THE ACCLIMATIZATION OF YEAST TO AMMONIUM FLUORIDE AND ITS REVERSION IN WORT

BY ELLIS I. FULMER

Addition of 0.4 decigram of hydrofluoric acid per litre (calc. as HF) to wort, while not injurious to yeast, kills bacteria and the lactic acid ferment and thus increases the yield of alcohol;[1] up to 0.15 decigram per litre are used in the manufacture of alcohol from beets[2] but a larger quantity is harmful to the fermentation. Klöcker[3] points out that the use of this substance is advisable only in distilleries, and Sykes[4] advises against its use in breweries, possibly because it seems to encourage the growth of wild yeasts.

Effront[5] found that in wort containing 3.0 decigrams of ammonium fluoride per litre yeast "seemed completely to lose its activity" but that if transferred to fluoride-free wort "it immediately recovered its fermentative power, and showed an extraordinary power of reproduction." He also discovered that by planting yeast in wort containing 0.2 decigram of ammonium fluoride per litre and repeatedly transferring it to higher concentrations of fluoride, he could obtain cells which caused active fermentation in wort containing 3.0 decigrams per litre: "The yeast had become accustomed, so to speak, to the antiseptic and had acquired a certain immunity which it did not possess originally." Effront's experiments were continued by Sorel[6] who finally got his yeast to grow in wort containing 10 decigrams of hydrogen fluoride per litre, which corresponds to 18.5 decigrams of the ammonium salt.

In the present paper are recorded the results of experiments on: I, the toxicity of concentrated fluoride solutions, with

[1] Sorel: Comptes rendus, **117,** 253 (1894).
[2] McIntosh: "Industrial Alcohol."
[3] Klöcker: "Fermentation Organisms."
[4] Sykes: "Principles of Brewing."
[5] Effront: Comptes rendus, **118,** 559 (1893).
[6] Sorel: Loc. cit.

and without alcohol; II, the delay in reproduction in wort containing fluoride; III, the effect of fluoride concentration on the delay; IV, the difference in behaviour towards ammonium fluoride of yeast acclimatized by different methods; V, the maximum concentration of fluoride in which yeast will reproduce; VI, the reversion of acclimatized yeast to normal when grown in fluoride-free wort; VII, the evidence of selection; and VIII, the evidence of adaptation during the process of acclimatization.

I. Toxicity of Ammonium Fluoride towards Yeast

The experiments were carried out in a rocker tube[1] at 25° C. with "normal yeast"[2] in a five percent aqueous solution of ammonium fluoride.

TABLE I

Time in poison	0	27	32	45	minutes
5% amm. fluor.	800	250	50	1	colonies on agar
Killed	0	68	93	100	percent

In a second series, the action of 4.8% ammonium fluoride in water was compared with that of 4.8% ammonium fluoride and 2.5% ethyl alcohol. (By % is meant grams per 100 cc of solution.)

TABLE II

Time in poison	0	10	13	17	23	28	38	minutes
Aqueous sol'n	77	30	—	23	16	4	1	colonies
ditto killed	0	60	—	70	80	95	100	percent
Alcoholic killed	—	—	100	100	100	100	100	percent

Similar experiments were carried out using solutions of ammonium fluoride to which a little methylene blue had been added; a purple precipitate is formed, and but few of the cells stain; a series was run with phenol to control the yeast.

[1] Fraser: Jour. Phys. Chem., **25,** 4 (1921).
[2] Fulmer: Ibid., **25,** 12 (1921).

TABLE III

Time in poison	5	6	8	9	20	26	minutes
0.658% ph. + MB	25	40	—	65	—	—	% stained
5% Am. fl. + MB	0	—	2	—	—	7	% stained
10% "	—	—	—	—	7	—	% stained
20% "	—	—	—	—	—	10	% stained

In another series, yeast was shaken in the rocker with ammonium fluoride solution, filtered off, and washed, before adding the MB; with 4%, 6%, and 8% ammonium fluoride not over 20% of the cells were stained in half an hour; and after 20 minutes in 20% ammonium fluoride solution only half the cells were stainable.

II. Delayed Fermentation

On repeating Effront's experiments it was found an easy matter to acclimatize yeast to ammonium fluoride in wort by the "stepping up" process employed by him; it soon appeared, however, that it is quite unnecessary to begin with a low concentration of fluoride; the cells can be planted at once in solutions of high concentration and will grow *provided they are left long enough.*

In the experiments, 50 cc wort was pipetted into a 250 cc Erlenmeyer flask, which was stopped with cotton wool and sterilized for 30 minutes in live steam at 100° C, where it loses 0.25 g by evaporation. After cooling, a small accurately measured volume of 24.07% ammonium fluoride solution was added, then enough water to bring the whole to 51.2 cc and finally the 1.0 cc of suspension of yeast in water count 150. The total volume was thus 52.2 cc, and if 0.218 cc fluoride solution had been added, the concentration would be 10 dg per litre; the "count" of yeast would be 3. The flasks were then let stand in the incubator at 25° C, and the time determined which elapsed between the addition of yeast and the appearance of flecks of foam on the surface.[1] Sykes[2] states that the "head" forms from 2 to 3 hours after carbon dioxide

[1] Fulmer: Jour. Phys. Chem., **25,** 10 (1921).

[2] Sykes: "Principles of Brewing."

is first given off; this was corroborated by the use of fermentation tubes. Whatever the concentration of the fluoride the number of cells per cubic centimeter of the medium when flecks first appear is about 50 x 250,000; the data in the following table were obtained at widely different times, with different yeasts and different worts; the count[1] was made in every case within half an hour of the first appearance of the flecks.

TABLE IV

Ammon. fluor.	0	10	10	10	10	20	20	20	20	30	40	dg per 1.
Count	74	64	57	49	52	53	49	56	30	47	40	(av. 51)

The interval between seeding and fleck formation depends on the concentration of the fluoride; but it depends also on the way the wort has been prepared, and on the nature of the yeast used.

Variation in the Wort.—The standard method of preparing the wort was to digest 360 g coarsely ground brewer's malt with 1150 cc distilled water for 24 hours at 55° C, filter through toweling and paper, and sterilize for settling for 15 or 20 minutes in an autoclave with steam at 15 lbs. After three days, it was filtered again and used undiluted. Its specific gravity was 1.07; 100 cc contained 16.2 g dry matter including 0.412 g ash, of which 0.023 g CaO and 0.017 g MgO. If the temperature of digestion rose to 65–70° C, the wort seemed as good as ever for the purpose of growing yeast, but ammonium fluoride added to such a wort in quantities up to 40 decigrams per litre had no effect on yeast; such wort has a higher density, its ash was not determined. The time of digestion at 55° C also affected the wort. A considerable precipitate is formed when ammonium fluoride is added to wort, but filtration had no effect on the time elapsing before active fermentation. Neither had the time (0 to 192 hours) which might elapse between adding the fluoride to the wort and inoculating with yeast. If, however, the wort be ster-

[1] Fraser: Jour. Phys. Chem., **25**, 3 (1921).

ilized after the fluoride has been added, the effect of the fluoride is much reduced.

Variations in the Yeast.—As already observed by Sorel, yeast which has grown in wort containing fluoride is apt to form large clusters, more like bunches of grapes than like the ordinary chains. Similar bunches, though smaller, may sometimes be obtained from the colonies on a plate made direct from a Fleischmann's yeast-cake. Ten flasks of fluoride-free wort were inoculated from different colonies of the same plate and were left to ferment; in one of them (No. 806) the yeast was very thickly bunched with but few single cells; in a second (No.804) there were many bunches but also a large number of chains; in a third (No. 808) there were some clusters but more double and single cells; the other seven were normal. Yeast from these ten cultures was planted in wort containing 10 decigrams of ammonium fluoride per litre, and the interval to fleck formation determined for each; the results showed that the greater the bunching, the shorter the interval.

TABLE V

Culture number	806	804	808	the 7 others
Flecks formed in	50	57	73	81 to 107 hours

This result was confirmed by another experiment for which a culture was selected because of its freedom from long chains or bunches of any kind; in this case 122 hours elapsed before the flecks appeared. That growth for long periods in (daily renewed) fluoride-free wort may increase the susceptibility of yeast to ammonium fluoride is shown by the data given in Table XII.

III. Influence of Fluoride Concentration on the Delay

That the time elapsing between seeding and fleck formation may be represented by a simple exponential function of the fluoride concentration is shown by the following results. (50 cc wort in flasks at 25° C; initial count 3; "conc. 40" means 40 decigrams of ammonium fluoride per litre of medium; it is in hours.)

TABLE VI

Conc.	0	3	6	9	12	13	20	25	30	35	40
t obs.	12	13	15	40	46	64	89	136	188	240	400
t calc.	(21	26	32)	40	50	63	91	132	190	275	400

The numbers after "t calc." are from the equation $\log_{10} t = 1.312 + 0.03225$ conc.

This relation has been verified repeatedly with various worts and yeasts, and though the constants of the equation vary considerably with these factors, yet the graph (log t against conc.) always gives a fairly straight line when the concentration lies between 10 and 45 decigrams per litre.

It being thus possible to acclimatize yeast to a high concentration of fluoride either by "stepping up" (Effront and Sorel's method) or directly, it seemed of interest to ascertain whether the method employed had any influence on the behaviour of the acclimatized yeast towards still higher concentrations of fluoride.

IV. Comparisons of Yeast Acclimatized by Different Methods

For this purpose two series of experiments were undertaken. In the first yeasts A to H were used, and in the second yeasts J to R. Yeast A was from a "normal" culture in pure wort; yeast B was taken from a solution containing 12 dg ammonium fluoride per litre which had been seeded with yeast A and let come to active fermentation; this life history may be synopsized by writing 0-12 after B. Yeast C (0-20) was from a solution of concentration 20 seeded with A. Yeast D (0-20-25) was from an actively fermenting sort containing 25 decigrams of fluoride per litre, which had been seeded with C; and similarly with the others. Each of these yeasts was used to seed a number of flasks of wort containing fluoride in various concentrations; the interval between seeding and fleck formation (in hours) for each case is given in the horizontal line which begins with the symbol identifying the yeast. The data from the second series are given in Table VIII; they started from a different "normal" yeast (Yeast J).

TABLE VII

Conc. of fluoride	15	20	25	30	35	40	45	50	55
A* (0)	64	89C	136	188	240	400	—	—	—
B (0-12)	17	17	66	—	—	—	—	—	—
C* (0-20)	—	43	48D	90	156	—	—	—	—
D* (0-20-25)	—	—	16	20	27E	60	—	—	—
E* (0-20-25-40)	—	—	—	—	16	20F	48	152	—
F (0-20-25-35-40)	—	—	—	—	—	20	48G	152	400
G (0-20-25-35-40-45)	—	—	—	—	—	—	96H	112	192
H (0-20-25-35-40-45-45)	16	16	—	—	—	72	90	97	—

TABLE VIII

Conc. of fluoride	10	15	20N	25	30	35	40	45
J* (0)	80L	106Q	180	159K	187	211	—	—
K* (0-25)	—	—	15	20	26	44	68	112
L* (0-10)	38	48M	66	134	112	168	—	—
M (0-10-15)	—	—	36	—	—	—	—	—
N (0-10-15-20)	—	—	—	16P	—	—	—	—
P* (0-10--15-20-25)	—	—	—	14	17	27	44	68
Q (0-15)	—	—	—	133R	—	—	—	—
R* (0-15-25)	—	—	—	42	59	86	112	136

These results show clearly that the "delay" is much less when the yeast used for seeding had previously been grown in a fluoride solution. Comparing Yeast G with Yeast H, it seems that a second period in concentration 45 somewhat further reduces the delay; acclimatization was evidently not quite completed in the first flask. Yeasts K, P and R, which had been acclimatized to concentration 25 by different routes, show considerable differences in their behaviour; P, which had lived longest in fluoride solutions, showed the least delay, but K showed less than R; no doubt the results are influenced to some extent by factors as yet undiscovered.

V. Maximum Concentration of Fluoride

Some of the results (those indicated by asterisks in Tables VII and VIII) are plotted in Figs 1 and 2. Omitting the experiments where the concentration of ammonium fluoride was lower than that in which the yeast had already been living, the others fall fairly well on straight lines converging to a point at concentration 80 in the first case and 72 in the second. If this

extrapolation were to be taken seriously, it would indicate that for concentrations above 80 decigrams ammonium fluoride per litre yeast already accustomed to fluoride would take longer to cause fermentation than would unacclimatized yeast. Attempts were made to grow yeast in these high concentrations. On May 1st, 1917, a number of solutions were seeded to a count of 3 with yeast grown in wort containing 36 dg fluoride per litre; on October 1st, five months later, the flasks were examined with the following results:

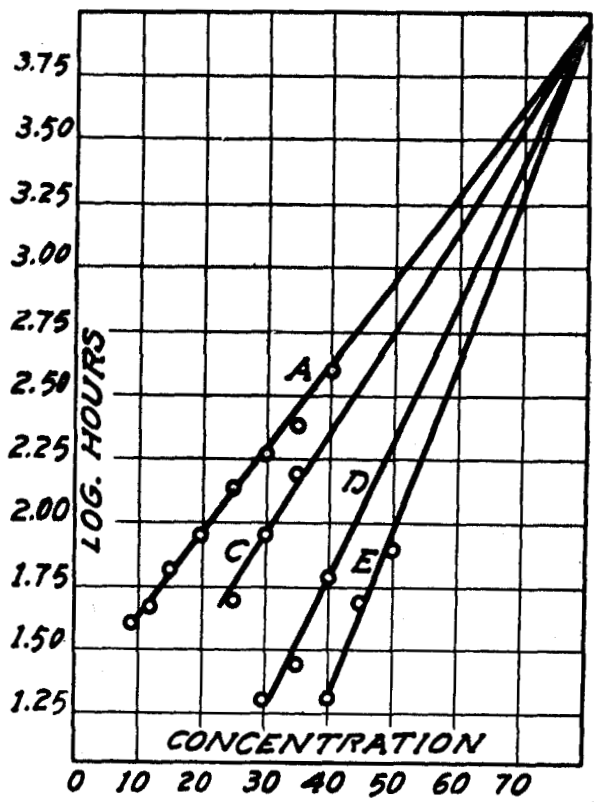

Fig. 1

TABLE IX

Conc.	0	36	50	65	80	dg per litre
Count	2000	4	320	3	3	

according to this, the highest concentration in which the yeast would grow lies between 65 and 80. A "normal" yeast from fluoride-free wort was then seeded into worts containing fluoride of the concentrations 65, 70, 75, 80 and 85 dg per litre; there was active fermentation in 65 after 760 hours, and in 70 after 1000 hours, the others were kept for four months without fermentation occurring. These results show that the highest concentration in which active fermentation will occur is about the same for the two yeasts, one acclimatized and the

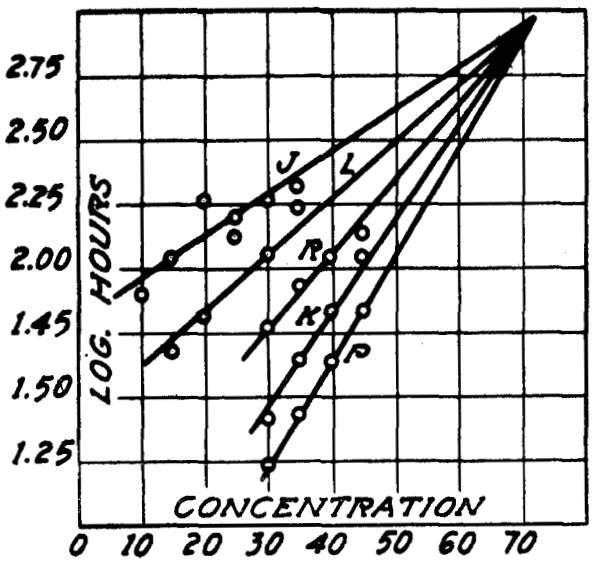

Fig. 2

other not; and that it lies close to 70 decigrams of ammonium fluoride per litre, that is, just below the crossing-points of the lines on the two graphs (Figs. 1 and 2). The "absurd" relations predicted for higher concentrations are thus unrealizable.

VI. Behaviour of the Acclimatized Yeast towards Phenol

To see whether the resistance towards fluorides, and the physiological changes noted by Effront and Sorel are accompanied by change of behaviour towards other reagents, yeast that had been grown in wort containing 30 dg ammonium fluoride per litre was used to seed wort containing 20 dg per litre, and when active fermentation took place (22 hours after seeding) the culture (Y 20-30) was compared with a normal culture (Y 0) from fluoride-free wort, with the following results (rocker, 25° C, 0.650% phenol in aqueous solution):

TABLE X

Tube 1 Y(0), phenol MB		Tube 2 Y(30-20) phen. MB		Tube 3 Y(0) phenol Plating		Tube 4 Y(20-30) Plating	
Min.	% std.	Min.	% std.	Min.	% killed	Min.	% killed
3	3	11	33	9	100	10	35
12	98	17	39			15	90
18	100	25	31			22	92
		40	34				

Whether ability to stain or inability to form colonies on wort agar be taken as criterion of death, yeast grown in fluoride solutions is more resistant to phenol than the other.

VII. Reversion

Plates.—Yeast was seeded from fluoride-free wort into wort containing 12 decigrams of ammonium fluoride per litre; when active fermentation occurred the culture was used to seed wort containing 25 decigrams of ammonium fluoride per litre, and so on, as follows; the concentrations are given in parentheses, and the intervals in hours between seeding and fleck formation are printed between them:

(0)-46-(12)-66-(25)-17-(30)-16-(30)-16-(30)

The delay when seeded into concentration 12 shows that the yeast used was similar to yeast A of Tables VI and VII.

From the last flask a wort-agar plate was seeded; three days later, four colonies on this plate were used to seed four flasks of wort; and when active fermentation was in progress

the cultures were used to seed (to a count of 3) four flasks of wort containing 30 decigrams of ammonium fluoride per litre. Flecks were formed 44, 48, 39, and 48 hours after seeding. As Yeast A required 188 hours, the yeast experimented with had obviously not lost all its acquired resistance by three days' growth on the plate followed by one transfer through fluoride-free wort.

Twelve days after the plate had been seeded, portions of two of the colonies already used were treated as before; the interval between seeding and fleck formation in this case was found to be 120 and 144 hours; so that even after twelve days' growth in the absence of fluoride the reversion was not complete.

Flasks.—Another yeast, taken from a wort containing 30 decigrams of ammonium fluoride per litre, was used to seed fluoride-free wort; every 24 hours a fresh flask of the same wort was seeded from the previous flask to a concentration of 2 or 3, thus the yeast was kept continually active. At intervals this culture was used to seed wort containing 30 decigrams of ammonium fluoride per litre (in duplicate) and the interval to fleck formation was determined, and is given after "delay" in Table XI.

TABLE XI

Hours in wort	0	23	48	71	95	117	140	165	189
Delay (1st flask)	38	42	65	70	90	117	140	144	170
Delay (2d flask)	41	47	65	72	90	125	140	144	170

When graphed (delay, hours in wort) these numbers fall not far from a straight line; in spite of the long delay of 170 hours in the last experiment, it would seem that the reversion was not complete after 189 hours in fluoride-free wort. The cells were still noticeably bunched, which points to the same conclusion.

Comparative experiments in which a normal yeast was grown in wort (under the same conditions of replanting) for 480 hours, and then used to seed fluoride worts of various

concentrations, showed that the "delay" in every case was greater than with the original yeast.

TABLE XII

Conc.	10	15	20	25	30	35	40	dg per l
Normal yeast	43	82	82	127	206	—	228	hours
After 480 hours	64	105	151	182	—	—	387	hours

In view of these results there is no way of predicting the degree of delay that would signify complete reversion.

VIII. Selection

When the foregoing experiments had established beyond all question that addition of ammonium fluoride to wort increased the interval between seeding (to count 3) and the appearance of flecks (count 50), the first explanation to suggest itself was that the fluoride may kill nearly all the 25 to 35 million cells (count of 3 in 50 cc wort) seeded into the flask, and that the extra time is needed for the remaining few to reproduce to a count of 50. Effront's "acquisition" of immunity would thus be reduced to a case of survival of the fittest, the process being "selection" by the poison; the experiments cited above under "variation of the yeast," which show that different cells from the same cake, and even different descendants of the same yeast-cell, may behave very differently towards ammonium fluoride, lend support to this view.

To test it, worts containing 9, 12, and 15 dg ammonium fluoride per litre, respectively, were seeded with yeast, and at noted intervals portions were removed, diluted, counted, mixed with wort agar, and poured into petri dishes. The following are the results obtained; time is measured in hours from the moment of seeding; duplicate plates were poured in each case and the number of colonies added; "active" means active fermentation in the flask; the experiments of each table were carried out at the same time with the same yeast, but the yeasts were not the same for the three tables.

TABLE XIII

Conc. Hours	9 Colonies	12 Colonies	15 Colonies
0	600 + 600 = 1200	600 + 600 = 1200	600 + 600 = 1200
1	20 + 24 = 44	20 + 24 = 44	27 + 24 = 51
16	10 + 14 = 24	0 + 0 = 0	0 + 0 = 0
23	25 + 26 = 51	3 + 2 = 5	0 + 0 = 0
41	active	352 + 370 = 723	33 + 34 = 67
45	——	active	slightly active

TABLE XIV

Conc. Hours	9 Colonies	12 Colonies	15 Colonies
0	600 + 650 = 1250	600 + 650 = 1250	600 + 650 = 1250
1	67 + 80 = 147	26 + 21 = 47	35 + 34 = 59
11.5	4 + 3 = 7	0 + 1 = 1	0 + 1 = 1
16.5	24 + 14 = 38	4 + 1 = 5	5 + 3 = 8
20.5	43 + 50 = 93	2 + 1 = 3	4 + 0 = 4
35	active	slightly active	4 + 3 = 7
40.5			35 + 40 = 75
45			active

TABLE XV

Conc. Hours	9 Colonies	12 Colonies	15 Colonies
0	313 + 335 = 648	300 + 300 = 600	250 + 300 = 550
16	50 + 35 = 85	31 + 32 = 63	35 + 32 = 67
23	11 + 6 = 17	0 + 1 = 1	2 + 2 = 4
39	active	28 + 28 = 56	24 + 24 = 48
46		38 + 45 = 83	25 + 50 = 75
61		active	active

If inability to form colonies on wort agar be regarded as evidence of death—the plates were kept at 25° C for a week to allow time for growth—it is clear that the fluoride kills a large proportion of the cells, and the proportion killed seems to increase with increase in the concentration of the fluoride. In Table XIII, for instance, not one cell in 1200 remained alive after 16 hours in wort containing 15 decigrams of ammonium

fluoride per litre. Whatever else it may do, the fluoride certainly acts as a selective agent.

IX. Adaption

To see whether anything beside selection is at work, measurements of the rate of reproduction of yeast were undertaken; the number of cells in the culture being counted at intervals by means of a haemocytometer.[1]

In a uniform medium, with plenty of food and the cells not too closely crowded, it is natural to expect that each cell would grow independently of the others, and that therefore the rate of increase of the number of cells in the flask at any moment would be proportional to the number of cells then present in the flask, i. e. (writing C for the "count"), $dC = k' C.\ dt$, whence $\log_{10} \frac{C_2}{C_1} = k(t_2 - t_1)$ where $k = 0.434\ k'$. If this assumption were true, the plot of log $\log_{10}$ C against t would be a straight line.

According to Euler[2] this formula was first proposed by Basenau[3] in 1895. Euler himself employed it in recording his experiments on the growth of yeast in media containing lactose, and if he had thought to omit the first point ($t = 0$) he would have obtained fairly straight lines—this neglect could be justified because of the probability that some of the cells with which he inoculated the lactose were unable to reproduce in that medium. Slator[4] and Carlson[5] have also employed this formula in their work on yeast.

My own measurements were made to see how closely this formula will enable one to calculate the initial count by extrapolation from counts made after many hours of growth. The results of four such experiments are here recorded in full; all were made with 50 cc fluoride-free wort in Erlenmeyers at 25° C.

[1] Fraser: Jour. Phys. Chem., **25,** 3 (1921).
[2] Zeit. physiol. Chem., **81,** 58 (1912).
[3] Chem. Centralblatt, **1895, I.**
[4] Biochem. Jour., **7,** 197 (1913).
[5] Biochem. Zeit., **57,** 313 (1913).

TABLE XVI

t	0	6.5	15.5	19.5	23	39.5 hours
C	(2)	13	100	275	350	300
log C	0.30)	1.11	2.00	2.44	2.54	2.78

TABLE XVII

t	0	15.25	17.0	18.75	hours
log C	(0.00)	2.16	2.45	2.60	

TABLE XVIII

t	0	16.25	18.25	19.0	20.0	21.0	22.0 hours
log C	−(0.30)	2.36	2.60	2.75	2.88	2.83	3.00

TABLE XIX

t	0	21.0	23.0	24.0 hours
log C	(−4.0)	0.96	1.34	1.56

Plotting these numbers (log C against t), but omitting the point for t = 0, it is obvious that the best line for Table XVI runs through t = 6.5 and t = 19.5; from these two values there follows for t = 0, C = 2.8, whereas the initial count in the actual experiment was 2.0. From Table XVII, t = 15.25 and t = 18.75 give by extrapolation an initial value C = 1.6, as against 1.0 actual. For Table XVIII, t = 16.25 and t = 20 give 1.4, the actual value was 0.5; and for Table XIX, t = 21 and t = 24 give 0.0006 instead of 0.0001. Nine series of this kind were carried out, with different yeasts and different worts; in all of them the extrapolated values of the initial concentration were higher than the true values, the ratio varying from 1 to 8; the method therefore can be relied upon to give at least the order of magnitude of the number of cells originally present in the solution.

Similar measurements with worts containing ammonium fluoride were then undertaken; for the present purpose it is sufficient to quote the results of one of them where the wort contained 40 dg ammonium fluoride per litre:

TABLE XX

t	402.5	403.5	405.5	407.5	hours
log C	1.80	1.90	2.02	2.14	

The graph gives a fairly straight line from which can be calculated k = 0.064 (about half the value with pure wort), and for t = 0, C = 10^{-24}, or 1.25 x 10^{-15} cells in the 50 cc. A similar experiment with another yeast in the same concentration gave 10^{-23} and a number of others with various concentrations of fluoride led to analogous results. As at least one cell must have been left alive to cause fermentation and reproduction, these extremely low values decisively negative the hypothesis that all but a few of the cells were killed by the fluoride and that these few reproduced regularly from the beginning. They show that in addition to a selective action the fluoride must have caused something analogous to paralysis in the cells left alive; on recovery they possessed the power to reproduce in the fluoride solution at about half the rate of normal cells in pure wort. This modification of the properties of the cells is what constitutes "adaptation;" so that adaptation as well as selection plays a part in the development of a fluoride-resisting yeast.

It seemed at first that a rough idea of the duration of the paralysis might be obtained by subtracting from the total interval between seeding and fleck formation, the time estimated to be needed for the yeast to increase in number to a count of 50; for normal yeast seeded to a count of 2 or 3 in fluoride-free wort, this is about 12 hours, probably longer in the presence of fluorides. If then 12 hours be subtracted from the total period, the remainder will certainly give a maximum value for the duration of paralysis, even though the calculation seems to involve the improbable assumption that recovery is instantaneous. The data of Table VII give means of calculating the maximum duration of paralysis when "Y 25" (i. e., a yeast that will actively ferment wort containing 25 decigrams of ammonium fluoride per litre) is evolved from normal yeast, either directly (136 — 12 = 124 hours), through

concentration 12 (46 + 66 — 24 = 88 hours) or through concentration 20 (89 + 48 — 24 = 113 hours); in the case of Y 40, the times are: direct (400 — 12 = 388 hours), through concentrations 20 and 25 (89 + 48 + 60 — 36 = 161 hours), or through concentrations 20, 25, and 35 (89 + 48 + 27 + 20 — 48 = 136 hours). Such calculations, however, only give the maximum values; either the total period of paralysis is shorter when the concentration is increased by steps, or a very much larger proportion of the cells are killed outright when concentrated fluoride acts on unadapted yeast; without further data it is impossible to decide; the figures of Tables XIII, XIV, and XV, however, show that a good deal of weight must be attached to the second factor.

These experiments were carried out in the chemical laboratory of the University of Toronto in the winters of 1917–1919 under the direction of Professor W. Lash Miller; my thanks are due to Mr. N. A. Clark for help with some of the later measurements.

Summary

(1) The toxicity towards yeast of a few aqueous and aqueous-alcoholic solutions of ammonium fluoride has been determined; alcohol increases the toxicity.

(2) In acclimatizing yeast to ammonium fluoride it is not necessary to begin with low concentrations (Effront's stepping-up method), the cells may be planted at once in the more concentrated solutions of fluoride and wort, and will grow if left long enough.

(3) The time elapsing between seeding and active fermentation depends on the method of preparing the wort, on the character of individual yeast cells, and on the previous history of the culture.

(4) Yeasts acclimatized to a given concentration of fluoride by different routes behave differently towards more concentrated fluoride solutions.

(5) If the logarithm of the interval (see III above) be plot-

ted against concentration, for various yeasts, acclimatized and unacclimatized, the experimental data fall on straight lines radiating from a point corresponding to about 70 or 80 decigrams of ammonium fluoride per litre.

The highest concentration in which yeast will grow is about seven grams ammonium fluoride per litre; the highest reached by Effront was about 0.3 g per litre; Sorel reached 1.0 g hydrogen fluoride per litre (corresponding to 1.8 g ammonium salt).

(6) Yeast acclimatized to ammonium fluoride is more resistant to phenol than is normal yeast.

(7) Acclimatized yeast may be grown on fluoride-free wort agar for 12 days, or in fluoride-free wort for 190 hours without completely reverting to normal.

(8) When yeast is planted in wort containing ammonium fluoride, a proportion of the cells die, i. e., will not reproduce on fluoride-free wort agar. The number of living cells decreases, passes through a mimimum, which may be less than one in 1200 of those originally present, and then increases. Selection plays a part in the acclimatization.

(9) Measurements of the rate of reproduction of yeast in wort with and without ammonium fluoride, show that the cells which are not killed outright undergo a period of paralysis, after which they reproduce, giving rise to fluoride-resistant cells. Adaptation plays a part in the acclimatization.

A maximum value for the duration of paralysis may be calculated from the experimental results.

The University of Toronto, May, 1921