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Immunohistochemical Localization of Pyruvate Kinase Isoenzymes in Chicken Tissues

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Summary. A method for the localization of pyruvate kinase isoenzymes type L, M_2 and M_1 in tissue sections is described. Mono-specific antibodies directed against isoenzymes of pyruvate kinase from chicken and the peroxidase antiperoxidase method were used. The following preferential localizations of the isoenzymes in chicken tissues were observed: Pyruvate kinase M_1 was found in skeletal muscle. The white muscle fibers were more intensely stained than the red. Some dark muscles (e.g., anterior latissimus dorsi) and the heart muscle showed no reaction with antiserum against pyruvate kinase M_1. Pyruvate kinase type L was found in the hepatocytes and in kidney cortex. Pyruvate kinase type M_2 was seen in the distal tubules of kidney, in hepatocytes and sinusoidal cells in liver, in lung, adipose tissue, and in the spleen mainly in the bursa dependent areas. Pyruvate kinase type M_2 was detected in high concentrations in the granulation tissue of regenerating liver after partial hepatectomy. Liver sections of a hen bearing a pancreatic tumor showed an unusually high content of pyruvate kinase type M_2 in some hepatocytes, which were each clustered to spots in the liver parenchyma. Thus, contrary to previous reports, the tissue distribution of isoenzymes in chicken is similar to that of other vertebrates.

Introduction

Pyruvate kinase (EC 2.7.1.40) of higher animals exists in at least 3 different isoenzyme forms (Tanaka et al., 1965, 1967; Hall and Cottam, 1978) which may form hybrid enzymes (Dyson and Cardenas, 1973; Strandholm et al., 1975, 1976). The L-type of pyruvate kinase is found as the major component in liver and as a minor component in kidney. Muscles and brain contain the M_1-type. The pyruvate kinase of most other tissues is of the M_2 (A or K)-type.

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The cellular distribution of the different isoenzyme forms has been studied in tissue homogenates of rat (Tanaka et al., 1965; Ibsen and Trippet, 1972; Osterman et al., 1973; Ibsen and Krueger, 1973), guinea pig (Faulkner and Jones, 1975a), frog (Schloen et al., 1974), chicken (Strandholm et al., 1975; Cardenas et al., 1978) and human (Bighley et al., 1968; Faulkner and Jones, 1975a, b) mainly by electrophoretic (Tanaka et al., 1965; Strandholm et al., 1975; Cardenas et al., 1978; Faulkner and Jones, 1975a, b; Schloen et al., 1974) or isoelectric focusing techniques (Ibsen and Krueger, 1973) or by analysis of kinetic properties (Strandholm et al., 1975; Faulkner and Jones, 1975a). Generally no differentiation was made in these studies between homopolymer and heteropolymer forms of the tetrameric enzyme. Only an approximate localization of isoenzymes in particular cells was possible by these methods. A histological classification of cells in tissue sections on the basis of isoenzyme content has not been reported so far.

Pyruvate kinase type L could not be detected by electrophoretic methods in chicken tissues (Strandholm et al., 1975; Ibsen et al., 1976), although this isoenzyme has been isolated from chicken liver (Eigenbrodt and Schoner, 1977). Kinetic studies to determine isoenzyme composition in chicken (Harris et al., 1977) have been criticized (Cardenas et al., 1978), because of the demonstration of hybrid forms of pyruvate kinase (heteropolymers of isoenzyme monomers) in some tissues (Cardenas et al., 1978; Strandholm et al., 1975, 1976). Therefore an immunohistochemical method was employed to obtain information on the localization and distribution of pyruvate kinase isoenzymes in chicken organs. Due to the specifity of the antibodies the term isoenzyme used in connection with immunohistochemical results in this paper refers to the nature of the monomer.

Materials and Methods

Materials. If not mentioned otherwise chemicals were from E. Merck AG, Darmstadt, Germany, and biochemicals from Boehringer Mannheim GmbH, Mannheim, Germany. Parablast was from Sherwood, St. Louis, USA; anti-rabbit-serum from swine, peroxidase-conjugated swine anti-rabbit-IgG and peroxidase antiperoxidase complex were from Dako-Immunoglobulins, Copenhagen, Denmark. Eukitt was purchased from Hecht-Mertens, Kiel-Hassee, Germany.

Organs. Organs were obtained from 35 "White Leghorn" chickens of both sexes, 1 day to two years of age. After sacrificing the animals in ether anesthesia by bleeding, thin slices of fresh organs were fixed immediately in precooled (8° C) acetone. After 3 days to 3 weeks at 8° C, the samples were embedded in paraffin (m.p. 42–44° C) at 46° C and then placed into a mould which was subsequently filled with a 1:1 mixture of the same paraffin with parablast. Sections of these samples were investigated with the unlabelled peroxidase antiperoxidase (PAP) method.

Cryostat sections of 10 µm thickness were obtained with a Leitz Histokryotom from unfixed tissue blocks snap-frozen in liquid nitrogen. They were analyzed with the indirect peroxidase method.

Similar cryostat sections of 20 µm thickness were used to determine the pyruvate kinase activity in tissue sections and its effusion out of sections during the several treatments.

Antigens. Pyruvate kinase type L and M_2 from chicken liver were purified as described previously (Eigenbrodt and Schoner, 1977). Pyruvate kinase type M_1 from chicken breast muscle was purified according to Cardenas et al. (1975).

Antisera. 1 mg of purified isoenzyme in 1 ml PBS (0.01 M phosphate buffer, pH 7.2, with 0.85% NaCl) was injected subcutaneously into one rabbit. For the first injection the isoenzyme was mixed with 1 ml of Freund's complete adjuvant, for the following injections with the same amount of incomplete adjuvant. 14 days after the first injection the animals were boostered, and they were bled one week later by puncture of the ear artery. Some animals were boostered again after one and two months. Preimmune sera from the same rabbits served as controls.

Immunization with pyruvate kinase type L was also done with trichloroacetic acid-treated enzyme: Enzyme which had been precipitated with 5% trichloroacetic acid was extensively dialysed against 50 mM Tris-HCl buffer pH 7.5 containing 4 mM [cyclohexylene]-(1,2)dinitrillo tetraacetic acid (CDTA), 100 mM urea and 100 mM mercaptoethanol. Antisera raised with this antigen preparation showed less unspecific reactions than those produced with the native enzyme.

Absorption of the Antisera. The antisera were absorbed with acetone-precipitated organ powder (Coons and Kaplan, 1950), until the sera exhibited no cross reaction against the different isoenzymes on at least one cell type in the immunohistochemical survey. The same cell type was checked to show positive reaction with at least one antiserum. Organ powder of skeletal muscle, liver, kidney, and lung of adult "White Leghorn" chickens were used. Anti pyruvate kinase type L serum (anti-L-Pk) and anti pyruvate kinase type M_2 serum (anti-M_2-Pk) were absorbed with acetone powder from skeletal muscle, anti pyruvate kinase type M_1 serum (anti-M_1-Pk) was absorbed with acetone powder of kidney, liver and lung. 10 to 100 mg of each organ powder were used to absorb 1 ml of the diluted antiserum. The sera were regarded specific if anti-L-Pk was bound to proximal kidney tubules and to hepatocytes, but not to distal kidney tubules and skeletal muscle; the anti-M_2-Pk reacted specific if the antiserum reacted with distal kidney tubules, but not with proximal tubules and skeletal muscle; the anti-M_1-Pk reacted specific if the antiserum was bound to skeletal muscle only but not to kidney or liver.

Unlabelled Peroxidase Antiperoxidase Method. 7–10 µm sections of paraffin embedded organs were transferred to slides covered with egg albumin: glycerol = 1:1 and deparaffinized in xylene. Endogenous peroxidase was inactivated by a 30 min incubation at room temperature in methanol containing 0.5% H_2O_2. The sections were then transferred through ethanol baths of decreasing concentration to TBS (0.05 M Tris-HCl pH 7.6 containing 0.8% NaCl). The isoenzymes were demonstrated by the peroxidase antiperoxidase (PAP) method according to Burns (1975) with two modifications. These were 1. to use undiluted serum from swine for the first incubation and 2. to dilute all antisera and the PAP-complex in porcine serum which itself had been diluted 1:5 in TBS (PTBS). The dilutions of the rabbit-anti-isoenzyme sera were 1:200 to 1:800; anti-rabbit-IgG from swine was diluted 1:20 and PAP 1:50. The demonstration of the peroxidase activity was performed in accordance with Graham and Karnovsky (1966). This was followed by a counterstaining in dilute 1% Giemsa solution for 10 minutes. The preparations were dehydrated in an ethanol dilution series and isopropanol, transferred to xylene, and the coverslips were then mounted with Eukitt.

Indirect Peroxidase Reaction. Cryostat sections were airdried, put into waterfree acetone at 8° C for 10 min, and then for 30 min into methanol containing 0.5% H_2O_2. After this treatment the sections undergoing indirect peroxidase reaction were airdried. They were then incubated with absorbed antiserum against the isoenzymes for 30 min in 1:10 to 1:20 dilution in PTPS. For the second step peroxidase-conjugated swine anti rabbit IgG was used in a dilution of 1:50 in the same buffer. The following procedures were as described for the unlabelled peroxidase antiperoxidase method.

PAS-Reaction. For the demonstration of glycogen the periodic acid-Schiff reaction was performed and its specifity controlled by digestion with diastase according to Pearse (1961).

Micrographs. Micrographs were taken with a Zeiss Photomikroskop III on Agfapan 25 Professional. All micrographs shown are from paraffin sections treated with the PAP method.

Agar Gel Precipitation Test. Agar gel precipitation tests were carried out according to standard procedures. The plates were stained with Coomassie Brillant Blue.

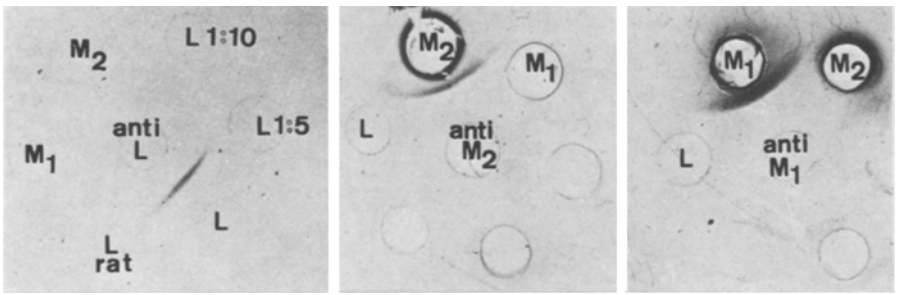

Fig. 1. Agar gel precipitation test of absorbed antisera and the purified isoenzymes (1 µg in 10 µl PBS)

Determination of Pyruvate Kinase Activity in Tissue Sections. Pyruvate kinase activity in tissue sections was determined (Eigenbrodt and Schoner, 1977) after homogenization in 0.2 ml PBS with a Potter Elvehjem tissue homogenizer. The sections (0.02 mm thick) were treated similarly as in the immunohistochemical survey to study the effect of the fixation and embedding procedures on the enzyme activity. The following types of sections were investigated:

1. Fresh cryostat sections of organs snap frozen in liquid nitrogen.
2. Sections as in 1. after airdrying.
3. Sections as in 2. after fixation for 30 min in precooled (8° C) acetone.
4. Sections as in 3. after incubation for 30 min in methanol containing 0.5% H_2O_2.
5. Sections as in 3. after incubation for 30 min at room temperature with 100 µl of the same antiserum as used as first antiserum in the indirect peroxidase method.
6. Sections of acetone fixed paraffin embedded organs as used in the PAP-method after deparaffinization in xylene.

Determination of the Effusion of Pyruvate Kinase Activity out of the Sections. 0.2 ml PBS were put on top of the same six types of sections as described above. After different time intervals the supernatant was removed and the pyruvate kinase activity determined. After 20 min of incubation, the sections were scraped off and the remaining pyruvate kinase activity was determined as described above.

Determination of Pyruvate Kinase Content of Organs and the Kinetic Properties in Homogenates. 1 g of tissue was homogenized in 10 ml of a solution consisting of 50 mM Tris-HCl pH 7.5, 4 mM CDTA, 100 mM urea, 10 mM β-mercaptoethanol, 100 mM NaCl, 2 mM ε-aminocapronate and 0.2 mM phenylmethylsulfonylfluoride. Pyruvate kinase activity determinations were done according to Eigenbrodt and Schoner (1977) or by the same test after replacing potassium phosphate buffer by 0.16 mM triethanolamine-HCl pH 7.1 and 30 mM KCl. One unit pyruvate kinase produces 1 µmol pyruvate in one minute at 37° C under these conditions (Eigenbrodt and Schoner, 1977).

For agar gel precipitation tests the homogenate supernatants were adjusted to 30 U = 0.1 mg pyruvate kinase per ml and dialyzed extensively against PBS.

Partial Hepatectomy. A 1 year old cock was anesthesized with phenobarbital and the left liver lobe (ca. 4.5 g) was removed. The wound was closed with Histacryl Blau. Antibiotica were given into the abdomen. The animal was sacrificed in ether anesthesia 4 days later by bleeding.

Results and Discussion

Characterization of Antibodies. Figure 1 shows an immunodiffusion assay according to Ouchterlony demonstrating that absorbed antibodies against pyruvate kinase type L from chicken liver neither react with pyruvate kinase type M_1

or M_2 from the same species nor with pyruvate kinase type L from rat. Furthermore, antibodies against pyruvate kinase type M_2 or M_1 do not cross-react with other purified isoenzymes from chicken. This finding is consistent with the specifity of the antisera in immunohistochemistry.

Studies on the Fixation of Pyruvate Kinase Isoenzymes in Sections. To demonstrate soluble cytoplasmic enzymes histochemically by their activity or immunohistochemically they must not effuse at a high rate from the sections during the various treatments. To determine this effusion rate the leakage of enzyme activity into PBS from sections treated differently was measured. The effect of 1. airdrying, 2. fixation in acetone, 3. incubation with antiserum as the first aqueous incubation medium, and 4. embedding in paraffin after acetone fixation and subsequent deparaffinization in xylene was investigated. The incubation of the sections with antiserum should produce insoluble immune complexes and thus inhibit the effusion of enzyme. Insoluble immune complexes formed by specific antibodies and an isoenzyme could be shown histochemically with the MAGIC-technique (Wachsmuth, 1973, 1976). But if this technique is to be sucessful the enzyme activity must not be inhibited in a high degree by the antibodies. Furthermore, all types of isoenzymes reacting in the histochemical test except the one type bound in the immune complex must be removable totally from the section by diffusion. Because this was not the case in our system and because the histochemical demonstration of pyruvate kinase activity is very difficult we made the immune complex visible by the indirect peroxidase method.

The total amount of enzyme activity in frozen sections immediately after sectioning (without airdrying) were 55 mU in kidney, 40 mU in liver, and 2,640 mU in the breast muscle. The total amount of enzyme activity in frozen, airdried sections were 30 mU in kidney, 9 mU in liver, and 1,950 mU in the m. pectoralis. Acetone fixed frozen sections had a total content of 30 mU in kidney, 9 mU in liver, and 1,200 mU in the m. pectoralis. The total content of paraffin sections of acetone fixed organs were 0 mU in kidney and liver, and 500 mU in the m. pectoralis.

Incubation of frozen sections of different tissues with PBS leads in most cases to a rapid release of pyruvate kinase into the supernatant whether the sections were acetone fixed or not (Fig. 2). In the presence of antibodies no pyruvate kinase activity is measurable. After preincubation of the sections with antiserum and replacing it by PBS, there is very little effusion of pyruvate kinase out of liver and kidney sections, but some leakage of the enzyme occurs out of skeletal muscle. It is also evident from Fig. 2 that pyruvate kinase isoenzymes M_2 and L are fixed tighter to the tissue than the isoenzyme M_1. About 30% of these enzymes are not released from untreated sections of kidney (Fig. 2A) and diffuse only slowly from liver (Fig. 2B). Only 50% of the total content effuse from acetone fixed frozen sections of liver and kidney. Because of the stability of the muscle pyruvate kinase, pyruvate kinase activity can still be measured in paraffin embedded muscle (Fig. 2C) in contrast to paraffin sections of other organs. However, almost no effusion of pyruvate kinase was seen from paraffin sections of skeletal muscle (Fig. 2C). Incubation of sections

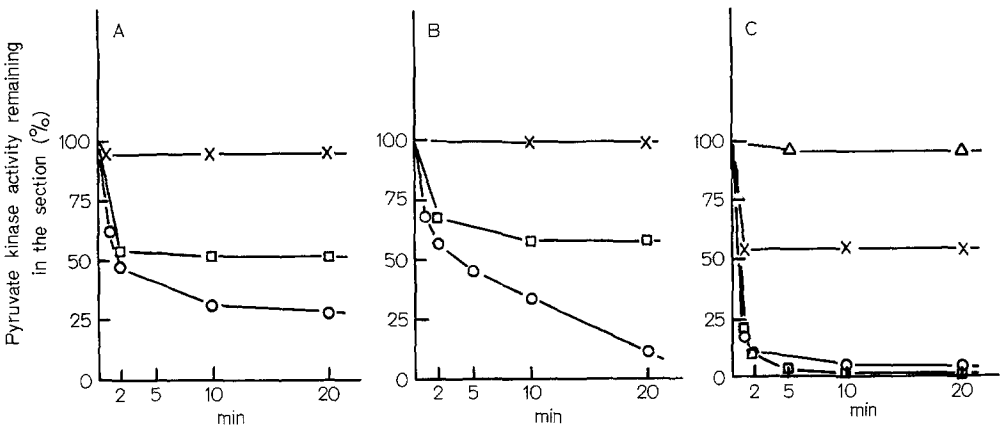

Fig. 2. Determination of the effusion of pyruvate kinase from $0.02 \times 0.4 \times 10$ mm tissue sections of kidney (A), liver (B), and the breast muscle (C) mounted on slides. o—o effusion of pyruvate kinase activity from untreated frozen sections into PBS; □—□ effusion of pyruvate kinase activity into PBS from frozen sections fixed in acetone after sectioning; ×—× effusion of pyruvate kinase activity into PBS from frozen sections treated with antisera (anti-M_2-Pk in A, anti-L-Pk in B, and anti-M_1-Pk in C) as the first auqueous incubation medium, △—△ effusion of pyruvate kinase activity into PBS from paraffin sections of acetone fixed organs

in methanol containing 0.5% H_2O_2, which destroys endogenous peroxidase activity, completely abolished any pyruvate kinase activity. If preparations of L, M_1 and M_2 pyruvate kinase (0.5 mg in 1 ml PBS each) were precipitated with 10 ml methanol containing 0.5% H_2O_2 for 30 min at room temperature, no protein was found in the supernatant. The precipitate was insoluble in TBS as determined by investigation of the supernatant by the method of Lowry et al. (1951). From this it is unlikely that the isoenzymes effuse from the sections after they had been treated with methanol containing 0.5% H_2O_2. Another evidence against effusion of pyruvate kinase type M_1 is the chessboard pattern found in some skeletal muscles (Fig. 6A). If one assumes that the isoenzyme M_1, which obviously diffuses best, effuses from each fiber type at the same rate, an equilibration of the concentrations should be found but not some totally negative fibers next to strongly positive ones. The same is true for the tubules in the kidney regarding L and M_2 type pyruvate kinase (Fig. 4). Paraffin sections could be incubated after deparaffinization over night in TBS prior to the immunohistological procedures without any change of the result. Furthermore, if snap frozen tissue sections and the indirect peroxidase procedure were used with the antiserum as the first aqueous incubation medium (which method should result in insoluble, precipitated immune complexes in the sections) an identical localization of the isoenzymes was seen as compared to the PAP method. However, the morphology was poorer than that of paraffin sections and the sensitivity was inferior to the PAP method.

There is also some evidence against the effusion of the isoenzymes during their fixation in acetone. First, all isoenzymes are precipitated repeatedly during the isolation procedure, so that only material insoluble in acetone is used for immunisation and thus demonstrated by the antibodies. This argues also against

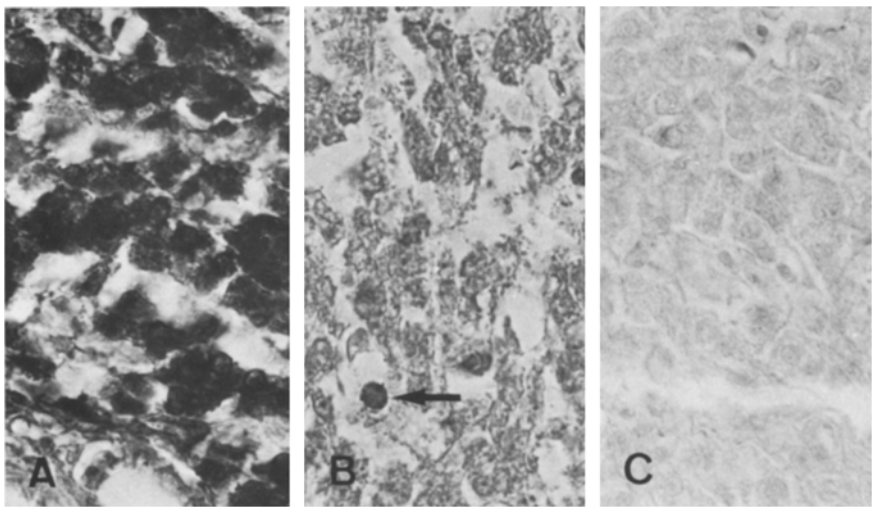

Fig. 3. Liver sections treated with antisera against pyruvate kinase type L (A), type M_2 (B), and type M_1 (C). Arrow=strong reacting cell adjacent to the sinusoid. Magnification: × 560

any loss of antigenic determinants during fixation. Second, fixation for more than one year in acetone at 8° C leads to no immunohistochemically detectable loss of antigen compared to samples from the same organs embedded after 4 days.

Localization of Pyruvate Kinase Isoenzymes in the Liver. Pyruvate kinase type L can be phosphorylated by a cAMP dependent protein kinase which leads to an inactivation of the enzyme. This reaction seems to be involved in the hormonal control of gluconeogenesis in liver (Engström, 1978). The isoenzyme is located in the hepatocyte of mammals and amphibians and is the major component of the liver pyruvate kinase in rat (65–97%) (Tanaka et al., 1967; Osterman et al., 1973), guinea pig (65%) (Faulkner and Jones, 1975a) and rana pipiens (62%) (Schloen et al., 1974).

Contrary to these animals, chicken liver appears to contain only 25% pyruvate kinase type L of total pyruvate kinase activity as judged from results obtained by enzyme purification (Eigenbrodt and Schoner, 1977). From studies with isolated Kupffer cells of rats it has been suggested that Kupffer cells contain only the M_2 type (van Berkel et al., 1972).

The immunohistochemical localization of pyruvate kinase isoenzymes in chicken liver is shown by the staining of hepatocytes after treatment of the sections with anti-L-pyruvate kinase antibodies (Fig. 3A) and anti-M_2-pyruvate kinase antibodies (Fig. 3B). Hepatocytes exhibit a strong reaction if treated with anti-L-Pk-serum and a modest reaction if anti-M_2-Pk-serum is used. Additionally some cells adjacent to the liver sinusoids react strongly positive with anti-M_2-Pk-serum. No M_1-Pk is detectable by this method in the liver (Fig. 3C).

These findings suggest that chicken hepatocytes contain both isoenzymes,

Table 1. Tissue content, immunological characterization and kinetic properties of pyruvate kinase in chicken tissue homogenates

tissue	content (U/g wet weight)	K_M-value for PEP (mM)		Immunoprecipitation of organ homogenates		
		no $FruP_2$	+0.2 mM $FruP_2$	anti-L	anti-M_2	anti-M_1
m. pectoralis major	3,055	0.027	0.043	−	−	+
anterior latissimus dorsi	184	0.027	0.043	−	−	−
posterior latissimus dorsi	2,699	0.027	0.043	−	−	+
m. gastrocnemius	43	0.027	0.043	−	−	+
heart muscle	135	0.043	0.043	−	−	−
lung	27	1.25	0.26	−	+	−
brain	230	0.047	0.053	−	+	+
adipose tissue	10	1.0	0.125	−	+	−
kidney	66	0.30	0.125	+	+	−
		1.02[a]	0.125[a]			
liver	40	0.114	0.040	+	+	−
		0.305[a]	0.040[a]			

The stimulation of pyruvate kinase activity by fructose-1,6-bis-phosphate ($FruP_2$) was used to distinguish between the different isoenzyme forms in addition to the determination of the K_M-values (Eigenbrodt and Schoner, 1977). The following signs are used: + = positive in immunoprecipitation, − = negative in immunoprecipitation, PEP = phosphoenolpyruvate, K_M = Michaelis constant

[a] K_M-value for the low affinity type in the homogenate

type L and type M_2. This was further supported by the demonstration of immunoprecipitates with anti-L-Pk and anti-M_2-Pk in chicken liver homogenates (Table 1) as well as by the demonstration of a heterogenous saturation curve for phosphoenolpyruvate allowing the calculation of a high affinity K_M-value of 0.114 mM (probably Pk-M_2) and of a low affinity K_M-value of 0.305 mM (probably Pk-L). Contrary to the rat (Guder and Schmidt, 1976; Reinacher, unpublished) no metabolic zonation of pyruvate kinase type L was seen in chicken liver of fed and of starved (up to 96 h) animals.

Localization of Pyruvate Kinase Isoenzymes in the Kidney. Chicken kidney contains pyruvate kinase type L in the cortex with the exception of some tubules and the medullary rays (Fig. 4A). Pyruvate kinase type M_2 is found in the tubules of the papillae and the medullary rays (Fig. 4B), which are negative with the anti-L-Pk-serum. Pyruvate kinase type M_1 could not be found in the kidney (Fig. 4C). The demonstration of pyruvate kinase type L in the kidney of chickens is in contrast to the report of Strandholm et al. (1975). From electrophoretic studies with the 10,000 × g supernatant of homogenates they concluded that pyruvate kinase type K (= M_2) is the only isoenzyme seen in spleen, lungs, erythrocytes, kidney, liver, and jejunum. Using the method of Dyson and Cardenas (1973) and of Strandholm et al. (1975) we could confirm their findings. However, the kinetic analysis on the affinity of pyruvate kinase for phosphoenolpyruvate in the 50,000 × g supernatant of a kidney homogenate clearly is consistent with the presence of two pyruvate kinases with two different affinities

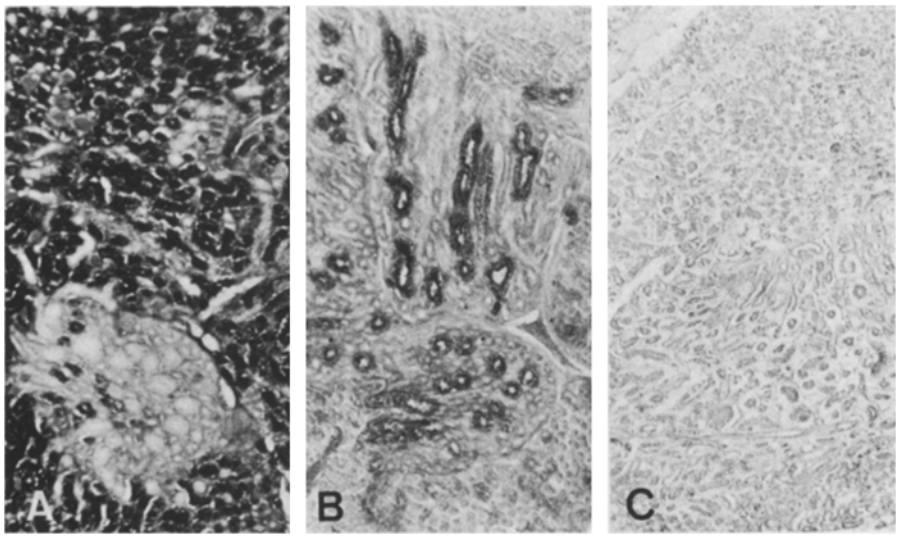

Fig. 4. Kidney sections treated with antisera against pyruvate kinase type L (A), type M_2 (B), and type M_1 (C). Magnification: ×35

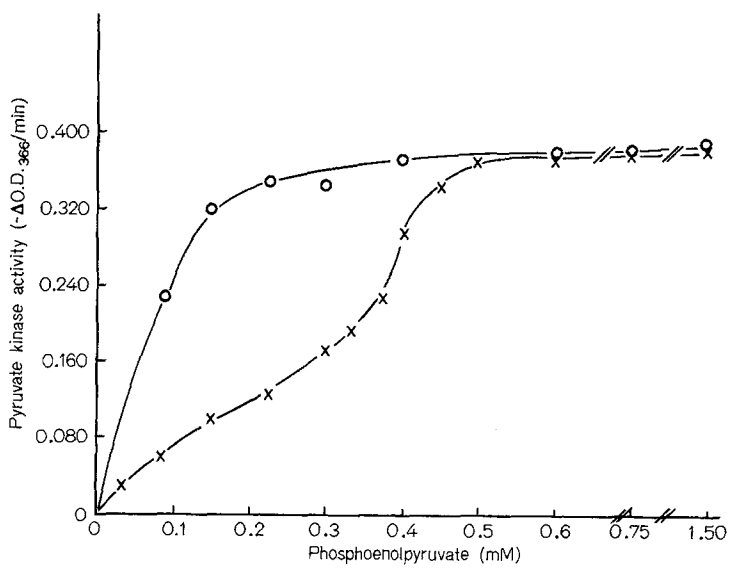

Fig. 5. Effect of phosphoenolpyruvate on pyruvate kinase activity in chicken kidney in the presence (o—o) and absence (×—×) of 0.2 mM fructose-1,6-bis-phosphate. The 50,000 × g supernatant of a homogenate was used

(Fig. 5, Table 1). Fructose 1,6-bisphosphate enhanced the affinity of both pyruvate kinase forms (Table 1), a finding which is to be expected from the isolated isoenzymes (Eigenbrodt and Schoner, 1977). Titration of the homogenate with antibody showed that pyruvate kinase type L was 30% of the total activity and pyruvate kinase type M_2 70%. Apparently the failure of Strandholm et al.

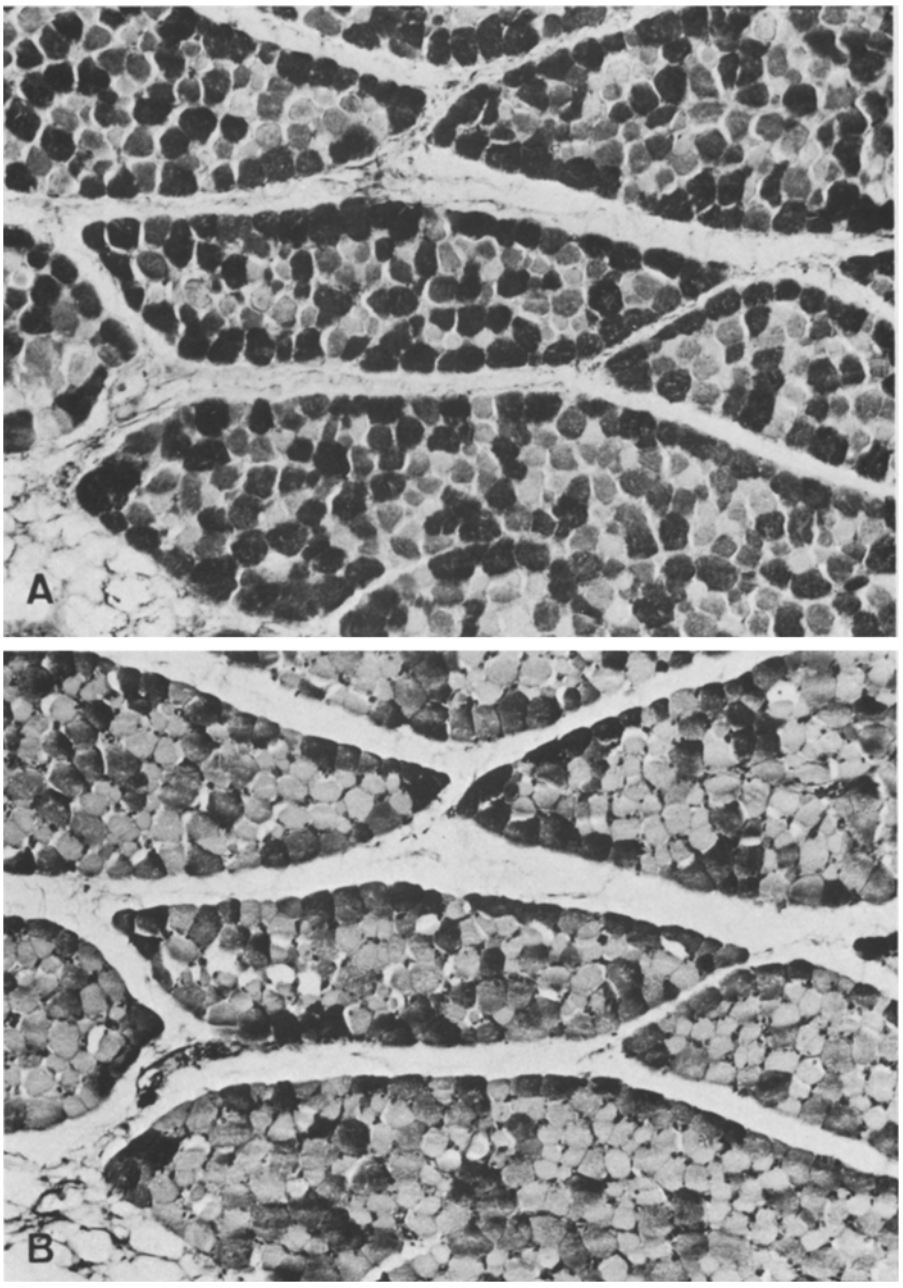

Fig. 6. Section of the m. pectoralis major, antiserum against pyruvate kinase type M_1 (A), and an adjacent section stained by the PAS-reaction for the demonstration of glycogen (B). Magnification: × 140

(1975) to demonstrate pyruvate kinase type L in kidney and liver is due to the fact that this isoenzyme is destroyed during the dialysis prior to electrophoresis.

The distribution of pyruvate kinases type L and M_2 is consistent with microdissection studies of others, who in the rat proximal tubules (Burch et al., 1974) and in bovine kidney cortex (Cardenas and Richards, 1977) found a pyruvate kinase activity which behaved kinetically like the L-type isoenzyme; however, an immunological characterization was not carried out in these studies. Since pyruvate kinase type L in the liver appears to be involved in the regulation of gluconeogenesis (Engström, 1978), it is of interest that the proximal tubule cells of rats containing pyruvate kinase type L have also the capacity for gluconeogenesis (Krebs, 1964; Irias and Greenberg, 1972).

Localization of Pyruvate Kinase Type M_1. There is a considerable difference in the amount of pyruvate kinase in the different muscle types (Table 1). Dark muscles contain less pyruvate kinase than pale muscles. These differences in the pyruvate kinase content can also be seen by the immunohistological method. Only M_1-type pyruvate kinase was detected in skeletal muscles. Figure 6A shows differences in the pyruvate kinase concentration of the different fibers of the pectoralis muscle. The fibers which are stained darker are the white ones as judged by their higher content of glycogen (Fig. 6B) (George and Berger, 1966). This result is consistent with our finding that the posterior latissimus dorsi, which posseses only white, twitch fibers (George and Berger, 1966), shows no chessboard pattern but only fibers positive for both M_1-type pyruvate kinase and glycogen. It should be noted, however, that some of the dark muscles (e.g., anterior latissimus dorsi) and the heart did not show a positive reaction with the immunohistochemical method described in this paper. The negative result of the anterior latissimus dorsi and the heart in the agar gel precipitation test is consistent with this finding (Table 1). The negative result in the agar gel precipitation test could be explained by an antigen unable to diffuse into agar, perhaps because it is bound to cell structures. If this were the case, however, the antigen should have been detected immunohistochemically. Another explanation would be the existence of isoenzymes which differ immunologically but are indistinguishable by common biochemical methods. They would not bind specific antibodies directed against the other isoenzymes but could be detected biochemically.

Localization of Pyruvate Kinase Type M_2 in Tissues Under Normal and Pathological Conditions. The findings reported above concerning the distribution of pyruvate kinase type M_2 in liver and kidney are consistent with the results obtained from kinetic (Faulkner and Jones, 1975a; Strandholm et al., 1975), electrophoretic (Tanaka et al., 1965; Schloen et al., 1974; Faulkner and Jones, 1975a, b; Strandholm et al., 1975; Cardenas et al., 1978), or microdissection studies (Burch et al., 1974) on the localization of pyruvate kinase isoenzymes. In addition to kidney, lungs, adipose tissue (Imamura et al., 1972; Strandholm et al., 1975) and the leukocytes (van Berkel and Koster, 1973) pyruvate kinase type M_2 is found also in the regenerating rat liver (Walker and Potter, 1973; Garnett

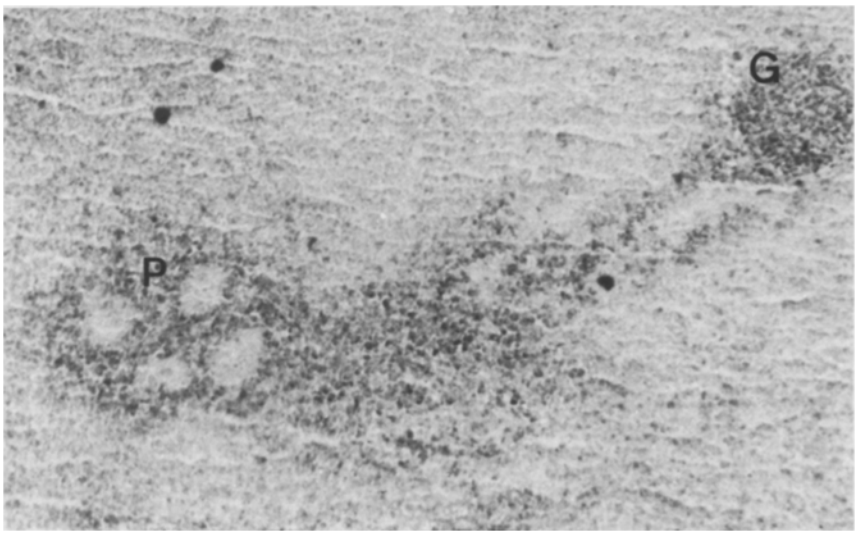

Fig. 7. Spleen section treated with antiserum against pyruvate kinase type M_2. *G* germinal center, *P* peri-ellipsoidal lymphatic tissue. Magnification: × 140

et al., 1974), foetal tissue (Osterman et al., 1973; Faulkner and Jones, 1975b; Strandholm et al., 1975), and tumor cells (Taylor et al., 1969; Imamura et al., 1972; Nakamura et al., 1972; Spellman and Fottrell, 1973; Weinhouse, 1973; Muroya et al., 1976; Irving and Williams, 1976; van Veelen et al., 1978). Therefore, a study on the distribution of pyruvate kinase type M_2 appears to be of considerable interest for a possible diagnostic application.

Figure 7 shows a spleen section which had been exposed to pyruvate kinase type M_2 antibodies. The positive areas reacting mainly with the anti-M_2-Pk-serum are known to be areas where B-lymphocytes are located. These are the periellipsoid tissue around the Schweigger-Seidel sheaths and the germinal centers.

With the immunohistochemical staining of sections for pyruvate kinase isoenzymes it is possible to distinguish every single intact liver cell from cells undergoing a degeneration. Figure 8 shows a liver four days after partial hepatectomy. The granulation tissue of the wound exhibits a high amount of pyruvate kinase M_2 as does the activated reticuloendothelial system of the organ.

In the liver of a hen bearing a pancreatic carcinoma, spots of hepatocytes are seen with an unusually high content of pyruvate kinase type M_2 (Fig. 9B). Staining for pyruvate kinase type L showed normal reaction in these areas. Apparently both isoenzymes of pyruvate kinase are in one cell in considerable concentrations. The reason for the high content of pyruvate kinase type M_2, which was not detected at all in normal liver (Fig. 3), is unknown.

Furthermore in an immunological, biochemical, and immunohistochemical survey, pyruvate kinase type M_2 was seen in the lungs and the adipose tissue (Table 1), the mucosa of the intestine and some parts of the brain.

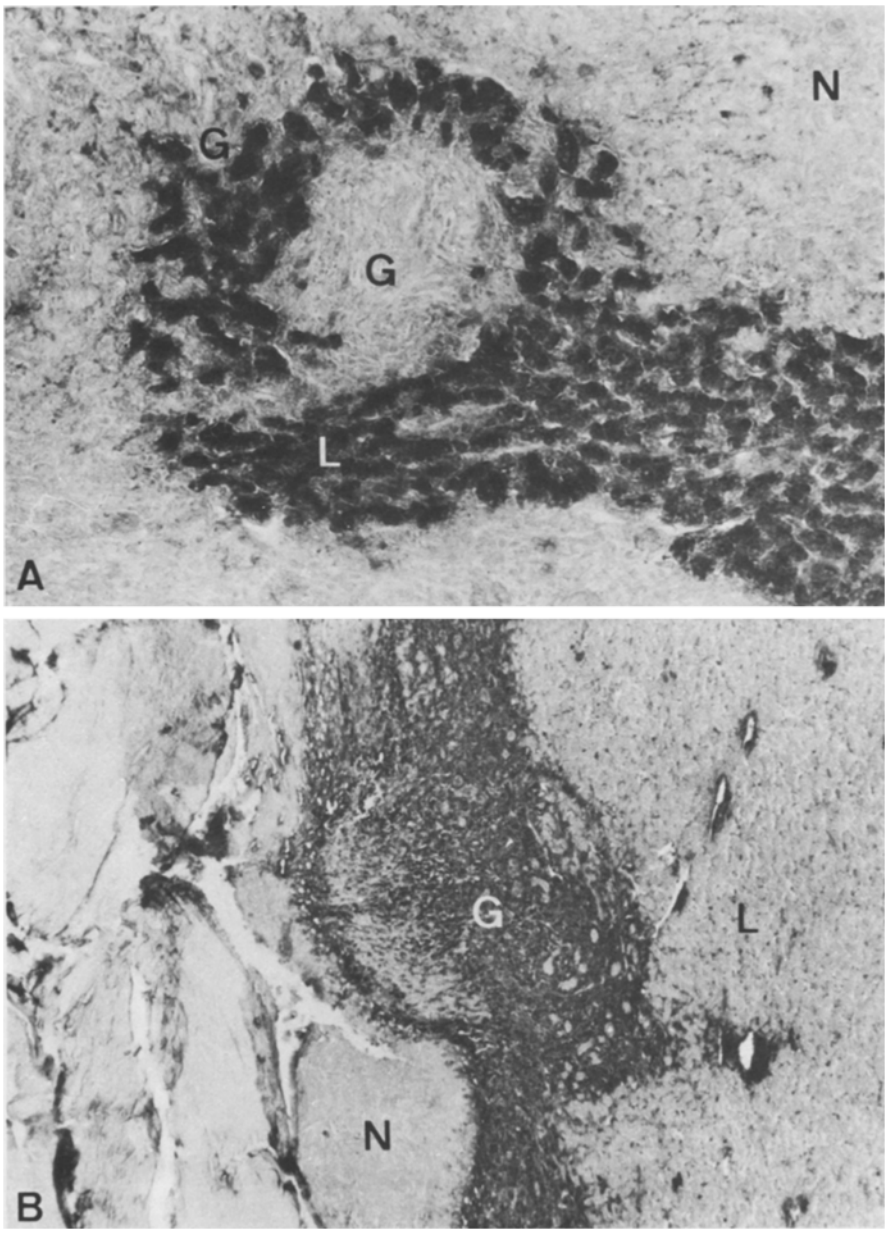

Fig. 8. Section of a liver four days after partial hepatectomy; antiserum against pyruvate kinase type L (A, magnification: ×140) and type M_2 (B, magnification: ×35); *L* liver tissue, *N* necrotic tissue, *G* granulation tissue

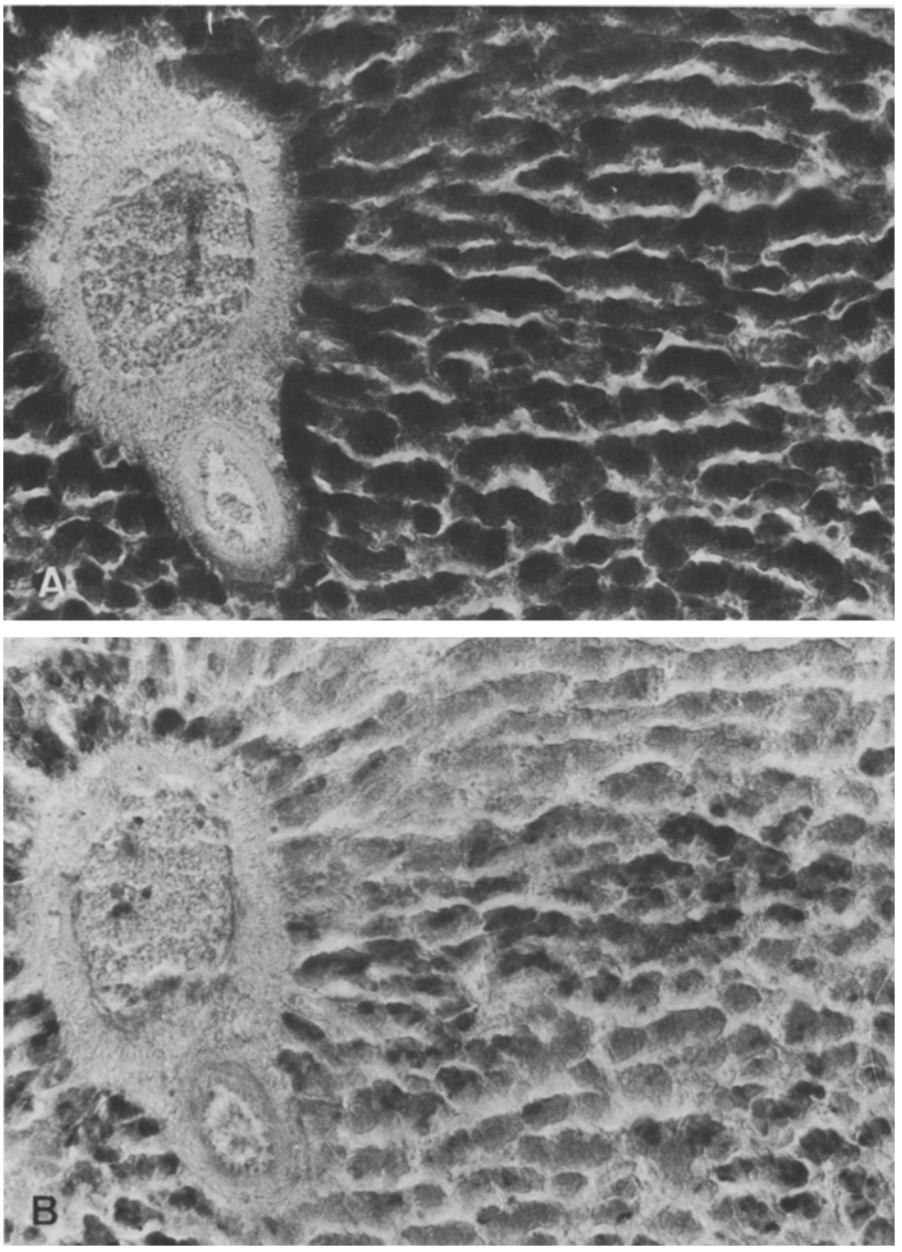

Fig. 9. Adjacent sections of a liver from a hen bearing a pancreatic carcinoma; antiserum against pyruvate kinase type L (A) and type M_2 (B). Magnification: ×224

General Discussion and Conclusions

In Figure 1 it was shown that each antiserum is specific after absorption for the type of isoenzyme used for immunization. The properties of the antisera differ considerably from those describes previously (Imamura et al., 1972; Eigenbrodt and Schoner, 1977). With these monospecific antisera it could then be shown that the tissue distribution pattern of the pyruvate kinase isoenzymes in chicken is similar to that of other vertebrates. These results refer to the isoenzyme nature of the monomer of pyruvate kinase but do not discriminate between possible homo- or heteropolymers in the same cell. The distribution pattern of pyruvate kinase isoenzymes is in contrast to the conclusions of Strandholm et al. (1975) and Ibsen et al. (1976), who were unable to detect pyruvate kinase type L in liver and kidney.

A peculiar observation in this study was that pyruvate kinase type L in the liver was stained more intensively by immunhistochemical means than pyruvate kinase type M_2 (Fig. 3). This is in contrast to the biochemical finding that only about 25% of the total extractable pyruvate kinase activity of chicken liver is of the L-type (Eigenbrodt and Schnoner, 1977). A possible explanation of this discrepancy would be that only part of the pyruvate kinase is extracted by homogenization of the liver and that most of the pyruvate kinase type L remains bound to some subcellular particles. Such an assumption is supported by the findings of Tamir et al. (1972) who reported that in rat brain pyruvate kinase is bound to the synaptosomal, microsomal and mitochondrial fractions by electrostatic forces (Knull, 1977). Preliminary studies in this laboratory indicate that pyruvate kinase type L behaves in a similar way in chicken liver (Fister, unpublished).

Increased concentrations of pyruvate kinase type M_2 in distinct hepatocytes of a tumor bearing hen have been found (Fig. 9). Since changes in the pyruvate kinase isoenzyme pattern have been noted in carcinomas (Taylor et al., 1969; Nakamura et al., 1972; Weinhouse, 1973; Muroya et al., 1976; Hammond and Bahinsky, 1978; van Veelen et al., 1978) the immunohistochemical method described here could be suitable to detect tissue alterations possibly related to cell transformation.

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References

Bighley, R.H., Stenzel, P., Jones, R.T., Campos, J.O., Koler, R.D.: Tissue distribution of human pyruvate kinase isoenzymes. Enzymol. Biol. Clin. **9**, 10–20 (1968)

Burch, H.L., Lowry, O.H., Perry, S.G., Fan, L., Fagioli, S.: Effect of age on pyruvate kinase and lactate dehydrogenase distribution in rat kidney. Am. J. Physiol. **226**, 1227–1231 (1974)

Burns, J.: Background staining and sensitivity of the unlabelled antibody-enzyme (PAP) method. Comparison with the peroxidase labelled antibody sandwich method using formalin fixed paraffin embedded material. Histochemistry **43**, 291–294 (1975)

Cardenas, J.M., Blachly, E.G., Ceccotti, P.L., Dyson, R.D.: Properties of chicken skeletal muscle pyruvate kinase and a proposal for its evolutionary relationship to the other avian and mammalian isozymes. Biochemistry **14**, 2247–2252 (1975)

Cardenas, J.M., Richards, T.C.: Distribution of pyruvate kinase, lactate dehydrogenase and aldolase isozymes in the bovine kidney. Fed. Proc. **36**, 809, Abstract 2830 (1977)

Cardenas, J.M., Bandman, E., Strohman, R.C.: Hybrid isozymes of pyruvate kinase appear during avian cardiac development. Biochem. Biophys. Res. Commun. **80**, 593–599 (1978)

Coons, A.H., Kaplan, M.H.: Localization of antigen in tissue cells. II. Improvements in a method for the detection of antigen by means of fluorescent antibody. J. Exp. Med. **91**, 1–13 (1950)

Dyson, R.D., Cardenas, J.M.: Bovine pyruvate kinases. III. Hybrids of the liver and skeletal muscle isoenzymes. J. Biol. Chem. **248**, 8482–8488 (1973)

Eigenbrodt, E., Schoner, W.: Purification and properties of the pyruvate kinase isoenzymes type L and M_2 from chicken liver. Hoppe-Seyler's Z. Physiol. Chem. **358**, 1038–1046 (1977)

Engström, L.: The regulation of liver pyruvate kinase by phosphorylation-dephosphorylation. Curr. Top. Cell. Regul. **13**, 29–51 (1978)

Faulkner, A., Jones, C.T.: Pyruvate kinase isoenzymes in tissues of the developing guinea pig. Arch. Biochem. Biophys. **170**, 228–241 (1975a)

Faulkner, A., Jones, C.T.: Pyruvate kinase isoenzymes in tissues of the human fetus. FEBS-Lett. **53**, 167–169 (1975b)

Garnett, E.M., Dyson, R.D., Dost, F.N.: Pyruvate kinase isoenzyme changes in parenchymal cells of regenerating rat liver. J. Biol. Chem. **249**, 5222–5226 (1974)

George, J.C., Berger, A.J.: Avian myology. London, New York: Academic Press 1966

Graham, R.C., Karnovsky, M.J.: The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney. Ultrastructural cytochemistry by a new technique. J. Histochem. Cytochem. **14**, 291–302 (1966)

Guder, W.G., Schmidt, U.: Liver cell heterogeneity. The distribution of pyruvate kinase and phosphoenol-pyruvate carboxykinase (GTP) in the liver lobule of fed and starved rats. Hoppe-Seyler's Z. Physiol. Chem. **357**, 1793–1800 (1976)

Hall, E.R., Cottam, G.L.: Isozymes of pyruvate kinase in vertebreates: Their physical, chemical, kinetic and immunological properties. Int. J. Biochem. **9**, 785–793 (1978)

Hammond, K.D., Bahinsky, D.: Isozyme studies of several enzymes of carbohydrate metabolism in human adult and fetal tissues, tumor tissues and cell cultures. Cancer Res. **38**, 1323–1328 (1978)

Harris, W., Days, R., Johnson, C., Finkelstein, J., Stallworth, J., Hubert, C.: Studies of avian heart pyruvate kinase during development. Biochem. Biophys. Res. Commun. **75**, 1117–1121 (1977)

Ibsen, K.H., Trippet, P.: Interconvertible and non-interconvertible rat pyruvate kinase. Biochemistry **11**, 4442–4450 (1972)

Ibsen, K.H., Krueger, E.: Distribution of pyruvate kinase isozymes among rat organs. Arch. Biochem. Biophys. **157**, 509–513 (1973)

Ibsen, K.H., Murray, L., Marles, S.W.: Electrofocusing and kinetic studies of adult and embryonic chicken pyruvate kinases. Biochemistry **15**, 1064–1073 (1976)

Imamura, K., Taniuchi, K., Tanaka, T.: Multimolecular forms of pyruvate kinase. II. Purification of M_2 type pyruvate kinase from Yoshida Ascites Hepatoma 130 cells and comparative studies on the enzymological and immunological properties of the three types of pyruvate kinases L, M_1 and M_2. J. Biochem. (Tokyo) **72**, 1001–1015 (1972)

Irias, J.J., Greenberg, R.R.: Relationship of renal gluconeogenesis to control of ammonia formation. Am. J. Physiol. **223**, 750–755 (1972)

Irving, M.G., Williams, J.F.: Kinetic properties of pyruvate kinase isolated from rat hepatic tumours. Biochem. Biophys. Res. Commun. **68**, 284–291 (1976)

Knull, H.R.: Pyruvate kinase binding to particles in brain. Neurochem. Res. **2**, 461–467 (1977)

Krebs, H.H.: Gluconeogenesis. Proc. R. Soc. London, Ser. B. **159**, 545–563 (1964)

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**, 265–275 (1951)

Muroya, N., Nagao, Y., Miyazaki, K., Nishikawa, K., Horio, T.: Pyruvate kinase isozymes in various tissues of rat, and increase of spleen-type pyruvate kinase in liver by injecting chromatins from spleen and tumor. J. Biochem. (Tokyo) **79**, 203–215 (1976)

Nakamura, T., Hosoi, K., Nishikawa, K., Horio, T.: Effect of injection of chromatins from rhodamine sarcoma into rats on pI-isozymes of liver pyruvate kinase. Gan **63**, 239–250 (1972)

Osterman, J., Fritz, P.J., Wuntch, T.: Pyruvate kinase isoenzymes from rat tissues. Developmental studies. J. Biol. Chem. **248**, 1011–1018 (1973)

Pearse, A.G.E.: Histochemistry, 2nd ed.. London: J. and A. Churchill 1961
Schloen, L.H., Kmiotek, E.H., Sallach, H.J.: Pyruvate kinase isozyme in adult tissues and eggs of Rana pipiens. Comparative studies on the properties of the different isozymes. Arch. Biochem. Biophys. **164**, 254–265 (1974)
Strandholm, J.J., Cardenas, J.M., Dyson, R.D.: Pyruvate kinase isozymes in adult and fetal tissues of chicken. Biochemistry **14**, 2242–2246 (1975)
Strandholm, J.J., Dyson, R.D., Cardenas, J.M.: Bovine pyruvate kinase isozymes and hybrid isozymes. Electrophoretic studies and tissue distribution. Arch. Biochem. Biophys. **173**, 125–131 (1976)
Spellman, C.M., Fottrell, P.F.: Similarities between pyruvate kinase from human placenta and tumours. FEBS-Lett. **37**, 281–284 (1973)
Tamir, H., Kaufmann, H., Rapport, M.M.: Subcellular Distribution of Pyruvate Kinase (EC 2.7.1.40) in Cerebral Cortex. J. Neurochem. **19**, 1759–1768 (1972)
Tanaka, T., Harano, Y., Morimura, H., Mori, R.: Evidence for the presence of two types of pyruvate kinase in rat liver. Biochem. Biophys. Res. Commun. **21**, 55–60 (1965)
Tanaka, T., Harano, Y., Sue, T., Mori, H.: Crystallisation, characterization and metabolic regulation of two types of pyruvate kinase isolated from rat tissues. J. Biochem. (Tokyo) **62**, 71–91 (1967)
Taylor, C.B., Morris, H.P., Weber, G.: A comparison of the properties of pyruvate kinase from Hepatoma 3924-A, normal liver and muscle. Life Sci. **8**, 635–644 (1969)
van Berkel, Th.J.C., Koster, J.F., Hülsman, W.C.: Distribution of L- and M-type pyruvate kinase between parenchymal and Kupffer cells in rat liver. Biochim. Biophys. Acta **276**, 425–429 (1972)
van Berkel, Th.J.C., Koster, J.F.: M-type pyruvate kinase of leucocytes: An allosteric enzyme. Biochim. Biophys. Acta **293**, 134–139 (1973)
van Veelen, C.W.M., Verbiest, H., Vlug, A.M.C., Rijksen, G., Staal, G.E.J.: Isozymes of pyruvate kinase from human brain, meningiomas, and malignant gliomas. Cancer Res. **38**, 4681–4687 (1978)
Wachsmuth, E.D.: An immunohistochemical method for localization of enzymes in tissue sections: The use of antibody bound to tissue antigen and its property of binding cross-reactive antigens. Histochemistry **37**, 251–263 (1973)
Wachsmuth, E.D.: The localization of enzymes in tissues sections by immuno-histochemistry. Conventional antibody and mixed aggregation techniques. Histochem. J. **8**, 253–270 (1976)
Walker, P.R., Potter, V.R.: Allosteric properties of a pyruvate kinase isoenzyme fro rat liver cells in culture. J. Biol. Chem. **248**, 4610–4616 (1973)
Weinhouse, S.: Metabolism and isozyme alterations in experimental hepatomas. Fed. Proc. **32**, 2162–2167 (1973)

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