transitions appearing in the solidification-point curves of binary mixtures of branched-chain acids with normal fatty acids. As is characteristic of *iso* acids, the melting points of these fractions are about 1° below the melting points of their corresponding normal acids (Weitkamp, 1945; Cason, 1948). Further melting-point evidence consistent with the fractions being *iso*stearic acid is that when they are mixed in equal quantities with *n*-stearic acid their mixed melting points were depressed by approximately 10° (cf. Weitkamp, 1945).

Before the present investigation only two high-molecular-weight even-numbered *iso* acids had been isolated from natural fats, namely the C_{14} *iso* acid 12-methyltridecanoic acid from butterfat (Hansen, Shorland & Cooke, 1954*b*) and the C_{16} *iso* acid 14-methylpentadecanoic from ox-perinephric fat (Hansen, Shorland & Cooke, 1955). In wool grease, however, Weitkamp (1945) separated the complete series of even-numbered *iso* acids from C_{10} to C_{28} inclusive. Of the low-molecular-weight even-numbered *iso* acids, *iso*butyric acid has recently been identified in the steam-volatile acids of mutton fat (McInnes, Hansen & Jessop, 1956) by means of the gas–liquid chromatogram of James & Martin (1952). Similarly, *iso*butyric acid has been reported to be present in wool grease (Sheng-Lieh Liu, 1952).

It is estimated that in the sample of mutton fat investigated, 16-methylheptadecanoic acid represents not less than 0·05 % of the total fatty acids.

SUMMARY

Hydrogenated mutton fat has been found to contain trace quantities of 16-methylheptadecanoic (*iso*-stearic) acid.

The authors are much indebted to Dr A. W. Weitkamp, Research Laboratories of the Standard Oil Co., Whiting, Indiana, U.S.A., for kindly supplying a sample of pure *iso*-stearic acid, and to Dr G. G. Claridge, Soil Bureau, Department of Scientific and Industrial Research, Wellington, for the X-ray measurements reported in this paper.

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Studies on Sulphatases

13. THE HYDROLYSIS OF SUBSTITUTED PHENYL SULPHATES BY THE ARYLSULPHATASE OF *ALCALIGENES METALCALIGENES**

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In a previous paper (Dodgson, Spencer & Williams, 1955) the effect of variaton of pH on the affinity of enzyme and substrate (cf. Dixon, 1953) was examined for the arylsulphatase of *Alcaligenes metalcaligenes* and the substrates *p*-nitrophenyl, *p*-acetylphenyl and 2-hydroxy-5-nitrophenyl sulphates. The results indicated that the formation of the enzyme–substrate complex involved an electrostatic attraction between the substrate and positively and negatively charged groups in the enzyme.

Further information on the mechanism of the enzyme action has now been obtained by correlating the effect of structural change in the substrate molecule with the kinetics of the hydrolytic reaction.

* Part 12: Dodgson, Spencer & Wynn (1956).

Examples of this approach can be seen in the work of Gawron, Grelecki & Duggan (1953) with phenyl acetate and wheat-germ lipase, and of Nath & Rydon (1954) with phenyl β-D-glucosides and emulsin. The method requires a knowledge of the distribution of electrons about the ester linkage of the substrate; thus aromatic esters are particularly suitable since the substitution constants (σ) of Hammett (1938, 1940) provide a quantitative assessment of the effect of substituent groups on the distribution of electrons in the environment of the aromatic ring. Increasing positive or negative σ values indicate respectively the increasing electrophilic or nucleophilic nature of the substituent groups. A preliminary account of this work has already appeared (Dodgson, Spencer & Williams, 1956).

MATERIALS AND METHODS

Preparation of the aryl sulphates. The ester sulphates of *o*-, *m*- and *p*-nitrophenol, *o*-, *m*- and *p*-cresol, *o*- and *p*-chlorophenol, *p*-methoxyphenol and phenol were prepared by the method of Burkhardt & Lapworth (1926). *o*-, *m*- and *p*-Hydroxyphenyl sulphates were prepared in a similar manner from catechol, resorcinol and quinol with sufficient chlorosulphonic acid to sulphate one hydroxyl group only. 2-Hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate) was prepared according to the instructions of Roy (1953), *p*-acetylphenyl sulphate according to Dodgson & Spencer (1953*a*), *o*-aminophenyl sulphate by the method of Boyland, Manson & Sims (1953) and *m*- and *p*-aminophenyl sulphates according to Burkhardt & Wood (1929). Potassium salts were prepared in all cases, and the purity was checked by gravimetric estimation of the sulphate released after hydrolysis with 2N-HCl for 15 min. at 100°.

The enzyme preparation. The electrophoretically concentrated arylsulphatase previously described (Dodgson *et al.* 1955) was used. After dialysis against tap water overnight, the enzyme was dialysed against three changes of distilled water. It was necessary to make several preparations during the course of the work and these varied in activity between 14000 and 18000 *p*-nitrophenol units/mg. of N (see Dodgson *et al.* 1955) when incubated with 0·0015M potassium *p*-nitrophenyl sulphate in the presence of 0·1M-NaH_2PO_4–Na_2HPO_4 buffer, pH 8·75, for 1 hr. at 37·5°.

Determination of enzyme activity. In most cases the phenols liberated by enzyme action were estimated by spectrophotometric methods. In practice, 0·6 ml. of enzyme solution of suitable concentration was incubated with 0·6 ml. of a solution of the appropriate substrate in 0·1M-NaH_2PO_4–Na_2HPO_4 buffer, pH 8·75, for 1 hr. at 37·5°. At the end of the incubation period the liberated phenols were estimated as follows: *p*-nitrophenol, *p*-hydroxyacetophenone and nitrocatechol by methods previously described (Dodgson, Melville, Spencer & Williams, 1954), *o*- and *m*-nitrophenols by the method of Robinson, Smith, Spencer & Williams (1952) and other phenols by use of the Folin–Ciocalteau reagent as described by Bray & Thorpe (1954). With some phenols it was convenient to obtain a reliable standard for the preparation of calibration curves by the hydrolysis of the corresponding aryl sulphates, which were in a high state of purity. These were hydrolysed with 2N-HCl for 15 min. in a boiling-water bath, volatile phenol being retained by a suitable trap containing N-NaOH. After hydrolysis the contents of the trap were added to the hydrolysate and the mixture was used to make up the standard phenol solutions.

The activity of the enzyme towards the various substrates varied considerably, and it was not possible to use the same concentration of enzyme for all substrates. In a few cases it was also necessary to increase the period of incubation, and specific mention is made of these in the text. In the determination of the relative maximum velocity ($V_{max.} = k_3 E_0$) the concentration of enzyme must be known. Since it was not possible to keep the enzyme concentration constant, and, moreover, since the enzyme preparations varied in activity, it was necessary to relate the activity of the enzyme, towards any particular substrate, to that obtained towards a reference substrate. Ideally, phenyl sulphate would be the most suitable reference compound, but the activity of the enzyme towards this substrate is relatively low. For this reason *p*-nitrophenyl sulphate was used, and whenever a new substrate was examined a reference experiment with the same enzyme concentration was made simultaneously with *p*-nitrophenyl sulphate as substrate (0·0015M in 0·1M-Na_2HPO_4–NaH_2PO_4 buffer, pH 8·75, the optimum conditions for this substrate).

With *o*-, *m*- and *p*-hydroxyphenyl sulphates it was not possible to measure the liberated dihydric phenols in the presence of their monosulphate esters, and it was necessary to determine liberated sulphuric acid by the benzidine method (Dodgson & Spencer, 1953*b*). This method cannot be used in the presence of phosphate buffer. Accordingly enzyme and substrate were incubated in the presence of 0·05M aminotrishydroxymethylmethane (tris), which had been adjusted to pH 8·75 with conc. HCl. Preliminary experiments showed that enzyme activity in the presence of this buffer was appreciably greater than in the presence of phosphate buffer. For this reason it was necessary to refer the enzyme activity towards *o*-, *m*- and *p*-hydroxyphenyl sulphates with tris buffer to that obtained with *p*-nitrophenyl sulphate in the same buffer. The increased activity appeared to be an activation by tris rather than an inhibition by phosphate since the activity of the enzyme in the absence of any buffer was the same as in the presence of phosphate.

Michaelis constants (K_m) and maximum velocity ($V_{max.}$). These were calculated from the plot of $[S]/v$ against $[S]$ (where $[S]$ is the molar concentration of substrate and v is the velocity of the enzymic reaction) at values of $[S]$ where inhibition by excess of substrate was negligible. The intercept of the curve is $K_m/V_{max.}$ and the slope is $1/V_{max.}$ (Lineweaver & Burk, 1934). The $V_{max.}$ values shown in Table 1 have been calculated relative to that for phenyl sulphate, which was taken as unity.

RESULTS

Table 1 shows the Michaelis constants

$$[K_m = (k_2 + k_3)/k_1]$$

and the maximum velocity values ($V_{max.} = k_3 E_0$) obtained for each aryl sulphate together with the appropriate substitution constant (σ) of the group present in the benzene ring. Fig. 1 shows $\log_{10} K_m$ plotted against the substitution constants

Table 1. *Michaelis constant (K_m) and maximum-velocity values ($V_{max.}$) for the hydrolysis of substituted phenyl sulphates by the arylsulphatase of* Alcaligenes metalcaligenes

$V_{max.}$ values have been calculated relative to that obtained for phenyl sulphate, which has been expressed as unity. Substitution constants (σ) are quoted from Hammett (1940), Mamalis & Rydon (1950) and Jaffé (1953), and the acidic hydrolysis velocity constants are those of Burkhardt *et al.* (1936) recalculated relative to that of phenyl sulphate. The incubations were carried out in 0·1 M-Na_2HPO_4–NaH_2PO_4 buffer, pH 8·75, at 37·5°.

Substituents	σ	$10^4 K_m$	Relative $V_{max.}$	Incubation time (hr.)	Relative velocity constants of acidic hydrolysis at 48·6°
p-NO_2	+1·270	4·75	16·6	1	5·0
p-$CO.CH_3$	+0·874	9·0	16·7	1	—
m-NO_2	+0·710	9·8	8·1	1	2·3
o-NO_2	+0·650	6·75	16·4	1	4·02
o-Cl	+0·325	13·1	6·4	1	1·46
p-Cl	+0·227	9·4	5·7	1	1·21
H	0	59·2	1	15	1
m-CH_3	−0·069	30·6	0·27	18	0·99
o-CH_3	−0·080	62·5	0·13	18	0·87
m-NH_2	−0·161	57·0	0·10	18	—
p-CH_3	−0·170	31·1	0·81	18	0·77
p-$O.CH_3$	−0·268	61·8	0·12	15	0·50
p-NH_2	−0·660	109·0	0·27	18	—
o-NH_2	—	111·0	0·11	18	—
o-OH, *m*-NO_2	—	1·68	2·4	1	2·1*
p-NO_2†	+1·27	2·33	16·5	1	—
H†	0	6·09	1	18	—
o-OH†	−0·002	9·4	0·21	18	—
m-OH†	−0·268	6·68	0·53	18	—
p-OH†	—	10·5	0·22	18	—
o-OH, *m*-NO_2†	—	1·25	2·2	1	—

* Determined in this Laboratory.
† These determinations were made in the presence of 0·05 M tris buffer, pH 8·75, at 37·5° (see text).

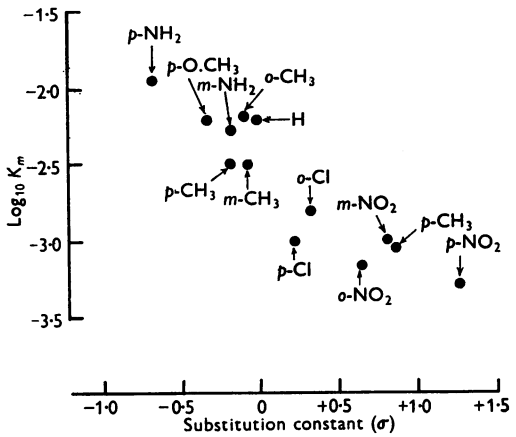

Fig. 1. Relationship between the substitution constant and the enzyme–substrate affinity ($1/K_m$) for the arylsulphatase of *A. metalcaligenes* acting on substituted phenyl sulphates in the presence of 0·1 M-Na_2HPO_4–NaH_2PO_4 buffer, pH 8·75.

(Hammett, 1940; Mamalis & Rydon, 1950; Jaffé, 1953). The results show that $\log_{10} K_m$ decreases with increase in the electrophilic nature of the substituent group and, if the usual assumption is made that $1/K_m$ is a measure of enzyme–substrate affinity, then it follows that the formation of enzyme–substrate complex is facilitated by the presence of electron-attracting substituents in the benzene ring. Strictly speaking, the affinity of enzyme for substrate in the reaction

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_3}{\dashrightarrow} E + \text{products}$$

is given by $1/K_s$ ($K_s = k_2/k_1$), and $1/K_m$ can only be used as a true measure of enzyme–substrate affinity when k_3 is negligible, in which case $K_m \doteq K_s$. Unfortunately it was not possible to determine the relative magnitude of k_3 in the present case by any of the usual methods. A method described by Gawron *et al.* (1953) for the direct determination of enzyme–substrate affinity could not be applied in the present case since the relationship between K_m and $V_{max.}$ was not linear.

Fig. 2 shows the plot of $\log_{10} V_{max.}$ against the substitution constants. The $V_{max.}$ values obtained serve as a relative measure of k_3 since $V_{max.} = k_3 E_0$ and the $V_{max.}$ for the various substrates have been related to one concentration of enzyme. It can be seen from Fig. 2 that the rate of hydrolysis of aryl sulphates is greatly increased by the presence in the benzene ring of electrophilic groupings. In this respect the hydrolysis of aryl sulphates by the

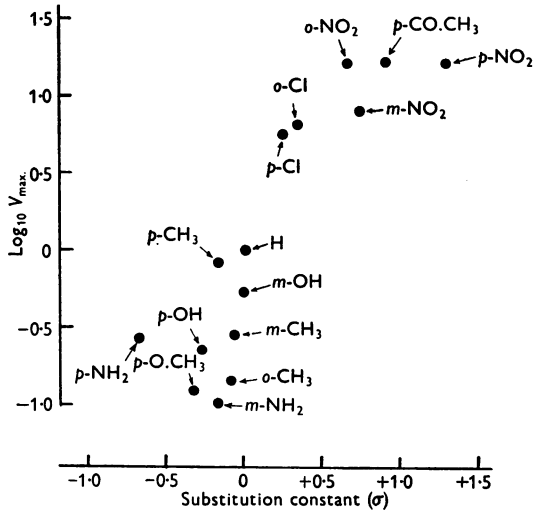

Fig. 2. Relationship between the substitution constant and the relative maximum velocity of hydrolysis ($V_{max.}$) for the arylsulphatase of *A. metalcaligenes* acting on substituted phenyl sulphates in the presence of 0·1 M-Na_2HPO_4–NaH_2PO_4 buffer, pH 8·75. The $V_{max.}$ values obtained for *m*- and *p*-hydroxyphenyl sulphates in the presence of 0·05 M tris buffer are also included.

Alcaligenes enzyme is very similar to the acidic hydrolysis of these compounds (see Table 1 and Burkhardt, Ford & Singleton, 1936).

DISCUSSION

Mechanism of the enzymic reaction

A possible method by which the enzyme interacts with, and aids the hydrolysis of, the aryl sulphates can now be proposed. It has already been suggested (Dodgson *et al.* 1955) that formation of the enzyme–substrate complex involves an electrostatic attraction between charged groups of both components and this has been assumed in the following hypothetical reaction mechanism:

$$\underset{(+)}{Ar.O.SO_2.O^-} + X^-.E.Y^- \rightleftharpoons \underset{(+)}{Ar.O.SO_2.O^-} \overset{H.OH}{\rightleftharpoons}$$

$$\begin{matrix} X^- & Y^+ \\ \diagdown & \diagup \\ & E \end{matrix}$$

$$Ar.OH + \underset{(+)}{HO.SO_2.O^-} \rightleftharpoons HSO_4^- + X^-.E.Y^+$$

$$\begin{matrix} X^- & Y^+ \\ \diagdown & \diagup \\ & E \end{matrix}$$

In this scheme, which is similar to that suggested for the hydrolysis of phenyl acetates by wheat-germ lipase by Gawron *et al.* (1953), X and Y denote the nucleophilic and electrophilic groups present at the active site of the enzyme (see Dodgson *et al.* 1955). The group X will tend to attract the sulphur atom of the substrate, which, by virtue of its semi-polar bonds, will possess some degree of positive charge. The group Y is formulated as attracting the ionized oxygen atom of the aryl sulphate. Support for this view is provided by the lack of pronounced *ortho* effect in the combination of enzyme and substrate (see Table 1 and Fig. 1). This absence of *ortho* effect suggests that interaction of enzyme and substrate occurs at a point some distance from the aromatic ring.

The introduction of an electron-attracting substituent group into the benzene ring results in an increased withdrawal of electrons from the sulphate group. In consequence there is an increase in the degree of positive charge on the sulphur atom which more than compensates for the simultaneous decrease in negative charge on the ionized oxygen atom, since the latter is further removed from the benzene ring. The increase in net charge should result in an increase in enzyme–substrate affinity ($1/K_m$), and this is in accord with the experimental results (Table 1).

In the breakdown of the enzyme–substrate complex it is not possible to say with certainty whether fission occurs at the oxygen–sulphur bond (1) or the oxygen–carbon bond (2), and this problem can be solved unequivocally only by experiments with ^{18}O:

$$Ar.O\text{-}SO_2.O^- \quad (1) \qquad Ar\text{-}O.SO_2.O^- \quad (2)$$

Of the two possibilities the rupture of the oxygen–sulphur bond is, however, the more likely. Burkhardt *et al.* (1936) concluded that the acidic hydrolysis of aryl sulphates, which is similar to the enzymic hydrolysis (see Table 1), did not involve the fission of the oxygen–carbon bond, since these compounds did not exhibit phenylating properties. The chemical stability of the aromatic nucleus–oxygen bond is well known.

Fig. 2 indicates that the breakdown of the enzyme–substrate complex into the products of the reaction is facilitated by the presence of electrophilic groups in the aromatic nucleus. On general grounds this might be considered to indicate that the complex decomposes under the attack of a nucleophilic reagent. However, the enzymic hydrolysis is similar to the acidic hydrolysis of the aryl sulphates (see Burkhardt *et al.* 1936), which involves an attack by the electrophilic hydroxonium ion. This suggests that the enzyme–substrate complex is similarly decomposed through the action of an electrophilic agent. If this is so, the increase in the rate of hydrolysis resulting from the introduction of electrophilic substituents is probably due to the greater stability of the product $Ar.\dot{O}$ formed as a result of the rupture of the oxygen–sulphur bond. The other product, $\overset{(+)}{SO_2}.O^-$, is presumably stabilized by combination with enzyme.

Although the mechanism postulated for the interaction of the arylsulphatase with its substrates is in agreement with the experimental results, alternative reactions cannot be excluded (see Gawron *et al.* 1953). Moreover, the general mechanism presented here does not explain the special properties of the enzyme which make it an arylsulphatase.

From the foregoing it would appear that there must be a strong electron-withdrawing influence on the sulphate group of an aryl sulphate before it can be appreciably hydrolysed by the arylsulphatase. The failure of the enzyme to attack other ester sulphates, such as ethyl and chondroitin sulphates, might thus be explained as due to the absence of a strong electrophilic group capable of influencing the sulphate radical.

The results obtained with nitrocatechol sulphate are worthy of comment. At the pH of the experiment, 8·75, this substrate has two contrasting substituents in the benzene ring: the electrophilic *m*-nitro group and the strongly electrophobic *o*-phenoxide group. The predominating influence on the sulphate group of this substrate might be expected to be that due to the *o*-phenoxide substituent, the net result being a low rate of enzymic hydrolysis and low affinity of enzyme for substrate. In fact the substrate is appreciably hydrolysed and the affinity is greater than that of *p*-nitrophenyl sulphate. Dodgson *et al.* (1955) have already indicated that the *o*-phenoxide group plays some part in the interaction of enzyme and nitrocatechol sulphate, and for this reason it is doubtful whether comparisons between this substrate and other aryl sulphates are permissible. The enzymic hydrolysis of catechol monosulphate (*o*-hydroxyphenyl sulphate) does not exhibit the anomalies shown by nitrocatechol sulphate. At pH 8·75 the free hydroxyl group of catechol monosulphate is present in the phenolic form, whereas that of nitrocatechol sulphate is present as the phenoxide. It should also be noted that comparison between the enzymic and acidic hydrolysis of nitrocatechol sulphate cannot be made, since the *o*-hydroxyl group is present in the phenoxide form during enzymic hydrolysis at pH 8·75 and in the phenolic form during acidic hydrolysis.

Boyland, Manson, Sims & Williams (1956) have recently shown that the arylsulphatases of Taka diastase and human urine, working at pH 6·0, are without appreciable effect on a number of *o*-amino-substituted arylsulphates, and suggested that this was related to the ability of these compounds to form zwitterions. The rate of hydrolysis of *o*-, *m*- and *p*-aminophenyl sulphates by the *Alcaligenes* enzyme is also very low; but the reason for this lies in the nucleophilic nature of the amino group rather than in the presence of zwitterions, since at the pH of the experiments (8·75) there is little tendency for zwitterion formation. The amino group of these aryl sulphates when participating in zwitterion formation contains a quinquivalent nitrogen atom and is, therefore, electrophilic in nature. In such cases, according to the arguments developed earlier, the aryl sulphates should be appreciably hydrolysed. If the mechanism of action of the Taka diastase and urine enzymes is similar to that of *Alcaligenes*, the fact that *o*-aminoaryl sulphates, when present in zwitterion form, are not hydrolysed suggests that a pronounced *ortho* effect is in some way involved. Unfortunately, Boyland *et al.* (1956) did not examine any aryl sulphates with highly basic substituent groups in the *meta*- and *para*-positions. Such a study would have indicated more clearly the extent to which the *ortho* effect is responsible for the resistance of the *o*-aminoaryl sulphates to enzymic hydrolysis.

The quantitative results obtained by Boyland *et al.* (1956) must be regarded with caution, since the observed rates of enzymic hydrolysis at an arbitrary substrate concentration of 0·01 M have been compared. Some of the substrates used, when present in excess, inhibit the arylsulphatases of Taka diastase and urine (Dodgson & Spencer, unpublished results), and in such cases the observed velocities are not related to the $V_{max.}$ values and are therefore not strictly comparable one with another. Moreover, the arylsulphatase of urine is acting in the presence of endogenous inhibitor (phosphate and sulphate), and this is a further complicating factor in attempting to make quantitative comparison between different substrates.

Arylsulphatases have been used to characterize urinary constituents as aryl sulphates (e.g. Butenandt & Hoffstetter, 1939). The present work and that of Boyland *et al.* (1956) have shown that the failure of an arylsulphatase to release a phenol from an unknown conjugate (e.g. Clarke, Akawie, Pógrund & Geissman, 1951) need not necessarily mean that the conjugate is not an aryl sulphate.

SUMMARY

1. The effect of substituents in the benzene ring on the affinity of enzyme for substrate and on the rate of hydrolysis of phenyl sulphate by the arylsulphatase of *Alcaligenes metalcaligenes* has been studied.

2. The affinity of enzyme for substrate (K_m) and the maximum velocity of hydrolysis ($V_{max.}$) are enhanced by the introduction of electrophilic substituents and decreased by nucleophilic substituents.

3. A possible mechanism for the enzyme reaction is discussed.

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The Chemical Composition of Microsomes and Mitochondria from Silver Beet

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Chemical analyses of the 'small granules' or microsomes isolated by the differential centrifuging of dispersions of animal tissues have been reported by a number of workers (see review by Schneider & Hogeboom, 1951). The results show that such microsomes contain a high proportion of ribonucleic acid (RNA) and lipid in addition to protein. About 50% of the total cellular RNA occurs in these particles. Mitochondria from animal tissues such as rat liver also contain a large amount of lipid (about 30% of the dry weight) but usually much less RNA than do microsomes (see Ada, 1949; Schneider & Hogeboom, 1951).

However, there are few published analyses of the cytoplasmic particles isolated from dispersions of plant tissues. Stafford (1951) found that the proportion of RNA in particles isolated from disrupted pea seedlings varied from 0·10% of RNA phosphorus in the larger particles to 0·24% in those which sedimented only when centrifuged at 60000 *g* for 30 min. The 'mitochondria' contained 30% of lipid and some deoxyribonucleic acid (DNA) (0·07–0·1% of DNA phosphorus), which was considered to arise as a contamination from disrupted nuclei. Brown, Jackson & Chayen (1953) analysed particles sedimented from dispersions of bean roots. The RNA (as mg./mg. of total nitrogen) varied from a value of 0·5 for particles sedimented at 1400 *g* for 1 hr. to 1·01 for particles sedimented between 25000 *g* for 1 hr. and 130000 *g* for 2 hr.

In a previous paper (Martin & Morton, 1956*a*) the isolation of microsomes and mitochondria from silver beet was described. These cytoplasmic particles were characterized in terms of their enzymic properties. The present paper describes analyses of these isolated cytoplasmic components to determine the lipid, RNA, DNA and protein contents.

To obtain a reliable estimation of both RNA and DNA, several different analytical procedures were compared. In agreement with Stafford (1951) and McClendon (1952), it has been found that procedures used by earlier workers do not give satisfactory values for RNA and DNA in the particles isolated from plant tissues. An improved analytical procedure is described. It has been found that the