Experiments on the prevention of agar contamination of the polysaccharides

Cellophan paper method. Agar medium (75 ml.) was poured into each of six large Petri dishes and, after they had cooled, sterile cellophan paper was transferred on to the agar surface by means of sterile forceps, so that the whole surface was covered with cellophan. The dishes were then inoculated with a heavy dose of the usual bacterial suspension of *Rhizobium radicicolum* Bartel A and kept at room temperature for 8 days. The growth which had formed on the cellophan was then scraped off with a glass rod, the cellophan rinsed with distilled water and, after filtration through cotton-wool, the polysaccharide was precipitated in the usual way. Yield, 3.6 g./l. of medium; ash, 15.1%; N, 3.7%.

Porous plate method. A large crystallization dish was filled with 75 ml. of agar medium and a sterile porous plate was embedded in the semi-solid agar by means of sterile forceps. After inoculation with a bacterial suspension, it was kept for 8 days at room temperature. The gummy growth on the porous plate was scraped off, the plate rinsed with distilled water and the polysaccharide obtained as in the usual way. Yield, 0.93 g./l. of medium; ash, 16.8%; N, 5.75%.

Kieselguhr method. Nutrient medium (25 ml.), containing the same contents as the usual lucerne root-agar medium with the exception of agar, was poured into each of eight conical flasks. Acid-free kieselguhr (25 g.) was stirred in until a thick paste was obtained. The flasks were sterilized on 3 successive days and then inoculated as above and kept at room temperature for 10 days. Distilled water (40 ml.) was added, the flasks warmed for 3 hr. at 40° and the mixture filtered and carefully washed out with warm distilled water. The combined filtrates were concentrated

in vacuo at 40–50° and the resultant syrup run into ethanol. Yield, 1.9 g./l. of medium; ash, 34.5%; N, 3.8%.

Large-scale production of polysaccharide by the Clover Bartel A strain

As had been mentioned above, strain Clover Bartel A was chosen for large-scale production. It remained active as gum-producer over a very long period and was sub-cultured every fortnight on slopes of the lucerne-agar metabolism medium. The gum which was obtained on precipitation from ethanol was purified by redissolving it in water and filtering the very dilute solution through kieselguhr. Final ash content, 38.4%.

Purified polysaccharide (60 g.) was obtained, and 200 g. of unpurified material with ash, 68.7%. It was decided not to purify the latter, since it was found that the impurities were separated during a process of methylation and dialysis, which will be described elsewhere.

SUMMARY

1. Various strains of *Rhizobium radicicolum* species were examined with regard to their ability to produce polysaccharides. Strain Clover Bartel A was found to be the most efficient for large-scale production.

2. Experiments on methods for elimination of agar impurities showed that the use of cellophan was the most satisfactory with regard to yield and purity of the polysaccharide formed.

The authors thank Prof. W. N. Haworth, F.R.S., for his interest in this work and Dr Hugh Nicol for providing cultures.

REFERENCES

- Birch-Hirschfeld, L. (1933–4). *Z. Immunforsch.* **8**, 260.
 Cooper, E. A., Daker, W. D. & Stacey, M. (1938). *Biochem. J.* **32**, 1752.
 Heidelberger, M. & Goebel, W. F. (1927). *J. biol. Chem.* **74**, 613.
 — & Hobby, G. L. (1942). *Proc. nat. Acad. Sci., Wash.*, **28**, 516.
 Heidelberger, M., Kabat, E. A. & Shrivastava, D. L. (1937). *J. exp. Med.* **65**, 487.
 Hotchkiss, R. & Goebel, W. F. (1937). *J. biol. Chem.* **121**, 195.
 Reeves, R. E. & Goebel, W. F. (1941). *J. biol. Chem.* **139**, 511.

The 'Q' Notation for Expressing Metabolic Activities in Tissue Slices. A Critical Study

By S. J. BACH, *Biochemical Laboratory, Cambridge*

(Received 24 November 1943)

Ever since Warburg introduced the Q_{O_2} in experiments with tissue slices it has been widely used as a convenient term for comparing experiments performed under different conditions. By expressing the quantity of any substance formed or consumed during a reaction in terms of

$$Q = \frac{\mu\text{l. substance (formed or consumed)}}{\text{mg. tissue (dry wt.)} \times \text{hours of incubation}},$$

it was thought possible to obtain a comparable index of the activity of the tissue even when the conditions of the experiments differed with respect to the amount of tissue used and the period of incubation. While 'Q' was originally designed to express gaseous changes it has become a general habit of workers to use the term also for changes of non-gaseous substances by assuming the compound formed or consumed to be a gas, of which

1 g. ml. = 22.4 l. In this way besides Q_{O_2} , the terms $Q_{\text{amino-N}}$, Q_{NH_3} , Q_{urea} , etc., were introduced.

This may be justifiable so long as the weights of tissue and times of incubation are not very different in the experiments to be compared. If, however, they vary widely the use of Q or even the expression of the result per time unit or per unit weight of dry tissue will lead to very considerable errors. It will be seen from the experiments below that no comparable data can be obtained by multiplying, for example, the result of one experiment by a factor x in order to compare it with the result of another experiment in which the amount of tissue used or the time of incubation was x times greater—a procedure frequently adopted. In view of the importance attributed to the Q of a reaction by some workers on problems of intermediary metabolism, a quantitative study of the possible errors involved and an attempt to readjust such erroneous conclusions seemed desirable.

The arginase present in liver slices served as a model in the experiments below, although the conclusions reached may also apply in principle to any work with tissue slices.

METHODS

Animals. Young rats were used, with an average weight of 150 g., fed on ordinary stock laboratory diet. They were killed and the liver was removed as quickly as possible.

Slices. The livers were sliced as uniformly as possible and weighed (wet weight). This procedure will be referred to later in detail.

Incubation of slices. The method described earlier (Bach, 1939) was closely followed. The total volume of the fluid in the Barcroft vessels varied between 3 and 6 ml. The substrate was added by tipping it from a side-bulb at suitable intervals after the equilibration of the system. At the end of the period of incubation a measured portion of the experimental fluid was taken, acidified with trichloroacetic acid (1% final conc.) and centrifuged. The neutralized supernatant fluid was used for the urea estimation.

Estimation of urea. This was carried out by incubation with urease, followed by ammonia distillation according to Parnas & Heller (1924) in the earlier experiments and with the Pregl apparatus modified by Markham (1942) in the later estimations. In many cases additional estimations were carried out by using the manometric method of Krebs & Henseleit (1932).

Reagents. $l(+)$ -Arginine hydrochloride was prepared from gelatin by the method of Whitmore (1932).

EXPERIMENTAL

The comparability of two results of experiments with tissue slices expressed as Q (see above), depends (a) on the linear relationship between time of incubation and action of the tissue, (b) on the linear relationship between quantity of tissue used and its action, (c) on the assumption that the dry weight of the tissue is independent of the time of incubation

so that tissue slices of identical wet weight will have the same dry weight after different times of incubation.

From the experiments shown in Figs. 1–3 it will be seen that none of these three assumptions applies if times of incubation and quantities of tissue are very different in the experiments to be compared, and the examples given below clearly demonstrate that the Q 's of two such reactions are far from comparable.

Fig. 1. Hydrolysis of arginine by liver slices. Progress curves with various enzyme and substrate concentrations. Curve 1, 50 mg. slices (wet wt.), 0.5% arginine HCl. Curve 2, 50 mg. slices (wet wt.), 0.075% arginine HCl. Curve 3, 200 mg. slices (wet wt.), 0.075% arginine HCl. Ringer's solution with bicarbonate, total volume 6 ml.

Comment on Fig. 1. The three curves represent three incubations of liver slices with arginine over a period of 60 min. Two experiments (2 and 3) differ in the amount of tissue used and two others (1 and 2) in the substrate concentrations. All three experiments show a similar course, i.e. almost complete linearity in the first 10–20 min. and a strong deviation from linearity when incubated for a longer period. Since sufficient substrate was added this deviation could not have been due to exhaustion of the substrate.

It will be seen that the quotient

$$K_1 = \frac{\text{urea-N production after 60 min.}}{\text{urea-N production after 30 min.}}$$

for all three experiments lies between 1.1 and 1.2, whereas it should be 2 if there were a linear relationship.

Comment on Fig. 2. The four curves represent four series of incubations of liver slices with arginine for four different periods and two different substrate concentrations. In each series several different portions of slices of varying weight were incubated for the same length of time. Thus the curves show urea production as a function of the quantity of slices incubated, for different periods of incubation. It

will be seen that while urea production approaches proportionality to the amount of tissue used with very short incubation periods, it is far from it with

Fig. 2. Hydrolysis of arginine by various quantities of liver slices. Curves 1 and 2, 0.5% arginine HCl. Curves 3 and 4, 0.15% arginine HCl. Ringer solution with bicarbonate (total volume 6 ml.).

60 and 90 min. periods, i.e. with incubation times widely used in experiments on intermediary metabolism. Thus

$$K_2 = \frac{\text{urea production with 100 mg. slices (wet wt.)}}{\text{urea production with 50 mg. slices (wet wt.)}} = 1.8, 1.5, 1.34, 1.26 \text{ for periods of 5, 40, 60 and 90 min., respectively.}$$

Fig. 3. Loss of weight of liver slices during incubation. Incubation at 37° in Ringer's solution containing bicarbonate, in presence of arginine. Curve 1, 200 mg. Curve 2, 100 mg. Curve 3, 50 mg. wet wt.

Comment on Fig. 3. The three curves show the loss in weight of various quantities of liver slices during incubation. A considerable loss is to be expected owing to the diffusion of proteins and other constituents out of the tissue (Elliot, Greig & Benoy, 1937); that such a diffusion occurs is easy to demonstrate by precipitating the proteins in the

fluid after the period of incubation. But that the loss of weight can be as large as 50% for 50 mg. slices after 60 min. incubation is surprising. There is a smaller proportional loss with larger quantities of slices.

Thus by taking the dry weights as a basis in the comparison of experimental data a considerable additional error is introduced if the times of incubation vary. Even if the slices were of equal weight before the experiment their dry weights would be different after the incubations.

Examples

The following examples may illustrate the order of magnitude of the errors introduced if the three points mentioned above are left out of consideration.

First example

The following experimental data are to be compared:

(a) 50 mg. liver slices (wet wt.) were incubated with their substrates for 30 min. and produced 1000 μg. urea-N (Fig. 1, curve 1) = 800 μl.

(b) 100 mg. liver slices after incubation for 60 min. gave rise to 1600 μg. urea-N (Fig. 2, curve 2) = 1280 μl.

Dry wts. for (a) and (b) taken from Fig. 3: 50 mg. wet wt. after 30 min. give 8 mg. dry wt. and 100 mg. wet wt. after 60 min. give 19.5 mg. dry wt.

The orthodox calculation gives for

$$Q = \frac{\mu\text{l.}}{\text{mg. dry wt.} \times \text{hours}}$$

$$Q_a = \frac{800}{8 \times 0.5} = 200 \text{ and } Q_b = \frac{1280}{19.5 \times 1} = 65.6.$$

$$\therefore Q_a : Q_b = \text{approx. } 3 : 1.$$

If, however, the conclusions drawn from Figs. 1 and 2 are allowed for, and if the wet weight is taken in place of the dry weight to avoid the errors illustrated in Fig. 3, the following calculation gives the true comparison of the two experiments:

$$\frac{50 \text{ mg. tissue incubated 60 min.}}{50 \text{ mg. tissue incubated 30 min.}} = \frac{1160}{1020} = \frac{1.14}{1}$$

(according to Fig. 1, curve 1) and

$$\frac{60 \text{ min. incubation of 100 mg. tissue}}{60 \text{ min. incubation of 50 mg. tissue}} = \frac{1550}{1158} = \frac{1.34}{1}$$

(according to Fig. 2, curve 2).

$$\therefore \text{ for } Q_a' = \frac{800}{1 \times 1}, \quad Q_b' = \frac{1280}{1.14 \times 1.34}.$$

$$\therefore Q_a' : Q_b' = 800 : 840 = \text{approx. } 1 : 1.$$

The error of 300% shown above is exceeded when the experimental conditions of the two experiments are still farther apart, as in the next example.

Second example

(a) As before: 50 mg. incubated 30 min. give rise to 1000 μ g. urea-N = 800 μ l.

(b) 200 mg. incubated 60 min. give rise to 2000 μ g. urea-N (Fig. 2, curve 2) = 1600 μ l.

Dry wt. from Fig. 3:

50 mg. after 30 min. = 8 mg.,

200 mg. after 60 min. = 33 mg.

$$Q_a \text{ calculated in the orthodox way} = \frac{800}{8 \times 0.5} = 200.$$

$$Q_b \text{ calculated in the orthodox way} = \frac{1600}{33 \times 1} = 48.5.$$

$$\therefore Q_a : Q_b = \text{approx. } 4 : 1.$$

Allowing for disproportionality (Figs. 1, 2):

$$\frac{50 \text{ mg. incubated } 60 \text{ min.}}{50 \text{ mg. incubated } 30 \text{ min.}} = \frac{1160}{1020} = \frac{1.14}{1}$$

(Fig. 1, curve 1) and

$$\frac{60 \text{ min. incubation of } 200 \text{ mg.}}{60 \text{ min. incubation of } 50 \text{ mg.}} = \frac{1992}{1158} = \frac{1.72}{1}$$

(Fig. 2, curve 2).

$$\therefore Q_a' : Q_b' = \frac{800}{1 \times 1} : \frac{1600}{1.14 \times 1.72} \\ = 800 : 816 = \text{approx. } 1 : 1.$$

Slicing and weighing of the tissue

If the weighing is done within 2-3 min. after slicing, the loss of water during weighing is negligible. Blotting the slices before weighing does not improve the accuracy. Table 1 shows a good agreement of wet weight and dry weight (with different periods of incubation) in several parallel weighings.

Table 1. *Reproducibility in the weights of slices*

Exp. no.	Wet wt. (mg.)	Dry wt. (mg.)	Period of incubation (min.)
I	50	8.8	20
I	50	8.8	20
	50	9.06	20
I	100	25.4	0
II	100	24.5	0
I	200	34.07	60
III	200	33.74	60
	200	34.07	60

DISCUSSION

Errors of 300-400% as calculated above are admittedly extreme examples of an erroneous application of the term *Q*. The errors will be less if the *Q*

of experiments with low tissue weight and a long period of incubation are compared with the *Q* of experiments with high tissue weight and a short period of incubation, because the errors may partially cancel out. It follows, however, that for a fair comparison of two tissue slice experiments one of three conditions must be fulfilled: *either* the times of incubation have to be kept below 30 min. and the weight of tissue below 50 mg. (wet weight) so that small variations in both time and tissue weight are linear; *or* if experimental conditions vary more widely, adjustments have to be made as shown above, based on preceding experimental work similar to that represented in Figs. 1 and 2. For while Fig. 3 may be generally applicable to tissue slices, the course of reactions as shown in Figs. 1 and 2 will probably have to be worked out for every particular case; *or* finally, equal wet weights of tissue and equal times of incubation have to be used in all comparative experiments.

The first requirement will be difficult to meet if slow reactions are involved, such as *in vitro* synthesis, necessitating long periods of incubation and comparatively large amounts of tissue in order to obtain significant effects. The second requirement involves laborious experiments in order to find the relations between time of incubation, weight of tissue, and formation of the products of reaction. But it should be possible to meet the third requirement and to use equal weights and times of incubation throughout the whole series of experiments, though the slicing and the weighing of the moist tissue will need special attention.

SUMMARY

1. It has been shown that the arginase activity of liver tissue is not proportional to the weight of tissue slices and the period of their incubation (if exceeding 20-30 min.) and that the dry weight of tissue slices decreases with the period of incubation.

2. Both findings contribute to cause considerable errors, amounting to more than 400% in extreme cases if

$$Q = \frac{\text{metabolic output}}{\text{mg. dry wt.} \times \text{hours of incubation}}$$

is used for the comparison of results from experiments with tissue slices under widely varying experimental conditions. It is suggested that the wet weight rather than the dry weight should be used and the time of incubation and weight of tissue kept constant. The accuracy of slicing and weighing of tissue is discussed.

REFERENCES

- Bach, S. J. (1939). *Biochem. J.* **33**, 1833.
 Elliot, K. A. C., Greig, M. E. & Benoy, M. P. (1937). *Biochem. J.* **31**, 1003.
 Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.

- Markham, R. (1942). *Biochem. J.* **36**, 790.
 Parnas, J. K. & Heller, J. (1924). *Biochem. Z.* **152**, 1.
 Whitmore, F. C. (1932). *Organic Syntheses*, **12**, 4.