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Note

Chromatography of melanogens from urine of hamsters with transplantable melanoma

I. Thormählen-positive melanogens

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The increased excretion of melanogens is one of the characteristic signs of malignant melanoma. Although several workers have reported the chromatographic behaviour of the so-called Thormälen-positive melanogens (TPM) in the urine of patients suffering from melanoma[1–8], nothing is known about the TPM in urine of animals with experimental melanoma. This paper describes the chromatographic behaviour of TPM in the urine of hamsters with transplantable melanotic melanoma and in the urine of hamsters without tumours.

EXPERIMENTAL

Collection of urine

Golden Syrian hamsters (*Mesocricetus auratus*) with transplantable melanotic melanoma (M-type, Bomirski, Poland) and hamsters without tumours were placed in metabolic cages and urine was collected during 24 h. The urine from individual groups of hamsters was pooled and stored at −20° for subsequent use.

Removal of urea

As the urine of hamsters contains a large amount of urea, it is necessary for it to be removed. One tenth of a volume of approximately 10% urease was added to the urine and the mixture was incubated at 40° for 2 h. During the incubation, the pH was adjusted to neutral with concentrated hydrochloric acid. The urine was then centrifuged (1500 *g*) for 15 min and the supernatant was evaporated under vacuum at 40° to half its volume.

Preparative paper chromatography of TPM

A 0.7–1.0-ml volume of the above supernatant from hamsters' urine was ap-

plied to Whatman No. 3 paper (width 23 cm, length 60 cm) and descending chromatography was commenced according to the method described by Anderson[3] (*n*-butanol–pyridine–water, 1:1:1). Both sides of the paper were treated in order to identify the position of TPM by the Thormälen reaction[5] or the Ehrlich reaction[9]. Each strip of chromatographic paper containing TPM was cut out and eluted with methanol for 1 h with continuous shaking. The eluates were evaporated to a small volume.

Chromatography on cellulose plates

Six solvent systems were used for the examination of the chromatographic mobility of TPM:

S1, collidine saturated with water;
S2, *sec.*-butanol saturated with water;
S3, *n*-butanol–pyridine–water (1:1:1);
S4, isopropanol–ammonia–water (20:1:2);
S5, *n*-butanol–acetic acid–water (12:3:5);
S6, isopropanol–ammonia–water (8:1:1).

Thin-layer chromatography (TLC) was performed on thin-layer plates of ion-exchange cellulose (DC-Fertigplatten, PEI-Cellulose F; Merck, Darmstadt, G.F.R.). For two-dimensional TLC solvents S3 and S6 were used. The spots were made visible by the Thormählen reaction[5], Ehrlich reaction[9], fluorindal reaction[10], reaction with ammoniacal silver nitrate and with a solution of barium chromate. For the last detection method, a solution of 0.01 *M* barium chromate in 0.5 *M* hydrochloric acid was prepared. For spraying with ammoniacal silver nitrate, a mixture (1:5) of 0.1 *N* silver nitrate solution and 5 *N* ammonia solution was prepared. It was found to be advantageous after the Thormählen reaction to spray the same chromatogram with Ehrlich reagent solution.

Administration of [2-^{14}C]-L-3,4-dihydroxyphenylalanine ([2-^{14}C]DOPA) to hamsters

In order to follow the distribution of carbon-14 in TPM in the urine of hamsters with transplantable melanotic melanoma and in healthy hamsters, [2-^{14}C]-DOPA (New England Nuclear, Boston, Mass., U.S.A.) was applied i.p. to both groups. Each hamster without tumours received $1.11 \cdot 10^5$ sec^{-1} (3 μCi) and hamsters with melanoma $2.96 \cdot 10^5$ sec^{-1} (8 μCi) of [2-^{14}C]DOPA. After the application of radioactive DOPA, the animals were placed in metabolic cages and collection of urine was commenced. The urines from both groups of hamsters were separately pooled and purification procedures were performed as described above.

Autoradiography

X-ray film was used for autoradiographic detection of TPM. Quantitative evaluation of the radioactivity on chromatographic plates was performed after scraping off the Thormählen-positive spots and after elution with liquid scintillator (SLS-31, Spolana).

RESULTS AND DISCUSSION

As shown in Fig. 1, three TPMs (X, Y and Z) were detected in the urine of hamsters with melanotic melanoma (solvents S1 and S3). The detection reactions

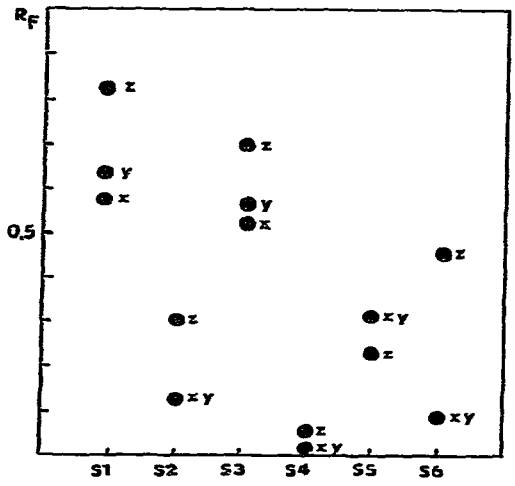

Fig. 1. Schematic representation of the separation of Thormählen-positive melanogens in six solvent systems. The numbers of the solvent systems correspond to those listed under Experimental.

are summarized in Table I. Although the chromatographic behaviour seems to be very similar for TPM X and Y, the Ehrlich and fluorindal detection reactions show considerable differences in these TPMs. In this connection, it should be noted that indoxyl glucuronides can give a positive fluorindal reaction[10].

TABLE I

COLOUR REACTIONS OF TPM

Reagent	*TPM*		
	X	*Y*	*Z*
Thormählen	Blue	Blue	Blue
Ehrlich	Pink	Violet → blue-grey	Violet?
Fluorindal	0	Red	?**
Ag^+*	0	0	?
Barium chromate	Blue → green	Blue → green	?

* Ag^+ = ammoniacal silver nitrate.
** ? = Unreliable for overlapping with indoxylsulphate; 0 = no colour reaction.

The evaluation of the colour reactions of melanogen Z is more difficult, as they are mostly obscured by those of indoxyl sulphate, which is present in large amounts in the urine of hamsters. The two-dimensional separation of the TPM of hamsters with transplantable melanoma and hamsters without tumours is shown in Fig. 2. The extracts of TPM of hamsters without tumours and those with melanotic melanoma were applied as a mixture; the same results were obtained when the extracts were applied separately. It seems that the differences in the extraction of TPM in these two groups of hamsters are only quantitative[11].

There is no doubt that melanin originates from tyrosine or 3,4-dihydroxy-

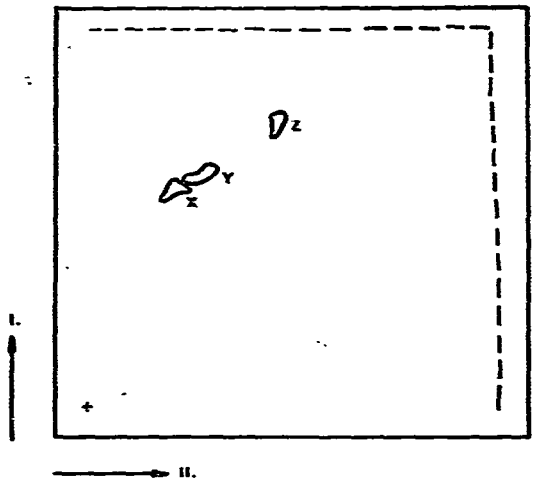

Fig. 2. Two-dimensional separation of Thormählen-positive melanogens from urine of hamsters with melanotic melanoma and without tumours. The sample was applied as a mixture of extracts of Thormählen-positive melanogens from both groups of hamsters.

phenylalanine. Although much information about the Thormählen-positive melanogens was collected, no proof of the origin of Thormählen-positive melanogens was obtained. To help to elucidate this question, the distribution of carbon-14 in the urine of hamsters after application of [2-^{14}C]DOPA was followed. Autoradiography and the quantitative measurement of radioactivity showed that all three Thormählen-positive melanogens contained carbon-14 (Table II). The detection of the chromatograms with diazotized *p*-nitroaniline showed that the spots of TPM did not overlap with those of Thormählen-negative melanogens. Nevertheless, for elucidation of the origin of Thormählen-positive melanogens, further studies are necessary.

TABLE II

RADIOACTIVITY OF TPM AFTER APPLICATION OF [2-^{14}C]DOPA

Hamsters	*TPM*	*Radioactivity (cpm)*
Without melanoma	Background	95.6
	X	979.5
	Y	1298.2
	Z	1386.6
With melanoma	Background	95.6
	X	3569.2
	Y	4188.1
	Z	2790.6

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