The alcohol was distilled until only a thick sirup remained in the flask. The last traces of solvent were removed *in vacuo* and the resulting crystals were placed in a vacuum desiccator for twelve hours. The salts were used without further purification.

Preparation of α-Bromoesters.—The dried, finely pulverized, potassium salt was mixed with 100 ml. of carbon tetrachloride. The ice-cold mixture was stirred vigorously while a solution of 25 g. (0.15 mole) of bromine in 50 ml. of carbon tetrachloride was added dropwise over a period of two to four hours. The bromine was decolorized rapidly at the start of the reaction, but persisted after all of the bromine solution had been added. The mixture was filtered and the solvent was removed in a current of air. The residue was distilled at reduced pressure to give in every case a colorless, strongly lachrymatory liquid. The yields and physical constants of the compounds prepared are given in Table I.

Summary

The reaction of the potassium salts of monoethyl alkylmalonates with bromine provides a new method of preparing α-bromoesters.

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[CONTRIBUTION FROM THE VIRUS LABORATORY, UNIVERSITY OF CALIFORNIA[1]]

The Apparent Sulfur in Cucumber Virus 4

BY C. A. KNIGHT

Rather extensive chemical analyses have been made of tobacco mosaic virus (TMV) and several of its strains; and cucumber viruses 3 and 4 (CV3 and CV4) have been included in these studies.[2] The consideration of CV3 and CV4 as strains of TMV has been based on their mutual possession of an uncommon size and shape, on weak but definite serological cross reactions, on a common unusual resistance to heat and desiccation, and on possession of apparently identical quantities of protein and pentosenucleic acid.[3-5] On the other hand, it has not been possible to perform one of the tests of strain relationship, the cross-protection test, for as yet no common host has been reported for TMV and the cucumber viruses. Furthermore, it appears that the particles of CV3 and CV4 differ slightly but significantly in size from those of TMV and some of its other strains.[6,7] However, whether they are actually distantly related strains of TMV as supposed, or whether they are distinct viruses, it seems evident that the facts revealed from their study could ultimately be brought to bear on problems of the relationship of biological, serological, and physical properties to the chemical composition and structure of the virus, or of biologically active proteins in general. As a consequence of this viewpoint, it seemed imperative to determine the nature of the sulfur in CV3 or in CV4, for, while the sulfur of TMV and of most extensively studied proteins has been shown to occur mainly in well-known sulfur-containing amino acids, the sulfur apparently present in CV3 and CV4 has eluded identification.[3,8]

It will be shown in the present report that CV4 differs decidedly from TMV and some of its strains in that CV4 probably contains no sulfur at all.

Materials and Methods

Preparation of Virus.—Highly purified preparations of CV4 were obtained from the expressed juice of appropriately diseased cucumber plants by differential centrifugation as previously described.[3] The salt content of the preparations was reduced to a very low level by handling the virus in distilled water during two or more cycles of centrifugation, by dialysis against flowing distilled water, or by electrodialysis. These preparations were then frozen and dried *in vacuo*, and then further dried to constant weight at 110° in a drying oven.

Sulfur Analyses.—The first sulfur values for highly purified, dried preparations of CV3 and CV4 were those of Bawden and Pirie in which a range of 0.0 to 0.6% sulfur was reported.[9] No explanation of the possible significance of this range of results was advanced, and the value recorded later by Bawden appears to be a mean of the earlier determinations, namely, 0.3%.[4] A figure of 0.85% sulfur was given by Knight and Stanley as the mean of 8 analyses made on 3 different preparations of CV4.[3] Since that time and including the individual analyses leading to the figure just given, a total of forty-one analyses have been made by three different analysts on thirteen of our preparations. The analyses were made by the customary Carius pressure-tube method and yielded an average of 0.6% sulfur, but the individual values obtained ranged from 0.07 to 1.26%. Furthermore, a given analyst obtained with the same preparations of virus, but on different occasions, sulfur values which differed by as much as 50%. Also, different analytical laboratories, analyzing portions of the same sample of virus, reported results equivalent to three- to ten-fold differences in sulfur content. No analyst was consistently high or low in his results.

Despite this confusing situation, the results seemed to indicate that there is some sulfur in CV4, but that it is for some unknown reason, inherently difficult to obtain a reliable estimate of its quantity.

Attempts to Identify Sulfur in CV4.—A series of tests made on numerous preparations of CV4 over a period of several years has established the absence from this virus of amounts greater than traces of the amino acids, cys-

(1) This investigation was begun in the laboratories of the Rockefeller Institute for Medical Research, Princeton, N. J. Presented in part before the Division of Biological Chemistry at the 115th Meeting of the American Chemical Society at San Francisco, March 28–April 1, 1949.

(2) C. A. Knight, *J. Biol Chem.*, **171**, 297 (1947).

(3) C. A. Knight and W. M. Stanley, *ibid.*, **141**, 29 (1941).

(4) F. C. Bawden, "Plant Viruses and Virus Diseases," second ed., Chronica Botanica Company, Waltham, Mass., 1943, p. 162.

(5) C. A. Knight and W. M. Stanley, *J. Biol. Chem.*, **141**, 39 (1941).

(6) J. D. Bernal and I. Fankuchen, *Nature*, **139**, 923 (1947).

(7) C. A. Knight and G. Oster, *Arch. Biochem.*, **15**, 289 (1947).

(8) C. A. Knight, Abstracts, Meeting of the American Chemical Society, September 8–12, 1941.

(9) F. C. Bawden and N. W. Pirie, *Brit. J. Exptl. Path.*, **18**, 275 (1937).

teine, methionine and thiolhistidine.[10] Cysteine and cystine were ruled out by the failure of CV4 to give a positive nitroprusside test either in the presence or absence of cyanide, the failure of CV4 to reduce uric acid reagent, and by the fact that these amino acids were not detected in the Baernstein determination of sulfur amino acids.[3,8,11-13] Methionine was eliminated from consideration by its failure to appear in the Baernstein separation, the Kolb and Toennies color reaction,[14] or in microbiological assays.[2] It should be noted also that the Baernstein method yielded no sulfate sulfur at all when applied to several of the samples. At one time, it was thought that positive color tests for thiolhistidine were obtained[8] but in more carefully controlled experiments these were not secured, which, coupled with the failure of thiolhistidine to appear in the Baernstein separation, make its presence in CV4 very dubious. Some microbiological tests for biotin, kindly made on a hydrolysate of CV4 by Dr. William Trager, indicated that this sulfur-containing vitamin was absent.

While the tests mentioned above did not preclude the possible presence in CV4 of some rarely occurring or previously unknown sulfur component, it nevertheless seemed advisable to test next for the possible presence of some element other than sulfur, but related to it, such as selenium.

Tests for Selenium in CV4.—When CV4 was digested and treated with codeine sulfate according to the procedure of Gortner and Lewis[15] or some modification of it, a blue color indicative of the presence of selenium was obtained. This color was not secured under comparable conditions with tobacco mosaic virus. However, application of the more specific distillation method of Klein[16] showed no detectable selenium in a 100-mg. sample of CV4. In a control experiment, using this method, the addition to a sample of the virus of 25 micrograms of selenium in the form of sodium selenite resulted in a recovery of 22 micrograms. The absence of selenium from CV4 was further confirmed by examination of the emission spectrum of the virus by Dr. G. I. Lavin. This test showed the absence of even traces of selenium from CV4. Therefore, it can be concluded that selenium is not present in CV4, although no explanation has yet been found for the positive codeine color reaction given by this virus.

The absence of tellurium, as well as selenium, was also indicated by failure to detect either of these elements when the test of Drew and Porter[17] was applied to 500 mg. of virus.

In view of the foregoing, it seemed pertinent to return to the potential sulfur in CV4 and to seek a decisive test which would indicate whether or not this element was present. The use of radiosulfur was selected for this purpose.

Experiments with Sulfur 35.—It had been found that radioactive phosphorus was incorporated into tobacco mosaic virus when mosaic-diseased tobacco plants cultured in sand were fed a nutrient solution to which had been added disodium phosphate containing radioactive phosphorus.[18] It seemed likely, therefore, that if CV4 contained sulfur, a radioactive isotope of that element could be introduced into the virus by a similar procedure. For this purpose, about 100 young cucumber plants growing in sand culture were inoculated with CV4, and starting the following day and continuing for about thirty days, the plants were watered with 17 parts of Spencer's medium nitrogen nutrient solution[19] to which had been added 1 part of potassium chloride solution containing S^{35}.[20] Each plant received about 100 ml. of solution every other day. A day after the last feeding, the plants were harvested, frozen and subsequently processed for CV4 by the usual methods.[3] The virus was handled in diminishing concentrations of phosphate buffer through successive cycles of centrifugation, starting with 0.1 *M* and working down through 0.05, 0.01 *M* and going finally to water, from which the preparation was frozen and dried. A portion of partially purified virus, containing a small but visible amount of green impurity, was reserved for radioactivity measurements. The highly purified, dried virus was white in color.

In order to compare the results obtained with CV4 to those procurable in the case of a strain of tobacco mosaic virus known to contain sulfur, an experiment similar to that just described was performed with tobacco mosaic virus grown in three Turkish tobacco plants.[21]

The sulfur in the various fractions was converted to sulfate by Carius oxidation and precipitated from the digests as benzidine sulfate.[22] Measurements of S^{35} activity were made in a Lauritzen electroscope[23] using the methods of Henriques and co-workers.[22] Carrier sulfur was used when necessary and all samples of benzidine sulfate measured for radioactivity came within the weight range of 6.7 to 7.1 mg.

The data, which are summarized in Table I, indicate that a negligible quantity of the S^{35} supplied to the cucumber plants appeared in the highly purified CV4. On the other hand, the partially purified virus, which contained a visible trace of green impurity, was found to possess 45 times the radioactivity of the highly purified virus. This, and the considerable amount of radioactivity found in the sap and in the plant residue after ex-

TABLE I

DISTRIBUTION OF S^{35} ACTIVITY IN CV4, TOBACCO MOSAIC VIRUS AND OTHER FRACTIONS

Mg. S supplied to plants in nutrient solution in CV4 expt.	17,292
Mg. S in 200 mg. isolated CV4 on basis of 0.6% S	1.2
Total units[a] S^{35} supplied to plants	132,000,000
Total units S^{35} found in 200 mg. of CV4	31
Per cent. of theory of S^{35} found in CV4	0.33
Total units of S^{35} in plant sap from which CV4 was isolated	2,170,000
Units of S^{35} found in dried plant residue after extraction of CV4	18,500,000
Mg. S supplied to plants in nutrient solution in TMV experiment	433
Mg. S in 27 mg. isolated TMV on basis of 0.2% S	0.054
Total units S^{35} supplied to plants	2,590,000
Total units S^{35} found in 27 mg. of TMV	178
Per cent. of theory of S^{35} found in TMV	55

[a] Results are expressed in terms of electroscope divisions per minute.

(10) A sample of thiolhistidine was kindly provided by Dr. V. du Vigneaud.

(11) H. D. Baernstein, *J. Biol. Chem.*, **115**, 25, 33 (1936).

(12) B. Kassel and E. Brand, *ibid.*, **125**, 145 (1938).

(13) M. L. Anson, *J. Gen. Physiol.*, **25**, 355 (1942).

(14) J. J. Kolb and G. Toennies, *J. Biol. Chem.*, **131**, 401 (1939).

(15) R. A. Gortner, Jr., and H. B. Lewis, *Ind. Eng. Chem., Anal. Ed.*, **11**, 198 (1939).

(16) A. K. Klein, *J. Assn. Off. Agr. Chem.*, **26**, 346 (1943).

(17) H. D. K. Drew and C. R. Porter, *J. Chem. Soc.*, 2091 (1929).

(18) W. M. Stanley, *J. Gen. Physiol.*, **25**, 881 (1942).

(19) E. L. Spencer, *Plant Physiol.*, **12**, 825 (1937).

(20) One irradiated unit no. **17** was obtained from the U. S. Atomic Energy Commission. The target material in this unit was 25 g. of potassium chloride. This was dissolved in 9 liters of distilled water and 8 liters of the resulting solution was employed in the CV4 experiment and a portion of the remaining liter was used in the TMV test.

(21) It was not feasible to make this parallel experiment in cucumber plants since no virus known to contain sulfur has been isolated from this source in a highly purified state.

(22) F. C. Henriques, Jr., G. B. Kistiakowsky, C. Margnetti and W. G. Schneider, *Ind. Eng. Chem., Anal. Ed.*, **18**, 349 (1946).

(23) The author is indebted to Dr. Roger Herriott for the use of this instrument.

traction of the virus, show that a significant portion of the S^{35} supplied to the plants was taken up and utilized. One can, therefore, conclude that if CV4 had contained S, even in the smallest amount shown by the analytical data, this would have been manifested by appreciable radioactivity in the isolated and highly purified CV4. Confirmation of this conclusion appears to be provided by the outcome of the TMV experiment where, as shown in Table I, 55% of the expected S^{35} activity was found in the highly purified TMV. The author was assisted in these experiments by Miss Jessie Mason McNeil.

Discussion and Summary

The fact that a highly purified preparation of cucumber virus 4 obtained from cucumber plants which had received a nutrient solution containing S^{35}, possessed much less than 1% of the potential, calculated radioactivity of a compound containing 0.6% sulfur, indicates that no sulfur is present in CV4. This conclusion is supported by the finding that 55% of the calculated radioactivity was present in a similar preparation of tobacco mosaic virus, which is known to contain 0.2% of sulfur.[24]

The sulfur values obtained by two different laboratories on portions of the CV4 of the S^{35} experiment illustrate the confusing results which made the isotope experiment imperative, for the analyst who had previously obtained high values for most preparations, reported essentially no sulfur present (0.07%), whereas a second analyst found an amount comparable to that present in TMV, *i. e.*, about 0.2%. The nature of this pseudo-sulfur, found in 90% of the analyses of CV4 thus far, remains to be solved. It seems clear, however, that it is an artifact.

As an incidental point, the S^{35} experiment demonstrated in a graphic manner how a virus can be separated from normal plant materials in a stepwise fashion by the technique of differential centrifugation. The clarified plant sap, from which the virus was separated by an initial high-speed centrifugation, possessed over 2 million units of S^{35} activity. After 3 cycles of centrifugation, the total S^{35} activity of the viral preparation was about 1500 units, and after 5 cycles, 31 units. It seems reasonable in view of these facts, to assign the relatively minute amount of radioactivity in the highly purified CV4 to a residual trace of impurity.

The demonstration that CV4 contains no sulfur constitutes a striking difference between this virus and the other strains of TMV which have been studied chemically thus far, for the latter all contain sulfur, mainly in the form of cysteine.

(24) A. F. Ross, *J. Biol. Chem.*, **136**, 119 (1940).

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY AND CHEMICAL ENGINEERING, UNIVERSITY OF PENNSYLVANIA]

The Effects of Substituents on the Dissociation Constants of Substituted Phenols. I. Experimental Measurements in Aqueous Solutions[1]

BY CHARLES M. JUDSON[2,3] AND MARTIN KILPATRICK[4]

The Sarmousakis modification[5] of the Kirkwood–Westheimer[6] electrostatic theory of the effects of substituents on the dissociation constants of benzenoid acids was originally tested by a comparison with the experimentally observed constants for the benzoic acids in several different pure solvents. To assist in providing a further test of the Sarmousakis treatment, the dissociation constants of a number of substituted phenols have been measured using a stepwise colorimetric method similar to that used previously in this Laboratory.[7] Complete data for the substituted phenols measured by a consistent method have been reported in the literature only for certain alcohol–water mixtures.[8] Values in a pure solvent are preferred for comparison with the electrostatic theory. The data obtained in the present investigation along with the previously available data have provided a satisfactory set of values for the substituted phenols in aqueous solution. These values have been compared with values calculated by the electrostatic theory and have been used to provide additional support for the theory.[9]

The ratio of the dissociation constant of an indicator A_i to that of an uncolored acid A may be defined by the equation

$$K_{A_iB} = c_{B_i}c_A/c_{A_i}c_B \qquad (1)$$

where B_i and B refer to the corresponding conjugate bases. By placing a small measured amount of the indicator in a buffer solution of known concentration of the uncolored acid with its sodium salt, the ratio of the dissociation constants can be determined from a colorimetric measurement of the concentration of one form of

(1) Taken from the dissertation presented by Charles M. Judson to the Faculty of the Graduate School of the University of Pennsylvania in partial fulfillment of the requirements for the degree of Doctor of Philosphy, August, 1947. Presented before the 112th meeting of the American Chemical Society held in New York, N. Y., September, 1947.

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(5) Sarmousakis, *J. Chem. Phys.*, **12**, 277 (1944).

(6) Kirkwood and Westheimer, *ibid.*, **6**, 506 (1938); Westheimer and Kirkwood, *ibid.*, **6**, 513 (1938); Westheimer and Shookhoff, THIS JOURNAL, **61**, 555 (1939); Westheimer, *ibid.*, **61**, 1977 (1939).

(7) Mason and Kilpatrick, *ibid.*, **59**, 572 (1937); Minnick and Kilpatrick, *J. Phys. Chem.*, **43**, 259 (1939); Kilpatrick and Mears, THIS JOURNAL, **62**, 3047, 3051 (1940).

(8) Schwarzenbach and Egli, *Helv. Chim. Acta*, **17**, 1176 (1934); Schwarzenbach and Rudin, *ibid.*, **22**, 360 (1939).

(9) Judson and Kilpatrick, THIS JOURNAL, **71**, 3115 (1949)