III. THE NITROPRUSSIDE REACTION OF BACTERIA.

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(*Received November 21st, 1924.*)

IN the present investigation an attempt has been made to obtain information as to the presence of glutathione in bacterial cells, as distinct from the culture medium, by means of the nitroprusside test [Hopkins, 1921]. Large quantities of various pure cultures of bacteria were grown in glucose broth, or on agar plates, and then freed from the culture medium. The proteins were removed by precipitation and the filtrate was investigated for the nitroprusside reaction by a quantitative method. Absence of the nitroprusside reaction indicates the absence of glutathione, but the positive reaction cannot be taken as final proof of the presence of this substance until more evidence is available.

Preliminary experiments on the nitroprusside reaction of bacteria were performed by one of us (A. B. C.) in 1922. At that time no quantitative method had been described, so the following method was devised. Pure cultures of bacteria were grown in glucose broth in Roux bottles. The broth was removed by centrifugalisation and the bacteria were washed twice with distilled water, and dried in a vacuum desiccator. The dried mass was powdered finely, and a weighed amount was mixed with 10 cc. of a saturated solution of ammonium sulphate and filtered. All the bacteria examined gave a clear colourless filtrate. In each case 0.2 cc. of a freshly prepared 5 % solution of sodium nitroprusside and 0.2 cc. of strong ammonia were added to the filtrate. The colour obtained was at once compared with a deep pink standard colour. If the filtrate gave a pink colour paler than that of the standard, a larger weight of bacteria was used, and in this way the weight of each organism required to give the standard colour was obtained.

Table I. *Preliminary Experiments.*

	Weight of dried organism which gave standard colour under standard conditions
	g.
<i>B. pyocyaneus</i>	0.04
<i>B. prodigiosus</i>	0.08
<i>B. proteus</i>	0.1
<i>B. alcaligenes</i>	0.1
<i>B. fluorescens</i>	0.1
<i>Staphylococcus aureus</i>	negative
<i>Sarcina aurantiaca</i>	"

The results of this experiment show that *Staphylococcus aureus* and *Sarcina aurantiaca* were negative, that is to say, that even large weights failed to give a pink as deep as the standard colour. On the other hand, the other bacteria all gave strong nitroprusside reactions. If the substance responsible for this reaction is glutathione, the actual percentage calculated as dry weight works out as 0.5 % for *B. pyocyaneus*, 0.25 % for *B. prodigiosus* and 0.2 % for the other three positive bacteria. These figures were obtained by matching the standard colour with a known solution of reduced glutathione, which had been treated with nitroprusside as described above.

Since the completion of the preliminary experiments, Tunncliffe¹ has described a quantitative method for the estimation of glutathione in muscle. Tunncliffe found that the SH group of reduced glutathione is quantitatively oxidised by iodine. He precipitated the proteins of muscle with trichloroacetic acid and titrated the filtrate with *N*/100 iodine, using the nitroprusside test as an outside indicator. We tried to apply this method to bacteria. *B. alcaligenes* was grown for fifteen hours on forty large agar plates, then carefully scraped off, and ground in a mortar with distilled water until an even emulsion was obtained. 1 cc. was placed in a small weighed centrifuge tube, centrifuged to remove excess water, then dried in a vacuum desiccator and weighed. In this way, the dry weight of bacteria in each cc. of emulsion was obtained. Another measured volume of the bacterial emulsion (usually 10 or 20 cc.) was precipitated with an equal volume of 20 % trichloroacetic acid. The mixture was ground in a mortar and then quantitatively transferred to a Buchner funnel, and filtered on a pump. The precipitate was removed, washed with distilled water, and again filtered. The two filtrates were added together and titrated with 0.00888 *N* iodine from a micro-burette, using the nitroprusside test as an outside indicator. Other bacteria were not so easy to manipulate, and solid trichloroacetic acid had to be used for precipitation. With *B. proteus* and *Sarcina aurantiaca*, filtration was so slow that it had to be abandoned in favour of centrifugation, the deposit being washed with distilled water, and the washings added to the liquid to be titrated. Several organisms, such as *S. acidi lactici* and the anaerobe *B. sporogenes* were grown on glucose broth instead of on agar plates, in order to obtain heavier growth. In all cases young cultures were used. The dry weight of the bacteria used for titration, calculated from the weight of organisms in 1 cc. of emulsion, is given with the results to show that negative results were not due to lack of material. From the titration result, the number of milli-equivalents of iodine required for 1 g. of each organism (dry weight) was calculated.

The results show that *B. alcaligenes* and *B. proteus* gave a strong nitroprusside reaction, compared with which the other bacteria examined were practically negative. This means that a drop of the trichloroacetic acid filtrate of the latter gave no nitroprusside reaction. If, however, 5 or 10 cc. of the filtrate was examined, most of the bacteria gave a very faint nitroprusside

¹ In the press.

Table II. *Trichloroacetic Acid Method.*

	Dry weight of bacteria used g.	Number of milli- equivalents of iodine for 1 g. bacteria (dry weight)
<i>B. alcaligenes</i>	1.5072	0.019
"	1.5885	0.018
<i>B. proteus</i>	0.3577	0.018
"	0.5890	0.020
<i>B. coli</i>	0.7260	0
<i>B. sporogenes</i>	0.462	0
<i>Streptococcus acidilactici</i>	0.9240	0
<i>Sarcina aurantiaca</i>	0.2639	0
<i>Staphylococcus aureus</i>	1.1235	0
Timothy Grass bacillus	0.7935	0

reaction. Since very small amounts of glutathione give the reaction, the amount of this substance present in these bacteria must be considered practically negligible. Even when 10 cc. of the filtrate were examined, the Timothy Grass bacillus gave no trace of the nitroprusside reaction, and it is possible that the fatty envelope of this organism may have prevented the escape of any glutathione that might be in the cell. Possibly also in the case of the other negative bacteria glutathione might be present in the cells and yet escape detection, but this is unlikely since the organisms were ground up with trichloroacetic acid. The negative result obtained for the anaerobe *B. sporogenes* agrees with McLeod and Gordon's observation that the anaerobe *B. welchii*, when scraped off the surface of a solid medium, gave no nitroprusside reaction [McLeod and Gordon, 1924].

Three strongly pigmented bacteria, *B. pyocyaneus*, *B. fluorescens* and *B. prodigiosus*, could not be investigated by Tunnicliffe's method because in each case the pigment interfered with the titration. The earlier experiments had shown that, when these organisms were precipitated with saturated ammonium sulphate, a colourless filtrate was obtained, so that this method of precipitation was now used in conjunction with the iodine titration. Control experiments showed that the sample of ammonium sulphate used contained no substance capable of reacting with iodine. However, an objection to the use of this reagent is that, although the saturated solution is acid, after being ground up with bacteria the resulting mass is not acid enough to ensure the stability of the SH group. Tunnicliffe and Dixon [1923] showed that the SH group autoxidises far more rapidly in neutral than in acid solution. In order to lessen the possibility of such a decomposition, a trace of sulphuric acid was added to the ammonium sulphate filtrate. Even with this precaution, however, this method has given us slightly lower results than the trichloroacetic acid method. A comparison of the two methods gave the following results for *B. alcaligenes*:

Trichloroacetic acid method: 0.020 milli-equivalents of iodine per g. of dried bacteria.

Ammonium sulphate method: 0.016 milli-equivalents of iodine per g. of dried bacteria.

In default of a better method, the highly-pigmented bacteria were examined by this method.

Table III. *Saturated Ammonium Sulphate Method.*

	Dry weight of bacteria used g.	Number of milli- equivalents of iodine per g. of bacteria (dry weight)
<i>B. pyocyaneus</i>	0.8586	0.019
<i>B. prodigiosus</i>	0.5475	0.018
<i>B. fluorescens</i>	0.6556	0.014
<i>B. coli</i>	0.6928	0

The results show that the three highly-pigmented bacteria, like *B. alcaligenes* and *B. proteus*, contain a considerable amount of the substance responsible for the nitroprusside test. If this substance is glutathione, the percentages can be worked out by Tunnicliffe's experimental data. He found that 1 cc. of *N*/100 iodine reacts with 0.0025 g. reduced glutathione. This gives a value of about 0.47 % for the glutathione content of *B. alcaligenes*, *B. proteus*, *B. pyocyaneus* and *B. prodigiosus*, and 0.35 % for *B. fluorescens*. It will be noticed that these results agree fairly well with those of the preliminary experiments. In each case the same five bacteria gave strong nitroprusside tests, and *Staphylococcus* and *Sarcina* were negative.

At this point we should like to emphasise the fact that at present we have not enough evidence to state that the nitroprusside reaction of bacteria is due to the presence of glutathione, since certain substances other than glutathione also give this reaction. Besides various substances which do not contain sulphur, cysteine, which like glutathione contains the SH group, gives the nitroprusside reaction. Also H_2S in dilute solution gives the pinkish-purple colour that is characteristic of reduced glutathione, although with stronger solutions a deep blue colour is obtained. For this reason, any nitroprusside test performed in the presence of H_2S cannot be considered as evidence of the presence of glutathione.

McLeod and Gordon¹ tested broth cultures of various bacteria for the nitroprusside reaction, but, except in the case of *B. welchii*, they do not appear to have freed the bacteria from the culture medium before performing the test. An obvious objection to this method is that broth cultures of many bacteria contain large quantities of H_2S . However, McLeod and Gordon consider that their positive results are not due to this substance, because the reaction was little, or not at all, reduced by prolonged boiling.

Our method of obtaining bacteria free from H_2S was, firstly, to separate them from the culture medium. Another precaution adopted was the addition of glucose to the broth, as we found that this appeared to lessen or inhibit the production of H_2S by certain aerobes. Moreover, in our experiments, after precipitation of the proteins, the strongly acid filtrate was allowed to boil

¹ Practically all the experiments described in this paper had been completed before the publication of McLeod and Gordon's paper.

under reduced pressure on the water pump, and in this way any trace of H_2S would be removed. Proof that this was actually the case is supplied by the results obtained for the anaerobe *B. sporogenes*. This organism formed large quantities of H_2S when grown on glucose broth, and traces of H_2S still remained even after the bacteria had been freed from broth and washed. Yet the trichloroacetic acid filtrate of this organism gave no trace of nitroprusside reaction. Further proof of this point was obtained in the following way. *B. alcaligenes* was grown on agar, the bacteria were scraped off the plates, precipitated with trichloroacetic acid and filtered. Two equal portions of the filtrate were placed in small flasks. Through one of these (Flask A) a stream of nitrogen was bubbled for two hours, the other (Flask B) being placed beside it as a control. Both solutions were then titrated with iodine with the following result.

Titration result.

Flask A (after nitrogen)	...	2.66 cc. 0.00888 N iodine.
Flask B (control)		2.62 ,, 0.00888 N ,,

If the nitroprusside test had been due to H_2S Flask A would have given a lower result, as some of the H_2S would have been removed by bubbling nitrogen through this acid solution. Since the results are practically identical, H_2S cannot be responsible for the nitroprusside reaction of *B. alcaligenes*.

DISCUSSION OF RESULTS.

It may be noted that the presence of a substance capable of giving the nitroprusside reaction bears no relation to the oxygen uptake [Callow, 1924] of the organisms investigated. Thus, although *S. acidi lactici* and the anaerobe *B. sporogenes*, which lack the nitroprusside substance, both show practically no oxygen uptake, yet *Staphylococcus aureus* and *Sarcina aurantiaca*, which also lack the substance, both take up large quantities of oxygen. An interesting point is that all the bacteria that give the nitroprusside reaction, under the conditions described, show particularly luxuriant growth on ordinary laboratory media; for instance, on the surface of an agar plate, the growth of these bacteria is considerably heavier than in the case of the other bacteria investigated.

McLeod and Gordon [1924] consider that their results show that the positive reaction obtained with certain *broth cultures* is merely due to the fact that these bacteria reduce the oxidised glutathione which they state is present in the uninoculated broth. They further state that they consider that bacteria are not themselves capable of producing glutathione, or substances allied to it that react with nitroprusside. We cannot yet state whether or not bacteria contain glutathione, but our results show that certain bacteria undoubtedly contain a substance which gives the nitroprusside reaction, and which, like glutathione, is soluble in trichloroacetic acid and can be oxidised by iodine. (Further experiments on this point are in progress and will shortly be

published.) On the other hand, we can state that our negative bacteria either lack glutathione, or else their glutathione escapes detection by the methods used in this investigation.

SUMMARY.

1. Bacteria were grown in bulk and separated from the culture medium. The proteins were precipitated with trichloroacetic acid or saturated ammonium sulphate, and the filtrate was examined for the nitroprusside reaction by a quantitative method.

2. *B. coli*, *B. sporogenes*, *Sarcina aurantiaca*, *Staphylococcus aureus*, *Streptococcus acidilactici* and the Timothy Grass bacillus gave practically no nitroprusside reaction and can therefore be considered to lack glutathione, unless this substance is present in the cells and escapes detection by the methods used.

3. *B. alcaligenes*, *B. proteus*, *B. pyocyaneus*, *B. prodigiosus* and *B. fluorescens* gave strong nitroprusside reactions, the iodine equivalents of which are given.

4. The substance which is responsible for the nitroprusside reaction has not yet been identified, but experiments with *B. alcaligenes* show that it is not H_2S .

In conclusion we gladly take this opportunity of thanking Professor F. Gowland Hopkins, F.R.S., for his encouragement and valuable criticism during the progress of this work. One of us (A. B. C.) is indebted to the Medical Research Council for a whole-time grant.

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