[CANCER RESEARCH 42, 4228–4232, October 1982]
0008-5472/82/0042-0000$02.00

Characterization of Some Glycolytic Enzymes from Retinoblastoma

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ABSTRACT

Glycolytic enzymes were studied from normal human retinas (both fetal and adult) and from retinoblastomas of eight patients and an established retinoblastoma cell line. No significant differences were found between the enzyme activities in the tissues investigated except for hexokinase and pyruvate kinase, which were significantly decreased in the tumor cells. In fetal retina, five different forms of pyruvate kinase could be detected by electrophoresis (K_4, K_3M, K_2M_2, KM_3, and M_4). In adult retina the K_4 isozyme is almost absent, while in retinoblastoma the M_4 isozyme is hardly present. In the retinoblastoma cell line, the M_4 isozyme is completely absent. Alanine inhibition of pyruvate kinase is in agreement with the electrophoretic pattern. Pyruvate kinase from the retinoblastoma cell line is more inhibited compared to the pyruvate kinase of fetal retina and retinoblastoma and is even more inhibited compared to adult retina.

Electrophoresis of aldolase from adult retina revealed the presence of all potential A-C hybrids (A_4, A_3C, A_2C_2, AC_3, and C_4). Fetal retina, however, is characterized by the predominance of the A type. The same patterns were observed in the retinoblastoma cell line and retinoblastoma. However, in other brain tumors, e.g., gliomas of adults, a five-membered A-C hybrid set is found.

Electrophoresis of hexokinase from normal fetal and adult retina revealed the predominance of hexokinase type I; retinoblastoma and retinoblastoma cell line are both characterized by the presence of considerable amounts of hexokinase type II.

The isozyme shifts in retinoblastoma result in an enzyme pattern identical to that of fetal retina except for the presence of hexokinase type II.

INTRODUCTION

Retinoblastoma is a malignant congenital tumor. In The Netherlands, its incidence is approximately 1:15,000 (18). Most cases are sporadic; some are hereditary. The mode of inheritance is autosomal dominant with about 90% penetrance (23); about 68% of inherited cases are bilateral (11, 23). Patients with retinoblastoma have an increased risk of developing secondary tumors (23). Other cancers have been reported in families of patients with retinoblastoma in a higher incidence than may be expected (5).

It is generally accepted that retinoblastoma stems from neuroectodermal tissue. The retina originates from the optic vesicle, a structure that is derived from the neural tube (15). Therefore, brain and retina may have much in common. The same primitive epithelium lining the cerebral vesicle in the embryo lines the optic vesicle (15). Both nerve tissue and supporting tissue develop from the same primitive neural cells. Tumors of the nerve system, *e.g.*, neuroblastoma, have much in common with retinoblastoma, not only histopathologically but also clinically; familial neuroblastomas also exist.

Neoplasia may be regarded as a disease of genetic regulation the phenotypic expression of which is a misprogramming of protein synthesis (17, 26). Evidence for this view is derived from the observation that, in tumors, enzymes and especially regulator enzymes may be altered (24, 25). Recently, we reported on enzyme alteration in brain tumors (20, 21). Particularly, pyruvate kinase was investigated.

In normal human brain, M_4-type pyruvate kinase is predominant; however, gliomas are characterized by the presence of mainly K_4 type. The shift in isozyme composition is directly correlated with histological grading and malignancy of the tumor. Therefore, the study of isozyme shift in human brain tumors has appeared to be of great diagnostic value (21, 22).

In view of our results on human brain tumors and the common origin of brain and retina tissue, it may be important to investigate some glycolytic enzymes from normal retina and retinoblastoma and to compare it with results of normal brain and brain tumors.

METHODS

Patients

Retinoblastomas of 8 patients (ages 2 months to 6 years) were studied; some of the data are summarized in Table 1. Fetal retinas were obtained from 2 fetuses ages 25 and 29 weeks. Normal adult retinas were obtained from eyes that had been used for cornea transplantation.

Sample Preparation

Normal human adult retinas were totally removed from eyes that were stored at −20° within 1 hr after enucleation. Storage time varied from 1 day to 1 month. Immediately after removal, the retina was stored at −70°. Storage thus far investigated showed no important influences on the enzyme activities.

Normal fetal retinas were totally removed within 4 weeks from the eyes that had been stored at −70°. After death, enucleation of the eyes had taken place within 4 hr.

Retinoblastoma tissue was taken from the eyes after enucleation within 4 hr. They were stored at −70° until enzymological investigations were performed. A part of the tumor (without normal tissue) was used for enzymological investigation.

The retinoblastoma cell line (the AG 1232) was purchased from the

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Received April 13, 1982; accepted June 25, 1982.

Table 1

Data of the 8 retinoblastoma patients studied

Patient	Age at diagnosis[a]	Sex	Ancestry	Presence of tumor	
				Unilateral or bilateral	Sporadic or familial
1	6 yr	F	Dutch	Unilateral	Sporadic
2	1 yr 7 mos.	M	Dutch	Bilateral	Sporadic
3	2 mos.	M	Dutch	Unilateral	Sporadic
4	7 mos.	M	Sicilian	Bilateral	Sporadic
5	4 yr	M	Turkish	Unilateral	Sporadic
6	3 yr 9 mos.	F	Dutch	Unilateral	Sporadic
7	7 mos.	M	Dutch	Unilateral	Familial (?)
8	3 yr	M	Dutch-Antillean	Unilateral	Sporadic

[a] Age at diagnosis is age at surgery.

Human Genetic Mutant Cell Repository, Camden, N. J.

Tissues were homogenized by mixing one part tissue with 5 parts extraction buffer containing 50 mM Tris-HCl (pH 8.0), 0.1 M KCl, 10 mM $MgCl_2$, 2 mM dithiothreitol, and 0.1 M sucrose. The mixture was rapidly minced (maximum duration, 1 min in a potter mincer).

After centrifugation (15,000 × *g* for 10 min), the clear supernatant was used for the experiments; when the enzyme preparation was stored (at −70°), sucrose was added up to a final concentration of 0.5 M (storage had no influence on the enzymes).

Preparation of Mitochondrial and Soluble Hexokinase

One part of the tissue was mixed with 10 parts of extraction buffer containing 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 1 mM dithiothreitol, 1 mM EDTA, 10 mM glucose, and 1 mM diisopropylfluorophosphate. The mixture was homogenized for 1 min. The homogenate was centrifuged at 800 × *g* for 5 min at 4°. The debris was washed with extraction buffer and then discarded, and the supernatant solution was centrifuged at 48,000 × *g* for 10 min at 4°. The activity in the supernatant was referred to as soluble hexokinase. The pellet containing the mitochondrial bound hexokinase was washed twice and resuspended in the extraction buffer supplemented with 10 mM $MgCl_2$ to prevent spontaneous solubilization. For determination of soluble and bound hexokinase activity, both fractions were incubated with 0.5% Triton X-100.

Electrophoresis

Pyruvate Kinase. The extracted enzymes were diluted to an activity of about 1.0 units/ml in the electrophoresis buffer containing 20 mM Tris-citrate (pH 7.7), 1 mM fructose 1,6-diphosphate, 1 mM disodium EDTA, and 0.05 mM dithiothreitol.

Electrophoresis was further carried out as described in Ref. 19.

Scanning of Electrophoretograms. The relative intensities of the bands in the electrophoretogram are quantitated at 540 nm with a densitometer (Helena Quickscan). The percentage of activity in K and M subunits, respectively, are calculated assuming: (*a*) a subunit distribution as indicated by the suffix in K_4, K_3M, K_2M_2, etc.; and (*b*) equal contribution of K and M subunits to the intensity of the stain.

Aldolase. Electrophoresis was performed on cellulose acetate in a 0.04 M sodium phosphate buffer, pH 7.0. The gels were run at room temperature and 10 V/cm during 1.5 hr. The staining mixture contained 0.06 M glycine (pH 8.2); 0.06 M sodium arsenate; 0.06 M sodium pyrophosphate; 5 mM EDTA; 0.4 mM NAD; 2 mM fructose 1,6-diphosphate, glyceraldehyde phosphate dehydrogenase (1 unit/ml), dimethylthiazolyldiphenol tetrazolium bromide (0.16 mg/ml), and phenazine methosulfate (0.04 mg/ml). Fructose 1,6-diphosphate was omitted for blank staining. Scanning was performed as described for pyruvate kinase.

Hexokinase. Electrophoresis on cellulose acetate was carried out at 4° and 20 V/cm (±2 ma/strip) during 45 min in Tris-Veronal buffer (Gelman high-resolution buffer), pH 8.8 (*I* ~ 0.05 mol/l), to which 2 mM glucose, 0.05 mM dithiothreitol, and 1 mM EDTA were added.

Staining for hexokii

(12). Staining solu

$MgCl_2$; 0.2 mM NADP; 0.001 M glucose; 0.0025 M ATP; and 0.21 unit glucose-6-phosphate dehydrogenase. Staining was performed at a glucose concentration of 2 mM and 0.1 M to identify hexokinase III, if present. Hexokinase III has a low K_m for glucose and is inhibited at high glucose concentrations (10). Blank stains in the absence of ATP were carried out.

Assay for Glycolytic Enzymes

Glycolytic enzyme activities were determined by the methods of Beutler (3) at 37° and expressed as units/mg protein. The protein content was determined by the method of Lowry *et al.* (14) with crystalline bovine serum albumin as standard. Alanine inhibition of pyruvate kinase was determined as described previously (19).

RESULTS

Enzyme Activities

Table 2 summarizes the activities of some glycolytic enzymes from fetal retina, adult retina, retinoblastoma, and retinoblastoma cell line. No significant differences are found between the activities of the enzymes from normal retina and tumor except for hexokinase and pyruvate kinase, which are decreased in the tumor.

Characterization of the Enzymes

Pyruvate Kinase. Three main types are known: L (liver), M (muscle), and K (kidney) type. Each of these isozymes is composed of 4 identical or nearly identical subunits (6). Recently, we could identify in white and gray matter of human fetal brain 2 isozymes, M and K types together with 3 hybrids composed of M and K subunits (21). These isozymes and hybrids are, according to their subunit composition, designated as M_4, KM_3, K_2M_2, KM_3, and K_4 (16). Fig. 1 shows the electrophoresis of pyruvate kinase extracted from fetal retina, adult retina, retinoblastoma, and retinoblastoma cell line. Fetal retina is characterized by the presence of 5 bands, representing the M_4 and K_4 type and the hybrids KM_3, K_2M_2, and K_3M. Adult retina contains the same set of isozymes but only a small amount of K_4, while in retinoblastoma the M_4 type is almost absent; in the retinoblastoma cell line, no M_4 type at all could be detected.

The percentage of K subunits is calculated from the electrophoretogram of Fig. 1 by scanning (see "Materials and Methods") and summarized in Table 3. The lowest percentage of K subunits is found for adult retina. Retinoblastoma and fetal retina show the same value, whereas the highest amount is present in the retinoblastoma cell line. Earlier, we demonstrated a close relationship between the percentage of K subunits and the alanine inhibition of pyruvate kinase (19). This is based on the strong inhibition of the K_4 type by alanine, whereas the M_4 type is not inhibited at all by this amino acid. The residual activity of pyruvate kinase in the presence of alanine is the same for fetal retina and retinoblastoma and the highest for adult retina, whereas the retinoblastoma cell line shows the lowest value (Table 3). The results of electrophoresis, percentage of K subunits, and alanine inhibition are in good agreement with each other.

Aldolase. Aldolase is also a tetramer of which 3 main isozymes are known (13). The homomeric isozyme A_4 (least

Table 2
Activity of glycolytic enzymes in normal fetal and adult retina, retinoblastoma,

	Activity (units/mg protein)							
	Hexokinase	Phosphoglucose isomerase	Aldolase	Triosephosphate isomerase	Glyceraldehyde phosphate dehydrogenase	Phosphoglycerate kinase	Enolase	Pyruvate kinase
Fetal retina (n = 2)	0.16-0.10	2.38-1.80	0.11-0.07	41.16-46.30	5.40-3.09	4.02-3.01	0.72-0.50	1.28-2.83
Adult retina (n = 11)	0.23-0.08	3.36-0.53	0.16-0.04	38.77-10.17	3.06-1.88	4.87-1.11	1.19-0.66	6.70-1.66
Retinoblastoma (n = 3)	0.05-0.015	1.82-0.45	0.20-0.15	26.71-14.17	1.24-1.76	2.57-0.29	0.57-0.14	2.88-0.84
Retinoblastoma cell line (n = 1)	0.06	1.60	0.08	17.6	1.05	1.82	0.57	2.37

Table 3
Percentage of K subunits calculated from Fig. 1 and residual activity of pyruvate kinase in the presence of alanine

	%	
	K subunits	Residual activity of pyruvate kinase in the presence of 4 mM alanine[a]
Fetal retina (n = 2)	60 ± 4[b] (2)[c]	45 ± 5 (2)
Adult retina (n = 11)	43 ± 4 (17)	62 ± 7 (19)
Retinoblastoma (n = 3)	58 ± 3 (8)	46 ± 9 (8)
Retinoblastoma cell line (n = 1)	69 (1)	30 (1)

[a] The 100% value is the activity without alanine.
[b] Mean ± S.D.
[c] Numbers in parentheses, number of determinations.

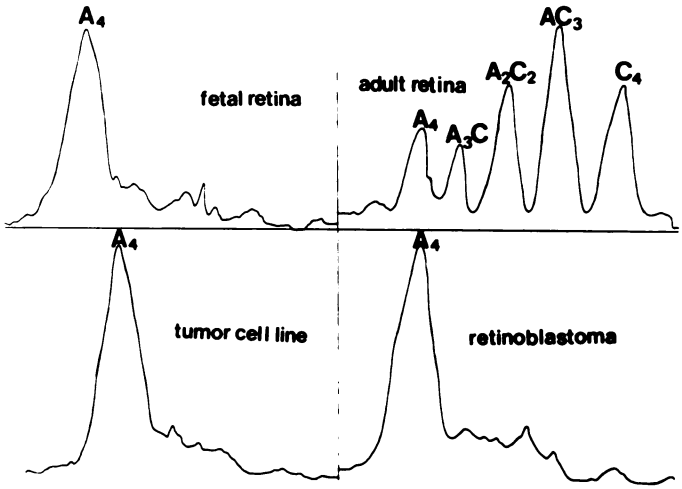

Chart 1. Scanning of the electrophoretogram of aldolase extracted from fetal retina, adult retina, retinoblastoma, and retinoblastoma cell line.

anodal) is predominant in adult skeletal muscle but also in most other tissues. Aldolase B_4 is usually confined to liver and kidney, and aldolase C_4 (most anodal) is the most principal form in nervous tissue were it occurs as part of a 5-membered A-C hybrid set.

Scanning of the electrophoretogram of aldolase extracted from fetal retina, adult retina, retinoblastoma, and retinoblastoma cell line is shown in Chart 1. In adult retina, all hybrids of aldolase A and C are present (A_4, A_3C, A_2C_2, AC_3, C_4). Fetal retina, however, is characterized by the predominance of type A_4. The same pattern is observed in both retinoblastoma and retinoblastoma cell line.

The percentage of A subunits can be calculated from the densitograms. For normal adult retina, this percentage is 51 ± 9.3 (S.D.); in fetal retina, 71 and 89%, respectively, was found, whereas in retinoblastoma 94.5 ± 6% and retinoblastoma cell line 90% was found. It is clear that in the tumor cells a shift in isozyme composition occurs towards the fetal pattern.

Hexokinase. Four isozymes of hexokinase are known, designated as I to IV in order of their increasing anodal electrophoretic mobility. Type IV, glucokinase, is specific for liver (19). It is known that hexokinase types I and II can bind to the mitochondrial outer membrane (4). The percentage of soluble hexokinase is: for fetal retina, 43 ± 2 (n = 2); for adult retina, 40 ± 12 (n = 15); for retinoblastoma, 38 ± 7 (n = 4); and for the retinoblastoma cell line, 35 (n = 1). No significant differences could be demonstrated. In all cases thus far investigated, about the same percentage was found. Fig. 2 shows the electrophoresis of soluble hexokinase from fetal retina, adult retina, retinoblastoma, and retinoblastoma cell line. Fetal and adult retina are characterized by the predominance of hexokinase type I. In some cases of adult retina, we observed a small amount of hexokinase type II as well as type I. From scanning plots (not shown), the percentage of hexokinase type I has calculated to be 61 (and 39% hexokinase type II) for retinoblastoma and 45 (55% hexokinase type II) for retinoblastoma cell line. Therefore, the high amount of hexokinase type II seems to be characteristic for the tumor cells.

DISCUSSION

There is strong evidence that a massive alteration in isozyme composition exists inside cancer cells as compared to its pattern in normal tissue. In particular, alterations in carbohydrate metabolism have been reported (1, 8, 9, 24, 26). Recently, we demonstrated that in human gliomas a shift in pyruvate kinase isozyme composition occurs (22).

In this paper, we report the characterization of pyruvate kinase, hexokinase, and aldolase from normal retina and retinoblastoma. The differences found for the activity of hexokinase and pyruvate kinase in normal retinas compared with retinoblastoma must be taken very prudently. First of all, we assayed only 3 tumors for enzyme activities, and standard errors are large. Also, the normal tissues were from adults, whereas the tumors were from children and infants.

With respect to pyruvate kinase, one may conclude that the observed isozyme pattern in retinoblastoma resembles the pattern found in fetal brain. Fetal brain is characterized by the presence of all the potential 5 forms (K_4, K_3M, K_2M_2, KM_3, M_4); the same is found for fetal retina. However, adult retina contains little K_4 while for retinoblastoma type M_4 is almost absent, the

other 4 forms being present. Although the total amount of K subunits in retinoblastoma is increased, the isozyme pattern is quite different from that in gliomas (mainly K_4 and little K_3M, but no other forms).

Imamura and Tanaka (8) found that the K type of pyruvate kinase was strongly predominant in young rat fetuses and identified it as the fetal form of pyruvate kinase. It has also been proposed that the M type is a modified K enzyme and that therefore the isozymes represent different molecular forms of the same gene (7).

We have no real explanation why sometimes (*e.g.*, Fig. 1, *Lane* 3) mainly K_3M and not K_2M_2 is present. It may be that different proportions of isozymes in different portions of the tissue are involved.

With respect to hexokinase, striking differences are found between retinoblastoma and normal retina: hexokinase type II is present in considerable amounts in the tumor. Therefore, in retinoblastoma, there is an isozyme shift towards hexokinase type II. It is well known that hexokinase type II is present in other tumors, for example, in gliomas (2, 9); however, the amount of hexokinase type II is much less than in retinoblastomas. Bennet *et al.* (2) reported that hexokinase type II is present in normal brain tissue only during the last 4 weeks of gestation. The fetal retinas studied by us were of an earlier period of gestation, and no hexokinase type II was observed.

The presence of hexokinase type II in retinoblastoma and the absence in fetal retina indicates that the appearance of this particular isozyme in the tumor may be an expression of an ordered alteration in gene expression rather than a dedifferentiation towards a more primitive undifferentiated form of the tumor.

Aldolase isozyme distribution has been reported to change in many tumors (17). Normal retina is characterized by the presence of the A–C hybrid set. In retinoblastoma, the synthesis of C subunits is depressed almost completely. However, in gliomas of adults, the 5-membered A-C hybrid set is present (16).[2] Therefore, with respect to aldolase, differences are found between retinoblastoma and gliomas of adults. The observed isozyme shift of aldolase may be explained by a shift toward the fetal stage. The isozyme shifts observed in retinoblastoma differ from those found in gliomas (20, 21). Therefore, it may be important to study pyruvate kinase, hexokinase, and aldolase in other neuroectodermal tumors such as neuroblastomas and medulloblastomas.

Preliminary results suggest that the enzymology of these embryonic tumors is in many respects the same as that of retinoblastoma, indicating a common origin. These results will be presented in another paper.

ACKNOWLEDGMENTS

For their aid in the preparation of this study, we are indebted to Drs. K. E. W. P. Tan and F. Dubois, University Eye Clinic, Utrecht; Dr. J. J. van der Harten, pediatric pathologist, Department of Histopathology, Free University, Amsterdam; Professor J. Huber, pe
Utrecht. M. de Jong a
also thank Drs. G. Jansen, E. D. Sprengers, and C. W. M. van Veelen for helpful discussions.

[2] Van Veelen, C. W. M., Rijksen, G., and Staal, G. E. J., unpublished results.

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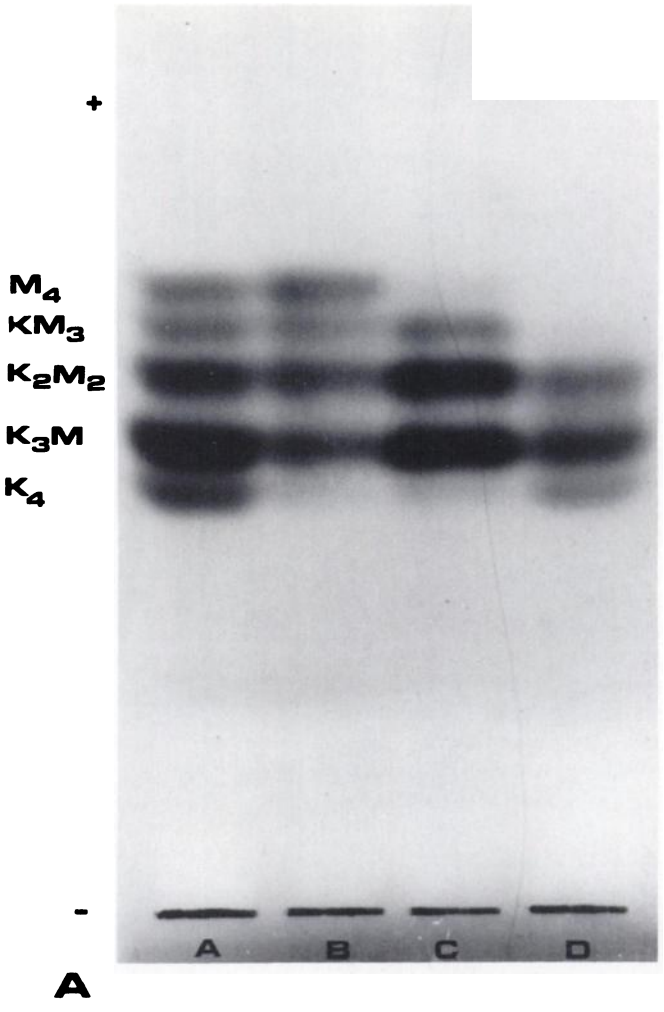

Fig. 1. Electrophoretic pattern of pyruvate kinase extracted from fetal retina (*A*), adult retina (*B*), retinoblastoma (*C*), and retinoblastoma cell line (*D*).

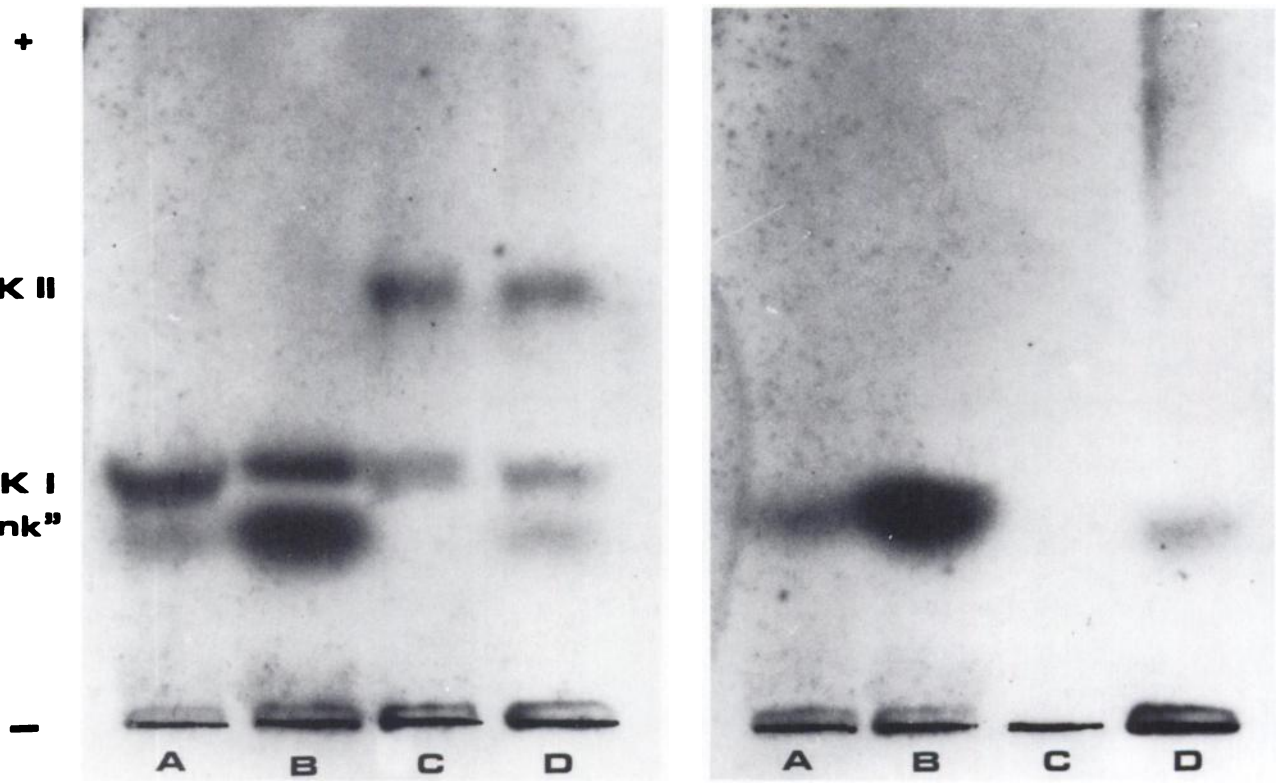

Fig. 2. Electrophoretic pattern of soluble hexokinase (*HK*) from fetal retina (*A*), adult retina (*B*), retinoblastoma (*C*), and retinoblastoma cell line (*D*) in the presence (*A*) and in the absence (*B*) of $MgATP^{2-}$.