AD NUMBER

AD842489

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov't. agencies and their contractors; Administrative/Operational Use; SEP 1968. Other requests shall be referred to Commanding Officer, Fort Detrick, Attn: SMUFD-AE-T, Frederick, MD 21701.

AUTHORITY

Biological Defense Research Lab ltr dtd 13 Sep 1971

TRANSLATION NO. 165

DATE: Sept 1968

(1)

(5)

DDC AVAILABILITY NOTICE

This document is subject to special export controls and each transmittal to foreign governments or foreign nationals may be made only with prior approval of Commanding Officer, Fort Detrick, ATTN: SMUFD-AE-T, Frederick, Md. 21701.

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

#16
Cy

Zeitschrift Viatiam Hormon Vermentforsch , 1951. Pages 278-304

Hanson, H. and Tannert, S. - Investigations on Sulfur-metabolism. 1. Reciprocal Action of Bacteria coli and Cystin, and a Discussion of Enzymatic Adaptation

As end-products of S-metabolism of S-containing amino acids in animals and man appear chiefly sulfate in the form of (a) free sulfates, (b) ester-sulfates, and (c) known sulfo acids (taurine), and in certain microorganisms H_2S also appears. It is now of interest that lately the evolution of H_2S has been established in isolated animal organs (liver-sections, liver-slurry, and liver-extract, and from kidneys) under anaerobic conditions (1, 2, 3). Under aerobic conditions, however, practically no H_2S appears from the same organs. Either the H_2S formed in vivo is rapidly oxidized, or else - and this would bring the situation into harmony with the "economic laws" of the metabolic intermediaries - no H_2S is evolved but rather an oxidation of the S has already occurred in the organic combination. A series of studies favors the latter possibility. Grace (4) Medes establishes that, in carbonate-buffer, liver-section and also kidney, heart-muscle and other organs are able to form inorganic sulfate rapidly from cysteine, cystine, sulfoxide and cystein-sulfinic acid ($COOH.CH(NH_2)CH_2.SOOH$). Diverse enzymes were held responsible for these activities. A similar mineralization of the S in feeding with cysteine-sulfinic acid with pronounced rise of the rhiosulfate liberation in the urine was observed in rabbits by Fromageot, et al (5). Rabbit-liver slurry can form no thiosulfate from cysteine-sulfinic acid, but very probably can form sulfite. According to the findings of Barrenscheen (6) an oxidation at the S to methionine-sulfoxide is necessary to permit the CH_3-donor-function of the methionine.

The above mentioned discoveries of the H_2S-formation from cysteine by liver-slurry and liver-sections, and the assumption of a liver-enzyme which transforms

the cysteine to pyroracemic acid, NH_3, and H_2S, do not jive with these ideas that a mineralization of the S occurs only after a resulting oxidation in the organic molecule. Smythe takes the stand that the H_2S from the liver under aerobic conditions partly oxidizes to sulfate, partly becomes polythionate, and partly enters into the formation of organic molecules, for example, alanine, in cysteine-formation. (C. V. Smythe) (7)

Whereas no doubt exists that the H_2S is a metabolic product in micro-organisms, accordingly, concerning the H_2S-formation in the animal organism it can only be stated that H_2S has been obtained up to now only from isolated, excised, and therefore more or less damaged organs, and thus perhaps is only the product of accessory metabolic pathways, which do not normally operate.

Purely formularily, in the case of the H_2S-generation from cysteine, in the assumption of reaction-events which biologically constantly occur (Hydrolysis I, Reduction II, Oxidation III), products are given whose occurrence in the intermediary metabolic processes are in part demonstrated (serine, alanine), and in part can only be inferred (aminoaldehyde carboxylic acid), and which in the course of metabolism can undergo further changes (deamination, etc.).

I. $CH_2(SH)-CH(NH_2)-COOH + H_2O$ yields $CH_2(OH)-CH(NH_2)-COOH + H_2S$

II. $CH_2(SH)-CH(NH_2)-COOH + H_2$ yields $CH_3-CH(NH_2)-COOH + H_2S$

III. $CH_2(SH)-CH(NH_2)-COOH + O$ yields $\begin{matrix} O \\ \| \\ C \\ / \\ H \end{matrix}-CH(NH_2)-COOH + H_2S$

When Smithe, for example, establishes that a liver enzyme permits the evolution of H_2S from cysteine, in addition to pyroracemic acid and ammonia, thus is shown no more the connection with alanine as the amination-product of the pyroracemic acid. Fromageot, et al (1), in the liver desulfurase activity, find no NH_3-formation; they establish variously, in the cysteine-breakdown, cystine, in addition to H_2S, and they maintain that an intermediate formation of alanine rather than serine is probable. According to Desnuelles and Fromageot (8) a cysteinase in Bact. coli breaks down cysteine and cystine, with the formation of H_2S, NH_3, formic acid, and acetic acid.

It is now of interest that in the investigation which Medes and Floyd (4) pursued concerning the break down of S-containing amino acids by the liver, H_2S did not appear; nor was it mentioned as an intermediate. The discovery by Smythe (7) that under anaerobic conditions the liver-section can form H_2S from cystine, led Medes and Floyd to the break down of intermediate thiosulfuric acid and H_2SO_2, without their actually having experimental evidence for this assumption. Their experiments, using the Warburg-method with rat-liver under aerobic conditions, lead them to the view that the SH-groups of the cysteine then would be oxidized, with water-deposition and concomitant dehydration, to cysteinesulfinic acid (RSOH), with the aid of a cysteine-oxidase which transforms itself to sulfinic acid (RSOOH) and cysteine (2 RSOH yields RSOOH + RSH). Cysteine sulfinic acid would then be split enzymatically (sulfinic-oxidase) into an organic residue and an inorganic sulfate (formed through further oxidation). Another pathway of cysteine-oxidation would be through the formation of cysteine-acid (cysteine-oxidase b). In in-vivo and in-vitro tests cystine forms sulfate slower than it does cysteine; also the formation of cysteine-acid occurs slower than that of cystine. The idea emerges that in contrast to previous assumptions the cystine is not turned reductively

into cysteine but hydrolytically to cysteine and cystinesulfinic acid (IV).

IV. CH_2-CH-COOH CH_2-CH-COOH

S NH_2 SH NH_2

H

+ O yields

S NH_2 H SOH NH_2 NH$_2$

CH_2-CH-COOH CH_2-CH-COOH yields H_2S + O C-CH . COOH

H

The authors assume a non-enzymatic hydrolysis. That must at first seem doubtful, if one considers the disregarded tests of A. Schoberl (10), K. Bloch and H. T. Clarke (11), and J. Routh (12) concerning the in-vitro hydrolysis of cystine and the changes of cysteine in aqueous solution. Cysteine shows itself to be more stable than cystine in heating in alkaline solution. However, under conditions (several-hour heating in strong alkali) the cystine would hydrolyze, at pH which would be far higher than any encountered in the organism. Then cystine-hydrolysis in-vivo, whether enzymatic or not, must remain as undemonstrated. Since, in the in-vitro hydrolysis of cystine, over the rapidly wider disproportioning of the succumbing sulfinic acid, H_2S is formed in addition to the aldehydic and keto-compounding, detection of in-vivo hydrolysis of cystine is perhaps more likely if the conditions of the H_2S-formation from cysteine-cystine in-vivo and the character of the compounds remaining after H_2S-splitting were more accurately sought to be grasped. The assumption of H_2S-formation from cysteine or cystine does not necessarily entail a special H_2S-splitting enzyme, but can be explained by the assumption that in-vivo or under aerobic conditions in the fully intact organ the sulfinic acid is further oxidized at the S to SO_4, but that in the use for example of liver-slurry the enzymatic break down to the

sulfinic-acid-stuff is the final step. In this case the sulfinic acid is disproportionate under H_2S-formation without special ferments. The otherwise very difficult-to-understand situation can be explained thus, whereupon the liver oxidizes cysteine at the sulfur, however, even still shall possess an enzyme to split H_2S from cysteine.

The previously published researches on living material do not permit certain conclusion regarding the role of sulfinic-acid or other reaction forms of S-containing amino acids. We are not certain about the first point of attack in the biological S-oxidation, nor do we know in which way the amino group or other certain intermediate product of cysteine, for example those with aldehyde- or keto-groups, passes in reaction with still S-containing intermediate products, and we do not know whether or to what extent the amino group in the cysteine or cystine is necessary for reaction at the S.

Before we go into our researches concerning the role of the liver in S-intermediary metabolism, we have to deal with the conditions of H_2S-formation from cysteine or cystine by Bact. coli. On the basis of data in the "Handbuch der Experimentellen Bakteriologie und Infektionskrankungen" (by Kolle and Hetsch (22)), and the "Handbuch der Pathogenen Mikroorganismen" (by Kolle, Kraus, and Uhlenhuth (23)) we could assume that this microorganism has no or quite limited capacity for H_2S production. But as we have shown with certainty, Bact. coli produces H_2S in considerable amounts from the cysteine and cystine. Here it is more proper to assume a specific H_2S-splitting enzyme, than it was according to the investigations of the liver. We presently report observations, which make it imperative that we go rather thoroughly into the question of the dependency of the enzyme-formation and enzyme-concentration of the substrate. The present first part, in addition to methodical data, concerns these investigations. We must

likewise bring out (what every bacteriologist knows) that the Bact. coli show a variability in their metabolic properties which sometimes make necessary time consuming investigations and testing as to whether the bacteria being used are "bonafide" Bact. coli.

METHOD

General Discussion

1. Obtaining and handling of the Bact. coli.

Coli-bacteria were used which were freshly isolated from human feces and which were tested for the characteristic coli biochemical properties. For the maintenance of the constance of their strain properties they were transferred to schrag-agar (slanted agar) from time to time. From this ground-strain were innoculated the incubation-flasks, which mostly contained 100 cc bouillon with 1% glucose, or ½% glucose, or sugar-free; they were incubated at 37° for 48 hours. So far as the nutrient-bouillon contained sugar, the pH of the solution fell to about 4.3, measured with Lyphan-paper. The growth in the Liebig-bouillon without sugar showed no pH drop. A variously observed inactivation of the coli with regard to the cystine-breakdown we traced to an injury due to too long exposure to the low pH. By regulating the pH during incubation by buffering with $NaHCO_3$ we stopped the loss of this activity. The first good growth must be conveyed to agar plates from want of material. After incubation the bouillon is centrifuged well, the liquid poured off in each test, where by more thorough washing is not remarked, in suspended in reduced Reichenbach-sol'n (5 g lactose, 5 g NaCl, 2.5 g Na_2HPO_4, water to 1000, no peptone, no asparagin and no szolithmin), later only still in m/30 phosphate-buffer. Eventually thorough washing with m/30 buffer took place. The suspending of the bacteria occurred in such a way that the micro-

organisms, which had developed in 100 cc nutrient, were suspended after centrifuging in 15-20 cc phosphate-buffer. For similar coli-concentration in the single starts a test series would thereby provide, that the contents of the single incubation flasks (100 cc) be centrifuged, the liquid poured off, and that the coli bacteria-containing sediment of the centrifuge tube be suspended in some phosphate buffer. The entire coli amount was then placed in a Mess-cylinder with the amount of phosphate-buffer directed by the above directions.

2. Test set-up with Bact. coli.

The coli-suspension prepared as directed above was tested as to its capability (and the conditions necessary) to break down (liberate) NH_3, H_2S, and variously CO_2 from various substrates, s. that these and certain other breakdown products of the substrates would be detectible in the media. To this end the bacterial suspension was pipetted into a pyriform 50 cc volumetric flask with ground stopper in which was added the substrate to be tested in substance or in phosphate buffer. At the same time as control tests set up (plus cysteine minus bacteria or plus bacteria minus cysteine) were set up by filling up with phosphate buffer sol'n similar volumes of the single test starts. The further test set-ups are shown in Figure 1.

For CO_2-determination, a third absorption flask with $Ba(OH)_2$ was inserted after thw WF_2, which then to be sure was loaded with acidified $CuSO_4$-sol'n for the absorption of H_2S and the better transmission of CO_2.

The apparatus was set up so that N_2 was bubbled through the flasks at the rate of 45-60 bubbles per minute. At this rate by the end of the 16 hour test time all the gaseous metabolic products had been liberated, without residue, and absorbed. We convinced ourselves of this by interchange of wash-flasks and by

interchange of 2 wash-flasks with the same absorption material. In the shorter test time in which the rate of bubbling was not sufficient to leave any residue, N_2 was passed through much faster at the end of the test time. Where the pH of the reaction flask was not favorable to the liberation of the gaseous metabolic products (i.e., pH too high in H_2S- and CO_2-tests), or when the metabolic process was to be cut off, the reaction flask was acidified with 5 cc 20% trichloroacetic acid and N_2 or air was bubbled through rapidly for 30 minutes. Also here we have convinced ourselves that one absorption flask is sufficient to take out the gaseous metabolic products. In model tests we found in the second absorption flask no more than a trace, for example of cadmium sulfide, which was not even measurable titrimetrically.

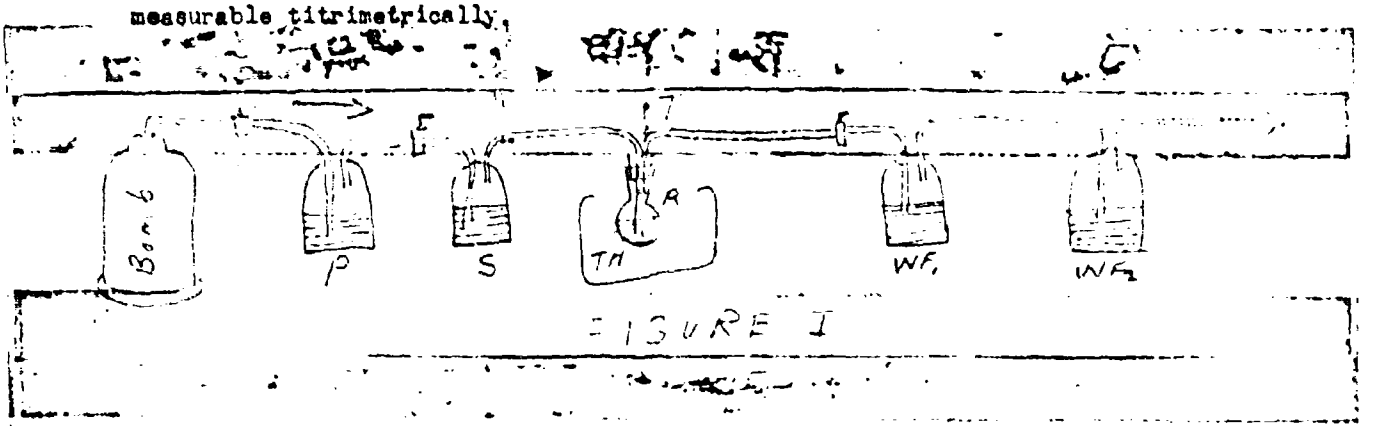

FIGURE I

Explanation -

P: is alkali pyrogallol-sol'n (for O_2- and CO_2-absorption).
S: is sulfuric acid (for NH_3-absorption).
TH: is thermostat to maintain 37°.
R: is reaction flask.
T: is funnel tube for adding substances (trichloroacetic acid, soda).
WF_1: contains n/20 sulfuric acid (for NH_3-determination).
WF_2: contains cadmium-acetate (for H_2S absorption).

3. NH_3-determination.

For the determination of the liberated NH_3, the NH_3 was distilled

(after alkalization of the test flask with 10 cc 15% soda sol'n, NaOH being avoided to avoid secondary NH_3-formation) in an absorption bulb with n/20 sulfuric acid at a temperature of 40° at the highest, and in this bulb was determined step-photometrically by the Nessler-process using a Filter S-43. The most favorable conditions for the quantitative NH_3-measurement were established in trials. It was found that under the conditions stated the NH_3 was to be determined quantitatively only in vacuum-distillation. This was accomplished using the methods of G. W. Pucher, H. P. Vickerey, and C. S. Leavenworth (13). (See Table I)

Table I

Trial Tests for NH_3-determination

Test-sol'n	est. NH_3-content in mg.	NH_3-determinat. in mg.	% of input	Remarks
Empty distillation value (UTC)	-	0.02	-	-
NH_4Cl 0.5 cc	0.333	0.330	99	-
NH_4Cl 1.0 cc	0.666	0.634	95	-
NH_4Cl 1.5 cc	0.999	0.946	95	-
NH_4Cl 2.0 cc	1.333	1.336	101	-
3 cc ferment-sol'n	-	0.218	-	Emp. dist-value not det.
3 cc ferment-sol'n plus 1 cc NH_4Cl	0.884	0.816	92.3	From found NH_3-content of ferment sol'n plus 0.666 mg. NH_3
5 cc ferment-sol'n	-	0.330	-	Emp. dist-value not det.
5 cc ferment-sol'n plus 1 cc NH_4Cl	0.996	0.912	92	From found NH_3-content of ferment sol'n plus 0.666 mg. NH_3
10 cc ferment-sol'n	-	0.609	-	Emp. dist-value not det.
10 cc ferment-sol'n plus 1 cc NH_4Cl	1.275	1.266	99	From found NH_3-content of ferment sol'n plus 0.666 mg. NH_3

By the single "empty-distillation-value" given in the Table is meant the value of the Nessler-reagent mixture alone, which was mixed up fresh for this test. It must again be pointed out that in the case of several measurements of the NH_3 evolution "empty-distillation-values already had occurred which in their dependence on the use of soda solution, gave values equal to distilled water or even a little less. It was thus common, above all when we used new soda solution and new phosphate buffer solution (or water which on the basis of the qualitative N-test was not entirely pure), to carry out NH_3-determinations using only the used solutions without adding Bact. coli or substrates under conditions similar to those of the primary test. That no NH_3 was liberated from cystine under the chosen conditions (24 hours at 37° in N_2 or air at various pH, then alkalization with soda solution at 40° bath temperature and vacuum of 12-15 mm Hg.) is obvious from the data in Table II, which gives similar values of the different empty-distribution values, and which demonstrates that when the reagent solutions are made up fresh the empty values should be redetermined and should be taken into consideration in computing the NH_3-liberation from cystine.

Table II

Experimental Ingredients	10 cc water	10 cc phosphate buffer pH 6.7*	2.4 mg cystine in 10 cc phosphate-buffer pH 6.7**	Cystine-empty-distillation-values without correcting for the H_2O- and PO_4-empty values					
				19.X.	23.X. ***	6.XII. ***	pH 3.8	pH 6.0	pH 8.0
NH_3 found in mg calculated on 100% NH_3-liberation from 2.4 mg cystine	0.039	0.003	0.001 .15	0.02	0.031	0.037	0.03	0.023	0.034

* Corrected for water values
** Corrected for Phosphate and water values
*** Using fresh soda solution

Nessler-test: contents of the WF (10 cc n/20 sulfuric acid) transferred to 50 cc volumetric flasks, almost neutralized with carbonate-free n/20 soda-lye, and filled to the mark with water. An aliquot was Nessler-tested. Then (depending on NH_3-content) 5 or 10 cc of this was transferred to a 25 cc volumetric flask, to which was added 2.5 cc of a mixture of the Nessler-reagent (1 part solution-A (potassium mercuric iodide) and 5 parts solution-B (2.78 n carbonate-free soda-lye)), filled up with water, and then with Filter S-43, using the 1 cm Cuvette in the step-photometer, a measurement of NH_3-content obtained. Although no screen-colloid was used in the Nesslerization to maintain the clarity of the solution, the determinations were only rarely messed up by turbidity. If the qualitative preliminary test showed relatively high NH_3-content, the solution was diluted with water so that a reading could be obtained in the photometer, and correction was made for the dilution. The dilution of the measuring solution was such that the extinctions, using the 1 cm Cuvette, lay between 0.1 and 0.5.

4. H_2S-determination.

As in NH_3-determination, N_2 was bubbled through the apparatus for the determination of H_2S. (See Figure 1) The NH_3-absorption flask was situated after the second absorption flask (with 10 cc 0.4-0.8% cadmium acetate solution) which we adjusted to pH 7-7.5 with a drop of soda-lye. In the tests with pH-values of 7-8.5 in the reaction flask, toward the end of the test the reaction flask was acidified with 5 cc 5-n HCl or 5 cc 20% trichloroacetic acid, and a rapid bubbling of N_2 or air for 30 minutes resulted in a complete recovery of the H_2S. The CdS-suspension of the absorption flask was transferred into accurately measured n/100 iodine solution (5-10 cc), which was acidified with

2 cc 2-n HCl, and was titrated with n/100 $Na_2S_2O_3$. In trials with Na_2S solution we ascertained the limits of variation, which we used in computing the values (Table III). When the test was continued 8 hours or more we observed deviations from the theoretical values, which induced us to assume certain divergences in the compared values only in the case of differences of more than 20%. The Cd-acetate preparation showed a slight iodine loss when it was left standing, and this was taken into account.

Table III

Solution used	S-content		S-content after Cd S-precipitation		H_2S precipitated by acidification							
					2 hrs. N_2 ***		4 hrs. N_2		8 hrs. N_2		21 hrs. N_2	
	cc n/100 iodine	mg S *	cc n/100 iodine	mg S **	cc n/100 iodine	mg S	cc n/100 iodine	mg S	cc n/100 iodine	mg S	cc n/100 iodine	mg S
1 cc	1.34	0.21	1.46	0.23	1.53	0.24	1.44	0.23	1.19	0.19	1.58	0.25
2 cc	2.58	0.41	2.65	0.42	2.42	0.39	2.57	0.41	2.58	0.40	2.35	0.37
4 cc	5.17	0.83	5.09	0.81	4.98	0.80	4.85	0.78	4.75	0.76	4.74	0.75

* By direct titration of the sulfide-solution without separation of the CdS.
** The Cd-acetate-empty-value was determined for example, using 10 cc Cd-acetate 0.24 cc n/100 iodine.
*** Separation of the H_2S by addition of 5 cc 5 n HCl, 30 minutes ventilation. Collection of H_2S in Cd-acetate solution.

5. Example of the production of an H_2S-liberating solution from Bact. coli.

1000 cc bouillon, variously thinned with 1000 cc water, and eventually (with addition of ½% dextrose) innoculated with Bact. coli from agar culture, and incubated 48 hours at 37°. Coli bacteria centrifuged 20 minutes at 3500 rpm, the liquid poured off, then the coli bact. (the residue) suspended in 250 cc m/30 PO_4-buffer pH 6.7. Allowed to work on cystine (4 mg) at 37° with N_2-bubbling for 24 hours. In most cases great H_2S-build-up, up to 99% of the S of the cystine

being recovered as H_2S. For example, when centrifuged two hours, 10 cc of the supernatant liquid put with 2.4 mg cystine and N_2 bubbled through for 24 hours at 37°, 0.351 mg of sulfur was recovered as H_2S (55% of the S in the cystine). Under the same conditions 10 cc of the supernatant without the addition of cystine produced no H_2S whatever.

It should be brought out that at times there were failures in the production of the "ferment solutions". We thought that at times the coli bact. form no H_2S. But then, through pH control, it was established that the incubation flask was too acid (pH less than 4.2), that also the coli, by lying too long in the acid solution, had apparently lost their H_2S-liberating function. Or, at least, if they had <u>indeed</u> produced H_2S, at least we were not able to detect it in the supernatant. We have not been able to explain this phenomenon.

Results

a. Direct effect of Bact. coli on cystine or cysteine.

In the described method, the coli bact. were at first suspended in Reichenbach's medium (but with <u>no</u> addition of a nitrogen source). Later we used exclusively m/15-m/30 phosphate-buffer (pH 6.7), since it became apparent that in lactose- or glucose-containing media (through the ability of the bact.) occurred a pH-drop which also stopped the break down of the cystine (which we had investigated), although, as shown especially by culture experiments, the growth-ability of the coli bact. was not impaired. This discovery (phenomenon) compares favorably with the brief observations of Thiele and Deckart (21), according to whom the very low pH impaired not only metabolism but also indole-formation, so that (to be sure) the authors speak also of a diminuation of the

growth and reproduction abilities of the microorganisms. The solution of cysteine or cystine in the substance or in phosphate buffer, and the addition of media, leads almost regularly to H_2S- and NH_3-buildup. An example is shown in Table IV. The H_2S-buildup occurs faster when cysteine is used. But definite significant distinctions could not be established with respect to the maximal H_2S-formation. In most of the tests cystine was used, proceeding on the assumption that when H_2S is liberated from cystine, the liberation must be much the same if cysteine is substituted for cystine. It remains still to be tested whether in the somewhat rare cases in which the coli bact. produce no H_2S from cystine, they could produce it from cysteine, or whether conditions can be found out under which coli bact. liberate H_2S only from cysteine, not from cystine. By the use of smaller amounts of cystine (instead of 24 mg only 2.4 mg) it may be that the liberated NH_3 can only partly come from the cystine, that a smaller proportion must come from other N-containing substances of the coli bact., but from these were then first liberated, when bact. coli can react with cystine. The H_2S comes always from the added cystine. Table V demonstrates that (measured as the N-content of their protoplasm) the coli bacteria in 7 times greater concentration liberated the same amount of H_2S as the bact. coli at lower concentration, but that the NH_3-formation rose as the coli concentration rose. The first cystine addition (supplement) appears as the initiator of the NH_3-liberation from no cystine-N-substances, because the coli suspensions without cystine form only insignificant amounts of NH_3 (see Table V).