XI. METABOLISM OF NORMAL AND TUMOUR TISSUE.

V. A NOTE ON THE CARBON DIOXIDE RETENTION OF SERUM: ITS USE IN THE MEASUREMENT OF TISSUE GLYCOLYSIS.

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IN manometric measurements of tissue metabolism in serum or similar fluids the problem of allowing for the CO_2 -combining properties of the medium is constantly arising. On the one hand the increase of CO_2 tension due to respiration leads to a portion of the extra CO_2 being bound by salts of weak acids (*BP*), including particularly the alkali proteinates, of the serum according to the equation:

On the other hand, if as a result of metabolic processes a strong acid (*e.g.* lactic acid, *HL*) is added to the medium, the CO_2 liberated from bicarbonate is less than that equivalent to the amount of acid added, since a portion of the lactic acid reacts with the salts of weak acids as follows:

These considerations have been made the basis of a theoretical treatment of the problem by Warburg [1925], in which for simplification he uses a "serum model" consisting of a solution of a single weak ampholyte (glycylglycine, acid dissociation constant, $K = 4 \times 10^{-8}$), in Ringer solution of the bicarbonate concentration of serum. Such a "model" was found by Warburg to bind CO_2 chemically to about the same extent as serum if the total concentration of dipeptide were made about equal to that of carbonic acid. Using this model, Warburg has developed two sets of equations representing the amount of CO_2 retained by this system for a given change in the pressure of free CO_2 . The former of these refers to the case when the acid added to the serum is carbonic acid, the latter to that when the acid is lactic or other strong acid.

Since in practice it is unusual to find either of these conditions divorced from the other, a compromise is necessary and one or other of the two sets of equations must be adopted as an approximation, according as the production

of carbonic acid (most normal tissues) or of lactic acid (tumours and certain pathological tissues) predominates.

In Part III of this series a method was described for the manometric measurement of respiratory CO₂, respiration, R.Q. and aerobic glycolysis of tissues in bicarbonate-media, including serum. The method there described for measurement of respiratory CO₂ was completely independent of any of the above considerations. That described for measurement of glycolysis in serum, however, was based on the Warburg [1925] principle. For each measurement of tissue glycolysis this method involves a separate control experiment in which the serum must be acidified with an amount of acid nearly equivalent to that produced in the main experiment. At the same time in another vessel an exactly equal amount of acid is added to a simple bicarbonate-Ringer solution. In the latter case the amount of CO₂ liberated is, of course, equivalent to the acid added, and is thus greater than with serum, the difference being a measure of the amount of the retained CO₂. This measurement of retention, however, as Warburg particularly emphasises, is valid only for the exact conditions of the acidification experiment; even the vessel used must be the same one as in the main experiment.

In practice it is convenient to employ the same specimen of serum for a number of measurements, and much time would be saved by a method involving no such separate determination of retention following every main experiment. For this purpose some linear function of the concentration of undissociated protein *HP*, the change of which (ΔHP or $-\Delta BP$), according to equations (1) and (2), is the measure of the retention, would possess the advantage of making possible a direct interpolation over the whole physiological range.

Such a relationship exists between *HP* and the p_H of normal serum. Van Slyke, Wu and McLean [1923], found that the empirical equation:

$$mM. (BP) = 0.068 P (p_H - 4.80) \quad \dots\dots(3),$$

holds for dialysed horse serum, where $P (= HP + BP)$ is the total concentration of protein (g. per litre serum).

Fig. 1.

A somewhat similar result follows from a consideration of Warburg's serum "model," containing a total concentration of dipeptide c , of acid dissociation constant K and at a hydrogen ion concentration H^+ . If in such a model H^+ changes to $H^+ + \Delta H^+$, the corresponding retention (*i.e.* increase in concentration of undissociated dipeptide, HP) is given [Warburg, 1925] by $\Delta HP = \Delta \rho \cdot c$; where $\rho = \frac{H^+}{H^+ + K}$ = Michaelis dissociation residue of the dipeptide.

Such a relationship, plotted graphically, gives a curve of the shape shown in Fig. 1.

Rewriting the above equations we have:

$$H^+ = K \cdot \frac{\rho}{1-\rho},$$

whence

$$\frac{d\rho}{dH^+} = \frac{(1-\rho)^2}{K},$$

and therefore

$$\begin{aligned} \frac{d\rho}{d(p_H)} &= -\frac{d\rho}{dH^+} \times \frac{dH^+}{\log_{10} H^+} = -\log_e 10 \cdot \frac{d\rho}{dH^+} \cdot \frac{dH^+}{d(\log_e H^+)} \\ &= -2.3 \frac{d\rho}{dH} \cdot H^+ \\ &= -2.3 \frac{(1-\rho)^2}{K} \cdot K \frac{\rho}{1-\rho} \\ &= -2.3 (1-\rho) \rho \end{aligned} \quad \dots(4).$$

Table I and Fig. 2 show that in the case of Warburg's "serum model" the

Table I.

ρ	p_H	Δp_H	$\frac{\Delta \rho}{\Delta p_H}$	$\frac{\Delta \rho}{\Delta H^+}$
0.40	7.574	-0.0890	-0.56	0.082
0.45	7.485	-0.0872	-0.57	0.069
0.50	7.398	-0.0871	-0.57	0.057
0.55	7.311	-0.0889	-0.56	0.045
0.60	7.222

Fig. 2.

value of $\Delta\rho/\Delta p_H$ reaches a maximum value at the point of inflexion of the ρ - p_H curve, but is nearly constant over a considerable range on either side of this point, the retention- p_H curve being nearly linear between about p_H 7.7 and 7.1.

The practical convenience of this logarithmic method of representation is clear. It is only necessary to determine the value of retention ($= \Delta\rho \cdot c$ in the case of the dipeptide model) corresponding to a suitable finite change of p_H in order to be able to interpolate with good accuracy for other values of Δp_H , a separate determination of retention for the conditions of each individual experiment being therefore unnecessary.

When the retention curve of serum is plotted in the same way as in the above example with the "serum model," the nature of the conditions necessary for the similar behaviour of the two systems in retaining CO₂ over the same range of p_H is clearly shown. The conditions required for the dipeptide in the "serum model" are that its point of half-neutralisation should lie at about the mid-point of the range required (*i.e.* its p_K should be nearly equal to the value of p_H at this point), and that the total concentration, c , of dipeptide should be so chosen that in this range the slope of its retention- p_H curve ($= c \cdot \frac{\Delta\rho}{\Delta p_H}$) is approximately equal to that of the HP - p_H curve for serum.

The linear nature of the retention- p_H curve for serum rests on experimental evidence. The measurements of Van Slyke, Wu and McLean [1923] were made upon serum which had been dialysed until free from salts, and to which known amounts of Na₂CO₃ had then been added, so that the concentration of total base was thus defined. The empirical equation (3) was found to hold between BP and p_H : the measurements applied to two different specimens of horse-serum and covered a range of p_H 7.07 to 7.74.

Fig. 3. Normal horse-serum.

In order to establish the applicability of this result for normal serum, we have carried out similar experiments on undialysed serum. In this case the total amount of base present was unknown, hence in the curves ΔHP is plotted against the p_H ; the results are shown graphically in Fig. 3. The measurements were made at 38° in vessels attached to Warburg manometers, the manometric

fluid being Clerici solution [Dickens and Šimer, 1931]. The serum (1.5 cc.) was previously equilibrated with gas-mixtures containing known percentages of CO_2 . By adding an excess of strong acid, the bicarbonate content was determined manometrically. In some vessels a weighed volume of suitably diluted tartaric acid, which had previously been evaporated in a side-bulb attached to the conical vessel containing the serum, was used for acidification [Warburg, 1925]. In this case the strength of the acid used was controlled by addition of the same volume of tartaric acid to NaHCO_3 -salt solution. From the resulting pressure readings the CO_2 liberated was calculated. The difference of these amounts gives the amount of carbonic acid combining according to equation (1) above, and hence is equal to the retention, ΔHP . The initial and final p_{H} of the serum were determined by (a) CO_2 analysis by Krebs's method of the original gas-mixtures used and (b) the bicarbonate determinations. p_{K} was taken as 6.10, and the p_{H} calculated by the Henderson-Hasselbalch equation. The curve (Fig. 3) shows that the linear relationship of HP and p_{H} holds for undialysed serum also, at least within the limits of accuracy of these experiments, which are similar to those prevailing in the actual manometric measurement of tissue metabolism [Dickens and Šimer, 1931].

Fig. 4. Dialysed horse-serum.

The measurements on normal serum are somewhat less accurate than those on dialysed serum (Fig. 4), at least for small values of ΔHP , since the accurate

measurement of a relatively small change in a large total amount of combined CO₂ is a matter of difficulty.

In the case of dialysed serum this difficulty can be artificially overcome by adding an abnormally low amount of base to the dialysed serum before equilibrating with the gas mixture. Under the conditions of our experiments, for example, this permits of the use of Brodie fluid (sp.gr. 1.033) as the manometric fluid instead of Clerici solution (sp.gr. approx. 4), with a corresponding fourfold increase in sensitivity. Fig. 4 shows that in the case of dialysed serum over the range of p_H studied the relationship between p_H and CO₂ retention is of similar type to that calculated for the "serum model" and also resembles that found for normal serum.

PRACTICAL APPLICATION TO MEASUREMENT OF GLYCOLYSIS
OR ACID CONSUMPTION BY TISSUES.

Determination of the retention- p_H curve.

Two methods are practicable: (1) the serum is saturated with gas mixtures containing different known percentages of CO₂, and the bicarbonate contents are determined by acidification with excess of strong acid (points thus determined are shown as squares in Figs. 3 and 4); or (2) the serum is acidified slightly with a known amount of strong acid and the CO₂ evolution is measured.

Of these methods the second is usually more convenient, since a liberal supply of gas mixture is necessary for saturation, and in this method only one sample of gas is required.

The same vessels are used as in the measurement of respiratory quotient in serum [Fig. 1, Dickens and Šimer, 1931]. The manometric fluid is Clerici solution. The vessels are first weighed clean and dry, without stoppers, and into the side-bulbs, *B*, suitable amounts of a dilute tartaric acid solution are pipetted and the amounts checked by weighing to the nearest milligram. The protocol at the end of this paper indicates some suitable amounts of acid. The solution is then evaporated by placing the vessels in the oven at 100°, and in the meantime the serum is approximately saturated with the gas mixture (oxygen with about 5 % vol. CO₂) from a cylinder of the compressed gas. 1.5 cc. of the serum is then carefully pipetted into each vessel (inner part *A*) and in order to control possible slight changes of vapour pressure on acidification a measured volume of saline may be measured into the outer compartment, *C*. Two further vessels are required, of which one is used to control the strength of the tartaric acid used, and contains instead of serum 1.5 cc. of a simple NaHCO₃-salt solution: the other vessel contains an excess of 50 % solution of tartaric acid in bulb *B*, and is used for determination of the initial bicarbonate concentration of the serum. All the vessels are connected to the gas supply and shaken in the bath at 38° in a stream of the moist gas for 20 minutes, as was described in detail in Part III. The gas stream is then stopped, the levels in the two limbs of the manometer are made equal and, after the

equilibration has been verified by the constancy of the manometer readings, the acid is washed thoroughly into the main contents of the vessels by removing them from the bath and tipping backwards and forwards.

An example showing the order of magnitude of the resulting pressures and the mode of calculation is given below.

It should be emphasised that the approximately linear shape of the curve should not be assumed except when normal serum is used as the medium. In any case, however, the construction of such a curve merely involves the determination of a few pairs of values of ΔHP and p_H , and is not more troublesome than the determination of retention by direct comparison, since once the curve has been constructed the applicability of the present method is much wider. Serious error may be introduced if the medium used is not kept free from bacteria and, if a large batch of serum is to be used for a series of experiments, it should be passed through a Berkefeld filter and stored in small sterile containers in a refrigerator until required for use.

Application to the measurement of tissue glycolysis.

By the method of measurement of tissue metabolism which has already been fully described [Dickens and Šimer, 1931] the following quantities are directly measured. (For convenience in calculation all pressures are measured in mm. Clerici solution of which $\frac{(760 \times 13.60)}{s} \text{ mm.} = P_0 = 1$ atmosphere, where s is the sp.gr. of the Clerici solution. Concentrations are measured throughout in mm.³ per 1.5 cc. serum.)

Initial partial pressure of total CO ₂ (at t_0)	$= H_0$
Final " " " (at t_1)	$= H_1$
Initial total BHCO ₂	$= B_0 = k_0 \cdot H_{B_0}$
Final " "	$= B_1 = k_1 \cdot H_{B_1}$

where k is the respective vessel constant for CO₂, and H_{B_0} , H_{B_1} are the actual pressure changes on liberation of bound CO₂ by acidification at times t_0 and t_1 .

From these figures the initial and final p_H of the solution may be at once calculated. For this purpose we assume $p_K^{38^\circ}$ for serum = 6.10 [Hastings, Sendroy and Van Slyke, 1928] and $\alpha_{\text{CO}_2}^{38^\circ} = 0.51$ [Van Slyke, Sendroy, Hastings and Neill, 1928].

The calculation of the amount of dissolved CO₂ follows simply from the relationship $H = H_{\text{gas}} + H_{\text{CO}_2} + H_B$. Since the partial pressure of free CO₂ ($H_{\text{gas}} + H_{\text{CO}_2}$) is equal to $H - H_B$, we may write at t_0 dissolved CO₂ = $\frac{H_0 - H_{B_0}}{P_0} \cdot 1500 \cdot 0.51$ (mm.³ per 1.5 cc. serum), and at t_1 dissolved CO₂ = $\frac{H_1 - H_{B_1}}{P_0} \cdot 1500 \cdot 0.51$.

Hence, initial p_H at t_0 (vessel 0) = $p_{H_0} = 6.10 + \log \frac{B_0}{\frac{H_0 - H_{B_0}}{P_0} \cdot 1500 \cdot 0.51}$,

and final p_H at t_1 (vessel 1) = $p_{H_1} = 6.10 + \log \frac{B_1}{\frac{H_1 - H_{B_1}}{P_0} \cdot 1500 \cdot 0.51}$,

$$- \Delta p_H = p_{H_0} - p_{H_1} = \log \frac{B_0}{B_1} + \log \frac{H_1 - H_{B_1}}{H_0 - H_{B_0}} \quad \dots\dots(5).$$

The value of Δp_H being thus known for every experiment, the corresponding retention, ΔHP , is read off at once from the correct region of the retention curve for the specimen of serum used. Here ΔHP is the change between t_0 and t_1 in the amount of base bound by buffers other than bicarbonate contained in the serum. In the absence of buffers other than bicarbonate in the medium, the amount of fixed acid, x_G , formed by the tissue would be exactly equivalent to the change in bicarbonate content x_b , according to the equation

In serum, however, the reactions shown in equations (1) and (2) occur. An amount of base equivalent to ΔHP is thus yielded, by which amount the fall in the bicarbonate content due to (6) is diminished. In this case, therefore, $x_b = x_G - \Delta HP$. The value of aerobic glycolysis, $Q_{CO_2}^{O_2}$ (mm.³ per mg. dry weight of tissue per hour), is then given directly by

$$Q_{CO_2}^{O_2} = \frac{x_G}{m \cdot t} = \frac{x_b + \Delta HP}{m \cdot t},$$

where m is the dry weight of the tissue in mg. and t the duration of the experiment ($t_1 - t_0$) in hours.

It should be noted that before the values of H_0 and H_1 are inserted in the above equations they should be corrected for the slight pressure change (about - 2 mm. of Clerici solution) found necessary in the use of Krebs's method for CO₂ analysis [Dickens and Šimer, 1931].

SUMMARY.

(1) The retention of CO₂ by serum shows an approximately linear relationship to the change of p_H over the range studied.

(2) This result applies to both normal and dialysed serum, and also to the "serum model" of Warburg.

(3) The application of the above observations to the measurement of production or consumption of organic acids by tissues in serum is described.

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PROTOCOLS.

(1) *Determination of retention-curve for serum.*

Horse-serum (inactivated) equilibrated with oxygen containing 4.5 % CO_2 ($H_{\text{gns}}^{\text{corr.}} = 110$ mm. Clerici solution of sp.gr. 3.976: $P_0 = 2600$; $P_0 - P_W = 2430$ mm.). Temperature 38° . See Fig. 3.

Vessel	...	11	7	9	10	11	11
Experiment		Bicarbonate determina- tion	Acidification experiment				Control on tartaric acid solution
Volume of serum (cc.)	1.5	1.5	1.5	1.5	1.5	1.5	1.5 cc. NaHCO_3 - Ringer solution
Contents of bulb	0.1 cc. 6N lactic acid	0.203	0.303	0.406	0.494	0.299	
		g. tartaric acid solution (approx. $N/20$): evapo- rated to dryness before experiment					
Vessel constant for CO_2 (k_{CO_2})	6.29	6.14	6.70	6.56	6.29	6.29	
Pressure on acidification (h mm.)	$H_{B_0} + 108.5$	$h + 27$	+40	+53.5	+66.5	+55.5	
ΔCO_2 (= hk_{CO_2} mm. ³)	—	166	268	351	418	349	
CO_2 equivalent to tartaric acid (mm. ³)	—	237	354	474	577	—	
ΔH_P (mm. ³)	—	71	86	123	159	—	
$B\text{HCO}_3$ (mm. ³ /1.5 cc.)	B_0 683	B 517	415	332	265	—	
p_{CO_2} (mm.)	110	137	150	163.5	176.5	—	
$\text{H}_2\text{CO}_3 = \frac{p}{P_0} \cdot 0.51 \cdot 1500$ (mm. ³ /1.5 cc.)	32.4	40.3	44.1	48.2	51.9	—	
$p_H = 6.10 + \log \frac{(B\text{HCO}_3)}{(H_2\text{CO}_3)}$	7.424	7.208	7.073	6.938	6.808	—	

(2) *Calculation of aerobic glycolysis of liver-tissue in serum.*

The following calculation is for the protocol at the end of Part III [Dickens and Šimer, 1931]. Time of experiment, 3 hours. The serum, gas-mixture and Clerici solution were the same as in (1) above.

Vessel	...	...	7	9	10	11
Vessel constant (k_{CO_2})			6.17	6.72	6.60	6.31
Dry weight of tissue (mg.)			15	18.7	21.2	21.3
$\Delta B\text{HCO}_3$ during experiment (x_b)			—	+3	+3	—
$H_0^{\text{corr.}}$ (mm.)			233	—	—	232.5
H_{B_0}			113	—	—	111
$H_1^{\text{corr.}}$			—	278	284.5	—
H_{B_1}			—	103.5	105.5	—
$H - H_B$			120	174.5	179	121.5
$\Delta p_H = -\log \frac{B_0}{B_1} - \log \frac{H_1 - H_{B_1}}{H_0 - H_{B_0}}$			-0.163		-0.171	
ΔH_P from curve (Fig. 3) mm. ³			+42		+44	
$x_G = x_b + \Delta H_P$			+45		+47	
Aerobic glycolysis, $Q_{\text{CO}_2}^{\text{O}_2}$ (mm. ³ per mg. dry weight per hr.)			+0.80		+0.74	