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BIOCHEMICAL STUDIES ON ACRYLAMIDE, A NEUROTOXIC AGENT

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Abstract—Acrylamide produces peripheral neuropathy in animals and therefore an examination has been made of its biochemical properties. It is inactive against the normal energy producing mechanisms. Using 1-[^{14}C]acrylamide it has been shown that it reacts *in vivo* and *in vitro* with non-protein sulphydryl and with proteins of rat tissue. Labelling of the proteins in the central nervous system persists for more than 14 days. The reactivity of many unsaturated compounds with glutathione has been measured. The reactivity of *N*-(hydroxymethyl)acrylamide is identical to that of acrylamide; after administration of 1-[^{14}C]-*N*-(hydroxymethyl)acrylamide and 1-[^{14}C]acrylamide to rats, tissues are labelled in a similar manner. In contrast to this similar reactivity, *N*-(hydroxymethyl)acrylamide does not produce peripheral neuropathy. The relevance of these observations to future work on the mechanism of the biological specificity of acrylamide is discussed.

MONOMERIC acrylamide produces weakness of the hindlimbs in various animals and human beings.[1–8] Examination of affected animals by electrophysiological techniques indicated that motor nerve conduction velocity is slightly reduced and by histological techniques that degeneration of axis cylinders and myelin sheaths was present in peripheral nerves, the distal parts of the longest fibres being most affected.[4] The damage has been classified as a "dying back" process[4] and acrylamide has been placed in a group of substances showing an organophosphorus pattern of degeneration.[9] However, acrylamide shows a remarkable specificity for the peripheral nerves and there is no evidence of spinal cord involvement.

Divided dosing of rats and cats[1, 4] at different time intervals has shown that the effects are cumulative. This cumulative effect is very striking in view of the high solubility of acrylamide in water, i.e. it would not be expected that it would persist in the tissues. Thus, it seems likely that acrylamide reacts with some component of nervous tissue, the product of the reaction persisting long enough for subsequent doses to increase the concentration of the modified component. If this is so then there is a chance to establish the nature of this component.

Many related compounds do not produce peripheral neuropathy[10, 12] although like acrylamide they contain a double bond capable of reacting with thiols. This suggests that work to establish the molecular basis of the action of acrylamide may have some chance of success. The present paper is an account of preliminary work designed to

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examine the biochemical and chemical properties of acrylamide and also to produce an experimental set-up suitable for a start on the problem of the primary chemical attack which leads to the peripheral neuritis in man and animals.

MATERIALS AND METHODS

Animals. Male albino rats of the Porton strain (200 ± 10 g body weight) fed on M.R.C. diet No. 41B were used unless otherwise stated.[13]

Determination of LD_{50}. The toxicity of *N*-(hydroxymethyl)acrylamide by intraperitoneal administration was carried out according to Weil[14] using four animals per dosage level and four different doses.

Subcellular fraction of rat brain. This was undertaken as previously described.[15]

Special chemicals. The following were obtained from the sources stated: acrylamide, acrylonitrile, ethyl crotonate, *N N'* methyl bisacrylamide (British Drug Houses Ltd.); allyl alcohol, crotyl alcohol, methylacrylamide (Aldrich Ltd.); acrolein (Hopkin & Williams Ltd.); ergothioneine (Sigma Chemical Co.); glutathione (Schwartz Bioresearch Ltd.); *N*-(hydroxymethyl) acrylamide (Vinyl Products Ltd.); methylvinylketone (Ralph Emanuel Ltd.). Ethylacrylate, *N*-methylacrylamide, senecionic acid amide and sodium acrylate were prepared by Dr. Mattocks in this laboratory.

[1-^{14}C] acrylamide was synthesized from K^{14}CN by a slight modification of the method of Weisgerber.[16] Final crystallization was carried out with petroleum ether (b.p. 40–60°) in ethyl acetate. The m.p. of the product was 83–85°.

[1-^{14}C] *N*-(hydroxymethyl)acrylamide was prepared using a slight modification of existing methods[17, 18] from the [1-^{14}C]acrylamide by incubating the latter with equimolar paraformaldehyde under slight alkaline conditions at 50° for 3 hr followed by standing at room temperature for 24 hr. The m.p. of the product was 70°.

S-carboxyethylcysteine was prepared by the method of Weil and Seibles.[19] The conjugates of acrylamide with *l*-histidine, *l*-lysine, *l*-methionine were prepared by incubating the two compounds in 0·1 M concentration at neutral pH for 24 hr. *l*-Arginine, *l*-tyrosine, *l*-phenylalanine and *l*-tryptophan were also incubated with acrylamide but none of them gave any conjugates at the conditions described above.

Nucleic acids. These were prepared from rat liver and brain according to existing methods[20, 21] with a slight modification for brain to prevent the degradation of DNA, i.e. after dissecting out from the animals, brains were immersed in a cold 0·3 M sucrose solution instead of being fixed with liquid nitrogen. The cooled brains were then homogenised within 10 sec with a Ultra Turrax homogeniser and were quickly added to the phenol reagent. RNA was estimated using *p*-bromo phenylhydrazine[22] and DNA using diphenylamine.[23]

Distribution and excretion of [^{14}C] *labelled compounds*

[1-^{14}C] acrylamide (100 mg/kg body wt.; specific activity 67·2 μc/m-mole or [1-^{14}C] *N*-(hydroxymethyl)acrylamide (140 mg/kg body wt.; specific activity 58·2 μc/m-mole) were injected by tail vein and animals were killed at various times. Radioactivity was counted in homogenates, in the solution after removal of material insoluble in cold 5% (w/v) trichloracetic acid or in protein. The protein was prepared by successive extraction with cold 5%, cold 10% and hot 5% (w/v) trichloracetic acid solutions followed by acetone neutralised using an internal indicator. Lipid was removed from this precipitated material by successive extraction with acetone–conc. hydrochloric

acid (100:1, v/v), anhydrous ethanol, chloroform–methanol (2:1, v/v), chloroform–methanol–conc. hydrochloric acid (200:100:1), twice with ethanol–ether (3:1, v/v) and twice with ether followed by drying. Expiration of [^{14}C] carbon dioxide from [^{14}C] acrylamide was measured according to Heath.[24]

Procedures for the counting of radioactivity. This was done using a Packard Model 4312 liquid scintillation counter. Activity in blood samples and in tissue homogenates was counted in a Dioxan scintillator with 4% cab-o-sil. Counts in trichloracetic acid and acetone extracts were measured in Dioxan scintillator. Protein powders (ca. 10 mg) were dissolved in 0·5 ml hyamine hydroxide solution at 50° for 1 hr followed by mixing with toluene scintillator and counting. Counting efficiency was calculated in every sample using a known amount of [^{14}C] toluene as an internal standard and gave a value of between 50–70 per cent depending on samples.

Determination of monomeric acrylamide. Since polymerisation will decrease the number of double bonds, monomeric acrylamide was determined using a modification of Hann's bromine method for unsaturation in lipids. Bromine solution (20 ml of 0·3% in glacial acetic acid) was added to 20 ml of a solution of acrylamide (10–80 mg). Reaction was allowed to take place for 60 min at room temperature in the dark. The excess bromine was then determined (after dilution with water) by reaction with potassium iodide and titration with thiosulphate. For complete bromination the ratio of water to glacial acetic acid must be 1:1 v/v. No decrease in double bonds was found after storage of concentrations of acrylamide used for *in vitro* experiments or for injection of animals. The solutions therefore contain little or no products due to polymerisation and probably contain only monomeric acrylamide.

Determination of non-protein sulphydryl in tissues and amino acid analysis. The method of Ellman[25] with 5 5′ dithiobis-(2-nitrobenzoic acid) reagent was used for non-protein sulphydryl. The reagent was dissolved in 1% sodium citrate.[26] The method of Benesch *et al.* [51] was used for protein sulphydryl. A vibrating platinum electrode and a glass reference electrode (Type RT 23 Electronic Instruments Ltd) were used. Amino acid analysis of tissue protein was made using an automatic amino acid analyser (Technicon Instruments Ltd.) after hydrolysing proteins with 6 N HCl at 108° for 24 hr. Standard buffer systems for the analyses were used.[27]

Determination of ergothioneine. The method of Hunter[28] was used. Reactivity of acrylamide with ergothioneine was studied incubating 0·33 mM acrylamide with 0·33 mM ergothioneine in 0·1 M phosphate buffer at pH 7·3 and 37° for 24 hr.

Reaction in vitro *of acrylamide and analogues with glutathione.* Both compounds were incubated in a 0·1 M phosphate buffer pH 7·3 at 37° in the presence of $1{\cdot}5 \times 10^{-4}$M KCN. KCN prevented the oxidation of glutathione during the incubation. In most cases except for highly reactive compounds 10 mM concentration was used for both test compounds and glutathione. Reaction rate was estimated measuring a decrease of sulphydryl in the mixture at intervals by DTNB method described above.

Reaction in vitro *of acrylamide with rat haemoglobin.* Crystalline haemoglobin was used.[29] A solution of reduced haemoglobin was prepared by repeated evacuation and filling with N_2 and was incubated with [^{14}C]-acrylamide (4·4 μc/m-mole) in 0·1 M tris-HCl buffer pH 8·0 under N_2 gas at 30°. Radioactivity bound to haemoglobin was estimated after precipitating with perchloric acid, washing sediment twice with acid acetone, twice with 3:1 EtOH–ether, twice with anhydrous ether and drying. The

concentrations of solutions of haemoglobin was measured using a molar extinction coefficient of $5{\cdot}96 \times 10^4$ for carbonmonoxyhaemoglobin at 540 mμ.[30]

Reaction in vitro *of acrylamide and* N-(*hydroxymethyl*) *acrylamide with protein sulphydryl.* Tissue homogenate (20% w/v) was incubated with an excess of acrylamide or *N*-(hydroxymethyl)acrylamide in 0·1 M phosphate buffer pH 7·3 at room temperature under N_2. Reaction was followed by the rate of decrease of protein sulphydryl.

Method of study of glucose metabolism in brain slices. Brain slices were prepared and the experiments carried out as previously described.[31] Lactic acid was determined according to Hohorst,[32] pyruvic acid according to Friedmann and Haugen[33] modified to increase the sensitivity.[34]

Measurement of oxidative phosphorylation. Rat liver mitochondria were isolated and examined according to Aldridge and Street.[35]

RESULTS

The effect of acrylamide on in vitro *and* in vivo *respiratory metabolism*

The results in Table 1 show that the addition of 10 mM acrylamide to the medium has no influence on the oxygen uptake of brain cortex slices with and without 30 μM 2:4 dinitrophenol nor on the concentrations of pyruvic or lactic acids at the end of the experiment. Assuming an even distribution throughout the body water 200 mg acrylamide/kg body weight would indicate an immediate concentration of approx. 4 mM. After this treatment or after feeding for 20 days on a diet containing 400 ppm acrylamide (rats on this treatment will just be developing abnormalities) the brain

TABLE 1. EFFECT OF ACRYLAMIDE *IN VITRO* OR ITS PRIOR ADMINISTRATION TO RATS ON THE OXYGEN UPTAKE, PYRUVIC ACID AND LACTIC ACID CONCENTRATIONS OF BRAIN CORTEX SLICES

Treatment	No. of expts.	O_2 uptake (μl/hr/mg protein)	Pyruvate (μmole/g dry wt.)	Lactate (μmole/g dry wt.)	Ratio lactate :pyruvate
Nil (Controls)	6	13·7 ± 2	20·5 ± 0·5	263 ± 6	11·3 ± 0·3
10mM acrylamide *in vitro*	4	14·3 ± 0·7	20·9 ± 1·1	275 ± 12	13·7 ± 0·6
30μM DNP *in vitro*	6	26·4 ± 1·4	29·6 ± 3·2	612 ± 19	21·3 ± 1·8
10mM acrylamide + 30μM DNP *in vitro*	4	26·6 ± 0·9	27·4 ± 1·2	560 ± 33	20·6 ± 1·1
24 hr after 200 mg acrylamide/kg by intraperitoneal injection	3	15·2 ± 0·5*	20·1 ± 1·0	298 ± 14	15·1 ± 0·9
24 hr after 200 mg acrylamide/kg by intraperitoneal injection + 30μM DNP *in vitro*	4	25·6 ± 1·0	25·8 ± 1·8	492 ± 43*	19·3 ± 1·5
20 days after feeding with 400 ppm acrylamide in the diet	3	12·4 ± 0·3	18·3 ± 1·3*	266 ± 12	15·4 ± 0·9

Oxygen uptake was measured over 1 hr and lactic and pyruvic acids were determined at this time.
DNP – 2:4-dinitrophenol. Results are expressed as Mean ± S.E.
* When compared with respective controls $0{\cdot}01 < P > 0{\cdot}05$.

cortex slices show little change from controls. These results indicate that it is unlikely that acrylamide either *in vitro* or *in vivo* affects mitochondrial metabolism and this is confirmed by the finding that the addition of 10 mM acrylamide to respiring rat liver mitochondria had no effect on their ability to carry out oxidative phosphorylation.

Reaction in vitro *of acrylamide and its analogues with glutathione and ergothioneine*

Acrylamide reacts with sulphydryl containing compounds and a comparison has been made of the reactivities of acrylamide and other related compounds with glutathione. Using the conditions described under "Methods" the reaction with acrylamide follows second order kinetics. For all compounds except acrolein, the rate constant (k) has been determined at 37° and pH 7·3 (Table 2). With acrolein the

TABLE 2. REACTIVITY OF ACRYLAMIDE AND ITS ANALOGUES WITH GLUTATHIONE AT pH 7·3 37° *IN VITRO*

Compounds	Formula	k l.mole^{-1}min^{-1}	Chronic toxicity
Acrolein	$CH_2 = CH - C(=O)H$	—	
Methyl Vinyl Ketone	$CH_2 = CH - C(=O)CH_3$	Approx. $3{\cdot}5 \times 10^4$	
Ethyl Acrylate	$CH_2 = CH - C(=O)O - C_2H_5$	22·6	
Acrylonitrile	$CH_2 = CH - C \equiv N$	2·42	None[12]
Acrylamide	$CH_2 = CH - C(=O)NH_2$	0·91	Ataxia[4]
N-(hydroxymethyl) acrylamide	$CH_2 = CH - C(=O)NHCH_2OH$	0·91	None (see text)
N-*N'*-Methylene-Bis-Acrylamide	$(CH_2 = CH - CONH)_2CH_2$	0·54	
Ethyl Crotonate	$CH_3CH = CH - C(=O)OC_2H_5$	0·24	
Methyl Methacrylate	$CH_2 = C(CH_3) - C(=O)OCH_3$	0·17	
N-Methyl Acrylamide	$CH_2 = CH - C(=O)NHCH_3$	0·058	Slight effect[12] on legs
N-*N'* Diethyl Acrylamide	$CH_2 = CH - C(=O)N(C_2H_5)_2$	0·058	None[12]
Sodium Acrylate	$CH_2 = CH - C(=O)ONa$	0·035	None[12]
Methacrylamide	$CH_2 = C(CH_3)C(=O)NH_2$	0·014	None[12]
Crotonamide	$CH_3CH = CH - C(=O)NH_2$	0·0	None[12]
Senecionic acid amide	$(CH_3)_2C = CH - C(=O)NH_2$	0·0	None[12]
Allyl Alcohol	$CH_2 = CH - CH_2OH$	0·0	
Crotyl Alcohol	$CH_3 - CH = CH - CH_2OH$	0·0	

See Methods section for details of procedure.

reaction was too rapid to follow and more than one molecule of glutathione reacted per molecule of acrolein, indicating reaction both at the double bond and the aldehyde group. The rate constants indicate a wide range of reactivity (Table 2).

There is no direct relationship between reactivity and toxicity. The rate of reaction of acrylamide with glutathione is rather slow—for example when both components are 10 mM, reaction is one half complete in 110 min. The concentrations of acrylamide *in vivo* are initially less than this and will rapidly decrease due to excretion. Out of the 18 compounds in Table 2 only acrylamide produces the characteristic peripheral neuropathy. *N*-(hydroxymethyl) acrylamide and acrylamide have identical reactivity with glutathione but *N*-(hydroxymethyl) acrylamide does not produce any neuropathy, (see later). According to Kuperman[1] acute dosing with high doses of acrylamide effects some physiological functions of the cerebellum. Crossland *et al.*[36] have produced evidence that the concentration of ergothioneine in the cerebellum is considerably higher than in other parts of the brain and other tissues. Therefore we examined whether acrylamide reacts with ergothioneine—no reaction was found after a 24-hr incubation *in vitro*.

TABLE 3. CONCENTRATIONS OF NON-PROTEIN SULPHYDRYL IN TISSUES AFTER A SINGLE ORAL DOSE OF ACRYLAMIDE OR *N*-(HYDROXYMETHYL) ACRYLAMIDE

		Non-protein sulphydryl (μmole/g tissue)		
		Brain	Spinal cord	Liver
Acrylamide 100 mg/kg	Control	1·70 ± 0·03	1·22 ± 0·07	6·95 ± 0·33
	4 hr	1·26 ± 0·07	0·83 ± 0·04	3·19 ± 0·36
		(74 ± 4·1)	(68 ± 4·1)	(46 ± 16)
	24 hr	1·32 ± 0·04	0·92 ± 0·08	6·56 ± 0·42
		(78 ± 2·2)	(75 ± 6·6)	(95 ± 11)
	48 hr	1·64 ± 0·09	1·15 ± 0·08	6·45 ± 0·43
		(96 ± 5·3)	(94 ± 6·6)	(93 ± 14)
	72 hr	1·72 ± 0·04	1·20 ± 0·12	5·70 ± 0·19
		(101 ± 2·2)	(98 ± 9·9)	(82 ± 6)
N-(hydroxymethyl) acrylamide 140 mg/kg	Control	1·70 ± 0·03	1·22 ± 0·07	5·61 ± 1·08
	4 hr	1·37 ± 0·05	0·90 ± 0·00	2·21 ± 0·76
		(81 ± 2·9)	(74 ± 0·0)	(39 ± 14)
	24 hr	1·43 ± 0·09	1·09 ± 0·10	3·78 ± 0·54
		(84 ± 5·3)	(89 ± 8·2)	(67 ± 9·6)
	48 hr	1·58 ± 0·05	1·13 ± 0·06	5·71 ± 0·66
		(93 ± 2·9)	(93 ± 4·9)	(102 ± 12)
	72 hr	1·69 ± 0·06	1·17 ± 0·06	5·30 ± 0·29
		(99 ± 3·4)	(96 ± 4·9)	(94 ± 4·8)

Acrylamide or *N*-(hydroxymethyl) acrylamide was given by oral route. Each value is the mean ± S.E. of the results for five rats. The values in parentheses are the percentages of the respective controls.

The effect of a single dose of acrylamide and N-(*hydroxymethyl*) *acrylamide on the non-protein sulphydryl content in the brain, spinal cord and liver*

After the administration of equimolar oral doses of acrylamide and *N*-(hydroxymethyl) acrylamide to rats, the non protein sulphydryl content falls rapidly (Table 3). The percentage fall is greatest in the liver and the decrease in brain and spinal cord is very similar for both compounds. The rate of return in the liver is rapid, the time for 50 per cent return is approx. 10 hr after acrylamide and 20 hr after *N*-(hydroxymethyl) acrylamide. The rate of recovery is slower for brain and spinal cord than for liver,

indicating a half life of between 20 to 30 hr for both nervous tissues after the administration of both compounds. These results agree with previous work which shows that the turnover of glutathione in the liver is faster than that in brain. The different turnover rates for glutathione in liver[37, 38] or in brain[39, 40] obtained with different techniques makes comparison with our results difficult.

Rats were dosed with acrylamide (100 mg/kg body wt.) on Monday and Friday of each of 4 consecutive weeks and determination of brain and spinal cord non-protein sulphydryl content was made on Thursdays. The concentrations found were always the same as those in the control rats and there was no indication of the development of an inability to resynthesize glutathione. The rats were ataxic after four doses ($2\frac{1}{2}$ weeks) until the end of the experiment (4 weeks).

Excretion of [^{14}C] from labelled acrylamide and its products in urine and by the lung

Acrylamide (100 mg/kg body wt.) labelled with [^{14}C] in the 1-position, was injected intravenously into two rats. The [^{14}C] CO_2 expired was measured and the cumulative

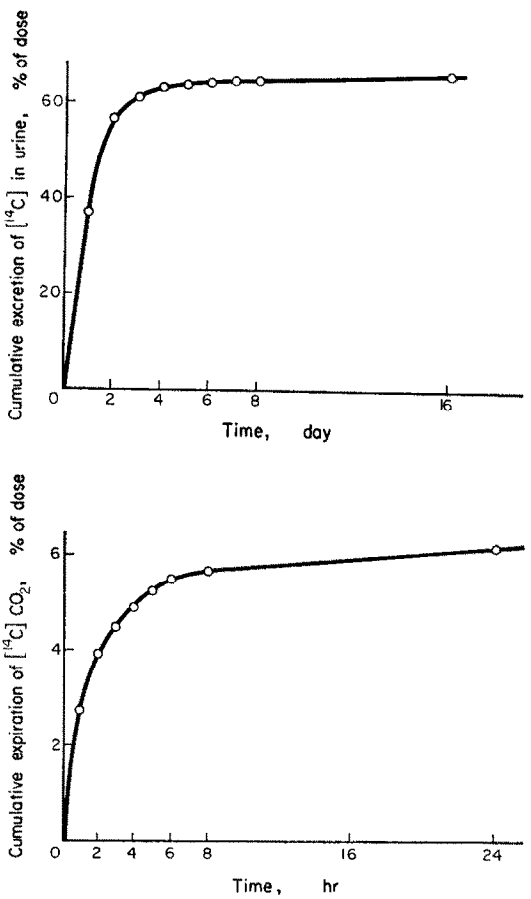

FIG. 1. Excretion of [^{14}C] in urine and expiration of $^{14}CO_2$ after a single dose of [1-^{14}C] acrylamide 100 mg/kg (7·3 μc) was administered intravenously. The results are the means of the results for two rats.

TABLE 4. DISTRIBUTION OF [^{14}C] IN TISSUES AFTER A SINGLE INTRAVENOUS DOSE OF [1-^{14}C] ACRYLAMIDE OR [1-^{14}C] *N*-(HYDROXYMETHYL)ACRYLAMIDE

Time after administration	Tissue	Homogenate	"Free and soluble"	Protein Bound
		(nmoles/g original tissue)		
Acrylamide (100 mg/kg body wt.)				
24 hr	Blood	1280	160	938
	Plasma	63	0·0	—
	Brain	262	105	156
	Spinal Cord	175	44	110
	Sciat. N.	149	60	—
	Liver	368	70	323
	Kidney	508	195	280
4 days	Blood	1360	7·4	920
	Plasma	26	0·0	—
	Brain	151	20	95
	Spinal Cord	131	19	89
	Sciat. N.	100	16	89
	Liver	142	20	158
	Kidney	248	66	181
14 days	Blood	772	1·8	920
	Plasma	6·0	0·0	—
	Brain	72	3·3	49
	Spinal Cord	71	2·8	44
	Sciat. N.	57	0·0	—
	Liver	57	1·6	61
	Kidney	91	1·2	76
N-(hydroxymethyl) acrylamide (140 mg/kg body wt.)				
24 hr	Blood	1200	73	1130
	Plasma	35	0·0	26
	Brain	157	39	104
	Spinal Cord	109	25	69
	Sciat. N.	101	0·0	93
	Liver	262	2·7	266
	Kidney	266	75	214
4 days	Blood	1370	0·0	1220
	Plasma	13	0·0	13
	Brain	95	0·0	79
	Spinal Cord	70	0·0	64
	Sciat. N.	42	0·0	46
	Liver	141	0·0	146
	Kidney	155	2·1	165

Each value is the mean from three experiments. The "free and soluble bound" is that remaining in solution after treatment with 5% trichloroacetic acid. See Methods for procedure.

excretion is shown in Fig. 1. Approximately 6 per cent of the injected dose was exhaled as CO_2 in 8 hr followed by a very slow excretion afterwards. This indicates a small amount of metabolism and means that the counts found attached to protein (Table 4) are due to reaction of the whole acrylamide molecule with protein and not due to incorporation via one carbon precursor derived from CO_2. Excretion of [^{14}C] in the urine is very rapid, 40 per cent of the injected dose being excreted over the 1st day and the maximum of just over 60 per cent being reached by day 3. The nature of the material excreted is not known.

The distribution of [^{14}C] *in tissues after injections of* [1-^{14}C] *acrylamide and* [1-^{14}C] N-(*hydroxymethyl*)*acrylamide*

The distribution of the radioactivity from each compound in tissues were studied

(Table 4). Radioactivity is found in every tissue with high counts in the blood. Most of the radioactivity is not extractable by 5% trichloroacetic acid and is presumably bound to protein. There was a considerable amount of radioactivity present 14 days after dosing. For brain, spinal cord and sciatic nerve this is a finding of some importance. The maximal interval between 100 mg/kg doses of acrylamide which will produce peripheral neuropathy is 14 days (it takes 30 weeks for symptoms to appear). Therefore, if the lesion is brought about by reaction with protein, some labelling must persist for 14 days.

TABLE 5. SUBCELLULAR DISTRIBUTION OF [^{14}C] IN RAT BRAIN AND LIVER AFTER A SINGLE DOSE OF [1-^{14}C] ACRYLAMIDE OR [1-^{14}C] *N*-(HYDROXYMETHYL)ACRYLAMIDE

Tissue	Fraction	Total	5% TCA Extract	Protein
		(nmoles/g original tissue)		(nmoles/mg)
Acrylamide, 100 mg/kg				
Brain	Homogenate	123	36·5	1·3
	Nuclear fraction	28·0	3·2	1·3
	Light Mitochondrial fraction	30·3	5·7	1·2
	Heavy Mitochondrial fraction	16·7	3·1	1·3
	Microsomal fraction	21·9	2·2	1·2
	Supernatant	49·7	31·5	1·1
Liver	Homogenate	214	31·8	1·2
	Nuclear fraction	45·7	3·4	1·0
	Light Mitochondrial fraction	15·5	5·5	0·8
	Heavy Mitochondrial fraction	13·0	3·2	0·9
	Microsomal fraction	26·8	2·1	0·9
	Supernatant	105	36·1	1·8
N-(hydroxymethyl) acrylamide 140 mg/kg				
Brain	Homogenate	210	47·2	1·5
	Nuclear fraction	30·7	4·6	1·3
	Light Mitochondrial fraction	39·9	9·2	1·2
	Heavy Mitochondrial fraction	15·8	4·1	1·3
	Microsomal fraction	17·7	2·9	1·2
	Supernatant	47·2	23·7	1·3
Liver	Homogenate	292	57·0	1·8
	Nuclear fraction	66·4	2·7	1·2
	Light Mitochondrial fraction	18·5	3·8	1·0
	Heavy Mitochondrial fraction	4·3	0·6	0·9
	Microsomal fraction	31·8	2·2	1·1
	Supernatant	127	33·2	2·4

The compounds were given intravenously and the animals killed after 24 hr. The results are expressed as the nmole equivalent of the compound administered. Each value is a fractionation of tissue from two rats.

The distribution of radioactivity in subcellular fractions of brain 24 hr after the administration of acrylamide and *N*-(hydroxymethyl) acrylamide is shown in Table 5. All fractions are labelled, the specific activity of the protein being very similar in all fractions.

The nucleic acids of brain and liver were isolated 24 hr after the administration of [1-^{14}C] acrylamide to rats (Table 6). Although the fractions were not pure the counts present were few and indicated an extremely low level of incorporation.

The high radioactivity in the blood, which is in the erythrocytes since the plasma

contains little label, seems likely to be due to reaction of acrylamide or *N*-(hydroxymethyl)acrylamide with rat haemoglobin. When reduced haemoglobin was incubated with [1-^{14}C]acrylamide, the bound radioactivity indicates that 4 moles acrylamide have reacted with 1 mole of haemoglobin. The rate constant for this reaction is approximately 18 l.mole^{-1}min^{-1} (the time for half reaction with acrylamide (0·1M) in large excess to the haemoglobin is 25 min). There is no further reaction until after 8 hr incubation, when some denaturation probably occurs. The molar ratio of 4:1 agrees with other published results for reactive sulphydryl groups in rat haemoglobin.[30] Amperometric titration of either haemoglobin solution or brain homogenate incubated with excess

TABLE 6. [^{14}C] IN NUCLEIC ACIDS ISOLATED FROM RAT BRAIN AND LIVER AFTER A SINGLE DOSE OF [1-^{14}C] ACRYLAMIDE TO RATS

		nmoles/mg
Brain	RNA	0·0075
	DNA	0·018
Liver	RNA	0·023
	DNA	0·016

[1-^{14}C] Acrylamide (100 mg/kg) was given intravenously and the six rats killed 24 hr later. The results are expressed as the nmole equivalent of the compound administered. Analysis showed that both fractions from brain contained RNA and DNA.

amount of acrylamide showed a decrease of protein sulphydryls. Amino acid analysis of the protein obtained from these samples indicated a peak corresponding to S-carboxyethyl-cysteine. The same product has been found for the reaction of acrylonitrile with α-lactoalbumin.[19]

The toxicity of acrylamide and N-*(hydroxymethyl)acrylamide*

The acute LD_{50} for *N*-(hydroxymethyl) acrylamide is 563 ± 20 mg/kg body wt. and it is 2·5 times less toxic than acrylamide (203 mg/kg body weight).[4] The growth rates for rats dosed twice weekly with acrylamide or *N*-(hydroxymethyl) acrylamide are shown in Table 7 (exp. 2). There was no difference in the rate of growth of controls and those treated with *N*-(hydroxymethyl) acrylamide whereas those treated with acrylamide lost weight. The acrylamide treated rats became ataxic after 2 weeks (four doses) whereas the *N*-(hydroxymethyl)acrylamide treated rats were normal after 10 weeks (20 doses). Histological examination of peripheral nerves showed no changes and no evidence was found of the changes in fibre diameter characteristic of acrylamide poisoning.[4]

In view of the similar chemical reactivity of acrylamide and *N*-(hydroxymethyl) acrylamide (Table 2) it might be possible to remove those acrylamide sensitive sites which are irrelevant to the lesion by treatment with *N*-(hydroxymethyl) acrylamide. Feeding of rats on a diet containing 700 ppm *N*-(hydroxymethyl)acrylamide makes the rats more sensitive to injected acrylamide, i.e. ataxia is produced by fewer doses and therefore in a shorter time. The rate of growth of these rats fed with a diet containing 700 ppm *N*-(hydroxymethyl)acrylamide was less than that of the controls (Table 8)

TABLE 7. THE EFFECT OF THE ADMINISTRATION *N*-(HYDROXYMETHYL) ACRYLAMIDE AND/OR ACRYLAMIDE ON THE GROWTH RATE OF RATS

Intraperitoneal injections twice weekly (mg/kg)		Mean increase in wt. (g)/day	
		Normal diet	Diet containing *N*-(hydroxymethyl) acrylamide
Acrylamide, 100	Expt. 1	1·24 ± 0·29	1·40 ± 0·27
Acrylamide, 50	Expt. 1	2·39 ± 0·28	1·50 ± 0·30
Acrylamide, 25	Expt. 1	3·31 ± 0·39	1·93 ± 0·21
Acrylamide, 12·5	Expt. 1	4·00 ± 0·15	1·91 ± 0·26
Controls	Expt. 1	3·32 ± 0·45	2·11 ± 0·17
Acrylamide, 100	Expt. 2	−0·75 ± 0·29	—
N-(hydroxymethyl) acrylamide, 100	Expt. 2	2·52 ± 0·17	—
Controls	Expt. 2	2·59 ± 0·41	—

Rats for expt. 1 were weighed twice and for expt. 2 once weekly and growth (derived from the regression line) is expressed as the mean ± S.E. increase in weight per day over the first 32 days of the experiment. The rats fed with a diet containing *N*-(hydroxymethyl) acrylamide and their control are the same animals described in Table 8 (cf. legend for details of the diet). In expt. 1 there were 4 rats in each group and in expt. 2 there were 5. The mean food consumption per week indicated that the rats in expt. 1 were receiving 500–600 mg *N*-(hydroxymethyl) acrylamide/kg. The reason for the difference in the rate of growth of the controls in the two experiments is not known.

TABLE 8. THE EFFECT OF THE ADMINISTRATION OF *N*-(HYDROXYMETHYL) ACRYLAMIDE ON THE ONSET OF ATAXIA CAUSED BY ACRYLAMIDE

Acrylamide injected twice weekly (mg/kg)	Time of onset of ataxia (days after first injection of acrylamide)			
	Weakness of hindlimbs	Slight ataxia	Moderate ataxia	Gross ataxia
N-(hydroxymethyl) acrylamide in the diet				
100	10 (3)	12 (4)	13 (4)	19 (6)
50	12 (4)	14 (4)	18 (6)	
25	14 (4)	18 (6)	32 (10)	
12·5	17 (5)	40 (12)		
0	—	—	—	—
Controls (No *N*-(hydroxymethyl) acrylamide in the diet)				
100	12 (4)	18 (6)	26 (8)	
50	17 (5)	40 (12)		
25	42 (12)			
12·5	—	—	—	—
0	—	—	—	—

The treated group were fed with a diet containing 1400 ppm *N*-(hydroxymethyl) acrylamide for 1 week before intraperitoneal injections of acrylamide were begun. For the rest of the experiment the diet contained 700 ppm *N*-(hydroxymethyl) acrylamide. The time of onset of ataxia is the mean of the times for four rats. In parentheses are the number of injections of acrylamide required to produce the indicated degree of ataxia. A dash indicates no ataxia was produced at the end of the experiment (50 days after the first injection of acrylamide). A blank space indicates that the degree of ataxia had not increased by the end of the experiment.

and this contrasts with the results obtained with twice weekly intraperitoneal doses of 100 mg/kg. However, in the former, calculation from the consumption of food indicate that the rats were ingesting 500–600 mg of *N*-(hydroxymethyl)acrylamide per week. Even with this increased dosage and the effects on the growth rate there was no histological evidence of damage in the peripheral nerves and they appeared completely normal.

DISCUSSION

The work in this paper demonstrates the contrast between the phenomenon of high biological specificity of toxic compounds and their general reactivity. An example is the delayed neurotoxicity shown by some organophosphorus compounds.[41] These organophosphorus compounds react with many proteins with esteratic activity. However, although an esterase has been shown to be involved in the genesis of the lesion[42] only a few organophosphorus compounds produce the lesion and it is likely that specificity resides in the stereochemical requirements of this particular esterase for inhibition to take place.[43] This will probably be also an indication of the stereochemical requirements for substrates of this enzyme.[44]

Other toxic substances combine a high biological activity with a chemical reactivity which seems to be limited to certain molecular groupings. The only enzyme systems which have been shown to be inhibited by low concentrations of triethyltin are oxidative phosphorylation and photosynthesis,[45] the chemical reactivity is likewise limited and appears to require two planar histidine residues the correct distance apart.[45, 46]

Acrylamide poses the extremes in both cases. It is biologically very specific. No other unsaturated compound related to acrylamide has been shown to produce this lesion. Two compounds produced marginal effects but the possibility that this was due to small amounts of acrylamide present as an impurity has not been excluded.[12] In contrast acrylamide in common with other similar unsaturated compounds reacts with glutathione (Table 2) and also with proteins containing sulphydryl groups.[47–50] Thus in general terms acrylamide qualitatively does not differ from other unsaturated compounds but it must be remembered that most of these studies have been made at high concentrations of a reacting compound. It is not known if the groups in protein to which acrylamide is attached after administration of acrylamide (Table 4) are sulphydryl groups or not; *in vitro* studies have shown that as well as sulphydryl other groups (lysine) may be alkylated.[47] Furthermore, we cannot ignore that enzymic catalysis depends upon the abnormal reactivity of particular groups in proteins and therefore we must retain an open mind about the groups which acrylamide may alkylate.

Faced with this problem of high biological selectivity and general chemical reactivity, are there any indications of ways in which the molecular basis for the development of the lesion may be examined? The results in this paper indicate a possible approach. The chemical reactivity of *N*-(hydroxymethyl)acrylamide (as judged by reaction with glutathione,) (Table 2) is identical with that of acrylamide and yet it does not produce peripheral neuropathy. The results of the toxicity studies show that lower doses of acrylamide are able to produce peripheral neuropathy in rats being fed on a diet containing *N*-(hydroxymethyl)acrylamide. This finding could be due to a sparing of the acrylamide from reaction with sites irrelevant to the production of the lesion or it could indicate the maintenance of a higher concentration of acrylamide due to inter-

ferences of *N*-(hydroxymethyl)acrylamide with its metabolism or distribution. Whatever the explanation, the technique has potentiality for further studying the basis of the genesis of this lesion.

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