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# USE OF FLUORESCENCE, CHEMILUMINESCENCE, AND SPECTROPHOTOMETRY IN MEDICINE

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A review is given of the research work carried out at the Institute concerning the use of fluorometry, luminometry, and spectrophotometry in toxicology and other specific fields of scientific and practical medicine. Fluorescence has been applied in the development of analytical methods for the determination of porphyrin in biological material. In this connection some important physico-chemical properties of porphyrin have been investigated. The fluorescence of the oxidation products of indole, as well as the chemiluminescence of luminol and lucigenin have been used for the determination of poisons based on the esters of phosphoric and thiophosphoric acids. Chemiluminescence has also been employed for the study of the properties of oximes in relation to their antidote action, for proving blood stains, and for differentiating different kinds of hemoglobin (Hb-A, Hb-F, cat Hb). A special form of spectrophotometry has been used for the quantitative determination of carbamate insecticides. – For all this work corresponding photoelectric fluorimeters and luminometers have been constructed at the Institute.

Optical measurement procedures are increasingly used in scientific and pratical medical work, either as analytical methods for the determination of the concentration of various substances in biological material or as specific procedures for the study of the properties of biologically and medically important substances. Out of a great number of optical procedures a special role is played by those based on the appearence of luminescence, especially fluorescence and chemiluminescence, as these manifestations are very specific on the one hand, and on the other, the substances producing them are measurable at very low concentration. Measurement procedures for various forms of luminescence have been developed almost invariably on the basis of sensitive photoelectric instruments. Nowadays they allow a simple, quick carrying out of the qualitative and quantitative analysis of biologically active matter. In addition, they allow recording of the duration of chemical and biochemical reactions and other processes. Corresponding photoelectric spectrophotometric (absorptiometric) procedures may well supplement fluorimetric and luminometric work.

In view of this a special working group at the Institute for Medical Research and Industrial Hygiene has for years been engaged in the application of the methods quoted to the special problems of practical medicine and allied fields. In so doing, a scientific approach to these problems has been employed, and special attention has been paid to the scientific background of the problem and the working method applied. Thus, a series of studies have been conducted on the use of fluorescence in medicine and toxicology, and just as many in connection with the use of chemiluminescence in toxicology, forensic medicine, and hematology. The same or similar problems have also been tackled by using spectrophotometric methods. Various photoelectric apparatures have been constructed at the Institute for conducting this work.

## 1. FLUORESCENCE OF PORPHYRIN

The finding of pathological amounts of *coproporphyrin* indicates a group of diseases which also include chronic lead poisoning (1), and this disorder has for long been in the focus of the Institute's scientific interest (2). In this connection the need existed for the fluorimetric determination of coproporphyrin by a suitable method. Thus, a convenient modification of the well-known method for the isolation of coproporphyrin has been evolved (3). Further studies related to the improvement of the equipment, the construction of the corresponding fluorimeter of adequate sensitivity, and the development of a method for the calibration of apparatures for coproporphyrin by the use of the Rodamin B dye (4, 5). On the basis of these studies the laboratories of Yugoslav hospitals and institutes carry out routine determinations of coproporphyrin in the urine for diagnostic purposes.

Work on the fluorescence of porphyrin was continued by the investigations into the fluorescence of hematoporphyrin in the *adsorbed* state (6, 7). It has been found that porphyrins adsorb well on the surface of various adsorbents, as well as in solid white oxides and hydroxides of earth alkaline metals. Under certain conditions hematoporphyrin in the adsorbed state produces a more intense fluorescence than in solutions. Methods have been developed for the fluorimetric measurement of the fluorescence of porphyrin adsorbates. They may prove of special importance for the determination of porphyrin in traces. On the basis of the experiments made possibilities of molecular conditions of porphyrin in adsorbates have been discussed.

In a study on the problem of photochemical fading of hematoporphyrin (8) it has been shown that hematoporphyrin in the alcohol solution – just as other porphyrins – photochemically autoxidatively fades under the effect of ultraviolet light. This reaction develops comparatively slowly, but its rate can very successfully be increased by adding organic compounds which act as oxygen transmitters, for instance, thiosinamine, diethylthiosinamine, and acetone. The effects observed were

studied by the methods of chemical kinetics, by which the change in the concentration of porphyrin during lighting was analysed spectrophotometrically. At the same time also changes in the whole absorption spectrum of reaction solutions in the visible and ultraviolet spectral range have been evidenced. It has been proved that the photochemical oxidation by the effect of oxygen, breaks the porphyrin ring, by which comparatively simple decomposition products of porphyrin are produced. When thiosinamines or acetone are used as oxygen transmitters, the reaction mechanisms are different. In the former case porphyrin and in the latter acetone act as the photoactive matter. By light absorption in the acetone molecule a triplet condition is created, producing peroxide which oxidatively decomposes porphyrin. The possibilities of a reaction mechanism in both types of oxygen transmitters have been discussed. The effect of various inhibitors on the oxidation decomposition of hematoporphyrin has been investigated in connection with a possible stabilization of porphyrin in solutions.

## 2. FLUORESCENCE OF THE OXIDATION PRODUCTS OF INDOLE

Indole in solutions shows no visible fluorescence, but the oxidation of indole produces indoxyl, then indigo white and finally indigo. The former two products of the indole oxidation give an intense fluorescence, by which it is allowed to follow the oxidation reaction of indole by fluorimetric measurements. This is of particular importance, because organophosphorus nerve poisons have proved to catalyse this reaction very officiently. On the basis of this fact the method for the determination of nerve poisons by the indole oxidation reaction and fluorescence intensity measurements as described in literature (9, 10) have been elaborated. This fluorimetric method for the determination of the concentration of nerve poisons has been used for the study of the *kinetics of the hydrolysis* of these poisons (11), by which the effect of solvents on the stability of sarin, tabun, and DFP has been studied in particular. Kinetic measurements have been carried out, at different temperatures, in water solutions in the presence of buffers, various alcohols, and inhibitors. Energy values of the hydrolysis activation of these poisons have been determined. The results are of pratical importance in the destruction of nerve poisons.

In connection with the use of the oxidation reaction of indole in the analysis of organophosphorus poisons the kinetics of this oxidation reaction itself, in the presence of methyl-paraoxon as the catalyser, has been investigated. It has been succeeded in interpreting the reaction according to the kinetic laws of the reactions katalyzed by enzymes, by which the process has been considered a model reaction of peroxidative effect. Maximum reaction rates, the Michaelis constant values, changes in the reaction enthalpy, and activation energy for this reaction have been established (12).

On the basis of the oxidation reaction of indole it has been succeeded in developing a method for the quantitative determination of the *carbamate insecticide* Sevin (1-naphthyl-N-methylcarbamate) (13). This method is likely to be applicable in the analysis of other carbamate insecticides.

All these investigations on the oxidation reaction of indole have been carried out by the fluorimetric apparatus of our own construction.

### 3. DETERMINATION AND STUDY OF ORGANOPHOSPHORUS POISONS BY CHEMILUMINESCENCE

The chemiluminescence of luminol (3-aminophthalhydrazide) was proposed by J. *Goldenson* (14) for the quantitative determination of nerve poisons, and sarin in particular. Our own studies have shown that this method can successfully be applied in the analysis of a whole series of the esters of phosphoric and thiophosphoric acid used as insecticides (15, 16). Experimental conditions, especially the composition of the reagent, should vary according to the nature of the ester studied (17). Work with the nerve poisons that easily get hydrolysed requires the use of a slightly alkaline reagent (potassium perborate and phosphate), while the insecticide esters of phosphoric acid that do not hydrolyze easily catalyze chemiluminescence successfully only in very alkaline solutions (NaOH and $H_2O_2$). It has been shown that a moderate addition of alkaline halogenides (KCl, KBr) to the luminol reagent may often considerably increase chemiluminescence intensity, by which the sensitivity of the method for the detection and determination of nerve poisons and insecticides by the luminol reaction is enhanced (17). A special study has been devoted to the mechanism of the effect of the esters of phosphoric acid on the chemiluminescence of luminol. These »catalysers« or activators of luminescence may be considered to act in two ways in relation to experimental conditions expressed in the concentration relations of the reactive agents. From the kinetic point of view, the reaction may develop by the mechanism basically corresponding to a *peroxidative* or *catalytic* action, but working conditions may also be such as to allow both mechanisms to come to the fore (18).

An attempt has also been made to apply the chemiluminescence of *lucigenin* (N,N-dimethyldiacridiliumnitrate) in the analysis of nerve poisons. This new reagent gives indeed very good results with tabun (19), whereas some other similar poisons have proved to produce no activity of the chemiluminescence of lucigenin.

Chemiluminescence has proved to be of value also in the study of *oximes* which serve as antidotes in poisonings by the esters of phosphoric acid. It has been found that the oximes that are good reactivators of cholinesterase in poisoning by organophosphorus compounds regularly produce the inhibition (quenching) of the chemiluminescence of both

luminol and lucigenin, by which nerve poisons or other known katalyzers of such processes serve as the activators of luminescence (20, 21, 22, 23, 24). It appears that between the reactivation of cholinesterase and the luminescence quenching by the oxime there is a close association when the cholinesterase is poisoned by the same ester of phosphoric acid that activates for chemiluminescence. In this way the possibility has been given for simple laboratory studies of the effectiveness of various oximes as antidotes.

## 4. EFFECT OF HEMOGLOBIN ON THE CHEMILUMINESCENCE OF LUMINOL

The luminol reaction has for long been used in forensic medicine for providing evidence of traces of blood (25). However, the composition of the reagent recommended for this purpose has proved inadequate, because the sensitivity it gives to the method is very poor, and the reagent itself remains stable only for a limited period of time. For this reason extensive studies have been conducted to explore the possibility of the use of the chemiluminescence of luminol for a more successful provision of evidence of blood stains. Emphasis was laid on the composition of the reagent (26, 27), and to this end a corresponding photoelectric apparatus with the automatic recording of measurement results (27, 28) has been constructed. This modified method allows the detection of blood (hemoglobin) in such a dilution as 1 : 10000000, regardless of whether hemoglobin (fresh blood) or methemoglobin (dried blood, stain) is in question. It has also been proved that during the activation of chemiluminescence the blood of an adult (HB-A) gives a basically different course of the chemiluminescence time curve than the blood of the fetus (H-F) or the cat. These kinds of hemoglobin can safely be distinguished by the simple luminol chemiluminiscence experiments. There are also further possibilities for the use of the luminol reaction in the field of the physical chemistry of hemin proteids.

## 5. SPECTROPHOTOMETRIC DETERMINATION OF CARBAMATE INSECTICIDES

Organophosphorus poisons can also be determined spectrophotometrically, by using the oxidation reaction of o-dianisidine (29). The attempt to apply this method also in the analysis of insecticide carbamates has proved successful in every respect (30). Calibration curves have been elaborated for seven carbamates commercially distributed under the name OMS. As little as 0.03 microgram of carbamate can be detected by this method.

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