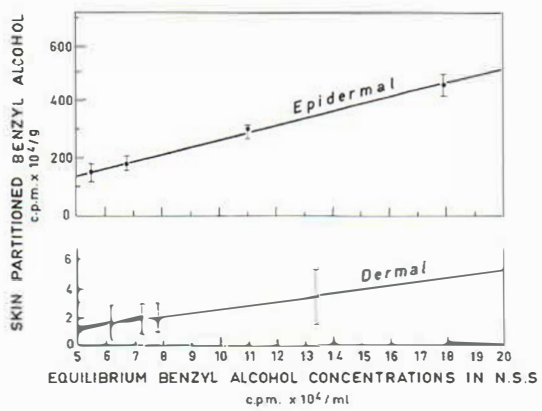

Fig. 2. Benzyl alcohol partitioning between Normal Saline Solution (N.S.S.) and skin compartments. ——, partition coefficient slopes (K of Eq. 3): dermal (+0.254) and epidermal (+24.93) respective to experimental points (filled circles) and their 95% confidence limits (vertical bars) of the regression line (12). Epidermal/dermal scale = 1 : 50. (Through scale amplifying, dermal partition coefficient slope is accentuated.)

was also estimated in the collateral layers of the epidermis exposed from both the epithelial surface and the stratum corneum.[1] The proportional distribution is listed in Table II. Predominantly, it is the stratum corneum which retains the major part of benzyl alcohol partitioned into the epidermis.

The chemical heterogeneity in the various compartments of the skin (1, 15, 16, 21) impedes the unbiased evaluation of partition–association interactions within the intact skin complex. Moreover, our findings with equilibrium dialysis, which is still the most advantageous method for binding investigations (20), should be substantiated by the other testing methods (18). In spite of these limitations, our data suggest that the skin components are affected by both partitioning and binding association with different chemicals. The skin binding of chemicals (with testosterone, in this case) is not to be considered as an exclusive characteristic of dermal tissue. The same binding mechanism (10) has been suggested for the stratum corneum proteins as inhibiting the percutaneous penetration of anionic surfactants (2). The results of this study warrant further investigation of those interactions with isolated and histochemically characterized dermal constituents.

The clinical significance of this *in vitro* observation is speculative. Percutaneous penetration and allergic contact dermatitis should be considered. The chronicity of halogenated salicylanilide and chrome dermatitis could involve such dermal binding of antigen (22). It has generally

[1] Trypsin digestion method was used to extract the drug from epithelial cells as described previously (8).

been assumed that when a chemical penetrates across the basement membrane, it is absorbed into the systemic circulation. If this *in vitro* data has *in vivo* correlation, dermal binding may require consideration in studies of percutaneous penetration kinetics. A thorough examination of various chemicals and their degree of dermal binding would be helpful in interpreting such possible clinical relevance.

It is possible that these *in vitro* skin preparations may have sufficient enzymic activity to metabolize the labeled testosterone; this was not investigated in the present study but has been demonstrated recently in another *in vitro* system (11).

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