The Isomeric Transformation of Urea into Ammonium Cyanate in Aqueous Solutions

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Walker & Hambly (1895) showed that the isomeric transformation of ammonium cyanate into urea (Wöhler, 1828) was spontaneously reversible in aqueous solutions. At equilibrium at 100°, a 0·1M-solution of urea was said to contain approximately 95% urea and 5% ammonium cyanate. Werner (1923) denied formation of cyanate from urea at temperatures below 60°. According to this author aqueous solutions of urea, kept for many months at room or body temperature under sterile conditions, did not contain traces of cyanate.

Since some pharmacological actions of cyanate were recently described (Schütz, 1945, 1946; Birch & Schütz, 1946), the question whether cyanate is formed in the mammalian organism was investigated. As the first step in studying this question, experiments were carried out with the sole aim of establishing whether cyanate was spontaneously formed in aqueous solutions of urea, at body or room temperature. Experiments dealing with living tissues will be reported separately.

The isomeric change in question could, theoretically, be demonstrated by showing a gradual decrease in the amount of urea originally present in the solution. Since, however, even at high temperatures, only a small percentage of the urea originally present is known to undergo the isomeric change into NH_4CNO, the expected decrease would probably be within the margin of error of the most sensitive quantitative methods for the determination of urea. On the other hand, the existing methods for the detection and quantitative determination of cyanate (Walker & Hambly, 1895; Hertig, 1901) were found to be too insensitive, especially when urea was present in higher concentrations.

In this paper two new methods are described for the qualitative and quantitative determination of cyanate in aqueous solutions. By means of these procedures evidence was obtained for the spontaneous formation of cyanate in aqueous solutions of urea, at body and room temperature.

METHODS AND EXPERIMENTAL

Spectroscopic method

Materials

Methaemoglobin. Human red cells from citrated blood were washed three times with 0·9% (w/v) NaCl solution. The white cells were removed and the red cells were lysed by adding 6 volumes of distilled water. 5% (v/v) of a freshly prepared saturated solution of $K_3Fe(CN)_6$ was added. The solution was left standing for 30 min. at room temperature, and was dialyzed overnight against running tap water through parchment paper, to remove surplus reagent. Thereafter the solution was centrifuged and buffered (10% v/v) with 0·1M-phosphate buffer (pH 7·3). Only fresh solutions were used.

Urea was recrystallized from ethanol. Sterile solutions were made by dissolving the weighed amount in ethanol; the solution was transferred into a sterile flask, closed with cotton wool, and the solvent removed by placing in a dry oven (100°). After cooling to room temperature, the desired amount of distilled water was added, sterile conditions being maintained throughout. The same results concerning the formation of cyanate were obtained, however, if the growth of micro-organisms was inhibited simply by saturating the solutions with chloroform or toluene.

Spectroscopy

A Hartridge reversion spectroscope was used in a dark room. If previously incubated, the solutions were brought to room temperature before the readings. A concentrated solution of methaemoglobin (metHb) was added drop by drop thereafter until the α-band became just clearly visible. Since the shift depended on the ratio CNO-metHb/metHb, care was taken not to add an undue excess of metHb to the urea solutions. For the readings, the solutions were transferred into containers of *c.* 2 cm. width. Samples which contained much carbonate, formed through decomposition of cyanate, were brought to pH 7·4 by a suitable addition of 0·4M-phosphate buffer.

Cyanate was found to combine readily with metHb in neutral solutions (Schütz, 1945). On saturation with cyanate the α-band of the absorption spectrum of metHb is shifted from 6325 to 6285 A. This shift can only be observed by means of small dispersion spectroscopes. Since freshly prepared solutions of urea did not alter the spectrum of metHb, the method consists in the determination of the position of the α-band after the addition of metHb solution to the urea solution. Details of the reaction of cyanate with metHb, and with other haemoglobin derivatives, will be reported in a future communication. The method described here is not applicable, without alteration, to solutions containing serum albumin or other substances which also combine with metHb.

Experimental

In preliminary experiments it was found that freshly prepared solutions of urea did not cause the slightest shift of the α-band of metHb added as described above. Neither did the buffer solution, nor

the chloroform nor toluene added as a bacteriostatic, cause any change in the position of the α-band.

It was found quite unsuitable to incubate the urea solutions with metHb, not only because the latter quickly coagulates, but also on account of its action as a cyanate-fixing substance. If left in the urea solution for hours, it 'traps' cyanate, thus shifting the equilibrium point of the urea $\rightleftharpoons$ NH_4CNO equilibrium in favour of NH_4CNO. The urea solutions were, therefore, incubated, with or without buffer, either sterile or saturated with a bacteriostatic. At intervals, samples were taken, cooled, and readings made immediately after the addition of metHb. Typical results are shown in Table 1.

at lower temperatures, is needed to show the presence of cyanate. The lowest concentration of cyanate which can be detected in this way was found to be of the order of 2×10^{-4}M. No quantitative assessment, however, was made with this method, since the manometric method, described below, was found to be superior for this purpose.

Since ammonium cyanate is formed from urea, control experiments were made on the possible action of ammonium ions on metHb, and on the reaction of cyanate with metHb. Under the conditions, and for the short periods, of the experiments shown in Table 1, no influence on either was found by ammonium chloride present in concentrations up

Table 1. *The position of the α-band of methaemoglobin immediately after its addition to fresh or incubated aqueous solutions of urea*

(α-Band of metHb, 6325 A.; α-band of cyanate-metHb, 6285 A.)

MetHb (2–3 drops) added at room temperature to	α-Band (average of several readings) (A.)
1·8M-Urea in sterile water, freshly prepared	6324 (±4)
1·8M-Urea in sterile water, after 41 hr. at 38°	6285 (±3)
1·8M-Urea in water, saturated with chloroform, freshly prepared	6325 (±5)
1·8M-Urea in water, after 41 hr. at 38°	6288 (±3)
1·8M-Urea in water, saturated with toluene, freshly prepared	6324 (±5)
1·8M-Urea in water, after 41 hr. at 38°	6289 (±4)
1·8M-Urea in 0·4M-phosphate buffer (pH 7·4), sterile, freshly prepared	6323 (±3)
Same solution, after 41 hr. at 38°	6285 (±2)
Same solution, after 41 hr. at room temperature	6318 (±4)
Same solution, after 5 days at room temperature	6308 (±3)
Same solution, after 41 hr. at 4°	6322 (±4)
Same solution, after 5 days at 4°	6322 (±5)
Controls	
Sterile water	6325 (±4)
0·05M-NaCNO, in water	6285 (±2)
Water saturated with chloroform, after 41 hr. at 38°	6326 (±5)
Water saturated with toluene, after 41 hr. at 38°	6325 (±4)
0·4M-Phosphate buffer (pH 7·4), sterile	6324 (±4)
0·4M-Phosphate buffer (pH 7·4), after 42 hr. at 38°	6325 (±3)

The figures show that with increasing time, and concentration of urea, the α-band of added metHb is shifted increasingly towards the point characteristic for CNO-metHb. Since the relevant shift was always very clearly observed with pure aqueous solutions of urea after incubation, the shift cannot conceivably be due to any other substance than cyanate. Moreover, when the maximum shift was observed, the α-band was situated as in the case of pure CNO-metHb.

It was also noted that the transformation, especially at the lower temperatures, is a very slow process. At least 6 hr. incubation at 38°, and more to 5% of the corresponding concentration of urea. Ammonium ions, in this range of concentration, had no influence on this qualitative method of detecting cyanate if the pH was maintained within the range 7·0–7·4.

Manometric method

Materials

Urea solutions were freshly prepared for each experiment and saturated with chloroform. Traces of the latter substance did not interfere with the determinations.

Sodium cyanate. This was made from urea according to Bader, Dupré & Schütz (1948). A pure sample of sodium cyanate was provided by Glaxo Laboratories Ltd.

It is known that cyanate decomposes on addition of acids into CO_2 and NH_3. This was studied with the manometric technique of Warburg. The solution of NaCNO was placed in the main compartment, and buffer solutions of different pH in the side arm. After equilibration at room temperature, the buffer was added to the NaCNO solution, and the CO_2 evolved was measured. The results are given in Fig. 1. It can be seen that only at pH < 5 does the decomposition of cyanate become obvious; only in very acid solutions does it decompose rapidly. Contrary to the common belief, cyanate was found to be relatively stable in moderately acid solutions (pH > 5).

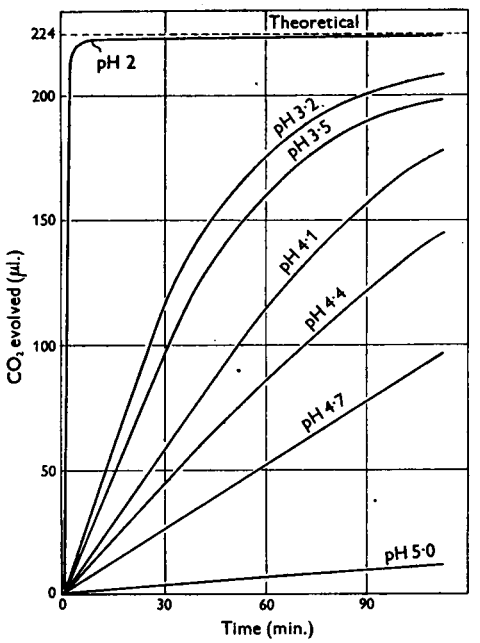

Fig. 1. Decomposition of sodium cyanate in aqueous solution at room temperature and various pH values.

In these experiments NaCNO was preferred to NH_4CNO because of the partial conversion of the latter into urea, even at room temperature. If NH_4Cl was added to NaCNO in equimolar amounts the same results were obtained as with NaCNO alone if the solution was not older than 0·5 hr. Similar results were obtained with different concentrations of NaCNO.

Since cyanate was found to be relatively stable at pH 5·0, it was possible to adopt the following procedure. Any carbonate or bicarbonate present was eliminated by the addition from one side arm of a buffer of pH 5·0. Within the time needed to re-establish equilibrium the decomposition of cyanate was found to be negligible at this pH and at room temperature. Then, after renewed equilibration, an appropriate amount of a strong acid was added from the second side arm, by which the pH of the mixture of cyanate and buffer was brought to 2, or less. The cyanate rapidly decomposed, and the corresponding amount of CO_2 evolved was measured.

Since the amount of CO_2 evolved after addition of the buffer solution (pH 5) corresponds to the carbonate and bicarbonate present in the solution, while that evolved after the addition of the strong acid measured the cyanate present in the mixture, carbonate and cyanate can be determined in the same sample and at the same time. This is useful since cyanate is known to decompose into carbonate to a varying degree according to the prevailing conditions.

Experimental

Incubated samples of solutions of urea were brought to room temperature, and 1–3 ml. of these transferred into the main compartment of a two-armed Warburg vessel, 0·2–0·3 ml. of 3M-acetate buffer being placed in one, and 0·2–0·3 ml. of 10% (w/v) H_2SO_4 in the other side bulb. After equilibration, the buffer was first added and, after re-establishment of equilibrium and noting of the pressure changes, the H_2SO_4 was added from the other bulb, and the CO_2 evolved again recorded.

Typical results are shown in Table 2. With known quantities of NaCNO, the results are in good agreement with the theoretical amounts, in the presence or absence of urea or phosphate buffer or both. It can further be seen that practically no CO_2 was evolved, after addition of the acid to a blank, which consisted of a freshly prepared solution of urea. This indicates that cyanate is not formed rapidly. The small pressure changes observed in this, and buffered blanks, are to be ascribed mainly to the altered CO_2 solubility and the small changes in vapour pressure which occur when the acid is added to the mixture of buffer and urea solution. These blank values are, however, so small in comparison with the data obtained with the same solutions after incubation, that only a very small correction factor would be needed.

Obviously the method is suitable to determine how much cyanate is formed in pure aqueous, or buffered, solutions of urea, since none of the substances present in such solutions interferes with the procedure; neither was any indication obtained of formation of cyanate from urea through manipulations required by the method.

Application of this method to biological material would yield results difficult to interpret, since there might be other substances present, besides cyanate, which would be stable at pH 5 and from which CO_2 would be liberated at pH 2.

With certain limitations, it can be applied, however, to mixtures of biological material and added NaCNO, more concentrated acetate buffer (3M) being required, and a higher concentration of H_2SO_4 to overcome the buffering capacity of biological materials. Results cited in this paper were also obtainable with these higher concentrations of buffer and acid.

Table 2. CO_2 *evolved from solutions after addition of acetate buffer* (pH 5), *showing amount of bicarbonate and carbonate*

(If cyanate is present subsequent addition of H_2SO_4 to the mixture causes a further evolution of CO_2 due to acid decomposition of cyanate.)

	CO_2 evolved (μl.)			
	After 0·3 ml. 3M-acetate buffer (carbonate, etc.)		After further addition of 0·3 ml. 10% H_2SO_4 (cyanate)	
	Found	Theoretical	Found	Theoretical
2M-Urea (fresh), 2 ml.	+ 16	—	+ 3	—
2M-Urea (fresh), 1 ml. 0·1M-Phosphate buffer, pH 7·4, 1 ml.	− 7	—	+ 12	—
0·1M-Phosphate buffer, pH 7·4, 2 ml.	− 7	—	+ 14	—
0·01M-NaCNO, 1 ml.	+ 9	—	+210	+224
0·01M-NaCNO, 1 ml. 0·1M-Phosphate buffer, pH 7·4, 1 ml.	− 6	—	+196	+224
0·01M-NaCNO, 1 ml. 0·1M-Phosphate buffer, pH 7·4, 1 ml. 2M-Urea, 1 ml.	− 13	—	+230	+224
0·01M-NaCNO, 1 ml. 0·01M-Na_2CO_3, 0·5 ml. 0·1M-Phosphate buffer, pH 7·4, 0·5 ml. 2M-Urea, 0·5 ml.	+108	+112	+218	+224

Table 3. CO_2 *evolved from urea solutions incubated for different periods and at different temperatures while saturated with chloroform*

(Carbonate and cyanate determined as in Table 2.)

	'Carbonate' (μl. CO_2)	'Cyanate' (μl. CO_2)	Urea converted (%)
1. 2M-Urea, freshly prepared, 2 ml. 'blank'	+ 16	+ 3	—
2. 1M-Urea, freshly prepared, 2 ml. 'blank'	+ 9	+ 4	—
3. 1M-Urea, 30 min. at 100°, 1 ml.	+ 59	+213	1·21
4. 1M-Urea, 1 day at 4°, 2 ml.	+ 9	+ 6	—
5. 1M-Urea, 2 days at 4°, 2 ml.	+ 4	+ 9	Trace ?
6. 1M-Urea, 4 days at 4°, 2 ml.	+ 4	+ 9	Trace ?
7. 1M-Urea, 2 days at room temperature, 2 ml.	+ 10	+ 10	Trace ?
8. 1M-Urea, 3 days at room temperature, 2 ml.	+ 4	+ 25	0·047
9. 4M-Urea, 24 hr. at 38°, 2 ml.	+ 50	+137	0·104
10. 2M-Urea, 24 hr. at 38°, 2 ml.	+ 31	+ 82	0·126
11. 1M-Urea, 24 hr. at 38°, 2 ml.	− 4	+ 46	0·102
12. 8·30M-Urea (7 days at 38°, 1 ml.)	+120	+322	0·242
13. 4·15M-Urea (7 days at 38°, 1 ml.)	+ 81	+245	0·359
14. 2·10M-Urea (7 days at 38°, 1 ml.)	+ 25	+165	0·412
15. 1·05M-Urea (7 days at 38°, 1 ml.)	+ 1	+ 99	0·462
16. 0·50M-Urea (7 days at 38°, 1 ml.)	− 9	+ 57	0·508
17. 0·25M-Urea (7 days at 38°, 1 ml.)	− 9	+ 42	0·750

In Table 3 representative results are shown, which provide evidence for the formation of cyanate in aqueous solutions of urea at 38°, and even at room temperature. The incubated samples were brought into the Warburg vessels without any further dilution and analyzed as described above. Dilution of the samples after incubation was found to introduce considerable error.

As expected, the formation of cyanate was found to proceed more quickly at 38° than at room temperature. As can be seen from Table 3, comparing Exps. 9–11, with Exps. 13–15, equilibrium at 38° was not obtained within a 24 hr. period of incubation. The amounts found to be formed at room temperature are very small, but are outside the margin of error, since the largest 'blank' ever obtained with pure aqueous solutions of urea, after addition of the acid, was 6 μl.

Similar results were obtained during incubation of urea in presence of 0·1M-phosphate buffer (pH 7·4), 10% (v/v). The formation of cyanate was not greatly altered, but more of it seemed to have decomposed into carbonate in presence of the buffer.

It is obvious that the formation of cyanate proceeds at a very slow rate at these lower temperatures. While at 100° equilibrium is reached within 30 min., several days are necessary at 38°.

Rate of isomerization as a function of concentration. As can be seen from Exps. 12–17 (Table 3) the relative amount of cyanate formed from urea increases rapidly on dilution. This is to be expected, since the thermodynamic equilibrium

$$(NH_2)_2CO \rightleftharpoons NH_4CNO \qquad (1)$$

between an undissociated (urea) and a dissociated component (cyanate) is, on dilution, shifted in favour of the latter; the transformation from right to left (1) is speeded up on concentration.

Both forward and reverse changes, moreover, are slowed down at lower temperatures, but the reaction from left to right (1) a little more so than the reverse; hence the shift of the equilibrium point in 1M-urea solution from 1·21% NH_4CNO at 100° to 0·46% at room temperature.

It will be seen that in Exps. 16 and 17 the values obtained after addition of the buffer solution were negative. This was only observed with samples which had been incubated for many days, and may have been due to the presence of small amounts of ammonia. The negative value (-9 μl.) observed with samples 16 and 17 was taken as zero point, and was added to all the 'carbonate' figures obtained in this series (Exps. 12–17).

The amounts of carbonate formed (corrected) in Exps. 12–17 were added to the amounts of cyanate, since obviously the former was formed by decomposition of the latter. The total was taken to represent the amount of urea converted during incubation. It can be seen that this amount, calculated in percentage of urea initially present, rises rapidly on dilution. This rise becomes steep at lower concentrations (0·005M) which represent the physiological range. The possible physiological significance of this observation will be discussed in a future communication.

SUMMARY

1. The formation of ammonium cyanate in aqueous solutions of urea by isomeric change has been studied by means of two new methods.

2. One method is based on the reaction of cyanate with methaemoglobin, the new compound, cyanate-methaemoglobin, being spectroscopically characteristic.

3. The other method is based on the acid decomposition of cyanate, and manometric measurement of the evolved CO_2; carbonate, present in the same solution, does not interfere, and is determined at the same time.

4. Evidence was obtained with both methods, that, contrary to the opinion of earlier workers, cyanate is formed spontaneously from urea in aqueous solution at 38° and also at 20°.

5. The isomerization is very slow at the lower temperature; equilibrium at 38° is reached after several days. The amounts of urea converted into cyanate at different temperatures, and in different initial concentrations of urea, have been determined.

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