Studies on Sulphatases

2. THE ASSAY OF THE ARYLSULPHATASE ACTIVITY OF RAT TISSUES

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The discovery of arylsulphatase activity in certain gastropods (Derrien, 1911) and extracts of *Aspergillus oryzae* (Neuberg & Kurono, 1923) was quickly extended to mammalian sources when Neuberg & Simon (1925) showed that liver, kidney, brain and muscle of rabbit, guinea pig and man were capable of hydrolysing potassium phenylsulphate and potassium *p*-cresylsulphate. The enzyme appears to be common to most animal tissues. In man arylsulphatase activity has been found in a large variety of tissues (Rosenfeld, 1925; Russo, 1947; Huggins & Smith, 1947), and various organs of the pig, calf (Hommerberg, 1931), dog, chicken (Morimoto, 1937), cat (Torda, 1943) and rat (Huggins & Smith, 1947) are also active.

It appears that the arylsulphatase activity of animal tissues is confined to substrates in which sulphuric acid is conjugated with hydroxyl groups of phenolic character, but the widespread distribution of the enzyme and its simultaneous occurrence with phosphatases and esterases (Hommerberg, 1931) have occasioned some doubt concerning the separate identity of the enzyme. Furthermore, the liver, muscle and kidney of horses and rabbits possess a myrosulphatase which releases inorganic sulphate from potassium myronate and, with the evidence available, it is not possible to decide the identity of myrosulphatase and arylsulphatase in animal tissues. No mammalian sources of gluco- or chondro-sulphatases have been reported. Tanaka (1938) has differentiated the phosphatase and arylsulphatase activities of rabbit tissues, but the lack of further work on the purification of the enzyme leaves its specificity ill defined.

Most investigations on mammalian arylsulphatase have been of a qualitative nature, and it is only recently that the introduction of sensitive colorimetric assay methods has enabled quantitative measurements of the enzyme to be made (Huggins & Smith, 1947; Robinson, Smith & Williams, 1951). In the rat considerable activity has been found in the liver and suprarenals whilst spleen and kidney are less potent. Decreased enzyme activity was shown in the liver after carbon tetrachloride damage, accompanied by a rise in serum arylsulphatase (Abbott & East, 1949), and certain tumours have been reported to have greater activity than their respective tissues of origin (Huggins & Smith, 1947). The methods employed by the above authors for rat tissues have been criticized (Dodgson & Spencer, 1953) on the grounds that tissue extracts, containing only a portion of the total enzyme, were used. To measure the total arylsulphatase present whole homogenates of the tissues must be used, but recovery of the liberated phenols (*p*-nitrophenol and 4-nitrocatechol), which are measured colorimetrically as an estimate of enzyme activity, is incomplete from such homogenates due to the action of reductases and other enzymes present.

Dodgson & Spencer (1953) have shown that the use of *p*-acetylphenylsulphate provides a reliable method for estimating arylsulphatase activity in whole homogenates of rat tissues and the development of an assay method using this substrate is presented in this paper.

EXPERIMENTAL AND RESULTS

Estimation procedure. Rats and materials have been previously described (Dodgson & Spencer, 1953).

Whole homogenates of rat liver were used in establishing optimum conditions for the enzyme. The rats were killed by a blow on the back of the head and the livers rapidly removed and dropped into ice-cold water. Small portions were homogenized in a glass homogenizer (Potter & Elvehjem, 1936) for 2 min. with ice-cold buffer and were suitably diluted.

The ice-cold enzyme solution (0·6 ml.), adjusted to the required pH, was pipetted into 15 ml. tapered centrifuge tubes and pre-incubated for 4 min. before addition of 0·6 ml. substrate solution (potassium *p*-acetylphenylsulphate dissolved in buffer at the same pH as the enzyme solution). The mixtures (1·2 ml.) were incubated at 37·5° for the desired period and the enzyme action stopped and protein precipitated by addition of 4·8 ml. ethanol. After centrifuging, 1 ml. N-NaOH was added to 5 ml. of the clear supernatant and the absorption of the liberated *p*-hydroxyacetophenone was measured in the spectrophotometer at 327·5 mμ. against an ethanol blank. The estimations were duplicated and controls, containing the enzyme solution with the substrate added after incubation followed immediately by ethanol, were run simultaneously. It is unnecessary to include controls in which substrate is incubated with buffer since there is no hydrolysis of potassium *p*-acetylphenyl-

sulphate at 37·5° under the experimental conditions used. Although the specific absorption by protein at 327·5 mμ. is very slight, the ethanolic precipitation has been adhered to in order to clarify the incubation mixtures for the final measurement.

The amount of *p*-hydroxyacetophenone liberated in the incubation mixture is given by

$$\mu g.\ p\text{-hydroxyacetophenone} = (E_t - E_c) \times 43{\cdot}86,$$

where E_t and E_c are the observed extinctions of the test and control solutions respectively (Dodgson & Spencer, 1953). The percentage error involved in neglecting the decrease in absorption due to hydrolysis of the substrate is 0·35% which can be ignored.

Optimum pH. The wide range of pH optima reported for arylsulphatase can be attributed largely to the use of different sources of the enzyme by the various authors. In some cases the buffer has not been named and this point assumes importance since our results indicate that the buffer can greatly influence the optimum pH of rat-liver arylsulphatase. Takadiastase (Dodgson & Spencer, unpublished observation) is also affected in this way.

The only optimum pH recorded for rat tissues is that for the enzyme hydrolysing potassium *p*-nitrophenylsulphate. Abbott & East (1949) found optimum activity with this substrate at pH 6·6, whereas with the takadiastase enzyme the corresponding value was 6·12 (Huggins & Smith, 1947). Seligman, Chauncey & Nachlas (1951) have used potassium 6-bromo-2-naphthylsulphate as a substrate for rat-liver arylsulphatase at pH 5, and Robinson *et al.* (1951) used a pH of 6·0 with potassium 2-hydroxy-4-nitrophenylsulphate, but the authors do not state whether these values are those of the optimum pH for the enzyme.

Preliminary experiments with potassium *p*-acetylphenylsulphate suggested that the optimum pH of rat-liver arylsulphatase in the presence of 0·5M-acetate was in the region 7·1–7·3 and that the optimum substrate concentration was 0·007M. At pH 7·2 acetate has slight buffering action only, but measurements of pH before and after 1 hr. incubation of enzyme extracts with substrate in 0·5M-acetate at pH 7·2 showed no significant difference. Water homogenates of most rat tissues, however, have a pH in the neighbourhood of 7·2 and possess a certain buffering power. It was decided to continue assays in the presence of acetate to allow comparison with work carried out simultaneously of the arylsulphatases of marine molluscs, bacteria and takadiastase (Dodgson, Lewis & Spencer, 1952; Dodgson & Spencer, unpublished). In 0·2M-phosphate buffer the optimum activity of the enzyme of rat-liver homogenates was at pH 7·75.

Rat liver was homogenized in 0·5M-sodium acetate and portions of the homogenate were adjusted to the required pH with 0·5M-acetic acid and kept ice-cold until placed in the water bath. Substrate (0·007M final concentration) was dissolved in 0·5M-acetate at the desired pH. Measurements of pH before and after incubation (1 hr.) agreed to within 0·02 pH, within the limits 4·3–7·5. Above 7·5 the final pH was significantly lower than the initial value and in Fig. 1 points marked at a pH greater than 7·5 represent the pH midway between the initial and final values.

The pH-activity curve shown in Fig. 1 indicates that the arylsulphatase of rat liver has maximum activity at pH 7·2 under the experimental conditions specified.

Optimum substrate concentration. Variation of the substrate concentration affected enzyme activity in the manner expected from the Michaelis-Menten equations (see Fig. 2). With 0·5M-acetate at pH 7·2 and 1 hr. incubation, optimal activity was shown at 0·007M-potassium *p*-acetylphenylsulphate; greater concentrations of substrate showed no inhibitory effect. The plot of $[S]/V$ against $[S]$ was a straight

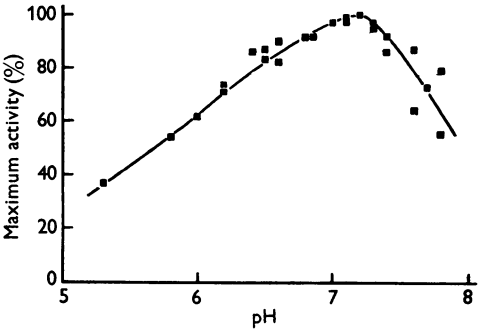

Fig. 1. pH-Activity curve for the arylsulphatase of rat-liver homogenates acting on potassium *p*-acetylphenylsulphate (0·007M) in 0·5M-acetate buffer at 37·5° during 1 hr.

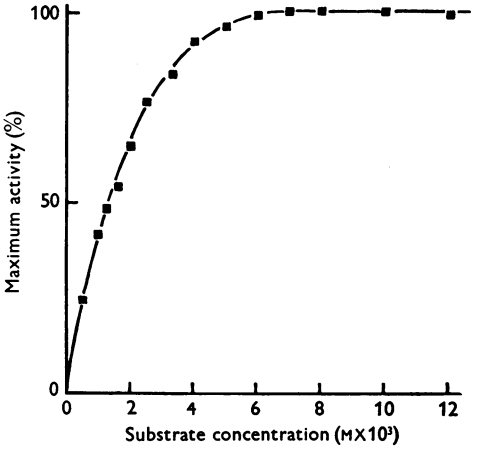

Fig. 2. Substrate concentration-activity curve for rat-liver arylsulphatase acting in 0·5M-acetate, pH 7·2, on potassium *p*-acetylphenylsulphate at 37·5° during 1 hr.

line and the Michaelis-Menten constant, K_m, was calculated to be $1{\cdot}57 \times 10^{-3}$M in a typical experiment. A second determination gave K_m $1{\cdot}54 \times 10^{-3}$M. No previous determination of K_m of rat-liver arylsulphatase has been reported.

Effect of time and enzyme concentration. The effect of incubating rat-liver homogenates with the substrate for various periods was studied under the optimum conditions of pH and substrate concentration. The typical curves shown in Fig. 3 demonstrate that a linear relationship exists between

liberation of *p*-hydroxyacetophenone and time up to 1·25–1·5 hr. but some decrease in activity occurs after this point. Fig. 4 shows that under the optimum conditions the release of *p*-hydroxyacetophenone bears a rectilinear relationship to the enzyme concentration even when large amounts of

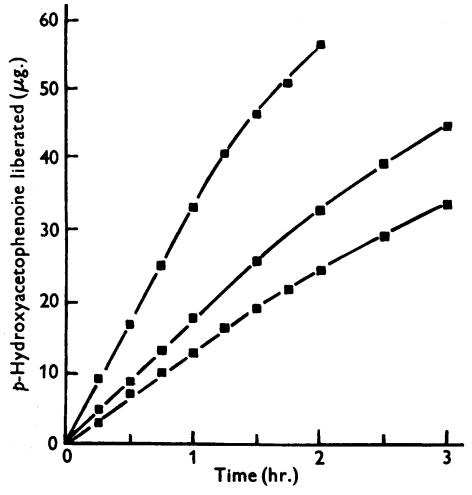

Fig. 3. Time-activity curves for rat-liver arylsulphatase acting at 37·5° on potassium *p*-acetylphenylsulphate in 0·5 M-acetate, pH 7·2. The results of three separate experiments, using different homogenates, are shown.

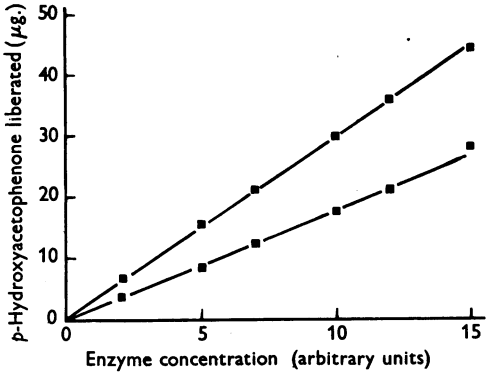

Fig. 4. Enzyme concentration-activity curves for rat-liver arylsulphatase acting on potassium *p*-acetylphenylsulphate (0·007 M) in 0·5 M-acetate at pH 7·2. Incubation was for 1 hr. at 37·5°. The results of two experiments, using different homogenates, are shown.

the phenol and inorganic sulphate are liberated. The decrease in activity of the enzyme with time often occurs when there is comparatively little hydrolysis and therefore it cannot be attributed to inhibition by the products of the reaction. In crude homogenates during incubation various enzymic reactions on natural substrates are taking place and the formation of products inhibitory to arylsulphatase is possible; proteolytic destruction of the enzyme may also take place.

The effect of pre-incubation. Since assay values were low when no precautions were taken to keep homogenates cold during manipulation prior to incubation with substrate, the loss of enzyme activity in crude homogenates incubated in the absence of substrate was investigated. Rat-liver homogenates in 0·5 M-acetate at pH 7·2 were incubated at 37·5° for various periods and subsequently assayed over 1 hr. with 0·007 M substrate. The results shown in Fig. 5 indicate that there is a large

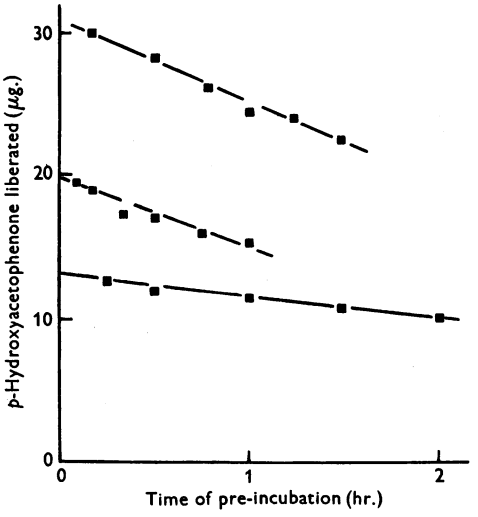

Fig. 5. The effect of pre-incubation at 37·5° on the arylsulphatase activity of rat-liver homogenates. The homogenate, in 0·5 M-acetate buffer, pH 7·2, was incubated at 37·5° for various periods and then assayed for arylsulphatase activity under the standard conditions. Three separate experiments, using different homogenates, are illustrated.

decrease in activity caused by pre-incubation at 37·5° and after 48 hr. all activity was lost. If enzyme solutions were kept ice-cold before incubation with substrate there was no loss of arylsulphatase activity even after 4–5 hr. but inactivation was still considerable at room temperature. For this reason it is essential to maintain all tissue preparations at 0° before assay.

The fractionation of arylsulphatase. In the previous paper (Dodgson & Spencer, 1953) the recovery of various phenols from rat tissues was investigated with respect to their suitability for arylsulphatase assay. Preliminary work indicated that supernatant solutions of centrifuged homogenates of rat tissues prepared in a similar manner to those described by Huggins & Smith (1947) did not contain the full arylsulphatase present and, for this reason, it was considered that the use of whole

homogenates in the recovery experiments was necessary.

Further work confirms this point. Freshly removed rat liver was homogenized in ice-cold water or acetate buffer. Portions were centrifuged under ice-cold conditions for 15 min. at 2200 rev./min., the supernatant separated and the residue suspended in the same medium as that used in the original homogenization. The whole homogenate, supernatant and residue were then adjusted to pH 7·2 and made 0·5 M with respect to acetate, and the arylsulphatase activities assayed. The results presented in Table 1 demonstrate that it is necessary to use whole homogenates of liver in order to realize the full activity. Fractionation by centrifugation leads to loss of the enzyme and the supernatant solutions contain only 60–80% of the total activity. Fractionation of rat-liver arylsulphatase is being further studied.

Table 1. *The fractionation of rat-liver arylsulphatase by centrifugation of homogenates*

(The figures quoted are the mean values from three female rats.)

Medium	pH	Activity (% of whole homogenate) Supernatant	Residue	Loss (%)
Water	—	78·5	10·8	11·7
0·5 M-Acetate	7·2	63·2	20·6	16·2

Method of assay

The preceding work established the optimum conditions for the arylsulphatase of rat-liver homogenates and a standard unit of activity may now be defined; one *p*-hydroxyacetophenone unit of arylsulphatase activity is that which liberates 1 μg. *p*-hydroxyacetophenone in 1 hr. from 0·007 M-potassium *p*-acetylphenylsulphate in the presence of 0·5 M-acetate, pH 7·2, at 37·5°.

The following procedure was adopted for assay work. The freshly killed adult rat was sectioned at the neck to allow free bleeding and the required organs dissected out and placed in ice-cold water. The organs were freed of fat, dried on filter paper, weighed and homogenized for 2 min. in ice-cold 0·5 M-acetate buffer, pH 7·2, using a glass homogenizer. Whole organs were used except in the case of intestine and liver where it was sufficient to use small samples. The tissue homogenates were then diluted with the buffer to suitable strengths so that the use of 0·6 ml. in the estimation procedure detailed earlier liberated 15–30 μg. *p*-hydroxyacetophenone. Owing to the fall in activity after 1·5 hr., 1 hr. incubations were adhered to. Ice-cold conditions were maintained for all solutions and apparatus throughout manipulation prior to incubation of the tissue with the substrate. With some tissues (brain, adrenals and ovaries) cloudiness develops in the final solution and further centrifugation is necessary before reading is made with the spectrophotometer.

Table 2. *The assay of arylsulphatase in liver samples from the liver (7·7 g.) of an adult female rat*

Sample	*p*-Hydroxyacetophenone liberated by 0·6 ml. in 1 hr. (μg.)	*p*-Hydroxyacetophenone liberated/g. tissue in 1 hr. (μg.)	Error from mean (%)
1	29·2	2040	−1·92
2	31·8	2030	−2·4
3	31·75	2090	+0·48
4	16·32	2150	+3·37
5	30·8	2150	+3·37
6	27·7	2020	−2·88

The use of small samples of liver rather than the whole organ was justified by showing that replicate samples from the same liver gave values which were within ±5% of the mean. The results of a typical experiment are given in Table 2.

A survey of the distribution of arylsulphatase in the tissues of rats has been made and the results are presented in Table 3.

DISCUSSION

The distribution of the enzyme in the various tissues compares broadly with that found by Huggins & Smith (1947) but differs from the results of Robinson *et al.* (1951). The latter authors report that spleen

Table 3. *Arylsulphatase content of rat tissues*

(Expressed as *p*-hydroxyacetophenone units of arylsulphatase activity per g. of wet tissue together with the standard error calculated with $n-1$ for small samples. The number of determinations appears in parentheses. *P* is the probability calculated by the '*t*' test.)

Organ	Male	Female	*P*
Liver	3630±161 (21)	2131±106 (26)	<0·01
Adrenals	1074±225 (6)	940± 78 (7)	0·40>0·30
Kidney	765± 36 (9)	722± 43 (8)	0·50>0·40
Heart	580± 42 (7)	518± 40 (7)	0·40>0·30
Spleen	550± 18 (9)	536± 19 (6)	0·70>0·60
Lung	450± 20 (8)	442± 42 (8)	0·90>0·80
Brain	203± 4 (5)	184± 10 (7)	0·20>0·10
Muscle	175± 10 (4)	178± 12 (6)	0·90>0·80
Intestine	166± 9 (4)	142± 12 (6)	0·30>0·20
Testes	432± 20 (8)	—	—
Ovaries	—	403± 34 (7)	—

is the most active organ, with liver only 10–20% as active, but since Robinson *et al.* and Huggins & Smith used tissue extracts and not whole homogenates and conditions of pH and substrate concentration that were optimal for the mould enzyme, their results are not strictly comparable with those of the present study. An added complication is that a strain difference appears to exist for arylsulphatase in rats (Dodgson & Spencer, unpublished). Although the numbers of samples in Table 3 are relatively small, the arylsulphatase activities of the livers were apparently normally distributed. The 't' test has therefore been applied and the results show that there is a significant difference between the enzyme activities of the male and female Medical Research Council hooded rats ($t=8{\cdot}16$, $n=45$, $P<0{\cdot}01$). A similar difference has been observed for the liver alkaline phosphatase of Sprague-Dawley rats (Lowe & Salmon, 1951) but not for liver β-glucuronidase of mice (Levvy, Kerr & Campbell, 1948). Since the values in Table 3 are based on wet weights of liver it is possible that the difference in enzyme activity may be accounted for by a difference in water content of the livers of the two sexes. However, the ratio wt. of animal:wt. of liver is the same in males (23·3 $\pm$ 0·44 (S.E.), $n=35$) and females (23·3 $\pm$ 0·48 (S.E.), $n=37$). The other organs of the rat showed arylsulphatase activities which were similar for both sexes (Table 3). Further investigation of these observations is in progress.

The function of arylsulphatase in the animal body is as yet unknown. There is some indication that its action may be purely hydrolytic since, after injection into rats, the ^{35}S-labelled ester sulphate group of sodium oestrone sulphate is recoverable in the urine to the extent of 75% as inorganic sulphate (Hanahan & Everett, 1950). On the other hand, sodium phenylsulphate can be quantitatively recovered unchanged from rabbit urine after oral administration (Garton & Williams, 1949). Alternatively, arylsulphatase activity may be the reverse process of what, in the intact animal, is normally a synthesizing action requiring a coupled energy-supplying system. Arylsulphate synthesis by rat tissues is known to be an endergonic reaction (DeMeio & Tkacz, 1952) which would not function under the conditions of assay employed in the present study.

In view of this possibility it is interesting to compare the distribution of the hydrolysing and the synthesizing action. The main points that emerge are that although the synthesizing ability of the liver and intestine are the same (DeMeio & Arnolt, 1944) the hydrolytic activities of these two organs are very different (Table 3) and that, whereas arylsulphatase appears to be common to all tissues, only liver, intestine and spleen were found to have synthesizing power (DeMeio & Arnolt, 1944). These differences do not necessarily show that different enzymes are responsible for synthesis and hydrolysis since there may be variations in other factors necessary for the synthesis of arylsulphates. Furthermore, the strain difference in rats used by DeMeio and in the present study must be taken into account since strain difference for both hydrolytic (Dodgson & Spencer, unpublished) and synthetic activities of rat tissues have been demonstrated (DeMeio & Arnolt, 1944).

SUMMARY

1. The optimum conditions for the arylsulphatase of rat-liver homogenates have been established using potassium p-acetylphenylsulphate as the substrate. In 0·5M-acetate the enzyme is optimally active at pH 7·2 and a substrate concentration of 0·007M.

2. Arylsulphatase activity varied linearly with the concentration of the enzyme but declined after incubation with the substrate for periods longer than 1·25–1·5 hr. Incubation of rat-liver homogenates in 0·5M-acetate at pH 7·2 in the absence of substrate caused considerable decrease in enzyme activity.

3. Fractionation of homogenates by centrifugation divided the enzyme between the supernatant and the residue but some loss of enzyme occurred.

4. A survey of the arylsulphatase content of rat tissues showed the greatest concentration in the liver. Adrenals, kidney, heart, spleen, lung, testes, ovaries, brain, muscle and intestine had lesser amounts, the values decreasing in the order given.

5. The arylsulphatase activity of the liver of male rats is significantly higher than that of females.

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The Effect of Zinc Deficiency on the Aldolase Activity in the Leaves of Oats and Clover

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It is now well established that zinc is an essential nutrient element for plants. When the supply of zinc falls short of the physiological requirements of green plants, the retarded growth, the chlorosis, and the morphological changes in the organized structures which supervene, all suggest serious metabolic disturbances. Several biochemical lesions have been described. An abnormal protein metabolism (Bean, 1942), an interruption of tryptophan synthesis and thus of the production of β-indole-3-acetic acid (Tsui, 1948), an accumulation of inorganic phosphorus and an impairment of carbohydrate metabolism (Reed, 1946), have all been observed in zinc-deficient green plants.

In fungi, the retardation of growth in zinc-deficient media has been claimed to be due to a failure of carbohydrate metabolism, in particular, to a break of one or more links in the chain of anaerobic glycolysis (Foster & Denison, 1950). Marked changes in the enzymic constitution of zinc-deficient *Neurospora crassa* have been described by Nason, Kaplan & Colowick (1951).

The accumulation of reducing sugars and the relative shortage of sucrose and starch, which have been observed in the leaves of zinc-deficient tomato plants, imply an impairment of a fundamentally important step in carbohydrate metabolism which might possibly be attributed to a partial failure of aldolase activity (Reed, 1946). There seems little reason to assume, however, that zinc is concerned directly with the aldolase activity in the tissues of green plants, since the activity, *in vitro*, of aldolases from different sources is not invariably influenced by reagents which form unionized complexes with heavy metals. As there is no experimental evidence to support the suggestion (Reed, 1946) that the disordered carbohydrate metabolism in zinc-deficient plants may be attributable to a partial failure of aldolase, the aldolase activity has been estimated in the leaves of normal and of zinc-deficient oats (*Avena sativa* var. Algerian) and of subterranean clover (*Trifolium subterraneum* var. Bacchus Marsh); and the estimations have been extended to include a series of determinations of the aldolase activity in the tissues of oat plants grown in copper-deficient media.*

EXPERIMENTAL

The water cultures. The main salts employed for the nutrient medium were freed from Zn and Cu by the exhaustive extraction of aqueous solutions at pH 7·0 with dithizone in CCl_4 and were subsequently recrystallized. The trace salts were purified by recrystallization.

The procedure adopted for growing the plants was briefly as follows: six plants were suspended through holes in the heavily paraffin-waxed wooden lids that covered each of the series of 3 l. Pyrex beakers containing the aerated culture solutions. Light was excluded from the solution by covering the outsides of the beakers with opaque paper. The solutions were aerated for 1 hr. each day with a stream of air, freed from dust, and saturated, by passing through a glass tower packed with glass Raschig rings immersed in glass-distilled water, and conveyed through plastic tubing. The water lost from the medium by transpiration and evaporation was replaced with glass-distilled water.

Each beaker held 3 l. of a culture solution containing: KNO_3, 1·0 g.; KH_2PO_4, 0·5 g.; $CaSO_4.2H_2O$, 0·5 g.; $MgSO_4.7H_2O$, 0·25 g.; ferric citrate, 0·04 g.; $MnSO_4.4H_2O$, 4 mg.; $Na_2MoO_4.2H_2O$, 0·25 mg.; H_3BO_3, 0·57 mg. (all/l.) Zinc sulphate and copper sulphate supplements were added to make the following final concentrations: for the zinc-deficient plants, 100 μg. Cu/l.; for the copper-deficient plants, 200 μg. Zn/l.; for the controls, 100 μg. Cu/l., 200 g.

* A brief note of the findings has been published (Quinlan-Watson, 1951).