blood stream would be exclusively mono-oleo- (or *iso*-oleo-) glycerides, amongst which oleo- (or *iso*-oleo-) distearin would be prominent. There is no evidence in this work that any desaturation of stearo-glycerides occurs during the transformation of hydrogenated groundnut oil to glycerides in the milk of the cow.

We are indebted to Mr A. H. Blissett for his assistance in the dietary trials and in the collection of the milk specimens, as well as for access to his records of milk yields, etc., of the individual cows; to Mr F. Burke and Miss E. H. Harrop for their assistance in the analysis of the fats and ester-fractions; and to the Directors of Messrs J. Bibby and Sons, Ltd., for permission to publish the results of this investigation.

REFERENCES

- Arnschlick, L. [1889]. *Z. Biol.* **26**, 434.
 Bloor, W. R. [1914]. *J. biol. Chem.* **16**, 517.
 Brown, W. C., Dustman, R. B. & Weakley, C. E. [1941]. *J. Dairy Sci.* **24**, 265.
 Cranfield, H. T. [1916]. *Analyst*, **41**, 336.
 Graham, W. R., Jones, T. S. G. & Kay, H. D. [1936]. *Proc. roy. Soc. B*, **120**, 330.
 Hilditch, T. P. & Jasperson, H. [1941]. *J. Soc. chem. Ind., Lond.*, **60**, 305.
 — & Meara, M. L. [1942]. *J. Soc. chem. Ind., Lond.*, **61**, 117.
 — & Sleightholme, J. J. [1930]. *Biochem. J.* **24**, 1098.
 — & Thompson, H. M. [1936]. *Biochem. J.* **30**, 677.
 Hill, O. J. & Palmer, L. S. [1938]. *J. Dairy Sci.* **21**, 529.
 Holland, E. B. & Buckley, J. P. [1918]. *J. agric. Res.* **12**, 719.
 — [1923]. *J. agric. Res.* **24**, 365.
 Langworthy, C. F. [1923]. *Industr. Engng Chem.* **15**, 277.
 Maynard, L. A., McCay, C. M., Ellis, G. H., Hodson, A. Z. & Davis, G. K. [1938]. *Mem. Cornell agric. Exp. Sta.* no. 211.
 Shaw, J. C. [1939]. *J. Dairy Sci.* **22**, 438.
 Shaw, J. C. & Petersen, W. E. [1938]. *J. Dairy Sci.* **21**, A, 122.
 Smith, J. A. B. & Dastur, N. N. [1938]. *Biochem. J.* **32**, 1868.

Sclerotiose—a Polysaccharide Metabolic Product of *Penicillium Sclerotiorum* van Beyma

By V. J. ALBERICCI, T. P. CURTIN AND D. REILLY, *From the National University of Ireland (University College, Cork)*

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During work on the metabolism of *Penicillium sclerotiorum*, disappearance of the carbohydrate constituent of the acid Czapek-Dox and other media employed was observed [Curtin & Reilly, 1940; Reilly & Curtin, 1943]. This, it was suggested, was probably the most important factor in the metabolic process. Had the disappearance of the carbohydrate been accompanied by a rapid and permanent decrease in the pH, the production of an acid in the substrate would have been indicated. It was invariably found, however, in batches grown on a number of different media, that though a drop in pH occurred initially, the pH of the fully grown substrate reverted towards neutrality. That there was no connexion between these variations in pH and (a) the initial pH of the medium or (b) the production of sclerotiorine by the mould, was proved by the fact that similar series of pH readings were obtained when the mould was incubated (1) on acid Czapek-Dox medium (pH 4.2) with the production of sclerotiorine, and (2) on Raulin-Dierckx medium (pH 7.0) (when sclerotiorine is not produced). The filtered substrate of the mould after a full period

of growth on acid Czapek-Dox medium contained no reducing carbohydrate. This, coupled with the fact that elimination of glucose caused no permanent drop in the pH value, suggested production by the mould of an insoluble glucose complex.

The mycelium was therefore extracted with boiling water and the flocculent precipitate which appeared on cooling was found to be a polyglucose, of specific rotation $\alpha_D = +244$ to $+284$, and for which we suggest the name 'sclerotiose'. In properties, it proved to be similar to other polysaccharides isolated from the lower fungi, being soluble only in hot water, giving no colour with iodine, and dissolving only to a slight extent in dilute NaOH, thus resembling mycodextran from *P. expansum* [Dox & Niedig, 1914], the polysaccharides from *P. digitatum* and *Fumago vagans* [Birkinshaw, Charles & Raistrick, 1931], and the polysaccharide from *P. javanicum* [Ward, Lockwood, May & Herrick, 1935] for which the specific rotations range from $\alpha_D = +217$ to $+299$. An examination has been made of sclerotiose and of some of its derivatives.

METHODS AND DISCUSSION

Over a period of 24 days, 40 bottles, each containing 100 ml. of substrate, were inoculated and grown as described earlier [Reilly & Curtin, 1943]. At intervals of 3 days, three representative cultures were filtered, adjusted where necessary to a vol. of 300 ml., and examined by the polarimeter in a 20 cm. tube. These figures are given in Table 1.

Table 1. *Influence of growth of P. sclerotiorum on optical activity of acid Czapek-Dox medium*

Days of incubation	Polarimeter reading	Days of incubation	Polarimeter reading
0	+5.7°	9	+0.4°
3	+5.0°	12	+0.1°
6	+1.3°	15-24	0.0°

A batch of cultures of *P. sclerotiorum* grown on Raulin-Dierckx medium was then grown for a similar period of 24 days, and pH readings taken as above for three representative cultures every 3 days. The figures are given in Table 2.

Table 2. *Influence of growth of P. sclerotiorum on pH of Raulin-Dierckx medium*

Days of incubation	pH (colorimetric)	Days of incubation	pH (colorimetric)
0	7.0	15	3.1
3	5.4	18	3.6
6	4.2	21	4.7
9	2.4	24	5.1
12	2.6		

This table shows that the gradual drop in pH, followed by a rise towards neutrality, is not determined by the initial pH of the medium used.

For the isolation of sclerotiose, the bulk of the mycelium was grown for 24 days at 25° on the acid Czapek-Dox medium (glucose, 50 g.; KH_2PO_4 , 1.0 g.; NaNO_3 , 2.0 g.; KCl, 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g.; distilled water to 1000 ml.). It was also grown on certain modifications of this medium.

The mycelium was repeatedly extracted with boiling water until, on cooling, there was no further separation of flocculent material. The red aqueous extract was allowed to stand overnight, the supernatant liquid was then decanted and the precipitate separated by centrifuging. The red-tinted slimy product was washed by suspending in 2000 ml. distilled water and filtering through a double filter, on which had been deposited a uniform layer of HCl-extracted, calcined kieselguhr. It was then removed from the filter by eluting with boiling distilled water. The coloured solution was treated with norit-charcoal at 100° for $\frac{1}{2}$ hr., the operation being repeated two or three times. The charcoal was finally removed by filtering through a silica plate. Sclerotiose now separated from the cooled liquid in a pure white flocculent mass. It was redissolved in fresh distilled water, allowed to settle and redissolved in fresh solvent, the procedure being repeated 10 to 15 times. The filtered product was washed with absolute ethanol, allowed to stand overnight in contact with the latter, and then washed with absolute ether and dried in a high vacuum. The pure, white, amorphous compound turned brown on heating above 260°, fused at 290-300°, and gave no residue

on ignition. The yield of polysaccharide was 2.8-3.0 g./100 g. mycelium. (Found: C, 40.4; H, 6.8; O, by difference, 52.8%. $(\text{C}_6\text{H}_{10}\text{O}_5)_n \cdot (\text{H}_2\text{O})_n$ requires C, 40.0; H, 6.7; O, 53.3%.) The molecule of water was removed by drying *in vacuo* over P_2O_5 at 90° for 2 hr. (Found: C, 44.1; H, 6.26%. $(\text{C}_6\text{H}_{10}\text{O}_5)_n$ requires C, 44.4; H, 6.17%.)

As already stated, the bulk of the mycelium extracted was grown on the acid Czapek-Dox medium. There were, however, some samples of mycelium, grown on modifications of this substrate, in stock, and these were extracted in the same way. On neutral Czapek-Dox medium (in which K_2HPO_4 replaces KH_2PO_4) the yield of polysaccharide was 10.1% of the extracted mycelium weight, and on the same medium, but with KBr replacing KCl, the yield was 10.0%. On acid Czapek-Dox medium, from which KCl was omitted, the quantity of sclerotiose was the same as when KCl was present, i.e. 2.9% of the mycelium weight. With the Raulin-Thom medium (glucose, 50 g.; NH_4 tartrate, 4.0 g.; K_2CO_3 , 0.6 g.; $(\text{NH}_4)_2\text{HPO}_4$, 0.6 g.; $(\text{NH}_4)_2\text{SO}_4$, 0.25 g.; MgCO_3 , 0.4 g.; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.07 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.07 g.; distilled water to 1500 ml.), the mould yielded 2.4% polysaccharide. On the Raulin-Dierckx substrate (glucose, 46.6 g.; $(\text{NH}_4)_2\text{SO}_4$, 0.16 g.; K_2CO_3 , 0.4 g.; $(\text{NH}_4)_2\text{HPO}_4$, 0.4 g.; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.04 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.04 g.; 66.7 ml. of a solution of 0.71 g. tartaric acid and 0.4 g. MgCO_3 in 100 ml. distilled water, adjusting to 1000 ml.), the yield was negligible. The contrast between the acid and neutral Czapek-Dox media in the production of sclerotiose finds a parallel in the different rates of KCl metabolism described in the recent communication of Reilly & Curtin [1943].

Properties of sclerotiose

Sclerotiose is insoluble in all organic solvents and in cold water, but dissolves readily in hot water. It is slightly soluble in warm dilute NaOH, and in formamide solution. The solution in water gives no coloration with iodine and does not reduce Fehling's solution on boiling. It gives Molisch's carbohydrate reaction but does not precipitate lead acetate from solution. A boiling solution of sclerotiose turns aniline-acetate-paper pink. With phloroglucinol, it gives a yellow coloration in the cold, which turns brown on boiling. The products of acid hydrolysis, after neutralization with NaOH, reduce both Fehling's and Pavy's solutions. On hydrolysing with dilute HCl, adding excess NaOH and heating, an aldose resin is formed.

If the moist solid is dried without using ethanol and ether, a grey-black, horny mass is obtained which is insoluble in water, even on prolonged heating. On testing with Pavy's solution, no hydrolysis could be detected when sclerotiose was treated with dilute NaOH at 70°.

The specific rotation was found to be $[\alpha]_D = +244$ at 70° in 6% NaOH and $[\alpha]_D = +284$ at 90° in formamide.

Hydrolysis of sclerotiose

This polysaccharide was shown to be a poly-glucose as follows: Sclerotiose (1 g.) was refluxed with 25 ml. $N \text{ H}_2\text{SO}_4$ for 6 hr., 25 ml. $N \text{ NaOH}$

were then added, together with 6 ml. phenylhydrazine and 3 ml. glacial acetic acid. The mixture was heated on the water-bath for 30 min. and the osazone which separated out in yellow needles was filtered off and recrystallized. It melted at 206° when heated quickly, and when 0.2 g. was dissolved in 4 ml. pyridine and 6 ml. ethanol, the rotation was $\alpha_D = +1.46$. Neuburg [1899] states that for glucosazone under similar conditions $\alpha_D = +1.50$.

Further confirmation was obtained as follows: sclerotiose (0.5 g.) was refluxed for 6 hr. with 25 ml. water and 25 ml. $N H_2SO_4$. The liquid was then titrated, with phenolphthalein as an indicator, with 24.7 ml. $N NaOH$ and the volume adjusted to 100 ml. The glucose content was then estimated (1) polarimetrically, which gave 0.52 g.; (2) by the iodine method of Willstätter & Schudel [1918] as modified by Judd [1920], the result then being 0.52 g. using the Baker & Houlton [1920] value of 1 g. glucose equivalent to 1.41 g. iodine; (3) by means of Fehling's solution when the value was 0.50 g.

If we assume the first two results to be correct, the glucose produced from 0.5 g. of pure, dry, ash-free sclerotiose is 0.52 g., the calculated figure being 0.55 g.

Nitration of sclerotiose

The method was that employed by Pictet & Reilly [1921] for the nitration of levulosan. Finely powdered polysaccharide (1 part) was added gradually with vigorous stirring at room temperature, and without appreciable rise, to 50 parts of a mixture of 3 parts by weight H_2SO_4 (sp. gr. 1.84) and 1 part by weight HNO_3 (sp. gr. 1.42). The mixture was allowed to stand overnight. The compound did not dissolve during the esterification process and showed little physical change. The nitrate was boiled for a short time with slightly acid water to decompose any small amount of sulphate formed, and was then washed with distilled water and finally dried *in vacuo* over concentrated H_2SO_4 when it gave a pure white solid, which became brown at 165° and decomposed at about 200° . (Found: C, 26.8; H, 3.1; N, 11.9; O, by difference, 57.8%. $C_{24}H_{31}O_{11}(NO_3)_9$ requires C, 27.2; H, 3; N, 11.9.)

Properties of sclerotiose nitrate. This nitrate is insoluble in boiling water, dilute acid and ethanol; it is soluble in acetone, amyl and ethyl acetates, and in nitrobenzene and is sparingly soluble in diacetone alcohol. Like nitrocellulose of the same percentage composition, it is completely soluble in a mixture of ether and ethanol. It is highly inflammable and gives no residue when ignited on copper foil. On slow evaporation of an acetone solution, the gelatinous product which separates first is followed by a slightly opaque celluloid-like modification. When slowly heated to the decomposition point, the nitrate liberates oxides of nitrogen. The specific rotation in acetone solution is $[\alpha]_D = +119$.

An attempt to form a higher nitrate, by the use of fuming HNO_3 , resulted in the production of sclerotiose endecanitate. As before, 1 g. of the polyglucose was added to a mixture of 25 ml. H_2SO_4 (sp. gr. 1.84) and 15 ml. fuming HNO_3 (sp. gr. 1.50), the nitrate being isolated and purified as above. (Found: N, 13.2%. $C_{24}H_{29}O_9(NO_3)_{11}$ requires

N, 13.5%.) It is similar in properties to the lower nitrate, but is sparingly soluble in a mixture of ethanol and ether. It decomposes at 132° with deflagration.

Treating a molecular mixture of the two nitrates with reducing agents, such as $(NH_4)_2S$ and H_2S , removes the nitrate groups. The regenerated sclerotiose so produced is not, however, absolutely pure, since it gives the diphenylamine test.

Sclerotiose diformate

0.9 g. of the pure, dry polysaccharide was added to 90 ml. of formic acid and 10 ml. concentrated H_2SO_4 with constant stirring. The polysaccharide quickly dissolved to give a slightly opaque brown solution without an appreciable rise in temperature. After standing overnight, the formate was precipitated by pouring into ice-water, was recrystallized from formic acid, and then dried *in vacuo*. This compound is soluble in pyridine and formic acid but insoluble in other organic solvents. It is also slightly soluble in thiocyanate solutions. The formic acid content was determined by slowly treating 0.5 g. of the dry formate with 75 ml. $N K_2Cr_2O_7$, and adding 20 ml. concentrated H_2SO_4 , the mixture being kept cool. Further cooling discourages the evolution of CO_2 . The mixture was then left for 8 hr. on a boiling water-bath and the excess of $K_2Cr_2O_7$ titrated with $N FeSO_4$ solution. (Found: 39.7% formic acid. $(H.COO)_2C_6H_8O_3$ requires 42.2%.)

Preparation of acetyl derivatives of sclerotiose

The method used was that of Reilly, Hayes & Drumm [1931]. 2 g. finely powdered polysaccharide were added to 20 g. acetic anhydride and 2 drops of concentrated H_2SO_4 . Most of the solid dissolved to give a brown solution with liberation of heat. The mixture was then heated nearly to the boiling-point when complete solution was effected. The crude acetate, precipitated on pouring the reaction mixture into ice-water, was washed free from acetic acid, dried and extracted with acetone. The insoluble fraction was dissolved in chloroform. Both fractions were then shaken with norit charcoal and allowed to stand for 24 hr.

Acetone-soluble acetate. The decolorized solution, filtered free from charcoal, was poured into ice-water, when the acetate separated as a fine white powder, which did not settle out on standing. It was gravity-filtered through a silica plate. When dry it was slightly coloured. Accordingly, it was redissolved in acetone, treated with charcoal, precipitated and dried again. The pure white acetate so obtained was insoluble in water, ethyl and amyl alcohols, carbon tetrachloride and benzene, but was soluble in acetone, pyridine and glacial acetic acid. On analysis, it was found to contain 48.2% acetic acid; $C_6H_8O_3(CH_3COO)_2$ requires 48.8%.

Chloroform-soluble acetate. The acetate was precipitated by pouring the faintly brown solution into ice-cold ether and the grey precipitate redissolved in $CHCl_3$ and then treated as above. On evaporating under reduced pressure microcrystals were obtained. These were further purified by dissolving in warm glacial acetic acid and precipitating with ice-water. The pure white crystalline acetate so obtained was soluble in chloroform, pyridine and glacial acetic acid but insoluble in water, acetone, ethanol, benzene and ether. It fused at $166-172^\circ$ to a gelatinous mass. The compound was found to contain 61.7% acetic acid; the compound $C_6H_7O_2(CH_3COO)_3$ requires 62.5%.

It was thought that the brown colour of the reaction mixture previously mentioned might indicate contamina-

tion by degradation products. The preparation was therefore repeated, using Barnett's method [1921] in which sulphuryl chloride is employed as a catalyst (thereby reducing degradation to a minimum) and was carried out according to the modification used by Irvine & Hirst [1922] for the preparation of cellulose tri-acetate. 2 g. sclerotiose were soaked in 10 ml. glacial acetic acid, through which a stream of dry sulphuryl chloride had been passed for 30 sec. After setting aside for $\frac{1}{2}$ hr., 122 ml. of acetic anhydride were added and SO_2 was then passed through the liquid for 1 min. At the end of 1 hr., during which the pasty mixture was vigorously stirred, the temperature was raised to 65° and maintained there until the polysaccharide had all dissolved to give a clear, colourless, viscous solution. After cooling to 30° , an equal volume of CHCl_3 was added, followed by a large excess of water. The mixture was continuously stirred and the CHCl_3 evaporated. During this process the acetate separated in the form of fine white granular particles, which were obtained as an aggregate of microcrystals on slow evaporation of the CHCl_3 solution. m.p. $165\text{--}168^\circ$ (decomp.). (Found: C, 49.6; H, 5.8; O, by difference, 44.6%. The tri-acetate $\text{C}_6\text{H}_7\text{O}_5(\text{OC}\cdot\text{CH}_3)_3$ requires C, 50.0; H, 5.5; O, 44.5%.)

There was no appreciable loss of material by this method. The filtrate had no action on Fehling's solution, and on evaporation to dryness gave a negligible residue; it was optically inactive. The specific rotation of sclerotiose tri-acetate, in CHCl_3 solution, was $[\alpha]_D = +205.7$, while the acetic acid content was 62.1% (calc. 62.5%).

Sclerotiose benzoate

Benzoylation was carried out by a modification of the Schotten-Baumann reaction. The final product had a benzoic acid equivalent of 65.6%. It was therefore probably the tri-benzoate, but contained some di-benzoate.

SUMMARY

1. Investigation of the rapid glucose consumption of *P. sclerotiorum*, on Czapek-Dox and Raulin-Thom media, led to the isolation of a polyglucose, somewhat similar to other polysaccharide metabolic products of the lower fungi.

2. The influence of the culture medium and its pH were amply demonstrated by the varying yield of sclerotiose. As expected the neutral medium gave the greater proportion of polyglucose, the more acid substrates having a hydrolysing effect [Reilly & Curtin, 1943]. Of the derivatives prepared, the primary or tri-derivative was always present in preponderating amount.

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REFERENCES

- Baker, J. L. & Houlton, H. F. E. [1920]. *Biochem. J.* **14**, 754.
 Barnett, W. L. [1921]. *J. Soc. chem. Ind., Lond.*, **40**, 8T.
 Birkinshaw, J. H., Charles, J. H. V. & Raistrick, M. [1931]. *Philos. Trans. A*, **220**, 355.
 Curtin, T. P. & Reilly, J. [1940]. *Biochem. J.* **34**, 1419.
 Dox, A. W. & Niedig, R. E. [1914]. *J. biol. Chem.* **18**, 167.
 Irvine, J. C. & Hirst, E. L. [1922]. *J. chem. Soc.* **122**, 1587.
 Judd, M. [1920]. *Biochem. J.* **14**, 754.
 Neuberg, C. [1899]. *Ber. dtsh. chem. Ges.* **32**, 3384.
 Pictet, A. & Reilly, J. [1921]. *Helv. chim. Acta*, **4**, 613.
 Reilly, D. & Curtin, T. P. [1943]. *Biochem. J.* **37**, 36.
 Reilly, J., Hayes, M. & Drumm, P. J. [1931]. *Proc. R. Irish Acad.* **8**, 102.
 Ward, G. E., Lockwood, L. B., May, O. E. & Herrick, H. T. [1935]. *Industr. Engng Chem.* **27**, 318.
 Willstätter, R. & Schudel, G. [1918]. *Ber. dtsh. chem. Ges.* **51**, 780.

The Excretion of a Metabolic Product of Salicylic Acid

By CECILIA LUTWAK-MANN (Fellow of Newnham College, Cambridge),
 From the Biochemical Laboratory, Cambridge

(Received 23 December 1942)

The experiments described below are a continuation of the work by Lutwak-Mann [1942].

METHODS

Alkali test in urine. It was shown previously that shortly after the injection of salicylate there appears in rat urine a substance, which on addition of fairly strong NaOH, and in the presence of air, causes the urine to turn brown to black. The following modifications of this test have now been worked out. When only very small amounts of urine

are available 1-2 drops of it are added to about 4-6 ml. NaOH ($M/50$ or stronger). After a 30-60 sec. lag a pink colour develops which persists for varying lengths of time (2-5 min.), after which the solution turns yellow to brown. Excess of either urine or alkali prevents the appearance of the pink phase and only the final brown colour is seen. A still more reliable result is obtained by acidifying a few ml. of urine with 10% HCl and shaking it with 3 vol. ether. The separated ether extract is then shaken with a few ml. dilute NaOH. The aqueous solution turns at once a bright pink colour which persists for a short while and then the