Evidence for the Existence of the Hexose Monophosphate Pathway for Glucose Metabolism in the Normal and Denervated Skeletal Muscle of Rats

By F. ROSSI, M. ZATTI AND A. L. GREENBAUM

Department of Biochemistry, University College London, and Department of General Pathology, University of Padua, Italy

(Received 27 August 1962)

It is generally believed that skeletal muscle uses, almost exclusively, the Embden-Meyerhof glycolytic pathway for glucose metabolism. This view is based on the finding that the activities of the enzymes glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in muscle are very low (Glock & McLean, 1954), and on experiments, performed mainly on the diaphragm with the use of radioactive labelled compounds, that have failed to show appreciable activity of the hexose monophosphate pathway (Bloom, Stetten & Stetten, 1953; Agranoff, Brady & Colodzin, 1954; Bloom, 1955; Cahill, Hastings, Ashmore & Zottu, 1958).

There is a considerable increase in the activity of the enzymes of the hexose monophosphate pathway in atrophic muscles (Rossi & Zatti, 1960; Zatti, Rossi & Tartarini, 1963; McCaman, 1961). The experiments reported in the present paper were designed to answer two questions: (1) Is it possible to define experimental conditions in which the occurrence of the hexose monophosphate pathway could be detected in skeletal muscle? (2) In the event of such an activity being detected, would it be possible to assess the relative importance of the two pathways of carbohydrate metabolism in atrophic muscle?

The results given below indicate that in the normal muscle the mechanism of [^{14}C]carbon dioxide production from [$1\text{-}^{14}\text{C}$]glucose may, to some degree, be different from that producing [^{14}C]carbon dioxide from [$6\text{-}^{14}\text{C}$]glucose, and suggest that, under the conditions employed here, the contribution of the hexose monophosphate pathway in muscles may be appreciable. It has not been possible, however, to show any differences in the activity of this pathway between normal and atrophic muscles.

METHODS

Animals. Wistar rats (150–180 g.) were used in all experiments. They were maintained on stock diet (M.R.C. no. 41B; Bruce & Parkes, 1946) and allowed free access to food. To produce atrophic muscles, the rats were anaesthetized with ether and the left sciatic nerve of each was cut. They were used 15–20 days after the operation.

Preparation of tissues. The animals were killed by cervical dislocation, and the gastrocnemius muscles were excised and placed in ice-cold 0.9% NaCl. The muscles were stripped lengthwise after the end of the fascia or of the tendon had been cut with a pair of fine scissors or a fine scalpel blade; this could be accomplished with little or no damage to the muscle fibres. The strips obtained in this way were about 1 mm. thick and weighed about 25 mg. The muscles were kept immersed in ice-cold 0.9% NaCl throughout the dissection. Strips prepared in this manner are probably the best preparation obtainable for the study of skeletal muscle *in vitro* (Richardson, Shorr & Loebel, 1930; Beatty, Peterson, Bocek & West, 1959; Beatty, Peterson & Bocek, 1960).

Incubation. About 300–400 mg. of muscle strips was put into flasks containing 4 ml. of Krebs-Ringer bicarbonate solution (Krebs & Henseleit, 1932) that had been previously gassed with either $\text{O}_2 + \text{CO}_2$ (95:5) or $\text{N}_2 + \text{CO}_2$ (95:5). The solution contained 4 or more μmoles of unlabelled glucose/ml. and ^{14}C -labelled glucose in the form of either [$1\text{-}^{14}\text{C}$] or [$6\text{-}^{14}\text{C}$]glucose. The specific activity used for each experiment is indicated in the Figure and Tables. Incubations were carried out at 37° for 120 min. with either $\text{O}_2 + \text{CO}_2$ (95:5) or $\text{N}_2 + \text{CO}_2$ (95:5) as the gas phase.

Collection of the carbon dioxide and measurement of radioactivity. At the end of the incubation 1 ml. of 5N-KOH was introduced into the centre well of each flask by injection through the rubber cap and, after the flasks had been shaken for a further 5 min., 2 ml. of 3N-HCl was injected into the outer well. Absorption of the CO_2 was allowed to continue for a further 2 hr., after which the flasks were removed from the bath and the KOH was recovered from the centre well. The centre well was repeatedly washed with small volumes of water and these were added to the KOH. The CO_2 was precipitated as BaCO_3 by adding 5% (w/v) BaCl_2 containing 0.5% of NH_4Cl . The BaCO_3 precipitate was washed twice with water, once with acetone and once with ether. It was then transferred quantitatively to planchets and counted at infinite thickness by using a conventional scaler with an end-window Geiger tube (EHM^{2a}) with a window weight of 1.7 mg./cm.²

RESULTS

The quotient ($[\text{C}^{14}\text{carbon dioxide produced from } [1\text{-}^{14}\text{C}]\text{glucose}]/[\text{C}^{14}\text{carbon dioxide produced from } [6\text{-}^{14}\text{C}]\text{glucose}]$) was greater than 1 for strips prepared from gastrocnemius muscles (Table 1). Since this value might be a property of the glucose

Table 1. *Effect of pyruvate under aerobic and anaerobic conditions and of arsenite on the incorporation of the ^{14}C of $[1-^{14}\text{C}]\text{glucose}$ and $[6-^{14}\text{C}]\text{glucose}$ into ^{14}C carbon dioxide*

The experimental conditions were as follows: 400 mg. of muscle strips was incubated for 2 hr. in 4 ml. of Krebs-Ringer bicarbonate medium containing 4 μmoles of glucose/ml. and 0.15 μC of ^{14}C glucose/ml.; the gas phase was $\text{O}_2 + \text{CO}_2$ (95:5), and the temperature 37°. The final concentration of pyruvate was 30 mM, and that of arsenite 0.3 mM. The $^{14}\text{CO}_2$ was counted as $\text{Ba}^{14}\text{CO}_3$, as described in the text. Results are given as means $\pm$ S.E.M. Each P value represents the probability of the quoted value differing from 100.

No. of animals	Incorporation of ^{14}C into $^{14}\text{CO}_2$ (values for treated slices as a percentage of the corresponding untreated slices)		$\frac{[1-^{14}\text{C}]\text{Glucose}}{[6-^{14}\text{C}]\text{Glucose}}$	
	$[1-^{14}\text{C}]\text{Glucose}$	$[6-^{14}\text{C}]\text{Glucose}$	Control	Plus pyruvate
(a) <i>Pyruvate (aerobic)</i>				
4	43.0 $\pm$ 3.7 (range of counts for untreated slices: 20 700–18 100 counts/15 min.) (P , not significant)	10.3 $\pm$ 1.8 (range of counts for untreated slices: 30 600–15 500 counts/15 min.) (P = 0.03)	1.26 $\pm$ 0.12 (P , not significant)	5.05 $\pm$ 0.18 (P < 0.001)
(b) <i>Pyruvate (anaerobic)</i>				
5	239.0 $\pm$ 38.4 (range of counts for untreated slices: 5300–1500 counts/15 min.) (P = 0.02)	90.3 $\pm$ 8.8* (range of counts for untreated slices: 300–510 counts/15 min.) —	—	—
(c) <i>Arsenite</i>				
6	77.8 $\pm$ 8.6 (range of counts for untreated slices: 22 300–16 300 counts/15 min.) (P , not significant)	21.7 $\pm$ 0.4 (range of counts for untreated slices: 20 300–10 500 counts/15 min.) (P < 0.001)	Control 1.40 $\pm$ 0.08 (P = 0.06)	Plus arsenite 5.20 $\pm$ 0.20 (P < 0.001)

* Three values only.

concentration used, experiments were performed in which the concentration of glucose was varied between 4 and 72 $\mu\text{moles/ml.}$ As shown in Fig. 1, the rate of oxidation of both C-1 and C-6 of glucose increased in parallel as the glucose concentration increased and the quotient remained substantially constant over the whole range tested. The effect of the addition of arsenite (a powerful inhibitor of pyruvate oxidation) on the quotient was also investigated and the results of this are shown in Table 1. With 0.3 mM-arsenite the oxidation of C-6 of glucose was inhibited by about 80 %, whereas the oxidation of C-1 was only inhibited by about 20 %. In the presence of arsenite, therefore, the quotient (^{14}C carbon dioxide from $[1-^{14}\text{C}]\text{glucose}$)/(^{14}C carbon dioxide from $[6-^{14}\text{C}]\text{glucose}$) increased more than fivefold.

Under aerobic conditions, pyruvate (final concn. 30 mM) and glucose (4 mM) decreased the rate of production of ^{14}C carbon dioxide from both C-1 and C-6 (Table 1), but the inhibition from C-6 was considerably greater than that for C-1.

Under anaerobic conditions the production of carbon dioxide from $[6-^{14}\text{C}]\text{glucose}$ was, as might be expected, virtually absent either in the presence or absence of pyruvate. There was, however, some production of ^{14}C carbon dioxide from $[1-^{14}\text{C}]\text{glucose}$.

Fig. 1. Effect of the glucose concentration in the medium on the production of ^{14}C from $[1-^{14}\text{C}]\text{glucose}$ and $[6-^{14}\text{C}]\text{glucose}$ by strips of normal gastrocnemius muscle of the rat. The experimental conditions were as follows: 300 mg. of muscle strips was incubated for 2 hr. in 4 ml. of Krebs-Ringer bicarbonate medium. The concentration of the glucose was varied as shown but the specific activity was kept constant at 0.07 $\mu\text{C/mg.}$ The $^{14}\text{CO}_2$ was counted as $\text{Ba}^{14}\text{CO}_3$, as described in the text. □, $[1-^{14}\text{C}]\text{Glucose}$; ▲, $[6-^{14}\text{C}]\text{glucose}$.

Table 2. *Effect of pyruvate under aerobic and anaerobic conditions on the incorporation of the ^{14}C of $[1-^{14}\text{C}]\text{glucose}$ into ^{14}C carbon dioxide by atrophic muscles*

The experimental conditions were as follows: 400 mg. of muscle strips was incubated for 2 hr. in 4 ml. of Krebs-Ringer bicarbonate medium containing $4\text{ }\mu\text{moles}$ of glucose/ml. and $0.15\text{ }\mu\text{C}$ of $[1-^{14}\text{C}]\text{glucose}/\text{ml.}$; the gas phase was either $\text{O}_2 + \text{CO}_2$ (95:5) or $\text{N}_2 + \text{CO}_2$ (95:5), and the temperature was 37° . The final concentration of pyruvate was 30 mM. The $^{14}\text{CO}_2$ was counted as $\text{Ba}^{14}\text{CO}_3$, as described in the text. Results are given as means $\pm$ S.E.M.

No. of animals	Incorporation of ^{14}C from $[1-^{14}\text{C}]\text{glucose}$ into $^{14}\text{CO}_2$ in the presence of pyruvate (as percentage of the incorporation in the absence of pyruvate)	
	Aerobic	Anaerobic
5	39.4 ± 7.02	202 ± 42.4

glucose although the rate was only about 10% of that found in aerobic conditions. Pyruvate stimulated this anaerobic production of ^{14}C carbon dioxide from C-1 (Table 1).

The experiments with atrophic muscles are summarized in Table 2. Only $[1-^{14}\text{C}]\text{glucose}$ was used in these experiments since it is assumed (see the Discussion section below) that the production of ^{14}C carbon dioxide from C-1 of glucose in the presence of pyruvate is derived entirely (under anaerobic conditions) or mostly (under aerobic conditions) from the operation of the hexose monophosphate pathway. Tables 1 and 2 show that there is no difference in the response between normal and atrophic muscles to the inhibitory (aerobic) or stimulatory (anaerobic) action of pyruvate. In the experiments with atrophic muscles the contralateral muscles were used as controls; the results from these latter were indistinguishable from those of normal muscles.

DISCUSSION

The previous reported values for the quotient (^{14}C carbon dioxide from $[1-^{14}\text{C}]\text{glucose}$)/(^{14}C carbon dioxide from $[6-^{14}\text{C}]\text{glucose}$) in muscle, all for diaphragm, approximate to 1 (Bloom *et al.* 1953; Agranoff *et al.* 1954; Emmelot, Bosch & Van Vals, 1955; Bloom, 1955; Cahill *et al.* 1958), and they have been used as evidence that there is virtually no hexose monophosphate pathway in skeletal muscle. The only exception to these are the values of 0.9, 1.27 and 1.55 reported by Dumont (1960) for slices of sheep-neck muscle. In the present experiment, in which strips of gastrocnemius muscles were used, values greater than 1 have also been found (Table 1). These high values

are not dependent on the glucose concentration. Racker (1956) has shown that the concentration of the glucose regulates the control of the alternative pathway of glucose oxidation in Ehrlich ascites-tumour cells, and McLean (1960) has shown that there is similar action in mammary gland. The present experiment on muscle (Fig. 1) shows that an increase in the concentration of the glucose from 4 to $72\text{ }\mu\text{moles}/\text{ml.}$ results in a parallel increase in the rate of ^{14}C carbon dioxide production from C-1 and C-6 of glucose. Similar results have been found in liver slices, with the difference that in this tissue 30 mM-glucose was sufficient to saturate all the pathways of glucose oxidation (Cahill *et al.* 1958). Although the yield of ^{14}C carbon dioxide from $[1-^{14}\text{C}]\text{glucose}$ relative to that from $[6-^{14}\text{C}]\text{glucose}$ is useful as a qualitative index of the occurrence of the hexose monophosphate pathway, the quantitative evaluation presents considerable difficulties. These problems have been reviewed by Wood (1955), Wood & Katz (1958) and Racker (1957). It is possible, however, to conclude from the results given above that, under the conditions employed, some of the ^{14}C carbon dioxide derived from C-1 of glucose passes by a route other than the Embden-Meyerhof pathway. Evidence in support of this conclusion has been obtained from experiments with inhibitors or acceptors of electrons from NADPH_2 , a line of approach that has been widely applied to the study of the hexose monophosphate pathway (Coxon & Robinson, 1956; Kinoshita, 1957; Cahill *et al.* 1958; Jolley, Cheldelin & Newburgh, 1958; Wenner, Hackney & Moliterno, 1958; Cohen & Noel, 1960; Hoskin, 1960; McLean, 1960). As a result of the present experiments three lines of evidence point to the existence of the hexose monophosphate pathway in muscle.

(1) The action of arsenite on the production of ^{14}C carbon dioxide from $[1-^{14}\text{C}]\text{glucose}$ and $[6-^{14}\text{C}]\text{glucose}$ has been tested since this compound prevents the oxidative decarboxylation of pyruvate (Peters, 1953). In a tissue using only the Embden-Meyerhof pathway of glucose catabolism, both C-1 and C-6 appear in C-3 of pyruvate in equal amounts. Thus the carbon dioxide derived from the Embden-Meyerhof pathway is derived equally from C-1 and C-6. Inhibition of pyruvate oxidation by arsenite should, therefore, decrease ^{14}C carbon dioxide production from $[1-^{14}\text{C}]\text{glucose}$ and $[6-^{14}\text{C}]\text{glucose}$ equally. But this is not the case. The production of ^{14}C carbon dioxide from $[6-^{14}\text{C}]\text{glucose}$ is inhibited to a greater extent than that from $[1-^{14}\text{C}]\text{glucose}$. Hence some part of the ^{14}C carbon dioxide derived from C-1 must arise from reactions that precede the decarboxylation of pyruvate, i.e. it is not produced in the citric acid cycle.

Similar results have been obtained by Hoskin (1960) in brain-tissue slices. Hoskin has pointed

out that cerebral tissue, which normally metabolizes glucose essentially by the Embden-Meyerhof pathway (Bloom, 1955; Sacks, 1957), can be made, by the action of arsenite or Synkavite, to utilize what must be a pre-existing, but unused, alternative route.

The present results do not support the suggestion of Cahill *et al.* (1958) (based on experiments with oxidation-reduction mediators such as pyocyanin and methylene blue) that, not only do muscles lack an active hexose monophosphate pathway, but there is not even a latent (but inactive) pathway present owing to the lack of an adequate electron-transport system.

(2) The effect of pyruvate in anaerobic conditions on the incorporation of the ^{14}C of the ^{14}C -labelled glucose into ^{14}C carbon dioxide has been tested. Under anaerobic conditions the appearance of ^{14}C carbon dioxide must be derived from C-1 via the hexose monophosphate pathway. Further, the fact that pyruvate stimulates the production of ^{14}C carbon dioxide can best be explained by assuming that pyruvate acts as a hydrogen-acceptor for re-oxidizing NADPH_2 via the NADP -linked lactate dehydrogenase, the NADP then being available again for the initial steps of the hexose monophosphate shunt. There is evidence for a NADPH_2 -dependent lactate dehydrogenase in the soluble fraction of the muscle cell (Rossi, Zatti & Tartarini, 1963), which, according to Navazio, Ernster & Ernster (1957), can couple with the dehydrogenases of the hexose monophosphate shunt in the soluble fraction. Similar effects of pyruvate have been demonstrated by Kinoshita (1957) in corneal epithelium, by Wenner *et al.* (1958) in ascites-tumour cells and by Beloff-Chain *et al.* (1962) in diaphragm.

Horecker, Gibbs, Klenow & Smyrniotis (1954) have pointed out that triose phosphate formed either in the Embden-Meyerhof or in the hexose monophosphate pathway can be reconverted into hexose phosphate by reversal of the aldolase reaction, with the possibility that C-6 of glucose might be oxidized via the hexose monophosphate pathway. But the fact that there is virtually no ^{14}C carbon dioxide from ^{14}C glucose under anaerobic conditions, either in the presence or absence of pyruvate, appears to preclude the possibility of randomization of this type under our conditions.

(3) It was also decided to study the effect of pyruvate under aerobic conditions, as in these circumstances it might be expected that pyruvate would provide isotope dilution for the Embden-Meyerhof pathway but not for the hexose monophosphate pathway to any appreciable extent, and would, therefore, lower the rate of production of ^{14}C carbon dioxide from ^{14}C glucose more than

from ^{14}C glucose if a hexose monophosphate pathway existed. In fact this was found: in the presence of pyruvate the quotient (^{14}C carbon dioxide from ^{14}C glucose)/(^{14}C carbon dioxide from ^{14}C glucose) increased about fourfold (Table 1). This also may be taken as supporting evidence in favour of the operation of the hexose monophosphate pathway in muscle, this time under aerobic conditions.

From an examination of the values obtained in the absence of either arsenite or pyruvate (Table 1a and 1c; ^{14}C glucose/ ^{14}C glucose, 1.34 ± 0.11 ; $n, 9$; probability that the ratio is greater than 1, less than 0.001) and, more important, those obtained in the presence of these two compounds, it may be estimated that even in aerobic conditions the hexose monophosphate pathway can account for a considerable proportion of the carbon dioxide production from glucose by gastrocnemius muscles. This is surprising in view of the low activity of the glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase found in muscle by Glock & McLean (1954). But, the great increase of the quotient (^{14}C carbon dioxide from ^{14}C glucose)/(^{14}C carbon dioxide from ^{14}C glucose) in the experiments with arsenite or pyruvate is hard to explain otherwise. It is possible that, under the experimental conditions used (anaerobiosis, arsenite or presence of pyruvate), the activity of the hexose monophosphate pathway is increased above the physiological level by altering the equilibrium conditions of some regulatory factors. This could be related to the accumulation of the pyruvate, which has been shown to act as an electron acceptor for the hexose monophosphate shunt. [The possibility that arsenite has a direct effect on the enzymes of the shunt pathway may be excluded on the basis of the experiments of F. Rossi & M. Zatti (unpublished work), who have examined the effect of arsenite on the activity of the enzymes of the shunt pathway in supernatants of muscle homogenates.] If this explanation is accepted, then the availability of pyruvate can be included, even in muscle, as one of the regulatory factors in the operation of the hexose monophosphate pathway. If this were so, then in many experimental or pathological conditions where the oxidation of pyruvate is impaired there would be a stimulation of the hexose monophosphate pathway. This increase would be most evident in those tissues, e.g. muscle or brain, where this metabolic route normally has a low activity.

The experiments on atrophic muscle were carried out by using only ^{14}C glucose in the presence of pyruvate under aerobic and anaerobic conditions. It may be assumed that under these conditions all the carbon dioxide formed anaerobic-

ally, and almost all the carbon dioxide formed aerobically, is derived from the hexose monophosphate pathway. In spite of the increase that has previously been reported in the activity of the dehydrogenases of the hexose monophosphate pathway (Rossi *et al.* 1963; Zatti *et al.* 1962; McCaman, 1961), the results of the radioactivity experiments showed no difference in either the stimulatory or inhibitory activity of pyruvate on atrophic and normal muscles.

Two explanations may be offered for this finding. First, and less likely, it may be suggested that the increase in the activity of the dehydrogenases *in vitro* are not an expression of an increase of the activity *in vivo*; this assumes that, during the preparation of the soluble fraction of the cells for assay, changes occur in the activities of the enzymes of atrophic muscles which are not paralleled in those of normal muscles. The nature of such changes cannot be guessed at. A second and perhaps more likely explanation would be that under the conditions employed, i.e. in the presence of either added pyruvate or where pyruvate has been allowed to accumulate, the activity of the hexose monophosphate pathway is increased in normal muscle above the physiological level to one approaching maximum potential activity. It is by no means certain that the same applies to atrophic muscle. Rossi *et al.* (1963) have shown that the NADP-linked lactate-dehydrogenase activity is considerably decreased below that of normal muscle, and it may be suggested therefore that in the present experiments the rate of re-oxidation of NADPH₂ by pyruvate in atrophic muscle was slower than that in normal muscle. It could then be that the activity of the hexose monophosphate pathway is limited by the availability of NADP and is therefore not maximal. Thus the enzyme measurements *in vitro* reported by Rossi & Zatti (1960), Rossi *et al.* (1963) and McCaman (1961) need not be related to the measurements made on intact tissue with radioactive compounds, and it is not surprising that the results of the two types of experiment give discordant results.

One fact does, however, emerge from these experiments and this is the very real existence of the hexose monophosphate pathway in muscle. It is not possible to make even an approximate estimation of the importance of this pathway *in vivo*, since, in the attempt to measure the activity of this route under conditions *in vitro*, fairly drastic interference with the normal regulatory mechanisms is inevitable. Before any assessment of the role of the hexose monophosphate pathway in either normal or atrophic muscles can be attempted, it will be necessary to devise a new method of approach to this problem which will necessitate less interference with the balance *in vivo*.

SUMMARY

1. The effect of a variety of conditions on the production of [¹⁴C]carbon dioxide from [1-¹⁴C]-glucose and [6-¹⁴C]glucose, with strips of rat gastrocnemius muscle incubated in Krebs-Ringer bicarbonate medium, has been measured.

2. The quotient ([¹⁴C]carbon dioxide from [1-¹⁴C]glucose)/([¹⁴C]carbon dioxide from [6-¹⁴C]glucose) was usually greater than 1.

3. When the concentration of labelled glucose in the medium was increased the oxidation of both C-1 and C-6 increased in parallel.

4. Arsenite, in aerobic conditions, inhibited the oxidation of C-6 to a greater extent than that of C-1.

5. Pyruvate, in aerobic conditions, also led to a greater decrease of the incorporation of C-6 into [¹⁴C]carbon dioxide than that of C-1.

6. Under anaerobic conditions [¹⁴C]carbon dioxide was produced only from [1-¹⁴C]glucose and this incorporation was stimulated by the addition of pyruvate.

7. No difference could be detected between normal and atrophic (denervated) muscles with respect to the aerobic (inhibitory) or anaerobic (stimulatory) action of pyruvate.

8. The results are discussed in relation to the existence of the hexose monophosphate pathway in skeletal muscle and to the possibility that this metabolic route can be stimulated in experimental or pathological conditions.

This work was financed by funds from the American Dystrophy Association, to whom we express our appreciation.

REFERENCES

- Agranoff, B. W., Brady, R. O. & Colodzin, M. (1954). *J. biol. Chem.* **211**, 773.
- Beatty, C. H., Peterson, R. D. & Bocek, R. M. (1960). *J. biol. Chem.* **235**, 277.
- Beatty, C. H., Peterson, R. D., Bocek, R. M. & West, E. S. (1959). *J. biol. Chem.* **234**, 11.
- Beloff-Chain, A., Catanzaro, R., Chain, E. B., Longinotti, L., Masi, T. & Pocchiari, F. (1962). *Biochim. biophys. Acta*, **56**, 153.
- Bloom, B. (1955). *Proc. Soc. exp. Biol., N.Y.*, **88**, 317.
- Bloom, B., Stetten, M. R. & Stetten, D., jun. (1953). *J. biol. Chem.* **204**, 681.
- Bruce, H. M. & Parkes, A. S. (1946). *J. Hyg., Camb.*, **44**, 491.
- Cahill, G. F., jun., Hastings, A. B., Ashmore, J. & Zottu, S. (1958). *J. biol. Chem.* **230**, 125.
- Cohen, L. H. & Noel, W. K. (1960). *J. Neurochem.* **5**, 253.
- Coxon, R. V. & Robinson, R. J. (1956). *Proc. Roy. Soc. B*, **145**, 232.
- Dumont, J. E. (1960). *Biochim. biophys. Acta*, **40**, 354.
- Emmelot, P., Bosch, L. & Van Vals, G. H. (1955). *Biochim. biophys. Acta*, **17**, 451.

- Glock, G. E. & McLean, P. (1954). *Biochem. J.* **56**, 171.
 Horecker, B. L., Gibbs, M., Klenow, H. & Smyrniotis, P. Z. (1954). *J. biol. Chem.* **207**, 393.
 Hoskin, F. C. G. (1960). *Biochim. biophys. Acta*, **40**, 309.
 Jolley, R. L., Cheldelin, V. H. & Newburgh, R. W. (1958). *J. biol. Chem.* **233**, 1289.
 Kinoshita, J. H. (1957). *J. biol. Chem.* **228**, 247.
 Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
 McCaman, M. W. (1961). *Fed. Proc.* **20**, 301.
 McLean, P. (1960). *Biochim. biophys. Acta*, **37**, 296.
 Navazio, F., Ernster, B. & Ernster, L. (1957). *Biochim. biophys. Acta*, **26**, 416.
 Peters, R. (1953). *Brit. med. Bull.* **9**, 116.
 Racker, E. (1956). *Ann. N.Y. Acad. Sci.* **63**, 1017.
 Racker, E. (1957). *Harvey Lect.* (1955-1956), Ser. **51**, 143.
 Richardson, H. B., Shorr, E. & Loebel, R. O. (1930). *J. biol. Chem.* **86**, 551.
 Rossi, F. & Zatti, M. (1960). *Boll. Soc. ital. Biol. sper.* **36**, 1113.
 Rossi, F., Zatti, M. & Tartarini, A. (1963). *G. Biochim.* (in the Press).
 Sacks, W. (1957). *J. appl. Physiol.* **10**, 37.
 Wenner, C. E., Hackney, J. H. & Moliterno, F. (1958). *Cancer Res.* **18**, 1105.
 Wood, H. G. (1955). *Physiol. Rev.* **35**, 841.
 Wood, H. G. & Katz, J. (1958). *J. biol. Chem.* **233**, 1279.
 Zatti, M., Rossi, F. & Tartarini, A. (1963). *G. Biochim.* (in the Press).

Biochem. J. (1963) **87**, 48

Cytochrome Oxidase and Cytochromes a and a_3 in Crab Mitochondria

By D. H. BURRIN AND R. B. BEECHEY

Department of Physiology and Biochemistry, The University, Southampton

(Received 13 July 1962)

Using a highly-purified mammalian cytochrome-oxidase preparation, Yonetani (1960) unequivocally demonstrated the spectrophotometric separation of cytochromes a and a_3 . In the present paper we give evidence for the presence of cytochromes a and a_3 in phosphorylating mitochondria obtained from the hepatopancreas of *Carcinus maenas* (Beechey, 1961*a, b*). Little detailed biochemical information is available for crustaceans.

Beechey (1961*a*) presented kinetic evidence for the presence of cytochromes a and a_3 in these mitochondria. In the present paper the responses of cytochromes a and a_3 and of cytochrome-oxidase activity to cyanide and carbon monoxide are reported and discussed. A preliminary report has been published (Burrin & Beechey, 1962).

METHODS

Animals. Specimens of *Carcinus maenas* were obtained from Southampton Water. They were maintained in the Laboratory in 75% artificial sea water (Lyman & Fleming, 1940). This water was continuously circulated through the aquarium at 13°.

Preparation of hepatopancreas mitochondria. The mitochondria were isolated in 0.25M-sucrose from the hepatopancreas of *Carcinus maenas* by the method of Beechey (1961*a*). The final pellet was suspended in 0.25M-sucrose or in one of the media shown in Table 1.

Respiratory activity. The consumption of O_2 was measured at 25° in Warburg manometers. Normally CO_2 was absorbed in 20% (w/v) KOH in the centre well. In those experiments where HCN was present CO_2 was absorbed in

a suspension of 10% (w/v) $Ca(OH)_2$, containing $Ca(CN)_2$, in the centre well (Robbie, 1948). Solutions of HCN were prepared before use by mixing and diluting equal volumes of 0.2M-KCN and 0.2N-HCl to give a neutral solution of HCN and KCl (0.1M-HCN-KCl). Rates of respiration are expressed as q_{O_2} (μ l. of O_2 consumed/mg. of protein/hr.).

Protein determinations. These were made by the biuret procedure of Cleland & Slater (1953). The biuret reagent was calibrated with crystallized bovine plasma albumin (Armour Pharmaceutical Co. Ltd.).

Gas mixtures for manometric purposes. These were freshly prepared before each experiment by displacement of water from 5 l. or 10 l. aspirators. The N_2 , CO and O_2 used in these mixtures were taken directly from cylinders. The required atmosphere in the manometric flask was obtained by the method of Umbreit, Burris & Stauffer (1957).

Difference spectra. These were obtained with a Zeiss spectrophotometer PMQ11 fitted with a double monochromator that had glass prisms. These gave half band-widths of 0.7, 1.2 and 1.7 $m\mu$ at 400, 445 and 500 $m\mu$ respectively. Samples were contained in cuvettes with a 1 cm. light-path. The constancy of a preparation during an experiment was checked by repeated reference to the extinction value of the preparation at 445 $m\mu$. If the value changed significantly the experiment was terminated. 445 $m\mu$ was chosen as the reference point since the extinction coefficient of the difference spectrum of cytochromes a plus a_3 is maximal at this wavelength. All experiments were performed at 4°. A thin film of detergent was used on the optical faces of the cuvettes to prevent condensation of atmospheric moisture. Cuvettes containing mitochondria treated with HCN or CO were sealed with a greased cap. At some stage in the experiment when the contents of both the experimental and test cuvettes had been treated identically the difference spectrum of the cuvettes was measured. The resulting