CXXVI. THE NATURE OF THE BACTERICIDAL SUBSTANCE IN THE URINE OF PATIENTS RECEIVING A KETOGENIC DIET.

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HELMHOLZ AND CLARK [1931] were the first to apply a ketogenic diet to the treatment of pyelitis. Providing a sufficiently intense ketosis can be obtained, the bacteria and pus often disappear from the urine within a few days, even in chronic cases. At first it was thought possible that the increased acidity alone caused the sterilisation. It soon became evident, however, that there was another factor, for Helmholz and Clark succeeded in producing sterile urines at p_H 5.5 by means of the ketogenic diet in cases where the use of acidifying salts yielding a urine of p_H 5.0 had been without effect.

This was also shown by experiments on the bactericidal properties of the urines *in vitro* at 37°. Helmholz [1932] demonstrated that acidity alone, or in combination with 0.5 % of acetoacetic acid, was not responsible for the bactericidal effect of ketonic urine, and concluded that some other bactericidal substance was present. Dick [1933] obtained similar results, showing that while normal urine at p_H 5.5 allowed a free outgrowth of *B. coli*, ketonic urines at this reaction were bacteriostatic, that is, they prevented an increase in the numbers of bacteria without necessarily killing them completely.

Thus, while it was evident that a change occurs in the urine making it bacteriostatic, and occasionally bactericidal, the nature of the substance responsible was unknown. The work described in this communication is an attempt to isolate and identify this bactericidal agent.

During the past 9 months the diet has been given to patients suffering from pyelitis and cystitis at the Isolation Block of Queen Charlotte's Hospital. Routine examinations of the p_H , acetoacetic acid content and *B. coli*-inhibiting power of the urine were carried out at frequent intervals on the patients under treatment.

Bacteriostatic test.

The bacteriostatic test was performed by implanting a measured volume of a *B. coli* culture into 3 cc. of filtered urine. A 10 mm.³ sample was explanted into nutrient agar immediately after mixing, and again after 5 hours and after 20 hours at 37°. The growth ratios at 5 hours and at 20 hours were determined by dividing the number of colonies obtained from these latter samples by that obtained from the first sample.

Determination of acetoacetic acid.

In a 4 × $\frac{3}{8}$ in. test-tube, 1 cc. of urine, 0.8 g. of ammonium sulphate, 0.05 cc. of 5 % sodium nitroprusside solution and 0.15 cc. of ammonia (sp-gr. 0.880) are placed. The tube is well shaken and kept in a water-bath at 20° for 7 minutes. If a deep purple colour is obtained, the contents of the tube are washed into a 10 × $\frac{3}{8}$ in. tube with water and quickly diluted until the colour

matches the standard solution contained in a similar tube. If a $\frac{3}{8}$ in. diameter measuring cylinder is available, this may be used, otherwise the volume is found by emptying into a measuring cylinder.

The standard solution is

- 8 cc. of 0.01 % methyl violet in alcohol;
- 2 cc. of 0.01 % basic fuchsin in alcohol;
- 2 cc. of 0.01 % chrysoidin in alcohol;

mixed and diluted to 40 cc. with alcohol.

(If only a pale pink tint develops, the tube should be compared directly with the second standard.)

This preliminary test gives fairly reliable results, but since it is empirical, the result may be confirmed as follows.

The volume to which the test sample has been diluted is noted. 1 cc. of urine is diluted to this volume, and a further Rothera test performed on the diluted urine, using the same conditions as before. This time the tube is compared with a tube of the same bore containing a dye standard which accurately matches the colour produced by 0.02 % acetone under the conditions of the test. The use of this standard obviates the necessity for preparing a fresh standard for each test. It is composed as follows:

- 1.84 cc. of 0.01 % methyl violet in alcohol;
- 0.48 cc. of 0.01 % basic fuchsin in alcohol;
- 2.14 cc. of 0.01 % chrysoidin in alcohol;

mixed and diluted to 20 cc. with alcohol.

If the colour is stronger or weaker than the standard, it is necessary to repeat the test on urine diluted to a greater or smaller extent respectively until the colour is matched. When the final dilution figure has been obtained, the concentration of acetoacetic acid in the urine is obtained by multiplying this figure by 0.004 %. The amount of total acetone *plus* acetoacetic acid is given by multiplying by 0.0045 %. The reason for this is as follows. Acetoacetic acid is five times as sensitive as acetone to the test, and so the 0.02 % acetone standard is equivalent to 0.004 % acetoacetic acid. The figure for acetone *plus* acetoacetic acid is based on the assumption that in fresh ketonic urine 90 % consists of acetoacetic acid and 10 % of acetone. β -Hydroxybutyric acid is not estimated by this method.

The colour standards are stable for several months if stored in the dark. If a number of urines are being tested at the same time, they should be put on test at 1-minute intervals, to allow sufficient time for the dilution of the colour before the next sample becomes due.

A large number of urines were tested both by this method and by the standard method in which interfering substances are removed, the acetone bodies distilled as acetone and estimated iodimetrically. The agreement between the two methods was good.

It soon became evident that in those cases in which a rapid cure was obtained, the urine had a pronounced bacteriostatic action *in vitro*, and contained a high concentration of acetoacetic acid. Normal urines allow a quick outgrowth of implanted *B. coli*, the growth ratio at 5 hours varying from 20 to 100. The growth ratios obtained with ketonic urines varied very greatly, but in general, urines containing up to 0.05 % of acetoacetic acid gave results very similar to those obtained with normal urine. Those containing up to 0.1 % often had a bacteriostatic action for 5 hours but allowed outgrowth at 20 hours, while those with up to 0.2 % frequently were bacteriostatic for 20 hours. Only very occasionally was complete killing of the bacteria obtained in 20 hours at 37°.

Fractionation of active urines.

A mixture of urines which were known to be actively bacteriostatic or which contained a high concentration of acetoacetic acid was fractionated in an endeavour to isolate the growth-inhibiting agent. The *coli*-inhibiting power of

each fraction was tested after removing interfering chemicals, by adding a small amount, representing a known volume of urine, to a filtered normal urine at p_H 5.5, and implanting with *B. coli*. The normal urine was used to supply the necessary nutrient material. In this way, the distribution of the active substance in the fractions was determined.

(1) A batch of 5 litres of urine was concentrated *in vacuo* below 50° to one-tenth of its volume. No detectable loss of activity occurred during concentration.

(2) Excess oxalic acid was then added, the mixture left in the refrigerator overnight and the urea oxalate filtered off. Barium hydroxide was added to the filtrate to remove oxalic acid, the mixture filtered and the filtrate neutralised with sulphuric acid and evaporated to low bulk *in vacuo*. No significant loss of activity occurred from the removal of the urea as oxalate.

(3) 20 % sulphuric acid was then added to the main bulk until it was acid to Congo red, and 5 volumes of acetone were added. After a few hours, the flocculent precipitate consisting of inorganic salts, lipoids and protein derivatives was filtered off. The acetone filtrate was neutralised with barium hydroxide and the acetone removed *in vacuo* without filtering.

The acetone precipitate contained no activity while the acetone-soluble fraction was bacteriostatic to the same degree as the original urine.

(4) The acetone-soluble fraction was again acidified with sulphuric acid to Congo red and 25 % phosphotungstic acid in 5 % sulphuric acid added till no further precipitation occurred. The precipitated phosphotungstates were filtered off and regenerated by grinding with water and barium hydroxide until alkaline to phenolphthalein, filtering and removing barium with sulphuric acid.

The filtrate from the phosphotungstates was treated with baryta until alkaline, filtered and the barium removed as sulphate. A part of each fraction was diluted to a volume equivalent to half the original volume (*i.e.* at twice the original concentration) and tested. The fraction precipitated by phosphotungstic acid was inactive, whereas the soluble fraction gave growth ratios of 0.7 at 5 hours and 1.0 at 20 hours.

(5) The extract at this stage consisted largely of organic acids. It gave no precipitate with mercuric sulphate or lead acetate. Lead acetate and ammonia produced a precipitate, but the soluble lead salt contained the activity. Similarly silver sulphate and also silver sulphate and baryta gave inactive precipitates, leaving the activity concentrated in the soluble fraction.

The lead salt fractionation was carried out on the main bulk.

The fraction from the soluble phosphotungstates was warmed on the water-bath with lead carbonate and then with lead hydroxide until neutral. Barium hydroxide was added until no more precipitation took place, and the precipitated lead salts were filtered off. Both the precipitate and the soluble fraction were freed from lead with hydrogen sulphide and barium with sulphuric acid, neutralised and tested at twice the original concentration. The growth ratios given below show that while the precipitated lead salts (largely lead lactate) had a slight activity, the greater part of the bacteriostatic substance was contained in the soluble fraction.

	Growth ratio 5 hours	Growth ratio 20 hours
Insoluble lead salts	1.3	∞
Soluble lead salts	0.5	0.5

This fraction now consisted very largely of β -hydroxybutyric acid, and the remaining fractionations consisted of separating this substance from impurities.

(6) β -Hydroxybutyric acid can be extracted by ethyl acetate from saturated ammonium sulphate solution. The solution was now saturated with ammonium sulphate, acidified to Congo red with sulphuric acid and extracted six times with ethyl acetate. The ethyl acetate extracts were washed with a small quantity of ammonium sulphate solution, and then with water, dried with sodium sulphate and the solvent removed *in vacuo*. A syrupy liquid was left. Both this and the ammonium sulphate fraction were tested in normal urine at twice the original concentration and gave the following growth ratios:

	Growth ratio 5 hours	Growth ratio 20 hours
Extracted by ethyl acetate	0.5	0.3
Not extracted by ethyl acetate	4.4	∞

Nearly all the activity had passed into the ethyl acetate. At this stage a large part of the material was lost by breakage and it was not possible to determine the equivalent of the fractions in terms of volume of original urine.

(7) Nearly all the salts of β -hydroxybutyric acid are very soluble in water. The zinc calcium salt is one of the least soluble [Shaffer and Marriott, 1913]. This salt was prepared by dividing the gummy acids into two equal portions, neutralising one with lime and the other with zinc carbonate, and mixing. In equivalent concentrations, the acid from the zinc calcium salt was much more active than that from the filtrate.

(8) The methyl ester [Fischer, 1909] is a characteristic derivative of β -hydroxybutyric acid. A small amount of the acid was esterified by dissolving in methyl alcohol, passing in hydrochloric acid gas and working up as described by Fischer. The ester boiled at $72^{\circ}/17$ mm. Fischer's ester boiled at $67-68^{\circ}/13$ mm. Another preparation yielded 0.98 g., boiling at $69^{\circ}/14$ mm. This was quantitatively saponified with cold $N/2$ KOH. Two determinations gave results of 116 and 120 for the equivalent weight (calculated for methyl hydroxybutyrate 118). The activity of the acid recovered from the ester by hydrolysis in 2 % solution in normal urine was as follows.

Growth ratio at 5 hours, 0.9; at 20 hours, 0.1.

(9) A new batch of urine was worked up to replace that lost. It contained 0.08 % of acetoacetic acid, and 0.2 % of β -hydroxybutyric acid by Van Slyke's method of estimation. The urine was not sufficiently ketonic to be bacteriostatic, giving growth ratios of 25 at 5 hours, and ∞ at 20 hours.

Eight litres were concentrated to low bulk, treated with lead carbonate and ammonia, filtered, the filtrate neutralised with sulphuric acid and evaporated to low bulk *in vacuo*. It was then saturated with ammonium sulphate, extracted with ethyl acetate and the solvent removed. The residue was tested at 3-10 times its concentration in the original urine, with the following results:

	Growth ratio 5 hours	Growth ratio 20 hours
3 times concentration	1.9	∞
5 times concentration	0.9	∞
10 times concentration	0.7	30

16 g. of gum were obtained from the 8 litres of urine. It appeared to be about 70 % pure from titration and optical activity measurements, so that the 5- and 10-fold concentrations were equivalent to 0.75 and 1.5 % of β -hydroxybutyric acid.

The zinc calcium salt of this crude acid was prepared and fractionally crystallised from aqueous alcohol. The first crop crystallising out was recrystallised three times, and other crops were worked up to an equivalent degree. The soluble fraction was concentrated and a further crop removed, the soluble fraction decomposed and the free acids isolated. 4.1 g. were obtained. This possessed no bacteriostatic action in normal urine at 0.5 % and 1 % concentration.

%	Growth ratio 5 hours	Growth ratio 20 hours
1	35	∞
0.5	44	∞

The purified zinc calcium salt of β -hydroxybutyric acid was converted into sodium salt. It was dissolved in water, zinc removed by passing in hydrogen sulphide, and after removing the excess sulphide, neutralised to phenolphthalein with NaOH. Sodium oxalate was then added to precipitate all the calcium. After removal of calcium oxalate, the filtrate was evaporated to dryness *in vacuo*, and 5.1 g. of sodium salt were obtained. This was recrystallised from a small quantity of alcohol.

The bacteriostatic powers of several concentrations of the purified sodium salt in normal urine at p_H 5.5 are tabulated below. The percentages are for free acid and not for sodium salt.

%	Growth ratio 5 hours	Growth ratio 20 hours
0.5	0.6	∞
1.0	0.7	0.6
2.0	0.7	0.5
2.0	0.4	0

The figures agree with those obtained for the acid derived from the methyl ester by hydrolysis.

The optical rotation of the sodium salt was $[\alpha]_D -13.8^\circ$. This determination was possible through the kindness of Dr Dudley of the National Institute of Medical Research. The figures quoted in the literature for the optical rotation of sodium β -hydroxybutyrate are $[\alpha]_D -13.8^\circ$ and -14.5° .

The purity of the sodium salt was also checked by preparing the silver salt, and determining the percentage of silver.

0.2489 g. of silver salt yielded 0.1278 g. of silver. This gives a value of 103.9 for the equivalent weight of the acid, the theoretical value being 104.0.

The quinine salt was sparingly soluble in water, and melted at 125° , which agrees with the published figure.

For purposes of comparison, the optically inactive acid was prepared according to the method of Wislicenus [1868] by the reduction of acetoacetic ester with sodium amalgam. The acid was isolated as sodium salt, the bacteriostatic properties of which are tabulated below. The percentages refer to free acid.

%	Growth ratio 5 hours	Growth ratio 20 hours
0.5	0.3	∞
1.0	0.9	∞
2.0	0.9	0.4

The acid was converted into zinc calcium salt, which was crystallised twice, and reconverted into the free acid. The methyl ester prepared from this distilled

at 67–68°/13 mm. and when hydrolysed showed an equivalent weight of 117. The recovered acid was still bacteriostatic, giving the following growth ratios:

	Growth ratio 5 hours	Growth ratio 20 hours
%		
1.0	2.7	∞
2.0	1.0	1.1

Thus both *l*- β -hydroxybutyric acid isolated from ketonic urine, and the *dl*-acid prepared synthetically have an inhibitory effect on the growth of *B. coli*, and indeed of many other organisms.

At the beginning of this investigation, it was not suspected that β -hydroxybutyric acid was the active agent, and no determinations of the amount of this substance in the ketonic urines were made. Since then, estimations of the β -hydroxybutyric acid content (by the method of Van Slyke) and of the bacteriostatic power of a few ketonic urines have been made. The results show that the growth ratios are of the same order as would be expected from the β -hydroxybutyric acid content.

Urine	β -hydroxybutyric acid content %	Acetoacetic acid content %	Growth ratios	
			5 hours	20 hours
A	0.6	0.08	0.6	∞
B	0.7	0.10	1.7	∞
C	1.0	0.12	1.3	∞
D	1.1	0.12	0.5	18
E	1.1	0.14	0.6	25

Since β -hydroxybutyric acid is the active agent it is obviously more rational to assay the urine for this acid rather than for acetoacetic acid. The estimation of β -hydroxybutyric acid is, however, a very lengthy process compared with the colorimetric method for acetoacetic acid, and since the concentrations of both these acids increase with the intensity of the ketosis, the acetoacetic acid content is a good guide to the progress of the treatment.

The above table illustrates that the bacteriostatic power increases with increasing acetoacetic acid concentration.

The effect of acidity on the bactericidal power of ketonic urine is of practical interest. The activity of urine containing β -hydroxybutyric acid at different reactions is shown in the following table:

Concentration of β -hydroxybutyric acid %	p_H	Growth ratios	
		5 hours	20 hours
0.5	5.0	0.1	0
0.5	5.0	0.8	0.3
0.5	5.5	0.6	∞
2.0	5.5	0.4	0
2.0	5.5	0.7	0.5
2.0	6.5	0.8	∞
2.0	7.4	20	∞

The inhibitory power increases considerably as the reaction becomes more acid. The results support to some extent the observation of Helmholz [1932], that ketonic urines are inactive at reactions more alkaline than p_H 5.6. Thus, while 2 % of β -hydroxybutyric acid is actually bactericidal at p_H 5.5, it exerts no appreciable inhibition of growth of *B. coli* at p_H 7.4.

The practical point which emerges is that acidifying salts should be given at the same time as the ketogenic diet, since the greater acidity helps moderate

concentrations to become effective. It is also evident that the presence of acetone bodies cannot give to blood at p_H 7.4 an effective bactericidal power.

The bacteriostatic power of other organic acids was determined, and it was found that they too inhibited the growth of *B. coli* in normal urine at p_H 5.5.

	Growth ratios	
	5 hours	20 hours
2 % acetic acid	0.9	0.4
2 % <i>n</i> -butyric acid	1.3	0.2
2 % lactic acid	4.3	∞
2 % acetoacetic acid	1.3	∞
1 % acetoacetic acid	2.3	∞
0.5 % acetoacetic acid	8.5	∞
2 % acetone	0.8	∞

Acetoacetic acid and acetone are much weaker in their action than β -hydroxybutyric acid, and since they are present in ketonic urine to a much smaller extent than the latter acid, they contribute only a small portion of the total activity.

SUMMARY.

The principal factor inhibiting the growth of bacteria in the urine from patients receiving the ketogenic diet is *l*- β -hydroxybutyric acid.

The activity of this substance increases in proportion to the acidity of the urine.

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REFERENCES.

- Dick (1933). *Proc. Roy. Soc. Med.* **26**, 227.
 Fischer (1909). *Ber. deutsch. chem. Ges.* **42**, 1222.
 Helmholtz (1932). *J. Amer. Med. Assoc.* **98**, 1710.
 — and Clark (1931). *Proc. Staff Meetings, Mayo Clinic*, **6**, 605, 609.
 Shaffer and Marriott (1913). *J. Biol. Chem.* **16**, 268.
 Wislicenus (1868). *Liebig's Ann.* **149**, 205.