Technical Papers

The Separation of Porphobilinogen and an Ehrlich Negative Precursor of Uroporphyrin[1]

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Hitherto it has been widely accepted that the Ehrlich aldehyde reacting porphobilinogen, excreted in the urine of acute porphyria cases, is the precursor of both uroporphyrin and the nonporphyrin pigment, porphobilin. Waldenström and Vahlquist (*1*) reported that the heating of porphobilinogen-containing urines at pH 4.0 favored uroporphyrin formation, whereas heating at pH 8.0 resulted in a greater formation of porphobilin. They observed the same behavior with porphobilinogen solutions obtained by electrophoresis and regarded as homogeneous entities. Evidence has now been obtained, however, that the Ehrlich-reacting porphobilinogen gives rise only to porphobilin once it has been separated from one or more non-Ehrlich–reacting precursors, which regularly accompany it in the urine of these cases. On the other hand, it has become clear that variable, but often large, fractions of the uroporphyrin in these urines are obtained from non-Ehrlich–reacting precursors. A relatively simple method of separating these substances has now been worked out and is described briefly here.

Urine samples from 8 patients suffering with intermittent acute porphyria were filtered and acidified to pH 5.6–6.0 with acetic acid. A column of Merck's alumina (for chromatographic analysis) was washed with 1% acetic acid until the eluate was acid to litmus. The filtered urine was then run through with gentle suction. Both pigments and chromogens were adsorbed on the column, which was washed first with distilled water and then with 1% acetic acid. The latter contains the porphobilinogen in a relatively concentrated solution. In some cases small amounts of uroporphyrin precursor are also eluted. This solution was brought to pH 6.0 and then irradiated in a Pyrex test tube for 3–5 min with filtered light from an ultraviolet lamp.[2] The irradiation converts any uroporphyrin precursor that may be present to porphyrin, leaving the porphobilinogen unaffected. The solution was barely alkalinized with dilute NaOH, and the free porphyrins were precipitated with $CaCl_2$ and Na_2HPO_4, according to the method of Sveinsson, Rimington, and Barnes (*2*). This final Ehrlich-reacting filtrate was usually free of porphyrin and porphyrin precursors. In certain instances it was necessary to repeat the radiation and $CaHPO_4$ adsorption of uroporphyrin in order to separate the porphobilinogen completely from uroporphyrin precursor.

Solutions of porphobilinogen obtained by the above method, after having been acidified to pH 4.0 and boiled for 30 min, gave rise only to porphobilin, a dark-brown pigment without characteristic absorption spectrum or any porphyrin characteristics.

Electrophoretic studies to be described in detail separately have revealed that porphobilinogen and the uroporphyrin precursor have a single homogeneous zone and cannot be separated on the basis of electrophoretic mobility. The solution of the single zone, however, can then be subjected to the above-described method with complete separation of the two chromogens.

The present observations are believed to explain an incorrect concept that porphobilinogen is a precursor of uroporphyrin as well as porphobilin. Further studies of these precursors are in progress.

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[2] Model BL-2, Black Light Products, Chicago, Ill.

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Ultraviolet Absorption Spectra as a Tool for Diagnosing Plant Virus Diseases[1,2]

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Methods developed in this laboratory for the detection of viruses in fruit trees by means of color or staining tests (*1–4*), although useful, are limited in that they are not critical in separating one virus from another. Moreover, these methods are not applicable to herbaceous plants. In a search for more refined methods of virus detection, the ultraviolet absorption spectra of various extracts from virus-diseased and healthy leaves were investigated. After considerable study it was found that acid hydrolysis of leaf tissue, subsequent to the removal of the alcohol-soluble constituents, produced absorption

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spectra that appeared to be characteristic of the virus diseases studied. More than 3000 healthy and diseased plants of various species have been studied by this procedure, which shows promise of being helpful in the study of virus diseases. Much of the work has been confined to virus diseases of stone fruits, but the method is suitable for virus diseases of herbaceous plants as well.

A relatively small sample is required for the procedure. Two leaf disks, ¼″ in diameter, are taken with a paper punch. Usually each disk is taken across the mid-vein or a large lateral vein to insure the inclusion of both phloem and xylem tissues. More than two leaf disks may be required for certain types of plants or for certain diseases. In some cases, one disk may be enough. Very young leaves or very old ones should not be sampled; the sampling should be confined to mature leaves. For small plants, a minimum of three samples of two disks each per plant is used from leaves of different ages. It is desirable to test leaves of different ages because the virus concentration may vary considerably from the apical to the basal portion of any current growth. Moreover, if virus mixtures are present, the relative proportions of the constituent viruses may vary with leaf age. It is easier to detect the constituent viruses if leaves of different ages are sampled. The three samples from any given plant are analyzed separately, and the absorption curves are plotted separately. Fresh material has been used in this work to avoid possible loss of labile constituents that might occur in dried material.

The two leaf disks are placed in a test tube, 1 ml 70% ethyl alcohol is added, and the tube is heated in a water bath at 80° C for 10 min. The alcohol is decanted from the sample, a fresh 1-ml portion is added, and the extraction procedure is repeated. Three such extractions are usually sufficient to remove all alcohol-soluble material. The leaf disks should be free of chlorophyll at the end of the extraction period. The final alcohol wash is drained from the sample, and 3 ml of an alcohol-hydrochloric acid mixture is added (10 ml HCl plus 90 ml 95% ethyl alcohol).[3] The sample is then heated at 80° C in a water bath for 30 min and cooled, and the alcohol-HCl mixture is decanted into a silica absorption cell (10 mm light path). A Beckman DU spectrophotometer was used in these studies, and absorption readings were taken at 2-mμ intervals in the range from 230 to 300 mμ. The alcohol-HCl reagent was used as a reference sample.

[3] Alcoholic HCl was used for hydrolysis rather than aqueous HCl because with virus-diseased stone fruit leaves a red color is produced by alcoholic HCl, and thus a simple color test as well as the ultraviolet absorption test is available from the same sample. The intensity of red color may be measured independently in a photoelectric colorimeter prior to the ultraviolet absorption measurements. This color test is based on the same principle as the tests reported previously (*1–4*). In this particular color test the HCl presumably produces furfuraldehyde from pentose sugars, and the furfuraldehyde condenses with phenolic compounds and yields the red color. Similar reactions have been used as color tests for pentose sugars (*5*).

In tests of leaf tissue that we consider to be healthy, whether from a herbaceous plant or a fruit tree, characteristic absorption curves for nucleic acid or its hydrolysis products are obtained (Fig. 1). The

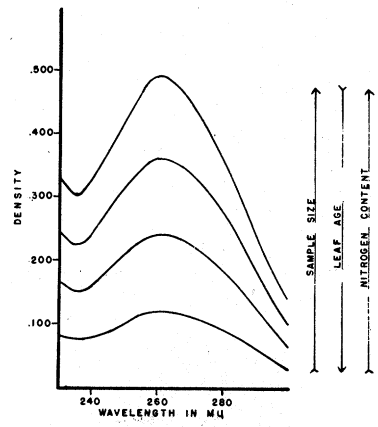

FIG. 1. Absorption curves from healthy plants, showing effect of sample size, leaf age, and nitrogen content.

height of the absorption peak at 260 mμ is a measure of the amount of nucleic acid present. The height of the peak may be increased by increasing the sample size or by using younger leaf tissue. Thus in comparing diseased tissue with healthy tissue it is necessary to use comparable samples.

The mosaic-type virus diseases that we have studied (tobacco mosaic and potato X) are not readily distinguished by this method because the principal hydrolysis products are nucleic acids (Fig. 2). Ring-

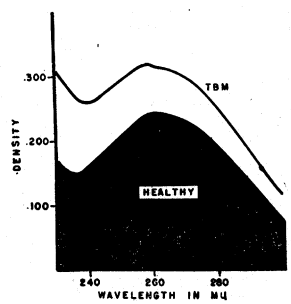

FIG. 2. Absorption curve from healthy *Physalis floridana* Rydberg compared with that of *P. floridana* affected with tobacco mosaic (TBM).

spot-type viruses, as represented by ringspot of stone fruit trees, produce a distinctive absorption curve, with a peak at or near 270 mμ (Fig. 3 *A*). The yellows-type virus diseases that we have studied, such as sour cherry yellows and Western X disease, also produce distinctive absorption curves, with peaks at or near 280 mμ, which appear to be characteristic (Fig. 3 *B, C, D*). Sour cherry yellows is considered to be caused by a complex of at least two viruses, ringspot virus and an unknown (*6*); this is confirmed by the absorption curves. The 270 and 280 mμ peaks are consistent for this disease, but the relative heights of the two peaks vary independently with a number of conditions (Fig. 3 *B* and *C*). Sometimes the ring-

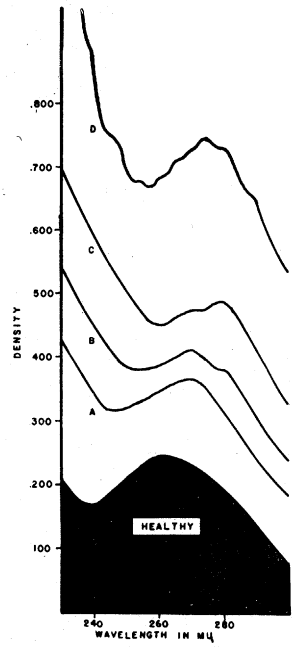

FIG. 3. Absorption curves from peach affected with various virus diseases compared with absorption curve from healthy peach: *A*, Ringspot; *B* and *C*, sour cherry yellows; *D*, Western X disease.

spot peak is dominant, and at other times the yellows peak is dominant.

Western X disease of peaches gives a very complex absorption curve (Fig. 3 *D*), which may be due to a mixture of several viruses. This disease from different sources often shows different secondary peaks, but the peaks at 274–276 mμ and at 280–282 mμ are consistent.

With the information on hand, it is not possible to determine whether each absorption peak is due to a single virus or whether a single virus may give more than one peak. If it were possible to study pure preparations of each of the viruses, this point could readily be settled; but so far none of the stone fruit viruses has been isolated in reasonably pure form. Likewise, it is not known whether the absorption peaks actually indicate hydrolysis products of the virus itself or end products of an interaction between the virus and the host. With a readily purified virus like tobacco mosaic, it is possible to show that the absorption peak obtained is probably from the virus itself. Fig. 4 shows the absorption curve of a purified preparation of tobacco mosaic virus along with the absorption curve of the hydrolysis products of the same preparation. A purified preparation of tobacco mosaic virus yields a nucleic acid absorption curve on hydrolysis that is similar to the curve obtained from leaves infected with the virus (*cf.* Fig. 4 and Fig. 2).

It has not been possible to determine whether disorders other than those caused by viruses produce materials in leaves that would interfere with this method. Nitrogen deficiency has been studied under controlled conditions and found to affect merely the height of the normal nucleic acid peak. The higher the nitrogen content of the leaf, the higher the nucleic acid peak (Fig. 1).

In some types of plant material in which virus mixtures occur, the concentration of one of the components may be very high and mask the absorption curves of other viruses that may be present. This is often true of sweet cherries, in which the 280 mμ yellows peak is very high.

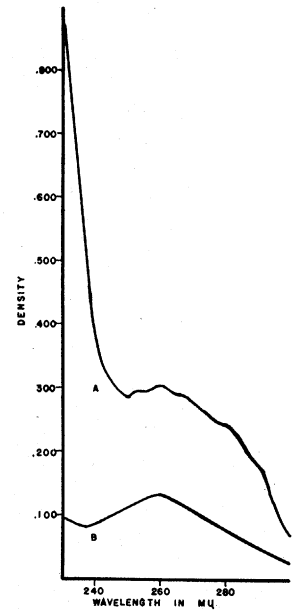

FIG. 4. Absorption curves of tobacco mosaic virus. *A*, 100 ppm purified tobacco mosaic virus in *M*/10 phosphate buffer at pH 7.0; *B*, alcoholic-HCl hydrolysis products of same preparation.

It should be emphasized that, as with all analytical methods, this one indicates only what is present at the time and place of sampling. Moreover, the virus concentrations must be high enough to produce an absorption curve that is distinctive from the curve from healthy tissue. Virus infection may be detected with this procedure before symptoms are obvious. For example, a stone fruit virus transmissible to cucumber usually produces visible symptoms in cucumber cotyledons in 4–5 days. With the spectrophotometer the disease can be detected in cucumber cotyledons 48 hr after inoculation.

Care must be exercised in associating any given symptom of a virus disease with a particular absorption peak if virus mixtures are present in a sample.

Although the method requires the use of a good spectrophotometer and is somewhat time-consuming (analyses of about 30 samples a day exclusive of plotting the absorption curves being possible), it promises to be very helpful in the diagnosis and study of virus diseases. It shows particular promise in the study of latent viruses, and virus mixtures and

complexes of stone fruits, and possibly may aid in arriving at a more rational virus nomenclature based upon chemical properties.

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Formation of 2,3-Butylene Glycol in Bacterial Fermentation of D-Glucosamine[1]

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There have been a few reports made with respect to the products of bacterial fermentation of D-glucosamine. According to these papers, acetic and butyric acids (*1*), propionic and D-lactic acids (*2*), D-lactic, L-lactic, and succinic acids (*3*), etc., have been shown to be produced by action of bacteria on D-glucosamine.

In our laboratory extensive investigations were recently carried out on the ability of microorganisms in the soil to make use of D-glucosamine; those that were observed to utilize it were isolated into pure cultures. From among them, a number of active strains of bacteria and of fungi were selected for studies on the fermentation of D-glucosamine. The bacteria of the coli-aerogenes group were found to utilize it vigorously.

A report is made here on the products of the fermentation of D-glucosamine by *Aerobacter cloacae* (Jordan) Bergey *et al.,* isolated from soil. A liquid culture medium containing D-glucosamine alone as the source of both carbon and nitrogen was employed; composition of the medium was as follows:

(*A*)	D-glucosamine hydrochloride			40.0 g	in 1 liter of water
(*B*)	NaCl	10.0 g,	KH_2PO_4	4.0 g	in 1 liter of water
	$MgSO_4 \cdot 7H_2O$	0.2 g,	$CaCO_3$	10.0 g	

where (*A*) and (*B*) were prepared apart, sterilized separately, and then combined aseptically.

A strain of *A. cloacae* was inoculated into the medium and incubated at 37° C for about 10 days. In the early stages of incubation, a vigorous generation of gas was observed.

After the incubation period, the liquid culture was filtered, concentrated *in vacuo* to about 300 ml, and extracted continuously with ether for 50 hr. The ether extract was dried, concentrated to a viscous liquid, and the residual liquid was distilled under diminished pressure to give 6.9 g (approx 20% of the D-glucosamine) of the main fraction at 89°–92° C/16–17 mm Hg after a small quantity of the foregoing fraction (0.5 g). The substance obtained was a colorless, clear, viscous liquid ($[\alpha]_D^{24°\,C} = +1.3°$ [1 = 1dm]), somewhat like glycerol, which crystallized in the cold. We confirmed it to be 2,3-butylene glycol by distilling it with 25% sulfuric acid, followed by separating the methylethyl ketone, which is to be derived from 2,3-butylene glycol, from the distillate, converting the ketone into the *p*-nitro-phenylhydrazone (yellow; mp, 126.5°–127.5° C), adopted under the Akabori method (*4*).

After the extraction of the glycol, the aqueous mother liquor was strongly acidified with sulfuric acid and extracted again with ether continuously for 40 hr. On concentration of the extract, there remained a considerable amount of colorless, clear, viscous liquid containing the crystals of succinic acid (1.1 g; approx 3% of the D-glucosamine) and giving out an acetic acid smell. The volatile acids contained were separated from it by steam distillation as usual; the total amount was estimated at 0.64 g as acetic acid. Lactic acid was also found in this acidic extract.

In addition, small amounts of ethyl alcohol and acids fixed in the precipitate, such as oxalic acid, were also observed to be produced in this fermentation.

Study as to when and how the amino group is split off in the process of the fermentation of D-glucosamine is in progress.

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The Influence of Skin Temperature upon the Pain Threshold as Evoked by Thermal Radiation—A Confirmation

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In a recent paper Hardy, Goodell, and Wolff (*1*) reported experiments on the effect of skin temperature on pain threshold evoked by thermal radiation. By a graphical extrapolation from their data, they were able to infer that "the skin in the areas tested must be raised [to a temperature of 44.9° C] to be noxiously stimulated, regardless of the initial level of skin temperature."

[1] This research was carried out while the author was at the Psycho-Acoustic Laboratory, Harvard University, Cambridge, Mass.

[1] Previously reported at the meeting of the Chemical Society of Japan in April 1947.

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