EFFECT OF pH INDICATORS ON VARIOUS ACTIVITIES BY CHROMATOPHORES OF *RHODOSPIRILLUM RUBRUM*

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SUMMARY

1. The effects of pH indicators on activities for ATP formation in the light, ATP hydrolysis in the dark and ATP-Pi exchange in the dark with chromatophores from *Rhodospirillum rubrum* were examined. Of thirty one kinds of pH indicators tested, eleven kinds (metanil yellow, 2,4-dinitrophenol, ethyl orange, bromocresol green, resazurin, neutral red, bromthymol blue, α-naphtholphthalein, *o*-cresolphthalein, phenolphthalein and alizarin yellow G) almost completely inhibited the activities for ATP formation and ATP-Pi exchange at 1 m*M*, and were studied further.

2. Of the eleven kinds pH indicators, the pH indicators other than α-naphtholphthalein, *o*-cresolphthalein and phenolphthalein, when assayed at appropriate concentrations of the dyes, inhibited the ATP-Pi exchange, but not the ATP hydrolysis. In the ATP-Pi exchange, these eight kinds of pH indicators at the concentrations described above were competitive against Pi, and non-competitive against ATP. The remaining three kinds of pH indicators were non-competitive against either Pi or ATP, when assayed at the

concentrations of the dyes that inhibited both activities.

3. The amounts of pH indicators bound with chromatophores were measured. No correlation was seen between the amounts of the bound dyes and the extents of their inhibitions on either the ATP formation or the ATP-Pi exchange.

4. Ethyl orange (pK_a = 4.1) and 2,4-dinitrophenol (pK_a = 3.9) were able to stimulate the ATP hydrolysis to higher extents than those of higher pK_a values.

5. The stimulatory effects of pH indicators on the ATP hydrolysis were hardly influenced by extraction of quinones from chromatophores.

6. Most of the pH indicators were able to stimulate both succinate-cytochrome $\underline{c}_2$ and NADH-cytochrome $\underline{c}_2$ reductions in the dark.

7. The mechanism for uncoupling the electron transfer system and the phosphorylation system by pH indicators was discussed, and hypothetical mechanism for the coupling was proposed.

INTRODUCTION

Chromatophores from Rhodospirillum rubrum are able to catalyze the formation of ATP from ADP and Pi, which is coupled to the photosynthetic, cyclic electron transport system (1,2). Okayama et al. (3) found that ubiquinone-10 is one of the essential components for the photosynthetic ATP formation. Kakuno et al. (4) showed that heavy water inhibits the ATP formation in the light,

the ATP-Pi exchange in the dark and the ATP hydrolysis in the dark. They showed also that the reduction of ubiquinone-10 is inhibited by heavy water, but other oxidation-reduction reactions are not. The results described above suggest the possibility that the protons incorporated from water to ubiquinone-10 simultaneously on reduction of the quinone by electrons and then liberated simultaneously on oxidation of the quinone may give rise the ATP formation.

The present paper deals with studies on effects of many kinds of pH indicators on ATP formation in the light, ATP hydrolysis in the dark, ATP-Pi exchange in the dark, and cytochrome $\underline{c}_2$ reductions by succinate and by NADH in the dark with chromatophores from R.rubrum.

MATERIALS AND METHODS

Cell culture and chromatophore preparation

The carotenoid-less mutant of R.rubrum (G-9) was used. The bacterial cells were incubated at 30° for a day anaerobically in the dark and then grown for 4 days under continuous illumination (150-foot candles) from tungsten lamps. The grown cells were harvested, washed with 0.1 M Tris-HCl buffer (pH 8.0) containing 10% sucrose and then disrupted by grinding with aluminum oxide powder (5). The disrupted cells were suspended in the buffer

described above, and chromatophores were collected by means of differential centrifugation described previously (1, 5). The chromatophores thus collected were suspended in such a volume of 0.1 M glycylglycine-NaOH buffer (pH 8.0) containing 10% sucrose that the suspension would show A_{873nm} = 50, which corresponds to a concentration of 0.355 mmole of bacteriochlorophyll/ml.

Activity assay of ATP formation, ATP hydrolysis and ATP-Pi exchange

Activities for ATP formation in the light, ATP hydrolysis in the dark and ATP-Pi exchange in the dark were measured by the same methods as described previously (5, 6).

The standard reaction mixture for the activity assay of ATP formation was composed of 0.50 ml of 0.6 M glycylglycine-NaOH buffer (pH 8.0) containing 10% sucrose, 0.10 ml of 0.1 M $MgCl_2$, 0.10 ml of 0.1 M ADP, 0.10 ml of 0.1 M [^{32}P]Pi (approximately 1 x 10^6 cpm), 0.10 ml of 1 M ascorbate, 0.15 ml of chromatophore suspension (A_{873nm}/ml = 50) and water to make the total volume 1.50 ml. The reaction was started by adding the chromatophore suspension, carried out at 30° for 4 min in the light (2,000-foot candles), and stopped by adding 0.50 ml of 30% trichloroacetic acid, previously cooled. The amount of ATP thus formed was estimated by measuring the radioactivity of [^{32}P]Pi incorporated into organic phosphate fraction according to the method of Nielsen and Lehninger (7) modified by Avron (8).

The standard reaction mixture for activity assay of ATP hydrolysis was composed of 0.50 ml of 0.6 M glycylglycine-NaOH

buffer (pH 8.0) containing 10% sucrose, 0.10 ml of 1.5 m$\underline{M}$ [γ-^{32}P]ATP (approximately 1 x 10^6 cpm), 0.10 ml of 0.1 $\underline{M}$ $MgCl_2$, 0.15 ml of chromatophore suspension (A_{873nm}/ml = 50) and water to make the total volume 1.50 ml. The reaction was started by adding the chromatophore suspension, carried out at 30° for 4 min in the dark, and stopped by adding 0.50 ml of 30% trichloroacetic acid, previously cooled. The amount of Pi liberated from ATP was estimated by measuring the radioactivity of the [^{32}P]Pi extracted in the organic fraction.

The standard reaction mixture for the activity assay of ATP-Pi exchange was the same as for the activity assay of ATP formation, except that the radioactivity of [^{32}P]Pi was approximately 1 x 10^7 cpm and 50 m$\underline{M}$ ATP was added instead of 0.1 $\underline{M}$ ADP. In some cases, [^{32}P]Pi concentrations were varied at a fixed concentration of ATP and ATP concentrations were varied at a fixed concentration of [^{32}P]Pi. The other experimental conditions were the same as those for the activity assay of ATP formation, except that the reaction was carried out in the dark.

Activity assay of reductions of cytochrome c_2 by succinate and by NADH

The standard reaction mixture for the activity assay of cytochrome $\underline{c}_2$ reduction by succinate was composed of 0.30 ml of 0.6 $\underline{M}$ glycylglycine-NaOH buffer (pH 8.0) containing 10% sucrose, 50 µl of 1 m$\underline{M}$ cytochrome c_2 in the oxidized form, 10 µl of 1 $\underline{M}$ succinate (succinate-cytochrome $\underline{c}_2$ reduction), 20 µl of chromatophore

suspension (A_{873nm}/ml = 50), and water tomake the total volume 1.0 ml. For the activity assay of cytochrome $\underline{c}_2$ reduction by NADH, 20 µl of 0.25 M NADH was added instead of succinate. The standard reaction mixture containing other than cytochrome $\underline{c}_2$ was incubated for 5 min in the dark at room temperature (24°), and the reaction was then started by adding cytochrome $\underline{c}_2$. The absorbace increase at 550.5 nm (the α-peak of cytochrome $\underline{c}_2$ in the reduced form) was measured.

Estimation of amount of pH indicator bound with chromatophores

The amount of a pH indicator bound with chromatophores was estimated as follows. Chromatophores were mixed with a pH indicator with the same components as the reaction mixture for assay of photosynthetic ATP formation, except that ADP and Pi were omitted. The mixture was centrifuged at 105,000 x g for 60 min at room temperature. The resulting suppernatant was measured of the absorbance at the wavelength for a peak of the pH indicator.

Some of the pH indicators such as o-cresolphthalein and phenolphthalein were bleached at pH 8.0. In these cases, an equal volume of 5 N NaOH was added to the suppernatant described above, and the absorbance was measured.

Preparation of quinone-free chromatophores

Quinone-free chromatophores were prepared according to the method of Okayama et al. (3) with some modifications. Chromatophores were washed twice with water, suspended in water to give A_{873nm} = 200, and lyophilized in the dark. The lyophilized chromatophores were

suspended in such a volume of isooctane that the A_{873nm}/ml would be 20 if they were suspended in the same volume of water. The resulting suspension was stirred at 0° for one hr. The other procedures were the same as those described by Okayama et al. (3).

Measurement of molar extinction coefficients and titration curves of pH indicators

Respective pH indicator (20 - 40 mg) was dried under vacuum in a dasiccator with phosphorus pentoxide until its weight became constant. The dye thus dried was weighed and dissolved in water with the use of a 100-ml volumetric flask. The resulting solution was measured of absorbance spectrum at visible region in 90 mM GTA buffer* (9) of various pH values.

Spectrophotometry

Absorbance and absorbance spectrum were measured at room temperature (24°) by a Cary model 17 spectrophotometer with the use of cuvettes of 1-cm optical path.

Reagents used

ADP and ATP were commercial preparations from Sigma Chemical Co., St.Louis, Missouri. NADH was purchased from Oriental Yeast

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* Mixture of equimolar concentration of 3,3-dimethyl glutaric acid, tris(hydroxymethyl)aminomethane and 2-amino-2-methyl 1,3-propandiol was adjusted by NaOH or HCl to desired pH value. The concentration of the buffer was represented by the total concentration of the components, except for that of NaOH or HCl.

Co., Ltd., Osaka. Isooctane was commecially obtained from E.Merk Ag, Darmstadt. [^{32}P]Pi obtained from Institute of Atomic Energy, Japan, was used without further purification. All of the pH indicators were obtained from BDH Chemicals Ltd., Poole, through Daiichi Pure Chemicals Co., Ltd., Osaka.

[γ-^{32}P]ATP was prepared according to the method of Horiuti et al. (10). Cytochrome c_2 was prepared from light grown cells of R.rubrum according to the method of Bartsch et al. (11). It was oxidized by mixing with an excess amount of potassium ferricyanide. The resulting mixture was passed through a column of sephadex G-25, and the cytochrome in the oxidized form was separated from the oxidizing reagent.

RESULTS

Effects of several pH indicators on activity for ATP formation in light

It is known that with a mixture of bromthymol blue and chromatophores in a buffer of sufficiently high concentration, the absorbance at 615 nm of the dye decreases to some extent in the light and then increases to the original level in the dark (12 - 15). The cause for this phenomenon has been interpreted mainly by the following two ways. 1) The absorbance decrease is due to the association of the dye with protons incorporated into chromatophre vesicles

vesicles (14), and 2) it is brought about by conformational changes of chromatophore membrane (15).

The effect of buffer concentrations on the absorbance change of bromthymol blue bound with chromatophores was measured at pH 8.0 (Fig. 1). It was found that the extent of the absorbance decrease

Fig. 1

of the dye at 615 nm reached a maximum when the concentration of the buffer was 0.1 M, was lowered at higher concentrations, and became constant at 0.3 M. It seems likely that the rise and fall in the extent of absorbance change at the low concentrations of the buffer were mostly invoked by bromthymol blue present at the free form in the medium, and that the absorbance change at 0.3 M or higher concentrations was mostly, if not all, due to the dye bound with chromatophore membrane. On the other hand, it was found that bromthymol blue was a potent inhibitor on the activity for ATP formation in the light with chromatophores, as well as with chloroplasts (16). Effect of other pH indicators than bromthymol blue were also examined (Table I). Of the thirty one kinds of

Table I

pH indicator tested, about half the kinds of pH indicator were inhibitory on the activity for ATP formation in the light at pH 8.0.

Effects of pH indicators on the activity for ATP-Pi exchange in the dark were also examined at pH 8.0 (Table I). Most of the pH indicators influenced both activities for ATP formation and ATP-Pi exchange in similar manners, However, for instance of exceptions, bromophenol blue (1 m$\underline{M}$) inhibited the activities for ATP formation and ATP-Pi exchange to 57% and 7%, respectively, whereas resazurin (1 m$\underline{M}$) to 7% and 48%, respectively. The eleven kinds of pH indicator, metanil yellow, 2,4-dinitrophenol, ethyl orange, bromocresol green, neutral red, bromthymol blue, α-naphtholphthalein, resazurin, phenolphthalein, $\underline{o}$-cresolphthalein and alizarin yellow G, which almost completely inhibited both activities at 1 m$\underline{M}$, were used in further experiments. These pH indicators have only one anionic group capable of dissociating one proton.

Some physical constants of pH indicators

The absorbance spectra of eleven kinds of pH indicator described above were measured in GTA buffer of various pH values. On the basis of the absorbance spectra thus measured, the percentage amounts of the pH indicators in the dissociated form were estimated, and plotted as a function of pH value (Fig. 2). The dissociation

Fig. 2

constants (pK_a) of pH indicators, which correspond to the pH values at which 50% of the dyes are in the dissociated form, were determined. The molar extinction coefficients of pH indicators were determined

as described above. Their physical constants are summarized in Table II. These values were used in the present study.

Table II

Inhibitory effects of pH indicators on activities for ATP formation in light and ATP-Pi exchange in dark

The eleven kinds of pH indicator, at various concentrations, were measured of effect on the activity for ATP formation in the light at pH 8.0 with chromatophores (Fig. 3). All the pH indicators

Fig. 3

except for 2,4-dinitrophenol almost completely inhibited the activity at 1 mM. Neutral red is known to be a pH indicator and also an oxidation-reduction dye. This dye slightly stimulate and significantly depressed the activity at low concentrations and at high concentrations, respectively. Any correlation is not seen between the pK_a values and the extents of inhibition. The pH indicators described above inhibited also the activity for ATP-Pi exchange in the dark at pH 8.0 (Fig. 4). Again, any correlation

Fig. 4

is not seen between the pK_a values and extents of inhibition.

The amounts of pH indicators bound with chromatophores were

measured by means of centrifugation of the mixture of chromatophores and respective pH indicators at pH 8.0, according to the method of Jackson and Crofts (14). The amounts of pH indicators bound with chromatophores per mole of bacteriochlorophyll are plotted as a function of concentrations of added pH indicators (Fig. 5). Since straight lines can be drawn in the cases with all

Fig. 5

the pH indicators used, it seems likely that the ratios of the amount of a pH indicator bound with chromatophores to the total amount of the dye added in the mixture were practically constant at the range of its concentrations tested. Any correlation is not seen between the ratios and the extents of the inhibition on either the activity for ATP formation or the activity for ATP-Pi exchange. It may be worth noticing that 2,4-dinitrophenol was not detectable to be bound with chromatophores.

The activity for ATP formation in the light was measured in the presence and absence of the pH indicaors with various concentrations of Pi and a fixed concentration (6.7 mM) of ADP (Fig. 6). It was found that all the eleven kinds of pH indicator described above were non-competitive inhibitors against Pi.

Fig. 6

With α-naphtholphthalein, o-cresolphthalein and phenolphthalein, the minimum concentrations required for the inhibition on the activity for ATP-Pi exchange were the same as those for the inhibition on the activity for ATP hydrolysis (cf. Figs. 4 and 9). On the other hand, with the other eight kinds of pH indicator, the minimum concentrations required for the inhibition on the activity for ATP-Pi exchange were lower than those required for the inhibition on the activity for ATP hydrolysis. At such concentrations of the pH indicators that the activity for ATP-Pi exchange would be inhibited, but not the activity for ATP hydrolysis, the activity for ATP-Pi exchange was measured in the presence and absence of the eight kinds of pH indicator with various concentrations of Pi and a fixed concentration (3.3 mM) of ATP (Fig. 7), and with

Fig. 7

various concentrations of ATP and a fixed concentration (6.7 mM) of Pi (Fig. 8). It was found that all these pH indicators were

Fig. 8

competitive inhibitors against Pi and non-competitive inhibitors against ATP. This indicates that these pH indicators interacted with the same site in chromatophores as that for Pi. At such concentrations of pH indicators that the activity for ATP hydrolysis

was inhibited, the former three kinds of pH indicator were non-competitive against Pi as well as against ATP. Although it was previously reported that they are also competitive inhibitors against Pi (6), repeated experiments revealed that this was not the case.

Properties of pH indicators as uncouplers of photosynthetic ATP formation

In the case of oxidative phosphorylation, reagents such as 2,4-dinitrophenol are classified into "uncoupler (uncoupling agent)" because of their characteristic property, in which the phosphorylation of ADP is inhibited without greately affecting the rate of electron transfer (19). Uncouplers stimulate the normally latent ATPase activity of intact mitochondria but inhibit the other partial reactions of oxidative phosphorylation such as ATP-Pi exchange. With chromatophores of R.rubrum, it is known that 2,4-dinitrophenol inhibits both activities for oxidative and photosynthetic ATP formations and the activity for ATP-Pi exchange but stimulates both activities for ATP hydrolysis and electron transfer (5, 10, 20).

The effects of the eleven kinds of pH indicator on the activity for ATP hydrolysis in the dark was examined at pH 8.0 (Fig. 9).

Fig. 9

It was found that the activity was stimulated by ethyl orange,

2,4-dinitrophenol, resazurin and metanil yellow higher than that in the absence of the dyes. Ethyl orange of pK_a = 4.1 stimulated the activity to the highest extent at 1 mM; the activity was three times as high as that in the absence of the dye.

Earlier, Yamamoto et al. (21) reported that when chromatophores are depleted of the quinones, ubiquinone-10 and rhodoquinone, the activity for ATP hydrolysis in the dark is depressed to some extent, and that the activity remaining after extraction of the quinones is no longer influenced by the oxidation-reduction potential of the reaction mixture.

It was found that the activity for ATP hydrolysis in the dark was significantly stimulated at pH 8.0 by metanil yellow, 2,4-dinitrophenol and ethyl orange, regardless of the extraction of the bound quinones and the readdition of ubiquinone-10 (Table III).

Table III

Parker (22) and Hemker (23, 24) reported that the effeciencies of reagents for uncoupling depend on their dissociation constants and solubilities in lipid. The former auther showed that with rat-liver mitochondria, the derivatives of nitro- and halogeno-phenols having low pK_a values inhibit the oxidative phosphorylation and stimulate the ATPase activity to higher extent than those having high pK_a values.

With chromatophores, all the eleven kinds of pH indicator

described above inhibited the activity for ATP-Pi exchange to higher extents than the activity for ATP hydrolysis (cf. Figs. 4 and 9). Doubtless, both activities originate from the same intermediary state of an enzyme. It is probable, therefore, that the activity for ATP hydrolysis remaining after the inhibition of the activity for ATP-Pi exchange by a pH indicator was resulted from the stimulation of the former activity by the dye. Of such the remaining activities for ATP hydrolysis in the presence of various concentrations of a pH indicator, the maximum remaining activity was plotted as a function of pK_a (Fig. 10). It may be

Fig. 10

seen that all the pH indicators stimulated the activity, regardless of their pK_a values, low or high. However, the extents of stimulation appears to have an intimate correlation with the pK_a values of pH indicators. The pH indicators tested have different chemical structures from one another, but they are common in having one group for dissociation-association of one proton. It is conceivable, therefore, that their pK_a values play an important role for the stimulation of the activity for ATP hydrolysis.

Jackson and Crofts (14) measured the ratios of the dissociated form to the total ("dissociated" plus "associated") of bromthymol blue in the presence and absence of chromatophores at various pH values, and found that the "apparent" pK_a value of the dye

("chromatophore-bound" plus "free") shifts to a more alkaline (higher) pH value. It was found that bromthymol blue was bound with chromatophores to decreasing extents with increasing (more alkaline) pH values (Fig. 11). The "true" pK_a value of the

Fig. 11

bromthymol blue bound with chromatophores was estimated as follows. The difference spectra, "chromatophores + bromthymol blue" minus "chromatophores", were measured at various pH values. The resulting spectra showed a peak at 615 nm due to the dye in the dissociated form. Differences in the difference spectra around 615 nm between the presence and absence of the dye were not detectable. Under the assumption that bromthymol blue would have the same molar extinction coefficient at 615 nm for the chromatophore-bound and the free forms, the percentage ratios of the amounts of the bound dye in the dissociated form to the total amount of the added dye at various pH values were calculated by the following equations.

$$A_b = \left(A_{b+f} - A_f \times \frac{F}{100} \right) \times \frac{100}{100 - F}$$

$$P = \frac{A_b}{A} \times 100$$

where the abbreviation used are

A_b, absorbance at 615 nm of dye bound with chromatophores (bound dye);

A_f, absorbance at 615 nm of dye in the free form (free dye);

A_{b+f}, total absorbance at 615 nm of free plus bound dye (total dye);

F, percentage ratio in mole of free dye to total dye;

P, percentage ratio in mole of bound dye in the dissociated form to total bound dye ("dissociated" plus "associated");

A, absorbance at 615 nm of the dye at a highly alkaline pH value, at which all the dye is in the dissociated form.

Bromthymol blue in the free form has a pK_a value of 7.3 (Fig. 2). In the absence of chromatophores, approximately 90% of the dye is in the dissociated form at pH 8.0. The pK_a value of the dye bound with chromatophores was obtained to be 8.8 (Fig. 11). At pH 8.0, 20% of the added dye was bound with chromatophores, and only 3.2% of the bound dye was in the dissociated form at pH 8.0 (Talbe IV).

Table IV

The activity for ATP hydrolysis in the dark was measured at pH 8.0, 8.5, 9.0 and 9.5 in the presence and absence of various concentration of bromthymol blue. The activities in the absence of the dye were respectively regarded as 100, and the difference percentage activities, "pH 8.5" minus "pH 8.0", "pH 9.0" minus "pH 8.0" and "pH 9.5" minus "pH 8.0", were plotted as a function of the concentration of the

of the concentrations of the dye (Fig. 12). Although the activity

Fig. 12

for ATP hydrolysis at pH 8.0 was stimulated by bromthymol blue to a slight extent (see above), the extent of stimulation was increased with increasing pH values. Either the amount of the bound dye in the dissociated form or that of the free dye in the dissociated form increased with incresing pH values (Table IV). These findings suggest that the dye in the dissociated form, but not the dye in the associated form, was responsible for the stimulation of the activity for ATP hydrolysis. With bromthymol blue, it is not certain which was more effective for the stimulation, the bound dye, or free dye. However, the finding that 2,4-dinitrophenol was hardly bound with chromatophores but stimulated the activity for ATP hydrolysis to a remarkable extent, indicates that at least the dye in the dissociated form was effective for the stimulation.

Effect of pH indicators on electron transfer systems in dark

Chromatophores catalyze the reductions of added cytochrome $\underline{c}_2$ by succinate and by NADH (25). According to Kakuno et al. (4), the cytochrome $\underline{c}_2$, cc' and B and non-heme iron protein bound with chromatophores are of 1-electron transfer, whereas the ubiquinone-10 bound with chromatophores is 2-hydrogen transfer; thus, the oxidation-reduction between one quinone molecule and two non-heme

iron atoms, besides electron transfer, incoporates and liberates two protons from and to water, so that heavy water inhibits the reactions involving the oxidation-reduction of ubiquinone-10.
It was found that not only pH indicators having low pK_a values but also those having high pK_a values stimulated the activities for succinate-cytochrome $\underline{c}_2$ reduction and NADH-cytochrome $\underline{c}_2$ reduction with chromatophores (Figs. 13 and 14). Of the eleven kinds of

Fig. 13

Fig. 14

pH indicator tested, metanil yellow stimulated the activity for succinate-cytochrome $\underline{c}_2$ reduction to the highest extent, which was three times as high as the activity in the absence of the dye, whereas $\underline{o}$-cresolphthalein stimulated the activity for NADH-cytochrome $\underline{c}_2$ reduction to the highest extent, which was ten times as high as the activity in the absence of the dye. Any correlation is not seen between the pK_a values and the extents of stimulation of both activities for cytochrome $\underline{c}_2$ reduction.

DISCUSSION

The competition between Pi and several kinds of pH indicator in the ATP-Pi exchange reaction in the dark with chromatophores (Fig. 7) indicates that the Pi and dyes competed to each other for the active center of the enzyme catalyzing the reaction. The chemical structures of these pH indicators are significantly different from one another, suggesting that the dissociation-association of proton, which is characteristic of the pH indicator, is responsible for the competition with Pi. The reaction mechanism in the active center may be represented by equations 1 and 2.

$$ERH + POH \longrightarrow ER\sim P + H_2O \qquad 1)$$

$$ERH + I^- \longrightarrow ER^- + HI \qquad 2)$$

where ER^-, ERH, $ER\sim P$, I^-, HI and POH are active center, protonated active center, phosphorylated active center, pH indicator in the dissociated form, pH indicator in the associated form and orthophosphate, respectively. Some of the pH indicators having high pK_a values such as alizarin yellow G (pK_a = 10.9) were also competitive inhibitors against Pi. A possible explanation for this may be that such pH indicators in the dissociated form present in a trace at pH 8.0, at which the reaction was carried out, were functional as described above.

It was previously reported by Yamamoto et al. (26) that when chromatophores are illuminated in the presence of Pi without addition of ADP, the ADP tightly bound with chromatophores is phosphorylated

to bound ATP, and when ADP is added, the bound ATP thus formed transphosphorylates the added ADP. They provided also evidences showing that the transphosphorylation is catalyzed by the NTP-NDP kinase (nucleosidetriphosphate:nucleosidediphosphate phosphotransferase) bound with chromatophores. In addition, Yamamoto et al. (27) highly purified the enzyme, and proved that the phosphoryl enzyme is formed as a reaction intermediate and hydrolyzed at rate as slow as the half life time is 6 min at 20°.

The mechanism for the formation of ER~P from ER^- and ATP (ADP-O-P) and that of the dissociation of ERH may be represented by equations 3 and 1, and by equation 4, respectively. ATP-ADP exchange

$$ER^- + ADP\text{-}O\text{-}P \rightleftharpoons ER\sim P + ADP\text{-}O^- \qquad 3)$$

$$ER\sim P + H_2O \rightleftharpoons ERH + POH \qquad 1)$$

$$ERH \longrightarrow ER^- + H^+ \qquad 4)$$

is brought about by equation 3, ATP-Pi exchange by equation 3 and 1, and ATP hydrolysis by equation 3, 1 and 4. These mechanisms suggest that in chromatophores, the inhibition of the ATP-Pi exchange by the pH indicators competitive against Pi was brought about by the stimulation of the ATP hydrolysis by them. It may be worth noticing that all these pH indicators inhibited the ATP-Pi exchange to higher extents than the ATP hydrolysis, and that the competition between Pi and pH indicators was obtained at such concentrations of the dyes that the ATP-Pi exchange, but not the ATP hydrolysis, would be inhibited. Recently, Yoshimura et al. (unpublished data) found, using chromatophores, that the ATP-Pi exchange is stimulated

by adding ADP, and that it does not proceed when all the ADP present, either bound or free, has been converted into ATP by the system of phosphoenol pyruvate and pyruvate kinase [EC 2.7.1.40]. All the pH indicators tested stimulated more or less the ATP hydrolysis, regardless of their pK_a values, acidic or alkaline (Fig. 10). There is a tendency suggesting that when the reaction carried out at pH 8.0, pH indicators having pK_a values significantly lower than 8 stimulated the ATP hydrolysis to higher extents than the other pH indicators. This indicates that the pH indicators in the dissociated form were functional in the stimulation as shown by equation 1. When bromthymol blue was bound with chromatophores, its pK_a value shifted from 7.3 to 8.8 (Fig. 11). In fact, it was found that the stimulation of the ATP hydrolysis is significantly increased with increasing (more alkaline) pH values of the reaction mixture (Fig. 12).

Earlier, it was reported that when quinones are extracted from chromatophores, the ATP-Pi exchange and the ATP hydrolysis are decreased, and that when ubiquinone-10 is readded, these activities are restored to the original levels (21). The activities with non-treated and readded chromatophores are influenced by oxidation-reduction potentials of the reaction medium, but not those with extracted chromatophores. This suggest that ubiquinone-10 is the coupling factor for the energy transduction. Kakuno et al. (4) reported, studying the electron transfer system, that two protons are incorporated from water to one molecule of bound ubiquinone-10

at the same time as the quinone molecule accepts two electrons from two atoms of the bound non-heme iron, and they are then liberated from the quinone molecule to the reaction medium at the same time as the quinone molecule gives two electrons to another pair of the non-heme iron atoms. This suggests that pH indicators are able to mediate the proton transport to and from the quinone, so that pH indicators stimulate the rate of the electron transfer. It was found that most of the pH indicators tested significantly stimulated the rate; the relative extents of stimulation by various pH indicators were remarkably different among the cytochrome $\underline{c}_2$ reduction by succinate, the cytochrome $\underline{c}_2$ reduction by NADH and ATP hydrolysis (Figs. 13 and 14). Together with the finding that the systems for the cytochrome $\underline{c}_2$ reduction by succinate and by NADH involve ubiquinone-10 (21), the present finding suggest that pH indicators were functional as proton mediators at the two different sites; one was the enzyme catalyzing the ATP hydrolysis (possibly, NTP-NDP kinase) and the other the ubiquinone-10. The reason why there are differences in the relative extents of stimulation by pH indicators between the cytochrome $\underline{c}_2$ reductions by succinate and by NADH is not known.

The reaction mechanism for coupling of the energy conservation system to the electron transport system is schematically shown in Fig. 15.

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TABLE I. Effect of pH indicators on activities for ATP formation in light and ATP-Pi exchange in dark. The experimental conditions were described in the text, except that the indicated amounts of various kinds of pH indicator were added. The pK_a values of pH indicators referred from the catalogue of BDH laboratory chemicals (15) were used, except for those listed in Table II. The activities for ATP formation in the light and ATP-Pi exchange in the dark in the absence of pH indicator were 4.8 moles of ATP formed/mole Bchl·min, and 0.55 mole of [^{32}P]Pi exchanged/mole Bchl·min, respectively. Abbreviation used; Bchl, bacteriochlorophyll.

		ATP formation (%)		ATP-Pi exchange (%)	
pH Indicator	pK_a	0.1 mM	1 mM	0.1 mM	1 mM
Metanil yellow	1.3	68	0	64	4
Tropaeolin 00	1.0 - 2.8(1.9)	68	3	61	0
Quinaldine red	1.4 - 3.2(2.3)	53	--	92	--
Methyl orange	2.8 - 4.6(3.7)	108	90	98	100
Bromophenol blue	3.0 - 4.6(3.8)	95	57	67	7
2,4-Dinitrophenol	3.9	87	36	71	26
Congo red	3.0 - 5.0(4.0)	55	4	31	0
Ethyl orange	4.1	80	3	77	3
Bromocresol green	4.6	59	0	28	1
2,5-Dinitrophenol	4.0 - 5.6(4.8)	110	108	97	54
Gallein	3.4 - 6.8(5.1)	108	74	90	37
Methyl red	4.2 - 6.3(5.3)	110	--	--	--
Ethyl red	4.5 - 6.5(5.5)	98	--	70	--
Resazurin	5.6	13	7	101	48
Bromophenol red	5.2 - 6.8(6.0)	99	60	93	24
Bromocresol purple	5.2 - 6.8(6.0)	105	66	73	11
4-Nitrophenol	5.7 - 7.0(6.0)	84	25	77	12
Neutral red	6.7	51	47	71	57
Bromthymol blue	7.3	5	0	1	0
3-Nitrophenol	6.8 - 8.4(7.6)	99	19	88	16
Phenol red	6.8 - 8.4(7.6)	106	20	82	3
α-Naphtholphthalein	8.0	58	0	0	9
o-Cresolphthalein	9.6	60	0	29	0
Phenolphthalein	9.7	70	--	57	--
Thymolphthalein	9.3 -10.5(9.9)	106	88	103	92
Alizarin yellow G	10.9	90	6	47	0
Alizarin yellow GG	10.0 -12.0(11.0)	61	9	47	0
Tropaeolin 0	11.1 -12.7(11.9)	97	106	102	90
Tropaeolin 000	11.0 -13.0(12.0)	93	14	70	9
Alizarin	11.0 -13.0(12.0)	111	82	102	33
Titan yellow	12.0 -13.0(12.5)	94	62	89	38

TABLE II. Physical constants of pH indicators

pH Indicators	pK_a	λisobestic point		λmax			Molecular
		nm	(ε)	nm	(ε)	pH	weight
Metanil yellow	1.3	468	(20.0)	435	(23.7)	7.7	375
				525	(59.3)	0	
2,4-Dinitrophenol	3.9	323	(6.6)	360	(16.1)	7.7	184
Ethyl orange	4.1	512	(21.6)	474	(37.4)	6.0	359
Bromocresol green	4.6	510	(7.2)	617	(47.4)	6.8	698
Resazurin	5.6	538	(12.3)	600	(35.8)	8.0	229
Neutral red	6.7	475	(14.6)	452	(17.5)	12.6	288
				525	(37.7)	2.6	
Bromthymol blue	7.3	498	(6.0)	615	(42.5)	13	624
α-Naphtholphthalein	8.0			653	(22.0)	11	419
o-Cresolphthalein	9.6			566			346
Phenolphthalein	9.7			552			318
Alizarin yellow G	10.9	410	(10.3)	372	(15.1)	9.8	287
				478	(18.2)	13	

TABLE III. Stimulatory effect of pH indicators on activity for ATP hydrolysis in dark with lyophilized, with extracted and with reconstructed chromatophores. The preparation of various kinds of chromatophores and the method of activity assay for ATP hydrolysis were described in the text. The concentrations of pH indicators were 1 mM. The activities for ATP formation in the light with lyophilized, with extracted and with reconstructed chromatophores were 0.73, 0.04 and 0.51 mole of ATP formed/mole Bchl·min, respectively.

	ATP hydrolysis (mole of ATP hydrolyzed/mole Bchl·min)		
pH Indicator	Lyophilized chromatophores	Extracted chromatophores	Reconstructed chromatophores
No addition	0.025	0.011	0.021
Metanil yellow	0.074	0.092	0.064
2,4-Dinitrophenol	0.054	0.052	0.057
Ethyl orange	0.27	0.30	0.22

TABLE IV. Amounts of free and chromatophore-bound bromthymol blue in dissociated form present in mixture with chromatophores. The amounts were calculated on the basis of Fig. 11.

pH	Amount of bound dye per total amount of added dye (%)	Amount of free dye in dissociated form per tota amount of added dye (%)	Amount of bound dye in dissociated form per total amount of added dye (%)
8.0	35	58	3.2*
8.5	20	77	6.4
9.0	12	87	6.6
9.5	9	91	7.1

* This valu is not so precise as the others.

Fig. 1. Effect of buffer concentrations on light-induced absorbance change of bromthymol blue with chromatophores. The reaction mixture comprised 67 m$\underline{M}$ ascorbate, 6.7 m$\underline{M}$ $MgCl_2$, 3.3% sucrose, chromatophore suspension (A_{873nm} = 5 in final concentration), indicated concentrations of glycylglycine-NaOH buffer (pH 8.0), and water to make the total volume 3.0 ml. Absorbance at 615 nm was measured at room temperature (24° C) with a Cary model 17 spectrophotometer with devices which enabled us to illuminate the sample cuvette crosswise with the measuring and the actinic light beams; the actinic light illuminated the whole sample solution uniformly. The actinic 880 nm light was obtained by means of an interference filter (the half bandwidth = 10 nm). Cuvettes used for cross illumination were made of quartz, being transparent at four sides (1 x 1 x 4 cm). The reaction was started by illuminating the actinic 880 nm light of 1 x 10^3 erg/sec·cm^2. —○—, with 50 μ$\underline{M}$ bromthymol blue; —□—, without bromthymol blue.

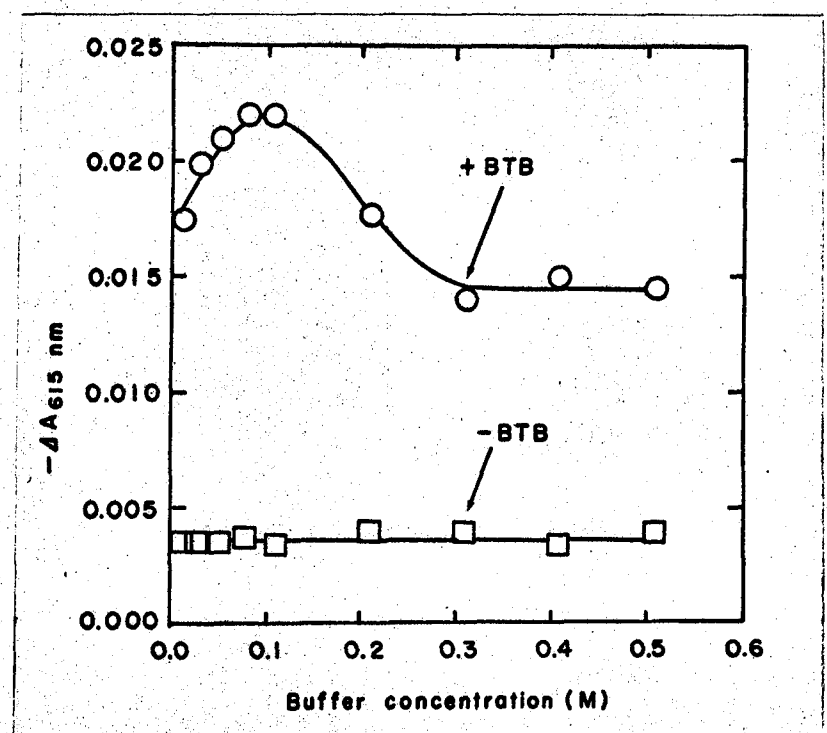

Fig. 2. Titration curves for pH indicators. Titrations were performed in 90 mM GTA buffer. The other experimental conditions and the methods for calculation were described in the text. 1, metanil yellow; 2, 2,4-dinitrophenol, 3, ethyl orange; 4, bromocresol green, 5, resazurin, 6, neutral red; 7, bromthymol blue; 8, α-naphtholphthalein; 9, o-cresolphthalein; 10, phenolphthalein, 11, alizarin yellow G.

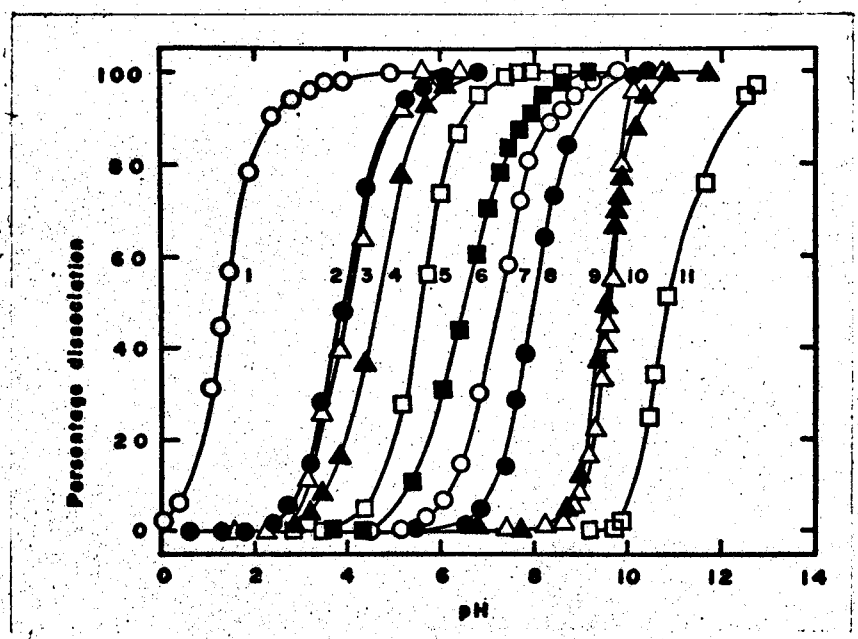

Fig. 3. Effect of concentrations of pH indicators on activity for ATP formation in light. The experimental conditions were described in the text, except that the indicated amounts of pH indicators were added. A, pH indicators having low pK_a values; B, pH indicators having high pK_a values.

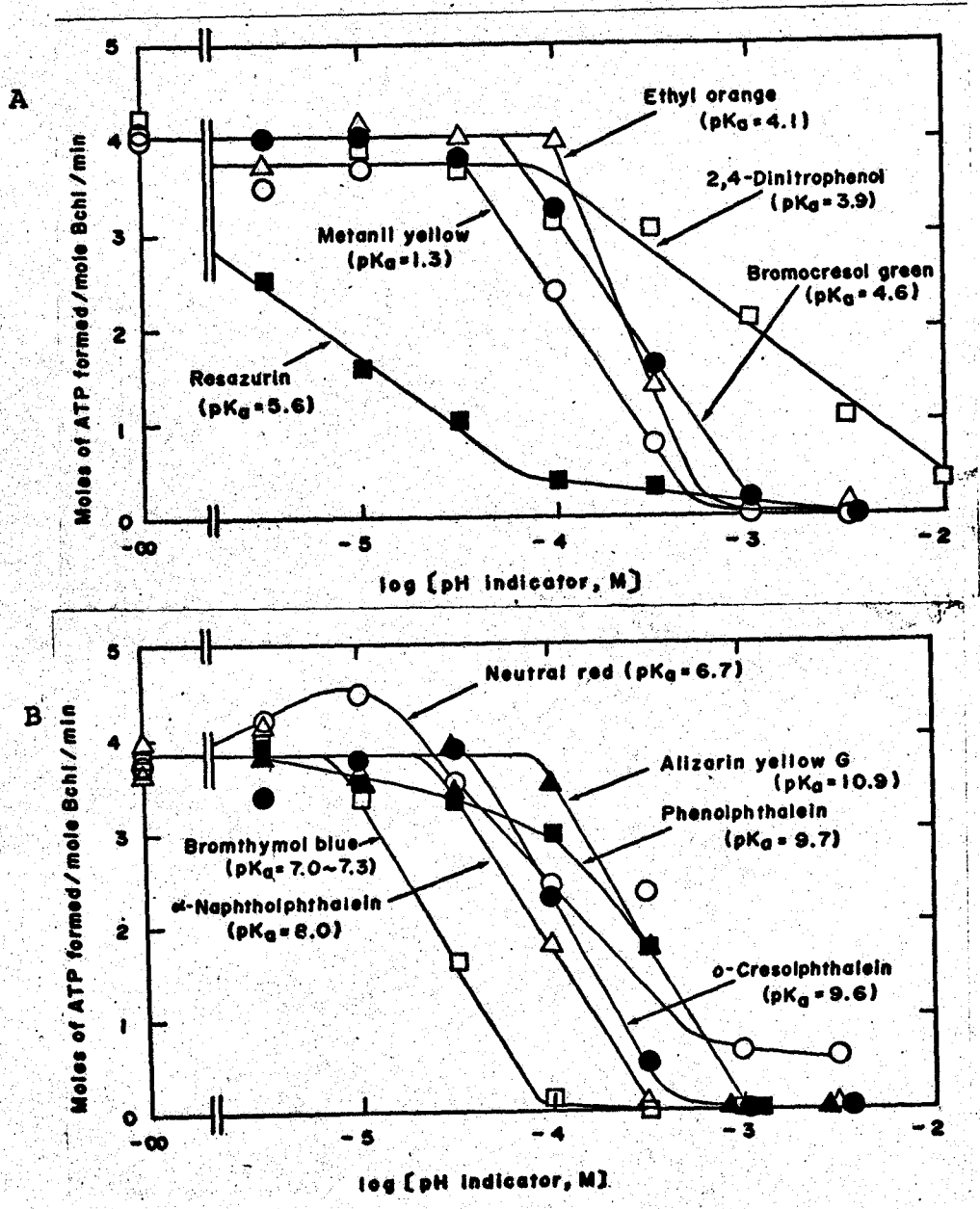

Fig. 4. Effect of concentrations of pH indicators on activity for ATP-Pi exchange in dark. The experimental conditions were descibed in the text, except that indicated amounts of pH indicators were added. A, pH indicators having low pK_a; B, pH indicators having high pK_a values.

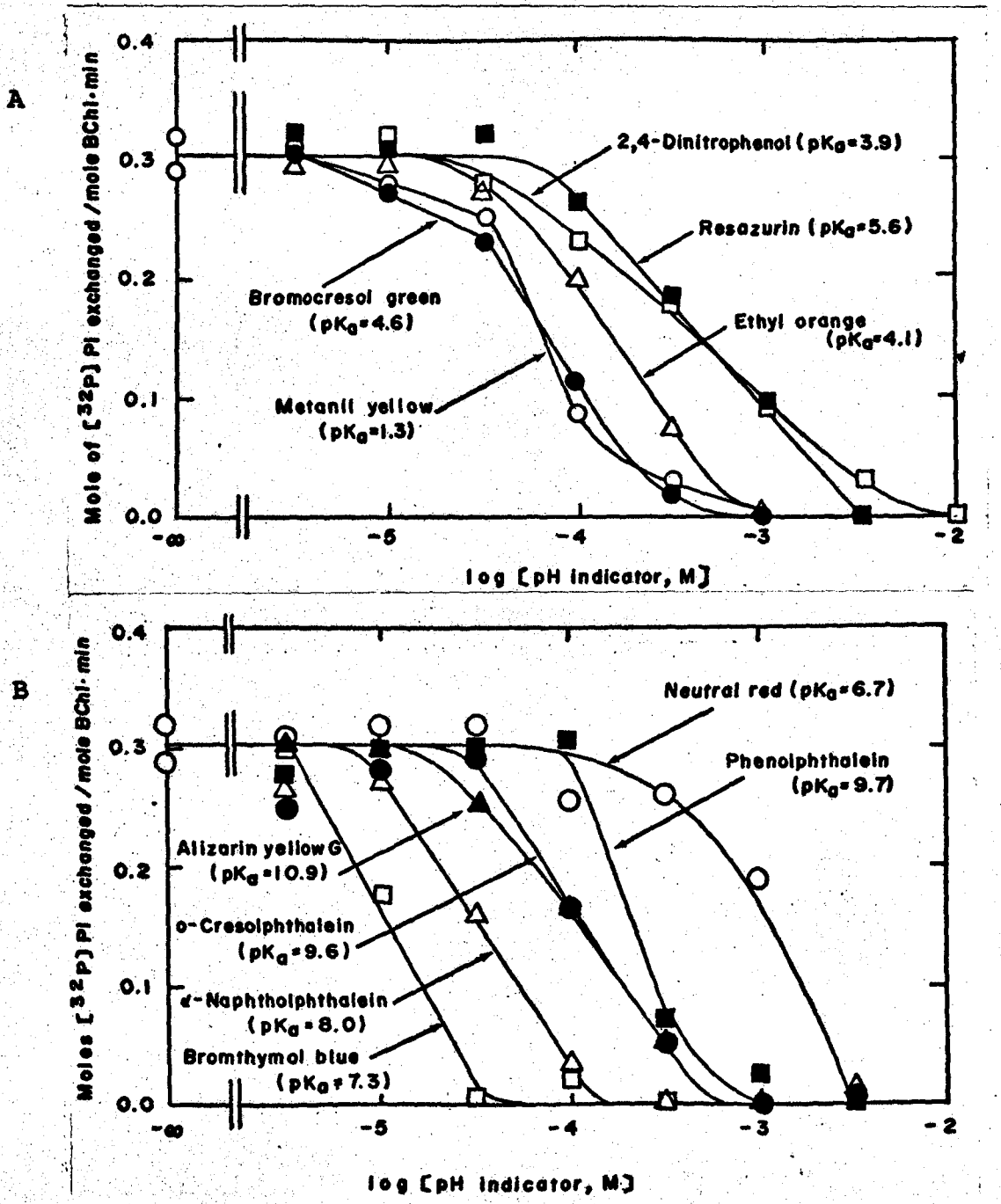

Fig. 5. Effect of concentrations of pH indicators on their amounts bound with chromatophores. The experimental conditions were descibed in the text. A, pH indicators having low pK_a values; B, pH indicators having high pK_a values; C, pH indicators slightly water-soluble.

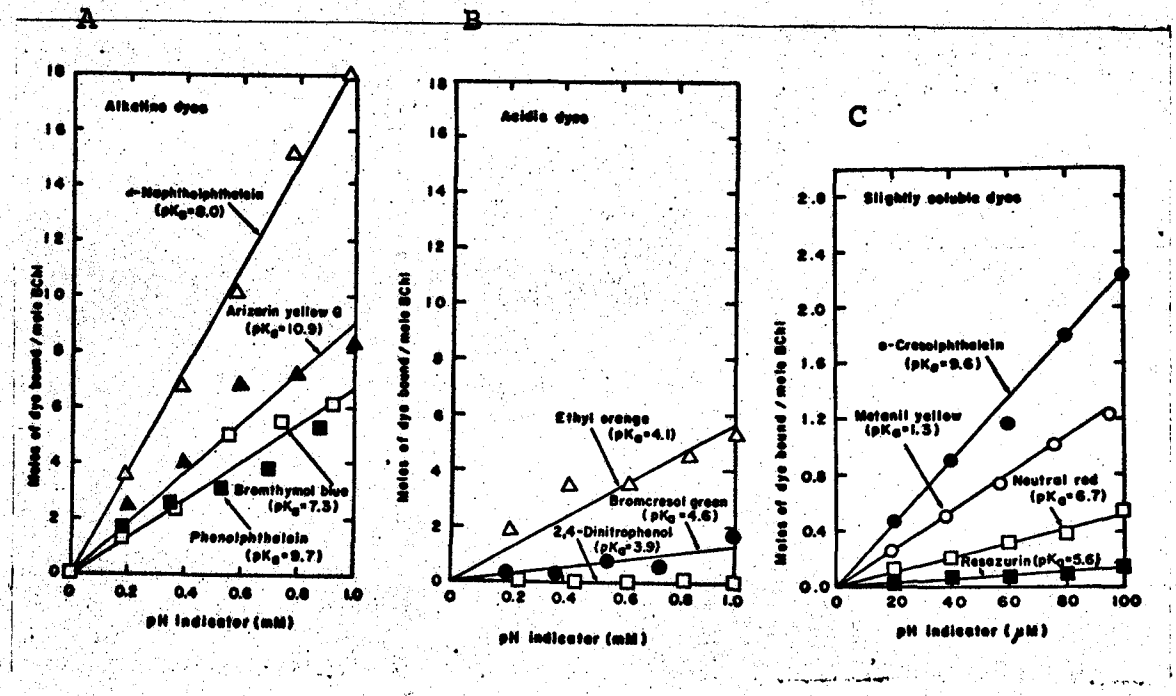

Fig. 6. Effect of Pi concentrations on activity for ATP formation in light in presence and absence of various pH indicators. The experimental conditions were described in the text, except that the concentration of [^{32}P]Pi was varied in the presence of 6.7 mM ADP. Metanil yellow, 0.1 mM; 2,4-dinitrophenol, 1 mM; ethyl orange, 0.2 mM; bromocresol green, 0.2 mM; resazurin, 10 μM; neutral red, 0.1 mM; bromthymol blue, 30 μM; α-naphtholphthalein, 0.1 mM; o-cresolphthalein, 0.1 mM; phenolphthalein, 0.3 mM; alizarin yellow G, 0.3 mM. A, pH indicators having low pK_a values; B, pH indicators having high pK_a values.

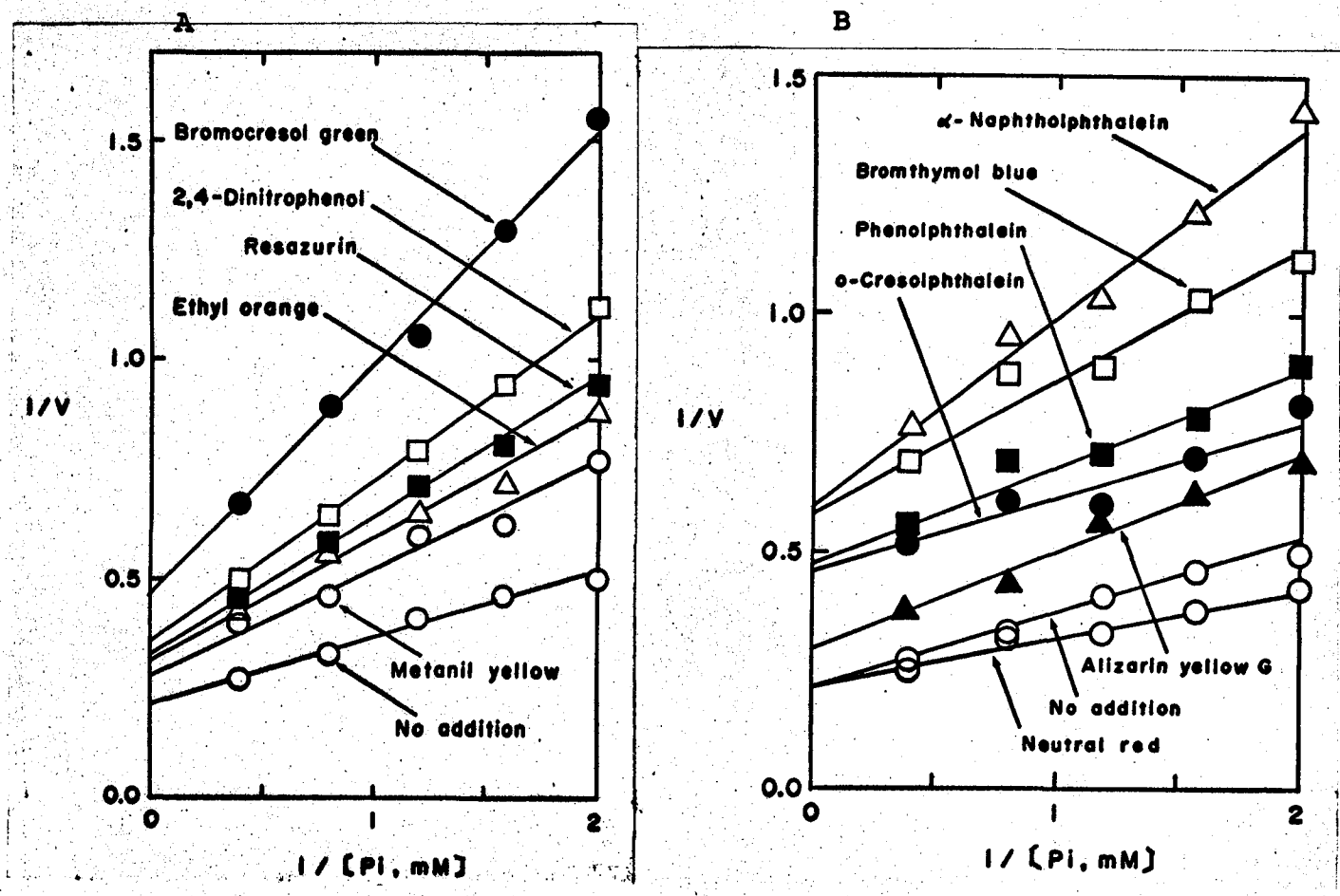

Fig. 7. Effect of Pi concentrations on activity for ATP-Pi exchange in dark in presence and absence of various pH indicators. The experimental conditions were described in the text, except that the concentrations of [^{32}P]Pi were varied in the presence of 3.3 mM ATP and that various pH indicators were added. 1, no addition; 2, 0.3 mM neutral red; 3, 0.3 mM resazurin; 4, 0.1 mM ethyl orange; 5, 0.3 mM 2,4-dinitrophenol; 6, 0.1 mM metanil yellow; 7, 50 μM bromocresol green; 8, 10 μM bromthymol blue; 9, 0.1 mM alizarin yellow G. The Km value for Pi in the absence of pH indicator was approximatelt 0.9 mM, practically the same as that in ATP formation in the light (18).

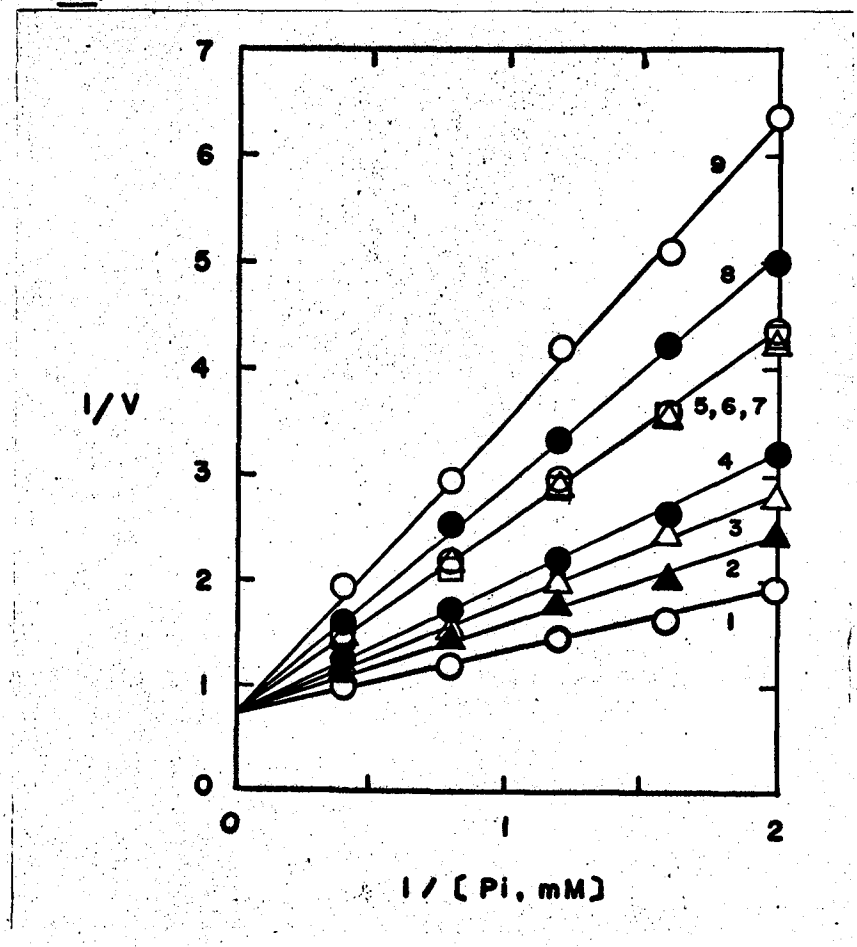

Fig. 8. Effect of ATP concentrations on activity for ATP-Pi exchange in dark in presence and absence of various pH indicators. The experimental conditions were the same as those for Fig. 7, except that the concentrations of ATP were varied in the presence of 6.7 mM [^{32}P]Pi. 2,4-Dinitrophenol, 0.3 mM; ethyl orange, 50 μM; bromocresol green, 50 μM; resazurin, 0.3 mM; neutral red, 0.3 mM; bromthymol blue, 10 μM; α-naphtholphthalein, 50 μM; o-cresolphthalein, 0.1 mM; phenolphthalein, 0.3 mM; alizarin yellow G, 0.1 mM. Except for the case in the presence of o-cresolphthalein, the Km values were approximately 0.3 mM, regardless of the presence and absence of pH indicator. A, pH indicators having low pK_a values; B, pH indicators having high pK_a values.

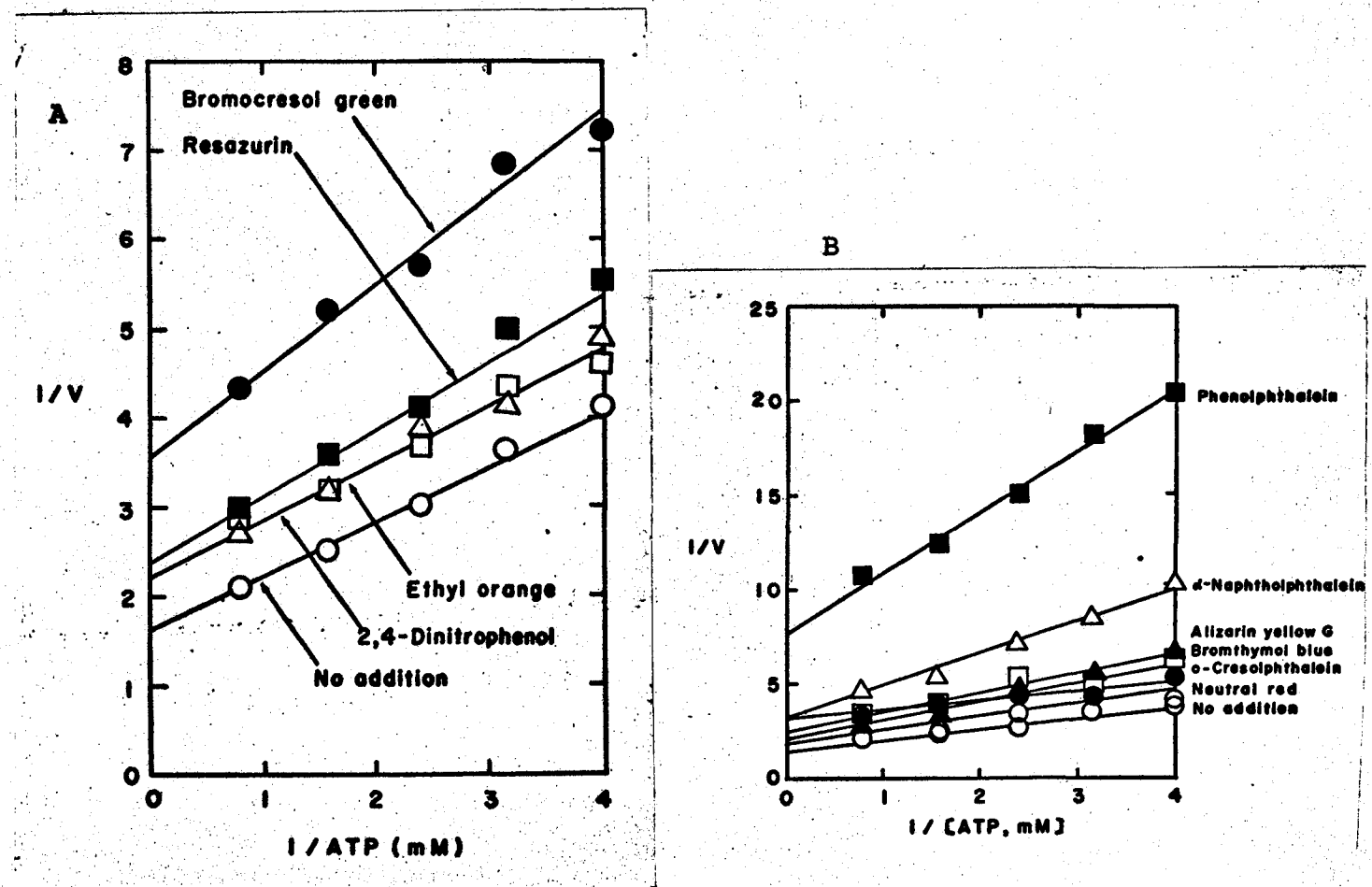

Fig. 9. Effect of concentrations of pH indicators on activity for ATP hydrolysis in dark. The experimental conditions were described in the text, except that indicated concentrations of various pH indicators were added. A, pH indicators having low pK_a values; B, pH indicators having high pK_a values.

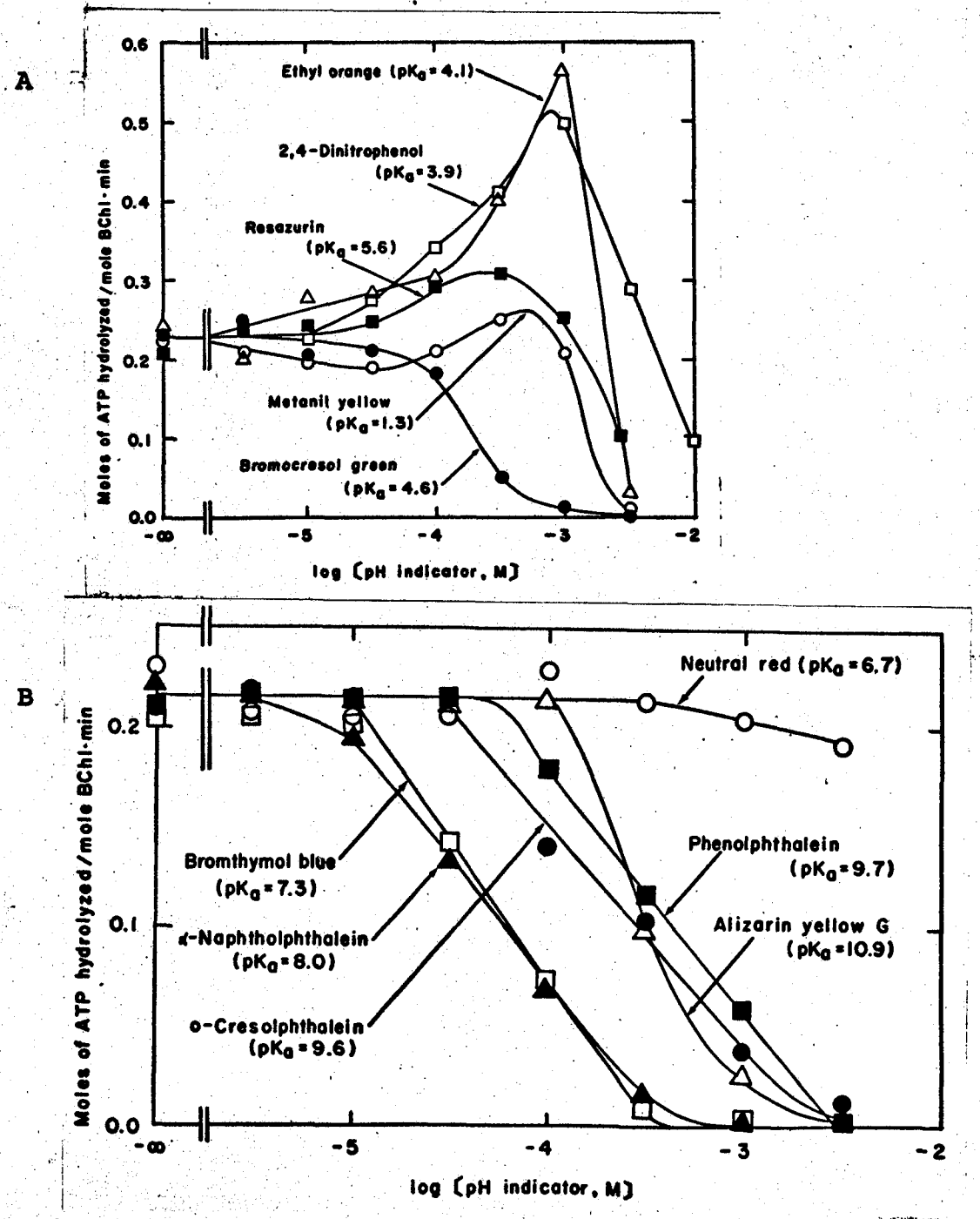

Fig. 10. Relation between activities for ATP hydrolysis stimulated by pH indicators and their pK_a values. The activities for ATP hydrolysis in the dark which were stimulated by various pH indicators, from the data of Fig. 9, were plotted as a function of their pK_a values, as described in the text. 1, metanil yellow; 2, 2,4-dinitrophenol; 3, ethyl orange; 4, bromocresol green; 5, resazurin; 6, neutral red; 7, bromthymol blue; 8, α-naphtholphthalein; 9, o-cresolphthalein; 10, phenolphthalein; 11, alizarin yellow G.

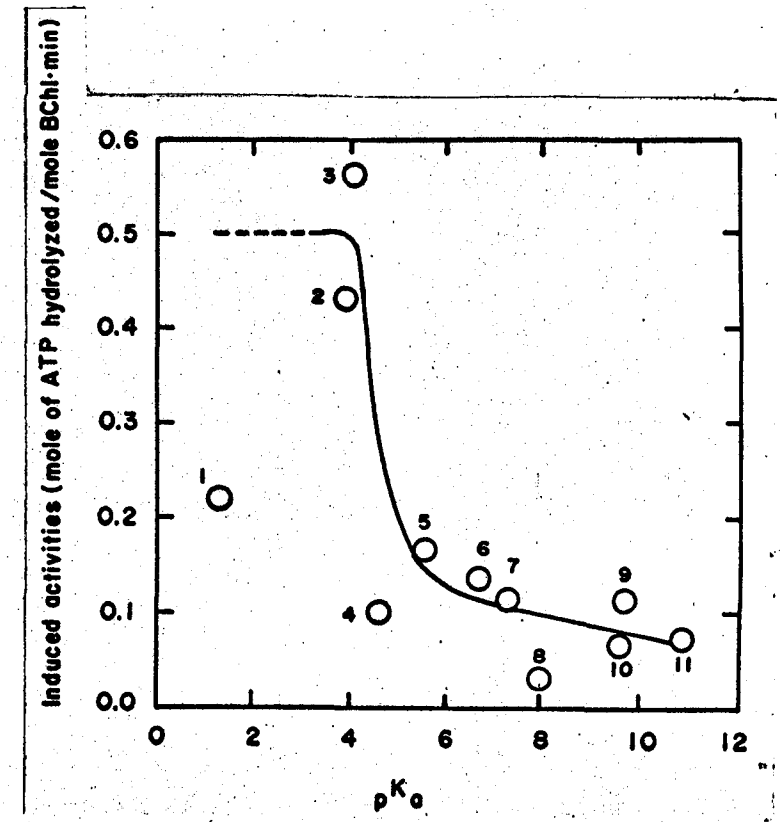

Fig. 11. Values for pK_a of bromthymol blue in free and chromatophore-bound forms. Titration of bromthymol blue was performed in GTA buffer of various pH values in the presence and absence of chromatophores. The amounts of the dye bound with chromatophores were estimated as described in the text. The calculation method for the pK_a value of the dye bound with chromatophores was described in the text.

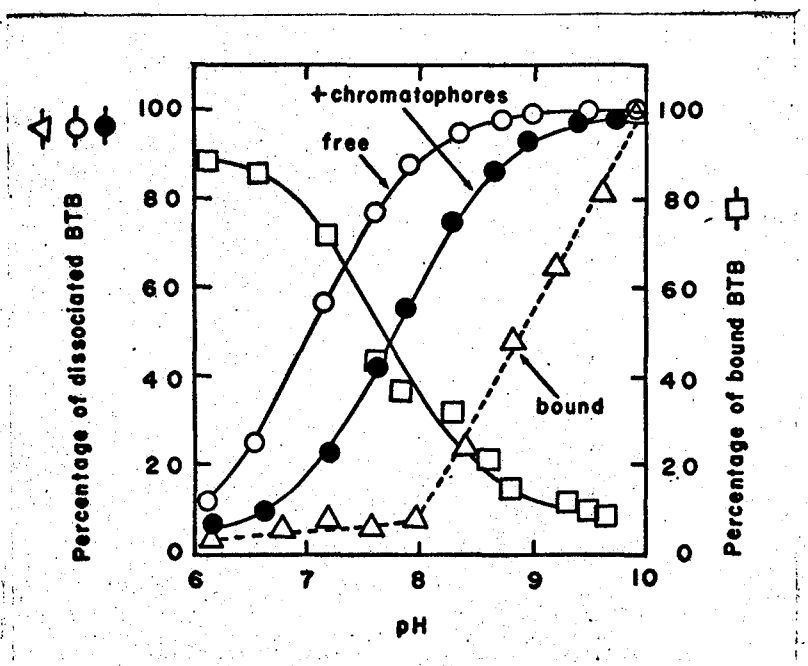

Fig. 12. Effect of bromthymol blue on activity for ATP hydrolysis at various pH values. The activity for ATP hydrolysis in the dark was measured as described in the text, except that 0.6 M GTA buffers containing 10% sucrose (pH 8.0, 8.5, 9.0 and 9.5) were used instead of glycylglycine-NaOH buffer, and that bromthymol blue was added as indicated. The method for plotting was described in the text.

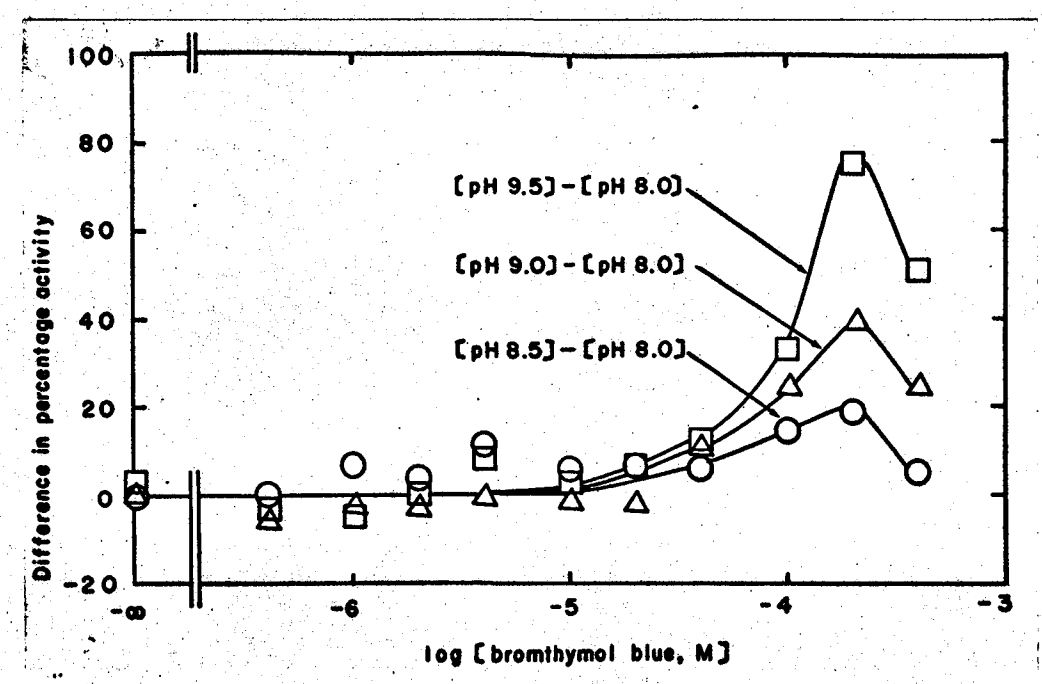

Fig. 13. Effect of concentrations of pH indicators on activity for succinate-cytochrome c_2 reduction. The experimental conditions were described in the text, except that indicated concentrations of pH indicators were added. The rate for succinate-cytochrome c_2 reduction in the absence of pH indicator was 0.61 mole of cytochrome c_2 reduced/mole Bchl·min. A, pH indicators having low pK_a values; B, pH indicators having high pK_a values.

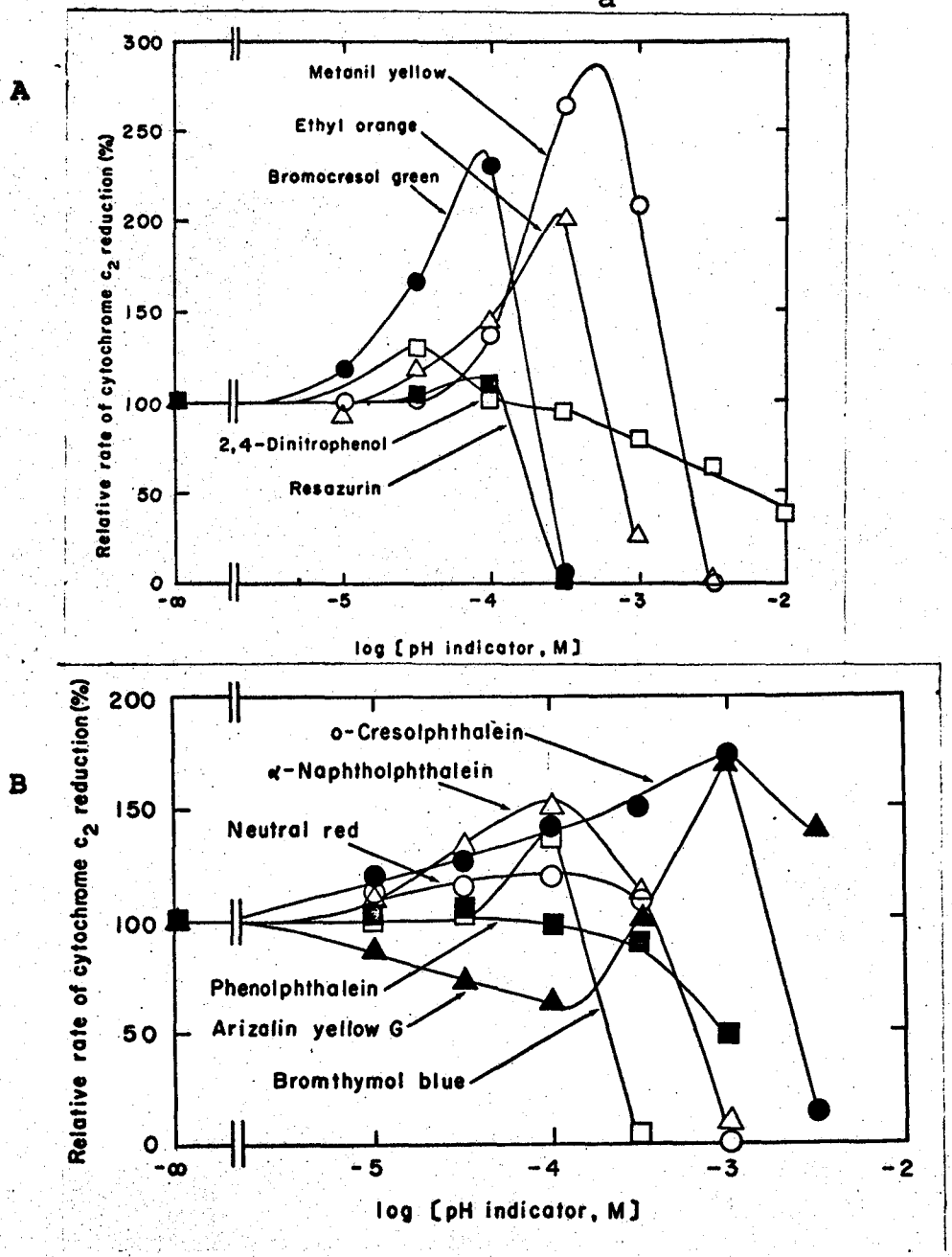

Fig. 14. Effect of concentrations of pH indicators on activity for NADH-cytochrome c_2 reduction. The experimental conditions were described in the text, except that indicated concentrations of pH indicators were added. The activity for NADH-cytochrome c_2 reduction in the absence of pH indicator was 1.5 mole of cytochrome c_2 reduced/mole Bchl·min. A, pH indicators having low pK_a values; B, pH indicators having high pK_a values.

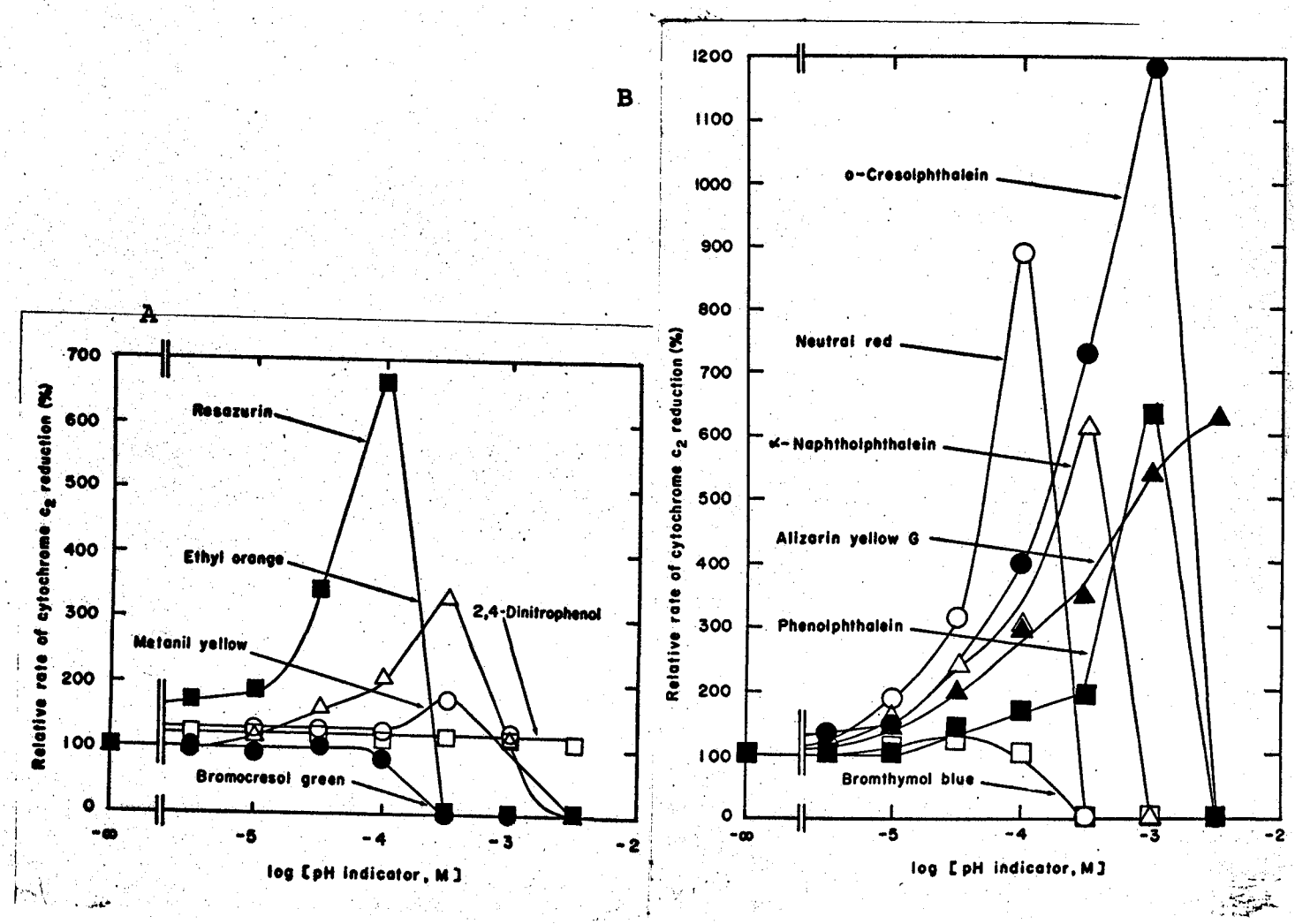

Fig. 15. Schematic mechanism for coupling of electron transport with energization of NTP-NDP kinase leading to ATP formation in chromatophore membrane. UQ, ubiquinone-10; Non-heme, non-heme iron protein; Cyt.c_2, cytochrome c_2; Liac, component responsible for light-induced abosorbance change (28); Bchl, antennal bacteriochlorophyll.