THE ESTIMATION OF RIBOFLAVIN (VITAMIN B_2) IN PLANT TISSUE

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The discovery of Warburg's "yellow-respiratory-enzyme" in 1932 (1), was a great stimulus to the study of cellular respiration. The significance of its properties in relation to biological activity in the living cell has been the basis for a number of important generalizations in regard to the metabolic functions of other such substances.

The elucidation of the composition and structure of the yellow enzyme (1, 2) has shown that it consists of a high molecular weight protein carrier to which is attached the chemically active group. This prosthetic group is known as riboflavin and is essentially an iso-alloxazine nucleus, methylated in the 6 and 7 positions and with a phosphorylated d-ribose side chain attached in the 9 position. The d-riboflavin–5′–phosphoric acid will dissociate from its protein carrier by dialysis but will reassociate with return of enzyme activity when again mixed with the protein solution (3). The prosthetic group appears to be attached to its protein at two points, i.e., through the phosphoric acid and the 3 amino group. It is interesting to note the extreme specificity of this compound in relation to its physiological activity. The closest homologs and isomers have only a fraction of the vitamin potency and enzyme activity that d-riboflavin exhibits.

Riboflavin occurs widely in nature and it has been found present in all living materials so far studied (4). Riboflavin will probably be found in all plants and it is reasonable to assume that it has physiological significance for their growth and metabolism. Riboflavin or vitamin B_2 (formerly called vitamin G) is an essential constitutent of the diet of animals.

Riboflavin is perhaps the most outstanding example of a group of biologically active chemical compounds capable of two-step oxidation-reduction. The free flavin has a high negative potential of –0.21 volts (5), which indicates that the leuco, dihydroxy form has a strong tendency to lose its hydrogen atoms and return to the colored, diquinoid form. The outstanding example of this function as hydrogen transporter is the dehydrogenation of certain hexose esters of phosphoric acid which Warburg and Christian (6) carried out in vitro and for which they proposed the following mechanism:

(1) Coenzyme II + hexosemonophosphoric acid $\rightleftarrows$ reduced coenzyme phosphohexonic acid.

(2) Reduced coenzyme + yellow enzyme $\rightleftarrows$ coenzyme + reduced yellow enzyme.

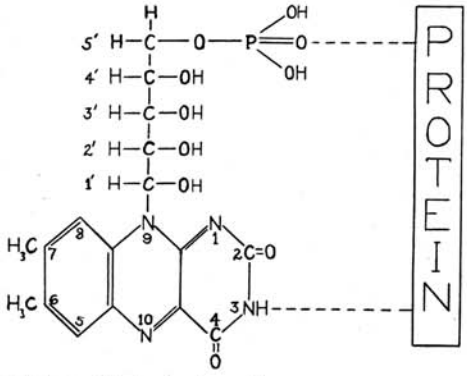
(3) Reduced yellow enzyme + O_2 (or cytochrome) $\rightleftarrows$ yellow enzyme + H_2O_2 (or red. cytochrome)

This system is one of the fundamental steps in the oxidation of sugar by respiring cells. The significance of riboflavin has recently been given increased emphasis through its role in several other enzymatic dehydrogenation systems by combining with different protein carriers and with adenine (7).

The methods which have been developed for the estimation of riboflavin fall into two general classes; physico-chemical and biological assay.

Preparatory to analysis by physico-chemical means, riboflavin must be extracted from the tissue and freed from impurities. This may be accomplished by the following methods: (1) refluxing the ground tissue with water, alcohol, alcohol-water mixtures or acetone, (2) adsorption on fuller's earth or other suitable adsorption materials and subsequent elution with pyridine, ammonia-water mixtures or alcohol-water mixtures, (3) Precipitation as a heavy metal salt, (4) combinations of these methods.

The riboflavin content of this extracted material is then determined by either of three general methods. First, the intensity of the yellow color may be colorimetrically compared with a known stand-



Riboflavine-5'-phoshoric acid

ard (8). This method is not entirely satisfactory because the color of riboflavin in low concentrations becomes too faint for accurate comparison. In a second method, the intensity of fluorescence of a standard riboflavin solution is compared with the fluorescence of the unknown flavin solution by means of a photoelectric photometer (9). The third method depends upon the characteristic and specific instability of riboflavin when exposed to light in alkaline solution (1). The decomposition product, lumiflavin (6, 7, 9 trimethyl isoalloxazine) is extracted in chloroform and gives a characteristically blue fluorescence measurable with a photometer. This method is now little used because the conversion is not quantitative. Technical errors may be encountered in any of these three methods as a result of incomplete extraction of other colored or fluorescing materials. Two prominent biological assay methods have been used up to the present time. The first was based on feeding tests with rats and the increase in weight compared to a control animal receiving no riboflavin. (11) This method is cumbersome, slow and expensive.

A promising microbiological assay method (10) employs the quantitative response of certain lactic acid bacteria to different amounts of riboflavin. The bacteria are cultured in tubes of flavin-free medium to which have been added varying amounts of the synthetic compound. The total metabolism at the end of three days is determined by titrating the lactic acid formed with standard alkali. When mls. of 0.1 N lactic acid produced are plotted against the micrograms of riboflavin added, a standard curve results, from which may be read the flavin content of unknown materials. The riboflavin is extracted from plant tissue by heating with water in an autoclave.

The authors claim that if cultures are kept free from contamination and care is taken with the standard solutions, flavin can be determined with an error of about ±5.0%. This method has several advantages over previously described methods: in many cases the crude product may be used thus eliminating preliminary extraction, only small amounts of material are required, the method is fairly rapid (3 to 5 days) and requires no expensive equipment.

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