Studies in Carotenogenesis

23. THE NATURE OF THE CAROTENOIDS IN THE PHOTOSYNTHETIC BACTERIUM *RHODOPSEUDOMONAS SPHEROIDES* (ATHIORHODACEAE)*

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French (1937, 1938, 1940*a, b*) first examined the pigment changes which *Rhodopseudomonas spheroides* undergoes when anaerobic cultures are allowed access to oxygen. The change in colour from yellow-brown to reddish purple is caused by the replacement of a yellow by a purple pigment.

van Niel (1947) isolated in crystalline form the two pigments responsible and demonstrated their carotenoid nature. As the amounts of pigment *R* formed when oxygen was allowed into the system equalled the amount of pigment *Y* disappearing, van Niel concluded that, in the presence of oxygen, *Y* was converted into *R*. Goodwin, Land & Osman (1955) confirmed van Niel's findings and also observed the presence of a pigment which was considered to be hydroxy-*Y*. From the changes observed in the amounts of *Y* and *R* in growing cultures and from studies with inhibitors, Goodwin *et al.* (1955) considered that it was possible that *Y* was not being converted into *R*. Further progress in elucidating the problem of the possible conversion of *Y* into *R* obviously depended on a deeper knowledge of their chemical structures, and this paper records an investigation designed with this end in view.

It was made possible mainly by the generosity of Drs C. B. van Niel and R. Y. Stanier. Dr van Niel presented us with small quantities of his original crystalline samples of *Y* and *R* and also with 630 g. of air-dried cells of *R. spheroides* (throughout this paper *R.* represents *Rhodopseudomonas*). Although these cells were 20 years old they still contained considerable amounts of both bacteriochlorophyll and carotenoids. Dr Stanier kindly supplied us with 58 g. of cells of the same organism freshly dried in air.

EXPERIMENTAL

Extraction of the pigments. As bacteriochlorophyll was required it was not possible to shorten the extraction procedure by a preliminary saponification of the crude lipid extracts. After a number of preliminary trials, the following extraction procedure was adopted: the dried cells were first ground to a fine powder in a coffee mill and then allowed to stand in the dark in a large mortar under methanol. After

15–20 min. the mixture was vigorously ground with a pestle and the suspension filtered through a large sintered-glass funnel (G3). The residue was returned to the mortar and the extraction procedure continued until the final residue was a very pale green. All the bacteriochlorophyll and considerable amounts of the carotenoids were thus extracted; the remaining carotenoids were then similarly extracted with successive portions of benzene (Polgár, van Niel & Zechmeister, 1944; Goodwin & Osman, 1954). The benzene (*A*) and methanol (*B*) extracts were separately reduced to small volume *in vacuo* by distillation with a slow stream of N_2 passing via the air leak through the apparatus.

The benzene extract (*A*) was saponified to remove neutral lipids (Goodwin & Osman, 1954). The unsaponifiable matter (containing the pigments) was dissolved in a 1:1 (v/v) mixture of benzene and light petroleum (b.p. 40–60°) (the light petroleum fraction which boiled between 40 and 60° was used throughout this investigation) ready for chromatography.

An equal volume of benzene was added to the methanol extract (*B*) and the pigments were transferred to the benzene by the cautious addition of water. After washing the benzene layer free from methanol and reducing its volume *in vacuo*, the bacteriochlorophyll was separated from the carotenoids on a column of deactivated alumina and the carotenoid fraction combined with fraction (*A*).

All the manipulations described above were carried out in a dark room lighted only with one low-intensity tungsten lamp.

Chromatography. Column chromatography was carried out with a number of adsorbents: (*a*) activated alumina (Grade 'O', P. Spence and Co. Widnes); (*b*) grade 'O' alumina deactivated with methanol (Goodwin & Srisukh, 1949); (*c*) MgO–Celite (2:1) mixture (Starr & Saperstein, 1953); (*d*) A. R. Ca(OH)₂–Celite (3:1) (Haxo, 1949).

Reduction with LiAlH₄. This was carried out in the cold in freshly distilled dry ethyl ether, the excess of reagent and the pigment-hydride complex being decomposed with ethanol.

Special tests. The reaction of the carotenoids with $CHCl_3$ –HCl was carried out as described by Wallcave & Zechmeister (1953). It was found, in agreement with the observations of these authors, that subdued light was necessary for the reaction to take place.

The reaction with boron trifluoride etherate was carried out according to the method of Zechmeister & Wallcave (1953*a, b*).

Isomerization. *Cis-trans*-isomers of the carotenoids were obtained by catalysis with I_2 (Zechmeister, 1944).

Absorption spectra. Ultraviolet and visible absorption spectra were measured with Unicam SP. 500 and SP. 600 instruments, respectively. Infrared measurements were

* Part 22: Goodwin (1956*b*).

most kindly carried out at the Thornton Research Centre of The Shell Refining and Marketing Co., and we are very grateful to Dr A. J. Ham for making the necessary arrangements.

Micro-analyses. These were kindly carried out in the Micro-analytical Laboratory of the Department of Organic Chemistry in this University under the direction of Mr A. S. Inglis. We are specially grateful to Mr Inglis for his interest in our problems and for the time and trouble he took personally to determine the *isopropylidene* contents of our specimens.

Isolation of pigments R and Y. The combined fractions *A* and *B* were chromatographed in three batches on deactivated alumina (Goodwin *et al.* 1955). Three main pigment fractions were obtained: *Y*, *R* and, in much smaller amounts, hydroxy-*Y*. After repeated chromatography *Y* and *R* were crystallized. The combined fractions containing *R* were dissolved in about 30 ml. of light petroleum and transferred to a 50 ml. centrifuge tube. The solvent was then carefully reduced under N_2 to about 5 ml. and placed at 0° under N_2 overnight. The supernatant was poured off and the crystals were washed rapidly with a small volume of cold light petroleum, which was returned to the mother liquor. The crystals were then centrifuged and dried in a vacuum desiccator. Further crops of crystals were obtained from the mother liquor and the combined yield was recrystallized from light petroleum; yield, 35 mg.

Y was similarly crystallized (yield, 13 mg.), but in the dry state it changes, even under N_2 , to a colourless material. In solution (light petroleum) it is stable in the dark at 0° for some considerable time and in later experiments it was kept in this form and crystallized just before use.

Because of this instability it was much more difficult to obtain reproducible analytical figures on *Y* than on *R*. Small batches of crystalline *Y* were also obtained from our own cultures of *R. spheroides* cultured in 1 l. bottles in the medium and under the cultural conditions described by Goodwin *et al.* (1955). Hydroxy-*Y* was similarly purified and obtained in a chromatographically homogeneous state; too little was available for crystallization.

Biological assay for vitamin A activity. Bio-assays were carried out on rats with a vitamin A-deficient diet (Lowe & Morton, 1953). When the animal showed definite signs of vitamin A deficiency (xerophthalmia together with either loss of weight or a stationary weight) the pigments were

added to the diet at a level of 30 μ g./day in 0.2 ml. of refined arachis oil. The controls were given 30 μ g. of β -carotene/day, also in 0.2 ml. of arachis oil.

Reference pigments. Crystalline lycoxanthin, required for comparative *isopropylidene* determinations and methylation experiments, was isolated from the berries of *Solanum dulcamara* collected in Northwest Cheshire during August 1955 (Zechmeister & Chlcnoky, 1936) and a chromatographically homogeneous specimen of capsanthin was obtained from red peppers bought commercially (Goodwin, 1956*a*).

RESULTS AND DISCUSSION

Pigment R

The general properties of two samples of *R*, that of van Niel (1947) and ours, together with our data on his original specimen, are recorded in Table 1. Their identity was confirmed by mixed chromatography on deactivated alumina. It would appear that the slight spectroscopic differences are mainly instrumental. The slightly increased $E_{1\text{cm}}^{1\%}$ value of our sample possibly indicates the absence of small amounts of ash which were present in van Niel's sample (van Niel, personal communication). The absorption spectra of *R* in a number of solvents are given in Fig. 1.

Elementary analysis of *R* indicated a formula $C_{41}H_{58\pm2}O_2$ (Found: C, 84.6, H, 10.3. $C_{41}H_{58}O_2$ requires C, 84.6; H, 10.0%) with one methoxyl group (Found: OMe, 5.85. One OMe requires OMe, 5.3%), confirmed by the presence of an infrared absorption band at 9.38 μ . (1066 cm^{-1}), and two *isopropylidene* residues (Found: *isopropylidene*, 10.0, 10.1. Two *isopropylidene* residues require *isopropylidene*, 14.4%). These low *isopropylidene* values are not unexpected in *isopropylidene* determinations (A. S. Inglis, personal communication), and as, under the same conditions, lycoxanthin, a similar type of compound, which is known to contain two *isopropylidene* residues (Zechmeister & Chlcnoky, 1936), gave a very similar result (Found

Table 1. Comparison of the properties of two specimens of pigment R

Property	van Niel's specimen		Our specimen 155.5–158° (corr.)
	van Niel's data 148° (corr.)	Our data 153° (corr.)†	
M.p.			
Absorption spectrum maxima (m μ .)			
Light petroleum	455, 487, 516.5*	~460, 482, 513	~460, 482, 513
Benzene	464.5, 499, 536	~475, 499, 530	~475, 499, 530
Chloroform	~462, † 493, 531.5	497	499
Carbon disulphide	486.5, 520, 557	—	~495, 519.5, 553
Ethanol	—	—	488
$E_{1\text{cm}}^{1\%}$ (benzene) at λ_{max} .	1855	—	2065

* van Niel (1947) used light petroleum, b.p. 60–70°; we use light petroleum, b.p. 40–60°. The absorption maxima of carotenoids are at higher wavelengths in higher-boiling light petroleum than in the lower-boiling fractions (see, for example, Goodwin, 1955).

† ~ Denotes inflexion.

‡ This was the m.p. recorded on the ampoule obtained from Dr van Niel.

9.4, calc. 15.2%), it is therefore justifiable also to conclude that *R* contains two such groupings. The presence of two isopropylidene groups is consistent with the results of bio-assay experiments, which showed that *R* was not active as vitamin A and therefore does not contain either an unsubstituted β -ionone residue or a 3-dehydro- β -ionone residue (Fig. 2).

The second oxygen atom is in the form of a conjugated ketone, because: (a) The marked absorption bands normally associated with an open-chain carotenoid (i.e. one with two isopropylidene residues), e.g. lycopene (Goodwin, 1955), are considerably damped down, which is characteristic of the presence of a keto grouping (Goodwin & Taha, 1950). (b) There is a shift to longer wavelengths of the spectrum in ethanol compared with that in light petroleum. The shift with a diconjugated keto carotenoid, e.g. rhodoxanthin, is 9 m μ . (Kuhn & Brockmann, 1933); with *R* the shift is only 5 m μ ., indicating one conjugated keto group. (c) The band observed in the infrared region at 5.99 μ . (1699 cm.⁻¹) is characteristic of a conjugated carbonyl grouping. (d) Reduction with LiAlH₄ converts *R* almost quantitatively into a compound with an absorption spectrum indistinguishable from that of *Y* or hydroxy-*Y* (Fig. 3), i.e. there is a shift of 30 m μ . to shorter wavelengths associated with a marked increase in the definition of the absorption bands, both changes being characteristic of the replacement of a conjugated keto grouping by a hydroxy grouping. The phasic reactions of reduced *R* also indicate the presence of one hydroxyl group, as does

its adsorptive behaviour; further, chromatographically it was inseparable from hydroxy-*Y* on deactivated alumina, MgO-Celite, or Ca(OH)₂-Celite. It is not, however, identical with hydroxy-*Y* (see below).

Fig. 2. Typical curves illustrating the growth of rats fed on β -carotene, *Y* and *R*. A, β -carotene; B, *Y*; C, *R*. Rats were maintained on a vitamin A-free diet until they began to fail to gain weight, when the pigment was added (indicated by arrow) as described in the Experimental section.

Fig. 1. Absorption spectrum of *R* in various solvents: (a) light petroleum, (b) benzene, (c) carbon disulphide.

Fig. 3. Absorption spectrum of reduced *R* (prepared by action of LiAlH₄ on *R*) (○), and the major fractions produced by the action of CHCl₃-HCl on reduced *R* (Table 2); zone 2 (continuous curve), zone 3 (broken curve).

Because of the hypsochromic shift on reduction of the keto grouping, the hydroxyl group in reduced *R* must be allylic, i.e. the ethylenic conjugated system begins in the α position relative to the hydroxyl group; it should, therefore, be possible to remove the elements of water from reduced *R* and introduce an additional double bond into the chromophoric system by treatment in the light with CHCl_3 -HCl. This type of reaction was first carried out by Karrer & Leumann (1951), who converted eschscholtzxanthin [absorption maxima (light petroleum) 442, 472 and 502 $\text{m}\mu$.] into anhydroeschscholtzxanthin, by the removal of two molecules of water [absorption maxima (light petroleum) 500, 531 $\text{m}\mu$.], and later by Wallcave & Zechmeister (1953) who converted isocryptoxanthin [(4-hydroxy- β -carotene) absorption maxima (light petroleum) 451, 479 $\text{m}\mu$.] and its methyl ether into dehydro- β -carotene [absorption maxima (light petroleum) 474, 504 $\text{m}\mu$.]. For example:

When reduced *R* was dissolved in CHCl_3 -HCl and allowed to stand for 20 min. in the light, there was a marked darkening of the solution. Chromatography of the reaction products on deactivated alumina yielded five zones (Table 2). Zones 1 and 4 are minor zones and are not discussed further; zone 5 is unchanged reduced *R*. Zone 2 (Fig. 3) has the properties expected if one hydroxyl group had been removed from reduced *R* and replaced by an ethylenic bond in an open-chain carotenoid. Its main maximum has been shifted by 12 $\text{m}\mu$. to higher wavelengths, which is characteristic of the insertion of an additional conjugated double bond into an acyclic carotenoid (e.g. for neurosporene to lycopene the shift is 32 $\text{m}\mu$., representing a shift of 16 $\text{m}\mu$. for one double bond).

Zone 3, however, which is generally by far the major product of the reaction, must, because of the bathochromic shift of 22.5 $\text{m}\mu$. compared with zone 2 (Fig. 3), contain two double bonds more than the pigment in zone 2. This can be achieved only if (a) the methoxyl group becomes allylic when the first double bond is inserted and (b) when methanol is

consequently removed a further isolated double bond is brought into conjugation.

All these observations are consistent with the formula 5:6-dihydro-4-methoxylycopene-6-one for *R*; it is proposed to name it spheroidenone. The reactions discussed above can then be accounted for as follows (the absorption maxima quoted are those of the main band, i.e. those with the highest *E* values):

Further points in favour of this structure are: (a) Fraction 3, with thirteen conjugated double bonds in an acyclic structure, should have an absorption spectrum very similar to that of spirilloxanthin, if one assumes that the auxochromic effect of the methoxyl group in this compound is slight. When carefully purified from *cis*-isomers the pigment does have the same absorption spectrum as spirilloxanthin. (b) In the parent *R*, the methoxyl group should not be allylic; this was demonstrated by treating *R* with CHCl_3 -HCl; only unchanged *R* and a small amount of its *cis*-isomers were obtained. There was no evidence of the formation of a pigment with absorption maxima at higher wavelengths than those of *R*, or with reduced adsorptive properties.

As *R* is spectrally very similar to capsanthin, which has an ill-defined absorption spectrum with λ_{max} at 475 $\text{m}\mu$. in light petroleum, it was possible

that it was structurally related to capsanthin, in which case the break in the terminal ring would be between C-5 and C-6 rather than between C-1 and C-6. Chromatographically pure capsanthin was reduced to capsanthol (Karrer & Hübner, 1936) with LiAlH_4 and this was dissolved in CHCl_3 . On

Capsanthin

addition of CHCl_3 -HCl no change in the absorption spectrum of the solution occurred, in spite of the allylic nature of the hydroxyl group. This is undoubtedly due to the absence of a free hydrogen from C-1. This experiment confirmed the absence of 5:6-scission in *R*.

Pigment Y

A comparison of the properties of van Niel's (1947) original specimen of *Y* with our specimen is given in Table 3. (Lack of material did not allow us to examine van Niel's sample in detail.) It will be seen that, as with *R*, the differences are minor. The absorption spectrum of *Y* in a number of solvents is recorded in Fig. 4. As indicated in the Experimental section, *Y* is much more unstable than *R*, rapidly changing into a wax on exposure to air; this accounts for the wide melting point recorded. It rapidly takes up oxygen; for example, in one crystalline specimen, the oxygen content increased

from 6.0 to 9.4 % on standing in an opened ampoule at room temperature for 2 hr. The elementary analysis obtained with the least oxygen content indicates a formula $\text{C}_{41}\text{H}_{60}\text{O}_2$ (Found: C, 84.4; H, 10.4. $\text{C}_{41}\text{H}_{60}\text{O}_2$ requires C, 84.2, H, 10.3 %), with one methoxyl group (Found: OMe, 6.7; 1 OMe group requires OMe, 5.5 %), confirmed by the presence of

Fig. 4. Absorption spectrum of *Y* in various solvents: (a) light petroleum; (b) benzene; (c) carbon disulphide.

Table 2. *Chromatography of the products obtained by treating reduced R with CHCl_3 -HCl*

The method of Wallcave & Zechmeister (1953) was used and the products were fractionated on deactivated alumina. The zones are listed in order of increasing adsorptive properties.

Zone no.	Colour	Spectral maxima in light petroleum (mμ.)	Concn. of ethyl ether required for elution (% v/v)
1	Yellow	456, ~475*	0
2	Orange	~445, 465, 493	1
3	Pink (major zone)	462, 487.5, 520	2
4	Pale yellow	397, 417.5, 444, 473	5
5	Yellow	425, 448, 478	15

* Denotes inflexion.

Table 3. *Comparison of the properties of two specimens of pigment Y*

Property	van Niel's specimen 132°	Our specimen 116-135° (decomp.)
M.p.		
Absorption spectrum maxima (mμ.)		
Light petroleum	426.5, 454, 486*	426.5, 452, 484
Benzene	438, 466.6, 499.7	438, 466, 498
Chloroform	436, 464, 497	437.5, 465, 497
Carbon disulphide	455.4, 487.1, 521.5	458, 485, 518
Ethanol	426, 454, 486.6	430, 454, 485
$E_{1\text{cm}}^{1\%}$ (benzene) at λ_{max}	2437	2630

* van Niel (1947) used light petroleum b.p. 60-70° (see note to Table 1).

an infrared absorption band at $9.4\ \mu$. ($1064\ \text{cm}^{-1}$). It is not biologically active (Fig. 2) and therefore does not contain an unsubstituted β -ionone residue. The position and shape of its absorption spectrum (Fig. 4) suggests a dihydrolycopene derivative, and Lijinsky & Zechmeister (1954) obtained a compound with a very similar absorption spectrum by partial hydrogenation of lycopene.

The nature of the second oxygen atom in *Y* (if present in the pure material) is obscure, but the following possibilities are ruled out: (a) A free hydroxyl: *Y* is epiphasic to 95% (v/v) aqueous methanol and is absorbed in the hydrocarbon region of the chromatographic spectrum for carotenoids. (b) A conjugated carbonyl grouping: *Y* has very sharp absorption bands (a conjugated carbonyl grouping always damps down these bands) and fails to react with LiAlH_4 . (c) Epoxide: *Y* does not alter on standing in ethanol containing 5% (v/v) of acetic acid (Strain, 1954) or give a blue colour with ether-HCl (Karrer & Jucker, 1950); further, epoxides are formed only from carotenoids containing a β -ionone residue, and the shape and position of the absorption spectrum of *Y* rule this out. It may be that an unconjugated carbonyl group exists, because the infrared spectrum of *Y* exhibits a small band at $5.75\ \mu$. ($1740\ \text{cm}^{-1}$), suggestive of either a carbonyl group or a $\text{C}=\text{C}$ stretching frequency, which occurs occasionally (Bellamy, 1954). If it is assumed that because of the instability of *Y* the second oxygen atom is an artifact, then the other recorded properties are consistent with the possibility that *Y* is a dihydrolycopene containing a non-allylic methoxyl group (no elimination of methanol on treatment with $\text{CHCl}_3\text{-HCl}$); by analogy with the structure of *R*, the methoxyl group may be at C-4. This possible structure does not, however, explain the instability of *Y*, which suggests that it may contain an acetylenic or allenic linkage; the latter has recently been observed in fucoxanthin (N. A. Sørensen, personal communication; Weedon, 1955). These possibilities appear to be ruled out because *Y* is quite stable in the presence of alkali and does not exhibit an infrared absorption band in the region $1950\ \text{cm}^{-1}$; further, extraction of the bacteria without the use of alkali still yields *Y*.

Hydroxy-Y

This pigment has not been obtained crystalline; it has the same absorption spectrum as reduced *R* but can be differentiated from this pigment by the $\text{CHCl}_3\text{-HCl}$ test, although the two pigments cannot be separated chromatographically. Hydroxy-*Y* is not labile to $\text{CHCl}_3\text{-HCl}$ but reduced *R* is. These two compounds must, therefore, bear a relationship one to the other similar to that which exists between

cryptoxanthin (3-hydroxy- β -carotene) and *iso*-cryptoxanthin (4-hydroxy- β -carotene) (Zechmeister & Wallcave, 1953*a*, *b*).

Biochemical significance of the proposed structures for Y and R

van Niel's (1947) suggestion that *Y* is converted into *R* when anaerobic cultures of *R. spheroides* are allowed access to oxygen was doubted by Goodwin *et al.* (1955), who could not detect an intermediate in the 'conversion'. The fact that reduction of *R* with LiAlH_4 gives a compound spectrally identical with *Y* indicates that the conversion of *Y* into *R* could take place essentially by the introduction of one allylic carbonyl grouping and that it is not necessary to postulate a stable intermediate.

SUMMARY

1. The two main pigments (*R* and *Y*) of *Rhodospirillum rubrum* have been isolated and their properties recorded.
2. Pigment *R* (*spheroidenone*) has the probable structure 5:6-dihydro-4-methoxylycopene-6-one.
3. Pigment *Y*, which is unstable in the crystalline state, may possibly be a dihydromethoxylycopene.

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Some Studies on Catalase in Mouse-Liver Slices

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Recent work from this laboratory has shown that liver catalase in the mouse is associated with a complex system which influences the level of its activity. So far, hormones, trace metals and riboflavin have been shown to be components of this system, and some indications regarding their possible interrelations have been obtained (Adams, 1952, 1953, 1955). Furthermore, a tumour constituent has been shown to depress catalase activity (Nakahara & Fukuoka, 1949, 1950; Adams, 1950, 1951*a*; Greenfield & Meister, 1951), and it has been suggested that this substance acts by interfering with the stimulating action of hormones on catalase activity (Adams, 1951*b*). In an attempt to open a new approach to the general problem of the nature of the system which controls catalase activity, it was decided to study the behaviour of the enzyme in liver slices. The present paper describes some results obtained by incubating slices in saline media.

EXPERIMENTAL

Animals. The early part of this work was done with the 'Schofield' stock albino strain of mice previously used in catalase studies from this laboratory, but the outbreak of 'Tyzzer's disease' referred to in a previous paper (Adams, 1955) necessitated a change over to other strains. Two such strains have been used, namely, Schneider albino mice (kindly supplied by the National Institute of Medical Research, Mill Hill, London), and a strain of Swiss albinos obtained commercially. Both these strains appear to be free from liver infection. Their catalase activity is similar to that of the albino strain previously used, and they show a marked sex difference in catalase level (greater in males than in females). In the Swiss strain, however, the catalase level in both sexes is rather higher than is usual in the other two strains. The mice were fed *ad lib.* on rat cubes (obtained commercially) and water.

Medium for incubation of slices. A phosphate medium of the following composition was used: NaCl (0.12*M*), KCl (0.006*M*), MgSO₄ (0.0012*M*), Na₂HPO₄ (0.02*M*), glucose

(0.01*M*), and potassium citrate (0.001*M*). The pH was brought to 7.0 by adding HCl.

Estimation of liver-catalase activity. Catalase was estimated as previously described, by titration of the excess hydrogen peroxide remaining after allowing 25 ml. of 0.025*M*-H₂O₂ to stand in contact with a measured sample of liver homogenate for 4 min. at 0°. Corresponding determinations of total nitrogen were made by the Kjeldahl method, and catalase results are expressed in arbitrary units/mg. of N (Adams, 1950, 1952).

Preparation and treatment of liver slices. Mice were decapitated, and the livers removed and separated into the individual lobes. One lobe from each liver was then cut with a tissue chopper (McIlwain & Buddle, 1953), giving slices of about 250 μ . in thickness. The slices were separated under ice-cold medium, and approximately 20 mg. (not weighed accurately) was placed in glass tubes which had a flat bottom (diam. 2.3 cm.). Each tube contained 1.5 ml. of medium, and slices for two or three sets of duplicate tubes were prepared from each liver. The tubes were then placed in a bath maintained at 38°, and shaken at 120 oscillations/min. Normally a slow stream of oxygen was passed continuously into the tubes. In experiments where anaerobic conditions were required, the slices were placed in Warburg flasks (gas phase, 100% N₂) and double quantities of slices and medium were used.

The slices were prepared for catalase estimation by tipping the contents of each vessel (i.e. slices and medium) into a Ten Broeck grinder (a glass-pestle homogenizer) and homogenizing. Part of the suspension (2 $\times$ 0.1 ml.) was then taken for duplicate determinations of enzyme activity and part (1.0 ml.) for nitrogen estimation.

After setting up the vessels and adding the liver slices and medium, a catalase estimation was done on the contents of one vessel from each mouse. The catalase level obtained at this point (i.e. after slicing but before incubation) will be referred to subsequently as the initial level. Tests made on slices and on uncut liver showed that substantially the same enzyme levels were obtained in each case. The initial level therefore corresponds to the catalase activity of the liver as normally measured. The remainder of the vessels were incubated as described above, and removed (in duplicate) at intervals (usually 2 and 4 hr.) for further catalase estimations.