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BIOCHEMICAL LESION IN RAT LIVER MITOCHONDRIA INDUCED BY HEXACHLOROPHENE*

RICHARD S. CALDWELL,† HARRY S. NAKAUE and DONALD R. BUHLER

Environmental Health Sciences Center and Department of Agricultural Chemistry, Oregon State University, Corvallis, Ore. 97331, U.S.A.

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Abstract—Mitochondria obtained from the livers of rats 18 hr after oral dosing with 20 and 100 mg/kg of 2,2′-methylenebis (3,4,6-trichlorophenol) (hexachlorophene; HCP) exhibited decreased oxidative phosphorylation, high adenosine triphosphatase (ATPase) activity, but no modification in the activities of electron transport enzymes when compared to controls. An inverse correlation between phosphorylation and the level of bound HCP was found, with 50 per cent uncoupling occurring at 0·1 mμmole HCP/mg of mitochondrial protein and complete uncoupling at approximately 0·6 mμmole/mg of protein. The concentration of HCP required for 50 per cent uncoupling *in vitro* was shown to be directly dependent on the mitochondrial protein concentration and was 0·5 μM when the latter was 2·5 mg protein/ml. Binding of HCP by mitochondrial protein was complete even when the ratio of bisphenol to protein in the incubation medium exceeded that necessary for 50 per cent uncoupling by 2000 times. Oxidative phosphorylation could be completely restored in HCP-inhibited mitochondria by the addition of a 200-fold molar excess of bovine serum albumin. In rats given a sublethal intraperitoneal injection of the drug, maximum uncoupling in liver mitochondria occurred within 15 min and complete recovery by about 6 hr. With a sublethal oral dose of HCP, maximal uncoupling occurred at 12–15 hr with complete recovery by about 36 hr. In both cases, the times for maximal uncoupling and subsequent recovery appeared to be nearly identical with the times for appearance and disappearance of gross symptomatology. Significant uncoupling of oxidative phosphorylation still occurred when less than 50 per cent of the oral or intraperitoneal LD_{50} doses were given. These observations suggest that uncoupling of oxidative phosphorylation may be the primary biochemical lesion in acute intoxication by HCP.

HEXACHLOROPHENE (HCP), 2,2′-methylenebis (3,4,6-trichlorophenol), is an antibacterial agent which has found widespread use during the last two decades in medicated soaps, powders and creams. Numerous studies dealing with the toxicity of HCP have been conducted, some of which have been recently reviewed.[1,2] However, little is known of the biochemical or physiological mechanisms through which HCP and related bisphenolic compounds are toxic. Gould *et al.*[3] reported that 0·001–0·1 mM concentrations of HCP and other bisphenols inhibited the succinoxidase activity of rat heart, liver and kidney preparations, and the same authors have shown that similar concentrations inhibited purified cytochrome oxidase and lactic acid dehydrogenase of animal origin. HCP has also been found to alter the permeability of membranes from plant and bacterial sources.[4,5] The latter results suggest that membrane dysfunction may be responsible for the spasmolytic action observed in smooth muscle.[6]

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† Present address: Pacific Fisheries Laboratory, Marine Science Center, Oregon State University, Newport, Ore. 97365.

In chronic studies, Kimbrough and Gaines[7] have recently reported extensive pathological changes in the brains of rats fed HCP in the diet.

While conducting preliminary toxicological studies of HCP in rats, we noted several symptoms similar to those displayed by rats poisoned with pentachlorophenol or other uncouplers of mitochondrial oxidative phosphorylation. These observations led us to investigate the effects of HCP on oxidative phosphorylation and associated reactions in rat liver mitochondria. A preliminary report of part of this study has been published elsewhere.[8]

MATERIALS AND METHODS

Treatment of animals. Female rats obtained from a closed colony of Wistar origin, weighing 175–225 g, were placed into separate cages without food 12 hr before initiating experiments. HCP was administered to test rats by stomach tube or intraperitoneally using corn oil as a vehicle, while control animals were given corn oil alone. All other aspects of care and handling were identical in both experimental and control rats.

Mitochondrial preparation. Rats were sacrificed by cervical fracture, and the livers were immediately excised and placed in ice-cold 0·25 M sucrose containing 0·04 M glycylglycine buffer, pH 7.4. Ten per cent homogenates were prepared using a glass homogenizer tube with a Kel-F pestle. After washing twice, mitochondria were resuspended in the homogenizing medium at a concentration of 20–25 mg protein/ml. All containers used for the preparation of mitochondria were prechilled before use and were removed from crushed ice only in a cold room to insure that the temperature of the preparation never exceeded 2°.

Determination of oxidative phosphorylation. Oxidative phosphorylation was determined by the conventional Warburg method.[9] The standard medium for oxidative phosphorylation contained the following concentrations of reactants in 3·0 ml: 43·3 mM glycylglycine buffer (pH 7·4), 13·3 mM potassium phosphate buffer (pH 7·4), 10 mM K pyruvate, 10 mM K malate, 1 mM NAD, 2·5 mM ATP, 0·027 mM K cytochrome-*c*, 5 mM $MgCl_2$, 20 mM glucose, 200 K.M. units hexokinase and 25 mM K sucrose. The center well contained a strip of pleated filter paper and 0·2 ml of 20% KOH to absorb the expired CO_2. The reaction temperature was 37°, the gas phase was air and the flasks were shaken at 110 strokes/min. The flasks were allowed to thermoequilibrate for 10 min and readings were taken for 15–30 min after tipping in the sidearm contents. After the respiration period, the flasks were quickly removed and chilled on ice, and 0·5-ml aliquots of the flask contents were pipetted into 0·5 ml of 10% trichloroacetic acid. The diluted tube contents were centrifuged and an aliquot of the supernatant was immediately used for determination of inorganic phosphate. In experiments with treated rats, mitochondria (6–8 mg protein) were placed in the sidearm and tipped in at the beginning of the respiration period. In other experiments, HCP was added directly to the phosphorylation medium and ATP, hexokinase and glucose were tipped in from the sidearm to begin the reaction. In the latter studies, the bisphenol was added to the medium in 30 μl of 95% ethanol.

Polarographic measurement of oxygen uptake. A Clark electrode was used for the polarographic determination of oxygen consumption essentially as described by Estabrook.[10] Mitochondria (6–9 mg protein) were incubated at 30° in 3 ml of a

medium containing 20 mM glycylglycine buffer (pH 7·4), 3·3 mM potassium phosphate buffer (pH 7·4), 120 mM KCl and 5 mM $MgCl_2$ to which were added microliter volumes of the reagents shown in Figs. 7 and 8. Oxygen consumption was expressed as millimicroatoms per minute. The ADP/O ratio was calculated from the millimicromoles of ADP added to the millimicroatoms of oxygen consumed during its utilization. Respiratory control indices (RCI) were defined as the ratio of oxygen uptake in the presence of ADP to that obtained after ADP exhaustion. The solubility of oxygen in the respiration medium was taken as 450 mμatoms/ml at 30°.

Determination of adenosinetriphosphatase activity. ATPase activity was determined by a modification of the method of Weinbach.[11] Each reaction tube contained 50 mM glycylglycine buffer (pH 7·4), 5 mM $MgCl_2$, 6 mM ATP, 125 mM sucrose and 0·4–0·5 mg mitochondrial protein in a 2-ml volume. The reaction was started by addition of enzyme, and the tubes were incubated for 10 min at 30°. At zero time and at 5-min intervals, a 0·5-ml aliquot was pipetted into 0·5 ml of 10% trichloroacetic acid, and the resulting mixture was diluted, centrifuged and the supernatant used for phosphate determination. All ATPase assays were done in duplicate and the results were averaged.

*Determination of cytochrome-*c *reductase activity.* Cytochrome-*c* reductase activity was determined spectrophotometrically by following the zero-order appearance of the α peak of reduced cytochrome-*c* at 550 mμ in the presence of either 0·1 mM NADH or 5 mM succinate as the electron donor.[12,13] Final concentrations of the remaining reactants were 66·7 mM potassium phosphate buffer (pH 7·4), 1 mM NaCN, 0·027 mM cytochrome-*c*, 0·167 mM sucrose and 0·04–0·05 mg mitochondrial protein in 3·0 ml. After a 10-min preincubation period, the reaction was initiated by the addition of ferricytochrome-*c*. The incubation temperature was 30°. Cytochrome-*c* concentrations were estimated from the extinction coefficients ϵ 550 mμ (reduced) = 27·7 mM^{-1} cm^{-1} and Δ^{ϵ} 550 mμ (reduced minus oxidized) = 18·5 mM^{-1} cm^{-1}.[14] Specific activity is expressed as micromoles cytochrome-*c* reduced per milligram of protein per minute.

Determination of cytochrome oxidase activity. Cytochrome oxidase activity was estimated as the first-order disappearance of the α peak of reduced cytochrome-*c* at 550 mμ.[15] Specific activity, expressed as micromoles cytochrome-*c* oxidized per milligram of protein per minute, was calculated as the product of the first-order velocity constant and the initial ferrocytochrome-*c* concentration.[16] Final concentrations of reactants were 73·4 mM potassium phosphate buffer (pH 7·4), 0·084 mM sucrose, 0·027 mM cytochrome-*c* and 0·020–0·025 mg mitochondrial protein in a final volume of 3·0 ml. Incubation temperature was 30°. The reaction was started by the addition of ferrocytochrome-*c* after a 10-min preincubation period. Cytochrome-*c* was reduced by the addition of stoichiometric amounts of sodium hydrosulfite.

Analytical methods. Mitochondria, suspended in either buffered sucrose or the oxidative phosphorylation medium, were centrifuged, washed with buffered sucrose in the latter case, and resuspended in 0·1 M glycylclycine buffer (pH 7·4) preparatory to analyzing measured aliquots, from protein and HCP.

In the *in vivo* studies, HCP was estimated as the dimethylether by a gas chromatographic assay (QF-1 on Chromsorb W) employing nitrogen as the carrier gas and a tritium electron capture detector.* Quantitative extraction of HCP was accomplished

* D. R. Buhler, to be published.

by incubation of the mitochondrial suspension with 8 vol. of benzene–95% ethanol–4 N NaOH (10:20:1) at 50° for 20 min. The entire extract was then washed into a separatory funnel with 1·7 vol. of benzene–ethanol (1:2), and an additional 1·7 vol. of hexane, 2·3 vol. of 2% Na_2SO_4 and 0·3 vol. of 1 N NaOH were added, producing a two-phase system in which HCP was retained in the aqueous phase. This phase and several 0·1 N NaOH washes were combined, acidified and extracted three times with dichloromethane. The solvent extracts were evaporated to dryness to recover HCP, which was immediately methylated with diazomethane. HCP recoveries averaged only 65 per cent in spiked samples, and appropriate corrections were applied to the unknown samples.

In the majority of the experiments *in vitro*, mitochondrial suspensions were incubated with methylene-^{14}C-HCP (specific activity, 2·71 mc/m-mole; Mallinckrodt Nuclear Company, St. Louis, Mo.) and the level of bisphenol bound to mitochondria was estimated from counting data. In this procedure, an aliquot of the washed mitocondria were solubilized in Hyamine 10-X[17] (Packard Instrument Co., Downers Grove, Ill.) and counted in a Packard model 3375 liquid scintillation counter. After eternal standardization, the amount of HCP bound to the mitochondria was calculated from the absolute radioactivity.

Protein was determined by the method of Lowry *et al.*[18] using crystalline bovine serum albumin as a standard. The method of Fiske and SubbaRow[19] was used for phosphate analyses.

Chemicals. Chemicals were either Mallinckrodt analytical reagent grade, or in the case of biochemicals, were obtained from Sigma Chemical Company. Technical or reagent grade solvents were glass-redistilled before use. Hexachlorophene, U.S.P. grade, a gift of the Givaudan Corp., was twice recrystallized from isopropanol–water.

RESULTS

The 24-hr and 72-hr oral LD_{50} values for 200-g Wistar rats receiving a corn oil solution of HCP were approximately 90 and 60 mg/kg.* Intoxicated rats showed signs of severe stress during the initial 24 hr, but most of the mortalities occurred during the second or third day after drug administration. Symptomatology included a marked elevation in rectal temperature, an increased respiratory rate, an erection and damp appearance of the pelage, listlessness, diarrhea and a paralysis of the hind extremities. Death usually occurred quietly without appreciable agitation, and rigor mortis was very rapid and complete. Rats receiving a sublethal oral dose (20 mg/kg) failed to show any visible signs of intoxication within 18 hr. The 72-hr LD_{50} for intraperitoneally administered HCP was about 25 mg/kg for 200-g rats.* Although the same symptoms of stress appeared, onset was very much more rapid and animals either died within 2 hr after injection or else survived. Similar symptoms were also observed in rats given a sublethal intraperitoneal dose (10 mg/kg), except that most of the animals had completely recovered by 24 hr.

Oxidative phosphorylation was uncoupled in liver mitochondria isolated from rats 18 hr after receiving orally administered HCP. The relationship between oral dose of HCP and the P/O ratio in isolated mitochondria is shown in Table 1. At a sublethal dose of 20 mg/kg, the P/O ratio was reduced by 59 per cent. A further reduction in P/O ratio was observed when the dose was increased to 100 mg/kg. Examination of

* H. S. Nakaue, F. N. Dost and D. R. Buhler, submitted for publication.

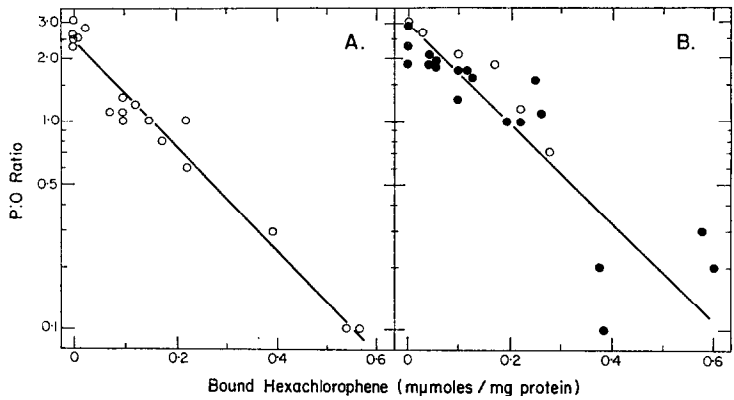

FIG. 1. Relationship between the P/O ratio and the amount of HCP bound to mitochondria. (A) Mitochondria were isolated from rats 18 hr after oral administration of HCP in corn oil. The P/O ratios were determined using the manometric procedure. HCP was analyzed in aliquots of the fresh mitochondrial suspensions by the gas chromatographic method. (B) Mitochondria obtained from untreated animals were incubated in the standard phosphorylation medium with increasing concentrations of HCP at 37°. After the phosphorylation period, the mitochondria were recovered by centrifugation, washed and analyzed for bound HCP by either the radioisotope procedure (●) or the gas chromatographic method (○).

mitochondria obtained from intoxicated rats used in these experiments revealed a correlation between the level of HCP bound to mitochondria and the observed P/O ratio (Fig. 1A). Fifty per cent uncoupling of oxidative phosphorylation by HCP occurred at about 0·1 mμmole/mg of mitochondrial protein and uncoupling was complete at about 0·6 mμmole/mg of protein.

TABLE 1. P/O RATIO AND ATPASE, CYTOCHROME OXIDASE, SUCCINATE–CYTOCHROME *c* REDUCTASE AND NADH–CYTOCHROME *c* REDUCTASE ACTIVITY OF LIVER MITOCHONDRIA ISOLATED FROM RATS 18 HR AFTER ORAL ADMINISTRATION OF HEXACHLOROPHENE*

HCP dose (mg/kg)	P/O ratio†	ATPase activity (mμmoles P_i/mg protein/min)	Cytochrome oxidase	Succinate–cytochrome *c* reductase	NADH–cytochrome-*c* reductase
			(μmoles/mg/protein/min)		
0	2·7 ± 0·2 (7)	6·4 ± 2·3 (5)	2·45 ± 0·24 (7)	0·51 ± 0·11 (7)	0·55 ± 0·06 (7)
20	1·1 ± 0·1 (6)	20·2 ± 3·0 (5)	2·04 ± 0·26 (7)	0·48 ± 0·08 (7)	0·52 ± 0·04 (7)
100	0·4 ± 0·4 (6)	18·7 ± 1·2 (5)	2·16 ± 0·26 (7)	0·51 ± 0·06 (7)	0·55 ± 0·06 (7)

* Data presented are means ± one standard deviation with the number of observations shown in parentheses.

†In these experiments, in which mitochondria were tipped in from the sidearm of the Warburg flasks to initiate the reaction, oxygen consumption rates were initially low but rapidly increased during the early measurement periods in all flasks. At each measurement period, the oxygen consumption rates of control mitochondria were highest, mitochondria from 20 mg/kg dosed rats intermediate, and those from the 100 mg/kg group lowest. We attribute these responses to permeability phenomena, rather than to an inhibitory effect on either the phosphorylating or respiratory enzymes, since they were never noted when mitochondria were preincubated with the complete respiration medium (less glucose, hexokinase and ATP) either with or without HCP.

In addition to the effect on oxidative phosphorylation, a substantial increase in ATPase activity was evident in liver mitochondria from rats intoxicated with HCP. In rats receiving HCP orally (20 mg/kg), the ATPase activity of liver mitochondria isolated 18 hr later was three times that observed in control rats. However, increasing the dose to 100 mg/kg did not result in further increase in ATPase activity (Table 1). The activities of cytochrome oxidase, succinate–cytochrome-*c* reductase and NADH–cytochrome-*c* reductase was also determined in these preparations. In contrast to the effect on oxidative phosphorylation, the activities of these three enzymes were not appreciably influenced at either dose level when compared to animals receiving only corn oil (Table 1).

In order to determine the rate of recovery of phosphorylating respiration, eight rats were given sublethal oral doses of HCP (30 mg/kg), sacrificed at various time intervals after dosing, and oxidative phosphorylation in the isolated mitochondria was then determined (Fig. 2). These results show that in animals receiving a sublethal oral dose, the maximal effect on oxidative phosphorylation occurred at about 12–15 hr after administration of the drug and that complete recovery of phosphorylation required up to 36 hr. There appeared to be a good correlation between the onset and disappearance of gross symptomatology in the dosed animals and the extent of uncoupling of the liver mitochondria.

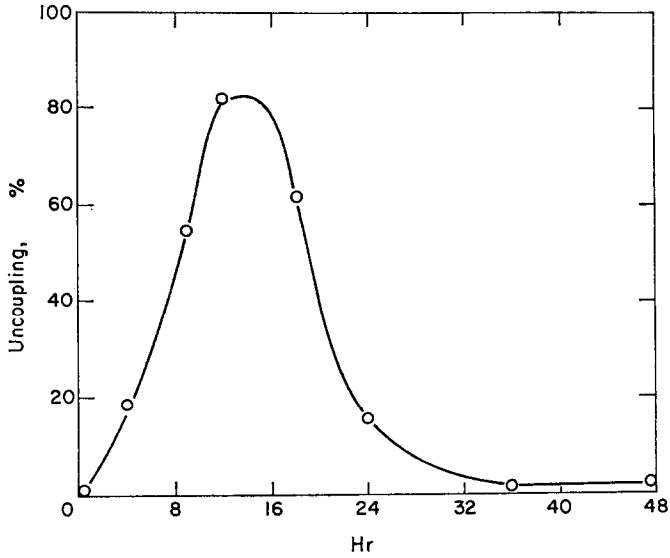

FIG. 2. Per cent uncoupling of liver mitochondria isolated from rats at various times after oral administration of 30 mg/kg of HCP. Oxidative phosphorylation was determined by the manometric procedure. Each point represents the response of one animal. The P/O ratio in mitochondria from a control rat was 2·7.

In another study, 20 rats were given intraperitoneal injections of HCP (10 mg/kg), while six control animals received only corn oil. Rats were sacrificed at various time intervals and the liver mitochondria subsequently isolated. The extent of uncoupling and the level of HCP bound to mitochondria are compared in Fig. 3. Both maximum binding of the bisphenol to mitochondria and maximum uncoupling occurred within

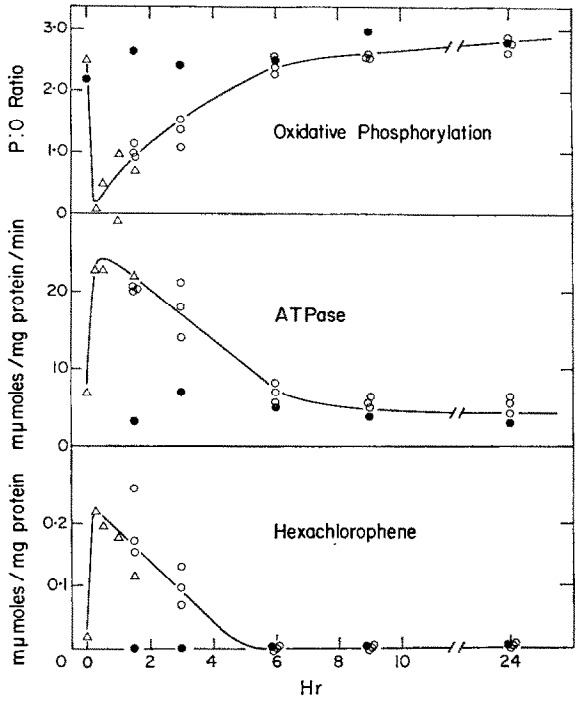

FIG. 3. Oxidative phosphorylation, ATPase activity and level of bound HCP in liver mitochondria isolated from rats at various times after intraperitoneal injection of 10 mg/kg of HCP. Each point represents the mean of duplicate or triplicate determinations on mitochondria from a single rat. The open circles (○) and triangles (△) represent the response of HCP-treated animals in two experiments, while the solid circles (●) are from corn oil-treated control rats.

15 min. Within 6 hr, phosphorylation had returned to normal and bound HCP could no longer be detected. Moreover, ATPase activity could also be correlated with HCP binding and with uncoupling in these experiments. Maximum ATPase activity was observed within 1 hr and activity had returned to normal 6 hr after injection of the drug (Fig. 3).

In the preceding experiments, it was shown that HCP administered parenterally or orally to rats led to uncoupling of oxidative phosphorylation in liver mitochondria and that the degree of uncoupling was related to the level of bound HCP. This relationship was confirmed in experiments *in vitro* employing liver mitochondria from untreated animals, and in addition the effect of mitochondrial protein level on the concentration of HCP causing 50 per cent uncoupling was elucidated. In several experiments, liver mitochondria representing more than a 5-fold range of protein concentration (0·75–3·85 mg/ml), were preincubated at 37° for 10 min in the phosphorylation medium with various levels of hexachlorophene and the P/O ratios were then determined. From these data, the concentration of HCP causing 50 per cent uncoupling of mitochondrial oxidative phosphorylation was determined graphically for each protein concentration.[20] The 50 per cent uncoupling concentration was found to be directly related to the concentration of mitochondrial protein in the incubation medium (Fig. 4), suggesting that under the conditions used here binding

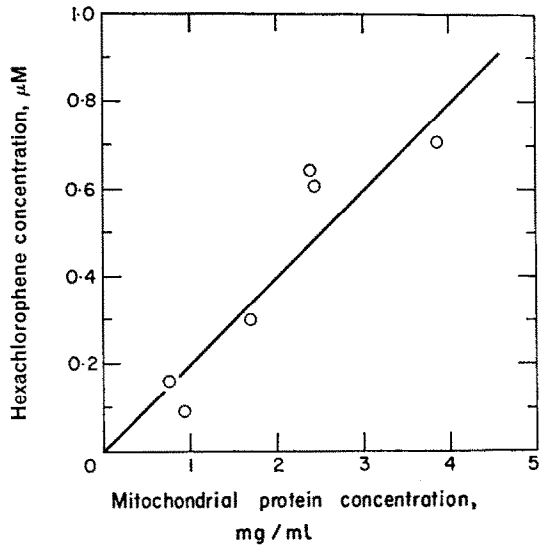

FIG. 4. Concentration of HCP causing 50 per cent uncoupling *in vitro* as a function of mitochondrial protein level. Experimental conditions are described in the text.

of HCP to mitochondria was complete. After the incubation period, mitochondria were recovered from the medium by centrifugation, washed and analyzed for bound HCP by either gas liquid chromatography or radioisotope procedures. The relationship between the level of bound HCP and the P/O ratio was essentially the same as that obtained in the previous *in vivo* studies (Fig. 1B).

Mitochondria representing a 4-fold concentration range (0·41–1·65 mg protein/ml) were incubated for 30 min at 37° in 3 ml of the phosphorylation medium with from 0·3–300 mμmoles of methylene-^{14}C-HCP in order to evaluate the completeness of the binding under these conditions. The mitochondria were recovered by centrifugation of aliquots of the reaction media, washed and then counted to determine the level of bound HCP. Even at the highest bisphenol concentration used, binding was complete at each protein level (Fig. 5). The quantity of HCP bound to mitochondrial protein in the most extreme case exceeded that required for 50 per cent uncoupling by over 2000 times.

The high affinity of liver mitochondria for the bisphenol was further indicated by the difficulty with which HCP was removed from mitochondria by simple washing (Fig. 6). Liver mitochondria were isolated in the usual manner, 60 min after intraperitoneal injection of a 215-g rat with a lethal dose of HCP (30 mg/kg). The washed mitochondrial suspension was then divided and one fraction was rewashed six times with the isolation medium while the other fraction was rewashed once with isolation medium containing 0·05 mM bovine serum albumin (BSA). Aliquots were examined for protein and HCP content after each wash. Even after six washes with 0·25 M sucrose, more than 60 per cent of the original HCP was retained with the mitochondrial fraction. After a single wash with the BSA-containing medium, however, only 36 per cent of the original HCP remained bound.

Since BSA is known to protect mitochondria from the effects of uncoupling phe-

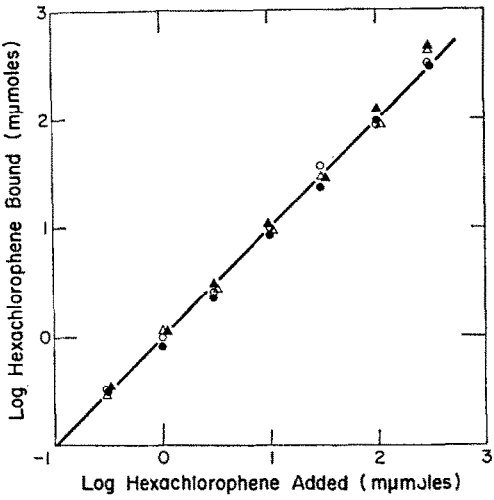

FIG. 5. Amount of HCP bound to mitochondrial protein (1·23–4·95 mg in 3 ml) as a function of the level of drug added. Experimental conditions were as described in Fig. 1B. The incubation period was 30 min and HCP was analyzed by the radioisotope method.

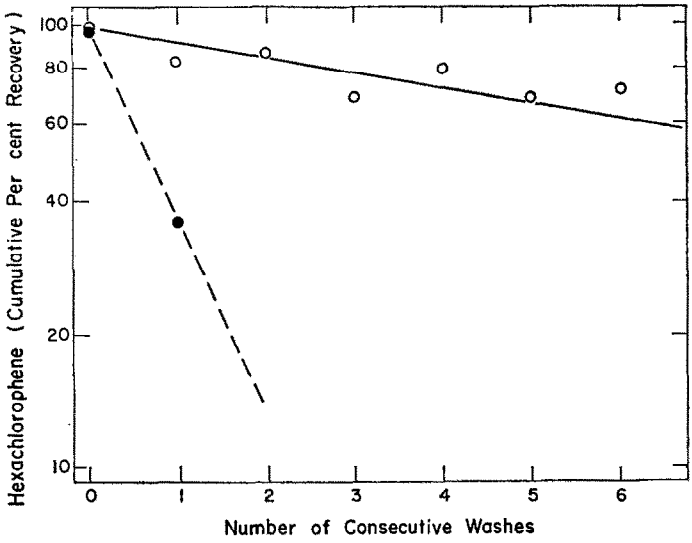

FIG. 6. Removal of HCP from mitochondria by consecutive washes with buffered 0·25 M sucrose with or without added bovine serum albumin. The wash medium represented by the open circles (○) contained 0·25 M sucrose and 0·04 M glycylglycine buffer (pH 7·4), while that represented by solid circles (●) contained 0·25 M sucrose, 0·04 M glycylglycine buffer (pH 7·4) and 0·05 mM bovine serum albumin. The initial level of bound hexachlorophene was 1·62 mμmoles/mg of mitochondrial protein.

nols[21] and restores phosphorylation to previously uncoupled mitochondria,[22] experiments were carried out to determine whether BSA was capable of reversing the uncoupling effect of HCP. Using the initial manometric procedures, mitochondria (7·5 mg protein) were preincubated for 10 min in the standard phosphorylation medium at 37° with 15 mμmoles HCP and from 0 to 203 mμmoles of BSA (mol. wt. 69,000) added in that order. Subsequently, the reaction was started by tipping in glucose, hexokinase and ATP from the sidearm, and the P/O ratios were than determined. Under these conditions no phosphorylation could be demonstrated, even with a 13·5 molar excess of BSA.

In order to examine this relationship in greater detail, a polarographic technique was employed which afforded a more convenient means of assessing stoichiometric relationships between HCP and BSA. A typical experiment, shown in Fig. 7, illustrates the effect of increasing concentrations of HCP on the rate of oxygen consumption of normal mitochondria deficient in phosphate acceptor. Only 1·6 mμmoles HCP increased the oxygen consumption rate 5·5 times. Subsequent additions of BSA were capable of restoring coupled respiration, but up to a 200-fold molar excess of BSA to HCP was required for complete restoration.

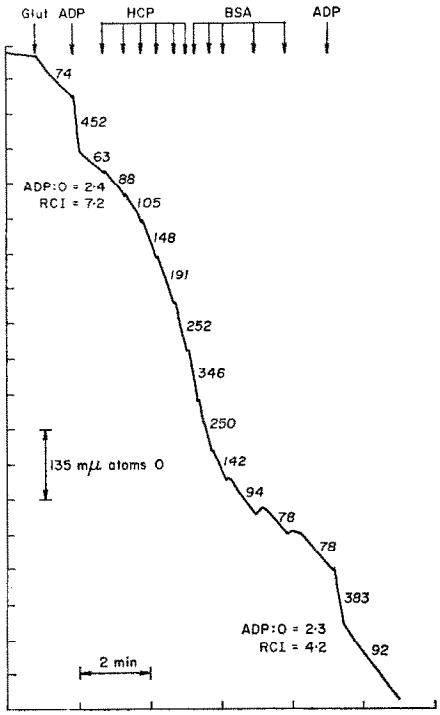

FIG. 7. Effect of bovine serum albumin on the respiration of mitochondria uncoupled by HCP. The arrows indicate the points of successive additions of 10 μmoles glutamate (Glut), 250 mμmoles ADP, four additions of 0·2 mμmole HCP, two additions of 0·4 mμmole HCP, two additions of 50 mμmoles bovine serum albumin (BSA), three additions of 100 mμmoles BSA and 250 mμmoles ADP to a suspension of mitochondria (8·7 mg protein) in 3 ml of the polarographic oxidative phosphorylation medium described in Methods. The numbers on the tracing indicate the oxygen consumption rate as millimicroatoms per minute. RCI is the respiratory control index.

The amount of BSA required to restore respiratory control to mitochondria treated with HCP proved to be much greater than that required with mitochondria uncoupled by pentachlorophenol.[22] Since HCP is known to bind strongly to BSA,[23] these results suggest that the affinity of mitochondria for the bisphenol is much greater than for pentachlorophenol. It is probable that this difference in binding affinity reflects the different structures of the two chlorinated phenols, while the actual binding site for uncoupling in the mitochondria remains the same. However, an alternative possibility could be that HCP combines and uncouples at a different site than that demonstrated for the classical uncoupling phenols. In order to examine this latter possibility, we attempted to locate the point of uncoupling relative to the locus of the oligomycin block by exposing liver mitochondria to oligomycin and examining the effect of added HCP (Fig. 8). After oligomycin treatment, normal mitochondria became refractory to added ADP, but HCP was capable of converting the inhibited mitochondria from the coupled state to an uncoupled condition. Subsequent addition of a 100-molar excess of BSA to HCP restored coupled respiration, but the mitochondria remained incapable of responding to ADP. This result is identical to that which is obtained with other uncoupling phenols[24] and indicates that HCP acts at a point in the coupling sequence between the respiratory chain and the site of oligomycin block.

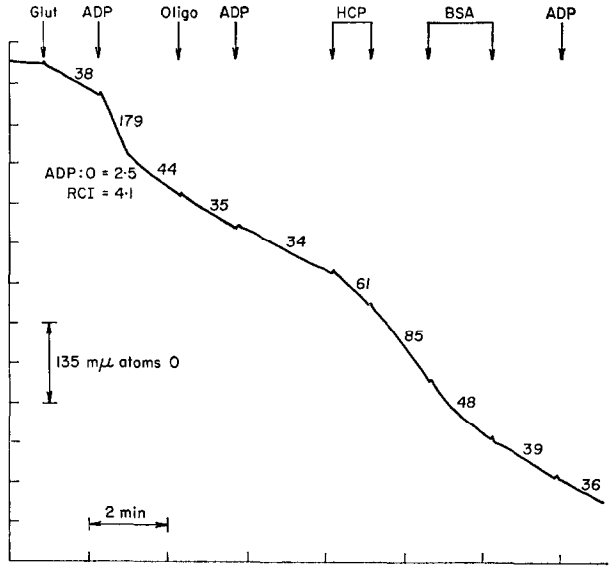

FIG. 8. Effect of oligomycin treatment on the response of mitochondria to HCP, bovine serum albumin and ADP. At the points indicated by arrows, successive additions of 10 μmoles glutamate (Glut), 250 mμmoles ADP, 2 mμmoles oligomycin (Oligo), 250 mμmoles ADP, two additions of 0·4 mμmole HCP, two additions of 50 mμmoles bovine serum albumin (BSA) and 250 mμmoles ADP were made to a suspension of mitochondria (6 mg protein) in 3 ml of the polarographic oxidative phosphorylation medium described in Methods. The numbers on the tracing indicate the oxygen consumption rate as millimicroatoms per minute. RCI is the respiratory control index.

Although uncoupling of oxidative phosphorylation was readily demonstrated in liver mitochondria isolated from orally dosed rats, we were unable to detect an effect on the electron transport enzymes under the same conditions (Table 1). However,

in agreement with earlier studies,[3] inhibition of these enzymes was found to occur *in vitro*. Liver mitochondria were incubated for 30 min at 37° in the phosphorylation medium with from 0·1–100 μM HCP. The isolated and washed mitochondria were subsequently examined for residual cytochrome oxidase activity. A gradually increasing inhibition of cytochrome oxidase was noted over the entire range of HCP concentrations tested (Fig. 9). Inhibition was estimated to be about 12 per cent at 0·1 μM HCP, increasing to 85 per cent at 100 μM. These results are virtually identical to those reported by Gould *et al.*[3] in studies employing similar concentrations of enzyme protein.

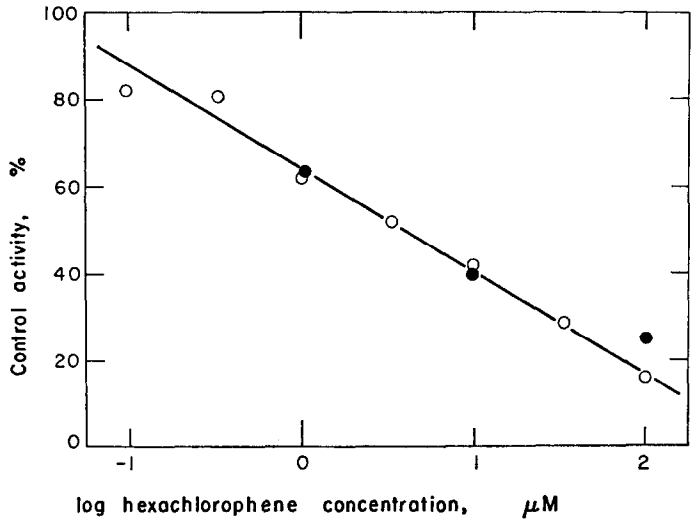

FIG. 9. Residual cytochrome oxidase activity in mitochondria incubated with increasing concentrations of HCP. The experimental details are given in the text. The open circles (○) represent a single experiment in which the concentration of mitochondrial protein was 1·65 mg/ml in 3 ml. The specific activity of control mitochondria was 0·911 μmole cytochrome-*c* oxidized/mg of protein/min. The closed circles (●) are data reported by Gould *et al.*[3] in an experiment in which the concentration of enzyme protein was 1·3 mg/ml in 3 ml.

Since we were unable to show a significant inhibition of the electron transport enzymes in mitochondria from orally dosed rats in which oxidative phosphorylation was markedly uncoupled (Table 1), it was of interest to compare the inhibition of these two systems as a function of the concentration of bound HCP. Liver mitochondria from normal rats were incubated with increasing concentrations of HCP as described previously. In five experiments the P/O ratios were determined and the mitochondria then recovered for analysis of HCP residues. In four additional experiments mitochondria were recovered after a 30-min incubation period and aliquots were analyzed for both residual cytochrome oxidase activity and bound HCP. The data for each experiment are shown as per cent of control activity and plotted as a function of the level of bound HCP (Fig. 10). It is clear from these results that cytochrome oxidase is less than 40 per cent inhibited at levels of bound HCP which completely abolish oxidative phosphorylation.

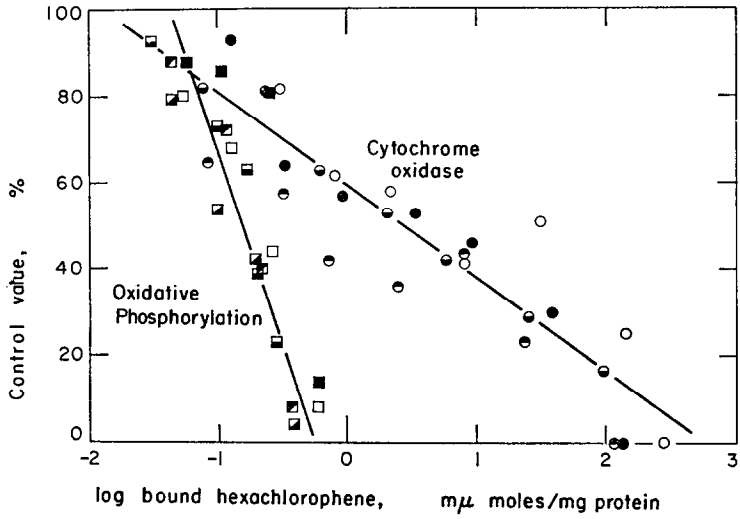

FIG. 10. Relative inhibition of oxidative phosphorylation and cytochrome oxidase activity as a function of bound HCP. The details of the experiment are given in the text. Per cent of control cytochrome oxidase activity is indicated in four experiments by the circles (○, ●, ◓, ◒) for which the specific activities of controls were 0·506, 0·694, 0·709 and 0·911 μmole cytochrome-*c* oxidized/mg of protein/min respectively. Oxidative phosphorylation values, expressed as per cent of control P/O ratios, are given for five experiments by the square symbols (□, ■, ◩, ◪, ⬓). Control P/O ratios in these experiments were 2·5, 2·1, 2·5, 2·4 and 3·0 respectively.

DISCUSSION

Many chemicals have been found to exert an uncoupling effect on mitochondrial oxidative phosphorylation. While the mechanisms by which these compounds uncouple are not entirely clear, there are a number of common effects which serve to characterize the group.[20] These include an increase in mitochondrial respiration rate when the incubation medium is deficient in phosphate acceptor or inorganic phosphate, a decrease in orthophosphate uptake, increased mitochondrial ATPase activity and inhibition of the [^{32}P]-phosphate–ATP exchange reaction.[20] Most uncoupling agents are weakly acidic and lipophilic and are presumed to exert their uncoupling effects through a nonspecific interaction with the mitochondrial membranes.

Two major classes of uncouplers are nitrophenols and halophenols, which are typified by 2,4-dinitrophenol and pentachlorophenol respectively.[25] The chlorinated bisphenols such as HCP have many physical and chemical similarities to these simple phenols. In addition, we have now shown that the chlorinated bisphenol, HCP, acts as an effective uncoupler of mitochondrial oxidative phosphorylation while also stimulating ATPase activity. The concentration of HCP resulting in a 50 per cent inhibition of phosphate uptake *in vitro* was found to be directly dependent on the protein level in the incubation medium and was 0·5 μM when the latter was 2·5 mg protein/ml. In similar experiments, Weinbach and Garbus[26] reported that 50 per cent uncoupling for pentachlorophenol and 2,4-dinitrophenol occurred at phenol concentrations of about 5 and 18 μM, respectively, when mitochondria were present at a concentration of 5 mg/ml. Fifty per cent uncoupling for these same two phenols occurred at 9 and 30 μM concentrations, respectively, according to Parker,[20] although the protein

concentrations were not reported in that study. These comparisons suggest that HCP is a more effective uncoupler than either of the classical uncoupling phenols. Quantities of bisphenol bound to mitochondria, however, were very similar to those previously found with pentachlorophenol and 2,4-dinitrophenol at uncoupling concentrations. Half and complete uncoupling with HCP required 0·09 and 0·25 μg/mg of mitochondrial protein respectively. Comparable values reported for pentachlorophenol and 2,4-dinitrophenol were 0·25, 0·75 and $<0·17$, 0·34 μg/mg of protein respectively.[26] HCP like pentachlorophenol cannot be readily removed from mitochondria by simple washing (Fig. 6). In contrast, 2,4-dinitrophenol bound to mitochondria is easily disassociated by one or two washings with sucrose.[26] Our results suggest that HCP may be even more tightly bound to mitochondria than is the case with pentachlorophenol. Thus, reversal of uncoupling by HCP can be accomplished with BSA (Fig. 7), but the amount of BSA required was much larger (50–100 times more) than that required with pentachlorophenol.[22] In other studies *in vitro*, HCP has been found to hydrogen bond very strongly to BSA[23] and synthetic polypeptides. In the present study, HCP was completely bound to mitochondria even when present at a 2000-fold excess over that required for 50 per cent uncoupling (Fig. 5). HCP was shown to reverse the inhibition of oxygen uptake (Fig. 8) which occurred after oligomycin treatment of mitochondria in the presence of ADP in a manner similar to that found with other phenolic uncouplers.[24] These observations provide conclusive evidence that HCP is an effective uncoupler of oxidative phosphorylation and suggest that the mechanism of uncoupling by this compound is similar to that of other uncoupling phenols.

Earlier work has demonstrated an inhibition of electron transport enzymes by HCP. Gould *et al.*[3] reported that HCP inhibited rat succinoxidase *in vitro* at concentrations as low as 1 μM. For lack of other studies, this work has often been cited, suggesting that inhibition of succinoxidase or other metal enzymes may be responsible for HCP toxicity. We have confirmed this earlier work in similar *in vitro* experiments. The inhibition of cytochrome oxidase incubated with HCP was gradually increased over the concentration range of 0·1–100 μM bisphenol with 50 per cent inhibition occurring at 4 μM (Fig. 9). However, HCP is more active in uncoupling oxidative phosphorylation. Using the same protein concentration (1·65 mg/ml) and incubation medium, 50 per cent uncoupling occurs at only 0·32 μM HCP (Fig. 4).

The relative sensitivity of these two systems to HCP is more clearly seen when inhibition is compared as a function of the level of bisphenol bound to the mitochondria (Fig. 10). The slope for inhibition of oxidative phosphorylation is much steeper than that for cytochrome oxidase. As a result both systems are slightly but similarly inhibited at 0·063 mμmole of the drug/mg of protein. However, the levels of bound HCP required for 50 per cent uncoupling of oxidative phosphorylation and inhibition of cytochrome oxidase, respectively, were 0·18 and 2·8 mμmoles of the drug/mg of protein. It would be expected, therefore, that in rats receiving increasing dosages of HCP an effect on oxidative phosphorylation would be observed prior to any effect on cytochrome oxidase. Our results confirm this expectation. When rats were exposed to an oral dose of HCP and liver mitochondria were subsequently isolated and examined for effects on oxidative phosphorylation and electron transport enzyme activity, the latter was only slightly inhibited while uncoupling of oxidative phosphorylation was nearly complete (Table 1). Caution must be used in extrapolating these results to other

tissues. Nevertheless, they suggest that inhibition of electron transfer reactions is not likely to be the critical biochemical site for HCP toxicity.

Demonstration that a compound uncouples oxidative phosphorylation *in vitro* cannot be considered *a priori* proof that such an effect is responsible for the toxicity of the compound or the response of the animal *in vivo*. A more convincing argument could be made, however, if it could be shown that the time sequence and extent of symptomatology, including the increase in body temperature, parallel significant mitochondrial uncoupling in an animal receiving a lethal or sublethal dose of the uncoupler. It has been suggested in the case of animals receiving lethal doses of pentachlorophenol that the actual cause of death is hyperpyrexia.[28,29] Buffa *et al.*[30] have recently shown that there is an excellent correlation between rectal temperature and the degree of uncoupling of liver mitochondria in rats treated with pentachlorophenol. However, they have appropriately cautioned against the conclusion that uncoupling of liver mitochondria alone is responsible for the elevation of body temperature. Although they were unable to demonstrate directly that muscle mitochondria from pentachlorophenol-injected rats are uncoupled, a reduction in the concentration of energy-rich phosphate compounds was shown in that tissue, suggesting that uncoupling of muscle mitochondria did occur *in situ*.[31]

Upon consideration of the dosage, route of administration, temporal relationships and symptomalogic responses in rats, there appears to be a strong indication that HCP may have a similar basis for toxicity. There is a good correlation between the effects on oxidative phosphorylation and ATPase activity in liver mitochondria and the elevation in rectal temperature and other symptoms of intoxication that follow intraperitoneal injection of HCP. The 24-hr intraperitoneal LD_{50} for HCP in rats is about 25 mg/kg, with death usually occurring within 2 hr and survivors appearing to be fully recovered within about 8–10 hr. Rectal temperatures of injected rats rapidly increased by 0·5–2·5° during the initial 20- to 40-min period after injection until death occurred.* Increases in rectal temperature were similar, although not as great, in animals given lower doses, and elevated temperatures declined to normal values within 6–8 hr after dosing. Similarly, uncoupling of oxidative phosphorylation in liver mitochondria was at a maximum 15 min after rats had been given an intraperitoneal dose of 10 mg/kg, while recovery was complete by 6 hr (Fig. 3). In the intraperitoneal experiments, the time relationships for the effect on oxidative phosphorylation were strikingly similar to those reported by Buffa *et al.*[30] for pentachlorophenol. In their studies, liver mitochondria from rats sacrificed only 15 sec after administering an intraperitoneal dose of pentachlorophenol already exhibited a significant reduction in P/O ratio. The maximum uncoupling was observed at 1 min in their work, with a gradual recovery occurring during the next 3–6 hr and complete recovery in 18–24 hr. Although no significant uncoupling was found in a single rat injected intraperitoneally with HCP and then sacrificed immediately (Fig. 3), we did not examine our remaining animals before 15 min. Consequently, no comparisons with the results of Buffa *et al.*[30] for this early period were possible. However, the rates of recovery of intoxicated animals were similar in both studies.

The same relationship appears to exist after oral administration of HCP. The 24-hr oral LD_{50} for HCP in rats under the conditions used in the present experiments was about 90 mg/kg. Rats receiving lethal or near lethal oral doses of HCP appeared to suffer maximum stress from about 12–36 hr after dosing. Death usually occurred

during 24–48 hr and signs of recovery in survivors were often visible by 30–36 hr. Oxidative phosphorylation in liver mitochondria isolated 18 hr after an oral dose of HCP was only 15 and 40 per cent of control values for doses of 100 and 20 mg/kg respectively (Table 1). Other data (Fig. 2) suggest that in orally dosed rats maximum uncoupling of liver mitochondria occurred at about 12–14 hr and that by 36 hr recovery was essentially complete.

While the effects of orally ingested HCP on oxidative phosphorylation in liver mitochondria and its subsequent recovery may slightly precede the onset and recovery of gross symptomatology in the rat, this inconsistency does not suggest a difference in the mechanism of toxicity between the orally dosed rat and those receiving the drug by intraperitoneal injection. In rats dosed intraperitoneally, absorption of HCP through the peritoneum would allow rapid circulation to all other tissues. However, absorption of orally administered HCP from the gut would result in transport first to the liver, by way of the hepatic portal system, before the drug entered the systemic circulation. Under these circumstances, assuming that uncoupling of oxidative phosphorylation in muscle and other tissues was mainly responsible for the rise in rectal temperature and other gross symptoms, the biochemical lesion would be anticipated in the liver slightly before the observation of stress by the entire animal. Thus, the observations in both orally and intraperitoneally dosed rats are compatible with the hypothesis that uncoupling of oxidative phosphorylation may be the principal biochemical lesion in acute intoxication with HCP. The actual cause of death is not known, but may be a result of thermal damage to critical biological systems by elevated body temperatures produced as a result of exposure to HCP. Alternatively, depletion of high energy phosphate reserves may result in failure of cellular maintenance reactions.

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