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Research Articles

Spectrophotometric Effect of Salicylic Acid and Selected Antiradiation Agents on Catalase and Lactic Dehydrogenase

By WILLIAM O. FOYE and PHILIP S. THIBODEAU*

Spectrophotometric measurements have been made of systems of catalase and of lactic dehydrogenase containing either salicylic acid, 2-mercaptoethylamine, 2-mercaptoethylguanidine, or sodium diethyldithiocarbamate. Absorption differences indicated complex formation between the dithiocarbamate and lactic dehydrogenase and at least salt formation with catalase. No definite indication of complex formation between these enzymes and either salicylic acid or the basic mercaptans was given by the method employed, although salt or weak field complex formation is a possibility with the latter.

COMPLEXATION of the metal constituents of enzymes by drugs or biologically active molecules has been postulated as a possible mechanism of drug action. Enzyme inhibition by such molecules is often believed to involve complexation of the active catalytic site, which is frequently the metal. Little definite evidence has been provided to demonstrate such complex formation, however, although Vallee has been able to demonstrate enzyme-chelating agent complexations involving the metal constituent by means of both ultraviolet absorption spectral changes (1) and optical rotatory dispersion studies (2). Keilin and Hartree (3) have shown complexation of catalase with such inhibitors as cyanide, sulfide, fluoride, and azide by means of ultraviolet absorption, and Chance (4) has been able to demonstrate the existence of short-lived catalase-peroxide complexes spectrophotometrically using a rapid-flow technique. In these cases, significant differences in either absorption maxima or absorbance of the enzyme were observed.

No less than 30 enzymes are known to be affected by the presence of salicylates *in vivo*, *in vitro*, or both, and many of these enzymes are now recognized as metal-containing. Nitzescu and Cosma (5) reported a powerful depressing action on the succinodehydrogenases by salicylate; von Euler and Ahlstrom (6) stated that salicylate inhibits the activity of lactic dehydrogenase (LDH). Baker (7) later found that salicylate inhibited the conversion of lactate to pyruvate by means of LDH catalysis. Tomimura (8) has described the inhibition of a peroxide-catalase system by salicylate, and

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Hagihara (9) has shown that the rate of denaturation of catalase by salicylate differed from its rate of inhibition by salicylate.

Accordingly, experiments have been carried out to find whether complex formation between salicylate and either lactic dehydrogenase or catalase can be demonstrated with ultraviolet or visible spectrophotometry. In addition, similar experiments were carried out with several of the more powerful antiradiation agents, 2-mercaptoethylamine (MEA), 2-mercaptoethylguanidine (MEG), and diethyldithiocarbamate (DDC) and the same two enzymes, since inhibition of radiation damage in the cells is believed in some cases to result from inhibition of cellular oxidations (10).

EXPERIMENTAL

Materials.—The enzymes, catalase (from beef liver) and lactic dehydrogenase (from beef heart muscle), were obtained from L. Light and Co., Ltd., England. Catalase was stored until use at 10° and LDH at −5° as concentrated suspensions. Salicylic acid was obtained from Merck and Co., Inc., and was recrystallized to constant m.p. from 95% ethanol. 2-Mercaptoethylamine hydrochloride and 2-aminoethylisothiuronium bromide hydrobromide were purchased from Chemicals Procurement Labs., Inc.; 2-mercaptoethylguanidine was obtained from the latter compound by rearrangement in tris buffer at pH 7.2. Sigma 121 primary standard grade tris-(hydroxymethyl)aminomethane hydrochloride, Sigma Chemical Co., was used to prepare the tris buffer, pH 7.2.

Methods.—The Beckman DU quartz spectrophotometer equipped with a constant voltage power supply and a photomultiplier tube was used with 1-cm. cells. Enzyme solutions of the desired concentrations were prepared by dilution of the stock suspensions with the tris buffer solution. All experiments were run at 25°, except for one at 10°. They were all completed within 1 hour after the enzyme solutions were prepared.

For measurement of complex formation, four matched cells were employed. In a typical experiment, cell *1* contained buffer only and served as blank. Cell *2* contained the enzyme solution. Cell *3* contained the complexing agent, prepared in tris buffer solution. Cell *4* contained both complexing agent and enzyme in precisely the same concentrations as cells *2* and *3*. Immediately after mixing, measurements of the absorption spectra were made. The spectra obtained from cells *2* and *3* were added and compared with the spectrum from cell *4*. A difference in shape or degree of the added spectra from the spectrum of cell *4* was assumed to indicate complex formation.

A modification of the method of infinite dilution was also made. In this procedure, cell *1* contained buffer only and served as blank for cell *2*, which contained the enzyme solution. Cell *3* contained the complexing agent and served as blank for cell *4*, which contained both the enzyme and complexing agent in the same concentrations as they existed in cells *2* and *3*. Nonequality of the spectra from cells *2* and *4* was taken to indicate complex formation.

Varying ratios of complexing agent to enzyme were employed from large molar excesses of complexing agent to stoichiometric amounts. In the case of large excesses of complexing agent, however, the possibility existed that its absorption could minimize or swamp any change in absorbance due to complex formation; but in the case of stoichiometric amounts, the other possibility existed—that of swamping by the amount of enzyme absorbance.

RESULTS AND DISCUSSION

Two of the more common metals in enzymes affecting cellular oxidations are iron and zinc. Lactic dehydrogenase is a pyridine nucleotide-linked enzyme containing zinc, and catalase is an iron-porphyrin containing enzyme. Little is known regarding the bonding of the zinc in LDH, but it is assumed that mixed complex formation of the zinc is possible by an extraneous agent. Vallee (1) has already demonstrated mixed complex formation of the zinc in liver alcohol dehydrogenase with 1,10-phenanthroline. In regard to catalase, the iron is *strongly* bound to the porphyrin nucleus; in addition, only one bond is available for coordination, normally with peroxide (11). Chelation of the iron by an external agent is thus considered impossible, although complex or salt formation with such an agent should be possible. In this light, the same type of reaction between metalloenzyme and complexing agent would not occur with the two enzymes. As far as assessing this method for demonstrating complexation, one enzyme serves as control for the other.

For salicylic acid in tris buffer solution maximum absorption was at 296 mμ. Catalase, at a concentration of 100 mg./L., showed no true maximum in the ultraviolet but did show a plateau at 256–268 mμ. Higher concentrations of catalase absorbed maximally in the visible at 404 mμ. Although Keilin and Hartree (3) reported a maximum at about 630 mμ for horse liver catalase at pH 6.0, this maximum did not appear in our enzyme preparation at pH 7.2. The lactic dehydrogenase exhibited peak absorbance at 278–280 mμ.

The spectrophotometric measurements made with catalase and salicylic acid in both the ultraviolet and visible range did not indicate complex formation. Optical differences between the sum of the absorbances of cells *2* and *3* and the absorbance of cell *4* or between the absorbances of cells *2* and *4* in the method of infinite dilution did not exceed 0.010, which probably may be considered the limit of experimental error. The differences reported in Table I represent only the maximum deviations found; however, the average extent of difference over the absorption range observed generally was less than 0.004.[1] It is possible then that a difference of greater than 0.010 may represent salt[2]

[1] Beaven and Johnson (12) point out, however, that under favorable circumstances variations of 0.002 in absorbance are experimentally significant.

[2] A weak field complex, such as described by Eichhorn (11), may also be possible. In this case, the electron "pairing energy" necessary for complex formation is not affected much by changes in the ligand (13). This "pairing energy" is opposed by the "separation energy" between *d* orbitals in the case of iron, but not in the case of zinc. On this basis, the absorption spectrum of catalase would show less variation on complexation than that of lactic dehydrogenase, which is shown in the experiments described here.

TABLE I.—SPECTROPHOTOMETRIC MEASUREMENTS OF ENZYME SYSTEMS, 25°

Compd.	Concn., mg./L.	Enzyme	Concn., mg./L.	Optical Range, mμ	Absorbance Difference
Sal. acid	25	Catalase	100	240–340	0.005
Sal. acid[a]	20	Catalase	300	240–340	0.005
Sal. acid[a,b]	20	Catalase	300	240–340	0.008
Sal. acid	2.2	Catalase	300	240–340	0.003
Sal. acid	50	Catalase	2,000	340–700	0.004
Sal. acid[a]	500	Catalase	4,200	340–600	0.008
Sal. acid	100	Catalase	17,000	480–700	0.010
Sal. acid	12.5	LDH[f]	125	240–340	0.016
Sal. acid[a]	12.5	LDH	125	240–340	0.015
Sal. acid	0.26	LDH	125	240–340	0.018
MEA[c]	100	Catalase	400	240–350	0.014
MEA	1.82	Catalase	300	240–350	0.022
MEA	100	LDH	125	240–350	0.034
MEA	0.2	LDH	125	240–350	0.015
MEG[d]	100	Catalase	200	235–350	0.009
MEG	100	LDH	125	235–350	0.029
DDC[e]	6	Catalase	200	235–350	0.045
DDC	6	LDH	125	235–350	0.363

[a] Method of infinite dilution. [b] Run at 10°. [c] 2-Mercaptoethylamine hydrochloride. [d] 2-Mercaptoethylguanidine hydrobromide. [e] Sodium diethyldithiocarbamate. [f] Lactic dehydrogenase.

formation or ionic bonding between the complexing agent and the enzyme, the small increase in absorbance being due to electrostatic attraction with no significant change in electron densities in either complexing species. The over-all shape of the absorption curves remained the same for the mixture as for the added curves of the individual components in this case, however.

The optical differences recorded for the combination of salicylic acid and lactic dehydrogenase did not exceed 0.018. While this difference is probably not indicative of covalent complex formation, it may represent salt (ionic bonding) formation. It has been pointed out by Martell and Calvin (14), for instance, that if the bonding is ionic, the absorption bands for the complex and complexing agent may coincide fairly closely. Similarly, in the experiments involving 2-mercaptoethylamine and the same two enzymes, no definite complex formation is apparent from the extent of the optical differences measured, although salt formation is again a possibility. In the case of 2-mercaptoethylguanidine, complex formation appears not to have taken place, although the somewhat greater optical difference shown in the mixture with lactic dehydrogenase again suggests salt formation.

Sodium diethyldithiocarbamate showed maximum absorption at 257 and 281 mμ. Addition of this compound to catalase produced a very definite disparity between the spectra of the individual components and that of the mixture (see Fig. 1). A difference in absorbance of 0.040 remained approximately the same from 235 to 300 mμ, after which it became insignificant. An even more striking difference in spectra was noted on the addition of diethyldithiocarbamate to lactic dehydrogenase (see Fig. 2). Here a maximum difference of 0.363 was observed, and the minimal absorbance of the added spectra at about 245 mμ and the increase in absorbance to about 265 mμ are completely eliminated in the absorption spectrum of the mixture. The disparity between the curves remains significant throughout the ultraviolet region, but diminishes as the visible range is approached.

This marked difference in absorbance and the dramatic change in the shape of the absorption curves for the mixture of DDC and LDH, compared to the sum of the absorption curves of the components, may be considered evidence of complex formation, as noted previously for absorption of enzyme-inhibitor complexes (3). It appears quite possible that a cyclic complex, or chelate, has been formed with the zinc still bound in the lactic dehydrogenase, whereas a more loosely bound linear complex only is possible with catalase. That disul-

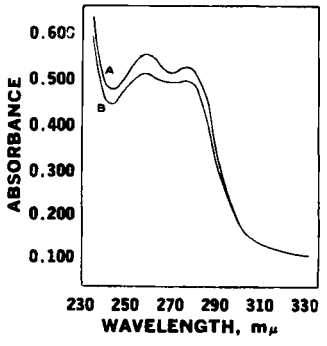

Fig. 1.—Absorption spectra at pH 7.2 of a mixture of sodium diethyldithiocarbamate and catalase (*A*) and of the sum of the spectra of the components (*B*).

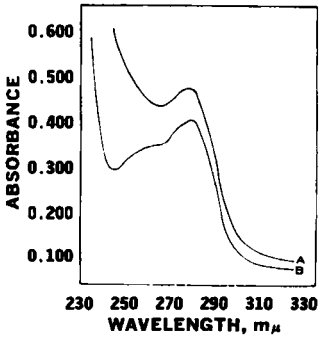

Fig. 2.—Absorption spectra at pH 7.2 of a mixture of sodium diethyldithiocarbamate and lactic dehydrogenase (*A*) and of the sum of the spectra of the components (*B*).

fide formation with protein mercaptan groups is not responsible for the change in absorption spectrum is indicated by the failure of the mercaptans MEA and MEG to cause a similar change.

However, it is again possible that salt formation has taken place with catalase since the over-all shape of the sum of the absorption curves has not been appreciably altered in the mixture, and the average difference in absorbance, 0.040, is not greatly different from those of the previous cases where salt formation is suspected. Moreover, it appears that complex formation of enzymes may be demonstrated by this method of absorbance differences in absorption spectra in certain cases at least.

CONCLUSIONS

Spectrophotometric evidence has been found that a known antiradiation agent, sodium diethyldithiocarbamate, undergoes complex formation with lactic dehydrogenase and either salt or weak field complex formation with catalase. No definite evidence for complex formation of these enzymes with either 2-mercaptoethylamine or 2-mercaptoethylguanidine was found, although salt or weak field complex formation is possible. Apparently, any complexation by the latter agents is more readily dissociable than that by the dithiocarbamate.

No spectrophotometric evidence of complex formation between salicylate and either lactic dehydrogenase or catalase was found, although both enzymes are known to be inhibited by salicylate. It is possible that any complex formed has dissociated too rapidly to be observed by this method.

Measurement of absorbance differences between the sum of the absorption spectra of the individual components and the spectrum of the mixture appears to be a possible method of demonstrating complex formation of enzymes in select cases. In these cases the binding is either irreversible or of sufficient strength to exist for a measurable period of time and thus be observable by the stationary, or nonflow, technique employed here.

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Assay of Iodine Content of Thyroid Extract by X-Ray Absorption Edge Analysis

By HARRY A. ROSE and DONALD E. FLICK

The assay of thyroid extract for iodine content by the X-ray absorption edge technique has been investigated. The method has been found to be applicable to the crude, U.S.P. powder, and uncoated Enseals. Excellent precision has been found for the assay of crude thyroid extract. It is also shown that because of the nature of the method the determination is specific for iodine. The instrumentation used is the same as that used for X-ray crystallography by the diffractometer method.

THE ASSAY of the iodine content of thyroid extract by X-ray absorption edge analysis was developed as a possible replacement for the chemical assay for this element.

The chemical assay that is used is prescribed by the "United States Pharmacopeia." The present procedure has undergone only minor improvements since the U.S.P. IX of 1916. Essentially, it is a potassium carbonate fusion to liberate the iodine from the organic matrix. After several intermediate steps, the determination ends with a sodium thiosulfate titration. It is about a 4-hour procedure with many opportunities for loss of sample.

X-ray absorption edge analysis has been applied in the analysis of molybdenum and zinc in hydrocarbons by Barieau (1). A recent review of the field has been given by Liebhafsky *et al.* (2). Because of the completeness of the description of the method given by Barieau, only a brief description will be given here.

X-ray absorption edge analysis is a very powerful tool because it is free from matrix effects and is specific for the element to be determined. This is so because the absorption of X-rays by a

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