SUMMARY

1. Pelligrino synthesizes phytofluene, β -, ζ - and γ -carotenes, leprotene, and three unknown xanthophylls, two of which also appear in various strains of *Mycobacterium phlei*.

2. Diphenylamine inhibits the synthesis of the more unsaturated polyenes but stimulates the production of phytoene, phytofluene, ζ -carotene, neurosporene and pigment X. The diphenylamine effect is not overcome by riboflavin, adenosine-5'-phosphate, Fe^{2+} , or Fe^{3+} .

3. Maximal carotenoid concentration occurs about 10 days after inoculation and with a glucose concentration of about 1% (w/v). Higher glucose levels increase growth but not carotenogenesis. Replacement of glucose by glycerol reduces growth and enhances carotenogenesis. Acetate reduces growth but has little effect on pigmentation.

4. Replacement of asparagine by $(\text{NH}_4)_2\text{SO}_4$ reduces growth and carotenogenesis equally, whilst NH_4NO_3 inhibits carotenogenesis much more than growth.

5. Concentrations of streptomycin less than 0.001% (w/v) tend to inhibit carotenogenesis to a greater extent than growth, whilst a number of common metabolic inhibitors when they did not inhibit growth completely did not have a preferential effect on carotenogenesis.

6. Tween 80 (0.02%, w/v) completely inhibits growth of Pelligrino; this is overcome when a lipid-soluble material (squalene or liquid paraffin, 0.1%, w/v) is dispersed in the medium with Tween.

7. Washed suspensions of Pelligrino did not synthesize additional amounts of carotenoids.

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The Metabolism of Pteroylglutamic Acid by the Rat

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From evidence published so far it appears that before pteroylglutamic acid (PGA)* can carry out its metabolic functions the organism must first

* As used by Welch & Nichol (1952) the term 'pteroylglutamic acid' has been restricted to the synthetic compound and 'citrovorum factor' to the substance(s) promoting growth of *Pediococcus cerevisiae* 8081 (*Leuconostoc citrovorum*); the term 'folic acid' has been considered a generic term applicable to substances which stimulate the growth of *Streptococcus faecalis*. The term 'unconverted folic acid' relates to the substance(s) enhancing the growth of *Strep. faecalis* but not of *Pediococcus cerevisiae*.

convert it into the tetrahydro form and that citrovorum factor (CF) may represent one of the metabolically active forms of folic acid (FA). Evidence has been presented (Nichol, 1950; Doctor *et al.* 1953; Doctor, Elam, Sparks, Lyman & Couch, 1954) for the existence in liver of an enzyme that is involved in the conversion of PGA into CF. Moreover, Nichol (1953a) has demonstrated that the liver is the main site of this conversion in the body. Of particular interest is the finding of Welch, Nichol, Anker & Boehne (1951) and of Broquist, Stokstad & Jukes (1951) that more CF is formed

when ascorbic acid is present. Since injection of ascorbic acid increases the urinary excretion of CF, it would appear that ascorbic acid brings about favourable reducing conditions essential for the formation of CF.

Since the conversion of PGA into CF by rat and chicken livers has hitherto been studied under normal nutritional conditions only, the question arose whether lack of nutrients or an unbalanced diet would impair this reaction. Before approaching this problem experimentally it was necessary to study the normal capacity of the liver to convert PGA into CF under adequate nutritional conditions. The first part of our study presents the urinary excretion and liver concentration of FA and CF of normal rats after treatment with varying amounts of PGA and ascorbic acid. In the second part liver FA and CF were determined in rats which were kept on diets of various fat and protein contents.

METHODS

Young male rats weighing 40–50 g. were used throughout. The diets employed are presented in Table 1.

reported here was in progress, the paper by Chang (1953) describing an improved method of liberation of CF in natural materials came to our attention. It was thought advisable, however, not to adopt another method in the midst of our experiments in order to make possible a comparison of all the results.

Liver N was determined by the conventional Kjeldahl method and ascorbic acid with 2:6-dichlorophenolindophenol (Mindlin & Butler, 1938).

RESULTS

Effect of PGA and ascorbic acid on urinary excretion and liver concentration of FA and CF. In the first series of experiments groups of ten to twelve rats were kept for 2 weeks on the control diet. During the last 3 days they were injected subcutaneously with different doses of PGA and ascorbic acid. Urine was collected, and at the end of the 3-day period the animals were killed and their livers assayed for FA and CF. The results are shown in Table 2.

It follows from the table that, with increasing doses of PGA, increasing amounts of both FA and CF are excreted in the urine. The upper excretion limit, however, seems to be reached with the

Table 1. *Composition of diets*

The diets were supplemented with the following vitamins (mg./100 g. ration): thiamine 0.2, riboflavin 0.3, pyridoxine 0.1, calcium pantothenate 1.6, niacin 5.0 and choline chloride 100. Each rat received 100 i.u. of vitamin A, and 4 i.u. of vitamin D twice weekly

Ingredient	Diet				
	Control	High-fat	Low-protein low-fat	Low-protein high-fat	Protein-free
Casein	18	18	8	8	0
Cornstarch	73	58	83	68	91
Vegetable oil	5	10	5	10	5
Lard	0	10	0	10	0
Salt mixture, U.S.P. 2	4	4	4	4	4

For production of choline deficiency, choline chloride was omitted from the low-protein, high-fat diet. The presence of choline deficiency was verified by determining the accumulation of fat in the liver (average: 14.8% in deficient rats, 5.7% in the livers of control rats).

FA and CF in urine and liver were determined with *Strep. faecalis* (Association of Vitamin Chemists, 1951) and *Pediococcus cerevisiae* 8081 (*Ln. citrovorum*) (Saubert & Baumann, 1948) respectively. Synthetic CF (Leucovorin, Lederle Laboratories, American Cyanamid Co.) in the form of the hydrated barium salt was used as standard. Since leucovorin is only half as active as the 'natural' CF occurring in horse liver (Keresztesy & Silverman, 1951; Cosulich, Smith & Broquist, 1952), microbiological assay values in terms of leucovorin were divided by two to give results as naturally occurring CF. For determination of FA and CF in liver, these organs were homogenized and autolysed as described by Dietrich, Monson, Gwoh & Elvehjem (1952). Values of 'unconverted FA' were obtained by subtraction of *Strep. faecalis* activity due to CF present from observed values of total *Strep. faecalis* activity. Whilst the work

administration of 5–10 mg. of PGA/100 g. body weight, since injection of 20 mg. of PGA does not raise the excretion of either FA or CF any further. With each level of PGA treatment excretion of FA surpasses by far (30- to 200-fold) that of CF.

In contrast to urine, the concentration of liver CF is much higher than that of 'unconverted FA' (after subtraction of *Strep. faecalis* activity due to CF). Values in the upper range which lie between 5.3 and 9.8 µg. are already reached with 0.10 mg. of PGA/100 g. body weight, whereas the content of unconverted FA does not exhibit any consistent variation over a wide range of PGA administration. Injection of 5 mg. of PGA/100 g. or more, however, increases significantly the liver concentration of unconverted FA and leads to a marked decrease in the ratio of CF to unconverted FA in the liver. It seems that this is the upper limit of the capacity of the liver to convert PGA into CF efficiently.

Injections of increasing doses of ascorbic acid, along with a constant dose of PGA (0.25 mg./100 g.), were found to induce increasing excretion of both FA and CF. With 25 mg. of ascorbic acid recovery of more than 90% of the injected dose of PGA was obtained as FA and CF in urine. Equally, treatment with ascorbic acid also raised the liver concentration of both these substances. Whereas no great variations were observed in the CF content, large doses of ascorbic acid seem to raise significantly the concentration of unconverted FA in the liver and thus to lower the ratio of CF to unconverted FA.

these conditions being unable to convert the PGA efficiently into CF stores an increased amount of unconverted FA. The consequence is a fall in the CF/FA quotient, and an apparent impairment of the capacity of the liver to produce CF.

Increased urinary excretion of CF was also observed as a consequence of increased endogenous ascorbic acid production (Table 3). In this experiment eleven rats were treated with sodium phenobarbital, which was incorporated into the control diet at a level of 1.5 g./kg. Rats receiving this drug excreted 3.43 mg. of ascorbic acid (standard error ± 0.32 mg.)/day as against 0.22 ± 0.07 mg.

Table 2. *Urinary excretion and liver concentration of folic acid and citrovorum factor after injection of different doses of pteroylglutamic and ascorbic acids*

Each group comprises 10–12 rats which were kept on a control diet during 2 weeks. During the last 3 days they were given subcutaneous injections of pteroylglutamic and ascorbic acid respectively. The figures in columns 1 and 2 indicate the daily doses per 100 g. body weight. The values for folic acid are the observed values for total folic acid minus *Strep. faecalis* activity due to citrovorum factor present. Figures in columns 3–7 are means $\pm$ S.E.M.

Compound injected		Urinary excretion/(\(\mu\text{g.}/100 \text{ g. body weight/day}\))		Liver			
Pteroyl-glutamic acid (mg.)	Ascorbic acid (mg.)	Folic acid	Citrovorum factor	Wt. (g.)	Folic acid (\(\mu\text{g.}\))	Citrovorum factor (\(\mu\text{g.}\))	Citrovorum factor Folic acid
—	—	2.7 ± 0.57	0.025 ± 0.004	3.3 ± 0.13	0.55 ± 0.24	3.3 ± 0.53	6.0
0.05	—	17.4 ± 2.68	0.11 ± 0.02	3.3 ± 0.21	0.50 ± 0.56	3.6 ± 0.85	7.2
0.10	—	17.7 ± 2.04	0.10 ± 0.02	3.5 ± 0.13	0.40 ± 0.28	5.8 ± 0.59	14.5
0.25	—	53.8 ± 3.43	0.19 ± 0.13	3.3 ± 0.21	1.15 ± 0.38	6.9 ± 1.45	6.0
0.50	—	102 ± 19.0	0.30 ± 0.07	3.3 ± 0.15	0.40 ± 0.25	5.3 ± 0.79	13.2
1.00	—	163 ± 22.7	0.61 ± 0.08	3.3 ± 0.19	1.00 ± 0.38	8.5 ± 1.36	8.5
2.00	—	267 ± 35.9	1.43 ± 0.31	3.2 ± 0.09	0.54 ± 0.28	6.9 ± 0.83	12.8
5.00	—	421 ± 53.6	2.44 ± 1.00	3.5 ± 0.16	2.10 ± 0.85	9.8 ± 1.20	4.7
10.00	—	456 ± 48.4	4.17 ± 1.43	2.8 ± 0.11	1.80 ± 0.60	5.3 ± 1.35	2.9
20.00	—	438 ± 30.0	3.03 ± 1.15	2.9 ± 0.16	8.70 ± 1.23	8.1 ± 1.15	0.9
0.25	0.25	130 ± 28.5	0.75 ± 0.20	3.6 ± 0.11	1.90 ± 1.04	13.7 ± 1.47	7.2
0.25	1.00	223 ± 64.2	1.27 ± 0.41	3.6 ± 0.10	2.31 ± 0.96	12.3 ± 1.75	5.3
0.25	5.00	207 ± 64.8	3.97 ± 1.09	3.7 ± 0.13	4.79 ± 1.51	15.5 ± 2.00	3.2
0.25	25.00	228 ± 77.0	3.59 ± 0.68	3.4 ± 0.07	6.80 ± 4.06	10.9 ± 1.63	1.5

Table 3. *Effect of ascorbic acid on urinary folic acid and citrovorum factor*

Folic acid and citrovorum factor excretion is given as mean values $\pm$ S.E.M. for 11 rats. Figures for folic acid are corrected for the presence of citrovorum factor (see Table 2).

Treatment	Excretion (\(\mu\text{g.}/100 \text{ g. body wt./day}\))	
	Folic acid	Citrovorum factor
None (control period)	1.16 ± 0.17	0.035 ± 0.009
Animals fed diet containing sodium phenobarbital (1.5 g./kg.)	0.94 ± 0.12	0.153 ± 0.024
Ascorbic acid (5 mg./day) added to urine-collection flasks	0.75 ± 0.15	0.044 ± 0.016

It would appear, therefore, that ascorbic acid protects not only CF from oxidation, leading to its increased concentration in both urine and liver, but has a similar protecting effect on FA, too. Ascorbic acid, given along with a small dose of PGA (0.25 mg./100 g.) leads to an increased PGA content of the liver, which is similar to that following a large dose of PGA (5–10 mg./100 g.) given without ascorbic acid. The liver under both

when untreated. Excretion of CF, too, rose significantly during a 3-day urine-collection period, whereas the FA concentration of the urine remained unchanged. In a further control experiment 5 mg. of ascorbic acid were added daily to the urine-collection flasks, and the rats did not undergo any treatment with either ascorbic acid or phenobarbital. No statistically significant changes of urinary concentration of FA or CF were noted.

'Endogenous' ascorbic acid, i.e. ascorbic acid formed in the body under the stimulation of phenobarbital, appears thus to increase urinary CF excretion as much as 'exogenous' ascorbic acid, whereas the *in vitro* addition of this substance to the collected urine is without any effect.

Effect of diet on the conversion of PGA into CF. In this series of experiments young rats were kept on different diets containing 0, 8 or 18 % of casein and 5 or 20 % of fat respectively. After 3 weeks on these diets, half of each dietetic group was dosed daily with 0.25 mg. of PGA and 5 mg. of ascorbic acid/100 g. body weight during 3 days. On the fourth day all rats were killed and their livers examined for FA and CF. The results obtained are presented in Table 4.

the capacity of the liver to synthesize CF from PGA, and in severe protein deficiency the ability of the liver to store injected PGA is impaired to a marked degree.

The depression of CF synthesis by rats kept on diets low or deficient in protein is accompanied by a similar decrease in liver nitrogen. Livers of twenty-one rats maintained on the control diet, of thirteen rats on the low-protein diet and of eleven rats on the protein-free diet were examined for nitrogen content. The following amounts (mg./g. of liver) with their standard errors were found: 30.8 ± 0.74 ; 25.9 ± 1.03 ; 23.9 ± 0.87 . Whereas the livers of rats on the low-protein diet exhibited significantly lower values than those of the controls, the difference between the two latter groups proved

Table 4. *Effect of diet on liver levels of folic acid and citrovorum factor*

Figures in columns 6-8 are means $\pm$ S.E.M. Each group comprises 10-12 rats which were kept during 3 weeks on the specified diets. Values for folic acid are corrected for presence of citrovorum factor (see Table 2). A + in column 5 indicates daily subcutaneous injections of 0.25 mg. pteroylglutamic acid (PGA) and 5.0 mg. ascorbic acid (AA)/100 g. body weight during the last 3 days of experiment; - indicates no injection.

					Liver			
Wt. change (g.)	Diet			PGA + AA	Wt. (g.)	Folic acid (μ g.)	Citrovorum factor (μ g.)	Citrovorum factor
	Casein (%)	Fat (%)	Choline (%)					Folic acid
+43	18	20	0.01	-	4.4 $\pm$ 0.27	0.35 $\pm$ 0.21	3.07 $\pm$ 0.07	8.8
+48	18	20	0.01	+	4.6 $\pm$ 0.20	2.43 $\pm$ 1.47	7.79 $\pm$ 1.97	3.2
+28	8	5	0.01	-	3.1 $\pm$ 0.21	0.50 $\pm$ 0.24	0.47 $\pm$ 0.15	0.9
+27	8	5	0.01	+	3.4 $\pm$ 0.15	2.45 $\pm$ 0.63	1.41 $\pm$ 0.44	0.6
+20	8	20	0.01	-	2.9 $\pm$ 0.25	0.40 $\pm$ 0.51	0.19 $\pm$ 0.12	0.5
+23	8	20	0.01	+	3.1 $\pm$ 0.15	2.51 $\pm$ 0.75	1.14 $\pm$ 0.50	0.5
+21	8	20	0	-	3.9 $\pm$ 0.21	0.66 $\pm$ 0.17	0.34 $\pm$ 0.10	0.5
+20	8	20	0	+	3.7 $\pm$ 0.32	2.38 $\pm$ 1.58	1.02 $\pm$ 0.33	0.4
-13	0	5	0.01	-	1.4 $\pm$ 0.05	0.40 $\pm$ 0.08	0.09 $\pm$ 0.02	0.2
-12	0	5	0.01	+	1.4 $\pm$ 0.05	0.63 $\pm$ 0.10	0.24 $\pm$ 0.04	0.4

As can be seen from Table 4, a moderately high fat diet with a sufficient protein content (18 %) does not significantly affect the FA and CF content of the liver. Similar values for FA, CF and CF/FA quotients were obtained as with a low-fat diet (Table 2). Reduction of the protein content of the diet from 18 to 8 %, however, significantly depressed the CF content of the liver, whereas the level of unconverted FA was not affected. Very low values of the quotient CF/unconverted FA were therefore noted. On the other hand, neither the fat content of the diet nor an accompanying choline deficiency had any influence on the FA and CF levels of the liver under these conditions. Rats maintained on a protein-free diet had a considerably lower liver level of CF than those on the low-protein diet, and, when treated with PGA, were not able to store unconverted FA in their livers. The quotient CF/unconverted FA was found to be lower than in livers of rats on low-protein diets. It seems, therefore, that lack of dietary protein lowers

to be statistically insignificant. Rats maintained on the low-protein diet, while growing much better than rats on the protein-free diet, have a similarly low level of liver nitrogen and impaired capacity to synthesize CF.

DISCUSSION

The experimental results presented above demonstrate that PGA, when introduced parenterally into the body, is to a large part excreted with the urine as FA. The recovery of the injected dose, however, decreases from about 30 % when 0.05 mg./100 g. body weight is given to less than 2 % with a 20 mg. dose. It is noteworthy that CF accounts for 0.5-3 % only of the total FA excreted in urine. Thus the percentage of the dose of PGA recovered in urine as CF decreases from 0.2 after injection of 0.05 mg./100 g. body weight to not more than 0.015 when 20 mg. of PGA are administered. On the other hand, the absolute amount of CF

excreted increases more than 100-fold when large doses of PGA are given. These figures show the limited ability of the organism to convert injected PGA into CF.

In contrast to urine most of the FA found in liver proved to be CF, and only 6–13% was unconverted FA. Treatment with PGA increased the liver content of CF by not more than a few micrograms; relatively very large doses of PGA, 5 mg. or more/100 g. body weight, increased the concentration of unconverted FA as well. Under these conditions the liver is apparently not able to convert PGA into CF efficiently.

Administration of ascorbic acid has been shown to increase CF excretion in urine of man (Welch *et al.* 1951; Broquist *et al.* 1951; Gabuzda, Phillips, Schilling & Davidson, 1952) and rats (Welch *et al.* 1951; Guggenheim, Leibowitz & Rakower, 1953) and to augment CF formation by liver of rats (Nichol, 1950; Broquist *et al.* 1951) and chicken (Doctor *et al.* 1954; Nichol, 1953b). The effect of the vitamin seems to be due to its reducing property, since glucoascorbic acid (Welch *et al.* 1951) and isoascorbic acid (Guggenheim *et al.* 1953), as well as cysteine and homocysteine (Doctor & Trunnell, 1954), likewise enhance urinary excretion of CF. In our experiments ascorbic acid increased the excretion not only of CF but also that of FA. When a dose of 0.25 mg. of PGA/100 g. body weight was injected without additional ascorbic acid, 22% of it was recovered in urine. The recovery rose to 52%, however, when the same amount of ascorbic acid was injected along with PGA, and even to 91% when the dose of ascorbic acid was increased 100-fold. Excretion of CF rose concomitantly, and in terms of the administered PGA without ascorbic acid it was 0.08%. After injection of 0.25 mg. of ascorbic acid/100 g. body weight, the excretion of CF rose to 0.3% and after injection of 25 mg. of ascorbic acid it rose to 1.4%.

The increase in liver FA and CF after administration of ascorbic acid is much smaller. Ascorbic acid was found to increase the CF level by 70–125%, and that of unconverted FA by 70–490%. It may be assumed that the amount of PGA available to the liver under the influence of large doses of ascorbic acid overtakes its capacity to convert it into CF, and as a consequence part of it is stored as unconverted FA.

Although variation of the fat content of the diet and even fat accumulation in the liver due to choline deficiency were not found to affect the conversion of FA into CF, protein deficiency had a marked influence. The liver concentration of CF decreases with diminishing protein content of the diet, and in severe protein deficiency the capacity of the liver to store injected PGA is also impaired. Protein deficiency therefore profoundly affects the

metabolism of PGA by impairment of its hepatic conversion into CF.

Since the most striking effect of PGA deficiency in man is macrocytic anaemia associated with megaloblastic erythropoiesis, the question arises as to whether the macrocytic anaemia that occurs in children suffering from Kwashiorkor (Walt, Wills & Nightingale, 1950; Brock & Autret, 1952; Joint FAO/WHO Expert Committee on Nutrition, 1953; Autret & Behar, 1954; Adams, 1954) is due to the impaired conversion of PGA into CF as a result of an insufficient intake of dietary protein. The observations of some workers (Pieraerts, 1949; Trowell, Davies & Dean, 1952) that this anaemia responds to protein supplementation of the diet but not to PGA administration are of special interest in the light of our findings reported above.

SUMMARY

1. Young rats when injected with pteroylglutamic acid (PGA) increase the urinary excretion of folic acid (FA) and citrovorum factor (CF), but the recovery decreases with increasing doses. At each excretion level, CF accounts for only 0.5–3% of the total FA.

2. In liver more CF than unconverted FA is found. Whereas administration of 0.05–2.0 mg. of PGA/100 g. body weight increases the CF content of the liver by only a few micrograms without increasing the concentration of unconverted FA, larger doses (5–20 mg./100 g.) lead to a higher storage of unconverted FA without further increases in the CF level. As a consequence, the ratio of CF to unconverted FA is decreased.

3. Ascorbic acid, when injected together with PGA, increases the urinary excretion of both FA and CF. It raises the liver level of CF and, when injected in larger doses, leads to an increased storage of unconverted FA as well. Under these conditions the ratio of CF to unconverted FA is again diminished.

4. Rats treated with phenobarbital increase their excretion of both ascorbic acid and CF. *In vitro* addition of ascorbic acid to collected urine is without any influence on its CF concentrations.

5. Low-protein and protein-free diets lower the liver content of CF and the ratio of CF to unconverted FA. In severe protein deficiency the ability of the liver to store injected PGA is also impaired. It is concluded that lack of dietary protein depresses the capacity of the liver to convert PGA into CF.

6. Both high-fat diets and fat accumulation in the liver due to choline deficiency do not impair its capacity to form CF.

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Changes in the Composition of the Fat of *Aspergillus nidulans* with Age of the Culture

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While studying linseed oil Ivanow & Klokow (1933) observed that as ripening of the oil-bearing seeds proceeded the proportion of the linolenic acid component increased, whereas those of the oleic and linoleic acid components decreased. From the results of feeding experiments with rats Boxer & Stetten (1944) formed the opinion that in these animals oleic acid was formed from stearic acid by dehydrogenation and that saturated acids are the primary products in fat biosynthesis. Coleman, Cefola & Nord (1952) studied formation of fat in *Fusarium lini* Bolley, and concluded that a mechanism for the dehydrogenation of fatty acids operates in this mould. Such micro-organisms, which can synthesize relatively large quantities of fat when grown on a simple defined medium of sugar and inorganic salts, may prove useful agents for the study of the reactions which give rise to fat, for both substrate and cultural conditions can be well regulated. Recently, we examined the fat of *Aspergillus nidulans* (Singh, Walker & Meara, 1955), and we have now conducted experiments

which were planned to throw light on the question whether the unsaturated acids that are components of the fat of this organism are formed by dehydrogenation of saturated acids or by some other mechanism.

EXPERIMENTAL

Aspergillus nidulans Eidam was grown as surface cultures from spores sown on a medium which contained (% w/v): NH_4NO_3 , 0.6; $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 0.73; K_2SO_4 , 0.044; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.016; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.005; sucrose, 34; the medium was adjusted to pH 3.8 and made up to volume with distilled water. In each experimental series a set of Pyrex penicillin flasks, each containing 175 ml. of this medium, was employed. After sterilization in steam at 10 lb. pressure for 15 min. the flasks were cooled, their contents inoculated and incubated at 25°. Cultures were harvested after 5, 7, 9, 14 and 21 days' incubation. The number of flasks taken at any given time depended upon the growth attained; thus at 5 days it was necessary to combine the contents of twenty flasks in order to obtain sufficient fat for detailed analysis. At the later periods, a smaller number of flasks yielded sufficient fat for this purpose, but the number of flasks withdrawn at any