REGULATORY MECHANISMS OF CELLULAR RESPIRATION

I. The Rôle of Cell Membranes: Uranium Inhibition of Cellular Respiration

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The large number of inhibitors of cellular respiration hitherto described have been reported to act by inhibiting the activity of enzyme systems. They do so (1) by combining with the activating protein, either through some groups essential for activity (for example, the —SH groups), through denaturation of the molecule, or by combination on the side chains where substrates or prosthetic groups form the protein-substrate complex (structural inhibitors); (2) by combining with the prosthetic groups of enzymes (diphosphothiamine, pyridoxal, pantothenic acid, etc.); (3) by combining with the series of oxidation reduction systems (pyridine nucleotides, flavins, cytochromes) which transfer electrons from oxidizable substrate to molecular oxygen. Besides this direct action on the components of enzyme systems, cellular respiration may be affected by alteration of the varied mechanisms which regulate in the living cells the rate and the direction of enzymatic reactions. One of these regulating mechanisms is the state of the cellular membrane. It is generally agreed that the cell membrane is a lipoid-protein system possessing varying degrees of permeability where penetration occurs by passage through the pores of the membrane or through solution in the lipoid portion. Any alteration of the solubility coefficient in the lipoid phase or changes of the pore size will bring forth alterations in the rate of passage of substrates, and as a consequence alterations in the metabolsim of the cell. We present in this paper experiments on the inhibition of cellular oxidations produced by uranyl nitrate, which have been interpreted as being due to combination of uranium with the protein layer of the cell membrane, bringing thus an increased impermeability to the passage of certain oxidizable substrates.

EXPERIMENTAL

The yeast cells used in these experiments were brewers' yeast from Keely Brewing Company, Chicago, and bakers' yeast from Fleischmann. The first fermented

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glucose to CO_2 and alcohol, but did not consume oxygen in air. Bakers' yeast, on the other hand, not only fermented glucose anaerobically but oxidized with great speed glucose, ethyl alcohol, and acetate. The cake of bakers' yeast (10 gm.) was washed twice with 250 cc. of distilled water and was aerated with oxygen for 20 hours at room temperature (25–26°). It was then centrifuged and suspended again in distilled water. Such yeast cells had little endogenous respiration (approximately 2 to 4 c.mm. O_2 per mg. dry weight per hour) and remained with unaltered metabolic activities for 7 days when kept at 3°. Gonococci were obtained from Dr. Phillip C. Miller's laboratory and *E. coli* from Miss Helen Van Sant's laboratory of the Department of Medicine. *Ps. aeruginosa*, *M. lysodeikticus*, and *M. creatinovorans* were obtained from the National Type Culture Collection, Georgetown University. Crystalline plasma albumin was obtained from Armour Company. The ultrafiltration experiments as well as the determination of uranium were made according to the methods described by Muntz and Barron (1). Nitrogen determinations were made by the micro-Kjeldahl method of Ma and Zuazaga (2), glucose by the method of Nelson (3). The cell-free brewers' yeast juice was prepared according to Nilsson and Alms (4). The clear, golden-brown, supernatant fluid fermented hexosediphosphate when glucose was present, maximum CO_2 production occurring when the substrates were added at a ratio of 4 moles of glucose per mole of hexosediphosphate. Hexosediphosphate or glucose alone was not fermented; apparently this preparation lacks the enzyme apyrase, which according to Meyerhof (5) is easily inactivated by the maceration procedure. The yeast juice prepared in this manner is 0.1 M in inorganic P so that no additional phosphate need be added in order to obtain maximum activity. Work with sea urchin (*Arbacia punctulata*) sperm was performed at the Marine Biological Laboratory, Woods Hole. Shed sperm were centrifuged for 5 minutes, the supernatant coelomic fluid was pipetted off, and the sperm were suspended in about 20 volumes of Ca-free artificial sea water. To this was added an equal volume of 0.1 M acetate buffer in artificial sea water (pH 6.5). The pH of the final suspension was then adjusted to give a pH value of 6.3 to 6.6. $UO_2(NO_3)_2$ was made up fresh just before use, in sea water–acetate buffer, pH 6.5. Dry weights of the sea urchin sperm suspension were determined as follows: 0.8 cc. of the suspension was centrifuged for 5 minutes in an air-driven high speed centrifuge at 75 pounds air pressure (16,000 *g*); the supernatant fluid was removed, and 0.7 cc. of the same suspension was added to the tube. This was centrifuged again for 10 minutes at the same 75 pounds air pressure. The supernatant fluid was removed as completely as possible, and the tubes were dried at 105° overnight. Dry weights of sperm were quite uniform, 18 to 20 per cent of the fresh weight.

Uranium and Proteins.—It is well known that uranyl nitrate, like most salts of heavy metals, precipitates proteins. In small amounts, however, U combines reversibly with proteins, as was shown by Muntz and Barron (1) in their studies on the combination of serum proteins with uranium. The U-protein complex, formed mostly with the albumin fraction, was split on addition of bicarbonate. The combination of plasma crystalline albumin with uranium and its reversibility was studied by the ultrafiltration technique of Muntz and

Barron (1) at two pH values: 7.3 and 3.8. At pH 7.3, veronal buffer, addition of uranyl nitrate (0.001 M) to the buffer produced aggregates distinguished by the formation of a Tyndall effect. When albumin was present there was no Tyndall effect on addition of uranyl nitrate, an indication that the uranium had combined with protein. On ultrafiltration, 27 per cent of U passed through the membrane in the uranyl nitrate—buffer system, and 16 per cent in the uranyl nitrate-buffer-albumin system. Almost all of the U became ultrafilterable on addition of citrate at a ratio of U:citrate of 1:10 (Table I). The combination of U with albumin was not reversed on addition of phosphate at the same ratio as that of uranium and phosphate. This was demonstrated by adding phosphate to the albumin-buffer-uranium mixture. No precipitation

TABLE I

The Combination of Uranium with Crystalline Plasma Albumin. Effect of pH on the Reversibility of the Reaction. pH 7.3

In all experiments 3 cc. of 0.001 M $UO_2(NO_3)_2$ to 10 cc. volume. Veronal buffer, 0.05 M, pH 7.3. (1) 3 cc. $UO_2(NO_3)_2$ + 7 cc. 0.154 M NaCl not ultrafiltered; (2) 3 cc. $UO_2(NO_3)_2$ + 3 cc. buffer + 4 cc. NaCl; (3) 3 cc. $UO_2(NO_3)_2$ + 3 cc. buffer + 250 mg. albumin + 4 cc. NaCl; (4) 3 cc. $UO_2(NO_3)_2$ + 3 cc. buffer + 250 mg. albumin + 0.3 cc. citrate (0.1 M) + 3.7 cc. NaCl. Ultrafiltration for 1 hour. Uranium analysis in 1 cc. of ultrafiltrate. Figures give log I_o/I values = E.

Experiment No.	E values	Uranium in ultrafiltrate
		per cent
1. Control	0.660	100
2. U in buffer	0.180	27.3
3. U + buffer + albumin	0.108	16.4
4. U + buffer + albumin + citrate	0.610	92.4

of uranyl phosphate appeared after centrifugation for 1 hour at 3,000 R.P.M. In absence of protein, the UO_2HPO_4 precipitate appeared immediately.

At pH 3.8, in hippurate buffer, the albumin-U complex was more strongly associated than at pH 7.3, as shown by the ultrafiltration experiments in Table II. The uranium was not released from the protein on addition of citrate or phosphate. Uranyl nitrate in this buffer went through the membrane on ultrafiltration to the extent of 87 per cent. Further indication of the combination of uranium with albumin was found in the change of pH when uranyl nitrate was added to the protein. There was liberation of base and the solution became more alkaline.

Uranyl Nitrate and Enzymes.—The UO_2^{++} cation has a great tendency to form complex compounds with a large number of organic substances (6–8). Since the uranyl ion can form complexes with carboxyl, hydroxyl, keto, and amino groups, it is possible that it may form reversible complexes with re-

active groups in the side chains of the protein moiety of enzymes. Uranium may also inhibit enzyme reactions by combination with the prosthetic groups of enzymes or with adenosinetriphosphate, the high energy phosphate compound essential for energy transfer in a large number of oxidation-reductions. In fact, Singer *et al.* (9) found that uranium as uranyl nitrate inhibited reversibly a large number of enzyme systems, among which succinoxidase, hexosemonophosphate oxidase, and lysozyme were most sensitive. Cytochrome oxidase, monoamine oxidase, pancreatic esterase, carboxypeptidase, cathepsine, and urease were partially inhibited. Hexokinase, pyruvate oxidase, and choline oxidase were slightly inhibited. Enzyme inhibition was reversible, and re-

TABLE II

The Combination of Uranium with Crystalline Plasma Albumin. Effect of pH on the Reversibility of the Reaction. pH 3.8

In all experiments 3 cc. of 0.001 M $UO_2(NO_3)_2$ to 10 cc. volume. Hippurate buffer, 0.05 M, pH 3.8. (1) 3 cc. $UO_2(NO_3)_2$ + 7 cc. of 0.154 M NaCl not ultrafiltered; (2) 3 cc. $UO_2(NO_3)_2$ + 3 cc. buffer + 4 cc. NaCl; (3) 3 cc. $UO_2(NO_3)_2$ + 3 cc. buffer + 250 mg. albumin + 4 cc. NaCl; (4) 3 cc. $UO_2(NO_3)_2$ + 3 cc. buffer + 250 mg. albumin + 3.7 cc. NaCl + 0.3 cc. phosphate (0.1 M) adjusted to pH 3.8; (5) 3 cc. $UO_2(NO_3)_2$ + 3 cc. buffer + 250 mg. albumin + 0.3 cc. citrate (0.1 M) adjusted to pH 3.8. Ultrafiltration for 1 hour. Uranium analysis in 1 cc. of ultrafiltrate. Figures give I_o/I values = E.

Experiment No.	E values	U in ultrafiltrate
		per cent
1. Control	0.690	100
2. U in buffer	0.600	87
3. U + albumin	0.130	18.8
4. U + albumin + phosphate	0.125	18.1
5. U + albumin + citrate	0.145	21

activation was achieved on addition of citrate, α-hydroxyaspartate, oxalacetate, malate, tartrate, malonate, or oxalate. These substances reactivated the enzyme by the formation of U-complexes more strongly associated than the U-enzyme complex. Enzyme-U complex was not, however, dissociated on addition of phosphate. Furthermore, the degree of reactivation on addition of the organic acids above mentioned varied with the enzyme system. An example of such inhibition and reversal is given in Fig. 1, where the degree of inhibition of succinoxidase by varying concentrations of uranyl nitrate and reactivation by varying concentrations of citrate is given.

Uranyl Nitrate and Yeast Maceration Juice.—To test the effect of uranium on fermentation produced by cell-free yeast juice, the production of CO_2 at pH 6 was measured. Phosphate and veronal-acetate were used as buffers. $MgCl_2$ (0.004 M) and acetaldehyde (0.01 M) were added to glucose. The sub-

strate added to each vessel consisted of 0.1 cc. of 0.1 M hexosediphosphate and 0.2 cc. of 0.2 M glucose to a total of 3 cc. Uranyl nitrate inhibited the activity of yeast maceration juice, the degree of inhibition depending upon the

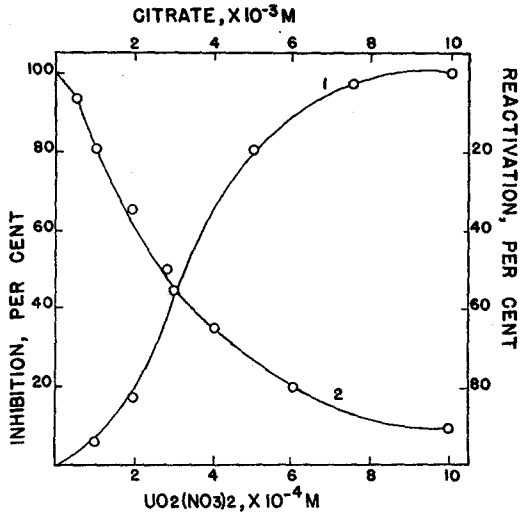

FIG. 1. Inhibition of succinoxidase (liver) by $UO_2(NO_3)_2$. Reactivation by Na citrate. (1) Inhibition of succinoxidase. (2) Reactivation of succinoxidase.

TABLE III

Inhibition by $UO_2(NO_3)_2$ of Yeast Juice Fermentation

Buffers, veronal-acetate, 0.032 M and phosphate, 0.01 M, pH 6.1. $UO_2(NO_3)_2$ brought to pH 6 before addition to the enzymes; final concentration, 0.001 M. Dry weight of yeast juice, 12.2 mg. per 0.1 cc. Temperature 23°. Final volume, 3 cc. Duration of experiments, 60 minutes.

Yeast juice	CO_2 production			
	Veronal buffer		Phosphate buffer	
	Control	U	Control	U
cc.	*c.mm.*	*c.mm.*	*c.mm.*	*c.mm.*
0.05	93	5	39	4
0.1	242	16	119	9.5
0.2	462	236	478	250

amount of yeast juice treated with a given concentration of U (Table III). The inhibition was essentially the same whether the experiment was carried out in veronal-acetate or in phosphate buffer. To test the reactivation of the enzymes on addition of phosphate, experiments were performed with 0.1 cc. of juice to which $UO_2(NO_3)_2$ was added to give a final concentration of 1 ×

10^{-3} M. After 10 minutes, increasing amounts of phosphate were added Phosphate produced a partial reversal; however, 23 moles of phosphate was required per mole of uranium to produce 22 per cent reactivation (Table IV). It can be concluded from these experiments that uranium inhibition of fermentation enzymes in yeast juice is similar to other enzyme inhibitions in that phosphate does not readily reactivate the enzymes.

Uranium and Cellular Metabolism.—It has been shown that the fermentation of glucose by cell-free brewers' yeast juice was inhibited by uranium and that

TABLE IV

Inhibition by $UO_2(NO_3)_2$ of Yeast Juice Fermentation. Partial Reactivation with Phosphate

Conditions as in Table III. Buffer, veronal-acetate. Phosphate added 10 minutes after addition of $UO_2(NO_3)_2$.

Phosphate	CO_2 production		
	Control	U	Inhibition
M	*c.mm.*	*c.mm.*	*per cent*
0.008	244	6.5	97
0.013	148	16	89
0.023	72	18	75

TABLE V

Inhibition by $UO_2(NO_3)_2$ of Glucose Fermentation in Brewers' Yeast Cells

Buffer, veronal-acetate, 0.05 M, pH 6.3. Gas phase, N_2. Yeast suspension 0.3 cc. of 5 per cent cells. Duration of experiment, 60 minutes. Temperature 23°.

$UO_2(NO_3)_2$	Glucose utilization
M	*mg.*
0	6.2
1×10^{-3}	0
3×10^{-4}	0
1×10^{-4}	0

this inhibition was only partially released on addition of phosphate. When the same experiments were repeated with intact brewers' yeast cells, no inhibition was obtained when fermentation was measured manometrically in bicarbonate buffer (pH 7.3) and N_2:CO_2 as gas phase. This striking contrast between enzyme experiments with cell-free extracts and with living intact cells was found to be due to reactivation of the inhibition by bicarbonate. Indeed, when veronal was used as buffer (pH 6.3) and N_2 as gas phase, 1×10^{-4} M uranyl nitrate produced complete inhibition in the fermentation of glucose, as measured by its utilization (Table V). In these experiments the CO_2 formed was absorbed by KOH in the center cup of the Warburg vessels.

The oxidation of glucose by bakers' yeast was equally sensitive to the inhibitory action of uranium at both pH values, 7.3 and 3.8 (Table VI). The inhibition at pH 7.3 was completely released on addition of phosphate or bicarbonate; addition of phosphate at a ratio of P:U of 5:1 produced 85 per cent reactivation; even a ratio of 1:1 produced 73 per cent reactivation. Furthermore, reactivation was achieved by adenosinetriphosphate, hexosediphosphate, citrate, and even by simply washing the cells with distilled water. However, inhibition by $UO_2(NO_3)_2$ at pH 3.8 (hippurate buffer) was not released on addition of phosphate, adenosinetriphosphate, or citrate (Table VII).

When the uranyl nitrate concentration was kept constant and the amount of yeast cells was varied, there was a close relationship between the inhibition of glucose oxidation and the ratio of yeast weight to U. Complete inhibition

TABLE VI

Effect of pH on $UO_2(NO_3)_2$ Inhibition of Glucose Oxidation by Yeast

Concentration of buffer 0.025 M. Glucose, 0.01 M. $UO_2(NO_3)_2$, 2×10^{-5} M. Yeast, 3.7 mg. dry weight. Temperature 28°.

Buffer	pH	O_2 uptake		Inhibition
		Control	$UO_2(NO_3)_2$	
		c.mm.	*c.mm.*	*per cent*
Veronal	7.3	257.5	23.5	91
Hippurate	3.8	218.5	58.2	73.5

TABLE VII

Effect of pH on the Reversibility of $UO_2(NO_3)_2$ Inhibition of the Oxidation of Glucose by Bakers' Yeast

Buffer, 0.05 M. Glucose, 0.01 M. $UO_2(NO_3)_2$ 0.00002 M; citrate, or phosphate, 0.002 M; adenosinetriphosphate or hexosediphosphate, 0.001 M, added 10 minutes after the addition of $UO_2(NO_3)_2$ and adjusted to the pH of the buffer.

Additions	pH	O_2 uptake		Inhibition
		Control	$UO_2(NO_3)_2$	
		c.mm.	*c.mm.*	*per cent*
Veronal	7.3	262	8.5	97
Veronal + phosphate	7.3	262	262	None
Veronal + citrate	7.3	263	263	None
Veronal + ATP	7.3	278	275	None
Veronal + hexose-diphosphate	7.3	280	278	None
Hippurate	3.8	218	58	73.5
Hippurate + phosphate	3.8	219	56.1	74
Hippurate + citrate	3.8	220	57	74
Hippurate + ATP	3.8	230	58	75

was reached with 7.7 micrograms of uranium per mg. dry weight of yeast cells (Table VIII). An attempt was made to measure the amount of uranium adsorbed by yeast cells (2 mg. per cc.). Since a concentration of 2×10^{-5} M of uranyl nitrate inhibited 98 per cent glucose oxidation by 1.86 mg. of yeast, an analysis of uranium in the supernatant fluid would give the amount adsorbed by the cell at the saturation level. 102.6 mg. of yeast was suspended in 4.6 cc. H_2O. To this suspension was added 17.5 cc. 0.1 M veronal buffer, pH 7.3, and 10 cc. 2×10^{-4} M $UO_2(NO_3)_2$. Half an hour later the suspension was centrifuged for 1 hour and uranium was determined in the supernatant fluid. An aliquot of the control sample (3 cc.) with no yeast contained in the supernatant 37 micrograms uranium, while the supernatant of the yeast suspension contained 16 micrograms. A total of 21 micrograms of uranium was taken by the

TABLE VIII

$UO_2(NO_3)_2$ Inhibition of Glucose Oxidation by Bakers' Yeast. Relation of Yeast Weight to Inhibition

$UO_2(NO_3)_2$, 2×10^{-5} M (14.28 micrograms per vessel). Buffer, veronal, 0.04 M, pH 7.3. Glucose, 0.01 M. Duration of experiments, 40 minutes. Temperature 28°. Figures are blank subtracted (blank, O_2 uptake = 2.2 c.mm. per mg. dry weight per hour).

Yeast Dry weight	O_2 uptake		Inhibition
	Control	$UO_2(NO_3)_2$	
mg.	*c.mm.*	*c.mm.*	*per cent*
7.44	337.5	306.4	9.4
5.58	244.0	211.5	13.3
3.72	164.0	64.0	60.8
1.86	84.3	1.5	98

yeast cells at the saturation level (complete inhibition of glucose oxidation); *i.e.*, 0.2 microgram U per mg. dry weight of yeast cells. Since 1 mg. of yeast cells contained 7.2×10^7 cells, 1 yeast cell would contain at saturation 7.06×10^6 molecules of uranium, not enough to cover the surface of the cell entirely (one yeast cell is from 73 to 388 square micra). One mg. of yeast cells contained 0.376 mg. protein. If the value of 100,000 is taken for the molecular weight of cell membrane protein, one molecule of protein would contain 0.232 molecule of uranium, an indication that only certain protein molecules of the cell membranes combine with uranium. Further evidence of this contention is given by the lack of inhibition of $UO_2(NO_3)_2$ on the activity of yeast saccharase (Fig. 2), an enzyme distributed at the surface of the cell.

Inhibition of glucose oxidation by uranium seemed to be confined to yeast cells. Thus the oxidation of glucose by bacteria such as *Ps. aeruginosa*, *E. coli*, and *M. lysodeikticus* was inhibited only 25 to 19 per cent, while oxidation by *M. creatinovorans* was not affected at all (Table IX).

If inhibition of glucose oxidation was due to combination of uranium with enzymes or coenzymes *in the cell* it would be possible to detect an induction period due to the rate of penetration. Of the numerous experiments devised

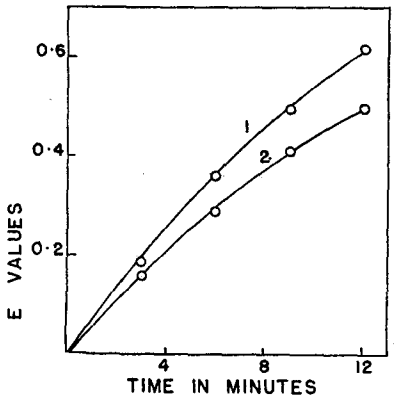

FIG. 2. Effect of $UO_2(NO_3)_2$ on the activity of bakers' yeast saccharase. Buffer, hippurate, 0.05 M, pH 3.8. Sucrose, 0.01 M. Temperature 0°. The E values are log $\frac{I_0}{I}$ values for the glucose colorimetric reaction of Nelson (3). (1) Control; (2) $UO_2(NO_3)_2$, 1×10^{-3} M.

TABLE IX

Comparative Effect of $UO_2(NO_3)_2$ on the Oxidation of Glucose by Yeast Cells and Bacteria

Buffer, veronal pH 7.3, 0.05 M. Glucose, 0.01 M; $UO_2(NO_3)_2$, 1×10^{-4} M. Temperature 28° for yeast, 38° for bacteria.

Cells	Weight	O_2 uptake		Inhibition
		Control	$UO_2(NO_3)_2$	
	mg.	*c.mm.*	*c.mm.*	*per cent*
Bakers' yeast	3.0	198	0	Complete
Ps. aeruginosa	4.4	400	300	25
E. coli	4.1	450	338	25
M. lysodeikticus	5.3	94	76.1	19
M. creatinovorans	6.2	53	65	None

to find this induction period all were negative. When glucose and uranyl nitrate were added simultaneously (in experiments with rapid rate of oxidation of glucose), inhibition set in instantaneously. When uranyl nitrate was added 3, 6, and 9 minutes after glucose oxidation had begun, inhibition started also immediately, and the oxygen consumption measured was probably that due

to the glucose which had penetrated into the cells prior to addition of uranium (Fig. 3).

Membrane Permeability and Cellular Oxidations.—Yeast cells are ideal for the study of the role of cell membranes as one of the regulatory mechanisms of cellular metabolism, because they can be kept suspended in buffer solutions varying from pH 2 to pH 8.5 with no change in the rate of their endogenous

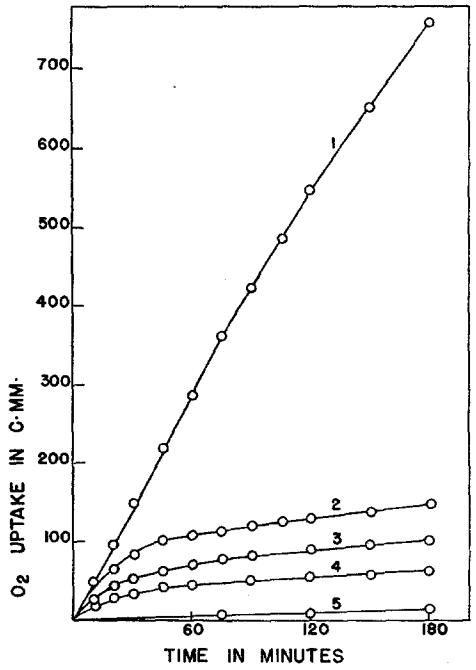

FIG. 3. $UO_2(NO_3)_2$ inhibition of glucose oxidation by bakers' yeast cells. Effect of time of addition of $UO_2(NO_3)_2$. Buffer, veronal, 0.05 M, pH 7.3. $UO_2(NO_3)_2$ 1 $\times$ 10^{-4} M. Glucose, 0.01 M. Temperature 28°. (1) Control; (2) $UO_2(NO_3)_2$ added 9 minutes after glucose addition; (3) $UO_2(NO_3)_2$ added 6 minutes after glucose addition; (4) $UO_2(NO_3)_2$ added 3 minutes after glucose addition; (5) $UO_2(NO_3)_2$ and glucose added simultaneously.

respiration. The membrane of the yeast cell was found to be easily permeable to pyruvic acid while the pyruvate ion did not penetrate, as can be seen by the effect of pH on the rate of oxidation of this acid. Pyruvic acid was oxidized at the highest rate at an H^+ ion concentration close to its pK value and oxidation did not become appreciable until 0.4 per cent of it was in the form of the undissociated acid. On the other hand, the behavior of the cell membrane towards acetic acid seemed to vary. While the rate of oxidation of acetic acid was independent of the degree of dissociation of the acid (for the O_2 uptake was

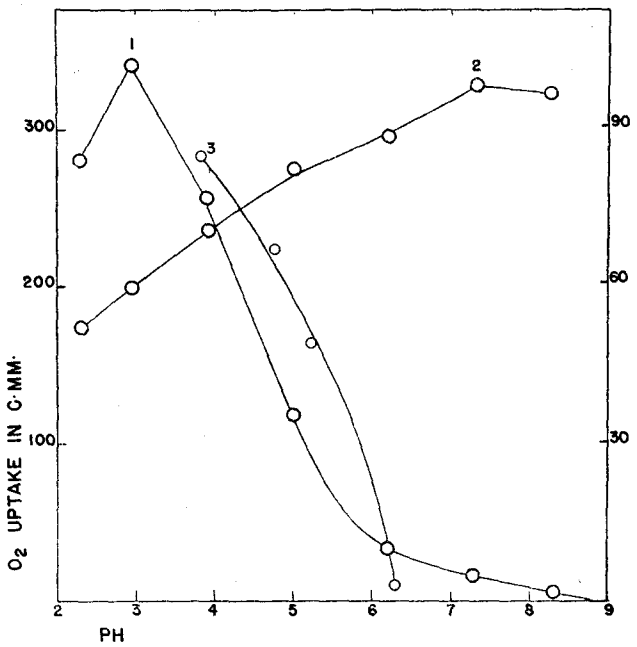

FIG. 4. Effect of pH on the rate of oxidation of pyruvic and acetic acids by bakers' yeast. Buffer, Theorell and Stenhagen's universal buffer; yeast, 10 mg. dry weight. Substrate, 0.01 M. Temperature 28°. Duration of experiment, 1 hour. The figures given are blank subtracted. Blank, 34 to 40 c.mm. O_2 uptake per hour. (1) O_2 uptake in the presence of pyruvic acid. (2) O_2 uptake in the presence of acetic acid. (3) Inhibition of acetate oxidation by fluoroacetic acid (2×10^{-6} M) (per cent inhibition on the right side of ordinate).

TABLE X

Effect of $UO_2(NO_3)_2$ on Oxidations Produced by Bakers' Yeast

Buffer, hippurate, 0.025 M, pH 3.8. Substrate concentration 0.01 M. $UO_2(NO_3)_2$, 0.00002 M. Duration of experiments, 60 minutes. Temperature 28°. All figures are blank subtracted.

Substrate	O_2 uptake		Inhibition
	Control	$UO_2(NO_3)_2$	
	c.mm.	*c.mm.*	*per cent*
Glucose	370	53	85.5
Lactate	100	70	30
Pyruvate	279	233	16
Acetate	170	207	None
Citrate	23.5	25.9	None
Ethyl alcohol	399	383	4
Malate	13.4	22.1	None

greater when 0.06 per cent of the added acetic acid was as the undissociated acid than when half of it was undissociated), the inhibition of acetate oxidation by fluoroacetic acid was proportional to the degree of undissociation of this acid, an indication that only the undissociated acid penetrates through the cell membrane (Fig. 4).

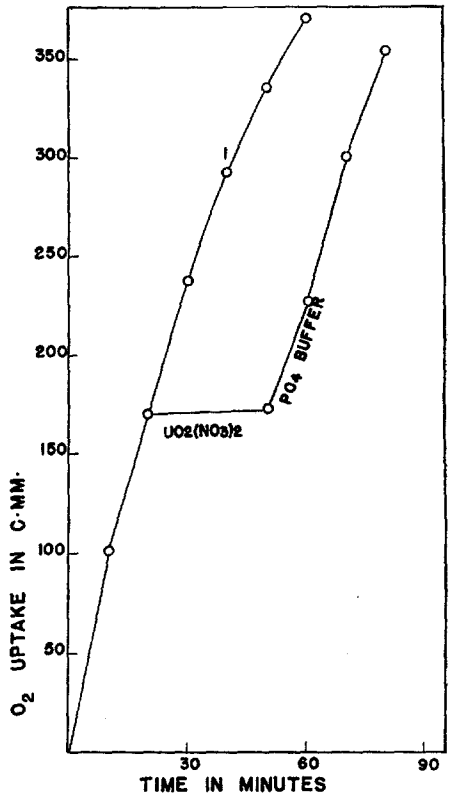

FIG. 5. $UO_2(NO_3)_2$ inhibition of the oxidation of lactate by gonococci. Reactivati n with phosphate. Buffer, veronal, 0.05 M. Lactate, 0.01 M. $UO_2(NO_3)_2$, 0.001 MO Phosphate, 0.01 M added 20 minutes later from the second side arm of the Warb.urg vessel. Temperature 38°. (1) Control.

Uranium and the Oxidation of Organic Acids.—The oxidation of acetate, citrate, ethyl alcohol, and malate at pH 3.8 was not inhibited at all by a concentration of uranyl nitrate strong enough to inhibit glucose oxidation 85 per cent. The oxidation of lactate was inhibited 30 per cent, and that of pyruvate 16 per cent (Table X).

The inhibition of glucose oxidation by uranium was at first thought to be

due to combination of U with adenosinetriphosphate distributed at the surface of the cell membrane. (The combination of uranyl with adenosinetriphosphoric acid was demonstrated by spectrophotometric measurements.) However, lactate oxidation by yeast, which does not require adenosinetriphosphate, was partially inhibited. The oxidation of lactate by gonococci was completely inhibited by 1×10^{-3} M uranyl nitrate, and the inhibition was completely released on addition of phosphate at a ratio of U:phosphate of 1:10 (Fig. 5). It must be recalled that the oxidation of lactic acid by the enzyme lactic dehydrogenase was not inhibited by U (9).

In cell-free tissue suspensions the oxidation of succinate was inhibited by uranium, an inhibition not affected by the addition of phosphate. The oxida-

TABLE XI

Effect of $UO_2(NO_3)_2$ on the Oxidation of Succinate by Liver Succinoxidase and by E. coli

Buffer, veronal, 0.05 M, pH 7.3. Succinate, 0.04 M. $UO_2(NO_3)_2$, 0.001 M. Temperature 38°. Duration of experiments, 60 minutes for the enzyme and 180 minutes for *E. coli*. Phosphate, 0.01 M, pH 7.3, added 10 minutes after the addition of $UO_2(NO_3)_2$.

System	O_2 uptake		Inhibition
	Control	$UO_2(NO_3)_2$	
	c.mm.	*c.mm.*	*per cent*
Liver Enzyme			
Succinate	213	23	89
Succinate + phosphate	220	25	89
E. coli			
Succinate (blank subtracted)	137.6	40.8	70
Succinate + phosphate	138	106	23

tion of succinate by *E. coli* was also inhibited by uranium, the inhibition in this case, however, being partially released on addition of phosphate 10 minutes after the addition of uranyl nitrate, an indication that inhibition in the intact cell was not due to combination of the enzyme with U (Table XI).

Uranyl Nitrate and Sea Urchin Sperm.—The inhibitory effect of uranyl nitrate is not confined to the metabolism of yeast cells and bacteria. To study the effect of uranium on isolated animal cells the spermatozoa of sea urchin (*Arbacia punctulata*) were chosen because of their high respiration. The respiration of these cells was inhibited by concentrations of uranyl nitrate, from 1×10^{-3} to 5×10^{-4} M. Half-inhibition was obtained with 1×10^{-4} M (Table XII). Some difficulty was found in the experiments where reversal by citrate and phosphate was studied, because addition of phosphate and citrate to sea water buffered with acetate (artificial sea water containing no calcium) inhibited respiration. To find the extent of release it was therefore necessary to compare

with the respiration of sperm suspended in sea water containing the same amount of citrate or phosphate as that added to the samples containing uranyl nitrate. Under those conditions both citrate and phosphate added at a ratio of 1:10 brought back sperm respiration to that of sperm in citrate or phosphate (Table XIII).

TABLE XII

Effect of $UO_2(NO_3)_2$ on the Respiration of Sea Urchin Sperm

Washed cells suspended in sea water buffered with acetate, 0.05 M. QO_2 values give c.mm. O_2 uptake per mg. dry weight per hour.

$UO_2(NO_3)_2$	pH	Weight of sperm	QO_2		Inhibition
			Control	$UO_2(NO_3)_2$	
M		*mg.*	*c.mm.*	*c.mm.*	*per cent*
1×10^{-3}	6.44	3.6	10	0.8	92
1×10^{-3}	6.50	2.2	31	3.0	88
1×10^{-3}	6.56	7.9	6.7	1.2	82
5×10^{-4}	6.38	6.6	11.0	1.1	90
5×10^{-4}	6.91	8.3	8.4	0.85	90
5×10^{-4}	6.50	6.25	6.9	0.87	87
1×10^{-4}	6.63	2.3	30.0	14.0	53
5×10^{-5}	6.60	3.7	13.0	10.0	23
1×10^{-5}	6.53	7.0	7.0	6.8	3

TABLE XIII

$UO_2(NO_3)_2$ Inhibition of Sea Urchin Sperm Respiration. Release with Citrate and with Phosphate

Phosphate (0.05 M), and citrate (0.02 M) added 10 minutes after the addition of $UO_2(NO_3)_2$ 5×10^{-4} M.

Conditions	QO_2		Inhibition
	Control	$UO_2(NO_3)_2$	
	c.mm.	*c.mm.*	*per cent*
Sperm cells in acetate	6.20	0.8	87
Sperm cells in phosphate	2.50	2.70	None
Sperm cells in acetate	8.4	0.85	90
Sperm cells in citrate	5.7	5.80	None

DISCUSSION

The experiments presented in this paper have shown that U in small concentrations can combine reversibly with certain proteins. With plasma albumin the dissociation of the protein-U complex at the alkaline side of the isoelectric point of albumin was readily accomplished on addition of citrate (pH 7.3), while there was no dissociation at pH 3.8. Albumin-U complex was,

however, strong enough not to be dissociated by phosphate at any pH. Similar results were found with enzymes: the inhibition of succinoxidase (from pigeon breast or liver) by uranyl nitrate was released on addition of citrate, while phosphate had no effect at all. When the glucose-fermentation enzymes of brewers' yeast were treated with uranyl nitrate there was inhibition of fermentation, an inhibition which was not abolished on addition of phosphate at a ration of U:P of 1:10.

The experiments with living cells of yeast showed striking differences from those described with uranium and enzymes. The fermentation of glucose by yeast inhibited by uranium was completely released on addition of phosphate or bicarbonate, substances which were unable to release the inhibition of fermentation in cell-free yeast juice. Uranium inhibition of the oxidation of glucose by living yeast was completely released at pH 7.3 on addition of phosphate, citrate, adenosinetriphosphate, and hexosediphosphate. Of these substances, phosphate does not release enzyme inhibitions and the last two do not penetrate across the membrane of yeast cells. If the uranium had penetrated into the cell no release of inhibition could have been produced on addition of adenosinetriphosphate or hexosediphosphate. It must be concluded therefore that inhibition is due not to combination of the metal with enzyme systems within the cell, but rather to adsorption of the metal into the cell membrane making it impermeable to glucose. Inhibition of glucose oxidation by combination with hexokinase or adenosinetriphosphate distributed at the surface of the cell cannot be accepted because (1) hexokinase is not appreciably inhibited by U (9), (2) adenosinetriphosphate addition did not release inhibition at pH 3.8, and (3) such a combination does not explain the inhibition of lactate oxidation.

The inhibition of lactate oxidation by uranium in gonococci, and complete reversal by phosphate, can only be explained as inhibition due to alteration of membrane permeability, because uranium did not inhibit lactate oxidase. The experiments with liver succinoxidase, and oxidation of succinate by *E. coli*, similarly can have no other interpretation. Inhibition of succinoxidase by uranium was not released on addition of phosphate, while phosphate did release partially such inhibition in the intact cell.

These experiments favor the assumption that U combines with the protein portion of the cell membrane and thus renders it less or completely impermeable to the passage of certain oxidizable substrates. This remarkable property of uranium of inhibiting cellular metabolism not by combination with enzymes but by combination with the cell membrane and alteration of its permeability is, we believe, the first example of a new kind of oxidation inhibition, surface inhibitions. Such inhibitions have been postulated by Clark (10), but none of the data presented in favor of this hypothesis were shown to be due to combination of inhibitor with the surface of the cell.

SUMMARY

Uranium as $UO_2(NO_3)_2$ combines reversibly with proteins. The degree of dissociation of this combination depends, among other factors, on the H^+ concentration. At pH 7.3 the U-albumin complex was easily dissociated on addition of citrate, while at pH 3.8 it was not. Uranium inhibited reversibly a number of enzyme systems.

Uranium enzyme inhibitions could be reversed on addition of certain hydroxypolycarboxylic acids (citric acid, α-hydroxyaspartic acid, malic acid); in no case, however, did phosphate have any effect.

In cell-free yeast juice, the fermentation of glucose-hexosediphosphate was inhibited by $UO_2(NO_3)_2$. Slight reactivation occurred on addition of phosphate.

In living yeast cells, the fermentation and oxidation of glucose was inhibited by small amounts of $UO_2(NO_3)_2$ (7.7 micrograms per mg. dry weight), while the oxidation of acetic acid, ethyl alcohol, malic and citric acids, was not affected at all. U inhibition in living yeast cells at pH 7.3 was completely released on addition of small amounts of phosphate, adenosinetriphosphate, and citrate, while at pH 3.8 U inhibition was not released by phosphate and citrate. At saturation, one yeast cell contained 7.06×10^6 molecules of uranium. Lactic dehydrogenase was not inhibited by U while the oxidation of lactic acid by gonococci was inhibited. Addition of phosphate released this inhibition. The U inhibition of liver succinoxidase was unaffected by phosphate, while the U inhibition of the oxidation of succinate by *E. coli* was released by phosphate.

It has been concluded from these experiments that U inhibition of cell metabolism is due to combination of the metal with the protein portion of the cell membrane. Uranium is presented as an example of surface inhibition.

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