CXLVIII. STRUCTURE IN RELATION TO CHROMIC OXIDATION OF NITROGENOUS SUBSTANCES.

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It was noted in an earlier communication [Acharya, 1936] that when nitrogenous substances were treated with a mixture of chromic and sulphuric acids, a part of the organic nitrogen was oxidised to nitrate while another was, in several cases, lost in gaseous form. A more detailed examination showed that the percentage of total nitrogen recovered as ammonia showed a higher and variable value with small amounts of substances but soon reached a constant figure on increasing the weight of substance taken. For amounts of substances of less than 5 mg. nitrogen content there seemed to be an interference, possibly from the high proportion of sulphuric to chromic acid in the oxidising mixture, which tended to raise the percentage recovery to nearly 100; whereas on increasing the weight of substance taken and consequently narrowing the chromic : sulphuric ratio, the curves representing the percentage of total nitrogen recovered as ammonia rapidly became horizontal, and continued to

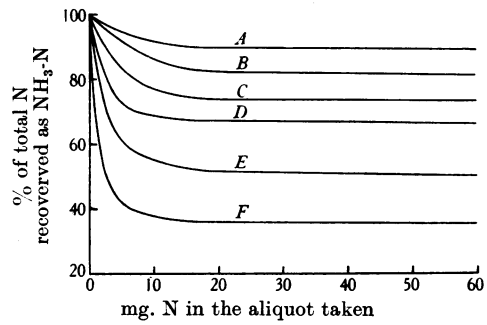

Fig. 1. The relation between oxidation constant and weight of substance taken. *A*, caseinogen; *B*, edestin; *C*, caffeine, theobromine; *D*, urea, creatine, uric acid; *E*, dicyandiamide; *F*, guanidine.

remain at that level. The percentage value at this constant level has been termed the "oxidation constant" [Acharya, 1935] and bears a definite relationship to the structure of the compound. The curves for some typical substances are given in Fig. 1 and are comparable to those obtained by Shewan [1935].

I. *Influence of structure on the nitrogenous products obtained.*

In order to explore the relationship between structure and the nature and quantity of nitrogenous products obtained, a large number (nearly 150) of organic nitrogenous substances of different types were treated with chromic-sulphuric mixture according to the previous procedure [Acharya, 1936]. In some cases commercial samples of the substances contained chloride either as impurity or

as hydrochlorides of nitrogenous bases, and it has been reported already that chlorides tend to increase the proportion of nitrate-nitrogen at the cost of ammoniacal nitrogen and thus interfere in the determination of the oxidation constants. In such cases the chlorides were removed by precipitation with silver sulphate.

With several substances, it was found that the sum of ammoniacal and nitrate-nitrogen formed accounted only for a fraction of the total nitrogen, the remaining portion being presumably lost in gaseous form. As the apparatus and procedure outlined before [1936] limited itself to the estimation of ammoniacal and nitrate-nitrogen only and no provision was made for the collection and estimation of gaseous nitrogen compounds evolved, the following modified apparatus shown in Fig. 2 was adopted to meet the difficulty.

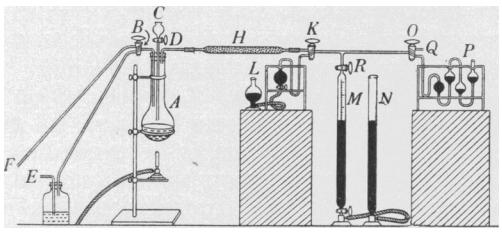

Fig. 2. Apparatus for the estimation of nitrogen by wet combustion.

A is a Kjeldahl flask of about 300 ml. capacity fitted with an inlet tube *B*, thistle funnel *C* and exit tube *D*. Tube *B* carries a three-way cock connected to an oxygen cylinder at *E* and water suction at *F*. The exit tube *D* is connected to a tube *H* about 20 cm. long and 0·5 cm. bore packed with Sofnolite (to absorb the CO_2 evolved) and thence to a Hempel's burette *M* *via* the three-way cock *K*. *L* is an explosion pipette, *O* a three-way cock connected to an absorption pipette *P* filled with alkaline pyrogallol, and also to an opening *Q* which serves either as an exit to the atmosphere or as inlet for the admission of hydrogen for purposes of explosion. The burette *M* is filled with mercury and the pressure inside can be manipulated by altering the position of the levelling tube *N*. The connections in the gas analysis apparatus are all of thick walled capillary tubing.

To start with, a known weight of the substance under examination is transferred into the flask *A* through a glass tube, the sides of which are washed with about 5 ml. of water. The flask is fitted into place, the mercury level in *M* is brought to *R* by raising the levelling tube *N*, cocks *R* and *O* are closed and the air inside the apparatus is replaced by oxygen, by alternately applying suction at *F* for a few minutes followed by connecting the tube *B* to the oxygen cylinder *E*. After repeating this operation three or four times, suction is applied at *F* for some minutes to ensure low pressure in the apparatus, the levelling tube *N* is lowered so as to keep the pressure at *R* about 3–4 in. of mercury and cock *R* is opened. The level of mercury in *M* shows a fall on opening *R* but slowly rises on continuing the suction at *F*. When the level reaches *R*, further suction is cut off by closing cock *B*; and chromic acid followed by sulphuric acid is added through the thistle funnel *C*, as described previously [Acharya, 1936]. The sulphuric acid is added in small amounts at a time so as to avoid excessive vigour of reaction and escape of gaseous products by back-bubbling through the thistle funnel *C*. After the addition of acid is completed the contents of the flask are mixed well and heated to gentle boiling with a micro-burner.

On the addition of sulphuric acid there is vigorous decomposition and gas evolution, as shown by the rapid fall of mercury level in *M*. The internal pressure in the apparatus is kept at about 10 in. of mercury by lowering the levelling tube *N* from time to time so as to keep the difference in levels between *M* and *N* at about 20 in. After about 25–30 min. heating (and longer in certain cases, *cf.* p. 1029), cock *R* is closed, cock *C* is opened, the levelling tube *N* is raised to equalise the levels in *M* and *N* and the volume of the gas collected in *M* is read off. This represents a mixture of oxygen (formed by decomposition of chromic acid) and gaseous nitrogen compounds evolved by the action of chromic acid on the nitrogenous substance taken. These latter could possibly be nitrogen and nitrous oxide only, since nitric acid in the amounts formed is retained in the digest itself by sulphuric acid and any trace of the higher oxides of nitrogen which might be evolved would be retained by the soda-lime tube *H* which, incidentally, also serves to remove the CO_2 formed.

The relative proportions of oxygen, nitrous oxide and nitrogen in the mixture in *M* were determined by two methods.

(1) Oxygen was removed from an aliquot by absorption in alkaline pyrogallol. This removed in the earlier stages some N_2O also, but after a few absorptions further absorption of N_2O was found to be negligible. The residual gas containing nitrous oxide and nitrogen was measured and analysed by explosion with hydrogen. The contraction in volume on explosion was equal to the amount of nitrous oxide present.

(2) An aliquot of the gaseous mixture containing O_2, N_2O and N_2 was exploded with a known volume of hydrogen, the contraction in volume noted and the amount of residual hydrogen determined by a second explosion with oxygen. If the volumes at N.T.P. of gases present in the given aliquot be $O_2 = X$, $N_2O = Y$ and $N_2 = Z$, the values of X, Y, Z could be determined from the following equations:

$X + Y + Z = a$ = original volume of aliquot;
$3X + Y = b$ = contraction on explosion with hydrogen;
$2X + Y = c$ = volume of hydrogen used up in the first explosion.

It was found that the two methods gave concordant results, the first being adopted for simplicity of procedure and consistency of values.

After completion of the gas analysis, the digested residue in the Kjeldahl flask *A* was analysed for ammoniacal and nitrate-N as previously [Acharya, 1936].

The values for nitrogen distribution (*i.e.* NH_3-N; NO_3-N; N_2O-N and N_2) by chromic oxidation of some typical groups of organic substances are given in Table I.

They show that nitrogenous substances in their relation to oxidation by chromic acid could be broadly subdivided under the following heads:

(1) Substances having their nitrogen atoms attached to different carbon atoms; these yield almost the theoretical recovery of nitrogen in the form of NH_3-N plus a small quantity of NO_3-N with the exception of hydroxylamine and hydrazine derivatives.

(2) Substances having two or more nitrogen atoms attached to the same carbon atom; these yield low recoveries of nitrogen as NH_3-N the proportion depending on the structure of the compound. The proportions of ammonia recovered from certain typical groupings are as follows: biuret group, 2/3 of total nitrogen; iminazole group, 4/5; guanidine group, 4/11; creatine group, 2/3. Of the fraction not recovered as ammonia, a small portion is oxidised to nitrate but the main bulk is lost in gaseous form as nitrous oxide.

(3) Hydroxylamine derivations are almost wholly converted into nitrous oxide and nitrate, while hydrazine derivatives lose almost the whole of their nitrogen as elementary nitrogen. In both cases the proportion of ammonia formed is inappreciable.

Table I.

Types of substances	% of total nitrogen as NH_3-N	NO_3-N	N_2O-N
I. Compounds with N atoms attached to different C atoms:			
(*a*) Tertiary amines including pyridine, quinoline, *iso*quinoline and alkaloids; quaternary ammonium derivatives including betaine and choline	100	—	—
(*b*) Secondary amines and substances with >NH groups	95	5	—
(*c*) Primary amines, amino-acids, amides and compounds easily hydrolysed to above, *e.g.* nitriles	97·5	2·5	—
II. Compounds with two N atoms linked to a C atom:			
(*a*) Compounds with —NH—CO—NH— grouping or easily hydrolysed to the above grouping by acids, *e.g.* cyanamide, urea, biuret, alloxan, allantoin, uric acid *etc.*	66·7 (2/3 of total N)	3·3	30
(*b*) Compounds with CH$\langle^{\text{N—}}_{\text{N<}}$ grouping, *e.g.* glyoxaline, histamine, histidine *etc.* (values for the α-amino-groups have been deducted)	80 (4/5 of total N)	—	20
(*c*) Substances with both the above groupings in the molecule, *e.g.* caffeine, theobromine *etc.*	74	—	26
III. Compounds with three N atoms linked to the same C atom:			
(*a*) Strongly basic substances absorbing CO_2 from the air, *e.g.* guanidine	36·5 (4/11 of total N)	2·5	61
(*b*) Arginine, deducting α-amino-group	50	2·5	47·5
(*c*) Creatine, creatinine, dicyandiamide (deducting end —CH group)	66·7 (2/3 of total N)	—	33·3
IV. Other nitrogenous compounds:			
(*a*) Hydroxylamine and its derivatives	0–5	35–40	As N_2O 55–60
(*b*) Hydrazine and its derivatives	0–5	—	As nitrogen 95–100

II. *Resistance of nitrogenous substances to chromic oxidation.*

While most nitrogenous substances, *e.g.* aniline, acetamide, hippuric acid, asparagine *etc.*, were rapidly oxidised by the chromic-sulphuric mixture and, under the experimental conditions adopted, theoretical values for carbon were obtained after 20–25 min. digestion, in the case of others, higher and varying degrees of resistance to oxidation were met with. Compounds having two or three nitrogen atoms attached to the same carbon atom, *e.g.* urea, biuret, caffeine, creatine, guanidine, dicyandiamide *etc.*, were more slowly attacked and correct values for carbon were obtained only after about 40–45 min. heating. Pyridine, quinoline and *iso*quinoline required more than an hour. Betaine, choline and the tetrammonium bases were very slowly attacked. The relative rates of oxidation of different types of nitrogenous substances are shown graphically in Fig. 3 and their possible physiological significance will be discussed later.

Shewan [1935] also noted that a much longer time of heating was necessary to obtain theoretical yields of nitrogen from betaine and quinoline derivatives,

but attributed it to a preliminary transformation of the nitrogen into some form before conversion into ammonia. But a simultaneous comparison of the yields of CO_2 obtained would show that the longer time necessary in these cases is due to resistance to oxidation.

It has been noted already [1936] that completion of oxidation of such resistant compounds could be tested either by weighing the Sofnolite tubes at half-hour intervals or better by having two sets of Sofnolite tubes in parallel and passing the CO_2 into each set alternately while weighing the other.

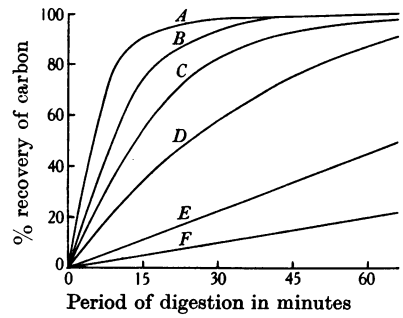

Fig. 3. Rate of oxidation of different nitrogenous compounds. *A*, aniline; *B*, pyrrole, piperidine; *C*, quinoline, *iso*quinoline; *D*, pyridine; *E*, betaine, choline; *F*, $(CH_3)_4N.OH$.

Discussion.

The relation between structure and chromic oxidation of nitrogenous substances has been hinted at by previous workers [Guyot and Simon, 1920; Shewan, 1935], but has not been systematically examined before. The data given above would show that the loss occurs in the form of nitrous oxide in the case of C-2N and C-3N linkages and with hydroxylamine derivatives; whereas free nitrogen is liberated from hydrazine and azo-derivatives. The fraction of total nitrogen recovered as NH_3-N, or the value of the chromic "oxidation constant", depends on the particular type of linkage, even among the C-2N and C-3N linkages, as shown in Table I.

In regard to the applicability of the chromic acid method for the estimation of nitrogen, the procedure as outlined previously [Acharya, 1936] was found to give only approximate results and the limits of applicability of the method for the determination of nitrogen in soils and plant materials were defined. For organic compounds containing the C-2N and C-3N linkages, or the hydroxylamine and hydrazine groups the method was inapplicable without further modification. The modified procedure given in this paper promises to be one of universal applicability for all types of nitrogenous substances. This improved wet combustion procedure could with advantage replace the traditional Dumas method for nitrogen, in view of its rapidity and ease of manipulation, and could be easily modified so as to make a simultaneous determination of carbon and nitrogen on the same sample.

It is interesting to note that substances which have the C-2N and C-3N linkages exert a marked physiological action on the human and animal systems, *e.g.* purine and pyrimidine bases, guanidine, creatine, dicyandiamide, arginine, histamine, histidine *etc.*, and all of them evolve substantial amounts of nitrous oxide on oxidation. It is not intended, at the present stage, to draw any causal relationship between such physiological activity and the formation of nitrous

oxide—at least until direct experimental evidence is forthcoming on the production of nitrous oxide by biological or enzymic oxidation of the above types of substances. At any rate, the technique of chromic oxidation appears capable of being developed into a rapid laboratory method for the detection of substances exerting marked physiological action.

It is also noteworthy that substances exhibiting a marked resistance to oxidation such as pyridine, quinoline, *iso*quinoline and the tetrammonium bases, *e.g.* betaine and choline, are either of no nutritional value or are highly harmful to the human and animal system. It is well known that administration of choline, betaine or the tetrammonium bases leads to a rapid depletion of the fat content of the liver. A possible explanation for this may be found in the above noted high resistance of these substances to oxidation and the effort made by the system to effect their oxidation and removal even at the cost of a large amount of fat.

The chromic oxidation method promises to be of value in the characterisation of different classes of proteins and in assessing the nutritional value of different types of food materials. It is well known that the quality of a food material improves with its content of diamino-acids, *e.g.* arginine and histidine, and these amino-acids yield a low recovery of NH_3-N on chromic oxidation. The values for "oxidation constants" for some typical proteins are given in Table II. There appears to be a rough inverse proportionality between the content of arginine and histidine and the loss of nitrogen on chromic oxidation. A more detailed examination of the behaviour of proteins, more widely differing in their contents of diamino-acids, and of protein hydrolysates and decomposition products towards chromic oxidation may yield information of great interest and value.

Table II.

Substance	Arginine and histidine as % of total nitrogen	Chromic "oxidation constant"
1. Wheat gliadin	9·0	97
2. Wheat gluten	12·2	95
3. Caseinogen	13·6	92·5
4. Glutenin (wheat)	15·0	92·5
5. Egg albumin	16·7	90
6. Blood albumin	17·7	90
7. Haemoglobin	18·8	90
8. Fibrin (blood)	18·7	90
9. Keratin	19·3	89
10. Edestin	32·7	82·5
11. Histidine	100	86·7
12. Arginine	100	66·7

The figures for the content of arginine and histidine are taken from the analytical data presented by Mitchell and Hamilton [1929].

Summary.

1. The qualitative and quantitative composition of the products obtained by chromic oxidation of nitrogenous substances vary with the structure of the compound. Compounds in which the nitrogen atoms are attached to different carbon atoms, with the exception of hydroxylamine and hydrazine derivatives, yield full recovery of nitrogen in the form of NH_3-N accompanied by small quantities of NO_3-N; those having two or three nitrogen atoms attached to the same carbon atom lose a portion of the total nitrogen in the form of nitrous oxide. Hydroxylamine derivatives are converted into nitrous oxide and nitrate,

while hydrazine derivatives yield mainly elementary nitrogen; in both cases, the amount of NH_3-N formed is inappreciable.

2. The fraction of total nitrogen recovered as NH_3-N is termed the chromic "oxidation constant" and is shown to depend on the structure of the compound.

3. The varying resistance to chromic oxidation of different types of nitrogenous compounds has been studied. Pyridine, quinoline, *iso*quinoline, betaine, choline and the tetrammonium bases are found to be highly resistant; the physiological significance of such resistance has been discussed.

4. An improved wet combustion apparatus has been described which includes the analysis of gaseous products and is applicable to the determination of nitrogen in all types of organic compounds.

5. The potentialities of the chromic acid method in offering a laboratory test for the detection of substances possessing marked physiological activity, in the characterisation of proteins and in assessing the nutritive value of different kinds of food materials have been pointed out.

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