We are most grateful to Dr A. T. Cowie, Dr F. H. Dodd, Dr S. J. Folley, and Dr A. S. Foot of the National Institute for Research in Dairying for providing the cows and taking the blood samples. This work was aided by a grant from the Agricultural Research Council.

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Metabolism of Polycyclic Compounds

10. ESTIMATION OF METABOLITES OF NAPHTHALENE BY PAPER CHROMATOGRAPHY*

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(*Received* 23 *September* 1955)

The examination by paper chromatography of the urine of rats dosed with naphthalene has shown that most of the metabolites of naphthalene could be detected in ultraviolet light. An advantage of this method is that urines can be examined without any previous chemical treatment. As quantitative elution from the paper chromatograms was difficult, the relationship between the spot area and amount of substances on paper chromatograms (Fisher, Parsons & Morrison, 1948) was investigated. Several authors have used this relationship to determine fatty acids (Reid & Lederer, 1951; Bryant & Overell, 1953), amino acids (Ohtsu & Mizuno, 1952) and indolyl-3-acetic acid (Bennet-Clark, Tambiah & Kefford, 1952). A method depending on spot areas similar to that used by Reid & Lederer (1951) for the estimation of fatty acids has been developed for the estimation of metabolites of naphthalene.

EXPERIMENTAL

Materials

1-Naphthol and 2-naphthol (A.R., British Drug Houses Ltd.) were each recrystallized from water before use. 1:2-Dihydronaphthalene-1:2-diol (m.p. 126°) was isolated from the urine of rats dosed with naphthalene by the method of Booth & Boyland (1949). 1-Naphthylsulphuric acid (potassium salt) was prepared by the method of Burkhardt & Lapworth (1926). 1-Naphthylglucosiduronic acid was isolated from the urine of rats dosed with 1-naphthol by the method of Berenbom & Young (1951).

1:2-*Dihydro-2-hydroxy-1-naphthylglucosiduronic acid.* A powder containing 80% of the barium salt of 1:2-dihydro-2-hydroxy-1-naphthylglucosiduronic acid was isolated from the urine of rats dosed with naphthalene. During the isolation of the triacetylmethyl ester of 1:2-dihydro-1-naphthylglucosiduronic acid Boyland & Solomon (1955) eluted 1:2-dihydro-2-hydroxynaphthylglucosiduronic acid from a charcoal column containing adsorbed metabolites of naphthalene with 50% aqueous acetone. The concentrated eluate was purified on a cellulose-powder partition column from which it was eluted with *iso*propanol–water (4:1). The fractions containing 1:2-dihydro-2-hydroxy-1-naphthylglucosiduronic acid were evaporated to low volume under reduced pressure, neutralized with $Ba(OH)_2$ solution and crystallized from 95% aqueous propan-2-ol. After a few days at 4° a solid separated which on recrystallization from 95% aqueous propan-2-ol yielded a white solid. Measurement of the light-absorption at 264 mμ. indicated that this powder consisted of the barium salt of 1:2-dihydro-2-hydroxy-1-naphthylglucosiduronic acid in 80% purity (based on $\epsilon_{max.}$ value of 1:2-dihydronaphthalene-1:2-diol component). Some of the powder was incubated at 37° with 10 Sigma units/ml. of bacterial glucuronidase (Sigma) at pH 6·0 for 5 hr.; the aqueous solution was extracted with benzene and the extract dried (Na_2SO_4). Evaporation of the benzene to small volume and crystallization from a benzene–light petroleum (b.p. 60–80°) yielded crystals of (−)-1:2-dihydronaphthalene-1:2-diol, m.p. 126°, $[\alpha]_D^{22} - 156°$ in ethanol (*c*, 0·5). The powder gave a strong naphtharesorcinol reaction and a precipitate of $BaSO_4$ with dilute H_2SO_4. Paper chromatography (solvent I, Table 1) showed that no other known metabolites of naphthalene were present.

* Part 9: Booth, Boyland & Manson (1955).

1:2-Dihydro-1-naphthylglucosiduronic acid was obtained in solution from the urine of rats dosed with naphthalene by the method of Boyland & Solomon (1955); the absence of any other metabolites of naphthalene was checked by paper chromatography. 1-Naphthylmercapturic acid was isolated from the urine of rats dosed with naphthalene by the method of Bourne & Young (1934).

Detection of metabolites on paper chromatograms

All the known metabolites of naphthalene can be detected with ultraviolet light (mainly 2536Å) with a Chance OX7 filter and a Hanovia Chromatolite. The metabolites possessing a 1:2-dihydronaphthalene structure can be seen as dark absorbent regions after drying the paper chromatograms. 1-Naphthol and 2-naphthol, and their ethereal sulphates and glucosiduronic acid derivatives, become fluorescent on exposure to NH_3, although small amounts of the glucosiduronic acids could not be reliably detected by this method.

1-Naphthol and 2-naphthol were detected by spraying chromatograms with a mixture of freshly diazotized 0·0005M *p*-nitraniline solution and ethanol (4:1). After the papers had dried at room temp. for 10 min. and the coloured acid *p*-nitraniline azo compounds were completely formed, they were converted into sodium salts by spraying with M-Na_2CO_3.

1:2-Dihydronaphthalene-1:2-diol, 1-naphthylsulphuric acid, 1-naphthylglucosiduronic acid and 1:2-dihydro-2-hydroxy-1-naphthylglucosiduronic acid (all acid-labile 1-naphthol precursors) were detected after acid hydrolysis on the paper chromatogram. The chromatogram was sprayed with a mixture of conc. HCl and ethanol (1:2) and heated in an oven at 70° for 5–10 min., when these substances gave mauve spots. The paper was then treated as for the free phenols with diazotized *p*-nitraniline. By this method 1:2-dihydronaphthalene-1:2-diol, 1:2-dihydro-2-hydroxy-1-naphthylglucosiduronic acid and 1-naphthylsulphuric acid yielded intensely coloured *p*-nitraniline azo compounds, whereas 1-naphthylglucosiduronic acid gave a faintly coloured azo compound.

The colours given by these metabolites of naphthalene are shown in Table 1. When the pink fluorescent spot from 1-naphthylmercapturic acid was sprayed with 5N-NH_3 soln., and heated at 60° for 2 min. an orange fluorescence was observed. A somewhat similar reaction was noticed by Patton, Foreman & Wilson (1949) when free amino acids were heated on paper. Estimations of 1-naphthylmercapturic acid were made by measuring the area of this orange fluorescent spot.

Chromatographic technique

Application of solutions to paper. The starting line was drawn 8 cm. from the end of each sheet of paper for downward development, and 1·5 cm. for those used for upward development. Four spots of each sample of urine were run alongside each other and the standard solutions (three different amounts of each metabolite) were applied near the centre of the paper. No spots were placed less than 5 cm. from the side of the paper, and only 15 or 16 spots were run on each sheet of paper (56 cm. × 45 cm.). The area of applied spots was about 1 cm.^{2}

As three of the metabolites of naphthalene are glucuronides which cannot be extracted into organic solvents without acidification, the urine had to be applied direct to the paper. The concentrations of metabolites of naphthalene in the urine of rats dosed with naphthalene were usually high enough to enable determinations to be made with 0·005–0·02 ml. of urine applied to the paper. To eliminate errors due to the effects of salt on the formation of the spots, all standard solutions were applied to the starting line on top of previously applied normal rat urine (volume approximately the same as the urine containing metabolites of naphthalene).

Solvent systems. The upper layers of the solvent mixtures given in Table 1 were used.

Measurement of spot areas. As the errors involved in the use of a planimeter have been quoted as ±2% (Fisher *et al.* 1948) and ±40% (Reid & Lederer, 1951), the error of the planimeter (Allbrit fixed-index pattern) was determined. Each area was measured ten times and the coefficient of variation (Table 2*a*) was as high as ±20% for small areas (0·5 cm.^{2}). To reduce this error the spot areas were magnified (about 20 times) on a photographic enlarger; this step would obviously be unnecessary if a more sensitive type of planimeter were used. Spots were outlined on the chromatograms with a sharp pencil and traced. The outlining

Table 1. *Colour reactions and R_F values of metabolites of naphthalene on paper chromatograms*

Direction of development (A, ascending; D, descending), paper and time are given in parentheses after solvents. Solvent systems: I, *n*-butanol–ethanol–water (17:3:20, by vol.) (D, Whatman no. 1, 16 hr.); II, 0·1N-NH_3 soln. (A, Whatman no. 3 MM, 2 hr.); III, *n*-butanol satd. with 2N-NH_3 soln. (D, Whatman no. 1, 16 hr.).

Compound	NH_3 and u.v. light	Diazotized *p*-nitraniline azo compound		R_F values in solvent system		
		Acid	Alkaline	I	II	III
1-Naphthol	Light blue	Orange	Blue	0·96	0·60	0·95
2-Naphthol	Dark blue	Pale green	Orange	0·96	0·53	0·95
1:2-Dihydronaphthalene-1:2-diol	Black	Orange*	Blue*	0·86	0·85	0·85
1-Naphthylsulphuric acid	Dark blue	Orange*	Blue*	0·69	0·95	0·51
1-Naphthylglucosiduronic acid	Dark blue	Orange*	Blue*	0·15	0·95	0·30
1-Naphthylmercapturic acid	Pink	—	—	0·55	0·95	0·37
1:2-Dihydro-1-naphthylglucosiduronic acid	Black (no NH_3)	—	—	0·22	0·95	0·23
1:2-Dihydro-2-hydroxy-1-naphthylglucosiduronic acid	Black	Orange*	Blue*	0·08	0·95	0·20

* Colours were produced after hydrolysis with ethanolic HCl as described in the text.

of the spots of unknowns and standards was carried out by the same observer. The tracings were then projected from a photographic enlarger at 20 times magnification. The magnified spots were outlined on paper and then measured with a planimeter.

Estimation of metabolites in urine

1-Naphthol and 2-naphthol in the urine of rats dosed with naphthalene were estimated by measurement of their separate spot areas on chromatograms as determined by fluorescence in the presence of NH_3 under ultraviolet light.

1:2-Dihydronaphthalene-1:2-diol, 1-naphthylsulphuric acid, 1-naphthylglucosiduronic acid and 1:2-dihydro-2-hydroxy-1-naphthylglucosiduronic acid were estimated on one paper chromatogram (solvent I) by measuring the spot areas of the acid azo compounds formed with diazotized *p*-nitraniline. 1:2-Dihydro-1-naphthylglucosiduronic acid was estimated by measuring the black absorbent area under ultraviolet light and 1-naphthylmercapturic acid by the area of the orange fluorescence described above.

RESULTS

Four observers tested the magnification method on one traced area (10 determinations); the human and the planimeter error by this magnification method is shown by a coefficient of variation of less than $\pm 0{\cdot}8$ % on an area which was 4 cm.^{2} before magnification (Table 2*b*).

Variation of the size of the applied spot

Berry, Sutton, Cain & Berry (1951) have indicated that the size of the original applied spot has an influence on the final spot areas of the developed chromatograms. This has now been investigated quantitatively by the use of metabolites with low, medium and high R_F values. Aqueous solutions containing 1:2-dihydronaphthalene-1:2-diol (10 μg.), 1-naphthylsulphuric acid (5 μg.) and 1:2-dihydro-2-hydroxy-1-naphthylglucosiduronic acid (20 μg.) were applied to the starting line so that the areas of the initial spots varied (no urine present). The paper was then developed with solvent I, and the spot areas were measured (Table 3). Results show that the areas of the spots after chromatographic development have a tendency to increase when the diameter of the applied spot of 1:2-dihydronaphthalene-1:2-diol and 1:2-dihydro-2-hydroxy-1-naphthylglucosiduronic acid was greater than about 1·6 cm., and when that of 1-naphthylsulphuric acid was greater than 1·3 cm. When the diameter of the applied spot was 3·2 cm. the spot areas of the glucuronide and diol after development were respectively 1·2 times and 1·4 times greater than the average values obtained from the smaller diameters of the applied spots; 1-naphthylsulphuric acid showed an increase of 2·2 times under these conditions. However, this effect is not relevant to the estimations of these metabolites as then the diameter of the applied spots is about 1 cm., and certainly not greater than 1·3 cm.

Variation in the chromatographic behaviour between sheets of paper

Although all determinations of a given metabolite of naphthalene were made on paper chromatograms in one tank, considerable variation in spot

Table 2. *Errors of planimetric measurement*

m = Standard error of the mean where $n=2$; V = coefficient of variation (standard error %) and n = number of determinations (10).

(*a*) *Planimeter errors in the measurement of small areas*

Mean area (cm.^{2})	$\pm\sigma m$	V
0·49	0·01	24·30
3·58	0·01	3·40
9·74	0·01	1·12
23·32	0·05	0·95
81·24	0·06	0·31

(*b*) *Reproducibility of the magnification method*

Observer	Mean area (cm.^{2})	$\pm\sigma m$	V
A	81·2	0·30	0·66
B	80·2	0·37	0·75
C	80·3	0·14	0·46
D	80·9	0·21	0·47

Table 3. *Influence of the size of the original applied spot on spot areas of a developed chromatogram*

The applied spots contained the same amount of metabolite in each case.

Applied spot		Magnified spot areas after development with solvent I (cm.^{2})		
Approx. diameter of spot before magnification (cm.)	Magnified area (cm.^{2})	1:2-Dihydronaphthalene-1:2-diol (10 μg.)	1-Naphthylsulphuric acid (5 μg.)	1:2-Dihydro-2-hydroxy-1-naphthylglucosiduronic acid (20 μg.)
1·1	18·2	42·7	93·1	24·4
1·1	20·8	42·7	82·7	28·0
1·2	24·9	43·0	88·4	25·4
1·3	27·2	41·0	92·7	27·8
1·6	40·4	42·5	114·5	26·8
1·9	55·8	51·3	117·2	32·6
2·8	122·7	55·0	157·2	33·6
3·2	165·7	54·5	196·9	38·1

areas on the chromatograms occurred between separate sheets of paper (cf. Bryant & Overell, 1953). The restriction on the possible number of spots that can be run on one sheet of paper would, in our view, decrease the precision with which a standard curve could be drawn from a very restricted number of observations more than it would increase the overall precision of the curve owing to the specific elimination of paper to paper variability. The method adopted for obtaining the standard curve (the linear regression line of best fit calculated from the pooled observations for each metabolite) does not eliminate this variable.

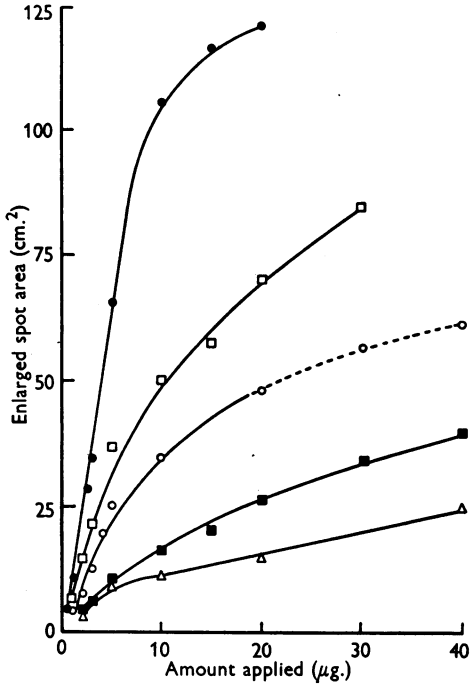

Fig. 1. Spot area–concentration curves of metabolites of naphthalene. ●, 1-Naphthylsulphuric acid; □, 1-naphthylmercapturic acid; ○, 1:2-dihydronaphthalene-1:2-diol; ■, 1:2-dihydro-1-naphthylglucosiduronic acid; △, 1:2-dihydro-2-hydroxy-1-naphthylglucosiduronic acid.

Spot area–concentration curves

Various amounts of each metabolite of naphthalene were applied to paper chromatograms and after development the spot areas were measured. Curves relating the amount of metabolite to its spot area are shown in Figs. 1 and 2. The limits to the amounts used were the amount that could be

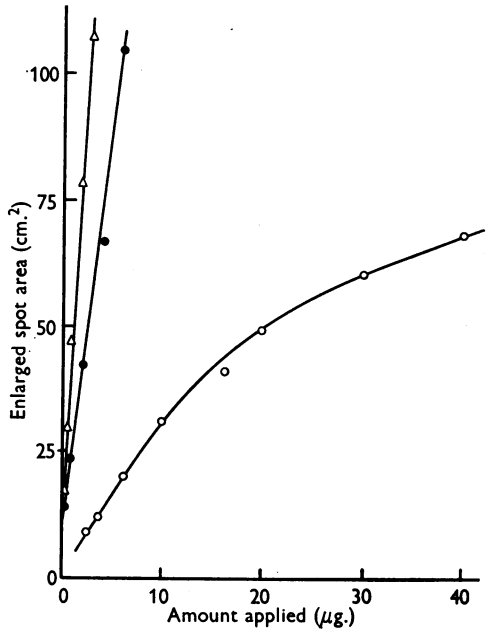

Fig. 2. Spot area–concentration curves of metabolites of naphthalene. △, 1-Naphthol; ●, 2-naphthol; ○, 1-naphthylglucosiduronic acid.

Table 4. *Constants of the regression lines of the form $y = a + bx$, with confidence limits, expressing the relationship between spot area and concentration of metabolites of naphthalene*

Metabolite	No. of determinations	Constants of the regression lines a	b	Standard error of estimate* (±μg.)	(±cm.²)	Minimum amount required for detection (μg./spot)	Concn. range (linear) for quantitative estimation (μg.)
1:2-Dihydronaphthalene-1:2-diol	48	−0·71	0·22	0·7	3·22	1	5–20
1:2-Dihydro-2-hydroxy-1-naphthyl-glucosiduronic acid	48	14·36	0·26	10·0	10·8	2	10–40
1:2-Dihydro-1-naphthyl-glucosiduronic acid	36	−0·19	0·23	1·0	4·24	2	5–20
1-Naphthylmercapturic acid	54	−2·11	0·10	0·4	4·20	1	5–20
1-Naphthol	24	−0·21	0·04	0·02	0·57	0·2	0·5–2·0
2-Naphthol	24	−0·38	0·04	0·01	0·30	0·2	0·5–2·0
1-Naphthylsulphuric acid	39	0·74	0·05	0·8	2·00	0·5	2·5–10
1-Naphthylglucosiduronic acid	48	2·87	0·29	1·1	3·82	1	5–20

* Ezekiel, 1947.

detected (Table 4) and the amount just before overloading and consequent streaking of the chromatogram. The standard curve of each metabolite for the purpose of estimation was chosen within a range where the relationship of spot area and concentration was substantially linear (Table 4). The amount of metabolites in the volume of urine applied to the paper (0·005–0·02 ml.) usually fell within these limits; but sometimes larger volumes of urine or a dilution was necessary to bring the amounts of metabolites present within the range of the standard curve.

The variation in spot area for a given amount of metabolite may be different for large and small amounts. As the standard error of estimation was calculated from the pooled spot areas given by three different amounts of metabolite, this error will apply over the whole regression line only if the coefficients of variation are the same for each different amount. The coefficients of variation at the three amounts of metabolite chosen to form each standard curve were measured by developing five spots at each of three amounts on one sheet of paper. The coefficients of variation of the spot areas for all the metabolites estimated were within the range $\pm$ 0·8–9·0 % except for low concentrations of 1-naphthylglucosiduronic acid (5 μg.), which was 26·0 %; this is due to the difficulty of detecting small amounts of this metabolite.

The standard curve used for the estimation was now obtained by calculating the linear regression line of best fit from the pooled observations made on each metabolite for the whole series of experiments. The equations for these lines, with the confidence limits (the standard error of estimate adjusted for the number of observations used in the calculation) are given in Table 4. The estimate was made from the mean of four estimates of the unknown and these standard curves.

DISCUSSION

This method enables a quantitative examination of naphthalene metabolism to be made. The great advantage of the method is that no previous chemical treatment of the urine is necessary before application to the paper so that acid-labile metabolites such as 1:2-dihydro-1-naphthylglucosiduronic acid can be measured. The spot area–concentration curves show a definite relationship between the amount of metabolite present in a spot and the spot area. Certain spot-area curves (e.g. those of 1:2-dihydronaphthalene-1:2-diol, 1-naphthylglucosiduronic acid and 1-naphthylmercapturic acid) show an exponential relationship to the amounts present over a wide range.

Generally the increase in spot area for increased amount of glucuronides is much lower than that for metabolites of naphthalene possessing higher R_F values. On the other hand, the very steep curves for 1- and 2-naphthol show a wide variation in spot area for small changes in amount present with consequent higher accuracy for the determinations of these phenols.

The confidence limits show that the method adopted is reasonably accurate for 1:2-dihydronaphthalene-1:2-diol, 1:2-dihydro-1-naphthylglucosiduronic acid, 1-naphthyl mercapturic acid, 1-naphthol, 2-naphthol, 1-naphthylsulphuric acid and 1-naphthylglucosiduronic acid; but that 1:2-dihydro-2-hydroxy-1-naphthylglucosiduronic acid cannot be estimated with any reasonable degree of precision.

SUMMARY

1. Some metabolites of naphthalene, including 1-naphthol, 2-naphthol, 1:2-dihydronaphthalene-1:2-diol, 1-naphthylsulphuric acid, 1-naphthylmercapturic acid, 1-naphthylglucosiduronic acid and 1:2-dihydro-1-naphthylglucosiduronic acid, can be estimated by the area of the spots which they form on paper chromatograms.

2. Confidence limits have been determined for the calculated regression lines of best fit relating spot areas with the amounts of each metabolite present.

This work was supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research, Royal Cancer Hospital) from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund, and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

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