3. Insulin did not alter the inactivity of rat mammary gland slices towards acetate as sole substrate, but in presence of glucose it considerably increased the ^{14}C found in the fatty acids. The incorporation of ^{14}C into the fatty acids of sheep udder slices was unaffected by insulin both in acetate alone and in acetate plus glucose.

4. Glycerol caused a slight incorporation of acetate carbon into the fatty acids of rat mammary gland slices in acetate alone, and in acetate plus glucose its stimulating effect on ^{14}C incorporation was as great as that of insulin. It stimulated the utilization of acetate for fatty acid synthesis by sheep udder slices in acetate alone but in acetate plus glucose its effect was slightly inhibitory.

5. Glyceride glycerol isolated as tri-*p*-nitrobenzoate from the neutral fat of the slices was not radioactive in any experiment. Mammary gland slices under our conditions do not incorporate acetate carbon into glycerol.

6. Deductions from respiratory and acid-change measurements as to the ability of mammary gland slices to utilize acetate for fatty acid synthesis *in vitro* and the effect of glucose, of insulin and of glycerol upon this process in ruminant and non-ruminant mammary tissue respectively have been confirmed. There is now little doubt that mammary gland slices are capable of effecting fatty acid synthesis from small molecules *in vitro*.

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D-Amino-acid Oxidase in the Molluscan Liver

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The enzyme D-amino-acid oxidase was first found in mammalian liver and kidney (Krebs, 1933); it occurs in all vertebrates that have been examined. A review on D-amino-acid oxidases by Krebs (1948) refers to the occurrence of an enzyme of this type in bacteria and in certain strains of the mould *Neurospora*.

We have recently briefly reported that D-amino-acid oxidase is present in the liver of *Octopus vulgaris* and of *Sepia officinalis* (Blaschko & Hawkins, 1951). This appears to be the first time that the enzyme has been found in invertebrates. We now wish to report some observations on the occurrence of D-amino-acid oxidase in other classes of molluscs and also to describe some of the properties of this enzyme.

MATERIALS AND METHODS

Animals. Specimens of *Sepia officinalis* and *Octopus vulgaris* were dissected in Plymouth. The livers were immediately frozen in solid CO_2 and taken to Oxford in a thermos flask. They were then stored at $-10°$.

In the other species examined (*Mytilus, Anodonta, Helix*) the liver is not as well defined a structure as in the two species of cephalopods, and tissue used included parts of the alimentary canal. *Mytilus edulis* was also obtained from

Plymouth and dissected in Oxford. A few specimens of *Anodonta* were collected locally, and specimens of a species of *Helix* (possibly *aspersa*) were collected in a garden in Oxford.

The enzyme preparation. Homogenates were prepared in 0·067 M-sodium phosphate buffer of pH 7·4; usually 1 g. of tissue was homogenized in a total volume of 5 ml. The preparation of an acetone-dried powder of *Octopus* liver will be described later.

Measurement of enzymic activity. This was determined manometrically. The main compartment of a conical flask contained 1·6 ml. of homogenate, the side bulb 0·4 ml. of either water or of a solution of the amino-acid to be tested. The concentration of the amino-acid solution was 0·05 M, when the D-amino-acid was used, and 0·1 M when the DL-amino-acid was used. The inner tube contained a filter paper and 0·3 ml. of N-KOH. The gas phase was O_2, and the temperature, unless otherwise stated, was 37·5°. Readings were usually taken at 5 min. intervals for 30 min.; the enzymic activity was usually calculated from the O_2 uptake during the first 15 min. period of incubation.

Some of the amino-acids did not dissolve completely; this was most marked with tyrosine, cystine and 2-amino-*n*-octanoic acid, which were added as suspensions. Solutions of the dicarboxylic acids, and of the dihydrochlorides of the basic amino-acids, were neutralized before use.

RESULTS

Cephalopods

The experiments on D-amino-acid oxidase of *Sepia* liver were carried out early in 1951, after the livers had been stored for about 4 months. It is therefore possible that the enzymic activity of the fresh organs had been higher. In the first experiment valine was used as substrate, and the oxygen uptakes (μl. O_2) recorded in 30 min. were: without added amino-acid, 38; additional uptake with D-valine, 28; additional uptake with L-valine, 3. In view of the large blank, the oxidation of L-valine cannot be considered significant.

D-Leucine was also oxidized by the *Sepia* enzyme, and in all the later experiments, one flask was included in which the oxidation of D-leucine was measured; this compound serves as basis of comparison. Table 1 contains results with all those amino-acids tested which can be considered as substrates of oxidation in *Sepia*; the figures in the last column give the relative rates of oxidation compared with that of D-leucine. A number of other amino-acids were tested, but these did not significantly raise the oxygen uptake above that found in the blank. They were: D-alanine, DL-α-amino-*n*-butyric acid, DL-serine, DL-threonine, DL-proline, DL-aspartic acid, DL-ornithine, DL-lysine, D-histidine, DL-cystine and DL-homocystine. It must be pointed out, however, that in view of the large oxygen consumption of the 'enzyme blank', it is quite possible that some of these compounds are oxidized, but at a slower rate.

The experiments on *Octopus* enzyme described here were carried out in the summer of 1951, on a liver which had been dissected only a few days earlier. The liver weighed about 35 g. Part of the organ was used in the preparation of several homogenates; the remainder, 12·8 g., was converted into an acetone-dried powder.

Altogether eight experiments were carried out, using different homogenates. The enzyme blank in the first 15 min. varied between 22 and 29 μl. O_2, the additional oxygen uptake with D-leucine, which again served as a standard of comparison, between 30 and 45·5 μl. These experiments are shown in Table 2.

It can be seen from Table 2 that all the unbranched monoamino-monocarboxylic acids were oxidized; the maximum rate of oxidation in this series occurred with α-amino-*n*-butyric acid. Of the branched acids, valine, leucine and isoleucine were oxidized, but α-amino*iso*butyric acid was not oxidized. It is interesting that histidine (the DL form) was oxidized by the *Octopus* preparation. The result with DL-lysine must be considered as doubtful. DL-Ornithine was not oxidized in one experiment, DL-threonine was not oxidized in two experiments.

Table 1. *Oxidation of* D-*amino-acids in homogenates of* Sepia *liver*

Exp. no.	1	2	3	4	5	6	7	Mean, as % of oxidation of D-leucine
Amino-acid	Additional O_2 uptake in 15 min. (μl.)							
DL-Norvaline	—	15·5	—	—	—	—	—	143
DL-Norleucine	—	11·5	7·5	—	—	—	—	110
D-Valine	—	4·5	3·5	—	—	—	—	48
D-Leucine	41·5	11	6·5	10	12·5	8	8	100
D-Isoleucine	28	6·5	4·5	—	—	—	—	65
DL-Glutamic acid	—	—	—	12·5	15	10·5	9	120
D-Tryptophan	—	16	7	—	—	—	—	126
D-Phenylalanine	—	13	7·5	—	—	—	—	117
DL-Methionine	—	18	14	—	—	—	—	189

In one additional experiment in which D-leucine was not used, the 15 min. figures for oxygen uptake (μl.) were: without added substrate, 29; additional uptake with D-phenylalanine, 75; additional uptake with DL-phenylalanine, 58·5; additional uptake with L-phenylalanine, 7.

Part of the same *Octopus* liver was converted into an acetone-dried powder; an extract was prepared from the powder following the procedure of Bender & Krebs (1950), except that the extract was not obtained by filtration but by centrifugation for 15 min. at 0°. This gave an almost clear supernatant fluid. We used the sodium pyrophosphate buffer of pH 8·4 described by Warburg & Christian (1938) for the extraction.

A sample (1 ml.) of this extract and 0·6 ml. of the pyrophosphate buffer were used in each flask. The genates were prepared by adding 2 ml. of cold 0·67 M-sodium phosphate buffer of pH 7·4 to each g. of tissue and grinding thoroughly. In the manometric experiment, each flask contained 0·5 ml. of the homogenate plus 1·1 ml. of the buffer. The temperature in these experiments was 17°; the gas phase was O_2.

Altogether five experiments were carried out with preparations from *Mytilus*. No significant oxygen uptake was noted with valine, leucine, proline, threonine, glutamic acid and tryptophan.

Table 2. *Oxidation of* D-*amino-acids in* Octopus *liver*

Exp. no.	1	2	3	4	5	6	7	8	Mean, as % of oxidation of D-leucine
Amino-acid	Additional O_2 uptake in 15 min. (μl.)								
DL-Alanine	—	44·5	—	—	—	—	—	35	100
DL-α-Amino-*n*-butyric acid	—	—	—	66·5	—	—	—	70·5	188
DL-Norvaline	49·5	—	—	—	—	—	—	56	165
DL-Norleucine	30	—	—	—	—	52	—	60	132
DL-2-Amino-*n*-octanoic acid	—	—	—	—	19	—	—	21	56
DL-Valine	—	—	—	32·5	—	—	16	—	64
D-Leucine	30	45·5	34·5	39	38	38·5	35·5	34	100
D-Isoleucine	—	—	—	25·5	—	—	26·5	—	70
DL-Aspartic acid	—	—	14	—	19·5	—	—	—	46
DL-Glutamic acid	38	—	—	—	—	—	31·5	—	108
DL-Serine	—	21	—	—	—	16·5	—	—	44
DL-Proline	—	—	30	—	—	—	—	29·5	87
D-Tryptophan	4	—	—	17·5	12·5	—	—	—	30
DL-Histidine	—	—	37	—	—	—	—	42·5	116
DL-Lysine	4	—	—	—	—	0	—	—	—
DL-Methionine	39	—	—	—	—	—	45·5	—	129

Table 3. *Oxidation of* D-*amino-acids in homogenates of* Helix *liver*

Exp. no.	1	2	3	4
Amino-acid	Additional O_2 uptake in 15 min. (μl.)			
DL-Alanine	—	—	22	11
DL-α-Amino-*n*-butyric acid	—	—	35·5	33·5
DL-Norvaline	—	—	44·5	47
DL-Norleucine	—	—	9	11·5
DL-2-Amino-*n*-octanoic acid	—	—	5·5	0
D-Leucine	78	72	59	44·5
D-Isoleucine	—	—	—	3
D-Phenylalanine	49·5	38·5	—	—
DL-Methionine	61·5	50	—	—

amounts of oxygen (μl.) consumed in 30 min. were: without added substrate, 7; additional uptake with DL-glutamic acid, 77; L-glutamic acid, 7·5; DL-tyrosine, 14·5; L-tyrosine, 6.

The experiment shows that the rapid oxygen uptake with glutamic acid occurred only when the D form was present. This makes it likely that the oxidation was catalysed by D-amino-acid oxidase and not by another enzyme. The oxygen uptake in the flask with DL-tyrosine was larger than in that with L-tyrosine; the slight oxidation of L-tyrosine may possibly have been due to tyrosinase which is known to occur in *Octopus*.

Lamellibranchs

Mytilus. The 'livers' from a number of specimens were dissected, weighed and frozen in a mortar at −10°. Homo-

There was an oxygen uptake in two experiments with DL-methionine (73·5 and 74·5 μl. O_2 respectively in 15 min.), and in one experiment with DL-serine (17·5 μl. O_2 in 15 min.). With the unbranched monoamino acids the oxygen consumed in 15 min. was:

	Exp. 1 (μl. O_2)	Exp. 2 (μl. O_2)
Blank	4	4
DL-Alanine	6·5	0
DL-α-Amino-*n*-butyric acid	13·5	27
DL-Norvaline	33	44
DL-Norleucine	47	43
DL-2-Amino-*n*-octanoic acid	0	11·5

Anodonta. Two experiments were carried out with the 'liver' homogenate. In the first experiment there was no oxygen uptake with D-leucine,

D-phenylalanine and DL-methionine; in the second experiment with D-leucine, DL-phenylalanine, DL-norvaline and DL-methionine there was again no oxygen uptake. The blank oxygen uptakes in 15 min. at 22° were 10·5 and 15·5 μl. respectively.

Gastropod (Helix)

The results of four experiments with the 'liver' homogenate are set out in Table 3. The blank oxygen uptake in these experiments varied from 4·5 to 14·5 μl., and many amino-acids were found to be oxidized. The result with 2-amino-*n*-octanoic acid and with isoleucine must be considered doubtful. In Exp. 4, DL-cystine, DL-homocysteine and DL-ornithine were also tested; they were not oxidized.

DISCUSSION

The experiments here reported show that in four of the five species of molluscs examined, the liver contains a D-amino-acid oxidase akin to that found in vertebrates. The enzyme occurred in all the classes examined: cephalopods, lamellibranchs and gastropods. It is of interest to compare the properties of the preparation studied in this paper with those of the mammalian enzyme. Bender & Krebs (1950) have recently examined the substrate specificity of the sheep-kidney enzyme. In many ways the molluscan enzymes resemble the sheep-kidney enzyme, but there are some differences not only between the sheep and the molluscs, but also differences from one mollusc to another. Points of similarity are that many monoamino-monocarboxylic acids are readily attacked by the sheep enzyme and by the molluscan enzyme; the basic amino-acids are not so readily oxidized. Serine is oxidized by the sheep and by *Octopus* and so is proline. Both methionine and phenylalanine are readily attacked in mammals and in molluscs. Of the differences, one may be noted: the *Octopus* enzyme oxidizes D-glutamic acid, which is not a substrate of the sheep enzyme.

There are differences between the different molluscan preparations in the relative rates of oxidation in the homologous series

$$CH_3(CH_2)_nCHNH_2COOH.$$

Of this series, alanine ($n=0$), α-amino-*n*-butyric acid ($n=1$), norvaline ($n=2$), norleucine ($n=3$) and 2-amino-*n*-octanoic acid ($n=5$) were available. In *Octopus* all members of the series were oxidized; the maximum rate of oxidation was found with α-amino-*n*-butyric acid ($n=1$); in *Sepia*, norvaline ($n=2$) and norleucine ($n=3$) were the only members that were oxidized at a measurable rate; in *Helix*, where all members were oxidized, the maximum rate was with norvaline ($n=2$). In *Mytilus*, norvaline and norleucine were oxidized most readily, and with both alanine ($n=0$) and 2-amino-*n*-octanoic acid ($n=5$) there was little or no oxygen uptake. These results show that at first there is a rise of the oxidation rate with increasing chain length, but that with a further increase in chain length the rate falls off. This might be explained by the lower solubility of the higher members of the series, but it is interesting that in each species the specificity pattern is different and the maximum rate of oxidation occurs with a different member of the series.

The structure and function of the liver in molluscs differs fundamentally from that in vertebrates. In fact, in different molluscan species the liver has an entirely different character. This subject is reviewed by Yonge (1937). In *Octopus* and *Sepia* the liver is a parenchymatous organ which is separated from the gut, as it is in vertebrates. In the other three species examined (*Mytilus*, *Anodonta* and *Helix*), the 'liver' is not separated from the gut, and each part of the tissue contains not only secretory elements, but also parts of the intestinal lumen. An interesting outcome of our observations is that the presence or absence of the enzyme does not coincide with these differences in structure and function, but that not only the cephalopods, but also *Mytilus* and *Helix*, contain the enzyme. The possibility that in the two latter species intestinal bacteria contribute to the enzymic activity cannot be excluded, but it is of interest that the enzyme in these two species is akin to the oxidase in vertebrates and in cephalopods where the danger of bacterial contamination seems remote.

The enzyme was not found in *Anodonta*. This may have been due to unsuitable experimental conditions, but these did not differ from those in which the presence of the enzyme in other species was easily demonstrated. It is of interest that in marine lamellibranchs (*Ostrea* and *Mytilus*) the concentration of non-protein amino-acids in the muscle tissue is high, but that in the fresh-water species, *Anodonta*, the amino-acid content is very low (Duchâteau, Sarlet, Camien & Florkin, 1952). This seems to make it worth considering whether the oxidase is in some way connected with the metabolism of the amino-acids in the tissues.

It would clearly be of interest to extend this survey of D-amino-acid oxidase to other phyla, as a better knowledge of its distribution may help towards an understanding of its function in the animal body, which is still unknown (see Krebs, 1948). It has been suggested (Warburg, 1948) that *in vivo* the enzyme is concerned in the synthesis of L-amino-acids rather than in the breakdown of the D forms. However, D-amino-acids are now known to occur in nature more commonly than had been believed and this makes it less surprising that an

enzyme dealing with these substances is so widespread. Possibly there are animals in which D-amino-acids are metabolized either as constituents of the diet or as products of intermediate metabolism.

SUMMARY

1. The enzyme D-amino-acid oxidase occurs in the liver of two cephalopods, *Sepia officinalis* and *Octopus vulgaris*. The substrate specificity of the enzyme has been studied in tissue homogenates. The *Octopus* enzyme is also present in the extract of an acetone-dried powder of the liver.

2. The enzyme was also found in the 'liver' of a gastropod species, *Helix*.

3. The 'livers' of two lamellibranchs were examined. The enzyme was found in *Mytilus edulis*, but it was not found in *Anodonta*.

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The Effect of Age on the Bulk Protein Composition of *Chlorella vulgaris*

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The effect of time of harvesting upon the proportions of the total protein nitrogen found as amide N, 'basic N' and monoamino N in the leaves of higher plants has been studied by Chibnall (1922, 1926) and Pearsall (1931). Such investigations have been continued by Lugg & Weller (1941, 1948) who determined the percentages of certain amino-acids (tryptophan, methionine and cystine-cysteine) in leaf-protein fractions. Methionine and cystine contents of the proteins of pasture plants harvested at different times have also been determined by Smith & Wang (1941).

The variation of protein composition with age in members of the lower plant orders has been little investigated. Several workers (Camien, Salle & Dunn, 1945; Freeland & Gale, 1947) have studied the protein composition of various micro-organisms, and the effects produced by variations in the culture media. The effect of the age of the culture upon the protein composition was not, however, investigated, the cultures normally being harvested during their exponential phase of growth. Fowden (1951*a*) has reported the composition of the bulk protein of the alga *Chlorella vulgaris* during its phase of exponential growth; Mazur & Clark (1938, 1942) earlier reported the protein composition of several algae at undefined stages of their growth.

The present paper presents the results of an investigation of the protein composition of *Chlorella* and includes complete analyses of protein fractions obtained from cultures of widely differing ages. In addition to contributing to knowledge of the nitrogen metabolism of this alga, the analyses can be expected to provide data for assessing the merits of *Chlorella* cultures of different ages if used as a source of food, as envisaged by several authors (Spoehr & Milner, 1948; Meier, 1949; Pearsall & Fogg, 1951; Geoghegen, 1951).

EXPERIMENTAL

Growth of alga. C. vulgaris was grown on the medium of Pearsall & Loose (1936) to which was added 1 ml./l. of trace-element solution A4 (Arnon, 1938) containing B, Mn, Cu and Zn. Into each one of a batch of penicillin-culture flasks were introduced 500 ml. of the medium; they were then autoclaved for 20 min. at a pressure of 12 lb./sq. in. The medium, which was heavily buffered with phosphate, usually had a pH of 6·1–6·2 at this stage. Each flask was then inoculated with 1 ml. of an actively growing *Chlorella* culture containing about 5000 cells/cu.mm. The flasks, now containing about 10 cells/cu.mm., were maintained at $20 \pm 2°$ under a continuous uniform illumination of 150 ft.c. Growth was allowed to proceed for various periods of time, the flasks being gently shaken twice per day.