Restoration by Albumin of Oxidative Phosphorylation and Related Reactions*

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SUMMARY

Bovine serum albumin prevented the effects of pentachlorophenol, 2,4-dinitrophenol, and various other reagents upon oxidative phosphorylation, the orthophosphate-adenosine triphosphate exchange reaction, adenosine triphosphatase, and respiratory control of rat liver mitochondria. More significantly, albumin restored the capacity for oxidative phosphorylation and the ancillary reactions, including respiratory control, to mitochondria previously reacted with these compounds. Inhibition by gramicidin, oligomycin A, antimycin A, and rotenone was not counteracted by albumin.

Oxidative phosphorylation of liver mitochondria uncoupled by the intraperitoneal injection of pentachlorophenol into rats was also restored by albumin. The circulating halophenol after injection was found associated with the albumin fraction of the plasma.

The ability of albumin to prevent the deleterious action of the uncoupling reagents, and to restore function to mitochondria treated with them, resides in its capacity to bind these reagents and thereby remove them from the medium or from mitochondria. The beneficial effect is specific for serum albumin. Various other proteins and compounds were found to be ineffective.

It was shown that 1 mole of bovine serum albumin can bind 5 moles of pentachlorophenol. Each of these 5 moles is not held with equal affinity, and reversible binding was readily demonstrated. Certain modified albumins, i.e. acetylated, or denatured by chemical or physical means, lost their capacity to bind pentachloro- or 2,4-dinitrophenol. Other modifications, such as blocking sulfhydryl or imidazole groups, did not alter the ability of albumin to bind pentachlorophenol. From these results, evidence is adduced that the native protein structure and especially free amino groups are involved in the protein-phenol interaction.

A possible physiological role for the reversible binding of uncoupling reagents to proteins is discussed, as is the use of the phenomenon in studying mitochondrial function and structure.

Bovine serum albumin has been found to be a useful adjuvant in media employed to demonstrate oxidative phosphorylation with freshly isolated insect flight muscle mitochondria (3-6) or to enhance phosphorylation in damaged mammalian liver mitochondria (7-10). It was shown that albumin exerts its salutary effect by binding liberated fatty acids which can act as endogenous uncouplers of oxidative phosphorylation (10-14).

It therefore became of interest to ascertain whether albumin exerted a similar effect toward exogenous uncoupling reagents whose structures differ considerably from that of long chain fatty acids. Previous studies in our laboratory had established that bovine serum albumin counteracted the effect of pentachlorophenol on oxidative phosphorylation in freshly isolated rat liver mitochondria (9). Studies by others have demonstrated a similar protective effect of serum proteins against certain concentrations of 2,4-dinitrophenol, Dicumarol, and sodium dodecyl sulfate (10, 15, 16).

The present report shows that bovine serum albumin if present in equimolar concentrations always protects mitochondria from the uncoupling and inhibitory effects of various substituted phenols. More significantly, it is shown that this protein restores oxidative phosphorylation, respiratory control, the orthophosphate-adenosine triphosphate exchange, and reduces adenosine triphosphatase activity of rat liver mitochondria previously reacted with these reagents. Furthermore, data are presented on the stoichiometry and the nature of the protein-phenol interaction which is responsible for the beneficial effect of the protein.

EXPERIMENTAL PROCEDURE

Mitochondria were isolated from rat liver as described previously (17). The preparations were washed twice and suspended in a sufficient volume of 0.25 M sucrose so that each milliliter contained 20 mg of protein.

Oxidative Phosphorylation—β-Hydroxybutyrate was used as substrate with the technique described earlier (9). Each flask contained, in a final volume of 2 ml, 50 μmoles of gycerylglycine buffer (pH 7.4), 50 μmoles of DL-β-hydroxybutyrate, 30 μmoles of F1 (pH 7.4), 5 μmoles of ADP, 2 μmoles of NAD, 0.08 μmole of cytochrome c, 50 μmoles of glucose, 0.5 mg of hexokinase (Sigma, type III), 10 μmoles of MgCl2, and 0.5 ml of mitochondria.

Restoration Experiments—For studying the restoration of oxidative phosphorylation sequential incubations were used as
follows. Mitochondria were incubated for 15 min at 30° in the phosphorylative medium described above, with and without uncoupling reagents. In most experiments, the contents of six flasks were combined and chilled; the mitochondria were removed in the cold by high speed centrifugation (10,000 × g; 10 min). Portions of the supernatant fractions were retained for later analyses of Pi (18), acetoacetate (19), and uncoupling phenols (20). The separated mitochondria were washed twice by resuspension in 0.25 M sucrose, recentrifuged as above, and resuspended in sufficient fresh 0.25 M sucrose to approximate the initial protein concentration. Samples of this suspension were analyzed for protein (21) and uncoupling phenols. The washed, resuspended mitochondria were reincubated for 15 min at 30° in fresh phosphorylative medium with and without bovine serum albumin. Oxidative phosphorylation was redetermined after this second incubation as described above, and the sedimented mitochondria were analyzed again for the presence of uncoupling phenols.

The Pi-ATP exchange reaction and ATPase activity were determined as described earlier (22). Sequential incubations also were used to study restoration of these reactions.

**Binding Studies**—Bovine serum albumin was reacted in aqueous, buffered media with various molar ratios of pentachlorophenol, extensively dialyzed, and analyzed for protein (21) and the halophenol (20). In a typical experiment, 4 μmoles of albumin in 5 ml of 40 mM glycylglycine buffer (pH 7.4) were reacted for 10 min at 25° with 20 μmoles of pentachlorophenol. Samples were removed for analyses, and the remainder was dialyzed against 2 liters of 0.15 M NaCl. The washed, resuspended mitochondria were reincubated for 15 min at 30° in fresh phosphorylative medium with and without bovine serum albumin. Oxidative phosphorylation was redetermined after this second incubation as described above, and the sedimented mitochondria were analyzed again for the presence of uncoupling phenols.

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**Binding Studies**—Bovine serum albumin was reacted in aqueous, buffered media with various molar ratios of pentachlorophenol, extensively dialyzed, and analyzed for protein (21) and the halophenol (20). In a typical experiment, 4 μmoles of albumin in 5 ml of 40 mM glycylglycine buffer (pH 7.4) were reacted for 10 min at 25° with 20 μmoles of pentachlorophenol. Samples were removed for analyses, and the remainder was dialyzed against 2 liters of 0.15 M KCl at 4°. The dialysis was continued for 48 hours, during which the dialyzing fluid was changed three times. The albumin solution was analyzed for protein and pentachlorophenol. Further dialyses, up to 140 hours, resulted in no additional loss of dialyzable material.

Similar procedures were employed for other molar ratios of pentachlorophenol.

**Modified Proteins**—Acetylation of bovine serum albumin was accomplished by the procedure of Fraenkel-Conrat (23). Tests of the acetylated protein showed that more than 90% of the initial ninhydrin-reacting groups had been blocked.

Imidazole groups of albumin were reacted with diazotized sulfanilic acid (Pauly reaction) as described by Greenstein and Winitz (24); sulfhydryl groups were reacted with p-chloromercuribenzoate (25) or with iodoacetamide (23). The reaction of albumin with 2-(4'-hydroxyphenylazo)benzoic acid was done by the procedure of Rutstein, Ingenito, and Reynolds (26).

For treatment with urea or guanidine, 900 mg of bovine serum albumin were shaken with 5 ml of 8 m urea or with 5 ml of 8 m guanidine hydrochloride for 12 hours at 30°. Configurational changes of albumin were also induced by treatment with 1% sodium dodecyl sulfate (27).

In all of the above procedures, the protein solutions were dialyzed against 0.15 M KCl in the cold (4°) for at least 18 hours to remove excess reagents. If not used immediately, they were lyophilized prior to storage at 4°.

Lipid-free albumin was prepared by the procedure of Goodman (28), or in a few experiments, by the method of Williams and Foster (29).

**Sources**—Crystalline bovine serum albumin was obtained commercially (Nutritional Biochemicals). The assumed molecular weight for this protein is 69,000. Human serum albumin (Fraction V) and the other human plasma proteins were generous gifts from Dr. John Finlayson, Division of Biologics Standards. Carboxyl cyanide m-chlorophenylhydrazone was supplied through the courtesy of Dr. P. G. Heytler, E. I. duPont de Nemours and Company. All other proteins and reagents were of the highest purities commercially available.

**RESULTS**

**Prevention Experiments**—The data collected in Table I show that albumin prevented the uncoupling and adverse action of pentachlorophenol upon net oxidative phosphorylation and two related reactions. The minimal amount of albumin found necessary for maximum protection of phosphorylation was equimolar with respect to that of the uncoupling phenol added (30). The basis for the requirement of equimolarity resides in the nature of the protein-phenol interaction as discussed later in this report.

Albumin was found also to be effective in completely preventing the uncoupling action of other substituted halophenols (cf. Reference 20) and 2,4-dinitrophenol. Likewise, the action of thyroxine, Dicumarol, carbonyl cyanide m-chlorophenylhydrazone, and Amytal was counteracted by this protein. As in the case with pentachlorophenol, full protection was obtained only when albumin was present in an amount at least equimolar to that of these reagents. On the other hand, the inhibitory action of gramicidin, antimycin A, oligomycin A, rotenone, and triethyltin was not abolished by albumin even when the protein was present in concentrations greatly exceeding that of the inhibitors.

**Restoration Experiments**—Albumin restored oxidative phosphorylation to mitochondria previously allowed to react with pentachlorophenol (Table II). The demonstration that albumin can restore this function was facilitated by the fact that the uncoupling action of pentachlorophenol, in contrast to that of 2,4-dinitrophenol, was not reversed by simple washing procedures (20). Analysis by a sensitive spectrophotometric technique (20) of mitochondria allowed to react with pentachlorophenol disclosed that it had remained tightly bound, despite the repeated washing (Table II).

With the procedure of sequential incubations, it was found that the presence of albumin in the media of the second incubation not only restored the capacity for net phosphate uptake but also had a beneficial effect on the ancillary reactions (Table

### Table I

**Restoration of effects of pentachlorophenol by albumin**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Oxidative phosphorylation</th>
<th>Pi-ATP exchange</th>
<th>ATPase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (Control)</td>
<td>3.0</td>
<td>0.34</td>
<td>0.30</td>
</tr>
<tr>
<td>Albumin</td>
<td>3.0</td>
<td>3.2</td>
<td>0.20</td>
</tr>
<tr>
<td>Pentachlorophenol</td>
<td>0.0</td>
<td>0.1</td>
<td>4.90</td>
</tr>
<tr>
<td>Pentachlorophenol + albumin</td>
<td>2.0</td>
<td>2.8</td>
<td>0.78</td>
</tr>
</tbody>
</table>

For treatment with urea or guanidine, 900 mg of bovine serum albumin were shaken with 5 ml of 8 m urea or with 5 ml of 8 m guanidine hydrochloride for 12 hours at 30°. Configurational changes of albumin were also induced by treatment with 1% sodium dodecyl sulfate (27).

In all of the above procedures, the protein solutions were dialyzed against 0.15 M KCl in the cold (4°) for at least 18 hours to remove excess reagents. If not used immediately, they were lyophilized prior to storage at 4°.

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III. It should be noted that the control preparation (i.e., mitochondria preincubated without pentachlorophenol) retained: (a) substantial capacity for net oxidative phosphorylation, (b) substantial ability to catalyse the Pi-ATP exchange, and (c) low ATPase activity, despite the attrition induced by the additional manipulations required for these experiments. Of particular interest was the marked effect of albumin in restoring the exchange reaction, a reaction which we had demonstrated earlier to be more labile than net phosphorylation (9). Albumin was also effective in restoring oxidative phosphorylation in sequential incubations to mitochondria previously uncoupled by those reagents whose activity it counteracted in prevention experiments. As Amytal and 2,4-dinitrophenol are easily removed by a single washing, restoration experiments with these reagents were not feasible.

The results of experiments with pentachlorophenol (Table IV) demonstrate that for full restitution, albumin must be present in an amount greater than equimolar to that of the halophenol retained by the mitochondria. It should be emphasized that in these restoration experiments, albumin is not binding pentachlorophenol free in the medium, but binding and thereby removing the uncoupling phenol which had been bound to mitochondrial sites. After the second incubation (and restoration) in the presence of albumin, these mitochondria no longer contained detectable amounts of pentachlorophenol.

The inclusion of albumin in the washing medium was also effective in restoring phosphorylative ability. Even after repeated washings with 0.25 mM succrose, mitochondria, which initially contained 1.8 µg of pentachlorophenol per mg of protein, retained sufficient reagent to cause uncoupling (20). However, a single washing with 0.25 mM succrose 0.05 mM albumin removed the entire initial content of pentachlorophenol and restored the capacity for oxidative phosphorylation.

Restoration of Respiratory Control—In order to determine whether albumin would also restore respiratory control (31) to mitochondria previously reacted with an uncoupling reagent, experiments of the type presented in Table V were conducted. It may be seen that in the absence of a phosphate acceptor, mitochondria containing bound pentachlorophenol oxidized β-hydroxybutyrate at a rate significantly greater than that of the control preparations. In the presence of albumin, such previously reacted mitochondria oxidized the substrate at the lower rate characteristic of the more tightly coupled control mitochondria. If phosphate acceptor was added together with albumin, oxidation of β-hydroxybutyrate was substantially enhanced. The extent of the return of respiratory control may be seen by the 2-fold increase in the respiratory control index. Similar results were obtained when pyruvate and malate were used as substrates.

Restoration of respiratory control was also obtained in polarographic experiments with succinate as substrate. The results showed that greater than equimolar amounts of albumin were needed for the complete return of respiratory control. Details of these experiments will be presented elsewhere.

Experiments in Vivo The effect of albumin in restoring oxidative phosphorylation to liver mitochondria isolated from rats injected intraperitoneally with pentachlorophenol is shown in Table VI. These mitochondria were uncoupled even though their isolation was initiated only 3 min after the injection of the halohenol.1 In most instances, the mitochondria contained

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Table II
Reversibility of uncoupled phosphorylation

The technique of sequential incubations as described in the text was used. The concentration of pentachlorophenol in the first incubation medium and that of albumin in the second was 0.05 mM.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>P:O</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td>Control</td>
<td>2.9</td>
</tr>
<tr>
<td>Pentachlorophenol</td>
<td>2.9</td>
</tr>
<tr>
<td>Pentachlorophenol in mitochondria (µg/mg of protein)</td>
<td>2.9</td>
</tr>
</tbody>
</table>

Table III
Reversal of pentachlorophenol effect by albumin

The technique of sequential incubations as described in the text was employed. Mitochondria were preincubated with and without 0.05 mM pentachlorophenol in the phosphorylative medium described in the text. After washing, the mitochondria were reincubated in the absence or presence of 0.05 mM albumin under conditions appropriate for each reaction.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Preincubated without pentachlorophenol; washed twice; reincubated with</th>
<th>Preincubated with pentachlorophenol; washed twice; reincubated with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No albumin</td>
<td>Albumin</td>
</tr>
<tr>
<td>Oxidative phosphorylation (P:O)</td>
<td>2.7</td>
<td>2.8</td>
</tr>
<tr>
<td>P:ATP exchange (%)</td>
<td>14</td>
<td>20</td>
</tr>
<tr>
<td>ATPase (µmoles of P, liberated)</td>
<td>0.4</td>
<td>0.2</td>
</tr>
</tbody>
</table>

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Table IV
Amount of albumin required to restore oxidative phosphorylation

Mitochondria were incubated with 0.05 mM pentachlorophenol as described in the text for sequential incubations. The 0.5-ml portions of mitochondria, washed once, used per flask in the second incubation contained 0.048 µmole of pentachlorophenol. The P:O ratio of the control mitochondria, i.e., preincubated in the absence of the halphenol, was 2.5.

<table>
<thead>
<tr>
<th>Albumin added per flask</th>
<th>P:O</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>1</td>
<td>0.044</td>
</tr>
<tr>
<td>2</td>
<td>0.029</td>
</tr>
<tr>
<td>3</td>
<td>0.044</td>
</tr>
<tr>
<td>4</td>
<td>0.058</td>
</tr>
<tr>
<td>5</td>
<td>0.072</td>
</tr>
<tr>
<td>6</td>
<td>0.087</td>
</tr>
<tr>
<td>7</td>
<td>0.102</td>
</tr>
<tr>
<td>8</td>
<td>0.116</td>
</tr>
</tbody>
</table>

* Based on an assumed molecular weight of 69,000.

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1 Buffa, Carafoli, and Muscetello (32) have shown that within 15 sec after the injection of pentachlorophenol into rats there is a...
an amount of pentachlorophenol sufficient to account for complete uncoupling. The possibility that these mitochondria bound free pentachlorophenol during the isolation procedure was tested by isolating mitochondria from perfused livers of animals treated by injection. Such mitochondria were also uncoupled and contained pentachlorophenol. In a few experiments, however, mitochondria isolated after the injection of pentachlorophenol were found to be uncoupled despite our inability to detect significant amounts of the halophenol. Albumin was also effective in restoring oxidative phosphorylation with such preparations. It is possible that in these instances, the effective uncoupling agents were fatty acids or other endogenous substances released by the action of the injected pentachlorophenol.6

Fractionation of the blood plasma proteins (33) of the animals treated by injection with pentachlorophenol (30 mg per Kg of body weight) disclosed that all of the circulating uncoupling reagent was bound to the albumin fraction. Analysis of this fraction showed that the halophenol was present in a molar ratio at least twice that of the albumin. However, it is clear that the binding of pentachlorophenol by the albumin fraction of the intact animal did not prevent its uncoupling action. This may reflect the fact that the amount of pentachlorophenol injected into these animals and bound to the albumin exceeded the estimated equimolar binding capacity of the circulating protein. Furthermore, incubation of liver mitochondria of control animals with the isolated albumin fraction of the pentachlorophenol-injected animals resulted in uncoupling of oxidative phosphorylation and the enhancement of ATPase activity.

Protein-Phenol Interaction as Basis for Albumin Effect—The experiments described thus far show that albumin exerted its effect by binding and thereby removing the uncoupling phenols from mitochondria. Consequently, it was important to determine the affinity of this protein for these reagents. Solutions of pentachlorophenol bound to bovine serum albumin were chromatographed on Sephadex G-25 (20). The protein, which was found to contain the halophenol in a 1:5 molar ratio, was readily eluted from the column. It required a larger volume and greater ionic strength of the eluant to remove the unbound pentachlorophenol. Analogous experiments showed that albumin bound 2,4-dinitrophenol in a 1:2 molar ratio.

In the experiments summarized in Fig. 1, bovine serum albumin was reacted with various molar ratio amounts of pentachlorophenol, exhaustively dialyzed, and analyzed for both protein and the phenol. These results also demonstrated that there are five binding sites per mole of serum albumin for pentachlorophenol. Albumin, allowed to react with 10 or 20 moles of pentachlorophenol, retained only 5 moles of the uncoupling reagent after dialysis.

It may be seen in Table VII that each of the 5 moles of pentachlorophenol bound to albumin was not held with equal affinity. Albumin preparations containing 1, 2, or 3 molar ratios of pentachlorophenol were incubated with mitochondria under conditions for determining oxidative phosphorylation. Measurement of the P:O ratios and the amount of halophenol in these mitochondria after incubation showed that the phenol was reversibly bound to albumin. Pentachlorophenol bound to albumin in excess of 1:1 molar ratio had greater affinity for the mitochondrial binding sites than it had for those of albumin. Under such conditions, the halophenol was removed from the albumin, and was bound to mitochondria, which resulted in uncoupling (Fig. 2).

Table V

Reversal of effects of in vivo-bound pentachlorophenol by albumin

7 Rats were injected intraperitoneally with 0.9% NaCl solution (controls) or with pentachlorophenol, 30 mg per Kg. Isolation of liver mitochondria was initiated 3 min after the injection. The mitochondria of the halophenol-injected animal contained 0.5 µg of pentachlorophenol per mg of mitochondrial protein. Conditions for the reactions studied are as described in Table I. The concentration of albumin was 0.05 mM.

Table VI

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Similarly, mitochondria isolated from the brains of rats injected with pentachlorophenol were fully uncoupled in vitro. Kidney and heart muscle mitochondria of these animals were only partially uncoupled. With all of the preparations, albumin was effective in restoring the decreased activity.
MOLES PCP ADDED PER MOLE ALBUMIN

FIG. 1. Binding of pentachlorophenol (PCP) to bovine serum albumin. Details are given in the text.

TABLE VII

Effect of albumin-pentachlorophenol complexes on oxidative phosphorylation

Oxidative phosphorylation was determined as described in the text. In Experiments 1 and 2, the concentration of albumin, and of the albumin-pentachlorophenol complex was 0.05 mM; in Experiment 3, it was 0.1 mM. The concentration of pentachlorophenol and of Dicumarol was 0.05 mM, that of 2,4-dinitrophenol was 0.1 mM.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Additions</th>
<th>P/O</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None (control)</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>Albumin</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>Pentachlorophenol</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>[Albumin]-[pentachlorophenol]</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>[Albumin]-[pentachlorophenol]</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>[Albumin]-[pentachlorophenol]</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>Dicumarol</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Albumin + Dicumarol</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>[Albumin]-[pentachlorophenol] + Dicumarol</td>
<td>0.9</td>
</tr>
<tr>
<td>3</td>
<td>2,4-Dinitrophenol</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Albumin + dinitrophenol</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>[Albumin]-[pentachlorophenol] + dinitrophenol</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Likewise, the P/O-ATP exchange was inhibited and ATPase activity enhanced. This is particularly noteworthy because albumin itself does not penetrate mitochondria to any significant extent (34). Furthermore, these experiments demonstrated the reversibility of the protein-phenol interaction.3

3 That pentachlorophenol was reversibly bound to serum albumin was also indicated by the observations that it could be readily removed by extraction of the albumin-pentachlorophenol complex with organic solvents such as chloroform. Also, it may be pertinent that iodinated albumin has been observed to inhibit aerobic phosphorylation (8). The explanation for this observation may reside in the ability of iodide to uncouple phosphorylation (35), and its possible reversible binding to albumin.

The single site which tenaciously retained pentachlorophenol presumably is the same site at which other uncoupling reagents are preferentially bound. The albumin-phenol complex in a 1:1 molar ratio no longer protected against the uncoupling action of 2,4-dinitrophenol and Dicumarol (Table VII). Relevant to these findings is the observation that an albumin-oleate complex failed to prevent uncoupling in “aged” mitochondria (10).

Experiments were conducted to elucidate the chemical nature of the sites on the albumin molecule involved in the protein-phenol interaction. Apparently, sulphydryl and imidazole groups are not the active sites, as modified albumins in which these groups were blocked by reaction with p-chloromercuribenzoate, iodoacetamide, or with diazotized sulfanilic acid retained their ability to protect mitochondria against the uncoupling action of pentachlorophenol. Likewise, the binding of the dye, 2-(4'-hydroxyphenylazo)benzoic acid, to albumin did not alter its effectiveness. On the other hand, acetylation of albumin completely abolished its beneficial effect. Similarly, acetylation of a protein isolated from mitochondria abolished its capacity to bind this uncoupling phenol (20). The native tertiary structure of albumin appears to be required for its maximum effectiveness, as albumin denatured by chemical (urea, guanidine, sodium deoxycholate) or thermal means was no longer beneficial.

Specificity of Albumin—A considerable number of proteins and other substances were examined for their ability to prevent the uncoupling activity of pentachlorophenol. Of all of the substances tested, only serum albumins were effective. Human and rat serum globulins, lactalbumin, ovalbumin, conalbumin, peptone, blood hydrolysate, ecdin, polylysine, imidazole, histidine, polyvinyl alcohol, and flavin nucleotides were ineffective. It is of particular significance that the globulin fractions of human plasma, which contained substantial amounts of lipids, were
completely ineffective. In contrast, the removal of lipid from commercial albumin (28, 29) did not alter its efficacy in these experiments. It has been demonstrated by Wojtczak and Wojtczak (13) that serum albumins are also specific for counteracting the endogenous uncoupling agent of insect mitochondria.

Albumins from various species were not equally effective. For example, although porcine albumin was equally as beneficial on a molar basis as bovine and rat albumins, equine albumin was 65%, and rabbit albumin only 16% as effective. This suggests that each albumin may contain different binding sites and thus differ in its avidity for the uncoupling phenol.4

**DISCUSSION**

The data presented in this report demonstrate that bovine serum albumin not only counteracts the effects of various uncoupling phenols initially present in the incubation medium but, more importantly, restores phosphorylation and related activities to rat liver mitochondria which had been previously reacted with these reagents and subsequently reinduced in media free of the uncoupling reagent. Of particular significance is the restoration of the more sensitive partial reactions, and of respiratory control.

Albumin exerts this effect by binding and thereby removing the inhibitory reagents from mitochondria. As this protein does not enter mitochondria to any significant degree (34), some type of uncoupling phenol concentration gradient across the mitochondrial membrane must be operative. It may be envisaged that as the phenol is removed by albumin from a site on or near the surface of the mitochondrion, additional phenol moves from distant loci to the membrane surface. Likewise, it may be envisaged that the various albumin-phenol complexes which uncouple phosphorylation (Table VII) envelope the mitochondrial surface. By an analogous concentration gradient, the uncoupling reagent may be transferred from albumin to the mitochondrial sites of phosphorylation.

The results also indicate the quantitative aspects of the protein-phenol interaction. It is evident that albumin must be present in amounts at least equimolar to that of the uncoupling phenol to counteract completely the effects of these compounds (cf. Reference 30). Unawareness of this stoichiometric requirement for albumin apparently accounts for earlier reports that the protein does not prevent the uncoupling action of 2,4-dinitrophenol (37),4 and that it is ineffective with high concentrations of the nitrophenol and of Dicumaroil (10).

We have previously demonstrated that a mitochondrial protein has the ability to bind uncoupling phenols (20, 39). However, serum albumin has at least one binding site of greater affinity for pentachlorophenol than do mitochondria. Consequently, in the protection experiments, the molar amount of albumin added to the medium must equal the molar amount of added phenol. In the reversal experiments, the amount of albumin required to restore oxidative phosphorylation is somewhat greater than the amount of phenol actually bound to the mitochondrion. This may mean that in addition to binding the uncoupling phenol,

4 Similarly species differences have been found for the binding of the dye, 2-(4'-hydroxyphenylazo)benzoic acid, to various serum albumins (36).

5 Interestingly enough, in a later report, Myers and Slater (38) in studying the effect of bovine serum albumin on 2,4-dinitrophenol-induced ATPase of mitochondria, employed an amount of the protein which approached an equimolar relationship with that of the phenol and thereby markedly inhibited the ATPase activity.

albumin is complexing endogenous substances released by the attrition incidental to the experimental procedures. On the other hand, it should be pointed out that it is not necessary to remove all of the pentachlorophenol from mitochondria swollen by this reagent in order to permit an ATP-induced contraction (30). In such experiments, the partial removal of the phenol may be sufficient to liberate sites essential for contraction which may differ from those sites involved in electron transport and coupled phosphorylation.

It appears that bovine serum albumin interacts most readily with those exogenous inhibitory reagents which have simple planar ring structures. It should be noted that albumin failed to counteract the inhibitory effects of polypeptide antibiotic reagents, and that of the polycyclic compound, rotenone. Presumably, these compounds are not bound preferentially by albumin.

The experiments designed to determine the chemical nature of the loci on the albumin molecule involved in the phenol binding indicate that sulfhydryl and imidazole groups are not the active sites. On the other hand, as acetylation abolished the beneficial effect of the protein, it is evident that free amino groups are necessary for the protein-phenol interaction. Similar treatment of the albumin molecule has been shown by Helinski and Cooper (10), and Preignitz and Wojtczak (14) to render it ineffective in countering liberated endogenous fatty acids which can uncouple oxidative phosphorylation. Moreover, albumin containing pentachlorophenol in a 1:1 molar ratio was not as effective as untreated albumin in restoring phosphorylation to mitochondria which had been stored for 96 hours for 4°C. Thus, it appears likely that similar sites on the albumin molecule are involved in interaction with either endogenous or exogenous uncoupling compounds.

The reversibility of the binding of uncoupling phenols by albumin may be considered as an analogue of one type of cellular regulatory control. For example, one of the controlling factors of mitochondrial oxidative phosphorylations may be the level of endogenous physiological uncoupling compounds. The concentration of such compounds at the active mitochondrial sites may in turn be regulated by their reversible binding to plasma proteins, to cytoplasmic proteins, and to mitochondrial proteins. Although the data in this paper provide some support for such speculation, more importantly, they provide a basis for studying diverse mitochondrial functions in the transition from the coupled to the uncoupled and to the recoupled states. This approach already has proven useful in a correlative study of morphological and functional changes accompanying both mitochondrial swelling and contraction (30) and mitochondrial interaction with various inhibitory reagents (40).

**REFERENCES**
