time a guide to the completeness of the digestion can be obtained although the figures have no absolute significance. Attention is drawn to these points as ignorance of these effects might lead to faulty conclusions being drawn from experimental results.

The failure to obtain a theoretical conversion factor with many amino-acids is at first sight puzzling, since it appears in some instances as if more copper were incorporated than the formation of the complex A_2Cu would allow. Borsook & Thimann (1932), however, showed that a number of copper complexes of both glycine and alanine can be obtained and can co-exist in solution; similar behaviour with other acids could account for the anomalous results obtained in the present work. Furthermore, it is not certain that the soluble copper salts estimated by the present method correspond to the isolated copper salts of the amino-acids.

SUMMARY

1. A method is described for determining micro amounts of α-amino nitrogen (1–50 μg.) and peptide amino nitrogen (1–25 μg.).

2. The method has been applied to single- and two-dimensional paper chromatograms of a number of α-amino-acids and an evaluation made of the losses involved during chromatography.

3. The destruction of amino-acids on heating the spots on paper chromatograms developed with ninhydrin has also been studied quantitatively. It is suggested that a decomposition product makes the reaction self limiting.

I am indebted to Dr C. E. Dent for samples of the non-α-amino-acids investigated. I also wish to thank Mr R. Knight for valuable technical assistance.

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Long-chain Unsaturated Fatty Acids as Essential Bacterial Growth Factors

SUBSTANCES ABLE TO REPLACE OLEIC ACID FOR THE GROWTH OF *CORYNEBACTERIUM* 'Q' WITH A NOTE ON A POSSIBLE METHOD FOR THEIR MICROBIOLOGICAL ASSAY

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Forty years ago, Fleming (1909) observed the stimulatory effect of oleic acid on the growth of *Corynebacterium acnes*, but it is only recently that the essential nature of long-chain unsaturated fatty acids for the growth of certain organisms has been firmly established. It is now generally accepted that linoleic acid is essential for the proper nutrition of rats (see Hansen & Burr, 1946), while Fraenkel & Blewett (1947) have shown that it (or linolenic acid) is necessary for normal development of the moth *Ephestia kuehniella*. Benham (1941) has reported that oleic acid is essential for the growth of the fungus, *Pityrosporum ovale*, while, amongst bacteria, oleic acid has been found necessary for the growth of some strains of *Corynebacterium diphtheriae* from a small inoculum (Cohen, Snyder & Mueller, 1941), for *Clostridium tetani* (Feeney, Mueller & Miller, 1943), for *Cl. sporogenes* in the absence of biotin (Shull, Thoma & Peterson, 1949), and for the unidentified *Micrococcus* 'C' (Dubos, 1947). There have also been reports, of which the most accurate and complete are those of Williams & Fieger (1946)

and Williams, Broquist & Snell (1947), proving the necessity of oleic acid or linoleic acid for some strains of lactobacilli, usually only in the absence of biotin.

During experiments on *Haemophilus pertussis*, a chance contaminant was isolated which was found to have the property of symbiotically stimulating the growth of *H. pertussis* in nutrient broth and other media without blood (Pollock, 1948*a*, 1949). This organism proved, on further investigation, to be unable to grow in a medium of known composition without the addition of oleic acid. It was found to be an obligatory aerobic diphtheroid, fermenting glucose, but not lactose, sucrose, mannitol or dulcitol, and has been provisionally labelled *Corynebacterium* 'Q'. Its general properties and its clear-cut response to oleic acid suggested that it might be a suitable test organism for a study of the function and metabolism of long-chain fatty acids.

METHODS

Glassware. All glassware was thoroughly cleaned with hot chromic acid to remove traces of fat, and the 50 ml. Erlenmeyer flasks used for growth experiments were plugged with special fine glass wool (Fibreglass Ltd.) previously extracted with methanol and acid-cleaned (Pollock, 1948*b*).

Medium. 'Vitamin-free' casein hydrolysate (Ashe), supplemented by tryptophan, cystine, salts and growth factors (pH 7·6) as follows. Basal medium (final concentrations): casein hydrolysate (Ashe, 'vitamin-free'), 5·0; KH_2PO_4, 4·5 mg./ml.; NaOH, to pH 7·6; L-cystine, 0·0024; L-tryptophan, 0·0008; $MgSO_4$, 0·02; $FeSO_4$, 0·0015 mg./ml. Growth factors 'A': biotin 0·01; nicotinamide, 1·0; Ca pantothenate, 0·75; pyridoxine, 2·5; riboflavin, 1·2 aneurin; HCl, 0·5; *p*-aminobenzoic acid, 0·45; haemin, 2·0 μg./ml. Growth factors 'B': guanine sulphate, 11·0; inositol, 9·0; uracil, 5·5; adenine sulphate, 10·0; cytosine, 5·5; pimelic acid, 0·8; folic acid, 1·0 μg./ml.

All tests classified in Table 1 were done in a medium consisting of casein hydrolysate, salts and growth factors 'A'. For the accurate curves depicting the relationship between growth opacity and fatty acid concentration (Fig. 1) both groups of factors 'A' and 'B' were included in the basal medium.

This was sterilized by Seitz filtration; the haemin was first autoclaved and added after the rest of the medium had been sterilized.

Inoculum. One drop of a 1:100 dilution of a standard suspension of cells grown on tryptic meat agar (without added oleic acid) for 24 hr. and once washed with water. This was found to contain on an average 50,000 viable cells.

Incubation. Unless otherwise stated, cultures were incubated aerobically at 37°, and growth was recorded by eye after 1, 2 and 3 days as tr., tr.+, +, + +, etc. Flasks were left for 7 days before recording a negative result.

Fatty acids. Trivial and systematic names of fatty acids mentioned in the text are as follows: brassidic, *trans*heneicos-12-ene-1-carboxylic acid; dihydroxystearic, 8:9-dihydroxyheptadecane-1-carboxylic acid; dibromostearic, 8:9-dibromoheptadecane-1-carboxylic acid; erucic, *cis*heneicos-12-ene-1-carboxylic acid; elaeostearic, heptadeca-8:10:12-triene-1-carboxylic acid; elaidic, *trans*heptadec-8-ene-1-carboxylic acid; lauric, undecane-1-carboxylic acid; linoleic, heptadeca-8:11-diene-1-carboxylic acid; linolenic, heptadeca-8:11:14-triene-1-carboxylic acid; myristic, tridecane-1-carboxylic acid; oleic, *cis*heptadec-8-ene-1-carboxylic acid; $\alpha\beta$-oleic, heptadec-1-ene-1-carboxylic acid; palmitic, pentadecane-1-carboxylic acid; palmitoleic, pentadec-8-ene-1-carboxylic acid; petroselinic, heptadec-5-ene-1-carboxylic acid; ricinoleic, 11-hydroxyheptadec-8-ene-1-carboxylic acid; stearic, heptadecane-1-carboxylic acid.

The fatty acids used were the purest that could be obtained. Palmitic and stearic acids and the first samples of linoleic and linolenic acids were gifts from Dr W. T. J. Morgan; lauric, myristic, petroselinic, elaeostearic and the second samples of linoleic and linolenic acids were supplied by Prof. T. P. Hilditch, the four latter as methyl esters. These esters were hydrolysed by heating with N-NaOH for 30 min. at 100° under N_2. Pure oleic acid was obtained by distilling a commercial sample (Hopkin and Williams 'Purified' grade), followed by purification through dibromostearic acid (Holde & Gorgas, 1926) and redistillation *in vacuo*. Methyl palmitoleate was isolated from distilled sperm oil fatty acids. The methyl esters of the liquid acids prepared by the Pb salt method (Hilditch, 1940) were fractionated *in vacuo* using an 8 in. Fenske column fitted with a heated jacket and reflux ratio head. The fraction boiling at 138–139°/1·6 mm. had an I_2 number of 102 (Trappe, 1938), and gave an acid with an equivalent of 256. The dihydroxystearic acids were prepared by the methods of Wittcoff, Moe & Iwen (1948) and Scanlan & Swern (1940). Elaidic acid was prepared by the method of Bertram (1936). Tween 80 (Atlas Powder Co.) was purified according to Davis (1947). Ricinoleic acid and erucic acid were purified by partition chromatography (Howard & Martin, unpublished).

All fatty acids were dissolved in the minimum amount of NaOH needed to give a stable solution or emulsion and added to the basal medium without further treatment, apart from sterilization by autoclaving at 15 lb. pressure for 20 min.

In experiments where growth was measured turbidimetrically, the Spekker absorptiometer was used, with neutral grey filter H 108, and the concentration of fatty acid added plotted against the opacity of the culture (expressed as $\log I_0/I$). The relationship between opacity and dry bacterial weight was established by making serial dilutions of a washed suspension containing a known weight of cells in the medium used for growth, and plotting a standard curve. The dry weight of organisms was measured by drying a suspension of cells twice washed with distilled water in an oven at 110° to constant weight.

RESULTS

Effects of different fatty acids

If strict precautions were taken to eliminate all traces of extraneous fat, no visible growth ever occurred in the basal medium. Attempts to simplify this medium were not very successful, although it was found that satisfactory growth occurred in a mixture containing eighteen amino-acids, Fe^{++}, Mg^{++}, and eight 'A' growth factors (see 'Methods') together with 10 μg./ml. oleic acid. After this confirmation that 'Q' would grow as well in a medium of known composition as in the casein hydrolysate,

and since no growth occurred in the latter in the absence of oleic acid, it was decided to use the casein hydrolysate as a basis for subsequent tests rather than the amino-acid mixture, for the sake of economy.

Table 1. *Ability of different long-chain fatty acids to support the growth of* Corynebacterium '*Q*'

(Medium: casein hydrolysate + growth factors (see 'Methods'); incubation at 37°, aerobically; inoculum, about 50,000 cells.)

Acid tested	Concentration (μg./ml.)	Growth after 1 day	2 days	3 days
Oleic	25	tr.	+	+ + +
	5	+	+ +	+ +
	1	tr. +	tr. +	+
Elaidic	25	+	+ +	+ + +
	5	+	+	+ +
	1	tr. +	tr. +	+
Petroselinic	25	? tr.	tr.	+ + +
	5	−	tr. +	+
	1	−	tr.	tr. +
Palmitoleic	25	−	−	−
	5	+ +	+ +	+ + +
	1	+	+	+
Linoleic	25	−	+ +	+ + +
	5	−	+ +	+ +
	1	tr. +	+	+
Linolenic	25	−	−	−
	5	−	+ +	+ +
	1	tr. +	+	+
Tween 80 (purified)	25	tr.	+ +	+ + +
	5	tr.	+	+
	1	−	−	tr.

Lauric	Brassidic	No growth up to 7 days, at conc. of 1, 5, and 25 μg./ml.
Myristic	αβ-Oleic	
Palmitic	*cis*Dihydroxystearic	
Stearic	*trans*Dihydroxystearic	
Ricinoleic	*cis*Dibromostearic	
Erucic	*trans*Dibromostearic	

The results are summarized in Table 1. For oleic, elaidic, petroselinic, linolenic, linoleic and palmitoleic acids, the relationship between concentration of fatty acid in the medium and total yield of organisms in dry weight after incubation for 3 days is expressed in the form of curves based on accurate opacity measurements (Fig. 1). None of the acids which failed to promote growth had any detectable inhibitory effect at a concentration of 25 μg./ml. in the presence of 10 μg./ml. oleic acid.

Three different preparations of oleic acid and two each of linoleic and linolenic acids gave substantially the same results. It will be noticed that the higher concentrations of palmitoleic, linoleic and linolenic acids were markedly inhibitory, and it was first supposed that this might be due to some toxic impurity present in relatively small amount, particularly since the first samples tested of linoleic and linolenic acids were nearly 20 years old. However, the second samples of these two acids, which were freshly prepared, kept as their methyl esters and tested on the same days as the hydrolysis to the free acid, gave exactly the same results as the original preparations. This 'double action' effect which characterizes the action of unsaturated fatty acids on a number of different bacterial species (see Pollock, 1949) is much more marked in the case of oleic acid acting on lactobacilli (Williams *et al.* 1947).

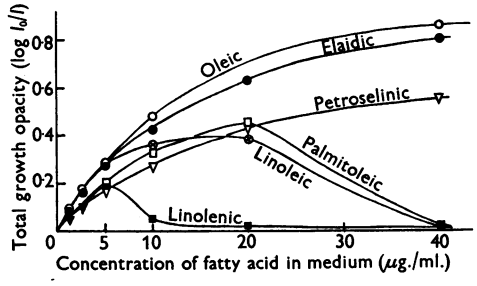

Fig. 1. Relation between total final growth of *Corynebacterium* 'Q' and concentration of different fatty acids in the medium. Basal medium: casein hydrolysate plus growth factors (see 'Methods'); incubation, aerobically at 37° for 3 days; inoculum, about 50,000 cells.

It can be seen that the quantitative response to the active compounds is nearly the same, although petroselinic acid is not apparently quite so active as oleic acid; while with palmitoleic, linoleic and (especially) linolenic acid the picture is complicated by the superposition of an inhibitory effect on the growth-promoting one. The most striking result is that shown by the 'unnatural' elaidic acid which gave almost exactly the same yield of cells as its isomer, oleic acid. However, with petroselinic acid it was observed that the rate of growth was considerably slower than with oleic acid (Table 1).

All saturated fatty acids tested were without effect. The action of purified Tween 80 (a polyoxyethylene derivative of sorbitan mono-oleate) shows conclusively that the organism can utilize the oleate in this esterified form, the slight delay in growth compared to free oleic acid possibly being due to the need for preliminary hydrolysis, or to slower diffusion through the cell wall. Utilization of purified Tween 80 by certain oleic acid-requiring lactobacilli has also been reported by Williams *et al.* (1947). Samples of ricinoleic acid and erucic acid gave, when first tested, slight growth in flasks containing the higher concentrations. This, however, was shown to be due to some residual impurity for, after passing these acids down a chromatographic column (Howard & Martin, unpublished), neither compound gave any growth whatever up to 25 μg./ml.

α-Elaeostearic acid was tested on several occasions, but the results were variable and have not been recorded. It was discovered that the iodine number of elaeostearic acid fell from 260 to 142 when the methyl ester was hydrolysed by heating 0·5 g. at 100° with 2N-NaOH, and continued to fall during incubation in M/30 phosphate buffer at pH 7·6 and 37°, reaching 32 in 5 days. Tests with this acid are therefore meaningless. This destruction of elaeostearic acid under aerobic conditions was also observed by Kodicek & Worden (1946).

Microbiological assay of oleic acid

The relative lack of specificity in the response of *Corynebacterium* 'Q' to fatty acids clearly reduces its value as an assay organism for oleic acid. Nevertheless, on occasions, the microbiological estimation of minute amounts of oleic acid (known to be the sole fatty acid present), or of oleic acid-like substances, has been of some value (see Pollock, 1949), particularly as there is yet no chemical method available. The basal medium employed was the same as that used in the ordinary growth experiments, with the addition of 'B' group of growth factors; and the technique of inoculation, incubation, etc. was similar. Satisfactory duplicates were obtained by incubating for 3 days in the usual way, but a linear response between concentration of fatty acid added and dry weight of cells produced was not obtained unless the flasks were shaken to promote maximal aeration. Table 2 gives the values obtained, with their standard deviations, up to a concentration of 10 μg./ml. of added oleic acid. It appears that concentrations of oleic acid between 2·0 and 10·0 μg./ml. could be assayed with an error of not more than 15%. Slight variations in the relationship between growth and concentration of oleic acid were found to occur from day to day. Flasks containing at least two concentrations of oleate were therefore set up and inoculated at the same time as the test, so that a fresh standard curve could be prepared each time. Tryptic meat broth and also 2% peptone water were found to be almost as satisfactory as the fat-free casein hydrolysate medium for such estimations, although, of course, allowance has to be made for the slight growth (equivalent to an oleic acid content of about 0·4 μg./ml. in both cases) which occurs in the basal medium without added oleic acid.

Table 2. *Microbiological assay of oleic acid with* Corynebacterium *'Q'*

(Flasks were incubated at 37° for 3 days on a mechanical shaker. Basal medium: 'Vitamin-free' casein hydrolysate with sixteen growth factors (see 'Methods'). Inoculum: about 50,000 cells.)

Oleic acid added (μg./ml.)	No. of flasks	Mean yield of cells (μg. dry wt./ml.)	Standard deviation
2·0	8	61	±3·6 (6%)
5·0	8	144	±4·2 (2·9%)
10·0	5	253	±6·8 (2·7%)

A crude biological extract was prepared by heating a washed suspension of *Escherichia coli* for 30 min. at 100° and spinning off the cell debris. The supernatant liquid was found to have a growth-stimulating action for 'Q' corresponding to an oleic acid content of 41·5 μg./ml., and the added oleic acid could be estimated satisfactorily in the presence of this extract diluted 1:9 (see Table 3). However, the assay of oleic acid in the presence of substances such as undenatured serum albumin or soluble starch, both of which have a high combining affinity for oleic acid, was found to be unsatisfactory. It is clear that an attempt to assay unsaturated fatty acids in biological material of unknown composition would have to be preceded by an extraction of all lipid with some fat solvent, or the demonstration that added oleic acid could be satisfactorily estimated in its presence.

DISCUSSION

In general, the results reported here for *Corynebacterium* 'Q' confirm and extend those of Williams & Fieger (1946) and Williams *et al.* (1947) with lactobacilli. The range of compounds able to replace oleic acid is relatively narrow. At least one double

Table 3. *Effect of crude biological extract on assay of oleic acid with* Corynebacterium *'Q'*

(The crude extract used was an aqueous infusion of a boiled cell suspension of *Escherichia coli*.)

Extract present (ml.)	Oleic acid added (μg./ml.)	Yield of cells (μg. dry wt./ml.)	Total assay in terms of oleic acid (μg./ml.)	Oleic acid 'recovered' (μg./ml.)	Oleic acid 'recovered' (%)
0	0	0	~	~	—
0	5·6	158	~	~	—
0	2·2	86	~	~	—
0·25	0	31	1·0	~	—
0·25	5·0	180	5·8	4·8	96
0·25	2·0	113	3·3	2·3	115
1·0	0	145	4·7	~	—
1·0	5·6	300	9·7	5·0	90
1·0	2·2	226	7·3	2·6	118

bond appears to be absolutely essential, but its exact position is less important. In the C_{18} series the 5:6 position* (petroselinic acid) is nearly as satisfactory as the 8:9 (oleic acid), although $\alpha\beta$-oleic acid (1:2 position) is inactive. The configuration around the double bond is apparently not critical, since elaidic acid has the same activity as its *cis*-isomer, oleic acid. A single hydroxyl group, however, in the 11 position (ricinoleic acid), abolishes activity. Shortening the chain length by two methylene groups, leaving the double bond in the same position relative to the carboxyl group (palmitoleic acid), does not greatly reduce the activity, but the C_{22} acids, erucic and brassidic, with the double bond in the 12:13 position, are quite inactive.

The same relative lack of importance of the position of the essential double bond has been reported by Shull *et al.* (1949), who found that *cis*-vaccenic acid ($\Delta^{10,11}$ isomer of oleic) would completely replace oleic acid for the growth of *Cl. sporogenes* in the absence of biotin. These workers also found that the *trans* isomers of both oleic and vaccenic acids were active, only less so than oleic acid itself. For lactobacilli, too, elaidic acid has been reported (Williams & Fieger, 1946) to be able to replace oleic acid.

The linear relationship between growth and fatty acid concentration of the medium suggests that oleic acid and its analogues are used to form some essential constituent of the cell protoplasm. The figures already quoted show that oleic acid consistently supports the growth of almost thirty times its own weight of cells of *Corynebacterium* 'Q'. Nevertheless, it must be emphasized that knowledge of the functions and metabolism of fatty acids in bacteria is scanty. The possibility that the growth-promoting effect of oleic acid may be due to some physicochemical effect (e.g. on the permeability of the cell wall, as suggested by Kodicek, 1949), or purely to its acting as a specific source of energy, cannot be completely ruled out. It is also conceivable, although very unlikely, that the difference in growth-promoting effect between active and inactive fatty acids is due possibly to very great differences in the ease with which they may be absorbed through the cell wall. Moreover, the constancy of cell composition in relation to the quantity and quality of the nutrients provided is not, in general, so marked in the case of lipids as it is with protein and carbohydrate.

It is thus not easy to speculate on such questions as, for instance, whether elaidic acid is absorbed and utilized as such, or is transformed into oleic acid. The latter would appear more likely since elaidic acid has not yet been found to occur naturally in any organism. Sinclair (1935), however, has shown that it can replace up to 30% of the natural fatty acids in the phospholipins of liver and muscle if fed to rats, and Paul & McCay (1942) claim that guinea pigs will absorb (and probably 'utilize') 95% of the elaidic acid fed in the diet.

Clearly, such questions will not be finally answered until it is possible to investigate how far and in what way the fatty acid in the growth medium affects the fatty acid composition of the cells when grown. It is hoped to carry out further research along these lines, as well as to extend the range of compounds tested for their ability to replace oleic acid for growth of this organism.

* In this discussion the numeration is on the basis of Chemical Society nomenclature, in which —COOH is a substituent on $C_{(1)}$.

SUMMARY

1. A number of long-chain fatty acids have been tested for their ability to support the growth of an oleic acid-requiring diphtheroid bacterium (*Corynebacterium* 'Q') in a medium of known composition.

2. None of the saturated fatty acids tested (lauric, myristic, palmitic, stearic) was active. Of the unsaturated acids, linoleic, linolenic, palmitoleic, elaidic and petroselinic acids were roughly equivalent to oleic acid, although the first three were inhibitory in the higher concentrations. Erucic, brassidic, $\alpha\beta$-oleic and ricinoleic acids, however, were all quite inactive.

3. A possible method for the microbiological assay of oleic acid in concentrations between 2·0 and 10·0 μg./ml. has been outlined.

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Phospholipin Metabolism in Rabbit-liver Cytoplasm

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The recent work of Fishler, Entenman, Montgomery & Chaikoff (1943) and of Entenman, Chaikoff & Zilversmit (1946) demonstrated that liver is the principal tissue in the body concerned with the production and removal of plasma phospholipins. However, it is not known whether synthesis and decomposition of phospholipins take place uniformly throughout the liver cell or whether these functions are associated with a particular type of particle in the cell. The present work deals partly with the technique of liver-cell fractionation and partly with the study of the rates of renewal of phospholipins in liver-cell fractions. Hevesy (1945) showed that the rate of renewal of phospholipin phosphorus in liver nuclei is slower than in the cytoplasm. His observation is confirmed, and it is shown that there are at least three different rates of renewal in different fractions of the liver cytoplasm.

1. ANALYSIS OF LIVER FRACTIONS

There have been numerous attempts to fractionate liver into components which can be directly related to visible granules in the liver cell. Several methods, e.g. those of Marshak (1941–2), Dounce (1943), Mirsky & Pollister (1946), have been used to prepare liver-cell nuclei, while methods have been described by Claude (1946*a*), and more recently by Hogeboom, Schneider & Pallade (1948) for the fractionation of liver cytoplasm. Claude described the preparation of three fractions: (1) large granules, corresponding to the mitochondria and secretory granules of the histologist; (2) microsomes (small granules), composed of particulate elements of submicroscopic size; and (3) a supernatant, containing the particles and molecules which remain in solution after the first two fractions had been removed. Hogeboom *et al.* (1948) have criticized Claude's (1946*a*) procedure on the ground that the granules isolated in fraction 1 are not identical morphologically or in staining reactions with the mitochondria seen in the intact cell, and they have described a method of isolating these intact.

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MATERIAL AND METHODS

In an earlier (unpublished) series of experiments normal rabbit liver was fractionated by Claude's (1946*a*) procedure. The presence of glycogen, however, interfered seriously with the fractionation, as has also been observed by Claude. In subsequent experiments this effect was largely eliminated by withholding food from the animals the night before the removal of the liver.

In view of the evidence of Hogeboom *et al.* (1948) that the large granules prepared by the use of a concentrated solution of sucrose more closely resemble the mitochondria of the intact cell, their method (in a slightly modified form) was used for most of the present investigation. In our work the first consideration has been the purity of the fractions, necessarily at the expense of information about the amount of each present.

Fully grown (2·3–2·8 kg.) rabbits were used. They were anaesthetized with nembutal and ether, and after withdrawal of blood from the aorta, the liver was excised. All fractionation procedures were carried out at or near 0°.

Liver fractionation. Hogeboom *et al.* used 0·88 M-sucrose solution in their procedure. For the sedimentation of the large and small granules in a reasonably short time from a solution of such high density, centrifugal forces of 24,000 and 41,000 *g* respectively are needed. A refrigerated centrifuge developing such forces was not available, and the following modifications were introduced to separate the particles at lower centrifugal forces.

The liver was pressed through a 1 mm. mesh screen; 15–20 g. of the resultant pulp were ground for 5 min. in a mortar and 100 ml. of 0·88 M-sucrose solution added over a period of 10 min. with continuous grinding. The suspension was centrifuged at 600 *g* for 10 min. and the deposit of intact liver cells, debris and nuclei discarded. The supernatant was centrifuged twice more at the same speed, the small sediments being discarded.

The suspension of cytoplasmic constituents, usually divided in two to four tubes, was now centrifuged at 18,000 *g* for 45 min. to throw down the large granules. The supernatant, SII, was set aside. The deposits were pooled with the help of the small amounts of SII left behind and