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Growth Inhibitory Effect of Extracts from Milt (Testis) of Different Fishes and of Pure Protamines on Microorganisms

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INTRODUCTION

In the course of investigation of the vitamin B_6 content of fish and fish organs according to the method of ATKINS et al. (1943) employing *Saccharomyces carlsbergensis* as test-organism, extracts from the milt (testis) or so-called "soft roe" from herring (*Clupea harengus*) and mackerel (*Scomber scombrus*) gave negative results. This indicated the absence of vitamin B_6 in this organ, a condition not likely to be encountered. Thus milt from cod (*Gadus morrhua*) gave normal analytical response, and showed a content of 0.11 μg vitamin B_6 per g fresh weight. The possibility of a growth inhibitor in the milt ("soft roe") from herring and mackerel thus had to be considered.

Compared with the milt from cod, this organ in herring and mackerel is relatively fat. Fatty acids are known to interfere markedly in assays with lactobacilli (SNELL, 1950), and similar effect of factors in the fat on *Saccharomyces carlsbergensis* might have been observed. Samples of milt from herring were tested fresh and extracted with acetone in the fresh state, as well as boiled and extracted with acetone after boiling. All four samples failed to give growth response in the vitamin B_6 assay with *Saccharomyces carlsbergensis*, thus an inhibitor specially related to the fat fraction could be excluded. A preliminary report has been given of these findings (BRÆKKAN & BOGE, 1959).

The next possibility was the possible effect of protamines or its breakdown products. A sample of clupeine was treated simultaneously with the samples of milt, i.e. with autoclaving at 120° C for 4 hrs. with about 0.055 N H_2SO_4. The extract from the clupeine did not at any concentration inhibit the growth, while the extracts from the milt as usual inhibited the growth in the vitamin B_6 assay.

The possibility of a competitive inhibition of vitamin B_6 by an antimetabolite had to be considered. Experiments, however, showed the inhibition to be non-competitive. Attempts to isolate the growth inhibiting substance was then carried out. After a series of experiments and employment of different techniques, a substance was isolated which showed to be a protamine. Considering the above referred experiment

with pure clupeine, the finding was unexpected. The isolation experiments led to a simple method for the extraction and precipitation of a fairly pure raw protamine. The procedure has been reported in detail. Inhibitory studies on *Saccharomyces carlsbergensis* indicated a possible method for the control of purity of a protamine-sample and for the assaying of protamine activity in milt from different species.

The growth inhibitory effect of protamines on *Saccharomyces carlsbergensis* further made it of interest to investigate the possible effect on other microorganisms, mainly such employed in vitamin assays. The results of these studies have been reported.

EXPERIMENTAL

a. Inhibitory studies with raw extracts.

All the inhibition studies with raw extracts were carried out on *Saccharomyces carlsbergensis* with the employment of the method described by ATKIN et al. (1943). The experimental volume, however, was reduced to 5 ml in tubes of the size 16 × 160 mm. We have found this volume to give practical advantages and precision at least equal to the original recommendations of 10 ml medium in tubes of the size 25 × 200 mm.

When the extracts of the milt from herring and mackerel were assayed, they showed no growth promotion for dilutions corresponding to a possible content of 0.1—1.0 μg vitamin B_6 per g sample.

Experiments were then carried out with addition of pyridoxine and pyridoxal in concentrations equal to the standard to different sample concentrations. In Fig. 1 is reported an experiment in which 2.5 mμg pyridoxine was added per ml of sample-extract of concentrations corresponding to 2400, 720, 240, 120 and 60 μg herring milt/ml. Each dilution was tried at six different levels. It may be pointed out that the proportion between sample and added pyridoxine is equal for all doses of each sample-concentration. No growth could be observed for the concentration 2400 and 720 μg milt. For the concentration 240 μg growth could be observed in the tubes with less than 120 μg milt, whereas for the concentration 120 μg growth was observed up to 180 μg milt per tube. Finally the concentration 60 μg gave normal growth, but somewhat higher than for the standard, with the exception of the doses 2.5 ml, equal to 150 μg milt in the tube. The results thus indicated that herring milt contain both growth stimulating and growth inhibiting factors, and that the growth stimulating factors was vitamin B_6 as this was the only factor omitted in the medium. Corresponding experiments with pyridoxal gave identical findings.

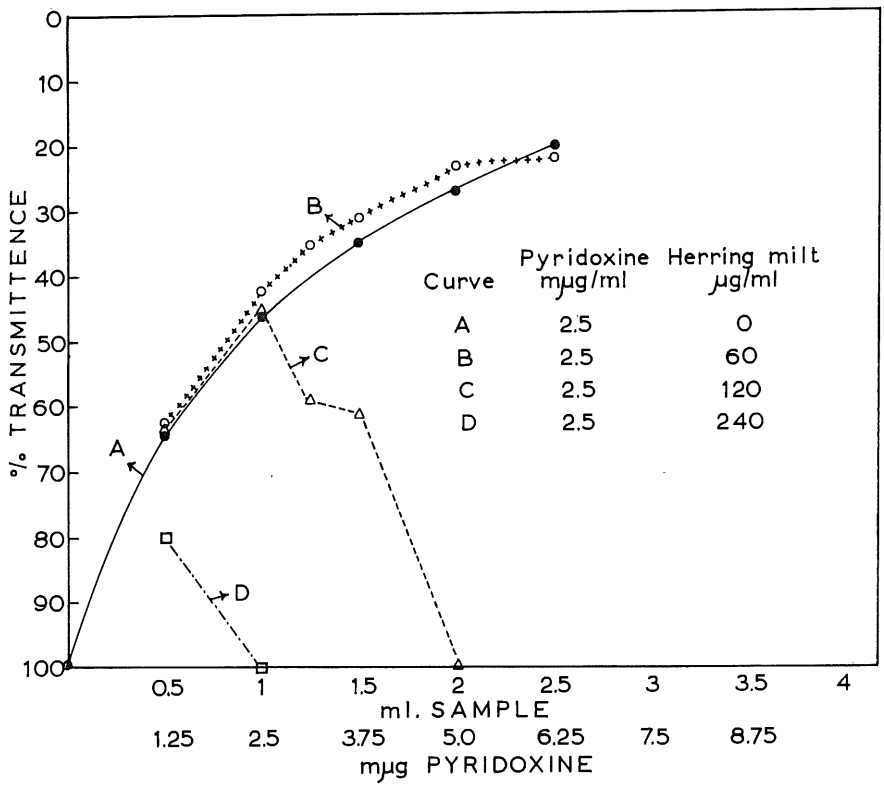

Fig. 1. Growth curves for *Saccharomyces carlsbergensis* grown on media containing 2.5 mμg pyridoxine per ml and different concentrations of herring milt extract.

Experiments were then carried out to find if large additions of pyridoxine could prevent the growth inhibiting effect of the milt-extracts. The results are reported in Fig. 2. A "growth blank", equal to 3 mμg pyridoxine per tube was included, and the response was measured against an inoculated medium blank. The results showed that addition of pyridoxine gave growth response for sample concentration up to 160—200 μg per tube. The growth response for the sample with addition of 3 mμg pyridoxine was higher than for the "growth blank", which indicated that the herring milt contained additional growth factor. It may further be noted that addition of up to 200 mμg pyridoxine failed to prevent growth inhibition for sample concentrations exceeding 160—200 μg per tube.

As a different approach similar studies were carried out in the Warburg-apparatus. The growth response measured as CO_2-production, might disclose special relations between inhibition and extract-concen-

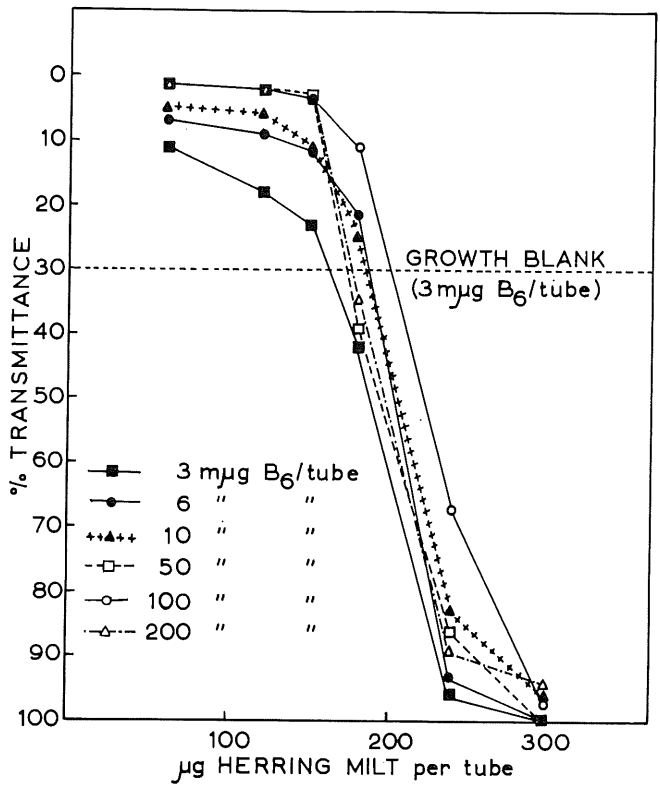

Fig. 2. Growth response of *Saccharomyces carlsbergensis* as related to different concentrations of pyridoxine and extracts of herring milt.

tration as related to the time. The substrate was the same as the basal medium in the micro-biological assay, and each flask was filled with 3 ml. *Saccharomyces carlsbergensis* was added as 0.3 ml dilute inoculum to each flask.

One experiment had the following design: The concentration of pyridoxine was kept at 3.75 mμg per flask, and the sample concentration applied at the doses: 20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 μg herring milt per flask. In addition was run a "growth blank" containing the vitamin but without addition of milt-extract, and a medium blank without addition of pyridoxine as well as sample. The results are summarized in Fig. 3. All flask gave the same CO_2-production for first 180 min. Concentration of 20, 40 and 60 μg herring milt per flask showed equal growth as measured by CO_2-production, concentration of 80, 100 and 120 μg gave response equal to the "growth blank", whereas higher

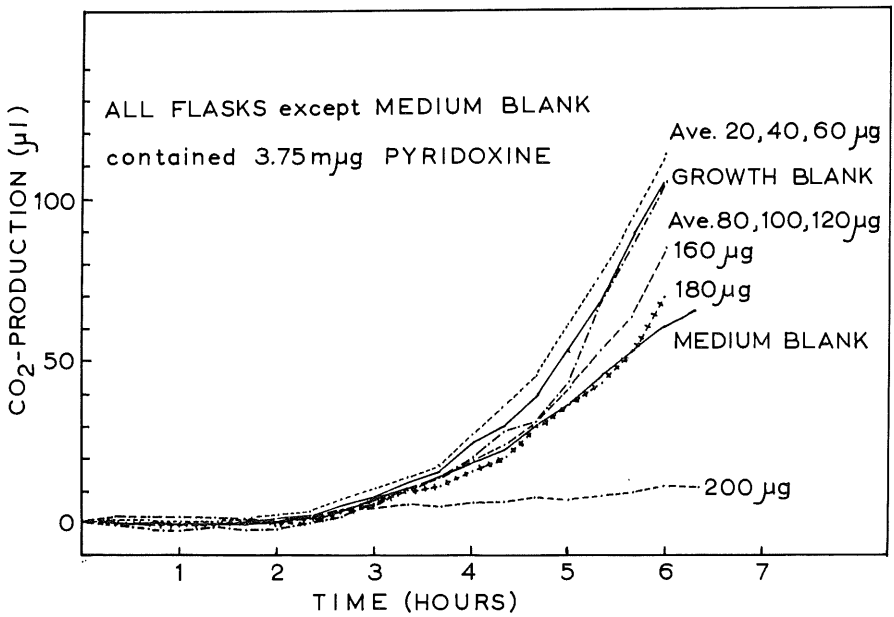

Fig. 3. Manometric measurement of growth response of *Saccharomyces carlsbergensis* in Warburg flasks containing 3.75 mμg pyridoxine and different concentrations of herring milt extract.

concentrations and especially 200 μg sample per flask gave stronger inhibitions. In general, the results agree with the finding in the ordinary microbiological assay.

Next was carried out a similar experiment in which the herring-milt extract was kept at 200 μg per flask, while the vitamin concentration was varied according to the doses: 10, 50, 100, 150, 200, 250, 300, 350 and 400 mμg pyridoxine per flask. In addition was run a "growth blank" and a "medium blank" as in the preceding experiment. The results are reported in Fig. 4. Up to a time of 9 hrs, there was growth inhibition in all flasks with herring-milt extract.

In general, from all these experiments, could be concluded that the herring milt extract contained a non-competitive growth inhibitor for yeast (*Saccharomyces carlsbergensis*). The results also indicated the presence of vitamin B_6 in the herring milt.

Another possibility for the assay of vitamin B_6 was the application of the method using *Streptococcus faecalis*, (RABINOWITZ & SNELL, 1947). This test-organism only responds to pyridoxal + pyridoxamine. Analyses at different concentrations showed normal response, and a content of about 1 μg pyridoxal + pyridoxamine per g milt was found.

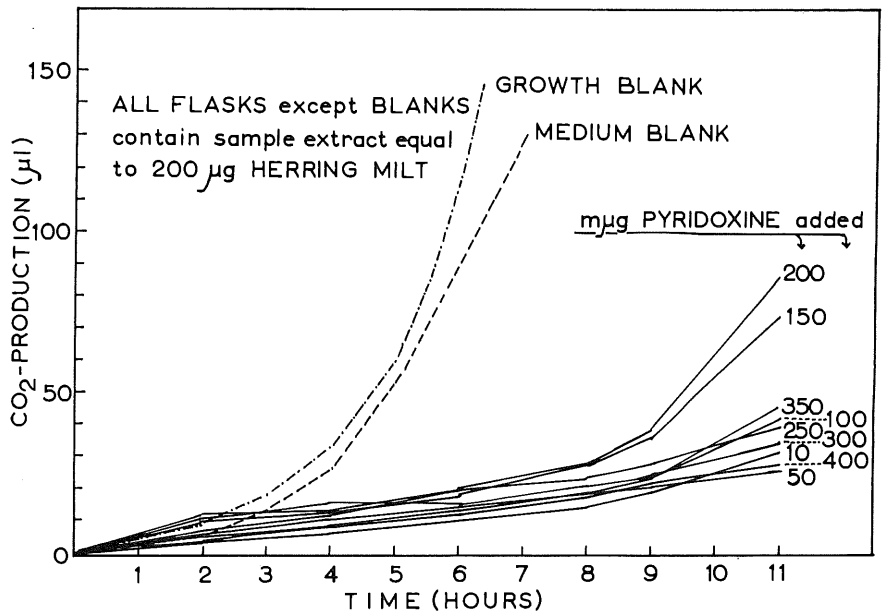

Fig. 4. Manometric measurement of growth response of *Saccharomyees carlsbergensis* in Warburg flasks containing extracts equal to 200 mµg herring milt and different concentrations of pyridoxine.

b) *Isolation of the inhibitory substance (protamine).*

Experiments were carried out to isolate the inhibitory substance in the milt extract. To gain some general information as to the character of the substance, different separation techniques were tried on the raw milt extracts. The normal extraction procedure involves addition of 100 ml 0.055 N hydrochloric acid or sulphuric acid per gm sample, followed by autoclaving at 15 lb pressure for 4 hours. To obtain a more concentrated extract with regard to the inhibitory substance, a ratio of one part milt to ten parts of acid was used. The raw extract used in the present study was prepared as follows: To 150 gm homogenized herring milt was added 1500 ml about 0.055 N sulphuric acid, and the mixture was autoclaved at 15 lbs pressure for 4 hrs. After cooling the pH was adjusted to 5.5, and then readjusted to pH 4.5. Thus a better precipitation of protein and other materials was obtained. The mixture was finally diluted to 1600 ml, and filtered through Schleicher & Schüll filter paper No 588. The clear raw extract thus obtained was used in the further studies.

Paper chromatography was tried on Whatman No 1 paper with the system tert.-butanol: 90% formic acid: water (70: 15: 15). Several spots

could be detected with ninhydrin reagent. Microbiological test of paper-strips showed that the inhibitory substance remained at the starting point, which showed no color reaction with ninhydrin. Thus the possibility of the inhibiting substance being an amino acid or a peptide could be excluded.

When 100 ml raw extract was treated by shaking with 5 gm Norit charcoal for 30 min, the filtrate showed no inhibitory properties, indicating a total absorption of the active substance.

Addition of ethanol to a final concentration of 80% gave a total precipitation of the inhibitory substance. Based on this finding stepwise precipitation was carried out (Table 1). Precipitates and filtrates were tested on growth inhibitory properties (Table 2). This experiment has been reported in detail, as it represents a convenient method for the preparation of a pure raw protamine. The results showed that the precipitates I—III from the 15—30% fractions gave strongest growth inhibition. Precipitate I showed total inhibition at a dilution to 100,000, precipitate II at a dilution to 50,000, whereas precipitate III showed partial inhibition at a dilution to 20,000. The sticky appearence of the precipitates incidated that the inhibitory substance was clupeine, as a sticky liquid had been described in the purification steps for protamines (KOSSEL, 1898; WALDERSCHMIDT—LEITZ et al., 1931, GUTERNATSCH, 1957). Assuming that the inhibitory substance was the protamine clupeine, a raw clupeine was prepared as follows; To a herring milt extract was added 96% ethanol to a final concentration 25% ethanol in the mixture. After stirring and storage over night in a cold room, a brownish, sticky precipitate had formed. The supernatant was decanted, and the precipitated clupeine was washed with 96% ethanol. A dehydration seemed to take place and a crystalline raw clupeine occurred. When it was ground in a mortar, a beige powder was obtained.

This raw clupeine was further purified as follows: 5 g raw clupeine was dissolved in 100 ml water of a temperature of 37° C, and the solution filtered. The clupeine was then precipitated by the addition of 300 ml of a sodium picrate solution saturated at 37° C. (pH = 7.0). After storage over night in a cold room the precipitate was filtered off on a Büchner funnel, and washed three times on the filter with 15 ml of a very weak solution of sodium picrate. The precipitate was then dissolved in 187 ml acetone and 65 ml water was slowly added. A slightly turbid solution resulted, which was filtered. To the clear filtrate was added half the volume of ethanol, and the clupeine precipitated with slow addition of 2 N H_2SO_4 (ca. 10 ml). Finally 300 ml 96% ethanol was added, and the mixture stored at —25° C over night. The precipitate was filtered off on a Büchner funnel, and redissolved in water of 37° C. After the

Table 1. *Precipitation of raw-clupein from a herring-milt extract.*

Fractionation 96% ethanol added*	Description of the precipitation	Labelling of the different fractions **
5% fraction 100 ml raw extract + 5.5 ml 96% ethanol	Slightly opalescent	Not separated Mixture I
10% fraction Mixture I + 6.1 ml 96% ethanol	Slightly opalescent The precipitate formed during the addition of alcohol redissolved.	Not separated Mixture II
15% fraction Mixture II + 6.9 ml 96% ethanol	Precipitation of a white substance which turned sticky and brownish.	Precipitate I Filtrate I
20% fraction Filtrate I + 7.8 ml 96% ethanol	Further white precipitation, but not very voluminous. A sticky substance precipitate on the wall.	Precipitate II Filtrate II
30% fraction Filtrate II + 19.2 ml 96% ethanol	A white precipitation formed slowly. Over night a thin layer of white substance had deposited on the wall.	Precipitate III Filtrate III
40% fraction Filtrate III + 23.3 ml 96% ethanol	A white precipitation formed slowly. Deposited as a greyish precipitate.	Precipitate IV Filtrate IV
50% fraction Filtrate IV + 37.5 ml 96% ethanol	The solution turned opalescent, but a precipitate could not be obtained.	Mixture III
60% fraction Mixture III + 58 ml 96% ethanol	—»—	Mixture IV
70% fraction Mixture IV + 102 ml 96% ethanol	—»—	Mixture V
80% fraction Mixture V + 231 ml 96% ethanol	A white precipitate occurred which precipitated bluisgrey upon centrifugation.	Precipitate V Filtrate V
85% fraction Filtrate V + 274 ml 96% ethanol	Precipitation as above, but less.	Precipitate VI Filtrate VI

* The addition of 96% ethanol was calculated by the formulae

$$\frac{(100 + x).\text{“\%”}}{100} = \frac{x \cdot 96}{100}$$

** All precipitates were dissolved in 30 ml 0.055 N H_2SO_4. Aliquots of the filtrates saved for microbiological control.

Table 2. *Growth inhibition on Saccharomyces carlsbergensis (ATCC 9080) of different precipitates and filtrates from alcohol precipitation of herring-milt extract.*

	Response measured as % turbidity at 660 mμ						
Precipitate diluted to:	200,000	100,000	50,000	20,000	1,000	500	200
Precipitate I (15% fraction):	44	110	110	110	—	—	—
— II (20% fraction):	44	44	110	110	—	—	—
— III (30% fraction):	—	43	83	110	110	110	110
— IV (40% fraction):	—	42	44	63	—	110	110
— V (80% fraction):	—	42	42	48	—	100	100
— VI (85% fraction):	—	42	42	42	42	42	48
Mediumblank-response %: 100							
Growth blank-response %: 41.5 42 43 42							

Mixture IV (60%) and Filtrate V (80%) and VI (85%) gave no inhibition at dilutions 1/370, 1/185 and 1/74.

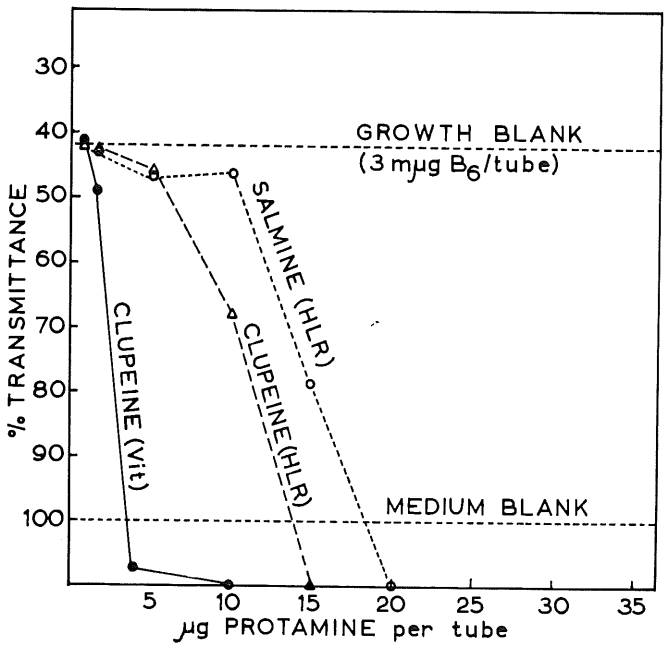

Fig. 5. Growth inhibition of different protamines on *Saccharomyces carlsbergensis*.

addition of ten volumes 96% ethanol, the clupeine sulphate was precipitated by slow addition of 2 N H_2SO_4. The mixture was stored over night at —25° C, and the precipitate filtered off on a Büchner funnel. This purification step was repeated twice, and finally a practically white clupeine sulphate was obtained in a yield of c. 3 g. This preparation is referred to as "clupeine (Vit.)" in the below studies.

c) Comparison of growth inhibition of different protamines on Saccharomyces carlsbergensis.

The growth inhibitory effect of clupeine (Vit) on *Saccharomyces carlsbergensis* was compared with commercial preparations of protamines available in our chemical stock. Two about 30 years old protamines were at hand, salmine (HLR) and clupeine (HLR). The results are reported in Fig. 5. A markedly stronger inhibitory effect was observed for clupeine (Vit) than for the clupeine (HLR) and salmine (HLR). Thus concentrations below 1 μg per ml medium gave total inhibition for our preparation, whereas c. 2 μg clupeine (HLR) and c. 3 μg salmine (HLR) were needed to produce the same effect.

The possibility that the fairly old commercial preparations were not

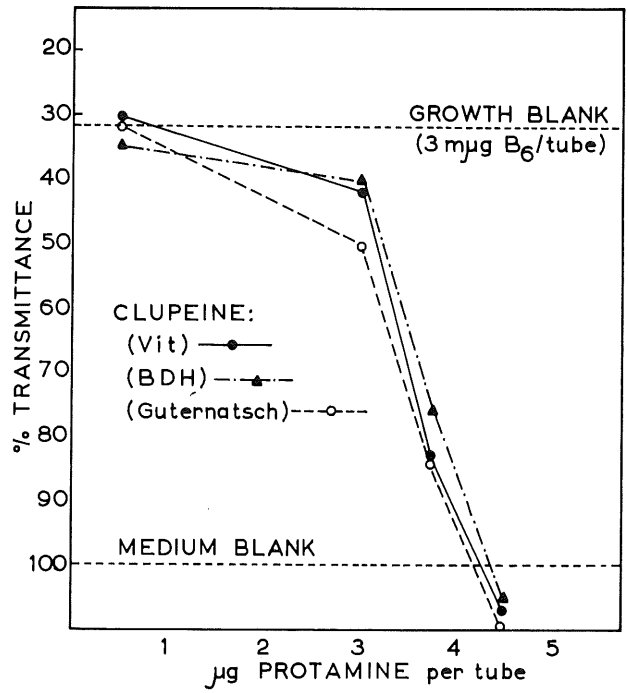

Fig. 6. Growth inhibition of different clupeine preparations on *Saccharomyces carlsbergensis*.

pure by todays standard had to be considered. With the courtesy of prof. WALDERSCHMIDT—LEITZ a sample of the purest clupeine prepared by GUTERNATSCH (1957) was obtained. This had been used for physical — chemical studies, and was considered highly pure. Further was obtained a commercial sample from British Drughouse (BDH). When our preparation was compared with these samples, a strikingly equal inhibition was observed (Fig. 6). For all three clupeines the growth inhibition was observed at 0.6 μg per ml medium, to be complete at concentration of 0.8 μg per ml. The close similarity of the slope on the inhibition curves may be pointed out.

At the time of this study samples of perch milt and mackerel milt were available. Raw extracts from these species were compared with a simultaneously produced herring milt extract (Fig. 7). Perch milt and herring extract gave both inhibition for concentration corresponding to about 40 μg organ per ml medium, but the slope of the inhibition curves differed. The raw extract from mackerel milt was weaker, and a concentration corresponding to 150 μg organ per ml medium was needed to obtain total growth inhitibion.

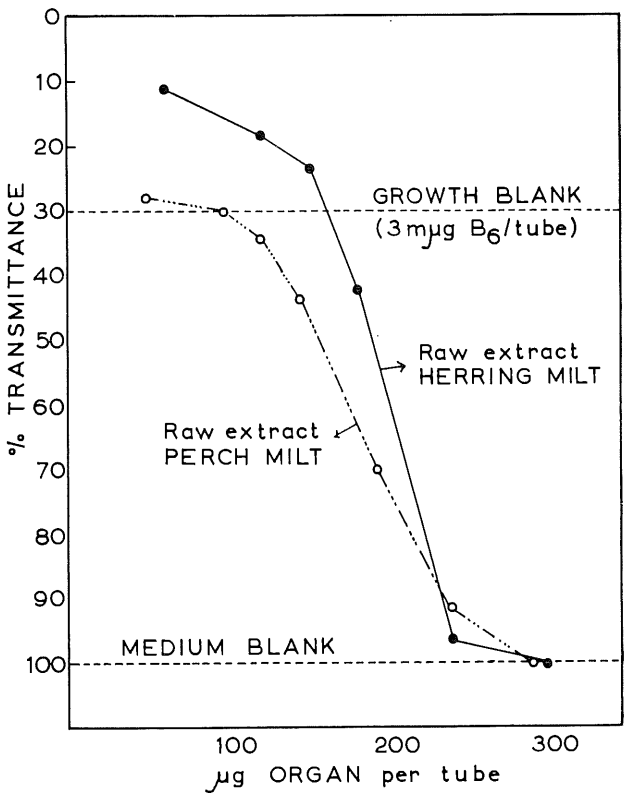

Fig. 7. Growth inhibition of raw extracts from the milts of herring (*Clupea harengus*)and perch (*Perca fluviatilis*).

d) The growth inhibition of clupeine on different microorganisms.

Microorganisms are today extensively employed for the determination of vitamins. The possible interference of clupeine in such assays was studied for selected microorganisms. The comparativ effect of our preparation, clupeine (Vit), and the apparently less pure old commercial preparation, clupeine (HLR), was included in this investigation, to establish the difference of the growth inhibiting properties regardless of microorganism. The results are reported in Tables 3—5. In these experiments all lactobacilli were grown on the medium of THOMPSON et al. (1950), supplemented with vitamin B_{12}. *Neurospora crassa* and *Aspergillus niger* were grown on the medium described by HOROWITZ & BEADLE (1943), supplemented with choline chloride.

The difference in the growth inhibition between clupeine (Vit) and clupeine (HLR) observed on *Saccharomyces carlsbergensis* was generally the same on the lactobacilli and *Neurospora crassa*. The strongest growth

Table 3. *Growth inhibition of protamines* on Lactobacillus plantarum and Lactobacillus casei.*

		L. plantarum (ATCC 8014)		*L. casei* (ATCC 7469)	
		% Transmittance (660 mμ)			
Growth blank		3		9	
μg protamine		Clupeine		Clupeine	
per tube	per ml	(Vit)	(HRL)	(Vit)	(HRL)
5	1	4	3	8.5	9
25	5	98	3	45	9
50	10	95	2.5	98	10
100	20	96	3	95	10
200	40	97	83	95	19
400	80	95	96	95.7	89
800	160	97	97	98	97

* Clupeine (Vit) = clupeine sulphate (own preparation). Clupeine (HLR) = clupeine (old commercial preparation (Hoffmann La Roche)).

Table 4. *Growth inhibition of protamines* on Streptococcus faecalis and Lactobacillus leichmannii.*

		S. faecalis (ATCC 8043)		*L. leichmannii* (ATCC 4797)	
		% Transmittance (660 mμ)			
Growth blank		14.5		26.5	
μg protamine		Clupeine		Clupeine	
per tube	per ml	(Vit)	(HLR)	(Vit)	(HLR)
5	1	13	14	28	26
25	5	12.5	14	96	26
50	10	11.5	13	95	25
100	20	15	13	93	28
200	40	17	12.5	94	93
400	80	28	12	96	97
800	160	60	12	97.5	96

* See foot-note Table 3.

inhibition was observed on *L. leichmannii* and *L. plantarum*, where c. 5 μg clupeine (Vit) or ca. 40 μg clupeine (HLR) per ml medium gave total inhibition. For *L. casei* the corresponding figures were 10 and 80 μg per ml, although growth inhibition is also observed for the concentrations 5 and 40 μg per ml medium. The growth of *S. faecalis* was slightly inhibited by clupeine (Vit) in concentration 80 μg per ml medium, and more pronounced when the concentration was increased to 160 μg per

Table 5. *Growth inhibition of protamines* on Neurospora crassa and Aspergillus niger.*

		*Neurospora crassa*** (ATCC 9277)		*Aspergillus niger****	
		Growth = mg mycelium			
Growth blank		20.4		65.6	
µg protamine		Clupeine		Clupeine	
per tube	per ml	(Vit)	(HLR)	(Vit)	(HLR)
10	1	19.3	21.0	74.4	77
50	5	18.7	20.8	85.4	97.8
100	10	18.0	20.4	93.3	88.0
200	20	11.9	23.0	96.6	92.0
500	50	2.4	14.8	95.2	90.8
1000	100	2.7	14.4	95.3	92.7

* See foot-note Table 3.

** *Neurospora crassa* Choline-less mutant.

*** Obtained from Botanical Laboratory, University of Bergen by the courtesy of dr. J. Goksøyr.

ml. Clupeine (HLR) failed to cause growth inhibition in concentrations up to 160 µg per ml medium. Growth on *Neurospora crassa* was slight at a concentration of 20 µg clupeine (Vit) per ml medium, pronounced at a concentration of 50 µg per ml, whereas a growth inhibition was first observed at a concentration of 50 µg clupeine (HLR). Both clupeine preparations failed to inhibit the growth of *Aspergillus niger*, instead a growth promotion was observed.

DISCUSSION

Since the early isolation of protamines by MIESCHER (1868) and its recognition as a protein by KOSSEL (1894), numerous papers have dealt with methods of preparation, purification and structural elucidation. The protamines are the main protein in the heads of the spermatozoa of many teleost fishes, and have also been found in the spermatozoa of some birds (FELIX et al. 1951; DALEY et al. 1951). The first step in methods described for the preparation of protamines from fishes involves isolation of the cell nuclei as originally recommended by KOSSEL (1894). From the cell nuclei the protamines can be extracted with dilute sulphuric acid (KOSSEL 1894, 1929; WALDERSCHMIDT—LEITZ et al. 1931; GUTERNATSCH 1957), or with hydrochloric acid (RASMUSSEN 1934; BLOCK et al. 1949; FELIX et al. 1951). Other methods involve the dissoci-

ation of the protamine from the nucleoprotamine with cuprous chloride, which precipitates the nucleic acid and leaves the protamine in solution (SCHMIEDEBERG 1899, MALENÜK 1908, NELSON—GERHARDT 1919). A direct extraction of the protamine from the cell nuclei with 1.5 M NaCl in 50 per cent ethanol has been reported by CALLANAN et al. (1957). When the raw protamine has been obtained, several methods for the purification and isolation have been proposed. KOSSEL (1894, 1929) used alcohol precipitation of the extract and purification over the picrate, with a final isolation of the protamine as the sulphate. This principle has been used by several authors and also in the present study. FELIX & DIRR (1929) developed a method for the isolation of clupeine in which the picrate was converted to clupeine methyl ester hydrochloride. This principle has been critisized by WALDERSCHMIDT—LEITZ et al. (1931) and by LINDERSTRÖM—LANG (1933), but was later applied by SORM & SORMOVA (1951) in their studies on the structure of clupeine.

It has been claimed that a direct extraction of the gonads is not possible (GUTERNATSCH 1957), as proteinases in the tissue may cause partial breakdown of the protamine (KLINGENBERG 1952). In the present procedure the extraction is carried out by autoclaving at 120° C, a temperature to high for any enzymatic activity. One should, however, expect acid hydrolysis of the clupeine, but such reactions seem not to take place with the fairly weak acid concentrations applied. Extractions have also been tried with water and with 1 N H_2SO_4, but in both cases the extracts showed no growth inhibition on yeast, indicating the absence of clupeine. Apparently 0.055 N H_2SO_4 is strong enough to liberate the clupeine from the nucleo-protamine, but not strong enough to promote hydrolysis of the protein in the mixture. Thus boiling of the extract at pH 4.5 did not result in losses of the growth inhibiting properties of normal extracts. Autoclaving of pure clupeine with 0.055 N H_2SO_4 resulted in destruction of the protamine. During the extraction of the milt conditions apparently occur which protect against the breakdown of liberated or dissolved clupeine. The basic properties of the organ most likely prevents that the pH falls below critical values.

The raw clupeine as well as the purified clupeine obtained by the present procedures, showed properties similar to the best commercial preparations, and also to the clupeine prepared by GUTERNATSCH (1957) and used in his studies on the composition. The only difference between the raw clupeine and the purified clupeine seemed to be the inclusion of colouring matters in the former. So far neither the isolation of a uniform clupeine, nor the final structure of this protamine have been reported in the literature. The procedure applied for the isolation of clupeine sulphate in the present study should, however, lend itself for

such investigations. The relatively few steps and mild extractions may have resulted in a clupeine which has not been partially broken down.

Few studies report on the effect of protamines on micro-organisms. McCLEAN (1931) compared the effect of clupeine sulphate upon the growth of *Eberthella typhosa* with that of the disinfectant phenol. When added to cultures in HARTLEY's broth, partial inhibition was observed up to 24 hrs for 1/100,000 clupeine sulphate as compared with 1/1000 phenol. On agar 1/1000 clupeine sulphate gave slight growth inhibition whereas 1/100 phenol showed no effect. REINER et al. (1942) found protamine to inhibit respiration of *Trypanosoma equiperdum*. MILLER et al. (1942) found in general salmine sulphate to give stronger respiratory inhibition to Gram-positive than to Gram-negative micro-organisms. They assumed the bactericidal effect to parallel the effect on metabolism. MIGUENS (1950) studied the antibiotic action of protamine and histone against dermatophytes and *Candida species*. During the first 4—5 days in culture 1/2500 protamine inhibited the growth of *Sporotrichum species* and of *Trichophyton mentagrophytus*. At 1/25,000 it inhibited the growth of *Epidermophyton floccosum*. After 8 days, growth occurred in all tubes, but weaker in those with the higher concentrations of protamine. There was no inhibition of *Microsporum canis* and *Candida parapsilosis*. Histone had only weak inhibitory powers. WOLF & BRIGNON (1954) reported on the influence and mode of action of protamine sulphate on the growth of micro-organisms. In neutral or weakly acid media protamine was found to exert a bacteriostatic action on some species of bacteria. This action could be antagonized by ribonucleic acid and by heparin, both of which were assumed stoichiometrically to displace protamine from its combination with the bacterial cell. Partial hydrolysis products of protamines excerted bacteriostatic action in relatively high concentration. ÅGREN (1954) investigated substances inhibiting or stimulating the growth of some lactobacilli. He found protamine in concentrations 0.1—1.0 mg per 5 ml medium to completely inhibit the growth of *L. casei* and *L. delbrückü*. KRASSE (1955) studied the inhibition of acid production by protamine, 5 fluor-nicotinamide and guanozole in pure cultures of oral micro-organisms. In both 4 and 48 hrs test protamine was found most effective in inhibiting acid production. His work comprised several of the lactobacilli investigated in the present study, and the result will be discussed below. BROCK (1958) studied bacteriostatic and bactericidal effects of salmine on various bacteria. Salmine showed more bacteriostatic activity against Gram-positive than against Gram-negative bacteria. It was bactericidal in water but not in broth, and was found to cause agglutination of washed suspension of certain bacteria without correlation to the Gram-stain. He further compared the effects of salmine

and polymyxin and concluded that salmine may also act by attachment to the bacterial surface.

The above refered papers seem to be the main contributions to the study of the effects of protamines on micro-organisms. When the present study was undertaken it was prompted by the need for a check on the possible influence of this growth inhibitor on the microbiological assays. The strong basic properties of the protamines have not always been taken into consideration in previous studies. Already in the medium protamine may precipitate or combine with substances, and the quantitative aspect of the effects be distorted. Thus McCLEAN (1931) used a one per cent solution of clupeine sulphate and ten fold solutions of this in HARTLEY's broth, and found the first and second dilutions to show a definite immediate precipitate in the tubes. The effect of the medium was also strikingly expressed in the above reported findings of BROCK (1958), who found salmine bactericidal in water, but not in broth. In experiments on the effect of protamines on micro-organisms, media should be employed which do not react with the protamines. The microbiological assays of vitamines and amino acids are usually carried out with such media, and they should thus lend themselves favourable to such studies. Such media were employed in all experiments in the present study.

The preliminary experiments were conducted to gain information with regard to the type of inhibition (Fig. 1—4). The growth inhibition proved a more suitable method than respiratory experiments, and was employed in the main studies.

The effect of the different protamines were studied on *Saccharomyces carlsbergensis*. The results indicate that it is important to distinguish between the different protamines (clupeine, salmine etc.). Unfortunately studies with protamines frequently fail to report which type was used. The very close agreement between the growth inhibition curves for the clupeines (Vit), (Guternatsch) and (BDH) as reported in Fig. 6, points toward the possible application of this principle for the assay of protamine. As the raw extracts (Fig. 7) give similar curves, it should be possible to follow the protamine formation during the ripening of the milt (testes) in fish. The high sensitivity of *Saccharomyces carlsbergensis* towards the growth inhibiting effect of protamines may be pointed out. Thus under the present experimental conditions clupeine sulphate started to give inhibition at 0.6 μg per ml, to give complete inhibition of the growth at about 0.8 μg per ml medium.

The growth inhibition of clupeine on different micro-organisms were studied with the preparations clupeine (Vit) and clupeine (HLR). The relative effects were the same for both preparations, and only the values

for the former, apparently purest sample, will be discussed. For the lactobacilli growth inhibition was observed at about 5 μg per ml medium for *L. plantarum* and *L. leichmannii*, and at about 10 μg for *L. casei*. For *S. fæcalis* slight inhibition was observed at 80, pronounced inhibition at 160 μg per ml medium. KRASSE (1954) employed a semi-synthethetic substrate described by LANDY and DICKENS (1942), but measured the acid production in a 48 hrs test. He found complete inhibition for *L. casei*, *L. arabinosus* (*L. plantarum*) and *L. brevis* with 10 μg protamine sulphate per ml medium. Our findings are in general agreement with these results. For *S. fæcalis* he failed to observe inhibition of 100 μg protamine per ml substrate, whereas our results indicate this as a level of inhibition in turbidimetric assays. BROCK (1958) observed a bactericidal effect of salmine on *S. fæcalis* in water at a level of 200 μg per ml by a method employing viable counts after 1 hour.

Neurospora crassa was inhibited at 50 μg per ml medium, whereas *Aspergillus niger* showed growth stimulus. The latter finding indicates that this fungi may excrete enzymes which digest the protamine, and the amino acids then causes a growth stimulation.

The mode of action of the protamines on microorganisms is an open question. BROCK (1958) pointed out that the protamines are basic polypeptides, and assumed an interaction with the bacterial surface as reported for the basic polypeptide antibiotic polymyxin. He found similar behaviour of these two substances in a comparative experiment. The possibility for increased permeability have also to be considered (MCCLEAN 1931). Thus MASSART & van den DAELE (1948) found protamine sulphate to inhibit the respiration of yeast up to 1 hr, and reverted the action with Al, Mg and Na ions with decreasing effect. They assumed a reaction between protamine and the nucleoproteins of the yeast. BLOOM et al. (1951) studied the inhibition of protamine and thymus basic protein on *Bacillus subtilis*, and found nucleic acids and desoxyribonucleic acids to reverse the effect. They also assumed that the mechanism of antibacterial action of basic proteins depends upon their capacity to combine with nucleic acid complexes essential in the processes of aerobic respiration. The possibility for an inhibition of special enzymes within the cells have also to be considered. Thus KREBS (1954) and MADSEN & CORI (1954) reported the inhibition of phosporylase with salmine. NYGAARD (1961, 1962) reported on the action of protamine on the yeast d- and l- lactic cytochrome c reductase. The acceptor specificity was changed by concentrations in the order of 2 μg protamine per ml. Thus the strong growth inhibition observed in yeast may be caused by vital interference with the metabolism of the cells.

SUMMARY

A method has been described for the isolation of protamine from the milt (testes) of fish, employing direct extraction of the organ with dilute sulfuric acid, followed by stepwise precipitation with ethanol. The growth inhibitory effect of different protamine preparations on *Saccharomyces carlsbergensis* have been studied. 0,7—0,8 μg clupeine sulphate per ml medium gave complete growth inhibition. It has been pointed out that the type (origin) of the protamine should be given in studies on these substances.

The growth inhibition of clupein sulphate on some microorganisms employed in vitamin assays have been tried. For *L. plantarum* and *L. leichmannii* growth inhibition was observed at about 5 μg per ml medium, for *L. casei* at 10 μg, for *S. fæcalis* at about 150 μg, and for *Neurospora crassa* at about 50 μg per ml medium

Aspergillus niger showed growth promotion, probably caused by enzymatic breakdown of the polypeptide and utilization of the free amino acids.

REFERENCES

Atkin, L., A.S Schultz, W. L. Willams & C. N. Frey, Ind. Eng. Chem. Anal. Ed. *15*, 141 (1943).

Bloom, W. L., M. G. Winters & D. W. Watson, J. Bacteriol. *62*, 7 (1951).

Brækkan, O. R. & Gj. Boge, Int. Symp. on B-Vitamins, Poznan 1959, p. 183.

Brock, T. D., Can. J. Microbiol. *4*, 56 (1958).

Callanan, M. J., W. R. Carrol & E. R. Mitchell, J. Biol. Chem. *229*, 279 (1957).

Daley, M. M., A. E. Mirsky & H. Ris, J. gen Physiol. *34*, 439 (1951).

Felix, K., H. Fischer, A. Krekels & R. Mohr, Z. physiol. Chem. *287*, 244 (1951).

Felix, K. & K. Dirr, Z. physiol. Chem. *184*, 111 (1929).

Guternatsch, H. «Über Clupein: Zusammensetzung und Reifegrad», Doktor Thesis, München 1957.

Horowitz, N. H. & G. W. Beadle, J. Biol. Chem. *150*, 325 (1943).

Klingenberg, H. G., Z. physiol. Chem. *290*, 139 (1952).

Kossel, A., Z. physiol. Chem. *25*, 165 (1898).

— «Protamine und Histone», Leipzig u. Wien, 1929.

Krasse, B., Acta Odont. Scand. *12*, 173 (1954).

Krebs, E. G., Biochem. Biophys. Acta *15*, 508 (1954).

Landy, H. & D. M. Dicken, J. Lab. and Clin. Med. *27*, 1086 (1942).

Lindstrøm—Lang, K., Erg. d. Physiol. *35*, 415 (1933).

Madsen, N. B. & C. F. Cori, Biochem. Biophys. Acta *15*, 516 (1954).

Malenük, W. D., Z. physiol. Chem. *57*, 99 (1908).

Massart, L. & P. van den Daele, Arch. Int. de pharmacodyn *76*, 424 (1948).

McClean, D., J. Path. Bacteriol. *34*, 459 (1931).

Miescher, F., Ber. *7*, 376 (1874).

Miguens, M. P., 5th Int. Congr. Microbiol., Abst. of Papers, Rio de Janeiro 1950, p. 136.

MILLER, B. F., R. ABRAM, A. DORFMAN & M. KLEIN, Science *96*, 428 (1942).
NELSON-GERHARDT, M., Z. physiol. Chem. *105*, 265 (1919).
NYEGÅRD, A., Acta chem. Scand., *15*, 1627 (1961).
— J. biol. Chem. *237*, 742 (1962).
RASMUSSEN, K. E., Z. physiol. Chem. *224*, 97 (1934).
RABINOWITZ, J. C. & E. E. SNELL, J. Biol. Chem. *169*, 631 (1947).
REINER, L., E. J. de BEER & M. GREEN, Proc. Soc. Exp. Biol. and Med. *50*, 70 (1942).
SCHMIEDEBERG, O., Arch. f. exp. Pathol u. Pharm. *37*, 100 (1896).
SNELL, E. E., In «Vitamin Methods» Edit. P. GYØRGY, Bd. I, p. 327, New York 1950.
SORM, F. & Z. SORMOVA, Coll. Cchecoslov. Chem. Communs *16*, 207 (1951).
WALDERSCHMIEDT—LEITZ, E., F. ZIEGLER, A. SCHÄFFNER & L. WEIL, Z. physiol. Chem. *197*, 219 (1931).
WOLF, R. & J. BRIGNON, Bull. soc. chim. biol. *36*, 1125 (1954).
ÅGREN, G., Acta path. et microbiol. Scand. *35*, 97 (1954).