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Inhibition of Wool Follicle DNA Synthesis by Mimosine and Related 4(1*H*)-Pyridones

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Abstract

The *in vitro* incorporation of [^{3}H]thymidine has been examined in thin slices of sheep skin. Most of the radioactivity (88%) was incorporated into the bulb cells of the wool follicles, and the technique is therefore suitable for the study of some aspects of wool follicle DNA synthesis.

The effect of mimosine and a number of related 4(1*H*)-pyridones on [^{3}H]thymidine incorporation into sheep skin slices was examined. Mimosine was shown to inhibit the incorporation at a concentration of 0·2 mM. At this concentration, the incorporation of [^{3}H]uridine or [^{14}C]leucine was not affected. The inhibition of [^{3}H]thymidine incorporation was time dependent, 2 h of incubation being required for maximal inhibition of DNA synthesis, and was readily reversible by removal of mimosine from the incubation medium.

The 3-hydroxyl-4-oxo function of the pyridone ring appears to be directly involved in DNA synthesis inhibition. The amino acid side chain is not a toxophoric centre, but changes in its polarity have been shown to affect the inhibitory activity.

The results suggest that the primary action of mimosine on the inhibition of wool biosynthesis *in vivo* is the inhibition of follicle bulb cell DNA synthesis and consequently of cell division.

Introduction

The amino acid mimosine is an inhibitor of wool biosynthesis *in vivo* (Hegarty *et al.* 1964) and has been suggested as a possible agent for the chemical defleecing of sheep (Reis *et al.* 1975). Little information is yet available, however, on the specific mode of action of mimosine on the wool follicle. The compound is known to inhibit a number of biochemical reactions. It competes with tyrosine to inhibit the activity of tyrosinase (Prabhakaran *et al.* 1969, 1973), and it also inhibits the activity of a number of pyridoxal-requiring enzymes (Lin *et al.* 1963). However, cytological evidence would suggest that the primary action of mimosine in the induction of alopecia is the inhibition of follicle cell division. Early degenerative changes have been observed in the structure of wool follicles (Hegarty *et al.* 1964; Reis *et al.* 1975) and mouse hair follicles (Montagna and Yun 1963) following mimosine treatment. The compound has also recently been shown to inhibit DNA synthesis *in vitro* in cultures of H.Ep-2 cells (Tsai and Ling 1971, 1972) and mouse bone marrow cells (M. P. Hegarty, personal communication).

In this investigation we have utilized sheep skin tissue slices to examine the effect of mimosine on wool follicle DNA synthesis. We have also synthesized a number of derivatives of mimosine which have clearly defined alterations of the ring structure or the side chain of the parent molecule, and have used these to determine the parts of the molecule which are involved in the inhibitory mechanism.

Materials and Methods

The sheep used in this study were adult Dorset Horn × Merino crossbred ewes of age 3–5 years, housed indoors in single pens and maintained on a diet of 6 parts chopped lucerne and 4 parts crushed oats (600–700 g/day).

Preparation of Skin Slices

The wool was removed from the mid-side of the sheep with Oster clippers (size 000), and the skin surface washed with Zephiran antiseptic solution (1 : 75 dilution) and then with water. The animal was placed under light general anaesthesia (sodium thiopentone, May and Baker, Australia, 20 mg/kg body weight) and skin slices, each 1 mm wide and about 5 cm long, were cut using two razor blades clamped firmly to a 1-mm thick steel spacer. The slices were gently freed of underlying tissue and used immediately.

Incubation of Skin Slices and Measurement of Radioactivity

Skin slices (10–30 mg wet weight of tissue) were incubated in 5 ml of Waymouth's medium MB752 (Waymouth 1959), pH 7·2–7·4 at 37°C, in a 95% O_2–5% CO_2 atmosphere for up to 4 h. Where appropriate, inhibitors were included in the medium. The slices were then transferred to 2·5 ml of fresh medium identical to the previous medium except that it also contained 2 μCi/ml of [*methyl*-^{3}H]thymidine or [5,6-^{3}H]uridine, or 0·2 μCi/ml of L-[U-^{14}C]leucine, and incubation was carried out for a further 30 min at 37°C. For the measurement of incorporated radioactivity, incubations were terminated by placing the skin slices in cold 5% (w/v) trichloroacetic acid (10 ml). Acid-soluble radioactivity was removed by washing three times with 10 ml of this solution, allowing 15 min between solution changes. The slices were then blotted dry on filter paper, weighed and dissolved in 0·4 ml of Soluene-100 (Packard Instrument, Australia) by heating for 2 h at 60°C. After addition of 4 ml of a toluene-based scintillant solution, radioactivity was measured in a Packard Liquid scintillation spectrometer. The number of counts per minute for each sample was corrected for variable counting efficiency by reference to a quench correction curve constructed using either n-[^{14}C]hexadecane or n-[^{3}H]hexadecane as a source of standard radioactivity.

Autoradiography

For autoradiographic examination, skin slices after incubation in medium containing [^{3}H]-thymidine were fixed in acetic acid–formalin–ethanol fixative (Davenport 1960) for 4–6 h and embedded in paraffin. Sections (8 μm) were coated with Kodak AR10 stripping film, exposed for 20 days at 4°C, developed and stained with haematoxylin and picric acid.

Inhibitors

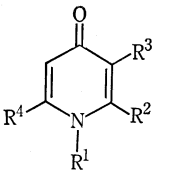
The following compounds were synthesized for the present study (details will be published elsewhere): DL-mimosine (2), DL-α-amino-β[5-hydroxy-4-oxo-2(4*H*)-pyridine]propionic acid ('iso-mimosine') (4), 3-hydroxy-4(1*H*)-pyridone (5), DL-2-methylmimosine (6), 3-hydroxy-2-methyl-4(1*H*)-pyridone (7), 1(2′-aminoethyl)-3-hydroxy-4(1*H*)-pyridone ('mimosinamine') (8), *N*,*N*-dimethylmimosinamine (9), DL-3-*O*-benzyl-2-methylmimosine (12), DL-3-deoxymimosine (13), DL-4-deoxyisomimosine (14), and DL-α-amino-β[5-hydroxy-4-mercapto-2-pyridine]propionic acid ('mercaptoisomimosine') (15). L-mimosine (1) was prepared by the method of Reis *et al.* (1975), and L-mimosine methyl ester (3), DL-α-hydroxy-desaminomimosine (10) and DL-*N*-benzoylmimosine (11) were obtained from other sources (see Acknowledgments). The authenticity and purity of the compounds was established by analysis, u.v., i.r., and n.m.r. spectroscopy, and by paper chromatography. Stock solutions of the compounds were freshly prepared at a concentration of 1 mM in water or, if necessary, in 0·1 M NaOH with adjustment of the solutions to pH 7·2 with 0·1 M HCl or 0·1 M NaOH as required.

Results

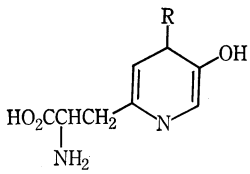
Incorporation of [^{3}H]Thymidine into Skin Slices

The use of sheep skin slices in the study of wool follicle function *in vitro* requires that the follicle cells contribute a substantial proportion of the biochemical activity of the slices. Some indication of the wool follicle contribution may be obtained by

examining by autoradiography the distribution of radioactively labelled precursors which have been incorporated by skin slices. An example of the results obtained using [^{3}H]thymidine is shown in Fig. 1*a*. Most of the cells which contain labelled nuclei were located in the follicle bulb, very frequently in close proximity to the dermal papilla. The remaining small numbers of labelled cells were distributed more generally throughout the skin slices, some being found in the outer root sheath, some in the sebaceous glands and some in the proliferative layer of the epidermis. The results suggested that most of the [^{3}H]thymidine was incorporated into the follicle bulb.



	R^1	R^2	R^3	R^4
(1)	L-$CH_2CH(NH_2)CO_2H$	H	OH	H
(2)	DL-$CH_2CH(NH_2)CO_2H$	H	OH	H
(3)	L-$CH_2CH(NH_2)CO_2CH_3$	H	OH	H
(4)	H	H	OH	DL-$CH_2CH(NH_2)CO_2H$
(5)	H	H	OH	H
(6)	DL-$CH_2CH(NH_2)CO_2H$	CH_3	OH	H
(7)	H	CH_3	OH	H
(8)	—$CH_2CH_2NH_2$	H	OH	H
(9)	—$CH_2CH_2N(CH_3)_2$	H	OH	H
(10)	DL-$CH_2CH(OH)CO_2H$	H	OH	H
(11)	DL-$CH_2CH(NHCOPh)CO_2H$	H	OH	H
(12)	DL-$CH_2CH(NH_2)CO_2H$	CH_3	OCH_2Ph	H
(13)	DL-$CH_2CH(NH_2)CO_2H$	H	H	H



(14) R=H
(15) R=SH

Similar results have been obtained using the precursors [5,6-^{3}H]uridine and L-[U-^{14}C]-leucine and these will be reported in detail elsewhere (K. A. Ward, unpublished data). To obtain a more accurate measure of the bulbar incoporation, advantage was taken of the property of the crossbred sheep used in this study that, in skin of the wool-bearing regions, most of the follicle bulbs are located at an approximately uniform depth from the epidermal surface (K. A. Ward, unpublished data). This enabled the portion of the dermis containing the follicle bulbs to be removed from the remainder of the skin slice. Six skin slices were incubated with [^{3}H]thymidine and each was then divided by cutting at a level just above the supra-bulbar region of the follicles. The amount of radioactivity in each part was then measured. The proportion of [^{3}H]thymidine in the portion of the skin containing the follicle bulbs was $88 \pm 0{\cdot}6\%$ (mean $\pm$ s.e.), a result which supports the autoradiographic evidence that most of the [^{3}H]thymidine incorporated *in vitro* by skin slices is in the follicle bulbs.

The rate of incorporation of [^{3}H]thymidine into skin slices in Waymouth's medium was linear for at least 4 h (Fig. 2). Furthermore, it was revealed that the distribution of radioactivity in the skin slices was not altered by incubation for at least 2 h in

unlabelled medium prior to labelling with [^{3}H]thymidine. These results indicate that skin slices may be used to study certain aspects of wool follicle DNA synthesis, particularly in relation to the action of compounds which affect such synthesis.

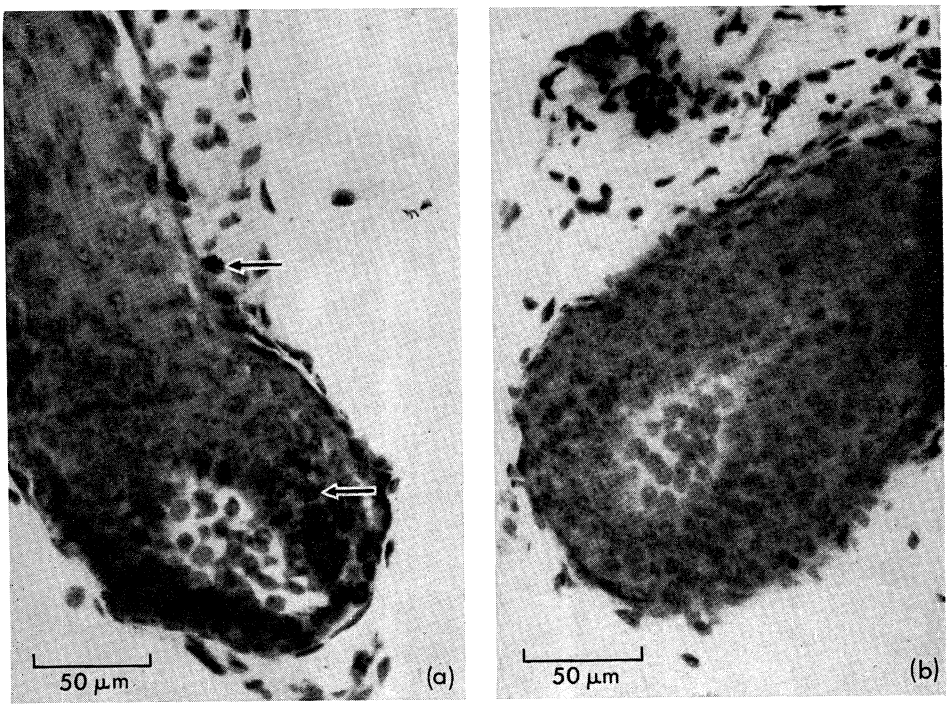


Fig. 1. Autoradiographs of skin slices labelled with [^{3}H]thymidine and incubated in Waymouth's medium (*a*) without mimosine and (*b*) containing 0·2 mM mimosine. Heavily labelled nuclei (indicated by arrows) are clearly seen in (*a*) but not in (*b*).

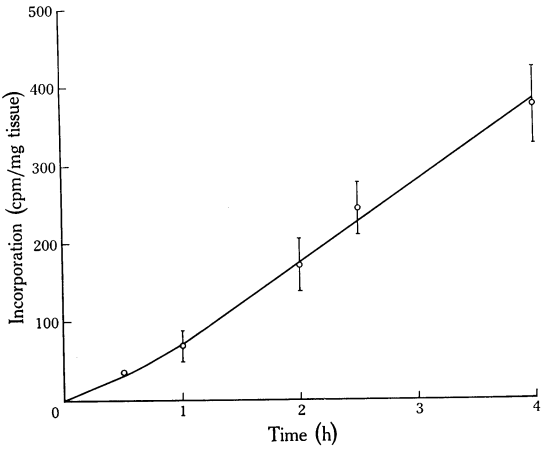


Fig. 2. Incorporation of [^{3}H]thymidine into skin slices. Each point is the mean of six determinations. Standard errors are indicated by vertical bars.

Effect of Mimosine on the Incorporation of [^{3}H]Thymidine into Skin Slices

The effect of mimosine on [^{3}H]thymidine autoradiographs of skin slices is shown in Fig. 1*b*. A great reduction in the concentration of silver grains over the follicle bulb

cells can be clearly seen in comparison with control skin (compare Fig. 1*a*). This observation is supported by the measurement of radioactivity in skin slices (Fig. 3). At a concentration of 0·2 mM, mimosine reduces the incorporation of [^{3}H]thymidine to about 20% of control values. The inhibition is a time-dependent process, so that at a mimosine concentration of 0·2 mM, a pre-incubation time of 2 h in unlabelled medium is required to maximally reduce the amount of [^{3}H]thymidine incorporated in a subsequent 30-min incubation. In contrast to the results obtained with [^{3}H]thymidine, 0·2 mM mimosine had no effect on the incorporation of L-[U-^{14}C]-leucine or [5,6-H^3] uridine into skin slices (Fig. 3).

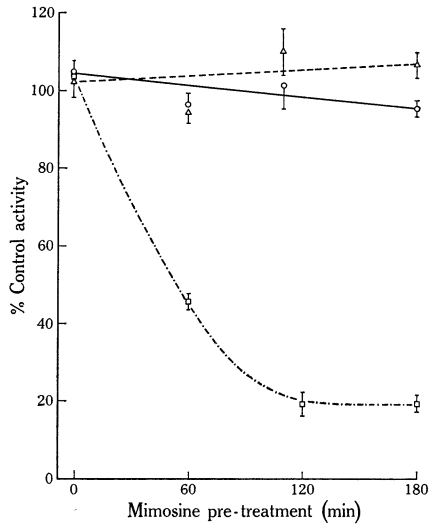


Fig. 3. Effect of mimosine on the incorporation of radioactively labelled precursors into skin slices. 0·2 mM mimosine was omitted from the incubation medium for control skin slices. Each point is the mean of six determinations. Standard errors are indicated by vertical bars. □ [^{3}H]Thymidine. ○ [5,6-^{3}H]Uridine. △ L-[U-^{14}C]Leucine.

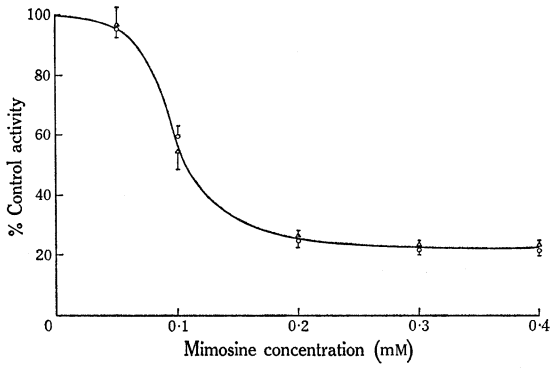


Fig. 4. Effect of various concentrations of mimosine on the incorporation of [^{3}H]thymidine into skin slices. Each point is the mean of 12 determinations. Standard errors are indicated by vertical bars. ○ L-Mimosine. △ DL-Mimosine.

The effect of different concentrations of mimosine on the incorporation of [^{3}H]thymidine into skin slices is shown in Fig. 4. The amount of radioactivity incorporated decreases rapidly from 95 to 25% of control values as the mimosine concentration increases from 0·05 to 0·2 mM. The further increase in mimosine concentration to 0·4 mM results in little additional change. Similar results were obtained both with the naturally occurring isomer L-mimosine, and with the chemically synthesized racemate DL-mimosine (Fig. 4).

The inhibition of [^{3}H]thymidine incorporation was rapidly reversed when mimosine was removed from the incubation medium (Fig. 5). As little as 60 min after the removal of mimosine, [^{3}H]thymidine incorporation had returned approximately to control values, and by 2 h had exceeded that of control tissue by about 50%. The incorporation then slowly declined to regain control values by about 4 h after removal of mimosine.

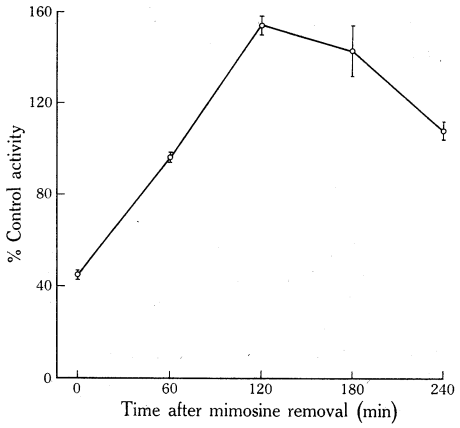


Fig. 5. Recovery of [^{3}H]thymidine incorporation into skin slices after removal of mimosine. Skin slices were incubated for 2 h in Waymouth's medium containing 0·2 mM mimosine and were then transferred to Waymouth's medium without mimosine and incubation continued. At the indicated times, slices were transferred to identical medium containing [^{3}H]thymidine and incubated for a further 15 min. Control skin slices were treated identically except that mimosine was omitted from the initial 2-h incubation. Each point is the mean of four determinations. Standard errors are indicated by vertical bars.

Effect of Mimosine Analogues on the Incorporation of [^{3}H]Thymidine into Skin Slices

The effect of a number of mimosine analogues on [^{3}H]thymidine uptake by sheep skin slices was studied in an attempt to define the structural features of the mimosine molecule that are essential for DNA synthesis inhibition in the wool follicle.

The results (Table 1) show that considerable variations in molecular structure are possible without appreciably affecting *in vitro* activity.

Table 1. Inhibition of wool follicle DNA synthesis by mimosine and related 4(1*H*)-pyridones

Skin slices were incubated in Waymouth's medium containing 0·2 mM of the appropriate inhibitor and radioactivity was determined as described in Methods. Each value is the mean of eight determinations ± s.e.

Compound No.	Name	% Control incorporation	Compound No.	Name	% Control incorporation
1	L-Mimosine	28±2	9	*N*,*N*-Dimethylmimosinamine	35±5
2	DL-Mimosine	28±3	10	DL-α-Hydroxy-desaminomimosine	92±4
3	L-Mimosine methyl ester	34±5	11	DL-*N*-Benzoylmimosine	104±10
4	DL-Isomimosine	27±2	12	DL-3-*O*-Benzyl-2-methylmimosine	100±14
5	3-Hydroxy-4-(1*H*)-pyridone	35±4	13	DL-3-Deoxymimosine	101±10
6	DL-2-Methylmimosine	28±2	14	DL-4-Deoxyisomimosine	100±18
7	3-Hydroxy-2-methyl-4(1*H*)-pyridone	21±1	15	DL-Mercaptoisomimosine	66±5
8	Mimosinamine	74±9			

Thus, racemization, esterification, shifting or removal of the amino acid side chain (as in compounds 2, 3, 4 and 5 respectively) does not diminish the activity, nor does the introduction of a methyl substituent into the pyridone ring (as in compounds 6 and 7). The presence in the molecule of side chains in which there is a basic function (compounds 8 and 9) still allows inhibitory activity; however, when an anionic (non-zwiterionic) carboxyl function is present (as in compounds 10 and 11) activity is completely eliminated.

When the 3-hydroxy group of the pyridone ring is either masked (compound 12) or absent (compound 13), the compounds are inactive, and on removal of the 4-oxo function (as in compound 14) activity is likewise abolished. Less drastic modification at this site (as in compound 15) reduces but does not completely destroy activity.

Discussion

The rapid proliferation of wool follicle bulb cells has been well documented (Schinckel 1961; Short *et al.* 1965; Downes *et al.* 1966; Chapman 1971), and it is therefore not surprising to find in this study that almost 90% of the incorporation of [^{3}H]thymidine into sheep skin slices takes place in the follicle bulbs. The high activity of the bulb allows certain aspects of wool follicle DNA synthesis to be studied *in vitro* in skin slices, thereby at least partially maintaining the association which exists *in vivo* between the follicle bulb and the dermal papilla. The extent to which the DNA synthesis measured in this manner approximates that which occurs *in vivo* has yet to be determined, but the linear incorporation of [^{3}H]thymidine for several hours *in vitro* suggests that the process has not been greatly disturbed.

It is clear that at a concentration of 0·2 mM, mimosine inhibits the *in vitro* synthesis of DNA in wool follicles, whereas it has no observable effect either on *in vitro* protein synthesis, measured by L-[U-^{14}C]leucine incorporation, or on *in vitro* RNA synthesis, measured by [4,5-^{3}H]uridine incorporation. The concentration necessary for DNA synthesis inhibition is similar to that required for mimosine to be effective *in vivo* (Reis *et al.* 1975), which suggests that the defleecing effects caused by the compound are due to the inhibition of follicle bulb cell DNA synthesis and hence the arrest of follicle cell division.

The present study of structure–activity relationships has emphasized the essential role of the 3-hydroxy-4-oxo moiety of mimosine and related compounds in DNA synthesis inhibition. Those compounds in which this function is masked or absent (compounds 12–14) are totally inactive and the compound with the related 3-hydroxy-4-mercapto moiety (compound 15) is active at a reduced level. The chelating ability of the 3-hydroxy-4-oxo function has been demonstrated (Tsai and Ling 1973) and has been implicated in the mechanism of inhibition by mimosine of other biological systems (Matsumoto *et al.* 1951; Lin and Ling 1961; Smith and Fowden 1966; Tsai and Ling 1972). Earlier evidence for the role of the chelate function has relied on the lack of inhibition exhibited by mimosine chelates, but the results are complicated by the slower rates of absorption and transport of chelated compounds. The inactivity of mimosine analogues (compounds 12–14) offers more direct evidence of the role of the 3-hydroxy-4-oxo function in the mechanism of inhibition.

The role of the amino acid side chain in mimosine toxicity is not so obvious. However, since activity is undiminished in its absence (e.g. compound 5), it cannot be a toxophoric centre. The only 3-hydroxy-4(1*H*)-pyridones tested with which no DNA synthesis inhibition was observed are compounds 10 and 11, both of which contain an anionic carboxyl group. It appears likely, therefore, that the loss of activity in these compounds may be a result of their altered side-chain polarity.

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