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THE RELATION OF RIBOFLAVIN TO THE EYE. A REVIEW ARTICLE

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RIBOFLAVIN became of immediate interest to ophthalmologists after the publication by Sydenstricker, Sebrell, Kleckley and Kruse (1940) of the paper in which they describe the ocular signs of ariboflavinosis in man. In fact, so much interest was aroused that a year later the Science News Supplement to Science (1941) carried a report from an eye specialist in Maine that 80 per cent. of people examined showed evidence of both past, chronic or new signs of deficiency of riboflavin. If the eye specialist made a return trip at the present time it is doubtful if he would reach the same conclusion, since the last two years have been spent in defining the true signs of riboflavin deficiency in man and animals.

Uncomplicated riboflavin deficiency in man probably never occurs. The diet is, in practice, always deficient in more than one of the vitamin B complex constituents. In the series of cases described by Sydenstricker, nicotinic acid, thiamin, ascorbic acid and cod liver oil were added to the diet in order to make the deficiency of riboflavin the dominating one. Ocular signs may be noticeable before other disorders. The patient seems usually to complain of photophobia and dimness of vision, and on examination circumcorneal injection is seen, and, to quote Sydenstricker *et al.*, " The earliest change that can be recognised with the slit-lamp is marked proliferation and engorgement of the limbic plexus with the production of great numbers of very narrow capillary loops which outline the extreme margins of the scleral digitations and obliterate the normal narrow avascular zone between the plexus and the sclerocorneal junction. . . . The cornea is actually invaded first by very small capillaries arising from the apexes of loops surrounding the scleral digitations. . . . Such capillaries lie just

beneath the epithelium and soon anastomose to form a tier of loops from which more single capillaries arise, extending centripetally." At a later stage the deeper layers of the cornea may be invaded by vessels and superficial and interstitial opacities may develop. After giving riboflavin, 5-10 mg./day, the photophobia may clear up within 24-48 hours and in this time also the engorgement of the limbal vessels subsides. If vessels have penetrated any distance on to the cornea 8-15 days treatment is necessary for their emptying. In all instances the vessels remained visible as greyish or refractile streaks.

Sebrell and Butler (1939) described in detail the general signs of ariboflavinosis in man. The lips become abnormally red and cracks may appear at the angles. There is seborrhoea of the nasolabial folds, eyelids and ears, and there may be seborrhoea and follicular keratosis of the forehead, malar eminences and chin. One of the very early signs is glossitis. The tongue is clean, purplish red and sometimes fissured, with large and flattened or mushroom shaped papillae. Sydenstricker *et al.* report that they have seen this type of tongue appear in pellagrins who had been cured of their pellagra with nicotinic acid, but remained deficient in riboflavin.

It is the attributes of superficiality and of symmetrical arrangement which mark off corneal vascularisation due to deficiency of riboflavin from the corneal vascularisation due to other vitamin deficiencies and pathological states. Johnson and Eckhardt (1940) reported that riboflavin improved rosacea keratitis, where the corneal vessels are irregular in distribution and in depth. Recently Fish (1943) has reported that in her hands riboflavin has no beneficial effect on rosacea keratitis. Kruse *et al.* (1940) found an improvement in four cases of syphilitic keratitis after riboflavin. Here again the vessels are of a different type from those seen in the ariboflavinosis of man and animals. Wagener (1941) commented on this and stated that he was unable to obtain any improvement of syphilitic keratitis with riboflavin therapy. This fundamental question of the diagnosis of riboflavin deficiency by an appraisal of the type of vascularisation seen is fully dealt with in a paper by Gregory (1943). It is not the purpose of this review to go deeply into the picture of riboflavin deficiency in man, but the papers already referred to show that there has been considerable confusion and it is possibly suitable to make a short survey of all the experimental data connecting riboflavin with the eye and to outline in brief the rôle it is thought to play in tissue metabolism.

Chemistry and Physiology of Riboflavin.—Riboflavin has now been firmly established as vitamin B_2 and has been found to be an essential part of the oxidising systems of animal tissues. It has been synthesised and its structure has been established as 6·7.

dimethyl 9. d-i ribityl iso-alloxazin. The work, which led up to the establishment of these facts has already been described in many reviews and the description will not be repeated here. References to such reviews are given at the end (Fig. 1).

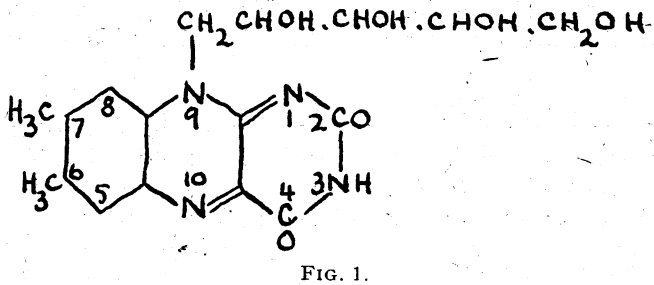

FIG. 1.

Riboflavin.

The recent advances in our knowledge of the relation between riboflavin and tissue oxidation must be described. The account will not be presented in strict historical sequence, but rather the present position will be described in terms of the earlier work.

Properties of Riboflavin.—Riboflavin is a bright yellow solid. It is slightly soluble in water, giving bright yellow solutions which show a very strong green fluorescence. The concentration of riboflavin in a solution can be estimated by determining the strength of this fluorescence. In solution riboflavin is heat stable, but it is fairly rapidly destroyed by light. The destruction is most rapid in ultra-violet light, but daylight is also slowly destructive, so that solutions of riboflavin must be preserved in the dark. In neutral solution the effect of light is to split the ribose side chain off the molecule. The resulting substance, lumichrom, still fluoresces weakly green-yellow, but has no vitamin activity and cannot replace riboflavin in the diet. The effect of light on alkaline solutions of riboflavin is different. Under these circumstances the four terminal carbon atoms of the ribose side chain are split off, giving a substance with a bright blue fluorescence, called lumiflavin, which again has no vitamin activity. The structure of riboflavin was partly elucidated from a study of these photolytically produced breakdown products of the vitamin.

Solutions of riboflavin can easily be reduced by sodium hydrosulphite or palladium and hydrogen. The reduced solution is no longer bright yellow in colour, but the yellow colour can be restored and the riboflavin re-oxidised by shaking the solution with air or oxygen. This process of reduction and re-oxidation can be carried out indefinitely. Reduced riboflavin has combined with 2 H atoms which are linked to the N atoms at positions 1 and 10

of the iso-alloxazin rings and when the solution is shaken with air these hydrogens are given up to oxygen. This reversible reduction of riboflavin serves as a model for its most important action in the body. In the tissues riboflavin compounds are constantly being reduced by one system of enzymes and re-oxidised by another system, so that they form a link in the chain of oxidation processes that are necessary for the complete oxidation of metabolites.

Occurrence of Riboflavin in the body.—In secretions such as milk and urine riboflavin occurs largely in the free state and is therefore easily dialysable. In tissues it is combined with adenylic acid, a phosphate group and specific proteins to form non-dialysable flavoproteins, most of which do not fluoresce. Pulver (1940) found that the press juice obtained from many tissues contained very little of the tissue riboflavin. Prolonged dialysis may release riboflavin from tissues but this is probably due to the breakdown of flavoproteins. Estimation of riboflavin in tissues depends either on its property of fluorescence, a typical method being described by Najjar (1941), or on the fact that the growth of certain bacteria is proportional to the concentration of riboflavin in their medium. This microbiological method worked out by Snell and Strong (1939) is the most satisfactory one at present available and enables 0·05γ riboflavin to be estimated accurately. In general, the liver, kidney and heart of most animal species are the tissues richest in riboflavin, but all tissues so far examined contain small amounts. It is also present in eggs, yeasts and bacteria, and possibly the virus of vaccina. It has been estimated that an adult man needs 3-5 mg. riboflavin per day.

Flavoprotein Enzymes.—Warburg (1938) isolated the first riboflavin containing enzyme from animal tissues. He had earlier (1932) isolated an oxidation enzyme containing riboflavin from yeast, which he had shown to be similar to a yellow enzyme preparation from muscle which Banga and Sz. Gyorgi had made in 1932. Warburg found that preparations of the *d*-amino oxidase from kidney contained a riboflavin compound and finally showed that the enzyme itself is a flavoprotein which contains flavin adenin dinucleotide as its active group. This flavin adenin dinucleotide (Fig. 2) can be separated from the specific enzyme protein. Separately, neither the protein nor the flavin is catalytically active, but they can be recombined by simply mixing the two solutions to give an active enzyme. This enzyme in the presence of *d*-amino acids—that is, the isomer which does not occur in proteins—will take up oxygen, being reduced by the amino acid and constantly re-oxidised by the oxygen of the air.

The fact that this enzyme can be, as it were, taken to pieces and then put together again has proved very useful in investigating

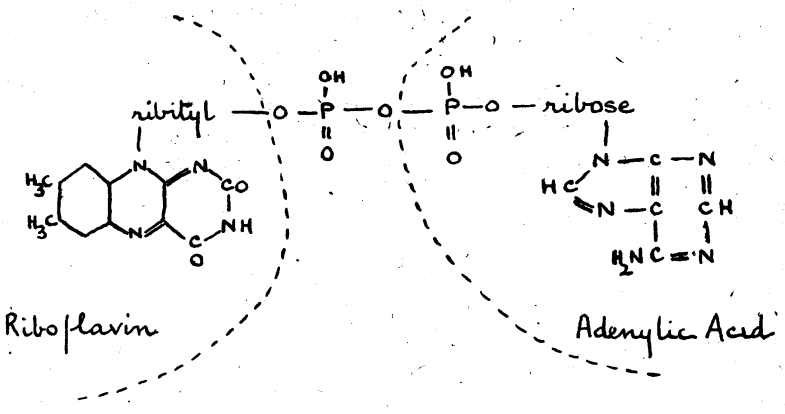

FIG. 2.

Flavin adenin dinucleotide

flavins in tissue extracts. Flavoproteins from other sources were dissociated into flavin plus protein and the effect of this flavin on the oxidation of *d*-amino acids in the presence of the specific kidney protein was then investigated. If the " foreign " flavin compound can replace the kidney flavin and combine with the kidney protein to give an active enzyme it is assumed that this " foreign " flavin is identical with the kidney flavin, namely flavin adenin dinucleotide. In this way flavin adenin dinucleotide has been found to be the active group of all well characterised tissue flavoproteins.

The most important function of the flavoprotein enzymes so far described is the oxidation of the nicotinic acid co-enzymes. Studies of the mechanism of tissue oxidation have shown that many substances are oxidised by specific enzymes, plus co-enzymes which are substances of relatively low molecular weight. Two of the most important co-enzymes contain nicotinic acid amide as the active group. This becomes reduced by the substance undergoing oxidation, but the mechanism whereby it is re-oxidised so that it can again take part in oxidation processes was unknown until Straub (1939) isolated a flavoprotein from pig heart which rapidly re-oxidised the nicotinic acid co-enzymes, becoming itself reduced in the process. We may picture the oxidation of a metabolite as taking place in the following way :—

1. Substrate + enzyme + co-enzyme → oxidised substrate + enzyme + reduced co-enzyme.
2. Reduced co-enzyme + heart flavoprotein → reduced flavoprotein + co-enzyme.
3. Reduced flavoprotein + (?) → flavoprotein + reduced (?).

The way in which reduced flavoproteins are re-oxidised is still unknown; under normal tissue conditions of 0_2 tension, most

flavoproteins, unlike riboflavin itself, are not re-oxidised by O_2. The cytochromes probably take part in their re-oxidation, but this gap in our knowledge of tissue oxidation is still waiting to be filled.

Other flavoprotein enzymes which have been isolated from tissues, in a state approaching purity, include the enzyme which catalyses the oxidation of xanthin and hypoxanthin to uric acid; an enzyme which occurs in liver and oxidises aldehydes and the kidney -amino oxidase which has already been described.

In riboflavin deficient rats Axelrod and Elvehjem (1941) found that the amount of xanthin oxidase and d-amino oxidase, both of which are flavoprotein enzymes, is reduced in the liver, and Ochoa and Rossiter (1939) found that the flavin dinucleotide content, and therefore presumably the content of flavoprotein enzymes, was significantly lowered in heart and liver tissue of riboflavin deficient rats, though not much affected in kidney and brain.

Synthesis of flavin adenin dinucleotide from riboflavin occurs very rapidly after the injection of riboflavin into the animal. Ochoa and Rossiter (1939) found that the flavin adenin dinucleotide content of the liver of riboflavin deficient rats increased half an hour after an injection of riboflavin. *In vitro* they found that liver slices did not synthesise the dinucleotide from added riboflavin but decomposed the dinucleotide already present in the tissue. Klein and Kohn (1940) found that human blood cells carried out the synthesis very slowly, but Trufanov (1941) found that a good many tissues, muscle in particular, could synthesise the dinucleotide. He found that there was a balance between synthesis and breakdown and the synthesis could only be shown if the tissue slices were incubated with riboflavin for very short periods. In longer periods breakdown was the dominating process.

RIBOFLAVIN AND THE EYE.

Occurrence.—Euler and Adler (1934) estimated riboflavin in the eyes of many species of fish and some mammals by a fluorimetric method. They found that the retinae of all species contained riboflavin but they could find none in the other parts of the eye. In some fish the retinae contained very large amounts of riboflavin, larger amounts than had previously been found in any mammalian tissue. Euler and Adler considered that the retinal riboflavin played some part in a light sensitive reaction owing to the fluorescence of free riboflavin. In mammalian tissues riboflavin occurs combined with adenylic acid and proteins and in general these flavoproteins do not fluoresce. In order to utilise riboflavin fluorescence the riboflavin must occur uncombined. Adler and Euler (1938) obtained evidence that free riboflavin does occur in cod retina. Frozen cod retina was extracted with ice water and the

extract shaken with benzyl alcohol, which dissolves free riboflavin. This gave a fluorescent solution. It is therefore possible that in fishes riboflavin acts as a photosensitiser by absorbing short-wave light and transmitting it as light of longer wave length. In mammalian retinae the total riboflavin present is in general about 1/100th that of the retinal riboflavin in fish. There is no evidence that it occurs as free riboflavin as Euler and Adler (1934) could not obtain fluorescent extracts from calf retinae. There is therefore no evidence that riboflavin does, in mammals, act as a photosensitiser in the retina, although from the number of times this has been suggested in the literature one might be excused for considering it a well established fact.

Riboflavin has recently been estimated in all parts of the ox eye, Philpot and Pirie (in the press), using the delicate microbiological method of Snell and Strong (1939). A parallel series of estimations of the flavin adenin dinucleotide content of the eye tissues was also run in order to see whether riboflavin occurred free or combined with adenylic acid. Only traces of riboflavin or flavin adenin dinucleotide were found in the vitreous or aqueous humours, in the lens and in the substantia propria of the cornea. The corneal epithelium, the ocular conjunctiva, the iris, the ciliary body and the choroid (plus pigment epithelium) all contained 2 – 3γ of total riboflavin per gm. wet weight of tissue. Probably all this riboflavin was combined with adenylic acid as flavin adenin dinucleotide. The retina contained 3 – 4γ total riboflavin per gm. wet weight. A large part of this occurred as the dinucleotide, but the agreement between the two different estimations was not as satisfactory as with the other tissues and there is a possibility that a very small amount of riboflavin may occur free in ox retina.

Meibomian glands, lacrymal glands and Meibomian secretion contained more riboflavin than any part of the eye, 4 – 6γ per gm. wet weight. Here again the riboflavin was combined with adenylic acid to give flavin adenin dinucleotide.

GENERAL EFFECTS OF DEFICIENCY OF RIBOFLAVIN IN ANIMALS.

As the complexity of the B vitamins has become greater and greater, so the signs of the deficiency of any one of the constituents of the complex have been narrowed down. But the present position is that members of the complex are thought to interact with one another, so that although one set of signs may be broadly diagnostic of—say riboflavin—deficiency, yet some of these signs may occur in animals receiving adequate riboflavin but deficient in pyridoxin, pantothenic acid or yet unknown factors. This overlap is described by Chick, Macrae and Worden (1940) who kept rats for long periods deficient in one or other of these members

of the B_2 complex. Simple riboflavin deficiency led to a failure in growth and the rats developed an eczematous condition of the skin affecting especially the nostrils and eyes. The hair round the eyes fell out and there was conjunctivitis, blepharitis and dullness of the cornea, the eyelids being stuck together. The fur also became scanty over the head and forelegs and appeared moist and matted with bald patches developing later. In a group of ten rats which received adequate riboflavin, but were deficient in other members of the B complex, seven animals showed—as well as the dermatitis characteristic of pantothenic acid and pyridoxin deficiency—riboflavin deficiency type of skin lesions with conjunctivitis and exudate from eyes and nostrils.

In dogs the general signs of riboflavin deficiency are lack of appetite, weakness and ataxia with sudden collapse and death after about four months on the diet. If riboflavin is given in the terminal stages it produces a rapid cure. In chicks, prolonged partial deprivation of riboflavin has been studied and it has been found to lead to " curled-toe paralysis " due to degeneration of the sciatic nerves. Acute riboflavin deficiency in the chick leads to paralysis and death as in the dog. The same picture has been described for the pig.

Effect of deprivation of Riboflavin on the Eyes of experimental Animals.—That changes occurred in the eyes of rats kept on a riboflavin deficient diet was first noticed by Day, Langston and O'Brien (1931). These workers noticed that there was vascularisation of the cornea and also found that nearly all their animals developed cataract. They considered that the cataract was due to the deprivation of riboflavin and in the next few years published a series of papers describing such cataracts in rats, mice, chickens and monkeys, and showing that they could be prevented or arrested by the addition of riboflavin to the diet, although a cataract could not be cleared up by riboflavin. These results caused great interest and workers in other laboratories started work on the same lines. In general, no one obtained 100 per cent. cataract as Day *et al.* had observed in their animals. Bourne and Pyke (1935) got 20-30 per cent. cataracts. Eckhardt and Johnson (1939) obtained an even smaller percentage and Gyorgy (1935) and Bessey and Wolbach (1939) found no cataracts in their experimental animals. Naturally each group of workers has used a slightly different diet and all the rat strains have not been the same. It is therefore possible that the divergent results are due to slight divergences of technique. At present it seems unlikely that such cataracts are caused by a simple deficiency of riboflavin. Recently Baum, Michaelree and Brown (1942) have claimed that rats on a diet absolutely free from riboflavin fail to develop cataract, whereas 70 per cent. of rats on the same diet plus very small amounts of riboflavin develop

cataracts. Stocksted and Manning (1938) found that chicks on a completely riboflavin free diet did not get " curled-toe paralysis " so frequently as chicks which received minimal amounts of riboflavin. They concluded that this was due to the stimulation of growth by these small amounts of riboflavin, the need for riboflavin and all other members of the B_2 complex becoming thereby much greater. It is possible that formation of cataracts in riboflavin deficient rats depends on imbalance between the different members of the B_2 complex.

Corneal vascularisation, first noticed by Day, Langston and O'Brien, has been found by Bessey and Wolbach (1939) to be one of the earliest and most constant signs of riboflavin deficiency in rats. They have described its development in detail and the parallelism between their description of the vascularisation of rat cornea and the vascularisation of human cornea in a-riboflavinosis described by Sydenstricker *et al.* (1940) is very striking indeed. The eye changes have not been described in such detail in other experimental animals, but Patek, Post and Victor (1941) described a clouding of the pig's cornea after five months deficiency of riboflavin. No slit-lamp observations were made, but histological preparations showed that the corneal epithelium was cornified on the surface with the lower cells swollen and irregular. The corneal stroma was more normal.

The cornea is not the only part of the eye affected by riboflavin deficiency. Nearly all observers have noted that, in rats and pigs at least, changes occur in the conjunctiva and lacrymal glands also. Sections of rats' lacrymal (?Harderian) glands were reported by Sherman and Sandel (1931) to show an " acute inflammatory process." Diminished lacrimation has been noted in rats by Shaw and Phillips (1941) and increased lacrimation in dogs by Street, Cowgill and Zimmerman (1941). In pigs the eyelids are swollen and the palpebral fissures narrowed. In severe deficiency in the rat there is conjunctivitis and blepharitis and the lids may stick together completely. Since the lacrymal and Meibomian glands and the Meibomian secretion, at least in the ox, contain a greater percentage of riboflavin compounds than other parts of the eye, it is reasonable to suppose that a deficiency should lead to pathological changes in them. The time relations between these changes and the vascularisation of the cornea has not been described.

It is interesting to notice the long time-lag between the first reports of eye changes in rats on riboflavin deficient diets, the application of this knowledge to human problems and the accurate detailed description of the lesion in experimental animals. Vascularisation of the rat cornea was first noticed in 1931, but was not described in detail until 1939. In 1940 an identical picture was

described in man. In 1939 descriptions of riboflavin deficiency in pigs and dogs make no mention of eye lesions, but in 1941 a description of the lesions in these species is given. No account has yet been given of eye lesions, if any, in riboflavin deficient chicks. There has been in the past a regrettable tendency on the part of experimentalists to neglect the detailed examination of eyes and of ophthalmologists to neglect the pointers given to them by experimentalists.

DISCUSSION.

We do not know how the riboflavin content of blood and tissues changes in riboflavin deficiency, so that it is impossible to speculate whether the ingrowth of vessels into the cornea is called forth by deficiency of riboflavin in the cornea itself or by deficiency of riboflavin in the blood. It has been assumed that the riboflavin of the cornea is derived from the blood, but it is possible that it is partly derived from the eye secretions which do, in the ox, contain flavin adenin dinucleotide. In other tissues riboflavin has been shown to take part in normal oxidation processes and we may assume that it fulfills the same function in the tissues of the eye, but no data of the flavoprotein enzymes of the eye are available. As it has a specific function in the cell it can only be of use when that specific function is upset and it is therefore useless to hope that riboflavin will help conditions where conjunctival hyperaemia or corneal vascularisation are due to other causes.

It is extremely interesting that the eye signs in riboflavin deficiency are some of the earliest to devolop and may be used—if possible in conjunction with skin and tongue changes—to make an early diagnosis of the deficiency. It is the early stages of deficiency which are most likely to be met with in this country, but in tropical countries reports of severe eye lesions associated with multiple vitamin deficiencies have been frequently reported. Pock Steen (1939) reported that sprue patients had lesions in their eyes which caused " twilight blindness " and that this condition was relieved by riboflavin. Landor and Pallister (1935) found that prisoners in Johore suffered from multiple deficiences and among their symptoms were photophobia, dimness of vision, " conjunctivitis and corneal ulceration," cured by Marmite or liver. Similar pictures have been reported by Moore (1939) from Nigeria, where school children were very largely affected, and Metivier (1941) from Trinidad and there is no doubt that eye lesions due to multiple vitamin B deficiencies are very widespread. The clear picture of riboflavin deficiency has been established by Sydenstricker and subsequent workers, but it yet remains to be seen what rôle, if any, is played by each of the other known and unknown members of the vitamin B complex.

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