BIRES CHANDRA GUHA MEMORIAL LECTURE, 1975

RECENT ADVANCES IN THE PHYSIOLOGY OF VITAMIN A

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The extensive information available in the literature on the effect of vitamin A deficiency in various species of animals is critically examined and it is pointed out that the effect of vitamin A deprivation is most pronounced in those cells which rapidly proliferate under normal conditions. These observations therefore lead to the general interpretation that vitamin A is probably involved in some steps in cell division. It is suggested that it might act like a hormone by sending a message through the membranes to the interior of the cells, and ultimately to the nucleus. It is also possible that it might function at the membrane itself by controlling the synthesis of glycopeptides which are usually present on the membrane.

The Indian National Science Academy has done me a great honour by awarding me this coveted Lectureship. I wish to thank the Academy for this Award.

The late Prof. Guha was not only father of Indian Biochemistry but he was also an inspiring leader and a *guru*. Although I did not have the privilege of learning biochemistry at his feet, I have always derived great deal of inspiration from him. He was especially responsible for settling me during the early phase of my career at Bangalore. Therefore, before I begin my lecture today I wish to express my most respectful homage to this great son of India.

We have been engaged in work on several aspects of metabolism of vitamin A for exactly three decades now. Today I do not wish to speak much about our earlier contributions in this field, and I shall try to confine myself to our more recent work which seems to point towards exciting possibilities regarding unravelling the mysteries of systemic mode of action of vitamin A.

Vitamin A is one of the earliest vitamins to be discovered. Soon after its discovery one of the areas of physiology where it functions viz., vision received considerable attention, because vitamin A was readily found in the eye. Slow but steady progress was made in this particular area and now we have some clarity about the manner in which it functions in the visual system. But we still know very little about colour vision, and moreover, the net amount of vitamin A required in the visual system is relatively very small compared to what is required for other areas of physiology. Therefore, we can safely say that, in spite of intensive work we still know very little about the major metabolic role of vitamin A. This is largely true in the case of all other fat-soluble vitamins also, which is quite different from our knowledge regarding the mechanisms of action of most of the water-soluble vitamins.

Our task was relatively easier in the case of such water-soluble vitamins, because they were found to fall into one pattern in that, they function as parts of enzyme-coenzyme systems. The normal physiological environment of the cell is aqueous, as a consequence of which these enzymes together with their coenzymes have adapted

themselves to function in such an environment. It is therefore natural that these water-soluble vitamins (excepting perhaps ascorbic acid) form part of such coenzymes. In sharp contrast, the mode of functioning of the fat-soluble vitamins appears to be rather complex and a search for their mechanism of action has offered formidable challenges and difficulties. While dealing with the fat-soluble vitamins one encounters a completely different situation in that, they are not readily soluble in the normal physiological environment of the cell. Therefore, newer approaches and orientations will be required in our attempts to unravel the mysteries of their mode of action. For example, the manner in which vitamin A functions in the visual system reminds one of the classical enzyme-coenzyme relationship. Just as in the case of a typical enzyme system the water-soluble vitamin becomes an integral part of the coenzyme and carries out the particular reactions together with the enzyme protein, here also the *cis*-retinal can be considered to be bound to the hypothetical enzyme-opsin and to undergo modifications of its structure in the reactions of the visual system under the influence of light. But this is as far as the analogy can go.

Now let us look at the information already available in the literature on vitamin A and try to see if some generalisation is possible. Ever since vitamin A was discovered experience spread over several decades and of numerous workers in many countries had led to the general conclusion that it is required in three distinct areas of physiology of higher animals viz., (*a*) Growth, (*b*) Reproduction, and (*c*) Vision. These conclusions were drawn from extensive observations of rather empirical nature which were made in higher animals, including man.

The logical approach to a study of the manner in which vitamin A functions would be to deprive an animal of all sources of vitamin A and then examine the effects of such deprivation on the animal system. In the recent past considerable amount of work has been done in many laboratories in this direction and many enzyme systems were found to be affected in the vitamin A-deficient animals. But the effects of the deficiency are so extensive and lead to such extensive pathological damages of almost all tissues of the animal that it became impossible to distinguish the direct effects of the deficiency from the secondary effects. To a certain extent such difficulties could be circumvented by using the compound retinoic acid. Retinoic acid can be formed in the animal body from the stored retinyl esters through successive actions on the esters by the enzymes, viz. retinyl ester hydrolase, retinol dehydrogenase and retinal oxidase. It is also available in the chemically synthesised form.

When weanling rats are put on a vitamin A-deficient diet they continue to grow till their initial reserves of vitamin A are exhausted when they cease to grow or reach the weight-plateau stage of the deficiency, after which they rapidly lose weight. Soon they become very sick and eventually die. If the A-deficient diet is supplemented with retinoic acid the rats do not lose weight but continue to grow like normal animals, excepting that they become completely blind and cannot reproduce. Retinoic acid was thus able to separate the physiological effects of vitamin A on growth from that on vision and reproduction. Now let us examine, in greater detail, how exactly vitamin A may function in these areas of physiology.

Growth — I have already mentioned how deprivation of vitamin A affects growth of weanling rats. Perhaps deficiency of no other essential nutrient exerts such profound effect on growth as we find in the case of vitamin A deficiency. Indeed, the

most reliable and accurate method of assay of very small amounts of vitamin A is still based on the growth response of vitamin A-deficient rats. I should also mention here that rats can be made deficient of vitamin A only if they are deprived of the vitamin at the very rapid phase of their growth and once they become adults it becomes virtually impossible to make them deficient. Therefore, it follows that vitamin A is essential for growth. In this context the interesting work of Bieri (1969) with rats under germ-free conditions deserves special attention. Rats raised on a vitamin A-deficient diet under germ-free conditions took much longer time to attain the weight-plateau stage. But once they stopped growing they continued to live for a considerable length of time without losing or gaining much weight. If, at this stage, they were given some supplements of vitamin A they again grew for some more time and reached yet another weight-plateau stage which was at a much higher level of body weight now. Clearly, these results also demonstrate that without vitamin A rats cannot grow.

Yet another example can be cited here and it is regarding work with developing chick embryo, which is a typical example of very rapid growth, and cell division and differentiation. When eggs laid by hens raised on a retinoate-supplemented diet and fertilised by normal roosters were incubated, the embryo developed upto 48 hr only, after which it died. But if retinyl acetate or methyl retinoate was injected into such eggs a good proportion of the embryos survived and hatched to baby chicks (Thompson 1969). Finally, I could cite the example of another type of cells, viz. the intestinal epithelial cells which regenerate every 24 to 36 hr in rats. It has been the universal experience that these cells are one of the earliest to be affected even at the very mild stage of vitamin A deficiency. As the deficiency progresses the effect becomes more pronounced so that at the terminal stages of the deficiency the intestine is virtually left with no such cells.

Reproduction — Several workers have reported that the testes and ovaries of rats are affected in vitamin A deficiency. But recent work with retinoic acid has brought to sharp focus absolute requirement of retinol for the normal reproductive performances of both male and female rats. Thus, when weanling male rats are raised on a vitamin A-deficient diet supplemented with retinoic acid their testes do not develop properly and become oedematous. The sperms develop only up to the spermatid stage, so that when they are allowed to mate with females of proven fertility no fertilisation takes place. The corresponding females on the other hand conceive when allowed to mate with normal males. But they cannot carry the pregnancy to full term and the pregnancy is terminated by fetal death and resorption beginning day 14 or 15 of the pregnancy (Juneja, Murthy and Ganguly 1964).

The effect of deprivation of retinol is very pronounced in rat testes and therefore it has been very easy to demonstrate such effects by histological techniques. But earlier workers had failed to find any such effects on rat ovaries and therefore it was widely accepted that the ovaries are quite normal in the retinoate-supplemented rats. Quite contrary to such well accepted belief we have produced convincing evidence establishing that the ovaries of such rats do not function normally. It was possible for us to demonstrate such effects in rats only under stress conditions like unilateral ovariectomy and pregnancy. We have also shown that synthesis in and secretion from such ovaries of the steroid hormones like pregnenolone, progesterone and 20-α-hydroxy-

progesterone are markedly less in the retinoate-treated rats. My point here is that the effect on the ovaries is very subtle and not easy to demonstrate.

I may also mention here the effect on the vaginal epithelial cells. Cornification of vaginal cells has been universally accepted as one of the criteria of vitamin A deficiency in female rats. But, our recent work indicates that the vaginal cells of rats receiving retinoic acid show rather irregular cycle and mostly stay at the cornified state.

A unified interpretation of these diverse observations

All these observations can be pieced together on the basis of a general theory that, wherever there is rapid cell proliferation, withdrawal of retinol affects such cells first. Thus the most accepted effect of vitamin A deficiency is cessation of growth. But this can be accomplished only if very young rats are subjected to deprivation of vitamin A and once the rats attain adulthood it becomes virtually impossible to deplete them of the vitamin. The experiments with germ-free animals also have established that rats will grow till the supply of vitamin A is exhausted, after which they maintain their weight for a considerable length of time and promptly resume growth on receiving fresh supply of the vitamin. The epithelial cells of the intestinal mucosa regenerate every 24 to 36 hr in rats and these cells are one of the earliest to be affected by vitamin A deficiency. Similarly the ovaries and vaginal cells cycle in rats every four days and here the effect is not so marked and consequently not easy to demonstrate. Finally, the chick embryo dies within 48 hr of incubation in absence of vitamin A and the foetus dies in the retinoate-supplemented pregnant rats.

On the basis of such numerous observations we have come to the general conclusion that vitamin A is involved in the overall process of growth in higher animals in a rather sophisticated manner (Ganguly 1974). But, here we must qualify our statement. In general, growth would require all essential nutrients, including essential amino acids. But the requirement of vitamin A is so small as compared to other essential factors and the effect of its deprivation is so pronounced that we feel that it must be placed in a special category as far as growth is concerned.

But, what is growth? Certainly it is a very vague and gross expression. To us it means rapid cell multiplication. But again, how much do we know about the basic mechanisms and processes which control cell division? Frankly speaking—very little. These are the limitations in further progress of our work and within these boundary conditions we have been actively engaged in producing some evidence in support of our working hypothesis. I shall now briefly discuss some of our recent results.

Effect of deprivation of vitamin A on RNAs of liver, testes and intestinal mucosa of rats

In a given rat the sperms regenerate very rapidly and the intestinal cells regenerate more slowly, while there is virtually no regeneration of the liver cells. We have attempted to investigate the synthesis of RNA and DNA in these tissues of rats during vitamin A deprivation. At the very mild stage of vitamin A deficiency or following retinoic acid supplementation the RNA of the liver, mucosa and testes were extracted and subjected to Sephadex G-100 chromatography, when it was found that the total RNA contents of the three tissues, when expressed as mg/g net weight, are not

affected by the deficiency. But, for the same amount of total RNA fractionated, there was a definite and consistent decrease in the ribosomal and tRNA of the deficient mucosa and testes with a concomitant increase in the degraded RNA materials, while the liver RNA remained unaffected.

Our more recent ^{32}P-incorporation studies have further shown that the specific radioactivities of the ribosomal and tRNA fractions of the mucosa and testes are significantly lower in the deficient animals, while those of the normal and deficient livers do not differ much. The poly A contents of the testes and mucosa also, but not of the liver, were similarly lower. These results have therefore provided definite evidence in support of the present hypothesis that vitamin A deficiency affects the rapidly proliferating cells first.

Regenerating rat liver, following partial hepatectomy, has been recognised as a model for growth and rapid cell proliferation (Bresnick 1971). After partial hepatectomy the cells of the remaining portions of the liver rapidly proliferate and consequently the regenerating liver provides one of the most reproducible systems for experiments on the processes of replication in cells. Marked increase in the biosynthesis of RNA is one of the earliest and necessary events in the process of regeneration of the liver. Almost immediately after partial hepatectomy there is an increase in the RNA synthesis, while at 16 to 18 hr after the operation DNA synthesis also increases.

Therefore it is obvious that such cells can be used for investigations of any role vitamin A might play in cell division, and by using such a model system we have attempted to investigate any role of retinol in cell proliferation (Jayaram, Sarada and Ganguly 1975). Weanling male rats were kept on a vitamin A-deficient diet till the rates of increase in their body weight slowed down and they gained about one gram per day. At this stage 30 per cent of their livers were removed. (If the rats were allowed to reach the weight-plateau stage, or if 70 per cent of the livers of the depleted rats were removed, invariably they could not withstand the operation). Immediately after the operation some of the deficient rats were given supplements of retinyl acetate or retinoic acid while another group received no such supplements. Along with the deficient rats a control group receiving sufficient supplements of retinyl acetate throughout was also subjected to partial hepatectomy (here 70 per cent of their liver was removed).

Regeneration of the liver in terms of net gain in tissue weight was markedly less in the deficient group and it was restored to normal by supplementation with retinyl acetate. The increases in DNA ran parallel to those of tissue weight, which conclusively proved that cell division was arrested in these cells in the absence of retinol. Retinoic acid supplementation on the other hand was not as effective, which is in general agreement with the earlier observations regarding the effect of this acid on the development of testes, ovaries and foetus in rats. The changes in proteins ran parallel to the changes in DNA and net weight gain, while RNA showed a marked increase immediately after the supplementation with retinyl acetate.

These very recent observations made in our laboratory are in full agreement with the general experience of earlier workers regarding the possible role of vitamin A in cell proliferation. But these preliminary results are unable to give any indications regarding the particular steps or locale where retinol might function during cell divi-

sion. It is possible that it could act like a hormone by sending a message through the membrane into the interior of the cell or it could function at the level of the membrane itself. In this context I may mention here that in recent years polyprenols have been shown to participate in sugar-transfer reactions. Since retinol is a tetraprenol, in attempts to implicate it in such mechanisms it has been shown that it can participate in sugar-transfer reactions, though much less efficiently than dolichol. But no specificity of retinol against other polyprenols has yet been demonstrated, nor has it been explained as to why retinol participates in such reactions in higher animals only. In case retinol functions in a similar manner in higher animals it is conceivable that it might regulate cell division by controlling synthesis of glycopeptides which are usually associated with cell membranes.

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