Biochemical Characterization of Actions of Chemotherapeutic Agents

5. LACK OF GROSS DISPLACEMENT OF PANTOTHENATE AND *p*-AMINOBENZOATE FROM MICRO-ORGANISMS, BY PANTOYLTAURINE AND SULPHANILAMIDE

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The bacteriostatic actions of sulphanilamide and pantooyltaurine have many characters in common, including those associated with their antagonism by compounds which are structurally related to the inhibitors and which are of natural occurrence. In the case of antagonism by *p*-aminobenzoate to the action of sulphanilamide, Woods (1940) and Fildes (1940) concluded that the interaction was by a competitive mechanism. A more quantitative demonstration of this was given by Wyss (1941); he, with later workers, based his analysis of the antagonism on equations which assumed equilibria between the reactants and some component of the micro-organisms. The equilibria implied that (in a sense to be discussed) the amount of free *p*-aminobenzoic acid in a system containing susceptible bacteria and a limited amount of the acid would increase on addition of sulphanilamide; Fildes (1940) and Harris & Kohn (1941) anticipated such 'displacement'. Similar characters have been shown, and mechanisms assumed, for the antagonism by pantothenate to the antibacterial action of pantooyltaurine (McIlwain, 1942, 1944). In neither case has the 'displacement' been directly observed, and the biological conditions under which it might occur, or the quantities of substances which it might involve, have not been specified. Although Green (1940) indicated that no considerable liberation of sulphonamide antagonists from *Brucella abortus* was caused by sulphanilamide, a displacement of *p*-aminobenzoic acid adsorbed on charcoal or a blocking of the adsorption, by sulphonamides, have been considered as models of the actions of the drugs by Hartmann & Druey (1943) and Eyster (1943).

The possible displacement of *p*-aminobenzoate and pantothenate from microbial cells has now been examined. The method adopted in the case of pantothenate permitted observation of the release, from a small quantity of cells, of about 1/1000 of their pantothenate content. As it was not found possible by Feinstein, Williams & Florestano (1942) to correlate the absorption of sulphonamides by bacteria with their biological action (as was the case with arsenicals: Hawking, 1937, 1938), any observation of displacement would have additional significance as affording a possible connexion between the changed biological behaviour of suscep-

tible organisms in the presence of the inhibitors and changes in their cell substance.

EXPERIMENTAL

Preparation of the streptococci. The Richards (group A) *Streptococcus haemolyticus* and a group G organism, maintained as previously described (McIlwain, 1944), were prepared for the present experiments by growth in a medium, based on a casein hydrolysate (McIlwain & Hughes, 1944), under conditions which yielded cells of varying pantothenate content. Pantothenate-poor cells were obtained from media initially containing 10^{-8} M-pantothenate, in which growth was allowed to proceed for 30–42 hr., with progressive neutralizing of the acid formed by glycolysis (McIlwain & Hughes, 1944). After such growth, the pantothenate concentration of the medium was $<10^{-8}$ M; these bacteria were examined without washing. Pantothenate-rich cells were produced by growth for 17–22 hr. in media whose initial pantothenate concentration of 10^{-5} M did not fall below 10^{-6} M when the organisms were harvested. This was done by centrifuging, and organisms from 100 ml. of medium were washed twice with 10 ml. of 0.5% NaCl, which did not remove all the pantothenate loosely associated with them (see below); they were used in the following experiments within 2 hr. of harvesting.

Effect of reagents on streptococcal pantothenate. Bacterial suspensions were divided into portions containing the equivalent of 15–60 mg. dry wt. of cells in M/18 phosphate (pH 7.6) and having a final volume of 2–5 ml. which contained the reagents whose effects were being studied (Tables 1 and 2). In the three cases examined, photometric measurement of the turbidity of portions of the suspension taken before and after such experiments showed no detectable differences; with the apparatus employed, this implied that any difference was $<1.5\%$. After exposure the suspensions were adjusted to 10 ml., the fluids separated by centrifuging, sterilized by filtration, and their pantothenate content determined. The cells, usually without washing, were digested to release pantothenate (McIlwain & Hughes, 1944), which in all cases was determined by growth of *Proteus morganii*, measured photoelectrically (McIlwain & Hughes, 1945). In a few experiments, using high pantooyltaurine concentrations and desoxycholate, cells were washed before digestion in case these reagents affected the liberation of pantothenate, but such effects were not found; pantothenate in the washings was negligible.

Effect of sulphanilamide on the liberation of its antagonists from streptococci. The Richards *Streptococcus* was grown for 17–20 hr. in the media described above, initially containing 10^{-6} M-pantothenate. Exposure to buffer and saline solutions, with and without sulphanilamide, was carried out

on batches of 1 g. (wet wt.) of the washed cells by the method described below; no release of sulphanilamide antagonists was detected. After exposure, antagonists were liberated by autolysis with ammoniacal solutions, as described by Stamp (1939). For further experiments yeast was chosen as a material which is accessible in larger quantities and whose sulphonamide antagonists have been much studied.

Effect of sulphanilamide on the liberation of its antagonists from yeast. Commercial yeasts were used; their growth was retarded by 0.02 M-sulphanilamide. A batch of bakers' and a batch of brewers' yeast were each suspended in 10 vol. of 0.5% NaCl, separated by centrifuging, and the washing repeated. Sulphanilamide (2 g.) and water (5 ml.) were added to 20 g. portions of the moist cells and the mixtures rubbed occasionally during 4 hr. at 37°. Similar portions of the yeasts were treated in the same way, but without addition of sulphanilamide. All were centrifuged, and the supernatants without sulphanilamide were saturated with the drug. All were kept at 0° overnight to remove the bulk of sulphanilamide. This arrangement of control experiments was chosen to avoid unequal losses (e.g. by adsorption or in crystals) from the different solutions, or unequal inhibition by them in testing. The recovery of added *p*-aminobenzoate from such a process was examined, and >90% was found to be recovered.

The total antagonists of the yeasts were determined by autolysis of portions for 12 hr. under Stamp's (1939) conditions; all solutions were assayed for antagonists by their effects on growth of the Richards Streptococcus in a peptone medium (McIlwain, Fildes, Gladstone & Knight, 1939; Woods, 1940) containing 3.3×10^{-4} M-sulphanilamide. Growth from a series of dilutions of the extracts was compared with that produced by a reference series of dilutions of *p*-aminobenzoate, and the results of assays expressed as the quantities of *p*-aminobenzoate which would be required

to produce the observed antagonism. Interaction between the material from yeast or streptococci, and sulphanilamide, was found to be competitive.

RESULTS

Behaviour of the pantothenate of streptococcal cells

Pantothenate content. Experiments attempting to displace pantothenate were first carried out with organisms grown in media whose initial pantothenate concentration (2×10^{-6} M) approximated to that of mammalian tissue fluids. The pantothenate content of the bacteria (Table 1) was about 30 μ mol./g. dry weight. These values are low in comparison with those found by Thompson (1942) in other organisms, though the method of extraction was similar to that used by Thompson and its applicability to the present streptococci has been confirmed (McIlwain & Hughes, 1944). It was then found that streptococci richer in pantothenate could be obtained by growth for shorter periods; these differences were correlated with the pantothenate content of growth media at the time of harvesting the cells. Although the medium first used contained about 50 times the quantity of pantothenate of the harvested cells, their inactivation of pantothenate (McIlwain & Hughes, 1944) had left the medium containing $<10^{-8}$ M-pantothenate at the time when the bacteria were collected. With shorter growth periods and higher initial pantothenate concentrations, cells containing up to 700 μ mol./g. dry weight were obtained (Table 2). Cells containing

Table 1. *Effect of pantoyltaurine on the pantothenate of pantothenate-poor streptococci*

Organism: Group G Streptococcus. Exposure to reagents for 4 hr. at 37° was in 2-5 ml. of phosphate buffer (pH 7.6) unless otherwise described (column 2). The casein hydrolysate was that of McIlwain & Hughes (1944).

Batch of organisms; dry wt. (mg.)	Reagents and conditions of exposure	Pantothenate			
		In solution after exposure		On later extraction of exposed organism	
		Total (μ mol.)	μ mol./g. dry wt. of organisms	Total (μ mol.)	Total, from solution and cells (μ mol./g. dry wt. of organisms)
I; 44	Buffer only	0.2	4.5	1.0	27.5
"	Pantoyltaurine, 200 μ mol.	0.2	4.5	0.95	26.5
"	Pantoyltaurine, 20 μ mol.	0.3	7	—	—
"	Desoxycholate, 1 mg.	0.25	6	—	—
II; 43	Buffer only	0.3	7	—	—
"	Pantoyltaurine, 100 μ mol. $\pm$ urea, 2 mmol.	0.25	6	—	—
"	Urea, 2 mmol.	0.35	8	—	—
IV; 44	(Not exposed)	—	—	1.25	28.5
"	Casein hydrolysate, 10 mg.; glucose, 0.25 mmol.	0.25	6	0.5	17
"	Casein hydrolysate, 10 mg.; glucose, 0.25 mmol. and pantothenate, 10 μ mol.	0.88	—	0.5	—
"	Casein hydrolysate, 10 mg.; glucose, 0.25 mmol. and pantothenate, 10 μ mol. and pantoyltaurine, 100 μ mol.	8.8	—	0.75	17
V; 48	At pH 9.0	0.13	2.5	—	—
"	At pH 7.6 $\pm$ pantoyltaurine, 100 μ mol.	0.13	2.5	1.20	27.5
"	At pH 5	1.02	21	0.45	30

the lower quantities of pantothenate remained viable, and lesser quantities were not found in freshly grown cells prepared with less pantothenate.

Stability of pantothenate in pantothenate-poor streptococci. Table 1 shows relatively little pantothenate to be liberated into solution from washed organisms of 'pantothenate-poor' type, under a variety of conditions. Pantooyltaurine was used in concentrations up to 50,000 times that of the total pantothenate; ratios of 100 and 20 inhibit respectively growth and pantothenate inactivation by the bacteria. Little inactivation of pantothenate was observed under the conditions of exposure of

pantothenate disappeared during exposure, but was protected by pantooyltaurine; phenomena of this type occur during glycolysis (McIlwain & Hughes, 1944).

Behaviour of sulphonamide antagonists of streptococci and yeasts

Conditions of extraction of sulphonamide antagonists from micro-organisms have not been investigated in the present study as fully as those of pantothenate, for the antagonists in streptococci (Stamp, 1939) and yeast (Woods, 1940) have already been examined with a view to obtaining them in maxi-

Table 2. *Effect of pantooyltaurine on the pantothenate of pantothenate-rich streptococci*

Strain and batch of organisms; dry wt. (mg.)	Pantooyltaurine (μ mol.) in 3 ml. (Not exposed)	Pantothenate				Total, from solution and cells (μ mol./g. dry wt. of organisms)
		In solution after exposure	On later extraction of exposed organisms	Total (μ mol.)	μ mol./g. dry wt. of organisms	
Group G, I; 27	(Not exposed)	—	—	4.6	170	170
"	0	2.3	85	0.44	16	100
"	50	4.5	165	0.43	16	180
Group G, II; 15.2	0	6.6	435	0.37	24	460
"	50	10.8	710	0.35	23	730
Group A (Richards); 4.7	(Not exposed)	—	—	3.38	724	724
"	0	2.7	580	0.23	49	629
"	25	3.0	640	0.22	47	685

batch I, Table 1, and the presence of pantooyltaurine would tend to protect any of the acid which it liberated to solution. Urea and desoxycholate did not increase the pantothenate extracted, either with or without pantooyltaurine. Together with previous results (Cheldelin, Eppright, Snell & Guirard, 1942; Thompson, 1942; McIlwain & Hughes, 1944) the present observations suggest the pantothenate to be chemically linked in the bacteria. Liberation by autolysis has been found maximal at acid pH; at pH 5, much pantothenate was freed, but pantooyltaurine did not increase that amount. Some of the pantothenate of streptococci disappeared in mixtures which permitted glycolysis (batch IV, Table 1) and pantooyltaurine decreased the quantity of pantothenate which disappeared but not the amount of pantothenate left in the cells.

Stability of pantothenate of pantothenate-rich streptococci. A large part of the pantothenate of such organisms, though remaining associated with them after washing at room temperature, was released into saline solutions at 37° (Table 2); the quantity remaining approximated to that shown by the pantothenate-poor preparations. The most sensitive indication of possible displacement of pantothenate with the present organisms was thus the amount remaining in their cells and liberated by later digestion. Large excesses of pantooyltaurine had little effect on these amounts (Table 2). Some

mum quantities. Results in which extractions with salt solutions are compared with extractions by salt solutions containing sulphanilamide are given in Table 3. The sulphanilamide was allowed to act for

Table 3. *Liberation of sulphanilamide antagonists from streptococci and yeasts*

Organism	Antagonists, as <i>p</i> -aminobenzoate (μ mol./g. dry wt. of organism) extracted by		
	Saline	Saline saturated with sulphanilamide at 37°	Autolysis
<i>Streptococcus haemolyticus</i> (Richards strain)	<8	<8	45
Yeast (bakers')	5.5	5.5	51
Yeast (brewers')	5.0	4.0	—

a time longer than the latent period in its growth inhibition of sensitive organisms, and in concentrations 100,000 times those of the '*p*-aminobenzoate' which appeared in solution. The antagonism shown by extracted material was similar to that shown by *p*-aminobenzoate in its competitive nature, and previous investigations have shown the bulk of such material from yeast to be *p*-aminobenzoate; it was not further characterized. The quantity of antagonists extracted was small and not appreciably altered by the presence of sulphanilamide; it was a small fraction of that obtainable by autolysis.

DISCUSSION

The foregoing experiments have demonstrated that there is not a 'displacement' by pantoyltaurine of the bulk of the pantothenate in streptococci, or of the bulk of substances behaving as *p*-aminobenzoate in streptococci and yeast, by sulphanilamide. This contrasts sharply with the effects to be anticipated from certain simple interpretations of the equilibria postulated to explain quantitative characters of the action and antagonism of the two inhibitors. If, however, the existence of equilibria during the functioning of pantoyltaurine and sulphanilamide be accepted, a divergence from the displacement which might be considered the immediate consequence of this hypothesis can be traced, by the following steps, to a definite feature in the action of the inhibitors.

(1) The divergence might be due to the bulk of the pantothenate or *p*-aminobenzoate of the organisms not functioning in the systems through which the inhibitors act. For this to be the case, > 80 % of the *p*-aminobenzoate of streptococci, > 95 % of the *p*-aminobenzoate of yeast, and > 95 % of the pantothenate of streptococci must belong to systems in which the inhibitors do not compete. The experiments with pantoyltaurine included some with streptococci containing minimal quantities of pantothenate, which might be supposed to be present in the most necessary systems, and with which very small quantities of pantoyltaurine interfere in growth. Against the view that pantothenate serves a multiplicity of essential functions, of which that sensitive to pantoyltaurine concerns only a small portion of the organisms' pantothenate, is the observation (McIlwain & Hughes, 1944) that a process of pantothenate metabolism, correlated with the action of pantoyltaurine on growth, is capable of a pantothenate turnover per hour some 50 times greater than that of the quantity of pantothenate in these cells, and is highly sensitive to pantoyltaurine. At least in this case, therefore, explanation (1) (with a reservation to be expressed later) appears to be inadequate; less direct evidence can be adduced to support a similar view concerning the *p*-aminobenzoate of streptococci.

(2) A further possibility is that in the present experiments the inhibitor may not reach the 'site of action', e.g. because of selective permeability. Pantoyltaurine, however, acts on a metabolic process of the organisms which is connected with its action on their growth, within 2 min. of its addition to suspensions identical with those of the present experiments (McIlwain & Hughes, 1944); and, indeed, one of these experiments (batch IV, Table 1) shows the process in operation. Sulphanilamide has been found to penetrate streptococcal

cells (Feinstone *et al.* 1942) and its action on growth is manifested within the period used in the present experiments.

(3) It is also possible that the *p*-aminobenzoate and pantothenate, though displaced from essential sites by the inhibitor, might remain otherwise attached to the organisms. In this case the substances would not appear in solution, but their functioning in non-proliferating cells would be disturbed by the inhibitor. Now although much evidence connects that functioning of pantothenate which is inhibited by pantoyltaurine with glycolysis (McIlwain & Hughes, 1944, 1945), non-proliferating streptococci continue their normal reaction with glucose in the presence of high concentrations of pantoyltaurine (McIlwain, 1944). Again, the many attempts which have been made to detect changes in major metabolic activities (such as O₂ uptake or CO₂ output) following addition of sulphonamides to non-proliferating suspensions of organisms whose growth is susceptible to sulphonamides, have largely failed to give results commensurate with the actions of the drugs on growth. Such negative evidence is, however, a less satisfactory basis for argument than the observation that metabolic defects have been demonstrated in cases in which some growth of the organisms in the presence of the drugs has been allowed (Kohn & Harris, 1941; Sevag & Shelburne, 1942; Henry, 1943).

(4) It is therefore suggested that, although *p*-aminobenzoate and pantothenate function in resting bacteria, these activities, when once the substances are incorporated in the organisms, are not influenced by sulphanilamide and pantoyltaurine, but that the reactions in which the inhibitors act are ones which result in the assimilation, or 'binding' in functioning form, of *p*-aminobenzoate and pantothenate. Evidence that such attachment is by chemical linkages is given by the stability of these substances in bacteria, and by the nature of the processes necessary to extract them. This suggestion agrees with all the foregoing evidence and also with the following points:

(a) *The existence of a latent period in the actions of pantoyltaurine and sulphanilamide on growth.* If only assimilation to give the functioning forms of *p*-aminobenzoate and pantothenate is affected, these 'bound' compounds will be available for daughter cells and will continue to perform their normal activities; but their quantity per cell will be reduced at each division which occurs in presence of the inhibitors. Effects are thus to be expected after a period of 'dilution' by growth, the period (which is found to be altered by many circumstances) depending on the adequacy of the original quantities of bound substance. This is consistent with Kohn & Harris's (1941) finding that the later effects of sulphonamides were not increased by prior exposure

of non-proliferating bacteria to the substances. This argument would apply both in the case of *p*-aminobenzoate, which is synthesized by many susceptible bacteria, and in the case of pantothenate, which is not; the production of compounds related to *p*-aminobenzoate can continue in the presence of sulphanilamide (Fox, 1942).

(b) *The bacteriostatic, as distinct from bactericidal, effect of the two inhibitors.* If the action of pantooyltaurine is to prevent assimilation of pantothenate, the organisms would be expected, when their growth is inhibited in the presence of both pantothenate and pantooyltaurine, to be in circumstances no more deleterious than those occasioned by absence of pantothenate. This results (McIlwain, 1944), with an otherwise adequate medium, in a gradual cessation of growth, but not in the rapid death produced by bactericidal agents. Although the extent to which streptococci might suffer from lack of *p*-aminobenzoate is unknown it would be unusual for a micro-organism to be killed rapidly by lack of a growth-promoting substance. Non-proliferating bacterial suspensions can show slow rates of 'natural' death (Wilson, 1922; Lodge & Hinshelwood, 1939), which may explain some apparent exceptions (e.g. Colebrook & Cawston, 1945) to the general observation that the sulphonamides are bacteriostatic.

(c) *Multiple antagonism to sulphonamide action.* Binding and functioning are here presented as two distinct and successive reactions of a possible series involving *p*-aminobenzoate, the first open to competition with sulphanilamide but the later ones not. Binding of *p*-aminobenzoate then becomes the 'primary reaction' of Harris & Kohn (1941) and bound *p*-aminobenzoate is to be looked upon as a product whose functioning can be partly aided or replaced by secondary antagonists, such as methionine and unknown compounds in peptone. The antagonism shown by these substances, as is necessary to the present scheme, has been found to differ considerably from that shown by *p*-aminobenzoate. Bound pantothenate, similarly, becomes the previously suggested (McIlwain & Hughes, 1945) product of pantothenate metabolism, normally formed in excess of the organism's minimal needs, and already believed to be part of the bacterial cell.

This conclusion respecting *p*-aminobenzoate unites two views of its functioning in susceptible micro-organisms, which have been regarded (Henry, 1943)

as mutually incompatible, namely, that it is both a substrate and plays a catalytic role. The first activity is now regarded as occurring primarily in growing cells and to be susceptible to inhibition by sulphonamides; the second activity is regarded as occurring independently of proliferation and to be insusceptible to sulphonamides. The dual aspects of the behaviour of *p*-aminobenzoate which have been separated here, perhaps artificially, make the proposition (1), above, true in one respect and untrue in another, according to whether or not growth is included in the functioning of bacteria; (2) is also ambiguous, as that activity of *p*-aminobenzoate which is inhibited by sulphanilamide is regarded as not operating in non-proliferating cells. Both propositions require the further specification, of a biological nature, which is provided by (3) and (4). An action of pantooyltaurine on the pantothenate of growing, as distinct from non-proliferating, cells has been demonstrated and will be reported later.

SUMMARY

1. Two strains of β -haemolytic streptococci, which needed added pantothenate for growth, normally contained 15–50 μ mol. pantothenate/g. dry matter, firmly bound to the bacterial substance. Larger quantities were more loosely associated with the organisms after their harvesting from pantothenate-rich media.

2. The firmly bound pantothenate was liberated on autolysis or enzymic digestion, but not by various reagents which included high concentrations of pantooyltaurine. The loosely attached pantothenate was released into saline solutions at 37°; pantooyltaurine did not decrease the quantity remaining with the organisms.

3. Sulphanilamide did not increase the small quantity of substances antagonizing sulphanilamide bacteriostasis, which were obtainable from streptococci and yeasts by saline solutions.

4. It is concluded, from this and previous evidence, that pantooyltaurine and sulphanilamide act as bacteriostatic agents by preventing the binding, in functioning form, of pantothenate and *p*-aminobenzoate respectively, by susceptible bacteria.

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Isolation from Liver of New Factors Essential for the Growth of *Lactobacillus helveticus* and *Streptococcus lactis* R.

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Snell & Peterson (1940), Stokstad (1941), Mitchell, Snell & Williams (1941), and O'Dell & Hogan (1943) have described the isolation, from various sources, of substances which stimulate the growth of *Lactobacillus helveticus* (*casei* ϵ) and *Streptococcus lactis* (*faecalis*) R. All of these workers used adsorption on norite and elution with ammoniacal solutions as an essential step in the process of isolation. The factor thus isolated by Mitchell *et al.* (1941) from spinach leaves was named folic acid.

Attempts have been made in the present work to prepare by similar methods a concentrate of folic acid, but using as starting material a precipitate obtained by the addition of ethanol to a concentration of 70% (v/v) during the manufacture of a commercial liver extract ('Plexan', made by Glaxo Laboratories Ltd.) by the method of Laland & Klem (1936). This precipitate was dissolved in water and the solution treated with norite. The adsorbate was eluted by ethanolic ammonia, following the procedure of Snell & Peterson (1940). The resulting eluate stimulated the growth of *L. helveticus* when, added to the medium of Landy & Dicken (1942) used for testing for folic acid.

The eluate was next adsorbed on Decalso (an ion-exchange adsorbent made by the Permutit Co. Ltd.), and the adsorbate eluted with hot 10% aqueous sodium chloride solution. Both eluate and filtrate stimulated growth of the test organism. It was also found that at least part of the active material was present in the precipitate formed when silver nitrate was added to the eluate at pH 7. By combining these three methods, there was prepared a concentrate of which 0.5 ml. gave good growth with *L. helveticus*; this concentrate contained under 100 μ g. total solids/ml.

Attempts to dissolve the active fraction in chloro-

form, preparatory to attempting partition chromatography (Martin & Synge, 1941), led to the discovery that most of the activity was extracted by chloroform from both acid (pH 3) and alkaline (pH 11) solutions, although folic acid is said to be insoluble in chloroform. Both the chloroform-soluble and chloroform-insoluble fractions were capable of stimulating the growth of *L. helveticus* and *S. lactis*, and the responsible factors are therefore provisionally designated factor HL1 and factor HL2 respectively. The latter may be identical with folic acid.

Extraction of a more refined liver extract ('Examen', made by Glaxo Laboratories Ltd.) by chloroform also gave an active fraction; this, on treatment with Decalso, gave an active filtrate and an eluate that was only slightly active. Similarly, when Examen was run through a column of Decalso and the filtrate and the hot 10% sodium chloride eluate were extracted with chloroform, the extract from the filtrate markedly stimulated the growth of *L. helveticus*, whilst the extract from the eluate gave only partial stimulation. The chloroform-soluble factors present in the Decalso eluate and filtrate are designated factors HL3 and HL4 respectively. When the two fractions were combined, growth equal to that given by the chloroform extract from Examen was obtained. It is possible that factor HL1 is a mixture of factors HL3 and HL4.

With one exception, factors HL1, HL3 and HL4 were not affected by acetylation, benzoylation, treatment with nitrous acid or boiling with water, dilute acid or dilute alkali. The activity of HL4 in the growth of *L. helveticus*, however, was largely destroyed by nitrous acid, but not the activity for *S. lactis*. Its synergistic action with factor HL3 towards either organism was not decreased.