Inhibition of p-*nitroanisole demethylation* in vitro. The demethylation of *p*NA to *p*-nitrophenol can be measured directly in a cuvette at 405 mμ in a photometer, thus allowing kinetic experiments. The incubation mixture was essentially the same as for amidopyrine demethylation except that the buffer was adjusted to pH 7·85 and the protein content was 0·5 mg/ml. Reaction velocity was measured by reading the optical density every minute. The data obtained were treated according to Dixon[15] (Fig. 2). Inhibition of *p*NA demethylation by disulfiram according to the reciprocal velocity–inhibitor plot was found to be competitive in nature. The inhibitor constant was calculated to be $0{\cdot}8 \times 10^{-5}$ M. Sodium diethyldithiocarbamate also inhibits competitively, the K_I being about one order of magnitude greater than that for disulfiram ($1{\cdot}3 \times 10^{-4}$ M).

The described results indicate that disulfiram under certain conditions inhibits the demethylation of amidopyrine *in vivo*. Incubation experiments with mouse liver microsomes demonstrate that this inhibition seems to be due to a competitive interference with the microsomal drug oxidizing enzymes.

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REFERENCES

1. N. O. KJELDGAARD, *Acta. pharmac. Tox.* **5**, 397 (1949).
2. W. D. GRAHAM, *J. Pharm. Pharmac.* **3**, 160 (1951).
3. M. GOLDSTEIN, E. LAUBER and M. R. MCKEREGHAN, *Biochem. Pharmac.* **13**, 1103 (1963).
4. J. M. MUSACCHIO I. J. KOPIN and S. SYNDER, *Life Sci.* **3**, 769 (1964).
5. O. V. OLESEN, *Acta pharmac. Tox.* **24**, 317 (1966).
6. T. C. BUTLER, *J. Pharmac. exp. Ther.* **119**, 1 (1957).
7. O. FOLIN and V. CIOCALTEU, *J. biol. Chem.* **73**, 627 (1927).
8. B. B. BRODIE and J. AXELROD, *J. Pharmac. exp. Ther.* **99**, 171 (1950).
9. K. J. NETTER and G. SEIDEL, *J. Pharmac. exp. Ther.* **146**, 61 (1964).
10. V. POPOVIC and P. POPOVIC, *J. appl. Physiol.* **15**, 727 (1960).
11. J. HALD, E. JACOBSEN and V. LARSEN, *Acta pharmac. Tox.* **5**, 179 (1949).
12. K. J. NETTER, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **238** 292 (1960).
13. J. AXELROD, J. REICHENTHAL and B. B. BRODIE, *J. Pharmac. exp. Ther.* **112**, 49 (1954).
14. K. J. NETTER, S. JENNER and K. KAJUSCHKE, *Naunyn-Schmiedebergs Arch. Pharmak. exp. Path.* **259**, 1 (1967).
15. M. DIXON, *Biochem. J.* **55**, 170 (1953).

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A spectrophotometric method for the estimation of the carcinostatic agent, 5-aziridino-2,4-dinitrobenzamide (CB 1954), in biological fluids

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IT HAS recently been shown that 5-aziridino-2, 4-dinitrobenzamide (CB 1954) has a highly selective tumour growth inhibitory action on the transplanted Walker rat carcinoma 256.[1, 2] Further studies of

the biological effects of this compound required a sensitive assay method which could be used to determine the concentration of active drug in various biological fluids.

Two main uses could be envisaged for any specific assay method. (1) The determination of the concentration of the drug in tissue extracts and fluids following various routes of administration with a view to establishing optimum dose regimes. (2) A comparison of the chemical assay with a biochemical assay (based on cytotoxic activity), of fluids from treated subjects which would indicate whether the drug was being converted into a more active metabolite.

A possible method of assay was suggested by the observation of a characteristic change in the ultra-violet absorption spectrum of solutions of CB 1954 on acidification. In neutral aqueous solution 5-aziridino-2, 4-dinitrobenzamide exhibits a broad absorption band with a maximum at 320 mμ (ϵ = 9750) and after acidification with dilute aqueous perchloric acid the spectrum changes with time and eventually reaches a steady state with a sharper maximum at 360 mμ (Fig. 1). The ratio of

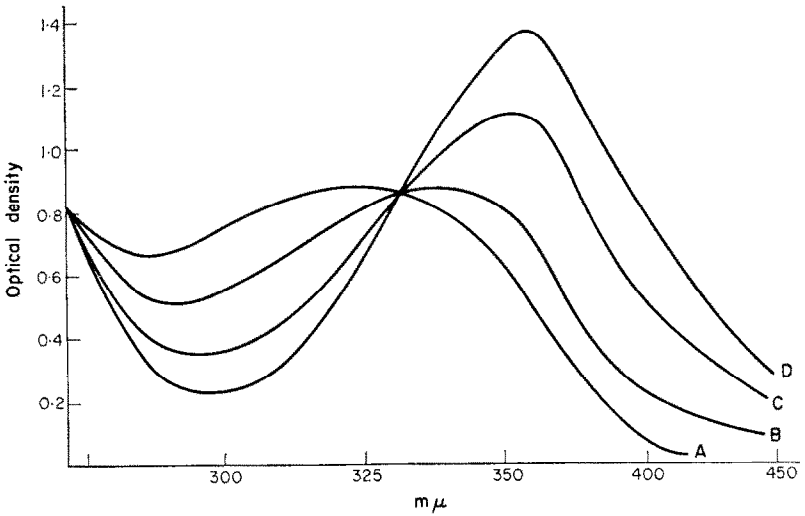
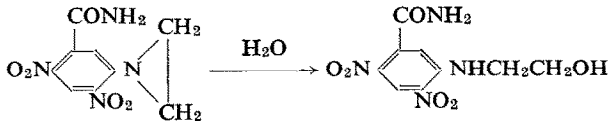


FIG. 1. Reference spectrum of CB 1954 in water (concentration = 30 γ/ml). A, Initial spectrum. B, 5 min after adding 0·05 ml N $HClO_4$ to 2 ml of solution. C, After 10 min. D, Steady curve after 30 min.

optical densities of the neutral and the acid peak is 1·62. It was decided to use the change in optical density at 360 mμ for the estimation of the drug since the change is due to the acid catalysed hydrolysis of the essential aziridine ring:



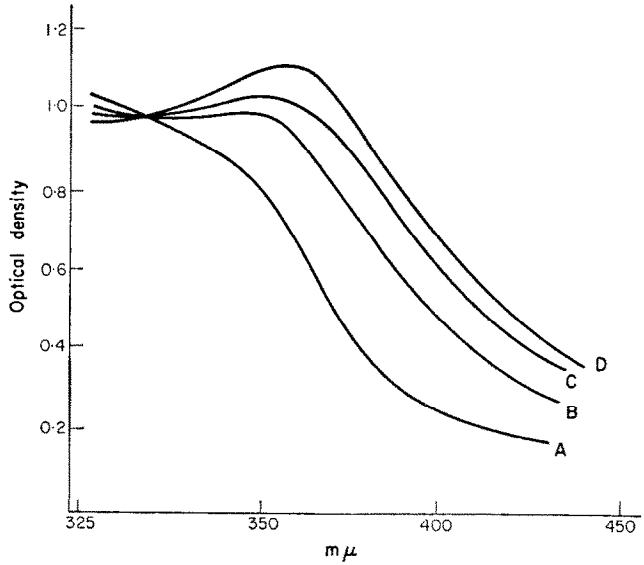
For subjects receiving doses of at least 10 mg/kg of CB 1954 it was convenient to use diluted aliquots of the fluid (urine or serum) but this technique was not sensitive enough when lower doses were administered. Following the finding that ethyl acetate effectively extracted the aziridine from fluids a more refined assay method was worked out and used for all subsequent estimations.

Methods

Estimation of CB 1954 *in diluted urine or serum.* 0·2 ml of the fluid was diluted to 2 ml with distilled water and the spectrum was determined using a Unicam SP 800 U.V. spectrophotometer with facilities for repeatedly scanning the region 250–400 mμ. The cell housing was thermostatted at 25°. When a steady reading had been obtained 0·05 ml of N aqueous perchloric acid was added and the spectrum was recorded at regular intervals. When the reading was again steady (about ½ hr) the change in optical density at 360 mμ (Δ_{360}) was read off.

libration graph had previously been obtained by adding known amounts of CB 1954 to urine ıtreated animals and determining the Δ_{360} values. It was thereby established that the concentra- the drug in the original fluid (in γ/ml) was given by dividing the Δ_{360} value of the diluted aliquot 32.

lid assay showed a progressive change in spectrum with time with a clear isobestic point at ι. Urine from untreated animals showed no such features. The absorption due to the drug is nposed on the background absorption of normal urinary constituents which does not alter ıe dependent manner on acidification except when there is an appreciable amount of blood in ıple. A typical assay is shown in Fig. 2. The Δ_{360} value here is 0·40, corresponding to a concen- of 0·40/0·0032 or 125 γ/ml of CB 1954 in the original sample.



Urine from rats receiving 10 mg/kg of CB 1954 (i.v. in 0·5 ml dimethylacetamide) diluted with water. A, Initial spectrum. B, Spectrum 5 min after adding acid. C, After 10 min. D, Steady curve after 30 min.

ive efficiencies of solvents for extracting CB 1954 *from aqueous solutions.* The partition coeffi- f CB 1954 between ether, benzene, chloroform and ethyl acetate on the one hand and water ɔther were determined as follows. Forty ml of an aqueous solution of CB 1954 containing was shaken with 20 ml of the solvent for 5 min and the partition coefficient (P) was calculated e expression:

$$P = \frac{2(A - A')}{A'}$$

ı is the initial optical density of the aqueous solution at 320 mμ and A′ is the optical density of ɔous phase after shaking with the solvent. The following values for the solvent/water partition ınts were obtained: ether, 0·50; benzene, 0·14; chloroform, 0·53; ethyl acetate, 9·1. It was ·e decided to use ethyl acetate as extracting solvent in the modified assay method.

ation of CB 1954 *in fluids (ethyl acetate extraction method).* A 10 ml sample of fluid was intro- ıto a stoppered tube. If an appreciable amount of sediment was present the fluid was centri- ıd the clear supernatant solution was used. Five ml of ethyl acetate was added and extraction cted by repeatedly inverting the tube for 5 min. Vigorous shaking produced emulsions which ficult to resolve. The mixture was centrifuged, if necessary, to obtain two distinct layers using apping rubber stopper in the tube. About 3 ml of the ethyl acetate layer was pipetted off and

dried over anhydrous sodium sulphate. Two ml of the dried extract was transferred to a stoppered cell and the spectrum was recorded at intervals to ensure that a steady reading at 25° was obtained. Then 0·05 ml of 0·1 N ethanolic perchloric acid (1 ml $HClO_4$, d 1·54, in 100 ml ethanol) was added, the solution was shaken, and the spectrum again recorded over the range 250–400 mμ until constant (about 15 min). The spectrum should not subsequently change on adding one drop of the same acid. The spectrum of CB 1954 in ethyl acetate shows a maximum at 320 mμ in neutral solution and 340 mμ in acid solution (isobestic point at 325 mμ) and so the change in optical density at 340 mμ (Δ_{340}) is determined in this instance.

A calibration graph previously obtained by assaying mixtures prepared as described above, showed that the concentration of CB 1954, in γ/ml, in the original fluid is given by dividing the Δ_{340} value by 0·035.

Results and discussion

Table 1 shows the results of assays of fluids from normal animals receiving CB 1954. The 1-hr specimen of urine from rats receiving 10 mg/kg (i.v.) contained 120 γ/ml of CB 1954. A bioassay on this urine against Walker tumour cells carried out by Dr. T. A. Connors, using a technique previously

TABLE 1. CONCENTRATION OF DRUGS IN FLUIDS FROM NORMAL ANIMALS RECEIVING CB 1954

Subject	Dose (mg/kg)	Route	Vehicle	Fluid	Time after admin. (hr)	Conc. of CB 1954 γ/ml
Rat	10	i.v.	DMA	urine	1	120*
(250 g)	..	..	..	..	2	115*
Rabbit	40	s.c.	DMSO	urine	24	180*
(3·5 kg)						
Pig	20	i.m.	DMSO	urine	2	80†
(50 kg)	..	..	..	serum	2	2·0†
	..	..	..	bile	2	1·5†

DMA, Dimethylacetamide.
DMSO, Dimethyl sulphoxide.
* Urine dilution method.
† Ethyl acetate extraction method.

described,[1] indicated that it had cytotoxic activity corresponding to a concentration of 100 γ/ml of 5-aziridino-2, 4-dinitrobenzamide. Thus practically all the carcinostatic activity contained in the urine could be accounted for by the CB 1954 present. This argues against there being a more active metabolite present in the urine.

As already reported[1] CB 1954 is concentrated in the urine of subjects since the uniform distribution of the drug in tissue fluids would result in concentrations of 10γ/ml (rat), 40γ/ml (rabbits) and 20γ/ml (pigs) (Table 1) whereas the urinary concentrations are 120, 180, and 80γ/ml respectively.

Table 2 shows the results of assays on urines of subjects with neoplastic disease. In the case of the dog an oral dose of 400 mg (13·3 mg/kg) gave the same urinary concentration as an intra-muscular dose of 100 mg (3·3 mg/kg). There is an indication that the same situation holds for the human patients for an oral dose of 100 mg to R. E. gives a similar urinary concentration of CB 1954 as an intravenous dose of 25 mg to J. F. This is not unexpected since aziridines are labile at the pH of gastric fluids.

Where serial samples have been taken it is clear that the drug is excreted quite rapidly: there being relatively little present in the 8 hr specimens. In a number of cases the total volume of urine passed was measured and it was possible to calculate the amount of active drug eliminated. This varied form between 1 and 5 per cent of the administered dose.

Conclusion

The spectrophotometric method described in this paper is convenient for determining the concentration of unchanged 5-aziridino-2, 4-dinitro-benzamide in various tissue fluids. There is no evidence

TABLE 2. CONCENTRATION OF DRUG IN URINES FROM PATIENTS WITH NEOPLASTIC DISEASE RECEIVING CB 1954

Subject	Condition	Dose (mg)	Route	Time after admin. (hr)	Urine vol. (ml)	[CB 1954] γ/ml*	% dose excreted
Samoyed dog	Bladder tumour	400	oral	3		9	
				6		26	
				20·5		5	
..	..	100	s.c.†	3	53	8·5	
				6	38	25	
				20·5	5	3	1·4
J.C. (male)	Bladder tumour	100	oral	24		< 1	
..	..	25	i.v.‡	¼	60	4	
				2	280	2	
				4	350	0·5	
				6	75	1	4·0
..	..	50	i.v.‡	2	114	8	
				4	78	3·5	
				6	83	1	
				8	73	1	2·4
J.F. (female)	Bladder tumour	10	i.v.‡	2		1·75	
				4		nil	
				6		nil	
				8		nil	
..	..	25	i.v.‡	2	60	8	
				4	70	6	
				6	60	5	
				8	45	2	5·0
..	..	50	i.v.‡	2	75	7·5	
				4	40	3	
				6	60	3	
				8	15	1	1·0
R.E. (male)	Hodgkins disease	100	oral	1	50	8	
				2	250	6	
				5	200	4	2·7

* Ethyl acetate extraction method.
† In 1 ml DMSO.
‡ In 1 part DMA, 9 parts isotonic saline.

that a more cytotoxic transformation product is present in the urines of treated animals. An oral dose of CB 1954 produces the same urinary concentration of drug as does one quarter of the same dose given by (i.v. or i.m.) injection.

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REFERENCES

1. L. M. COBB, T. A. CONNORS, L. A. ELSON, A. H. KHAN, B. C. V. MITCHLEY, W. C. J. ROSS and M. E. WHISSON, *Biochem. Pharmac.* **18**, 1519 (1969).
2. A. H. KHAN and W. C. J. ROSS, *Chemico-Biol. Interact.* (in press) (1969).

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A daily rhythm in the rate of depletion of brain norepinephrine by reserpine

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IN THE course of investigations using reserpine in the rat, variation in mortality rate with time of day was observed. Several regions of the rat brain are subject to a circadian* cycle in endogenous norepinephrine content,[1,2] and it has been observed that metabolism[3] and efficacy[4] of some drugs oscillate daily. This communication describes a circadian rhythm in the rate of depletion of brain norepinephrine by reserpine and reports an environmental factor capable of entraining the cycle.

Female, Sprague–Dawley (160-200 g) rats were housed in clear, plastic cages and provided with Purina chow and water *ad libitum*. The animals were subjected to 50–75 ft-c. of cool white fluorescent light from 5 a.m. to 7 p.m., except where otherwise noted, for 7 days prior to each experiment.

All drugs were administered in aqueous solution by the intraperitoneal route in a volume of 1 ml. Reserpine was obtained from Ciba. Alpha-methyl-paratyrosine (α-m-t) was kindly donated by Dr. Clement Stone (Merck, Sharpe & Dohme). Rats were killed at various times over a 24 hr period by cervical dislocation and tissues were immediately removed and frozen on dry ice. Catecholamines were isolated and determined by methods previously described.[5,6] No significant differences in either whole brain or heart endogenous norepinephrine was observed among control groups killed throughout the day. Consequently, the degree of depletion by reserpine in each experiment was computed on the basis of pooled data from controls killed along with treated groups over the experimental period. DL-7-^{3}H-norepinephrine was employed in the study of turnover in brain norepinephrine. The isotope (specific activity, 9·7 c/m-mole), obtained from New England Nuclear Corp., was purified before use by elution from alumina columns. After appropriate dilution with Elliot's "B" solution (Baxter), the tracer was administered by the intracisternal route[7] to rats anesthetized with ether. All rats received 0·5 μc ^{3}H-norepinephrine (9 ng) in a volume of 25 μl.

Groups of rats were treated with 1 or 2 mg/kg of reserpine throughout the day and killed, together with controls, 4 hr later. Reduction of whole brain norepinephrine varied with time of day. Maximal depletion was observed during the dark period (Fig. 1). Reduction of endogenous norepinephrine varied significantly at a dose of 1 or 2 mg/kg from a trough at 10 a.m. to 2 p.m., to a peak at 2 a.m. With a dose of 0·5 mg/kg, no significant norepinephrine rhythm was apparent (Fig. 1).

To determine whether the observed rhythm was restricted to the central nervous system or involved organs throughout the body, heart and salivary gland were examined. With doses of 0·5, 1·0 or 2·0 mg/kg of reserpine, no statistically significant rhythm in depletion of cardiac norepinephrine was observed. Depletion of salivary gland norepinephrine with reserpine showed no consistent rhythm.

To examine whether the observed rhythm reflected differences in the rates of norepinephrine

* The term circadian is here used to denote a rhythm with a period of 24 hr without implying either an endogenous or exogenous nature.