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Dermal Absorption and Metabolism of the Antipsoriatic Drug Dithranol Triacetate[1)]

By W. Wiegrebe*, A. Retzow*, E. Plumier*, N. Ersoy*, A. Garbe**, H.-P. Faro**, and R. Kunert**

Summary: *Percutaneous absorption, excretion kinetics, and metabolism of dithranol triacetate (2) have been investigated in Wistar rats. By the use of two differently labelled molecules – ^3H in the anthracene nucleus and ^{14}C in the acetoxy groups of* **2**, *resp. – the fate of the different parts of the dithranol triacetate molecule could be followed. After injection, large amounts of* **2** *are cleaved under the influence of enzymes into acetate and dithranol. These deacetylated metabolites lose half their ^3H label with formation of ^3H_2O. In urine, 1,8-diacetoxy-9-anthrone, 1-acetoxy-8-hydroxy-9-anthrone, 1,8-dihydroxy-9,10-anthraquinone and its diacetate were found as metabolites.*

After dermal application, unchanged **2** *is practically not absorbed at all. Arylesterases which, according to in vitro studies, are present in or on the skin, hydrolyse dithranol triacetate to give free dithranol. Up to 33% of the latter are absorbed from under an occlusive dressing. Dithranol triacetate, therefore, shows pro-drug characteristics for the treatment of psoriasis.*

Zusammenfassung: *Dermale Resorption und Metabolisierung des Antipsoriatikums Dithranol-triacetat*

Die perkutane Resorption, die Ausscheidungskinetik und der Metabolismus von Dithranol-triacetat **(2)** *wurden bei Wistar-Ratten untersucht. Durch die Verwendung zweier verschieden markierter Verbindungen – ^3H im Anthracen-Teil und ^{14}C in den Acetat-Resten – konnte das Schicksal der verschiedenen Molekülteile verfolgt werden. Nach Injektion wird* **2** *in großem Umfang durch enzymatische Einwirkung in Acetat und Dithranol gespalten. Die desacetylierten Metaboliten verlieren zur Hälfte die ^3H-Markierung unter Bildung von ^3H_2O. Als Stoffwechselprodukte wurden nach iv. Injektion von ^3H-***2** *im Urin 1,8-Diacetoxy-9-anthron, 1-Acetoxy-8-hydroxy-9-anthron, Dithranol, 1,8-Dihydroxy-9,10-anthrachinon und dessen Diacetat gefunden. Bei der dermalen Verabreichung wird unverändertes* **2** *praktisch nicht resorbiert. Die in der Haut in vitro nachweisbaren Arylesterasen hydrolysieren jedoch* **2** *zu Dithranol, das unter einem Okklusiv-Verband bis zu 33% resorbiert wird. Dithranol-triacetat erweist damit seine Prodrug-Eigenschaften für die Psoriasis-Behandlung.*

Key words: *Antipsoriatic drugs · Dithranol acetate, dermal absorption, metabolism*

1. Introduction

Dithranol (1,8-dihydroxy-9-anthrone) **(1)** is a proven antipsoriatic usually to be applied topically. Since it irritates healthy skin and causes brown discolouration [1], its use is mostly restricted to in-hospital treatment. For these reasons the colourless and less aggressive 1,8,9-triacetoxyanthracene (dithranol triacetate) **(2)** has been used instead, although it acts more slowly and more weakly. In a previous publication [1] we investigated the metabolism of dithranol triacetate in serum, the results indicating both hydrolytic and oxidative pathways.

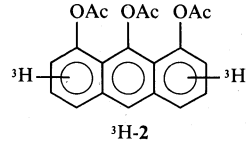
The present work is concerned with dermal absorption and metabolism of ^3H- and ^{14}C-dithranol triacetate in rats, as well as with metabolic reactions in vitro by homogenates of fresh mouse and rat skin.

2. Materials and equipment

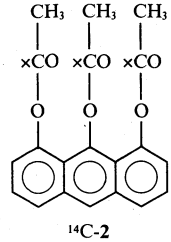
2.1. Chemicals (see formula diagram)

1,8,9-Triacetoxyanthracene (dithranol triacetate, **2**); 1,8-dihydroxy-9-anthrone (dithranol, **1**); 1-hydroxy-8-acetoxy-9-anthrone (dithra-

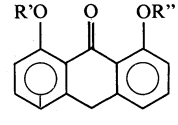
[1)] Preliminary communication: Arch. Derm. Res. **273**, 163 (1982).



^3H-2



^{14}C-2



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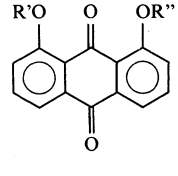
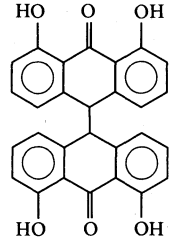
3: R'=R"=Ac
4: R'=Ac; R"=H



5: R'=R"=H
6: R'=R"=Ac
7: R'=Ac; R"=H



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nol monoacetate, **4**); 1,8-diacetoxy-9-anthrone (dithranol diacetate, **3**); 1,8-dihydroxy-9,10-anthraquinone (dantron, **5**); 1-hydroxy-8-acetoxy-9,10-anthraquinone (dantron monoacetate, **7**); 1,8-diacetoxy-9,10-anthraquinone (dantron diacetate, **6**); 1,1',8,8'-tetrahydroxy-10,10'-bisanthrone (**8**); aryl sulfatase/glucuronidase (12 U/ml from Helix pomatia (Merck No. 4114)).

Radiolabelled compounds

1,8,9-Tri-[1-^{14}C]acetoxyanthracene (^{14}C-**2**) [3]; 12.5 mCi/mmol [3]; 1,8,9-triacetoxy-[G-^{3}H]anthracene (^{3}H-**2**) [3]; 400 mCi/mmol [3]; sodium [1-^{14}C]acetate; 50 mCi/mmol (New England Nuclear, Boston, USA).

2.2. Thin-layer chromatography

TLC-Plates: Silica Gel 60 F_{254} 20 × 20 cm (Merck No. 5715).

2.3. Measurement of radioactivity

Liquid scintillation spectrometer: Packard Tri-Carb, model 3380/544.

Results were calculated as dpm with an external standard-channel ratio method. Samples were counted 3 times for 5 min.

Radioactivity measurements on TLC plates were performed on a Berthold TLC Scanner II model-LB 2721 or LB 2723. Samples were oxidized before liquid scintillation counting with a Packard Tri-Carb 306 Sample Oxidizer.

2.4. Scintillation solutions

a) Dioxane-based scintillator: 7 g PPO; 0.3 g dimethyl-POPOP; 100 g naphthalene in 1000 ml dioxane; 16 ml per sample.

b) Omniscintisol® (Merck No. 15386), 10 ml per sample.

3. Methods

3.1. In vitro stability of radiolabelled compounds

[^{3}H]-**2** and [^{14}C]-**2** were tested for both radiochemical homogeneity and chemical stability in protic solvents. Neither compound lost any amount of radioactivity in the form of [^{14}C]acetic acid or $^{3}H_2O$.

3.2. Experimental animals

Mice: male, weighing approx. 20 g (used in the experiments described in 3.8.); origin: EMD NMRI (SPF).

Rats: male, weighing approx. 200 g (used in the experiments described in 3.3–3.8); origin: EMD Wi-AF/Han (SPF).

3.3. Administration

3.3.1. i.v. and p.o.

[^{3}H]-**2** and [^{14}C]-**2** were both used undiluted with inactive material.

The labelled compounds were stored as standard solutions in benzene. The required amount of standard solution was evaporated to dryness in a stream of nitrogen, and the residual material dissolved in PEG 200. 0.1 ml of this solution was injected into one of the caudal veins of each animal.

For oral application, 0.5 ml of PEG 200 solution were given by gavage.

3.3.2. Percutaneous application

a) An aliquot of the standard solution containing approx. 40 μCi (1.1 mg) of [^{14}C]-**2** was evaporated in a stream of nitrogen. After addition of 50 mg of petroleum jelly (white petrolatum) [4] the mass was melted and subsequently stirred until cooled off.

Homogeneity of the preparation was ascertained by exact radioactivity measurements on samples taken from four different locations in the mixing vessel.

The concentration mentioned above corresponds to approx. 2.2% of **2** in white petrolatum.

[^{3}H]-**2**-ointment was prepared in the same way.

Approx. 30 mg of this ointment (determined accurately by reweighing the unused portion) were applied to a 2 × 2 cm area of the skin on the back while massaging gently for 1 min.

To protect the ointment from being licked off by the animal, and to optimise absorption through the skin, the treated area was covered by a piece of polyethylene foil [5].

b) 20 μl of a DMSO-solution [6] containing 16 μCi (0.4 mg) of [^{14}C]-**2** were applied to a 2 × 2 cm area and covered by a piece of polyethylene foil as above.

3.4. Animal experiments

During the experiments the rats were kept in closed metabolism cages through which a constant stream of air was drawn by a suction pump. The animals had unlimited access to standard feed and water.

Urine and feces were collected separately, expired $^{14}CO_2$ was absorbed in a trap containing 40% aqueous ethanolamine solution as ^{14}C-carbamate [7]. The traps were changed at 24-h intervals. At the end of the experiment, after 72 h, the animals were sacrificed.

Mice were sacrificed upon arrival from the breeding station.

3.5. Sample preparation and radioactivity measurements

Samples of urine and plasma (0.2 ml in each case) were measured directly after addition of scintillator. A sample of the ethanolamine solution was diluted with 5 ml methanol, and 10 ml of scintillator were added. Solid samples (feces and tissues) were homogenized in 3 times their volume of water, subsequently, an aliquot (1 g) was dried and oxidized. Skin was carefully dissolved in 20% NaOH and decolourized with a few drops of H_2O_2. Radioactivity in the polyethylene foil was measured after oxidation.

3.6. Determination of $^{3}H_2O$ in urine and plasma

3 ml urine or plasma were freeze-dried. Co-distilled volatile organic material was removed from the distillate by absorption on activated charcoal. Approx. 1.8 ml of the water were redistilled, aliquots of 0.5 ml of the final distillate were measured.

3.7. Separation of urinary metabolites

After administration of [^{3}H]-**2**, 3 ml of the 24-h urine were shaken with 30 ml methylene chloride. After 15 min, the phases were separated by centrifugation (3000 rpm). 20 ml of the organic layer were evaporated, and the residue dried by azeotropic distillation with 3 portions of 0.2 ml chloroform and applied to a silica gel thin-layer plate. After development with toluene-methanol-glacial acetic acid (85 : 10 : 5, v/v) the plate was scanned with a radio-TLC-scanner. Identification of radioactive zones was done by comparison with zones of the corresponding non-radioactive materials on the same plate.

2 ml of urine were hydrolyzed by 24 h incubation at 37 ° with 0.5 ml β-glucuronidase/arylsulfatase. The mixture was worked up and chromatographed as described above.

3.8. Arylesterase activity in mouse and rat skin homogenates

The skins of 4 mice were freed from fat, blood vessels, and hair, immersed into 30 ml ice-cold barbital buffer solution (0.1 mol/l, pH 8.6) and cut into pieces. These were homogenized with an Ultra-Turrax (5 × 20 s) at 0 °C and the resulting homogenate centrifuged at 100,000 g and 0 °C for 30 min.

The skin of a male rat (weight of the skin approx. 22 g) was homogenized in 58 ml of buffer solution as above.

After 1, 18, and 24 h at room temperature the esterase activity of the supernatant was determined according to the method of Pilz [8] as follows: 5–6 samples ranging from 0.05 to 1 ml each were diluted to 1 ml with barbital-EDTA buffer. After addition of 25 ml substrate solution (phenyl acetate), they were incubated for 30 min at 37 °C in an N_2 atmosphere.

Between 1 and 24 h at room temperature, the esterase activity of the homogenate remained constant, whereas 15 min heating at 80 °C resulted in deactivation. In experiments with dithranol triacetate and dithranol diacetate, these were added to the supernatant as a solution in dimethyl formamide (10 mg/ml) in a ratio of 1 : 100. The incubation was terminated by addition of twice the volume of chloroform. After acidification, the mixture was extracted twice more with chloroform. In the combined chloroform extracts, dithranol triacetate, dithranol diacetate, and dantron were determined densitometrically according to Retzow et al. [9].

4. Results and discussion

Due to the two different radioactive labels, we were able to determine the excretion pathways of the ^{3}H-labelled anthracene nucleus and the ^{14}C-labelled acetoxy substituents separately.

4.1. Elimination kinetics after i.v. and p.o. administration

Data on the fate of the acetyl substituents of dithranol triacetate (**2**) can be obtained from ^{14}C-measurements in the excretion products when compared with data from analogous experiments with authentic [1-^{14}C]acetate.

After i.v. injection of [^{14}C]-**2**, an essential part (72% of the dose) is recovered as $^{14}CO_2$ in expired air already within 1 day. Within 3 days, only 8% of the dose are excreted with urine and 3% with feces (Table 1), and approx. 9% are still left in the organism.

The experiments with [1-^{14}C]acetate gave similar results (Table 2): 83% of the dose appear as $^{14}CO_2$ in expired air, a

small percentage is present in urine, and 11% are still in the organism after 1 day.

These data indicate that already during the first day after injection the three acetate groups are removed from the molecule almost completely. They obviously enter the acetate pool of the body where they are readily metabolized to $^{14}CO_2$. A small portion is incorporated into endogenous substances, resulting in a longer residence time in the body.

After oral administration of [^{14}C[-**2**, rate and mode of excretion are virtually the same as after i.v. injection, the mode of administration being reflected only in a somewhat more pronounced excretion with feces (Table 3).

After i.v. injection of the material labelled with ^{3}H in the anthracene ring system, degradation of the label resulted in the formation of a substantial amount of ^3H_2O (42%). Only 40% of the dose are excreted, in equal parts in urine and feces, as non-volatile products within 3 days. After this period, 6% of the dose are still present in the body as non-volatile material and 19% as ^3H_2O (Table 4).

Table 1: Excretion of ^{14}C after i.v. injection of [^{14}C]dithranol triacetate (**2**) into a rat (in % of radioactive dose).

Collection period (h)	0–24	24–48	48–72	0–72
$^{14}CO_2$ in exhaled air	72.1	5.1	1.8	79.0
^{14}C in urine	6.4	1.0	0.6	8.0
^{14}C in feces	1.5	0.7	0.8	3.0
Residual ^{14}C in the organism after 72 h				8.6
Total recovery of ^{14}C	80.0	6.8	3.2	98.6

Table 2: Excretion of ^{14}C after i.v. injection of [1-^{14}C]acetate into a rat (in % of radioactive dose).

Collection period (h)	0–3	3–24	0–24
$^{14}CO_2$ in exhaled air	74.6	8.1	82.7
^{14}C in urine	–	4.1	4.1
^{14}C in feces	–	0.8	0.8
Residual ^{14}C in the organism after 24 h	–	–	10.9
Total recovery of ^{14}C	74.6	13.0	98.5

Table 3: Excretion of ^{14}C after oral administration of [^{14}C]dithranol triacetate (**2**) to a rat (in % of radioactive dose).

Collection period (h)	0–24	24–48	48–72	0–72
$^{14}CO_2$ in exhaled air	58.6	4.6	1.2	64.4
^{14}C in urine	3.6	0.7	0.3	4.6
^{14}C in feces	10.5	8.9	0.4	19.8
Residual ^{14}C in the organism after 72 h				6.1
Total recovery of ^{14}C	72.7	14.2	1.9	94.9

Table 4: Excretion of ^{3}H as non-volatile compounds and ^3H_2O after i.v. injection of [^{3}H]dithranol triacetate (**2**) into a rat (in % of dose; n.v. = non-volatile labelled material; ^3H_2O = tritiated water).

Collection period (h)		0–24	24–48	48–72	0–72
^{3}H in exhaled air	n.v.				–
	^3H_2O				7.9
^{3}H in urine	n.v.	15.5	1.5	0.8	17.8
	^3H_2O	3.6	3.6	2.9	10.1
^{3}H in feces	n.v.	12.2	6.6	3.7	22.5
	^3H_2O	1.9	1.3	1.6	4.8
Residual ^{3}H in the organism	n.v.				5.8
	^3H_2O				18.9
Recovery of ^{3}H	n.v.	27.7	8.1	4.5	46.1
	^3H_2O	5.5	4.9	4.5	41.7
Total recovery of ^{3}H-dose					87.8

Apparently, [^{3}H]-**2** loses, after complete or partial enzymatic cleavage of the acetate groups, its ^{3}H-label in an S_E-reaction with ^1H_2O, i.e. in reverse to the tritiation reaction with which it had been obtained originally.

4.2. Identification of urine metabolites

Derivatives of anthrone can be metabolized in Phase 1 reactions, i.e. oxidation and hydroxylation. These metabolites, in turn, can be further conjugated in Phase 2 reactions with sulfuric and glucuronic acid [10]; they are subsequently excreted as such. For identification of dithranol triacetate metabolites, urine samples obtained in the previous balance experiments were both extracted directly and after hydrolysis with aryl sulfatase/glucuronidase with methylene chloride and investigated by TLC. Thus, only 10% of the total urine radioactivity were found to be present as non-conjugated metabolites.

Fig. 1 shows the TLC and the distribution of ^{3}H-radioactivity in untreated urine, a small proportion of which can be attributed to unchanged **2**, dithranol diacetate (**3**) and dantron diacetate (**6**).

The by far largest part remains close to the origin, presumably as polar conjugates. After enzymtic hydrolysis, additional metabolites can be identified: dithranol (**1**), dantron (**5**) and dithranol monoacetate (**4**) (see Fig.2). A rather large peak occurs at the same R_f as unchanged **2**, and another one can be located in the lower third of the chromatogram. The latter does not correspond to any of the known potential metabolites of **2**. It may represent one of the hydroxylated species referred to earlier, but could not be identified due to lack of material.

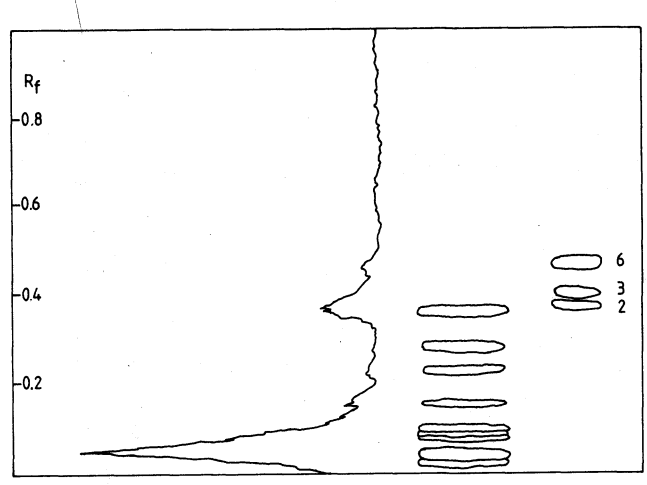

Fig. 1

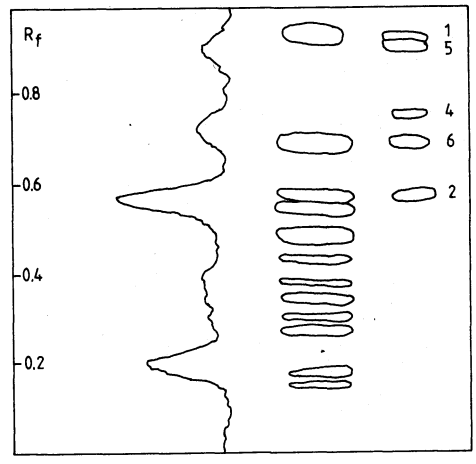

Fig. 2

4.3. Percutaneous absorption

To test the absorption through the skin of dithranol triacetate, balance studies were performed after dermal application of both the ^{14}C- and ^{3}H-labelled forms, analogous to

those described for i.v. injection. By comparison of these data with those obtained for 100% absorption (i.e., i.v. data), and considering the differences of metabolic fate of the two labels, it should be possible to draw conclusions regarding the absorption rate and nature of the absorbed material.

After dermal application of a preparation of ^{14}C-**2** in white petrolatum with occlusive protection, 1.9% of the radioactive dose were exhaled as $^{14}CO_2$ within 3 days, and 0.3% were excreted in urine. An additional 2% were found absorbed in the body. After dermal application of ^{14}C-**2** in dimethyl sulfoxide (DMSO) solution, slightly more radioactivity was excreted with urine and feces (Table 5).

A similar experiment with [1-^{14}C]acetate in 50% aqueous DMSO was performed to obtain basic information (Table 6). From the amount of radioactivity in expired air, urine, feces and organism, an absorption rate of approx. 33% for acetate can be calculated. A comparison between these experiments clearly shows that intact **2** is absorbed to a very limited extent only. It might even be possible that **2** is enzymatically deacetylated, the resulting free acetate being the only absorbed species.

This question could be answered by an experiment with ^{3}H-**2**, i.e. with a dithranol triacetate labelled in the anthracene ring system (Table 7). After dermal application in 50% aqueous DMSO with occlusive protection a total of 27% of the radioactive dose was recovered within 3 days in excretion products (expired air, urine, and feces) and in the carcass. This part of the ^{3}H-**2** dose then must have been absorbed through the skin. Considering the results with acetate-labelled ^{14}C-**2** where only 4.3% had been absorbed, it can be concluded that **2** is metabolized in or on the skin with formation of free dithranol (or partially acetylated derivatives) which in turn is absorbed.

Table 5: Excretion of ^{14}C after dermal administration of [^{14}C]dithranol triacetate in white petrolatum (A) and in dimethylsulfoxide (B) (in % of radioactive dose) to a rat.

Collection period (h)	A				B			
	0–24	24–48	48–72	0–72	0–24	24–48	48–72	0–72
$^{14}CO_2$ in exhaled air	0.8	0.6	0.5	1.9	1.7	0.4	0.3	2.4
^{14}C in urine	0.1	0.1	0.1	0.3	0.3	0.1	0.1	0.5
^{14}C in feces	0.0	0.1	0.1	0.2	0.2	0.2	0.2	0.6
Residual ^{14}C in the organism after 72 h				2.0				0.8

Table 6: Recovery of ^{14}C after dermal administration of [1-^{14}C]acetate to 2 rats. Dose: 0.25 mg in 0.1 ml DMSO/H_2O 1:1 on 4 cm² skin (in % of radioactive dose, mean of two experiments).

Collection period (h)	0–8	8–24	0–24
$^{14}CO_2$ in air	14.1	8.9	23.0
^{14}C in urine	–	–	2.3
^{14}C in feces	–	–	1.1
^{14}C in skin (area of administration)	–	–	11.2
Residual ^{14}C in the organism after 24 h			6.6
^{14}C in the dressing material			41.9

Table 7: Excretion of ^{3}H after dermal administration of [^{3}H]dithranol triacetate in dimethylsulfoxide (in % of radioactive dose) to a rat.

Collection period (h)	0–24	24–48	48–72	0–72
^{3}H in exhaled air				4.2
^{3}H in urine	4.4	2.2	1.6	8.2
^{3}H in feces	4.2	1.3	2.9	8.4
Residual ^{3}H in the organism after 72 h				6.8

Table 8: Incubation of dithranol triacetate (**2**) and dithranol diacetate (**3**) with homogenates of rat skin (100,000 g supernatant). TLC-separation of metabolites.

Metabolite	Substrate					
	Dithranol triacetate (**2**)			Dithranol diacetate (**3**)		
	I	II	III	I	II	III
Dithranol triacetate (**2**)	+++*)	+++	+++			
Dithranol diacetate (**3**)	+	+	–	+	++	+++
Dantron diacetate (**6**)	++	+	–	+++	++	+
Dithranol monoacetate (**4**)	–	–	–	++	+	–
Dantron monoacetate (**7**)	+	+	–	+	+	–
Dantron (**5**)	++	+	–	+++	+++	+

*) +++ denotes intense, ++ medium, + weak, and – no spot on the thin layer chromatogram with the Rf of the metabolite in question.
I: Homogenate dil. 1:5 with barbital buffer.
II: Homogenate dil. 1:10 with barbital buffer.
III: Buffer solution only (no homogenate added).

4.4. Formation of metabolites by skin homogenates

To test the hypothesis of ester cleavage by enzymes present in or on the skin, we subjected ^{14}C-**2** to the action of skin homogenates in vitro. Cell-bound, oxidative enzyme systems were excluded from the experiments by use of only the 100,000 g supernatant.

With phenyl acetate as substrate under our incubation conditions, we found an aryl esterase activity of 6.2–7.8 U/g of mouse skin and 4.6–6.0 U/g or $1.14–1.46 \times 10^2$ U/mm² in rat skin.

With dithranol triacetate (**2**) as substrate and the 100,000 g supernatant of rat skin homogenate, a mixture of a large amount of unchanged substrate (**2**) in addition to dantron (**5**), dantron monoacetate (**7**), dantron diacetate (**6**), and dithranol diacetate (**3**) was formed (see Table 8).

Dithranol diacetate as substrate gave the same metabolites, the difference being the presence of dithranol monoacetate (**4**) and very little unchanged **3** among the metabolites.

In the enzyme free buffer, dithranol triacetate (**2**) is stable, whereas dithranol diacetate (**3**) decomposed slowly to give some dantron (**5**).

Incubation of mice skin homogenates with the two dithranol esters gave a similar composition of metabolites.

These results show the central acetoxy group in the dithranol triacetate molecule to be preferentially hydrolyzed by the action of enzymes. The resulting dithranol diacetate is quite sensitive to non-enzymatic oxidation reactions, in contrast to the original triacetate.

After administration, the latter is converted to active metabolites only after it has penetrated into the skin thus showing a pronounced pro-drug character for the treatment of psoriasis.

5. References

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