ISOENZYMES OF ASCITES TUMOR CELLS

IN VIVO AND IN VITRO

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1. INTRODUCTION

Cancer cells are different from normal cells in that their growth is not limited by the host. In general, normal cells in adult individuals are continuously dying off and are continuously replaced by new cells. A fairly constant equilibrium is maintained between the death rate of old cells and the rate of multiplication for new cells. During repair of a wound the cells multiply more rapidly and once the repair is completed the equilibrium between death and multiplication of the cells will be reinstated. Such an equilibrium serves as a control exerted by the body to prevent the overgrowth of one type of cells so that harmony is maintained in the body. Somehow, cancer cells multiply continuously faster than they die off, and such a growth is not limited by the body. What makes this growth unlimited has not been demonstrated. Since cancer and normal cells are different in their mode of growth a difference somewhere in the cell is also suspected to exist between them. The difference may be minute, but sufficient to effect the unlimited growth of cancer cells. The search for a difference is then logical with the hope of throwing some light on the nature and the origin of cancer.

At present, cancer cells are considered to arise from normal somatic cells (9). A genetic alteration in a normal cell may lead to the rise or deletion of some enzymes and may

thus alter the cellular metabolism. Alteration of cellular metabolism may possibly result in cancerous growth. Since chromosomes are considered to be the apparatus for the transfer of hereditary information, chromosomal change would be a good evidence for a genetic alteration. Indeed, chromosomal anomalies of cancer cells of different species have been repeatedly reported (18,36,51,56). The anomalies can be arbitrarily classified into three groups; namely, variations in chromosome morphology, variations in gene arrangement and variations in chromosome number, and these may overlap to some extent. However, there is no evidence that the chromosome alterations are directly responsible for the cancerous growth. Furthermore, similar alterations can be induced by carcinogens and viruses without the production of tumors, and tumors can be produced without any apparent chromosome alterations (56).

A tremendous amount of research has been done in an attempt to find some biochemical differences between normal and cancer cells. Warburg (104) discovered that a high level of glycolysis and a high production of lactic acid are generally characteristic of malignant cells. He has proposed that respiration in tumors is defective. This hypothesis has been disputed by Weinhouse (106) on the basis that there is simply no evidence for defective or damaged respiration. Greenstein (29,30) found apparent biochemical similarities among tumors from tissues of different origin. While he recognized the

great diversity in the biology of tumors, he suggested the "hypothesis of convergence" that tumors of different origin tend to have a similar biochemical pattern. But a unique characteristic cannot be established. Some enzymes have been shown to be absent or present at very low level (6,7,11, 12). Potter (76) has proposed the "deletion hypothesis" that deletion of catabolic enzymes in one way or another may lead to more efficient use of substrates via anabolic pathways and may then cause a loss of growth restraint which could lead to neoplasia. But so far there is no conclusive evidence to correlate the deletion of enzymes and the neoplastic growth. Numerous exceptions have been encountered whenever a general explanation has been proposed to distinguish cancer cells from normal cells.

Perhaps the anomalies of cancer cells lie at the genic level, which may be difficult to detect. Alteration of a deoxyribonucleic acid (DNA) molecule may give rise to a new gene, and hence a new enzyme which may alter the metabolic control mechanism and lead to malignant growth. Pauling et al. have observed that the abnormal electrophoretic behaviour of sickle cell hemoglobin is due to a single amino acid substitution in the protein molecule (71). It became evident that abnormal biochemical structure due to gene mutation at the molecular level may indeed cause metabolic diseases (38). An analogy of this kind to cancer cells would help to elucidate the origin and nature of malignancy. With the advent of

the discovery that enzymes may exist in multiple molecular forms, or isoenzymes (37,85,101,111), the detection of qualitative biochemical marker(s) in cancer cells can be approached in a more sensitive way.

The concept of multiple molecular forms of enzymes has been well established in the last five years. The advancement of this concept may be attributed to the development of the starch gel electrophoresis by Smithies (86) for the separation of closely related proteins, and to the development of histochemical methods (37,57) for visualizing the isoenzymes directly on starch gels. This investigation is a search for distinctive biochemical marker(s) in cancer cells at the isoenzyme level and to correlate malignancy with the isoenzyme patterns of lactate dehydrogenase (LDH), glucose-6-phosphate dehydrogenase (G-6-PD), malate dehydrogenase (MDH) and non-specific esterases in ascites tumor cells. It is hoped that by studying the isoenzyme patterns of the enzymes chosen, information might be obtained regarding possible alteration of the main catabolic pathways in cancer cells.

Ascites tumor cells have been found to produce high concentrations of lactic acid (104). Lactate dehydrogenase is thus selected to represent the enzymes in the Embden-Myerhof--Parnas pathway (28). It requires the coenzyme nicotinamide adenine dinucleotide (NAD) for catalyzing the reaction of the interconversion of lactate and pyruvate:

$$\text{pyruvate} + H^+ + \text{NADH} \xrightleftharpoons{\text{LDH}} \text{lactate} + NAD^+$$

Malate dehydrogenase is an enzyme in the tricarboxylic acid cycle which is the main pathway for producing energy under aerobic conditions. It also requires NAD for the reaction:

$$\text{malate} + NAD^+ \xrightleftharpoons{\text{MDH}} \text{oxaloacetate} + H^+ + \text{NADH}$$

Glucose-6-phosphate dehydrogenase leads into the pentose phosphate shunt which is the main pathway for the biosynthesis of ribose phosphate (28). Beaconsfield and Reading (8) have reported that the utilization of the pentose phosphate shunt in glucose catabolism is in parallel with the biosynthesis of nucleic acid. Since ascites tumor cells are fast growing, they should have a high rate of nucleic acid synthesis, and may have a parallel high activity of G-6-PD. For this reason, G-6-PD would seem to be important in relation to the rapid growth of malignant cells. This enzyme requires the coenzyme nicotinamide adenine dinucleotide phosphate (NADP) and magnesium for the following reaction:

$$\text{glucose-6-phosphate} + NADP^+ \xrightleftharpoons{\text{G-6-PD, } Mg^{++}} \text{6-phosphogluconate} + \text{NADPH} + H^+$$

Non-specific esterases are believed to consist of a variety of closely related enzymes using a broad spectrum of

substrates. Although the substrate specificity for the different esterases may vary, they all catalyze the reaction of breaking the ester bond of the substrate. Esterase was the first enzyme to be identified histochemically on starch gel (37).

In the experimental work to be described, conventional starch gel electrophoresis with a discontinuous buffer system (77) has been used to determine the isoenzyme patterns of non-specific esterases, LDH, G-6-PD and MDH in the cells from a variety of ascites tumors and their sublines maintained by serial passage in mice. Similar studies have also been made with established tissue culture lines of several varieties of ascites tumor cells. Several of these lines were established in tissue culture for more than five years and had lost their tumorigenic capacity; some recently established lines still retained this capacity. Back transplantation of the cells of the tumorigenic tissue culture lines into mice was undertaken and the isoenzyme patterns were also studied.

2. HISTORICAL REVIEW

Similar enzymes isolated from different organisms may have wide variations in their physical properties although they catalyze the same reactions (3,5,19,93). Freudenberg in 1940 (25) first reported the presence of other proteolytic enzymes associated with pepsin in gastric juice and that they had different pH optima. This observation has been supported by Buchs (14), Ryle and Porter (82) and Tang et al. (91).

In 1950, Meister (62) found two protein fractions of crystalline beef heart lactate dehydrogenase (LDH) in the Tiselius electrophoresis. This was confirmed by Neilands (66) with the further observation that both the fractions possessed LDH activity. Thus, a purified enzyme may exist in more than one active form. However, these forms might have been derived from a single-component enzyme due to the purification procedure. When human serum was found to contain multiple protein fractions with lactate dehydrogenase activity by Sayre and Hill (83) and by Vesell and Bearn (101), it became quite clear that an enzyme from a single organism can exist in electrophoretically distinct forms. Unfortunately, there was no evidence for the direct origin of the different forms of lactate dehydrogenase. The possibility still remained that only a single form was synthesized in the cells but appeared as multiple active forms in the blood. This

doubt was soon removed when various enzymes from different organs and tissues of an organism, and from cells of established tissue culture lines, were found to consist of multiple forms (37,57,74,98,107). Finally, enzymes from single cell organisms, such as lactate dehydrogenase (31,68), hexokinase (40) and fumarase (35) in yeast, aspartokinase (87) in Escherichia coli and esterase in Tetrahymena pyriforms (2), were also shown to have more than one component. With all this evidence, it has become obvious that enzymes commonly exist in multiple molecular forms within the cells of a single organism or even within a single cell.

Markert and Møller in 1959 (58), realizing the limitation of the classification of enzymes based on substrate specificity, proposed to use the term "isozyme" to describe the different molecular forms in which proteins from an organism may exist with the same enzymatic specificity. The Standing Committee on Enzymes, of the International Union of Biochemistry (105) has recommended a system for the nomenclature of multiple enzyme forms. The system is:

"Multiple enzyme forms in a single species should be known as isoenzymes; the term isozyme is acceptable since it has been widely used. Multiple enzyme forms may be distinguished from one another by any of several means, e.g. electrophoresis, chromatography, salt fractionation, ultracentrifugation, immunochemistry and reaction kinetics. When multiple forms of an enzyme are identified by electrophoretic

separation, they should be given consecutive numbers, the form having the highest mobility towards the anode being numbered one."

Accordingly, the term isoenzyme shall be used throughout this report. About one hundred enzymes have thus far been shown to be separable in multiple molecular forms with similar substrate specificity (85,103). And the phenomenon of isoenzymic forms has been found to be widespread in the whole biological kingdom including plants, animals and unicellular organisms.

Among the numerous enzymes which have been observed to exist in multiple forms, lactate dehydrogenase is the one most extensively studied. Wieland and Pfleiderer (67) first demonstrated five distinct isoenzymes of LDH with paper and starch gel electrophoresis and showed that the isoenzyme patterns are species and tissue specific based on the distribution of the isoenzymes. This was confirmed by Markert and Møller (58), who compared the LDH isoenzyme pattern of beef heart with the patterns of isoenzymes from the hearts of sheep, pig, mouse and rabbit. Each pattern was found to be unique. Although the electrophoretic positions of the isoenzymes remained unchanged, the number of isoenzymes and the relative amount of the different isoenzymes present may be different for the same organ from different species. For example, mouse heart has all five LDH isoenzymes with the number 3 and number 4 predominant; whereas pig heart has only

two isoenzymes (nos. 1 and 2) with no. 1 predominant. When the LDH isoenzyme patterns of seven different tissues of the pig were examined (58), each of these patterns was shown to be different to some degree from all the others and was thus tissue-specific. Furthermore, embryonic tissues were found to contain isoenzyme patterns differing from the patterns of the corresponding adult tissues. The heart from a 12 cm embryonic pig, an active functioning organ at this embryonic stage, contains a larger number of LDH isoenzymes (three isoenzymes) than adult pig heart (two isoenzymes). Embryonic skeletal muscle, on the other hand, contains fewer isoenzymes than adult muscle. The final adult pattern is reached by both gains and losses in the isoenzyme repertory of embryonic tissues. This observation has been confirmed by Markert and Ursprung (59) and Fine et al. (24) and also in chick embryo by Cahn et al. (16), Lindsay (54) and Nebel and Conklin (65).

The variations of the LDH isoenzyme pattern have raised many questions with regard to phylogeny, ontogeny, cell differentiation, genetics, physiology and metabolic control mechanisms. Why should a cell contain more than one form of an enzyme to give the similar function? Does the presence of more than one form of an enzyme offer any advantage to an organism concerning the physiology and the metabolic control mechanism? How do the isoenzymes evolve? What is the relationship of isoenzymes to phylogeny and ontogeny? Are the isoenzymes synthesized individually and controlled by

different genes or are they all derived from one fundamental form controlled by a single gene? How do the isoenzymes differ chemically and structurally from each other? The numerous studies conducted in the attempt to answer some of these questions in the last five years appear to have led to some understandings of certain fundamental problems in biology, such as cell differentiation and metabolic control mechanisms.

In analyzing the amino acid composition of the five different LDH isoenzymes from the rat, Wieland and Pfleiderer (108) have shown that although all the isoenzymes have the same molecular weight (130,000), they differ from each other in their protein primary structure and in the ratio of acidic to basic side chains, on which the electrophoretic mobility depends. The LDH isoenzymes have also been found to be different with respect to their pH optima, influence of pyruvate concentration, antibody-inhibition (75), and the use of coenzyme analogues (42). Based on all these observations, Appella and Markert (4) proposed the subunit theory of two distinct genes for the biosynthesis of the LDH isoenzymes. They treated crystalline LDH from beef heart with 5M guanidine-HCl. The LDH molecule was dissociated into four subunits of equal molecular weight, as determined by sedimentation studies. These subunits, or polypeptide chains, were shown to exist in two different electrophoretic varieties which were designated as subunit A and B. They theorized that the five isoenzymes are the five different tetramers that would be obtained by

associating these two subunits in all possible combinations of four (4,59). The subunit formula for the LDH isoenzymes has the relationships: LDH-1 = BBBB, LDH-2 = BBBA, LDH-3 = BBAA, LDH-4 = BAAA and LDH-5 = AAAA. Cahn et al. (16) and Lindsay (54), using chicken LDH, showed that the two "pure" tetramers (LDH-1 and LDH-5) are immunologically distinct and, further, that the three intermediate isoenzymes (LDH-2, 3 and 4) are cross-reactive with both "pure" types. Then, Markert (60) dissociated LDH-1 and LDH-5 into subunits by freezing in 1M NaCl; after thawing, the subunits recombined at random in groups of four to yield all five isoenzymes in approximately the expected ratio of 1:4:6:4:1 with a corresponding reduction in the quantities of LDH-1 and LDH-5. Thus, the subunit theory is well established.

In advancing the subunit theory, Markert et al. (4,59) have suggested that the two different polypeptides, A and B, are under the control of two separate genes. This hypothesis was confirmed by Shaw and Barto (84) with genetic data from crossing homozygous and heterozygous deer mice.

Since the isoenzyme patterns of LDH are different during the course of ontogenic development, it may imply that the expression of the genes is also changing (15,24,54,58,59, 65) at the same time. The possible relation between cellular differentiation and the change of the LDH isoenzyme patterns during embryonic development was excellently discussed by Markert (61). The embryonic patterns of mouse with LDH-5

predominant in all tissues (59) suggest that the gene for the subunit polypeptide A first becomes active. Later the shift in pattern with the appearance of the other isoenzymes that occurs in most tissues implies that the gene for the subunit B is progressively activated while the A gene is relatively suppressed. This sequence of gene activation and inhibition is not true in all tissues, skeletal muscle being a notable exception with LDH-5 predominant in the adult pattern. In general, those tissues subject to relative anaerobiosis tend to be richer in LDH-5 while those abundantly supplied with oxygen have more LDH-1 at the other end of the spectrum. These distributions are also correlated with the synthesis of lactic acid. Wherever lactic acid tends to accumulate, LDH-5 is likely to be the most abundant isoenzyme. This is true for adult skeletal muscle and also for the tissues of the early mammalian embryo which has little supply of oxygen. It is clear that by regulating the rate of production of the two subunits the tissues can accomplish the appropriate isoenzyme patterns to suit their physiological functions at various stages.

Although all the LDH isoenzymes catalyze the same reaction for the interconversion of pyruvate and lactate, their catalytic efficiencies vary with such conditions as pH and substrate concentration. Plagemann et al. (75) and Cahn et al. (16) have demonstrated that LDH-1 (predominant in heart) is maximally active at low concentrations of pyruvate

and is strongly inhibited by excess pyruvate, whereas LDH-5 (predominant in skeletal muscle) maintains its activity at relatively high pyruvate concentrations. These facts may be related to function in the following way. In skeletal muscle there is a requirement for sporadic, sudden release of energy in the relative absence of oxygen. This energy is supplied by glycolysis, which produces large amounts of pyruvate and requires its reduction to lactate. Muscle LDH (LDH-5) allows this reaction to take place despite high levels of pyruvate and temporary accumulation of high levels of lactate which may be toxic to the cell. In the heart, a steady supply of energy is favourable, and this is maintained by the complete oxidation of pyruvate and lactate in mitochondria. The inhibition of heart LDH (LDH-1) by high level of pyruvate favors this oxidative pathway and also protects the heart from toxic concentrations of lactic acid. It is clear that the LDH isoenzyme patterns are significant in the metabolic control mechanism in relation to the physiological functions of the different tissues.

Isoenzymes can be used as a genetic marker to identify certain genotypes which are different from normal. Deficiency in erythrocytic glucose-6-phosphate dehydrogenase (G-6-PD) activity is well recognized as an enzyme defect which has been found in all major races of man in varying frequencies (81). It has been shown that primaquine causes hemolysis in the otherwise healthy individuals having this

enzyme defect. Boyer et al. (12,13) reported two electrophoretic forms (fast and slow) of erythrocytic G-6-PD in American Negroes. The G-6-PD in deficient Negro males usually migrates to the fast position. The slow form of the enzyme is the common type found in Caucasians. Studies in humans by Kirkman and Hendrickson (45) and in Drosophila by Young et al. (114) have shown that the different G-6-PD phenotypes distinguishable by their electrophoretic isoenzymes follow a sex-linked mode of inheritance. The genes responsible for G-6-PD are linked with the X chromosome. Thus, the examination of the isoenzyme pattern of G-6-PD may serve as a tool to diagnose a congenital metabolic error. Furthermore, Davidson et al. (21) using tissue culture technique, demonstrated the existence of two distinct populations of cells in women heterozygous for qualitative G-6-PD variants. From the same tissue of the heterozygous female, one type of cell has the fast moving G-6-PD isoenzyme, and the other type has the slow moving one. This would indicate that, as far as the gene locus for G-6-PD is concerned, only one X chromosome in each cell is functional. This seems to be direct evidence to support the "Lyon Hypothesis" which proposes that in each somatic cell of the female, one of the two X chromosomes is genetically inactive.

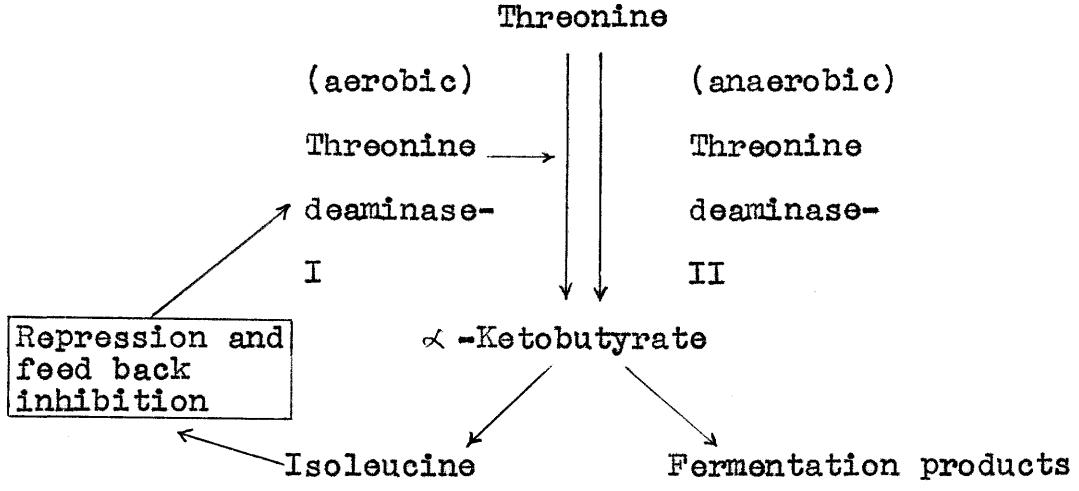
With regard to evolution, Kaplan et al. (41,42,110) have studied the lactate dehydrogenases and malate dehydrogenases of about one hundred species. By determining the

molecular weight, amino acid composition and fingerprint pattern, and by measurements of temperature stability, electrophoretic mobility and catalytic activity with coenzyme analogs, they were able to conclude that the dehydrogenases are phylogenetically related and the changes of the dehydrogenases appear to be associated with evolution. It seems that the studies of isoenzymes are also valuable in comparative biochemistry and in taxonomy.

Normal human serum was found to have a characteristic LDH isoenzyme pattern (112). Characteristic alterations in the distribution of the isoenzymes were found in a variety of diseases associated with elevated serum LDH levels (20,109, 112). For example, there is an increased level of the fast moving LDH isoenzymes in the serum of patients suffering from myocardial infarction, hemolytic anemia and renal injury, and an increase of the slow moving LDH isoenzyme in liver disease (109) and muscular dystrophy (43,72). The elevation of the serum LDH isoenzyme levels generally reflects the sites of organ injury. Thus, the LDH isoenzyme patterns of serum can be used as a means of diagnosis in clinical pathology.

Studies by Umbarger and Brown (99) reveal that Escherichia coli has two different threonine deaminases, or two isoenzymes of this enzyme, one of which is under the selective feed back control by isoleucine, whereas the other is formed only under anaerobic conditions in the absence of fermentable sugars, and is not subject to end-metabolite

regulation. It is best explained in the following illustration.



The above scheme shows how the isoenzymes control the common steps both in synthetic and degradative processes. When fermentable sugars are available under aerobic conditions, the pathway for the synthesis of isoleucine is favoured without the over accumulation of isoleucine due to the feed back inhibition. When fermentable sugars are not available under anaerobic conditions, the other pathway is favoured by using a different isoenzyme for the manufacture of sugars for energy. The two different isoenzymes help the cell to avoid conflict between end-product regulation and energy metabolism. This is another mode of action of isoenzymes in the metabolic control mechanism. Umbarger proposed the principle (100) that "whenever a chemical reaction in a cell is catalyzed by an enzyme whose formation and activity are under the rigid control of repression and end-

product inhibition, a second enzyme will be needed by the cell if there is some other essential role for that reaction to play in the cell." This principle might explain the significance of the existence of multiple active forms of an enzyme.

Malate dehydrogenase (MDH) catalyzes the reaction of the interconversion of malate and oxaloacetate. It has been established that it exists in two main forms, namely, the supernatant MDH, and the mitochondrial MDH (23,32,48,94). The number of MDH isoenzymes is different depending on the species. Kun and Volfin (49) recently reported that oxaloacetate above 10^{-4}M causes marked substrate inhibition of mitochondrial MDH isolated in pure form from beef heart, but not the supernatant MDH. The degree of substrate inhibition of the mitochondrial MDH by oxaloacetate was found to be a function of the concentration of the coenzyme reduced nicotinamide adenine dinucleotide ($NADH^+$). This shows that isoenzymes may exist at different sites in the cell. It is conceivable that different parts in the cell would have different conditions with regard to pH and the concentrations of substrates and cofactors. One single form of enzyme may not be able to carry out its function efficiently under different conditions. Perhaps this is why a cell needs more than one form of an enzyme so that the appropriate function of the enzyme can be accomplished under different conditions at different loci in the cell. The compartmentation of isoenzymes in a cell appears to add another advantage to the metabolic

control mechanism.

The studies of isoenzyme patterns with tissue culture methods are also of notable interest. Paul and Fottrell (70) and Vesell et al. (102) have observed that the isoenzyme patterns may or may not change and are species-specific when cells are grown in tissue culture and this specificity is retained even after long term culturing (e.g. Hela cells in tissue culture for ten years). The isoenzyme patterns of esterases are very stable and no essential difference has been found between the pattern in fresh tissue, short-term and long-term tissue culture cells (9,47,70). The LDH isoenzyme patterns were found to be unstable in tissue culture. When cells were propagated in vitro, there was a progressive loss of the more rapidly migrating anodal band(s) (67,73,79). Cahn et al. (16) and Cahn (17) have shown that during the development of the chick embryo breast muscle, the LDH-5 subunit characteristic of adult breast muscle makes its appearance abruptly at approximately 6-10 days and no similar change in embryonic chick heart muscle occurs. The adult heart LDH pattern is almost identical to the pattern of the heart prior to 6-10 days, which is predominant with the LDH-1 isoenzyme. Heart cells never synthesize LDH-5 in any significant amounts in vivo; however, when heart cells were put in tissue culture, within a few days, LDH-1 was found to decrease and LDH-5 increase greatly, although the heart cells were still beating. Citric acid cycle intermediates, coenzyme A

and high oxygen culture conditions retarded the appearance of this newly acquired synthetic capacity. Cahn suggested that the act of explantation and culturing of the heart tissue in itself caused a rapid "turning on" of the gene controlling the functioning of the LDH-5 subunit. How tissue culture conditions cause the "turning on" of a gene remains unanswered.

With regard to cancer, since the level of LDH in cancer cells has long been known to be generally higher than in normal cells (104), studies of the isoenzyme patterns of LDH in malignant tissues appear to be valuable in attempting to understand the nature of malignancy. Starkweather and Schoch (38), comparing the LDH isoenzyme patterns of many normal and neoplastic human tissues, have reported that there is a shift of the pattern in all the malignant tissues examined by them. Tumor LDH tends to lose the high degree of heterogeneity observed in normal human tissues and there is a significant increase in the LDH-3 in all malignant tissues. The Michaelis constants for the LDH-3 in different malignant tissues are about the same but differ markedly from the Michaelis constant for the LDH-3 in normal tissues. The data suggest that LDH-3 associated with neoplastic tissues may represent a structurally different protein characteristic of neoplastic tissue. Other workers have also shown the shift of the LDH isoenzyme patterns of cancer tissues with increased levels of the slow moving LDH-5 (27,80,81,113). Benign tumors

were found to be essentially indistinguishable from their normal tissue of origin in the comparison of the LDH isoenzyme patterns (27). Prout et al. (78) reported also the increased levels of LDH-4 and LDH-5 in sera from patients with prostatic carcinoma, and removal of the tumor suppressed the level to normal. It seems that, when a tissue undergoes malignant transformation, the lactate dehydrogenase isoenzyme distribution changes from that characteristic of the normal tissue towards the uniform pattern of cancer with a preponderance of the slow-moving LDH isoenzymes.

Certain viruses are known to be associated with the production of malignancy in animals. There is a possibility that the same will prove true in man. Adenovirus type 12 has been shown to produce malignant tumors when inoculated into newborn hamsters (97). Latner et al. (50) infected primary cynomologus-monkey kidney cells and human thyroid cells in tissue culture with adenovirus type 12 and poliovirus (not found to be oncogenic). They found that there was a significant increase of the total LDH in the adenovirus-infected cultures but not in the poliovirus-infected ones which had similar or lower total LDH in the uninfected cultures. Furthermore, the adenovirus infection resulted in a complete disappearance of the fastest-moving LDH isoenzyme demonstrated in the uninfected and the poliovirus-infected cultures, and in a marked increase of the slow-moving LDH isoenzyme. It appears that adenovirus causes the LDH

isoenzyme pattern to shift from normal to malignant pattern. The correlation of isoenzyme patterns with malignant transformation may possibly shed some light on the mystery of cancer.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Regular ascites tumor cell lines (in vivo)

Ehrlich carcinoma in ascites form was developed from solid form by Loewenthal and Jahn in 1932 (89) and has been carried by mouse transplantation in different laboratories throughout the world. Its tissue origin cannot be traced.

TA_3 originated as a spontaneous mammary adenocarcinoma in a female A strain mouse in 1949 (118). After 16 passages in A strain mice, it was gradually converted to the ascites form by Klein (118) and has been maintained in A strain mice.

$6C_3HED$ is a lymphoma that was originated in 1940 by Gardner in a female C_3H mouse treated with estradiol benzoate. It was transformed into the ascites form in 1951 also by Klein (118) and has been maintained in C_3H mice.

SA_1 is a spontaneous ascites tumor in a Swiss mouse, Connaught strain, discovered in this laboratory by Dr. J.F. Morgan in 1963. Its tissue origin is not known. It has been maintained in Swiss mice, Connaught strain.

To determine if the environment of regular maintenance of the tumors would influence the isoenzyme patterns, ascites tumors of the same strains were collected from other laboratories. Table 1 shows the types and sources of all the regular ascites tumors used. The ascites tumors regularly

TUMOR	SOURCE
Ehrlich carcinoma	Dr. J.F. Morgan, this Department
Ehrlich carcinoma	Dr. D.G.R. Blair, this Department
Ehrlich carcinoma	Dr. G.A. LePage, Stanford Research Institute, Menlo Park, Calif.
Ehrlich carcinoma (Lettré)	Dr. M.J. Fraser, Dept. of Biochemistry, University of Manitoba, Winnipeg
Ehrlich carcinoma (hyperdiploid)	Dr. S.C.J. Fu, The Children's Cancer Research Foundation, Boston
Ehrlich carcinoma (hypertriploid)	Dr. S.C.J. Fu
Ehrlich carcinoma (hypotetraploid)	Dr. S.C.J. Fu
TA3 mammary carcinoma	Dr. J.F. Morgan
TA3 mammary carcinoma	Dr. G.A. LePage
$6C_3$HED lymphosarcoma	Dr. J.F. Morgan
$6C_3$HED lymphosarcoma	Dr. G.A. LePage
$6C_3$HED lymphosarcoma	Dr. J.D. Broome, University of Alberta Cancer Research Unit, Edmonton

Table 1. Regular ascites tumor lines

maintained in this laboratory were used throughout this investigation except where otherwise mentioned.

3.1.2 SN and INSN ascites tumor lines

The SN and INSN lines are variants developed from the regular ascites tumors maintained in this laboratory by Dr. J.F. Morgan. These ascites tumor lines are characterized by the presence of a large number of peculiar enlarged balloon cells (95,96) shown to have different chromosomal number (80-155) from the cells of the mother line (about 78). The SN lines are different from the INSN lines in that inositol was used for the development of the INSN lines but not for the development of the SN lines. The procedure for the development of the SN and INSN lines will be described in the method section. Table 2 shows all these cell lines used and the mother cell lines from which they were derived.

3.1.3 Mice used for the maintenance of the ascites tumors

All the ascites tumors have been maintained by serial passage in Swiss mice, Connaught strain, except the regular 6C3HED lymphosarcoma for which C3H mice were used. Adult mice of both sexes and of 9-13 weeks of age were used for regular passage of the ascites tumors. The weights of the adult Swiss mice were between 23-30 grams, and of the C3H mice were between 20-25 grams.

For the experiment to compare the isoenzyme patterns of the ascites tumor cells grown in adult and in immature

Tumor Variant	Mother Ascites Tumor Line
SN-Ehrlich-576	Ehrlich carcinoma
SN-Ehrlich-578	Ehrlich carcinoma
SN-TA_3	TA_3 mammary carcinoma
SN-SA_1	SA_1 spontaneous ascites tumor
INSN-Ehrlich	Ehrlich carcinoma
INSN-TA_3	TA_3 mammary carcinoma
INSN-$6C_3$HED	$6C_3$HED lymphosarcoma

Table 2. SN and INSN lines

mice, three female adult Swiss mice of 11 weeks old (about 26 grams) and three immature female Swiss mice of 4 weeks old (about 15 grams) were used.

For the comparison of the isoenzyme patterns of the regular 6C3HED lymphosarcoma from different laboratories, both adult female Swiss mice and C3H mice were used.

3.1.4 Tissue culture cell lines

Tissue culture lines of Ehrlich, TA3 and 6C3HED ascites tumors were established in Dr. J.F. Morgan's laboratory in Ottawa (33,34). These lines have been kept in tissue culture for more than five years and have lost the capacity of producing tumors when back transplanted into mice. New lines of the same ascites tumors were recently established in tissue culture for the comparison of isoenzyme patterns with the old lines and with the ascites cells grown in mice. These new lines retain the tumorigenic capacity after six months in tissue culture, and their establishment will be described in a later section. Table 3 shows all the tissue culture lines used in the in vitro experiments.

3.1.5 Apparatus

Ordinary centrifugation was performed with an International Centrifuge Model H, with rotor number 23; ultracentrifugation was performed with a Spinco ultracentrifuge, model L, with Type 40 rotor.

Tissue Culture Lines		
Old (non-tumorigenic)	New (tumorigenic)	tissue origin (in vivo ascites tumor)
Ehrlich	Ehrlich	Ehrlich carcinoma
TA_3	TA_3	TA_3 mammary carcinoma
$6C_3HED$	$6C_3HED$	$6C_3HED$ lymphosarcoma
	SA_1	SA_1 spontaneous ascites tumor

Table 3. Tissue culture lines

For the sonication of ascites tumor cells, a Type MT20 MSE Ultrasonic Oscillator, set at 125 volts, was used.

For the X-ray irradiation of Ehrlich ascites tumor cells, a Picker's X-ray machine with a Type OEG-60 X-ray tube was used.

The inositol (myo) treatment of Ehrlich ascites tumor cells was performed in a Dubnoff Metabolic Shaker.

Protein determinations on the cell-free extracts of ascites tumors were made with a Bausch and Lomb Colorimeter using Spectronic 20 cuvettes, employing the Lowry method (55).

3.1.6 Solutions and media

Hanks' balanced salt solution and Puck's balanced salt solution were used for washing and suspending ascites tumor cells. Trypsin solution (0.05%) was used to detach cells from the glass surface in tissue culture experiments. Balloon cells and regular-sized cells were separated by gradient centrifugation for which Ficoll solution was used. To induce ascitic fluid in normal mice, Freund's complete adjuvant (Difco Laboratories, Detroit, U.S.A.) was used. Tissue culture media, M150 and Waymouth's MB572/1 were prepared in this laboratory. All chemicals used were commercial preparations. The formulae of these solutions and tissue culture media are presented here.

Hanks' Balanced Salt Solution (g/litre) (HBSS)

sodium chloride	8.0
potassium chloride	0.4
calcium chloride	0.14
magnesium sulfate ($7H_2O$)	0.1
magnesium chloride ($6H_2O$)	0.1
disodium phosphate	0.048
monopotassium phosphate	0.06
D-glucose	1.0
sodium bicarbonate	1.4
double distilled water to	1000 ml

Puck's Balanced Salt Solution

The salt contents of Puck's balanced salt solution are the same as Hanks' balanced salt solution, but calcium and magnesium ions were omitted.

Trypsin Solution

A trypsin (1:250, Difco Laboratories, Detroit, Michigan) solution of 0.05% was prepared in Puck's balanced salt solution.

Ficoll Solution

Ficoll was purchased from Pharmacia Company, Uppsala, Sweden. The Ficoll solution was prepared with 23.4 g. of Ficoll in HBSS to make up 100 ml solution. This solution gave a specific gravity of 1.08.

Compositions of Media (milligrams per litre) (64,69)

Chemical	M150	Waymouth's MB752/1*
DL-alanine	50	
L-arginine•HCl	70	75
DL-aspartic acid	60	60
L-cysteine•HCl	0.1	90
L-cystine	20	15
DL-glutamic acid	150	150
L-glutamine	100	350
glycine	50	50
L-histidine•HCl	20	150
hydroxy-L-proline	10	
DL-isoleucine	40	25
DL-leucine	120	50
L-lysine•HCl	70	240
DL-methionine	30	50
DL-phenylalanine	50	50
L-proline	40	50
DL-serine	50	
DL-threonine	60	75
DL-tryptophan	20	40
L-tyrosine	40	40
DL-valine	50	65
p-aminobenzoic acid	0.05	0.02
biotin	0.01	1.0
calcium pantothenate	0.01	250

Chemical	M150	Waymouth's MB752/1*
choline chloride	0.50	0.40
folic acid	0.01	1.0
inositol	0.05	
niacin	0.025	
niacinamide	0.025	1.0
pyridoxal•HCl	0.025	
pyridoxine•HCl	0.025	1.0
riboflavin	0.01	1.0
thiamine•HCl	0.01	10.0
vitamin B_{12}		0.2
vitamin A	0.10	
ascorbic acid	0.05	17.5
α-tocopherol phosphate	0.01	
calciferol	0.10	
menadione	0.01	
adenine	10.0	
guanine•HCl	0.3	
hypoxanthine	0.3	25.0
thymine	0.3	
uracil	0.3	
xanthine	0.3	
adenylic acid	0.2	
2-deoxy-D-ribose	0.5	
D-ribose	0.5	
Tween 80 (oleic acid)	5.0	

Chemical	M150	Waymouth's MB752/1*
cholesterol	0.2	
D-glucose	1000.0	5000.0
glutathione (reduced)	0.05	
sodium acetate	50.0	
adenosinetriphosphate	10.0	
NaCl	8000.0	6000.0
KCl	400.0	150.0
$CaCl_2$	140.0	90.0
$MgCl_2 \cdot 6H_2O$	100.0	240.0
$MgSO_4 \cdot 7H_2O$	100.0	200.0
Na_2HPO_4	48.0	300.0
KH_2PO_4	60.0	80.0
$NaHCO_3$	1400.0	2240.0
$Fe(NO_3)_3 \cdot 9H_2O$	0.1	
phenol red	20.0	20.0

Note: Both media were supplemented with 20 percent whole calf serum. Double distilled and deionized water was used for the preparation of all media.

* Those amino acids indicated in DL-form were used for M150 but only L-forms were used for Waymouth's MB752/1 medium.

3.1.7 The following abbreviations have been used throughout this report:

HBSS - Hanks' balanced salt solution

PBSS - Puck's balanced salt solution

LDH - lactate dehydrogenase

MDH - malate dehydrogenase

G-6-PD - glucose-6-phosphate dehydrogenase

NAD^+ - oxidized nicotinamide adenine dinucleotide

NADH - reduced nicotinamide adenine dinucleotide

$NADP^+$ - oxidized nicotinamide adenine dinucleotide phosphate

NADPH - reduced nicotinamide adenine dinucleotide phosphate

PMS - phenazine methosulfate

MTT - 3(4,5 - dimethyl thiazolyl 1-2)2,5 - diphenyl tetrazolium bromide

Tris - tris(hydroxymethyl)aminomethane

Fast blue B - commercial name of tetrazotized O-dianisidine

3.2 Methods

3.2.1 Regular passage of the ascites tumors

Maintenance of the regular ascites tumor lines has been carried out by conventional methods. About five million ascites tumor cells in 0.25 ml HBSS were injected intraperitoneally in a healthy mouse. A group of three mice was used for each regular passage.

SN and INSN lines have been maintained by another method. Freshly harvested ascites were subjected to ordinary centrifugation for 15 minutes at 2,500 r.p.m. in the model H

International Centrifuge (rotor number 23). The majority of the cells settled on the bottom and a layer of balloon cells floated on the top with the supernatant fluid in between. Some balloon cells remained in suspension in the supernatant. The supernatant was carefully drawn out with a Pasteur pipette and was subjected to ordinary centrifugation twice more as just described to remove as many cells as possible. The resulting supernatant fluid was finally centrifuged at high speed, 35,000 r.p.m., for one hour in the Spinco Model L Ultracentrifuge (Type 40 rotor). At this stage, the supernatant fluid still contained some balloon cells of which the number was not counted; 0.25 ml of this supernatant fluid was injected intraperitoneally in each of three mice.

3.2.2 Development of the SN and INSN ascites tumor lines

The method of developing the SN lines was reported by Tolnai *et al.* (95). Tumor cells were removed from freshly harvested ascitic fluid by ordinary centrifugation once and high-speed centrifugation once as described. The clear supernatant was then inoculated intraperitoneally into mice (0.25 ml per mouse). If ascites tumor developed, a large number of balloon cells would be seen in the ascitic fluid from which the supernatant was again used by the same method to inoculate other mice. By the third or fourth serial passage of the supernatant fluids, the incidence of ascites tumor formation reached 100%.

To develop INSN lines, tumor cells from regular ascites tumor lines were spun down from freshly harvested ascites by ordinary centrifugation as described. The supernatant was removed, and the cells were washed with HBSS and were spun down as before. The washing was repeated twice more. Then the cells were suspended with two volumes of 5% aqueous myo-inositol solution. The cell suspension in inositol solution was left to stand at room temperature (approximately 25°C) for one hour. The cells were removed by ordinary centrifugation as described. The supernatant obtained was ultracentrifuged at 35,000 r.p.m. for one hour in the Spinco Model L Ultracentrifuge to remove any remaining cells. Of this final supernatant 0.25 ml was injected intraperitoneally into each of five Swiss mice of the Connaught strain. If tumor formed, it was passed to other mice with the supernatant fluid from the tumor ascites with the same method of maintaining the SN lines. The characteristic of the presence of a large number of balloon cells was maintained in the subsequent regular passages of the INSN lines.

3.2.3 Establishment of new tissue culture lines of the ascites tumors

Tissue culture lines of Ehrlich, TA3 and 6C3HED ascites tumors were established by Guerin and Morgan (34), and Guerin and Kitchen (33). These lines have been kept in tissue culture for more than five years and have lost the capacity of producing ascites tumors when back transplanted

into mice from tissue culture. It was felt that new tissue culture lines of the same ascites tumors should be established in order to compare the isoenzyme patterns.

The new $6C_3HED$ tissue culture line was established in a modified medium M150 (64) supplemented with L-asparagine (100 mg/litre), and the new Ehrlich, TA_3 and SA_1 lines in Waymouth's medium MB752/1 (69). Both media were supplemented with 20% calf serum.

To grow cells in tissue culture, approximately 10-15 million ascites cells (three drops of ascitic tumor fluid with a Pasteur pipette) were cultured in 10 ml of medium in a T-60 flask. The majority of the cells died out gradually over a period of two weeks. The cells were fed with fresh medium when the change in colour of the medium from pink to yellow indicated acidity. But some cells attached to the glass surface and became spindle-shaped, and some of these cells began to multiply to form colonies. When multiplication of the cells was continuous, eventually a monolayer was formed and it was necessary to transfer the cells to other flasks to maintain cell multiplication. Cells were fed with fresh medium once or twice a week depending on the degree of change of the pH of the medium.

At the time of the isoenzyme study, the new cell lines, namely the new Ehrlich, TA_3, $6C_3HED$ and SA_1 lines had been kept in tissue culture with Waymouth's medium MB752/1 for six months and were at their fifth passage. When cells

(approximately one million/mouse) of these new lines were injected into mice from tissue culture, typical ascites tumors developed after 2-3 weeks. Thus, these new tissue culture lines were tumorigenic.

3.2.4 Methods of harvesting the ascites tumor cells for isoenzyme study

(a) Regular cell lines

Ascites tumors developed rapidly with a tremendous increase of tumor cells and fluid in the peritoneal cavity. Within nine to fourteen days after inoculation the ascitic fluid was withdrawn with a hypodermic needle and was subjected to ordinary centrifugation as described previously. The clear supernatant was removed and was either discarded or kept for isoenzyme study. The packed cells were washed with HBSS by repeated pipetting. The HBSS was then removed by centrifugation. The washing was repeated twice more, after which the packed cells were considered to be essentially free from ascitic fluid. At least 0.2 ml of packed cells was required to prepare sufficient cell-free extract for isoenzyme detection.

(b) SN and INSN lines

Because of the presence of the large balloon cells in these lines, the ordinary centrifugation would sediment a mixed cell population of both the balloon cells and cells of regular size. In order to obtain a good separation of the two types of cells in a convenient way, density gradient

centrifugation was used. Approximately 1.5-1.8 ml (one Pasteur pipetteful) of Ficoll solution was mixed with 5-6 ml of the ascitic tumor fluid. After the ordinary centrifugation as described previously, the balloon cells formed a layer on top of the supernatant and the cells of regular size sedimented at the bottom with the clear supernatant in between. The regular-size cells at the bottom were obtained by removing the supernatant after the balloon cells were withdrawn. Cells of both kinds were washed with HBSS three times as previously described before being used for the preparation of cell-free extract. It should be noted that the balloon cells would sediment in HBSS by ordinary centrifugation.

3.2.5 Tissue culture methods for obtaining cells for the isoenzyme studies

Seven ascites tumor cell lines in tissue culture were used. As mentioned before, they were the three old non-tumorigenic lines, namely, Ehrlich, TA_3 and $6C_3HED$, and the four new tumorigenic lines, namely, Ehrlich, TA_3, $6C_3HED$ and SA_1.

Attempts were made to propagate all the cell lines in both media M150 and MB752/1 supplemented with 20% calf serum. Conventional tissue culture methods and sterile techniques were employed. Cells were grown in T-60 flasks until a monolayer was formed. In order to obtain sufficient numbers of cells, it was necessary to transfer the cells to other flasks for further propagation. Passage was done by trypsinizing

the monolayer cells. The medium was first removed and the monolayer was washed twice with PBSS. Five to six ml of 0.05% trypsin in PBSS was added to each T-60 flask. After about 15 minutes at 37°C, the cells detached from the glass surface and were dispersed by gentle trituration with a Pasteur pipette. The cell suspension was then divided into three approximately equal portions, each of which was transferred to a new T-60 flask with 10 ml of culture medium. The cells were found to multiply rapidly and to form a new monolayer in about two weeks. For each cell line at least five heavy monolayer cultures in T-60 flasks were required to provide sufficient cells for the isoenzyme study. About 0.3 ml of packed cells was obtained from five such monolayers. The cells obtained were washed three times with HBSS before being used for the preparation of cell-free extract.

To examine if trypsin would affect the isoenzyme pattern, a control experiment was done by using a rubber policeman to scrape off the cells from the glass surface instead of using trypsin.

3.2.6 Preparation of cell-free extracts

Two parts of double distilled water were mixed with one part of packed cells. The cell suspension was subjected to sonication for one minute with a Type MT20 MSE ultrasonic oscillator. The cell debris and any unbroken cells were removed by centrifugation at 17,500 r.p.m. in the Spinco Model Ultracentrifuge for 30 minutes. The clear supernatant

obtained was the cell-free extract and was stored at -20°C. The cell-free extracts thus obtained were the samples used for the isoenzyme studies. The samples were thawed immediately before use and were re-stored at -20°C immediately after use so that the enzyme activity would not decrease appreciably.

To examine if sonication would affect the isoenzyme pattern, the method of repeated freezing and thawing was also used for the disruption of ascites tumor cells. A cell suspension was divided into two equal portions, one was subjected to sonication, the other to freezing and thawing. After freezing and thawing for three times, the cell suspension was homogenized with a Potter and Elvehjem homogenizer before being subjected to ultracentrifugation for the cell-free extract.

3.2.7 Protein determination of the cell-free extracts

The amounts of the enzymes applied for electrophoresis were not controlled. It was felt later that protein concentrations of the cell-free extracts should be examined. Protein concentration was measured colorimetrically with the method developed by Lowry *et al.* (55). Crystalline bovine serum albumin (Mann Research Laboratories, New York) was used as the standard protein and a Bausch and Lomb Colorimeter was used for the colorimetric measurement of protein. The protein concentrations of the cell-free extracts were found to be in a wide range between 12 and 47 mgm of protein per 0.1 ml of cell-free extract.

3.2.8 Ascitic fluid induced by Freund's adjuvant

Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan) was injected intraperitoneally into a group of five adult female Swiss mice with 0.5 ml per mouse. Another similar dose was given after ten days. Within a month after the first injection, a large volume of fluid accumulated in the peritoneal cavity and a clear ascitic fluid was obtained. Cells were rarely found in this ascitic fluid when it was examined under the microscope. This fluid was used for a comparison with the isoenzyme studies of the ascites tumor cells.

3.2.9 Treatment of Ehrlich ascites tumor cells with inositol

Ehrlich ascites tumor cells freshly harvested from an adult female Swiss mouse were divided into two equal parts. The ascitic fluid was removed by the ordinary centrifugation procedure. The cells in one tube were washed once with HBSS and those in the other tube with 5% inositol (myo) solution. The cells were then resuspended in the corresponding solutions in 1:10 dilution (1 part of cells in 9 parts of HBSS or inositol solution). The cell suspensions were gently shaken at 37°C in a Dubnoff Metabolic Shaker for one hour with a speed of 1 cycle/second. Finally three mice were inoculated with each cell suspension (about five million cells per mouse). Cells left over from inoculation were washed once with HBSS as the controls for the isoenzyme study. When the ascites tumors developed in the mice, cells were harvested for a

comparison of the isoenzyme patterns.

3.2.10 Irradiation treatment of Ehrlich ascites tumor cells

Ehrlich ascites tumor fluid was subjected to ordinary centrifugation at 2500 r.p.m. in the Model H International Centrifuge to remove the supernatant. The cells were washed twice and then diluted with HBSS to about 25,000,000 cells per ml. Five ml of this cell suspension were placed in 60 x 15 mm tissue culture Petri dishes for X-ray irradiation. The radiation doses given to cells were 500, 1000, 1500, 2000, 2500 and 3000 rads with a Picker's 50KVP X-ray tube at the dose rate of 43 rads per second. A sample of the same cell suspension which was not irradiated served as the control. Each irradiated cell suspension was diluted to 4,000,000 cells per ml and 0.25 ml of this cell suspension containing 1,000,000 cells was injected into each of a group of five mice. The mice used were female about the same weight (26 grams). When the ascites tumors developed, cells were harvested from the mice inoculated with cells treated with X-rays of different doses. The cells harvested were used for the isoenzyme study.

3.2.11 Cloning of the ascites tumor cells of the tumorigenic tissue culture lines

Cloning of the cells of the tumorigenic Ehrlich, TA3 and 6C3HED tissue culture lines was undertaken. A T-60 flask of healthy monolayer cells was selected. The culture was fed 36 hours prior to use. Cells were harvested and dispersed by

using 0.05% trypsin as described previously and were counted with a Neubauer cell counting chamber. Two separate counts (about 185 cells per total area) were made and the difference between the two counts was found to be less than 20%. The average of the two counts was used. Cell clumps were found to be less than 3%. The cell suspension was diluted serially with the culture medium (Waymouth's MB752/1) until a final dilution of 10 cells per ml was reached. Six ml of the final cell suspension were placed into each of three tissue culture Petri dishes (60 x 15 mm) to give 60 cells per dish, and 3 ml into each of another three dishes to give 30 cells per dish. To make up the total volume of 6 ml per dish, 3 ml of the medium were added into each of the latter three dishes. The cultures were incubated for 14 days in 5% CO_2 in air to maintain the pH. By microscopical examination a well isolated clone was selected from each of the six dishes.

The clonal cells were obtained with the following method. A siliconed sterilized glass cylinder was placed over the clone selected to isolate it from the cells of other clones nearby. The cylinder was pressed down firmly so that it would stick (by the silicone) to the bottom of the dish. With a fine Pasteur pipette the medium was removed from within the cylinder. The clone was washed twice with PBSS. Then the cylinder was filled with 0.05% trypsin. The clonal cells were detached from the dish after about 20 minutes of incubation at 37°C, and were transferred to another Petri dish containing

6 ml of the medium. After two weeks of incubation in 5% CO_2 in air, the clonal cells were transferred to a T-30 flask. From then on, cells were propagated by conventional tissue culture methods until five heavy T-60 flask cultures were obtained for the isoenzyme study.

3.2.12 Starch gel electrophoresis

Horizontal starch gel electrophoresis in a discontinuous buffer system (77) was employed to separate the isoenzymes. Thirteen grams of hydrolysed starch (Connaught Medical Research Laboratories, University of Toronto) per 100 ml of buffer was heated to a clear sol, de-aired with vacuum and then poured in plastic molds with a trough size of 30 x 1.9 x 0.6 cm. To obtain an even surface of the gels, a sheet of plastic was pressed down on the starch before it solidified. The gels were then cooled at 4°C for 2-3 hours. A piece of Whatman No. 3 filter paper (1.5 x 0.4 cm) was used to absorb 25 microliters of cell-free extract and was then inserted in the gel at a point 5 cm from the cathodal end of the gel. Electrophoresis was performed at 4°C by connecting the gels to the bridge buffer by two layers of ordinary flannel. The gels were then covered with Saran Wrap to prevent loss of moisture. After 20 minutes of electrophoresis, the filter papers were removed from the gels, and the gels were again covered with the Saran Wrap. Electrophoresis was resumed until the appropriate length of time was reached.

Different systems of electrophoresis were employed for different enzymes in order to obtain the best possible separation of the different molecular forms of each enzyme.

For the separation of isoenzymes of LDH, G-6-PD and MDH, the buffer for making up the gels was 0.05 M Tris adjusted to pH 8.6 with HCl; the bridge buffer contained 0.3 M boric acid adjusted to pH 8.6 with NaOH. Electrophoresis was run at 10 V/cm for seven hours for LDH, and two hours for G-6-PD and MDH. The same gel was used to separate G-6-PD and MDH. After the electrophoresis the gel was sliced horizontally into two halves, one for identifying G-6-PD and the other for MDH.

For the separation of isoenzymes of esterases, gels were prepared with Tris-citrate buffer at pH 8.6 (0.076 M Tris, 0.0057 M citric acid). The bridge buffer used was the same as for LDH, G-6-PD and MDH. Electrophoresis was run at 6 V/cm for 5 hours.

3.2.13 Identification of the isoenzymes

After electrophoresis the location of enzyme activity on the starch gel was visualized by incubating slices of the gels in the following media at room temperature.

For LDH:

Tris buffer, pH 7.2	0.1 M
Sodium lactate (DL)	0.1 M
NAD^+	0.1 mg/ml
MTT	0.2 mg/ml
PMS	0.1 mg/ml
KCN	0.3 mg/ml

Incubation time: 2-5 hours

For G-6-PD:

Tris buffer, pH 7.2	0.1 M
Glucose-6-phosphate (sodium salt)	1.0 mM
$NADP^+$	0.1 mg/ml
MTT	0.2 mg/ml
PMS	0.1 mg/ml
$MgCl_2$-$6H_2O$	0.1 mg/ml

Incubation time: 4-7 hours

For MDH:

Tris buffer, pH 7.6	0.1 M
Malic acid (DL)	0.01 M
NAD^+	0.1 mg/ml
MTT	0.2 mg/ml
PMS	0.1 mg/ml
KCN	0.3 mg/ml

Incubation time: 2-5 hours

For esterases:

Phosphate buffer, pH 5.8, 0.1 M (1000 ml KH_2PO_4, 0.1 M + 96 ml Na_2HPO_4, 0.1 M)	100 ml
Fast blue B	20 mg
Alpha-naphthyl acetate (1% in acetone)	2 ml

Incubation time: about 6 hours

3.2.14 Washing and storage of the gels

Starch gels with coloured bands were washed for about three hours in a solution containing 10 parts glacial acetic acid, 50 parts methanol and 50 parts distilled water. The gels were finally kept in water in the dark at room temperature. The colour of the bands faded only slightly after six months of storage. The slight fading of the colour did not significantly affect the resolution of the bands.

3.2.15 Photography of the starch gels

A Zeiss Contarex camera with a Tessar lens (1:3.5, f = 115 mm) was used to photograph the gels. Kodak High Contrast films (35 mm) were used and were developed in D-11 developer for 6 minutes. The pictures were printed with Kodak Bromide papers (single weight) developed in Dektol (1 Dektol: 2 water).

4. OUTLINE OF EXPERIMENTAL WORK

4.1 *In vivo* experiments

Conventional starch gel electrophoresis and histochemical stains were used to study the isoenzyme patterns of LDH, G-6-PD, MDH and esterases in mouse ascites tumors of different tissue origin. The samples for comparison were the ascitic fluids of the ascites tumors after the tumor cells were removed and the ascitic fluid induced by Freund's complete adjuvant in Swiss mice.

To obtain uniform and reproducible results, the effects of the age and the sex of the mice used, the age of the ascites tumors, the storage of the cell-free extracts, and alternative methods for disrupting the cells were studied.

The isoenzyme patterns of LDH, G-6-PD and MDH in the regular cell lines and the derived SN and INSN lines were compared. In addition, the same regular ascites tumors were acquired from other laboratories to check the isoenzyme patterns.

The effects of X-irradiation and of inositol on the isoenzyme patterns of Ehrlich ascites tumor cells were also studied.

4.2 *In vitro* experiments

Ehrlich, TA_3 and $6C_3HED$ mouse ascites tumor cells have been maintained in tissue culture for more than five years. These are designated old lines and have lost the capacity to produce tumors. The same three ascites tumors and the SA_1 spontaneous ascites tumor were again established in tissue culture and have been maintained up to now. These new lines still retained the capacity to produce tumors when they were back transplanted into mice. The isoenzyme patterns of the old lines and the new lines *in vitro*, of the new lines on back transplantation and of the regular lines *in vivo* were compared.

It was suspected that the new tissue culture lines might consist of a heterogeneous population of different cell types which might have different isoenzyme patterns. Accordingly, cloning of the new cell lines was undertaken and the isoenzyme patterns of the different clones obtained were studied.

Since the ascites tumor cells may lose their tumorigencity after long term culturing *in vitro*, it would be interesting to check the isoenzyme patterns of the tumorigenic cell lines from time to time. The LDH isoenzyme pattern of the SA_1 tissue culture line was examined at two different times after the cell line was established.

5. EXPERIMENTAL RESULTS

5.1 General

All the results are presented in photographs or diagrams. Each band shown in the pictures represents an isoenzyme. It is unfortunate that the bands are not delineated as clearly as they actually occur in the starch gels. The cathode end of the starch strips was the point at which the samples were inserted. This point was the origin from which the isoenzymes travelled towards the anode. In all cases, no isoenzymes were observed migrating to the cathode side of the origin.

The numbering of the isoenzymes is according to the system recommended by the Standing Committee on Enzymes, of the International Union of Biochemistry (105). The system is: 'When multiple forms of an enzyme are identified by electrophoretic separation, they should be given consecutive numbers, the form having the highest mobility towards the anode being numbered one.'

Coloured bands appeared at the areas containing enzyme activity after 30 minutes of incubation in the staining solution. The colour deepened as incubation was continued. There was not a fixed period of time for the development of the bands to the right intensity because the amount of enzymes applied for electrophoresis was not uniform. Prolonged

incubation with the staining solutions gave a deep background stain to the gels. And the bands closely separated might coalesce with their individuality becoming less distinct. This was particularly true with LDH. If the incubation time was not long enough, some isoenzymes with slight activity might not be visualized at all. Thus, the incubation times given for the enzymes are quite arbitrary.

Due to the different dyes used, the bands of the dehydrogenases are deep blue, the esterases deep brown.

5.2 Control work

5.2.1 Isoenzyme patterns of the ascitic supernatant fluids

It is currently postulated that LDH in normal mouse tissues exists in five molecular forms. The present study does not seem to agree fully with this prevalent concept. In Fig. 1 the ascitic fluids from mice with various ascites tumors and from mice treated with Freund's complete adjuvant have basically six LDH isoenzymes, five close together and one with much higher mobility towards the anode. The 5 bands in a group are not well shown in the diagram. TA_3 appears to contain 3 bands, Ehrlich, SA_1 and Ehrlich-SN-576 four bands in the group. During the colour development of the bands, actually, 5 distinct bands were observed. The washing solution decolourized some bands of very light intensity. Occasionally, 6 main bands in the group instead of five were observed. This is poorly shown in the starch strips of SN-TA_3 and of Freund's adjuvant in Fig. 1. The number of bands may

vary slightly, but their positions of mobility are consistent. The fastest moving band is very faint in Ehrlich and in Ehrlich-SN-576. Tumor cells were immediately removed from these fluids after they were withdrawn from the mice. And the ascites induced by Freund's adjuvant contained an insignificant number of cells. The characteristic LDH isoenzyme pattern of these fluids, shown in Fig. 1, may serve as a reference to compare with the LDH isoenzyme patterns of the ascites tumors.

Furthermore, the serum of healthy Swiss mice, obtained by heart puncture, was examined. It was found to possess the characteristic LDH isoenzyme pattern as shown in Fig. 1.

The enzyme activities of MDH and G-6-PD in these ascitic fluids were extremely low so that no bands or only very, very faint bands could be detected on the starch gels.

5.2.2 Effects of sex and age of the mice

The physiological conditions of the host may influence the growth and the metabolism of tumor cells, particularly those due to the hormonal and immunological factors involved in tumor transplantation. It was felt that the age and the sex of the mice used should be checked in order to see if the isoenzyme patterns might be affected by these factors. As shown in Fig. 2, the isoenzyme patterns of G-6-PD in Ehrlich ascites tumor cells are the same when the tumor was grown in male and female adult (both 11 weeks old) and immature female (4 weeks old) mice; the same results were found for MDH and LDH. It

appears that the age and sex of the mice used have no effect on the isoenzyme patterns of the ascites tumors.

5.2.3 Effect of age of the ascites tumors in the hosts

It is possible that the isoenzyme patterns may be different when cells are harvested at different growth phases. If there is a difference, the age of the tumor must be controlled in order to obtain uniform and reproducible results.

Fig. 3 shows that the MDH isoenzyme patterns of Ehrlich ascites tumor cells, harvested 8, 11 and 14 days after inoculation, were the same. Thus, the age of the tumor did not seem to affect the isoenzyme patterns. This would imply that it does not matter when the ascites tumors are withdrawn from the mice, since the isoenzyme patterns should remain the same.

5.2.4 Effect of the methods used for disrupting the cells

Heat is generated during the time of sonication. This could lead to the denaturation of some enzymes or isoenzymes. To check if the isoenzyme patterns would be affected by the sonication, the cells were also broken by freezing and thawing. In Fig. 4 the MDH isoenzyme patterns of Ehrlich ascites tumors are shown to be similar when the cells were disrupted by either method. In the starch strip from freezing and thawing, the separation does not appear to be clear. But the pattern of one fast-moving band and one slow-moving diffused zone is apparent. The slow-moving zone appears to have two bands.

It was also found that the isoenzyme patterns of LDH and G-6-PD were not affected by the two methods for disrupting the cells.

5.2.5 Effect of storage of the cell-free extracts

It was felt that when it was possible, electrophoresis for the isoenzymes of the different tumors should be run at the same time so that a comparison of the positions of the bands would be more accurate. The cell-free extracts of different tumors could not be obtained at the same time due to the extensive manipulations involved. Consequently, many extracts had to be stored in a commercial deep freezer (-20°C) until the other samples were prepared for analysis. Enzymes may be denatured considerably during storage. Therefore, it was necessary to ascertain whether the isoenzyme patterns would be changed by storage.

As shown in Fig. 5, the isoenzyme pattern of MDH of the $6C_3$HED new tissue culture line remained the same even after 142 days of storage at -20°C. Only G-6-PD appeared to have a slight decrease of activity. But again its isoenzyme pattern was not altered.

In fact, it was observed that the enzymes studied were reasonably stable during 6 months of storage at -20°C. Further examination for longer periods was not carried out.

5.2.6 Effect of trypsin

In the in vitro experiments, cells were harvested from the tissue culture flasks by using trypsin to detach the cells from the glass surface. The proteolytic activity of trypsin could conceivably cause a leaking of enzymes from the cells and the isoenzyme patterns might be changed. To test this possibility, cells grown in tissue culture were also harvested by using a rubber policeman to rub off the cells from the glass surface. Fig. 6 shows that trypsin has no effect on the isoenzyme pattern of MDH of the TA_3 new tissue culture line. This was also true for LDH and G-6-PD.

5.3 In vivo experiments

5.3.1 Comparison of the isoenzyme patterns of the regular tumor lines

The isoenzyme patterns of LDH, G-6-PD, MDH and esterases in the four ascites tumors and the ascitic fluid induced by Freund's adjuvant are shown in Fig. 7 which consists of five pictures. In Fig. 7a, LDH in the tumor cells grown in mice has a similar pattern of five main bands in spite of the different tissue origins of the tumors. Sometimes a band may be observed between bands 3 and 4, bands 4 and 5, and another band may occur after band 5. This will be shown in other pictures later. The results were not uniform. It is indeed difficult to number the bands due to the slight irregularity of the results. However, the relative position of the bands remains quite constant. The numbering of the bands is thus based on

the constancy of the positions of the bands. It is the pattern of the bands which is of primary importance. And it is clear that the isoenzyme pattern of the ascitic fluid induced by adjuvant is different from that of the tumor cells. It was also observed that the serum from healthy Swiss mice had LDH isoenzyme patterns similar to those shown by the ascitic fluid.

In Fig. 7b, the G-6-PD isoenzyme patterns of the four kinds of tumors are the same with two distinct bands while the ascitic fluid has practically no enzyme activity.

In Figs. 7c and 7d, MDH also has two main bands. Band 1 is always very distinct and band 2 is a big diffused one. Sometimes two bands may be observed in the band 2 position and this is seen in Ehrlich cells in Fig. 7c when the electrophoretic time was extended. Again almost no enzyme activity of MDH was observed in the ascitic fluid induced by Freund's adjuvant.

In Fig. 7e, as was found in the case of the other enzymes, the four ascites tumors all had the same esterase isoenzyme pattern characterized by seven bands, a pattern which was entirely different from the pattern of the ascitic fluid.

From the five pictures of Fig. 7, it is clearly demonstrated that the respective isoenzyme patterns of LDH, G-6-PD, MDH and esterases in ascites tumors of four different tissue origins were the same when the tumor cells were grown in mice.

5.3.2 Comparison of the isoenzyme patterns of the same ascites tumors from different laboratories

(A) Lactate dehydrogenase

The LDH isoenzyme patterns of Ehrlich, TA_3 and $6C_3HED$ ascites tumors from different laboratories are shown respectively in Figs. 8a, 8b and 8c. There was no major difference in case of the Ehrlich carcinoma (Fig. 8a), even the three sublines from Dr. Fu, which had different chromosome numbers. In the TA_3 mammary carcinoma, there was also no great difference between the two lines tested, although the tumor from Dr. Morgan's laboratory appeared to have three bands at the band 1 region, while the tumor from Dr. LePage's laboratory showed only one (Fig. 8b). Three bands at the band 1 region were rarely observed.

In case of the $6C_3HED$ lymphosarcoma, the tumor obtained from Dr. LePage seemed to have a different pattern from that of the tumors maintained in Dr. Morgan's and Dr. Broome's laboratories, as shown in Fig. 8c. This was one of the earliest experiments done and the labelling was different from that in the other pictures. The two starch strips on the left showed the isoenzyme pattern of the $6C_3HED$ tumor from Dr. Morgan. $6C_3HED$ ascites tumor originated from C_3H mice and has been regularly passaged only in C_3H mice. However, it has been successfully transplanted in Swiss mice in this laboratory. An extra band which moved much farther towards the anode was observed in the $6C_3HED$ tumor obtained

from Dr. LePage. This extra band was consistently shown regardless of whether this ascites tumor was grown in C_3H mice or in Swiss mice. No extra band was observed for the same tumor from Dr. Morgan and Dr. Broome.

(B) Glucose-6-phosphate dehydrogenase

Figs. 9a, 9b and 9c show that G-6-PD had two bands for all the tumors. The second band was very faint for the Ehrlich ascites tumor from Dr. LePage and from Dr. Fu (2N) (Fig. 9a), and for the $6C_3HED$ from Dr. LePage; and the pictures (Figs. 9a and 9b) do not appear to show it at all due to the deficiency of photography. In this case, there may be some quantitative difference, but no qualitative difference in the isoenzyme pattern of G-6-PD.

(C) Malate dehydrogenase

Malate dehydrogenase had basically similar isoenzyme pattern for all the ascites tumors from different laboratories, as shown in Figs. 10a, 10b and 10c. The pattern shows two heavy bands of which the second one diffused out in a large area. A distinct small band was sometimes observed between the two heavy bands, such as shown for the Ehrlich ascites tumors from Morgan, Lettré and Fu (3N) in Fig. 10a. It would appear that there is more than one isoenzyme at the band 2 region, which could not be separated by the electrophoretic system used in these experiments. It should also be noted that the second band of Fu's Ehrlich (4N) diffused out very near band 1. Of the TA_3 (Fig. 10b) and $6C_3HED$ (Fig. 10c)

ascites tumors from different laboratories, the MDH isoenzyme patterns were extremely uniform and no small band between the two heavy bands was found.

To summarize the results presented, it appears that the respective isoenzyme patterns of LDH, G-6-PD and MDH were quite similar in all the mouse ascites tumors obtained from different laboratories, even though the chromosome numbers and tissue origin were different.

5.3.3 Comparison of the isoenzyme patterns of the regular ascites tumor lines with those of their derived supernatant lines

(A) Lactate dehydrogenase

It has been mentioned that the SN and INSN ascites tumor cell lines are characterized by having a large number of enlarged balloon cells in addition to the tumor cells of regular size. Upon gradient centrifugation with Ficoll, the balloon cells floated on top of the ascitic supernatant fluid and the cells of regular size sedimented to the bottom of the centrifuge tubes. The isoenzyme patterns of these two cell types were examined; in the pictures "Top" simply designates the balloon cells and "bottom" the cells of the regular size.

As shown in Figs. 11a, 11b, 11c and 11d, the results were very irregular. It appears that, in general, the LDH isoenzyme patterns of the SN and INSN lines were similar to the patterns of their mother lines. One characteristic of these supernatant lines, with the exception of SN-Ehrlich-576,

is that the cells on the bottom had a fast moving band towards the anode, which was not found in the mother cell lines (Fig. 11a). Fig. 11b shows that the INSN-TA3 balloon cells had only two bands. In Fig. 11c, the balloon cells of INSN-6C3HED also had the fast-moving band.

For better comparison, the gels of all the SN lines and of all the INSN lines respectively were put together and were photographed. Fig. 11e shows that the SN lines of different tissue origin had similar LDH isoenzyme pattern; the same was true with the INSN lines shown in Fig. 11f.

(B) Glucose-6-phosphate dehydrogenase

The G-6-PD isoenzyme patterns of the SN and INSN lines were compared with the patterns of their respective mother lines, as shown in Figs. 12a, 12b, 12c, and 12d. It is obvious that the balloon cells of all the lines had less enzyme activity so that the second band was shown very faintly or was not shown at all.

Of all the sub-lines, the cells of regular size at the bottom after centrifugation had the same two bands as the mother cell lines. Fig. 12e shows that the balloon cells of all SN lines were missing the second band. Of the INSN lines, all the balloon cells possessed the second band, though very faint, except the INSN-6C3HED lines, as shown in Fig. 12f.

(C) Malate dehydrogenase

Figs. 13a, 13b, 13c, 13d, 13e and 13f show the MDH isoenzyme patterns of the SN and INSN lines. No apparent

difference was shown between the mother lines and the SN and INSN sub-lines. In Figs. 13a and 13b, a small band was found between the two large bands in the Ehrlich and TA_3 mother lines and in the cells of regular size of the SN and INSN sub-lines with the exception of SN-Ehrlich-576 (bottom). This small band was not found in any balloon cells. As shown in Figs. 13c and 13d, the $6C_3HED$ and SA_1 lines had two large bands, and of their sub-lines both the balloon cells and the cells of regular size had the same MDH isoenzyme pattern.

It appears that the SN and INSN lines had similar MDH isoenzyme patterns, as shown in Figs. 13e and 13f.

5.3.4 Effect of radiation on the isoenzyme patterns of Ehrlich ascites tumor

Ehrlich ascites tumor cells were irradiated with different X-ray doses and then injected into groups of mice. When the tumors developed in the mice, cells were harvested for isoenzyme studies.

Figs. 14a, 14b and 14c respectively show the isoenzyme patterns of LDH, G-6-PD and MDH in the Ehrlich ascites tumor cells which were the progeny of the cells previously treated with X-rays of different doses. It appears that the isoenzyme patterns remained relatively stable even after the treatment of 2500 rads. The only small difference observed was a fast moving band towards the anode, which appeared in LDH in the cells with the 2500 rad. treatment, as shown in Fig. 14a. Cells with X-ray treatment of 1500 rads. and 3000 rads. were

not collected for isoenzyme studies.

It may be concluded that X-ray treatment of up to 2000 rads. had no effect on the isoenzyme patterns of Ehrlich ascites tumor cells developing in the mouse after the irradiation treatment.

5.3.5 Effect of exposure to inositol on the isoenzyme patterns of Ehrlich ascites tumor cells

Ehrlich ascites tumor cells were suspended in HBSS or in 5% aqueous inositol for one hour. Part of the cells were used to inoculate mice and the rest of the cells were used for isoenzyme studies. When tumors developed in the mice, cells were harvested for comparative isoenzyme studies. Figs. 15a, 15b and 15c show the isoenzyme patterns of LDH, G-6-PD and MDH. In Fig. 15a, after the Ehrlich ascites tumor cells had been treated with 5% inositol, the LDH isoenzyme pattern was the same as the pattern of the cells suspended in HBSS, which served as a control. The treated cells gave rise to the ascites tumor as rapidly as in normal passage. When cells were harvested from the mice, the previously HBSS-treated tumor had the same LDH isoenzyme pattern, whereas the previously inositol-treated tumor appeared to have only three bands, as shown by starch strip no. 4 in Fig. 15a. The fastest-moving and the slowest-moving bands were missing in the LDH pattern. The isoenzyme patterns of G-6-PD and MDH remained constant (Figs. 15b and 15c).

5.4 *In vitro* experiments

5.4.1 Comparison of the isoenzyme patterns of the tissue culture cell lines

Tissue culture lines of Ehrlich, TA_3 and $6C_3HED$ cells had been maintained for five years *in vitro* and had lost their tumorigenic capacity. New tissue culture lines of these ascites tumors were now established and when these tissue culture cells were injected into mice, tumors were produced. In other words, the newly established tissue culture lines still retained their tumorigenicity. The isoenzyme patterns of the old lines and the new lines were compared in order to determine whether there was any difference.

Figs. 16a, 16b, 16c and 16d respectively show the isoenzyme patterns of LDH, G-6-PD, MDH and esterases of all the tissue culture cell lines.

In Fig. 16a, it is obvious that the LDH isoenzyme pattern of the tumorigenic lines was distinctively different from that of the non-tumorigenic lines. There were more bands in the new tumorigenic lines, particularly one or two fast-moving bands towards the anode, which were never found in any of the non-tumorigenic lines. All the tumorigenic lines had the same LDH isoenzyme pattern and all the non-tumorigenic lines had another type of pattern. Although the 3 non-tumorigenic lines appear to have the same LDH isoenzyme pattern, the bands of the Ehrlich line did not move as far as those of the TA_3 and $6C_3HED$ lines.

The isoenzyme patterns of G-6-PD in the tissue culture lines are shown in Fig. 16b. The tumorigenic TA_3 and $6C_3HED$ lines had an extra band which was the fastest-migrating one towards the anode. This band was not observed in the tumorigenic Ehrlich and SA_1 lines and in any of the non-tumorigenic lines.

In Fig. 16c, all the tumorigenic tissue culture lines had an extra band for their MDH isoenzyme pattern, except the Ehrlich line. Again, this extra isoenzyme was the fastest moving one towards the anode in the electrophoretic separation and was never found in the non-tumorigenic tissue culture lines.

Fig. 16d shows the isoenzyme pattern of the esterases. It appears that all the tissue culture lines, either tumorigenic or non-tumorigenic, had the same isoenzyme pattern. However, when the tumorigenic lines were back transplanted into mice, in all cases a slow-moving band appeared which was never found in any cell line in tissue culture. Also on back transplantation, the fastest moving esterase disappeared and band-4 became quite prominent. The old Ehrlich tissue culture line had also lost band-1.

5.4.2 Comparison of the isoenzyme patterns of the ascites tumors *in vivo* and *in vitro*

(A) Lactate dehydrogenase

The LDH isoenzyme patterns of the ascites tumor cells grown *in vivo*, *in vitro*, and on back transplantation into the

mice for two passages are compared in Figs. 17a, 17b, 17c and 17d.

As shown in Fig. 17a, the Ehrlich ascites tumor cells had different LDH isoenzyme patterns when the cells were grown in vivo and in vitro. Furthermore, the two tissue culture lines, of which one has lost the tumorigenicity and the other still retained this property, had completely different LDH isoenzyme patterns. When cells of the tumorigenic line in tissue culture were back transplanted into mice, the LDH isoenzyme pattern remained the same as that in tissue culture. The results of TA_3 and $6C_3HED$ were about the same in that the LDH isoenzyme pattern of the non-tumorigenic lines resembled that of the in vivo lines, and that the tumorigenic lines and their cells on back transplantation into mice had a similar LDH isoenzyme pattern among themselves but different from that of the in vivo and the non-tumorigenic lines, as shown in Figs. 17b and 17c.

In case of SA_1, as shown in Fig. 17d, a non-tumorigenic tissue culture cell line has not been established. The observation was again true that back transplantation into mice did not change the LDH isoenzyme pattern of the tumorigenic tissue culture line. It should be pointed out that on the second passage of the back transplantation experiment, a very slow-moving band towards the cathode was found and this was also observed in TA_3.

In summary, the results show that the LDH isoenzyme pattern of the tumorigenic tissue culture cell lines was distinctively different from that of the non-tumorigenic tissue culture lines with the characteristic of having one or two extra isoenzymes which migrated faster than the other isoenzymes on starch gel electrophoresis. Back transplantation did not alter the LDH isoenzyme pattern significantly.

(B) Glucose-6-phosphate dehydrogenase

Figs. 18a and 18b show that Ehrlich and SA_1 ascites tumor cells had the same isoenzyme pattern of G-6-PD disregarding their tumorigenicity. In vivo or in vitro growth conditions and back transplantation did not affect the isoenzyme pattern in any detectable manner.

With regard to TA_3 and $6C_3HED$ ascites tumors, an extra band was occasionally found at the anode end of the starch strips as shown in Fig. 18c and 18d. This band was generally quite small in comparison to the two main bands. Sometimes this extra band was observed in the TA_3 cells grown in vivo (Fig. 18c). The extra band of G-6-PD in the tumorigenic line disappeared on back transplantation into the mice. Whether C_3H or Swiss mice were used, the effect was the same (Fig. 18d).

An extra isoenzyme of G-6-PD was found in the tumorigenic TA_3 and $6C_3HED$ tissue culture lines, but not in the tumorigenic Ehrlich and SA_1 tissue culture lines. This extra isoenzyme disappeared on back transplantation.

(C) Malate dehydrogenase

Figs. 19a, 19b, 19c and 19d show the MDH isoenzyme patterns of the ascites tumor cells grown *in vivo* and *in vitro*. Again, an extra MDH isoenzyme was found in all the tumorigenic cell lines in tissue culture with the exception of the tumorigenic Ehrlich line. The Ehrlich ascites tumor *in vivo*, its two tissue culture lines and the tumorigenic line on back transplantation had the same MDH isoenzyme pattern (Fig. 19a). The extra MDH isoenzyme was distinctly shown in the tumorigenic TA_3, $6C_3HED$ and SA_1 lines in tissue culture and it was at the anode end of the starch strip as in the cases of LDH and G-6-PD. Again, back transplantation caused the disappearance of this extra band (Fig. 19b, 19c and 19d).

5.4.3 Effect of different tissue culture media on the isoenzyme patterns

(A) The tumorigenic (new) tissue culture lines

All tumorigenic lines were quite new in tissue culture. The Ehrlich, TA_3 and SA_1 lines were established in Waymouth's MB752/1 medium and the $6C_3HED$ line in a modified M150 medium, both supplemented with 20% calf serum. At the time of the experiments on isoenzymes, the cell lines had been maintained in culture for only about six months. The morphology of the cells of all these tissue culture lines was fibroblastic-like. They all grew very well in Waymouth's MB752/1 medium. In M150, the SA_1 line did not grow at all,

the Ehrlich line grew very poorly, and the TA3 and 6C3HED lines did not grow as well as in Waymouth's MB752/1 medium. Consequently, only the TA3 and 6C3HED lines could yield enough cells for the comparison of the isoenzyme patterns when the cells were cultured in M150 medium.

Figs. 20a, 20b and 20c show that the isoenzyme patterns were the same when different media were used for culturing the cells. The LDH patterns (Fig. 20a) show relative uniformity. The extra band of G-6-PD and MDH in 6C3HED cultured in M150 was relatively small (Figs. 20b and 20c). But in all cases, the extra isoenzyme or isoenzymes stood out sharply as the fastest-moving band towards the anode end of the starch strips.

It is concluded that the isoenzyme patterns were the same with Waymouth's MB752/1 and M150 media.

(B) The non-tumorigenic (old) tissue culture lines

These tissue culture lines were fairly old (five years) in comparison to the newly established lines which were still tumorigenic. The morphology of the cells of these old lines in tissue culture was epithelial-like. They grew well both in Waymouth's MB752/1 and M150 media.

As shown in Figs. 21a, 21b and 21c, the respective isoenzyme patterns of LDH, G-6-PD and MDH were the same when cells were cultured in Waymouth's MB752/1 or in M150 medium. It was again true that the medium used had no effect on the isoenzyme patterns.

5.4.4 Isoenzyme patterns of cells from clones

Cloning of the cells was done for all the tumorigenic tissue culture lines. Unfortunately, the procedure did not prove successful except for the Ehrlich lines. Cells of the other tumorigenic lines died out in the cloning experiments. Four clones of the Ehrlich lines were obtained for the isoenzyme study. They all had the same type of isoenzyme patterns as shown in Figs. 22a, 22b and 22c. Also, the general cell population from which the clones were isolated had the similar isoenzyme patterns to the clones.

5.4.5 Effect of the length of time after the establishment of the cell line in tissue culture on the isoenzyme pattern

The SA_1 ascites tumor cells from mouse were cultured in Waymouth's MB752/1 medium. After five weeks and one subcultivation, cells were harvested for isoenzyme study. The LDH isoenzyme pattern is shown with the starch strip 1 (Fig. 23); an extra fast moving band was found. This cell line has still been kept in tissue culture. After another four months, the cells were again harvested when it was in the seventh passage. The extra band did not disappear. In fact, instead of one extra band, two were observed. These results indicate that the LDH isoenzyme pattern of the SA_1 ascites cells was changed when the cells were put in tissue culture, but once the pattern was changed, it remained quite similar for four months at least.

6. DISCUSSION

6.1 Choice of enzymes

This investigation is based on the assumption that some enzyme or enzymes in cancer cells may have isoenzyme patterns different from those of normal cells. It is also assumed that not all the enzymes would change their isoenzyme pattern once a cell becomes malignant, for change of every enzyme might upset the cellular metabolism to such an extent that a lethal effect would result. Thus, one of the problems in this investigation is to choose several enzymes for study whose isoenzyme patterns might yield direct information in relation to malignancy. Since ascites tumor cells are fast growing, it would seem promising to work on some enzymes in the biosynthetic pathways of nucleic acid, but adequate methods for separating and visualizing these enzymes have not yet been developed. Most of the current research on isoenzymes is carried out on dehydrogenases, probably due to the availability of methods for visualizing their enzyme activity directly on starch gel after separation by electrophoresis. This is one of the reasons that three dehydrogenases, namely lactate, malate, and glucose-6-phosphate, were chosen for this isoenzyme-pattern study.

The three dehydrogenases chosen are enzymes involved in different pathways of glucose catabolism. Lactate

dehydrogenase has been of considerable interest in the biochemistry of cancer since Warburg's classical observation (1930) that malignant cells produced a large amount of excess lactic acid under both aerobic and anaerobic conditions. Its isoenzyme pattern in cancer tissues has also been found to be different from the pattern of normal tissues (27,80,81,113). Apart from this, the LDH isoenzyme pattern has been found to be related to evolution (110), ontogeny (58,59), genetics (21,84), cellular differentiation (61), metabolic control mechanisms (16,44) and pathology (109,112). For all these reasons, LDH was chosen for this isoenzyme study.

Malate dehydrogenase is an enzyme in the tricarboxylic acid cycle. It will serve as a comparative parameter in conjunction with LDH as far as energy production is concerned. The conversion of malate to oxaloacetate by MDH appears to be an important reaction since oxaloacetate is involved with several pathways such as the synthesis of amino acids through aspartate, energy production through either the TCA cycle or the glyoxylate by-pass, and conversion to pyruvate (28). Malate dehydrogenase was thus chosen.

Glucose-6-phosphate dehydrogenase is the enzyme which initiates the pentose phosphate shunt through which five-carbon sugars are produced for the synthesis of 5-phosphoribosyl-α-1-pyrophosphate, an important intermediate for the de novo synthesis of nucleic acid. This enzyme is also genetically interesting as mentioned previously (21,45,114).

The isoenzyme study of G-6-PD might conceivably yield some valuable information about cancer cells.

Esterase was chosen for two reasons. Firstly, it is easily separated and visualized in starch gel. Secondly, the isoenzyme pattern of non-specific esterases has been shown by Paul and Fottrell (70) to be genetically stable and to be essentially the same even after cells have been maintained in tissue culture for several years. In searching for a biochemical marker due to gene mutation, the stability of the isoenzyme pattern of esterases would be of interest, especially with regard to those ascites tumor cells in tissue culture which had lost their tumorigenic capacity.

In summary, the four enzymes studied were selected because of the involvement of three of them in three different pathways of carbohydrate metabolism, namely, LDH in glycolysis for energy production under anaerobic conditions, MDH in TCA cycle for energy production under aerobic conditions, and G-6-PD in pentose phosphate shunt for the biosynthesis of five-carbon sugars which are essential for nucleic acid synthesis. The fourth one (esterase) has been found to be genetically stable and was thus not expected to have altered its isoenzyme pattern.

6.2 Electrophoretic systems

One of the problems in this investigation was to find a suitable electrophoretic system for separating the different molecular forms of the enzymes studied. No single system is

suitable for all four enzymes; a system may be good for separating the isoenzymes of one enzyme but not for the others. In preliminary studies, the most widely used buffers such as tris-citrate, phosphate, veronal and borate were tested, with both continuous and discontinuous systems at different pH's and different ionic strengths. All were found to be unsatisfactory for the separation of LDH from ascites tumor cells. Poulik's discontinuous buffer system (77) gave good separation of the isoenzymes of esterases, but when higher voltage was applied for LDH, the gels tended to break off at the slit where the cell-free extract was inserted. Tris buffer of 0.05 M at pH 8.6 appeared to be most satisfactory for preparing the starch gels for several reasons. First, it gave fairly good separation of the isoenzymes of the three dehydrogenases. Secondly, the gels prepared with this buffer had suitable consistency for easy handling. Thirdly, higher voltage could be applied without producing too much heat in the gel. Thus, the buffer system finally selected was 0.05 M tris (pH 8.6) for the preparation of starch gels and 0.3 M borate (pH 8.6) for connecting the current. This system was used for separating the isoenzymes of the three dehydrogenases and only the time of electrophoresis was different (7 hours for LDH, and two hours for G-6-PD and MDH). The electrophoretic time was relatively short in comparison with the 10-20 hour periods reported by most other workers (24,58). Another advantage of the present method was that G-6-PD and MDH could

be studied simultaneously with one gel. Half of the gel was used for identifying G-6-PD and the other half for MDH. In this way, much time was saved. Also, in the preliminary studies, the coloured bands of LDH were often found to be distorted. This discrepancy was later found to be due to the little piece of filter paper used for absorbing the cell-free extract. When this filter paper was removed from the gel after 15-20 minutes of electrophoresis, the bands were seldom observed to be distorted. For consistent results, the removal of the filter paper was found to be necessary.

6.3 Staining for enzyme activity

The isoenzymes were not identified by staining them directly on the gels; indirect methods for visualizing them were used. The methods were adopted from histochemistry.

For the staining of the dehydrogenases on starch gel, a modification of the method developed by Markert and Ursprung (59) was used. This method has general applicability to any electrophoretically mobile enzyme using NAD^+ or $NADP^+$ as coenzyme. When a dehydrogenase catalyzes a reaction, the substrate is oxidized and loses two hydrogen atoms. The coenzyme NAD^+ is in turn reduced to become NADH. In this staining method, phenazine methosulfate is used to transfer the electron from the reduced coenzyme NADH to the tetrazolium salt (MTT) to form the insoluble formazan in the gel. Formazan has a deep blue or purple colour and, thus, the enzyme activity can be localized directly on the gel. By using different

substrates, the isoenzymes of different dehydrogenases can be identified.

In the preliminary tests, specifically, NAD^+ was found to be used by LDH and MDH, and $NADP^+$ by G-6-PD. All three dehydrogenases were stained between pH 7.0 - 8.2, but the background stain of the gel was more intense at higher pH. This made it very difficult to distinguish the bands, especially for LDH. For this reason, relatively low pH was used for the staining media, 7.2 for LDH and G-6-PD, and 7.6 for MDH. Magnesium ions were not found to be essential for the activity of G-6-PD but it did enhance the activity. Potassium cyanide, used for LDH and MDH, is to block the electron transfer from the NADH to the cytochrome system so that the tetrazolium salt is reduced more readily.

The intensity of the colour of the bands reflects the accumulated activity of the enzyme during the incubation period, and it is dependent on the concentration of the active enzyme present and on the length of the incubation time. For this reason, longer incubation time is required in order to visualize very faint bands.

Various tetrazolium salts, e.g. nitro blue tetrazolium and neo-tetrazolium chloride, were tested and MTT was found to be the best dye for two reasons. It is readily soluble in the buffer solutions. It produces formazan in the gel faster than other tetrazolium salts. The widely used nitro blue tetrazolium was proved less satisfactory than MTT.

For the staining of the non-specific esterases, the commercial diazonium salt, Fast blue B, was used as the dye coupler and α-naphthyl acetate as the substrate. Esterase breaks the ester bond of α-naphthyl acetate and the naphthol released is allowed to couple with the dye to form a brown precipitate at the site of the enzyme. No other substrates were used to test the substrate specificity.

6.4 Reproducibility of the results

In the preliminary experiments, attention was given to examining whether the isoenzyme patterns observed were reproducible. The amount of cell-free extract applied was not found to be critical. Similar isoenzyme patterns were obtained with 15-35 microlitres of sample. The mobility of the isoenzymes was not affected by more sample used. However, for LDH, over 25 microlitres of sample seemed to cause trailing and intense background stain, and thus prevent separation of the bands.

In the in vivo experiments, the age and sex of the mice used appear to have no influence on the isoenzyme patterns as shown in Fig. 2. The age of the ascites tumor, the methods used for breaking the cells and storage of the cell-free extracts up to 142 days also do not seem to affect the isoenzyme patterns as shown in Figs. 3, 4 and 5. In the in vitro experiments, the use of trypsin to collect the cells from the tissue culture flasks gave the same isoenzyme pattern as when a rubber policeman was used (Fig. 6). None of these

factors appeared to have any effect on the reproducibility of the results.

It should be pointed out that slightly irregular results were observed from time to time. For example, the second band of MDH in Ehrlich cells (Fig. 3) appears to have split into two distinct bands as shown in Fig. 7c. In regard to LDH, sometimes band 1 was not observed and a slow-migrating band may appear behind band 5, and sometimes a small band may appear between bands 3 and 4, as well as bands 4 and 5 with the conditions used. But the relative positions of the bands were very constant. The reasons for the small irregularity of the results were not investigated further, since minor inconsistency did not appear to affect significantly the general interpretation of the results. The isoenzyme pattern of G-6-PD was found to be most consistent except that the second band was very faint on rare occasions. Since the amount of enzyme which was applied for electrophoresis was not controlled precisely, perhaps slight irregularity of the results is unavoidable.

6.5 Lactate dehydrogenase

Ehrlich, TA_3, $6C_3HED$ and SA_1 ascites tumor cells grown in mice are shown to have a similar LDH isoenzyme pattern (Fig. 7a) under the experimental conditions described. Although the pattern consists mainly of five discrete bands, as many as eight bands were observed occasionally as shown in $6C_3HED$ (Morgan) from Swiss mice (Fig. 8c). There was no

obvious explanation for this variation. Notwithstanding, the relative positions of the five main bands are consistent, and the number of bands, though found to be variable on rare occasions, does not necessarily alter the pattern. In comparison with the pattern of the ascitic fluids from both the tumor-bearing and non-tumor bearing mice, the pattern of the ascites tumor cells appears to be different in that the ascitic fluids always show a distinct fast-moving band and the other bands are closer to each other and form a group. Since ascitic fluid is a physiological fluid of the host and the LDH in this fluid comes from tissues of the mouse, its isoenzyme pattern may serve as a reference to the isoenzyme pattern of the host. This may imply that the LDH isoenzyme pattern of the ascites tumor cells is different from the pattern of the mouse tissues. However, the ascitic fluids from the tumor-bearing mice may have contained some LDH originally from the tumor cells that had died in the peritoneal cavity. Ascitic fluids induced by Freund's adjuvant were obtained from the mice in abnormal physiological conditions. It is debatable whether the LDH isoenzyme pattern of these ascitic fluids represents the true pattern of the normal physiological fluid from the mice. This doubt was removed when sera from normal healthy Swiss mice were found to have the same LDH isoenzyme pattern as the ascitic fluid used.

The LDH isoenzyme pattern of the ascitic fluids is itself interesting with regard to the origin of the fast-moving

band. No literature reference has been found to contain a description of such a band. Using a different electrophoretic system, Allen reported (1) a slow-moving band migrating towards the cathode in mature mouse testis. Indeed, sperm-specific LDH isoenzymes have been found in a variety of animals (10,26,115,116). But the fast-moving band found in the ascitic fluid does not seem likely to be related to the sperm-specific isoenzymes.

The four ascites tumors may represent different tissues of the mouse although the tissue of origin of Ehrlich carcinoma and SA_1 is not known. TA_3 mammary carcinoma and $6C_3HED$ lymphosarcoma have different tissue origins. The similarity of the LDH isoenzyme patterns of these tumors agrees with the observation that LDH isoenzyme patterns of malignant tissues of different origin from the human tend to converge to a common pattern in spite of the difference among the patterns shown by the respective normal tissues (27,81,88). The similarity of the LDH isoenzyme patterns of the ascites tumors cannot be related to malignancy without further studies of the individual isoenzymes. Also, the occasional appearance of eight LDH bands for the ascites tumors cannot be explained with respect to genetics and malignancy.

For the X-ray irradiation experiment, the LDH isoenzyme pattern of Ehrlich ascites tumor cells remained similar up to 2500 rads of treatment with a small exception that the progeny of the 2500 rad-treated cells gave a fast-moving band.

Such a fast-moving band has also been found in the cells of regular size of the SN and INSN lines. Since the experiment was not repeated, it is questionable whether the appearance of this fast-moving band is due to the effect of the high dosage of radiation.

The treatment of the Ehrlich ascites cells with 5% inositol (myo) for one hour did not alter the LDH isoenzyme pattern. But when the treated cells were propagated in mice, two main bands were missing and a faint fast-moving band appeared. This fast-moving band has also been found in the regular-sized cells of the INSN-Ehrlich line which was developed with the treatment of 5% inositol. Perhaps this fast-moving band is related to the development of the peculiar balloon cells.

With regard to the in vitro experiments, the tumorigenic cell lines and the non-tumorigenic cell lines had two distinctly different LDH isoenzyme patterns, although these two kinds of tissue culture line have the same tissue origin. The tumorigenic pattern has several more bands of which one or two are fast moving. The fast-moving bands are quite prominent and were never found in the non-tumorigenic lines. The slow-moving bands appear to be more prominent in the non-tumorigenic lines which had lost the fast-moving bands. It seems difficult to relate the loss of these electrophoretically fast-moving LDH isoenzymes to the loss of malignancy because these fast-moving LDH isoenzymes were seldom found

in the ascites tumor cells grown in mice.

It is also difficult to relate the fast-moving LDH isoenzymes to malignancy for the same reason that they were seldom observed in the ascites tumor cells grown in mice. If these fast-moving isoenzymes are necessary for the metabolism of malignant cells, they should have also shown up consistently in the ascites tumor cells grown in mice as well as grown in tissue culture. It appears that the tumorigenic ascites cells possess the potential to synthesize some LDH isoenzymes of different electrophoretic mobility, which have not been found in the non-tumorigenic cells of the same tissue origin. This potentiality alone is interesting although its importance in relation to malignancy is beyond the present speculation.

Enzyme activity levels in various organs and tissues of an animal show relatively large variations (58). Lieberman and Ove (53) have found that human cells of different tissues in tissue culture have marked similarity in their enzyme activity levels. In general, as far as enzyme activity levels are concerned, few conspicuous differences were noted between tissue culture cell lines of the same species, whether derived from normal or malignant tissues (52). At the isoenzyme level, the present results also indicate that the convergence to a common pattern is true when cells of different tissue origin are cultured <u>in vitro</u> since all the tumorigenic tissue culture lines show a similar LDH isoenzyme pattern (Fig. 16a).

This similarity between the tumorigenic lines is not surprising for the cells of the different ascites tumors grown *in vivo* have already shown a common LDH isoenzyme pattern. The LDH isoenzyme patterns of the four ascites tumors are remarkably uniform in each type of condition and at each time of alteration. Cells of the different ascites tumors had a common LDH isoenzyme pattern when they were grown in mice (Fig. 7a). If ascites cells were removed from the mice and propagated in tissue culture, a new LDH isoenzyme pattern appeared which was also common to all the ascites tumors (Figs. 17a, 17b, 17c). This new pattern due to the *in vitro* cultivation remained unchanged after the cells were back transplanted into mice for two passages. The *in vivo* conditions did not appear to reverse the *in vitro* pattern, at least for two passages in mice. Furthermore, the non-tumorigenic tissue culture lines originated from the same ascites tumors showed another type of LDH isoenzyme pattern which was different from the *in vitro* pattern of the tumorigenic lines. Since the non-tumorigenic lines had been kept in tissue culture for five years and the tumorigenic lines for only six months, it may imply that the *in vitro* LDH isoenzyme pattern of the tumorigenic lines would shift to the non-tumorigenic pattern after a longer time of cultivation *in vitro*. On close examination the non-tumorigenic pattern of TA_3 and $6C_3HED$ is similar to the pattern of the tumor cells serially grown in mice (Figs. 17b, 17c). The above observations raise a number of questions of their

biological implications. Why do the different ascites tumors have a similar LDH isoenzyme pattern? Why did they change to a new pattern when cultivated *in vitro*, and why did this new pattern not change back to the original *in vivo* pattern when the cells were transplanted back into mice? Why did they change to another new pattern after a long time of cultivation *in vitro*? How did they lost their malignancy in tissue culture? How did all these changes come about? What is the relationship between all these changes of LDH isoenzyme pattern and malignancy? Would the non-tumorigenic pattern continue to change after even longer time of cultivation *in vitro*? Would the non-tumorigenic cells change back to be tumorigenic ones? Much will have to be done if these questions are to be answered satisfactorily.

The similarity of the LDH isoenzyme patterns of the ascites tumors may be significantly related to malignancy. Since the common pattern is shown consistently in different types of malignant cells, it would seem that such a pattern is essential for the type of metabolism characteristic of malignant growth. The origin of the common pattern could be achieved during the transformation of cells from normal to malignant. Whether such an LDH isoenzyme pattern causes malignancy or whether malignancy causes the advent of such a pattern, or whether they are actually two independent events, cannot be resolved at the present time. Starkweather and Shoch (88) reported that the LDH isoenzyme in human tumors

had different Michaelis constants for lactic acid when compared with the LDH isoenzymes for normal tissues. They suggested that a structurally different protein characteristic of neoplastic tissue may have evolved.

The change of the LDH isoenzyme pattern due to *in vitro* conditions could be essential for the adaptation of the cells to the new environment. It should be emphasized that the influence of hormones and of other unknown factors from the host is removed once cells are put in tissue culture. The appearance of the new isoenzymes may then be caused by the loss of this influence from the host. Jacob and Monod (39) have proposed that the synthesis of individual proteins may be provoked or suppressed within a cell under the influence of specific external agents or conditions, and such an operation is generally essential to the survival of the cell. The change of the growth conditions may not be a direct cause for the change of the LDH isoenzyme pattern. It is possible that some metabolic pathways may have to operate more actively, some remain about the same, and some become less active when cells are transferred from mice to tissue culture. The alteration of activity of one pathway may lead to the alterations of some others. Thus, the alteration of the LDH isoenzyme pattern could be caused by the alteration of some other pathways. The consistent appearance of a few more isoenzymes *in vitro* may occur due to the operation of one or more suppressed gene(s) which is or are not supposed to function. Cahn (17)

has observed that embryonic chick heart cells never synthesize muscle-LDH in any significant amounts if left in ovo, but when they were put in tissue culture for six days, the muscle-LDH increased from less than 0.01% to 41% with a corresponding decrease of heart-LDH from 65% to 1%. He suggested that the in vitro conditions caused the "turning on" of gene expression for the muscle-LDH. Perhaps the appearance of the extra LDH isoenzymes in the tumorigenic ascites cells is due to this mechanism of the "turning on" of gene expression when the cells are grown in tissue culture.

Nitowsky and Soderman (67) reported that the electrophoretic LDH isoenzyme pattern of fetal mouse liver cells in primary culture showed the appearance of a new, faster-moving anodal isoenzyme after 13 days of growth. This seems to agree with the results described in the present study. No extra electrophoretically fast-moving isoenzyme in mouse tissue has been reported. This is particularly interesting because fetal cells were used instead of adult cells. This may imply that the ascites tumor cells resemble the fetal cells as far as the LDH isoenzyme pattern is concerned. This will lead to another question as to whether tumor cells really reverse to the more primitive stage, that is, less differentiated than their original cells. Nebel and Conklin (65) reported that chicken liver transplanted to the chorioallantoic membrane of the chick embryo changed its LDH isoenzyme pattern to a more primitive type. This may seem to suggest that the reversion to a less differentiated cell type would

be shown by the LDH isoenzyme pattern. Whether this is the case in the ascites tumors is not known.

Tsao (98), using a different buffer system for starch gel electrophoresis, found only one LDH band in mouse fibroblast cells and mouse ascites lymphoma cells in tissue culture. The results of this study do not agree with his observations. Perhaps his electrophoretic system was not good enough to resolve the isoenzymes. Perhaps the cell lines he used did have only one LDH isoenzyme. It is unfortunate that the names of the cell lines used were not mentioned in his report.

The tumorigenic cells in tissue culture persist in having the new LDH isoenzyme pattern when back transplanted into mice. This is an intriguing phenomenon. Theoretically, the new pattern should change back to the original in vivo pattern when cells are transplanted back into mice. If the new pattern was caused by the "turning on" of gene(s) under tissue culture conditions, perhaps the gene(s) cannot be turned off once the operation is turned on. Penicillinase is induced in Bacillus cereus when penicillin is added in the medium (39). However, the synthesis of penicillinase continues for a long time, at a decreasing rate, after removal of inducer (penicillin) from the medium. Perhaps two passages of the tissue culture cells in mice were not long enough to reverse the LDH isoenzyme pattern back to the original in vivo pattern. It would be most interesting to carry on the mouse passage further following back transplantation of the

cells into mice; the persistence of the new pattern after back transplantation from tissue culture may then be better explained.

The change of the LDH isoenzyme pattern after long term (five years) cultivation in tissue culture presents another intriguing problem. Ehrlich ascites cells had their pattern changed to a distinctly new pattern after long term cultivation, but the pattern of TA3 and 6C3HED cells changed to a pattern similar to the original pattern for ascites tumor cells serially propagated in mice. Thus, the change of the in vitro tumorigenic LDH isoenzyme pattern to another new pattern does not seem to be related to the loss of tumorigenic capacity of the old tissue culture cell lines. The change of LDH isoenzyme pattern and the loss of tumorigenic capacity may be two independent events occurring in the cells during long term cultivation. There are other alternatives to explain the change of the isoenzyme pattern in vitro. First, the initial cultures may consist of more than one type of cell. The culture conditions may favour the growth of a certain cell type and thus selectively outgrow the other types of cells after a long time in tissue culture. It is possible that the cell type selected gives the new isoenzyme pattern. Secondly, the inactivation of gene(s) due to the cultural conditions could lead to a new isoenzyme pattern. This is unlikely to be the case for why should the cells shift their isoenzyme pattern again to another pattern after

longer time (five years) under similar cultural conditions. Thirdly, gene mutation can cause the change of the isoenzyme pattern. It would be interesting to examine the chemical and immunological properties of the LDH isoenzymes of the non-tumorigenic cells in tissue culture. If they are immunologically different from those isoenzymes of the tumorigenic cells, inference could be made that a gene mutation may have occurred in the cells during the long time of cultivation in vitro.

The purpose of cloning of the tumorigenic tissue culture lines was to see if the culture consisted of a mixed population of different cell types as far as isoenzyme pattern is concerned. Clones of TA_3 and $6C_3HED$ lines were not obtained since the cells did not grow in the cloning experiments. But the four clones of the tumorigenic Ehrlich tissue culture line did not show any significant difference in

their isoenzyme patterns of LDH (Fig. 22a). Clone a and clone b seem to have fewer bands than clone c and clone d, but the pattern appears to be similar. It is possible that the tissue culture line has a homogeneous cell population. On the other hand, if the cell line has a heterogeneous cell population, the randomly isolated clones could still show a similar isoenzyme pattern for the following reasons. Since only four clones were isolated, they could be of the same type of cells which was predominant in the mixed cell population. Or even if the clones were of different cell types, they could still show a similar isoenzyme pattern for

the tissue culture conditions may make the closely related cell types converge their isoenzyme patterns to a common pattern, or different closely related cell types may indeed be similar with regard to their isoenzyme pattern.

The growth of the ascites tumor cells in tissue culture depends greatly on the medium used. Waymouth's MB752/1 medium with 20% calf serum appears to be most suitable for the growth of these cells. The recently established cell lines grew poorly in M150 medium. This is understandable since the cell lines were established in Waymouth's medium. Perhaps these new cell lines need a period of time of adaptation before they can be propagated well in another medium, or M150 is simply not as suitable for the growth of these ascites cells. However, the LDH isoenzyme pattern of the tissue culture lines remained the same when M150 was used, although the cell growth was not as good as in Waymouth's medium (Figs. 2a, 21a). No correlations were observed between cell morphology or nutrient medium and LDH isoenzyme pattern.

In the SA1 tissue culture line, a fast-moving band was observed in the LDH isoenzyme pattern after five weeks and one subcultivation (Fig. 23). Another new band appeared at a similar position after another 4 months and six subcultivations. The appearance of the second new band may be due to further adaptation of the cell in tissue culture. The true nature of the rise of new isoenzymes as cultivation continues is not known.

6.6 Glucose-6-phosphate dehydrogenase

The isoenzyme patterns of G-6-PD in the regular ascites tumor lines in vivo are similar to each other though some of the tumors were obtained from other research laboratories. No qualitative difference was found. With regard to the supernatant lines, it appeared that the G-6-PD enzyme activity was generally lower than the regular lines on gross examination of the bands. But quantitative analysis of the enzyme activity was not carried out. Balloon cells of all the SN lines appeared to lose the second band (Fig. 12e) which was quite faint for the regular-sized cells. It is possible that the balloon cell SN lines have lost the second band due to the transformation of cell type (at least morphologically different). On the other hand, the loss of the second band may simply be due to the presence of less active enzyme in the cell-free extract. Although the same dilution (1 part of packed cell; 2 parts of water) was used for obtaining cell-free extract, since the balloon cells contain large vacuoles, the same volume of packed balloon cells may not give as much enzyme as the same volume of packed regular cells to the cell-free extract. In fact, the protein determination (data not presented herein) indicates that the cell-free extracts of the balloon cells have a lower protein concentration. Conclusion cannot be made that the second band of G-6-PD is missing in balloon cells of the SN lines. Besides, this second band is shown in the balloon cells of the INSN-Ehrlich

and INSN-TA_3 lines. Inositol and X-irradiation were found to have no effect on the isoenzyme pattern of G-6-PD.

In the *in vitro* experiments, the G-6-PD isoenzyme pattern was the same in all cases except that a fast-moving band was shown in the tumorigenic TA_3 and $6C_3HED$ tissue culture lines. It should be emphasized that this extra fast-moving band was never observed in any of the non-tumorigenic tissue culture lines and in the tumorigenic Ehrlich and SA_1 tissue culture lines (Fig. 16b). Furthermore, cells when back transplanted into mice from tissue culture did not show this extra band (Figs. 18b, 18c). In other words, the isoenzyme pattern of G-6-PD in the tumorigenic TA_3 and $6C_3HED$ lines reversed back to the *in vivo* pattern when back transplanted into mice from tissue culture. Here a different picture is seen when compared with the LDH isoenzyme patterns. With regard to LDH, all the ascites tumor lines changed to the same pattern when the cells were grown in tissue culture, whereas with regard to G-6-PD, Ehrlich and SA_1 lines remained unchanged but TA_3 and $6C_3HED$ changed to a new pattern with an extra fast-moving band. These results were observed in three experiments. Only on one occasion the extra fast-moving band was faintly shown in cells grown in mice, that is in TA_3 (Fig. 18b). The loss of the extra band of G-6-PD in the tumorigenic TA_3 and $6C_3HED$ tissue culture lines on back transplantation into mice was probably due to the adaptation to the former environment.

The extra fast-moving band does not seem to be related to malignancy for the same reasons as mentioned in the case of LDH. If this band is essential to the metabolism of malignant growth, it should consistently show up in the in vivo experiments, and Ehrlich and SA_1 should also possess such a band when their cells from mice are grown in tissue culture. Moreover, the non-malignant tissue culture lines have the same pattern as the regular lines in vivo. Nevertheless, this band found in TA_3 and $6C_3HED$ would serve as a difference from SA_1 and Ehrlich. It is possible that SA_1 and Ehrlich are of a similar or more closely related cell type, and TA_3 and $6C_3HED$ of another cell type.

6.7 Malate dehydrogenase

The isoenzyme patterns of MDH were similar throughout the in vivo experiments with two large bands of which the second (slower-moving) one was sometimes shown as a large diffused zone. A very small band between the two main large bands was frequently observed (Figs. 10a, 13e, 13f) in the regular cell lines and in the cells of regular-size of the SN and INSN lines, but never in the balloon cells. Whether this small band was truly missing is again the question of the enzyme concentration applied for electrophoresis, which may not have been high enough to show the band. This small band is unlikely to be related to malignancy since it is not observed consistently.

The results of the MDH isoenzyme patterns for the in vitro experiments are similar to those of G-6-PD. An extra fast-moving electrophoretic band was observed in the tumorigenic TA_3, $6C_3HED$ and SA_1 tissue culture lines (Fig. 16c). This is slightly different from the results of G-6-PD in that the extra band was never shown in the isoenzyme pattern of G-6-PD in SA_1. This would serve to distinguish the two ascites tumors, SA_1 and Ehrlich. Again, as in G-6-PD, the extra band was missing when the cells were back transplanted into mice from tissue culture. Since the extra band was not observed in the non-tumorigenic tissue culture lines and in the regular in vivo lines, it would appear that the extra band is not significantly related to malignancy. The loss of the extra band when cells were back transplanted into mice from tissue culture was probably due to the adaptation of the cells to the former conditions. No difference of the MDH isoenzyme pattern was shown in the four clones from the tumorigenic Ehrlich line in tissue culture.

6.8 Non-specific esterases

The isoenzyme pattern of non-specific esterases has been found to be essentially no different in fresh tissue, in primary cell cultures and in cells grown for many years in tissue culture (9,47,70). This finding has suggested a high degree of genetic stability of the esterases. Attempts to induce changes of the isoenzyme pattern of non-specific esterases by culturing them in the presence of a substrate

(an aromatic ester) failed to produce a change (70). In this investigation, studies of esterases had not been carried out as extensively as with the three dehydrogenases. Nevertheless, the results obtained demonstrate convincing evidence of changes of the isoenzyme patterns of these enzymes (Figs. 7e, 16d).

The four regular ascites tumors grown in vivo had the same esterase isoenzyme pattern characterized by seven bands (Fig. 7e). Ascitic fluids had an entirely different pattern. All tissue culture lines had a similar pattern with an exception of missing band-1 in the non-tumorigenic Ehrlich line. The tissue culture pattern is different from the pattern of the regular in vivo cell lines in that band-4 is very faint and band-6 and band-7 are missing. When cells of the tumorigenic tissue culture lines were back transplanted into mice, band-1 was missing, band-4 became prominent again, band-6 and band-7 did not reappear, and a very distinct slow-moving band appeared which was not found in other conditions. These results do not agree with Paul and Fottrell's observations that esterases are genetically stable (70). The marked difference between the patterns of the tumor cell lines, either in vitro or in vivo, and the pattern of ascitic fluids could be important with regard to malignancy. Bands-1,5,6,7 were not found in ascitic fluids which had very prominent bands not found in tumors. Whether the extra esterases found in tumors exist in normal tissues of the mouse is not known.

It would be most interesting to examine the isoenzyme patterns of non-specific esterases in different normal tissues. If these esterases are shown to be missing in normal tissues, they may then be significant in relation to malignancy. The lack of the two esterases found in ascitic fluids could be important to the metabolism of malignant growth. Potter's "deletion theory" (63,76) suggests that the loss of an enzyme could lead to the accumulation of its substrate, which could lead other pathway(s) to operate at a higher rate. And if the pathway(s) being driven are of anabolic nature, uncontrollable growth of the cell might result. It would be too audacious to suggest such a relation between the missing of esterases and malignant growth with the present preliminary results.

It is most intriguing that band-1 should have been missing when cells of tumorigenic tissue culture lines were back transplanted into mice for this band was consistently shown in regular in vivo lines and in tissue culture lines. No reasonable explanation was found for this observation. The appearance of a new esterase on back transplantation into mice presents similar problems for an explanation with regard to the physiological significance of this new enzyme.

7. SUMMARY

1. Respective isoenzyme patterns of lactate, glucose-6-phosphate and malate dehydrogenases, and non-specific esterases were essentially similar in Ehrlich, TA_3, $6C_3HED$ and SA_1 ascites tumors grown in mice. The enzyme activities of G-6-PD and MDH in ascitic fluids were too low for visualization of their isoenzyme patterns in starch gels. Ascitic fluids had different isoenzyme patterns of LDH and non-specific esterases from the four ascites tumors.

2. Ascites tumors of the same origins but from other laboratories had similar isoenzyme patterns of the three dehydrogenases.

3. Balloon cells appeared to be different from the regular ascites tumors, and the cells of regular size of SN and INSN ascites tumor lines, with regard to isoenzyme patterns of the three dehydrogenases.

4. X-ray treatment up to 2500 rads to Ehrlich ascites tumor cells did not change the isoenzyme patterns of the three dehydrogenases in the progeny of the treated cells.

5. Treatment of 5% inositol solution to Ehrlich ascites cells for one hour had no effect on the isoenzyme patterns of G-6-PD and MDH in the progeny of the treated cells, but led to a change in the LDH isoenzyme pattern with the appearance of an extra fast-moving band and the loss of two original bands.

6. Ehrlich, TA_3, $6C_3HED$ and SA_1 ascites tumors were established in tissue culture in Waymouth's MB752/1 medium supplemented with 20% calf serum. These cell lines were found to retain tumorigenic capacity when their cells were back transplanted into mice from tissue culture after six months and at least five subcultivations in tissue culture.

7. Comparisons of isoenzyme patterns of the ascites tumor cells were made between the regular cell lines in vivo, the recently established tumorigenic tissue culture lines, the long-term cultured non-tumorigenic lines of the same tissue origins, and the cells on back transplantation into mice from tissue culture.

8. Isoenzyme patterns of LDH in all the tumorigenic cell lines were similar and characterized by having

one or two extra fast-moving electrophoretic isoenzyme bands which were not found in cells of the non-tumorigenic tissue culture lines and the regular ascites tumor cells *in vivo*. The LDH isoenzyme patterns of the tumorigenic lines and of the non-tumorigenic lines in tissue culture were different from each other. Back transplantation of cells of the tumorigenic lines into mice from tissue culture did not reverse the LDH isoenzyme pattern to the original pattern of the regular lines *in vivo*.

9. Isoenzyme patterns of G-6-PD in the tumorigenic TA_3 and $6C_3HED$ tissue culture lines had an extra fast-moving electrophoretic isoenzyme band which was not found in the tumorigenic Ehrlich and SA_1 lines, all the non-tumorigenic lines in tissue culture and the regular lines *in vivo*. Back transplantation of cells into mice caused deletion of this fast-moving band and the reversion of the pattern to the original pattern of the regular lines *in vivo*.

10. Isoenzyme patterns of MDH in the tumorigenic TA_3, $6C_3HED$ and SA_1 tissue culture lines had an extra

fast-moving electrophoretic isoenzyme band as in the case of G-6-PD. This extra band was not found in the tumorigenic Ehrlich line and all the non-tumorigenic lines in tissue culture, and the regular lines *in vivo*. Back transplantation of cells into mice caused deletion of this extra band and the reversion of the pattern to the original pattern of the regular lines *in vivo*.

11. Tissue culture lines of Ehrlich, TA_3, and $6C_3HED$ ascites tumor cells had a similar isoenzyme pattern of non-specific esterases with an exception of missing band-1 in the non-tumorigenic Ehrlich tissue culture line. The tissue culture pattern was different from the pattern of the regular lines *in vivo* in that band-4 became very faint and bands-6 and 7 were missing. Back transplantation of cells into mice caused the disappearance of bands-1, 6 and 7 and the appearance of a slow-moving band which was never observed in other cases; such changes led to an entirely new esterase isoenzyme pattern which was different from the tissue culture pattern and the original *in vivo* pattern of the regular lines.

12. Four clones isolated from the tumorigenic Ehrlich tissue culture lines showed no difference in the respective isoenzyme patterns of LDH, MDH and G-6-PD.

13. Cells propagated in tissue culture in Waymouth's MB752/1 and M150 media supplemented with 20% calf serum had similar isoenzyme patterns.

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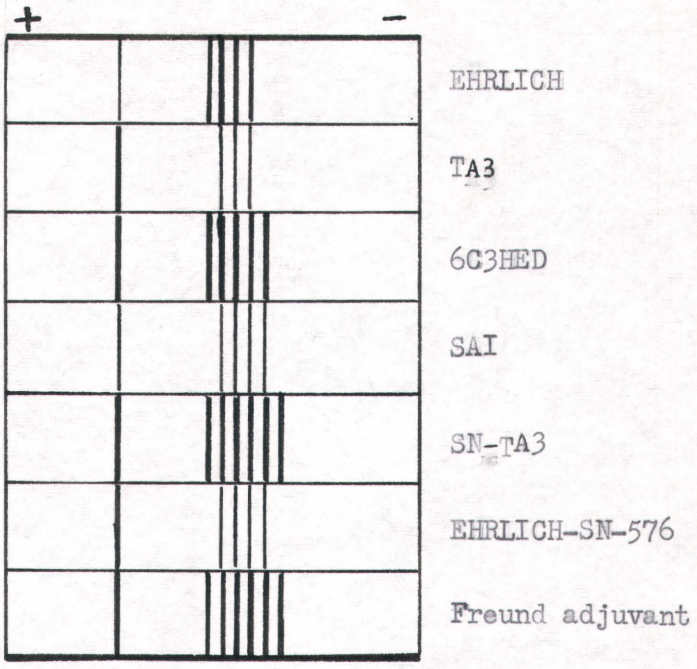
Figure 1. LDH isoenzyme patterns of the ascites fluids from mice bearing different kinds of ascites tumor. The ascitic fluid induced by Freund's complete adjuvant served as the comparison. The cathodal end of the starch strips was the origin at which enzyme samples were inserted.

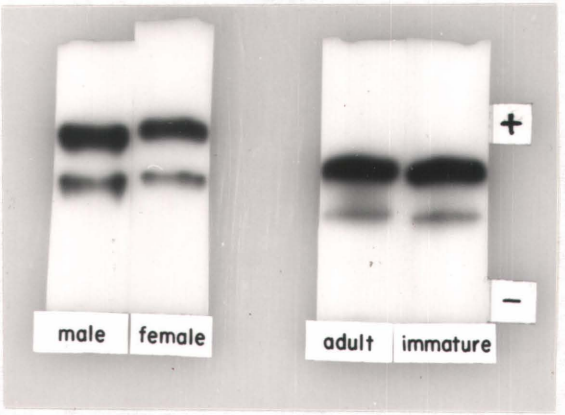
Figure 2. G-6-PD isoenzyme patterns of Ehrlich ascites tumor grown in male and female mice, and in adult and immature female mice.

Figure 3. MDH isoenzyme patterns of Ehrlich ascites tumor cells harvested 8, 11 and 14 days after inoculation.



ASCITIC FLUID

(1)



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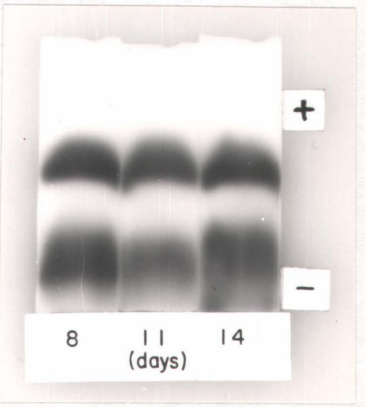


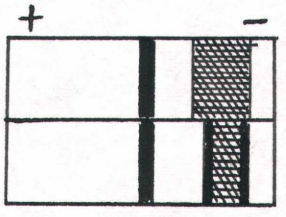
(3)

Fig. 1

Figure 4. MDH isoenzyme patterns of Ehrlich ascites tumor cells. The cells were disrupted by two different methods: freezing and thawing, and sonication.

Figure 5. MDH isoenzyme patterns of the tumorigenic $6C_3HED$ line in tissue culture; the same cell-free extract was used after 14 and 142 days of storage at -20°C.

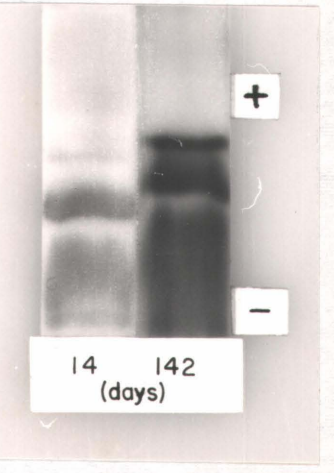
Figure 6. MDH isoenzyme patterns of the tumorigenic TA_3 line in tissue culture. Trypsin had no effect on the isoenzyme pattern.



Freezing and thawing

Sonication

(4)



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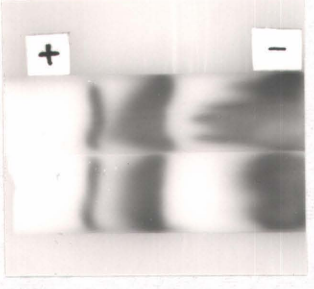


Cells removed by trypsin

Cells removed with rubber policeman

(6)

fig 4

Figure 7. Comparison of the isoenzyme patterns of the regular ascites tumors *in vivo*. The ascitic fluid was induced by Freund's complete adjuvant in mice bearing no tumor.

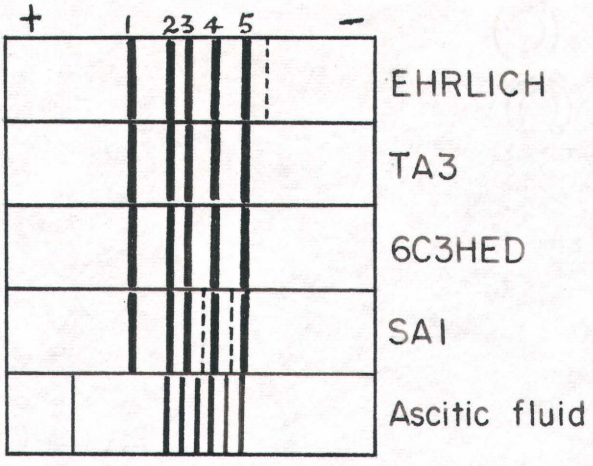
a. LDH

b. G-6-PD

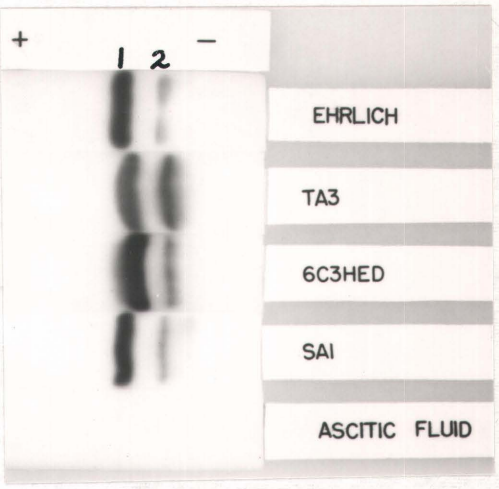
c. MDH - electrophoretic time: 2 hours

d. MDH - electrophoretic time: 90 minutes

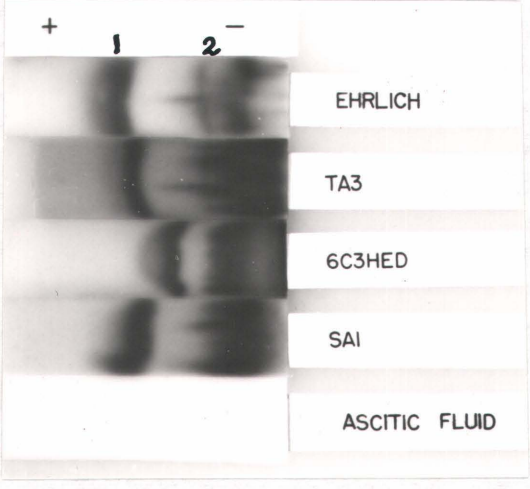
e. Esterases



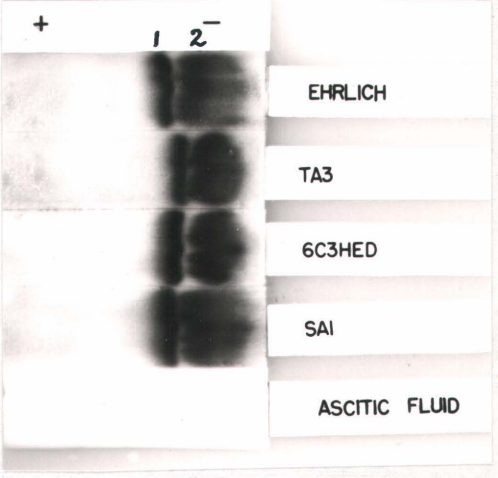
(7a)



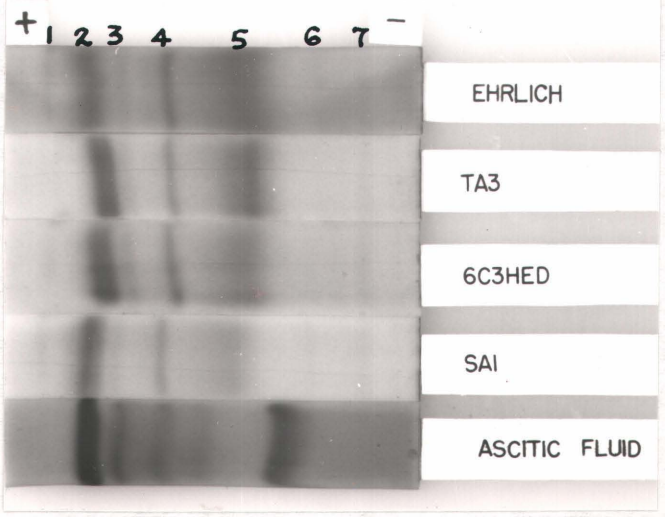
(7b)



(7c)



(7d)



(7e)

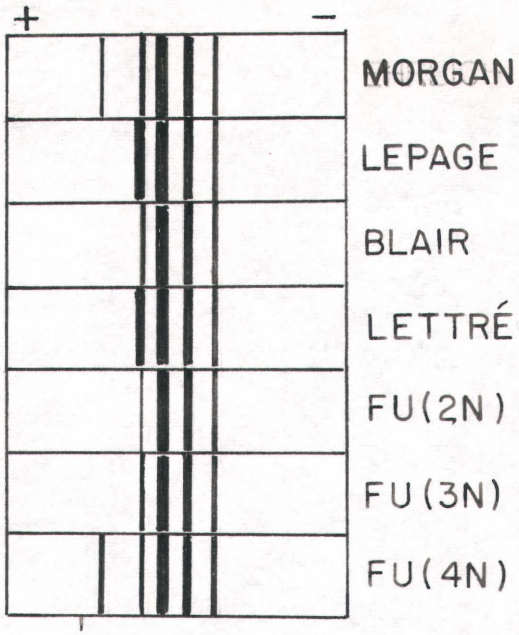
Fig 7

Figure 8. Comparison of the LDH isoenzyme patterns of the same ascites tumors from different laboratories. The names are the research workers from whom the tumors were obtained; Fu (2N) designates the hyperdiploid tumor line from Dr. Fu, 3N the hypertriploid line, and 4N the hypotetraploid line.

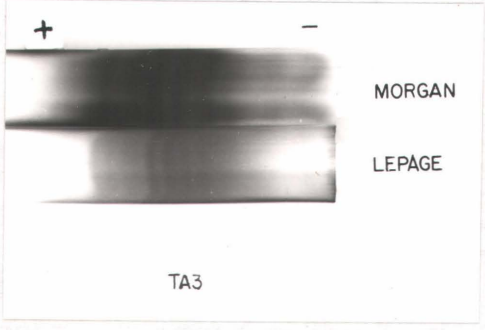
a. Ehrlich carcinoma

b. TA3 mammary carcinoma

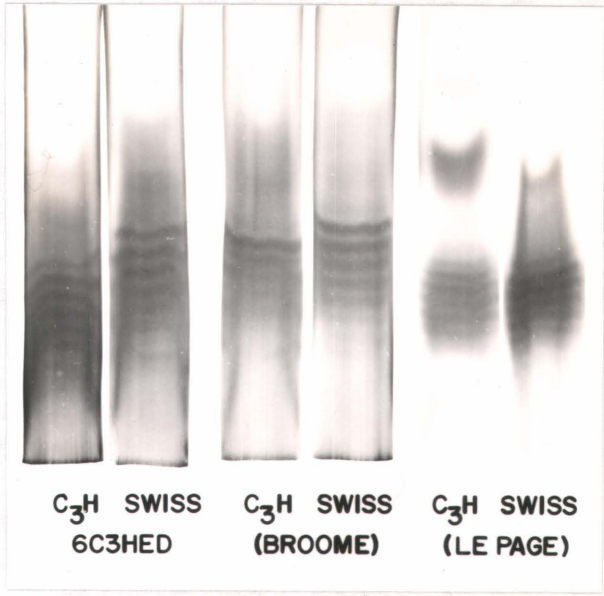
c. $6C_3HED$ lymphosarcoma


+
−
MORGAN
LEPAGE
BLAIR
LETTRÉ
FU(2N)
FU(3N)
FU(4N)
EHRLICH

(8a)


+
−
MORGAN
LEPAGE
TA3

(8b)


C3H SWISS
6C3HED
C3H SWISS
(BROOME)
C3H SWISS
(LE PAGE)

(8c)

Fig 8

Figure 9. Comparison of the G-6-PD isoenzyme patterns of the same ascites tumors from different laboratories.

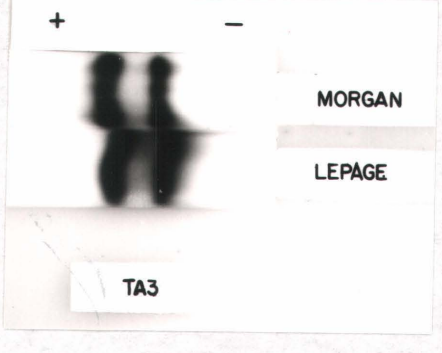
a. Ehrlich carcinoma

b. TA_3 mammary carcinoma

c. $6C_3HED$ lymphosarcoma


+
–
MORGAN
LEPAGE
BLAIR
LETTRÉ
FU (2N)
FU (3N)
FU (4N)
EHRLICH

(9a)


+
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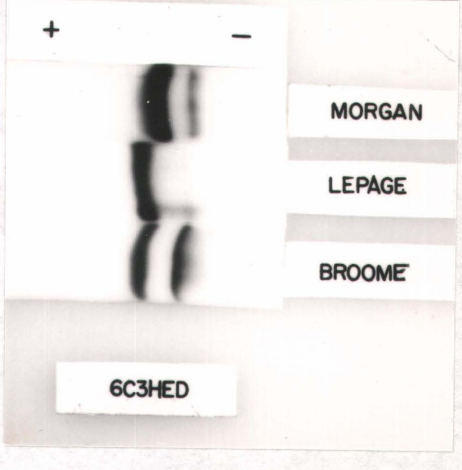
MORGAN
LEPAGE
TA3

(9b)


+
–
MORGAN
LEPAGE
BROOME
6C3HED

(9c)

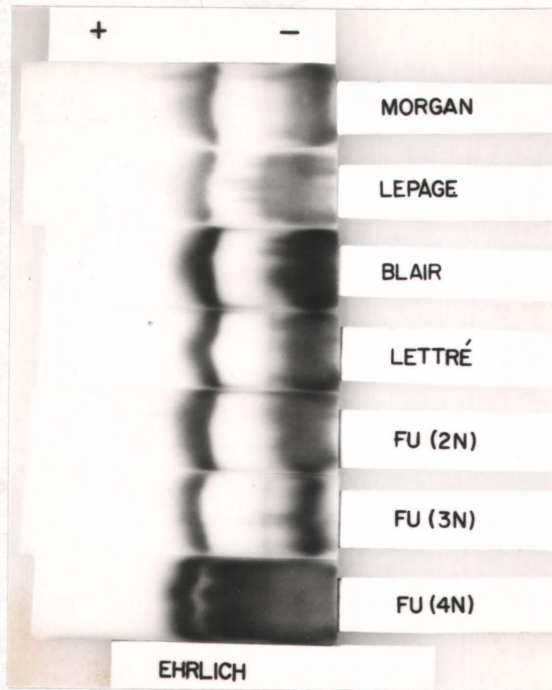
Fig 9

Figure 10. Comparison of the MDH isoenzyme patterns of the same ascites tumors from different laboratories.

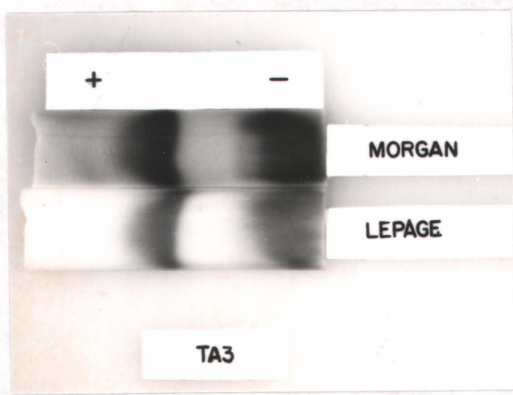
a. Ehrlich carcinoma

b. TA_3 mammary carcinoma

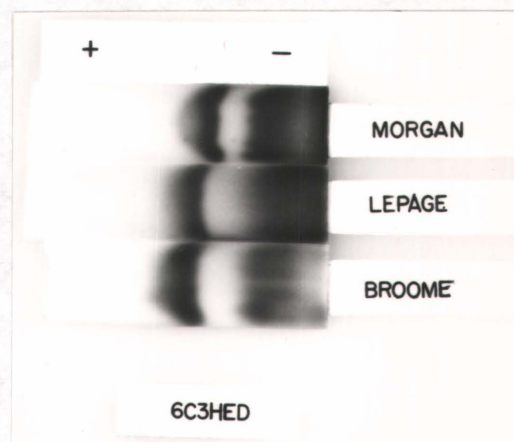
c. $6C_3HED$ lymphosarcoma



(10a)



(10 b)



(10c)

Fig 10

Figure 11. Comparison of the LDH isoenzyme patterns of the regular ascites tumors with those of their supernatant lines. "Top" designates the balloon cells, "bottom" the cells of regular size.

a. Ehrlich and its supernatant lines

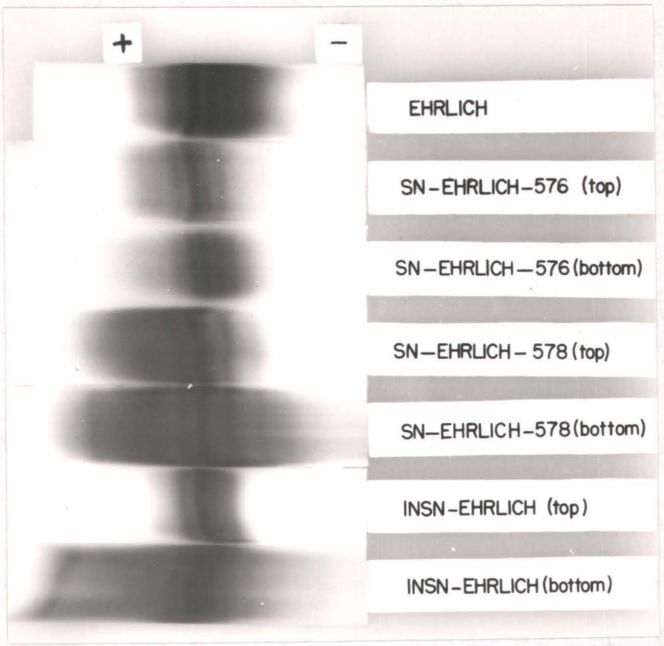
b. TA_3 and its supernatant lines

c. $6C_3HED$ and its supernatant line

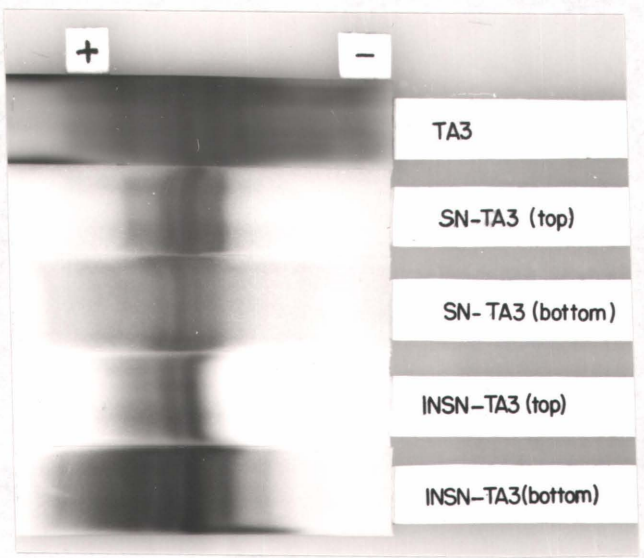
d. SA_1 and its supernatant line

e. All the SN (supernatant) lines

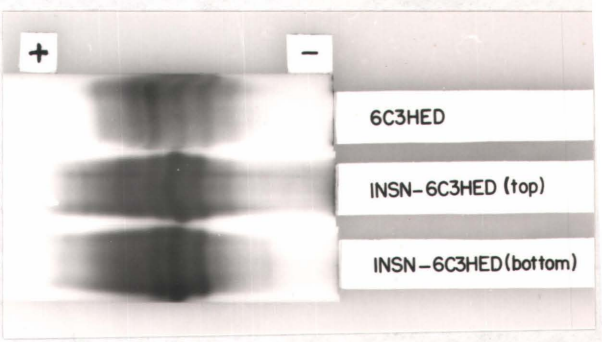
f. All the INSN (inositol supernatant) lines



(11a)



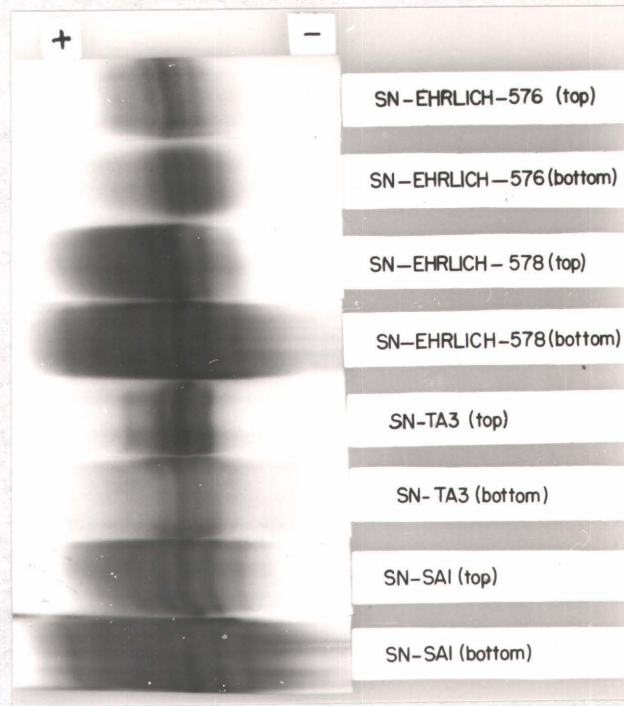
(11b)



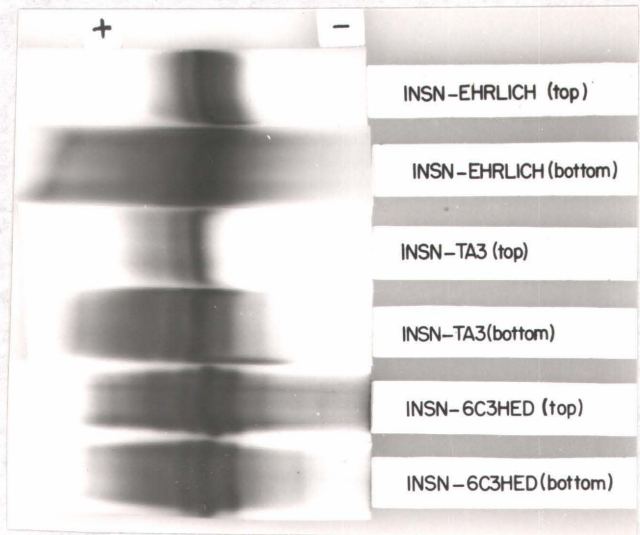
(11c)

Fig 11

(11d)



(11e)

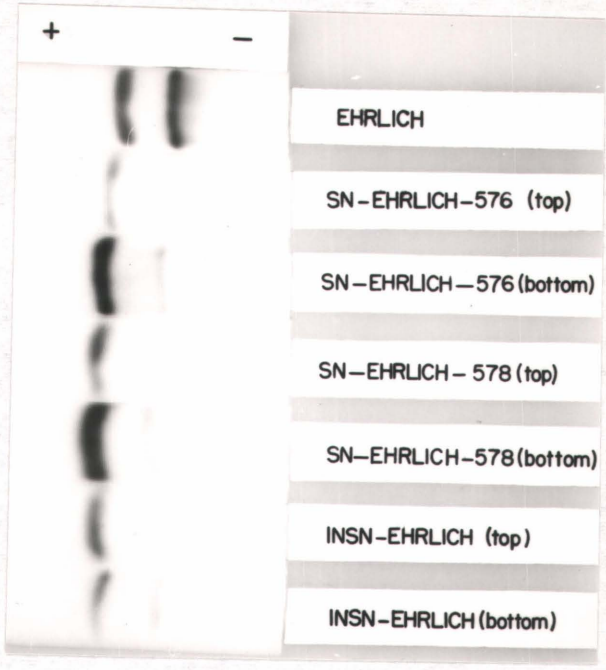


(11f)

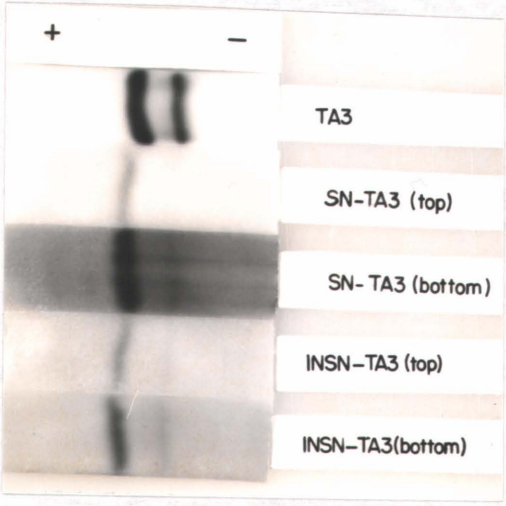
Fig 11

Figure 12. Comparison of the G-6-PD isoenzyme patterns of the regular ascites tumors with those of their supernatant lines.

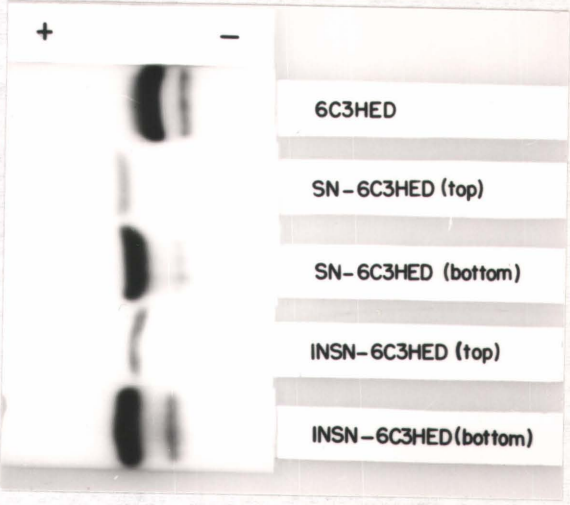
a. Ehrlich and its supernatant lines
b. TA_3 and its supernatant lines
c. $6C_3HED$ and its supernatant line
d. SA_1 and its supernatant line
e. All the SN lines
f. All the INSN lines



(12 a)

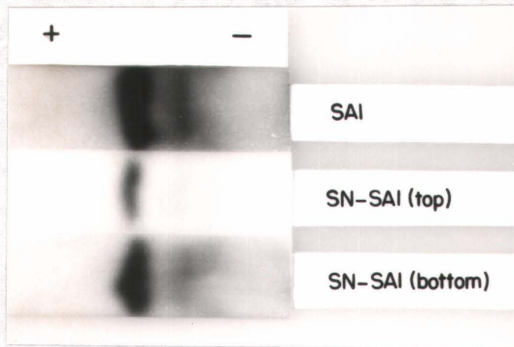


(12b)



(12c)

Fig 12



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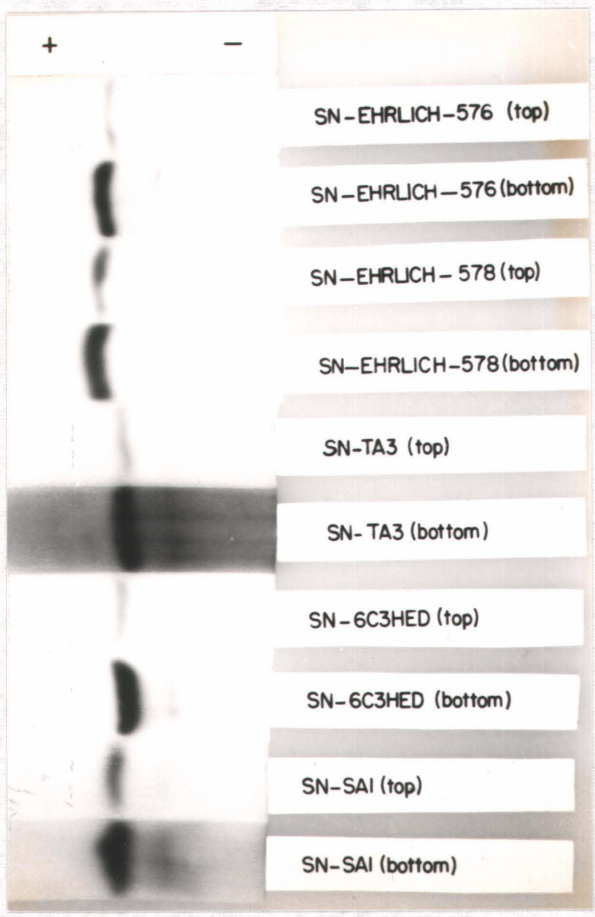
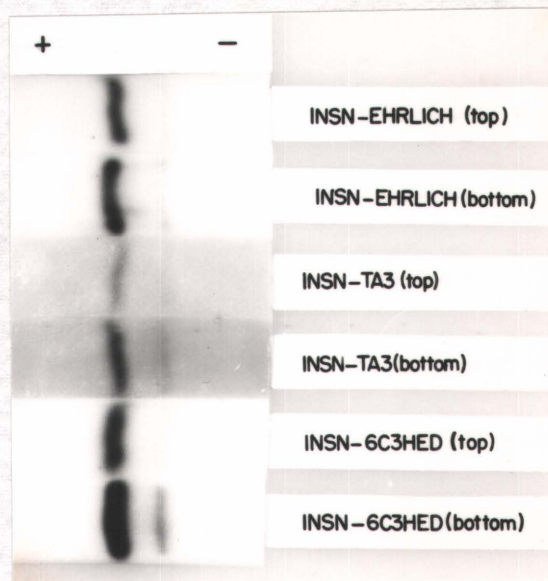


(12e)



(12f)

Fig 12

Figure 13. Comparison of the MDH isoenzyme patterns of the regular ascites tumors with those of their supernatant lines.

a. Ehrlich and its supernatant lines

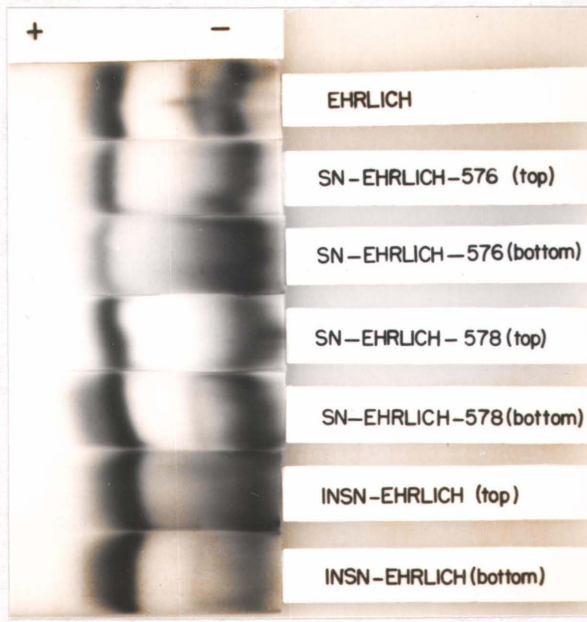
b. TA_3 and its supernatant lines

c. $6C_3HED$ and its supernatant line

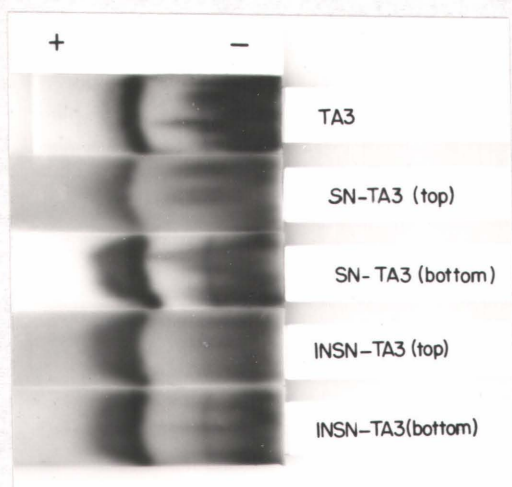
d. SA_1 and its supernatant line

e. All the SN lines

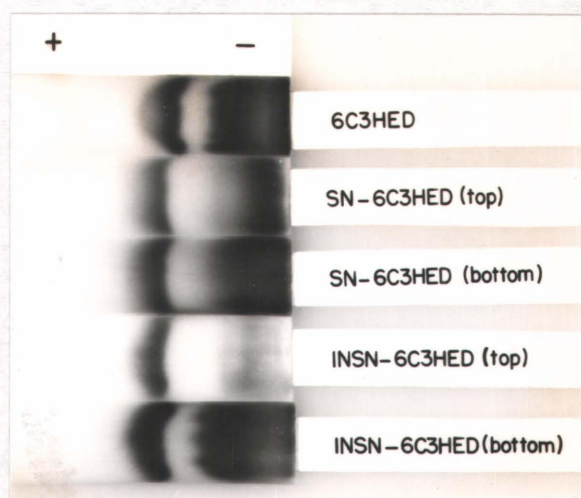
f. All the INSN lines



(13a)

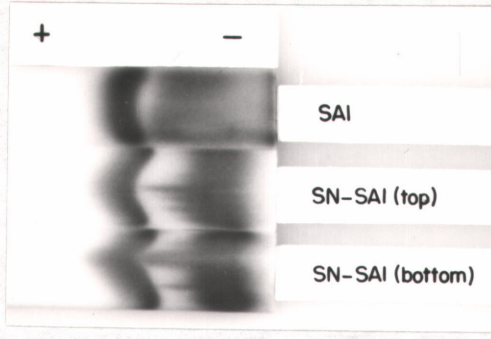


(13b)

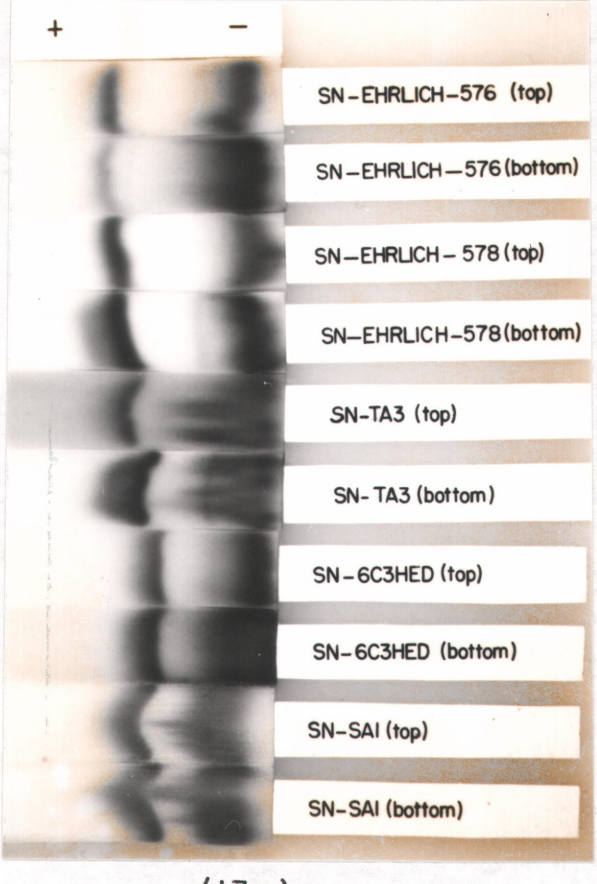


(13c)

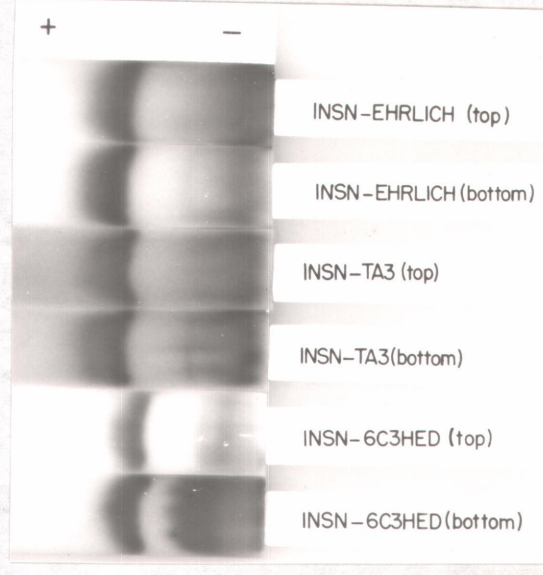
fig 13



(13d)



(13e)



(13f)

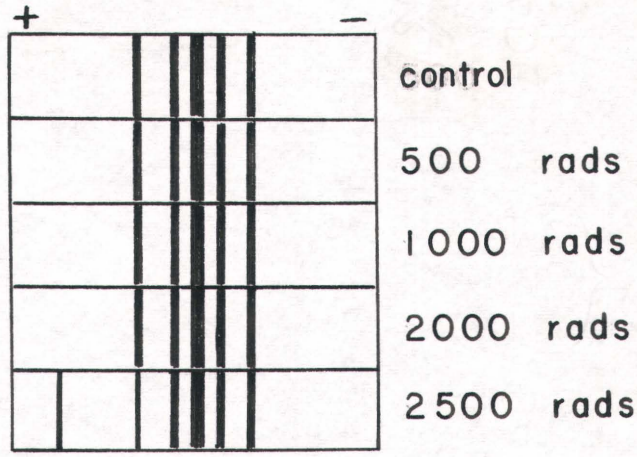
fig 13

Figure 14. Effect of X-irradiation on the isoenzyme patterns of Ehrlich ascites tumor cells.

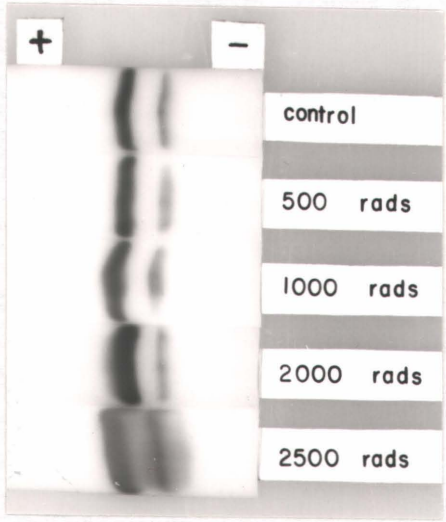
a. LDH

b. G-6-PD

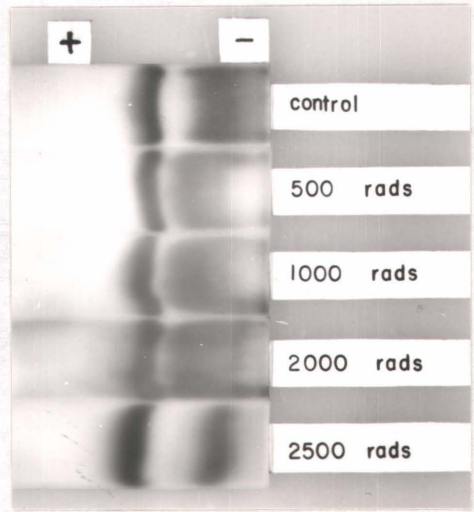
c. MDH



(14a)



(14b)



(14c)

fig 14

Figure 15. Effect of inositol on the isoenzyme patterns of Ehrlich ascites tumor cells.

1 - cells treated with Hank's balanced salt solution for one hour

2 - cells treated with 5% inositol for one hour

3 - cells treated with HBSS grown in mice

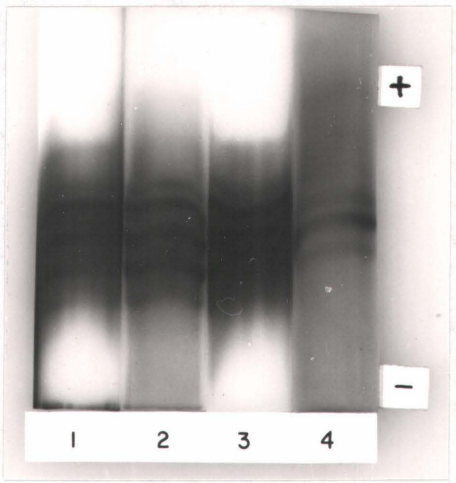
4 - cells treated with 5% inositol grown in mice

a. LDH

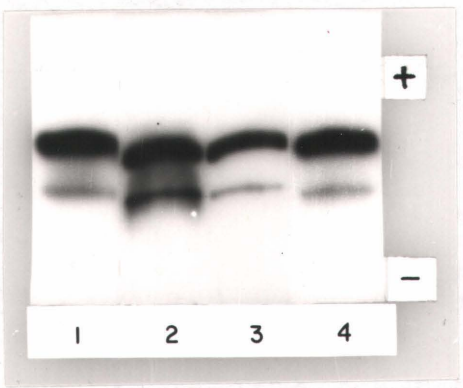
b. G-6-PD

c. MDH

Fig 15



(15a)



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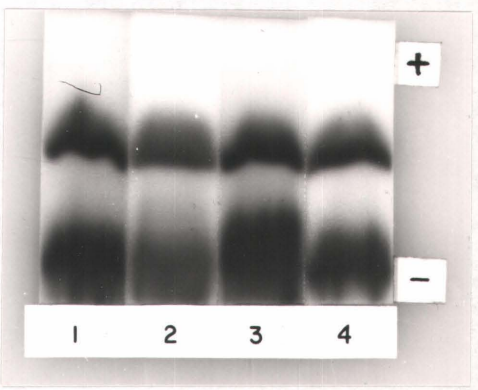


(15c)

Fig 15

Figure 16. Comparison of the isoenzyme patterns of the tissue culture lines of the ascites tumors.

Old (non-tumorigenic) T.C. - the old tissue culture lines, non-tumorigenic

New (tumorigenic) T.C. - the new tissue culture lines, tumorigenic

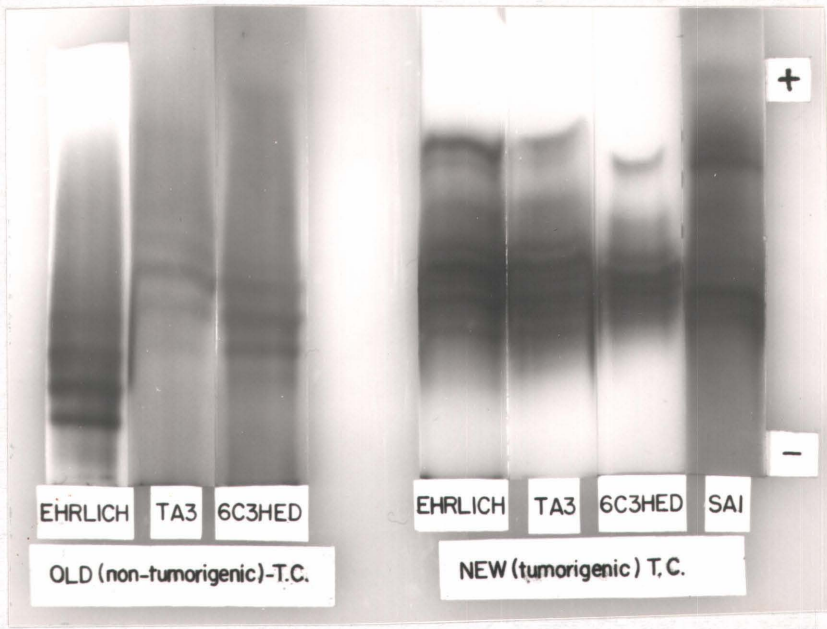
New (B.T.) - back transplantation of cells of the new lines into mice

a. LDH

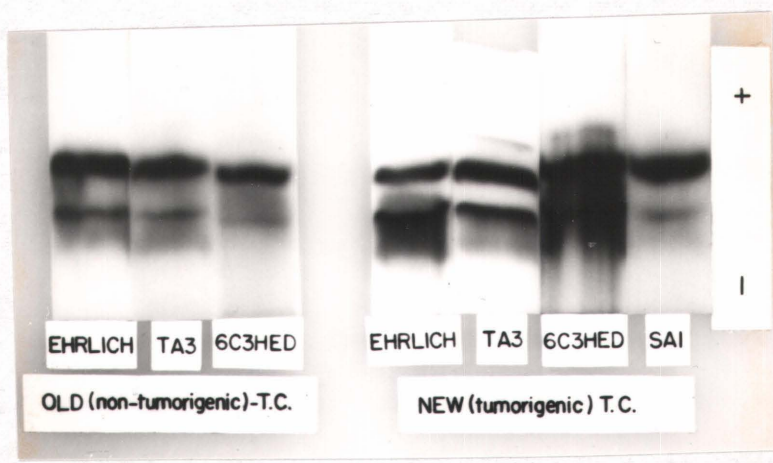
b. G-6-PD

c. MDH

d. Esterases

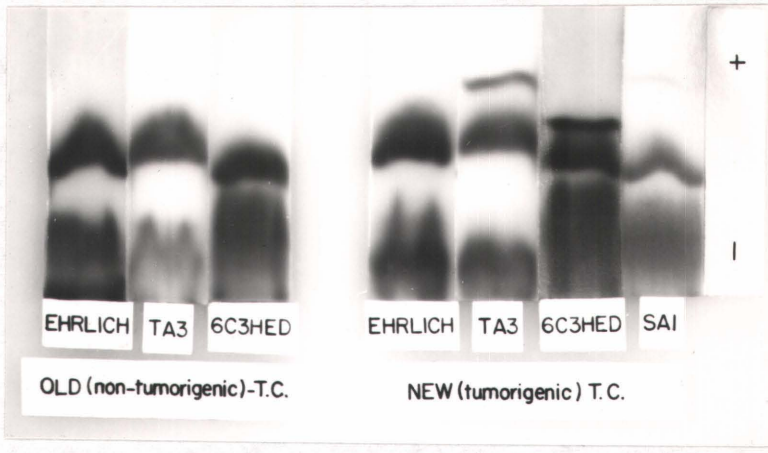


(16a)

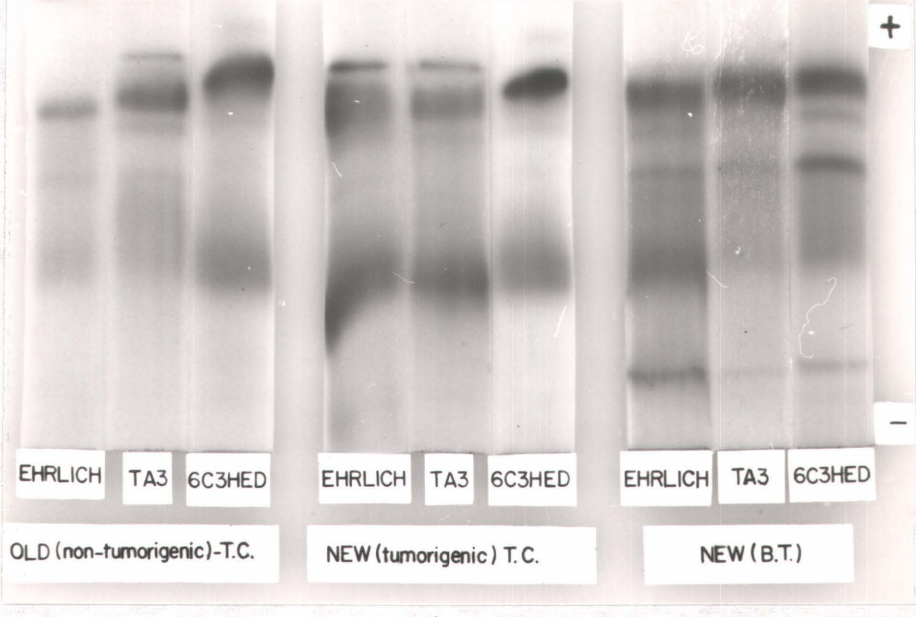


(16b)

Fig 16



(16c)



(16d)

Fig 16

Figure 17. Comparison of the LDH isoenzyme patterns of the ascites tumors *in vivo*, *in vitro* and following back transplantation from tissue culture.

New (B.T.)-1 - The first mouse passage of the cells of the new lines in back transplantation, i.e. from tissue culture into mice

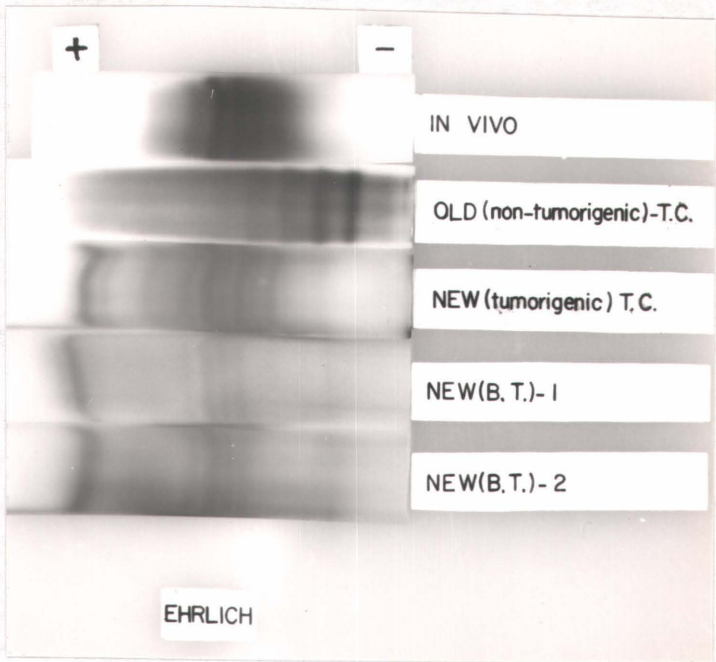
New (B.T.)-2 - The second mouse passage in back transplantation, i.e. from mouse to mouse

a. Ehrlich carcinoma

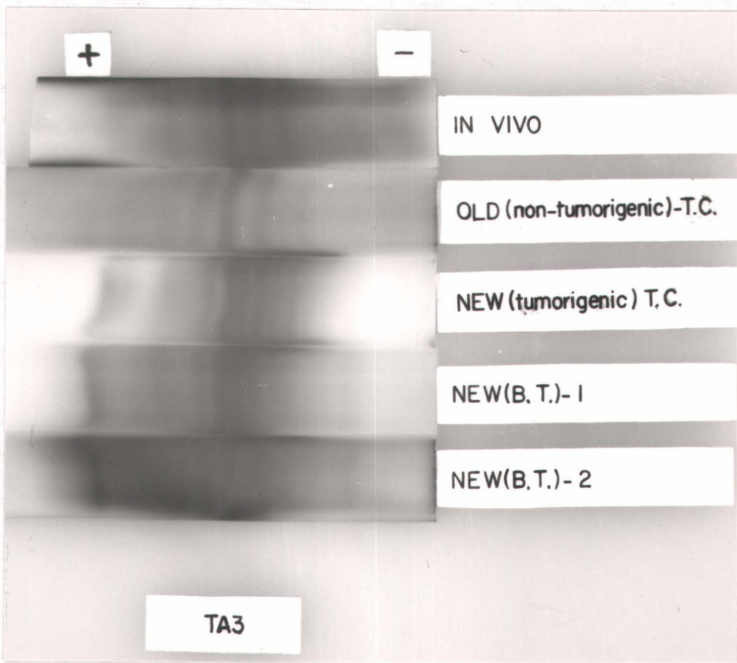
b. TA_3 mammary carcinoma

c. $6C_3HED$ lymphosarcoma

d. SA_1 spontaneous ascites tumor

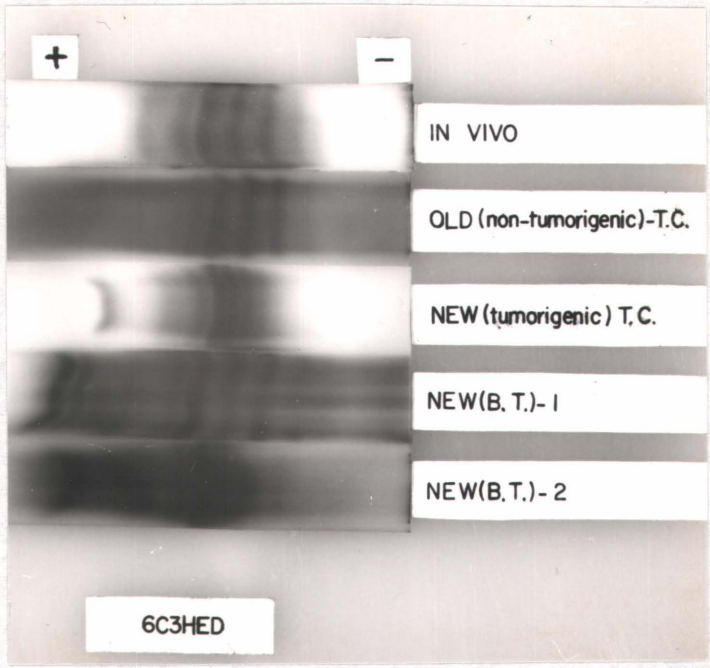


(17a)

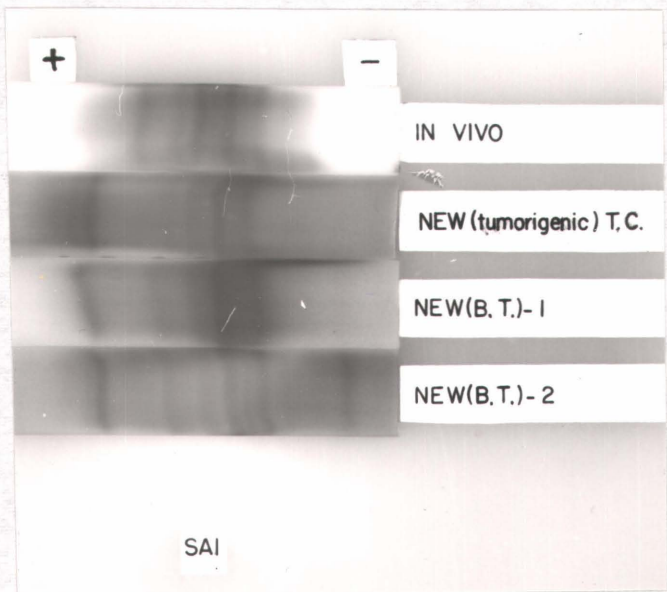


(17b)

Fig 17



(17c)



(17d)

fig 17

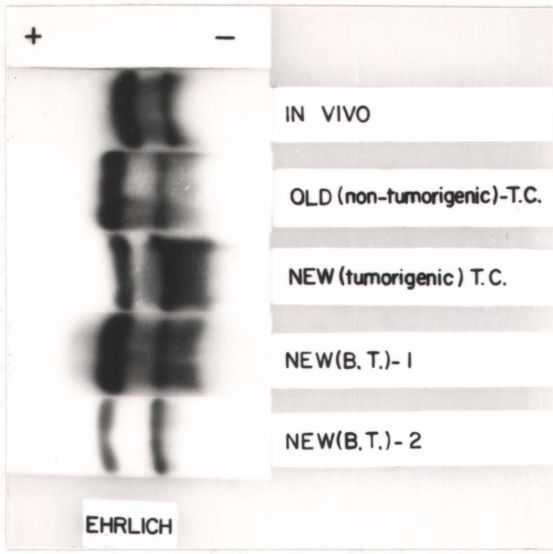
Figure 18. Comparison of the G-6-PD isoenzyme patterns of the ascites tumors *in vivo*, *in vitro* and following back transplantation into mice from tissue culture.

a. Ehrlich carcinoma

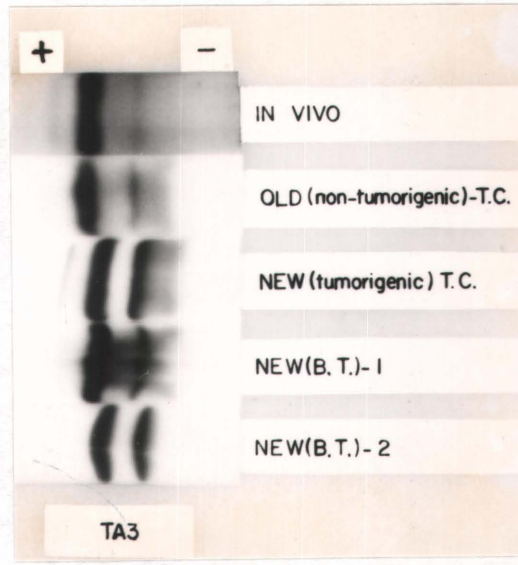
b. TA_3 mammary carcinoma

c. $6C_3HED$ lymphosarcoma

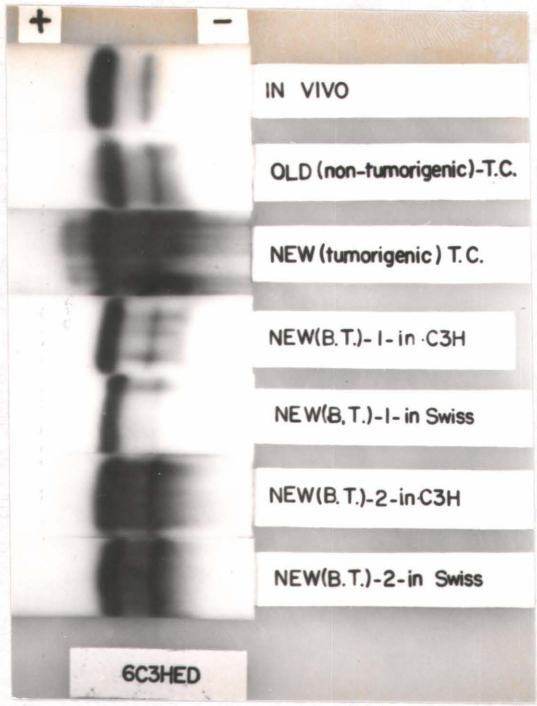
d. SA_1 spontaneous ascites tumor



(18a)



(18b)



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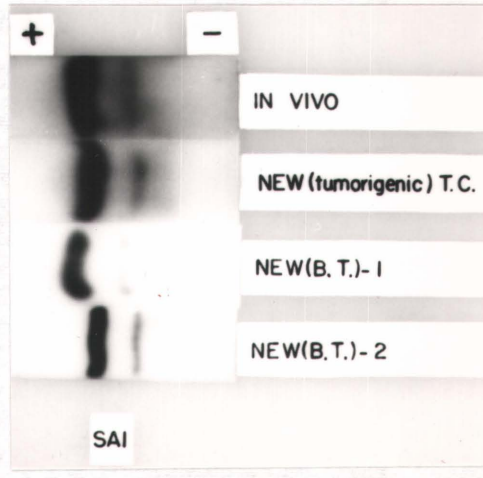


(18d)

fig 18

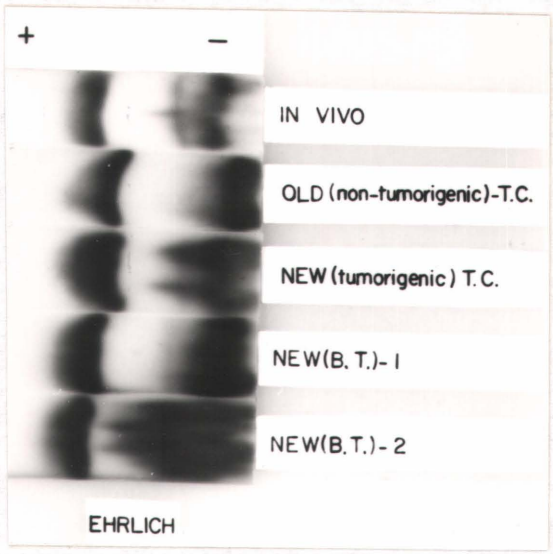
Figure 19. Comparison of the MDH isoenzyme patterns of the ascites tumors *in vivo*, *in vitro* and following back transplantation into mice from tissue culture.

a. Ehrlich carcinoma

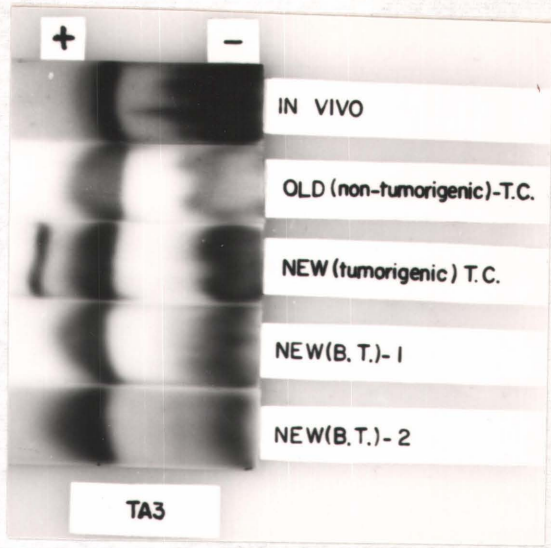
b. TA_3 mammary carcinoma

c. $6C_3HED$ lymphosarcoma

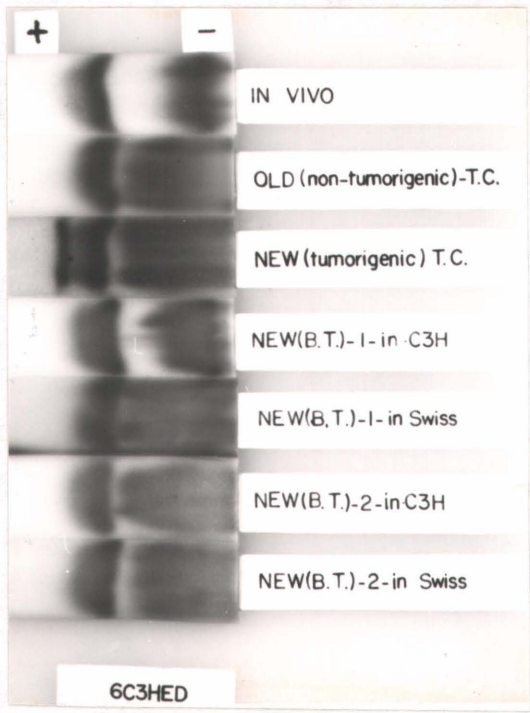
d. SA_1 spontaneous ascites tumor



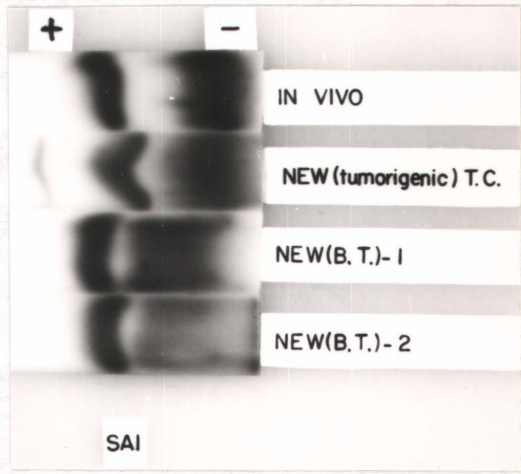
(19a)



(19b)



(19c)



(19d)

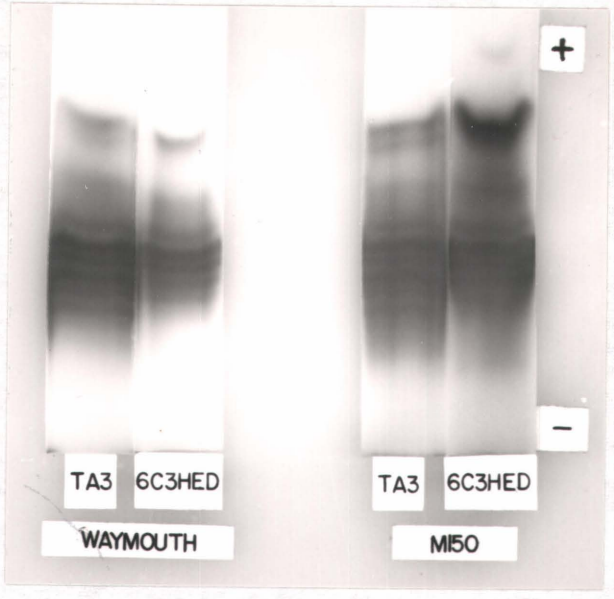
fig 19

Figure 20. Effect of different media on the isoenzyme patterns of the tumorigenic lines of ascites tumor cells in tissue culture.

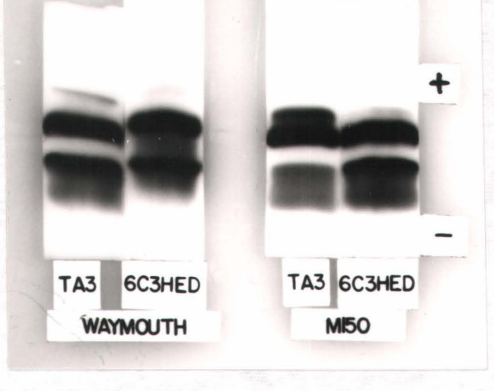
a. LDH

b. G-6-PD

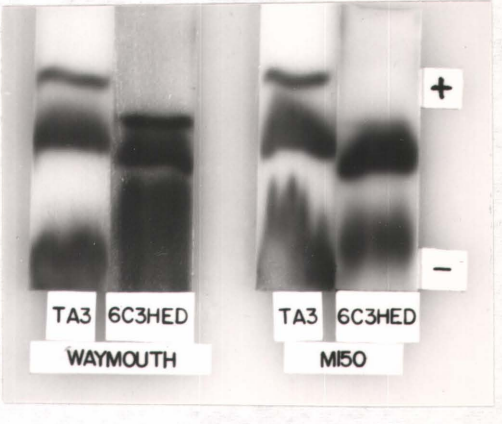
c. MDH



(20 a)



(20b)



(20c)

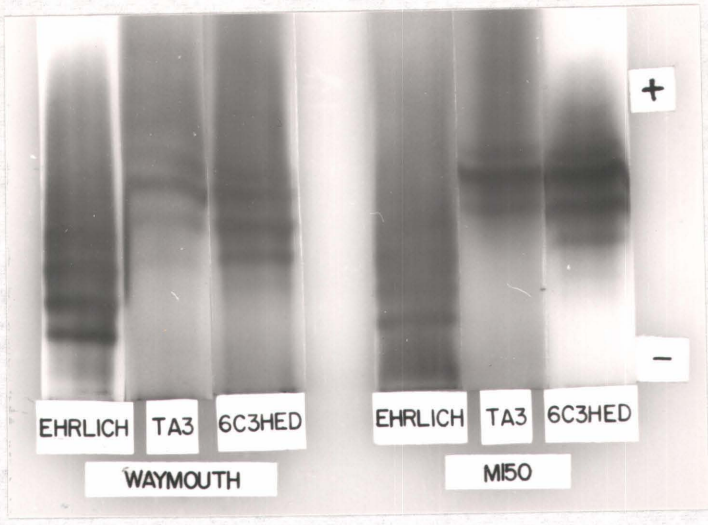
fig 20

Figure 21. Effect of different media on the isoenzme patterns of the non-tumorigenic lines of ascites tumor cells in tissue culture.

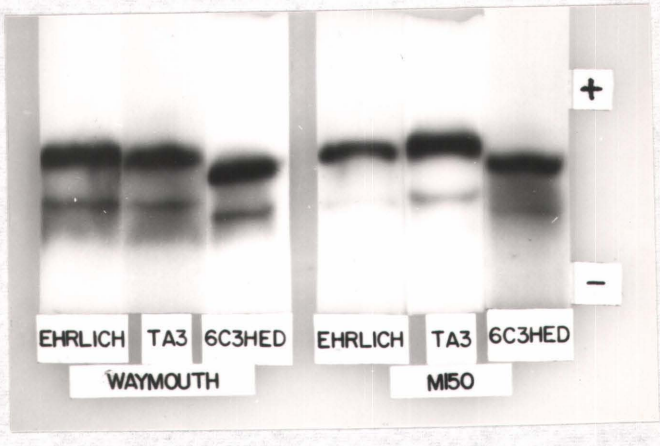
a. LDH

b. G-6-PD

c. MDH



(21a)



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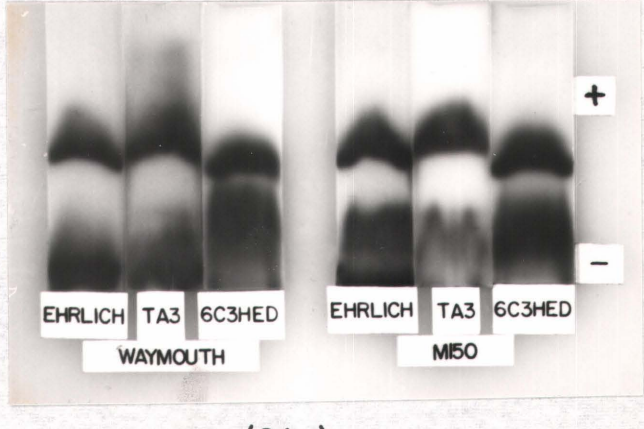


(21c)

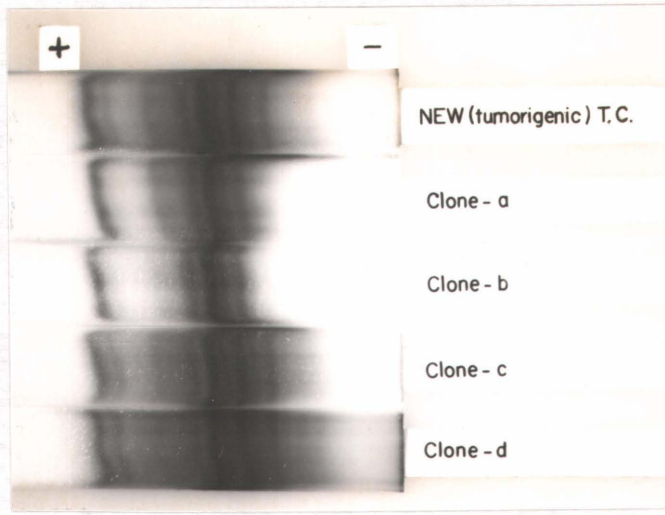
Fig 21

Figure 22. Comparison of the isoenzyme patterns of the clonal cells from the tumorigenic Ehrlich tissue culture line.

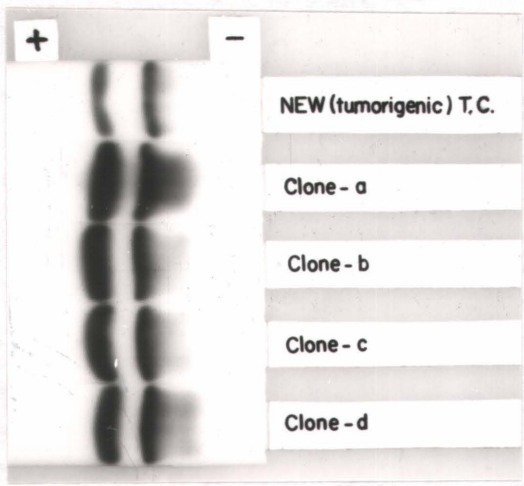
a. LDH

b. G-6-PD

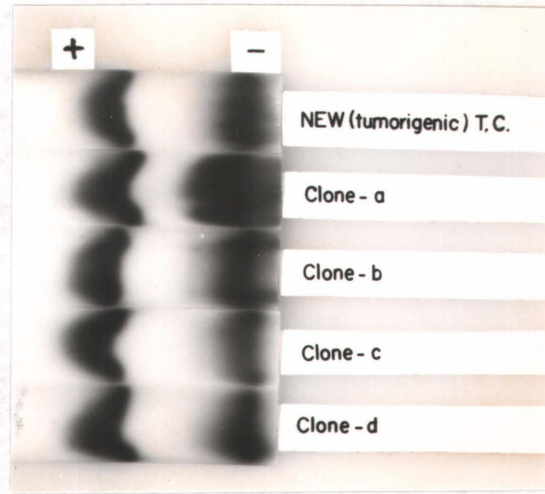
c. MDH



(22a)



(22b)



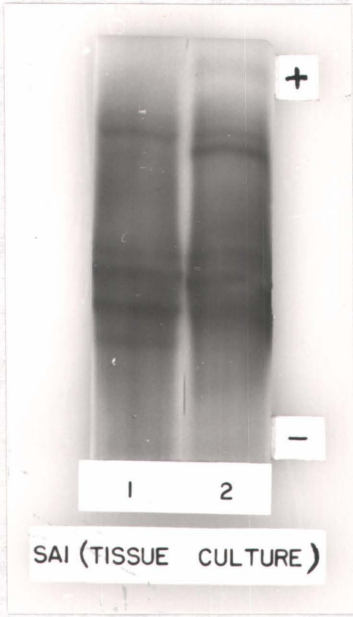
(22c)

fig 22

Figure 23. The LDH isoenzyme patterns of the SA_1 tissue culture line at two different times after the cell line was established.

1 - Cells harvested after five weeks in tissue culture in the first passage

2 - Cells harvested after four months in tissue culture in the seventh passage.



(23)

fig 23