Short Communications and Preliminary Notes

IDENTIFICATION OF ISONICOTINOYLHYDRAZONES OF PYRUVIC AND α-KETOGLUTARIC ACID IN RAT URINE AFTER TREATMENT WITH ISONICOTINIC ACID HYDRAZIDE (ISONIAZID)

by

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During a quantitative study of the metabolic fate of isoniazid in albino rats by paper chromatography, three metabolites were detected and identified together with unchanged isoniazid (INI) in the urine of animals treated intraperitoneally or subcutaneously with a single dose of INI: these are isonicotinoylglycine (AINU), isonicotinic acid (AIN) and 1-isonicotinoyl-2-acetylhydrazine (AcINI)[1].

On the chromatograms of urine specimens, developed with *iso*propanol and water (85:15), besides the bands due to the four above-mentioned compounds, we noted two further spots giving a strong absorption in the short-wave U.V. light (Mineralight Lamp): the faster one had an R_F value a little higher than that of AIN, while the slower had a very low R_F. By subsequent treatment with benzidine and cyanogen bromide (CNBr) they gave a gray-green colour similar to that of INI, with a brilliant blue fluorescence in U.V. light, which increased when the chromatogram was left in an atmosphere of CNBr for several hours. With this treatment the presence of another metabolite was detected: while the colour of the AINU spot faded after prolonged contact with CNBr vapours, a blue fluorescent spot appeared at about the same place on the chromatogram. The presence of this metabolite was not always detected nor was its position definite nor constant.

The substances eluted from these bands and treated with *p*-dimethylaminobenzaldehyde according to the method of KELLY AND POET[2] gave a positive reaction for hydrazine; they were also endowed with a remarkable antitubercular activity when tested against the *M. tuberculosis* strain ATCC 607 by the agar diffusion method.

These results, together with the fact that the unknown spots were not detected when an acidic solvent was used as developer (*iso*amyl alcohol-acetic acid-water), led us to suppose that the three metabolites were isonicotinoylhydrazones. The chromatograms gave no coloured spots with aniline hydrogen phthalate[3] (negative sugar reaction), but when sprayed with a solution of 0.05% *o*-phenylenediamine in 10% aqueous trichloracetic acid and heated for 2 minutes at 100° C, a yellow colour, with a strong yellow fluorescence, appeared in correspondence to the three unknown bands (characteristic reaction of α-ketoacids)[4]. Such a fact induced us to examine the isonicotinoylhydrazones of the α-ketoacids involved in the KREBS cycle, and we found that pyruvic acid isonicotinoylhydrazone gives two spots with R_F values very close to those of two unknown bands, while the value of the third unknown spot corresponded to that of α-ketoglutaric acid isonicotinoylhydrazone. The two spots given by the pyruvic acid isonicotinoylhydrazone are probably due to two geometrical isomers (*syn-* and *anti-*).

Table I summarizes the R_F values of all urine metabolites compared with those of synthetic tandard compounds added to urine of untreated animals (control urine). A chromatogram is shown n Fig. 1.

The presence of α-ketoacids in the bands with R_F values of 0.06, 0.20 and 0.50, was confirmed after elution with 0.05 *M* aqueous sodium carbonate. The eluates were treated with 2,4-dinitrophenylhydrazine and the 2,4-dinitrophenylhydrazones (DNP-hydrazones) obtained were extracted and examined by paper chromatography according to the method of EL HAWARY AND THOMPSON[5] (using a mixture of *n*-butanol-ethanol-NH_3 0.5 *N* as solvent). The R_F values found are in agreement with those of DNP-hydrazones prepared in the same way from standard solutions of the two α-keto-acid isonicotinoylhydrazones and with those obtained after chromatography of standard solutions of pyruvic and α-ketoglutaric acid DNP-hydrazones.

The absorption spectra of the DNP-hydrazone spots, extracted with 1 *N* NaOH, are also in perfect agreement with those of the DNP-hydrazones, prepared from the synthetic isonicotinoyl-hydrazones, separated with paper chromatography, and eluted with 1 *N* NaOH. Absorption maxima are slightly shifted towards the short wavelengths when compared with the spectra of the DNP-hydrazones prepared by synthesis. This is probably due to the fact that a small quantity of dinitro-

phenylhydrazine, released during the extraction process, is present in the eluted compounds.

The presence of pyruvic acid in the eluates of chromatographic bands with R_F 0.20 and 0.50 and of α-ketoglutaric acid in the eluate obtained from the band with R_F 0.06, was confirmed by transforming the α-ketoacids into nitroquinoxalinol derivatives according to the method of HOCKENBULL[6,7] and by chromatography of these compounds with a mixture of *iso*amyl alcohol-ammonia-water.

TABLE I

R_F VALUES OF ISONIAZID METABOLITES OF RAT URINE, COMPARED WITH THOSE OF SYNTHETIC SUBSTANCES

Solvent: *iso*propanol-water (85:15); paper: Whatman 1; ascending technique.

Substances	*Color with benzidine + CNBr*	*RF values*	
		1	2
Acetyl isoniazid (AcINI)	blue	0.76 (± 0.03)	0.76 (± 0.03)
Isoniazid (INI)	gray-green (Fl + + +)	0.66 (± 0.03)	0.68 (± 0.04)
Pyruvic acid isonicotinoylhydrazone*	gray-green		
(Two spots) (INI P_2)	(Fl + +)	0.52 (± 0.03)	0.50 (± 0.03)
(INI P_1)		0.20 (± 0.03)	0.20 (± 0.03)
Isonicotinic acid (AIN)	purple	0.38 (± 0.03)	0.37 (± 0.03)
Isonicotinoylglycine (AINU)	violet-blue	0.22 (± 0.02)	0.23 (± 0.02)
α-Ketoglutaric acid isonicotinoylhydrazone* (INIKG)	olive-green (Fl + +)	0.06 (± 0.02)	0.06 (± 0.02)

1. Synthetic compounds added to control urine.

2. Urine metabolites. Urine samples were treated with a water solution of Na_2CO_3 (10%) in order to shift the pH to about 8; at this pH value pyruvic acid isonicotinoylhydrazone gives two well differentiated spots and α-ketoglutaric acid isonicotinoylhydrazone a single narrow spot.

(Fl). Intensity of fluorescence in U.V. light.

* Spotted as sodium salt.

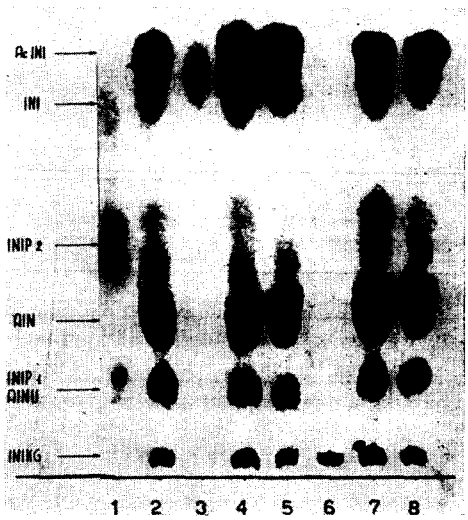

Fig. 1. Chromatograms of rat urine collected 4 hours after a single subcutaneous injection of isoniazid (100 mg/kg); Paper Whatman No. 1; ascending technique; *iso*propanol-water 85/15 as solvent. All the urine samples were treated with some drops of Na_2CO_3 10% solution (pH *ca.* 8). 1. Synthetic pyruvic acid isonicotinoylhydrazone added to urine of untreated animals (control urine); 2. Synthetic pyruvic isonicotinoylhydrazone added to urine of isoniazid treated animals; 3. Standard isoniazid added to urine control; 4. Synthetic pyruvic and α-ketoglutaric acid isonicotinoylhydrazones added to urine of treated animals; 5, 8. Urine of treated rats; 6. Synthetic α-ketoglutaric acid isonicotinoylhydrazone added to control urine; 7. Synthetic α-ketoglutaric acid isonicotinoylhydrazones added to urine of treated animals. AcINI = Acetyl isoniazid; INI = isoniazid; INI P_1 and INI P_2 = two spots given by pyruvic acid isonicotinoylhydrazone; AIN = isonicotinic acid; AINU = isonicotinoylglycine; INIKG = α-ketoglutaric acid isonicotinoylhydrazone.

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A NEW DEVICE FOR MICRORADIOGRAPHY AND A SIMPLIFIED TECHNIQUE FOR THE DETERMINATION OF THE MASS OF CYTOLOGICAL STRUCTURES

by

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Microradiography is increasingly becoming a tool of importance for several research fields such as biology, medicine, mineralogy and metallurgy. In the field of histochemistry quantitative microradiography has given new information on composition and function of cells and tissues[1].

The dry weight (mass) of a histological or cytological structure in a biological tissue can be determined from its capacity to attenuate extremely soft X-rays[2]. The method is based on measurement of the photographic density in a microradiogram of the specimen and a simultaneously microradiographed reference system. The microradiogram is registered on a fine grained photographic emulsion such as Lippmann emulsion, Kodak Maximum Resolution Plate or Eastman Kodak Spectroscopic Plates 548 or 649. The resolution of the best of these fine grained emulsions is more than 1000 lines per mm, but granularity varies from one batch to another. Quantitative microradiography permits the determination of the absorption of soft X-rays in cytological structures as small as a few microns in diameter. The theory for the cytological X-ray weighing procedure has been described elsewhere[2].

In order to calculate the dry weight of a cytological structure the microradiogram has to be recorded with extremely soft X-rays[1,2]. The first equipment for such weight determination worked with 8 A and softer X-rays and the specimen was in the high vacuum of the X-ray tube. The tube was evacuated during the exposure. Other types of apparatus for weight analysis have been described[3,4,5] and they all have in common relatively complicated X-ray tubes that have to be pumped continuously.

Recently, sealed-off miniature X-ray tubes with very thin Be-windows have been developed at Philips X-ray laboratories, Eindhoven. Earlier technical development of these tubes for therapeutic treatment has been described[6]. The purpose of this note is to show how a newly developed X-ray tube provides us with very simple equipment for quantitative historadiography. Fig. 1 shows a photograph of one the models of this small tube. It is provided with a Be-window 50 μ in thickness and has a 0.3 mm focal spot. This tube could be energized with max. 3 mA at 5 kV using a simple small high voltage generator. The sample-film holder (camera) is attached directly to the tube; the advantage is that the sample is outside the high vacuum of the tube. If necessary the camera can be evacuated. The output of this particular tube is such that the exposure time varies with the present technique between 2 and 20 minutes in the 2000 to 5000 volts range.

The resolution of the microradiographic technique using this sealed-off miniature tube is highly satisfactory. Fig. 2 shows an optical enlargement of the microradiogram of a silver grid kindly supplied by Dr. V. E. COSSLETT. The width of each bar is 3 μ and it is clearly demonstrated that structures even smaller than 0.5 μ are resolved. Of course this resolution is not reached for objects

Fig. 1. Laboratory model of a miniature X-ray tube. A, anode; C, cathode; W, window.
Fig. 2. Microradiogram of a silver grid. Each bar is 3 μ thick.
Fig. 3. Microradiogram of a microtome section of a blood vessel (2500 volts, 50 μ Be-window).
Fig. 4. Microradiogram of a thin ground section of bone showing the distribution of mineral salts (5000 volts, 200 μ Be-window).
Fig. 5. Microradiogram of a section of epiphyseal cartilage with beginning calcification (top of the fig.) of the cartilage cells (3000 volts, 50 μ Be-window).
Fig. 6 and 7. Microradiograms of sections from a rabbit ear and a pathological kidney. In Fig. 7 the individual cells of the tubuli are clearly seen (2500 volts, 50 μ Be-window).
Fig. 8. Microradiogram of a section through a kidney with pathological calcification (4000 volts, 200 μ Be-window).