and on pouring into cold water a brownish yellow substance separated in good quantity. This was apparently a normal condensation product or 5-thio-4-benzalhydantoin, but it could not be purified because of its insolubility. After trituration with boiling water and alcohol it was dried and analyzed. A nitrogen determination gave 14.4% of nitrogen, while the calculated for the benzalthiohydantoin is 13.73%. The product had no definit melting point and was decomposed by digestion with acids and alkali forming hydrogen sulfide and alkali sulfide, respectively.

NEW HAVEN, CONN., July 8, 1912.

[FROM THE RUDOLPH SPRECKELS PHYSIOLOGICAL LABORATORY OF THE UNIVERSITY OF CALIFORNIA.]

ON A NEW GLUCOLYTIC FERMENT OF YEAST.

BY VICTOR BIRCKNER.

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1. The Ferment as First Observed and Recognized.

In the early part of 1911, following a suggestion of Dr. T. Brailsford Robertson, I found it necessary for a certain purpose to prepare the ferment maltase. I tried to obtain it from yeast by the well known method of Croft Hill[1] or with a slight modification according to O. Emmerling.[2] The material chosen was the yeast of the so called California "steam beer," a local brew, which although a bottom fermentation beer, differs a good deal from the common lager beers.

The differences between lager beer and steam beer depend in the first place on the difference in temperature at which the fermentation of the wort is being carried on, and they are therefore a direct function of the respective metabolic activity of the yeast. The following table will serve to illustrate these relations:

	Temp. at start.	Temp. maximum which is allowed.	Time required for the whole ferm. process.
Lager beer.....	5°	10–11°	8 to 10 days
Calif. "steam".....	13°	18°	3 days

Besides the temperature it is probably the more extensive aeration of the steam beer yeast which in part causes its high activity. After having reached the temperature of 18°, instead of being cooled down by artificial means in the fermenting vat itself, the whole brew is transferred to large wooden pans, the so-called "clarifiers," where in a layer about one foot deep the fermentation process is carried to the end. Although in this way, by giving the mixture a large surface, the rise of temperature is checked, the fermentation process still proceeds with considerable speed on account of the ample aeration. The use of these clarifiers is a typical feature in the manufacture of the California steam beer.

The yeast of the steam beer has accommodated itself to these conditions to such an extent, that it can no longer be employed for the preparation of lager beer, while lager beer yeast may without difficulty be used for the manufacture of steam beer. The cells of the typical steam beer yeast are somewhat smaller than those of lager beer yeast.

[1] A. Croft Hill, *J. Chem. Soc.*, **73**, 634 (1898).

[2] O. Emmerling, *Ber.*, **34**, 602 (1901).

The yeast used for the most part in the experiments which I am about to describe, was furnished by the California Brewing Co. of San Francisco. I am highly indebted to Mr. G. Woehrle of this firm, who very kindly supplied me with the necessary material on many occasions.

After following the directions of Hill[1] and Emmerling[1] in every detail, I arrived at the conclusion, after numerous trials, that with my particular material (California steam beer yeast), it was impossible to obtain an active maltase preparation by the method of Hill.[2]

At the time when I still had some hope of having maltase in my solution, part of the extract was tried on a strong solution of glucose, on which maltase according to Croft Hill[1] and Emmerling[1] exerts a synthetic action, yielding a disaccharide.

I noticed very peculiar changes taking place. After standing in the 70° incubator for one day, the samples showed a reddish brown coloration, which, after 2–3 days, had changed into dark crimson, the liquid, which was still perfectly clear having acquired an aromatic odor. An acid reaction to litmus paper was also observed. After prolonged standing in the 70° incubator in tightly stoppered test tubes, the samples turned almost black, and a brownish carbon-like substance settled out along the edges of the glass very gradually. No gas formation was noticed.

The samples which had been kept in the 30° incubator, underwent no visible changes for a very long time, after which they finally became faintly yellow. They, too, gradually gave an acid reaction.

The fact that a solution of glucose alone in the same concentration, did not undergo any similar changes, could be easily ascertained. Nor did either maltose or the ferment extract, or both together, show any such colorations for at least two weeks. It was therefore natural to assume that a catalytic agent of some kind was bringing about these changes in the glucose-extract mixture.

[1] *Loc. cit.*

[2] It is not at all surprising that in working with living material, methods that have been found useful for a certain operation in one locality may not be successful, if employed at a different locality. Experiences of that sort have frequently been made, much more frequently perhaps than could be concluded from the literature alone. The lower forms of life, such as yeasts and bacteria, are especially sensitive to slight variations of the external conditions. Such changes must necessarily affect the one cell organism much more deeply in its whole structure and organization than they would influence higher forms, where those variations may only affect the function of one special organ. It is of interest in this connection that with yeast material of this locality I am not the first one to report a complete failure of a method that is generally found successful in other places. Taylor (A. E. Taylor, "On Fermentation," Univ. of Calif. Publications in Pathology, 1, 212) after many unsuccessful trials to obtain by Büchner's well known method an active zymase preparation from San Francisco yeast, is led to the statement, that "the commercial *Saccharomyces Cerevisiae* of this city is worthless for the preparation of a yeast-powder; the glycogen content is high, the proteolytic ferment active, the zymase weak."

That the substance which causes the crimson coloration is actually formed at the temperature of 70° and not simply in the time during which the mixture is gradually being heated up to this point, was shown by placing each of the two solutions (glucose and extract) in the 70° incubator separately at first, and mixing them when warm. The coloration appeared in the same way as before.

On transferring some of the colorless samples, which had been kept in the 30° incubator for weeks, to the 70° incubator, the change in color took place readily, and it could be rendered twice as intense by boiling the sample for a moment just before transferring it. The respective compound, therefore, had possibly been formed at the lower temperature too, and only assumed that different color at the elevated temperature. At any rate, it seemed important to know what was the nature of the substance which caused these characteristic transformations with such regularity.

I was at first inclined to think that really a synthetic change from glucose into isomaltose had taken place, an assumption which seemed well justified with regard to what is known about the properties of this disaccharide.[1] Very soon, however, I could convince myself that this conclusion was erroneous.

All disaccharides are known to have a higher optical rotation and a lower copper-reducing power than *d*-glucose. In some preliminary experiments I had already noticed, however, that there was taking place in my mixtures not only a decrease in reducing power, but also a decrease in optical activity. These results had been obtained with a ferment preparation that was more than 2 months old; they therefore needed confirmation. With a fresh preparation, the result was largely the same, as we shall see presently.

To 160 cc. of a 40% solution of pure glucose (Kahlbaum) 40 cc. of yeast extract and a few drops of toluene were added, the liquid well mixed, and measured from a buret into test tubes in portions of exactly 10 cc. The tubes were tightly stoppered and divided into two sets. One set was placed in an incubator at 30°, the other into the 70° incubator. From time to time, one test tube of each set was taken for analysis, its contents being washed into a one-liter graduated flask. After adding 10 cc. of a 2.5% solution of sodium carbonate to stop the reaction, the liquid was made up to mark with distilled water, filtered, if necessary, and an aliquot used for the determination of the reducing power, while the remainder was available for the polariscope reading. The liquid had to be used in such a high dilution on account of the coloration, and because it is not advisable, with white light, to obtain readings that would

[1] See E. v. Lippmann, "Die Chemie der Zuckerarten," Third edition. Fr. Vieweg & Sohn, Braunchweig, 1904, p. 1513.

exceed the value of 3°.[1] The white light of a 50-candle power globe was used with the polariscope, as I could not procure a sodium light of sufficient intensity. The variations in temperature were slight, and their influence on the reading was negligible in this preliminary work. The readings refer to a tube length of 400 mm.

The determinations of the reducing power were all made by the method of Bertrand.[2] In the tables below, which are intended to give an approximate idea of the progress of the reaction, only the number of cc. of the standard $KMnO_4$ solution which is used in this method have been inserted. Each cc. is equivalent to 8.72 mg. Cu. The mixture at the beginning was composed of 40 cc. yeast extract, 80 cc. glucose (40%), and toluene. Ten cc. were made up to 1 liter for each determination.

Rotation. Polariscope reading.			Reduction. Cc. $KMnO_4$ required.	
30°.	70°.	Time in hours.	30° C.	70° C.
0.60°	0.60°	0	11.62	11.62
0.57°	0.48°	64	11.70	11.55
0.57°	0.45°	110	11.65	11.35
0.53°	0.42°	190	11.58	11.25
0.54°	0.41°	254	11.65	11.15

The yeast extract, as already mentioned, gave a precipitate in the 70° incubator if kept there for several hours. Both fractions, the clear fluid and the precipitate, were tested separately for their action on glucose. The precipitate showed no action whatever, while the clear liquid had preserved its fermentative qualities almost unweakened. In the following table the composition of the mixture was exactly the same as in the preceding one, except that for the ordinary yeast extract this clear liquid, which has just been described, was substituted

Rotation. Polariscope reading.			Reduction. No. of cc. $KMnO_4$ required.	
30°.	70°.	Time in hours.	30° C.	70° C.
0.55°	0.55°	0	11.63	11.63
0.53°	0.48°	$63\frac{3}{4}$	11.58	11.60
0.54°	0.42°	$142\frac{1}{4}$	11.60	11.35

From both these tables it follows clearly that there is no synthetic process going on, but that sugar is being broken down. On these figures alone we could, however, not base a reasonable interpretation either of the rate of the reaction or of its products, for the following reasons:

Although highly diluted for the analysis, the color of the liquid deepens to such an extent as the reaction proceeds that this color alone causes a marked depression of the polariscope reading, so that the actual loss of

[1] See Landolt-Long, "The Optical Rotating Power of Organic Substances," Easton, Pa., 1902, The Chemical Publishing Co., p. 417.

[2] *Bull. soc. chim.*, [3] 35, 1285 (1906).

rotatory power is probably not nearly as great as it would appear from the tables. On the other hand, the fermentation products may be optically active substances themselves, so that in no case would the polariscope reading give an accurate idea of what transformations are really taking place. Hence although from the last two tables it seems as if the fall in optical activity proceeds much faster than the fall of the reducing power, we can not be at all sure that such is really the case.

The destruction of glucose may, on the other hand, yield substances which themselves have a high reducing power against Fehling's solution. Hence this method of determination might be just as inaccurate and misleading in its results as the optical method.

I therefore had no accurate means of measuring the progress of the reaction, nor was there much chance of finding out, within a short time, what the products of transformation would be. Moreover, as long as I was following the method of Hill, I could prepare only a small quantity of yeast extract at a time. My particular working material dried only very slowly in a vacuum over sulfuric acid, and had to be spread in very thin layers, thus giving only small yields.

2. Experiments with Hydroquinol.

Fortunately, while in these difficulties, I happened to gain a new aspect of the problem by observing that the ferment showed a certain activity against hydroquinol, hastening its oxidation to quinone. Hence it appeared to be a typical oxydase.[1]

It was decided to prepare a larger quantity of it, and if possible to find a somewhat simpler method of preparation.

The yeast material was, at this time, procured from a different, and larger brewery; it was, as in the previous cases, typical steam beer yeast. My expectation that this material would yield a highly active ferment preparation, was, however, not fulfilled. In fact the new extracts showed so little activity towards glucose that they could not be used to any advantage for the study of this ferment. As the reason for this peculiar behavior, I later recognized the fact that this particular ferment is greatly damaged by low temperatures and does not develop to any extent in yeasts which, like all the lager beer yeasts, are ordinarily kept in refrigerated localities. The California Brewing Company, which devotes itself exclusively to the manufacture of steam beer, does not possess an ice-plant, and it is most probably due to this fact that their yeast contains this glucolytic agent to such a remarkable extent. Naturally, under

[1] Grüss and Issajew had already described the presence of an oxydase in yeast: Grüss, "Ueber Oxydaseerscheinungen der Hefe," *Wochschr. Brauerei*, **18**, 310, 335 (1901); cf. Kastle, "The Oxydases, Etc," *Bull.* **59**, Hyg. Lab. U. S. Pub. Health and Marine Hosp. Serv., Washington, 1910; W. Issajew, "Ueber die Hefe-Oxydase," *Z. physiol. Chem.*, **42**, 132 (1904).

these circumstances, I had to return to the use of this yeast material. The first aim, as already stated, was to find out which way of preparing the yeast powder would have the least obnoxious effect on the ferment.

The experiments with those weak extracts, mentioned in the preceding paragraph, unsatisfactory as they were on the whole, had shown with fair certainty that the acetone yeast as well as the methyl alcohol yeast (Dauerhefe), on extracting, yield ferment preparations which are in every respect equivalent, if not superior, to those prepared by Hill's method. They had shown, moreover, that the ferment in question is most likely not zymase, as zymase is rapidly destroyed by methyl alcohol.[1] They had finally shown another, not unimportant fact. One of the powders, namely the CH_3OH powder, happened to be already neutral to litmus paper in its aqueous suspension, while the others, being acid, were neutralized with dilute Na_2CO_3, when first brought in contact with water. Now I observed that the extract which was already neutral by itself, not only gave practically no precipitate at 70°, but together with glucose, the mixture kept much clearer in appearance than in the samples which contained the neutralized extracts, notwithstanding the coloration process. As, besides, the ferment did not seem to be very sensitive to a slightly acid reaction of the medium (it was naturally formed in a medium of pronounced acidity), I have in all subsequent cases avoided the addition of alkali. By thoroughly washing the yeast all the acid can be removed, so that the resulting powder is, on extracting, practically neutral to litmus.

Above all, however, the tedious method of Hill[2] could now be discarded entirely. A new batch of yeast was all prepared as "Dauerhefe." Three different fixing agents were tried once more, however, in order to determine definitely, which of them could finally be used with the greatest advantage.

In preparing the three powders, I followed closely a method recently employed by Herzog and Saladin.[3] As fixing agents methyl alcohol, ethyl alcohol, and acetone were used. The two alcohol preparations were white, dust-like powders, while the acetone preparation was heavier, and of a somewhat sandy character.

On extraction with water, all except the CH_3OH preparation, which reacted slightly acid, were neutral to litmus paper. The CH_3OH suspension settled quickly and was easy to filter, while the other two, especially the acetone preparation, settled very slowly, and were very difficult to filter.

All three preparations, after passing through a Chamberland filter by

[1] E. Büchner, H. Büchner und M. Hahn, "Die Zymasegärung," 1903: cf. W. Zaleski, *Biochem. Z.*, 31, 195 (1911).

[2] *Loc. cit.*

[3] R. O. Herzog and O. Saladin, *Z. physiol. Chem.*, 73, 263 (1911).

means of a pressure pump, were not used directly, but were submitted to a process of repeated precipitation with alcohol and solution in water, a process which I shall describe later in the section on purification methods. The ferment was finally obtained in the form of a crisp powder which was partly dried in the vacuum after washing with alcohol, partly after additional washing with ether. To my knowledge, ether has never been used so far in this connection. But as the evaporation of the alcohol is a slow process, even in a vacuum over sulfuric acid, I tried the possible effect that washing with ether might have on the ferment.

Finally, a small portion of each of the three raw extracts was slowly boiled for 6 minutes with animal charcoal[1] and filtered before being precipitated.

All these different preparations were now tested for their action on hydroquinol. The apparatus employed for this purpose was the same, in principle, as that described by Euler.[2] I have, however, applied a few alterations, which, I believe, assure a higher accuracy of the readings without complicating the method as such. Thus, instead of a buret I used a thin glass tube 100 cm. long, to which I attached a measuring scale, in order to secure a smaller cross-section. The capacity of the full length of the tube (100 cm.) was only 17.72 cc. As I did not have a vessel of the kind described by Euler[3] I used an ordinary wide mouth bottle of rather strong glass, having an opening of 3.7 cm. in diameter and a capacity of about 500 cc. Inside of this bottle was first placed a small weighing flask into which the ferment solution was measured with a pipet. Then, the respective amount of hydroquinol was introduced into the larger bottle, and the latter closed with a tightly fitting rubber stopper which contained two glass tubes, one connecting with the gasometer, the other provided with a glass stopcock, as outlet for the air. After the bottle had been filled with oxygen and the stopcock closed, the level of the mercury was read on the scale (bringing the mercury in both branches of the glass tube to the same level), then the small weighing flask tipped over, and the bottle put on the shaking machine, which was already in motion. The motor was arranged so as to give the shaker about 90 movements per minute, which was found sufficient to accelerate the reaction notably. A reading was taken every minute for half an hour. The temperature was 18° in all experiments. The total amount of liquid in each case was 50 cc. and it was always 0.2 normal with regard to hydroquinol. Equal amounts of the respective ferment solutions of equal

[1] Specially purified with HCl. See Euler, as below.

[2] H. Euler and I. Bolin, "Zur Kenntnis biologisch wichtiger Oxydationen," *Z. physiol. Chem.*, **57**, 80 (1908); "Ueber die chemische Zusammensetzung und die biologische Rolle einer Oxydase," *Z. physik. Chem.*, Arrhenius Jubelband, **69**, 187 (1909).

[3] *Loc. cit.*

strength (0.2 g. purified ferment powder for each case) were employed in all experiments, so as to make possible an exact comparison between the different preparations. The amount of oxygen absorbed, as expressed by the rising of the mercury in the tube, gave a measure of the respective rate of oxidation.

What interests us here is chiefly the total average amount of oxygen absorbed in half an hour by the hydroquinol solution under the influence of the different ferment preparations. The following table expresses these amounts as measured by the respective total differences in centimeters in the height of the mercury level in the tube. The corresponding average oxygen absorption of 50 cc. of a 0.2 normal solution of hydroquinol alone, was 25 cm. on the scale, corresponding to 4.43 cc. of oxygen.

	Nature of the fixing agent.		
Previous treatment of ferment extract.	Methyl alcohol, cm.	Ethyl alcohol, cm.	Acetone, cm.
Final washing with alcohol	42.2	54.0	55.0
Final washing with ether	47.8	52.0	63.0
Boiled for 6 min	16.4	26.0	24.4

For all three preparations the influence of the ferment was therefore readily noticeable. The process under the influence of its action proceeded with at least twice its original velocity. No alkali was added in any case. The washing with ether, it is noticed, had a favorable rather than an obnoxious effect on the ferment. Boiling for six minutes had destroyed the oxidative agent entirely. Compared with the others, the acetone preparation proved to be the most active against hydroquinol.

I may add, that in some qualitative experiments, I convinced myself that the addition of a trace of $MnSO_4$ greatly accelerated the action of the ferment on hydroquinol, and that it also accelerated appreciably its action on glucose. Hence the ferment, in this respect, resembled Bertrand's laccase.[1]

On trying the three different preparations on glucose, I found that their relative activity against this substance was not simply in parallel with their respective activity on hydroquinol.[2] While against hydroquinol, the extract of the acetone powder caused the strongest reaction,

[1] G. Bertrand, "Sur le pouvoir oxydant des sels manganeux et sur la constitution chimique de la laccase," *Bull. soc. chim.*, [3] **17**, 753 (1897); "Sur l'action oxydante des sels manganeux et sur la constitution chimique des oxydases," *Compt. rend.*, **124**, 1355 (1897); "Sur l'intervention du manganèse dans les oxdations provoquées par la laccase," *Ibid.*, **124**, 1032 (1897).

[2] Similar observations had led Euler to unite all oxidative ferments which at the same time act on glucose, under the new heading "Gärungsenzyme," thus expressing their important relationship to Büchner's zymase and at the same time separating them from both the oxidases and the hydrolytic ferments. H. Euler, "Grundlagen und Ergebnisse der Pflanzenchemie," Vieweg & Sohn, Braunschweig, 1909, Vol. II, p. 68; "Allgemeine Chemie der Enzyme," Bergmann, Wiesbaden, **1910**, p. 37.

this extract was the weakest when acting on glucose (judging from the coloration). Both alcohol preparations acted much stronger in the latter case. But ethyl alcohol seemed to be even slightly in advance of the methyl alcohol preparation. This result, in consequence, furnished the standard method for the preparation of the yeast powder. The following is the way in which I proceed.

3. Method of Preparing the Yeast Powder.

The yeast, after removing from the clarifiers (see above), is allowed to drip in the brewery for about 2 days, so as to become fairly dry. About 50 lbs. may now be taken to the laboratory. The mass is at once distributed in several large glass tanks, so as to fill not more than one-fifth of their content. Then while stirring,[1] a stream of tap water is turned in, and the vessel filled to the edge. After the mass has settled, the liquid is siphoned off, and new water added while stirring. This is repeated a third time with tap water, and, after this, twice with distilled water. Great care should be exercised in this work in order to remove all the acid, and to secure a neutral reaction of the resulting powder. After the last washing, the settled yeast is poured on big porcelain funnels (Büchner form, the bottom of which consists of porous clay), and the liquid drained off by suction. The sticky compressed mass is now, in portions, placed in a large porcelain dish with absolute alcohol and stirred (best with the clean hand). The suspension is poured back on the funnel and the alcohol removed by suction. The mass is now again placed in a dish, and treated in the same way with ether. The process is then repeated, once with alcohol and once with ether, care being taken that the substance does not stay in contact with these liquids longer than necessary. After the second treatment with ether, the whitish mass is spread on filter paper until dry. The powder is to be kept in closed vessels in a warm and dry place.

4. Method of Extracting the Ferment.

The method finally adopted for getting an active ferment preparation, is the following:

A portion of the yeast powder is ground in a mortar to a fine dust. A weighed portion of this is placed in a bottle and thoroughly shaken with 5 to 10 times its weight of distilled water and about 1% of toluene. The bottle is stoppered and kept at a temperature of between 30° and 40° or at room temperature. At the end of 36 hours the bottle is transferred to the 70° incubator for another 36 hours. During this whole period of extraction, the bottle is shaken from time to time. At the end of the second 36 hours the contents of the bottle are filtered, at first through paper, and through a small porcelain funnel with clay bottom, using

[1] I usually use the fingers of one hand (well cleaned) as the most effective means of crushing all the small clods of yeast.

suction. The dark yellow, strongly opalescent filtrate contains the ferment. It may be kept in solution indefinitely, if saturated with toluene, and placed at room temperature. No precipitation will occur. Any addition of water, however, during or after the filtration process should be avoided, as a strong autodigestion of the extract may set in on dilution.

5. Attempts of Further Purification.

Although the raw extract showed a distinct activity toward both glucose and hydroquinol, the progress of the action on glucose, to judge from the coloration, seemed to be rather slow, considering the usual rapidity of fermentative accelerations. I had noticed at the beginning that the yeast gave a strong reaction with iodine, indicating great richness in glycogen. Apparently the extract contained also a good many gummy substances; and it seemed not improbable that by using some kind of a purification process, I might be able to eliminate part of these substances.

At first the method of repeatedly precipitating the ferment with alcohol and redissolving in water was tried, a method which has frequently been used, especially when working with oxidases. I followed closely the method as described by Euler[1] for the Medicago laccase. The freshly prepared ferment extract was poured into 3 times its volume of 98% alcohol, the precipitate collected on a hardened filter and dried in a vacuum over sulfuric acid. When dry, it formed a brownish, gluey mass. This was now redissolved in water, filtered,[2] and precipitated again by pouring the filtrate slowly into three times its volume of absolute aclohol. The precipitate was again collected on a filter, washed with alcohol and dried. This manipulation was repeated a third time. But even after this, the resulting substance was not a white powder, as Euler's Medicago laccase, but a very brittle, porcelain-like mass, showing that there were still a good many gummy substances contained in it. The process can be somewhat improved by using ether for washing the precipitate, in which case the operation of drying is hastened considerably, while the ether, as can be seen from the last table, has no injurious effect on the ferment.

The final purification product, if dissolved in water and filtered, is a colorless, opalescent fluid, which contains the ferment. It was with preparations of this kind that the hydroquinol experiments[3] were carried out. Later on, I observed, however, that toward glucose, these preparations showed far less activity than the original raw extracts.

I have also tried with these extracts the method employed by Oscar Loew[4] for the purification of diastase, but with no success.

[1] *Z. physik. Chem.*, 69, 190 (1909).

[2] There is always a part left which does not redissolve, upon which fact the purification process is based.

[3] See above, p. 1220.

[4] Oscar Loew, *Pflüger's Archiv.*, **27**, 203 (1882).

6. Properties of the Yeast Extract.

It has become evident in the course of this investigation that the above yeast extract contains at least two different ferments: An oxidase (active toward polyphenols) which is destroyed by boiling for several minutes. A glucolytic ferment, which is not destroyed by boiling, not even by boiling for 15 minutes in a pressure flask.

If a small portion of the extract or of the purified powder (alcohol purification) is added to a dilute solution of hydroquinol, and the mixture shaken, it turns red after a few minutes' standing. A similar action takes place with pyrogallol, the mixture turning yellow on short standing. No color change occurs with guaiacol, nor with tincture of guaiacum, not even after adding some hydrogen peroxide. In this respect, the ferment resembles Euler's Medicago laccase.[1] As we have already seen,[2] it also resembles Bertrand's Rhus laccase[1] in being accelerated in its action by manganese salts.

The indophenol test (Röhmann-Spitzer's reagent)[3] was negative; the same was also the case with Tollens' orcin reaction and Goldschmidt's test.[4] Tollens' reaction with naphthoresorcin[5] was also negative. No coloration occurred with α-naphthol, nor with tannic acid, the latter, however, giving a precipitate. Molisch's test[6] with α-naphthol as well as Neuberg's pyrrol reaction[7] was distinctly positive, even with the preparation which had been precipitated with alcohol, and redissolved, for three times. The carbohydrate group is therefore possibly in firm combination with the ferment molecule and may be regarded as one of its essential constituents.

The purified ferment in aqueous solution is slightly dextrorotatory; it does not reduce Fehling's solution. It causes no color change on being added to a solution of tyrosine, and therefore contains no tyrosinase. It has a slight action on sodium lactate, liberating an acid, without the formation of gas. The ferment gives all protein reactions; boiling for one hour does not cause any precipitation; it is precipitated by alcohol and ether.

7. Studies on the Products of Glucose Fermentation.

Numerous attempts have been made to obtain some knowledge about

[1] *Loc. cit.*

[2] See above, p. 1220.

[3] F. Röhmann und W. Spitzer, "Ueber Oxydationswirkungen tierischer Gewebe," *Ber.*, **28**, 567 (1895).

[4] G. Goldschmidt, "Eine neue Reaction auf Glucuronsäure," *Z. physiol. Chem.*, **65**, 390 (1910).

[5] *Ber.*, **41**, 1788 (1908).

[6] H. Molisch, "Zwei neue Zuckerreaktionen," *Monatsh.*, **7**, 198 (1886).

[7] C. Neuberg, "Ueber den Nachweis der Bernsteinsäure," *Z. physiol. Chem.*, **31**, 574 (1900); "Zur Kenntnis der Pyrrolreaktion," *Chem. Centr.*, **1904**, I, 1435.

the substances into which glucose is transformed under the influence of this ferment.

If glucose-ferment mixtures are filled into Schrötter's fermentation bulbs, and placed in the 70° incubator, the progress of the reaction can be observed very plainly. The liquid, if containing a fairly active extract, became distinctly acid within a few hours, and by and by the coloration took place. No gas formation was observed in any case. The iodoform reaction for alcohol was negative, but with Pasteur's droplet test, if applied in the manner as recently described by Klöcker,[1] the presence of traces of alcohol or of a similar substance, was ascertained.

The sugar is therefore mainly transformed into acids. The access of air causes the reaction to proceed slightly faster, but it is not at all necessary, as I have convinced myself in special experiments. In tightly stoppered flasks, which were filled nearly to the top, leaving only about 1 cc. of air space, the darkening and the formation of the dark residue occurred almost as readily as in other flasks that were provided only with cotton plugs. As a matter of course, toluene was added to all cultures, although the high temperature alone would nearly suppress bacterial action.

I have tried several ways of decolorizing the dark mixture with the object of rendering possible an application of the polariscope method. All these trials were unsuccessful so far. In the meanwhile I have found, however, that a complete decolorization of the mixture is possible by means of a combined precipitation with mercuric acetate and phosphotungstic acid in the manner recently recommended by Neuberg.[2]

All color reactions with this mixture as such naturally had to be unreliable, partly in direct consequence of the color, partly perhaps on account of the many substances present. Tollens' orcinol reaction alone, indicated with some certainty the presence of pentoses.

The dark mixture, if added to Fehling's solution, reduced it rapidly in the cold. As according to Neuberg[3] only a few sugar derivatives show this behavior, namely, glucuronic acid, glycerose, dioxyacetone and glycolose, I was led to believe, also, with regard to the recent results of Jolles,[4] that glucuronic acid was one of the products. As at that time I did not have at hand the reagents to make sure of this by a qualitative color reaction, I started a distillation process, following largely the sug-

[1] A. Klöcker, "Nachweis kleiner Alkoholmengen in gärenden Flüssigkeiten," *Centr. Bakteriol.*, **31**, II, 108 (1911).

[2] C. Neuberg und M. Ishida, "Die Bestimmung der Zuckerarten in Naturstoffen," *Biochem. Z.*, **37**, 142 (1911).

[3] C. Neuberg, "Die Physiologie der Pentosen und der Glucuronsäure," *Ergebn. der Physiol.*, **3**, 387 (1904).

[4] *Biochem. Z.*, **34**, 242 (1911).

gestions given by C. Tollens.[1] The mixture, after filtering and cooling, was precipitated with basic lead acetate (no ammonia being added on account of the glucose), the precipitate washed with water, and boiled directly with hydrochloric acid (sp. gr. 1.060) in the distilling apparatus. Part of the filtrate from the lead precipitate was also distilled over with hydrochloric acid, as this portion would contain any pentose that might possibly have been formed. If the distillate gave the furfurol test with aniline acetate[2] the distillation was continued, until about 450 cc. had passed over. At the end, a solution of pure phloroglucinol (Kahlbaum) in hydrochloric acid was added, and the amount of furfurol phloroglucide estimated after 16 hours' standing. In no case did the lead precipitate, if properly washed, give even a trace of this substance, while the filtrate quite regularly gave a fair amount of phloroglucide. This filtrate, of course, still contained a large amount of unaltered glucose, and it seemed not absolutely certain that the furfurol was really derived from pentose.[3] To investigate this point, I have in one case, treated the filtrate of the lead precipitate with H_2S, thus removing all the lead. The H_2S was driven out of the dark yellowish filtrate by a current of air, and the liquid fermented with yeast. The solution soon turned dark crimson, and, although perfectly clear, assumed a strong odor, similar almost to that of indol and skatol. The tests for these substances, however, gave negative results, as was to be expected. At the end of the fermentation, the reducing power of the liquid against Fehling's solution was strongly diminished, and the Cu_2O formed had a peculiar crimson color. That the reducing agent in this case was not glucose, was ascertained by preparing the osazone. A sample of the liquid was boiled in the water bath for one hour with phenylhydrazine hydrochloride and sodium acetate. It was then allowed to cool very gradually. On examining the substance under the microscope after several hours, I observed only traces of glucosazone crystals, while the liquid was filled with masses of small, brownish, globule-like crystals of an oily appearance. This is exactly the manner in which the arabinosazone is said by von Lippmann[4] to appear when first formed in the presence of foreign bodies. In fact, on prolonged standing, a solid, yellowish brown sediment of osazone crystals separated out. On examining, the long, needle-shaped crystals of a light yellow color were easily seen, although they were densely covered with a rust brown, amorphous substance. On distilling the liquid over with hydrochloric acid, and adding phloroglucinol to the distillate, an ample precipitation took

[1] C. Tollens, "Quantitative Bestimmung der Glucuronsäure im Urin mit der Furfurol HCl Destillations Methode," *Z. physiol. Chem.*, 61, 95 (1910).

[2] C. Tollens, *Loc. cit.*

[3] 100 grams glucose may yield on distillation with HCl up to 0.222 gram furfurol. Stoklasa, cf. v. Lippmann, *Loc. cit.*, p. 103.

[4] Von Lippmann, *Loc. cit.*, p. 91.

place. Hence the formation of pentose seemed sufficiently assured; while any formation of glucuronic acid had to be denied.

Quantitative studies of this pentose formation are in progress.

With one of the early digests, which contained 40% glucose, I had made an ether extraction, shaking the mixture with ether in a separating funnel, and using fresh ether several times. The first portions of this extraction were unfortunately lost. With the dark oily syrup at the bottom and ether on top, the funnel was put aside for a long time. Finally, on testing, I found that the ether had become strongly acid. Still this acid apparently was not very soluble in ether, as it had not been removed by the first 3 or 4 portions of ether. Besides, when I tried to obtain a similar extraction from an ether digest, which did not contain such a high concentration of glucose, the acid, for the most part, stayed in the aqueous solution, and the ether became only very faintly acid.

The strongly acid ether fraction, on evaporating, yielded a yellowish, oily fluid of a peculiar odor. This liquid was taken up in water and carefully distilled. The distillate was a colorless, neutral fluid of aldehydic odor, which did not give precipitates with $CaCl_2$, $FeCl_3$, or alcohol. It gave, however, a fine bluish white precipitate with $AgNO_3$, which turned grayish brown on heating. The tests for aldehyde gave negative results. This substance was not identified.

The acid residue, in the distilling flask, did not give any precipitate except with lead salts. It was optically inactive and did not reduce Fehling's solution. Tollens' orcinol reaction, as well as the naphthoresorcinol reaction and Goldschmidt's test[1] was negative. Molisch's test with α-naphthol was positive. On evaporating in a vacuum over sulfuric acid, the acid liquid did not crystallize, but yielded a dark syrup.

If to this syrup a concentrated solution of potassium acetate was added, crystals were formed almost immediately. These colorless granular crystals could also be obtained in dilute solution in a test tube, if, after adding potassium acetate the walls of the test tube were rubbed with a glass rod.

I obtained just enough of these crystals to make a few melting point determinations. The substance did not melt until above 300°. I prepared the acid potassium salts of saccharic acid and of tartaric acid in order to compare their respective melting points with the one obtained. The melting point (uncorrected) of the saccharate was found to be 186.5°, that of the tartrate about 270°, while the melting point of the acid potassium oxalate, which salt is rather insoluble too, was found to lie above 350°. I presume that the crystals which I had obtained on addition of potassium acetate, were the result of some sort of secondary transforma-

[1] *Loc. cit.*

tion of the original acid into oxalic acid. Primarily, the ether soluble extract did certainly not contain oxalic acid, as no crystals were obtained on evaporation and as the calcium precipitate was readily soluble in acetic acid (see table below).

If the free acid was just neutralized with ammonia, several characteristic reactions could be obtained, the results of which I have arranged in the following table:

Free acid.	Reagent.	Ammonia salt.
No precipitate....	$AgNO_3$	*Yellow* precipitate; soluble in the cold in NH_3. On heating, slight reduction.
No precipitate....	$FeCl_3$	*White*, gelatinous precipitate, which dissolves slowly in 0.1 *N* HCl, more rapidly in strong HCl. Also dissolves in excess of NH_3 to a dark yellow solution (of $FeCl_3$ color).
No precipitate....	$CaCl_2$	*Thick white* precipitate. Insoluble when heated, and in cold NaOH. Dissolves in 0.1 *N* HCl, and also on adding a few drops of glacial acetic acid.
No precipitate....	Alcohol	*White* precipitate insoluble in ether.

On evaporating the solution of the ammonia salt, a grayish, crystallin mass of caramel like odor was obtained. Under the microscope, it appeared to consist largely of rhombic plates. No exact melting point could be obtained with this residue. Probably it was not a single compound, but a mixture of several salts. Above 180° the substance in the capillary tube turned brownish, but it did not melt even at a temperature of 250°.

A portion of the dark residue of the ether extraction which I mentioned above, was diluted with water and carefully distilled, the receiver being cooled by ice-water. The colorless, clear distillate had a strong odor and was distinctly acid to litmus paper. It gave the following reactions:

(*a*) It reduced ammoniacal silver solution.

(*b*) After acidifying a small portion with H_2SO_4, it was shaken in a separating funnel with pure chloroform. The chloroform was drawn off; and on adding to it 0.5 cc. of Nessler's reagent (freshly prepared), a strong yellow coloration of the latter occurred at once.

(*c*) A crystal of resorcinol was dissolved in a few drops of the liquid, and the mixture allowed to run down slowly along the side of a test tube containing concentrated sulfuric acid. At the zone of contact a bright, orange red color developed very soon, which gradually turned darker red.

(*d*) The distillate gave Schryver's[1] formaldehyde test very plainly. The reaction was carried out in the following way: To 5 cc. of the liquid

[1] S. B. Schryver, "The Photochemical Formation of Formaldehyde in Green Plants," *Proc. Roy. Soc.*, **82**, 226 (1910).

were added 1 cc. of a 1% solution of phenylhydrazine hydrochloride (freshly prepared and filtered), 0.5 cc. of a freshly prepared 5% solution of potassium ferricyanide, and 2.5 cc. of strong HCl (sp. gr. 1.19).

The mixture showed a distinct, though not very intense red coloration. It was now shaken with pure ether in a small separating funnel, and the lower layer drawn off. To the ethereal extraction were then added 1–2 cc. of strong hydrochloric acid. The latter assumed almost immediately a very intense red color, indicating the presence of formaldehyde.

In addition to this substance, the distillate, as already mentioned, contained also a volatil acid, possibly formic acid, which, however, I have not yet identified.

It is apparently of some interest that the products resulting from the action of this yeast glucase, namely, acids, formaldehyde and pentose, are identical with those recently obtained by Walther Löb in his studies on the electrolysis of grape sugar.[1]

Conclusions.

1. A ferment has been described which occurs in the California steam beer yeast under certain conditions, and which has the property of accelerating the decomposition of glucose at an elevated temperature.

2. This new ferment is not identical with zymase. It is very active at a temperature of 70°. It causes no gas formation, and yields no alcohol.

3. Its action on glucose at 70° manifests itself by a rapid darkening of the mixture, a strongly acid reaction, a gradual formation of a carbon like, solid deposit, and the development of an odor similar to that of caramel.

4. The ferment may be extracted from a yeast powder (Dauerhefe) which is best obtained by treating the cells with ethyl alcohol.

5. From the watery extract the yeast glucase may be obtained and purified by repeated precipitation with alcohol; but this process always involves a weakening of the ferment.

6. Yeast glucase is very stable in aqueous solution, if kept at room temperature under sterile conditions. Boiling does not destroy its activity.

7. Yeast glucase shows activity in neutral or acid solution against glucose, polyphenols and lactates. The preparation does not contain tyrosinase, nor does it act as a peroxidase against glucose.

8. The ferment solution gives a strong pyrrol reaction (Neuberg).

9. Yeast glucase shows some relationship to the oxidases, but with regard to its main function it is to be classed together with zymase in a group which stands separately from the oxidases and the hydrolytic

[1] Walther Löb, "Zur Kenntnis der Zuckerspaltungen III–VII," *Biochem. Z.*, **17**, 132, 343 (1908); *Ibid.*, **20**, 516 (1909); *Ibid.*, **22**, 103 (1909); *Ibid.*, **23**, 10 (1909); *Z. Elektrochem.*, 16, 1 (1910).

ferments, and to which Euler has applied the term "Gärungsenzyme" (see footnote on page 1220).

10. The transformation products of glucose resulting from the action of this ferment are largely acids, none of which has so far been definitely identified. However, among the cleavage products of the sugar the presence of pentose and of formaldehyde could be ascertained.

I wish to express my thanks to Dr. T. Brailsford Robertson for his valuable counsel and continuous interest in this investigation, and also to Mr. C. B. Bennett for kind suggestions in carrying out some of the chemical work.

SOME ANALYSES OF URIN COMPOSITS.[1]

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Several years ago, in the course of some metabolism experiments carried out in this laboratory, a large number of urin examinations were made daily with special reference to the nitrogen factors and the sulfur. At the same time composits were saved for a more detailed examination regarding certain constituents, and especially for the determination of the inorganic bases.

Much of the investigation was concerned with a study of the effects of certain diets on the composition of the urin, but the composites in question were collected during the preliminary or fore periods of the studies, and represent, therefore, urins corresponding to the usual or ordinary diets. The length of the fore periods from which urin could be saved from the same individuals amounted to a month or more, as will be shown in the detailed statements below.

The men whose urins were the objects of the investigation lived under the same general conditions and consumed a diet qualitatively the same in all cases. There were appreciable quantitative variations, however, in some of the factors of the food, which variations will be shown in the tables below, along with certain other data regarding the men, which may be considered as preliminary to the presentation of the special details of the urin analyses. The fecal excretion for two important factors is given also.

While the inorganic constituents of the food have not been directly determined, it will be recognized that the relative distribution of these bodies might be approximately found by calculation from the relative nitrogen distribution.

[1] Paper presented at the annual meeting of the American Chemical Society, Washington, December 27, 1911.