2. The number of protein disulphide groups reduced was greater with sodium tetrahydroborate than with sodium sulphite. In an extract of seeds which had been soaked for 16 hr., sodium tetrahydroborate did not reduce all the disulphide groups.

3. The results suggest that chemical reactions take place *in vivo* between glutathione and protein sulphydryl groups.

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REFERENCES

- Association of Official Agricultural Chemists (1950). *Methods of Analysis of the Association of Official Agricultural Chemists*, p. 103. Washington: A.O.A.C.
- Bailey, J. L. & Cole, R. D. (1959). *J. biol. Chem.* **234**, 1733.
- Benesch, R. E., Lardy, H. A. & Benesch, R. (1955). *J. biol. Chem.* **216**, 663.
- Brachet, J. (1959). *Nature, Lond.*, **184**, 1074.
- Burton, H. (1958). *Biochim. biophys. Acta*, **29**, 193.
- Carter, J. R. (1959). *J. biol. Chem.* **234**, 1705.
- Hatch, M. D. & Turner, J. F. (1959). *Biochem. J.* **72**, 524.
- Hopkins, F. G. & Morgan, E. J. (1943). *Nature, Lond.*, **58**, 288.
- Hopkins, F. G., Morgan, E. J. & Lutwak-Mann, C. (1938). *Biochem. J.* **32**, 1829.
- Huggins, C., Topley, D. F. & Jensen, E. V. (1951). *Nature, Lond.*, **167**, 592.
- Hughes, C. & Spragg, S. P. (1958). *Biochem. J.* **70**, 205.
- Liener, I. E. (1957). *J. biol. Chem.* **225**, 1061.
- Mazia, D. (1954). In *Glutathione*, p. 209. Ed. by Colowick, S. P. et al. New York: Academic Press Inc.
- Moore, S., Cole, R. D., Gundlach, H. G. & Stein, W. H. (1958). *Proc. 4th int. Congr. Biochem., Vienna*, **8**, 52.
- Narahara, H. T. & Williams, R. H. (1959). *J. biol. Chem.* **234**, 71.
- Rapaport, S. & Scheuch, D. (1960). *Nature, Lond.*, **186**, 967.
- Roberts, E. & Rouser, G. (1958). *Analyt. Chem.* **30**, 1291.
- Ryle, A. P. & Sanger, F. (1955). *Biochem. J.* **60**, 535.
- Sluyterman, L. A. (1957). *Biochim. biophys. Acta*, **25**, 402.
- Spragg, S. P. (1955). Ph.D. Thesis: Bristol University.
- Spragg, S. P. & Yemm, E. W. (1959). *J. exp. Bot.* **10**, 409.
- White, F. H. (1960). *J. biol. Chem.* **235**, 383.

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Porphyrins from Congenitally Porphyric (Pink-Tooth) Cattle

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Of all types of porphyria, congenital or erythropoietic porphyria is the most rare. Very few cases have been reported. Information on the excretion of porphyrins is meagre and sometimes even confused by results from cutanea tarda cases (Watson, Perman, Spurrell, Hoyt & Schwartz, 1959). Ever since Fourie (1936) and Rimington (1936) reported detailed studies of a herd of affected cattle, more reports and also more controversial findings have appeared. Whereas Fourie (1936) concluded that congenital porphyria is inherited as a Mendelian recessive characteristic in cattle, Jorgensen & With (1955) reported it to be a dominant character in swine. Most investigators have agreed that porphyrins from cases of the congenital disease are mainly those of the I series. Ellis, Barner, Madden, Melcer & Orten (1958) reported a predominance of coproporphyrin III and uroporphyrin III in bovine cases, whereas Rhode & Cornelius (1958) found no uroporphyrin III in samples from porphyric heifers.

This report is concerned with biochemical features of congenital porphyria (pink tooth) of a herd of cattle in Michigan, U.S.A. Besides the well known copro- and uro-porphyrin, special attention has been paid to the isolation and characterization of the penta-, hexa- and hepta-carboxylic porphyrin from samples of urine, blood and post-mortem materials. Their properties have been compared with the corresponding porphyrins isolated from cutanea tarda cases.

MATERIALS AND METHODS

Materials. Blood and urine samples were collected from more than ten cattle, including nine (b, e, g, j, l, m, n and r) affected, one (Q) suspected to be the carrier of the disease, and several young and adult animals. Post-mortem tissues, including liver, muscle, teeth and bones, were obtained from the heifer (g). All samples were kept under refrigeration and sent to us by air. Most of them were analysed within 3 days after collection.

The pedigree information, supplied by Dr D. E. Madden, is shown in Scheme 1.

Samples from (D) and (A) were not available. The breeding history of other cattle is not known.

Analysis of urine. Urine samples, about 150 ml. each, were coloured brown or cherry red with a purple fluorescence under ultraviolet light. Samples from cattle (l), (m) and (n) showed absorption bands at 505, 540, 570 and 615 $m\mu$ through a light-path of 2 cm. The porphyrins were first adsorbed on to talc and finally isolated as their methyl esters.

The quantitative determination of urinary porphyrins was done according to a procedure previously described (Chu & Chu, 1958). The accuracy of the determination varies with concentration of the sample. For an average concentration, it is within $\pm 3\%$. Because of lack of material, the colorimeter has not been calibrated with penta-, hexa- and hepta-carboxylic porphyrin. A larger error is involved in their values read from the calibration curves of copro- and uro-porphyrin. Porphyrins from other tissues were determined by paper chromatography with an error of $\pm 15\%$.

Analysis of blood. After centrifuging, each blood sample (about 100 ml.) yielded a pale-yellow plasma with a very faint pink fluorescence, and erythrocytes without much red fluorescence. They were respectively extracted with ethyl acetate-acetic acid (4:1, v/v), after Schwartz & Wikoff (1952). The purified ethyl acetate extracts were dried and esterified in the same manner as the urinary porphyrins.

Analysis of tissues. Liver (47 g.) and muscle (20 g.), without any fluorescence, were separately minced before extraction with ethyl acetate-acetic acid. A sample of bone marrow (35 g.), with a white rather than pink fluorescence, was cleaned from bone powder under a magnifier and ultraviolet light before removal of fat. It was then extracted with ethyl acetate-acetic acid.

The bones and teeth showed pink to red discoloration with a red fluorescence. They were respectively filed or drilled into powder without drying. A sample of 8.5 g. of powdered shank bone was thoroughly washed with ether and then soaked in 10% HCl overnight. The mixture was filtered on a sintered-glass filter and the residue was washed several times with 10% HCl. The combined HCl extract was diluted and the pH adjusted to 3.2 with sodium acetate. Then the porphyrins were adsorbed on to talc and processed by the usual procedure. Similarly, the powdered jaw bone (1.3 g.), dentine (0.67 g.) and cement (0.14 g.) of teeth were extracted for porphyrins.

Chromatographic separation of porphyrins. For the separation of methyl esters of porphyrins, a Hyflo Super-Cel column (Chu & Chu, 1957) was used. A solvent mixture of chloroform (U.S.P.)-light petroleum (b.p. 30-60°)

(1:2, v/v) was used for separating porphyrins from urine, plasma and bony materials. For porphyrins from erythrocytes, bone marrow and muscle, chloroform-light petroleum-benzene (2:30:1, by vol.) was used. The development of the chromatogram was stopped when the solvent front reached almost the bottom of the column. The monocarboxylic porphyrin ester was located near the bottom of the chromatogram, protoporphyrin ester at about the middle, coproporphyrin ester near the top and uroporphyrin ester on the top of the column.

For the separation of the I and III isomers of the uroporphyrin fraction, the procedure described by Chu & Chu (1957) was employed.

The free porphyrins and their methyl esters were also examined by paper chromatography at various stages of experiments. References for procedures will be given at appropriate places in the text.

Analysis of porphobilinogen. This was according to the method of Mauzerall & Granick (1956).

Infrared absorption. The experiment was done on a sample in the form of solid film (Chu & Chu, 1959c) with a Beckman IR5 double-beam instrument.

RESULTS

The results of the analyses of urinary porphyrins are given in Table 1. Urine samples, about 150 ml. each, from a normal female Holstein, a suspected Ayrshire female and the full brother to heifer (b) were also examined and were found to contain no porphyrins.

The uroporphyrin esters obtained from primary columns were respectively chromatographed on long Hyflo columns for the separation of isomers (Chu & Chu, 1957). The methyl esters of uroporphyrin I (m.p. 296-297°) and uroporphyrin III (m.p. 258-260°), thus isolated, showed their characteristic infrared absorption values to be the same as those of authentic samples (Chu & Chu, 1959c). Both uroporphyrins were decarboxylated to the corresponding coproporphyrins (methyl esters of copro-I, m.p. 254°, and copro-III, m.p. 145°) for further confirmation.

The hepta-, hexa- and penta-carboxylic porphyrin revealed absorption values identical with those from human cases of porphyria cutanea tarda (Chu & Chu, 1959a). Their melting points and crystalline forms, however, differed. The methyl ester of the heptacarboxylic porphyrin isolated from congenital porphyric cattle easily crystallized in fine needles, that of the hexacarboxylic porphyrin in long needles, and that of the penta-carboxylic porphyrin in curly fibres. These properties, together with relatively high melting points, suggest their resemblance to the corresponding porphyrin esters prepared by stepwise decarboxylation of uro-I (Chu & Chu, 1959b). Therefore a sample of the crude uroporphyrin (m.p. 292-294°) separated from the urine of heifer (l) was accordingly decarboxylated. The methyl

Table 1. *Urinary porphyrins of cattle*

For description of separation procedure see Materials and Methods section. Compounds were determined as methyl esters. Percentages are of the total porphyrin content. F, Female; M, male; tr., trace.

Animal	Sex	Age (months)	Total porphyrins (mg./100 ml.)	Copro-porphyrins			Penta-carboxylic porphyrins		Hexa-carboxylic porphyrins		Hepta-carboxylic porphyrins	Uroporphyrins			Other unidentified porphyrins (%)
				(%)	M.p.	(%)	(%)	M.p.	(%)	M.p.	(%)	M.p.	(%)	Isomer ratio (I:III)	
b	F	17	0.520	48.1	252°	9.7	225°	0.1	0.2	0.2	41.2	294°	41.2	92:8	0.7
e	M	64	0.365	55.2	251	10.5	226	0.2	0.7	0.7	33.0	292	33.0	91:9	0.4
g*	F	7	0.830	53.8	250	10.1	224	0.3	1.2	1.2	33.8	288	33.8	90:10	0.8
q*	M	7	0.060	48.1	254	3.1	226	0.4	1.5	1.5	46.0	294	46.0	89:11	0.9
j	M	13	0.283	52.1	252	5.4	226	0.1	0.6	0.6	41.5	288	41.5	95:5	0.3
l	F	2	6.700	50.4	254	10.5	228	0.6	1.1	1.1	36.4	295	36.4	92:8	1.0
m	F	4	3.750	44.0	254	12.5	222	1.4	250	1.1	40.4	294	40.4	91:9	0.6
n	F	17	1.410	47.9	254	10.1	227	0.1	0.6	0.6	40.6	293	40.6	85:15	0.7
r	F	22	0.305	51.7	249	5.8	222	0.4	1.0	1.0	40.8	286	40.8		0.3
Q	M	.	tr.	.	.	.	.	.	.	.	.	.	.	.	.

* Twins.

esters of hepta-, hexa-, penta- and copro-porphyrin, thus obtained, melted respectively at 247°, 230–240°, 224° and 249°, and showed a close resemblance to isomers of the I series by paper chromatography. Hepta-, hexa- and penta-carboxylic porphyrin directly isolated from urine samples also showed the same paper chromatogram with the kerosene (paraffin)–chloroform and kerosene–propanol system (Chu & Chu, 1959b). The relatively wide melting-point range of the hexacarboxylic porphyrin ester indicates the possible presence of two isomers of the I series, as previously discussed.

After separation on a secondary column, the combined heptacarboxylic porphyrin (m.p. 250°) from all the affected urine samples was decarboxylated to coproporphyrin. The methyl ester melted at 245–248°, consisting of about 90 % of copro-I.

The coproporphyrin fractions, comprising about 50 % of the total porphyrins, yielded long needles with melting points, paper chromatograms and infrared absorption values closely resembling those of coproporphyrin I ester.

The analytical results of blood porphyrins are listed in Table 2. In some instances, the data are given semiquantitatively, because of the low concentrations of porphyrins and small sizes of available samples. Nevertheless, some important constituents were obtained in crystals for final identification. By paper chromatography the crude plasma porphyrins showed the same pattern as urinary porphyrins except that they contained a higher percentage of the coproporphyrins. After purification, the crystalline esters of coproporphyrins, pentacarboxylic porphyrins and uroporphyrins were found to be identical with those prepared from urine samples. A trace of protoporphyrin was also detected in most of the plasma samples. This may have its source from traces of contaminating erythrocytes. In the erythrocytes, protoporphyrin was identified by its characteristic absorption values at 502, 540, 572 and 630 m μ in ethyl acetate. It was finally converted into crystalline methyl ester (m.p. 218°) and identified as protoporphyrin IX by paper chromatography (Chu, Green & Chu, 1951). Some samples also yielded crystalline esters of coproporphyrins (m.p. 254°) besides protoporphyrin.

A monocarboxylic porphyrin was found to be present in erythrocytes. By column chromatography the product, from animal (q), was obtained in crystalline plates. A similar product was also obtained from other animals in a smaller quantity. Its methyl ester melted at 225°. The amount was too small for observation of the absorption in the visible region. However, a Soret peak at 400 m μ was located in a chloroform solution of the porphyrin, and the protoporphyrin fraction from the same column showed a Soret band at 408 m μ . In

Table 2. Blood porphyrins of cattle

Compounds were determined as methyl esters for plasma (1) and erythrocytes (2). For details see text. Percentages are of the total porphyrin content. +, ++, ++++, Semiquantitative indications of amounts.

Animal	Total porphyrins (μg./100 ml.)	Mono-carboxylic porphyrin (%)	Protoporphyrin		Coproprophyrins		Pentacarboxylic porphyrins		Hexa-carboxylic porphyrins (%)	Hepta-carboxylic porphyrins (%)	Uroporphyrins	
			(%)	M.p.	(%)	M.p.	(%)	M.p.			(%)	M.p.
b 1	9	.	+	.	85	.	4	.	+	+	10	.
2	34	30	60	.	10	.	.	.	.	.	.	.
e 1	9	.	++	.	75	.	4	.	+	+	20	.
2	67	15	75	.	10	.	.	.	.	.	.	.
g* 1	12	.	.	.	84	250°	5	.	+	++	10	.
2	30	10	75	.	15	.	.	.	.	.	.	.
q* 1	2	.	+	.	85	.	2	.	+	+	12	.
2	38	70	25	.	5	.	.	.	.	.	.	.
j 1	4	.	1	.	85	.	2	.	+	+	12	.
2	250	2	62	.	36	.	.	.	.	.	+	.
l 1	25	.	+	.	82	254	5	225°	+	+	12	293°
2	410	2	55	219°	42	248	.	.	.	.	1	.
m 1	50	.	.	.	75	254	7	224	0.4	0.7	15	288
2	265	2	50	210	42	250	.	.	.	.	6	.
n 1	18	.	+	.	84	254	5	.	+	++	10	293
2	104	5	85	.	10	.	.	.	.	.	.	.
r 1	3	.	1	.	85	.	2	.	+	+	12	.
2	83	5	90	.	5	.	.	.	.	.	.	.
Q 1	.	.	.	.	.	.	.	.	.	.	.	.
2	.	+	++	.	+	.	.	.	.	.	.	.

* Twins.

paper chromatography it ran ahead of proto-, meso- and deuterio-porphyrin, either in the ester form with the kerosene method or as the free porphyrin with the modified lutidine method of Nicholas & Rimington (Chu & Chu, 1955). The results indicate that it is probably a monocarboxylic porphyrin. It was also detected in normal erythrocytes of man and cattle.

The results on the analyses of post-mortem tissues of the heifer (g), which was killed at the age of 10 months, are listed in Table 3. The porphyrin concentrations were generally low. The bladder-urine sample was analysed for porphobilinogen. Its concentration was found to be 0.65 mg./100 ml. The presence of porphobilinogen was further demonstrated by the formation of uroporphyrins when the porphyrin-free filtrate was heated. Its presence is not surprising, because Mauzerall & Granick (1956) have found it in normal urine.

The liver and muscle from the slaughtered animal showed no abnormal appearance. A sample of normal calf liver was also analysed for comparison. The porphyrin content of the normal liver tissue was found to be 5 μ g./g. Both the normal and affected tissues showed absorption values mainly of protoporphyrin at 500, 540, 570 and 628 m μ in ethyl acetate. After esterification and column chromatography, the methyl ester of protoporphyrin melted at 220°. The bone marrow and muscle yielded the same varieties of porphyrins with lower concentrations.

The bones and teeth showed brown and pink discoloration with pink to red fluorescence. The porphyrins accumulated were mainly uroporphyrins. From the melting points of their esters, it is assumed that the isomeric composition is about the same as that of the urinary products. Hepta- and hexa-carboxylic porphyrin stayed at the same concentration as those in the urine, but concentrations of penta- and copro-porphyrin were greatly reduced.

DISCUSSION

Porphyrins were isolated as methyl esters for better separation and more precise identification. Free porphyrins with similar absorptions and hydrochloric acid numbers are hardly distinguishable either spectrophotometrically or fluorimetrically. For instance, although pentacarboxylic porphyrin represents about 10% of the total urinary porphyrins, it has not been isolated before. It might have been determined with the copro-porphyrin fraction because of its identical hydrochloric acid number.

All the affected cattle, from 2 months to more than 5 years of age, irrespective of severity of the

disease, excrete urinary porphyrins, the methyl esters of which have higher melting points and apparently differ from those isolated from human cases of porphyria cutanea tarda (Chu & Chu, 1959a). When any porphyrin fraction from congenital porphyria is compared with the corresponding fraction from porphyria cutanea tarda, the former contains much less III isomer than does the latter.

Jorgensen & With (1955) reported the presence of tri-, penta-, hexa- and hepta-carboxylic porphyrin in urine samples from congenitally porphyric pigs. With (1958) detected hepta- and hexa-carboxylic porphyrin in the uroporphyrin fraction from urine samples from a congenitally porphyric steer, and hexacarboxylic porphyrin in the coproporphyrin fraction without mentioning any penta-carboxylic porphyrin. He considered the hexacarboxylic porphyrin to be a characteristic compound of bovine congenital porphyria and one that is difficult to purify. The pentacarboxylic porphyrin comprises about 10% of the total urinary porphyrins, and the hexacarboxylic porphyrin has the lowest concentration in all samples examined in this work. The efficiency of Hyflo and other column chromatography in separating porphyrins (Chu & Chu, 1957) might explain this discrepancy.

A comparison of the percentage composition of urinary porphyrins of congenitally porphyric cattle with that of cutanea tarda products (Chu & Chu, 1959a) shows that cattle excrete, besides copro- and uroporphyrin, more pentacarboxylic porphyrins, containing about 90% of the I series; the patients with cutanea tarda excrete more heptacarboxylic porphyrins, containing about 90% of the III series. The hexacarboxylic porphyrins apparently play a minor role in both diseases. Whether this distinction will be observed in human cases of congenital porphyria remains to be seen.

Although the relative abundance of urinary copro- and uroporphyrin is in agreement with that reported by Rimington, Roets & Fourie (1938), Amoroso, Loosmore, Rimington & Tooth (1957) and Watson, Perman, Spurrell, Hoyt & Schwartz (1958) for congenitally porphyric cattle, the result differs from that of human patients (Aldrich, Hawkinson, Grinstein & Watson, 1951; Watson *et al.* 1958). According to Aldrich the urinary uroporphyrin of a 4-year-old porphyric girl is ten times the amount of the urinary coproporphyrin.

An animal with higher plasma-porphyrin concentration excretes more urinary porphyrins. The only exception is the animal (m), whose erythrocytes: plasma ratio, 1:3, is also unusual (Tables 1 and 2). However, the relative concentration of

porphyrins in the blood differs from that in the urine. The high concentration of coproporphyrin in the plasma has led to congenital porphyria also being known as erythropoietic porphyria. The serum porphyrins of a patient with cutanea tarda show a different pattern: coproporphyrin 7%, pentacarboxylic porphyrin 5%, hexacarboxylic porphyrin 13%, heptacarboxylic porphyrin 42% and uroporphyrin 33%.

The observation that a major portion of the total porphyrins in erythrocytes is protoporphyrin agrees with Watson *et al.* (1959), and is in contrast with uroporphyrin in human cases. The protoporphyrin ester isolated in this experiment is closely similar to protoporphyrin IX. Erythrocytes from normal man and cattle also contain monocarboxylic as well as proto- and coproporphyrin.

Fourie & Roets (1939) reported a seasonal variation in urinary porphyrins of cattle. From Table 3, the fluctuation of the output of urinary porphyrins is noticeable; the output is almost directly proportional to the concentration of plasma porphyrins.

The liver-porphyrin pattern of the heifer (g) is not very different from normal. However, when compared with that of a cutanea tarda sample, it reveals a difference in these two types of porphyria. The data for the latter case are: coproporphyrin 0.02%, pentacarboxylic porphyrin 0.01%, hexacarboxylic porphyrin 0.13%, heptacarboxylic porphyrin 18.8% (m.p. 212°) and uroporphyrin 76.2% (m.p. 278°), with a total of 345 µg./g.

The presence of porphyrins, other than uroporphyrins, in bones and teeth has been scarcely noted previously. Rimington (1936) detected no coproporphyrin in the skeleton of an affected steer. Jorgensen & With (1955) and With (1955) found only uroporphyrin in bones and teeth of affected cattle and pigs.

Paul, Engstrom & Engfeldt (1953) found about 1.4 µg. of uroporphyrin/g. in the femur bone of a patient with cutanea tarda and considered it to be the methyl ester of uroporphyrin III (m.p. 266°). That all the uroporphyrin fractions from urine, blood and bones of congenitally porphyric cattle have no appreciable difference in the crystalline form, m.p. and isomeric proportion suggests that the uroporphyrin fraction isolated from different tissues of a patient with cutanea tarda should be about the same. Urinary uroporphyrins from cutanea tarda cases examined in this laboratory contain about 60–80% of uro-I (m.p. 260–280°). If the uroporphyrin fraction is contaminated with the heptacarboxylic porphyrin, which is mainly the III series in cutanea tarda cases, then the melting point will be even lower, such as the 'Waldestrom porphyrin' described by Chu & Chu (1957).

Further, according to the melting-point curve of mixtures of uroporphyrin I and III reported by Nicholas & Rimington (1953), a product of m.p. 266° could also consist mainly of uroporphyrin I.

SUMMARY

1. Samples of urine, blood and post-mortem tissues from congenitally porphyric cattle have been analysed for porphyrins. The results have been discussed and compared with those from human patients with porphyria cutanea tarda.

2. In addition to copro- and uro-porphyrin, penta-, hexa- and hepta-carboxylic porphyrin have also been isolated. All the porphyrins are mainly type I isomers. The uroporphyrins contain 85–95% of uroporphyrin I.

3. The possibility of the existence, in Nature, of two isomeric hexacarboxylic porphyrins of the I series has been mentioned.

4. A monocarboxylic porphyrin has been found in erythrocytes of the affected and normal cattle.

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REFERENCES

- Aldrich, R. H., Hawkinson, V., Grinstein, M. & Watson, C. J. (1951). *Blood*, **6**, 685.
- Amoroso, E. C., Loosmore, R. M., Rimington, C. & Tooth, B. E. (1957). *Nature, Lond.*, **180**, 230.
- Chu, T. C. & Chu, E. J.-H. (1955). *J. biol. Chem.* **212**, 1.
- Chu, T. C. & Chu, E. J.-H. (1957). *J. biol. Chem.* **227**, 505.
- Chu, T. C. & Chu, E. J.-H. (1958). *Analyt. Chem.* **30**, 1678.
- Chu, T. C. & Chu, E. J.-H. (1959a). *J. biol. Chem.* **234**, 2741.
- Chu, T. C. & Chu, E. J.-H. (1959b). *J. biol. Chem.* **234**, 2747.
- Chu, T. C. & Chu, E. J.-H. (1959c). *J. biol. Chem.* **234**, 2751.
- Chu, T. C., Green, A. A. & Chu, E. J.-H. (1951). *J. biol. Chem.* **190**, 643.
- Ellis, D. H., Barner, R. D., Madden, D., Melcer, I. & Orten, J. M. (1958). *Veterinarian, Mich.*, **18**, 89.
- Fourie, P. J. J. (1936). *Onderstepoort J. vet. Sci.* **7**, 535.
- Fourie, P. J. J. & Roets, G. C. S. (1939). *Onderstepoort J. vet. Sci.* **13**, 369.
- Jorgensen, S. K. & With, T. K. (1955). *Nature, Lond.*, **176**, 156.
- Mauzerall, D. & Granick, S. (1956). *J. biol. Chem.* **219**, 435.
- Nicholas, R. E. H. & Rimington, C. (1953). *Biochem. J.* **55**, 109.
- Paul, K. G., Engstrom, A. & Engfeldt, B. (1953). *J. clin. Path.* **6**, 135.

- Rhode, E. A. & Cornelius, C. E. (1958). *J. Amer. vet. med. Ass.* **132**, 112.
 Rimington, C. (1936). *Onderstepoort J. vet. Sci.* **7**, 567.
 Rimington, C., Roets, G. C. S. & Fourie, P. J. J. (1938). *Onderstepoort J. vet. Sci.* **10**, 421.
 Schwartz, S. & Wikoff, H. M. (1952). *J. biol. Chem.* **194**, 563.

- Watson, C. J., Perman, V., Spurrell, F. A., Hoyt, H. H. & Schwartz, S. (1958). *Trans. Ass. Amer. Phycns*, **71**, 196.
 Watson, C. J., Perman, V., Spurrell, F. A., Hoyt, H. H. & Schwartz, S. (1959). *A.M.A. Arch. intern. Med.* **103**, 108.
 With, T. K. (1955). *Biochem. J.* **60**, 703.
 With, T. K. (1958). *Biochem. J.* **68**, 717.

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The Qualitative Analysis of the Cell Walls of Selected Species of Fungi

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Extensive studies have already been made on the chemistry, morphology and biosynthesis of bacterial cell walls. In view of their importance as pathogens both of plants and animals, it seems equally pertinent to make a study of the composition of the cell walls of fungi.

Much of the earlier work on fungal cell walls was concerned with attempts to detect chitin and cellulose by cytochemical techniques [see Foster & Webber (1960), and Fuller & Barshad (1960) for references]. In some investigations extraction procedures have been used, either to attempt selective removal of specific components (Garzuly-Janke, 1940*a, b*), or to prepare a basic cell-wall residue free from cytoplasmic contamination, which was then submitted to chemical analysis (Daoud & Ling, 1931; Norman & Peterson, 1932), X-ray analysis (Frey, 1950; Blank, 1953, 1954; Blank & Burke, 1954; Aronson & Preston, 1960; Fuller & Barshad, 1960; Winkler, Douglas & Pramer, 1960) or to electron microscopy (Aronson & Preston, 1960). X-ray diagrams and electron micrographs have been prepared of the residues left after sequential solubilization of various components from mechanically isolated cell walls of yeasts (Houwink, Kreger & Roelofsen, 1951; Houwink & Kreger, 1953; Kreger, 1954) and filamentous fungi (Kreger, 1954).

To gain more extensive information, hydrolysates of mechanically isolated cell walls have been prepared and examined by several authors from yeasts (Northcote & Horne, 1952; Roelofsen, 1953; Falcone & Nickerson, 1956; Eddy, 1958; Miller & Phaff, 1958; Shifrine & Phaff, 1958) and from filamentous fungi (Cummins & Harris, 1958; Aronson & Machlis, 1959). Hydrolysates of cell walls isolated by mild chemical and enzymic

cleaning procedures have also been examined (Rzucidlo, Stachow, Nowakowska & Kubica, 1958).

The following investigation of the hydrolysates of the mechanically isolated cell walls of selected species was undertaken to gain some idea of the types of components to be expected in a wide range of species.

MATERIALS AND METHODS

Total nitrogen. This was estimated by the micro-Kjeldahl procedure with $\text{Na}_2\text{SeO}_4 \cdot 10\text{H}_2\text{O}$ - $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ - HgSO_4 - K_2SO_4 (2:3:10:85, by wt.) as catalyst.

Fungi. These are listed in Table 1.

Media. For yeasts the medium of Kilkenny & Hinshelwood (1951) was used, with yeast extract (Difco; 3 g./l. of medium) as a source of vitamins and CaCO_3 (1.5 g./l.) as a buffer where required. For *Ustilago maydis* a glucose- $(\text{NH}_4)_2\text{SO}_4$ medium (Haskins, 1950; designed to give no crystal production) was used. For *Phytophthora cactorum* the complete medium of Hall (1959) was used. *Saprolegnia ferax* was grown in the following medium: glucose, 25 g., NH_4NO_3 , 1.6 g., K_2HPO_4 , 0.2 g., $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.16 g., DL-methionine, 25 mg., NaCl, 2.5 mg., together with the constituents of Reischer's (1951) inorganic basal medium (excluding K_2HPO_4 , KH_2PO_4 and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), made up to 1 l. with water and the pH adjusted to 7.0. For *Nematospora gossypii* the following medium was used: glucose, 20 g., KH_2PO_4 , 1.0 g., $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g., asparagine, 5.0 g., trace-element soln. (Kilkenny & Hinshelwood, 1951), 1 ml., biotin, 5 μg ., thiamine, 100 μg ., inositol, 0.2 g., water to 1 l. For *Polystictus sanguineus* 2.5% (w/v) malt extract (Difco) was used. For all other fungi, the basal medium of Lilly & Barnett (1953) was employed, with 1 ml. of the solution of Kilkenny & Hinshelwood (1951) to supply trace elements, and for the growth of the strain of *Neurospora sitophila*, 0.25% of yeast extract (Difco) was included.

Culture conditions. The yeasts, *Ustilago maydis* (which grew in the yeast form) and *Nematospora gossypii* were grown in their respective media to prepare inocula. For *Chaetomium globosum*, a suspension of ascospores, and for