THE EFFECT OF CYANATE ON THE STABILITY OF PROTEINS

by

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MILLINGTON AND SCHÜTZ[1] recently found that the diuretic action of cyanate, as previously described by SCHÜTZ[2] and BIRCH AND SCHÜTZ[3], was abolished when cyanate was incubated with serum. This may have been due to an enzyme capable of destroying cyanate. This question is being investigated and will be discussed separately.

An alternative explanation for the loss of biological activity of cyanate on incubation with serum would be the binding of the former by serum proteins. A reaction of this kind seems probable because of the known great reactivity of cyanate, *e.g.*, with amino acids[4], methaemoglobin[5, 6] and cytochrome a_3[7].

This paper describes an effect of cyanate, consisting in stabilizing proteins against a number of denaturing agents or procedures.

HEAT COAGULATION

Methods and materials

Blood was taken from human volunteers, and the serum obtained in the usual way. Samples of serum were kept at 4° for not longer than 1–2 days. Before use it was centrifuged.

Serum proteins. A purified fraction of serum albumin was prepared as follows. Solid ammonium sulphate (250 g/litre) was added to fresh horse serum, and the precipitate rejected. A further 150 g/litre of ammonium sulphate were added to the supernatant and the precipitate dissolved in the smallest possible quantity of water. After dialysis against distilled water at 4° the lipoids of this solution were extracted by precipitating the proteins in ethanol below −15°, according to HEWITT'S[8] modification of HARDY AND GARDINER'S method. The ether washed precipitate was then extracted in a Soxhlet for 48 h with ether containing metallic sodium, both of which were once renewed. The extracted material, a white powder, was dried *in vacuo* until the sample no longer smelled of ether. The powder was then made up into a paste with water and dialysed at 4° for several days against frequent changes of distilled water. A precipitate of insoluble material was centrifuged off and rejected before use.

Sodium cyanate was prepared from urea according to BADER, DUPRÉ, AND SCHÜTZ[9]. Only freshly prepared solutions were used. Since higher concentrated solutions of sodium cyanate were very alkaline (p_H 8.0–9.0), the p_H was brought to that of serum in equilibrium with air (p_H 7.8–8.0), by the addition of a few drops of acetic acid or primary potassium phosphate. Equivalent amounts of the acid or potassium phosphate were added to the controls, using sodium bicarbonate instead of cyanate.

Ammonium cyanate was prepared as follows. A solution of sodium cyanate was precipitated by silver nitrate, the precipitate washed with cold water and acetone. A solution of ammonium chloride in equimolecular amounts was added to the dry powder, and the suspension shaken in a WARBURG shaker for 30 min at room temperature. The filtered solution was used at once to avoid greater losses through isomerization into urea.

Caffeine was recrystallized from alcohol. It was dissolved in serum or the protein solution, or used as caffeine citras, B.P.

p_H. The p_H of the solutions which were added to serum samples, was adjusted to that of serum. Where that was not possible, the p_H nearest to 7.8 was aimed at. Usually the small amounts of substances to be tested did not greatly alter the p_H of the serum. When, however, this was altered, a similar sample of serum was brought to the same p_H by addition of small amounts of acetic or citric acid, of phosphate buffer solutions, or of sodium bicarbonate, and used as control.

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

Bilateral nephrectomy. Both kidneys of rabbits were removed under ether anaesthesia by the usual dorsal approach. After the operation the animals were given no food, but plenty of water to drink. The animals usually survived 3 to 5 days, sometimes 7 days, if no food was given during the first 24 h after the operation. Later they spontaneously abstained from food. Blood, taken by cardiac puncture, was left 1 h at room temperature. The clot was then detached from the glass wall, left standing for further 1–3 h at room temperature, and centrifuged. The serum was again centrifuged after separation from the clot, and used within 4 h.

Addition of substances to serum. Whenever possible the substances to be tested for their action on proteins, were added to the serum or protein solutions, in solution, except when very high concentrations of caffeine and cyanate were added. In this case a part of the added quantities was dissolved directly in the serum. Urea was always added in solution; in order to prevent higher concentrations of urea from developing locally, the liquid was kept agitated while the urea solution was added drop by drop.

Determination of heat coagulation temperature. A number of procedures have been used to determine the effect of heat on serum proteins[10, 11, 12]. While some of these methods are undoubtedly of great sensitivity, they either are lengthy procedures, or not independent of individual factors of the observers.

The simple method described below, was developed solely for the purpose of this investigation. Although it is probably less accurate than some of the known methods, it allowed rapid and fairly accurate comparisons of the effect of different substances on samples of the same batch of serum. The method is independent of individual factors of different observers, and gave repeatable results. One determination took *circa* 15 min.

The principle of the method was to record the sudden change of heat conduction when the serum coagulated. The device is shown in Fig. 1. A test tube (*A*) containing serum, or serum protein solution, was placed in a groove of an electric heater (*B*). The heating coil was covered by a cement layer. A little sand, placed in the groove, assured uniform contact. The heated area of the test tube did not vary substantially in different experiments. A motor driven mixer of the glass ring type (*C*) and a thermometer (*D*) were placed into the test tube as shown in Fig. 1. Care was taken to immerse the thermometer to equal depths in all experiments. The mixer was lifted 1 cm high, and fell to the bottom of the tube by its own weight, 140 times/min. A circular draught excluder (*E*) was placed around the lower part of the test tube. The test tube was covered with loosely packed cotton wool The heating was arranged to bring the contents of the test tube from room temperature to 70° in *circa* 8–9 min. The heating current was kept constant by means of a resistance. The rate of heating varied very little. The current, for the heating and the mixer, was switched on when the serum was filled in and the arrangement was rigidly set up.

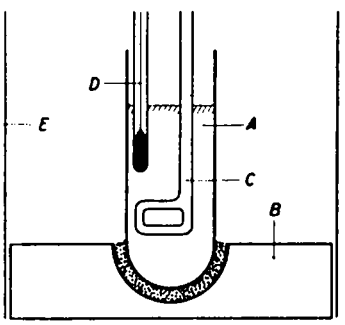

Fig. 1. Device used for the determination of coagulation-temperature and -time. The protein solution in test tube *A* is heated by the hot plate *B*, contact being made through sand. *C* = stirrer. *D* = thermometer. *E* = circular draught excluder. When coagulation occurs, the heat conduction from *B* towards the thermometer is reduced, causing a sudden fall in temperature, as shown by the heating curves (Figs. 2 and 4).

Temperature readings were taken at intervals of 15 sec and plotted against time. From a typical curve, shown in Fig. 2 it can be seen that the temperature suddenly fell, when coagulation occurred.

While the serum remained liquid, the heat supplied from the bottom of the tube, was evenly distributed by the mixer. When coagulation took place, the conduction of heat towards the thermometer was much decreased; thus the heat loss into the surrounding air from the thermometer was not any more made good by heat conduction from the heated area upwards. Hence the sudden fall of temperature.

To obtain the effect, the heating should be confined to one area only, and an appreciable heat loss should take place from the remainder of the surface. When heated in a waterbath no drop of temperature occurred. The drop of temperature seemed simultaneous with the sudden increase in viscosity. It was found best to place the thermometer near the wall, which caused a more rapid and sudden fall of temperature when coagulation occurred. The lower end of the thermometer was at a distance of 1.6 cm from the bottom of the tube. The same thick walled test tube was used for all experiments. 3.6 ml serum were added to the tube, and 0.2 ml, if not otherwise mentioned, of the various solutions tested for their activity. In control experiments a similar volume, containing equimolecular amounts of sodium chloride, or of a buffer solution, was added.

The period from the time when the serum reached 20°, till coagulation occurred, was read from

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the heating curve (Fig. 2). Obviously coagulation takes place at lower temperatures when the heating is prolonged, and vice versa. In Fig. 3 the temperature, when coagulation occurred, is plotted against the time needed to heat different samples from 20° until coagulation occurred. It can be seen that normal human sera showed small variations in this respect.

By addition to serum, or serum proteins, of a substance capable of stabilizing proteins against heat, both the temperature of coagulation is raised, and the time of heating lengthened. Obviously it was desirable to express the results with one figure only. Trials to heat at a constant temperature and record only the time needed for coagulation, gave such huge differences of coagulation times, due to the very great temperature coefficient of heat denaturation, that the above described method seemed more advantageous. *E.g.*, a serum sample without addition took 22 min to coagulate at 70.9°. Additions of cyanate increased the time to many hours or days, when heated at the same temperature. With the above described method, however, a result was obtained within 20 min. Moreover, the time needed to raise the temperature of the samples from 20° to the coagulation temperature ("heating time"), did not vary greatly, usually less than ± 20%.

By applying the following simple correction it was found possible to express the results contained in this paper with one figure only. The accuracy was sufficient for our purpose.

By extrapolating the rising and falling parts of the heating curve (see Fig. 2) a crossing point is obtained, indicating the coagulation temperature, and the heating time. *E.g.*, a serum sample coagulated at 72.8° with a heating time of 9 min 15 sec. On addition of the substance to be tested the same serum coagulated at 77.4°, with a heating time of 11 min 45 sec. Once it was assured that the coagulation temperature and heating time of the untreated sample corresponded well with the values usually obtained with normal sera (Fig. 3), the temperature corresponding to the heating time of 11 min 45 sec was obtained from the standard curve (Fig. 3), thus: 71.4°. The difference between this temperature and the temperature, when coagulation actually occurred (77.4°), is 6.0°, which, in this paper, would represent the result, *i.e.*, the rise of the heat coagulation temperature corrected for time of heating. If not otherwise mentioned, this correction was applied to the data of heat coagulation temperatures given in this paper.

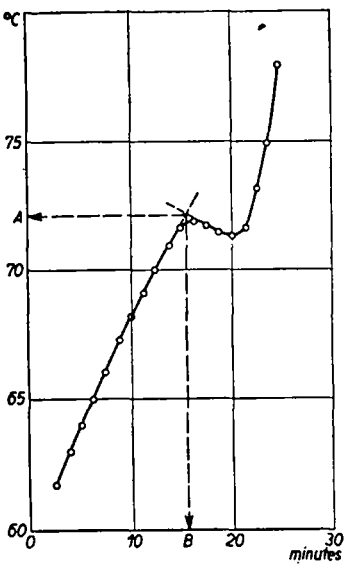

Fig. 2. Heating curve of serum. The serum was heated as shown in Fig. 1. Temperature readings were made every 15 sec. When coagulation occurred, a sudden fall of temperature was recorded. The coagulation-temperature (*A*) and coagulation-time (*B*) were obtained from the curve, after extrapolation of the rising and falling parts of the heating curve.

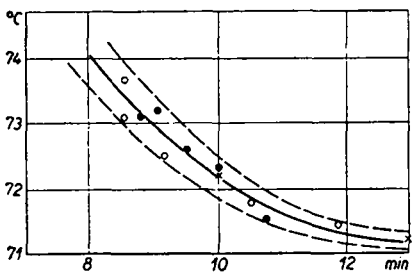

Fig. 3. Relation of the coagulation temperature to the time of heating needed to produce coagulation. Normal human sera.

When several determinations of the coagulation temperature were made on different serum samples the results showed a maximum variation of ± 0.35° from the standard curve (Fig. 3). The standard deviation from the graphically obtained mean for different sera was ± 0.18° (n = 11). with an extreme range ± 1.3° (n = 11).

Only such sera were used for tests, which normally gave coagulation temperatures within the limits shown by a dotted lines in Fig. 3. This was the case with most sera. It enabled the application of the correction for the time of heating to be applied by means of the standard curve, and made the construction of a new curve for each serum unnecessary.

EXPERIMENTAL

From typical results shown in Table I, it can be seen that cyanate was very potent in stabilizing proteins against heat coagulation.

With high concentrations of cyanate no coagulation occurred at all, even when the temperature reached boiling point. This result may, at least partly, be due to the

TABLE I

THE EFFECT OF THE ADDITION OF DIFFERENT SUBSTANCES ON THE HEAT COAGULATION OF HUMAN SERUM AND SERUM ALBUMIN

Final molar concentration in serum	Rise of heat coagulation temperature (corrected for time of heating), above that observed with a sample of the same serum, or protein solution, without addition °C
Sodium cyanate, 0.043	1.6, 1.8, 1.0, 2.2, 1.1
,, ,, 0.086	3.4, 4.0, 4.2, 5.0
,, ,, 0.166	8.0, 7.1, 8.2
,, ,, 0.191	no coagulation, 13.5
Potassium thiocyanate, 0.040	± 0
,, ,, 0.086	1.5, 1.3
,, ,, 0.166	2.8, 3.2
,, ,, 0.191	4.1, 4.6
Urea, 0.16	± 0
,, 0.19	± 0
,, 1.0	± 0
,, 3.0	2.2
,, 4.0	2.8
,, 6.0	no coagulation
Sodium cyanide, 0.16	± 0
Potassium ferricyanide, 0.16	1.8, 1.1
Urethane, 0.11	± 0
Pilocarpine, 0.10	1.8, 1.8
Picrotoxin, 0.08	± 0
Strychnine hydrochloride, 0.1	± 0
Caffeine, 0.10	2.8
,, 0.12	3.1, 3.5
Theobromine sodium acetate, 0.1	± 0, ± 0
Theophylline sodium acetate, 0.1	± 0, ± 0
Sodium salicylate, 0.1	2.5, 3.2
Final concentration in a purified fraction of serum albumin	
Sodium cyanate, 0.04	1.6, 2.0, 2.1
,, ,, 0.086	3.0, 3.1

fact that on heating a solution containing relatively large amounts of cyanate, the reaction soon becomes alkaline, unless the solution is very strongly buffered. Alkali is, of course, also capable of preventing heat coagulation of serum. When the p_H was controlled by adding a highly concentrated buffer solution, a very marked difference in the coagulation temperature of the sample and control was still observed. The addition of concentrated buffer tended to lower the heat coagulation temperature of the controls.

On several occasions the p_H of the clot was measured with a glass electrode, after the clot was cooled to room temperature. With final concentrations of cyanate in serum up to 0.04 *M*, the p_H was not much altered in comparison with the control, both being in the range of p_H 8.0. The p_H of serum containing higher concentrations of cyanate was higher, (0.2–0.8 units), than that of the control, although strongly buffered. When, however, a similar p_H change was produced in controls, through addition of sodium

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bicarbonate, the coagulation temperature was not as much affected as through cyanate. It must be concluded that the observed effect was due to cyanate, and not to a change of p_H.

Comparing the action of different substances on heat coagulation, no substance was found quite as active as cyanate at or near the usual p_H of serum, when the latter was in equilibrium with air (p_H 7.8–8.0). Caffeine and salicylate seemed nearly as active, thiocyanate, though still active, was much less potent than cyanate, salicylate and caffeine. Ferricyanide seemed slightly active, while cyanide had no influence at all (Table I).

Many effects of cyanate were found to be similar to those of caffeine. Not only have both substances a diuretic action[2, 3], but they are also strikingly similar in their reaction with haemoglobin derivatives. J. KEILIN[13, 14] drew attention to a group of effects of caffeine on a number of haemoglobin derivatives. This "caffeine effect" was characterized by the dispersion and solution, and by preventing the precipitation and aggregation of these pigments, and by the reinforcement and shift of the absorption bands. BADER, DIRNHUBER, AND SCHÜTZ[7] recently observed that cyanate was also capable of producing all these effects.

The action of cyanate on heat coagulation described above was therefore compared with the action of a number of other substances on heat coagulation, which were either found by J. KEILIN to produce the "caffeine effect", or were closely related to substances capable of producing this effect.

It seems remarkable, as can be seen from Table I, that caffeine raised the heat coagulation temperature nearly as much as cyanate, but that substances closely related to caffeine, like theobromine and theophylline, did not show any obvious activity in this respect. The only other substance found active in this regard, though much less than caffeine and cyanate, was pilocarpine, which was found by J. KEILIN also to show the "caffeine effect". Picrotoxin, strychnine, urethane, and uric acid had no effect whatsoever in the range of concentrations in which cyanate was very active.

Essentially the same effects were obtained with ammonium cyanate as with the sodium salt. Since, even at room temperature, appreciable amounts of the ammonium salt undergo isomerization into urea, the slightly less pronounced effects obtained with this salt are probably due to the fact that the solutions of ammonium cyanate, when used, are already less concentrated with regard to cyanate.

Aspect of the coagulum. When serum, with an addition of cyanate, was heated until coagulation occurred, the clot was of a very different appearance than that obtained from the control. While the coagulum of the control was firm, and somewhat brittle, that obtained from the same serum containing cyanate was more fluid and jelly-like than that of the control. It was, of course, more viscous than normal serum. In the sample containing cyanate the change in viscosity seemed to precede slightly the change in colour and turbidity. The sample containing cyanate appeared more yellow and almost transparent after coagulation, while the controls were greyish-white and quite turbid.

PREVENTION OF HEAT DENATURATION BY CYANATE

The effect of cyanate in raising the heat coagulation temperatures of proteins could be due to cyanate being capable (1) of preventing heat denaturation, or (2) of preventing the coagulation only of heat denatured protein, or (3) of redissolving heat coagulated

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protein. The following experiment was made to obtain information regarding the first of the above mentioned possibilities.

A solution of *M*-sodium cyanate, or *M*-sodium chloride respectively, was added to two samples of the same batch of horse serum (1:9 v/v). Since the addition of sodium cyanate caused a slight shift of the p_H, the serum sample containing sodium chloride was brought to the same p_H by careful addition of *N* NaOH (final p_H of both samples was 8.0–8.2). After standing for circa 1 h at room temperature, the 2 samples were immersed simultaneously into a water bath at 72°. Already after 3–4 min the control, containing sodium chloride, became very turbid and slightly more viscous. When the samples were removed after 8 min incubation, the control was nearly completely clotted, while the sample containing cyanate was quite fluid and transparent.

In order to ascertain whether and how much undenatured protein was left in the sample containing cyanate, the following procedure was adopted. Both samples were cooled in a freezing mixture and then brought to room temperature. Solid ammonium sulphate was added until saturation, the precipitate was filtered off and washed with a saturated solution of ammonium sulphate.

While the precipitate thus obtained from the control did not dissolve in water, appreciable amounts of the precipitate obtained from the sample containing cyanate proved to be readily soluble in water. The precipitates were suspended in water and, after thorough mixing, centrifuged and filtered. A clear supernatant liquid was obtained from the sample containing cyanate. The redissolved protein present in this solution could be re-precipitated with ammonium sulphate, and redissolved in water in the usual way.

The amount of redissolved protein was determined by precipitation with trichloracetic acid; the precipitate was centrifuged, twice washed with distilled water and dried at 105°. Unheated samples of serum, whether containing cyanate or chloride, showed the same amount of re-soluble protein (5.420 g dry weight/100 ml) after saturation with ammonium sulphate. 0.996 g/100 ml was obtained from heated serum containing cyanate, while no re-soluble protein was obtained from the heated control, containing sodium chloride.

Thus 18.4% of the amount of protein present was apparently protected from heat denaturation in the sample containing cyanate. Essentially the same results were obtained with solutions of purified fractions of serum albumin. It is now being investigated whether the "protected" protein in these heated solutions can be re-crystallized in the usual way, and whether its crystalline habit and other properties are identical with those of non-denatured protein.

UREA AND HEAT COAGULATION OF SERUM

EXPERIMENTS WITH SERA FROM ANIMALS AFTER BILATERAL NEPHRECTOMY

The addition of urea to serum had no effect whatsoever on the heat coagulation of the latter, in concentrations equimolar to those in which cyanate was highly active. It can be seen in Table I that *circa* 80–100 times the amount of urea was needed to produce an effect similar to that of cyanate.

It should be recalled that on heating aqueous urea solutions, considerable amounts of cyanate are formed through isomerization from urea. The rate of formation of cyanate greatly increases with temperature. Thus *circa* 5% of the urea present in a 0.1 *M*-solu-

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tion, are transformed into ammonium cyanate at 100° within 30 min [15]. At lower temperatures the equilibrium is attained much more slowly, *e.g.*, at 38° it is reached only in several days, when *circa* 0.8% of the urea present in a 0.1 *M*-solution are transformed[16].

When serum, containing an excess of urea, is heated, the formation of appreciable amounts of cyanate must, therefore, be expected. Our method of determining the coagulation temperature had an advantage in this respect, because the samples were heated for very short periods only. The time, during which the samples were at temperatures above 60°, was only of the order of 3–4 min. Nevertheless, at these temperatures, cyanate is formed fairly rapidly from urea. As mentioned above, *circa* 80–100 times the amount of urea was needed to produce the same effect as cyanate, with regard to heat coagulation. This ratio would correspond very well with the percentage of the amount of urea initially present which could be expected to be transformed into cyanate.

Thus a considerable part of the action of urea on heat coagulation is most probably due to the cyanate formed from this substance during the heating, and not to urea itself, unless it could be shown that the isomeric transformation urea → cyanate was completely suppressed in serum. An indication to the contrary was found, as will be shown below.

It appears that the question of exactly how much of the effect of higher concentrations of urea on the heat coagulation of serum, was due to urea itself, and how much to cyanate formed from the latter, would have to be studied separately, after establishing the kinetics of the reactions involved.

Heat coagulation of sera with urea content raised in vivo

Since the isomeric transformation of small amounts of urea into ammonium cyanate under physiological conditions, must be considered as highly probable[17, 18, 19], all states characterized by a raised concentration of urea in the body, become of interest in connection with cyanate. The possibility that cyanate may play a role in the intoxication of uraemia is discussed elsewhere[20]. The marked influence of cyanate on the heat coagulation temperature of serum was thought to be a possible tool to help to decide whether a reverse WÖHLER reaction occurred under physiological conditions. The heat coagulation temperatures of serum and plasma of normal rabbits was therefore compared with that of serum samples obtained from the same rabbits after bilateral nephrectomy, when a high urea concentration had developed.

On all occasions the coagulation temperature of serum or plasma obtained after bilateral nephrectomy was very markedly raised (Table II), if the blood was taken not earlier than 2 days after the operation.

The results would seem in harmony with the assumption that more cyanate is formed when the concentration of urea is maintained at a raised level over relatively long periods of time. The results cannot be ascribed to artificial formation of cyanate from urea, produced by the heat applied during the determinations of the coagulation temperature, as discussed above; the urea content was raised to 10–15 times the normal concentration. Even if as much as 1% of the urea present had undergone artificial isomerization during the short heating period of the coagulation experiment, which seems extremely unlikely, this could not account for the very considerably raised heat coagulation temperatures observed after bilateral nephrectomy. If the raised coagulation temperatures were due to cyanate, the bulk of it must have been formed *in vivo*.

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TABLE II

HEAT COAGULATION TEMPERATURES, AND TIMES OF HEATING NEEDED TO PRODUCE COAGULATION OF PLASMA FROM RABBITS, BEFORE AND AFTER BILATERAL NEPHRECTOMY

	Coagulation temp. °C	Time of heating
Rabbit 1		
before bilateral nephrectomy	80.15	10′ 15″
2 days after bilateral nephrectomy	84.5	12′ 30″
3 days after bilateral nephrectomy	85.5	13′ —″
Rabbit 2		
before bilateral nephrectomy	79.4	9′ 45″
1 day after bilateral nephrectomy	80.1	10′ 15″
2 days after bilateral nephrectomy	82.4	12′ 12″
3 days after bilateral nephrectomy	85.8	13′ 45″
Rabbit 3		
before bilateral nephrectomy	80.9	10′ —″
1 day after bilateral nephrectomy	80.6	11′ —″
2 days after bilateral nephrectomy	83.5	12′ 45″
Normal rabbit 4	79.8	10′ 15″
Normal rabbit 5	81.2	11′ 15″

Considerable quantities, however, of many other substances are retained after bilateral nephrectomy, many of which may affect the heat coagulation temperature of serum. Moreover, bilateral nephrectomy is also regularly followed by a very pronounced dilution of the blood (hydraemia), which, as can be seen from Fig. 4, is capable of markedly raising the heat coagulation temperature of serum. It cannot therefore be decided whether the results obtained after bilateral nephrectomy can be ascribed to an excess formation of cyanate from urea, although the results do not contradict such an assumption.

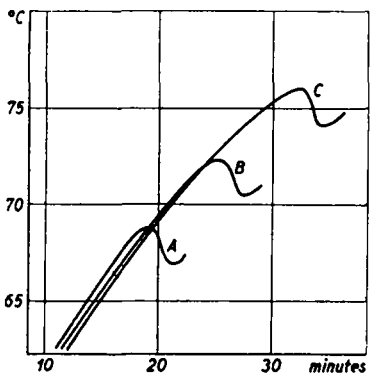

Fig. 4. Heating curves of a sample of serum; undiluted (*A*), and after dilution with 0.9% NaCl; *B* = serum: NaCl solution = 10:2, *C* = 10:5.

A striking indication, however, that the isomeric transformation of small amounts of urea into ammonium cyanate can procede in serum at physiological temperature and p_H, was obtained by the following experiment.

Heat coagulation of sera incubated with an excess of urea

The effect of cyanate on heat coagulation of serum made it possible to investigate whether urea can undergo isomeric transformation into ammonium cyanate in serum under physiological conditions. Since it is known that, in pure aqueous solutions of urea, isomerization takes place at an increasing rate with rising temperature, and is practically at a standstill at 4°, an excess of urea was added to a sample of serum, and one half was immediately cooled to 4°, while the other half was incubated at 38°. The time needed to attain heat coagulation at constant temperature was determined after 40 h.

Since at 4° no isomerization could be expected to occur, any delay of heat coagula-

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tion or rise of the coagulation temperature of the sample incubated at 38° could be regarded as a strong indication for the formation of cyanate from urea, provided that ordinary serum, with its usual low urea content, would show no, or less, difference between the two parts, previously incubated at 4° and 38° respectively. This was indeed found in several experiments to be the case.

Since it was essential in these experiments to compare the heat coagulation of the two corresponding samples under exactly equal conditions, the determination of the coagulation time at constant temperature was found more suitable. Because of the very high temperature coefficient of heat denaturation the differences in time of samples of different behaviour towards heat, are then very great.

Urea was added, in a freshly made solution, to horse serum at room temperature, establishing a final concentration of 0.08 *M*, in excess of the amounts of urea naturally present in the serum. Immediately after mixing the urea solution with the serum, the sample was halved. One part was immediately cooled to 4° in an ice water bath, and thereafter kept in the refrigerator at an average temperature of 4°. The other half was incubated at 38° for the same period of time (40 h). Chloroform was added to the samples, as a bacteriostatic. On other occasions benzoic acid was added in sufficient amounts, which, beside acting as a bacteriostatic, brought the p_H of the serum to 7.4. The results were essentially the same, when the p_H of the 38°-sample was brought to 7.4, by keeping the serum in equilibrium with a gas phase of oxygen, containing 5% CO_2.

After incubation at 4° and 38° respectively, the samples, *circa* 15 ml, were placed in stoppered boiling tubes, bound together by rubber-bands. These pairs were immersed into a water bath at 70.8° (± 0.05°). Provision was made to tilt the samples gently from time to time without removing them from the waterbath. This was done slowly, since brisk movements are known to influence coagulation. By tilting, the increase in viscosity and the final coagulation was observed. A light source was placed behind the tubes, so that also the turbidity was observed. An effective stirrer in the water bath, and continuous agitation of the solutions for the first 2 min assured that both halves rapidly reached the temperature of the water bath.

Two parts of a sample of horse serum, *without* any further addition of urea, previously incubated at 4° and 38° respectively, showed practically no difference on being heated at 70.8°. If anything, the sample previously incubated at 38° became sooner opalescent than that previously kept at 4°, but the difference was small, and they coagulated at practically the same time (19.5 min). The final aspect of the clot of both halves was the same.

To another batch of serum, sodium chloride, to a final concentration of 0.08 *M* was added, in excess of the amounts naturally present. Again one half was kept 40 h at 4° and 38° respectively. On heating at 70.8°, no marked difference was observed. The sample previously incubated at 38°, coagulated even slightly earlier than that previously kept at 4° (16.0 and 16.5 min). The clots of both parts looked alike.

When, however, an excess amount of urea was added to serum, and two batches subsequently incubated at 4° and 38° respectively, very great differences on heating the samples together at 70.8° were seen. A typical experiment is described below.

The sample kept at 4° became strongly opalescent after 9 min at 70.8°, and was clotted completely after 15 min, while the other half, previously incubated at 38°, was, even after 15 min heating, less opalescent than the 4°-half was after 9 min only. While the 4°-sample was coagulated after 15 min, the 38°-sample was then still quite fluid.

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After 17 min the difference was still marked; after 19 min, though still significant, the difference was smaller. Only after 22 min the 38°-sample coagulated.

There was a difference of at least 7 min (32%) between the coagulation times of the two samples. The difference is significant, since ordinary serum samples without an excess of urea, previously incubated at 4° and 38°, never showed an obvious difference. Any difference which could be observed was in the opposite direction, *i.e.*, the 38°-sample coagulated slightly earlier than the 4°-sample. This difference, however, was $< 6\%$ of the coagulation time (3 experiments).

The significance of the result is, moreover, strongly supported by the observation that the clot obtained with the 38°-sample of the serum, containing an excess of urea, was always less firm and far more transparent than that of the same serum-urea mixture, previously kept at 4°. Thus also in this respect, *the 38°-sample showed all the characteristics of serum to which cyanate was added before heating.*

It should be noted that urea, in the range of concentrations used in the above described experiments, did not influence the heat coagulation of serum directly (see Table I). On prolonged incubation, however, at 38° and at p_H 7.4, a very marked rise of the heat coagulation temperature was obtained, whereas no marked influence on the heat coagulation of the samples similarly incubated at 4° and 38° was obtained, when the urea content was not raised before.

It was shown, therefore, that neither urea alone, nor the incubation at 38° was the reason for the altered stability of serum towards heat. Since, however, an excess of urea *and* incubation produced this change, it is most probable that appreciable amounts of cyanate were formed from urea on incubation in serum, with all the consequent characteristics of enhanced stability towards heat.

SOLUBILITY OF COAGULATED SERUM PROTEINS IN SOLUTIONS OF CYANATE

10 ml portions of serum from the same batch were heat coagulated by immersion for 15 min in a boiling water bath. After cooling, the contents were broken up, cut, and ground with sand. This material was suspended in equimolar solutions of cyanate, or sodium chloride respectively. The controls, in sodium chloride solutions, were adjusted to the same, slightly more alkaline p_H of the sodium cyanate solutions. The samples, in conical flasks, were then shaken in a *Warburg* apparatus at room temperature for 3 h, centrifuged, and the supernatant liquid filtered through a retentive filter (Whatman No. 5). The nitrogen content in the liquid was then determined (*micro-Kjeldahl.*)

Already after centrifugation it was quite obvious that some of the precipitate had dissolved in the more concentrated solutions of cyanate, because they frothed. It can be seen in Table III that cyanate was capable of dissolving appreciable amounts of coagulated protein. Urea, in the same range of concentrations, was quite inactive in this respect.

THE STABILIZING EFFECT OF CYANATE AGAINST VARIOUS PRECIPITATING AGENTS

Cyanate was found to have a stabilizing effect against a variety of precipitating agents. In Tables IV and V the effect of cyanate against the precipitating action of alcohol and mercuric chloride is shown. There was a range of concentrations at which

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TABLE III

SOLUTION OF HEAT DENATURED AND COAGULATED SERUM PROTEINS AFTER SHAKING PARTICLES IN SOLUTIONS OF SODIUM CYANATE, UREA AND SODIUM CHLORIDE, AT THE SAME p_H AND AT ROOM TEMPERATURE (3 h)

The amounts of dissolved protein are expressed as N (*micro-Kjeldahl*), found in excess of the N-content in the solution due to the addition of cyanate, of urea.

Solvent	Dissolved μg N/10 ml
Sodium cyanate, 0.05 *M*	380
,, ,, 0.1 *M*	595
,, ,, 0.2 *M*	910
Sodium chloride, 0.05 *M*	26
,, ,, 0.1 *M*	12
,, ,, 0.2 *M*	18
Urea, 0.05 *M*	45
,, 0.1 *M*	40
,, 0.2 *M*	62

cyanate prevented or diminished the precipitating effect of these agents. No similar effect of cyanate was found on trichloracetic acid precipitation, if the amount of acid neutralized by the decomposition of cyanate was taken into account. The p_H of the controls was adjusted to that of the samples containing cyanate, which caused a slight shift towards the alkaline side. A similar protecting effect of cyanate against urea and surface denaturation will be described separately[23].

A typical experiment with alcohol was carried out as follows. To three portions of the same batch of horse serum, sodium chloride, sodium cyanate, or ammonium carbonate were added respectively, to give a final concentration of 0.1 *M*. The solutions were added in 1.0 *M* concentrations, in the proportion of 1:9 (v/v) to the serum samples,

TABLE IV

THE PROTECTING EFFECT OF CYANATE AGAINST PRECIPITATION OF SERUM PROTEINS BY ETHYL ALCOHOL

Serum *A* = containing 0.1 *M*-sodium cyanate; final concentration in the mixture: 0.071 *M*. Serum *B* = containing 0.1 *M*-sodium chloride, adjusted to the same p_H as serum *A*, by addition of 0.1 *N* NaOH

	1	2	3	4	5	6	7
0.9% NaCl, ml	0.9	0.7	0.5	0.4	0.3	0.2	0.1
96% ethanol, ml . . .	0.7	0.9	1.1	1.2	1.3	1.4	1.5
Serum *A*, ml				4			
Precipitation	—	—	—	—	+	++	+++
Serum *B*, ml				4			
Precipitation 1.	—	+	++	++	+++	+++	+++
Final conc. of ethanol, %	12	15	19	21	23	25	27

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TABLE V

THE PROTECTING EFFECT OF CYANATE AGAINST PRECIPITATION OF SERUM PROTEINS (HORSE) BY MERCURIC CHLORIDE

Serum *A* = containing 0.1 *M*-sodium cyanate
Serum *B* = containing 0.1 *M*-sodium chloride, adjusted to the same pH as serum *A*, by addition 0.1 *N* NaOH

	1	2	3	4	5	6	7	8
$HgCl_2$, 2 %	1.0	0.5	0.25	—	—	—	—	—
$HgCl_2$, 1 %	—	—	—	0.25	0.12	—	—	—
$HgCl_2$, 0.5 %	—	—	—	—	—	0.12	0.06	—
NaCl, 0.9 %	—	0.5	0.75	0.75	0.88	0.88	0.94	1.0
Serum *A*, ml	1							
Precipitation	+++	++	+	±	—	—	—	—
Serum *B*, ml	1							
Precipitation	+++	++	+	+	+	±	±	—

and left standing for several h. 10.7 ml ethyl alcohol was then added to 10 ml of each sample. The precipitate (*p*) was centrifuged off. The supernatant (*s*) was filtered and saturated with ammonium sulphate which brought down a very much greater quantity of precipitate (*ps*) in the sample containing cyanate. These precipitates (*ps*) were then each suspended in 10 ml water to determine how much could be redissolved. After thorough mixing, the samples were centrifuged and the supernatant filtered. The amount of dissolved protein in this supernatant was then determined by precipitation with trichloracetic acid, centrifugation of the precipitate, twice washing the latter with water, and drying it at 105°. Thus, 16.7 mg/10 ml serum dry weight was obtained from the sodium chloride sample, while 175.0 mg/10 ml was obtained from the sample containing sodium cyanate.

Since the sample containing sodium cyanate was slightly more alkaline, than the control, containing sodium chloride (0.1 p_H unit), a further control solution was used, as mentioned above, by adding an equivalent amount of ammonium carbonate to serum before alcohol precipitation. This sample was even more alkaline (p_H 8.6) than that containing cyanate (p_H 7.8–8.0). 111 mg/10 ml of resoluble protein was obtained from this sample. This was significantly less than what was obtained from the sample containing sodium cyanate.

The precipitate (*p*), obtained immediately after the addition of alcohol to the serum samples, was apparently of much greater volume in the sample containing sodium cyanate, than in both the chloride or ammonium carbonate controls. When water was added to these precipitates the amounts of resoluble protein recovered from them were practically equal in the three cases. The sample containing sodium chloride gave 30 mg/10 ml dry weight resoluble protein, that containing ammonium carbonate 31.6 mg/10 ml, while that containing sodium cyanate gave 36 mg/10 ml.

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DISCUSSION

The cyanate effect

The known effects of caffeine in preventing precipitation and aggregation, promoting solution and dispersion of haemoglobin derivates, as well as reinforcement and shift of absorption bands, are reactions, which were described by J. KEILIN and discussed as the "caffeine effect". Most of these effects can also readily be produced by cyanate instead of caffeine[7]. The above described experiments show that also with regard to the effect on heat coagulation of proteins, cyanate and caffeine behave similarly. The two near relatives of caffeine, theobromine and theophylline, which are, as discovered by J. KEILIN, unable to produce the caffeine effect, were also found in the above described experiments to have no effect on heat coagulation. Moreover, both caffeine and cyanate are diuretics. Both caffeine[21, 22] and cyanate[23] increase the ultra-filtration rate of dilute, buffered protein solutions. Both, as mentioned below in greater detail, have anti-mitotic activity.

Since thus, beside the "caffeine effect", cyanate shares with caffeine this remarkable number of pharmacological and physico-chemical properties, it seems likely that the underlying mechanisms of these effects are intimately related. Since strong indications were recently found for the formation of small amounts of cyanate from urea (see above, and [16, 17, 18, 19]), the above mentioned effects most probably have physiological significance.

Counteraction of heat denaturation

It has apparently not been pointed out before that the effect of a substance in raising the temperature of heat coagulation of protein solutions, often goes parallel with the effectiveness of the same substance to dissolve coagulated protein. Salicylate and urea, in high concentrations, were both found capable of raising the temperature of heat coagulation and are also known to promote solution of denatured and precipitated proteins (see ANSON[24]).

Because of this parallelism, it would seem conceivable that the effect of raising the temperature of coagulation was merely due to the prevention of the visible change *i.e.*, of coagulation, by redissolving the protein while, or immediately after coagulation; cyanate would thus not hinder heat denaturation, but the precipitation of heat denatured protein only. An interpretation of this kind seems very unlikely since apparently undenatured protein could be precipitated with ammonium sulphate from a heated protein solution, and a significant part of the precipitate proved readily soluble in water. This experiment suggests that, to a considerable extent, denaturation itself was prevented by the addition of cyanate. The delay of heat coagulation, or rise of the coagulation temperature, cannot only be ascribed, therefore, to a mere inhibition of coagulation or precipitation of heat denatured protein.

Since blood pigments were also found to be protected by cyanate against precipitation and aggregation, it seems probable that this effect is intimately connected with the caffeine effect. Because of the change in the absorption spectra a combination of the pigments with cyanate can be assumed. Similarly it becomes probable that cyanate also combined with proteins in forming new compounds, and that the newly formed protein-cyanate complex showed the increased stability towards denaturing agents.

The stabilizing effect on proteins of cyanate against solutions of heavy metal salts,

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is probably partly due to a formation of heavy metal complexes with cyanate. It seems, however, improbable that this could account for the whole stabilizing effect in these cases, since the effect is also produced by cyanate against a variety of other agents or procedures (alcohol, heat, surface denaturation, etc.), when no combination of cyanate with some of the precipitating agents could be assumed.

Mechanism of solution of coagulated protein by urea and cyanate

It was pointed out above that approximately 80–100 times the amount of urea, than that of cyanate, was needed to achieve similar effects on the heat coagulation of proteins. The possibility was discussed that urea itself may not be directly responsible at all for the observed effect, and that this may have been due to cyanate formed from urea at the high temperature necessary to promote heat coagulation. The other observation, namely the well known fact that higher concentrations of urea are capable of redissolving denatured protein, cannot be ascribed to a formation of cyanate during the experiment, since this dissolving property of urea can be observed at relatively low temperatures, at which the formation of cyanate from urea is negligible.

The underlying mechanisms of dissolving coagulated protein in solutions of urea or cyanate respectively, seem to differ fundamentally. Practically no denatured protein is dissolved in a urea solution, except when the latter is very highly concentrated. A huge excess of molecules of urea seems to be needed for one molecule of denatured protein to go into solution, or to prevent it from aggregating with the remainder of denatured protein present in the solution.

Cyanate seems to act differently, since very much smaller concentrations are active in dissolving coagulated protein. With increasing concentration of cyanate more protein is dissolved, suggesting a stoichiometric reaction, whereas the dissolving effect of high concentrations of urea appears to be due rather to a radical change of the physical nature of the solvent.

The possible physiological significance of cyanate

The above described experiment, in which, the heat coagulation of serum was significantly raised after incubation with a small amount of urea, strongly suggests that a reverse WÖHLER reaction can indeed take place in serum at the physiological temperature and p_H. DIRNHUBER AND SCHÜTZ[18, 19] recently found evidence for the presence of small amounts of cyanate in incubated brain brei suspensions. Both these findings make it appear highly probable that cyanate is normally formed in the mammalian organism, and that, therefore, the above mentioned effects of cyanate concerning the stability of proteins have physiological significance.

It becomes of interest in this connection that, as was pointed out by J. KEILIN, native proteins also have a pronounced dispersing effect on certain blood pigments, an effect, which is also followed by reinforcement and shift of the absorption bands. It may thus be said that proteins exhibit the "cyanate", or J. KEILIN's "caffeine-effect", since these effects are practically identical. After having discovered that globin and serum proteins had effects on porphyrin[25, 26], similar to those of caffeine on these tetrapyrrolic compounds, J. KEILIN carefully investigated whether any of a great number of the known amino acid contents of these proteins were capable of producing the "caffeine-effect". None of these amino acids was found capable of producing this effect.

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One of the possible explanations offered by J. KEILIN for this finding, was that "proteins may contain an additional, not yet isolated constituent".

Since the effects of cyanate are so similar to those of proteins in this respect, it seems conceivable that cyanate may indeed be the "not yet isolated constituent", predicted by J. KEILIN. There are, of course, a number of other possible explanations for the similarity of action of proteins, cyanate and caffeine in this respect. Since, however, the formation of cyanate in the warm blooded organism from the ubiquitous urea must now be regarded as very probable, the above mentioned possibility of small amounts of cyanate being normally present in solutions of native proteins, or as natural constituents of these proteins, must be taken into consideration.

The influence of cyanate on protein denaturation and mitosis

Following the finding of the weak hypnotic action of cyanate in rats[3], and the observation that it stopped normal growth of cats and rats[1], it was suggested to Professor HADDOW and Dr SEXTON, that cyanate may have an antimitotic action, especially in view of the remarkable discovery of these authors that urethane effectively reduced the number of leucocytes in leucaemia[27]. Thereupon P. DUSTIN[28], in HADDOW's laboratory, discovered that cyanate had indeed a potent antimitotic action.

It seems of interest in this connection that in the above described experiments urethane was not capable of raising the heat coagulation temperature of proteins, nor is it known to cause any of the other physico-chemical effects with protein solutions, which are so marked with cyanate and caffeine. Since caffeine is also an antimitotic substance, the results reported in this paper suggest that the underlying mechanisms of the antimitotic action of cyanate and caffeine on the one hand, and of urethane on the other, may be different. This would be under the assumption that the reactions with proteins of these substances are connected with their antimitotic activity.

That a connection of this kind exists is somewhat supported by the fact that thiocyanate was also found to have an antimitotic action, though this was less powerful than that of cyanate[28]. Thiocyanate also showed an effect on heat coagulation of proteins; again it was less potent than cyanate, also in this respect. The antimitotic activity of a number of substances, and their stabilizing effect on proteins, thus seem to show a certain parallelism.

RAPKINE[29, 30, 31] discussed mitosis in connection with an assumed denaturation of proteins *in vivo*, with a consequent unmasking of SH-groups. It seems of interest in this connection that cyanate has 1) a pronounced antimitotic activity, 2) effectively counteracts denaturation, and 3) combines with amino- and SH-groups[4]. The possible existence of an equilibrium between native and denatured protein *in vivo*, has been discussed by JOHNSON *et al.*[32]. If further work should finally establish the existence of an equilibrium of this kind in the warm blooded mammalian organism, cyanate would, most probably, be involved.

The fact that the relatively large number of physico-chemical and pharmacological effects mentioned above could all be produced by so simple an organic compound as cyanate, suggests that the underlying mechanisms of these effects may be intimately related. Thus, the antimitotic, hypnotic, diuretic, etc., actions of cyanate may ultimately be found to be connected with the effects of cyanate on proteins described in this paper.

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SUMMARY

1. The heat coagulation temperature of serum and protein solutions was determined by means of heating curves.

2. Cyanate was found effective in stabilizing proteins to various extents against heat, heavy metal salts, and alcohol. It was more potent in some of these respects than caffeine, salicylate, thiocyanate, etc.

3. Small amounts of urea had no effect on heat coagulation, but on incubation in serum at 38° the heat coagulation temperature rose, suggesting that isomerization of urea into cyanate took place in serum at the physiological temperature and pH.

4. Cyanate protected a part of the proteins from becoming irreversibly insoluble through heat or alcohol.

5. Cyanate was found capable of dissolving denatured and precipitated protein.

6. The effect in raising the heat coagulation temperature of proteins, produced by a number of substances, seems to go parallel with the potency of these substances to promote solution of denatured protein.

7. In view of the probable formation of cyanate from urea in the organism, the heat coagulation temperature of sera with raised urea content was studied.

8. The possible physiological significance of the results, and their bearing on the antimitotic of cyanate, is discussed.

RÉSUMÉ

1. La température de coagulation par la chaleur du sérum et de solutions de protéines a été déterminée au moyen de courbes de chauffage.

2. Le cyanate s'est montré efficace pour protéger les protéines vis-à-vis de la chaleur, des sels de métaux lourds et de l'alcool. Son efficacité est souvent supérieure à ce point de vue, à celle de la caféine, du salicylate, du thiocyanate, etc.

3. De petites quantités d'urée n'ont aucune action sur la température de coagulation; mais après maintien dans du sérum à 38°, l'urée provoque une élévation de la température de coagulation, ce qui semble indiquer que dans les conditions de température et de pH physiologiques, l'urée s'ets transformée en cyanate dans le sérum.

4. Le cyanate protège une partie des protéines dissoutes contre la dénaturation irréversible par la chaleur ou l'alcool.

5. Le cyanate provoque la solubilisation des protéines dénaturées et précipitées.

6. L'élévation de la température de coagulation par la chaleur des protéines, produite par différentes substances, semble aller de pair avec l'aptitude de ces substances à solubiliser les protéines dénaturées.

7. Du fait de la formation probable de cyanate à partir de l'urée chez les organismes, la température de coagulation par la chaleur du sérum a été étudiée en fonction de sa teneur en urée.

8. La signification physiologique possible de ces résultats est discutée, de même que leurs relations avec l'action antimitotique du cyanate.

ZUSAMMENFASSUNG

1. Die Temperatur der Hitzekoagulation von Serum und Eiweisslösungen wurde mit Hilfe von Erhitzungskurven bestimmt.

2. Es wurde festgestellt, dass Cyanat auf Eiweiss eine stabilisierende Wirkung in verschiedenem Ausmass gegen Hitze, Schwermetallsalze und Alkohol ausübte. In verschiedenen dieser Fälle hatte es eine stärkere Wirkung als Kaffein, Salicylat, Rhodanid usw.

3. Kleine Harnstoffmengen hatten keine Wirkung auf die Hitzekoagulation, aber nach Inkubation in Serum bei 38° stieg die Temperatur der Hitzekoagulation, was darauf hinweist, dass in Serum bei physiologischem pH und Temperatur Isomerisierung von Harnstoff zu Cyanat auftrat.

4. Cyanat schützt einen Teil der gelösten Eiweisstoffe gegen irreversible Denaturierung durch Erhitzen oder Alkohol.

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5. Cyanat hat, wie festgestellt wurde, die Fähigkeit, denaturierte und gefällte Eiweissstoffe in Lösung zu bringen.

6. Die Erhöhung der Hitzekoagulierungstemperatur von Eiweisskörpern, die von einer Anzahl Stoffen hervorgerufen wird, scheint mit der Fähigkeit dieser Stoffe, das Lösen denaturierten Eiweisses zu fördern, parallel zu laufen.

7. Wegen der wahrscheinlichen Cyanatbildung aus Harnstoff im Organismus wurde die Hitzekoagulierungstemperatur von Sera mit erhöhtem Harnstoffgehalt untersucht.

8. Die mögliche physiologische Bedeutung der Resultate und ihre Beziehung zur antimitotischen Wirkung von Cyanat wird besprochen.

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