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Suitable Indicators and an Altered Empirical Equation for Calculating the CO_2 Concentration in Colorimetric Determinations of Photosynthetic Rate

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Abstract. Cresol red was found to be the most suitable of the six colour pH indicators tested for the colorimetry of CO_2 concentration within the range of 100 to 2000 p.p.m. In the region near pH 8·2 (corresponding approximately to 300 p.p.m. CO_2) this indicator is most suitable in view of its low unit concentration, although somewhat greater differences in absorbancy can be found on changing pH by one unit if an approximately six-fold higher concentration of thymol blue is used. For higher concentrations of CO_2 (pH below 7·5) phenol red is most appropriate. Tropaeolin 000 and aurin (rosolic acid) are not suitable. It is more convenient in spectrophotometry and colorimetry using filters with narrow spectral ranges to apply any of the above indicators in the green-yellow-orange spectral region rather than in blue radiation.

When the air to be measured is bubbled through a bicarbonate solution, an equilibrium between the CO_2 concentration in the air and pH value is established in less than 10 min for differences in CO_2 concentration from 100 to 400 p.p.m. Partial pressure of CO_2 and the experimentally established pH value are related by the altered equation: $pH = a - 0{\cdot}934 \log P$. The values of a at different temperatures were determined.

The limiting factor of accuracy of the colorimetric estimation of CO_2 in air lies in the precision of determining the pH (ČATSKÝ and SLAVÍK 1958, SLAVÍK and ČATSKÝ 1965). This precision is particularly important for the colorimetric determination of photosynthetic rate where the maximum of a 10 to 20% difference between CO_2 concentrations in control air and in air after passage through a leaf chamber with the assimilating object is measured. Even if it is not attempted in colorimetry to achieve the same accuracy as that afforded by modern physical methods (e.g. infra-red analyzers), it is necessary to check the principles of measurement, to calibrate the method most carefully and to evaluate the results most cautiously, in order not to add further sources of error to those inherent in all gazometric methods (errors in estimating air flow, leaf area, effect of chamber microclimate etc.).

The fundamental prerequisite of exact pH measurement by visual and especially by objective colorimetry is the selection of the colour indicator.

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A suitable indicator of pH change is characterized by (1) sensitivity, i.e. a small change of pH brings about a considerable change in absorbancy in the desired widest range, (2) inertness; hence it does not change the pH and composition of the solution, (3) stability. For objective colorimetry and spectrophotometry it must be determined at which wavelengths the absorption maxima of the indicator are located, how pronounced they are and whether their position changes with the change of pH.

The accuracy of the determination of changes in CO_2 concentration in the air then depends on the accuracy of converting the observed pH to CO_2 concentration. For this purpose LANGE (1956) uses an equation which was experimentally arrived at with the aid of two calibration gases for a solution of 0·001N-$NaHCO_3$ + 0·099N-KCl without any admixture of a colour indicator, viz.

$$pH = a - 1{\cdot}02 \log P \qquad [1]$$

where P is the partial pressure of CO_2 in the assayed air and a is a temperature-dependent coefficient. LIETH (1958) and ČATSKÝ (1960) checked the applicability and accuracy of the colorimetric method (using LANGE's equation) by an infrared analyzer and observed some deviation of data, in particular of the marginal values of the range used (0·02—0·1 vol.% CO_2).

In this paper it is intended to determine the characteristics of applicable colour pH indicators, to achieve the highest possible accuracy in calculating CO_2 concentration from measured pH values of the bicarbonate solution employed and to find further data on the applicability of the colorimetric method of CO_2 determination, in particular of the response time and stability of the measuring solution.

Methods

Reagents were all from Lachema, Brno, Czechoslovakia, except for tropaeolin 000 which was from Gurr, London, Great Britain.

Stock solutions of colour pH indicators were prepared by dissolving 100 mg of each indicator (cresol red, phenol red, thymol blue, bromothymol blue, aurin [rosolic acid] and tropaeolin 000) in 96% ethanol and the volume made up to 100 ml. The indicators were not neutralized.

Bicarbonate stock solution: 840·1 mg $NaHCO_3$ + 73·807 g KCl ad 1000 ml water; normality of the solution was checked by titrating with hydrochloric acid using methyl yellow.

Bicarbonate measuring solution: 100 ml (time factor) of the stock solution + 10 ml indicator mixture (100 mg cresol red + 200 mg thymol blue in 100 ml 96% ethanol) made up to 1 litre with water.

Tetraborate-boric acid buffers according to ZELLER (1951) were used as the basis for measurement. Their pH was checked on a pH-meter. A mixture of N_2 + CO_2 served as a gas of known composition for calibrating the infrared analyzers from The Infra Red Development Co., Ltd. (Welwyn Garden City, Great Britain).

Apparatuses: Recording spectrophotometers Unicam SP. 700 (Cambridge, Great Britain) and Beckman DB (Fullerton, Calif., U.S.A. and Munich, F.R.G.), Pulfrich's photometer with an Elpho photoelectric attachment

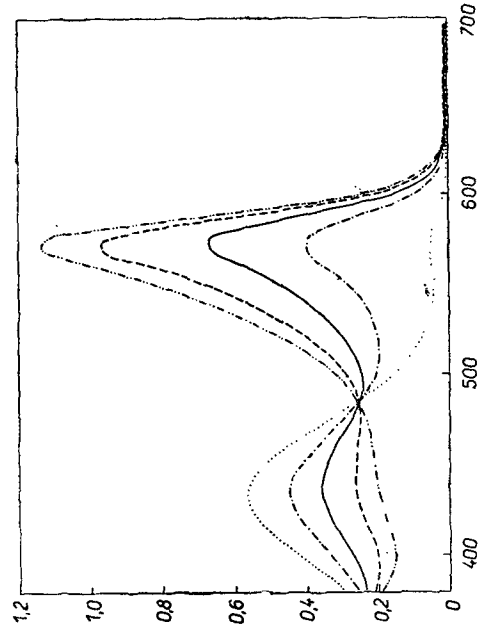

Fig. 1

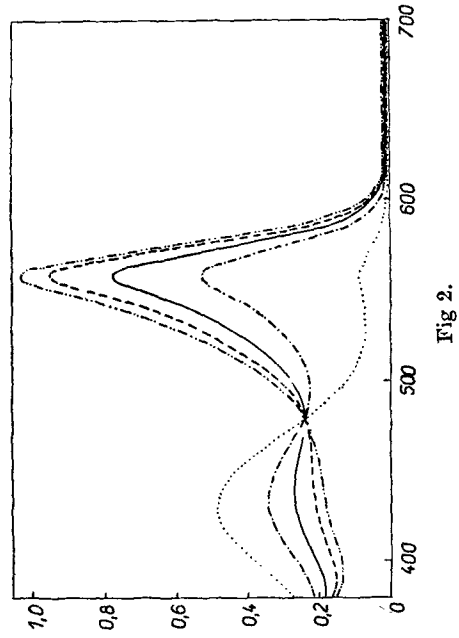

Fig 2.

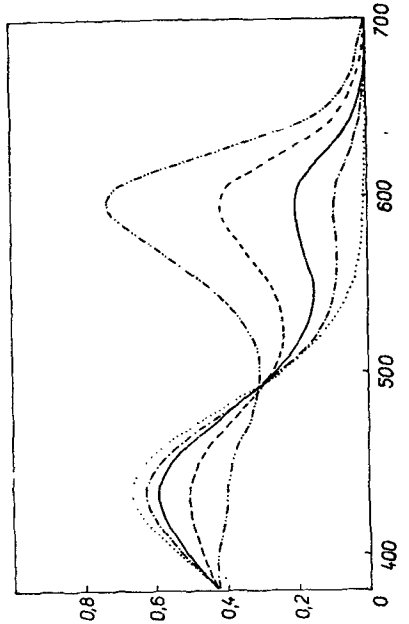

Fig. 3

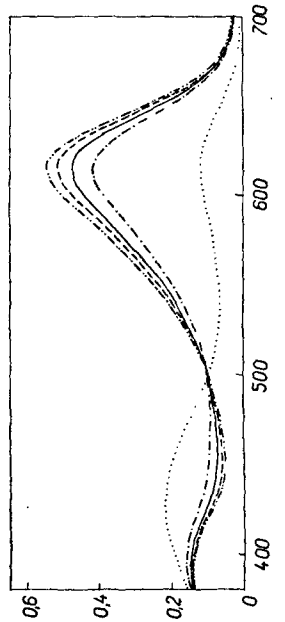

Fig. 4

(Zeiss, Jena, G.D.R.). Cuvettes with 10 mm optical path were used throughout, set against a pure solvent.

pH-Meters PHM 3ik and PHM 4c of Radiometer (Copenhagen, Denmark) with glass electrodes G 100B and G 200B, calibrated with buffers by Cambridge Instrument Co. Ltd. (London).

Results and Discussion

A. Selection of pH Colour Indicator

1. Sensitivity of the Indicator

Spectra of four suitable indicators (of the six tested) are shown in Figs. 1—4 for the wavelength range from 380 to 700 nm, as measured in borate buffers of pH 6·52, 7·66, 8·05, 8·45 and 8·85. The chosen pH values correspond to the range of CO_2 concentrations in air, as they may occur during CO_2 exchange measurements. Spectra for aurin (rosolic acid) are not shown, as the colour of solution changes rather rapidly (absorbancy increased in its maximum by more than 10% within 10 min). Tropaeolin 000 is also of little value for objective colorimetry or spectrophotometry, as its absorbancy maximum shifts on increasing pH, from about 475 nm at pH 6·52 to about 510 nm at pH 8·85,

Table 1

Sensitivity of cresol red, phenol red, thymol blue and bromothymol blue in individual pH ranges. For indicator concentration equal to 10 mg/litre it is expressed by the difference in absorbancy (times 10) for limiting pH of the given range of green-yellow-orange and blue radiation. A Beckman DB spectrophotometer, cuvette path 10 mm.

Indicator	Wavelength of measurement, nm	pH range				
		6·52—7·66	7·66—8·05	8·05—8·45	8·45—8·85	6·52—8·85
Cresol red	572 433	+345 −120	+280 − 90	+295 − 95	+170 − 65	+1090 − 370
Phenol red	558 432	+440 −140	+250 − 75	+175 − 60	+ 80 − 30	+ 945 − 305
Thymol blue	595 434	+ 40 − 20	+ 55 − 20	+105 − 40	+160 − 55	+ 360 − 135
Bromo-thymol blue	614	+305	+ 60	+ 35	+ 30	+ 430

Figs. 1—4. Spectral curves of absorbancy (ordinate — A) in the wavelength range 380—700 nm (abscissa) obtained on a Beckman DB spectrophotometer (cuvette optical path 10mm) for indicator solutions at concentrations of 10 mg/litre (thymol blue at 20 mg/litre) in borate buffers of pH 6·52 (. . . .), 7·66 (– · – · –), 8·05 (———), 8·45 (– – – –) and 8·85 (– · · – · · –). Fig. 1, Cresol red; Fig. 2, phenol red; Fig. 3, thymol blue; Fig. 4, bromothymol blue.

the absorbancy itself decreasing somewhat at first and rising then at pH above 8·45.

The sensitivity of unit amounts of the individual indicators can be determined for the given pH ranges from the shape of absorbancy curves, as well as the suitability of the individual spectral regions for colorimetry. Differ-

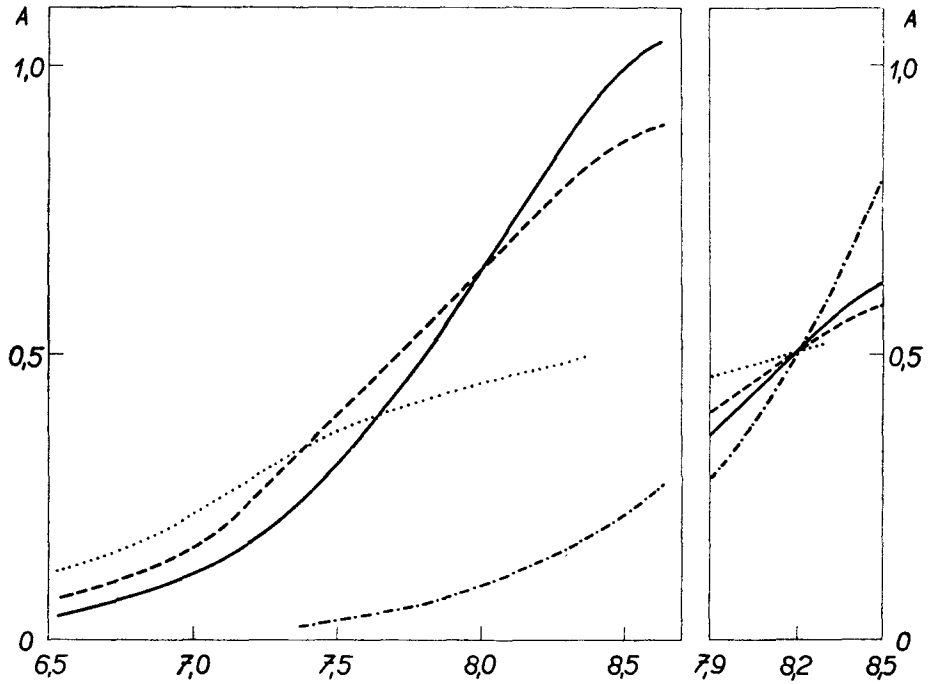

Fig. 5a. Dependence of absorbancy at the wavelength of the absorption maximum (ordinate) on the pH (abscissa) for unit concentration of the indicator (10 mg/litre) in borate buffers. Measured on a Beckman DB spectrophotometer in a 10 mm cuvette. ——— cresol red; – – – phenol red; – · – · – thymol blue; . . . . bromothymol blue.

Fig. 5b. Extrapolation of values from Fig. 5a to absorbancy at pH 8·2 = 0·5 for the pH range from 7·9 to 8·5.

ences in the blue part of the spectrum are less than in the green-yellow-orange one for all four indicators (Tab. 1). Less sharp maxima in the blue section could be suitable only for colorimetry using filters with a wide spectral transmittancy. In addition, the character of the absorbancy curve changes for all indicators at higher pH values, in particular for bromothymol blue.

It appears without doubt that the very universal cresol red is most suitable for all the pH ranges examined and it is most frequently used for measuring the intensity of CO_2 exchange in plants. Phenol red (Fig. 2) is most suitable for the range from pH 6·5 to 7·7 where bromothymol blue is also applicable (Fig. 4), but bromothymol blue is completely useless for higher pH. Thymol blue (shown in Fig. 3 at a two-fold higher concentration) produces relatively high differences for pH 8·45—8·85. The course of these changes is more ac-

curately determined for the same concentration of the indicator when measuring in the green-yellow-orange absorption maxima (Fig. 5a).

Heretofore, the reaction of a unit amount of indicator was considered. The absorption values and hence the size of their differences increase, however, proportionally to indicator concentration. If the indicator concentration is disregarded, the sensitivity of the reaction is determined by the relative changes in absorbancy per unit change of pH regardless of the absolute absorbancy value. Fig. 5b indicates changes in absorbancy within the pH range of 7·9—8·5 at such indicator concentrations as would produce absorbancies of 0·5 at pH 8·2, which is an important point for measuring photosynthesis at normal CO_2 concentration in air. Greatest differences and steepest increase of absorbancy in dependence on pH are then produced by thymol blue at a concentration of 37·3 mg/litre, i.e. almost six times higher than cresol red (6·28 mg/litre) or phenol red (6·67 mg/litre). Bromothymol blue is the least sensitive (10·56 mg/litre). This type of indicator selection is important for comparative measurements in colorimetry when the change of absorbancy is determined with respect to a pre-set standard value, or possibly to the amount of CO_2 in the control air sample. If the amount of indicator added is of no concern, thymol blue would be most suitable.

2. Inertness and Stability of Indicator

The stability of indicator colour was examined for 4 weeks. Buffers of two different pH values were stained with the four indicators studied, their absorbancy was determined on a Pulfrich photometer using a S 57 filter and their pH estimated on a pH-meter. The solutions were then divided into three parts each, one of them placed in daylight, the second in the dark at room temperature and the third in the dark at +2 to +3° C. The pH and absorbancy were checked after 6 and 28 days. The minute differences observed lay within the range of experimental error and thus the indicators can be considered as stable.

While individual colour indicators are more suitable for objective colorimetry and spectrophotometry (more pronounced maxima and greater absorbancy changes), a mixture emphasizing the change in colour is more suitable for visual colorimetry. It goes without saving that in a mixture the colour indicators used must not chemically interact. This may be determined by mixing equal amounts of solutions of the individual indicators: absorbancy of the resulting spectrum must correspond to one-half of the sum of absorbancies for individual solutions throughout. This is borne out for pH 7·55 and 8·25 in Fig. 6a, b for a mixture of cresol red and thymol blue suggested by Čatský and Slavík (1960).

B. Rate of Equilibration and Reversibility of the Measuring Solution

Colorimetry is particularly suitable for serial determinations of CO_2 concentration in the air. In such cases visual pH indication is usually employed, using an indicator mixture when particularly the colour transition is emphasized rather than changes in colour intensity for which the eye is less sensitive and where even small differences in layer thickness can play a role. For

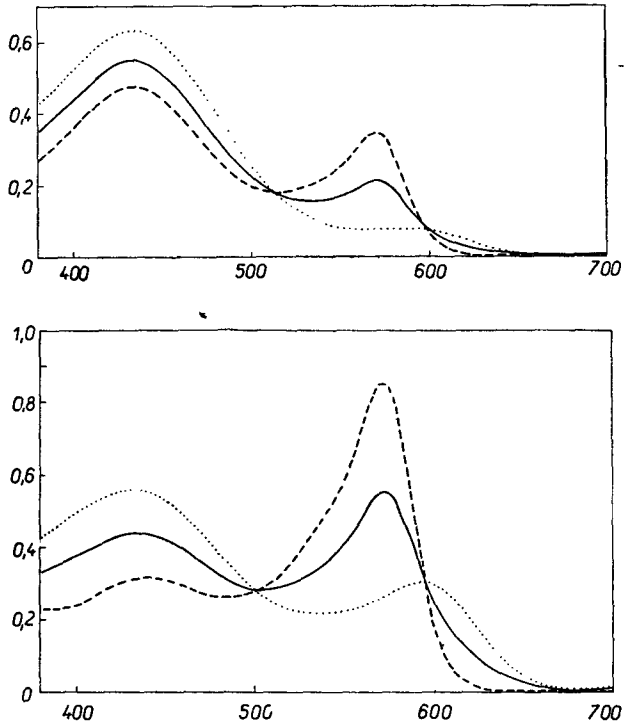

Fig. 6a, b. Spectral curves of absorbancy (ordinate) for solutions of cresol red at concentrations of 10 mg/litre (– – –) and thymol blue at 20 mg/litre (. . . . .) in borate buffers and for a solution obtained by their 1 : 1 mixing (———). Abscissa: wavelength in nm. Fig. 6a, pH 7·55; Fig. 6b, pH 8·25.

estimating the reversibility of the measuring solution and exactifying the conversion formula, a mixture of indicators (20 mg thymol blue and 10 mg cresol red in 1 litre bicarbonate solution) was therefore used. As was found earlier (ČATSKÝ 1960), the add tion of such a small amount of indicator (even if not neutralized) has a negligible effect on the pH of the bicarbonate solution. The established relationships can thus be considered valid even for a measuring solution containing a different (low) concentration of indicator.

When a gas mixture of known CO_2 concentration was bubbled through 15 ml measuring solution containing the indicator mixture (flow rate 5 litres/h), a pH-meter was used for determining the rate of reaching equilibrium (Fig. 7). It was found that equilibrium is reliably reached at differences of CO_2 concentrations smaller than 600—700 p.p.m. after 10 min (differences of only 100 p.p.m. are commonly encountered in estimating photosynthesis), even when the air flow is very slow. At great differences of CO_2 concentration and a low rate of flow equilibrium is attained within 15—20 min.

In another experiment we measured the attainment of equilibrium with a view to the reversibility of the reaction. In this experiment we selected a spectrophotometric determination at the wavelength of highest sensitivity of the used indicator mixture, viz. 575 nm. The air was bubbled through 5 m

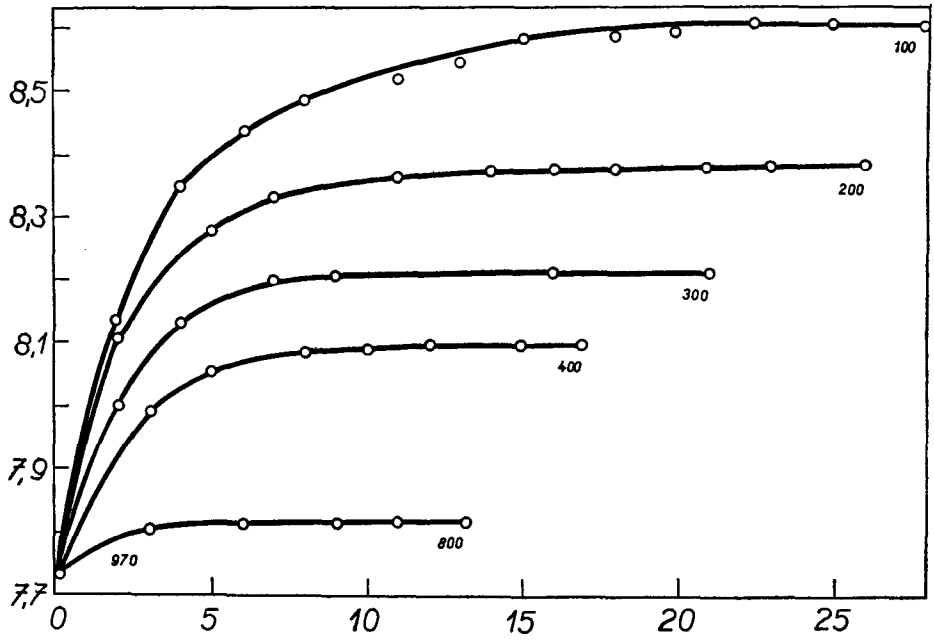

Fig. 7. Equilibration between CO_2 concentration in air (numbers at individual curves — p.p.m. CO_2) and the pH of bicarbonate solution (ordinate). Abscissa: time in min.

bicarbonate solution at a constant rate (2 litres/h) directly in a spectrophotometric cuvette at constant temperature (25° C). This determination also bore out the sufficient rate of reaching equilibrium even at great differences (400 p.p.m.) in CO_2 concentration in the passing air. The results (Fig. 8) point to an important source of error during spectrophotometric or objective colori-

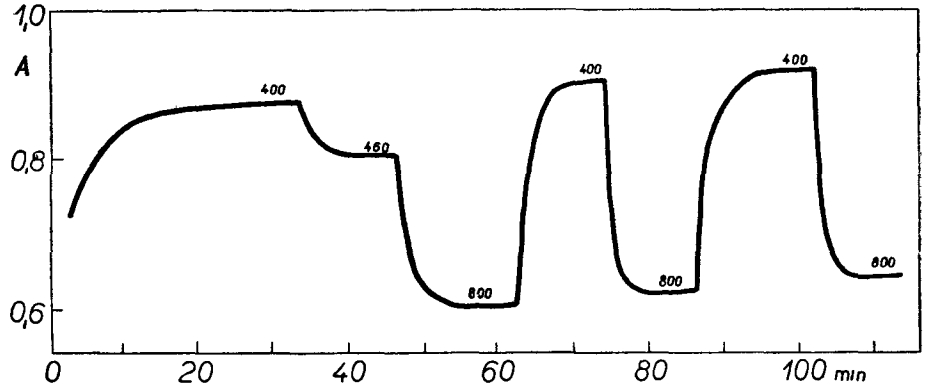

Fig. 8. Absorbancy of the bicarbonate solution with indicator (ordinate) during bubbling with air with different concentrations of CO_2 (numbers at the curve — p.p.m. CO_2). Abscissa: time in min.

metric estimation of CO_2 concentration, namely, evaporation of the measuring solution during bubbling with the air sample which alters the indicator concentration (and hence its absorption) as well as the normality of bicarbonate solution (and hence pH dependence on CO_2 concentration). In visual colorimetry the first-named error (more important) is negligible, since the eye is far more sensitive to differences in colour quality than in its intensity. KARPUSHKIN (1963, personal communication) recommends, therefore, to place a vessel with $KMnO_4$ solution in front of the measuring solution to saturate the analyzed air with water and to remove impurities which might change the solution colour after prolonged bubbling.

Sometimes the indicator (especially thymol blue) will concentrate in the foam in the upper part of the cuvette (ŠESTÁK 1961). This difficulty can be removed by adding a grain of paraffin to the solution (KARPUSHKIN 1963, personal communication). Both errors can be removed by renewing the solution after 1—3 h (for details see ŠESTÁK, ČATSKÝ et al. 1966).

C. Determination of pH Dependence of the Bicarbonate Solution on CO_2 Concentration at Different Temperatures

Fifteen ml bicarbonate solution with an admixture of indicator (20 mg thymol blue and 10 mg cresol red in 1 litre solution) were bubbled with a mixture of N_2 and CO_2 of known composition (Fig. 9) at constant temperature at a rate of 30 litres/h and the pH value estimated after reaching equilibrium at the atmospheric pressure of 760 torr. Results of measurements are shown in Figs. 10 and 11.

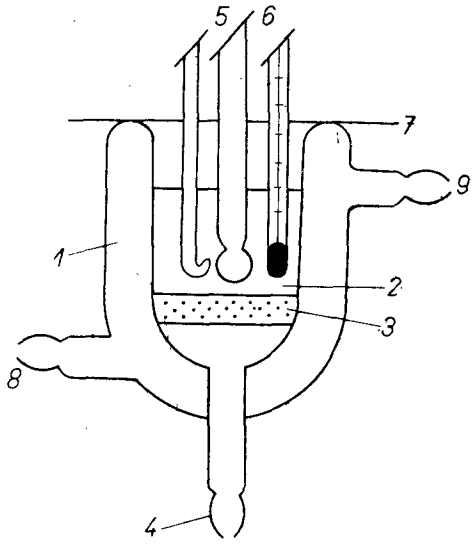

Fig. 9. Apparatus for measuring the pH of bicarbonate solution in equilibrium with passing air. 1 — glass vessel with a water jacket, 2 — bicarbonate solution, 3 — sinter, 4 — inlet of air, 5 — pH-meter electrodes, 6 — thermometer, 7 — polyethylene foil, 8, 9 — inflow and outflow of water at the ultrathermostat.

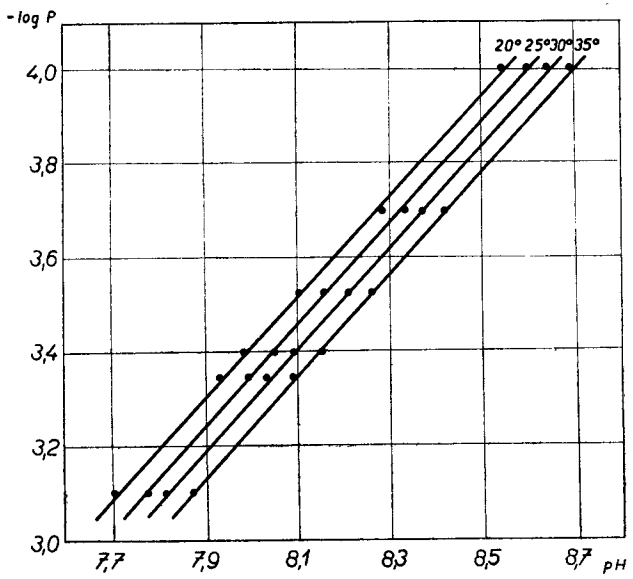

Fig. 10. Dependence of the pH value of bicarbonate solution with indicator (20 mg thymol blue and 10 mg cresol red in 1000 ml) on partial pressure of CO_2 in air with which the solution is in equilibrium, at different temperatures. Abscissa: pH, ordinate: $-\log P$.

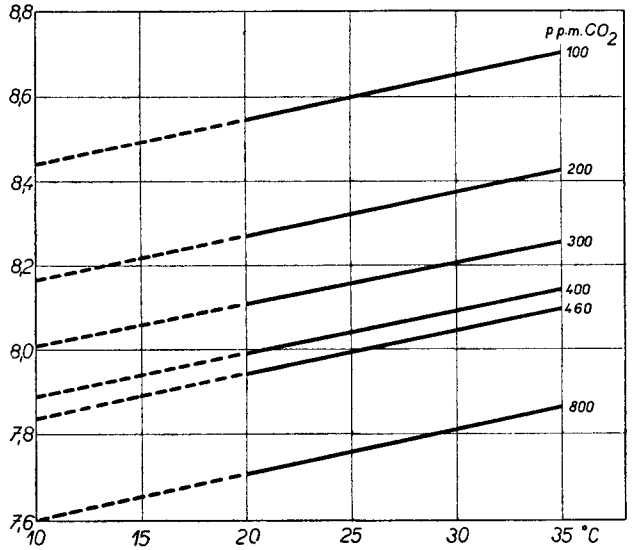

Fig. 11. Dependence of the pH value of the bicarbonate solution with indicator on temperature of the solution at different concentrations of CO_2 in air with which the solution was in equilibrium. Abscissa: temperature in °C, ordinate: pH.

The observed relationship makes possible a new equation and values of the coefficient a. (The procedure and graphical representation are identical with those in LANGE's paper (1956).

It holds generally that

$$n \log P = a - \mathrm{pH}\,, \qquad [2]$$

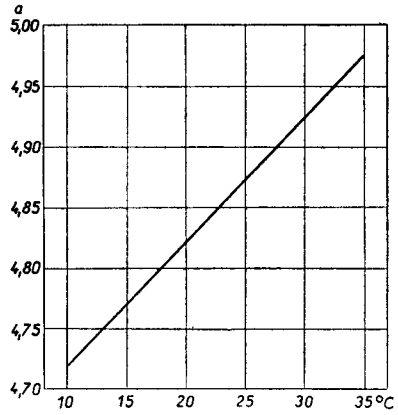

Fig. 12. Values of coefficient a at different temperatures. Abscissa: temperature in °C, ordinate: a.

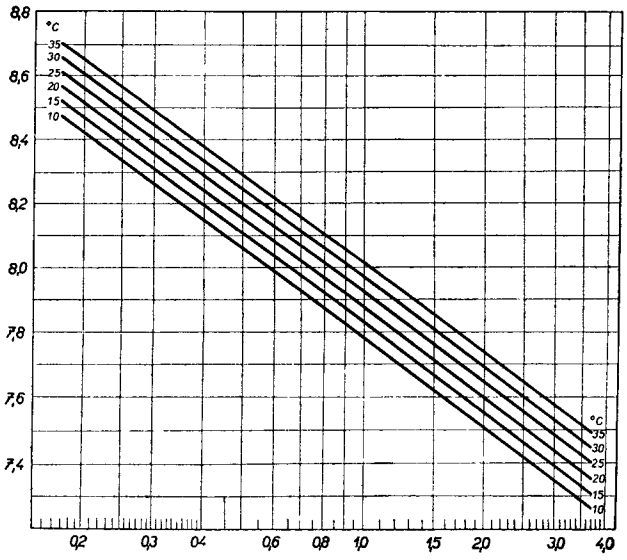

Fig. 13. Dependence of pH of the bicarbonate solution with indicator (ordinate) on CO_2 concentration in air (mg CO_2/litre — abscissa) with which the solution was in equilibrium, at different temperatures (numbers at the individual curves).

where P is the partial pressure of CO_2 in air with which the bicarbonate solution is in equilibrium, n is a factor and a a temperature-dependent coefficient. The factor n can be determined from the slope of experimentally determined curves in Fig. 10 as the reciprocal value of the tangent of the angle bound by the curve and the abscissa. The value of a and dependence on temperature are readily computed from Fig. 10 or 11 (cf. Fig. 12). In this manner the following equation was derived:

$$0{\cdot}934 \log P = a - \mathrm{pH} \qquad [3]$$

and hence

$$\mathrm{pH} = a - 0{\cdot}934 \log P \qquad [4]$$

From this equation the dependence of CO_2 concentration (in mg CO_2/litre air) on the pH of the bicarbonate solution was calculated for different temperatures (Fig. 13). For practical purposes a conversion table was constructed (ŠESTÁK, ČATSKÝ et al. 1966).

The absolute accuracy of the conversion equation (4) is determined by the absolute accuracy of the pH-meter and the standard mixture of nitrogen and carbon dioxide used. The absolute accuracy of modern pH-meters is usually 0·02 pH, even if the relative accuracy may be greater. This corresponds to an error of CO_2 determination amounting to c. $\pm$ 5%. The accuracy of composition of $N_2 + CO_2$ mixtures supplied by The Infra Red Development Co. is $\pm$ 5%. For this reason, 6 mixtures of different CO_2 concentrations were used for determining the new equation so as to reduce the second-named error to a minimum. On the other hand, however, it must be borne in mind that in measuring photosynthetic rate small differences in absolute values are negligible, since they compensate each other to a certain extent in calculating the differences in CO_2 concentrations in the air before and after passage through the leaf chamber. Even the differences in photosynthetic rate between that computed from Lange's equation and from ours amount only to several per cent as a rule, although in absolute terms the equations differ markedly.

Acknowledgement

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J. ČATSKÝ, Z. ŠESTÁK, Ústav experimentální botaniky ČSAV, Praha: **Vhodné indikátory a upravená empirická rovnice pro výpočet koncentrace CO_2 při kolorimetrickém stanovení intenzity fotosyntézy.** — Biol. Plant. **8** : 60—72, 1966.

Ze šesti vyzkoušených barevných indikátorů pH je pro kolorimetrické měření koncentrace CO_2 v rozmezí 100 až 2000 p.p.m. nejvhodnější kresolová červeň. V oblasti okolo pH 8,2 (odpovídající cca 300 p.p.m. CO_2) je tento indikátor nejvhodnější s ohledem na malou jednotkovou koncentraci, i když poněkud vyšší rozdíly extinkce s jednotkovou změnou pH získáme použitím cca šestinásobné koncentrace thymolové modři. Pro vyšší koncentrace CO_2 (pH nižší než 7,5) je nejlepší fenolová červeň. Nevhodné jsou tropaeolin 000 a aurin (kyselina rosolová). Pro spektrofotometrii a kolorimetrii s filtry úzkých spektrálních rozsahů je výhodnější měřit se všemi doporučenými indikátory v oblasti zelenožlutooranžového záření než v oblasti modré.

Při probublávání bikarbonátového roztoku měřeným vzduchem se ustavuje rovnováha mezi koncentrací CO_2 ve vzduchu a hodnotou pH při změnách koncentrace CO_2 100 až 400 p.p.m. po méně než deseti minutách. Pro vztah mezi parciálním tlakem CO_2 a experimentálně zjištěnou hodnotou pH byla změněna přepočtová rovnice: $pH = a - 0{,}934 \log P$ a byly zjištěny hodnoty konstanty a při různých teplotách.

И. ЧАТСКИ, З. ШЕСТАК, Институт экспериментальной ботаники ЧСАН, Прага: **Подходящие индикаторы и преобразованное эмпирическое уравнение для расчета концентрации CO_2 при колориметрическом определении интенсивности фотосинтеза.** — Biol. Plant. 8 : 60—72, 1966.

Из шести испытанных цветных индикаторов pH для колориметрического определения концентрации CO_2 от 100 до 2000 p.p.m. самым подходящим оказался крезоловый красный. В области около pH 8,2 (соответствует приблизительно 300 p.p.m. CO_2) этот индикатор является лучшим по низкой единичной концентрации, хотя немного более высокую разницу в экстинкции при изменении pH на единицу получим применив шестикратную концентрацию тимолового синего. Для более высоких концентраций CO_2 (pH ниже 7,5) лучшим является феноловый красный. Неподходящими являются тропеолин 000 и аурин (росоловая кислота). При спектрофотометрии и колориметрии с фильтрами узких спектральных областей более выгодным является измерение со всеми рекомендованными индикаторами в области зеленожелтооранжевого излучения, чем в синей области.

При продувании бикарбонатного раствора измеряемым воздухом равновесие между концентрацией CO_2 в воздухе и значением pH при изменениях концентрации CO_2 от 100 до 400 p. p. m. устанавливается после менее чем десяти минут. Для отношения между парциальным давлением CO_2 и экспериментально определенным значением pH было преобразовано расчетное уравнение : $pH = a - 0{,}934 \log P$ и были определены значения постоянной a для разных температур.