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Studies on the uptake of benzanthrone by rat skin and its efflux through serum

A. JOSHI, S. K. KHANNA, G. B. SINGH and C. R. KRISHNA MURTI
Industrial Toxicology Research Centre, Mahatma Gandhi Marg, Lucknow 226 001

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Abstract. The uptake of benzanthrone by rat skin showed saturation kinetics and was dependent upon the weight of skin and time, temperature and pH of the incubation medium. Heating of segments above 50°C caused significant lowering of the uptake. The uptake was irreversibly inhibited by $HgCl_2$ and not by sodium arsenate, KCN, NaF, *p*-chloromercuriben zoate, N-ethyl-maleimide, cycloheximide, iodoacetic acid and 2,4-dinitrophenol suggesting that the uptake was not energy-dependent. Lipid micelles of the skin accounted for a part of the binding. Most of the benzanthrone taken up by the skin was effluxed through serum proteins.

Keywords. Benzanthrone; dye intermediate; uptake by the skin; serum transport.

Introduction

Benzanthrone [7H-benz(de)anthracen-7-one], an anthraquinone derivative, is a dye intermediate commonly used for the synthesis of a number of vat and disperse dyes. Workers exposed to benzanthrone during its manufacture, pulverization and storage develop itching, burning sensation, erythema and pigmentation of the exposed as well as covered parts of the skin (Singh *et al.,* 1967; Trivedi and Niyogi, 1968; Singh and Zaidi, 1969; Singh, 1970). In acute stages, the skin becomes tender, rough and peals off easily. Singh *et al.* (1967) showed that prolonged exposure to benzanthrone in experimental animals stimulates cell growth of all the skin layers together with increased vascularity and fibrosis. These histopathological reports indicate that the skin is equipped with a mechanism for the uptake of benzanthrone. In the present investigation, an attempt has been made to study the *in vitro* kinetics of benzanthrone uptake by skin and its transport into the serum.

Materials and methods

Chemicals

Benzanthrone, obtained from Indian Dyestuff Industries, Kalyan, was purified on a neutral alumina column using 1,2-dichloroethane as an eluant. The pooled active

Abbreviation used: KRB, Krebs-Ringer bicarbonate buffer.

fractions were concentrated in a Buchi Rotavapor and finally dried in a vacuum oven. It gave one spot (Rf value, 0.58) by thin layer chromatography on silica gel-G plates using toluene as solvent system (Swicki *et al.,* 1966). The purity of benzanthrone was further assessed according to the Indian Standard specifications (IS: 5044-1969), infra-red (IR), nuclear magnetic resonance (NMR) and Mass spectroscopy. Iodoacetic acid and N-ethylamaleimide were products of Sigma Chemical Co., St. Louis, Missouri, USA. *p*-Chloromercuribenzoate and glutathione were procured from V.P. Chest Institute, New Delhi. 2,4-Dinitrophenol and 2-mercaptoethanol were the respective products of B.D.H., Poole, England and E. Merck, Dermstadt, Germany. All other chemicals used were of the Analytical Reagent grade.

Preparation of skin segments

Male albino rats, weighing approximately 200 g, were drawn from Indian Toxicology Research Centre's stock colony. The dorsal surface of the skin was shaved with an Oster electric clipper (model A5-00) and the animals sacrified by decapitation. Skin pieces from the shaved area were cut and soaked in chilled 150 mM KCl solution as described in the procedure of Sahib and Krishna Murti (1969).

Uptake studies

To each 25 ml Erlenmeyer flasks containing 0.3-0.4g portions of skin segments and 5.0 ml of Krebs Ringer bicarbonate (KRB) buffer, pH 7.4, 200 µg of benzanthrone (from 1 mg/ml ethanolic stock) was added. The blanks contained equal volume of ethanol. The flasks covered with aluminium foils were shaken in a metabolic shaker (120 strokes/min) for the required period. The effects of increasing weight of skin segments (0.1-0.5 g), varying concentrations of benzanthrone (50-500 µg) and time (10-180 min); temperature (25-70°C); inhibitors, namely, sodium arsenate, KCN, NaF, $HgCl_2$, *p*-chloromercuribenzoate, N-ethylmaleimide, cycloheximide, iodoacetic acid and 2,4-dinitrophenol (at 0.01, 0.1 and 1.0 mM levels); reducing agents, namely, ascorbic acid, cysteine, sodium dithionite and sodium metabisulphite all at 1.0 mM concentration and pH 5.0-10.0 of the incubation medium, on the uptake of benzanthrone were studied. After incubation, the skin segments were washed, homogenized and used for the determination of benzanthrone. Alternatively, the washings were mixed with the supernatant and benzanthrone in the supernatant was extracted with 1,2-dichloroethane and estimated. The reliability of the extraction procedure was confirmed by recovery experiments in which known amounts of benzanthrone were added to the KRB-buffer and extracted with 1,2-dichloroethane in exactly the same manner as that of experimental animals. The recovery was between 98-100%.

Efflux studies

Skin segments preincubated with 200 µ*g* benzanthrone were washed twice with chilled KRB-buffer, pH 7.4 and transfered to flasks containing 5.0 ml of rat serum and shaken for 2 h. The skin segments were then removed and homogenized in 1,2-dichloroethane for the estimation of the remaining bound benzanthrone. Its transport into serum was calculated. The effect of serum dilutions (0.0, 2.5, 5.0, 7.5 and 10.0 fold) and contact period (15-180 min) on the transport of benzanthrone was studied.

Preparation of homogenate

Skin segments were thrown into liquid air, the frozen pellets thawed at room temperature and the operation repeated to get a pulpy mass that could be homogenized in a power-driven Potter-Elvehjem homogenizer with teflon pestle The homogenate was centrifuged at 2000 *g* for 15 min in a Remi T8 centrifuge. The homogenate was prepared in 1,2-dichloroethane to extract the bound benzanthrone from the skin segments completely.

Preparation of lipid micelles

Lipids were extracted by homogenizing the skin segments with a chloroform-methanol (2:1) mixture according to the procedure of Folch *et al.* (1957). The lipids thus extracted were dispersed in 50 mM phosphate buffer pH 7.5, and exposed to ultrasonic waves in a Vibronics Ultrasonic Processor (model VPL-P2) for 10 min at 20 KHz and 150 mA current. After sonic disruption samples were centrifuged at 5°C at 1,00,000 *g* for 60 min in an ultracentrifuge (MSE superspeed 75). The supernatant was used as the source of lipid micelles (Kapoor *et al.*, 1972).

Binding of benzanthrone to lipid micelles

Skin lipid micelles (1-10 mg) were allowed to interact with 50 µg of benzanthrone in 50 mM phosphate buffer pH 7.5 for 1 h at 37°C. The unbound benzanthrone was recovered by centrifugation at 2000 *g*, in a Remi T8 centrifuge for 15 min extracted with 1,2-dichloroethane and estimated against suitable blanks.

Estimation of benzanthrone

Benzanthrone was estimated by measuring the absorbance at 385 nm in an Unichem SP-500 spectrophotometer. The molar extinction coefficient (ε) of benzanthrone was 1.175×10^4 litre mol^{-1} cm^{-1} at 385 nm in 1,2-dichloroethane.

Results

Uptake studies

Effect of varying concentrations of benzanthrone: The rate of uptake as affected by concentration of benzanthrone in the medium is shown in figure 1. Increase in the concentration of benzanthrone upto 300 µ*g* gives a linear relationship on the uptake per g of skin. Further increase in concentration does not enhance the uptake.

Effect of skin weight: The uptake of benzanthrone by skin from the medium increases linearly on increasing the weight of the skin as illustrated in figure 2.

Effect of incubation time: Figure 3 shows the effect of time of incubation of skin segments in the medium containing benzanthrone. Maximum uptake was observed at 70 min. Further increase in incubation time showed no effect on the total uptake of benzanthrone.

Effect of incubation temperature: The uptake (after 70 min of incubation) is initially temperature-dependent. maximum uptake is observed between 40-45°C, after which further increase in temperature decreases the uptake of benzanthrone (figure 4).

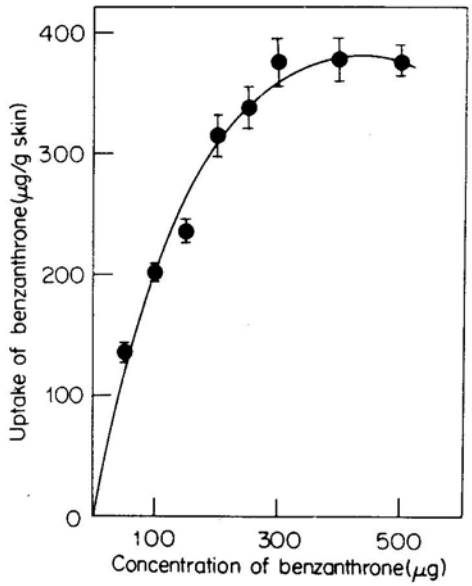

Figure 1. Concentration dependence in relation to benzanthrone uptake.

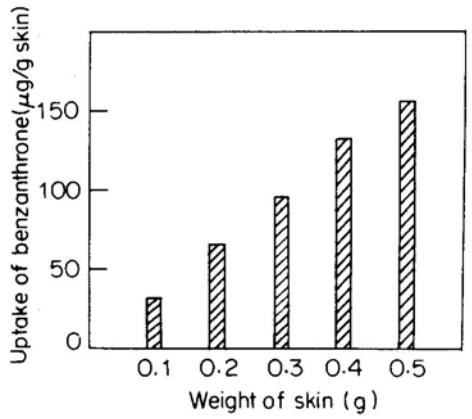

Figure.2 Effect of skin weight on the uptake of benzanthrone

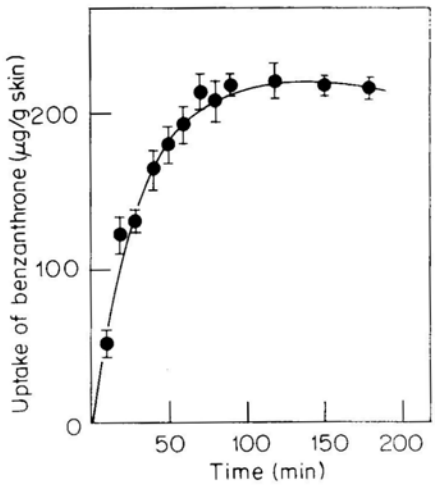

Figure 3. Uptake of benzanthrone in response to exposure time.

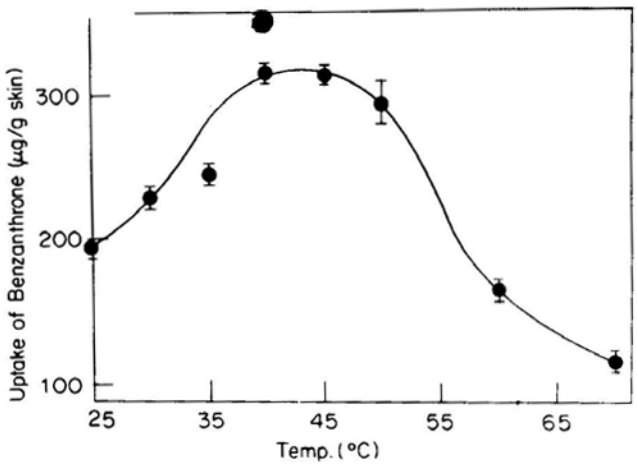

Figure 4. Effect of temperature on the uptake of benzanthrone.

Effect of pH: The uptake of benzanthrone is increased as the pH of the incubation medium is increased from acidic to neutral. The optimum pH range is 7.2-7.6. Further increase towards the alkaline region leads to a slow decrease in the uptake (figure 5).

Effect of inhibitors: Sodium fluoride, iodoacetate, sodium arsenate, KCN, dinitrophenol and cycloheximide do not inhibit the uptake of benzanthrone at

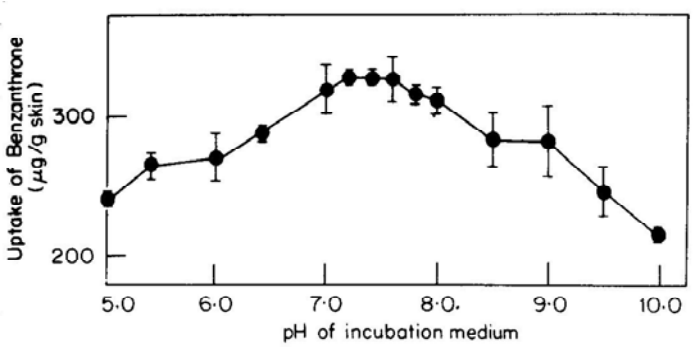

Figure 5. Influence of pH on the uptake of benzanthrone.

0.01, 0.1 and 1.0 mM levels. $HgCl_2$ has no effect at 0.01 and 0.1 mM. An inhibition of 43% in the uptake was observed at the highest concentration tested (1.0 mM) (table 1). The inhibition is not reversed by adding reduced glutathione and 2-mercaptoethanol to a final concentration of 1.0 mM.

Table 1. Effect of inhibitors on the uptake of benzanthrone by rat skin.

	Uptake of benzanthrone (μg/g skin)		
	Concentration of inhibitor (mM)		
	0.01	0.1	1.0
Nil	–	312	–
Sodium arsenate	310	308	303
KCN	316	311	312
NaF	311	309	307
$HgCl_2$	311	294	176
p-Chloromercuribenzoate	318	299	303
N-Ethylmaleimide	305	305	307
Cycloheximide	310	308	308
2,4-Dinitrophenol	308	306	306
Iodoacetic acid	310	303	305

Effect of reducing agents: Ascorbic acid, cysteine, sodium dithionite and sodium metabisulphite have no effect on the uptake at the concentration tested (1.0 mM) (table 2).

Table 2. Effect of reducing agents on the uptake of benzanthrone.

Reducing agent (1.0 mM)	Benzanthrone uptake (μg/g skin)
Control	320
Ascorbic acid	316
Cysteine	318
Sodium dithionite	317
Sodium metabisulphite	324

Transport studies

Effect of serum concentration: Undiluted serum can transport out 84% of the skin-bound benzanthrone (in 120 min of incubation). With 5 and 10 fold dilution, 40 and 13.5% benzanthrone is realised respectively indicating thereby that higher the dilution of serum lesser is its transport (table 3).

Table 3. Relationship between benzanthrone transport and serum concentration.

Fold dilution of serum	Transported benzanthrone	
	(μg/g skin)	(% transport into the serum)
No serum	321	0
0.0	270	84
2.5	199	62
5.0	128	40
7.5	86	27
10.0	43	13.5

Effect of contact time on transport: The transport of benzanthrone into serum gradually increases with contact time. Maximum transport is encountered at 120 min after which further increase in contact time has no influence on the total transport (figure 6).

Binding study with isolated lipid micelles: As illustrated in table 4, binding of benzanthrone is linearly dependent on the amount of skin lipid micelles up to 10.0 mg levels as tested in the present study.

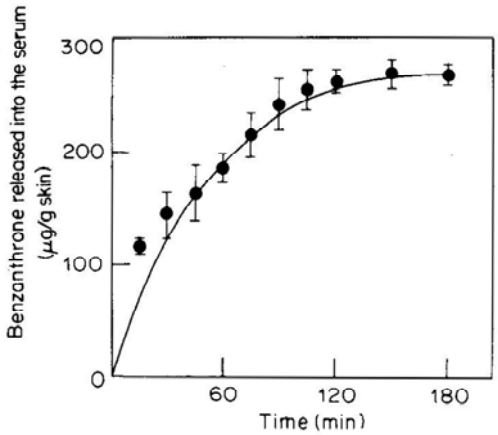

Figure 6. Relationship between benzanthrone transport and contact time.

Table 4. Binding of benzanthrone to rat skin lipid micelles.

Skin lipid micelles (mg)	Benzanthrone bound (µg)
1.0	4.6
2.5	9.2
5.0	18.3
7.5	28.8
10.0	38.3

Discussion

The present study shows that rat skin could pick up benzanthrone from the medium and the skin bound benzanthrone could then be transported through serum.

The uptake is temperature-dependent initially and increases exponentially with rising temperature. This may be largely due to changes in the membrane diffusion constant (Blank *et al.,* 1967) causing a decrease in the activation energies for diffusion across the stratum corneum (Scheuplein and Blank, 1971). The binding is sensitive to temperature above 50°C as the uptake is significantly decreased. Collagen, the major structural protein of skin, is known to undergo thermal denaturation at 52-53°C (Gross 1964). This may suggest that collagen is possibly one of the factors involved in the binding. Separate experiments with reconstituted collagen fibrils and the effect of temperature are in agreement with this finding (Joshi *et al.,* personal communication). Within a pH range of 5.0-10.0, maximum uptake occurs around pH 7.0-7.6. This behaviour is somewhat different from that of albumin, the binding property of which is limited to a narrow pH range (Hargreaves, 1968).

The uptake of benzanthrone is decreased by $HgCl_2$ at 1.0 mM concentration. The inhibition is not likely to be due to the involvement of –SH groups because -SH binding agents such as *p*-chloromercuribenzoate and N-ethylmaleimide had no effect on the uptake; glutathione and 2-mercaptoethanol when added to the final concentration of 1.0 mM were without effect on the uptake. The inhibition by $HgCl_2$ may be either due to the irreversible binding at the same site on the skin at which the benzanthrone binds, or due to its interaction with some other site on the skin not directly involved in the binding of benzanthrone which may result in a conformational change due to which the binding ability is decreased. The uncouples and inhibitors of oxidative phosphorylation showed no change on the benzanthrone uptake kinetics indicating it was not an energy dependent process. The absence of metabolic processes in the dead keratinizing layers precludes any role of active transport processes (Malkinson and Gehlmann, 1977) and molecules may diffuse across the stratum corneum by purely passive means (Treager,1966).

No work on the metabolism of benzanthrone by skin has been reported this far. However, this possiblity can not be ruled out under *in vivo* conditions. In order to confirm whether metabolism of benzanthrone takes place under our experimental condition and whether such metabolites might also be included in the estimation procedure, we performed a separate experiment with a view to identify the metabolites formed, if any by chromatography. Results of this study indicated the presence of only one spot corresponding to benzanthrone confirming that no metabolism occurs in skin segments.

Serum proteins appear to have an affinity for benzanthrone as the skin-bound benzanthrone was transported into serum. The release of benzanthrone increased with increasing serum protein concentration. Acton (1968) showed that the fraction of a bound chemical increase with increasing protein concentration. A likely competition for benzanthrone between binding sites of the skin and serum proteins may exist. When all the binding sites of serum proteins are saturated there is no more efflux. A separate study (Joshi et al., personal communication) in which the protein bound benzanthrone was fractionated on Sephadex G-200 column, revealed that benzanthrone has more affinity for albumins than the globulin fractions.

Part of the uptake of benzanthrone by the skin could also be attributed to lipids present in the skin, as is evident from the binding of benzanthroneitoskin lipids micelles *in vitro.*

On the basis of the above preliminary investigation, it may be assumed that benzanthrone due to its lipophilic nature is readily absorbed through the skin preferably crossing the lipoidal barrier of the epidermis and gets bound to certain specific sites in the skin. The skin-bound benzanthrone is subsequently released into circulation.

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References

Anton, A. H. (1968) *Clin. Pharmac. Ther.*, **9,** 561.
Blank, I. H., Scheuplein, R. J. and Macfarlance, D. J. (1967) *J. Invest. Dermatol.,* **49,** 582.
Gross, J. (1964) *Science,* **143,** 960.
Hargreaves, T. (1968) *Liver and bile metabolism,* (New York: North Holland Publishing Co., Amsterdam) p. 69.
Kapoor, C. L., Krishna Murti, C. R. and Bajpai, P. C. (1972) *Indian J. Med. Res.,* **60,** 918.
Malkinson, F. D. and Gehlmann, L. (1977) *Cutaneous toxicity,* eds V. A. Drill and P. Lazar (New York: Academic Press) p. 64.
Sahib,M.K.and Krishna Murti,C. R. (1969) *J. Biol. Chem.,* **244,** 4730
Sawicki, E., Johnson, H. and Kosinski, K. (1966) *Microchem. J.,* **10,** 78
Scheuplein, R. J. and Blank, I. H. (1971) *Physiol. Rev.,* **51,** 702.
Singh, G. B. (1970) *J. Indian Med.,* **16,** 122.
Singh, G. B., Sharma, S. N. and Zaidi, S. H. (1967) *Indian J. Med. Sci.,* **21,** 727.
Singh, G. B. and Zaidi, S. H. (1969) *J. Indian Med. Assoc.,* **52,** 558.
Trivedi, D. H. and Niyogi, A. K. (1968) *Indian J. Indust. Med.,* **14,** 13.
Treager, R. T. (1966) *Physical functions of skin,* (New York: Academic Press).