during the test period. Of particular interest was the finding that the increase in mean weights of sham-operated, infected and thymectomized (partial), non-infected animals was less than that observed in sham-operated, non-infected mice. Moreover, there was a still greater reduction in the mean weights of thymectomized (partial), infected animals. Thus, it would appear that partial thymectomy or infection with the LDH virus have an effect on mouse weight and when both factors are present in the same animal these effects are additive.

The results shown in Table III indicate that: (a) plasma virus titers ($MgCl_2$-resistant fraction) were not affected by neonatal thymectomy or time of infection; (b) the total virus titer (PBS) in the plasma of 5-week-old mice, thymectomized and infected after birth, was higher than the mean infective titer observed in sham-operated, infected animals of the same age; and (c) plasma virus titers (PBS) in sham-operated or neonatally thymectomized offspring born to mothers infected before conception or during gestation were similar. These findings are presented as additional evidence in support of a model system involving 2 particles: (a) a smaller virus or S particle which is resistant to heat inactivation in the presence of $MgCl_2$ and non-antigenic; and (b) a larger virus or L particle which is susceptible to heat inactivation in the presence of magnesium ions and antigenic[2,7,10].

Zusammenfassung. Die gegebenen Befunde lassen vermuten, dass neonatale Thymektomie und/oder Infektion mit LDH-Virus einen Effekt auf eine Anzahl von Faktoren wie LDH-Plasma-Spiegel, Gewichtserhöhung und Lebensdauer in Mäusen hervorrufen können. Der Virustiter im Plasma ist vom Zeitpunkt der Infektion abhängig.

CH. G. CRISPENS, JR. and IRIS F. REY

Department of Anatomy, University of Maryland School of Medicine, Baltimore (Maryland 21201, USA),
9th December 1966.

[10] C. G. CRISPENS JR., Virology *24*, 501 (1964).

STUDIORUM PROGRESSUS

Heterogeneity of Lactate Dehydrogenase in the Developing and Adult *Xenopus laevis* Daud[1]

Experiments to determine the isoenzymes of amphibian lactate dehydrogenase (LDH) showed either 2 distinct components[2,3], or 4[4], as opposed to the pattern of 5 isoenzymes found in most vertebrate classes. These 5 components have shown to be tetramers resulting from the combination of 2 types of protein subunits designated as A and B, or H and M, and the synthesis of the 2 different polypeptide subunits is regarded as being under the control of separate genes[5].

Xenopus laevis was selected because of its unique larval development, which shows at least 20 differences, most of them radical, from the typical anuran pattern[6]. An extensive study of the LDH development, from fertilized egg to sexually mature adult, has been in progress in this department. Results proved so challenging that it was decided to give this preliminary report.

The adult (6 years old) animals were taken from laboratory stock. To examine the developmental stages, toads were injected with 400 IU of the choriogonadotropic hormone Pregnyl (Organon). The spawns, thus obtained, were raised at 22 °C; the food used was nettle powder (Herba urticae) during the larval period and ox liver after metamorphosis. Unfertilized eggs were produced by injecting isolated females with the same hormone. The LDH-isoenzymes were separated electrophoretically on agar-gel using the high voltage technique of WIEME[7] (pp. 68, 148, 157) and on starch-gel, as previously described[3], and subsequently stained with iodoneotetrazolium (INT) and Neotetrazolium respectively. Samples were applied in various ways. Agar-gel: (a) Specimens were homogenized in twice their volume of bi-distilled water, centrifuged under cooling for 20 min at 16,000 rpm and 3 λ of the supernatant applied to a narrow slit. (b) Small fragments were pushed into a cut made in the gel (direct tissue electrophoresis)[7]. Starch-gel: Samples were homogenized in *Tris* buffer, pH 8.9, centrifuged for 20 min at 16,000 rpm at 7 °C, and the supernatant placed on a filter paper strip and inserted into the gel.

In the results (Figures 1 and 2) the relative activity of the isoenzyme bands is expressed by shading, and the bands are numbered from 1–9 in their order of migration towards the anode. -Agar-gel: 9 bands were observed in organs taken from the adult toad (the only exception being the liver), the corresponding bands in each occupying the same position but differing in strength (Figure 2). Changes observed on total homogenates during development are the following. Unfertilized eggs, extruded into water, yield bands 1 and 2. Fertilized eggs at the 2-cell stage, up to neurulation (day 1 and 2), show a pattern of 4 bands – No. 1–4. At the tailbud stage (end of 2nd day) band 9 makes its appearance. During the following 2 days (day 3–4) – which are characterized by the outgrowth of the tail, pigmentation of eyes, appearance of external gills, beginning of heart beat and hatching of the embryo – band 8 shows up; bands 2, 3 and 4 disappear temporarily but 2 and 4 reappear within 1 day. From the 5th–7th day, the external gills are absorbed and replaced by internal gills; the animals previously attached by cement glands

[1] This paper is dedicated to Prof. Dr. A. PORTMANN on the occasion of his 70th birthday.
[2] I. HAUPT and H. GIERSBERG, Naturwissenschaften *45*, 268 (1958).
[3] J. N. R. GRAINGER and Y. W. KUNZ, 2nd Intern. Symp. Quant. Metabol. Helgol. wiss. Meeresunterschg. *14*, 335 (1966).
[4] G. W. NACE, T. SUYAMA and N. SMITH, Symp. Germ Cells and Dev. (Inst. Internat. d'Embryol. Baselli, Pavia 1961), p. 564.
[5] J. H. WILKINSON, *Isoenzymes* (Spon, London 1965), p. 47.
[6] P. D. NIEUWKOOP and J. FABER, *Normal Table of Xenopus laevis (Daudin)* (North Holland Publ. Co. Amsterdam 1956); P. B. WEISZT, J. Morph. *77*, 163 and 193 (1946).
[7] R. J. WIEME, *Agar Gel Electrophoresis* (Elsevier, New York 1965), pp. 73, 74, 129, 157.

now swim, fill their lungs with air and start feeding. During this stage band 6 makes its appearance. At 12 days – when hindlimb buds become visible and oral tentacles grow – band 7 appears and bands 2, 4 and 6 increase in strength. During the following 4 weeks (days 15–45), characterized by extensive growth in size and development of the limbs, no striking changes in pattern take place: bands 2 and 4 become stronger and bands 7 and 8 separate clearly, giving a total of 7 distinct bands. At position 3, faint indications of a reappearance of the band are seen.

During the 15–45 day stage the larvae, which may still be examined as total homogenates, now reach a stage of development which allows the testing of single organs for LDH-activity. From this stage through metamorphosis to the young toad stage, experiments were continued on single organs. Metamorphosis requires 15 days (days 45–60), and is marked by the sudden appearance of the forelimbs and the gradual resorption of the oral tentacles and the tail. Sexual maturity is reached as early as 6 months of age[6].

The brain and eye of the 15–45 day period have the adult complement of 9 bands, as opposed to 7 bands already established in the homogenate of total larvae. Both the tail and leg muscles have essentially the same pattern, the former, however, attaining the full number of bands only towards the end of metamorphosis, i.e. when $^2/_3$ of the tail are resorbed. The leg and heart muscles of the 15–45 day period differ from the adult muscles both in the number and intensity of the bands – they still have not reached the adult pattern in a 3-month-old toad. The liver pattern so far has been too inconsistent to permit any definite conclusion to be drawn (Figure 2).

Starch-gel: all adult organs examined, with the exception of the heart, revealed 7 bands at the same positions but differing in intensity (Figure 2). The results obtained with liver extracts varied considerably; a cathodically moving band was observed in many cases. Development is accompanied by the following changes in LDH-pattern of total homogenates (Figure 1): unfertilized eggs and fertilized eggs up to the 4th day reveal only band 1. At the onset of feeding (day 5) faint activity at positions 2 and 3 appears. The next change in pattern is observed at 12 days, when bands 5 and 7 are developed and bands 2 and 3 are clearly stronger. For the following 40 days the pattern remains unchanged. About the 40th day – 5 days before metamorphosis – band 6 is detected, bringing the total to 6 bands. Individual organs tested from the 15–45-day-old larvae, through metamorphosis, to the 3-month-old toad stage, gave the following results: in the brain of the 15–45-day-old larva bands 1, 2, 3 and 6 are present. The full complement of 7 bands (which is not yet present in the animal immediately after metamorphosis) shows up clearly in the 3-month-old toad stage. The larval eye has all bands except 7, which is only added in the adult stage. In the tail 7 bands are never achieved, through the absence of band 4. During the last stages of resorption band 7 seems to be about to resolve into two, but complete separation is never accomplished. In the larval leg muscle pattern (day 15–45) bands 4 and 7 are missing; the full number of bands appears at metamorphosis, but the band intensities in the 3-month-old toad do not correspond to those in the adult. The heart of the 15–45-day period exhibits band 1 only; 3 further bands – 2, 3 and 5 – are found after metamorphosis, with band 1 predominating. The complete number of 7 bands is not yet present in a 3-month-old toad. The liver pattern gives various results.

The method of starch-gel electrophoresis which had been used formerly[3] proved unsatisfactory for detailed study of both embryological material and larval organs of *Xenopus*. The rate of cleavage, for example, is too fast to permit the collection of sufficient material at each stage. A laborious microdissection of large numbers of larvae is involved in order to obtain enough organs for analysis; during the long preparation the LDH-enzyme – especially the slow isoenzymes which are known to be labile – may become affected. The method of agar-gel high voltage electrophoresis on microscope slides[7] was used as well,

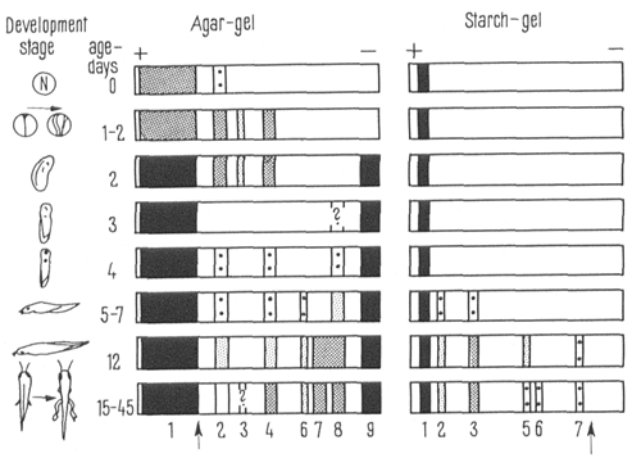


Fig. 1. Diagrammatic representation of LDH-isoenzymes during early development. Agar-gel and Starch-gel electrophoresis. N, unfertilized eggs, →, point of application.

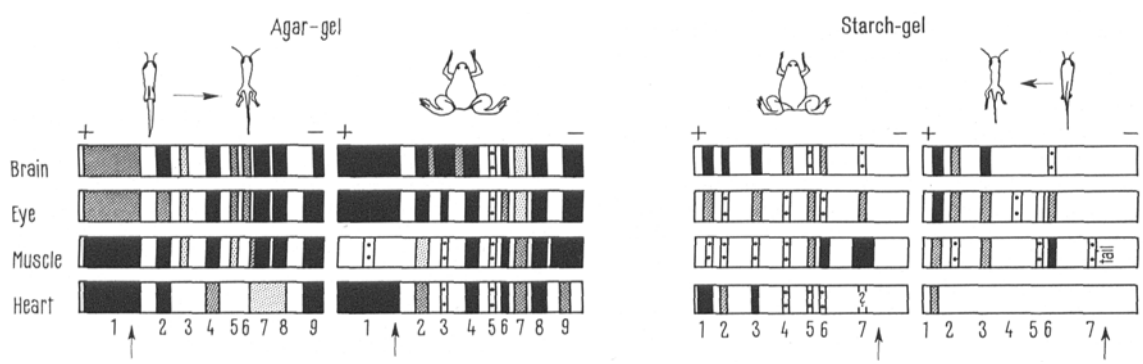


Fig. 2. LDH-isoenzyme pattern of different organs in the larval and adult stage.

because it allows tests of homogenates as small as 3 λ. For the study of even smaller samples direct tissue analyses, which are possible by this method, seem to be the answer[7], (p. 73). This method is so sensitive that isoenzyme patterns of dorsal and ventral halves of an embryo at the tailbud stage and even animal against vegetal halves of single eggs (diameter 1 mm) were achieved (observations unpublished). Other advantages of WIEME's agar-gel method are that electrophoretic separation is accomplished in 1 h (as against 16 h in the starch-gel method), and that only small quantities of the very expensive reactants are needed.

In all experiments agar-gel yielded more bands than starch-gel. The difference in buffer systems, as an explanation, was ruled out by control experiments transferring the agar-gel buffer to starch-gel. VESELL and BRODY[8] have demonstrated that water-extracts of rat kidney yield more bands on starch-gel than do extracts prepared in *Tris* buffer pH 8.7. In the experiments described in this paper, water extracts were used in the agar-gel and *Tris* buffer extracts in the starch-gel method; it was not possible to produce more bands on starch-gel by using water extracts. The fact that more isoenzyme bands are obtained by using the agar gel method is probably due to the higher sensitivity of the electron acceptor INT over Neotetrazolium.

The LDH-pattern, followed through various stages of early development (Figure 1), seems to suggest 2 patterns following one another. The faster isoenzymes in both methods appear first and are followed by a set of more cathodic components. Agar-gel electrophoresis exhibits after the tailbud stage (days 2/3) a temporary loss of 3 bands. This is in agreement with results on *Rana temporaria*[3] and the findings of NACE[4] on *Rana pipiens*. It should be noted that the tailbud stage is characterized by early organogenesis. Why then do some isoenzymes fail to be present at this stage? Embryonic mouse tissues show a shift in activity from the cathodic to the anodic isoenzymes during development, whereas in the developing chicken the transition occurs in the opposite direction[9]. According to MARKERT and URSPRUNG[9] it is uncommon to observe a concentration of LDH-activity at opposite ends of the electrophoretic spectrum as is the case in the results with agar-gel presented here.

Observations of the organ pattern during ontogenesis show that the adult pattern of the different organs is attained at different stages of the development of the animal. Differentiation of the LDH-pattern of brain and eye, as revealed by agar-gel, is clearly in advance of the other organs tested. Heart and skeletal muscle change into adult pattern only after metamorphosis. The development of the liver does not show a regular pattern; the change-over after metamorphosis from herbivorous to carnivorous feeding habits does not give a corresponding change in isoenzyme pattern. Amphibian liver cells retain embryonic yolk longer than do the cells of other developing organs, and it has been shown that the liver is the main haemopoietic organ in premetamorphic stages[10]. Detailed histological study not only of the liver, but also of the other organs during ontogenesis is needed in order to understand the varying developments of organ patterns.

The results from experiments on adult heart and skeletal muscles fit into the LDH-pattern of other vertebrate classes. In the heart muscle the most anodic band predominates, while in skeletal muscle the cathodic components are the principal bands.

Although it is by now well established that LDH occurs in 5 molecular forms in most vertebrate classes, additional bands have been established[11], and have been explained by some of these authors as being due to a third polypeptide subunit. In only 2 instances have 9 bands been observed. CROISILLE[11] described 9 isoenzymes (2 of these transitory) in the embryonic chick liver; ALLEN[11] distinguished 9 electrophoretically distinct sites of LDH-activity in mice, but no single tissue or organ possessed all 9.

LDH-isoenzymes from the same species have been shown to be of equal molecular weight[5]. Starch-gel electrophoresis separates the LDH-fractions according to their electrical charge and molecular dimensions. The pore size of agar-gel, however, is such that the molecular sieve effect is negligible – separation in this case, therefore, is only according to charge[7]. In the experiments described above, both in the starch-gel and agar-gel, the bands are in no case equally spaced, as would have been expected. In none of the developing or adult organs tested, nor in any of the early developmental stages (0–15 days) is there a sequence of 5 equally distributed bands. Some pilot experiments (stage 15–45 days) were carried out using the

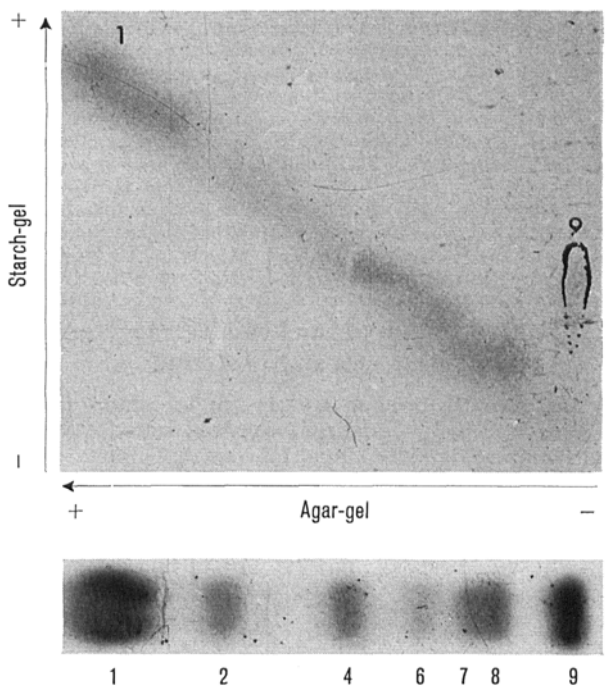


Fig. 3. Two-dimensional electrophoresis experiment. Total homogenate of 19-day-old larva.

[8] E. S. VESELL and I. W. BRODY, Ann. N.Y. Acad. Sci. *121*, 544 (1964).

[9] R. CAHN, N. O. KAPLAN, L. LEVINE and E. ZWILLING, Science *136*, 962 (1962); C. L. MARKERT and H. URSPRUNG, Devl Biol. *5*, 363 (1963).

[10] E. M. DEUCHAR, *Biochemical Aspects of Amphibian Development* (Methuen, London 1966), p. 32.

[11] J. M. ALLEN, Ann. N.Y. Acad. Sci. *94*, 937 (1961); M. C. BLANCHAER, Pure appl. Chem. *3*, 403 (1962); L. A. COSTELLO and N. O. KAPLAN, Biochim. biophys. Acta *73*, 658 (1963); Y. CROISILLE, Carnegie Instn. Wash. Year Book *62*, 434 (1963); E. GOLDBERG, Science *148*, 391 (1965); E. GOLDBERG, Science *151*, 3714 (1966); A. L. KOEN, and C. R. SHAW, Biochem. biophys. Acta *96*, 231 (1965); TH. WIELAND and G. PFLEIDERER, Ann. N.Y. Acad. Sci. *94*, 691 (1961).

2-dimensional electrophoresis, agar to starch-gel, method [7], (p. 129). All bands separated by agar-gel, except No. 9, appeared in starch-gel on an oblique straight line passing through the origin (Figure 3). This would indicate that component 9 is of different molecular weight and/or shape from the other ones. The fact that all fractions separated on agar-gel reappear when applied on starch-gel shows that none of the bands of this stage can be due to interactions with the electrophoretic medium. It also follows from this result that, apart from difference in number, bands on starch-gel and agar-gel cannot be compared directly.

At this stage of the investigation it is impossible to present any hypothesis which would incorporate these results within the framework of the general subunit theory of LDH-structure. Further extensive experiments with bidimensional electrophoresis, tests on differential inhibition and thermal stability of the isoenzymes and dissociation-recombination experiments[12] are envisaged, and these should eventually allow an interpretation of the multiplicity of Xenopus-LDH[13].

Zusammenfassung. Das Isoenzymmuster der Laktatdehydrogenase (LDH) wurde mittels Agar-Gel- und Stärke-Gel-Elektrophorese untersucht. Unbefruchtete Eier und Entwicklungsstadien vom 2-Zell-Stadium bis zum 15. Tag wurden als Totalextrakte analysiert; vom 15. Tag an wurden einzelne Organe geprüft und bis ins Adultstadium verfolgt. Die Entwicklung der LDH-Muster ist gekennzeichnet durch Zu- und vorübergehende Abnahme der Anzahl Isoenzyme und durch Verlagerungen der Aktivitäts-Intensitäten. Adultorgane weisen 7 Isoenzyme auf mit Stärke-Gel-Elektrophorese und deren 9 mit der Agar-Gel-Methode; dies steht im Gegensatz zu Untersuchungen an den übrigen Vertebraten-Gruppen, die in der Regel ein LDH-Muster von 5 Komponenten aufweisen.

YVETTE W. KUNZ and J. HEARN

Department of Zoology, University College, Dublin (Ireland), 30th March 1967.

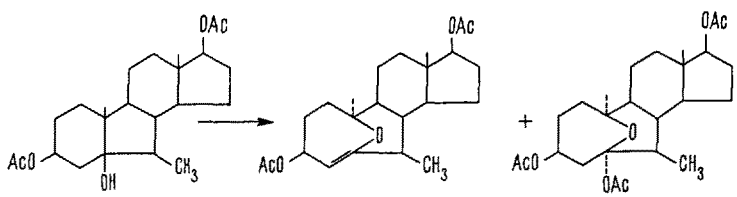
[12] A. BLANCO and W. H. ZINKHAM, Science *139*, 601 (1963); W. ZINKHAM, Science *142*, 1303 (1963).

[13] The authors are much indebted to Professor Dr. R. J. WIEME, Ghent, for demonstrating his agar-gel method and allowing them to work in his laboratory.

DISPUTANDUM

The Mechanism of the Lead Tetraacetate Oxidation of a B-Norsteroid

It has recently been shown[1] in our laboratories that the B-norsteroid I is oxidized with lead tetraacetate to form the bridged oxides II and III.



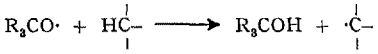
The direct formation of enol ethers or hemiketal acetates from tertiary monohydric alcohols by lead tetraacetate oxidation is unprecedented, and we should like here to present a brief review of the literature pertinent to this reaction, with some of our ideas regarding possible mechanisms for this transformation.

There is general agreement that the first stage in the oxidation of monohydric alcohols with lead tetraacetate is the formation of lead triacetate alkoxides[2], although such intermediates have rarely been isolated[3].

The alkoxide may decompose by either homolytic or heterolytic fission of the O-Pb bond. In the case of primary or secondary alcohols in polar solvents, heterolytic fission with concomitant elimination of a proton to form a carbonyl compound is the favored pathway[4]. On the other hand, in benzene solution, lead alkoxides of tertiary alcohols have been shown to undergo homolytic fission to form alkoxy and lead triacetate radicals[5,6].

Our reaction was carried out using an excess of lead tetraacetate in anhydrous benzene solution at reflux in the presence of calcium carbonate. Under these conditions we may assume that the alcohol I was initially converted to an alkoxy radical[7]. It is the fate of this tertiary alkoxy radical which we wish to discuss.

Aliphatic tertiary alkoxy radicals are known to react by 2 different pathways. The first involves abstraction of a hydrogen atom to reform the tertiary alcohol and to give a new radical.



If this reaction occurs with the solvent, the starting alcohol is regenerated and can react anew to form a lead alkoxide. Where sterically feasible, intramolecular hydrogen atom transfer from carbon to oxygen is a favored pathway and the resulting radical alcohol may be transformed into a cyclic ether[8]. A second and more common route for reactions of tertiary alkoxy radicals is by β cleavage to yield carbonyl compounds.