ment of motor coordination) or the cardiovascular system (*9–11*).

Numerous experimental studies have been made with dantrolene sodium and various mammalian and nonmammalian models in efforts to determine the site of action of this spasmolytic agent. The consensus is that the drug does not act on the central nervous system (*6, 10, 12–14*), the neuromuscular junction (*2, 8, 10, 11, 14*), or the muscle sarcolemma (*10, 12, 15, 16*). Because of its generally accepted role in the modulation of intracellular Ca^{2+} for muscle contractility through excitation-contraction coupling, the sarcoplasmic reticulum (SR) has been suggested as a site at which dantrolene sodium may act to reduce the amount of Ca^{2+} released from membranes for the subsequent contractile event (*7, 13, 15, 17–20*).

We have isolated SR from a number of mammalian sources (including canine heart) and examined the effect of dantrolene sodium on the Ca^{2+}-sequestering parameters of these fractions. The SR was isolated from rabbit back muscle; cat soleus, caudofemoralis, and tibialis anterior; and dog heart by a modification of the procedure of DeMeis and Hasselbach (*21*) using a Polytron PT20 for the initial homogenization. The capacities of the SR fractions for Ca^{2+} binding and uptake (in the presence of oxalate) were monitored by a rapid assay technique employing dual-wavelength spectrophotometry, as previously described (*22, 23*). In this system, Ca^{2+} movements, such as sequestration and release by SR vesicles, are reflected in absorbance changes in the presence of the Ca^{2+}-sensitive chelometric dye murexide. Adenosine triphosphate (ATP)–dependent Ca^{2+} binding to SR results in a downward deflection of the trace (see Fig. 2) followed by a spontaneous release of the bound Ca^{2+} (upward deflection). The reaction conditions are given in the legend to Fig. 1.

Two consecutive Ca^{2+} binding experiments (in the absence of oxalate) are shown in Fig. 1. The effect of dantrolene sodium is on the release phase only of the reaction, which is shown quantitatively in Fig. 2. Not only are the three release phases altered with respect to rates, but, more importantly, the total amount of Ca^{2+} released is much lower than in the control preparations. It is interesting to note that complete abolition of muscle twitch cannot be effected with dantrolene sodium (*11, 19*). Similarly, using higher doses in the in vitro experiments does not completely inhibit Ca^{2+} release from the SR. It is evident that none of the binding parameters, such as the amount of Ca^{2+} bound or the time required for binding, are affected by dantrolene sodium. Furthermore, the rate of Ca^{2+} uptake (in the presence of oxalate) is not significantly altered by dantrolene sodium (data not shown). It is of significance that the Ca^{2+} binding and release phases of cardiac SR (*24*) are not affected by the drug. This is the first report, to my knowledge, of such a specific difference between skeletal and cardiac muscle SR.

It may be extrapolated from the in vitro findings reported here and the recent electrophysiological data of Morgan and Bryant (*25*) that, in the intact skeletal muscle, dantrolene sodium acts at a site on the SR which suppresses but does not completely inhibit the release of Ca^{2+} necessary for the activation of the contractile apparatus. This drug would thus seem to be useful, not only clinically but as an experimental tool for probing SR energetics and differences in Ca^{2+} modulating systems in fast, slow, and cardiac muscles.

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Photochemotherapy: Identification of a Metabolite of 4,5′,8-Trimethylpsoralen

Abstract. *A compound, 4,8-dimethyl,5′-carboxypsoralen (DMeCP), has been identified in mouse urine as a major metabolite of the photoactive drug, 4,5′,8-trimethylpsoralen (TMeP). This drug is widely used in the treatment of vitiligo and psoriasis. DMeCP is fluorescent, and nonphotosensitizing when tested on guinea pig skin. DMeCP also occurs in the urine of human patients receiving TMeP orally.*

Psoralens, which are particular isomers of furocoumarins, are a ubiquitous group of naturally occurring compounds which include certain potent photosensitizers such as psoralen, 8-methoxypsoralen (8-MOP), and 4,5′,8-trimethylpsoralen (TMeP) (Fig. 1) (*1*). In the presence of long-wave ultraviolet light (320 to 400 nm) these photoactive compounds stimulate tanning (melanogenesis) of the skin (*2*). Some of these compounds are present in common food plants such as citrus fruits, celery, and figs (*1*). Extracts of other psoralen-containing plants, such as *Psoralea corylifolia* (Leguminosae) and *Ammi majus* (Umbelliferae), have been used for centuries in India and Egypt in conjunction with sunlight for treating vitiligo, a clinical condition of depigmentation in which parts of the body lose melanocytes and melanin pigmentation of the skin (*3*). Recently, psoralens as photochemotherapeutic agents have received attention in Western countries because they are useful in the treatment of psoriasis, vitiligo, and mycosis fungoides when used in combination with long-wave ultraviolet light (*4*). In the United States, the two most commonly used psoralens are 8-MOP and TMeP.

Little is known of the metabolism of psoralens in man or other animals (*5*). We have now isolated and identified a major metabolite of TMeP from mouse urine, and have found the same material

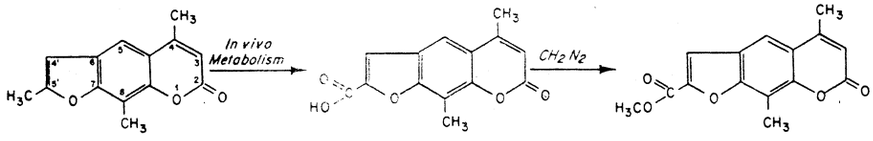

Fig. 1. Structure of (1) TMeP (4.5′,8-trimethylpsoralen), (2) DMeCP (4.8-dimethyl.5′-carboxypsoralen), and (3) the methyl ester of DMeCP.

in the urine of humans who have ingested TMeP.

To isolate psoralen metabolites, we placed 20 male albino mice (strain CD-1, Charles River Breeding Laboratories) in metabolic cages and administered TMeP orally (150 mg/kg) by intubation as a suspension in 0.1 ml of peanut oil. Urine samples were collected for the following 8 hours, during which time the mice were denied food, but had access to water. We had previously established that more than 80 percent of the TMeP administered is excreted within 8 hours (*5, 6*). The urine samples were pooled and frozen until used.

Urine was acidified to *p*H 2 with 1*N* HCl and extracted three times with 1.5 volumes of anhydrous diethyl ether. During subsequent evaporation of the ether, a white precipitate was formed which proved to consist primarily of metabolites of TMeP. This precipitate was washed several times with small volumes of cold diethyl ether, and once with 1 ml of cold absolute ethanol before being dried under vacuum and stored in a desiccator. No precipitate could be detected in the urine of 20 control mice given only peanut oil.

More than 90 percent of the precipitate consisted of a fluorescent material hereafter referred to as 4,8-dimethyl,5′-carboxypsoralen (DMeCP) (Fig. 1). The following studies to determine the structure of DMeCP were performed on this precipitate. The remainder of the precipitate consisted of a fluorescent metabolite apparently closely related to DMeCP. Studies in which radioactive TMeP was administered orally to mice showed that about half the radioactivity recovered in the urine appeared as DMeCP (*6*).

The precipitate was somewhat soluble in water and very soluble in sodium bicarbonate, which suggested that it contained a carboxyl group; TMeP, on the other hand, is soluble in chloroform and ethanol and almost insoluble in aqueous solutions (see Table 1).

A mass spectrum of the precipitate showed a substantial peak at 258, and a smaller, variable intensity peak (about 2 percent of that at 258) at 274. To determine whether the material peaking at 274 was a contaminant of that peaking at 258, the precipitate was applied as a line to several silica gel plates (Bakerflex SG IB_2), which were developed with chloroform, ethyl acetate, and glacial acetic acid (6 : 3 : 1 by volume) as a solvent. Under ultraviolet light, most of the material (DMeCP) appeared as a blue fluorescent band with an R_F value of 0.45, and a small amount of material was present with an R_F value of 0.22. Both substances were eluted with 0.1*M* sodium bicarbonate buffer, *p*H 9.5; the solutions were then acidified and extracted with ether. After removal of the ether, both substances were reexamined by chromatography and mass spectroscopy, and both appeared to be pure.

The mass spectrum of pure DMeCP showed the following major ions (with relative intensities expressed as percentages of the molecular ion): 258 (100 percent), 230 (32 percent), 229 (35 percent), 185 (10 percent), 167 (11 percent), and 149 (23 percent). The material with the R_F value of 0.22 had a mass spectrum similar to that of DMeCP with major ions at 274 (100 percent), 246 (41 percent), 245 (36 percent), 229 (26 percent), 218 (20 percent), and 217 (16 percent).

About 1 mg of the precipitate was reacted with diazomethane at room temperature in diethyl ether to form the methyl ester derivative (Fig. 1). Mass spectroscopy of the product showed a strong peak at 272 and no material of molecular weight 258. In addition, a small peak at 288 was present and the variable small peak at 274 was gone. These data indicate that both DMeCP and the compound of molecular weight 274 contain a carboxyl group.

Spectra obtained by nuclear magnetic resonance (NMR, 100 Mhz) with the material dissolved in deuterated chloroform ($CDCl_3$) provide evidence that the ester group of DMeCP ester is on C-5′. The H-3 and H-5 resonances of TMeP [δ = 6.18 and 7.45 parts per million (ppm), respectively] are shifted downward by 0.09 and 0.29 ppm, respectively, in the ester, while H-4′ (δ = 6.37 ppm in TMeP) is shifted down by 1.17 ppm in the ester. If the C-4 methyl group of TMeP had been oxidized, substantial downward shifts in the resonances of H-3 and H-5 would have occurred in the product; if the C-8 methyl group had been oxidized, no large resonance shifts would have been seen in the product NMR spectrum. The three methyl signals in TMeP (δ = 2.45, 2.48, and 2.50 ppm) are reduced in DMeCP to two methyl signals in deuterated pyridine (C_5D_5N solution). In the ester ($CDCl_3$ solution) the two ring methyl groups are at δ = 2.50 and 2.65 ppm, while the ester methyl group appears at δ = 3.98 ppm.

From NMR and mass spectral analysis of the metabolite and its methyl ester, we conclude that the major compound in the precipitate is DMeCP. Ultraviolet absorption spectra are given in Fig. 2, and information concerning the fluorescence, solubility, and photosensitizing characteristics of DMeCP and its methyl ester are shown in Table 1. It is of interest that on topical application to guinea pig skin

Table 1. Some properties of DMeCP and its methyl ester.

Compound	Fluorescence* (nm)		Skin photosensitivity†	Solubility properties
	Excitation maximum	Emission maximum		
DMeCP	350	430	No	$NaHCO_3$ > H_2O > ethanol > $CHCl_3$
DMeCP methyl ester	355	410	No	$CHCl_3$ > ethanol > H_2O, $NaHCO_3$
TMeP	360	430	Yes	$CHCl_3$ > ethanol >> H_2O, $NaHCO_3$

*Fluorescence measurements were performed in ethanol on an Aminco-Keirs spectrophosphorimeter. †Photosensitizing properties of these agents were determined on the epilated back skin of guinea pigs (*7*). Skin sites (2.0 by 2.0 cm) received topical applications of 10 to 100 μg of test substances. One hour after application the skin sites were exposed to a total irradiance of 12 joule/cm^2 of wavelengths 320 to 400 nm emitted by a high-pressure mercury lamp equipped with a Wood's filter (Glo Craft, model 70, Switzer). Test sites were read at 24 and 48 hours after exposure for the degree of erythema (redness) and edema.

and subsequent irradiation, neither DMeCP nor its ester derivative causes the photosensitization reaction so characteristic of TMeP and other photoactive psoralens (Table 1) (*7*).

Urine specimens obtained from human volunteers and patients with vitiligo or psoriasis receiving TMeP orally contained a major fluorescent metabolite very similar to DMeCP. Volunteers took 40 mg of TMeP orally, urine samples were collected during the following 8 hours, and a portion was extracted as described for mouse urine. The residue after ether evaporation was dissolved in ethanol and examined by paper chromatography (Whatman 3MM) with water being used as a solvent. A compound with the mobility of DMeCP (R_F = 0.6) was found which, after elution, had the same mobility as DMeCP in two thin-layer chromatography systems, and the same ultraviolet absorption spectrum as DMeCP. Further analysis by NMR and mass spectroscopy confirmed the presence of DMeCP, thus demonstrating that DMeCP is formed when TMeP is given in therapeutic doses to humans. No DMeCP was found in the urine of control patients who did not receive TMeP.

The compound DMeCP is the major fluorescent metabolite of TMeP in the urine of mice given TMeP orally; we have not yet determined whether nonfluorescent metabolites are present. We have detected a minor fluorescent metabolite of molecular weight 274 which could be a hydroxylated derivative of DMeCP, but have not found unchanged TMeP in the urine.

Oxidation of a methyl group to a carboxyl group is apparently an uncommon reaction in the biotransformation of drugs (*8*). In addition to TMeP, one of the few examples of such a transformation involves the anti-inflammatory drug tolmetin (1-methyl-5-*p*-toluoylpyrrole-2-acetic acid), where a toluene derivative is metabolized to a benzoic acid derivative (*9*). However, in the case of TMeP, the oxidized methyl group is on a furan ring rather than on a benzene ring.

The phototherapeutic effects of psoralens are related to their chemical structure and their photoactivity; minor structural modifications of psoralen derivatives are known to result in major changes in their phototherapeutic properties (*7*). Exploitation of such structure-activity relationships led in early 1960 to the synthesis of TMeP and its safe and effective use in the treatment of vitiligo. The therapeutically useful properties of 8-MOP and TMeP are believed to result from their binding specifically to double-stranded nucleic acids through intercalation and cross-linking between adjacent strands (*4, 10*). This photoconjugation of psoralens to DNA in vivo has been demonstrated in guinea pig skin, human fibroblasts, and other biologic systems (*11*), and is believed to inhibit DNA synthesis and cell division, thereby leading to subsequent clinical improvement in disease conditions such as psoriasis (*4, 10*).

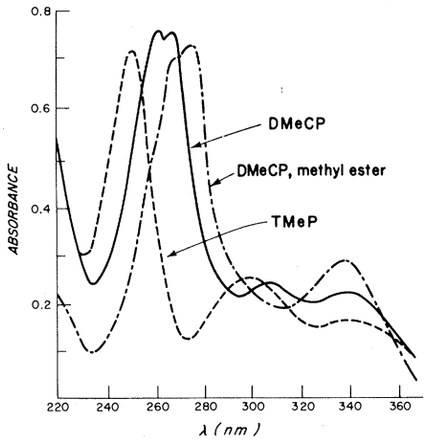

Fig. 2. Absorption spectra in ethanol of TMeP (4,5′,8-trimethylpsoralen), DMeCP (4,8-dimethyl,5′-carboxypsoralen), and the methyl ester of DMeCP.

In establishing a relationship between the binding of a photoactive drug such as TMeP to DNA and its therapeutic effectiveness, we have found two phenomena that have a direct bearing on our observations. When TMeP is applied topically to guinea pig skin or to human skin in low concentrations (0.2 to 1.0 μg/cm^2), it is very reactive in the presence of long-wave ultraviolet light and causes skin photosensitization which is manifested by an augmented sunburn reaction (erythema), edema (even vesiculation), and subsequent desquamation and hyperpigmentation. Contrary to this, when TMeP is given orally (0.3 to 1.2 mg per kilogram of body weight) to normal volunteers, or to patients with vitiligo or psoriasis, or even to experimental guinea pigs in high doses (10 mg/kg), its ability to induce cutaneous photosensitization reactions is markedly diminished, and at times it is nonphotoactive. This decreased photoactivity of orally administered TMeP is not apparently related to poor absorption or low concentration in the extracellular fluid that bathes the skin cells, but more probably to its rapid biotransformation to nonphotosensitizing moieties, one of which has now been identified as DMeCP. In addition, studies in vitro show that when TMeP or DMeCP undergo photoreactions with calf thymus DNA, they form fewer cross-links than do the strongly photoactive 8-MOP and psoralen (*12*). These observations are consistent with our clinical experience, where patients with generalized psoriasis were treated either with 8-MOP and long-wave ultraviolet light or with TMeP and long-wave ultraviolet light. Photochemotherapy with oral 8-MOP (0.6 mg/kg) and ultraviolet light was very effective in clearing psoriasis rapidly; TMeP (0.7 mg/kg) with ultraviolet light under identical conditions was distinctly less effective, and at times virtually inactive, in clearing psoriasis. Although at present 8-MOP appears to be the drug of choice in the treatment of psoriasis, TMeP is still a potentially useful drug in the treatment of other diseases such as vitiligo and certain types of psoriasis.

When mice or human volunteers are given 8-MOP orally, at least four fluorescent metabolites can be detected in the urine. Although the major metabolites of 8-MOP have not been structurally characterized, the biotransformation pathway of 8-MOP differs significantly from that of TMeP, since 8-MOP lacks any oxidizable methyl groups. These studies on the metabolism of the psoralens should provide a better understanding of how the photosensitizing drugs work in vivo, and how safe and effective therapy can be instituted in this newly emerging field of photochemotherapy.

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H-Y (Male) Antigen: Detection on Eight-Cell Mouse Embryos

Abstract. *The H-Y (male) antigen is expressed on 50 percent of eight-cell embryos (2 days old) of the mouse, strain C57BL/6. This work provides additional evidence that H-Y expression is not dependent on male differentiation.*

The male-specific (H-Y) antigen (*1*) has been detected on all male tissues that have been tested in the mouse (*2*). Although some evidence suggests that H-Y expression is dependent on male hormonal differentiation, most of the data reported indicate that H-Y is expressed whenever the Y chromosome is present. Bennett *et al.* (*3*) have demonstrated that H-Y is expressed on cells of Tfm/Y mice which are lacking all testosterone-dependent characters, suggesting that the presence of H-Y is dependent on the expression of a gene or genes on the Y chromosome. Wachtel *et al.* (*4*) demonstrated that the H-Y antigen continues to be expressed on male hemopoietic cells after their transfer to female recipients, indicating that the maintenance of H-Y is independent of a male environment. By using a two-step cytotoxicity assay we have detected the H-Y antigen on eight-cell mouse embryos (day 2 of development). We thus provide additional evidence that the expression of H-Y is dependent on the presence of the Y chromosome.

We used mice of strain C57BL/6 (B6) (Jackson Laboratory). Antiserum to H-Y antigen was prepared by inoculating intraperitoneally B6 females with 25×10^6 B6 male spleen cells. The serums obtained from inoculated mice on days 7, 10, and 14 following the sixth or seventh inoculation were cytotoxic for sperm in the presence of complement. Guinea pig serum (Gibco) was used as the complement source. Our tests showed that either antiserum or guinea pig serum alone did not cause lysis of eight-cell mouse embryos. All manipulations and incubations were performed in Whitten's medium (WM) (*5*) with the following modifications: NaCl, 5.14 g/liter; sodium pyruvate (Schwarz/Mann), 0.025 g/liter; and purified bovine serum albumin (Miles Laboratories), 3.4 g/liter. To obtain eight-cell embryos, B6 females were mated with B6 males. Females were examined daily for vaginal plugs, with the morning of plug detection being defined as day 0 of pregnancy. On day 2 the pregnant females were killed by cervical dislocation. The oviducts were removed and placed in watch glasses containing WM supplemented with bovine serum albumin (WM-BSA). This and all subsequent steps were performed at 37°C. Embryos flushed from the oviducts with WM-BSA by means of a glass micropipet were collected in a small droplet (50 to 100 μl) of WM-BSA contained in a 35 by 10 mm petri dish (Falcon Plastics) coated with paraffin oil. The embryos were washed one time by transferring them to a fresh droplet of WM-BSA. The eight-cell embryos were selected and transferred to a droplet of WM containing 1 percent Pronase (Calbiochem) to partially digest the zona pellucida. Removal of the zona pellucida was achieved by repeated passage through a glass micropipet. The embryos were washed three times by successive transfer to droplets of WM-BSA, and transferred to serially diluted (in halving dilutions) antiserum to H-Y antigen in WM. The embryos were incubated in a droplet of antiserum for 30 minutes at 37°C in a humidified atmosphere of 5 percent CO_2 and 95 percent air. The embryos were then transferred to guinea pig serum (diluted 1 : 2 by volume in WM) and incubated for an additional 30 minutes under identical conditions. Embryos were scored as being H-Y^+ when one cell or more of the embryo was lysed in the presence of H-Y antibody and complement. Cell lysis was determined by light microscopy (Fig. 1).

The results in Fig. 2 indicate that 50 percent of the eight-cell embryos were affected by antiserum to H-Y antigen (diluted 1 : 4 or 1 : 8 by volume) and complement. The number of cells lysed in an affected embryo ranged from one to five.

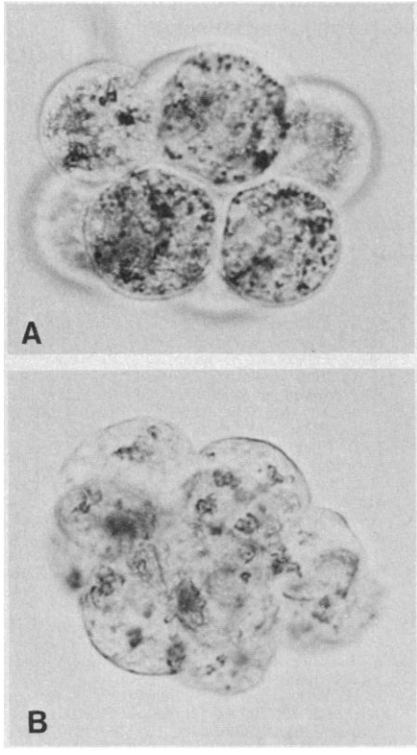

Fig. 1. Demonstration of lysis of eight-cell C57BL/6 embryos with antiserum to the H-Y antigen and complement. (A) H-Y^- embryo. (B) H-Y^+ embryo.

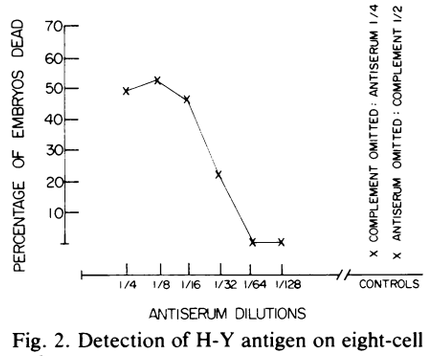

Fig. 2. Detection of H-Y antigen on eight-cell embryos.

Table 1. Specificity of embryo lysis by antiserum to H-Y antigen. Serum was used at a dilution of 1 : 8 by volume in all tests. Guinea pig serum diluted 1 : 2 by volume was used in each test as the source of complement. All tests were performed on eight-cell C57BL/6 embryos. Cytotoxic antiserum to H-Y antigen was absorbed one time with either B6 male or B6 female spleen cells and tested on eight-cell C57BL/6 embryos at a dilution of 1 : 8.

Absorption	Total number of embryos	Dead embryos	
		N	Percent
None	49	25	53
B6 male cells	30	0	0
B6 female cells	27	12	46