CCXLII. THE NATURE OF THE "SPOROGENES VITAMIN", AN ESSENTIAL GROWTH FACTOR FOR *CL. SPOROGENES* AND RELATED ORGANISMS.

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IN a series of papers on the nutritional requirements of bacteria [Knight and Fildes, 1933; Fildes, 1935; Fildes and Richardson, 1935] it has been shown that *Cl. sporogenes* and certain other obligate anaerobes may be grown on media containing salts and known amino-acids only when a trace of an unknown ether-soluble acid, which has been called the "sporogenes vitamin", is present.

The present paper gives a further study of the chemical properties of this essential growth factor, carried out in collaboration with Dr Fildes and Mr Knight at the Middlesex Hospital. Although we are unable to report the successful isolation of the "vitamin" in crystalline form, we have succeeded in effecting substantial purification and have obtained preparations of constant composition and properties and of uniformly high activity in promoting growth on otherwise deficient media.

EXPERIMENTAL.

Biological testing.

The tests on the activity of various preparations containing the sporogenes growth factor were carried out in the Department of Bacterial Chemistry, Middlesex Hospital. Fivefold dilutions of the solution to be tested were added to a series of tubes containing the deficient medium, which were then inoculated with a standard suspension of *Cl. sporogenes* spores. The deficient medium used for most of the testing was an acid hydrolysate of gelatin with the addition of 0.02% tryptophan, 0.2% thiolacetic acid *plus* the requisite salts [Knight and Fildes, 1933]. Later tests were made using a medium containing only amino-acids, most of which were synthetic, *plus* thiolacetic acid and the necessary salts [Fildes and Richardson, 1935]. Results with the latter medium were more clear cut and must be regarded as more significant. The reason for this appears to be that the gelatin contains traces of substance having the biological activity in question, but in inactive form, not activated by acid hydrolysis but possibly activated by bacterial growth. These traces sometimes permit very slight growth which does not increase. This does not occur with the amino-acid medium.

Materials.

Initial attempts to isolate the essential growth factor were carried out with a commercially prepared fraction from pregnant women's urine. An oil was obtained which was active in concentrations of 0.01γ per ml. in promoting growth on the gelatin hydrolysate medium. As will become apparent from evidence given later, this corresponds to an activity of about 0.1γ per ml. on the amino-acid medium. Unfortunately this product quickly lost its activity on

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standing, before much could be learned of its properties. Subsequent preparations have all been kept as the methyl ester, which shows no diminution in activity after long standing in the cold.

Since no further supply of this urine concentrate was available, we next attempted to isolate the factor from yeast. A laborious fractionation of 120 kg. of brewer's yeast yielded about 0.5 g. of impure methyl ester of the active substance, which could not be distilled without destruction. The great instability of the yeast preparations suggests that the growth-promoting activity may be due to a different substance from that in urine.

It therefore became necessary to search for a new source of material. Tests on fresh human urine showed that the commercial concentrate which we had previously used only contained about 1% of the active factor present in the original fresh urine. Further, the urine of two herbivorous animals (horse and cow) was found to be about ten times more active in promoting the growth of *Cl. sporogenes* than the fresh urine of man. Through the courtesy of Dr A. Girard of Paris we were able to test some by-products from the commercial preparation of oestrogenic hormones from pregnant mare's urine. These were found to be highly active. To our surprise, we found that the active factor had been completely extracted from a 50% pyridine-ether solution of the saponifiable *plus* non-saponifiable material of mare's urine by concentrated hydrochloric acid. Accordingly, Dr Girard, to whom we wish to express our sincere thanks, kindly sent us 10 litres of this highly active solution, containing the growth factor from 1300 litres of mare's urine, and this was used in the work to be described.

Method of separation.

Table I shows the steps followed in separating the sporogenes growth factor from the mare's urine concentrate. Activity at each stage was followed by

Table I. *Preparation of sporogenes growth factor from mare's urine.*

(1) 10 l. mare's urine concentrate (contains HCl, pyridine). Make alkaline to litmus with solid Na_2CO_3 (7 kg.). Steam distil.	distillate
	Residue 15 l. (active)
(2) Concentrate <i>in vacuo</i> to 2.5 l., acidify to Congo red with H_2SO_4 and continuously extract with ether (24 hours)	acid aqueous residue
	Ether extract 1000 ml. (active)
(3) Concentrate ether extract to 200 ml. and precipitate with 400 ml. light petroleum	supernatant
	Precipitate 25 g. (active)
(4) Esterify with 3% $\text{HCl}-\text{CH}_3\text{OH}$. Extract ether solution of esters with NaHCO_3 and NaOH	NaHCO_3 and NaOH fractions
	Ether residue (active on hydrolysis) (Methyl esters)
(5) High vacuum distillation	Fraction distilling below 90° (inactive)
	Middle fraction $90-100^\circ$ (active on hydrolysis)
(6) Redistillation	7 g. purified methyl ester: activity on hydrolysis 0.04 γ /ml.

determining the minimum amount of material necessary to add to 1 ml. of deficient medium, in order to produce just visible growth in twenty-four hours.

Step 1. After steam-distillation, a considerable amount of tar was removed by filtration.

Step 2. During concentration of the filtrate the salt which separated was removed from time to time by filtration. After acidifying the concentrate and filtering off precipitated tar the filtrate was coloured light orange. Peroxide-free ether was used for the subsequent extraction and throughout this work.

Step 3. The oil precipitated with light petroleum was dissolved in 150 ml. water and kept in the cold as a stock solution.

Step 4. 30 ml. of this solution were extracted ten times with a total of 500 ml. of ether and the extract was dried over Na_2SO_4 . After removing the ether, the residue was refluxed for one hour with 50 ml. 3% $\text{HCl}-\text{CH}_3\text{OH}$. The alcohol was removed *in vacuo*, and the residue, dissolved in ether, shaken out twice with 5% NaHCO_3 , twice with 0.5% NaOH , and finally with dilute $(\text{NH}_4)_2\text{SO}_4$ solution.

Steps 5-6. The residual esters, freed from phenolic substances, were then distilled in a high vacuum (*ca.* 0.001 mm. Hg). The first fraction (0.87 g.) coming over below 90° was inactive on hydrolysis and was discarded. The middle fraction ($90-100^\circ$) contained practically the entire activity (after hydrolysis) and weighed 2.2 g. 0.3 g. dark residue remained in the distilling flask. The middle fraction was then redistilled and the fraction coming over between 94° and 96° collected. The yield of pale yellow oil was 1.53 g., 0.001-0.005 γ of which was sufficient to activate 1 ml. of gelatin hydrolysate medium after alkaline hydrolysis. The activity on the amino-acid medium was somewhat less (0.04 γ /ml.) but the titration was far more reproducible. The total yield of redistilled esters from 10 litres of mare's urine concentrate was about 7 g.

Properties of distillate.

The methyl ester failed to crystallise even when kept at the temperature of solid CO_2 for two days. The oil was very soluble in ether, alcohol and benzene, slightly soluble in water, but insoluble in light petroleum. Millon, SbCl_3 and Liebermann-Burchard reactions were negative. With *p*-dimethylaminobenzaldehyde a violet-pink colour of moderate intensity was obtained on addition of excess concentrated HCl . A burnt orange colour was produced on a pine splint moistened with HCl . The oil contains no nitrogen or sulphur. It has no appreciable optical rotation in 4% solution.

Analysis of methyl ester. Three independent distillates gave the following analyses. (Found C, 62.65, 62.43, 62.51; H, 7.00¹, 6.87¹, 6.77%. Calculated for $\text{C}_{11}\text{H}_{14}\text{O}_4$, C, 62.85; H, 6.67; Calculated for $\text{C}_{11}\text{H}_{16}\text{O}_4$, C, 62.26; H, 7.55%.)

Mol. wt. by freezing point of benzene, mean value found, 209; calculated for $\text{C}_{11}\text{H}_{14}\text{O}_4$, 210.

Hydrolysis of methyl ester. The methyl ester itself is only 1/25 as active as the free acid. This slight activity is probably due to partial hydrolysis in the culture medium during test.

268 mg. of ester were refluxed for one hour with 5 ml. of *N* NaOH . The solution was saturated with CO_2 , extracted twice with ether and finally acidified with dilute sulphuric acid and thoroughly extracted with peroxide-free ether. After drying the ether extract over Na_2SO_4 and removing the ether, 240 mg. of

¹ For the first and second analyses we are indebted to Mr A. W. Spratt; the third was made by Roth (Heidelberg).

yellow oil remained which rapidly darkened on standing in air or in a vacuum desiccator.

The free acid was very soluble in alcohol and ether, moderately soluble in cold water and benzene, but insoluble in light petroleum and *cyclohexane*. The acid gave no colour with Millon's reagent or with ferric chloride. Permanganate was readily reduced in both acid and alkaline solutions in the cold, and the silver mirror test with Tollen's reagent was positive on heating. Neither the free acid nor its methyl ester gave any colour reactions for aldehydes on oxidation with lead tetra-acetate [Criegee, 1931], nor could any ketone be detected. The acid retained its full activity after treatment with boiling *N* HCl or *N* NaOH for three hours, but was destroyed by treatment with norite charcoal at 100°.

Titration of free acid. Duplicate determinations of the acid equivalent of the freshly prepared free acid gave values of 206 and 209. A monobasic acid, $C_{10}H_{12}O_4$, requires an acid equivalent of 196. One of the above titrations was done electrometrically using the glass electrode¹. The points agree well with the theoretical curve for a single substance with $p_K' = 4.7$ (Fig. 1).

Fig. 1. Titration curve of a highly active "sporogenes vitamin" preparation. Crosses represent back-titration with 0.1N HCl.

Attempts to obtain a crystalline derivative of the active factor. All attempts to crystallise the acid and its methyl ester and to prepare crystalline derivatives have met with failure. The ester reacts with hydrazine and phenylhydrazine to form acid hydrazides and with ammonia to form the amide. From these derivatives the active free acid can be quantitatively regenerated by alkaline hydrolysis. It can be esterified with 3:5-dinitrobenzoyl chloride in pyridine and other reagents which generally yield crystalline derivatives with hydroxy-compounds. Although reaction takes place and products are obtained with entirely different

¹ We are indebted to Dr W. J. Elford for the use of the glass electrode.

solubilities, all have been oils. Benzoyl- and tolylsulphonyl-derivatives have been prepared from the free acid by the Schotten-Baumann reaction. However, benzylation and acylation of the free acid in dry solvents such as pyridine yield only tars and the active principle is destroyed. We have also prepared strychnine, brucine and other alkaloidal salts from the acid, all of which resist attempts to crystallise them. Even the *p*-phenylphenacyl derivative fails to crystallise. Lead, zinc and barium salts are hygroscopic and form resins on drying.

Bromine titration of methyl ester. 47.9 mg. of oil required 9.54 ml. of 0.1 *N* bromine [Reindel and Niederländer, 1929]. On addition of 1 % KIO_3 , a further quantity of bromine equivalent to 4.30 ml. of 0.1 *N* $\text{Na}_2\text{S}_2\text{O}_3$, was set free. This indicates that the equivalent of 5.24 ml. of 0.1 *N* bromine had been added to a double bond. Calculated for one double bond and molecular weight 210, 4.56 ml. Found, 1.15 double bonds + 1.9 atoms bromine substituted.

As a check on the above method, 50.8 mg. of ester were titrated by the pyridine-sulphate-dibromide method [Rosenmund and Kuhnenn, 1923]. 5.92 ml. 0.1 *N* bromine were used up. 50.8 mg. require 4.84 ml. for one double bond assuming a molecular weight of 210. Found 1.23 double bonds.

Hydroxyl determinations on methyl ester. The method of West *et al.* [1934] was used. The ester was acetylated with acetic anhydride-pyridine (1 : 7) and the acetic acid liberated after decomposition with water titrated with 0.1 *N* NaOH and compared with a blank. 51.5 mg. required 1.61 ml. 0.1 *N* NaOH less than the blank. 63.7 mg. required 2.06 ml. 0.1 *N* NaOH less than the blank. OH found 5.29, 5.45 %. Calculated for one hydroxyl and molecular weight 210, 8.09 %.

As a check on this method, active hydrogen determinations by the Zerevitinoff method were carried out (Roth, Heidelberg). 5.362 mg. gave 0.72 ml. CH_4 at 25° in pyridine.

7.005 mg. gave 0.98 ml. CH_4 at 25°. OH found, 6.90, 6.58 %.

Catalytic reduction. No measurable quantity of hydrogen was absorbed by 250 mg. of ester using a highly active platinum catalyst.

Oxidation of the methyl ester with alkaline permanganate. 91 mg. of the methyl ester in 6 ml. of *N* NaOH were oxidised with 0.8 g. of KMnO_4 at 100°. The slight excess of KMnO_4 was reduced with CH_3OH and the precipitate filtered off and washed. The filtrate was acidified to Congo red with dilute H_2SO_4 and extracted ten times with ether. On removing the ether 26 mg. of a crystalline residue were obtained which was purified by sublimation at 100° under the water-pump. The anhydrous sublimate melted at 182° and was identified as oxalic acid. The calcium salt was analysed. Found Ca, 26.40, 26.38. Calculated for CaC_2O_4 , H_2O , Ca, 27.39 %.

DISCUSSION.

We have not, as yet, succeeded in crystallising the purified preparations of the sporogenes growth factor or in obtaining a single crystalline derivative. Until we are able to accomplish this, it is not possible to be certain that the characteristic properties of our active oil are those of the sporogenes factor itself. However, we have obtained considerable evidence that the redistilled ester of the active substance is relatively homogeneous. The preparations, after hydrolysis, show a uniform activity (0.04 γ /ml.) in stimulating the growth of *Cl. sporogenes* on the amino-acid-deficient medium. Different preparations show a constant elementary composition suggesting a formula of $\text{C}_{11}\text{H}_{14}\text{O}_4$ or $\text{C}_{11}\text{H}_{16}\text{O}_4$. This elementary composition agrees well with the molecular weight determination by freezing point of benzene, with the acid equivalent by titration, with the

bromine titrations indicating the presence of one double bond and with the active hydrogen determinations indicating one hydroxyl group. One oxygen atom is left unaccounted for. Although definite evidence for the presence of a furan ring is still wanting, this possibility is not excluded.

From the evidence presented, it would seem certain that the sporogenes growth factor is an acid of molecular weight about 200 containing at least one hydroxyl group. From the bromine titrations at least one double bond is indicated, although the substance is not reduced by catalytic hydrogenation. Although the elementary composition might suggest the presence of a benzene ring, the sole oxidation product with alkaline permanganate is oxalic acid and no benzoic acid could be detected. Since neither the free acid nor its methyl ester gives aldehydes or ketones on oxidation with lead tetra-acetate, the presence of a glycol or α -hydroxy-acid may be excluded. The latter possibility is also rendered improbable by the failure of the acid to give a colour reaction with ferric chloride and by the p_K' value of 4.7, which would suggest that the carboxyl group is at the end of an aliphatic chain.

Relation of the sporogenes vitamin to other growth factors.

Recent work on the growth requirements of lower organisms has revealed the existence of a number of accessory factors necessary for their growth. The present study, however, indicates clearly that the sporogenes growth factor is distinct from any of the other well-characterised factors which have been hitherto described.

In their original paper on the "sporogenes vitamin", Knight and Fildes [1933] called attention to the similarity of its properties with those of the plant growth hormones, the auxins [Kögl *et al.* 1933; 1934; Kögl, 1935]. Since that time, however, pure auxin- α -lactone and "heteroauxin" from Prof. Kögl's laboratory have been tested and found to be inactive¹. We have prepared synthetic "heteroauxin", *i.e.* β -indoleacetic acid, by the method of Majima and Hoshino [1925]. It had no activity when tested in place of the sporogenes growth factor. The present factor is also distinguished from the "auxins" by its great stability to boiling mineral acid and alkali.

Recently a series of papers has appeared from the laboratory of R. J. Williams and his collaborators [1932; 1934] on the nutritional requirements of the "Gebrüder Mayer" strain of yeast. A widely distributed hydroxy-acid of low molecular weight, not yet isolated, is essential for the growth of this and certain other strains of yeast. They have called this factor "pantothenic acid". The concentrated preparations of sporogenes "vitamin" obtained by Knight and Fildes [1933] from yeast appeared to promote growth of the Williams strain of yeast [Edwards, 1933]. We have tested our own active fractions on the Williams strain of "Gebrüder Mayer" yeast, obtained through the National Collection of Type Cultures, and found them to be without growth-promoting effect. An acid factor extractable by butyl alcohol from urine, which stimulates the growth of this strain of yeast, was found to be alkali-labile as reported for "pantothenic acid" by Williams *et al.* [1932].

The sporogenes growth factor is also distinct from the "staphylococcus factor" [Hughes, 1932; Knight, 1935] and from the "bios" whose isolation has just been announced by Kögl [1935]. The last two factors appear to be basic and have altogether different properties from the sporogenes factor.

¹ Private communication from Dr Fildes.

SUMMARY.

1. A highly active preparation of the sporogenes growth factor, an essential nutrient component for *Cl. sporogenes* and other obligate anaerobes has been obtained. This preparation has the properties of an unsaturated hydroxy-acid of molecular weight about 200. Its methyl ester may be distilled in a high vacuum. The purified product, liberated from the inactive distilled methyl ester by alkaline hydrolysis, invariably promotes visible growth on a medium of otherwise known composition when added in quantities as small as 0.04 γ per ml.

2. The relation of the sporogenes growth factor to other accessory growth factors of lower organisms has been discussed.

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