that necrotic livers from tocopherol-depleted rats fed baker's yeast diet had an even lower glutathione content of about 25 mg./100 g. liver.

In conclusion, the nutritive properties of baker's and brewer's yeasts differ in respect to both growth-promoting and necrogenic properties. The amino-acid contents of the two yeasts could explain the difference in growth-promoting properties, the diet containing baker's yeast being the more deficient in several amino-acids. We cannot, however, say whether the small differences in the sulphur-containing amino-acid content account entirely for their different necrogenic properties. Further experiments are needed to establish this point.

SUMMARY

1. A comparison was made between the dietetic properties of British baker's yeast and American brewer's yeast, both of which were used as the sole source of protein in the production of dietetic liver necrosis in rats. Diets containing 7–8 % of yeast protein were used.

2. When fed to tocopherol-depleted weanling rats the baker's yeast diet proved to be more necrogenic than the brewer's yeast diet. Growth experiments on normal weanlings also showed the inferior biological value of baker's yeast.

3. Rats fed the yeast diets for 33 days had liver glutathione contents which were 50 % lower than those of normal rats. There was no difference between the composition of livers of rats fed either type of yeast.

4. Different dietetic deficiencies exhibited by the two yeasts are discussed in relation to their contents of sulphur, nitrogen and amino-acids. Brewer's yeast contains 38 % more cystine and glutathione than baker's yeast. Comparison with Rose's (1937) figures for minimum essential amino-acid requirements for normal growth showed that both yeast diets were also deficient in other essential amino-acids.

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The Use of 'CO_2 Buffers' in Manometric Measurements of Cell Metabolism

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Pardee (1949) has recently devised a simple manometric method for the measurement of the oxygen absorption of cells respiring in the presence of carbon dioxide. The method is generally applicable to systems where carbon dioxide and a second gas are formed or used and where the pressure changes of the second gas are to be measured. It is based on the use, in a centre well of the manometric vessel, of a 'CO_2 buffer' capable of maintaining a virtually constant pressure of carbon dioxide in the gas phase. A suitable 'CO_2 buffer' is an aqueous solution of diethanolamine which binds carbon dioxide reversibly, mainly according to the equation

$$\mathrm{NH(CH_2.CH_2OH)_2+CO_2+H_2O \rightleftharpoons HCO_3^- + \overset{+}{N}H_2(CH_2.CH_2OH)_2}.$$

Any carbon dioxide formed by metabolic processes is removed by this reaction proceeding from left to right, and any carbon dioxide used is replaced by the reverse process. Warburg (1919) was the first to use this principle in the form of carbonate-bicarbonate buffer; but only very low carbon dioxide pressures of the order of a few mm. mercury can be maintained by this system, whilst diethanolamine can maintain carbon dioxide pressures of 25 and more mm. mercury.

In applying Pardee's method a complication was encountered which led to a fuller study of the principle. It was observed that solutions of diethanolamine, even after repeated recrystallizations of the amine, absorb oxygen. This raised the question whether the autoxidation was a factor in Pardee's results. The effectiveness of diethanolamine and similar reagents as carbon dioxide buffers was therefore systematically examined. In addition the theoretical basis of the method was studied.

Theory of 'CO_2 buffers'

Calculation of buffering capacity. To calculate the capacity of a CO_2 buffer it is necessary to know the relationship between the CO_2 pressure and the concentration of bound CO_2 in the solution. This relationship can be calculated in simple systems based on the reaction

$$\text{monoacidic weak base} + CO_2 \rightleftharpoons HCO_3^- + \text{monoacidic base cation}, \quad (1)$$

if the electrolytic dissociation constant of the base (K_B), the apparent first electrolytic dissociation constant of carbonic acid ($K_{H_2CO_3}$), the dissociation constant of water (K_W), the total concentration of the base (undissociated base + cation) c, the Bunsen absorption coefficient of CO_2 for the solution (α_{CO_2}) are known and if some simplifying assumptions are made, namely that no other reactions between the base and CO_2 (especially no formation of carbamate) occur and that the second stage of dissociation of carbonic acid is negligible.

It follows from the equations defining K_B, $K_{H_2CO_3}$, K_W and from the assumption that, in accordance with reaction 1, the concentration of bicarbonate in the solution equals that of the base cation, that

$$[HCO_3^-] = \sqrt{\left\{\frac{cK_B K_{H_2CO_3}[CO_2]}{K_W} + \left(\frac{K_B K_{H_2CO_3}[CO_2]}{2K_W}\right)^2\right\}} - \frac{K_B K_{H_2CO_3}[CO_2]}{2K_W}, \quad (2)$$

where the square brackets refer to the concentrations in the solution. With this formula the bicarbonate concentration of a base solution can be calculated for any CO_2 pressure. If a salt of a weak acid which reacts with CO_2 according to the equation

$$\text{Anion}^- + CO_2 + H_2O \rightleftharpoons HCO_3^- + \text{acid}$$

is used instead of the base solution, $[HCO_3^-]$ can be calculated by substituting K_{acid} for K_B/K_W in equation 2.

The validity of equation 2 has been tested experimentally for dilute solutions of triethanolamine, and the agreement between calculated and measured data was found to be satisfactory (see p. 353). However, in the concentrations of 3 or 4 M strength, required for buffering purposes, major deviations from the theory occur which are due to the occurrence of significant quantities of carbonate and of carbamate and to additional factors not operative in dilute solution. Mason & Dodge (1936), who have measured the bound CO_2 of concentrated amine solutions at different CO_2 pressures, have come to the conclusion that the observations cannot be accounted for in terms of a simple theory.

Warburg (1925) has shown how data correlating the increase in CO_2 pressure with the increase in bound CO_2 in the solution can be used for calculating manometric pressure changes due to CO_2 in the presence of substances combining with CO_2. In accordance with Warburg's terminology the binding of CO_2 by the solution is referred to as 'retention' of CO_2. If d [retained CO_2] is the increase in the concentration of retained CO_2 (in μl./ml.) produced by the increase in CO_2 pressure dp_{CO_2} (in mm. of manometric fluid), then $\frac{d\,[\text{retained } CO_2]}{dp_{CO_2}}$ is the 'retention' of CO_2/mm. change in CO_2 pressure/ml. of solution.

Assuming, by first approximation, that

$$\frac{d\,[\text{retained } CO_2]}{dp_{CO_2}}$$

is, within a limited range of p_{CO_2}, practically constant, the pressure changes in a manometric vessel due to the formation or disappearance of CO_2 can be calculated by the formula

$$h_{CO_2} = \frac{x_{CO_2}}{k_{CO_2} + v_F \dfrac{d\,[\text{retained } CO_2]}{dp_{CO_2}}},$$

where h_{CO_2} is the pressure change (in mm.) produced by the amount x_{CO_2} (in μl.), k_{CO_2} the vessel constant for CO_2 and v_F the volume of the retaining fluid (in ml.). It will be seen that for a given value of x_{CO_2} the value for h_{CO_2} will be the smaller, i.e. the retention will become the more effective, the larger v_F; further that for a given value of $\frac{d\,[\text{retained } CO_2]}{dp_{CO_2}}$ the percentage retention

$$\frac{v_F \dfrac{d\,[\text{retained } CO_2]}{dp_{CO_2}}}{v_F \dfrac{d\,[\text{retained } CO_2]}{dp_{CO_2}} + k_{CO_2}} \times 100,$$

will be the greater the smaller k_{CO_2}. The ideal system for retaining CO_2 is one in which the above term approaches the value of 100.

In dilute solutions of an amine d [retained CO_2] is practically identical with d $[HCO_3^-]$, and $\frac{d\,[HCO_3^-]}{d[CO_2]}$ can be calculated by differentiating equation 2 with respect to $[CO_2]$:

$$\frac{d\,[HCO_3^-]}{d\,[CO_2]} = \frac{\dfrac{cK_BK_{H_2CO_3}}{K_W} + \left(\dfrac{K_BK_{H_2CO_3}}{K_W}\right)^2 \dfrac{[CO_2]}{2}}{2\sqrt{\left\{\dfrac{cK_BK_{H_2CO_3}[CO_2]}{K_W} + \left(\dfrac{K_BK_{H_2CO_3}[CO_2]}{2K_W}\right)^2\right\}}} - \frac{K_BK_{H_2CO_3}}{2K_W}. \quad (3)$$

$[HCO_3^-]$ and $[CO_2]$ are here expressed in the same units as the dissociation constants which are customarily molar. For manometric work it is convenient to use different units, namely μl./ml. for $[HCO_3^-]$ and mm. of manometric fluid for $[CO_2]$. It follows from the definition of the Bunsen absorption coefficient α, and the value for the molar volume of CO_2 (22·26 l.), that a CO_2 concentration of 1 M is equivalent to a CO_2 pressure of 222,600/α mm. manometric fluid. The conversion factor for transforming

$$\frac{\text{g. mol. } HCO_3^-/\text{l.}}{\text{g. mol. } CO_2/\text{l.}} \text{ into } \frac{\mu\text{l. } HCO_3^-/\text{ml.}}{\text{mm. } CO_2}$$

is thus $\frac{22,260}{222,600/\alpha} = 0{\cdot}1\,\alpha$. The conversion factor obviously depends on the temperature, solute concentration and other factors which affect the value of α.

Equation 3 allows the calculation of the retention at varying CO_2 concentration for the conditions to which the simplifying premises used in the derivation of equation 2 apply. Although the conditions in the solution actually used are more complex, it is of interest to calculate how changes in the values of the constants and of the CO_2 pressure affect the retention.

Effect of $[CO_2]$ *on retention.* When $[CO_2]$ is very small all terms in equation 3 containing $[CO_2]$ become very small and the numerator, which contains the only term without $[CO_2]$, becomes very large compared with the denominator. $\frac{d\,[HCO_3^-]}{d\,[CO_2]}$ approaches ∞ as $[CO_2]$ approaches 0. When $[CO_2]$ is very large $\frac{d\,[HCO_3^-]}{d\,[CO_2]}$ becomes 0. Thus the retention is the more effective the lower the concentration of CO_2 in the absorbing solution. It follows as a practical conclusion that the retention for a given CO_2 pressure in the gas mixture can be improved if the absorption coefficient α can be reduced.

Effect of K_B on retention. Fig. 1 is a plot of the concentration of HCO_3^- in base solutions against the concentration of CO_2 for different values of K_B. The data were calculated from equation 2. It will be seen that when K_B is large (10^{-2} M) most of the base reacts with CO_2 according to 1 at very low CO_2 concentrations (below 10^{-4} M). On the other hand, if K_B is very small (10^{-6} M) very little HCO_3^- is formed at the CO_2 concentrations which are of interest, say, between 10^{-3} and 10^{-4} M. There must be a value for K_B at which, for a given CO_2 concentration, the retention will be maximal, i.e. the slope of the plot of $[HCO_3^-]$ against $[CO_2]$ steepest. The K_B value giving maximum retention at a given CO_2 concentration can be calculated by differentiating $\frac{d\,[HCO_3^-]}{d\,[CO_2]}$ with respect to K_B and equating to zero. This leads to the following equation:

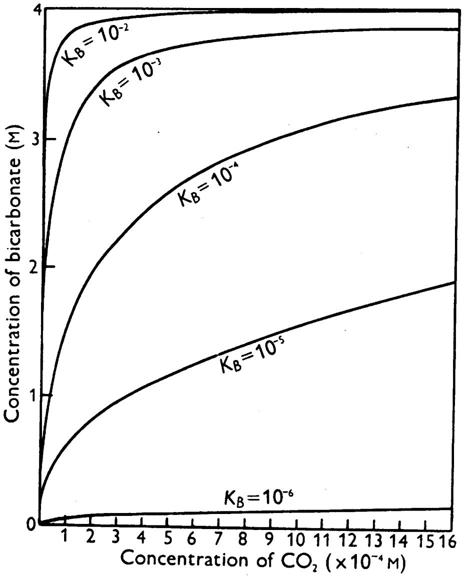

Fig. 1. Concentration of bicarbonate at various CO_2 concentrations in solutions of bases with different dissociation constants (calculated according to equation 2).

$$K_B^{\text{max.}} = \frac{2K_W c\,(\sqrt{2}-1)}{[CO_2]\,K_{H_2CO_3}}, \quad (4)$$

where $K_B^{\text{max.}}$ is the dissociation constant giving maximum retention at the CO_2 concentration $[CO_2]$. With the help of this equation K_B values giving maximum retention can be calculated for each CO_2 concentration. It follows from equation 4 that $K_B^{\text{max.}}$ is directly proportional to the concentration of the retaining base (c) and inversely proportional to the concentration of CO_2. A few examples are shown in Table 1. It is especially important to use a favourable K_B value at the higher concentrations of CO_2.

Table 1. *Values for* $K_B^{\mathrm{max.}}$ *calculated from equation* 4

($K_{\mathrm{H_2CO_3}} = 10^{-6}$; $K_W = 10^{-14}$; all concentrations M)

CO_2	c	$K_B^{\mathrm{max.}}$
10^{-4}	4	$3{\cdot}32 \times 10^{-4}$
10^{-3}	4	$3{\cdot}32 \times 10^{-5}$
10^{-3}	2	$1{\cdot}66 \times 10^{-5}$
10^{-3}	1	$0{\cdot}83 \times 10^{-5}$

In Fig. 2 the retention $\left(\frac{\mathrm{d}\,[\mathrm{HCO_3^-}]}{\mathrm{d}\,[\mathrm{CO_2}]}\right)$ is plotted against $-\log K_B$ for various concentrations of base and for a mixture of two bases. The curves show that the peak giving maximum retention is fairly sharp at higher concentrations of base.

Retention in mixtures of bases. A relatively simple case which may be considered is the mixture of an amine with a much stronger base, e.g. sodium hydroxide. In this case CO_2 will react at very low pressures until virtually all the strong base is converted into the bicarbonate. On the assumption that the strong base reacts in fact quantitatively with CO_2 to form bicarbonate before the retaining amine reacts with CO_2, and that the simplifying premises made before are valid, the bicarbonate concentration in the mixture can be calculated from the following equation:

$$[\mathrm{HCO_3^-}] = \sqrt{\left\{\frac{(c+b)\,K_BK_{\mathrm{H_2CO_3}}\,[\mathrm{CO_2}]}{K_W} + \left(\frac{b-\dfrac{K_BK_{\mathrm{H_2CO_3}}\,[\mathrm{CO_2}]}{K_W}}{2}\right)^2\right\}} + \frac{b-\dfrac{K_BK_{\mathrm{H_2CO_3}}\,[\mathrm{CO_2}]}{K_W}}{2}, \qquad (5)$$

where b is the concentration of a monoacidic strong base. Differentiation of equation 5 with respect to $[\mathrm{CO_2}]$ gives:

$$\frac{d\,[\mathrm{HCO_3^-}]}{\mathrm{d}\,[\mathrm{CO_2}]} = \frac{\dfrac{(c+b)\,K_BK_{\mathrm{H_2CO_3}}}{K_W} - \dfrac{b\,K_BK_{\mathrm{H_2CO_3}}}{2K_W} + \left(\dfrac{K_BK_{\mathrm{H_2CO_3}}}{K_W}\right)^2 \dfrac{[\mathrm{CO_2}]}{2}}{2\sqrt{\left\{\dfrac{(c+b)\,K_BK_{\mathrm{H_2CO_3}}\,[\mathrm{CO_2}]}{K_W} + \left(\dfrac{b-\dfrac{K_BK_{\mathrm{H_2CO_3}}\,[\mathrm{CO_2}]}{K_W}}{2}\right)^2\right\}}} - \frac{K_BK_{\mathrm{H_2CO_3}}}{2K_W}.$$

This equation is applicable, for example, to (dilute) solutions containing, as does Pardee's (1949) reagent, sodium bicarbonate in addition to diethanolamine. The theoretical effect of the addition of bicarbonate as calculated by this formula is seen in Fig. 2. Addition of a strong base increases the retention and raises the value of $K_B^{\mathrm{max.}}$. The increase in retention is smaller than that brought about by an equivalent increase of the concentration of the weak base.

EXPERIMENTAL

Autoxidation of diethanolamine

Solutions of diethanolamine were shaken in air or in O_2 in manometer vessels at 25 or 40°. Measurable quantities of O_2 were always absorbed, but the rates showed considerable variations under apparently identical conditions. For example, at 40° 1 ml. of 4M-diethanolamine, in equilibrium with 5 % CO_2 in O_2, absorbed between 15 and 100 μl. of O_2 per hr. The rate usually increased with the time of incubation. Solutions made from freshly recrystallized diethanolamine showed a very low uptake during the first 30 min., but the rate gradually rose to values similar to those with the commercial material. Addition of *sec.*-octanol increased the rate of oxidation. Among the many substances tested for inhibitory activity, dithizone (*sym.* diphenylthiocarbazone) and thiourea were found to be most effective. Examples of observations made at 40° are given in Table 2. At 25° the rate of oxidation was about 12 % of that at 40°.

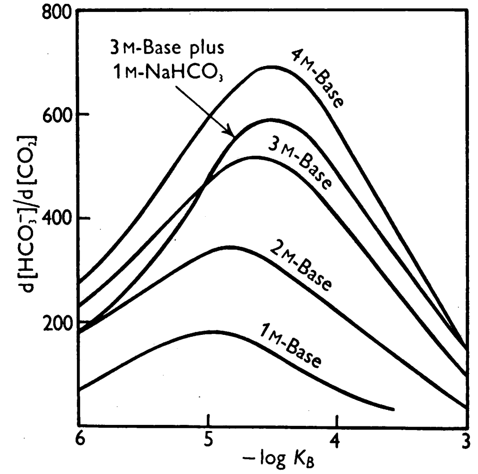

Fig. 2. Dependence of retention d $[\mathrm{HCO_3^-}]$/d $[\mathrm{CO_2}]$ on K_B for various concentrations of base (calculated according to equation 3; $[\mathrm{CO_2}] = 10^{-3}$; $K_{\mathrm{H_2CO_3}} = 10^{-6}$; $K_W = 10^{-14}$).

At 40° dithizone (0·02 %) abolished the autoxidation almost completely for 1 or 2 hr., but on prolonged incubation at 40°, or on storage for several days at room temperature, the inhibitory effect gradually disappeared. This resulted in a gradually

Table 2. *Autoxidation of diethanolamine*

(3 ml. 4 M-diethanolamine in equilibrium with 5% CO_2 in O_2; 40°.)

Additions	0	0·02% Dithizone	30 mg. *sec*-Octanol	30 mg. *sec*-Octanol + 0·02% dithizone	0·1% Thiourea	0·1% Thiourea + 30 mg. *sec*-octanol
	O_2 absorbed after equilibration period of 1 hr. (μl.)					
First 20 min.	15	2	54	2	7	7
Second 20 min.	23	2	61	2	8	7
Third 20 min.	37	3	77	3	7	7

rising rate of O_2 absorption. With 0·1% thiourea the initial rate of autoxidation was a little higher than with dithizone (Table 2), but the rate remained steady for more than 24 hr. at 40° or for several weeks at room temperature. Thiourea is therefore the more useful general antioxidant.

Experimental test of equation 2

Manometric flasks of the Dickens & Šimer (1931) type were filled as follows:

Inner vessel	0·0100 M-Triethanolamine, 3 ml.
Outer vessel	Na_2CO_3 solution of various strengths, 2 or 3 ml.
Side arms (both)	0·5 M-H_2SO_4, 0·5 ml.
Gas	N_2

The triethanolamine used was freshly purified according to Germann & Knight (1935). The vessels were shaken at 25·0° until the manometric readings were constant. The acid was then added to the carbonate in the outer vessel and the manometers were shaken until the readings indicated that equilibrium between gas phase and solution had been established. The increase in pressure—the CO_2 pressure over the triethanolamine solution—was read and the acid was then added to the triethanolamine solution to measure the concentration of bound CO_2 in this solution.

In Fig. 3 the concentration of bicarbonate in the amine solution is plotted against the CO_2 pressure. The curve is that calculated on the basis of equation 2, using for $K_B^{25^\circ}$ the value $5\cdot97\times10^{-7}$ (Hall & Sprinkle, 1932), for $K_W^{25^\circ}$ $1\cdot01\times10^{-14}$ (Harned & Owen, 1944), for $K_{H_2CO_3}^{25^\circ}$ $5\cdot00\times10^{-7}$ (Roughton, 1943) and for $\alpha_{CO_2}^{25^\circ}$ 0·760. The crosses in the figure represent the measured data. The agreement between theory and measurement is very satisfactory.

Another test of the theory is as follows. According to the theory it is to be expected that

$$\frac{[CO_2]\,[\text{Undissociated base}]}{[HCO_3^-]\,[\text{Base cation}]}=\frac{K_W}{K_{H_2CO_3}K_B}.$$

As the concentration of the base cation is virtually equal to that of bicarbonate and the concentration of undissociated base equals $[C-HCO_3^-]$ where C is the total concentration of triethanolamine, the above equation becomes

$$\frac{[CO_2]\,[C-HCO_3^-]}{[HCO_3^-]^2}=\frac{K_W}{K_{H_2CO_3}K_B}=K'.$$

Data for K' are given in the last column of Table 3. The values obtained are constant. The experimental error is expected to be relatively high at low CO_2 pressures (because a small error in the manometer reading causes a large percentage error in p_{CO_2}) and at high bicarbonate concentrations (because a small percentage error in $[HCO_3^-]$ causes a large error in $[C-HCO_3^-]$). If the data below 50 mm. CO_2 and above 0·008 M-HCO_3^- are omitted for the calculation of the average, the average value of K' is $3\cdot23\times10^{-2}$; the average of all values is $3\cdot16\times10^{-2}$. The value calculated from the data for the constants quoted above from the literature is $3\cdot39\times10^{-2}$.

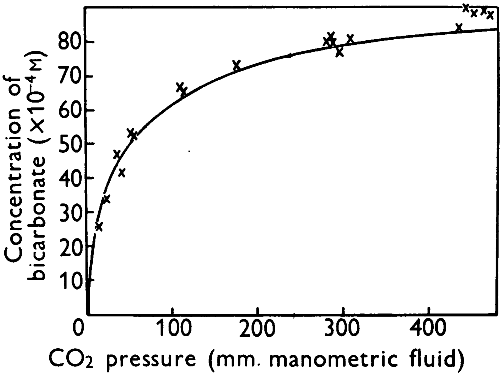

Fig. 3. Concentration of bicarbonate in 0·0100 M-triethanolamine solutions at different CO_2 pressures. (Experimental test of equation 2. The curve is calculated from equation 2; the crosses are the data measured. For details of procedure see text, p. 353).

It is evident that if K_{CO_2} and K_W are known the dissociation constant of a base can be ascertained by this procedure. The value obtained in this way for triethanolamine ($6\cdot25\times10^{-7}$) agrees reasonably well with that reported by Hall & Sprinkle (1932) ($5\cdot97\times10^{-7}$).

Table 3. *Concentration of bicarbonate in triethanolamine solutions at various* CO_2 *pressures*

(0·0100 M-triethanolamine; 25°; initial gas N_2; $\alpha^{25^\circ}_{CO_2}=0{\cdot}76$; for significance of K' see text, p. 353.)

CO_2 pressure (mm. manometric fluid)	Concentration of CO_2 in solution ($\times 10^{-3}$M)	HCO_3^- found in 3 ml. (μl.)	Concentration of HCO_3^- ($\times 10^{-2}$M)	K'
12	0·0410	176	0·262	0·0440
13·5	0·0461	174	0·258	0·0507
22	0·075	232	0·344	0·0446
37	0·126	314	0·466	0·0306
40·5	0·138	286	0·425	0·0436
55	0·188	356	0·529	0·0313
56	0·191	354	0·526	0·0327
111	0·380	446	0·664	0·0287
112·5	0·384	441	0·656	0·0302
175	0·597	493	0·733	0·0295
282	0·962	541	0·805	0·0286
287	0·980	545	0·810	0·0280
288	0·983	529	0·785	0·0337
296	1·010	519	0·770	0·0385
309	1·056	545	0·810	0·0301
435	1·485	566	0·844	0·0327
443	1·515	608	0·905	0·0175
455	1·555	595	0·885	0·0227
464	1·585	601	0·895	0·0208
471	1·610	591	0·880	0·0247
726	2·48	606	0·902	0·0298

Measurement of retention

A known amount of CO_2 was liberated in a manometric flask containing the retaining fluid and the amount of CO_2 not absorbed by the retaining fluid was measured. Dickens & Šimer (1931) flasks, provided with valve stoppers, were filled as follows:

Inner compartment	Retaining solution to be tested, 0·5 ml.
Outer compartment	0·010 M-$NaHCO_3$ solution, 2 ml.
Side arm of outer compartment	3 M-H_2SO_4, 0·25 ml.

Before the amine solution was measured into the flask a rapid stream of CO_2 was passed through it for about 10 min. to neutralize about half the amine with CO_2. This expedited the subsequent equilibration of the retaining solution with the gas mixture.

The manometers with their flasks were placed into the water bath and a gas mixture containing CO_2 in N_2, saturated with water by passage through a wash bottle immersed in the water bath, was sent through the vessels whilst they were shaken at a rapid rate in the water bath. This was continued until the gas equilibrium was reached, usually for 30–60 min. To test the equilibrium the tap and the valve stopper were closed; on further shaking the pressure changes should not exceed a few mm. N_2 rather than O_2 was used to avoid complications from autoxidation.

This procedure was adopted after many preliminary trials. When larger quantities of amine solution were used the time required for equilibration was greatly prolonged.

When equilibrium had been established and the pressure read, the acid in the side arm was mixed with the bicarbonate solution and the manometers were again shaken until constancy of pressure was attained. If a = the quantity of CO_2 liberated from bicarbonate in the outer compartment (in μl.); h = the CO_2 pressure remaining when absorption by the amine solution was completed (in mm. manometric fluid); b = the quantity of CO_2 not absorbed by the base in the inner vessel (in μl.), and v_F = the volume of the amine solution (in ml.) the average retention $\left(=\frac{\Delta\,[\text{retained } CO_2]}{\Delta p\ CO_2\ v_F}\right)$ for the pressure increase h was $(a-b)\,hv_F$.

Over two hundred measurements were made in the search for the best CO_2 buffers for varying CO_2 pressures at 25 and 40°. The number of suitable bases is limited by three requirements: the solubility must be high enough to allow the preparation of concentrated solutions; the substance must not be volatile lest it should distil into other compartments of the manometric vessel (this excludes ammonia and many alkyl amines); and K_B must be of the order of 10^{-5}. The amines tested included monoethanolamine, diethanolamine, triethanolamine, mono*iso*propanolamine, di*iso*propanolamine, tri*iso*propanolamine, ethylenediamine, diethylenetriamine, triethylenetetramine and 1:3-diaminopropanol. In addition to pure solutions of bases, mixtures of bases were tested as well as the solutions recommended by Pardee (1949), prepared by mixing diethanolamine, powdered potassium bicarbonate and hydrochloric acid.

Table 4. *Retention of* CO_2 *by amine solutions*

(For procedure see text, p. 354; the solutions according to Pardee (1949) varied with the CO_2 pressure and were prepared for use at 40 and 25° according to the directions given by Pardee for 38°.)

Temp. (°)	Initial CO_2 content of gas mixture per cent (v/v)	Solutions tested	Retention
40	0·94	4 M-Diethanolamine	50
		5 M-Diethanolamine	45
		4 M-Diethanolamine, 12 % (w/v) $KHCO_3$; 6 % (w/v) KCl	49
		Pardee's solution	51
40	1·98	4 M-Diethanolamine	28
		5 M-Diethanolamine	31
		4 M-Diethanolamine, 1 M-monoethanolamine	31
		4 M-Diethanolamine; 12 % (w/v) $KHCO_3$; 6 % (w/v) KCl	37
		Pardee's solution	41
40	3·09	4 M-Diethanolamine	16·7
		5 M-Diethanolamine	23·3
		4 M-Diethanolamine, 1 M-monoethanolamine	13·0
		4 M-Diethanolamine; 4 % (w/v) $KHCO_3$; 12 % (w/v) KCl	22·4
		Pardee's solution	22·0
40	3·93	4 M-Diethanolamine	12·3
		5 M-Diethanolamine	12·3
		4 M-Diethanolamine, 1 M-monoethanolamine	10·5
		4 M-Diethanolamine; 12 % (w/v) $KHCO_3$; 6 % (w/v) KCl	11·3
40	5·74	3·75 M-Diethanolamine	10·2
		4 M-Diethanolamine	10·5
		4·5 M-Diethanolamine	9·5
		4 M-Diethanolamine, 1 M-monoethanolamine	11·4
25	0·94	4 M-Diethanolamine	29
		4 M-Diethanolamine; 12 % (w/v) $KHCO_3$; 6 % (w/v) KCl	44
		Pardee's solution	140
25	1·98	4 M-Diethanolamine	25·6
		5 M-Diethanolamine	19·6
		4 M-Diethanolamine, 1 M-monoethanolamine	17·8
		Pardee's solution	25·3
25	3·09	4 M-Diethanolamine	16·6
		5 M-Diethanolamine	13·8
		4 M-Diethanolamine, 1 M-monoethanolamine	13·0
		Pardee's solution	17·3
25	3·93	4 M-Diethanolamine	10·3
		5 M-Diethanolamine	9·2
		4 M-Diethanolamine, 1 M-monoethanolamine	8·2
		Pardee's solution	6·6
25	5·74	4 M-Diethanolamine	9·5

Neutral salts were added to the amine solutions in the expectation that this might increase retention by reducing the solubility of CO_2 in the solution, the expected 'salting out' effect being the greater the smaller the ion and the greater the charge (see Glasstone, 1941). As the salt employed must be very soluble and not form sparingly soluble compounds with the amine or with bicarbonate, the range of promising substances was limited. Several halides and sulphates of the alkali metals were tested. Some effects in the expected direction were noted (see below), but no substance was found to be generally effective.

Only a few selected results are given in Table 4. Omitted are data showing low retention as these are not of interest in connexion with the object of this paper. Diethanolamine in various combinations gave throughout higher retention values than other substances tested. The main points emerging from the data are the following:

(1) In general the retention for the same solution, e.g. 4 or 5 M-diethanolamine, fell as the CO_2 pressure rose. At 40° retention was greater than at 25°, as is theoretically expected because of the fall in α with rising temperature.

(2) At 40° the highest retention values were given under most conditions by 4 or 5 M-diethanolamine. The solutions recommended by Pardee (1949) gave similar values with 1 and 3 % CO_2 and somewhat higher retention with 2 % CO_2. Addition of potassium bicarbonate or potassium chloride increased retention with 2 and 3 % CO_2, but not with 1 or 4 %.

(3) At 25° exceptionally high retention was observed with Pardee's solution with 1 % CO_2. At this gas pressure the addition of potassium bicarbonate and potassium chloride to 4 M-diethanolamine increased retention considerably. An unexplained discrepancy was observed between the solution of Pardee (prepared from diethanolamine, potassium bicarbonate and potassium chloride), which gave a value of 140, and a solution expected to have the same composition prepared from diethanolamine, potassium bicarbonate, potassium chloride and CO_2, which gave 44.

(4) 4 M-Diethanolamine is the best reagent for most conditions, the only major exception being for 1 % CO_2 at 25°. In the majority of cases 4 M-diethanolamine is as effective as are Pardee's mixtures (which contain about 3·8 M-diethanolamine). No solutions appreciably more effective than those recommended by Pardee were found.

Procedure for the use of CO_2 *buffers*

Choice of experimental arrangements. The data in Table 4 may be used for calculating the effectiveness of retention under different conditions, the percentage retention being

$$\frac{v_F R}{v_F R + k_{CO_2}} \times 100,$$

where v_F is the volume of the retaining fluid, k_{CO_2} the vessel constant for CO_2 and R the retention given in the last column of Table 4. For Pardee's solutions as used by Pardee ($v_F \doteq 0{\cdot}6$; $k_{CO_2} = 1{\cdot}3$) the following values result: 1 % CO_2, 96 % retention; 2 % CO_2, 94 % retention; 3 % CO_2, 83 % retention. As Pardee has already pointed out, his conditions may be regarded as satisfactory for CO_2 pressures up to 3 %.

To increase retention with a given value for the retaining capacity of the solution, it is necessary to reduce k_{CO_2} or to increase v_F. The effects of variation in k_{CO_2} and in the volume v_F may be seen from Table 5, where data are shown for the volume required to give 90 and 95 % retention for two values of k_{CO_2}. v_F was calculated according to the formula

$$v_F = \frac{k_{CO_2}}{R} \times \frac{a}{100 - a},$$

where a is the percentage retention. It will be seen that, except with 1 % CO_2, larger volumes of retaining fluid are required than can be used in the centre well of the conventional conical manometer flask. On the other hand, the vessels designed by Dickens & Simer (1931), already recommended by Pardee, or those described by Warburg, Kubowitz & Christian (1931), are suitable; either the centre compartment or the outer ring can be filled with several ml. of fluid. In these vessels sufficiently large volumes of retaining fluid can be used to make 98 % retention practicable at 1 and 2 % CO_2, but not at higher CO_2 pressures. It is clear that 'complete' absorption of CO_2 is not always obtainable, but in many types of experiment 90 or 95 % retention may be satisfactory.

In order to test whether diethanolamine may distil from one manometer compartment into another under the experimental conditions, Dickens-Šimer vessels containing 3 ml. of 4 M-diethanolamine (prepared from 'diethanolamine technical' of British Drug Houses Ltd) in one compartment and 3 ml. of 1 % potassium dihydrogen phosphate solution in the other, were rapidly shaken for 4·5 hr. at 40°. The gas space contained 3 % CO_2 in O_2. After incubation the phosphate solution was wet ashed with sulphuric acid. No measurable quantities of ammonia, i.e. less than 0·005 mg. nitrogen, were found. The danger of contamination with diethanolamine by distillation is therefore negligible.

Preparation of CO_2 *buffers.* It is essential to equilibrate the buffer solution (before it is pipetted into the vessel) with the same CO_2 mixture as is used in the experiment. The equilibration has to be carried out at the temperature at which the solution is finally used, but a stock of equilibrated buffer may be kept for several weeks at room temperature if stored in a stoppered cylinder.

Table 5. *Volume of retaining fluid* (4 M-*diethanolamine*) *required for* 90 *and* 95 % *absorption of* CO_2

			Volume required (ml.)			
			$k_{CO_2} = 1{\cdot}5$		$k_{CO_2} = 2{\cdot}5$	
CO_2 concentration (%)	Retention	Temp. (°)	90 % absorption	95 % absorption	90 % absorption	95 % absorption
1	50	40	0·27	0·57	0·45	0·95
2	28	40	0·48	1·02	0·50	1·79
3	17	40	0·80	1·68	1·32	2·80
4	12	40	1·10	2·33	1·88	3·95
1	29	25	0·47	0·98	0·78	1·64
2	26	25	0·52	1·10	0·87	1·83
3	17	25	0·80	1·68	1·32	2·80
4	10	25	1·35	2·85	2·35	4·75

Stock solutions of 4M-diethanolamine buffer are prepared by passing first a rapid stream of 100 % CO_2 through one-third of the desired quantity of solution until saturation is obtained. This is tested with phenolphthalein (pH ~ 8) and can be achieved in 30 min. if a sintered glass distributor of the type used for aerating aquaria is used. The remaining two-thirds of the untreated solution are then added and the gas mixture to be used in the manometer is passed through the solution at the appropriate temperature for 1 or 2 hr., again through a sintered glass distributor. Whether equilibrium has been reached may be tested as follows: the solution (3 or 4 ml.), together with a few glass beads of 4–5 mm. diameter, is placed in a manometer provided with a valve stopper (Warburg & Kubowitz, 1929) and the gas mixture is passed through the vessel whilst it is being shaken in the bath. The further procedure is as described on p. 354.

Diethanolamine 'technical' was found to be as satisfactory as a recrystallized sample, provided that thiourea (0·1 %) was added. Thiourea should be dissolved before the solutions are equilibrated with the gas mixture.

If the solutions of Pardee are used, thiourea (0·1 %) should also be added and equilibrating and testing should be carried out as here described except for the omission of the primary treatment with 100 % CO_2. This procedure is recommended because the CO_2 pressures stated by Pardee are only approximately correct.

Controls. A manometer vessel without the metabolically active material but containing the buffer solution and the same gas mixture is required in each series of experiments. This vessel may also serve as the thermobarometer.

Test of the method

Anaerobic fermentation of baker's yeast. To test whether the absorption of CO_2 in the presence of CO_2 buffers is of the expected order, yeast suspensions steadily producing CO_2 were placed in manometer vessels, with and without a CO_2 buffer. Dickens-Šimer flasks were used. The outer compartment contained 2 ml. of yeast suspension in 0·05M-potassium dihydrogen phosphate solution and the side arm attached to it 1 ml. of 0·1M-glucose solution. The CO_2 buffer (3 ml. of 4M-diethanolamine) and five glass beads were placed in the centre compartment. The side arm attached to this compartment contained yellow phosphorus; the gas mixture consisted of 3·6 % CO_2 and 96·4 % N_2. The bath temperature was 25°. The vessels were gassed in the bath for about 20 min. After readings had shown that gas equilibrium was established, the glucose was mixed with the yeast cells. The gas evolution observed after mixing is shown in Table 6. It will be seen that the absorption of CO_2 was less than expected during the first two 10 min. periods, but afterwards it was of the expected order. In other words, after about 20 min. a steady state developed where the CO_2 pressure was about 15 and 30 mm. respectively above the equilibrium pressure. Once the steady state was attained, absorption was according to the theory.

In further experiments of this type the level of excess CO_2 pressure in the steady state was found to vary with the shape and size of the vessel, the rate of shaking and especially the rate of CO_2 production. In the experiment shown in Table 6 where the two vessels containing CO_2 buffer were of identical size ($k_{CO_2} = 2·36$) the quantity of non-absorbed CO_2 was about proportional to the rate of CO_2 production. It made no difference to the efficiency of the CO_2 buffer if it was placed in the outer compartment instead of the centre. From the practical point of view it is important that in all experiments the steady state is established within 20–40 min. If a continuous reaction is being studied most of this time would fall within the usual period of gas equilibration.

Respiration of Escherichia coli. In this experiment the rates of O_2 consumption of a bacterial suspension as measured by the new method and by the standard method (sodium hydroxide in centre well) were compared. *Esch. coli* was grown on an agar medium containing 1 % casein digest, 0·2 % yeast extract and 2 % glycerol. The cells were washed with water and suspended in 0·01M-phosphate buffer, pH 6·8.

Table 6. *Effectiveness of* CO_2 *buffer. Absorption of* CO_2 *produced by fermenting yeast cells*

(The weight of yeast refers to fresh weight. Rate of shaking: 100 oscillations/min.; amplitude 5 cm.; $k_{CO_2} = 2·36$. For further particulars see text, above)

	5 mg. yeast/vessel			10 mg. yeast/vessel		
	CO_2 evolved			CO_2 evolved		
Time period (min. after addition of glucose)	Without CO_2 buffer (µl.)	With CO_2 buffer (µl.)	CO_2 absorbed (%)	Without CO_2 buffer (µl.)	With CO_2 buffer (µl.)	CO_2 absorbed (%)
0–10	29	24	17	53	38	28
10–20	40	10	75	90	24	73
20–40	91	4	95	198	23	88
40–60	87	6	93	196	10	95
60–120	234	8	97	453	18	96

Table 7. *Respiration of* Escherichia coli *in the presence and absence of* CO_2

(Duplicate measurements; readings began 20 min. after addition of substrate; for further particulars see text, p. 357.)

Time of observation	Substrate (final concn. 0·033 M)	Q_{O_2} 3 % CO_2 in O_2; CO_2 buffer	Q_{O_2} O_2; 2 N-NaOH
First 40 min.	Glucose	32·6, 36·7	31·1, 30·7
Second 40 min.	Glucose	36·7, 42·4	34·1, 33·0
First 40 min.	Sodium fumarate	63·4, 63·0	66·0, 65·7
Second 40 min.	Sodium fumarate	69·2, 65·5	63·7, 62·6

Table 8. *Respiration of Fleischmann's yeast. Effect of metabolic rate on efficiency of* CO_2 *buffer*

(Temp. 25°. The data for weights refer to fresh weight. Rate of shaking: 90 oscillations/min.; amplitude 5 cm.; the data for O_2 absorption in the presence of CO_2 buffers are not corrected for incomplete absorption. With the higher yeast cell concentrations the pressure changes exceeded the scale length. When the bottom of the scale was approached the manometer level was re-adjusted by injection of O_2 into the vessel. Readings were resumed after 5 min. and the O_2 uptake for this interval was calculated by interpolation.)

Period of observation (min. after addition of substrate)	O_2 absorbed (µl.)							
	3·6 % CO_2 in O_2; CO_2 buffer				O_2, NaOH			
	64 mg. cells	32 mg. cells	16 mg. cells	8 mg. cells	64 mg. cells	32 mg. cells	16 mg. cells	8 mg. cells
0–10	32	22	14	7	—	50	32	9
10–20	152	80	42	19	214	104	47	18
20–30	233	115	61	29	285	135	69	32
30–50	540	266	134	64	687	329	161	81
50–70	624	319	160	80	689	338	177	86
70–90	627	313	163	79	707	338	160	81

The manometric arrangement was the same as in the previous experiment, except that the yeast suspension was replaced by 2 ml. of bacterial suspension containing 4 mg. (dry weight) of cells. The bath temperature was 40°. Dickens-Šimer cups were also used for the standard method, the CO_2 buffer being replaced by the same volume of 2 N-sodium hydroxide, and 3 % CO_2 in O_2 by O_2. The results are shown in Table 7. No correction was made for incomplete (about 96 %) absorption of CO_2. It will be seen that the presence of 3 % CO_2 had no major effect on the rate of respiration. Owing to incomplete CO_2 absorption the rates in the presence of CO_2 buffer would be expected to be slightly lower; in fact they are slightly higher than in the presence of sodium hydroxide.

Respiration of baker's yeast. Graded quantities of Fleischmann's yeast were shaken in 3 ml. of 0·07 % potassium dihydrogen phosphate solution containing 0·033 M-glucose. In one series the gas was O_2; CO_2 was absorbed by 3 ml. 2 M-sodium hydroxide placed in the centre compartment of a Dickens-Šimer vessel. In the other, the gas was 3·6 % CO_2 in O_2; CO_2 was absorbed by 3 ml. of 4 M-diethanolamine buffer. The general experimental arrangements were the same as in the previous two experiments. The substrate was added to the yeast suspension from the side arm after gas equilibrium had been established. The results are shown in Table 8. In the first 10 min. after the addition of the substrate diethanolamine was much less efficient than sodium hydroxide as a CO_2 absorbent at all four yeast concentrations tested. From the second 10 min. period onwards the O_2 uptakes observed with diethanolamine and with sodium hydroxide were about the same at the two lower yeast concentrations. At the next higher yeast concentrations the rates of O_2 absorption became approximately equal 50 min. after the addition of the substrate (taking into consideration a correction factor for incomplete absorption). At the highest yeast concentration, where the rate of CO_2 production rose to over 30 µl./min., the absorption of CO_2 was below the expected value throughout the period of observation. It follows that except for a short initial period diethanolamine buffer is as efficient an absorbent for CO_2 as sodium hydroxide as long as the rate of CO_2 production does not exceed, say, 10 µl./min.

DISCUSSION

The study of the theory and the measurement of retention confirms the practicability of the principle introduced by Pardee. It must be stressed that careful equilibration of the buffer with the gas mixture and an adequate rate of shaking are essential in the use of the method; further that trials must be carried out for each set of experiments to test whether the absorption of CO_2 by the buffer is adequate at the rate of CO_2 production at which it is used. In general it is advisable to adjust the quantity of material in the flasks so that the rate of CO_2 production does not exceed 6–10 μl./min., the upper limit being determined by the design of the vessels and the rate of shaking.

For the measurement of gas exchanges in the presence of CO_2 Pardee's method is simpler and more accurate than the older methods, e.g. the 'two-vessel method' of Warburg (1924). The new method, however, is applicable only to CO_2 pressures below, say, 6 %, whilst the 'two-vessel method' has no limitations of this kind.

As the presence of CO_2 may influence the metabolic activity of cells and tissues, the absorption of CO_2 by strong alkali, as commonly practised in the measurement of the O_2 uptake, is often undesirable and in these cases Pardee's method will be of great value. The measurements reported in this paper show that 'complete', say over 98 %, absorption is difficult to obtain except at CO_2 pressures below 2 %. But even incomplete absorption of CO_2, although it might result in inaccuracies of the measurement of the O_2 uptake, is not necessarily a serious drawback as the magnitude of the error can be assessed. Moreover, in many investigations the measurement of the oxygen consumption is a side issue serving as a measure of the intactness of the energy-giving systems, the main aim being the study of other cell activities such as synthetic processes, absorption or secretion. These processes are often affected by the presence of CO_2 and bicarbonate.

SUMMARY

1. A mathematical theory is given of Pardee's (1949) 'CO_2 buffers' which are designed to maintain an approximately constant carbon dioxide pressure in manometer vessels in which CO_2 is produced or absorbed. The theory is based on simplifying assumptions and indicates how the dissociation constants and the concentration of the base, and the CO_2 pressure, affect the efficiency of the buffer.

2. The buffering capacity or 'retention' of various bases has been measured at 25 and 40° for CO_2 pressures between 1 and 5·7 %. Diethanolamine solutions were found to be the best general CO_2 buffers. Their effectiveness may be increased by various additions.

3. Diethanolamine solutions absorb measurable quantities of oxygen. The autoxidation is inhibited by dithizone or thiourea. Controls are required in each experimental series in order to allow for the O_2 uptake of the CO_2 buffer.

4. Absorption of CO_2 exceeding 95 % is obtainable at low CO_2 concentrations (1 or 2 %) when traditional Warburg manometer vessels are used. With the vessels of Dickens & Šimer, allowing the use of several ml. of CO_2 buffer, absorption over 95 % is possible for gas pressures up to 4 %.

5. Particulars concerning the preparation and use of CO_2 buffers and examples showing their efficiency are given.

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