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# ON THE BINDING OF THE BISQUATERNARY AMMONIUM COMPOUND PARAQUAT TO MELANIN AND CARTILAGE *IN VIVO*

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**Abstract**—The bisquaternary ammonium compound paraquat has been shown to accumulate in melanin-containing tissues and in cartilage *in vivo*. The present study was intended to elucidate if ionic binding is involved in the mechanism for the affinity of paraquat to these tissues *in vivo*. Ionic binding has previously been shown to be involved in the binding of paraquat to melanin *in vitro* and in the binding of other bisquaternary ammonium compounds to cartilage *in vitro*. [$^{14}$C]paraquat was given to mice. Whole-body tissue sections were taken and incubated in solutions with different cationic composition. The ability of the cations to displace [$^{14}$C]paraquat from melanin of the eye and intervertebral cartilages was then determined by autoradiography, using densitometric measurements for the quantitations. It was found that the cations in the incubation solutions were able to displace [$^{14}$C]paraquat from both melanin and cartilage. The [$^{14}$C]paraquat was more effectively displaced from cartilage than from melanin. In both tissues divalent cations were more effective than $K^+$ and $Na^+$; and $H^+$ was also effective. The results indicate that ionic binding is involved in the binding *in vivo* of paraquat both to melanin and cartilage. The binding sites may be carboxyl groups present in the subunits in melanin and ester sulphate and carboxyl groups of chondroitin sulphate in cartilage. The stronger binding of paraquat to melanin may depend on extra interactions due to conjunctions of the aromatic rings of the paraquat and the melanin polymer.

Paraquat, a member of a group of substances referred to as dipyridylium bisquaternary ammonium compounds, is widely used as a herbicide [1]. In mammals, paraquat produces progressive lung damage [2]. The basic mechanism of the damage is not known. It has been shown that no metabolism of paraquat takes place when it is administered parenterally and that a retention of the compound occurs in the lungs [3, 4]. In addition, autoradiographic distribution studies have revealed a preferential localization of paraquat in cartilage [3]. It has been shown that several other bisquaternary ammonium compounds localize in cartilage *in vivo* [5–9]. A binding to cartilage and to chondroitin sulphate has also been shown *in vitro* [10–12]. On the basis of the experiments, *in vitro*, the authors suggested that ionic binding to anionic sites of chondroitin sulphate or acid mucopolysaccharides in general explains the accumulation of these drugs in cartilage *in vivo*.

Recently we have shown a high uptake of paraquat in melanin-containing tissues of mice [13]. Experiments *in vitro* with pigment from beef eyes indicated that ionic binding was involved in the uptake of paraquat on melanin [13], and this in turn can be ascribed to a cation-exchange activity of melanin [14]. Thus, ionic binding may explain the accumulation of paraquat both in melanin and cartilage. However, there are still no direct experimental data available to show that the mechanism of binding *in vitro* is valid also for the situation *in vivo* neither for the binding of paraquat to melanin, nor for the binding of the bisquaternary ammonium compounds to cartilage. The present study is intended to elucidate if ionic binding is involved in the mechanism for the affinity of paraquat to melanin and cartilage *in vivo*. [$^{14}$C]paraquat has been given to mice. Whole-body tissue sections of the mice have then been incubated in solutions with different cationic composition. The ability of the cations to displace the [$^{14}$C]paraquat from melanin and cartilage has then been determined by autoradiography using densitometric measurements for the quantitations.

## MATERIALS AND METHODS

*Isotope.* [$^{14}$C]paraquat dichloride (*N*,*N*$^1$-dimethyl-4,4$^1$-dipyridylium dichloride [$^{14}$C]methyl), sp. act. 125 $\mu$Ci/mg, was obtained from the Radiochemical Centre, Amersham, England.

*Experiments.* Two pigmented C57-Bl-mice, body wt about 25 g, were used. [$^{14}$C]paraquat was dissolved in physiological saline and 0.2 ml containing 5 $\mu$Ci [$^{14}$C]paraquat (1.6 mg/kg body wt) was i.v. injected into each animal. The mice were killed after 24 hr and 4 days by anaesthetizing with ether, embedding in a mixture of carboxymethyl cellulose and water and freezing in hexane cooled to −78°C with solid $CO_2$. The embedded mice were sectioned in a microtome on tape (tape No. 688, Minnesota Mining and Manufacturing Co.) according to Ullberg [15].

A series of 20 $\mu$m thick whole-body sagittal sections were taken from the animal with 4 days survival. As a consequence of being quite close to each other, the sections became almost identical. They were cut so that one eye and several intervertebral cartilages were present in the same section. After being freeze-dried in a temperature of −20°C, each

section was incubated in distilled water or in distilled water supplemented with various amounts of HCl or monovalent or divalent cation-chloride salts. The pH of the incubation solutions was determined by means of a PHM62 Standard pH-meter with a glass-electrode (Radiometer, Copenhagen).

The incubation volume was 20 ml per section. After 15-min incubation, the sections were rinsed in distilled water for 1 min. The sections were then air-dried. The whole procedure was carried out at room temperature.

The contents of radioactivity in the uveal pigment of the eye and in the intervertebral cartilages were analyzed by autoradiography. To make a quantification possible, standard isotope staircases were used [16]. Such a staircase consists of a sequence of polyeten-supported gelatin layers containing different concentrations of $^{14}C$ in a geometric series where the radioactive contents of adjacent steps is related in the ratio 2:1, thus forming a gradually decreasing scale.

The sections, together with isotope staircases, were attached to X-ray film (Industrex C, Kodak) under light pressure. The exposure time was 4 months.

The quantitative evaluation of the autoradiograms was carried out by means of a densitometer (Schnell–photometer GII, Zeiss, Jena). A standard curve was prepared by plotting the photographic density of each step of the autoradiograms of the standard staircases against the relative isotope concentration of the corresponding staircase step. The photographic densities (which correspond to the contents of radioactivity) in the melanin of the eye and in the intervertebral cartilages respectively, could then be estimated by means of the standard curve. The concentration in the sections solely incubated in distilled water was assigned the relative value 100 per cent, and the concentrations in the other sections were then expressed in per cent of this value.

To evaluate the general distribution of radioactivity at 24 hr and 4 days, tissue sections were

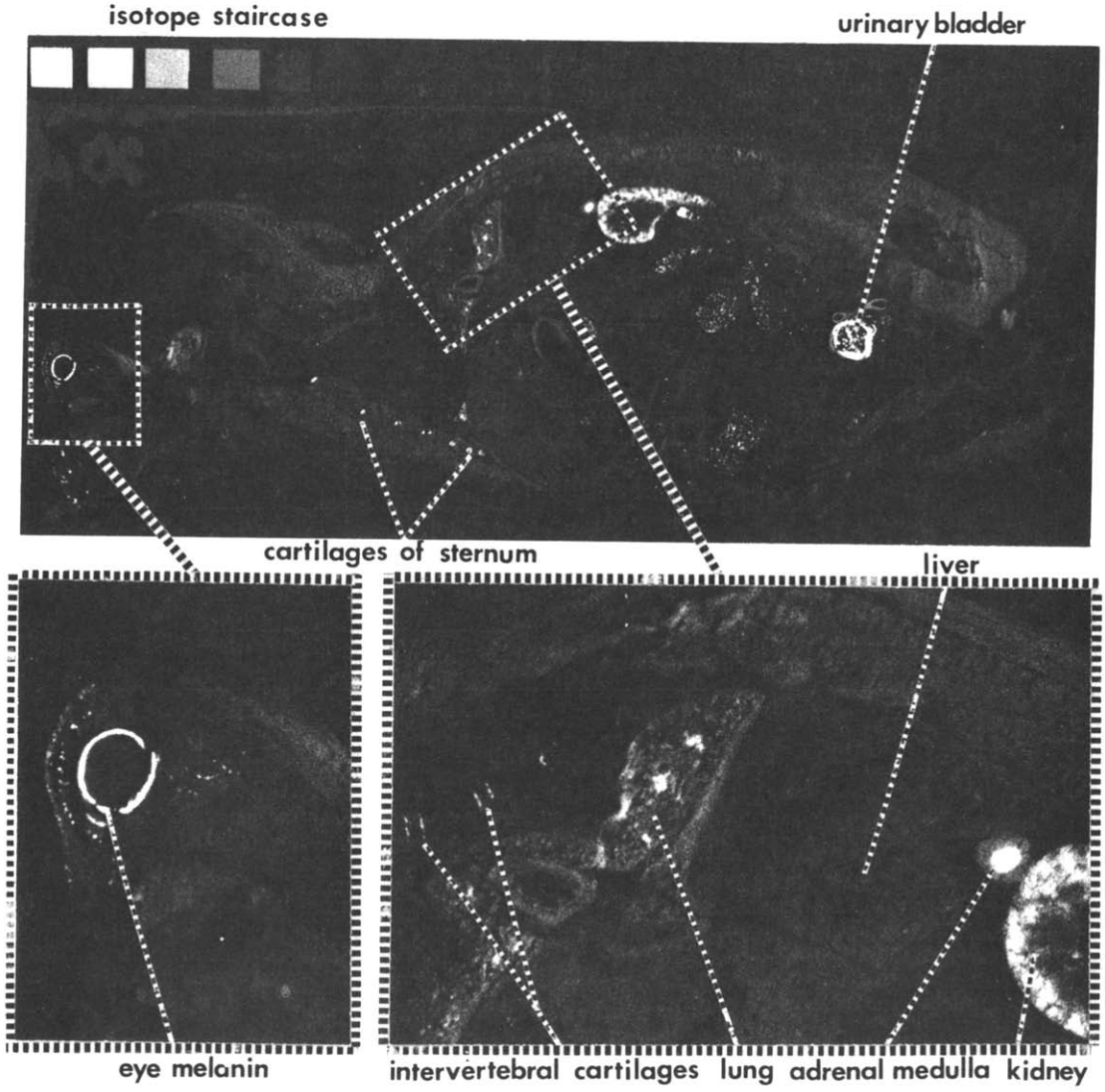


*Fig. 1. Whole-body autoradiogram of a mouse 24 hr after an i.v. injection of [$^{14}C$]paraquat with enlargements of the indicated areas. A high radioactivity (white areas) is present in the melanin of the eye, in cartilages, in the adrenal medulla, in the lung, and in the kidney and urinary bladder.*

Table 1. The ability of cations to displace [$^{14}$C]paraquat from eye melanin and intervertebral cartilages*

| Cation | Conc. (M) | pH of incubation solution | Per cent [$^{14}$C]paraquat bound after incubation | |
|---|---|---|---|---|
| | | | Eye melanin | Intervertebral cartilages |
| — | — | 6.5 | 100 | 100 |
| $H^+$ | 0.001 | 3.0 | 58.1 ± 2.9 | 36.2 ± 13.4 |
| $Ba^{2+}$ | 0.01 | 5.5 | 50.0 ± 3.6 | 55.3 ± 16.9 |
| $Ba^{2+}$ | 0.1 | 5.3 | 44.6 ± 2.3 | 0 |
| $Mg^{2+}$ | 0.1 | 5.4 | 60.8 ± 2.8 | 0 |
| $Ni^{2+}$ | 0.1 | 5.4 | 58.1 ± 2.5 | 4.3 ± 0.1 |
| $Ca^{2+}$ | 0.1 | 6.9 | 56.8 ± 3.0 | 7.4 ± 1.4 |
| $K^+$ | 0.1 | 5.5 | 81.1 ± 3.9 | 36.2 ± 7.2 |
| $Na^+$ | 0.1 | 5.5 | 74.3 ± 0.7 | 45.7 ± 11.6 |

* [$^{14}$C]-paraquat was injected i.v. into a mouse. Four days later, whole-body tissue sections (20 $\mu$m thick) were taken and freeze-dried. They were then incubated in distilled water or different cation-chloride solutions. After subsequent autoradiography, a relative densitometric analysis of the autoradiograms with respect to the [$^{14}$C]paraquat concentration of the eye melanin and the intervertebral cartilages was performed.

The radioactivity in the eye and the cartilages of the water-incubated sections is assigned the value 100 per cent, and the radioactivities in the cation chloride-incubated sections are expressed in per cent of this value (mean ± S.E; $n = 12$).

taken and used for autoradiography without previous incubations.

## RESULTS

*The distribution pictures.* At 24 hr, a high radioactivity was present in pigmented tissues such as the uveal pigment of the eye (Fig. 1). A high radioactivity was also present in cartilage. In addition, the cells of the adrenal medulla contained a high radioactivity. A considerable radioactivity was also present in the lungs, the kidneys and the urinary bladder. At 4 days, a considerable radioactivity was still present in the melanin-containing tissues and cartilage, while the radioactivity in other tissues had decreased to a low level.

*The binding of* [$^{14}$C]*paraquat to melanin and cartilage.* The cations in the incubation solutions were able to displace the radioactivity from both melanin and cartilage (Table 1). However, the [$^{14}$C]paraquat was more effectively displaced from cartilage than from melanin. In both tissues, the divalent cations were more effective than the $K^+$ and $Na^+$; $H^+$ was also found to displace the radioactivity. The pH of the latter incubation solution was 3.0. Small variations in the pH-values were found in the other incubation solutions. It was obvious that these small variations had no relation to the displacement of [$^{14}$C]paraquat from melanin and cartilage.

## DISCUSSION

Since it has been reported that no metabolism occurs of parenterally administered paraquat [3, 4], it is assumed that the radioactivity in the autoradiograms represents the unchanged compound. It was found in the present study that inorganic cations and $H^+$ displaced the *in vivo* bound paraquat from melanin and cartilage. It has been shown that binding *in vitro* of inorganic cations to melanin [14, 17] and to cartilage [18, 19] occurs by an ion-exchange process. Melanin contains free carboxyl groups [20], which may behave analogously to ion-exchange resins in the binding of cations. The binding-sites in cartilage may be the ester sulphate and carboxyl groups of chondroitin sulphate. Characteristics of weak acid cation exchangers are increasing affinity for the cations with increasing valence and a high affinity for $H^+$ [21]. In the present study, $H^+$ was found to be effective in displacing paraquat from melanin and cartilage. The divalent cations were found to be more effective than $Na^+$ and $K^+$. Therefore, the results of the present study indicate that ionic binding is involved in the uptake *in vivo* of paraquat both in melanin and in cartilage. It was obvious, however, that the binding to melanin was stronger than the binding to cartilage. It has been shown that cationic dyes are more strongly bound to DNA and RNA than to polyphosphates in general [22]. It was proposed that extra interactions due to the presence of aromatic rings in the dyes and the DNA and RNA (purines and pyrimidines) explain the enhanced binding. Melanin is a polymer with an aromatic indole-nucleus as the main building-stone [20]. It is probable that the stronger binding of paraquat to melanin than to cartilage depends on conjunctions of the aromatic rings of the paraquat and the melanin polymer. We have found radioactivity in the pigment of the eye still 5 weeks after a single injection of [$^{14}$C]paraquat [13].

It is reasonable to suggest that the mechanism of the binding *in vivo* to cartilage of other quaternary ammonium compounds is the same as for paraquat. While cartilage is an extracellular tissue which may be reached by diffusion through the extracellular space, the binding to melanin *in vivo* requires an

uptake in the melanin-containing cells. This may limit the ability for cationic compounds, such as the bisquaternary ammonium compounds, to reach the melanin granules *in vivo* even if a binding to melanin can be demonstrated *in vitro*. Paraquat is bound to melanin *in vivo*. It was also found in the present study that a high uptake occurred in the adrenal medulla. The melanocytes and the cells of the adrenal medulla have a common embryological origin and common morphological and cytochemical characteristics [23], and they belong to the so called APUD-cell series [24]. It is possible that the accumulation of the bisquaternary amine paraquat in the melanocytes and the adrenal medula takes places because it is accepted by a common transport mechanism in these cells. However, due to the binding to the melanin, paraquat will be retained in the melanocytes for a longer time.

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## REFERENCES

1. A. A. Akhavein and D. L. Linscott, *Residue Rev.* **23**, 97 (1968).
2. D. G. Clark, T. F. McElligott and E. W. Hurst, *Br. J. ind. Med.* **23**, 126 (1966).
3. M. H. Litchfield, J. W. Daniel and S. Longshaw, *Toxicology* **1**, 155 (1973).
4. R. E. Murray and J. E. Gibson, *Toxic. appl. Pharmac.* **27**, 283 (1974).
5. K. Asghar and L. J. Roth, *J. Pharmac. exp. Ther.* **176**, 83 (1971).
6. H. Shindo, I. Takahashi and E. Nakajima, *Chem. pharm. Bull., Tokyo* **19**, 1976 (1971).
7. H. Shindo, E. Nakajima, N. Miyakoshi and E. Shigehara, *Chem. pharm. Bull., Tokyo* **22**, 2502 (1974).
8. O. Wassermann, *Naunyn-Schmiedebergs Arch. Pharmak.* **270**, 419 (1971).
9. O. Wassermann, *Naunyn-Schmiedebergs Arch. Pharmak.* **275**, 251 (1972).
10. K. Asghar and L. J. Roth, *Biochem, Pharmac.* **20**, 3151 (1971).
11. G. D. Olsen, E. M. Chan and W. K. Riker, *J. Pharmac. exp. Ther.* **195**, 242 (1975).
12. H. Shindo, E. Nakajima and E. Shigehara, *Chem. pharm. Bull., Tokyo* **24**, 2327 (1976).
13. B. Larsson, A. Oskarsson and H. Tjälve, *Expl Eye Res.* **25**, 353 (1977).
14. L. P. White, *Nature, Lond.* **182**, 1427 (1958).
15. S. Ullberg, *Acta radiol. suppl.* 118 (1954).
16. M. Berlin and S. Ullberg, *Archs Envir. Hlth* **6**, 589 (1963).
17. F. W. Bruenger, B. J. Stover and D. R. Atherton, *Radiat. Res.* **32**, 1 (1967).
18. E. S. Boyd and W. F. Neuman, *J. biol. Chem.* **193**, 243 (1951).
19. J. R. Dunstone, *Biochem. J.* **72**, 465 (1959).
20. R. A. Nicolaus, in *Melanins* (Ed. E. Lederer), Hermann, Paris (1968).
21. W. C. Bauman, in *Cation Exchange Resins with Weakly Acidic Groups* (Ed. F. C. Nachod), p. 61. Academic Press, NY (1949).
22. J. E. Scott, *Histochemie* **32**, 191 (1972).
23. F. W. D. Rost, J. M. Polak and A. G. E. Pearse, *Virchows Arch. (Zellpathol.)* **4**, 93 (1969).
24. A. G. E. Pearse, *Proc. R. Soc.* **170**, 71 (1968).