Microbiology. — *Some remarks on the reduction intensity of living cells.* By A. J. KLUYVER and J. C. HOOGERHEIDE.

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Ever since micro-organisms have been studied attention has been drawn to the reduction phenomena which are caused by their activities. Already in 1844 HELMHOLTZ [1]) observed that litmus was decolorized in a medium containing putrifying proteins and he clearly proved the reduction character of this change by showing that on shaking the liquid with air the original colour was again obtained.

The classical researches of EHRLICH [2]) on the avidity for oxygen of the various organs of the animal body mark a milestone in the development of our knowledge of biological reductions, because they gave a convincing demonstration of the usefulness of certain dyes for the differentiation of the reduction intensities of different cells.

From this time on numerous observations regarding the behaviour of living cells towards various dyes have been recorded, but a just evaluation of the results obtained has been only made possible by the fundamental studies of CLARK and collaborators [3]) on the principles underlying the oxidation and reduction of a great number of so-called redox indicators.

Moreover, building on some preliminary observations made by POTTER [4]) and by GILLESPIE [5]), the last mentioned investigators have emphasized that a more exact quantitative determination of the reduction intensity of living cells could probably be based on the direct electrometric measurement of oxidation-reduction potentials which manifest themselves in suspensions and in culture media of these cells.

The work of CLARK et al. has greatly stimulated the interest in this field and in later years very numerous publications have appeared which deal with oxidation reduction potentials in their relation with biological systems; we may refer here to the monographs of MICHAELIS, WURMSER and HEWITT in which a survey of this work has been given [6]).

A closer inspection of the literature in question shows, however, that

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1) H. HELMHOLTZ, Journ. f. prakt. Chem. **31**, 429 (1844).

2) P. EHRLICH, Das Sauerstoff-Bedürfnis des Organismus. Eine farben-analytische Studie. Berlin (1885).

3) W. MANSFIELD CLARK et al., Studies on Oxidation-Reduction I—X, U. S. Public Health Service, Hyg. Lab. Bull. **151** (1928) and subsequent papers.

4) M. C. POTTER, Proc. Royal Soc. Ser. B., **84**, 260 (1911).

5) L. J. GILLESPIE, Soil Science, **9**, 199 (1920).

6) L. MICHAELIS, Oxydations-Reductions-Potentiale. 2te Aufl., Berlin (1933); R. WURMSER, Oxydations et réductions. Paris (1930); R. WURMSER, L'électroactivité dans la chimie des cellules. Paris (1935); L. F. HEWITT, Oxidation-reduction potentials in bacteriology and biochemistry. 3rd Ed., London (1935).

by far the greater part of these publications bears a more or less empirical character. They reveal that in culture media containing growing micro-organisms remarkable changes in oxidation-reduction potentials occur. Yet, as a rule, the authors fail to connect the results of their observations with any characteristic property of the cells under consideration. If we may ascribe to the potentials observed any significance for the evaluation of the reduction intensity of cells then the conclusion can only be that this property is liable to considerable variations under the influence of several badly known factors.

There is, however, a restricted number of investigations in which a direct relation between the potentials observed and the chemical nature of the metabolism of the cells is established beyond doubt. We will cite here only the papers of QUASTEL and WHETHAM[1]), LEHMANN[2]), BORSOOK and SCHOTT[3]), WURMSER and MAYER[4]) and of SZENT-GYÖRGYI[5]).

Since recent development of our knowledge of biochemical conversions has thrown a clear light on the preponderant rôle of oxidation-reductions in these processes, it seems indeed probable that the metabolic processes will play an essential part in the establishment of the potential difference between a noble metal electrode and the medium of the cells.

A more systematic study of the relation between the metabolism of various cells and redox potentials occurring in their media appeared, therefore, to be of real interest.

It is obvious that for studies of this nature it will offer many advantages if the activities of the cells are examined in a medium of a simple, well-defined composition. Under these conditions the nature of the prevalent metabolic processes can be established with certainty and only then a clear answer to the question whether these processes determine the value of the redox potentials in the media can be expected.

Experiments performed in this laboratory by ELEMA[6]) have shown definitely, that indeed in suspensions of denitrifying bacteria in a medium containing only nitrate, phosphate and a simple organic compound like ethyl alcohol reproducible potentials can be determined and that the value of these potentials is clearly dependent on the nature of the katabolic processes of the bacteria.

A second proof for the existence of such a relation was given by the present authors[7]) in an investigation of the redox potentials occurring in

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1) J. H. QUASTEL and M. D. WHETHAM, Biochem. J., **18**, 519 (1924).

2) J. LEHMANN, Skand. Arch. f. Physiol., **46**, 339 (1925).

3) H. BORSOOK and H. F. SCHOTT, Journ. Biol. Chem., **92**, 535 (1931).

4) R. WURMSER et N. MAYER, Compt. rend. Acad. Sc., **195**, 81 (1932).

5) A. SZENT-GYÖRGYI, Zeitschr. f. physiol. Chem., **217**, 51 (1933).

6) B. ELEMA, De bepaling van de oxydatie-reductiepotentiaal in bacteriëncultures en hare beteekenis voor de stofwisseling. Delft (1932); B. ELEMA, A. J. KLUYVER and J. W. VAN DALFSEN, Biochem. Zeitschr., **270**, 317 (1934).

7) A. J. KLUYVER und J. C. HOOGERHEIDE, Biochem. Zeitschr., **272**, 197 (1934); J. C. HOOGERHEIDE, Bijdrage tot de kennis van de reactie van Pasteur. Delft (1935).

suspensions of yeast cells under conditions of full metabolic activity. Here it was found that the values of the potentials observed were determined by the relative intensities of the two katabolic processes of the yeast, i.e., fermentation and respiration.

The question now arises in how far the potentials observed in these experiments may be considered as characteristic for the reduction intensity of the cells under the conditions of the experiment, i.e., for the ability of the cells to reduce or not to reduce under these conditions reversible redox systems with normal potentials situated respectivily either higher or lower than the potentials measured.

Although in our preceding publications no definite opinion has been expressed as to this point, yet it is easily understood that such an assumption has been tacitly made. At first we too have been inclined to subscribe to this view. The results of a recent investigation by FROMAGEOT and DESNUELLE [1]) — more particularly the demonstration of the ability of fermenting yeast to reduce nil blue — have, however, at once thrown considerable doubt as to the validity of the said conclusion. Therefore we have decided to examine this point in more detail. The results of our researches are briefly discussed below.

It seems unquestionable that, if a noble metal electrode on immersion in an aqueous medium, shows a constant and reproducible potential difference against this medium, the latter will contain a reversible redox system in a state of partial reduction [2]). Now in the majority of the experiments with the denitrifying bacteria and with the yeast cells referred to above no such redox indicator had been added, the only constituents of the media employed being either substances like ethyl alcohol, nitrates and phosphates (denitrifying bacteria) or sugar and phosphates (yeast cells). If, therefore, in these media too characteristic potentials manifest themselves, it must be concluded that in these cases reversible redox systems have been excreted by the cells in the suspension. This conclusion has indeed been drawn by ELEMA (l.c.) who at the same time introduced the term "bio-indicator" to denote the systems in question. Experimental proof for the correctness of this assumption was brought in the paper by ELEMA, KLUYVER and VAN DALFSEN (l.c.) and later also by YUDKIN [3]).

When on the other hand one observes that the potentials measured at the electrode vary with the metabolic activities of the cells present in the

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[1]) CL. FROMAGEOT und P. DESNUELLE, Biochem. Zeitschr., **279**, 34 (1935).

[2]) It is a fact of common experience that a given redox system only determines a reproducible potential at a noble metal electrode as long as the equilibrium between the oxidized and reduced component of the system is not too much shifted to one side. In practice, working with low concentrations of the redox system, at least about 5% of the system should be present either in its oxidized or in its reduced state. This requirement limits the potential determining activity of a given redox system to a range of about 30 millivolt above or below its normal potential.

[3]) J. YUDKIN, Biochem. Journ., **29**, 1130 (1935).

medium, one cannot escape the conclusion that the electro-active system responsible for the potential observed enters the cells in question and that the state of equilibrium between the oxidized and the reduced component of the system will be changed on the surface of the cell catalysts.

These considerations imply that an electrometrically determined potential will only then be an expression for the reduction intensity of the cells under the conditions of the experiments, in so far as the medium contains a redox system answering the two requirements formulated above, namely, that the system is present in a partially reduced state and is capable of entering the cells.

Now the results obtained in the yeast experiments, showed that indeed yeast cells do excrete such redox systems, the normal potentials of which obviously cover a wide range. For at a $p_H = 5{,}4$, dependent on the degree to which either respiration or fermentation prevailed, values of E_h had been observed varying between + 300 and + 60 millivolt.

Nevertheless there seems to be no reasonable ground why living cells should excrete such systems over the whole range covered by the variations in reduction intensity of these cells. This consideration has induced us to repeat our experiments on the redox potentials observable in the media of yeast cells under conditions of full metabolic activity but this time after addition of small quantities of suitable redox indicators (dyes of known normal potentials) to the medium.

In the first place attention has been given to the redox potentials occurring in yeast suspensions in phosphate-buffer ($p_H = 5{,}4$) containing 5 % glucose under completely anaerobic conditions. It was ascertained beforehand that the addition of the various redox systems in the very low concentration employed did not alter the rate of fermentation to any appreciable extent.

It seemed desirable to test the influence of a large series of redox indicators, the normal potentials of which at $p_H = 5{,}4$ varied between + 150 and —236 millivolt.

The results of these experiments are collected in the following table to which may be remarked that the final p_H in the media proved to be 5,2.

At first sight one is struck by the diversity of the potentials measured in the various experiments. A closer inspection shows, however, that in the majority of cases a potential is reached which lies in the region between +40 and +90 millivolt, that is the same region which was found in our former experiments in the absence of added indicators. It is especially noteworthy that this applies for all cases in which indicators with high normal potentials (from 80 millivolt upwards) or with low normal potentials (from —74 millivolt downwards) are used.

This indicates that these dyes in the potential region as determined by the fermentation occur in the nearly completely oxidized or in the nearly completely reduced state respectively and for this reason are unable to control the electrode.

Redox indicator	E_0' in Millivolt at $p_H = 5.4$	E_h in Millivolt (after 20 hours)
Prune	150	90
Thionine	118	78
Gallocyanine	116	42
Toluidine blue	86	67
Methylene blue	80	49
Pyocyanine	56	— 6
Janus green	(46)—144	—12
Indigo tetrasulphonate	36	118
Indigo trisulphonate	6	83
Chlororaphine	— 18	—40
Nil blue	— 34	—43
Gallophenine	— 36	67
Indigo disulphonate	— 36	73
Brilliant alizarine blue	— 74	88
Anthrachinone 1—5 disulphonate	— 84	90
Phenosafranine	—159	—
Rosinduline	—185	42
Neutral red	—236	50

Obviously the lowest values observed, viz. those in the presence of chlororaphine and of nil blue, must be considered as the limit of the reduction intensity of the yeast cells under the condition of alcoholic fermentation. For if indeed lower potentials than —43 millivolt (at $p_H = 5{,}2$) could be brought about by the yeast there is no reasonable ground why these should not be indicated by the electrode since both indicators (especially nil blue) are still able to control it at markedly lower potentials (Cf. note 2, page 243).

If we accept this view part of the remaining abnormal potentials can be readily explained. For at this particular reduction level indicators like pyocyanine, Janus green (first reduction step) and indigo tetrasulphonate will occur in a too highly reduced state to be still able of control of the electrode.

Only the behaviour of indigo trisulphonate, gallophenine and indigo disulphonate seems to contradict the statement made. The explanation for this apparent deviation was, however, soon found in the experimental

proof that these indicators — in contrast to chlororaphine and nil blue — do not enter the cells; they do not belong to the group of vital stains as is corroborated by the findings of other investigators.

The same value for E_h of about —40 millivolt (at $p_H = 5{,}2$) has since approximately been found back in numerous experiments, in which different yeast species and also other sugars than glucose have been applied. The investigation of *Pseudomonas Lindneri,* a bacterium which brings about a nearly pure alcoholic fermentation of glucose, led to the same result.

All this seems to warrant the conclusion that the said value, corresponding to a $r_{H_2} = 9{,}0$, is characteristic for the reduction intensity of all cells under the conditions of alcoholic fermentation.

Since the foregoing experiments had shown convincingly that in determining reduction intensities it is inadmissible to rely in all instances on the natural redox systems excreted by the cells, we have also repeated our former experiments regarding the potentials occurring in suspensions of yeast cells in contact with oxygen, i.e. under conditions in which besides fermentation also respiration proceeds.

It was found that in these cases addition of suitable redox indicators did not affect the potential levels observed in the absence of these indicators to an appreciable extent. Apparently in this, much higher situated, potential region the natural redox systems excreted by the yeast cells suffice to control the electrode. The potentials observed may, therefore, be considered to be characteristic for the reduction intensities of the yeast cells under the special metabolic conditions of each experiment.

It seemed of interest to make similar observations in suspensions of cells which are characterized by another, yet also simple, way of anaerobic sugar breakdown.

As such we have chosen homofermentative lactic acid bacteria, of which numerous species belonging to various morphological groups (genera: *Thermobacterium, Streptobacterium* and *Streptococcus*) have been examined. All these bacteria convert glucose almost quantitatively into lactic acid.

The results will only very briefly be reported here.

In phosphate buffer with 2 % glucose but without addition of redox systems the potentials sank only gradually and the levels attained proved to be not well reproducible. This seemed to indicate that in this case too for the determination of the reduction intensity of the cells it was not permitted to rely on the natural redox systems excreted by the bacteria. Therefore the experiments were repeated but now after addition of a suitable mixture of redox indicators with decreasing normal potentials. Under these conditions a very definite reduction level was quickly attained.

In presence of a mixture of nil blue, Janus green, phenosafranine and neutral red (in a concentration of 0,00025 % each) the potentials listed

in the following table were observed. Since owing to the production of a rather considerable quantity of lactic acid, it proved impossible to maintain fully the initial p_H, attention should be given more especially to the recalculation of the reduction intensity in terms of r_{H_2} (last column).

Bacterial species	E_h in millivolt at the end of the experiment	Final p_H	r_{H_2}
Thermobacterium Delbrücki a	—163	5.5	5.6
„ „ *b*	—156	5.5	5.8
Thermobacterium bulgaricum			
Streptobacterium plantarum a	—190	5.9	5 5
Streptobacterium casei b	—186	5.9	5.6
Streptococcus lactis a	—168	5.4	5.2
„ „ *b*	—208	6.0	5.4
Streptococcus cremoris a	—192	5.8	5.2
Streptococcus faecium a	—228	6.3	5.0
„ „ *b*	—180	5.6	5.2
Streptococcus bovis a	—189	5.6	4.9
„ „ *b*	—222	6.3	5.2
Streptococcus amylolactis a	—151	5.0	5.0
„ „ *b*	— 95	4.1	5.0
Streptococcus mastitidis a	—210	6.0	5.0
„ „ *b*	—214	6.1	5.1
Streptococcus liquefaciens	—201	6.1	5.5
Streptococcus agilis	—173	5.8	5.8

The figures obtained do not leave any doubt that the reduction intensity of all cells under the conditions of active lactic acid fermentation can be represented by a value of r_{H_2} between 5,0 and 6,0. These values contrast well with the values of r_{H_2} between 8,4 and 9,0 as found for cells under the conditions of alcoholic fermentation.

The result that all lactic acid bacteria examined, independent of their specific properties, lead to practically one and the same reduction level may be deemed to be rather remarkable. For in bacteriological literature several instances can be found which seem to contradict this statement.

Thus SHERMAN and ALBUS [1]), AVERY [2]), RUDOLPH [3]), a.o. have applied the different behaviour of various *Streptococcus* species in milk containing either litmus, or methylene blue, or neutral red for the differentiation of humane and bovine streptococci from those of other groups. CLARK [4]) in his fundamental paper on methylene blue refers to these investigations and states explicitly that the differences observed should now be expressed in numerical values for reduction intensity of the various species.

In order to elucidate this contradiction we have made a special inquiry into this matter. Although we must refrain from giving any details here, we may state that the results obtained show clearly that in no instances are the differences observed visually due to differences in reduction intensity of the cells in question.

From the experiments reported and the considerations given we wish to conclude:

1. The reduction intensity of the living cell is only in a restricted sense a specific property of the cell, in so far as this intensity is at any moment determined by the nature of the metabolic processes occurring in the cell.

2. The oxidation-reduction potentials in media of living cells under well-defined metabolic conditions may be considered characteristic for the reduction intensity of the cells under these conditions, provided that the medium contains a partially reduced redox system, capable of penetrating the cells.

3. Although, as a rule, living cells excrete into their medium several reversible redox systems which together often are able to control an electrode over a wide region of potentials, yet is is not permitted to accept that in all cases such systems will cover the whole range of the reduction intensity of the cells. Therefore, a redox potential measured electrometrically in the medium is only then characteristic for the reduction intensity of the cells under the conditions of the experiment, if the addition of a small quantity of a redox system answering the requirements formulated under 2 does not alter the potential.

A more extensive publication, in which full particulars regarding the experiments will be given, will appear elsewhere.

Laboratorium voor Microbiologie der
Technische Hoogeschool te Delft.

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[1]) J. M. SHERMAN and W. R. ALBUS, Journ. of Bact., **3**, 168 (1918).

[2]) R. C. AVERY, Bact. Abstracts, **6**, 31 (1922).

[3]) J. RUDOLF, Centralbl. f. Bakt. I Abt., **100**, 147 (1926).

[4]) W. MANSFIELD CLARK, Studies on Oxidation-Reduction, VIII, Public Health Reports, Washington **40**, 1131 (1925).