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Cyanate

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WALKER and HAMBLY[2] showed that the isomeric transformation of ammonium cyanate into urea (WÖHLER[3]) was spontaneously reversible in aqueous solutions. At equilibrium at 100°, a 0·1 *M* solution of urea was said to contain approximately 95% urea and 5% ammonium cyanate. WERNER[4] 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.

Whether cyanate is formed in the body from urea by a reverse WÖHLER reaction, thus: $(NH_2)_2CO \rightarrow NH_4CNO$, does not appear to have been investigated. At one time ammonium cyanate was thought to be a precursor of urea in the mammalian organism[4,5], a possibility which could finally be discarded through the work of KREBS[6]. MONTGOMERY[7] claimed to have found evidence for the presence of cyanate in the blood of rabbits. The results reported by this author were however well within the limits of error of the procedure used. Moreover, using this procedure, BADER and SCHÜTZ[8] could not confirm his results. Also NICLOUX and WELTER[9] in a careful study, and applying similar methods, found no evidence for the presence of cyanate in the body.

The reason why the formation of cyanate from urea in the body, and a possible physiological role of cyanate, has apparently never been taken into consideration, is probably due to the fact that the few authors who briefly mentioned the pharmacological effects of injected cyanate, usually reported it to be very toxic ("cyanate poisoning"). Undoubtedly the results were often due to impurities. Most commercial samples of cyanate contain appreciable amounts of cyanide. Only VŒGTLIN, JOHNSON, and DYER[1] noted the low toxicity of potassium cyanate in the intact animal, and HOLMES and WATCHORN[2] found it of low toxicity when added to embryo kidney tissue cultures. VOIGT[3] described increased reflex activity and convulsions, following high and mostly lethal doses.

In the following a summary is given of several investigations, which were all started more or less simultaneously, with a view to investigating with newly developed, highly sensitive methods, the presence and formation of cyanate in the mammalian organism, its pharmacological actions and its possible physiological significance.

Preparation and properties of pure sodium cyanate from urea

When prepared from cyanide, or by heating alkali metal hydroxides or carbonates with NH_3 and CO_2, sodium cyanate samples contain impurities (cyanide, carbonate, etc.), which preclude their use for biological experiments. When prepared by isomerization from urea, by boiling a solution of the latter substance in water or alcohol, in the presence of alkali hydroxide, the samples contain large amounts of carbonate, due to hydrolysis of cyanate in the presence of water[4]. BADER, DUPRÉ, and SCHÜTZ[5] based their method on isomerization of urea to NH_4CNO under anhydrous conditions. Sodium metal, and not NaOH, is dissolved in dry butanol and urea is then added in equimolecular quantities; the mixture is refluxed. Insoluble sodium cyanate separates in almost theoretical yield, which contains carbonate as the only detectable impurity (0·5–1·5%).

Is cyanate formed spontaneously in aqueous solutions of urea at body temperature?

As the first step in studying the problem of cyanate formation in the organism, experiments were carried out with the sole aim of establishing whether cyanate was still spontaneously formed in aqueous solutions of urea at body temperature. This, as mentioned above, was previously denied by WERNER.

[1] Medical School, Department of Pharmacology, Birmingham.

[2] J. WALKER and F. J. HAMBLY, J. Chem. Soc. *67*, 746 (1895).

[3] F. WÖHLER, Ann. Chim. Phys. *37*, 330 (1828).

[4] E. A. WERNER, The Chemistry of Urea (London, Longmans, Green & Co., 1923).

[5] L. SALKOWSKI, Hoppe-Seyl. Z. *1*, 1 (1874). – F. HOFMEISTER, Arch. exp. Path. Pharmak. *37*, 426 (1896). – W. R. FEARON and E. G. MONTGOMERY, Biochem. J. *18*, 576 (1924).

[6] H. A. KREBS and K. HENSELEIT, Hoppe-Seyl. Z. *210*, 33 (1932). – H. A. KREBS, Ergebn. Enzymforsch. *3*, 247 (1934).

[7] E. G. MONTGOMERY, Biochem. J. *19*, 71 (1925).

[8] R. BADER and F. SCHÜTZ, unpublished experiments.

[9] M. NICLOUX and G. WELTER, C.R. Acad. Sci., Paris *174*, 1733 (1922).

[1] C. VOEGTLIN, J. M. JOHNSON, and H. A. DYER, J. Pharmacol. *27*, 467 (1926).

[2] B. E. HOLMES and E. WATCHORN, Biochem. J. *23*, 199 (1929).

[3] F. VOIGT, Arch. exp. Pathol. Pharmak. *164*, 215 (1932).

[4] A. HALLER, Ann. Chim. (Phys.) *9*, (6), 275 (1886). C.R. Acad. Sci., Paris *102*, 974 (1886).

[5] R. BADER, D. J. DUPRÉ, and F. SCHÜTZ, Biochim. biophys. acta *2*, 543 (1948).

The isomeric change in question could, theoretically, be demonstrated by showing the gradual decrease in the amount of urea originally present in the solution. Since, however, even at high temperature only a small percentage of urea isomerizes into ammonium cyanate, the expected decrease of urea would probably be near or within the margin of error of the most sensitive quantitative methods for the determination of this substance. On the other hand the existing methods for the determination of cyanate[1] were found to be too insensitive, especially when urea was present in higher

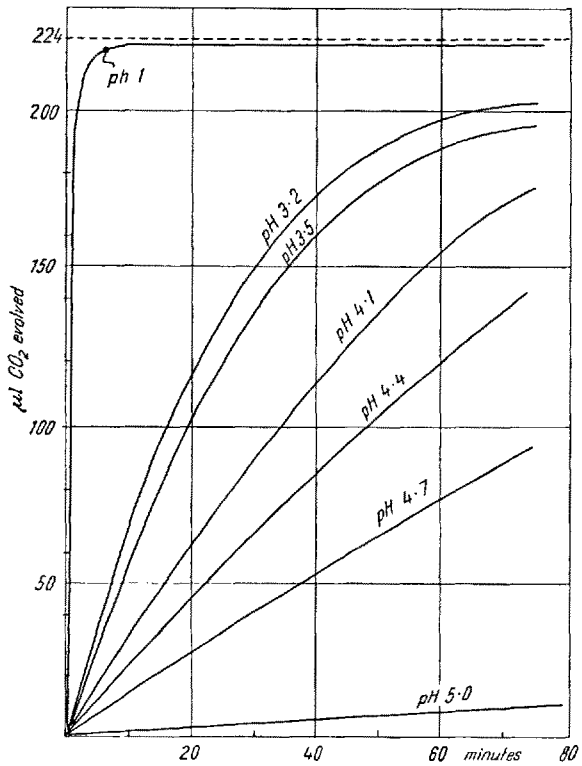

Fig. 1. – *Decomposition of cyanate at various* p_H. 0·2 ml 0·05 *M* NaCNO was added from the side-arm to 2·5 ml buffer solutions (0·8–3 *M*) of various p_H, contained in the main-compartment of Warburg vessels at 18° C (DIRNHUBER and SCHÜTZ[2]).

concentrations. Two new and highly sensitive methods for the detection and quantitive determination of cyanate in aqueous solutions were developed.

Spectroscopic method. In the course of a study of the reaction of cyanate with blood pigments it was found that cyanate combined readily with methæmoglobin. When a sufficient amount of cyanate is added to methæmoglobin, the α-band, normally at 6325 Å, is shifted to 6285 Å; the absolute intensity of light absorption at these wave-lengths is also much increased[3]. Intermediate readings between the two extremes (6325 and 6285 Å) can only be made by means of small dispersion spectroscopes, most conveniently by means of a Hartridge reversion spectroscope. Urea, even in *M* solution does not alter the spectrum of methæmoglobin. Neither is the development of the spectrum of cyanate- methæmoglobin inhibited by the presence of urea.

Manometric method. It is known that cyanate decomposes into CO_2 and NH_3 on addition of acids. DIRNHUBER and SCHÜTZ[1] studied the acid decomposition of cyanate by means of the Warburg technique. Buffer solutions of different p_H were added to a cyanate solution and the amount of CO_2 evolved was measured manometrically. They found that weak acidification ($p_H > 5$) at room temperature resulted in very slow hydrolysis of cyanate. Only below p_H 5 was the decomposition more rapid (Fig. 1).

Since carbonate is very rapidly decomposed at p_H 5, where cyanate is relatively stable, both substances could be determined in the same experiment by adding first a buffer solution of p_H 5 from one side-arm and, immediately after renewed equilibration, a strong acid from the other side-arm. The amounts of CO_2 evolved after each addition were recorded separately; the respective concentrations of carbonate and cyanate were calculated therefrom.

Evidence was obtained by both methods, that contrary to the opinion of earlier workers, cyanate is formed in pure aqueous urea solutions, at p_H 7·0–7·4, at body and even at room temperature. The transformation urea → ammonium cyanate is very slow at the low temperatures.

Equilibrium at 38° is reached only after several days. After 27 h at 38° only about $^1/_3$ was formed of the amount formed after 7 days. The amounts of urea found to be converted into cyanate, at equilibrium, vary according to the initial concentration of urea in the solution. A 0·28 *M* urea solution contained 0·75% of the amount of urea initially present as cyanate, while only a fraction of this percentage was found in solutions of a higher initial urea concentration.

It thus became clear that, in aqueous solutions at physiological temperature and p_H, urea undergoes spontaneous isomerization into ammonium cyanate. It became therefore more probable, that this reaction should also take place in the body.

The distillation of cyanic acid from aqueous solutions of cyanate and from tissues

Both, the spectroscopic and manometric methods for the determination of cyanate were suitable for pure aqueous solutions of urea, but considerable difficulties were encountered in trying to apply these methods to biological material. Another method was therefore developed.

Distillation *in vacuo* of cyanic acid, set free from aqueous solutions of cyanate through acidification, was apparently never carried out successfully. This is due to the fact that, on acidification, cyanate is known to decompose rapidly into CO_2 and NH_3.

[1] A. T. HERTIG, Z. angew. Chem. *14*, 585 (1901).
[2] P. DIRNHUBER and F. SCHÜTZ, Biochem. J. *42*, 628 (1948).
[3] F. SCHÜTZ, Nature, Lond. *155*, 759 (1945). – R. BADER, P. DIRNHUBER, and F. SCHÜTZ, Biochim. biophys. acta, in the press (1949).

[1] P. DIRNHUBER and F. SCHÜTZ, Biochem. J. *42*, 628 (1948).

Since, however, cyanate was found to be relatively stable at p_H 5[1], it seemed possible that distillation was hitherto unsuccessful, not because mild acidification *in vacuo* would necessarily cause complete decomposition of the liberated cyanic acid, but because cyanic acid was lost on condensation in an aqueous milieu, through decomposition and polymerization. On this assumption DIRNHUBER and SCHÜTZ[2] developed a new technique by which cyanic acid could successfully be distilled.

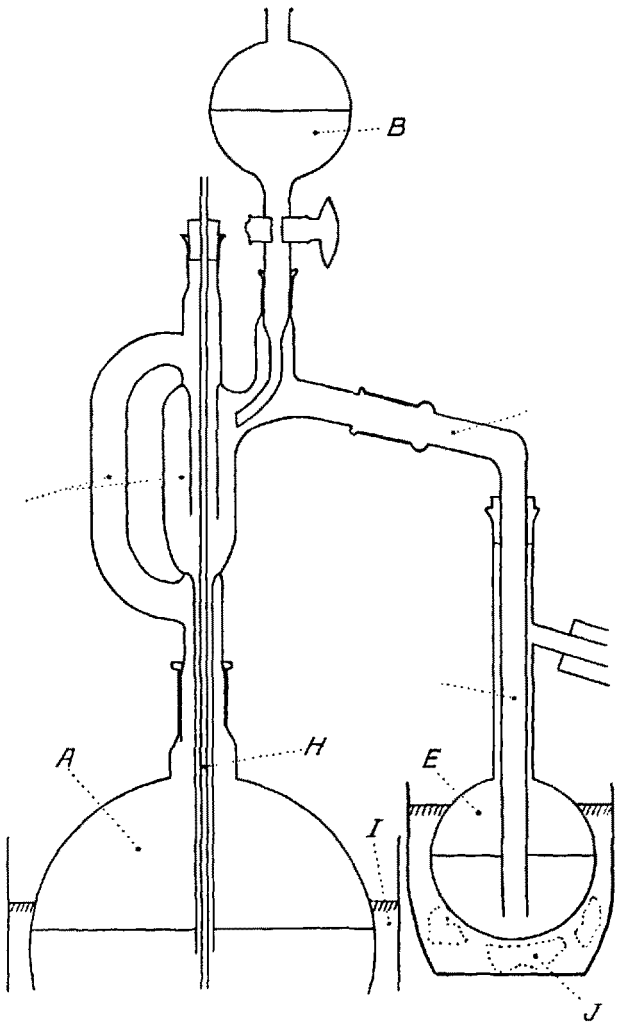

Fig. 2. – Distillation of cyanic acid by means of acidification to p_H 5 in a high vacuum. Condensation of HCNO in NaOH at 0°. For explanation see text (DIRNHUBER and SCHÜTZ[2]).

By means of the arrangement shown in Fig. 2, aqueous solutions of pure sodium cyanate were brought to p_H 5·0–5·3 in a high vacuum. Without special arrangements for condensation, the acid undergoes rapid and complete decomposition in a neutral aqueous medium, even at 0°. If, however, the distilled acid was condensed in 0·1–0·2 *N* sodium hydroxide at 0°, varying amounts were transformed into the more stable sodium cyanate, thus:

$$HCNO + NaOH = NaCNO + H_2O \quad (1)$$

Sodium cyanate present in the receiver was converted into urea by incubating with an excess of ammonium ions, thus:

$$2\,NaCNO + (NH_4)_2\,SO_4 = Na_2SO_4 + 2\,(NH_2)_2CO \quad (2)$$

Yields of 30–37% of the equivalent urea were regularly obtained from distillations under optimal conditions.

A part of the cyanic acid was hydrolysed during the procedure, yielding one mol of ammonia which, with another mol of cyanic acid, gave one mol of urea, thus:

$$HCNO + H_2O = CO_2 + NH_3 \quad (3)$$

$$HCNO + NH_3 = NH_4CNO \rightarrow (NH_2)_2CO \quad (4)$$

Whilst two mols of cyanate yield one mol of urea according to (3) and (4), only one mol of cyanate is needed to yield one mol of urea according to (1) and (2).

Some urea is therefore found in the distillate on mild acidification (p_H 6), even without previous incubation with ammonium ions. On mere acidification usually one-fifth of the amount was found, which was obtained after incubation with ammonium ions. Thus, still less than theoretically to be expected was obtained from the samples which were incubated at p_H 6 in the absence of NH_4^+. This must be ascribed to increased amounts of cyanate having been hydrolysed.

When the above-mentioned method of distillation of cyanic acid was applied to biological material[1], it was soon found that, if at all, the amounts of cyanate present were very small. Larger quantities of brain from freshly killed cows, etc., were ground with sand. This was done uniformly, but not too thoroughly in order to keep a fair amount of cells undamaged. The ground material, in Krebs-Ringer solution (5:1 v/v), containing bicarbonate and glucose, was shaken in a Warburg apparatus at 38° under physiological aerobic conditions; a stream of oxygen, containing 5% CO_2, was bubbled through the mixture. After 1–3 h the material was subjected to the high vacuum distillation as described above. Significant amounts of urea were found in the distillate after the latter was incubated with NH_4-ions.

A number of control experiments excluded the possibility of the yields being due to "entrained" urea, carried over in minute droplets. Artificial production, under the conditions of the experiment, of the urea-yielding volatile substance was also excluded. No yields were obtained from liver tissue under conditions which gave regular yields from brain tissue. These

[1] P. DIRNHUBER and F. SCHÜTZ, Biochem. J. *42*, 628 (1948).

[2] P. DIRNHUBER and F. SCHÜTZ, Biochem. J. *41*, liv (1947); Biochim. biophys. acta *2*, 362 (1948).

[1] P. DIRNHUBER and F. SCHÜTZ, Biochim. biophys. acta *2*, 522 (1948).

negative results seem to increase the significance of the positive results obtained with brain tissue, although the yields from brain tissue were also very small.

Through a number of experiments it was shown that it could have been no other volatile substance than cyanic acid which was distilled from the brain tissue, yielding urea in the distillate. That it was indeed urea which was found in the distillate was ascertained by incubating one half of the distillate with crystalline urease, when a complete blank was obtained.

No other substance than cyanic acid is known which, distilled under the described conditions (p_H 5 and at a temperature $< 10°C$), would, after condensation in NaOH at 0°C, yield urea in a mildly acid medium p_H 6 at relatively low temperatures (40–60°C), and in short periods of time (1–3 h).

Also the following peculiar characteristics of the volatile substance, distilled from surviving brain tissue, are identical with those of cyanic acid:

(1) The volatile substance could not be distilled when the acidification of the tissue was omitted ($p_H > 7$), nor after strong acidification (p_H 1). At p_H 5·0–5·3 regular yields were obtained. In the first case cyanic acid would not be set free from cyanate; in the second, it would be hydrolysed.

(2) The volatile substance was "lost", when the vapour was condensed in acid or water, even at 0°C.

(3) None of the volatile substance could be distilled from brain tissue which was previously incubated with an excess of ammonium ions. The latter, in excess, would rapidly convert into urea the greater part of cyanate present.

(4) 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 tissue suspensions.

Significant, though still smaller yields were obtained from brain tissue, frozen in liquid air soon after the death of the animal. Possible losses of cyanate through isomerization into urea at low temperatures are discussed by DIRNHUBER and SCHÜTZ[1].

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

Incubation of tissue with sodium cyanate increased the yields only when very large quantities of cyanate were added. Small amounts seemed to react rapidly with some constituent of the tissue, thus becoming unavailable for distillation. Similarly, the urea concentration had to be increased to about 100 times the physiological concentration before the tissue suspension was incubated for 3 h, until the yields were significantly increased.

It cannot be stated whether the found amounts of cyanate can be derived from urea. This would certainly seem a probable explanation, especially, since HOLTHAM and SCHÜTZ[1] (see below) were able to show that the reaction urea → ammonium cyanate does proceed in serum under physiological conditions. Obviously, experiments with isotopes are indicated.

It would seem premature to speculate on the amount of cyanate present in incubated brain tissue. Probably losses occurred during the adopted procedures. The experiments are believed to show that very small amounts of cyanate were present but, in view of the highly reactive nature of this substance, it seems more probable that cyanate is removed about as quickly as it is made. The question of the amount of cyanate actually present, and how much could be intercepted by distillation, seems of less interest than its actions and reactions.

Actions of cyanate

BIRCH and SCHÜTZ[2] found the lethal dose of pure sodium cyanate for 50% of a colony of rats (L. D. 50) as high as 310 mg/kg. A marked hypnotic effect was observed which could be recorded by means of suspended cages[3], after a dose of 40–50 mg/kg was given just before the hours of their normal wakefulness after sunset. No narcosis, however, could be produced. Higher doses even showed an increase in excitability and reflexes, and convulsions.

Medium doses were also followed by a marked diuresis in rats and rabbits[2,4].

To human beings the injection of cyanate in effective doses can only be given intravenously, since a 5% solution is hypertonic and its injection painful. Using an isotonic solution, the volume would become too large. 2–4 g in 5–7% solution were given intravenously to more than 50 human beings without any untoward effect. A short-lasting blindness or dimming of vision was regularly observed during the injection. No subject complained about it. Often a mild and transient drowsiness was observed which consisted of a slight alteration in speech and facial expression, of which the subject was usually unaware. Both effects lasted a few seconds only.[5] The diuretic effect in man is small and sometimes absent[6].

[1] P. DIRNHUBER and F. SCHÜTZ, Biochim. biophys. acta, *2*, 522 (1948).

[1] S. B. HOLTHAM and F. SCHÜTZ, Biochim. biophys. acta, *3*, 65 (1949).

[2] K. M. BIRCH and F. SCHÜTZ, Brit. J. Pharmacol. *1*, 186 (1946).

[3] F. SCHÜTZ, J. Physiol. *105*, 20, P (1946).

[4] F. SCHÜTZ, J. Physiol. *105*, 17, P (1946).

[5] M. B. MILLINGTON and F. SCHÜTZ, in preparation.

[6] R. F. A. DEAN, Department of Experimental Medicine, Cambridge. Personal communication to the author (1947).

The hypnotic action, so obvious in rats, is practically absent in man. In this respect cyanate resembles urethane which, as is well known, is a potent narcotic for some animals but not for man. Cyanate also shares another property with urethane, i.e. an antimitotic action (see below).

The injection of medium and large doses into rats and rabbits is followed by lachrymation, salivation, an extreme narrowing of the pupil, diarrhœa, diuresis and drowsiness. The dimming of vision, observed in man, may be due to the extreme miosis[1]. Some effects of cyanate are reminiscent of those of cholinergic drugs, but since the doses needed to produce these effects are rather large, it seems uncertain whether these actions have physiological significance.

Only very large doses of cyanate had any action on the heart and circulation. Respiration is stimulated in the decerebrated cat and the blood pressure is slightly raised after $^3/_4$–$^4/_5$ of the lethal dose. The blood pressure of rabbits (urethane) fell slightly after a similar dose. Cyanate depressed the isolated mammalian heart slightly and stimulated the isolated guinea-pig uterus a little more than the isolated gut of the same animal. After a lethal dose the respiration stops before the heart. Many of these effects seem to be non-specific and due to osmotic or p_H changes.

A small, transient rise of the blood sugar and a small, transient fall of body temperature was observed after single large doses in rabbits. After daily injections of small doses, given for 8–21 days, the blood sugar and body temperature were both low, but within normal limits[2]. A marked antipyretic effect of cyanate was observed in rats and rabbits after production of fever, following injections of milk and yeast. No analgesic action could be ascertained in rats[3].

On the ground of a number of experiments it was concluded that the observed actions were due to the CNO-group, and not to any substance into which cyanate may conceivably be transformed in the body (urea, ammonia or thiocyanate). Reduction, in the body, to cyanide was excluded[2].

Antimitotic action

When sodium cyanate was given to growing rats and cats in daily doses of 40–70 mg/kg the most obvious effect was a complete standstill of growth (see Fig. 3). The cat whose growth was stopped through cyanate was otherwise apparently not greatly affected. It ate less than the control, and did not move about much. No diabetes developed. The blood picture was within the normal range, only the erythrocyte-count was sometimes slightly raised; this was probably due to the rather marked dehydration which followed the cyanate diuresis[1]. Macroscopically, the complete absence of fat and a slight congestion of the kidneys were noted. Histological examination of the organs is in progress.

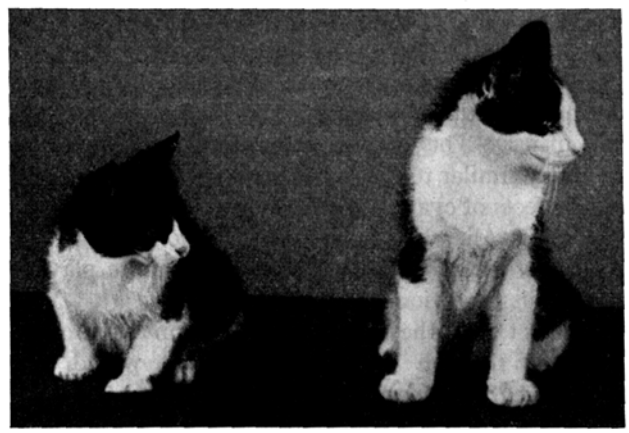

Fig. 3. – *Two cats of the same litter.* The smaller one received daily intramuscular injections of sodium cyanate (50–70 mg/kg) over a period of 52 days. The larger cat received simultaneously a similar volume of 0·9% NaCl over the same period. Growth was practically arrested in the former (MILLINGTON and SCHÜTZ[2]).

When the hypnotic and growth-delaying actions of cyanate were found, the author suggested to Professor A. HADDOW, Director of the Chester Beatty Cancer Research Institute, London, that cyanate may have an antimitotic action. P. DUSTIN, in a paper from the above mentioned Institute[3], reported that cyanate had indeed a very pronounced antimitotic activity.

Cyanate seems the first natural constituent of the body, capable of poisoning mitosis. It is of very low toxicity, did not greatly diminish the number of leucocytes, but had a marked antimitotic action on intestinal cells[2].

In case a mechanism exists, capable of regulating mitoses, partly by means of a "physiological antimitotic substance", cyanate would probably be connected with this mechanism. Besides its antimitotic action, it combines two further properties which both have been discussed in connection with cell division: (1) It reacts with SH-groups, (2) it is capable to a certain degree of counteracting denaturation (see below). RAPKINE[4] was the first to draw attention to the now widely discussed connection of the unmasking of SH-groups and denaturation on the one hand and mitosis on the other.

[1] I am greatly indebted to Professor I. MANN, professor of ophthalmology in the University of Oxford, for having confirmed this fact, and for invaluable advice with regard to the effects of cyanate on vision.

[2] K. M. BIRCH and F. SCHÜTZ, Brit. J. Pharmacol. *1*, 186 (1946). – M. B. MILLINGTON and F. SCHÜTZ, in preparation.

[3] M. MUSTAFA and F. SCHÜTZ, in preparation.

[1] K. M. BIRCH and F. SCHÜTZ, Brit. J. Pharmacol. *1*, 186 (1946). – M. B. MILLINGTON and F. SCHÜTZ, in preparation.

[2] M. B. MILLINGTON and F. SCHÜTZ, in preparation.

[3] P. DUSTIN, Nature, Lond. *159*, 794 (1947).

[4] L. RAPKINE, Ann. Physiol. Physicochim. biol. *7*, 381 (1931); J. Chim. phys. *33*, 493 (1936); *34*, 416 (1937).

Antimitotic action and ionizing radiations. A. P. DUSTIN[1] pointed out the remarkable similarity of the action of mitotic poisons with those of ionizing radiations (*radiomimetic* substances). Since then the idea has been discussed that radiations may cause the formation of an antimitotic substance (P. DUSTIN[2]). Whether radiations are capable of catalysing the isomerization urea cyanate is now being investigated.

Intoxication of uræmia and cyanate

It is well known that the symptoms of uræmia cannot be produced by raising in a normal animal the level of urea. The drowsiness produced by cyanate is strikingly similar to that existing in uræmia; since also other effects of cyanate were reminiscent of symptoms of uræmia (diarrhœa, miosis, convulsions, etc.), the possibility must be pointed out that cyanate plays a role in this condition.

The fact that the symptoms of uræmia cannot be produced through injections of urea, would not contradict the formation of cyanate in the body from urea, since the isomeric change, urea → cyanate, is a very slow process. Only after many days was equilibrium reached at 38°C in pure aqueous solutions of urea[3]. When the concentration of urea is raised in a normal organism to the level existing in uræmia, the excess amounts are excreted before a substantial quantity of cyanate could be formed.

Cyanase. Obviously, not only an increased production of cyanate, whether derived from urea or not, may lead to an accumulation of this substance, but also its impaired destruction or elimination. The recently described enzyme, cyanase, present in liver, kidney, and erythrocytes, which catalyses the hydrolysis of cyanate[4], seems of interest in this connection.

Mitotic poisons in uræmia

ZYLBERSZAC[5] found that bilateral nephrectomy was followed by an arrest of mitoses. This fact, as was also pointed out by P. DUSTIN[6] is relevant in connection with our supposition that cyanate may be responsible for some of the symptoms in uræmia. In two mice, which survived the operation for 25 and 29 h, P. DUSTIN[6] found the intestinal mitoses not affected. He concluded that this result did not suggest that cyanate accumulated "as a consequence of acute renal failure". Since however, the transformation urea → cyanate is a very slow process, probably more than *acute* renal failure is needed to promote sufficient amounts of cyanate to be formed. It would have been indeed surprising if, during 25–29 h following nephrectomy, a substantial amount of cyanate were formed. Further work is needed to ascertain which role cyanate plays in uræmia, and if indeed bilateral nephrectomy produces the same uræmic state, as when diseased kidneys are present.

Many cases exist with high blood urea but without marked uræmic symptoms; other cases with low blood urea may display marked symptoms. This suggests that, if some of the symptoms are due to cyanate, an accumulation of the latter substance may also be due to other factors beside the rise of urea in blood and tissues.

The reaction of cyanate with proteins. The stabilizing effect of cyanate against denaturation

Cyanate lost its hypnotic and diuretic activity in rats, when previously incubated with serum or serum proteins. This loss could not be ascribed to catalysed hydrolysis of cyanate into NH_3 and CO_2. No gas was evolved or taken up, during incubation of serum with sodium cyanate. Moreover, serum had no cyanase activity[1]. A direct reaction with serum proteins was assumed ("bound cyanate").

Other indications for such a reaction were as follows. Addition of cyanate very markedly raised the temperature and time of heating needed for the heat coagulation of serum; it protected proteins, to a certain degree, against the action of various precipitating

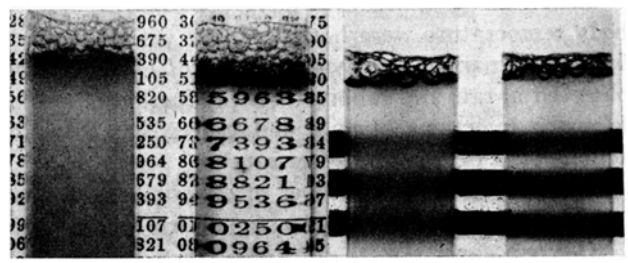

Fig. 4. – Serum (diluted 1:1 with 0·9 % NaCl), containing a final concentration of 0·14% mercuric chloride in tubes *1* and *2*, and a final concentration of 23% alcohol in tubes *3* and *4*. These additions caused much less precipitation of proteins when sodium cyanate was present in a final concentration of 0·025–0·072 *M* (tubes *2* and *4*), than in the control tubes (*1* and *3*), to which a molecular equivalent of NaCl was added instead of NaCNO. Cyanate was similarly found to stabilize protein solutions against a number of other agents, like acids, heat, urea, etc. (HOLTHAM and SCHÜTZ[2]).

agents. If added in sufficient quantity cyanate kept many proteins in solution which otherwise precipitated on heating or on addition of salts of heavy metals, etc.[2] (see Fig. 4). Cyanate also increased the ultrafiltration rate of diluted buffered solutions of proteins.

Cyanate was also found to react with a number of hæmoglobin derivatives, causing dispersion and

[1] A. P. DUSTIN, C.R. Soc. Biol. *93*, 465 (1925); Arch. Anat. micr. *25*, 37 (1929); Le Sang *12*, 677 (1937); Arch. exp. Zellforsch. *22*, 395 (1939).
[2] A. P. DUSTIN, Nature, Lond. *159*, 794 (1947).
[3] P. DIRNHUBER and F. SCHÜTZ, Biochem. J. *42*, 628 (1948).
[4] S. B. HOLTHAM and F. SCHÜTZ, Exper. *4*, 398 (1948).
[5] S. ZYLBERSZAC, C.R. Soc. Biol. *115*, 411 (1934).
[6] A. P. DUSTIN, Nature, Lond. *159*, 794 (1947).

[1] M. B. MILLINGTON and F. SCHÜTZ, in the press (1949).
[2] S. B. HOLTHAM and F. SCHÜTZ, Biochim. biophys. acta *3*, 65, (1949).

solution of these pigments, as well as reinforcement and shift of absorption bands. When a dialysed solution of methæmoglobin is incubated at 38° the pigment soon aggregates and precipitates. Cyanate-methæmoglobin, however, remained completely dispersed and in solution[1].

The question arose whether the action of cyanate against precipitation or coagulation, following denaturation could be ascribed to one or more of the following reasons: (1) to a faculty of cyanate to redissolve denaturated protein, (2) to prevent the denaturated protein from being precipitated, or (3) whether denaturation itself was counteracted by cyanate. While it was found that cyanate promoted the slow solution of heat denaturated protein in water[2], the following experiments suggested that cyanate directly interferred with the process of denaturation (alcohol, etc.). From the aspect of the precipitate, e.g. after addition of ethanol, in just sufficient concentration to cause the formation of a precipitate, it also appeared that cyanate counteracted the dehydration of the protein. The supernatant contained about 5–10 times the amount of dissolved protein, when cyanate was present, than in the sample without cyanate which, however, contained the same amount of ethanol[2].

When serum albumin was incubated at 4°C for 18 h in the presence of 6 *M* urea, complete denaturation took place, and no protein-crystals could be obtained. When, however, 1–2 h before the addition of urea, cyanate was added (final concentration 0·1 *M*), protein crystals were obtained.

This shows that cyanate is in fact capable of counteracting denaturation itself, not only precipitation. The crystal habit of the proteins which resisted various denaturing agents, or procedures (alcohol, urea, heat, surface denaturation, etc.) is now under investigation, as well as the stabilizing action of cyanate on certain enzymes.

Isomerization urea → cyanate in serum

The effect of cyanate on heat coagulation was used to investigate whether the reaction: urea → NH_4CNO does take place in serum under physiological conditions.

Since the isomerization is known to proceed more rapidly with increasing temperature, and to be practically at a standstill at 4°C, a small amount of urea was added to the serum (0·08 *M* in excess of that naturally present), and one half of the sample was cooled to 4°C, the other brought to 38°C. After 40 h incubation at these temperatures, both halves were brought to room temperature, and then immersed into a water bath at 70·8°C. When, in a similar experiment *no* urea was added in addition to that naturally present, no difference was observed in the behaviour towards heat of the two samples, previously incubated at 4 and 38°C respectively.

When urea was added as described above, a very marked difference was regularly observed. E. g., the sample, previously kept at 4°C became very opalescent after 9 min. and was completely coagulated after 15 min. The other half, previously kept at 38°C, became slightly opalescent after 15 min. and coagulated after 22 min. Even then the clot was less firm and more transparent than that of the half kept at 4°C. *The 38°C-sample behaved exactly as if cyanate had been added before heating.*

Urea, in the low concentrations used in these experiments, did not directly influence the heat coagulation of serum. If incubated at 38°C with serum for 40 h, it markedly delayed heat coagulation.

Thus, neither the addition of urea alone, nor the incubation at 38°C, altered the stability of serum proteins towards heat. A small excess of urea *and* incubation, however, produced this change. The most probable explanation for this effect is that cyanate was isomerized from urea, on prolonged incubation in serum, with all the consequent characteristics of enhanced stability towards heat[1].

These experiments strongly suggest that the reaction "urea → ammonium cyanate" does proceed in serum under physiological conditions. Some proteins, in catching cyanate, may alter the urea-cyanate equilibrium, in favour of cyanate.

Nothing can be said yet about the precise nature of the reaction of cyanate with proteins. Cyanate was found to react readily with amino- and SH-groups of a number of amino acids, between p_H 5 and 8[2]. Probably a reaction takes place similarly to the known reaction of isocyanate with the amino groups of proteins, thus:

$$R{-}CNO + H_2N\text{-protein} \rightarrow R{-}NH{-}CO{-}NH\text{-protein}$$

Antigen-antibody reactions

Since antigen-antibody reactions are highly specific, the reaction of cyanate with proteins was thought to make possible an interference with these reactions *in vivo*.

Cyanate protected guinea-pigs, to a certain extent, against anaphylactic shock, but the protection was not absolute. When the shock dose was given soon after the cyanate injection, the latter was not, or nearly not, effective. Several cyanate injections and an interval of

[1] R. BADER, P. DIRNHUBER, and F. SCHÜTZ, in the press.
[2] S. B. HOLTHAM and F. SCHÜTZ Biochim. biophys. acta, *3*, 65 (1949).

[1] S. B. HOLTHAM and F. SCHÜTZ, Biochim. biophys. acta, *3*, 65 (1949).
[2] S. B. HOLTHAM and F. SCHÜTZ, Biochim. biophys. acta in the press (1949).

about 2·5–5 h between the last cyanate and the shock dose, reduced the number of deaths and symptoms significantly. The anaphylactic reaction *in vitro* (DALE Experiment), however, was not altered, neither was the effect of histamine; but the precipitin reaction was suppressed by traces of cyanate[1]. Similarly the typhoid-O-agglutinin reaction was suppressed by cyanate[2].

Further analysis, with various antigens, and varying the doses and times of cyanate injections, may show whether the effect *in vivo* was due to the weak sedative action of cyanate in guinea-pigs, since narcosis is known to prevent anaphylactic shock; or whether the assumed reaction of cyanate with some of the proteins involved, plays a role. Its "point of attack" would obviously differ from that of antihistamine drugs.

Actions on enzymes

Cyanate had a slight depressing effect on the respiration of brain slices, but still less on liver and kidney slices. It combined with cytochrome a_3. Urease was not inhibited, except in very high concentrations. Serum cholinesterase was not inhibited; it was even made more stable. This investigation is being continued.

Actions against various poisons in vivo

Spectroscopically, it was found that cyanate had a greater affinity for the iron of methæmoglobin, than fluoride[3]. If given before the fluoride, cyanate reduced the mortality of rats poisoned with fluoride. If given 1 h after the fluoride, the mortality was not significantly reduced[4]. Subsequently it was found that cyanate prevented the development of a number of symptoms in chronic heavy metal poisonings[5].

Further work is needed to analyse the mechanism of these effects. These may be due to one, or more, of the following reasons: (1) A complex-formation of cyanate with the heavy metal. (2) The stabilizing effect of cyanate on proteins, as observed with agents (heat, alcohol, urea, surface-denaturation, etc., see Fig. 4) of which a direct reaction with cyanate could be excluded. (3) The diuretic effect of cyanate which may have aided elimination of the toxic substance.

Thiocyanate

Some effects of cyanate could be reproduced by thiocyanate, although cyanate was always more potent. P. DUSTIN[6] found that also thiocyanate had a certain antimitotic action, though, also in this respect CNS′ was less potent than CNO′[1].

Discussion

Is cyanate a normal constituent of native proteins?

Many of the described actions of cyanate on proteins and tetrapyrrolic compounds show striking similarity with effects of caffeine. D. KEILIN[2] discovered that caffeine reacted with hæm, and J. KEILIN[3] studied the reaction of caffeine with proteins and hæmoglobin derivatives. This author summarized the following effects of caffeine under the heading of "caffeine effect": Dispersion and solution of a number of hæmoglobin derivatives, reinforcement and shift of absorption bands, prevention of spontaneous aggregation and precipitation. All these effects on methæmoglobin can be produced also by cyanate.

There are other striking similarities between the effects of cyanate and caffeine. Both stabilize proteins against heat; they increase the ultrafiltration rate of buffered, diluted protein solutions, and both are diuretic and antimitotic agents[4].

Also native proteins are known to have a pronounced dispersing effect on tetrapyrrolic compounds, causing reinforcement and shift of absorption bands, and inhibition of aggregation and precipitation[5]. J. KEILIN found that globin and serum proteins reacted with porphyrins in a similar manner as caffeine.

One possible explanation for this fact would be that native proteins may be normally in a combination with cyanate, or contain cyanate as an adsorbed admixture; the removal of cyanate, in the course of purification of proteins, may be connected with the changes in the physico-chemical behaviour, stability, etc., of protein solutions with progressing purification.

J. KEILIN investigated whether any of a considerable number of the amino acid contents of proteins was capable of causing the "caffeine effect". But none of those tested was found to produce the effect. One of the alternative explanations put forward by J. KEILIN was that "the proteins may contain an additional, not yet isolated constituent". It is possible that cyanate is this constituent.

There remain other possible explanations, e.g. similar to J. KEILIN's explanation in the case of the caffeine effect of proteins, that amino acids in their

[1] M. MUSTAFA and F. SCHÜTZ, in preparation.
[2] K. A. BISSETT and F. SCHÜTZ, in preparation.
[3] R. BADER, P. DIRNHUBER, and F. SCHÜTZ, Biochim. biophys. acta in the press (1949).
[4] M. B. MILLINGTON and F. SCHÜTZ, in preparation.
[5] M. MUSTAFA and F. SCHÜTZ, in preparation.
[6] P. DUSTIN, Nature, Lond. *159*, 794 (1947).

[1] Since reports on the use of thiocyanate in hypertension are persistently appearing[4], it is regretted that it was not yet possible to arrange adequate clinical trials for cyanate. The antimitotic action, low toxicity, its stabilizing effect on proteins, its reactions with heavy metals, etc., and the fact that cyanate (as ammonium salt) is the isomer of urea, should suggest clinical trials in a variety of relevant conditions.
[2] D. KEILIN, Ergebn. Enzymforsch. *2*, 239 (1933).
[3] J. KEILIN, Biochem. J. *37*, 281 (1943); Nature, Lond. *154*, 120 (1944).
[4] R. E. FORSTER, Amer. J. Med. Sci. *206*, 668 (1943). – K. S. ALSTAD, Brit. Med. J. (7. Feb. 1948), p. 250.
[5] R. HILL and H. F. HOLDEN, Biochem. J. *20*, 1326 (1926). – F. HAUROWITZ and H. WAELSCH, Hoppe-Seyl. Z. *182*, 82 (1929).

natural configuration as polypeptides, may have properties similar to cyanate, without the latter group being actually incorporated in the molecule.

Physiological significance

If a substance, not foreign to the body, is found to have certain pharmacological actions, it would appear that those of its effects have physiological significance which can be produced by the smallest doses. In the case of cyanate, its reaction with proteins and amino acids would thus seem to have physiological significance, since it is obvious with very small amounts of cyanate.

In this connection the question becomes of interest whether the reactions of cyanate with proteins, etc., are reversible. So far, only the combination of methæmoglobin and cyanate was found to be reversible by dialysis against water at 4°C[1].

Relatively large doses are needed to produce the other pharmacological effects. 30–50 mg/kg are the minimum effective doses, producing the antimitotic, diuretic, and the weak hypnotic effect. Also the effect on O_2-uptake of tissue slices is achieved in relatively high concentrations only; the ratio of the molecular concentrations of cyanide and cyanate respectively which cause a similar depression of O_2-uptake, is of the order of 1:40.

If the above-mentioned argument of the smallest effective doses is applied, it would seem unlikely that the last mentioned effects should have physiological significance. It should, however, be considered that injected cyanate is probably reacting rapidly with a variety of substances in the body, and that, therefore, a small part only of the injected amount may reach the effector cells (kidney, for diuresis; intestinal cells, for antimitotic action, etc.). Moreover, injected cyanate is, at first, always outside cells; it may often not reach or penetrate into cells. Cyanate formed *within* cells, perhaps from urea, may have a far greater effect. It seems, therefore, that certain actions of cyanate should not be dismissed as being without physiological significance, because they can be produced with relatively large amounts only.

Since cyanate is a very reactive substance, it is probable that some of its effects are due to reactions with different constituents of the body. There are indications, however, that the reactions with proteins, with its various consequences, may be the underlying mechanism for a number of its pharmacological effects. It was mentioned above that, through RAPKINE's work[2], SH-groups and denaturation of proteins were linked with mitosis. The stabilizing effect of cyanate on proteins, and its reaction with SH-groups, may well be connected with its antimitotic effect.

[1] R. BADER, P. DIRNHUBER, and F. SCHÜTZ, Biochim. biophys. acta, in the press (1949).

[2] L. RAPKINE, Ann. Physiol. Physicochim. biol. *7*, 381 (1931); J. Chim. phys. *33*, 493 (1936); *34*, 416 (1937).

Similarly, another consequence of the reaction of cyanate with proteins may be connected with its diuretic effect: Cyanate increases the ultrafiltration rate of certain protein solutions, and altered the dehydration (denaturation) of proteins through alcohol.

The protein effect and that on mitosis seem to go parallel also in the case of thiocyanate. Both are less pronounced than those produced by cyanate.

Via the reaction with proteins and amino acids, cyanate seems capable of influencing fundamental functions of the organism. It probably plays a role in stabilizing proteins *in vivo*, and in the physiological regulation of mitosis; it may be a factor in the regulation of the water balance, and be a weak physiological depressor of cell respiration.

It seems of interest that it readily reacts with certain proteins *in vivo*, and yet is of very low toxicity. Finally, two facts should be considered in connection with all actions of cyanate: (1) That it is the simplest organic compound, capable of producing most of the above-mentioned effects. Some of its actions may perhaps be due to the CNO-group acting as a "competing analogue" of another naturally occurring group. (2) That the formation of cyanate, under physiological conditions from the ubiquitous urea in the warm blooded mammalian organism is highly probable.

Zusammenfassung

Mittels neu ausgearbeiteter spektroskopischer und manometrischer Methoden wurde gezeigt, daß sich noch bei 38° in wässeriger Lösung ein kleiner Prozentsatz Harnstoff in Ammoniumcyanat isomer umsetzt. Dies berechtigt zur Annahme, daß im Säugetierorganismus eine umgekehrte Wöhler-Reaktion stattfinden könnte. Nach Inkubation von Gehirnbrei unter physiologischen Bedingungen konnten kleine Mengen von Cyanat nachgewiesen werden. Da Cyanat eine sehr reaktive Substanz ist, sind wahrscheinlich normalerweise nur Spuren vorhanden.

In einigen Tierarten ruft Cyanat mehrstündigen Schlaf und Diurese hervor. Ähnlich dem Urethan hat es jedoch keine narkotische Wirkung beim Menschen. Seine antimitotische Wirksamkeit stellt eine weitere Ähnlichkeit mit Urethan dar. Die Möglichkeit, daß manche Symptome der Urämie durch Cyanat hervorgerufen werden, wird erwogen.

Cyanat reagiert mit Serumproteinen und mit Hämoglobinderivaten. Es schützt Pigmente und Proteine gegen eine Anzahl präzipitierender und denaturierender Lösungen oder Verfahren, z. B. gegen Hitze, Säuren, Schwermetallsalze, Harnstoff, Oberflächen-Denaturierung usw. Nach Absättigung der Aminogruppen reagiert Cyanat auch mit den SH-Gruppen der SH-Aminosäuren. Die Möglichkeit wird besprochen, daß Cyanat ein normaler Bestandteil nativer Proteine sei. Cyanat ist imstande, wahrscheinlich durch seine schnelle Reaktion mit Eiweiß, anaphylaktische Reaktionen *in vivo* zu beeinflussen.

Dem Cyanat kommt vermutlich physiologische Bedeutung zu, insbesondere da einige seiner Reaktionen umkehrbar sind, z. B. mit Methämoglobin. Cyanat ist höchstwahrscheinlich ein normaler Bestandteil des Körpers und zugleich die einfachste organische Verbindung mit den beschriebenen Wirkungen.