The observation that monoamine oxidase activity is present only in part of the circulating reticulocyte population can be explained by a more rapid rate of decrease of monoamine oxidase activity than of the substantia reticulo-filamentosa (which is responsible for the staining characteristics) during the course of red cell maturation. In this respect, monoamine oxidase obviously shares common characteristics with other enzymes, especially with the mitochondrial marker enzyme, cytochrome oxidase: it has been shown by ROSENTHAL et al.[19] that there is a similar nonlinear correlation between cytochrome oxidase activity and RNA content in rabbit reticulocytes. The latter finding is in agreement with the results of morphological investigations[20,21] which indicate a very rapid loss of mitochondria during the maturation of reticulocytes.

It may therefore be concluded that reticulocyte monoamine oxidase is a mitochondrial enzyme; this conclusion has recently been confirmed in preliminary experiments in which acetyl-phenylhydrazide was used as an inductor of reticulocytosis[22]. With respect to its substrate and inhibitor specificities[5,22], and also with respect to its mitochondrial location, the reticulocyte enzyme is classified as a 'classical'[6] monoamine oxidase.

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Anionic Activation of Human Salivary Amylase

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Summary. In all earlier studies on α-amylase, the influence of different ions were studied in phosphate buffer. The present report shows the effect of different ions individually with *Tris* and amino acid. Though it has been claimed recently that sodium ion is an activator of α-amylase, this study reconfirms that sodium ion does not activate human salivary amylase.

The activation of mammalian α-amylases by chloride ion, and less effectively by certain other monovalent anions has long been known[1,2], whereas microbial and diastatic α-amylases do not require chloride ion as activator[3]. The various aspects of salt activation of α-amylases mostly of pancreatic and salivary origin have been studied by different workers. MYRBÄCK[1] had pointed out that the pH optimum of the enzyme shifts from 6.0 to 6.9 on being activated in the presence of Cl^-. It is also reported that the pH optima shift occurs by the addition of other anions; a few of them activate the enzyme partially. BERNFELD et al.[4] showed that, after removal of such activators by exhaustive dialysis of both the enzyme and the substrate, the activity is reduced to 15% of its original value, which is entirely restored instantaneously by the inclusion of 0.01 *M* NaCl to the reaction mixture. Using various chlorides and other anions, they concluded that Cl^- is essential to mammalian α-amylases for full activation and the cation has got no role in the process. Calcium ion is believed to be firmly bound to the protein molecule and contributes to the structural stability against higher pH and proteolytic degradation[2]. In contrast to the above findings, recently it has been shown that sodium ion and not the chloride ion is sole activator for pancreatic α-amylases[5] of toad, reptile, pigeon and rat. However, the literature fails to provide enough information about the role of different activators independently, particularly anionic, since all of the earlier experiments were done with phosphate buffer which itself is likely to activate to some extent.

In order to study the activation by anions independently and by their influence on the shift of pH optimum, the present investigation was carried out using suitable buffers with and without phosphate at two different pH values viz. 6.0 and 6.9. This report simultaneously throws light upon certain observed discrepancies in earlier reports.

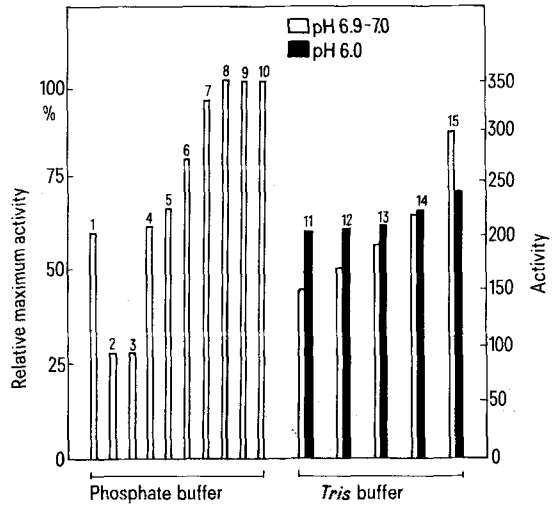

Fig. 1. Activation of α-amylase by ions (salts). Numbers 1 to 10 all in phosphate buffer. 1. buffer alone; 2. Na_2CO_3; 3. $CuCl_2$; 4. $NaNO_3$; 5. CH_3COONa; 6. NaBr; 7. $CsCl_2$; 8. NH_4Cl; 9. KCl; 10. NaCl. Numbers 11 to 15 all in *Tris* buffer. 11, aspartate; 12, acetate; 13, phosphate; 14, oxalate; 15, chloride.

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Materials and methods. Amylopectin, *Tris*, $CsCl_2$ and aspartic acid used were of highest purity. The rest of the chemicals were of analytical grade. The substrate was prepared by dissolving amylopectin in 0.02 *M* of 5 different *Tris* buffers, viz. aspartate, acetate, phosphate, oxalate and hydrochloride of pH 6.0 and 6.9 at a concentration of 1%. Saliva was collected from normal healthy persons and centrifuged for $^1/_2$ h at 7000 rpm at 10 °C. The supernatant was separated and stored at 0–5 °C until use. The supernatant from the centrifuged saliva, which showed specific activity between 125–150 units per mg of protein, was diluted 200–250 times with respective buffer and used as enzyme.

The α-amylase activity was measured as outlined by BERNFELD[6] with minor modifications; 1 ml of the substrate was incubated with 1 ml of the enzyme for 3 min at 25 °C. The reaction was stopped by adding 2 ml of 3,5-dinitrosalicylic acid reagent. The mixture was kept in boiling water-bath for 5 min, cooled and made up to 25 ml with water. The colour developed was measured by double wavelength method[7], which pertains to a modification of optical measurements without changing the principle. Our standard curve was drawn by taking ΔA (difference in absorbances at 570 and 700 nm) against maltose concentration (0.5–3.0 mg/25 ml). In a separate set of experiments, the same was repeated with 0.02 *M* phosphate buffer instead of *Tris* buffer at pH 6.9 containing varying concentrations of NaCl, NaBr, $NaNO_3$, CH_3COONa, Na_2CO_3, KCl, NH_4Cl, $CuCl_2$ and $CsCl_2$. The unit of enzyme activity was defined as mg of maltose liberated per 3 min at 25 °C.

Results and discussion. The effect of addition of different salts in the reation mixture consisting of exhaustively dialyzed substrate and enzyme has been thoroughly investigated by BERNFELD[4] and we avoided the repetition of the same. The probability of denaturation by exhaustive dialysis cannot also be ruled out, and in the present work both the substrate and the enzyme were taken undialyzed; *Tris* replaced phosphate buffer. It is noteworthy here that in our investigation the total Na^+ and Cl^- concentration present in the reaction mixture through reagents were calculated and found to be 1.8 and 0.8 microequivalents per reaction mixture, which are far below the anticipated activation level.

From Figure 1, it is seen that at pH 6.0 the influence of different anions in *Tris* buffer are comparatively less. At pH 7.0, all other anions except chloride showed decline in activity. However, from MYRBÄCK's observation[1] we find that the activity declined from pH 6.0 to 7.0 in phosphate buffer alone, whereas it increased for the same change of pH in presence of other anions. Since in our work, the particular anion is present independent of phosphate, namely *Tris*-aspartate, *Tris*-acetate, *Tris*-oxalate etc. the possible reason for the enhanced activity at pH 7.0 by different anions as suggested by MYRBÄCK and others[1,2] should not be attributed to the effect of particular ion but a combined effect of the same with phosphate. This is also supported by the fact that the reaction mixture containing phosphate buffer and chloride shows a little higher activity than the reaction mixture in *Tris*-hydrochloride (Figure 1, column 10 and 15).

The activity of salivary α-amylase observed by adding different salts of varying concentration to the reaction mixture are presented in Figure 2. It is evident from the figure that all the chloride salts tested, except cupric chloride, activated the enzyme almost to the same extent. These results are in perfect agreement with those of BERNFELD and others[1,2,4]. The presence of $CuCl_2$ inter-

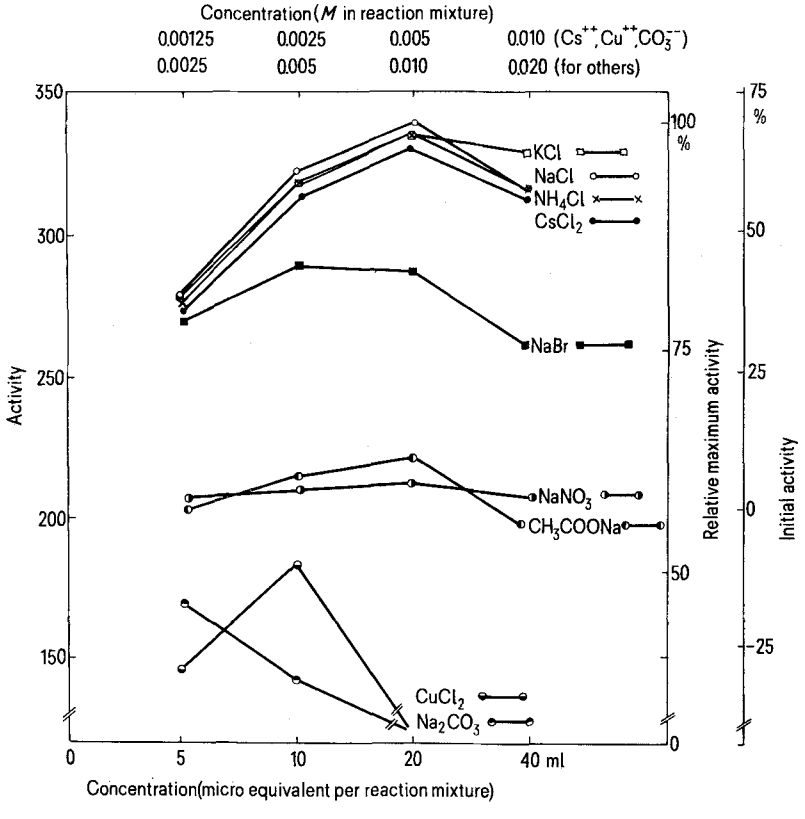

Fig. 2. Effect of different salts on salivary α-amylase.

fers with the reduction of 3,5-dinitrosalicylic acid, the chromogen by reducing sugars, product of amylolysis; and hence remains inconclusive. The size of the hydrated cation seems to be irrelevant, since $CsCl_2$ could activate the enzyme almost to the same degree as by sodium, potassium and ammonium chlorides. Sodium bromide activated the enzyme to some extent, while both $NaNO_3$ and CH_3COONa had no such action. The enzymic activity was inhibited by Na_2CO_3 as expected, since the pH of the reaction mixture was raised. These observations regarding bromide and acetate are also in agreement with the earlier findings. According to BERNFELD[4], NO_3^- exhibits some activation but we failed to get the same as MYRBÄCK and others.

In a recent report[5], perhaps the only one so far, sodium ion has been claimed to be the activator of pancreatic α-amylases of toad, reptile, pigeon and rat. Using NaCl, Na_2CO_3, KCl and $CuCl_2$ in phosphate buffer, they had shown that while NaCl and Na_2CO_3 activated the enzyme both $CuCl_2$ and KCl did not. It is already reported in the literature[8] that rat salivary and liver amylases are activated by KCl, as also confirmed by us. The failure to get any activation by $CuCl_2$ is rather to be expected and the reasons are discussed earlier. Unlike the findings of these authors, activation by Na_2CO_3 could not be confirmed in our laboratory since additions of Na_2CO_3, as low as 2.5 μmoles in the 2 ml of reaction mixture caused a change in pH. However, with *Tris*-carbonate buffer at pH 7.0 similar activity was obtained as with *Tris*-phosphate. It can be mentioned here that methodology adapted and concentrations of KCl, $CuCl_2$ and Na_2CO_3 used by us were the same as reported by them[5].

In view of the above, the following conclusions have been drawn. Chloride ion shows increased activation from pH 6.0 to 7.0 while most other anions including phosphate do the reverse. Activation by chloride ion is higher compared to other ions at both the pH values. The presence and size of the cation is irrelevant. Essentiality of Ca^{++} as anticipated through earlier investigation can evidently be ruled out as shown by the results of oxalate, acetate and aspartate in *Tris* buffer.

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The Oxidative Metabolism of α-Chlorohydrin and the Chemical Induction of Spermatocoeles

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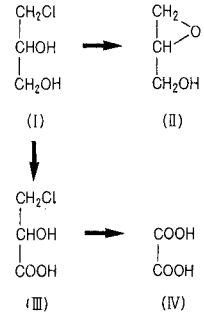
Summary. α-Chlorohydrin (I) is oxidatively metabolized to β-chlorolactic acid (III) and oxalic acid (IV). Deposition of calcium oxalate within the renal tubules is responsible for the toxic effects of α-chlorohydrin and a similar action on the epididymis or epididymal blood vessels could initiate the formation of spermatocoeles from this and other male antifertility agents.

The male antifertility agent[2] α-chlorohydrin (3-chloropropan-1,2-diol, I) has two effects on the male rat reproductive tract. Consecutive low daily doses (5 × 10 mg/kg) cause an immediate and reversible phase of infertility[3] by inhibiting sperm glycolysis[4], whereas a single high dose (100 mg/kg) produces epididymal lesions or spermatocoeles[5]. These lesions occlude the ductuli efferentes, block the passage of testicular sperm and produce prolonged or even permanent infertility. As the main metabolite[6] of α-chlorohydrin, the epoxide glycidol (2,3-epoxypropan-1-ol, II) has a similar antifertility action to the low dose regime of α-chlorohydrin[3], but does not induce spermatocoele formation, the possibility arose that α-chlorohydrin may be converted by another route to a metabolite responsible for the epididymal lesions.

Chromatograms of rat urine collected within 8 h of either oral or i.p. administration of ^{36}Cl-α-chlorohydrin (100 mg/kg) revealed the presence of 3 radioactive components. These were identified as Cl^-, unchanged α-



The metabolism of α-chlorohydrin in the Rat. β-chlorolactic acid (BCLA, III) was isolated from the acidified urine of male Wistar rats dosed with α-chlorohydrin (100 mg/kg), by continuous ether extraction and chromatography on a column of Merck silica gel G, elution with ethyl acetate: 40–60° petroleum ether (2:1) giving white plates, m.p. and mixed[20] m.p. 78–79°. Characterization was by reverse isotope dilution, mass spectroscopy {M^+ 125/127, base peak 79/81 $(M-H-CO_2)$} and GLC (retention time of a spiked sample 11.30 m on a 1 m × 3 mm i.d. 5% FFAP on 80–100 mesh Chromosorb G at 150° with N_2 inlet pressure of 14 psi). Urinary α-chlorohydrin (I) was identified as the *bis*-benzoyl derivative as previously described[6], chloride ion by the method of SEILER and KAFFENBERGER[21] and oxalic acid (IV) according to PARKE and WILLIAMS[22]. ^{36}Cl-BCLA and uniformy-labelled ^{14}C-BCLA were prepared from ^{36}Cl-α-chlorohydrin[6] and uniformly-labelled ^{14}C-α-chlorohydrin[23], respectively, by mild nitric acid oxidation[24]. Rf values (Merck pre-coated silica gel G TLC plates, 0.25 mm) were α-chlorohydrin (0.73), BCLA (0.30), calcium oxalate (0.10) and Cl^- (0.01–0.15) in chloroform:methanol (7:3).

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