BBA 3926

RESPIRATION AND GLYCOLYSIS IN LIVER SLICES PREPARED FROM RATS OF DIFFERENT FOETAL AND POST-NATAL AGES

G. D. V. VAN ROSSUM*

Laboratory of Physiological Chemistry,
University of Amsterdam, Amsterdam (The Netherlands)

(Received October 29th, 1962)

SUMMARY

1. The rates of respiration and glycolysis of liver slices prepared from rats of different foetal and post-natal ages have been determined *in vitro*.

2. Liver slices from foetuses of 17–20 days gestational age respired actively. There was a fall in the rate of respiration at 21–22 days gestation, which was reversed within the first day of post-natal life.

3. The effects of 2,4-dinitrophenol suggest that the fall in the rate of respiration at 21–22 days gestation was due to controlling effects of the reactions of oxidative phosphorylation upon respiration.

4. There was little aerobic glycolysis at any age. Glycolysis was stimulated several-fold at all ages by the addition of cyanide to the incubation medium. The rate of anaerobic glycolysis was greatest in liver slices prepared from foetuses at 17–18 days gestation, and fell continuously throughout the period of growth studied.

5. The respiration of the liver slices at all ages was largely dependent upon endogenous substrate. The dependence of glycolysis in the foetal liver upon the presence of glucose in the incubation medium varied inversely as the glycogen content of the tissue.

INTRODUCTION

The foetal and post-natal development of rat liver is accompanied by considerable changes in respiratory and glycolytic activity. POTTER, SCHNEIDER AND LIEBL[1] and DAWKINS[2] have shown that there is a several-fold increase in the activity of respiratory enzymes in the rat liver during the last five days of gestation and the first few days of post-natal life. At the end of this period the activity of the enzymes studied was at the adult level. However, these changes in the activities of individual enzymes during growth differ from the changes in the rate of respiration of whole cells. Thus, VILLEE AND HAGERMAN[3] found that liver slices prepared from rat foetuses 5–6 days before birth respired at almost the same rate as slices prepared from the adult tissue. Shortly before birth there was a small decrease in the rate of respiration, which was followed by a return to the earlier level within a few hours after parturition.

* Present address: Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pa., (U.S.A.).

Whole rat foetuses show a high rate of anaerobic glycolysis and this activity decreases during development[4,5]. The results of VILLEE AND HAGERMAN[3] suggest that this is also true of the rate of anaerobic glycolysis of the developing rat liver, but DAWKINS[2] could not confirm this finding.

In a study of cation movements in renal cortex tissue from new-born and young animals, WHITTAM[6,7] found that the ability of the tissue to transport cations under anaerobic conditions was closely related to the rate of anaerobic glycolysis. In a similar study of cation movements in liver slices prepared from rats of different foetal and post-natal ages, it has been found that the ability of the liver tissue to transport sodium and potassium under aerobic and anaerobic conditions varies considerably during growth[8]. In order to relate the ability of the tissue to transport cations to its metabolic activity, measurements of the respiratory and glycolytic activity of the slices were made simultaneously with observations of the ion movements. The observations on the metabolic activity of the liver slices are described below.

In agreement with the results of VILLEE AND HAGERMAN[3], it was found that the rate of respiration of the liver slices decreased during the later stages of foetal development and that this decrease was reversed after parturition. These changes were due to a control of respiration by oxidative phosphorylation. The rate of anaerobic glycolysis fell throughout the period of growth studied.

A preliminary account of some of these results has been published[9].

METHODS

the treatment of the animals, preparation of liver slices and details of the incubation procedure are those described previously[8].

Measurement of oxygen uptake

Liver slices were incubated in a conventional Warburg manometric apparatus. Since it was desired to determine the ability of the slices to transport cations simultaneously with the measurements described below, the slices were pre-incubated at 1° before being incubated at 38°. During the last 15 min of the incubation at 1° the manometers were gassed with O_2. After a 10-min equilibration period at 38°, the oxygen consumed by the slices was measured during a further 50-min incubation at 38°. The oxygen uptake was usually linear throughout this time.

After incubation the slices were separated from the incubation medium. When measurements of the lactate or protein contents of the tissue were to be made, the slices from each manometer flask were divided into two groups. The fat-free solid and cation contents of one group were determined by methods already described[8]. The second group was weighed wet and then analysed by methods described below. From the wet weight and solid content of the first group of slices and the wet weight of the second group it was possible to deduce the total weight of fat-free solids responsible for the oxygen uptake of the slices in the flask.

Lactate production

The lactate produced by the liver slices durirg incubation at 38° was taken as a measure of the glycolytic activity. Some lactate was found to be present in the flask

contents after the incubation at 1°; this had probably been formed between the death of the animal and cooling of the liver during which time the tissue must have been relatively anoxic. In determining the amount of lactate formed by the slices during incubation at 38°, the amount of lactate present in each flask at the end of the cold incubation was calculated from the lactate content of control flasks which had only been incubated in the cold bath, and was deducted from the total lactate contained in the flask after incubation at both 1° and 38°.

After incubation, the liver slices were homogenized in water and proteins were precipitated with $HClO_4$, which was then removed by neutralization with KOH. Samples of the remaining solution were then analysed for lactate by the lactate dehydrogenase (EC 1.1.1.27) method of HOHORST[10] as modified by OEI[11]. Samples of the incubation medium, taken after removal of the slices, were also treated with $HClO_4$ and analysed for lactate. It was necessary to measure the lactate content of both tissue and medium since it was found that while most of the lactate formed diffused into the medium, a considerable part nevertheless remained within the tissue. Further, the lactate became distributed differently between the tissue and the medium under different experimental conditions, the ratio of the concentration of lactate in the tissue water to its concentration in the medium being much lower in the presence of the nhibitors, cyanide and iodoacetate, than in their absence (Table I).

TABLE I

DISTRIBUTION OF LACTATE BETWEEN THE TISSUE WATER OF RAT-LIVER SLICES AND THE INCUBATION MEDIUM

The values given are the mean ± standard error of mean (number of observations) of the ratio: $\frac{\text{Concentration of lactate in tissue water}}{\text{Concentration of lactate in medium}}$. The results from liver slices of all age groups have been pooled. For incubation conditions, see text.

	Concentration ratio
After incubation at 1°	13.4 ± 2.2 (22)
After incubation at 1° followed by incubation at 38° with:	
No inhibitor	10.3 ± 1.6 (19)
KCN (1 mM)	3.6 ± 0.3 (32)
KCN (1 mM) + iodoacetic acid (1 mM)	3.7 ± 1.0 (14)

Protein

Liver slices were homogenized in water. Samples of the homogenate were analysed for their protein content by the biuret method[12] as described by CLELAND AND SLATER[13]. The haem moiety of haemoglobin absorbs light at the wavelength used for the protein determination (*i.e.* 540 mμ) and after the protein precipitation procedure of CLELAND AND SLATER the haem moiety of the haemoglobin content of the foetal liver[8] accounted for 3–5 % of the total extinction obtained in the determination of the liver protein. Unless otherwise stated, no allowance was made for this extinction in calculating the protein content of the liver.

RESULTS

Respiration

The rate of uptake of oxygen by liver slices prepared from rats of different ages is shown in Table II. Slices of the two youngest age groups (17–18 and 19–20 days gestation) showed a high rate of respiration in the absence of inhibitors. The rate fell by some 50 % to a minimum value at 21–22 days gestation, but within the first day after birth the rate of respiration again approached the value seen in the youngest slices. This high rate was maintained at least until the fourth day of post-natal life (Table III) but the rate fell somewhat during later growth since the Q_{O_2} of liver slices prepared from adults was significantly lower than that of slices from rats 4 days old.

TABLE II

RATE OF RESPIRATION OF RAT-LIVER SLICES *in vitro*, IN THE ABSENCE AND PRESENCE OF INHIBITORS

Values given are the mean ± standard error of mean (number of observations). For incubation conditions, see text.

Age (days)	*Q_{O_2} (µl/mg fat-free dry wt./h)*					
	No inhibitor	*0.1 mM KCN*	*1 mM KCN*	*5 mM KCN*	*10 mM KCN*	*1 mM KCN + 1 mM iodoacetic acid*
Foetal						
17–18	17.5 ± 1.1 (10)		1.2 ± 0.2 (7)			2.5 ± 0.3 (5)
19–20	17.6 ± 0.6 (15)		1.7 ± 0.1 (19)			3.1 ± 0.5 (5)
21	8.4 ± 0.3 (5)		1.6 ± 0.1 (6)			2.7 ± 0.4 (3)
22	8.4 ± 0.2 (19)	2.9 ± 0.3 (5)	1.6 ± 0.2 (18)			2.4 ± 0.4 (5)
Post-natal						
1 h	9.6 ± 1.3 (4)		2.0 ± 0.1 (4)			2.7 (2)
4–16 h	16.1 ± 0.5 (8)	4.0 ± 0.2 (7)	2.5 ± 0.1 (12)			3.1 ± 0.2 (7)
Adult	11.2 ± 0.5 (11)	4.8 ± 0.2 (7)	2.8 ± 0.1 (13)	3.1 (2)	2.4 (2)	2.7 ± 0.2 (9)

TABLE III

PROTEIN CONTENT AND RATE OF RESPIRATION OF RAT-LIVER SLICES INCUBATED *in vitro*

For incubation conditions see text. The non-haemoglobin protein content was calculated on the basis of the mean values for the haemoglobin content of fresh liver tissue[8]. In this calculation an allowance was also made for the interference of the haem moiety of haemoglobin in the determination of tissue protein. Values given are the mean ± standard error of mean.

Animal age (days)	*No. of observations*	*Protein content*		*Oxygen uptake*		
		Total	*Haemoglobin-free*	*µl/mg fat-free dry wt./h*	*µl/mg protein/h*	*µl/mg haemoglobin-free protein/h*
		(g/kg fat-free dry wt.)				
Foetal						
17–18	6	716 ± 48	590	21.1 ± 2.3	29.4 ± 1.4	35.8
19–20	8	601 ± 34	505	14.5 ± 0.7	24.3 ± 0.9	28.7
21	3	416 ± 11	323	9.2 ± 0.1	22.1 ± 0.9	28.5
22	9	421 ± 11	383	7.3 ± 0.3	17.4 ± 0.5	19.1
Post-natal						
1.5	3	693 ± 55	645	16.5 ± 1.7	23.7 ± 0.5	25.6
4	6	668 ± 38		15.0 ± 0.6	23.5 ± 0.5	
Adult	5	700 ± 34		12.3 ± 0.4	17.2 ± 0.4	

The addition of KCN to the incubation medium resulted in a large inhibition of respiration, a maximal effect being given by a cyanide concentration of 1 mM (Table II). However, a fraction of the oxygen uptake, amounting to 7–25 % of the total (according to the age group), persisted even when the concentration of cyanide was increased to 10 mM. The addition of iodoacetic acid (1 mM) to the medium containing 1 mM cyanide had little further effect on the respiration, although there was a tendency for the inhibition to be released.

During the last few days of gestation there is a large increase in the glycogen content of rat liver, which is followed after birth by a rapid decrease[3,14]. It seemed possible that the changes in oxygen uptake per unit fat-free solids, in the absence of inhibitors, during the peri-natal period of growth were simply due to the unsuitability of the fat-free solids as a reference basis during the large changes in the contribution of glycogen to the tissue solids. Accordingly, in the series of experiments shown in Table III the protein contents of samples of the slices from each manometer flask were determined after measurement of the oxygen uptake. As anticipated from the large changes in the glycogen content of the tissue during growth, there was a fall in the amount of protein per unit fat-free solids towards the end of the gestation period, followed by an increase after birth. Nevertheless, the changes in the respiratory activity of the tissue during growth were similar whether the oxygen uptake was expressed relative to the fat-free dry solids or to the tissue protein. The percentage changes were, however, somewhat smaller in the latter case.

Changes in the amount of haemopoietic tissue in the liver (as measured by changes in the haemoglobin content) were too small to account entirely for the changes in respiratory activity per unit protein in the peri-natal period of growth (Table III).

The rate of respiration which is coupled to the formation of adenosine triphosphate may be controlled by factors concerned in the reactions of oxidative phosphorylation[15–17]. The controlling effect is eliminated by 2,4-dinitrophenol since this substance uncouples respiration from oxidative phosphorylation[18]. The effect of dinitrophenol on the respiration of liver slices of the different age groups was therefore examined in order to see if the changes in the rate of respiration during growth were due to controlling effects of oxidative phosphorylation. Fig. 1 shows that concentrations of dinitrophenol between $5 \cdot 10^{-6}$ and 10^{-4} M stimulated the respiration of liver slices of all the age groups studied, while higher concentrations were inhibitory. Maximal stimulation was usually given by $5 \cdot 10^{-5}$ or $7 \cdot 10^{-5}$ M dinitrophenol. The percentage stimulation tended to fall as the age of the rats from which the slices were prepared increased (Table IV). The maximal rate of respiration in the presence of dinitrophenol, expressed relative to the tissue protein, decreased throughout the period of growth studied. The small decrease between 19–20 days gestation and parturition was not statistically significant ($P > 0.05$). It is therefore concluded that the fall in the rate of respiration in the absence of dinitrophenol shortly before birth, and the subsequent increase, were due to controlling effects of the reactions of oxidative phosphorylation. The fall in the rate of respiration between the age of 1 day and adulthood was still seen in the presence of dinitrophenol and was thus due to other factors.

Glycolysis

Table V shows that the amount of lactate formed by liver slices incubated under aerobic conditions was small and showed little change with age. The addition of KCN

(1 mM) to the incubation medium led to a large increase in the lactate production. The amount of lactate formed per unit fat-free solids during incubation at 38° for 60 min was greatest with slices prepared from foetuses at 17–18 days of gestation and decreased continuously as the age of the animals increased. A similar pattern was obtained when the lactate production was expressed relative to the tissue protein. The extent to which the lactate production was stimulated by cyanide fell considerably during growth.

The stimulation of lactate production by cyanide was prevented by the addition of iodoacetic acid to the medium at a concentration of 1 mM.

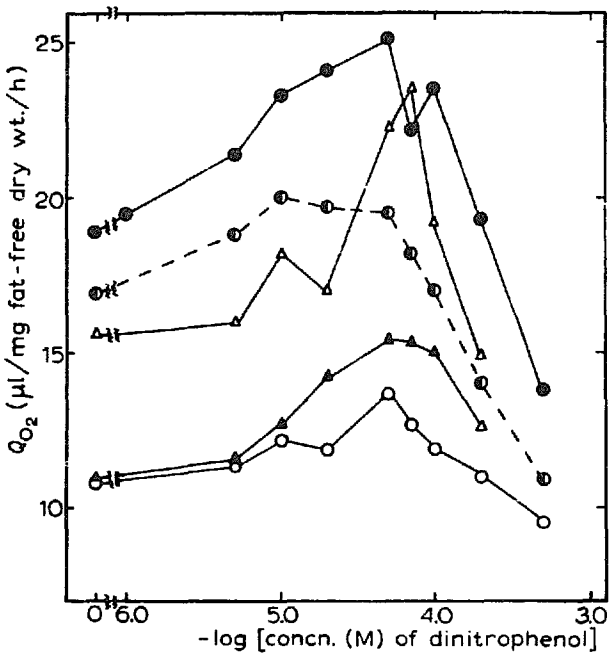

Fig. 1. Effect of 2,4-dinitrophenol on the respiration of liver slices prepared from rats of different ages. Each point represents the mean of 2–7 observations. For incubation conditions, see text. Ages of rats: △—△, 19–20 days gestation; ▲—▲, 22 days gestation and 1 h after birth; ●—●, 4–16 h after birth; ◐—◐, 5 days after birth; O—O, adult.

TABLE IV

EFFECT OF 2,4-DINITROPHENOL ON THE RESPIRATION OF RAT-LIVER SLICES INCUBATED *in vitro*

The values are taken from the results shown in Fig. 1 and represent the rates of respiration of liver slices in the presence of the concentration of 2,4-dinitrophenol giving maximal stimulation of respiration. The values of Q_{O_2} relative to tissue protein were calculated with the aid of data on the tissue protein contents shown in Table III. Values given are the mean ± standard error of mean.

Animal age	*No. of observations*	*Maximal stimulation of respiration by dinitrophenol (%)*	*Maximal Q_{O_2} in the presence of dinitrophenol*	
			(µl/mg protein/h)	*(µl/mg haemoglobin-free protein/h*
19–20 days gestation	3	58 ± 10	41.2 ± 0.9	46.5
22 days gestation and 1 h after birth	5	48 ± 5	36.1 ± 2.1	40.2
4–16 h	7	36 ± 6	36.3 ± 2.3	39.0
4 days	4	35 ± 5	32.9 ± 0.7	
Adult	7	25 ± 4	19.5 ± 0.5	

TABLE V

FORMATION OF LACTATE BY SLICES OF RAT LIVER DURING INCUBATION *in vitro*

The values are the mean ± standard error of mean (number of observations). They represent the total amounts of lactate formed during the 60-min incubation period at 38°. The amount of lactate formed relative to the tissue protein was calculated with the aid of data from Table III. For further details of incubation conditions and calculations, see text.

Animal age (days)	*Lactate formed (mmoles/kg fat-free dry wt.) in the presence of:*			*Lactate formed (mmoles/kg protein) in the presence of KCN (1 mM)*	*Anaerobic lactate / Aerobic lactate (Ratio of columns 3 and 2)*
	No inhibitor	*KCN (1 mM)*	*KCN (1 mM) + iodoacetate (1 mM)*		
Foetal					
17–18	73 ± 21 (3)	947 ± 214 (4)	83 (2)	1320 ± 298 (4)	13.0
19–20	105 ± 39 (6)	811 ± 61 (7)	175 ± 72 (3)	1345 ± 101 (7)	7.7
21	32 (2)	375 ± 31 (4)	73 ± 15 (3)	900 ± 74 (4)	11.7
22	54 ± 14 (3)	315 ± 38 (9)	8 (1)	748 ± 90 (9)	5.8
Post-natal					
4–16 h	69 (2)	227 ± 21 (4)	21 (2)	327 ± 30 (4)	3.3
Adult	21 ± 24 (3)	137 ± 39 (4)	12 ± 9 (3)	196 ± 56 (4)	6.5

Substrate requirements

In the experiments described above, the incubation medium always contained glucose (10 mM). A few experiments were also done with a glucose-free medium in order to see whether the respiration and glycolysis of the liver slices used the exogenous glucose or endogenous materials as substrate. The results (Table VI) suggest that the respiration of the liver slices of most of the age groups examined was dependent upon endogenous substrate. This was also true of liver slices from adult rats which had been fasted for the 48 h preceding the experiment; this treatment is known to reduce the liver glycogen to very low levels[19]. The respiration of liver slices prepared at 17–18 days

TABLE VI

EFFECT OF EXOGENOUS GLUCOSE ON THE RESPIRATION AND ANAEROBIC GLYCOLYSIS OF RAT-LIVER SLICES

Values are the mean ± standard error of mean (number of observations). For incubation conditions, see text.

Animal age (days)	Q_{O_2} *(µl/mg fat-free dry wt./h)*		*Lactate formed (mmoles/kg fat-free dry wt.) in presence of KCN (1 mM)*	
	10 mM glucose	*0 Nc glucose*	*10 mM glucose*	*0 Nc glucose*
Foetal				
17	15.7 (2)	12.0 (1)	908 (2)	298 (1)
20			785 ± 75 (3)	547 ± 150 (3)
22			388 ± 83 (3)	314 (2)
Post-natal				
0.5–1.5	14.7 ± 1.1 (4)	14.2 ± 0.6 (5)		
Adult—fed	11.6 ± 1.2 (3)	11.7 ± 1.3 (3)		
Adult—fasted 48 h	10.4 ± 1.3 (3)	10.0 ± 0.8 (3)		

gestation (which also contained little glycogen[3,14]) was reduced by 24 % in the absence of glucose.

The anaerobic glycolysis of liver slices from foetuses at 17–18 days gestation was reduced by some 70 % in the absence of glucose. The requirement for exogenous glucose as a substrate for glycolysis became less during later foetal development, probably as a result of the increasing glycogen content of the foetal liver towards term.

DISCUSSION

Liver slices prepared from rat foetuses of the youngest age studied in the present work had a high rate of respiration. During the last two days of gestation there was a fall in the rate of respiration which was followed after birth by a rapid increase. These results are similar to those of VILLEE AND HAGERMAN[3]. The fact that these changes were not seen in the presence of dinitrophenol ($5–7 \cdot 10^{-5}$ M) suggests that they were due to variations in the availability of phosphate or phosphate acceptor[15–17].

POTTER, SCHNEIDER AND LIEBL[1] and DAWKINS[2] have shown that there is an increase in the activity of respiratory enzymes in rat liver throughout the period of development studied in the present work. This is probably due to an increase in the number of mitochondria per cell[2]. It has been seen above that the maximal rate of respiration of the liver slices in the presence of dinitrophenol did not increase after birth. In contrast, there was a fall in the rate of respiration in the presence of dinitrophenol between the age of 4 days and adulthood, although POTTER *et al.* and DAWKINS found no fall in the activity of the respiratory enzymes at this period. It must therefore be concluded that the rate of respiration of the rat-liver slices, even in the presence of dinitrophenol, is controlled by factors other than the enzyme concentration. In the presence of dinitrophenol the controlling effects of oxidative phosphorylation upon respiration are eliminated. It therefore seems probable that it is the availability of substrate which is limiting and which prevents the maximal activity of the respiratory enzymes from being manifest in slices utilizing endogenous substrate. The endogenous substrate may be present at insufficient concentrations to allow the maximal rate of respiration or the uncoupling effects of dinitrophenol may limit the supply of ATP which may be needed for the activation of the substrate. It is clear that changes during growth in the rate of respiration of rat-liver slices, under the conditions used in the present work, cannot be taken as evidence of a change in the concentration of respiratory enzymes, as was earlier suggested[9].

The increase in the rate of lactate production by foetal liver under anaerobic conditions, compared to the rate under aerobic conditions, is largely a reflection of an increase in the rate of glucose utilization via the glycolytic path, rather than of an inhibition of the removal of pyruvate by the tricarboxylic acid cycle[20]. However, it has been shown by VILLEE AND HAGERMAN[3] that slices of rat liver prepared at 20–21 days gestation can rapidly use pyruvate for the synthesis of fats under both aerobic and anaerobic conditions. At this stage of growth, therefore, the amount of lactate accumulating may to some extent underestimate the true rate of glycolysis.

It is nevertheless of interest to consider the relation between the rates of glycolysis of the foetal liver *in vitro* (as measured by lactate production) and *in utero*. From the results of Table V it is clear that the rate of glycolysis of the liver slices *in vitro* is very sensitive to the availability of oxygen to the respiratory chain. The results of BARCROFT

et al.[21,22], suggest that some days before the end of gestation the foetus receives an ample supply of oxygen. However, inequalities in the growth of the foetus and the placenta shortly before birth result in a sharp decline in the availability of oxygen to the foetus, and hence to a reduction in the oxygen consumption per unit foetal tissue. From the results presented above it may be anticipated that such a reduction in the rate of respiration, if it is shared by the foetal liver, will result in an increase in the rate of glycolysis of the foetal liver tissue. Simultaneously with the decline in the oxygen supply, there is presumably also a fall in the availability of substrates brought to the liver in the blood, but the glycolysis of the liver is independent of exogenous substrates at this stage of development (Table VI). Thus it may be concluded that, at the earliest period of foetal development studied in the present work, the rate of glycolysis of the liver *in utero* will be much lower than the maximal rate of glycolysis found under anaerobic conditions *in vitro*. Shortly before birth the rate of glycolysis *in utero* will tend to approach the rate observed in the slices incubated *in vitro* in the presence of cyanide.

ACKNOWLEDGEMENTS

I wish to thank Professor E. C. SLATER for his advice and hospitality. This work was carried out while the author was in receipt of a research fellowship from the North Atlantic Treaty Organization.

REFERENCES

1 V. R. POTTER, W. C. SCHNEIDER AND G. J. LIEBL, *Cancer Research*, 5 (1945) 21.
2 M. J. R. DAWKINS, *Proc. Roy. Soc. (London) B*, 150 (1959) 284.
3 C. A. VILLEE AND D. D. HAGERMAN, *Am. J. Physiol.*, 194 (1958) 457.
4 E. NEGELEIN, *Biochem. Z.*, 165 (1925) 122.
5 E. J. BOELL, in J. T. LANMAN, *Physiology of Prematurity, Transactions of the 2nd Conference, March 25-27, 1957, Princeton, N.J.*, Josiah Macy Jr. Foundation, New York, 1957.
6 R. WHITTAM, *J. Physiol. (London)*, 153 (1960) 358.
7 R. WHITTAM, *Biochim. Biophys. Acta*, 54 (1961) 574.
8 G. D. V. VAN ROSSUM, *Biochim. Biophys. Acta*, 74 (1963) 1.
9 G. D. V. VAN ROSSUM, *Biochim. Biophys. Acta*, 54 (1961) 403.
10 H. J. HOHORST, *Biochem. Z.*, 328 (1957) 509.
11 T. L. OEI, *Clin. Chim. Acta*, 7 (1962) 193.
12 A. G. GORNALL, C. J. BARDAWILL AND M. M. DAVID, *J. Biol. Chem.*, 177 (1949) 751.
13 K. W. CLELAND AND E. C. SLATER, *Biochem. J.*, 53 (1953) 547.
14 H. J. SHELLEY, *Brit. Med. Bull.*, 17 (1961) 137.
15 H. A. LARDY AND H. WELLMAN, *J. Biol. Chem.*, 195 (1952) 215.
16 B. CHANCE, *Ciba Foundation Symposium on the Regulation of Cell Metabolism*, J. and A. Churchill, Ltd., London, 1959, p. 91.
17 E. C. SLATER AND W. C. HÜLSMANN, *Ciba Foundation Symposium on the Regulation of Cell Metabolism*, J. and A. Churchill, Ltd., London, 1959, p. 58.
18 W. F. LOOMIS AND F. LIPMANN, *J. Biol. Chem.*, 173 (1948) 807.
19 W. O. FENN, *J. Biol. Chem.*, 128 (1939) 297.
20 C. A. VILLEE, in J. T. LANMAN, *Physiology of Prematurity. Transactions of the 2nd Conference, March 25-27, 1957, Princeton, N.J.*, Josiah Macy Jr. Foundation, New York, 1957.
21 J. BARCROFT, W. HERKEL AND S. HILL, *J. Physiol.*, 77 (1933) 194.
22 J. BARCROFT, L. B. FLEXNER, W. HERKEL, E. F. MCCARTHY AND T. MCCLURKIN, *J. Physiol.*, 83 (1935) 215.