CXXXVII. THE LIBERATION FROM YEAST OF SUBSTANCES GIVING THE NITROPRUSSIDE REACTION.

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THE observations recorded here were made in the course of a study, of which one part is described elsewhere [Barry *et al.* 1928] of the action of arsenic upon oxidation-reduction systems. We endeavoured at first to examine the fixation of arsenic by glutathione which has been described in several papers by Voegtlin and his fellow-workers [1923, 1925]. To this reaction they have assigned great importance in the detoxication of arsenical compounds, and the process should be of considerable interest in connection with arsenic cancer. Our experiments were made with the skin and muscle of the rat, the Jensen sarcoma, the liver of the calf and fowl, and yeast. We could not find the expected constant difference in the strength of the nitroprusside reaction between extracts of these materials which had, or had not, been treated with solutions of arsenites. Evidently the effect upon the nitroprusside reaction of the combination of arsenic with glutathione, which Voegtlin seems to regard as beyond doubt², requires investigation. However, in the course of these experiments we were led to note the liberation by a number of physical and chemical agents of substances giving the nitroprusside reaction, and these observations are recorded here.

TECHNIQUE.

The experiments, begun with mammalian cells, have been carried on up till now with yeast, a very convenient material. 2 g. compressed yeast were suspended in 20 cc. of fluid, which was either Pasteur's solution, 10 % sucrose or dextrose in water, or distilled water alone. We have not checked every result given below with yeast suspended in each one of these four media, but in general the effects appear to be of the same relative order whichever medium is used. As a rule the order of increasing strength of nitroprusside

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² Apparently the only direct evidence which Voegtlin and his co-workers [1923] give for the suggestion that "the —SH group of glutathione may be regarded as the so-called arsenic receptor of mammalian protoplasm" consists in the preparation *in vitro* of arsenic compounds of thioglycolic acid. The numerous results showing the detoxication of arsenic *in vivo* by glutathione do not prove the formation in the body of similar compounds.

reaction given by yeast in these various media is as follows: (1) Pasteur's solution, (2) sugar solution, (3) water (*e.g.* Tables I and X). Thus weakly acting reagents, such as 0.05 *N* organic acids, may give at first a negative reaction in the presence of sugar and a positive reaction in its absence; but after some hours' incubation the two suspensions tend to give the same intensity of reaction, no doubt because most or all of the sugar has been removed by fermentation.

In a series of test-tubes were placed 1. or 2 cc. of the suspension and generally an equal volume of the chemical reagent to be tested; or a suspension of yeast was made up in bulk in the solution under test (*e.g.* a saturated salt solution), either with or without the addition of sugar, and from this samples were withdrawn at intervals. The mixtures were incubated at 37° for varying times¹. For the colour test, 1 cc. of suspension was mixed with ammonium sulphate (crystals, or 0.5 cc. of saturated solution), and four drops of strong ammonia (0.880) and of fresh 1 % sodium nitroprusside solution were added. In every case control tubes were set up and incubated, containing (1) yeast suspension alone, the amount of colour given by this varies considerably with different samples (see below, "Autolysis"); and (2) each reagent, with Pasteur's or sugar solution, but without yeast; for the possible presence in the various reagents, or production from them during incubation, of acetone, must always be considered. In most cases, a sample of the suspension after exposure to a physical or chemical agent was examined with a 2 mm. objective for evidence of cytolysis. The comparative strength of the colours produced by nitroprusside is shown roughly below by + signs; it was found most convenient to describe a certain colour, similar in depth to 0.0005 *N* KMnO_4 , as "+," and others as fractions or multiples of this (faint +, $\frac{1}{4}$ +, $\frac{1}{2}$ +, ++, 4 +, etc.). A standard colour-scale could be used, but this seemed unnecessary for the comparative results recorded here; the colours in many cases fade so quickly that there is no time for any elaborate method.

Different samples of yeast under the action of the same reagents give nitroprusside reactions of different degrees of intensity; thus with a yeast which gives weak reactions the less strongly acting salts, such as Na_2SO_4 or KNO_3 (Table II), may not cause the production of any colour at all with nitroprusside. Similarly a sample of compressed yeast which is obtained and tested in the morning will be found to give weaker results if it be kept at room temperature till the afternoon and then used again. But the different reagents maintain on the whole their relative positions in the scale of potency; with different yeasts the results shift to and fro over a certain range of intensity.

¹ This temperature is about 10° above the optimum for the growth of yeast, but this does not affect the comparative value of the results, and the investigation brought forward here was concerned with a search for cytolytic agents rather than with the growth of yeast.

AUTOLYSIS.

The nitroprusside reaction given by many samples of yeast, and by all after sufficient incubation (Table I), may no doubt be attributed to autolysis. The strongest reaction observed in this series is that recorded in Table X. If yeast be autolysed in the ordinary way, by placing a jar filled with it in a warm place for a week or two, the liquefied material will be found to give a very strong colour. Many, if not all, of the positive reactions described below may be due to an acceleration of autolytic processes.

Table I.

Time	In H ₂ O	In H ₂ O + 10 % sucrose
0 min.	—	—
30 mins.	? ft +	—
60 "	ft +	—
180 "	++	+
240 "	++	++
24 hrs.	+++	+++

1 g. yeast in 10 cc.

ft + = faint + in this and subsequent tables.

SOURCES OF ERROR.

1. *False positives.* These would be detected by the control tubes containing no yeast. Thus commercial butyl alcohol cannot be used on account of its acetone content, and sulphites themselves give the reaction. Solutions of caprylic alcohol and of sodium oleate give faint pink or orange colours with nitroprusside, but these are by no means sufficient to account for the reactions observed in the presence of yeast.

2. *False negatives* are more difficult to detect, and may arise from more than one cause. (1) With many reagents the positive reaction does not appear until after a period of incubation of an hour or more (see, for instance, Tables I, V, VI, VII, IX, X, XII) and disappears again later (Tables III, VI, IX, XII). Hence if no test be made during a certain interval of time the reagent may be set down wrongly as negative. After 24 hours' incubation many positive reactions become fainter, or may cease altogether, this being due perhaps to oxidation of glutathione [cf. Harris, 1923]. This source of false negatives can be avoided by testing a sufficient number of times. (2) The reagent itself may interfere with the nitroprusside reaction, and this is more troublesome to detect. If suspensions of yeast be made in saturated solutions of potassium nitrite, and of sodium nitrite, a striking difference will be observed; the former will give a very strong nitroprusside reaction (Table III), the latter none at all however long the incubation be continued. Further tests show, however, that one cannot thereupon reckon sodium nitrite as a negative reagent; one cannot tell whether it does, or does not, liberate reducing substances from yeast because the colour test by which these would be detected is inhibited. It is very difficult, and perhaps not worth while, to devise a perfectly satisfactory means of detecting these false negatives. Two methods

have been used. (a) A suspension of yeast with the reagents in question is heated to 70° (see under "Heat" below); if after cooling it then gives as strong a colour as a control, one may suppose there is no interference. Thus suspensions in water, and in saturated KNO_2 and NaNO_2 , when heated in this way give a strong colour in the first named, a stronger colour in the second, and no colour at all in the third. This method is not fitted for very volatile substances (acetaldehyde, methyl alcohol) and heating in strong salt solutions is not desirable. (b) A suspension of yeast giving a strong nitroprusside reaction is prepared by the action of phenol, salicylic acid, or thymol (see under "Organic compounds" below); samples are mixed with the reagents under examination, and the colour tests compared with those given by suitable controls. This serves to show the various degrees of inhibitory action exerted by methyl and ethyl alcohols and acetaldehyde and formaldehyde. All the substances recorded as negative below have been examined by one or both of these methods.

Evidently the nitroprusside test is sensitive to the interfering action of many substances, and this should perhaps make one cautious in concluding from a negative test that a tissue extract contains no glutathione. Hurltley [1913], when working with ethyl acetoacetate and acetoacetic acid, found that "quite small quantities of the ester completely inhibited the test when applied to the acid." Probably there are all grades of such interference, ranging from complete suppression, as with saturated NaNO_2 , to simple decreased sensitivity.

While some substances can thus prevent the production of the colour, others can remove it after its development. If to a few cc. of a suspension in which a strong nitroprusside colour has been produced one adds 1 cc. of toluene, or methyl alcohol, the colour disappears instantly on shaking. One might expect that the precipitates formed when some of the reagents (*e.g.* MgCl_2) mix with those ($(\text{NH}_4)_2\text{SO}_4$ and NH_3) required for the test might interfere with the colour by adsorption, but we have not been able to detect this effect except perhaps in some instances (HgCl_2) which have been excluded below.

PHYSICAL AGENTS.

Grinding. If a 10 % suspension of yeast be ground in a mortar with powdered silica, which is a much more effective abrasive than sand, there is no liberation of substances giving the nitroprusside reaction as long as the mixture is of semi-fluid or creamy consistence, even though the grinding be carried out very vigorously; but when enough silica has been added to make the material semi-solid, a very intense colour is obtained, both in the solid mass and in the supernatant fluid of a portion suspended in water and centrifuged. Microscopical examination of samples taken at intervals during the grinding shows that the disruption of the yeast cell is far more difficult to bring about than one would expect; after prolonged grinding, when the nitro-

prusside reaction is very strong, one still finds large numbers of cells showing no visible sign of injury. However, an interesting difference is shown by staining with watery methylene blue; in the control suspension only a very small proportion of the cells take up the dye, whereas in the ground material in which reducing substances have been liberated a large number of the apparently uninjured cells stain deeply. In view of the very interesting observation of Harris [1923] that frothing in an albumin solution produces a strong nitroprusside reaction, it would perhaps be worth while to try whether the reaction could be obtained from albumin by any process of grinding.

Heat. If a 10 % suspension of yeast giving no, or a very faint, nitroprusside reaction be boiled and cooled it will then be found to give a very strong reaction. This change takes place sharply at about 61° ; as a rule yeast heated to 59° – 60° gives no, or a very faint, colour, but after exposure to 62° – 63° the reaction is quite distinct. The change reaches a maximum at 65° – 66.5° ; yeast exposed to this temperature gives as much colour as does a boiled sample. In all these experiments the time of exposure to any given temperature was 2 minutes. Euler and Lindner [1915] give the following data for the lethal temperature for four different yeasts: (1) 68° , (2) 64° , (3) 62° – 64° , (4) 58° – 60° . The temperature at which the nitroprusside reaction appeared in our experiments is thus within the range covered by these figures.

Freezing and thawing. If a suspension of yeast in test-tubes be frozen solid by immersion of the tube in ice and salt, a strong nitroprusside reaction (3 +) is obtained on thawing both in the whole mixture and in the clear centrifuged fluid; cooling which does not freeze the suspension gives negative results.

Ultra-violet light. 5 cc. of a yeast suspension (2 g. in 20 cc. 10 % sucrose) were poured into each of two Petri dishes, which were placed side by side 35 cm. from a mercury vapour lamp; the glass lid was replaced on one of the two dishes, which then served as a control on the warming effect of the lamp. After 50 minutes' exposure, the yeast in the uncovered dish showed a very distinct reaction ($1\frac{1}{2}$ + in the whole emulsion and $\frac{1}{2}$ + in the centrifuged fluid), while that from the covered dish gave no reaction at all.

X-rays. The effects of both (1) hard and (2) soft rays were tested; neither produced any change detectable by the nitroprusside test. (1) 10 cc. 10 % yeast suspension in a Petri dish were placed for half an hour immediately beneath a large Coolidge tube, with no filter but with a paraffin wax plate 4 cm. thick interposed to prevent heating. The current was 4 ma. at 160 kv. and the distance from the anticathode to the suspension 15.5 cm. (2) A similar suspension was exposed for 19 or 38 minutes immediately below a universal Coolidge tube working at 130 kv. with current of 4 ma., no filter being used.

SALTS.

If to a series of test-tubes of yeast suspension one adds more than enough of various salts to produce saturation, or if one suspends yeast in various salt

solutions already saturated, some curious differences in the nitroprusside reactions of the different mixtures can be observed; with some salts a very strong reaction develops, with others none at all, or no more than is given by a control suspension of yeast in water. The table below shows roughly the comparative activity of a number of salts in this respect. The test was made usually after from 1 to 3 hours' incubation.

Table II. 1 g. yeast in 10 cc. of 10 % dextrose or sucrose with salt added to saturation. Incubation at 37° for 1-3 hours.

Salt		Nitroprusside reaction
CaCl ₂	Na thiosulphate	++ to 8+
MgCl ₂	Na salicylate	
Na and K arsenite	Na benzoate	
NaNO ₃	(NH ₄) ₂ NO ₃	
NaCl	NH ₄ Cl	
KNO ₃	Y(NO ₃) ₃	+ to 4+
KH phthalate		
Na ₂ SO ₄	(NH ₄) ₂ SO ₄	+ to -
NaH ₂ PO ₄	KCl	
NaF	KNO ₃	
Na acetate	KH ₂ PO ₄	
Na citrate	BaCl ₂	
(Na and K arsenate)	Ba acetate	
Na ₂ HPO ₄	Na oxalate	—
Na tartrate	KClO ₃	

Table III. 1 g. yeast in 10 cc. salt solution without sugar.

		KNO ₂		
		9.5 M	5M	
Time (mins.)				
0		+++	ft+	
15		+++	ft+	
30		++	ft+	
45		+++	ft+	
60		++	ft+	
90		+	+	
120		++	+	

Solutions saturated at 37°					
g. in 100 cc.*	Molar conc.	KNO ₂	KNO ₃	NaNO ₃	Control in water
		126	45.9	71.2	
Time		14.8	4.55	8.4	
0 min.		++++	—	ft+	—
30 mins.		6+	—	++	ft+
60 "		6+	ft+	+++	ft+
120 "		6+	—	+++	ft+
21 hrs.		—	+	6+	+

* These data were obtained by weighing 10 cc. of the solution saturated at 37°, and then interpolating for this temperature from the tables in the "Chemiker-Kalender" giving g. salt in 100 g. solution saturated at various temperatures.

It does not seem possible to offer any simple explanation of these results. Possibly all arsenites and salicylates would give similar strong positive results but not many of these salts have been available. The contrast between arsenites and arsenates is very striking in this as in many other biological

reactions; the arsenates, however, have to a certain extent the character of false negatives and have hence been marked with a parenthesis in Table II. The inhibitory action of potassium arsenate is most distinct in the heating test, and that of the sodium salt in the salicylic acid test (see under "Sources of error" above).

One factor which one would expect to influence the results is the molar concentration of the saturated solution but the results show that this is not so.

If one takes any salts, such as KNO_3 , NaCl , or CaCl_2 , which when saturated give a strong positive reaction, and prepares a series of dilutions of the saturated solution, one finds that the activity diminishes rapidly as the concentration of the salt is lessened. Thus there is a large difference between 9.5 M and 5 M solutions of KNO_3 (Table III). A fairly uniform gradation is shown by solutions of NaCl (see Table IV) and of CaCl_2 (see Table V).

The presence of solid salt in a saturated solution seems to increase the effect, but this was tested in one experiment only. In this, 2 g. yeast suspended in 20 cc. 6 M NaCl (solution A), which is a nearly saturated solution, gave distinctly less colour than did the same quantity of yeast in 20 cc. of water containing an excess (16 g.) of the salt (solution B, see Table IV).

Table IV. 1 g. yeast in 10 cc. No sugar.

Time	NaCl				Solution B saturated + excess salt
	6 M	5 M	4 M	Solution A 6 M	
0 min.	ft +	-	-	$\frac{1}{2}$ +	+
90 mins.	ft +	-	-	ft +	$\frac{1}{2}$ +
120 "	ft +	? ft +	-	$\frac{1}{2}$ +	+
135 "	$\frac{1}{2}$ +	ft +	v. ft +		
150 "	$\frac{1}{2}$ +	$\frac{1}{2}$ +	-		
180 "	$\frac{1}{2}$ +	ft +	-		
270 "	$\frac{1}{2}$ +	ft +	-	$\frac{1}{2}$ +	+
22 hrs.	++	+	$\frac{1}{2}$ +	+	+

Table V. 1 g. yeast in 10 cc. No sugar.

Time	CaCl_2			Control in H_2O
	1 M	0.5 M	0.25 M	
0 min.	? ft +	? ft +	-	
90 mins.	+++	ft +	-	ft +
120 "	+++	ft +	-	-
270 "	++	ft +	-	-
330 "	++	v. ft +	-	-
23 hrs.	+++	+	ft +	-
	1 M	0.75 M	0.5 M	
0 min.	$\frac{3}{4}$ +	$\frac{1}{2}$ +	ft +	
60 mins.	++	+	$\frac{1}{2}$ +	
120 "	+++	++	+	
180 "	++	+	$\frac{1}{2}$ +	
20 hrs.	+++	++	+	

Of the inorganic salts tested, CaCl_2 seems to be the most potent. With the samples of yeast used, a strong reaction was given by a molar solution (about 11 % CaCl_2), and the strength of the salt had to be reduced to 0.25 M before

the treated and control samples ceased to show any difference. With KNO_2 or NaCl , in contrast, the action usually becomes very slight at strengths of 5 or 4 M . One may have here another instance of the great physiological activity of calcium.

ACIDS AND BASES.

These have given far more variable results than have any other agents, physical or chemical. With 0.05 N solutions containing 10 % of sugar, one finds as a rule that HCl and HNO_3 give stronger reactions than H_2SO_4 , while the organic acids (acetic, tartaric, lactic, oxalic) are less active than the inorganic, and give with many samples of yeast negative results. An example of the type of change observed is shown in Table VI. With 0.1 N solutions in the absence of sugar much stronger reactions are obtained from both inorganic and organic acids. There is an interesting difference between acetic and trichloroacetic acids, the latter being much more powerful than the former.

Table VI.

Time (mins.)	HCl	H_2SO_4	Acetic acid	Tartaric acid	Oxalic acid	Lactic acid	Control
30	++	++	-	-	+	-	-
60	++	++	ft +	-	+	-	-
120	+++	+++	+	ft +	++	ft +	-
180	+++	+	ft +	+	++	ft +	-
240	+++	+	+	ft +	++	ft +	-

1 cc. yeast suspension (1 g. yeast + 1 g. sucrose in 10 cc. H_2O) + 1 cc. 0.1 N acid.
Control, 1 cc. yeast suspension + 1 cc. water.

In contrast to the inorganic acids the bases NaOH , KOH , NH_3 may, in 0.05 N strength, in the presence of sugar, cause only very slight or no liberation of substances giving the nitroprusside reaction within 3 hours (Table VII). One might suspect that this was due to the conversion of GSH liberated first of all into G_2S_2 during the period of incubation in alkaline solution. That this is not the reason is shown by the following considerations: (1) if the test be made within 1 or 2 minutes only of the addition of alkali the result is likewise negative; (2) if yeast were very sensitive to the action of alkalis in this respect the nitroprusside reaction would never be negative, for the strength of NH_3 reached in the colour test as carried out in these experiments is from 1.2 to 2.5 N ; (3) greater concentrations of alkali (0.1 N NaOH or KOH without sugar, or half-saturated Na_2CO_3 or K_2CO_3), which might be expected to promote still more the oxidation of glutathione, can produce strong positive reactions (Table VII). With 0.1 N solutions NaOH is generally more active than KOH , and KOH more active than NH_3 .

Perhaps the reason for the great variability of the results obtained with the weaker acid and alkaline solutions is that these affect the interchanges between GSH and G_2S_2 , which are dependent upon the conditions for oxidation, which again are dependent upon the particular method by which the experiment is carried out (large or small quantity of suspension in narrow or wide vessels, frequency of stirring).

Table VII.

Time (mins.)	NaOH 0.05 <i>N</i>	NH ₃				
		3 <i>N</i>	0.5 <i>N</i>	0.1 <i>N</i>	0.05 <i>N</i>	0.01 <i>N</i>
15	ft +	ft +	ft +	—	—	—
35	ft +	ft +	ft +	—	—	—
60	—	++	+	ft +	ft +	—
120	—	+++	++	+	ft +	ft +
180	—	+++	+++	+	ft +	ft +

1 g. yeast + 1 g. sucrose in 20 cc. alkali solution.

NaHCO ₃	A	—	KHCO ₃	A	—
	B	—		B	—
Na ₂ CO ₃	A	+++	K ₂ CO ₃	A	+++
	B	—		B	—
(NH ₄) ₂ CO ₃	A	++	Control		—

Results after 60 minutes' incubation.

A = saturated solution.

B = 1.25 % solution. This concentration was tested as a control on experiments with alkali salts of bile acids described below.

1 cc. yeast suspension (1 g. yeast + 1 g. sucrose in 10 cc. H₂O) + 1 cc. A or B.

ORGANIC COMPOUNDS.

Table VIII represents an attempt to summarise the results which have been obtained with various organic compounds. The + signs are intended to represent the maximum nitroprusside reaction usually observed in a series of tests carried out at various times during 3 to 5 hours' incubation. The table shows that the sodium salts of the mixed bile acids, and of cholic and deoxycholic acids, have a powerful action even in concentrations as low in some cases as 0.04 %. Many compounds of the class of phenols (phenol, *p*-cresol, thymol, salicylic acid and its two isomers, hexylresorcinolcarboxylic acid, α - and β -naphthol) and aniline, and the naphthylamines and their tetrahydro-derivatives are active in concentrations from 0.1 to 0.01 *M*. Hexylresorcinolcarboxylic acid is of interest on account of its solvent action upon Gram-negative micro-organisms [Bleyer, 1927]. Of the alcohols tested only caprylic alcohol (saturated solution in water filtered from excess of alcohol) gave a satisfactory positive result. Ethyl alcohol has an increasing inhibitory effect upon the nitroprusside reaction in concentrations from 20 vols. % onwards, until at 50 vols. % the colour reaction (of yeast in thymol-water or salicylic acid solution) is inhibited almost completely; but amounts of this alcohol which might occur in sugar solutions (10 to 15 vols. %) have no perceptible effect upon the colour. Methyl alcohol in concentrations of 1 to 50 vols. % produces weak positive reactions, but can be shown to have at the same time an inhibitory effect; and glycerol (25 vols. %) and formaldehyde (5 to 10 vols. %) suppress the colour test; these three compounds are therefore excluded from the table as possible false negatives. Acetaldehyde (3 vols. %, about 0.5 *M*) gives a negative result, and in this concentration has only a very slight inhibitory effect; while the trichloro-derivative, chloral hydrate, gives a positive result in much weaker solutions (0.066 *M*; cf. the relative potency of trichloroacetic acid and acetic acid, under "Acids" above).

Table VIII.

	Concentration		
	%	Molar approx.	
Na cholate	4.0	—	4 +
Na deoxycholate	2.0	—	4 +
Na salts, mixed bile acids, ox bile	2.0	—	4 +
Hexylresorcinolcarboxylic acid ...	0.6 as Na salt	0.025	4 +
Phenol	1.0-0.1	0.1-0.01	} see Table XII
Salicylic acid	0.2	0.014	
<i>m</i> - and <i>p</i> -Hydroxybenzoic acid ...	0.2	0.014	
<i>p</i> -Cresol	0.5	0.05	5 +
Thymol saturated	0.07	0.005	2 + to 6 +
α -Naphthol	1.44 as Na compound	0.1	+ +
β -Naphthol			
Aniline	1.0	0.1	2 + to 4 +
Caprylic alcohol saturated	?	—	+ +
Inositol	5.0	0.3	—
Acetaldehyde	—	0.5	—
Chloral hydrate	1.0	0.066	+ +
Chloroform saturated	—	—	+ to 4 +
Ether saturated	—	—	+ +
Ethyl acetate	6.0	0.7	3 +
Urea saturated	—	—	4 +
Urea half-saturated	—	—	+
Urethane	1.8	0.2	+ +
Na oleate	1.0-0.1	0.03-0.003	} see Table XIII
Na stearate	0.5	0.015	
Quinine-HCl	3.0	—	
Saponin	1.0	—	+ +
KCN	—	0.1	—

1 g. yeast in 10 cc. watery solution. No sugar.

A test with saturated urea was suggested by Ramsden's [1902] discovery of the macerating action of this solution; the result was strongly positive and even the half-saturated solution is active with some yeasts. The allied compound urethane gives a positive result in much lower concentration (0.2 *M*) and perhaps for quite different reasons.

There is a curious difference between the actions of naphthalene and of its half-reduced form tetrahydronaphthalene; after 24 hours the former gives a strong positive, the latter a negative result. This is shown in Table IX together with the effects of three other naphthalene derivatives. Sodium oleate appears to be about 20 times more active than sodium stearate.

Salicylic acid and phenol are of interest on account of the very low concentrations in which they are active (Table XII). Salicylic acid (0.2 %), which is a nearly saturated solution, is very effective, and with some yeasts one-tenth of this concentration, or 0.0014 *M*, produces a distinctly stronger nitroprusside reaction than that seen in a control. We have made a number of experiments with the three isomers, salicylic acid and *m*- and *p*-hydroxybenzoic acids; the usual result, at 0.2 % strength, is that the *o*-acid is more active than the *p*-, and this again more active than the *m*-acid; evidence was obtained that at a lower concentration (0.1 %) this relation is altered, so that salicylic acid becomes the weakest of the three; but there were some irregularities and this interesting point requires further experiments. From the pharmacological inertness of the *p*- and *m*-compounds one might expect a very

distinct difference. Lafar [1906] states that, at 0.1 % strength, salicylic acid arrests the multiplication of yeast, while the two isomers have no effect. Sodium salicylate, though very effectual in saturated solution (Table II), is less active in dilute solution (0.2 %) than the free acid.

Table IX.

Time	α -Naphthyl- amine-HCl	Naphthalene	Tetrahydro- naphthalene	Tetrahydronaphthylamine		Control in gum
				Ar- α	Ac- β	
0 min.	+++	-	-	ft+	+++	-
60 mins.	+++	ft+	-	+++	+	-
120 "	+++	$\pm$	-	+++	+	-
210 "	+++	-	-	+++	ft+	$\pm$
270 "	+++	-	-	+++	-	ft+
23 hrs.	Solution too dark for test	6+	-	ft+	-	ft+

Solutions. α -Naphthylamine 1.43 g. + 10 cc. *N* HCl $\rightarrow$ 100 cc. = 0.1 *M*.
Naphthalene 0.13 g. in 2.6 cc. alcohol + 7.4 cc. gum = 0.1 *M*.
Tetrahydronaphthalene 1 cc. + 10 cc. gum = approx. $\frac{2}{3}$ *M*.
Tetrahydronaphthylamine 0.15 g. in 10 cc. gum = 0.1 *M*.
1 g. yeast suspended in 10 cc. of each solution.
Control 1 g. yeast in 10 cc. gum.
Gum = 5 % gum-acacia in water.

A result of the same type is obtained when the naphthalene and tetrahydronaphthalene are in the same molar concentrations.

Table X.

Time	In H ₂ O			In H ₂ O + 10 % sucrose		
	Control	KCN		Control	KCN	
		0.1 <i>M</i>	0.01 <i>M</i>		0.1 <i>M</i>	0.01 <i>M</i>
0 min.	$\frac{1}{2}$ +	-	$\frac{1}{2}$ +	-	$\pm$	-
60 mins.	+	-	-	-	-	-
120 "	+++	$\frac{1}{2}$ +	$\frac{1}{2}$ +	$\frac{1}{2}$ +	$\frac{1}{2}$ +	-
180 "	++	$\frac{1}{4}$ +	$\frac{1}{4}$ +	$\frac{1}{4}$ +	-	-
240 "	+++	+	$\frac{1}{2}$ +	+	-	ft+
300 "	++	$\frac{1}{2}$ +	$\frac{1}{4}$ +	$\frac{1}{2}$ +	-	ft+
28 hrs.	+++	++	+++	+++	+	+++

The effect of potassium cyanide is to be seen in Table X; this compound causes a considerable lessening of the natural autolytic reaction of the yeast. It so happened that the yeast used in this experiment gave by itself, in the absence of sugar, an exceptionally strong (up to 3+) colour, hence the inhibitory action of the cyanide was well shown. Voegtlin, Johnson and Dyer [1926] find that glutathione, cystine and cysteine act as antidotes to NaCN, which changes the first two of these three compounds to the reduced forms.

INTRACELLULAR AND EXTRACELLULAR LIBERATION.

When the positive results recorded above are considered, the question arises whether the reducing substances liberated are retained within the yeast cell, or pass out into the surrounding fluid. If a series of suspensions of yeast in different media be prepared and incubated and the nitroprusside test be

applied to each emulsion and to the clear fluid obtained from it by centrifuging, some remarkable differences are observed; under the action of some reagents the reducing substances are confined to the cells, the surrounding fluid giving no colour at all with nitroprusside, whilst with other reagents the clear fluid, and the whole emulsion, show colours of equal intensity. Thus a suspension of yeast in 0.2 % salicylic acid will give after from 1 to 4 hours' incubation a very strong (3 +) reaction, while the clear supernatant fluid gives no colour whatever; on the other hand a suspension in 1 or 2 % aniline or phenol will show the same depth of tint in the whole emulsion and in the centrifuged fluid. Thus some reagents cause chiefly an intracellular, and some also an extracellular, liberation of substances giving the nitroprusside reaction. No claim is made here that these distinctions are absolute; the differences are those of degree, and the important factor is of course the duration of exposure to the reagent. When incubation is continued for many hours extravasation from the cells increases and positive tests begin to be obtained from fluids previously negative; yet even after 24 hours' incubation the contrasts may still be very distinct. Different samples of yeast may give more or less variable results. With some agents (inorganic acids, saturated urea solution, chloroform and thymol water) which produce an effect of the intracellular (A) type there is an especial tendency to change to the B type as incubation is continued. Table XI summarises the range of results obtained in over 800 colour tests, on about 25 samples of yeast.

Table XI.

A. Intracellular type	B. Extracellular type
Phenol 0.5 % or less Salicylic acid 0.2 % <i>m</i> - and <i>p</i> -Hydroxybenzoic acid Na cholate Na deoxycholate Na oleate 1.0 to 0.02 % Caprylic alcohol saturated in H ₂ O Chloroform saturated in H ₂ O Ether saturated in H ₂ O Thymol saturated in H ₂ O Chloral hydrate 1 % Urea saturated or half-saturated in H ₂ O Urethane 0.2 <i>M</i> Saponin 1 % KNO ₃ saturated HNO ₃ , H ₂ SO ₄ , HCl, 0.1 <i>N</i> (at first) Autolysis	Phenol 1 % Aniline 2 % β-Naphthol 0.1 <i>N</i> as Na compound α-Naphthylamine-HCl 0.1 <i>N</i> Hexylresorcinolcarboxylic acid, 1.25 % Na salt Saturated Na benzoate Quinine-HCl 3 % Ethyl acetate 6 % Trichloroacetic acid 10 % to 0.8 % (0.05 <i>M</i>) CaCl ₂ , 1 <i>M</i> , 0.75 <i>M</i> , 0.5 <i>M</i> NaCl 6 <i>M</i> NH ₄ Cl saturated Na arsenite 1.5 <i>M</i> Heat (100°, 65°) Grinding Freezing

1 g. yeast in 10 cc. watery solution. No sugar. Tests made usually after 30 minutes' to 5 hours' incubation.

The demonstration of the nitroprusside reaction in the cells separated from the fluid was not attempted since this would require a process of washing and centrifuging which might cause secondary changes. The whiteness of the yeast lessens the pink colour in the whole suspension, and some allowance must be made for this in recording comparisons with the supernatant fluid.

Table XII.

		Hours							
		1-5		3		4-5		23	
		F	E	F	E	F	E	F	E
Phenol	1 %	4+	4+	4+	4+	4+	4+	ft+	$\frac{1}{2}$ +
	0.5	-	+	-	$\frac{1}{2}$ +	-	ft+	4+	3+
	0.2	-	++	-	++	-	+	-	3+
	0.1	-	+	-	+	-	+	-	3+
Salicylic acid	0.2 %	-	3+	-	3+	?	4+	++	4+
Control in H ₂ O		-	ft+	-	ft+	-	ft+	-	++

F=supernatant fluid after centrifuging.

E=whole emulsion.

1 g. yeast in 10 cc. solution or H₂O. No sugar.

Table XII represents a series of changes which has been observed with no great variations in each one of six samples of yeast tested. The figures show (1) that phenol in 1 % concentration produces the extracellular, and salicylic acid in very nearly saturated solution the intracellular, type of change; (2) that phenol in 0.5 % or weaker solutions produces the intracellular liberation only; (3) that 0.2 % phenol is more effective than the 0.5 % solution—we have noted other instances where the lesser amount of a reagent produced the greater effect, and some of these are under investigation; (4) that after a whole day's incubation the change, at first intracellular, may become extracellular (0.5 % phenol, and salicylic acid); (5) that the reducing substances formed in autolysis (control in H₂O) are not detected outside the cell; this has been the case in all of 16 controls examined in this way. Table XIII illustrates the intracellular type of action of sodium oleate, and its activity in high dilutions, 0.1 % (0.003 *M*) or less.

Table XIII.

		30 mins.		90 mins.		150 mins.		20 hrs.	
		F	E	F	E	F	E	F	E
Sodium oleate	1 %	-	++	ft+	+++	$\frac{1}{2}$ +	+++	+	++
	0.1	-	$\frac{1}{2}$ +	-	+++	-	+++	++	++++
	0.02	-	-	-	ft+	-	+	-	+++
Control in H ₂ O		-	-	-	-	-	-	-	ft+

F=supernatant fluid of centrifuged emulsion.

E=whole emulsion.

1 g. yeast in 10 cc. solution or H₂O. No sugar.

SERA AND ENZYMES.

Yeast treated with an antiserum, prepared by giving six intravenous injections of yeast suspension to a rabbit, gave no nitroprusside reaction, although there was considerable agglutination. Peptic digestion usually gives a positive, and tryptic digestion a negative, result, but the experiments were not controlled fully by corresponding acid and alkaline solutions. Digestion with takadiastase was likewise negative in result.

MICROSCOPICAL EXAMINATION.

The microscopical examination of yeast exposed to the various physical and chemical agents enumerated here has given almost wholly negative results (the changes brought about by grinding are described separately above). Fragmentation of cells, and cell débris, such as are seen in abundance when mammalian cells are exposed to some bile salt preparations, were not seen in one single instance. Occasionally some effects were noted (some shrinkage in 10 % trichloroacetic acid and in saturated ammonium chloride; a glassy appearance in saturated sodium benzoate; clumping in a tryptic digest and in anti-yeast serum) but no appearance of cytolysis. The two classes of reagents which seem to produce chiefly intracellular, or extracellular, liberation of reducing substances showed no differences.

DISCUSSION.

When the results presented in the tables above are considered, the number and great variety of agents which cause yeast to give a positive nitroprusside reaction may suggest that this effect can be brought about by anything which does considerable damage to the cell, and that the findings therefore do not indicate any specific processes and are hence lacking in interest. But when one regards some of the agents which give a negative or only very weak positive result (heating for 2 minutes at 59°; X-rays in the doses stated; 0.1 *M* KCN; a large number of saturated salt solutions) it is difficult to believe that these are not injurious. Hence there seem to be two classes of harmful factors: those which do, and those which do not, cause a considerable liberation of reducing substances, and this points to a field for further experiments. To decide whether yeast is or is not killed by the various conditions giving the positive and negative results would require a lengthy investigation by methods of subculture which we did not wish to pursue, since we were not interested in the biology of yeast itself so much as in suggestions for work on mammalian cells.

The experiments described above were not capable of showing to what extent substances other than glutathione contribute to the nitroprusside reactions observed; the slight differences in tint of the colour test with some reagents, *e.g.* a reddish tinge with CaCl_2 , suggest that the processes occurring are not always quite the same in nature. In this connection, one may note that Tunnicliffe's method for the estimation of glutathione employs two factors, namely, grinding, and trichloroacetic acid, which are among those found in the present investigation to be most effective, and to cause extracellular liberation. Tunnicliffe's [1925] method was checked by parallel estimations of organic sulphur. It would be of some interest to compare the amount of glutathione obtained from a sample of yeast by this method with the amount of nitroprusside-reacting substance liberated by such agents as heat or phenol.

Although no cytolysis was observed microscopically the reactions described here have no doubt some relation to those of haemolysis; some of the most active factors (freezing, bile salts, sodium oleate, saponin, ether, chloroform) are well-known haemolytic agents. It is noteworthy that some substances which have a powerful effect in lowering surface tension (bile salts, sodium oleate, saponin, caprylic alcohol, ether) nevertheless produce the intracellular rather than the extracellular type of liberation.

The results of most interest seem to be those in which the amount of reagent required to cause a liberation of reducing substances is very small. Thus phenol, salicylic acid, thymol and sodium oleate are active in strengths from 0.014 *N* to 0.003 *N*. An experiment with thymol water, such as those which provided the figures given in Table VIII, consists in the addition of about 7 mg. of thymol to 1000 mg. of compressed yeast, and this gives a very strong reaction. Further experiments with these and with still more dilute solutions are in progress.

These experiments were almost completed when we learned of the work of Harris [1923] on the denaturation of proteins; many of his results obtained with egg-albumin, such as the action of heat, of incubation with acids and alkalis, and of ultra-violet light, and the greater apparent effect of peptic as compared with tryptic digestion, are very similar to some of those described here. It is perhaps worth noting that some of the substances (sodium salicylate, sodium oleate, bile) stated by Vincent [1928] to have a remarkable neutralising action upon bacterial toxins are among those which produce a nitroprusside reaction; possibly both phenomena depend upon a process of denaturation.

SUMMARY.

A large number of agents have been examined with regard to their power to liberate from yeast substances giving the nitroprusside reaction. Positive results were obtained by a variety of methods, some physical (heating above a certain temperature, freezing, grinding, ultra-violet light), some chemical (*e.g.* exposure to certain saturated salt solutions, or to many organic compounds of the phenol and amine classes in solutions of 0.1 to 0.01 *M* strength). Negative results are more difficult to establish, because the nitroprusside test is inhibited by many compounds (*e.g.* sodium nitrite, ethyl alcohol, toluene); hence an apparent negative result with yeast may be in reality a positive one in which the colour test is masked. The factors which produce from yeast substances giving the nitroprusside test can be separated to a certain extent into two classes; in one class the liberation is chiefly intracellular, in the other it is extracellular also. Many of the positive agents are known to be haemolytic (bile salts, saponin, sodium oleate, freezing) but lysis of the yeast cell was not observed in any instance.

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