THE CHEMICAL NATURE OF ENZYMES

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Efforts to elucidate the chemical nature of enzymes date back many years, and the results of these earlier investigations, as well as those of today, may be grouped into two classes. One indicates that enzymes are of protein nature, and the other attempts to show that these same enzymes are non-protein substances. It will be seen that many of the earlier as well as recent reports have been the basis of erroneous interpretations, and that it is quite clear now that many enzymes are proteins.

THE CHEMICAL NATURE OF PEPSIN

Pepsin is the proteolytic enzyme secreted by certain cells of the gastric mucosa of adult mammals. It is characteristic in being active only in a distinctly acid medium. In the mucosa, however, it is present as a precursor, an inactive form, called pepsinogen or propepsin. Pepsin digests proteins to the proteose–peptone stage. Like other proteases, it splits peptide linkages.

In 1885 Sundberg (76) prepared a pepsin solution which gave negative protein color tests. He extracted the mucosa of the calf's stomach with saturated sodium chloride solution for a few days and autolyzed the extract for about two weeks. Then he precipitated the pepsin with calcium phosphate and redissolved it in hydrochloric acid. After dialysis, this pepsin solution lost most of its activity, but gave no protein color tests. According to Sundberg, however, his pepsin gave a very strong odor, when heated on platinum, resembling that of burning horn. Similar results were reported in 1861 by Brucke (6).

In 1902 Pekelharing (61) published a very interesting paper on pepsin. He purified gastric juice and acid extracts of the gastric mucosa of the pig by dialysis and found that the pepsin separated in refractive globules. These preparations of Pekelharing were very active.

Northrop (54) found that the precipitate which formed in the dialyzing sac when the procedure of Pekelharing was followed, appeared in more or less granular form and filtered rather easily, as though it were on the verge

of crystallization, and that "this precipitate dissolved on warming the suspension and it was eventually found that it could be induced to crystallize by warming to 45°C., filtering, and allowing the filtrate to cool slowly." Northrop has succeeded in working out a method by which pepsin can be obtained in crystalline form in large quantities. Precipitation by magnesium sulfate in acid medium and solution in alkali with subsequent acid precipitation is the principle of his method. Northrop has recrystallized pepsin seven times and has found by chemical and physical tests that the enzyme is a protein. A typical experiment is the heat inactivation of

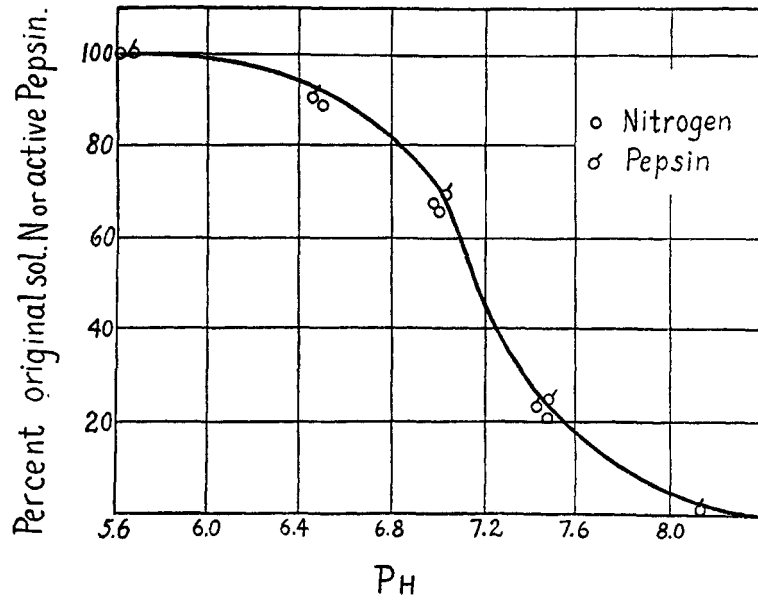

FIG. 1. PERCENTAGE INACTIVATION AND PERCENTAGE DENATURIZATION OF CRYSTALLINE PEPSIN AT VARIOUS pH VALUES AT 20°C.

(Northrop: J. Gen. Physiol. **13**, 739 (1930))

pepsin. The latter parallels the denaturization of the protein. Percentage activity left after heat inactivation is the same as percentage of soluble nitrogen remaining (see figure 1). Northrop (57) has also obtained crystalline pepsin from the bovine gastric juice.

According to Levene and Helberger (41) crystalline pepsin is in the main an individual protein, but it probably contains a small admixture of an extraneous protein.

A separation of crystalline pepsin into two fractions having different gelatin-liquefying power has been reported by Holter (20). That crystalline pepsin contains a small amount of an enzyme with high gelatin-liquefying power was first stated by Northrop (55).

Loughlin (45) has prepared crystalline pepsin by Northrop's method. This product was identical with that of Northrop, both in general properties and in specific proteolytic activity.

Recently, Willstätter and Rohdewald (98) verified the findings of Sundberg and those of Brucke on the preparation of a protein-free pepsin solution. They, too, state that the activity of their solution decreased considerably in the course of preparation, resulting in a very slightly active *solution*, but that this solution gave no protein color tests. Tauber (78) has shown that these findings of Willstätter and Rohdewald were based on an erroneous interpretation, since active enzyme solutions may be too dilute to give positive protein color tests. As to Northrop's crystalline pepsin, Willstätter and Rohdewald (98) do not believe that these crystals are identical with the enzyme pepsin. Willstätter has been a staunch supporter of the carrier theory. Generally by a "carrier" is meant any colloid of high molecular weight, such as a protein or a carbohydrate which carries the active enzyme. This carrier is said to be exchangeable for other carriers. The enzyme itself, according to this theory, is a substance which cannot be classified with any of the known chemical compounds. However, Willstätter and Rohdewald have now modified the carrier theory by saying that enzymes have a "necessary colloidal carrier," and that the chemical nature of the *latter* is still unknown.

Glutathione acts in every way like an enzyme, and if it were inactivated upon heating its aqueous solution, it would be classified as an enzyme. In that case, one might say that the tripeptide is only the carrier of the active principle. Colloidal platinum has an enormous catalytic effect as compared to solid platinum. Is the colloidal platinum a carrier of an active substance or principle? It should be noted that the carrier theory is not new. J. Perrin (62) proposed it as early as 1905, and Mathews and Glenn (48) wrote in favor of it in 1911.

Dyckerhoff and Tewes (9) and Waldschmidt-Leitz and Kofranyi (90) published experiments purporting to show that crystalline enzymes are really crystalline proteins carrying the non-protein enzymes. They have compared Northrop's crystalline pepsin with edestin and other crystalline plant proteins which have been in suspension in pepsin solution. These proteins were found to be just as active as the crystalline pepsin, and accordingly these workers maintain that the active principle has exchanged its carrier. Northrop (58) repeated the work of these authors and found that, "The pepsin protein is taken up as such and the quantity of protein taken up by the foreign protein is just equivalent to the peptic activity found in the complex." Results similar to those of Northrop were reported by Sumner (71), who absorbed crystalline pepsin on casein and ovalbumin.

Dyckerhoff and Tewes stated that rennin acts in a similar manner, i.e..

it may be absorbed on crystalline edestin in active form, exchanging its carrier. However, they do not furnish experimental evidence concerning the absorption of rennin. Tauber and Kleiner (84), interested especially in the nature of rennin, have attempted to prepare "crystalline edestin–rennin," using methods similar to those of Dyckerhoff and Tewes and of Waldschmidt-Leitz and Kofranyi. Preparations of only slight activity were obtained, indicating that no exchange of the protein carrier took place.

THE RENNIN–PEPSIN CONTROVERSY AND THE CHEMICAL NATURE OF RENNIN

The controversy as to whether rennin and pepsin are separate enzymes is of early date. Hammarsten on the one hand represented those who believed in the existence of a separate enzyme, rennin having only milk-clotting power; Pavlov, on the other hand, was the leader of a school which maintained the existence of a mammoth molecule with side chains acting proteolytically at an acid pH and rennetically at the neutral point. The latter view has been supported by the fact that all proteases are able to clot milk.

According to Linderström-Lang (1928) the casein of the milk is a system of three components, one of which acts as a protective colloid for the other two. Any proteolytic enzyme has the ability to attack the protective colloid and under proper conditions (presence of calcium, pH, etc.) coagulation of the casein takes place. The clot is called calcium paracaseinate, and is insoluble. An attack on the casein, however, takes place also in the absence of calcium. Then paracasein, a soluble complex, forms, which subsequently may be precipitated on the addition of soluble calcium salts. In contrast to the very acid pH required for peptic activity (i.e., the digestion of other proteins by pepsin), pepsin clots milk at a practically neutral pH. It is not known whether the milk-clotting activity of rennin is a proteolytic process or not, i.e., whether opening of peptide linkages takes place.

Luers and Bader (46) have recently reported an attempt to separate rennin from pepsin using the adsorption method. These authors found their preparation to be nearly free of protein, containing only 0.678 per cent nitrogen, possessing high rennet activity as well as relatively high peptic activity. They state that their results do not allow them to decide in the favor of two distinct enzymes, rennin and pepsin.

Edie (10) obtained a pepsin preparation free of milk-coagulating power.

Tauber and Kleiner (81) have been able to isolate by isoelectric precipitation an extremely active rennin preparation, from fresh fourth stomachs of the calf. This possessed the highest activity so far reported. The minced mucosa was extracted for 8 minutes with 0.04 *N* hydrochloric acid.

The clear filtrate was dialyzed against distilled water until the pH was about 5.4. Then alcohol was added up to 50 per cent, which precipitated the rennin. The precipitate was dissolved in water freed from insoluble matter and precipitated as before. This procedure was repeated several times.

For peptic activity determination, the formol titration method was employed, and the increase of nitrogen not precipitated by 10 per cent trichloroacetic acid was measured by the micro-Kjeldahl method. Coagulated egg albumin of pH 2.0 was used as a substrate. This rennin contained no pepsin, since it did not produce an increase in formol titration and only a negligible increase in nitrogen not precipitated by trichloroacetic acid. It clotted 4,550,000 times its weight of milk (pH 6.2) in 10 minutes at 40°C. This is more than 100 times as active as Parke, Davis and Company's 1:30,000 preparation, and 2000 times as active as the original extract of the calf's mucosa. Crystalline pepsin has less milk-clotting power than concentrated rennin.

The vacuum-dried rennin differs chemically from crystalline pepsin in many ways. It is very readily soluble in slightly acidified water; it is not coagulated by heat, whereas pepsin is. It gives a pink biuret test, whereas pepsin gives a violet color. The Millon and Hopkins-Cole tests are not given. In contrast to pepsin, rennin diffuses quickly through dialyzing membranes. The isoelectric point of rennin is at 5.4, whereas that of pepsin is at 2.7. Pepsin contains phosphorus, whereas rennin does not. *Pepsin is an albumin; rennin a thioproteose.* These results of Tauber and Kleiner on the purification of rennin have been verified by Holter (21) working in the laboratory of Sörensen.

Thus, it is now obvious that rennin, the protease of the young mammal's gastric mucosa, and pepsin, the protease of the adult mammal's gastric mucosa, are distinct entities. It should be noted, however, that the precursors of these enzymes and not the active enzymes are present in the mucosa. (See also below.) Rennin is a milk-clotting enzyme; pepsin a typical protein-hydrolyzing enzyme with considerable milk-coagulating power.

THE CHEMICAL NATURE OF TRYPSIN

Trypsin as obtained from and secreted by the pancreas is almost completely inactive, and as such it is called trypsinogen (precursor). It can be activated, however, to an extremely powerful enzyme, if an activator, called "enterokinase" and prepared from the mucosa of the small intestine, is added to it. Trypsinogen, however, becomes active ("autoactivation") without the addition of enterokinase on standing at room temperature for several days. This may be due to activation by slightly active tissue

proteases. Trypsin digests practically all proteins, including proteoses and peptones, to polypeptides. The polypeptides are digested by specific peptidases, produced mainly by the intestinal mucosa. The optimum pH of trypsin, as also of all other enzymes, varies slightly with the substrate used. It is at about 8 to 9. Trypsin is also active in slightly acid solution.

A crystalline protein possessing tryptic properties has been obtained by Northrop and Kunitz (56, 59) from beef pancreas. A large number of experiments have been carried out, which showed that the protein crystals are identical with the proteolytic enzyme. This trypsin does not become more active on the addition of enterokinase. It is completely inactive towards peptides, has a slight milk-clotting power, an isoelectric point of about 7.0, and an optimum pH of 8.0 to 9.0 when casein is used as a sub-

TABLE 1

Showing that trypsin can clot milk only within a certain range of concentration

Tauber and Kleiner (85)

TRYPSIN	RESULTS AFTER 10 MINUTES	TRYPSIN	RESULTS AFTER 10 MINUTES
per cent		*per cent*	
2.00	No clot	0.10	Slight clot
1.00	No clot	0.08	Heavy curd
0.75	No clot	0.05	Heavy curd
0.50	Slight clot	0.03	No clot
0.20	Slight clot		

To 10-cc. samples of clacium chloride-milk, 1 cc. of each of the various trypsin solutions was added. The pH of this milk was 6.4, as determined by the electrometric method. Temperature, 40°C.

After 5 hours results remained the same.

strate. The milk-clotting of this trypsin, according to Northrop and Kunitz, is due to a contamination of the trypsin by a milk-coagulating enzyme. Tauber and Kleiner (85), however, have shown that the trypsin itself can clot milk, but only within a certain limited range of concentration; if too dilute or if too concentrated, no coagulation will occur (see table 1). When concentrated trypsin solutions, like those used in protein digestion experiments, are employed, trypsin changes the casein molecule so rapidly beyond the paracasein stage, that the milk will not clot even after the subsequent addition of a very active rennin solution (see table 2). Thus, Tauber and Kleiner have definitely established that the milk-clotting power is a function of the trypsin molecule. They also found that the velocity of milk coagulation in all three cases—rennin, pepsin, and trypsin—is proportional to the hydrogen-ion concentration, and if the milk is adjusted to a low pH so that the proteolytic activity of the (concentrated) trypsin is depressed, coagulation may occur (see table 3).

Kleiner and Tauber have recently obtained from pancreatic tissue, by complete autolysis (eighteen months), dialysis, and acetone precipitation,

TABLE 2

Showing rapid effect of trypsin on casein

Tauber and Kleiner (85)

EXPERIMENT NO.	PER CENT OF TRYPSIN SOLUTION	RESULTS AFTER 10 MINUTES	AFTER 10 MINUTES OF TRYPTIC DIGESTION 1 CC. RENNIN SOLUTION CONTAINING 13,000 UNITS* PER CC. WAS ADDED	REMARKS
1	0.015	No clot	Clot in 8 min.	Trypsin too dilute to affect casein molecule (or complex)
2	0.030	No clot	Clot in 7 min.	
3	0.300	Clot	No rennin added	At this pH (6.4) and concentration trypsin readily clots milk
4	0.600	No clot	No clot in 5 hrs.	Trypsin solutions of these concentrations change casein to such degree that insoluble Ca paracaseinate cannot form, even on addition of active rennin
5	1.000	No clot	No clot in 5 hrs.	
6	4.000	No clot	No clot in 5 hrs.	

To 10 cc. of calcium chloride–milk of pH 6.4, 1 cc. of the trypsin solution was added. Temperature, 40°C.

* A unit is the amount of milk (pH 6.2) in mg. clotted by 1 mg. of rennin.

TABLE 3

Milk-clotting activity of rennin, pepsin, and trypsin at various pH values

Tauber and Kleiner (85)

pH	CLOTTING TIME OF RENNIN	CLOTTING TIME OF PEPSIN	CLOTTING TIME OF TRYPSIN
	minutes	*minutes*	*minutes*
5.6	3.5	1.5	3.0
5.9	4.5	2.0	4.0
6.1	8.0	3.0	6.0
6.4	10.0	11.0	No clot in 40 min.*
6.6	29.0 very slight	No clot in 40 min.	No clot in 40 min.*
6.8	36.0 very slight	No clot in 40 min.	No clot in 40 min.*

The pH of the milk was adjusted with hydrochloric acid or sodium hydroxide and electrometrically controlled. To 10-cc. samples of milk, 0.5 cc. of each of the enzyme solutions was added. The trypsin solution was 1 per cent. The pepsin solution contained 18,000 rennet units per cubic centimeter. The rennin solution had an activity of 16,000 units per cubic centimeter. Temperature, 40°C.

* No clot occurred after adjustment to pH 5.6.

a trypsin preparation which does not resemble a protein of the ordinary type. The activity of this preparation is about the same as that of crystal-

line trypsin. The dry enzyme is very soluble in water and dilute alkali, slightly soluble in dilute mineral acids, and insoluble in organic solvents. It dialyzes readily.

The protein color tests as obtained with a 1 per cent solution were as follows: Xanthoproteic test, slightly positive; Folin-Denis test, positive; various modifications of biuret test, negative; Millon's test, negative; heat coagulation test, positive only on prolonged boiling, when a slight precipitate was obtained. The isoelectric point is at pH 6.2. This trypsin may be either a broken-down product of protein, or a protease differing from that obtained directly from fresh pancreatic tissue. The elementary composition differs considerably from that of crystalline trypsin. The milk-clotting power is about one-thousandth that of rennin. The milk-coagulating power is a function of the trypsin molecule.

CHYMOTRYPSIN

An interesting crystalline proteolytic enzyme has been recently isolated by Kunitz and Northrop (38) from fresh beef pancreas. The enzyme as first obtained is in an inactive form and cannot be activated by enterokinase. This inert form is called chymotrypsinogen. If, however, a very small amount of active trypsin is added, the inactive chymotrypsin becomes very active. The gelatin-hydrolyzing power of this enzyme is less than that of trypsin, but its milk-clotting power is greater. The chymotrypsinogen has been recrystallized ten times with unchanged optical activity and constant proteolytic activity when activated. The preparations are pure proteins and "the proteolytic activity is a property of the protein molecule."

Thus another enzyme has been obtained in crystalline form and another controversy has been settled, since it had previously been uncertain whether trypsinogen may be activated by a small amount of active trypsin.

THE CHEMICAL NATURE OF PAPAIN AND CATHEPSIN

Papain is a plant protease obtainable from the fruit or milky juice of the melon tree (*Carica papaya*). Its optimum pH is about 7.0. Cathepsin is an animal tissue protease, having also an optimum pH of about 7.0.

Papain is not completely active when prepared from the plant material, but is readily activated with hydrogen sulfide. Bersin and Logenmann (4) explain the activation of papain by reduced glutathione as a reduction of the oxidized form of the enzyme having the S—S linkages. They inactivated papain with hydrogen peroxide and reactivated it with hydrogen sulfide or with other reducing agents such as sodium sulfite and reduced glutathione. A possibility of an active hydrogen group in the enzyme is suggested, since diazomethane inactivates the enzyme.

Bersin (3) offered further evidence that the activation of a glutathione-free papain is due to the reduction of the S—S groupings present in the enzyme–protein complex to SH. He activated papain by a number of reducing agents such as hydrogen sulfide, glutathione, hydrogen cyanide, sulfur dioxide, cysteine, and succinate plus dehydrogenase. Ultra-violet irradiation likewise produced an activation which may be explained by the recent observation that cysteine was formed from cystine by similar treatment. Organic arsenic compounds and such inorganic arsenic compounds as have the ability to reduce the S—S group to the SH group, also activate papain. Ascorbic acid, however, could not activate papain, which Bersin explains to be due to the fact that it does not form thiol derivatives from disulfides. Contrary to Waldschmidt-Leitz (89), Bersin could not activate phosphatase with glutathione. Purr (64) activated cathepsin, using a carcinoma–glycerol suspension, by ascorbic acid with practically the same degree of activation as was obtained when cysteine was used as an activator. He also activated liver arginase with ascorbic acid; ascorbic acid–iron was found to be the best activator.

Karrer and Zehender (28) freed liver cathepsin from its natural activators and were likewise able to reactivate it with ascorbic acid. The degree of activation was of the same magnitude as that of hydrogen sulfide activation.

These findings are interesting, since papain cannot be activated by ascorbic acid. From a physiological point of view, therefore, ascorbic acid appears to be more than only an antiscorbutic. Combined with glutathione, it catalyzes important oxidation and reduction systems, which may be reversibly oxidized and reduced establishing equilibrium between the S—S and SH compounds. Without these combined effects, certain intracellular enzymes would not be able to carry out their metabolic function, as indicated by the experiments of Purr and those of Karrer and Zehender.

THE CHEMICAL NATURE OF UREASE

Urease is found in large amounts in the seed of *Leguminosae*, and has been obtained in small amounts from various animal organs as well as from certain bacteria. The best sources of urease are jack bean and soy bean. It hydrolyzes urea into ammonia and carbon dioxide, and has an optimum pH of 7.

Urease was the first enzyme to be obtained in crystalline form. The rather simple method for the crystallization of this enzyme was described by Sumner in 1926 (70). The principle of his procedure is as follows: Jack bean meal is extracted for a short time with 30 per cent acetone and left to filter overnight in a refrigerator. Octahedric, microscopic crystals which

separate may be recrystallized from either dilute acetone or dilute alcohol. The activity of the crystals is considerable. The yields of urease crystals are small, however. Since the method of crystallization is very convenient, it should be used whenever a pure preparation of urease is desired. Sumner's results on the preparation and activity of crystalline urease were confirmed by Tauber in 1930 (77).

Crystalline urease gives all the protein color tests and the test for unoxidized sulfur. It is a globulin having an isoelectric point of about pH 5.0 (72).

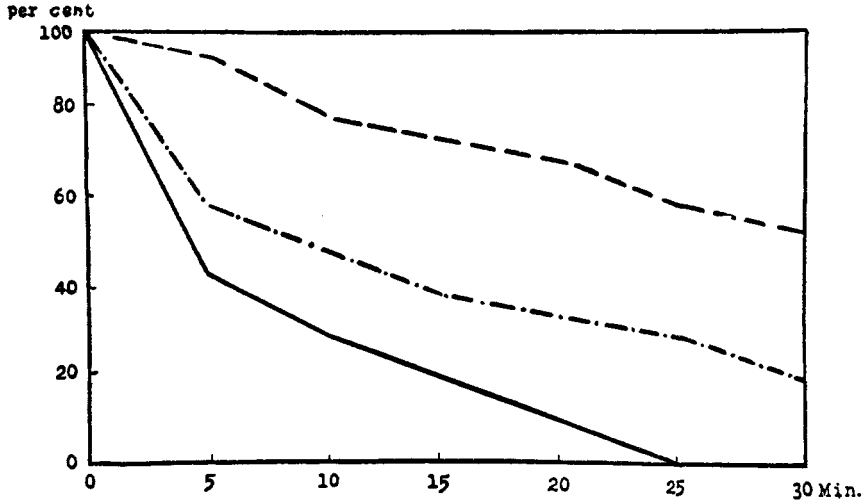

FIG. 2. EXPOSURE OF CRYSTALLINE UREASE SOLUTIONS TO ULTRA-VIOLET RAYS

———— represents 5 cc. of urease, without eosin, at 41°C. exposed at 25 cm.; ---------- represents 5 cc. of urease, without eosin, at 30°C. exposed at 50 cm.; -·-·-·-·- represents the same plus 0.05 cc. of 0.01 per cent eosin. The function of the eosin is that of a photodynamic agent. (Tauber: J. Biol. Chem. **87**, 625 (1930).)

A controversy as to the protein nature of urease between Waldschmidt-Leitz and Steigerwald (92) and Sumner and coworkers (73, 74) appears to be settled in favor of the latter workers, i.e., crystalline urease is a protein.

Ultra-violet irradiation inactivates crystalline urease (figure 2), which is apparently due to a molecular rearrangement of the protein molecule (77).

Hotchkiss and Tauber (22) have shown that crystalline urease solutions produce agglutination of erythrocytes. Agglutination did not inactivate the urease. With whole blood from dead rabbits no agglutination could be demonstrated, since serum tends to inhibit this reaction. Crystalline urease was found to be a highly purified form of a toxic hemagglutinin. The minimum effective dose was 0.00014 mg. of the enzyme in 1 cc. The

hemagglutinin obtained from the navy bean by Goddard and Mendel (15) was effective with a minimum dose of 0.0006 mg. in a total volume of 1 cc.

The toxicity of urease when injected into the animal body was ascribed to various causes. The results of Tauber and Kleiner (79), who also studied the toxicity of ammonium carbonate, point directly to ammonia poisoning, as was also suggested by Carnot and his coworkers (8) and by Lövgren (42). The minimum lethal dose for mice was determined by Kleiner and Tauber, and was found to be 0.09 Sumner units of urease per gram of body weight.

An immunity to urease was acquired by the experimental animals (79), and Sumner (30) described a method for the preparation of antiurease. They also found urease poisoning to be the result of ammonia intoxication. According to Howell (24) the hen is not poisoned by urease since it has only 2 mg. of urea per 100 cc. of blood; antiurease is produced, however.

Jacoby (27) studied the effect of cupric chloride, mercuric chloride, and silver nitrate on urease and its reactivation with potassium cyanide. At low temperatures the inactive urease–metal compound can be completely reactivated with potassium cyanide; if, however, the temperature is raised to 37°C., the inactivation is irreversible. Sumner, Lloyd, and Poland (75) recrystallized urease several times and found that it gave the same amount of red color in the nitroprusside test, indicating a sulfhydryl group in the urease molecule. According to these authors, this is the reason for the rapid destruction of urease by silver, mercury, and copper ions and by quinone and other oxidizing agents. The protection of urease by sulfhydryl compounds is thus explained.

Hellerman, Perkins, and Clark (19) subjected urease to oxidation and reduction. Urease solution, completely inactivated (by cuprous oxide), was reactivated by hydrogen sulfide to 83 per cent. Benzyl mercuric chloride inactivated a urease solution completely. An excess of hydrogen sulfide effected complete reactivation. If urease solutions are aerated in the presence of Cu^{++} rapid destruction of the enzyme takes place; sulfhydryl groups, however, cause reactivation. When urease is inactivated by iodine, potassium cyanide does not restore activity, but hydrogen sulfide does. The authors offer the following explanation for the above results: " . . . the more drastic action of iodine might involve a deep seated change due to a permanent substitution in the protein molecule as well as an oxidation of thiol sulfur beyond the dithio stage, in which case irreversible inactivation of a labile, highly specific protein enzyme molecule would be anticipated. On the other hand, more superficial substitutions in the protein molecule accompanying the oxidation of thiol to dithio, might be wholly reversed by such a reagent as H_2S but not necessarily by KCN."

Similar studies of active groups in enzyme molecules and their inactiva-

tion and reactivation may throw light on the constitution of enzymes. Extensive investigations of such a nature are highly suggestive.

THE CHEMICAL NATURE OF THE YELLOW OXIDATION ENZYME

Warburg and Christian (93) prepared oxidation enzymes from various sources such as anaerobic bacteria, chlorella, and bottom yeast. Unlike the pheohemin of aerobic cells, oxidation enzymes are not poisoned by carbon monoxide or hydrogen cyanide. The oxidation enzyme of bottom yeast has been obtained in pure form and extensively studied by Warburg and Christian. It does not dialyze through cellophane and is destroyed when heated above 60°C. The aqueous solutions of the enzyme have a yellow-red color. The color disappears if treated with reducing agents, but it reappears if the solution is shaken with oxygen. The reduced form does not absorb in the visible spectrum and is called the "leuco form." The oxidation of the leuco enzyme is much quicker when methylene blue is used instead of molecular oxygen, and by titration with the former the concentration of the active group may be determined. The "enzyme" has the ability to transfer oxygen to Robison's hexosemonophosphate ester, the colored form being reduced by this process. Molecular oxygen reoxidizes the leuco compound *by direct chemical reaction. Since, like hemoglobin, this oxidation enzyme combines chemically with oxygen, it is obvious that it cannot be called an enzyme.* The reduction of the colored compound by hexosemonophosphate, however, is, according to Warburg and Christian, a catalytic effect. It requires a second enzyme and a coenzyme. The hexosemonophosphate, the second enzyme, and the coenzyme, are colorless and are called the "reducing system."

The coenzyme may be prepared from red blood cells and when purified until it is free of protein, it does not give a precipitate with trichloroacetic acid. It should be noted, however, that certain derived proteins, like peptones, do not give a precipitate with the latter. The coenzyme is sensitive to acid and alkali, but is stable in neutral solution.

The second enzyme (the enzyme of the reducing system) may be obtained from baker's yeast extracts prepared according to Lebedew's method. This enzyme does not dialyze through cellophane membranes. It is destroyed when heated at 60°C. for 15 minutes.

The oxidizing agents in the aerobic cell are molecular oxygen and ferric iron of pheohemin. The ferric iron reacts more rapidly with the leuco form of the colored pigment than the molecular oxygen, and consequently there is no direct reaction between molecular oxygen and the leuco form of the yellow-red dye, and the oxidation follows this course: $O_2 \rightarrow$ pheohemin (Fe^{++}) $\rightarrow$ pheohemin $\rightarrow$ leuco form of dye $\rightarrow$ pigment $\rightarrow$ "reducing system" (I). (Fe^{+++})

If, however, the pheohemin is inactivated by carbon monoxide or hydrogen cyanide then the course is the following: $O_2 \rightarrow$ leuco form $\rightarrow$ pigment $\rightarrow$ "reducing system" (II). Generally a decrease in the rate of oxidation takes place, if I is replaced by II.

Warburg and Christian (93) state that they have separated the yellow-red oxidation enzyme into a crystalline pigment (enzyme) "component" and a "protein carrier." Both, however, were obtained in an inactive form. They report: "In dem Ferment ist der Farbstoff an Eiweiss gebunden. Man kann Farbstoff-und Eiweiss Komponente trennen, indem man das Ferment bei 38° mit Methanol–Wasser (1 Vol. Wasser, 3 Vol. Methanol) schüttelt. Dann wird dass Eiweiss denaturiert, der Farbstoff geht in Lösung. Die eiweissfrei Lösung des Farbstoffs fluoresziert grün. Sie ist *katalytisch unwirksam*, aber in Bezug auf ihr Absorptionsspectrum (im sichtbaren) der Fermentlösung sehr ähnlich."[1]

THE CHEMICAL NATURE OF LIPASE

Pancreatic lipase is secreted by the pancreas. It hydrolyzes fats into fatty acids and glycerol. A specific lipase is present in the gastric mucosa, which hydrolyzes only emulsified fats such as the butter fat of milk and the fat of the egg yolk. Plants, too, contain lipases; *Ricinus communis* is one of the best sources.

Glick and King (14) used 10 per cent sodium chloride for the extraction of lipase from dried pancreas tissue and obtained more powerful preparations than were obtained by solvents used in earlier work. The lipase may be salted out quantitatively by saturation with magnesium sulfate. Magnesium salts activate the lipase. Expression of enzyme activity in units per milligram of nitrogen is suggested. The purified enzyme shows the general characteristics of a globulin.

Bamann and Laeverenz (2) described an accidental observation of a "crystalline lipase protein." They found that if a certain dry lipase preparation was extracted with 25 per cent glycerol and allowed to filter slowly for 21 days, active colorless needles, 2 mm. long, separated from the dark yellow filtrate. The chemical nature of this crystalline lipase preparation has not been further studied by these authors.

THE CHEMICAL NATURE OF PANCREATIC AMYLASE

Amylases are starch- and glycogen-splitting enzymes, and the end-product of hydrolysis is maltose. Saliva, the pancreas, and the liver are good sources of animal amylase. In plants, the germinating seed contains large amounts of this enzyme. The optimum pH of amylases differs

[1] For an extended discussion of the respiratory enzymes see H. von Euler's recent book,—Die Katalasen und die Enzyme der Oxydation und Reduction. J. Springer, Berlin (1934).

greatly; for instance, pancreatic amylase has an optimum pH of about 7, salivary amylase of 6.0, and malt amylase of about 5.0. These enzymes are peculiar in being inactive in the absence of neutral salts.

A crystalline protein with high amylolytic activity has been obtained from pancreatin by Caldwell, Booher, and Sherman (7). Waldschmidt-Leitz and Reichel (91), however, state that they have obtained an active protein-free amylase from pancreatic tissue. Salivary amylase is digested by trypsin, pointing to the protein nature of this amylase (Tauber and Kleiner).

Sherman, Caldwell, and Doebbeling (67) have purified malt amylase, which yields products of much higher activity than any previously reported. These authors attempted to purify their enzyme by means of adsorption by alumina gel and subsequent elution. This method was introduced at various stages in the purification process. However, it was found that this adsorption procedure was relatively inefficient when compared with precipitation by ammonium sulfate and alcohol. When both the precipitation and the adsorption procedures were used in conjunction, there was no increase in the activity of the final preparation. The purified product contains about 16 per cent nitrogen, shows all the protein color tests, and is precipitated and denatured as is a typical protein. It was found that denaturation of the protein was proportional to the loss of enzymic activity. The authors believe that their purified material appears to correspond to the β-amylase discussed by Kuhn (34).

CHEMICAL NATURE OF CARBONIC ANHYDRASE

In 1932, Roughton and Meldrum isolated from ox blood a white substance of which 1 part in 10,000,000 is able to catalyze the formation of carbon dioxide from a buffered bicarbonate solution of a physiological pH. The dry substance appears to be free from hemoglobin and heme compounds. It has the typical characteristics of an enzyme, but differs from any of the known enzymes of the blood. As to the chemical nature of the purest preparation, it resembles that of a protein and it gives all the protein color tests. Its molecule, however, seems to be of a small size judged by the fact that the enzyme tends to pass through ultrafiltration membranes. The physiological function of the carbonic anhydrase is to accelerate the carbon dioxide formation from bicarbonate in the pulmonary blood vessels. The possibility of numerous other functions within the animal body is suggested, such as the removal of carbon dioxide from active tissue, catalysis of decarboxylation, and the formation of urea in the liver. An account of the discovery of the new enzyme, purification, activity determinations, etc., and review of the literature has been given in an article by Roughton (65).

THE CHEMICAL NATURE OF CATALASE

Catalase is found in abundance in animal and plant tissues. It decomposes hydrogen peroxide into water and oxygen, but it does not decompose other peroxides. Physiologically it is very important, since it destroys hydrogen peroxide (a toxic intermediate product of oxidation) within the living tissue. Another biological function of catalase is its dehydrogenation function. It furnishes oxygen for dehydrogenation.

Michaelis and Pechstein (51) and Stern (68) describe the enzyme catalase as an ampholyte of a high molecular weight, with an isoelectric point of about pH 5.5. Waentig (88) found catalase to be a protein containing 14 per cent nitrogen. Stern (69) determined the diffusion velocity of the catalase particle and states that it is similar to that of hemoglobin. According to Zeile and Hellstroem (100), catalase is a heme (ironporphyrin) compound. Kuhn, Hand, and Florkin (35) claim peroxidase to be a heme compound. This could not be corroborated, however, by Elliot and Sutter (11). Boyd (5) confirmed Howell's (23) earlier finding that hematoporphyrin in the presence of light hydrolyzes fibrinogen, so that it loses its power to clot with thrombin or coagulate on heating; nor does it precipitate upon half-saturation with sodium chloride. Furthermore, he was able to digest serum albumin in the presence of light with hematoporphyrin. Oxygen is essential for hydrolysis. "Hematoporphyrin unites with the protein, becomes excited through the absorption of light energy, combines with oxygen, and passes a part of the energy over to the protein molecule, thereby activating the latter and causing it to become hydrolyzed." Boyd calls hematoporphyrin an artificial proteolytic enzyme.

DIGESTION OF ENZYMES BY PROTEASES

As shown above, the carrier theory, based largely on theoretical considerations, has been found to be untenable. Obviously, some method must be employed to throw light on this problem, and the digestibility of enzymes by proteases has recently been used to a considerable extent.

Tauber (77) found crystalline urease to be digestible by trypsin in the presence of a colloid such as gum arabic. The time required for complete inactivation of the urease was three days. Tauber and Kleiner (80) found gum arabic or gum ghatti to be essential for the digestion of urease by trypsin and found that no digestion of the urease took place if the addition of the gum was omitted, thus confirming the finding of Waldschmidt-Leitz and Steigerwald (92), that urease cannot be digested by trypsin in the absence of a gum. If trypsin is added to urease before the addition of the gum, no inactivation takes place. An inhibitive (antitryptic) group of the urease molecule combines with trypsin to form an irreversible inactive

trypsin compound which cannot digest the urease but has unchanged ureolytic activity. In the presence of a gum, this compound cannot form because the gum unites with a part of the urease molecule. Tryptic activity is unimpaired and the urease is digested. Tauber and Kleiner suggested that crystalline urease may possess "antitryptic" properties. Woodhouse (99) reported that gum arabic and gum tragacanth were able to augment definitely the hydrolysis of olive oil by lipase. According to Sumner, Kirk, and Howell (72) urease is rapidly inactivated by pepsin and by papain–hydrogen sulfide, and inactivation coincides with the digestion of the protein crystals.

It has also been shown by Tauber and Kleiner (82) that maltase is readily digested by trypsin. Tauber (78) found emulsin to be indigestible by pepsin, trypsin, and pancreatin during several weeks incubation at 40°C. Protein-free emulsin, however, could not be obtained by methods of earlier investigators, nor could the active principle be freed from the protein by other procedures. At the present time, no explanation can be given for the indigestibility of emulsin. It should be noted, however, that not all proteins are digestible by trypsin and pepsin, and that emulsin may not be a protein of the ordinary type. As an example, the heminproteose of Haurowitz (18) may be mentioned. Haurowitz digested oxyhemoglobin, CO-hemoglobin, and reduced hemoglobin by pancreatic extracts, all at approximately the same rate. Putrefactive bacteria have an inhibitive effect on the digestion. About 95 per cent of the globin is split off as dialyzable products and the residual 5 per cent forms a new combination with the protohemin fraction, which is amorphous and which Haurowitz calls heminproteose. This protein component is resistant to pepsin and trypsin. Rennin, as found by Tauber and Kleiner (81), is digested by trypsin with amazing rapidity. Pepsin digests rennin also with a fair degree of rapidity, but erepsin does not. The digestion of rennin by pepsin supports earlier evidence which Tauber and Kleiner (81) brought forward for the protein nature of rennin, and for the identity of rennin as an entity distinct from pepsin. Therefore, the claim that rennin exists in the adult mammal's stomach is quite paradoxical. Moreover, it has been experimentally proven that there is no rennin in the adult mammal's stomach (83). Another point of differentiation between these two enzymes is the difference in the pH of activation of their respective zymogens. Recent experiments of Dr. I. S. Kleiner and the author, to be published soon, corroborate their earlier findings as to the existence of individually differing zymogens of rennin and pepsin.

Schulman and Rideal (66) studied enzyme inactivation and distinguished between two activities,—bulk phase reaction and surface reaction. Pancreatin solutions were digested by pepsin at pH 2, and the pH of the solu-

tion was adjusted to pH 8. For "bulk phase reaction" no tryptic activity was observed; monolayers ("surface reaction") of egg albumin, however, are readily digested. Trypsin solutions which have been boiled at pH 8.0 behave in a similar manner. The authors' conclusion is: "In the tryptic enzymes, the 'free enzymes' can be separated from their protein-like carriers, but when free can only react with proteins when presented to them in a suitable form such as a monolayer at an air–water interface." They found, however, that proteases inactivated by acids or alkalies or poisoned by heavy metals lose both bulk and surface activities equally.

Tauber and Kleiner (86) subjected pepsin and trypsin to reciprocal digestion. At pH 2.0 pepsin completely digests trypsin, leaving the former unchanged. This takes place in a relatively short time. At this pH the trypsin is inactive but not destroyed. If, however, a mixture of the two enzymes is kept at pH 5.5, which does not permit peptic activity (but does not inactivate pepsin), the pepsin is digested by trypsin. That trypsin may be digested by pepsin had also been found by Long and Johnson (44), by Long and Hull (43), and by Northrop and Kunitz (60).

Salivary amylase is slowly inactivated by proteases (86). Euler and Josephson (12) claimed that yeast invertase is a protein, offering important evidence for their assumption. Willstätter (96), however, found that this enzyme could be obtained free or almost free from protein, carbohydrates, and phosphorus. This preparation contained varying amounts of nitrogen which did not parallel the activity of the invertase. Willstätter, Kuhn, and Graser (97) explain the tryptic inactivation of invertase, as carried out by Euler and Josephson, as due to the digestion of the protein carrier. According to Willstätter and associates, as has been pointed out above, and contrary to Euler and others, enzymes are associated with carriers which have nothing to do with the catalytic effect of the enzyme per se.

We have seen that a great many enzymes have been found to be digested by proteases and some of them with amazing rapidity. Many of these enzymes have been reported to contain little nitrogen or else to be "free of protein." If this is the case, their inactivation by proteases must be attributed to digestion of their protein carriers. The question arises: Is the carrier, if it really exists, always, or almost always a protein? If this is the case it is contrary to the basic principle of the carrier theory, i.e., that the carrier may be *any* colloid of high molecular weight.

THE SPECIFICITY OF CARBOHYDRASES

The theory of Leibowitz (1925) (39, 40), states that there are two kinds of *maltases*. One, which hydrolyzes both maltose and α-methylglucoside, and another, which attacks only maltose. The latter is found in certain moulds, the former in yeast. Fischer and Niebel (13) found, as early as

1896, that the maltase of horse serum would hydrolyze only maltose and not α-methylglucoside. Weidenhagen (95), however, denies the existence of a specific sucrase, maltase, or α-methylglucosidase. His theory is that sucrose is split by α-*n*-glucosidase because it is an α-*n*-glucoside and by β-*h*-fructosidase because it is also a β-*h*-fructoside; maltose, however, being only an α-*n*-glucoside is hydrolyzed only by α-*n*-glucosidase. Weidenhagen extends the same view to other saccharides. Much interest has been aroused in this new theory, but it has not been generally accepted.

For example, Karström (29) found that the enzyme of a certain strain of *Bacterium coli* hydrolyzes maltose but no sucrose, as would be expected according to Weidenhagen's theory. This was confirmed by Myrback (52) and by Tauber and Kleiner (82). Others, like Pringsheim, Borchard, and Loew (63), found malt extracts and certain moulds to be inert toward α-methylglucoside but not toward maltose. Ivanoff, Dodonowa, and Tschastuchin (26) obtained from mushrooms a maltase which hydrolyzed maltose but not sucrose. Schubert[2] prepared in Nelson's laboratory an invertase from honey, which hydrolyzed maltose but not α-methylglucoside (53). A maltase inactive to sucrose and α-methylglucoside has been extracted by Kleiner and Tauber (31) from mammary tissue. Recent experiments of Grassmann and associates (16, 17), using various sugars and glucosides as substrates and working with different carbohydrases, point to the invalidity of Weidenhagen's theory.

More recently Tauber and Kleiner (87) have tested the theory of Weidenhagen once more. Since he has stated that α-phenylglucoside is more readily hydrolyzed by maltase than α-methylglucoside, Tauber and Kleiner employed the new substrate. They used the enzyme obtained from the tropical plant *Solanum Indicum*. This maltase did not hydrolyze α-methylglucoside. It did, however, slowly split α-phenylglucoside, and maltose was very rapidly hydrolyzed. Therefore, it was suggested that the maltases be divided into two groups. The first, or *true α-glucosidases*, split all α-glucosides and maltose, e.g., yeast maltase; the second, or *pseudo α-glucosidases*, split maltose, are only slightly active to certain α-glucosides, and are inactive to all others. To the latter group belong maltase of *B. coli*, maltase of the mammary gland, malt extracts, and the maltase of *Solanum Indicum*.

Regarding the *specificity of the saccharases*, there is no general accord. Kuhn and Rohdewald (36) maintain that there are two types of saccharases. Some taka-saccharase preparations do not hydrolyse raffinose, while others (37) do. Leibowitz and Mechlinski (40) repeated these experiments and found that some taka-saccharase preparations contain

[2] An extensive review on the chemistry of invertase has recently been published by Nelson (53).

melibiase, which is responsible for the hydrolysis of raffinose, while others do not. In the former instance, the trisaccharide was hydrolyzed by the melibiase to galactose and sucrose, and the sucrose freed in this manner was then split by the taka-saccharase. Weidenhagen (94) contradicts this. He found that such taka-saccharase preparations as contained no melibiase could attack raffinose.

Tauber and Kleiner (87) obtained an enzyme preparation from *Solanum Indicum* which readily split raffinose, but both melibiase and sucrase were present.

THE EFFECT OF HIGH PRESSURE ON ENZYMES

Macheboeuf, Basset, and Levy (47) studied the effect of very high pressures upon enzymes. At 6000 atmospheres enzymes are not destroyed; at 17,000 atmospheres (ultrapressure), however, varying destruction took place, which depended on the pH of the solution, the duration of exposure, and other factors. Enzymes in general are less resistant than bacterial spores, more resistant than non-spored forms, viruses, or bacteriophage, and approximately as resistant as bacterial toxins. Certain protein solutions were found to coagulate under similar conditions. It appears that the inactivation of certain enzymes by high pressure is due to the destruction of the colloid molecule, which in most cases is a protein.

ENZYMIC HYDROLYSIS OF THE DIKETOPIPERAZINE RING

All proteolytic enzymes split peptide linkages. This has been explained by the fact that the amount of amino groups formed is equal to the amount of carboxyl groups liberated. That this is the case in peptic hydrolysis has been definitely established recently (R. K. Cannan (1930)). However there are exceptions, and, contrary to the earlier general belief that the diketopiperazine ring cannot be split by proteases, Abderhalden and Schwab (1) succeeded in hydrolyzing leucylglycyltyrosine anhydride, leucylglycylleucine anhydride, and leucylglycinserine anhydride by erepsin and by trypsinkinase, respectively. The above diketopiperazine compounds have been obtained in stable form for the first time. Matsui (49) has reported similar results using asparagyldiglycyltyrosine, a synthetic tetrapeptide, as a substrate. Pepsin, trypsin, and erepsin did not split this compound, but trypsinkinase hydrolyzed it very rapidly. The same author (50) found that synthetic diketopiperazinecarboxylic acid was hydrolyzed by trypsin and trypsinkinase, but not by pepsin nor by erepsin. Ishiyama (25) extended these observations by studying a series of diketopiperazine compounds, such as glycylaspartic anhydride, glycylglutamic anhydride, aspartic anhydride, glycylglutamine anhydride, glycine anhydride, pyrrolidonecarboxylic acid, and pyrrolidonecarboxamide. The

first three compounds were not split by erepsin nor by pepsin, whereas trypsin and especially trypsinkinase were very active in hydrolyzing them. The remainder of these compounds were not hydrolyzed by any of the proteases or by the erepsin. It appears that in the diketopiperazine molecule a free carboxyl group is essential if it is to be hydrolyzed by trypsin; this was the case in the first three compounds. This corroborates earlier findings. Amidation of the carboxyl group is sufficient to make these compounds which would ordinarily be digested, indigestible; for example, glycylglutamine anhydride is very resistant (see also experiments of M. Bergmann).

In view of the increasing number of the various biochemical catalysts and to assure a distinct differentiation between enzymic and non-enzymic catalysts, the author proposes the following classification:

CLASSIFICATION OF BIOCHEMICAL CATALYSTS

1. *Specific, cell-independent, biochemical catalysts or enzymes:*—catalysts produced by the living cell, but whose action is independent of the living cell, and which are destroyed if their solutions are sufficiently heated. Examples: pepsin, trypsin, maltase, lipase.

2. *Specific, cell-dependent, biochemical catalysts:*—catalysts produced by the living cell, active in vitro as well as in vivo, their activity, however, depending on the unimpaired cell. They are destroyed on heating and their activity ceases on mechanical destruction of the cell. Example: the catalyst effecting the synthesis of urea in the liver. Discussed by Krebs (33).

3. *Non-specific biochemical catalysts:*—catalysts elaborated by the living cell, their action being independent of the life processes of the cell. They are not destroyed when their solutions are heated. Examples: glutathione, ascorbic acid.

Considering the existing evidence it may be said that, with a few possible exceptions, enzymes are proteins or broken-down products of proteins. As has been seen, there are enzymes which are simple proteins (pepsin, an albumin; urease, a globulin), and some are derived proteins (rennin, a thioproteose). The preparation of certain enzymes by prolonged autolysis and further purification, their subjection to proteolytic digestion, and the study of chemically active groups in the enzyme molecule may throw further light on the chemical nature of enzymes.

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