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The Accumulation and Utilization of Asperuloside in the Rubiaceae

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The significance of glycosides in plant metabolism is obscure, and the present work has been undertaken as a contribution to its elucidation. Large amounts of glycosides are present in many plant cells and although these substances vary widely in their chemical constitution many of them exhibit a common pattern of distribution in the plant. They are accumulated in the vacuoles of young parts of plants during the period of cell expansion, and afterwards may be partly or completely removed as the part ages.

The plants investigated were: *Galium aparine* (cleavers, or goose-grass), *Asperula odorata* (wood-ruff) and *Rubia tinctorum* (dyer's madder), which belong to the tribe Stellatae of the family Rubiaceae. Most plants produce glycosides of a number of aglycone types, and the Stellatae are known to produce anthocyanins and related glycosides, which are universal among higher plants, anthraquinone glycosides, coumarin glycosides and asperuloside, which probably has a substituted furan structure (Trim & Hill, 1952).

Asperuloside was chosen for study because it occurs in very large quantities in the plants investigated, and because its chromogenic properties provide very simple means for its detection and estimation.

EXPERIMENTAL

The methods of growing and collecting the different plant materials will be described with the experiments in which they were used. The time of collecting was mid-morning. Extracts were made from fresh, weighed material unless otherwise stated. Dry weights were determined after drying equivalent batches of the material for several days at 37° and then over concentrated H₂SO₄ and solid NaOH *in vacuo*. The parts to be extracted were ground with an equal weight of 0.1 N-HCl and a little sand. A few drops of CHCl₃ were added and the mush allowed to stand for about 15 min. The ground tissues were then mixed with kieselguhr, filtered on a Büchner funnel and washed with water until the runnings were free from asperuloside. The extract was made up to an

appropriate volume with water, neutralized with CaCO₃ and filtered.

Estimation of asperuloside. 1 ml. samples of the extracts were mixed with 11 ml. of reagent A (Trim & Hill, 1952) and heated on a boiling-water bath for 5 min. with occasional shaking. The characteristic blue derivative of asperuloside developed. The cooled solution was compared with a standard in a Duboscq-type visual colorimeter. Beer's Law applies over a wide range of concentrations as may be seen in Table 1. The effect of the addition of a plant extract is

Table 1. *Colorimetric estimation of known amounts of asperuloside*

Asperuloside (mg.)	Recovery, using a 1 mg. standard (%)
0.25	108
0.50	100
0.75	100
1.0	100

Table 2. *Colorimetric estimation of known amounts of asperuloside in the presence of an extract of Galium aparine*

Extract (ml.)	Asperuloside added as 0.1% solution (mg.)	Total found (mg.)	Recovered (mg.)
0.5	0.0	0.21	0.0
0.5	0.10	0.31	0.10
0.5	0.25	0.46	0.25
0.5	0.50	0.71	0.50
0.0	0.50	0.50	0.50

shown in Table 2. The colour developed under these conditions has an absorption spectrum with bands at 645 and 600 mμ. Early in the season, when most parts of the plants were young, very little extraneous colour was developed. Later, when the bulk of the tissues were older, the plants produced sufficient quantities of interfering substances to necessitate the use of a red filter when making the colorimetric measurements. The nature of the interference by substances such as phenols, ketoses and heterocyclic N compounds, which are likely to occur in plants, has been

described (Trim & Hill, 1952). Since relatively large proportions of these substances must be added to cause a marked effect, the interference was rarely serious.

Reagent *A* was also used for the detection of asperuloside in portions of plant tissues. The blue colour develops in sections and fragments of plant heated with reagent *A* on a slide or in a test tube. This procedure was used to detect the localization of asperuloside.

Galium aparine

The material was collected at random from one large plot of plants. Ripe seeds were collected from the plot at the end of the season 1946 and used for experiments during the following winter and spring.

Seeds and seedlings. Batches of 30 newly ripened seeds were analysed and found to contain 3.5% asperuloside on a dry-weight basis. After storing until the following spring the value had fallen to 2.5% and the seeds remained viable. Qualitative tests on sections which had been cut from seeds soaked in water for 16 hr. showed that the endosperm and the whole of the embryo contained asperuloside. As germination proceeds the endosperm shrinks and the amount of asperuloside in it declines. The embryo develops at the expense of the endosperm. Asperuloside was found in all parts of the developing embryo and was in particularly high concentrations in the early embryonic hypocotyl and cotyledons. These parts gave a purple colour with reagent *A*, which suggested the presence of a large proportion of the interfering substances mentioned above. The interfering material disappeared during the development of the seedling. As the seedlings grew they were dissected into their parts and each part was tested separately. This confirmed the observation that asperuloside occurs in all parts of the very young seedling and that there is a high concentration in the cotyledons. The glycoside appears to be absent from the growing tips of the roots.

The following measurements show how asperuloside accumulates during the period of germination. Two batches of about 500 seeds were sown on wet sand in two shallow dishes covered with sheets of glass, slightly raised above the tops of the dishes to admit air. Both dishes were kept in a greenhouse at 18° and one of them was covered to exclude light. At intervals two samples of 30 seeds were removed from each dish and ground with sand and a few ml. 3% HCl to extract the asperuloside. This was estimated, and the values obtained are plotted in Fig. 1. The initial fall in the amount of asperuloside per individual takes place while the sprouting radicle has still not emerged through the seed coat. By the time the minimum has been reached sprouting is just visible among the seeds which have been kept in the dark. These seedlings then accumulate asperuloside until the amount per individual reaches the level in the ungerminated seed, and if

the seedlings are kept dark this level is maintained for many days. By the time the maximum has been reached the plant has fully emerged. Exposure of these seedlings to the light results in an immediate marked increase of asperuloside, due presumably to photosynthesis. The sprouting of the seedlings grown in the light is retarded by about 8 days and the accumulation of asperuloside is slower, but by the time the cotyledons appear the amount of asperuloside has reached the level of the maximum for seedlings grown in the dark. There is then a rapid increase in the amount of asperuloside due to the supervision of photosynthesis.

Fig. 1. The production of asperuloside in germinating seeds of *G. aparine*: (i) ●—●, seeds germinated in the dark; (ii) ○—○, seeds germinated in the light.

This experiment was repeated a month later and the same changes were observed. The amount of asperuloside in the seedlings germinated in the dark reached 0.34 mg. per plant after 12 days and remained constant for another 10 days. Then it fell off slowly, reaching 0.26 mg. per plant 32 days from the initiation of germination. Some of the seedlings germinated in the dark were exposed to the light 18 days after the beginning of the experiment and immediately began to produce more asperuloside. After 2 days' exposure there was 0.4 mg. per plant and after 15 days 0.59 mg. per plant.

It is concluded from these experiments that asperuloside is continuously accumulated by the germinating embryo in the absence of external nutrients other than water, the initial rapid fall in the amount of glycoside being due to the utilization of the endosperm. This accumulation is terminated abruptly at a certain level unless photosynthesis is permitted. The effect of feeding through the roots was not tested.

Asperuloside formation in seedlings grown in soil. Twelve 25 cm. pots of soil were each sown with 40 seeds of *G. aparine* and kept in a greenhouse at 18°. After a month, when the seedlings were well developed, they were carefully removed from two of the pots, washed, counted and each plant divided into four parts: roots, hypocotyl, cotyledons and shoots. The parts from one pot were weighed and the asperuloside was extracted. The parts from the other pot were used for the determination of the dry weights. Further samples were taken as the seedlings developed. Some of the results are plotted in Fig. 2.

Fig. 2. The accumulation of asperuloside in seedlings of *G. aparine* grown in soil with normal lighting; (A) leaves, (B) roots, (C) cotyledons, (D) hypocotyl.

As the plant develops there is continuous steady accumulation of asperuloside in the roots and shoots. At the same time, the amount in the cotyledons falls to a constant low level. While there is a possibility of transport of glycoside from the organs which are losing it to those which are gaining it, at least part of it is synthesized anew and accumulates in the growing parts, for the amount of glycoside which is gained by the roots and shoots separately is greater than the sum of the amounts lost by the hypocotyl and cotyledons over the period of the experiment. Figures for dry weights (Table 3) show that in the course of this experiment the cotyledons and

hypocotyl were no longer growing, while the shoots and roots were rapidly increasing in weight.

Table 3. *Dry weights of parts of Galium aparine seedlings*

Day	Dry weight (mg.)			
	Hypocotyl	Cotyledons	Shoots	Roots
0	3.3	6.6	7.4	17.0
12	2.6	5.2	27.0	23.0
21	1.2	5.2	58.0	58.0

The seasonal variation of the amounts of asperuloside. Individual shoots from different plants at the same stage of growth varied considerably in their asperuloside content. It was found that significant values for comparative purposes could be obtained by analysing batches of six or more shoots picked at random from the experimental plot. The values for six batches of six shoots picked at the same time are tabulated in Table 4.

Table 4. *Asperuloside in the shoots of Galium aparine*

(Batches of six shoots.)

Batch no.	Wet wt. (g.)	Asperuloside (% of dry wt.)
1	8.9	4.8
2	10.7	4.8
3	10.9	4.8
4	11.9	5.3
5	8.2	4.8
6	6.4	5.3

The formation of the glycoside throughout the season was followed. Batches of 20 shoots were taken for as long as the rapidly increasing size of the shoots permitted. After that the number was reduced. The amount of glycoside per shoot gradually rises and then falls sharply as the shoot becomes senescent. When different parts of the shoot were tested with reagent A it was found that asperuloside is present in large amounts in all parts of the very young leaf, with the possible exception of the epidermis and chlorenchyma. There is much less in old leaves and what they do contain is confined to the vascular tissues, showing that the decline, already observed in the ageing hypocotyl and cotyledons, also occurs in the leaves.

The manner in which the glycoside content of the plant changes with age was shown more clearly by the following experiments with growing shoots, which were cut up into successive nodes, defined as follows. The leaves grow in whorls about the nodes. Each node and the internode above it develop together so that their parts have the same average age and may be taken as a unit composed of tissues of approximately the same age. Dry weights were determined on material from a similar shoot from

the same plant and a corresponding node. The results in Fig. 3 show that where rapid, exponential growth is taking place there is a high, constant concentration of asperuloside; after exponential growth has ceased there is a sharp downward trend in asperuloside concentration towards a lower, slowly drifting level.

Fig. 3. The concentration of asperuloside in successive nodes of a shoot of *G. aparine*. Node 1 is the most apical. The black columns indicate the relative dry weights of the nodes.

If the change in asperuloside concentration is considered in relation to the development of an individual mesophyll cell the change from the predominance of accumulation to the predominance of utilization (or transport away from the leaf)

appears to be sharp and to coincide with the end of vacuolation and cell expansion. In this case (and all the others described in this paper) the change does not occur in a particular node, say 3 or 4, counting from the apex of the shoot downwards, but appears to be at a hypothetical point between node 3 and 4 (in this case). This is probably to be explained by the fact that all the cells in a particular node are not at the same stage of development. In this case all the cells in node 4 have passed through the stage of exponential growth; but while some of those in node 3 have also passed through that stage many of them are still in it. In this situation it is logical to expect the asperuloside concentration in node 3 to be on the higher level, although growth has already begun to slow down.

It seemed that during the stage of exponential growth the accumulation of asperuloside was directly proportional to the increase in dry matter. This was tested by estimation of asperuloside in shoots of *G. aparine* over a period of 2 weeks. The shoots were chosen so that the whole of the tissues were well within the stage of exponential growth. They were picked at random from the experimental plot and divided into three batches of 30.

Asperuloside was estimated in two batches and the third was used for dry-weight determinations. The results in Table 5 show that over a period during which the bulk of the average shoot increased nearly threefold the amount of asperuloside remained directly proportional to the fresh and dry weights.

Accumulation of asperuloside in the fruit. The accumulation of the glycoside during the period of the swelling of the fruit was also observed. Fruit was collected at three successive stages. As is shown in Table 6, the amount of glucoside is approximately proportional to the dry weight.

Table 5. *Proportion of asperuloside in the shoots of Galium aparine in the stage of exponential growth*

Sample	Date of picking	Wet wt. (g.)	Dry wt. (g.)	Dry matter (%)	Asperuloside (% of dry wt.)
1a	14. iv. 47	10.5	1.33	—	3.25
b		12.4	1.57	—	3.35
c		12.4	1.58	12.7	—
2a	18. iv. 47	12.3	1.54	—	3.25
b		12.5	1.57	—	3.35
c		12.1	1.51	12.5	—
3a	22. iv. 47	14.8	1.67	—	3.20
b		16.4	1.84	11.3	—
4a	29. iv. 47	27.8	3.6	—	3.20
b		29.2	3.8	—	3.30
c		31.1	4.0	12.9	—

Table 6. *Asperuloside in the fruit of Galium aparine*

Condition of fruit	Wet wt. of 30 fruit	Dry matter (%)	Asperuloside per fruit (mg.)	Asperuloside (% of dry wt.)
Small and soft	0.766	31	0.25	3.2
Beginning to harden	0.992	33	0.39	3.7
Hardened	0.977	37	0.44	3.6

Table 7. *Asperuloside in the leaves of Rubia tinctorum*

Whorl no.	No. of leaves	Length of internode (cm.)	Dry matter per leaf (g.)	Asperuloside (% of dry wt.)
1	6	2.6	0.003	10.4
2	6	7.0	0.015	10.8
3	6	8.0	0.054	8.4
4	7	7.0	0.055	5.0
5	6	7.5	0.045	3.7
6	6	6.5	0.047	2.4

Table 8. *Asperuloside in the phloem of Rubia tinctorum*

Strip	Wet wt. (mg.)	Dry wt. (mg.)	Dry matter (%)	Asperuloside (% of dry wt.)
1	12.0	2.35	—	23
2	10.0	1.95	—	24
3	13.3	2.60	19.5	—
4	10.2	2.0	19.5	—

Rubia tinctorum

Determinations of asperuloside in the growing shoots of the dyer's madder, *R. tinctorum*, showed that the same process of accumulation and subsequent utilization of asperuloside occurs in this

Fig. 4. The concentration of asperuloside in successive internodes of a shoot of *R. tinctorum*. Node 1 is the most apical.

plant. In this case the initial concentration of glycoside in the young leaves was much higher (10–15 % of the dry weight) than in *Galium aparine*. Figures for successive whorls of leaves, numbered from the apex of the shoot downwards, are tabulated in Table 7. The length of the accompanying internode is given as an additional index of the stage of growth.

The values for the asperuloside concentration in successive internodes of a developing shoot, numbered from the apex downwards, are recorded in Fig. 4. The arrow in this figure marks the ninth internode below which the asperuloside concentration rises. Comparison of these internodes with

higher ones is scarcely permissible, since they had begun to develop characteristics of the subterranean parts of the plant. This was indicated by the growth of adventitious rootlets from some of the nodes and the appearance of anthraquinone glycosides, which are characteristic of the roots and underground stems of *Rubia*. The observations in the following section relate to the form of the curve in Fig. 4.

Asperuloside in the vascular tissues of the stem. A fully developed internode of *R. tinctorum* was divided into three parts: cortex, inner bark or phloem and xylem combined with pith. Tests showed that there is scarcely any asperuloside in the cortex and woody regions, but there is a very high concentration in the phloem. Fresh strips of the latter were weighed rapidly on a torsion balance, dropped into a known volume of reagent A and the asperuloside estimated. Other strips were taken for dry-weight estimations. More than 20 % of the dry matter was found to be asperuloside. The values are recorded in Table 8. If younger, still growing internodes are tested much asperuloside is found in the cortex and pith.

Asperula odorata

The pattern of distribution of asperuloside in *A. odorata* was found to be very similar to that in the two plants already described. An experiment with this plant was devised to confirm the observations already made. It was arranged to cover the period of flowering to see if the development of the inflorescence is accompanied by asperuloside accumulation. The experiment was started when it was just possible to separate the developing flower buds from the shoot and continued until the flowers were just beginning to die. Sixty shoots were picked at random from one large clone at intervals of 3 days over a period of 4 weeks. These batches were divided into two equal parts and the nodes of each part

separated. The leaves were separated from the stems and the inflorescence was counted as one node, number 1. The parts from one batch of 30 shoots were used for dry-weight determinations and those from the second for asperuloside determinations. In this way it was possible to follow the course of asperuloside accumulation and disappearance in each unit. The results recorded in Figs. 5a and b

cells appears to be as follows: after the primary meristematic stage, the cells enter the period of exponential growth, during which the glycoside accumulates in amounts directly proportional to the increase in dry matter. This may be the result of simple storage of the product but may equally be due to the accumulation of glycoside, resulting from the balance between opposed processes of synthesis

Fig. 5 (a, b). The concentration of asperuloside in components of successive nodes of shoots of *Asperula odorata* during flowering. The nodes are numbered from the apex downwards: (a) leaves, number 1 is the inflorescence; (b) inter-nodes, number 1 corresponds to leaves number 2.

show that the parts of *A. odorata* accumulate and utilize asperuloside according to the same developmental pattern as the two plants already investigated. In the inflorescence the glycoside level remains high throughout the experiment. However, this was the only part which continued to form new elements, thus compensating for the loss of glycoside in the senescent elements.

DISCUSSION

While the study of individual cells has not been possible in this work, it appears that many of them accumulate asperuloside during an early stage of their existence. In this respect the history of such

and removal, in which there is a preponderance of synthesis over removal. The disappearance of the glycoside could occur by further metabolism at or near to the site of formation, by translocation, or by a combination of both processes. At the end of exponential growth of the cells increase in volume virtually ceases and a decline in glycoside accumulation sets in, so that finally the cells become devoid of it. This change would be explained either by a cessation of synthesis or by a decline of synthesis sufficient to be completely overbalanced by a continued steady removal of glycoside, or by an increased rate of removal. Whatever the explanation, the cessation of growth is accompanied by an abrupt change in glycoside metabolism.

The chlorenchyma may not accumulate asperuloside and the epidermal cells also appear to be exceptional in that very few, if any of them, accumulate the glycoside. Since the methods employed were crude these conclusions are only tentative. In all organs part of the vascular system appears to be exceptional in continuing to accumulate asperuloside until the organ becomes moribund. The technique available did not permit a distinction between individual cells, but it is certain that this phenomenon is confined to the inner bark, that is, to the phloem and cambium. But this is a region of continued growth, containing young cells which would be expected to contain the maximum amount of asperuloside. Consequently, it might be suggested that the exceptional behaviour of these parts, in respect of asperuloside accumulation, is more apparent than real. However, it should be added that the amounts of asperuloside found in the inner bark were in excess of what might have been expected if it were composed entirely of very young cells, which is not the case. In addition, it has to be remembered that the phloem is the main path of transport for organic material, so that the large amounts of asperuloside found there may be in process of transport.

As far as the author is aware this is the fullest investigation of its kind. But there are many other recorded observations which support the view that the distribution of asperuloside in the rubiaceae plant is typical of the distribution of glycosides and many other metabolites throughout the whole range of higher plants. It is frequently stated in the literature that young, actively growing parts of a particular plant are the most abundant source of some of its characteristic products. The young shoots of many plants are pigmented by anthocyanins in the developing epidermal cells, and the pigmentation often disappears after these cells have become fully grown. In his observations on the living rose leaf, Guilliermond (1913) showed that anthocyanins accumulate in the developing vacuoles of the young epidermal cells, and thus provide a visible parallel to the first stage of the process which has been postulated to explain the present observations on asperuloside. Hurry (1930) showed that the largest amount of the indigo-producing glycoside of *Isatis tinctoria* is present in the very young leaves and that the older leaves contain much less. The classical work of Treub (1896, 1904, 1907) on cyanogenesis in *Pangium edule* and other plants provides further examples of the same developmental process.

Certain exceptional cases have been observed. Bourquelot & Danjou (1905), and others, have

shown that sambunigrin, D-mandelonitrile glucoside, which occurs in the elder, *Sambucus nigra*, remains in the leaves even after they have fallen. Treub (1904) noticed the same phenomenon with the cyanogenetic glycoside of *Indigofera galeoides*. There is a suggestion in such cases that the appropriate hydrolysing enzyme is absent, but it has yet to be shown that such phenomena are not also dependent upon the absence of all of a number of different modes of utilization of the glycoside concerned. At the other extreme, Treub (1904) found that in *Phaseolus lunatus* there is a marked diurnal variation in the amount of cyanogenetic glycoside stored in the leaves.

The balance of evidence clearly favours the view that glycosides are rapidly synthesized and broken down in the plant and that in some cases at least large amounts of glycoside are involved. The characteristic distribution of these substances in the plant is related chiefly to different metabolic stages in its development. If, as it seems, glycosides enter into the metabolic processes of plants, they cannot be regarded as mere end products of metabolism, and it is important to determine the reactions in which they participate.

SUMMARY

1. The accumulation and utilization of asperuloside in the tissues of three rubiaceae plants have been studied.
2. The glycoside accumulates in young tissues such as new leaves in the stage of exponential growth and cells in the neighbourhood of the cambium.
3. The accumulation stops abruptly with the completion of the stage of exponential growth and is followed by a period of glycoside depletion, which may involve translocation to another part of the plant.
4. Nearly all the cells in the plant, with the possible exception of most of those in the epidermis and chlorenchyma of the aerial parts and some of those in the roots, appear to accumulate asperuloside when in the early stage of growth.
5. The significance of these results in relation to metabolism and translocation is discussed.
6. The general pattern of production and utilization of asperuloside appears to be characteristic of many glycosides and other metabolic products throughout the higher plants.

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A Modified Medium for *Lactobacillus casei* for the Assay of B Vitamins

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Snell & Wright (1941) were the first to point out that their method for the assay of nicotinic acid could be applied to the determination of biotin and pantothenic acid. Shortly afterwards, Landy & Dicken (1942) published a general procedure for the estimation of six vitamins, using *Lactobacillus casei* as the test organism. Roberts & Snell (1946) devised a medium for microbiological assays with *L. casei*, which was an improvement on the original medium of Landy & Dicken (1942).

On investigating the various factors affecting the growth of *L. casei*, we have modified the medium of Tepley & Elvehjem (1945) with the aim of developing a suitable medium which could be used for assays of various vitamins.

MATERIALS AND METHODS

Stock solutions

Unless otherwise mentioned, the stock solutions were prepared according to techniques described by Snell (1950).

Salt solution E (Kodicek & Pepper, 1948a).

MgSO₄·7H₂O, 60 g.; FeSO₄·7H₂O, 4 mg.;
 MnSO₄·4H₂O, 1.5 g.; CuSO₄·5H₂O, 4 mg.;
 NaCl, 0.5 g.; ZnSO₄·7H₂O, 4 mg.

were dissolved in 250 ml. of water and 3 drops of conc. HCl added.

Casein hydrolysate: H₂SO₄-hydrolysed, vitamin-free 'Labco' casein. 'Labco' casein (200 g.) with 1 l. 25% (w/w) H₂SO₄, was autoclaved for 15 hr., at 15 lb. pressure. A litre of water was added, sulphate precipitated with about 850 g. Ba(OH)₂, the mixture autoclaved for 10 min. at 10 lb. pressure, and filtered hot through a Büchner funnel. The BaSO₄ cake was thoroughly broken up in 1 l. water, autoclaved and filtered hot. The precipitate was washed once more as before. The combined filtrates were heated and the excess barium was carefully precipitated with 25% H₂SO₄. The suspension was filtered hot, and the filtrate concentrated on a water bath to about 2500 ml. Dry weight determination gave a recovery of a little over 90%.

The hydrolysate at pH 6.0 was stirred for 1 hr. with 100 g. Norite charcoal (British Drug Houses Ltd.), filtered

and the pH adjusted to 3.8 with glacial acetic acid. The charcoal treatment was repeated, the solution filtered and brought to pH 6.8–7.0 with 40% KOH. The hydrolysate was finally treated with Norite, 40 g., for 1 hr. The residue from each filtration was washed with 50–100 ml. of water and the washings added to the filtrate. The filtrate was concentrated on a water bath to contain 10% (w/v) casein solids. Recovery was 73% (casein solids). Before use, new batches of hydrolysate were always tested at several levels and the optimum concentration determined. An amount of 50 mg./10 ml. medium was usually found adequate. We have found it necessary to restandardize the adsorption treatment when a different brand of charcoal was used.

Peptone treated with Norite charcoal. 'Difco' bacto-peptone (10 g.) was dissolved in about 80 ml. water, pH adjusted to 3.0 with HCl and made up to 100 ml. The solution was stirred with 5 g. Norite for 1 hr., filtered, and the charcoal treatment repeated twice with 2 g. Norite.

Pteroylglutamic acid (PGA). During the early part of the work 'Folvite' (Lederle Laboratories) was used as the PGA standard. Later, when a 97% pure and aldehyde-free preparation (Lederle Laboratories) became available it was used instead.

A stock solution of PGA containing 100 µg./ml. was prepared in 1% K₂HPO₄. It was renewed twice weekly. From the stock solution weaker standards were prepared for immediate use.

Bacteriological procedure

The double-strength medium, 5 ml., the composition of which is shown in Table 1, was diluted to 10 ml. with distilled water after adding the test solutions. The test substances were assayed usually in triplicate at each level. The tubes were placed in Petri dish canisters and autoclaved for 6 min. at 15 lb. pressure. Autoclaving for a longer period resulted in caramelization and consequent deterioration of the medium. On autoclaving, a slight precipitate resulted, but this redissolved on shaking at room temperature.

Stock culture. Cultures of *L. casei*, A.T.C.C. no. 7469, with characteristics reported by Kodicek & Pepper (1948b) were maintained on slopes of 'Difco' yeast-dextrose-agar supplemented with 'Hepamino' liver powder (Evans) 1% and glucose 0.5%. They were subcultured every 2–3 weeks. Each subculture consisted of two transfers from liquid to