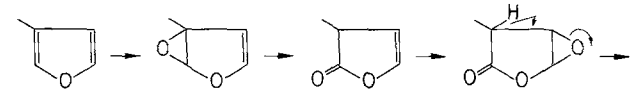
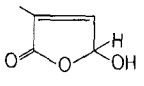
that this fraction is a mixture of a least two components. This was confirmed by the MS, which showed the same molecular ion (*m/e* 362; 7%) as that of the γ-hydroxy--α, β-butenolides and an identical fragmentation pattern with strong peaks at *m/e* 213 (80%), 181 (75%) and 150 (100%)[11]. Attempts at separation by SiO_2 chromatography resulted in the isolation of the mixture of (**2–5**), as indicated by IR and NMR. Acetylation of (**6–9**) gave the mixture of γ-acetoxy-α, β-butenolide acetates. All these data are constitent with structures (**6–9**), and the NMR-signals, listed above, can be reasonable assigned to the β, γ-epoxybutenolide moieties as indicated in formulae (**6**) and (**9**). Protons at δ 5.44 and 3.70 are coupled to each other, as confirmed by decoupling experiments.

Terpenoids with different states of oxidation of the furan ring are known[4,12,13], but their status as natural products is uncertain. It is established[14–16] that alkylated furans give autoxidation products of type (**10**), and evidence has been accumulated that such photo-oxidations proceed via ozonides, which, in a few cases, have been isolated. Moreover, the co-occurrence in a specimen of *Cedrela odorata* of gedunin and photogedunin, in which latter the furan ring is oxidized to a γ-hydroxy-α, β-butenolide, has been reported recently[4].

The mode of isolation of photogedunin, and its absence from other samples of *Cedrela* in which gedunin was present, led the authors[4] to suggest that the photo-oxidation of the furan ring might occur in vivo.

Extraction of a sample of *Spongia officinalis* in the dark gave both γ-hydroxy- and β, γ-epoxybutenolide fractions, while from a methanolic solution of furospongin-**1** (**1**) we were unable to detect any of the above oxidized derivatives after exposure to light. This indicates that at least the β, γ-epoxybutenolides (**6–9**) are genuine natural products and that possibly the oxidation of the furan rings might occur through epoxidation steps as indicated in Scheme 1, in contrast to photo-oxidation in vitro[15] (the spontaneous isomerization of arene oxides to phenols is well documented[17]).





(**10**)

Finally we want to emphasise that β, γ-epoxybutenolides are compounds of a type not previously encountered.

Riassunto. L'isolamento dalla *Spongia officinalis* di otto C_{21} monofuranoterpeni, strettamente correlati alla furospongina-1 (**1**), il componente terpenico più abbondante della stessa spugna, per quattro dei quali si dimostrano le strutture di γ-idrossi-α,β-butenolidi (**2–5**) e per gli altri quattro le strutture di β,γ-epossibutenolidi (**6–9**), sembra rilevante in connessione col problema della ossidrilazione dei substrati aromatici in vivo.

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[10] N. S. BHACCA, L. F. JOHNSON and J. N. SHOOLERY, *NMR Spectra Catalog* (Varian Associates, Palo Alto, California 1962), vol. 1, No. 51.

[11] The MS of this fraction also showed significant peaks at *m/e* 135 (40%) and 195 (20%), the former associated with (*6*) and (*8*) arising by cleavage of the 8,10-bond, and the latter is associated with (*7*) and (*9*) originating by cleavage of the same 8,10-bond.

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[17] J. W. DALY, D. M. JERINA and B. WITKOP, Experientia *28*, 1129 (1972) and references therein.

[18] Acknowledgements. We are indebted to Professor R. H. THOMSON (University of Aberdeen, Scotland) for his interest and advice on this problem. We also wish to thank Professor E. LEDERER and Dr. B. C. DAS (Gif-sur-Yvette, France) for some mass spectra. The technical assistance of Mr. C. DI PINTO (NMR) and G. SCOGNAMIGLIO is also acknowledged.

Hemin as a Catalyst for Chemiluminescence

The chemiluminescence produced by the reaction of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) in a basic solution in the presence of hydrogen peroxide and catalyzed by protohemin (protoporphyrin IX iron III chloride) has been well studied. The actual mechanism of the reaction, however, is not thoroughly understood. The purpose of this investigation was to determine the effect of the chemical modification at the 2 and 4 positions of protohemin on its ability to catalyze the decomposition of luminol.

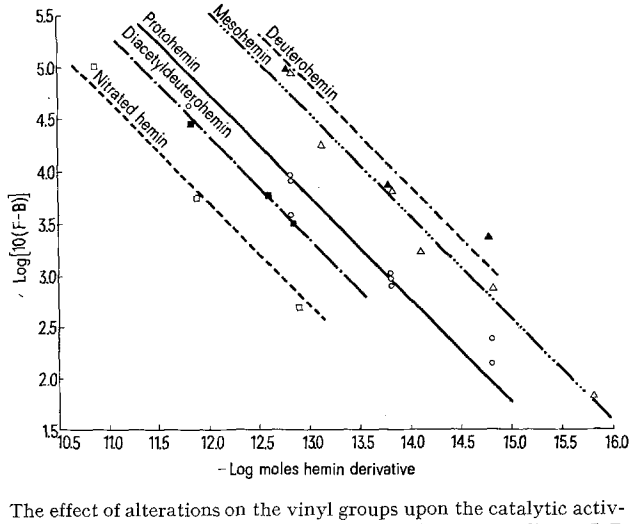
When the vinyl side chains of hemin are modified, various electronic effects have been observed through examination of the absorption spectra in solvents of varying polarity, at different pH values and as pyridine hemochromogens. Changes in both intensity and position of the absorption maxima occur and the degree of correlation between catalytic and absorptive changes is considered.

The assays for chemiluminescence were done according to the method of NEUFELD et al[1]. The salient features of the instrument used are: a holder for the test tube which is light-tight, a camera shutter that permits changing sample tubes without turning off the high voltage to the phototube, and a housing for the phototube. We used either a RCA IP28, IP21, or an EMI 9635 phototube; amplification was provided by a Sanborn preamplifier model 150–1500. The data were recorded on a Sanborn 151 recorder. The reaction is triggered by the injection of H_2O_2 into a test tube containing alkaline luminol, EDTA, and the catalyst.

[1] H. A. NEUFELD, C. J. CONKLIN and R. D. TOWNER, Analyt. Biochem. *12*, 303 (1965).

The intensity of the light emission produced when hydrogen peroxide is injected into the reaction mixture is directly proportional to the concentration of the hemin catalyst[1]. Thus a plot of the log of the flash height versus the log of the concentration of catalyst should yield a straight line with unitary slope. The data have been fitted to a line with a fixed slope of 1 through the point representing the mean values. Since some light is emitted when hydrogen peroxide is injected into a luminol solution with no hemin present, a blank was measured daily and subtracted from each experimental flash height.

The catalytic capacity of four derivatives was compared to that of protohemin (Sigma Chemical Company, Type I, Bovine): 1, the vinyl groups reduced to ethyl groups (mesohemin, a gift of Dr. A.H. CORWIN); 2, the vinyl groups replaced with hydrogen atoms (deuterohemin, prepared by the method of FISCHER and ORTH[2] and purified by recrystallization according to FISCHER'S[3] procedure); 3, the vinyl groups replaced with acetyl groups (diacetyldeuterohemin, a gift of Dr. W.S. CAUGHEY); and 4. with hydroxyl and nitro groups added to the double bonds of the vinyl groups (nitrated hemin, prepared by reaction with tetranitromethane according to the procedure of ATASSI[4]. The results are shown in the Figure. If we assign a value of 1 to the catalysis with protohemin, ratios of flash height minus the blank at equal concentrations yield the following activities: deuterohemin 12, mesohemin 6.6, protohemin 1, diacetyldeuterohemin 0.39, nitrated hemin 0.088. There is obviously an excellent correlation between the degree of electronegativity and the ability to catalyze luminol chemiluminescence. Reduction of the vinyl groups increases light production and their removal even more strongly. Diacetyldeuterohemin is more electrophilic than protohemin and as one would expect introduction of the two hydroxyl and two nitro groups is considerably more effective in decreasing the catalysis. Resonance with the pyrrole rings does not seem to be of great significance.



The effect of alterations on the vinyl groups upon the catalytic activity of hemin in the decomposition of luminol. On the ordinate F–B is the flash height minus the blank. Experimental details are described in the text.

By analogy with the proposal by LINSCHITZ[5] for the porphyrin-catalyzed decomposition of peroxide, we suggest that the first step in this reaction is a complexing of the peroxide with the central metal ion. Then since free radicals[6] are involved, the next step postulated is the formation of a heme-peroxy free radical. The reaction may be written formally thus:

$$\text{Por–Fe}^{3+}_{|}{}_{\text{OH}} + \text{OOH}^- \rightarrow \text{Por–Fe}^{3+}_{|}{}_{\text{OH}}\text{—OOH}^- \rightarrow \text{Por–Fe}^{2+}_{|}{}_{\text{OH}}\text{—OOH}\cdot$$

This radical could then act as the primary oxidant of luminol and a decrease in electrophilicity at the 2 and 4 positions could function by facilitating the formation of the free radical, or by increasing its reactivity. In view of the extreme rapidity of the reaction (msec) we favor the latter alternative.

Alternatively the heme-peroxy free radical could dissociate to form a peroxy free radical which would function as the primary oxidant. The effect of the electrophilic substitution in this mechanism is to bind the peroxide radical more tightly to the central metal ion. Conversely, the decrease in electrophilicity would facilitate the dissociation of the peroxide radical. It has been noted by many investigators that at a high pH, the addition of peroxide to luminol in the absence of a hematin catalyst results in the production of some light. We would ascribe this to the non-catalytic formation of a small concentration of peroxy radicals.

BROWN et al.[7] have observed that deuterohemin is 10 to 100 times more effective than protohemin in the decomposition of peroxide. They assumed that the relative catalytic activities are independent of the composition of the peripheral substituents of the porphyrin ring and interpret their data in terms of the degree of dimerization of the heme derivatives. We believe that this does not apply to our system since the concentrations of monomer are not stoichiometric with total concentration. The slope of the log concentration vs. log flash height plot would not be unitary if the monomer were the only reactive form.

Considerable attention has been given to the relation between electron-withdrawing effects of peripheral substituents upon the wavelengths of absorption maxima of deuteroporphyrin IX, and some authors have suggested that one does in fact exist[8,9]. Most recently, however, CAUGHEY et al.[10] in a very careful study, have reported that such a relationship does not obtain. We examined the absorption spectra of our 5 hemin compounds and cannot find a consistent relationship with their electrophilicity. There is some indication that groups conjugated with the porphyrin residues tend to shift some of the visible bands to longer wavelengths. CLEZY and MORELL[11] have observed a similar phenomenon in the Soret region

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[10] W. S. CAUGHEY, W. Y. FUJIMOTO and B. P. JOHNSON, Biochemistry *2*, 3830 (1964).
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in non-polar solutions. But the correlation with the effect upon catalysis of luminol decomposition is only fair.

Examination of the catalytic ability of a larger series of derivatives with changes in other than the 2 and 4 positions should be illuminating. We have found heme *a* to be an extremely poor catalyst.[12]

[12] Acknowledgments. We are indebted to Mr. R. D. TOWNER for some of the chemiluminescence assays. We are grateful to Dr. J. R. TOTTER for helpful discussions.

[13] Reprint requests to be addressed to U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick (Maryland 21701, USA), 23 August 1973.

Zusammenfassung. Untersuchungen über den Wirkungsmechanismus der Chemolumineszenz von Luminol.

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Activities of Decarboxylases of Histidine and Ornithine in Young Mice after Injection of Epidermal Growth Factor

Epidermal cell proliferation, initiated by surgically inflicted skin wounds, has been found associated with a striking increase in histamine formation, i.e. histidine decarboxylase activity, in the growing wound tissues of rats and man. On artificially lowering or elevating the histamine formation of rat skin, the rate of healing was retarded or accelerated, respectively. It thus appears that a causal relationship between growth processes in the skin wound and histidine decarboxylase activity exists. Some rapidly growing tissues generate ornithine decarboxylase activity (for references see KAHLSON and ROSENGREN[1,2]). An 'epidermal growth factor' (EGF), occurring in the submaxillary gland of adult mice[3], stimulates epidermal growth in several species[4]. These observations led us to investigate EGF for possible effects on activities of histidine and ornithine decarboxylases of skin and some other tissues.

Material and methods. EGF was obtained by procedures designed to simplify existing published methods and to increase the overall yield and reliability of the extraction from male mouse submaxillary glands. Complete details of the methodology will be described in a forthcoming publication and therefore only a summarized account will be presented here. The method consists of a first stage extraction similar to that described by COHEN[3] and also TAYLOR, MITCHELL and COHEN[5], up to and including the precipitation with ammonium sulphate. The resolubilised material is then subjected to acidic conditions at pH 3.0, followed by dialysis at pH 5.5. The precipitate is removed at each stage. The liquor is then applied to a column of DEAE cellulose at pH 5.5. After batch elution with 0.09 *M* salt, the eluate is re-applied to an identical column and eluted with a salt gradient. The active peak is dialysed, concentrated and subjected to molecular filtration on a column of Sephadex G-75 at pH 5.5.

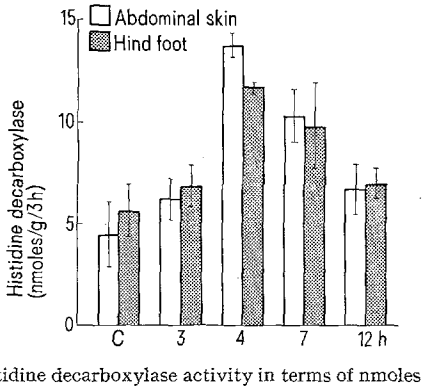

Fig. 1. Histidine decarboxylase activity in terms of nmoles histamine formed in abdominal skin and hind foot after injection of EGF (6 µg/g). C, controls; h, time after injection. The EGF-induced elevation is significant for the time course studied, $p < 0.001$ by Student's *t*-test.

The EGF obtained by the above procedure is found to be as active as that prepared according to the method of COHEN[3], when tested in vivo on the eye opening response of neonatal mice. However, the product differs in its molecular weight (5,750 by ultracentrifugation) and in its absorbance at 280 nm, a value for $E^{1\%}_{1\,cm}$ of 8.4 being obtained, compared to a reported value of 25.9 or 30.9 for COHEN's preparation[3,6]. The lower value is reflected in the presence of only trace amounts of tryptophan in the product. The amino acid, phenylalanine, is also absent in agreement with COHEN's findings but the presence of 2 other amino acids, lysine and alanine, is consistently shown. These amino acids have been claimed to be absent in the EGF molecule[3,5,6]. During the preparation of this manuscript, the extraction of EGF by a comparable technique involving low pH treatment of crude homogenates was reported[7,8]. At pH values in the range 3.2 to 3.5 a product designated EGF-2 was obtained in which the COOH-terminal leucine-arginine residue was absent, whereas the product described in this publication must be lacking a minimum of five COOH-terminal residues in order to account for the absence of tryptophan residues located at positions 49 and 50 on the native EGF molecule[8]. Therefore it is probable that the preparation presently employed represents the derivative EGF_{1-48} or an even lower molecular weight form.

The experiments were done on tissues of 6–9-days-old female mice, strain NMRI. EGF was injected s.c. in the back of the neck and the animals were killed at different times thereafter. Controls were injected with the solvent 0.9% NaCl solution. The appropriate tissue from 2 animals was pooled, finely cut and a sample removed for assay of enzymic activity.

Determination of histidine decarboxylase activity was made by incubating excised tissues with radioactive histidine and measuring the amount of histamine formed[9].

The ornithine decarboxylase activity of minced tissue samples was determined by measurement of the release of $^{14}CO_2$ from DL-1-^{14}C ornithine[10].

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[7] C. R. SAVAGE and S. COHEN, J. biol. Chem. *247*, 7609 (1972).
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