learning (*6*, *7*). Different doses of SP can produce different effects also. Frederickson found that smaller SP doses, alone, produced analgesia, while higher doses, given after prior treatment with naloxone, resulted in hyperalgesia. We also observed dose-dependent analgetic or hyperalgetic effects of SP. We found, furthermore, as did Frederickson *et al.* and others, that SP analgesia was reversible by naloxone. This supports a role for endogenous opioid peptides in this action. A dual action of SP on nociception suggests that there might be more than one receptor type for SP (*2*). Some further evidence for the possible existence of such different receptors has been provided by Bergmann *et al.* (*7*) who investigated SP action on smooth muscle. The presence of several signatures in one molecule determining different functions is referred to in information theory as the principle of ambiguity or indeterminancy of effect. That principle can be biologically important when molecules have conformationally flexible structures, such as the linear flexible SP undecapeptide (*4*).

Thus we suggest that the dual actions of SP result from this flexibility of the molecule and that both the dose of the peptide and condition of the animal determine which configuration and effect predominate. Thus, the resultant effect is a "normalization" of the responsivity of the animal to painful stimuli. Accordingly, we propose that the term regulatory peptides or "regulides" be applied to such peptides.

PETER OEHME
HEINZ HILSE
Institute for Drug Research, Academy of Sciences of the G.D.R., 1136 Berlin, G.D.R.
EVELINE MORGENSTERN
ERHARD GÖRES
Pharmacological Research Institute, 1136 Berlin, G.D.R.

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8. We thank Dr. R. C. A. Frederickson for suggestions and assistance in the preparation of this manuscript.

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Cadmium Increases Tissue Factor (Coagulation Factor III) Activity by Facilitating Its Reassociation with Lipids

Abstract. *The coagulant activity of partially purified and delipidated tissue factor (TF) (coagulation Factor III) has previously been recovered by dialysis of the apoprotein after addition of mixed brain lipids and deoxycholate. Inclusion of cadmium chloride in the relipidation mixture greatly increases the recovered activity of highly purified TF from human placenta by promoting incorporation of TF into phospholipid vesicles; TF that had not been incorporated into vesicles showed no coagulant activity. Thus, TF must be present in a lipid bilayer for expression of coagulant activity. In vitro, cadmium induces fusion of lipid vesicles and may contribute to the incorporation of proteins in membranes.*

The proteolytic activation of coagulation Factor X by Factor VIIa (*1*) is dependent on the presence of calcium and is accelerated by tissue factor (TF) (coagulation Factor III) complexed with phospholipids (*2*). The activity of TF purified from human placenta (*3*, *4*) is lost as lipids are removed during fractionation, but can be recovered by relipidation of the apoprotein. The coagulant activity of TF (*5*), reactivated by dialysis with a lipid preparation from bovine brain in deoxycholate (*6*), is increased by adding $CdCl_2$ to the protein-lipid mixture. The $CdCl_2$ does not replace calcium, which is required for the interactions of vitamin K–dependent clotting enzymes with phospholipids.

Since the TF-accelerated cleavage of Factor X is the first of several enzymatic steps leading to coagulation in the two-stage clotting assay (*5*), we determined the rate of activation of Factor X (*7*) by measuring the time-dependent release of the activation peptide. When TF was relipidated in the presence of cadmium, the rate of Factor X activation was greater than it was in the absence of cadmium (Fig. 1). Furthermore, the difference in activation rate increased as TF was added to the reaction, demonstrating that cadmium had specifically increased the TF activity.

Tissue factor activity recovered after relipidation depends on the amount of phospholipid present in the relipidation mixture (*2*). We therefore conducted experiments to determine whether cadmium affected the optimum concentration of mixed brain lipids for recovery of TF coagulant activity. In the absence of cadmium, the optimum lipid concentration was near 0.3 m*M* [determined as phosphate (*6*)], resulting in a TF activity of 6.8 U/ml. This lipid optimum was not measurably different when $CdCl_2$ was included at 10m*M*, 15 m*M*, or 20 m*M*, although the TF coagulant activity was increased tenfold after relipidation with 10 m*M* $CdCl_2$. In addition, the $CdCl_2$ optimum remained constant between 5 m*M* and 7 m*M* when the relipidation mixture contained (determined as phosphate) 0.1 m*M*, 0.2 m*M*, 0.3 m*M*, or 0.4 m*M* mixed brain lipids. Thus, lipids and cadmium have distinct effects on the recovery of TF activity which are amenable to independent optimization.

The three primary components of the relipidation mixture (TF, mixed brain lipids, and $CdCl_2$) were dialyzed together in various combinations to ascertain whether cadmium was acting on the apoprotein or on the lipids. Incubation and dialysis of $CdCl_2$ with either TF or mixed brain lipids, prior to addition of the third component and a second dialysis, failed to increase the TF activity. Thus cadmium was not removing potential inhibitors (such as sulfhydryl compounds) from either component, nor was it selectively activating the apoprotein or altering the state of the lipids to increase their ability to promote coagulation. Furthermore, functionally significant amounts of $CdCl_2$ were not being retained by either the lipids or TF apoprotein, since dialysis of $CdCl_2$ with either component failed to increase TF activity. Increased activity was observed when $CdCl_2$ and TF were added to dialyzed lipids, or when $CdCl_2$ was added to TF dialyzed with lipids, followed by additional dialysis. This series of experiments indicated that the increased activity was the result of interactions between apoprotein, cadmium, and lipids.

The conditions we use for TF relipidation produce phospholipid vesicles (*8*); we therefore used sucrose density-gradient ultracentrifugation to examine the effect of $CdCl_2$ on the TF-phospholipid complexes. After centrifugation, activity in the preparations relipidated with and without cadmium was present in the top half of the density gradient (Fig. 2). However, the TF relipidated with $CdCl_2$ produced a sharp peak of activity between 13 and 16 percent sucrose, which was not observed in the sample relipidated without $CdCl_2$. Relipidation of each density-gradient fraction with additional lipids and $CdCl_2$ produced addi-

tional TF activity only in the sample originally relipidated without $CdCl_2$, and only in fractions near the bottom of the gradient (sucrose concentration, ≥ 30 percent). Hence the $CdCl_2$ present during relipidation increased the recovery of TF activity by facilitating the incorporation of apoprotein into phospholipid vesicles. Furthermore, TF activity is expressed only when the apoprotein is present in phospholipid vesicles, an important consideration in studies of TF function.

The action of $CdCl_2$ in increasing TF coagulant activity is not a general effect attributable to the presence of 5 m*M* divalent cations during relipidation. Relipidation mixtures incorporating $CuCl_2$, $NiCl_2$, $HgCl_2$, or $ZnCl_2$ decreased the recovered tissue factor activity whereas $SrCl_2$ and $BaCl_2$ were ineffective, and $CoCl_2$ produced only a twofold increase in activity. Because of their potential for inducing fusion of vesicles composed of various phospholipids (*9*), the effects of $CaCl_2$, $MgCl_2$, and $MnCl_2$ on relipidation of TF were examined at concentrations of 2 m*M* to 40 m*M*. No effect was observed with either $CaCl_2$ or $MgCl_2$, but $MnCl_2$ above 4 m*M* resulted in a fourfold increase in activity.

Experiments demonstrating that the optimum concentrations of mixed brain lipids and $CdCl_2$ were independent of each other suggest that the association of TF apoprotein with individual phospholipids and its incorporation into vesicles may be distinct events. As proposed by Eytan and Broza for cytochrome oxidase (*10*), the hydrophobic domains of TF apoprotein may bind phospholipid molecules which correspond to "annular lipids." This interaction should be dependent on the concentration of free phospholipids and represents the first step in recovery of TF activity. The second step, leading to expression of activity, would then involve incorporation of the protein-lipid complex into a vesicle. This step requires binding of the protein-lipid complex to the vesicle followed by coalescence. Divalent cations can induce aggregation and fusion of phospholipid vesicles, and the effectiveness of different cations in producing vesicle-vesicle fusion varies with their phospholipid composition (*9*, *11*); $MnCl_2$ induces fusion of vesicles prepared from soybean lipids (*9*) and $CdCl_2$ induces fusion of mixed lipid vesicles composed of phosphatidic acid and phosphatidyl choline (*11*). The increase in TF activity when $CdCl_2$ is added after prior relipidation in the absence of cadmium is consistent with the proposed model in which cadmium mediates binding and fusion of the protein-lipid complex with phospholipid vesicles.

Our results demonstrate that cadmium increases the activity recovered after relipidation of highly purified TF obtained from human placenta. This increased activity results from cadmium-facilitated incorporation of the apoprotein into phospholipid vesicles. The data are consistent with a model in which cadmium induces fusion of an apoprotein-annular lipids complex with phospholipid vesicles to produce active TF. These results are pertinent not only to expression of TF activity, but to more general studies of vesicle-vesicle fusion and the incorporation of macromolecules into phospholipid membranes. In summary, the use of cadmium provides a new approach for studying protein assembly into membranes.

STEVEN D. CARSON
WILLIAM H. KONIGSBERG
Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06510

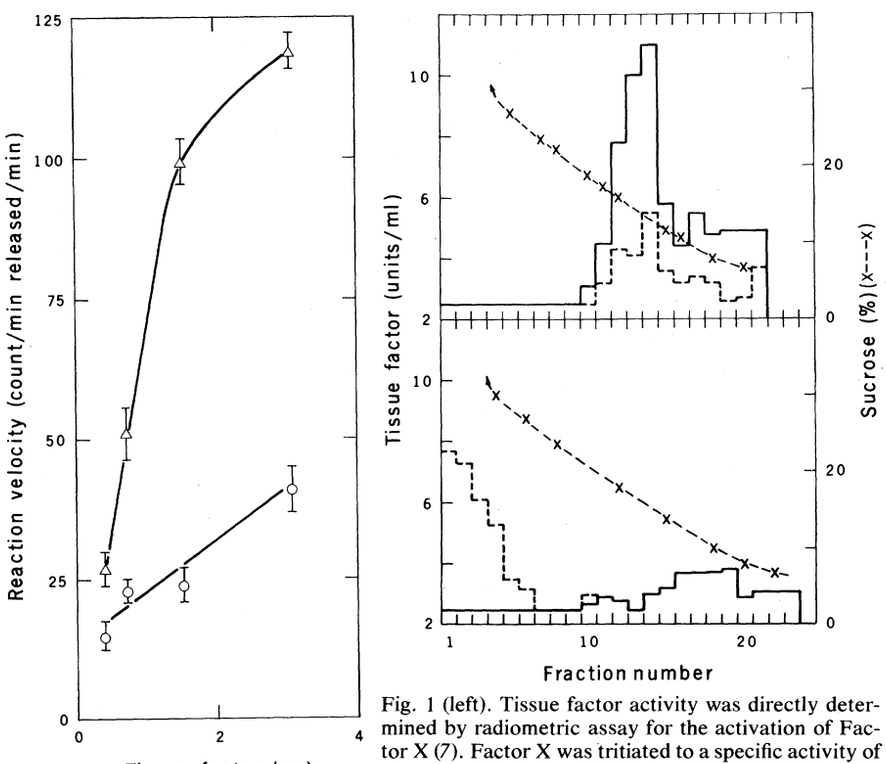

Fig. 1 (left). Tissue factor activity was directly determined by radiometric assay for the activation of Factor X (*7*). Factor X was tritiated to a specific activity of 148,500 cpm/μg. In addition to TF, the reaction mixture contained 6.86 ng of Factor VIIa and 4.6 μg of ^{3}H-labeled Factor X in 200 μl. At intervals, 20-μl samples were withdrawn and added to 140 μl of the stopping solution at 0°C. Proteins were precipitated with 75 μl of 15 percent trichloroacetic acid. Duplicate 80-μl samples of the supernatant, containing released activation peptide, were combined with 4 ml of formula 936 (New England Nuclear) scintillant for counting. The TF apoprotein was relipidated with 0.3 m*M* mixed brain lipids alone (○) and with 5 m*M* $CdCl_2$ (△). Bars indicate 1 standard deviation in the measurement of released radioactivity. Fig. 2 (right). Ultracentrifugation of TF relipidated with 5 m*M* $CdCl_2$ (upper panel) and without $CdCl_2$ (lower panel). Density gradients of 5 to 30 percent sucrose were poured in 4.5-ml nitrocellulose tubes (1.25 by 6 cm) on top of 200 μl of 50 percent sucrose. Tissue factor relipidated with 0.3 m*M* phospholipids was layered in 200-μl amounts on top of the gradients, and the tubes were centrifuged (Beckman L5-65 ultracentrifuge in an SW50.1 swinging bucket rotor at 40,000 rev/min and 22°C for 26.5 hours). Fractions were collected by puncturing the tube bottom, and the sucrose concentrations were determined by measuring the refractive index. The fractions were assayed for TF coagulant activity without further treatment (——). The fractions were then individually relipidated by combining 100 μl of each with 12 μl of 0.5*M* tris-HCl, 1*M* NaCl, *p*H 7.6, with bovine serum albumin (10 mg/ml), 10 μl of 50 m*M* $CdCl_2$, and 12 μl of the mixed brain lipids, then assayed again for TF coagulant activity (- - -).

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3. Acetone powders of full-term human placentas were prepared and extracted with a mixture of heptane and butanol (*4*). The TF was extracted with Triton X-100 and purified to a specific activity of 1.7×10^5 U/mg [S. D. Carson and W. H. Konigsberg, *Int. Congr. Biochem. Abstr.* **11**, 207 (1979)]. The protein concentration of the TF preparation was estimated by the method of Lowry *et al.* [O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951)] as modified by Hartree [E. F. Hartree, *Anal. Biochem.* **48**, 422 (1972)] with incorporation of sodium dodecyl sulfate to reduce interference by Triton X-100 [C.-S. Wang and R. L. Smith, *Anal. Biochem.* **63**, 414 (1975)].
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mate; General Diagnostics). Bovine Factor X and Factor VIIa were provided by Dr. Yale Nemerson and were used at 6.48 μg/ml and 0.158 μg/ml, respectively. Bovine plasma and cephalin were prepared as described (*4*). A typical standard curve generated with bovine brain TF had clotting times of 15.7 seconds for 100 TF units per milliliter and 48.5 seconds for 6.25 TF units per milliliter. All coagulation times were determined twice and averaged.

6. Tissue factor (10 μl, 38 μg/ml), mixed bovine brain lipids [2 mg/ml in 0.01*M* tris with 0.25 percent sodium deoxycholate (*4*)], 50 μl of a mixture of 0.5*M* tris-HCl, 1*M* NaCl (*p*H 7.6) with bovine serum albumin (10 mg/ml), test cations, and water to 0.5 ml were combined in 1.5-ml conical polypropylene tubes. The tubes were covered with dialysis membrane and floated inverted on tris-NaCl at 4°C (*4*, *8*). The lipid concentration (milligrams per milliliter) was calculated on the basis of a phosphorus content of 4 percent (*4*).
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Hepatocarcinogenicity of Glandless Cottonseeds and Cottonseed Oil to Rainbow Trout (*Salmo gairdnerii*)

Abstract. *Glandless cottonseed kernels are available for purchase and consumption by the general public. These kernels contain no gossypol but still have a full complement of naturally occurring cyclopropenoid fatty acids, which in rainbow trout are active as synergists with aflatoxins and primary liver carcinogens. Diets containing glandless cottonseed kernels or a lightly processed cottonseed oil produced significant numbers of hepatocellular carcinomas in rainbow trout after 1 year. The much greater incidence of cancer induced by the kernel than by the oil indicates that synergists or other carcinogens may be present in the kernel in addition to the cyclopropenoid fatty acids.*

By-products of the seeds of the cotton plant *Gossypium hirsutum* are constituents of both animal and human foodstuffs. Cottonseed meal is used extensively as a protein supplement in cattle, swine, poultry, and fish rations, and cottonseed oil is an important ingredient of margarines and cooking oils for human use. Cottonseed, however, contains two toxic compounds that limit its use. Pigment glands of cotton plants contain the polyphenolic compound gossypol, which is toxic to livestock (*1*), swine (*2*), poultry (*3*), dogs (*4*), rodents (*5*), and trout (*6*). The presence of gossypol in cottonseed meal has been the primary limitation to its use in human food.

Cottonseed also contains malvalic acid (MA) and sterculic acid (SA) as glycerides in the oil. These cyclopropenoid fatty acids (CPFA) produce several undesirable effects in birds and mammals, including pink discoloration of stored egg whites (*7*); inhibition of the fatty acyl desaturase system (*8*); increased saturated fatty acids in body lipids (*9*); delayed sexual maturity in female rats (*10*); lung, liver, and kidney abnormalities and high prenatal and postnatal mortalities in rat pups (*11*, *12*); increased cholesterol levels, aortic atherosclerosis, and liver damage in rabbits (*13*); and mitogenic activity in rat liver and pancreas (*14*, *15*).

In rainbow trout, SA and, to a lesser degree, MA are powerful synergists in combination with aflatoxin B_1 and its metabolites, greatly increasing the incidence and severity of liver cancer (*16*–*18*). Both SA and MA are also primary hepatocarcinogens in rainbow trout (*18*–*20*). However, neither synergistic nor carcinogenic properties of SA have been unequivocally demonstrated in mammals (*21*, *22*).

Selective breeding of the cotton plant has resulted in varieties that produce glandless seeds that are free of gossypol but still contain a full complement of CPFA. The CPFA content is approximately 0.5 to 1.0 percent of the total lipid content of the seed. Of this, about 65 percent is MA and 35 percent SA (*23*). Nevertheless, glandless cottonseeds have been approved by the Food and Drug Administration as a nut substitute and snack item for human consumption.

We designed experiments to test the effects of glandless cottonseeds on rainbow trout. A mildly processed cottonseed oil was also fed to rainbow trout for comparison. (Heavy processing of cottonseed oil for use in margarine or salad oils destroys much of the CPFA content.)

Roasted glandless cottonseed kernels (GCK) were ground and added to semipurified trout feed (the control diet) (*24*) so that they constituted 25 percent of its dry weight. Protein, carbohydrate, lipid, and mineral components of the control diet were reduced to keep the two diets isonitrogenous and isocaloric. Cottonseed oil containing 0.35 percent CPFA (*22*) was added to the control feed (*24*) at 7.5 percent of its dry weight, and carbohydrate and lipid levels were again reduced to accommodate the increased level of lipid. Duplicate lots of 80 rainbow trout fingerlings (3 months old) were given free access to the control diet or one of the two experimental diets for 12 months and were sampled at 6, 9, and 12 months to determine histopathology—in particular, liver cancer incidence. Food consumption, weight gain, and ratios of liver weight to body weight were also recorded.

The CPFA content of the GCK was determined with the nuclear magnetic resonance (NMR) method of Pawlowski *et al.* (*25*). The GCK contained 39.8 percent lipid, as determined by extraction with ethyl ether. Electronic integration of the terminal methyl and cyclopropene region of the GCK lipid on a Varian FT-80 NMR spectrograph demonstrated that 0.73 percent of the fatty acids contained an isolated 1,2-disubstituted cyclopropene ring (MA and SA). These values permit estimation of the total CPFA content of the diets. The GCK diet contained 9.95 percent cottonseed lipid, of which 0.73 percent, or 726 mg/kg, was CPFA. Therefore, 35 percent, or approximately 250 parts per million (ppm), was SA. In the cottonseed oil diet the cottonseed oil (7.5 percent of the diet) contained 0.35 percent CPFA, which means there were 262 mg of CPFA per kilogram of the diet, or about 90 ppm SA.

Duplicate 25-g samples of GCK were analyzed for aflatoxins with the method described in (*26*): none were detectable. The level of any aflatoxin B_1 present would have been < 0.1 part per billion (ppb) in the GCK or < 0.025 ppb in the diet.

Another GCK sample was prepared with the method of Goodhead and Gough (*27*) and was analyzed by combined gas chromatography and thermal energy analysis for volatile *N*-nitroso compounds (*28*); the sensitivity of this method is 0.1 ppb. No *N*-nitroso compounds were present.

Growth of the trout was significantly ($P < .001$, Student's *t*-test) reduced by the diet containing GCK. At 12 months of age, the fish fed the GCK diet had an average body weight of 424 ± 110 g compared with 565 ± 164 g for the control fish. The fish fed the cottonseed oil diet weighed slightly less than the control fish (533 ± 140 g), but the difference was not significant. Ratios of liver weight to body weight indicated the presence of CPFA toxins. The ratio was significantly higher ($P < .001$) in the cottonseed oil–fed fish (1.28 ± 0.26) than in the controls

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