4-Hydroxy-4'-cyanostilbene (28). 4-Cyanophenylacetic acid [Jaeger & Robinson, 1941] (2.0 g.), 4-hydroxybenzaldehyde (1.0 g.) and piperidine (1 ml.) were heated at 140° for 1½ hr. The semi-liquid product was mixed with 50% ethanol, when a yellowish solid was rapidly deposited. This was collected, washed with fresh 50% ethanol, crystallized twice from glacial acetic acid and finally from 70% ethanol: 4-hydroxy-4'-cyanostilbene was obtained in faintly coloured prisms, m.p. 221–223°. (Found: C, 81.3; H, 5.0; N, 6.4%. $C_{15}H_{11}NO$ requires C, 81.4; H, 5.0; N, 6.3%.)

4-Hydroxy-4'-amidinostilbene hydrochloride (29). This was prepared from 4-hydroxy-4'-cyanostilbene according to the general procedure of Ashley, Barber, Ewins, Newbery & Self [1942]. 4-Hydroxy-4'-amidinostilbene hydrochloride crystallized from hot water in long yellowish needles, m.p. 316–317° (decomp.). (Found: C, 65.6; H, 5.4; N, 9.9; Cl, 12.5%. $C_{15}H_{10}ON_3Cl$ requires C, 65.6; H, 5.5; N, 10.2; Cl, 12.8%.)

SUMMARY

1. A series of compounds of the stilboestrol type has been prepared and tested *in vitro* for bactericidal activity against staphylococci and

streptococci. Several have been found to possess exceptionally high activity, the most active being

which is some 40 times as active as the mould metabolic product citrinin, and as active as chloroxylenol under the conditions employed.

2. A comparison of the bactericidal and oestrogenic activities of these compounds shows that the two actions are not necessarily concurrent, though both probably depend on the ready acceptance by the cell of molecules of the cyclopentanophenanthrene and stilboestrol types.

3. *In vivo* the compounds tested showed little or no activity, possibly on account of their very low solubility.

The authors thank Dr Sydney Smith and Dr J. W. Trevan for their interest in this work, and Mr Bennett of the Wellcome Chemical Works, Dartford, for the microanalyses.

REFERENCES

- Aschheim, S. & Hohlweg, W. [1933]. *Dtsch. med. Wschr.* **59**, 12.
 Ashley, J. N., Barber, H. J., Ewins, A. J., Newbery, G. & Self, A. D. H. [1942]. *J. chem. Soc.* p. 103.
 Auwers, K. v. & Noll, W. [1938]. *Liebigs Ann.* **535**, 245.
 Carlisle, C. H. & Crowfoot, D. [1941]. *J. chem. Soc.* p. 6.
 Ciamician, G. & Silber, P. [1914]. *Ber. dtsh. chem. Ges.* **47**, 1809.
 Cullinane, N. M. [1923]. *J. chem. Soc.* **123**, 2053.
 Dodds, E. C., Golberg, L., Lawson, W. & Robinson, R. [1939]. *Proc. roy. Soc. B*, **127**, 140.
 Fildes, P. [1940]. *Lancet*, **1**, 955.
 Freund, M. & Remse, P. [1890]. *Ber. dtsh. chem. Ges.* **23**, 2865.
 Hewitt, J. T., Lewcock, W. & Pope, F. G. [1912]. *J. chem. Soc.* **101**, 604.
 Jaeger, R. & Robinson, R. [1941]. *J. chem. Soc.* p. 744.
 Janssen, H. [1889]. *Liebigs Ann.* **250**, 132.
 Kharasch, M. S. & Kleiman, M. [1943]. *J. Amer. chem. Soc.* **65**, 9.
 Mellwain, H. [1943]. *Nature, Lond.*, **151**, 270.
 Moritz, C. & Wolfenstein, R. [1899]. *Ber. dtsh. chem. Ges.* **32**, 2533.
 Oxford, A. E. [1942]. *Chem. Ind.* **61**, 48.
 Petrenko-Kritschenko, P. [1892]. *Ber. dtsh. chem. Ges.* **25**, 2242.
 Ramart-Lucas & Anagostopoulos [1928]. *C.R. Acad. Sci., Paris*, **186**, 1626.
 Rising, M. M. & Tsoh-Wu Zee [1928]. *J. Amer. chem. Soc.* **50**, 1706.
 Späth, E. [1913]. *Mh. Chem.* **34**, 2009.
 Stoermer, R. & Kippe, O. [1903]. *Ber. dtsh. chem. Ges.* **36**, 4009.
 Womack, E. B., Campbell, N. & Dodds, G. B. [1938]. *J. chem. Soc.* p. 1402.

Growth-promoting Substances from Bacteriological Filter Pads

By G. J. E. HUNTER (Travis Research Fellow), *Travis Laboratory, Medical School, University of Otago, New Zealand*

(Received 7 October 1943)

During an investigation of the nutritional requirements of acid-fast saprophytes [Edson & Hunter, 1943], synthetic media were employed, solutions of possible growth stimulants being added aseptically to a basal salt mixture. The methods of sterilization were selected according to the nature and stability of the chemical compounds involved. It soon be-

came evident that the results varied with the mode of preparation and filtration of the test substances. It was shown that solutions, both in water and in saline, were able to extract materials from filter pads in a concentration sufficiently high to stimulate the growth of acid-fast organisms, so that false positive results were sometimes recorded.

MEDIA AND METHODS

The salt mixture, at pH 7.5, was the one described by Edson & Hunter [1943]; the medium and the inoculum were prepared, and growth estimated, as before. Stock solutions of the source of carbon (glucose) and solutions (usually 10 ml. quantities) of substances to be tested were sterilized by filtration through Seitz E.K. or Ford S.B. pads clamped in the glass and steel holders described by Knight & Fildes [1936].

By these methods clear-cut results were obtained, and it was shown that of over 200 different compounds tested, those which were active belonged to four classes: (1) citric acid, (2) α -amino-acids or compounds in which the amino groups were bound in peptide linkages, (3) histamine, (4) ions of calcium and iron.

Growth of Mycobacteria in media sterilized by different methods

Anomalous results were first clearly found when solutions of 2:5-diketopiperazine were sterilized by different methods. The results for *Myc. phlei* are set out in Table 1. When growth occurred, it was

evident that the anhydride was inactive (flask 5), and that the methods of preparation and sterilization were responsible for the difference in flasks 3 and 4.

Similar examples of 'false' stimulation were encountered when it became necessary to filter the final medium containing other test substances or when the solubility of some compounds precluded the preparation of concentrated stock solutions. Whenever heat-stable substances were added to the medium and autoclaved, no false results were obtained. 'False' stimulation occurred, however, if small quantities of the test substance were added to the basal salt mixture and the whole passed through a 3 or 6 cm. filter pad. Many of the unstable compounds under investigation proved to be sufficiently free from bacterial contamination to permit their use without previous sterilization, and thus it was possible to prove that the stimulation in filtered solutions was 'false'.

Filtration of the basal salt mixture alone through a pad caused stimulation of the growth of acid-fast saprophytes in the presence of a suitable source of carbon. Table 2 shows results of tests in media under different conditions. Growth in a filtered

Table 1. *Effect of method of sterilization of synthetic medium on growth of Myco. phlei*

	Flask no.				
	1	2	3	4	5
Source and treatment of 2:5-diketopiperazine	0.4 ml. of 0.5% autoclaved stock solution added aseptically to autoclaved salt mixture	2 mg. solid added to salt mixture and autoclaved	0.4 ml. of 0.5% filtered stock solution added aseptically to autoclaved salt mixture	2 mg. solid added to salt mixture and filtered	2 mg. unsterilized solid added to autoclaved salt mixture
Growth at 7 days	+	+	Nil	+	Nil

Medium: 20 ml. basal salt mixture containing 1% of glucose and 0.01% 2:5-diketopiperazine. Inoculum, 0.01 mg. Temp. 38°.

copious and was checked by microscopic examination of stained smears, and by subculture of a small portion of surface film on Dorset's egg medium.

Autoclaving of aqueous and saline preparations of 2:5-diketopiperazine (flasks 1 and 2) probably induced hydrolysis to glycyl-glycine, which was subsequently shown to be a growth stimulant. It was

basal medium is compared with true stimulation by citrate and by *dl*-alanine.

The stimulant effect was progressively greater as successive 20 ml. portions of salt mixture were passed through the same pad.

One other organism, *Esch. coli* (laboratory stock strain), was also tested, the same basal salt mixture

Table 2. *Growth of saprophytic Mycobacteria in different media*

Inoculum: 0.01 mg. seeded in 20 ml. medium. Time, 7 days. Temp. 38°.

Organism	Growth (mg. dry wt.) on				
	Autoclaved basal salt mixture + 1% glucose	Filtered basal salt mixture + 1% glucose	Autoclaved basal salt mixture + autoclaved citrate (0.01%) + 1% glucose	Basal salt mixture + <i>dl</i> -alanine (0.01%) autoclaved + 1% glucose	Broth controls
<i>Myc. phlei</i>	Nil	6.4	9.6	11.8	46.0
<i>Myc. stercoris</i>	Nil	6.7	9.5	8.0	37.1
<i>Myc. smegmatis</i>	Nil	6.2	9.6	13.2	50.2
<i>Myc. sp. Karlinski</i>	Nil	6.5	8.0	8.0	33.4
<i>Myc. sp. leprous Kedrowsky</i>	Nil	1.4	Trace	Trace	5.8

being employed, with an inoculum of one drop of a slightly opalescent suspension of washed bacilli. The results were read within 48 hr. and were unchanged after 3 days. No growth occurred on inoculation when glucose or citrate was the source of carbon in the autoclaved basal mixture, but the addition of glucose (1%) and citrate (0.01%) to autoclaved salt mixture stimulated heavy growth. When the basal salt mixture containing glucose was filtered through a pad, strong stimulation equal to that obtained by citrate addition was observed. The growth-promoting quality of Seitz filtrates was therefore not confined to species of the genus *Mycobacterium*.

The nature of the growth-promoting substances

The active materials were more easily extracted by water or saline mixture when these were hot than when cold and hence the substances were readily extracted by autoclaving portions of a pad in 20 ml. of basal mixture. Ether and alcoholic extracts were inactive, while tests for citric acid in aqueous extracts were consistently negative within the limits of the micro-tests employed. The pads appeared to consist mainly of asbestos and paper pulp; average ash content, 36.0%. Sodium silicate, tested as a possible stimulant, was ineffective. Although the water-soluble portion of pad ash (completely ashed in an electric furnace) caused little, if any, growth stimulation, the neutralized acid-soluble (HCl Analar) fraction produced a growth averaging 7.6 mg.

The results indicated that the stimulating inorganic material remained after ashing in a form soluble in dilute HCl. Of the numerous inorganic elements tested [Edson & Hunter, 1943], all were ineffective with the exception of Ca and Fe. It was found that Ca^{++} (50 $\mu\text{g.}$) and Fe^{+++} (20 $\mu\text{g.}$), produced growth stimulation approximately equal to that given by pad ash.

The filtered basal medium was very slightly turbid, and its behaviour on heating and cooling was suggestive of the presence of calcium phosphate. A positive test was given with ammonium oxalate, indicating, by comparison against known standards, the presence of about 100 $\mu\text{g.}$ Ca^{++} /20 ml. of medium. The Prussian blue test [Feigl, 1937] revealed a trace of Fe^{+++} , 1–10 $\mu\text{g.}$ /20 ml., but the $\alpha\alpha'$ -dipyridyl test for Fe^{++} was negative. Filter pads, extracted with HCl (Analar Fe-free) gave strong positive tests for Fe^{+++} with thiocyanate reagent, and analysis showed the presence of 250 $\mu\text{g./g.}$, or about 730 $\mu\text{g.}$ /6 cm. pad. The pads contained only a trace of Fe^{++} ($\alpha\alpha'$ -dipyridyl). The iron, however, was not soluble to any great extent in the basal salt mixture. Pad ash gave very strong tests for Fe^{+++} (KCNS) and Ca^{++} (oxalate). It is also of interest to note that acid extracts of pads gave positive tests for copper, determinations based on the diethyl-dithio-carbamate colour reaction [Callan & Henderson, 1929] showing the presence of 10–15 $\mu\text{g./6 cm. pad.}$

Since previous experiments [Edson & Hunter, 1943] had shown that the growth of acid-fast sapro-

phytes was stimulated by low concentrations of Ca and Fe, it appeared that the presence of these ions was responsible for the results obtained with solutions which had been filtered through the pads. It was further shown that the filtered stock 10% glucose solutions contained Ca^{++} and traces of Fe^{+++} . In practice 2 ml. of 10% glucose were added to the salt mixture to give 1% sugar in the final medium. It was found, however, that 20 ml. of salt mixture, containing 2 ml. glucose solution, gave negative tests for Ca^{++} (by the method used), and that small additions did not supply a sufficiently high concentration of calcium ions to stimulate growth during the period of the test. Similarly the aseptic addition of small volumes, usually less than 0.5 ml., of preparations of test substances (as in Table 1) to autoclaved synthetic medium did not convey sufficient material to affect the growth of *Mycobacteria* under the conditions employed.

DISCUSSION

The findings reported show the possibility of unwittingly adding extraneous materials during filtration of media employed in investigations of bacterial nutrition. Ions of calcium and iron, which have been shown to be growth stimulants for members of the genus *Mycobacterium*, were extracted from filter pads. By comparison of the type of growth stimulated by Ca^{++} and Fe^{+++} separately with that promoted by pad ash and extracts, it was evident that calcium ions were the main stimulating agents and that not enough iron was extracted to affect growth. The growth produced with pad materials and by Ca was relatively unpigmented and somewhat slimy in character, while that with Fe was granular and deeply pigmented. It was noted, also, that whenever the pad filtrates were very slightly turbid, i.e. when the calcium phosphate or traces of fibre which the pads yielded were just visible, the growth of *Myco. phlei* was more profuse. This is analogous with the findings of Bigger & Nelson [1941], on the effect of talc and other insoluble inorganic substances on the growth of coliform bacilli in water.

It is not suggested that the results eliminate the presence of organic growth factors in pads, but in view of the fact that the stimulation obtained in filtered media was of the same order and appearance as that given by pad ash, it seems likely that, for the *Mycobacteria* at least, the ions of Ca and possibly of Fe are the principal growth-stimulating substances extractable from ordinary bacteriological filter pads.

SUMMARY

In a study of the nutritional requirements of various species of the genus *Mycobacterium*, Seitz filtration

was employed for the purpose of sterilizing certain preparations of possible growth stimulants. It was found that Ca^{++} and Fe^{+++} were extracted from filter pads in a single filtration. These ions were growth stimulants for the acid-fast organisms under the conditions used, and their presence in filtrates

accounted for anomalous results obtained with some preparations.

The author desires to express his gratitude to Dr N. L. Edson for helpful advice and criticism. Acknowledgement is also made to the W. H. Travis Trustees for their interest in the work, and to Dr H. A. Krebs for correcting the proofs.

REFERENCES

- Bigger, J. W. & Nelson, J. H. [1941]. *J. Path. Bact.* **53**, 189.
 Callan, T. & Henderson, J. A. R. [1929]. *Analyst*, **54**, 650.
 Edson, N. L. & Hunter, G. J. E. [1943]. *Biochem. J.* **37**, 563.
 Feigl, F. [1937]. *Qualitative Analysis by Spot Tests*. New York: Nordemann Publishing Co.
 Knight, B. C. J. G. & Fildes, P. [1936]. *Biochem. J.* **30**, 1138.

The Mechanism of Coprosterol Formation *in vivo*

2. ITS INHIBITION BY SUCCINYL SULPHATHIAZOLE AND BY CARBARSONE

BY O. ROSENHEIM AND T. A. WEBSTER, *National Institute for Medical Research, Hampstead, N.W. 3*

(Received 30 September 1943)

The discovery that brain contains a substance, probably identical with or allied to the cerebroside phrenosin, which enables the organism to convert large amounts of cholesterol, added to the diet, into coprosterol [Rosenheim & Webster, 1941], offers a convenient experimental method for investigating the effect on coprosterol formation of drugs which act specifically on the intestinal flora or fauna. In view of the surprising lack of evidence for the theory, advanced on purely theoretical considerations by the early investigators [Bondzynski & Humnicki, 1896; Müller, 1900], and held ever since, that putrefactive intestinal bacteria are responsible for the conversion of cholesterol into coprosterol by direct hydrogenation, we selected in the first instance the sulphonamide derivative succinyl sulphathiazole (S.S.) for investigation. This drug exerts a powerful bacteriostatic action on *Escherichia coli* and other coliform bacteria of the intestine and has the additional advantage of low toxicity, owing to its poor absorption from the alimentary tract.

As the outcome of this investigation, together with other experimental evidence, made it improbable that intestinal bacteria have a share in coprosterol formation, the possibility was considered whether the fauna, and especially the protozoan population, of the intestine rather than its flora was concerned in the process. This possibility was investigated by examining the coprosterol excretion of rats treated with the amoebicidal drug carbarsone (*p*-carbamino phenylarsonic acid). This arsenical also frees the intestinal tract from the flagellate *Trichomonas*, a non-pathogenic parasite occurring

in the intestinal canal of man and animals. A drug specifically acting on *Trichomonas* was chosen for the test, since it has been claimed that cholesterol is an essential growth factor for four species of *Trichomonas* [Cailleau, 1937; 1939].

EXPERIMENTAL

Methods

The technique of the animal experiments, the composition of the diet, and the analytical methods used have previously been described [Rosenheim & Webster, 1941]. S.S. was administered by intimately mixing the drug with the diet to the amount of 5% [Welch, Mattes & Latven, 1942]. The S.S. contents of the faeces were determined by the method of Bratton & Marshall [1939]. Carbarsone was given in the drinking water, which consisted of a 0.5% solution of the drug in a 1% solution of sodium hydrogencarbonate in distilled water [Gabalson, 1936]. *Esch. coli* counts were made by the usual method, using poured MacConkey's plates. The faeces and intestinal contents were examined for *Trichomonas* microscopically and by cultivation in the medium of Dobell & Laidlaw [1926]. The protozoological examinations were kindly made by our colleague, Dr Clifford Dobell.

Experiments with succinyl sulphathiazole

In a preliminary experiment two groups each of three male rats were used. During a period of 3 days they were fed on the stock diet, the experimental group receiving S.S. as well. Bacteriological examination of the faeces on the fourth day showed that *Esch. coli* had been almost eliminated in the experimental group, whilst the faeces of the control group contained 50×10^6 organisms/g.