CLXIV. ON THE RESPIRATORY METABOLISM OF *HELIX POMATIA*

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THE results brought forward in this paper have been accumulated during the last three years in the course of experiments on the respiratory metabolism of the edible snail, *Helix pomatia*. This paper does not purport to give a complete picture of the mechanisms concerned in the metabolism of this animal, but rather to summarize the situation as it appears at present, to indicate some metabolic peculiarities which have come to light and to draw attention to some interesting problems which still await solution. The work arose out of an attempt to obtain some information as to the general metabolism of carbohydrates in *Helix*, and it seemed worth while to extend the experiments to cover some other substrates of oxidation.

MATERIAL AND METHODS

The tissue used was the so-called "liver" or hepatopancreas of *Helix pomatia*, an organ which, according to Biedermann [1911], is responsible for the digestion, absorption and storage of the food. There can be little doubt of the metabolic importance of this organ. It is a large, dark brown structure which accounts for the greater part of the whole visceral mass, and produces numerous enzymes, including cellulase, α - and β -glucosidases, α - and β -galactosidases, fructosaccharase and other but less well-defined carbohydrases such as lichenase [see Krüger, 1933; Vonk, 1937]. Proteinases and aminopolypeptidase [Rosén, 1934] and one or more lipases [Kuntara, 1934] have also been found either in the secretion or in the substance of the gland itself.

The main stock of animals was kept in the refrigerator and samples of a dozen or so were taken out often enough to keep up a supply of experimental animals. The use of manometric technique and tissue slices were the principal methods employed, the directions of Dixon [1934] being followed with only minor alterations. Slices were cut from the hepatopancreas of animals which had been starved for 2 to 4 weeks at room temperature; this preliminary starvation went far towards removing stored foodstuffs and made it possible to cut thin slices from the tissue which, when full of stored materials, has the consistency of a soft rubber sponge and is exceedingly difficult to manipulate. The slices were washed in the phosphate-buffered saline described below (agitated by an air blast) till the washings were no longer turbid. No definite indications of a loss of coenzymes or carriers were encountered at any stage in this work.

After the slices were freed from surplus fluid by draining on filter paper, they were weighed on the torsion balance before being placed in the manometric flasks. The dry wt. was determined on a separate small sample of the same tissue when sufficient material was available. In all, 47 such determinations were carried out, the results varying from 14 to 22 % and averaging 18 % of the wet wt. This value was assumed in cases where a separate estimation of the dry wt. was not made.

The solution used for washing and suspending the tissue slices had the composition given below. The various substances the effect of which was to be tested were added to the stock solution, usually to give a final concentration of $M/100$:

	g./l.
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	16.120
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	0.690
NaCl	0.141
KCl	0.443

This solution is allowed to stand for at least 3 days before use since, for some reason as yet undetermined, the use of perfectly fresh preparations occasionally leads to atypical results. The medium contains $M/20$ phosphate, a concentration which lies between the $M/10$ solution used by Dixon & Elliott [1929] and which van Heyningen [1935] believes to be detrimental to animal tissues, and the $M/40$ solution which van Heyningen himself recommends. It was found necessary to use a fairly high concentration of buffer since the tissue itself has a distinctly acid reaction, and as much as 500 mg. of it had sometimes to be suspended in only a few ml. solution. The depression of the freezing point (0.31°), the $p\text{H}$ (7.73 at 28° , electrometric), and the ratio Na^+/K^+ of this medium are the same as those of the haemolymph of normal active snails [see Baldwin & Needham, 1934].

The O_2 uptake of slices suspended in this solution fell off slowly with time, but the same thing happens in CO_2 - NaHCO_3 Ringer [Baldwin & Needham, 1934], and seems to be due to fragmentation of the somewhat fragile tissue rather than to any deleterious effect of the suspension medium. Every care was taken to minimize fragmentation by keeping the shaking machine well lubricated and reducing the rate of shaking to about 70 oscillations per min. As a rule readings were taken only over the first $\frac{1}{2}$ hr., and very seldom over more than 1 hr., and during this period the respiration remained practically linear in nearly every experiment.

The "direct" technique described by Dixon [1934] was used for measuring the rate of respiration, the flasks being filled with pure O_2 and shaken in an electrically controlled bath maintained at 28° . This temperature has been repeatedly used for molluscan tissues and seems perfectly satisfactory [see for example Baldwin, 1935]. Where other methods have been employed they will be referred to in the text.

RESULTS

It was found that there is considerable variation in O_2 consumption from one hepatopancreas to the next, and this made it necessary that all the slices used in any given experiment should be prepared from one and the same animal. Since it is often impossible to cut uniform slices of the tissue, and as the organ is too small to allow of the preparation of a large number of slices from which the best could be selected, the measurements were usually made in duplicate. The results then showed a mean variation of the order of 10%, and consequently each experiment was repeated several times and the results averaged. Each experiment mentioned in the protocols therefore refers to duplicate determinations on one and the same individual except in a very small proportion of cases. The rates of O_2 uptake are expressed throughout the paper in the usual Q_{O_2} notation, with reference to $p\text{H}$ 7.7 and 28° . The quotients found for the control and experimental samples are represented by Q^c and Q^e respectively as a matter of convenience.

The effect of saccharides and other substances upon respiration

It has long been known that large quantities of glycogen can be stored in the hepatopancreas and that it disappears during starvation [von Fürth, 1903; Biedermann, 1911]. Probably much is used in muscular activity, for recent advances in muscle chemistry indicate that all muscles probably make use of closely similar mechanisms for their energy production. Very little is known about carbohydrate metabolism in *Helix*, although this form has been studied more than any other gastropod type. Schwartz [1935] and Wolf-Heidegger [1935] studied the effects of injecting adrenaline and insulin into snails, but could find no significant changes in the blood sugar level. Ermakov *et al.* [1935] on the other hand find that persistent hyperglycaemia develops after extirpation of the peripharyngeal ganglia or the injection of nicotine, while transient effects follow piqure of the ganglia. These workers are of the opinion that the blood sugar is under nervous control.

In addition to glycogen a second polysaccharide, galactogen, is present in the snail. This substance, to which we must refer again, has been studied by May [1931-34], May & Kordowich [1932], and Schlubach & Loop [1937]. Investigations which began before the paper of Schlubach & Loop appeared are also in progress in this laboratory; the results lead us to believe that from the purely chemical point of view May's conclusions are not entirely reliable, and we hope to have definite evidence regarding this in the near future. In the most recent of his papers May has brought together the pertinent observations of earlier workers, and comes to the conclusion that galactogen is a polysaccharide consisting entirely of galactose units and having a peculiar importance in reproductive processes. There are indications in the work of Levene [1925] upon the slimy secretions of *H. aspersa* and *H. pomatia* that a polygalactose or perhaps an acetylated polygalactose may also be important in connexion with slime formation. May has shown that whereas glycogen is present in practically every part of the body of *Helix*, only the albumin gland contains galactogen, and here glycogen is absent. Galactogen accumulates in the albumin gland during the period prior to egg laying and is formed in quantities larger than can be accounted for by the amounts of galactose ingested, presumably being formed from some other sugar, such as glucose [May, 1934, 2]. The eggs themselves are remarkably rich in galactogen and contain no glycogen [May, 1932, 2], but if the animals are starved galactogen may disappear again and be utilized by the organism, but no eggs are then laid [May, 1934, 4]. It is likely therefore that glycogen (or glucose) and galactogen (or galactose) are interconvertible in the organism.

It should be mentioned in passing that one of the two samples of galactogen used in the present work was prepared from the albumin glands of a number of snails. The glands were crushed in alcohol and allowed to harden, boiled with 30% KOH, precipitated with alcohol several times and finally purified by precipitation with alkaline CuSO_4 [May, 1934, 1], followed by repeated precipitation with alcohol. The other sample, kindly given to me by Dr N. W. Pirie, was obtained from the whole bodies in a similar way and freed from glycogen by treatment with salivary amylase. Both preparations were free from reducing sugars and contained over 90% of galactogen.

The carbohydrates studied were added to the tissue in the form of a 2.5% solution, the final concentration in the flask being c. 0.25%. The results are summarized in Table I. It will be seen that most of the sugars added were without effect, since changes of less than 10% cannot be regarded as significant on the basis of only a small number of experiments. Apart from the results

Table I. *Effect of carbohydrates on respiration of snail hepatopancreas*

Carbohydrate	Averages		Increase %
	Q^c	Q^e	
Glycogen (5)	3.44	3.35	- 3
Galactogen (4)	3.51	4.68	+33
Inulin (3)	3.62	3.49	- 4
Raffinose (2)	2.54	2.63	+ 4
Maltose (2)	2.85	2.62	- 8
Lactose (2)	3.00	2.89	- 4
Sucrose (2)	4.42	4.11	- 7
Fructosediphosphate (2)	3.48	3.31	- 5
Glucose (5)	2.93	3.01	+ 3
Mannose (4)	3.76	3.87	+ 3
Galactose (10)	2.66	3.85	+44
Fructose (4)	3.89	4.64	+19
Xylose (6)	2.93	3.42	+17
Arabinose (3)	3.00	2.94	- 2

Note: The figures in brackets refer to the number of experiments carried out with each substrate. None of the experiments was carried out during the "winter period" (Dec.-Feb.); experiments performed at this time are considered separately.

obtained with galactose and galactogen, the general picture is not unlike that given by mammalian liver. Fructose and xylose cause small increases in the rate of respiration, but glucose, glycogen and fructosediphosphate are not oxidized. Correlated with this there is a very low rate of anaerobic glycolysis; a $Q_M^{N_2}$ value of about 1 was found at 28°, and Chappeau [1932] has found results of the same order (0.8-1.0) for the hepatopancreas of another mollusc, the oyster. These figures are comparable with those of 2-3 at 38° recorded for mammalian livers [see Krebs, 1933, 1]. The rates of respiration too are of the same order as for mammalian liver, for the Q_{O_2} values found in all the 92 control samples quoted in this paper (i.e. those to which no substrate was added) varied from -1.13 to -5.19 at 28°, with an average of -2.93, which is very like Chappeau's figure of c. -2 at 28° for the oyster hepatopancreas, and may be compared with the values of -3.4 to -18.7 for mammalian liver at 38° [Krebs, 1933, 1]. There is thus a general qualitative and quantitative resemblance between the activities of the molluscan hepatopancreas and the mammalian liver. Dorman's [1928] contention, that the invertebrate "hepatopancreas" and the vertebrate liver are homologous, could thus claim a certain amount of biochemical support.

But the parallel breaks down when we consider the large effects produced by galactose and galactogen which contrast interestingly with those of glucose and glycogen. It is possible that galactogen, which has a smaller effect than its parent sugar, may be converted into galactose before being oxidized. Neither galactosemonophosphate nor glucosemonophosphate has yet been tried, but it seems unlikely in view of the other results that the latter would be oxidized. Although the galactose ester has been shown not to be attacked by a yeast trained to act on galactose [Grant, 1935], it might be attacked here if phosphoric esters play any part in the suggested conversion of galactogen into galactose. The existence of an enzyme which catalyses the oxidation of galactose is very interesting since it seems that, in general, galactose is converted into glucose before being metabolized [see Grant, 1935]. The presence of such an enzyme in *Helix* must presumably be correlated with the fact that galactose is a major metabolite in this species.

Attempts to demonstrate the presence of dehydrogenase systems with the aid of the methylene blue technique were wholly unsuccessful. Whether the tissue was used in the form of a brei or as slices, the reduction time ran into many

hours in most cases, even in the presence of added substrates. It was therefore necessary to have recourse to manometric measurements again, and the effects of pyruvate, lactate, succinate and α -glycerophosphate have been studied. As Table II shows, only the last three of these substances produced considerable

Table II. *Effect of various substances on the rate of respiration of snail hepatopancreas*

Substrate (<i>M</i> /100)	Averages		Increase %
	Q^c	Q^e	
Pyruvate (3)	2.02	1.86	- 8
Lactate (3 + 5*)	2.51	3.04	+ 21
Succinate (3 + 4*)	2.80	4.00	+ 43
α -Glycerophosphate (7*)	2.33	3.32	+ 43
Alanine (3*)	3.26	4.16	+ 27

Note: The figures in brackets refer to the number of experiments carried out. Where the figure is marked with an asterisk the corresponding number of experiments was carried out during the "winter period".

increases in the rate of respiration. That pyruvic acid is without effect indicates that it is not converted by the tissue into succinic acid at any considerable rate, though this transformation is known to take place in moulds [Butkewitsch & Fedoroff, 1929] and in yeast [Wieland & Sonderhoff, 1932]. The transformation also occurs in mammalian kidney, but not in liver [Elliott, 1937].

In one experiment in which the Thunberg technique was used, 500 mg. fresh slices were set up with buffer, *M*/100 succinate and 2 ml. 1/20,000 methylene blue at 28°, and no less than 80 min. were required for decoloration of the dye. Nevertheless, as we have seen, the rate of O_2 uptake of the slices is of the same order as for slices of mammalian liver. From the data of the experiment just quoted a Q value of about -0.03 can be calculated to correspond to the rate of reduction of the methylene blue, whereas the Q of normal respiration is about -3. O_2 is therefore reduced about 100 times as fast as methylene blue. In the case of yeast [Wieland & Claren, 1932] there is a factor of about 10, and the slowness of methylene blue reduction is due to the failure of the dye to penetrate into the cells. Methylene blue, indeed, is used as a diagnostic reagent for determining the viability of yeast cells. If these cells are disintegrated and their contents thereby made accessible to the dye, a rapid reduction begins at once. Similarly, mammalian liver reduces methylene blue more rapidly in brei than in slice form, but in the case of the snail tissue no more rapid reduction was obtained by using brei preparations.

It seemed worth while to test the hepatopancreas for the presence of a possible inhibitory substance, and this was done by comparing the rates of reduction of methylene blue by washed, chopped frog muscle in the presence of succinate, with and without the addition of aqueous extracts of hepatopancreas, but the reduction times were identical within the limits of experimental error. Although methylene blue is known to inhibit most dehydrogenases to a greater or less extent [Meldrum, 1934, p. 39], it seems unlikely that inactivation of this kind could account for the large difference found here.

The addition of methylene blue at concentrations up to 1/5000 had but little effect on the O_2 uptake of surviving hepatopancreas slices, and cresyl blue was not much more active in this respect (Table III). The mean increase found was of the order of only 20 %, which is very different from the admittedly exceptional increases of 20-30 times found by Barron [1929] for the respiration of red blood cells. Now the addition of an auxiliary O_2 carrier such as methylene blue can

only bring about an increase in the rate of O_2 absorption of a tissue if the rate of action of the reducing systems of that tissue is limited by that of the normal O_2 -carrying systems, suggesting that in the present case the rate of O_2 transport in the tissue is not the limiting factor. This possibility was investigated by reducing the rate of respiration to about one-fifth by poisoning with $5 \cdot 10^{-3} M$ cyanide. The experiments showed conclusively that even then the respiration is not stimulated to any significant extent (+8%) by the addition of 1/5000 methylene blue. Dixon & Keilin [1936] have pointed out, however, that the inhibition produced by cyanide is not reversible under all conditions, but it is very improbable that irreversible inhibition can have played a significant part here since, in experiments in which no precautions were taken to prevent the distillation of HCN from the suspension medium into the alkali used for CO_2 absorption, the inhibition fell off very markedly in the course of time.

Table III. *Effects of dyestuffs on respiration of hepatopancreas*

Dye and concentration	Substrate	Averages		Increase %
		Q^e	Q^e	
1/50,000 methylene blue	Galactose (3)	2.88	2.95	+ 2
1/5000 methylene blue	Succinate (3)	3.60	4.22	+17
1/5000 cresyl blue	Succinate (3)	3.04	3.93	+29

(Concentration of substrate $M/100$ in all cases.)

The failure of methylene blue to exert its usual effect when added to hepatopancreas tissue need not, however, be taken as conclusive evidence that the mechanisms of cellular respiration here are essentially different from those found in other tissues. It must be supposed that methylene blue fails to reach the reducing systems for some reason, though not, apparently, because it fails to penetrate into the cells. As we shall see, there is reason to think that the oxidation of succinic acid, at any rate, is catalysed by a typical succinic dehydrogenase in this as it is in other tissues.

A complete survey of the individual dehydrogenases has not been attempted. The data of Table II suggest that powerful succinic and α -glycerophosphate dehydrogenases are present, together with a rather weaker lactic enzyme, while those of Table I may indicate the presence of a powerful dehydrogenase for galactose. In view of the recent work of Szent-Györgyi [1937] and Krebs & Johnstone [1937], for example, it seemed desirable to carry the study of the supposed succinic dehydrogenase a little further. This was done by studying the influence upon the respiration of surviving hepatopancreas slices in the presence of succinate of (a) narcotics, which inhibit the majority of dehydrogenases, and of (b) malonate, which inhibits the succinic enzyme competitively and rather specifically.

The concentrations of various narcotics required to reduce to 50% the O_2 uptake of washed frog muscle in the presence of succinate have been determined by Sen [1931], and in the present case four different narcotics were used at the concentrations given by this author. Well washed slices of the tissue were suspended in the usual phosphate solution in the presence of $M/100$ succinate, with and without the addition of phenylurea, symm. diethylurea, urethane and phenylurethane. The data in Table IV (a) show that, although different individual narcotics produce varying degrees of inhibition, the average of all the results is very close to the expected 50% inhibition, and it therefore seems reasonably likely that the hepatopancreas oxidizes added succinate by means of a typical succinic dehydrogenase. More conclusive results were obtained

Table IV. *Effects of certain respiratory inhibitors*

Inhibitor	Molarity	Averages		Increase %	
		Q^c	Q^e		
(a) Phenylurea (2)	0.028	4.71	2.78	-41	} Av. 46 %
Diethylurea (2)	0.350	3.67	1.79	-51	
Urethane (2)	0.670	3.67	1.02	-72	
Phenylurethane (2)	0.003	3.53	2.79	-21	
(b) Malonate (1)	0.010	4.31	4.08	-5	
„	0.020	4.41	3.54	-20	
„	0.050	4.49	3.11	-31	
„	0.100	4.52	2.40	-47	
(c) Malonate (3)*	0.100	2.09	1.61	-23	

Substrate *M*/100 succinate in all cases except that marked with an asterisk, in which no substrate was added.

in experiments in which malonate was used. Of the respiration taking place in the presence of succinate, nearly 50 % is due to the addition of that substance (Table II), and about the same percentage is inhibited by *M*/10 malonate (Table IV (b)). Quastel & Wooldridge [1928] found that the addition of *M*/14 malonate inhibits more than 95 % of the activity of the succinic dehydrogenase of *Bact. coli* in the presence of *M*/140 succinate. The fact that the relative concentrations of substrate and inhibitor are the same in both these cases very strongly suggests that the respective dehydrogenases must also be similar. The effects of *M*/10 malonate upon the basal respiration of hepatopancreas slices was also tested (Table IV (c)) and it was found that, even in the absence of added succinate, 20–25 % of the respiration is inhibited, suggesting that the succinic dehydrogenase system may be in some way concerned in a considerable proportion of the basal respiration of this tissue.

Metabolism during the winter period

It was found in the course of experiments carried out between December 1936 and February 1937 that the usual effect of galactose upon the rate of respiration cannot in general be observed at this time of year. The more or less complete disappearance of this effect during what might be called the “winter period” is interesting from several points of view. Table V (a) gives a series of confirmatory data obtained during the winter period of 1937–38, and it will be observed that although a fairly, though not typically, large increase occasionally follows the addition of galactose to the tissue slices (Exps. 146, 148), the effect is absent in most cases. These data may be compared with those obtained at other times of year and shown in Table IV (b). The average increase for the whole series shown in Table V (a) is only 10 %, a value which is just on the borderline of significance. There is no reason to suppose that the winter period is associated with any peculiarities of metabolism so far as the other systems which were studied are concerned, for all these were found still to be active (Table VI). This suggests that the suppression of the galactose effect is not attributable to a breakdown on the part of the O_2 -carrying system (i.e. the cytochrome + cytochrome oxidase system or its counterpart). The breakdown of the mechanisms by which galactose is ordinarily oxidized might be due (a) to the absence of some special carrier or coenzyme, or (b) to the absence of the enzyme itself. The first of these possibilities has not been tested since it was not found possible to extract any of the enzymes from the tissue itself.

Table V. *Effect of galactose on respiration of snail hepatopancreas*

Exp. no.	Q ^c	Q ^a	Increase %
(a) During winter			
143	4.24	4.20	-1
144	2.42	2.50	3
145	1.76	1.85	5
146	1.97	2.78	41
147	1.88	2.06	10
148	1.13	1.35	19
149	2.32	2.52	9
150	2.23	2.54	14
151	1.58	1.70	8
Averages	2.17	2.39	10
(b) At other periods			
8	3.85	5.31	38
15	2.51	2.62	4
19	2.40	3.52	47
22	2.18	4.06	86
30	3.38	5.92	75
64	2.22	3.10	40
65	3.22	4.79	49
66	2.45	2.91	19
152	2.45	3.33	36
153	1.95	2.93	50
Averages	2.66	3.85	44

Table VI. *Metabolism during winter period*

Substrate	Mean increases %		Data in table no.
	"Summer"	"Winter"	
Galactose	44	10	V (a, b)
Alanine	—	27	II
Succinate	52	38	II
Lactate	19	23	II
α-Glycerophosphate	—	43	II

The alternative view involves consideration of the galactose enzyme as an "adaptive" one, disappearing during the winter for some reason as yet unknown, and reappearing in the spring, perhaps in response to the presence of galactose in the food which is then ingested. There is now a fair body of evidence to show that bacteria can develop enzymes of this kind in response to the presence of certain particular substances in the culture media, and even among Metazoa there are examples of the production of adaptive enzymes [Stephenson, 1937; Yudkin, 1938].

It seems possible that the suppression of the oxidation of galactose by the hepatopancreas during the winter period may be a physiological, seasonal change, correlated with the fact that the snail normally spends this part of the year in hibernation. It is not known whether the seasonal changes in metabolism which take place in certain animals are associated with corresponding "adaptive" changes in enzymic activity, even in the extreme cases in which hibernation takes place. Indeed, we know very little about the metabolism of hibernation [Gorer, 1930]. It is generally believed that in the cases of the hedgehog and the marmot, the metabolism of carbohydrate is almost entirely suppressed in favour of that of fat during the winter sleep, but whether there are any changes in the enzymes concerned we cannot say. Considerable metabolic differences exist between winter and summer frogs [see Tigerstedt, 1914, and Adler, 1921, for example], and seasonal variations are known even in animals which do not pass

the winter in a state of inanition. Sherwood [1936], for instance, has found small but perfectly definite seasonal changes in the basal metabolic rate of rats. Seasonal variations such as these may well be associated with changes in enzymic activity, and the disappearance, or at any rate the suppression, of the oxidation of galactose in "winter" snails may perhaps be an example of this.

There is reason to believe, however, that galactose can be utilized in some way by winter snails, for galactogen may disappear from the albumin glands of starved specimens after the stores of glycogen have been depleted [May, 1934, 4]. If, as seems to be the case, we must regard galactogen as a substance which is stored up in preparation for the egg-laying season, suppression of the oxidation of galactose during hibernation might be regarded as a device for conserving this polysaccharide as far as possible.

Oxidase systems

The presence of xanthine oxidase in the hepatopancreas of *Helix* has already been demonstrated by Przyłeczki [1926] and confirmed by Baldwin & Needham [1934], but it could not be shown by the technique used in the present experiments. No increase in the rate of respiration (-2%) could be found following the addition of $M/100$ hypoxanthine to tissue slices, or to a preparation obtained by grinding the fresh tissue with half its own weight of sand and five volumes of phosphate buffer, centrifuging, half-saturating the centrifugate with ammonium sulphate, centrifuging again and finally taking up the precipitate in phosphate buffer. It is known, however, that xanthine oxidase has a great affinity for its substrate. For the xanthine oxidase of milk, Dixon & Thurlow [1924] give a Michaelis constant of $3 \cdot 10^{-5} M$, and it is quite possible that in the present experiments the enzyme was already saturated with its substrates, considerable amounts of which are believed to be present in the hepatopancreas tissue [Wolf, 1933].

The presence of amino-acid deaminase was readily demonstrated. Since pyruvate had already been found to have no effect upon the rate of respiration, alanine was a suitable substrate for these experiments, and a $M/100$ solution of the *DL*-acid was employed. The results are shown in Table II. The presence of the deaminase was also detected by shaking 5.23 g. washed slices with 500 mg. alanine in 25 ml. $\text{CO}_2\text{-NaHCO}_3$ Ringer [see Baldwin & Needham, 1934] to which 1 ml. saturated As_2O_3 had been added [cf. Krebs, 1933, 2]. The shaking was continued for 5 hr. at 28° and the fluid then deproteinized with 5 ml. 30% trichloroacetic acid. When cold, the filtrate received 10 ml. saturated 2:4-dinitrophenylhydrazine in 2*N* HCl, and crystallization began at once. The whole was left in the refrigerator till next day and the crystals were then collected on the centrifuge, washed twice with $N/4$ HCl and three times with distilled water and finally dried *in vacuo*: m.p. 208° . After recrystallization from ethyl acetate the purified product came out in hexagonal plates, m.p. 216° . Found: C, 40.4; H, 3.1; N, 21.0%. Calc. for the 2:4-dinitrophenylhydrazone of pyruvic acid; C, 40.3; H, 2.99; N, 20.9%. An entirely similar product was obtained by allowing an acetone powder preparation of the hepatopancreas to act upon alanine [cf. Krebs, 1935].

Colour tests were carried out on *p*-phenylenediamine, *o*-phenylenediamine, catechol, 3:4-dihydroxyphenylalanine and tyrosine. When well washed slices of the hepatopancreas were added to dilute aqueous solutions of these substances a deep purple coloration was produced within $\frac{1}{2}$ hr. in the case of *p*-phenylenediamine, but the other reagents showed no appreciable colour change after 24 hr.

If the slices were not thoroughly washed, weak positive reactions were given also by catechol and 3:4-dihydroxyphenylalanine, and Pugh [1930] has found that these substances, but not monohydric phenols, are also oxidized by extracts prepared from the large black slug, *Arion ater*. In both these cases tyrosinase must be regarded as absent. But it seems difficult to attribute the powerful and decidedly specific oxidation of *p*-phenylenediamine to any enzyme other than the cytochrome (indophenol) oxidase.

Confirmation of the presence of this enzyme was obtained manometrically by measuring the increase in O_2 uptake resulting from the addition of a few mg. solid *p*-phenylenediamine to slices of hepatopancreas respiring in $M/100$ succinate. Characteristically large increases (average +360%) followed the addition of this reagent. The effect was counteracted to the extent of 70–80% by the simultaneous addition of $10^{-2} M$ cyanide, and the presence of cytochrome oxidase thus seemed to be very probable. Further confirmation of this was obtained by taking advantage of the fact that cytochrome oxidase is powerfully inhibited by cyanide. In the majority of animal tissues it seems that 60–80% of the total respiration is inhibited by $10^{-3} M$ cyanide and therefore mainly due to the cytochrome system.

The results of much early work on cyanide inhibition were invalidated because no precautions were taken to prevent distillation of cyanide from the suspension medium into the alkali used for CO_2 absorption, but Krebs [1935] has described equilibrating solutions which overcome this difficulty. These were used in the present experiments, and with very satisfactory results, for whereas the inhibition passed off with time if this precaution was not taken, no such effect was detectable when Krebs's solutions were employed. The results of the experiments, which were carried out in the presence of $M/100$ succinate, are shown in Fig. 1. They closely resemble those obtainable with many other animal

Fig. 1. Effect of cyanide on respiration of hepatopancreas.

tissues. A maximal inhibition of 80–85% is reached at a cyanide concentration of $10^{-3} M$, and the concentration of cyanide required to inhibit 50% of the total cyanide-sensitive respiration is about the same as that for cytochrome oxidase. It therefore seems reasonably certain that cytochrome oxidase is present in the hepatopancreas.

Perhaps there has been no more fundamental contribution to the subject of cellular respiration than the discovery by Keilin [1925] of cytochrome and of the part which it plays in living tissues [Keilin, 1929; 1933]. It seems, indeed, that

the cytochrome + oxidase system is of almost universal occurrence in aerobic organisms, but its functional importance has been definitely established in only a few particular tissues. Although various unicellular organisms and some mammalian tissues have now been examined it must be owned that the lower Metazoa have scarcely been studied at all from this point of view, although Keilin [1925] has studied the biological oxidation and reduction of cytochrome in the wing muscles of the wax-moth, *Galleria*.

An attempt was made to demonstrate the presence of cytochrome by micro-spectroscopic examination of slices of the hepatopancreas. Whereas Keilin's [1925] finding of cytochrome in the muscles of the radula was readily confirmed, no trace of cytochrome could be found in the hepatopancreas. In this respect again the hepatopancreas resembles mammalian liver. The tissue is so darkly coloured that only small thicknesses of it could be examined, and it is therefore not impossible that minute amounts of cytochrome may have been present but evaded detection. The only visible band corresponded to the α -band of reduced heliocorubin, and this was visible even after the tissue, which was used in the form of thin slices piled on top of each other, had been subjected to very vigorous washing.

This pigment, heliocorubin, is very widely distributed. It occurs in the gut juice of starving specimens of *Helix*, and according to MacMunn [1885], who described it under the name of enterohaematin, it is present also in the gut contents of many other gastropods and also in some crustaceans. Pantin [1932] states that it is to be found also in the gut of an annelid worm, *Aphrodite*. It appears to be made in the hepatopancreas and poured out into the gut, and "the heliocorubin in the liver of the crayfish appears to be exactly the same as molluscan heliocorubin" [Anson & Mirsky, 1925]. In *Helix* at any rate it must probably be of physiological importance, for if a starving snail be fed on filter paper, no trace of heliocorubin can be detected in the faecal mass. This suggests that the pigment is not to be regarded simply as a waste product, analogous perhaps to the bile pigments of the mammals, but that it is actively retained in the body. In view of the fact that it is autoxidizable at the rather acid pH of the gut contents, Anson & Mirsky [1925] suggested that it might act as a respiratory pigment, but since it is found in the gut and the hepatopancreas it is a little difficult to see how it could play any important part in the respiration of the animal as a whole.

It seems not inappropriate at this point to put forward the tentative hypothesis that, while cytochrome is in all probability the chief ultimate H acceptor in many tissues, it is replaced by heliocorubin in the hepatopancreas of *Helix*, and perhaps elsewhere. Experiments are in progress with a view to testing the possible truth of this hypothesis, and in the meantime there is some apposite information. It is already known that cytochrome is replaced by other haematin compounds in certain tissues, among which mammalian liver is included. Keilin [1929] has shown that its place is taken by another type of haemochromogen in facultative anaerobes, the liver cells of mammals and in the cells of some gastropods. The actiniohaematin of the *Actinia* is also a pigment of this kind [Roche, 1936, 2; see also the review by Roche, 1936, 1]. Heliocorubin itself is a haematin pigment, the haem of which is identical with that of haemoglobin [Dhr & Vegezzi, 1917; Anson & Mirsky, 1925] and very like that of cytochrome *c*, if not identical with it [Hill & Keilin, 1930]. In slightly acid solutions it is autoxidizable, a property which it shares only with haemoglobin, and with cytochrome in rather strongly alkaline solutions. Near the neutral point it loses this property but is then oxidizable in the presence of cytochrome oxidase,

just as is cytochrome itself under physiological conditions [Keilin, 1925, 1933]. These properties fit it, more than any other known haematin compound, to take the place of cytochrome.

Evidence for the presence of cytochrome oxidase in the hepatopancreas of *Helix* has already been presented and there can be little doubt therefore that helicorubin, which is certainly present in this tissue, can be oxidized there under the conditions obtaining *in vivo*. With regard to its possible reduction under biological conditions, some preliminary experiments have already shown that it is very rapidly reduced by traces of cysteine, and rather more slowly by certain biological systems. Further work in this direction is in progress.

SUMMARY

1. The results of a number of experiments on the respiratory metabolism of the hepatopancreas of *Helix pomatia* show that on the whole this tissue resembles the liver tissue of vertebrates fairly closely, but differs markedly from it in that its rate of respiration is much increased by galactose and galactogen.

2. Succinate, lactate, α -glycerophosphate and alanine also increase the rate of respiration.

3. During the winter the oxidation of galactose is largely suppressed, though succinate, lactate, α -glycerophosphate and alanine still produce their usual effects. It is suggested that this may be physiological seasonal change.

4. The oxidation of succinate appears to be due to a typical succinic dehydrogenase. This is inhibited by narcotics and by malonate in the same way and to the same extent as the succinic dehydrogenase of other tissues.

5. No rapid reduction of methylene blue by the tissue could be demonstrated under any of the conditions tried, nor could the respiration of normal or cyanide-poisoned tissue be accelerated by means of methylene blue.

6. Cyanide in $M/1000$ solution inhibits 80 % of the respiration of the hepatopancreas, and the inhibition seems to be attributable to the poisoning of cytochrome oxidase. The presence of this enzyme seems to be highly probable.

7. No cytochrome could be detected spectroscopically in the tissue; the only haematin compound detectable in this way is helicorubin. A possible function of this pigment is tentatively suggested and discussed.

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