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Effect of Hexacarbons on Selected Lipids in Developing Rat Brain and Peripheral Nerves

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The effects of neurotoxic solvents, i.e. 2,5-hexanedione (2,5-HD), 2,5-hexanediol (2,5-HDiol) and the non-neurotoxic solvent, 2,4-hexanedione (2,4-HD) (500 mg/kg body wt./day, i.p.), have been studied on the lipid composition of brain and sciatic nerves in weanling rats. Five-day-old rats were administered a solvent daily for 21 days. Clinical signs of peripheral neuropathy appeared in 2,5-HD and 2,5-HDiol treated groups. Absolute weights of brain, spleen, thymus significantly decreased with 2,5-HD. Cholesterol content in whole brain homogenates and myelin was significantly reduced with 2,5-HD and 2,5-HDiol treatment. There was also a significant reduction in ubiquinone content of brain with 2,5-HD and 2,5-HDiol treatment. On exposure to neurotoxic chemicals to weanling rats, significant alteration in lipid profile was observed in the brain, which may be one of the key factors in the development of neuropathy.

INTRODUCTION

Hexacarbon neuropathy has been extensively studied.[1] Morphological changes induced by neurotoxic hexacarbons include giant axonal swellings, focal accumulation of 10-nm neurofilaments and paranodal myelin retraction in the central and peripheral nervous system.[2] The biochemical mechanisms underlying these morphological alterations are not understood. Neurotoxicity of 2,5-HD is dependent in the 1,4 (gamma) spacing of the diketone and compounds that lack the 1,4 spacing (such as 2,4-HD) do not cause peripheral neuropathy.[3] Graham *et al.*[4] have shown that the neurotoxicity of 2,5-HD is related to its chemical reactivity with the E-amine of axonal neurofilaments to form the substituted pyrrole derivatives. Some authors have indicated that 2,5-HD irreversibly inhibits certain glycolytic enzymes which are necessary to support energy dependent axonal transport.[5] Others have proposed that hexacarbon neuropathy is associated with defective lipid metabolism, particularly a deficiency in sterol metabolism.[6] It is, however, not established whether defective lipid metabolism is the important biochemical lesion or merely a reflection of nerve fibre degeneration in hexacarbon neuropathy. If defective lipid metabolism is involved in hexacarbon neuropathy, a profound reduction in the accumulation of certain lipids would be expected in the nervous tissue of 2,5-HD treated weanling rats.

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METHODS

Animals and exposure

Five-day-old weanling rats (Wistar) were used. Pups were pooled and then randomly redistributed to the nursing mother according to size, sex and weight. The litter size was limited to ten pups in each group. Water and food were available *ad libitum*. Animals were divided into four groups. An equal volume of normal saline was administered to the first group. In the second group, 2,5-hexanedione (CH_3COCH_2 CH_2COCH_3) Cat. No. 1167097, 97% supplied by Eastman Kodak Co., New York was interaperitoneally administered, at a dose level of 0.5 ml/kg after appropriate dilution in normal saline. The third group received 2,5-hexanediol (CH_3CHOH CH_2 $CHOHCH_3$) Cat. No. 4320 E. Merck, Darmstadt 99%, and the fourth group received 2,4-hexanedione ($CH_3COCH_2COCH_2CH_3$) Cat. No. 110752 Eastman Kodak Co., New York. The animals were treated daily for 21 days postnatally. Body weight and clinical signs were monitored daily.

Tissue preparation

Animals were killed by cervical dislocation. The whole brain along with liver, kidney, spleen, thymus, adrenal and segments of the sciatic nerve were carefully excised, stripped of adhering connective tissue, washed with physiological saline and weighed.

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Extraction of lipid from tissue

Lipid was extracted from brain and sciatic nerve following the method of Folch *et al.*[7] Tissues were homogenized in chilled chloroform: methanol (2:1) and filtered. A phase separation in the filtrate was induced by the addition of 0.2 vol. of 0.05 M sodium chloride and further washed twice.

Isolation of myelin membrane

Myelin from brain and sciatic nerve was isolated and purified using the methods of Sabri *et al.*[8] and Greenfield *et al.*[9] Tissues were homogenized in 0.29 M sucrose. Crude myelin was isolated from the interface of 0.29 M and 0.85 M sucrose after centrifugation at 82 500 *g* for 45 min. Crude myelin was washed three times with distilled water and stored at −20°C until use. Myelin lipid was extracted according to the procedure of Folch *et al.*[7]

Biochemical assays

Estimation of uniquinone was carried out as described by Bishop *et al.*[10] using a molar extinction coefficient (oxydized–reduced) of 12250 at 275 nm. The method of Marsh and Wenstein[11] was followed for the colorimetric determination of total lipids. Tissue triglycerides and cholesterol content were estimated according to the procedures of Fletcher[12] and Zlatkis *et al.*[13] respectively. For total phospholipid estimation, duplicate aliquots of lipid extract were dried and digested with perchloric acid. Liberated inorganic phosphorus was determined by the method of Bartlett.[14] The results were obtained as lecithin phospholipid after multiplying the inorganic phosphorus value by a factor of 25. The protein content was estimated by the procedure of Lowry *et al.*[15]

Statistical analysis

Student's *t* test for comparing control samples was used; *P* values were derived from a two-tailed table of student's value for *t*. The level of significance was chosen as $P = \text{or} < 0.05$.

RESULTS

Rats receiving 2,5-HD or 2,5-HDiol had reduced body weight gain and developed clinical signs of neuropathy (i.e. ataxia, and hind limb weakness) within 2 weeks. These clinical signs were not observed in 2,4-HD or saline-treated rats (Fig. 1).

The results in Table 1 show body and tissue weights following treatment with saline or the hexacarbon compounds. A significant reduction in wet weight was observed in the spleen, thymus and brain of 2,5-hexanedione-treated animals. However, weight reduction in 2,5-HDiol and 2,4-HD was not that pronounced (Table 1).

Table 2 summarizes the results of the lipid profile of whole brain. There appeared to be a selective depletion of certain lipids. For example, ubiquinone

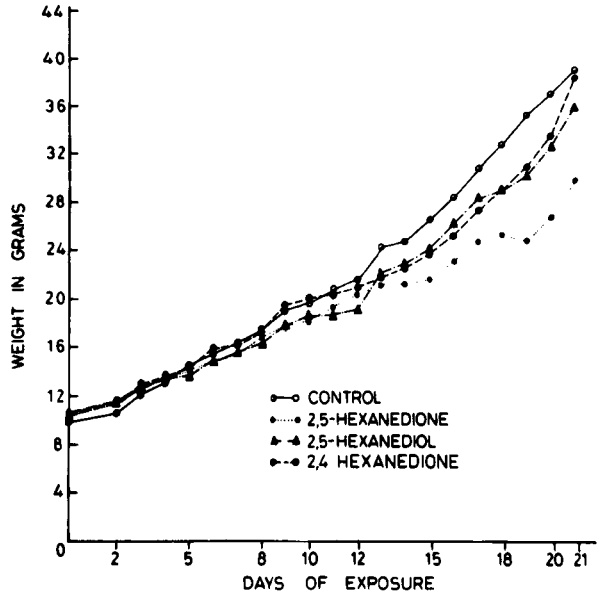

Figure 1. Effect of 2,5-hexanedione, 2,5-hexanediol and 2,4-hexanedione on body weight of rats.

(UQ) content was decreased significantly in 2,5-HDiol- and 2,5-HD-treated animals. The decrease of 2,4-HD group was not that pronounced. A significant depletion in total cholesterol content was observed in 2,5-HD-treated animals.

In the lipid profile of the sciatic nerves none of the solvents produced any alteration in total lipid, triglycerides, phospholipid or cholesterol content (Table 3). However, brain myelin lipids showed a significant decrease in phospholipid content with 2,5-HDiol treatment and the cholesterol content was significantly decreased with 2,5-HD- and 2,5-HDiol treatment. Conversely, triglyceride content was increased in 2,4-HD-treated animals (Table 4).

Hexacarbon treatment produced a significant increase in triglyceride content of sciatic nerve myelin. While the phospholipid and cholesterol contents were significantly decreased with 2,5-HD treatment (Table 5).

DISCUSSION

Weanling rats treated with either 2,5-HD or 2,5-HDiol showed signs of hexacarbon neuropathy within 2 weeks but 2,4-HD failed to produce clinical effects. This observation is consistent with the structure–activity relationship shown in adult rats.[1] A greater reactivity of gamma-diketones might be related to the slower rate of elimination from the blood and nervous tissues as compared to 2,4-HD.[16] In addition gamma diketones form pyrrole protein adducts; a pathogenic step in gamma-diketone neuropathy.[4]

Singh *et al.*[17] found severe thymus atrophy and reduced lymphoid organ weights of adult rats given 2,5-HD orally for 6 weeks. This finding is in agreement with our observations on the thymus and spleen and can be attribtuted to lymphoid organ toxicity of the solvents. The mode of action of these compounds on lymphoid organs is unknown.

Table 1. Effect of neurotoxic and non neurotoxic hexacarbon on organ weights in developing rats (after continuous daily administration 0.5 ml/kg body weight from 5th to 21st day).

Treatment	Body weight	Liver abs.	Kidney abs.	Spleen abs.	Brain abs.	Adrenals abs.*	Thymus abs.*
Saline	38.74 ± 1.64	1.69 ±0.08	0.44 ±0.02	0.142 ±0.01	1.26 ±0.02	10 ± 0.7	133 ± 14
2,5-Hexanedione	29.94 ± 2.21	1.27 ±0.09	0.36 ±0.03	0.077[a] ±0.01	0.99[a] ±0.02	8 ± 0.7	84[a] ± 10
2,5-Hexanediol	35.94 ± 1.36	1.59 ±0.08	0.43 ±0.02	0.097[a] ±0.01	1.14[a] ±0.02	9 ± 0.9	87[c] ± 10
2,4-Hexanedione	38.22 ± 3.64	2.01 ±0.13	0.46 ±0.04	0.097[a] ±0.01	1.06[a] ±0.02	10 ± 0.7	87[c] ± 10

Values are expressed as mean ± SE of ten animals.
P values: [a] ≤ 0.001, [b] ≤ 0.01, [c] ≤ 0.02, [d] ≤ 0.05
Abs. = Absolute (expressed in g) * expressed in mg.

Table 2. Effect of hexacarbon on lipids in whole brain homogenate of developing rats (after continuous daily administration 0.5 ml/kg body weight from 5th to 21st day).

Treatment group	Total lipids (mg/g wet tissue)	Triglycerides (mg/g wet tissue)	Phospholipids (mg/g wet tissue)	Cholesterol (mg/g wet tissue)	Ubiquinone (μM/g tissue)
Control	69.44±1.33	3.7 ±0.55	47.45±1.41	12.68±0.67	0.272±0.013
2,5-Hexanedione	65.89±1.78	5.33±0.60	47.25±1.27	9.69[b]±0.32	0.151[a]±0.021
2,5-Hexanediol	65.89±1.43	3.90±0.16	46.43±1.37	10.44±0.80	0.149[d]±0.033
2,4-Hexanedione	66.61±1.60	4.38±0.58	46.40±1.88	11.97±0.58	0.206±0.031

Values are expressed as mean ± SE from six rats.
P values, [a] ≤ 0.001, [b] ≤ 0.01, [c] ≤ 0.02, [d] ≤ 0.05

Table 3. Effect of hexacarbon on lipid content of sciatic nerves in weanling rat (mg/g wet tissue) (after continuous daily administration 0.5 ml/kg body weight from 5th to 21st day).

Treatment group	Total lipids	Triglycerides	Phospholipids	Cholesterol
Control	119.22±16.91	19.25±1.90	44.42±7.52	27.89±1.70
2,5-Hexanedione	118.42±11.97	18.03±3.64	44.26±8.64	31.80±3.81
2,5-Hexanediol	109.50±17.31	17.52±3.43	34.21±4.83	32.64±1.98
2,4-Hexanedione	114.11±20.07	21.16±0.80	43.77±5.79	26.79±1.07

Values are expressed as Mean ± SE of six animals.

Table 4. Effect of hexacarbon on myelin lipids in brain homogenates of weanling rats (after continuous daily administration 0.5 ml/kg body weight from 5th to 21st day).

Treatment group	Total lipids (μg/mg of protein)	Triglycerides (μg/mg of protein)	Phospholipids (μg/mg of protein)	Cholesterol (μg/mg of protein)
Control	1645±144	235±33	735±19	237±32
2,5-Hexanedione	1362±97	225±37	724±60	127[c]±15
2,5-Hexanediol	1059[a]±74	221±18	498[a]±34	154[d]±17
2,4-Hexanedione	1543±161	336[d]±32	758±72	276±37

Values are expressed as mean ± SE of six rats.
P values, [a] ≤ 0.001, [b] ≤ 0.01, [c] ≤ 0.02, [d] ≤ 0.05

Table 5. Effect of hexacarbon on lipid of sciatic nerve myelin (μg/mg protein) of weanling rats (after continuous daily administration 0.5 ml/kg body weight) from 5th to 21st day).

Treatment group	Total lipids	Triglycerides	Phospholipids	Cholesterol
Control	1991±186	318±37	843±127	306±30
2,5-Hexanedione	1950±268	460[d]±63	561±54	171[c]±19
2,5-Hexanediol	2343±180	483[a]±55	976±94	259±13
2,4-Hexanedione	2118±594	456[b]±41	726±89	225±47

Values are expressed as Mean ± SE of six rats.
P values: [a] ≤ 0.001, [b] ≤ 0.01, [c] ≤ 0.02, [d] ≤ 0.05

Nervous tissue is rich in lipids, particularly cholesterol. Any alteration in cholesterol metabolism could induce neurotoxicity.[18] The present data show reduction in cholesterol content in the brain and sciatic nerves of 2,5-HD and 2,5-HDiol treated weanling animals. However, cholesterol content was not significantly reduced in weanling rats treated with the non-neurotoxic solvent. The increase in triglycerides contents in sciatic nerve myelin of all three groups indicates a general reaction with neurotoxic and non-neurotoxic hexacarbon solvents.

The precise relationship between the inhibition of sterologenesis and the development of peripheral neuropathy is unknown. Infact inhibition of sterol biosynthesis has been reported in 2,5-HD-treated animals.[6] Cholesterol is a lipid component of neurofilaments which accounts for most of the lipid in myelin-free axons.[19] Decreased cholesterol may alter the membrane fluidity of the nerve which in turn influences the activity of NA^+, K^+-ATPase,[20] the generation of action potentials[21] and cause accumulation of 10-nm neurofilaments, which is a characteristic feature of many neuropathies[22] including hexacarbon neuropathy.[2]

Since cholesterol and ubiquinone have several common enzymes in their biosynthetic pathways, factors which influence cholesterol biosynthesis often influence ubiquinone biosynthesis. It is therefore not surprising that decreased cholesterol content is accompanied by a reduced ubiquinone content in 2,5-HD and 2,5-HDiol-treated weanling animals (Table 2). However, the reduction of UQ content was not that pronounced in 2,4-HD-treated weanling rats. A depletion in ubiquinone content is potentially more serious than inhibition of cholesterol synthesis because ubiquinone is vital for energy transformation. Gillies *et al.*[6] suggested that the inhibition of UQ biosynthesis might contribute to 2,5-HD neuropathy via disruption of oxidative phosphorylation within the nerve.[23] Since the cholesterol and ubiquinone contents in brain and nerve lipids are similarly decreased in weanling rats or adult animals treated with 2,5-HD, the altered lipid composition may be the toxic event after exposure to neurotoxic hexacarbon solvents.

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