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SHORT COMMUNICATIONS

Mixed disulphides in liver extracts

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IN A previous paper[1] it was shown that a ninhydrin-positive sulphur-containing peptide (known as thiol peptide X) appeared in electropherograms of extracts of livers of male albino rats which had been injected intraperitoneally with hepatocarcinogens such as 3′-methyl-4-dimethylaminoazobenzene (3′-MeDAB). The material was found in traces or not at all in extracts of livers of control rats. An apparently similar material occurred in extracts of normal male guinea pig liver. Acid hydrolysates of peptide X from 3′-MeDAB rat liver and from normal guinea pig liver appeared to contain only glutamic acid, cyst(e)ine (detected on chromatograms as cysteic acid) and glycine. Addition of the thiol reagent, N-ethylmaleimide (NEM), to liver extracts prior to electrophoresis prevented the appearance of X and glutathione (GSH) in the electropherograms. Thus X seemed to be a thiol peptide closely related to GSH, but attempts to isolate the thiol as its cuprous mercaptide failed. Only GSH could be recovered when the mercaptide precipitation procedure was applied to livers of 3′-MeDAB-treated rats or normal guinea pigs.

The possibility of producing the peptide *in vitro* by incubating GSH with homogenates of various tissues has now been examined. These experiments led to the recognition that guinea pig liver peptide X is not a thiol peptide but is most probably the mixed disulphide of GSH and cysteine (CySH). Similarly the X peptide detected in liver extracts of 3′-MeDAB-treated rats may also be a mixed disulphide with GSH as one of the components. It is less certain in this case that the other component is cysteine.

EXPERIMENTAL

Glutathione-tissue incubation experiments

A substance with the properties of peptide X was obtained by incubating at 38° under nitrogen a solution of GSH (6·1 mg) in borate-KCl buffer (1 ml H_2O + 2 ml borate–KCl buffer[2] nominal pH 8·6, actual pH 6·5–7·0) with supernatant (0·1 ml) of normal rat kidney homogenate (1 g of kidney homogenised in ice-cold deionised water, diluted to 10 ml with water and centrifuged for 10 min at 3000 rev/min). Samples of incubation mixture (0·5 ml) were removed at intervals, mixed with 2 ml Sorensen phosphate buffer, pH 7·2, and 0·01 ml aliquots of the dilutions examined electrophoretically in barbitone buffer (pH 8·6) on Whatman No. 1 paper for 3 hr at 350 V as already described.[1] Ninhydrin-positive material with the same mobility as peptide X appeared after about 30 min incubation attained its maximum concentration after 1 to 1½ hr and then gradually disappeared until after 3 to 4 hr, only glycine, glutamic acid and cysteine could be detected in the incubation mixture.

In attempts to modify the GSH-rat kidney incubation mixture so as to produce larger amounts of peptide X, we tested the effect of adding various reaction products such as glycine, glutamic acid and L-cysteine to the incubation mixture, adjusting the pH of the incubation mixture to 7·0 when necessary. Addition of L-cysteine led to greatly increased amounts of X. However, control experiments showed that the formation of this extra product had nothing to do with enzyme activity since appreciable amounts of an X-like substance could be detected in electropherograms of a freshly prepared mixture of GSH and CySH at pH 7 to which no kidney extract had been added.

Toennies's nitroprusside reagent[3] revealed that none of the three ninhydrin-positive spots gave the characteristic red colouration for thiol groups until the paper had been treated with sodium cyanide. Two of the spots are known to be due to CySSCy and GSSG. The X spot must be due to the mixed disulphide of GSH and CySH, namely GSSCy. This substance has in fact been detected by Livermore and Muecke[4] in chromatograms of a phosphate (pH 7) solution containing GSH and CySH.

GSSCy however cannot be present as such in fresh phosphate solutions of GSH and CySH,

otherwise traces of it should be detectable by ninhydrin at position X after electrophoresis of the thiol mixture to which NEM has been added. This was not the case.

When solutions containing oxidised glutathione (GSSG) and CySH or cystine (CySSCy) and GSH at pH 7 are examined electrophoretically, X spots as well as CySSCy and GSSG spots were obtained which could not be entirely removed by the addition of NEM. These residues must represent GSSCy, CySSCy and GSSG which have formed in the phosphate solution of disulphide plus thiol prior to electrophoresis. Electrophoresis of a mixture of the two disulphides GSSG and CySSCy generated no GSSCy, nor were the intensities of ninhydrin-positive spots due to the disulphides affected by the addition of NEM. Thus the X product arising from GSH–CySH–pH 7 solution is apparently the artifact GSSCy which is formed together with GSSG and CySSCy when the solution is placed on the barbitone-saturated paper (pH 8·6) at the start of electrophoresis.

The X spot found in GSH–rat kidney incubation mixtures can be considered to be due to GSSCy. During hydrolysis of GSH, CySH is formed and at a certain time sufficient CySH and GSH will be present in the incubation mixture to form a maximal amount of artifact GSSCy. As destruction of GSH continues, the amount of artifact will diminish until finally it disappears altogether. However the X substance found in these mixtures may not be due solely to GSSCy. Thus a phosphate solution of synthetic γ-L-glutamylcysteine and L-cysteine gave rise on electrophoresis to a mixed disulphide glucySSCy which had the same mobility as GSSCy. γ-L-glutamylcysteine by itself had the same mobility as glutamic acid. This dipeptide is probably formed during hydrolysis of GSH by rat kidney supernatant. At any rate, a disulphide-reactive spot has been detected among the hydrolysis products in the region of the spot due to glutamic acid.

Nature of guinea-pig liver peptide X

This peptide is most probably the mixed disulphide GSSCy. An appreciable amount of cyst(e)ine (as a typical pair of crimson and orange–brown ninhydrin spots) is easily detected in chromatograms of phosphate (pH 7·2) extracts of guinea-pig liver. Since this extract also contains much GSH, conditions are favourable for the appearance of a large X spot due to GSSCy in electropherograms. Neubeck and Smythe[5] noted that the GSH level in normal guinea-pig liver declined rapidly from the time the tissue is removed from the animals. As GSH disappeared, cyst(e)ine accumulated.

Nature of peptide X in the livers of rats injected with 3′-MeDAB

In spite of the close similarity between this peptide and the one found in guinea-pig liver extracts, we do not think that it is identical with GSSCy although it is probably a closely related mixed disulphide. Thus we have been unable to detect cyst(e)ine in chromatograms of extracts of either normal of 3′-MeDAB-treated rat liver, or in autoradiographs of similar chromatograms prepared from extracts of the livers of normal and 3′-MeDAB-treated rats injected with small amounts of DL-cystine ^{35}S. On the other hand, Plaquet *et al.*[6] have stated that alcoholic extracts of normal rat liver contain GSSCy as well as CySH, GSSG and another unidentified disulphide.

Smythe[7] has noted that, in contrast to guinea-pig liver, rat liver shows no tendency for its GSH to be converted to cysteine. Moreover, he found that normal rat liver contains 100 times more of a cysteine-degrading enzyme than does guinea pig liver. It is not surprising therefore that CySH and GSSCy can be detected readily in extracts of guinea pig liver but not in extracts of normal rat liver.

That nature and mode of formation of peptide X in 3′MeDAB-treated rat liver extracts is being investigated further.

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