The results presented here suggest a similarity of "coagulase-thrombin" and thrombin. In addition, prolongation of coagulase clotting time by heparin may provide a useful tool in studying the role of coagulase in pathogenesis of staphylococcal infections. The possible influence of heparin upon staphylococcal infections was suggested by the studies of Borgstrom *et al.*(6) who found that it made experimental staphylococcal skin infection more amenable to treatment with penicillin.

Summary. Heparin has been shown to prolong coagulase clotting time of 30 strains of staphylococci. This finding suggests that coagulase may interact with an "accessory factor" of plasma to produce a substance analogous or identical to thrombin.

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1. Elek, Stephen D., *Staphylococcus Pyogenes and Its Relation to Disease,* Livingston Ltd., London, 1959.

2. Walston, H. D., *J. Hyg.,* 1935, v35, 549.

3. Rigdon, R. H., PROC. SOC. EXP. BIOL. AND MED., 1942, v50, 1324.

4. Rigdon, R. H., Haynes, A., *Ann. Surg.,* 1942, v116, 430.

5. Miale, J. B., *Blood,* 1949, v4, 1039, 1317.

6. Borgstrom, S., Muren, A., Erici, I., *Acta Chir., Scand.,* 1954, v108, 114.

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Antifungal Activity of Decanoic Hydroxamic Acid. (27148)

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During an investigation of the pharmacologic properties of hydroxamates of certain carboxylic acids, it was noted that decanoic hydroxamate had a marked inhibitory effect on growth of certain fungi. This paper is a report of *in vitro* and *in vivo* studies of this compound.

Methods. Decanoic hydroxamic acid (DHA) was synthesized by the method previously described(1) and was recrystallized from 95% ethanol by addition of water. Assays of growth inhibition were carried out in liquid media, using Bacto-nutrient broth for bacteria, Kelley's broth(2) for *Cryptococcus neoformans, Blastomyces dermatitidis, Sporotrichum schenkii,* and *Histoplasma capsulatum,* and Bacto-Sabouraud broth for filamentous fungi and *Candida albicans.* Measurement of growth of the yeast-like organisms and bacteria was done turbidimetrically in a Coleman spectrophotometer; dry weight determinations were made of shaken cultures of the filamentous fungi. The former were grown at 37°C and the latter at room temperature. For respirometric studies a 24-hour broth culture of *C. albicans* grown at 37°C was washed with 0.1 M phosphate buffer, pH 7.4, and finally made to a 2.0% suspension (v/v) in this buffer. One ml of this was used in each Warburg vessel. The center well contained 0.2 ml 20% KOH to absorb CO_2. The final reaction volume was 2.0 ml and temperature was 37°C.

Results. Some degree of specificity of the inhibitory action of DHA against both filamentous and yeast-like fungi was evident (Table I); concentrations required for inhibition of certain bacteria were up to 20 times those required for fungi. Even though the hydrogen ion concentration of Sabouraud broth was about 1 pH unit lower than nutrient broth, this pattern bears some similarity to the results obtained by Wyss *et al.*(3) and Prince(4). The former authors, in their survey of antifungal properties of fatty acids, tested both fungi and bacteria at the same hydrogen ion concentrations. Whereas they found 2 dermatophytes to be inhibited by

TABLE I. Inhibitory Concentrations of Decanoic Hydroxamic Acid for Certain Microorganisms.

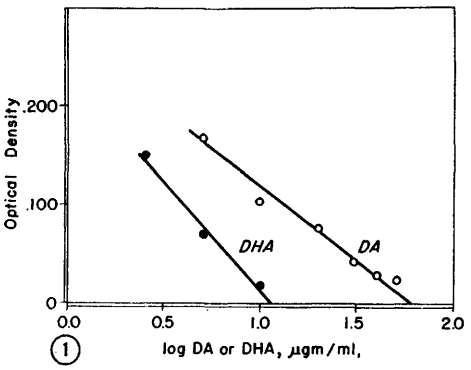
Organism	Inhibitory conc., μg/ml
Candida albicans	12
Cryptococcus neoformans	5
Histoplasma capsulatum	10
Sporotrichum schenkii	10
Blastomyces dermatitidis	10
Epidermophyton floccosum	5
Trichophyton mentagrophytes	5
Rhizopus oryzae	20
Penicillium sp.	15
Aspergillus fumigatus	10
Aerobacter aerogenes	>100
Pseudomonas aeruginosa	50
Staphylococcus aureus	>100
Bacillus subtilis	80
Escherichia coli	>100

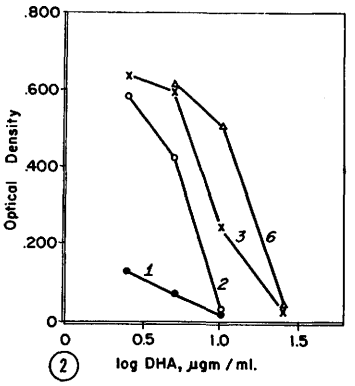
concentrations of decanoic acid (DA) in the range of 0.005 to 0.0075% (50 to 75 μg/ml), *Staphylococcus aureus* required 0.015% (150 μg/ml). Their *Aspergillus niger* required a concentration of 0.06% for complete inhibition, unlike the *A. fumigatus* used in these tests which was inhibited at 0.001%. None of the compounds tested by those authors was completely inhibitory at a concentration of 0.0005%; DHA inhibited *C. neoformans*, *Epidermophyton floccosum*, and *Trichophyton mentagrophytes* completely at this concentration.

To compare the unsubstituted acid with the hydroxamate directly, parallel culture tubes were set up with each compound in equal percent concentrations. Fig. 1 shows the difference in inhibitory pattern using *C. albicans* as test organism. Both the slopes of the response curves and the intercepts on the abscissa were different, indicating approximately a 4-fold increase in activity by converting the free acid to the hydroxamate.

Cells of *C. albicans* which had been exposed to 10 times the minimal inhibitory concentration of DHA for up to 3 hours grew normally when washed by centrifugation and streaked on Sabouraud agar plates. Within these time-concentration limits the effect was fungistatic rather than fungicidal. The low solubility of DHA in aqueous media at room temperature precluded testing at higher concentrations.

Prolonged incubation of cultures of *C. albicans* in presence of DHA yielded the results shown in Fig. 2. Ten μg/ml was virtually completely inhibitory up to 2 days incuba-





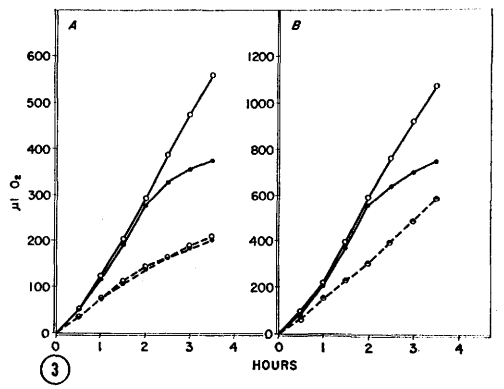


FIG. 1. Growth of *Candida albicans* as a function of concentration of decanoic hydroxamic acid (DHA) and decanoic acid (DA).

FIG. 2. Growth of *Candida albicans* as a function of concentration of decanoic hydroxamic acid when measured at 1, 2, 3, and 6 days after inoculation of cultures.

FIG. 3. Effect of decanoic hydroxamic acid (DHA) on respiration of *Candida albicans* (A) without and (B) with 25% serum. Solid lines, without DHA; dotted lines, with 100 μg/ml DHA. ●, without acetate (endogenous); ○, with 0.01 M acetate.

tion; on the third day there was considerable growth at this concentration. Twenty-five μg/ml suppressed growth up to 6 days at which time observations were terminated. Due to the relatively low sensitivity of the colorimetric assay for hydroxamates with resulting inability to measure concentrations below about 20 μg/ml with accuracy, it cannot be stated with certainty that growth upon continued incubation in the presence of DHA was not due to its destruction by the organism. However, when 0.5 ml packed cells was incubated in the presence of DHA at 100 μg/ml in phosphate buffer at pH 7.4, no significant reduction in concentration of $FeCl_3$-reactive substance was noted after 18 hours when measured by centrifugation of an aliquot, addition of HCl followed by $FeCl_3$, and comparison with a standard at 540 λ. Although this does not rule out degradation of the C_{10} chain to a less toxic hydroxamate of C_{10-n}, it appears to be evidence that degradation, if it occurs, is not through formation of hydroxylamine and the free C_{10} acid. Conversely, this may also be taken as evidence that the inhibitory effect of DHA on the organism is not related to a release of free hydroxylamine from the bound state.

Experiments to determine if serum would reverse the inhibitory effects of DHA were evaluated visually because of the clumping of growth of *C. albicans* when grown in presence of fresh human serum which made spectrophotometric determinations impractical. In Sabouraud broth prepared with serum at a final concentration of 25%, complete inhibition of growth occurred at a DHA concentration of approximately 40 μg/ml as compared with only 12 μg/ml required in absence of serum.

To determine if cell respiration was likewise affected by DHA, conventional Warburg vessels of approximately 15 ml capacity were set up to measure effects on endogenous respiration and oxidation of an added substrate. Results are shown in Fig. 3A. As with growth, there was also some reversal by serum of the depression of respiration (Fig. 3B).

For determination of acute toxicity of DHA, young albino mice of mixed sex were divided into groups of 6, all mice in each group receiving the same dosage intraperitoneally. DHA was prepared in a 40 mg/ml suspension by homogenizing 30 seconds in a Waring blendor in 0.2% deoxycholate solution. Results shown in Table II illustrate the delayed death which occurred. A direct comparison of the toxicity of DHA with other hydroxamates tested by Epstein and Freeman(5) is not possible on the basis of these data since those authors observed their mice only 18 hours after injections and used only those deaths which occurred within 2 hours in the calculation of the LD_{50} for each compound. It appears that under the conditions of the present experiment the LD_{50} of DHA in mice is approximately 600 mg/kg. Surviving animals were sacrificed and autopsied after 19 days. Liver, kidney, and bone marrow specimens were fixed in buffered formalin, embedded, sectioned, and stained with hematoxylin-eosin. Microscopic examination showed that the livers of both the controls, which received deoxycholate alone, and the mice receiving DHA developed some pericentral monocytic infiltration with occasional foci of parenchymal cell variation and necrosis. The kidneys showed moderate perivascular monocytic infiltration. Degree of changes in both experimental and control livers and kidneys was of approximately the same magnitude; consequently, the principal site of lethal damage with the higher dose levels was probably other than on these 2 organs. Bone marrow showed no alterations in either group.

The backs of 2 adult male albino rabbits were shaved and 10% DHA in propylene glycol was applied to one site on each and propylene glycol alone to another site daily for 56 days. Some erythema developed at sites

TABLE II. Acute Toxicity of DHA in Mice. Numbers indicate survivors in each group of 6 mice after period shown in column at left.

	Dose, mg/kg					
Days	0	100	200	300	400	500
2	6	6	6	6	6	6
3	6	6	6	6	6	5
7	6	6	6	6	6	4
15	6	6	6	5	5	4
19	6	6	6	4	5	4

of application of DHA after approximately 14 days but did not progress with further applications. This rapidly cleared when applications were stopped and there was no residual scarring or other gross evidence of injury. Growth of hair at sites of application of DHA was normal throughout the period of observation.

Chronic toxicity in one rabbit was determined by injection of DHA in deoxycholate intramuscularly daily for 24 days at a dosage of 100 mg/kg/day; the control rabbit received the appropriate volume of deoxycholate solution alone. Neither rabbit lost weight during the observation period. Within a few days areas of necrosis appeared at the injection sites of both animals, but there was no other gross evidence of toxicity. Both animals were sacrificed and autopsied on the 24th day. Specimens of kidney, liver, and spleen were fixed, sectioned, and stained as above. Microscopically, findings were virtually the same as with mice. There was some interstitial nephritis and scarring of tubules in the kidney from both animals; the livers showed moderate perivascular inflammatory infiltration. Occasional inflammatory cells were observed around bile ducts and portal areas. There was no change in the spleen from either animal.

Citric, succinic, and malonic hydroxamates rapidly produce methemoglobin *in vitro* and *in vivo* (Bernheim, M.L.C., personal communication). To determine if this was also a property of DHA, one drop of human blood was added to 1.0 ml distilled water in each of two 19 × 105 mm Coleman spectrophotometer tubes to induce hemolysis. To one tube was then added 9.0 ml water containing DHA to give a final concentration of 50.0 μg/ml; to the other was added water alone. No change in the absorption spectrum of the hemoglobin in either was noted up to 18 hours incubation at room temperature. This concentration of DHA also did not induce any hemolysis *in vitro* of intact red cells in 1.5 hours incubation at 37°C.

To determine whether DHA would have any beneficial effects on *C. albicans* infections of the skin and nails, Dr. J. Lamar Callaway of the Duke Medical Center Dermatology Division undertook preliminary clinical trials with patients with such infections. For this purpose DHA was dissolved in 95% ethanol and recrystallized by addition of 0.01 M phosphate buffer, pH 7.4. After removal of the supernatant solution by filtration and drying at room temperature, the compound was prepared as a 1% solution in propylene glycol and patients were instructed to apply this topically twice each day to the involved areas. Results with 10 patients receiving it thus far appear encouraging. Two of these who had failed to respond to amphotericin B intravenously and nystatin topically showed definite regression of lesions within one week. To date there has been no evidence of sensitivity or primary irritation. Due to the fact that patients chosen for study lived up to 200 miles from Duke Medical Center, a propylene glycol placebo was not used in this preliminary test. However, since patients were chosen who had chronic dermatomycoses which were refractory to other therapeutic agents, the rapid, marked improvement observed was considered significant. It is anticipated that a detailed evaluation of these and other clinical trials will be reported.

Discussion. Even though the antifungal properties of fatty acids have been recognized since the past century(6), the fundamental mechanisms of action are still not completely understood. These acids are generally considered to be more active in an acid than in an alkaline medium, the implication being that the anion is not the toxic component. The recent observations of Prince(4), however, appear to rule out any simple relation between concentration of undissociated acid and inhibition. He found that minimal inhibitory concentration of undecylenic acid for *T. mentagrophytes* varied from 3 μg/ml at pH 4.5 to 1500 μg/ml at pH 9.0. However, when he calculated the actual concentration of undissociated acid at each hydrogen ion concentration tested, the amount required for complete inhibition was fairly constant at a value of about 2.2 μg/ml between pH 4.5 and pH 6.0, but dropped to a value of approximately 0.2 μg/ml at pH 9.0. He stated that the increased susceptibility of the organisms to the undissociated acid at

high pH values was not a result of any "weakening" of the cells, since both growth rate and respiratory activity were at or near their maxima at pH 8.0 to pH 9.0. Certainly a "weakening" of the cells need not be a required factor for an increased susceptibility to a drug, and he no doubt approaches the true nature of the problem when he mentions unknown effects of pH-dependent ionizations at the cytoplasmic membrane on response to a drug which itself undergoes ionic changes.

Of considerable interest is the difference in susceptibility of *C. albicans* to DHA compared to undecylenic acid. Both compounds have virtually identical activities against dermatophytes, but Wyss *et al.*(3) reported 70 μg/ml undecylenic acid was required to inhibit their strain of *Monilia* (*Candida*) *albicans*, and Prince(4) reported 200 μg/ml of either free undecylenic acid or its calcium salt was required with his strain. This is in contrast to 12 μg/ml required to inhibit either of 2 strains of *C. albicans* in these tests. This suggests the possibility that the mechanism of action of DHA may differ somewhat from that of the free fatty acid. A systematic study of physical properties of a fatty acid along with its corresponding hydroxamate which, in the case of decanoic acid, is about 4 times as potent as the unsubstituted acid, may yield correlations helpful in inferring a mechanism of action.

The encouraging results obtained with DHA on 10 patients with superficial *C. albicans* infections suggest that further clinical trials should be undertaken. The sensitizing capability of free hydroxylamine should be continually borne in mind, however, and every attempt made to remove this compound completely by repeated crystallization. Since the inhibitory effects of DHA were reversed by serum, and evidence of toxicity was noted in experimental animals after parenteral administration, it is quite doubtful that treatment of systemic mycoses with this compound would be beneficial.

Summary. Decanoic hydroxamic acid (DHA) was markedly inhibitory to growth of certain fungi, both mycelial and yeast-like. Quantitatively it was approximately 4 times as active as the unsubstituted acid on *Candida albicans*. Activity was apparently not mediated through a release of free hydroxylamine. Toxicity upon parenteral administration to experimental animals was not primarily to liver, spleen, bone marrow, or kidney. Topical application of 1% DHA in propylene glycol on patients with cutaneous *C. albicans* infections caused considerable regression of lesions in one week. No evidence of sensitization or primary irritation was observed in this group.

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1. Bernheim F., Stoops, J. K., PROC. SOC. EXP. BIOL. AND MED., 1960, v104, 542.
2. Kelley, W. H., *J. Infect. Dis.*, 1939, v64, 293.
3. Wyss, O., Ludwig, B. J., Joiner, R. R., *Arch. Biochem.*, 1945, v7, 415.
4. Prince, H. N., *J. Bacteriol.*, 1959, v78, 788.
5. Epstein, M. A., Freeman, G., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 660.
6. Clark, J. F., *Botan. Gaz.*, 1899, v28, 378.

Received October 11, 1961. P.S.E.B.M., 1962, v109.