Acta vet. scand. 1971, *12*, 551—559.

From the Department of Food Hygiene and the Department of Medicine, College of Veterinary Medicine, Helsinki, Finland.

THE EFFECT OF EXPERIMENTAL METHYL MERCURY POISONING ON THE NUMBER OF SULFHYDRYL (SH) GROUPS IN THE BRAIN, LIVER AND MUSCLE OF RAT

By

Timo J. Pekkanen and *Markus Sandholm*

Alkyl mercury compounds are considered to react rapidly in vitro with certain free and exposed protein sulfhydryl (henceforth SH) groups (*Irukayama et al.* 1965, *Kominz* 1961, *Webb* 1966 a). *Goto & Fujino* (1969) studied the in vivo effect of an organic pesticide, phenyl mercuric acetate, fed with and without added methionine on SH groups of rat liver. The number of SH groups was higher in the livers of rats fed the mercuric compound with methionine than in the livers of rats fed a similar diet without added methionine.

The in vivo effects of the well-known methyl mercury poisoning as far as SH groups are concerned have not been investigated. The poisoning causes symptoms which are mainly of a neurological character, and the pathological damage to the brain is serious (*Irukayama et al.*). Methyl mercury poisoning is also considered to affect the stability of the liver lysosomal membranes (*Pekkanen* 1971), which is known to be in relation to the function of the membrane SH groups (*Hegner* 1968).

The aim of the present investigation was to study the effect of experimental methyl mercury poisoning on the SH groups of the brain, the liver and for comparison also the muscle of the rat. In order to evaluate mercury binding to local SH groups, the mercury content of these organs was investigated.

MATERIALS AND METHODS

Test animals and their diets

Fifteen healthy adult male rats, the "experimental" rats, with weights varying between 388 and 445 g, were once a day fed a diet which contained methyl mercury in liver homogenate. Six similar rats, the "controls", received the same diet without methyl mercury. All the test animals were of the same strain and age.

The rats were kept in separate cages during the experiment and their condition was checked daily.

The methyl mercury was fed mixed with liver homogenate, which was prepared as described previously (*Pekkanen* 1971). The final homogenate fed to the rats contained 1.8 mg of Hg per g.

One g of the liver homogenate was embedded in about 5 g of minced beef, wrapped in plastic film and stored at — 25 °C. This was 1 daily rat dose. Before administration to the rats, the minced beef with the liver homogenate was kept at room temperature for 12 hrs. In addition 5—8 g of commercial rat food pellets (Orion, Helsinki, Finland) was given to each of the rats after they had eaten the minced beef with the liver homogenate. Water was available ad libitum.

When 2 of the experimental rats were found dead and the others had developed symptoms typical of the methyl mercury poisoning (*Morikawa* 1961, *Kai* 1963, *Irukayama et al.* 1965, *Ushikusa* 1965), all the rats were sacrified by decapitation; this was done on the 15th day of the experiment.

Determination of the mercury content of brain, liver and muscle of the experimental animals

After decapitation of the animals and removal of the brains, livers and the caudal femoral muscles, the mercury determination was carried out by instrumental neutron activation analysis with a Triga Mark II reactor, the neutron flux being 2×10^{12} neutrons per $cm^2 \times$ second (*Häsänen* 1970). The determinations were made from the left halves of the sagittally divided brains, the left lobes of livers and the left caudal femoral muscles of the experimental rats. For the determination the left halves of the brains were pooled and homogenized with an Ultra-Turrax homogenizer (Janke & Kunkel KG, Staufen, Germany) for a period of 2 min. From each experimental rat 1 2-g sample was taken from the lobus sinister of the liver and 1 from the left caudal femoral muscles. These were kept in separate vials and homogenized as described for the brain halves. The mercury content of these homogenates was determined.

Determination of the SH groups of the brains, livers and muscles of the test animals

The determination of the SH groups from the tissue homogenates was made by the method of *Hamm & Hoffmann* (1966), in which the SH groups are first bound with excess $AgNO_3$, reduced glutathione is then added, and finally the SH groups left are titrated amperometrically by $AgNO_3$.

Preparation of the samples

Immediately after removal of the brain, liver and caudal femoral muscles, the right half of the sagittally divided brain and an approx. 1 g sample of the liver and of the muscle was dropped in pre-weighed dark vials containing 50 ml of 0.001 M-$AgNO_3$ solution in 0.15 M Tris buffer (pH 7.4). The time between sacrification of the animals and the dropping of all the samples in $AgNO_3$ solution never exceeded 2 min.

The weights of the samples were determined and the samples were homogenized for 2 min. with an Ultra-Turrax homogenizer. The vial with the mixture was kept in ice water during homogenization. Because of the labile character of the reduced glutathione SH groups of the brain (*McIlwain* 1959), the removal and homogenization of the brain were done first, before the removal of the liver and the muscle. The removal and homogenization of the brain were done within 90 sec. after decapitation. The $AgNO_3$ with the homogenized tissue was then shaken for 1 hr. at room temperature and during this procedure protected from light.

The titration procedure

For the final analysis, a 1 ml aliquot was added to 35 ml of 0.15 M Tris buffer, pH 7.4. One ml of 0.001 M reduced glutathione in 0.00075 M-Na_2-EDTA and 0.03 M-KCl were added immediately before the titration. The SH groups which remained free were titrated at room temperature with 0.001 M-$AgNO_3$ solution. The standards were titrated by adding 1 ml of 0.001 M reduced glutathione solution to 35 ml of the Tris buffer, from which mixture the SH groups were titrated amperometrically with 0.001 M-$AgNO_3$. The titration apparatus was the same as that used by *Hamm & Hoffmann*, except that the change in the current was recorded by a writer synchronized to an $AgNO_3$ pump. By this arrangement the whole titration was performed in less than 5 min.

RESULTS

The data concerning the feeding of the methyl mercury diet and the results of the mercury determinations performed on the brains, livers and muscles of the experimental animals are given in Table 1. The toxic dose of methyl mercury to rat given orally was found to be 58.1 ± 6.1 mg Hg per kg body weight. The highest

Table 1. Experimental methyl mercury poisoning in rat. The rats were fed a diet which contained methyl mercury hydroxide in liver homogenate until they developed symptoms typical of methyl mercury poisoning.

Number of animals	Feeding of methyl mercury, mg Hg/kg body weight (mean ± s)	Hg-content, mg Hg/kg wet weight		
		brain	liver	muscle
15	(58.1 ± 6.1)	26.0	124.6	39.1

Table 2. The effect of experimental methyl mercury poisoning on the number of SH groups in the brain, liver and muscle of the rat.

	Number of animals	Number of SH groups, μM/g tissue; mean ± s		
		brain	liver	muscle
Controls	6	17.63 ± 1.12	44.75 ± 5.60	29.24 ± 1.43
Methyl mercury-fed rats	15	14.95 ± 1.97	35.89 ± 4.10	29.82 ± 4.60
Difference between means of groups		2.68	8.86	0.58
P		< 0.001	< 0.01	$(0.5 < P < 0.6)$

average mercury concentration, 124.6 mg Hg per kg wet weight, was found in the livers. The average mercury concentration in the muscles and the brains was found to be 39.1 and 26.0 mg Hg per kg wet weight respectively.

The results of the SH group determinations made on the brains, livers and muscles of the test animals are given in Table 2. The mean number of SH groups in the brains and livers of the methyl mercury fed rats was significantly*) ($P < 0.001$ and $P < 0.01$ respectively) lower than that of the controls. The mean number of SH groups in the muscle tissue did not significantly differ between the animal groups ($0.5 < P < 0.6$).

DISCUSSION

The toxic dose of methyl mercury fed to the rats, which was found to be 58.1 ± 6.1 mg Hg per kg body weight, was somewhat lower than that in the corresponding Japanese experiments, in which the toxic dose of methyl mercury for rat was found to

*) The statistical calculations were made using Student's t-test.

vary between 60 and 130 mg Hg per kg body weight (*Irukayama et al.* 1965).

When the mercury concentrations of the samples are relatively high, as in this work, repeated mercury determinations from the same samples by instrumental neutron activation analysis differ from the mean by less than ± 5 %, according to *Häsänen* (1970). The reliability of the actual mercury determinations were of the same order.

The relatively large differences between the mercury concentrations of the brains, livers and muscles are in accordance with previous results (*Takeda et al.* 1968).

To avoid oxidation of the SH groups after sacrification of the animals, the tissue samples were rapidly removed and dropped into Tris buffered 0.001 M-$AgNO_3$ solution, where they were homogenized immediately.

Because the affinity of Ag^+ seems to be greater to the SH groups of reduced glutathione than to the SH groups of proteins (*Hamm & Hoffmann* 1966), the 0.001 M reduced glutathione, which was used in the titration mixture, was added immediately before the titration. In order to avoid interference by tissue chloride, a 0.15 M Tris buffer, pH 7.4, was used during titration.

During the preliminary experiments, all procedures with the tissues were carried out under nitrogen (N_2) whenever possible to avoid oxidation of SH groups. The results of the experiments, however, proved this to be unnecessary. The results of repeated amperometric titrations of 1 μM of reduced glutathione in 34 ml of Tris buffer differed from each other by less than 2 %.

When 0.1, 0.3 and 0.4 μM of reduced glutathione was added per ml of rat liver homogenized in the $AgNO_3$ solution, the "recovery" of the added glutathione by amperometric titration was 98, 98 and 100 % respectively. Thus the method of SH determination is considered reliable enough for the present purpose.

When varying amounts, less than 1 μM, of methyl mercury hydroxide were mixed with 1 μM of reduced glutathione and the SH groups of the mixtures titrated amperometrically with $AgNO_3$, the combination of methyl mercury hydroxide with SH groups of reduced glutathione was always found to occur in a 1:1 M ratio.

The combination of $AgNO_3$ to protein SH groups is generally considered to occur in a 1:1 M ratio (*Benesch & Benesch* 1962). To investigate how the combination of CH_3HgOH with tissue SH groups occurs, 1 g of rat liver was homogenized in 50 ml of 0.15

M Tris buffer, pH 7.4. One of the 2 1-ml aliquots of this homogenate was mixed with 1 μM of CH_3HgOH and the other with 1 μM of $AgNO_3$ in 35 ml of the Tris buffer.

After a 60 min. reaction time at room temperature, during which the vials were protected from light and shaken constantly, 1 μM of reduced glutathione was added to both mixtures, and the SH groups of reduced glutathione remaining free were titrated immediately amperometrically.

As could be assumed because of the monovalent character of CH_3HgOH, since 1 of the valences of the mercury is occupied by a covalent bond with carbon, the combination with tissue SH groups was found to occur in the same 1:1 M ratio as did the combination of the $AgNO_3$.

If the combination of methyl mercury with SH groups occurs in the same 1:1 M ratio in vivo in the brain and in the liver, the average mercury content observed, 26.0 p.p.m. (parts per million) in the brain and 124.6 p.p.m. in the livers, corresponds to only 0.12 μM of SH per g of brain and 0.62 μM of SH per g of liver respectively. Thus the discovered difference between the mean number of SH groups — 2.68 μM per g of brain and 8.86 μM per g of liver — in the 2 animal groups is mainly due to other reasons than to direct binding of methyl mercury to brain and liver tissue SH groups.

The equillibrium 2 SH $\leftrightarrows$ S-S in the organism is dependent partly on glutathione reductase and also on the occurrence of reduced glutathione (*Jocelyn* 1959). The inhibition of glutathione reductase by methyl mercury, which inhibition has been found to occur in vitro by Hg^{++} and by parachloromercurybenzoate (*Webb* 1966 b), could partly explain the differences observed.

The amount of reduced glutathione in the brain is about 3 μM per g (*McIlwain* 1959) and from 3.8 to 9.65 μM per g in the liver (*Jocelyn*). Because the affinity of mercury is obviously greater to glutathione SH groups than to protein SH groups (*Webb* 1966 c), it seems reasonable to assume that all the mercury is bound to glutathione SH at least in the brains and possibly also in the livers of the experimental animals.

There is, however, some evidence that the binding of methyl mercury in the brain differs from that in the liver (*Irukayama et al.*). Here it is to be noted that the amount of reduced glutathione (*Jocelyn*) and the activity of glutathione reductase (*Roll & Lehninger* 1952) is negligible in muscle when compared to

brain and to liver, and that the mean number of SH groups in the muscles of the methyl mercury-fed rats did not differ significantly from that of the controls.

REFERENCES

Benesch, R. & R. Benesch: Determination of -SH groups in proteins. *In* Methods of Biochemical Analysis, ed. D. Glick. Interscience Publishers, New York, London 1962, vol. 8, 43—70.

Goto, T. & M. Fujino: Counteraction of sulfhydryls (SH) in rats against mercury. I. Influence of methionine on the growth of rats given a diet containing mercury pesticides, and SH in liver. J. Jap. Soc. Fd Nutr. 1969, *22,* 73—77.

Hamm, R. & K. Hoffmann: Schwefelhaltige Verbindungen des Fleisches. III. Mitteilung. Bestimmung von Sulfhydryl- und Disulfidgruppen in Myofibrillen und Muskelgewebe mit Hilfe der amprometrischen Titration. (The sulphur compounds of the meat. III report. The determination of the sulfhydryl and disulfide groups of the myofibrils and muscle using the amperometric titration). Z. Lebensmittel-Untersuch. 1966, *130,* 133—144.

Hegner, D.: Funktionelle Bedeutung von SH-Gruppen in Membranen von Lysosomalen Granula aus polymorphkernigen Rinderleucocyten. (Functional importance of sulfhydryl groups in membranes of lysosomal granules of bovine polymorphonuclear leucocytes). Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak. 1968, *261,* 239—251.

Häsänen, E.: Determination of mercury in biological material by neutron activation analysis. Acta chem. fenn. 1970, *43* B, 251—254.

Irukayama, K., F. Kai, T. Kondo, S. Ushikusa, M. Fujiki & S. Tajima: Toxicity and metabolism of methyl mercury compounds in animals — especially in relation to Minamata disease. Jap. J. Hyg. 1965, *20,* 11—21.

Jocelyn, P. C.: Glutathione metabolism in animals. *In* Glutathione. Biochemical Society Symposia No. 17, ed. E. M. Crook. Univ. Press, Cambridge 1959, 43—65.

Kai, F.: Metabolism of mercury compounds in animals administered with the shellfish from Minamata bay and methylmercuric compounds. J. Kumamoto med. Soc. 1963, *37,* 673—700.

Kominz, D. R.: Molecular changes in myosin caused by methylmercuric hydroxide. Biochim. biophys. Acta (Amst.) 1961, *51,* 456—463.

McIlwain, H.: Glutathione and neural tissues. *In* Glutathione. Biochemical Society Symposia No. 17, ed. E. M. Crook. Univ. Press, Cambridge, 1959, 66—78.

Morikawa, N.: Pathological studies on organic mercury poisoning. I. Experimental organic mercury poisoning in cats and its relation to the causative agent of Minamata disease. Kumamoto med. J. 1961, *14,* 71—86.

Pekkanen, T. J.: The effect of experimental methyl mercury poisoning on the distribution of acid phosphatase during autolysis in cat liver. Acta vet. scand. 1971, *12*, 523—535.

Roll, T. W. & A. L. Lehninger: Glutathione reductase of animal tissues. J. biol. Chem. 1952, *194*, 119—130.

Takeda, Y., T. Kunugi, O. Hoshino & Y. Ukita: Distribution of inorganic, aryl and alkyl mercury compounds in rats. Toxicol. appl. Pharmacol. 1968, *13*, 156—164.

Ushikusa, S.: Supplementary experiments on the toxicity of methylmercuric chloride in cats administered per os. Kumamoto med. J. 1965, *39*, 33—39.

Webb, J. L.: Mercurials. Chapter 7. *In* Enzyme and Metabolic Inhibitors. Acad. Press, New York and London 1966, Vol. II, a 746—768; b 773, 841; c 753.

SUMMARY

Fifteen adult male rats, the "experimental" rats, were fed a daily diet which contained methyl mercury hydroxide combined with liver homogenate. The daily dose of methyl mercury in terms of metallic mercury was 1.8 mg per rat. Six similar rats, the "controls", were fed the same diet without methyl mercury. After the experimental rats had developed symptoms typical of methyl mercury poisoning, which occurred on the 15th day of the experiment, all the test animals were decapitated. The average total dose of methyl mercury producing toxic symptoms was 58.1 ± 6.1 mg Hg per kg body weight. The sulfhydryl groups and the mercury content of the brains, livers and caudal femoral muscles of the animals were determined. The SH determinations were made by amperometric titration and the mercury determinations by neutron activation analysis.

The mean number of SH groups in the brains (14.95 ± 1.97 μM/g) and livers (35.89 ± 4.10 μM/g) of the methyl mercury-fed rats was found to be significantly ($P < 0.001$ and $P < 0.01$ respectively) lower than the corresponding means of the livers (17.63 ± 1.12 μM/g) and brains (44.75 ± 5.60 μM/g) of the controls. The number of SH groups in the muscles did not differ significantly between the animal groups ($0.5 < P < 0.6$). The mean mercury content of the brains, livers and muscles was found to be 26.0, 124.6 and 39.1 p.p.m. respectively, corresponding to 0.12, 0.62 and 0.19 μM per g of tissue. The decrease in the number of SH groups in the brains, 2.68 μM/g, and in the livers, 8.86 μM/g, of the methyl mercury-fed rats is thus considered to be due mainly to other effects of methyl mercury than to the direct binding of this compound to the tissue SH groups.

SAMMANFATTNING

Inverkan av experimentell metylkvicksilverförgiftning på mängden av sulfhydrylgrupper (SH) i hjärna, lever och muskel hos råtta.

Femton hanråttor utfodrades dagligen med ett foder som innehöll metylkvicksilverhydroksid blandat med leverhomogenat. Sex råttor,

„kontrollerna", fick samma foder utan metylkvicksilver. Den dagliga dosen av metylkvicksilver uttryckt i Hg per råtta var 1,8 mg. Då djuren efter femton utfodringsdagar visade för metylkvicksilverförgiftning typiska symptom, avlivades de och deras hjärnor, levrar och delar av kaudala femorala muskler tilvaratogs för kvicksilver- och sulfhydrylgruppbestämning. Den åt råttorna utfodrade totala medelmängden metylkvicksilver var då 58,1 ± 6,1 mg Hg per kg kroppsvikt.

Sulfhydrylbestämningarna utfördes genom amperometrisk titration och kvicksilveranalyserna medels neutronaktiveringsanalys.

Hjärnornas genomsnittliga mängd av SH grupper var 14,95 ± 1,97 μM/g och levrarnas 35,89 ± 4,10 μM/g hos de med metylkvicksilver utfodrade råttorna. Motsvarande genomsnittsvärden hos kontrollerna var 17,63 ± 1,12 μM/g i hjärnan och 44,75 ± 5,60 μM/g i levern. Differensen mellan genomsnittsvärden för hjärnorna (2,68 μM/g) och för levrarna (8,86 μM/g) var statistiskt signifikant ($P < 0{,}001$ för hjärnorna och $P < 0{,}01$ för levrarna), vilket ej var fallet med musklernas SH grupper, där metylkvicksilver ej förorsakade någon statistiskt signifikant förändring i SH gruppernas mängd ($0{,}5 < P < 0{,}6$). Hjärnornas genomsnittliga Hg mängd var 26,0, levrarnas 124,6 och musklernas 39,1 mg/kg hos metylkvicksilvergruppen motsvarande 0,12, 0,62 och 0,19 μM per g vävnad. Förminskningen av hjärnornas och levrarnas mängd av SH grupper hos metylkvicksilverråttorna (2,68 μM/g resp. 8,86 μM/g) beror alltså på andra verkningar av metylkvicksilver än på den direkta bindningen av denna förening till SH grupperna.

(Received November 19, 1970).