The Hepatotoxic Action of Allyl Formate

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The hepatotoxic action of allyl formate on rat liver has been investigated. Biochemical changes can be detected in the liver cell many hours before the histological changes and it would appear that the toxin has a direct action on the liver parenchymal cell. The results suggest that allyl formate is not the toxic agent but that it is converted via allyl alcohol into acrolein. This reaction requires the presence of alcohol dehydrogenase. Histochemical studies have shown that this enzyme is localized in the periportal region of the liver lobule, and may explain why allyl formate solely produces a periportal necrosis. As glutathione and 1,4-dithiothreitol protect against the early biochemical changes produced by the poison, it is probable that acrolein alkylates proteins and nucleic acids.

In the nineteenth century it was observed that workers engaged in the industrial production of allyl alcohol developed toxic symptoms. Miessner (1891) showed that the inhalation of allyl alcohol was toxic to white mice, and Piazza (1915) showed that a hepatic periportal necrosis developed after subcutaneous administration of allyl formate to mice, rabbits, cats and dogs. Eppinger, Kaunitz & Popper (1935) studied the effect of allyl formate on dog's liver; they considered that it was a specific capillary poison and that the liver necrosis was secondary to endothelial damage. Heinemann (1937) considered that the liver lesion preceded the vascular damage in rats and rabbits. Rosin & Doljanski (1946) reinvestigated the hepatotoxic effect of allyl formate and concluded that the site of the primary damage was at the capillaries, but their results were not sufficient to exclude a direct action on the parenchymal cell.

Since the histological approach had not yielded clear evidence on the site of action of allyl formate it was considered that the problem might be resolved by biochemical methods. In the present investigation the early effects of allyl formate on hepatic protein synthesis have been examined. It appears that allyl formate itself is not the toxic agent but that it is probably metabolized to acrolein. The localization of the pathological lesion can be explained in terms of this metabolic pathway.

METHODS

Animals. Male albino rats weighing approx. 150g. and fed on stock diet M.R.C. 41B without restriction were used throughout.

Treatment of animals. Allyl formate was dissolved in

0.9% NaCl (0.015 ml./ml.) and the solution was administered intraperitoneally at a dose rate of 0.075 ml. of allyl formate/kg. body wt. Control rats received 0.9% NaCl.

Chemicals and reagents. Allyl formate was obtained from Eastman Organic Chemicals, New York, N.Y., U.S.A., phosphoenolpyruvate, pyruvate kinase and yeast alcohol dehydrogenase were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, and allyl alcohol, acrolein and acrylic acid were from British Drug Houses Ltd., Poole, Dorset. Cytochrome *c* and ADP were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A.

Preparation of tissue fractions. (1) Mitochondrial preparations for respiration studies were isolated from 10% (w/v) liver homogenates in 0.25M-sucrose by the method of Schneider (1948).

(2) Liver homogenates, postmitochondrial fractions and microsomes for amino acid-incorporation experiments were prepared by homogenizing the liver in twice its weight of a medium containing MgCl₂ (10 mM), KCl (25 mM), tris-HCl buffer, pH 7.8 (35 mM), and sucrose (0.15 M) (Rendi & Hultin, 1960). The homogenate was centrifuged for 10 min. at 10000g to remove cell debris, nuclei and mitochondria, leaving the 'postmitochondrial fraction'. For the preparation of microsomes the supernatant was centrifuged in a Spinco model L preparative centrifuge (no. 40 rotor) for 50 min. at 105000g. After the supernatant had been decanted the microsomal pellet was resuspended in the initial preparative medium to give a final volume of 0.4 ml./g. wet wt. of liver.

Respiration studies. Mitochondrial respiration and oxidative phosphorylation were studied in a Clark-type oxygen electrode by the procedure of Clark, Greenbaum & Slater (1965).

Radioactive experiments. DL-[1-¹⁴C]Leucine was obtained from The Radiochemical Centre, Amersham, Bucks. Its specific activity was 36.6 mc/m-mole.

Incorporation of DL-[1-¹⁴C]leucine into the proteins of liver slices. Liver slices (total about 200 mg.) were incubated in 3 ml. of Krebs-Ringer phosphate at 37° in Warburg flasks, the gas phase being oxygen. After 5 min. equilibration the

abelled compound was added from the flask [$1.0\mu\text{C}$ in 0.2ml. of Krebs-Ringer phosphate (Umbreit, Burris & Stauffer, 1959)]. The reaction was stopped with 20% (w/v) trichloroacetic acid (2ml.) and the protein extracted by the method of Rees & Rowland (1961).

Incorporation of [^{14}C]leucine into liver proteins *in vivo*. Control and poisoned rats were given a caudal-vein injection of 0.9% NaCl (0.2ml.) containing DL-[^{14}C]leucine ($100\mu\text{C/kg.}$ body wt.). After 15 min. the animals were killed and the livers removed and homogenized in 0.25M -sucrose. Portions of the tissue suspension were treated with an equal volume of 20% (w/v) trichloroacetic acid and the precipitated proteins were washed and dried by the method of Rees & Rowland (1961).

Incorporation *in vitro* of DL-[^{14}C]leucine into proteins of the homogenate and of the postmitochondrial fraction. Each incubation tube contained DL-[^{14}C]leucine ($1\mu\text{C}$), ATP ($2\mu\text{moles}$), phosphoenolpyruvate ($10\mu\text{moles}$) and pyruvate kinase ($100\mu\text{g.}$), to which was added 0.7ml. of the homogenate, 0.7ml. of the postmitochondrial supernatant, or 0.4ml. of the resuspended microsomes and 0.3ml. of final supernatant. The final volume was 1.5ml. , the gas phase was air and incubation was carried out at 37° for 20 min. The reaction was stopped with an equal volume of 20% (w/v) trichloroacetic acid and the proteins were extracted as described above for liver slices.

Preparation of dried protein residues for measurement of radioactivity. The protein residue was ground in a pestle and mortar and then compressed with a stainless-steel pestle on a planchet of 1cm.^2 surface area. All samples were counted for 5 min. in an end-window Geiger-Müller counter (type EHM 2/5; General Electric Co.). The results were corrected for background and adjusted for infinite thickness.

Chemical determinations. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Histochemical demonstration of alcohol dehydrogenase in normal albino-rat liver. The rat was killed with chloroform and a portion of the middle lobe of the liver removed, embedded in aq. 5% (w/v) CM-cellulose and frozen on to a metal chuck at -20° for use in the cryostat. The incubating medium was made up in 0.05M -phosphate buffer, pH 7.0, containing ethanol (0.05M), NAD (0.5mM), NaCN (5mM) and MgCl_2 (2mM), with nitro-blue tetrazonium (0.2mg./ml.) as final electron acceptor as described by Hess, Scarpelli &

Pearse (1958). The final pH was adjusted to 7.0 with 0.1N-HCl and the solution warmed and filtered. Cryostat sections about 6μ thick were cut, picked up on coverslips, dried for 30 sec. and placed in the incubation medium at 37° for 10–35 min. The sections were washed and mounted in water-mounting medium for inspection.

RESULTS

The previous findings of Rosin & Doljanski (1946) that rats killed 24 hr. after an intraperitoneal injection of allyl formate (0.075ml./kg. body wt.) had a severe hepatic haemorrhagic periportal necrosis have been confirmed. In contrast, at 1 hr. after poisoning no histological changes were observed in the liver. Thus any early disturbances of biochemical activities, thought to be mainly localized in the hepatic parenchymal cells, might indicate that the poison was having a direct action on these cells.

The effect of allyl formate on protein synthesis was examined. Slices and homogenates prepared from the livers of control rats and those poisoned with allyl formate were incubated *in vitro* with [^{14}C]leucine. The results (Table 1) show that preparations from the livers of poisoned rats had a reduced ability to incorporate the amino acid into the liver proteins. There was a similar inhibition of amino acid incorporation into liver proteins in the poisoned rat *in vivo*. Similarly, addition *in vitro* of allyl formate to preparations from the livers of control rats inhibited the incorporation of amino acid into liver proteins (Table 2).

It was observed in the experiments *in vitro* (Table 2) on incorporation of amino acid that the postmitochondrial fraction was considerably less sensitive to the addition of allyl formate than was the liver slice or homogenate. One explanation of this could be that the toxic agent was a metabolite of allyl formate and that in the preparation of the postmitochondrial fraction the system metabolizing

Table 1. *Incorporation of [^{14}C]leucine in vitro and in vivo into liver proteins of rats poisoned with allyl formate*

The results of each experiment are the mean of duplicates from either two control or two poisoned rats. The rats were killed 30 min. after poisoning. The experimental conditions and incubation medium for the experiments *in vitro* are given in the Methods section. Postmitochondrial fractions were incubated for 20 min. and slices for 60 min. The conditions for the incorporation *in vivo* are also given in the Methods section.

Expt. no.	Type of incorporation	Treatment of rat	Tissue preparation	Counts/min. at infinite thickness	Inhibition (%)
1	<i>in vivo</i>	None	—	305	—
	<i>in vivo</i>	Allyl formate	—	77	75
2	<i>in vitro</i>	None	Postmitochondrial fraction	1453	—
	<i>in vitro</i>	Allyl formate	Postmitochondrial fraction	582	60
3	<i>in vitro</i>	None	Slices	1625	—
	<i>in vitro</i>	Allyl formate	Slices	740	55

Table 2. *Effect of allyl formate in vitro on the incorporation of [¹⁴C]leucine into the proteins of liver preparation*

Tissue preparations from the livers of control rats were prepared as described in the Methods section, as were the conditions of incubation. The results are the mean of duplicate samples.

Type of preparation	Duration of experiment (min.)	Final concn. of allyl formate (mM)	Counts/min. at infinite thickness	Inhibition (%)
Homogenate	20	0	320	—
Homogenate	20	1.0	66	80
Postmitochondrial fraction	20	0	402	—
Postmitochondrial fraction	20	2.6	425	Nil
Postmitochondrial fraction	20	4.6	323	21
Postmitochondrial fraction	20	18.0	98	77
Slices	60	0	1735	—
Slices	60	0.5	1131	30
Slices	60	1.0	350	84

Table 3. *Effect of NAD on the inhibition by allyl formate and allyl alcohol of incorporation of [¹⁴C]leucine into protein by a postmitochondrial fraction of rat liver*

The postmitochondrial fraction was prepared from the liver of a control rat. The incubation period was 20 min. The results are the mean of duplicate samples.

Addition (final concn.)	Counts/min. at infinite thickness	Inhibition (%)
None	968	—
NAD (70 μ M)	1010	Nil
Allyl formate (6 mM)	588	39
Allyl formate (6 mM) + NAD (70 μ M)	71	93
Allyl alcohol (6 mM)	887	8
Allyl alcohol (6 mM) + NAD (70 μ M)	178	82

allyl formate had been largely removed. It seemed likely that the metabolism of allyl formate would be to the alcohol followed by an NAD-dependent oxidation to the aldehyde (acrolein). The effect of the addition of NAD on the action of allyl formate and allyl alcohol on the postmitochondrial fraction was measured. The results (Table 3) show that the addition of NAD increased the inhibitory action of both these agents.

These results are in accordance with the hypothesis that an oxidative product of allyl alcohol is the toxic agent. If this were correct, either acrolein or acrylic acid would be toxic and their toxicity would not be increased by the addition of NAD. Consequently, the effects of acrolein and acrylic acid were tested on protein synthesis in the postmitochondrial fraction, and only acrolein was inhibitory (Table 4).

To investigate further the inhibition of protein synthesis by allyl formate, microsomal and postmicrosomal fractions were prepared from the livers of rats poisoned with allyl formate. Similar fractions were isolated from the livers of control rats. The effect on protein synthesis of the addition of a postmicrosomal fraction of a control rat to that of microsomes from a poisoned rat was compared with the addition of a postmicrosomal fraction from the poisoned rat to a microsomal fraction from a control rat. The result of such a 'cross microsome' study is given in Table 5; the major defect in the protein-synthesizing system produced by allyl formate lies in the microsomal fraction rather than in the postmicrosomal fraction. This result might be due to a reduced microsomal content in the livers of poisoned rats. To examine this possibility, the RNA content of the microsomal fraction and postmicrosomal fraction from the livers of poisoned and control rats was compared. At 1 hr. after poisoning no differences were found, the values being 253 ($\pm$ S.E.M. 24) and 58 ($\pm$ 11) μ g. of RNA/g. wet wt. of liver for microsomal fraction and postmicrosomal fraction respectively.

Acrolein is a highly reactive aldehyde that would be expected to react with many nucleophilic compounds in the cell. Glutathione and Cleland's reagent (1,4-dithiothreitol) might therefore be expected to protect against the effects of allyl formate. The effect of the addition of these compounds was tested against the inhibitory action of allyl formate on amino acid incorporation by a postmitochondrial fraction. The results (Table 6) show that both these compounds afford complete protection.

The effect of acrolein on mitochondrial respiration was also examined. Concentrations of acrolein that inhibited protein synthesis *in vitro* (1 mM)

Table 4. *Effect of acrolein and acrylic acid on the incorporation of [¹⁴C]leucine into protein by a postmitochondrial fraction of rat liver*

The postmitochondrial fraction was prepared as described in the Methods section from the liver of a control rat. NAD (final concn. 70 μ M) was added where indicated. The incubation period was 20 min. The results are the mean of duplicate samples.

Expt. no.	Addition (final concn.)	NAD	Counts/min. at infinite thickness	Inhibition (%)
1	None	—	591	—
	Allyl formate (1 mM)	—	617	Nil
	Allyl formate (1 mM)	+	101	83
	Acrolein (1 mM)	—	88	85
	Acrolein (1 mM)	+	92	84
2	None	—	766	—
	Acrylic acid (1 mM)	—	751	Nil
	Acrylic acid (1 mM)	+	795	Nil

Table 5. *'Crossed microsomes' experiments: incorporation of [¹⁴C]leucine into protein in vitro*

The postmitochondrial supernatant fractions of rat liver from normal and poisoned animals were separated into microsomal and cell-sap components by differential centrifugation as described in the Methods section. These were then recombined as shown below, and the amino acid-incorporation ability of the resulting system was measured. The results are the mean of duplicates of a typical experiment from a group of three.

Tissue fractions	Counts/min. at infinite thickness	Inhibition (%)
Control supernatant + control microsomes	1425	—
Control supernatant + poisoned microsomes	633	56
Poisoned supernatant + control microsomes	1035	28
Poisoned supernatant + poisoned microsomes	410	71

inhibited respiration of mitochondrial preparations from the livers of control rats oxidizing succinate by 20%.

It was suggested above that, in the conversion of allyl formate via allyl alcohol into acrolein, the final step would be carried out by a dehydrogenase present in the postmicrosomal fraction. The following experiment suggests that alcohol dehydrogenase plays a role in the metabolism of allyl formate to a more toxic product. Allyl formate was added to a mitochondrial suspension in the presence of a postmitochondrial fraction or a boiled postmitochondrial fraction or a boiled postmitochondrial fraction to which purified alcohol dehydrogenase had been added. The results (Table 7) show that in the presence of the postmicrosomal fraction the

Table 6. *Effect of thiol compounds on the inhibition of protein synthesis caused by allyl formate in a post-mitochondrial fraction of rat liver*

Preparation of tissue fraction and incubation conditions are as described in the Methods section.

Addition (final concn.)	Counts/min. at infinite thickness	Inhibition (%)
None	1288	—
Glutathione (100 mM)	1333	Nil
Cleland's reagent (100 mM)	1250	Nil
Allyl formate (1 mM)	669	48
Allyl formate (1 mM) + glutathione (100 mM)	1215	9
Allyl formate (1 mM) + Cleland's reagent (100 mM)	1279	Nil

oxidation of succinate was inhibited, and in the presence of the boiled fraction there was no inhibition of oxidation, whereas the addition of alcohol dehydrogenase to this latter system again produced an inhibition of oxidation.

In view of the probable role of alcohol dehydrogenase in the metabolism of allyl formate the distribution of this enzyme in the liver lobule was investigated. In a histochemical study it was observed that the sections showed a periportal increase in the density of the reaction product, indicating that the alcohol dehydrogenase was mainly localized in this region of the lobule. These results confirm the observations of Greenberger, Cohen & Isselbacher (1965).

DISCUSSION

The results of this investigation demonstrate that very early biochemical changes occur in the liver parenchymal cells before any histological

Table 7. *Effect of adding postmitochondrial fraction to the incubation medium on the respiratory rate of isolated rat liver mitochondria in the presence of 1.7 mM-allyl formate*

The preparation of rat-liver mitochondria and the postmitochondrial fraction were as described in the Methods section. A portion of the postmitochondrial fraction was heated in a tube in a boiling-water bath for 10 min. and centrifuged to remove coagulated protein.

Expt. no.	Addition	Respiratory rate (μ moles of O_2 /hr./ mg. of mito- chondrial N)	Inhibition of respiration (%)
1	None	3.70	—
	Postmitochondrial fraction (0.25 ml.)	1.97	47
2	None	4.32	—
	Boiled postmitochondrial fraction (0.25 ml.)	3.90	10
3	None	3.18	—
	Boiled postmitochondrial fraction (0.25 ml.) + purified yeast alcohol dehydrogenase (2 μ g.)	2.16	32

changes are detectable in the liver. Since these changes can also be demonstrated in liver slices and homogenates by the addition of the toxic agent *in vitro*, it seems very likely that allyl formate has a primary action on the parenchymal cell. There is no doubt that endothelial lesions also occur in the livers of poisoned rats and may play a role in the development of the necrosis.

It would be expected that the concentration of free NAD in the homogenate and in the postmitochondrial fraction would be the same. Thus NAD should not be a limiting factor in the metabolism of allyl formate. One explanation for the stimulatory effect of added NAD in the postmitochondrial fraction may be that the separation of mitochondria has removed systems capable of oxidizing the $NADH_2$, which is formed by the conversion of the alcohol into the aldehyde. In this case the extra addition of NAD permits a greater formation of aldehyde.

Most hepatotoxic agents that have been studied produce a centrilobular necrosis, e.g. carbon tetrachloride (Cameron & Karunaratne, 1936), thioacetamide (Gupta, 1956) and dimethylnitrosamine (Barnes & Magee, 1954). In contrast, the lesion produced by allyl formate is periportal. From the present study this may now be explained by the localization of the activating enzyme, alcohol dehydrogenase, within the liver lobule. It may well be that the centrilobular cells are intrinsically more sensitive to the action of toxic agents, and that the few cases in which periportal necrosis develops reflect a specifically periportal activation of the toxin. Alternatively, centrilobular cells may specifically activate the toxins that attack them.

There are many analogies between the effect of

acrolein and that of dimethylnitrosamine, which alkylates nucleic acids and protein *in vivo* (Farber & Magee, 1960). If this is so it would be expected that allyl derivatives, like the nitrosamines, may be both mutagenic and carcinogenic.

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