SPECIAL ARTICLES

THE ISOLATION OF TOBACCO RING SPOT AND OTHER VIRUS PROTEINS BY ULTRACENTRIFUGATION

A HIGH molecular weight crystalline protein, which available evidence indicates is tobacco mosaic virus,[1] was isolated from mosaic-diseased plants by chemical methods.[2] These same methods, when applied to plants affected by some of the less stable viruses, such as those causing tobacco ring spot, latent mosaic of potato, cucumber mosaic and severe etch, were successful only to the extent that partial purification and a limited degree of concentration were accomplished. In no instance was a crystalline protein isolated. The results, which were probably due to the instability of these viruses, and possibly to their low concentration, indicated that new methods would have to be developed in order to work successfully with such viruses. The observation that ultracentrifugation of the clarified juice from tobacco mosaic-diseased plants resulted in the separation of a pellet at the bottom of the tube,[3] and the demonstration by means of x-ray analysis[4] and virus activity measurements[5] that this pellet consisted of crystalline tobacco mosaic virus protein suggested the new methods that have been employed. If other viruses are high molecular weight proteins, it should be possible to concentrate and isolate them by ultracentrifugation.

This possibility was explored by ultracentrifuging the juice from leaves of Turkish tobacco plants diseased with tobacco ring spot virus, in 7 to 17 cc celluloid tubes, for about 3 hours in a maximum field of about 60,000 times gravity. Ring spot virus is unstable and becomes almost completely inactivated on merely standing at room temperature for one day,[6] hence it was necessary to keep the preparations cold during the entire manipulation. This was accomplished by working with the material in a room held at about 2° C. and by carrying out the ultracentrifugation in a quantity head[7] precooled to about 0° C. Since the centrifuge head spins in a vacuum, it absorbs but little heat, and during a 3-hour run usually warms up less than 5°. On ultracentrifugation of tobacco ring spot juice, which had been previously clarified by low-speed centrifugation in a Swedish angle centrifuge or by filtration through filter paper, very small pellets, less than 1/50th the size customarily found with tobacco mosaic juice, were obtained. Although about 80 per cent. of the amount of protein originally in the juice was found in the supernatant liquid, this protein was inactive and all the virus activity was concentrated in the pellets. Thus one ultracentrifugation served to separate the virus activity from the major portion of the protein. Since these pellets were so small and were found to contain much pigment and colloidal matter, several were combined, well suspended in 0.1 M phosphate buffer at pH 7 and spun on a Swedish angle centrifuge for 15 minutes. This served to further purify the protein, for much of the pigment and colloidal matter sedimented to the bottom of the tube. The supernatant liquid, which contained the soluble protein and a small amount of finely dispersed colloidal matter, was then ultracentrifuged and the whole process of alternate ultracentrifugation, re-solution of the protein and low-speed angle centrifugation was repeated 2 times. Each ultracentrifugation served to separate the high molecular weight from the low molecular weight material and to aggregate colloidal matter, and each low speed angle centrifugation separated this aggregated colloidal matter from the soluble material.

No protein could be demonstrated in the supernatant liquid from the third ultracentrifugation. The pellet which was obtained was found to contain but a trace of insoluble matter and to consist of crystalline protein. In the analytical ultracentrifuge a solution of this protein gave a sharp boundary characteristic of a single molecular species with sedimentation constant S_{20° = ca 115×10^{-13} cm. sec.$^{-1}$ dynes^{-1}. A total of about 10 mg of this high molecular weight protein has been prepared on 4 different occasions and the yield has varied from about 0.005 to 0.01 mg of protein per gram of starting material. This indicates that diseased plants contain about one part of this protein per 100,000 parts of plant material and hence that the virus activity of the protein concentrate might be expected to approach about 100,000 times that of the starting material. The virus activity of the protein has been tested on 10 different occasions and in every instance solutions containing but 10^{-7} grams of the protein per cc were found to be active and capable of causing necrotic lesions on cowpea,[8] *Vigna sinensis* Endl. Since the juice from plants diseased with tobacco ring spot virus, when similarly tested, was not found to be

[1] W. M. Stanley, *Amer. Jour. Bot.*, 24: No. 2, 1937.
[2] W. M. Stanley, SCIENCE, 81: 644, 1935; *Phytopath.*, 26: 305, 1936; *Jour. Biol. Chem.*, 115: 673, 1936.
[3] R. W. G. Wyckoff, J. Biscoe and W. M. Stanley, *Jour. Biol. Chem.*, 117: 57, 1937.
[4] R. W. G. Wyckoff and R. B. Corey, SCIENCE, 84: 513, 1936.
[5] W. M. Stanley, *Jour. Biol. Chem.*, 117: 755, 1937.
[6] C. N. Priode, *Amer. Jour. Bot.*, 15: 88, 1928.
[7] R. W. G. Wyckoff and J. B. Lagsdin, *Rev. Sci. Instr.*, 8: no. 3, 1937.
[8] W. C. Price, *Contrib. Boyce Thompson Inst.*, 4: 359, 1932.

active at dilutions greater than 1 to 1,000, the protein isolated by the ultracentrifugal method is about 10,000 times more active than the starting material. This tremendous concentration of virus activity and the very small yield of protein are in striking contrast to the results obtained in the case of mosaic-diseased Turkish tobacco plants, which were found to contain about one part per 500 of crystallizable tobacco mosaic virus protein, the activity of which was, therefore, only about 500 times that of the starting material.

As indicated by the great difference in sedimentation constants, the properties of tobacco ring spot virus protein are quite different from those of tobacco mosaic virus protein. The latter does not become denatured and loses practically no virus activity on short exposures to hydrogen ion concentrations between pH 2 and 3 or to temperatures up to about 70° C. It does not denature and its activity is not lost on standing for several days at room temperature. In marked contrast, tobacco ring spot virus protein is almost completely denatured and inactivated after one hour at pH 3, is completely denatured and inactivated after a 5-minute exposure to a temperature of 64° C., and is partially inactivated after one day and almost completely inactivated after 6 days at room temperature. Of interest is the fact that it may be more stable than tobacco mosaic virus protein towards alkali, for ring spot virus protein loses only a small amount of activity on standing for one hour at pH 9.6. It is, however, completely denatured and inactivated after standing for one hour at pH 10.8.

The serological properties of the 2 proteins are also quite different for, although the sera of animals injected with tobacco mosaic virus protein give a precipitate when mixed with solutions containing only 10^{-6} gm of mosaic protein per cc, they fail to give a precipitate when mixed with solutions containing as much as 10^{-3} gm of tobacco ring spot virus protein per cc. This property was put to practical use in the purification of one sample of ring spot virus containing a trace of tobacco mosaic virus protein as a contaminant. Antiserum to mosaic virus protein was added to the contaminated preparation, the precipitated mosaic virus protein was removed by low-speed angle centrifugation, and the ring spot protein was then separated from the excess antiserum by ultracentrifugation. It was thus possible to separate and remove mosaic protein from ring spot protein. These results demonstrate, as might have been expected from work with the crude juices,[9] that the mosaic and ring spot proteins are distinct serological entities. Further evidence indicating that the two proteins are different is found in the fact that the x-ray diffraction pattern of crystalline ring spot virus protein differs from that of crystalline tobacco mosaic virus protein. It has been possible, therefore, by a method involving use of the newly developed quantity ultracentrifuge, to isolate from Turkish tobacco plants diseased with ring spot virus a high molecular weight protein possessing the properties of ring spot virus and differing markedly from tobacco mosaic virus protein in its concentration in the plant and in its physical, chemical and serological properties.

This method has also been used in examining the juices of Turkish tobacco plants diseased with latent mosaic of potato (X-virus), severe etch and cucumber mosaic viruses, respectively. In the case of latent mosaic virus the pellets which were obtained were about 1/20th to 1/50th the size customarily obtained with mosaic juice and were found to contain all the virus activity. The protein in these pellets was also of a single molecular species with a sedimentation constant, S_{20} = ca 110, close to that of the ring spot virus protein. With severe etch virus the pellets were larger than those of latent mosaic virus and were also found to contain all the virus activity. The protein in these pellets sedimented more diffusely than did the ring spot and latent mosaic virus proteins, but the heterogeneity this indicates was probably the result of decomposition that occurred before the ultracentrifugal analysis was carried out. Although the boundaries obtained were too diffuse for accurate measurement, it was obvious that severe etch virus protein sediments at a rate comparable with that of the tobacco mosaic virus protein. When the juice from cucumber mosaic-diseased plants was ultracentrifuged, insufficient soluble protein for physical and chemical tests was obtained, despite the fact that all the virus activity was concentrated at the bottoms of the tubes. If, as seems to be the case, the dilution end-points of viruses may be used as a rough criterion of the amount of virus protein present in the host, then, since the dilution end-point of juice from cucumber mosaic-diseased plants is about 1 to 100, the amount of virus protein would be expected to be about 0.001 mg or less per gram of plant material. It would be necessary, therefore, to ultracentrifuge a liter or more of juice in order to secure a milligram of virus protein. Attempts to concentrate the juice before ultracentrifugation by means of precipitation with ammonium sulfate and solution in from 1/5th to 1/15th the original volume, although quite successful and useful in the cases of ring spot and latent mosaic viruses, were not successful in the case of cucumber mosaic virus, possibly because of its extreme instability.

The tremendous difference in the concentration of the various virus proteins in well-diseased Turkish tobacco plants is especially noteworthy. The concentration in the host ranges from one part per 500 for tobacco mosaic virus protein, through latent mosaic and severe etch to ring spot virus protein which occurs

[9] K. S. Chester, *Phytopath.*, 25: 686, 1935.

in about one part per 100,000, and to cucumber mosaic virus protein which possibly may occur in less than a part per million. It seems likely that, with respect to concentration in the host and to instability, certain of these viruses are much more nearly comparable to many animal viruses than is the very stable and abundant tobacco mosaic virus. As a whole, the results demonstrate that high molecular weight proteins are characteristic of these various virus diseases, and that the physical, chemical and serological properties and the concentration in the host of these proteins differ widely.

SUMMARY

A high molecular weight crystalline protein, possessing the properties of ring spot virus and differing markedly from tobacco mosaic virus protein in its physical, chemical and serological properties, has been isolated by means of an ultracentrifuge from Turkish tobacco plants diseased with tobacco ring spot virus. Ultracentrifugal methods were also used to demonstrate that high molecular weight proteins are characteristic of other virus diseases. The concentration of the different virus proteins in the host was found to differ greatly. The quantity ultracentrifuge, used in conjunction with an analytical ultracentrifuge, has proven to be a powerful tool for the concentration, purification and crystallization of high molecular weight virus proteins and to be indispensable in the case of unstable viruses existing in low concentration in the host.

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VITAMIN B_1 A GROWTH FACTOR FOR HIGHER PLANTS

IN experiments to be reported in detail elsewhere, we have found that vitamin B_1 is an important "growth factor" or "growth hormone" for growth *in vitro* of isolated roots. It seems probable that vitamin B_1 is the active principle of yeast extract, shown by Robbins[1] to be beneficial for the growth of isolated corn roots, and by White[2] to be necessary for the continued growth of isolated tomato roots.

After an extensive search for optimal conditions and optimal composition of the nutrient solution it was first found possible to grow freshly isolated pea roots in a pure synthetic medium containing inorganic salts and sucrose. Additions of yeast extract had no stimulating effect upon this initial culture or "passage" and, in fact, yeast extract concentrations higher than 0.01 per cent. were slightly inhibitory, due probably to heteroauxin present in the yeast. If such roots were subcultured by the removal of 10 mm tips into fresh medium and particularly if this procedure were repeated several times, yeast extract was, however, found to be essential for growth. Thus in the third passage pea roots, cultivated in nutrient medium but without yeast, ceased growth completely, whereas roots in the same medium but with the addition of 0.01 per cent. yeast extract may be carried through many passages with an average growth rate of 6 to 9 mm per root per day. The pea root as cut from the seedling plant contains thus sufficient "growth factor" to permit of growth for some time and the initial culture is not influenced by yeast extract, since this growth factor is not limiting. After two or more passages this initial supply is, however, used up and the root responds to growth factor present in the yeast.

It was next found that vitamin B concentrates are considerably more active as a source of the root growth factor than is yeast. This suggested that vitamin B_1 itself might be the active principle and experiments carried out with Merck's crystalline preparation have shown that this is the case. Table I shows that 0.2 gamma per cc is able to replace the optimal yeast extract concentration completely and is in fact superior to it.

TABLE I

GROWTH RATE OF EXCISED PEA ROOTS IN MM PER ROOT PER PASSAGE

Passage	I	II	III	IV	V
No addition	65	10	0	0	0
0.01 per cent. Yeast ext.	64	43	45	40	55
Cryst. B_1 0.2 γ/cc	65	72	65	66	65

Much smaller concentrations of crystalline vitamin B_1 than 0.2 gamma per cc suffice. Thus 0.002 gamma per cc still has a marked stimulating effect upon the growth of these roots. Two gamma per cc has on the other hand no more effect than does 0.2 gamma per cc.

We have as yet no indication that substances other than vitamin B_1 (for example, amino acids in small amounts[3]) are necessary as "growth substances" for pea roots. It is possible, however, that over larger numbers of passages such co-growth substances may be indispensable.

Vitamin B_1 is then not only an animal vitamin and a growth substance for fungi and bacteria, but it is also a growth substance for higher plants. Kögl and Haagen-Smit[4] in a paper published while the above

[1] W. J. Robbins, *Bot. Gaz.*, 74, 59, 1922.
[2] P. R. White, *Plant Physiol.*, 9, 585, 1934.
[3] P. R. White. Paper read at the annual meeting of the Amer. Soc. of Plant Physiologists, Atlantic City, December, 1936.
[4] F. Kögl and A. Haagen-Smit, *Zeit. Physiol. Chemie*, 243, 209, 1936.