VI. THE NITROGENOUS CONSTITUENTS OF LIME-JUICE.

By CASIMIR FUNK,
Research Scholar, Lister Institute.

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*From the Biochemical Department, Lister Institute of
Preventive Medicine.*

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In connection with the work previously published on beri-beri [Funk, 1911, 1912, 1], in which the physiological importance of certain nitrogenous substances, belonging most probably to the pyrimidine group, was demonstrated, a similar fractionation of lime-juice was undertaken for the investigation of scurvy.

From the classical work of Holst and Fröhlich [1907, 1912] on scurvy we know that this disease has a close analogy to beri-beri and is also due to a lack of some substance in the food. They specially advanced our knowledge of this disease by inducing in animals (rabbits and guinea-pigs) a disease which possesses a great similarity in symptoms to human scurvy. They produced the experimental scurvy by an exclusive diet of oats or autoclaved vegetables and were able to prevent it by an addition to this diet of fresh vegetable-juice, unboiled milk or lime-juice. Lime-juice itself has been known for many years as an excellent remedy for scurvy.

Some time ago I was able to show [1912, 2] that lime-juice contains an anti-neuritic substance curing polyneuritis in birds, produced by an exclusive diet of polished rice, and a substance, present in another fraction, which is only capable of prolonging the life of these birds. Further experiments have shown that pyrimidine substances in general possess this life-prolonging action and a paper on this subject will shortly appear. No anti-scorbutic substance however was obtained from lime-juice.

I had at first intended to try each fraction, obtained during this

investigation, on guinea-pigs fed on oats, but in this I found considerable difficulties. The animals refused all food after a period of three weeks and even an addition of freshly made potato-juice or lime-juice was not able to save them from starvation. The same negative result was obtained with fractions of lime-juice which might have been expected to contain the anti-scorbutic substance in a more concentrated form.

The only positive results were obtained with milk, 50 c.c. daily of unboiled milk in addition to the oats diet preventing loss of weight and onset of scurvy. Even after the elimination of caseinogen and the other proteins present in milk, the latter keeps its preventive power. In doing this extreme care must be taken not to destroy the substance, and all boiling must be carefully avoided. The caseinogen is precipitated by addition of acetic acid at 50° and lactalbumin by dialysed iron-solution, kaolin however carries the substance down. The solution is then concentrated by freezing it and taking the supernatant liquid. This subject is being further investigated. This unstable character of the anti-scorbutic substance renders it unlikely that the usual method of separation, with the unavoidable use of alkali, would lead to its isolation. Although no anti-scorbutic substance was obtained from lime-juice, several new compounds have been detected and are described below.

Besides a substance, which apparently belongs to the terpene group and which has only been analysed and not further investigated, as having no direct connection with the subject, three nitrogenous substances have been isolated. One belongs to the purine group, the second to the pyrimidine group and the third to the choline group. The small yield obtained did not allow however of a complete investigation, so that this paper must be regarded as preliminary.

EXPERIMENTAL.

407 litres of commercial lime-juice were precipitated in portions of 15 litres each with 2800 grms. of neutral lead acetate. The bulky precipitate was filtered off on a large Buchner funnel and the residue pressed out in a hydraulic press. The combined filtrates were freed from the excess of lead, first by an addition of sulphuric acid, then by sulphuretted hydrogen, and were then concentrated *in vacuo* at low temperature. During the distillation crystals separated out, which were filtered off, and weighed when dry 258 grms. These were recrystallised from 20 % acetic acid and 162 grms. were obtained in needles. The substance proved to be free from nitrogen and was recrystallised for analysis once more from water. It is

nearly insoluble in all other solvents, except acetic acid, and is volatile with steam; M.P. 97° – 100° (uncorr.).

0.1357 grm. gave	0.3400 CO_2 and	0.1312 H_2O .
Found	68.33 % C;	10.73 % H.
Calc. for $\text{C}_{13}\text{H}_{24}\text{O}_3$;	68.42 % C;	10.52 % H.

This substance probably belongs to the terpene group; it possesses a very characteristic agreeable smell when heated. The nature of this compound, which has no direct relationship with the scurvy problem, was not further investigated.

To the filtrate from this substance enough sulphuric acid was added to make a 5 % solution and then a 50 % solution of phospho-tungstic acid until precipitation was complete. The precipitate obtained amounted to nearly 12 kg., and consisted chiefly of potassium salt.

INVESTIGATION OF THE PHOSPHO-TUNGSTATE PRECIPITATE.

The precipitate was decomposed in portions of 1 kg. each with $2\frac{1}{2}$ kg. baryta in a mortar and the mixture was shaken on the shaking machine for one hour. The precipitate was then filtered off, suspended in water and again shaken. The excess of baryta was eliminated from the combined filtrates by the careful addition of dilute sulphuric acid. The solution obtained was then neutralised with nitric acid, as it was strongly alkaline, and evaporated *in vacuo* to about two litres. In this solution the usual fractionation with silver nitrate was performed and three fractions were obtained. The first fraction came down on addition of a silver nitrate solution and contained the substances of the purine group. This precipitate was filtered off and to the filtrate a saturated solution of silver nitrate was added until a drop of the liquid gave with a cold baryta solution a brown precipitate which indicates an excess of silver. A saturated aqueous baryta solution was then added until a drop of the clear liquid gave only a small precipitate with silver nitrate and ammonia. The second precipitate consists of pyrimidine bases and substances of the histidine type. To the filtrate pulverised baryta was added until the whole of the silver was precipitated. The fraction thus obtained contains, in addition to traces of pyrimidine substances, compounds of the arginine group. The last filtrate contains substances of the choline group. All the fractions were investigated separately.

INVESTIGATION OF THE FIRST SILVER FRACTION.

The bulky precipitate obtained by a simple addition of silver nitrate was filtered off, well washed with water, suspended in water and the double silver nitrate salts converted into the silver salts by heating them on the water-bath with ammonia. The precipitate was filtered off and washed with water, until the filtrate was free from nitric acid. It was then suspended in water and decomposed with sulphuretted hydrogen. The solution on evaporation yielded 1 gram. of substance, which however contained a little ash. It was recrystallised from water and gave 0.7 gram. of crystals in the form of plates. After drying *in vacuo* at 110° they became brown at 240° and melted at 282° (corr.). The substance is precipitated by mercuric acetate and partially by gold chloride. It differs from the known purine bodies by the great solubility in hot water and the absence of all known reactions for these derivatives. Heated with nitric acid it becomes yellow, and brown when more strongly heated, no change of colouration being produced on the addition of alkali.

0.3855 gram. loses at 110° (*vacuo*) 0.0190 gram. H₂O
 0.1176 „ requires 32.7 c.c. N/10 H₂SO₄
 0.1372 „ „ 38 c.c. „ „
 0.1226 „ gave 0.1788 CO₂ and 0.0196 H₂O

Calc. for C ₈ H ₇ O ₂ N ₅ (Mol. wt. = 181)	Found
C 39.78 %	39.77
H 3.86 %	3.55
N 38.67 %	38.77 38.92
$\frac{1}{2}$ Mol. H ₂ O 4.73 %	4.92

INVESTIGATION OF THE SECOND SILVER FRACTION.

The second silver nitrate precipitate was filtered off, washed well with water and decomposed with sulphuretted hydrogen. In the filtrate the last traces of baryta were taken out with a very dilute solution of sulphuric acid and the solution was evaporated *in vacuo*. As the residue did not deposit any crystals, the solution was precipitated with mercuric sulphate. The solution obtained by decomposition of the precipitate showed no tendency to crystallisation, but on addition of a picric acid solution an oil separated, which was decanted and dissolved in acetone. After evaporation a solid powder was obtained which amounted to 0.35 gram. This was recrystallised from a mixture of alcohol and acetone and yielded about 0.1 gram. of brown prisms, which decomposed at 205°–210°. An amount of this fraction

corresponding to 0.01 grm. of the picrate was decomposed and given to a polyneuritic pigeon which showed a considerable improvement and died only after four days.

The mercuric sulphate filtrate, freed from mercury and sulphuric acid, was evaporated *in vacuo* and the residue left in a desiccator after addition of alcohol. After a few days a colourless substance separated out which amounted to 0.7 grm. After recrystallisation from dilute alcohol 0.25 grm. was obtained in the form of microscopical spherulites, which after drying at 110° *in vacuo*, melted and decomposed at 188° – 189° .

0.0958 grm. subst. required 7.5 c.c. N/10 H_2SO_4 ; 10.96% N.

0.1034 „ gave 0.1625 CO_2 and 0.0636 H_2O ; 42.86% C; 6.83% H.

Calc. for $\text{C}_9\text{H}_{18}\text{O}_8\text{N}_2$ (Mol. wt. = 250) 43.2% C; 7.2% H and 11.2% N.

The third silver fraction yielded but little substance. On evaporation of the solution obtained by decomposing the precipitate no crystalline compound separated out. Picric acid solution however gave a precipitate which after standing formed crystalline, yellow spherulites, which began to decompose at 260° ; yield 0.1 gr.

INVESTIGATION OF THE SILVER NITRATE FILTRATE.

Of this filtrate, which amounted to 4600 c.c., only 220 c.c. were taken for further investigation. To this liquid after elimination of silver and baryta, phospho-tungstic acid was added and 420 grms. of a dry precipitate were obtained, which was decomposed in the ordinary way. The solution of the bases belonging to the choline group was neutralised with hydrochloric acid and evaporated *in vacuo* to dryness. To get rid of the last traces of water the residue was redissolved in alcohol and the alcohol evaporated *in vacuo*. Finally an alcoholic solution was made and an alcoholic solution of sublimate was added as long as a precipitate was formed. This was recrystallised from hot water with the addition of a little sublimate. It was found to be very slightly soluble in water. In this way 10.5 grms. of crystals were obtained in the form of cubes. These were redissolved in water and decomposed with sulphuretted hydrogen. The filtrate was evaporated *in vacuo* to dryness, the residue dissolved in alcohol and evaporated again. The alcoholic solution finally obtained was precipitated with an alcoholic platinic chloride solution, 1.3 grms. of a pale yellow precipitate being obtained which was recrystallised from dilute alcohol; 0.85 grm. was thus obtained in the form of needles, which, after drying at 110° *in vacuo*, melted at 220° (uncorr.). The substance gave the following figures on analysis, after being dried *in vacuo* at 110° .

0.1666 grm. gave 0.0451 grm. Pt
 0.1622 „ required 4.2 c.c. N/10 H_2SO_4
 0.2173 „ gave 0.2104 CO_2 ; 0.0878 H_2O ; 0.0589 Pt and 0.2586 AgCl.
 Calc. for $(\text{C}_8\text{H}_{16}\text{O}_2\text{NHCl})_2\text{PtCl}_4$ (Mol. wt. 724). Found:
 C: 26.52; 26.40
 H: 4.42; 4.48
 N: 3.86 3.62
 Pt: 26.94; 27.07; 27.1
 Cl: 29.55 29.44

On igniting the platinum salt a peculiar smell was noticed, which was quite different from that given by choline.

A substance of this formula, with similar properties (m.p. 219°), was described by v. Braun [1908] as the platinichloride of methylpiperidylacetic betaine. The substance is being further investigated.

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