molarity of such solutions was about twice that existing in the saccharin-ferrihemoglobin solution. No visible or spectroscopically detectable alteration was produced.

On the basis of the facts that soluble saccharin is the sodium salt of a hydro-acid, o-benzoic sulphinide; that it reacts with ferrihemoglobin in solution to produce a colored entity which is similar in its spectroscopic picture to that of the class of blood pigment derivatives termed ferridehemoglobins, the saccharin derivative is designated as saccharin- or o-benzoic sulphinide ferridehemoglobin.

Like certain other ferridehemoglobin formers (fluoride, fulminate, and cyanate) saccharin does not form ferridehemochromogens. The apparently high degree of dissociation of saccharin ferridehemoglobin which would seem to be indicated by the relatively high concentrations of saccharin required for the complete conversion of ferrihemoglobin is similar to that exhibited by the ferridehemoglobins of cyanamide, cyanate, and thiocyanate while it differs markedly from that observed in the cases of the ferridehemoglobins formed by the highly toxic anions, azide, cyanide, and fluoride. There appears to be some parallelism in the degree of association of hydro-acid anions with ferrihemoglobin and their inhibitory effect on the blood catalase. In this connection it is found that saturation with soluble saccharin is without appreciable effect on the catalase activity of whole blood, plasma or hemoglobin solutions. From the high concentrations of soluble saccharin found necessary for the completion of its reaction with the prosthetic nucleus of ferrihemoglobin, it is doubtful if this reaction is of any toxicologic importance.

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The Anti-Bacterial Action of the Xanthine Oxidase System.*†

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Notatin, an anti-bacterial substance isolated from *Penicillium notatum,* has been shown by Raistrick and coworkers[1] and others[2,3] to be a flavoprotein which catalyses the aerobic oxidation of glucose to gluconic acid with production of H_2O_2. According to these authors H_2O_2 is the actual anti-bacterial agent and the function of the flavoprotein-glucose-oxygen system is merely that of providing a continuous albeit minute supply of H_2O_2. It follows that any flavoprotein system which gives rise to H_2O_2 should have at least qualitatively the same anti-bacterial action as notatin. We have tested this hypothesis with the flavoprotein from milk which catalyses the oxidation of hypoxanthine to uric acid, and the results clearly indicate that there is little to choose between the flavoproteins from cow's milk and *Penicillium notatum* respectively as far as anti-bacterial action is concerned. The sole difference is that notatin is a somewhat more stable enzyme.

Experimental. Milk flavoprotein was prepared in purified form by the method of

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† Since our article was submitted for publication on June 25, 1943, there has appeared in *Science* for September 10, 1943, an article by Lipmann and Owen who made observations comparable to those which we are reporting.

1 Coulthard, C. E., Short, W. F., Michaelis, R., Sykes, G., Skrimshire, G. E. H., Standfast, A. F. B., Birkinshaw, J. H., and Raistrick, H., *Nature,* 1942, **150**, 634.

2 Van Bruggen, J. T., Reithel, F. J., Cain, C. K., Katzman, P. A., Doisy, E. A., Muir, R. D., Roberts, E. C., Gaby, W. L., Homan, D. M., and Jones, L. R., *J. Biol. Chem.,* 1943, **148**, 365.

3 Kocholaty, W., *Science,* 1943, **97**, 186.

TABLE I.

Anti-bacterial Action of the Xanthine-oxidase System on the Growth of *Staphylococcus aureus*. Experiments Were Carried Out Aerobically at 38° in 1% Peptone Medium (Difco No. 13123). Final Concentration of Hypoxanthine Was 1 mg in 5 cc of Medium.

Additions to medium	Production of uric acid at 16 hr	Growth after hr				
		16	24	40	64	88
None	0	++++	++++	++++	++++	++++
Hypoxanthine	0	++++	++++	++++	++++	++++
Xanthine oxidase (0.03 unit)	0	++++	++++	++++	++++	++++
Hypoxanthine + xanthine oxidase (0.03 unit)	++++	0	0	0	0	0
Hypoxanthine + xanthine oxidase (0.003 unit)	+	0	0	0	0	0
Hypoxanthine + boiled xanthine oxidase (0.03 unit)	0	++++	++++	++++	++++	++++

TABLE II.

Concentration-activity Relations for Anti-bacterial Action of the Xanthine Oxidase System on Aerobic Growth of *Staphylococcus aureus* in Casein Hydrolysate-Beef Broth at 38°.

Final conc. of enzyme (parts pure enzyme to parts of water)	16 hr	20 hr	24 hr	40 hr	64 hr
0	++++	++++	++++	++++	++++
$1:10^4$	0	0	0	0	0
$1:10^5$	0	0	0	0	0
$1:10^6$	0	0	0	0	+
$1:10^7$	0	0	0	+	++++
$5:10^8$	0	0	+	++++	++++
$1:10^8$	0	+	++	++++	++++
$1:10^8$ (renewed every 12 hr)	0	0	0	0	0

Corran *et al.*[4] One Corran unit of xanthine oxidase activity is equivalent to 600 γ flavoprotein containing 1.6 γ flavinphosphate. Thus by estimating the enzymic activity of our purified, sterile solutions in terms of Corran units we were able to determine the precise concentration of active flavoprotein. It is important to note that sterilization by filtration through a Seitz filter led to great losses of activity owing to contamination by heavy metal. Chamberland candles proved more satisfactory.

The substrate for the milk enzyme (xanthine or hypoxanthine) is present in abundance in the meat broth media commonly used in bacteriological studies. Further addition of substrate is therefore superfluous. Peptone water or hydrolysates of purified proteins, however, contain neither hypoxanthine nor xanthine and were used for the experiments demonstrating that the anti-bacterial action of the xanthine oxidase is dependent upon the presence of its substrate.

[4] Corran, H. S., Dewan, J. G., Gordon, A. H., and Green, D. E., *Biochem. J.*, 1939, **33**, 1694.

Oxygen must be present in the medium for the enzymic oxidation to take place. We have found in practice that an adequate oxygen tension obtains in the growth medium when the layer of fluid does not exceed 2 cm in depth.

Table I shows that the aerobic growth of *Staphylococcus aureus* in peptone water is inhibited by milk flavoprotein only when hypoxanthine is added. The boiled enzyme in presence of substrate is inactive. That the anti-bacterial action is due to the functioning of the xanthine oxidase is shown by the fact that uric acid is formed in appreciable quantity only when the complete enzyme system is present. This coincides with the suppression of bacterial growth. Under conditions of anaerobic growth which do not permit the xanthine oxidase to function, no anti-bacterial action is observed. The anti-bacterial action of milk flavoprotein on the growth of *Staphylococcus aureus* is demonstrable at a final dilution of 10^8 over a 16-hour period (Table II). The enzyme is unstable under the experimental conditions, and at the end of a

TABLE III.

Reversal by Catalase of Anti-bacterial Action of the Xanthine Oxidase System on Growth of *Staphylococcus aureus*. The experiments were carried out aerobically at 38° in casein hydrolysate-beef broth.

Addition to medium	Growth after 16 hr	40 hr	64 hr
None	++++	++++	++++
Xanthine oxidase (0.05 unit)	0	0	0
" " + catalase sol. diluted 1:25	++++	++++	++++
" " + " " " 1:250	++++	++++	++++
" " + " " " 1:2500	+	++++	++++
" " + " " " 1:25,000	0	++++	++++
" " + " " " 1:250,000	0	0	0

TABLE IV.

Reversal by Azide and Hydroxylamine of Catalase Inhibition of Anti-bacterial Action of Xanthine Oxidase (X.O.) on Growth of *Staphylococcus aureus*.

Additions to medium	Growth after 40 hr	80 hr
Xanthine oxidase (0.05 unit)	0	0
X.O. + catalase (diluted 1:2500)	++++	++++
X.O. + " + M/100 hydroxylamine	0	0
X.O. + " + M/200 "	0	0
M/100 hydroxylamine	+	++++
M/200 "	++	++++
X.O. + catalase + M/1000 azide	0	0
X.O. + " + M/2000 "	0	0
M/2500 azide	++	++++
M/5000 "	++++	++++

few hours only a small fraction of the original activity survives. The effective concentration of enzyme during the experimental period is therefore much less than the initial.

The anti-bacterial effect of milk flavoprotein at the lower limits of concentration is one of bacteriostasis. Growth is suppressed so long as the enzyme system produces H_2O_2. As soon as the activity of enzyme ceases for whatever reason, growth of the bacteria proceeds normally. Thus a concentration of enzyme at the lower limit of effectiveness will stop growth for 16 hours. If the concentration of enzyme is renewed every 12 hours growth can be suppressed for days. However, growth starts promptly as soon as one renewal is missed out.

That H_2O_2 is indeed responsible for the bacteriostasis is shown by the fact that minute concentrations of catalase, an enzyme which decomposes H_2O_2, reverse the anti-bacterial action of the xanthine oxidase (Table III). In turn by adding azide or hydroxylamine as catalase inhibitors the anti-bacterial action is restored. The conditions for obtaining this azide and hydroxylamine inhibition of the catalase reversal are critical and are given in Table IV. We have never been able to demonstrate the anti-bacterial action of milk flavoprotein on the growth of *Staphylococcus aureus* in serum but this failure has been correlated with the presence of high concentrations of catalase in serum.

Milk flavoprotein inhibited the growth of *M. catarrhalis, Bact. shiga, Proteus vulgaris, Pseudomonas pyocyanea, M. sarcina lutea, Streptococcus haemolyticus* (5 strains), and *B. subtilis* in addition to some 10 strains of *Staphylococcus aureus* and *albus*. It had no action even at high concentrations on the growth of *Bact. coli, Bact. friedlanderi, Bact. lactis aerogenes, Bact. paratyphosus* B, *Streptococcus pneumonia* A 66, *Bact. flexneri* and *Corynebacterium diphtheriæ*.

Summary. The anti-bacterial action of the milk xanthine oxidase system has been shown to be due to formation of H_2O_2.