INTRACELLULAR OXIDATION-REDUCTION STUDIES.

I. Reduction Potentials of Amœba dubia by Micro Injection of Indicators.*

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INTRODUCTION.

The reductive power of living cells, tissues and bacterial cultures, as evidenced by their action on colored indicators, has been the subject of much study since the observation by von Helmholtz in 1843. With the passage of years there has accumulated a large amount of evidence, more or less contradictory in some respects but quite unanimous as to the cardinal fact that the living cell exerts a truly remarkable reducing power. In general, the earlier observers, and a number of recent ones, made no sharp distinction between the intensity of reduction, the quantity and *time*, which are the dimensions of reducing *power*.

It was not until 1920 that Gillespie presented data suggesting that the reducing intensity of bacterial cultures might be measurable in terms of electrode potential. Shortly thereafter Clark (1920), by measuring the equilibrium potentials between methylene blue and methylene white and between indigo blue and indigo white, gave quantitative values to the different reduction intensities indicated by these systems; and at about the same time (1920) he also presented a comprehensive basis for interpreting, in terms of electrode potential, the results given by the biological reduction of reversible oxidation-reduction systems. This pioneer work opened the way to the study and elaboration at the Hygienic Laboratory of a number of reversible oxidation-reduction indicators. The assortment of com-

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pounds now available is, as we shall see, by no means exhaustive nor entirely satisfactory; but it appears to be adequate for a preliminary survey of certain limited aspects of the biological problem.

Following the hint given by Gillespie, a considerable amount of work has been done by Clark and Cohen (1922, 1925, 1928) in an effort to correlate the reduction potentials attained in bacterial cultures and cell suspensions with certain phases of metabolism and life history. Some of the results have been summarized in the paper by Cannan, Cohen and Clark (1926). In this contribution a general correlation was shown between the reduction potential of a cell suspension, the cellular reduction of a particular dye and the reduction potential of the same dye as determined in pure solution. It was also found that cell suspensions are poorly poised with respect to the electromotively active material present at any moment; but that active material is slowly mobilized by cell activity from some large reserve, and the electromotive activity is paralleled by a reductive activity toward appropriate indicators.

A more direct attack upon the problem of cellular reduction is recorded in the admirable studies made by Needham and Needham (1925, 1926). They were the first to inject reversible oxidation-reduction indicators into living cells and to observe microscopically the color changes undergone by these compounds. In observations on *Amœba proteus* they report that this cell is capable not only of reducing the oxidized forms of indicators of more positive potential but also of oxidizing the leuco form of indicators of oxidation-reduction potential lower than its own. This led to the conclusion that this organism is able to maintain a fairly constant reduction potential at a zone lying somewhere between rH 17 and 19.[1] Needham and Needham claimed that when the ameba was studied in atmospheres of nitrogen, hydrogen or oxygen, they could discover no essential change in the picture, and they therefore concluded that the rH of the ameba is probably widely independent of the concentration of oxygen in the external atmosphere.

Other organisms and cells were studied by these authors and by Rapkine and Wurmser (1926, 1927), and additional support was adduced for the thesis that each type of cell maintains a more or less characteristic and independent internal reduction potential.

This announcement of the existence of a fairly well poised oxidation-reduction system within the cell stands in contrast to the conditions found by Cannan, Cohen and Clark in bacterial cultures which showed an increasing reduction intensity as the neutralizing effect of oxygen was eliminated. A further study of the problem therefore seemed appropriate.

In the experiments to be described we sought to learn how far the

[1] The reader is referred to the papers by Clark and Cohen for discussion of the elementary aspects of reversible oxidation-reduction equilibria.

microinjection technic in its present stage of development could be applied to the problem, and to what degree we would repeat and possibly extend the observations of the Needhams upon the ameba. To this end each of a series of indicators, in its oxidized or reduced state, was injected under controlled conditions of oxygen access. It was also of importance to determine the relative toxicity of the indicators, to provide a guide for purification of the compounds and for the synthesis of new ones.

EXPERIMENTAL.

Amebæ.—The experiments reported in this paper were upon *Amœba dubia* principally. In a subsequent communication experiments will be reported on certain marine ova, fertilized and unfertilized. A number of observations were made upon *A. proteus* and the results were always in agreement with those obtained with the other species. We confined our attention largely to *A. dubia* because its interior is less granular and its pellicle is more easily pierced by the micro pipet.

The amebæ were supplied by Dr. J. A. Dawson of Harvard University; as soon as received, the original culture was diluted with glass-distilled water to which were added 2 or 3 boiled wheat grains. The cultures were stored in the ice box and were thus maintained in excellent condition.

The cells were washed once with distilled water and usually placed in groups of about 4 to 8 in hanging drops. We found it convenient to mark off with paraffin wax two rows of contiguous circles on the cover-slip and thus to keep separate different lots of cells as well as drops of reagents and wash water.

The internal pH of both *A. dubia* and *A. proteus* has been found to be 6.9 ± 0.1 by Chambers, Pollack and Hiller (1927), who state that these cells maintain this internal pH as long as they are in normal condition, and that they possess an appreciable buffering capacity. Significant change in internal pH is associated with death of the cell. Thus the natural buffering ability of the protoplasm made it unnecessary to employ buffered dye solutions for injection. Some injections were tried with indicator dissolved in buffer, but disturbing salt effects were encountered. We shall see that even in aqueous solution, certain compounds produced salt effects due to inorganic ions occasionally present as impurities.

Injection Apparatus.—The injections were made with the improved double micromanipulator apparatus (Chambers, 1928). One manipulator carried a micro needle to move the ameba into proper position and to hold it in place and the other carried a micro pipet for injecting the cell.

The apparatus with a straight pipet, described by Chambers (1922–23), was employed for injections made in the presence of air.

For injections under strict anaerobiosis, a moist chamber was used which was provided with a mercury seal; in this case the shank of the pipet was given a

U-shape (see Fig. 1) to dive under the mercury seal in the manner described by Barber (1914) and by Needham and Needham (1926).

The whole system, from the syringe to the shank of the micro pipet, was filled with deaerated water; only a small air space was left in the pipet proper. The oxygen contamination from this source was eliminated later when necessary in the sealed chamber by replacing the air with the gas of the chamber and washing the pipet several times with the injection fluid.

The hermetic moist chamber for injections under anaerobiosis was made of glass, the joints being cemented together with balsam (see Fig. 1). It consisted of two troughs, one to hold mercury for sealing the chamber and the other to serve as the moist chamber proper. The latter contained a gas inlet and an outlet. Through the axis of the inlet there passed a capillary tube used for delivering the reduced dye into the moist chamber under complete exclusion of oxygen. The chamber was 30 × 40 mm., and 15 mm. high; and the total volume, including the gas space over the mercury seal, was about 25 cc. Purified nitrogen was passed through at a rate varying from 50 to 250 cc. per minute. The chamber was roofed over by a cover-slip (40 × 60 mm.) and sealed by strips of mica, all heavily coated with vaseline and pressed firmly on the broad contact surfaces. The seal was tested for tightness and found to withstand a pressure of at least 2 to 3 cm. of water. The diffusion of oxygen through the vaselined seals was of a negligible order, or else it was effectually eliminated by the constant stream of purified nitrogen passing through the chamber, for drops of reduced indicator often remained uncolored on the cover-glass for several hours during an experiment.

Purification of Nitrogen.—An essential part of the experimental technic is the particular care to eliminate leakage of atmospheric oxygen into the anaerobic system. Unprotected rubber connections are dangerous because oxygen diffuses through the rubber in quantities sufficient to upset conditions and lead to erroneous conclusions. This point needs emphasis.

Commercial Linde process nitrogen containing about 0.5 per cent oxygen was used. The oxygen was removed by passing the gas through an electrically heated (between 650° and 750°C.) Pyrex glass tube, filled with copper gauze and wire which had been previously reduced with hydrogen. The issuing stream of purified nitrogen was moistened by passage through a bubbler of distilled water. The gas was conveyed through narrow copper tubing to the moist chamber. All joints in the gas line were of metal-to-metal or glass-to-metal, the latter being sealed with deKhotinsky cement. Some difficulty occurred in sealing the joint at the moist chamber for it was necessary to maintain a flexible and sliding tight fit for the few moments when a drop of reduced dye was delivered to the cover-glass. This was finally solved by cracking the seal for a moment (with nitrogen flushing the system), depositing the drop of dye on the slide and quickly resealing the joint. By providing a long, well greased sliding fit at this joint, the latter may be safely left without further sealing.

Indicators and Reagents.—The amebæ were injected with the compounds listed

in Table I. For convenience in designating and referring to the indicator compounds in our list, we have assigned to each a letter as shown in the table. Opposite each compound is given its characteristic E_0' value (the potential on the hydrogen scale) at pH 7.0, that is, the electrode potential difference between the normal hydrogen electrode and an equimolecular mixture of oxidant and reductant at pH 7.0. These values may be converted into rH values which are independent of pH. The compounds are thus placed on a graduated scale of reducing (or oxidizing) intensity irrespective of their other chemical characters. The potentials of the hydrogen and theoretical oxygen and air electrodes are included as orienting reference points. Compounds on the positive end of the scale are "more oxidizing" than those on the negative end; and conversely, those on the negative end are "more reducing."

In addition to the indicators, there is included in this scale the ferricyanide system, because we have depended in critical experiments upon injections of potassium ferricyanide to restore the color of intracellularly reduced dyes and thus to make sure that the compound under investigation was still present in the cell and potentially available. We have found that small and moderate[2] injections of aqueous 1 per cent potassium ferricyanide are tolerated by the normal ameba. Large injections cause the cell to round up and take on a yellow color; the granules sink in the cytoplasm and the ameba becomes motionless and dies. At no time have we found the freshly made ferricyanide solution to produce a blue coloration within an ameba which had not been treated with a blue dye. This point is emphasized for we are aware of the possibility of the formation of Prussian blue and similarly colored iron compounds. The results were frequently checked by noting the effect of air or of potassium chromate on the reduced dye within the cell. Instances of apparent disagreement proved to be due to a time factor involved; because the dye was possibly undergoing decomposition and one of the tests was performed soon enough to give a positive result while the next test was done too late. In other words, injection of fresh ferricyanide promptly after decoloration of the reversible indicators always restored the color of the dye or intensified it; if the oxidizing agent was injected long after the dye had been reduced, the color might or might not be restored depending upon secondary effects.

The oxidation-reduction indicators used were 25 in number, all but one of which were synthesized at the Hygienic Laboratory. Phenosafranin was a fairly pure commercial product (Safranin B extra, Badische). Three sulfonated indophenols (compounds *A1*, *A2* and *B* in the tables) were specially synthesized[3] to test the validity of Dixon's (1926) suggestion that the toxicity of the sulfonic acid might yield a false index of the reducing ability of the cell. There is also included in this group compound *M*, a disulfonated indophenol. The list contains two indicators, phenol blue (dimethylaniline indophenol) and *m*-toluylene diamine

[2] See p. 593.

[3] We wish to acknowledge our indebtedness to Mr. W. L. Hall of the Hygienic Laboratory who made these compounds for us.

indophenol, amphoteric compounds which have been studied by Cohen and Phillips but which have not yet been reported. Finally there is the group comprising the last three compounds in the tables, namely, neutral red, dimethylaminomethylphenazine and phenosafranin. These are rather unsatisfactory for pur-

TABLE I.

List of Compounds Injected.

	Name of oxidant	E'_0 at pH 7.0 (volts)	rH
	(Oxygen electrode)	+0.81	41.0
	(Oxygen in air)	+0.80	40.7
	Potassium ferricyanide	+0.43	28.4
	Potassium chromate	?	?
A1	Phenol *m*-sulfonate indo-2,6-dibromophenol	0.273	23.1
B	Phenol *m*-sulfonate indophenol ... approx.	0.25	22.4
C	*m*-Bromophenol indophenol	0.248	22.3
A2	Phenol *o*-sulfonate indo-2,6-dibromophenol	0.235	21.9
D	*o*-Chlorophenol indophenol	0.233	21.8
E	*o*-Bromophenol indophenol	0.231	21.7
F	Phenol blue chloride	0.227	21.6
G	Bindschedler's green zinc chloride	0.224	21.5
H	Phenol indo-2, 6-dichlorophenol	0.217	21.3
I	" " " dibromophenol	0.217	21.3
J	*m*-Cresol indophenol	0.210	21.0
K	*o*-Cresol "	0.195	20.5
L	*o*-Cresol indo-2, 6-dichlorophenol	0.181	20.1
M	*1*-Naphthol-2-sulfonate indophenol *m*-sulfonic acid	0.135	18.5
N	*m*-Toluylene diamine indophenol chloride	0.127	18.3
O	*1*-Naphthol-2-sulfonate indophenol	0.123	18.1
P	*1*-Naphthol-2-sulfonate indo-2,6-dichlorophenol	0.119	18.0
Q	Toluylene blue chloride	0.115	17.9
R	Methylene blue chloride	+0.011	14.4
S	K_4 indigo tetrasulfonate	−0.046	12.5
T	K_3 " trisulfonate	−0.081	11.3
U	K_2 " disulfonate (also Na_2)	−0.125	9.9
V	Neutral red iodide ... approx.	−0.30	4.0
W	Dimethylaminomethylphenazine chloride	?	?
	(Hydrogen electrode)	−0.421	0.0
X	Phenosafranin ... approx.	−0.525	−3.5

The potential and rH values listed are those found at 30°C. Our experiments were performed at room temperatures ranging from about 20° to 25°; the small corrections applicable have been ignored for present purposes.

poses of studying the cell potential because they do not seem to possess, in the physiological range of pH, the labile reversibility demanded by our experiments. They were included, however, for such incidental information as they might yield.

Preparation of Reduced Dye Solutions.—The apparatus for reducing the indicator solutions and storing them is shown in Fig. 1. The dye was dissolved, filtered if necessary, and then placed in the reducing flask *F* together with washed, platin-

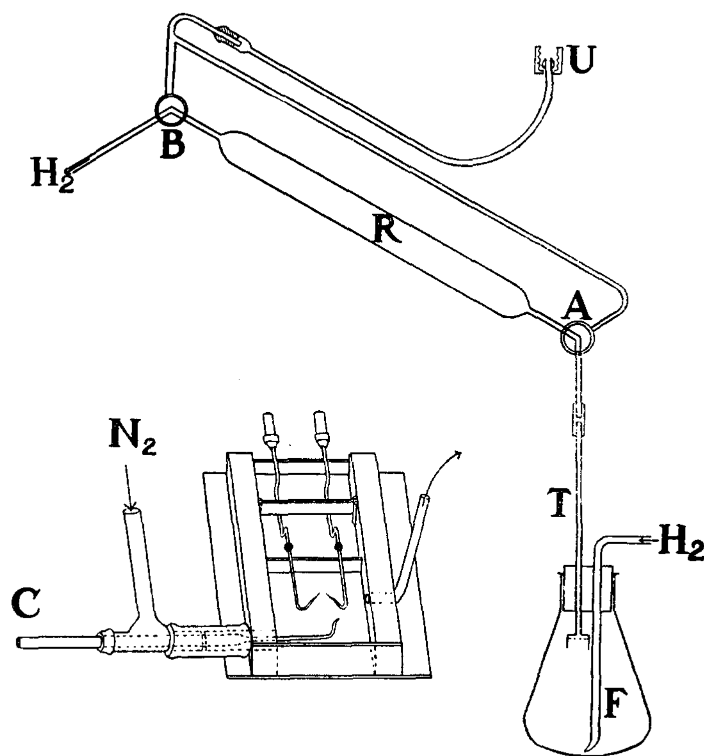

FIG. 1.

ized asbestos. Moist hydrogen was then bubbled through the suspension until the dye was decolorized. Meanwhile the escaping hydrogen passed through the filter tube *T* and the reservoir *R* and washed out the oxygen contained in them. The filter tube *T* was provided at its lower end with a perforated platinum disk upon which was laid a thin mat of washed asbestos. Its upper end was connected with the inlet to the reservoir. When the dye was reduced, the flask *F* was raised, the filter tube *T* sliding down through the rubber stopper until its lower end reached the flask bottom. The pressure of hydrogen then forced the reduced in-

dicator solution through the filter and up into the reservoir. When filtration was completed, the cocks *A* and *B* were turned so as to isolate the reservoir from the exterior, and *T* was detached from the reservoir.

To remove residual hydrogen purified nitrogen was bubbled through the solution in the reservoir. In the case of photosensitive compounds like leucomethylene blue, the reservoir was covered with black paper.

If furnished with properly ground and well greased cocks, the reservoir will preserve the solutions indefinitely. There is still on hand, at this writing, one containing reduced naphthol sulfonate indophenol which was prepared in July, 1927, and which shows not the slightest visible trace of change.

To deliver the reduced dye to the moist chamber, the procedure is as follows: The moist chamber is thoroughly deaerated with purified nitrogen as indicated by the arrows. Meanwhile the reservoir is fixed (*A* upward and *B* down) in close proximity to the moist chamber and is joined to the nitrogen supply by a small gas-tight, bronze union *U*. Nitrogen is now allowed to flush through cocks *A* and *B* alternately until the system, excepting *R*, is deaerated. The lower outlet (from cock *B*) is then joined by a short rubber connection to the outer end (*C*) of the capillary delivery pipet leading into the moist chamber, and the delivery pipet is deaerated.

This rubber connection is only a temporary one and is eliminated as a source of oxygen contamination by our subsequent procedure. After blowing nitrogen through the capillary for 15 to 30 minutes for thorough deaeration, cock *A* is turned to open the reservoir to the nitrogen supply, and cock *B* is carefully turned to let reduced dye solution run slowly through the capillary delivery tube. The solution washes forward any residual oxygen contamination from the short rubber connection, and the first few drops of solution are wasted on absorbent paper placed for this purpose along the back and bottom of the moist chamber.

The wasted dye solution serves still another purpose, for it acts as an oxygen absorbent in the chamber. We may state that with ordinary careful technic even the first drop of the entering solution of reductants, such as leucomethylene blue and leuco-indigo carmine, was entirely devoid of tell tale signs of reoxidation.

The tip of the capillary delivery tube is then raised and a drop of clear reductant deposited on the under side of the cover-glass of the moist chamber. During all this manipulation, a steady stream of nitrogen is running through the chamber. As soon as the drop of reductant is delivered to the slide, the wax seal (cracked while depositing the reductant) is restored and cock *B* is shut off. Since the capillary delivery tube remains filled with reductant, any subsequent infiltration of oxygen through the rubber connection is effectually neutralized. In fact, the rubber connection may be detached and the end of the delivery tube *C* safely left exposed to the air, for the rate of oxygen diffusion in the capillary is exceedingly low. Leaks in the chamber are quickly betrayed by the coloring of the indicator drop; we have frequently kept experiments going for several hours without signs of coloration of the reductant.

Solutions.—The solutions were freshly made on the day used, and in the case of unstable compounds like methylene blue they were prepared within a few minutes before use. The indicators were made up in 1 per cent strengths in cold water, filtered if necessary, and the solutions were well stoppered and protected from light and heat. A few of the basic compounds were insoluble in water and to these was added the calculated amount of HCl to produce the chloride. The solubility of some of the substances is low, leucomethylene blue, for instance, is very slightly soluble. Therefore the 1 per cent strength represents merely the maximum possible calculated concentration. Unless otherwise stated, an aqueous solution of the sodium salt of each acid indicator was employed.

Amounts Injected.—The terms small, moderate and large are frequently used here when referring to the volume of reagent injected (*cf.* Chambers and Reznikoff, 1926). The amount was estimated in comparison to the volume of the nucleus or of the ameba. A small amount corresponds approximately to the volume of the nucleus; a moderate amount equals a quarter to a third, and a large amount, one-half the volume or more of the whole ameba.

Dimensions vary a good deal, but if we assume for orientation a spherical shape of 0.2 mm. diameter for the average ameba and 0.01 mm. for its nucleus, the corresponding volumes would then be 0.004 c. mm. and 0.000,000, 5 c. mm. Therefore, a "small" injection would be of the order of magnitude of 5×10^{-7} c. mm., a "moderate" injection, 1×10^{-3} c. mm. and a "large" injection, 2×10^{-3} c. mm.

Microscopic Equipment.—Most of the injections were performed under a Leitz No. 5 or a Zeiss No. 20 objective and a 10× or 15× ocular. The No. 3 Leitz double demonstration eyepiece was also employed; in practically all the experiments, the observations recorded were seen by at least two observers; the experiments were repeated until the observers agreed upon the findings. The illumination was the same as that employed and described by Chambers and Pollack (1927).

EXPERIMENTAL RESULTS.

In view of the interpretations involved, it is considered necessary to report certain details of the observations. These will be described as briefly as possible. The essential results are summarized in Table II.

The injections were made mostly within the first 2 hours after the amebæ had been transferred from the culture to the cover-slip. Injections under anaerobiosis were not done until after a preliminary deaeration of the moist chamber for 15 to 30 minutes.

A1. Phenol m-Sulfonate Indo-2,6-Dibromophenol.—The oxidant was injected aerobically. The greenish blue solution decolorized instantly, the injected region was then pinched off, breaking within 2 or 3 minutes. The ameba often survived 5 or 10 minutes before breaking up. With rather large injections, the pinched off

portion remained blue for 1 minute, fading slightly or completely before bursting within 3 minutes. The compound was toxic.

A2. Phenol o-Sulfonate Indo-2,6-Dibromophenol.—The blue oxidant was injected aerobically. In the cytoplasm, the dye decolorized within 10 seconds; it took 30 to 60 seconds to disappear from the nucleus when the latter was stained by the injection. The compound was toxic on injection. Amebæ immersed in the diluted dye remained alive as long as 3 days, without showing internal coloration.

B. Phenol m-Sulfonate Indophenol.—The oxidant was injected anaerobically. The greenish blue solution faded instantly in the cytoplasm, the injected region then pinched off and the ameba broke. The compound was unpurified and pronouncedly toxic.

C. m-Bromophenol Indophenol.—On injecting the oxidant aerobically, the blue solution from the pipet as it entered the ameba showed a pink flash followed by instantaneous decoloration. The ameba recovered.

D. o-Chlorophenol Indophenol.—The oxidant was injected aerobically. The deep blue solution from the pipet on entering the ameba showed an evanescent, purplish pink flash of color which instantly faded. The compound was apparently non-toxic.

E. o-Bromophenol Indophenol.—The pink oxidant was injected aerobically. The dye on entering the ameba assumed a faint pink which faded rapidly.

F. Phenol Blue.—The dye base was treated with an equivalent amount of HCl to produce the soluble chloride. The purple oxidant was injected aerobically and the color faded in the ameba within 3 seconds. The compound is non-toxic; one ameba was injected 5 times without apparent injury. As an indicator it appears unsatisfactory because of instability.

G. Bindschedler's Green.—The zinc chloride salt of the oxidant was injected aerobically and was found to be extremely toxic. The ameba lost all activity and appeared injured. The injected dye did not fade in 3 to 5 minutes, and appeared to accumulate on the nucleus and the granules in the cytoplasm. The oxidant is unsatisfactory also because of its low tinctorial power.

The reductant (free base) was dissolved anaerobically in an equivalent amount of HCl and several amebæ were immersed in the drop anaerobically. The amebæ survived the immersion but showed no signs of coloration.

H. Phenol Indo-2,6-Dichlorophenol.—The blue oxidant injected aerobically or anaerobically into the ameba faded rapidly. The decoloration was so rapid that the color faded at the site of injection before the oxidant could diffuse through the whole organism. The compound is non-toxic, for the amebæ recovered quickly from the injections.

Potassium ferricyanide injected shortly after decoloration of the dye brought back the blue color; when injected 1 hour after decoloration the color was not restored.

I. Phenol Indo-2,6-Dibromophenol.—The oxidant was injected aerobically and the blue color faded from the cytoplasm in about 5 seconds. The fading was

somewhat slower from the nucleus (about 10 seconds). The compound gave evidences of toxicity; but this was an unpurified preparation.

J. m-Cresol Indophenol.—The red oxidant was injected aerobically and decolorized rapidly. The compound is non-toxic.

K. o-Cresol Indophenol.—The red oxidant was injected aerobically and the color faded within 5 seconds. The compound is non-toxic.

L. o-Cresol Indo-2,6-Dichlorophenol.—The oxidant was injected aerobically and anaerobically. In both instances the blue color diffused rapidly through the cell, faded within 5 seconds and the ameba became very active. The compound is not very toxic except for a tendency to disrupt the surface of the cell.

Ferricyanide injected after decoloration of the indicator gave a pronounced return of color.

M. 1-Naphthol-2-Sulfonate Indophenol m-Sulfonic Acid.—This was an impure specimen contaminated with almost 50 per cent NaCl. The red oxidant injected aerobically in small and medium amounts in an active ameba faded within 10 seconds. If injected into a quiescent ameba or if in large amounts, the color persisted. The compound was toxic, and the injected portion was pinched off, as a rule.

The oxidant injected anaerobically decolorized within a few seconds (up to 45 seconds).

Injection of ferricyanide restored the color.

N. m-Toluylene Diamine Indophenol.—This amphoteric compound is practically insoluble in water, and an equivalent amount of HCl was added to produce the soluble chloride. The brown-red oxidant injected aerobically or anaerobically decolorized in 5 to 15 seconds: fading from the nucleus was a little slower. Injection of potassium ferricyanide or chromate restored the brown-red tint.

Although the nucleus and cytoplasm became colorless shortly after injection of the dye, certain inclusion bodies gradually took on a distinct brown-red which persisted at least 24 hours in the active ameba. Brief immersion in a solution of the oxidant caused certain granules in the ameba to take on the color, which faded shortly and which was restored on the granules by the injection of chromate.

The colorless reductant injected anaerobically remained colorless in the ameba. There was no sign of recoloration.

Both the oxidant and reductant are non-toxic, the amebæ recovering completely 1 minute after an injection.

O. 1-Naphthol-2-Sulfonate Indophenol.—The red oxidant was injected aerobically and the intensity of color immediately diminished. A distinct pink tinge persisted for 5 seconds up to 1 minute, according to varying amounts injected, and eventually decolorized. In small amounts the dye was immediately decolorized. The compound is largely non-toxic. In those cases where the color persisted the ameba rounded up, its granules sank, the nucleus took on the color and the organism never recovered.

P. 1-Naphthol-2-Sulfonate Indo-2,6-Dichlorophenol.—This sample was contaminated with about 30 per cent sodium chloride, and exhibited toxic effects.

The oxidant injected aerobically changed to a paler blue and faded completely from the cytoplasm in 1 to 3 (up to 10) seconds, and from the nucleus in 3 to 5 (up to 30) seconds. The nucleus stained a deeper blue than the cytoplasm. With small injections no color remained after 30 seconds; with larger injections, the color persisted for 1 to 2 minutes. On injection of large amounts, the color often remained localized, and the injected area was pinched off. With small injections there was no pinching off and the color disappeared within 5 seconds.

Injection of the oxidant anaerobically caused a localized blue which faded from the cytoplasm within 10 seconds and from the nucleus within 2 minutes. In the active ameba the reduction was rapid; but in the quiescent cell the color persisted.

The colorless reductant was injected under the usual strict anaerobiosis. Many of the amebæ broke while exhibiting a strong sodium ion effect but there occurred no sign of coloration. The injection of partly colored reductant was followed by an immediate fading of the color, which could be restored by ferricyanide. This restored color, which faded again shortly, was most pronounced on the nucleus.

The reductant was toxic and tended to fluidify the interior of the ameba and to cause its surface to break (Chambers and Reznikoff, 1926).

Q. Toluylene Blue Chloride.—The oxidant was injected aerobically. The purple solution from the pipet, on entering the ameba localized at the site of injection as a brilliant purple coagulum before diffusing throughout the cell. The color changed to blue in 10 to 15 seconds and, except for some scattered granules, faded in 30 seconds. The nucleus turned blue and faded in 40 seconds. Some blue and purple granules persisted; and a blue tinge often persisted in the hyaline cytoplasm for many minutes. The amebæ recovered.

Injection of potassium chromate accentuated the blue on the granules and slightly in the cytoplasm.

The oxidant, on injection anaerobically, caused a local solidification which was sometimes extruded; and some of the dye accumulated on the granules. When the injected region was not extruded and the cell was sluggish, the region remained stained for a long time. The active cell decolorized the dye rapidly. When injected on the surface of the ameba, the color penetrated the cell wall. Exposure of the ameba to air or oxygen caused a temporary return of the purple color of the oxidant. The compound appeared to be somewhat toxic and acted on the ameba like methylene blue.

R. Methylene Blue Chloride.—The oxidant injected aerobically usually produced a local blue coagulum; and the nucleus took on an intense blue, while the contractile vacuole remained uncolored. After 1 minute, the cytoplasm became a very pale blue, which sometimes persisted. Occasionally, the stained region was pinched off. Following the local solidification there ocurred some diffusion of the color. The blue coagulum dispersed in fragments most of which decolorized in about 2½ minutes. With time, some color accumulated on certain granules in the almost decolorized cytoplasm. A very small injection of the 1 per cent solution decolorized within 1 minute. Injection of ferricyanide brought back the color especially on the granules.

Injection of the oxidant anaerobically produced a localized blue coagulum and the ameba became quiescent. In the active portion of the ameba, the dye faded quickly (within 1 minute); in the coagulated portion the decoloration took 3 to 4 minutes. In one case, injection of oxidant anaerobically caused the nucleus to take on a green color which began to fade in 10 minutes and decolorized completely in 14 minutes.

Injection of ferricyanide or exposure of the amebæ to air restored some of the color. After transfer from nitrogen to air, the ameba became more active and assumed a pale blue, after which the color accumulated on certain granules and the cytoplasm became colorless.

The oxidant injected anaerobically into *dead* amebæ remained blue indefinitely.

The *reductant* was a saturated aqueous solution of the chloride. It was troublesome to manipulate because it crystallized out and clogged the pipet; and also because it is light-sensitive. The reductant on anaerobic injection produced a local, dark blotch which, upon inspection, proved to be a mass of crystals (apparently leucomethylene blue). There was no local coagulation. The amebæ remained colorless, and readily recovered from the injection. Injection of colorless reductant always left the ameba uncolored. Subsequent injection of ferricyanide or exposure of the ameba to air restored the color of the oxidant temporarily; and the nucleus appeared distinctly bluer than the cytoplasm.

S. K_4 Indigo Tetrasulfonate.—The oxidant is toxic. Injection of the purple oxidant aerobically in very small amounts was followed by a quick disappearance of the color. In large amounts, the ameba colored distinctly blue, which persisted for an hour with only slight fading. The color was localized at the moment of injection and then spread quickly giving the cytoplasm and nucleus a distinct pale blue tinge. In some cases, the blue tinge, before spreading, localized in a large blister-like elevation which became incorporated within 1 to 2 minutes. After 6 minutes the color had paled appreciably especially in the nucleus which seemed to lose the color completely. The contractile vacuole remained uncolored at all times. Injection of ferricyanide brought back no increase of color.

Injection of the oxidant anaerobically caused quiesence; the dye diffused rapidly and stained the nucleus. The color disappeared in from 1 to 7 minutes. With very small injections the color disappeared immediately. Subsequent injection of ferricyanide or chromate or exposure to air restored the purple color in the ameba.

Injection of the reductant anaerobically caused quiescence followed by active movements. The yellow reductant remained yellow in the ameba provided air was excluded. On exposure to air the ameba turned purple; and injection of ferricyanide also restored the purple color. The reductant appeared non-toxic in contrast to the oxidant.

T. K_3 Indigo Trisulfonate.—The blue oxidant is toxic and the injected amebæ exhibited a marked potassium effect. When injected aerobically it imparted a blue color which persisted.

The oxidant injected anaerobically decolorized in the ameba within 4 to 7 minutes (2½ to 15 minutes in extreme cases). Injection of ferricyanide or exposure to air restored the color.

The reductant injected under strict anaerobiosis imparted only a yellow color to the ameba and a slightly greenish tinge to the nucleus. Partly oxidized reductant injected into the ameba produced a blue color which was quickly reduced. The reductant appeared to be toxic in moderate amounts. Injection of ferricyanide or exposure to air restored the color in the cytoplasm which also exhibited blue granules not previously visible.

U. K_2 Indigo Disulfonate, Also the Na_2 Salt (Indigo Carmine).—The oxidant is very toxic and seemed also to toughen the pellicle. The blue oxidant injected aerobically was not reduced as long as the amebæ were kept under observation (maximum time 45 minutes). On injection, the dye solution did not appear to diffuse through the cell, but rather seeped through channels in the cytoplasm. When the nucleus was near the site of injection it took on a deep blue color. When the nucleus was beyond the site of injection it gradually assumed a blue color while still surrounded by colorless cytoplasm.

The oxidant injected *anaerobically* showed evidences of toxicity and usually caused pinching off. The injection fluidified the ameba and produced a blue cytoplasm and a deeper blue nucleus. In some cases (of apparently injured amebæ) the color was partly reduced, but not completely. When the membrane of the ameba broke, the nucleus lost its intense color and assumed the same tint as the cytoplasm. However, when the ameba survived, the color was gone in 3 to 4 minutes. Injection of ferricyanide restored the blue color which faded again under anaerobiosis. Exposure of the cells to air also restored the color. In air the restored color remained for about 15 to 20 minutes and then seemed to fade as the ameba became more active.

The reductant injected under strict anaerobiosis imparted a greenish yellow tinge to the ameba but no signs of blue. Exposure to air caused the injected cell to assume a deep blue; and injection of ferricyanide also restored the blue color.

V. Neutral Red Iodide.—This basic dye does not seem to possess a labile oxidation-reduction equilibrium at pH 7.0.

The oxidant injected aerobically tends to produce a localized coagulum which stains deep red. With concentrations more dilute than 1 per cent, the color diffused through the cytoplasm giving it a reddish tint with no visible sign of coagulation. When the nucleus was in the immediate vicinity of the injected region, it took on a red color. If the color persisted in the nucleus, the ameba eventually either died or expelled the nucleus by a pinching-off process. The diffuse coloration of the cytoplasm lasts only a few seconds after which the color accumulates on or in granules and vacuoles.

W. Dimethylaminomethylphenazine Chloride.—This compound differs from neutral red by the lack of one amino group.

The oxidant injected anaerobically produced a flash of brilliant orange-red which disappeared at once and left a shower of crystals of the dye inside the ameba. Some of the spear-like crystals stuck out of the ameba. The cell responded by attempting to pinch off the injected portion. The oxidant was toxic. In one case, after pinching off the injected region, the ameba recovered with a pink coloration persisting.

The yellow solution of reductant remained unoxidized in air. Injection of the reductant aerobically and anaerobically colored the ameba yellow, and the color persisted unchanged. The reductant was non-toxic. It did not behave like a basic dye, did not stain the nucleus nor did it coagulate the cytoplasm. The injected yellow compound tended to accumulate in particles, leaving the background a paler yellow.

Subsequent injections of ferricyanide did not color the ameba but merely caused the nucleus to clear up and the granules to sink.

X. Phenosafranin.—The oxidant was very toxic and accumulated in the nucleus. Injected anaerobically, it produced a localized coagulum from which the red color diffused into the rest of the cell. The nucleus was intensely stained and the color persisted. In one case, a small amount was injected and the color seemed to disappear within 2 minutes. There was a pronounced staining of the amebæ coming into contact with escaped oxidant in the hanging drop.

The yellow reductant solution is relatively stable in air at pH 7.0; and when injected it imparted a yellow coloration to the ameba. The reductant was relatively non-toxic, did not produce coagulation in the cell and did not accumulate in the nucleus. When the injected ameba was exposed to air, the granules in the cell took on a pinkish tinge. Injection of ferricyanide reddened the ameba which had previously received a moderate injection of reductant; in 14 minutes the granules became salmon-colored, and in 30 minutes they were a distinct pink. In one case, injection of ferricyanide resulted, within 2 minutes, in the appearance of a diffuse pink in the ameba.

RÉSUMÉ OF EXPERIMENTAL RESULTS.

Table II gives the results in summary. The main aspects of the experimental findings may be briefly stated as follows:

1. Under anaerobiosis, *A. dubia* was able to reduce completely all the *reversible* oxidation-reduction indicators tried.

2. Under anaerobiosis, the ameba was unable to reoxidize six of the most easily oxidizable indicators in the list.

3. Under aerobiosis, the ameba was able to reduce completely all but one (a very toxic member) of the reversible indicators from *A1* to *P* (*i.e.*, down to and including 1-naphthol-2-sulfonate indo-2, 6-dichlorophenol). Compounds *Q* and *R* were on the border-line, the reduction under aerobiosis being sometimes complete and sometimes only partial. Compound *S* was only slightly reduced, if at all, while *T* and *U* remained apparently unreduced. The irreversible indicators *V*, *W* and *X* showed questionable reduction.

4. The time of reduction varied approximately with the amount of injection.

5. In general, the speed of reduction was greater under anaerobiosis than under aerobiosis; and there is a suggestion that reduction was more rapid in the active ameba than in one which remained quiescent after the injection.

6. Details of the *speed* of color disappearance should be noted. With the average injection under aerobiosis complete fading was instantaneous or of the order of 5 seconds for all of the indicators from *A1* to *L* (excepting toxic *G*). It required about 10 to 30 seconds for *M*, *N*, *O* and *P;* and it took up to 10 minutes or more to accomplish partial fading of *Q*, *R* and *S*.

7. Toxic compounds as a class were reduced somewhat more slowly than adjacent non-toxic compounds on the rH scale.

8. Toxicity varied considerably, owing partly to inaccurate control of dosage and to impurities. Compound *G* (zinc chloride salt of Bindschedler's green) was immediately fatal. The simple indophenols were generally non-toxic. The sulfonated compounds as a class were toxic to different degrees. The reduced compounds were usually less toxic than the oxidants.

TABLE II.

Results of Injections of Oxidation-Reduction Indicators into the Cytoplasm of Amœba dubia.

Indicator	Aerobic injection	Anaerobic injection		Toxicity
	Oxidant	Oxidant	Reductant	
A1	Reduced	—	—	Toxic
A2	Reduced	—	—	Toxic
B	—	Reduced	—	Very toxic
C	Reduced	—	—	Non-toxic
D	Reduced	—	—	Non-toxic
E	Reduced	—	—	—
F	Reduced	—	—	Non-toxic
G	Not reduced*	—	—	Extremely toxic
H	Reduced	Reduced	—	Non-toxic
I	Reduced	—	—	Toxic
J	Reduced	—	—	Non-toxic
K	Reduced	—	—	Non-toxic
L	Reduced	Reduced	—	Slightly toxic
M	Reduced	Reduced	—	Toxic
N	Reduced	Reduced	Not oxidized	Non-toxic
O	Reduced	—	—	Slightly toxic
P	Reduced	Reduced	Not oxidized	Toxic
Q	Reduced partly	Reduced	—	Slightly toxic
R	Reduced partly	Reduced	Not oxidized	Slightly toxic
S	Slightly or not reduced	Reduced	Not oxidized	Oxidant toxic; reductant non-toxic
T	Not reduced	Reduced	Not oxidized	Oxidant toxic; reductant slightly toxic
U	Not reduced	Reduced	Not oxidized	Toxic
V	Not reduced	—	—	—
W	Not reduced	Not reduced?	Not oxidized	Oxidant toxic; reductant non-toxic
X	Not reduced	Slightly reduced?	Not oxidized	Oxidant toxic; reductant non-toxic

* In moribund or dead cells; zinc salt of dye used.

Sources of Error.

Before proceeding to an interpretation of the experimental results, it is necessary to consider a few of the sources of error.

Critique of Microinjection Method.—With the recent refinements in construction of the apparatus, the manipulations incident to micro

injection can be performed with ease, and furnish an elegant method of attack on various problems in cellular biology. For direct observation of certain qualitative changes, the method is quite unexcelled in its delicacy and the definiteness of approach. As will be seen from the experiments and in the discussion to follow, certain quantitative aspects of the problem became of paramount importance for a proper interpretation of the results. The mere observation that a dye is reduced within the cell is clearly only a first step; when we come to compare the reduction of different dyes and note differences in the *speeds* of reduction, we face at once the need for strict quantitative comparisons. This is where the microinjection technic in its present stage of development fails to satisfy except in a very gross way. It is as yet impossible to gage precisely, as ordinarily understood in quantitative work, the volume of solution injected intracellularly.

One must not leave unmentioned in this connection the microscopic equipment and the illumination, nor yet the personal factor. The observations were primarily those of color change and fading in the microscopic field. Obviously the lens system must be of a type which will reduce intrinsic coloration to a minimum; and more important still, the source of illumination and screening should be satisfactory from the same standpoint. Not the least important is the condition of the observer's eye and his acuity of color perception. Prolonged and excessive observation through the microscope, even under almost ideal conditions, induces eye strain and fatigue which definitely inhibit quick and accurate color perception, especially when one must decide if a color is entirely faded. The observer must also be on his guard to avoid misinterpretations of color due to artefacts such as those caused by varying depth of the drop, shadows cast by the micro needles and colors due to natural pigment or inclusion bodies in the cell under observation.

Possible Injury to Amebæ.—In considering our results, one may ask to what extent the observations were affected by possible injury to the organisms studied. A full discussion of the question would take us too far afield. To assert that the manipulation of the cells left them in "normal" condition would depend largely on the definition of the term normal.

The amebæ remain alive for at least 24 hours in a hanging drop in

the moist chamber with free access of air. At the end of that period, the cells appear to be quite active and show no *visible* signs of abnormality. As for amebæ injected with water, the observations of Howland and Pollack (1927) show that the cells usually recover quite completely from the injury incident to injection.

One may urge that the ameba cannot be "normal" after suffering injury from the injection needle. This criticism may perhaps be valid if applied to the case of highly differentiated cells; but we believe that it can apply, if at all, only to a very limited degree to *A. dubia* and then only for a relatively short period immediately following the injection. The ameba exhibits very remarkable powers of readjustment and recovery. Pricking increases the internal flowing movements and there is a quick renewal of the plasmalemma, following which the cell continues to subsist without visible signs of permanent injury.

The situation is somewhat different when the amebæ are kept for short periods in an atmosphere devoid of oxygen. Instead of the typical stellate shape spread out on the under side of the cover-glass, the organism after an exposure of about 30 to 50 minutes assumes a *Limax* form and no new, extended pseudopodia are evident such as are to be seen in the resting stage. Occasionally, there may be seen an ameba with 10 to 15 short lobate pseudopodia. However, except for these changes the cells give no evidence of injury. We have maintained them for as long as 9 to 10 hours under anaerobiosis and then on exposure to air, the cells became more active, lost their *Limax* form and seemed none the worse for the experience.

The introduction of foreign material by gross assault on the cell is no doubt drastic treatment. However, with respect to most of the compounds which we have injected, this criticism loses its force because a number of the indicators (some of which can be taken up by staining) are vital dyes and the cells appear to tolerate them in their interior indefinitely. Moreover, toxic quantities of the compounds simply kill the cells or render them moribund, and our observations and conclusions are based mainly on the response of cells which have survived the operation.

It is easily demonstrable that the injection of a suitable reducible dye into a moribund ameba or into the dead débris is followed by no

reduction of the dye. If the color disappears, the dye is obviously washed out. On the other hand, injection of the dye into a *living* cell is followed by a fading of color which can be restored with a ferricyanide injection. The important fact is that the living ameba exhibits a reducing intensity and capacity equivalent to its ability to perform the work necessary for the intracellular reduction of appropriate compounds. That is, a cell capable of causing the reduction is, if not normal, at least living and still capable of performing work.

Toxicity of the Indicators.—Due to the inherent limitations in the technic the doses injected varied probably very widely. In addition, some of our compounds were known to be more or less contaminated. Another variable factor was the general condition of the different lots of amebæ used in the various experiments. However, with obvious reservations, certain conclusions as to the toxicity of the injected indicators may be made.

Compound G was almost instantly fatal, undoubtedly because of the zinc chloride present. There is no theoretical reason for believing that the free base is any more toxic than methylene blue. The simple indophenols as a class were non-toxic; and when contaminated with much salt they produced typical sodium ion effects (Chambers and Reznikoff, 1926).

The different basic indicators exhibited different degrees of toxicity. The amphoteric amino-indophenol N was non-toxic. Methylene blue and toluylene blue were slightly toxic. The diazines W and X were decidedly toxic; in this connection, mention should be made of Marston's (1923) observation that diazines precipitate proteolytic enzymes.

We observed also that toxicity on injection is not necessarily paralleled by toxicity on immersion in the indicator. Attention is drawn to the interesting fact that the reductants of toxic oxidants were usually non-toxic. The sulfonated compounds as a class were more or less toxic; but so far as could be observed, there was no parallelism between the degree of toxicity and the number of sulfonate groups in the compounds studied.

Effect of Sulfonic Acid Radicals on Reduction.—Dixon (1926) found in the case of xanthine oxidase, which he believes is the type of system

responsible in large measure for the reducing power of all living cells, that the velocity of reduction of certain of the oxidation-reduction indicators was appreciably retarded by those indicators possessing sulfonic acid radicals in their structure. Consequently, he suggests, the apparent reductions of such indicators within the cell may depend not so much on the intrinsic reducing power of the cell as upon whether the indicator is sulfonated or non-sulfonated.

In this connection our observations may be of interest. There were nine compounds in the present series which contained one or more sulfonic acid radicals (compounds *A1*, *A2*, *B*, *M*, *O*, *P*, *S*, *T*, *U*). Indicators *A1*, *A2* and *B* were on the most easily reducible end of the series and although definitely toxic, they were reduced in the ameba with considerable rapidity. Their rates of reduction however appeared to be slower than those of adjacent non-sulfonated dyes.

Compounds *M*, *O* and *P* were in the middle range of the series, approximately in the zone where the Needhams observed the critical reduction level of *Amœba proteus*. *M* has two sulfonic acid radicals, and *O* and *P* contain one each. *M* and *P* were toxic, but they were heavily contaminated with NaCl; while *O* was only slightly, if at all toxic. These dyes were completely reduced in the surviving cells within a minute or two.

Compounds *S*, *T* and *U* (indigos) contained 4, 3 and 2 sulfonic groups respectively, and they all exhibited marked toxicity. Under aerobiosis only *S*, the most heavily sulfonated compound, gave evidences of a slight reducibility; but under anaerobiosis, all three were reduced in 5 to 7 minutes (maximum, 15 minutes).

Judged by their speeds of reduction, the sulfonated indicators behaved very much like the non-sulfonated ones, *e.g.*, those on the positive end of the scale decolorized in a few seconds, those in the middle in a minute or two and those on the negative end in 5 to 15 minutes. A slight retardation in the reduction of the sulfonates was noted, but it was of a much lower order of magnitude than that reported by Dixon for the xanthine-oxidase system.

Therefore, despite a certain degree of toxicity which undoubtedly influences cellular processes, the sulfonated indicators were not found to yield a false index of the gross reducing *intensity* in the surviving ameba.

DISCUSSION AND CONCLUSIONS.

We may now consider the results of our experiments as a whole and seek an interpretation of the facts. In this connection, the review by Needham and Needham (1926) and the paper by Cannan, Cohen and Clark (1926) are of interest. Three major factors may be discerned in the reduction processes under our experimental conditions. These are the *intensity* factor, the *capacity* factor, and the *time* or *rate* factor. They have been ignored, confused or inadequately considered in certain recent papers on the subject, which cannot be discussed here. The importance of the matter, however, merits more careful and critical consideration.

Reduction Intensity.—As regards the intensity or potential factor of reduction inside the cell of *Amœba dubia*, the following facts seem clear. The living ameba generates a reduction potential which is sufficiently high to condition, under anaerobic environment, the complete reduction of all the reversible oxidation-reduction indicators down to and including indigo disulfonate. The latter when 99 per cent reduced corresponds to rH 7.5 at pH 7.0, or rH 7.6 at pH 6.9.

The presence of a high intracellular reducing intensity is corroborated by the failure to secure under anaerobiosis even partial reoxidation of six of the most easily oxidizable compounds (namely, *N*, *P*, *R*, *S*, *T* and *U*).

In the aerobic ameba, we find a reducing intensity ranging from rH 13 to 18; the Needhams report for the same species the narrow zone, rH 17 to 19. When there is present the neutralizing effect of atmospheric oxygen, the *apparent* level of intracellular reducing potential rises to a broad zone covered by as many as three indicators, *viz.:* toluylene blue, methylene blue and indigo tetrasulfonate, depending on the capacity and rate factors as discussed below. The particular level found appears to vary with the amount of indicator injected and the duration of observation. One may therefore question if any restricted *portion* of this range represents a unique physiological attribute of the ameba. In general, we find in the aerobic ameba the well known effect found in aerated suspensions of cells and "metabolites," namely, a gross reducing intensity stabilized between 0.1 and 0.2 volts at pH 7. The probable physiological significance of this has

already been discussed by Needham and Needham, and by Cannan, Cohen and Clark (1926).

Our results show that the series of satisfactory indicators is incomplete, and must be extended down to and beyond the hydrogen electrode potential before a final conclusion can be drawn, from this type of experiment, as to the limiting reduction potential attainable inside the ameba. In this connection, nothing satisfactory can be elicited from the behavior of the three irreversible compounds in our series.

This much however may be stated with confidence. The ameba develops in its interior a high, primary, reducing potential with a value certainly less than rH 7.6. In the presence of the neutralizing effect of atmospheric oxygen, the internal rH appears to assume any value between 13 and 18 depending on secondary factors.

If the rate of oxygen activation in the cell or at the cell surface is a function of the pressure, we can predict that the apparent internal rH will depend also on the oxygen pressure; it should certainly do so at very low oxygen tensions.

Reducing Capacity.—The quantity of material that the ameba can reduce will depend primarily on the reduction potential it can generate, and secondarily on the quantity of reducing substance present or generated. The evidence offered by these experiments on the capacity factor is only indirect because the use of accurate quantities of solutions was precluded. We found it possible to swamp out the reducing capacity of an ameba by five successive injections of a non-toxic, easily reducible compound. The result was a living ameba colored for several minutes by an indicator representing a high, "unnatural" rH.

Another aspect of the capacity factor appears in the case of certain basic dyes which tend to accumulate upon granules in the cytoplasm. This matter deserves further study.

If the speed of reduction is a criterion, it seems that even under aerobiosis the ameba ordinarily has available a quantity of active reducing material sufficient to completely reduce moderate injections of 1 per cent solutions of the various simple indophenols (compounds *A* to *M*). The behavior of the rest of the reversible indicators is more involved but, making allowance for factors of toxicity and granular accumulations, etc., there appears to exist a retarded rate of reduction

as if material of high reducing potential were not immediately available in sufficient quantity.

The fact that under anaerobiosis even small injections of completely reduced indicators remained completely reduced within the ameba, and that partly reduced compounds were quickly decolorized points quite clearly to an important conclusion: namely, there was not available an appreciable quantity of cellular oxidation-reduction substances poised at any rH point covered by the indicators employed. Such a level may exist, but for the particular organisms studied, namely, *A. dubia* and *A. proteus*, it lies below the range of indigo disulfonate and in the direction of the hydrogen electrode level.

How does this absence of poising ability in the rH range investigated harmonize with our finding that even under aerobiosis the ameba has available an appreciable quantity of active reducing material? In this connection one must remember that poising ability (the capacity to maintain a particular reduction potential level) depends on the presence of finite quantities of both oxidant and reductant in equilibrium with each other. Consequently, the apparent absence of poising ability and the failure to oxidize reduced dyes indicates that, in the rH range studied, the anaerobic ameba contains *only active reductants* in appreciable amount. (This refers, of course, only to the over-all effect in the cell, and may not apply to localizations.) Interference of oxygen complicates the picture by introducing oxidants of all shades of activity and elaborated presumably at varying rates.

The Rate Factor.—On this point also, our observations are necessarily indirect. We have already noted the retardation of reduction by toxic compounds. Our observations suggest that a kinetic study of intracellular reduction is experimentally feasible and of important promise.

It is of course precarious to apply to the dynamic activity of the heterogeneous system of the cell some of the criteria established for the essentially static, homogeneous indicator systems. In the latter the postulates of complete, labile reversibility and practically instantaneous equilibrium are important criteria. To apply these to the cell system requires the introduction of the time factor and possibly a host of phase and phase boundary equilibrium factors which are as yet only vaguely understood.

Despite the complexity of the subject, it is not difficult to discern a sequence of events which is reproducible, and which seems logically attributable to the high reducing intensity of the living cell interior, to the quantity factor and the time factor.

The almost instantaneous reduction of the indicators on the electropositive side is strong evidence for the existence of a virtual labile, reversible equilibrium state. The slower reduction rate of indicators on the electronegative side may merely indicate an inadequate capacity factor rather than any essential difference in the degree of lability of the equilibrium.

These considerations necessarily assign to molecular oxygen a secondary, neutralizing rôle dependent on its rate of diffusion and activation. This secondary effect may become appreciable as soon as its magnitude equals or exceeds the net biological effect. From the work of Brooks (1926), Rapkine and Wurmser (1926) and Cannan (1926) on chlorophyll-bearing cells, it is now known that relatively high reducing potentials can be maintained apparently concurrently with an active production of molecular oxygen by the chloroplasts within the cell. To what extent this phenomenon and the rate factor are influenced by the action of "antioxidants" remains an interesting question to be determined.

The present indicator studies on the ameba show an almost complete parallelism with the reduction electrode studies on bacterial suspensions reported by Cannan, Cohen and Clark, with the very important difference that the center of interest has been shifted from the mass culture to the individual cell. These results stand somewhat in contradiction to those reported by Needham and Needham for *A. proteus*, and render it impossible to accept the validity of a number of their conclusions. However, it is possible that their organism was entirely different from our *A. dubia* and *A. proteus*, since the internal pH of the Needham strain is reported at about 7.6 while Chambers, Pollack and Hiller found for our two strains a pH of about 6.9. Our criteria and technic were also somewhat different from those employed by the Needhams, therefore a critical comparison would be out of place. However, if the strains in Europe and America are comparable, our results indicate that the conclusions of the Needhams with regard to ameba will have to be modified in several important

respects to include the wide rH range under aerobiosis, the high reducing potential under anaerobiosis and the capacity and time factors, as we have shown above.

It is well known that ameba feeds on bacteria and other small organisms, and it is not improbable that certain bacteria may live symbiotically in the interior of the ameba. This possibility has a significant theoretical bearing and it might, perhaps, account for part of the discrepancy of our findings and those of the Needhams. However, direct experimental evidence is needed.

SUMMARY.

Twenty-five oxidation-reduction indicators were injected in oxidized or reduced form into *Amœba dubia* and *Amœba proteus* under controlled conditions of oxygen access. (1) Under anaerobiosis the ameba was able to reduce completely all the reversible oxidation-reduction indicators down to and including indigo disulfonate. (2) Under anaerobiosis the ameba was unable to reoxidize six of the most easily oxidizable indicators. (3) Under aerobiosis the ameba was able to reduce completely all the indicators down to and including 1-naphthol-2-sulfonate indo-2, 6-dichlorophenol. Toluylene blue, methylene blue and indigo tetrasulfonate were sometimes completely and sometimes only partly reduced, depending on the quantity of indicator injected and the duration of observation. (4) The time of reduction varied approximately with the size of the injection. Reduction was more rapid under anaerobiosis than under aerobiosis, more rapid in active than in sluggish cells and was retarded by toxic compounds. (5) Sulfonated compounds were somewhat toxic, as a rule.

In interpreting reduction phenomena of micro injection, it is necessary to take into consideration the intensity, capacity and rate factors. It then becomes apparent that the ameba has a high reducing potential lying on the rH scale below the zone of indigo disulfonate. The reducing capacity of the ameba seems to be relatively great in the region of the simple indophenols and of a progressively diminishing magnitude as the zone of the indigos is approached. Material of high reduction potential appears to be generated within the ameba at a measurable rate. These phenomena, observed in the interior of the

cell with the aid of indicators, parallel very closely those found in reduction electrode studies on bacterial cultures.

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