The antifertility action of α-chlorohydrin: Enzyme inhibition by α-chlorohydrin phosphate

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Summary. Preparations of the enzymes glyceraldehyde-3-phosphate dehydrogenase and triosephosphate isomerase are shown to be inhibited by α-chlorohydrin phosphate **(II)** in a competitive and non-competitive manner, respectively. α-Chlorohydrin **(I)**, glycidol and epi-chlorohydrin have no inhibitory activities suggesting that their antifertility actions are due to their metabolism in vivo to α-chlorohydrin phosphate.

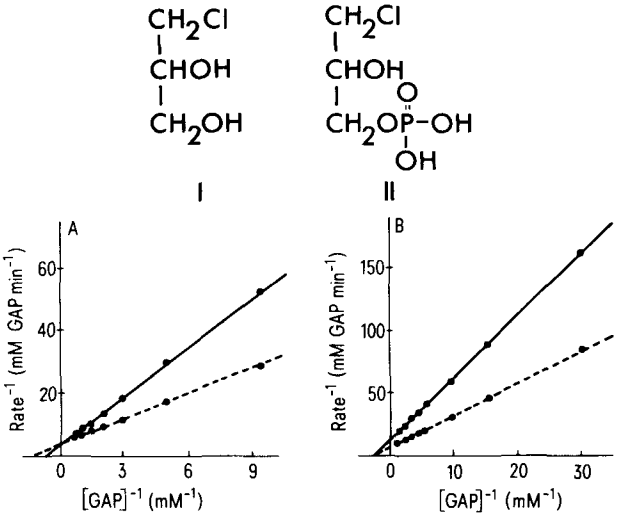
The immediate and reversible antifertility activity of α-chlorohydrin (3-chloropropan-1,2-diol, **I**) in a number of species of male animals has been well documented over the past decade[1]. At the cellular level, α-chlorohydrin has been shown to inhibit the 2 glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPdeH) and triose phosphate isomerase (TPI) in sonicates of ejaculated ram sperm, resulting in a decrease in the utilization of fructose and a lowering of the level of ATP[2]. As these inhibitory actions were apparent only after a period of pre-incubation of α-chlorohydrin with the sperm sonicate, it was proposed that α-chlorohydrin required conversion to a metabolite which acted as the true glycolytic inhibitor. The suggestion that a phosphorylated derivative, α-chlorohydrin phosphate (α-CP, **II**) was the inhibitory metabolite appeared correct as it was reported to have an immediate action on both of the enzymes in ram sperm sonicates[2]. As 2 subsequent communications[3,4] mention α-CP as the active form of α-chlorohydrin, but with no experimental details, we wish to report our observations on the nature and kinetics of the action of α-CP on pure preparations of these 2 enzymes.

There is no effect of α-chlorohydrin on the pure enzymes GAPdeH and TPI even after long periods of pre-incubation. This confirms that the effects seen on these enzymes in sperm sonicates must be due to a metabolite of α-chlorohydrin. When α-CP is added[5] to the reaction cuvette containing GAPdeH and its substrate, inhibition is immediate and occurs to extents of 16, 52 and 76% at inhibitor concentrations of 20, 75 and 150 mM respectively. A double reciprocal plot of the inhibition, compared to glycerol controls (figure, A) shows that α-CP acts by competitive inhibition. Similarly, TPI is immediately inhibited by α-CP to extents of 26, 45 and 50% at inhibitor concentrations of 20, 75 and 150 mM respectively. A double reciprocal plot of this inhibition, again compared to glycerol controls, reveals that the action is one of non-competitive inhibition (figure, B). Further confirmation of the classification of inhibition was obtained by dialysis of the inhibited enzymes; whereas the activity of GAPdeH was restored, that of TPI remained inhibited.

That the source of these pure enzymes was rabbit muscle[6], and knowing that α-chlorohydrin has no antifertility activity in this species[7,8], it was necessary to determine whether α-CP inhibited the enzymes in the sperm of a susceptible species. As the boar is susceptible to the antifertility action of α-chlorohydrin in vivo[9] and in vitro[10], the enzymes were isolated from mature boar sperm[11]. When the purified sperm enzymes were adjusted in concentration to activites analogous to those of the rabbit muscle preparations, the degree and type of inhibitions observed with α-CP were identical, α-chlorohydrin itself having no inhibitory activity.

These results, together with those published[1], enables an overall mechanism of action for α-chlorohydrin to be postulated. α-Chlorohydrin is widely distributed after administration[13] and gains access to mature caudal sperm[14]. Since α-chlorohydrin is a competitive inhibitor of glycerol kinase[15], it could be converted to α-CP by this enzyme which would explain the observation that the presence of glycerol protects sperm from the action of α-chlorohydrin in vitro[16]. The α-CP would competitively inhibit GAPdeH and non-competitively inhibit TPI to reduce the rate of glycolysis[17]. This would lower the production of ATP[19,20] to such an extent that fertilization could not be successful. Furthermore, the recent finding[21] that the S(−)-isomer of α-chlorohydrin produces the antifertility and antiglycolytic effects, whereas the R(+)-isomer is inactive, is added evidence towards a stereospecific involvement of enzymes and indicates that the active male antifertility agent is S(−)-α-CP.

Both epi-chlorohydrin (1-chloro-2,3-epoxypropanol) and glycidol (2,3-epoxypropan-1-ol), compounds related to the structure of α-chlorohydrin and possessing similar types of antifertility activity[22], have no effect on GAPdeH or TPI isolated either from rabbit muscle or boar sperm. This would appear to confirm that their biological actions are due to their metabolic conversion in vivo to α-chlorohydrin[23] and, consequently, to α-CP. The actions of other antifertility agents on glycolytic and other enzymes is at present under investigation.



Typical double reciprocal plots of the inhibitory action of α-chlorohydrin phosphate (α-CP) at 35 °C on glyceraldehyde-3-phosphate dehydrogenase (A) and triose phosphate isomerase (B) isolated either from rabbit muscle or boar sperm. The inhibitions shown by α-CP (-●-●-●-●-) are compared to glycerol controls (-●- -●- -●-) at concentrations of 67 mM, specific activities of the enzymes being 1.33×10^{-4} M GAP min^{-1} mg $protein^{-1}$ for GAPdeH and 1.61×10^{-3} M GAP min^{-1} mg $protein^{-1}$ for TPI, where GAP = glyceraldehyde-3-phosphate. The GAPdeH assays were performed in a glycine-hydrazine buffer at pH 9 according to Velick[12] and the TPI assays at pH 7.5 in triethanolamine buffer by the method of Beisenherz[24]. The coupling enzyme in the TPI assay, glycerophosphate dehydrogenase, is inhibited by α-CP but only at very high concentrations and in a competitive manner so that the observed action in this assay is directed at TPI. Dialyses were performed on enzyme solutions of activity 36 units ml^{-1} in the presence of 90 mM α-CP. An initial 3 h dialysis at 4 °C against distilled water was followed by a 15 h period before assay.

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Partial purification and some properties of a nucleoside phosphotransferase of chick embryos

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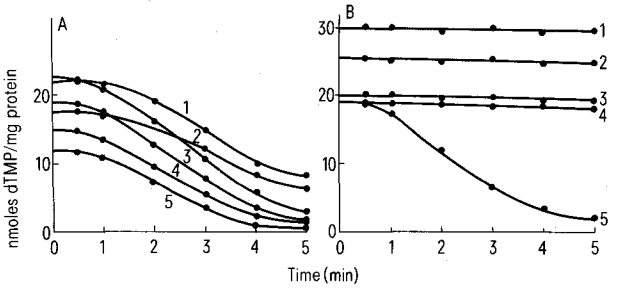
Summary. A nucleoside phosphotransferase purified about 40fold from chick embryos utilizes efficiently as phosphate donors deoxyribonucleoside and pyrimidine ribonucleoside monophosphates, whereas the pyrimidine deoxyribonucleoside appear to be the preferred acceptors of phosphate. The enzyme is very unstable to heat, dilution and dialysis. A marked enhancement in the stability is caused by nucleotides and it seems associated with the formation of an aggregated state of the protein.

Previously[1] we have found in the retina of the chick embryos 2 different non-specific forms of nucleoside phosphotransferase which are able to phosphorylate thymidine, and we have hypothesized that these forms could be an expression of the same enzyme at different aggregation states. We describe now some properties of a nucleoside phosphotransferase activity purified about 40fold from 12-day-old chick embryos.

Methods. 50 chick embryos were homogenized with 300 ml of 5 mM tris-HCl buffer pH 8.0. The homogenate was centrifuged at 105,000 × g for 30 min and the supernatant was collected. Protamine step was performed by 2 successive additions of 1% protamine sulfate to this supernatant. At first 6.25 ml/g of protein were added and the resulting mixture was stirred and centrifuged, and the precipitate discarded. Successively a new aliquot of 1% protamine sulfate (16.6 ml/g of protein) was added and the precipitate was homogenized with 50 ml of 0.2 M KH_2PO_4, pH 8.0 (protamine I). This homogenate was centrifuged and the supernatant collected. Proteins were then precipitated by solid ammonium sulfate to 30%, resuspended in 40 ml of 5 mM tris-HCl pH 8.0 and dialyzed for 12 h at 4 °C against 400 ml of the same buffer. After dialysis, 4 ml of 1% protamine sulfate were added and the precipitate was homogenized with 20 ml of 0.2 M KH_2PO_4 (protamine II). After centrifugation, solid ammonium sulfate to 50% saturation was added to supernatant. The precipitate obtained was resuspended in 10 ml of tris-HCl pH 8.0 and dialyzed for 12 h at 4 °C against 50 ml of the same buffer. This dialyzate (ammonium sulfate II) was generally utilized for the incubation samples.

The standard reaction mixture contained, in a final volume of 500 µl, 40 mM tris-HCl buffer pH 8.8; 20 µM (0.5 µCi) (Me-^{3}H)thymidine; 5 mM nucleotide (phosphate donor); 10 µl of enzyme or 50 µl of homogenate or 105,000 × g supernatant. After incubation at 37 °C for 30 min, the reaction was stopped by addition of 0.2 ml of 10% trichloroacetic acid. The nucleoside phosphotransferase activity was evaluated as previously reported[1]. Protein was estimat-



Stability of nucleoside phosphotransferase at 37 °C. *A* Stability in relation to the phosphate donor employed. For each sample about 90 µg of purified enzyme were preincubated at 37 °C in a volume of 0.3 ml with 2.5 µmoles of $MgCl_2$ and 20 µmoles of tris-HCl buffer pH 8.8. At the time intervals indicated, 10 nmoles were (0.5 µCi) of (Me-^{3}H) thymidine and 2.5 µmoles of phosphate donor were added and the incubation was carried out at 37 °C for 30 min in a final volume of 0.5 ml (1, d-UMP; 2, d-TMP; 3, d-AMP; 4, UMP; 5, d-GMP; 6, CMP). *B* Protective effects of various nucleotides. The preincubation mixture as in *A*, except that a nucleotide protector was added. At the indicated time intervals, 10 nmoles (0.5 µCi) of (Me-^{3}H) thymidine and 2.5 µmoles of UMP were added and the incubation was carried out at 37 °C for 30 min (1, addition of 1 nmole of d-TTP; 2, 1 nmole of UDP; 3, 2 nmoles of d-UMP; 4, 2 nmoles of d-TMP; 5, no addition).