Flies were kept at -20 °C until they were used for analyses. The method of Pant and Agrawal[12] was used for the preparation of amino acid extracts from adult fruit flies. Free amino acids in the tissue extracts were separated and determined quantitatively by 2-dimensional paper chromatography according to the method detailed in the preceding paper by Boctor and Salem[13]. For each sample, 6 chromatographic separations were carried out, and the average and experimental errors were calculated (table).

Results and discussion. Few studies have been made regarding the effect of radiation on the free amino acids of insects. Radiation affects the rates and patterns of protein synthesis and would thus be expected also to alter free amino acid pools. In the present study, 16 amino acids have been identified on the 2-dimensional paper chromatogram of both normal and irradiated adult fruit flies. In 6-day-old female fruit flies, irradiation was seen to have caused a decrease in the quantities of glycine, serine, proline, lysine, histidine and methionine and an increase in the amounts of threonine, valine, aspartic acid, glutamic acid, glutamine, tyrosine, ornithine and cystine. The results also show that levels of alanine and leucine were not changed by irradiation. In 2-day-old female fruit flies, the radiation effect on the free amino acids was an increased concentration of both individual amino acids and the total pool. Methionine was the only amino acid which was lower (18.3%) in the irradiated group.

Several investigators demonstrated that X- and γ-irradiation have been found to cause variations in the amounts of amino acids in organisms, but there seems to be little consistency. For instance, in yellow mealworm embryos *Tenebrio molitor* arginine, serine, histidine, phenylalanine, threonine, tryptophan and valine increased due to X-irradiation, and alanine, aspartic acid, cystine, glutamic acid, methionine and proline decreased[15]. Richardson and Myser[16] showed that large doses of irradiation (20 krad) increased the total concentration of amino acids in the haemolymph pools of prepupae of the honeybee and last-instar larvae of the greater waxmoth, *Galleria mellonella* (L.); lesser amounts raised the levels of particular amino acids such as lysine, the most radiosensitive in their study. Recently Meyer et al.[17] reported abnormalities in the free amino acids of adult hornflies irradiated as pharate adults. They found no effect of irradiation on the total concentration of amino acids in either their physiological or total amino acid analyses.

In the present investigation, most of the free amino acids of 6-day-old female fruit flies changed in titre as a result of irradiation, but the total concentration of amino acids was almost the same in both normal and irradiated flies. In 2-day-old female fruit flies, irradiation caused a higher increase in the total concentration of amino acids. The increased free amino acid concentration after γ-irradiation may be attributed to a decreased capacity of the tissues to utilize the free amino acid pool particularly with respect to protein synthesis.

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2 M. Hafez and A. Shoukry, Z. angew. Ent. *72*, 59 (1972).
3 M. Hafez, A.A. Abdel-Malek, A.M. Wakid und A. Shoukry, Z. angew. Ent. *73*, 230 (1973).
4 A. Shoukry, Z. angew. Ent. *74*, 366 (1973).
5 A. Shoukry, Z. angew. Ent. *75*, 109 (1974).
6 L.F. Steiner and L.D. Christenson, Proc. Hawaiian Acad. Sci. *3*, 17 (1956).
7 M. Feron, Report in the panel on the 'Advances in insect control by the sterile male technique', Vienna, July 1964, IAEA Tech. Ser. No.44.
8 K.P. Katiyar and S.J. Valerio, 5th Int. Am. Symposium on the peaceful application of nuclear energy, p. 197, 1964.
9 H.S. Ducoff, Biol. Rev. *47*, 211 (1972).
10 R.D. O'Brien and L.S. Wolfe, in: Radiation, Radioactivity and Insects. Academic Press, New York 1964.
11 N.F. Shehata, Thesis, Cairo University, 1974.
12 R. Pant and H.C. Agrawal, J. Insect Physiol. *10*, 443 (1964).
13 I.Z. Boctor and S.I. Salem, Comp. Biochem. Physiol. *45B*, 785 (1973).
14 I.Z. Boctor, Zool. Jb. Physiol. *82*, 349 (1978).
15 D.S. Po-Chadley, Nuclear Sci., abstr, *19*, 10 (1964).
16 B.L. Richardson and W.C. Myser, Radiation Res. *54*, 274 (1973).
17 R.T. Mayer, J. Cooper, F.M. Farr and R.H. Singer, Insect Biochem. *5*, 35 (1975).

A logical approach to the isolation of lactate dehydrogenase isozyme X from human testes: A general rationale for the isolation of homotetrameric LDH isozymes[1]

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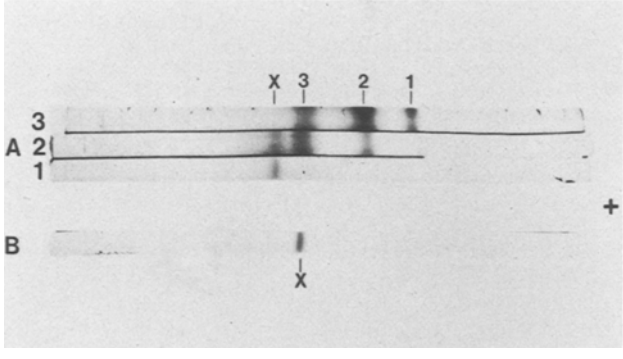
Summary. Immunoadsorbent and oxamate-Sepharose chromatography were used to isolate electrophoretically homogeneous LDH-X from human testes with a final specific activity of 125 IU/mg and good yields: other applications of this approach are discussed.

Lactate dehydrogenase isozyme X^{2-4} (LDH-X) is found only in the mature testes and spermatozoa of mammalian and avian species, and differs from the 5 major isozymes (LDH-1-LDH-5) in its kinetic[5], chemical[5] and immunological properties[6]. These properties make LDH-X a possible target for fertility regulation and in this connection, active[7] or passive[8] immunization of female mice with mouse LDH-X has been shown to decrease fertility. Although human LDH-X has been separated from other LDHs by us using DEAE-cellulose chromatography[9] and by others using AMP-Sepharose chromatography[10] the differences in charge properties and affinity for nucleotides are not sufficient to predict such separations a priori. Here we present a generally applicable procedure for the isolation of LDH-X from human testes, using immunoadsorbent chromatography to separate LDH-X from other LDHs and oxamate-Sepharose affinity chromatography to free LDH from other proteins.

Materials and methods. Human tissues (heart, liver and testes) were obtained by autopsy from accident victims with the cooperation of the Police Hospital, Bangkok. Homogenous LDH-1 (sp. act. 168 IU/mg) and LDH-5 (sp. act. 283 IU/mg) were isolated from human heart and from human liver as described[11]. Antisera to these proteins were obtained by immunizing rabbits (LDH-1 was acetylated prior to immunization[12]). Gamma globulins were isolated from antisera by the method of Fleischman et al.[13] and were coupled to Sepharose (1:30 by wet weight) by the cyanogen bromide procedure[14]. The immunoadsorbent used in the present experiments was an equal volume mixture of anti-

LDH-5-Sepharose and anti-acetyl-LDH-1-Sepharose. The oxamate-Sepharose affinity column was prepared by coupling amino-hexyl-Sepharose to oxalate, pH 4.7[15]. The procedures for gel electrophoresis, protein determination and LDH assay have been described[11]. 1 IU of LDH is defined as the amount of enzyme that will oxidize 1 µmole of NADH per min at 25 °C.

Results. The following procedure, carried out at 4 °C and using 1 mM EDTA and 1 mM 2-mercaptoethanol in all buffers, was found to be successful for the isolation of LDH-X from human testes. The crude extract, prepared by homogenizing human testes in 10 mM sodium phosphate buffer, pH 7.0 (1.5 ml/g testis) and centrifugation at 12,000 × g for 1 h, was further fractionated by ammonium sulfate precipitation. The precipitate obtained at 40–70% saturation was resuspended in 10 mM sodium phosphate buffer, pH 7.0, dialyzed and applied to the anti-LDH-5/antiacetyl LDH-1-Sepharose immunoadsorbent column (1.4 × 6.5 cm), equilibrated with the same buffer. Unadsorbed enzyme and protein was washed out with 6 column volumes of starting buffer, and then adsorbed enzyme was eluted out with 4 column volumes of 1 M NaCl. These 2 pools were analyzed by polyacrylamide gel electrophoresis, followed by activity staining (figure, A). Compared to testis crude extract (figure, A: gel 2), the unadsorbed material was shown to contain LDH-X free of other isozymes (figure, A: gel 1), while the adsorbed pool was essentially free of LDH-X (figure, A: gel 3).



Electrophoretic analysis of LDH pools from immunoadsorbent column (A) and oxamate-Sepharose column (B). Electrophoresis was carried out in 5.25% polyacrylamide gels in 0.02 M glycine-NaOH buffer, pH 9.8 at 20 V/cm for 3 h at 4 °C. Some gels were stained for LDH activity (A) in 0.075 M Tris-HCl, pH 8.0 containing 10 mM DL-lactate, 0.15 mM NAD, 1 mg/ml phenazine methosulfate and 1 mg/ml nitroblue tetrazolium, while another gel (B) was stained for protein with amido black. *A* Stained for activity: 1. unadsorbed pool from immunoadsorbent; 2. testis crude extract; 3. adsorbed pool from immunoadsorbent. *B* Stained for protein: adsorbed pool from oxamate-Sepharose column.

The LDH-X containing fraction (the unadsorbed pool from the immunoadsorbent) was made 0.2 mM in NADH and 0.5 M in NaCl and applied to an oxamate-Sepharose column (1.4 × 3.3 cm), equilibrated with 0.2 mM NADH, 0.5 M NaCl in 10 mM sodium phosphate buffer, pH 7.0. The column was washed with 6 column volumes of starting buffer to remove contaminating non-LDH protein. Then bound LDH-X was eluted out with 4 column volumes of 1 mM lactate in 10 mM sodium phosphate buffer, pH 7.0. This adsorbed pool showed a single band on polyacrylamide gel electrophoresis, followed by protein staining (figure, B), indicating that the isolated LDH-X was electrophoretically homogenous. Consistent with the properties of human LDH-X[9], the reaction rate ratio with 1 mM α-ketobutyrate as substrate to that with 0.3 mM pyruvate as substrate was 1.50, compared to ratios of 0.78 and 0.16 obtained for LDH-1 and LDH-5. The LDH-X purified here had a sp. act. of 125 IU/mg, higher than the 80 IU/mg previously obtained by us from testes[9] and of similar magnitude to the 146 IU/mg obtained by others from frozen semen[10], given possible differences in methodology. Estimates of LDH-X content as a percent of total LDH vary, possibly due to differences in staining conditions. Using our value[9,11] of 20% for LDH-X content, the yield in the present procedure is 40% compared to the 20% previously obtained by us[9,11]. But taking the value of 10–15% for LDH-X content of human frozen semen and testes reported by Kolk et al.[10], the yield in the present studies (60–80%) is comparable to that obtained (70–80%) by the same authors.

Discussion. The isolation of any LDH isozyme requires separation of LDH from other proteins and separation of the desired isozyme from other isozymes. We have made a minor modification to the powerful technique of oxamate-Sepharose chromatography by including 1 mM lactate in the elution buffer to improve the dissociation of the ternary complex. But our major innovation has been to introduce immunoadsorbent chromatography for separating LDH isozymes: such columns are reusable, but depending on the antisera, may require other conditions for regeneration (e.g. low pH) rather than the 1 M NaCl effective here. Such a procedure should be consistently applicable to the isolation of LDH-X from most species. This contrasts with other affinity chromatographic methods, where the special conditions for separating LDH-X from other LDHs in each species must be worked out by trial-and-error. Since antisera for a given LDH subunit type of 1 species are expected to cross-react with the same (but not with a different) subunit type of another species[16,17], it may be possible to use antisera against a commercially available LDH-1 or LDH-5. For LDH-X isolation, antisera to a mixture of H and M subunits should be sufficient. The rationale may be applied to LDH-1 or LDH-5 isolation from other tissues using antisera to LDH-5 and to LDH-1 respectively.

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2 A. Blanco and W.H. Zinkham, Science *139*, 601 (1963).
3 E. Goldberg, Science *139*, 602 (1963).
4 W.H. Zinkham, A. Blanco and L.J. Clowry, Ann. N.Y. Acad. Sci. *121*, 571 (1964).
5 E. Goldberg, J. biol. Chem. *247*, 2044 (1972).
6 E. Goldberg, Proc. nat. Acad. Sci., USA *68*, 349 (1971).
7 E. Goldberg and J. Lerum, Science *176*, 686 (1972).
8 J. Lerum and E. Goldberg, Biol. Reprod. *11*, 108 (1974).
9 J. Svasti and S. Viriyachai, in: Isozymes, vol. 2, p. 113. Ed. C.L. Markert. Academic Press, New York 1975.
10 A.H.J. Kolk, L. Van Kuyk and B. Boettcher, Biochem J. *173*, 767 (1978).
11 J. Svasti and S. Viriyachai, J. Sci. Soc. Thailand *1*, 57 (1975).
12 K. Rajewsky, Biochim. biophys. Acta *121*, 51 (1966).
13 J.B. Fleischman, R.H. Pain and R.R. Porter, Archs Biochem. Biophys., suppl. *1*, 174 (1972).
14 M. Wilchek, V. Bocchini, M. Becker and D. Givol, Biochemistry *10*, 2828 (1971).
15 P. O'Carra and S. Barry, FEBS Lett. *21*, 281 (1972).
16 J.F. Burd, M. Usategui-Gomez, A. Fernandez de Castro, N.S. Mhatre and F.M. Yeager, Clinica chim. Acta *46*, 205 (1973).
17 E. Goldberg, J. exp. Zool. *186*, 274 (1973).