Biochemical Studies on Beer

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Among numerous drinks, beer is one of the favorite beverages for the summer season and is quite commonly drunk in large quantities. This popularity is probably due to its low content of alcohol and the fact that like other refreshing drinks, it contains carbonic acid.

A few glasses of refreshing beverage such as lemonade which contains carbonic acid, awaken the feeling of a full stomach and also an unpleasant belching, but beer is free from such disagreeable consequence. This is the reason why beer may be indulged in so freely.

Since the addition of alcohol to lemonade in the proportion in which it is contained in beer does not produce the qualities we have noted, the characteristics of beer cannot be attributed to the carbonic acid contained but must be regarded as due to its foam-retaining capacity.

Foam-retaining capacity, called by brewers[1] "Schaumhaltigkeit" or "Schaumbegünstigkeit", is one of the most important properties of beer and is employed as the standard for deciding the relative merits of beer.

From what physical or chemical qualities may this property originate? Helm and Rechard[2] ascribed it to the content of carbonic acid in beer and R. Hopkins[3] pointed out that this property is intimately related to surface tension and also the content of hydrophyllic colloid in beer. Although the real solubility[4] of carbon dioxide in beer is lower than in pure water and in dilute alcohol of the same concentration as beer, beer of superior quality always dissolves the gas in the degree of extreme supersaturation,[5] showing a value of 0.35~0.5% against that of true solubility 0.16~0.17% at 15°C; and the surface tension of excellent beer indicates 40~45 dyne/cm,[6] while pure water shows 73 dyne/cm. Therefore the viscosity of the former is about twice as large as the latter.

Since, besides water, alcohol, and carbon dioxide, beer contains numerous kinds of carbohydrates, proteins, other nitrogen-containing matter and inorganic salts, certain constituents or systems formed by

them may be supposed to be closely concerned with its foam-retaining capacity.

K. Ueno[7] attributed these characteristics to the molecular weight and also the amount of dextrin contained in beer and he assumed that this substance controls the viscosity of the beverage and consequently its foam-retaining capacity. H. Lüers[8] argued that the substances which affect surface tension of beer might be such constituents as nitrogen-containing substances, hop-tannins and volatile organic acids.

But a comparison of the constituents of some representative brewages (*v.* Table I) shows that they may differ slightly in the content of dextrin, proteins and volatile organic acids. The above-mentioned explanations of the characteristics of beer seem therefore less than satisfactory. Moreover, according to A. Findlay's investigation,[9] hydrophyllic colloidal solutions of dextrin and albuminoid indicate a lower solubility of carbonic acid than beer and this may be contrary to the opinion of Ueno and Lüers.

Table I

	Beer	Japanese Saké	Wine
Sp. gr.	1.01	0.99	1.00
Alcohol (Vol. %)	3 8	17.6	10.0
Dry substance	4.26	2.92	2.34
Sugar	1.08 (as maltose)	0.50 (as maltose)	2.18 (as glucose)
Dextrin	2.10	0.36	—
Volatile acids	0.02	0.15	0.13
Non-volatile acids	?	0.03	0.16
Crude protein	0.35	0.96	trace
Total ash	0.14	0.05	0.25

From the biochemical view point, the writers disposed to attribute these differences to the raw materials from which the brewages have been made and also to the process of brewing, namely, the employment of malt was assumed to be the most significant factor in causing the characteristics now under consideration.

Now, in the germination of barley seeds, the various enzymes contained in them become remarkably active, and consequently vigorous metabolic changes[10] may take place and the substances reserved in the grains begin gradually to decompose. The major parts of starch are hydrolyzed into maltose and glucose and further burnt down into water

and carbon dioxide setting free enormous amounts of energy for the growth[11] and maintenance of life, while at the samt time, another portion is converted into carbohydrates such as cellulose and hemicellulose[12] which construct the cellular wall.

The chemical difference between the grain of barley[13] and the brewing malt[14] produced from the former, may be seen in Table II.

The chemical changes which occur in the germination of seeds are presumed to resemble closely those taking place in the growing tips[15] of plants where parts of the polysaccharides are oxidized by action of respiratory enzymes into uronide carbohydrates such as mucilages, gummy substances and pectic substances which contain uronic acid in their molecules, after which some of these products in turn convert into pentosan[16] by slipping off carbon dioxide and the others change into various non-volatile organic oxy-acids.

Table II

	Grain of barley	Brewing malt
Starch	63%	58%
Reducing sugar	2	4
Sucrose	—	5
Soluble pentosan Insoluble pentosan	9	1 9
Cellulose	4.8	6
Crude protein	10	10
Leucosin Edestin Hordein Glutelin	0.4 3.1 3.6 2.9	2.5 5 2 3
Fat	2.8	2.5
Total ash	2.9	2.5

Therefore, it may be assumed that in the brewing process of beer, most parts of fermentable carbohydrates formed by hydrolysis of starch in the malting process have been converted into alcohol by fermentation, but non-fermentable uronide carbohydrates remain in the beer. And from the fact that the aqueous solution of uronide carbohydrates such as mucilages and pectic substances always shows a lower value in surface tension and reversely a higher degree of viscosity than pure water does, the writers considered that the above-mentioned characteristics of beer ought to be attributed to its high content of uronide carbohydrates.

In lager beer, especially in bottled beer, a gradual denaturation

occurs during a few months' storage in summer. The original yellow colour turns slowly to reddish yellow with the diffusion of a disagreeable odour, (the so-called " Sonnegeruch ") and in the worst cases turbidity arises. These properties may be also regarded as one of the characteristics of beer in which it differs from other brewages and it has distressed the brewers for a long time.

Three different opinions have been set forth to explain the colouring of beer. The first[17] is that the cause of the formation of colouring substances may be due to the secondary chemical reactions brought about between maltol and furfurol which may be produced from the husk of barley grain during the kilning process.

The second[18] opinion attribute the colour to the formation of phlobaphane by oxidation of tannins contained in the raw materials, especially in the hops. This opinion has been derived from the fact that the tone of the colour turns into marked reddish brown with the addition of a large amount of hops and especially when the hopped wort is boiled in contact with the air.

The last is an opinion referring to the formation of beer melanoidine[19] and is considered by the brewers[20] a most noteworthy one. Melanoidine is the general name given to the colouring matters formed by the reaction detween reducing sugars and amino acids at temperatures of 110°~150°C.[21] During the kilning process, an analogous chemical reaction may take place between the hydrolyzed products of malt carbohydrates and proteins according as is argued from the observation of a gradual decrease of amino-nitrogen content[22] in the malts and also from the vigorous evolvement of carbon dioxide during this process.

But even the structure of melanoidine synthesized from alanin and arabinose has not yet been made clear and is presumed to be a heterocyclic compound or a mixture of such compounds containing pyrol or imidazol rings[24] in the molecules. Accordingly the colouring substances in beer, which are presumed to be melanoidine, are of course uncertain.

Thus, there is as yet no generally accepted opinion as to the colouring substances contained originally in beer, much less the discolouring matters formed by its denaturation. H. Leberle[25] considered the discolouration to be due to chemical changes, similar to those brought about during the kilning process occurring under the high pressure of carbon dioxide in the bottle; and H. Lüers[26] attributed it to the slow changes of the decomposition products of carbohydrates and proteins as well as some of the labile fermented products. On the other hand,

E. Emslander[27] presumed that discolouration might occur on account of the chemical changes in tannic substances adsorbed in albumin.

Recently, C. Enders and A. Lother[28] analyzed the colouring substances in beer by the chromatographic method.[29] They found that the yellowish colouring matter adsorbed in the lower layer of calcium carbonate might be presumed to be pigments such as flavine and xanthophyll; and those adsorbed in the middle layer they attributed to melanoidine; while the deep red coloured matter adsorbed in the upper layer was designated phlobaphane. They concluded that the last one might be produced by autoxidation of tannic substances and that it caused the worst colour in beer.

However the writers differ completely with these preceding investigators in regard to formation of the colouring matters in beer.

The discolouration of beer is promoted remarkably by various external influences such as heating to too high temperature at pasteurization, the employment of bottles showing strong alkali reaction and especially irradiating with sunshine[30] in the bottle.

These facts lead to the presumption that by action of light or other means certain constituents of beer may be activated, with discolouration following. Since, as was mentioned above, the system constructed by the chemical constituents of beer may be similar to those of the living cell saps, denaturation brought about in beer may be referred to changes analogous to those of fruit-juice or of sea-weed when exposed to the sunshine and the air.

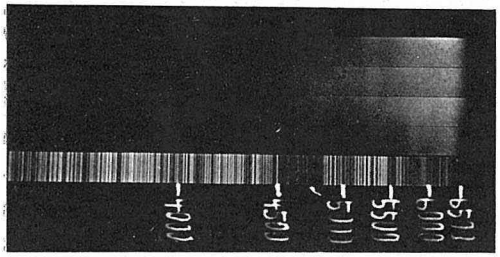
The writers' hypothesis was partially confirmed by the following observation: during the storage of beer for half a year, the changes occurring in its constituents, head formation, foam-retaining capacity and degree of discolouration were precisely investigated with the results shown in Table III.

As may be seen in the table, in spite of discolouration and changes in taste and in foam-retaining capaity observed after three months' storage and an added light turbidity at the end of six months' storage, only slight changes are recognized in the results of the analysis of its chemical constituents by the general analytical method for brewages. It is to be noted that in the amount of carbonic acid evolved at 25° and 100°C marked differences were evident after a few months' storage. These facts indicate that the marked changes may take place in some chemical constituents retaining carbonic acid, which the general analytical method was inadequate to measure.

Table III.

	Aug.	Sept.	Oct.	Nov.	Dec.	Feb.
Balling	2.30	2.32	2.32	2.32	2.32	2.38
Sp. gr.	1.0090	1.0092	1.0086	1.0089	1.0090	1.0099
Dry substance	3.95	3.94	3.93	3.91	3.92	3.93
Total acids	16.30	16.50	16.65	16.40	16.40	16.25
Volatile acids	2.99	2.82	2.56	3.04	3.44	2.84
Carbonic acid	2.464	2.578	2.562	2.759	—	2.759
Reducing sugar	1.1545	1.1075	1.1178	1.1455	1.1730	1.1640
Dextrin	1.7870	1.8768	1.8113	1.8510	1.8300	1.8530
Total N	0.0494	0.0412	0.0476	0.0463	0.0473	0.0478
Amino N	0.0043	0.0042	0.0048	0.0048	0.0049	0.0051
pH	—	4.18	4.28	4.26	4.25	4.28
Colour (c.c.N/10) Iodine	7.14	7.14	8.33	8.47	8.33	7.69
Iodine value	9.79	9.92	9.48	9.36	9.41	10.24
Foam retaining capacity (time)	12′53″	12′38″	12′06″	10′21″	10′09″	9′18″
Foam mass	140	142	130	100	90	90
Remark	no change	no change	changes in taste and colour	changes in taste and colour	changes in taste and colour	light turbidity
CO_2 evolved at 25°C for 3 hrs.	0.168%	0.173%	0.148%	0.208%	—	0.115%
CO_2 evolved at 100°C for 3 hrs.	0.212	0.233	0.256	0.246	—	0.316
Sum	0.380	0.406	0.404	0.454	—	0.431

Fig. 1.



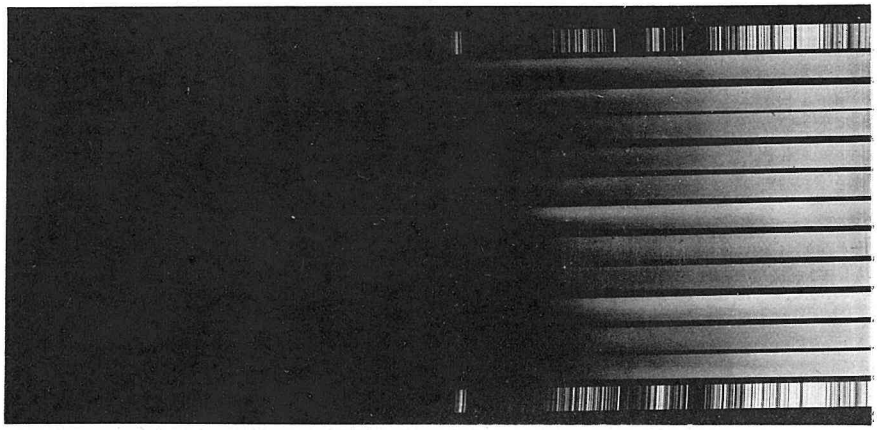
The absorption spectrum of beer.
(visible region)

To search for these constituents, spectroscopical investigation was begun. As shown in Figs. I and II, beer showed four distinct absorption bands at the frequency of 6000 Å, 5400 Å, 3400 Å and 2900~2700 Å. The first three bands are identical with those of respiratory enzymes such as cytochrome and the yellow enzyme, while the last may be considered as the characteristic band shown by carbonyl compounds. Tentatively ascribing the last band to a carbonyl group in uronide carbohydrates, the absorption spectrums of mannuronic acid, orange peel pectin, and achro-

dextrin were examined and the results are shown in Figs. III, IV and V. As may be seen, the writers' presumption is partly proved by the fact that the compounds which contain uronic acid rest in the molecule showing a band similar to that of beer but no sign of the characteristic band of achrodextrin.

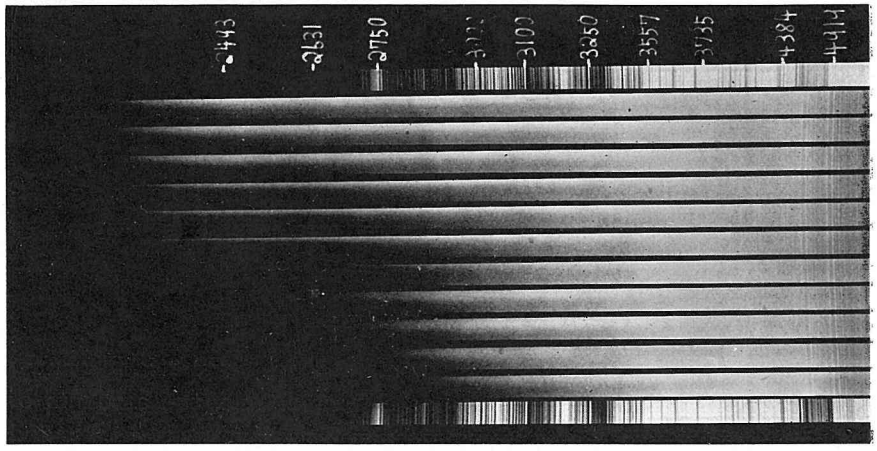
Thus the writers came the conclusion that from the biochemical point of view the characteristics of beer such as foam-retaining capacity may be attributed to the speciality of the system of its chemical constituents, and that the most important substances may be uronide

Fig. II.



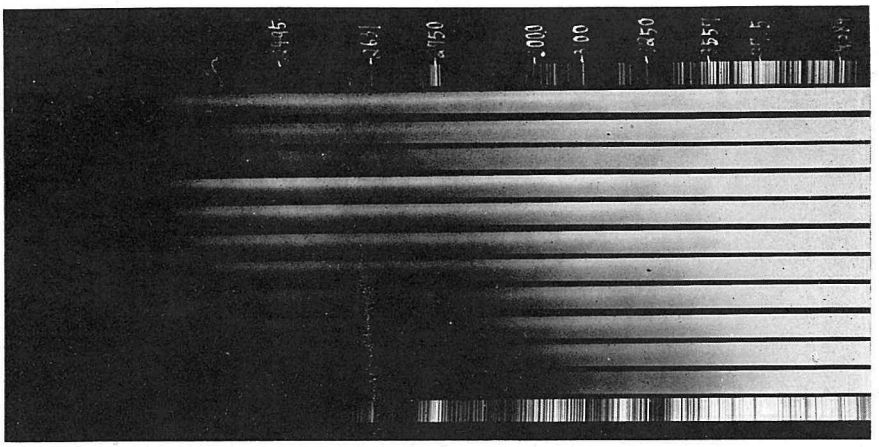
The absorption spectrum of beer. (ultra-violet region)

Fig. III.



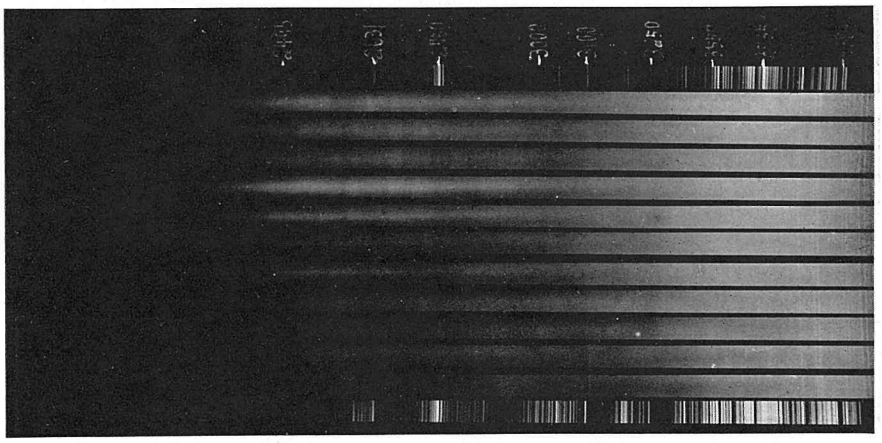
Mannuronic acid.

Fig. IV.



Orange peel pectin.

Fig. V.



Achrodextrin.

carbohydrates and the respiratory enzymes which promote the oxidation of the former. Therefore next, carbohydrates and the respiratory enzymes contained in beer were investigated.

(A) Carbohydrates contained in beer

With regard to carbohydrates contained in beer, relatively few investigations have been made and as a general rule, the amounts of reducing sugars before and after the hydrolysis have been designated as maltose and dextrin respectively.[31]

C. Lintner and G. Düll[32] confirmed the presence of isomaltose which may be formed from starch by action of diastase and may remain in beer because of being less fermentable than maltose. O. Jung[33] analyzed the constituents of München beer with the addition of pentosan to maltose and dextrin, as shown in Table IV. H. Fink[34] observed that though a small amount of pectic substances or pentosans was taken into the wort from malt[34] and hop[35] during the boiling, major parts of them were decomposed in the fermenting process. But no one has noticed the presence of uronide-carbohydrates in beer.

Table IV.

Maltose	11.67%
Pentosan	6.79
Dextrin	70.26
Crude protein	7.75
Total ash	3.57

For the purpose of the confirming the existence of uronide carbohydrates in beer, after releasing carbon dioxide as much as possible by vigorous agitation, fresh beer was evaporated into a small volume under diminished pressure. The concentrated beer thus obtained was treated according to the process indicated in Fig. VI. Of the portions soluble and insoluble in 80% methanol, dry substances, total nitrogen, total ash, phosphoric acid, reducing sugars and non-reducing sugars were estimated. The results of the translocation of these constituents are shown in Table V.

Fig. VI.

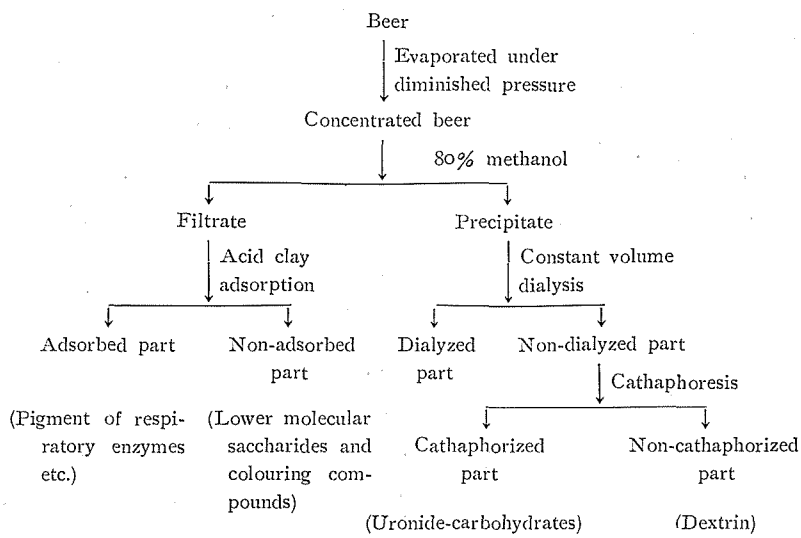


Table V.

	Concentrated beer	Insoluble part in 80% methanol	Soluble part in 80% methanol
Dry substance (% for dry sub. of beer)		55%	45%
Reducing sugars	8.68%	7.11	10.16
Non-reducing sugars	39.60	57.70	22.28
Total nitrogen	1.25	0.53	1.92
Crude protein	7.83	3.37	11.50
Total ash	3.64	3.22	4.02
Phosphoric acid	1.86	2.29	1.54

Fig. VII.

80% methanol insoluble part.

I. Substances insoluble in 80% methanol— Uronide carbohydrates and Dextrins

Substances insoluble in 80% methanol was a pale brown-coloured hygroscopic powder, easily dissolved in water to make a very viscous solution which continues to retain the foam for a long time after strong shaking, while achrodextrin of similar solubility in water, shows no such properties.

In order to carry on further investigation of these substances, the aqueous solution was dialyzed in the vesical bag against a constant volume of water for a long time, occasionally changing the outside solution to fresh water, and the results of the translocation of the

components were also investigated with the results shown in the following table.

As seen in the table, the non-dialyzed part still possesses the reducing power which may depend on polysaccharide since it gives no sign of the formation of osazone.

Table VI.

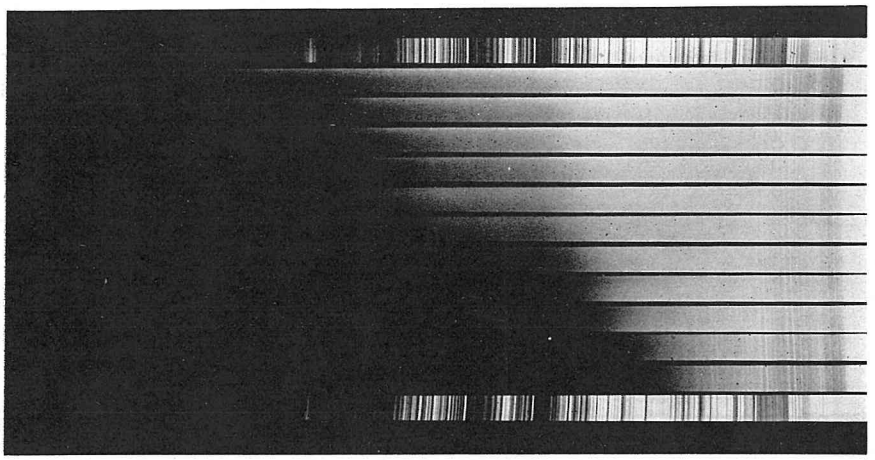
	Non-dialyzed part	Dialyzed part
Dry substance	47%	8%
Reducing sugars	6.72	11.10
Non-reducing sugars	65.50	58.70
Total-nitrogen	0.65	1.44
Crude protein	3.44	9.11
Total ash	1.82	—
Phosphoric acid	1.30	6.44
Iodine test	(−)	?
pH (8% aqueous solution)	5.8	
Fermenting test (by Saccharomyces cerevisiae)	(−)	(−)

The aqueous solution of the non-dialyzed part showed a weak acidic reaction against litmus paper and with the addition of basic lead acetate solution or baryta precipitated the corresponding salts, from which it was again separated by dilute acid. These facts show the intermixing of compounds of acidic character in the non-dialyzed part.

Now were this acidic nature due to the existence of uronic acid, an equal number of molecules of carbon dioxide and furfurol[36] should be produced by distillation with concentrated hydrochloric acid. The result of distillation gave 1.8% of carbon dioxide and 2.8% of furfurol for the non-dialyzed substance which meant 1 mol. of furfurol against 1.08 mol. of carbon dioxide. Consequently, the presence of uronic acid radical in beer was partially proved. The absorption spectrum of the aqueous solution proved to be also favourable to the assumption.

For the purpose of isolating uronide carbohydrates, the aqueous solution of the non-dialyzed part was cathaphorized by means of Pauli's electro-dialyser, and a carbohydrate of acidic nature was isolated from anodic solution, which proved to be uronide carbohydrate since it showed intensive naphthoresorcine reaction and also by the elementary analysis; and this is confirmed by its ultraviolet absorption spectrum as shown in Fig. VIII.

Fig. VIII.



Cathaphorized part.

Table VII.

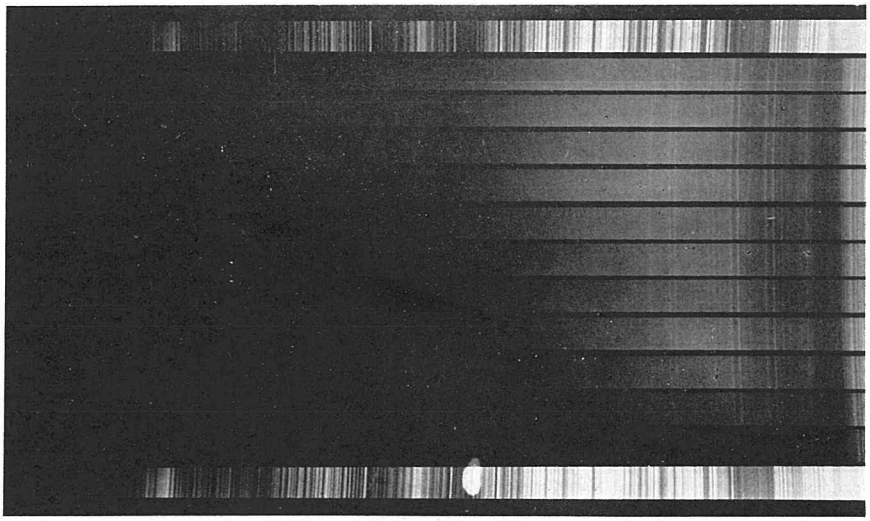
	Cathaphorized part	Non-cathaphorized part
Dry substance	27%	20%
Reducing sugars	5.23	5.49
Non-reducing sugars	66.10	71.60
Total nitrogen	0.60	0.70
Crude protein	3.81	4.36
Total ash	1.54	3.27
pH (8% aqueous solution)	4.2	6.5
Fermenting test	—	—
Naphthoresorcine reaction	Intensive violet	weak

	C%	H%	O% (from difference)
Cathaphorized part	43.0	6.9	50.9
Non-cathaphorized part	43.7	6.9	49.4
$(C_6H_{10}O_5)_n$	44.5	6.2	49.4

II. Substances soluble in 80% methanol—Achrodextrin, Disaccharides and the Colouring Compounds

The filtrate from an 80% methanol precipitation was evaporated to expel the solvent and was shaken with acid clay several times to adsorb the respiratory enzymes. Then the residual solution was again concentrated to a small volume under reduced pressure and the precipitate (A) was obtained by means of a 90% alcoholic solution. The filtrate

Fig. IX.



Non cathaphorized part.

separated from the precipitate (A) was evaporated to a small volume and absolute alcohol was added to make it up to 95% alcoholic solution, on which the precipitate (B) was formed and the alcoholic filtrate was evaporated to a thick syrup (C). Thus the soluble part in 80% methanol was separated approximately into three crops by fractional precipitation.

Of these crops, the rotatory power and reducing power of the aqueous solution before and after hydrolysis were determined on the one hand, and on the other, the formation of osazone or phenyl hydrazide with phenyl hydrazine before and after hydrolysis, and the fermenting test were studied. The results are shown in Table VIII.

Table VIII.

Soluble substances in 80% methanol

Dry substance	43%
Reducing sugars	14.4
Non-reducing sugars	23.3
Total nitrogen	1.1
Crude protein	6.5
Total ash	4.4
Phosphoric acid	1.4
Relative acidity	34.1

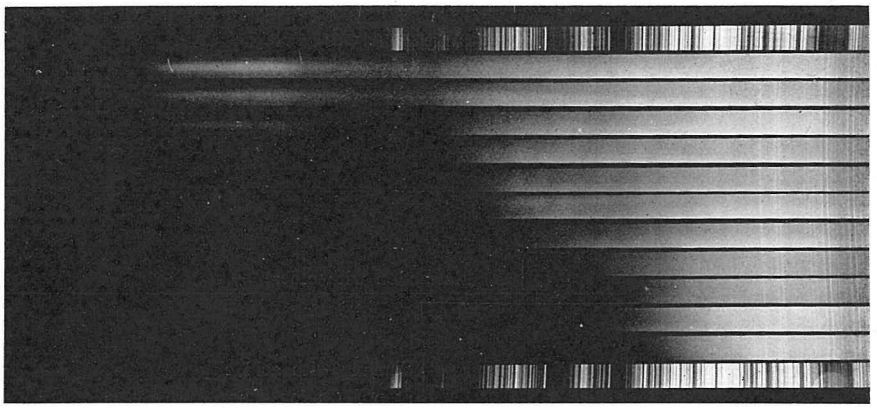
	(A) Insoluble in 90% C_2H_5OH	(B) Insoluble in 95% C_2H_5OH	(C) Soluble in 95% C_2H_5OH
Dry substance	20%	9%	14%
Colour of aq. solution	pale brown	pale brown	thick reddish brown
Reducing sugars	9.2%	24.9%	13.8%
Non-reducing sugars	62.6	36.7	—
$[\alpha]_D$	+157.6°	+98.9°	+37.2°
Fermenting test	(−)	(−)	(−)
Formation of osazone or hydrazide	(−)	(−)	Phenyl hydrazide M. p.=130°-131°. N%=12.5
Formation of osazone after hydrolysis	Glucosazone M. p.=205°-206°	Glucosazone M. p.=204°-205°	

As seen in the table, the first precipitate (A) seems to consist of achrodextrin, and the second (B) was ascertained from their properties to consist of disaccharide, probably isomaltose. The residue (C) was a syrupy substance of thick reddish brown colour, insoluble in ether, and its aqueous solution indicated a strong acidic character. From the facts that it shows comparatively small content of nitrogen and gives no sign of the formation of glucosazone but does form phenyl hydrazide (phenyl hydrazide of this substance N%=12.5, glucosazone N%=15.6, maltosazone N%=10.8) and also from the results of the examination of the following characteristic reactions, this substance was supposed probably to consist of unsaturated polyoxy ketonic acid and was presumed to be intimately concerned in the discolouration of beer owing to its giving a deep colouration by the action of oxidising agents.

(1) The formation of phenylhydrazide.
(2) Brownish colouration with ferric chloride solution.
(3) Slow reduction of ammoniacal silver oxide solution with the formation of silver mirror.
(4) Negative recolouration of the Schiff's reagent.
(5) Wine red colouration with sodium nitroprusside and sodium hydroxide solution.
(6) Negative iodoform reaction.
(7) Negative colouration by naphthoresorcine reaction.
(8) Immediate decolouration of bromine water and the formation of white precipitate.
(9) Change into thick reddish brown by oxidation with dilute potassium permanganate solution.

(10) Manifestation of the characteristic band of carbonyl group (2800Å) in the ultraviolet absorption spectrum as shown in Fig. X.

Fig. X.



The colouring matter.

(B) Respiratory enzymes in beer

It is well known that as plant seeds respire extraordinarily at germination, the content of respiratory enzymes in the embryo of the germinating seeds always predominates over resting ones. With regard to respiratory enzymes in barley malts, the presence of dehydrogenase,[37] cytochrome[38] and the yellow enzyme[39] has been reported. During the malting process of beer brewing, rapid increase in these enzymes appears after the 2nd day of germination and they reach the maximum amount after the 5th or 8th day with of 8 times the amount of oxidase,[40] 20 times the yellow enzyme[41] and twice the dehydrogenase[42] respectively. As beer is brewed generally from malt of 8 days old, the respiratory enzymes are supposed to come up to each of these maximum contents.

On the other hand, there is also a large amount of these enzymes contained in the brewing yeast cell[43] and they are exuded into the autolysate which contains about 32 mg. of cytochrome[44] and 30 mg. of the yellow enzyme[45] for 1 kg. of dry yeast.

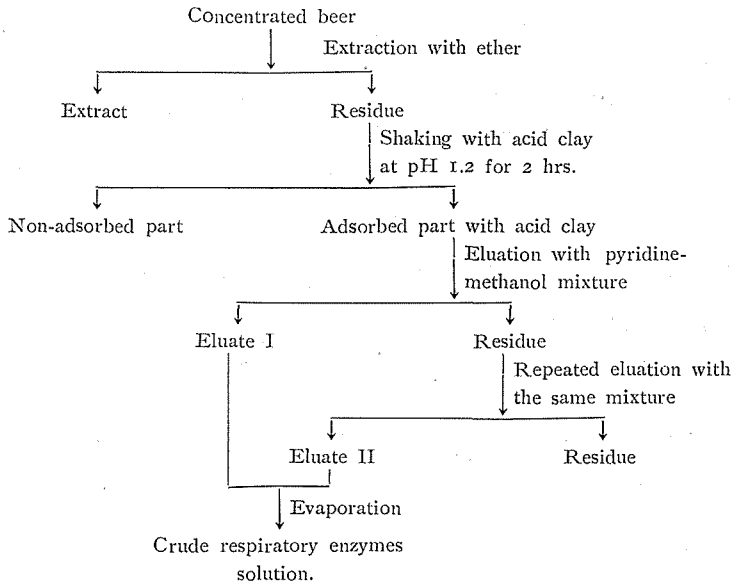
Consequently, notwithstanding the fact that some of enzymes in the malt may be destroyed during the mashing and kilning processes, yet the larger number are probably extracted into the wort and moreover, not a few of the enzymes are supposed to be exuded into it by the unavoidable autolysis of yeast in the fermenting process.

In investigations on the respiratory enzymes in beer, only H.

Fisher and H. Hilmer[46] proved spectroscopically the existence of porphyrin compound, the pigment constituents of cytochromes, and R. Kuhn, J. Wagner-Jauregg and H. Kaltschmidt[47] estimated the content of flavine, the pigment constituent of the yellow enzyme by the fluorescence method. But no one has yet undertaken the confirmation and the isolation of respiratory enzymes of beer.

As mentioned above, the presence of cytochrome and flavine was suggested by the absorption spectrum of fresh beer, and the writers intended to isolate them from beer by the following process:

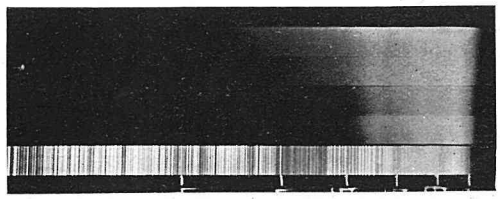
Fig. XI.



The concentrated beer was extracted with ether to separate fat and the resinous matter and was shaken for two hours with acid clay after adjusting its pH at 1.2 with hydrochloric acid. The enzyme adsorbed in acid clay were eluated with pyridine-methanol mixture (pyridine 1, methanol 2 and water 3) and the eluate was evaporated into a small volume under diminished pressure. From this solution, after adjusting its pH at 5.2 which is the isoelectric point of flavine, cytochrome was adsorbed on the fine powder of beaten cellophane. Cytochrome thus obtained is an orange coloured powder and shows two characteristic bands at frequencies of 6000 Å and 5400 Å as shown in Fig. XII.

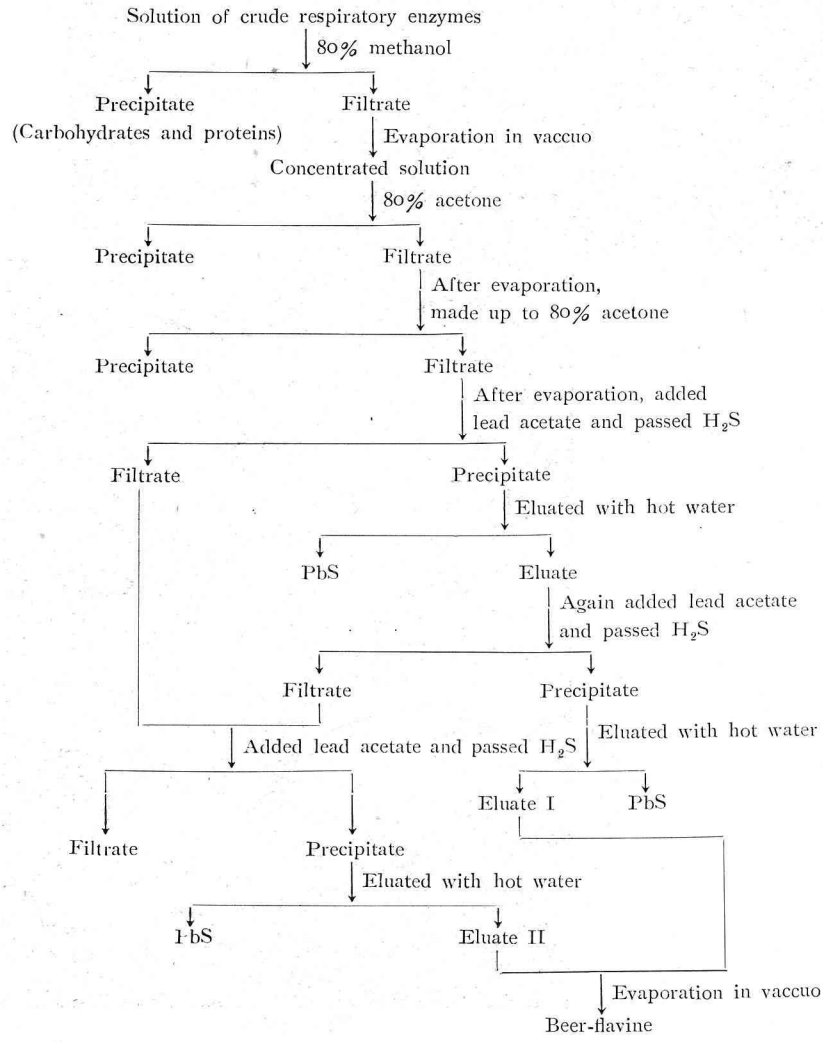
The yellow enzyme in beer was decomposed by treating with an 80% methanol into flavine pigment and colloid trager,[48] the latter was filtered off and the filtrate was treated by the process shown in Fig. XIII.

Fig. XII.



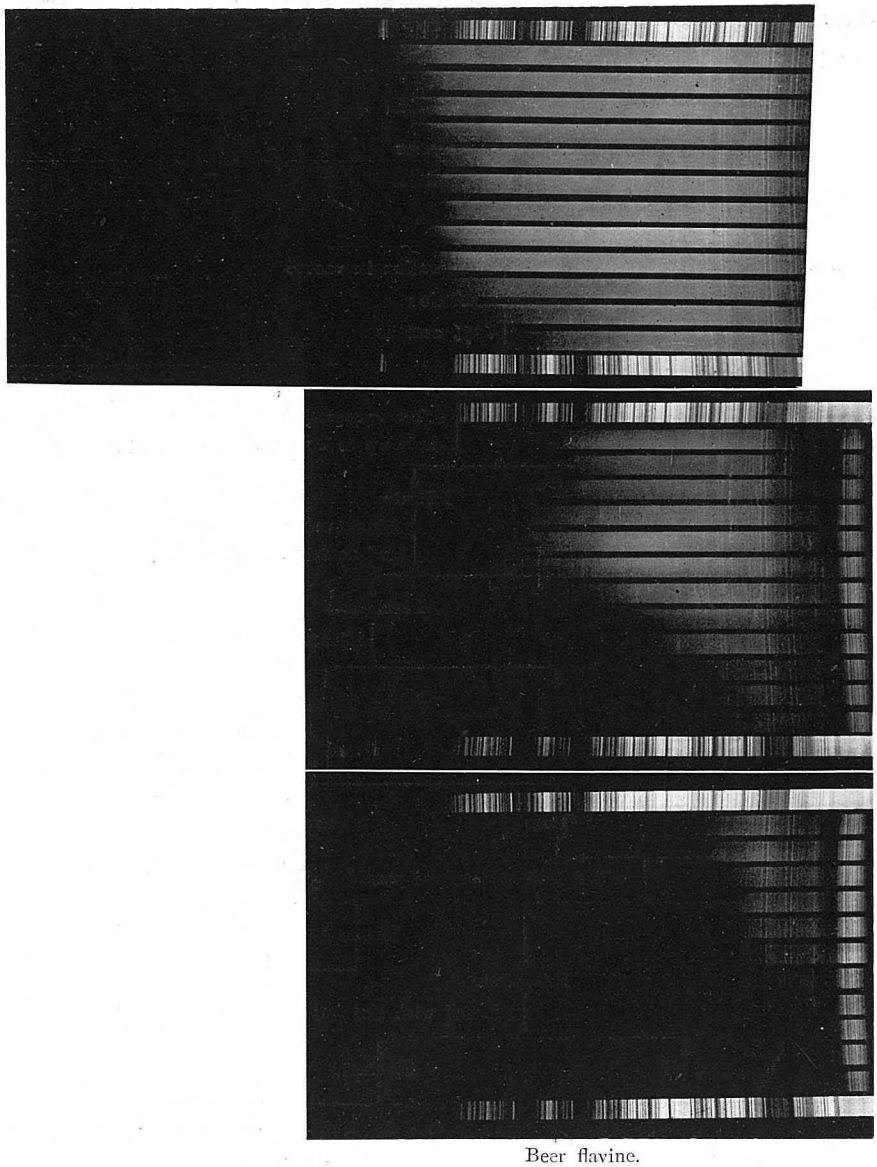
Cytochrome.

Fig. XIII.



By the repetition of precipitation with acetone[49] and of adsorption with lead sulphide, finally the lemon yellow coloured solution with intensive greenish fluorescence was obtained, which showed a characteristic absorption spectrum closely resembling that of the lactoflavine as shown in Fig. XIV.

Fig. XIV.



Beer flavine.

In these separating processes of beer flavine, the amounts of dry substance, the total nitrogen, the total ash, and phosphoric acid were estimated at every step of the treatment with the results shown in Table IX, and the final eluate from lead sulphide was found to contain almost pure flavine. The aqueous solution thus obtained was evaporated to a small volume and left to stand for a long time in the desiccator until the crystal of flavine was separated out. From 60 L. of fresh beer, 500 mg. of raw flavine was obtained, which gave 15.4 mg. of pure flavine, melting at 269°~270°, after recrystallisation from dilute acetic acid solution. The content of pure flavine is estimated at 0.26 mg. per 1 liter of beer, which is almost the same as that of German Pilsner[50] estimated by the fluorescence method.

Table IX.

	per 1 liter of beer				
	Dry sub.	Total-N	Ash	Phosphoric acid	N%
Concentrated beer	43.11 g	0.438 g	3.184 g	1.133 g	1.01
Soluble part in 80% methanol	19.25	0.368	0.774	0.296	1.90
Non-adsorbed part with acid clay	16.16	0.166	0.684	0.212	1.03
Eluate with pyridine-methanol mixture	0.158	0.020	0.006	0.001	12.4
Insoluble part in 80% acetone	0.025	0.002	0.003	trace	7.9
Soluble part in 80% acetone	0.099	0.016	0.003	trace	15.0
Non-adsorbed part with lead sulphide	0.062	0.014	—	—	19.9
Adsorbed part with lead sulphide	0.022	0.004	—	—	12.4
Lacto-flavine $C_{17}H_{20}O_6N_4$					14.9

This crystal found to be identical with yeast flavine,[51] melting at 270°, which was obtained from the autolysate of the species of Saccharomyces cerevisiae employed in brewing of the present beer, but it was impossible to decide whether it is the same as the malt flavine,[52] which melting at 282°, or not.

In order to make clear that flavine thus obtained was contained in beer as the flavine pigment or as a form of the yellow enzyme, the concentrated beer was dialyzed to separate free flavine pigment. The residual solution was treated according to the previously described method and the amount of total nitrogen at every step of the treatment and

also the intensity of the absorption band of flavine were estimated.

As the results showing in Table X, the total amount of nitrogen in the final solution is almost equal to that in non-dialyzed beer. This means that flavine is scarcely lost by dialysis,—in other words, in beer major parts of flavine are contained as a form of the yellow enzyme in combination with colloid trager.

Table X.

	gr. of total N per 1 liter of beer	
	Dialyzed beer	Non-dialyzed beer
Fresh beer	0.550 gr.	0.550 gr.
Non-dialyzed residue	0.284	—
Condensate	0.266	0.550
Residue from ether extraction	0.230	0.490
Residue from acid clay adsorption	0.152	0.360
Eluate with pyridine-methanol mixture	0.046	0.050

(C) Denaturation of beer

It is a well-known fact that plant cell sap containing rich carbohydrates, such as fruit juices, the macerated juices of beet or sweet potato and the gummy matters secreted from a wound on the bark, turns reddish brown immediately in the air, and also the black discolouration of seaweed in the sun with its disagreeable odour, is often observed on the sea-shore.

W. Palladin[53] has offered the opinion that the discolouration of the plant cell sap is due to the oxidation into colouring matter, of the respiratory chromogen contained in the cells, by the action of respiratory enzymes.

As repeatedly mentioned above, beer shows a close resemblance to plant cell saps, especially those of fruit or the seaweed, in chemical constituents, such as the uronide carbonhydrates and the respiratory enzyme, as is shown in the accompanying table (XI).

From the writers' view-point, discolouration of bottled beer often noticed in summer is presumed to be caused by a change similar to the colouration of the plant cell sap, which may be attributed to the chemical reactions in the system containing uronide-carbohydrates and the respiratory enzymes.[57]

Therefore, in the present experiment, the promotion of discoloura-

tion of beer with the addition of the respiratory enzyme-system was investigated.

Table XI.

	Beer	Apple juice[54]	Summer-orange[55] juice	Seaweed[56] (Gelidium Amandi) cell sap.
Moisture	95.8%	86.0%	91.6%	82.9%
Dry substance	4.2	10.6	8.4	17.1
Total ash	0.15	0.01	0.4	0.7
Total N	0.06	0.03	0.1	0.3
Crude protein	0.4	0.2	0.6	1.9
Reducing sugars	0.8	7.1	3.3	0.5
Non-uronide carbohydrates	2.6	2.9	3.8	0.4
Uronide-carbohydrates	1.1	0.4	0.3	6.8

According to the results of a series of investigations, the system of respiratory enzymes has been presumed to consist of the linkage of three important enzymes; namely cytochrome[58]—oxidase, the yellow enzyme[59] and dehydrogenase,[60] as follows:

Substrates—Dehydrogenase—Yellow enzyme—
Cytochrome—(Oxidase)—Oxygen molecule.[61]

For this experiment three kinds of samples were employed: unaffected beer, beer free from carbon dioxide, and beer in which the flavine enzyme had been destroyed[62] by irradiation of light of frequency 5400~3300 Å identical with the absorption band of flavine.

With the addition of the solution of flavine pigment and of Lebedew's extract which contains the same amount of flavine as the original yellow enzyme content, the samples were left standing in the thermostat and changes in the tint of colour were estimated colorimetrically at definite intervals. The results are shown in Table XII.

Table XII.

Relative intensity of colour against the sample without enzyme solution.

Untreated beer			
hr. / Sample	0	24	48
With Lebedew's extract	1.10	1.24	1.27
With beer flavine	1.04	1.08	1.11
Beer free from CO_2			
With Lebedew's extract	1.09	1.21	1.22

With beer flavine	1.02	1.08	1.11
Flavine enzyme destroyed beer			
With Lebedew's extract	1.10	1.21	1.23
With beer flavine	1.04	1.09	1.11

As will be seen, rapid discolouration was observed in the case of the addition of Lebedew's macerate containing the total system of the respiratory enzyme, but this occurred only slightly when beer flavine was mixed.

The aqueous solution of uronide carbohydrates from beer was also coloured by exposure to light after mixing with Lebedew's extract.

From these results, the writers' presumption with regard to denaturation of beer may be proved and the slow discolouration occurring in the bottle in practice is supposed to be attributable to a diminishing partial pressure of oxygen.

Summary

(1) The polysaccharides isolated from beer were proved to be a mixture of dextrin and uronide-carbohydrates. The latter is presumed to be concerned in the foam-retaining capacity and also in the denaturation of beer.

(2) Instead of maltose, the presence of a non-fermentable disaccharide, probably iso-maltose, has been confirmed.

(3) As one of the colouring matters of beer, a reddish brown coloured acidic substance, soluble in 95% alcohol, has been isolated, which is supposed to be polyoxy ketonic acid and is presumed to be connected with the discolouration of beer, since it gives a deep colouration by the action of oxidizing agents.

(4) The presence of a small amount of cytochrome in beer has been proved.

(5) A relatively large amount of flavine, estimated at 0.26 mg. per liter of beer, has ben proved to be contained in beer, mostly in a form of the yellow enzyme.

(6) Discolouration of beer is promoted by the addition of the respiratory enzyme-system.

(7) The aqueous solution of uronide-carbohydrates from beer is also coloured with the addition of Lebedew's extract.

(8) From the biochemical view-point, the characteristics of beer are attributed to the reaction-system which consists of uronide-carbohydrates and the respiratory enzymes.

(9) Denaturation of beer may be supposed to follow the oxidation which occurred in its reaction-system.

Experimental part

(A) Carbohydrates contained in beer.

Dry substance of beer:—6.7 liter of bottled beer was agitated vigorously to expel carbon dioxide as completely as possible and evaporated in vaccuo to 1.6 liter. The amounts of dry substance, the total nitrogen, total ash, phosphoric acid, reducing sugars and non-reducing sugars were then estimated by the usual methods and the results are shown in the following table.

	The content for	
	67 liter of beer	1 liter of beer
Dry substance	251.40 g	37.25
Total N	3.14	0.47
Crude protein	19.63	2.93
Reducing sugars	21.82	3.25
Non-reducing sugars	98.20	14.65
Total ash	9.15	1.36
Phosphoric acid	4.68	0.70

Fractional precipitation of concentrated beer by 80% methanol:—1.6 liter of concentrated beer was poured into 6.4 liter of methanol with vigorous stirring. The mixture was left to stand over night and the pale brownish precipitate thus obtained was filtered off. After washing it twice with 100 c.c. of 80% methanol, the precipitate was again dissolved in 200 c.c. of water and reprecipitated by pouring it into 800 c.c. of methanol. The filtrates and washings were combined and evaporated to 500 c.c. The results of analysis on the precipitate and the filtrate are shown in the following table.

	gr. per 1 liter of beer	
	Insoluble part in 80% methanol	Soluble part in 80% methanol
Dry substance	20.76	18.25
Total N	0.11	0.32
Crude protein	0.70	2.01
Reducing sugars	1.44	1.70

Non-reducing sugars	11.97	3.65
Total ash	0.67	0.67
Phosphoric acid	0.47	0.25

Dialysis of the insoluble part in 80% methanol:—130 gr. of insoluble substances in 80% methanol were dissolved in 50 c.c. of water in the vesical bag and dialyzed against 2 liters of water for 50 hours exchanging the outside solution for fresh water at intervals of 10 hours. The dialyzed and non-dialyzed solutions were concentrated to 300 c.c. respectively and also analyzed. The results are shown in the following table:

	gr. per 1 liter of beer	
	Dialyzed part	Non-dialyzed part
Dry substance	16.57	2.83
Total N	0.09	0.04
Crude protein	0.57	0.25
Reducing sugars	1.11	0.31
Non-reducing sugars	10.85	1.66
Total ash	0.30	—
Phosphoric acid	0.21	0.19

The non-dialyzed solution showed neither colour reaction for iodine solution nor the formation of osazone; and the precipitate from this solution with an 80% methanol gave the following results of elementary analysis:

Sample	CO_2	H_2O	C%	H%
0.0717 gr.	0.1173 gr.	0.0474 gr.	44.62	7.34
0.1073	0.1671	0.0704	42.48	7.29
Average			43.58	7.32
$(C_6H_{10}O_5)_n$			44.44	6.17

By distillation with 12 N. hydrochloric acid, this precipitate produced carbon dioxide and furfurol. The former was measured directly by B. Tollens and Leferve's method[63] and the latter was estimated by the phloroglucine method.[64] The results of the estimation calculating the amount of furfurol as pentose, are shown in the following table:

Sample	CO_2	Furfurol	Pentose	Pentose : CO_2
0.9700 gr.	0.0172 gr.	0.0269 gr.	0.0481 gr.	1 : 1.20
0.5393	0.0075	0.0157	0.0278	1 : 0.92
Average				1 : 1.06

From the non-dialyzed solution an amorphous lead salt was precipitated with a saturated solution of basic lead acetate. The salt after washing several times with distilled water was again decomposed by dilute sulphuric acid and after filtering off lead sulphate, the filtrate was neutralized carefully with baryta solution. In this treatment, barium sulphate was first precipitated from the solution, while, at the range of pH 3~4 the formation of precipitate was interrupted for a while and again precipitation occurred in a more acidic degree. This later precipitate was filtered and analyzed with the following results:

Sample (ash free)	CO_2	H_2O	C%	H%
0.1381 gr.	0.2036	0.1002	40.35	8.10
0.1211	0.1729	0.0799	39.00	7.38
0.1119	0.1583	0.0747	38.59	7.46
Average			39.31	7.65

Sample (ash free)	N mg (by Kjeldahl's method)	N%
0.0856 gr.	1.15	1.34
0.1109	1.60	1.44
Average		1.39

Sample (ash free)	N c.c. (by Duma's method)	N%
0.1265	1.50 (758.6 m.m., 22.8°C)	1.33
0.1304	1.60 (757.6 „ , 23.5°C)	1.44
Average		1.39

Cathaphoresis of the non-dialyzed solution:—43 gr. of the non-dialyzed precipitate were dissolved in 250 c.c. of water and by electric current of 0.1 ampere, cathaphoresis of the solution was carried on for 20 hours in Pauli's electro-dialyzer with platinum electrode. The anodic and the non-cathaphorized solution was concentrated to a small volume and analyzed with the following results:

	gr. per 1 liter of beer	
	Cathaphorized part	Non-cathaphorized part
Dry substance	3.67	2.65
Total N	0.02	0.02
Crude protein	0.14	0.11
Reducing sugars	0.19	0.14
Non-reducing sugars	2.43	1.90
Total ash	0.05	0.08

Cathaphorized substance

Sample	CO_2	H_2O	C%	H%
0.1721 gr.	0.2708 gr.	0.1051 gr.	42.92	6.78
0.1380	0.2169	0.0853	42.87	6.87
0.1571	0.2481	0.0978	43.07	6.90
Average			42.95	6.85

Non-cathaphorized substance

Sample	CO_2	H_2O	C%	H%
0.1497 gr.	0.2414 gr.	0.0927 gr.	43.98	6.88
0.2098	0.3343	0.1346	43.46	6.97
0.1500	0.2492	0.0977	43.57	6.96
Average			43.67	6.94
$(C_6H_{10}O_5)_n$			44.44	6.17

Fractional precipitation of substances soluble in 80% methanol :— 9 liters of a combined filtrate and the washing from the insoluble part in 80% methanol was concentrated to 1.2 liters and shaken for 2 hours with 20 c.c. of hydrochloric acid and 40 gr. of acid clay. The filtrate from acid clay was again concentrated in vaccuo and analyzed. The results are shown below :

	gr. per 1 liter of beer		gr. per 1 liter of beer
Dry substance	8.04	Non-reducing sugars	1.98
Total N	0.08	Total ash	0.34
Crude protein	0.52	Phosphoric acid	0.11
Reducing sugars	1.13		

70 c.c. of the concentrated solution was poured into 1400 c.c. of 95% ethyl alcohol with vigorous stirring and left to stand overnight.

The white amorphous precipitate thus formed was filtered off and refined by repetition of the same treatment. The precipitate insoluble in 90% ethyl alcohol shows the following properties and analytical results and is presumed to be achrodextrin :

	gr. per 1 liter of beer		gr. per 1 liter of beer
Yield	25.51 gr.	Reducing power after hydrolyzing with 1% HCl for 1.5 hrs. (as glucose)	12.79 gr.
Reducing power (as glucose)	2.35	Reducing power after hydrolyzing with 3% HCl for 3 hrs. („)	18.32

$[\alpha]_D$ (0.6169 gr. in 100 c.c. H_2O) +157.5°

Sample	CO_2	H_2O	C%	H%
0.0976 gr.	0.1572 gr.	0.0608 gr.	43.93	6.92
0.1344	0.2155	0.0809	43.87	6.71
	Average		43.90	6.82
	$(C_6H_{10}O_5)_n$		44.44	6.17

A 90% alcoholic solution separated from achrodextrin was evaporated to 30 c.c. in vaccuo and poured into 570 c.c. of absolute alcohol. The precipitate thus obtained was refined twice by the same treatment. This precipitate shows no sign of the formation of osazone and is not fermented by Saccharomyces cerevisiae. The properties and the analytical results are shown as follows :

	gr. per 1 liter of beer		gr. per 1 liter of beer
Yield	10.79 gr.	Reducing power after hydrolyzing with 1% HCl for 1.5 hrs. (as glucose)	6.65 gr.
Reducing power (as glucose)	2.69		

$[\alpha]_D$ (3.083 gr. in 100 c.c. H_2O) +98.9°

Sample	CO_2	H_2O	C%	H%
0.1134 gr.	0.1178 gr.	0.0679 gr.	42.76	6.83
0.2015	0.3172	0.1195	42.93	6.71
	Average		42.85	6.77
	$C_{12}H_{22}O_{11}$		42.11	6.58

The filtrate from a 95% alcoholic precipitate was evaporated to a thick syrup, which gave white crystal of phenyl hydrazide, melting at

130°~131°C, with phenyl hydrazine solution. The analytical results of the syrup are shown below:

	gr. per 1 liter of beer		gr. per 1 liter of beer
Yield	17.60 gr.	Total nitrogen	0.52 gr.
Reducing power (as glucose)	2.43		
$[\alpha]_D$ (5.028 gr. in 100 c.c. H_2O) +37.2°			

(B) Respiratory enzymes contained in beer

Separation of enzymes from the concentrated beer by adsorption:—3.35 liter of bottled beer was evaporated to 600 c.c. and shaken vigorously with 10 gr. of concentrated hydrochloric acid and 20 gr. of acid clay for 2 hours and the clay which adsorbed the respiratory enzymes was separated from the solution by means of a centrifugal machine. After washing the clay twice with 100 c.c. of water, the enzymes were again eluated with the mixture of 100 c.c. of pyridine, 200 c.c. of methanol and 300 c.c. of distilled water and the solvents were distilled from the eluate.

Isolation of cytochrome:—after adjusting pH at 5 with hydrochloric acid, 100 c.c. of the eluate was shaken for a long time with 10 gr. of cellophane powder, which was made of sliced cellophane after digesting with super-heated water at 130° and under 3 atmospheric pressures for 18 hours in an autoclave and grinding down into powder. Cytochrome adsorbed in cellophane powder was eluated twice with 50 c.c. of N/2 ammonia water, the eluate after neutralizing with sulphuric acid was dialyzed against running water to remove the inorganic salts and then evaporated to dryness. The orange coloured powder thus obtained shows the characteristic absorption bands of cytochrome as shown in Fig., but further investigation could not be made owing to the minute amount of the sample.

Isolation of beer flavine:—300 c.c. of the enzyme solution were poured into 1.3 liter of methanol and left to stand overnight. After filtering off the precipitate of protein, the filtrate was concentrated to 20 c.c. under diminished pressure, then mixed with 80 c.c. of acetone and left to stand overnight. By the repetition of the same treatment, 50 c.c. of solution of raw beer flavine was obtained. 10 gr. of lead acetate was dissolved in this solution, to which hydrogen sulphide gas was passed to precipitate lead sulphide. Flavine pigment adsorbed in lead sulphide was eluated three times with hot water as shown in Fig.

XIV. The same treatment was repeated several times to obtain a solution of pure beer flavine. 80 c.c. of the refined solution were evaporated to dryness, dissolved again in 20 c.c. of 1 N. acetic acid, the insoluble impurities filtered off and the remainder evaporated to dryness. Thus 354 mg. of crude beer flavine was obtained. 300 mg. of crude flavine was dissolved in 4 c.c. of 1 N. acetic acid and left to stand in the desiccator to become crystalline pure flavine. In this way, 7 mg. of the crystal, melting at 269°~270° (decomposition), were obtained. The absorption spectrum and the analytical results with this substance are shown in Fig. XIII, and in the following table:

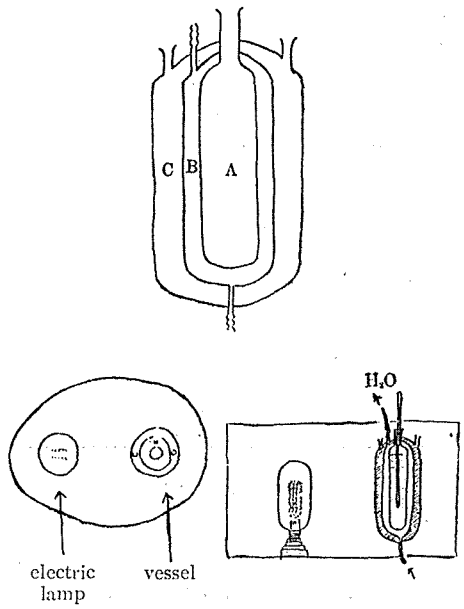
Sample	CO_2	H_2O	C%	H%
4.316 mg.	8.693 mg.	2.254 mg.	54.81	5.82
5.203	10.408	2.725	54.54	5.86
Average			54.67	5.84
Lactoflavine $C_{17}H_{20}O_6N_4$			54.25	5.36

(C) Denaturation of beer

Photochemical destruction of the yellow enzyme in beer:—In order to prepare the sample for the examination of discolouration, photochemical destruction of the yellow enzyme in beer was first investigated.

For this purpose, the vessel shown in Fig. XV was employed, of which, A is a glass cylinder of 2.5 cm. diameter and 20 cm. length, where the sample was taken in, B is the cooler 1 cm. thick and C is the space for containing the light-filter solution. The vessel was set at one of the foci of an elliptical cylinder of brightly polished brass, which has diameters of 40 and 35 cm., and at the other focus an electric lamp of 500 W. was placed. With the object of preventing possible de-

Fig. XV.



composition of other constituents of beer, the light-filter through which only the ray of wave length corresponding to the absorption bands of flavine might pass, was selected and from the following results the solution of ammoniacal copper oxide was chosen as the most suitable one.

Light filter	Thickness of layer	Frequency of ray passed	Relative intensity of light against non-filtered light
30% Cupric sulphate solution	20 m.m.	5900—3400 Å	45
	16	6200—3300	50
	12	6400—3100	55
	8	6500—3000	60
Ammoniacal copper oxide solution ($CuSO_4 \cdot 5H_2O$ 12.5 gr., 30% NH_4OH 46 c.c.) H_2O 509 c.c.	28	4900—3600	15
	21	4950—3450	25
	14	5100—3350	30
	7	5400—3300	40
Cobalt glass 0.2		5600 and 5000—3400	30

As preliminary tests, 0.02% solution of beer flavine of different alkalinity was irradiated in the apparatus and after acidifying the solution with 1 N. sulphuric acid, lumiflavine produced by photochemical destruction was extracted twice with chloroform and again with ether and the amount of non-decomposed flavine was measured spectroscopically. As shown in the following table, in N/20 caustic soda solution the major parts of flavine were destroyed by irradiation for 2 hours.

Alkalinity	Temperature	Irradiating time	Percentage of decomposition
N/1000	11°—13°	5 hrs.	0
„	12°	10	?
„	11°—13°	20	20
N/20	12°	5	38
„	12°	7	50
„	11°	12	90
N/2	12°	5	50

Photochemical destruction of the yellow enzyme in beer was performed in the light of the above results. After the ejection of carbon dioxide, beer was made to N/20 alkaline solution by the addition of dilute caustic soda and irradiated and again restored to the original pH of beer with phosphoric acid. The solution thus obtained was

tinted a deeper red than the original colour tone, but against this colour tone, relative increase in red colouration was measured and compared.

Promotion of discolouration in beer with the addition of respiratory enzymes :—With the addition of a definite quantity of flavine solution and of the Lebedew's extract from Saccharomyces cerevisiae, 15 c.c. of the sample was left to stand in the thermostat adjusted at 25°C and at definite intervals the degree of discolouration produced was measured by means of a colorimeter. The results are shown in the following table :

(1) Irradiated beer

No.	c.c. of sample	Water added	Yeast flavine soln.	Lebedew's extract
I	15 c.c.	8.0 c.c.	— c.c.	— c.c.
II	„	„	—	—
III	„	7.7	0.3	—
IV	„	„	„	—
V	„	6.0	—	2.0
VI	„	„	—	2 0

(Note : 0.3 c.c. of flavine solution contains 0.03 mg. of flavine
2.0 c.c. of Lebedew's extract contains 0.02 mg. „)

Relative intensity of the tint deepening measured against I and II as the standard.

hr. / No.	0	24	72
III	1.20	1.55	1.49
IV	1.18	1.67	1.63
Average	1.19	1.62	1.56
V	1.20	2.05	2.00
VI	1.24	2.05	2.00
Average	1.22	2.05	2.00

(2) Irradiated beer

No.	Sample	Water added	Yeast flavine soln.	Lebedew's extract
I	15 c.c.	8.0 c.c.	— c.c.	— c.c.
II	„	7.9	0.1	—
III	„	„	0.1	—
IV	„	7.0	—	1.0
V	„	„	—	1.0

Relative intensity

No. \ hr.	0	24	48
II	1.02	1.06	1.11
III	1.02	1.06	1.14
Average	1.02	1.06	1.13
IV	1.13	1.42	1.59
V	1.10	1.42	1.50
Average	1.12	1.42	1.55

(3) CO_2-released beer

No.	sample	Water added	Yeast flavine soln.	Lebedew's extract
I	15 c.c.	8.0 c.c.	— c.c.	— c.c.
II	„	7.9	0.1	—
III	„	„	0.1	—
IV	„	7.0	—	1.0
V	„	„	—	1.0

Relative intensity

No. \ hr.	0	24	72
II	1.02	1.05	1.14
III	1.02	1.05	1.21
Average	1.02	1.05	1.18
IV	1.11	1.50	1.63
V	1.14	1.50	1.54
Average	1.13	1.50	1.59

(4) Untreated beer

No.	Sample	Water added	Yeast flavine soln.	Lebedew's extract
I	15 c.c.	8.0 c.c.	— c.c.	— c.c.
II	„	7.9	0.1	—
III	„	„	0.1	—
IV	„	7.0	—	1.0
V	„	„	—	1.0

Relative intensity

No. \ hr.	0	24	72
II	1.04	1.06	1.10
III	1.02	1.07	1.10
Average	1.03	1.06	1.10
IV	1.11	1.31	1.46
V	1.11	1.31	1.38
Average	1.11	1.31	1.42

(5) Irradiated beer

No.	Sample	Water added	Beer flavine soln.	Lebedew's extract
I	15 c.c.	0.7 c.c.	— c.c.	— c.c.
II	„	0.65	0.05	—
III	„	„	0.05	—
IV	„	„	—	0.7
V	„	„	—	0.7

(Note 0.05 c.c. of beer flavine soln. contains 0.005 mg. of flavine
0.7 c.c. of Lebedew's extract contains 0.005 mg. „)

Relative intensity

No. \ hr	0	24	48
II	1.04	1.08	1.11
III	1.04	1.11	1.11
Average	1.04	1.09	1.11
IV	1.11	1.21	1.24
V	1.09	1.21	1.22
Average	1.10	1.21	1.23

(6) CO_2-released beer and unaffected beer

	No.	Sample	Water added	Beer flavine soln.	Lebedew's extract
CO_2-released beer	I	15 c.c.	0.7 c.c.	— c.c.	— c.c.
	II	„	0.65	0.05	—
	III	„	0.65	—	0.7
Untreated beer	I	„	0.7	—	—
	II	„	0.65	0.05	—
	III	„	0.65	—	0.7

Relative intensity

	No. \ hr	0	24	48
CO_2-released beer	II	1.04	1.08	1.11
	III	1.10	1.24	1.27
Untreated beer	II	1.02	1.08	1.11
	III	1.09	1.21	1.22

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Literature cited

1) E. Leyser, Malz u. Bierbreitung, Bd. II, 135 (1910).
H. Lüers, Chemie des Brauwesens, 381 (1929).
H. Leberle, Die Bierbrauerei, Bd. II. 576, 613 (1930).
2) J. Instit. Brewing. **42**, 191 (1932).
3) R. Hopkins and C. Krause, Biochemistry applied to Malting and Brewing, 306 (1937).
4) A. Findlay and B. Shen, J. C. S. **99**, 1313 (1911).
5) T. Langer and W. Schultz, Z. f. gesamt. Brauwesen **2**, 369 (1879).
F. Emslander and H. Freundlich, Z. physik. Chem. **49**, 317 (1904).
6) R. Hopkins and C. Krause, Loc. cit. 303 (1937).
7) K. Ueno, J. C. S. Japan, **20**, 376 (1898).
8) Z. f. gesamt. Brauwesen **43**, 185 (1920).
9) A. Findlay and H. Creighton, J. C. S. **97**, 536 (1910).
10) S. Komatsu, Outline of biochemistry, **12**, 239 (1937).
11) E. Lohmann and P. Aichele, Keimungsphysiologie, 550 (1931).
12) W. S. Tao, Memoir Coll. Sci. Kyoto Imp. Univ. **14**, 287 (1931).
13) Russel and Bishop, J. Instit. Brewing, **39**, 287, 292, 297 (1933).
14) Hopkins, Hind and Day, Ibid., **40**, 445 (1934).
15) S. Komatsu, Anniversary Vol. Prof. Chikashige 127 (1930).
H. Spoehr, The Carbohydrate Economy of Cacti, 55 (1919).
16) K. Bernhauer, Grundlagen der Chemie u. Biochemie der Zuckerarten, 91 (1933).
S. Komatsu, Outline of Biochemistry, 40 (1937).
17) H. Leberle, Die Bierbrauerei, Bd. II. 567 (1931).
Brand, Z. f. gesamt. Brauwesen 41 (1907).
18) Hayduck; Wochensch. f. Brauerei, 813 (1893).
409 (1894).
Reichard, Z. f. gesamt. Brauwesen, 277 (1906).
H. Lüers, Wochensch. f. Brauerei, 100 (1927).
19) A. Ling, J. Instit. Brewing, 594 (1904).
W. Duncan, Ibid., 164 (1910).

20) H. Lüers, Chemie des Brauwesen. 240 (1929).
H. Leberle, Die Bierbrauerei. Bd. II, 380 (1930).
21) C. Lintner, Z. f. gesamt. Brauwesen 545 (1912).
L. Mailard, C. R. **154**, 66 (1912) **156**, 1159 (1914).
L. Grünhut and J. Weber, Bioch. Z. **121**, 109 (1921).
22) W. Ruckdeschel, Z. f. gesamt. Brauwesen, 435 (1914).
23) H. Lüers and Nishimura, Ibid., 61 (1924).
24) H. Lüers, Wochensch. f. Brauerei, 704 (1936).
25) Die Bierbrauerei. Bd. II. 620 (1930).
26) Chemie des Brauwesens 375 (1929).
27) Brewing Trade Review (1929).
28) Wochensch. f. Brauerei, 305 (1936).
29) Hess, Z. f. gesamt. Brauwesen 315 (1936).
30) E. Emslander, Wochensch. f. Brauerei, 65 (1937).
31) H. Lüers, Chemie des Brauwesens, 396 (1929).
32) Ber. **26**, 2533 (1893).
33) Wochensch. f. Brauerei 74 (1923).
34) Ibid. 401 (1935).
35) A. Heiduschka and J. Segl, Z. f. gesamt. Brauwesen 1 (1933).
36) G. Klein, Handbuch der Pflanzenanalyse II. III/1, 121 (1932).
37) A. v. Herke, Sv. Vet. Akad. Arkiv. f. Kemi **11**, A. 22 (1935).
38) A. Foder and L. Frankenthal, Bioch. Z. **225**, 414 (1930).
O. Schumm, Z. f. physiol. Chem. **154**, 192 (1926).
39) P. Karrer and K. Schöpp, Helv. **17**, 1013 (1934).
40) A. Bach and A. Oparin, Bioch. Z. **148**, 476 (1924).
41) H. v. Euler and O. Dahl, Ibid., **282**, 235 (1935).
42) H. v. Euler and R. Weichert, Z. f. physiol. Chem. **233**, 81 (1935).
43) O. Warburg and W. Christian, Bioch. Z. **254**, 438 (1932).
257, 496 (1933).
263, 228 (1933).
266, 122 (1935).
44) H. v. Euler and H. Fink, Z. f. physiol. Chem. **164**, 69 (1927).
J. Wagner-Jauregg, Ibid **231**, 55 (1935).
H. v. Euler and H. Adler, Ibid. **235**, 122 (1935).
45) O. Warburg and W. Christian, Bioch. Z. **266**, 377 (1933).
46) Z. f. physiol. Chem. **153**, 204 (1926).
47) Ber. **67**, 1452 (1934).
48) O. Warburg and W. Christian, Bioch. Z. **257**, 492 (1933).
258, 496 („).
263, 228 („).
266, 377 („).
49) R. Kuhn, R. Györgyi and J. Wagner-Jauregg, Ber. **66**, 317, 1034, 1577, 1950 (1933).
R. Kuhn and J. Wagner-Jauregg, Ibid., **67**, 1770 (1934).
R. Kuhn and H. Kaltschmidt, Ibid., **68**, 128 (1935).
P. Karrer and K. Schöpp, Helv. **17**, 419, 735, 1013 (1934).
50) R. Kuhn, J. Wagner-Jauregg and H. Kaltschmidt, Loc. cit.
51) S. Komatsu, S. Otsuka, S. Tanaka and T. Suzuki, unpublished.
52) P. Karrer and K. Schöpp, Helv. **17**, 1013 (1934).
53) Ber. deutsch. bot. Ges. **269**, 378, 389 (1908).
270, 101 (1909).

54) S. Komatsu, S. Ozawa and S. Tanaka, J. C. S. Jap. **52**, 732 (1931).
55) S. Kamatsu, S. Tanaka, S. Ozawa, R Kubo, Y. Ono and Z. Matsuda, Ibid., **51**, 278 (1930).
56) S. Komatsu and H. Ishizawa, unpublished.
57) R. Kuhn, Ber, **67**, 1454 (1934).
58) Mac. Mann, J. physiol. Chem. **13**, 497 (1889).
D. Keilin, J. Proc. Roy. Soc. (B) **98**, 318 (1925).
104, 206 (1930).
106, 418 (1930).
107, 289 (1930).
109, 209 (1931).
H. Theorell, Bioch. Z. **279**, 463 (1935).
285, 207 (1936).
59) O. Warburg and his collaborators, Bioch. Z. **275**, 112, 464 (1934).
282, 157 (1935).
285, 156 (1936).
286, 81 (1936).
287, 291 (1936).
60) A. Harden and W. Young, J. Proc. Roy. Soc. (B). **78**, 369 (1906).
H. v. Euler, K. Myrbäck and their collaborators,
Z. f. physiol. Chem, **203**, 143 (1931).
212, 7 (1932).
217, 247 (1933).
220, 173 (1933).
225, 125, 131 (1934).
233, 87, 95, 148, 154 (1935).
234, I, 254, 259 (1935).
241, 148, 223 (1936).
Nature, **138**, 968 (1936).
Naturwiss., **50**, 794 (1936).
O. Warburg and his collaborators, Bioch. Z. **254**, 438 (1933).
260, 499 (1933).
257, 492 (1933).
258, 496 (1933).
263, 228 (1933).
266, 377 (1933).
272, 155 (1934).
275, 37, 344 (1934).
Naturwiss., **20**, 688, 980 (1932).
22, 289 (1934).
H. v. Euler, Z. f. physiol. Chem. **226**, 87 (1934).
233, 105 (1934).
61) H. v. Euler and his collaborators, Z. f. physiol. Chem. **226**, 195 (1934).
232, 6, 10 (1935).
233, 120 (1935).
235, 122, 154, 164 (1935).
238, 233, 261, 269 (1936).
239, 241 (1936).
241, 239 (1936).
H. Theorell, Bioch. Z. **288**, 317 (1936).

62) R. Kuhn and H. Rudy, Ber. **67**, 1298 (1934).
P. Karrer, H. Salomon, K. Schöpp, F. Schlitter and H. Fitztche. Helv. **17**, 1010 (1934).
63) Ber. **40**, 4513 (1907).
64) B. Tollens, (G. Klein, Handbuch der Pflanzenanalyse, II. III/I, 33 (1932).