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Electrophoretic Analysis on the Protein Alteration in the Brain of Actylamide Administered Mouse

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To investigate the neurological effect of acrylamide, whole brain of intoxicated mouse induced early hindlimbs ataxia was examined by using the methods of SDS-PAGE and two-dimensional electrophoresis. In the gel patterns by SDS-PAGE, when the patterns of each group were compared relatively, there were no remarkable changes but in the patterns of 2D-PAGE, some protein alterations were observed. Especially, the spots containing 20 (14,500, 5.64) and 21 (19,900, 6.78) were disappeared, and the spots 9 (31,300, 5.82), 11 (31,300, 5.36) and 19 (16,400, 5.42) showed marked decrease relatively in the case of treatment group. Among these changed spots, the spot 20 (14,500, 5.64) showed higher quantity than that of control group but several spots containing the spots 11 (31,300, 5.36), and 19(16.400, 5.42) were identical or equal to those of control in quantity in the case of recovery group. It seems that acrylamide might already inhibit the brain protein synthesis mechanism at the time of onset of distal neuropathy by participation in the protein metabolism so as to impair the brain regulation ability followed by a malfunction of mouse central nervous system (CNS) and recovery is gradually progressed with the dose and duration dependence after the cessation of acrylamide administration.

KEY WORDS: Acrylamide, Brain, Neurotoxicity, Electrophoresis

Acrylamide monomer ($CH_2 = CHCONH_2$) has found many uses in the mining, paper and polymer industries but data from occupational accidents have shown that acrylamide is also a neurotoxicant. It induces a slowly progressive distal axonal degeneration in many and animal subjected to chronic exposure (Fullerton and Barnes, 1966). The polymer is water soluble and believed to be non-toxic (Kuperman, 1958). It is when handling the monomer or during the process of polymerization that there is a hazard (McCollister *et al.*, 1964).

The experimental intoxication of animals with acrylamide is one of the best investigated model systems for polyneuropathies of the dying-back type. Acrylamide is often considered as specifically neurotoxic. Nervertheless, a variety of systemic effects on growth rate, food intake and faeces production has been described (Gipon *et al.*, 1977).

It was suggested that in the dying-back process a malfunction of neuronal cell bodies would result in a gradual decrease in the production of materials required for the maintenance of axonal integrity (Cavanagh, 1969). Research on metabolic changes in neuronal cell bodies of intoxicated animals has centered upon protein. Protein synthesis, determined by in vivo incorporation of leucine into proteins of brain stem, was decreased prior to onset of neuropathy (schotman *et al.*, 1978) that was related to the development of and recovery from the acrylamide neuropathy (Schotman *et al.*, 1977).

In the present study, to investigate the protein alteration caused by acrylamide intoxication the

whole brain of intoxicated animal was analyzed by electrophoretic separation using SDS-PAGE and high resolution two-dimensional gel electrophoresis.

Materials and Methods

Animal and Treatment

Male mice (10 weeks old) of an inbred strain weighing approximately 34 g were used. They were divided into three groups and housed individually in separate cages.

Acrylamide was dissolved in sterile physiological saline to deliver the desired dose and injected intraperitoneally with a dose of 50 mg/kg daily in the afternoon 6 days per week with no injection on Sunday for 2 weeks; control animals were received and equivalent volume of saline. The third group, recovery group, was further housed for 3 weeks after the final injection. Animals were allowed free access to food and water throughout the experimental period.

Tissue Taken for Electrophoretic Analysis

When animals exhibited splaying of the hindlimbs as the earlist clinical sign, they were judged to be neuropathic. On the 12th day (12 doses of 50 mg/kg = 600 mg/kg C. D.), when neurotoxicity was confirmed for the first time, the mice were sacrificed by cervical dislocation and the chest was opened and a cannula was passed through the left ventricle into the ascending aorta for perfusion. To remove all blood in the wanted tissue, perfusion was performed for 15 min with a vascular rinse solution (pH 7.4) using a peristaltic pump (Gilson Co.).

The whole brain, from olfactory lobe to medulla oblangata, was excised as possible as quickly, weighed, frozen and stored at -70°C in a deep freezer until analyzed.

Preparation of Soluble Protein

Tissues were thawed and placed in an ice-cold 10 mM tris-HCl buffer (pH 7.4) containing 5 mM $MgCl_2$. After cleaning the tissue from any adhering tissue, they were blotted dry, weighed and minced with fine scissors. All subsequent steps were carried out at 0-4°C. Tissues were homogenized in 3 volume of buffer (10 mM tris-HCl, pH 7.4; 5 mM $MgCl_2$) using a Pyrex homogenizer and sonicated in three strocks for 20 sec each at microtip limit intensity with an ultrasonic system (Model W 385) and 30 sec intervals were arranged for cooling.

The sonicated suspension was centrifuged at 4,000 g for 15 min and the supernatant fluid was further centrifuge at 15,000 g for 45 min. The obtained supernatant fluid was finally centrifuged at 10,000 rpm for 10 min. Aliquots of supernatant fluid were immediately used for electrophoresis and the remaining portions were stored at -70°C in a deep freezer.

The protein concentraion of the obtained protein solution was determined by the method of Lowry *et al.* (1951) with an UV-visible recording spectrophotometer (UV-260, Shimadzu).

SDS-polyacrylamide Gel Electrophoresis

For the electrophoretic analysis in this study, LKB protein analyzing system was used.

Discontinuous gel electrophoresis was carried out according to the method of Laemmli (1970) with 5-12.5% T linear gradieng gel (16 × 18 × 0.75 mm). The amount of 40 μg of soluble proteins, 1% SDS, 5% mercaptoethanol, 0.02% bromophenol blue, 25% glycerol and 125 mM tris--glycine buffer (pH 6.8) were applied into the well after boiling for 60 sec in a water bath. Electrophoresis was performed with a constant current of 15 mA per slab at 10°C until the tracking dye reached the bottom of the gel.

The gel was stained for 2 hrs in a staining solution containing 0.2% Commassie brilliant blue R-250, 45% methanol and 10% acetic acid , then destained for 2 hrs in a destaining solution containing 45% methanol and 10% acetic acid, and further destained for overnight in a destaining solution containing 7% acetic acid and 2% glycerol. The relative concentration of each fraction was measured by TLC recording scanner (Shimadzu) at 570 nm.

Two-dimensional Gel Electrophoresis

For the high resolution two-dimensional electrophoretic seperation, 2D-PAGE was performed according to the method of O'Farrell (1975) ex-

cept minor modifications.

Isoelectric focusing gel was prepared with ampholines (pH 3-10 and pH 5-8) as carriers and polymerized in glass tube (150 × 2.3mm, inner diameter). Then, prerunning was carried out as following schedules, 200 volts for 15 min; 300 volts for 30 min; 400 volts for 45 min. The amount of 500 μg of soluble proteins were applied to each gel tube and running was performed at 400 volts for 19 hrs, followed by a run at 800 volts for 1 hr. After the IEF was finished, the gel was gently shaken in a 5 ml of SDS equilibrium buffer for 30 min prior to being run on the SDS slab gel, then loaded on the 2D-slab gel. The remaining gels were placed in a 5 ml of same buffer and stored at -70°C for up to 1 week before being run in the second dimensional electrophoresis.

To measure pH gradient, unstained and unequilibrated IEF gel which was runned without sample solution was cut into 0.5 cm pieces and soaked overnight in a 2 ml of degassed, deionized distilled water and the pH value of each segment was measured with a pH meter.

For the second dimensional electrophoresis, all conditions were as same as those of SDS-PAGE. The second dimensional gels were stained and destained by the same methods as SDS-PAGE. All gels obtained by electrophoresis were dried with a gel drier (LKB) for further uses.

Results

SDS-polyacrylamide Gel Electrophoresis

The protein pattern obtained by SDS-PAGE in the molecular weight range of between about 250,000 and 10,000 dalton are shown in Figs. 1-3. The standard curve for the molecular weight estimation of the present study is shown in Fig. 4.

When the protein patterns of control group and of both treatment and recovery group were compared, the bands of 44,000 dalton (actin) was slightly decreased in quantity in the case of treatment goup but this band was recovered as same quantity as that of control group in the case of recovery group (Figs. 2, 3).

In general, there were no markedly changed bands but the minor quantitative changes were observed throughtout the detected bands. Thus, the portein patterns in SDS-PAGE were further examined in the spot patterns by two-dimensional electrophoretic analysis.

Two-dimensional Gel Electrophoresis

The major spots shown remarkable changes in quantity in the spot patterns obtained by 2D-PAGE are shown and numbered in Figs. 5-7. Table 1 showed the molecular weight, approximate isoelectric point and changed degree of each major spot. When the spot patterns of control group and of treatment group were compared, many spots showed quantitative changes. Especially, the spots 2, 15, 16, 20 and 21 in spot number were disappeared in the spot pattern of treatment group (Fig. 6).

In the case of recovery group, many spots among the changed spots were almost recovered as same as those of control group in quantity but the spots 1 and 20 were higher than those of control group in quantity (Fig. 7).

In addition to the quantitatively altered spots mentioned above, many other spots detected by 2D-PAGE showed minor quantitative changes, so the general biochemical sign of the acrylamide administrated mouse whole brain in the present study was the quantitative loss of protein (Figs. 5-7).

Discussion

In the first description of the pharmacological effects of acrylamide, Kuperman (1958) made the interesting observations that in cats, ataxia developed when the total dose of about 200 mg/kg of acrylamide had been reached regardless of whether it had been given in a few large doses or in many small doses over an extended period. He suspected, on physiological grounds, that the lesion responsible for the ataxia might lie in the brain stem region but made no pathological studies. By the dosing schedule in this study, the mice began to show early signs of ataxia at the 12th day of dosing when they had been given approximately 600 mg/kg.

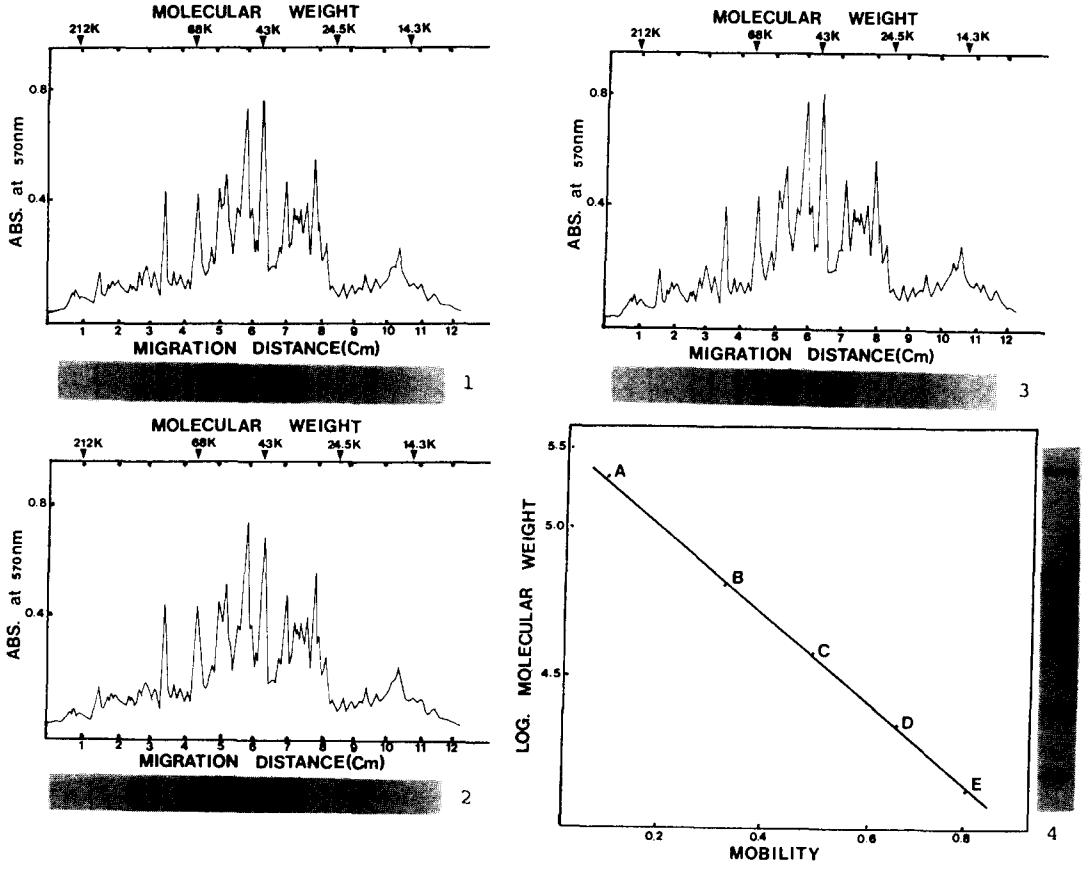


Fig. 1. SDS-polyacrylamide gel electrophoresis pattern of the mouse whole brain of control group.
Fig. 2. SDS-polyacrylamide gel electrophoresis pattern of the mouse whole brain of treatment group.
Fig. 3. SDS-polyacrylamide gel electrophoresis pattern of the mouse whole brain of recovery group.
Fig. 4. Stnadard curve for molecular weight estimation. Marker proteins were: myosin, 212,000; bovine serum albumin, 68,000; chicken egg albumin, 43,000; bovine pancreatic trypsinogen, 24,500; lysozyme, 14,300.

Cavanagh and Nalon (1982) reported that during the early phase of the intoxication there is no Wallerian degeneration of the peripheral axons, but there is a rapid loss of Purkinje cells as shown by cell counts and by the increasing numbers of necrotic cells that come to represent as much as 10% of the remaining cells and the dramatic changes in enzyme activity begins to dominate the pathological picture. In the results of present study, the marked decrease of total portein in the acrylamide administrated mouse whole brain showes the effect of acrylamide on the CNS at the time of onset of peripheral neuropathy and the recovery of altered protein in the brain of recovered mice after final injection suggested that the neuropathy is usually reversible after the cessation of acrylamide exposure with the dose and duration of exposure being the limiting factors, and long periods are required for functional recovery although incomplete.

In the previous studies, a good correlation was found between the development of a polyneuropathy during chronic acrylamide intoxication and the incorporation of leucine into porteins of spinal cord and brain stem (Gipon *et al.*, 1977). Decreases in protein systhesis rates were measured ranging from 45% for plasma proteins to 11% for liver and a similar decrease in protein synthesis rates was found in rats treated with methylenebisacrylamide (MBA), and the effect of MBA or acry-

Table 1. Molecular weight, approximate isoelectric point and degree of each major spot compared with whole brain of control group and of both treatment group and recovery group by two-dimensional gel electrophoresis.

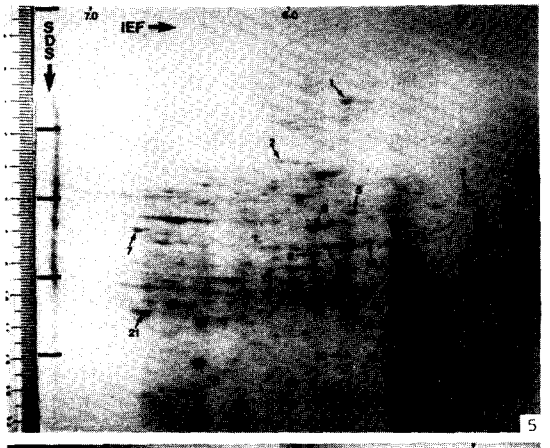
Spots (number)	Molecular weigh (dalton)	Isoelectric point (pI)	Changed degree (treatment G.)	(recovery G.)
1	86,500	5.65	−	++
2	58,800	6.01	D	R
3	45,600	4.98	−	R
4	45,100	4.96	+	+
5	39,700	5.65	−	R
6	35,500	5.83	−	R
7	34,800	6.78	−	R
8	29,900	5.74	−	R
9	31,300	5.82	−−	+
10	32,400	5.52	−−	+
11	31,300	5.36	−−	R
12	26,800	5.96	−	R
13	23,900	5.94	−	+
14	23,200	5.55	−	+
15	23,900	5.03	D	R
16	20,900	5.15	D	R
17	17,300	5.31	−−	R
18	19,900	5.65	−	R
19	16,400	5.42	−−	R
20	14,500	5.64	D	++
21	19,900	6.78	D	R
22	10,300	5.97	−	+

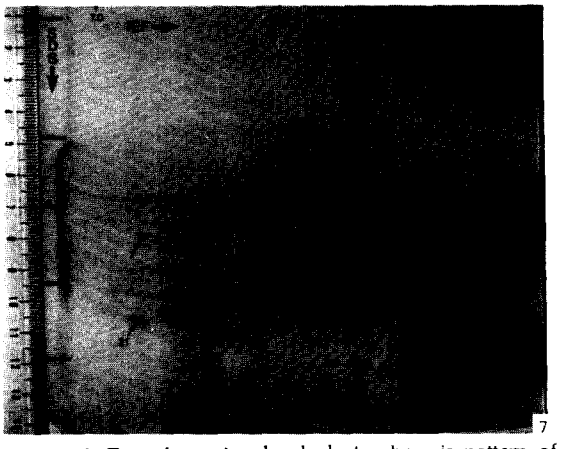
*The signs used in this table indicate the relative changes in quantity. +; slight increase, −; slight decrease, ++; marked increae, −−; marked decrease, D; disappeared, R; recovered

lamide on protein synthesis is not mediated by the lower food intake in intoxicated animals (Schotman *et al.*, 1977). The general sign of the retardation of body growth in intoxicated animals, however, is due only partly to the decrease in food consumption, since pair-fed controls showed a greater increase in body weight than their dosed counterparts (Gipon *et al.*, 1977). This may suggests that acrylamide and MBA also hamper body growth by an as yet unidentified mechanism.

It is conceivable that binding of acrylamide to tissue and plasma proteins underlies the cumulative effects of acrylamide (Hashimoto and Aldridge, 1970). The possibility that acrylamide does not act by itself as neurotoxic agent but does so indirectly via an intermediate compound or metabolic process has to be considered as well. In this respect, it is interesting that acrylamide excerted an effect on the incorporation of leucine first, and subsequently a functional inability developed at 5 and 8 hrs after a single dose of 100 mg/kg, respectively (Schotman *et al.*, 1978). A similar sequence has been observed in the chronic experiments, and following recovery from chronic intoxication an increase in leucine was detected (Schotman *et al.*, 1977).

In the further investigation of the level at which acrylamide interferes with mechanisms of protein synthesis (O'Donghue, 1985), further studies on the interference with translational processes are needed to understand the detailed effects of the neurotoxin.





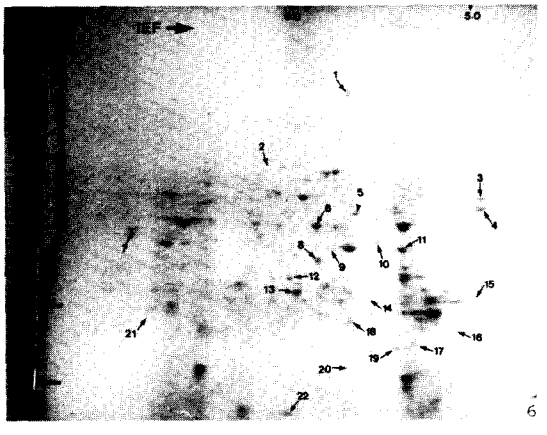


Fig. 5. Two-dimensional gel electrophoresis pattern of the normal mouse whole brain.

Fig. 6. Two-dimensional gel electrophoresis pattern of the acrylamide administrated mouse whole brain.

Fig. 7. Two-dimensional gel electrophoresis pattern of the mouse whole brain recovered after acrylamide administration.

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Acrylamide에 의한 생쥐 뇌단백질의 변화양상에 관한 전기영동적 분석

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Acrylamide의 신경독성에 대해 조사하기 위하여 초기 후지마비 증상이 유도된 생쥐의 뇌를 SDS-PAGE와 이차원 전기영동법으로 분석한 결과, SDS-polyacrylamide gel 전기영동에 의해 처리군, 대조군 및 회복군을 비교하여 보았을때 두드러진 양상의 차이를 나타내지 않았으나 이차원 전기영동상에서 실험군간의 다소 상이한 단백질 양성이 관찰되었다. 특히, 처리군에서 spot 20 (14.500, 5.64), 21 (19.900, 6.78) 및 수개의 단백질은 소실되었으며 spot 9(31.300, 5.82), 11(31.300, 5.36) 및 19(16.400, 5.42)들은 비교적 많이 감소되었다. 처리군에서 양적 변화를 보인 이들 spot중에서 spot 20 (14.500, 5.64)는 회복군에서 대조군 보다 현저히 증가하였으나 spot 11(31.300, 5.36), 19(16.400, 5.42) 및 다수의 spot들은 회복군에서 대조군과 대등한 양상을 보여주었다. 위와같은 실험결과는 acrylamide가 초기적 말초신경 장애를 나타내는 시기에 이미 뇌의 단백질대사에 관여함으로써 단백질합성이 저해되고, 이로 인하여 뇌의 조절능력이 부진하게 되어 결과적으로 중추신경계의 이상이 일어나게 된다는 것을 시사한다. 또한 손상에 대한 회복은 투여된 농도 및 기간에 의존하여 acrylamide에 대한 노출이 중지된 후 점진적인 자구적 복구에 의해 이루어지는 것으로 사료된다.