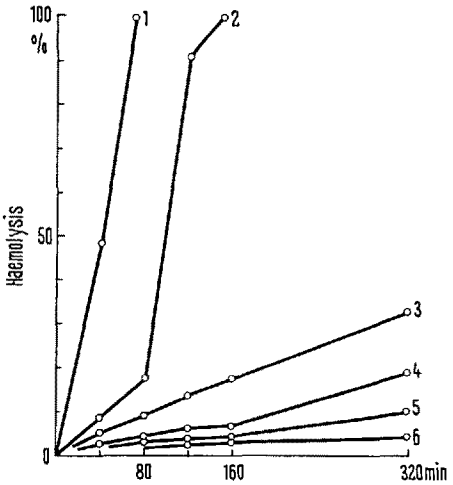
mixtures were incubated at 37 °C and portions of 1 ml each were taken at various time intervals for photometric estimation of haemolysis.

As seen from the Figure, viscotoxin B has a weak direct haemolytic effect when acting in concentrations of 10^{-4} or 5×10^{-4} g/ml for prolonged times. Phospholipase A at the final concentration of 10^{-6} g/ml is practically non-haemolytic. When both substances are present simultaneously, complete haemolysis occurs in 80–160 min. Thus a pronounced potentiation is apparent, similar to the synergistic effect of phospholipase A and DLF.



Effect of phospholipase A (final concentration 10^{-6} g/ml) and varying concentrations of viscotoxin B on washed guinea-pig erythrocytes. Ordinate: % haemolysis. 1. Phospholipase A + 5×10^{-4} g/ml viscotoxin. 2. Phospholipase A + 10^{-4} g/ml viscotoxin. 3. 5×10^{-4} g/ml viscotoxin alone. 4. 10^{-4} g/ml viscotoxin alone. 5. Phospholipase A alone. 6. Control without haemolysins.

By demonstrating that viscotoxin B has the anticipated enhancing action, our results support the view[3] that the phospholipids in the red cell membrane, normally protected from attack by phospholipase A, can be exposed by structural changes induced through reaction of S–S-groups with membrane constituents. The cationic charge, which has been found earlier also to be essential[1,3], may serve to attract the peptides to the proper membrane sites where they can react.

Zusammenfassung. Viscotoxin B, ein basisches Peptid mit Disulfidgruppen, zeigt in der Hämolyse den gleichen synergistischen Effekt mit Phospholipase A wie der direkt lytische Faktor des Kobragiftes. Dieses Ergebnis stützt die Hypothese, dass Zellmembranen durch Reaktionen ihrer Protein-SH-Gruppen so verändert werden können, dass Phospholipase A sonst unzugängliche Membranphospholipide angreift.

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Oxidation of Vanillin by Cream Xanthine Oxidase and its Inhibition by Allopurinol

Recent research has directed attention of biochemist and pharmacologists to aldehydes, which rather than inactive transitory intermediates of oxidation of alcohol, and of amines, have come into prominence of their own, and are now considered important metabolites, endowed with significant biochemical activity[1–5]. Several enzymes (alcohol dehydrogenase, aldehyde oxydase and dehydrogenase, and xanthine oxidase) can metabolize aldehydes to the corresponding acids; other pathways are also available for metabolizing aldehydes[6].

In connection with an investigation dealing with the metabolism of 'seroton-aldehyde' and other metabolites of biogenic amines[7], the oxidation of aldehydes by purified cream xanthine oxidase (E.C. 1.2.3.2; Worthington) was studied. The selected substrate was vanillin (3-methoxy-4-hydroxy-benzaldehyde) which is of special interest due to its similarity to catecholamines (Figure 2). This compound has been shown to be a substrate for xanthine oxidase by several investigators[8–10], but its behavior in the tetrazolium reduction assay for xanthine dehydrogenase[11,12], nor the effect of the specific competitive inhibitor of xanthine oxidase, allopurinol[13], has not been investigated.

Vanillin presents a broad absorption peak at 345 nm, which is absent in vanillic acid (Figure 1); the disappearance of this peak can be utilized for spectrophotometric assay of xanthine oxidase activity with vanillin as substrate (Figure 2).

Allopurinol (4-hydroxypyrazolo-[3,4-d]pyrimidine) is a structural analog of hypoxanthine, which explains its inhibiting activity with that substrate. It is well known,

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that xanthine oxidase shows high specificity with purines as substrate, but has no specificity at all for aldehydes[14].

It was therefore of interest, to test, whether allopurinol can also block oxidation of vanillin and other aldehydes by xanthine oxidase, although these substrates are substantially different from the purines.

In order to determine possible non-enzymatic interaction between these two reagents, vanillin alone or 0.375 µmoles of vanillin + 0.1 µmoles of allopurinol were dissolved in 3.0 ml of 0.1 *M* sodium phosphate, pH 7.8, and incubated for 30 min at 37 °C. No change in absorbance at 345 nm was observed. This indicates no loss of vanillin by oxidation to vanillic acid.

In presence of xanthine oxidase, vanillin is oxidized, as shown by decrease in A_{345}; when allopurinol is added to this reaction system, the rate of decrease is lowered (Figure 3). It is abolished completely by higher concentrations of allopurinol. Thus, allopurinol also inhibits oxidation of aldehydes by xanthine oxidase, although significantly less efficiently than xanthine oxidation. This may be due to the high level of enzyme required for oxidation of vanillin. Frequently, a rise at A_{345} is observed during the first 3-min interval, especially when allopurinol is also present; the experiment just described above indicates, that this must be enzymatic effect, the mechanism of which is not known.

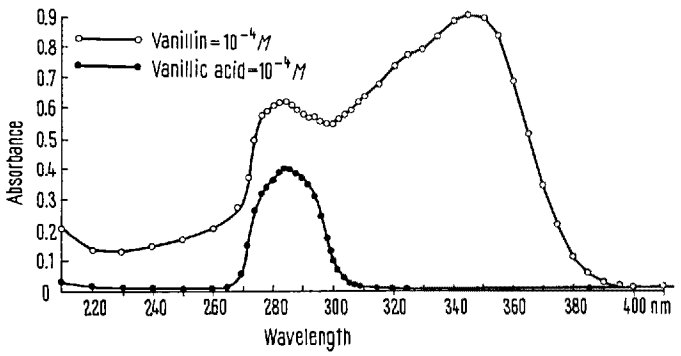

Fig. 1. Absorption spectrum of vanillin and vanillic acid.

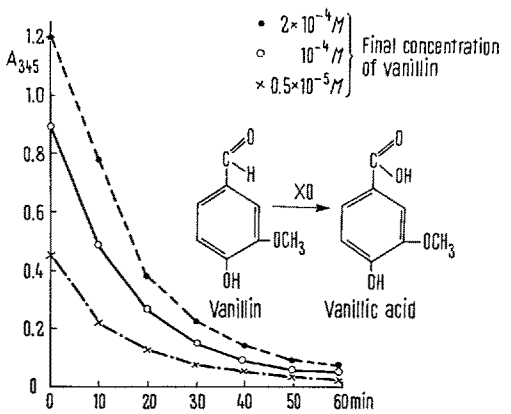

Fig. 2. Oxidation of vanillin by cream xanthine oxidase. λ = 345 nm. 1 cm lightpath, vol. 3.0 ml. Reaction started by addition of substrate.

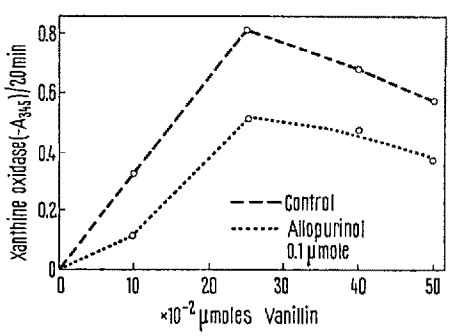

Fig. 3. Inhibition of oxidation of vanillin by allopurinol (Figures represent cumulative decrease at 345 nm). Volume = 5.0 ml.

Vanillin causes substrate inhibition at high levels, which is also shown by xanthine. Due to this effect, a graphic analysis of inhibition by allopurinol by means of the Lineweaver-Burke method is inconclusive, and cannot be interpreted by simple kinetics.

Oxidation of vanillin by cream xanthine oxidase was also assayed by reduction of Nitro-Blue tetrazolium[11,12]. No color was produced when vanillin (5–10 µmoles) was incubated for 30 min at 37 °C with 2.0 ml of tetrazolium assay solution, containing also phenazine methosulfate and gelatin, but without xanthine oxidase.

When cream xanthine oxidase was added to the solution of vanillin and chromogen mixture, rapid color formation was observed. Much higher concentrations of aldehyde are required for tetrazolium reduction than are necessary for xanthine or allopurinol oxidation:

xanthine, 0.4 µmoles	1.13 A_{540}/10 min at 25 °C
allopurinol, 0.4	0.43
vanillin, 1.5	0.06

5–10 higher concentration of enzyme are recommended for vanillin oxidation than for xanthine oxidation, to obtain reasonable reaction rates in both types of assays.

Attempts were made to study the initial rates of vanillin oxidation in presence of allopurinol and tetrazolium. However, these experiments, repeated frequently, gave a very erratic pattern, showing flucuating increase and decrease in A_{540} for the first 5 min at 25 °C, which could not be interpreted. The interaction was analyzed, by incubation for 10 min at 37 °C and determination of the formazan produced (Figure 4).

Allopurinol was rapidly oxidized by xanthine oxidase in the tetrazolium reduction assay, which also contained catalytic amounts of phenazine methosulfate as auxiliary electron carrier. JOHNS[15] had previously shown, that phenazine methosulfate itself could serve as electron

acceptor in this reaction, allopurinol being oxidized about 50 times more rapidly than with oxygen as electron acceptor.

An examination of Figure 4 shows that allopurinol and vanillin interfere with each other in the enzymatic reduction of tetrazolium; the higher the concentration of the 2 reagents, the greater is the proportional inhibition of tetrazolium reduction. A consequence of this is, that inhibition of enzymatic oxidation of vanillin requires high levels of allopurinol; proper allowance must be made for the enzymatic oxidation of allopurinol. The data could be interpreted as indicating that vanillin is an inhibitor of allopurinol oxidation by xanthine oxidase.

The mutual interference of vanillin and allopurinol, which is probably a competition for the active site of xanthine oxidase, is similar to the mutual interference of allopurinol and xanthine in the tetrazolium reduction assay[16] with purified cream xanthine oxidase: xanthine (0.0001 M) = 0.68; allopurinol (0.0001 M) 0.09; combined = 0.01 A_{540}/10 min at 25 °C. Due to very different rates of oxidation, interference between xanthine and vanillin in this reaction could not be tested; other experimental conditions will be required for this study.

Xanthine oxidase is considered to be highly specific for purine or pteridine structures as substrates, and to show no specificity at all for aldehydes; these substrates range from acetaldehyde to aromatic aldehydes such as vanillin[8,14]. With one exception in the literature[17] these 2 types of activity are considered properties of the same enzyme, which cannot be separated. The inhibition of both substrates by allopurinol indicates that either the regions of substrates bound to the enzyme are similar, or that allopurinol (in addition to being a competitive inhibitor due to being a structural analog of hypoxanthine) also acts as allosteric inhibitor, causing a conformational change in the enzyme. Vanillin can be assayed enzymatically, either by change in absorption in the UV region, or by colorimetric tetrazolium assay.

The initial interaction between allopurinol, vanillin and tetrazolium may be related to the lag phase observed in other enzymatic reductions of tetrazolium salts[11,18,19]. When this method is used for metabolic studies, dealing with aldehydes or biogenic amines, it should be kept in mind that indolyl-3-acetaldehyde and 5-hydroxyindolyl-3-acetaldehyde, as well as norepinephrine and certain phenols reduce tetrazolium non-enzymatically[19-21]. Acetaldehyde and aromatic aldehydes[21], as well as vanillin do not reduce tetrazolium in absence of enzymes.

In an important publication (received after the present paper had been submitted) MASSEY et al.[22] have shown, that allopurinol inactivates xanthine oxidase by combining with the Molybdenum. This portion of the enzyme must therefore be also concerned with the oxidation of aldehyde substrates as well as with the electron transport to phenazine methosulfate and tetrazolium as electron acceptors. The inactivation of the enzyme by allopurinol may explain, why this compound, a structural analog of hypoxanthine, is also an inhibitor in the aldehyde-oxidizing pathway of xanthine oxidase.

Vanillin, which was used in the present study as a model for enzymatic reaction mechanisms with purified cream xanthine oxidase, is itself oxidized by the rat and rabbit after oral or parenteral administration, and is excreted in urine mainly as free or conjugated vanillic acid[23,24]. No study has a yet been carried out with purified enzyme systems, but oxidation of vanillin was suppressed by treatment of the experimental animals with disulfiram[24], and inhibitor of NAD-linked aldehyde dehydrogenase. This compound also inhibits xanthine oxidase[16,25] and other enzymes[26,27], so that a clear decision of which enzyme or enzymes are blocked cannot yet be made. In the present report vanillin oxidation was shown to be inhibited by the specific xanthine oxidase inhibitor allopurinol; thus this enzyme probably participates in the over-all metabolism of vanillin by the rat and rabbit. It is of interest to note, that vanillic acid itself has been identified as a minor metabolite of catecholamines (see [24]).

Zusammenfassung. Es wird gezeigt, dass Vanillin durch Xanthinoxydase oxydiert und dass diese Reaktion durch Allopurinol gehemmt wird.

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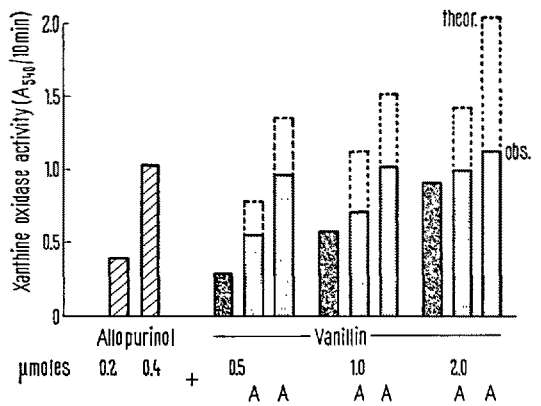

Fig. 4. Oxidation of allopurinol and vanillin by cream xanthine oxidase. Tetrazolium (1.2 mg/tube) reduction in presence of catalytic amounts of phenazine methosulfate (20 μg/tube). Reaction started by addition of vanillin, or allopurinol, where indicated. A, addition of 0.2 μmoles (left column) and 0.4 μmoles (right) of allopurinol. Dotted line indicates theoretical value (sum of absorbance of allopurinol + vanillin, tested singly). 10 min at 37 °C, volume = 5.0 ml.

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