DISCUSSION
NON-NUCLEAR PROTEINS OF EXCRETORY
ORGANS IN FIVE SPECIES OF AMPHIBIA.

A. KIDNEY:

The term "Protein" was derived from a Greek word 'Proteios' meaning prime which again derived from the word 'Protos' means first. Proteins are intimately concerned with all physiological events of a living organism. Thus they are related to the structural and functional aspects including catalysis, metabolic regulation, contractile functions and defense mechanisms of higher organisms. These polymers (proteins) are ubiquitous in distribution throughout the living systems. These can best be designated as primary and direct gene products. An organism can be conceived of as an interacting system of genes and a representative sample quantum of a gene pool of a species. The genome of a species exhibits a high degree of uniformity within the members of it's own. It is known from the DNA content (Mirskey and Ris, 1951; Gerzeil et al., 1956; Vendenly 1958) and the measurements of nuclear diameters (Reichert and Frown 1909) that the amphibia have a marked tendency towards polyploidy as mentioned by Dessauer (1974) after Brawner and Shapiro (1962). England and Mayer (1957) have confirmed Boivin vendrely theory of the constancy of DNA in cells of the same

The phenotypic potentialities of an individual are determined by the genetic information in his chromosomes. On the other hand, the phenotypic picture presented by an organism is the summation of the effects produced by the complement of protein molecules characterizing the species in question. Thus the metabolic and adaptive mechanisms may be related to the concentration of proteins and their qualitative differences in different species.

We studied only five species from the class Amphibia, of which two species belong to the same genus Bufo and three other from three different genera from three different families of two distinct orders. The species *Bufo melanostictus*, *B. himalayensis* of family Bufonidae, *Rana tigrina* of family Ranidae and *Ranoborus maculatus* of family Polypedaliidae, *Tylototriton xanthophthalmus* all belong to the order Anura and the species *Tylototriton verrucosus* of the family Salamandridae belong to the order Urodela.

The protein content of kidney of the two species of the genus Bufo though showed apparent quantitative differences but such differences were not significant. Significant quantitative differences of protein content of kidney was observed among the species of different genera of different families. Such quantitative differences of proteins may be
the result of the genetic differences of such widely different species, and determined by the physiological and environmental influences. Choogoonov et al. (1965) compared the serum proteins of low land and mountain great spotted woodpeckers (Dendrocopos leucolus) and observed quantitative differences in their albumin. Sibley et al. (1974) commented that quantitative and environmentally determined qualitative variation may be a value in the separation of populations. The two species of the genus Bufo inhabit two distinctly different habitats, still they did not exhibit significant quantitative differences in the protein content of their kidneys. Bufo melanostictus is a plain land species whose environment is characterized by moist soil with a varying temperature of 7 to 36°C from winter to summer in a year. Average rain fall of the B. melanostictus environment is 1,581.8 mm. On the other hand B. himalayanae is a mountain inhabiting and water loving species. The environmental temperature varies from -4°C to 20°C in the winter to summer months. The average rain fall in the environment of this harpooning species is 3,267.0 mm yet the observed insignificant difference of non-nuclear proteins of kidney of these two species is not surprising. This difference in the nature of the environment poses the characteristic problems of adaptation to the individuals of these species which is probably related to the qualitative differences of their proteins.
It is not always an essential condition that the amount of protein in an organ of distantly related species should vary significantly. In the cases of *Bufo melanostictus*, *Rana tigrina* and *Tylototriton verrucosus* such phenomena were observed. The two species *Bufo melanostictus* and *Rana tigrina* inhabit the same geographical region but they occupy two distinctly different environmental niches. *Bufo melanostictus*, a terrestrial species while *Rana tigrina* is an aquatic species, but still they exhibited the same amount of proteins in their kidneys. In case of *Tylototriton verrucosus*, a high altitude species whose environment is characterized by a relatively temperate climate where the temperature varies from 0°C to 22°C according to the different seasons of the year. Where as the two other species *Bufo melanostictus* and *Rana tigrina* were of tropical climate. In such cases, the main key to the problem of adaptation lies not on the quantitative differences of proteins but to their qualitative differences which ultimately helped in the finer adjustments i.e., in the micro-adaptation of these species to their immediate environment. *Rhacophorus maculatus* and *Tylototriton verrucosus*, both are temperate zone species. *Rhacophorus maculatus*, the tree frog of Eastern Himalayan region lives on small harvest and trees. They attach themselves on the undersurface of green leaves of the plants, their body sizes are comparatively small.
**Tylototriton verrucosus**, the only Indian salamander is an aquatic species lives in the small mountain pools and water area on mountain valleys. The kidney non-nuclear proteins of these two species vary both quantitatively as well as qualitatively.

Qualitative studies by electrophoresis of non-nuclear kidney proteins of amphibians showed some interesting results. Kidney proteins of *Bufo melanostictus* were separated into twelve electrophoretically distinct protein bands while *Bufo himalayana* kidney proteins were separated into fifteen components. Thus three new protein bands were present in *Bufo himalayana* which were absent in *Bufo melanostictus*. A few bands also showed differences in their mobilities in *Bufo himalayana* from that of *Bufo melanostictus*. *Bufo melanostictus* is a widely distributed species in India ranging from subhimalayan region to sea coast. It may be conceived that this species may have gradually differentiated to the other *Bufo* species of more localized distribution. Thus the variation and increased no of non-nuclear kidney protein bands of *Bufo himalayana* indicate the probable splitting of this species from the *Bufo melanostictus* stock. Such qualitative differences of proteins may help *Bufo himalayana* to adjust with the specific highly humid temperate environment in contrast to that of *Bufo melanostictus*. With regard to the identity value of the protein
bands' where the 100% identity is expressed with 0.5 value, a 0.33 value of protein identity for these two species of the same genus indicate a moderately early intrageneric divergence.

*Rana tigrina*, the mostly aquatic largest Indian frog distributed throughout India, showed 19 non-nuclear protein bands of which 7 bands were identical with those of *Bufo melanostictus* and 8 bands were identical with those of *B. himalayanas*. A calculated kidney non-nuclear protein identity value of 0.22 and 0.23 indicate a closer relation of this genus with *Bufo* than *Rhacophorus maculatus* or *Tylototriton verrucosus*, where the values are 0.18 and 0.21 respectively. The large variation in number of proteins of *Rana tigrina* from that of *Bufo himalayanas* certainly corresponds to their placement in different genera and families. This picture of protein variation of kidney which is related to their environmental conditions and physiological functions also justify their inclusion into different taxa of higher categories above species level.

*Rhacophorus maculatus* showed 19 non-nuclear protein bands of kidney. They shared 5 common bands with *Bufo melanostictus*, 6 common bands with *B. himalayanas*, 7 bands common with *Rana tigrina* and 5 bands with *Tylototriton verrucosus*. The protein identity values of *Rhacophorus maculatus* vs *Bufo melanostictus* was 0.16, *Rhacophorus maculatus*
V/S Bufo himalayanus was 0.17 Rhacophorus maculatus V/S Rana tigrina was 0.18. All these values indicate a divergence of this species from the other two groups at a very early stage. The relationship of Rhacophorus maculatus with the two other anuran species being more or less the same. Thus the genus Rhacophorus diverged from a stock which gave rise to Rana and Bufo so early and so distinctively at their evolutionary history that their inclusion in a separate family may be taken to be justified.

Tylototriton verrucosus showed 20 non-nuclear protein bands. It shared 5 common bands with Bufo melanostictus, 7 common bands with Bufo himalayanus, 8 common bands with Rana tigrina and 6 common bands with Rhacophorus maculatus. The protein identity values being 0.18 with Bufo melanostictus, 0.20 with Bufo himalayanus, 0.21 with Rana tigrina and 0.15 with Rhacophorus maculatus which indicate that Urodela and Anura had a common ancestry, from which Rhacophorus first diverged and then Bufo and Rana probably. This Indian salamander group which posses the distinctive protein pattern very different from the other four species, ultimately became highly specialised to their highly localized mountainous environment and could not spread over the diverse habitat of the large Indian continent.

During the processing of the data and writing the discussion, we were aware of the fact that proteins of
identical electrophoretic mobility are not necessarily of identical structure as pointed out by Dessauer and Fox (1964). Dessauer (1974) also commented that the possession of such proteins, when of common occurrence, was suggestive of a close relationship of the species involved. Lanza and Anatoniini, (1955); Paulov and Kmetová (1964); Hebard (1964); Manwell (1966); Chen (1967) and Salthe (1969) used electrophoresis of plasma and tissue proteins to distinguish many species of Rana from Europe and North America. Cei et al. (1968) presented a phylogenetic arrangement of species grouping of American toads by incorporating biochemical and immunological data. Guttman (1965) concluded that study of electrophoretic properties of tissue proteins offers a much needed additional means of characterizing salamanders. All these published accounts of the earlier authors, throw some light on the possibility and validity of the relationship and divergence of the amphibian species mentioned in the few earlier paragraphs of this section.
Like all other tetrapod vertebrates, most of the amphibian species possess lungs. In plethodontid salamanders, the lunglessness is a general character. Noble (1931) remarked that the simplicity of the amphibian lungs can be explained either as a retention of larval structure in the adult stage or as a secondary degeneration and not the mark of primitivism. The lunglessness of plethodontids are probably secondary adaptations. It is also known that the amphibian lungs serve hydrostatic functions more than respiratory. The lungs are more intricately developed in land-dwelling forms than in the aquatic ones. In amphibians, there has been a gradation in complexity of the structure and function of the lung; it seems possible that the proteins of this organ might also manifest qualitative and quantitative differences in relation to the importance of this organ to perform a hydrostatic or respiratory function. Such variations in the non-nuclear proteins may help the species to exploit the environmental conditions more successfully. Among the five species of amphibia we studied, _B. melanostictus_ and _B. himalayanas_ showed the highest amount of non-nuclear proteins. These two closely related species of the genus _Bufo_ inhabit to distinctly different environment and possess different habits and habitats. The tropical climatic land living form
B. melanoostictus shows well developed lung and depend on pulmonary respiration considerably along with the skin and buccopharyngeal respiration. While the temperate climatic toads B. himalayanae of Darjeeling inhabits a relatively colder and humid environment and depends largely on cutaneous respiration. These toads of Darjeeling were collected from small pools where they were seen to float in water, keeping their head out of the surface. Thus although it seems that for these species the pulmonary respiration is less economic than the skin respiration still they utilize the lung because considerable number of blood capillaries were found to be prominently displayed in the tissue. Thus the relative importance of the lung respiration of these two Bufo species is related to the more or less same amount of non-nuclear proteins of lung in these two species. Lungs of Rana starts functioning much early during their ontogenic development than the Bufo species which utilize the lungs not before metamorphosis (Savage, 1952). Rana tigrina lives in the small pools and ponds and are mostly aquatic. Thus the aquatic habit and habitat of this tropical species compelled them to utilize the skin surface for respiration than the lungs. It is also known from the works of Szopek (1955b) that skin of Rana possess more than 30% blood capillaries while the skin of Bufo possess only about 20% capillaries as determined by Bieniak and Watka (1962).
These findings indicate the relative importance of the skin of *Rana* in respiration than that of *Bufo* species, and a lesser dependence on lung respiration. This is also reflected on the lesser amount of lung proteins in *Rana tigrina* than that of the *Bufo*.

*Rhaeophorus maculatus* showed apparently low non-nuclear protein content but such difference in lung protein was not statistically significant. The members of this species live in temperate regions where the atmosphere is humid. Most of the specimens we examined were juvenile ones. These tree frogs possibly use their lungs as *B. melanostictus* do. From the functional point of view it may be expected that quantitative variation of non-nuclear proteins in the lungs may not be the usual case, whatever problems the species will find in their specific environment regarding lung functions in microadaptation level, qualitative variation of proteins may be enough to 'co-adapt' the species in their own environment. This aspect of qualitative protein difference will be dealt with later.

In *Tylototriton verrucosus* the non-nuclear protein content of lung was found to be significantly lower than that of *B. melanostictus*. *Tylototriton verrucosus* is a purely aquatic species living in mountain streams and bogged mountain pools, where cutaneous respiration seems to be more facilitative.
than pulmonary respiration. On the other hand the lungs of these species are elongated sac-like structures though highly vascularized in the internal epithelium lining the lumen as seen in histological sections. Possibly the lungs perform hydrostatic function than respiratory. Thus it is not unlikely that this secondarily reduced organ will contain significantly lesser amount of non-nuclear protein.

By polyacrylamide gel electrophoresis the non-nuclear proteins of *Bufo melanostictus* lung showed 17 bands while *B. himalayana* showed 16. Thus one protein band was absent in the lung extract of *B. himalayana* which was present in *B. melanostictus*. Three bands of *B. himalayana* lung had differences in mobilities from the corresponding bands of *B. melanostictus*. Such qualitative variations in proteins indicate differences in genotypes of these two species which were probably favoured by their respective environments. While the lung carries out the function of CO2 excretion, the kidney of *Bufo melanostictus* performs major functions in excretion metabolites and preservation of water balance. Thus the lung is functionally effective in a gaseous environment. The lung alveolar epithelium on its one side is in contact with gaseous environment of the atmosphere, while the other side is in contact with the fluid filled tissues. The lung also absorbs and excretes gaseous matters through its alveolar epithelium and the endothelial lining of its subepithelial capillaries. Thus the nature of functions and the immediate environments in which
lungs functions for both the species *B. melanostictus* and *B. himalayanus* is not so variable as it is for the kidney. *B. himalayanus* kidney excretes more water along with other substances than *B. melanostictus* kidney. This is because *B. himalayanus* live in humid or aquatic environment of the temperate zones, while *B. melanostictus* live in relatively dry and tropical environment. Thus the kidney of *B. himalayanus* shows the possible difference in functions from that of *B. melanostictus*. The lung on the other hand shows relatively little difference in their function in both these two species. This functional aspect of these two organs are associated with their protein patterns. The comparatively greater difference in function of the kidneys demand a relatively greater difference in their protein patterns for these two species. Since the lung functions follow a conservative attitude in the two *Bufo* species in question, the demands of the lungs of these two species regarding their proteins does not seem to be different. Such a picture is reflected in the non-nuclear protein patterns of the lungs of *B. melanostictus* and *B. himalayanus*. However, it can be said that the greater difference in non-nuclear protein patterns of kidney than that of the lungs between the species *B. melanostictus* and *B. himalayanus* may be explained in the light of the above discussion. The non-nuclear protein identity value of *B. melanostictus* and *B. himalayanus* was found to be 0.39 which indicate a closer relationship
among these two species. This identity value of lung appears to be higher than the kidney identity value. This may be co-related to the lesser divergence of function of the lungs of these two species and thus the preservation of the greater number of identical proteins of the lungs of these two species as demanded by the ecophysiological conditions.

*Rana tigrina* lung non-nuclear proteins showed 13 bands and a gross resemblance to the *Bufo* pattern, though differences exist in their details. There were 9 identical bands in the lung of *Rana tigrina* and *Bufo melanostictus* while about same number of bands showed difference in this organ of the two species. *Rana tigrina* is an aquatic species, though considerably depend on lung respiration but still in lesser extent than the *Bufo* species. Thus the developmental (i.e. early functioning of lung) and functional difference of the organ may perhaps be related to the difference in their structural level. Besides the developmental and structural differences these two species show notable differences in their morphology which was the reason of their inclusion into two different genera and families. The non-nuclear protein banding pattern and their calculated identity value of 0.26 corresponds to their developmental, function and structural differences and also support their taxonomic positions as mentioned earlier.
Rhacophorus maculatus lung non-nuclear proteins were fractionated into 20 bands. The general banding pattern was very different from that of Bufo and Rana. There were 10 identical bands in Rhacophorus maculatus and Bufo melanostictus out of total 37 bands and 10 bands were identical with Rana tigrina out of total 39 bands. This tree dwelling temperate zone species appears to depend largely on lung respiration as their ecological opportunities permit. The non-nuclear protein identity of this species with B. melanostictus and Rana tigrina show a calculated value of identity as 0.27 and 0.26 respectively. These values indicate their probable place in the taxonomic scale at a position of about same distance from both the Bufo and Rana group. Possible divergence of this Rhacophorid from a basic stock which gave rise to Bufo and Rana groups are supported by equidistance of their values on the identity scale.

Tylototriton verrucosus lung non-nuclear proteins showed 19 bands on polyacrylamide gels. The general banding pattern was quite distinct from all the other species, studied. Of all these 10 bands only 8 were identical with Bufo melanostictus, while 3 bands were identical with Rana tigrina and 7 bands were identical with Rhacophorus maculatus. The calculated identity values were 0.22, 0.21 and 0.18 respectively. The lung of Tylototriton is more a hydrostatic organ than respiratory. Thus the distinctly different pattern showed by
the proteins of *Tylototriton verrucosus* is in partial compliance at least if not full, with the distinctive functional aspect of the organ and support their inclusion in a different order, *Urodela* from the rest of the genera studied i.e. *Bufo*, *Rana* and *Rhacophorus* of the order *Anura*. 
Unlike reptiles, birds and mammals, where the body surface is covered with a waterproof skin which helped them to suit the terrestrial habitat and withstand the process of desiccation, amphibia possess a specialized skin to suit life in water as well as on land. Besides the protective, excretory and temperature regulatory functions of skin of higher vertebrates, the amphibian skin has to perform some special functions. These are respiratory and regulatory function with respect to water balance. Anurán skin has also been reported to perform special protective function against desiccation which is not found in other groups of amphibia. Such special functions of anurán skin has been reviewed by Elkán (1976).

The mode of life of different groups of amphibia show variability in their degrees of exploitation of the aquatic and terrestrial habitat. Some species are more aquatic than others, while some are mostly terrestrial. Thus the variation in habit and choice of specific habitat may be related to the histo-anatomical structures of different organs and their molecular components. The type of habitats i.e. whether aquatic or terrestrial imposes special threats from problems of respiration, excretion, water balance and protection. To combat these problems the amphibian skin takes major part in these functions. To perform the varying functions of the skin...
in different groups of amphibia, there is a possibility of occurrence of variation in the molecular components of this organ. Michl and Kaiser (1962, 1963); Cei and Erspamer (1966) used skin components for characterizing anuran families. Bagnara and obika (1965) are of opinion that the similarities in skin pteridines of Bufonidae and Pelobatidae indicate a more closer relationship among the two than present taxonomy shows. Differences in patterns of distribution in skin of the various metabolites like biologically active polypeptides, imidazole amines, phenylalkyl amines, variety of indole amines, pteridines and other toxins are also useful in distinguishing certain genera (Cei et al., 1968); Hungser et al. (1961); Wittliff (1962, 1964); Porter (1964); Cei and Erspamer (1974); Cei et al. (1968); Low (1968) showed that the skin in each group of Bufo possess a fairly unique spectrum of indole alkylamines and other metabolites. In the present study intrafamilial, intrageneric and intraspecies differences of non-nuclear proteins have been observed. In non-nuclear protein content of skin of Bufo melanostictus and Bufo himalayanas there was no significant quantitative difference; qualitatively, however, these proteins differ. although a close similarity in general pattern was seen to occur. The number of electrophoretic bands of skin non-nuclear proteins in Bufo melanostictus was 17 and in Bufo himalayanas was 19. There were two extra bands in Bufo himalayanas which were completely absent
in *B. melanostictus*. Moreover two more bands showed variations in their mobilities. Thus the electropherograms of these two species showed characteristic differences of protein patterns. Such differences in non-nuclear proteins of skin may probably be due to genetic differences and might help these species to suit their specific environments. The specific habit and habitats are remarkably different. One is more terrestrial and tropical in distribution and the other is more aquatic and inhabits temperate zones. In the first case, the members of the species *B. melanostictus* is liable to face desiccation and higher metabolic rate than the second. In the second case, the environment is either more humid or aquatic, thus the animal has less chance to face desiccation, and due to the lower temperature its metabolic rate also may be lower. It is also worth mentioning that gases are more soluble in water in comparatively low temperatures, thus facilitating respiratory and excretory functions through the skin. These non-nuclear protein electrophoretic patterns of skin of these two *Bufo* species follow the findings on kidney non-nuclear protein patterns and support the idea of separation of the species *B. himalayensis* from *B. melanostictus* stock. The non-nuclear protein identity value of skin is 0.41 and thus indicate a closer relationship among these two species. This also indicates a more conservative nature of this organ than kidney,
regarding genetic changes in non-nuclear protein differentiation
during speciation.

*Rana tigrina* skin possess significantly lower
non-nuclear protein content than the skin of the other four
species studied. There were 22 electrophoretically separated
non-nuclear protein components in *R. tigrina*. Among these
protein bands, 12 were identical with *Bufo melanostictus*
and *Bufo himalayanas*. A good number of bands showed differences
in their mobilities while a number of other bands were found
in the case of *R. tigrina* which were not present in *Bufo
melanostictus* and *Bufo himalayanas*. Such a difference in
protein pattern justify the inclusion of this widely distributed
aquatic species of India not only in a separate genus but in a
separate family also. An identity value of 0.50 indicates a
closer relation of this species with *Bufo* and their separation
on a much higher taxonomic level, i.e., in the family taxa
from a common stock from which the family Bufonidae diverged.

*Rana tigrina* skin contained significantly
lower non-nuclear proteins than *Bufo himalayanas* but higher
than *Rana tigrina*. This may be related to the juvenile
condition of these tree frogs and their specific mode of life.
These frogs live on the undersurface of leaves as mentioned
earlier and reflect a distinctly different mode of life than
*Bufo himalayanas* or *Rana tigrina*. The two latter mentioned
species are aquatic in habit, one of them i.e. Bufo himalayanas is a temperate zone living species and the other i.e. Rana tigrina being a tropical zone species. Thus this difference in mode of life may be related to the characteristic protein content of the skin of these species. There were 20 non-nuclear protein bands whose pattern on electropherograms was different from the patterns of Bufo and Rana. The electrophoretic identity values of proteins of Rhacophorus maculatus with Bufo and Rana were more or less same i.e. 0.27 with Bufo melanostictus, 0.23 with Bufo himalayanas and 0.26 with Rana tigrina. These indicate the identical relation of Rhacophorus with Bufo and Rana, which is in full agreement with the studies from kidney and lung of these species.

Tylototriton verrucosus contained more or less the same amount of protein in skin as it was found in Bufo melanostictus, Bufo himalayanas and Rhacophorus maculatus. But this amount was significantly higher than that of Rana tigrina. Qualitatively the non-nuclear protein of skin of this species was separated into 24 components, whose banding pattern was distinctly different from other four species studied. This very characteristic protein pattern may be a specialized form of skin protein differentiation. This findings is in agreement with the inferences drawn from the findings of non-nuclear
proteins of kidney and lung of this species. The calculated identity values of non-nuclear proteins of skin of when compared *Nectophryne verrucoglossa* with *Bufo melanostictus* and *Bufo himalayana* was 0.26 and 0.23 respectively, and with *Rana tigrina* was 0.28 and with *Rhacophorus maculatus* 0.29. All these data support the findings of kidney and lung and indicate more or less identical relations with all the anuran species studied.
COMPARISON OF NON-NUCLEAR PROTEINS OF KIDNEY,
LUNG & SKIN IN AMPHIBIA.

From the results of the studies of non-nuclear proteins of the three tissues kidney, lung and skin, it is evident that the kidney contained the highest amount of non-nuclear protein than the two other organs. The skin had the lowest non-nuclear protein content in all the cases. The difficulty in homogenization and toughness of skin may be one of the factors responsible for the lesser values of non-nuclear proteins of this organ as compared to the other two organs. The kidney tissues of amphibia were soft and easier to homogenize than the lung tissues. In Rhaeophorus maculatus the kidney and lung had comparable non-nuclear protein content while the skin had less. This may somewhat be related to the soft nature of the juvenile lung which facilitated complete homogenization of this organ. Further the organs of these juvenile frogs may not have completed their full structural and functional differentiation.

The cells of the different organs differentiated in their own way although their genetic constitution is the same. Such structural and functional differentiation of the organs are thus epigenetic in nature. The highest or lowest amount of protein of an organ is not related to the quality of proteins of that organ. An organ containing highest amount of
non-nuclear protein may contain the lowest number of electrophoretic bands of proteins as it was seen in case of kidneys of *Bufo melanostictus*. *Rana tigrina* skin showed lowest amount of non-nuclear proteins but it contained 22 electrophoretic bands. The kidney of this species showed 19 bands although it had sufficiently high amount of non-nuclear proteins. *Tylotriton verrucosus* showed highest number of bands i.e. 24, while it has shown comparably lower amount of non-nuclear proteins than the two other organs. Thus the quantitative variation of non-nuclear proteins of the different organs of the same species, where the genetic constitution is the same, may be due to differences at the transcription level of protein synthesis. For living process itself certain enzymes are necessary, e.g. the enzymes of TCA cycle or the enzymes required for membrane stability and possibly these are common to all tissues whether kidney, lung or skin. Yet the different organs have to perform different specific functions which demand specific enzymes or enzyme systems for that organ, what is reflected in the qualitative variation of proteins of the various organs. At last it may be worth mentioning to remind again the quantitative variation of non-nuclear proteins of different organs within the same species may be due to structural differences of the organs which created relative difficulties in homogenization and extraction of proteins.
The renal carbonic anhydrase provides $H^+$ ions from carbonic acid to exchange for cations flowing through the tubules and helps in the regulation of both the electrolyte concentration and acid-base balance of the extracellular fluid. As in the mammals, acidification of the urine in amphibia is partially dependent upon carbonic anhydrase. The five amphibian species we have studied, are varied in their distribution and consequently in their habit and habitat. The relative dependence on aquatic and land habitat of the different species is thus associated with special problems of regulation of water balance and excretion. The availability of water from the immediate environment get uncontrolled entry into the body of the amphibia through the extensive body surface. The responsibility of regulation lies on the kidneys, and so different amounts of liquid are excreted through the kidneys in different species of amphibia. Naturally the relatively terrestrial species excretes less water through their kidneys under normal conditions than the aquatic species.
Hence there is a possibility of variation in excretion and regulation of electrolytes along with the variation in the excretion of water through the kidneys in the different species of amphibia. Thus the study of carbonic anhydrase in the excretory organs of the amphibia becomes a matter of concern. Remarkable variations of renal carbonic anhydrase activity was observed among the five amphibian species. From the results as shown in table 19 and 20, no intrageneric variation in renal carbonic anhydrase activity among the two closely related species Bufo melanostictus and Bufo himalayana was evident. These two species inhabit two distinctly different environmental niches and with different habits as mentioned earlier during our discussion on non-nuclear proteins. Although significant quantitative variations were not seen in renal carbonic anhydrase activity in these two species, qualitative variations like electrophoretic patterns of the enzyme proteins were seen which will be discussed later on. Significant differences were observed in distantly related species of different genera from different families and even from different orders. Among the anuran species it was observed that relative body size of the individuals of different species may have some apparent relation to the specific activities of renal carbonic anhydrase. In general the body size of the Rana tigrina members were largest among the species we studied. These were about 6 to 7 inches
from snout to vent. Specimens of *Bufo melanostictus* and *Bufo himalayana* were mostly about 5 inches from snout to vent, while the specimens of *Rhacophorus maculatus* were within 1 inch from snout to vent. These were mostly juvenile ones. The average specific activities of carbonic anhydrase differed significantly (from 64 to 65 μl CO₂/min/mg protein for both the species *Bufo melanostictus* and *Bufo himalayana* and 46 μl CO₂/min/mg protein for *Rana tigrina* which is about 68% of the *Bufo* activity. Expressed per mg protein the activity was 111.16 μl CO₂ liberated per min for *Rhacophorus maculatus* which was about 170% of that of the activity in *Bufo*. The kidneys of *Tylototriton verrucosus* had low enzyme activities, (only one third of the activity of *Bufo*). In some specimens no appreciable activity at all could be detected. It may be conceived that such a small amount of CO₂ liberation may be due to the activity of the erythrocytic carbonic anhydrase present in the blood content of the kidney tissues. This chiefly aquatic species is very sluggish in nature and also showed appreciably low O₂ consumption and CO₂ elimination which indicate a low metabolic rate. Thus the total absence or presence of a low activity of carbonic anhydrase in amphibia, at least, if not in other animal groups, may be related to the metabolic rate and habit of the animal. In case of absence of the renal enzyme, the species probably carries out the usual function of the organ at a lower rate by the uncatalyzed
reaction, which incidentally is quite high for carbonic anhydrase. Such instances are reported by Maren et al. (1963) in the alkaline glands of the Skate, R. Stabuloforis. Moren (1967) also mentioned a report of Dr. Goldstein in a personal communication that *Xenopus laevis*, the African clawed toad showed no renal response to acetazolamide. He concluded that the enzyme appears to be present, but like the marine fishes it may be due to the haematopoetic tissues. From our studies we can conclude that all the anuran species we have studied surely contain renal carbonic anhydrase, but doubt exists for the renal carbonic anhydrase of the Indian salamander (*Tylototriton verrucosus*). We also observed that the Tylototriton kidney glomerulus and the renal tissue in general were poorly vascularised, when the histological sections of *Tylototriton* and *Bufo* were compared. (Plate II a & b; 13 a & I).

2. Isozymes:

There is no satisfactory method of demonstration of carbonic anhydrase activity on proteins separated by disc electrophoresis. The usual histochemical method used by various authors in tissue slices was that of Hausler's modification (1958) of the Kurata technique (1953). Following this technique, the gel was incubated in a medium containing $\text{HCO}_3^-$ and $\text{Co}^{++}$. The immediate precipitation of basic cobalt salts was prevented by adding $\text{SO}_4^{2-}$ to the medium and keeping the pH at about 7.4.
β-naphthyl acetate esterase isozyme pattern of kidney in amphibia. (i) Without diamox; (ii) with diamox.
(a) *B. melanostictus*. (b) *B. himalayanua*. (c) *R. tigrina*.
(d) *R. maculatua*. (e) *T. verrucosus*. 

![Image of gel electrophoresis](image-url)
β-naphthyl acetate esterase isozyme pattern of lung in amphibia. (i) Without diamox; (ii) with diamox. (a) *B. melanostictus*, (b) *B. himalayanus*. (c) *R. tigrina*. (d) *R. maculatus*. (e) *T. verrucosus*. 
β - naphthyl acetate esterase isozyme pattern of skin in amphibia. (i) Without diamox; (ii) with diamox. (a) *B. melanostictus*. (b) *B. himalayanus*. (c) *R. tigrina*. (d) *R. maculatus*. (e) *T. verrucosus*. 
**Plate - 11 a.**

*T. verrucosus* kidney section showing poorly vascularized glomerulus and renal tissue. H.E. x 320.

**Plate - 11 b.**

*B. melanostictus* kidney section showing relatively well vascularized glomerulus and renal tissue. H.E. x 320.
Plate - 12 a.
Same as fig. 11 a. H.E. x 800.

Plate - 12 b.
Same as fig. 11 b. H.E. x 800.
Plate - 13 a.

_T. verrucosus_ kidney section showing relatively few erythrocytes in the glomerulus and renal tissue. Feulgen with Mallory's -II. x 320.

Plate - 13 b.

_E. elanostictus_ kidney section showing relatively large number of erythrocytes packed in the glomerulus and renal tissue. Feulgen with Mallory's- II. x 320.
Plate 14 a.
Same as fig. 13 a. Feulgen with Mallory's-II x 800.

Plate 14 b.
Same as fig. 13 b. Feulgen with Mallory's-II x 800.
Pl. 15 a.
*T. verrucosus* showing long tube-like lung.

Pl. 15 b.
*B. melanostictus* showing sac-like lung with honey comb appearance.
Plate - 16 a.
Section of *T. verrucosus* lung at the distal portion of the respiratory tube showing multiple lumen lined by epithelial cells which are flanked containing erythrocytes. H.E. x 32.

Plate - 16 a'.
Section of *T. verrucosus* lung at the middle portion of the respiratory tube showing the main air passage with intercalated extensions lined by epithelial cells which are flanked at place by capillaries containing erythrocytes. H.E. x 32.
Section of *B. melanostictus* lung showing alveolar formations lined by thin epithelium flanked with extensive capillaries containing erythrocytes. H.E. x 80.
Plate - 17 a.

*T. verrucosus* lung section showing erythrocytes, few in number lie just adjacent to the epithelium lining the air sac. H.E. x 320.

Plate - 17 b.

Section of *B. melanostictus* lung showing multiple air sacs lined by epithelium which have extensions into the air cavity. Erythrocytes are numerous in the capillaries adjacent to the epithelium, some times jutting into the air sacs. H.E. x 320.
Section of *B. melanostictus* lung from which air was squeezed out. Feulgen with Mallory’s - II. x 320.
Plate - 18.
Same as Fig. 16 a. H.E. x 800.
Section of *T. verrucosus* skin showing the epidermis pierced by capillaries containing erythrocytes which also shows melanophores and various glands. H.E. x 80.

Section of *B. melanostictus* skin showing epidermis which is thinner than that of *T. verrucosus*. The capillaries are seen to infiltrate the epidermis and lie close to the surface. It also shows melanophores and various glands. H.E. x 80.
Plate - 20 a.

*T. verrucosus* skin section showing erythrocytes adjacent to epidermis. H.E. x 800.

Plate - 20 b.

Section of *B. melanostictus* skin showing erythrocytes adjacent to the epidermis. H.E. x 320.
Near the protein bands, containing carbonic anhydrase activity, there was localised liberation of \( \text{CO}_2 \) by dehydration of \( \text{HCO}_3^- \) and consequent formation of \( \text{CO}_2 \text{CO}_3^- \). The carbonate was changed to \( \text{CO}_3^- \) after a brief incubation with \( (\text{NH}_4)_2\text{S} \). The specificity of the reaction was challenged by Faund et al. (1959) and Diamanstein et al. (1964). Hausler's method was further modified by Hanson (1967, 1968) which was further challenged by Muther (1972). Roše and Musser (1972) checked the specificity of the Hanson's histochemical method and met the objections raised by Muther. Lonnerholm (1974) also studied critically the cobalt phosphate method of Hanson and observed the specific nature of the reaction, thus supporting the claims of Hanson.

When we used the method of Hausler to locate the carbonic anhydrase bands on polyacrylamide gels after electrophoresis we got six bands for the kidney tissues of *Bufo melanostictus* having Rf's of 0.64, 0.53, 0.34, 0.17 and 0.06. The 3rd and 4th bands were stained the most, of which the 4th band appeared as a broad black region. When incubated with acetazolamide at a concentration of 0.25 mM some bands were completely inhibited while others were inhibited only partially. When we tried to demonstrate the enzyme activity by other colour reactions of the enzyme we observed differences in the banding patterns. In some colour reactions difficulties arose in identifying and photographing the bands. Tashian (1965)
utilized acetazolamide inhibitable \(\beta\) naphthyl acetate esterase to demonstrate erythrocytic carbonic anhydrase. But Maren (1967) mentioned that tissue carbonic anhydrase, notably kidney, lacked esteratic properties. In our study also we could not find satisfactory acetazolamide inhibition in all the cases, when compared to the Häusler’s method.

Using the \(\beta\)-naphthyl acetate esterase method on polyacrylamide gel we detected seven bands with the \(Rf\)'s 0.64, 0.57, 0.51, 0.35, 0.17, 0.11 and 0.04. In this case there was only about 50% inhibition with acetazolamide, having no discrimination for any particular band. About four of the seven esterase bands showed different mobilities from those of the Häusler’s carbonic anhydrase bands.

We also employed Pihar’s (1965) method of staining carbonic anhydrase bands after electrophoresis with bromothymol blue. This method is based on the hydrating properties of the enzyme. Tashian (1969) advocated that Pihar’s technique is the correct method of demonstrating carbonic anhydrase bands on gels. After separating the soluble proteins on polyacrylamide gels by electrophoresis we could detect only three bands which mostly corresponded to the three slow moving bands of Häusler’s reaction. But we had difficulties of photographing the bands produced by Bromothymol blue staining reaction.
The extract, which was used to demonstrate the isozyme bands by Hausler's, Tashian's, and Pihar's method was separated by polyacrylamide electrophoresis in triplicate. After electrophoresis the gels were divided in equal 3 mm. pieces. These pieces were then extracted in phosphate buffer to elute the isozymes. Each of the eluted extracts was then assayed for carbonic anhydrase activity with the Warburg's apparatus as detailed in the section on methods. Most of the enzyme activity was present in the middle of the gel. Results showed 5 main peaks of activity. When we compared the graphical representation of the enzyme activity of the eluates with those of the three histochemical methods of demonstration of carbonic anhydrase, it was observed that the bands of Hausler's method represented carbonic anhydrase bands, most closely. Pihar's CO₂ hydration bands also represented carbonic anhydrase but it was not possible to detect all the bands. This may be due to the transparent nature of the polyacrylamide gel medium. β-naphthylacetate esterase bands of the tissues did not represent carbonic anhydrase. Where some inhibition with acetazolamide was observed, it could be due to the nonspecific interference of this drug with the compounds used in the histochemical reaction, resulting in a reduction of staining of β-naphthol. Lastly it can not be fully denied that chances of artifact formation may prevail during processing and staining of the gels in all the methods
discussed above to demonstrate isozyme bands on polyacrylamide gels. Hodgen and Gomes (1969) utilized the CoS staining method on polyacrylamide gels but cleared them with 7% acetic acid after staining. He concluded from his studies that CoS staining positively represent acetazolamide inhibitable carbonic anhydrase isozyme bands on polyacrylamide gels. But in our study we used 0.5% $\text{H}_2\text{O}_2$ as a destainer and compared it with 7% acetic acid cleared gels. We observed that 0.5% $\text{H}_2\text{O}_2$ cleared the gels more effectively than 7% acetic acid. Moreover acetic acid converted the gel background opaque. We also followed Hanson’s modification and compared it with Häsler’s method but we found no qualitative difference in the results of the two methods. Ultimately throughout our study we followed the Häsler’s modification of Kurata method to demonstrate carbonic anhydrase isozymes on polyacrylamide gels although we were not, even now, fully sure which of the methods truly demonstrate carbonic anhydrase isozymes on polyacrylamide gels.

The enzyme proteins showing carbonic anhydrase activity have been shown to be polymorphic on polyacrylamide gels, stained with CoS according to Häsler’s technique in the kidney tissues of four species of Anura and one species of Urodela. In *Bufo melanostictus* there were six bands of carbonic anhydrase which mostly corresponded to the regions of the gels showing carbonic anhydrase activity when assayed with
Raughton & Meldrum's manometric technique. Yet the control gels which were treated with acetazolamide presented confusing results. Among the six bands only three bands were inhibited completely while the other three bands showed incomplete inhibition. For all the amphibian species we studied, such confusing results were obtained. While in manometric techniques acetazolamide behaved as an active inhibitor to all the eluted enzyme proteins, why it failed to do so on the gels is not clear. *Bufo himalayana* the relatively cold adapted water loving high altitude species showed 9 bands of which four were completely inhibited by acetazolamide while the other five bands were incompletely inhibited. This multiplicity of the carbonic anhydrase enzyme proteins in this species showing highly specialized distribution may have some adaptive significance.

There were six bands of CoS stained carbonic anhydrases in *Rana tigrina*, the widely distributed frog species in Indian sub-continent. This banding pattern and the position of the diamox inhibitable bands are different from those of *Bufo melanostictus*, the widely distributed toad species of Indian sub-continent.

*Rhacophorus maculatus* pattern was also different from those of *Bufo* and *Rana* both. Here the fast moving bands mostly showed complete inhibition when the gels were treated.
with diamox. It is also noticeable that Tylototriton verrucosus like Bufo himalayanae and Rhacophorus maculatus a temperate zone, high altitude species, contained 10 renal CoS stained bands. These data indicate a probable relation of number of bands to the specific environmental conditions. The high altitude temperate zone species showed larger no. of bands that the tropical zone plain land living species. It is also perplexing to note that Tylototriton verrucosus kidney exhibited significantly lower amount of carbonic anhydrase activity, but very prominent and a large number of bands were seen from 100 μg. of tissue proteins when added on polyacrylamide gels and were subjected to electrophoresis. The reasons of such an occurrence of bizzazy behavior of staining carbonic anhydrase bands from samples of significantly low quantitive carbonic anhydrase activity were not clear to us.

while studying the enzyme carbonic anhydrase in the excretory organs like the kidneys in in amphibia, we were interested about the differences of the enzyme patterns among the different amphibian species. In course of this investigation we also tried to find out whether the isozyme patterns of the different excretory organs in the same species and in the same individual differ significantly. To cover all these aspects we had to deal with a large no. of individuals from different species and ultimately tried to detect the species
specific pattern of carbonic anhydrase isozymes. We hope this may help in identifying species and thus in the study of taxonomy and systematics of amphibians. For such purposes, we did not consider the blood contamination of the organs as a very important one, because the differences observed are much too high for a blood content of around 10%. We, therefore, did not try to perfuse the organs to make them free of blood. Wistrand (1975) showed that in perfused human kidney there was only one form of carbonic anhydrase which was similar to the erythrocytic form of carbonic anhydrase 'C'. This was in contradiction of his earlier reports that human kidney contain 3 isozymes of carbonic anhydrase, as it is found in the erythrocytes. In his earlier studies he used unperfused kidney tissue for the detection of carbonic anhydrase isozymes. Thus there is a possibility that perfused organs may show different patterns of isozymes in these amphibian organs, studies of which have been included in our future programmes.
B. **LUNG.**

1. **Activity:**

Erythrocytes are nowhere exposed directly to the air for gas exchange. It is only through various membranes and films that the exchange can occur between the gas phase of the outside air and the hemoglobin of the erythrocytes. For example, in a mammalian lung, the hemoglobin is separated from the exchangeable air within the alveolus by the membrane of the erythrocyte, the basement membrane of the alveolus, the stretched alveolar cytoplasm, and the membrane of the alveolus. If the process of gas exchange had to depend only on the partial pressure of the gases between the two phases, it would have taken a long time for the diffusion to occur. But the process is greatly facilitated by the enzyme carbonic anhydrase. This is so, for two reasons. One is that the process can be facilitated, and the second is because of the nature of the enzyme. CO₂ can be hydrated and dehydrated according to the particular situation and demand.

Fisher (1961) and Maren (1967) expressed that the activity found in homogenates of adult mammalian lung was due to erythrocyte contamination. Chinard et al. (1962) showed that when HCO₃⁻ was injected into the trachea, it was rapidly converted to expired CO₂ which was slowed down after the
administration of acetazolamide. On the basis of these findings, Maren further expressed that lung carbonic anhydrase clearly exists, though its role was not defined. From the biochemical studies of perfused dog lung, Chinard (1969) clearly showed that nonerythrocytic pulmonary carbonic anhydrase existed. In the lungs, the erythrocytic carbonic anhydrase dehydrates $\text{H}_2\text{CO}_3$ to $\text{CO}_2$ which becomes hydrated by pulmonary carbonic anhydrase from the luminal side of the capillary through endothelial lining to alveolar side. At the alveolar side again dehydration occurs and gaseous $\text{CO}_2$ escapes to the alveolar air. The complicated series of events are regulated by the partial pressure of carbon dioxide in the different compartments. Regarding the significance of carbonic anhydrase in the pulmonary endothelium of amphibians and reptiles, Pain and Rosen (1973), commented that large erythrocytes and lower temperatures in these animals impede maximal red cell carbonic anhydrase function, which is compensated, at least partly, by the endothelial carbonic anhydrase of the lungs. We studied the activity of carbonic anhydrase in five species of the class amphibia. Results showed some remarkable variations among some of the species of &bull; amphibia. *Bufo melanostictus* and *Bufo himalayanus* showed comparable activity, having no significant difference between the two mean values. These activities were $50.18/\mu\text{l CO}_2/\text{min/mg protein}$ and $44.73/\mu\text{l CO}_2/\text{min/mg protein}$.
respectively. Rana tigrina lung homogenates showed an activity of 40.30/ul CO₂/min/mg protein which was not significantly lower than that of Bufo melanostictus. But this mean value of carbonic anhydrase activity of Rana tigrina and its relation with Bufo melanostictus differed from the data published by Pain and Rosen who obtained only 10% of Bufo marinus activity for Rana pipiens lung per gm. wet weight. They argued, in favour of their data, that biochemical activity of carbonic anhydrase in different animals per gm. dry weight corresponds to the extent of pulmonary capillary masses of the animals. Foxon (1964) mentioned in a tubular form that a number of species of Rana contain only 10% less capillaries in the lung than that of Bufo species. Rahn et al. (1976) quoted that data published by a number of authors, where it was shown that the relative percentage of CO₂ removed through the lung varied from 24% to 43% in different species of Rana while it was 17% to 31% in different species of Bufo. These data may throw light on some possibility of the occurrence of a comparable carbonic anhydrase activity in Bufo melanostictus and Rana tigrina. Rhacophorus maculatus showed apparently 30% high carbonic anhydrase activity than that of Bufo melanostictus, but it had significantly higher carbonic anhydrase activity than that of Bufo himalayanus, Rana tigrina and Tylototriton verrucosus. Such a high activity may be related to the smaller
body size of the species, their high altitudinal distribution and temperate climate. *Tylototriton verrucosus* lung had only 18% of *Bufo melanostictus* pulmonary carbonic anhydrase activity. Such a small activity may actually be due to erythrocytic content of the lung. These sluggish animals, whose CO₂ elimination and O₂ consumption rate is comparatively lower than that of *Bufo melanostictus*, may contain a lower amount of protein having carbonic anhydrase activity in the pulmonary tissue specially in the capillary endothelium. The *Tylototriton* lung is a long sac like organ with scanty pocket like infoldings but there was no alveolar formation when compared with *Bufo* as was seen in the histological sections. (Plate 15b). Thus the total amount of pulmonary capillary endothelium was lower in *Tylototriton* than in *Bufo*. This may be the cause of occurrence of lower amount of pulmonary endothelial protein having carbonic anhydrase activity in the lung of *Tylototriton verrucosus*. 
2. **ISOZYMES.**

When supernatants of lung homogenates of *Bufo melanostictus* were subjected to electrophoresis and stained with Hāusler's method, 6 bands corresponding to those of kidneys were seen. The pattern of diamex inhibition on gels was same as that of kidney. In *Bufo himalayensis* there were 8 bands on the gels and the pattern was somewhat different from that of the kidney of the same species. There was a superficial resemblance with that of *Bufo melanostictus*. Thus the isozyme pattern of lung varies from that of kidney of the same. Wright and Mayer (1966); Eppenberger et al. (1967); Chen (1968); Ma and Fisher (1968) have already shown that isozyme patterns often vary from tissue to tissue as quoted by Dessauer (1974). Such variations are dependent upon the number of gene loci active in different tissues, the tendencies of the polypeptide gene products to aggregate, the number of polypeptide subunits per isozyme and the resolving power of the electrophoretic method, as pointed out by Dessauer (1974).

In case of *Rana tigrina* there were six CoS stained carbonic anhydrase bands on the gels electrophoresed with lung homogenates. The mobilities and relative positions of the bands varied from that of *Bufo melanostictus* and *Bufo*
himalayanus. The isozyme pattern of lung carbonic anhydrase was similar to that of kidney and thus there was no tissue variation of isozyme pattern of carbonic anhydrase in this species, at least for the two tissues, kidney and lung. *Rhaeophorus maculatus* lung had 12 bands, this pattern was different from that of kidney where there were 10 bands. Some bands showed differences in their mobilities. The relative stainabilities of the different bands of lung carbonic anhydrases also differed from that of kidney. Thus the tissue variation of carbonic anhydrase isozymes were observed in *Rhaeophorus maculatus*.

*Tylototriton verrucosus* showed 8 bands and the banding pattern was similar to that of kidney. The *Tylototriton* carbonic anhydrases showed a distinctly different type of banding on the gels, from the carbonic anhydrase isozyme patterns of all other anuran species, we studied. There was a gross similarity in the isozymograms of carbonic anhydrases of the four anurans described here. Like the kidney carbonic anhydrases, the lung carbonic anhydrases also showed a tendency to increased number of bands in the temperate zone species than in the tropical zone ones. Thus in amphibia, relatively greater number of bands may have some relation to the temperature of the
Further it is interesting to note that the supernatants from lung homogenate of Tylototriton showed significantly lower activity which was not more than 18% of that of Bufo melanostictus. When the same supernatant was applied on acrylamide disc gels containing 100 μg protein per tube, exhibited a clear and prominent zymogram like the other amphibian species with considerably higher carbonic anhydrase activity. The reason for such a peculiar phenomenon is not clear.
Amphibia have a dual system of gas exchange. The lung exchanges with air, while the gill and/or skin exchanges gases with water (Rahn et al. 1976). In reality the amphibian skin has a double role in gas exchange, i.e., skin serves its gas exchange function in air as well as in water. Rahn et al. (1976) also showed that at about 17°C water and air are equally efficient in removing CO₂ from the skin. Below 17°C water becomes more efficient in removing CO₂ than air while above 17°C air acts as a more effective medium for the transport of CO₂. They also pointed out that assuming a similar convective flow over the skin in air and water where diffusion of CO₂ in the medium plays a minor role, then little morphological changes are demanded to adopt the changes in its environment. However skin is the primary CO₂ eliminator for the aquatic and terrestrial species, making no difference between the two medium. About 75% of the CO₂ is eliminated by the skin (Foxon 1964; Rahn et al. 1976).

In amphibian skin the capillaries extend their supply to very near the external body surface leaving only one or two layers of epidermal cells outside. The erythrocytic carbonic anhydrase dehydrates H₂CO₃ to CO₂ which is hydrated in the capillary endothelial cells to its luminal
side and dehydrates towards the epidermal cells, which in turn is hydrated and dehydrated within the epidermal cells to escape to the outside air or water. The complicated series of events are regulated by the partial pressure of carbon dioxide in the different compartments and facilitated by the catalytic action of carbonic anhydrase. Maren (1967) expressed that frog skin lacks carbonic anhydrase, but Rosen et al. (1973) detected carbonic anhydrase in Rana pipiens skin, both biochemically and histochemically. For the failure of the previous authors to detect carbonic anhydrase in amphibian skin Rosen et al. argued that this may be due to the diluting of the low activity of carbonic anhydrase in the whole skin homogenate preparation. Smith (1974) found that total gas exchange, pulmonary and cutaneous CO₂ removal rates, and the ratio between pulmonary and cutaneous exchange rates in Rana marina were unaffected by acetazolamide. He could not detect any carbonic anhydrase in the skin homogenates. In our study, while we used homogenates grinding 1 gm of tissue in 10 ml buffer for kidney and lung, for the skin we used homogenate having 1 gm tissue in 5 ml buffer. The presence of carbonic anhydrase in the skin is significant for the functions like acidification, CO₂ elimination and chloride transport as postulated by Rosen and Friedley (1973).

The importance of frog skin in physiological functions emphasizes a need for studying the various enzymes present in the amphibian skin. We studied the enzyme carbonic
anhydrase in the skin of five species of amphibia. The results showed that *Bufo melanostictus*, *Bufo himalayanus* and *Rhacophorus maculatus* had comparable activity. *Rana tigrina* skin homogenates showed significantly lower carbonic anhydrase activity than the three above mentioned species. It is not known whether *Rana tigrina* excretes less amount of CO$_2$ through the skin than the other three species examined, or whether *Rana tigrina* skin contains lesser amount of capillaries than the three other anuran species studied. Thus the significance of the relatively lower amount of carbonic anhydrase in *Rana tigrina* skin is not clear. Such studies when correlated and supported with comparative histochemical observations may help to arrive at some meaningful conclusions. *Rhacophorus maculatus* and *Bufo himalayanus* both are temperate zone species, and showed a comparable amount of carbonic anhydrase activity, which was not quite different from that of *Bufo melanostictus*. These probably indicate a very similar role of skin in CO$_2$ excretion in these three species. *Tylototriton verrucosus* skin showed only 24% of *Bufo melanostictus* activity which was significantly lower than the four anuran species we studied. This may be related to the lower metabolic rate of this sluggish aquatic animal, if not due to blood contamination of the skin homogenate. Regarding the functional aspect of salamander skin, the blood capillary distribution at the superficial skin layers, penetrating below one or two epidermal cell layers like that of toad, indicate a possible role in respiration (Plate 19-20).
2. Isozymes

The skin carbonic anhydrase isozyme patterns of the five amphibian species we studied, were grossly similar to that of the kidneys and lungs. But differences in the mobilities of the CoS stained individual bands was seen on the gels of the different organs of the same species. Relative stainabilities also varied among the individual bands of the different organs like skin, kidney and lung of the same species. The number of bands of skin carbonic anhydrase showed variations among the different species of amphibia. *Bufo melanostictus* skin showed six bands while *Bufo himalayensis* skin showed eight bands, thus intrageneric variations of skin carbonic anhydrase isozyme bands was observed in *Bufo melanostictus* and *Bufo himalayensis*. *Rana tigrina* skin had six bands, *Rhexophorus maculatus* had 12 bands while *Tylototriton verrucosus* had eleven bands. Although *Bufo melanostictus* and *Rana tigrina* both showed six bands, their relative position on the gels were different. Thus intergeneric variations in skin carbonic anhydrase isozyme patterns was evident in amphibia. The minor variations in the isozyme patterns of the different tissues we observed was probably due to tissue variations and thus being dependent upon the active gene loci, the tissue of the particular organ and the characters of the polypeptide
subunits of the particular proteins. Like the kidney and lung of *Tylototriton verrucosus* it is also not clear why the skin homogenate was resolved into distinct eleven bands stained with CoS while the same homogenate either showed no appreciable activity or showed very feeble activity when assayed biochemically following manometric methods. It is also not clear why acetazolamide did not exhibit complete and effective inhibition of all the CoS stained bands on acrylamide gels in all cases, as it did in monometric estimations of carbonic anhydrase activity in the same tissue homogenates of kidney, lung and skin of all the amphibians studied.
A number of new non-physiological substrates was found to be split by red cell carbonic anhydrases in invitro experiments by a number of authors. Schneider and Lieflander (1963) was pioneer among them, who used p-nitrophenyl acetate as the substrate. Tashian et al. (1964) observed the diamox inhibitable β-naphthyl acetate splitting activity of human erythrocytic carbonic anhydrase. Human and bovine red cell carbonic anhydrases showed esteratic properties towards a number of non-physiological substrates like o-nitrophenyl acetate, ethyl p-nitrophenyl carbonate besides those mentioned earlier. But Marsh (1967) mentioned that tissue carbonic anhydrase, notably kidney, lacked esteratic properties. In the previous chapter on kidney carbonic anhydrase we have already mentioned that in our study we could not unequivocally demonstrate diamox inhibitable esteratic properties for the amphibian renal carbonic anhydrases. It is also known that carbonic anhydrase act as a general acid base catalyst, which indicates the possibility of the existence of
any new physiological substrate which is still unknown. In our study we also tried to find out whether any relation existed between carbonic anhydrase and β-naphthyl acetate esterase of the excretory organs of amphibia. β-naphthyl acetate is one of the substrates of non-specific esterases. The naphthyl acetate esterase activity was measured in this investigation using β-naphthyl acetate as the substrate. In the biochemical estimations acetazolamide showed no significant inhibition of the hydrolysing activity of nonspecific esterases to the β-naphthyl acetate substrate.

The functions of the various types of esterases are not clear. Skanes et al. (1968) attributed detoxification activity of calf spleen esterases as one of the functions. Li Lem and Yam (1973) proposed that the esterases of human neutrophils may have some debris digesting function. However, it is not unlikely to think that the non-specific esterases of the excretory organs of amphibia hydrolyse various toxic esters produced as intermediate metabolite or metabolic end products either ported to the excretory organs of amphibia via blood or produced within the cells of the tissues of the excretory organs involved, or both.

In our study we found that the β-naphthyl acetate esterase activity of kidney varied in different species of amphibia. Highest specific activity was found in Bufo himalayanus which was about three times higher than that of
*Bufo melanostictus*. The next higher activity was found in *Rhacophorus maculatus*, which was comparable to that of *Bufo himalayanus*. Thus intrageneric variations were observed in amphibia as exemplified by the two species of *Bufo*, *Bufo melanostictus* and *Bufo himalayanus*. Unlike carbonic anhydrase, $\beta$-naphthyl acetate esterase activity was significantly high in *Tylototriton verrucosus*. Thus a general tendency of high esterase activity is seen in temperate zone high altitude species than the low land tropical zone amphibian species. The tropical low land species like *Rana tigrina* and *Bufo melanostictus* showed significantly lower activity of $\beta$-naphthyl acetate esterases in kidneys. All these data indicate some relation of the esterase activity to the distribution of the species in specific environments and specially to the environmental temperatures of the species concerned.
2. Isozymes:

Not only the activity of the $\beta$-naphthyl acetate esterase of amphibian kidney varied but their isozyme pattern also varied from species to species of the same genus as well as in different genera. The esterase pattern was seen to differ from the CoS stained carbonic anhydrase bands. It is also important to note that kidney carbonic anhydrases showed increased number of bands in the species of the high altitude temperate climate, while such phenomena were not observed in the case of $\beta$-naphthyl acetate esterases of the same organ. Unlike carbonic anhydrase, esterases showed no difference among the bands in responding to acetazolamide when used as an inhibitor. Acetazolamide showed partial and indiscriminate inhibition to all the bands. Number and position of bands of $\beta$-naphthyl acetate esterases in kidneys were different in different species of the same genus like Bufo. Bufo melanostictus kidney $\beta$-naphthyl acetate esterases were resolved into seven bands while in Bufo hirumalayanus there were eight bands with different Rf values on acrylamide disc gels. The most active deeply stained bands also occupied different positions on the gels and their number also varied in different species. Thus species variation of renal esterases within the same genus was observed. Rana tigrina the largest Indian frog of tropical climate
showed six bands, the pattern of which on the gels were different from Bufo. The temperate climatic tree frog Ranaophorus maculatus showed eight bands, which also occupied different position on the gels. Tylototriton verrucosus showed six bands with its distinctive distribution on the gels. All these findings indicate the intraspecific and intrageneric variations of renal esterases in amphibia. From all these data the adaptive significance of renal esterases is not clear although the intraspecific and intrageneric variations in esterase electrophoretic patterns surely indicate protein polymorphism of considerable biological importance as suggested earlier by Augustinsson (1959) and Holmes et al. (1963). Botte et al. (1973) studied non-specific esterases in the kidney and interrenal area of a number of anurans and urodela. They observed intraspecific differences in isozymes of nonspecific esterases in a number of amphibians of the genus Rana, Bufo, Eula, Bombia & Triturus. Sexlinked differences of isozymes were also detected by them in Rana esculenta and Bufo viridis. All these variations of renal isozymes of esterases and carbonic anhydrases (discussed earlier) probably reflect the environmental selective pressures more directly on protein polymorphism as the amphibians like other heterotherms experience greater extremes in oxygen tension, pH, salt content, water balance and temperature than do those of mammals and birds as suggested by Dessauer (1970).
B. LUNG:

1. Activity:

The lung /β-naphthyl acetate esterase activity was different from that of the kidney in five species of amphibia. The maximum esterase activity of the lung tissue was observed in Bufo himalayanus next to which was that of Tylototriton verrucosus. This result did not correspond to the carbonic anhydrase activity of this organ as minimum carbonic anhydrase activity was observed in the lung of Tylototriton verrucosus Rhacophorus maculatus lung /β- naphthyl esterase activity was lower than that of kidney or skin. This may probably be due to the juvenile condition of the specimens used in our experiments, or it may be a case of tissue variation. Bufo melanostictus lung showed more or less the same type of /β-naphthyl acetate esterase activity to that of kidney. Rana tigrina lung showed lowest /β-naphthyl acetate esterase activity among the five amphibian species we studied. Thus other than /β-naphthyl acetate esterase activity of the lung of Rhacophorus maculatus the two temperate climate adapted species, like Bufo himalayanus and Tylototriton verrucosus showed remarkably high enzyme activity than the two tropical climate adapted amphibians, Bufo melanostictus and Rana tigrina.
2. Isozymes:

Like the kidney $\beta$-naphthyl esterases, lung $\beta$-naphthyl esterases also varied from species to species. In most cases, the number of bands of lung $\beta$-naphthyl esterases was same as that of kidney but their pattern was different with variation in mobilities and intensity of staining of individual bands. *Rana* *maculata* lung showed ten bands of $\beta$-naphthyl esterases while kidney had eight. In this case variation in pattern of banding of these two tissue esterases with variation of electrophoretic mobilities and intensity of staining of individual bands were seen. Thus like the renal esterases, the variation in esterases of the lung according to species and tissues raises the question of considerable biological significance which may have some adaptive value in relation to the animal's environmental conditions and the specific nature of the tissue and their microenvironment.
1. **Activity:**

The skin β-naphthyl acetate esterase activities of the five amphibian species we studied, were unlike the lungs but were comparable to that of kidneys. The highest activity among the five species was in *Bufo himalayanus* followed by *Tylototriton verrucosus*, *Rhacophorus maculatus*, *Rana tigrina* and then *Bufo melanostictus*. Thus like the kidneys same activity pattern was followed, where the activities were higher in the temperate zone species than the tropical zone ones. It is also interesting to note that relatively aquatic species showed higher activities than the non-aquatic ones. These findings suggest a probable adaptive relation of activity of the esterase enzyme with the environmental temperature. The notably high cutaneous esterase activity in *Bufo himalayanus* is puzzling, whose significance is not clear. The significantly high β-naphthyl acetate esterase activities of the kidney, lung and skin of *Tylototriton verrucosus* were very interesting while carbonic anhydrase activities in these organs of this species were almost negligible. Thus the occurrence of a group of enzymes like esterases having hydrolytic and esteratic functions in these excretory and respiratory organs surely bear some
important biological significance. The comparative study of esterase activities of the skin and lung of five amphibian species indicate that amphibian skin is not physiologically and biologically a less important organ than the lungs, which is in agreement with the occurrence of lunglessness in some amphibians.

2. Isozymes:

When \( \beta \)-naphthyl acetate esterase isozyme patterns of skin are compared among the two species *Bufo melanostictus* and *Bufo himalayana*, the pattern differed from one another. The difference in the patterns were both in the number of bands and in the relative mobilities of the individual bands of the esterase electropherograms of the two species. The skin pattern of these two *Bufo* species also differed from the kidney and lung esterase patterns discussed earlier. The skin esterases varied both in the number as well as in the relative positions of bands from those of kidney and lung. *Rana tigrina* skin showed only three esterase bands on acrylamide gels, while kidney and lung showed six bands. *Ephalophorus maculatus* showed ten skin esterase bands. The kidney of this species had eight bands while the lung had ten like the skin. Differences in mobilities of the individual bands in kidney, lung and skin were seen in both *Rana Tigrina* and *Ephalophorus maculatus.*
Tylototriton verrucosus skin showed one more band than lung and kidney. Differences in band positions were also seen in these three organs. Thus in all these cases intergeneric and interspecific differences of esterase electrophoretic patterns are pronounced. The occurrence of tissue variation of esterase pattern is another important thing to notice. All these findings suggest some important biological significance of protein polymorphism of esterases in the skin. It is also important to note that the relation of skin esterase isozyme multiplicity to the environmental temperatures is more apparent than the kidneys and lung in amphibian. Lastly all the studies so far mentioned and discussed reminds us to recollect the valuable conclusions drawn by Ehrlich and Raven (1963) and mentioned by Denamier (1974) regarding the significance of protein polymorphism, that 'perhaps the uniform, more invariant proteins 'fit'-adapt the species for high average fitness in a slowly, but inevitably, changing environment, whereas the more variant proteins, controlled by genes more sensitive to environmental feed-back, provide 'fine adjustment' to the immediate environment.