Journal & Proceedings of the Royal Society of New South Wales, vol. 151, part 2, 2018, pp. 125–134. ISSN 0035-9173/18/020125-10

Adventures with sugars

John Warcup Cornforth*

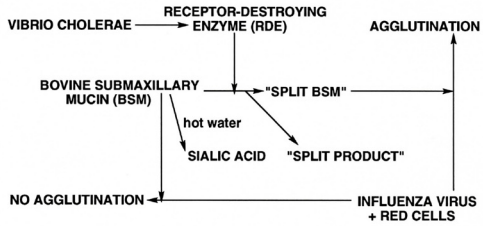
*The late Sir John "Kappa" Cornforth, FRS, is the only NSW-born Nobel Laureate.

Abstract

This is a presentation given at the Alan Johnson Memorial Lecture on 23 July 1999 at the School of Chemistry, University of Sussex. It is the last publication of Sir John Cornforth's (1917–2013) [http://oa.anu.edu.au/obituary/cornforth-sir-john-warcup-17375]. It has not previously been published (the transcript has been lightly edited by Robert Marks).

Like the rest of you, I am looking forward to the presentations of Professors A. G. Barrett and K. C. Nicolaou. They are very capably engaged in creating the present and future of organic chemistry. My own present and future are limited, but I have a rather extensive past and I propose in my talk to revisit one aspect of this. My excuse is that I have never lectured about it before. Those of you who are digesting a good lunch should feel free to snooze peacefully before the real business begins. But wake up by three o'clock — I know my duty as first speaker and I shall not overrun.

The title of this talk might suggest that I am a sugar[1] chemist. Nothing could be further from the truth. But 43 years ago I was a scientist at the National Institute for Medical Research at Mill Hill. Within the constraints of time and resources I, and my colleagues, were free to form whatever collaborations we chose, and my burden of accountability was discharged by a brief annual report to my Director. It happened about this time that the biochemist Alfred Gottschalk (1894–1973),[2] from Macfarlane Burnet's Walter and Eliza Hall Institute in Melbourne, was a visiting worker at Mill Hill. He was deeply interested in mucins, the slimy substances in saliva and in many other secretions. Because these contain amino-sugars he was interested in them too, and he endlessly discussed their chemistry with me and anyone else who would listen.



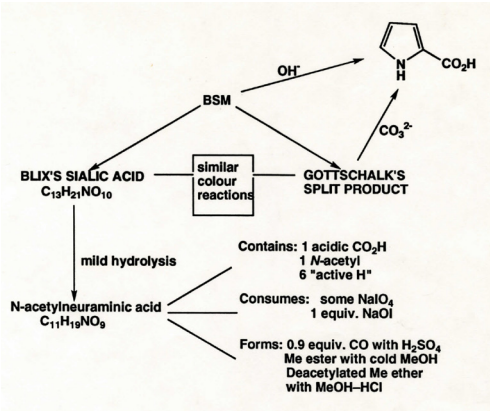
Macfarlane Burnet FRS (1899–1985)[3] had asked Gottschalk to study the interaction between mucins and the influenza virus (Figure 1). Some mucins were readily available, notably from the submaxillary glands of slaughtered cattle, and it had been found that these would inhibit the agglutination, by the influenza virus, of red blood cells. If the mucin had previously been treated with an enzyme preparation, then called receptor-destroying enzyme, from the cholera Vibrio, it lost this inhibitory

[1] As a chemical term, "sugar" usually refers to all carbohydrates of the general formula $C_n(H_2O)_n$.

[2] http://adb.anu.edu.au/biography/gottschalk-alfred-10336

[3] http://rsbm.royalsocietypublishing.org/content/roybiogmem/33/99

power and dialysable substance appeared in the medium. Gottschalk never got crystals from his preparation of this substance, but on the basis of three different colour reactions, he concluded that it was a sialic acid. He also made a significant observation about its chemistry. On heating in alkaline solution, bovine submaxillary mucin yielded pyrrole-2-carboxylic acid, and the same acid was formed from his "split product" in much milder alkali and in a yield consistent with its being the sole source of the acid from mucin (Figure 2).



The history of the sialic acids (the name is from the Greek word for saliva or spittle) began in 1936 when Gunnar Blix (1894–1981)[4] obtained a crystalline acid by heating bovine submaxillary mucin with water. Similar compounds were later isolated by Ernst Klenk (1869–1971)[5] from brain tissue and by Richard Kuhn (1900–1967)[6] from cow colostrum. They were all given different names and it took some time for this confusion to settle. Nowadays, sialic acid is the generic term and neuraminic acid (Klenk's name) is the parent molecule. The sialic acids are various acyl derivatives of neuraminic acid.

I must try to give you a picture of carbohydrate biochemistry as it was half a century ago. For sugar chemists, ion-exchange resins had taken a lot of drudgery out of the isolation of sugars. But one of the most liberating events had been the invention of paper chromatography. You could usually find a biochemistry department on a campus just by following your nose: you would be guided unerringly by the reek of isobutyric acid, which is a component of farmers' muck-heaps and of a useful mobile phase in sugar chromatography. And because the spots on the paper needed to be made visible, all kinds of more or less specific spray reagents came into use. Biochemists in those days relied much more on colour reagents both for detection and measurement, and the invention of reliable spectrophotometers was another liberating event. When I was a post-doctoral student, ultra-violet spectra had to be plotted from successive exposures on a glass photographic plate. The little Beckman DU spectrophotometer came like the gift of a Stradivarius to a virtuoso.

For chemists, infra-red spectroscopy was fairly well developed as a means of identification and detection of functional groups. X-ray crystallography was a sure way to identify a known compound but was slow and uncertain for structural elucidation. Mass spectrometry was still in its childhood and NMR newly born. On the other hand, organic chemists already had fairly satisfactory theories of structure and reactivity. As

[4] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4463483/

[5] https://www.encyclopedia.com/science/dictionaries-thesauruses-pictures-and-press-releases/klenk-ernst

[6] https://www.encyclopedia.com/people/science-and-technology/chemistry-biographies/richard-kuhn

a student of Robert Robinson (1886–1975)[7] I had been heavily exposed to these, whereas most biochemists had learned their chemistry as a separate subject taught by contemporaries or students of Emil Fischer (1852–1919).[8] Given a crystalline substance, their first care was elementary analysis. For Blix's sialic acid, this led eventually to an empirical formula $C_{13}H_{21}NO_{10}$. There was an O-acetyl group, very easily hydrolysed, and another acetyl group thought to be *N*-acetyl. Removal of the labile acetyl led to the compound now called *N*-acetylneuraminic acid, also available directly from sheep submaxillary mucin, for which the formula $C_{11}H_{19}NO_9$ was established. One obstacle in the way of this finding was the spontaneous formation of a methyl ester in dry methanol. The successful solvent for crystallization, found by Blix, was the strangest I ever heard of: methanol-water-ether-petrol. All four were essential.

Nowadays, the structure would be found out in a few days by crystallography or by pulsed NMR. As it was, this was one of the last natural products whose structure was divined from its chemistry alone. For a classic example from the same period, Robert B. Woodward's (1917–1979)[9] solution of the Terramycin structure[10] is supreme, but I reconstruct my own logical process here for its historical interest.

Little more of the chemistry of *N*-acetylneuraminic acid was known at the time. The substance was oxidizable by Willstätter's hypoiodite, and also by periodate since its spots on paper could be made visible by a treatment beloved of sugar chemists: periodate followed by Schiff's reagent. With hot methanolic HCl it gave a methyl ether with concomitant loss of the acetyl group. A now forgotten analytical procedure gave it six "active hydrogens:" that is, it liberated six equivalents of methane from methylmagnesium iodide. Discounting the carboxyl and acetamido hydrogens, that indicated four hydroxyls. But it also gave nearly one equivalent of carbon monoxide on warming with concentrated sulfuric acid.

Gottschalk prepared a note[11] to *Nature*, attempting to reconcile the data in terms of a partial structure. I forget the structure proposed; it was a pyrroline of some kind. But I saw the note before publication and I began to read the published work and to think independently. Blix's finding of carbon monoxide intrigued me. I knew that tertiary carboxylic acids readily yielded it on warming with sulfuric acid. But tertiary carboxylic acids are difficult to esterify; on the one hand, α-oxo acids form methyl esters very easily. Then the intellectual leap came: suppose that the substance was both!

It had sugar-like reactions: could it be a pyranose-carboxylic acid tautomeric, like sugars, with an open-chain α-oxo acid? This fitted many observations neatly. The formation of pyrrole-2-carboxylic acid placed the acetamido group on position 5 of the pyranose, excluding the furanose alternative, and it required an additional hydroxyl at the 4-position to provide, by dehydration, the second double bond. This left three carbons and three hydroxyls to be accounted for. Position 6 was the only place for them, and they had to be together in a chain because of

[7] https://www.encyclopedia.com/people/science-and-technology/chemistry-biographies/robert-robinson

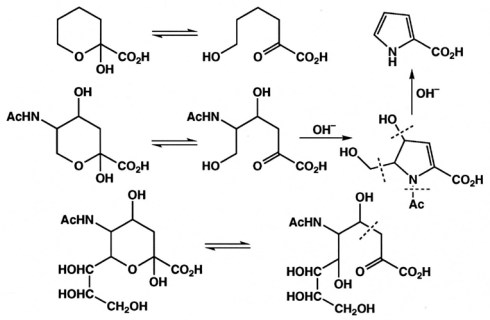
[8] https://www.encyclopedia.com/people/science-and-technology/chemistry-biographies/emil-fischer

[9] https://www.encyclopedia.com/people/science-and-technology/chemistry-biographies/robert-burns-woodward

[10] Stephens et al. (1952).

[11] Gottschalk (1954)

the periodate consumption. Suddenly I saw that I was looking at a potential aldol condensation product of an acetyl-hexosamine and pyruvic acid; both well-known natural products (Figure 3).



I wrote to Gottschalk suggesting this structure. He rewrote his note to adopt it, added a line at the end thanking me for helpful advice, and sent the note[12] to *Nature* without showing it to me. He also reported a new experiment: some pyrrole-2-carboxylic acid could be obtained by heating glucosamine with pyruvic acid in alkaline solution. When the note appeared, he explained that he too would have arrived at my structure in time.

I am less surprised now by Gottschalk's action than I was then. He had been studying this stuff for years and I was an interloper. And since he had published the structure without ever having seen an isolated specimen of the acid, I suppose that he in turn was an interloper to Blix, Klenk and Kuhn. But I am still surprised that Richard Kuhn, an excellent chemist who had won the Nobel Prize for Chemistry in 1938 for his work on carotenoids and who had all the information long before me, did not get the structure first.

Gottschalk and I had already agreed to collaborate on the synthesis of acetylneuraminic acid and I enlisted the help of Mary Daines[13] who was doing a London Ph.D. with me. We wanted to try the condensation of *N*-acetylhexosamine with pyruvic acid. Acetyl-glucosamine was the only one commercially available and it was prohibitively expensive. So the first step of the synthesis was to Scott's Restaurant in Piccadilly Circus, from which we obtained bucketsful of evil-smelling crab shells. We made several hundred grams of glucosamine hydrochloride from these, and put the *N*-acetyl group on most of it. Then we tried condensation with pyruvic acid in alkaline solution and this was unsuccessful.

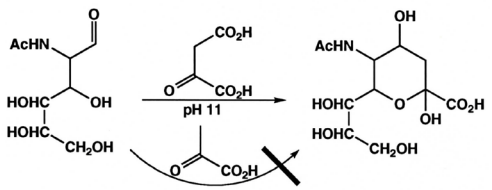
At this point I remembered a classic experiment by Robert Robinson.[14] He put together the alkaloid derivative tropinone in aqueous solution from methylamine, butanedial and acetone: a double Mannich condensation. But I also remembered what text-books omit: the yield of tropinone was so poor that he had had to isolate it as an insoluble derivative, whereas the condensation with acetonedicarboxylic acid instead of acetone yielded 40% of tropinone.

So we substituted oxaloacetic acid for pyruvic acid and tried again. After 2–3 days at pH 10–11, the solution showed promising colour reactions. We separated the acidic fraction and chromatographed it on activated carbon. This gave us fractions which were monitored by paper chromatography. Some of them crystallized, and the exotic solvent system of Blix then gave us pure *N*-acetylneuraminic acid, identical with natural material (Figure 4).

[12] Gottschalk (1955)

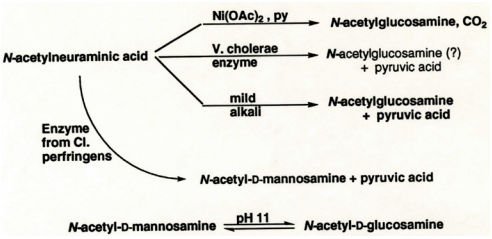
[13] Cornforth, Daines, Gottschalk (1957).

[14] Robinson (1917).

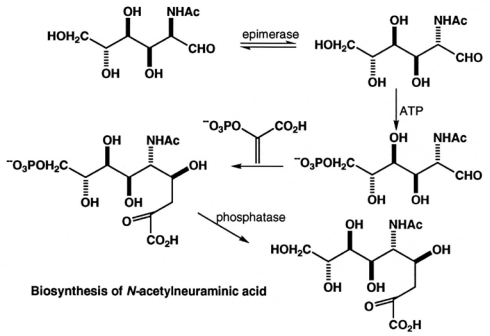


Synthesis of *N*-acetylneuraminic acid

Was this the end of the matter? No, not quite. During the work, Kuhn had reported that *N*-acetylneuraminic acid when heated in pyridine with nickel acetate yielded *N*-acetylglucosamine, and other workers showed that the acid is degraded to *N*-acetylglucosamine and pyruvic acid by mild alkali treatment. There was even a claim to have effected the same fission enzymically. But although *N*-acetylglucosamine was identified rigorously from the chemical fissions, identification with the enzymic fission was less secure; and Roseman and Comb[15] later showed that enzymic fission yields *N*-acetylmannosamine, which in alkali is equilibrated with *N*-acetylglucosamine. So our synthesis had depended on this equilibration occurring in the alkaline medium and its success contained a dollop of luck (Figure 5).



Actually, the synthesis imitates quite closely the biosynthesis, in which *N*-acetylglucosamine is epimerized to the mannosamine which, after phosphorylation at the 6-hydroxyl, is condensed with a different form of activated pyruvic acid: phosphoenol-pyruvate. The superfluous phosphate is then removed (Figure 6).



Biosynthesis of *N*-acetylneuraminic acid

One can speculate that Nature uses this intermediate phosphorylation because it is impossible to obtain a good yield of the mannosamine from the glucosamine without disturbing the thermodynamic equilibrium by continuously removing the product. A phosphotransferase specific for *N*-acetylmannosamine does just that. Later Pat Carroll and I[16] found a preparative procedure for making *N*-acetylmannosamine from the glucosamine. We did it by selective crystallization and recycling, and we showed that *N*-acetylmannosamine yielded around five times more product on condensation with oxaloacetate. Even so, that was barely 10% and I regret having left this problem without devising a decent yield. As it is, the commercial supply of *N*-acetylneuraminic acid depends on a Chinese delicacy, bird's-nest soup. A species of sparrow[17] living on the Eastern shores of China makes small cup-shaped nests almost entirely from its own spit. These are the source of the soup, and thence of *N*-acetylneuraminic acid.

As time has passed, the sialic acids have become recognized as important compo-

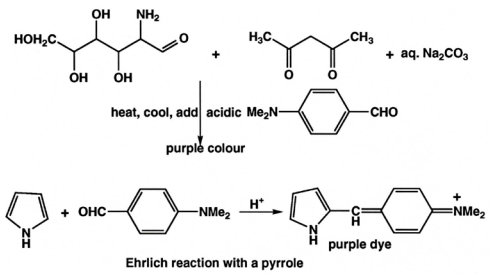
[15] Comb and Roseman (1960).

[16] Carroll and Cornforth (1960)

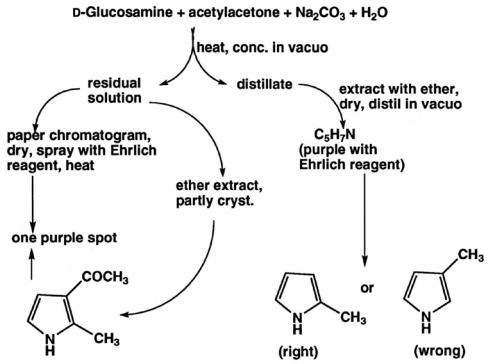
[17] The edible-nest swiftlet or white-nest swiftlet (*Aerodramus fuciphagus*).

nents of glycoproteins and glycolipids. The "receptor-destroying enzyme" is now called a neuraminidase and the influenza virus itself is known to produce a neuraminidase which it uses to liberate itself from infected cells and which is consequently a target in the chemotherapy of influenza.

Gottschalk's interest in amino-sugars extended to their colour reactions. One of these is the Elson-Morgan reaction which was widely used for quantitative analysis of hexosamine in biological fluids and hydrolysates. Essentially, one heats the solution with acetylacetone and sodium carbonate at a pH around 9.8 and then adds the Ehrlich reagent, a solution of 4-dimethylaminobenaldehyde in ethanol and hydrochloric acid. A purple-red colour develops and in standard conditions its intensity is a measure of the hexosamine content. Gottschalk, a paper chromatography addict as many biochemists were at the time, took a rather concentrated reaction mixture from glucosamine and acetylacetone and chromatographed it on paper. He then dried the paper, sprayed it with the Ehrlich reagent, and heated it. A single purple spot developed. He found that ether extraction of the reaction mixture removed the precursor of this spot (Figure 7).



I agreed to look at the chemistry of this finding, again with Mary Daines's help.[18] We examined the literature on the Elson-Morgan reaction and found a 1951 paper which showed that at least one of the chromogens — that is, products generating the colour with Ehrlich's reagent — was volatile with steam, so we looked at this first. Gas-liquid chromatography at the time was in its infancy although its parents, Archer Martin and Tony James, were both working at Mill Hill; so we isolated the volatile component the hard way, by extraction of distillates with ether and final purification of the product by distilling it in vacuum at room temperature through a tube packed with magnesium perchlorate to dry it. It had the composition of a monomethylpyrrole (Figure 8).



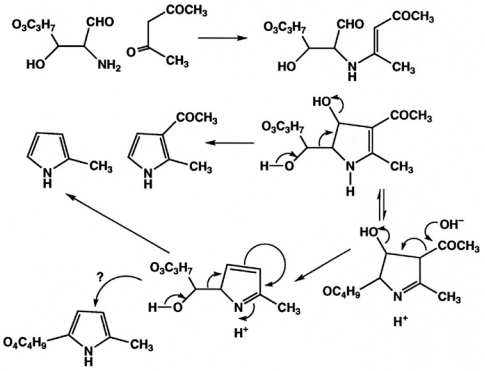
We had to make both 2- and 3-methylpyrrole synthetically in order to be sure which one it was, because virtually no physical data were available and there was a mix-up of the two pyrroles in Hans Fischer's standard monograph.[19] The description of the two pyrroles in this book has the distinction that every single fact in it is wrong. But the problem induced me to devise new syntheses of 3-methylpyrrole and the fact that Sigma can sell this pyrrole today is basically because Fischer got it wrong 65 years ago. We cleaned up other parts of this mess and that led to

[18] Cornforth and Firth (1958).

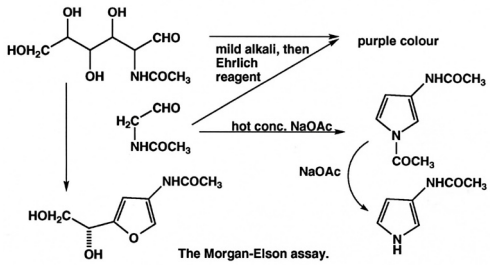
[19] Fischer and Orth (1940).

some interesting chemistry, but to recount it would take us too far from the sugars. For the glucosamine work it had negative value because the chromogen turned out to be identical with 2-methylpyrrole. The chemical yield of this pyrrole in the reaction with acetylacetone in analytical conditions is around 10%, and in the usual assay for hexosamine it is responsible for nearly 70% of the colour.

Gottschalk's single spot on paper chromatography turned out to be a red herring, or perhaps one should say a purple herring. We repeated the ether extraction of a concentrated reaction mixture and purified his crystalline product. It turned out, as he had suspected, to be 3-acetyl-2-methylpyrrole. It was a new compound and we verified its structure by a Knorr synthesis from aminoacetaldehyde and acetylacetone (Figure 9).



But the colour that it gave with the Ehrlich reagent in the conditions of analysis was so faint and so slow to develop that it could not have been responsible for as much as 1% of the colour in an Elson-Morgan assay (Fig 10). On the other hand, when it was spotted on paper and heated with the Ehrlich reagent it gave an intense colour. In Gottschalk's experiment he lost the main chromogen, 2-methylpyrrole, by evaporation when the paper was dried. When it was then heated after spraying with Ehrlich reagent, the hydrochloric acid in the reagent would have been concentrated sufficiently to effect acid-catalysed deacetylation, already known for other acetylpyrroles. The 2-methylpyrrole so formed would react fast with the colour-producing reagent, too fast to allow evaporation or polymerization. Thus, a minor actor had been promoted to the principal role.

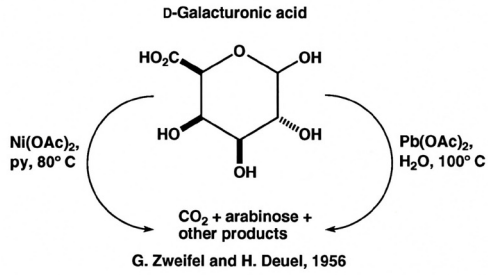


The chemistry of chromogen formation is of some interest. In both 2-methylpyrrole and the 3-acetyl analogue, four of the six carbons in glucosamine had been lost. This parallels the formation of pyrrole-2-carboxylic acid from sialic acids in similar conditions: only two carbons of the parent mannosamine remained in the product. As we shall see, these fissions can be formulated essentially as retroaldol cleavages. The loss of an acetyl group from acetylacetone in the process leading to 2-methylpyrrole is less obvious. Although an acetyl group can be cleaved by strong alkali from acetylpyrroles and even from acetylacetone, this does not happen fast enough at the pH used. Hence the acetyl group must be lost at an intermediate stage. The most rapid reaction between glucosamine and acetylacetone is likely to be the formation of the enamine, which then undergoes internal aldol condensation to a hydroxypyrroline. This can already give rise to an acetylpyrrole by the elimination shown, but the preferred mode, by about 5

to 1, seems to be the addition of hydroxyl anion to the acetyl carbonyl, leading to loss of acetic acid. This gives 5H-pyrrole which can generate the normal 1H-pyrrole not by prototropy but by a cleavage of the retroaldol type leading to loss of erythrose. There are minor ether-soluble chromogens in the concentrated reaction mixture and it is possible that some of them are 1H-pyrroles formed by 5H-prototropy and retaining the tetrahydroxybutyl group of the hexosamine. My analysis suggests that the favoured species should be 2-methyl-5-tetrahydroxybutylpyrrole, but nobody has purified the minor chromogens. There is always some unfinished business.

Around that time I became interested in the parallel Morgan-Elson assay, in which *N*-acetylhexosamines on heating in mild alkali solutions generating a purple colour with the Ehrlich reagent. Walter Morgan (1900–2003),[20] then working at the Lister Institute, had found that acetamidoacetaldehyde, which can be regarded as the parent *N*-acetylamino sugar, gave a similar colour after similar treatment; and he encouraged me to explore the chemistry. It turned out that the chromogen is formed in two steps. When acetamidoacetaldehyde is heated in concentrated aqueous sodium acetate and the solution is periodically extracted with ethyl acetate, colourless air-stable Ehrlich-negative crystals are obtained. If the heating is prolonged and extraction is carried out only at the end, a different crystalline compound is obtained, highly air-sensitive and Ehrlich-positive. The sodium acetate not only acts as a mild base but assists the extraction by a salting-out effect. The two compounds, both new, were identified as 1-acetyl-3-acetamidopyrrole and 3-acetamidopyrrole respectively; the latter was also obtained by Curtius degradation of the known pyrrole-3-carboxylic acid. 3-aminopyrroles are rare and this is still the simplest one ever made. It was also the first example of a Knorr synthesis succeeding with an acylated aminocarbonyl compound. I have a specimen of my 3-acetamidopyrrole in a sealed evacuated tube. It is still white after 42 years. But as an indication of Morgan-Elson chemistry it was another purple herring. Richard Kuhn and his collaborators did a fine bit of work on this, and they showed that the chromogens are not pyrroles but furans. They actually isolated and synthesized one of the chromogens.

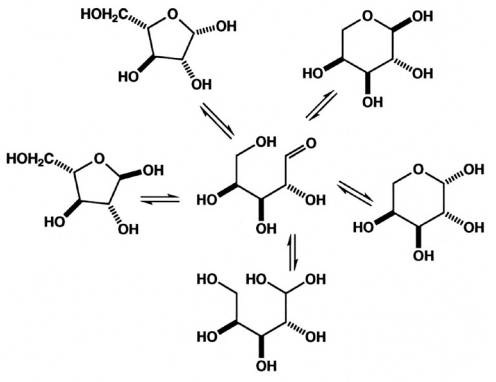
I thought that with this work I had finished with sugars. But during the last two years they have come back into my life. In the course of a survey of decarboxylation reactions I found two processes that I could not understand; one chemical, one enzymic. The chemical one is the catalysed decarboxylation of hexuronic acids. At about the same time as the work I have described, Zweifel and Deuel at the ETH in Zürich showed that galacturonic acid could be decarboxylated in astonishingly mild conditions: most remarkably, by heating with nickelous acetate in pyridine or with lead acetate in water (Figure 11).[21]



[20] https://en.wikipedia.org/wiki/Walter_Thomas_James_Morgan

[21] Zweifel and Deuel (1956).

Both reactions had been shown by Zweifel and Deuel to yield arabinose, which is nominally the product of simple removal of carbon dioxide from galacturonic acid. I have been investigating these catalyses. The nickel acetate reaction is showing every sign of being a monstrous red herring so far as the decarboxylation is concerned. The lead acetate reaction remains mysterious. But while I was preparing this lecture I re-read Kuhn's work[22] on the degradation of *N*-acetylneuraminic acid to acetyl-glucosamine. This was done with nickel acetate in pyridine and Kuhn actually mentions Zweifel and Deuel's work! Formally, the other product of this cleavage is pyruvic acid. But the only other product isolated was carbon dioxide and in a separate experiment Kuhn showed that pyruvic acid with nickel acetate and pyridine gave carbon dioxide but not acetaldehyde, the formal decarboxylation product. I had completely forgotten this experiment and I shall now repeat it and find out what is happening. But the work has also presented me with the problem of separating and identifying complex mixtures of sugars at the pentose level and this has led me to realize the deficiency of sugar analysis in its present state.



The Six Faces of L-Arabinose

[22] Kuhn and Brossmer (1956)

Figure 12 is of the commonest aldopentose, L-arabinose, in aqueous solution. As you can see, the problem of converting quantitatively a hydrophilic, transparent, non-fluorescent, non-volatile, dynamic mixture of six components into a derivative that behaves as an individual and is measurable by UV absorption or fluorescence is not trivial, especially when one has up to six of these dynamic mixtures in the same solution. And if I succeed in my present attempt to do something about this, I shall at last have done something to please sugar chemists as well as to satisfy a continuing and probably lifelong curiosity about decarboxylations.

Acknowledgements

The Royal Society of New South Wales thanks the Royal Society of London (and its archivist, Jonathan Bushell), and Philippa Cornforth and the Cornforth family, for their kind permission to publish this paper. The editor thanks David Black and Dean Popovski for their proof-reading of his transcription.

References

Carroll, P. M. and Cornforth, J. W. (1960) Preparation of *N*-acetylneuraminic acid from *N*-acetyl-D-mannosamine. *Biochim Biophys Acta*, 39: 161-162.

Comb D. G. and Roseman S. (1960) The sialic acids. I. The structure and enzymatic synthesis of *N*-acetylneuraminic acid. *J. Biol. Chem*. 235: 2529-2537.

Cornforth, J. W, Daines, M. E., Gottschalk, A. (1957) Synthesis of *N*-acetylneuraminic acid (lactaminic acid, O-sialic acid). *Proc. of the Chem. Soc.*, 25-26. DOI: 10.1039/PS9570000001

Cornforth, J. W. and Firth, M. E. (1958) Identification of two chromogens in the Elson-Morgan determination of hexosamines. A new synthesis of 3-methylpyrrole. Structure of the "pyrrolene-phthalides." *J. Chem. Soc.*, 1091-1099. DOI: 10.1039/JR9580001091

Cornforth, J. W., Firth, M. E., Gottschalk, A. (1958) The synthesis of *N*-acetylneuraminic acid. *The Biochemical J.* 68: 57-61.

Fischer, H. and Orth, H. (1940) *Die Chemie des Pyrrols* Leipzig, Akademische Verlagsgesellschaft.

Gottschalk, A. (1954) The precursor of 2-carboxy-pyrrole in mucoproteins. *Nature*, 174: 652-653.

Gottschalk, A. (1955) Structural relationship between sialic acid, neuraminic acid and 2-carboxy-pyrrole, *Nature*, 176: 881-882.

Kuhn, R. and Brossmer, R. (1956) Abbau der Lactaminsäurelactose zu *N*-Acetyl-D-glucosamin, *Chem. Ber.*, 89: 2471.

Robinson, R. (1917). LXIII. A synthesis of tropinone. *J. of the Chem. Soc.*, *Trans.*. 111: 762–768. DOI:10.1039/CT9171100762

Stephens, C. R., Conover, L. H., Hochstein, F. A., Regna, P. P., Pilgrim, F. J., Brunings, K. J., and Woodward, R.B. (1952) Terramycin. VIII. Structure of aureomycin and terramycin, *J. Am. Chem. Soc.*, 74 (19), 4976-4977. DOI: 10.1021/ja01139a533

Zweifel, G. and Deuel, H. (1956) Über die Decarboxylierung von Galakturonsäure mit Schwermetallionen, *Helvetica Chimica Acta*; 39: 662-667.