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THE INFLUENCE OF LIMONENE ON INDUCED DELAYED HYPERSENSITIVITY TO CITRAL IN GUINEA PIGS. II. LABEL DISTRIBUTION IN THE SKIN OF ^{14}C-LABELLED CITRAL

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Abstract. ^{14}C-labelled citral was applied on four groups of guinea pigs: (*a*) one sensitized to citral alone; (*b*) one sensitized to a citral-*d*-limonene 1:1 molar mixtie; (*c*) FCA-treated controls; (*d*) controls. The most important results concern the amount of labelled material in soluble compared with insoluble skin protein extracts (SPE). In citral-sensitized animals, more label was found in the soluble SPE when citral +limonene was applied to the skin; in citral+limonene sensitized animals, the same trend (i.e. more label in soluble SPE) was found. The possible role of limonene in alleviating the allergic reaction to citral is discussed.

Key words: Allergic contact dermatitis to citral; Limonene-modulating effect in ACD, ^{14}C-labelled citral

Whereas experimental sensitization to citral in man is successful, it is not possible to induce sensitization to lemongrass, a natural citral + limonene 4:1 mixture (5). This "quenching" effect, also noted for other sensitizing aldehydes such as cinnamaldehyde and phenylacetaldehyde when "inhibitors" such as eugenol and phenylethanol respectively were added, could, if confirmed, be of great importance in the problem of sensitization to perfumes and fragrances.

In a histological study of the effect of limonene added to citral (2), we found that, in fact, the allergic reaction to citral–limonene mixtures was weaker than that to citral alone in citral-sensitized animals. Also worthy of note was the weaker response to citral in citral+limonene sensitized animals as compared with citral-sensitized ones.

In order to confirm this difference, we undertook a study of ^{14}C-label distribution in the skin, urine and feces in two groups of animals painted with either citral alone or with citral + limonene mixture. The results of this study are reported here.

MATERIAL AND METHODS

The study was made on 8 female Hartley albino guinea pigs (the same as those used in biopsy experiments, reference 2). Four groups of animals were used: (i) citral-sensitized animals, (ii) citral + *d*-limonene sensitized animals (sensitization methods have been described previously, see reference 2), (iii) intact controls, and (iv) FCA treated controls (2).

1,2-^{14}C-labelled citral (specific activity 83 μCi/mg) was kindly provided by Dr M. Montavon from Hoffmann-La Roche Basle. Experiments were conducted in the laboratory of Dr G. Klecak, Hoffmann-La Roche, Basle.

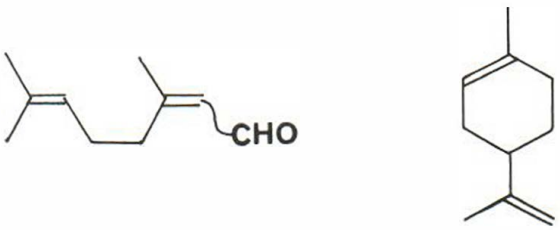
Cold citral from *Litsea Cubeba* and *d*-limonen (from lemongrass) were kindly supplied by Dr W Pilz from Haarman und Reiner, GmbH, West Germany. The purity of these substances was tested by GC, NMR and IR. 0.5% ethanol solutions were used, each containing 0.025 mg labelled citral, per 100 ml.

The general procedure for depositing solution on the skin was as follows. The flanks of the animal were shaved. On each flank a circular 15 cm^2 area was delineated with an ink stamp. With a micropipette 188 μl was deposited on each 15 cm^2 area.

The animal was placed in a metabolism cage where urine and feces could be collected separately; water was provided, but no food. The animal was sacrificed by an intracardial nembutal injection 16 h after deposit was made on the skin.

The skin was collected and wiped with a cotton swab containing methanol, which was then placed in 100 ml toluene, stirred, and radioactivity measured in two aliquots (Sample C).

The skin was stretched on a board in such a way as to cover its initial area. The 15 cm^2 marked area was punched with 28 mm^2 drills (twice) which were placed in soluene (2 ml) and heated at 60° for 4 hours. After cooling to rt, acetic acid (100 μl), aquasol II (10 ml) and distilled water (5 ml) were added and radioactivity counted (D).



Citral Limonene

Fig. 1. Chemical structures of citral and limonene.

Table I. *Label distribution (% of initial deposit) of ^{14}C-citral in sensitized guinea pigs and controls*

Animal sensitized to[a]	Elicitation with[b]	A Feces	B Urine	C Cotton swab	D Total skin	E Stratum corneum	F Stripped skin	G Insoluble skin	H Precipitate
1) C	C	0.3	11.6	8.3	27.5	12.2	10.5	5.3	0.1
2) C	C+L	0.6	11.1	7.5	26.2	11.0	9.0	4.1	0
3) C+L	C	1.0	17.4	4.1	21.5	11.2	5.9	2.6	0
4) C+L	C+L	0.6	15.0	6.2	22.3	11.1	6.5	2.3	0.1
FCA-treated control	C	0.1	14.1	7.1	17.1	10.0	7.2	3.2	0.2
FCA-treated control	C+L	0.3	14.6	9.8	2.6	10.3	8.0	2.9	0.8
Control	C	0.4	11.7	6.1	23.9	10.8	6.4	2.5	0.4
Control	C+L	0.1	11.9	6.0	28.0	12.1	9.2	3.7	0.6

[a] C = citral, L = limonene, C+L = 1 : 1 molar mixtures.
[b] All solutions are 0.5% in citral.
[c] FCA = Freund's complete adjuvant.
[d] Percentages are expressed relative to the total label deposited on the skin.

The skin was then stripped with a cellulose tape (15 strips) until the wet layer appeared. The tapes were collected in toluene (4×10 ml) stirred for 1 h and radioactivity counted (E, stratum corneum). The stripped skin was counted as above (F).

The remaining skin was cut into small pieces (area <1 cm²) dipped in liquid nitrogen and blended. The preparation was introduced into an Erlenmeyer flask containing 350 ml phosphate buffer (pH 7.2, ref. 1), stirred, and placed in a cold room (5°C) for 40 h with stirring. After that time, the buffer was centrifuged and filtered, giving sample I.

The insoluble portion was homogenized with a spatula, weighed, and 200 mg aliquots were dissolved in soluene (2 ml) and heated for 4 h at 60°C. This is sample G.

The filtered buffer (5) was then placed in dialysis bags (10 000 daltons exclusion), 100 ml per 1.2 bag, and dialysed against 5 l de-ionized water. Dialysis water was changed after 4 h and this was repeated each 24 h for 4 days. An aliquot (5 ml) of the dialysate was placed in 10 ml aquasol II and radioacitivity measured. This is sample K. The radioactivity of the content of the dialysis bag (dialysed) was counted (sample J).

Radioactivity of urine was measured directly (sample B) by dissolving 100 µl in 15 ml aquasol II.

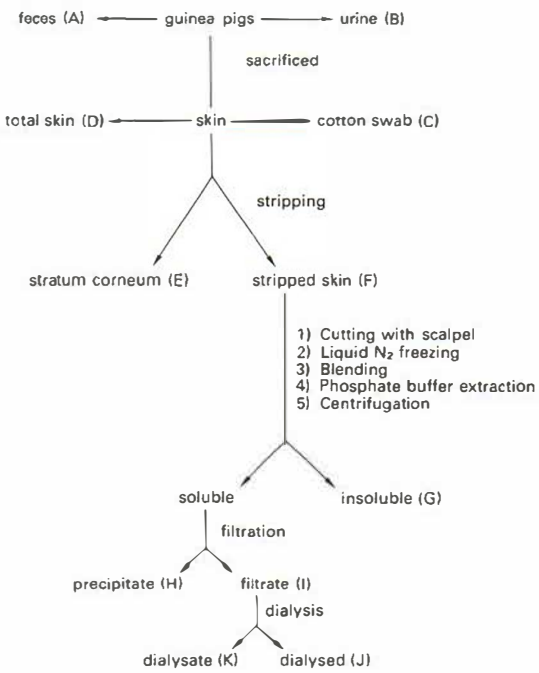
Feces were ground, weighed, and aliquots (20 mg) dissolved in *X* ml soluene and left at 60°C for a few hours. An aliquot of this solution (300 µl) was dissolved in a mixture of aquasol II (10 ml) and soluene (5 ml).

RESULTS AND DISCUSSION

The general procedure for the treatment of skin extracts is depicted in Scheme 1. Results are presented in Table I. Recovered radioactivity ranged from 39 to 48% of the total deposited on the animal skin.

Preliminary in vitro ^{14}C-citral skin penetration was performed by using stretched skin placed on glass chambers and measuring the amount of label passing into the receiving flask (3). These experiments showed that about 50% of the labelled citral was disappearing by evaporation into the atmosphere.

The concentration used in all experiments (8



Scheme 1. Treatment of guinea pigs 16 h after deposit on the skin of citral or citral + limonene solutions.

I Soluble skin before dialysis	J Unfiltered dialysed	K Dialysate	L $100 \times \frac{J}{J+G}$	Total recovered
5.1	0.6	4.6	10.2	47.7
4.9	0.8	3.9	16.3	45.2
3.3	0.4	2.4	13.3	43.9
4.1	0.5	1.8	17.8	44.1
3.8	0.8	3.0	20	38.4
4.3	0.7	3.4	19.4	47.4
3.5	0.6	2.4	19.3	42.1
4.9	0.9	3.3	19.6	45.9

animals) was 0.5% in citral. The animals were sacrificed after 16 h: the preliminary in vitro skin penetration showed that most of the label had penetrated after that time.

"Total" skin contains, after 16 h, 17–18% labelled compounds. By "soluble skin" we mean the soluble skin proteins extracted with phosphate buffer, after centrifugation. By "insoluble skin" we mean the insoluble skin protein extract from the above separation. Although the exact composition of the "soluble skin protein extract" is not known, purification through a G-75 Sephadex column showed a major peak in the 12 000 dalton range.

Since a fair amount of radioactivity was found in urine (11.1 to 17.4%, column B), this shows that the chosen time for observing label distribution (i.e., 16 h after desposit) was probably enough to allow for all metabolic pathways (including binding of the hapten to a carrier) to take place. The very small amount of label in feces (0.1 to 1.0%), however, was to be expected from the relatively short duration of the experiment.

Also to be noted is the rather constant amount of radiolabel in the *stratum corneum* in all animals: from 10.8 to 12.2% (column E). Since penetration through the *stratum corneum* is a passive phenomenon (7) one could have expected such a finding. The "precipitate" (column H) was formed during dialysis of the soluble protein extracts and probably corresponds to some protein denaturation, and since the amount of label is small (0 to 0.8%), the results are not considered significant.

Probably, the most significant results appear in the column showing the ratio of "soluble skin protein extracts" (column J) over "soluble + insoluble skin protein extracts" (columns J + G).

In the elicitation tests to citral + limonene there is 4.5 (17.8 vs. 13.3) to 6.1% (16.3 vs. 10.2) more label in the soluble skin protein extract. This trend was evident in sensitized guinea pigs. In controls, there was no difference between citral and citral + limonene solutions (19.3 to 20.0%).

Limonene could act as a tolerogen, i.e. according to current theories (6), it could be responsible for the increase in suppressor cell populations. Normally this would operate *at the induction* level. Indeed when one compares the results of biopsies in elicitation tests for citral (2) in guinea pigs, the group which had been sensitized to a 1 : 1 molar mixture of citral + limonene reacted in a weaker way. However, this "hyposensitizing" effect of limonene is also evident at the *elicitation stage*.

Another possible effect can be deduced from radiolabel experiments described here. When a mixture of ^{14}C-citral and limonene was applied to the skin, more citral (or its metabolites) passed into the "soluble" skin protein extracts. In other words, the was less labelled citral in the "insoluble skin protein extracts" when limonene was added to the sensitizing solution. This is particularly striking when one compares animal 1 (sensitized to citral, elicited with citral) with animal 4 (sensitized to a citral + limonene mixture). There is 7.6% (17.8 vs. 10.2, column L) more label passing into the "soluble" protein fraction. In a seris of experients with guinea pigs, Macher & Chase (4) had shown that a minimal amount of sensitizer (DNCB in that case) remaining in the skin was required to induce sensitization. The effect of limonene could be to induce more citral to pass into the "soluble" fraction (and hence less remaining in the insoluble skin protein extract).

In conclusion, the quenching effect of limonene in citral-sensitization can be detected to some extent in biopsies and radiolabel experiments. The reasons for this effect are still unclear.

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