Chapter 3

Micro-Determination of Phospholipids

General Remarks

As phospholipids are determined fluorometrically, rigorous standards of purity and cleanliness for the solvents, reagents, and cuvettes employed must always be observed. Any fluorometer can be used, so long as it is possible to record the spectra either directly or by a coupled pen-recorder. No fluorometric measurement, even if it is only a routine measurement with a well-established procedure, should be carried out without recording the spectrum. It is not enough simply to measure and record a single pen deflection at a known fluorescence maximum; under such conditions an alteration in a spectrum, due perhaps to an impurity which could falsify the measurement, can be overlooked very easily. A salutary example from our own laboratory experience serves to illustrate this. In the quantitative estimation of 3-acetylpyridine, an anti-metabolite of nicotinamide, large numbers of extractions of organs were carried out, with methyl ethyl ketone, NaOH, and finally with 5 N HCl, to isolate an intensely fluorescent product (Neuhoff and Herken, 1962; Herken and Neuhoff, 1963; Willing, Neuhoff and Herken, 1964). At times, series of 50 to 100 preparations were made. The 5 N HCl was prepared freshly each week in a 1 l measuring flask with a ground-glass stopper. In one of the excitation spectra, recorded routinely for each single measurement, an appreciable broadening of the spectrum in one of the experimental series was observed, which brought all the measured values into question. On investigation it was found that the ground-glass stopper of the HCl-flask had accidently come into contact with the cement of a tiled bench surface, and an unknown substance had been introduced into the HCl. This substance had an emission maximum at a similar wavelength to that of the actual reaction product, and had falsified the readings appreciably. This impurity would not have been noticed if the spectra had not been recorded.

Recording the spectrum is necessary not only to detect accidental impurities, but also to detect fluorescent compounds in inadequately purified extracts, as is shown by the following example. On recording the excitation spectrum of extracts of rat kidney, after treatment with 6 N NaOH for NAD and NADP determination according to Lowry et al. (1957, 1961), an indication of an additional maximum at the excitation wavelength of 300 m\(\mu\) (uncorrected) was observed; no such maximum was detectable after treatment of pure NAD or NADP with 6 N NaOH (Fig. 1). This additional maximum was not present in the excitation spectra of other organ extracts of rat produced similarly (brain, liver,
spleen, lung, heart, muscle, pancreas, aorta, blood); it was also not demonstrable in kidney extracts of man, rabbit, guinea pig, or mouse. The substance responsible for this fluorescence maximum had therefore to be traced. Since the substance interfered with the quantitative estimation of NAD or NADP in rat kidney, it was isolated chromatographically. The excitation spectrum of the purified substance is shown in Fig. 1; it possesses three distinct maxima, of which two practically coincide with those of NAD and NADP after treatment with 6 N NaOH. Contamination of NAD or NADP solutions with this substance results in spuriously high measurements for the concentration of nucleotides because of superposition. It was shown that about 15% of the observed total fluorescence of an extract of rat kidney, after treatment with 6 N NaOH, may be traced to this substance, which is at present unidentified (HERKEN and NEUHOFF, 1964). Such incidents emphasize the correctness of the first basic rule, that in every kind of fluorometric analysis the spectra must be recorded, and only such spectra should be used for evaluation.

Only the purest quality (spectrally pure) solvents should be used, and in addition the emission and excitation spectra of each solvent should be recorded; the spectra should be recorded at the wavelength at which the actual measurements are to be carried out.

If water is used at any stage of the assay, only tap water which has been distilled four times from quartz should be used; this is easy to produce and has very little fluorescence. Water from ion-exchangers is unsuitable, as, even after several distillations from quartz it often contains traces of fluorescent impurities which co-distill on distillation. Pure distilled water was already a qualitative concept at the turn of the century, as illustrated by the ice-delivery wagon shown in Fig. 2; this is now one of the rarities housed in the National Museum of History and Technology, Smithsonian Institution, U.S.A.