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Metabolism of excised embryos of *Lupinus luteus* L.

VI. An electrophoretic analysis of some dehydrogenases in cultured embryos as compared with the normal seedling axes

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Abstract

The electrophoretic patterns (disc electrophoresis) of the studied dehydrogenases: glucose-6-phosphate — (*A*), malate — (*B*), glutamate — (*C*), alcohol — (*D*) and lactate dehydrogenase (*E*), in the axial organs of isolated *Lupinus luteus* embryos and seedlings cultivated over 12 days are characterized by great similarities. With time, after the third day of cultivation the patterns begin to become less developed. Analyses performed during the first 10 hours of imbibition of seed parts indicate that the maximal development of isozyme patterns occurs during the third hour after which the patterns become poorer. The most uniform type of pattern and the lowest number of isozymes was shown by glutamate dehydrogenase, the richest pattern was shown by malate dehydrogenase. No band common for all the 27 experimental elements was found.

INTRODUCTION

In the previous studies it has been shown that decotyledonized embryos of yellow lupine (cultivated *in vitro*) are incapable of normal growth and development primarily as a result of the abnormal utilization of the nitrogen source supplied to the medium (Czosnowski, 1962 b; Czosnowski and Michejda, 1964). It also appeared that the nitrogen supplied in the form of an extract from young etiolated seedlings is more favourable in many ways than the mineral nitrogen (particularily NO_3^-). It appears that the degree of difficulty of utilizing nitrogen sources supplied from outside by isolated embryos rather than as normally from the cotyledons depends primarily on the

effects associated with the compartmentation of the substrate pool and with a deviation in the functioning of individual enzyme types. It is the aim of the present paper to follow the behaviour of several dehydrogenases in the organs of cultivated isolated embryo axes compared with the organs in an intact seedling during 12 days of its development in the darkness.

MATERIALS AND METHODS

Plant material. Seeds of *Lupinus luteus* cv. Express were obtained from the Poznań Plant Breeding Station in Kosieczyn. For the main experiment (series I, see below) the seeds were treated as in the previous paper (Czosnowski, 1968). The conditions for the cultivation of the isolated axes were identical except for the nitrogen dose, which in comparison with the normal Heller (1954) medium has been tripled.

Normal seedlings were cultivated for 12 days in the darkness on distilled water in germinators.

Series I. For analysis samples were taken after 1 day of soaking the seed (on the graphs Fig. 1.: 1^d) and after 3, 7 and 12 days of cultivation of the isolated embryo axes on the medium or of normal seedlings on water (in the graphs Fig. 1 respectively: 3^d, 7^d, and 12^d).

The isolated embryos starting from the third day of cultivation were divided into a root and a shoot and the seedlings into a root, stem with a developing apical bud, and the cotyledons. In this way 17 experimental elements were obtained, which were subjected to enzymatic analysis and designated on the diagram in Fig. 1 by numbers *1* to *17*.

Series II. An additional experiment concerned the elements of a seed deprived of its seed coat during ten hours of its swelling. For the enzymatic analyses material in an air dry condition was used (0^h on the graphs in Fig. 2) and after 3, and 10 hours (3^h and 10^h) of soaking in distilled water under conditions of good aeration. Two experimental variants were employed: a) an embryo axis not separated from its cotyledons in 0^h (upper row of Fig. 2) and b) an embryo axis separated from its cotyledons in 0^h (lower row of Fig. 2). In this series 10 experimental elements were obtained designated in the diagram (Fig. 2) by numbers *18—27*.

Preparation of extracts. For the extraction of proteins each time the whole organs were taken. 1 g of material was ground in a mortar in a frozen condition after which 5 ml of a cooled buffer was added: HEPES 0.05 M (SIGMA), dithiothreitol 0.01 M (SIGMA) and 1N NaOH to a pH of 7.2. After 30 min of extraction at a temperature

of +2°C the material was centrifuged at the same temperature for 30 min at 16.000 g. To the supernatant 0.75 g of Sephadex G25 (coarse) was added and after 10 min the material was filtered under slight pressure through a sintered filter G3. In the filtrate the total content of proteins soluble in the given extraction conditions was estimated by the standard method of Lowry, and finally it was diluted to such an extent with the above mentioned buffer, till it contained 200 µg of proteins in 100 µl. The solutions of proteins were stored at a temperature of —20° C until the moment of electrophoretic analyses.

In the case of Series II for the extraction of proteins 500 mg of cotyledons were used and 30 embryo axes (125—135 mg).

Electrophoresis. We used the disc electrophoresis procedure of Davis (1964). Polyacrylamide gel 7%, pH 8.9, reservoir buffer: TRIS — glycine was at pH 8.3. Electrophoresis was performed in tubes 65 × 5 mm at +4° C and at 4 mA per tube until the marker dye (bromophenol blue) was about 3 mm from the bottom of a separate gel which was not used for enzymatic reactions.

200 µg of proteins have been separated on each gel. The solution of the proteins was pipetted to about 4 mg of Sephadex G-200 and placed on top of the upper gel and after swelling it was layered with the reservoir buffer:

Enzymatic assays.

The following dehydrogenases were assayed:

(E.C. 1.1.1.49)	*A* glucose-6-P-dehydrogenase
(E.C. 1.1.1.37)	*B* malate dehydrogenase
(E.C. 1.4.1.2)	*C* glutamate dehydrogenase
(E.C. 1.1.1.1)	*D* alcohol dehydrogenase
(E.C. 1.1.1.27)	*E* lactate dehydrogenase

The letters *A* — *E* have been used to designate the individual enzymes on the diagrams in Figs. 1 and 2 and in the text below. (Thus for example "*C-9*" indicates a glutamate dehydrogenase in a root isolated from an embryo on the 7th day of cultivation.)

The assay for dehydrogenases based on the coupling of the enzymatic activity to a reduction of a tetrazolium salt to a coloured diformazan.

The solution in which the gel was incubated after electrophoresis contained: 1) appropriate substrate, 2) $MgCl_2$, 3) NAD (Reanal) or NADP (Boehringer), 4) phenazine methosulphate (Koch-Light), 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl-tetrazolium-chloride (BDH) at concentrations employed by Honold et al. (1966). The final concentration of the buffer TRIS-HCl pH 8.3 was 0.1 M. The gels have been incubated for 20 hours at room temperature.

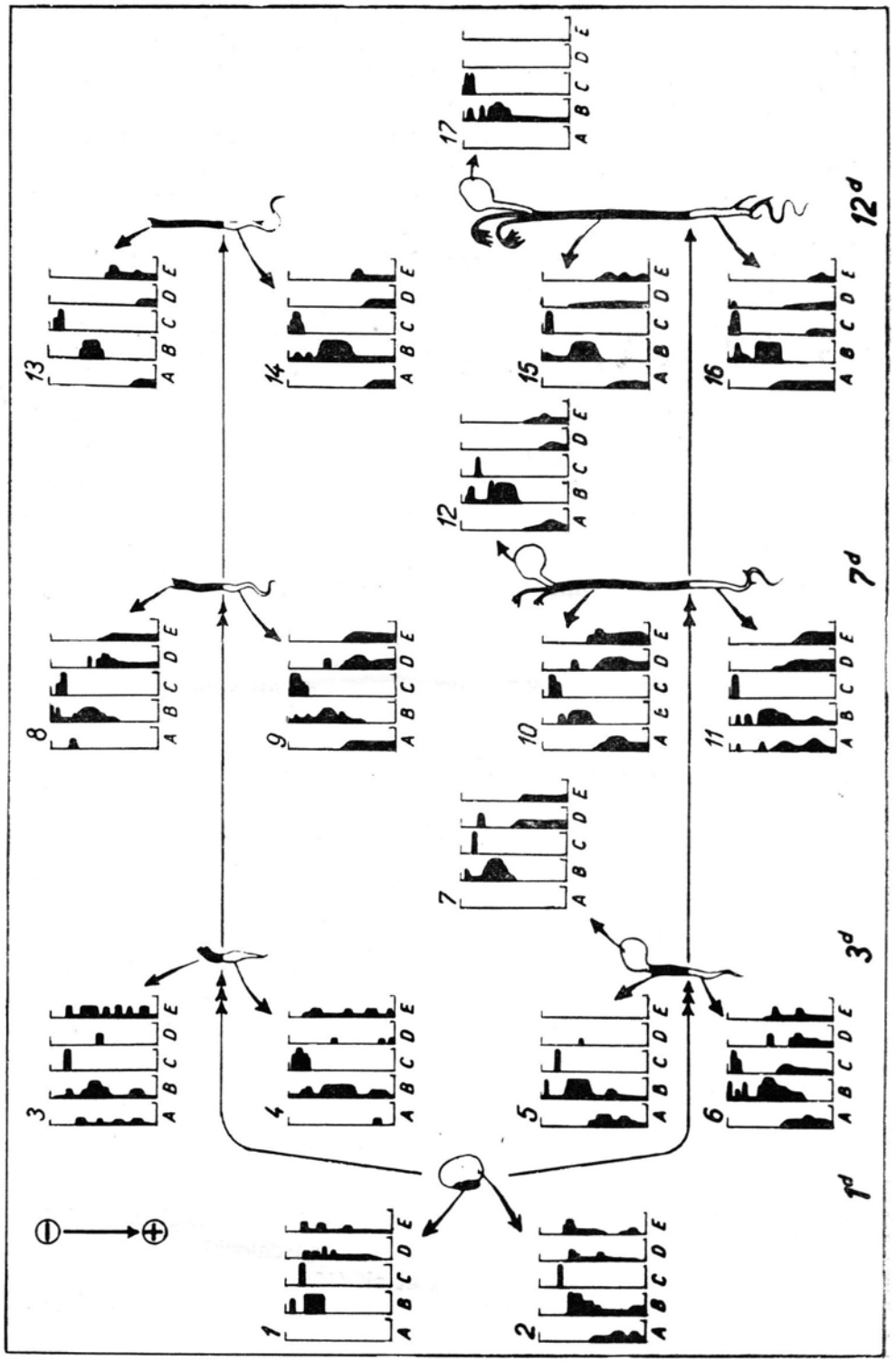

Fig. 1

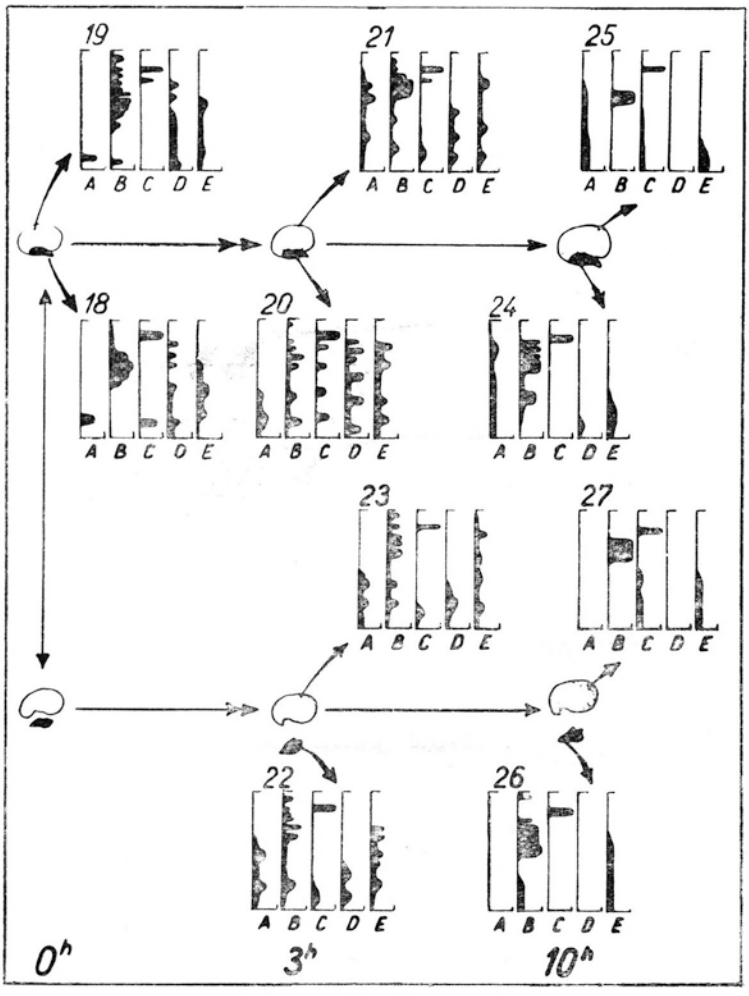

Fig. 2

Fig. 1. Isozyme patterns of 5 dehydrogenases:

A — glucose-6-phosphate-, *B* — malate-, *C* — glutamate-, *D* — alcohol- and *E* — lactate dehydrogenases, in 24 hours swollen seeds, in isolated cultured embryonal axes (upper row) and in normal seedlings (lower row) on the 3rd, 7th, and 12th day.

Fig. 2. Isozyme patterns of dehydrogenases (as in fig. 1).

Upper row: in dry decoated seed (0 h) and after 3 and 10 hours of imbibition. Lower row: in separated cotyledons and embryos after 3 and 10 hours of swelling.

All experiments were conducted in duplicate, and a complete agreement of results was obtained. The controls were reaction mixtures without the substrates.

When evaluating the results a visual estimation was employed and the individual graph (e.g. *B-3*) represents 1 electrophoregram as a visual imitation of densitometric readings. It appears that this type of graphical interpretation of an isozyme electrophoregram demonstrates the true picture better than the usually presented solid or stippled bands.

RESULTS AND DISCUSSION

Series I

The seeds after 24^h of swelling (1^d)

a) embryo (*1*). No *A*; rich *D* and *E* patterns

b) cotyledon (*2*). All enzymes present, a rich pattern of *B* at *Rf* regions beyond these for *B-1*.

Embryo 3^d, 7^d, 12^d

a) shoot (*3*, *8*, *13*). The richest patterns on the 3rd day of cultivation, particularily rich are the isozymes of *E*. With time the total isozyme pattern becomes reduced, there are considerable shifts in the pattern of *B*.

b) root (*4*, *9*, *14*). General picture similar to that in the shoot.

Seedling 3^d, 7^d, 12^d

a) shoot (*5*, *10*, *15*). In contrast to the shoot of the embryo 3^d there is a complete absence of *E*, it reappears, however, in later development. In the shoots 7^d and 12^d apart from some shifts, the patterns are similar.

b) root (*6*, *11*, *16*). The richest spectral pattern is to be seen on the third day of cultivation. In contrast to the shoot *E* is present. With time the patterns become poorer.

c) cotyledons (*7*, *12*, *17*). *A* is absent on the 3rd day of cultivation. With time the spectrum becomes poorer; on the 12th day of cultivation *A*, *D* and *E* can not be found in the cotyledons.

Generally speaking the patterns of the studied dehydrogenases in axial tissues of the isolated embryo and in the seedling are characterized by considerable similarity in that over a period of 12 days the patterns have a tendency to become poorer. An interesting exception is the occurrence of 6 isozymes of *E* easily distinguishable in the axis of the embryo but completely lacking in the shoot of the seedling on the third day of cultivation.

Series II

Because in series I, in which the starting point was a seed swollen in water for 24^h, a rich group of active isozymes was observable and it reached its maximum on the 3rd day of cultivation, it was decided to study the spectra of the analyzed dehydrogenases in the first 10 hours of imbibition. The method of preparing the material was described under "Plant material".

Dry seeds

a) embryo (*18*). All enzymes present

b) cotyledons (*19*). All enzymes present; *B* richly developed. *C* occurs in both organs in the form of two isozymes but at very different Rf regions.

Swollen seeds 3^h and 10^h

a) embryo (*20*, *24*). After 3^h the isozyme complement is much richer than in a dry seed; *B*, *C*, *D*, *E* in numerous forms strongly distributed in *Rf*. *C* has the maximal number of isozymes here (5). After 10^h of imbibition the patterns of all enzymes become much poorer, the only exception being *B*.

b) cotyledons (*21*, *25*). After 3^h of imbibition the isozyme complement is richer than in a dry seed (*A*, *C*, *E*). After 10^h of imbibition the pattern becomes much poorer, *D* disappears completely and *B* has a much poorer complement.

Seed parts swelling independently 3^h and 10^h

a) embryo (*22*, *26*). After 3^h of imbibition the complement is rich, but poorer than in an embryo of an intact seed (*20*), particularily in *C* and *D*. After 10^h of imbibition *A* and *D* are lacking completely, and the remainder are similar to *24*.

b) cotyledons (*23*, *27*). After 3^h of imbibition the complement is rich, similarily as in the free embryo (*22*). After 10^h *A* and *D* are lacking as in the free embryo. *B* is poorly developed, similarily as in *25*.

In general after 3 hours of imbibition a further development of isozyme patterns is observable which were already rich in dry seeds. After 10 hours of swelling, both in the organs of a normal seed and in the organs separated from one another a rapid decline in the patterns is observable, and there is a tendency for partial or complete disappearance of *A* and *D*. The isolation of the cotyledons from the embryo axes results in all instances in a decline of enzymatic patterns.

The results obtained indicate that during the 12 days of cultivation *in vitro* (series I) the organs of isolated yellow lupine embryos behave very similarily to the organs of normal seedlings as regards the development of activity patterns of the studied dehydrogenases. These facts permit the formulation of the conclusion that the course of the

processes in which the studied dehydrogenases are directly involved should not have a limiting effect on the nitrogen nutrition of the isolated embryos. Further studies which are being conducted now will probably permit the formulation of appropriate conclusions concerning other groups of enzymes.

A closer analysis of the electrophoretic paterns provides us with some data on the dynamics of appearance, disappearance and activity of the individual studied enzymes.

C. Glutamate dehydrogenase. This enzyme has the most uniform character of occurrence and activity among those studied. It occurs throughout the studied material giving sharp, strong and narrow basic bands in the R_f region of 0.05—0.25. One can observe here either 1 band, which appears at various R_f values within the range given above, or several (up to 4) bands lying close to each other (*C-17*) or touching each other (eg. *C-9*). Beyond these basic bands, there appear sometimes sharp or more washed out bands at higher R_f values, the maximal number of isozymes appearing at 3^h in an embryo from a whole seed (*C-20*).

Macko et al. (1967) have shown that in dormant wheat grains in the first phase of germination a slight activity of *C* has been also localized at low R_f values. This poor activity may possibly be associated with the starchy nature of the grain.

B. Malate dehydrogenase. In contrast to *C*, this dehydrogenase demonstrates a complete instability in the patterns of isozyme complements. Within the R_f range of 0.18 to 0.60 the widest and most intensive bands are to be found. Several other bands, narrower but frequently just as intense are distributed throughout the remaining parts of the gels, however this happens more frequently at the lower R_f values. In extreme cases it is possible to observe only one band (*B-25*) or as many as 11 (*B-19*). Also Ovcharov et al. (1972) and Macko et al. (1967) have found numerous isozyme bands in the resting and germinating seeds of corn and wheat respectively.

A. Glucose-6-phosphate dehydrogenase. This enzyme occurs in 1 to 4 isozymes, which can constitute sharply defined and compact bands, or wide ones with indistinct delimitations. In some cases it is possible to see very distinct relations in the patterns of *A*. Thus for example after having one distinct band at a high R_f in *18* and *19* it develops into several and is maintained there in *20*, *21* and *24*, *25*, while they are developed in *22* and *23*, but completely disappear in *26* and *27*.

D. Alcohol dehydrogenase. Here the pattern of isozymes extends for the whole range of R_f values, the majority of activity being concentrated in the upper half. In experimental series II (Fig. 2) the developmental tendencies for *D* are more distinct than in series I

(Fig. 1). In a dry seed several bands exist, after 3^h of imbibition they strengthen and increase in number to 6, which is followed by a decline in activity during the following hours. In separated embryos and cotyledons (*22*, *26*, *23*, *27*) the decline is even greater. The decline and disappearance of the activity of *D* can be attributed to the rapid shift of all respiration to the aerobic form (Macko et al., 1967; Ovcharov et al., 1972).

E. Lactic dehydrogenase. In the majority of cases it occurs in bands at R_f values above 0.35. Its activity, has been demonstrated in a resting seed, it increase during the first 3 hours of imbibition (*20-23*) and then drops substancially to 10^h (*24-27*). After 24 hours of imbibition there is an increase in the number of bands (*1*, *2*). After 3 days of development strong differences develop. In the shoot of an isolated embryo 6 very distinct bands are distributed throughout the R_f range, while in the shoot of a seedling there is a complete absence of activity *(3, 5)*. Starting from the 7th day of development the situation in the isolated embryos and in the axes of seedlings is very similar. There is considerable activity of *E* in both shoot and root.

Pooling the results of series I and II together, one can say in general that the composition of isozyme complements and frequently of their acivity are not in a linear relation, nor are they on a uniform level during the 12 days of development of normal seedlings or isolated embryo axes. During the first 10 hours of imbibition of the decoated seeds the maximum of the development of the studied enzymes occurs around the third hour, after which a decline is observable until the 10th hour. The next increase in intensity occurs between the 1st and 3rd day of cultivation, after which there is a slow decline (with some exceptions) till the 12th day. At the moment it is difficult to interpret this type of relationships without further studies. Possibly there are some associations with the initial lack and later onset of mitotic activity and all the associated processes in the apical meristems as well as with the processes of differentiation. It is not unlikely that there are also correlations with the specifity and type of active and non-active imbibition by tissues (Czosnowski, 1962).

In the case of none of the enzymes was it possible to find a common band for all the 27 experimental elements. This is further evidence of the considerable lability and the possibility of shifting the nature of the activity of isozyme systems, which undoubtedly is directly associated with the processes of differentiation and development.

Weimberg (1968) when studying the isozymes of malate dehydrogenase in several plants did not found an isozyme band common to all organs or to all analysed plants. Racusen (1966) has estab-

lished changes in the isozyme spectra of peroxidases during development of bean leaves. Shaio-Lim Chen et al. (1970) have shown that, in *Xanthium pennsylvanicum* the isozyme patterns of several enzymes in the leaf tissues depend both on leaf and plant age.

The changes observed by the authors mentioned above, in the isozyme patterns have taken place over long periods of time. The results of the present study indicate that at least in the early phases of development of a young plant the changes in patterns of individual enzymes in the same organ can take place very rapidly, during hours (eg. *C-18, 20, 24*; disappearance: *D-18, 22, 26*; *B-19, 21, 25*). In this respect these results agree with those obtained by Ovcharov et al. (1972) with respect to seeds of maize.

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Metabolizm izolowanych zarodków Lupinus luteus L. VI.

Streszczenie

Badano przy zastosowaniu elektroforezy na żelu poliakrylamidowym spektra 5 dehydrogenaz (glukozo-6-fosforanowej, jabłczanowej, glutaminianowej, alkoholowej i mleczanowej) w organach izolowanych zarodków łubinu żółtego, hodowanych na pożywce Hellera oraz organach siewek hodowanych na wodzie w okresie 12 dni. Spektra izozymów poszczególnych dehydrogenaz wykazują w obydwu typach materiału duże podobieństwo. W miarę upływu czasu, po trzecim dniu hodowli, spektra te mają tendencję do stopniowego ubożenia. Analizy przeprowadzone w ciągu pierwszych 10 godzin pęcznienia elementów nasion wykazały maksymalne rozwinięcie spektrów enzymatycznych w trzeciej godzinie i następnie ich ubożenie. Najbardziej jednolity typ spektrów i najmniejszą liczbę izozymów wykazywała dehydrogenaza glutaminianowa, zaś największe bogactwo spektrów objawiała dehydrogenaza jabłczanowa. W przypadku żadnego z enzymów nie stwierdzono pasma wspólnego dla wszystkich 27 elementów doświadczalnych.