APPENDIX

The Identification of Spleen Propionylcholine by Infrared Microspectroscopy

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As a preliminary to the study of the sample of spleen propionylcholine, the spectra of synthetic acetylcholine and propionylcholine perchlorates were recorded, together with those of two possible impurities in the biological material, choline perchlorate and sodium perchlorate. The spectra of the pure compounds were measured with a Perkin-Elmer Model 21 double-beam infrared recording spectrophotometer, using standard mulling techniques for all but the choline perchlorate sample. The mulling agents were Nujol (paraffin oil) and hexachlorobutadiene; since one agent is clear where the other absorbs, the sample bands obscured by one agent could be observed in the mull with the other. The sample of crystalline choline perchlorate was observed to be unusually malleable, and was rolled out into thin self-supporting films, or alternatively, rolled on optical silver chloride sheets to the thickness required for spectroscopic samples.

The spectra of the four compounds recorded in this way are shown in Fig. 1. The curves have been redrawn so that the bands of the mulling agents do not appear. It will be noted that there are differences in the spectra of the two esters which are remarkable in view of the slight difference in their structure. In particular the differences in the region from 7.5 to 12 μ . should be noted, since these are the bands which were used in making the identification of the spleen-extract ester sample. Below the spectra of the esters are shown the spectra of choline perchlorate and sodium perchlorate. It will be noted that the strong band of the free hydroxyl group of choline which appears at 2.87 μ . is absent from the spectra of the ester sample shown above, indicating that choline is not an impurity in the synthetic samples. Since hydrated sodium perchlorate (bottom curve in Fig. 1) has an OH frequency due to the water of hydration lying very close to that of the OH frequency of choline (2.82 and 2.87 μ . respectively), it is not possible to detect the presence of choline in ester samples containing sodium perchlorate as an impurity, using this band as a criterion. There are other bands, however, by which choline can be detected in the presence of sodium perchlorate, and although these are much weaker and the criterion correspondingly less critical, no evidence for the presence of choline in the spleen-extract sample was found.

The spectrum of sodium perchlorate shown at the bottom of Fig. 1 indicates that the broad band near 9 μ . common to all the perchlorate spectra is due to the perchlorate ion. This band does not obscure the most important differences between the spectra of the two esters in the top two curves. It was concluded that neither choline nor sodium perchlorate would interfere in the spectroscopic identification of propionyl- or acetylcholine perchlorates. To establish that the identification could be made in the presence of a considerable excess of sodium perchlorate, two pairs of mixtures of the two esters and sodium perchlorate were prepared by grinding in a mortar. The spectra of these samples run as mulls in the double-beam spectrometer showed that a mixture containing 4% of ester was too dilute to discriminate between the acetyl and propionyl esters. In 18% samples, however, it was quite easy to tell which was which and the differences in the spectra agreed fairly well with those recorded from mulls of the pure compounds, although the agreement was not complete, indicating compound formation between the ester and the sodium perchlorate.

Fig. 1. Infrared spectra of acetylcholine (ACh), propionylcholine (PrCh) and choline (all as perchlorates) and of sodium perchlorate monohydrate.

With this established, the spectra of the mixtures of ester and sodium perchlorate were recorded again using an infrared microspectrometer previously described (Wood, 1950). The microspectra show somewhat less resolving power than the double beam spectra and the region showing the spectroscopic differences between the esters is given in

Fig. 2. Microspectra of *A*, synthetic acetyl- and propionylcholine perchlorates as mixtures with sodium perchlorate (19 and 16.5% ester respectively) and *B*, spleen ester sample. —, acetylcholine perchlorate mixture; - - - -, propionyl perchlorate mixture.

Fig. 2. The other regions of the spectrum where differences between the two esters also occurred are not included, because for various reasons the microspectra were considered unreliable (e.g. water vapour in the 6–7.5 μ . region). In comparing the two upper curves in Fig. 2 it will be noted that the acetyl ester has the band at 8.05 μ . lacking in the propionyl ester and the propionyl ester has strong absorption at 8.35 μ . where the acetyl ester has a region of maximum transmission. On the other side of the broad 9 μ . band due to perchlorate the acetyl ester has a broad multiple band near 10.5 μ . while the propionyl ester has a single sharp peak at 10.50 μ .

The curve *B* in Fig. 2 is the microspectrum of the sample of the biological extract thought to be propionylcholine perchlorate, and containing approximately 100 μ g. of active material. This curve lacks the acetyl ester band at 8.04 μ ., but has the propionyl ester band at 8.35 μ . (as a shoulder). In addition, on the other side of the intense perchlorate band at 9 μ . the natural ester curve has a single peak at 10.50 μ . similar to that of the synthetic propionyl ester, and in contrast to the broad multiple band of the acetyl ester in the same region. With the exception of the small peak at 10.75 μ . in the natural extract the spectrum of the latter is identical with that of the synthetic propionylcholine perchlorate. The 10.75 μ . band is attributed to an unidentified impurity from the extraction procedure. On the basis of the infrared spectrum, the conclusion is that the extracted ester is indeed propionylcholine.

REFERENCE

Wood, D. L. (1950). *Rev. sci. Instrum.* **21**, 764.

The Application of Four Methods for Assessing Protein Homogeneity to Crystalline β -Lactoglobulin: an Anomaly in Phase Rule Solubility Tests

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In order to follow the purification of a protein a reliable and sensitive method for determining the number and amount of components present in different samples must be available. Ultracentrifugal, electrophoretic, immunological and solubility tests were carried out with crystalline β -lactoglobulin in order to find such a method for following

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further purification of this protein. The results of these tests are presented in the first part of this paper and the usefulness of each is considered.

In the second part of the paper the occurrence under certain conditions of an anomaly in the 'variable solvent' solubility test for protein homogeneity (Falconer & Taylor, 1946; Roche & Derrien, 1947; and Derrien, 1952) is described and discussed. This appears to be the first time that such an