THE SUCCINOXIDASE SYSTEM IN OYSTER MUSCLE

BY GEORGE FREDERICK HUMPHREY
Department of Biochemistry, University of Sydney, Australia

(*Received* 19 *March* 1947)

(With One Text-figure)

The importance of the role which the C_4-dicarboxylic acids play in tissue respiration was pointed out by Szent-Györgyi in 1937. It is now known that succinic dehydrogenase is one of the few dehydrogenases which react directly with the cytochromes, and the 'succinoxidase' system has been investigated in a large number of vertebrate tissues. The action of succinate on the oxygen uptake of a few invertebrate tissues has been studied, but no detailed consideration has been given to the possible occurrence of a succinoxidase system. This system becomes especially important in view of the varied response of such tissues to agents like cyanide and azide, which have a marked effect on cytochrome oxidase.

The occurrence of cytochrome in lower organisms has not been widely investigated, although it is known that cytochrome oxidase is of frequent occurrence. Also, in the snail, there occurs helicorubin (Dhéré & Vegezzi, 1917), a pigment whose chemical properties resemble those of cytochrome (Keilin, 1933). Baldwin (1938) found that in this animal, cytochrome occurs only in the radula.

In the present paper, some properties of the succinoxidase in oyster muscle have been investigated, particularly with reference to the action of inhibitors. The effect of the same inhibitors on the oxygen uptake of homogenates has been determined.

MATERIALS

The oysters used were 2–3 years' old specimens of *Saxostrea commercialis*, from the C.S.I.R. Experimental Farm at George's River, Sydney, Australia.

Cytochrome C was prepared from pig heart by the method of Keilin & Hartree (1937) but was dialysed against distilled water, following the recommendation of Potter (1941). The concentration was determined by measuring the absorption at 550 mμ after oxidation with ferricyanide and reduction with hydrosulphite. The results for both forms agreed within 5%.

Sodium succinate was used as a neutralized (pH = 7·1) solution of succinic acid. The inhibitors were similarly neutralized sodium salts.

METHODS

Homogenates were prepared as previously described (Humphrey, 1946) but at room temperature. The same manometric technique was employed. When cyanide was used as an inhibitor the centre-well contained 0·2 ml. of KOH-KCN solution (Krebs, 1935).

Inhibitors were always placed in the main compartment when preparing the flasks and would therefore be in contact with the enzyme for about 20 min. before the initial reading. The succinate was always tipped in from a side-arm. When added, cytochrome was present in a final concentration of $10^{-5}M$.

Succinoxidase was prepared from homogenates (200 mg./ml. in water). The suspension was diluted with an equal volume of water, centrifuged, and the residue stirred with water equal in volume to the discarded supernatant. After centrifuging again the residue was diluted with Sörensen phosphate buffer (pH=7·5) to give the same volume and a final phosphate concentration of $0{\cdot}05M$. The pH of this suspension is 7·1.

RESULTS

Effect of cyanide, azide and methylene blue

It can be seen from Table 1 that the muscle is very sensitive to cyanide, and also that azide in higher concentration depresses the oxygen uptake. The addition of a small amount of methylene blue can increase the activity of the uninhibited homogenate and also restore the activity inhibited by cyanide.

Table 1. *Effects of cyanide, azide and methylene blue on the whole homogenate*

Figures are percentage of control with no methylene blue or inhibitor. 600 mg. of muscle and $0{\cdot}02M$ (final concentration) phosphate in each flask. pH=7·3. 0·1 mg. methylene blue in last three flasks.

Cyanide	$10^{-3}M$	10
	10^{-4}	10
	10^{-5}	35
Azide	$10^{-1}M$	40
	10^{-2}	65
	10^{-3}	75
Methylene blue	—	130
	$10^{-2}M$ cyanide	100
	10^{-3} cyanide	98

These inhibitions suggest the presence of cytochrome oxidase. The results in Table 2 show that this enzyme is present.

Table 2. *Presence of cytochrome oxidase*

2 ml. succinoxidase preparation per flask; additions to give final concentrations of $10^{-5}M$ cytochrome, $10^{-2}M$ ascorbic acid (tipped in from side-bulb), $0{\cdot}04M$ phosphate. Final pH=7·1.

	μl. O_2/hr.
Enzyme	0
+cytochrome	0
+ascorbic acid	5
+cytochrome+ascorbic acid	48
+cytochrome+ascorbic acid+$10^{-2}M$ $AlCl_3$	24
+cytochrome+ascorbic acid+$3\times10^{-3}M$ $AlCl_3$	12

Following the report of Horecker, Stotz & Hogness (1939), regarding the stimulating effect of aluminium in small concentration, Schneider & Potter (1943) recommend the use of this in cytochrome oxidase assays. However, the values in Table 2 show an inhibitory effect and therefore it was never used.

Detection of cytochrome

An attempt was made to estimate the amount of cytochrome in the muscle by a micro-isolation procedure (Potter & Dubois, 1942). After dissolving the final precipitate from 10 g. of muscle in 0·3 ml. NaOH, no bands could be observed with a Zeiss ocular micro-spectroscope, even after addition of hydrosulphite. Ground muscle added to benzidine-peroxide-acetic acid mixture have faint blue specks on the surface of the particles, indicating the presence of an oxidase, peroxidase or haem compound. The benzidine test was repeated as follows. Muscle was ground with 5*N* acetic acid, and the suspension extracted with ether; the ethereal layer was evaporated down and any residue dissolved in a little water. This solution was boiled, and when added as a top layer to the benzidine mixture, gave a definite blue-green ring.

The positive benzidine reaction suggests the presence of some haem compound. Also, it seems that cytochrome, if present, is in very small amount.

Oxidation of succinate

Increased oxygen consumption is observed in the presence of succinate (Table 3). This indicates that the system is quickly saturated with succinate, especially in the absence of cytochrome.

Table 3. *Oxidation of succinate*

Each flask contained 0·02*M* phosphate and 600 mg. of muscle. Final pH = 7·3. Values are μl.O_2/hr.

Concentration of succinate	Cytochrome	Without cytochrome
—	22	15
0·01*M*	60	30
0·02	83	33
0·05	85	35

Little loss of succinic dehydrogenase occurs during the preparation of the succinoxidase (Table 4).

Table 4. *Distribution of succinic dehydrogenase*

Each flask contained the equivalent of 500 mg. of muscle. Also cytochrome, 0·03*M* succinate, 0·02*M* phosphate and $5 \times 10^{-3}M$ $CaCl_2$.

	μl. O_2/hr.
Whole homogenate	94
First supernatant	38
Second supernatant	53
Succinoxidase	98

Calcium is included because of the observations of Keilin & Hartree (1940), Schneider & Potter (1943), and Axelrod, Swingle & Elvehjem (1941), demonstrating the relationship between calcium, oxalacetic acid, and diphosphopyridine nucleotide. Since Ochoa & Ochoa (1937) have found the latter compound in the muscles of

Ostrea edulis, it is not surprising that calcium is needed (Table 5) to give maximum activity. Fig. 1 gives a pH curve for the system, and Table 5 shows the changes in activity due to altered substrate concentration.

Table 5. *Effect of* Ca *and succinate concentration*

2 ml. succinoxidase suspension in phosphate buffer in each flask. Also cytochrome.

Succinate	$CaCl_2$	μl. O_2/hr.
0·03 *M*	—	9
0·03 *M*	5×10^{-3} *M*	27
0·03 *M*	10^{-2} *M*	18
—	5×10^{-3} *M*	0
0·01 *M*	5×10^{-3} *M*	19
0·02 *M*	5×10^{-3} *M*	25
0·03 *M*	5×10^{-3} *M*	27
0·10 *M*	5×10^{-3} *M*	30

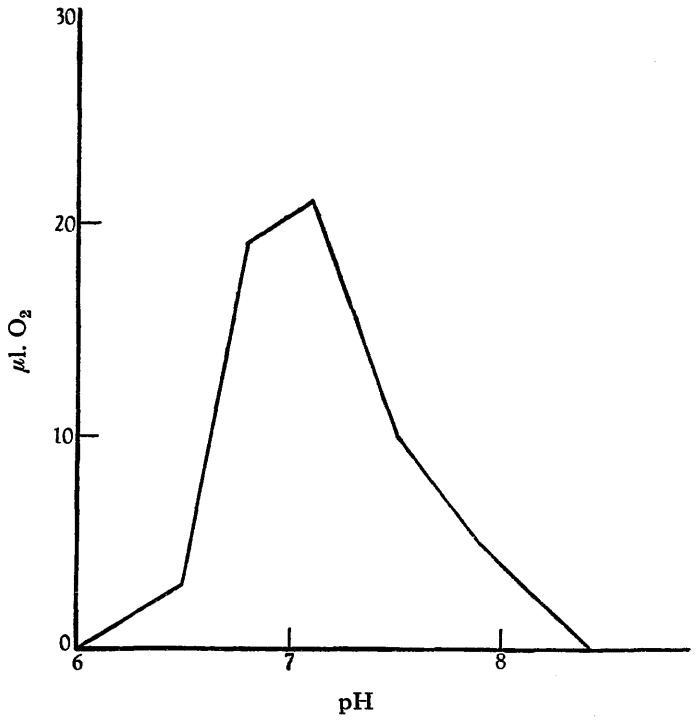

Fig. 1. The effect of pH on the oxygen uptake of the succinoxidase preparation. Each flask contained 2 ml. of succinoxidase; cytochrome, 5×10^{-3} *M* $CaCl_2$ and 0·03 *M* succinate were also added. The figures represent μl. O_2/hr.

Effects with inhibitors

A number of inhibiting agents were tried on the succinoxidase suspension in the presence of 0·01 or 0·03 *M* succinate.

Apart from cyanide, selenite was the most powerful inhibitor of succinoxidase (Table 6), but arsenite was comparatively weak. On the whole homogenate (Table 7), arsenite had a much greater effect, and 0·01 *M* fluoride brought about a stimulation.

This is perhaps due to combination with inhibiting metals, or a shunting of the metabolism into another pathway (Borei, 1945). As in Table 1, azide is less effective than cyanide.

A comparison of the results in Tables 6 and 7 seems to indicate that the succinoxidase system is not the chief one responsible for the respiration of the muscle. The conditions with regard to substrate concentration and presence of co-factors are so different, however, that this suggestion must be interpreted with caution.

Table 6. *Effect of inhibitors on succinoxidase*

2 ml. succinoxidase per flask. Cytochrome, $CaCl_2$ ($5 \times 10^{-3} M$) also present. Figures are percentage inhibition observed over 2 hr.

Inhibitor	Succinate concentration							
	0·03 *M*				0·01 *M*			
Concentration (*M*)	10^{-1}	10^{-2}	10^{-3}		10^{-1}	10^{-2}	10^{-3}	
Malonate	60	43	17		84	68	15	
Iodoacetate	92	55	34		76	48	44	
Pyrophosphate	87	53	15		78	63	23	
Fluoride	57	8	0		75	30	0	
Arsenate	55	6	10		18	0	0	
Arsenite	29	29	29		51	35	21	
Selenite	90	94	80		94	70	64	
Azide	90	80	40		90	95	45	
Cyanide	—	90	85	80 ($10^{-4} M$)	—	90	100	82 ($10^{-4} M$)

Table 7. *Effect of inhibitors on the whole homogenate*

Each flask contained 600 mg. muscle in 0·02*M* phosphate (pH = 7·3). Figures are percentage inhibition observed over 2 hr.

Inhibitor concentration (*M*)	10^{-1}	10^{-2}	10^{-3}		
Malonate	40	24	22		
Iodoacetate	69	65	70		
Pyrophosphate	69	50	47		
Fluoride	45	−12	19		
Arsenate	85	60	48		
Arsenite	70	68	40		
Selenite	89	82	70		
Azide	60	35	25		
Cyanide	85	88	90	90 ($10^{-4} M$)	65 ($10^{-5} M$)

DISCUSSION

Only a few of the invertebrates have been examined for their ability to metabolize added succinate. Baldwin (1938) demonstrated that slices of hepato-pancreas from *Helix pomatia* showed increased respiration after the addition of succinate. Using the Thunberg technique with washed tissue suspensions, Ball & Meyerhof (1940) found succinic dehydrogenase in *Limulus polyphemus*, *Busycon canaliculatum*, *Homarus americanus*, *Loligo pealii* and *Venus mercenaria*. The activity was most pronounced in the heart and faint or doubtful in white and adductor muscles. Navez, Crawford, Benedict & Du Bois (1941), using the chopped heart of *V. mercenaria*, found that added succinate raised the oxygen utilization by 10%. Methylene blue gave 150%, a value which was not further increased by the addition of succinate. *Saxostrea* seems

to be much richer in succinic dehydrogenase and/or those systems which can metabolize succinate than the animals used by either Ball & Meyerhof or Navez.

Loew (1891) found that azide was toxic to various worms, crustaceans and molluscs. Since Keilin (1936), and later, Keilin & Hartree (1940) showed that azide was as effective as cyanide in preventing the oxidation of cytochrome by cytochrome oxidase, workers have tended to assume that these two inhibitors have more or less the same effect on tissues. However, a comparison of their effects shows differences which are not easily explained. It seems that among the lower animals, there occur all possible combinations of cyanide sensitive and insensitive, and azide sensitive and insensitive respiratory systems (Stannard, 1939; Spiegelman & Moog, 1945). Working with *Ostrea edulis*, Chapheau (1932) investigated the effect of KCN on the oxygen consumption of chopped muscle. The Q_{O_2} values he obtained, in the presence of 10^{-5}, 10^{-4}, and $10^{-3}M$ KCN, respectively, were: 0·20, 0·12 and 0·10. No control figure is given, and no KCN was added to the centre-well of the Warburg flasks which he used. From other values given in his paper, the control value can be estimated at 0·25; i.e. at a concentration of $10^{-3}M$ KCN, 40% of the respiration is cyanide stable. Navez *et al.* (1941), using 'high concentrations of KCN' on *Venus mercenaria* heart, obtained no more than 40% inhibition of oxygen uptake, even in the presence of *p*-phenylene diamine or succinate.

Table 7 shows that cyanide produces very marked inhibition with *Saxostrea* muscle; at $10^{-5}M$ KCN, only a third of the normal oxygen consumption is obtained. To cause a similar effect, $10^{-1}M$ azide must be used, and its efficacy drops sharply at lower concentrations; the difference in inhibition given by cyanide from 10^{-1} to $10^{-4}M$ cannot be regarded as significant. Unfortunately, azide could not be tried at a pH where it might be more effective (about pH 6·5) since the normal respiration is depressed at this pH (Humphrey, 1946). From Table 6, it can be seen that cyanide is a very powerful inhibitor of the succinoxidase system, and again, that azide gives similar effects at a higher concentration.

Fluoride appreciably inhibits the succinoxidase in high concentration, but the effect decreases rapidly with dilution (Table 6). These results contrast with those of Navez *et al.* (1941), who found that fluoride had little or no effect on succinate oxidation by *Venus*. The action on the homogenate is not so marked, and the accelerating effect cannot be satisfactorily explained. Stotz & Hastings (1937) found that $0{\cdot}06M$ fluoride inhibited the succinic dehydrogenase from beef-heart about 45% and the oxidase, only 16%. Borei (1945) states that the action of fluoride is a competition with cytochrome oxidase for the cytochrome, and that fluoride may form a fluorophosphoprotein complex with cytochrome.

Iodoacetate has a marked inhibitory effect on succinoxidase and also on the homogenate even at $10^{-3}M$. Again this contrasts with the work of Navez *et al.* (1941). Inhibition may be explained as due to reaction with —SH groups (Hopkins, Morgan & Lutwak-Mann, 1938).

Dixon & Elliott (1929) found that pyrophosphate inhibited liver respiration by 30–70%. Leloir & Dixon (1937) showed that of a number of dehydrogenases tested, succinic dehydrogenase was the only one affected. Stotz & Hastings (1937) could

find no effect on the dehydrogenase with 0·06*M* pyrophosphate, but obtained 38% inhibition of the succinoxidase. The action on oyster muscle is very strong and the succinoxidase activity is significantly decreased even at 0·01*M*.

Szent-Györgyi (1930) showed that 0·002*M* arsenite inhibited the oxygen consumption of liver by 67%; there was no inhibition of succinate oxidation. Potter & Elvehjem (1937), working with chicken kidney, found 87% inhibition in the presence of 0·01*M* arsenite, while, in the case where succinate was added, arsenite caused 70% inhibition; using 0·01*M* selenite, they obtained 95% inhibition of both succinate oxidation and endogenous respiration. With a beef-heart preparation, Stotz & Hastings (1937) showed that while 0·02*M* selenite did not affect the oxidase, succinic dehydrogenase was completely inhibited. Navez *et al.* (1941) found no inhibition of *Venus* heart by selenite. Both arsenite and selenite strongly inhibit the respiration of oyster homogenates; with the succinoxidase preparation selenite is again very powerful, but arsenite has only a moderate effect.

Arsenate, according to Potter & Elvehjem (1937), inhibits respiration and succinate oxidation only 20%. The results in Table 6 do not conflict sharply with this, but those in Table 7 give a much higher inhibition, only 15% of the normal oxygen uptake remaining in the presence of 0·1*M* arsenate.

Quastel & Woolridge (1928) showed that 0·01*M* malonate completely inhibited succinic dehydrogenase in bacteria. With pigeon-breast muscle, Stare & Baumann (1936) obtained 25% inhibition of respiration in the presence of 0·007*M* malonate. With the same concentration of malonate, Potter (1940) found complete inhibition of succinoxidase from pig-heart. Thus, malonate has come to be regarded as a very efficient inhibitor of succinic dehydrogenase. Its effect on the whole homogenate (Table 7) is not very marked, whereas, with low succinate concentration, it exerts a marked inhibition on succinoxidase. This could indicate that only a small part of the normal respiration goes through succinic dehydrogenase.

Recently, Ball & Meyerhof (1940) have called attention to the presence of haem compounds in animals possessing haemocyanin as a respiratory pigment. They say: 'Why an animal should employ haemocyanin for a blood pigment and yet possess muscles rich in the pigment myoglobin is indeed puzzling.' No satisfactory investigation of the presence of haemocyanin in oysters seems to have been made. Boyce & Herdman (1897) found only two instances where the plasma acquired a faint blue tinge on exposure to oxygen. In 1899, Herdman & Boyce recorded similar observations and pointed out that this occurred only in specimens showing a green leucocytosis. Maloeuf (1937) cites *Ostrea* as an animal with no type of 'oxygen transporter in internal medium and isolated organs'. Earlier, MacMunn (1886) had observed a faint but definite histohaematin spectrum before and after treating various tissues of *O. edulis* with ammonium sulphide; this indicates the presence of a haem compound and is supported by the positive benzidine test given by *Saxostrea* muscle. The low intensity of these last two reactions shows that the concentration of cytochrome must be very small; and this is interesting when compared with the relatively strong cytochrome oxidase and succinoxidase activities. It may be that this aspect of the metabolism of *Saxostrea* muscle is similar to that

occurring in *Arbacia* eggs; here, Krahl, Keltch, Neubeck & Clowes (1941) said that 'it seems safe to conclude that cytochrome C cannot carry a significant fraction of the oxygen consumption'. The positive benzidine reaction observed in *Saxostrea* might indicate the haemochromogen precursor mentioned by Keilin (1929). It is interesting to note that in insects, brown muscles give a strong benzidine reaction, whereas white muscles give scarcely any (Keilin, 1925). It seems, then, that *Saxostrea* can be classified as an animal with no respiratory pigment and with a very small concentration of cytochrome or cytochrome precursor.

CONCLUSIONS

The large increase in respiration (about 300%) observed in the presence of 0·01 *M* succinate indicates that the muscle possesses very active systems for the metabolism of succinate. It is not possible, as yet, to say whether the increase is direct succinate oxidation or is brought about by the catalytic effect of succinate on metabolism as a whole. The small malonate inhibition may be regarded as an indication that the endogenous respiration does not proceed very much through the succinate system.

The inhibition brought about by cyanide is such as to indicate very little cyanide-stable respiration. There is little evidence to support the suggestion of Navez *et al.* (1941) that the lamellibranchs use a flavoprotein or glutathione metabolism in contrast to the cytochrome-cytochrome oxidase systems. The low concentration of cytochrome found is to be expected in a white muscle; this may be correlated with the lack of oxygen-transporting pigment in the blood, i.e. there is a low metabolism of the organism as a whole. The increased oxygen uptake observed in the presence of methylene blue also indicates a lack of carriers in the experimental system, and the small rate of respiration can be explained by the sessile nature of the animal.

SUMMARY

Succinoxidase was prepared from the adductor muscles of *Saxostrea commercialis* by homogenizing in water and suspending the washed pulp in phosphate buffer. The effect of various inhibitors on this succinoxidase and on the oxygen consumption of homogenates was determined.

The respiration of the whole homogenate is almost completely inhibited by cyanide, selenite, and arsenate. Azide, arsenite, pyrophosphate and iodoacetate also inhibit strongly. Malonate and fluoride inhibit only about 40%. Methylene blue and cytochrome increase the oxygen uptake, the former being able to offset the inhibition caused by cyanide. Succinate brings about a 300% increase in respiration.

Calcium but not aluminium is needed for the action of succinoxidase, which is almost wholly inhibited by cyanide, azide, iodoacetate, pyrophosphate and selenite. Malonate and fluoride cause marked inhibition, whereas arsenate and arsenite are relatively inactive.

Cytochrome oxidase was detected in the succinoxidase preparation by using ascorbic acid as reductant.

Although cytochrome could not be isolated, the presence of a haem compound was shown by a positive benzidine reaction in an ethereal extract of a suspension of the muscle in acetic acid.

REFERENCES

AXELROD, A. E., SWINGLE, K. F. & ELVEHJEM, C. A. (1941). *J. Biol. Chem.* **140**, 931.
BALDWIN, E. (1938). *Biochem. J.* **32**, 1225.
BALL, E. G. & MEYERHOF, B. (1940). *J. Biol. Chem.* **134**, 483.
BOREI, H. (1945). *Ark. Kemi Min. Geol.* 20 A, no. 8.
BOYCE, R. & HERDMAN, W. A. (1897). *Proc. Roy. Soc.* **62**, 30.
CHAPHEAU, M. (1932). *Bull. Soc. sci. Arcachon*, **29**, 85.
DHÉRÉ, C. & VEGEZZI, G. (1917). *J. Physiol. Path. gén.* **17**, 44.
DIXON, M. & ELLIOTT, K. A. C. (1929). *Biochem. J.* **23**, 812.
HERDMAN, W. A. & BOYCE, R. (1899). *Lancs. Sea-fish Mem.* no. 1.
HOPKINS, F. G., MORGAN, E. J. & LUTWAK-MANN, C. (1938). *Biochem. J.* **32**, 1829.
HORECKER, B. L., STOTZ, E. & HOGNESS, T. R. (1939). *J. Biol. Chem.* **128**, 251.
HUMPHREY, G. F. (1946). *Aust. J. Exp. Biol. Med. Sci.* **24**, 261.
KEILIN, D. (1925). *Proc. Roy. Soc.* B, **98**, 312.
KEILIN, D. (1929). *Proc. Roy. Soc.* B, **104**, 206.
KEILIN, D. (1933). *Ergebn. Enzymforsch.* **2**, 239.
KEILIN, D. (1936). *Proc. Roy. Soc.* B, **121**, 165.
KEILIN, D. & HARTREE, E. F. (1937). *Proc. Roy. Soc.* B, **122**, 298.
KEILIN, D. & HARTREE, E. F. (1940). *Proc. Roy. Soc.* B, **129**, 277.
KRAHL, M. E., KELTCH, A. K., NEUBECK, C. E. & CLOWES, C. H. A. (1941). *J. Gen. Physiol.* **24**, 597.
KREBS, H. A. (1935). *Biochem. J.* **29**, 1620.
LELOIR, L. F. & DIXON, M. (1937). *Enzymologia*, **2**, 81.
LOEW, O. (1891). *Ber. dtsch. chem. Ges.* **24**, 2947.
MACMUNN, C. A. (1886). *Philos. Trans.* **177**, 267.
MALOEUF, N. S. R. (1937). *Z. vergl. Physiol.* **25**, 43.
NAVEZ, A. E., CRAWFORD, J. D., BENEDICT, D. & DU BOIS, A. B. (1941). *Biol. Bull. Woods Hole*, **81**, 289.
OCHOA, S. & OCHOA, G. (1937). *Nature, Lond.*, **140**, 1097.
POTTER, V. R. (1940). *J. Biol. Chem.* **134**, 417.
POTTER, V. R. (1941). *J. Biol. Chem.* **141**, 775.
POTTER, V. R. & DUBOIS, K. P. (1942). *J. Biol. Chem.* **142**, 217.
POTTER, V. R. & ELVEHJEM, C. A. (1937). *J. Biol. Chem.* **117**, 341.
QUASTEL, J. H. & WOOLRIDGE, W. R. (1928). *Biochem. J.* **22**, 689.
SCHNEIDER, W. C. & POTTER, V. R. (1943). *J. Biol. Chem.* **149**, 217.
SPIEGELMAN, S. & MOOG, F. (1945). *Biol. Bull. Woods Hole*, **89**, 122.
STANNARD, J. N. (1939). *Amer. J. Physiol.* **126**, 196.
STARE, F. J. & BAUMANN, C. A. (1936). *Proc. Roy. Soc.* B, **121**, 338.
STOTZ, E. & HASTINGS, A. B. (1937). *J. Biol. Chem.* **118**, 479.
SZENT-GYÖRGYI, A. (1930). *Biochem. J.* **24**, 1723.
SZENT-GYÖRGYI, A. (1937). *Acta med. Szeged.* **9**, 1.