Biochemical Pharmacology, 1967, Vol. 16, pp. 295–308. Pergamon Press Ltd. Printed in Great Britain

# PLASMA ALBUMIN AS A CATALYST IN CYCLIZATION OF DIARYL *o*-(α-HYDROXY)TOLYL PHOSPHATES

MORIFUSA ETO, YASUYOSHI OSHIMA

Department of Agricultural Chemistry,
Kyushu University, Fukuoka, Japan

and

JOHN E. CASIDA

Division of Entomology,
University of California, Berkeley, Calif., U.S.A.

(*Received* 15 *June* 1966; *accepted* 3 *October* 1966)

**Abstract**—Diaryl *o*-(α-hydroxy)tolyl phosphates are cyclized with liberation of an aryl group to yield aryl saligenin cyclic phosphates, which usually are potent antiesterase agents. This cyclization is catalyzed by various plasmata, but not by liver homogenate. The cyclizing activity of plasma is associated with the albumin component when this fraction is prepared either by salting-out with ammonium sulfate or by using Cohn's method and ethanol (fraction V). The activity of the cyclizing enzyme could not be separated from albumin either by Sephadex G-100 or by DEAE-cellulose column chromatography. *p*-Nitrophenyl acetate, which is also hydrolyzed by the albumin fraction, inhibits the cyclization of diaryl *o*-(α-hydroxy)tolyl phosphates.

TRI-*o*-TOLYL phosphate (tri-*o*-cresyl phosphate or TOCP) is metabolized to saligenin cyclic phosphates having neurotoxic and antiesterase activities.[1–3] Some homologous cyclic phosphates are insecticidal.[4–6] The metabolic activation of TOCP and its homologs *in vivo*, having at least one *o*-tolyl group and an additional aryl group, occurs in rats,[1, 2] chickens,[2] and houseflies.[7] Rat liver microsomes and reduced nicotinamide adenine dinucleotide ($NADH_2$), in the presence of air, are effective in activation of TOCP *in vitro*.[2, 8] Liver microsome systems, in the presence of $NADH_2$ or the 5′-phosphate analog ($NADPH_2$), are known to be active in oxidation or hydroxylation of organic compounds foreign to the body. Thus, the first step in TOCP activation is probably hydroxylation to yield di-*o*-tolyl *o*-(α-hydroxy)tolyl phosphate. The conversion *in vivo* of this hydroxylated intermediate to the corresponding cyclic phosphate has been demonstrated.[2] This report presents evidence for the action of plasma albumin as a catalyst for the cyclization reaction. The metabolic pathway of TOCP homologs is shown in Fig. 1; it is the last reaction in this series, that of II cyclizing to form III, which is specifically considered.

## EXPERIMENTAL

*Synthesis of o-(α-hydroxy)tolyl phosphates.* One molar equivalent of diphenyl phosphorochloridate was added, with stirring, to a chilled aqueous solution containing 1 M equivalent of each of saligenin and sodium hydroxide. After 15 min at 5°, the reaction mixture was extracted with benzene, the benzene phase was washed with

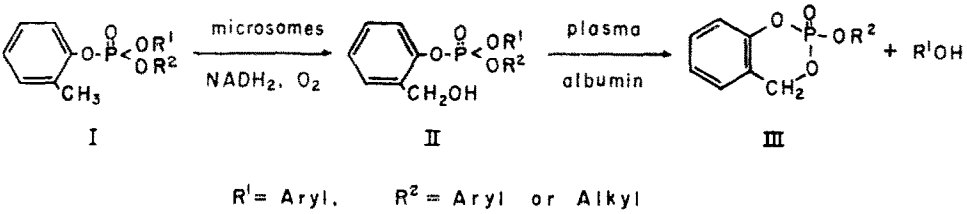



FIG. 1. Enzymes and reactions involved in formation of antiesterases from TOCP analogs.

dilute aqueous sodium hydroxide and saturated sodium chloride solutions, dried with sodium sulfate, the solvent evaporated, and the residue purified on a silicic acid column.[2] The crude product generally contained some cyclic phosphate and other impurities. The purified product, an undistillable oil, was negative to the amino-antipyrine test for phenol (described later), unless it was previously hydrolyzed. The

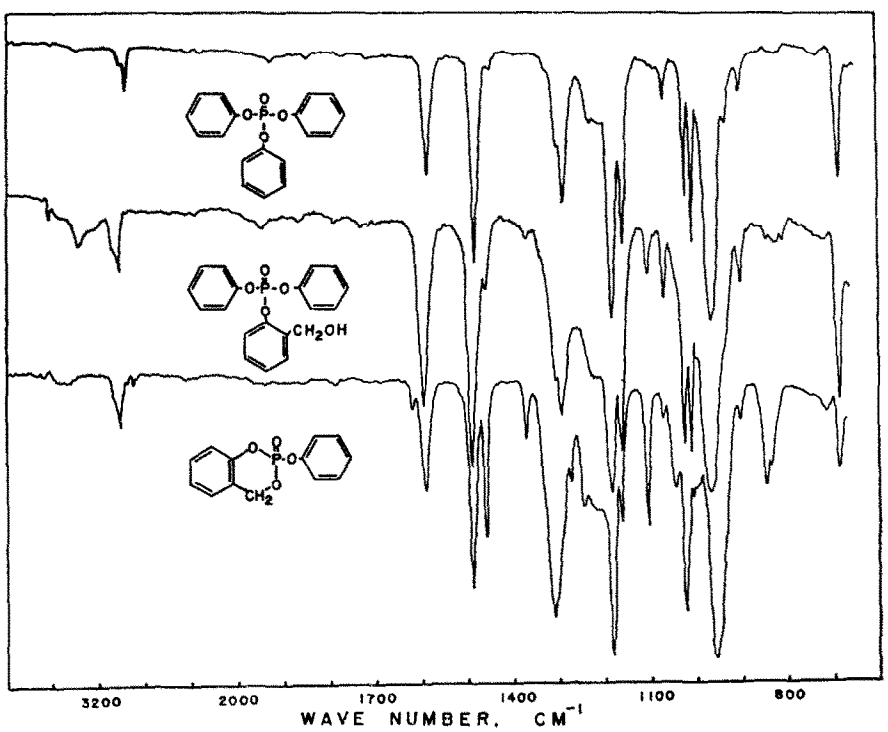
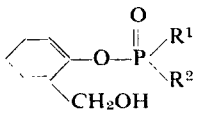



FIG. 2. Infrared spectra of triphenyl phosphate, diphenyl *o*-(α-hydroxy)tolyl phosphate, and 2-phenoxy-4H-1,3,2-benzodioxaphosphorin-2-oxide (5% solutions in chloroform).

criteria of product purity were a single component on thin-layer chromatography and an appropriate infrared spectrum. As seen in Fig. 2, the spectrum of diphenyl *o*-(α-hydroxy)tolyl phosphate is almost identical with that of triphenyl phosphate, except for a broad associated OH-stretching vibration at 3360 $cm^{-1}$, but is quite different in

many regions from that of its corresponding cyclic phosphate, 2-phenoxy-4H-1,3,2-benzodioxaphosphorin-2-oxide. Three analogs were prepared in a similar manner, and these are listed in Table 1.

TABLE 1. SUBSTRATE SPECIFICITY OF SWINE PLASMA FOR CYCLIZATION



| $R^1$ | $R^2$ | μmole Phenol or cresol/g plasma/ 10 min | Relative activity |
|---|---|---|---|
| $OC_6H_5$ | $OC_6H_5$ | 3·1 | 100 |
| $OC_6H_4$-*o*-$CH_3$ | $OC_6H_4$-*o*-$CH_3$ | 2·5 | 81 |
| $NHCH_3$ | $OC_6H_5$ | 0·2 | 6 |
| $OC_2H_5$ | $OC_2H_5$ | | 0 |

The reaction conditions for the first three compounds were as indicated in Table 3. In the case of the last compound, the reaction mixture contained 80 μmoles substrate and 0·5 g plasma in 2·4 ml of 0·025 M veronal buffer, pH 8·2; after 1 hr at 30°, the reaction mixture was extracted with chloroform and the products in the chloroform were examined by thin-layer chromatography.

Saligenin cyclic phosphates were prepared by published procedures.[2, 9]

*Chromatography.* Silicic acid columns[2] developed with either benzene–ether or chloroform–ether gradients were used for purification of the *o*-(α-hydroxy)tolyl phosphates. Thin-layer chromatography (TLC) with silicic acid on microscope slides (2·6 × 7·7 cm) and reversed-phase paper chromatography on silicone-impregnated paper[2] were employed for separation of phenols from their phosphate esters; the silica gel was Wako gel B-5 from Wako Pure Chemicals Industries, Ltd., Osaka, and the silicone was Toshiba 953H from the Tokyo Shibaura Electric Co., Ltd., Kawasaki. In order to characterize them more definitely, the phenols were converted to their aminoantipyrine derivatives prior to paper chromatography. Solvent systems used for chromatography were: chloroform for TLC; acetone:dioxane:water (2:2:5) for paper chromatography of the phenols and phosphate esters, the upper layer from a benzene:acetone:water (5:1:4) mixture for paper chromatography of the aminoantipyrine derivatives. By developing the TLC plates with chloroform, the order of the spots in ascending $R_f$ values was saligenin, *o*-(α-hydroxy)tolyl phosphates, phenols, and saligenin cyclic phosphates; more complete resolution of the first two compounds resulted on addition of small amounts of ether to the chloroform.

Diazotized sulfanilic acid was used to detect phenols liberated on hydrolysis of the esters as a result of spraying with an alcoholic solution of potassium hydroxide and heating for a short period.[2]

*Enzyme preparations.* Homogenates of whole adult oriental houseflies, *Musca domestica vicina* Macquart, or of liver from male mice were prepared in water and centrifuged at 250 *g* for 5 min. The supernatant fraction was used for assays.

Plasma proteins were separated into three fractions by salting-out with ammonium sulfate. The "globulin" fraction was the precipitate obtained at 50% saturation, and

the "albumin" fraction was the precipitate obtained by adjusting to pH 4·8 the supernatant separated from the globulin fraction; the "soluble" fraction was that remaining after removing the albumin. Egg albumin was also prepared by the salting-out method. Each protein fraction was reprecipitated three times for purification. The precipitates from the salting-out procedure were dialyzed through Visking tubing against water or subjected directly to a Sephadex G-100 column (2 × 30 cm) and eluted with 0·033 M phosphate buffer, pH 8. Bovine plasma albumin fraction V (50 mg) was also chromatographed on this column, as well as on a DEAE-cellulose column (1 × 25 cm). Elution of the 50-mg sample from the DEAE-cellulose column was accomplished with a gradient of 0·006 M to 0·333 M phosphate buffer, pH 8. The position of protein elution was determined by measuring the absorbance at 280 mμ ($E_{280m\mu}$).

The other proteins used were from the following sources: bovine plasma albumin fraction V (as prepared by Cohn's method with ethanol) was purchased from Armour Pharmaceutical Co., Kankakee, Ill.; crystalline ribonuclease [E.C. 2.7.7.16: ribonucleate pyrimidinenucleotido-2′-transferase (cyclizing)] and crude snake venom were provided by Dr. J. I. Mukai of Kyushu University; *Streptomyces* protease (pronase) was a commercial preparation from Kaken Kagaku Co., Tokyo; casein according to Hammersten was from Merck A.G., Darmstadt; gelatin was from Wako Pure Chemicals Industries, Osaka.

*Assay of enzyme activity.* In most of the studies the reaction was followed by analysis for liberated phenols. Each enzyme preparation was buffered with veronal or phosphate and adjusted to the desired pH. A 1·0-ml aliquot was placed in a glass-stoppered test tube in a water bath; then 1–2 μmoles substrate [*o*-(α-hydroxy)tolyl phosphate derivative] in 0·1 ml acetone was added. After 10-min incubation, 10 ml of xylene, 0·5 ml of 0·2% aqueous 4-aminoantipyrine solution, and 3 ml of 0·033 M phosphate buffer at pH 8 were immediately added to the tube. The mixture was vigorously shaken for 30 sec, 0·5 ml of 0·8% aqueous potassium ferricyanide solution was added, and the mixture was shaken for 4 min. The xylene solution was drawn off, dried over sodium sulfate, and the absorbance measured with an electrophotometer (Hitach EPO-B) with a blue filter( No. 43). Correction for spontaneous or nonenzymatic reactions was made in each experiment. In certain cases, the specific activity of the preparations is expressed on the basis of μmoles phenol or cresol liberated per $E_{280}$ of protein solution per ml/10 min.

For characterization of reaction products, a mixture containing 1 ml of horse plasma, 1 ml of 0·033 M phosphate buffer (pH 8·0), and 16 μmoles of diphenyl *o*-(α-hydroxy)tolyl phosphate in 1 ml phosphate buffer (pH 8) was incubated at room temperature for 1 hr, and the reaction mixture was then extracted with 1 ml chloroform. The extract was submitted to thin-layer and paper chromatography. In another experiment, a mixture containing 5 mg bovine plasma albumin and 0·1 μmole diphenyl *o*-(α-hydroxy)tolyl phosphate in 1 ml phosphate buffer (pH 8) was incubated at 30° for 10 min, and then extracted with 1 ml chloroform. A 0·1-ml aliquot of the chloroform was evaporated, and 2 mg chymotrypsin in 1 ml of 0·033 M phosphate buffer (pH 7) was added and incubated at 10° for 10 min. Inhibition of the esteratic activity of chymotrypsin was assayed with *p*-nitrophenyl acetate.

Esterase activity of plasma and other proteins was assayed by using 1 μmole *p*-nitrophenyl acetate as the substrate. The liberation of *p*-nitrophenate ion was measured at 400 mμ with the Hitachi EPU-2 spectrophotometer.

## RESULTS

*Formation of 2-phenoxy-4H-1,3,2-benzodioxaphosphorin-2-oxide from diphenyl o-(α-hydroxy)tolyl phosphate by the catalytic action of plasma*

Phenol and the corresponding cyclic phosphate, 2-phenoxy-4H-1,3-2-benzodioxaphosphorin-2-oxide, are the reaction products of diphenyl *o*-(α-hydroxy)tolyl phosphate with bovine plasma (Table 2). These products are also produced spontaneously,

TABLE 2. PRODUCTS FORMED BY THE ACTION OF HORSE PLASMA ON DIPHENYL *o*-(α-HYDROXY)TOLYL PHOSPHATE

| Sample | Chromatography, $R_f$ | |
|---|---|---|
| | Thin-layer* | Paper |
| Known compounds | | |
| Diphenyl-*o*-(α-hydroxy) tolyl phosphate | 0·06 | |
| 2-Phenoxy-4H-1,3,2-benzo-dioxaphosphorin-2-oxide | 0·43 | 0·69† |
| Phenol, free | 0·26 | 0·90† |
| Phenol, as aminoantipyrine derivative | | 0·95‡ |
| Reaction products | | |
| A | 0·44 | 0·68† |
| B | 0·26 | 0·90† |
| B, As aminoantipyrine derivative | | 0·95‡ |

* Silica gel, chloroform.
† Silicone, acetone:dioxane:water (2:2:5).
‡ Benzene:acetone:water (5:1:4), upper layer.

but at only about one tenth of the rate for the reaction as catalyzed by the plasma, based on the amount of phenol liberated. It appears that the amount of cyclic phosphate produced in the presence of plasma is smaller than the amount of phenol liberated, based on the intensity of the yellow color on TLC plates resulting from reaction of the resolved products with diazotized sulfanilic acid. However, when the plasma is heated at 90° for 10 min prior to assay, larger amounts of cyclic phosphate are recovered than without this heat treatment, even though the phenol liberation is somewhat less.

The production of a saligenin cyclic phosphate by a plasma component, fraction V, is also established by the inhibitory activity of the product for the esteratic activity of chymotrypsin. The cyclic phosphate produced on incubation with fraction V results in 75 per cent inhibition of the normal activity of chymotrypsin for hydrolysis of *p*-nitrophenyl acetate. The degree of esterase inhibition found in this study from the cyclic phosphate recovered after incubation with fraction V is based on comparison with that recovered from another reaction mixture identical in all constituents except that albumin was omitted.

*Activity of various plasmata*

The amount of phenol liberated from diphenyl *o*-(α-hydroxy)tolyl phosphate increases with both time and concentration of swine plasma (Fig. 3). The amount of phenol liberated in 10 min at 30° is proportional to the concentration of plasma. However, the rate of liberation of phenol decreases during the course of the reaction.

The activity of plasmata from various mammals, including human, cow, swine, and horse, is almost twice as high as that of chicken plasma (Table 3). Only very weak activity for phenol liberation (and, therefore, for catalyzing cyclization) is observed for homogenates of houseflies and mouse liver.

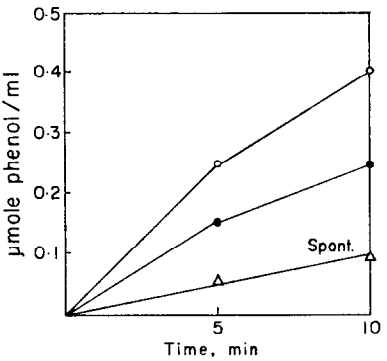



FIG. 3. Effect of swine plasma on liberation of phenol from diphenyl *o*-(α-hydroxy)tolyl phosphate. Reaction constituents in each ml of 0·025 M veronal buffer, pH 8·2, consisted of 1·6 μmoles substrate and no (Spont.) △——△, or 46 μl ●——●, or 92 μl ○——○ plasma. Reaction was in 2·2 ml total volume at 30°.

*Fractionation of the active component in plasma*

When plasma is fractionated with ammonium sulfate, the most active component for the release of phenol from diphenyl *o*-(α-hydroxy)tolyl phosphate is the albumin precipitated at pH 4·8 and at half saturation. Results obtained with swine plasma, with the *o*-tolyl homolog as the substrate are given in Table 4; similar results are also

TABLE 3. ACTIVITY OF VARIOUS PLASMATA AND HOMOGENATES OF MOUSE LIVER AND HOUSEFLIES FOR LIBERATION OF PHENOL FROM DIPHENYL *o*-(α-HYDROXY)TOLYL PHOSPHATE

| Enzyme | (μmoles phenol/g/10 min) |
|---|---|
| Human plasma | 3·5 |
| Swine plasma | 3·1 |
| Bovine plasma | 3·0 |
| Horse plasma | 2·8 |
| Chicken plasma | 1·6 |
| Mouse liver | 0·13 |
| Houseflies | 0·36 |

Each reaction mixture contained 1·6 μmoles substrate and 0·1 g (fresh weight) of plasma or homogenized tissue in 1·1 ml of 0·025 M veronal buffer, pH 8·2. Incubation was for 10 min at 30°.

obtained with human, bovine, and chicken plasmata. Chromatography on either Sephadex or DEAE-cellulose of the albumin fraction obtained from salting-out of bovine and chicken plasma gives only a single protein peak, and the catalytic activity is associated with this peak (Table 5). Bovine plasma globulin and egg albumin, from salting-out and purification on the Sephadex column, are without appreciable catalytic activity (Table 5). Bovine plasma albumin fraction V is even more active than the

albumin fraction of bovine plasma obtained by the salting-out procedure (Table 5). Fraction V of bovine plasma consists of about 95–98 per cent albumin and shows only one protein peak, which is coincidental with that of the catalytic activity, when chromatographed on the Sephadex column (Fig. 4) or on the DEAE-cellulose column

TABLE 4. ACTIVITY OF SWINE PLASMA AND FRACTIONS OBTAINED WITH AMMONIUM SULFATE FOR LIBERATION OF *o*-CRESOL FROM DI-*o*-TOLYL *o*-(α-HYDROXY)TOLYL PHOSPHATE

| Fraction | Activity recovery (%) | μmole cresol/ $E_{280}$/ml/10 min | Purification |
|---|---|---|---|
| Whole plasma | 100 | 0·024 | 1 |
| "Globulin" fraction | 0–21 | 0·005 | |
| "Albumin" fraction | 50–68 | 0·055 | 2·3 |
| "Soluble" fraction | 0–18 | 0·015 | |

Each reaction mixture contained, in addition to the enzyme, 1·6 μmoles substrate in 1·1 ml of 0·025 M veronal buffer, pH 8·2. Incubation was for 10 min at 30°.

TABLE 5. ACTIVITY OF SEVERAL PROTEIN PREPARATIONS FOR LIBERATION OF PHENOL FROM DIPHENYL *o*-(α-HYDROXY)TOLYL PHOSPHATE

| Protein | Preparation method | (μmoles phenol/ $E_{280}$/ml/10 min) |
|---|---|---|
| Bovine plasma albumin | Fraction V | 0·172 |
| | Fraction V Sephadex G-100 | 0·172 |
| | Fraction V DEAE-cellulose | 0·186 |
| | Salting-out Sephadex G-100 | 0·131 |
| Bovine plasma globulin | Salting-out Sephadex G-100 | 0·0007 |
| Chicken plasma albumin | Salting-out Sephadex G-100 | 0·099 |
| Egg albumin | Salting-out Sephadex G-100 | 0·0008 |

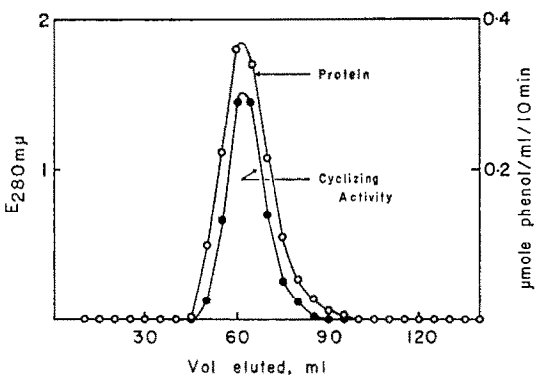



FIG. 4. Elution patterns for protein and cyclizing activity resulting from chromatography of bovine plasma albumin fraction V on a Sephadex G-100 column.

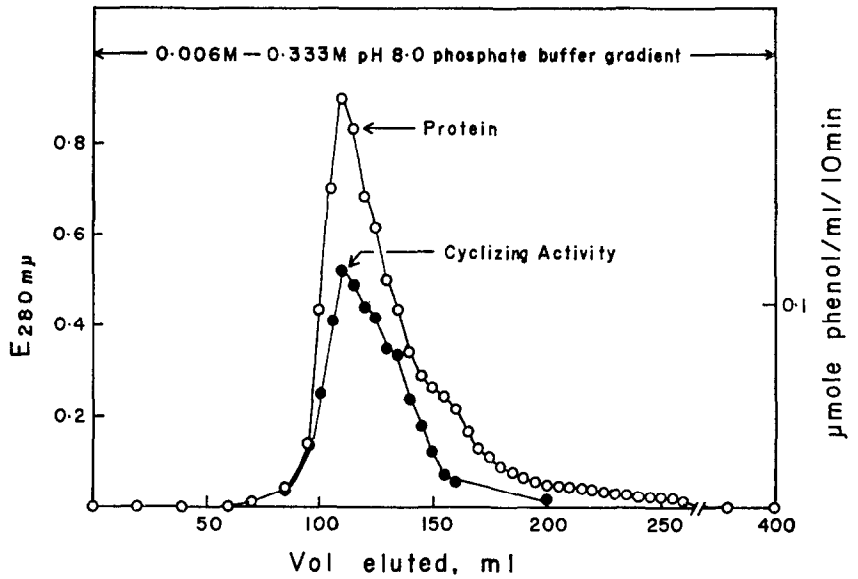



FIG. 5. Elution patterns for protein and cyclizing activity resulting from chromatography of bovine plasma albumin fraction V on a DEAE-cellulose column. Correction was made for the phosphate-catalyzed nonenzymatic liberation of phenol.

(Fig. 5). The activity of fraction V is not appreciably altered by further purification on these columns (Table 5). It appears that the albumin component of plasma is responsible for the catalysis in releasing phenol; therefore it is probably the component active in cyclization of diphenyl *o*-(α-hydroxy)tolyl phosphate.

Among the other proteins assayed, casein and gelatin are not active.

*Characteristics of plasma albumin in the catalytic action*

The amount of phenol liberated from diphenyl *o*-(α-hydroxy)tolyl phosphate increases with the concentration of bovine plasma fraction V, but the increase is not linear at high protein concentrations (Fig. 6). The pH optimum for release of phenol

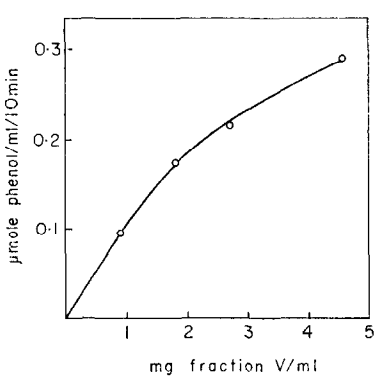



FIG. 6. Effect of bovine plasma albumin fraction V on liberation of phenol from diphenyl *o*-(α-hydroxy)tolyl phosphate. Reaction constituents and analysis were the same as indicated for Fig. 3 except that 2 μmoles substrate was used per 1·1 ml reaction mixture.

by plasma albumin is about 8·5; however, in the high pH regions, the spontaneous reaction contributes a high proportion of the total phenol liberated in the presence of fraction V (Fig. 7). Therefore, to minimize the spontaneous reaction, pH 8 is the most suitable for the assay. The optimal temperature for assay of the catalysis is 30° (Fig. 8).

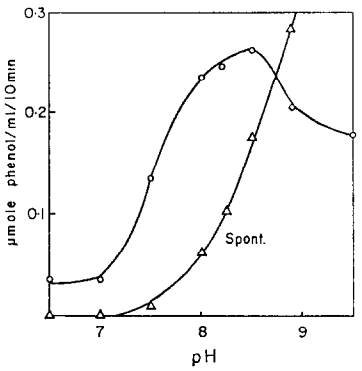



FIG. 7. Effect of pH on the activity of bovine plasma albumin fraction V for liberation of phenol from diphenyl *o*-(α-hydroxy)tolyl phosphate. Each reaction mixture contained 2 μmoles substrate and 3 mg fraction V in 1 ml of 0·017 M veronal buffer. Incubation was for 10 min at 30°.

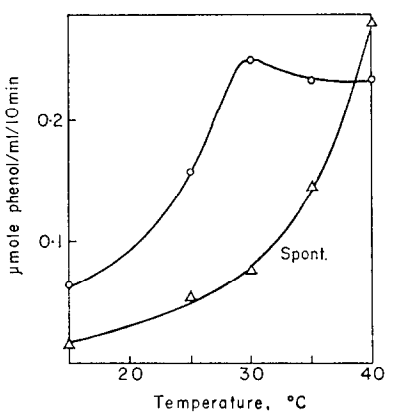



FIG. 8. Effect of temperature on the activity of bovine plasma albumin fraction V for liberation of phenol from diphenyl *o*-(α-hydroxy)tolyl phosphate. Conditions the same as those indicated in Fig. 7, with buffer pH of 8·0.

The catalytic activity of fraction V from bovine plasma is decreased to a greater extent by heating than is that of whole swine plasma. Heating solutions of swine plasma and bovine plasma albumin (fraction V) at pH 8 and 95° for 10 min destroys 28 and 59 per cent, respectively, of their catalytic activities (Table 6). Incubation of fraction V with *Streptomyces* protease at pH 8 and 27° for 16 hr completely destroys the activity for liberation of phenol from diphenyl *o*-(α-hydroxy)tolyl phosphate.

The rate of phenol liberation from diphenyl *o*-(α-hydroxy)tolyl phosphate, as catalyzed by bovine plasma albumin fraction V, varies with substrate concentration, as illustrated in Fig. 9. This Lineweaver–Burk plot indicates the formation of an unstable enzyme–substrate complex with an apparent dissociation constant ($K_s$) of

TABLE 6. HEAT-SENSITIVITY AND SUBSTRATE-SPECIFICITY FOR ESTERASE, PHOSPHATASE, AND CYCLIZING ACTIVITIES OF SNAKE VENOM, SWINE PLASMA, AND BOVINE PLASMA ALBUMIN FRACTION V*

| Enzyme preparation | Substrate, relative activity† | | | | |
|---|---|---|---|---|---|
| | *p*-Nitrophenyl acetate | Phenyl phosphate | Diphenyl phosphate | Triphenyl phosphate | Diphenyl *o*-(α-hydroxy)-tolyl phosphate |
| Snake venom | 740 | 0 | 150 | 0 | 100 |
| Snake venom heated‡ | 0 | 0 | 17 | 0 | 24 |
| Swine plasma | 1315 | 2·2 | 0 | 0 | 100 |
| Swine plasma heated‡ | 208 | 0 | 0 | 0 | 72 |
| Bovine plasma albumin | 276 | 0 | 0 | 0 | 100 |
| Bovine plasma albumin heated‡ | 138 | 0 | 0 | 0 | 41 |

* Each reaction mixture contained 1·6 μmoles substrate and the enzyme in 1·1 ml of 0·025 M veronal buffer, pH 8·2. The reactions with *p*-nitrophenyl acetate and diphenyl *o*-(α-hydroxy)tolyl phosphate were carried out for 10 min at 30°, those with the other compounds had a somewhat longer reaction time and involved higher enzyme concentrations.

† Activities, as μmoles of phenol or *p*-nitrophenol/g/10 min, are related to that for diphenyl *o*-(α-hydroxy)tolyl phosphate, which is set at 100 with each unheated enzyme source. Actual values for the standard with each enzyme were 29·4 for snake venom, 3·1 for plasma, and 95 for albumin.

‡ Heated for 10 min at 95°.

$4{\cdot}0 \times 10^{-4}$ M. The effect of *p*-nitrophenyl acetate on the activity of albumin fraction V for liberating phenol from diphenyl *o*-(α-hydroxy)tolyl phosphate is also shown in Fig. 9. *p*-Nitrophenyl acetate gives a mixed type of competitive and noncompetitive inhibition.

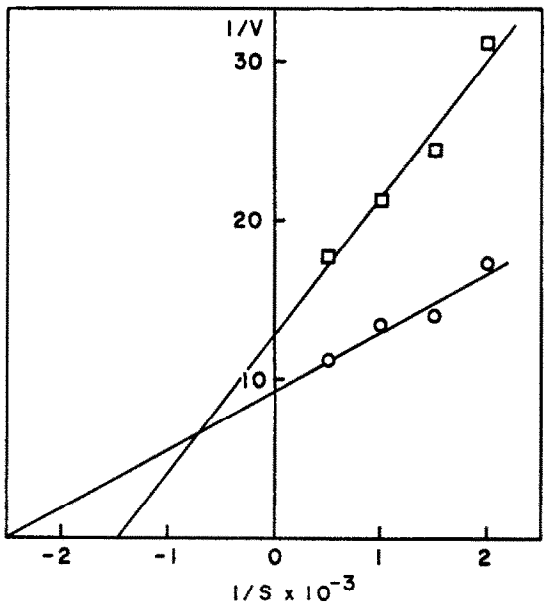



FIG. 9. Effect of concentration of diphenyl *o*-(α-hydroxy)tolyl phosphate on the activity of bovine plasma albumin fraction V for liberation of phenol in the presence (□——□) and in the absence (○——○) of *p*-nitrophenyl acetate. Reaction conditions were the same as those indicated in Fig. 6 except that fraction V was used at 2 mg/ml and *p*-nitrophenyl acetate, when present, was at 1·67 μmoles/ml.

No noticeable effect on the activity of swine albumin for liberating phenol from diphenyl *o*-(α-hydroxy)tolyl phosphate is observed when the following possible inhibitors or activators are used: metal ions at 1 mM, including $Al^{3+}$, $Ca^{2+}$, $Cu^{2+}$, $Fe^{2+}$, $Mg^{2+}$, and $Zn^{2+}$; various organic compounds at 0·2 mM, including ethylenediamine tetraacetic acid, iodoacetic acid, *p*-chloromercuribenzoate, and N-bromosuccinimide; certain organophosphorus compounds active as esterase inhibitors, including tetraethyl pyrophosphate (TEPP), diisopropyl phosphorofluoridate (DFP), and 2-methoxy-4H-1,3,2-benzodioxaphosphorin-2-oxide (salioxon, one of the saligenin cyclic phosphates).

Diphenyl *o*-(α-hydroxy)tolyl phosphate is the best one of a series of homologous phosphates examined as potential substrates for swine plasma (Table 1). Di-*o*-tolyl *o*-(α-hydroxy)tolyl phosphate is also a good substrate, while *o*-(α-hydroxy)tolyl phenyl N-methylphosphoramidate is a poor one. No cyclic phosphate is produced from diethyl *o*-(α-hydroxy)tolyl phosphate, as is shown by thin-layer chromatography of the reaction mixture. No liberation of phenol occurs on incubation of triphenyl phosphate with swine plasma or the albumin fraction of bovine plasma (Table 6), even in the presence of added benzyl alcohol.

Table 6 compares the activity of swine plasma and bovine plasma albumin fraction V with that of snake venom for hydrolysis or liberation of a phenol from several substrates. Pancreas ribonuclease is not included in the tabulation because it shows no activity on diphenyl *o*-(α-hydroxy)tolyl phosphate. However, crude snake venom shows activity on *p*-nitrophenyl acetate, diphenyl phosphate, and diphenyl *o*-(α-hydroxy)tolyl phosphate; with each of these substrates, almost all the activity is destroyed by heating. Swine plasma shows high activity in hydrolyzing *p*-nitrophenyl acetate, and 84 per cent of this activity is destroyed by heating at 95° for 10 min; however, only 28 per cent of the activity for release of phenol from diphenyl *o*-(α-hydroxy)tolyl phosphate is destroyed by this heating procedure. The plasma activity for catalyzing hydrolysis of phenyl phosphate is completely destroyed by heating; no activity for hydrolysis of either diphenyl phosphate or triphenyl phosphate is observed for the plasma.

The reaction rate for liberation of phenol from diphenyl *o*-(α-hydroxy)tolyl phosphate relative to that for liberation of *p*-nitrophenol from *p*-nitrophenyl acetate is greater with the purified bovine plasma albumin than with the whole swine plasma. The stability to heat of the bovine plasma albumin is almost the same when assayed with these two substrates. The activity of the fraction V albumin acting on either of the substrates is not at all inhibited by organophosphate antiesterases at 0·2 mM. Phosphatase activity is not found in the albumin fraction when mono-, di- or triphenyl phosphates are used as the substrate.

## DISCUSSION

From earlier studies it is known that TOCP is activated in living rats[2] and houseflies,[7] and that the activation is catalyzed by liver microsomes and $NADH_2$.[2, 8] Liver and housefly homogenates, however, are not active for cyclization of di-*o*-tolyl *o*-(α-hydroxy)tolyl phosphate, which is the probable intermediate in TOCP activation. The production of cyclic phosphates from TOCP, on incubation with microsomes and $NADH_2$, probably results from spontaneous cyclization of the hydroxylated intermediate, because diaryl *o*-(α-hydroxy)tolyl phosphates are so labile that spontaneous cyclization slowly proceeds with the liberation of an aryl group. Similar spontaneous cyclization of phenyl N,N-bis-(β-chlorocthyl)-N′-(β-hydroxyethyl)phosphorodiamidate is known.[10]

The present study concerns the enzymatic catalysis of the cyclization of diphenyl *o*-(α-hydroxy)tolyl phosphate and related compounds. These compounds cyclize with liberation of a phenol to form saligenin cyclic phosphates. In certain of the studies the cyclic phosphate is directly or indirectly assayed but, in most cases, the reaction is followed by determining the liberation of the phenol. The use of xylene as an extraction solvent in the assay for liberated phenol or cresol is important for accurate assays because it stops the reaction immediately by extracting the substrate and gives a clear solution. Xylene is preferred to chloroform for this use because it is less volatile, yields fewer emulsions, and extracts less of the aminoantipyrine dye formed from saligenin, which is released on decomposition of the cyclized products.

Other types of enzymatically catalyzed cyclization reactions for phosphorus compounds are known; for example, the cyclization of a phosphoric acid ester during the course of hydrolysis of ribonucleic acid by the action of pancreas ribonuclease.[11] However, ribonuclease is a specific phosphodiesterase and does not catalyze the

cyclization of diphenyl *o*-(α-hydroxy)tolyl phosphate. Also, no cyclization activity is found for many other purified proteins assayed. Only snake venom and various plasmata are found to be active in the cyclization. The activity of snake venom in cyclization of diphenyl *o*-(α-hydroxy)tolyl phosphate is not due to enzymes that hydrolyze *p*-nitrophenyl acetate or diphenyl phosphate, because the cyclizing enzyme is more stable to heat than are the enzymes hydrolyzing the other substrates.

Plasma from many animal species is active in cyclization of diaryl *o*-(α-hydroxy)tolyl phosphates. The catalytic activity of plasma cannot be separated from the albumin fraction by many procedures, including the following: salting-out with ammonium sulfate, ethanol fractionation by Cohn's procedure, chromatography on Sephadex or DEAE-cellulose, various combinations of these procedures. It is important to use more than one procedure for such a study because it is known that the arylesterase (E.C. 3.1.1.2:aryl-ester hydrolase) of rabbit serum, which hydrolyzes diethyl *p*-nitrophenyl phosphate (paraoxon), appears in the globulin fraction by Cohn's procedure, whereas it appears in the albumin fraction by the ammonium sulfate method.[12] A greater variation occurs in activity of various plasmata than of the albumin fractions from these plasmata in cyclization of diphenyl *o*-(α-hydroxy)tolyl phosphate. Whole chicken plasma is lower in activity than mammalian plasmata, particularly that of humans. The amount of albumin in chicken plasma is about half (1·8–2·0%)[13] that in human plasma (3·5–4·9%),[14] and this difference partially explains the low cyclizing activity of chicken plasma.

Other enzymes are also present in plasma, but these are different from the one involved in cyclization of diaryl *o*-(α-hydroxy)tolyl phosphates. Cholinesterases (E.C. 3.1.1.7 and 3.1.1.8) and aliesterases (E.C. 3.1.1.1:carboxylic-ester hydrolase) are inhibited by organophosphorus esters such as TEPP and DFP, but the cyclizing activity is not inhibited by these agents. No phosphatase activity is found in the albumin fraction, which contains the cyclizing activity. One of the arylesterases of plasma, which is referred to as A-esterase or paraoxonase,[12, 15] differs from the present enzyme in the following points: A-esterase is heat-unstable[16] and is found in Cohn's fraction I + II + III,[12] whereas the activity for the liberation of phenol from diphenyl *o*-(α-hydroxy)tolyl phosphate is relatively heat-stable and is found in fraction V.

Several studies[17–21] have shown arylesterase activity associated with serum albumin; this activity cannot be differentiated from the activity for liberating phenols from diaryl *o*-(α-hydroxy)tolyl phosphates. Furthermore, one of the substrates for arylesterase, *p*-nitrophenyl acetate, inhibits competitively, at least in part, the liberation of phenol from diphenyl *o*-(α-hydroxy)tolyl phosphate. These findings suggest that serum albumin arylesterase is responsible for the cyclization of diaryl *o*-(α-hydroxy)-tolyl phosphates.

The turnover number of the plasma albumin enzyme for the cyclization is calculatedto be 0·67 mole/min for diphenyl *o*-(α-hydroxy)tolyl phosphate. Though the efficiency of the enzyme is very low, the rate of cyclization of material circulating in the living animal possibly is high, owing to the high concentration of albumin in blood.

Studies with the albumin fraction and either a mixture of triphenyl phosphate and benzyl alcohol or various *o*-(α-hydroxy)tolyl phosphate esters as substrates indicate that this fraction does not catalyze hydrolysis of phosphoric triesters or intermolecular transphosphorylation between triaryl phosphates and benzyl alcohol; only intra-

molecular transphosphorylation between the aryl phosphate ester group and the alcohol group yields a cyclic phosphate. Thus, the *o*-(α-hydroxy)tolyl group and another aryl group are necessary in a molecule for liberation of phenols.

Saligenin cyclic phosphates, the products from the cyclization reaction, inhibit esterases by phosphorylation of their active site.[8] Therefore, if whole plasma is used as the enzyme source, the amount of cyclic phosphate recovered is less than that resulting from incubation with only the albumin fraction, because a portion of the product is lost on reaction with other organophosphorus-sensitive esterases in the whole plasma. This difficulty can also be minimized by heat treatment of the whole plasma, because the cyclizing enzyme is more stable to heat than the enzymes which are phosphorylated by the products. The amount of phenol liberated relative to the amount of cyclic phosphate that can be isolated is therefore higher with the unheated plasma than with the heated plasma or albumin component of the plasma. It is for this reason, as well as for ease of determination, that phenol anlaysis rather than cyclic phosphate analysis was used for routine comparisons in most of the present studies.

Thus the metabolic activation mechanism for tri-*o*-tolyl phosphate and related compounds is now more completely understood, as indicated in Fig. 1. An *o*-methyl group is hydroxylated by the action of the liver microsome hydroxylation system, and the product then cyclizes, mainly owing to the action of the plasma albumin, to yield the corresponding saligenin cyclic phosphate by intramolecular transphosphorylation with liberation of one aryl group.

*Acknowledgements*—We wish to thank Dr. J. I. Mukai of Kyushu University for the gift of several enzymes and for useful discussions, and Dr. L. Lykken of the University of California for helpful suggestions.

## REFERENCES

1. J. E. CASIDA, M. ETO and R. L. BARON, *Nature, Lond.* **191**, 1396 (1961).
2. M. ETO, J. E. CASIDA and T. ETO, *Biochem. Pharmac.* **11**, 337 (1962).
3. R. L. BARON, D. R. BENNETT and J. E. CASIDA, *Br. J. Pharmac.* **18**, 465 (1962).
4. M. ETO, Y. KINOSHITA, T. KATO and Y. OSHIMA, *Nature, Lond.* **200**, 171 (1963).
5. M. ETO, Y. KINOSHITA, T. KATO and Y. OSHIMA, *Agric. biol. Chem., Tokyo* **27**, 789 (1963).
6. M. ETO, K. KOBAYASHI, T. KATO, K. KOJIMA and Y. OSHIMA, *Agric. biol. Chem., Tokyo* **29**, 243 (1965).
7. M. ETO, S. MATSUO and Y. OSHIMA, *Agric. biol. Chem., Tokyo* **27**, 870 (1963).
8. M. ETO, Y. OSHIMA and J. E. CASIDA, 16th Symposium of Enzymology, 1964, Tokyo, *Abstracts of Papers*, p. 143.
9. M. ETO and Y. OSHIMA, *Agric. biol. Chem., Tokyo* **26**, 452 (1962).
10. H. ARNOLD and F. BOURSEAUX, *Angew. Chem.* **70**, 539 (1958).
11. R. MARKHAM and J. D. SMITH, *Biochem. J.* **52**, 552 (1952).
12. W. N. ALDRIDGE, *Biochem. J.* **53**, 117 (1953).
13. P. D. STURKIE, *Avian Physiology*, p. 32. Comstock, Ithaca, N.Y. (1954).
14. C. LONG (Ed.), *Biochemists' Handbook*, p. 1081. Spon, London (1961).
15. A. R. MAIN, *Biochem. J.* **74**, 10 (1960).
16. A. R. MAIN, *Biochem. J.* **75**, 188 (1960).
17. E. L. RONGONE, B. C. BOCKLAGE, D. R. STRENGTH and E. A. DOISY, *J. biol. Chem.* **225**, 969 (1957).
18. J. E. CASIDA and K.-B. AUGUSTINSSON, *Biochim. biophys. Acta* **36**, 411 (1959).
19. W. K. DOWNEY and P. ANDREWS, *Biochem. J.* **96**, 21c (1965).
20. C. E. WILDE and R. G. O. KEKWICK, *Biochem. J.* **91**, 297 (1964).
21. H. HARRIS, D. HOPKINSON and E. B. ROBSON, *Nature, Lond.* **196**, 1296 (1962).