0.016 *M* β-glycerophosphate where it might be assumed that substrate formation is fast enough so that it is no longer rate-controlling.

In addition to the cases above, Ross and his associates[3] have reported variation in pH optima for alkaline phosphatase activity with substrate concentration for phenyl disodium phosphate, adenylic acid, fructose-1,6-diphosphate, fructose-6-phosphate, and ribonucleic acid. The same analysis may be applied to these; and the "true" substrate may be selected as that which demonstrates an optimum concentration invariant with pH change. It was found that the optimum concentration was constant when the un-ionized acid form was picked as the substrate for all cases but ribonucleic acid, where the monovalent ion showed invariance.

SUMMARY

In the enzymic hydrolysis of β-glycerophosphate, the species on which the alkaline phosphatase acts is the un-ionized acid. Supporting evidence for this includes: (a) ^{32}P specific activity of the hydrolysis product corresponding to the specific activity of the un-ionized acid in the reaction medium; (b) an increase in reactivity with enzyme dilution which disappears with lower pH; and (c) experimental data from the literature.

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FLUORESCENCE SPECTROPHOTOMETRY OF REDUCED PHOSPHOPYRIDINE NUCLEOTIDE IN INTACT CELLS IN THE NEAR-ULTRAVIOLET AND VISIBLE REGION

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INTRODUCTION

It is necessary to study enzymic systems *in vivo* in order to verify or correct conclusions obtained with *in vitro* systems. Such a study may also reveal unknown reactions and mechanisms.

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In this paper an apparatus is described with which it will be possible to study certain fluorescing substances *in vivo* by means of fluorescence spectrophotometry. We developed this apparatus, having in mind as a first application the study of reduced phosphopyridine nucleotide (DPNH), especially in photosynthesizing cells.

So far studies on pyridine nucleotide *in vivo* have been carried out by means of absorption spectrophotometry. Extensive studies have been done on DPNH in yeast by CHANCE and co-workers[1] and by LUNDEGARDH[2], who measured changes in the absorption spectra of yeast suspensions in the region 300–400 mμ, under various conditions. "Reducing conditions" generally caused an increase in absorption in the region 300–380 mμ. Both authors interpreted these changes as being caused by reduction of phosphopyridine nucleotide or formation or dissociation of complexes of pyridine nucleotide and an (apo)dehydrogenase.

In the chlorophyll *a*-containing photosynthesizing organisms, the green alga *Chlorella* and the red alga *Porphyridium*, an increase in absorption was observed in the region 300–380 mμ upon illumination. This increase, which was rather difficult to measure because it was about hundred times smaller than in yeast, was also interpreted as being caused by reduction of pyridine nucleotide.

The interpretation that the absorption changes in yeast and photosynthesizing cells were caused by pyridine nucleotide is plausible, since under the conditions of the experiments such changes in this coenzyme may be expected. It is, however, possible that other substances cause spectral changes in the region 300–380 mμ, which would make the interpretation of difference spectra uncertain. In photosynthesizing organisms especially, the changes caused by cytochromes and perhaps by other pigments are not negligible. Thus the development of a more specific method than absorption spectrophotometry would presumably be of great value for the investigation of pyridine nucleotide *in vivo*.

Fluorescence spectrophotometry is, at least in principle, such a method since only a small fraction of all known substances is fluorescent. Therefore it would be expected that a fluorescent substance such as DPNH, could more readily be detected and identified by its fluorescence than by its absorption.

Substances that are fluorescent in solution generally fluoresce also *in vivo*, although the intensity of fluorescence and the location of the fluorescence spectra may be different. For instance, the quantum yield of fluorescence of chlorophyll *a* in *vivo* is about ten times smaller than that in organic solution and the major fluorescence peak is shifted a few hundred Angstrom to longer wavelengths; the shape of the spectrum, however, is not markedly altered[4].

A complete fluorescence analysis requires the measurement of fluorescence spectra with exciting light of a great number of wavelengths and of known intensity and the analysis of these spectra in terms of the fluorescence spectra of single fluorescing substances. Such an analysis may enable the fluorescing substances present to be identified and their quantity at any moment determined, and provide some evidence concerning their interaction with neighbouring molecules

Until now such an analysis has been performed only for a number of photosynthesizing species in the region 600–1400 mμ, where the photosynthetic pigments fluoresce[4] (*cf.* also[5]).

By means of the apparatus described in this paper it is possible to use shorter wavelengths for the fluorescence analysis of living cells. As exciting radiation the

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mercury "lines" 313 mμ and 366 mμ were used; the measuring regions were 350–600 and 400–600 mμ, respectively. When 366 mμ was used, the fluorescence spectrum in yeast, and presumably also in the other cell suspensions, was for the main part caused by reduced pyridine nucleotide.

APPARATUS AND METHODS

A schematic diagram and description of the apparatus used for the measurement of the fluorescence spectrum is given in Fig. 1. The mercury arc is a medium pressure Philips HP 125 Watt. The lenses l_1 and l_2 and the vessel were of fused quartz, the mirror m was an aluminized surface mirror. The vessel for the DPNH solution had a depth of 1.3 mm, that used for cell suspensions a depth of 10 mm. The exciting radiation fell on the side of the vessel from which the measured fluorescence emerged. This minimized the loss of incident and fluorescent light by absorption in the cell suspension, which is of great importance when strongly scattering or strongly absorbing suspensions or specimens are used.

For isolating wavelength 366 mμ the filter set f_1 was Schott UG 2, 2 mm, UG 11, 2 mm, and the GAB interference filter R-UV 361 mμ, half-width 30 mμ; set f_2 was Schott WG 1, 6 mm, and GG 13, 3 mm. We estimated from the transmission of the filters and given relative intensities of the mercury lines that the intensities of the lines 334 and 313 mμ were less than 2 % of that of the line 366.

For isolating "wavelength 313 mμ": f_1 was Schott UG 11, 2 mm and a quartz vessel of 1 cm thickness with a solution of 10^{-3} M K_2CrO_4 and $5 \cdot 10^{-2}$ M KOH in water and f_2 was Schott WG 2, 1 mm. The relative intensities of the lines 313, 302, 297 and 289 mμ were estimated at 100, 18, 3 and 0.05 respectively.

The filter combinations f_1 and f_2 reduced scattered exciting light in the measuring regions mentioned above to a negligible value. This was proved by the fact that the recorder did not show a deflection after moving filter WG 2 or WG 1 from f_2 to f_1.

The fluorescence of the vessel, filled with medium (or water), was not negligible when weakly fluorescent organisms such as *Chlorella* cells, were studied. When necessary, an approximate correction for this fluorescence was made by subtracting the fluorescence spectrum of the vessel filled with medium from that of the suspension.

The monochromator was a Bauch and Lomb 600 mm 1200 lines/mm grating monochromator. The slits of the monochromator were set at equal widths. The half-width of the transmitted band was 4.5 mμ or less.

The light-detector was a RCA 1P 21 multiplier. The electronic part of the apparatus was

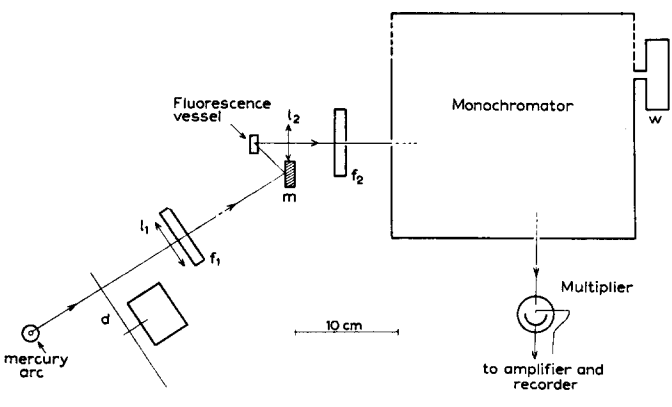

Fig. 1. Apparatus for measurement of "fluorescence spectra". By means of a lens l_1 and a mirror m the mercury arc was imaged in a vessel containing the fluorescing solution or cell suspension. The fluorescing image was focussed by lens l_2 on the entrance slit of the monochromator. Filter set f_1 isolated the spectral region of the exciting light, filter set f_2 absorbed the exciting radiation and transmitted light of longer wavelengths. The wavelength drum was driven by a synchronous motor. A sectioned disc d, mounted on another synchronous motor, modulated the incident light. The a.c. component of the current caused by the light falling on the 1P 21 multiplier was amplified, rectified and fed into the recorder.

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essentially the same as that described elsewhere[6]. The main modification was the addition of a faster four-stage smoothing filter, in order to make full use, if desired, of the response time of a one-second Philips recorder.

The recorder chart and the wavelength drum of the monochromator were driven by synchronous motors. Thus the fluorescence plotted on the recorder paper had a linear wavelength scale. The paper was marked by means of a magnetic pen-relay, which was actuated each time when a 50 and 100 mμ mark on the wavelength drum passed a fixed line. The velocity of wavelength recording was 120 mμ per minute. The wavelength scale was calibrated by recording mercury lines. All recordings were run forward and backward through the spectrum. The spectra were corrected for the small shifts (± 1 mμ) caused by the rapid recording.

The energy calibration of the fluorescence spectrum (energy emitted per unit of wavelength interval in relative units) was for both sets *f* carried out by replacing the fluorescence vessel and mirror (see Fig. 1) by freshly prepared MgO surfaces, and the mercury arc by a calibrated tungsten ribbon filament lamp with quartz envelope. The precision of the calibration of the energy was probably within 3% from the middle to the limits of the spectral region used.

The abscissa scales of the fluorescence curves of the various spectra for incident light of 366 mμ are only comparable as regards the order of magnitude.

RESULTS

DPNH spectrum

The fluorescence spectrum of $6 \cdot 10^{-4}$ *M* DPNH (Sigma) dissolved in distilled water, is shown in Fig. 2. The shape of the spectrum obtained by excitation with 366 mμ is apparently, within experimental error, the same as that excited by radiation of 313 mμ. The maximum of the spectrum is at approximately 462 mμ, its half-width is 97 mμ. The maximum was found to be the same in distilled water as in 0.05 *M* phosphate buffers of pH 8.0, 7.0, 5.6 and 3.3.

Yeast spectrum

Commercial baker's yeast was washed twice by suspending in distilled water and centrifuging. The final sediment was suspended in a solution of 0.005 *M* phosphate buffer of pH 5.6, 0.01% $MgSO_4$ and 0.003% KCl, and continuously aerated. The solution was mistakenly prepared with lower concentration of salts than originally intended. Fluorescence spectra of 2% suspensions of this yeast, excited by 366 mμ, are shown in Fig. 3. The lower spectrum was measured for a suspension after three

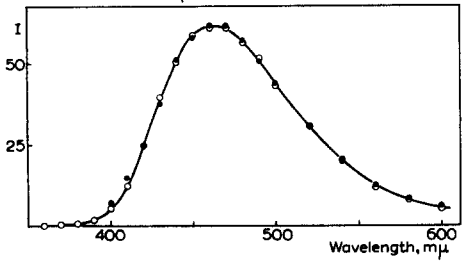

Fig. 2. Fluorescence spectra of DPNH. The open circles are for excitation by wavelength 313 mμ, the black ones for excitation by 366 mμ. The spectrum for 313 mμ has been multiplied by a certain factor to make its maximum of equal height as the maximum of the spectrum excited by 366 mμ. The spectra appear to be identical; the maximum is at about 462 mμ.

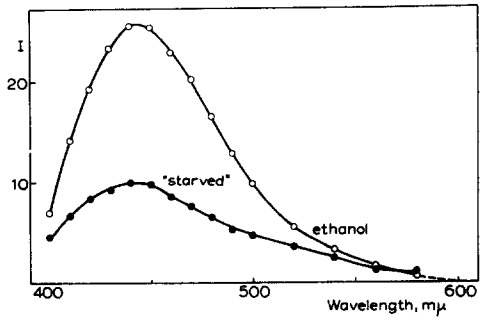

Fig. 3. Fluorescence spectra of "starved" baker's yeast and of the same suspension in 10% ethanol for excitation with 366 mμ. The maxima of both spectra are at about 443 mμ.

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hours of aeration (or "starvation"), the higher one for the same "starved" suspension after addition of one tenth volume of ethanol immediately before measurement. Both spectra have a maximum at 443 mμ. Except for the wavelength shift of 19 mμ to shorter wavelengths, the spectra are similar to but somewhat narrower than the fluorescence spectrum of DPNH. The spectra are apparently not caused solely by free DPNH, but may be caused, at least in part, by a complex of DPNH with a cell constituent (see DISCUSSION). There is in the spectrum of the starved yeast (Fig. 3) a relatively higher fluorescence at 520 mμ than in the spectrum with ethanol, which may be caused by a flavin.

When excited by 313 mμ, the fluorescence spectrum of the suspension of baker's yeast (Fig. 4) is completely different from that excited by 366 mμ. The fluorescence of the substance that is mainly excited by 366 mμ is apparently masked by another strongly fluorescent substance. The assumption that the first substance is DPNH is consistent with the ratio of fluorescence intensities, measured for a very dilute suspension and for a DPNH solution for exciting wavelengths 313 and 366 mμ, respectively.

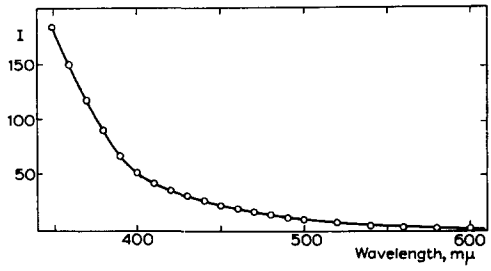

Fig. 4. Fluorescence spectrum of baker's yeast for excitation with 313 mμ, in anaerobic 0.9% sodium chloride.

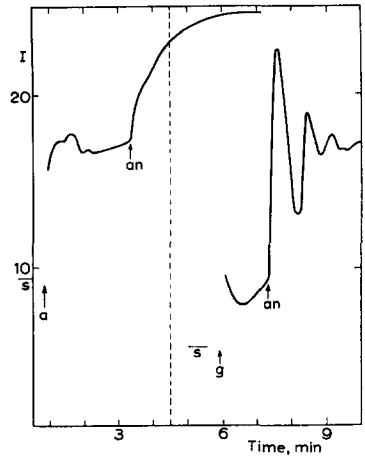

Fig. 5. Time course of fluorescence of a 2% yeast suspension at 450 mμ, excited by 366 mμ. The left-hand spectrum is for a two day starved suspension. The fluorescence level is *s*. At *a*, 0.01 ml ethanol is added to 3.5 ml of the suspension. An increase in fluorescence occurs, which is followed at *an* by a further increase. The right-hand spectrum is for a suspension starved for one day. The fluorescence increases from the steady state *s* upon addition at *g* of glucose to 0.02 *M*, and then decreases. At *an* a rapid second increase in fluorescence occurs, which approaches after a damped oscillation to a steady-state level, which is appreciable higher than that before the addition of glucose. The abcissa values of the two graphs are not comparable.

Some kinetic and other orientating experiments with yeast

Upon addition of ethanol up to a final concentration of 10%, the fluorescence of a yeast suspension that had been starved for one day increased about 4 times. The yeast fluorescence was compared with the increase of fluorescence upon addition of DPNH to the suspension. The fluorescence at 450 mμ of the one-day-starved suspension in the absence of ethanol was doubled by adding 0.75 mg and in the presence of ethanol by adding 3.1 mg DPNH/g wet yeast.

Time courses (Fig. 5) of fluorescence of starved yeast at 450 mμ, excited by 366 mμ, upon addition of ethanol (*a*) and glucose (*g*), indicate that this fluorescence is caused by DPNH or DPNH-complexes (see DISCUSSION).

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Spectra of Photobacterium phosphoreum and Photobacterium splendidum

These bacteria were grown in 1% peptone and 3% sodium chloride. Their fluorescence was measured in 0.3% glycerol and 3% sodium chloride. The maximum of the fluorescence spectrum of the luminescent bacterium *Ph. phosphoreum* (Fig. 6) is at about 445 mμ and its half-width is 115 mμ. The spectrum of *Ph. splendidum* (Fig. 7) has a maximum at about 450 mμ and a half-width of 107 mμ. In a similar way as with yeast, the fluorescence of *Ph. splendidum* was found to be the same as the fluorescence of 0.6 mg DPNH/g wet cells. The spectra of *Ph. phosphoreum* and *Ph. splendidum* resemble that of DPNH, but are somewhat broader and displaced for 17 and 12 mμ, respectively, to shorter wavelengths.

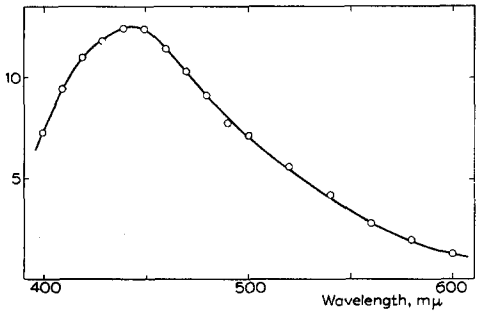

Fig. 6. Fluorescence spectrum of *Ph. phosphoreum* excited by 366 mμ.

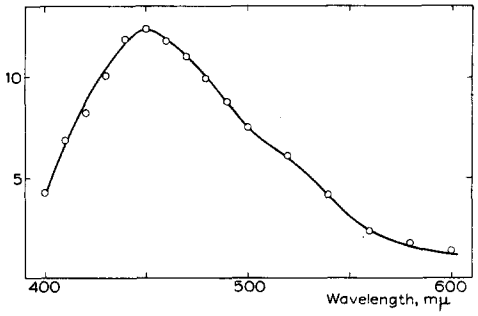

Fig. 7. Fluorescence spectrum of *Ph. splendidum* excited by 366 mμ.

Chlorella

The fluorescence spectrum of *Chlorella* is shown in Fig. 8. The quartz fluorescence, for which a correction was applied, was of the same order of magnitude as that of the suspension itself, which caused an uncertainty in the spectrum in the region 420 mμ. No correction is made for the "self-absorption" of the fluorescent light by the suspension. We estimated, however, that the increase in *Chorella* absorption from 520 to 480 mμ is at least partly responsible for the apparent fluorescence maximum at 510 mμ. The wavelength of the other maximum is only slightly influenced by self-absorption. The location of this maximum suggests that it is caused by the same substance as in yeast and *Photobacterium*.

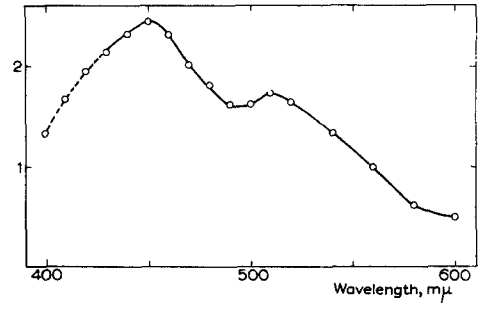

Fig. 8. Fluorescence spectrum of *Chlorella*. The maximum is at about 455 mμ. The maximum at 515 mμ is at least partly an artifact (see text).

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DISCUSSION

As we remarked, the fluorescence spectra of yeast and *Photobacterium*, for excitation with 366 mμ, are similar to but not identical with the fluorescence spectrum of free DPNH. The shift of the maximum in cells to a shorter wavelength, is presumably caused by complex formation of DPNH with cell constituents. This is suggested by experiments of THEORELL *et al.*, which indicated that the maxima of both absorption[8] and fluorescence[9] spectrum of DPNH when bound to alcohol dehydrogenase from horse liver shift to shorter wavelengths.

So far no fluorescence spectra of complexes of DPNH and isolated yeast enzymes have been measured. However, absorption spectra of these complexes are, according to CHANCE[7], as far as studied, no different from that of free DPNH. It is theoretically possible that the fluorescence spectra of bound and of free DPNH are different, although the absorption spectra are the same. It seems that definite analysis of the fluorescence spectra and also of absorption spectra (*cf.*[2]) in yeast in terms of DPNH-enzyme-complexes has to await determination of the spectra of complexes of DPNH with isolated yeast enzymes.

Although the type of complexes is uncertain, the time course of fluorescence upon addition of glucose or ethanol (Fig. 5) is what one would expect if DPNH in one form or another were the main cause of fluorescence. The changes in fluorescence parallel the changes in absorption observed by CHANCE[7] around 340 mμ, which were plausibly interpreted as being caused by DPNH. According to CHANCE, the suspension becomes anaerobic after a second increase in absorption, which corresponds to our second increase *an* in fluorescence (Fig. 5). The approach to the anaerobic steady state upon glucose addition (right-hand side of Fig. 5) is in our time course, but not in CHANCE's, a damped oscillation with a period of about one minute.

If the fluorescence yield of the hypothetical DPNH complex or complexes in yeast is the same as that of free DPNH, then 3 g DPNH would be present per kg wet yeast in 10% ethanol. This is in satisfactory agreement with the finding that, by extraction, 1–1.5 g DPN can be obtained per kg (*cf.* SCHLENK[10]). If a more precise estimation is to be made of the DPNH in yeast, then the fluorescence yield of the DPNH complex in yeast should be determined. Observations[10] on the fluorescence of DPNH bound to alcohol dehydrogenase of horse liver suggest that the yield of bound DPNH may be higher than that of free DPNH.

The fluorescence spectra of the two species of *Photobacterium* have a shape resembling that of yeast, which suggests that they also are mainly caused by reduced pyridine nucleotide; *Chlorella* has in addition to the maximum at 450 a minor maximum at 510–520 mμ. As calculation shows, the minor maximum is caused, at least in part, by self-absorption of fluorescent light; in part it may be caused by a flavin.

Free flavin is strongly fluorescent. THEORELL AND NYGAARD[11] observed quenching of the fluorescence of FMN in the old yellow enzyme upon binding. Thus the weakness or absence of flavin fluorescence in the fluorescence spectra of cells indicates that most of the flavin is bound to cell constituents.

Our results show that the fluorescence excited by 366 mμ can be used for measuring reduced pyridine nucleotide *in vivo*. Fig. 4 shows that wavelength 313 mμ causes strong fluorescence by a substance different from DPNH. This fluorescence is stronger than that of DPNH, and masks the latter. In order to keep fluorescence

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by other substances than DPNH small, light of wavelengths shorter than 366 mμ should be carefully filtered out.

Scattering of the cells has only a secondary effect in fluorescence spectrophotometry. In absorption spectrophotometry scattering and absorption by other substances make direct measurements of reduced pyridine nucleotide impossible, when cell suspensions are used; it is necessary to measure difference spectra. The method described in this paper, in contradistinction to that of absorption spectrophotometry, can also be used for measuring reduced pyridine nucleotide in the surface layer of large intact organisms or organs such as large muscles.

SUMMARY

An apparatus is described with which it is possible to measure precisely fluorescence spectra, not only of clear solutions, but also of a scattering weakly fluorescent suspension of living cells or pieces of tissue, in the region 350–600 mμ by excitation with the mercury line 313 mμ and in the region 400–600 mμ by excitation with 366 mμ.

Fluorescence spectra of free DPNH, suspensions of baker's yeast, *Photobacterium phosphoreum* and *Photobacterium splendidum*, and the alga *Chlorella* were determined. When excited by carefully filtered light of 366 mμ, all three species showed fluorescence spectra presumably caused mainly by reduced pyridine nucleotide. Measurements of the time course of the fluorescence of starved yeast upon addition of ethanol and glucose indicated that this fluorescence was for the main part caused by reduced phosphopyridine nucleotide.

The method appears to be more specific and more generally applicable than absorption spectrophotometry for the measurement of reduced pyridine nucleotide *in vivo*.

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