40° C. The whole was evaporated in vacuum and the alcohol was removed as far as possible at the lower temperature. The residue, after dilution with water, was extracted with ether six times and the ether extract, after being dried with sodium sulphate, was evaporated to a small volume. Benzene was added to it, and a white precipitate settled out. The whole mixture was passed through a column of alumina (BL-6) in order to remove impurities and pigments. The effluent was evaporated at a temperature below 60° C to remove the solvent, giving 4.5 grams of an orange wax, which contained plenty of bombicesterol. 3 grams of the extract was dissolved in a small amount of methanol by warming slightly. Bombicesterol separated on standing in a refrigerator, was filtered and the mother liquor evaporated gradually in a desiccator in the dark, when a crop of bombicesterol crystals again separated out. After filtration, the mother liquor, protected from oxidation in air by the addition of a drop of D,L-α-tocopherol, was evaporated gradually in order to remove methanol. The crystals of bombicesterol from the second and further crystallization contained the adhering sex attractant and attracted the male moth strongly. These crystals were treated with methanol and the methanolic extracts were united to the mother liquor. This procedure was repeated in order to remove bombicesterol as much as possible, and when the quantity of mother liquor reached about 1 ml the whole was evaporated strongly under reduced pressure to remove as much methanol as possible. The resulting brown syrup was subjected to the molecular distillation. The portion which distilled between 100° C and 110° C under 0.06 mm Hg pressure was taken separately. It is a thick faintly yellowish oil and 0.0005 γ of it has clearly the ability to excite the male moth and cause him to dance in a circle; 0.00025 γ of it is further able to make him vibrate his wings. On a paper chromatograph developed with butanol/acetic acid/H_2O (ascending method), the R_F of bombixin was about 1.0, with 85% phenol, 0.98, and with abs. benzene/abs. methanol (6:4), 0.82. In this case it was very interesting to use a male moth as the detector, in the following way. After the paper strip was developed with abs. benzene/abs. methanol and dried, it was passed slowly in front of the male moth. When the spot of the sex attractant approached the male, the insect became excited and went round the spot shaking his wings violently (Fig. 1).

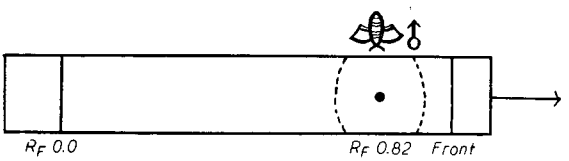

Fig. 1.

The elementary analysis showed that our bombixin had the following composition; C, 86.41%; H, 12.05%. In the spectral analysis this substance showed no remarkable specific absorption in the ultraviolet region. In the infrared spectral analysis there was some indication of the existence of an primary hydroxyl group. A portion of the sample was purified by chromatography in the absence of air and light and in a favourable case a sample active in $2 \sim 4 \cdot 10^{-5}$ γ was obtained. But the yield was very minute.

When our study had proceeded so far, we were informed by a friend that there had been a report on the same subject by A. BUTENANDT, who isolated in 1938 a male attracting substance from the female of *Bombix mori* by a method different from ours (*Angew. Chem.*, 54 (1941) 89), which was said to be physiologically active in 0.01 γ. But, as described above, our bombixin is far more active than BUTENANDT's substance.

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Activation of cytochrome c reductases by a lipid bound to crystalline bovine serum albumin*

It has already been shown that the marked decrease in activities of diphosphopyridine (DPN)- and succinate-cytochrome *c* reductases resulting from *iso*octane extraction can be completely reversed by the addition specifically of tocopherol[1]. The naturally occurring lipid material which is removed from the purified enzymes by *iso*octane extraction can also reactivate, although it contains no free tocopherol[1]. During a study of these systems it was observed that crystalline bovine serum albumin in relatively high concentrations could substitute for tocopherol in

restoring the activities of the above enzymes as well as that of reduced diphosphopyridine nucleotide (DPNH) oxidase. The experiments reported in the present paper demonstrate that a lipid material, bound to crystalline bovine serum albumin and extractable with *iso*octane, is responsible for the ability of the albumin to reactivate the above *iso*octane-extracted enzyme systems.

A 15- to 25-fold purified particulate fraction possessing DPNH oxidase and DPN- and succinate-cytochrome *c* reductase activities was prepared from rat skeletal muscle[1] and extracted 2 to 4 times where indicated with spectral grade *iso*octane (2,2, 4-trimethylpentane) as previously described[1]. The activities of the DPN- and succinate-cytochrome *c* reductases were followed spectrophotometrically by observing the rate of reduction of added cytochrome *c* at 550 mμ in the presence of cyanide. DPNH oxidase was determined by measuring the rate of decrease in optical density at 340 mμ in the absence of cyanide. Crystalline and amorphous bovine serum albumin were obtained from the Armour Laboratories; and 3- to 5-times recrystallized bovine serum albumin, human serum albumin and human serum γ-globulin were generously provided by Dr. WALTER L. HUGHES. The various protein solutions were made up with 0.1 *M* phosphate buffer, pH 7.5, and, where indicated, extracted twice by shaking each time with an equal volume of isooctane for 1 minute, followed by removal of the *iso*octane layer.

The curves in Fig. 1 represent a comparison of the abilities of various proteins to activate the *iso*octane-extracted DPN-cytochrome *c* reductase system. Bovine serum albumin, whether amorphous or crystalline (or recrystallized several times), characteristically restores enzyme activity, whereas crystalline egg albumin, and human serum γ-globulin have little or no effect. Crystalline human serum albumin at higher concentrations shows some activity.

Fig. 2 shows that *iso*octane-extracted crystalline bovine serum albumin** is no longer able to reactivate *iso*octane-extracted DPN-cytochrome *c* reductase. The *iso*octane-extracted albumin, however, can still be used to suspend tocopherol in the usual manner[1] as shown in Fig. 2. The small oily residue remaining after vacuum distillation of the *iso*octane extracts of the crystalline bovine serum albumin, when suspended in the extracted and, therefore, inactive albumin, can partly restore the activity of the enzyme. Similar results have been obtained using succinate-cytochrome *c* reductase and DPNH oxidase in place of DPN-cytochrome *c* reductase. Complete restoration by the lipid residue has not been obtained thus far. It appears that the lipid material is in part unavailable after extraction and resuspension, perhaps as a result of destruction, since its total activity is considerably less than an equivalent amount of unextracted albumin. The effect of added lipid residue, though small, is reproduceable in each of the above enzymic assay systems.

The above data indicate that a lipid material originally bound to bovine serum albumin and extractable with *iso*octane is the responsible component in the activation of enzyme activity by the albumin. In view of the similarity of the ultra violet absorption spectrum and biological activity of the albumin lipid with that of the partially purified lipid cofactor*** obtained from beef heart and rat skeletal muscle, it is not unlikely that they may prove to be identical. KEILIN AND HARTREE[2] have attributed the restoration in activity of succinoxidase preparations by calcium phosphate gel and proteins to a non-specific effect on the colloidal structure of the enzyme, presumably by reorienting the catalytic components, especially endogenous cytochrome *c*, within the colloidal particles.

Lysine and glyoxaline-4-5-dicarboxylic acid[3] and histidine[4] have also been shown to activate preparations of KEILIN-HARTREE horseheart succinoxidase in unfavorable environments. ALTMAN AND CROOK[5], however, have ascribed the action of the above materials to their ability to adsorb or chelate heavy metals which are present as contaminants in the reaction mixture. They showed that the use of purified succinate resulted in a marked increase in activity and that a sharp rise is also obtained when various chelating agents, especially versene, are added to the reaction mixture. BONNER[6] who has also reported activation by the above chelating agents and adsorbents of the succinate-cytochrome system has interpreted his results as indicating that "the chemical combining properties of these activating agents play a role in drawing together the various components and in this manner ensuring their mutual accessibility, one to the other".

The results reported here with the bound lipid of bovine serum albumin and the lipid cofactor obtained from beef heart and rat skeletal muscle in reactivation of enzyme activity are different from that indicated in the above literature. The enzyme used in these experiments was inactivated as a result of removal of a lipid cofactor by *iso*octane extraction. Restoration

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** It should be noted that relatively high concentrations of albumin (10 to 40%) are first extracted with *iso*octane and then diluted at least 4-fold before addition to the reaction mixture. Albumin solutions (0.2 to 40%) when extracted with *iso*octane and used as such without dilution are inhibitory. *iso*Octane-extracted phosphate buffer, however, is not inhibitory.

*** In preparation.

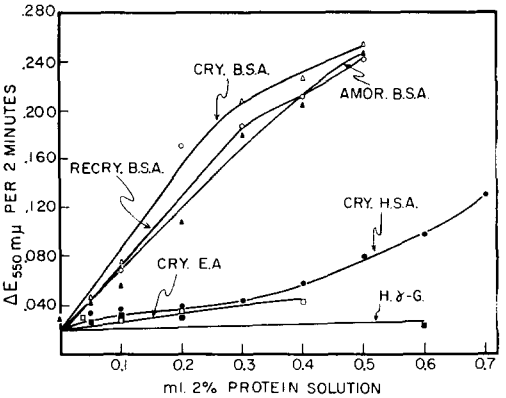

Fig. 1. Effect of various crystalline proteins in reactivating *iso*octane-extracted DPN-cytochrome *c* reductase. The reaction mixtures contained 0.05 ml of enzyme (about 130 μg protein), 0.05 ml of 2% aqueous cytochrome *c*, 0.1 ml of $10^{-2}M$ KCN, and $10^{-1}M$ phosphate buffer, pH 7.5, and 2% protein solutions to give a final reaction volume of 1.0 ml. Crystalline bovine serum albumin (Armour Laboratories), △—△: amorphous bovine serum albumin (fraction V powder, Armour Biochemicals), ▲—▲; recrystallized bovine serum albumin, ○—○; crystalline human serum albumin, ●—●; crystalline egg albumin, □—□; human serum γ-globulin, COHN procedure, fraction II (7), ■—■.

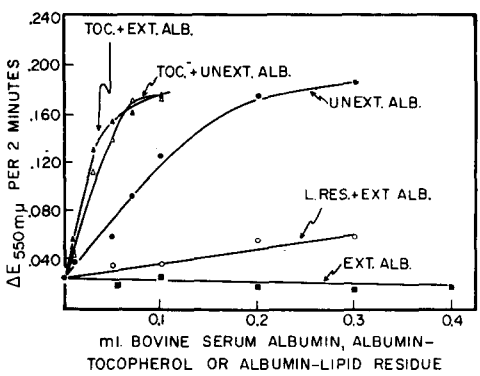

Fig. 2. Effect of unextracted and *iso*octane-extracted crystalline bovine serum albumin and added tocopherol or lipid residue in reactivating *iso*octane-extracted DPN-cytochrome *c* reductase. The reaction mixture contained 0.05 ml of enzyme (about 200 μg protein), 0.10 ml of 2% aqueous cytochrome *c*, 0.3 ml of $10^{-2}M$ KCN, 0.1 ml of DPNH (6.1 μmole/ml), and $10^{-1}M$ phosphate buffer, pH 7.5, and the indicated solutions of crystalline bovine serum albumin to give a final volume of 3.0 ml; *d*-α-Tocopherol (4 μmole/ml) as a 15% ethanol-0.2% bovine serum albumin suspension was added in increasing amounts as shown. The lipid residue obtained from *iso*octane extraction of 10 ml of a 2% crystalline bovine serum albumin solution was suspended in 1.0 ml *iso*octane-extracted albumin and also added in increasing amounts as shown. Tocopherol in unextracted 0.2% albumin, △—△; tocopherol in *iso*octane-extracted 0.2% albumin, ▲—▲; 10% unextracted albumin ●—●; 10% *iso*octane-extracted albumin, ■—■; lipid residue in 2% *iso*octane-extracted albumin, ○—○.

of activity can only be achieved by the addition of the removed lipid, the bound lipid of bovine serum albumin, or tocopherol*. The latter dissolved in ethanol**, instead of suspended in albumin, can also restore the activities of DPN- and succinate-cytochrome *c* reductases and DPNH oxidase. Calcium phosphate gel or versene had no activity. The possible role of this lipid in oxidative phosphorylation and electron transport as well as its possible relationship to tocopherol are now being studied.

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** The use of tocopherol dissolved only in ethanol is limited in view of the removal of tocopherol from solution upon addition to the aqueous reaction mixture, as well as the sensitivity of the albumin or some other protein such as γ-globulin is necessary as a vehicle in the aqueous reaction mixture.