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CUMULATIVE EXCRETION OF SUCCINONITRILE IN MICE

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Abstract—Mice were given [^{14}C]succinonitrile intraperitoneally at a dose of 0·5 mg (1 μCi) per 20 g in single and multiple dose experiments. Cumulative excretion of total ^{14}C, and of four radioactivity fractions, two of which contained unchanged succinonitrile, and cyanide excreted as thiocyanate, were recorded. Unmetabolized succinonitrile was virtually absent after 24 hr, but metabolites persisted longer. Evidence for accumulation of cyanide was obtained. The possible nature of unidentified metabolites was considered.

A number of authors have investigated the metabolism and excretion of aliphatic nitriles. Stern *et al.* [1] reported a general belief that cyanide is liberated from malononitrile *in vivo*, and demonstrated cyanide production in rats *in vitro*. However, this reaction apparently occurred with only two examples from a further series of ten related compounds. In particular, cyanide-like effects, and cyanide production, were not observed with succinonitrile. In contrast, Meskow *et al.* [2] found small amounts of cyanoacetic acid and thiocyanate following succinonitrile administration to rats. Cavanna and Pocchiari [3] reported preliminary results on the metabolic fate of succinonitrile in mice, noting the production of cyanide as a metabolite, excreted as thiocyanate. Smith and Foulkes [4] showed that cyanide is generally excreted as thiocyanate. Conversion of cyanide to thiocyanate is catalysed by the enzyme rhodanese. Contessa and Santi [5] demonstrated release of cyanide from succinonitrile *in vivo* and *in vitro* in rabbits and rats. They estimated that 60 per cent of the dose was converted to cyanide and excreted as thiocyanate *in vivo*. These authors also referred to detection of cyanide in viscera of patients who had died during succinonitrile treatment, and recommended further investigation of the metabolism and excretion of the drug. The purpose of the present study was to investigate the excretion kinetics of succinonitrile and its metabolites in mice.

METHODS

Materials. Non-radioactive succinonitrile was obtained from Koch-Light Laboratories, Colnbrook, England. [1,4,^{14}C]succinonitrile was obtained from New England Nuclear Corporation, Boston, Mass., by courtesy of Professor M. Carissimi of Maggioni, Milan. The labelled material was 1·00 mCi as 1·47 mg of succinonitrile in benzene.

Injection solutions were prepared from 100 mg of unlabelled succinonitrile, plus a sufficient volume of the radioactive material to give a radioactive solution at 1 μCi per 0·1 ml, dissolved in water to a final volume of 20 ml.

Reagents were analytical grade, purchased from British Drug Houses, Poole, U.K., and Hopkin and Williams, Chadwell Heath, U.K.

Procedure. Brown, male mice, mean weight 30 g, were given intraperitoneal doses of the injection solution, 0·1 ml per 20 g, the succinonitrile dose being 25 mg/kg. The injected mice were stored in groups of 6–8, in metabolic cages designed to separate urine and faeces. The mice were given their normal diet throughout the experiment, which was conducted at room temperature (18–21°). In single dose experiments urine was collected at 2, 6, 24, 48 and 72 hr after the dose. Faeces were collected at 24 and 72 hr. Two multiple dose experiments were conducted. In the first, mice were given doses (25 mg/kg) of unlabelled succinonitrile daily for three doses. A fourth, radioactive, dose was then given, and urine was collected for 24 hr following this dose. The timetable for the second multiple dosing experiment was similar, but all four doses were radioactive, and urine was collected in each 24-hr period. Control experiments were carried out with mice given injections of water 0·1 ml/20 g.

Chemical analyses. Faeces were homogenized with three times their weight of distilled water, and solid material was allowed to settle out. Samples (0·1 ml) of urine or supernatants of faecal homogenates were taken for total radioactivity determinations. Further samples (0·1 ml) were examined as follows: (1) 0·1 ml of HCl (1 N) was added, and the mixture was extracted with 2 ml of chloroform (10 min mechanical shaking), the mixture was centrifuged, and a 1 ml sample of the chloroform layer was retained; (2) the mixture was further extracted with 2 × 2 ml of chloroform, and

2 ml of each chloroform layer was retained; (3) an aliquot of the 5 ml total chloroform extract (Fraction A) was examined for radioactive content; (4) the extraction was repeated on the residue (aqueous layer + 1 ml of chloroform) using three portions of water saturated amyl alcohol (1, 2 and 2 ml) yielding a total of 5 ml of combined extracts (Fraction B) (4·1 ml amyl alcohol and 0·9 ml chloroform) which was examined as for Fraction A; (5) 0·1 ml of a 23% aqueous solution of ammonium thiocyanate was added to the residue, followed by 0·4 ml of an 8% aqueous solution of ferric chloride (slight excess) to form ferric thiocyanate which was extracted as described for Fraction B into a total of 5 ml of water saturated amyl alcohol (Fraction C); (6) Fraction C was examined as for Fractions A and B and a 0·1 ml sample of the aqueous residue was also assessed (Fraction D).

Radioactivity estimations. Samples of the various fractions were assessed in a Tracerlab Corumatic 200 liquid scintillation spectrometer, using scintillation fluid containing: PPO 10 g, POPOP 0·25 g, naphthalene 100 g/litre in dioxane. Quench corrections were made by means of the channels ratios method and by introduction of internal standard aliquots of radioactive succinonitrile solutions into the experimental counting vials. All calculations were in terms of mass of labelled succinonitrile, at the specific activity of the injection solution.

RESULTS AND DISCUSSION

The fractionation process, which was essentially that of Cavanna and Pocchiari [3], resulted in 95 per cent of the unmetabolized succinonitrile being extracted into Fraction A. This was checked with authentic succinonitrile. Fraction B contained isoamyl alcohol extractable material plus 5 per cent of the unmetabolized succinonitrile, for which a calculation correction was made. Fraction C contained any thiocyanate present, plus 5 per cent of Fraction B for which a calculation correction was also made. Fraction D contained unextractable material. The nature of the materials in Fractions B and D remains to be determined.

In single dose studies, the injected radioactivity was lost quite rapidly from the body (Fig. 1). Almost 80 per cent was found in urine within 72 hr. A further 6·5 per cent was found in faeces. Assuming that excretion in urine and faeces represented the sum total of the excretory processes for succinonitrile, the loss of material was at first rapid with 60 per cent of the dose being lost by 24 hr, and later relatively slow, with 78 per cent by 48 hr, and 83 per cent by 72 hr.

The four excretion fractions showed a consistent pattern (Table 1). The proportion of the radioactivity detected as unmetabolized succinonitrile was highest early on and lowest later. Fractions B and C reached their maxima in the 2–24 hr, with some fluctuation. Fraction D reached its maximum at 72 hr. Since drug excretion generally occurs by first order kinetics, at

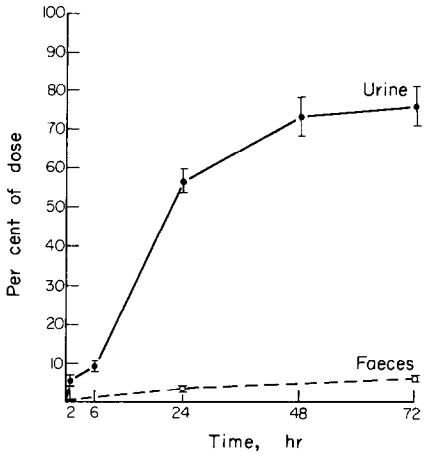

Fig. 1. Cumulative excretion of radioactivity in urine and faeces of mice given intraperitoneal doses of 0·5 mg succinonitrile/1 μCi per 20 g. Data are as per cent of administered dose. Each point is the mean ± S.E. from six groups of 6–8 mice.

rates dependent on the amount present in the body, it can be assumed that these relationships indicate that succinonitrile was almost entirely excreted unchanged or converted to metabolites in the first 24 hr. The highest rate was during the first two hours. Excretion of the metabolites took longer. The highest rate for cyanide was in the period 2–6 hr. From 48 hr onwards, excretion was principally of water soluble material, the excretion of which persisted beyond the excretion of cyanide. Thus the water soluble material presumably persisted in the body for longer than did the cyanide. Faeces tended to contain only cyanide and the water-soluble material.

In the first multiple dose experiment, in which mice received three unlabelled doses and then one of [^{14}C]succinonitrile, the mean 24-hr urinary radioactivity yield was 52 per cent, as opposed to 56 per cent

Table 1. Distribution of radioactivity from mouse urine and faeces into four fractions, following administration of [^{14}C]succinonitrile

Time after dose (hr)	Material	Fraction A	B	C	D
0–2	Urine	37	23	32	8
2–6	Urine	18	9	68	4
6–24	Urine	9	62	22	6
24–48	Urine	2	15	33	49
48–72	Urine	2	8	16	74
0–24	Faeces	2	3	13	82
24–72	Faeces	2	5	12	80

Figures are as percentages of the means from output data from six groups of 6–8 mice. Fraction A is unmetabolized succinonitrile. Fraction B is amyl alcohol extractable material. Fraction C is cyanide. Fraction D is water-soluble residue.

Table 2. Distribution of radioactivity from mouse urine following administration of [^{14}C]succinonitrile in four doses

Time (dosage period of 24 hr)	Fraction A	B	C	D
1	21	27	45	7
2	18	28	48	6
3	12	30	52	6
4	10	31	54	5

Figures are as percentages of daily output as means from a group of six mice. See Table 1 for identification of fractions.

following the single doses. These figures were not significantly different. The percent distribution of radioactivity among the fractions in the multiple dose experiment was: A 20; B 27; C 48; D 5. The percent distribution among the fractions in a 24-hr cumulative collection following single doses was: A, 22; B, 31; C, 41; D, 6. These data are clearly very similar, there being slightly less in Fraction B and slightly more in Fraction C, during multiple dosing, in accordance with reports of other workers [3].

In the second multiple dosing experiment, in which all doses were radioactive, in total amount 4·2 mg/day in six mice, the fact that approximately 50% of the body content of radioactivity was excreted in each 24-hr time period led to the recording of means of 1·6, 1·4, 2·7, and 3·3 mg yields over 4 days. A steady accumulation was thus revealed. This accumulation was obviously of metabolites, not of succinonitrile, shown by the distribution of the radioactivity amongst the four fractions. The proportion of succinonitrile fell steadily, although the amount remained constant (Table 2). This resulted from the fact that succinonitrile contributed almost nothing to urinary radioactivity at times beyond 24 hr, unless new doses were given. There was therefore no carry-over into later dosage periods. The corresponding increase was most obvious in regard to cyanide, as thiocyanate, again in accord with observations of other workers [3]. It seems that to a small extent, accumulation of cyanide occurs. Presumably something approaching a constant level would be reached at some point. There is however no evidence for any enzyme induction phenomenon, leading to unexpectedly increased cyanide levels. This possibility has been proposed by others [3].

At this stage it is only possible to speculate about the nature of the materials in Fractions B and D, but careful speculation is in order. There are two reactive centres in the succinonitrile molecule. One possible reaction is conversion of cyanide groups to carboxyl groups (II) (Fig. 2). Alternatively, the methylene groups can react, with loss of cyanide groups, to form diethylene cyanhydrin (III), or with conversion to aldehyde groups to form what has been called earlier a keto intermediate (IV). Compound IV can be hydrated to

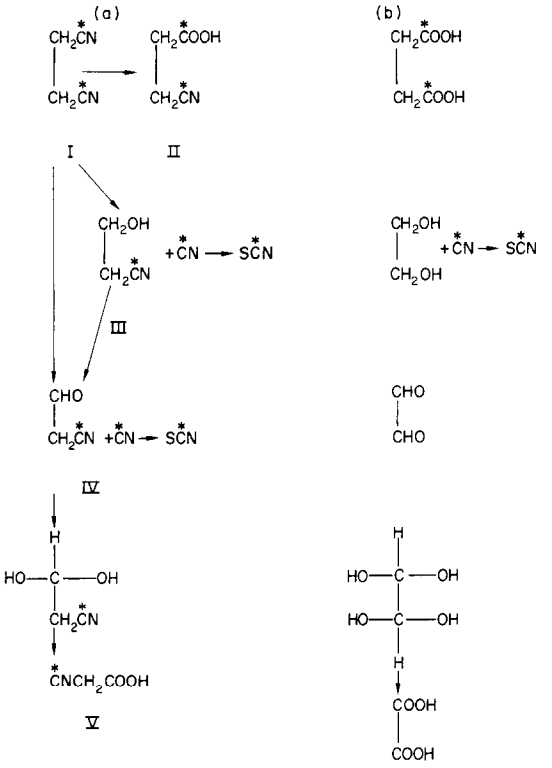

Fig. 2. Possible metabolic reactions of succinonitrile. An asterisk (*) indicates ^{14}C. (a) If one cyanide or methylene group is affected; (b) If two groups are affected.

form an unstable acetal (V) which spontaneously converts to cyanoacetic acid (VI), a known metabolite. Reaction of both of the cyanide or ethylene groups is possible [3].

Since cyanide (as thiocyanate) and two other radioactive compounds are formed, reaction of both ends of the molecule can be ruled out, as it would yield only two radioactive products. Since formation of cyanoacetic acid has been reported, albeit in rats, and since the acetal is unstable, it seems that the possibilities for the products in Fractions B and D are compounds II to V. Since the water soluble material occurs in greatest abundance at a time later than that for thiocyanate, Fraction D must contain materials from points further down the metabolic chain than those involved in cyanide production. Hence, Fraction D probably contains cyanoacetic acid. As regards Fraction B, it is most unlikely that compounds II and V would have greatly different polarities and extraction properties, so compounds III and IV as constituents of Fraction B are favoured. However, this speculation is no substitute for the obviously essential further experiments, of a different type from those of the present study, with a view to metabolite identification.

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