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Phosphorylation of Rat Liver Nuclear Acidic Phosphoproteins after Administration of α-1,2,3,4,5,6-Hexachlorocyclohexane *in Vivo*

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SUMMARY

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α-1,2,3,4,5,6-Hexachlorocyclohexane (α-HCH) is known to induce mixed-function oxidases in rat liver endoplasmic reticulum and to stimulate liver cell proliferation. During the 12 hr after administration of α-HCH the phosphorylation of nuclear acidic proteins *in vivo* increased to about twice the control level. Essentially all of the increased phosphorylation after α-HCH *in vivo* involved preferential ^{32}P incorporation into fractions of phenol-soluble acidic chromatin proteins, resolved by polyacrylamide gel electrophoresis into proteins with estimated molecular weights of 15,000–25,000, 35,000–50,000, and 60,000–90,000. Although no qualitative changes in the electrophoretograms of phenol-soluble acidic chromatin proteins were observed, the incorporation of radioactive amino acids into the acidic protein fraction of rat liver nuclei *in vivo* increased 6 hr after α-HCH, suggesting a change in turnover or an increased number of phosphate acceptor sites at the time of maximal phosphorylation of acidic nuclear phosphoproteins.

INTRODUCTION

The administration *in vivo* of hormones, cyclic 3′,5′-AMP analogues (1–4), and various drugs (5–12) is known to alter normal metabolic pathways and lead to the induction of several enzymes. α-1,2,3,4,5,6-Hexachlorocyclohexane has been reported to induce mixed-function oxidases in the endoplasmic reticulum of liver cells (10–12). Considerable cellular proliferation was also seen in the livers of animals receiving α-HCH[4] (10, 13). Inhibitors of mixed-function oxidases, such as CFT 1201 (β-diethylaminoethyl diphenylpropylacetate HCl) and SKF 525 (α,β-diethylaminoethyl diphenylpropylacetate HCl), as well as other substrates (hexabarbital, cyclohexane, aminopyrine, etc.), can prevent the liver proliferation caused by α-HCH (14).

The mechanism of enzyme induction may involve changes in the tissue-specific ex-

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[4] The abbreviation used is: α-HCH, α-1,2,3,4,5,6-hexachlorocyclohexane.

pression of genetic templates in the chromatin of the affected cells (15–17). According to recent reports, changes in the phosphorylation of nuclear phosphoproteins can be correlated with experimentally induced alterations in the transcriptional activity of chromatin (18–20). Furthermore, nuclear non-histone phosphoproteins can change the transcription of isolated chromatin *in vitro* both quantitatively (19, 21) and qualitatively (18, 19). To relate the enzymatic induction to the anticipated activation of chromatin templates, the phosphorylation of acidic phosphoproteins in chromatin was studied in rat livers following the administration of α-HCH *in vivo*.

METHODS

Animals.[5] Male Sprague-Dawley rats (180–250 g), fasted for 12 hr, received orally 250 mg/kg of α-HCH (K & K Laboratories) in 5 ml of USP peanut oil. Control rats were given the same volume of peanut oil. (In short-term experiments, in which the effect of α-HCH was checked 1 and 2 hr after administration, the drug was given orally to one group and intraperitoneally to another group of rats. No significant differences between the effects of these two methods were observed.) α-HCH and oil were administered between 8 and 11 a.m.

Preparation of rat liver nuclei and chromatin. Livers were removed under ether anesthesia and immediately homogenized in ice-cold 0.32 M sucrose and 3 mM $MgCl_2$ in 0.02 M Tris-HCl buffer, pH 7.5 The homogenate was centrifuged at 800 × *g* for 10 min at 4°, and the resulting pellet was used to isolate nuclei according to Fleischer-Lambropoulos and Reinsch (22). The isolation of chromatin and criteria for its purity were given previously (23, 24). The ultraviolet absorption ratios of chromatin preparations, $A_{320}:A_{260}$ and $A_{280}:A_{260}$, were determined routinely and yielded values less than 0.1 and between 0.6 and 0.7, respectively. Protein to DNA ratios were between 2.0 and 2.2.

Phenol-soluble acidic proteins were extracted from chromatin freed of histone by the method of Teng *et al.* (19).

Labeling of acidic nuclear phosphoproteins in vivo. [^{32}P]Orthophosphoric acid (carrier-free, Schwarz/Mann), neutralized in 0.5 ml of 0.9% NaCl, was injected intraperitoneally at a dose of 2 mCi/100 g of body weight 60 min before removal of the livers.

Nuclear RNA synthesis in vitro. This was assayed by a slight modification of the method of Roeder and Rutter (25). Nuclei (40–80 μg of DNA) were incubated in a final volume of 250 μl containing 10 μmoles of Tris-HCl (pH 8.0), 0.2 μmole of $MnCl_2$, 1.0 μmole of $MgCl_2$, 0.4 μmole of 2-mercaptoethanol, 0.75 μmole of NaF, 0.2 μmole each of ATP, GTP, and CTP, 0.02 μmole of [^{3}H]UTP (specific activity 20 μCi/nmole), and either 1.0 μmole of KCl (low salt reaction) or 60 μmoles of $(NH_4)_2SO_4$ (high salt) for 10 min. The reaction was stopped by adding an excess of 10% (w/v) trichloracetic acid. The acid insoluble material was washed four times with 5% (w/v) trichloracetic acid containing 1% $Na_2P_2O_7$, then dissolved in 2,5-diphenyloxazole and *p*-bis-[2-(5-phenyloxazolyl)]benzene in toluene scintillation fluid ("NCS"), and counted in a Packard Tri-Carb liquid scintillation spectrometer.

Chromatin template activity. The reaction mixture contained the following in a final volume of 250 μl: 200 nmoles each of ATP, GTP, and CTP, 50 nmoles of [^{3}H]UTP (specific activity, 125 μCi/nmole), 10 μmoles of Tris-HCl (pH 8.0), 30 μmoles of KCl, 25 μmoles of dithiothreitol, 25 μmoles of EDTA, 625 nmoles of $MgCl_2$, 10 μl of RNA polymerase [approximately 5 units, prepared from *Escherichia coli* according to Burgess (26)], and DNA as rat liver chromatin in the amounts shown in Table 1. The mixture was incubated at 37° for 10 min, and the reaction was stopped with ice-cold 10% (w/v) trichloracetic acid–1% (w/v) pyrophosphate. After five washes with 5% (w/v) TCA with 1% (w/v) pyrophosphate, the acid-insoluble product was dissolved in the scintillation fluid described above and its radioactivity was determined.

Polyacrylamide gel electrophoresis was

[5] Animals were maintained in animal care facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care.

performed in the presence of 0.1% (w/v) sodium dodecyl sulfate, according to Wilhelm *et al.* (24).

Protein and DNA were determined according to the methods of Lowry *et al.* (27) and Burton (28), respectively.

[γ-^{32}P]ATP was prepared as described by Glynn and Chappell (29) and Weiss *et al.* (30), using [^{32}P]phosphoric acid (carrier-free, International Chemical and Nuclear Corporation). The radioactivity in the alkali-labile phosphate fraction was determined according to Kleinsmith *et al.* (31).

RESULTS

Time course of phosphorylation in vivo. The time course of phosphorylation of rat liver nuclear acidic phosphoproteins after application of α-HCH (Fig. 1) was studied by determining the specific activities 60 min after the intraperitoneal injection of $^{32}P_i$. The incorporation of $^{32}P_i$ into nuclear acidic phosphoproteins of control rats showed only minor variations. In α-HCH-treated animals $^{32}P_i$ incorporation rose gradually beginning at 2 hr, reached twice the control level at 6 hr, then declined to the control level at 24 hr, with a second, smaller increase at 40 hr. The incorporation of $^{32}P_i$ into liver nuclear acidic phosphoproteins of control rats as well as rats treated with α-HCH 6 hr before death increased linearly with pulse time up to 2 hr, reached a peak at 4 hr, and thereafter declined gradually. There was no difference in decay of radioactivity between liver nuclear acidic phosphoproteins from control rats and rats treated with α-HCH 6 hr previously, if the nuclear acidic phosphoproteins had been labeled *in vivo* with $^{32}P_i$ for 3 hr before treatment. In a series of experiments the radioactivity in the 0.2 N perchloric acid supernatant after centrifugation of liver homogenates from control rats and rats treated with α-HCH 6 hr before was determined 0.5, 1, 2, 4, 6, and 9 hr after the injection of $^{32}P_i$ and showed no difference between the treated and control groups. This result eliminated the possibility that the increase in $^{32}P_i$ incorporation after α-HCH treatment was due to an increased accumulation of ^{32}P in the liver, caused, for example, by changes in blood circulation.

In order to compare the time course of the phosphorylation of nuclear acidic phospho-

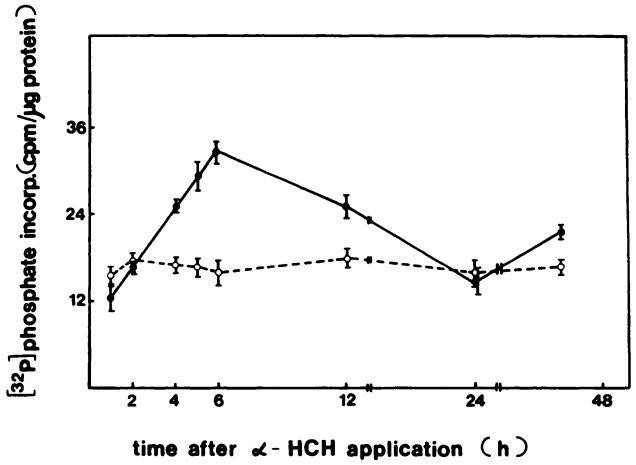

FIG. 1. *$^{32}P_i$ incorporation into acidic proteins of rat liver nuclei at different times after α-HCH* (●——●) Controls (○- - -○) received the same volume of peanut oil at the specified time. Labeling *in vivo* with 666 μCi of $^{32}P_i$ per 100 g of rat body weight and the isolation of nuclei are described under METHODS. Nuclei were washed twice with ice-cold 0.14 M NaCl, extracted twice with ice-cold 0.25 N HCl, and washed once with ice-cold chloroform- methanol (1:1 v/v) containing 0.2 N HCl and once with chloroform-methanol (2:1 v/v) containing 0.2 N HCl. The washed material was heated in 15% (w/v) trichloracetic acid at 90° for 15 min and washed four times with 5% (w/v) trichloracetic acid. The radioactivity of the alkali-labile phosphate fraction was determined and correlated with the amount of protein in the acid-insoluble pellet. Each point represents at least three assays of two to four pooled rat livers.

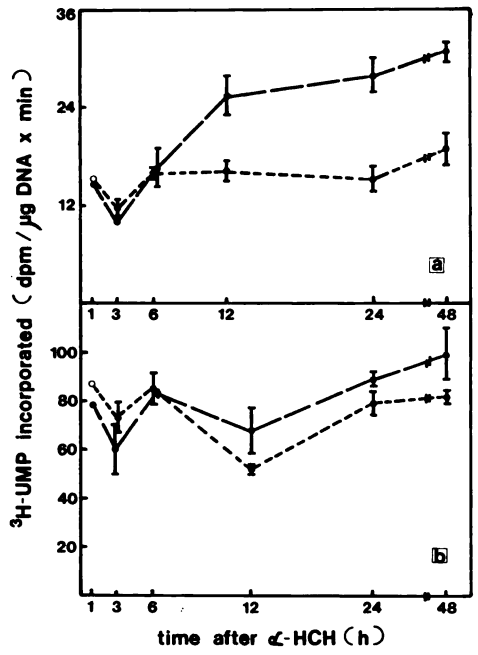

FIG. 2. *Nuclear RNA synthesis in vitro*
α-HCH or peanut oil was given to two or three rats at each time point. Liver nuclei were isolated from control rats (O-----O) and rats already treated with α-HCH (●– – –●) and incubated under low salt conditions (a) and high salt conditions (b) as described under METHODS. Points and vertical bars are arithmetic means ± one standard deviation for three experiments. [³H]UMP incorporation with enzyme inactivated by ice-cold 10% (w/v) trichloracetic acid was subtracted. The acid-insoluble radioactivity has been shown to be labile to treatment with 15% (w/v)trichloracetic acid at 90° for 15 min.

proteins after α-HCH with the anticipated changes in the restriction of genetic templates, nuclear RNA synthesis *in vitro* (Fig. 2) and the capacity of isolated rat liver chromatin to act as a template for RNA synthesis *in vitro* (Table 1) were determined.

There were variations in nuclear RNA synthesis *in vitro* from the livers of control rats as well as in the capacity of chromatin to act as a template for RNA synthesis *in vitro* (Fig. 2 and Table 1). Since the control values derived 1, 24, and 48 hr after administration of oil showed little variance, the fluctuations observed at 3, 6, and 12 hr probably reflect a circadian rhythm, as has been reported for rat liver nuclear RNA

TABLE 1

Template capacity of rat liver chromatin for RNA synthesis in vitro (V_{max}) after α-HCH

Template activity was determined as described under METHODS. Each calculated V_{max} value is based on the amount of [³H]UMP incorporated at three chromatin DNA concentrations (4, 8, and 20 μg) and represents the average of three experiments, each including two pooled rat livers for every time point. Blanks, i.e., [³H]UMP incorporation without RNA polymerase and without chromatin, were subtracted to obtain these values. Figures are arithmetic means ± one standard deviation.

Time after α-HCH or oil administration	[³H]UMP incorporated	
	Control	α-HCH
hr	*dpm/10 min*	
1	58.5	42.3
3	50.7 ± 5.5	40.6 ± 4.4
6	70.2 ± 4.7	79.5 ± 5.9
12	45.2 ± 3.0	53.3 ± 2.9
24	54.1 ± 3.3	69.8 ± 4.3
48	58.1 ± 5.3	64.9 ± 3.7

polymerase activities (32), mouse liver chromatin template activity (33) and DNA synthesis (34), rat liver histone phosphorylation (35), chromatin-bound protein kinase activity of rat kidney (36), and rat liver protein synthesis (37).

The increase in nuclear RNA synthesis *in vitro* under low salt conditions (Fig. 2) began 12 hr after α-HCH administration and was maximal at 24 and 48 hr. The increase in nuclear [³H]UMP incorporation under high salt conditions between 12 and 48 hr after α-HCH administration was insignificant. At 24 hr after α-HCH treatment a 29% increase over the control values was found in the capacity of chromatin to act as a template for RNA synthesis *in vitro* (Table 1). This is in agreement with the findings of Oberdisse *et al.* (9, 38), who reported that maximal nuclear RNA polymerase activity occurred between 24 and 48 hr after α-HCH.

Increased phosphorylation of acidic nuclear phosphoproteins, as observed during the first 12 hr after α-HCH administration, could be partially caused by an increased amount of these proteins in the chromatin. In order to examine this possibility the

TABLE 2

Incorporation of [^{14}C]amino acids into acidic nuclear (non-histone) proteins of rat liver at different times after α-HCH

[^{14}C]Protein hydrolysate, 30 μCi (Schwarz/Mann), was injected 30 min before removal of livers. Values are arithmetic means ± one standard deviation for three experiments that included two pooled rat livers at each time point. Preparation of nuclear acidic proteins was the same as described in the legend to Fig. 1. The nuclear acidic proteins were dissolved in a tolune-based scintillation mixture as described in the text, and the radioactivity was determined at the times shown, with an efficiency for ^{14}C of 65%.

Treatment	^{14}C radioactivity incorporated			
	2 hr	4 hr	6 hr	12 hr
	dpm/μg protein			
Control	3.61 ± 0.24	3.95 ± 0.38	3.82 ± 0.28	3.67 ± 0.39
α-HCH	3.74 ± 0.36	5.10 ± 0.26	5.72 ± 0.27	4.22 ± 0.26

amino acid incorporation into rat liver acidic nuclear (non-histone) proteins at different times after α-HCH treatment was determined (Table 2) and found to be increased 4 and 6 hr after α-HCH. Preliminary experiments indicated an increase in the ratio of non-histone protein to DNA ratio at those times.

To determine whether preferential phosphorylation of certain acidic nuclear proteins could result from the administration of α-HCH, the time of maximal phosphorylation (6 hr) was selected for electrophoretic analysis of the ^{32}P-labeled, phenol-soluble acidic chromatin proteins, which were isolated according to Teng *et al.* (19). The densitometric tracings of this class of chromatin proteins from α-HCH-treated (6 hr) and control rats showed no major qualitative differences (Fig. 3a). However, the distribution of ^{32}P radioactivity associated with the electrophoretically resolved fractions (Fig. 3b) showed greater phosphorylation of certain protein fractions after α-HCH treatment as compared with the controls. These fractions have estimated molecular weights of 15,000–25,000, 35,000–50,000, and 60,000–90,000 (Fig. 3b) and account for essentially all the increase in phosphorylation after α-HCH. The highly phosphorylated fractions in the molecular weight range of 15,000–25,000 represent proteins with the lowest molecular weights of the phenol-soluble acidic chromatin proteins.

DISCUSSION

The administration of α-HCH *in vivo* produces biochemical and morphological changes characteristic of liver cell proliferation (10, 13). These are preceded by changes in the expression of genetic templates in chromatin, which may be associated either with induction of several enzymes (9, 11–13, 38) or with the induction of liver growth.

More specifically, the ability of liver chromatin to support RNA synthesis *in vitro* is increased 24 hr after administration of α-HCH (Table 1). This is accompanied by increased nuclear RNA synthesis *in vitro*, with maximal values between 24 and 48 hr (Fig. 2). Similarly, nuclear rat liver RNA polymerase activity is maximal between 24 and 48 hr after α-HCH, as reported by Oberdisse *et al.* (9, 38). The increased transcriptional and proliferative activities are preceded by increased phosphorylation of acidic nuclear phosphoproteins *in vivo*.

The increased amino acid incorporation into acidic nucleoproteins after α-HCH treatment suggests that changes in the number of phosphate acceptor sites, controlled by the turnover rates of phosphoproteins, also contribute to the phosphate content of phosphoproteins. The phosphate content of chromatin phosphoproteins was shown *in vitro* to be proportional to the transcription rate (18, 20). Additionally, phosphoprotein phosphorylation *in vivo* is almost certainly influenced by protein

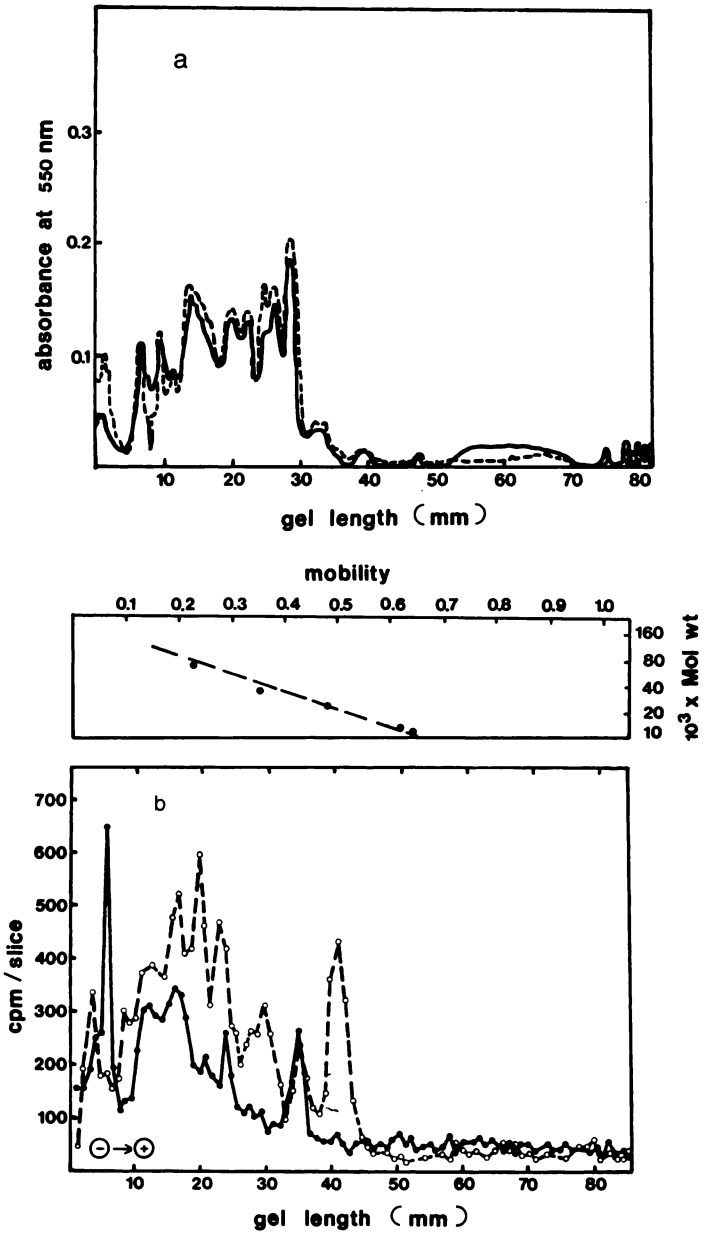

FIG. 3. *Densitometric tracings (a) and distribution of radioactivity of ^{32}P-labeled phenol-soluble acidic proteins (b) fractionated by polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate.*

Averages of pooled livers of three rats treated with α-HCH 6 hr before death (- - -) and of three control rats (——) are shown. Equal amounts of protein (160 μg/gel) were subjected in triplicate to electrophoresis for 10 hr at 8 mamp. For labeling *in vivo*, the rats received 2 mCi of $^{32}P_i$ per 100 g of body weight as described under METHODS. The upper part of Fig. 3b shows the position of marker proteins in a reference gel and the correlation between their molecular weights and mobilities. Mobilities were calculated according to Weber and Osborne (39). Bovine serum alumin (mol wt 68,000), pepsin (frog stomach, mol wt 35,000), trypsin (bovine pancreas, mol wt 13,700), and cytochrome *c* (horse heart, mol wt 12,500), all from Sigma Chemical Company, served as markers.

kinase and phosphatase activities, phosphate and nucleotide pools, cellular permeability to essential precursors, etc. It seems unlikely, however, that these factors would change in concert with a preferential increase in the amount of $^{32}P_i$ incorporated into certain acidic phosphoproteins of chromatin. The incorporation of $^{32}P_i$ into acidic (non-histone) phosphoproteins was measured 60 min after its injection when the phosphorylation of such phosphoproteins from control rats as well as from rats treated with α-HCH is expected to increase linearly. This result, together with the finding that there is no difference in decay of ^{32}P radioactivity in previously labeled liver acidic nuclear phosphoproteins from control rats and from rats treated with α-HCH for 6 hr, indicates that the observed increase in $^{32}P_i$ incorporation after α-HCH administration is due to increased phosphorylation rather than to decreased dephosphorylation.

From the distribution of incorporated ^{32}P radioactivity in the electrophoretically resolved phenol-soluble chromatin phosphoproteins, it appears that protein fractions with estimated molecular weights of 15,000–25,000, 35,000–50,000, and 60,000–90,000 account primarily for the increased phosphorylation of total acidic chromatin phosphoproteins following α-HCH treatment. Similar results were obtained recently by Johnson and Allfrey (40) after administration of cyclic AMP *in vivo*, which, by analogy with α-HCH, is known to induce several enzymes (1, 2) and to increase nuclear RNA synthesis (41). The fraction of phenol-soluble phosphoproteins with the lowest molecular weight exhibited the highest $^{32}P_i$ incorporation after cyclic AMP and after α-HCH. The turnover of acidic chromatin proteins and the possible correlation between activation of certain protein kinases and phosphorylation of distinct fractions of the acidic chromatin proteins needs to be studied.

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