CCLXXXVIII. INHIBITORY ACTION OF THE LOWER ALIPHATIC ACIDS AND ALDEHYDES ON CYTOCHROME REDUCTION IN YEAST.

By LYLE VIBERT BECK.

From the Cancer Research Laboratory, Graduate School of Medicine, University of Pennsylvania, Philadelphia, Pa., and Lilly Research Laboratories, Marine Biological Laboratory, Woods Hole, Massachusetts.

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KEILIN [1929; 1933] has demonstrated the important rôle which cytochrome plays in cellular respiration as carrier between activated oxygen and activated substances. He has opened up the possibility of making use of the rate of cytochrome reduction as a measure of intracellular dehydrogenase activities, in much the same way in which methylene blue has been used by Thunberg and his associates [1930; Ahlgren, 1925]. Keilin has further shown that the state of oxido-reduction of cytochrome is of value in deciding which part, if any, of the indophenol oxidase-cytochrome-dehydrogenase respiratory mechanism is affected by various respiratory inhibitors. For example cyanide, hydrogen sulphide and carbon monoxide inhibit respiration by blocking the indophenol oxidase system whilst the narcotics act by blocking the dehydrogenases.

Beck and Robin [1934] have found that Fleischmann's yeast suspended in acetate buffers requires a much longer time to reduce cytochrome and oxidation-reduction indicators than does yeast suspended in citrate-phosphate and phosphate buffers covering the same p_H range (4.0–7.0). In parallel experiments on yeast vitally stained with methyl red the acetate buffers were found to induce rapid intracellular acidification whilst the other buffers had no such effect. The inhibitory effects of the acetate buffers were therefore attributed to intracellular acidification acting on dehydrogenase systems.

These findings were of sufficient interest to suggest further experimentation, to determine, if possible, whether the action of the acetate buffers might be due to factors other than increase in intracellular acidity. Experiments were therefore performed in which the first six aliphatic acids were compared with one another and with hydrochloric and citric acids, the last two agents having been selected because of their slow penetration into cells. The acids were also compared with some of the chemically homologous aldehydes, alcohols and urethanes, as to their action on cytochrome reduction. The actions of acetic and butyric acids, each in the presence and absence of its sodium salt, have been determined with particular care, because of the possible bearing of the data on the problem of penetration into cells of the salts of penetrating acids.

EXPERIMENTAL.

Materials.

Fleischmann's yeast¹ was employed, since Keilin has found that commercial yeast constitutes very excellent material for the study of cytochrome oxidation and reduction, and since yeast cells are not injured by external p_H values so low (2.0–5.0) as to be toxic to most cells [Richards, 1932].

¹ Fleischmann's yeast contains more than one strain of yeast and many bacilli and cocci, as well as starch. The author feels that its use is justified in experiments of the kind reported in this paper, since consistent results were always obtained.

Methods.

A 10 % by weight suspension of Fleischmann's yeast in tap-water was aerated vigorously for 20–30 min. and then kept for 2 hours. Samples for testing the rate of cytochrome reduction were prepared in amounts of 4.5 ml. of yeast suspension in each of a series of test-tubes. To determine a normal or basic rate of cytochrome reduction 0.5 ml. of tap-water was added to the yeast suspension in one of the test-tubes. Its contents were shaken together and poured into a small test-tube placed between a good source of light and a hand spectroscope. The suspension was then aerated in the small test-tube at a constant pressure for exactly 1 min., and the time required to secure reappearance of the D band of reduced cytochrome determined with a stop-watch. The value so obtained is termed the "2-min." value, since approximately 2 min. are required for manipulation and aeration. Two hours later this same suspension was tested again, in exactly the same manner, giving another value, called the "2-hour" value. By this method, the basic reduction time values for different samples were found to agree satisfactorily with one another, as may be seen from the tables.

The same procedure was employed to test the reaction of the yeast to a given chemical solution, except that 0.5 ml. of a solution of the chemical in ten times the desired concentration was substituted for the 0.5 ml. of tap-water.

Effects of aliphatic acids and aldehydes on rate of cytochrome reduction.

The effects on cytochrome reduction of the six lowest fatty acids, HCl and citric acid, in 0.01 and 0.001 *M* concentration, are shown in Table I. The comparative effects on cytochrome reduction of the acids, formic to butyric, and their corresponding aldehydes, again in 0.01 and 0.001 *M* concentration, are shown in Tables II and III.

Table I. *Cytochrome reduction in yeast as affected by various acids.*

Agent to which yeast sample was exposed	Seconds required to secure return of D band of reduced cytochrome					
	Values for yeast exposed to acids in 0.01 <i>M</i> conc.			Values for yeast exposed to acids in 0.001 <i>M</i> conc.		
	Values after 2-min. exposure	Values after 2-hour exposure	Values after washing and addition of glucose	Values after 2-min. exposure	Values after 2-hour exposure	
Controls (tap-water)	19, 19	18, 21	11, —	19, 24	28, 28	
Hydrochloric acid	22	34	8	29	32	
Citric acid	24	30	10	30	34	
Formic acid	(0)*	(0)†	9, 10	30	28	
Acetic acid	38	45	10	27	26	
Propionic acid	73	77	9	28	31	
Butyric acid	94	96	10	28	33	
Valeric acid	123	142	9	29	36	
Hexanoic acid	123	154	14	28	38	
Citrate-PO ₄ buffer, pH 4.0	17	24	10	24	31	

* Formic acid, in 0.01 *M* concentration, differs markedly from the other fatty acids in its action on cytochrome, which it brings into a condition where weak bands of the reduced form are seen; a condition not affected by long and vigorous aeration or long periods of standing without aeration.

† The citrate-phosphate buffer was used in 1/10 the standard concentration.

Table II. *Comparison of aldehydes with fatty acids in their effects on cytochrome reduction.*

Agent to which yeast sample was exposed	Seconds required to secure return of D band of reduced cytochrome				
	Values* for yeast exposed to agents in 0.01 <i>M</i> conc.			Values* for yeast exposed to agents in 0.001 <i>M</i> conc.	
	Values after 2-min. exposure	Values after 2-hour exposure	Values after washing and addition of glucose	Values after 2-min. exposure	Values after 2-hour exposure
Controls (tap-water)	47	47	33	63	66
Formaldehyde	65	(0)†	32	—	—
Acetaldehyde	61	59	35	—	—
Acetic acid	74	108	33	—	—
Propaldehyde	67	64	31	—	—
Propionic acid	127	131	32	—	—
Butyraldehyde	97	108	34	68	71
Butyric acid	140	159	34	77	73
Valeraldehyde	—‡	—‡	—	76	69
Valeric acid	—‡	—‡	—	83	73

* Each value, as given, is the average to the nearest whole number of seconds of three separate determinations.

† Like formic acid, formaldehyde in 0.01 *M* concentration brings cytochrome into a condition where weak bands of the reduced form are seen; a condition not affected by long and vigorous aeration or long periods of standing without aeration.

‡ Valeraldehyde is not sufficiently soluble to permit the preparation of a 0.1 *M* stock solution, as required by the technique employed with all the other agents, hence no attempt was made to compare valeraldehyde with valeric acid and with the lower acids and aldehydes at 0.01 *M* concentration.

Table III. *Statement of results with aldehydes and acids, in 0.01 M concentration, in terms of relative velocities.*

Agents		Aldehydes		Acids	
		Velocity* of cytochrome reduction	% inhibition of cytochrome reduction	Velocity* of cytochrome reduction	% inhibition of cytochrome reduction
Controls	2-min. values	21.3	—	21.3	—
	2-hour values	21.3	—	21.3	—
2-carbon compounds	2-min. values	16.4	23	13.5	37
	2-hour values	16.9	21	9.3	56
3-carbon compounds	2-min. values	14.9	30	7.9	63
	2-hour values	15.7	26	7.6	64
4-carbon compounds	2-min. values	10.3	52	7.15	66
	2-hour values	9.3	56	6.3	70

* Velocity of cytochrome reduction is defined as $1000/t$, where t = no. of seconds required to secure return of the D band of reduced cytochrome (see Table II).

The effects of these various agents in 0.01 *M* concentration may be summarised as follows.

(a) Formic acid and formaldehyde differ from all the other agents in bringing cytochrome into a peculiar condition characterised by the appearance of weak bands of the reduced form. This condition is not affected by vigorous and long-continued aeration or by long keeping without aeration. Formic acid acts more quickly than does formaldehyde in bringing cytochrome into this condition.

(b) The remaining aliphatic acids and aldehydes strongly inhibit cytochrome reduction, each of the acids being considerably more effective than the corresponding aldehyde.

(c) The greater the length of the aliphatic chain of an acid or aldehyde the greater is its effectiveness in inhibiting cytochrome reduction.

(d) The aliphatic acids are far more effective than are the stronger but much more slowly penetrating hydrochloric and citric acids.

(e) The effects of all these agents on cytochrome reduction may be completely reversed by thorough washing and addition of the substrate glucose.

These various agents have relatively little effect on cytochrome reduction when used in $0.001M$ concentration.

Effects of aliphatic alcohols and urethanes on rate of cytochrome reduction.

The alcohols methyl to amyl and the urethanes ethyl, propyl and butyl were tested in $0.01M$ concentration. At this concentration, the urethanes were found not to affect cytochrome reduction, whilst of the alcohols only methyl and ethyl had any effect, and their effects were acceleration rather than retardation of cytochrome reduction.

Relative penetrations of the various acids into yeast cells.

Since the relatively weak aliphatic acids were far more effective in inhibiting cytochrome reduction than were hydrochloric and citric acids, it was thought of interest to compare these various acids, at least qualitatively, as to rate and degree of penetration into the yeast. This was done by a study of colour changes in yeast vitally stained with propyl red¹ and then treated with the various acids.

The tests were carried out by adding propyl red in 0.002% concentration to phosphate buffer of p_H 5.4. After 3 min., samples of the stained yeast were centrifuged, washed once with fresh citrate-phosphate buffer of p_H 5.4 to remove as completely as possible the propyl red which had failed to penetrate into the cells and treated with one of the acids in 0.01 , 0.0033 or $0.001M$ concentration. The colour changes in each sample during the next 10 min. were noted with the naked eye by comparison with a control sample of stained yeast.

With the exception of formic, all the fatty acids and in all the concentrations employed induced a very rapid change in colour of the stained yeast; this method was unsatisfactory for detecting differences in rate of penetration within the series. Formic acid induced a pronounced change in colour when used in $0.01M$ concentration and a faint colour change when used in $0.0033M$ concentration. Hydrochloric and citric acids in $0.01M$ concentration induced a slow faint change of colour of the stained yeast, an effect quite possibly due to colour changes of dye outside the yeast. In the two lower concentrations neither agent produced any colour changes in the yeast suspensions. The order of penetration of these acids into Fleischmann's yeast, is, by this method: valeric to acetic, formic, citric, hydrochloric.

¹ Like methyl red [Chambers, 1930], propyl red stains cells only from acid media. Propyl red is more satisfactory than methyl red for studying acid penetration into yeast cells, since it gives a deeper stain, and the fading of its colour is slower. This fading of methyl red and propyl red in the presence of yeast has not been observed with other cells, such as marine eggs and amoebae. It seems likely that it is the result of an irreversible reduction of these compounds. The effect is not reversible with oxidising agents, e.g. ferricyanide.

Counteracting effects of Na acetate and Na butyrate on the inhibition of cytochrome reduction by acetic acid and butyric acid respectively.

In so far as the salts of the fatty acids penetrate they may be expected to buffer the intracellular reaction back towards its original condition and so may be expected to counteract in some degree any part of the action of the acids on cytochrome reduction due to intracellular acidification.

Yeast treated with 0.01M acetic acid was compared with yeast treated with 0.01M acetic acid + 0.03M sodium acetate and with yeast treated with 0.01M acetic acid + 0.09 M sodium acetate as to rate of cytochrome reduction. The principle of keeping the concentration of the acid constant while varying that of the salt is adopted from Lillie [1926] and Howard [1932]. These experiments show that as a rule the acid alone inhibits cytochrome reduction to a greater degree than the acid *plus* its sodium salt. This differential effect can be detected within 2 min. after beginning treatment of the yeast with the acid or acid-salt solution. In part A of Table IV are summarised the results of a lengthy experiment carried out to secure sufficient data on this difference for the application of statistical methods. The results of this experiment demonstrate that the observed counteracting effects of sodium acetate are not due to chance.

An experiment carried out in exactly the same manner with 0.01M butyric acid, and 0.01M butyric acid + sodium butyrate gave the data shown in summary form in part B of Table IV, from which it is evident that sodium butyrate is considerably more effective in counteracting butyric acid than is sodium acetate in counteracting acetic acid.

Table IV.

Part A—Comparison of (a) 0.01 M acetic acid, (b) 0.01 M acetic acid + 0.09 M Na acetate in their effects on cytochrome reduction.

Part B—Comparison of (a) 0.01 M butyric acid, (b) 0.01 M butyric acid + 0.09 M Na butyrate in their effects on cytochrome reduction.

Agent to which yeast samples were exposed	Seconds required to secure return of D band of reduced cytochrome (each value for (a) and (b) is the average of 10 determinations $\pm$ its standard error)	
	Values for 2-min. exposure period	Values for 2-hour exposure period
Part A		
Controls (tap-water)	19.3, 22.0	29.1, 29.4
0.01 M acetic acid (a)	43.37 $\pm$ 0.80	48.07 $\pm$ 0.37
0.01 M acetic acid + 0.09 M Na acetate (b)	40.14 $\pm$ 0.73	40.13 $\pm$ 0.32
Differences and standard errors of differences between (a) and (b)	3.23 $\pm$ 1.27	7.94 $\pm$ 0.49
Part B		
Controls (tap-water)	34.7, 36.8, 34.1	38.0, 40.3, 33.4
0.01 M butyric acid (a)	90.05 $\pm$ 1.59	94.10 $\pm$ 1.65
0.01 M butyric acid + 0.09 M Na butyrate (b)	66.33 $\pm$ 0.89	64.07 $\pm$ 0.91
Differences and standard errors of differences between (a) and (b)	23.72 $\pm$ 1.83	30.03 $\pm$ 1.88

These results do not establish the mode of action of sodium acetate and sodium butyrate in counteracting the corresponding acids. The action of the salts might be due, in part or in whole, to any of the following: (I) the considerably greater osmotic pressures of the solutions containing both the acid and the salt, (II) the considerable difference in external p_H in the two cases (p_H 3+ for

0.01 *M* acetic acid or butyric acid; p_H 6— for 0.01 *M* acetic or butyric acid *plus* the corresponding salt in 0.09 *M* concentration), (III) utilisation of sodium acetate or sodium butyrate as substrate for cytochrome reduction, (IV) penetration of the salt to a degree sufficient to overcome in some degree the intracellular acidification induced by the acid. Experiments were therefore performed to determine to what extent (I), (II) and (III) may account for the observed counteracting effects of the fatty acid salts.

(I) *The considerably greater osmotic pressures of solutions containing both acid and salt.* Yeast was treated with (a) 0.01 *M* acetic acid, (b) 0.01 *M* acetic acid *plus* 0.09 *M* sodium chloride, (c) 0.01 *M* acetic acid *plus* 0.09 *M* sodium acetate. The results of these experiments were that reduction times for yeast treated with (a) and (b) were identical, within the limits of error of the method, whilst those for yeast treated with (c) were consistently smaller than those for yeast treated with (a) or (b). These experiments therefore rule out the difference in osmotic pressure in the two cases as the responsible factor.

(II) *The considerable difference in external p_H in the two cases.* Two experiments were performed to test the effects of variation in external p_H on cytochrome reduction. In the first of these experiments the yeast was exposed to citrate-phosphate buffers of p_H 3.0, 4.0, 5.0 and 6.0 in 1/10 the standard concentrations. In the second experiment the inhibiting agent ethylurethane was added in concentration of 3 % to each of the buffer solutions. The data secured in these two experiments are shown in Table V which show that for a treatment period

Table V. *Cytochrome reduction in yeast as affected (a) by citrate-phosphate buffers* of varying p_H , (b) by 3% ethylurethane in citrate-phosphate buffers* of varying p_H .*

Agent to which yeast samples were exposed	Seconds required to secure return of D band of reduced cytochrome		
	Values for 2-min. exposure period	Values for 2-hour exposure period	Values for 3-hour exposure period
Controls (tap-water)	38, 37, 39, 42	37, 32, 36, —	—
Buffer of p_H 3.0	41, 41	55, 53	—
Buffer of p_H 4.0	40, 42	43, 39	—
Buffer of p_H 5.0	38, 41	38, 37	—
Buffer of p_H 6.0	39, 41	34, 33	—
Controls (tap-water)	39, 42, 45, 42	46, 48, 46, 49	41, 48, 39, 43
Urethane in p_H 3.0 buffer	64, 66	81, 84	92, 92
Urethane in p_H 4.0 buffer	64, 66	74, 77	78, 82
Urethane in p_H 5.0 buffer	66, 62	69, 72	66, 68
Urethane in p_H 6.0 buffer	66, 67	80, 77	65, 70

* The citrate-phosphate buffers were employed in 1/10 the standard concentration.

of approximately 2 min., variation in the p_H of the external medium over the range 3.0–6.0 has no effect on cytochrome reduction; for a treatment period of 2–3 hours there is definite inhibition of cytochrome reduction in the yeast treated with the buffers of p_H 3.0 and 4.0. This action is quite possibly due in part at least to slow penetration of citric acid into the yeast; the magnitude of the difference is not nearly so great as that between butyric acid and butyric acid *plus* its salt, but is quite comparable with the difference in action of acetic acid and acetic acid *plus* its salt.

(III) *Utilisation of sodium acetate and sodium butyrate as substrates for cytochrome reduction.* Experiments on (a) untreated yeast and (b) yeast treated with 0.09 *M* sodium acetate or sodium butyrate gave the results shown in Table VI, from which it is evident that, under the experimental conditions employed

Table VI.

Part A—Cytochrome reduction in (a) untreated yeast, (b) yeast exposed to 0.09 M sodium acetate.

Part B—Cytochrome reduction in (a) untreated yeast, (b) yeast exposed to 0.09 M sodium butyrate.

Seconds required to secure return of D band of reduced cytochrome (each value for (a) and (b) is the average of 8 determinations $\pm$ its standard error)

Agent to which yeast samples were exposed	Values for 2-min. exposure period	Values for 2-hour exposure period
Part A		
(a) Tap-water	41.7 $\pm$ 1.11	44.6 $\pm$ 1.14
(b) 0.09 M Na acetate	38.4 $\pm$ 1.17	41.8 $\pm$ 1.30
Differences and standard errors of differences between (a) and (b)	3.3 $\pm$ 1.61	2.8 $\pm$ 1.73
Part B		
(a) Tap-water	42.1 $\pm$ 0.63	37.3 $\pm$ 0.32
(b) 0.09 M Na butyrate	40.3 $\pm$ 0.49	36.4 $\pm$ 0.47
Differences and standard errors of differences between (a) and (b)	1.8 $\pm$ 0.8	0.9 $\pm$ 0.57

in the acid-salt experiments, it is very unlikely that sodium butyrate acted to any appreciable degree as substrate for cytochrome reduction, and certainly not to an extent sufficient to account for any substantial part of the sodium butyrate-counteracting effect. On the other hand, the findings with sodium acetate suggest possible utilisation of this substance for cytochrome reduction to an extent sufficient to account for at least part of the sodium acetate-counteracting effect.

It should be noted in passing that utilisation of a compound as substrate for cytochrome reduction should not be interpreted as indicating non-penetration of the compound, but rather the reverse. The reducing mechanisms of the cell are in the main intracellular, as shown by the inability of starfish eggs to reduce non-penetrating oxidation-reduction indicators unless these are micro-injected [Chambers *et al.*, 1929; 1931] and the inability of yeast to reduce non-penetrating indicators [Beck and Robin, 1934].

DISCUSSION.

The comparative ineffectiveness of hydrochloric and citric acids is to be attributed to their poor powers of penetration into the cells, it being generally known that strongly dissociated acids penetrate cells much less readily than do weakly dissociated, lipoid-soluble acids, *e.g.* the fatty acids. This relation was confirmed for the yeast cell by a study of colour changes in yeast vitally stained with propyl red and then treated with the various acids.

The experimental findings indicate that formic acid and formaldehyde act on the cytochrome reduction process in a manner quite different from that of any of the other agents employed or any of those employed by Keilin. In the presence of 0.01 M formic acid cytochrome is immediately, and in the presence of 0.01 M formaldehyde eventually, brought into a peculiar condition, in which weak bands of the reduced form are present, and in which the cytochrome is resistant both to oxidation and reduction. Tentatively these results may be interpreted to indicate (a) reaction of formic acid and of formaldehyde with cytochrome, the formic acid-cytochrome and the formaldehyde-cytochrome complexes both being far

more resistant to changes in state of oxidation-reduction than is cytochrome itself, or (b) marked inhibition of both oxidase and dehydrogenase systems by formic acid and formaldehyde.

A comparison of the effects on cytochrome reduction exercised by the remaining fatty acids and by the corresponding aldehydes, alcohols and urethanes indicates that the carbonyl group in combination with an aliphatic chain is partly responsible for the inhibitory action exercised by the acids and entirely responsible for that exercised by the aldehydes. An aliphatic chain in combination with an alcohol or carbamate group is completely ineffective when such compounds are used in 0.01 *M* concentration. The manner in which the effectiveness of the acids and aldehydes increases with increase in length of the aliphatic chain is entirely analogous to that found by Warburg [1928] for the action of urethanes on respiration, and the same type of mechanism may be postulated, *i.e.* blocking of (dehydrogenase) centres concerned in cytochrome reduction by specific adsorption of the acids and aldehydes at these centres. As with the urethanes, the adsorption would be considered as increasing with increase in length of the aliphatic chain. The carbonyl group would be considered as a key group whose presence in the molecule is required if it is to be adsorbed, and it should be present in compounds used as substrates, as well as those which exercise an inhibiting effect.

The greater effectiveness of the fatty acids as compared with the corresponding aldehydes is the only part of the action of the acids attributable to the increase in intracellular acidity which they induce. However, this part of the total acid inhibition is considerable (see Tables II and III).

The counteraction of butyric acid by sodium butyrate and of acetic acid by sodium acetate constitutes additional evidence that part of the inhibitory effects of fatty acids on cytochrome reduction is due to the intracellular acidification which they induce. The counteracting effect of sodium butyrate can certainly not be attributed to differences in osmotic pressure or external p_H or to its utilisation as substrate for cytochrome reduction. The most plausible interpretation of this rapid counteracting effect appears to be that sodium butyrate and sodium acetate penetrate yeast cells in the presence of the corresponding acid at a rate and to an extent sufficient to overcome in some degree that portion of the inhibitory effects of the acid due to intracellular acidification. This interpretation is in accord with the relatively high penetrating power attributed to the sodium salts of rapidly penetrating acids by Smith and Clowes [1924], Lillie [1926] and Howard [1932]. For discussion and literature of the salt penetration problem the reader is referred to Howard.

SUMMARY.

1. A method is described for bringing a 10 % suspension of Fleischmann's yeast into such condition that different control samples give values for rate of cytochrome reduction which agree with one another satisfactorily.

2. When used in 0.01 *M* concentration the various agents employed were found to have the following effects on the behaviour of cytochrome.

(a) Formic acid and formaldehyde bring cytochrome into a condition where weak bands of reduced cytochrome are maintained, whether the yeast be vigorously aerated or kept for a long time without aeration.

(b) Cytochrome reduction is markedly inhibited, within 2 min. and in a completely reversible manner, by the weak but rapidly penetrating aliphatic acids, acetic to hexanoic.

(c) Acetaldehyde, propaldehyde and butyraldehyde also inhibit cytochrome reduction, each to a less degree than the corresponding acid.

(d) The greater the length of its aliphatic chain the more effective is an acid or aldehyde in inhibiting cytochrome reduction.

(e) The very rapidly penetrating alcohols methyl to amyl and the urethanes ethyl, propyl and butyl do not inhibit cytochrome reduction when used in this concentration (0.01 *M*).

(f) The strong but slowly penetrating acids, citric and hydrochloric, inhibit cytochrome reduction more slowly and much less than do the aliphatic acids.

(g) The effects of all of these agents may be completely reversed by washing and addition of the substrate glucose.

3. Employing the same methods, sodium acetate and sodium butyrate were found rapidly to counteract in part the inhibitory action of the corresponding acids on cytochrome reduction.

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