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STUDIES ON THE PERCUTANEOUS ABSORPTION OF PARATHION AND PARAOXON

II. Distribution of ^{32}P-Labelled Parathion within the Skin

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In recent years, autoradiographic techniques frequently have been used to study the cutaneous distribution of various compounds applied topically, occasionally in order to determine routes of absorption (Witten, 1951; Scott and Kalz, 1956; Blank *et al.*, 1958; Fredriksson, 1958; Blank and Gould, 1959). However, there have been no previous investigations of this kind concerned with parathion, an organophosphorus insecticide which is known to produce occupational intoxications by percutaneous absorption, as reviewed earlier, Fredriksson *et al.*, 1961. Since this compound is of great practical importance, it was decided to study the distribution of ^{32}P-labelled parathion within the skin of various species following topical application, with special attention paid to artefacts produced by diffusion, time of exposure, and type of radiation.

Methods

^{32}P-labelled parathion (E 605, or diethyl 4-nitrophenyl thiononophosphate) with an initial specific activity of 1.75 mC per mM, furnished by Volk Radiochemical Company, Chicago, Ill., was used in all the experiments. When tested for purity (Fredriksson *et al.*, 1961) it was found to contain less than 0.1 percent of an unidentified contaminant.

Skin from man, cat, rabbit, and rat was used. Human sternal skin was obtained at autopsy, performed within 10 hours of death. These human skin samples, which were from adult individuals of both sexes, had evidently not been washed or otherwise treated and showed no evidence of disease. Skin samples were taken from a clipped area on the back of cats and rabbits under sodium pentobarbital anaesthesia. Rats (Sherman strain) were killed by a blow on the head, and immediately after death clipped skin was removed from the back.

Stripping experiments

Pieces of human skin were nailed to a board, and with the aid of a glass rod, 6 μl of labelled parathion were evenly distributed on a round area of

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approximately 1.5 cm^2. The treated skin was then placed in a humid chamber where the temperature was maintained at 23 to 24° C. The parathion was allowed to remain on the skin for 30 minutes and for 1, 2, 4 and 24 hours. At the end of the exposure period, the excess parathion was removed by one gentle blotting with a high grade, smooth surface, medium speed filter paper (Scientific Products, No. 63200). Twenty-five consecutive strippings then were made of the treated area with the aid of 1″ transparent cellulose tape. Each set of strips consisting of cellulose tape and attached skin, was immediately attached to a 1—1/4″ wide band of cellophane, which had a thickness of approximately 40 μ. This band was then run in a Nuclear-Chicago model C 100 A Actigraph II chromatogram scanner with the cellophane, which had a more uniform thickness than the tape, facing the detector. The scanning speed was 3″ per minute, and the slit opening was set at 1/16″. A Nuclear-Chicago model D 34 thin mica window (1.4 mg/cm^2) GM-tube, operated at 950 volts, was used as detector together with a Nuclear-Chicago recording rate meter model 1620 B, with the time constant set at 2 seconds.

With the cellophane facing the film, autoradiograms were then obtained by apposition to Eastman-Kodak No Screen Medical X-ray film. The autoradiograms were subsequently developed and fixed as described below. All experiments were run in duplicate.

Autoradiograms from sections

Skin samples from all the species were included in these experiments, and the labelled parathion was applied in the same manner and for the same exposure period as above. At the end of the exposure period, the skin samples were placed immediately on dry ice and transferred to a freeze room (— 10° C). In one series of experiments the excess parathion was left on the skin surface, and in another series it was removed by blotting with filter paper as above. The frozen pieces of skin were sectioned transversely in the freeze room at 10, 25 and 100 μ, particular care being taken to avoid radioactive contamination of the knife or the sections. The following four autoradiographic techniques were used: 1) sections were mounted directly on photosensitive slides (Eastman-Kodak Autoradiographic Plates Type A and Type No Screen); 2) sections were mounted on glass slides and autoradiograms were obtained by apposition to the same photosensitive slides as above and to Eastman-Kodak No Screen Medical X-ray film; 3) sections were attached to transparent cellulose tape, according to Ullberg, 1954, and autoradiograms were obtained by apposition as above; and 4) sections were mounted on glass slides and covered with small pieces of Eastman-Kodak Autoradiographic Safety Stripping Film Type NTB, essentially according to Boyd, 1955. Control studies were performed *in vivo* with cats and rabbits under sodium pentobarbital anaesthesia to learn whether there was any difference in the distribution of parathion in excised and intact skin; the parathion in these cases was left on the skin for 1 hour. In all cases, controls were run also with sections from skin that had not been exposed at all to labelled material. Exposure of photosensitive material took place in the freeze room or at room temperature. In the freeze room, the sections were allowed to reach temperatures above the freezing point only when they were momen-

tarily warmed against the back of the bare hand in order to make them adhere to the slides. This was not necessary when the sections were attached to the cellulose tape and brought in contact with the film under pressure. All the autoradiograms were developed with Eastman-Kodak D 19 developer for 5 minutes at 21° C, transferred to an acid stopping bath, and fixed for 30 minutes with Kodak fixing bath F 5. The final rinsing water contained 0.5 percent of Eastman-Kodak Hypo-flo solution. The sections were mounted and examined in phase contrast.

Results

Stripping experiments

The results are illustrated by Figs. 1 and 2. As can be seen from Fig. 1, there was a rapid drop in activity in the first four strips followed by a much slower but steady decrease in activity. However, even in strip number 25, the activity peak was significantly higher than the background. This general pattern was the same regardless of the exposure time.

Following relatively short exposure (2 hours or less) to the X-ray film, the autoradiograms, with the partial exception of the first three or four strips, showed an uneven distribution of the labelled material, which corresponded to the follicular pattern of the piece of skin in question. This follicular arrangement of the activity was obscured by prolonged exposure to the photosensitive film. There was a tendency towards more uniform distribution of the material when it had been in contact with the skin for 24 hours, but even in this case there was a distinct follicular arrangement.

Autoradiograms from sections

The results are illustrated by Figs. 3 and 4. The various factors studied and the procedures that influenced the autoradiograms and their interpretation are listed below.

Controls without labelled material. Occasionally, artefacts appeared that were produced by mere contact of the unexposed sections with the photosensitive material. Such darkening usually followed the border of the sections, and also showed some predilection for such structures as hair follicles and sweat glands.

Control experiments in vivo. The control studies, performed on cats and rabbits *in vivo*, did not produce autoradiograms that differed in any respect from those obtained *in vitro* from the same species and with the corresponding exposure period.

Removal of the excess parathion. When the excess parathion was not removed, the autoradiograms were obscured by an intense darkening corresponding to the skin surface, and the photosensitive material could not be exposed sufficiently long to detect the comparatively low activity at more deeply located sites. Furthermore, under this condition, radioactive contamination of the microtome knife or the sections frequently occurred. However, when the excess was removed by blotting with filter paper longer exposure of the photosensitive material was possible, and thus, a more detailed picture of the actual distribution of the labelled material could be obtained.

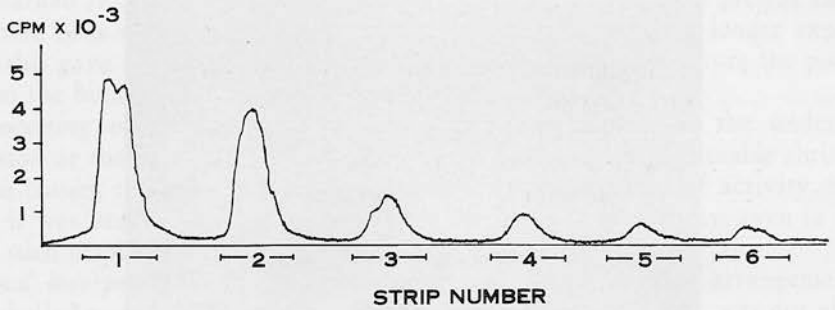

Fig. 1. Radioactivity as obtained from recorder in the first 6 tape strips from human skin, taken 1 hour following application of 6 μl of ^{32}P-labelled parathion. Scanning speed 3″ per min.; slit opening 1/16″; time constant 2 seconds.

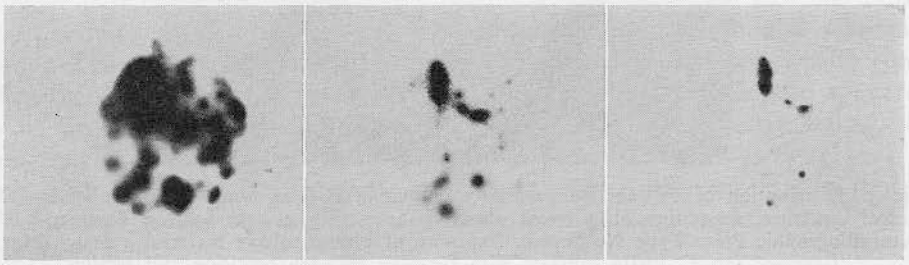

Fig. 2. Autoradiograms of strip no. 3, 10, and 25 from the same experiment as in Fig. 1. Magnification: 2 ×. Exposure of photosensitive material 2 hours.

Thickness of sections. As a rule, the most detailed pictures were obtained with the thinnest sections, which had relatively less activity on the surface, and, therefore, could be allowed longer exposure to the photosensitive material.

Temperature during exposure of photosensitive material. There were seldom any evident diffusion artefacts in the autoradiograms obtained by exposure at room temperature, probably due to the rather short time of exposure — generally less than 4 hours. However, as a precaution, only freeze-room exposed autoradiograms were used for the final evaluation. The momentary elevation of the temperature when the sections were mounted did not seem to produce any artefacts, since the sections, which were attached to the cellulose tape and which were kept at — 10° C during the whole exposure period, gave identical pictures.

Autoradiographic techniques. The four autoradiographic techniques used gave essentially the same results. Direct mounting on photosensitive slides or the use of stripping film allowed, however, much higher resolution than the apposition techniques, and autoradiograms so obtained were therefore used for the final interpretation.

Exposure time of photosensitive material. It already has been indicated that the time of exposure to the photosensitive material must be carefully selected in order to obtain accurate pictures of the distribution, particularly when there

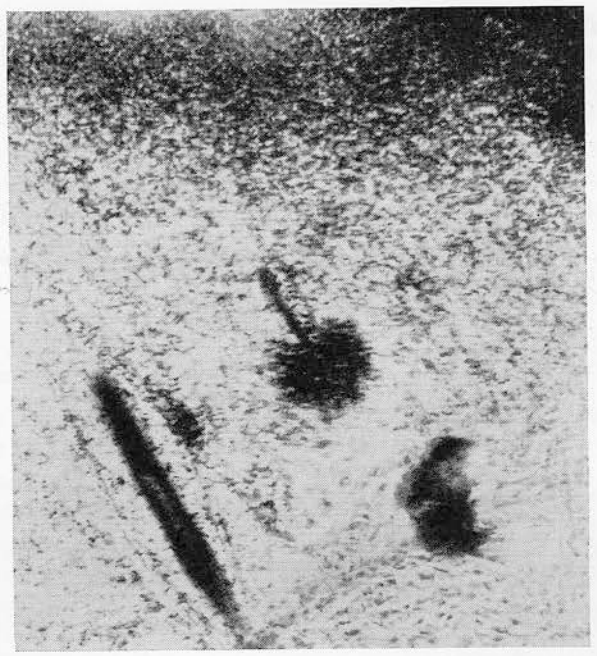

Fig. 3. Distribution of ^{32}P-labelled parathion in human skin 24 hours after application. The central structure, suggestive of a sweat gland, is in reality a hair follicle. Eastman-Kodak Autoradiographic Plate Type No Screen. Exposure of photosensitive material 2 hours. Magnification: 100 ×. Phase contrast with focusing between emulsion and section.

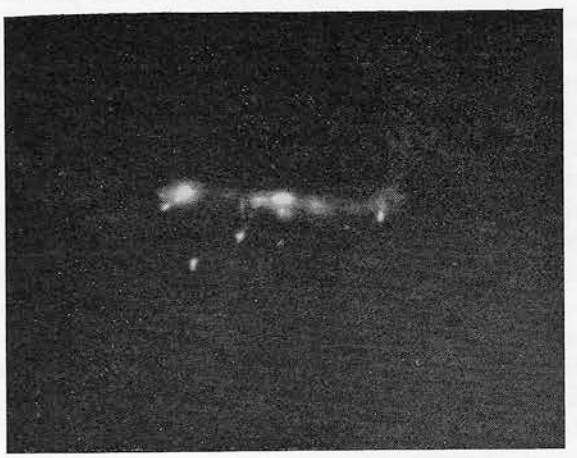

Fig. 4. Distribution of ^{32}P-labelled parathion in cat skin 2 hours from application. Two barely noticeable pressure artefacts from hairs in left part of the autoradiogram. Eastman-Kodak No Screen Medical X-ray Film. Exposure of photosensitive material 2 hours. Magnification: 5 ×.

are marked relative variations in activity in the sections. In the present case an exposure of 2 to 4 hours gave the most detailed pictures, and longer exposure than this gave no further information but rather tended to obscure the pictures due to the high activity on the skin surface.

Processing of sections. Occasionally, the development of the underlying emulsion or the processing of the tissue sections produced a noticeable shrinkage of the tissues, resulting in a relative displacement of the sites of activity. However, it was usually possible to deduce the original site of activity, even in slides with such displacement.

Final interpretation of the autoradiograms. The follicular arrangement of the labelled material, demonstrated in the stripping experiments, was not always evident in the autoradiograms from the sections, particularly when the photosensitive material had only been exposed for a short period of time. As could be expected from the stripping experiments, there was much higher activity corresponding to the skin surface than anywhere else in the sections, even when a major part of the labelled material had been removed by the blotting. However, activity could always be demonstrated in hair follicles and sebaceous glands. The amount in the follicles seemed to increase progressively with time; when the skin had been exposed to parathion for 24 hours, there also appeared to be an increased spreading around the follicles and directly below the epidermis. There seemed to be no increase in activity around sweat glands or ducts in human skin. There were no apparent species differences, even though it was easier to demonstrate the penetration into follicles in animal skin than in human skin.

Comments

It has been pointed out repeatedly that the interpretation of autoradiograms from sections of the present kind must be done very carefully, particularly when they have been obtained with a high energy β-emitter such as ^{32}P, since the particles may travel some distance before darkening the photosensitive material. (Blank *et al.*, 1958; Blank and Gould, 1959, Griesemer, 1959). Also, the present study indicates that various forms of artefacts constitute a most important limiting factor for definite conclusions regarding routes of percutaneuos absorption. It is the opinion of the present author that the pictures obtained reveal affinity of parathion for certain structures rather than actual pathways of absorption. After all, it is an obvious fact that autoradiograms of this kind show only what has *not* been absorbed.

However, the autoradiograms from the sections combined with the results obtained with the rather crude stripping method indicate that parathion penetrates into hair follicles to some extent. This can easily be overlooked in case the amount of labelled material on the surface is large and the exposure of the sections to the photosensitive material is short. The progressively increasing amounts in the follicles and the spreading around them does not necessarily mean that this is the main route of absorption, as Blank has pointed out in the discussion of a paper presented by Griesemer, 1959. The increasing radioactivity below the epidermis makes transepidermal absorption just as likely.

It does not seem probable that the blotting used to remove part of the excess

on the skin surface will produce any rearrangement of the labelled material. At least, the risks of such artefacts appear to be much less than it is when the excess is removed by washing, which sometimes is necessary with more toxic compounds like sarin (Fredriksson, 1958).

Even if no certain conclusions can be drawn from the present study regarding routes of absorption or, for that matter, the location of the epidermal barrier, the results have other immediate implications. Thus, the penetration into follicles helps to explain the shape of disappearance curves obtained by measurements of rates of percutaneous absorption (Fredriksson, 1958; 1961), and may also explain the difficulties in decontaminating skin from parathion (Fredriksson, 1961).

SUMMARY

The distribution of ^{32}P-labelled parathion (E 605, or diethyl 4-nitrophenyl thiononophosphate) within the skin following topical application for various periods of time was studied in excised skin from man, rat, rabbit, and cat. Two different approaches were chosen: 1 a) determination of radioactivity in 25 consecutive cellulose tape strips from the surface of human skin, 1 b) autoradiography of the same strips; and 2) autoradiography of skin sections with the use of four different techniques. Various factors influencing the autoradiograms were studied, special attention being paid to artefacts due to diffusion, type of radiation, and exposure time of the photosensitive material. It was found that parathion penetrates into hair follicles and sebaceous glands to some extent, but it was concluded that this is not necessarily the main route of absorption. There also was increasing activity below the epidermal layers, and transepidermal absorption is as likely. The results were discussed with special attention to the interpretation of the autoradiograms and to the implications for other studies of the percutaneous absorption of parathion.

RÉSUMÉ

La répartition du parathion (E 605 ou diéthyl 4-nitrophényl thiononophosphate) marqué au P^{32} se trouvant à l'intérieur de la peau, à la suite d'une application locale et après des temps variés, a été étudiée dans la peau excisée de l'homme, du rat, du lapin et du chat. Deux méthodes différentes ont été adoptées: 1 a) la détermination de la radioactivité de 25 strip-tests pris successivement à la surface de la peau humaine, 1 b) les autoradiographies de ces mêmes strip-tests et 2) les autoradiographies de coupes de peau, au moyen de 4 techniques différentes. Les divers facteurs susceptibles d'influencer les autoradiographies ont été étudiés, en prêtant spécialement attention aux artefacts provenant de la diffusion, au type de radiations, et au temps d'exposition du matériel photosensible. On a trouvé que le parathion pénètre dans une certaine mesure dans les follicules pileux et les glandes sébacées, mains on a pu conclure qu'il ne s'agit pas nécessairement de la voie principale d'absorption. Il existe aussi une augmentation de l'activité dans les couches sous-épidermiques qui rend l'absorption transépidermique plus facile. Ces résultats ont été discutés en portant spécialement attention à l'interprétation des autoradiogrammes et à l'utilisation de l'absorption percutanée du parathion en vue d'études ultérieures.

ZUSAMMENFASSUNG

Es wurde die Verteilung von P^{32}-markiertem Parathion (E 605, Diäthyl-4-nitrophenyl-thiononophosphat) in excidierter Haut von Menschen, Ratten, Kaninchen und Katzen in verschiedenen Zeitabständen nach der Excision untersucht. Zwei verschiedene Versuchsanordnungen wurden gewählt: 1 a) Bestimmung der Radioaktivität von 25 aufeinander folgenden, mit Hilfe eines Cellulose-Streifens vorgenommenen Abzügen der Hautoberfläche; 1 b) Autoradiographie der gleichen Abrisse; 2) Autoradiographie von Hautschnitten mit 4 verschiedenen Techniken. Verschiedene Faktoren, die die Autoradiogramme beeinflussen, wurden untersucht, wobei besonderer Wert auf Artefakte infolge Diffusion, Typ der Strahlung und Expositionszeit des photoempfindlichen Materials gelegt wurde. Es zeigte sich, dass Parathion zu einem gewissen Ausmass in die Haarfollikel und Talgdrüsen eindringt; aber es wurde geschlossen, dass dieses nicht notwendigerweise der Hauptweg der Resorption ist. Es fand sich eine gesteigerte Aktivität unterhalb der epidermalen Schichten; transepidermale Resorption ist ebenso wahrscheinlich. Die Ergebnisse werden diskutiert mit besonderer Berücksichtigung der Interpretation der Autoradiogramme und der Folgerungen für andere Untersuchungen der percutanen Resorption von Parathion.

RESUMEN

Se estudió la distribución en la piel del paration (E 605, o dietil 4-nitrofenil tiononofosfato) marcado con ^{32}P, en aplicaciones tópicas en períodos de tiempo variables, a piel escindida de hombre, rata, conejo y gato. Se han seguido dos caminos: 1 a) determinación de radioactividad en 25 tiras consecutivas de celulosa aplicadas a la superficie de la piel humana, 1 b) autorradiografía de dichas tiras; y 2) autorradiografía de secciones de piel utilizando cuatro diferentes técnicas. Se estudiaron varios factores que influyen los autorradiogramas, atendiendo especialmente a los artefactos debidos a la difusión, tipo de radiación y tiempo de exposición del material fotosensitivo. Se comprobó que el paratión penetra en cierto grado folículos pilosos y glándulas sebáceas, pero se sacó la conclusión de que éstas no constituyen forzosamente la principal vía de absorción. También había aumento de actividad por debajo de las capas epiteliales y la absorción transepidérmica es pues probable. Se discutieron los resultados sobre todo en la interpretación de los autorradiogramas y respecto a las deducciones para otros estudios de la absorción percutánea del paration.

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