Biochemical Pharmacology, Vol. 18, pp. 693–700. Pergamon Press. 1969. Printed in Great Britain

ACUTE BIOCHEMICAL CHANGES IN RAT LIVER INDUCED BY THE NATURALLY OCCURRING AMINO ACID INDOSPICINE

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(*Received* 11 *June* 1968; *accepted* 11 *October* 1968)

Abstract—Preliminary observations have been made on the hepatotoxicity to female rats of indospicine (L-2-amino-6-amidino hexanoic acid) the toxic principle of the seed of *Indigofera spicata* Forsk, previously shown to be hepatotoxic to mice. A dose of 2 g/kg given by stomach tube produced a marked rise in the levels of liver triglycerides which reached a maximum at 24 hr and thereafter declined. Progressive enlargement of the liver occurred and continued even when, at 40 hr, liver lipid levels were falling. Although small increases in the liver content of protein, DNA and RNA were found, about 75 per cent of the increment in liver weight was due to water. The increase in water content was paralleled by an increase in cations, though the relative proportions of the cations to each other were unchanged. Liver ATP levels were not decreased. A significant inhibition of incorporation of leucine-1-^{14}C into liver and serum protein was observed at 5, 12, and 24 hr after treatment. At 40 hr incorporation was enhanced. The inhibition of protein synthesis preceded the rise in liver lipid.

THE POTENTIAL value of the legume *Indigofera spicata* Forsk as a convenient source of nitrogen in tropical pasture improvement has been limited by the hepatotoxic and abortifacient effects encountered in feeding trials under Australian conditions. Hegarty and Pound[1] reported the isolation of a toxic amino acid (L-2-amino-6-amidino hexanoic acid), which they named indospicine, from the seed. This reproduced in mice the fatty liver characteristic of feeding the seed to mice. Pearn[2] reported specific teratogenic effects in foetal rats after administration of a single dose of an extract of the seed to pregnant females, and noted that extracts of Indigofera have been used medicinally in man for various ailments.

Dosage variation made it difficult to obtain consistent analytical data from experiments in which seed was fed. Crude concentrates of seed contained excessive amounts of K, Ca and Mg. The isolation of an active constituent from the seed made it possible to study the effects of this component, but as the supply was limited and it was desirable to use the rat rather than the mouse in order to obtain adequate samples of blood and liver, it was decided to study the results of administration of a single dose. The present work is preliminary, but as even the seed is in short supply, a report appears justified. The indospicine content of the seed is about 1 per cent of the dry weight.

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MATERIALS AND METHODS

Indospicine was isolated from the seed of *Indigofera spicata* by a modification of the method of Hegarty and Pound.[1] The aqueous extract of the seed obtained by the adsorption-dialysis method was desalted on a column of "Dowex 50" cation-exchange resin at 1°. The organic bases were eluted from the resin with 2N triethylamine and the eluate was concentrated to a small volume at about 5° and adjusted to pH 5 with 4N HCl. Addition of flavianic acid to this solution at 80° resulted in the formation of the sparingly soluble monoflavianate. This was purified by repeated recrystallization from hot water and finally converted to the monohydrochloride. The sample used in this study was shown to be 97–98 per cent pure by analysis in an automatic amino acid analyzer; arginine was the main impurity.

Animals. Female rats of the Sprague–Dawley strain weighing 180–220 g, were used. Two control and 2 indospicine-treated rats were used at each time interval. Both groups were fed standard laboratory pellets but were deprived of food overnight before killing. Water was always available *ad libitum*. In the first series of experiments the treated animals received by stomach tube 2 g indospicine/kg body weight as a 20% (w/v) aqueous solution. At 5, 12, 24, or 40 hr after the dose of toxin, both the treated and control rats were injected via the tail vein with 100 μc/kg DL-leucine-1-^{14}C as a solution in 0·9% (w/v) NaCl. (The DL-leucine-1-^{14}C, from the Radiochemical Centre, Amersham, contained 34 mc/m-mole.) After 35–40 min, the animals were anaesthetized with ether. Blood was obtained by severing cervical vessels and the liver was rapidly removed. Samples were immediately weighed out and stored at − 20° for determination of triglycerides, RNA and DNA, protein, and incorporation of radioactivity into protein. The blood samples were allowed to clot at room temperature; then centrifuged to obtain serum. Aliquots from each serum sample were taken for determination of incorporation of radioactivity into protein.

Protein. Liver samples were homogenised in 9 vol. of ice-cold deionized water. A 0·5 ml aliquot (50 mg wet liver) was transferred to a centrifuge tube and protein precipitated by adding 0·5 ml 10% (w/v) trichloroacetic acid. The proteins were purified by repeated washing with hot (90°) and cold 5% trichloroacetic acid, ethanol-acetone (1/1) and ether as described by Radding and Steinberg.[3] To each 0·2 ml aliquot of serum was added 0·3 ml deionized water. Proteins were precipitated by the addition of 0·5 ml 10% trichloroacetic acid and purified as for liver protein. Finally, the liver and serum proteins were dissolved in 1·7 ml 12% (w/v) NaOH by warming at 60° for 30 min. After cooling, aliquots (1 ml) were taken for protein analysis by further addition of 4 ml biuret reagent.[4] Solutions of crystalline bovine serum albumin (Commonwealth Serum Laboratories, Australia) were used as standards. Other aliquots (0·5 ml) were transferred to counting vials, dried at 130° for 4 hr, extracted with 0·5 ml hydroxide of Hyamine 10-X (Packard) and 0·5 ml methanol at 37°.[3] After the addition of 6 ml 0·6% (w/v) 2, 5 diphenyloxazole (PPO) in toluene, radioactivity was measured with a liquid scintillation counter (Type 6012A, Isotope Developments Limited). Corrections for quenching were made by use of an internal standard.

Liver triglycerides. The method of Butler, Maling, Horning and Brodie[5] was used.

RNA and DNA. The extraction of nucleic acids and determination of RNA at 260 mμ were carried out as described by Munro and Fleck.[6] DNA was determined by the method of Ceriotti.[7] Standards were prepared from DNA (Sigma, Type 1: Sodium salt).

Liver ATP. The rats were killed 7 hr after receiving indospicine. During this period they were deprived of food. The liver extracts were prepared as described by Hyams, Taft, Drummey and Isselbacher[8] except that the homogenates in 0·1 M NaF were transferred to 250 ml Erlenmeyer flasks containing 50 ml boiling water and boiling was continued for exactly 10 min. ATP was assayed by the luciferin-luciferase method (C) described by Strehler.[9] Results were calculated from both internal and external standard solutions of ATP (Sigma), assayed similarly to the samples.

Liver cations. Tissue samples for the measurement of K^+, Mg^{2+}, Na^+, Ca^{2+} were prepared by the methods described by Judah, Ahmed, McLean and Christie.[10] K^+ and Na^+ were determined by flame photometry, Ca^{2+} and Mg^{2+} were determined by atomic absorption spectrophotometry (Techtron AA-100).

Liver water content. Thin slices were cut freehand, gently blotted, weighed (200–300 mg), dried at 105° for 24 hr, cooled in a desiccator and weighed again. The wet weight minus the dry weight enabled liver water to be calculated.

Liver total lipids. After extraction and purification by the method of Folch, Lees and Sloane-Stanley,[11] total lipids were determined gravimetrically.

RESULTS

Initial experiments were carried out with histological control to determine the optimum dose and route of administration of indospicine. Female rats were found to be more susceptible than males, and administration by stomach tube produced more rapid and consistent effects on the liver than subcutaneous injection, which was also complicated by congestion and oedema at the injection site. A dose of 1 g/kg body weight was effective in producing histological fatty liver. Rats given 5 g/kg body weight survived.

A dose of 2 g/kg body weight was considered optimum and no deaths occurred; pallor and some enlargement of the liver were first apparent visually in rats killed 16 hr after receiving this dose. A few focal necroses occurred in the inner parts of the lobules between 12 and 36 hr but extensive necrosis was never observed. The morphological changes will be reported elsewhere. Weight loss in the indospicine-treated groups only slightly exceeded that in the corresponding control groups.

An increase of triglyceride (Table 1) was established by 12 hr, reached a maximum at 24 hr and thereafter decreased. A larger dose of indospicine (5 g/kg body weight) did not accelerate the onset of triglyceride accumulation.

TABLE 1. EFFECT OF INDOSPICINE ADMINISTRATION ON LIVER TRIGLYCERIDE CONTENT

Duration of treatment	Liver triglyceride	
(hr)	Control	Treated
5	8·9 (29·6)	18·0 (53)
12	15·8 (47·9)	47·6 (165)
24	6·4 (20·5)	99·2 (476)
40	10·3 (34·5)	55·4 (288)

Values are expressed as mg/g wet wt. and, in parentheses, as mg/100g body wt. Methods as described in text.

Since several toxins which produce fatty liver also impair protein synthesis,[12] the *in vivo* incorporation was observed in rats treated with indospicine 5 hr, 12 hr and 24 hr previously, but after 40 hr incorporation in the treated rats was much greater than in controls. Incorporation into serum proteins was also depressed at 5, 12 and 24 hr and slightly increased at 40 hr.

Figure 1 shows the time relationships of the liver changes indicating that impairment of amino acid incorporation was established before an increase of fat was apparent, that the inhibition continued whilst fat was increasing (12 hr, 24 hr), and that clearance of the fat coincided with enhanced incorporation.

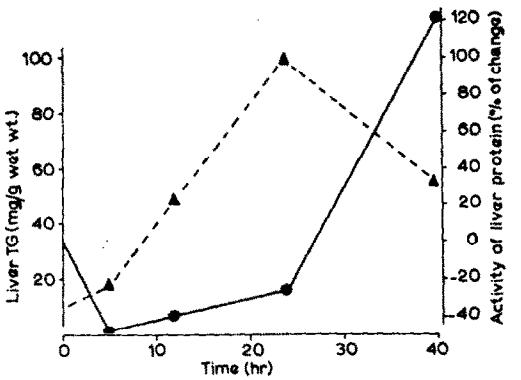

FIG. 1. Time relationship and degree of inhibition of incorporation of leucine-1-^{14}C into liver protein (●—●) and increase in liver triglyceride levels (▲—▲). Rats were treated with indospicine as described in text.

Liver DNA, RNA and protein content were estimated to ascertain whether the inhibition of amino acid incorporation could be accounted for by cell destruction (Table 3). Although an apparent fall of DNA (expressed per g wet weight) was observed, the total liver DNA was not decreased at any time. RNA levels showed similar behaviour so that at each time interval the RNA/DNA ratios were within the range of the control values. During the period of maximum inhibition of leucine incorporation the total liver protein content was little changed. At 40 hr the protein content of the livers of the indospicine-treated rats was higher than the control value.

TABLE 2. EFFECT OF INDOSPICINE ADMINISTRATION ON INCORPORATION OF DL-LEUCINE-1-^{14}C INTO LIVER AND SERUM TOTAL PROTEIN

Duration of treatment	Liver			Serum		
(hr)	Control	Treated	Per cent difference	Control	Treated	Per cent difference
5	575	308	− 47	558	202	− 64
12	575	338	− 41	558	123	− 78
24	651	401	− 38	840	219	− 74
40	230	427	+ 81	105	126	+ 20

Values are expressed as specific activities (cpm/mg protein) methods as described in text.

TABLE 3. EFFECT OF INDOSPICINE ADMINISTRATION ON LIVER DNA, RNA AND PROTEIN

Duration of treatment (hr)	No. of rats	DNA* (mg)	RNA* (mg)	Protein† (mg)
0	8	2·67 ± 0·26 (8·2 ± 0·6)	5·81 ± 0·31 (19·0 ± 1·6)	197 ± 3 (633 ± 17)
5	2	2·90 (8·6)	5·92 (17·6)	189 (560)
12	2	2·69 (9·4)	5·67 (19·5)	172 (594)
24	2	1·85 (8·9)	4·50 (21·6)	160 (766)
40	4	1·95 ± 0·30 (9·9 ± 1·4)	4·65 ± 0·33 (23·6 ± 1·2)	144 ± 2 (805 ± 24)‡

Results are expressed as mg/g wet weight of liver, and, in parentheses, as mg liver protein/100 g body wt.
* Data represent means + S.D.
† Data represent means + S.E.
‡ $P < 0·01$.
Methods as described in text.

Since indospicine is an atypical amino acid, and the abnormal amino acid ethionine also causes fatty liver and decreased protein synthesis, which are probably consequent on a decreased ATP level resulting from adenine nucleotide sequestration,[13] the effect of indospicine administration on liver ATP was determined. At 7 hr, when amino acid incorporation was already inhibited and triglyceride levels had started to rise (Fig. 1) ATP levels were normal (0·86 μmole/g wet wt.; control value 0·82 μmole/g wet wt.).

The increase of liver weight was apparent at 24 hr. The increased amount of triglyceride in the liver at 24 hr (476 mg/100 g body wt., Table 1), did not account for the whole of the weight increment (1·58 g, Table 4). At 40 hr the enlargement of the liver was maintained although the triglyceride content was less than at 24 hr, accounting for only 14 per cent of the weight increment.

TABLE 4. EFFECT OF INDOSPICINE ADMINISTRATION ON LIVER WEIGHT

Duration of treatment	Liver wt. (g/100 g body wt.)	
(hr)	Control	Treated
5	3·32	2·97
12	3·04	3·45
24	3·21	4·79
40	3·23	5·19

Methods as described in text.

The ability of indospicine to cause liver enlargement was confirmed in a second series of experiments, performed at a reduced dose level (1 g/kg body wt., by stomach tube). The changes occurred more slowly, but were similar in degree (see fat content, Table 5). All the main constituents of the cytoplasm (water, cations, fat, non-fat solids) increased, but the increase of the water and cations exceeded that of the NFS. The fat began to fall from its peak level whilst the levels of the other components were still maintained. At all time intervals the cation level was closely related to the total liver water, and was similar to, and slightly below the control value. The distribution pattern of the individual cations was normal.

DISCUSSION

The purpose of this study was to make initial observations on a new toxin. It is obvious that further work is required to explain the primary mechanism of action and its secondary consequences. It would be of interest to know: (a) the factors involved in the inhibition of leucine incorporation, (b) whether the fatty change is related to (a), and (c) the factors leading to liver enlargement.

In regard to (c) some conclusions can be drawn. The enlargement was not regenerative, because cell destruction was slight, and the enlargement was in excess of normal. Even when fat accumulation had reached a maximum, lipid was not the principal component of the increase of liver weight. Water contributed most to the weight increment, and was somewhat in excess of solids (enlarged liver 72 per cent water, control liver 67 per cent water). Liver protein, nucleic acid and cation levels

TABLE 5. EFFECT OF INDOSPICINE ADMINISTRATION ON LIVER COMPOSITION

Duration of treatment (hr)	Liver wt. (g/100 g body wt.)	Liver total non-fat solids (g/100 g body wt.)	Liver tri-glyceride (g/g non-fat solids)	Liver water (g/g non-fat solids)*	Liver total lipid (g/g non-fat solids)*	Liver cations							
						μmole/g non-fat solid				μmole/g liver water			
						K^+	Na^+	Ca^{2+}	Mg^{2+}	K^+	Na^+	Ca^{2+}	Mg^{2+}
0	3·18	0·86	0·04	2·49 (2·14)	0·20 (0·18)	373	84	4·1	46	141	33	1·6	18
20	4·47	1·07	0·12	2·93 (3·13)	0·27 (0·28)	404	80	3·4	39	141	28	1·2	14
30	5·43	1·09	0·42	3·45 (3·76)	0·53 (0·58)	450	116	5·6	44	131	33	1·6	13
45	5·13	1·11	0·18	3·34 (3·67)	0·32 (0·35)	444	112	3·8	46	131	33	1·1	14

The data were derived from a series of experiments in which the treated rats received 1 g/kg body wt. of indospicine by stomach tube. The control group comprised 3 rats, the group at each time interval comprised 2 rats.

* Values in parentheses represent g/100 g body wt.

were all increased relative to body weight, despite the slight excess of water on a wet weight basis.

The cation levels indicated that selective accumulation had occurred, related to the increase of liver water, and maintaining the normal proportions of the cations relative to each other. Adequate ATP levels were available to enable energy-requiring cation transport to function normally. It was concluded that the increase of liver weight was not due to a disturbance of cation transport, which would have caused Na^+ and Cl^- to accompany the water, but rather that the cation pattern was adjusted to a new water content. Since water is not independently transported into or out of cells,[14] the increase of cell water and cations suggests an increased intracellular content of osmotically active units with anionic properties.

Acknowledgements—This work was made possible by financial support from The Wellcome Trust and the Trustees of the Sir A. E. Rowden White and Edward White Fund (University of Melbourne).

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