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Alteration of Cholesterol Biosynthetic Pathways in the Skin of Mice Administered Polycyclic Aromatic Hydrocarbons[1]

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ABSTRACT

When polycyclic aromatic hydrocarbons were applied solely or together with a tumor promoter (12-*O*-tetradecanoylphorbol-13-acetate) to the skin of mice, a marked decrease in the level of lathosterol was observed, reflecting a significant change in the metabolism of sterols. Yet the total amount of cholesterol was not changed.

When diazacholesterol (a metabolic inhibitor) was administered to mice, both desmosterol and 5α-cholesta-7,24-dien-3β-ol accumulated in the skin, whereas the level of lathosterol decreased. These results seem to suggest that a significant portion of lathosterol is formed via 5α-cholesta-7,24-dien-3β-ol in addition to the pathway through methostenol. When polycyclic aromatic hydrocarbon was applied to the skin of the mouse treated with diazacholesterol, a significant increase of desmosterol and a marked drop of the level of 5α-cholesta-7,24-dien-3β-ol were observed.

These results strongly suggest that polycyclic aromatic hydrocarbons perturb the metabolism of sterol in the skin of mice while keeping the total amount of cholesterol unchanged. A similar metabolism also seems to be operating in tumor tissue itself.

INTRODUCTION

Since *de novo* sterol synthesis is needed for a discrete period of time prior to DNA synthesis and cell proliferation, sterol metabolism seems to be important for cell proliferation (1, 2). In fact, some abnormalities in sterol concentration and its metabolism in cancer cells have been demonstrated (3–5).

More than three decades ago Kandutsch and Baumann observed that the level of fast-acting sterols, *i.e.*, sterols which develop color faster (1.5 min) than slow-acting ones (30 min) during the Liebermann-Burchard reaction, were lowered by application of polycyclic aromatic hydrocarbons to mouse skin (6). In 1981 Yoshiga *et al.* (7) reinvestigated the phenomenon employing a more sophisticated analytical method and clearly identified the sterol that was depressed by carcinogens as lathosterol,[3] an intermediate in one of the biosynthetic pathways for cholesterol (a pathway proposed by Kandutsch and Russel). This work was then followed by Morita *et al.* who showed that when a subcarcinogenic amount of 3-MC was applied, the initially lowered lathosterol level was gradually restored to the normal level, whereas such a recovery was repressed when the single application of 3-MC was followed by repeated applications of TPA, a tumor promoter (8). It was also shown in this laboratory that sterol metabolism was changed by chemical carcinogens in the cultured skin tissue of chick embryo and mouse epidermal keratinocytes (9, 10).

However, the mechanism of the lathosterol depressive action of polycyclic aromatic hydrocarbons remains to be established, due to the fact that the entire pathway for cholesterol biosynthesis in animal tissue has not been established at the enzyme level. As an approach to solving this problem we have been studying the metabolic pathway of cholesterol biosynthesis in the skin of mice treated with diazacholesterol, a metabolic inhibitor known to block the conversion of desmosterol to cholesterol. In the course of this study we observed that an unknown sterol other than desmosterol accumulates in the skin of mice administered diazacholesterol. The level of this compound was greatly lowered by the application of the polycyclic aromatic hydrocarbon whereas that of desmosterol was markedly increased. Identification of the chemical structure of this compound provided insight into the events occurring in mouse skin administered the chemical carcinogens.

MATERIALS AND METHODS

Chemicals. 3-MC, DMBA, BaP, and β-naphthoflavone were purchased from Katayama Chemical Co. (Osaka, Japan) and TPA was from Sigma Chemical Co. (St. Louis, MO). Diazacholesterol was kindly supplied by Searle Laboratories (Chicago, IL) 3-MC, DMBA, BaP, β-naphthoflavone, and TPA were used after dissolving in acetone.

Sterol Analysis. The animals used were male CD-1 mice (Charles River, Hino, Japan) weighing about 25 g. Rat chow and water were given *ad libitum*. The skin of the back was shaved with a surgical clipper 1 or 2 days before treatment, and chemicals were applied to the shaved area three times each week for 2 weeks in a volume of 0.2 ml.

Extraction of Sterols. At the 15th day from the first treatment, the animals were sacrificed by cervical dislocation, and mouse back skin was removed and the epidermis was prepared according to the method described by Van Scott (11). The epidermis was hydrolyzed with 20% ethanolic KOH for 4 h under an atmosphere of nitrogen. The hydrolysate was extracted with *n*-hexane, and the solvent was evaporated to dryness using a rotary evaporator. The residue was dissolved in 2.0 ml of *n*-hexane and used as the sample.

Gas-Liquid Chromatography of Sterols. The gas-liquid chromatograph (model GC-8A; Shimadzu Co., Kyoto, Japan), was equipped with a flame-ionization detector. The column used was the chemical-bonded type capillary column of methylsilicone (model CBP1-M50-025, 0.25 mm x 50 m; Shimadzu). The pressure of the carrier gas (N_2) was 23.1 cm/s; the temperature of the column was kept constant at 320°C, and that of the injection port was kept at 350°C. Samples were applied as trimethylsilyl ether derivatives. For quantitation, a known amount of hexatriacontane was added to the sample as an internal standard, and the height of the individual peaks was compared with that of hexatriacontane.

Experiments with Diazacholesterol. Animals that had been treated with chemicals were administered 20,25-diazacholesterol (10 mg/kg/day) dissolved in drinking water for 2 weeks. AT the 15th day, the back skin was removed and their sterols were analyzed as described above.

Gas-Liquid Chromatography-Mass Spectrometry. The mass spectrum of the skin sterol was taken by a gas-liquid chromatography equipped

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[3] The abbreviations and trivial names used are: DMBA, 7,12-dimethylbenz(*a*)anthracene; BaP, benzo(*a*)pyrene; 3-MC, 3-methylcholanthrene; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; cholesterol, 5-cholesten-3β-ol; lathosterol, 5α-cholest-7-en-3β-ol; desmosterol, cholesta-5,24-dien-3β-ol; lanosterol, 4,4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol; methostenol, 4α-methyl-5α-cholest-7-en-3β-ol and/or 4α-methyl-5α-cholest-8-en-3β-ol; diazacholesterol, 20,25-diazacholesterol; 7-dehydrocholesterol, cholesta-5,7-dien-3β-ol.

with a mass spectrometer (model GCMS-QP1000; Shimadzu), using the same column as described above. The temperature of the column was programmed gradually from 260 to 310°C (2.5°C/min) and that of the injection port was kept at 300°C. The ionization voltage of the mass spectrometer was 70 ev, and the temperature of the ionic source was 250°C.

RESULTS

Sterol Composition in the Skin of Mice Treated with Polycyclic Aromatic Hydrocarbons and TPA. The effect of application of polycyclic aromatic hydrocarbons, alone or with tumor promoter, on sterol metabolism in mouse skin was studied. As shown in Fig. 1, the nonsaponifiable fraction of normal mouse skin revealed four peaks on a gas-liquid chromatogram (Table 1, *Experiment 1*). The observed peaks were identified as cholesterol, desmosterol, lathosterol, and lanosterol. In the nonsaponifiable fraction of mouse skin treated with 3-MC, DMBA, and BaP (*Experiments 2–4*) the lathosterol peak was much smaller than that of the normal mouse. However, the peak of cholesterol did not seem to be largely different from that of the normal mouse. Desmosterol and lanosterol peaks were too small to quantitate precisely. When a subcarcinogenic amount of 3-MC was once applied (*Experiment 6*) and subsequent application of TPA was not performed, the lathosterol level dropped at first, then was gradually restored. However, sequential application of TPA following the initial application of 3-MC (*Experiment 7*), suppressed such a restoration and kept the sterol content at a very low level. The dose-responsive effect of various carcinogens are shown in Fig. 2. Apparently the more potent carcinogen seemed to reveal the more potent lathosterol depressive effect. In contrast, β-naphthoflavone which is known to induce aryl hydrocarbon hydroxylase like 3-MC, but is not carcinogenic itself, reduced lathosterol only slightly. These results were essentially the same as those described by Yoshiga *et al.* (7) and Morita *et al.* (8) who used a classical, low resolution packed column.

Sterol Composition in Skin Tumors Developed by 3-MC and TPA. To clarify that similar alterations of sterol composition to those observed with skin treated with polycyclic aromatic

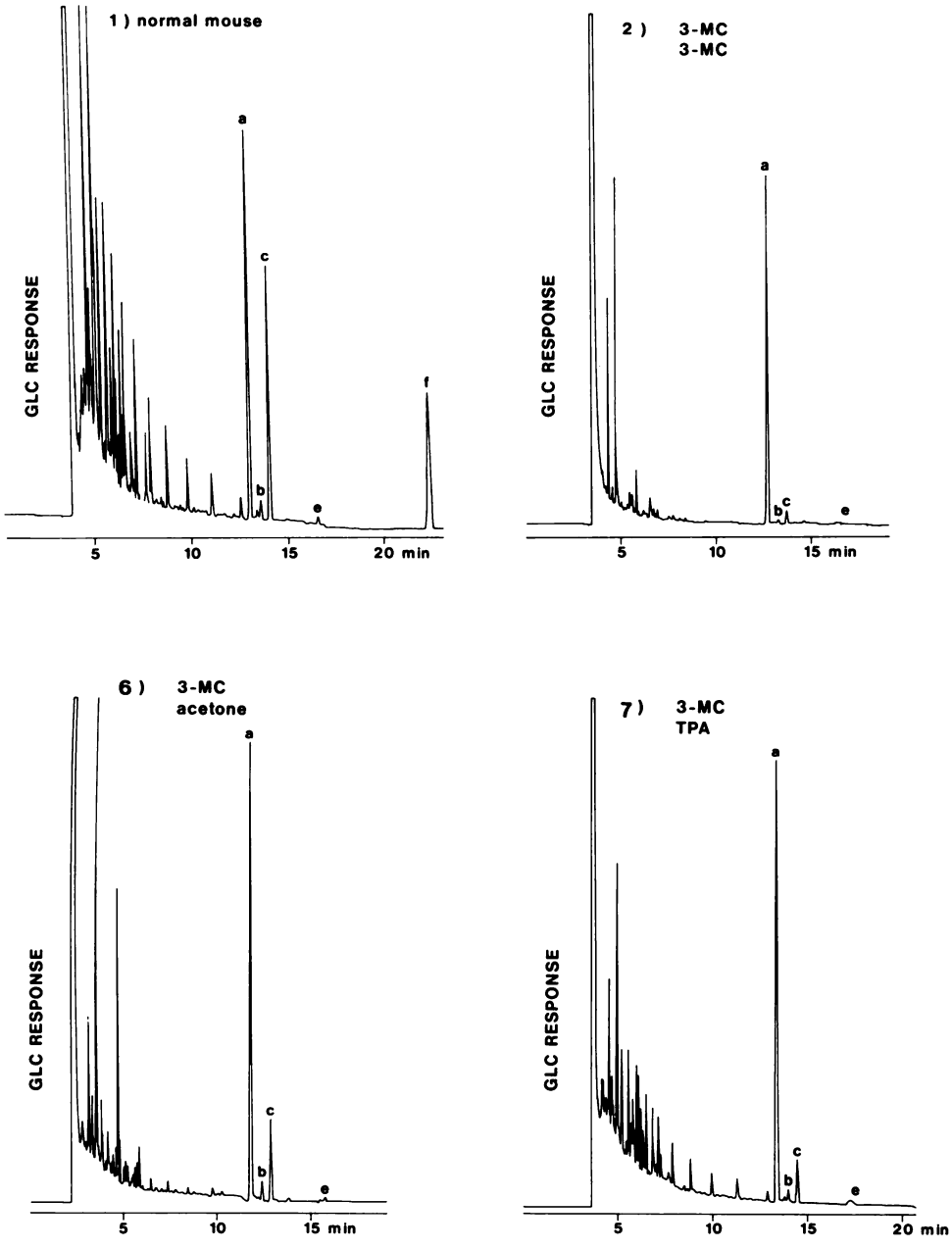

Fig. 1. Gas-liquid chromatograms of skin sterol of normal mice and those treated with 3-MC and TPA. Numbers shown in the top of the individual charts correspond to experiment numbers in Table 1. Chemicals were applied three times each week for 2 weeks. The first treatment (*top*) and the following (*bottom*) were described at the top of each chart. *a*, cholesterol; *b*, desmosterol; *c*, lathosterol; *e*, lanosterol; *f*, hexatriacontane; column, chemically bonded type capillary column of methylsilicone (0.25 mm x 50 m); column temperature, 320°C; nitrogen gas pressure, 23.1 cm/s. *1*, normal mouse; *2*, mouse treated with 3-MC three times each week for 2 weeks; *6*, mouse treated with 3-MC once in the beginning, then with acetone three times each week for 2 weeks; *7*, mouse treated with 3-MC once in the beginning then with TPA three times each week for 2 weeks.

Table 1 *Changes of sterol metabolism of mouse skin treated with carcinogens*

Experiment	Treatment schedule[a] A	B	Lathosterol/cholesterol (%)	Cholesterol (mg/g of skin)	Lathosterol (mg/g of skin)
1	normal mouse		53.7 ± 3.1[b]	2.18 ± 0.06	1.37 ± 0.05
2	3-MC	3-MC	3.6 ± 1.4	2.19 ± 0.07	0.09 ± 0.03
3	DMBA	DMBA	1.1 ± 0.3	2.85 ± 0.30	0.02 ± 0.01
4	BaP	BaP	5.4 ± 0.9	2.40 ± 0.16	0.16 ± 0.03
5	β-Naphthoflavone	β-Naphthoflavone	32.7 ± 3.8	2.11 ± 0.39	0.82 ± 0.1
6	3-MC	Acetone	15.9 ± 1.7	2.85 ± 0.16	0.54 ± 0.04
7	3-MC	TPA	9.7 ± 0.5	2.50 ± 0.07	0.29 ± 0.009

[a] Chemicals were applied three times each week for 2 weeks. The first treatment (*A*), following treatments (*B*). The amounts of 3-MC, DMBA, BaP, and β-naphthoflavone applied at a time were 600 μg; the amount of TPA was 20 μg.
[b] Mean ± SE.

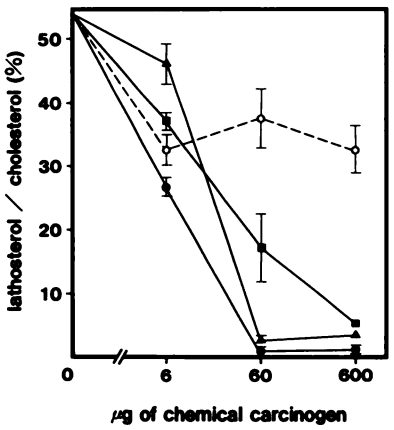

Fig. 2. Changes in sterol metabolism in the skin of mice treated with chemical carcinogens and β-naphthoflavone. Chemicals were administered three times each week for 2 weeks. *Abscissa*, amounts of chemicals applied at a time. ○, β-naphthoflavone; ■, BaP; ▲, 3-MC; ●, DMBA.

Fig. 3. Gas-liquid chromatogram of mouse skin tumor developed by 3-MC and TPA. Experimental conditions were as described in Fig. 1. Symbols as in Fig. 1.

hydrocarbons was also observed in the tumor, some mice were treated with 3-MC and TPA for a longer time (24 weeks) to develop tumors (mostly papillomas) and the sterol composition of the tumor tissue was studied. As shown in Fig. 3, the gas-liquid chromatogram of the sterols in tumor tissue was quite similar to that of skin treated with the chemical carcinogens, *i.e.*, a markedly low peak of lathosterol was observed, whereas the peak of cholesterol looked similar to that of normal mouse.

Studies with the Use of Diazacholesterol. To study the mechanism of lathosterol-depressive effect of polycyclic aromatic hydrocarbons a metabolic inhibitor, diazacholesterol which is known to block mainly the conversion of desmosterol to cholesterol (12), was employed. The experiments were performed according to a protocol described in Table 2. The gas-liquid choromatograms of sterols in the skin of mice treated with diazacholesterol are shown in Fig. 4. Apparently the peak height of desmosterol and that of an unknown substance (*Peak d* substance) were increased remarkably in the mice treated with diazacholesterol compared to that of normal mouse skin (*Experiment 8* in Fig. 4). When 3-MC (*Experiment 9*) or 3-MC and TPA (*Experiment 10*) were applied to the skin of mice administered diazacholesterol, the level of desmosterol increased about twofold to that of the control animal (Table 2). In contrast, lathosterol (*peak c*) and the *peak d* substance, the peak of which was only observed in the skin of mice treated with diazacholesterol, were decreased, but lanosterol (*peak e*) was not significantly altered.

Identification of the Chemical Structure of the *Peak d* Substance. The mass spectrum of the sterol corresponding to *Peak d* is shown in Fig. 5. The molecular peak of the sterol of the trimethylsilyl ether derivative was observed at *m/e* 456, suggesting that the molecule is a C_{27} sterol possessing two double bonds. In addition, the base peak at *m/e* 343 suggested that the unknown could be a sterol with a double bond at C_{24}. Unsaturation in this position would lead to transfer of two protons from ring D to this double bond with concomitant loss of the side chain to form the intense M-113 ion, as described by Wyllie and Djerassi (13). (An alternative possibility that the base peak at *m/e* 343 could be obtained by loss of the saturated side chain was ruled out from the existence of Δ^{24}-double bond from the data of nuclear magnetic resonance as described below.) However, the position of the other double bond was not determined from the gas chromatography-mass data, although the presence of the double bond at C_5 was ruled out since it lacks the intense peak at *m/e* 129, which was considered as the important diagnostic peak indicating the presence of the double bond at C_5 (14).

To determine the position of the double bond in the nucleus the *Peak d* substance was purified by silicic acid column chromatography using the solvent system benzene:ethyl acetate. The *Peak d* substance was eluted by the solvent mixture benzene:ethyl acetate (95:5, v/v). After evaporation of solvents the *Peak d* substance was crystallized from ethyl acetate-methanol to give needles. The nuclear magnetic resonance spectrum of *Peak d* substance showed the following chemical shifts: 0.538

Table 2 *Changes of sterol metabolism of the skin of diazacholesterol administered mouse*

Experiment	Treatment schedule[a] A	Treatment schedule[a] B	Desmosterol/ cholesterol (%)	Peak d/ cholesterol (%)	Desmosterol (mg/g of skin)	Peak d sterol	Cholesterol (mg/g of skin)	Lathosterol (mg/g of skin)
8	No treatment		59.0 ± 9.8[b]	42.7 ± 4.2	0.55 ± 0.04	1[c]	1.00 ± 0.1	0.14 ± 0.05
9	3-MC	3-MC	123.4 ± 7.0	9.2 ± 2.6	1.07 ± 0.05	0.19 ± 0.05	0.84 ± 0.02	0.01 ± 0.006
10	3-MC	TPA	154.9 ± 16.9	27.6 ± 3.0	1.31 ± 0.09	0.57 ± 0.06	0.83 ± 0.04	0.04 ± 0.006

[a] Chemicals were applied three times each week for 2 weeks. The first treatment (A), following treatments (B). The amounts of 3-MC and TPA applied at a time were 600 and 20 μg, respectively. All experimental animals were administered diazacholesterol (10 mg/kg/day) for 2 weeks.
[b] Mean ± SE.
[c] Expressed as ratio of peak height to that of no treatment mouse.

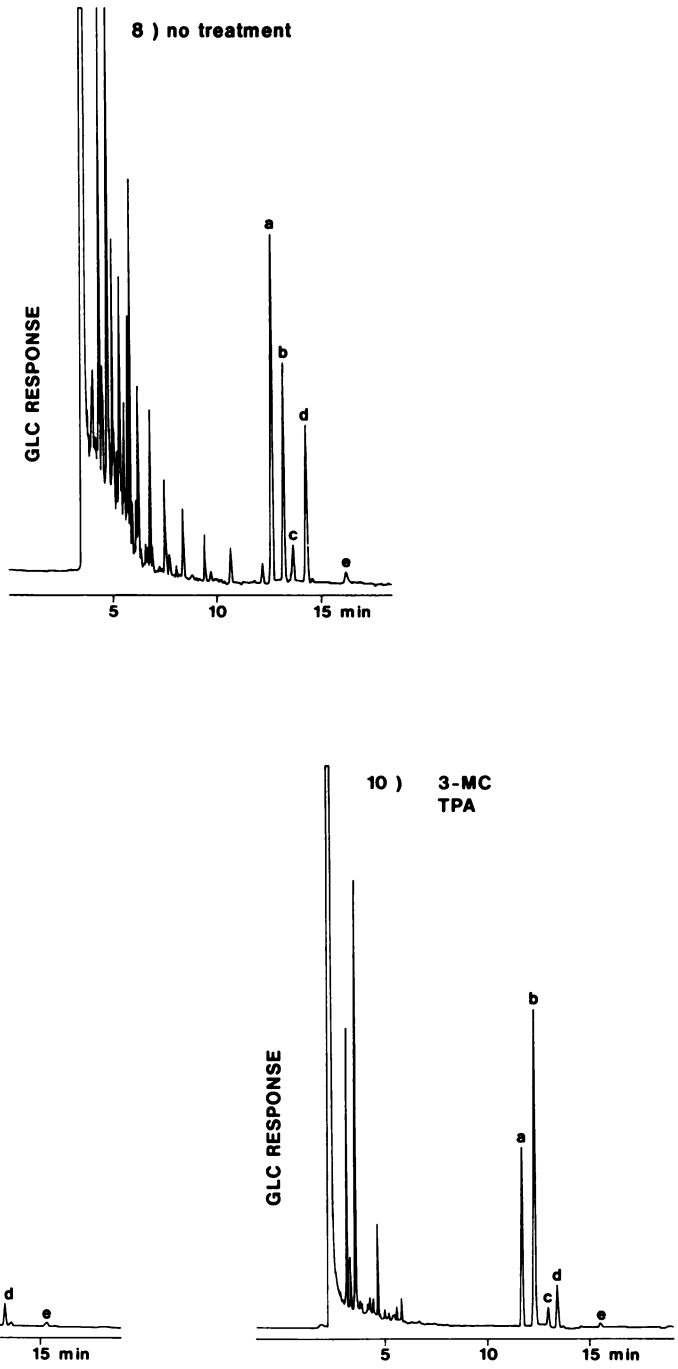

Fig. 4. Effect of 3-MC and TPA on mouse skin sterols treated with diazacholesterol. Numbers correspond to experiment numbers shown in Table 2. Symbols as in Fig. 1 except *d*, the structure of which is described in the text. Experimental conditions were as described in Fig. 1.

(s, 18-CH_3); 0.799 (s, 19-CH_3); 0.958 (s, 20-CH_3); 1.605 (s, 25-CH_3); 1.686 (s, 25-CH_3); 3.61 (s, 3α-H); 5.11 (m, 24-H); 5.17 (m, 7-H). Since signals due to 7-H and 24-H were observed at δ5.17 and δ5.11, respectively, the existence of double bonds at C_7 and C_{24} was established. From these results the chemical structure of the *Peak d* substance was identified as 5α-cholesta-7,24-dien-3β-ol (15). This structure was also corroborated by comparing the methylene unit of the *Peak d* substance (32.83) with those of other C_{27} sterols (cholesterol, 31.98; desmosterol, 32.28; lathosterol, 32.52; 7-dehydrocholesterol, 32.28) (16).

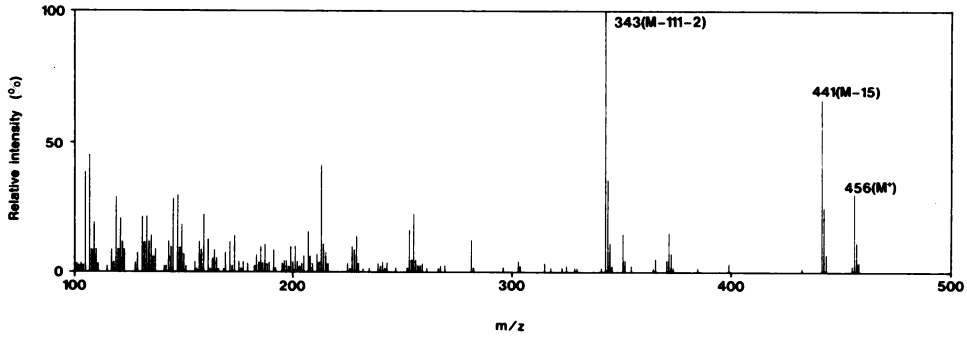

Fig. 5. Mass spectrum of the sterol corresponding to *peak d* in Fig. 4.

DISCUSSION

The use of the chemically bonded capillary column markedly increased the resolution of sterols in gas-liquid chromatography compared to the previous analyses by packed column, and for that matter we could analyze most, if not all, of the thermostable sterols in a single column. Since with classical packed column we had to use two or three different columns to separate all of the sterols in the skin, the new method also significantly increased the accuracy of quantitation. Although we used such a highly sophisticated analytical method, our older finding that lathosterol is depressed by application of polycyclic aromatic hydrocarbons to mouse skin, was shown to be essentially correct. However, it was found that a correction was needed as to the identity of some compounds in the skin of mice administered diazacholesterol. Although we have tentatively identified methostenol in the skin of mice treated with diazacholesterol based on the retention time without comparing with the authentic sample, it has now been unequivocally established as 5α-cholesta-7,24-dien-3β-ol. Furthermore, our previous concept that the pathway proposed by Kandutsch-Russel was repressed by the chemical carcinogens while that proposed by Bloch was activated, must be modified, since the 5α-cholesta-7,24-dien-3β-ol is not a member of the Kandutsch-Russel pathway.

The function of diazacholesterol seemed to block the steps of 24 reduction either at the level of desmosterol and 5α-cholesta-7,24-dien-3β-ol since desmosterol and 5α-cholesta-7,24-dien-3β-ol accumulated in the skin of mice administered diazacholesterol (Fig. 6). However, diazacholesterol did not seem to affect the 24 reduction of lanosterol, since the amount of lanosterol in the skin was not changed by treatment with diazacholesterol. The fact that lathosterol was depressed by treatment with diazacholesterol seemed to suggest that a large part of lathosterol in the skin is formed via 5α-cholesta-7,24-dien-3β-ol bypassing from Bloch's pathway to the Kandutsch-Russel pathway. Treatment of mice administered diazacholesterol with polycyclic aromatic hydrocarbons significantly changed the relative concentration of sterols in the skin compared to that of mice administered diazacholesterol only. Although the chemical carcinogen depressed the amount of lathosterol and 5α-cholesta-7,24-dien-3β-ol in the skin of mice administered diazacholesterol, it did not lower the amount of desmosterol, but rather, markedly increased it. Furthermore, this increase of desmosterol seemed to compensate the reduction of cholesterol synthesis through lathosterol (5α-cholesta-7,24-dien-3β-ol→lathosterol→7-dehydrocholesterol→cholesterol), since the cellular level of cholesterol was unchanged despite the marked change in synthetic pathway (Table 1 and Ref. 17).

One possible explanation for this could be that in the skin of carcinogen-treated mice the synthesis of cholesterol via lathosterol and 7-dehydrocholesterol was repressed, while the other pathway (5α-cholesta-7,24-dien-3β-ol→desmosterol→cholestrol) was activated, keeping the tissue level of cholesterol constant. An alternative explanation is that the lathosterol produced was rapidly converted to cholesterol, via 7-dehydrocholesterol, while in the control case the amount of lathosterol could build up to where Δ^5-desaturase is rate limiting. However, the latter possibility may be ruled out since, according to our recent results, the enzymatic activities of Δ^5-desaturase and Δ^7-reductase were not significantly changed by treatment with polycyclic aromatic hydrocarbons despite the fact that the level of 7-dehydrocholesterol decreased markedly (17).

The effect of polycyclic aromatic hydrocarbons seems to elicit a perturbation in biosynthetic pathways of cholesterol while keeping the cellular level of cholesterol unchanged. It may therefore be interesting to see whether any inhibitors which would eliminate the activation of the compensatory pathway

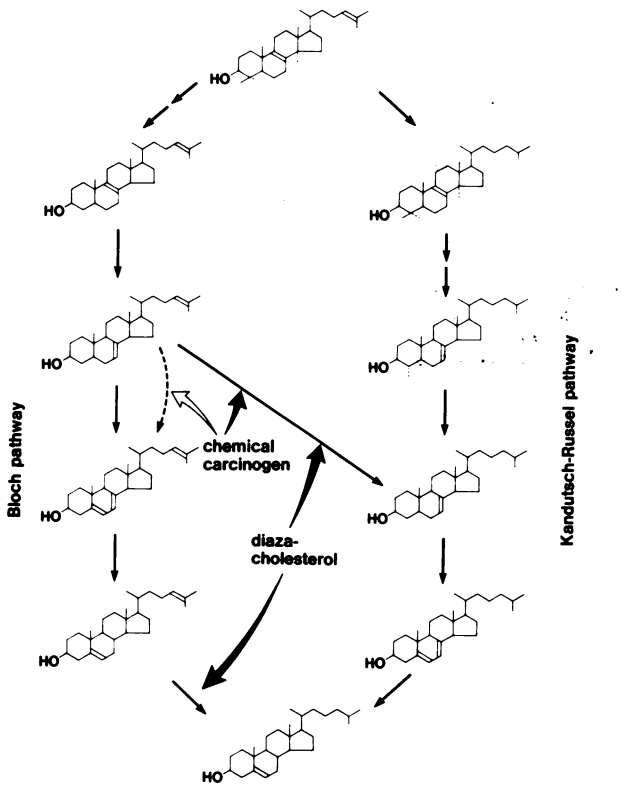

Fig. 6. A proposed mechanism for the action of diazacholesterol and chemical carcinogens in the postlanosterol biosynthetic pathways in mouse skin. *Closed arrows*, inhibition of the reaction; *open arrow*, acceleration of the reaction; *broken arrow*, a hypothetical activation of the pathway induced to compensate the decrease of cholesterol biosynthesis due to blockage of normal pathway by chemical carcinogen.

might repress the carcinogenesis or tumor development, since a constant level of cholesterol is essential for cell viability (18).

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