THE ESTIMATION OF NITROGEN.

Part I.—The Nitrogen Factor for Casein.

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THE object of this investigation was to ascertain whether the factor 6·37, used for the milk proteins, was applicable to both casein and albumin when the nitrogen determination was made by the Kjeldahl method.

The starting-point was some observations made many years ago, that whilst preparations of lactalbumin gave results for nitrogen very closely agreeing with those required by the above factor—*i.e.*, 15·7 per cent. N—several preparations of casein gave results seriously below this, the minimum being 14·2 per cent. N. It was noticed that the more carefully the purification of the casein was carried out, the higher were the results, but the highest was but slightly over 15·0 per cent.

It was thought that this might indicate that Kjeldahl's method did not give the whole of the nitrogen as ammonia, and a special investigation was made to test this; as, however, it has been proved that this is not the case, it will not be necessary to give the experiments in full.

The first series of experiments was made by estimating the nitrogen by the various modifications of the Kjeldahl method, using several reducing agents, but no difference in the result was obtained.

The second series consisted of estimations of the nitrogen by Dumas' method, and by Kjeldahl's method in an atmosphere of carbon dioxide. The gases evolved from the Kjeldahl estimation were passed over a layer of manganese dioxide, then over a long layer of heated cupric oxide and in some cases a layer of lead chromate, and collected over caustic potash solution, the unabsorbed gas being measured and calculated as nitrogen. It was observed that an appreciable amount of permanent gas was evolved quite early in the heating with strong sulphuric acid.

The results were calculated for dry and ash-free casein:

		Per Cent.		Per Cent. Mean.
Nitrogen by Dumas ...	...	16·26, 16·67, 16·71	... ...	16·55
Nitrogen by Kjeldahl ...	...	15·26, 15·26	... ...	15·26
Gas as nitrogen ...	...	1·20, 1·40	... ...	1·30

The agreement of the sum of the Kjeldahl nitrogen and the gas evolved with the Dumas nitrogen appeared to show that nitrogen was evolved as gas, but on testing the gas it was found to burn with a blue flame.

On heating 5 grams of casein with strong sulphuric acid in an atmosphere of carbon dioxide, 65·5 c.c. of gas were collected after absorbing the carbon dioxide, and of this 0·5 c.c. were absorbed by alkaline pyrogallol, and 63·0 c.c. by ammoniacal cuprous chloride; the residue was taken as nitrogen, as it did not explode with oxygen, and as it was four times the oxygen, it was evidently due to atmospheric air, which had not been removed from the apparatus. Carbon monoxide was also identified in the gas by the spectroscopic examination of blood treated therewith.

It is evident that no nitrogen is evolved as gas in the Kjeldahl method, and that

the high result in the Dumas method was due to unburnt carbon monoxide. I conclude that the Kjeldahl method is better and more reliable for the estimation of nitrogen in casein, and probably in all proteins, than the Dumas method.

As the casein used had been extracted with ether, it was not previously thought necessary to look for fat as an impurity; but as a possible explanation of the low results, estimations of fat by the Gottlieb and Werner-Schmid methods were made, and in the specimen which gave the above results 1·9 per cent. of fat was found, and the nitrogen corrected for this impurity was 15·56 per cent.

It has been observed (Miller and Richmond, ANALYST, 1906, **31**, 321) that milk proteins take up small quantities of aldehyde from ether; by estimating the aldehyde figure of casein treated with ether, and comparing it with that of casein which has not been treated, it appears that a small correction should be made for this.

The following is an analysis of a carefully purified sample of casein :

Moisture	...	...	...	...	11·76 per cent.
Fat	...	...	...	...	1·46 ,,
Milk-sugar	...	...	...	...	None
Ash	...	...	...	...	1·75 per cent.
Aldehyde condensed		...	...	0·41 ,,
Nitrogen	...	...	...	...	13·26 ,,
Nitrogen corrected for impurities			...	15·67 ,,

The experiments give a mean value of 15·65 per cent. nitrogen in casein, which corresponds to the factor of 6·39.

I wish to acknowledge the assistance I have received from Mr. E. H. Miller in this investigation.

Part II.—Triazo Nitrogen.

Forster and Fierz have described a series of compounds containing the triazo group (*Trans. Chem. Soc.*, 1905, **87**, 826; 1907, **91**, 855, 1350, and 1942; 1908, **93**, 72), and they found that, although camphorylazoimide and the aromatic azoimides yielded two-thirds of the azidic nitrogen quantitatively as gas when treated with sulphuric acid, triazo-acetic ester gave abnormal results, only 16·3 per cent. instead of 21·7 per cent. being set free. The explosive nature of the aliphatic azoimides renders their combustion an inconvenient, and sometimes a dangerous, process, making it desirable to modify the treatment with sulphuric acid in such a way as will furnish quantitative results, and so dispense with the more usual method of estimating nitrogen.

When the action of sulphuric acid on aromatic azoimides is normal an amine is produced, usually an amino-phenol. Moreover, Curtius and Darapsky (*Journ. pr. Chem.*, 1901, ii., **63**, 428) have shown that benzylazoimide is converted into imino-benzaldehyde, methylene aniline, and benzylamine. Forster and Fierz (*loc. cit.*) consider that with the aliphatic ketones an unstable ring is formed containing the three nitrogen atoms and the – CO group, and that this breaks down into an imine, though they obtained evidence that a portion of the nitrogen was split off as hydrazoic acid. Accordingly, it appeared to me possible that addition of form-

aldehyde, tending as it does to undergo condensation with amines and imino-compounds, would so regulate the action as to cause two-thirds of the azidic nitrogen to be removed in the elemental form, leaving the remainder to be estimated by the Kjeldahl process.

In the case of compounds of the type $R - CH(N_3)_2$, and especially

$$\frac{R}{R_1} \!\!>\!\! C(N_3)_2 \text{ or } \frac{R}{R_1}\!\!\!\underset{R_{11}}{\longrightarrow}\!\!\!>\!\! CN_3,$$
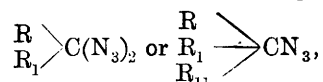
it would be obviously necessary to have a reducing agent present, and formaldehyde would act thus.

It has been found that, while compounds of the types $R - CH_2N_3$ and $\frac{R}{R_1}\!\!>\!\!CHN_3$ yield two-thirds of their nitrogen when shaken up in alcoholic solution with sulphuric acid and mercury, and more readily when addition of formaldehyde is made, bis-triazo-acetic ester, which is of the type $RCH(N_3)_2$, gives but little more than one-third of its nitrogen with sulphuric acid, but yields readily two-thirds if formaldehyde be added. In this case evidence that the formaldehyde has acted as a reducing agent is afforded by the formation of formic acid, a portion of which yields carbon monoxide, small quantities of which were found in the gas.

As mentioned above, Forster and Fierz found that only 16·3 per cent. of nitrogen was liberated by the action of sulphuric acid alone on triazo-acetic ester, while in the presence of alcohol and mercury I have succeeded in getting nearly 21 per cent. It is curious that the small difference in procedure should have led to this divergence. I am inclined to attribute it to the reducing action of the mercury ; there is always a mercurous salt present in the sulphuric acid. Incidentally, I may mention that this forms a delicate test for traces of halogens. A precipitate is produced in the acid, whitish in the presence of chlorides and bromides, and green with some red coloration of the acid in the presence of iodides, and the presence of small quantities of halogen derivatives as impurities in some of the compounds experimented upon was at once shown by the formation of a precipitate.

On suggesting this method to Dr. Forster, he kindly furnished me with specimens of a number of compounds. The method of analysis was to weigh about 0·1 to 0·2 gram into the cup of a Lunge nitrometer, and wash this in with several successive quantities of about 0·2 c.c. of alcohol ; in many experiments 2 drops of 40 per cent. formaldehyde solution were added. Strong sulphuric acid was added little by little, and the nitrometer shaken between each addition. Brisk effervescence set in after each addition till about 2 c.c. had been added, 10 c.c. being added in all. The nitrometer was shaken, and usually slightly warmed till all frothing ceased ; the apparatus was allowed to stand till the volume was constant, when it was read off, the level of the mercury in the side-tube being adjusted to the level of the mercury in the nitrometer. An allowance was made for the pressure of the column of acid (amounting to 6 mm. and 8·5 mm. in the two nitrometers employed) based on calculation from the density and from actual measurement, the two values agreeing. The gas was taken as dry, and several observations were made of the volumes under greatly differing pressures. As the value of $p.v.$ was sensibly constant, it was proved

THE ANALYST.

that there was no appreciable vapour pressure from the acid mixture. The following is an example :

(i.) $35 \cdot 5$ c.c. at $16 \cdot 5°$ and 757 mm. $p.v. = 26 \cdot 87 \times 10^3$
(ii.) $25 \cdot 5$,, ,, $16 \cdot 5°$,, 1053 ,, ,, $= 26 \cdot 85$
(iii.) $60 \cdot 3$,, ,, $16 \cdot 5°$,, 445 ,, ,, $= 26 \cdot 83$
(iv.) $35 \cdot 5$,, ,, $16 \cdot 5°$,, 757 ,, ,, $= 26 \cdot 87$

A vapour pressure of 2 mm. would certainly have been detected, and this would have been within the limits of experimental error.

The acid was transferred to a flask, and the nitrometer washed out with $5 + 3 + 2$ c.c. of acid. About 2 grams of zinc-dust were added, and a determination of the nitrogen by Kjeldahl's method made.

The following results were obtained :

Triazo-acetic ester, $N_3CH_2COOC_2H_5$—

		N as Gas.	N as Ammonia.	Total N.	Ratio.
Without formaldehyde	...	20·86	10·33	31·19	2·02
,, ,,	...	20·73	10·20	30·93	2·03
With formaldehyde	...	20·81	10·65	31·46	1·95
Calculated... ...	...	21·71	10·85	32·56	2·00

a-Triazo-propionic ester, $CH_3.CHN_3.COOC_2H_5$—

		N as Gas.	N as Ammonia.	Total N.	Ratio.
Without formaldehyde	...	19·00	10·21	29·21	1·87
,, ,,	...	18·96	10·36	29·32	1·83
With formaldehyde	...	19·28	9·82	29·10	1·965
Calculated... ...	...	19·58	9·79	29·37	2·00

Second specimen, which contained a little $CH_3.CHBr.COOC_2H_5$—

		N as Gas.	N as Ammonia.	Total N.	Ratio.
Without formaldehyde	...	18·65	9·50	28·15	1·965
With formaldehyde	...	18·83	9·59	28·42	1·97

β-Triazo-propionic ester, $N_3CH_2.CH_2.COOC_2H_5$—

		N as Gas.	N as Ammonia.	Total N.	Ratio.
Without formaldehyde	...	18·78	9·65	28·43	1·95
With formaldehyde	...	18·77	9·59	28·36	1·96

a-β Bis-triazo-ethane, $N_3CH_2.CH_2N_3$—

		N as Gas.	N as Ammonia.	Total N.	Ratio.
With formaldehyde	...	48·95	25·02	73·97	1·96
,, ,,	...	49·04	24·77	73·81	1·98
Calculated... ...	...	50·00	25·00	75·00	2·00

Bis-triazo-acetic ester, $(N_3)_2CH.COOC_2H_5$—

		N as Gas.	N as Ammonia.	Total N.	Ratio.
With formaldehyde	...	32·98	16·52	49·50	2·00
,, ,,	...	32·81	16·50	49·31	1·99
Calculated... ...	...	32·94	16·47	49·41	2·00

THE ANALYST.

These results were corrected for the presence of CO in the gas. The following results were obtained without correcting for the presence of CO ; and in the absence of formaldehyde :

			N as Gas (uncorrected).	N as Ammonia.	Total N.
With formaldehyde ...	...	...	33·2	16·5	49·7
,, ,, ...	...	...	33·8	16·5	50·3
,, ,, ...	...	...	33·7	—	—
Without formaldehyde	...	...	19·5	16·3	—
,, ,,	...	...	19·5	16·5	—

The gas obtained without formaldehyde smelt strongly of hydrogen cyanide. By diluting the acid solution (2 c.c.) with water and distilling, a solution was obtained which on titration with silver nitrate and thiocyanate indicated 13·85 per cent. of nitrogen as HCN.

It is seen that where two triazo groups are attached to one carbon atom the use of formaldehyde is absolutely necessary, and the results must be corrected for carbon monoxide. Where only one triazo group occurs in combination with a carbon atom the results do not greatly differ with and without formaldehyde ; the use of formaldehyde, however, seems to make the reaction more regular, and it is remarkable that in seven experiments where it was used the ratio of the two forms of nitrogen should only have varied from 1·95 to 1·98. The average of these is 1·962, which indicates that 1·9 per cent. of the azidic nitrogen is not evolved as gas. It would appear legitimate to multiply the readings of nitrogen as gas by 1·02, and, applying this correction, the results are—

$$N_3CH_2.COOC_2H_5 \quad ... \quad 21·23 + 10·65 = 31·88 \text{ calculated } 32·56$$
$$CH_3.CHN_3.COOC_2H_5 \quad 19·65 + \ 9·82 = 29·47 \quad ,, \quad 29·37$$
$$N_3CH_2.CH_2.COOC_2H_5 \quad 19·13 + \ 9·59 = 28·72 \quad ,, \quad 29·37$$
Forster and Fierz found by combustion 28·83
$$N_3CH_2.CH_2N_3 \quad ... \quad 49·93 + 25·02 = 74·95 \quad ,, \quad 75·00$$
$$,, \quad ... \quad 50·02 + 24·77 = 74·79 \quad ,, \quad 75·00$$

DISCUSSION.

Dr. LEWKOWITSCH suggested that it might be possible by means of the chloramine reaction, which had lately been investigated by Cross, Bevan and Briggs (see p. 198), to differentiate the proteins, or at any rate a portion of them, into different amino compounds.

Dr. DYER said that the figures obtained by Mr. Richmond with the Dumas process seemed to represent nitrogen *plus* an unknown quantity of something which was not nitrogen. The experiments apparently showed that the Kjeldahl process was the only one that gave correct results.

Mr. CHAPMAN said that these experiments seemed to afford another indication of what, he thought, was now generally recognised—namely, that the Dumas process ought never to be taken as a standard process. It had been shown that with some substances the process yielded more correct results when cuprous chloride was mixed with the substance, and this might possibly effect some improvement with casein.

Mr. L. M. NASH asked how it was that, the casein having been thoroughly

THE ANALYST.

extracted with ether, something over 1 per cent. of fat was found in the extracted casein.

Dr. RIDEAL said that Mulder (*Berzelius Jahresb.*, 1840, **19**, 734) had precipitated a number of proteins with chlorine, and determined the nitrogen in the precipitates, and in a paper which he (Dr. Rideal) and Mr. Stewart had read before the Society (ANALYST, 1897, **22**, 228) the subject was also dealt with; while Allen and Searle (*ibid.*, 1897, **22**, 258), using bromine for precipitation, had obtained further results.

Mr. RICHMOND, in reply, said that he had not made any experiments on the determination of the amino groups in casein by the chloramine reaction, but thought that this was a line of research which it would be worth while to follow up. He had, however, done what amounted to practically the same thing—namely, estimated the α-amino-acid groups by means of the aldehyde reaction, a little over 5 per cent. of the total nitrogen existing in this form in casein. He had also tried to estimate the total amino groups by treatment of the casein with nitrous acid, but this had not been very successful, because the casein, being insoluble, held back some of the gas. With regard to the nitrogen determinations, he had, perhaps, not made it sufficiently clear that he considered the discrepancies to be due, not to any fault in the Kjeldahl method, but to errors in the other methods. Owing to the comparative coarseness of its particles, the casein, even after extraction with ether, still contained some fat, which could be determined by either the Werner-Schmid or the Gottlieb method, the latter by preference.

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