A Comparison of Frozen and Reconstituted Cattle and Human Skin as Barriers to Drug Penetration

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Abstract □ An *in vitro* study of the permeabilities of frozen and reconstituted cattle skin and human skin to levamisole was done. Cattle skin was 400 times more permeable to levamisole from an organic solvent (largely 2-ethoxyethanol) than was human skin. The diffusion coefficient value of levamisole in cattle skin and the partition coefficient value of levamisole from the organic solvent into the skin suggested that a relatively large amount of drug passed through skin appendages such as hair follicles or sweat/sebaceous ducts. Transcellular transport across the stratum corneum was rate-determining in human skin penetration.

Keyphrases □ Permeability—frozen and reconstituted cattle and human skin *in vitro*, levamisole □ Levamisole—*in vitro* permeability through frozen and reconstituted cattle and human skin □ Dosage forms, topical—levamisole, *in vitro* permeability through frozen and reconstituted cattle and human skin

The use of topical dosage forms for the systemic delivery of drugs to domestic animals was foreshadowed by Rogoff and Kohler (1) when they showed that cattle grubs could be controlled by applying a small volume of a concentrated crufomate solution to a cow's skin. Such dosage forms, which are known as pour-ons or spot-ons, have subsequently been proposed for the systemic delivery of a variety of drugs to sheep and cattle (2–6).

The present report concerns the rate and mechanism by which the anthelmintic levamisole is transported across samples of excised cattle skin that were frozen and reconstituted.

The work was undertaken as part of a program to develop an *in vitro* screen for pour-on formulations. The development of topical drug delivery systems for humans has been greatly facilitated by the use of *in vitro* screens which are based on the realization that strong qualitative agreement exists between the rate at which drugs penetrate excised human skin in *in vitro* experiments and *in vivo* penetration (7). In addition, it was established (8) that it does not matter whether the skin used in the *in vitro* experiments is fresh or frozen or whether stratum corneum, epidermis, or whole skin is used.

EXPERIMENTAL

Preparation of Cattle Skins—Skin was harvested in mid-March (*i.e.*, early fall in Australia) from an 8-month old Hereford calf. As soon as the animal had been sacrificed its hair was clipped[1] as close as possible to the skin without damaging it. Strips of skin were removed from the dorsal thoraco-lumbar region using a dermatome[2] set at 1.1 mm. The strips of skin were immediately labeled, wrapped, and placed on ice. Within 3 hr the skins were placed in a freezer at −30° until required for an experiment. The permeability of skin handled in this way did not change appreciably after at least 12 months storage.

Before starting a skin permeability experiment, a sample was thawed at room temperature in a sealed jar containing paper that was soaked in normal saline. This procedure was adopted to minimize skin dehydration.

Preparation of Human Skin—Skin, and its accompanying subcutaneous fat, was obtained from the upper thigh of humans following leg amputations or from breasts following cosmetic surgery. The skin was frozen, stored, and thawed in the manner described for cattle skin. When the human skin had thawed, the subcutaneous fat and the lower section of the dermis were removed with a scalpel.

Materials—Levamisole[3], mp 61°, was used without further purification. the solvents used in the levamisole formulations were an aqueous borate solution (pH 8.9 buffer) and an organic solvent (solvent A) containing nonaromatic hydrocarbons (15%), polyoxypropylene 15-stearyl ether (12%), and ethoxyethanol (73%). The receptor phase was normal saline.

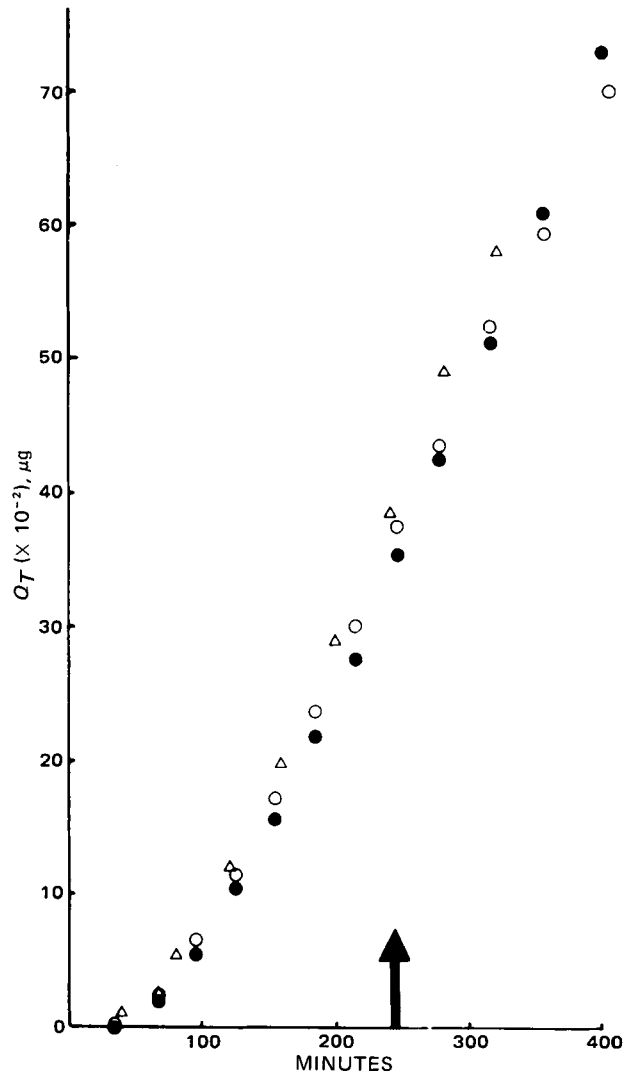

Figure 1—*The quantity of levamisole penetrating 1.45 3 cm² of cattle skin from a 10% solution in solvent A* versus *time using a constant stirring rate throughout (△), a constant stirring rate for 245 min and then half the rate (●), and a constant stirring rate for 245 min and then no stirring except for 4 min prior to taking a reading (○).*

[1] Andis R 400 or Oster A5 Clippers.
[2] Brown Electro Dermatome, Model 902.

[3] ICI Australia Ltd.

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Table I—Permeability of Cattle Skin to Levamisole from a 10% Solution in Solvent A

$10 \times r^a$, cm	$10^6 \times k_p{}^b$, cm/min	$10^6 \times k_p\, r$, cm^2/min	L^c, min	$10^5 \times D^d$, cm^2/min	$10 \times PC^e$	$10^{-2} \times Q_{200}{}^f$, μg
1.69 (0.10)	42	7.1	118	4.0	1.8	5.1
1.31 (0.03)	94	12.3	104	2.8	4.4	12.8
1.24 (0.05)	93	12.5	96	2.7	4.3	13.9
1.23 (0.05)	110	13.5	99	2.5	5.4	16.1
1.15 (0.03)	113	13.0	100	2.2	5.9	16.1
1.14 (0.08)	120	13.7	80	2.7	5.1	20.1
1.09 (0.03)	164	17.9	76	2.6	6.9	29.7
1.08 (0.02)	141	15.2	63	3.1	4.9	27.1
1.04 (0.05)	165	17.2	79	2.3	7.5	28.9
1.01 (0.03)	144	14.5	90	1.9	7.6	23.2
0.98 (0.05)	164	16.1	72	2.2	7.3	30.2
0.95 (0.04)	187	17.8	68	2.2	8.1	35.3
0.87 (0.02)	177	15.4	43	2.9	5.3	41.4
0.86 (0.06)	187	16.1	62	2.0	8.1	38.3
0.86 (0.03)	219	18.8	65	1.9	9.9	43.7
0.80 (0.03)	206	16.5	58	1.8	9.2	41.8
0.65 (0.03)	286	18.6	30	2.3	8.1	72.1

[a] r = skin thickness and number in parentheses is the spread; [b] k_p = permeability constant; [c] L = lag time; [d] D = diffusion coefficient; [e] PC = partition coefficient; [f] Q_{200} = quantity crossing 1.453 cm^2 in 200 min.

Measurement of Skin Permeability and Calculation of Permeability Constants—The method used for measuring skin permeability and the chemical assay of levamisole have been described previously (9). Skin thickness was measured at the conclusion of an experiment with a tension micrometer. These measuresments were made at the end of an experiment rather than at the beginning to avoid causing mechanical damage to the skin prior to making permeability measurements. The average of five readings taken over the entire surface was recorded.

The permeability constant, k_p (cm/min), was calculated using:

$$k_p = \frac{S \times V}{1.453 \times C} \qquad \text{(Eq. 1)}$$

where S (μg/ml/min) is the slope of the steady-state plot of drug concentration in the receptor compartment *versus* time, V (cm^3) is the volume of the receptor compartment, 1.453 cm^2 is the surface area of the skin, and C (μg/ml) is the concentration of drug applied to the skin surface.

The lag time, L (min), was taken as the intercept on the x-axis of the steady-state plot of drug concentration in the receptor compartment *versus* time.

RESULTS AND DISCUSSION

Permeability of Cattle Skin to Levamisole—*Effect of Rate of Stirring of Receptor Phase*—Figure 1 shows the amounts of levamisole that had penetrated samples of cattle skin (Q_T) from a 10% solution in solvent A. The stirring rate of the receptor phase was different in each experiment. In one experiment, a constant stirring rate was maintained from start to finish. In subsequent experiments, the same rate was maintained for 245 min, and then the stirring rate was either halved or stopped (except for 4 min immediately prior to the removal of an aliquot for assay).

The results of Fig. 1 indicate that the rate of appearance of levamisole in the receptor phase was independent of the stirring rate. This result suggests that transport of levamisole through the skin is the *in vitro* rate-determining process rather than clearance from the skin into the receptor phase (10). Increasing the agitation rate of the receptor phase would decrease the thickness of the unstirred layer of receptor phase adjacent to the skin and would increase the rate of appearance of levamisole in the receptor phase if this were the rate-determining process.

The value of the present report to an understanding of the *in vivo* topical absorption process in domestic animals depends on whether penetration of drug through the skin rather than clearance into a receptor phase is the rate-determining step in both *in vitro* and *in vivo* situations. If this were not the case, the difference in character between stirred normal saline (*in vitro* experiments) and flowing blood (*in vivo* experiments) as receptor phases would reduce the likelihood of correlation between the results of the two types of experiment.

Effect of Skin Sample Thickness—The skin samples from the calf consisted of the epidermis together with varying thicknesses of dermis.

Table II—Permeability of Human Skin to Levamisole from a 0.85% Solution in an Aqueous Buffer at pH 8.9

Donor	$10 \times r^a$, cm	$10^6 \times k_p{}^b$, cm/min	L^c, min	$10^{-2} \times Q_{200}{}^d$, μg
6	1.28 ± 0.04	131	83	2.2
6	1.23 ± 0.06	145	84	2.4
6	0.98 ± 0.19	117	98	1.7
9	1.09 ± 0.18	161	75	1.7
9	0.99 ± 0.08	135	98	2.0
8	0.91 ± 0.05	101	107	1.3
8	0.81 ± 0.01	118	150	0.9

[a] r = skin thickness. [b] k_p = permeability constant. [c] L = lag time. [d] Q_{200} = quantity crossing 1.453 cm^2 in 200 min.

Studies of the penetration of levamisole from its 10% solution in solvent A indicated that steady-state penetration was achieved by 100 min in all cases and was maintained for at least 5–6 hr. Table I shows the permeability constants, lag times, and quantity of levamisole which had penetrated in 200 min through samples of cattle skin 0.65–1.69 mm thick.

There was a strong negative correlation between skin thickness and permeability constant ($\gamma = -0.95$; $0.01\% > P$) and a positive correlation between skin thickness and lag time ($\gamma = +0.90$; $0.01\% > P$). Furthermore, the product of permeability constant and total skin thickness ($k_p r$) was essentially constant for skins whose thickness varied from 0.65–1.1 mm.

These results suggest that the outer 1 mm or so of skin acts like a homogeneous barrier to levamisole penetration. For diffusion of molecules through a homogeneous barrier, the values of k_p and L are related to the skin thickness, r, in the following manner (11):

$$k_p = \frac{D(PC)}{r} \qquad \text{(Eq. 2)}$$

$$L = \frac{r^2}{6D} \qquad \text{(Eq. 3)}$$

where D (cm^2/min) is the diffusion coefficient of the diffusing molecule in the barrier, and PC is the partition coefficeint for the diffusing substance between the barrier and the solvent.

Under conditions where Eqs. 2 and 3 apply, values of the terms $k_p r$ [$= D(PC)$] and $r^2/6L$ ($=D$) should be constant. Values of these terms are included in Table I.

While values of $k_p r$ and $r^2/6L$ do vary with r when it is changed from 1.69 to 0.8 mm, it appears that these terms are essentially constant for skin specimens <1.1 mm thick. This suggests that the portion of the skin from the surface of the stratum corneum down to a depth of ~1 mm can be considered to be a homogeneous barrier when considering levamisole penetration. The mean value of the term $r^2/6L$ throughout this thickness range, 2.1×10^{-5} cm^2/min, is the apparent diffusion coefficient of levamisole in the homogeneous barrier.

The mean value of the term $k_p r$ [$D(PC)$] throughout the barrier with a thickness 0.8–1.09 mm was 17.2×10^{-6} cm^2/min. Thus, on the basis that D was 2.1×10^{-5} cm^2/min, the apparent value of PC between the barrier and the solvent A was 0.84.

Permeability of Human Skin to Levamisole—It has been established (11, 12) that the stratum corneum is the rate-determining barrier to penetration of human skin by most nonelectrolytes of small to medium size molecular weights. This was confirmed for levamisole penetration in human skin by considering the effect of skin sample thickness and the removal of the stratum corneum on permeability.

Table II contains the calculated permeability constants and lag times for penetration of human skin at 37° by levamisole from its 0.85% solution in an aqueous buffer, pH 8.9.

Table III—Effect of Removal of Stratum Corneum on Human Skin Permeability to Levamisole from a 0.85% Solution in an Aqueous Buffer at pH 8.9

Donor	$10 \times r^a$, cm	Treatment	$10^6 \times k_p{}^e$, cm/min	L^f, min	$10^{-2} \times Q_{200}{}^g$, μg
9	1.09	*b*	161	75	1.7
9	0.97	*c*	108	92	1.7
9	0.93	*d*	720	33	17.5
9	0.91	*d*	692	46	15.5

[a] r = skin thickness. [b] treated as in Table II. [c] heated at 60° for 2 min and cooled. [d] as in *c* but stratum corneum peeled off. [e] k_p = permeability constant. [f] L = lag time. [g] Q_{200} = quantity penetrating 1.453 cm^2 in 200 min.

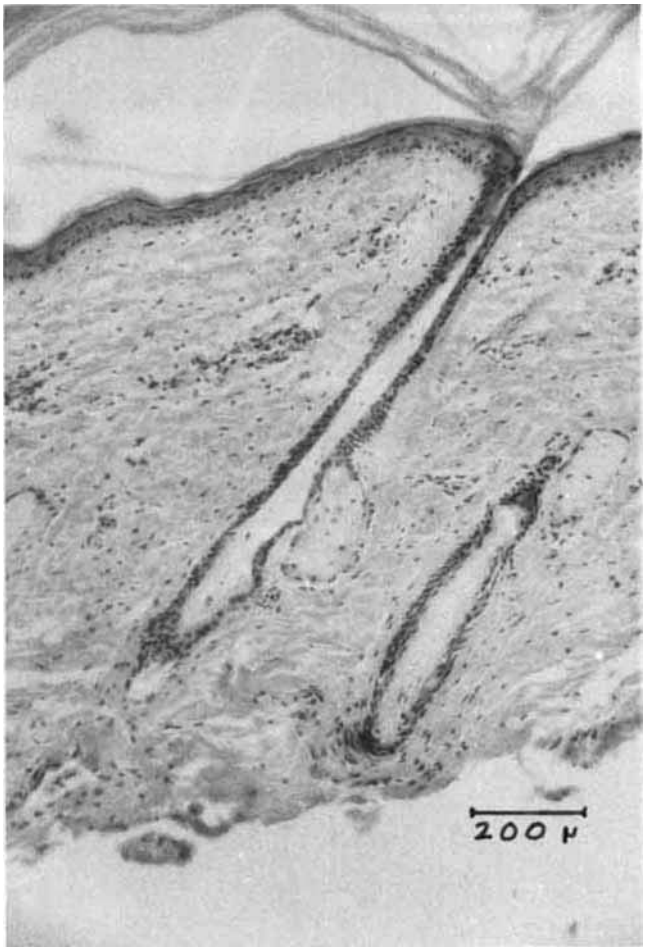

Figure 2—*Micrograph (×80) of a vertical section of calf skin stained with hemotoxylin and eosin.*

The aqueous buffer was used in these experiments instead of solvent A because levamisole only penetrated human skin very slowly from the latter solvent. This is in marked contrast to the situation with cattle skin penetration. Whereas 4180 μg of levamisole penetrated a 0.8 mm thick sample of calf skin in 200 min from solvent A, only 10 μg penetrated through a 0.81-mm sample of human skin in the same time. Only 121 μg of levamisole had penetrated the human skin in 12 hr. Steady-state penetration from the aqueous solution was achieved within 100 min in most cases and was maintained for at least 5 hr.

One problem encountered in interpreting the data in Table II arose because only limited amounts of skin could be obtained from each donor. Hence, in contrast to the situation with cattle skin penetration, insufficient data to permit a statistical analysis of the effect of skin thickness was obtained on skin from a single donor. However, the data suggest that no strong correlation with a negative correlation coefficient exists between human skin thickness and the permeability constant when the former is varied between 0.81 and 1.28 mm. The small amount of data on skin from each donor is more consistent with the conclusion that permeability constants and lag times for diffusion are independent of skin sample thickness. This is in contrast to the cattle skin permeability and is consistent with the proposal that the stratum corneum (*i.e.*, the outer 10 μm of the epidermis) is the rate-determining barrier to penetration.

Table III supports this conclusion. The data relate to the permeability of a skin sample that was heated in 60° water for 2 min prior to the penetration experiment and a similarly treated skin sample whose stratum corneum was peeled off after heating and prior to the permeability experiment.

The results show that heating the skin to 60° and cooling prior to a permeability experiment did not significantly effect the skin permeability. However, removal of the stratum corneum led to a dramatic increase in permeability which confirmed the postulate that this layer is rate determining for penetration of levamisole through human skin which has been frozen and reconstituted.

An estimate of the diffusion coefficient of levamisole in human stratum corneum, D (cm^2/min), and of the partition coefficient of levamisole between water at pH 8.9 and human stratum corneum can be made using Eqs. 1 and 2, a value of 10^{-3} cm for the thickness of the stratum corneum, and values of k_p (cm/min) and L (min) in Table II. For the purpose of this exercise, the data in Table II that were obtained on skin sample 6 were used, *i.e.*, $k_p = 131 \pm 14 \times 10^{-6}$ cm/min and $L = 88 \pm 10$ min. This led to values of $D = 1.9 \times 10^{-9}$ cm^2/min and $PC = 69$.

The value of D is similar to the values that have been reported (11) for water and low molecular weight nonelectrolytes ($3–4 \times 10^{-8}$ cm^2/min) in human stratum corneum.

Levamisole has a pKa value of 7.94 at 37° (9) and, thus, exists to the extent of 90% as a neutral molecule at pH 8.9. The calculated value of PC(69) suggests that the stratum corneum has a much lower polarity than water and is a better solvent for levamisole than water.

Mechanism of Absorption of Levamisole across Cattle Skin—The apparent constancy of the product $k_p r$ for levamisole absorption across cattle skin samples with a thickness of 0.65–1.1 mm suggests that this total skin thickness, rather than the 30 μm stratum corneum (13), is the rate-determining barrier. Values of $k_p r$ tended to decrease when skin with a thickness >1.1 mm was used. It will be argued subsequently that the first 1.1 mm of skin is richly supplied with blood and that drug molecules that penetrate into it would be rapidly absorbed into the blood. Consequently, it is proposed that controlling the rate at which drug molecules pass through the first 1.1 mm will result in control of the rate at which they enter the blood in the *in vivo* situation.

It may be argued that the stratum corneum was removed from the skin samples investigated by either the method of the skin preparation or by solvent A. The first possibility is considered unlikely since fine clipping of hair was shown (14) to remove no more than 4 of 30 layers of bovine stratum corneum. The second possibility is discounted because $k_p r$ was also constant for levamisole penetration through cattle skin from aqueous solutions.

It is proposed that the first 1.0–1.1 mm of the cattle skin used in these experiments is made up of the epidermis with its stratum corneum (60 μm thick) (13) and the highly vascular papillary layer of the dermis.

Histological studies of a variety of cattle skins revealed (15) that the first 1 mm is usually epidermis plus papillary layer of the dermis, and the lower 6 mm is the collagenous reticular layer of the dermis. McEwan-Jenkinson (16) has suggested that the papillary and reticular layers of the dermis of bovine skin meets in the area of the sebaceous gland. Confirmation of this proposition comes from inspection of the micrograph of a dehydrated, paraffin-embedded section of cattle skin (Fig. 2). This section, which is typical of the skin studied, shows that the first 1 mm of skin contains most of the hair follicle and the sebaceous gland associated with it.

It is not clear whether the bulk of the drug is transported through the first 1 mm or so of skin *via* the cells (*i.e.*, transcellular) as in human skin penetration (11) (although in this case it is only the stratum corneum which is rate determining) or *via* the appendages such as the hair follicle, sweat duct or sebaceous duct.

The value of the diffusion coefficient for levamisole in the first 1.0 mm of cattle skin (2.1×10^{-5} cm^2/min) is much closer to those of water and low molecular weight nonelectrolytes through human hair follicles ($0.3–1.2 \times 10^{-5}$ cm^2/min) or sweat ducts ($6.0–12.0 \times 10^{-5}$ cm^2/min) than to the value calculated for levamisole passing through human stratum corneum cells (1.9×10^{-9} cm^2/min). Consequently, it is tempting to postulate that most of the drug is transported *via* the skin appendages in transport across cattle skin.

A major reason why transport *via* skin appendages has only been assigned a minor role in human skin transport is because their density per unit area of skin is very low (40–70 cm^{-2} for hair follicles and 200–250 cm^{-2} for sweat glands) (11). Thus, it would be reasonable to predict that this route would be more favorable in cattle skin penetration because the density of hair follicles is higher ($\simeq$890 cm^{-2}) (17), and each follicle has a sweat gland, sebaceous gland, and the ducts associated with it. However, the greater density of hair follicles in cattle (~15 times the density in human skin) cannot on its own account for the 400-fold increase in the rate at which levamisole crosses cattle skin as compared to human skin. It is possible that penetration *via* the cattle skin appendages is facilitated by the emulsified sebum that is associated with them (18). This emulsion may be a better solvent for levamisole than the sebum which is associated with human hair follicles or the sweat associated with human sweat glands. Studies concerning the role that emulsified sebum plays in cattle skin penetration are currently underway.

If the above postulate is correct, it is likely that the drug would rapidly enter the systemic circulation in live animals because the appendages are richly supplied with blood vessels.

CONCLUSIONS

The first 1 mm or so of frozen and reconstituted cattleskin[4] acts as a homogeneous barrier to penetration of the skin by chemicals. The diffusion coefficient of levamisole in this barrier is close to its expected value in hair follicles or sweat ducts. The results of the present study suggest that polar molecules such as levamisole will penetrate cattle skin much more rapidly than human skin.

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[4] It should be noted that freezing the skin could very well alter its cellular geography to such an extent that the results of this study may not reflect properties of intact fresh skin. If this is true, then the value of the present results to *in vivo* behavior will only be realized when *in vivo* experiments are completed. However, the results clearly indicate that frozen and reconstituted cattle skin has very different barrier properties to similarly treated human skin and that the stratum corneum of the former skin does not appear to be the rate-determining barrier to penetration.

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Antitumor Agents XLVIII: Structure–Activity Relationships of Quassinoids as *In Vitro* Protein Synthesis Inhibitors of P-388 Lymphocytic Leukemia Tumor Cell Metabolism

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Abstract □ A series of brusatol, bisbrusatol, and bruceantin esters were examined for their ability to inhibit protein synthesis in P-388 lymphocytic leukemia cells. Compounds which produced high T/C % values (170–272) resulted in ID_{50} of 5.4–15.5 μM for inhibition of whole cell protein synthesis, ID_{50} of 1.3–13 μM for inhibition of endogenous protein synthesis in cell homogenates, and ID_{50} of 1.9–6 μM for inhibition of polyuridine directed polyphenylalanine synthesis using "runoff" ribosomes and a "pH 5" enzyme preparation. The polyuridine directed polyphenylalanine synthesis requires neither initiation nor termination factors, suggesting that quassinoids are exclusively elongation inhibitors. Bruceantin, brusatol, and bisbrusatolyl malonate allowed a runoff of the polyribosomes to 80S free ribosomes. However, formation of the ternary complex and 80S initiation complex were not inhibited by the quassinoids. Thus, these agents do not affect the individual steps leading to the formation of a stable 80S initiation complex in P-388 cells. Brusatol, bruceantin, and bisbrusatolyl malonate inhibited the formation of the first peptide bond between puromycin and [^{3}H]methionyl-transfer RNA bound to the initiation complex, indicating peptidyl transferase activity is inhibited by the quassinoids in P-388 cells. These studies also suggest that the free 80S ribosome is the site of binding by the quassinoid. Ribosomes actively conducting protein synthesis will continue protein synthesis and terminate before the quassinoids bind. This proves quassinoids are elongation inhibitors of tumor cells. A strong correlation was observed between potent antileukemic activity and the ability to inhibit protein synthesis in P-388 lymphocytic leukemia cells.

Keyphrases □ Protein synthesis—inhibition by quassinoids, P-388 lymphocytic leukemia cells □ Quassinoids—inhibition of protein synthesis, P-388 lymphocytic leukemia cells □ Structure–activity relationships—quassinoids, inhibition of protein synthesis, P-388 lymphocytic leukemia cells □ Antitumor agents—quassinoids, inhibition of protein synthesis, P-388 lymphocytic leukemia cells

Bruceantin, a quassinoid now in phase II clinical trials, was first isolated from *Brucea antidysenterica* (1, 2). Subsequently, bruceoside A was isolated from *Brucea javanica* (3) and brusatol was derived chemically from bruceoside A. It was demonstrated (4) that bruceantin inhibited protein synthesis in HeLa cells by 90% at 2 μM, whereas DNA and RNA synthesis were inhibited 60 and 15%, respectively. Protein synthesis was inhibited 79% in rabbit reticulocytes by bruceantin at 0.1 μM (4). Liao (4) postulated that bruceantin was an initiation inhibitor of

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