

## Difficulties at the Infrared-Spectrophotometric Identification and Estimation of Pure Compounds and Mixtures by Use of Potassium Bromide Disk Technique

BØRGE BAK and DANIEL CHRISTENSEN

*Chemical Laboratory of the University of Copenhagen, Copenhagen, Denmark*

Spectra of a number of biologically active compounds such as urea, D-glucose, D-fructose, amino acids etc. have been studied in the infrared, using pressed KBr-disk technique. Examples show that identification of the compounds of even simple mixtures of compounds of this type meets with difficulties of unexpected magnitude.

With the advent of the KBr-disk technique<sup>1,2</sup> new fields of research were opened up. Since strong, discrete spectra are obtainable from a mixture of 0.2–2.0 mg crystalline material and 500 mg KBr, evaporation residues from rather small amounts of reaction mixtures, blood serum, tissue liquids etc. can now be examined spectrophotometrically in the infrared. In this connection it is of interest to know how well-defined a KBr-disk spectrum of a *pure compound* is. Also, the spectra of *mixtures* may or may not obey the 'superposition principle' to the same extent as for mixtures of liquids and mixtures of gases. We want to illuminate these problems by practical examples from an investigation of blood serum crystalloids in

which we are engaged. No complete treatment of the subject is intended.

*Experimental.* All our spectra were recorded on a Beckmann IR 2 instrument with NaCl optics. Water vapor and CO<sub>2</sub> had effectively been removed from the spectrograph by flushing with air from a liquid-air container. The quantity of sample to be mixed with 500 mg KBr never exceeded ca. 2 mg. Table 1 gives the results of seven experiments with glycine. The method of disk preparation marked with an 'a' in Table 1 consisted in mixing the quantity of crystalline glycine indicated with 500 mg KBr, shaking the mixture in a conventional von Ardenne vibrator<sup>3</sup>, and finally pressing the disk *in vacuo*. The recorded spectra were quite comparable in quality with spectra of liquids and gases. Lines in the region where the spectrograph has high resolving power (667–1 000 cm<sup>-1</sup>) appear typically with a width of about 10 cm<sup>-1</sup> at the 'foot' and very sharp maxima with *D* (optical density) = 0.3–0.4 for 1 mg samples.

In many types of experiment it is, however, impossible or impractical to use the 'a' procedure. In such cases we dissolve 300 mg KBr in 1–2 ml of an aqueous solution of the compound(s) to be investigated, whereafter the water is removed by freeze-drying. The resulting voluminous crystalline powder is slightly compressed in order to decrease the volume, 200 mg KBr is added, a five minutes treatment in the vibrator follows and a disk is pressed (procedure *b*).

The results in Table 1 show that method *b* gives spectral lines at 894 and 912 cm<sup>-1</sup> of equal peak intensity in contrast to the lines of *different* intensity observed with

Table 1. Optical density at maximum of some of the spectral lines observed for KBr-disks containing glycine.

Experiment number	Glycine quantity (mg)	Method of disk preparation	Optical density of disk at top of absorption line at (cm <sup>-1</sup> )			
			700	894	912	934
1	1.170	a	0.25	0.32	0.26	No line
2	1.210	b	0.25	0.31	0.30	, ,
3	1.180	b	0.33	0.37	0.36	, ,
4	1.150	c	0.37	0.38	0.38	, ,
5	0.818	b	0.21	0.27	0.26	, ,
6	0.414	b	0.06	0.13	0.11	0.05
7	0.400	b	0.06	0.12	0.10	0.04

the *a* procedure. In method *c* an aqueous glycine solution (1 ml) was freeze-dried. The resulting glycine was mixed with 500 mg KBr, vibrated and pressed. The spectrum only deviates from *b*-spectra in the astonishing high intensity of the band at  $700\text{ cm}^{-1}$ . Since the glycine particle size is probably more uniform in freeze-drying experiments (*b*) than by 'dry mixing' (*a*), freeze-drying is likely to mean a step towards a standardization of the disk-spectra. But a glance at the results in expts. Nos. 2 and 3 (Table I) shows that no simple relationship between spectral band intensity and compound quantity exists. Expts. 3, 5 and 6 in which the freeze-drying was carried out simultaneously do show a very rough Beer's law agreement. But as will be noted, expt. 6 (successfully repeated in expt. 7) presents the surprising feature that a new line is observed! The remaining part of the glycine spectrum has the same not too well defined character but we chose the examples in Table I in order to speak of bands that could be observed with optimum resolving power. Since we know now with what kind of uncertainties we are dealing in the disk-spectrum of a pure compound we shall see what happens with mixtures.

*Glycine and L-alanine* form relatively well-behaving mixtures. Spectra of disks prepared by *a* and *b* procedure are almost identical. Roughly, they are a superposition of a glycine and an alanine spectrum. But the absorption intensities are wrong. For the line at  $894\text{ cm}^{-1}$  in the spectrum of a mixture (glycine 0.87 mg; alanine 0.619 mg) we would expect  $D = 0.27$  at the maximum (Table I). We found  $D = 0.20$ . But in spite of similar defaults throughout the spectrum nobody would doubt that one sees the spectrum of a mixture of glycine and alanine since no bands of medium and high intensity from these components are missing and no extra bands are found.

In the disk-spectrum of mixtures of *urea* and *D-glucose* prepared by method *a* and *b*, no bands corresponding to the strong absorption at  $1481$  and  $1716\text{ cm}^{-1}$  in urea occur (urea quantity 0.5 mg; *D-glucose* 1.5 mg). In return, both disks have an extra line at  $1460\text{ cm}^{-1}$  (which may be a displaced  $1481$ -band (urea)). A band, corresponding to the urea band at  $1169\text{ cm}^{-1}$  is observed in the 'dry' disk (proc. *a*), but hardly in the 'wet' (*b*). The freeze-dried disk has a band at  $1083\text{ cm}^{-1}$  neither to be found in the spectrum of pure *D-glucose*,

nor in the urea spectrum. A superficial glance at the disk-spectrum of the urea-glucose spectrum immediately makes one suspect what the components are. But closer inspection reveals quite incompatible with the superposition principle, even if this is reduced to being of only qualitative validity.

A third case may be mentioned in which the identification of the components is even more difficult. We studied mixtures of *glycine* and *L-aspartic acid*. Between  $863$  and  $953\text{ cm}^{-1}$  glycine has bands at  $894$  and  $912\text{ cm}^{-1}$ . Aspartic acid has bands at  $875$  ( $D = 0.06$ ),  $900$  ( $D = 0.11$ ), and  $940$  ( $D = 0.03$ ) (sample quantity 1.156 mg). A 'dry-mixed' disk with 0.987 mg aspartic acid and 0.532 mg glycine roughly shows the four bands to be expected from superposition, namely, at  $875$ ;  $(894 + 900)/2 = 897$ ;  $912$ , and  $940\text{ cm}^{-1}$ . If  $3/5$  of the disk is treated by the 'wet' procedure (*b*), a spectrum results with only one pronounced maximum at  $904\text{ cm}^{-1}$ . Also a band at  $700\text{ cm}^{-1}$  which would indicate the presence of glycine, is completely missing. From the spectrum of the freeze-dried disk one can probably conclude that the disk contains two or more *amino acids*. But it seems impossible to deduce that a simply glycine-aspartic acid mixture is present.

No simple explanation covers the glycine-aspartic acid case. If it is suggested that hydrogen ions are transferred from the latter (being the stronger acid) to the former this would mean that we should 'see' the ion  $\text{H}_3\text{N}^+ - \text{CH}_2 - \text{COOH}$ . But the spectrum of this ion we know from a study of glycine hydrochloride which has dominant lines at  $807$  and  $859\text{ cm}^{-1}$  where no lines are observed by us in any glycine-aspartic acid mixture.

*Discussion.* Ebert and Gottlieb<sup>4</sup> found pronounced differences in the spectra of *polymorphic* compounds (in Nujol), but it seems impossible to assume any kind of polymorphism in our cases. Kirkland<sup>5</sup> has emphasized that caution must be used when attempting to apply the disk technique to systems containing solid solutions or mixed crystals, but no examples of the effect to be expected were given by him. The findings by Brockmann and Musso<sup>6</sup> seem more interesting in the present connection. They demonstrated that differences exist between the spectra of optical isomers and the spectrum of the racemate, provided that the optical isomer and the racemate differ sufficiently in melting

point and solubility. Also, they claim that mixtures of optical isomers can be converted to the racemate if the KBr disk technique is applied (see also Right<sup>7</sup>). Therefore, the optical isomers can 'find' each other in spite of the great quantity of KBr, consistent with our result on the 'dry' D-glucose-urea mixture. Brockmann and Musso state that probably hydrogen bonding is responsible for the effects they found. The same explanation may be valid here, but further experiments are undoubtedly necessary to verify this interpretation. Therefore, although KBr disk spectra are of great value to the analyst they must be used with some reservation in view of the present incomplete experimental coverage of the field and the lack of adequate theoretical insight.

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## On Sialic Acid in Brain Tissue

LARS SVENNERHOLM

*Department of Medical Biochemistry, University of Gothenburg, Gothenburg, Sweden*

Gangliosides were isolated from brains by exhausted extraction with methanol-chloroform (2:1, v/v). After evaporation of the solvent *in vacuo* and drying the residue with ethanol the lipids were redissolved in waterfree solvent of the same composition. An insoluble rest remained, and it was considerably larger when the tissue was extracted directly with methanol-chloroform without previous drying with acetone. It was composed of low molecular substances, such as amino acids, purines and salts but also substances of high molecular weight. The low molecular substances were removed by repeated dialysis against distilled water. The total amount of carbohydrates (hexose, hexos-

amine and sialic acid) was high (20–50 % in different samples) in the dialysed material. After hydrolysis several amino acids were also identified with paper chromatography. If strandin prepared according to procedure C of Folch, Arsove and Meath<sup>1</sup> was redissolved in methanol-chloroform (2:1) a substance containing hexosamine and sialic acid remained undissolved.

Only a few similar observations have been found in the literature. Klenk and Lauenstein<sup>2</sup> isolated a substance from methanol-chloroform extract of erythrocyte stroma which was only soluble in hot pyridine and contained a high amount of hexosamine. Rosenberg, Howe and Chargaff<sup>3</sup> have recently identified several amino acids after hydrolysis of strandin prepared by procedure C of Folch *et al.*

The observations described indicate that amino sugar and sialic acid are not only occurring in the lipopolysaccharides (gangliosides) but also in other substances in brain.

A quantitative estimation of sialic acid with Bial's reagent<sup>4</sup> in brain tissue, from which the lipids had been extracted, demonstrated that the amount of 'protein-bound' sialic acid was higher than that of lipid-bound. The amount of sialic acid was higher in old brains than in young ones and about twice as high in grey as compared with white matter. In adult brain the content was about 0.6 %, calculated on lipid-free brain tissue. N-Acetylsialic acid has now been isolated from lipid-free brain tissue and its X-ray diagram compared with that of sialic acid from gangliosides and serum proteins.

*Analytical methods.* Nitrogen was determined by the Kjeldahl micromethod. Hexose was determined with an orcinol-sulphuric acid method<sup>5</sup> and calculated as galactose. Hexosamine was assayed with a modified Elson and Morgan method<sup>6</sup>, and glucosamine was separated by the procedure outlined by Gardell<sup>7</sup>. The determination of sialic acid was performed with Bial's reagent and calculated as N-acetyl sialic acid (mol.wt. 309)<sup>4</sup>. The X-ray powder diagrams were taken with a Guinier camera using copper K $\alpha$  radiation.

*Materials.* Brains from old people were freed from membranes and blood vessels. Grey matter was dissected free from the main part of white matter and water removed by two successive treatments with 4 vol. of acetone at 0°. The dried brain powder was extracted with