4. From the above results it is calculated that the degree of hydrolysis of the carbamyl group of salicylamide is 4-7 and of *m*-hydroxybenzamide 4-16%.

5. 2- and 3-carbamylphenylglucuronides have been isolated as major metabolites of salicylamide and *m*-hydroxybenzamide respectively.

6. Gentisic acid (4-5%) has been isolated as a metabolite of salicylic acid and gentisamide of salicylamide.

7. Evidence has been obtained of the formation of gentisic acid (or amide) as a metabolite of *m*-hydroxybenzoic acid and amide.

8. Glycine conjugates of *o*- and *m*-hydroxybenzoic acids were not detected.

9. The fact that salicylic acid is hydroxylated to an extent greater than is salicylamide is discussed.

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Growth Factors for *Lactobacillus casei*

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We have described elsewhere (Chattaway, Happold, Lythgoe, Sandford & Todd, 1943; Chattaway, Happold & Sandford, 1944) the presence of a growth factor for *Corynebacterium diphtheriae gravis*, sub-type Dundee, in the amyl alcohol insoluble fraction obtained by extracting liver residues with this solvent at pH 2, and the separation of this factor from material stimulating the growth of *Lactobacillus casei* at pH 3, the *Lb. casei* factors being in the cresol extract.

The present paper describes the further concentration and separation of the factors promoting acid production by *Lb. casei* (Chattaway, Dolby & Happold, 1944), and Factor 3 of Dolby, Happold & Sandford (1944), and considers their relationships to

factors reported as stimulatory for this organism by other workers. Among these factors are fractions from liver, fermentation residues and yeast, which have been shown to be members of the pteroyl-glutamic acid (PGA) group of components, differing from one another in having one, three or seven glutamyl residues respectively (Angier, Boothe, Cosulich, Fahrenbach, Hultquist, Hutchings, Kuh, Mowat, Northey, Seager, Semb, Sickels, Smith, Stokstad, Subbarow & Waller, 1945, 1946; Bloom, Calkins, O'Dell & Pfiffner, 1946). The material known as folic acid on the other hand (Mitchell, Snell & Williams, 1941) is probably different from these, as also is the '*Streptococcus lactis* R' factor (Keresztesy, Riekes & Stokes, 1943) which has been shown

to be 4-*N*-formyl-*N*-(2'-amino-4'-hydroxyl-6'-pteridylmethyl) aminobenzoic acid (Anderson, Arth, Folkers, Harris, Kaczka, Mazingo, Southwick & Wolf, 1947), but it has been claimed (Angier *et al.* 1946) that PGA represents the sole remaining unknown growth factor for *Lb. casei*. The following observations by various workers indicate that this is probably not so:

- (1) Williams (1944) has stated that folic acid probably consists of a number of closely related compounds with differing physiological activity, a statement supported by Hall (1947*a*) in this Department.
- (2) Mitchell, Snell & Williams (1944) reported the absorption spectrum of folic acid as indicating the presence of a xanthopterin nucleus, while that of PGA does not.
- (3) Hall (1947*b*) has demonstrated differences in the response of *Strep. faecalis* R to folic acid, vitamin B₉ and PGA.
- (4) Elvehjem & Teply (1945) found the need for unidentified factors other than vitamin B₉ in the growth of *Lb. casei*.
- (5) Dolby *et al.* (1944) showed the *C. diphtheriae* factor to be present in casein and to be required for the growth of *Lb. casei*. They also reported the presence of a growth factor (Factor 2) distinct from the *C. diphtheriae* factor, which did not affect the amount of acid production by *Lb. casei*, but which did increase its growth. It appears to us possible that this factor might be related to the strepogenin factor of Woolley (1941).
- (6) Barton-Wright, Emery & Robinson (1944, 1945) describe two chloroform-soluble fractions obtained from liver which are synergistic for *Lb. casei* and not identical with folic acid.

Evidence will be presented in this paper to show that on our basal medium the addition of either PGA or 'folic acid' (7% concn. kindly supplied by Prof. R. J. Williams) produces little response, either in the growth or acid production of *Lb. casei*, thus demonstrating the need of this organism for further growth factors.

EXPERIMENTAL

Organisms and media. The medium used for investigation with *Lb. casei* has been previously described (Chattaway, Happold & Sandford, 1943); the techniques of culture and subculture were unchanged. Readings were made after 72 hr. and recorded as ml. 0.1 *N*-acid produced.

In the determination of 'folic acid' activity, *Strep. faecalis* R was used; the medium employed was that of Guirard, Snell & Williams (1942) with the addition of leucine and isoleucine (cf. Dolby & Waters, 1944), and using an acid hydrolysate of casein prepared according to the method of Johnson & Mueller (1941). Acid production was determined after 18 hr. and recorded as ml. 0.05 *N*-acid produced.

Preliminary concentration of active material. The starting material was that fraction from the liver residues (Boots Pure Drug Co., Ltd., Nottingham) which was not adsorbed on fuller's earth at pH 3, but which was subsequently adsorbed on norite at this pH, and then eluted with an ammonia-ethanol-water mixture (containing 12% (w/v)

NH₃ in 50% (v/v) ethanol). The eluate was neutralized, concentrated, acidified to pH 2 and extracted repeatedly with amyl alcohol. The amyl alcohol-insoluble material (water soluble) was then extracted with *p*-cresol at pH 3, and the cresol extract used for the work described below.

RESULTS

Separation of two synergistic fractions by treatment with baryta

Active concentrates prepared in the above manner gave a considerable precipitate on saturation with baryta, additional quantities being obtained by the addition of 3 volumes methylated spirits to the baryta-saturated material. The baryta-soluble material was found, after removal of excess barium, to show little loss in activity compared with the original material when tested with *Lb. casei*. The insoluble barium salts, after removal of barium, showed little intrinsic activity, but when the two fractions were tested together at low concentrations it became apparent that the original activity was due to two components. A typical set of results is shown in Table 1.

Table 1. *Acid production by cultures of Lactobacillus casei on inclusion of baryta-soluble and insoluble fractions*

Additions (ml./20 ml. medium)		Acid production after 72 hr. (ml. 0.1 <i>N</i> -acid/20 ml. medium)
Baryta soluble	Baryta insoluble	
1.0	—	6.3
0.5	—	5.2
0.05	—	2.6
0.005	—	1.1
0.0005	—	0.2
—	1.0	1.4
—	0.3	0.9
—	0.1	0.6
—	0.01	0.2
—	0.001	Nil
0.01	0.01	4.4
0.001	0.01	2.9
0.0001	0.01	1.8
—	—	Nil

Fractionation of the baryta-soluble material

(a) *Phosphotungstic acid.* The material was treated with excess of 50% (w/v) phosphotungstic acid in 5% (w/v) H₂SO₄, the precipitate removed and the filtrate freed from phosphotungstic acid with Ba(OH)₂. There was some loss of activity (20–30%) in the process, but practically the whole of the remaining activity was in this filtrate.

(b) *Picric acid.* On treatment of the Ba(OH)₂-soluble material with saturated picric acid, small amounts of a picrate separated which always showed slight activity, yet there was no detectable loss of activity in the soluble fraction after removal of excess picric acid by ether extraction.

(c) *Silver nitrate*. Treatment of the $\text{Ba}(\text{OH})_2$ -soluble fraction with AgNO_3 at acid reactions effected a considerable fractionation of the active material, the following technique being adopted. The solution was acidified with excess HCl in the cold and NaCl , free HCl and silver salts of some organic acids were removed by stepwise addition of solid AgNO_3 (ppt. 1), the reaction remaining at pH 1.0 (approx.). An excess of AgNO_3 was then added and the reaction adjusted cautiously to pH 4.0 with NaOH ; a flocculent precipitate, which later darkened, formed and was removed (ppt. 2). With further additions of NaOH a white, heavier precipitate (ppt. 3) which was inactive began to appear at pH 4.5, increasing in amount at more alkaline reaction. The whole activity of the $\text{Ba}(\text{OH})_2$ -soluble material for *Lb. casei* was found in ppt. 2; the active material was recovered by treatment with a mixed solution of saline and HCl carefully adjusted to give a neutral supernatant readily separated from the precipitated AgCl . The 'folic acid' activity measured on cultures of *Strep. faecalis* R was concentrated mainly in ppt. 1, although some remained in ppt. 2. There was no measurable activity in ppt. 3 and later residue fractions.

On addition of ethanol (50 to 60%, v/v) to the aqueous solution of ppt. 2 an inactive white crystalline precipitate separated at 0°. Addition of ether (50%, v/v) gave a further separation of white inactive material in the ethanol phase. From the ether-water phase, which was orange in colour, a reddish-brown oil began to separate, the activity being distributed between these two components.

(d) *Lead acetate*. Attempts to fractionate the $\text{Ba}(\text{OH})_2$ -soluble material by treatment with lead acetate at pH 3 followed by basic lead acetate at pH 7.6 were unsuccessful. The active material was found to be unevenly and variably divided between the precipitates and some slight activity remained in the filtrate.

(e) *Adsorption on alumina*. The alumina used was 'Alumina purified for chromatographic purposes' (British Drug Houses Ltd.). It was suspended in 40% methanol at pH 3 and kept at 37° for 3 days, filtered, air-dried at room temperature for 4 days, suspended in 40% methanol at pH 3 and poured into a column 30 cm. by 2.5 cm. The column so prepared was washed through with 40% methanol at pH 3 using approximately 250 ml. A slight positive pressure of N_2 was maintained on the column during this preliminary washing and during the subsequent run with the active material. The material to be treated, dissolved in 40% methanol at pH 3, was added to the top of the column, and washed through with acidified 40% methanol and the filtrate collected until quite colourless and inactive for *Lb. casei*. Elution was carried out using 2% (w/v) NH_3 . Filtrate and eluate fractions were collected and concentrated *in vacuo* as soon as possible, dissolved in a small volume of water and adjusted to pH 6.8.

The usual procedure adopted with the baryta-soluble material was to separate it into filtrate and 2% (w/v) NH_3 -eluate fractions as described above, and then to pass both of these fractions, separately, down further alumina columns, finally combining all filtrate fractions and all eluate fractions. All fractions were tested for acid-producing activity using *Lb. casei* and *Strep. faecalis* R. The filtrate fractions were found to be almost inactive while the eluate fraction contained considerable active material for *Strep. faecalis* R, indicating that this material probably contains some or all of the components of 'folic acid' (Mitchell *et al.* 1944). Both

fractions contained material active for *Lb. casei* and they were shown to be synergistic in their action. Typical results are shown in Tables 2 and 3.

Table 2. Separation of baryta-soluble material into two active fractions by treatment with alumina

(Baryta-insoluble fraction 0.01 ml. was added to all flasks in *Lb. casei* tests. Concentrations tested: Al_2O_3 -filtrate fraction, 310 mg./ml.; Al_2O_3 -eluate fraction, 153 mg./ml.)

Al_2O_3 filtrate (ml./20 ml. culture)	Al_2O_3 eluate (ml./20 ml. culture)	Acid production	
		<i>Lb. casei</i> (ml. 0.1 N- acid/20 ml. medium; 72 hr. incubation)	<i>Strep. faecalis</i> R (ml. 0.05 N- acid/10 ml. medium; 18 hr. incubation)
1.0	—	7.25	1.2
0.1	—	3.45	0.25
0.01	—	2.25	Nil
0.001	—	1.7	Nil
—	1.0	4.45	—
—	0.1	2.5	—
—	0.01	1.5	4.6
—	0.001	—	3.05
—	0.0001	—	0.95
Nil	Nil	1.4	Nil

Table 3. Synergism between Al_2O_3 -filtrate and eluate fractions for *Lactobacillus casei*

(No lactic acid was produced when fractions were tested individually as follows in presence of baryta-insoluble fraction. Al_2O_3 filtrate, 0.01, 0.001, 0.0001 ml.; Al_2O_3 eluate, 0.01, 0.001 ml. All figures refer to quantities/20 ml. medium and 72 hr. incubation.)

Al_2O_3 filtrate (ml.)	Al_2O_3 eluate (ml.)	0.1 N-acid (ml.)
0.01	0.01	1.7
0.01	0.001	1.65
0.005	0.005	1.3
0.005	0.0005	1.05
0.0001	0.001	0.15
0.00005	0.0005	Nil
Nil	Nil	Nil

Fractionation of material from silver precipitate 2 in alumina column

The orange-yellow pigment and the reddish-brown oil obtained from precipitate 2 (see above) were bulked, and examined by chromatography on Al_2O_3 after solution in 40% (v/v) methanol at pH 3.0. As each fraction was collected it was concentrated to dryness *in vacuo*, and redissolved in water. The fractions were all tested several times with *Lb. casei* on the basal medium, with and without both pteroylglutamic acid and the baryta-insoluble fraction at the lower limit of its activity. They were tested for 'folic acid' activity with *Strep. faecalis* R. The results are not completely conclusive, but Table 4, which shows the treatment each fraction received, also gives a summary (columns 4 and 5) of their activity for both the organisms employed.

Table 4. *Activities of fractions produced from the pigmented fractions of the silver separation by chromatography on alumina*(None of the fractions showed any activity for *Lb. casei* when administered alone or with the baryta-insoluble fraction.)

No. of fraction	Colour	Treatment producing fraction	Activity	
			<i>Strep. faecalis</i> R	<i>Lb. casei</i> with PGA
I	Pale yellow	1st filtrate fraction	Equivalent to 0.001 μ g. PGA/ml.	Slight activity
II	Colourless	2nd filtrate fraction	Inactive	Active
III	Pale yellow	1st eluate, 20% (v/v) methanol	Inactive changing to active	Growth, but no acid
IV	Pale yellow	2nd eluate, water for 12 hr.	Slightly active	Inactive
V	Colourless	3rd eluate, 0.5% (w/v) NH_3 . Collected prior to appearance of first pigmented band	Inactive	Inactive
VI	Dark brown	4th eluate, 0.5% (w/v) NH_3 ; pH of fraction 6.8-7.0	Active	Very active
VII	Dark brown	5th eluate, 0.5% (w/v) NH_3 ; pH 7.2 upwards	Active approx. $\equiv$ 1 μ g. PGA/ml.	Very active
VIII	Pale yellow	6th eluate obtained after 6 hr. washing with 0.5% (w/v) NH_3	Active	Slightly active

These fractions were tested again later and fraction III, which had previously been inactive, had acquired measurable activity, so possibly some 'folic acid-like' component had slowly hydrolyzed from a more complex peptide. In the cultures where the basal medium had aged somewhat, and where the controls were consequently positive—a finding previously reported by us and explicable on the basis of the findings of Hall (1947c) in this laboratory that 'folic acid-like' activity develops in amino-acid-sugar media containing histidine—the above fractions, which in fresh media gave growth and acidity only in the presence of PGA, did so without the addition of this component.

These results suggest the presence in the filtrate fraction of an active component for *Lb. casei* which is probably not the yellow substance present since activity is measurably greater in the colourless fraction II. Another active component is present in association with the pigments present in fractions VI and VII. Attempts to show synergistic action between fractions I-VII were not very successful though the critical interpretation of such experiments at each stage in an investigation is often difficult. The possibility must not be overlooked, however, that the same or similar compounds may be combined with other groups, in say, fractions II or VI and VII, which would modify their behaviour on the alumina.

In previous work, with repeated ethanol-ether precipitations from a silver salt similar to precipitate 2, a fraction had been obtained which it was expected would correspond roughly to fraction VII. This material was similarly developed upon an Al_2O_3 chromatogram. The colourless filtrate fraction was inadvertently thrown away, but a colourless water eluate A (cf. fraction IV, Table 4); a colourless

fraction B (cf. fraction V) after treatment with 0.5% (w/v) NH_3 ; the main pigment, fraction C (pH 6.8-7.0, cf. fraction VII); and the colourless eluate D were obtained. Fraction C was the only one to give unequivocal positive results with *Lb. casei*, but its activity was increased by fraction D although this fraction alone was inactive. These results suggest that more than one component is present in the alumina-eluate fraction from the baryta-soluble material.

Properties of Al_2O_3 filtrate and eluate fractions

The effect of the following reagents on the activity of the above fractions for *Lb. casei* and *Strep. faecalis* R was determined, the filtrate fraction being tested with *Lb. casei* only and the eluate fraction with both organisms.

Table 5. *Effect of nitrous acid treatment of Al_2O_3 -eluate fraction, tested with Lactobacillus casei*

Material added	(ml./20 ml. medium)	Acid production in 72 hr. (ml. 0.1 N-acid)
Al_2O_3 eluate	0.1	3.8
	0.075	4.5
	0.05	2.6
HNO_2 -treated Al_2O_3 eluate	0.1	1.5
	0.075	1.4
	0.05	1.1
HCl -treated Al_2O_3 eluate	0.1	3.6
	0.075	4.0
	0.05	2.5

(a) *Nitrous acid.* 0.5 ml. of each of the fractions was taken, together with 10 ml. distilled water, 40 mg. NaNO_2 and 1 ml. dilute HCl and the solution left to stand for 24 hr. at room temperature, evaporated to dryness, redissolved in

water and adjusted to pH 7 (volume to 25 ml.). A control was carried out omitting the NaNO_2 . Some inhibitory substance is obviously present in the Al_2O_3 eluate which is apparent in the highest concentration of eluate used. The Al_2O_3 filtrate fraction showed no inactivation while the eluate fraction showed partial inactivation for *Lb. casei* (see Table 5), and complete inactivation for *Strep. faecalis* R. These results accord with the previous observation that at least two components are present in the eluate fraction.

(b) *Acetic anhydride*. To 1 ml. fraction were added 3.2 ml. 2N-NaOH and 0.2 ml. acetic anhydride. The mixture was shaken and cooled in ice. Five further additions of 2 ml.

material being removed from the control by chloroform. However, when tested against *Lb. casei* the results were difficult to assess, as components active for this organism were removed by the chloroform extraction.

(d) *Methanolic HCl*. The solid residue from 0.5 ml. of each fraction was refluxed for 4 hr. with dry methanol saturated with dry HCl. Excess reagent was removed *in vacuo*, and the residue dissolved in water, adjusted to pH 7, and made up to 25 ml. The activity for *Lb. casei* was almost completely destroyed by the treatment and attempts to regenerate it by hydrolysis were unsuccessful. A summary of the above results is given in Table 7.

Table 6. *Effect of acetylation of Al_2O_3 -eluate fraction on acid production by Streptococcus faecalis R*

Al_2O_3 -eluate fraction (ml./ 10 ml. medium)	Acid production (ml. 0.05N-acid)		Acetylated Al_2O_3 - eluate fraction (ml./10 ml. medium)	Acid production (ml. 0.05N-acid)	
	16 hr. incubation	72 hr. incubation		16 hr. incubation	72 hr. incubation
0.02	4.1	9.75	0.02	Nil	9.3
0.01	3.9	9.7	0.01	0.4	8.1
0.003	2.9	8.45	0.003	1.5	6.5
0.001	2.2	7.2	0.001	0.9	4.85
0.0003	1.1	5.5	0.0003	0.25	3.4
0.0001	0.4	4.0	0.0001	Nil	2.65
Basal medium	Nil	2.7	—	—	—

Table 7. *Effect of various reagents on the activity of fractions from the baryta-soluble material*

Reagent	Al_2O_3 filtrate <i>Lb. casei</i>	Al_2O_3 eluate	
		<i>Lb. casei</i>	<i>Strep. faecalis</i> R
HNO_3	Full activity	Partial loss	100% loss
Acetic anhydride	100% loss	? little loss	No loss
Ninhydrin	Indeterminable	Indeterminable	25% loss
Methanolic HCl	100% loss	100% loss	—

2N-NaOH and 0.2 ml. acetic anhydride were made at 1 min. intervals. After 10 min. at room temperature the mixture was acidified with H_2SO_4 to pH 6.8 and diluted to a final volume of 15 ml. The Al_2O_3 -filtrate fraction was completely inactivated by this treatment. The eluate fraction, tested against *Strep. faecalis* R, was apparently inactive if the cultures were examined after 16 hr. incubation, but the organism could slowly utilize the acetylated material which, at 72 hr. incubation, was almost as active as the original material (see Table 6). By an oversight, the acetylated eluate fraction was not tested with *Lb. casei*, but crude preparations so treated have not shown any great loss of activity, and since growth is continued for 72 hr. with this organism, it is likely that no great inactivation would have been demonstrated.

(c) *Ninhydrin*. 10 ml. of one third saturated KH_2PO_4 solution were added to 0.5 ml. of each fraction and 120 mg. ninhydrin. After boiling for 15 min. sufficient alanine was added to react with excess ninhydrin. The mixture was again boiled, extracted with chloroform to remove the coloured products, the insoluble material centrifuged off and the aqueous solution adjusted to pH 6.8 and diluted to 25 ml. 0.5 ml. of the same fraction in 10 ml. of the phosphate solution was extracted with chloroform and used as a control. The Al_2O_3 filtrate showed 80% inactivation with ninhydrin, no active material being extracted from the control by chloroform. The eluate fraction tested against *Strep. faecalis* R showed 25% inactivation, no active

Fractionation of the baryta-insoluble material

At pH 4 a large proportion of the active material could be extracted from aqueous solution by chloroform. Some active material was extracted at pH 7 which appeared to be similar to that extracted at pH 4. Portions of this material, prior to chloroform extraction, were treated with the same reagents as the baryta-soluble fractions, the results being shown in Table 8.

Table 8. *Effect of various reagents on baryta-insoluble material*

Reagent	Effect on activity	
	<i>Lb. casei</i>	<i>Strep. faecalis</i> R
HNO_3	100% loss	100% loss
Acetic anhydride	100% loss	100% loss
Ninhydrin	Full activity	Full activity
Methanolic HCl	100% loss	100% loss

Response of *Lactobacillus casei* to baryta-soluble fraction, pteroylglutamic acid and 'folic acid'

The three materials were tested separately, and PGA and the baryta-soluble fraction were also examined for synergism. Typical results are shown

in Table 9. Neither PGA nor folic acid tested on our medium show an activity comparable with that reported by Angier *et al.* (1945), and Mitchell *et al.* (1941), since PGA is claimed to be 50% active in 1×10^{-6} $\mu\text{g./ml.}$ medium. This fact and the higher titrations obtained with the baryta-soluble fraction demonstrate the existence of unidentified factors essential for the growth of *Lb. casei* on purified media. There is some evidence for synergism between PGA and the baryta-soluble fraction, but this effect was not expected to be very pronounced as the latter contained traces of PGA.

Table 9. *Effect of baryta-soluble fraction, pteroyl-glutamic acid and folic acid on acid production by Lactobacillus casei*

PGA (1×10^{-3} $\mu\text{g./}$ ml. medium)	Folic acid ($\mu\text{g./ml.}$ medium)	Baryta- soluble fraction (1×10^{-3} ml./ ml. medium)	Acid production after 72 hr. incubation (ml. 0.1 N- acid/20 ml. medium)
—	—	—	Nil
—	—	2.50	4.9
—	—	0.025	2.0
5.0	—	—	2.9
0.50	—	—	2.0
0.05	—	—	0.3
0.005	—	—	0.4
—	0.6	—	0.7
—	0.1	—	0.6
—	0.01	—	0.2
5.0	—	2.50	6.6
5.0	—	0.025	2.4
0.05	—	2.50	5.9
0.05	—	0.025	2.8

DISCUSSION

There are considerable discrepancies in the claims made as to the growth requirements of *Lb. casei*. The variations in basal media used in different laboratories may account for many of these differences. It is generally recognized that such materials as casein and peptone contain quantities of vitamins sufficient to affect micro-organisms, and no standard procedure is adopted for the removal of these contaminants. In particular, emphasis may be placed on the methods of preparation of casein digests. In these laboratories, digestion is carried out with hydrochloric acid followed by precipitation with litharge to pH 4 and then with barium sulphide. It can be demonstrated that most of the *Lb. casei*-active material is removed from liver concentrates in lead precipitates at this pH. Several groups of American workers, however, prepare a digest with sulphuric acid followed by neutralization with baryta, a process which does not remove appreciable *Lb. casei* activity from liver fractions.

Using our casein digest as the medium for the experiments, the growth factors present in liver extract, additional to the *C. diphtheriae* factor and the chloroform-soluble factors of Barton-Wright *et*

al. (1944), have been separated into the following components: (1) a factor or factors insoluble in saturated baryta; and (2) at least three factors soluble in saturated baryta and in silver nitrate at pH 1.0—a filtrate fraction which is not adsorbed on alumina at pH 3, and two fractions which are adsorbed, one of which is eluted with 20% (v/v) methanol or water and the other with 0.5% (w/v) ammonia. These three components have properties dissimilar to both PGA and the folic acid of Mitchell *et al.* (1941). The greatest concentration of folic acid-like material was found in the silver salts insoluble at pH 1 (from the baryta-soluble material) which contained none of the components discussed above.

The differential action of ninhydrin and nitrous acid, and of esterification and acetylation upon the activity of the 2% (w/v) ammonia-eluate material for *Strep. faecalis* R and *Lb. casei*, confirms that there are two components present in this material. The fact that the above chemical treatment affects the filtrate fraction and the baryta-insoluble fraction dissimilarly to one another and to the above ammonia-eluate material is evidence that we have four separate and distinct factors.

The possibility that folic acid-like compounds might be split during fractionation from more complex components, which themselves show no activity immediately on separation, complicated studies such as these, and, indeed, there is some evidence to support such a view. This does not, however, invalidate the claim that several active and synergistic compounds are present in the starting material. The difficulty of obtaining adequate supplies of material, which had caused work to cease on these fractions for over two years, makes the publication of these results desirable.

SUMMARY

1. The presence is demonstrated of further unidentified growth factors for *Lactobacillus casei*, present in liver.
2. The crude material contains a factor or factors insoluble in baryta, and at least three factors soluble in saturated baryta.
3. The baryta-soluble factors may be further separated by treatment with alumina; two components are adsorbed and may be separated by fractional elution, and one is present in the filtrate.
4. The effect of a number of chemical reagents on the activity of these components has been studied and shown to distinguish them from the pteroyl-glutamic acid group of factors and from folic acid.

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Lipids of Normal Brain

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In most of the early studies on the distribution of the lipids in the brain large samples of tissue were used, and the various fractions isolated, more or less quantitatively, by the classical differential solubility techniques. Extensive reviews of this work have been given by Page (1937) and Bloor (1943).

To investigate experimentally degenerative conditions in the nervous system, methods must be available which require only small samples of tissue. In recent years many such micro methods have been reported. In the experiments described in this paper the micro methods at present in use in this laboratory have been applied to normal brain tissue.

METHODS

Reagents. All reagents used were of A.R. grade, and all solvents were freshly redistilled on the day of use.

Extraction of lipids

The brain was dissected out as soon as possible after the animal had been killed. A small sample of tissue (1–2 g.) was rapidly weighed, and extracted by a modification of the

method of Bloor (1929). It was ground in a mortar with a small quantity of acid-washed, ether-extracted sand, and successively extracted with 50 ml. ethanol (2 portions), 50 ml. 1:1 ethanol-ether mixture (8 portions) and 50 ml. ether (1 portion). Each extract was filtered in turn through an ether-extracted Whatman no. 2 filter paper into a 500 ml. volumetric flask, and the contents of the flask finally made up to volume with ether. By this procedure, in which the proportion of solvent to tissue was greater than that usually adopted, the lipids were completely extracted from brain tissue. To verify this, the insoluble residue was extracted for 6 hr. with ether in a continuous extraction apparatus, followed by a further 6 hr. with methanol. The combined extract was found to contain neither cerebroside nor cholesterol and only negligible quantities of phospholipid. When the same procedure was applied to liver tissue, however, it was found that a considerable phospholipid fraction was not extracted.

Three separate 100 ml. samples of the ether-ethanol extract were placed in a 60° water bath, and evaporated just to dryness under reduced pressure and in an atmosphere of N₂. The residue was extracted successively with eight 2 ml. portions of light petroleum (b.p. 40–60°) and the light petroleum evaporated down to 1 ml. on a 60° water bath. The phospholipids were precipitated by adding 30 ml.