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ALTERATION OF AMINO ACID INCORPORATION INTO PROTEINS OF THE NERVOUS SYSTEM *IN VITRO* AFTER ADMINISTRATION OF ACRYLAMIDE TO RATS

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Abstract—Incorporation *in vitro* of either L-[U-^{14}C]lysine or L-[^{35}S]methionine into proteins in isolated nervous tissues from rats treated with acrylamide has been determined. Incorporation of the amino acids was different in different tissues. Both potassium cyanide and 2,4-dinitrophenol inhibited the incorporation, suggesting that the incorporation is dependent on aerobic energy metabolism. In the brain cortex and liver the incorporation of amino acids was not affected by acrylamide, while in the spinal cord it started to increase after the rats became paralytic, and it reached the maximum value after acrylamide was withdrawn. In contrast, in sciatic nerve, lysine incorporation was suppressed at the early stage but increased later as in the spinal cord. Methionine incorporation into sciatic nerve was not decreased. Equilibration of lysine between the suspending medium and the tissue both in control and treated rats was established within at least 10 min after the incubation was begun. Autoradiographically a large number of silver grains due to [^{14}C]lysine was visible in the anterior horn cells of the spinal cord and in the Schwann cells of the sciatic nerve both in normal and treated rats. A possible mechanism for the altered protein metabolism is discussed.

IT HAS been shown in a previous report[1] that acrylamide (CH_2=CH—$CONH_2$) which produces peripheral neuropathy in animals and human beings,[2] reacts with glutathione and protein in the central and peripheral nervous systems. Studies *in vitro* have also shown that acrylamide combined with cysteine sulphydryls in protein to produce *S*-carboxyethyl cysteine after hydrolysis.[1]

A disturbance of the axoplasmic flow of proteins labelled with radioactive leucine down the nerve roots in cats dosed with acrylamide has been shown by Pleasure, Mishler and Engel.[3] The present study was undertaken to examine whether acrylamide affected the protein metabolism of the nervous tissues. The effect of acrylamide on the *in vitro* incorporation of radioactive amino acids into the proteins of nervous tissues was investigated.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley albino rats were obtained from the Laboratory of Experimental Animals, Osaka University.

Dosing of acrylamide. Rats (8 weeks old) were fed on a commercial powder diet (Oriental M powder) containing 500 ppm acrylamide for 4 weeks, followed by 4 weeks without acrylamide, and water *ad lib.* The control group was fed powder only.

Incubation of nervous tissues with radioactive amino acids. The rats were killed by decapitation under ether anesthesia at varied times, and the brains, spinal cords,

sciatic nerves and livers were rapidly removed. Slices of 0·5 mm thickness were prepared from the brain cortex and the liver using a mechanical chopper made in this laboratory. The spinal cord was separated from the arachnoides and was divided into three parts, i.e. upper, middle and lower, each of the same length (corresponded approximately to the vertebral levels of $C_1 \sim T_2$, $T_3 \sim T_9$ and $T_{10} \sim S_5$, respectively), and each part was cut into small pieces of about 1 mm thickness by free hand. The sciatic nerve of both sides was removed from the roots to the peripheral end and the perineurium was carefully removed. About 100 mg of each tissue was incubated in 2 ml of Krebs–Ringer phosphate buffer containing 0·011 M-glucose in a small flask with a glass stopper at 37° under the normal atmosphere. After a preincubation period of 5 min 0·5 μCi of either L-[U-^{14}C]lysine (198 mCi/mmole, Daiichi Chemical Co.) or L-[^{35}S]-methionine (maximum 49·3 mCi/mmole, Comissariat A L'Energie Atomique) were added to the flask. After 2 hr incubation reaction was stopped by adding ice-cold 20% TCA. Tissue was removed from the flask into a tube containing 10% TCA, homogenized by Ultra-Turrax and the protein fraction was extracted as previously described.[1] The protein sample was then dissolved in 1 N-NaOH solution at 80° in 15 min. An aliquot of the protein solution was used for the estimation of radioactivity by scintillation counting using a dioxane–toluene–Cab-O-Sil gel scintillator, and another for protein content by the method of Lowry *et al.*[4] The specific activity was expressed as dis/min/milligram protein. Time course of the penetration of [^{14}C]lysine from the suspending medium to the tissues was also determined by counting the radioactivity in the incubation medium and in the first 10% TCA extract. The rate of penetration was expressed as follows: dis/min in the TCA extract from 1 g wet tissue/dis/min in millilitre medium.

To see the effect of metabolic inhibitors on the amino acid incorporation, either potassium cyanide (1×10^{-3} M) or 2,4-dinitrophenol (3×10^{-5} M) was added to some of the incubation flasks immediately before the labelled amino acid was added.

Autoradiographic study. About 5 mm lengths of the three parts of the spinal cord and the sciatic nerve were incubated with [^{14}C]lysine as already described. After the incubation they were fixed in Bouin solution for one night. Autoradiography of paraffin embedded sections was undertaken by the dipping method using Sakura NR-M2 emulsion. Exposure was for 5 months at 5°.

RESULTS

Incorporation of [^{14}C]lysine into the protein fraction of nervous tissues of the control rats. Figure 1 showed time course of the incorporation of [^{14}C]lysine into proteins of the brain, spinal cord, sciatic nerve and liver of control rats. The incorporation proceeded in each tissue, linearly, for up to 3 hr. The incorporation per unit/protein weight was highest in the brain cortex followed by the sciatic nerve, liver and spinal cord. In the spinal cord the middle part showed the highest incorporation of the three (see Table 3), while protein content per unit weight of tissue was the lowest in the same part. Difference of the incorporation of the amino acid among different ages of rats observed by Matheson[5] was not found among the rats aged between 8 and 16 weeks in the present experiment as shown below in Table 3. Both potassium cyanide (1×10^{-3} M) and 2,4-dinitrophenol (3×10^{-5} M) showed inhibitory effects on the amino acid incorporation as shown in Table 1, though the incorporation gave essentially straight lines.

TABLE 1. INHIBITION OF [^{14}C]LYSINE INCORPORATION INTO THE TISSUE PROTEINS OF NORMAL RATS BY METABOLIC INHIBITORS

	Dis/min/mg protein					
	Incubation time (min)	Without inhibitor (control)	+ Potassium cyanide (1×10^{-3} M)	Control (%)	+2,4-Dinitrophenol (3×10^{-5} M)	Control (%)
Brain cortex	60	766	320	42	574	75
	120	1510	665	44	1140	76
Spinal cord middle	60	467	187	40	379	81
	120	923	386	42	838	80
Sciatic nerve	60	562	219	36	410	73
	120	1110	402	36	855	77
Liver	60	533	260	47	394	74
	120	1030	474	46	742	72

Incubation at 37° in 2 ml Krebs–Ringer phosphate buffer containing 0·011 M-glucose and 0·5 μCi L-[U-^{14}C]lysine. Values are means of three experiments.

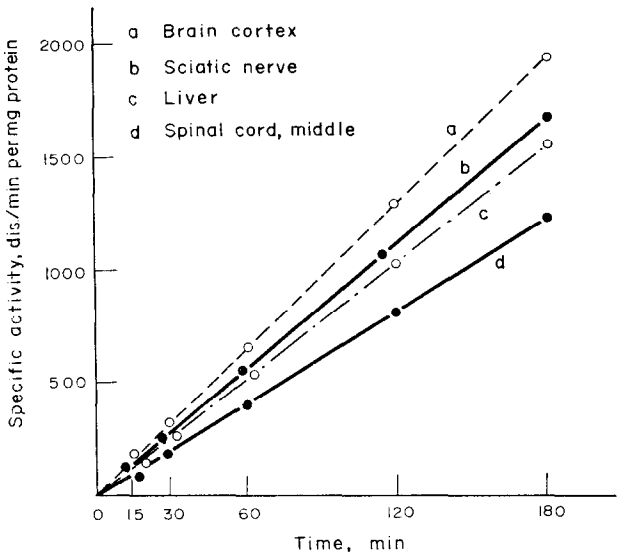

FIG. 1. Time course of [^{14}C]lysine incorporation into tissue proteins of normal rats. Tissues from 15 rats aged 8 weeks were incubated at 37° in 2 ml Krebs–Ringer phosphate buffer containing 0·011 M-glucose and 0·5 μCi L-[U-^{14}C]lysine. Each point represents the means of three experiments.

Time course of the penetration of the amino acid from the suspending medium into the tissue was measured in the sciatic nerve. As shown in Table 2 the equilibrium between them seemed to be established within 5 min after the incubation. Neither potassium cyanide nor 2,4-dinitrophenol affected the process.

Incorporation of [^{14}C]lysine in the treated rats. Under the dosing condition of acrylamide described in Materials and Methods, the animal began to show a weakness of the hindlimbs at 2 weeks, slight disturbance in walking at 3 weeks and paralysis at 4 weeks. After removing acrylamide from the diet the animal clinically recovered from paralysis in 5–6 weeks.

TABLE 2. TIME COURSE OF THE PENETRATION OF [^{14}C]LYSINE FROM THE SUSPENDING MEDIUM INTO THE SCIATIC NERVE OF NORMAL RATS, AND THE EFFECTS OF METABOLIC INHIBITORS ON THE PENETRATION

Incubation time (min)	Rate of penetration		
	Without inhibitor (control)	+Potassium cyanide (1×10^{-3} M)	+2,4-Dinitrophenol (3×10^{-5} M)
5	1·46	1·58	1·46
15	1·63	1·79	1·72
30	1·70	1·84	1·80
60	1·58	1·62	1·55
120	1·60	1·64	1·64
180	1·69	1·77	1·70

Incubation at 37° in 2 ml Krebs–Ringer phosphate buffer containing 0·011 M-glucose and 0·5 μCi L-[U-^{14}C]lysine. Rate of penetration was calculated as; dis/min in the first 10% TCA extract/dis/min in millilitre suspending medium. Results are means of three experiments.

TABLE 3. INCORPORATION OF [^{14}C]LYSINE INTO THE TISSUE PROTEINS OF RATS DOSED WITH ACRYLAMIDE

Week after commencing acrylamide		Dis/min/mg protein					
		Brain cortex	Spinal cord Upper	Spinal cord Middle	Spinal cord Lower	Sciatic nerve	Liver
1	Control (5)	1310 ± 218	414 ± 93	708 ± 114	375 ± 30	1260 ± 193	1190 ± 170
	Treated (5)	1300 ± 256	395 ± 66	725 ± 96	376 ± 62	1030 ± 155	1070 ± 219
2	Control (5)	1020 ± 109	451 ± 53	715 ± 130	403 ± 14	1140 ± 117	980 ± 162
	Treated (5)	1100 ± 104	457 ± 87	694 ± 50	403 ± 84	959 ± 127*	900 ± 214
3	Control (5)	1320 ± 122	433 ± 60	689 ± 92	380 ± 33	1050 ± 153	1140 ± 146
	Treated (5)	1240 ± 177	448 ± 37	747 ± 105	418 ± 81	818 ± 112	1070 ± 91
4	Control (5)	1210 ± 107	460 ± 75	760 ± 117	410 ± 44	1060 ± 137	900 ± 200
	Treated (5)	1330 ± 256	640 ± 78†	1160 ± 195†	770 ± 104†	1270 ± 242	802 ± 134
6	Control (5)	1170 ± 191	410 ± 39	779 ± 89	461 ± 42	985 ± 130	944 ± 128
	Treated (5)	1300 ± 118	656 ± 107†	1400 ± 169†	990 ± 266†	1810 ± 304†	912 ± 83
8	Control (5)	990 ± 162	402 ± 83	671 ± 66	372 ± 39	900 ± 139	1240 ± 154
	Treated (5)	1140 ± 190	653 ± 101†	1200 ± 228†	587 ± 108†	1290 ± 195†	1300 ± 210

Acrylamide 500 ppm in a powder diet was given until the end of the 4th week. Incubation for 2 hr at 37° in 2 ml Krebs–Ringer phosphate buffer containing 0·011 M-glucose and 0·5 μCi L-[U-^{14}C]lysine. Number of observations in parentheses. Values are mean ± S.D.

* $P < 0.05$.

† $P < 0.01$.

The incorporation of [^{14}C]lysine into tissue proteins at various times after treatment was begun as shown in Table 3. In Fig. 2, the percentage incorporation of the control values after feeding acrylamide is shown. In the brain and liver no differences in the incorporation between normal and acrylamide-treated rats from the beginning to the end of the experiments were found. In the spinal cord, however, more lysine was incorporated in treated rats than in controls after 4 weeks of dosing. The incorporation continued to increase until 6 or 8 weeks and then tended to decrease of the normal level. The increase was most evident in the lower part. In the sciatic nerve, a slight decrease of the incorporation was observed after 2 and 3 weeks followed by a larger increase from 4 weeks. The maximum value was at 6 weeks as seen in the spinal cord.

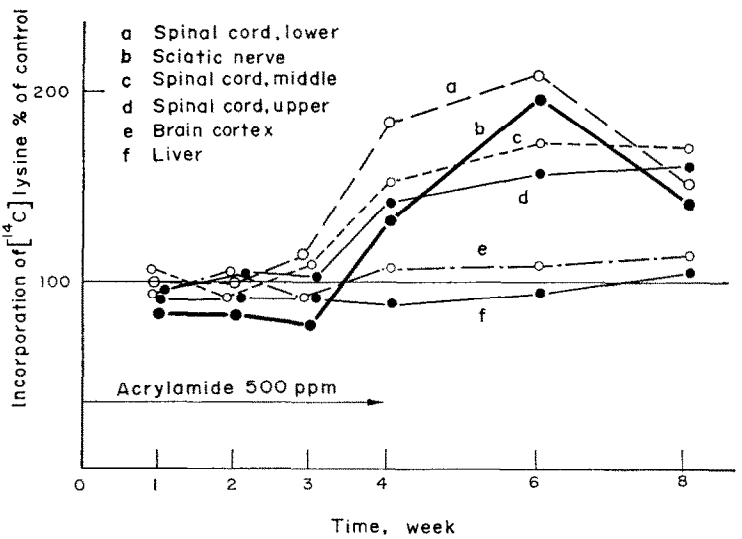

FIG. 2. [^{14}C]Lysine incorporation into tissue proteins of rats dosed with acrylamide for 4 weeks expressed as per cent of controls at various time.

The time course of the incorporation and the effect of either potassium cyanide or 2,4-dinitrophenol on it were determined in the sciatic nerve of rats treated for 4 weeks. As shown in Fig. 3 the elevated incorporation proceeded linearlly. Both potassium cyanide and 2,4-dinitrophenol inhibited the incorporation to the same degree as in the control rats.

The time course of penetration of the amino acid into tissue was also measured with sciatic nerve of rats after 4 weeks of dosing. As shown in Table 4 the concentration of the amino acid in the tissue and suspending medium reached an equilibrium within at least 10 min of incubation. Neither potassium cyanide nor 2,4-dinitrophenol affected the process.

Incorporation of [^{35}*S*]*methionine into protein.* Table 5 shows the incorporation of [^{35}S]methionine into the protein fraction of each tissue and the effect of acrylamide on the incorporation from the second to sixth week of the experiment. The control incorporation was by far the highest in the sciatic nerve followed by in the liver, the brain cortex and the spinal cord. This order of incorporation was not coincident with

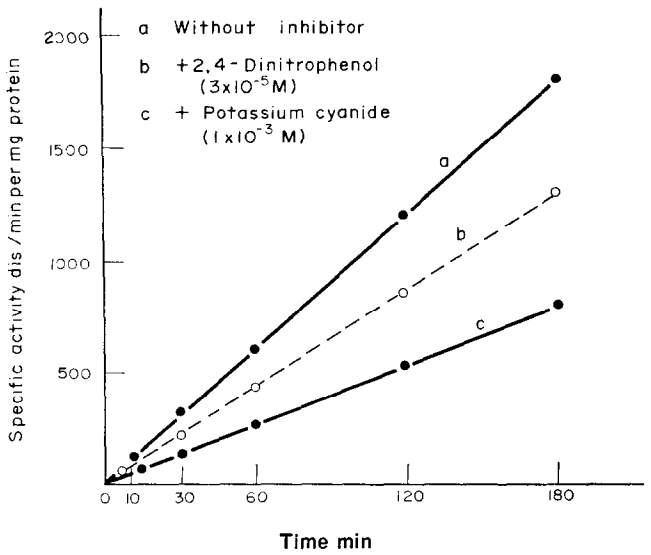

FIG. 3. Time course of [^{14}C]lysine incorporation into sciatic nerve protein of rats dosed with acrylamide for 4 weeks and the effects of metabolic inhibitors on the incorporation.

that of Richter *et al.*[6] obtained by *in vivo* study. After treatment with acrylamide for 4 weeks, a significant increase in the incorporation was also demonstrated in the spinal cord and sciatic nerve but not in the brain and liver. No decreased incorporation of methionine into the sciatic nerve protein was found at the early stage, in contrast with the results obtained with lysine.

Autoradiographic study of [^{14}C]*lysine in tissues.* After the autoradiography of the incorporated [^{14}C]lysine in tissues, a large number of silver grains were visible in the anterior horn cells in the spinal cord and in the Schwann cells in the sciatic nerve, although tissue structures were considerably broken after 2 hr incubation *in vitro* as

TABLE 4. TIME COURSE OF THE PENETRATION OF [^{14}C]LYSINE FROM THE SUSPENDING MEDIUM INTO THE SCIATIC NERVE OF RATS DOSED WITH ACRYLAMIDE FOR 4 WEEKS, AND THE EFFECTS OF METABOLIC INHIBITORS ON THE PENETRATION

Incubation time (min)	Rate of penetration		
	Without inhibitor (control)	+Potassium cyanide (1×10^{-3} M)	+2,4-Dinitrophenol (3×10^{-5} M)
10	1·48	1·65	1·43
30	1·52	1·37	1·68
60	1·38	1·63	1·46
120	1·45	1·46	1·51
180	1·53	1·47	1·39

Acrylamide 500 ppm in a powder diet was given until the end of the 4th week. Incubation at 37° in 2 ml Krebs–Ringer phosphate buffer containing 0·011 M-glucose and 0·5 μCi L-[U-^{14}C]lysine. Rate of penetration was calculated as; dis/min in the first 10%TCA extract/dis/min in millilitre suspending medium. Results are means of three experiments.

TABLE 5. INCORPORATION OF [^{35}S]METHIONINE INTO THE TISSUE PROTEINS OF RATS DOSED WITH ACRYLAMIDE

Week after commencing acrylamide		Dis/min/mg protein					
		Brain cortex	Spinal cord Upper	Spinal cord Middle	Spinal cord Lower	Sciatic nerve	Liver
2	Control (3)	1990 ± 212	1260 ± 188	1510 ± 220	1250 ± 191	7670 ± 474	2480 ± 303
	Treated (3)	2130 ± 305	1130 ± 203	1620 ± 181	1220 ± 148	7740 ± 516	2120 ± 164
3	Control (5)	1750 ± 118	1100 ± 94	1410 ± 205	1290 ± 140	8170 ± 1986	2060 ± 250
	Treated (5)	1820 ± 199	1230 ± 147	1570 ± 92	1510 ± 96	9420 ± 1780	1990 ± 81
4	Control (5)	1870 ± 105	1070 ± 180	1540 ± 246	1050 ± 166	6470 ± 903	2140 ± 135
	Treated (5)	1880 ± 206	1670 ± 243*	2500 ± 272*	1700 ± 190*	7800 ± 939	2810 ± 651
6	Control (3)	1690 ± 287	1060 ± 226	1300 ± 10	1060 ± 167	5770 ± 910	2250 ± 486
	Treated (3)	2340 ± 421	2000 ± 339*	2440 ± 81*	1910 ± 221*	10700 ± 930*	2450 ± 268

Acrylamide 500 ppm in a powder diet was given until the end of the 4th week. Incubation for 2 hr at 37° in 2 ml Krebs–Ringer phosphate buffer containing 0·011 M-glucose and 0·5 μCi L-[^{35}S]methionine. Number of observations in parentheses. Values are mean ± S.D.

* $P < 0.01$.

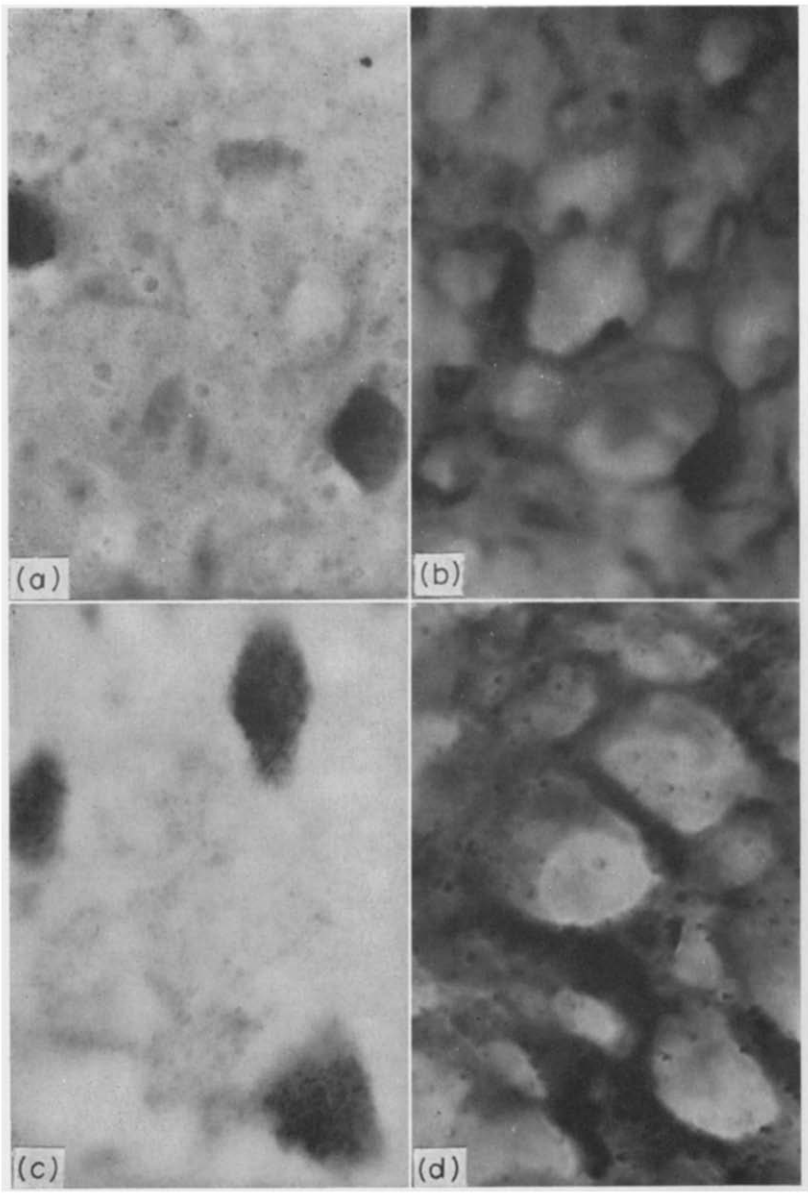

FIG. 4. Autoradiograms of [^{14}C]lysine incorporated into tissue *in vitro*. After 2 hr incubation with [^{14}C]acrylamide tissues were fixed in Bouin solution. Autoradiography of paraffin embedded sections was done by the dipping method. (a) Lower part of the spinal cord, (b) sciatic nerve, from a control rat, (c) lower part of the spinal cord, (d) sciatic nerve, from a rat dosed with acrylamide for 4 weeks.

shown in Fig. 4. No difference in mode of distribution of the silver grains was demonstrated between the control and the acrylamide-treated rats. Quantitative comparison of grains between them was not possible in this experimental condition.

DISCUSSION

In a previous report[1] it was found that acrylamide reacted *in vivo* and *in vitro* with nonprotein sulphydryls and with proteins of rat tissues, and that labelling of the protein in the nervous system with [^{14}C]acrylamide persisted for more than 14 days. These results suggested the possibility that acrylamide might affect protein metabolism in tissues and might relate to the specific cumulative nature of this compound in the body.[7] According to Simpson[8] disturbances in protein metabolism are commonly seen in peripheral neuropathy. Cavanagh and Chen[9] have recently demonstrated that amino acid incorporation into the nervous proteins is greatly altered by organomercury and bromophenyl compounds.

In the present study the incorporation of amino acids into tissue proteins has been investigated in rats poisoned with acrylamide. The incorporation was shown to be different in different tissues, being highest in the brain cortex for lysine and in the sciatic nerve for methionine among tested, and to be dependent upon aerobic energy metabolism as shown by the inhibitory action of potassium cyanide and 2,4-dinitrophenol on the incorporation. After dosing with acrylamide the incorporation was altered in the spinal cord and sciatic nerve.

The decrease of [^{14}C]lysine incorporation in the sciatic nerve at the early stage may be connected with a biochemical mechanism of the neuropathy, such as the interruption of the axoplasmic flow of protein in nerve roots shown by Pleasure *et al.*[3] and the decreased metabolism of proteins in the axons and Schwann cells. It is unclear, however, why methionine incorporation was not reduced in the same stage.

The increased incorporation of amino acids in the spinal cord may be due to an increase of protein metabolism in the anterior horn cells, in which a large number of silver grains due to [^{14}C]lysine were visible in autoradiograms. From the present study it is not easy to decide whether the increased metabolism of protein is due to a regenerating process[10] after lesions in the tissue, such as demonstrated electron-microscopically by Prineas,[11] or to the direct action of acrylamide on protein metabolism. It seems likely, however, that the increased metabolism is connected more with the regenerating process because the incorporation was elevated from the later stage of the neuropathy to the recovery stage after the withdrawal of acrylamide. Engh *et al.*[12] have recently shown by autoradiography an increased perikaryal synthetic function following peripheral axon injuries. In the present study it was also demonstrated with lysine that acrylamide did not affect the penetration of amino acid into tissues. The altered incorporation of amino acid into protein may, therefore, be attributed to the change in the protein metabolism.

The increased incorporation of amino acids into the sciatic nerve at the later stage of the neuropathy might be related to the proliferation and increased metabolism of the Schwann cells[13] in which many silver grains were demonstrated. Fullerton[14] suggested by electrophysiological and histological studies on peripheral nerves that degeneration began from the early stage of the acrylamide poisoning.

In the present study the amino acid incorporation was determined in total proteins. Clouet and Waelsch[15] showed an increased incorporation of [^{14}C]lysine into brain

protein, especially of microsomal fraction, in rats dosed with an organic phosphorus inhibitor. Caston and Singer[16] observed that amino acids were incorporated into varied macromolecules in peripheral nerves. It may, therefore, be of interest to study the effect of acrylamide on varied subfractions of nervous tissues, above all of the spinal cord and sciatic nerve.

From the fact that neither in the brain nor in the liver was the amino acid incorporation affected by acrylamide, it seems that the compound has a specific effect on the spinal cord and peripheral nerve. If the reaction of acrylamide with tissue proteins[1] plays any role in causing the lesions in the latter tissues, there should be specific structures and/or functions vulnerable to acrylamide in their proteins. The interruption of axoplasmic flow shown by Pleasure, Mishler and Engel[3] might be an evidence of this view.

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