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THE EFFECT OF SULFUR MUSTARD ON GLUCOSE PHOSPHORYLATING ENZYMES AND LIVER CELLS IN MALE RATS

M. Fazilati, M. Taghikhani and G. H. Riazi

Institute of Biochemistry and Biophysics, University of Tehran, P. O. Box 13145-1384 Tehran, Islamic Republic of Iran

Abstract

A significant decrease of hepatic high-Km glucose- phosphorylating enzyme glucokinase (ATP- D- glucose 6- phosphotransferase, EC 2.7. 1.2, GK) and low-Km hexokinases (EC 2.7. 1.1. HK) activities were noticed in liver treated with 2, 2'-dichlorodiethyl sulfide (sulfur mustard, HD) when injected interaperitoneally to male rats in 1/4 LD50. This decrease occurred 1.5 to 5.5 hours after the injection. Simultaneously, a significant decrease in concentration of soluble protein and glycogen was also noted after 2 hours. In addition some morphological changes on liver cells were noticed.

Introduction

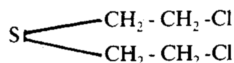
The circumstances concomitant to the HD, cause of a loss of enzyme activity, concentration of soluble protein, glycogen and morphological changes in time function, have not been previously described.

HD is a radiomimetic alkylating agent that has mutagenic [1, 2] carcinogenic [3, 4, 5, 6] and a powerful cytotoxic effect [3, 4, 7]. The effect of the compound on the body tissue and its enzymatic importance was studied. HD* is a stable compound and also a powerful vesicant that produces incapacitating injuries at the site of exposure [8, 9, 10].

In spite of 60 years of intensive research, the mechanism of action of HD is still not understood and no effective treatment for preventing HD induced lesions is available.

We have proposed a new and unique biochemical hypothesis, a mechanism that links enzymes and protein to liver cell damage. In order to validate the portions of this biochemical hypothesis linking to a decrease in enzyme activity, the concentrations of glycogen and soluble protein were studied.

* Formula for Sulfur Mustard, HD



Key Words: Hexokinase, Glucokinase, Sulfur mustard

In this paper it has been demonstrated that sulfur mustard causes a decrease in the activity of glucose phosphorylating enzymes, diminishes the concentration of glycogen and soluble protein and some histological variations are observed after injection into male rat liver.

Materials and Methods

Chemicals, coenzymes, nucleotides, triethanolamine, hydrochloride, sucrose, EDTA, glycerol were purchased from Aldrich Ch. Co., and HD was obtained from the Bio-organic Lab, at Tehran University.

Glucose 6- phosphate dehydrogenase (D- glucose 6-phosphate- $NADP^+$ Oxidoreductase EC, 1. 1. 1. 49 G6 PDH, from yeast, type VII (345, units/ mg)) was purchased from Sigma Chemical Co. All other chemicals were supplied by BDH chemicals pool, Dorset, U.K.

The rats used in this investigation were all healthy male Wister rats ranging from four to six weeks of age weighing 240-290 g. HD was administered interaperitonealy (I. P.) as a solution of propylene glycol in a dose of 1/4 LD50 [11]. The volume of the injected solution was 0.25 ml/kg body weight. Groups of animals (60, at least three for each case) were sacrificed at 1 hr, 2 hrs, 2.5 hrs, 5.5 hrs, 16 hrs, 24 hrs, 49 hrs, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 11 days and 12 days, post administration.

The collected livers were placed in ice, weighed and 20% (W/V) homogenates were prepared in 100 mM triethanolamine hydrochloride, 1.25 M sucose, 2.5 mM EDTA and 10% glycerol, using a glass potter Elvehjem homogenizer for three min. in 1500 stroke. The method was based on *Parry* and *Walker's* [12].

The homogenates were then centrifuged at 40,000 rpm for 60 min at 4°C in a L 5-50 Beckman Ultralcentrifuge. The clear supernatant was collected by Pasteur Pipette. Glucokinase activity was measured at 32°C in a total volume of 0.75 ml by the coupled assay of *Parry* and *Walker* [12] as described by *Storer* and *Cornish-Bowden* [13].

Low-Km Hexokinase activities were estimated in the presence or absence of GK [14].

G6PDH activity was also measured by the increase in E340 as $NADP^+$ was reduced in a total volume of 0.75 ml containing the same reagents as used for the GK assay except that the glucose, ATP and glucose 6-phosphate dehydrogenase were replaced by 20 mM glucose 6- phosphate.

Enzyme activity was calculated on the basis of (per g whole liver and per mg of supernatant protein), but these data have not been included, because no additional calculation can be made. Results are given as means ± S. E. M. for three animals per experiment.

One unit of enzyme activity is defined as that which catalyses the formation of 1μ mol of either glucose 6-phosphate/ min (GK, HK's) or 6- phosphogluconate (G6PDH) at 32°C.

Glycogen concentration in supernatant was measured using the filter paper technique [15] on samples of liver homogenate (10%) which was made in 0.03 NHCL [16].

Protein concentration in the supernatant was determined by the Tannin micro method [17], using tannin reagent and gum arabic in warm water.

For morphological studies a piece of normal and treated rat liver was fixed in 10% formaline and sections (6μ) of paraffin embedded tissues were stained with haematoxylin and eosin for histological study.

Since HD is a powerful vesicating agent in addition to its mutogenic, carcinogenic and cytotoxic actions, all experiments were performed in strict adherence to local safety regulations concerning the handling of carcinogens and other hazardous materials.

Results

Rats of 4-6 weeks of age were used for most of our experiments. At this age the rat is physically much more mature than a few weeks earlier. It should be noted therefore that in all in experiments reported, the normal control animal has maximum glucose phospholylating enzyme activity.

The effect of HD on enzyme activity

The time course of glucose phosphorylating enzyme activity in HD treated rat liver is shown in figures 1 and 2. The maximum decrease of liver GK activity was observed 2 hours post administration. This is comparable to the activity of control GK which is 1.78 unit/g liver.

The maximum level of decreased low-Km hexokinase activity was reached 1.5 hrs post administration (Fig 2). Total HK activity of controls were 0.83 unit/g of liver.

This difference between glucose phosphorylating enzyme activity in Fig 1 and 2 is due to the predominant GK in the liver which is accompanied by an increase in activity of up to three other low-Km hexokinases [18, 19].

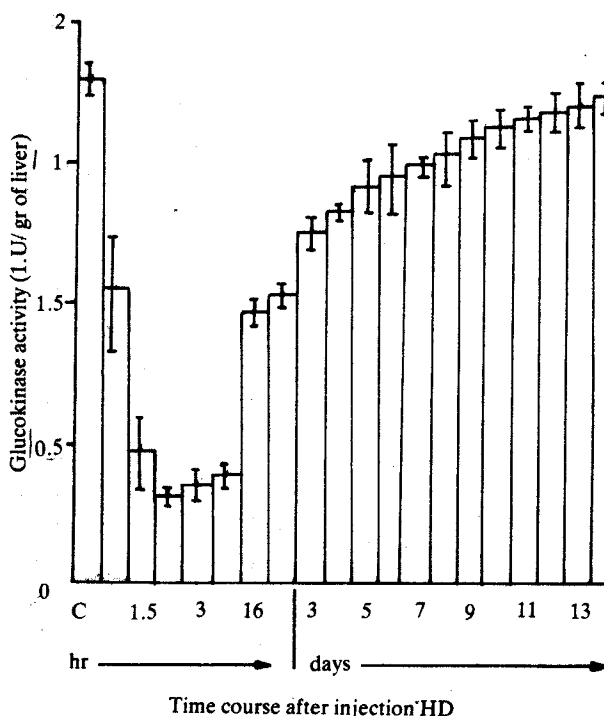


Fig. 1- Variation in GK activity in HD treated male rat liver.

However, enzyme activity in the animals treated with 0.14 mg/Kg body weight as mentioned above were decreased during the first hours (Fig 1, 2). During the ensuing 2 hrs of treatment, the liver showed a remarkable decrease of glucose phosphorylating enzyme activity, when compared to the control activity levels (Fig 1, 2).

The fall of enzyme activity occurs immediately after treatment. The time for the determination of enzyme activity seems to be sufficient, and the reaction between HD and enzyme will occur immediately, resulting in the inactivation of the enzyme.

Concentration of soluble protein and glycogen is dependent on the decrease in enzyme level

Figures 3 and 4 show the glycogen concentration and soluble protein in HD rat liver treatment. Protein and glycogen levels decreased significantly, 45% at the same time after treated with HD (Fig. 3, 4).

The concentration of protein and glycogen concentration decreased 40% (Fig. 4) of control and remained at this level similar to enzyme levels (Fig. 1, 2).

The maximum decrease in glycogen, protein concentration occurred between 1.5 hrs and 3 hrs after HD administration. The amount of glycogen concentration in normal rats was about 72 g/mg of liver. This is in comparision with 40 g/mg of glycogen after treatment with HD after 1.5 hrs which is 50% of the normal amount. Also the decrease in protein concentration, 1.5 hrs after the injection will reach its amount with the ratio of 45 to 85 mg/gr liver is about 53%.

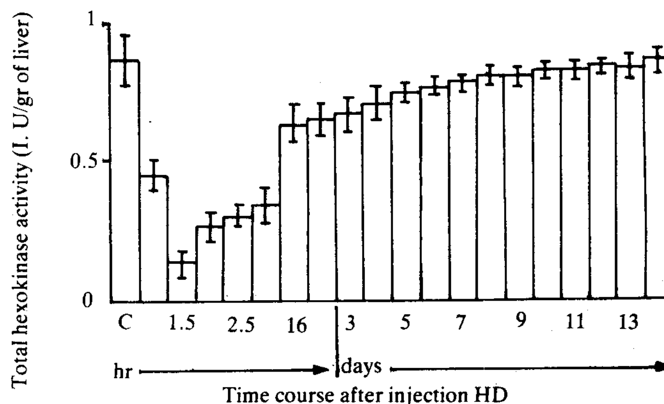


Fig. 2- Effect of HD on total HK activity following a single interaperitoneal injection of 0.14 mg/Kg body weight in male rat liver.

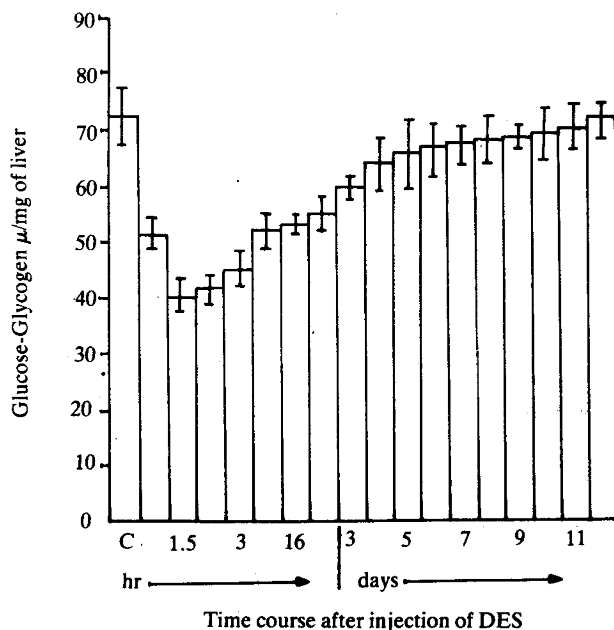


Fig. 3 Effect of HD upon rat male liver, glycogen concentration following interaperitoneal injection 0.25 ml/Kg body weight.

The recovery takes place after thirteen days in the case of GK, HK's and twelve days in the case of glycogen and ten days in the case of protein. However, the difference in the number of days is not significant.

The decrease in glucose phosphorylating enzyme activity could not be explained by reduction of protein and glycogen concentration after treatment by sublethal doses of HD. It is possible that the drop in enzyme activity is the result of alkylating HD with fractions mentioned before (Fig. 1, 2).

The effect of HD on liver cells

The relationship of HD on enzyme levels and concentration of protein and glycogen, apparently has similar destructive effects on liver cell morphology.

The morphological appearance of the liver cell at all times after administration of HD, e. g between 1 hr to 24 hrs, shows some variation.

Fig. 5 shows after 1.5 hr after interaperitioneal injection of HD in male rat, swelling, shrinkage of cell membrane, nucleus, and some vacuelear in the nucleus and cytoplasm. Also in figures 6, 7, 8 and 9 after 2, 3, 5.5 and 16 hrs cell death or necrosis and alkylation in chromatin was realized.

Recovery of morphological change in rat liver cells may occur after 10 days of administration of HD (Fig. 10).

Discussion

Alkylating compounds such as HD react with the cellular components randomly. Alkylation of proteins in water occurs on carboxyl amino sulfydril and imidazole groups. The process depends on the dissociation constant (k) of the reacting groups [20, 21]. The site of alkylation in DNA double helix and RNA molecules in nitrogen at position 7 of guanine [22, 23]. HD was showed to affect the levels and activity of a wide variety of enzymes in many tissues in a wide range of organisms.

On the other hand, the relationship between the inhibition of glycolytic pathway, cell division, inhibition of DNA synthesis, the ability of these agents to cross link DNA and their mutagenic effects were not fully appreciated up until 1962, although the involvement of DNA in mutagenesis had been suggested [24]. The dynamics of activity changes and recognition of glucose phosphorylating enzyme activity, and amount of glycogen and protein concentration from the liver of rats depend presumably on several mechanisms. The decrease in high, low Km hexokinase activity is probably due to the alkylation of sulfydrill group by HD [25].

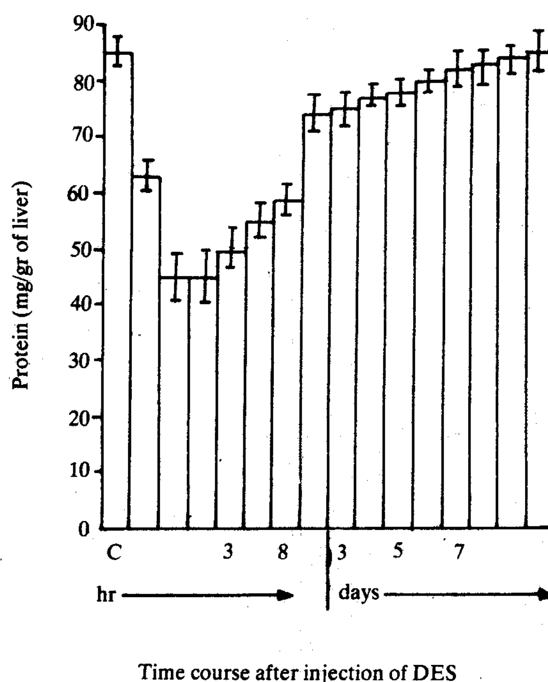


Fig. 4- Variation in protein concentration in HD treated male rat liver.

* Dichloro di Ethyl Sulfide

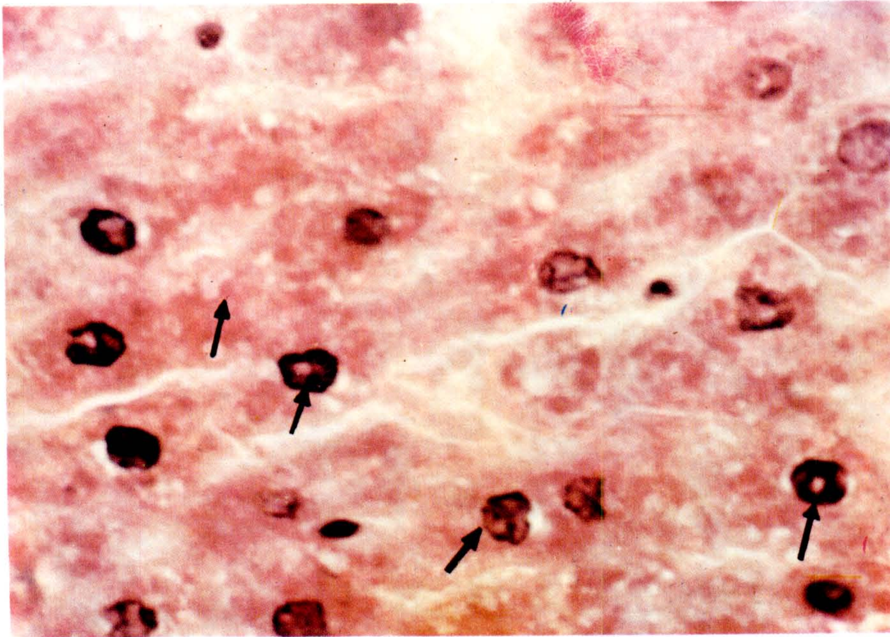


Fig. 5 Microscopic view of rat liver cells 1.5 hrs after interapreritoneal injection of HD Some of the liver cells show degenerative changes (⟶). Pyknosis (⟶) and intense eosinophilic of the liver cells are present. (H & E x 40).

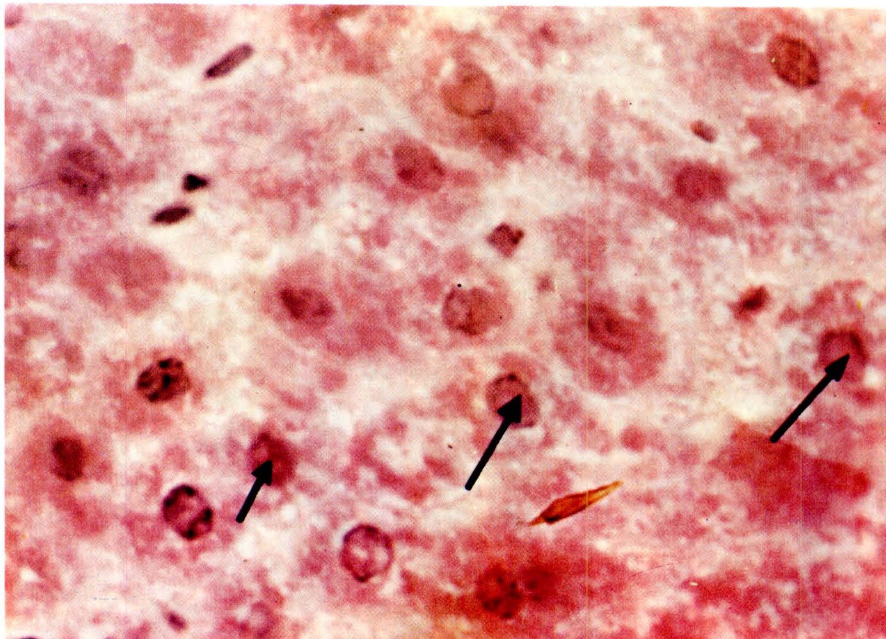


Fig. 6 Microscopic appearence of rat liver cells 2 hrs after I.P. injection of sulfur mustard in addition to microscopic changes designed for Fig. 5, accumulation of interavaculear eosinophilic proteinous materials (⟶) and margination of chromatin could be seen. (H & E x 40).

It seems that most, if not all, of these enzymes have SH-groups. [26], which is readily inactivated by oxidation and reactivated by cysteine and is sensitive to HD. This is in agreement with observations that the reactions of HD with SH-groups in proteins are faster than with other groups such as amine and carboxyl groups [27]. However, among the highly sensitive enzymes were hexokinase, pyruvate kinase, pyruvate phosphokin-

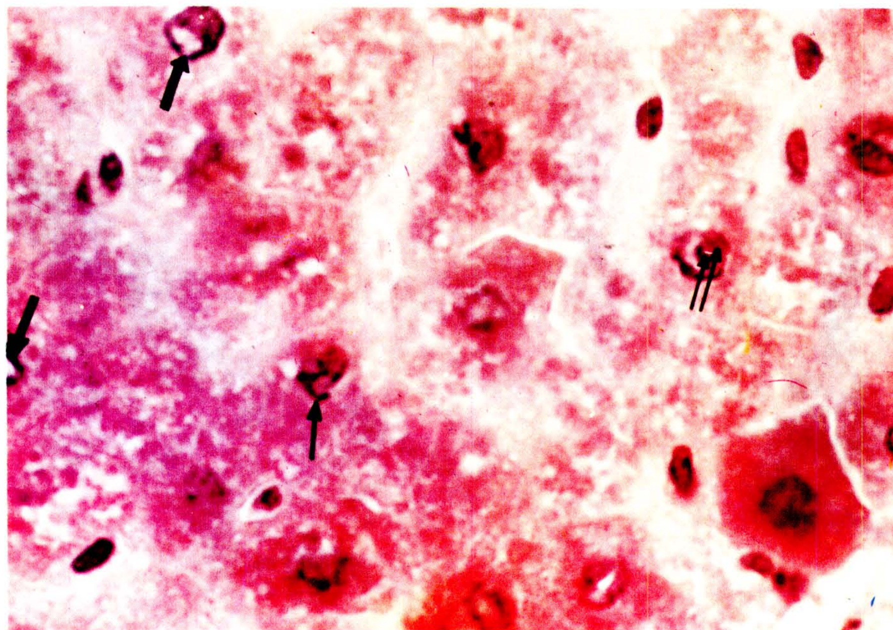


Fig. 7- Rat liver cells 3 hrs after I.P. injection of HD. Clouding, swelling, eosinophilic of cytoplasm, pyknosis (——►) enlargement of nucleolus (——►) and margination of nuclear chromatin (——►) of liver cells are present. (H & E x 40).

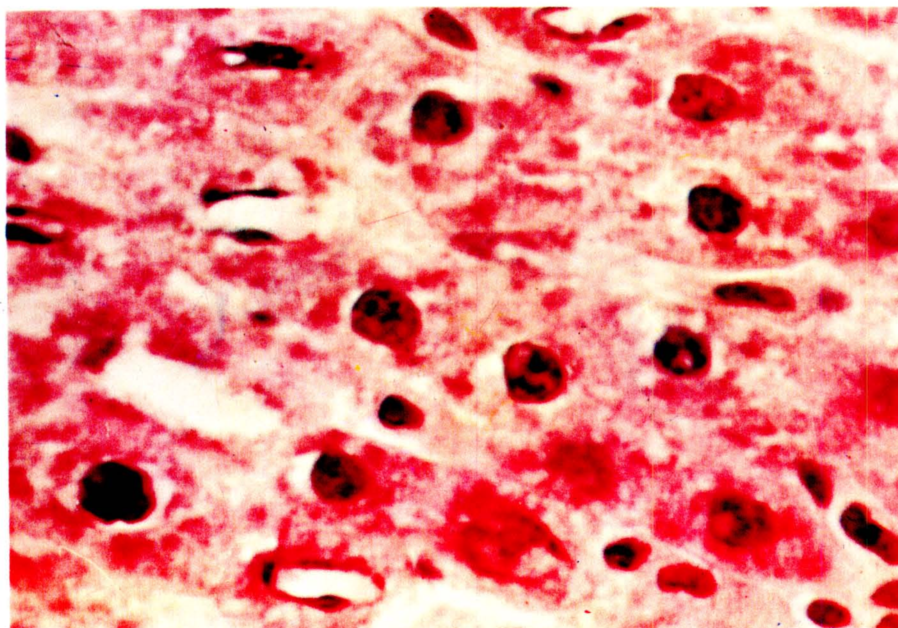


Fig. 8- Microscopic view of rat liver cell 5.5 hrs after I.P. injection HD. The changes are much less than figures 5, 6, 7 (H & Ex 40).

ase, inorganic pyrophosphatase, adenylic acid deaminase, chicken pepsins, kidney pepsinase, and peptidases of serum and skin epithelial cells [28].

It is also possible that another cause of the decrease in glucose phosphorylating enzyme activity may be the loss of glycogen and protein concentration of liver cells

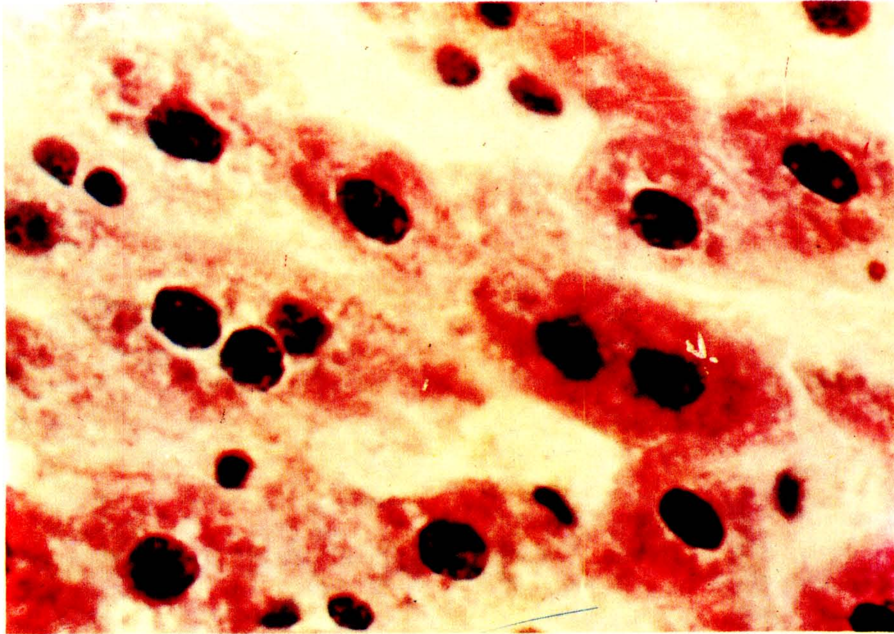


Fig. 9 Rat liver 16 hrs after I.P. injection of HD. The liver cells appear to be normal, probably due to the process of cellular regeneration and detoxification of HD (H & E × 40).

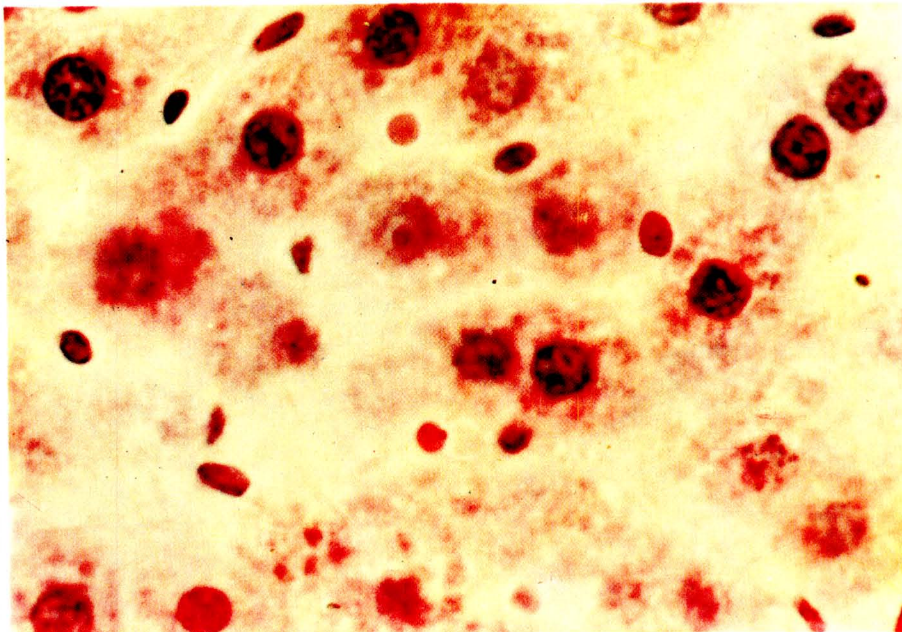


Fig. 10- Microscopic appearance of rat liver cells 10 days after I.P. injection of HD. The liver cells have returned to their normal cellular characteristics. (H & E x 40).

being damaged by HD. The last of the detectable mechanisms concomitant to that mentioned above is the temporary inhibition of GK, HK's, protein synthesis. Futhermore, there is a possible relationship between the decrease of GK, and HK's, activities and the inhibition of the enzymes of glycogen and protein synthesis.

Finally maximum decreases occurred between 1.5 hrs and 5.5 hrs, post administration which is similar to maximum binding of HD to kindney cytoplasmic proteins (2 hrs and 5.5 hrs) and liver cytoplasmic maximum binding occurred before 2 hrs [29]. The histological appearance of the liver at all times after administration of HD also supports the above findings.

Microscopic examination revealed some morphological changes in the liver cells (swelling, shrinkage, disappearance of chromatin). There was also the appearance of nuclear damage in chromatin of HD treated cells which precedes cytoplasmic damage and necrosis.

These studies are consistent with the initial steps of the effect of HD on glucokinase activity, and decrease of protein and glycogen concentration and change in liver cells morphology. Other studies show that the interaction of sulfur mustard, nitrogen mustard on these parameters [30, 31].

The present results may demonstrate that an alkylating agent of HD is capable of reacting covalently with glucose phosphorylating enzyme, glycogen and proteins to an extent similar to a known hepatocarcinogen and liver toxin, but apparently have similar destructive effects on liver morphology.

In conclusion, the ability to prevent enzyme loss even 1.5 hrs, after HD exposure may be in the interaction to cell and enzyme. The elucidation of responsible mechanisms must wait until definitive studies are carried out in isolated cell hepatocytes preparations.

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References

1. Auebach. C and Robson, *J. M. Nature*, **157**. 302, (1946).
2. Gilbert, R. M. Rowland, S. Davision, C. L. and Papirmeister. *B Mutat. Res*, **28**, 257, (1975).
3. Wheeler, G. P. *Cancer. Rev.*, **22**, 651, (1962).
4. Fox. M and Scott., D. Mutat. Res,. **75**, 131, (1980).
5. Hoston, W. E. *J. Natl. Canc. Inst.* **11**, 415, (1950).
6. Wadd, S. Nishimoto. Y, Miyanishi. M. Kambe, S and Miller, R. M. *Lancet*, **1**, 1161, (1968).
7. Papirmeister, B and Davison, C. L. *Biochem Biophys. Acta*, **103**, 70, (1965).
8. Andrezjewski, S and Scianowski, J. Bulletin Delacà demie polonais des sciences Vol. XXIII, 773, (1975).
9. Warthin, S. W and Weller, C. V., The Medical Aspecto of Mustard GJS poisoning, Mosby, St. Louis, Mo, (1919).
10. Voft,. R. F. Djnnenberg, Jr. Schofield,. BH. Hynes, H. A. and Papirmeister, P. Fundam Appl. Toxicol 4 (2), 571, (1984).
11. Aslow. W. P, Karnofsky, D. A. Jager, B. V. and Smith, H. W. *J. Pharm. Exp. Ther*, **93**, 1, (1948).
12. Parry. M. J. and Walker, D. G. *Biochem. J.* **99**, 266, (1966).
13. Storer, A. C. and Cornish-Bowdem, A., *Biochem. J.* **141**, 205 (1974).
14. Holroyde, J. J. Chesher, M. E. Trayer, I. P., and Walker, D. G. *Biochem, J.* **153**, 351, (1975).
15. Timothy. M. C, and John, Exton, H. *Biochem. Biophys. Acta*, **71**, 96, (1976).
16. Passonneau, J. V. and Lauderdale, V. R, 60: 405, (1974).
17. Katzenellenbogen, W. M. and Dobryzycha, W. M. Clinica, *Clinica Acta*, 05: 515, (1959).
18. Bonney, R. J. Walker, P. R. and Potter, P. R. *Biochem. J*, **136**: 947, (1973).
19. Sapay-Hagar, M. R. and Marcoo, A. S. *Febs Lett*, **3**, 68, (1969).
20. Djvis, S. Band Ross, F. W. *J. Amer. Chem. Soc.* **69**, 1177, (1947).
21. Moore, S. Stein, W. H. and Fruton, J. S. *J. Org. Chem.* **11**: 675, (1946).
22. Lawley, O. D, *Biochem. Biophys. Acta.* **26**: 450, (1957).
23. Trams. E. G. Vadkarni, N. V. and Smith, P. K. *Cancer. Res.* **21**: 560, (1961).
24. Loveless. A. and Stock, J. C. Proc. Roy. Soc. London, S. B. 150: 423, (1959).
25. Dixon. W. Report, No. 27, (1944).
26. Dixon. M. and Needham, D. M. *Nature*, **158**, 432, (1946).
27. Peters. R. A. and Walker, R. W. *Biochem. J.* **41**: 550, (1947).
28. Gilman. A and Philips, F. S. *Science*, **103**: 409, (1946).
29. Warwick, G. P. *Biochemical, Pharmacology*,. **18**: 538, (1968).
30. Barron, G. Bart Lett, G. Baker, Miller, Z, Meyer, J and Seegmiller, J. E, *J. Exper. Medicine*, **87**: 503, (1948).
31. Scianowski, *J. Acta. Med. Pol*, **2** (18): 97, (1977).