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Studies on Sulphatases

3. THE ARYLSULPHATASE AND β-GLUCURONIDASE OF MARINE MOLLUSCS

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Arylsulphatase was first demonstrated in molluscs by Derrien (1911) who found that extracts of a large marine gastropod, *Murex trunculus*, could hydrolyse indoxyl sulphate. Soda & Egami (1933) and Soda (1936) studied the distribution of the enzyme in a large number of tropical marine molluscs common to Japanese waters. Considerable activity was observed amongst members of the Gastropoda, Pelecypoda, Cephalopoda and Amphineura. It was also found in some Arthropoda. In all cases the enzyme activity was mainly concentrated in the digestive glands. Information regarding the properties of the mollusc enzyme is scanty. Soda (1936) recorded an optimum pH of 7 for the arylsulphatase of *Charonia lampas* (*Tritonium nodiferum*) when potassium phenylsulphate was the substrate, although Soda & Egami (1941) used pH 5·2 for enzyme assay. The substrate concentration used by these workers (0·05 M) was apparently arbitrary and not necessarily optimal. The high arylsulphatase activity of extracts of the digestive glands of *Haliotis gigantea* (Soda & Koyama, 1935) was confirmed by Morimoto (1937) using potassium *p*-nitrophenylsulphate as substrate under arbitrary experimental conditions. Tanaka (1938) found that the maximum hydrolysis of potassium *p*-nitrophenylsulphate by aqueous extracts of digestive glands of various marine organisms occurred at pH 5·0–5·2 when the substrate concentration was 0·0005 M. The optimum substrate concentration was not recorded.

During preliminary studies (Dodgson, Lewis & Spencer, 1952) the digestive organs of many marine molluscs common to British waters were found to be rich in arylsulphatase and promising as starting material for the preparation of enzyme concentrates. It was noted that the organisms also possessed marked β-glucuronidase activity. Nagaoka (1951) used the β-glucuronidase of certain terrestrial snails, but there appears to be no previous record of the occurrence of this enzyme in marine molluscs. In view of the desirability of

removing β-glucuronidase from preparations of arylsulphatase, and because of the comparative dearth of information regarding the optimum conditions for the activity of the two enzymes in invertebrate organisms, it was decided to investigate these conditions. The large periwinkle (*Littorina littorea*) and the limpet (*Patella vulgata*) were selected for this study since they were both readily accessible locally and rich sources of both enzymes. Using the optimum conditions established for these two organisms, the arylsulphatase and β-glucuronidase activities of a number of other marine molluscs have been examined in order to throw further light on the problem of the physiological significance of the two enzymes. A preliminary account of this work has already been published (Dodgson *et al.* 1952).

MATERIALS AND METHODS

Supplies of *Littorina* and *Patella* were obtained locally from the Bristol Channel area; other organisms were obtained from the Marine Biological Station, Plymouth. In the laboratory they were kept alive in aerated sea-water or, for shorter periods, in the ice-chest.

Substrates. Potassium *p*-acetylphenylsulphate, free from *p*-hydroxyacetophenone, was prepared as previously described (Dodgson & Spencer, 1953). *p*-Chlorophenylglucuronide monohydrate was prepared biosynthetically (Spencer & Williams, 1951).

Tissue suspensions. These were prepared using a glass homogenizer of the Potter-Elvehjem type (see Potter & Elvehjem, 1936; Brown, Dodgson, Sherwood & Spencer, 1952).

Determination of arylsulphatase activity. The spectrophotometric method of Dodgson, Spencer & Thomas (1953) using potassium *p*-acetylphenylsulphate as substrate was modified by omission of ethanol. When ethanol was used as a protein precipitant with mollusc digests the addition of NaOH gave cloudy solutions which could not be clarified by centrifugation. Complete removal of protein from the digests was not necessary since protein solutions do not absorb strongly at 323 mμ. Enzyme action could be stopped by 0·2 N-NaOH and, at the concentrations of tissues used in these experiments (usually 0·1% wet wt./vol.), solutions sufficiently clear for spectrophotometric measurement could be obtained by centrifuging at 2000 *g* for 15 min. The procedure was identical with that of Dodgson *et al.* (1953) except that 4·8 ml. of 0·2 N-NaOH were used instead of 4·8 ml. of ethanol. After centrifuging, the absorptions of the solutions in 1 cm. cells were measured on a Hilger Uvispek spectrophotometer at 323 mμ. without further treatment. Under these conditions the expression $(E_t - E_c) \times 40{\cdot}84$ (where E_t and E_c are the observed extinctions of the test and control solutions respectively) gives the amount of the liberated *p*-hydroxyacetophenone in μg.

Determination of β-glucuronidase activity. The method was based on that of Spencer & Williams (1951) using *p*-chlorophenylglucuronide monohydrate as substrate. Ethanol was omitted for the reason given in the preceding section. At the tissue concentrations used for the measurement of β-glucuronidase activity (approx. 1% wet wt./vol.), neither 0·2 N-NaOH nor KOH could be substituted for ethanol since the cloudy solutions obtained could not be clarified by centrifugation but 0·35 N-$Ba(OH)_2$ gave clear solutions providing precautions were taken to prevent the formation of $BaCO_3$ during the centrifuging and subsequent spectroscopic procedure. The amount of $Ba(OH)_2$ was sufficient to convert the *p*-chlorophenol to the anionic form. Any traces of protein which remained in solution did not greatly interfere with the determinations since protein does not absorb very strongly at 298 mμ.

To each of four 15 ml. centrifuge tubes was added 0·4 ml. of tissue suspension in 0·5 M-acetate buffer of the required pH. All suspensions were kept at 0° before incubation (see p. 256). The four tubes were stoppered and placed at 1 min. intervals in a water bath at 37·5°. After allowing 4 min. for each suspension to attain the temperature of the bath, 0·4 ml. of a suitably buffered solution of *p*-chlorophenylglucuronide monohydrate was added to the first two tubes (tests). After incubating each tube for exactly 1 hr., 5·0 ml. of 0·35 N-$Ba(OH)_2$ were added to the first two tubes; 0·4 ml. substrate, followed immediately by 5·0 ml. of 0·35 N-$Ba(OH)_2$, was added to the second two tubes (controls). After clarifying the solutions by centrifugation, the extinctions of these solutions at 298 mμ. were measured on the spectrophotometer using 1 cm. cells. The amount of *p*-chlorophenol (μg.) liberated by the enzyme is given by the expression

$$(E_t - E_c) \times 286{\cdot}6,$$

where E_t and E_c are the observed extinctions of the test and control solutions respectively.

RESULTS

Arylsulphatase

The characteristics of arylsulphatase. Suspensions of both *Littorina* and *Patella* viscera exhibited maximum enzyme activity at pH 5·5 in 0·5 M-acetate buffer (Fig. 1). At this pH the optimum concentration of substrate was 0·065 M for Patella and 0·04 M for *Littorina* (Fig. 2). Under these optimum conditions the release of *p*-hydroxyacetophenone by both enzyme preparations was not rectilinearly related to the time of incubation (Fig. 3). The decrease in activity with time is apparently not due to the formation of inhibitory end-products of the reaction since for both preparations there was a rectilinear relationship between the extent of hydrolysis of *p*-acetylphenylsulphate and the concentration of enzyme.

Effect of incubation in the absence of substrate. Dodgson *et al.* (1953) have shown that preliminary incubation of rat-liver suspensions in the absence of substrate results in a loss of arylsulphatase activity. *Littorina* and *Patella* preparations also lost enzyme activity when incubated before addition of substrate. In a typical experiment the activities of *Littorina* and *Patella* suspensions had declined to 79·5 and 70·5% respectively of the initial value after incubation for 1 hr. at 37·5°. This inactivation was still appreciable at room temperature, but there was no loss of enzyme activity in enzyme preparations

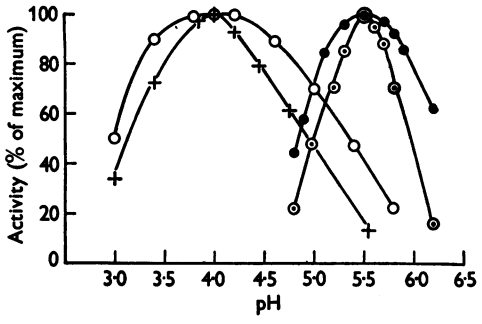

Fig. 1. pH activity curves for the arylsulphatases and β-glucuronidases of visceral-hump suspensions of *Patella* and *Littorina*. For arylsulphatase the respective concentrations of potassium *p*-acetylphenylsulphate were 0·065 M and 0·04 M in 0·5 M-acetate buffer. For β-glucuronidase the concentration of *p*-chlorophenylglucuronide was 0·045 M in 0·5 M-acetate buffer. Incubations were for 1 hr. at 37·5°. ●—●, *Patella* arylsulphatase; ⊙—⊙, *Littorina* arylsulphatase; ○—○, *Patella* β-glucuronidase; ×—×, *Littorina* β-glucuronidase.

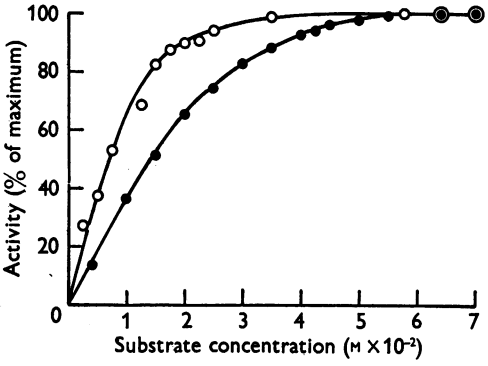

Fig. 2. Substrate concentration-activity curves for the arylsulphatases of *Patella* and *Littorina* acting on potassium *p*-acetylphenylsulphate in 0·5 M-acetate buffer, pH 5·5, during 1 hr. at 37·5°. ●—●, *Patella*; ○—○, *Littorina*.

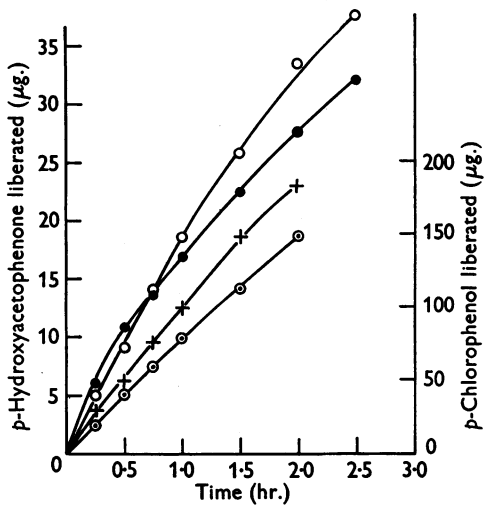

Fig. 3. Time-activity curves for the arylsulphatases and β-glucuronidases of *Patella* and *Littorina* at 37·5° using optimum concentrations of potassium *p*-acetylphenylsulphate in 0·5 M-acetate buffer, pH 5·5, and *p*-chlorophenylglucuronide in 0·5 M-acetate buffer, pH 4·0. ●—●, *Patella* arylsulphatase; ○—○, *Littorina* arylsulphatase; ⊙—⊙, *Patella* β-glucuronidase; ×—×, *Littorina* β-glucuronidase.

maintained at 0° for several hours. For this reason all enzyme preparations were kept at 0° before assay.

The fractionation of arylsulphatase in buffered suspensions. Dodgson *et al.* (1953) noted that whole suspensions of rat liver must be used in the assay of rat-liver arylsulphatase, since only about 60–80% of the total enzyme activity is present in supernatant solutions after the suspensions have been centrifuged at 2000 *g* for 20 min. This also applies to mollusc preparations (Table 1). As with rat arylsulphatase the total activity of mollusc suspensions is not obtained when the supernatant

Table 1. *The fractionation of mollusc arylsulphatase and β-glucuronidase*

(Suspensions were centrifuged at 2000 *g* for 15 min. at 0°, and the supernatant solutions and debris assayed separately using the optimum conditions for enzyme activity. The activities of the whole suspensions were determined simultaneously. Values are expressed as percentage of activity of whole suspension.)

Organism	Suspension medium	Activity: Supernatant	Activity: Residue	Loss (%)
	Arylsulphatase			
Littorina littorea	Water	80·5	7·4	12·1
	0·5 M-acetate buffer, pH 5·5	79·0	6·8	14·2
Patella vulgata	Water	87·6	3·5	8·9
	0·5 M-acetate buffer, pH 5·5	71·5	16·8	11·7
	β-Glucuronidase			
Littorina littorea	Water	73·6	31·2	—
	0·5 M-acetate buffer, pH 4·0	66·7	31·5	1·8
Patella vulgata	Water	92·0	9·5	—
	0·5 M-acetate buffer, pH 4·0	78·3	21·5	0·2

solution and the debris are separated and assayed independently. This point is being investigated further.

β-Glucuronidase

The characteristics of β-glucuronidase. Both *Patella* and *Littorina* preparations showed greatest β-glucuronidase activity at pH 4·0 in 0·5 M-acetate buffer (Fig. 1). At this optimum pH both enzyme preparations were most active when the concentration of substrate was 0·045 M. Greater concentrations of substrate showed no inhibitory effect (Fig. 4).

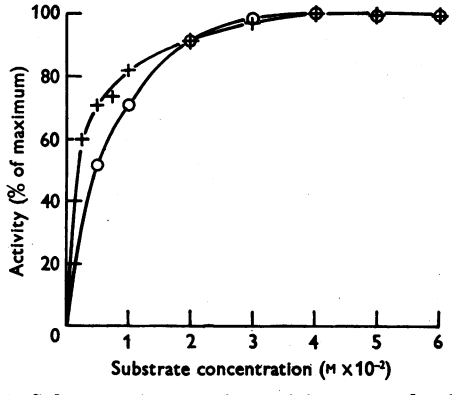

Fig. 4. Substrate concentration-activity curves for the β-glucuronidases of *Patella* and *Littorina* acting on *p*-chlorophenylglucuronide monohydrate in 0·5 M-acetate buffer at pH 4·0 at 37·5° during 1 hr. ×—×, *Patella*; O—O, *Littorina*.

There was not a straight-line relationship between the time of incubation and β-glucuronidase activity of either *Patella* or *Littorina* preparations, using the optimum conditions of pH and substrate concentration, although over the period of the first hour the curves were very nearly rectilinear (Fig. 3). A graph relating the enzyme concentration with the degree of hydrolysis of *p*-chlorophenylglucuronide gave the expected rectilinear curves.

Effect of incubation in the absence of substrate. Incubation of the enzyme preparations in the absence of substrate resulted in a small but steady loss of enzyme activity. In typical experiments the activity of *Littorina* and *Patella* preparations after incubation for 1 hr. had declined to 84 and 97% respectively of the initial activities. It is thus necessary to keep all enzyme preparations at 0° before assay.

Fractionation of the enzyme in buffered suspensions. The β-glucuronidase activity of both organisms is distributed between supernatant solution and debris when suspensions are centrifuged at 2000 *g* for 15 min. (Table 1). It is therefore necessary to use whole suspensions in order to realize maximum enzyme activity. In contrast to arylsulphatase there appears to be no loss in β-glucuronidase activity when the supernatant solution and the debris are separated and assayed independently.

The distribution of arylsulphatase and β-glucuronidase in various marine molluscs

It has been shown that the optimum conditions for β-glucuronidase activity in *Littorina* and *Patella* are identical. These conditions have been used when assaying β-glucuronidase activities of other marine organisms. The optimum conditions for the arylsulphatase activity of the two organisms, however, are not identical, for although the pH optima are the same (pH 5·5) the optimum substrate concentration of *Patella* (0·065 M) is higher than that of *Littorina* (0·045 M). In neither case was there any inhibition by excess of substrate and for the assay of arylsulphatase activity of other molluscs the higher substrate concentration has been used. Since Dodgson *et al.* (1952) indicated that the arylsulphatase activity of marine molluscs can vary with their nutritional state, and Soda (1936) suggested possible seasonal variations of enzyme activity, organisms were assayed as soon as possible after collection and only those results obtained during the month of November are quoted. As far as possible within each species organisms of the same weight were selected.

The required organs were dissected out and washed in ice-cold distilled water for a few seconds in order to remove contaminating sea water. After being dried on filter paper, they were weighed and suspended in chilled distilled water for 2 min. using a chilled glass homogenizer. A portion of each suspension was diluted with distilled water for arylsulphatase assay to give a solution, 0·6 ml. of which would liberate between 15 and 30 μg. *p*-hydroxyacetophenone; the remainder was diluted to give a solution, 0·4 ml. of which would liberate between 100 and 200 μg. *p*-chlorophenol for β-glucuronidase assay. The appropriate amount of diluted suspension was then incubated for 1 hr. at 37·5° with an equal amount of the substrate in 1·0 M-acetate buffer of the required pH.

The results recorded in Table 2 show that all organisms tested were highly active with respect to both enzymes.

DISCUSSION

Although the present survey of the distribution of arylsulphatase and β-glucuronidase in the *Mollusca* is not comprehensive, there is no doubt that many of these organisms are particularly rich sources of the two enzymes. In order to make a balanced distribution survey several important factors would have to be considered. Thus during the present work it has been noted that the activities due to both enzymes increase considerably if the organisms are starved. This may be an apparent increase due to the elimination of material from the alimentary canal

Table 2. *Arylsulphatase and β-glucuronidase activities of various molluscs*

(Arylsulphatase activity was determined at pH 5·5 using 0·065M-potassium *p*-acetylphenylsulphate in 0·5M-acetate buffer. β-Glucuronidase activity was determined at pH 4·0 using 0·045M-*p*-chlorophenylglucuronide monohydrate in 0·5M-acetate buffer. Activities are expressed as μg. phenol liberated/g. wet tissue/hr. Ranges are given in parentheses.)

Organism	Organs taken	No. of expt.	β-Glucuronidase activity	Arylsulphatase activity
Calliostoma zizyphinum	Visceral hump	6	8 138 (2 950–15 480)	37 592 (27 900–45 600)
Nucella lapillus	Visceral hump	5	9 084 (1 350–17 500)	13 450 (1 476–36 000)
Osilinus lineatus	Visceral hump	5	53 180 (42 300–71 000)	284 200 (164 000–382 500)
Littorina littorea	Visceral hump	5	19 780 (14 400–25 200)	54 140 (42 800–82 500)
Patella vulgata	Visceral hump	5	44 925 (18 420–57 400)	98 500 (57 600–141 000)
Gibbula umbilicalis	Visceral hump	5	43 608 (31 700–68 000)	127 360 (64 000–202 000)
G. cineraria	Whole organism	5	4 988 (2 448–7 616)	27 540 (12 400–38 600)
Buccinum undatum	Posterior digestive gland	3	8 950 (5 220–13 950)	66 633 (49 200–89 400)
	Anterior digestive gland	3	1 490 (910–2 070)	10 576 (3 850–21 300)
	Ovary	1	2 990	4 120
Scaphander lignarius	Visceral hump	3	2 336 (1 248–3 022)	12 970 (7 110–17 800)
Mytilus edulis	Digestive glands	5	13 912 (4 740–30 600)	49 900 (28 700–76 000)
Ostrea edulis	Digestive glands	4	10 078 (7 850–17 080)	12 935 (5 140–20 800)

during starvation, or a true increase. It would seem important to select organisms of similar age, sex and size. This is not easy for, although Moore (1936, 1937) has outlined methods for determining the sex and age of *Littorina*, these are not generally applicable to other organisms. Finally, it would be necessary to locate the enzymes more precisely within each species. The term 'visceral hump' is vague but it usually includes the digestive glands, the ovaries or testes, the alimentary canal and the kidney. In the larger organisms it is comparatively easy to recognize and dissect out the individual organs, but in the smaller organisms this is often impracticable. Moreover, although the work of Soda (1936) shows that the arylsulphatase activity of tropical molluscs is confined mainly to the digestive glands, this may not necessarily apply to the present study. Indeed it is possible that enzyme activity might be partially microbial in origin. Karunairatnam & Levvy (1951) have shown that the β-glucuronidase activity of sheep rumen is due to bacteria, and Marsh, Alexander & Levvy (1952) have suggested that the high β-glucuronidase activity found in the omasum, rumen, caecum and colon of many animals might well have a similar origin. Buehler, Katzman, Doisy & Doisy (1949) and Barber, Brooksbank & Haslewood (1948) have also revealed the potent β-glucuronidase activity of certain bacteria and Whitehead, Morrison & Young (1952) have found arylsulphatase activity in certain strains of salmonellae and mycobacteria. The possibility that the activity of β-glucuronidase and arylsulphatase in molluscs is due in part to bacteria or other parasites cannot be excluded, and is being studied further.

In a study of glucosulphatase and arylsulphatase in molluscs, Soda (1936) noted a correlation between the distribution of the two enzymes. The results of the present work suggest that the distribution of arylsulphatase and β-glucuronidase may be similar. Thus the activity of both enzymes appears to be very much greater in *Littorina*, *Patella*, *Osilinus* and *Gibbula umbilicalis* than in *Ostrea*, *Scaphander*, *Mytilus* and others. However, there is an insufficient number of experiments to justify such a conclusion.

Among the organisms studied enzyme activity cannot be correlated with habitat or feeding habits since some are relatively active carnivora living permanently under water (e.g. *Buccinum* and *Scaphander*) whereas others are relatively inactive herbivora periodically exposed to the atmosphere

Table 3. *The optimum* pH *and substrate concentrations for arylsulphatases of different origin, when potassium* p*-acetylphenylsulphate is the substrate*

Source	In 0·5M-acetate buffer		In 0·2M-phosphate buffer	
	Opt. pH	Opt. substrate concentration ($M \times 10^{-3}$)	Opt. pH	Opt. substrate concentration ($M \times 10^{-3}$)
Fungal				
Taka diastase (Parke, Davis and Co.) containing added lactose	6·2–6·4	4	7·6	3·5
Molluscan				
Patella vulgata	5·5	65	No activity	
Littorina littorea	5·5	40	No activity	
Mammalian				
Rat	7·2	7	8·2*	3·5*
Man	6·9	5	7·3	8

* Results from an acetone-dried preparation of rat liver.

by the retreating tide (*Patella*, *Littorina* and *Mytilus*). No clue is thus afforded as to the physiological significance of the two enzymes in these organisms. It is possible that β-glucuronidase may play a digestive role since many of the marine algae on which some of these organisms feed contain polysaccharide material which is rich in uronic acid residues. The function of arylsulphatase is even more obscure since the existence of naturally occurring substrates of the enzyme in the marine environment has not been established.

The high arylsulphatase and β-glucuronidase activities found in molluscs during this work probably do not represent the true picture of the activities of the enzymes in the intact organism in their marine environment. For instance the activity of the arylsulphatase of *Littorina* and *Patella* is inhibited to the extent of about 15 and 33% respectively in the presence of 50% sea water, although β-glucuronidase activity of the organisms is largely unaffected. Moreover, the activity of all the enzyme preparations is increased seven or eight times by raising the temperature of incubation from 10° to 37·5°. However, these facts do not lessen the possible value of molluscs as starting material for the preparation of highly active enzyme concentrates. Dodgson *et al.* (1952) have obtained mollusc arylsulphatase concentrates with activities many times greater than those from other sources, and it seems likely that highly active β-glucuronidase preparations could be prepared from the same material.

Table 3 shows the optimum pH values and optimum substrate concentrations for arylsulphatases of different origin which have been studied in this laboratory using potassium *p*-acetylphenylsulphate as substrate. The optimum conditions for the arylsulphatases of the rat, *Aspergillus oryzae*, and man are similar but very different from those for the enzyme from molluscs. This is true whether the buffer be acetate or phosphate. Unfortunately, mollusc β-glucuronidase is not comparable with β-glucuronidases from other sources which have been studied using substrates other than *p*-chlorophenylglucuronide. It is of interest, however, that there is as yet no evidence that the mollusc enzyme possesses more than one pH optimum. This is in contrast to the findings of Mills (1948), Mills & Paul (1949) and Spencer & Williams (1951) for ox-spleen β-glucuronidase, and Kerr, Campbell & Levvy (1949) for the mouse-liver enzyme.

SUMMARY

1. Using potassium *p*-acetylphenylsulphate and *p*-chlorophenylglucuronide monohydrate as substrates, the conditions necessary for the activity of the arylsulphatases and β-glucuronidases respectively of *Patella vulgata* and *Littorina littorea* have been studied.

2. Using the optimum conditions established for these organisms a short survey of the distribution of the two enzymes in other molluscs has been made.

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The Effect of Cobalamin on the Quantitative Utilization of Serine, Glycine and Formate for the Synthesis of Choline and Methyl Groups of Methionine

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It was observed by Du Vigneaud, Chandler, Moyer & Keppel (1939) that rats generally lost weight when kept on a synthetic diet containing homocystine but deficient in methionine and choline. The favourable effect on growth of the addition of choline or betaine indicated the occurrence of transmethylation which was conclusively proved later by isotope experiments carried out in Du Vigneaud's laboratory. In these early experiments it was noticed that occasionally some animals on the deficient diets grew, but this was ascribed to the action of the intestinal flora. These observations were greatly extended by Bennett and co-workers (Bennett, Medes & Toennies, 1944; Bennett & Toennies, 1946) who also obtained evidence suggesting that factors present in liver extract are involved in the synthesis of labile methyl groups. That such a synthesis indeed occurred was shown with the aid of deuterium by Du Vigneaud, Simmonds, Chandler & Cohn (1945). However, the relatively small amount of deuterium in the choline methyl groups indicated that only about 8% of them was derived from the body water, the rest presumably originating from the methionine also present in the diet. This incorporation was again ascribed to micro-organisms.

More precise information about this synthesis of methyl groups in the rat was obtained by the use of carbon isotopes. Thus, one of us (Arnstein, 1950, 1951) showed that formate, methanol, the β-carbon atom of serine and the α-carbon atom of glycine can be precursors of choline methyl groups. Similar results were reported about the same time by various groups of American workers (Jonsson & Mosher, 1950; Sakami & Welch, 1950; Weissbach, Elwyn & Sprinson, 1950; Du Vigneaud, Verly & Wilson, 1950). Sakami (1950) also demonstrated that the feeding of acetone labelled with ^{14}C in the methyl group gives rise to radioactivity in the methyl group of choline. Du Vigneaud, Verly, Wilson, Rachele, Ressler & Kinney (1951) used methanol labelled both with ^{14}C and deuterium and showed that the hydrogen was diluted to a greater extent than the carbon, indicating that the methyl group of methanol is not directly converted to that of choline. These workers obtained results similar to those reported for formate on feeding [^{14}C]formaldehyde, [^{14}C]formylphenylalanine and [^{14}C]methyl stearate. *In vitro* formation of choline methyl groups from various precursors has also been reported (Sakami & Welch, 1950; Siegel & Lafaye, 1950; Berg, 1951). These experiments and the important observations