CHEMICAL PRESERVATIVES IN FOODSTUFFS. III

Hexamethylenetetramine as mold inhibitor and the antagonistic action of amino acids

REINO R. LINKO and OLAVI E. NIKKILÄ

Central Laboratory of Tukkukauppojen Oy, Turku

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Hexamethylenetetramine is extensively used as a food preservative, primarily in fish products, where it has been found a particularly effective inhibitor of the growth of micro-organisms. NIKKILÄ (5), for example, established that hexamethylenetetramine at a concentration as low as 0.01 per cent completely arrests at both pH 5 and 7 the growth of bacteria (*Micrococcus, Sarcina, Achromobacter, Flavobacterium* and *Corynebacterium*) that are responsible for the spoilage of fish preserves. In earlier studies it has been reported that molds are much less susceptible to the action of hexamethylenetetramine than bacteria and yeasts. v. SCHELHORN (7) found that *Penicillium sp.* continues to grow in malt-peptone-broth at pH 7 even though the concentration of hexamethylenetetramine is as high as 5 per cent. Recent investigators have, however, presented results that are divergent in this respect. Thus, for example, NIKKILÄ and LINKO (6) observed that hexamethylenetetramine is a relatively strong inhibitor of many molds although differences were noted in the sensitivity of various strains to this agent.

The inhibitory properties of hexamethylenetetramine are due to the formaldehyde that is liberated when it decomposes. The decomposition proceeds very slowly in neutral media but is greatly speeded up when the acidity of the medium increases (9). The action of hexamethylenetetramine against microbes is also decisively influenced by the composition of the medium (6). For example, molds tolerate much higher hexamethylenetetramine concentrations when they grow in wort medium than when they grow in a synthetic medium. The greater resistance of molds may be due to the existence of various organic nutrients and growth factors in wort.

YAMADA (10) noted the antagonistic action of cysteine on the inhibition of growth of *Escherichia coli* by hexamethylenetetramine. He later demonstrated

that cysteine reacts with hexamethylenetetramine to form thiazolidine-4-carboxylic acid (11). According to the same author, other sulphydryl compounds such as thioglycollic acid and ethyl mercaptan do not have this antagonistic effect against hexamethylenetetramine in the same degree since they form addition compounds and not condensation compounds with formaldehyde.

The work described below relates to the antagonistic action of amino acids and some other compounds on the inhibition of mold growth by hexamethylenetetramine.

Methods

The isolation, cultivation and identification of the molds were described in a previous publication by the authors (6). The following four molds were employed in the study: A_5 *Aspergillus niger*, C_1 *Cladosporium herbanum*, P_1 *Penicillium expansum*, P_5 *Penicillium viridatum*.

Spore suspensions were prepared in the usual manner. The number of spores was determined by the Ellerman method in a Bürker counting chamber (3). The spore suspension was then diluted to contain about 5 million spores per milliliter.

Czapek medium buffered to pH 4.55 with McIlvaine's phosphate-citrate buffer was used in all the experiments. The antagonistic effects of the laevorotatory forms or racemic mixtures of 21 amino acids, glutathione and thioglycollic acid on the inhibition of mold growth and respiration by hexamethylenetetramine were studied. The hexamethylenetetramine was employed at the so-called threshold concentration, the concentration which is just sufficient to prevent the growth of the mold or in which no respiration is observed.

In the growth experiments each of the amino acids was added to give a concentration of 0.05 per cent to the medium containing the threshold concentration of hexamethylenetetramine and the resulting medium was inoculated with the spore suspension. The cultures were incubated in the dark at room temperature for 12 days. The degree of growth was graded from 0 to 3 and the occurrence of spore formation indicated by a plus (+) sign.

The respiration studies were carried out with *P. viridatum* in the presence of hexamethylenetetramine at a respiratory threshold concentration of 0.0007 molar (0.01 per cent). Since six biologically active formaldehyde molecules are liberated when hexamethylenetetramine decomposes, the potential formaldehyde concentration in the solutions was 0.0042 molar. The amino acids and the other antagonistic agents were added to the solutions in amounts that gave the same molar concentration (0.0042 M) of each. Exceptions were two series of experiments in which the influence of the amino acid concentration was particularly studied. The respiration experiments were conducted in a Warburg apparatus. The manometer liquid was ethyl lactate dyed with Brilliant Green. The total volume of culture medium was 3.2 ml including 0.2 ml of 10 per cent potassium hydroxide to absorb the carbon dioxide formed. The gas space was filled with pure oxygen. The temperature of the thermostat was 25°C. The quantity measured was the total oxygen consumption or the rate of oxygen consumption during the germination.

Table 1. The effect of amino acids and sulphydryl compounds on the growth of molds in a medium containing the threshold concentration of hexamethylenetetramine.

Culture medium: Czapek's solution + 3 per cent saccharose + 0.047 M phosphate + 0.027 M citrate (pH = 4.55). Incubation: 12 days at 20° C.

Amino acid [1] (0.05 per cent)	*Aspergillus niger* (*A5*)	*Cladosporium herbanum* (*C1*)	*Penicillium expansum* (*P1*)	*Penicillium viridatum* (P5)
	Threshold concentration of hexamethylenetetramine			
	0.02 %	0.05 %	0.03 %	0.03 %
Control (no amino acid added)	0	0	0	0
d, l-Alanine			0	0
l-Arginine			0	0
d, l-Aspartic acid		0	0	0
l-Cysteine	2+	2+	2+	1+
l-Cystine			0	0
l-Glutamic acid		0	0	0
Glutathione	0	1	0	0
Glycine			0	0
l-Histidine		(1)	0	0
d, l-Isoleucine	0	0	0	0
l-Leucine		0	0	0
l-Lysine	0	1	0	0
d, l-Methionine			0	0
d, l-Norleucine			0	0
l-Ornithine	0	0	0	0
l-Phenylalanine		0	0	0
d, l-Proline	0	0	0	0
d, l-Serine			0	0
Thioglycollic acid	2	0	2	1
d, l-Threonine	0	0	0	0
l-Tryptophan	2	3+	1+	1
l-Tyrosine			(1)	0
d, l-Valine			0	0

[1] l-Cystine 0.01 per cent

Results

Growth experiments. The results of the growth experiments with *Aspergillus niger, Cladosporium herbanum, Penicillium expansum* and *P. viridatum* are collected in Table 1. From the table it will be seen that all strains exhibited a strong growth after a 12-day incubation in the media containing the threshold concentration of hexamethylenetetramine and l-cysteine or l-tryptophan; most of them had also formed spores in abundance. Thioglycollic acid was an effective antagonist in the case of all molds except *C. herbanum*. Glutathione, l-histidine, and l-lysine led to a weak growth of the last-mentioned mold and l-tyrosine to a

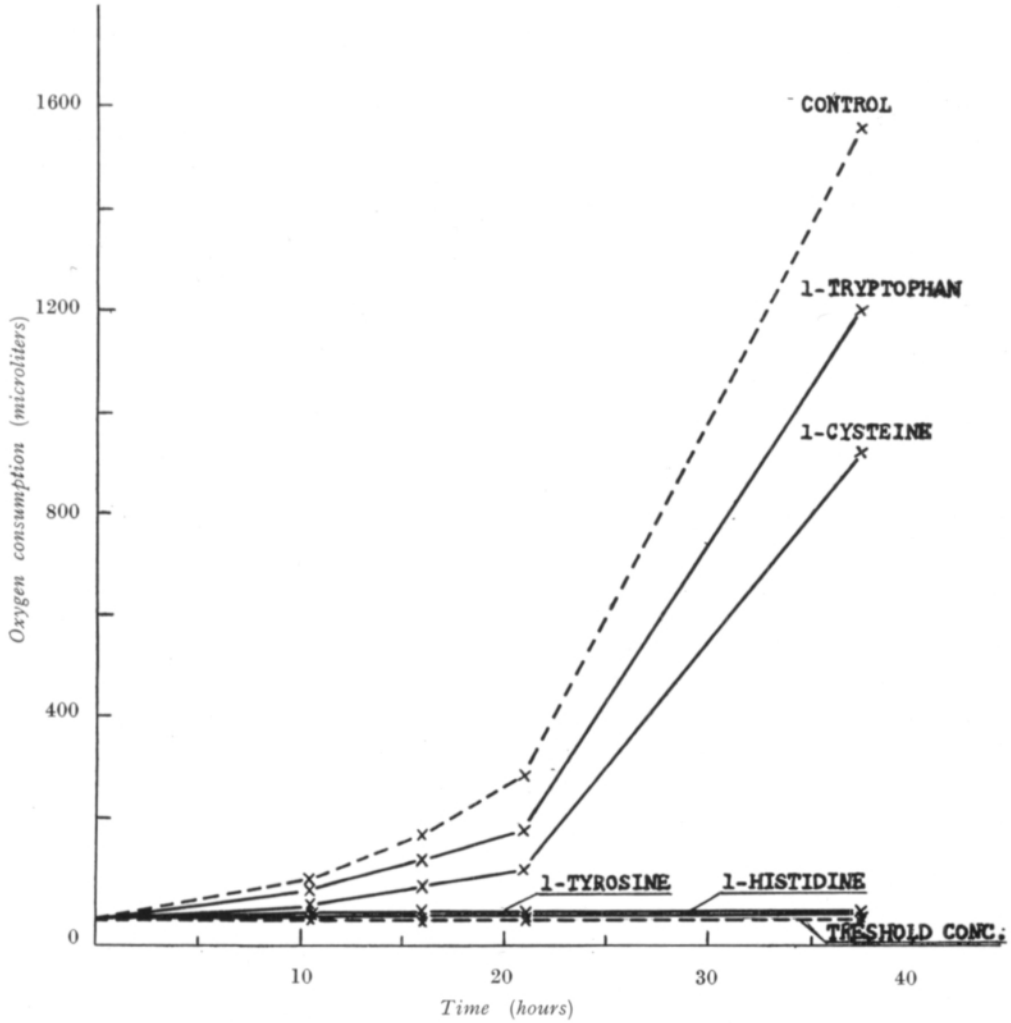

Fig. 1. The effect of amino acids on the respiration of P. viridatum in the presence of the threshold concentration of hexamethylenetetramine. Culture medium: Czapek's solution + 3 per cent saccharose + 0.094 M phosphate + 0.054 M citrate (pH = 4.55). Concentration of hexamethylenetetramine 0.0007 M; concentration of amino acids 0.0042 M. Temperature 25°C.

weak growth of *P. expansum*. The other amino acids, on the other hand, were unable to counteract the inhibitory action of hexamethylenetetramine.

Respiration experiments. The respiration studies were carried out to determine the effects of the antagonistic agents on the inhibition of germination of *P. viridatum* by hexamethylenetetramine. Fig. 1 shows the variation of the total oxygen consumption by *P. viridatum* in the presence of l-tryptophan, l-cysteine, l-tyrosine or l-histidine and, in all cases, the threshold concentration of hexamethylenetetramine. For comparison, curves showing the rate of oxygen consumption in the absence of inhibitor and antagonist (control curve) and in the

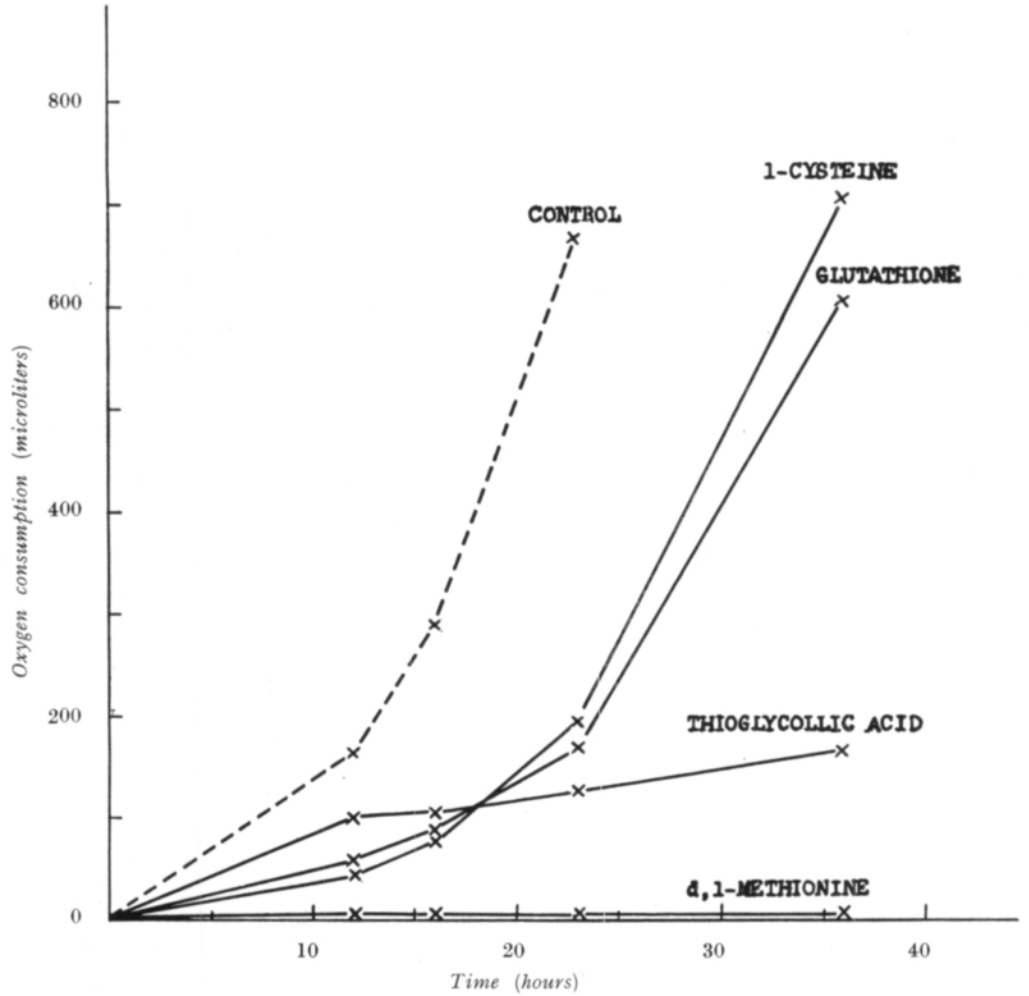

Fig. 2. The effect of organic sulphur-containing compounds on the respiration of P. v i r i d a t u m in the presence of the threshold concentration of hexamethylenetetramine. Culture medium: Czapek's solution + 3 per cent saccharose + 0.094 M phosphate + 0.054 M citrate (pH = 4.55). Concentration of sulphur-containing compound 0.0042 M. Temperature 25°C.

presence of the threshold concentration of the inhibitor are also drawn in the figure. In the latter case the respiration was completely inhibited. The addition of l-tryptophan has, however, eliminated the inhibition by hexamethylenetetramine in such a degree that the oxygen consumption curve runs almost as high as the control curve, just as if no inhibitor had been added. l-Cysteine was almost as effective as l-tryptophan, whereas l-tyrosine and l-histidine were unable to counteract the effect of hexamethylenetetramine.

Other sulphur-containing compounds than l-cysteine that were studied were glutathione, thioglycollic acid and d,l-methionine.

The curves in Fig. 2 show that thioglycollic acid has the greatest antagonistic effect against the influence of hexamethylenetetramine during the first twelve

Table 2. The effect of l-tryptophan and sulphydryl compounds on the respiration of *P. viridatum*. No inhibitor added. Culture medium: Czapek's solution + 3 per cent saccharose + 0.094 M phosphate + 0.054 M citrate (pH = 4.55). Concentration of added compound: 0.0042 M. Temperature 25°C.

Time in hours	Difference in total oxygen consumption (control minus test) microliters of oxygen			
	l-Tryptophan	l-Cysteine	Glutathione	Thioglycollic acid
11	— 1	— 91	— 14	+ 112
15		— 202	— 71	+ 97
20		— 372	— 150	+ 40
24	— 240	— 608	— 262	— 59

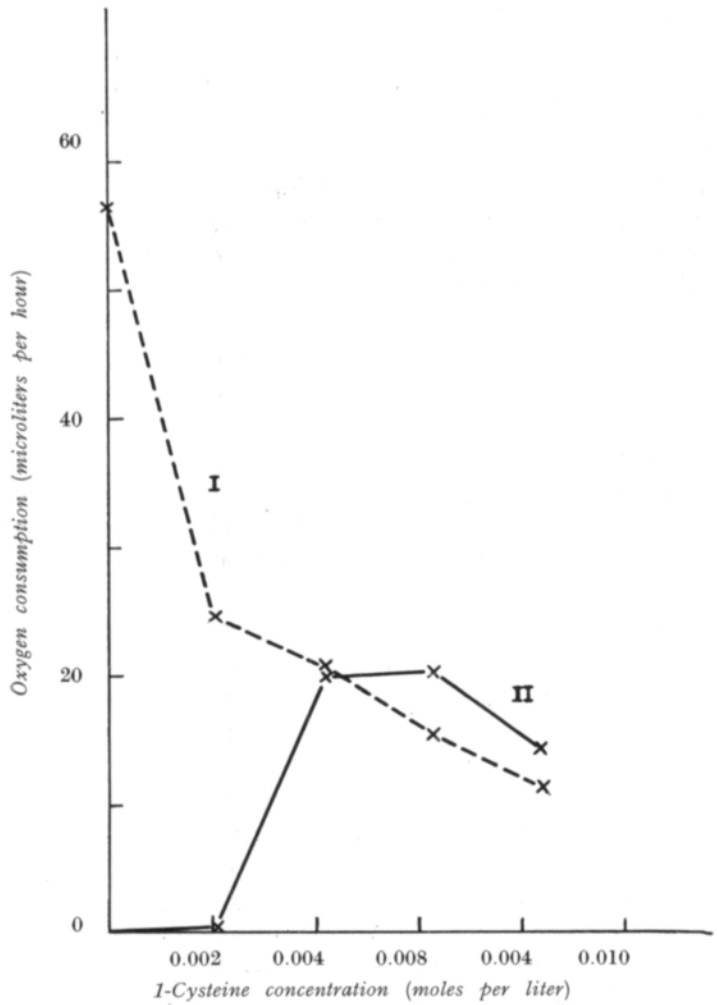

Fig. 3. The variation of the respiration of P. v i r i d a t u m with increasing cysteine concentration in the presence (curve II) and absence (curve I) of hexamethylenetetramine. Culture medium: Czapek's solution + 3 per cent saccharose + 0.094 M phosphate + 0.054 M citrate (pH = 4.55). Concentration of hexamethylenetetramine 0.0007 M. Temperature 25°C.

hours. This effect then rapidly diminishes until after 18 hours it equals that of l-cysteine and glutathione. Subsequently the respiration increases very slowly in the medium containing thioglycollic acid, and rapidly in the media containing l-cysteine and glutathione. Addition of methionine was unable to counteract the inhibition of mold growth by hexamethylenetetramine.

The effect of l-tryptophan, l-cysteine, glutathione and thioglycollic acid on the respiration of spores of *P. viridatum* was also studied without an inhibitor being present. The data in Table 2 give the differences in the total oxygen consumptions as compared with the control experiments after various intervals during 24 hours. It will be seen that the respiration remained the same in the medium containing tryptophan as in the control medium during eleven hours. After 24 hours the respiration was slightly weaker in the former medium than in the control medium. Thioglycollic acid strongly promotes respiration in the very first stages of germination, but its effect gradually weakens until after 24 hours the respiration is weaker in its presence than in the control medium. Cysteine and glutathione retard respiration from the beginning, the former more than the latter.

The results of the study of the respiration of the spores of *P. viridatum* during 24 hours in media containing various concentrations of l-cysteine both with and without hexametylenetetramine are plotted in Fig. 3. Curve I shows that l-cysteine retards the respiration even when no inhibitor is present. The intensity of the respiration diminishes rapidly until the l-cysteine concentration rises to 0.002 M after which it becomes slower and more linear. When the cysteine concentration is 0.008 M, the respiration is only 20 per cent of that in its absence. With hexamethylenetetramine present the respiration curve II for cysteine has an altogether different form. Low concentrations of cysteine have no visible effect and the respiration is practically at a standstill up to a cysteine concentration of 0.002 M. At concentrations higher than this the curve begins to rise abruptly until the cysteine concentration reaches that of the formaldehyde available from the hexamethylenetetramine when the respiration attains a maximum; a further increase in the cysteine concentration leads to a respiration curve that resembles the curve with no inhibitor present.

The corresponding respiration curves recorded with l-tryptophan are drawn in Fig. 4. Also in this case the curve (I) plotting the respiration with increasing amino acid concentration with no inhibitor present gradually falls, but the effect of l-tryptophan is weaker that that of l-cysteine. The respiration intensity no longer decreases appreciably after the tryptophan content has been increased to 0.006 M. With hexamethylenetetramine present at the threshold concentration (curve II), the antagonistic effect of l-tryptophan becomes apparent only after the concentration of the latter has been increased to 0.002 M; the respiration rate rises slowly up to a concentration of 0.004 M and very rapidly up to a concentration of 0.006 M, but then gradually diminishes as the amino acid concentration is increased further. At the highest tryptophan concentrations (0.006—0.0125 M) the respiration rate is still higher in the medium containing inhibitor than in the medium containing no inhibitor.

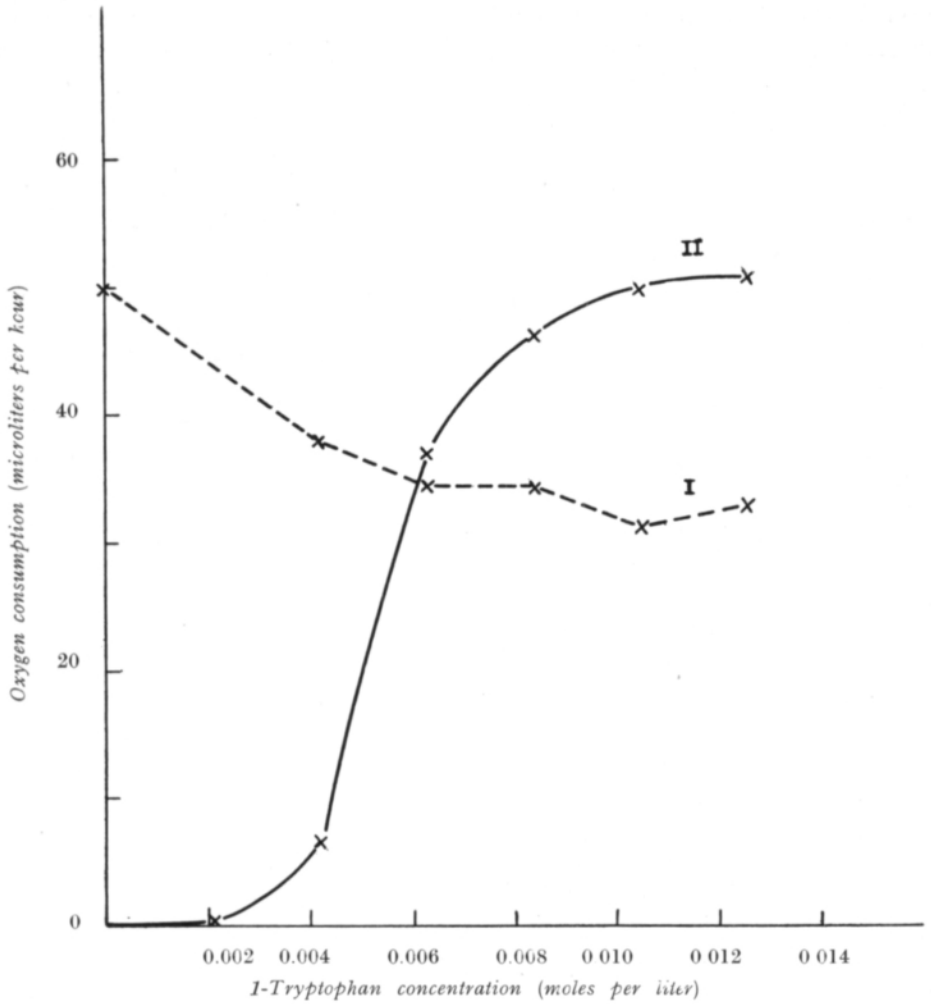

Fig. 4. The variation of the respiration of P. viridatum with increasing tryptophan concentration in the presence (curve II) and absence (curve I) of hexamethylenetetramine. Culture medium: Czapek's solution + 3 per cent saccharose + 0.094 M phosphate + 0.054 M citrate (pH = 4.55). Concentration of hexamethylenetetramine 0.0007 M. Temperature 25°C.

Discussion

The results of the present study show that the respiration and growth of all the examined mold strains in Czapek's medium at pH 4.55 are inhibited by fairly low concentrations of hexamethylenetetramine. The experiments in which the effect of various amino acids and sulphur-containing compounds on the inhibition of respiration and growth by hexamethylenetetramine was studied revealed that only l-tryptophan and the sulphydryl compounds l-cysteine, glutathione and thioglycollic acid effectively counteracted the inhibition by hexamethylenetetramine present at the threshold concentration. Of the four active agents cysteine and

tryptophan promoted the growth of all the examined molds in the presence of the inhibitor (Table 1), glutathione only the growth of *C. herbanum* and thioglycollic acid the growth of all the molds except the last-mentioned. In the study of the respiration rate of *P. viridatum*, l-cysteine and glutathione were found equally effective growth promoters, while thioglycollic acid was more effective than either of these sulphur containing compounds in the early stages of germination but less effective in the later stages (Fig. 2). These differences are readily understood when the respiration in the absence of the inhibitor is examined (Table 2). Of the three compounds only thioglycollic acid was able to accelerate the respiration rate from the level seen in the control experiment, but this effect gradually weakened with time until after 24 hours when the respiration rate was retarded. l-Cysteine and glutathione suppressed the respiration of the mold already from the beginning. The suppression by cysteine increased with its concentration in the medium (curve I, Fig. 3). This inhibition of mold growth by cysteine has been observed by other investigators. For instance, Steinberg (8) found in extensive studies that certain amino acids, and cysteine in particular, inhibit the growth of *A. niger*, even in the presence of ammonium nitrate. No satisfactory explanation for this action has been advanced although it is known that a large excess of an amino acid interferes with the participation of other amino acids in the nitrogen metabolism of an organism. Since, despite its ability to suppress mold growth, cysteine counteracted the inhibition of mold growth by hexamethylenetetramine, it must be concluded that these two compounds react with each other whereupon both compounds are inactivated. This conclusion is supported by curve II in Fig. 3, which shows that the inhibition of the respiration of the mold spores by hexamethylenetetramine is counteracted by cysteine, but only as long as the concentration of the latter remains below that of the formaldehyde derived from hexamethylenetetramine. When the cysteine concentration is increased further, the inhibitory properties of the compound become evident.

The observations of this study thus indicate clearly that cysteine and the other compounds containing a sulphydryl group check the inhibition of mold growth by hexamethylenetetramine. On the other hand, sulphydryl groups play an extremely important role in the metabolism of micro-organisms. Many enzymes such as dehydrogenases and transaminases require sulphur in the reduced state before they can function (1). This is suggested also by the observation made in this study that thioglycollic acid promotes the respiration of mold spores in the early stages of germination. It is thus possible that the inhibitory effect of hexamethylenetetramine or its decomposition product formaldehyde on the growth of the mold cell involves at least partly the inactivation of the sulphydryl centers of the protein of the enzyme and that this inactivation is prevented by adding sulphydryl compounds.

It has already been noted that also l-tryptophan greatly suppresses the inhibitory action of hexamethylenetetramine. This was seen in the growth experiments with *A. niger*, *C. herbanum*, *P. expansum* and *P. viridatum* (Table 1) and in the respiration studies carried out with the last-mentioned mold (Figs. 1 and 4). Tryptophan alone did not promote the respiration of spores of *P. viridatum*; on the contrary,

it reduced the respiration rate in proportion to its concentration (curve I, Fig. 4). In this case an excess of amino acid appears to be detrimental. The antagonistic effect of tryptophan against hexamethylenetetramine differs, however, decisively from that of cysteine. The rate of oxygen consumption in the presence of tryptophan rises continuously with increasing tryptophan concentration to that in the control experiment with no hexamethylenetetramine or antagonist present (curve II, Fig. 4). In the presence of cysteine, however, the maximum respiration rate does not rise higher than to one-third of the rate in the control experiment (curve II, Fig. 3). In the latter case the respiration rate is at a maximum when the cysteine concentration is equal to that of the formaldehyde derived from hexamethylenetetramine. With tryptophan the respiration rate at this concentration is only about one-tenth of the maximum rate attained at higher concentrations.

The reason for the antagonistic effect of tryptophan may, as in the case of cysteine, be attributed with its reaction with hexametylenetetramine or, preferably, with the biologically active decomposition product of the latter. Tryptophan is known to react with formaldehyde over a fairly wide pH range with the formation of 3,4,5,6-tetrahydro-4-carbolin-5-carboxylic acid (2).

The reaction with the inhibitor cannot alone explain the antagonistic effect of tryptophan since in the first place the antagonistic effect of this compound increases manyfold even after its concentration has exceeded the formaldehyde concentration. Secondly, histidine could be expected to behave like tryptophan (4), but it was found not to have counteracted the effect of hexamethylenetetramine in the experiments that were carried out (Table 1 and Fig. 1).

The observed antagonism between tryptophan and hexamethylenetetramine implies, however, that the latter compound disturbs the tryptophan equilibrium within the micro-organism. This observation may be of considerable importance since tryptophan is a precursor of nicotinic acid, which in turn is essential for the function of dehydrogenases.

Summary

Growth and respiration tests carried out on *Aspergillus*, *Cladosporium* and *Penicillium* molds have shown that certain amino acids such as l-tryptophan and l-cysteine and certain sulphydryl compounds such as glutathione and thioglycollic acid counteract the inhibitory action of hexamethylenetetramine. In the absence of hexamethylenetetramine, these compounds inhibit the growth of the molds.

The dependence of the antagonistic action on the amino acid concentration differed for l-cysteine and l-tryptophan. The respiration of the mold was a maximum when equimolar concentrations of l-cysteine and of the formaldehyde formed by the decomposition of hexamethylenetetramine were present in the culture media. The antagonistic effect of l-tryptophan increased continuously with increasing concentration until the rate of oxygen consumption was the same as in the control test with no hexamethylenetetramine or antagonist present.

The antagonistic effect of these four compounds on hexamethylenetetramine or on formaldehyde is evidently due to chemical interaction.

The inhibitory effect of hexamethylenetetramine on the microbial cell may at least partly be due to its ability to inactivate enzymes such as the dehydrogenases.

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SELOSTUS:

ELINTARVIKKEIDEN KEMIALLISISTA SÄILÖNTÄAINEISTA. III.

Heksametylentetramiini homeinhibiittorina ja aminohappojen antagonistinen vaikutus

REINO R. LINKO ja OLAVI E. NIKKILÄ

Tukkukauppojen Oy, Turku

Uusimmat tutkimukset ovat osoittaneet, että heksametylentetramiini on melko voimakas homeinhibiittori (6). Sen inhibitiovaikutuksen tehokkuus riippuu mediumin happamuusasteesta ja ravintoainekoostumuksesta. Määrättyjen aminohappojen ja eräiden rikkiyhdisteiden todettiin olevan heksametylentetramiinille antagonistisia aineita. Tutkimukset suoritettiin *Aspergillus-*, *Cladosporium-* ja *Penicillium* -sukuihin kuuluvilla neljällä homekannalla heksametylentetramiinin ns. rajaväkevyydessä, jossa kantojen kasvu tai hengitys oli estynyt. Antagonistiset aineet voitiin tällöin todeta homeiden kasvun tai hengityksen elpymisenä. Tutkituista 23 yhdisteestä, joista 21 aminohappoa, olivat heksametylentetramiinille selvästi antagonistisia aineita vain l-tryptofaani ja sulfhydryyliryhmän sisältävät l-kysteiini, glutationi ja tioglykolihappo. Näistä tryptofaani ja kysteiini aikaansaivat kaikkien tutkittujen homekantojen kasvun heksametylentetramiinin rajaväkevyydessä, glutationi vain *C. herbanum*in ja tioglykolihappo kaikkien muiden paitsi mainitun kannan kasvun. Hengityskokeissa *P. viridatum*illa olivat tryptofaani, kysteiini ja glutationi likipitäen yhtä tehokkaita antagonisteja heksametylentetramiinille, mutta tioglykolihappo oli näitä huomattavasti heikompi.

Kaikki edellä mainitut neljä antagonistista ainetta estivät ilman heksametylentetramiinia homeiden hengityksen, kysteiini voimakkaimmin, seuraavina glutationi ja tryptofaani. Ainoastaan tioglykolihappo kiihdytti aluksi homeiden hengitystä, mutta sen vaikutus heikkeni kuitenkin vähitellen ja muuttui 24 tunnin kuluttua hengitystä hidastavaksi.

Tutkittaessa aminohappoväkevyyden vaikutusta *P. viridatum*in hengitykseen heksametylentetramiinin läsnäollessa todettiin, että hengitysintensiteetti oli maksimissaan kun kysteiinin ja heksametylentetramiinin hajoamistuloksen formaldehydin väkevyydet olivat ekvimolaariset. Suuremmat kysteiinimäärät hidastivat homeen hengitystä. Tryptofaani suhtautui kuitenkin toisella tavalla. Sen antagonistinen vaikutus kohosi nimittäin jatkuvasti väkevyyden kasvaessa aina alkuperäisen, inhibiittoria ja sen antagonistia sisältämättömän kontrollin tasolle asti pysyen senjälkeen muuttumattomana.

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