THE ENDO- AND EKTOINVERTASE OF THE DATE.[1]

BY A. E. VINSON.

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In a recent paper the writer[2] called attention to the fact that the invertase of the date could not be extracted from the active tissues by solvents until the fruit ripened. In other words the invertase changes suddenly from an endo to an ektoenzyme.[3] The invertase of the green date thus corresponds very closely, in its behavior towards solvents, to the invertase of certain yeasts,[4] of immature *Penicillium*,[5] of *Monilia candida*,[6] and of various parts of growing plants,[7] notably the rootlets of seedlings; to the urease in the torula causing alkaline fermentation of urine;[8] to Buchner's[9] zymase of beer yeast; and finally to several zymogens, notably that of diastase which occurs in the scutellum of ungerminated grain[10] and which hydrolyzes soluble starch only, the diastase of translocation of Brown and Morris.[11] It probably corresponds also to the zymogen or proferment of the proteolytic enzymes observed by Vines[12] in yeast, beans, and other plant tissues, and possibly mistaken by him for vegetable ereptase. After presenting the general facts relating to the insoluble invertase of the green date the application of a new hypothesis will be made which seems to harmonize these various phenomena without assuming differences in permeability of the cell wall.

The action of green date tissue in inverting cane sugar, while no active extract can be prepared, might be considered as due to the living cells, under the influence of cane sugar, secreting invertase[13] or activating some

[1] Read at the Chicago meeting of the American Chemical Society.

[2] *Bot. Gaz.*, **43**, 393 (1907).

[3] M. Hahn, *Z. Biol.*, **40**, 172; see also Green, Soluble Ferments, Cambridge, 1899, p. 116; Oppenheimer, Die Fermente, Leipzig, 1903, p. 72.

[4] Beer yeast, O'Sullivan and Tompson, *J. Chem. Soc.*, **57**, 873 (1890); **61**, 593 (1892); Fernbach, Thesis, Paris, 1890, through Pottevin and Napias; Pottevin and Napias, *Compt. rend. soc. biol.*, **50**, 237; Milk sugar yeast, lactase, E. Fischer, *Ber.*, **27**, 3481, 1894, etc.

[5] *Penicilium glaucum*, Bourquelot 1886, through Green, p. 115; *P. duclauxi*, *Bull. soc. mycol.*, **8**, 147 (1891).

[6] Hansen, through Fischer and Lindner; E. Fischer and Lindner, *Ber.*, **28**, 3034 (1895); Buchner and Meisenheimer, *Z. physiol. Chem.*, **40**, 167; Fischer, *Z. physiol. Chem.*, **26**, 77 (1898).

[7] Brown and Morris, *J. Chem. Soc.*, **1893**, 633; Brown and Heron, *Proc. Roy. Soc.*, **1880**, 393; J. O'Sullivan, *J. Chem. Soc. Proc.*, **16**, 61; *Abst. Centb.*, **1900**, 773; *J. Chem. Soc.*, **77**, 691, 1900.

[8] Sheridan Lea, *J. Physiol.*, **1885**, 136.

[9] *Ber.*, **30**, 117, 1110, 2668; **31**, 209, 568, 1084, 1090, 1531; **32**, 127.

[10] Reed, *Ann. of Bot.*, **1904**, 267.

[11] *J. Chem. Soc.*, **1890**, 458; Vines, *Ann. of Bot.*, **1891**, 409.

[12] *Ann. of Bot.*, **16**, 10; **17**, 237, 597; **18**, 289; **19**, 149; **20**, 113.

[13] Effront, p. 69–70.

zymogen, either soluble or insoluble, already existing in the cell; that is, the inversion was due to some activity of the living protoplasm.[1] This point, however, was readily answered in the negative by a short series of tests in the presence of ordinary killing reagents. The inversion curves for equal weights of green and of ripe date pulp were determined and found to be practically identical. Similar amounts of green and of ripe pulp from the same sample as was used for the blank, were allowed to act upon equal quantities of cane sugar under the same conditions, excepting that 1 per cent. of chromic acid, $^1/_2$ per cent. of picric acid and 2 per cent. of formaldehyde were added to the respective samples. If the inversion by the green date were due to living protoplasm, its inverting power would be inhibited to a much greater degree than that of the ripe date, where the invertase is known to exist in its soluble form. In all cases there was considerable inhibition, but the curves for both green and ripe pulp were approximately parallel and of the same order. These results were fully confirmed by later experiments, designed to break down the resistance of the plasmotic membrane, by treating the green pulp with chloroform, ether, toluene, and other reagents. These killed the protoplasm but left the inverting power of the tissues unaffected. The inverting action of chromic acid under the same conditions was determined. The following abridged table shows the effect in a general way of these protoplasmic poisons on the rate of inversion.

[1] NOTE.—Whatever our personal beliefs concerning the mechanism of biochemical processes may be, it is not always an easy matter to differentiate clearly between enzymatic and protoplasmic agencies. This is abundantly exemplified in the literature: thus Hugo Fischer (*Cent. Bakt.*, 1903, 453) says:

"Unrichtig ist es aber darum auch, enzymatische Vorgänge nun als 'rein chemische' grundsätzlich von denen zu trennen, die man nicht in zellenfreier Lösung sich abspielen lassen kann; die Eiweissynthese ist gerade so gut ein chemischen Vorgang wie die alkoholische Garüng. Denn, dass das Agens der letzteren sich in Wasser löst, das der ersteren nicht, ist zwar für die Laboratoriumstechnik wesentlich, viel weniger aber für die theoretische Physiologie." Bokorny (*Pflüger's Arch.*, **90**, Heft 11–12, autoabst. *Centb. Bakt.*) "Die wirkliche Entscheidung über Protoplasma oder Enzymnatur konnte hier wie immer nur durch den Nachweis der bestimmten Organization oder des Fehlens einer solchen hierbei geführt werden. * * * * Die Löslichkeit in Wasser spricht gegen die Protoplasmanatur, da das Protoplasma nach den bisherigen Beobachtungen nie ein wirkliche Lösung darzustellen scheint. Doch ist auch bei einer Lösung, wenn dieselbe eine, "micellare" ist, Organization, das ist bestimmte specifische aneinanderreihung der Molecüle möglich." M. Hahn (*Ber.*, **31**, 200) quotes Neumeister (*Lehrb. d. physiol. Chem.*, 137): "Für die tierischen Zellen nimmt Neumeister an dass die cellulare Verdauung ohne Enzyme lediglich durch eine eigenartige Thätigkeit des lebenden Protoplasma zu Stande kommt." Wills (Review of Buchner's book, Die Zymasegärung, *Centb. Bakt.*, **1903**, 464): "Die Tatsache, dass die beiden Erscheinungen augenscheinlich durch dieselben Momente beinflusst werden, legt den Gedanken nahe, dass auch die Reduktionswirkung auf der Wirkung enzymatige Körper beruht, der für gewöhnlich in der Leibessubstanz der zellen eingeschlossen ist."

EFFECT OF PROTOPLASMIC POISONS ON THE RATE OF INVERSION OF CANE SUGAR BY GREEN AND BY RIPE DATE TISSUES.

	No poison.		Chromic acid.			Picric acid.		Formaldehyde.	
Time.	Ripe.	Green.	Ripe.	Green.	Alone.	Ripe.	Green.	Ripe.	Green.
Nov. 27	+4.81	+6.02	+4.81	+5.41	+4.75	+4.72	+6.43	+4.65	+5.73
Nov. 28	+2.65	+1.63	+2.59	+1.09	+4.61	+5.12	+5.83	—0.06	+0.93
Nov. 29	+0.10	—0.56	+1.03	+0.07	+4.20	+3.61	+5.08	—1.00	—0.67
Nov. 30	—1.76	—1.81	—0.66	—0.85	+4.04	+3.32	+4.67	—1.81	—1.36
Dec. 1	—2.32	—2.25	—1.93	..	+3.95	+2.93	+4.23	—2.10	—1.99
Dec. 3	—2.07	—2.24	—1.10	—0.98	+4.14	+1.91	+2.81	—2.01	—1.92
Dec. 12	—2.45	—2.44	—1.32	—1.95	+4.13	—1.29	—0.85	—2.05	—1.99
Dec. 22	..	..	..	..	..	—2.04	—1.95	..	..

Since the invertase becomes soluble at the time of ripening and the tannin becomes insoluble at the same period, it would appear entirely possible that, on crushing the green fruit, the soluble tannin, which is confined to specific cells, would precipitate the invertase and prevent its extraction. It has been shown by Brown and Morris[1] that a decoction of hops will not extract diastase from malt until after the tannin has been removed by treatment with hide powder. They find also that the diastase of the hop strobile cannot be extracted with water and that those plants whose aqueous extracts show weak diastatic powers are rich in tannin.[2] This is especially true of *Hydrocharis*.[3] Warcolier[4] belives the presence of abnormal quantities of starch in bruised apples to be due to the action of escaped tannin on the diastase. These considerations led the writer to investigate the relations between tannin and invertase in general, and especially the possibility of the phenomenon of insoluble invertase in the green date being due to soluble tannin. I have not been able to connect the tannin with enzymic action, although its disappearance at the time the invertase becomes soluble and its presence in those parts of plants where metabolism is rapid, as in the tips of shoots like those of the rose, seem to be suggestive.

The first point to determine is whether tannin in relatively large amounts is in any way inimical to the action of invertase. The effect of added tannin was studied both with date pulp and date extract. Twenty grams of ground date were disintegrated with 100 cc. of water, varying amounts of tannin added, then 500 cc. of sugar solution. The values in the following tables are the readings given on the sugar scale of the saccharimeter, when double the normal weight of the sugar solution was used. The variations in initial reading are due to inversion before weighing off the quantities for polarization.

[1] *Trans. Inst. of Brewing*, 6, 94 (1893).
[2] *J. Chem. Soc.*, 63, 604, 640.
[3] *Ibid.*, 653.
[4] *Compt. rend.*, 141, 405 (1905).

EFFECT OF TANNIN ON RATE OF INVERSION OF CANE SUGAR BY DATE TISSUE.

	No tannin.	0.25 gram.	0.50 gram.	0.75 gram.	1 gram.
Initial reading.....	+14.5	+14.2	+12.8	+13.2	+13.0
24 hours later.....	— 4.0	— 4.0	— 4.7	— 4.7	— 4.9

The presence of added tannin seems to slightly increase the rate of inversion rather than retard it.

The extracts used in the following table were made from 250 grams of ground date with 400 cc. of water and with 200 cc. of water and 200 cc. of glycerol respectively. In the experiments 500 cc. of sugar solution were treated with 50 cc. of date extract.

EFFECT OF TANNIN ON THE RATE OF INVERSION OF CANE SUGAR BY DATE EXTRACT.

	Water extract.		Glycerol extract.	
	No tannin.	1 gm. tannin.	No tannin.	1 gm. tannin.
Initial reading.....	+14.9	+14.2	+15.2	+14.3
24 hours later.....	— 5.2	— 5.5	— 5.0	— 6.0

The effect of tannin on the solubility of the enzyme, however, is a very different matter, as shown by the references cited above. A short series of experiments was planned to study this effect on the solubility of date invertase in both water and glycerol. 250 grams of ground date (Amari and Amhat mixed, one year old) were treated with 400 cc. of water, with and without the addition of 5.0 grams of tannin, and also with 200 cc. each of water and glycerol with and without tannin. 50 cc. of each of the extracts were then added to 500 cc. of sugar solution and the rate of inversion determined as before.

EFFECT OF TANNIN ON THE SOLUBILITY OF DATE INVERTASE IN WATER AND GLYCEROL AS DETERMINED BY THE RATE OF INVERSION.

	Water alone.		Water + glycerol.	
Date.	Without tannin.	With tannin.	Without tannin.	With tannin.
June 21.....	+14.85	+15.8	+15.2	+15.2
June 22.....	— 5.2	+14.6	— 5.0	— 1.3
June 25.....	— 5.6	+ 9.5	— 5.1	— 5.5

The press residues appeared about as active as the original ground date. The glycerol solution, being somewhat turbid, was allowed to settle several days, the clear upper portion decanted off, and the solid residue separated and washed in a small centrifugal. 50 cc. of the clear upper portion gave a result almost identical with that obtained from 50 cc. of the unsettled solution. The solid residue from the entire extract showed only a weak inverting action.

This solubility of the tannin enzyme compound in glycerol, as compared with its behavior toward water alone, seemed so unusual that I thought it desirable to change the conditions somewhat. Accordingly two samples, each containing 250 grams of the same date material and 5 grams of added tannin, were treated with 200 cc. of water, the one receiving 200 cc. of

glycerol at once and the other receiving a similar quantity two days later. Both were macerated 24 hours longer, pressed, and the turbid extracts allowed to stand in long narrow tubes for two weeks. The sample which had stood two days with the tannin before the glycerol was added, settled out clear in a few hours; but the other, which received the glycerol at once, remained slightly cloudy, tending rather toward opalescence. Considerable residue had separated out in both cases, from which the supernatant solution was carefully siphoned. These solutions were then tested as before, using 50 cc. of both the clear upper portion and the turbid lower portion.

EFFECT OF THE TIME OF ADDING GLYCEROL ON THE SOLUBILITY OF DATE INVERTASE IN THE PRESENCE OF TANNIN.

	Glycerol added at once.		Glycerol added after two days.	
Date.	Upper portion.	Lower portion.	Upper portion.	Lower portion.
July 16.....	+15.2	+14.3	+15.9	+15.7
July 17.....	— 5.6	— 5.6	+11.1	+ 4.5
July 18.....	— 5.65	— 6.0	+ 6.7	— 2.7
July 19.....	...	....	+ 3.7	— 4.3
July 24.....	...	....	— 5.0	— 6.0

These results indicate a marked solubility of the tannin-invertase compound in glycerol, especially when the glycerol was added at once. If the inversion had been due to a suspended insoluble invertase compound the first two columns of the table would show a marked difference, such as is apparent in the case where glycerol was added later. From these observations, we may conclude that if green date contained any soluble invertase its extraction would not be seriously hindered by the tannin liberated when the fruit was crushed, provided the glycerol was added at once. Numerous samples of green dates from several different invert-sugar varieties, treated in this way, have given negative results, although the press residues themselves were always very active, even after prolonged washing with water. While this test in itself should suffice to demonstrate that the invertase of the green date is not prevented from going into solution by the soluble tannin present, the distribution of the tannin in the fruit is such that it can be dissected away readily and leave a considerable mass of tannin-free tissue. For this purpose one of the larger varieties, such as Rhars or Amraayah, serves best. The tannin in the ovulary of the date exists during all stages of its growth in a layer of large tannin cells just beneath the skin and easily visible to the naked eye, so that it can be pared away as readily as the peel of any other fruit. After removing the seed, the fibrous envelope, which also contains tannin, is easily scraped out. By carefully comparing the inverting power of equal weights of pulp from the outer tannin-bearing and the inner tannin-free portions of the same sample, no appreciable difference could be detected. Extracts of the two portions were invariably inactive. This shows, beyond doubt, that the insolubility of the

invertase in the green date is not the result of the presence of soluble tannin, and the change in the solubility of the invertase and the disappearance of soluble tannin are not directly connected, although they coincide very closely in time.

It remains yet to show whether green date tissue, in the act of inverting cane sugar, does or does not secrete invertase into the surrounding medium as some yeasts are known to do, or whether a pre-existing soluble proinvertase, a zymogen, such as Pantanelli[1] has lately shown to exist in *Mucor*, was rendered active. To disprove this, portions of tannin-bearing and of tannin-free date tissue were allowed to act on cane sugar solution several hours. After the sugar was inverted, the juice was pressed off and more cane sugar added to the extract, but no further inversion took place even after standing several days. Thus no appreciable quantity of invertase passes from the green date into the surrounding medium during the process of inversion; the inversion must take place within the cell.

This brings us face to face with the long-accepted theory governing all these cases; namely, that it is a matter of the impermeability of the cell wall to the enzyme. This theory was first advanced in 1871 by Hoppe-Seyler,[2] who had observed that the invertase could be extracted from yeast cells after treating them with alcohol, the assumption being that such treatment broke down the resistance of the protoplasm to the passage of the enzyme. This same theory has worked well in a great number of cases and has been used by Lea[3] in explaining the solubility of urease after treating the torula of urine with alcohol; by Buchner[3] for the release of zymase from yeast cells by grinding with sand and kieselguhr; by Albert[4] for the same result by treatment with acetone; by Pottevin and Napias[3] to explain the discrepancies between Fernbach and O'Sullivan with regard to the liberation of invertase by various yeasts; and by many others, but always to explain the same phenomenon of an enzyme not being yielded to the solvent, until after some special treatment of the cells containing it. Later I shall discuss some of these cases in detail.

For the purpose of this investigation we may consider the plant cell to be made up of two layers. The outer cell wall is composed chiefly of hemicellulose, true cellulose, lignin, and cutin, the lignin, according to König,[5] increasing with the age of the cell and at the expense of true cellulose. This outer wall acts much like a filter, allowing practically any substance in solution to pass. Within this outer cellulose wall and closely adherent

[1] Proinvertase reversibilita dell invertasi nei Mucor, *Rend. Accad. Lincei. Roma*, **15**, I Sem., 587 (1906); Abst. in *Bot. Centb.*, **105**, 245 (1907).

[2] *Ber.*, **1871**, 810. Report of the Rostock meeting by Victor Meyer.

[3] *Loc. cit.*

[4] *Ber.*, **35**, 2378 (1902).

[5] *Landw. Ver. Sta.*, **65**, 55, 65 (1907).

to it, probably secreting it, lies the outer protoplasmic layer, the plasmotic membrane or *Hautschicht.*[1] So long as the protoplasm is living, this plasmotic membrane acts like a semipermeable membrane, allowing the free passage of water but tenaciously holding most other substances. On the death of the protoplasm the plasmotic membrane loses its semipermeable properties and allows various substances to pass it freely; that is, the protoplasm no longer plasmolyzes. It would seem then, that any treatment which killed the protoplasm would liberate the enzyme, and that appears to take place in many cases, although not in all.[2]

At this point we must take into consideration the work of Iscovesco[3] on the passage of colloids through colloids as applied to cell specificity. Iscovesco has shown that one colloid may penetrate another when they bear electric charges of the same sign, but not when the signs are opposite. The sign may be changed by changing the chemical composition of the surrounding medium. It is thus plausible that treatment with various agents might change the sign of the charge borne either by the protoplasmic wall or by the enzyme, and thus render the cell permeable.

Pantanelli[4] also has recently studied the permeability of the cell wall by observing the effects of colloids on the formation and excretion of invertase, and by comparing the permeability of the wall to certain salts at the time when enzyme secretion was at its maximum. He holds that the secretion of the enzyme into the surrounding medium is a function of the living cell, being made possible by autoregulation of the permeability of the protoplasmic cell wall, and that this change is reversible. Thus he finds the invertase of yeast and of *Mucors* to be the only true ektoenzymes, since in others the escape of the enzyme from the cell is conditioned by the dying of the cell itself. Some colloids, as gum arabic and peptone, in the culture medium stop the intracellular formation and secretion of invertase. The secretion is at a maximum during the time of maximum fermentation, and at the same time the permeability of the cell wall to certain salts is also greatest. In the case of *Mucor stolonifer*, the appearance of the invertase in the surrounding medium is due rather to the enzyme being given off by the dead tissue, because it corresponds in time with spore formation and is wanting in the earlier stages of development, at which period cane sugar as such is taken up. The general facts here correspond closely with those for the date as well as for *Penicillium*, the invertase passing into the solution readily at the time of maturity. In the case of *Mucor* there seems to be no adequate ground for supposing that increased permeability of the cell wall to inorganic salts would indicate increased permeability to inver-

[1] Jost, Pflanzenphysiologie, Vorl. 2.
[2] Buchner and Meisenheimer, *Z. physiol. Chem.*, **40**, 167.
[3] *Compt. rend. soc. biol.*, 62, 625.
[4] *Ann. di Bot.*, **3**, 113 (1905); Abst. in *Bot. Centb.*, **105**, 185

tase, although it might be true. There is, however, no ground to believe that the invertase, prior to its time of secretion, did not exist in an insoluble state. While I am not in a position to disprove impermeability in every case, it certainly does not conform to the facts for the green date.

It is significant that observations heretofore on the persistency with which cells often retain the enzyme have been made, with few exceptions, on unicellular plants. In these cases the cell walls are more resisting than those of soft complex tissues, and a process similar to Buchner's would be required to break open any considerable number of them. The conditions in the interior of a green date, however, are quite different. Here the heavy cellular walls found in the yeasts and other unicellular plants are not needed for protection; therefore grinding and crushing by ordinary means should allow the escape of at least detectable amounts of invertase. Especially is this true, since the cells of a complex tissue are more or less torn and cannot glide about freely among one another like yeast cells. The juice pressed from such bruised tissues contains a very large percentage of all the water present in the fruit, and with it, sugar, protein, and other soluble cell constituents, which are otherwise held by the semipermeable plasmotic membrane. Under these conditions it is hardly possible to believe that invertase exists in solution in the cell sap. The retention of a great amount of invertase by the ripe date pulp which cannot be removed by prolonged washing with water, a similar partial retention of invertase by the rootlets of seedlings and of catalase by cured tobacco, also speak against retention by impermeability.

Another fact which points to the insolubility of the invertase is the behavior of green date tissue toward such substances, as ether, chloroform, toluene,[1] etc., with regard to the subsequent solubility of the enzyme. It was mentioned above that, on the death of protoplasm, the plasmotic membrane ceases to be semipermeable and allows the passage of most substances as freely as does the cellulose wall. If the enzymes in general are in solution in the cell sap, then by treatment with protoplasmic poisons they should be released. The instances already cited of various enzymes being released by treatment with alcohol, acetone, toluene, etc., might well be due to this effect. Fischer and Lindner,[2] however, failed to liberate invertase from *Monilia candida* by this process. The green date also fails to give up its invertase after killing with these substances.

Samples of ground green date, from which the inactive juice had been largely removed by pressure, were treated over night in closed vessels with acetone, ether, toluene, and chloroform, respectively. After exposing in the air until all traces of the added material had disappeared, they were macerated with water for two days and the aqueous extracts tested for

[1] E. Fischer, *Z. physiol. Chem.*, **26**, 75 (1898).

[2] *Ber.*, **28**, 3034.

nverting power with negative results in all cases. The residues were extracted seven days longer with very frequent changes of water, and their nverting power determined. It did not seem to be in any degree impaired. A microscopic examination showed the protoplasm drawn away from the ellulose wall and collected in small dense masses within the dead cell. A econd test on the inner tannin-free portion of Rhars date from which the ugar had been extracted with water was made by digesting two weeks in . large volume of ether. After decanting the ether, the pulp was dried in he air and then soaked in water several days. The extract obtained in his way was found to be inactive, but the residue showed very active properties. An attempt was also made to liberate the invertase by heat.[1] A portion of the inner tannin-free material was extracted to remove sugar nd insoluble materials, dried *in vacuo* over sulphuric acid and finally eated to 145-150° C. in a hot air oven for 45 minutes. The activity of the ulp was much impaired, but no active extracts could be prepared from it. n all these cases the protoplasm must have been killed and more or less roken down, so that the retention of the invertase could not have been lue to impermeability of the cell wall. Considering all these facts we find ny other explanation, than that the invertase is insoluble, very difficult to naintain.

The acceptance of insoluble invertase is not so easy because the concept, nzyme, carries with it the idea of solubility. This undoubtedly follows rom the use of the term enzyme synonymously with soluble ferment or norganized ferment, in contradistinction to organized ferment which involves the idea of living matter and is necessarily insoluble.

Before further consideration of the subject it will be well to recall the condition under which an enzymic action may take place. Barring temperature, alkaline or acid reaction, etc., the essential condition is to establish molecular contact. There are four cases:

First, soluble enzyme and soluble substance to be acted upon; reaction follows. Invertase and cane sugar.

Second, soluble enzyme and insoluble substance to be acted upon. Diastase and starch.

Third, insoluble enzyme and soluble substance to be acted upon. Green date invertase or artificially precipitated invertase and cane sugar.

Fourth, insoluble enzyme and insoluble substance to be acted upon; no reaction. Diastase of ungerminated grain and starch granules, and probably many enzymes which have been recognized as existing in the zymogen state.

Insoluble Compounds of Invertase.

That invertase in the green date exists in an insoluble condition but retains all the catalytic properties of the soluble enzyme seems entirely possi-

[1] Buchner, *Ber.*, **30**, 117, 120.

ble. Loew[1] has shown catalase also to exist in two modifications. The insoluble catalase he believes to be a nucleoprotein compound of the soluble form. The writer has already called attention to the fact that tannin does not retard the action of date invertase, although in aqueous solutions the tannin-invertase compound may be filtered off. The tannin-enzyme precipitate, whatever its nature, retains its original catalytic properties. A similar result was observed in the case of the lead subacetate precipitate of date extract. A number of samples of date extract—cane sugar solution mixture was prepared as follows: 40 cc. of glycerol extract of Birket el Haggi date were mixed with 1000 cc. of sugar solution, samples of 85 cc. each were removed with a pipette, placed in 100 cc. flasks, 1 cc. of lead subacetate added to each flask and the mixed contents diluted to 100 cc. Half the samples were filtered at once and the remaining half allowed to stand in contact with the precipitate. One sample from each of the sets was polarized from time to time. Those which stood in contact with the lead precipitate were inverted rapidly, while those from which the precipitate had been removed by filtration were unaffected. The retardation is scarcely more than would be expected from the poisonous effect of the lead on the enzyme as noted by Bokorny for metallic salts in general.

RATE OF INVERSION OF CANE SUGAR BY THE LEAD SUBACETATE DATE EXTRACT PRECIPITATE.

Hours.	Filtered.	Unfiltered.
Initial reading	+13.96	+13.96
18	+14.02	+11.64
42	+14.02	+ 9.62
90	+14.08	+ 4.80
234	+13.70[2]	— 2.22
330	+13.35	— 4.96

The chemical nature of the tannin and lead subacetate precipitates is not known. They may be true chemical compounds with the enzyme itself, such as are formed with the other protein matter, or the removal of the enzyme may have been mechanical, due to adsorption. In either case the enzyme was removed from the solution, but ceased its catalytic activities only after the removal of the precipitate. It is not improbable that compounds of the enzyme could be formed which, while insoluble, would retain all the catalytic properties of the original, provided the substrat was soluble. In other words, the chemical nature of the enzyme molecule as a whole could be changed easily so as to affect its solubility without affecting that portion in which the catalytic properties reside.

[1] Catalase, *U. S. Dept. Agr. Report*, 68, 1901.

[2] The filtered mixture did not remain sterile but became quite turbid after about one week, hence the slight inversion after that time. Had the slight inversion noticed been due to invertase not precipitated by the lead subacetate, its effect would have been most marked in the first hours of action instead of first becoming noticeable after ten days. Microscopic examination showed great numbers of *cocci*.

Hedin[1] has observed that casein solution in 0.2 per cent. sodium carbonate is digested by contact with trypsin adsorbed in charcoal. He finds that the casein actually takes up trypsin from the charcoal and that the trypsin can then be removed by filtration, retaining its proteolytic properties. This he believes to be due to a non-adsorbable compound of the enzyme with the substrat. Such a compound does not seem to exist between cane sugar and date invertase, because the lead subacetate removes all the invertase, even in the presence of cane sugar. If such an invertase-cane sugar compound does exist, it is carried down completely with the lead subacetate precipitate.

Hedin[2] has also studied the selective adsorption of the enzymes and has separated α-and β-protease of ox spleen by adsorption in charcoal. Application is then made of this principle to explain why sometimes more enzyme is obtained by Buchner's process than by others. He suggests that probably kieselguhr and perhaps also the cell residues act adsorptively. One might infer from this, that in the case of the green date, the invertase was held back by simple adsorption. The retention of the enzyme by the green cells, however, is too complete to admit of explanation in this way. Furthermore, if adsorption by the cell residues were responsible, we should not expect so profound a change in the adsorption powers at the time of ripening. I am inclined to believe that the retention of the enzyme as an endoenzyme up to the moment of ripening, has a deeper physiological significance than to be merely accidental to our methods of extraction. I do not contend that no adsorption takes place, for it probably does; but I do not believe that adsorption in the sense used by Hedin could take place almost momentarily and completely.

Insolubility of the invertase during the green stages thus seems to be the only theory which explains the actual facts observed. To determine the mechanism of this insoluble state, however, will require much further investigation. We suggest, tentatively, two general ways in which it could be accomplished by the plant. The enzyme may exist in combination with some other substance and move about freely in the cell sap without being in solution. This hypothetical compound might take the form of dense, minute, or even ultramicroscopic particles, or it might be indefinitely expanded until it occupies the same limits as the medium in which it is suspended, similar to the way caseinogen in milk is supposed to be expanded by the help of calcium phosphate. Along this line, but seemingly less probable, is the recent theory held by Hofmeister and Jacoby.[3] They believe the endoenzyme is localized in vacuoles which are kept isolated by a thin layer of colloid. I fully agree with this idea in so far as it concerns localization,

[1] *Biochem. Jour.*, 1, 484 (1906).

[2] *Ibid.*, 2, 81.

[3] Oppenheimer, Die Fermente, p. 73.

but rather in the manner to be described later under the second possibility. Such a compound would be under the direct control of the cell and could be broken down, leaving the free enzyme in true colloidal solution and capable of passing the protoplasmic layer, which, heretofore, had acted as a filter. In this way there could exist simultaneously both soluble and insoluble enzyme exhibiting the same properties.

The second possibility is that the enzyme is held in some insoluble combination by the protoplasm. This theory was first proposed by E. Fischer for the invertase of Monilia, and seems to the writer to be the most tenable of all, after certain modification. As is well known the protoplasm is exceedingly complex, histologically, being made up of innumerable fine threads and granules. Certain of the granules may be the seat of one enzyme and others of another. This accomplishes the same purpose as the vacuoles suggested by Hofmeister and Jacoby. The enzyme-protoplasm compound which I have assumed to exist, whether it be simple adsorption or a loose chemical combination does not necessitate living protoplasm. The writer therefore suggests the following theory for those cases where endoenzymes exist.

The enzyme is in combination with some constituent of the protoplasm. This combination modifies the enzyme molecule so as to render it insoluble, without, however, affecting its catalytic properties, provided the substrat is soluble. The enzyme-protoplasm compound may or may not break down on the death of the protoplasm. The protoplasm may and usually does liberate the enzyme about the time of maturity of the cell. Slow decomposition, autolysis, or external chemical or physical influence may render the enzyme soluble. The latter possibly act by destroying the integrity of the cell, thus allowing more intimate mixing of the contents and the consequent liberation of the enzyme before its proper time.

Zymase.

The theory outlined above seems to explain adequately the phenomena observed with zymase. Buchner[1] advances and successfully answers three objections which might possibly be raised against his theory of fermentation without living yeast cells. Firstly, the fermentation may have been due to bacteria or yeast cells. Secondly, the evolution of carbon dioxide may have been due to some process other than alcoholic fermentation. Thirdly, the fermentation may have been due to small pieces of living protoplasm passing through the filters.

The idea that zymase exists within the cell as an insoluble compound is not at variance with the observed facts, nor does it invalidate the theory of cell-free or life-free fermentation. If Hans Buchner[2] had maintained that

[1] Lecture before Deutschen Chem. Gesell., *Ber.*, **31**, 568.

[2] *Münchener medic. Wochenschr.*, **1897**, 322.

the fermentation was due to a ferment held in combination by the protoplasm instead of attributing it to particles of the living protoplasm, his objections would not have been answered so easily. Microscopic examinations have shown the entire protoplasmic content of many of the cells to be forced out into the juice by Buchner's process. Thus his results with yeast cells may be explained in two ways other than assuming soluble zymase to exist within the unbroken cell. Some of the insoluble compound may have been easily comminuted till it passed the finest filters. The fact that Buchner sometimes observed 90 per cent. of the activity of his juice to be removed by filtration, speaks for this theory. He attributed the fact to adsorption. Other observers[1] have found the juice to be rendered inactive by similar treatment. Another explanation of Buchner's results may be that by destroying the integrity of the cell, some other substance, probably a protease which has been held isolated by the living protoplasm, comes into contact with the enzyme-protoplasm compound and splits it up. The fact that zymase is rapidly destroyed by a protease after it is extracted from the cell and that it is shielded by adding another protein, such as serum albumin, favors this view.[2] Furthermore the green date which yields no soluble invertase when killed by acetone, chloroform, etc., was found by Slade, while working upon the identification of the enzymes of the date, to contain no, at least only occasionally, traces of protease.

Vegetable Ereptase.

The work of Vines[3] on the vegetable protease requires a somewhat different interpretation when we consider the probability of an insoluble protease. All the phenomena that Vines attributes to vegetable ereptase in his earlier papers can be explained quite as readily by assuming an insoluble protease. As he states, at that time he had never gotten the peptic reaction unaccompanied by the tryptic, but frequently the latter without the former.[4] The peptic reaction is determined by the disappearance of a clot of fibrin, an effect which could never be brought about by an insoluble enzyme. The ereptase reaction is determined by the production of tryptophane from peptone, which could take place easily with an insoluble enzyme. His main indication of the existence of ereptase depends upon the difference in the time of appearance of the two reactions.[5] A clot of fibrin is suspended over ground bean; no reaction takes place till about the 9th day, after which the clot quickly disappears. The same bean material gave the tryptophane reaction with peptone at once. This is exactly what would occur if the bean contained an

[1] Stavenhagen, *Ber.*, **30**, 2422.
[2] Hardin, *Ber.*, **36**, 715.
[3] *Loc. cit.*
[4] Vines, *Ann. of Bot.*, **1905**, 169, 175.
[5] *Ibid.*, **20**, 113, 118 (1906).

insoluble protease which became soluble later. Another indication he finds in the different effect of sodium carbonate on the two reactions when yeast was used.[1] The tryptophane reaction took place readily with a small amount of yeast and relatively large amount of sodium carbonate. On the other hand, a fibrin clot was first appreciably attacked in the presence of 2 per cent. sodium carbonate when 20 per cent. of yeast was present. This is to be expected if an insoluble protease were present which was slowly passing into the solution or even if a soluble protease existed within and was escaping slowly from the yeast cells. Even though the speed of reaction by which sodium carbonate destroyed the protease were very great, some tryptophane would be produced from the peptone, since the chances for molecular contact are very great, because both substances are soluble. On the other hand, contact between protease and fibrin clot would take place only with the molecules on the surface of the latter, a relatively small number, and furthermore the enzyme must travel through a layer of sodium carbonate solution before reaching the fibrin. Thus only when protease molecules in overwhelming number are present can there be any appreciable effect on the fibrin clot.

Recently Vines[2] has succeeded, however, in isolating a purely peptic enzyme from hemp seed. This admittedly renders probable the existence of vegetable ereptase, but does not answer the foregoing objections to his former experiments.

Summary.

The invertase of the date remains insoluble in all ordinary solvents throughout its green stages but becomes readily soluble on ripening. The change in the behavior of the invertase toward solvents coincides very closely in point of time with the passage of the tannin into the insoluble form. Tannin in relatively large amounts does not retard the action of date invertase either in the extract or in the pulp. Soluble tannin, however, hinders the solution of date invertase in water but the invertase can be extracted by glycerol, provided the glycerol is added at the same time the tannin is added. Green date invertase cannot be extracted by crushing and macerating the green fruit with glycerol, therefore the invertase is not rendered insoluble by the escape of soluble tannin on crushing the tannin cells. This conclusion is confirmed by the behavior of the invertase in the tannin-free portion of the date after the tannin-bearing tissues have been completely removed. There is no direct connection between the change in the state of the tannin and that of the invertase.

The inversion of cane sugar by the green date pulp is not accomplished by the living protoplasm liberating a soluble ferment from a pre-existing insoluble zymogen or by rendering active a proferment. The rate of inver-

[1] *Ann. of Bot.*, **289**, 305 (1904).

[2] *Ibid.*, **22**, 103.

sion by equivalent amounts of green and ripe pulp of the same variety are practically identical. Protoplasmic poisons—picric acid, chromic acid, and formaldehyde—retard the action of green and ripe pulp but to approximately the same degree. If the living protoplasm were in any way connected with the inversion, the retardation would be greatly intensified in the case of green date. Green date tissue is not rendered inactive by soaking in ether, chloroform, acetone, etc.

The inner tissues of the green date are composed of relatively tender walled cells, which, unlike those of yeast and unicellular plants, are fixed and cannot glide about freely among themselves; consequently by ordinary grinding, appreciable numbers are broken up and yield their soluble contents to water or glycerol. The press juices contain most of the water in the fruit and large amounts of soluble substances which are usually retained by the healthy and unbroken semipermeable plasmotic membrane. These juices and extracts are invariably free from invertase until the fruit ripens, although the press residues are always very active.

Treatment of the tannin-free green date tissue with chloroform, ether, toluene and acetone causes the protoplasm to collect in granules but does not alter the behavior of the invertase towards solvents. Moreover, the invertase is not liberated by heat. This treatment should destroy the semipermeable nature of the plasmotic membrane. From these observations, the theory of the impermeability of the cell wall for the invertase of green date is untenable.

Enzymic action will take place whenever either enzyme or material to be acted upon is soluble; that is, molecular contact must be established. Tannin removes the invertase of green dates from solution, but inversion is not checked unless the precipitate is filtered off. Date extracts precipitated by lead subacetate still invert cane sugar, but on removing the precipitate inversion is stopped. It is then possible to invert sugar by means of invertase artificially rendered insoluble.

In the place of impermeability of the cell wall to the enzyme, the writer proposes the following theory. It is highly probable that green date invertase and possibly other endoenzymes are held in an insoluble combination by some constituent of the protoplasm. In some cases this combination may be broken down and the enzyme pass into solution while the protoplasm is living, but in others the combination may persist, even after the death of the protoplasm. The enzyme may be rendered soluble also by external chemical or physical influence. These probably act by destroying the integrity of the cell and allowing the contact of substances which have been localized by the living protoplasm. On maturity of the tissues the enzyme is generally liberated, possibly by autodigestion or other profound change in the protoplasm.

In order to establish the impermeability of the cell wall to the enzyme in

any given case it must be shown that the enzyme is in solution in the cell sap and not held in combination by the protoplasm.

UNIV. OF ARIZONA AGR. EXP. STA.,
TUCSON, ARIZONA.

NOTE ON THE DYER METHOD FOR THE DETERMINATION OF PLANT FOOD IN SOILS.[1]

BY FRANK T. SHUTT AND A. T. CHARRON.

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During the past twenty years or so the problem of soil fertility has received much attention from chemists, physicists and biologists, and as a result there is to-day a clearer understanding as to what constitutes productiveness in a soil—its several factors and their relative importance—than there was a quarter of a century ago. We did not then recognize that the chemical data formed but one factor in soil diagnosis; that texture, moisture-holding capacity, bacterial life, drainage, precipitation, etc., must all be considered before drawing any conclusion as to a soil's probable productiveness. It was this neglect that caused doubt on all sides to be entertained as to the practical value of soil analysis, and indeed the agricultural chemist himself had well-nigh abandoned all hope of being able to interpret his own data for the benefit of the farmer. Though we are yet far from having soil diagnosis on a thoroughly satisfactory basis, a distinct advance has been made and the writers feel that one of the most prominent and valuable features of this diagnosis lies in the determination and recognition of the soil's store of more or less available plant food.

In so far as the soil's crop-producing power or the need of special fertilization can be determined by chemical means, the estimation of the plant food present in a condition more or less available for crop use must be a matter of considerable importance. The stores of insoluble, inert plant food, no matter how large, can be of but little value to the growing crop; it is rather those small percentages of potash, phosphoric acid, etc., that are at once capable of being utilized by plants that serve to measure the soil's immediate ability to sustain vegetable life. In these two classes we have represented what might be termed latent and potential fertility, though, of course, no strong line of demarkation can be drawn between them—the former being always converted into the latter, gradually but with varying rapidity, according to a number of conditions which we need not now discuss.

In 1894 Dr. Bernard Dyer, working on soils from the Rothamsted Experiment Station, the history of which as regards manuring and crop yields was well known, proposed the use of a 1 per cent. solution of citric acid as a solvent for the available phosphoric acid and potash.

[1] Read at the Chicago meeting of the American Chemical Society.