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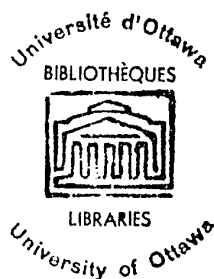
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EFFECT OF HYALURONATE AND ITS DERIVATIVES ON TRYPTOPAN-INDUCED
METAMORPHOSIS OF TADPOLES AND THE ACTIVITY OF LACTATE DEHYDROGENASE
ISOZYMES DURING TRYPTOPAN-INDUCED TADPOLE METAMORPHOSIS.

By

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THESIS

Submitted to the Faculty of Medicine in partial fulfillment
of the requirements for the degree of Doctor of Philosophy.

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INTRODUCTION

During the past few years the pathogenic action of thalidomide on pregnant animal has been amply demonstrated. The induced malformation of the embryo, and the pathogenic action of thalidomide on the embryo occur after implantation and during organogenesis. Since many enzymes show a marked alteration during thyroxine-induced metamorphosis of tadpoles, and furthermore, as these biochemical changes are correlated with morphological transformation of tadpole, the thyroxine-induced anuran metamorphosis might represent an excellent system for testing the effect of thalidomide and its derivatives, and for studying the mechanism of differentiation of tadpole per se. Owing to its sedative effects, thalidomide might also be concerned to the metabolism of catecholamines in amphibian. Our studies described in the first part of the present thesis have been mainly concerned with the study of the thyroxine-induced metamorphosis of tadpole and the effect of thalidomide and its derivatives on this metamorphosis from the point of view of morphological changes, several enzymatic activities, and the amount of catecholamines and amount of glucosamine in various organs.

In the second part of the thesis, LDH pattern during thyroxine-induced amphibian metamorphosis is described. Generally, the LDH isozymes are separated by starch-gel-electrophoresis and the separated enzymes are visualized by using tetrazolium dyes as elution carriers. Generally the enzyme activity is estimated by either

elution or scanning devices. Both these methods are time-consuming and semi-quantitative. In this manuscript we describe a new technique for dissolving the electrophoretic strip together with the colored spots of LDH isozyme. This method is simple, accurate and rapid and has thus been applied for the determination of lactic dehydrogenase isozymes during thyroxine-induced tadpole metamorphosis. The effect of thalidomide on the distribution pattern of this enzyme is also studied.

REVIEW OF LITERATURE

A) Thyroxine-induced metamorphosis of tadpole.

The morphological changes during metamorphosis are carefully described, especially by Taylor and Kollros (1), Etkin (2), and Lynn and Wachowski (3), and many others (4-4). Alterations are observed in nearly every organ system. Prior to the onset of metamorphosis, the frog tadpole is an aquatic animal with well developed gills, flattened tail, lidless eyes, horny and raspy teeth, and long coiled intestine. The frog, in contrast, is a lung breathing animal with no tail, well-developed limbs, eye-lids and other structures adapted to its carnivorous habits. Even structures which persist into the adult life undergo extensive alterations. The skin thickens, becomes more glandular, and attains an outer cornified layer, the intestine decreases in length, and the brain becomes more highly differentiated. A partial list of morphological development which is likely to involve marked cellular modifications during metamorphosis is; the development of limbs, eyelids, lungs, tongue, tympanic membrane, and opercular perforation, the alterations in skin and pigments, liver, pancreas, intestine, loss of teeth, and horny beak, and the atrophy of tail and cloaca.

Only brief mention will be made here on the fact that the morphological and biochemical changes and responses in induced metamorphosis are not always identical with the slower, apparently more

ordered changes of spontaneous metamorphosis, a point emphasized by Etkin (2). However, stimulation of metamorphosis by the elaboration of endogenous hormones or by exogenous thyroid hormone constitute the extrinsic factor in anuran differentiation. Probably of even greater importance are the intrinsic factors contributed by the mosaic of sensitive tissues. It is thus more accurate to speak of metamorphosis as resulting from the interaction of thyroid hormone with undifferentiated tissue factor because it emphasizes that the many phenomena of metamorphosis depend not only on the action of thyroxine but also on the presence of an inherent tissue sensitivity. The discovery of the relationship between the thyroid gland and amphibian metamorphosis by Gudernatsch in 1912 (4) provided an important biochemical development in the study of metamorphosis. This observation facilitated research on this phenomenon by shortening the transitory period from the larval stage to the adult under controlled conditions. In the absence of added thyroxine, a *Rana catesbeiana* tadpole may remain in the larval form for 2 to 3 years under certain environmental conditions. The identification of thyroxine as the active agent in the thyroid gland gave impetus to these studies. The intrinsic susceptibility of tadpole tail tissue is dramatically illustrated by Schwind (5). Schwind transplanted an eye cup to a tadpole tail. During metamorphosis the eye moved gradually forward during tail resorption, and finally came to rest in the sacral region at the termination of metamorphosis. Similarly transplanted limbs are unaffected by the degenerative processes in the surrounding tail tissues. The impressive degenerative changes

of the tail have afforded an extremely convenient tool for bioassay for thyroid hormones using tadpoles, although certain other structure may be even more sensitive to T_3 and T_4 .

The acute and differential sensitivity of amphibian tissues was also shown by Hartwig (6). He obtained local metamorphic changes by thyroxine implants in salamander larvae. This technique has been improved and extended by Kollros (7), and Kaltenbach (8-9). Using thyroxine-cholesterol pellet implants, these workers have reproduced locally many of the individual tissue changes relatively independent of general systemic effects. Kaltenbach implanted thyroxine-cholesterol pellets into early larval tadpoles and induced prematurely and unilaterally in the vicinity of the pellets such effects as change in head outline, resorption of labial fringe, lengthening and ossification of bones of the hind limbs, complex changes in eye structure and tail resorption, depending on the site of implantation. Thus, thyroxine is shown to be the direct initiator of a wide variety of cellular differentiation in a variety of tissues. Kaltenbach, in 1959, (9) also studied that thyroxine-cholesterol pellets containing 20%, 40%, and 60% thyroxine by weight and pieces of fresh mouse or frog thyroid glands (some of which contained I^{131}) were implanted into the dorsal and ventral tail fins of anuran larvae in early stages of development. Cholesterol control pellets were similarly implanted. No local metamorphic response was induced in the fins of tadpoles with cholesterol control implants, and general metamorphic progress was not accelerated. No local metamorphic response was induced in the fins of 90% of the tadpoles with implants containing 20% thyroxins, yet general metamorphic progress was somewhat accelerated.

A striking local resorption of the tail fin was induced prematurely to general metamorphic events by implants containing 1.0% thyroxine. This was accelerated by darkening of the fin at the site of the implant and by some acceleration of general metamorphic progress. Also fin resorption was induced by implants containing 60% thyroxine and by implant of fragments of fresh thyroid glands; however, the localized response was not as sharply delimited, and its precocity in relation to other metamorphic events was lessened by an accompanying rapid acceleration of general metamorphic events. Premature local resorption of dorsal and ventral tail fins was brought about directly by local concentration of thyroxine. It may be concluded that resorption of the dorsal and ventral tail fins occurring during normal metamorphosis of anuran larvae is probably caused by the direct action of thyroid hormone upon the fins.

An additional approach emphasizing inherent tissue response has come from Kollros and his associates (7). They exposed hypophysectomized *R. pipiens* to extremely low doses of thyroxine or tri-iodothyronine. At extremely low immersion levels of exogenous hormone, disappearance of skin tissue in the opercular region, and skin pigment alterations were noted. Under the identical conditions, little change in tail tissue, limb development, or certain other morphological features were detected.

The reference to the biochemical alterations which occur during anuran metamorphosis have been in the earlier review of Etiken (2), Lynn and Wachowski (3), and Wald (15). A more detailed review has

been given by Urbani (10). In the latter review, biochemical changes during anuran metamorphosis were discussed as pertinent to an organism transforming from a free swimming, aquatic larval form to a terrestrial, air breathing, adult form. Thus, during metamorphosis, biochemical alterations may be considered to: (a) Have an apparent direct adaptive value, or (b) Serve as a basis for morphological, biochemical, or other changes which have adaptive value. It is not always easy to decide whether a significant chemical change is directly adaptive or only indirectly. Possibly, all the transitions observed will prove to be directly adaptive to land living when properly appreciated. Therefore, they emphasize the arbitrary and temporary nature of this distinction and anticipate a steady migration of the less well understood chemical alteration into the primary adaptive category.

In relating biochemical changes to the anuran adjustment to land, it is not meant to deny the many useful description of metamorphosis as an extended embryological process. Indeed, many embryologists consider metamorphosis as merely a terminal phase of embryonic development. Witschi includes three chapters on this metamorphosis in his book, "Development of Vertebrates" (11). Many of the biochemical changes during metamorphosis parallel foetal to adult changes including the increase in serum albumin, the reduction in oxygen binding by the prevailing hemoglobins, and the development of the urea cycle enzymes. Some of the most useful arguments in support of the recapitulation theory are drawn from the events of anuran metamorphosis. The tadpole to frog transition illustrates many of the essential features of

biochemical evolution. Since these two outlooks are well known, an equally general but less used view point based on chemical adaptation has been preferred to an attempt to integrate the biochemical changes which have been observed during anuran metamorphosis. The biochemical changes accompanying anuran metamorphosis which contribute to land adaptation may be considered as follows:

1. The shift from ammonotelism to ureotelism during metamorphosis.
2. The increase in serum albumin.
3. The change in the structure and biosynthesis of the hemoglobins.
4. The changes in the digestive enzymes.
5. The augmentation of respiration.
6. The metabolic changes for adaptation.

It is axiomatic in the classical approach to comparative biochemistry, particularly as developed by Needham (12) and Baldwin (13), that the nature of an animal's nitrogenous excretory product is a function of its environment and phylogenetic position. As early as 1931, Needham (14) emphasized that ammonia is the principal end product of protein metabolism of aquatic animals, but is supplanted by urea or uric acid in amphibians, birds, reptiles and mammals. An abundance of water is required for the efficient elimination of ammonia because of its great toxicity. Terrestrial animals have urea or uric acid as the predominant nitrogen excretion product. Mammals are ureotelic, urea being the main end-product, whereas birds and reptiles are primarily excretors of uric acid, uricotelic. Between these permanently aquatic vertebrates and strictly terrestrial animals are the amphibians,

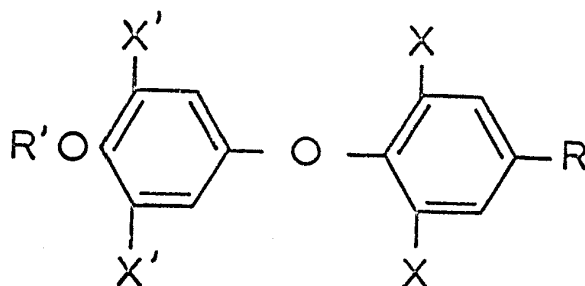
vertebrates making a transition from an aquatic to a terrestrial environment in their life cycle. From these results, it is apparent that the nitrogen excretion pattern of amphibians should fluctuate between that of their aquatic, ammonotelic pattern and terrestrial, ureotelic relatives. At the outset of metamorphosis or under the influence of the metamorphic stimulus, T_H , alterations in the nitrogen excretion pattern begin to occur(15).

William et al (16) reported that forty three different substances structurally related to thyroxine were assayed for their metamorphosing effect on *Rana pipien* tadpoles. Triiodinated compounds with iodines at the 3,3',5 positions were found more active than tetraiodinated compounds. The 3,5 and 3,3' diiodinated compounds tested also had some activity except for the 3,5 diiodothyroformic acid. None of the moniodinated compounds assayed showed biological activity. The most active compound studied was 3,3', 5-triiodo-thyropropionic acid which was 300 times more potent than I-thyroxine. The next most active compound was 3,5', 5,5'-tetraiodothyropropionic acid which was 80 times more active than thyroxine. The 3,3',5-triiodothyroacetic acid and 3,3',5,5'-tetraiodothyroacetic acid followed closely, being 24 and 10 times more active respectively than thyroxine. In general their data for potency agree quite well when comparison can be made with data reported by others (17-20). Roche et al (17) reported that Antigoitrogenic activity and its effect on the metamorphosis of tadpoles of *Rana esculenta* have been studied with sixteen iodothyronines and substances structurally similar to them. The

3,5,3'-triiododerivatives show themselves to be more active than their homologues 3,5,3',5'-tetraiodinated substances, whether the carbon side chain is an alanine(iodothyronines), propionic acid or acryloyl-residue or carboxyl group. In the iodothyronine series, the D,L-3,3'-diiododerivatives show as great an activity as that of the D,L-thyroxine. That of the D,L-3,3',5'-triiodothyronine is very small in contrast to that of the D,L-3,5,3'-triiodothyronine, previously defined as being five times greater than that of D,L-thyroxine. The presence of one atom of iodine ^{at} 3 and one at 3' is a necessary condition for the existence of activity; this activity is strengthened by a substitution at 5 and reduced by a substitution at 5'. Its effect on the metamorphosis of tadpoles does not always run parallel with its effect on the development of experimental goiter. This activity is very great in the case of thyropropionic and thyroacryloyl derivatives, and in the case of 3,5,3'-triiodothyropropionic acid reaches a value 290 times that of DL-thyroxine. Bruice et al studied (18) a correlation of structure vs. thyroxine-like activity for 47 analogs of thyroxine. The thyromimetic activities of these analogs in amphibia and mammal are related to the electronic character of the diphenyl ether nucleus, as affected by the abilities of the groups X, X' and OR' to attract or release electrons, with electron-releasing groups favoring higher activity. The abilities of the substituents X and X' to form hydrogen bonds influence thyromimetic response, activity increasing with enhanced ability to form such bonds. Activity is related to the pK of the side chain, R. The empirical nature of the correlation is stressed

and its efficiency and possible uses are noted.

Fig. 1



Michel and Pitt-Rivers reported that (17) when thyroxine analogues are compared with thyroxine, it is often found that these analogues exert a much greater effect on amphibian metamorphosis than on various tests in mammalian organism. This observation also applies to the rate of metamorphosis where various thyroxine analogues seem to accelerate metamorphosis much more than thyroxine (20-27). It is rather rare that the potencies of thyroxine-like compounds are higher, relative to thyroxine, in mammals.

Kaltenbach (28) in studies on tadpole metamorphosis using

thyroxine implants suggested that diffusion of thyroxine into the surrounding tissues was indicated. The accompanying slight acceleration of other metamorphic events, such as hind limb development, is attributed to the action of small quantities of thyroxine which entered the circulatory system. In general a pellet containing 20% thyroxine elicited no local metamorphic response in the fin, yet it did stimulate overall metamorphic progress to some extent, as judged by accelerated hind limb development. Furthermore, when implanted into various other regions of body, such pellets were effective in eliciting premature local metamorphic responses in structures such as skin, eyelids, and operculum. Hind limb growth and ossification were accelerated by pellets containing an even lower concentration of thyroxine. The responsiveness of the fin to a relatively high concentration of thyroxine and its failure to respond to a lower concentration suggested that the fin has a high threshold of response to thyroxine (29-31). This is further substantiated by the occurrence of generalized fin resorption rate in the normal sequence of metamorphic events; in a few days after the onset of metamorphic climax eyelid formation, opercular thinning and perforation, limb formation, etc. occurred. These changes coincided with a period of great thyroid activity (32). The late and early metamorphic events probably have different thresholds of response to thyroxine; those for late events being high, and those for early events, such as hind limb development, being low. This view is in accord with that expressed by Kollros (33). On the other hand, according to the hypothesis of Etkin (33-34), all events of metamorphosis will respond to low

concentrations of thyroxine given sufficient time, the rate of response depending upon the concentration of hormone. Clausen (35) (36) demonstrated that, when implant contained high concentrations of thyroxine or consisted of pieces of fresh thyroid glands, the locally resorbed area in the fin was often large, but not sharply delimited. Moreover, such resorption was accompanied by a general acceleration of metamorphic events, including reduction in the height of both fins. This indicates that diffusion of thyroxine from the implants was rapid, thereby allowing relatively high concentration of thyroxine to diffuse not only into the adjacent fin tissues but also into the circulatory system. The fin was more reduced in height anterior to the resorbed area than posterior to it may have been due in part to the circulatory pattern in the fin or to the anterior-posterior gradient of fin susceptibility. Evidence for the rapid diffusion of hormone from gland implants was further afforded by radioautographs of tadpoles with I^{131} containing implants in the tail fin or beneath back skin (14), for within a few days very little I^{131} was present in the implants or surrounding tissues, but large amounts were concentrated in the tadpole's own thyroid gland. When glands were dried, mixed with cholesterol, and compounded into pellets in order to decrease the rapid rate of hormone diffusion from them, they were ineffective in stimulating metamorphosis. This was probably due to destruction of effective substances during drying of the glands or to insufficient concentrations of thyroxine in the pellets. The same concentration of thyroxine were probably caused by early expulsion of some pellets from fins due to pressure

atrophy (37), variations in the size firmness, and thyroxine contents of the pellets and hence in the rate of hormone diffusion from them, and variations in the individual responses of tadpoles. The induction of localized fin resorption by thyroxine-containing implants negates the early hypothesis concerning the dependence of tail resorption upon the growing urostyle and its interference with the blood supply of the tail. The results, therefore, are in accord with those of Morse (38) and Helff (39). The lack of metamorphic response in the fin both anterior and posterior to the implant, and its resorption locally at the site of implant, indicate that resorption was not initiated by a factor which influenced the tail as a whole. For similar reasons, and in accord with the findings of Brown (40), degeneration of the nervous system of the tail cannot be considered as the factor causing fin resorption. Since resorption was sharply localized and the tail as a whole functioned normally, any degenerative changes which may have occurred in the nervous system must have been localized in the resorbing area of the fin only and have been part of the general syndrome of histological changes occurring locally near the thyroxine implant. Furthermore, underlying atrophying muscle must be ruled out as the causative agent of fin resorption, since serial sections revealed intact muscle beneath locally resorbing regions. This is consistent with the view of Lindeman (41-42) that tail skin is able to undergo histolysis independently of underlying atrophying tail muscle.

That the tail fin is specific in its response to thyroxine was indicated by the localized resorption which occurred near thyroxine

implants in the fin and by the variety of other specific local metamorphic responses which occurred near thyroxine implants in other regions of the body, such as maturation of back skin and growth of mesencephalic V nucleus cells (43-44). Specificity of tail tissues to histolytic factors had been indicated earlier by transplantation experiments of Lindeman (41-42), Helf and Clausen (45), and Geigy (46).

In contrast to the amount of information available on the physiological function of the thyroid hormones in amphibian metamorphosis, relatively little is known about their effects on growth and differentiation in other classes of lower vertebrates such as tunicates, acranians and fishes, or invertebrates. Contradictory findings have been reported but it appears that the effects of the thyroid on growth and differentiation is less spectacular than in amphibians. Although so many studies have been made on the effects of thyroid hormone on metamorphosis, none of the theories put forward to explain the mechanism of action of thyroid hormones on metamorphosis has gained wide acceptance. It has been suggested that the thyroid controls metamorphosis by influencing metabolism. However, many investigators who did observe an increase in metabolic rate of tadpoles during metamorphosis have expressed the belief that such a rise in metabolism is incidental rather than a cause of metamorphosis as first suggested by Huxley (47) and Aleschin (48).

Champy (49) believed that the thyroid hormone causes an increase in mitotic activity in those organs which are characteristic of terrestrial life, i.e. lungs, limbs and intestines, while it has

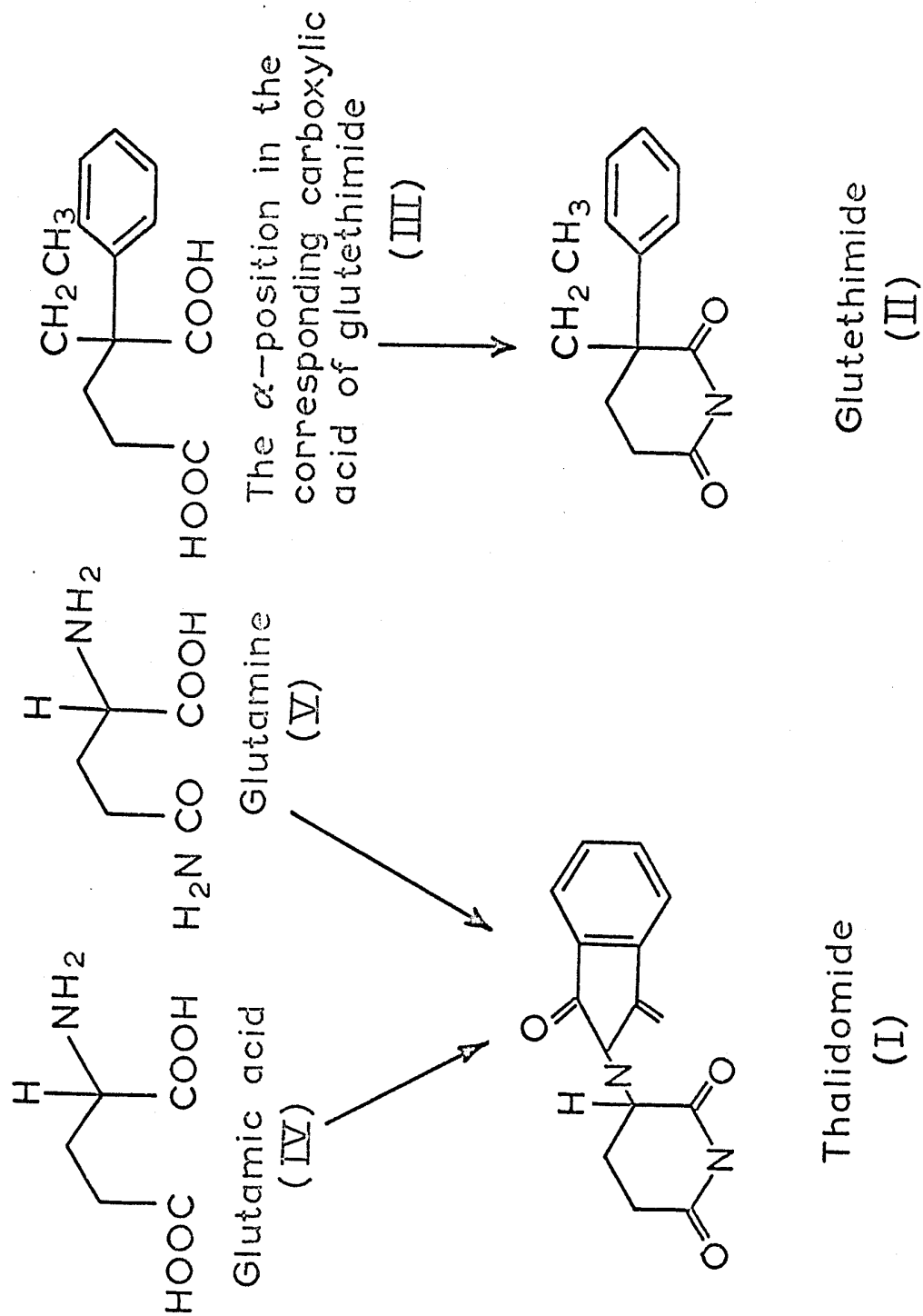
effects on characteristics of aquatic existence, such as the tail, gills and the horny beak. Aleschin considers that impregnation of larval tissues with thyroid hormone leads to autolysis, especially in the gills and intestines; autolysis would lead to a general acidosis and this would promote further autolysis. This would occur even when the thyroid is in a resting state towards the end of metamorphosis(43). The principal objection to this theory is that it only accounts for the disappearance of certain tissues, but provides no explanation for the appearance of new ones (limb buds). Lynn and Wachowski (5) concluded that the characteristic responses of different tissues to the hormone result from inherent properties of the tissues themselves. This leads us back to the basic problems in embryology of "differentiation" and "determination", and Pitt-Rivers and Tata (50) concluded that the diverse changes which occur during metamorphosis are predetermined and are only triggered off by the thyroid hormone.

B) The effects of thalidomide and its derivatives on thyroxins..
induced metamorphosis of tadpole.

The peculiarity of thalidomide (51) becomes particularly evident when its structural formula is compared with that of other sedatives. The most of the known drugs exerting central-sedative effects (hypnotics, sedatives and anticonvulsive agents) are grouped according to their structural features and to the ring system they contain. These substances are derived from carboxylic acids substituted at the α -carbon atom with two hydrocarbon residues. The type of ring system into which this grouping is incorporated seems to be of minor importance. As will be seen from the structural formula of thalidomide (I), it bears, in the α -position, an acylated amino group instead of two hydrocarbon residues. Of all the sedatives known to date, thalidomide, a derivative of glutamic acid (IV) or glutamine (V), is the only preparation derived from a natural endogenous α -amino acid. Superficially, the formula of thalidomide resembles that of glutethimide (II), (Glutethimide generic name for the hypnotic Doriden), with an additional two hydrocarbon residues on the corresponding carboxylic acid (III).

It is not only in its chemical structure, but also with regard to its pharmacological properties that thalidomide displays differences as compared with the other known sedatives and hypnotics. In animal experiments thalidomide was found to be a mild sedative with a rapid onset of effect (51-54). Its central-sedative action merely induces a reduction in spontaneous movement without any initial

Fig. 2



excitatory phase. Unlike preparation exerting an anesthetic effect (barbiturates, glutethimide, ethynyl-cyclohexyl carbamate, and methylpentynol), thalidomide does not interfere with coordination of movement, even given in very high doses (5/). Nor does it show any anticonvulsant action. One particularly striking feature of thalidomide is its extremely low toxicity in acute experiments. Even when the drug is administered in the highest possible dosage, it was impossible to determine the lethal dose or even to establish the dose at which an anesthetic effect occurs - an observation which contrasts with the properties of the sedative and hypnotics. The findings emerging from animal experiments was confirmed in the course of clinical trials. When the drug was taken in extreme over-dosages, it exerted no anesthetic effect in man (55-59). In view of these facts it appears that thalidomide constitutes a unique type of sedative (5/). However, during the year 1961, reports appeared in the medical press referring to neurotoxic side effects caused by thalidomide (33,61-70). In 1961, Lenz (71) voiced the suspicion that treatment with thalidomide during early pregnancy might be responsible for the occurrence of Wiedmanns syndrome (72) in Germany. With the publication of further papers on this subject (73-85) the opinion became more and more firmly established that thalidomide, if taken by pregnant women during the critical phase of limb development (4th-6th week of pregnancy) could be the causative agent of deformities of this type. It would appear that during this phase, even very small quantities of thalidomide are sufficient to cause congenital malformations (86-91). Since in adults an oral

therapeutic dose of 100 mg thalidomide yields at the utmost a blood concentration of 0.9 $\mu\text{g/ml}$ (91), it appears that only a few micrograms of the active substance may suffice in the embryo to produce a teratogenic effect. Thus, it seems rather difficult to reconcile with the very low acute toxicity of thalidomide in man and animal, and raises the question whether it is the preparation itself or, more likely, one of its metabolites that is the factor adversely affecting the embryo.

In 1964, Delahunt reported that they could induce malformations in monkey fetus using thalidomide, as observed in man (92). Fourteen female *Cynomolgus* (*Macaca irus philippinensis*) were used in their study. Thalidomide (10 mg/kg) was given by an oral tube from the 32nd to the 42nd day of pregnancy. These days were chosen for treatment because implantation only occurs 9 to 11 days after mating. The dosage schedule took into account that limb development occurs 26 days after conception. They performed Caesarean sections on pregnant females in order to avoid losing any fetuses by unobserved abortions. All fetuses were examined. The thalidomide syndrome in the monkeys was manifested by amelia, phocomelia, internal hydrocephaly, facial capillary hemangioma, hypogenesis of the metatarsal bones, and anotia. Cross dissection revealed that the conceptus was a teratoma. The malformations described in these monkeys were anatomically identical to the deformities reported in human children whose mothers had taken thalidomide during pregnancy (70).

The anomalies found in monkeys are of such distinctive character that it would be highly unlikely that they could have occurred, even in these limited number of studies, merely by chance. The findings

by Lapin and Yakovleva (93) would further strengthen the report of Delahunt et al that thalidomide produced the skeletal defects in the fetus of monkey (92). Delahunt et al reported that the skeletal system in both man and the monkey was the primary target for malformation induced by thalidomide. In 1964, Fabro et al investigated the effect of thalidomide and its derivatives on the pregnancy in rabbits (94). They reported that thalidomide exerts, in the two strains of rabbits, a clear teratogenic and embryotoxic effect as is evidenced by high incidence of resorptions and malformations. Substitution in the phthalimide ring of thalidomide by a nitro group as in 3-(1',3'-dioxo-4'-nitroisindolinyl-2')-2,6-dioxopiperidine (3-nitrothalidomide), appears to reduce teratogenic activity markedly; however, the incidence of resorptions remains high. In 1964, Joyce et al (95), studied the teratogenicity of thalidomide and its derivative compounds in the developing chick embryo. Whereas doses of thalidomide up to 10 mg had little or no effect on the 4-day old embryo, 0.5 mg injected into the yolk sac just prior to incubation produced micromelia and edema, which were rarely seen in the controls. Other abnormalities observed were rumplessness, cerebral hernia, and defects of the eyes and beak, which were also present, but to a lesser extent, in the controls. Metabolites of thalidomide which are found in the urine of rabbits after oral administration of this drug were also investigated. α -N-phthaloylglutamine and α -N-phthaloylglutamic acid had little effect, suggesting that hydrolytic cleavage of the glutarimide ring of thalidomide results in decreased teratogenicity. However, increased teratogenicity was shown by hydroxylated derivatives of these compounds and of thalidomide in

which the hydroxyl group was located in the 3- or 4-position of the phthalimide group, the 4-hydroxylated derivatives being more effective.

The malformation induced by thalidomide in rabbit, chicken, monkey and mouse were well established. However, there is another interesting effect of thalidomide. Kemper et al., and others (96-101) showed that in treating animals with thalidomide before implantation of the zygote resulted in still births. They advanced the hypothesis that the thalidomide killed the embryo prior to its implantation. Kemper et al. (96), in 1962, reported that they carried out some experiments on chick eggs using thalidomide. In their experiments on more than 600 eggs, they used those of an inbred hybrid-strain which were injected once under sterile condition with 10 mg., 2.5mg., or 0.5mg. thalidomide either before starting the artificial breeding, or 144 hours (6th day) after beginning the breeding. In the thalidomide-treated groups, a number of gross malformations (encephalocele, anencephalia, microphthalmia superior), deformities of the jaw and of the legs) even in the group which was injected with only 0.5 mg. ^{were observed.} The abnormalities occurring in 20% were not dependent upon the dose of thalidomide, and only macroscopic and external malformations were noted. The interesting part of this finding is that, in a large number of eggs injected before breeding, the development of the germ per se ceased or was delayed, whereas those injected on the 6th day of breeding showed far better germ development although malformations could be observed.

Giroud et al. (97) also studied the effect of thalidomide on pregnant animals of three species, mice, rabbit, and rat. The mice used

were Swiss Albino and the Black and A/HO strains. Daily doses of 125-250 mg./kg. of thalidomide mixed with the food and given from day 1 to day 14 or day 6 to day 14 of the pregnancy produced 40-50% fetal resorption and 5-11% gross malformation of the survivors. The commonest abnormalities were harelip, cleft palate, cataracts, and various tail deformities. Malformation of the axial skeleton associated with a reduction of the general size and a case of craniorachischisis have been found in mice given lower doses (50-75mg. per kg. orally from day 1 to 14 of gestation). In rabbits, daily doses of 125-250 mg. per kg. mixed in food from day 6 to 14 produced fetal resorptions, and 30% of the surviving fetuses showed malformations of the central nervous system, the limbs and the axial skeleton; encephalocele, anencephaly, clubbed feet, deformities of the spinal column associated with leg defects. In rats, the three strains investigated were Wistar, Long Evans, and August. Thalidomide was added to food from day 1 to 14 or from day 6 to 14 in doses ranging from 250 to 500mg. per kg. This treatment resulted in 20-40% of fetal resorptions but no gross malformations. These findings in the rat agree with the findings of Somers (88) and Plicke (98).

In 1963, Lucey et al (99) administered thalidomide to 44 female rhesus monkeys immediately after they had mated. They made a daily list of females to be mated. During the one month period of this investigation 101 females were listed and mated. The first ^{two} or three on the daily list were arbitrarily started on thalidomide immediately after separation from the male. There were 44 animals in the group. Thalidomide usually given for 33 to 45 days, but three animals

received it only 6, 7 and 8 days after their second mating. Fifty seven females from the list constituted the control group. If a female in the treated group menstruated, they were continued on the tenth to twelfth days after menses. Similarly, females in the control groups remained. There were no live births from these thalidomide group animals, whereas there were 11 live births in 57 untreated control animals. The results are statistically significant. Thus, they advanced the hypothesis that thalidomide killed the embryo prior to implantation.

In 1963, Villa et al (100) reported that chick embryos before and after incubation were injected with thalidomide dissolved in egg-yolk in varying doses. Some of the embryos were allowed a long incubation to observe survival rate or growth defects. Others were terminated at the 96th or 120th hours of incubation and besides the survival rate, mitosis of blood cells were determined. So far as long-term observation is concerned, thalidomide injected embryos after eighteen days of incubation showed poor growth compared to controls, and with larger doses, complete arrest of maturation. In the short-term experiments, the results obtained point to a ~~distinctive~~ depression of the mitotic activity of chick embryo blood cells. Their observation showed that thalidomide, besides its gross effect on growth and maturation, can affect the mitotic activity of chick embryo blood cells. A slight inhibiting effect of the egg yolk was observed only when it was injected before incubation, presumably because of mechanical impact. The anti-mitotic

activity of the compound seems to be directly related to the time of administration, since it can not be elicited after a few days of incubation(101).

In 1962 Paigle et al reported that (102) when thalidomide is administered orally (100 mg/Kg) in the rat, approximately 50% is absorbed, while in the dog approximately 30% is absorbed. Similar findings have recently been published by Mackenzie and McGrath in rats (103). According to these authors, when single oral doses of 10, 100, 1000 mg/Kg were given, the portion excreted in the urine amounted to 40.8%, 39.4% and 28.8%, respectively. Thus, over a wide range of dosage, the percentage of the substance absorbed is independent of size of the dose administered. Absorption takes place fairly rapidly, maximum concentrations being attained in the blood and organs after only four hours. The substance circulating in the blood at this time, as shown by Mackenzie and McGrath, consists mainly of unchanged thalidomide. Their results indicate that thalidomide or their metabolites become evenly distributed throughout the various tissues and organs. The fact that higher concentrations are encountered in the gastrointestinal tract and the kidneys are not surprising, as these organs are directly involved in the processes of absorption and excretion. One remarkable feature is the strikingly low affinity of thalidomide for the fat depots. It is a well known fact that the short-acting barbiturates as well as nonbarbiturates such as glutethimide show a marked tendency to become rapidly but transiently concentrated in the adipose tissues (104). Once absorbed, thalidomide is

relatively quickly excreted in the urine, almost entirely in the form of metabolites. The metabolites in the dog are products of thalidomide hydrolysis and consist chiefly of derivatives of glutamic acid. In this respect, the metabolism of thalidomide differs markedly from that of the other known hypnotics and sedatives. Thalidomide is the only known sedative whose metabolites are glutamic acid derivatives, and which possesses neurotoxic and possibly teratogenic properties. This gives rise to a number of considerations and speculations which might serve as a guide to further experiments. Glutamic acid is known to be involved in a wide variety of biochemical processes (105). Apart from its importance as a component of protein, it occupies a key position at many points within the network of intermediary metabolism. Via α -ketoglutaric acid it is concerned in the citric acid cycle, and thereby with carbohydrate metabolism. In amino acid and protein metabolism it plays a major role as an $-NH_2$ donor during transamination. The important function which fulfills in connection with the physiology of nervous tissue is evidenced by the fact that it is the precursor of γ -aminobutyric acid (106). Finally it should be mentioned that biological substances closely related to glutamic acid, such as glutamine, glutathione, carbamyl-glutamine and folic acid, likewise play important physiological roles. Glutamic acid has also proved of limited clinical interest in certain neurological disorders (107-110).

Thus it is quite possible that thalidomide may in vivo, through its hydrolytic metabolites, in some way interfere with the biochemical and physiological functions of natural glutamic acid, glutamine

or other glutamic acid derivatives. Kemper (111) had demonstrated that thalidomide interferes with the growth and sexual development of cockerels and has drawn attention to the possibility that thalidomide or its metabolites may be folic-acid antagonist. Robertson (68) and Tewes (112) reported independently that they had successfully treated thalidomide neuropathies with vitamin B. Murphy, Dagg and Karnofsky have investigated (113) the effects of various compounds in chicken and rat embryos. They found that the teratogenic effect of the nicotinamide antagonist, 6-amino-nicotinamide, could be countered by the simultaneous administration of nicotinamide. Leck and Millar (114) have suggested that thalidomide may interfere with the metabolism of riboflavin, as riboflavin deficiency is capable of producing malformation in rats. The findings reported a number of years ago by Abderhalden (115) and Fishman and Arton (116) should be mentioned here. It was found that vitamins of the B group reduced the toxicity of D,L-amino acids. It may be recalled that the vitamins of the B group constitute cofactors for numerous enzymes such as the oxidases, the transaminases, and glutamic acid decarboxylase. In recent years, several review articles dealing with the problem of congenital malformations have appeared (116-117). So far, some 70 different methods are known by which malformations can be produced from a metabolic disorder in the pregnant animal during the sensitive phase of embryonic development. These metabolic disorders can be produced experimentally by a wide variety of methods, e.g. by supplying a diet deficient in vitamins or by administering specific vitamin antagonists or metabolites. It is significant that in most instances where a vitamin deficiency has

a teratogenic effect, the vitamins in question belong to the B group, including particularly riboflavin and folic acid. A deficiency of the latter, their blockade by antagonists such as galactoflavin, x-methyl-folic acid, or methopterin, leads to a severe malformation affecting the limb and various organs. Among the antimetabolites, the glutamine antagonist azaserine (O-diazoacetyl-L-serine) and DON (6-diazo-5-oxo-L-norleucine) have attracted particular interest as potent teratogenic factors (116).

c) Isozymes of lactate dehydrogenase.

(1) The multiforms of the lactate dehydrogenase isozymes.

When subjected to a variety, (118-121) of chemical (122-124) or serological tests (125-129), enzymes from different organisms are commonly found to be different from each other even though catalyzing the same chemical reaction. In view of the demonstrated genetic control of protein syntheses (130), it is not surprising that differences should exist. The structure of homologous enzymes or protein synthesized by animals of different species (131-135) or even by animals of different genotype within the same species (136-140). Rather surprising, however, is the evidence demonstrating that several enzymes exist in multiple molecular forms not only within a single organism but also even within a single tissue.

In 1957 Wieland and Pflieger (119) reported that a correlation had been found between the electrophoretic mobility of the components of the LDH of tissue and their degree of sulfite inhibition. In 1958 different components have been found to have distinctive pH optima of lactate dehydrogenase by Vesell and Bearn (141). In 1959 Markert and Möller (142) reported that from tissue homogenates three dehydrogenases (lactate dehydrogenase, malic dehydrogenase, and isocitrate dehydrogenase) were resolved into their component isozymes by zone electrophoresis on starch gels. They proposed first to give name these molecular types, "isozymes" which many different enzymes have been resolved by physicochemical techniques into several distinguishable molecular types. The lactate dehydrogenase isozymes, malic

dehydrogenase isozymes, and isocitrate dehydrogenase isozymes were then localized on the starch by a new method that should be applicable to any electrophoretically mobile enzyme used NAD or NADP as a cofactor. They also reported that lactate dehydrogenase obtained from the tissues of several different species was electrophoretically separated into several molecular types (isozymes) each with the same substrate specificities. The pattern of LDH isozymes is species- and tissue-specific and changes during the embryological differentiation of the tissue.

They also reported that (172) crude homogenates of beef heart were resolved electrophoretically into essentially the same components as the crystalline beef heart enzyme. Kaplan, Ciotti and Stolzenbach (173) compared the activities of 3-acetylpyridine and pyridine-3-aldehyde analogues of NAD APNAD and PY3ALNAD with NAD in a number of dehydrogenase systems. APNAD reacts with yeast alcohol dehydrogenase at approximately 10 per cent the rate of NAD. PY3ALNAD reacts at a considerably slower rate. With horse liver alcohol dehydrogenase, APNAD and PY3ALNAD reacts more rapidly at the saturation levels than does the natural coenzyme. The K_m values for the two analogues are approximately the same as that of NAD with the yeast alcohol dehydrogenase. However, the affinities of the two analogues for the horse liver enzymes are considerable lower than that of NAD. PY3ALNAD reduced by beef heart lactate dehydrogenase at a faster rate than APNAD, whereas with the same enzyme from rabbit skeletal muscle the reaction is more favorable with APNAD.

Pfleiderer and Jackel (174) showed that pig heart, rabbit skeletal, and rat skeletal muscle LDH showed different degrees of

inhibition with 1-fluoro-2,4-dinitrobenzol. The difference in enzymatic behavior, even though slight, raises the question of the sharpness of the boundary separating a family of the isozymes from distinctly different enzymes. In an attempt to characterize homologous enzyme from different sources, investigators have resorted to serological methods. Generally, antisera prepared against an enzyme from one species will not cross-react with homologous enzymes from distantly related species (^{26, 27}). However, some cross-reaction is commonly observed when the species are closely related (²⁵). Gregory and Wroblewski (²⁷) using antisera obtained from chickens injected with rabbit muscle LDH, demonstrated varying degrees of cross-reaction between these antisera and LDHs obtained from five different mammalian species. A similar cross-reactivity was shown by Herion and Sutherland (²⁸) for phosphorylases extracted from the tissues of the dog, rabbit, and cat. These investigators also demonstrated that the phosphorylases from different organs of the same animal are immunologically different, although cross-reactive in varying degrees. Serological distinctions have also been shown with alkaline phosphatases extracted from different human tissues (²⁹). They reported that wide variation was noted in the degree of cross-reaction between heterologous antisera and the phosphatase from different tissues. The results of these tests suggested that human serum phosphatase was a mixture of types found singly in other tissues. These results with the LDH, phosphorylase, and phosphatase antisera were in the light of isozymic patterns merely reflecting the degree to which the different tissues share a common repertory

of isozymes. Failure of complete cross-reaction may indicate merely the existence of additional isozymes in the tested tissue that were not present in the immunizing extract. This seems to be particularly probable as an explanation of partial cross-reaction obtained with enzymes from different tissues of the same species.

In 1961 Kaplan and Clotti (143, 144) observed that two types of lactate dehydrogenase exist in most animals. These two types appear to be controlled by separate genes and are different in their amino acid compositions, catalytic characteristics, and immunologic reactions (143). They have designated one type as M, since it occurs largely in skeletal muscle, and the second, which is usually present in cardiac muscle, as H. Both types appear to exist as tetramers consisting of identical polypeptide units. Kaplan et al (143) have designated these aggregate forms as M_4 and H_4 . In addition to the two homologous forms, hybrids between the two types also containing four units have been identified as intermediate electrophoretic entities. They have designated these hybrids M_3H_1 , M_2H_2 and M_1H_3 (143). The form M_3H_1 contains three M subunits and one H unit, M_2H_2 is composed of two units of each type, and M_1H_3 contains one M peptide chain and three of the H type. These hybrids have intermediate immunologic, catalytic, and physical properties (144). Kaplan et al (144) reported that the lactate dehydrogenase of the heart of newborn rat was different from that of the adult. This difference was determined by comparing the rates of reaction with analogs of NAD (144). Furthermore hybrids of these types appear and progress characteristically

during development of the embryo as well as during maturation after birth. Thus, they suggested that the hybrids are formed at random and their concentrations follow a binomial distribution. They again emphasized that the occurrence of the hybrids appear to be due to the fact that both genes are operating in one cell during the change of lactate dehydrogenase from the embryonic form to the adult form. Chan et al (45) reported that the embryonic form in the chicken is the H type. In contrast, the three mammals that they have studied - rat, rabbit and beef - all appear to have the M form as the embryonic type. Markert et al have reported that the mouse and pig also appear to have M unit as the embryonic form. Most unexpected was the finding that in the human embryo the H type of lactate dehydrogenase is exclusively present. Whether the human is unique among the mammals remains to be elucidated, since only a limited number of mammals have been studied (42). Fine et al reported that their data showed the great variability with which different species select the type of lactate dehydrogenase in the liver (46). The early human embryonic liver is entirely H type, but the adult liver contains only a very small percentage of this type. In the rat, on the other hand, the embryonic form in the liver, even in the earliest embryo studied, is the M subunit, and there is relatively no changes in the composition of the lactate dehydrogenase of the adult rat liver.

The shifts in types of lactate dehydrogenase appear to be similar to those which take place in hemoglobin; these shifts, appear to be species-specific. It seems that in different species the characteristics of repression and de-repression of the genes controlling the

synthesis of the two types of lactate dehydrogenase are different and that these characteristics may be of considerable importance for the development as well as the survival of species. Fine et al, suggested that all of the cells of one animal have the genetic capacity to produce both types of lactate dehydrogenase, but their expression is controlled by unknown factors which are associated with the underlying mechanism involved in differentiation and development (/46). They have further advanced the view that the two types of lactate dehydrogenase have different physiological roles. Because of their differences with respect to inhibition by excess pyruvate, they have ascribed a function to the M type enzyme in tissues where glycolysis is important such as most mammalian skeletal muscle, and the H type in tissues which have a relatively constant rate of metabolism and which are more aerobic, such as heart. The M type enzymes of vertebrates show fewer properties of substrate inhibition than the corresponding H form. The results obtained by Wilson et al (/47) gained further support for the functional significance of the two forms; birds that are poor fliers have the M type of enzyme in their breast muscle, whereas in those birds whose flight is extensive and sustained a large percentage of H type is present (/47). Wilson et al (/47) have shown that there is an almost perfect correlation between the strength of sustained flying and the amount of H type of lactate dehydrogenase present in the pectoral muscle.

The functional distinction between the two types of subunits has been established by determination of optimal substrate concentrations, comparative K_m values, analogue ratios, and turnover numbers. This

functional distinction appears to be of considerable importance in the metabolism of the tissue involved. It is now clear that the synthesis of the two types of subunits is under the control of separate genes (143,149,144). In 1964 Kaplan et al showed that independent regulation of these genes can give rise to varying proportions of H and M subunits, thereby controlling the relative distribution of the five LDH forms within a given tissue. The distinctive catalytic properties of the two types of subunits are adapted to the metabolic environment of the tissues in which each subunit predominates (143). The operation of a sulfhydryl group at the active-site of LDH has been established by several workers (151-157). The observation that the M enzyme from bullfrog muscle has only one or two sulfhydryl groups per subunit has permitted identification of the region of the primary sequence that contains the active site thiol. A dodecapeptide containing the essential thiol group labelled with iodoacetate-1-C¹⁴ has been isolated from tryptic digest of the frog muscle enzyme. A sulfhydryl peptide of virtually identical sequence has also been isolated from tryptic digests of four other LDH's. These four LDH's included M_H enzymes from chicken and dogfish and H_L enzymes from chicken and beef. The amino acid compositions of the sulfhydryl peptides are common to these four LDH's and to the active-site of frog M_H LDH. After partial acid hydrolysis, identical peptide mixtures were obtained for each of the five peptides. This fact demonstrates that the sequences of these peptides are also identical (157-158).

The comparison of this peptide to active site thiol peptides previously identified in triosephosphate dehydrogenase (TPD) (157-160), and in yeast alcohol-dehydrogenase (Y-ADH), and horse liver alcohol

dehydrogenase (HL-ADH) (161-162) are also reported and they described data in detail (163). In vitro hybridization of H and M subunits were reported by several workers (164-165), and it is believed that the ability of H and M subunits from a single species to associate into tetrameric form of the active enzyme is implicit in the concept of hybrid LDH's. The association of the two types of subunits from the same species into the intermediate forms of LDH has been achieved in vitro by freezing and thawing under appropriate conditions. This ability of H and M subunits to associate into the tetrameric forms of LDH extends also to subunits from quite distinct species. Very recently, functional significance of H and M subunits were reported by several workers in 1963 and 1964 (166, 167-170). Aside from their optimum substrate concentration, K_m values, reactivities towards coenzyme analogues and turnover number previously mentioned, the concept of such a functional role is supported by the differential repression of synthesis of H subunits under conditions of increasing oxygen tension (143, 166, 171, 174). This suggested functional role as well as this differential repression of synthesis, may be reflected in the fact that the proportion of M subunits are higher in these tissues that function by means of an aerobic metabolism.

Thus, it can be summarized on the basis of structural characteristics that the lactate dehydrogenase may be divided into two types; the H type and M type. The structural distinctions between these two basic types extended to their amino acid compositions, to their primary sequences as measured by comparative finger print patterns and immunological cross reactions, and to their temperature stabilities. In spite of the

marked differences between the two types of LDH, subunits are similar in molecular weight and shape. They possess the identical active site sulfhydryl peptide, and they can associate with LDH subunits from a wide variety of species into the hybrid forms of active LDH. The H and M subunits of lactate dehydrogenase show distinctive catalytic properties. The M type are adapted to function at higher substrate concentration than are the H types. The synthesis of M type is stimulated by low oxygen tension, whereas the synthesis of H subunits is not affected in this manner. The M type of LDH predominates in tissues which operate in a relatively anaerobic condition.

(2) The lactate dehydrogenase isozymes in the developmental animals.

It is clear that the genetic potential is realized through the processes of cellular differentiation. Each tissue has its own characteristic pattern of isozymes and this pattern changes during embryonic development until the adult configuration is reached. Embryonic tissues, depending upon the one selected, may possess either more or fewer isozymes than corresponding adult tissues, and the relative amounts of the isozymes also vary in accord with the stage of development. This tissue specificity of isozyme pattern may be based on populations of different kinds of cells with each single cell producing only a single LDH isozyme or alternatively a single cell may produce several isozymes. No evidence is yet available to distinguish critically between these alternatives but the second one seems more probable in view of the observation that embryonic pig heart contains a larger number of isozymes than the adult heart (⁴²). Moreover, the relative proportions of the various isozymes in embryonic and adult tissues is conspicuously different. If changing cell populations are to account for changing isozymic patterns, then these shifts in cell populations would have to be much greater than direct observations of embryonic development would suggest. More plausible is hypothesis that the enzymatic pattern of a tissue reflects the state of differentiation of cells. If isozymic multiplicity is viewed as a function of cellular differentiation rather than as a rigid expression of genetically specified multiple enzyme-forming sites, then several hypothesis may be considered (⁴²). During the course of cellular metabolism the same enzyme-forming site may produce a variety of

closely related but distinguishable protein molecules depending upon the raw material made available to it, or perhaps the protein-forming mechanism itself is subject to slight structural variations with a consequent variation in its products (/42). After an enzyme molecule has been synthesized, it is integrated into the structure of the cell. Various modes of attachment within the cell may account for changes in reactive groups on the molecule, thus changing the net molecular charge and hence the electrophoretic mobility of the molecule. Electrophoretic mobility is a function of molecular size as well as charge and the presence of several isozymes might be interpreted as reflecting merely different aggregates or polymers of a single molecular species.

A comparison of the isozymic pattern of embryonic tissues with homologous adult tissues were shown by Markert et al (/42). It is apparent that the final adult pattern is reached by both gains and losses in the isozyme repertory of embryonic tissues. Embryonic heart, an active functioning organ at this embryonic stage, contains a larger number of LDH isozymes than adult pig heart. Skeletal embryonic muscle, on the other hand, contains fewer isozymes than adult muscle. Particularly, the single isozyme that migrate toward the cathode at pH.8.6 and is characteristic of adult skeletal muscle is not present at this embryonic stage. Other differences between embryonic adult tissue, particularly in the relative enzymatic activity disposed in each isozymic band, are the changes in numbers of bands. Characteristics of several embryonic pig tissues is the presence of an isozyme at position lll. Curiously, this enzyme later disappears in adult pig tissues although adult tissues of other species retain an isozyme at this position (/42).

In 1962 Markert and Ursprung (1962) described that the lactate dehydrogenase was shown to exist as five electrophoretically distinct molecular varieties, or isozymes, within tissues of the mouse. Nearly every tissue or organ of the adult mouse contains all five isozymes of LDH, but in proportions that are highly specific for the tissue. The pattern of isozyme distribution in embryonic tissues differs from the adult pattern. Most embryonic tissues initially contain principally LDH-5, as development proceeds LDH activity is gradually transformed to LDH-1. The LDH isozymes differ in kinetic properties, for example, optimal substrate concentration; these differences apparently enable the isozymes to fulfill distinct metabolic roles in cellular metabolism. LDH-5 is more abundant in tissues subject to relative anoxia, and the isozymes at the other end of the spectrum are more abundant in highly oxygenated tissues. The isozyme patterns in the mouse are satisfactorily explained by the previously elaborated subunit hypothesis as mentioned before, which pictures the LDH molecule as composed of four polypeptide subunits that may be separated into two distinct varieties, A and B, each presumably under the control of a separate gene. Assortment of these subunits in all possible combinations of four makes the five isozymes of LDH and the relative proportions of A and B synthesized by a cell would determine the tissue-specific patterns of LDH isozymes either in the adult or during the course of embryonic or neonatal development (1962).

This investigation (1962) clearly demonstrates the tissue and ontogenetic specificity of LDH isozyme patterns in the mouse. Such specificity implies that the different isozymes have different roles to play in the metabolism of the cell. Moreover, the changing needs of differentiating

cells are met by rapid alterations in the relative rates of synthesis of the isozymes so that the synthetic mechanisms of the cell are also clearly not random products of the synthetic process, nor can they be artifacts of the analytical procedure used to reveal them. Although, tissues exhibit remarkable specificity in their patterns of isozymes, it is obvious that this specificity is based upon the relative amounts, rather than upon the presence or absence, of particular isozymes. Nearly every tissue, in fact, contains all five isozymes in measurable amounts. LDH-5 is the most abundant in many of the tissues that were tested, but each isozyme is the principal one in some tissue (170). These observations are obviously inconsistent with general conception of a tissue-specific enzyme. What is specific is the pattern or relative abundance of several isozymes, each of which probably fulfills its own characteristic function in the cell metabolism. This function, of course, involves the same catalytic activity, and hence all these isozymes are forms of the enzyme, lactate dehydrogenase, but this general classification does not exclude the possibility that individual isozymes may differ somewhat kinetic, or other properties. In fact, some difference is essential, if isozymes are to play the distinctive role implied by the specificity of their distribution (172).

The subunit hypothesis also explains the shift in isozyme patterns during ontogeny in terms of differential function of the two hypothetical genes for subunits A (M) and B (H) (145). It is assumed that gene A is first active in the early embryo and gives rise to LDH-5 (A^4B^0). Later, gene B becomes increasingly active and as its contribution to the subunit pool increases, a progressive shift in isozyme pattern occurs until LDH-1 (A^0B^4) becomes the most abundant isozyme. By adjusting the relative

contributions of the genes for the A and B polypeptides most of the isozyme patterns can be generated by simply assuming a random assortment of these polypeptides in groups of four. For example, the patterns for the kidney and heart would be obtained if subunit B were three or four times as abundant as A. The pattern for skeletal muscle would require about twenty-five times as many A chains as B. Tongue would contain about equal quantities of A and B. Deviations from randomly generated patterns (diaphragm, for example) can be explained by assuming mixtures of distinct cell types, each contributing its own quite different pattern to the tissue homogenate. This basic control mechanism needsto regulate only the relative rates of production of two polypeptides (A and B) within each cell in order to generate all the isozyme patterns observed in mouse tissues, either during development or in the adult (145).

It seems reasonable, even necessary, to assume that each isozyme plays a distinct metabolic role. This can be achieved if the physicochemical differences which distinguish isozymes also confer on them either topographic specificity within the cell or distinct kinetic properties in discharging catalytic functions. Both conditions may be true. It is important to realize that the pyruvate $\rightleftharpoons$ lactate reaction is accompanied by a corresponding $\text{NADH}_2 \rightleftharpoons \text{NAD}$ oxidoreduction. Lactate is essentially a hydrogen storage reservoir which is needed during periods of relative anaerobiosis, but the pyruvate $\rightleftharpoons$ lactate reaction can also be used to regulate the ratio of NADH_2/NAD . This ratio is important in controlling the rate of many different reactions in the cell, and it may well be that the regulation of this ratio in different metabolic pathways is the principal function of the isozymes which exhibit different kinetic properties. Kaplan and his

collaborators (193, 195) have demonstrated that the different molecular forms of LDH have different reaction rates for NAD analogs and different substrate optima for pyruvate. This latter observation has been confirmed by Lindsay (194) for chicken LDH isozymes and by Markert and his associates for the mouse isozymes. The latter reported that the catalytic activity of LDH-1 compared to LDH-5 is relatively reduced at higher pyruvate concentrations (190). The other isozymes, LDH-2, LDH-3 and 4 show intermediate properties, so that the whole spectrum of isozymes composes a regular gradient from LDH-1 to LDH-5. The relative abundance of the different isozymes corresponds with the metabolic requirements of the tissue. Embryonic tissues in general and adult skeletal muscle, for example, function under conditions of a relatively low oxygen supply and produce substantial quantities of lactate. Such tissue contains predominantly those isozymes at the LDH-5 (190) end of the spectrum. Highly oxygenated tissues, producing little lactate, such as heart muscle, have most of their LDH activity disposed among isozymes at the LDH-1 end of the spectrum. During periods of high metabolic activity and increased glucose utilization, a larger amount of pyruvate would be produced, and this would normally lead to more lactate. Tissues relying on LDH-1 or LDH-2, however, would be prevented from converting the larger quantities of pyruvate to lactate by virtue of the progressive increase in inhibition of those isozymes by the pyruvate. Thus, in these tissues, lactate production is maintained at a relatively constant low level even in the face of fluctuating pyruvate concentrations. On the other hand, tissues rich in LDH-5 are able to convert larger quantities of pyruvate to lactate to store hydrogen while simultaneously generating needed NAD from NADH₂ (190).

The metabolic utility of each LDH isozyme would seemingly be enhanced if it were located within the cell at the site where its catalytic products were needed. Little direct evidence exists on this point, and the great solubility of LDH makes an examination of the intracellular localization difficult. However, studies of Allen (1975), Nace et al (1976), and Walker and Seligman (1977) all point to the attachment of LDH at specific sites within the cell. It is easy to understand that the charge differences by which one usually identifies isozymes enable them to be loosely bound at the different positions in the cell. Such topographical specificity coupled with the known difference in kinetic properties provides an attractive and plausible explanation for the physiological significance of isozymes in the metabolism of the cell. This explanation, pictures isozymes as carrying on the same general catalytic activity, but at different locations in the cell and as integral parts of distinct metabolic pathways.

Wiggert and Villée subjected the non-particulate fraction of homogenates of heart, liver, kidney, lung and cerebral cortex from human fetuses of varying gestational ages to electrophoresis on starch gel (1978). Five lactate dehydrogenases and two malic dehydrogenases with characteristic rates, in presence of pyridine nucleotide analogs, were found in all tissues at all ages. However, quantitative differences in the relative proportions of these peaks were observed as gestation proceeded. These different ratios could be due to varying amounts of two or more enzymes, each with characteristic APDPN/DPN ratios. In the rat the embryonic form of lactate dehydrogenase is the M form; during development and maturation in rat heart, there is a shift towards the H type (1979). The rabbit and bovine embryonic forms are shown to be the M type of lactate dehydrogenase. In contrast,

the human embryonic type is the H form. Great variation in the composition of the two types of lactate dehydrogenase in the liver of adult mammals as well as in other animals have been found. It was also reported that all of the cells of one animals have the genetic capacity to produce both type of LDH, but their expression is controlled by unknown factors which are associated with the underlying mechanisms involved in differentiation and development of living organisms (49).

Smith and Kissane (49) measured total lactate dehydrogenase activity in dissected portions of the nephron of fetal and adult rat kidney, and the proportion of heart and muscle subunits has been determined by means of the differential inhibition of the two forms by excess pyruvate. The undifferentiated mesenchyme and the glomeruli both have a relatively low total activity and contain principally muscle subunits. Cortical tubules have higher activity and contain chiefly heart subunits. In the adult kidney, the medulla contains approximately equal number of heart and muscle subunits while the LDH of the papilla is almost entirely of the muscle type. Glomeruli of fetal kidney have essentially the same total activity and proportion of the heart and muscle subunits as they do in the adult kidney. During the differentiation of the nephron, the cortical tubule, once formed and recognizable, has the same high total LDH activity and the same proportion of heart subunits as the corresponding structure of the adult kidney. In the whole kidney during development there is a large increase in both total LDH activity of the heart-like subunit fraction. This change is a reflection of the increasing relative mass of cortical tubules as development proceeds (49). Thus, they concluded that the

formation of the proximal tubules from the neck of the glomerulus appears to be accompanied by increased synthesis of H-form subunits, presumably reflecting an increased expression of the gene for this protein. The stimulus for this phenomenon accompanying the histologic differentiation is not known.

In 1964 Hinks and Masters reported that developmental progressions of ruminant lactate dehydrogenase have been studied in terms of electrophoretic patterns, substrate-activity ratios, and the percentage of subunits types present. The evidence demonstrated that early embryonic form in bovine tissues is predominantly "A" (H type), and the two genes controlling the synthesis of lactate dehydrogenase may both be active in the newly fertilized ovum (180). Possibly, the nature of the initial enzymic forms is related to the environment during the gestation period (178). While the subsequent developmental progress of lactate dehydrogenase may still be visualized as an expression of cellular differentiation correlated with enzyme repression and derepression and the different functional significance of the multiple-enzyme forms (181-182), it is evident that such detailed investigation is necessary in order to ascertain the exact nature of interspecies variation with regard to these enzymic changes during gestation.

(3) Methods for the determination of lactate dehydrogenase isozymes.

1. The separation methods.

Chromatography on ion-exchange cellulose derivatives, as developed by Peterson and Sober, is a method that has been used to purify many enzymes from various sources (183). Soluble proteins of liver, brain, and other organs have been successfully fractionated by modifications of this method and the variety and resolution of which have been demonstrated by the localisation of enzyme activities as sharp peaks in the chromatograms by Moor et al (184-185).

In 1950, Meister (186) observed that during electrophoretic analysis of crystalline beef heart lactate dehydrogenase in the Tiselius instrument there appeared two components, a faster moving component, which accounted for most of the enzymatic activity, and a slower-moving protein which amounted to 15% of the total protein. Two years later, Neillands (187) noticed that this second protein (called component C) also possessed LDH activity.

In 1956 Wieland et al started (188) an extensive investigation of physical and biochemical differences between LDHs, after having seen that many preparations of LDH from different origins can be resolved into several, up to five, proteins with the activity of LDH, by electrophoresis on paper or in a layer block of starch. Later (189), it was found that minimal amounts of enzyme mixtures can be quickly separated in the electrical field on a foil of acetyl cellulose. The individual enzymes then can be eluted and evaluated quantitatively in an optical test conducted to determine the rate of hydrogenation of pyruvic acid by NADH. Thus they

succeeded in showing that nearly all organs of an animal contain the same number of LDH proteins, usually five. The corresponding components have the same migration rate but are distributed in a manner which is characteristic for each organ. (49) The distribution patterns of LDHs of several rat tissues (48-49) are reported, as mentioned in the previous chapter.

The first biological application of LDH heterogeneity utilizing the technique of starch block electrophoresis was introduced by Vesell and Bearn (91). The electrophoretic method, including chromatography (92-93) and ultracentrifugation (94-95) have subsequently also been successfully employed. However, the starch gel electrophoresis is most widely used for LDH isozymes (92-93, 96-100). This is largely due to the improved power of resolution that starch-gel electrophoresis affords, to the development of convenient histochemical methods for exhibiting lactate dehydrogenase isozymes on the gel, and to the capacity of the gel for separating multiple specimens simultaneously. The extreme sensitivity of the starch gel and its capacity to reveal small differences in electrophoretic mobility have, however, presented certain problems of procedures. For example, the appearance of tailing could not be prevented, also up to eighteen hours of running time was needed to achieve separation.

In 1957, Kohn et al (301) began to use cellulose acetate as a support medium. In this technique, now called zone electrophoresis, the mixture to be separated is applied in a narrow zone on a strip of paper or cellulose acetate support media whose ends dip into a buffer solution. This media is selected to offer little resistance to current and to be inert to the proteins and dyes used in electrophoresis. Gelman Sepraphore III cellulose polyacetate support strips were found to be better for

electrophoresis procedures and preparation of support media is not needed in this method. The pH of the buffer solution is selected so that it will help separation of the proteins in the mixture by favoring migration at both electric poles. When a current is passed through the buffer and support strip, the charged molecules in the protein are pulled toward one of the poles. Each type of molecules in the substance that is being separated moves a certain distance along the strip according to its charge and its molecular weight. The resulting separation consists of bands or zones representing different components of the substance being analyzed. The protein bands are stained with various dyes to aid in the interpretation and in the quantitative analysis.

While paper electrophoresis is still used, cellulose polyacetate Sepraphore 111 affords many advantages over paper and other support media (202). It cuts the running time from 16-24 hours to 1-2 hours and reduces also the time spent in preparation, staining and drying to about one half or one quarter that required for paper and other methods. Because Sepraphore 111 does not absorb protein, it produces clear, distinct separation of bands with no tailing and gives larger number of bands.

2. Preparation of the dye-substrate for the determination of lactate dehydrogenase isozymes.

Markert and Möller (42) were the first to report a dye substrate mixture for the localization of lactate dehydrogenase isozymes, after starch gel electrophoresis. They utilized nitotetrazolium as terminal electron acceptor in a medium containing substrate, cofactor, methylene blue, diaphorase and hydrazine. When Tsao (203) modified this method to

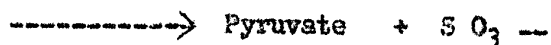
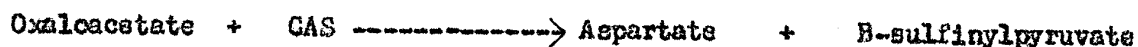
use the same tetrazolium he omitted diaphorase and replaced hydrazine with cyanide. The use of neotetrazolium necess¹⁵ed to overlay with an agar (42) or starch (43) layer to obtain anaerobic conditions and also required long periods of incubation. Dewey and Conklin reported (207) an improved method for demonstration of lactate dehydrogenase after starch gel electrophoresis based on the use of the more sensitive tetrazolium, nitroblue tetrazolium, and substitution of phenazine methosulfate for exogenous diaphorase. In addition, the standard incubating medium was varied in an attempt to characterize more closely the lactate dehydrogenase fractions obtained from homogenates of kidneys of adult rats.

The method by Dewey and Conklin (207) offered distinct advantages over those previously employed by Markert et al (42). The use of nitroblue tetrazolium afforded a greatly reduced incubation time and eliminates the necessity for anaerobiosis. Thus, the method of Dewey and Conklin system does not require use of an agar or starch gel overlay. Cyanide was used as a carboxylic acid trap since hydrazine nonspecifically reduced nitroblue tetrazolium under the conditions employed. However, cyanide was not essential for the reaction to proceed but did facilitate the reaction rate. The first use of phenazine methosulfate was reported by Singer and Kearney (205) and followed by many others. Singer and Kearney proposed that succinate oxidation could be reliably measured in soluble extracts of mitochondria with phenazine methosulfate as an electron carrier (205-206), whereas other dyes commonly used for the assay of succinic dehydrogenase proved to be ineffective. The oxidation was further stimulated by providing a means to pull the reaction by continuous removal of fumarate. This was ineffectively accomplished by the addition of L-cysteine-sulfate

(CSA) and NAD to the extracts resulting in the overall reaction:



Reaction (1) was shown to proceed by the following sequence;



Since this method was modified by Dewey and Conklin for the determination of LDH, detailed explanation of the Singer and Kearney's method is required. By utilizing phenazine methosulfate as a dye to link the dehydrogenase to O_2 and reaction (1) to provide a rapid and continuous removal of fumarate, an assay system was obtained which enabled the authors to show that the succinic dehydrogenase activity of rat liver and beef heart mitochondria is quantitatively extracted from acetone powder by mildly alkaline buffers. In beef mitochondrial extracts phenazine methosulfate cannot be replaced by methylene blue, 2, 6-dichlorophenol-indophenol, or cytochrome C, and ferricyanide has only a fraction of the activity of the phenazine dye as a catalyst of succinate oxidation. In homogenates of rat liver mitochondrial acetone powder methylene blue effectively replaces phenazine methosulfate, but in extracts only the latter accepts electrons from the dehydrogenase. The data (Singer and Kearney) indicate that amongst the dyes tested phenazine methosulfate is the only suitable one for the assay of the primary dehydrogenase and may, therefore, in part, explain

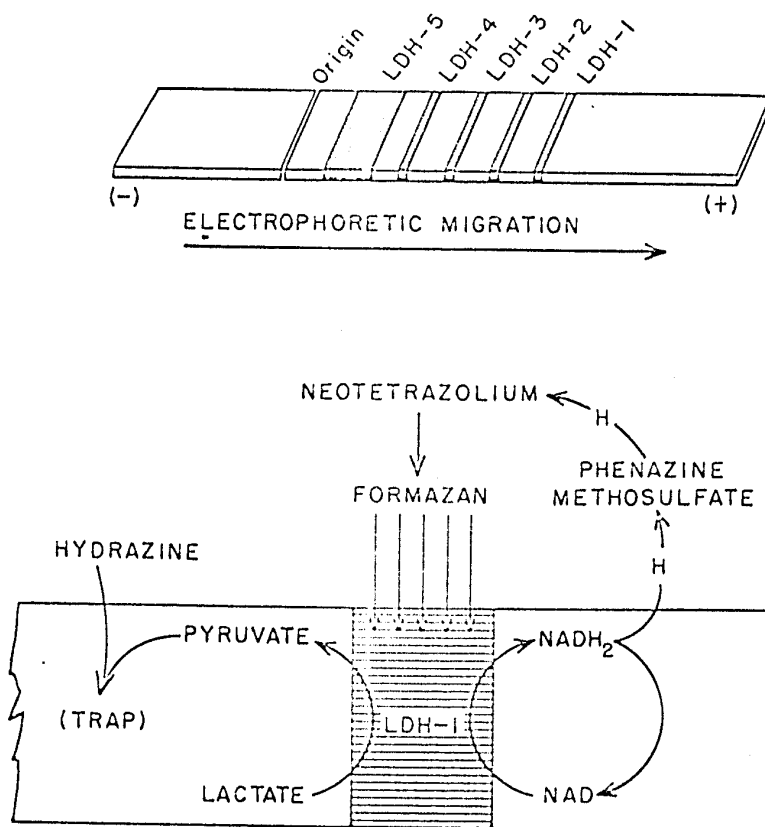
previous apparent failures in solubilization and purification of the enzyme.

When succinate alone (286) is present as the substrate in the soluble preparations, the stoichiometric accumulation of fumarate is readily demonstrated. In a typical experiment the oxygen uptake indicated the disappearance of 3.5 μ M of succinate. After deproteinization, 3.36 μ M of fumarate were determined by means of crystalline fumarase and malic dehydrogenase. The one step oxidation of succinate to fumarate as well as the coupled reaction (1) are competitively inhibited by malonate and oxaloacetate. When succinate alone is the substrate, the rate of O_2 uptake declines after the first five to ten minutes and ceases altogether within a short time. When the reaction is pulled by means of the sequence shown (reaction 1) linearity of O_2 uptake with time is maintained much longer and the oxidation does not come to a halt. In the coupled reaction (1) the initial rate of O_2 uptake is double that observed in the succinate-fumarate step. While the initial mitochondrial extracts contain all of the enzymes required for the conversion of succinate to aspartate, purified preparations of the dehydrogenase require besides CSA and NAD, fumarate, malic dehydrogenase, and transaminase as supplementary enzymes (285-286).

There is good reason to believe that the dehydrogenase discussed above is an essential component of the particulate succinic dehydrogenase system and that, in fact, it is the first enzyme in the electron transport chain from succinate to cytochrome C. The soluble dehydrogenase may be extracted from the particulate preparations of Green et al (287) after treatment with acetone. The kinetics of substrate oxidation, in either the one-step oxidation to fumarate or in the coupled reaction (1) are

identical in the purified soluble and particulate preparations. The main difference between the two systems is that while the soluble enzyme can donate electron efficiently only to phenazine methosulphate, the particulate preparation also reacts readily with ferricyanide, 2,6-dichlorophenol-indophenol, and cytochrome c. Dewey and Conklin (206) and Markert et al (142) modified those methods for the visualization of lactate dehydrogenase isozymes patterns. They proposed following reaction sequence:

Figure 3



The upper part of the figure illustrates a strip containing the five LDH isozymes after electrophoresis. The lower part of the figure is a diagrammatic representation of the reaction occurring during the procedure for visualizing LDH activity on electrophoretic diagram. The formazan which marks the site of enzyme activity is a highly colored insoluble precipitate.

MATERIALS AND EXPERIMENTAL METHODS

(A) Thyroxine and thalidomide and its derivatives on amphibian metamorphosis.

Tadpole of giant bull frog (*Rana catesbeiana*) in premetamorphic stages were supplied by the North Carolina Biological Supply Company and maintained in a water bath at 23° C. The water was changed every two days. Canned boiled spinach was fed as the sole food source. L-thyroxine supplied by Sigma Chemical Company was dissolved in the minimum amount of 1 N NaOH solution and diluted with water to a final concentration of 1 mg/ml. Sufficient amount of this thyroxine solution was added directly to the water bath to give the desired final concentration. The adequate concentration of thyroxine to induce metamorphosis as reported in the literature (208) is 2.6×10^{-8} M. Thalidomide and its derivatives were dissolved in the thyroxine solution already present in the water bath. Sixteen to twenty individual determinations were carried out for each mean value of the content of adrenaline, noradrenaline, glucosamine and the activity of several enzymes reported herein. Materials pooled for four to six tadpoles were used for each different determination.

(B) The assay methods.

- (1) Measurement of the effects of thalidomide and its derivatives on the morphological changes during thyroxine-induced metamorphosis of tadpole.

In all the following experiments, morphological changes of

metamorphosis were quantitatively estimated by measuring the ratio of leg length in mm to tail length in mm and were expressed at L/T ratios. These ratios were found to be readily correlated with the criteria used by Taylor and Kollros (7). The L/T ratio of animals treated only with thyroxine was considered as 100% morphological change.

(2) The determination of proteins.

Protein was determined by the method of Lowry et al (1959).

i) Principle of the method.

This method is based on the color reaction of proteins with phospho-molybdictungstic acid (phenol reagent). The tyrosine molecules of the proteins in solution react with this reagent. When phenol reagent is added to the copper treated protein, a maximum intensity of coloration is produced in alkaline solution.

ii) Reagents.

- (1) Reagent A = 2% Na_2CO_3 in 0.1 N NaOH.
- (2) Reagent B = 1.0% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$.
- (3) Reagent C = 2% Sodium or potassium tartrate.
- (4) Reagent D = 1 N phenol reagent (Fisher Scientific Company, New York).

iii) Procedure.

50 ml of reagent A, 0.5 ml of reagent B and 0.5 ml reagent C

are mixed well immediately before use, 5 ml of this solution is added into 1 ml of protein solution and mixed thoroughly. This mixture is allowed to stand at room temperature for ten minutes and 0.5 ml of 1 N phenol reagent is added quickly into the solution and mixed. After thirty minutes the optical density was read at 700 mμ in a Coleman Spectrophotometer. It was found that (200) one unit of optical density was equivalent to 260 γ of protein/ml as can be seen in Fig. 4. This calibration curve was obtained using bovine albumin as standard protein.

(3) The determination of adrenaline and noradrenaline contents.

Catecholamines were determined by a modification of the Euler and Floding (210) method.

i.) Principle of the method.

Catecholamines from tissues are selectively absorbed on alumina at pH 8 and eluted with 0.5 N acetic acid. This eluted material was further treated with iodine at a weakly acidic pH to form reaction products which in alkali solution form fluorescent derivatives. These are trihydroxyindoles in the case of adrenaline and noradrenaline. The fluorophores of adrenaline and noradrenaline are relatively stable if formed in the presence of a reducing agent such as ascorbic acid (or sodium sulfates). They exhibit relatively high specific fluorescence in the pH range around 3 for adrenaline and pH 6 for noradrenaline. The fluorophore of catecholamines can then be determined with a fluorometer at specific wavelengths of activation and emission.

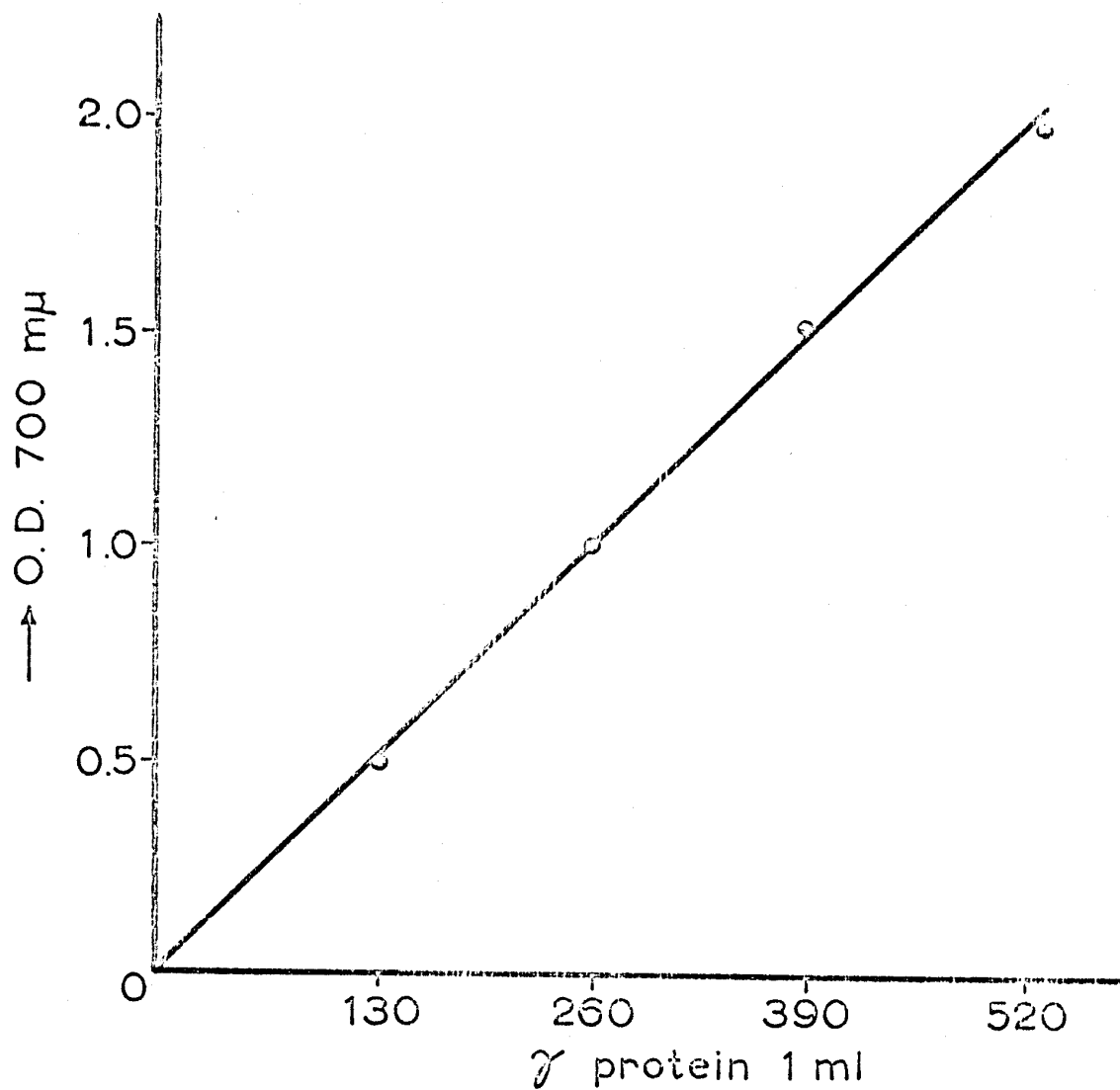


Fig. 4 The relationship between optical density and amount of standard protein.

ii) Reagents.

- (1) 10% Trichloroacetic acid.
- (2) 10% EDTA.
- (3) 1% Phenolphthalein.
- (4) Ascorbic acid.
- (5) Alumina (chromatographic grade).
- (6) 5 N and 2.5 N Sodium hydroxide solution.
- (7) 0.5 N Acetic acid.
- (8) 1 M Acetate buffer, pH 6.
- (9) 0.1 M Glycine buffer, pH 3.
- (10) 0.01 N Iodine solution.
- (11) 0.01 M Nuthiosulfate.
- (12) 1-Adrenaline and 1-noradrenaline (Eastman Organic Chemicals).

iii) Extraction and Adsorption.

The tissues of tadpole were removed quickly and rinsed free of visible blood and blotted on damp filter paper. If the tissue is to be extracted at once, tissues were weighed, washed in H_2O and homogenized with 10% TCA within the next two hours, using glass homogenizer suitable for small volumes. If it were not extracted at once, then tissues were individually wrapped in parafilm with identification tag and were deep-frozen until use. The volume of extract including washing solution did not exceed 15 ml. These were transferred to a 50 ml test tube, and 2 mg ascorbic acid in 1 ml water were added followed by 2.5 ml of 10% EDTA and

1 drop of 1% phenolphthalein. In order to adsorb the amines 0.5 g of alumina was added to the solution. The sample was brought to pH 8 (phenolphthalein end point) by addition of NaOH 5 N and 2.5 N with constant stirring. Sample tubes were stoppered and shaken for ten minutes. If the colour disappeared after two or three minutes the pH was readjusted and the tube was further shaken for another ten minutes.

After alumina had settled, the supernatant was removed by suction with a water pump. The alumina was washed twice with 5 ml aliquots of double-distilled water centrifuged and the supernatant again removed by suction with a water aspirator. Finally the alumina was stirred in presence of 5 ml 0.5 N acetic acid. The tube content was shaken with a mechanical shaker for fifteen minutes and then centrifuged. The supernatant eluate was collected using a Pasteur pipette. The supernatant had to be very clear for the accurate estimation of catecholamine.

iv) Procedure for adrenaline and noradrenaline determination.

1 ml of eluate was added into 2 ml of glycine buffer, mixed well and the final pH was adjusted to pH 2.9. Catecholamines are oxidized by 0.5 ml of 0.01 N iodine solution. At exactly 3 min. 0.5 ml of 0.01 M Nethiosulfate was added to the sample with shaking. The iodochrome was then isomerized to the iodolutin by adding 1.0 ml of 5 N NaOH containing 2 mg of ascorbic acid. This alkaline-ascorbate solution is prepared immediately before the oxidation. After 45 minutes, fluorescence is activated at 410 mμ and the sample was read at 510 mμ against a reagent blank. Noradrenaline was measured by the procedure described for adrenaline except that the acetate buffer, pH 6, was utilized.

v) Calculation.

The following equations were applied for the differential estimation of adrenaline and noradrenaline.

$$(1) \text{ Formula for noradrenaline } \frac{100 \times C}{N \times D} - \left(\frac{H \times 100}{N \times D} \right) \frac{A}{M}$$

$$(2) \text{ Formula for adrenaline } \frac{100 \times A}{F \times B}$$

where, A is fluorescence of sample at pH 3.

B is fluorescence of adrenaline standard at pH 3.

C is fluorescence of sample at pH 6.

D is fluorescence of noradrenaline standard at pH 6.

F is percentage of adrenaline oxidized at pH 3.

H is fluorescence of adrenaline standard at pH 6.

N is percentage of noradrenaline oxidized at pH 6.

M is concentration of adrenaline in the cuvette at pH 3.

(L) The determination of glucosamine contents.

Glucosamine was measured by the Elson-Morgan's modified method of Mylie and Johnson (211).

1) Principle of the method.

The production of colour in this method depends upon two

successive reactions. The formation of an intermediate compound, a glucosazone (2/2-213), formed by heating the acetylglucosamine with alkali, and the reaction of this intermediate with dimethylaminobenzaldehyde for the development of colour in an acidic medium.

ii) Reagents.

- (1) 2N HCl solution.
- (2) Dowex-50 W (H+).
- (3) 0.5 N HCl solution.
- (4) Condensing reagent (2N carbonate-bicarbonate buffer, pH 9.9 with 5% acetylacetone added immediately before use).
- (5) Stock color reagent (1.5% p-dimethylaminobenzaldehyde (British Drug House Ltd.) in a mixture of 35 ml 95% ethanol and 65 ml concentrated HCl).
- (6) 30 µg/ml glucosamine standard solution. (Nutritional Biochemicals Corp.).
- (7) 95% ethanol.
- (8) 2 N carbonate-bicarbonate buffer, pH 9.9.

iii) The preparation of hydrolysates.

Samples were hydrolyzed with 2 N HCl for three hours. The hydrolysate was dried in vacuo, the residue was extracted several times with small amounts of boiling water, filtered, and the filtrate was placed on a 12x40 mm column of Dowex-50 (H+). The column was washed with water and glucosamine was eluted with 20 ml of 0.5 N HCl; the eluate was

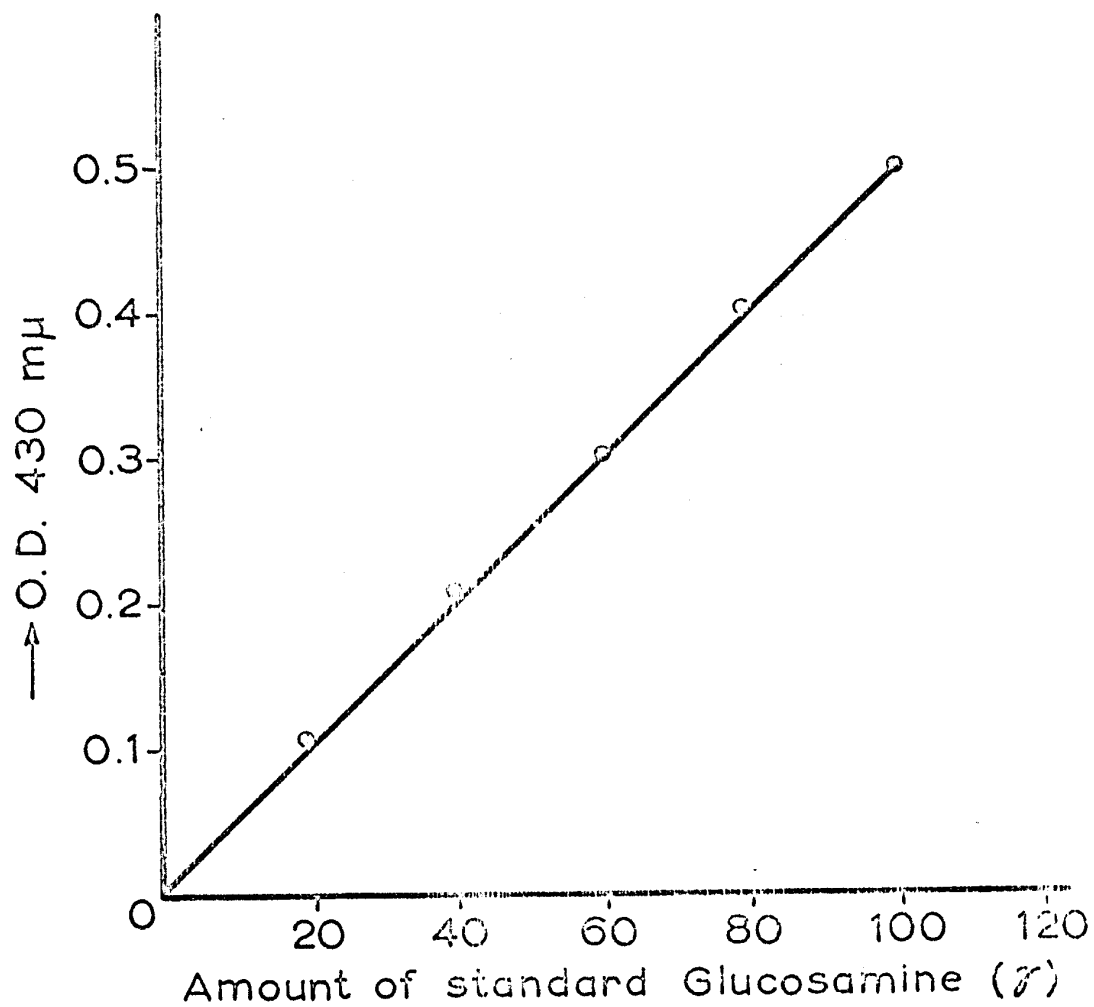


Fig. 5. The standard calibration curve for glucosamine content determination.

dried in vacuo and redissolved in double distilled water for the determination of glucosamine content.

iv) Colorimetric procedures.

1 ml of samples containing 4 to 40 μ g of glucosamine in 18 x 150 mm tubes were mixed with 1 ml of condensing reagent; the tubes were heated for twenty minutes in a boiling water bath, cooled, and 10 ml of a mixture of 2 parts stock color reagent and 8 parts 95% ethanol were added. Absorbance was determined at 430 m μ after five minutes against a reagent blank in a Coleman spectrophotometer. A 1 ml solution standard of glucosamine was carried through the above procedure and constituted the standard for every determination.

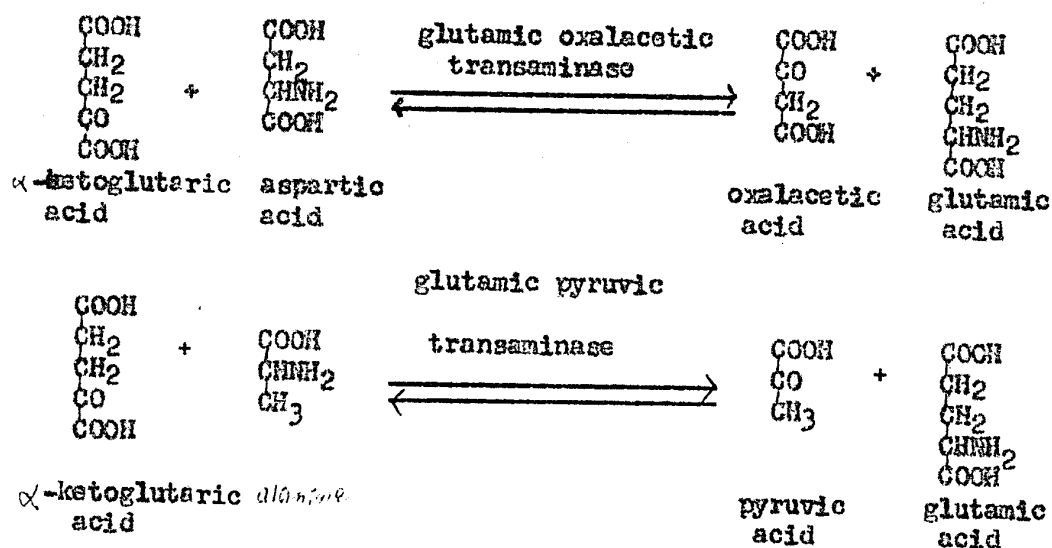
v) Calculation.

The following equation was applied for the calculation on the content of the glucosamine. $\frac{C \times B}{A} \times D \times F$, where, A is O.D. of standard glucosamine, B is reading of O.D. of unknown, C is concentration of standard glucosamine, D is the dilution factor, and F is the rate of recovery. As can be seen in Fig. ⁵, the relationships between the concentration of glucosamine and the optical density are directly proportional. However, while preparing the hydrolysates, a small amount of glucosamine (in the tissues) was lost during the preparation of hydrolysates. The per cent recovery is rather critical for the calculation.

(5) The determination of glutamic-oxalacetic transaminase activity.

i) Principle of the method.

Both glutamic-oxalacetic transaminase activity and glutamic-pyruvic transaminase activity can be measured by this method. The reactions involved are as follows:



The method is based on the advantage derived from the great differences in the absorption of an alkaline solution of the 2,4-dinitrophenylhydrazones of α -ketoglutarate, oxalacetate, and pyruvate at 505 m μ (Fig. 6). With an increase in pyruvate or oxalacetate, there is a concurrent decrease in α -ketoglutarate, the resulting increase in optical density is proportional to the amount of pyruvate or oxalacetate produced (Fig. 6). Mathematically, this is expressed as

$$D = C_p E_p - C_{ak} E_{ak}$$

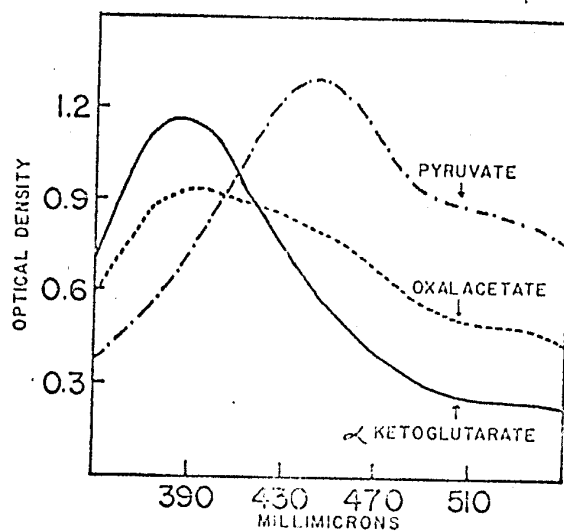
where D is the increase in optical density, C_p is the concentration of the product which is pyruvate in the case of GPT, and a mixture of

oxalacetate and pyruvate (by breakdown of oxalacetate) in the case of GOT. E_p is the extinction of the hydrazone from the resulting product and E_{ak} is the extinction of the hydrazone of α -ketoglutarate. Moreover, since ΔD is proportional to the yield, and the yield is a function of transaminase activity, then

$$\text{Transaminase activity} = f \Delta D$$

A method designed to determine optimal condition for the procedure revealed that optimal colour production was observed when 1 μ mole of 2,4,-dinitrophenylhydrazine was used (in a volume of 12.2 ml). 2,4,-Dinitrophenylhydrazine reacts with the α -keto acids of the substrate as well as of the products. For this reason the keto acids produced had to represent a significant percentage of the total keto acids. For the assay of transaminase a production of less than 1 μ mole of keto acid fulfilled this requirement under the present condition. In as much as the rate of transamination is dependent upon the concentration of α -ketoglutarate in the specified range, the maximum of 2 micromoles was selected. Maximal stability of the colour was obtained by the use of 0.4 N sodium hydroxide (10 ml for each tube). One of the most important facts in the present method is that pyruvate, instead of oxalacetate, was used for the standard curve because it is known that oxalacetate is unstable in this medium and converted to pyruvate..

Fig. 6 Absorption spectra of equimolar alkaline solutions of the 2,4-dinitrophenylhydrazones of α -ketoglutarate, oxalacetate, and pyruvate.



ii) Reagents.

- (1) 0.1 M Phosphate buffer, pH 7.4.
- (2) 2 mM Pyruvate.
- (3) 2 mM α -ketoglutarate and 200 mM d,l,-aspartate.
- (4) 1 mM 2,4-dinitrophenylhydrazine.
- (5) 0.4 N Sodium hydroxide solution.

iii) Procedures.

Tadpole liver, tail and rest of the body were weighed and homogenized in 20 volumes of 0.1 M phosphate buffer, pH 7.4 in an ice bath, and the homogenate was diluted ten times with the buffer. One ml

of the GOT substrate is pipetted into each test tube, and the tube was placed in a water-bath metabolic shaker at 38°C for ten minutes. Following addition of 0.2 ml of tissue homogenate to the tubes, the contents were mixed, and after an incubation period of exactly sixty minutes, the tubes were removed from the water bath. One milliliter of 2,4-dinitrophenylhydrazine reagent was immediately added into each tube, thereby stopping the reaction. After the tubes were allowed to stand at room temperature for a minimum of twenty minutes, 10 milliliters of 0.4 N sodium hydroxide solution were added, and the contents was mixed well. At the end of thirty minutes, the optical density of the solution was measured at 505 mμ, using water as the blank. While the assays were incubated, a control for each sample was also prepared. For the control preparation, one ml of the substrate, 0.2 ml of each sample, and 1 ml of 2,4-dinitrophenylhydrazine reagent are mixed well in a test tube. After a minimum of twenty minutes, 10 ml of 0.4 N sodium hydroxide solution are added, and the optical density of the solution is measured as described previously.

iii) Calculation.

The procedure as described above expressed the values as the change in optical density. These values were plotted against the activity of transaminase in the tissue, as measured by the spectrophotometric method. Activity of transaminase was correlated with micromoles of pyruvate in the prepared standard (Fig. 7). Preparation of a standard curve for pyruvate permits the conversion of the change in optical density into micromoles

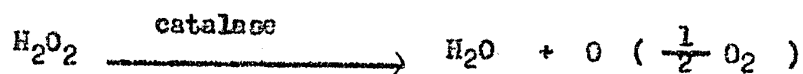
of pyruvate; the standard curve is prepared by mixing known amounts of pyruvate, such as 0.2, 0.4, 0.6, 0.8, and 1.0 micromoles, with the hydrazine reagent and alkali solution as described above. As illustrated in Fig. 7, the change in optical density is plotted against micromoles of pyruvate. The activity of transaminase was expressed in micromoles of converted pyruvate per hour per mg protein.

(6) The determination of catalase activity.

Catalase activity was determined by the method of Morgulis and Rabkin (215).

1) Principle of the method.

Catalytic reaction of catalase is greatly favoured at low temperature. Because, the destruction of the catalase by the peroxide is accelerated at high temperature, thus leading to rapid cessation of the catalytic reaction. The destruction of the catalase is an oxidation phenomenon (215). Greater catalytic activity in the cold is, therefore, due to less enzyme destruction through oxidation reaction. At 2°C such destruction either does not occur or is very negligible. The enzymatic reaction is as follows:



The oxygen produced by catalase can be titrated with KMnO_4 to a point of permanent rose color at 2°C (usually run in the ice bath).

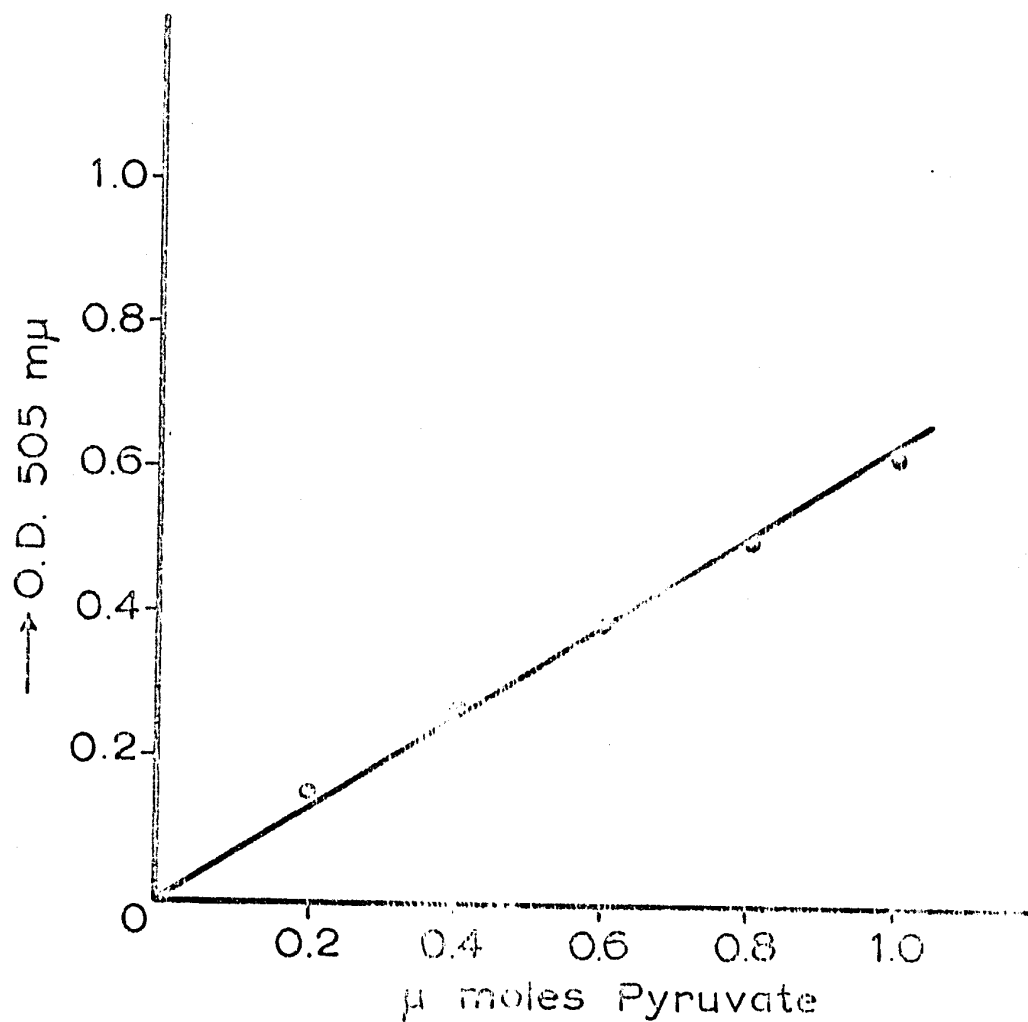


Fig. 7 Changes in optical density associated with an increase in concentration of pyruvate, as measured by their 2,4-dinitrophenylhydrazonol.

ii) Reagents.

- (1) M/15 Na_2HPO_4 solution.
- (2) M/15 KH_2PO_4 solution.
- (3) M/150 H_2O_2 substrate solution.
- (4) 0.05 N KMnO_4 standard solution (Fisher Scientific Company Ltd).
- (5) 20% H_2SO_4 solution.
- (6) 0.2 M phosphate buffer pH 7.2.

iii) Procedures.

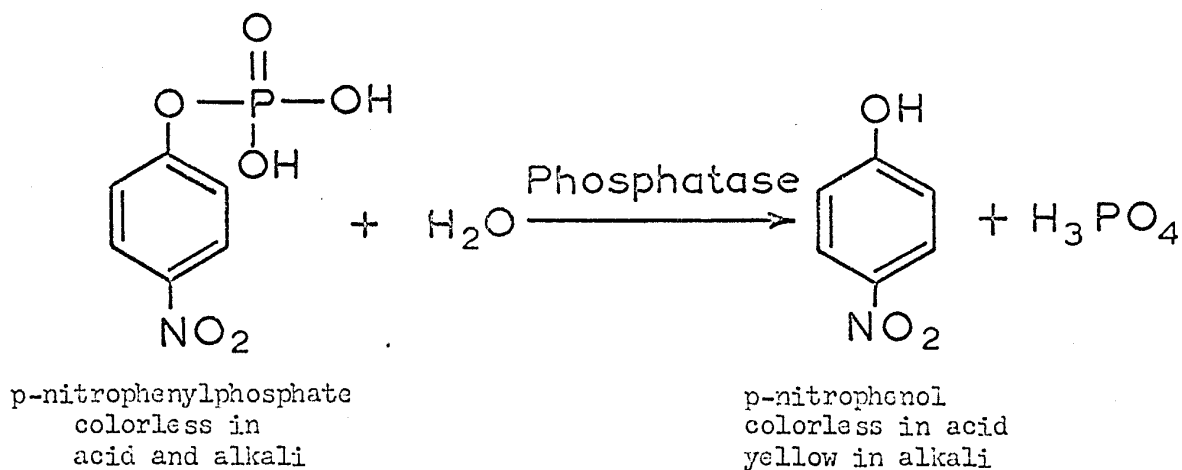
Tadpole liver was weighed in 20 volumes of 0.2 M phosphate buffer at pH 7.2. 90 ml of M/150 H_2O_2 solution and 10 ml of a mixture of equal parts of M/15 Na_2HPO_4 and M/15 KH_2PO_4 were put into three 250 ml flasks and the flasks were placed in an ice bath at 2°C. At zero time the following reagents were added to the various flasks: flask 1, 1 ml of enzyme solution, flask 2, 1 ml of 0.2 M phosphate buffer, flask 3, no addition. After ten minutes of incubation at 2°C, 20% sulfuric acid was added to each flask to stop the reaction. The contents of the flasks were titrated with 0.05 N KMnO_4 to a point of permanent rose color. One ml of 0.05 N KMnO_4 represents 0.0004 g or 0.28 ml of available oxygen.

(7) Determination of alkaline phosphatase activity.

The activity of alkaline phosphatase was determined by a modification of the Sigma procedure (1954).

i) Principle of the method.

The compound, p-nitrophenyl phosphate, is colorless, but hydrolysis of the phosphate group liberates the yellow salt of p-nitrophenol (absorption maximum, 400 mμ). Thus, the substrate is itself an indicator of the amount of phosphate which is split off and hence is a measure of phosphatase activity, as indicated by the following reaction:



The tissue homogenate is incubated with the buffered substrate, the reaction stopped by the addition of alkali, and the amount of color developed is measured without further treatment.

ii) Reagents.

- (1) Alkaline buffer solution; Glycine buffer pH 10.5
(stored in refrigerator, stable at least 6 to 12 months).
- (2) Stock substrate solution. Dissolve 100 mg p-nitrophenyl phosphate in 25 ml of water. This solution in a stoppered container can be frozen and is stable for 4 to 6 weeks.
(Sigma Chemical Company).

- (3) p-Nitrophenol standard solution; 10.0 ml per liter, in water solution when protected from light and kept in the cold is stable for about one year. (Sigma Chemical Company).
- (4) Borate buffer at pH 9.2.
- (5) 0.02 N sodium hydroxide solution.
- (6) Concentrated hydrochloric acid.
- (7) 0.2 M phosphate buffer, pH 7.2.

iii) Procedure.

Tadpole liver, tail, and rest of the body were homogenized in 10 volumes of 0.2 M phosphate buffer at 7.2 and the homogenate was diluted in 10 volumes of 0.2 M phosphate buffer pH 7.2. 0.5 ml alkaline buffer solution and 0.5 ml of stock substrate solution were pipetted into each of two tubes and placed in a water bath at 38° C. After five minutes temperature equilibration, 0.1 ml of water was added into one tube and 0.1 ml of enzyme (sample) solution into the other tube. The tube content is mixed gently and quickly replaced into the water bath. Exactly thirty minutes after addition of the enzyme, 10 ml of NaOH solution was added into the tubes, and the contents of tubes are mixed well. Thus, the enzyme action was stopped and colour was developed by the addition of 0.02 N NaOH solution (in alkaline medium, p-nitrophenol solutions became yellow). This colour was stable for several hours. The optical density was read at 410 mμ against a reagent blank. (Reading A). This value is corrected by subtracting from it the optical density of the endogenous blank. The

endogeneous blank value is obtained by adding to all tubes 0.1 ml concentrated HCl following reading A. This procedure fades the yellow colour of p-nitrophenol and only leaves the endogeneous colour due to reagents and tissues. This second reading B is also carried out at 410 mμ against a reagent blank. The difference between reading A and B is alkaline phosphatase activity and provides a standard curve illustrated in Figure 9.

iv) The preparation of the standard calibration curve.

0.5 ml of p-nitrophenol standard solution was pipetted into 100 ml volumetric flask and made up to volume with 0.02 N NaOH. The content was mixed well and the amount of standard solution indicated in columns 2 and 3 of the following table was pipetted into six tubes.

Composition of tubes for calibration curve

(1)	(2)	(3)	(4)
Tube Nos.	ml of st. sol.	ml of NaOH	Units
1	1.0	10.0	1.0
2	2.0	9.0	2.0
3	4.0	7.0	4.0
4	6.0	5.0	6.0
5	8.0	3.0	8.0
6	10.0	1.0	10.0

For each of these six tubes the optical density was read at 410 mμ using 0.02 N NaOH as a blank. The standard calibration curve using the above standard solutions is illustrated in Figure 9 .

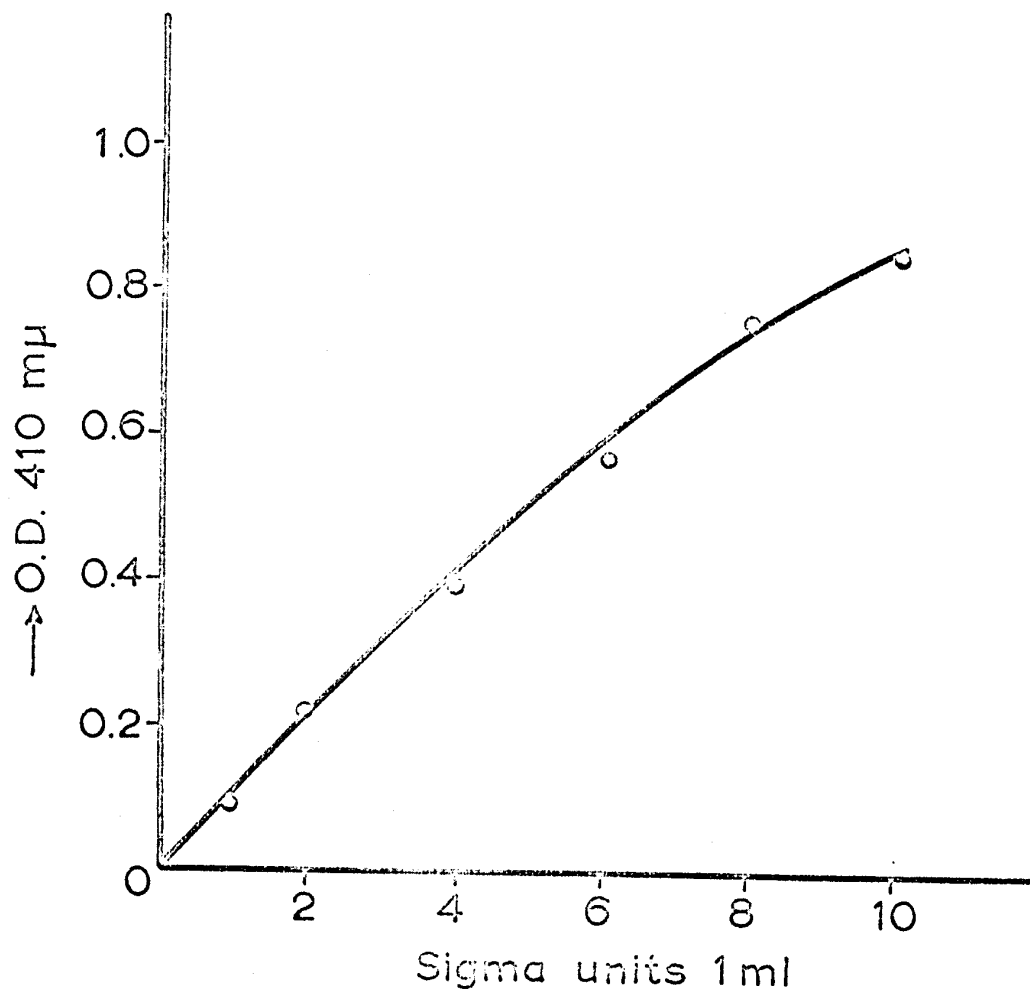


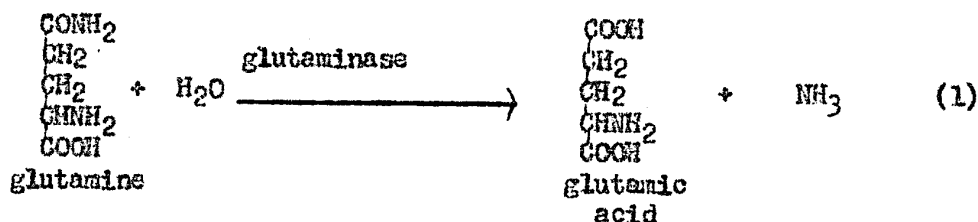
Fig. 9 The standard calibration curve for alkaline phosphatase activity measurement.

(8) Determination of glutaminase activity.

The activity of glutaminase was determined by the method of Seligson (218).

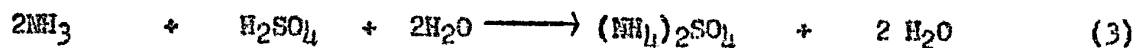
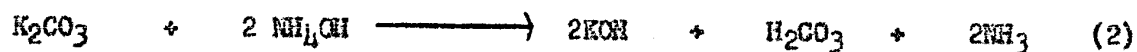
i) Principle of the method.

This method is based on the determination of the rate of ammonia liberated from substrate (L-glutamine) by glutaminase. The reaction scheme can be illustrated as follows:



The ammonia formed is separated by diffusion method, and trapped as $(\text{NH}_4)_2\text{SO}_4$ and determined by Nesslerization.

The reaction are as follows:



ii) Reagents.

- (1) Tris buffer pH 7.2 (Tris aminomethane).
- (2) D,L-glutamine (Fisher Scientific Company Ltd.).
- (3) Saturated potassium carbonate solution.

- (4) Standard ammonium solution containing 100 µg per milliliter (0.4716g of $(\text{NH}_4)_2\text{SO}_4$ per liter).
- (5) 1N H_2SO_4
- (6) Nessler's solution diluted 1:10 before use. (Fisher Scientific Company Ltd.).

iii) Procedure.

The tadpole tissues were excised placed in aluminum foil containers in ice, were then rinsed free of visible blood with tris buffer and blotted on filter paper. Tissues were rapidly homogenized with tris buffer. One ml of glutamine substrate solution is introduced into 30 ml bottles and placed in a water bath at 38° C and shaken for ten minutes. 0.2 ml of homogenate was pipetted into each bottle, the content was mixed well and was incubated sixty minutes with shaking. After sixty minutes incubation, the bottles were removed from the water bath and one milliliter of saturated potassium carbonate was added into each bottle immediately, thereby stopping the reaction. The bottles are immediately stoppered with a stopper which carries through it a glass rod the end of which is spoon-shaped. Before stoppering 0.3 ml of 1N H_2SO_4 has been deposited in the spoon. The bottles are then shaken mechanically for two hours thus permitting evolution of ammonia which is trapped in the solution of sulfuric acid.

The contents of the spoon is collected and the spoon is rinsed with water; both rinsings and original collection are pooled and made up to constant volume and reacted with 10 ml of dilute Nessler's reagent.

After standing five minutes at room temperature, the optical density was measured in a Coleman Spectrophotometer, at 420 mμ. The blank was prepared by replacing the sample with distilled water and Nesslerizing the diffusate. The standard were carried out by the same procedures as samples by replacing the sample with ammonium sulfate solution standard.

iv) Preparation of a standard curve.

In Figure 10, curve A illustrates the relationship between color intensity and quantity of ammonia, ranging from 5 to 30 μg per 10 milliliters of final solution. In curve B, the same quantities of ammonia were isolated by diffusion. It can be seen that the recovery by diffusion method is almost complete.

(9) An investigation on lactate dehydrogenase isozymes during thyroxine-induced metamorphosis of tadpole and study of the effect of thalidomide.

The total LDH activity was measured with Bausch and Lomb Spectronic 505 automatic recording Spectrophotometer following the appearance of NADH (19). The electrophoretic separation was carried out by the method described in "Manual 51199" (Gelman Instrument Company) (22) and the quantitative analysis for each isozyme pattern was carried out by an improved colorimetric method described below.

i) Reagents.

- (1) 0.2 M phosphate buffer at pH 7.2.
- (2) 0.1 M phosphate buffer at pH 7.2.

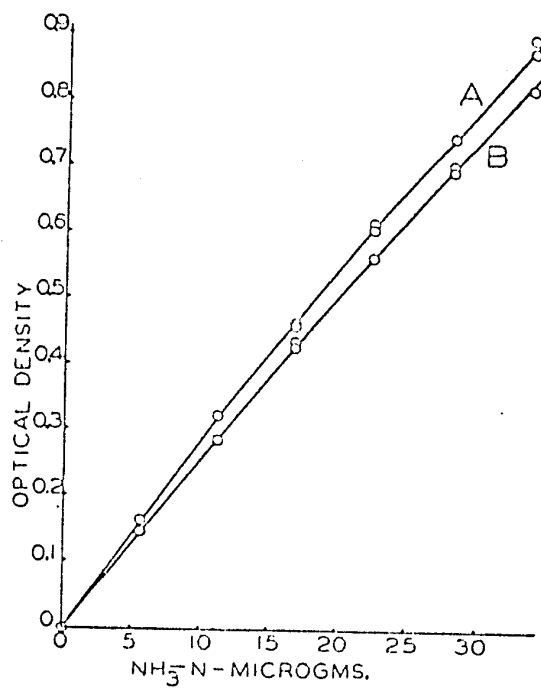


Fig. 10 Relationship between O.D. and the amount of $(\text{NH}_4)_2\text{SO}_4$.

A-curve obtained by Nesslerization of ammonium sulfate standard.

B-curve obtained by Nesslerization of ammonium sulfate trapped following two hour diffusion.

- (3) 0.02 NAD solution at pH 6.0 (Nutritional Biochemical Corporation).
 - (4) 0.1 M glycine- NaOH buffer at pH 10.0.
 - (5) Standard enzyme solution, 5.0 μ moles/ml. (Sigma Chemical Company).
 - (6) 1 M sodium lactate solution (Sigma Chemical Company).
 - (7) NAD 20 mg/20 ml. (prepared immediately before using) (Nutritional Biochemical Corporation).
 - (8) 0.1 M NaOH solution.
 - (9) 0.005 M HgCl_2 solution.
 - (10) 0.5 M phosphate buffer, pH 7.4.
 - (11) Nitroblue-tetrazolium, 1mg/ml (Nutritional Biochemical Corporation).
 - (12) Phenazine methosulfate, 1mg/ml (Nutritional Biochemical Corporation).
 - (13) 5% Acetic acid.
 - (14) Ionic strength 0.05 M Barbitol buffer, pH 8.6 (Fisher Scientific Company Ltd.).
 - (15) N-N-Dimethyl Formamide and benzene solvent (1:1) (Fisher Scientific Company).
 - (16) Absolute ethanol.
- ii) The measurement of the total activity of the lactate dehydrogenase.

Tadpole liver, tail and brain were homogenized in 2 to 4 volumes of 0.2 M phosphate buffer at pH 7.2 and the homogenate was

centrifuged at 15,000g for 1 hour. The clear supernatant was taken for the following enzyme assay. The total LDH activity was determined with a Spectronic 505 automatic recording Spectrophotometer following the appearance of NADH (219). A thermoregulator was used to maintain the temperature of the cell compartment at 28° C. 1.8 ml of glycine buffer, 0.1 ml of lactate solution and 0.1 ml of NAD solution are introduced into a cuvette, the contents stirred and allowed to stand for five minutes in order to let the temperature equilibrate with that of the cell compartment. A blank was similarly prepared without the addition of NAD. 0.02 ml of the supernatant is now added into the cuvette and the optical density is recorded at 340 mμ. The optical density change 0.03 at 340 mμ corresponds to the formation of 9.7 millimicromoles of NADH in 2 ml of the test solution per minute. Specific activity is expressed as micromoles NADH converted per minute.

iii) The separation of lactate dehydrogenase isozymes by cellulose polyacetate membrane electrophoresis.

Tissues were homogenized in 2 to 4 volumes of 0.2 M of phosphate buffer at pH 7.2 and the homogenate was centrifuged at 15,000 g for one hour. For the separation of the LDH isozymes of tadpole liver, tail, and brain, it was found desirable to apply 0.005 ml of the clear supernatant on the cellulose polyacetate membrane (Sepraphore 111, Gelman Co.) for the electrophoresis. Electrophoretic separation of LDH isozymes were carried out by the method described (222) at 200 volts. Owing to the fact that the tadpole LDH isozymes moved very slowly, the

electrophoresis was carried out for 4 hours at 23° C (room temperature) instead of the usual 1 hour (202). While electrophoresis was carried out, the following dye-substrate solution mixture was prepared.

1 M sodium lactate	2 ml
20 mg NAD	2 ml
0.1 M NaOH	2 ml
0.005 M MgCl ₂	2 ml
0.5 M phosphate buffer, at pH 7.4	5 ml
5 mg Nitroblue-tetrazolium	5 ml
0.5 mg Phenazine methosulfate	0.5 ml
Total volume	18.5 ml

A strip of Sepraphore 111 was soaked for five minutes in the above dye-substrate solution, it was then layered over the membrane which had been subjected to electrophoretic separation. The areas of enzyme activity appear as purple and there is diffusion of the coloured bands on the dye-substrate carrying membrane.

iv) Colorimetric assay procedures.

After dying of strips, the individual bands were cut out from both membranes. Each pair of pieces were put into a tube which contained 4 ml of a mixture in equal volume of N-N-dimethylformamide and benzene. The test tube was shaken in order to facilitate the dissolution of the membrane and 0.5 ml of absolute alcohol (ethanol) was added after the membrane was completely dissolved. In order to

secure enough material for accurate colorimetric estimation, separation was conducted on 4 to 6 strips at once and all identical isozyme bands pooled. The content of the test tube was transferred to a colorimetric tube (12X100 mm) and the optical density was read at 540 mμ against the mixture of N-N-dimethyl-formamide, benzene and absolute ethanol as a blank. The electrophoretic membrane had to be completely dry if complete solution was to be achieved.

Figure 11 illustrates the unfractionated total lactate dehydrogenase activity determined by the rate of appearance of NADH at 340 mμ (determined by automatic Spectrophotometer on abscissa versus the enzyme activity, as measured by dissolving the Sepraphore 111 strips and the coloured isozymes, on ordinate. It can be seen that there is a good linear relationship between the activities measured by the two methods. It was found that the same amount of the enzyme which gave an O.D. (at 540 mμ) of 0.14 by the present dissolving method caused a 0.13 (at 340 mμ) O.D. change per minute by the usual automatic recording performed for one minute. Thus the sensitivities of the two methods are quite comparable. It is also seen that intensity of the colour after dissolving the electrophoretic membrane increased as time elapsed and it was found that after 24 hours the colour reached its maximum intensity. Even though the colour intensity increased according to time, the relationship between the amount of the enzyme and optical density remained proportional. In the following series of experiments, the optical density of the colour was measured one hour after dissolving the membranes.

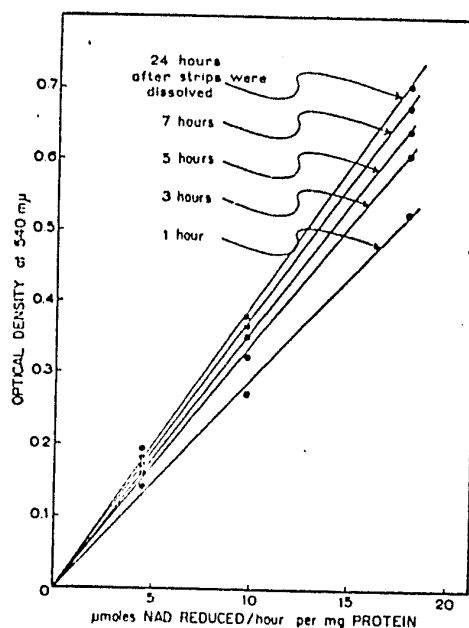


Fig. 11 Stability of color developed on and dissolved from electrophoretic membranes. The enzymatic activity represented on the abscissa was determined with fresh enzyme preparation by the appearance of NADH at 340 mμ by a spectrophotometric method; and the enzymatic activity presented on the ordinate, by the new method with identical aliquots from the same enzyme preparation.

RESULTS

- (1) The effect of thalidomide and its derivatives on the morphological changes during thyroxine-induced metamorphosis of tadpole.

In all of these experiments, morphological changes of metamorphosis were quantitatively estimated by measuring the ratio of leg length in mm to tail length in mm and expressed throughout as L/T. This ratio was found to be readily correlated with the criteria used by Taylor and Kollros (7). Inhibition was measured by considering the L/T ratio of animals treated only with thyroxine as 100%.

As shown in Figure 12 thalidomide at a concentration of $10^{-4}M$ inhibits the growth of hind legs and resorption of tail during thyroxine-induced tadpole metamorphosis. The inhibition became evident only after five consecutive days of treatment with thalidomide. This latent period is followed by the appearance of morphological change. If the treatment was carried on for sixteen days with thalidomide at $10^{-4}M$, the percentage of inhibition of L/T ratio was approximately 40%.

Table 1 lists percentage of inhibition of thalidomide and its derivatives on L/T ratio during thyroxine-induced metamorphosis of the tadpole. Thalidomide at the concentration of $1 \times 10^{-4} M$ inhibits the change of L/T as mentioned above. Further increase in thalidomide concentration does not increase the degree of inhibition. When thalidomide is hydrolyzed in vitro, possibly also in vivo, and one ring is opened, this will give rise to N-phthalyl-D,L-glutamic acid, N-phthalyl-D,L-

glutamine, and N-phthalyl-D,L-isoglutamine. If both rings are opened, N-(O-carboxybenzoyl)-D,L-glutamic acid, N-(O-carboxybenzoyl)-D,L-glutamine, and N-(O-carboxybenzoyl)-D,L-isoglutamine are formed. Very recently, Williams (1960) reported that 15 metabolites were found in the urine of rabbit, rat and man previously treated with thalidomide. Twelve of these have been identified after they were isolated in crystalline form by column chromatography on alumina. Those identified are as follows: α - (N-phthalyl)-isoglutamine, α - (N-phthalyl) - glutamine, α - (N-phthalyl) - glutamic acid, α - (O-carboxyl) - amino acid, α - (O-carboxybenzoyl) - isoglutamine, α - (O-carboxybenzoyl) - glutamine, α - (O-carboxybenzoyl) - glutamic acid, α - aminoglutarimide, phthalic acid, glutamine, isoglutamine, and glutamic acid. Some of the above compounds or other closely related derivatives were tested for their effect on the L/T ratio, and the results are also presented in Table 1. It is seen that 3-hydroxyphthalyl-D,L-glutamine is the most potent inhibitor among the compounds tested and it inhibits approximately 50% at the concentration of 5×10^{-5} M. This concentration is the lowest ever reported to be effective in biological systems: for example, 0.5 to 2.7 mg/ml thalidomide in the case of protozoa (22) and 10 mg thalidomide per Kg body weight in monkey (9). The other compounds tested in Table 1 have rather high inhibiting potency of the L/T ratio. In general, it can be concluded that the derivatives of thalidomide tested are more potent than the mother compound in inhibiting the morphological changes occurring during thyroxine-induced metamorphosis of tadpole.

In Figure 13 the duration of treatment with thalidomide is plotted against the percentage of inhibition of L/T ratio as observed

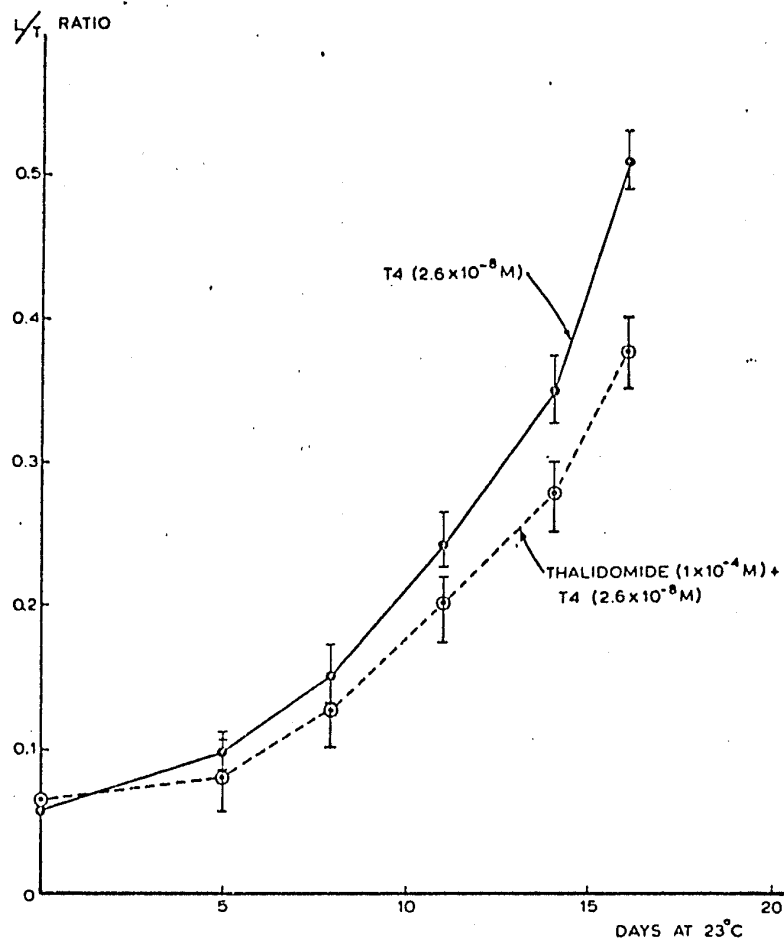


Fig. 12. The effect of thalidomide on the ratio of leg length to tail length. The ratio of the hind leg length (mm) and tail length (mm) is plotted against the number of days of treatment. Closed circles are for the control group (thyroxine-treated). Double circles are for thalidomide plus thyroxine.

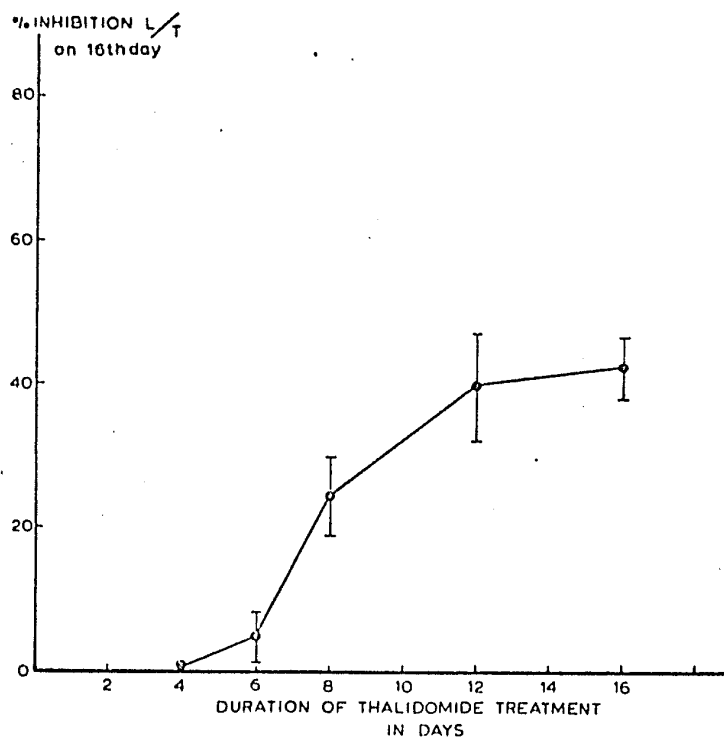


Fig. 13. The influence of duration of thalidomide treatment on thyroxine-induced metamorphosis. The L/T ratio in every case represents the final development at the end of 16 days. The abscissa represents the number of days during which the tadpoles were treated with thalidomide, 5×10^{-4} M.

TABLE 1

Inhibitory effect of thalidomide and its derivatives on
thyroxine-induced metamorphosis of the tadpole

Compound	Concentration used	% of Inhibition of L/T ratio
Thalidomide	5×10^{-4} M	43.0±8.3
	1×10^{-4} M	41.3±8.8
	5×10^{-5} M	26.3±7.4
	5×10^{-6} M	16.2±6.6
	1×10^{-6} M	7.2±6.8
	5×10^{-7} M	0.
3-OH-Phthalyl-D,L-glutamine	5×10^{-5} M	48.0±10.4
	5×10^{-6} M	26.7±7.6
	1×10^{-6} M	9.0±6.6
	5×10^{-7} M	0.
4-OH-Phthalyl-D,L-glutamine	5×10^{-5} M	39.3±9.2
	5×10^{-6} M	23.1±6.1
	1×10^{-6} M	7.3±6.7
Phthalyl-D,L-glutamins	5×10^{-5} M	36.7±8.6
	5×10^{-6} M	23.4±8.0
	1×10^{-6} M	13.7±5.4
Phthalyl-D,L-glutamic acid	5×10^{-5} M	32.7±7.4
	5×10^{-6} M	15.4±7.1
	1×10^{-6} M	8.6±6.3

on the 16th day of thyroxine-induced metamorphosis of tadpole. Animals were treated with thalidomide for the number of days indicated and the solution in the water bath was then changed to contain only thyroxine up to a period of 16 days, when the L/T ratio of all animals was measured. As seen in the figure, an 8-day treatment was necessary for the observable inhibition to occur, while a 12-day treatment gave nearly full inhibition.

- (2) The effect of 3-hydroxyphthalylglutamine on the glutamic oxalacetic transaminase activity during thyroxine-induced metamorphosis of tadpole.

The activities of glutamic-oxalacetic transaminase in the liver, tail and rest of the body during thyroxine-induced metamorphosis of tadpole are shown in Table II. It can be seen that significant differences were observed between the thyroxine-treated group and thyroxine plus 3-hydroxyphthalyl glutamine. Chan and Cohen recently reported that thyroxine treatment increases glutamic-oxalacetic transaminase activity in tadpole liver (22). Similar results were obtained in our experiments (Table II). When 3-hydroxyphthalylglutamine was added together with thyroxine, a slight but significant inhibition occurred in all tissues. This result thus supports the contention that thalidomide and its hydrolytic metabolites might act as an antimetabolite for glutamic acid as mentioned earlier in the introduction. However, whether these compounds act^{at} the level of biosynthesis of the enzyme or

TABLE II

The specific activity of glutamic-oxaloacetate transaminase in the tadpole liver, tail, and body (whole body without liver and tail) versus the number of days of exposure to thyroxine and thyroxine plus 3-hydroxyphthalylglutamine

Duration of exposure	Thyroxine			Thyroxine plus 3-OH-phthalylglutamine		
	Liver	Tail	Body	Liver	Tail	Body
1 day	6.2±0.6	5.2±1.1	4.0±1.3	6.5±0.9	5.3±1.2	4.5±1.1
5 days	6.3±1.1	5.7±0.9	4.7±0.8	6.2±1.2	5.2±0.9	4.3±0.8
10 days	8.2±0.6	6.5±0.9	5.2±0.8	8.1±1.2	6.3±1.1	5.1±0.8
13 days	10.4±1.2	7.3±0.9	5.6±0.8	9.2±1.1	6.8±0.9	4.9±0.6
16 days	12.4±1.39	9.4±1.1	7.1±0.9	10.3±1.2	8.1±0.9	6.0±0.7

The specific activity of glutamic-oxaloacetate transaminase of tadpole liver, tail, and body is expressed in micromoles converted per hour per mg protein. The concentration of 3-OH-phthalylglutamine was 5×10^{-6} M. Each value represents the mean of 8 to 12 experiments.

the level of the enzymic activity is not yet certain until more detailed investigation is carried out.

(3) Effect of 3-hydroxyphthalylglutamine on the liver catalase activity during thyroxine-induced metamorphosis of tadpole.

Effects of thalidomide on the liver catalase during thyroxine-induced metamorphosis of tadpole are shown in the Fig. 14 . The specific activity of the liver catalase is plotted against the number of the days of exposure to thyroxine and thyroxine plus 3-hydroxyphthalylglutamine. Thyroxine treatment increases liver catalase activity of tadpole, confirming the results obtained previously (208). Also illustrated in the figure is the fact that 3-hydroxyphthalylglutamine inhibits the increase of catalase activity caused by thyroxine treatment. Even though the implication of this finding in the term of mechanism of thalidomide is not clear at present, the fact that inhibitory action of this drug on the thyroxine-induced metamorphosis of the tadpole is selective will become evident from the data presented.

(4) Effect of 3-hydroxyphthalylglutamine on the alkaline phosphatase activity during thyroxine-induced metamorphosis of tadpole.

The effects of 3-hydroxyphthalylglutamine on the liver alkaline phosphatase are shown in Table III. During thyroxine-induced metamorphosis, alkaline phosphatase activity in the liver, tail and "residual body"

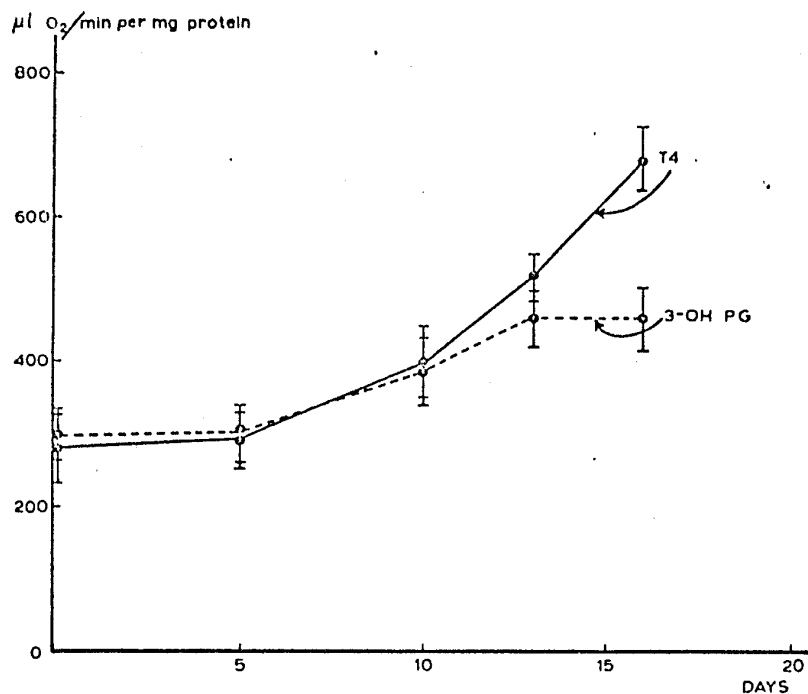


Fig. 14. The effect of 3-hydroxyphthalylglutamine, 5×10^{-4} M, on the specific activity of tadpole liver catalase. The specific activity of the tadpole liver catalase, in $\mu\text{l O}_2/\text{mg protein}$, is plotted against the number of days of exposure to thyroxine and thyroxine plus 3-hydroxyphthalylglutamine.

TABLE 111

The specific activity of alkaline phosphatase in the tadpole liver, tail, and body (whole body without liver and tail) versus the number of days of exposure to thyroxine and thyroxine plus 3-hydroxyphthalylglutamine

Duration of exposure	Thyroxine			Thyroxine plus 3-hydroxyphthalylglutamine		
	Liver	Tail	Body	Liver	Tail	Body
1 day	3.8±0.3	0.6±0.2	1.8±0.3	3.6±0.2	0.6±0.4	1.7±0.2
5 days	3.7±0.6	0.6±0.2	1.8±0.4	3.6±0.5	0.6±0.2	1.8±0.3
10 days	4.4±0.6	0.8±0.2	1.9±0.3	4.6±0.8	0.7±0.2	1.8±0.3
13 days	6.1±0.6	1.2±0.3	2.2±0.4	5.4±0.6	0.9±0.2	2.0±0.4
16 days	8.1±0.9	1.6±0.3	2.9±0.7	6.9±0.6	1.2±0.3	2.4±0.6

The specific activity of alkaline phosphatase is expressed in Sigma Units per hour per mg protein. The concentration of 3-OH-phthalylglutamine was 5×10^{-5} M. Each value represents the mean of 5 to 9 experiments.

increases. However, simultaneous presence of 3-hydroxyphthalylglutamine with thyroxine inhibits this increase slightly but significantly. The magnitude of the inhibition in the case of alkaline phosphatase activity by this derivative of thalidomide is rather small.

- (5) Effect of thalidomide on the liver glutaminase activity during thyroxine-induced metamorphosis of the tadpole.

The specific activities of the liver glutaminase versus the number of days of exposure to thyroxine and thyroxine plus thalidomide, are shown in Fig. 15 . During thyroxine-induced metamorphosis, liver glutaminase activity of tadpole increases about fourfold. However, simultaneous treatment with thalidomide reduces this increase to about twofold. This result is very similar to the observation made by Fabro et al (1964), that one of the metabolites of thalidomide, 2-(0-carboxybenzamide)-glutaric acid, was found to inhibit the activity of glutamine synthetase, glutamate decarboxylase, and glutamate dehydrogenase of rat brain. The concentration of 1×10^{-3} M of thalidomide was used to inhibit 50% of the activities of these enzymes. These suggested that thalidomide and its derivatives in vivo might act as antimetabolites of glutamic acid and glutamine.

- (6) Effect of 3-hydroxyphthalylglutamine on the adrenaline and noradrenaline contents during thyroxine-induced metamorphosis of tadpole.

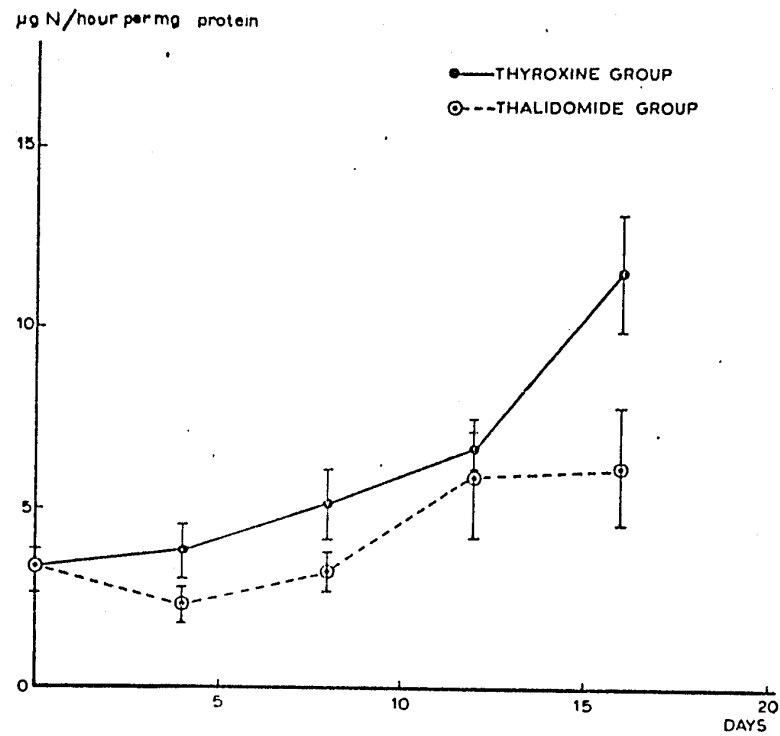


Fig. 15• The specific activity of the liver glutaminase vs. the number of days of treatment with thyroxine and thalidomide, 10^{-4} M.

Fig. 16 shows changes of the contents of kidney adrenaline and noradrenaline with thyroxine treated and thyroxine plus 3-hydroxyphthalylglutamine treated tadpole. Fig. 17 shows the changes of the contents of brain adrenaline and noradrenaline during thyroxine treated and thyroxine plus 3-hydroxyphthalylglutamine treated tadpole. The adrenaline and noradrenaline contents are plotted against the days of exposure.

Thyroxine treatment does not change the contents of these catecholamines in the case of organs to any significant degree. Furthermore, simultaneous treatment with 3-hydroxyphthalylglutamine does not bring any significant change from that observed in the presence of thyroxine alone. This might indicate that catecholamines do not play any direct important role in the differentiation of tadpole.

(7) Effect of thalidomide on the liver glucosamine content during thyroxine-induced metamorphosis of tadpole.

Since glucosamine is part of the biologically important series of aminated polysaccharides (chitin, heparin, hyaluronic acid, etc.), it was felt that the investigation on the amount of this compound in tadpole liver during metamorphosis might be interesting, particularly in the presence of thalidomide.

The contents of liver glucosamine are plotted against the number of days of exposure in Fig. 18. As seen in this figure, thyroxine treatment increases the content of glucosamine in the liver of tadpole

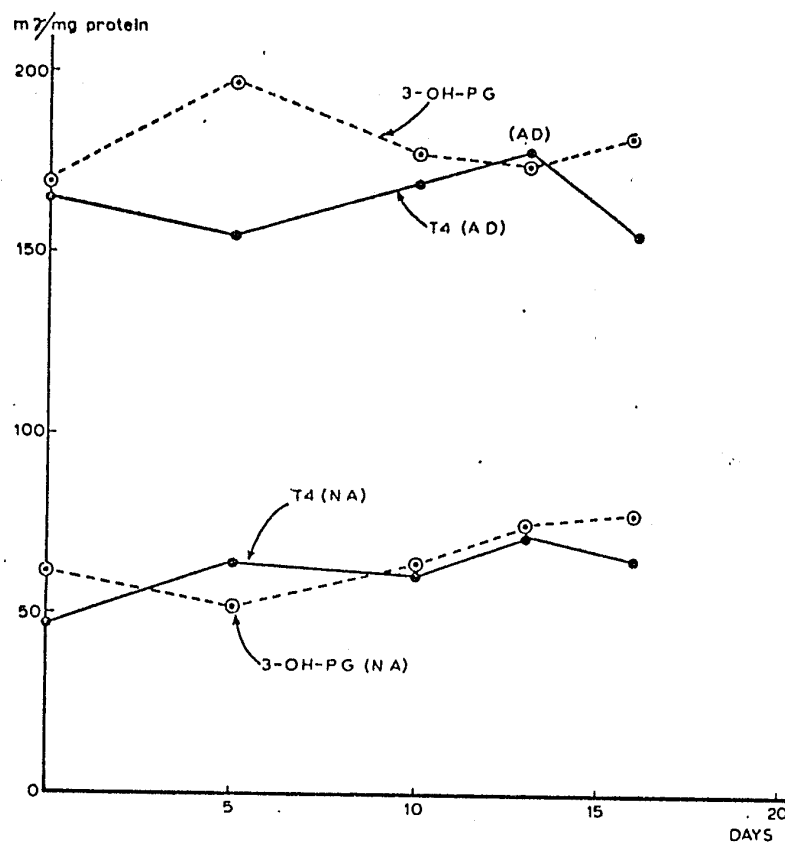


Fig. 16. The influence of 3-OH-phthalylglutamine, 5×10^{-6} M, on the changes in the adrenaline and noradrenaline contents of the tadpole kidney. AD, Adrenaline; NA, noradrenaline; 3-OH-PG, 3-hydroxyphthalylglutamine; and T_4 , Thyroxine.

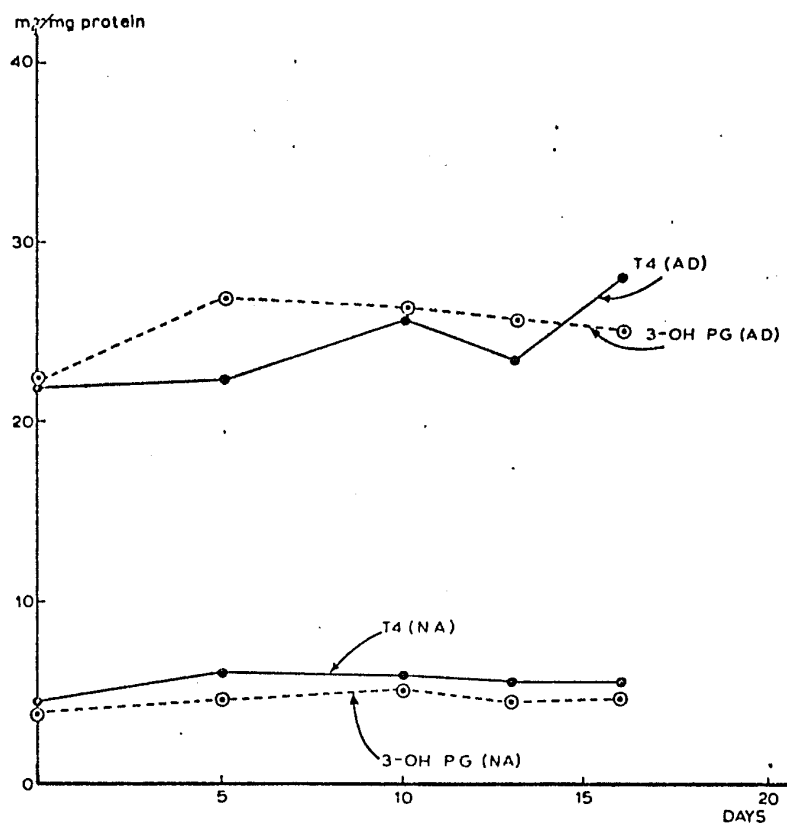


Fig. 17. The influence of 3-hydroxyphthalylglutamine, 5×10^{-6} M, on the changes in the contents of the brain adrenaline and noradrenaline of the tadpole.

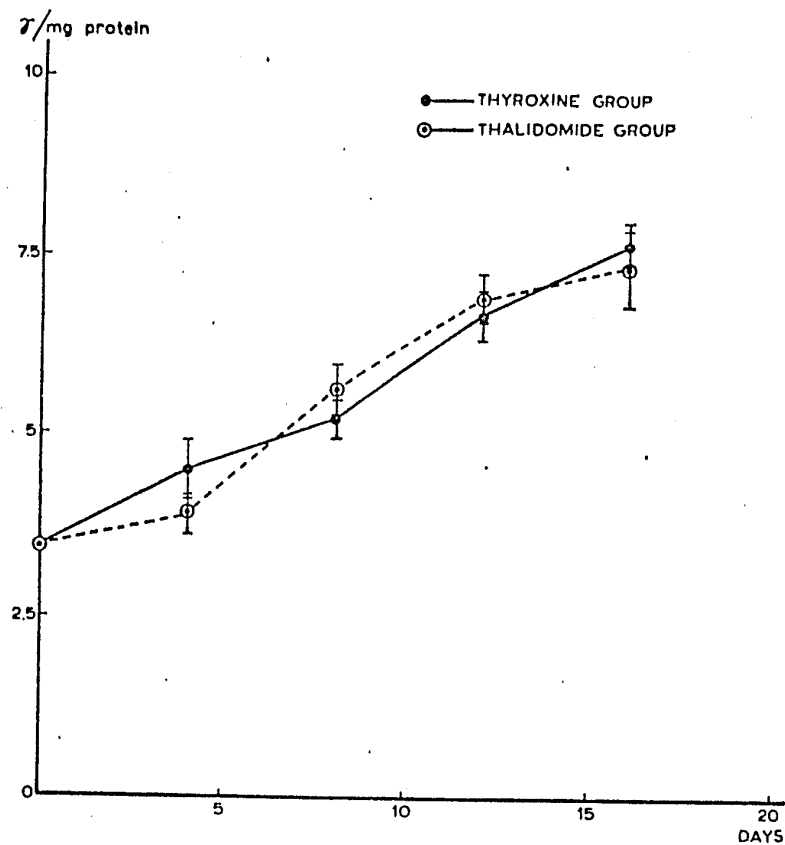


Fig. 18. Influence of thalidomide, 10^{-4} M, on the liver glucosamine. The contents of tadpole liver glucosamine are plotted against the number of the days of treatment.

approximately twofold. However, thalidomide does not affect the change brought about by thyroxine alone.

(8) The activity of lactate dehydrogenase isozymes during thyroxine-induced metamorphosis of tadpole.

(1) Effect of thalidomide on the total lactate dehydrogenase activity during thyroxine-induced metamorphosis of tadpole.

It was reported by Degroot (223) that thyroxine treatment of tadpole decreased the liver total lactate dehydrogenase activity. The results illustrated in Fig. 19 show the effects of thalidomide on the liver total lactate dehydrogenase activity during thyroxine-induced metamorphosis of tadpole. The data confirm the previous findings (224) and further indicate that thalidomide exerts no significant effects on the decrease of lactate dehydrogenase. Practically similar results were observed with tadpole tail and brain. Since thalidomide has been shown to inhibit, selectively, some of the biochemical and morphological changes of thyroxine-induced tadpole metamorphosis (224), lactate dehydrogenase activity constitutes an example of one of the activities not affected by thalidomide treatment.

(2) The lactate dehydrogenase isozymes patterns of various tadpole tissues.

When electrophoresis of the supernatant of tadpole liver homogenate was carried out as usual (222), no separation into the isozymes

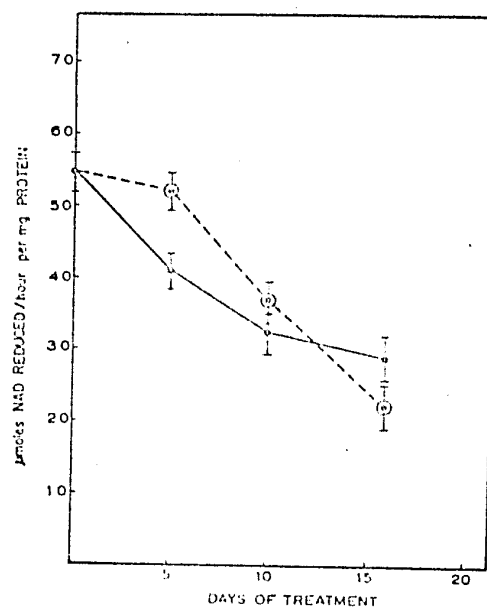


Fig. 19. Specific activity of tadpole liver lactate dehydrogenase vs. number of days of treatment with 2.6×10^{-6} M thyroxine (○), and 2.6×10^{-6} M thyroxine plus 5×10^{-4} M thalidomide (⊙).

was obtained. However, with tail and brain the separation was very good, as shown in Fig. 20 . Thus, it is quite possible to examine quantitatively the pattern of each isozyme during thyroxine-induced tadpole metamorphosis. The results obtained are listed in Table ^{IV} and Table V . First of all, it can be seen that the values obtained by the present method are reproducible in all cases. Furthermore, the results in both Tables confirm the previous observation that thyroxine or thyroxine plus thalidomide treatment decreased the lactate dehydrogenase in both tail and brain of tadpoles. However, close examination of the results indicate that the decrease in the total lactate dehydrogenase activity is not due to the overall decrease in the individual isozyme activity. That is, thyroxine treatment decreased only the F3 isozyme but not the other isozymes. The relative amounts of isozyme F1 and F2 rather increased. Moreover, it can be seen that simultaneous treatment with thalidomide and thyroxine produces changes similar to those observed with thyroxine treatment alone.

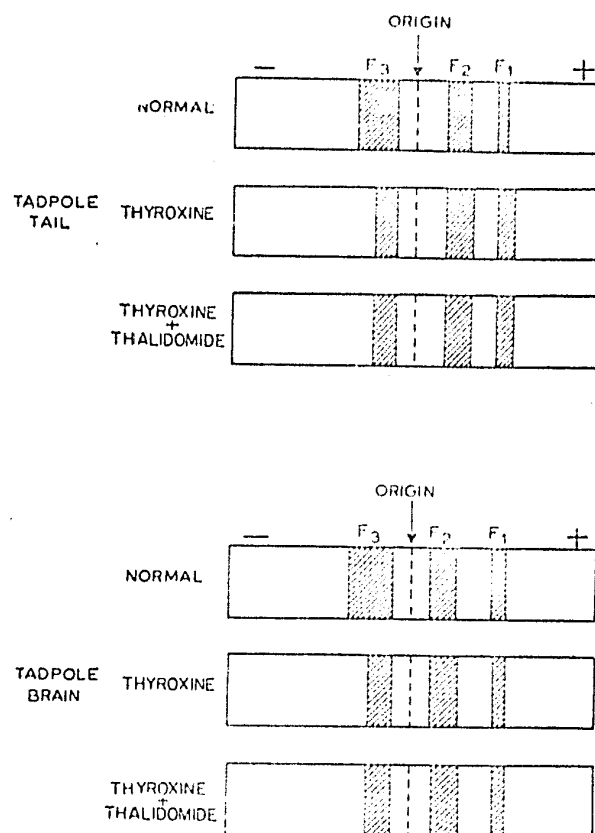


Fig. 20 Electrophoretic separation patterns of LDH isozymes from tail and brain of normal tadpole, and tadpole treated with thyroxine and thyroxine plus thalidomide (concentrations of thyroxine and thalidomide the same as given in Fig. 19).

TABLE IV

Per Cent of the Various Lactate Dehydrogenase Isozymes of Tadpole Brain

Frac- tion No. of Exp.	NORMAL				T ₄ *			T ₄ + THA *				
	Total ** Activity	Per cent			Total ** Activity	Per cent			Total ** Activity	Per cent		
		F3	F2	F1		F3	F2	F1		F3	F2	F1
1a	6.8	47.92	40.87	11.19	4.1	33.45	49.76	16.78	3.8	32.88	50.10	17.01
1b	6.1	48.41	37.74	13.84	3.2	34.21	49.09	16.68	3.1	34.53	47.23	17.12
1c	5.9	48.90	38.63	10.45	3.6	33.87	49.32	16.80	3.6	33.86	47.52	18.61
2a	6.2	48.69	38.60	12.69	4.1	31.53	50.56	17.89	3.8	31.77	49.37	18.85
2b	5.6	49.12	38.46	12.41	3.2	31.83	50.58	17.58	3.1	35.63	46.81	17.55
2c	5.6	50.50	37.44	12.05	3.6	31.28	51.47	17.24	3.6	30.72	51.85	17.42
3a	5.8	50.70	37.17	11.11	4.1	31.84	50.72	17.43	3.8	30.53	51.13	18.32
3b	6.3	48.47	38.95	12.57	3.2	30.88	52.17	14.94	3.1	32.11	50.31	17.57
3c	5.8	49.20	37.89	12.89	3.6	30.67	52.55	16.76	3.6	29.02	53.12	17.85
Mean and S.D.	6.01 ± 0.88	49.10 ± 0.77	38.41 ± 1.0	12.46 ± 0.74	3.63 ± 0.45	32.17 ± 1.6	50.69 ± 1.1	17.12 ± 1.4	3.56 ± 0.23	32.00 ± 1.2	50.04 ± 0.3	17.82 ± 0.7

* Tadpoles were treated with thyroxine (2.6×10^{-8} M) or thyroxine (2.6×10^{-8} M) and thalidomide (5×10^{-4} M) for 16 days.

** values had reduced/hr/mg/protein.

a, b and c represent 3 aliquots of one pool from 10 tadpoles.

TABLE V
Per Cent of the Various Lactate Dehydrogenase Isozymes of Tadpole Tail

No. of Exo.	Frac- tion	NORMAL			T _L *			T _L + THA *					
		Total ## Activity	Per cent			Total ## Activity	Per cent			Total ## Activity	Per cent		
			F3	F2	F1		F3	F2	F1		F3	F2	F1
1		4.2	57.61	31.72	10.65	2.3	39.13	45.08	15.77	2.0	28.92	56.14	14.92
2		4.1	60.50	30.01	9.84	2.6	29.04	55.15	15.79	2.4	29.94	55.93	14.11
3		3.6	56.79	33.42	9.78	2.2	38.78	47.39	13.81	2.3	36.94	50.66	12.39
4		3.7	51.64	38.14	9.86	2.3	28.64	56.28	15.56	2.0	33.50	52.19	14.30
5		3.7	51.10	41.38	7.51	2.3	29.38	57.73	12.88	2.1	36.60	48.00	15.38
6		4.1	58.26	32.69	9.03	2.4	34.99	52.14	12.86	2.2	37.01	48.06	14.91
7		4.1	60.40	30.45	9.13	2.4	24.09	59.39	16.50	2.1	28.34	58.29	13.36
8		3.6	52.10	37.88	10.01	2.6	27.92	56.40	15.66	2.0	29.33	57.85	12.80
9		3.8	53.27	35.95	10.77	2.5	29.17	53.39	17.42	2.3	37.76	51.29	10.94
10		4.2	50.96	36.98	12.04	2.2	34.75	50.46	14.78	2.1	37.91	46.66	15.41
11		4.1	49.68	36.38	13.92	2.4	33.96	48.56	17.46	2.3	32.95	51.07	15.96
Mean and S.D.		4.1 ± 0.24	54.75 ± 3.44	35.03 ± 4.03	10.19 ± 1.29	2.3 ± 0.26	31.80 ± 4.08	52.90 ± 4.19	15.04 ± 1.78	2.1 ± 0.23	33.56 ± 3.86	52.37 ± 4.09	14.04 ± 1.52

* Tadpoles were treated with thyroxine (2.6×10^{-6} M) or thyroxine (2.6×10^{-6} M) plus thalidomide (5×10^{-4} M) for sixteen days. Tissues from 2 or 3 tadpoles were pooled for each experiment.

** samples RAD reduced/hr/mg protein.

DISCUSSION

- (a) Effect of thalidomide and its derivatives on the morphological changes during thyroxine-induced metamorphosis of tadpoles.

Since the reports of Macbride (79) and Lenz (71) of congenital malformations from thalidomide, there have been numerous attempts to reproduce the human malformations in experimental animals. Various animals have been used for this purpose (77, 78, 79), but the rabbit (225-226) and the mouse have been the only mammals in which gross malformations of the fetus were observed. Somers produced fetal abnormalities with thalidomide in a rabbit (225), and Lucey and Behrman (227) showed that treating monkeys before implantation of the zygote resulted in still births. He suggested that the drug killed the embryo prior to its implantation. Delahunt and Lassen (72) showed that pregnant monkeys were treated with thalidomide after implantation but before formation of the fetal limbs. Two fetuses that were recovered from the treated females had congenital abnormalities. The thalidomide syndrome in the monkeys was manifested by amelia, phocomelia, internal hydrocephaly, facial capillary hemangioma, hypogenesis of the metatarsal bones, and anotia. They administered thalidomide (10 mg/Kg) by an oral tube from day 32 to 42. Fabro et al (227) reported that when thalidomide at the concentration of 300-400 mg/Kg body weight was administered orally or interperitoneally to rats, pregnant New Zealand white rabbits or Himalayan rabbits, the fetal toxic effects were observed in all strains. Dipaolo and Wenner reported that 750 µg in 3 ml of fresh

medium of Ehrlich Ascites Tumor cells was effective (234). In the case of protozoa, 0.5-2.7 mg/ml was sufficient to inhibit growth (227).

The study presented in this thesis reveals that thalidomide at a concentration of 10^{-4} M inhibits the growth of hind legs and the resorption of tail during thyroxine-induced tadpole metamorphosis. The inhibition became evident only after five consecutive days of treatment with thalidomide. This latent period is followed by the appearance of morphological changes. If the treatment was carried on for 16 days with thalidomide at 1×10^{-4} M, the percentage of inhibition of L/T ratio was approximately 40%. Thus this concentration would be the lowest ever reported to be effective in biological systems.

When thalidomide was administered to rabbit, rat or man, 15 metabolites were found in the urine (235). Twelve of these have been identified after they were isolated in crystalline form by column chromatography on alumina. Whether thalidomide itself or its metabolites in vivo is the main responsible agent for producing abnormality is not yet settled. Thus, some of these and other derivatives were tested for their effect on the L/T ratio, and the result is also presented in Table 1 in this thesis. It is seen that 3-hydroxyphthalyl-D,L-glutamine is the most potent inhibitor among the compounds tested and it inhibits approximately 50% at the concentration of 5×10^{-5} M. The other compounds tested in Table 1 have a rather high inhibiting potency in the L/T ratio. In general, it can be concluded that the derivatives of thalidomide tested are more potent than the mother compound in inhibiting the morphological changes occurring during thyroxine-induced tadpole metamorphosis.

Several authors have investigated the mechanism of action of thalidomide. It was observed that, inhibitory effect of thalidomide on protozoa was countered by nicotinic acid, nicotinamide, nicotinamide adenine dinucleotide, and vitamin K₁ (22/). Thus, the authors postulated that the mechanism of toxicity may be due to an interference with cellular oxidation. They have also found that thiamine, riboflavin, choline, inositol, thymine, orotic acid, p-aminobenzoic acid, and folic acid could not reverse the toxicity of thalidomide for all organisms tested. Since tryptophan is a precursor of nicotinic acid, metabolites along the pathway of synthesis of nicotinic acid from tryptophan were tested (22/). 5-hydroxytryptophane, kynurenic acid, xanthurenic acid and quinolinic acid partly countered the toxicity of thalidomide in *O. malhamensis*. Quinolinic acid slightly inhibited the toxicity of thalidomide in *O. danica*, but none of the compounds mentioned above lessened the action of thalidomide in *E. gracilis* or *T. pyriformis* (22/). Martius (23/) also reported that NAD inhibited the toxicity of thalidomide action and vitamin K₁ (menadione) inhibits the toxicity of thalidomide action. They hypothesized that thalidomide acts at the level of cellular oxidations. Thus, it is quite possible that thalidomide acts at the point where nicotinic acid is connected to NAD, or it may interfere with the utilization of NAD in cellular oxidations (23/). If the conclusion derived above could be applicable to man, thalidomide might interfere with cellular oxidations during morphogenesis and cause abnormalities (24, 232). It is of interest to note here that thalidomide toxicity was not reversed by riboflavin nor folic acid in the protozoa system, although deficiencies of these two vitamins have been implicated in causing fetal malformation (24).

Robertson has described the development of polyneuritis and glossitis upon prolonged therapy with large doses of thalidomide in human (68). These effects were controlled by the prophylactic use of vitamin B-complex. Interestingly, chick embryo injected with nicotinic acid antagonists had a high incidence of rumpleness, ectrodactylism and ectromelia. These findings are the only actual indication of the mode of action of thalidomide in the living organism.

In 1965, the effect of thalidomide on the metamorphosis of tadpole was independently reported by Tata (69), who used 1-3,5,3'-triiodothyronine for inducing metamorphosis of tadpole. He observed that thalidomide inhibits the growth of hind legs and the resorption of tail during 1-3,5,3'-triiodothyronine-induced metamorphosis of tadpole. This data is in agreement with ours.

In conclusion, it can be said that thalidomide and its derivatives affect gross morphological differentiation to the highest degree during thyroxine-induced tadpole metamorphosis. Attempts to demonstrate the direct interaction of thalidomide or its derivatives with thyroxine at various pH did not reveal any change in absorption spectra. The extreme sensitivity of tadpole to the various substances reported here suggests that anuran metamorphosis is an extremely useful tool in drug testing.

(B) Effect of thalidomide and its derivatives on the several enzymatic activities during thyroxine-induced metamorphosis of tadpole.

During the last decade or so, there has been a gradual shift in the thinking of many embryologist as to the role of enzymes in developmental

processes. Over this period there has also developed a great deal of interest in the description of enzymatic phenomenon in developing tissues, thus paralleling a general revival of descriptive biochemistry. Since amphibian metamorphosis is a process which bridges the transition from an aquatic to terrestrial evolutionary form, this transformation occupies, as such, an important place in comparative biochemistry. Moreover, the hormonal control of this differentiation, via the pituitary thyroid axis, offers additional reasons for exploring more fully the biochemical nature of tadpole metamorphosis.

Thus, one approach to study the biochemical mechanism of tadpole metamorphosis should be the attempts to characterize this process enzymatically. A study of arginase activity during metamorphosis and changes in other enzymes has been previously reported (233-234). Shen has emphasized the significance of the appearance and development of enzymatically active and specific proteins during any morphogenetic processes (235). Specific enzymes are undoubtedly involved in the synthesis and transformation of specific proteins. In tissue culture of muscle a large increase in "free ribonucleoprotein granules" occurred as cellular proliferation increased (236). Electron microscopic studies on rat embryos (237) indicated an increase in "RNP particles" during early cleavages, and a qualitative change in sea urchin ribosomes after fertilization could be detected (238). These "activated ribosomes greatly enhanced in vitro protein synthesis.

Increasing number of experimental data has been accumulating to indicate that the egg and early embryos of *Rana pipiens* contain relatively

small amounts of soluble protein and cytoplasmic ribosomes (237). However, both these moieties start to increase simultaneously at about Shumway stage 18 (after the first muscular contraction), when the majority of enzymes and specific proteins either appear or are augmented (241-242). Furthermore, the rate of synthesis of ribosomal RNA increases greatly at this time. Brown et al (239) suggested that study on the development of *Rana pipiens* may provide an excellent experimental opportunity to correlate the synthesis of ribosomes with protein synthesis and embryogenesis. This could be possible due to the fact that the egg and early embryo contain a low level of RNP particles which does not increase for several days and cytoplasmic ribosomes start to increase only after (stage 18 as mentioned above) 3-4 days after fertilization. Embryos increase in its soluble protein content, and begin to concentrate magnesium from the medium, 25% of which is bound to the newly formed ribosomes. The evidence for a general increase in the synthesis of proteins and enzymes after thyroid treatment in the rat or in the metamorphosis of amphibians is also well documented (243, 244). Some authors maintain the opinion that this enhancement of protein synthesis is directly related to the action of the thyroid hormones (245). This was further supported by recent studies showing the interference of thyroid hormone effects in the rat with puromycin and actinomycin D by Tate et al (246-247), on enzyme synthesis in the tadpole with chloramphenicol by Paik and Cohen (248) and on tail response in tadpole with actinomycin D by Wahlborg and Frieden (249).

The work of Sokoloff and Kauffman (249) and their associates suggested a stimulation of amino acid uptake produced by thyroid hormone

both in vivo and in vitro. Later, Sokoloff et al (251) reported that thyroxine can only increase amino acid incorporation into an optimal, or below optimal, concentration of ribosomal particles. However, exploration into various aspects of the amino acid entry into protein synthesis in the metamorphosing amphibian have not presented a convincing rationale for the effect of thyroid hormones. Degroot and Cohen (223) found no increase in amino acid activating enzymes in either the soluble or particulate fraction during thyroxine-induced metamorphosis of tadpole. No increase in glycine-2-C¹⁴ incorporation into tadpole liver protein during the first 24 hours after 3',3,5-triiodothyronine treatment could be demonstrated by Dashman and Frieden (251). While the literature contains numerous examples of greatly increased enzyme and protein synthesis in the tadpole during T₄ or T₃-induced metamorphosis, these changes do not appear to result directly from the alteration of amino acid metabolism or the amino acid activating systems (253, 252).

It was to be expected that workers in this field would turn to the nucleic acid segment of the protein biosynthetic plan as the site of thyroid hormone action in the tadpole. Finamore and Frieden (253) found that the gross nucleotide composition of liver and tail RNA was essentially unchanged after T₃ injection. Of course, altered sequences or traces of unusual nitrogenous bases might not have been detected in these early experiments. Thus, it seemed more likely than the effect of thyroid hormone might be reflected in the metabolism of nucleic acid. A substantial increase in p³²O₄ incorporation into liver RNA and protein within two days after T₃ treatment was observed (253). In contrast, phosphate incorporation into tail DNA, RNA and protein was less than in the control

group as early as 24 hours after T_3 administration. Later, Paik, Metzenberg and Cohen (254) observed a decreased rate of incorporation of adenine-8- C^{14} into the liver RNA during thyroxine-induced metamorphosis of tadpole. Numerous other alterations in the metabolism of RNA components have been recently reported by Akamatsu, Lindsay and Cohen (255) including an increase in uridine phosphorylase and a deficiency of pyrimidine bases during thyroxine-induced metamorphosis of tadpole.

Studies have been made on many enzymes involved in nitrogen metabolism, also liver cathepsin and trypsin have been shown to decrease during metamorphosis (256). Urbani (10) reported an increase in the dipeptidase for leucylglycine with a decrease in alanylglycine and glycylglycine. No particular trend could be seen for the tripeptidase for glycylglycylglycine. Weber (256) observed that acid proteinase increases whereas alkaline proteinase shows an almost unchanged activity, and the activity of tail cathepsin increases 30-fold during metamorphosis of tadpole. A marked increase in activity in liver carbamyl phosphate synthetase and catalase during thyroxine-induced metamorphosis of tadpole has been reported, whereas succinic acid dehydrogenase decreased in the liver (257). Thus, as mentioned earlier, the metamorphosis of tadpole is accompanied by a complex series of biochemical events including a marked change in activities of various enzymes, in one way or the other, to provide pathways for the specialized biochemical functions of adult organism. Our studies described in this thesis have been mainly concerned with the study of the enzymes, glutamic oxaloacetic transaminase, catalase, alkaline phosphatase, and glutaminase during thyroxine-induced metamorphosis of tadpole and the effect of thalidomide and its derivatives on these

enzymes during thyroxine-promoting metamorphosis of tadpole.

- (1) The effect of 3-hydroxyphthalylglutamine on the alkaline phosphatase activity during thyroxine-induced metamorphosis of tadpole.

The effect of metamorphosis on the activity of a representative group of phosphatase was reported by Yanagisawa in 1954 (1954). He tested tadpole of the *Phacophorus schlegelii araborea* with an acetone-dried powder of bovine thyroid gland. The metamorphosis was accelerated upon treatment. Liver acid phosphatase increased 200% and tail acid phosphatase approximately 180% by this treatment. Also liver and tail alkaline phosphatase increased 210% and 150% respectively by thyroid hormone treatment. He also reported an increase in inorganic phosphate in the liver and tail by thyroid hormone treatment. Moog et al (1958) and others (1957-1964) reported a similar results to those of Yanagisawa. In 1964, Kubler and Frieden (1965) confirmed the increase of alkaline phosphatase activity in the liver and tail during thyroxine-induced metamorphosis of tadpole.

Data reported in the present thesis showed that alkaline phosphatase activity in the liver, tail and "residual body" increased markedly during thyroxine-induced metamorphosis of tadpole. However, simultaneous presence of 3-hydroxyphthalylglutamine with thyroxine inhibits this increase slightly but significantly. Since the magnitude of the inhibition in the case of alkaline phosphatase activity by this

derivative of thalidomide is small, the biochemical significance of these data in relation to the mode of action of thalidomide can not be decided.

The versatility of the metamorphosing tadpole for the study of differentiation arises not only from the presence of numerous anabolic tissues such as liver and limbs, but also from variety of tissues such as tail and gut in which numerous catabolic reactions predominate. The possibility that tail resorption is a controlled expression of the activity of intracellular catabolic enzymes has long been explored. Most tissues seem to contain the enzymes which are responsible for their destruction when cells die or assume certain pathological or controlled physiological states. The numerous metabolism for the control and regulation of the metabolic action of some intracellular enzymes have been described by many authors. The most attractive hypothesis proposes that the regulation of the intracellular catabolic enzymes may be determined by the action of lysosome, the intracellular activity first proposed by DeDuve and his associates (33⁹) and hydrolytic enzymes during tail resorption such as β -glucuronidase, and some phosphatases.

To assess the role of hydrolytic enzymes during tail resorption, Kubler and Frieden (26⁵) have recently studied the fate and properties of tail β -glucuronidase during anuran metamorphosis. It was found that the specific activity of soluble and particulate β -glucuronidase increase over 30 fold in the regressing tadpole tail during spontaneous metamorphosis, while the total activity appears to increase 3 times. The increase in soluble enzyme activity occurs simultaneously in the skin, fin and muscle at the beginning of metamorphosis. During induced metamorphosis, the soluble

β -glucuronidase activity increase to about 30% of that found during spontaneous metamorphosis of tadpole (245).

As our data and others indicated, the activity of alkaline phosphatase in the tail during thyroxine-induced metamorphosis of tadpole is increased markedly. It is of particular interest that even in a tissue undergoing degeneration, there appears to be an initial synthesis, not just a release, of certain specific enzymes which might be involved in the destruction of that tissue. These evidences, as mentioned above, suggest that the synthesis of these hydrolytic enzymes not only persists but is also stimulated during tail resorption. The data on $P^{32}O_4$ incorporation into DNA, RNA and protein of the tail also suggest that while the incorporation is less than controls it does persist for the first 48 hours after T_3 administration (253). During this period, the emphasis of protein synthesis could be shifted to the numerous catabolic enzymes which appear to increase in the tail during metamorphosis.

One of the metabolic derivatives, 3-hydroxyphthalylglutamine, countered this increase in alkaline phosphatase activity slightly by thyroxine but significantly, as mentioned under results in this thesis. Thus, it appears that the action of alkaline phosphatase and the effect of thalidomide might have some common target. However, further study would be required to know the exact mechanism of these factors.

- (2) Effect of 3-hydroxyphthalylglutamine on the activity of liver catalase during thyroxine-induced metamorphosis of tadpole.

Catalase in liver of tumor bearing animal decreases dramatically (266) and this enzyme can be formed adaptively by yeast as a response to aeration (267). Paik and Cohen studied the relationship between the increased activity of catalase and of carbamyl phosphate synthetase (268). Up to the time when the activity of carbamyl phosphate synthetase has increased about fourfold, the catalase remains unchanged. After this time interval, the activity of catalase begins to increase and reaches a plateau at the same time as does carbamyl phosphate synthetase. The final level of the enzyme activity is about five times that which is observed in the premetamorphic tadpole (268). This was confirmed in our present experiment.

Significant increase in oxygen consumption after T_3 or T_4 injections were reported by Lewis and Frieden (29, 266). T_3 or T_4 produces a greater oxygen uptake, calculated on the basis of the individual animal and per unit of wet weight. This increased resorption might stimulate the activity of catalase during thyroxine-induced metamorphosis of tadpole. However, 3-hydroxyphthalylglutamine inhibits the increase of catalase activity caused by thyroxine treatment. It suggests that 3-hydroxyphthalylglutamine might inhibit cellular oxidation during thyroxine-induced metamorphosis of tadpole. This contention is in accordance with the finding of Frank et al (227) and others (226, 229, 231-232), as previously described, that thalidomide inhibits growth of protozoa, and this inhibition was countered by nicotinic acid, nicotinamide, nicotinamide adenine dinucleotide, and vitamin K. They also described that thalidomide may possibly act at the point where

nicotinic acid is connected to NAD, or it may interfere with the utilization of NAD and vitamin K in cellular oxidations. Even though the implication of these findings in the term of mechanism of thalidomide action is not clear at present, the fact that the inhibitory action of this drug on the thyroxine-induced metamorphosis of tadpole is not non-discriminatory will be evident from the data presented in this thesis.

- (3) Effect of 3-hydroxyphthalylglutamine on the glutamic oxaloacetic transaminase activity during thyroxine-induced metamorphosis of tadpole.

Studies have been made on many enzymes involved in the nitrogen metabolism. An impressive 30-fold increase in tail cathepsin was found by Weber (256), and 15-fold increase in liver carbamyl phosphate synthetase during thyroxine-induced metamorphosis of tadpole was reported by Paik and Cohen (208). There is a remarkably complete correlation of the alteration in principal nitrogenous metabolism with enzyme development during anuran metamorphosis; the important metabolic sequences involving the conversion of ammonia to urea are markedly increased during induced or spontaneous metamorphosis of tadpole. The key enzymes in the urea formation cycle are carbamyl phosphate synthetase, arginase, transaminase (glutamic oxaloacetic transaminase), ornithine transcarbamylase, and arginosuccinate synthetase. Munro (257-272) and Frieden (247) made a thorough study of liver arginase activity during induced and spontaneous metamorphosis of tadpole. They reported that in both the toad and the

swamp frog, a direct relationship was obtained between the morphological stage and an increased arginase activity. The arginase activity is reported more rapidly to T_3 and T_4 than did the morphological conversion. The increase in arginase has been confirmed subsequently by Brown and Cohen in another species, *Rana catesbeiana* (27).

The enzymes, carbamyl phosphate synthetase, ornithine transcarbamylase, arginosuccinate synthetase, glutamic oxaloacetic transaminase, and arginase increased markedly during induced or spontaneous metamorphosis and remain at an elevated level in the adult frog (Chan and Cohen also reported that a rise of glutamic oxaloacetic transaminase activity begins at onset of metamorphosis of tadpole in both spontaneous and thyroxine-induced metamorphosis of tadpole. The highest activity of the glutamic oxaloacetic transaminase was obtained in the liver of the adult frog, which showed a 4-fold increase as compared with that in the premetamorphic stage (22). Similar results were also obtained in our experiment as described previously. It was observed that the enzyme started to increase at the onset of metamorphosis of tadpole in our experiment and it increased in activity 2.0-fold, 2-fold, and 1.5-fold in the liver, tail and "residual body" respectively at the completion of thyroxine-induced metamorphosis of tadpole. Increased urea formation from ammonia is directly correlated with the increased activity of glutamic oxaloacetic transaminase activity.

The enigmas posed in attempting to find out meaningful relationship between the thyroid hormone and numerous differentiating processes have been discussed at the morphological and physiological level by many authors. In addition to metamorphosis, numerous characteristics associated

with maturation and sexual development in mammals are regulated by the thyroid hormone. At this time it will be most important to consider the possible intimate biochemical mechanism that might be involved in the hormonal control of differentiation in general and the particular role of the thyroid hormone in these processes.

However, when 3-hydroxyphthalylglutamine was added together with thyroxine, a slight but significant inhibition of glutamic-oxaloacetic transaminase activity occurred in all tissues tested. In the present stage of knowledge no conclusion can be derived about whether these compounds act at the level of biosynthesis of the enzyme or at the level of the enzymic activity.

(4) Effect of thalidomide on the activity of glutaminase during thyroxine-induced metamorphosis of tadpole.

Paigle et al (102) reported that various methods for concentrating the metabolites present in the urine after administering radioactive thalidomide invariably yield highly radioactive fraction containing substances of an acidic nature which, following total hydrolysis with hydrochloric acid, gave rise to phthalic acid and glutamic acids which were identified by quantitative paper chromatography. After methylation of the concentrated metabolites and separation of the resultant esters by thin layer chromatography, it was found that glutamic acid was conjugated with other compounds (102). Thalidomide is the only known sedative whose metabolites are glutamic acid derivatives, and which possesses

neurotoxic and possibly teratogenic properties. Thus it gives rise to a number of considerations and speculations which might serve as a guide to study the action mechanism of thalidomide.

Apart from its importance as a component of protein, glutamic acid occupies one of the most important positions within the network of intermediary metabolism. The biological substances closely related to glutamic acid such as glutamine, glutathione, carbamyl-glutamine and folic acid also play important physiological roles. Glutamic acid has also proved of limited clinical interest in certain neurological disorders (61-70). Thus it is quite possible that thalidomide may in vivo through its hydrolysis in some way interfere with the biochemical and physiological function of natural glutamic acid, glutamine or other glutamic acid derivatives, as mentioned previously.

Degroot and Cohen (223) reported that the level of glutamic acid dehydrogenase activity is 6 times higher in adult frog than in tadpole liver, and 1.5 times higher during thyroxine-induced metamorphosis of tadpole than in tadpole liver. It was observed in our experiment that thyroxine treatment results in about a fourfold increase in liver glutaminase activity of tadpole. However, simultaneous treatment with thalidomide reduces this increase about two fold. This result is very similar to the observation made by Fabro et al (229) that one of the metabolites of thalidomide 2-(O-carboxybenzamine)-glutaric acid, was found to inhibit the activity of glutamine synthetase, glutamine decarboxylase, and glutamate dehydrogenase of rat brain. The concentration of 1×10^{-3} M of thalidomide was used to inhibit 50% of activities of

these enzymes. These results suggested that thalidomide and its hydrolytic products invivo might act as antimetabolites to glutamic acid and glutamine.

(C) Effect of 3-hydroxyphthalylglutamine on the biological amines during thyroxine-induced metamorphosis of tadpole.

(1) Effect of 3-hydroxyphthalylglutamine on the adrenaline and noradrenaline contents during thyroxine-induced metamorphosis of tadpole.

It is now well established that noradrenaline is present together with adrenaline in most mammalian suprarenal glands, even though its relative amount varies widely. In the rabbit, for example, only traces are found (272), whereas in the cat up to half the amount of total amine may be present as noradrenaline.

The detection and isolation of epinephrine from the parotid secretion of the tropical toad, *Bufo marinus*, was first accomplished by Abel and Macht in 1919 (273). In 1929 Chen and Jenson (274), and in 1933 Chen and Jensen (275-276) succeeded in isolating epinephrine from Ch'an Su, a commercial preparation of the Chinese toad venom employed in medicine in China for centuries. The source of Ch'an was finally proven to be the parotid secretion of *Bufo gargarizans* (277). Evidence for the presence of epinephrine was also obtained from the parotid secretions of *Bufo regularis* (278). Loewi in 1936 (279) and Euler in 1946 (280)

demonstrated adrenaline in extracts from frog's heart. In 1949 the identification of norepinephrine in adrenal glands by Euler and Hamberg was promptly followed by the isolation of the same substance by Berstrom and his associates (282), and by Tullar (283) from U.S.P. standard epinephrine. However, Bacq and Lecomte (284) and Fischer and Lecomte (285) found no trace of norepinephrine in the parotid secretion of *B. arenarum*, *B. marinus*, and *B. mauritanicus*, although in a later publication Fischer and Lecomte (286) admitted that the quantity of norepinephrine present in the parotid secretion of *B. marinus* might be so small that it could not be detected easily. Finally, in 1951 Lee and Chen have found norepinephrine in toad venom by paper chromatographic method (287). In 1953 Gregerman (288), using the paper chromatographic method, demonstrated both adrenaline and dopamine in the secretion of the skin glands of the toad. Lasagna (289) reported that chemical and chromatographic studies have revealed the presence of a substance, possessing characteristics of norepinephrine, in a preparation of epinephrine extracted from the parotid *Bufo agui*. They also reported that the presence of norepinephrine in toad secretions demonstrates the ability of these animals to form amines. This raises the possibility that other tissues of toad, including those of the nervous system, may possess this ability, and that norepinephrine may play an important role in the normal physiology of these animals, perhaps analogous to that suggested for higher animals. The presence of adrenaline in the toad adrenal gland was first reported by Houssay (290-291), and in the frog adrenal gland by West (324). These authors also demonstrated the existence of noradrenaline by paper chromatographic method.

In view of the fact that catecholamines in amphibian could be changed by respiratory metabolism (293-294), Locker (295-296) reported that oxygen consumption of amphibian tissues is related by catecholamines contents, and Smith (297) observed that glycemia is caused by catecholamines in toad. Mazzoco (298 - 301) observed that heart glycogen content of toad is related to catecholamines, and that this may be the case with the yearly variations in vasomotor reactivity and arterial blood pressure in toad, with changes in circulating catecholamines (298), as reported^b Segra and D'Agostino in 1964 (302). The presence of adrenaline was demonstrated by paper chromatography (303). Noradrenaline was present in minute amounts and could only be detected in ultraviolet light.

The origin of plasma catecholamines in the toad remains to be elucidated. Very few studies have been conducted in this area. Adrenaline is the main catecholamine in amphibian tissues innervated by sympathetic fibers, but in the adrenal gland both amines are present. Euler and Lishajko (304-305) found only traces of noradrenaline in heart, spleen and liver of *Rana temporaria*. Bertler and Rosengren (306) showed that adrenaline is the principal catecholamine in brain of *Rana temporaria* and *B. vulgaris*. Similar results have also been reported in brain and heart of *Rana pipiens*, *H. cinerea*, *Bufo americanus*, and *Bufo marinus* by Bogdanski (307) in 1963. It is of great interest that peripheral sympathetic neurons in the frog contain adrenaline instead of noradrenaline (308). Our data also showed that the content of epinephrine is larger than that of nor-epinephrine in the brain of tadpole. These evidences seem to indicate a marked difference between amphibian and mammals, since in the latter the

sympathetic system contains larger amounts of noradrenaline, whereas in the amphibian noradrenaline is much lower (504-507). Since the brain of fish contains norepinephrine, it is possible that modern amphibia arose from primitive stock whose brain catecholamine was norepinephrine. The reason why epinephrine has become the main catecholamine in amphibian brain is not well understood. Strangely enough, norepinephrine is the main catecholamine in the peripheral organs of salamanders but not of frog and toads.

With the above background knowledge on catecholamines, we have found that thyroxine treatment seems to have some action on the contents of catecholamines in the tadpole brain and kidney; however, the individual variations were such that statistically there is no difference between thyroxine-treated and control group, at this concentration of thyroxine treated. Furthermore, simultaneous treatment with 3-hydroxyphthalyl-glutamine does not bring any difference from that observed in the presence of thyroxine alone.

(2) Effect of thalidomide on the glucosamine contents during thyroxine-induced metamorphosis of tadpole.

Effect of thyroxine upon the mucopolysaccharide of blood vessel wall have been studied by various workers; Aschoff (509), Danisch (510) and Friedland (511) reported that thyroid hormone produced some damage on the wall of the blood vessel. In 1939 Van Balo treated adult rabbits with high dose of thyroxine and found extensive gross and microscopic changes

in the aorta. These findings agreed with the findings by Fischer in 1905 (313). Comparing the hexosamine/hydroxyproline ratio in epinephrine-treated and epinephrine-thyroxine-treated rabbits, a significantly higher value was found in the latter group. The fact that the concentration of hexosamine and hydroxyproline were somewhat lower in control as well as in experimental animals of the epinephrine group is presumably due to a more quantitative determination of the water content in the epinephrine-thyroxine animals when using the vacuum dessicator. The H/Hp ratio for the control was the same in both experiments. The alteration in the hexosamine/hydroxyproline ratio may be taken to represent an accumulation of mucopolysaccharides in relation to quantity of collagen which has been found to be present in wound healing (314).

Biochemical and histological changes in the aortae wall of rabbits were studied following injection of adrenalin and l-thyroxine for 2 weeks (315). It was observed that the wide spread gross and microscopic changes were accompanied by increase in hexosamine content, an increased uptake of ^{35}S labeled sodium sulphate, and an increased calcium content (316). There was a clear-cut increase in hexosamine content and uptake of ^{35}S sulphate, most pronounced in the first part of the aorta, from the aortic valve to the first intercostal arteries, and in the abnormal aorta. These changes were of the same nature as those seen following injection of adrenalin plus l-thyroxine-induced sensitization to endogenous adrenalin, and the accumulation of mucopolysaccharide probably indicates a related process. From these evidences they postulated that possibly the damaging effects of adrenalin on the vessel wall is exerted through the variations in the blood pressure which accompanied

the adrenaline injection (316-319). There may also be a question of anoxic damage via a constriction of the vasa vasorum. Thyroid extracts are able to increase the susceptibility of the tissues to adrenaline. Also the possibility exists that this sensitization is perhaps explicable by the inhibitory effects of thyroxine on the monoamine oxidase^{and catecholamine-methyl-transferase} which are assumed to be the important factors in the breakdown of catecholamines (321-328).

As described under results glucosamine increased 2-fold during thyroxine-induced metamorphosis of tadpole, in the present experiments. Thus, our results are in good agreement with the hypothesis that thyroxine increases hexosamine contents in the tadpoles, as mentioned previously. Furthermore, it is quite understandable that collagen should be needed during metamorphosis of tadpole for the formation of limbs. Since glucosamine is one of the important components of collagen, these two factors, such as thyroxine administration and development processes, are expected to exert an increased glucosamine synthesis in the liver during thyroxine-induced metamorphosis of tadpole.

Simultaneous presence of thyroxine and thalidomide does not affect the changes brought about by thyroxine alone. Thalidomide and its derivatives inhibit the activities of several enzymes, such as catalase, transaminase, glutaminase, and alkaline phosphatase during thyroxine-induced metamorphosis of tadpole as mentioned previously, and they also inhibit the activity of glutamine synthetase, glutamine decarboxylase, and glutamate dehydrogenase in rat brain (327). Thus, it is quite possible that thalidomide and its derivatives do not play any direct effect

on the glucosamine content during thyroxine-induced metamorphosis of tadpole, and that the mode of action of thalidomide and its derivatives is quite selective in the living organisms.

- (D) The activity of lactate dehydrogenase isozymes during thyroxine-induced metamorphosis of tadpole.

Over 60 years have elapsed since Nutall et al showed by immunological methods that serum proteins differ in structure from species to species (329). Further studies of species differences have since been made, usually with antisera to protein mixtures from serum or egg white. Landsteiner (330) and Boyden (331) have summarized these studies, which showed that the degree of immunological resemblance between proteins of different species is often a function of how closely related the species are. Genetic control of the synthesis of specific proteins has been repeatedly demonstrated and in a few cases changes in the specific properties of a protein have been correlated with corresponding changes in a gene. Several variations of adult and fetal hemoglobin have been identified and shown to be under genetic control with heterozygous individuals producing the two alternative types of hemoglobin (332). There have been studies of species difference with antisera directed to enzymes and they suffice to show that enzymes also exhibit the phenomenon of species specificity (332-334).

The existence of isozymes raises the question of whether the synthesis of each of these also is controlled by a separate gene or

whether they are due to modification of a single gene. However, it is implausible to consider the multiplicity of isozymes as reflecting a heterozygous genetic constitution since many animals examined thus far could scarcely have been so uniformly heterozygous; for example, the mice used were from highly inbred colonies of independent genes for the synthesis of fetal and adult hemoglobins. Especially relevant is the fact that these genes for hemoglobin synthesis function at different periods during ontogeny. Certain isozymes also appear to be characteristic of specific stages of development. The genetic basis for the isozyme synthesis cannot be settled completely at present and must await the discovery of isozymic differences in the organisms that can be subjected to genetic analysis. However, it is suggested that each species has its own characteristic isozymic pattern, presumably reflecting the genetic makeup of the species. The over-lapping but distinct patterns of isozymes in genetically different animals are amenable to either of two genetic interpretations; each isozyme attributed to a single gene, with species differences due to the particular genes possessed, or alternate forms of gene may produce somewhat different constellation of isozymes, each species then having a uniquely characteristic gene for synthesizing its own array of LDH isozymes.

Fourteen samples of pure M_4 enzymes from various species have been subjected to quantitative amino acid analysis (335). The histidine content varies widely, from 70 residues per mole in the turkey enzyme to below 30 in the frog. Furthermore, it is evident that closely related vertebrate classes are grouped in the same patterns of isozymes in the same

organ. Thus, all birds have a high histidine content. The mammals examined exhibit similar values. It is noted that the caiman, which is a reptile related to the crocodile, has an M_H isozyme with a histidine content intermediate between that of birds and other tetrapods. This is in good agreement with the well-known theory that birds have evolved from reptiles and, in particular, from a reptilian group allied to the crocodilians (336 - 338). In contrast to histidine, phenylalanine does not vary in content among M_H LDH (339). Eight crystalline H_H enzymes have been analyzed for the amino acid contents (339). Several amino acids vary significantly in content from species to species; for instance, the arginine content of the frog enzyme is lower than in birds and mammals. The isoleucine contents of the mammalian enzymes are similar to each other and are distinct from those of birds and frog. On the other hand, the lysine content is rather similar in all eight of the H_H isozymes. The H_H LDH data leads to conclusions as the M_H LDH data; namely, that the amino acid compositions varies considerably among various classes of vertebrates, and that closely related species have similar compositions. Although amino acid analysis show that variations take place in the chemical structure of LDH's among species, such data cannot be expected to give a clearcut picture of the extent to which the amino acid sequence are variable. However, the finger print method has some advantages in this regard. Fondy et al in 1964 began to examine the finger print patterns of several crystalline LDH's amino acid analysis (152, 339). They reported that the hybrid lactate dehydrogenase H_2M_2 from chicken liver and HM_3 from chicken leg muscle have been isolated and crystallized and the pure H_H

form of the enzyme has been isolated and crystallized from chicken liver, and these were compared with the H_4 obtained from heart. Finger print patterns of tryptic digests of the hybrids have been compared to that obtained from a 1:1 mixture of the H_4 and M_4 and have shown the hybrids to be a combination of the pure forms. Amino acid analysis, particularly of histidine, place the hybrids at points intermediate between the two pure types. All the evidences such as analysis of amino acids composition, tryptic peptide mapping, immunological analysis (157), as well as recent genetic experiments (45, 340), support the "enzyme subunits hypothesis".

- (1) The patterns of lactate dehydrogenase isozymes during thyroxine-induced metamorphosis of tadpole.

Kaplan and his coworkers (45, 341-342) have demonstrated different catalytic efficiencies for the different LDH isozymes. It has been suggested that the distinguishing properties of various isozymes may allow them to be integrated into distinct metabolic pathways in different locations in the cell. Such an arrangement might prove biologically advantageous because of a more precise and localized control of cellular function (343, 179). Markert et al investigated the LDH isozymes pattern of the various tissues and ontogenic specificity in mouse (170), and the results indicated that the changing need of the differentiating cells is met by rapid alteration in the relative rates of synthesis of the isozymes. Kaplan and his co-workers demonstrated that the different forms of LDH have different reaction rates for NAD analogues and different optimal

substrate concentrations for pyruvate (1.25). Very recently, the M_H and H_H isozymes of many species have also been investigated by the use of coenzyme analogues by Wilson et al (1967). The data suggested that, the more closely related the species are, the more similar patterns of LDH isozymes, as mentioned previously. However, our results demonstrate that tadpole liver has only one isozyme and the tail and brain have three isozymes. As reported in the Results in this thesis the mobility of LDH of tadpole liver, tail and brain is generally slow, the H_H isozymes of rat kidney moved 4.3 cm in 2 hours whereas corresponding fraction F1 of tadpole tail and brain moved 1.2 cm and 2.6 cm in 4 hours, respectively, under identical conditions.

Electrophoretic mobility measurements can also be made with safety using crude extracts and a specific standard method for LDH. In 1962 Vessel and Bearn reported that erythrocytes from representatives of the 5 classes of vertebrates revealed a marked species variation in the number of LDH isozymes, in their electrophoretic mobilities (1962). Starch gel electrophoresis of hemolysates followed by direct histochemical demonstration of LDH activity with nitroblue tetrazolium as dye and phenazine methosulfate as the electron carrier showed that closely related species exhibited similar LDH patterns. The rhesus monkey had LDH isozymes of similar pattern to those of human hemolysate but slightly slower in electrophoretic mobility. They also observed that the goat and sheep each had 1 band of LDH activity in their erythrocytes of identical electrophoretic mobility, whereas the single band in steer hemolysate migrated slightly faster. The five bands of chicken hemolysate were

quite similar in pattern to the 5 bands of duck hemolysate but migrate slightly faster and exhibited a different distribution of the LDH activity. The two species of snake had 1 band each of LDH activity with identical mobility (344). Examination of more than 380 human hemolysates failed to reveal any difference among individuals in the main LDH bands. However, it was quite often found that the closely related species exhibit significant differences. As an outstanding example, H_L enzymes of bullfrog and Leopard frog can easily be distinguished by electrophoretic methods (344). Although there is considerable variation in electrophoretic mobility of the H_L enzymes among species of a vertebrate class, certain general trends are observed, when species of each class are investigated (344). The distance moved is in the range of 5-10 cm for the great majority of fishes and frogs. Most mammals have an enzyme that moves 12-15 cm under the same conditions. Evidently, a significant increase in electrophoretic mobility took place when mammals evolved from reptiles (344). Birds have an H_L LDH that moves only 1.5-3 cm under the same conditions. Apparently, a reduction in electrophoretic mobility took place when birds evolved from reptiles. It is interesting to note, however, that certain birds, such as the ostrich, rhea and tinamou, which are termed palaeognathous birds, have an H_L enzyme whose mobility is like that of reptiles. These birds have been considered on anatomical grounds to be related to each other and to be primitive among birds (336-338). Thus, from the foregoing consideration, our data again confirmed that tadpole is more closely related to birds than mammals as

far as the mobility of isozymes are concerned; frog is more like fish than birds from the viewpoint of mobilities of isozymes on the electrophoretic diagram.

(2) Effect of thalidomide on the lactate dehydrogenase activity during thyroxine-induced metamorphosis of tadpole.

The effect of thyroxine on the level of individual enzymes has been studied a great deal as mentioned previously. Vestling and Knoepfelmacher (345) observed the decrease in the total LDH activity in the liver of rats fed with thyroxine. Degroot and Cohen (223) reported that in the liver of thyroxine-stimulated metamorphosing tadpole the total LDH activity decreased approximately 60%. Marvin et al (200) reported that the administration of L-5-3'-5'-triiodothyronine to rabbits resulted in the decreased lactate dehydrogenase activity in the liver. Similar results as described above were obtained in our experiment, if one compares the patterns of changes between control (non-treated animal) and thyroxine-treated tadpole LDH activity. As it can be seen in Fig. 19 and Table IV^{and V}, in this thesis, thyroxine treatment caused a decrease of LDH activity in liver, tail and brain of tadpole. A change in the activity of an enzyme during development as measured may be due to many factors; that is, it may represent a change in the amount of enzyme, the removal or formation of an inhibitor, changes in the stability of the enzyme, etc. However, little could be said with certainty on the mechanism of inhibition of thyroxine on the lactate dehydrogenase activity at present,

even though numerous workers have studied on the effect of thyroxine on the living organisms.

Although a large amount of work had been carried out on the isozymes of lactate dehydrogenase, very few observations have been made on the relationship between thyroxine and lactate dehydrogenase isozymes. The results reported in the present thesis, demonstrate that thyroxine decreased LDH activity. However, it can also be seen that the relative amount of the LDH isozymes activity does not follow the overall pattern; among the isozymes, as seen in Fig. 20 and Table IV^{and V}, F3 is relatively and absolutely reduced whereas F2 and F1 are reduced only relatively. Thus, thyroxine seems to act very selectively on only one of the isozyme fraction during thyroxine-induced metamorphosis of tadpole.

The results, illustrated in Fig. 20 and Table IV^{and V}, show that thalidomide exerts no significant effect on the decrease in both total LDH activity and any of the amount isozymes patterns in the liver. Similar results were obtained with tadpole tail and brain. Since thalidomide has been shown to inhibit, selectively, some of the biochemical and morphological changes of thyroxine-induced metamorphosis of tadpole, as described previously (22⁹), lactate dehydrogenase activity constitutes an example of one of the activities not affected by thalidomide treatment.

- (3) An improved colorimetric method for the cellulose poly-acetate electrophoresis.

The first biological application of LDH heterogeneity utilizing

the technique of starch block electrophoresis have been reported by several authors (87, 92). The starch-gel electrophoresis is most widely used for lactate dehydrogenase isozymes separation (196 - 200). This is largely due to the improved power of resolution that starch-gel electrophoresis affords, to the development of convenient histochemical methods for exhibiting lactate dehydrogenase isozymes on the gel, and the capacity of the gel for separating multiple specimens simultaneously. The extreme sensitivity of the starch gel and its capacity to reveal small differences in the electrophoretic mobility have, however, presented certain problems as mentioned in detail previously in the Review of Literature.

After staining, two methods have been reported for the quantitative analysis of lactate dehydrogenase isozymes separated on the electrophoretic diagram; one of the methods is to elute the starch-gel electrophoretic diagram and the LDH activity was determined by measuring the oxidation of NADH or the reduction of NAD (219). The other method is the scanning procedure by ^{an} appropriate densitometer. The former method is a modification of Smith's method (196) by Fine and Kaplan (196). Agar-saturated KCl bridges were used to connect the electrode chambers to the chambers in which the wicks were immersed. Separation was carried out 18-20 hours with a voltage gradient of 7.5-12.5 volt/cm across the gel and a constant current of 24-27 milliamperes. After the separation the gel of electrophoregram was cut into 3 slices. The center slice, as colored carrier, 2-3 mm thick was incubated in the dye-substrate reaction mixture for 2 hours at 37°C in the dark. This staining method is a modification of Dewey and Conklin method by Markert et al (1962).

Each isozyme band was then cut by the carrier of center slice electropherogram and was eluted with 0.2 M, phosphate buffer solution pH 7.2. The LDH activity in the eluates from the starch-gel electropherogram was then determined by measuring the oxidation of NADH or the reduction of NAD. Even though this quantitative method is used by many workers, certain problems are inherent to this procedure; the carrier of center slice is not always shown at an exact area for the band, because in some cases the separation bands are not appeared as straight line band. The area cut, therefore, is not always accurate. One of the other problems is the elution procedure. The loss of LDH isozymes activity was unavoidable in this elution procedure.

The scanning method is another widely used procedure. However, certain problems are tied with this scanning method for the quantitative analysis of the LDH isozyme activity; the LDH isozymes bands are not always separated in straight line, as mentioned previously. Furthermore, the intensity of color development in the isozyme band is not homogenous, specially on the starch-gel electrophoregram. Thus, the cellulose polyacetate was introduced into the use. However, before the cellulose polyacetate electrophoregrams can be scanned, cellulose polyacetate membrane must be made transparent. Since Sepraphore 111 is not transparent membrane, the scanning can not be recorded clearly for the quantitative analysis of each LDH isozyme separated by electrophoretic procedures without prior clearing. In order to clear the membrane the cellulose polyacetate membrane is floated on the top of liquid petrolatum until the membrane is completely transparent, and the transparent membrane

is sandwiched between two glass slides to remove excess petrolatum, and the slide is taped at either end with transparent tapes (202). This procedure, however, in many cases in our own experience, results in certain opacity. Thus, the values of scanning records might be greatly affected by this opacity. The other fault of this clearing procedure is the loss of LDH isozymes stained *during the* clearing process.

The special electrophoretic migration room and facilities are required for the starch-gel electrophoresis, and the special scanner is needed for the cellulose polyacetate membrane electrophoregram analysis. In comparison with the values obtained and the facilities needed, it was felt that the investigation of colorimetric method for the quantitative analysis of LDH isozymes stained on the cellulose polyacetate membrane electrophoregram would greatly simplify the procedure and facilitate the study on the isozymes of the enzymes. Errors described above are eliminated by the present dissolving procedure improved, and described in the present thesis. According to the method described in the present thesis, the membrane was stained, the individual bands were cut and each piece of membrane was put into a test tube which contained 4 ml of a mixture of equal volumes of N-N-dimethyl-formamide and benzene. The test tube was shaken to facilitate the dissolution of the membrane and 0.5 ml of absolute ethanol was added after the membrane was completely dissolved. The electrophoregram together with each colored isozyme band is dissolved in the solvent. Therefore, the loss of activity and the elution problem can be avoided completely.

It can be seen in Fig. // that there is good linear relationship between the activities measured by the present method and often used spectrophotometric method. It was found that the same amount of the enzyme which gave an optical density at 540 mμ of 0.14 by the present dissolving method, caused an optical density change of 0.13 per minute at 340 mμ by the usual automatic recording spectrophotometry. Thus, when the automatic recording is carried out for 1 minute, the sensitivities of the two methods are quite comparable. It is also seen that the intensity of the color after the electrophoretic membrane was dissolved increased as time elapsed, and it was found that after 24 hours the color reached its maximum intensity. Even though the color intensity increased with time, the relationship between the amount of the enzyme and the optical density remained proportional. This method is reproducible, accurate and less time-consuming.

SUMMARY OF ORIGINAL WORK

Thalidomide and its derivatives are inhibitory to both morphological and biochemical changes occurring during thyroxine-accelerated metamorphosis of the tadpole. In general, the morphological changes are more affected than the biochemical differentiation. The activities of glutamic oxaloacetic transaminase, alkaline phosphatase, and catalase were inhibited by 3-OH-phthalylglutamine, and the activity of glutaminase was inhibited by thalidomide during thyroxine-induced metamorphosis of tadpole. Thyroxine treatment increases the amount of the liver glucosamine, while simultaneous treatment with thalidomide has no effect on the content of this compound. Treatment of the tadpoles with thyroxine alone or thyroxine and thalidomide does not affect the adrenaline and noreadrenaline contents of kidney and brain. Thus, the effect of thalidomide on the metamorphosis of tadpole induced by thyroxine is quite selective. These changes were brought about at a thalidomide concentration of 1×10^{-4} M, which is the lowest ever reported to be effective in biological systems.

A new improved method to quantitate the amount of the various isozymes of lactate dehydrogenase (LDH) has been devised. The method is sensitive and very reproducible. This method has been employed for studies on LDH isozymes during thyroxine-induced tadpole metamorphosis. Only one isozyme is present in tadpole liver whereas three isozymes are present in tadpole tail and brain. Thyroxine treatment produced a decrease of the total LDH activity of tadpole liver, tail, and brain; however, the

relative amount of the isozyme F3 in tail and brain is the only fraction which shows a decrease. The F1 and F2 fractions show an increase in relative amount. Thalidomide has no influence on the isozyme pattern of LDH in thyroxine-induced metamorphosis of the tadpole.

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