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THE ELEVATION OF RAT LIVER GLUTATHIONE-*S*-TRANSFERASE ACTIVITY BY ALPHA-HEXACHLOROCYCLOHEXANE

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Abstract—(1) Single intraperitoneal dose (30–200 mg/kg) of alpha-hexachlorocyclohexane elevated the activity of glutathione-*S*-transferases for 1,2-dichloro-4-nitrobenzene and 3,4,6/5 pentachlorocyclohex-1-ene in rat liver cytosol by 50–60 per cent. The activity for 1,2-epoxy-3-(p-nitrophenoxy)-propane remained unchanged. Under similar conditions, the activity of mitochondrial glutathione-*S*-transferases was also elevated, the activity of the microsomal enzyme was not. (2) Single peroral dose (200 mg/kg) of α-hexachlorocyclohexane had only little effect on the hepatic, and no effect on the intestinal glutathione-*S*-transferases. (3) Oral application of 0.2–1.0 mg/kg of alpha-hexachlorocyclohexane daily for 41 days had no effect on hepatic glutathione-*S*-transferases. (4) The inducibility of glutathione-*S*-transferases by alpha-hexachlorocyclohexane changed during the development. (5) The activity of cytosolic glutathione-*S*-transferases was also elevated by γ-hexachlorocyclohexane and 3,4,6/5-pentachlorocyclohex-1-ene.

The conjugation of glutathione with a number of electrophilic substrates is catalyzed by glutathione-*S*-transferases (RX:glutathione-*R*-transferase, EC 2.5.1.18), a family of multifunctional enzymes which were found in numerous organs of experimental animals [1]. Hepatic glutathione-*S*-transferase activity is predominantly (> 90%) localized in cytosol but smaller amounts of this enzyme were also detected in mitochondria and endoplasmic reticulum [2, 3]. The activity of glutathione-*S*-transferases was elevated by pretreating the animals with phenobarbital [4–8], 3-methylcholanthrene [5, 6, 8], 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [8, 9], 3,4-benz-(α)pyrene [6], acetaminophen [10] isosorbide dinitrate [11], DDT [12], polyhalogenated biphenyls [12, 13] and some commonly used insecticides [14].

In the present study we examined the effects of hexachlorocyclohexanes. They are known to stimulate the growth and proliferation of liver cells [15–17] and to increase the activity of some microsomal P-450-dependent enzymes [18–20]. Our previous work [21] provided evidence that the glutathione-dependent biotransformation of α-hexachlorocyclohexane is stimulated by pretreating the experimental animals with the same compound. Therefore we assumed that hexachlorocyclohexanes may be inducers for glutathione-*S*-transferases and we attempted to support this hypothesis experimentally.

MATERIALS AND METHODS

Reagents. 1,2-dichloro-4-nitrobenzene and 1-chloro-2,4-dinitrobenzene were obtained from Merck; 1,2-epoxy-3-(*p*-nitrophenoxy)-propane from Eastman. 3,4,6/5-Pentachlorocyclohex-1-ene was synthesized from α-hexacylorocyclohexane as described by Münster *et al.* [22]. Human gamma-globulin and reduced glutathione were obtained from Serva.

Determination of enzymic activity. All incubations were done at 37°. The activity of glutathione-*S*-transferase with 1,2-dichloro-4-nitrobenzene, 1-chloro-2,4-dinitrobenzene and 1,2-epoxy-3-(*p*-nitrophenoxy)-propane was determined by a slightly modified method [23] of Habig *et al.* [24]. The determination of activity for 3,4,6/5 pentachlorocyclohex-1-ene was also as described [3].

Other analytical methods. Hexachlorocyclohexane in organ extracts was determined gas-chromatographically [25] after extraction with hexane. Proteins were determined by the method of Bradford [26] using human gamma-globulin as a standard.

Animals. The experiments were done on Wistar rats. Unless otherwise stated, male rats (180–220 g) were used. The animals had free access to Altromin R pelletted diet and tap water.

The compounds which were tested as inductors for glutathione-*S*-transferases were dissolved in olive oil so that the amount of the substance required for 100 g body weight was contained in 1.0 ml of the solution. At the proper time, the animals were killed by decapitation and the required organs were excised. The preparation of cytosol [21], microsomes [3] and mitochondria [3] was as described. Intestinal mucosa which was obtained by the method of Pinkus *et al.* [27] was homogenized with an approximately three-fold volume of 0.05 mole/l *tris*-acetate, pH 7.4 and centrifugated for 1 hr at 105,000 *g*. The supernatant was used for the determination of enzymic activity.

Statistics. The results were statistically evaluated with use of the Student's *t*-test at 5 per cent level of significance.

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Table 1. The effect of a single intraperitoneal dose (200 mg/kg) of α-hexachlorocyclohexane on the activity of glutathione-*S*-transferases in liver cytosol (mean ± S.D., $n = 5$)

Interval* (days)	Group	RLW†	Specific activity‡ for 1,2-Dichloro-4-nitrobenzene	Specific activity‡ for 3,4,6/5-Pentachloro-cyclohex-1-ene	Specific activity‡ for 1,2-Epoxy-3-(*p*-nitro-phenoxy)propane
2	control	4.0 ± 0.55	8.3 ± 0.28	21.0 ± 2.05	310 ± 36
	treated	4.9 ± 0.71	11.1 ± 0.64	25.4 ± 2.61	314 ± 12
	(diff.)	+12%	+34%¶	+21%	+1%
4	control	4.1 ± 0.60	9.4 ± 0.55	20.4 ± 2.25	310 ± 36
	treated	5.4 ± 0.66	15.0 ± 0.67	32.8 ± 3.81	270 ± 25
	(diff.)	+32%¶	+59%¶	+61%¶	−13%
6	control	3.7 ± 0.17	8.1 ± 0.82	21.4 ± 3.06	310 ± 36
	treated	4.5 ± 0.43	12.9 ± 1.87	35.7 ± 2.37	381 ± 22
	(diff.)	+19%¶	+59%¶	67%¶	+22%
10	control	4.2 ± 0.20	6.9 ± 1.0	21.6 ± 3.21	
	treated	4.9 ± 0.50	7.9 ± 1.2	23.1 ± 3.98	
	(diff.)	+17%	+14%	+7%	

* Between the pretreatment and the determination of enzymic activity.
† RLW, i.e., relative liver weight (liver weight × 100: body weight).
‡ In nanomoles. $min^{-1} \cdot mg^{-1}$ of protein.
¶ Denotes a significant difference ($P < 0.05$) between the control and treated groups.

RESULTS

The effect of a single dose of α-hexachlorocyclohexane on cytosolic glutathione-S-transferase of rat liver. Two to six days after the intraperitoneal application of 200 mg/kg of α-hexachlorocyclohexane, the relative liver weight, as well as the activity of glutathione-*S*-transferases for 1,2-dichloro-4-nitrobenzene and 3,4,6/5-pentachlorocyclohex-1-ene was significantly increased. The degree of stimulation was relatively constant: in five experiments, the mean ± S.D. of the increase was 50 ± 8.8 per cent for 1,2-dichloro-4-nitrobenzene, and 61 ± 16.4 per cent for 3,4,6/5-pentachlorocyclohex-1-ene. Ten days after the application of hexachlorocyclohexane, the activity of glutathione-*S*-transferases in treated animals was usually the same as in the controls; however, in some experiments, there was still a significant difference between both groups.

In contrast, the specific activity for 1,2-epoxy-3-(*p*-nitrophenoxy)-propane was not affected by the pretreatment with α-hexachlorocyclohexane. (Table 1).

Perorally administered α-hexachlorocyclohexane was less effective. There was a similar increase of relative liver weight but only the activity for 3,4,6/5 pentachlorocyclohex-1-ene was increased 6 days after the pretreatment. The activity for other tested substrates was not affected (Table 2).

In an attempt to find out the explanation for the different effects of oral and parenteral hexachlorocyclohexane, we also measured the concentrations of the inducer in the liver. However, it turned out that two days after the application of 200 mg/kg of α-hexachlorocyclohexane, the mean concentration of that substance in the livers of 5 i.p.-treated rats was 112 ± 12.6 ng per 1 g tissue, and 105 ± 20.7 ng/g tissue in orally treated animals.

Assuming that the direct contact of highly concentrated inducer with the target organ may have some effect on enzyme biosynthesis we also measured the activity of glutathione-*S*-transferases in the intestinal mucosa of orally treated animals. For this purpose we selected two electrophilic compounds [1-chloro-2,4-dinitrobenzene and 1,2-epoxy-3-(*p*-nitrophenoxy)propane] which are known to be good

Table 2. The effect of a single peroral dose (200 mg/kg) of α-hexachlorocyclohexane on the activity of glutathione-*S*-transferase in liver cytosol (mean ± S.D., $n = 5$)

Interval* (days)	Group	RLW†	Specific activity‡ for 1,2-Dichloro-4-nitrobenzene	Specific activity‡ for 3,4,6/5-Pentachloro-cyclohex-1-ene	Specific activity‡ for 1,2-epoxy-3-(*p*-nitro-phenoxy)propane
2	control	3.7 ± 0.16	8.7 ± 1.10	13.5 ± 2.06	248 ± 18
	treated	4.9 ± 0.34	8.5 ± 1.13	16.0 ± 2.23	231 ± 71
	(diff.)	+31%	−2%	+19%	−9%
6	control	3.7 ± 0.16	8.7 ± 1.10	13.5 ± 2.06	294 ± 53
	treated	4.8 ± 0.38	9.2 ± 1.51	21.0 ± 4.57	242 ± 46
	(diff.)	+21%¶	+6%	+56%¶	−17%
10	control	3.7 ± 0.16	8.7 ± 1.10	13.5 ± 2.06	258 ± 44
	treated	4.6 ± 0.17	9.1 ± 1.13	15.0 ± 2.97	239 ± 26
	(diff.)	+21%¶	+5%	+11%	−8%

*†‡¶—see footnotes to Table 1.

Table 3. Dose–response relationship (mean ± S.D.)

Dose* (mg/kg)	*n*	Specific activity† for 1,2-Dichloro-4-nitrobenzene	Specific activity† for 3,4,6/5-Pentachlorocyclohex-1-ene
0‡	10	9.9 ± 0.65	20.0 ± 3.20
3	5	9.4 ± 0.67	18.9 ± 1.55
10	5	9.3 ± 1.24	21.8 ± 3.15
30	5	11.9 ± 1.75¶	25.4 ± 3.54¶
50	5	15.0 ± 0.80¶	27.1 ± 6.03¶
100	5	14.3 ± 0.93¶	25.7 ± 4.23¶
200	5	15.0 ± 0.67¶	32.8 ± 3.80¶

The activity of glutathione-*S*-transferases was determined in liver cytosol 4 days after the pretreatment with α-hexachlorocyclohexane.

* α-Hexachlorocyclohexane was given i.p.

† In nanomoles·min^{-1}·mg^{-1} of protein.

‡ Control.

¶ Denotes a statistically significant difference ($P < 0.05$) to the control.

substrates for intestinal glutathione-*S*-transferases [1]. However, there was no difference in the enzymic activity between treated and control animals.

Dose–effect relationship. Various amounts of α-hexachlorocyclohexane (3–200 mg/kg) were injected i.p. and the activity of cytosolic glutathione-*S*-transferases was measured with 1,2-dichloro-4-nitrobenzene and 3,4,6/5-pentachlorocyclohex-1-ene as electrophilic substrates. In each case, the lower effective dose was 30 mg/kg (Table 3).

Repeated stimulation. At the beginning of the experiment, all animals received 200 mg/kg of α-hexachlorocyclohexane i.p. The rats were killed 6, 10 and 16 days later and the activity of glutathione-*S*-transferases in liver cytosol was measured. Another group of animals received a second dose of α-hexachlorocyclohexane at the 10th day. The rats of this group were killed 6 days later, i.e., 16 days after the start of the experiment.

The results which are summarized in Table 4 demonstrate that the first and second dose of α-hexachlorocyclohexane produced the same degree of elevation of activity of cytosolic glutathione-*S*-transferases.

Long-term application. The animals were treated with low oral doses (0.2 and 1.0 mg/kg) of α-hexachlorocyclohexane for 41 days. Two days after the last application, the rats were killed and the activity of hepatic glutathione-*S*-transferases was determined.

However, it turned out that under such experimental conditions, the tested substance had no effect on the activity of hepatic glutathione-*S*-transferases.

The effect of α-hexachlorocyclohexane on hepatic particle-bound glutathione-S-transferases. The activity of mitochondrial glutathione-*S*-transferases was significantly increased by pretreatment with a single dose (200 mg/kg) of α-hexachlorocyclohexane. On the other hand, the pretreatment had no effect on the microsomal enzyme (Table 5).

The effect of age and sex. As may be seen in Table 6, the activity of glutathione-*S*-transferases was low in newborn rats. Then, it increased continuously and at the age of 42 days, it was nearly as high as in adult animals. The specific activity was usually higher in males, but this difference was seldom statistically significant.

The susceptibility of cytosolic glutathione-*S*-transferases to the stimulation with α-hexachlorocyclohexane depended on the age. The pretreatment had no effect in 8-day-old animals but the response to the stimulation increased markedly in later stages in development so that in 14- and 21-day-old rats the degree of elevation of enzymic activity was 2 to 5 times higher than in adult animals. There were no essential differences in the responses of male and female rats.

The effect of some related compounds. In addition, two other stereoisomers of hexachlorocyclohexane (β and γ) and a metabolite of α-hexachlorocyclo-

Table 4. The effect of repeated application of α-hexachlorocyclohexane on the activity of glutathione-*S*-transferases in rat liver cytosol (mean ± S.D., $n = 7$)

Day	Treatment* Group 1	Treatment* Group 2	Specific activity† for 1,2-Dichloro-4-nitrobenzene Group 1	Specific activity† for 1,2-Dichloro-4-nitrobenzene Group 2	Specific activity† for 3,4,6/5-Pentachlorocyclohex-1-ene Group 1	Specific activity† for 3,4,6/5-Pentachlorocyclohex-1-ene Group 2
0†	200 mg/kg	200 mg/kg	6.2 ± 0.67		15.4 ± 1.77	
6			8.4 ± 1.69¶		20.6 ± 3.66¶	
10		200 mg/kg	6.8 ± 1.23		20.3 ± 3.63¶	
16			6.1 ± 0.87	7.6 ± 0.96¶	16.0 ± 1.70	19.7 ± 4.68¶

* α-Hexachlorocyclohexane was given i.p.

† Control.

‡ In nanomoles·min^{-1}·mg^{-1} of protein.

¶ Denotes a significant difference ($P < 0.05$) to the control.

Table 5. The effect of α-hexachlorocyclohexane on the particle-bound gluthatione-*S*-tranferases. The activity is given in nanomoles·min^{-1}·mg^{-1} of protein (mean ± S.D.)

Interval* (days)	Group	Mitochondrial GST† ($n = 6$) Electrophilic substrate		Microsomal GST ($n = 5$) Electrophilic substrate
		1-chloro-2,4-dinitrobenzene	3,4,6/5-pentachloro-cyclohex-1-ene	3,4,6/5-pentachloro-cyclohex-1-ene
	control			0.71 ± 0.098
1	treated			0.84 ± 0.320
	(diff.)			+18%
	control	73.8 ± 16.06	1.1 ± 0.41	0.71 ± 0.098
6	treated	93.3 ± 6.85	1.8 ± 0.08	0.64 ± 0.032
	(diff.)	+26%¶	+64%¶	−10%
	control			0.62 ± 0.050
10	treated			0.75 ± 0.16
	(diff.)			20%

The rats received 200 mg/kg of α-hexachlorocyclohexane i.p.
* between the treatment and the determination of enzymic activity.
† GST, i.e., gluthatione-*S*-transferase.
¶ Denotes a significant difference ($P < 0.05$) to the control.

hexane, 3,4,6/5-pentachlorocyclohex-1-ene [28] were examined as possible inductors of hepatic glutathione-*S*-transferases. The dosage schedules were adapted with respect to the elimination rates and toxicity of the tested compounds. The results are summarized in Table 7.

It may be seen that β-hexychlorocyclohexane has no effect on the activity of hepatic glutathione-*S*-transferases. On the other hand, repeated applications of 10 mg/kg of the γ-isomer increased the activity for 3,4,6/5-pentachlorocyclohex-1-ene.

A single dose of 600 mg/kg of 3,4,6/5-pentachlorocyclohex-1-ene had no effect but the same amount of the inductor divided into three daily doses increased significantly the activity for 1,2-dichloro-4-nitrobenzene.

DISCUSSION

These studies are a part of our effort to recognize the hazards which arise from the exposition to organohalogens. We examined the effect of various

Table 6. The effect of age and sex on the stimulation hepatic glutathione-*S*-transferases by α-hexachlorocyclohexane (mean ± S.D.)

Age (days)	Sex	Group	n	RLW†	Specific activity‡ for 1,2-dichloro-4-nitrobenzene	Specific activity‡ for 3,4,6/5-pentachlorocyclohex-1-ene
		control	6	3.3 ± 0.57	2.1 ± 0.29	3.9 ± 1.27
3	N.D.	treated	6	3.5 ± 0.16	2.0 ± 0.24	3.7 ± 0.9
		(diff.)		+6%	−5%	−5%
		control	6	2.7 ± 0.15	4.8 ± 0.80	5.3 ± 0.49
14	N.D.	treated	6	3.9 ± 0.39	17.7 ± 2.18	7.6 ± 1.07
		(diff.)		+44%¶	+268%¶	+43%¶
		control	6	3.2 ± 0.73	4.3 ± 0.64	5.4 ± 0.69
21	M	treated	6	4.8 ± 0.37	8.2 ± 1.07	13.5 ± 2.22
		(diff.)		50%¶	+90%¶	+150%¶
		control	6	3.3 ± 0.14	3.6 ± 0.54	4.7 ± 1.05
21	F	treated	6	4.5 ± 0.17	7.3 ± 0.44	11.2 ± 1.99
		(diff.)		+36%¶	+102%¶	+138%¶
		control	5	4.6 ± 0.18	7.3 ± 0.61	18.1 ± 2.98
42	M	treated	5	5.3 ± 0.92	9.9 ± 1.50	27.3 ± 4.48
		(diff.)		+15%¶	+36%¶	+51%¶
		control	5	4.8 ± 0.30	6.1 ± 0.36	15.4 ± 1.63
42	F	treated	5	6.6 ± 0.76	8.6 ± 0.42	23.7 ± 4.05
		(diff.)		+37%¶	+41%¶	+55%¶

The rats received 200 mg/kg α-hexachlorocyclohexane i.p. and were killed 6 days later
N.D., for 'not determined'; M for 'males'; F for 'females'.
* When killed.
† RLW, i.e. relative liver weight (liver weight × 100: body weight).
‡ In nanomoles·min^{-1}·mg^{-1} of protein.
¶ Denotes a significant difference ($P < 0.05$) to the control.

Table 7. The effect of some selected substances on the activity of glutathione-*S*-transferases in rat liver cytosol (mean ± S.D., $n = 5$)

Substance	Dosage	Interval* (days)	Group	Specific activity† for 1,2-dichloro-4-nitrobenzene	Specific activity† for 3,4,6/5-pentachloro-cyclohex-1-ene	Specific activity† for 1,2-epoxy-3-(*p*-nitrophen-oxy) propane
β-Hexachlorocyclohexane	100 mg/kg	4	control	6.6 ± 1.33		
			treated	6.9 ± 0.28		
			(diff.)	+7%		
γ-Hexachlorocyclohexane	5 × 10 mg/kg‡	2	control	8.5 ± 0.52	14.8 ± 4.63	
			treated	10.2 ± 2.58	20.4 ± 2.92	
			(diff.)	+12%	+38%¶	
3,4,6/5-Pentachlorocyclohex-1-ene	600 mg/kg	3	control	10.9 ± 1.22	23.6 ± 2.32	
			treated	12.4 ± 2.79	21.7 ± 8.14	
			(diff.)	+14%	−8%	
	3 × 200 mg/kg‖	1	control	7.5 ± 0.36	21.3 ± 3.06	
			treated	8.6 ± 0.81	27.2 ± 6.68	
			(diff.)	+15%¶	+13%	

All substances given i.p.

* Between the last doses and killing the animals.

† In nanomoles·min^{-1}·mg^{-1} of protein.

‡ On five consecutive days.

‖ On three consecutive days.

¶ Denotes a significant difference ($P < 0.05$) to the control.

substances on glutathione-*S*-transferases since these enzymes are known to play an important role in the detoxification of xenobiotic compounds. For the evaluation of the presented results, practical and general aspects are to be considered separately.

It has been demonstrated that some glutathione-*S*-transferases could be stimulated by α-hexachlorocyclohexane but this occurs only under special conditions. Doses as high as 30 mg/kg at least were required and the full effect was achieved only when the inducer was given parenterally. The poor effect of oral α-hexachlorocyclohexane cannot be explained by the organ distribution of the inducer. Since the tissue concentrations of α-hexachlorocyclohexane were nearly the same in intraperitoneally and orally treated animals, some other mechanisms should be considered.

Repeated application of effective doses neither exhausted nor enhanced the capacity of the organism to increase the activity of glutathione-*S*-transferases upon stimulation with α-hexachlorocyclohexane, but a long-term oral application of subeffective quantities of the same substance has brought about almost no positive result. The rats which received 1 mg/kg of α-hexachlorocyclohexane daily for 41 days, developed an increase in relative liver weight but no significant change in the activity of glutathione-*S*-transferases.

Under given experimental conditions intestinal glutathione-*S*-transferases were entirely insensitive to the stimulation with α-hexachlorocyclohexane. In repeated experiments, we never obtained significant differences of enzymic activity in treated and control animals.

Summarizing this part of our results from the practical point of view it may be concluded that the environmental pollution with α-hexachlorocyclohexane probably does not significantly influence the activity of glutathione-*S*-transferases, for the following reasons:

(a) the threshold dose can be hardly achieved;
(b) the drug is received orally and therefore less effective;
(c) even under optimal conditions, the degree of stimulation is relatively low (about 50 per cent in the adults).

Apart from this, some data of general importance were obtained. Alpha-hexachlorocyclohexane appeared to be a selective inducer which elevates the activity of glutathione-*S*-transferases for 1,2-dichloro-4-nitrobenzene and 3,4,6/5-pentachlorocyclohex-1-ene but not for the epoxide.

3,4,6/5-Pentachlorocyclohex-1-ene also elevated the activity of some glutathione-*S*-transferases when repeatedly administered in sufficiently large doses. Nevertheless, we do not suppose that it is the active metabolite of α-hexachlorocyclohexane. The conversion of the latter substance of 3,4,6/5-pentacholorocyclohex-1-ene proceeds very slowly and the product of the reaction is rapidly metabolized [28]. Thus, only minimal tissue concentrations of 3,4,6/5-pentachlorocyclohex-1-ene were found in the animals treated with α-hexachlorocyclohexane [29] and it is not probable that such low amounts of the inducer suffice to elicit the elevation of glutathione-*S*-transferase activity.

As with the stimulation of microsomal enzymes (30), there were some differences in the effect of various isomers of hexachlorocyclohexane. Gamma-hexachlorocyclohexane increased significantly the activity of glutathione-*S*-transferases for 3,4,6/5-pentachlorocyclohex-1-ene but not this for 1,2-dichloro-4-nitrobenzene. However, any quantitative comparison is difficult since the dosage schedule must have been adapted to the high toxicity of the tested substance. Beta-hexachlorocyclohexane appeared to have no effect on hepatic glutathione-*S*-transferases.

The sensitivity of hepatic glutathione-*S*-transferases to the stimulation with α-hexachlorocyclohexane changed during the ontogenesis. The stages of areactivity, hyperreactivitiy and normoreactivity may be recognized. They coincide roughly with the phases of sexual development (infancy, prematurity, maturity) so that it may be supposed that the hormonal status plays an important role in the inducibility of glutathione-*S*-transferases by α-hexachlorocyclohexane. There were no sexual differences in the inducibility of glutathione-*S*-transferases, but, in agreement with the findings of other authors [31], we observed lower activity in untreated female rats than in the males.

Some other results (dose–response relationship, repeated stimulation, and, in part, long term application) allow us to pronounce a hypothesis concerning the mechanisms involved in the elevation of glutathione-*S*-transferases by hexachlorocyclohexane. It may be supposed that a certain threshold concentration of α-hexachlorocyclohexane (or its metabolite) in a relevant organ is required. Once achieved it triggers other biological reactions which proceed independently on the concentration of the primary inducer and result in the enhanced activity of glutathione-*S*-transferases.

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