DISTILLATION OF CYANIC ACID FROM TISSUES

by

P. DIRNHUBER AND F. SCHÜTZ

Department of Pharmacology, The Medical School, University of Birmingham (England)

In a previous paper DIRNHUBER AND SCHÜTZ[1] submitted evidence that the isomeric transformation in aqueous solution of urea into ammonium cyanate was still taking place spontaneously at body temperature. This fact made it more probable, that a reverse WÖHLER reaction, $(NH_2)_2CO \rightarrow NH_4CNO$, could occur in the mammalian organism. This possibility seems not to have been considered hitherto.

At one time cyanate was thought to be the precursor of urea in the mammalian organism[2, 3, 4], a possibility which could finally be discarded through the work of KREBS[5, 6]. It seems that only MONTGOMERY[7] claimed to have found evidence for the presence of cyanate in rabbits blood. The results reported by this author were however well within the limit of errors of the procedure used. Moreover, in preliminary experiments to this study BADER AND SCHÜTZ[8] were unable to confirm MONTGOMERY's results. Also NICLOUX AND WELTER[9], applying similar methods, found no evidence for the presence of cyanate in blood and lymph.

The new and highly sensitive spectroscopic and manometric methods, recently developed to show the presence and amounts of cyanate formed from urea[1] were most suitable for pure aqueous solutions, but when applied to biological material great difficulties were encountered. A method of distillation of cyanic acid from aqueous solutions of cyanate was therefore developed (DIRNHUBER AND SCHÜTZ[10, 11]) by which yields of the order of 30% were regularly obtained. This paper reports the application of this method to mammalian tissues, and experiments designed to ascertain whether the obtained yields were indeed due to cyanate present in the tissues.

METHODS AND MATERIALS

Most experiments were carried out with larger quantities of organs, since the yields were small. *Cows and sheeps brains* were obtained within 20–30 minutes after death of the animal. The brains were cleaned of the larger blood vessels and placed into vessels containing KREBS RINGER solution (KREBS AND HENSELEIT[5]). These vessels were used for the transport to the laboratory in order to prevent undue cooling of the organs. In the laboratory, usually within a further 20 minutes, the brains were cut with scissors and ground with sand. The latter was previously washed with acids and water. Before use it was moistened with KREBS RINGER solution, to give a thick paste. The brains, and similarly the other organs used for the same purpose, were not ground too thoroughly, since it was desired to keep a fair amount of cells undamaged. The grinding was aimed to produce an equivalent of tissue slices in large quantities. The brei was then transferred into conical flasks and suspended in a small volume of KREBS RINGER solution, then containing $NaHCO_3$ and glucose. This solution was added to the brain brei usually in a proportion of 1 : 5 (v/v). The suspension was then shaken in a WARBURG shaker at 38°, and O_2 or N_2, both containing 5% CO_2, was slowly bubbled through. The bulk of sand was left behind in the mortars, by allowing the mixture to settle for 1–2 minutes, but care was taken to transfer into the flasks for incubation all particulate matter as well as the small amount of liquid. After various times of incubation the tissue suspension was transferred into the distillation flask, and the distillation carried out as described before[11].

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Rabbits were anaesthetised with ether, or killed by a blow on the neck, and thoroughly bled. The organs were removed as quickly as possible, cleaned of the larger blood vessels and treated similarly to the brains mentioned above. Muscles were frozen in liquid air, passed through a Latapie mincer, and treated as the other frozen organs described below.

Cats were bled under anaesthesia and the organs treated as described above.

Dogs were bled from the carotid under ether anaesthesia into Dewar vessels containing 0.4 *M*-citrate buffer (pH 7.4). The blood was used at once for distillation.

Frozen organs. Sometimes organs were frozen in liquid air as soon as possible after death, ground with moistened cooled sand, and suspended in cooled 0.1 *M*-phosphate buffer (pH 7.4). The brei suspension was then subjected to the distillation procedure as soon as possible. Cooling was applied throughout, but it was found difficult to keep the organs below an average temperature of 4° during the stages leading up to the distillation, and during the first stages of the latter. These lasted ca 1 h, because of the persistent foaming of the brei suspensions during evacuation.

Urease solution consisted of a freshly made, centrifuged and filtered, aqueous extract of benzene-extracted jack bean meal.

Sodium cyanate was prepared according to BADER DUPRÉ AND SCHÜTZ[12].

DISTILLATION

Foaming. Most tissues, especially brain, frothed strongly in the initial stage of the evacuation. The foaming was not much diminished when octyl alcohol was added. Since transformation of cyanate into urea proceeds at a very considerably higher speed in an alcoholic milieu than in aqueous solution (WALKER AND KAY[13]), considerable losses occurred when octanol was added. When it was discovered that by means of the procedure described below the interference of foaming could be overcome, no more octanol was added. In most of the experiments reported below, no octanol was used.

When the foam had risen to the neck of the flask, the evacuation was stopped by closing the connection with the pump. This connection was only opened again when most of the foam had spontaneously broken. From time to time a little air was let in, which caused an immediate collapse of the foam. In the case of previously incubated tissue the flask was warmed by a waterbath at 55°. A vacuum of approximately 0.5 mm Hg was usually reached within 40–60 minutes by this slow, stepwise evacuation. Once this low pressure was reached the bubbles broke very rapidly, and the evacuation was always continued without further interruption or interference through foaming. The foam never rose again as far as the neck of the flask.

The acidifying buffer was then added, as previously described, using 2 *M*-citrate buffer (pH 5.0) 80–100 ml/100 g tissue, if not otherwise mentioned. Most of the subsequent steps concerning conversion in the distillate of cyanate into urea, and the determination of the latter, with and without addition of urease, were carried out as described in the foregoing paper. The vacuum finally reached, 30 minutes after the addition of the acid buffer solution, was of the order of 0.1–0.05 mm Hg.

In the course of this work the impression was gained that 55° was to be preferred to 50° as the temperature of the waterbath containing the distillation flask. The acidifying buffer was of pH 5.0, instead of 5.3, as used in some of the earlier experiments reported in this paper. On some occasions pure citric acid solution was added during the distillation, instead of the buffer solution, in order to obtain finally a more acid pH. The acid was added very slowly when the pressure was < 0.4 mm Hg.

Conversion of cyanate into urea

When the present investigation was in course a considerable improvement was found in the method of converting into urea the cyanate obtained in the distillate. It is known that cyanate is converted into urea in the presence of ammonium ions. Ammonium acetate was added to the distillate instead of $(NH_4)_2SO_4$, as described in the foregoing paper. It was advantageous that acetate was soluble in methanol and did not precipitate during the subsequent washings of the precipitate of dixanthylurea with methanol.

When, as in our earlier experiments $(NH_4)_2SO_4$ was used for the conversion, a part of the large excess of this salt was precipitated on addition of methanol, together with any dixanthylurea, eventually present. In the subsequent 5–6 centrifugal washings of the dixanthylurea crystals, according to ENGEL AND ENGEL[14] the precipitated $(NH_4)_2SO_4$ was removed, but the use of ammonium acetate made this laborious and perhaps even uncertain removal unnecessary. Both salts were only used to provide ammonium ions for the isomerization of NaCNO into urea.

In one experiment, when samples of NaCNO were incubated with ammonium sulphate, and with ammonium acetate respectively, the rate of urea formation was found equal in both cases. The latter salt was therefore preferred, since it did not precipitate on addition of methanol. In most experiments reported below, when the distillate was incubated with ammonium salts, NH_4-acetate was used.

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Recovery of small amounts of urea from the distillate

With small yields it was essential to carry through the whole procedure of conversion, addition of xanthydrol and addition of methanol, etc., as previously described[11], even if no obvious precipitate of dixanthylurea was seen after the addition of glacial acetic acid and xanthydrol. The mixture was allowed to stand at room temperature for ½–1 h, and at 4° for 24 h. Methanol, saturated with dixanthylurea (wash solution *A*), was then added, according to the xanthydrol method of urea determination of ENGEL AND ENGEL[14]. Any suspended crystals of dixanthylurea were then precipitated. Whatever NH_4-salt was used for conversion, the micro-crystals of dixanthylurea did not all precipitate spontaneously, and were often not clearly visible before the addition of methanol, when the yields of urea were small. Any turbidity, caused by suspended micro-crystals, disappeared completely after the addition of methanol and centrifuging.

It was, indeed, only the addition of methanol, which enabled the very small amounts to be recovered. When 0.2 mg pure NaCNO in water was distilled, no crystals were visible after addition of glacial acetic acid and xanthydrol to the distillate, which was previously incubated with NH_4-ions for conversion. On addition of methanol the suspended dixanthylurea was precipitated, and the colour, in measurable intensity, was subsequently obtained by adding conc. H_2SO_4 to the washed crystalline precipitate, as described by ENGEL AND ENGEL. It was found possible to increase the specificity of this colour reaction still further by splitting the sample and treating one half with urease, as described below.

An alternative to the photo-electric determination of the colour was found in the course of this investigation. One reason for this alteration was that the samples, even after centrifugation, contained a varying amount of "fluffy" turbidity, which remained suspended in the 50% H_2SO_4. Moreover, the yellow colour was, as is well known, the least suitable for photo-electric determination when colour filters are used. When the differences in the yellow colour between two samples were very striking to the eye in the light of a mercury lamp, the result with the photo-electric colorimeter was often found somewhat misleading. A considerably greater degree of accuracy and sensitivity was achieved, especially with smaller yields, when the samples were matched with a series of known concentrations of dixanthylurea under a mercury lamp, using the DUBOSCQ principle. All the differences of colour on which conclusions are based in this paper, were, however, obvious both to the naked eye and from the readings made with the photo-electric colorimeter. The smallest amount of urea which gave in our hands a measurable colour under the mercury lamp, was 0.1 μg.

Evidence for the presence of urea in the distillate from tissues, after incubation of the distillate at p_H 6.5 with ammonium ions

The following experiments were carried out to decide whether the precipitate, obtained in the distillate on addition of xanthydrol, was due to urea.

After the distillation of brain brei suspension, 2 g HN_4-acetate were added to the contents of the receiver flask, which originally contained 5 ml 2 *N*-NaOH in ca 100 ml water, cooled to 0° during the distillation. The contents of this flask were then brought to circa p_H 7.5 by addition of dilute acetic acid, while being mechanically stirred. A further excess of 2 g NH_4-acetate was then added, and the solution again adjusted to p_H 6.5 by careful addition of acetic acid, while keeping the liquid mechanically stirred. The mixture was then incubated at 65° for 6 h in the presence of a few drops of octanol. The volume was then reduced to ca 10 ml by distillation *in vacuo*, with an outside waterbath at 65°. Half of the reduced volume was incubated at 50° for 1 h with a small amount of urease solution, after adjusting the p_H to 7.0, with dilute NaOH.

After incubation of the solution with urease, the latter was destroyed by acidification to p_H 5 through addition of acetic acid, and immersion in a boiling waterbath for 10 min. Ethanol was then added (6–8 v/v), which completed the precipitation. The alcohol was removed from the centrifuged and filtered solution, by distillation *in vacuo*, the outside waterbath being at 60°. This was found to be a suitable method of eliminating the urease; other protein precipitants interfered with the subsequent xanthydrol reaction. The urea content was then determined as previously described.

Usually no urease was added to the other half of the distillate. In some experiments, however, a similar amount of a urease solution, previously inactivated by incubation

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for ½ h in a boiling water bath, was added, and the mixture was then treated similarly to the other half, which was incubated with active urease.

When the distillation was made from surviving brain tissue, which had previously been incubated at 38°, the half of the distillate incubated without urease, or with inactivated urease, gave a significant crystalline precipitate, and a measurable colour, when the washed crystals were treated with H_2SO_4. From the other half of the distillate, incubated with active urease, practically no precipitate or colour was obtained (Table I, No. 1, 2, 5, 10, 13). When in one experiment urea was added after the urease was inactivated and removed, the added amounts were quantitatively shown by the following xanthydrol procedure.

These experiments provide evidence that after incubation with NH_4-ions the distillate from tissues contained significant amounts of urea.

"*Entrained urea*". Since the material frothed considerably at the beginning of the evacuation, the possibility was investigated of a direct transference into the receiver of urea, by splashing or by inclusion in small droplets. The possibility of a transport of this kind has been excluded for pure aqueous solutions without organ brei. Although the "splash head" (see Fig. 1, in foregoing paper) excluded a direct transference of drops of the visible size, the following experiments were carried out, to ascertain whether any transference in other than the vapour state was likely to occur with the strongly foaming tissues.

Cow's brains, previously frozen, or their brei previously incubated at 38°, were distilled as usual, except that the acidification of the tissue was not carried out. A similar quantity of 0.4 *M*-phosphate buffer of p_H 7.4 introduced instead of the usual acid buffer. The receiver flask contained 0.1 *M*-citric acid (p_H 5.0), instead of NaOH, but was cooled as usual to 0°. Urea, carried over in droplets, would have been quite stable in the acid, but at p_H 7.4 no cyanic acid could be expected to distill over. Moreover, cyanic acid would have undergone immediate decomposition on condensation in citric acid. No other changes were made in the procedure that followed, except that, for obvious reasons, the distillate was not incubated with NH_4-ions. No trace of urea was found in the distillate in these experiments. The positive results shown in the tables cannot, therefore, be ascribed to "entrained" urea.

This conclusion is strongly supported by the fact that negative yields were consistently obtained from a certain other tissue (liver, see Table V) which also frothed strongly. If a transference of urea in droplets occurred in these experiments, it should have been observed to some extent, with breis of all tissues.

In other experiments the distillation was made first without acidification of the tissue. No yield was obtained in the distillate ("1st distillate") obtained during this period (30 min). The receiver flask was then exchanged with a similar one, again containing the usual amounts of NaOH. The liquid which had distilled over from the tissue suspension during the first period, was replaced by an addition to the tissue suspension of a similar quantity of water, added at the start of the second period of distillation. When the vacuum was again of the order of 0.4 mm Hg, the acidification of the tissue suspension was this time carried out as usual, and the distillation continued for 30 min ("2nd distillate"). Thereafter once again the receiver flask was exchanged, and the liquid distilled over from the tissue suspension was again made good by addition of water, and the distillation continued for a further 30 min ("3rd distillate"). Practically no yield was obtained in the 1st and 3rd distillate, while a significant amount

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TABLE I

Distillation of cyanic acid from brain brei suspensions (5 : 1 in KREBS RINGER solution, containing glucose and bicarbonate), previously incubated for 2–2½ h at 38°, in equilibrium with O_2, containing 5% CO_2. In expts 18, 19 and 20 the brains were frozen in liquid air and subjected to distillation without previous incubation. Acidification *in vacuo* liberated cyanic acid, which was condensed at 0° in NaOH and converted into urea in the presence of an excess of NH_4-ions

No.	Brain brei g	Acidification of the tissue suspension *in vacuo*	Condensation of vapour in	Treatment of distillate	Yield (dixanthylurea µg)	Yield/kg tissue (µg dixanthylurea/kg)
1	500 (sheep)	citrate buffer (pH 5)	NaOH	+ NH_4-acetate ½ + urease	< 2	< 4
				+ NH_4-acetate ½ — urease	15	60
2	800 (cow)	citrate buffer (pH 5)	NaOH	+ NH_4-acetate ½ + urease	< 1	< 1
				+ NH_4-acetate ½ — urease	24	60
3	760 (cow)	omitted	NaOH (1st distillate)	+ NH_4-acetate	5	7
		later: citrate buffer (pH 5)	NaOH (2nd distillate)	½ + NH_4-acetate	38	100
				½ — NH_4-acetate (pH 6)	9	24
			NaOH (3rd distillate)	+ NH_4-acetate	< 2	< 3
4	750 (cow) acidified to pH 1 after incubation	citrate buffer (pH 5)	NaOH	+ NH_4-acetate	< 4	< 5
5	720 (cow) as expt 4	citrate buffer (pH 5)	NaOH	+ NH_4-acetate ½ + urease	9	—
				+ NH_4-acetate ½ — urease	13	—
6	450 (sheep)	citrate buffer (pH 5)	NaOH	½ + NH_4-acetate	30	130
				½ — NH_4-acetate (pH 6)	8	33
7	630 (cow)	citrate buffer (pH 5)	NaOH	½ + NH_4-acetate	38	121
				½ — NH_4-acetate (pH < 3)	< 2	< 6
8	850 (cow)	slow addition of citric acid (final pH 3.2)	NaOH	½ + NH_4-acetate	53	124
				½ — NH_4-acetate (acidified to pH 2)	< 2	< 4
9	550 (cow)	citrate buffer (pH 5.0)	NaOH	+ NH_4-acetate	20	36
10	720 (cow)	slow addition of citric acid (final pH 3.7)	NaOH	+ NH_4-acetate ½ + urease	< 2	—
				+ NH_4-acetate ½ — urease	13	36

11	640 (cow)	slow addition of citric acid (final pH 3.7)	NaOH	+ NH_4-acetate	53	83
12	580 (cow) incubated in equilibrium with N_2 (containing 5% CO_2)	citrate buffer (pH 5)	NaOH	+ NH_4-acetate	28	48
13	715 (cow)	citrate buffer (pH 5)	NaOH	+ NH_4-acetate { ½ + urease ½ — urease	< 2 13	— 36
14	820 (cow)	citrate buffer (pH 5)	NaOH	+ NH_4-acetate	34	41
15	760 (cow)	citrate buffer (pH 5)	NaOH	+ NH_4-acetate	19	25
16	700 (cow)	citrate buffer (pH 5)	0.01 *M*-citrate buffer (pH 5)	+ NH_4-acetate	< 2	—
17	695 (cow)	citrate buffer (pH 5)	0.005 *M*-citrate buffer (pH 5)	+ NH_4-acetate	< 2	—
18	270 (cow) frozen in liquid air, and not incubated	citrate buffer (pH 5.1)	NaOH	+ $(NH_4)_2SO_4$	34	126
19	340 (cow) as expt 18	citrate buffer (pH 5.1)	NaOH	+ $(NH_4)_2SO_4$	15	44
20	980 (cow) as expts 18 and 19	citrate buffer (pH 5.1)	NaOH	{ ½ + $(NH_4)_2SO_4$ ½ — $(NH_4)_2SO_4$ (pH 6)	13 5	26 10
21	640 (cow) incubated with 50 ml saturated $(NH_4)_2SO_4$	citrate buffer (pH 5.1)	NaOH	+ NH_4-acetate	< 1	—
22	800 (cow) incubated with 10 ml saturated $(NH_4)_2SO_4$	citrate buffer (pH 5.1)	NaOH	+ NH_4-acetate	< 3	—

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was found in the 2nd distillate (see Table I, expt 3, also Table III, expt 7).

These experiments suggest that it was a volatile substance which was distilled from tissue, immediately after acidification of the latter *in vacuo*, yielding urea in the distillate, after the latter was incubated with NH_4-ions. The results are shown in Tables I, II, V and VI.

TABLE II

Distillation of cyanic acid from citrated whole blood (citrate buffer p_H 7.4). Approximately ½–1 h elapsed from the death of the animal until distillation was started. The blood was kept at room temperature during this period. The blood was acidified *in vacuo* to p_H 5, the vapour condensed in NaOH, and the distillate treated with NH_4-acetate for conversion of cyanate into urea, as in Table I

	Blood, citrated, whole (ml)	Yield (μg dixanthylurea)	Yield/100 ml μg dixanthylurea/ 1000 ml
1	500 (sheep)	19	38
2	600 (ox)	13	22
3	600 (calf)	17	28
4	600 (dog)	6	10

Was the volatile substance artificially produced?

Having obtained evidence for the presence of urea in the distillate, and that it seemed to be due to a volatile substance distilled from acidified tissues, the question arose whether this substance was naturally present in the tissue, or an artefact. A non-physiological production of the substance in question could have occurred during one of the stages of the preparation of the tissues in the course of a post mortem change, or through the acidification *in vacuo*.

If the volatile substance, or its precursor, was made artificially during one of the post-mortem stages of preparation of the tissue, a prolongation of this stage might be expected to favour a considerably greater yield. The procedure was therefore divided into four stages, and in a series of experiments with frozen muscle tissue from freshly killed rabbits, one stage was prolonged each time, while the rest was carried out as usual. The yields were compared.

The stages were: 1. From the time of death of the animal until the tissue was frozen. 2. From the time of mincing and grinding, till the vacuum was established. The tissue was then usually at 4–6°. 3. Distillation of the suspended brei until acidification at ca 4°. 4. Distillation *in vacuo* at p_H 5, and with a surrounding temperature of 55°.

Stage 1 was prolonged by removing the muscle 1 h after the rabbits death. Stage 2 was altered by leaving the minced tissue 40 min at room temperature. Stage 3 was prolonged to 60 min, the usual period being ca 25–30 min. The amount of liquid which had distilled over in this case during the first 30 min was replaced by water, and the distillation continued for further 30 min. Stage 4 was prolonged by distilling 1 h, instead of ½ h, the greater loss of liquid having been made good again by water.

As can be seen from Table III, no obvious increase of the yields was obtained by the alterations of any of the 4 stages. Indeed, prolongation of stage 1 and 2 resulted in a blank or in a very small yield, suggesting a loss of the substance in question rather than an artificial production. Any increase above the usual yields (Table III, expts 1–3) would have been easily observed.

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It must be regarded as highly improbable that the substance in question could have been due to artificial production through acidification (stage 4). Prolongation of this stage gave no further yield (Table III, expt 7). All the substance in question had distilled over during the first 30 min, while nothing was recovered from a distillate obtained after this period. If the substance in question had been made artificially from a precursor, the latter must be assumed to be present in very small quantities. A substantial production of cyanate from urea in the brei, due to acidification to p_H 5, can thus be excluded, since urea was present in relatively large amounts. If the cyanate were made from urea during the distillation, a significant amount should have distilled over in the second period (Table III, expt 7), since any such formation would be expected to continue as long as some urea was left. Moreover, it was previously found by DIRNHUBER AND SCHÜTZ[1] that the amounts of cyanate formed spontaneously from urea at the temperature of this experiment (< 10°) in aqueous solutions are negligible.

TABLE III

Distillation of cyanic acid from rabbit muscle, frozen in liquid air, minced in a LATAPIE mincer, ground with sand, and suspended in 0.1 *M*-phosphate buffer solution (p_H 7.4). Cooling was applied throughout. The distillates were incubated with $(NH_4)_2SO_4$ for conversion of cyanate into urea.

	Muscle g		Yield (dixanthylurea) μg	Yield/kg tissues (μg dixanthylurea/kg)
1	565		30	53
2	370		15	41
3	300		17	57
4	340	Muscles frozen 1 h after death of the animal	< 2	< 6
5	300	Prolonged distillation before acidification *in vacuo*	17	57
6	300	Muscles left 40 min at room temp. after mincing	8	27
7	520	Distillate collected during ½ h following acidification *in vacuo*	18	35
		Distillate collected thereafter	< 2	< 4

In the above described experiments no indication was found that the volatile substance in question was made artificially during a post-mortem change.

Distillation from acidified and neutral tissues

It was previously reported that cyanic acid could be set free and successfully distilled from pure aqueous solutions of cyanate, only if the solution was mildly acidified. The slow addition of a non-volatile buffer of p_H 5.0 *in vacuo* seemed the optimal procedure. At $p_H > 7.0$ no detectable trace of cyanic acid could be distilled from even concentrated solutions of cyanate. Nor could any yield be expected if the cyanate was hydrolysed at $p_H < 2$ before distillation. Thus, if the volatile substance obtained from tissues was cyanic acid, no yields could be expected, if the acidification of the tissue was omitted, or, if the tissues were strongly acidified.

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Several experiments were carried out omitting acidification of the tissues. One has been described above, which also furnished evidence that no urea was being "entrained" during the distillation. In this experiment, however, the receiver contained acid instead of the usual NaOH, so that any cyanic acid distilling over would have been instantly hydrolysed.

In several other experiments, similarly to expt 3 (Table I), which was described above (*e.g.*, Table VI, expts 9, 10) no yields were ever obtained from the distillates from neutral tissue. The usual significant yields, however, were obtained from the same brei after acidification (Table I, expt 3; Table VI, expt 9); smaller amounts were also found in distillates from freshly drawn citrated blood (Table II).

When distillations were carried out as usual, but the method of condensation of the vapour distilled from the tissue suspension was changed by placing a mild acid into the receiver flask, instead of the usual NaOH, no yields were obtained (Table I, expts 16 and 17). This would again be consistent with the behaviour of cyanic acid.

It is well known that cyanate, or cyanic acid, is not stable in a strongly acid aqueous milieu. When a concentrated NaCNO solution was acidified with 25% H_2SO_4 to p_H 1–2, not only the violent evolution of CO_2, and the precipitation of polymerised HCNO took place, but for a few seconds, the pungent smell of HCNO was clearly observed. This showed that even at these low p_H values a small part of HCNO evaporated, which, as is well known, decomposed or polymerised completely within a few seconds.

The following experiment was made to see whether the volatile substance distilled from brain, shared with HCNO the instability at $p_H < 2$. Incubated brain suspension was brought to approximately p_H 1 by addition of 10% H_2SO_4, and the mixture shaken for 20 min at room temperature. Care was taken that the mixture was not heated above 20° during the acidification. Only very small yields were obtained from the following distillations. Moreover, a large part of these small yields was not due to urea, since the half of the distillate treated with urease after conversion gave very nearly the same yield as the other half not previously treated with urease (Table I, expts 4 and 5). These traces are due to an unidentified substance giving a xanthydrol precipitate. This substance seemed to distil over from brain brei only after very strong acidification.

The acidification to p_H 1 had to be carried out *before* and not during the distillation, since as shown by the pungent, shortlasting smell, observed after strong acidification of pure aqueous and concentrated solutions of NaCNO, some HCNO is set free even on addition of very strong acid. Since it was the object of this experiment to show that the substance in the tissue, from which the volatile substance was distilled on acidification to p_H 5, would, like cyanate, undergo decomposition on acidification to p_H 1, the latter had to be carried out before the distillation. When acidified to p_H 2 and not shaken, small yields were still obtained, probably because the acid does not readily penetrate the tissue particles.

That the absence of any yield from tissues, acidified to p_H 1 20–30 min before distillation, is significant, was shown by an experiment, where the acidification to the usual p_H 5.0–5.3 was carried out 20 min before evacuation; the yield then obtained from brain brei was smaller than that usually obtained when acidification was carried out *in vacuo*, but it was still significant.

The experiments described above provide evidence that the volatile substance distilled from brain, yielding urea on incubation with NH_4-ions, could only be distilled from mildly acidified tissue suspensions. No yields were obtained either from tissue at

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p_H 7.0–8.0 (Table I, expt 3), or from tissues brought to p_H 1.0 shortly before distillation (Table I, expt 4,5).

These results suggest that the volatile substance was an acid, set free from a non-volatile salt on mild acidification, and that the salt and/or the acid were destroyed at p_H 1, while the acid was not set free from the salt at $p_H > 7$. Cyanic acid, set free from cyanate, would possess exactly these characteristics.

Incubation of the distillate with, or without ammonium salts

If the volatile substance was cyanic acid, the latter was transformed into NaCNO, on condensation in NaOH at 0°. Sodium cyanate, present in the distillate, was then converted, by incubation with NH_4-ions into NH_4CNO, which spontaneously isomerised into urea. Further evidence for the presence of cyanate in the distillate was provided by the fact that very different yields of urea were obtained from two halves of the same distillate, both treated in the same way, except that one half was previously incubated *with* NH_4-ions for conversion into urea, while the other half was incubated *without* NH_4-ions.

It should be recalled here, that even when pure NaCNO was distilled, as shown in the previous paper[11], and the distillate was not incubated with NH_4-ions, a certain amount of urea was nevertheless obtained in the mildly acidified distillate. When incubated at p_H 6.0–6.5 without NH_4-ions, a part of the free HCNO hydrolysed and liberated one NH_4^+/mol, which, with another mol of HCNO isomerised via NH_4CNO into urea. When acidified to $p_H < 2$ very little urea is formed directly from cyanate in the absence of NH_4-ions, since practically all the cyanate is hydrolysed at this low p_H.

Small amounts of urea could therefore be expected also in that half of the distillate from tissues, which was mildly acidified and not incubated with NH_4-ions; but a significant difference in the yields in the two halves of the same distillate, the one incubated *with*, the other *without* NH_4-ions, would supply further evidence for the presence of cyanate.

Brain brei suspensions were distilled as usual, and the distillate was halved. NH_4-acetate and acetic acid were added to one half as described above, thus establishing a final p_H 6.0–6.5. After incubation at 65° for 6 h in the presence of a few drops of octanol, the volume was reduced *in vacuo* to approximately 10 ml, and the urea content determined. Incubation with NH_4-acetate for shorter periods, and at lower temperatures (1 h at 45°) gave smaller but still quite significant yields.

The other half of the distillate was brought immediately to p_H 2.0 with acetic acid. At this p_H most of the cyanate would be rapidly hydrolysed, but urea would be stable. Similarly to the other half, this sample was incubated, and its volume equally reduced. Great care was taken to prevent NH_3, inevitably present in the laboratory air, from coming into contact with this half of the sample. It was kept under air traps containing conc. H_2SO_4, both during the incubation and during the reduction of volume *in vacuo*.

From typical results, shown in Table I, expts 3, 6, 7, 8, it can be seen that the yields were invariably significant when the distillate was incubated with NH_4-ions. If no ammonium salt was added, and atmospheric NH_3 was excluded, no significant amount of urea was found, if these halves of the distillates were acidified to p_H 2 before incubation. When, however, one half of the distillate was brought only to p_H 6.0–6.5 with acetic acid, incubated for 6 h at 38°, and reduced in volume, both with exclusion NH_3, a significant yield of urea was obtained. The amounts found were in the range of

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20–25% of the amounts found in the other half, which was previously incubated with NH_4-ions at the same p_H.

Theoretically it must be expected that only at an acid p_H could small amounts of urea be formed directly from NaCNO without special addition of NH_4-ions, since part of the NaCNO must hydrolyse to provide the necessary NH_4-ions. On the other hand not too much may be hydrolysed, since when all NaCNO was decomposed into NH_3 and CO_2, no urea is formed from these substances under the conditions of the experiments. The optimal p_H for this reaction has not yet been determined, but it is likely to be in the range where the hydrolysis of cyanate is known to proceed slowly (p_H 5–p_H 7), (DIRNHUBER AND SCHÜTZ[1]), rather than at p_H 2, where cyanate is rapidly hydrolysed.

The above results agree therefore in every detail with the assumption that cyanate was present in the distillates from tissues. The results show that the volatile substance distilled from brain, and condensed in NaOH at 0°, yielded small, but significant quantities of urea directly on incubation at p_H 6.0–6.5, without NH_4-ions; but that many times the amount of urea was obtained after previous incubation with NH_4-ions at the same p_H.

No substance other than cyanate is known to yield urea under the conditions of p_H, temperature and time of the described experiments, and no other explanation seems available for these results than that cyanate was present in brain brei.

Incubation of brain brei with an excess of ammonium ions

Since the isomerisation of ammonium cyanate into urea is greatly speeded up in the presence of an excess of NH_4-ions, less, or no cyanic acid would be expected to be distillable from a tissue suspensions previously incubated with an excess of an ammonium salt. To a suspension of cow's brain brei, prepared and incubated as usual, were added 50 ml, and 10 ml respectively, of a saturated $(NH_4)_2SO_4$ solution, soon after incubation was started. On both occasions the yields obtained from these tissues by distillation were equal to those obtained from blanks. (Table I, expts 21 and 22; and Table VI, expt 8).

These results further strongly support the evidence submitted above, for the presence of cyanate in tissue suspensions.

CONTROLS

Blanks. A blank of the usual contents of the receiver flask was made up as follows.

In addition to the usual amount of 5 ml 2 *N*-NaOH in ca 100 ml water, 0.2 g Na_2CO_3 was added, since the latter substance was certainly formed by the CO_2 evolved from acidified tissue. When 1 ml 0.1 *N*-NH_4OH was added to the above mentioned blank, the solution cooled to 0°, similarly to the contents of the receiver flask, and thereafter treated similarly to the distillate, no yield was obtained (< 1 μg dixanthylurea).

The addition of NH_4OH was made, since traces of this substance are assumed to occur under physiological conditions. NH_3, present in the tissue, would have evaporated before the latter was acidified. Since, however, the receiver flask contained NaOH, NH_3 would not have been retained there. It could have been condensed in the tube leading into the cooled contents of the receiver flask, and the blank experiment was made to test whether, under the conditions of the described procedure, detectable amounts of urea were formed from NH_3 and carbonate, although this seemed extremely unlikely.

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On several occasions no trace of urea was obtained from the above mentioned blanks.

The experiments in which the distillation was carried out at neutral reaction without acidification, or with previous strong acidification (p_H 1), both giving negative results, were reported above. Similarly, investigations which gave negative results, *e.g.*, when water or acid, instead of NaOH, was placed into the receiver flask (Table I, expts. 16 and 17), can also be regarded as controls, showing that the amounts of urea found in the distillate were not due to irrelevant conditions of the adopted procedure.

Non-specific formation of cyanate from amino acids, etc.

The question of a non-specific formation of cyanate due to the conditions of the procedure, was further investigated as follows. From the work of FOSSE[15, 16] a greater number of substances are well known to produce cyanic acid and urea, in strongly alkaline solution, in the presence of permanganate, or other oxidising agents. Although it seemed extremely unlikely that under the conditions of our experiments cyanic acid or cyanate could be formed from any of the substances present in the suspensions, the following experiment was made.

Most of the substances and amino acids reacting as described by FOSSE, were added to 1 l KREBS RINGER solution, in the following order and quantities: 0.1 g of isoleucine, serine, norleucine, arginine mono-HCl, cysteine-HCl, tyrosine, adenine-HCl, histidine mono-HCl, guanine-HCl, aspartic acid, leucine, sodium glutamate, norvaline, methionine, d-1-valine, tryptophane, uracil, threonine, glycine, amino propionic acid, alanine, and phenylalanine, NH_4Cl, glucose, "soluble" starch, and 0.1 ml of glycerol, acetaldehyde, propaldehyde; 0.5 ml solution 1% phenol and 0.5 ml 5% α-naphthol in ethanol. The mixture was incubated at 38° for 2½ h, while O_2, containing 5% CO_2, was bubbled through. It was then distilled, and the distillate treated as usual. The result was faintly positive, equivalent to 6 μg dixanthylurea. Subsequently this reaction was shown to be due to some other substance than urea, since it was not abolished by urease.

Non-specific isomerisation of urea into cyanate during incubation

Since it was recently shown that small amounts of ammonium cyanate are still spontaneously formed from urea in aqueous solution at 38°, experiments were made to assess how much of the obtained yields from tissues could be ascribed to a non-specific isomerisation of urea → NH_4CNO occurring during the time of incubation.

In the case of frozen organs, which were kept always below 6°, and usually at an average temperature of 4°, until distillation, no special control experiment was needed, since the amounts of cyanate formed from urea at these temperatures in so short a time are quite negligible, and would not be detectable with the methods used.

Controls to the incubations of tissue brei at 38° were made as follows. Dry urea was freshly dissolved in a few ml water and added to 1 l KREBS RINGER solution, immediately before incubation (2½ h at 38°), while O_2, containing 5% CO_2 was bubbled through. The solution was then distilled, and the distillate treated as usual.

In the usual experiments with brain brei, a volume of 1 l was made up by approximately 80% brei and 20% KREBS RINGER solution (v/v). On the assumption that brain contained 70% water, and that in the aqueous phase there was an average of 30 mg urea/100 ml, the final urea concentration in the aqueous phase of the tissue suspensions used, was thus of the order of 22 mg/100 ml. Higher concentrations of urea in KREBS RINGER solution were used for control experiments, in order to obtain measurable yields.

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1 l containing 40 mg urea/100 ml, and on another occasion 1 l containing 400 mg urea/100 ml were incubated and distilled as usual. The yields were 14 and 65 μg dixanthylurea respectively.

If one disregards the fact that with decreasing initial concentration of urea, relatively larger amounts of cyanate are isomerised from urea, it can be calculated from the above mentioned results that yields of the order of 3.6–7.7 μg could be expected after 2½ h incubation at 38°, from an aqueous solution containing 22 mg urea/100 ml. This latter urea concentration corresponds to the average concentration present in the tissue suspensions of the experiments reported in this paper. Since, however, the average yield obtained from incubated brain brei was 69 μg dixanthylurea ($\pm$ 10.5 s.d.m., n = 13)*, only a small amount, probably 10%, of these yields could be assumed to be due to a non-specific transformation which took place during the period of incubation of the tissue brei.

This is in harmony with the fact that also substantial amounts were obtained from frozen brain without previous incubation (Table I, expts 18–20, Table VI, expts 7, 13).

Difference between frozen and incubated tissue

The question arose, why greater yields were usually obtained from brain tissue which was previously incubated at 38°, than from tissue which was frozen soon after death of the animal, and subsequently kept at low temperature.

With regard to the equilibrium between urea and ammonium cyanate, thus:

$$(NH_2)_2CO \rightleftharpoons NH_4CNO$$

it is known that lowering the temperature of the aqueous solutions considerably slows down the transformation from left to right far more so than that from right to left (WALKER AND HAMBLY[25]).

Now, when tissues are frozen, the reaction would obviously be stopped in both directions. It was, however, found impossible, to get relatively large amounts of tissue cut, ground, suspended and distilled without the temperature of the substance and suspension being raised a few degrees. As described above, a stepwise and slow evacuation had to be adopted to avoid interference from frothing which, together with the other steps of the preparation of the tissue, made it necessary to keep the tissues for 2–3 h at an average temperature of 4°. In order to find out whether, and how much, NH_4CNO was lost by isomerisation into urea under these conditions, the following experiment was made.

A freshly prepared solution of NaCNO and NH_4-acetate, both 0.1 *M*, was halved, and both halves incubated for 10 min at 38°. One half (*A*) of the solution (2.5 ml) was mixed with glacial acetic acid (1 : 1) at exactly the same time, when the other half (*B*) was frozen in an alcohol bath containing solid CO_2. The cyanate, not yet isomerised into urea, was instantaneously hydrolysed by the glacial acetic acid in the first half (*A*). At the same time the addition of the glacial acetic acid was the first step in the determination of urea, according to the method of ENGEL AND ENGEL[14]. The frozen sample (*B*) was thawed to 4° and kept at this temperature for 65 h. After this period and while still cold, glacial acetic was added to *B*, and its urea content deter-

* Standard deviation of the mean $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$

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mined. From the difference of the urea contents of both samples, the amount of urea formed at 4° by isomerisation from NH_4CNO in 65 h was found to be 1200 μg.

This shows that considerable losses of ammonium cyanate through isomerisation into urea must be expected to occur even at these low temperatures. It is true that losses of cyanate by isomerisation of this kind would, of course, be far greater at 38°, if this reaction occurred in tissues. KREBS[5,6] found no indication that this reaction did occur in tissues. If however, in tissue brei suspensions, at 38°, very small amounts of cyanate are isomerised into urea, this loss seems to be made good, possibly by an isomeric change in the opposite direction. Such a reaction, however, could be excluded at 4°. Any loss of cyanate by isomerisation into urea would not be made good at this temperature by an isomerisation in the opposite direction.

It appears that keeping tissues at low non-freezing temperatures may result in a greater-post mortem change with regard to cyanate, than when the tissues were kept at 38° under physiological conditions. It is not possible, however, to make a quantitative assessment of the losses of cyanate sustained when the tissues were kept at 4°, since it is well known that the rate of isomerisation cyanate → urea increases with concentration, and much higher initial concentrations of cyanate were used in the above described experiment than could be expected to occur in tissue suspensions.

Recovery of added cyanate

When less than 1–2 mg NaCNO in aqueous solution was added to the usual amounts of brain brei suspensions, either before incubation, or immediately before distillation after the incubation, the yields were not greater than those usually obtained from the same tissue under similar conditions.

When larger amounts of NaCNO were added, however, the yields were significantly increased (see Table IV). Markedly more cyanate was recovered when addition was made immediately before distillation, than when a similar amount of cyanate was incubated for 2½ h with the brei suspension. The recovery yield of the added cyanate

TABLE IV

Distillation of cyanic acid from brain brei suspensions previously incubated with added amounts of sodium cyanate or urea. Incubation and distillation of the tissue suspensions, and conversion into urea of cyanate found in the distillate, as in Table I

	Brain brei, g	Addition	Yield (μg dixanthylurea)	Added NaCNO recovered (%)
1	770	1.3 mg NaCNO added before incubation	15	—
2	700	1.3 mg NaCNO added after incubation	52	—
3	780	5.6 mg NaCNO added before incubation	154	0.26
4	700	5.6 mg NaCNO added after incubation	560	1.3
5	820	1400 mg % urea added before incubation	175	—

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was calculated by deducting an average value of what was obtained from brain brei (69 μg dixanthylurea/kg tissue) from the actual yields obtained from the brei suspensions with added cyanate. It can be seen from Table IV that amounts of the order of 0.3 to 1.3% only were recovered.

Incubation of brain brei suspensions with an excess of urea

Several experiments were made by adding a freshly prepared urea solution to the brain brei suspension before incubation. It was not surprising that relatively small amounts of urea did not increase the yield to an obvious extent. Even if appreciable amounts of urea had isomerised into cyanate during the short period of incubation (2.5 h), the small amounts of cyanate could not be expected to increase the yield to an obvious extent, since, as shown above, cyanate was found to disappear on incubation with brain brei.

When, however, the concentration of urea was raised above 1000 mg/100 ml, the yields were significantly increased, already after 2.5 h incubation at 38° (*e.g.*, Table IV, expt 5; Table VI, expts 14, 15).

TABLE V

Distillation of cyanic acid from liver. Tissue frozen in liquid air soon after death of the animal, or incubation of suspensions of freshly ground tissue. Incubation, distillation and treatment of distillate, as in Table I

	Brei g		Yield (μg dixanthylurea)	Yield/kg tissue (μg dixanthylurea/kg)
1	120 (cat)	frozen, ground	< 1	—
2	120 (rabbit)	frozen, ground	< 1	—
3	650 (cow)	incubated 2.5 h (38°, pH 7.4, aerobic)	< 6	< 9

Acid decomposition of cyanate, obtained in the distillate from tissues, with following determination of the nitrogen content

While the conversion into urea of the cyanate found in the distillate from tissues, and the variations of the procedure, were an essential part of the evidence for the presence of cyanate in the tissues, another procedure for estimating the amounts of cyanate present in the distillate was developed.

One of the reasons for this alteration was that the conversion, by means of incubation at 65° with an excess of ammonium ions, was never complete. 100% yield could have been obtained by boiling down to dryness the mixture of cyanate and ammonium salt. For obvious reasons this was avoided with distillates from tissues, to exclude any possibility of urea or cyanate formation, which could eventually take place at this temperature from another source. Indeed, the formation of urea at relatively low temperatures (40–65°), and in a short period of time, was a valuable part of the evidence for the presence of cyanate in the distillate, although only a part of the cyanate present was converted.

The following procedure, though less specific, entailed no losses and was much simpler.

The principle consisted of hydrolysing the cyanate present in the distillate into

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NH_3 and CO_2 by acidification to p_H 2 at room temperature, and determination of the N-content in the steam-distillate, obtained from this solution after it was again rendered alkaline.

The procedure was as follows. The distillate, of which a part was always frozen immediately after the distillation, was thawed at room temperature, and while still cold ca 10 ml 25% H_2SO_4 were added carefully, keeping the liquid well agitated, until the mixture was at p_H 2. Care was taken that the temperature remained < 30°. This mixture was left standing for ca 3–6 h at room temperature. Thereafter its volume was reduced *in vacuo*, with an outside waterbath at 60°. The reduced volume, usually ca 10–12 ml, was transferred into a flask, connected with a steam distillation apparatus of the KJELDAHL type. Immediately after start of the steam distillation 4 ml 10 *N*-NaOH were added, and the steam distillation carried out as usual, collecting ca 20–30 ml of the distillate in a vessel containing 10 ml 0.01 *N*-HCl. 0.2 ml of a 2% gum ghatti solution was added to this solution, followed by 2 ml NESSLER reagent. The colour was determined by photoelectric colorimetry[17].

Since the found quantities were small, care was taken not to expose the solution unnecessarily to the atmosphere of the laboratory, in which, moreover, smoking was avoided. The distillation of the samples was not started until preliminary blanks were obtained.

TABLE VI

Distillation of cyanic acid from different tissues. The tissue was either frozen in liquid air soon after the death of the animal, or freshly ground and the suspension incubated. Incubations and distillations as in Table I. The cyanate in the distillate was decomposed by acidification to p_H 2 (H_2SO_4) at room temperature, and the nitrogen content was determined in the steam-distillate from this solution, previously rendered alkaline (NESSLER)

		Yield μg	Recovery-yield (%)
1	1 mg pure NaCNO in ca 400 ml H_2O		
	½ distillate — NH_4-acetate, for conversion cyanate into urea	83 urea	17
	½ distillate acid decomposed, N-content determination	300 NH_4Cl	30
2	0.5 mg pure NaCNO in ca 400 ml H_2O; distillate acid decomposed, N-content determination	175 NH_4Cl	35

		Yield μg (NH_4Cl)	Yield/kg tissue (μg NH_4Cl/kg)
3	Cows brain (690 g) suspension incubated	25	36
4	,, ,, (860 g) ,, ,,	25	28
5	,, ,, (680 g) ,, ,,	20	29
6	,, ,, (700 g) ,, ,,	28	40
7	,, ,, (500 g) frozen suspension distilled while cold	20	40
8	,, ,, (500 g) suspension incubated with $(NH_4)_2SO_4$	—	—
9	,, ,, (620 g) suspension incubated		
	a) distillation without acidification of tissue *in vacuo*	< 2	—
	b) distillation of the same tissue after acidification *in vacuo* (p_H 5.1)	28	45
10	Cows brain (610 g) suspension incubated, acidification of distillate omitted	—	—
11	Liver (790 g) suspension incubated	< 2	—
12	Kidney (500 g) suspension incubated	31	62
13	Kidney (480 g) frozen, suspension distilled while cold	10	21
14	Cows brain (600g) incubated with urea, final concentration 1000 mg %	9	15
15	Cows brain (550g) incubated with urea, final concentration 3000 mg %		
	a) first ½ h distillate	92	(167)
	b) second ½ h distillate	5	(9)

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It can be seen from Table VI that the yields obtained by distillation of cyanic acid were 30 and 35% respectively. In expt 1 (Table VI) it can be seen that a considerable loss occurred through the usual conversion of cyanate into urea, compared with the result obtained with the above described determination of the N-content, following acid decomposition of the cyanate.

The main experiments, formerly done by converting the cyanate found in the distillate into urea, were repeated with this new procedure.

It can be seen from Table VI that consistent yields were obtained from brain, while liver again gave a blank (expt 11). When the acid decomposition of the distillate was omitted a blank was obtained (expt 9*a*; 10). This suggests that all, or very nearly all of the N-content found in the distillate from brain brei after acid decomposition, was due to cyanate, especially since the acidification was carried out at low temperature.

When the tissues were not acidified in the high vacuum distillation, the distillate obtained gave a blank also with this procedure. When, in a continuation of this experiment, the same tissue brei was later acidified *in vacuo*, the distillate obtained thereafter gave an appreciable yield (expt 9*b*). This is in harmony with the results reported above and with the characteristics of cyanic acid.

When in another experiment (No. 8) the tissue was incubated with an excess of an ammonium salt, a blank was obtained. This is again in harmony with the previous results and the assumption of cyanate being present in the tissue suspension; cyanate, in the presence of an excess of ammonium ions, would be converted into urea.

DISCUSSION*

All results obtained from distillations, of either incubated or frozen brain tissue, agree in every detail with the assumption that cyanate was present in this tissue.

It is unlikely that the amounts found were due to a post-mortem change, since appreciable amounts were also found in brains which were frozen in liquid air soon after death. This result seems to gain in significance by the fact that not more, but much less, or no cyanate was found, when brains were left at room temperature for longer periods after death. This suggests a loss of cyanate, rather than an artificial production.

It cannot yet be stated whether the found amounts of cyanate can be derived from urea through isomerisation. This would certainly seem the most probable explanation, but final proof at present can only be expected from experiments with tracer elements.

The experiments in which increased quantities of cyanate were distilled from brain brei suspension, after incubation of the latter with added amounts of urea, strongly suggest that the isomerisation urea → cyanate can proceed in brain tissue under physiological conditions. It will be noted that the incubation of the tissue brei in these experiments was under conditions closely resembling the physiological milieu. Apart from the prevailing physiological p_H and temperature, the brei was only very little diluted, *i.e.*, 5 : 1 with KREBS RINGER solution, and a fairly large amount of cells was left intact. The period of incubation (2.5 h) of urea with brain brei must be considered as very short, with regard to the isomerisation of urea → cyanate, since it was previously found (DIRNHUBER AND SCHÜTZ[1]) that in pure aqueous solutions at 38° the equilibrium point is reached only in a number of days.

* Most of the results obtained in the various experiments described above were briefly discussed in the respective section, in order to make each following section easier understood.

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These experiments only show that urea, at a high concentration outside the physiological range, but within the short period of 2.5 h, markedly raised the amounts of cyanate present in incubated brain brei. It could be argued that cyanate may only occur, when the urea concentration is so high; that in normal tissue, however, in and outside the cells, a mechanism would exist, capable of completely suppressing the formation of cyanate, and that when the concentration of urea was raised markedly, this mechanism would be destroyed, with a consequent non-specific isomerisation of urea into cyanate.

Such an explanation would not seem likely, since no marked effects on tissue metabolism are known to be produced by urea at the concentration in question (0.5 *M*), which was capable of raising significantly, after 2 ½ h, the amounts of cyanate obtained from tissue. It becomes very unlikely that such a concentration should so thoroughly have altered the physiological conditions, that the isomerisation urea → cyanate would only then be possible, and not at all when the concentration of urea is lower.

The fate of cyanate. The fact that small amounts of cyanate incubated with brain brei could not be accounted for, may be related to the recent observation of MILLINGTON AND SCHÜTZ[18], that the diuretic action of cyanate (SCHÜTZ[19]; BIRCH AND SCHÜTZ[20]) was lost on incubation of cyanate with serum. Isomerisation of cyanate into urea was excluded as a possible reason for this effect. Both observations may possibly be due to enzymatic destruction of cyanate, which is now being investigated, or they may be explained by the binding of cyanate to one or more of the constituents of brain or serum. The latter explanation appears more probable at present. Apart from the known fact that cyanate is a very reactive substance, it was found that it combined with methaemoglobin[21,22], with a number of haemoglobin derivatives (BADER, DIRNHUBER, AND SCHÜTZ[23]), with the amino groups and the SH-groups of a number of amino acids (HOLTHAM AND SCHÜTZ[24]).

It seems, therefore, that there are many possibilities for small amounts of cyanate to react with one or the other normal constituent of the body. Which of these reactions of cyanate is reversible under physiological conditions, seems important in this connection. So far only the reaction with methaemoglobin was found reversible by dialysis against water[23].

The fact that a certain amount of cyanate was regularly found in brain brei, while added amounts soon disappeared, suggests the possible existence of a regulating mechanism in this tissue. Such a mechanism may also be connected with the fact that no trace of cyanate could be found in liver, under conditions which regularly gave positive yields from brain.

We are indebted to the Medical Research Council and to the ELLA SACHS PLOTZ-Foundation, Boston, U.S.A., for grants to one of us (F.S.) in aid of equipment of this laboratory, to the Board of Mental Disease Research for financial assistance, and to Professor A. C. FRAZER for his help given in the presentation of the results contained in this paper. We are also indebted to the veterinary authorities of the slaughter house, especially to veterinary surgeon, Mr DAVISON, for having generously provided us with the organs, and to Dr T. F. MACRAE, D.Sc., Glaxo Laboratories, Ltd, for the gift of samples of a number of amino acids, used in our experiments. We are also grateful to Messrs J. DAVIS and W. DUNN for technical assistance.

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SUMMARY

1. A method, recently developed for the distillation of cyanic acid from aqueous solutions of cyanate (see foregoing paper), has been applied to tissue suspensions.

2. Brain brei in KREBS RINGER solution (5 : 1 v/v) was incubated at 38° under physiological aerobic conditions, and distilled. Significant amounts of urea were found in the distillate, after incubation with NH_4-ions.

3. Instead of conversion into urea of the cyanate present in distillates from tissues, the cyanate was decomposed by acidification to p_H 2 at room temperature, and the N-content determined.

4. No indication of a post-mortem origin of the urea-yielding volatile substance was obtained.

5. No other volatile substance than cyanic acid is known, which distilled under the described conditions (p_H 5 and at a temperature $< 10°$) and would yield urea in a mildly acid medium (p_H 6), at relatively low temperatures (40–60°), and in short periods of time (1–3 h).

6. The following characteristics of the volatile substance are also identical with those of cyanic acid:

a) The volatile substance could not be distilled when the acidification of tissues was omitted ($p_H > 7$), nor after very strong acidification ($p_H < 2$). At p_H 5 regular yields were obtained.

b) None of the volatile substance in question could be distilled from tissue, incubated with an excess of ammonium ions.

c) When a pure aqueous solution of sodium cyanate was distilled, the distillate halved, and one half incubated at p_H 6 with an excess of NH_4-ions, the other half without NH_4-ions, the yields of urea in both halves were approximately 5 : 1. Very similar ratios were obtained in the corresponding halves of the distillates obtained from brain brei.

7. Significant, though smaller yields were obtained from brain tissue, frozen in liquid air soon after the death of the animal. Possible losses of cyanate through isomerisation into urea at low temperatures are discussed.

8. More cyanate was found in brain brei after 2–3 h incubation under physiological conditions, than could be accounted for by non-specific isomerisation during the incubation from the amounts of urea present.

9. No yields were obtained from liver under conditions which gave regular yields from brain tissue. Very small yields were obtained from muscle and whole blood.

10. Incubation of brain brei suspensions with an excess of urea increased the yields of cyanate, even after short periods of incubation. The possible occurrence of urea → cyanate isomerisation in tissues is discussed.

11. The possible existence of a regulating mechanism is discussed.

RÉSUMÉ

1. Une méthode récemment mise au point pour la distillation de l'acide cyanique à partir de solutions aqueuses de cyanate (voir le mémoire suivant) a été appliquée à des suspensions de tissus.

2. Un broyat de cerveau dans une solution de KREBS-RINGER (5 : 1 v/v) a été incubé à 38° dans des conditions physiologiques, en aérobiose, puis distillé. Le distillat renfermait des quantités notables d'urée après incubation avec des ions NH_4.

3. Au lieu d'être transformé en urée, le cyanate présent dans des distillats provenant des tissus a été également décomposé par acidification à p_H 2, à la température du laboratoire, et la teneur en azote a été déterminée.

4. Il n'existe aucune indication d'une origine postmortem de la substance volatile capable de fournir de l'urée.

5. On ne connaît aucune substance volatile, autre que l'acide cyanique, qui, distillée dans des conditions indiquées (p_H 5 et température $< 10°$), fournirait de l'urée en milieu légèrement acide (p_H 6), à des températures relativement basses (40–60°) et dans un temps court (1–3 h).

6. Les caractéristiques suivantes de la substance volatile sont également identiques à celles de l'acide cyanique:

a) La substance volatile ne peut pas être distillée sans acidification préalable des tissus ($p_H > 7$), ou après une acidification très forte ($p_H < 2$). A p_H 5, on obtient des rendements réguliers.

b) Aucune trace de la substance volatile en question ne peut être distillée d'un tissu après incubation avec un excès d'ions NH_4.

c) Quand une solution aqueuse pure de cyanate de sodium est distillée, que le distillat est séparé en deux moitiés, et que l'une des moitiés est incubée à p_H 6 avec un excès d'ions NH_4, l'autre moitié étant traitée sans ions NH_4, les formations d'urée dans les deux fractions sont approximativement de 5 : 1. Des rapports tout à fait comparables ont été obtenus dans les moitiés correspondantes de distillat provenant de broyats de cerveau.

7. Une production de cyanate notable, quoique plus faible, a été obtenue à partir de tissus de

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cerveau congelé dans l'air liquide rapidement après la mort de l'animal. Les pertes possibles en cyanate par isomérisation en urée à basse température sont discutées.

8. On trouve plus de cyanate dans un broyat de cerveau après une incubation de 2–5 heures dans des conditions physiologiques, que ne pourrait l'expliquer une isomérisation non spécifique de l'urée présente au cours de cette incubation.

9. Le foie, traité dans les mêmes conditions, ne donne aucune production de cyanate. Le muscle et le sang ne fournissent que de très faibles quantités.

10. L'incubation de suspensions de broyats de cerveau avec un excès d'urée accroît la productlon de cyanate, même après des périodes d'incubation très courtes. La posibilité d'une isomérisation de l'urée en cyanate dans les tissus est discutée.

11. La possibilité de l'existence d'un mécanisme régulateur est discutée.

ZUSAMMENFASSUNG

1. Eine neuerdings ausgearbeitete Methode zur Destillation von Zyansäure aus wässrigen Zyanatlösungen (siehe vorhergehende Arbeit) wurde auf Gewebesuspensionen angewandt.

2. Gehirnbrei in KREBS-RINGERlösung (Volumenverhältnis 5 : 1) wurde bei 38° unter physiologischen aeroben Bedingungen inkubiert und destilliert. Bedeutende Harnstoffmengen wurden nach Inkubieren mit NH_4-ionen im Destillat angetroffen.

3. Anstatt es in Harnstoff zu überführen, wurde das in den Destillaten vorhandene Zyanat durch Ansäuren auf p_H 2 bei Zimmertemperatur aufgespalten und der N-Gehalt bestimmt.

4. Es wurden keinerlei Anzeichen eines post mortem Ursprungs der flüchtigen harnstoffbildenden Substanz erhalten.

5. Ausser Zyansäure ist kein flüchtiger Stoff bekannt, der nach Destillation unter den beschriebenen Umständen (p_H 5 und Temperatur $< 10°$), in schwach saurem Milieu (p_H 6), bei ziemlich niedriger Temperatur (40–60°) und in kurzer Zeit (1–3 Stunden) Harnstoff liefern würde.

6. Die folgenden charakteristischen Eigenschaften der flüchtigen Substanz sind ebenfalls mit denen von Zyansäure identisch:

a) Die flüchtige Substanz konnte bei Weglassen des Ansäurens ($p_H > 7$) nicht destilliert werden, und auch nicht bei sehr starkem Ansäuren ($p_H < 2$). Bei p_H 5 wurden gute Ausbeuten erhalten.

b) Keiner der in Frage kommenden flüchtigen Stoffe konnte aus Geweben, die mit überschüssigen Ammoniumionen inkubiert waren, destilliert werden.

c) Wenn eine reine wässrige Lösung von Natriumzyanat destilliert wird, wenn das Destillat in zwei Hälften geteilt wird, von denen eine bei p_H 6 mit einem Überschuss von Ammoniumionen, die andere ohne Ammoniumionen inkubiert wird, verhalten sich die Harnstoffmengen in beiden Hälften ungefähr wie 5 : 1. Sehr ähnliche Verhältnisse wurden in den entsprechenden Hälften der aus Gehirnbrei erhaltenen Distillate erhalten.

7. Nennenswerte, doch kleinere Mengen wurden aus Gehirngewebe, das kurz nach dem Tode des Tieres in flüssiger Luft zum Gefrieren gebracht wurde, erhalten. Mögliche Verluste an Zyanat durch Isomerisierung bei niedriger Temperatur werden diskutiert.

8. Nach 2–5-stündiger Inkubation unter physiologischen Bedingungen wurde im Gehirnbrei mehr Zyanat gefunden, als durch unspezifische Isomerisierung aus Harnstoff entstanden sein könnte.

9. Unter den Bedingungen, die bei Gehirnbrei regelmässige Ausbeuten ergaben, wurde aus Leber kein Zyanat erhalten. Aus Muskel und Gesamtblut wurden sehr geringe Mengen erhalten.

10. Inkubation von Gehirnbrei mit Harnstoffüberschuss erhöhte die Zyanatmenge, sogar nach kurzen Inkubationsperioden. Das eventuelle Auftreten der Harnstoff-Zyanatisomerisierung in Geweben wird diskutiert.

11. Die mögliche Existenz eines Regulierungsmechanismus wird besprochen.

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Received June 9th, 1948