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A preliminary study in this laboratory on the growth of 34 species and strains of lactobacilli showed that Lactobacillus gayoni 8289 alone would not respond without the presence of a hitherto unrecognized substance found in natural products. The existence of this substance, named the Gayoni factor, was detected by means of microbiological assay as used for measurement of B vitamins. A satisfactory growth medium for L. gayoni 8289 was devised which made possible a measurement of growth resulting from graded additions of the Gayoni factor present in liver extracts.

A test of 71 organic compounds and growth factor concentrates showed that none could replace the Gayoni factor for L. gayoni growth. These compounds included 26 amino acids plus the tripeptide glutathione, 21 pyrimidine derivatives, and several growth factors obtained from other laboratories.

Partial characterization of the substance indicated that it is present in natural products "bound" to protein and can best be released by enzyme hydrolysis. The free form is necessary for L. gayoni growth. In addition, the factor displays properties indicating both acid and basic groups in the molecule.

A 40-fold concentration of the material was made by use of its adsorption from acid solution on Norite and Lloyd's reagent and its elution from these agents with hot 28% NH_4OH and 0.5 N NaOH. This scheme appeared to remove inhibitors to L. gayoni growth and thus made possible a greater sensitivity of the bacterium to the factor.

Initial classification of the substance is attempted from the experimental data obtained. In addition, a comparison shows it to be distinct from growth factors recently reported in the literature and as yet unidentified. Work is continuing on concentration of the material in an attempt to identify it with, or distinguish it from known compounds and biological growth factors.

A GROWTH FACTOR FOR *LACTOBACILLUS GAYONI* 8289

by

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CONTENTS

TABLES

TABLES (cont.)

FIGURES

A GROWTH FACTOR FOR *LACTOBACILLUS GAYONI* 8289

INTRODUCTION

In a preliminary study of the growth response of 34 species and strains of lactobacilli, it was found (8) that *Lactobacillus gayoni* 8289 alone failed completely to respond. Upon addition of yeast or liver extracts, *L. gayoni* 8289 grew abundantly. It appeared evident that these natural materials supplied a substance (or substances) required by this bacterium which was not found in any of the growth media used. This thesis reports experiments which demonstrate the non-identity of this substance with known growth promoting agents, together with methods used in preliminary characterization and concentration of the factor. For convenience, it is referred to throughout as the Gayoni factor.

EXPERIMENTAL

Organism

Lactobacillus gayoni 8289, American Type Culture Collection, is kept on stab cultures in tubes containing 1 percent glucose, 1 percent Difco yeast extract, and 2 percent agar. Stabs are incubated at 37° C. until heavy growth is obtained (24 to 36 hours) and then kept in a refrigerator. Stock cultures are transferred every month, while stabs for daily use are prepared weekly,

Cultures for inoculum are made by transferring cells from the stab to a liquid medium suitable for growth. Such media are prepared by adding 5 mg. of a liver extract to 5 ml. of the basal medium (described later) and diluting to 10 ml. with water. The culture is then grown for 24 hours at 37°.

Basal Medium

The medium used in discovery of the Gaynni factor was essentially that of Snell and Wright (21) modified to contain 40 grams glucose and 40 grams sodium acetate per liter (23). With this medium, fair growth was obtained in response to graded additions of liver or yeast extracts. However, the best response to the Gayoni factor was observed in the presence of additional yeast and

peptone extracts which had been freed of the factor by adsorption on Norite or Lloyd's reagent. The final basal medium used throughout is given in Table 1.

TABLE 1

Basal Medium for L. Gayoni

Casein, vitamin-free, H_2SO_4 hydrolyzed		10 gms.
Peptone, Norite ads. 2 times at pH 1.5		5 gms.
Yeast, Lloyd's ads. 2 times at pH 1		2 gms.
Sodium acetate		40 gms.
Glucose		40 gms.
Cystine		200 mg.
Tryptophane		200 mg.
Adenine, Guanine, Uracil	each	20 mg.
Riboflavin		400 γ
Thiamin, Nicotinic acid, Calcium pantothenate, Pyridoxin, p-Amino benzoic acid	each	200 γ
Biotin		0.8 γ
Salt solution A*		10 ml.
Salt solution B#		10 ml.

Distilled water to 1 liter
pH to 6.7

*Salts A: 25 gms. each of K_2HPO_4 and KH_2PO_4 per 250 ml. H_2O

#Salts B: 10 gms. $MgSO_4.7H_2O$, 0.5 gm. each of NaCl, $FeSO_4.7H_2O$, and $MnSO_4.4H_2O$ per 250 ml. H_2O

Lloyd's treated yeast is prepared by dissolving 16 grams of dried yeast extract (Difco) in 400 ml. of distilled water, adjusting the pH to 1 with concentrated HCl, and adding 16 grams of Lloyd's reagent. The mixture is shaken mechanically for 1 hour. The Lloyd's reagent is then filtered off, another 16 grams are

added, and the shaking repeated. The reagent is again filtered off and the pH is returned to 6.7. The solution is steamed, filtered, and kept under toluene in the refrigerator.

Norite treated peptone is made by adding 3 grams Norite to 20 grams peptone in 400 ml. distilled water adjusted to pH 1.5 with concentrated HCl. The material is agitated 1 hour, filtered, and the process is repeated with a fresh 3 grams of the charcoal. The final solution is adjusted to pH 6.7 before using. It is kept under toluene in the refrigerator.

Assay Procedure

Throughout this study, detection and measurement of the Gayoni factor was made by microbiological assay as used for quantitative determination of B vitamins. In general, the method consists of measuring bacterial response to varying amounts of the vitamin. Cultures of L. gayoni are grown on a liquid medium which contains sufficient amounts of all components required for growth with the exception of the Gayoni factor. In the absence of the factor, little detectable growth is measured. With graded amounts, a corresponding increase in growth is noted up to the maximum required. Samples are arranged in test tubes containing 5 ml. of a water solution of the sample and 5 ml. of the

liquid medium adjusted to pH 6.7 (suitable for growth). A standard curve, which is included with each series of assays, contains 0, 0.25, 0.5, 0.5, 1.0, 1.0, 1.5, and 2.0 mg. of liver powder per tube (10 ml.). Samples to be assayed are usually tested at varying levels equivalent to 0.2 to 0.8 mg. liver powder.

Tubes are cotton-plugged and sterilized by autoclaving 15 minutes at 15 pounds pressure. Each tube is then cooled and inoculated aseptically with 1 drop of a dilute inoculum prepared by adding 0.5 to 1.0 ml. of the 24-hour culture (previously described) to 10 ml. of sterile 0.9% NaCl solution. The tubes are incubated at 37°C. in a water bath. Growth is measured by turbidimetric readings after 36 to 45 hours by use of a suitable turbidimeter, or by titration of the lactic acid produced with 0.1 N NaOH after 68 to 72 hours. Comparative activities of samples are obtained by reference to the standard curve. Typical standard curves are illustrated in Figures 1 and 2.

Sources of the Factor

The most satisfactory source of the Gayoni factor was found to be the liver powder concentrates supplied by the Lederle Laboratories or by the Wilson Laboratories.*

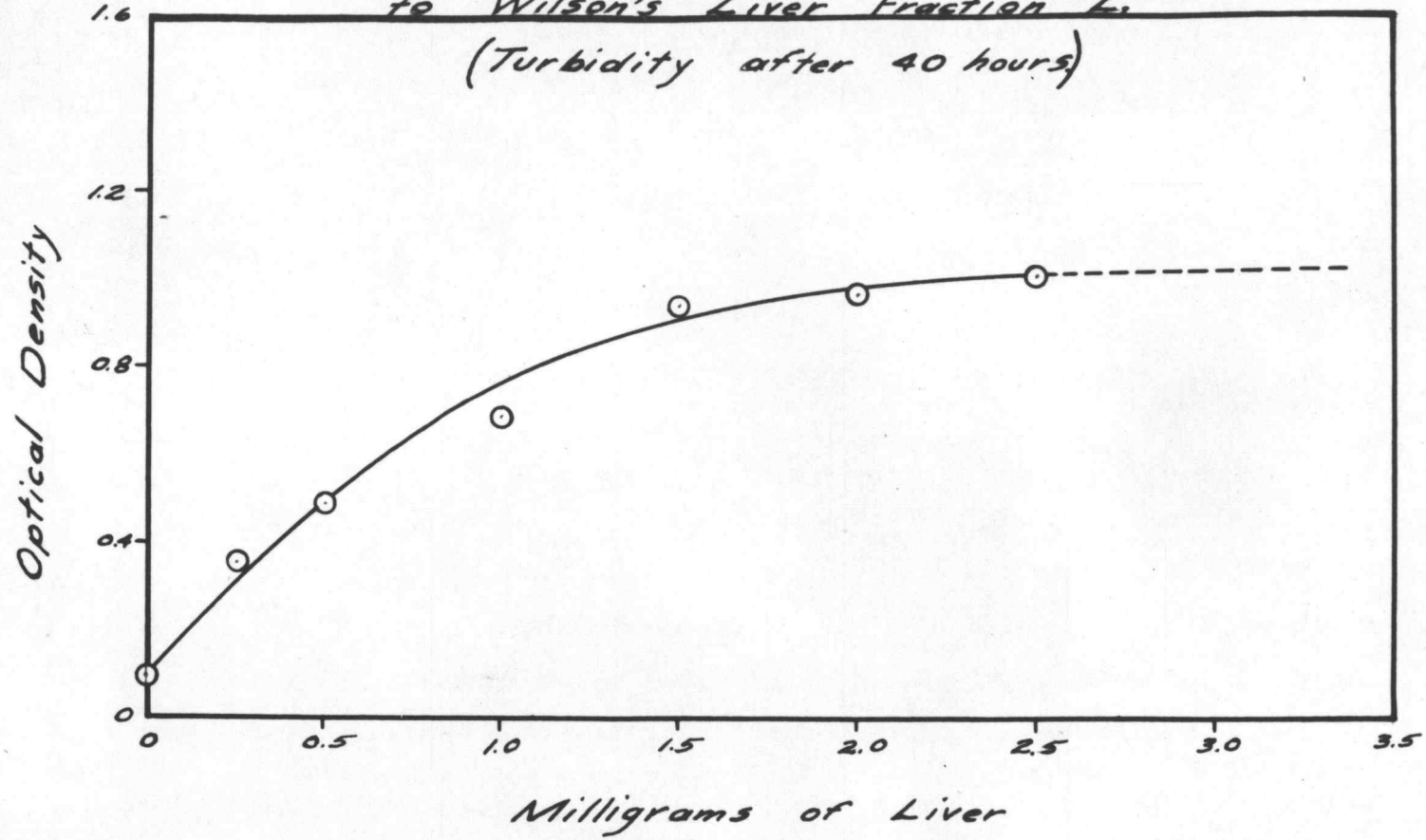
*Lederle liver concentrate 1:15.6 is a hot water-soluble extract of fresh liver evaporated to dryness.
Wilson liver fraction L is the 70% ethyl alcohol-insoluble fraction of whole liver solution which is redissolved in hot water and evaporated to dryness.

Figure 1

Response of L. Gayoni 8289

to Wilson's Liver Fraction L

(Turbidity after 40 hours)



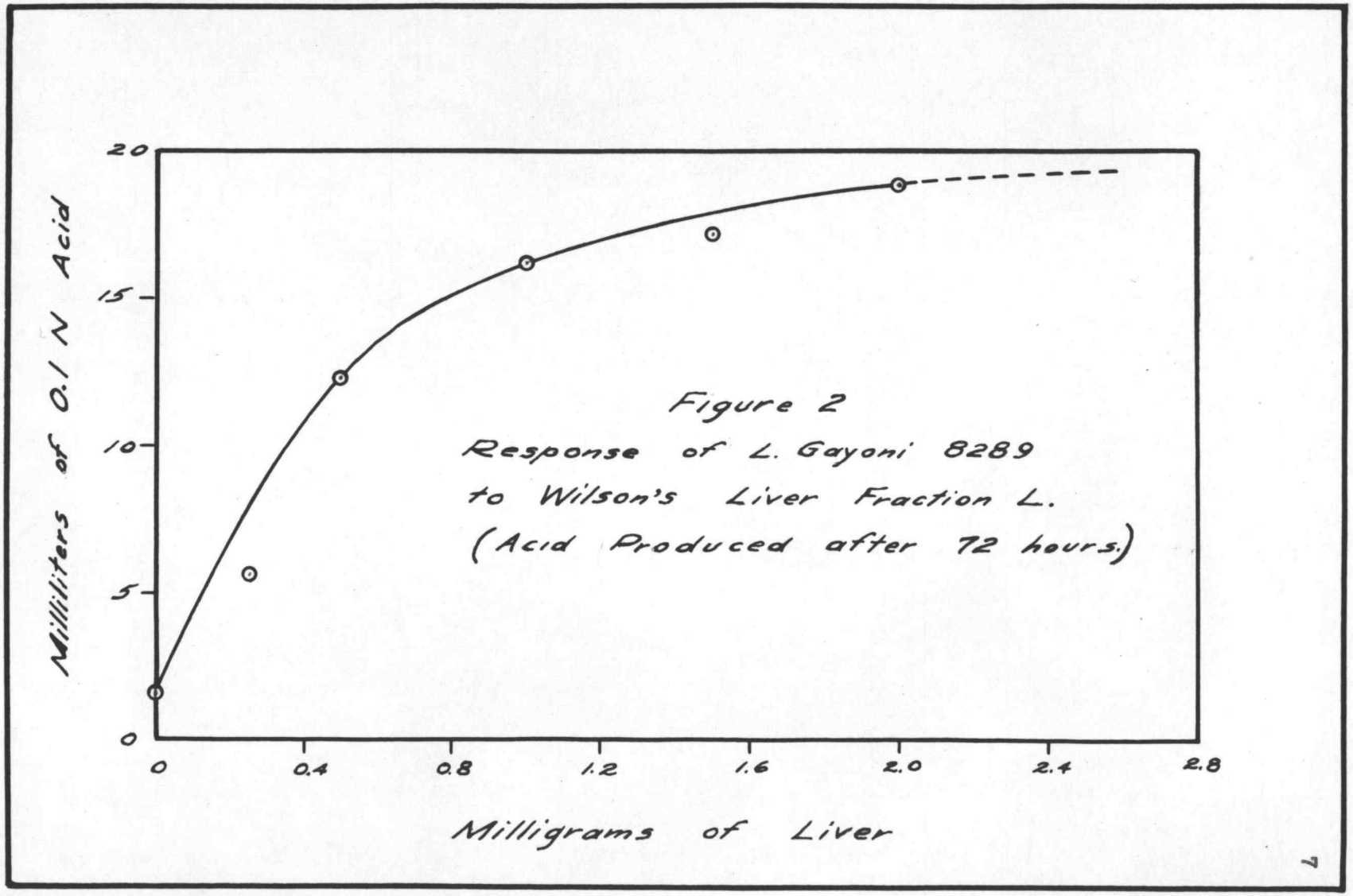


Figure 2
Response of L. Gayoni 8289
to Wilson's Liver Fraction L.
(Acid Produced after 72 hours.)

Since these powders are water-soluble, they can easily be used as standard sources. Consequently, all experiments were carried out using these as starting materials.

The Gayoni factor seems to occur in all natural products, as do the B vitamins. Liver and yeast are the richest sources, followed by various other materials as shown in Table 2.

TABLE 2

Sources of the Gayoni Factor

Source	Method of Preparation	Relative Activity
Liver powder (Lederle or Wilson L)	Water solution	1
Beef liver (fresh)	Papain & takadiastase	0.15
Pork liver (fresh)	Papain & takadiastase	0.14
Pork liver (fresh)	Water hydrolyzed	0.017
Pork liver (fresh)	0.1 N H_2SO_4 hyd.	0.033
Hamburger	Papain & takadiastase	0.035
Peptone	Water solution	0.20
Yeast extract (Difco)	Water solution	0.83
Vit.-free casein	1N HCl hyd., 1 hour	0.04-0.12
Vit.-free casein	Trypsin hyd., 2 days	0.06-0.2
Dehyd. white potato	Acid - Papain & takadiast.	0.1-0.15
Dehyd. cabbage	Acid - Papain & takadiast.	0.23-0.33
Dehyd. tomato	Acid - Papain & takadiast.	0.14-0.22
Tomato juice	0.1 N acid hydrolyzed	0.042

Vegetables are relatively rich sources of this factor; cabbage contains almost half as much as fresh liver when calculated on the dry weight basis.

The mode of extraction of foods is seen to have a marked effect upon the yield of the factor. Incubation of fresh liver with papain and takadiastase at pH 4.5 results

in nearly a ten-fold increase in activity over that obtained by hot water extraction.

Response of L. Gayoni to Known Compounds

Before intensive efforts were made to concentrate the Gayoni factor, a large number of potential growth factors were tested in the medium for L. gayoni. These are listed in Table 3. In preliminary studies, addition of inositol (5 mg./l.), choline (2 mg./l.), asparagine (1 gm./l.), and a folic acid concentrate (5 γ/l.) together with the 7 other B vitamins of the medium did not stimulate growth. Similar negative results were obtained when concentrations of these eleven vitamins were trebled. The other substances tested which showed no activity were 26 amino acids plus the tripeptide glutathione, 21 pyrimidine derivatives, numerous pyridine carboxylates, including Coenzymes I and II, and several concentrates of growth factors from other laboratories which have not been fully characterized.

Characterization Studies

Reaction to Various Treatments. Hydrolysis. Although the Gayoni factor in liver powder is stable to digestion with takadiastase and papain at pH 4.5, it is rather easily destroyed by heating with strong acid or alkali. Destruction is more rapid in the presence of alkali, being about

TABLE 3

Compounds Tested For Gayoni Activity

Amino Acids

Alanine
a-Amino-butyric acid
a-Amino-i-butyric acid
a-Amino-caproic acid
a-Amino-caprylic acid
a-Amino-a-methyl butyric acid
a-Amino-valeric acid
d-Amino-n-valeric acid
Arginine
Asparagine
Aspartic acid
e-Benzoylamino caproic acid
Glutamic acid
Glutamine
Glutathione
Histidine
Isoleucine
Leucine
Lysine
Methionine
Ornithine
Phenyl alanine
Phenyl glycine
Proline
Threonine
Tyrosine
Valine

Pyrimidines

Nucleic acid
Nucleic acid, base hyd. 1 hr.
Nucleic acid, base hyd. 3½ hrs.
Nucleic acid, acid hyd. 10% 2 hrs.
Nucleic acid, acid hyd. 25% 5 hrs.
Hypoxanthine
Xanthine
2-Amino-4-diethylamino pyrimidine
2-Amino-4-hydroxy-5-methyl pyrimidine
4-Amino-5,6-dimethyl pyrimidine
2-Amino-pyrimidine
6-Amino-pyrimidine
2,4-Diamino pyrimidine
2,5-Dimethyl-6-amino pyrimidine
5-Ethyl-6-amino pyrimidine
2-Methyl-6-amino pyrimidine
4-Methyl-6-amino pyrimidine
2-Methyl-5-ethoxy-methyl-6-amino pyrimidine
4-Methyl uracil
Isocytosine
Xanthopterin

Miscellaneous Compounds

Arecaidine hydrobromide
Arecoline hydrobromide
Choline
Inositol
N-Methyl nicotinamide chloride
Nicotinamide
Nicotinuric acid
Pimelic acid
Pyridoxal
Pyridoxamine
Trigonelline

Natural Products

Coenzyme I
Coenzyme II
Coenzyme I plus Coenzyme II
Coenzyme I, acid hyd.
Coenzyme I, base hyd.
Coenzyme II, acid hyd.
Coenzyme II, base hyd.
Folic acid
Folic acid, acid hyd.
Folic acid, base hyd.
L.casei factor (13)
Orange peel factor (29)

two-thirds complete after steaming for 10 minutes with 0.1 N NaOH. Comparable treatment with HCl, on the other hand, results in no loss of activity. These and other hydrolysis figures are shown in Table 4.

TABLE 4

Destruction of the Gayoni Factor

Reagent	Treatment	% Activity Remaining
Enzymes	Acetate buffer 24 hrs. 37°	100
0.1 N HCl	Steam 10 minutes	100
1 N HCl	Autoclave 15 minutes	100
6 N H_2SO_4	Autoclave 15 minutes	3
8 N H_2SO_4	Reflux 24 hours	0
8 N HCl	Reflux 24 hours	0
NaOH, pH 9.5	Autoclave 15 minutes	100
0.1 N NaOH	Steam 10 minutes	65
0.5 N NaOH	Autoclave 30 minutes	50
1 N NaOH	Stand in cold for 24 hours	50

Oxidation. Oxidation by cold neutral $KMnO_4$ or 6% H_2O_2 destroys approximately 20% of the activity. The permanganate was added dropwise until excess was noted and the solution was filtered. For the peroxide treatment, 1 ml. of the 6% reagent was added to 5 ml. of liver solution (20mg./ml.) and allowed to stand at room temperature for 30 minutes. Excess H_2O_2 was steamed out.

Bromination. Excess bromine water was added dropwise to the liver solution. The solution was then boiled and filtered free of insoluble material. Only 40% of the

original activity remained.

Nitrous Acid Treatment. Approximately 70% of the total activity was destroyed by reaction with nitrous acid. 0.8 grams $NaNO_2$ and 1 ml. glacial acetic acid were added to the liver solution and allowed to stand at room temperature for 24 hours. The solution was then carefully boiled to dryness twice, redissolved, and tested.

Esterification. Attempts to esterify the Gayoni factor were made by treating the Lederle liver powder with 1% fuming H_2SO_4 in methanol at 37° C. for 30 minutes. Precipitated material was filtered, the pH adjusted, additional precipitate filtered, and the samples steamed. 37% of the activity was recovered in the filtrate, 13% in the first residue, and 20% in the second residue, or a total of 70% from the treatment. Attempts to recover the remaining 30% of the activity by hydrolysis with KOH failed due to apparent poisoning of the organism from an undiscovered source.

In an effort to dissolve the factor in an organic solvent, HCl gas was passed into dry methanol to concentrations of 0.3 N and 0.45 N. Treatment of the dry liver powder with these solutions showed 32% activity dissolved and 10% insoluble in the former, and 20% dissolved and 16% insoluble in the latter. The disappearance of the remaining 58 to 64% suggests that esterification may have

occurred. No recovery by hydrolysis was tried.

Dialysis. 24-hour dialysis of the factor removed about 60% of the activity. A liver solution of 20 mg./ml. in water was placed in a cellophane bag in a beaker and cold tap water was run into the beaker for the 24-hour period.

Heat. Several samples which were heated excessively during steam drying underwent considerable loss of activity. In most such cases, the liver was apparently denatured, for much of the solid material would not redissolve in water. With careful drying, however, complete re-solution was possible, and total activity remained.

The results of the special treatments just described are summarized in Table 5.

TABLE 5

Special Treatment of the Gayoni Factor

Reagent	Treatment	% Activity Remaining
Potassium permangante	Excess dropwise, boiled	80
6% H_2O_2	1ml./5ml. 30 min.,steamed	80
Bromine water	Excess dropwise, boiled	38
Nitrous acid	$NaNO_2$-HOAc, 24 hrs.,boiled	29
Fum. H_2SO_4 in CH_3OH	1% acid 30 min., 37°	70*
0.3 N HCl in CH_3OH	20 mg./ml. of dry powder	42#
0.45 N HCl in CH_3OH	10 mg./ml. of dry powder	36#
Dialysis	Cellophane, run. H_2O 24 hrs.	40
Heat	Dry sample with steam	100
Heat	Char sample	0

*Total of three portions
#Total of two portions

Solubility in Various Organic Solvents. Although the Gayoni factor is readily soluble in water, an attempt to find another solvent by treatment with common organic liquids proved generally unsuccessful. These solubilities are listed in Table 6.

TABLE 6

Solubility of the Gayoni Factor

Solvent	% Volume	Times Treated	% Activity Insoluble
Ether	50	3	85
i-Butyl alcohol	50	3	65
n-Butyl alcohol	Continuous 24 hrs. (Dakin) (9)		100
i-Amyl alcohol	Continuous 24 hrs. (Dakin) (9)		100
n-Amyl alcohol	50	4	96
Ethyl alcohol	95	2	65
Acetic acid	100	1	50
Acetone	100	1	90

For the liquids immiscible with water, the indicated volume was shaken with the liver solution. The remaining water solutions were steamed to eliminate the organic solvent. The n-butyl and i-amyl alcohol extractions were made by continuous treatment of the liver solution for 24 hours by means of the Dakin extractor (9). The water-miscible solvents were stirred with dry liver powder, filtered, the liquid steamed away, and the remaining material dissolved in water. Solubility in water-solvent (miscible) mixtures is given in Table 17.

Concentration Studies

Adsorption and Elution Experiments. Norite. Norite adsorptions were tried under a variety of conditions, but most satisfactory adsorption-elution results were obtained by using 20% Norite at pH 3. Two treatments with this amount, shaken 1 hour each time, gave greatest adsorption. One treatment with 40% Norite resulted in comparable adsorption but elution after such a process was not as satisfactory. The concentration of the liver was generally 20 mg./ml. Table 7 summarizes these results.

TABLE 7

Adsorption of the Gayoni Factor on Norite

pH	% Wt.	Time Shaken	No. Times Ads.	% Activity Ads.
1.0	15	15 min.	1	61
10	15	15 min.	1	34
11	15	15 min.	1	55
1.5	20	15 min.	1	48
4.0	20	15 min.	1	44
7.0	20	15 min.	1	43
11	20	15 min.	1	46
1.5	20	15 min.	2	70
1.5	30	15 min.	1	55
1.5	40	15 min.	1	82
1.0	20	1 hour	2	82
1.5	20	1 hour	2	94
2.0	20	1 hour	2	90
3.0	20	1 hour	2	96
4.0	20	1 hour	2	71

Quantitative elution of the activity from Norite may be obtained by exhaustive treatment with hot 28% NH_4OH.

Less thorough elution fails to release near-comparable amounts, although somewhat less solid material is freed by use of such compounds as aniline and pyridine. The concentrated NH_4OH is driven off by adding a little NaOH (to free NH_3 from NH_4Cl) and evaporating the solution to a small volume. A complete list of elution results is given in Table 8.

TABLE 8

Elution of the Gayoni Factor from Norite

Material	Treatment	Activity Eluted: % of Total Ads.
HCl at pH 3	Boiled with cake	0
HCl at pH 1	Boiled with cake	0
0.1 N Acetic acid	Boiled with cake	0
0.1 N HOAc in CH_3OH	Boiled with cake	0
Glacial acetic acid	Boiled with cake	12
5% Aniline	Boiled with cake	57
10% Pyridine	Boiled with cake	25-50
100% Aniline	Boiled with cake	18
100% Pyridine	Boiled with cake	25
50% Methyl alcohol	Boiled with cake	40-70
$NaHCO_3$ and pH 11	Boiled with cake	5
0.1 N NaOH	Boiled with cake	16
10% NH_4OH	Boiled with cake	14
20% NH_4OH	Boiled with cake	33
6% Amyl alcohol	Two portions hot	33
15% $NaHCO_3$	Shake 4 times in cold	55
5% NH_4OH	Shake 4 times in cold	65
10% NH_4OH	Shake 4 times in cold	60
28% NH_4OH	Exhaustive in cold	100
28% NH_4OH	Exhaustive boiled	100
28% NH_4OH	Exh. hot, each of 2 cakes separately	69) 42) 111

These values are based on the amount of activity freed compared to the amount adsorbed.

Lloyd's Reagent. Earlier experiments indicated adsorption of the factor on Lloyd's reagent in the acid range. A variety of conditions was tested using equal weight of the reagent in 20 mg./ml. liver solutions. Best adsorption occurred at pH 1 to 1.5 and was nearly as complete after one treatment for 15 minutes as after two treatments for one hour each time. Alkali was again required to remove the factor, the best results being obtained by shaking the Lloyd's reagent for a few minutes with cold 0.3 or 0.5 N sodium hydroxide. Adsorptions and elutions by use of Lloyd's reagent are given in Tables 9 and 10.

TABLE 9

Adsorption of the Gayoni Factor on Lloyd's Reagent

pH	% Wt.	Time Shaken	No. Times Ads	% Activity Ads
1	100	15 min.	1	93
2	100	15 min.	1	60
3	100	15 min.	1	75
5.5	100	15 min.	1	63
1	100	2 hours	2	90
3	100	2 hours	2	75
5.5	100	2 hours	2	50

TABLE 10

Elution of the Gayoni Factor from Lloyd's Reagent

Material	Treatment	Activity Eluted: % of Total Ads.
100 % Aniline	Boiled with cake	6
100 % Pyridine	Boiled with cake	21
0.1 N Na_2CO_3	Boiled with cake	10
20 % NH_4OH	Boiled with cake	45
28 % NH_4OH	Exhaustive hot	63
0.1 N NaOH	Boiled with cake	48
0.3 N NaOH	Shaken in the cold	88
0.5 N NaOH	Exh. shaking cold	94

Other adsorbents. A variety of other adsorbing agents was tested for ability to separate the factor from whole liver powder. Dry aluminum oxide powder in a column adsorbed 95% activity at pH 3 and gave up 65 to 70% with either cold or boiling NH_4OH (20%). Three samples of fullers earths from different sources adsorbed 85% of the factor at pH 1 and gave up about 50% with cold 0.3 N NaOH. Norite A and Darco G-60 charcoals adsorbed 95% at pH 3 and slightly less at pH 2 under conditions similar to Norite adsorption. They released about 70% upon exhaustive elution with hot 28% NH_4OH. Other adsorbents showing less efficiency of separation at a variety of pH values were Decalso, Florisil, the Amberlites IR-1 and IR-4, and Anex. The Al_2O_3 adsorption-elution results are given in Tables 11 and 12, while other adsorption data are shown in Tables 13 and 14.

TABLE 11

Adsorption of the Gayoni Factor on Aluminum Oxide

pH	Treatment	% Activity Ads.
1	Run once through column	0
3	Run once through column	95
5	Run once through column	90
5.5	Run once through column	90
7	Run once through column	35
9	Run once through column	28
11	Run once through column	50
1	200% shaken 15 min.	32
3	200% shaken 15 min.	26

TABLE 12

Elution of the Gayoni Factor from Aluminum Oxide

Material	Treatment	Activity Eluted: % of Total Ads.
5% Aniline	Column in cold	4
10% Pyridine	Column in cold	20
100% Aniline	Column in cold	5
100% Pyridine	Column in cold	4
HCl at pH 1	Column in cold	7
Glacial acetic acid	Column in cold	8
10% NH_4OH	Column in cold	0
6 N NH_4OH	Column in cold	35-70
20% NH_4OH	Column in cold	25
100% Aniline	Boiled in two portions	25
100% Pyridine	Boiled in two portions	30
20% NH_4OH	Boiled in two portions	65

TABLE 13

Adsorption of the Gayoni Factor on Other Adsorbents

Material	pH	Treatment	% Activity Ads.
Fuller's earth 1.	1	100% shaken 15 min.	86
Fuller's earth 2.	1	100% shaken 15 min.	85
Fuller's earth 3.	1	100% shaken 15 min.	85
Darco G-60	2	20% 2 times, 1 hr. each	90
Darco G-60	3	20% 2 times, 1 hr. each	96
Norite A	2	20% 2 times, 1 hr. each	87
Norite A	3	20% 2 times, 1 hr. each	95
Decalso	4	Run through column	30
Decalso	4.5	Run through column	30
Decalso	7	Run through column	35
Decalso	3	Run through column twice	0
Decalso	5.5	Run through column twice	0
Florisil	3	Run through column	0
Florisil	5.5	Run through column	0
Anex	3	Run through column	30
Anex	5.5	Run through column	0
Anex	9	Run through column	62
Anex	5	250% shaken 15 min.	22
Anex	8	250% shaken 15 min.	12
Amberlite IR-1	5	200% shaken 15 min.	59
Amberlite IR-1	8	200% shaken 15 min.	50
Amberlite IR-1	5.5	Run through column	0
Amberlite IR-4	5.5	Run through column	0

Precipitation of the Factor with Salts and Acids. A number of inorganic and organic (acetate) salts as well as acids were added to the liver solution, the precipitate thus formed filtered off, and the residue treated for recovery of any precipitated activity. Both the original filtrate and the material recovered from the residue were tested to determine separation. The results obtained with several types of treatment are listed in Tables 15 and 16. In many cases, much difficulty was encountered in clearing

TABLE 14

Elution of the Gayoni Factor from Other Adsorbents

Adsorbent	Eluting Agent	Treatment	Activity Eluted: % of Total Ads.
Fuller's earth 1.	0.3 N NaOH	Shake in cold	56
Fuller's earth 2.	0.3 N NaOH	Shake in cold	40
Fuller's earth 3.	0.3 N NaOH	Shake in cold	52
Darco G-60	28% NH_4OH	Hot exhaustive	66
Norite A	28% NH_4OH	Hot exhaustive	73
Anex	100% Pyridine	Boil in 2 portions	55-100
Anex	20% NH_4OH	Boil in 2 portions	95-100
Amberlite IR-1	100% Aniline	Boil in 2 portions	18
Amberlite IR-1	100% Pyridine	Boil in 2 portions	25
Amberlite IR-1	20% NH_4OH	Boil in 2 portions	30

TABLE 15

Precipitation of the Gayoni Factor with Salts and Acids

Material	Treatment	% Act. in Filt.
$HgNO_3$	Excess pptd. with HCl	3
$Hg(OAc)_2$	Five fractions in HOAc	0
$Pb(OH)OAc$	150%, ppt. excess w. H_2SO_4	10
$Pb(OH)OAc$	Riboflavin-free method (20)	15
$Pb(OH)OAc$	Three fractions	20
$AgNO_3$	Excess pptd. with HCl	20
Phosophotungstic acid	Excess pptd. with $Ba(OH)_2$	5
Flavianic acid	Excess pptd. with $Ba(OH)_2$	33

TABLE 16

Release of the Gayoni Factor from Insoluble Salts

Pptd. Salt	Release Treatment	% Act. Released from Ppt.
$HgNO_3$	Excess HCl, boiled	34
$Hg(OAc)_2$	H_2S (from fractions)	70
$Pb(OH)OAc$	Excess H_2SO_4 stirred	34
$Pb(OH)OAc$	Excess H_2SO_4 boiled	28
$Pb(OH)OAc$	H_2S (from fractions)	85
$AgNO_3$	Excess HCl, boiled	29
Phosphotungstic acid	$Ba(OH)_2$ - H_2SO_4 hot	23
Flavianic acid	$Ba(OH)_2$ - H_2SO_4 hot	10

the test solution of precipitation reagent or reagent used to decompose the precipitate. This was particularly true in decomposition with H_2S where colloidal sulfur was often present in the solutions. Also, adsorption of the factor on some of the precipitates was sometimes noted.

Mercury Salt Precipitation. Either $HgNO_3$ or $Hg(OAc)_2$ precipitated the factor nearly completely. Excess $HgNO_3$ solution was added to the liver solution and filtered. The filtrate was cleared with HCl. The original precipitate was decomposed by boiling with excess HCl. Only 34% of residual activity was recovered in this manner.

Mercuric acetate precipitation was carried out fractionally according to the method described by Warburg (26). A solution of 1 gram of liver powder in 50ml. of water was made acid with 0.1 ml. of 2 N acetic acid and 1 ml. of 10% mercuric acetate was added. The precipitate was filtered off as Fraction 1. An additional 1 ml. of the 10% acetate was added to the filtrate, the precipitate being Fraction 2. The procedure was repeated until five fractions were precipitated. Each fraction was decomposed separately by suspending it in water and passing in an excess of H_2S. About one-third of recoverable activity remained with the precipitated HgS and could be separated from it by boiling in water and filtering it while hot. The final filtrate,

cleared of mercuric ion with H_2S, showed 0 to 2% original activity, while a total of the precipitated fractions, tested separately, gave 50 to 70% recovery.

Lead Salt Precipitation. Basic lead acetate, Pb(OH)OAc, appeared to be nearly as efficient as mercury salts in precipitating the Gayoni factor. When fractionation and H_2S recovery were used comparable activity was likewise obtained. As complete precipitation of activity (80 to 90%) was obtained by simple addition of 150% by weight of the basic salt as was found by the more complete method used in preparing riboflavin-free natural products (20). Hot sulfuric acid recovery amounted to only about 30 to 35%, however. Lead precipitation in three fractions removed 80% of the activity. This procedure was carried out as follows: To 400 mg. of liver powder in 20 ml. water, 1 ml. of 5% Pb(OH)OAc was added, the precipitate filtered, and the process repeated on the filtrate. The residues were tested separately after treatment with H_2S. In each case the PbS precipitate was boiled with water and the filtrate added to the original PbS filtrate.

Silver Salt Precipitation. Excess silver nitrate precipitated about 80% of the factor, but only 30% of this amount was recovered by boiling the residue with excess HCl. The precipitated silver chloride was not tested for

possible retention of activity, however.

Phosphotungstic Acid Precipitation. Excess phosphotungstic acid solution was added to the liver powder solution at pH 5.5. Barium hydroxide and sulfuric acid were used to decompose the precipitate and clear the filtrate. Although tests showed 95% activity precipitated, only 25% of this was recovered from the residue.

Flavianic Acid Precipitation. About two-thirds of the active factor came down as a flavianate. The acid was added in excess and the filtrate cleared with barium hydroxide. Sulfuric acid was used to remove excess barium. Hot $Ba(OH)_2$ decomposed the residue to release 10% of the precipitated activity.

Precipitation of the Factor with Water-miscible Liquids. Water mixtures of either ethyl alcohol or acetone precipitated solid matter and some activity from the liver. However, separation of the factor from inactive solid material was not successful, whether carried out in a single step or by fractionation with progressively greater concentrations of the solvent. (See Table 17.)

Extraction of the Factor with Water-immiscible Liquids. The immiscible solvents of Table 6 were used in trying to fractionate the factor from water solution. The results indicate the failure of such operations when applied to liver powder.

TABLE 17

Precipitation of the Gayoni Factor
with Water-miscible Liquids

Organic Liquid	% Org. Liq.	Act. Sol.	% Act. Recov. from Ppt.
Ethyl alcohol	35	100	6
Ethyl alcohol	50	100	12
Ethyl alcohol	70	14	75
Ethyl alcohol	85	53	20
Acetone	35	53	17
Acetone	70	37	55
Acetone	90	15	15
Fractionations:			
Ethyl alcohol	50	70	0
	50, 85	50	25
	50, 95	15	50
Acetone	55	42	34
	55, 70	18	24
Acetone	60	62	48
	60, 73	36	74
	60, 73, 80	27	83

Application of Concentration Methods to Higher Potency Liver Samples

In order to develop a suitable method of concentrating the Gayoni factor from the original liver powder, the processes just considered were tried in a combination of sequences. Norite has been used in the first step since it is cheap and readily available in the quantities necessary for treatment of large amounts of liver. A number of these schemes gave higher potency solutions, whereas others failed. The final plan used for concentration was devised

from the best of the successful schemes.

Since the Gayoni factor has not been purified, it is necessary to use arbitrary units to designate the weights and concentrations present. The degree of concentration is therefore given here in terms of "potency" (π), which is a measure of the activity of solid material at any step. All samples are compared to Wilson's liver Fraction L, which is assigned a "potency" of 1. The measure of total activity is given in terms of "milligram units." One milligram unit is the amount of activity present in one milligram of Wilson's Liver Fraction L.

Concentration Failures. A number of treatments of Norite eluates gave potency values less than the original. Decalso adsorption through a column was no better than on original liver. Hot 28% NH_4OH elution of Lloyd's reagent also failed. A n-amyl alcohol extract of this eluate had the same potency as the solution treated. Alcohol or acetone precipitations also gave unsatisfactory yields. An exception was the hot 5% acetic acid solution of a 50% acetone residue.

Preparation of Concentrates of the Factor. A number of treatments are outlined in Table 18 which appeared promising for concentration of the Gayoni factor. By combination of some of these the following concentration procedure has been devised as outlined in Figure 3.

FIGURE 3

Scheme for Concentration of the Gayoni Factor

(Total initial activity = 1,000,000 Mgu.)

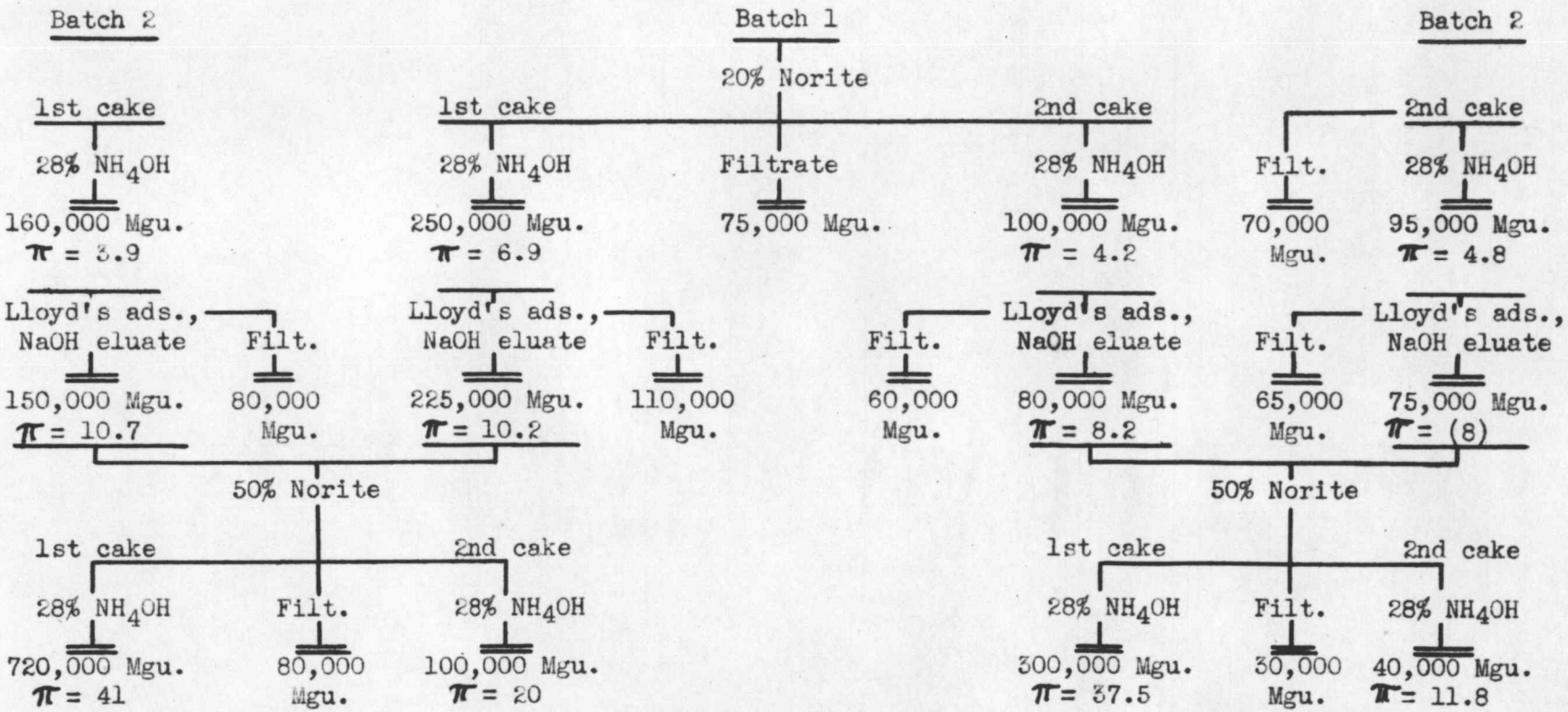


TABLE 18

Methods for Concentrating the Gayoni Factor

Sample No.	Treatment	No. Sample Treated	Yield Mgu.	Potency
1	3 $Hg(OAc)_2$ fract. - H_2S	orig. (1000 Mgu)	460	2.1
2	Lloyd's 15 min. 0.3 N NaOH	1	320	
3	20% Norite, 2 eluates comb.	orig. (1000 Mgu)	900	7.7
4	200% $Hg(OAc)_2$, H_2S, HgS boil	3	800	12
5	150% $Hg(OAc)_2$, H_2S, HgS boil	3	400	16
6	20% Norite, 2 eluates comb.	orig. (1000 Mgu)	500	5.1
7	3 $Hg(OAc)_2$, H_2S, HgS boiled	6	340	12.5
8	3 $Hg(OAc)_2$ fractions, filt.	6	130	6.5
9	20% Norite once, eluate	orig. (1000 Mgu)	330	7.7
10	50% Norite once, eluate	9	170	14.8
11	50% Norite, 2nd eluate	filt. from 10	70	9.3
12	100% Norite once, eluate	9	110	19
13	50% acetone res., hot 5% HOAc	9	50	14.5
14	20% Norite, 2nd eluate	filt. from 9	350	4.0
15	40% Norite once, eluate	14	300	12
16	Lloyd's 15 min. 0.5 N NaOH el.	14	510	74

500 grams of Wilson's Liver Fraction L were dissolved in about one liter of distilled water by careful stirring. The pH was brought to 2.8 to 3.0 with concentrated HCl and the solution was transferred to a 12 liter round-bottom flask. Distilled water was added to make 10 liters of

solution, 100 grams of Norite were added, and the mixture was stirred mechanically for 45 minutes to 1 hour. At the end of this time, the solution was filtered through Celite at about 50 pounds pressure. The Norite was washed while in the filter with 1 to 2 liters of water. The charcoal (with the Celite) was eluted immediately by boiling with 200 to 300 ml. of 28% NH_4OH and filtering. This process was repeated until the filtrate showed very little coloration. The total volume of filtrate varied slightly with each batch treated. In the meantime, the original filtrate from charcoal treatment was adsorbed on a fresh 100 gram batch of Norite and eluted in similar manner. The eluates were steamed separately to small volume to remove ammonia, a little NaOH being added to release it completely. The pH of such solutions after steaming was found to be around 6. The solutions were then made to convenient volume (usually 250 ml.) with distilled water and filtered free of any charcoal still present. Small aliquots were removed for dry weight determinations.

For immediate Lloyd's treatment, the remaining solution was adjusted to pH 1.5 with concentrated HCl. After the total weight of solids was obtained, an equal weight of Lloyd's reagent was added and the mixture was shaken for 45 minutes. The Lloyd's reagent was removed by centrifuging and eluted with approximately 100 ml. of 0.5 N NaOH.

The elution was repeated 3 to 5 times with 50 to 100 ml. portions of NaOH and all eluates combined. Solids were determined on the supernatants and solids in the eluates were assumed by difference, since they contained large amounts of NaOH. Potencies calculated from these weights may therefore be somewhat unreliable.

Following this, identical Lloyd's eluates from two 500-gram batches were combined and adsorbed on 50% Norite at pH 3 two times as previously described. Again, the charcoal cakes were eluted separately with hot 28% NH_4OH and the solutions steamed to small volumes.

Using the procedure outlined in Figure 3, the bulk of the factor was obtained as a concentrate of potency approximately 40. An overall apparent yield of 114% of the original activity was obtained in all fractions. This did not include an additional 50 to 60% which was lost in the filtrates from the various adsorption treatments. The apparent increase in activity which accompanies adsorption and elution has been observed frequently, both with Norite and Lloyd's reagent. It seems likely that these adsorbents may remove growth inhibitors from the liver, thus increasing the sensitivity of the organism to the Gayoni factor.

DISCUSSION

Interpretation of Experimental Results

A study of the properties of the factor makes possible a rough preliminary classification of this substance. Among the most significant observations are those concerning hydrolytic procedures and dialysis. The fact that the substance is best released from fresh liver and casein by enzymic digestion indicates that it is connected to protein in natural materials (Table 2). However, since it passes through cellophane, the active component itself appears not to be of protein proportion; in fact, the dissociated form possesses maximum growth promoting activity for L. gayoni. In this form, it is much more stable to hydrolysis than is the protein complex from which it is derived.

The active component is probably both acidic and basic in nature, judging from its adsorption on Norite from both acid and alkaline solutions. Since the best adsorption occurs at about pH 3, the acid properties would seem to predominate. It seems only weakly acid, however, since alumina adsorbs it nearly quantitatively at pH 3 but not at all at pH 1. Attempts to demonstrate the presence of acid groups by esterification experiments were not decisive, but destruction by nitrous acid indicates a primary amino group.

Heavy metal precipitations suggest the presence of

active hydrogen atoms. Flavianates and phosphotungstates, on the other hand, are common base precipitants.

Solubility behavior of the factor points to a highly polar structure. Glacial acetic acid is the only organic solvent which shows appreciable solvation power.

Non-identity of the Gayoni Factor with Other Reported Growth Factors

With the preliminary characterization and concentration methods made available, the Gayoni factor may be compared with other recently reported growth factors which have not as yet been fully identified. Several of these compounds were available for testing, and, as shown, previously, possessed no activity for L.gayoni. Published reports of others point out properties which distinguish them from the Gayoni factor.

The L. casei factor isolated from yeast by Hutchings et al.(13) showed no activity when tested by L.gayoni. A crude concentrate of Williams' folic acid (17) failed to replace the Gayoni factor. The latter may thus be regarded as different from vitamin B_c (18), Peterson's Norite eluate factor (12), and the L. casei factor from liver (24) since these are all considered identical with folic acid.

The vitamin M which is necessary to prevent leucopenia, anemia, diarrhea and death in the rhesus monkey was

successfully replaced by the L. casei yeast factor. (10). Vitamin M is not folic acid or xanthopterin, however (25), but the yeast factor appears to be a conjugate of folic acid. Keresztesy's S. lactis growth factor (14) was shown to produce folic acid and be replaceable by it forall bacteria which need it (22). Again, this factor may be the L. casei yeast factor and thus not identical with the Gayoni factor.

The two chick factors B_{10} and B_{11} have been shown to be distinct from folic acid (3) and vitamin B_c (4). However, B_{10} and B_{11} are stable to dry heat or autoclaving with 1 N NaOH and are only partially precipitated with silver nitrate. In addition, both seem more easily eluted from common adsorbents than is the Gayoni factor. Neither factor is removed from original solution by dialysis, a fact which leads the investigators to suggest that the various "folic acids" may be fragments of the larger molecule or molecules of B_{10} and B_{11}. Again, the Gayoni factor appears distinct from these vitamins and from large molecule "conjugates."

Vitamin B_{11} may be similar to the factor R required by the chick and reported by Hill and coworkers (11). Hill's factor S (11) is not readily adsorbed on common adsorbents and may be identical with the kidney residue factor of Mills et al. (16). Both Briggs (4) and Hill (11)

report evidence of an additional factor necessary for preventing anemia in chicks but not identical with B_{10}, B_{11}, R, S, B_c, folic acid, or the L. casei liver factor provided the last three are actually the same. No properties of this supposed factor are reported, however.

Other factors which differ distinctly from the Gayoni factor in one or more respects are: 1) Two similar growth factors reported by Woolley (27, 28), termed "strepogenin" and GPF-3, which are not precipitated by lead and are not adsorbed by Norite; 2) The S. lactis factor of Smith (19) which is not precipitated by lead or mercury and is not adsorbed on Darco or fuller's earth; 3) Kuiken's tomato juice factor (15) which withstands 24-hour refluxing with 8 N H_2SO_4; 4) The diphtheria bacillus factor of Chattaway (6, 7) which fails to adsorb on fuller's earth, is not precipitated by lead, mercury, or phosphotungstic acid, and is not destroyed by excess bromine; 5) An L. casei growth factor reported by Chattaway (5) which fails to adsorb on fuller's earth at pH 3; 6) The growth factor reported by Ballentine (1) for a gas gangrene clostridium, which is not adsorbed on Norite at pH 3, is not precipitated by silver or phosphotungstic acid, and fails to be oxidized with $KMnO_4$ at pH 7.5. 7) Barton-Wright and co-workers (2) report isolation of four growth factors from liver which are necessary for L. helveticus and S. lactis R. Of these,

only HL 4 (as distinguished from HL 1, 2, and 3) was affected by nitrous acid treatment. HL 4 is, however, a portion of the fraction soluble in chloroform--a behavior which seems highly improbable for the Gayoni factor in view of its reaction to non-polar solvents similar to chloroform.

CONCLUSION

Summary

Evidence is here presented of an unknown factor essential for growth of the bacterium Lactobacillus gayoni 8289. A microbiological assay method has been developed for the substance, which is designated as the Gayoni factor. It is distinct from 71 organic compounds and growth factor concentrates tested. Experimental work has led to preliminary characterization of the compound. A 40-fold concentration has been made possible by use of its adsorption of Norite charcoal and Lloyd's reagent and its elution from these materials with hot 28% ammonium hydroxide and 0.5 N sodium hydroxide. On the basis of the evidence obtained, a preliminary classification of the compound is made. It displays properties which distinguish it from any of the growth factors recently reported in the literature and as yet unidentified. Work is continuing on the concentration of the material in attempts to further identify it with, or distinguish it from known compounds and biological growth factors.

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