OIL SPLITTING BY CASTOR SEED LIPASE.

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Part I. Lipolytic Enzymes

By J. J. Sudborough, H. E. Watson and P. S. Varma.

Introduction.

In 1855 J. Pelouze (C. R. 1855, *40*, 605) drew attention to the fact that seeds, such as linseed, poppy, cameline, rape, mustard, sesame, ground nut, and sweet and bitter almonds, contain a substance which is capable of producing comparatively rapid hydrolysis of the oil present in the seeds when these are crushed. The oil expressed from freshly crushed seeds is practically free from acid, but, if the oil is removed several days after crushing, appreciable quantities of free acid can be detected, and the amount tends to increase when the crushed seeds are kept for several months in closed jars. In the case of sesame seed the amounts of oil hydrolysed are 6, 17 and 47·5 per cent at the end of 8 days, 1 month and 3 months, respectively. According to the author the field poppy gives the greatest yield of free acid *viz.* 85 to 90 per cent at the end of four months. The decomposition was attributed to the presence of a ferment which has since been termed "lipase". Similar observations had been made by Chevreul (*loc. cit.* 611) in 1853 when working on an African seed, Pentadesma.

W. Siegmund (Monats., 1890, *11*, 272; also Sitz. Kais. Akad. Wiss. Wien, 1901, *100*, 328) investigated the following seeds a little more in detail :— rape (*Brassica napus* var *annua* and *oleifera*); castor, (*Ricinus communis* and *R. Major*); poppy *Papaver somniferum;* hemp (*Cannabis sativa*), linseed (*Linum usitatissimum*); pumpkin (*Curcubito pepo*) and maize (*Zea Mays*). The seeds were ground with water and the resulting emulsions tested for free acids after given intervals of time up to the end of two days. He also extracted the active enzyme by means of water or glycerine and precipitated with alcohol and found that the precipitate when emulsified with a vegetable oil and water was capable of hydrolysing the oil. The statement is made that pure egg-albumen can also produce slight hydrolysis.

In the same year J. Reynolds Green (Proc. R. S., 1890, *48*, 370) described a series of elaborate experiments on the enzyme present in castor seed, mainly from the plant physiologist's point of view. Green gives a summary of work of a similar nature carried out by Sachs (Bot. Zeitung, 1859, 178), Peters (Land. Versuchsstat., 1861, vol 3), Fleury (Annales de Chim., 1865, (iv) *4*, 38), Muntz (Ibid 1871, *22*, 472), Schutzenberger (Vol. on Fermentation), Detmer (Vol. on Vergleichende Physiologie des Keimungs-processes der Samen 1880). The chief object of the work was the elucidation of the chemical changes which occur during germination and the fate of the oil in the seed. Green's conclusions are:—1. The reserve material of the seeds of *R. communis* consist of oil and protein matter, the latter consisting of a mixture of globulin and albumose. 2. The changes during germination are partly due to ferment action and three distinct ferments are present *viz* a proteolytic or tryptic, a fat splitting or lipolytic and a rennet ferment. 3. At least two of the three and probably all three are in a zymogen condition in the resting seed and become active in consequence of the metabolic activity set up in the cells by the conditions leading to germination *e. g.* warmth and moisture. 4. The proteins are converted into peptone and finally into asparagin. 5 The oil is split into fatty acids and glycerol; the latter gives rise to sugar and the former to a crystalline vegetable acid soluble in water and ether. 6. The appearance of starch and of oil in the embryo or young plant is due to secondary reactions and not to a simple translocation of either. (cf. Nature, 1909, *82*, 100).

Constein, Hoyer and Wartenberg (Ber., 1902, *35*, 3989) were the first to recognise the technical importance of vegetable lipases from a chemical point of view. Their first experiments consisted in grinding together a given weight of castor seed with its own or twice its weight of a one per cent. chloral hydrate solution The results proved that in the latter case 95 per cent of the oil present in the seed was hydrolysed within four days at 35°. In their later experiments they added small amounts of acid to the mixture of water, oil and ground seed, and found that the reaction was much accelerated They also showed that many other oils besides castor oil can be saponified by the lipase present in castor seed, but that some oils are not readily hydrolysed.

The amount of work described by Constein, Hoyer and Wartenberg in this and in other papers and the large number of publications bearing upon vegetable lipases which have appeared since 1902 render it desirable to put all the more important results which have been obtained in as clear but succint a manner

as possible, and with this object in view we have deemed it advisable to collect together all the results obtained by previous workers and group them under the following headings:—

I. The Enzyme. II. Co-ferments. III. Oils which can be split. IV. Temperature limits. V. Dynamics of the reaction. VI. The products and their separation. VII. Animal lipases.

I. The Enzyme.

(a) *The Enzyme from castor seed.* The seed to which most attention has been paid is the castor seed or castor bean and so far this appears to be the material which gives the best results. There are many varieties of Castor (*Ricinus communis*) but all the varieties so far examined appear to produce similar effects. It is possible to use the ground seed after decortication or the fresh cake obtained by cold pressing the seed or some preparation of the seed *e. g.* the product obtained after removal of oil, aleurone &c.

According to Constein, Hoyer and Wartenberg the proportion of seed or enzyme to oil is important. On the whole an increase in the amount of seed accelerates the hydrolysis, but the velocity of the decomposition is by no means proportional to the amount of seed used, as small quantities act relatively more energetically than larger (*cf.* Fokin J. S. C. I, 1904, *23*, 1152). Much discussion has taken place as to the advisability of using resting or germinating seeds. Sigmund (Sitz. Kais. Akad, Wiss., 1892, *100*, 328) states that germinating seeds are more active. Constein and his co-workers deny this and Walker and Bourne (Tech. Quart., 1904, *17*, 284) claim that germination lessens the activity of the seed. According to Hoyer (Ber., 1904, *37*, 1436) in seeds in process of germinating the enzymatic activity is weaker in the portions of seed adjoining the germ than in those further removed. Compare also S. Fokin (Chem, Rev. Fett & Harz Ind, 1906, *13*, 130). The oil and husk have no lypolytic activity and treatment with water, salt solutions or glycerol lowers the activity of the crushed seed. This was proved in the case of water by grinding the oil-free seeds with water and removing the water by heating to 30°—35° for some time, when it was found that the residual solid was less active than the original (Constein, Hoyer & Wartenberg, compare also H. E. Armstrong Proc, R. S., 1905, B*76*, 606). If the same batch of crushed seed is used a number

of times its activity tends to diminish. According to Jalander (Biochem. Zeitsch., 1911, *36*, 435) the activity of the enzyme decreases considerably even in 48 hours although Nicloux states the contrary. Like most enzymes lipase cannot withstand high temperatures : many emulsions of the enzyme rapidly lose their activity when heated at 60°, but dry castor beans can be heated for 24 hours at 100° and it is found that the crushed seed is almost as active as the meal from beans which have not been heated. For poisons and substances which retard the activity of the enzyme see section II—Co-ferments (p 223).

(b) Other seeds which contain lipase. Numerous other seeds also contain lipolytic enzymes. The well known fact that groundnut oil, cocoanut oil and palm-kernel oil rapidly develop acidity when kept, unless they are carefully refined, indicates the presence in these oils of small amounts of enzymes derived from the meal from which they are extracted, The extent to which such oils can undergo spontaneous hydrolysis is well illustrated by the fact that crude, country-pressed cocoanut oil has been bought and the free glycerine extracted by washing with warm water. The original researches of Pelouze (page 213) indicate the presence of such enzymes in all the seeds he examined. Lipases have also been shown to exist in the following :—Jequirity or Indian liquorice, *Abrus precatorius* (Braun and Behrendt, Ber., 1903, *36*, 1142); Celandine, *Chelidonium majus;* Toadflax, *Linaria vulgaris* and *L. reticulata* (S. Fokin, J. S. C. I. 1904, 23 259, 614, compare also Bournot, Biochem. Zeitsch., 1913, *52*, 172; 1914, *65*, 140); rice bran (C. A. B. Browne (Junr), J. Am. Chem. Soc., 1903, *25*, 950); kola nut (H. Mastbaum, J. S. C. I., 1907, *26*, 262); horse chestnut, *Aesculus hippocastanum* (W. Sigmund, Monats., 1910, *31*, 657); sweet almonds (M. Tonegutti, J. S. C. I., 1911, *30*, 221); soya bean, *Glycine Soja* Benth., (K G Falk, J. Am. Chem. Soc., 1915, *37*, 649); ginestra berries, *Sparteum junceum* (M. Raffio, Annali Chim. appl. 1917, *7*, 157; J. S. C. I., 1917, *36*, 657); paper mulberry, *Broussonetia papyrifera* (Gerber, C. R., 1911 *152*, 1611). The seeds of *Cherianthus cheiri* have only sight hydrolysing power whereas the myrosin obtained from the leaves and stalk is much more active; on the other hand pure abrin from the seeds of *Abrus precatorius* hydrolyses fats less readily than the seeds themselves. Both croton seeds and crotin are unable to hydrolyse oils (Braun, Ber., 1903, *36*, 3003).

S. Fokin (Chem. Rev. Fett und Harz Ind., 1904, *11*, 30 48, 69; 1906, *13*, 130, 163, 191, 219; J. S. C. I. 1904, *23*, 259, 614; 1906, *25*, 994) examined 60 plants belonging to 30 families and although many of these effected the hydrolysis of 10—16 per

cent of oil, the conclusion was drawn that the decomposition is not due to a lipase as there is no quantitive relationship between the yield of fatty acid and the amount of seed used and also the same seeds when old produce no hydrolysis. He states that all plants yet known to contain a lipase are poisonous but that not all seeds known to contain poisonous alkaloids are capable of hydrolysing oils, e. g. *Heliotropum europeum, Cynoglossum officianalis, Digitalis purpurea, Buxus sempervirens.*

Certain small seeds have their lipolytic power doubled or trebled by the process of germination, the stimulating effect is greatest with small seeds e. g. *Linaria marsocana*, then *L. purpurea*, and celandine in decreasing order. (cf. part III).

According to Dunlap and Seymour (J. Am. Chem. Soc. 1905, *27*, 935) the seeds of the following have very little hydrolytic activity in the resting stage :—groundnut, *Arachis hypogea ;* flax, *Linum usitatissimum* ; celandine, *Chelidonium majus* ; toad flax, *Linaria vulgaris*; sweet almond, *Prunus amygdalus* var. *dulcis.* With germinated groundnuts the activity is somewhat greater.

H Mastbaum (Rev. Fett und Harz Ind., 1907, *14*, 5, 31, 44; J. S. C. I., 1907, *26*, 262, states that small amounts of lipolytic enzymes are present in maize, chestnuts and mace, and large quantities in black pepper; on the other hand, coffee, cocoa, almonds, wheat, rye, barley and beans are free from such an enzyme A comparison of the ferments present in castor and in celandine seeds has been made by Bournot (1913) and by Armstrong and Gosney (Proc. R. S., 1914, B88, 176).

(*c*) *Lipase in bacteria and moulds.* N. L Söhngen (Koninkl. Akad. van Wetensch. Amsterdam, 1910, *19*, 689, 1263; 1911, *20*, 126 ; J. S. C. I. 1911, *30*, 140, 812, 1124) has shown that numerous bacteria and moulds are able to hydrolyse fats under anaerobic conditions and to oxidise them under aerobic conditions. The hydrolysis is due to the secretion of a lipase by the bacteria and the glycerol and fatty acids formed undergo further decomposition. Certain micro-organisms secrete two lipases α and β, of which the former diffuses more rapidly than the latter and decomposes fats in an acid as well as in an alkaline medium. β-Lipase will not produce hydrolysis until the medium has been rendered alkaline with sodium carbonate. An acidity greater than 0·02 N entirely inhibits hydrolysis. The lipase appears to form compounds with the acids which diffuse through gelatine or agar cultures in much the same

way as lipase, but which are incapable of decomposing fats. The lipolytic enzyme obtained from *Bacterium lipolyticum*, *B. fluorescens non liquefaciens*, *B. Stutzeri*, *Oidium lactis* aërogenes, *Aspergillus niger*, *Penicillium glaucum*, *Cladosporium butyri* is destroyed when heated at 80°, but a lipase which can be heated for 5 minutes at 100°C without decomposition can be obtained from cultures of *B. fluorescens liquefaciens*, *B. punctatum*, B. *pyocyaneous* and B. *liquefaciens albus*. The fungus, fly agaric, *Amarinta muscaria*, which grows in Upper Styria and S. Bohemia, also contains a lipolytic ferment which can split the oils present in the fungus and also added oils such as rape, olive, castor, or tallow. The decomposition is slow and the ferment is best used in the form of the freshly dried and ground fungus (J. Zellner, Monats., 1905, *26*, 792).

(d) *Lipolytic activity of amino-acids.* K. G. Falk and J. M. Nelson (J. Am. Chem. Soc., 1912, *34*, 735) and M. L. Hamlin, (*ibid.* 1913, *35*, 624, 1897) have drawn attention to the fact that amino-acids such as glycine, alanine, phenylalanine, aspartic acid and glutamic acid are capable of hydrolysing esters such as methyl acetate, ethyl butyrate, phenyl acetate, triacetin and glycerides present in olive oil, but the conclusion is drawn that there is no evidence to show that the hydrolytic activity of lipase is due to the presence of amino-acids or polypeptides. Products obtained by shaking proteins such as caseinogen or gelatin, with *3* N alkali solutions for 24 hours and neutralising the turbid solutions with hydrochloric acid possess the property of hydrolysing esters (Hulton-Frankel, J. Biol. Chem., 1917, *32*, 395) This property is only slightly impaired by dialysis and is unaffected by boiling the solutions.

(e) *The two lipolytic enzymes of castor.* The researches of Falk (J. Am. Chem. Soc., 1913, *35*, 1904) and of Falk and Suguira (*ibid.*, 1915, *37*, 217) show that castor beans contain two distinct lipolytic enzymes. One of these is soluble in water and has a comparatively greater hydrolysing effect on esters like ethyl butyrate than on glycerides like triacetin, the other is insoluble and is more active towards triacetin than towards ethyl butyrate. The experiments were made by using a castor meal from which all oil had been removed by extraction with carbon tetrachloride or chloroform, grinding the extracted meal to pass a 40 mesh and again repeatedly extracting with ether. This powder was extracted with small amounts of water for a definite length of time and the solution filtered by means of a Gooch crucible using a pad of long fibre asbestos and the activity of both filtrate and residue were tested, care being taken to make all the

necessary blank experiments. The soluble enzyme is termed *esterase* and the insoluble one *lipase*. Their actions are selective. In preparing the esterase from the dry castor seed preparation by extraction with water it is advisable to use a relatively large number of small quantities of powder rather than a small number of large amounts. In most cases 0·5 gram of the preparation and 60 c. c. of water were used and the extraction allowed to proceed for 24 hours. If the clear solution is dialysed in a collodion bag against running water it becomes turbid and nearly neutral to phenol-phthalein. This turbid solution is less active as a hydrolysing agent, but the activity returns practically to the original value if the acidity is restored by the addition of the requisite amount of acetic acid. A solid active preparation can be obtained by precipitating the solution with three times its volume of alcohol, allowing to stand overnight, filtering, grinding repeatedly with fresh acetone and drying in a vacuum desiccator. The suggestion is made that esterase is identical with Plimmer's glycerophosphatase. (Biochem, J., 1913, 7, 43). The lipase proper is soluble to a certain extent in common salt solution, more especially in 1·5 N. solution. If such solution is dialysed against running water a preciptate is formed and if this be removed by filtration the solution is quite inactive whereas the precipitat- retains its activity, (Compare however statement p. 215). A coferment does not appear to be present.

(f) *Concentrated preparations of lipase.* Numerous methods have been devised for obtaining a product with a hydrolysing power greater than that of the crushed castor seed: these all consist in removing from the seeds the materials, such as oil and albuminoid substances, devoid of hydrolysing properties, The reasons for such treatments are largely the isolation of good yields of products of high purity and are referred to in detail in section VI dealing with the products and their separation.

Methods of Nicloux. M. Nicloux (C. R., 1904, *138*, 1112, 1175, 1288, 1352) was able to demonstrate that the lipolytic activity of the castor seed resides in the fine granular cytoplasm, and that the oil, cell-membrane and aleurone grains are devied of activity, and he devised the following process for removal of the aleurone grains. The decorticated seeds are crushed and then ground with a relativly limpid oil such as cotton-seed and the homogenous mass is filtered first through wire gauze and then through cloth. The filtered oil which is turbid, contains in suspension a mixture of grains of aleurone and cytoplasm with a few fine particles of cellular membrane and is then centrifuged in an apparatus of high power when two distinct layers

are found in the tubes: the lower whitish layer is made up of grains of aleurone together with a few particles of cellular membrane, the upper greyish layer contains the cytoplasm together with a few aleurone grains and particles of husk. This upper oily layer can be used as such for hydrolytic purposes or the oil can be removed by means of a solvent and the cytoplasm obtained in a dry state; the average amount of this cytoplasm is 5 per cent of the weight of the decorticated seed, and 1 part in the presence of 500 parts of cotton-seed oil can hydrolyse 80 per cent. of the oil in 15 hours at 20°, provided a little free acetic acid is present. In the absence of water the cytoplasm can be heated with oil for 20 hours at 100° or for 15 minutes at 100° without its activity being diminshed; after 15 minutes at 150° the activity is diminished to 1/10th its original value. The dry cytoplasm is very sensitive to various reagents: simple treatment with water destroys the activity and aqueous solutions of acetic acid and salt have the same effect. If the cytoplasm is mixed with oil and then dilute acetic acid added, saponification takes place readily, but if the ferment is mixed first with the acidified water and the oil added last hydrolysis does not occur. (A summary of all Nicloux' results is to be found in "Contribution a l'etude de la saponification des corps gras" 1906).

Hoyer's methods. E. Hoyer (Ber., 1904, *37*, 1436) states that it is impossible to prepare a solution of the enzyme with solvents such as water, common salt solution or glycerine, the clear liquid is always inactive and the seed residue is less active than the original material. Buchner's press method also gives negative results. The turbid oil obtained by pressing castor seeds is active and the suspended matter settles slowly; if however ether or carbon disulphide is added and the mass filtered, a fine powder is obtained which is very rich in enzyme. An experiment made by grinding for 30 minutes castor seed with cotton seed oil and its own weight of sand and filtering the product through linen cloth gave a turbid oil which showed high activity in the presence of dilute acetic acid. If however the turbid oil is filtered and the clear oil is mixed with dilute acetic acid no saponification occurs. Another method described by Hoyer is to grind the seed with sand and acidified water and press through cloth when an active, milky emulsion is formed. When the mixture is centrifuged three layers are obtained: (a)an aqueous liquid containing proteins in solution but devoid of activity (b) an active creamy emulsion (c) a solid residue which is also active. A third method consists in taking the ground seed and mixing with ether or a limpid oil like cotton-seed and allowing the mixture to settle in layers when it is found that the upper layer, consisting of the finest particles, is

the most active, and the lowest layer, containing the coarsest particles, least active. By such processes it is claimed that the active material contains only 10 per cent of the proteins originally present in the seed. (Seifensieder Zeit., 1905, *32*, 509; J. S. C. I., 1905, *24*, 977). In another paper (Zeitsch. physiol. Chem., 1907, *50*, 414) the same author describes the formation of an active turbid product by treating the crushed seeds with a solvent for oil. Freshly expressed castor oil before filtration also contains a large proportion of the enzyme. Another process is also described in the same paper for preparing the product termed "ferment". The seeds are ground in an Excelsior mill with water and expressed, when an active emulsion is obtained. On leaving this to ferment at 24° a thick scum forms on the surface, this scum contains about 38 per cent ricinoleic acid, 4 per cent of proteins and 58 per cent of water. This concentrated ferment is very sensitive to the action of acids and of salts such as manganous sulphate, which stimulate its activity when added in small quantities. The product keeps well in the cold, but it was not found possible to obtain it in the form of dry powder.

Taylor's extraction method. A. E. Taylor (J. Biol. Chem., 1906, *2*, 87) describes the preparation of an active powder by crushing fresh, fully ripe castor seeds free from husks and extracting the material with anhydrous ether until free from lipoids. To accomplish this the crushed seed must be removed repeatedly and reground during the extraction Ultimately a light fluffy powder is obtained which keeps indefinitely in the dry state. An appreciable amount of enzyme is lost during the extraction as although insoluble in ether it dissolves to an appreciable extent in a mixture of ether and oil. The author recommends the process as a suitable one for preparing small amounts of a homogeneous ferment. Other products present in the seed are amylase, invertase, maltase and an endotrypsin but no peroxydase, also globulin, albumin, nucleoalbumin and a glycoprotein. It is possible to separate the lipase from most of these substances, but it is not desirable, as the purer the lipase preparation the less stable it is. The dry powder can be heated to 100° without injury, but when suspended in water it is immediately destroyed at that temperature.

Armstrong and Ormerod's extraction method. H. E. Armstrong & Ormerod (Proc. R. S., 1906, B78, 376) use a method somewhat similar to Taylor's and Armstrong & Gosney (*ibid* 1913 B86, 586) adopt the method of extracting the decorticated seeds with light petroleum and ether and digesting the residue for 15 minutes with 0·1 N. acetic acid. The liquid is decanted, the residue washed, filtered and dried under reduced pressure, then ground

and sifted through fine muslin. The yield is about 9 per cent of the weight of the original seed. The enzyme is affected by dilute acids and easily rendered inactive by excess of acid and its inferior activity towards esters other than fats is probably due to the solubility of the acids produced from such esters and the retarding action of concentrated aqueous solution of acids. The natural oils, on the other hand, give rise to acids which are practically insoluble in water.

Tanaka's dry powder. Y. Tanaka (Eighth Int. Cong. of Applied Chemistry, 1912, Sec. Vd., *11*, 37; J. S. C. I., 1912, *31*, 884,) describes the preparation of an active dry powder. 100 grams of pressed or extracted castor seeds are triturated with 600—700c.c. of 0·1 N acetic acid at 30—35° for 30 minutes, after which the milky liquid is filtered and the residue thoroughly washed with water and dried at a temperature not exceeding 40°. The lipase powder thus obtained is white, odourless and quite free from soluble matter. The composition of one sample was: water 5·33, oil 37·2, nitrogenous substances 46·3, mineral matter 1·2 and non-nitrogenous 10·0 per cent. The powder produces rapid hydrolysis in the presence of water.

Tancov's method. The method recommended by N. V. Tancov (J. Russ. phys-chem. Soc., 1914, *46*, 33 5) consists in grinding the decorticated seed with its own weight of castor oil, centrifuging, removing the upper fine layer by means of ether, washing with water and drying over calcium chloride. Its emulsion in ether is next filtered by means of a pump and the residue washed with ether and dried to a fine white powder. The preparation loses its activity after a fortnight.

Falk's method has been described on p. 218.

Concentrated Lipase from Chelidonium seeds. The attempts of K. Bournot (Biochem. Zeitsch., 1913, *52*, 172; J. S. C. I., 1913, *32*, 758) to isolate the active enzyme from chelidonium seeds were unsuccessful, the enzyme is almost insoluble in alcohol, water or glycerol, but dissolves to a certain extent in the oil extracted from the seeds or in a mixture of oleic acid and alcohol. The same author (*ibid.*, 1914, *65*, 140; J. S. C. I. 1914, *33*, 797) states that the uncrushed chelidonium seeds are inactive and that the activity of the powdered seeds increases with the fineness of the powder; the expressed and filtered oil is inactive although the one obtained by disgesting the ground seeds with light petroleum at the ordinary temperature is somewhat active. An active preparation of the lipase is best obtained by extracting the crushed seeds for 2-3 hours with ether or petroleum

ether in a Soxhlet apparatus and sifting the residue through a hair sieve; if ground for a longer time the product becomes less active.

II. Co-ferments.

In the earlier experiments of Constein, Hoyer and Wartenberg (see p. 215) when the crushed seeds were ground with a one per cent chloral hydrate solution and left for some time at 35°, it was found that the hydrolyisis did not proceed regularly as shown by the following numbers:—

Days	0	1	2	3	4
per cent. of ricinoleic acid in the oil	3	5	58	85	95

The cause of this break was attributed to the stimulating action which small amounts of acids, for example the acids produced by the hydrolysis, have on the activity of the seeds. By replacing the chloral hydrate by toluene,* Armstrong (Proc R, S., 1905, *76* B, 606) was able to show that no such break as that described above occurs, and he attributed the break in the case of chloral hydrate to the liberation of a small amount of hydrochloric acid during the reaction. In their later experiments Constein, Hoyer and Wartenberg always added small amounts of some acid as an activator, or co-enzyme as it is sometimes termed. The acids used were acetic, sulphuric, phosphoric, sodium bisulphate and fatty acids such as oleic, and experiments were made using different concentrations of the acid. Two important points established by these chemists were, 1. The amount of water compared with seed and oil plays an important part in the rate of hydrolysis. They state that at least three times the theoretical amount of water should be used. The following numbers, which give the amounts of free fatty acids present after given intervals of time when 5 grams of ground seed and 6·5 grams of castor oil are mixed with different quantities of water containing chloral hydrate and acetic acid (2 per cent), illustrate the effects produced by different quantities of water.

Water	*3*	*18*	*24*	Hours.
2 grams	68	74	74	per cent of free acid.
4 „	74	80	84	„ „
10 „	76	86	86	„ „

2. The neccessity for obtaining a good emulsion. They point out that it is better to grind the oil and seed together and then add the acidified water, as the emulsification is thus much

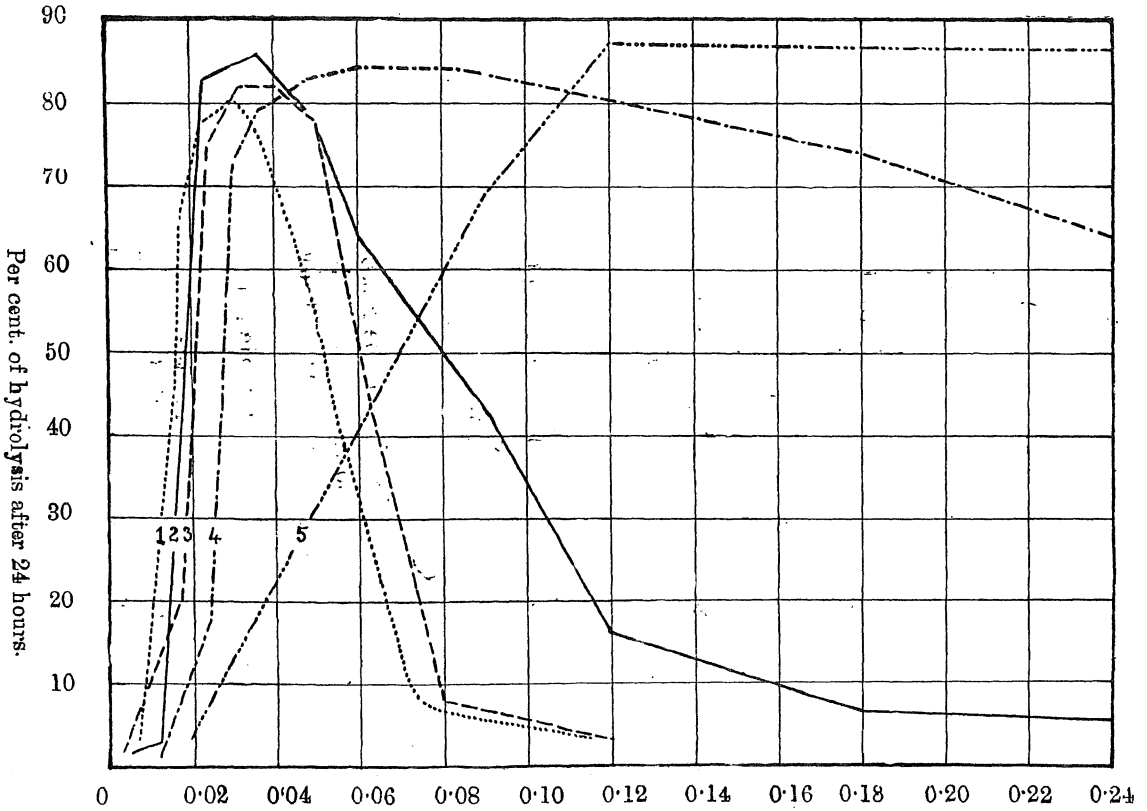
*The chloral hydrate or toluene is added as an antiseptic to prevent the growth of bacteria, moulds and other micro-organisms, so that the possibility of the hydrolysis being due to the bacterial or similar action is excluded. Such mild antiseptics do not interfere with the activities of enzymes.

better. As a rule the mixture should be well stirred during the early stages of the saponification, but after a short time when small amounts of fatty acids have been formed stirring is no longer necessary as the emulsion is permanent.

The low values obtained with small quantites of water are explicable when it is remembered that the reaction is a reversible one (compare section V, Dynamics of the reaction p. 232); Fokin (*loc. cit.* or J. S. C. I., *23*, 1152) also draws attention to the importance of the amount of water as compared with the quantity of seed; if too much water is used there is a great tendency for the emulsion to break and for the reaction to stop.

Influence of acids. A detailed study of the influence of the different acids on the reaction was made by Hoyer (Ber., 1904, *37*, 1436). The acids used were sulphuric, oxalic, formic, acetic and butyric and he was able to show that in order to obtain the optimum decomposition, the ratio *weight of seed/weight of acid* must be kept within certain limits, or, in other words, for any particular acid the total weight of acid used, and not its actual concentration, is the important factor. For example with acetic acid solution using 60 per cent of water calculated on the weight of the seed the optimum concentration of acid is 0·06, whereas with only 30 per cent of water the optimum concentration of acid is nearly 0·11. (compare also Tanaka J. Coll. Eng. Imp. Univ., Tokyo, 1910, *5*, 25) The limits within which the absolute quantity of acid may vary are different for the individual acids and appear to have a rough relationship to the dissociation constants of the acids but are by no means propotional to the concentration of hydrogen ions. Strongly ionised acids such as sulphuric and oxalic can be used within narrow limits of concentration only, but with feeble acids there is a much wider range of concentration. The curves given on p. 225 are taken from Hoyer's paper and illustrate this relationship.

The view is put forward that acid enters into combination with the seed or enzyme. A similar view is also held by Tanaka (*loc. cit.*) who concludes that the enzyme is originally present as a zymogen, the acid liberates the lipase but the liquid does not become acidic as the acid combines with certain protein substances present. Higher fatty acids can take the place of acetic acid, for example buytric acid or the saponified oil from a


90
80
70
60
50
40
30
20
10
Per cent. of hydrolysis after 24 hours.
1 2 3 4
5
0
0·02
0·04
0·06
0·08
0·10
0·12
0·14
0·16
0·18
0·20
0·22
0·24
grams of acid with 3·3 grams of crushed seed and 100 grams of linseed oil
1=oxalic acid. 2=formic acid. 3=sulphuric acid. 4=acetic acid and 5=butyric acid.

previous experiment, but the amount required is large. Various authorities e. g. Hoyer (*loc. cit.*) Nicloux (C. R., 1906, *139*, 143) and Fokin state that carbon dioxide dissolved in water acts as accelerator, and Armstrong (1905 *loc. cit.*) states that glutamic and aspartic acids have high values as accelerators. E. Lombard (Fr. pat., 346415 of September 24th 1904; J. S. C. I., 1905, *24*, 141) states that pure water, ground castor seed and one part of ethyl acetate in 10,000 parts of oil gives as complete hydrolysis as a solution of aceticacid. According to Hoyer (1907) when crushed castor seed is mixed with water a development of acids takes place as the result of enzymatic activity and this formation of acid precedes the hydrolytic action of the lipase on the oil. The production of the acid is promoted by adding to the mixture a suitable acid preferably those formed in the seeds themselves—the "cell acids", which appear to be mainly lactic acid together with small amounts of formic and acetic acids.

Influence of manganous sulphate. Another co-ferment which has been suggested is manganous sulphate which has been recommended by Hoyer (1907) Nicloux (1906) and Tanaka (1912) in the form of very dilute solution. Falk and Hamblin (J. Am. Chem. Soc., 1910, *35*, 210) have examined the action of manganous sulphate and they are able to show that after the activity of Falk's lipase preparation (p. 218) has been largely destroyed by heat or by keeping it can be restored by means of dilute manganous sulphate solution especially if air is blow through at the same time. The conclusion they draw is that the inactive zymogen of castor seed lipase is converted into an active enzyme by an oxidation reaction for which the presence of an oxygen carrier or catalytic agent is necessary. Probably zymogen, enzyme and inactivated enzyme are protein in character. Heat destroys the enzyme, but not all the zymogen, hence the addition of the manganous sulphate is able to convert the undestroyed zymogen into enzyme.

Accelerators with lipase preparations. The addition of a co-ferment or accelerator such as acetic acid, sulphuric acid or manganous sulphate is essential when the crushed castor seeds are used as the hydrolysing agent, if rapid saponfication is required; but some of the lipase or ferment preparations described on pp. 219—222 are not activated by the addition of acid. This holds good for Tanaka's powder which is rendered less active by the addition of acid; (compare Jalander, Biochem. Zeitsch., 1911, *36*, 435 also G. Kita, J. Chem. Ind., Tokyo 1918, *21*, 1; Y. Tanaka *ibid*, 112.) and quite inactive by the addition of alkali; the

lipase is less stable towards alkalis than is its zymogen. Armstrong's preparation is not accelerated by the addition of acid and the experiments with Falk's preparations were made without the addition of a co-enzyme. This difference between the activity of the seeds themselves and of certain preparations is due to the fact that the activator is required in the case of the seed to produce the liberation of the lipase from its zymogen, whereas in many of the lipase preparations the lipase has already been set free during the production of the preparation, (compare however Constein, Hoyer and Wartenberg *loc cit.* p. 400). Activators are essential when Hoyer's or Nicloux' preparation is used. Nicloux (loc. cit. p. 66) has shewn that, in addition to various acids and manganous sulphate, a saturated solution of calcium sulphate is an excellent activator. Numerous other salts also produce appreciable acceleration.

For purified castor seed lipase the best effects are obtained with quantities of acetic acid varying from 0·3 to 1·5 grams for the ferment obtained from 100 grams of seed—a higher concentration is unfavourable. For powdered castor seeds freed from oil the best results are obtained with 1·5 grams of acid per 100 grams of crushed seed. In this connection the experiments made by D. Sommerville (Biochem. J., 1912, *6*, 203) are of interest. An emulsion of ground castor seed and water is incubated at 25° until hydrolysis of the oil in the seed has been definitely established. The emulsion is then mixed with oil and water and shaken for a few minutes at intervals of quarter of an hour during several hours and then left. In 3 days at the ordinary temperature 80—85 per cent. of the oil has been saponified and neither acetic acid nor manganous sulphate has any effect. The emulsion when mixed with two or three volumes of water, alcohol or acetone rapidly loses its activity but not when mixed with benzene or ether. Armstrong and Ormerod (Proc. R. S., 1906, B 78, 376) state that long digestion of the oil-free seed with water or dilute acetic acid causes only a gradual loss of lipolytic activity, but the mass loses to a large extent its property of producing an emulsion with oil and water. (Compare Nicloux).

Influence of acids on seeds other than castor. Although an activator is required for castor seeds, it does not follow that other seeds with lipolytic activity also require activators. The activity of the ground kola nut is diminished by the addition of acid (Mastbaum *loc. cit.*) as are also the activities of the powdered seeds of *Chelidonium majus*, (Fokin 1906, Bournot 1913, Armstrong and Gosney, Proc. R. Soc., 1914, 88 176 of *Abrus precatorius* (Brown and Behrendt 1903) and of

seeds of *Sparteum junceum* (Raffio 1917). In this last case the addition of sodium carbonate has a favourable effect.

Lipase poisons, The effects of various chemicals on the activity of different lipases have been noted by several investigators. Alcohol, alkalis, soaps, formaldehyde, sodium fluoride and mercuric chloride all act as poisons to castor seed lipase, whereas salts such as sodium chloride or the sulphates of iron, sodium, magnesium, manganese and ammonium in quantities of 0·1 gram per 10 grams of acid solution have no action. (Constein, Hoyer & Wartenberg 1902, compare also Armstrong & Ormerod, Proc. R. S., 1906, *78*, 376).

In the case of kola nut lipase Mastbaum states that potassium cyanide, calcium chloride, bismuth nitrate, arsenic, alcohol, chloroform, acids, alkalis all have an inhibiting effect on the activity, but that potasium chromate, salicylic acid, ether and light petroleum have a stimulating effect. Van den Driessen-Mareeuw (Pharm. Weekblad., 1909, *46*, 346, J. S. C. I., 1909, *28*, 612), on the other hand, states that potassium cyanide has no action and that potassium chromate, uranyl nitrate, mercury chlorides, benzene and many organic acids have a deleterious action and potassium ferrocyanide, salicylic acid and sodium carbonate a favourable effect.

The following substances accelerate the activity of bacteria lipase ;—hydroxyl ions, calcium, magnesium and trimethylammonium salts and sodium glycollate ; but mono-hydric alcohols have a retarding action and sugar and glycerol are without effect.

Influence of salts. A more detailed examination of the influence of various salts on the activity of castor seed lipase has been made by Falk (J. Am. Chem. Soc., 1913, *35*, 601) using ethyl butyrate as the ester to be hydrolysed. In the presence of most univalent salts or of the chlorides of magnesium, calcium and barium there is a decrease in the activity of the ferment ; with very dilute solutions of barium chloride or calcium chloride and with solutions of magnesium sulphate, sodium sulphate (0·1 to 0·5 molar) manganous chloride and manganous sulphate there is increased activity. Methyl and ethyl alcohols and acetone also exert an inhibiting action using concentrations of 2 N downward, solutions of glucose (2 N) or glycerol (25 per cent) produce no retarding effect (compare, however, Nicloux p. 232). The esters of monohydric alcohols also produce a retarding effect but glyceryl esters such as triacetin do not (compare section III Oils p. 229).

Nature of the Emulsion. A careful examination of the emulsion formed by the enzyme, oil and water has been made by Y. W. Jalander (Biochem. Zeitsch., 1911, *36*, 435; J. S. C. I., 1911, *30*, 1321) the enzyme used being Nicloux' cytoplasm (p 219). When a mixture of oil and ferment is shaken with a relatively large volume of 0·1 N acetic acid an emulsion of the ordinary type is formed, i. e. the water is the disperse medium, but the dispersed oil globules are filled with innumerable minute particles of the ferment swollen by absorption of water. This emulsion is not stable and after a comparatively short time the oil gloubles coalesce, the water is adsorbed by the lipase and the stable reversed emulsion produced. The presence of neutral glycerides appears to be essential for the formation of this reversed emulsion. A stable emulsion is best prepared by mixing one gram triolein with 0·005 gram lipase, adding 0·6 c. c. of 0·01 N acetic acid and then slowly rotating the mixture. An emulsion is produced after 1·5 minutes and this on vigorous shaking becomes creamy in consistency. In an emulsion prepared in this way the hydrolysis of the oil proceeds to the equilibrium point even on standing, but the velocity of hydrolysis is increased by agitation, best by a slow, continous rotation of about 25—40 turns per minute.

III. The Oils or Hydrolysable Substances.

Selective action of enzymes. In many cases of hydrolytic decomposition by means of enzymes it is found that a particular enzyme is selective in its action and can only hydrolyse specific compounds. Maltase hydrolyses maltose and invertase cane sugar; glucase hydrolyses α glucosides and emulsin β glucosides. Castor seed lipase is capable of hydrolysing most natural oils and fats i. e. the glycerides of the higher fatty and unsaturated acids. A selective activity is exhibited, however, in the case of certain esters of optically active acids: Dakin shows (J. Physiol., 1903, *30*, 84) that in the hydrolysis of an ester of racemic mandelic acid by means of lipase the dextro ester is hydrolysed more readily than the laevo, the unsaponified ester is found to be laevo-rotatory and the free acid dextro-rotatory. Similar results have been obtained with other racemic compounds (compare Dakin, ibid, 1905, *32*, 199; Mayer, Biochem, Zeitsch., 1906, *1*, 39; O. Warburg, Z. Physiol. Chem., 1906, *48*, 205; C. Neuberg and E. Rosenberg, Biochem Zeitsch., 1907, *7* 191.

Action of castor seed lipase on glycerides. The early experiments of Constein, Hoyer, and Wartenberg show that most natural oils and fats are readily hydrolysed by the castor seed ferment; in most cases using acetic or sulphuric acid as accelerator, 75—85 per cent of the oil is hydrolysed at the end of

24 hours. The decomposition, however, does not proceed with the same readiness with all oils. Cocoanut oil and palm-kernel oil are not hydrolysed as readily as most other oils, butter is still more resistent and also triolein and tributyrin, and esters of mono hydric alcohols, for example ethyl acetate, iso-butyl acetate, amyl acetate, benzyl benzoate, amyl nitrate, show practically no hydrolysis at the end of three days under the conditions of the experiments.

A series of experiments made by E. Urbain, L. Sangon and A. Fiege (Bull. Soc. Chim., 1904 (iii) *31*, 1194) on the hydrolysis of cocoanut oil by means of Nicloux' cytoplasm (p. 219) in the presence of acetic acid proves that an appreciable amount of free acids present in this oil tends to retard hydrolysis: an oil containing no free acid gave 90 per cent hydrolysis after 24 hours whilst an oil containing 13 per cent of free fatty acid gave only 75 per cent hydrolysis during the same time. The free fatty acids present in the oil appear to be due to the hydrolysis of the glycerides, the production of unsaturated acids and the subsequent oxidation of these, and for the same degree of acidity the retarding effect is greater the lower the mean molecular weight of the acids present. The addition of butyric acid has an inhibiting effect and with 10 per cent present the amount of cocoanut oil decomposed after 24 hours is nil. The different glycerides present in cocoanut oil appear to be hydrolysed at much the same rate.

Action of the lipase on simple esters. Fokin (1914) states that most glycerides are hydrolysed at much the same rate except those derived from fatty acids of low molecular weight, but according to Taylor (1905) even triacetin is readily hydrolysed by his lipase preparation. Hoyer (1907) claims that the amount of ferment necessary appears to be directly proportional to the saponification value of the oil or fat; tallow requires 8-10, cocoanut oil 8 and linseed 5-6 per cent. Armstrong and Ormerod Proc. R. S., 1906, B *78*, 376) state that the dry powder obtained after extracting the seeds by ether can hydrolyse ethyl butyrate but not ethyl acetate and comparing the esters ethyl succinate, ethyl malate and ethyl tartrate the first is hydrolysed most readily and the tartrate least readily. Falk and Nelson's experiments (J. Am. Chem. Soc., 1912, *34*, 735) show that with methyl acetate the results vary with the amount of ester and the amount of ferment, but with ethyl butyrate the amount of ester has not the same effect, probably due to its being sparingly soluble; with olive oil the amount of oil has still less effect. Falk suggests that simple esters exert an inhibiting action similar to that produced by methyl and ethyl alcohol but that glyceryl esters such as

triacetin and oils have a much smaller inhibiting action. The lipolytic activity of castor seed preparations was tested by means methyl acetate, ethyl acetate, ethyl butyrate and triacetin using concentrations from 0.01 to 1·0 N. The results show a very small increase in the rate of hydrolysis for a very large increase in the concentrations of the added esters in the case of methyl acetate, ethyl acetate or ethyl butyrate. With triacetin, on the other hand, an increase in the concentration of the esters produces a large increase in the rate of decomposition. With very dilute solutions ethyl butyrate and triacetin undergo nearly equal amounts of decomposition and much greater than in the case of the acetate. Tanaka's experiments (J. Coll. Eng. Imp. Univ. Tokyo, 1912, *5*, 152, J. S. C. I., 1912, *31*, 1084) show that oxidised oils are hydrolysed more slowly than the original oils from which they are derived and the effect is most marked with drying and least marked with non-drying oils. Rancid oils are also hydrolysed less readily than fresh oils. Polymerised oils obtained by heating oils in a current of nitrogen are also only slowly hydrolysed by lipase.

Comparison of the action of castor and chelidonium lipases. Experiments made with the lipase of chelidonium majus prove that its action is not similar to that of castor seed lipase in all respects. According to Fokin (1906) butter and cocoanut oil are hydrolysed more readily by chelidonium lipase than by castor ferment. Celandine seeds can hydrolyse trilaurin to 91 per cent and tricaprylin to 78.4 per cent., the same seeds hydrolyse the glyceride of sebacic acid which is unaffected by castor lipase. The esters of polyvalent alcohols e.g. glycol, glycerol, mannitol, can be hydrolysed by celandine seeds, whereas the esters of monohydryic alchols act as poisons, their effect increasing with their solubility in water. The glycerides of aromatic acids are unaffected by celandine seeds.

The lipase of jequirity seed also differs somewhat from castor seed lipase (Braun and Behrendt Ber., 1903, *36*, 1900) Lanolin is more readily hydrolysed by jequirity than by castor lipase and somewhat similar results have been obtained with Carnauba wax at 80° and with simple esters of aliphatic and aromatic acids.

IV. Temperature Limits.

As in the case of most enzymes the temperatures at which castor seed lipase can produce its optimum effects lie within narrow limits. According to Constein, Hoyer and Wartenberg a temperature of 35° is better than one of 15°, but with

ordinary liqiud oils at a temperature of 35° there is a greater tendency for the emulsion to break and the oil and the water to separate, and in order to avoid constant stirring they recommend a temperature of 15—20°. At 50° the reaction is extremely slow and at 100° is nil. Somewhat similar results were obtained by Nicloux.

In a later paper Hoyer (1905) states that the best results are obtained by using a temperature of 23° and that the ferment loses its activity above 42°. Nicloux' results indicate that the maximum saponification is attained at a temperature of 30° (*loc. cit.* p. 29).

With solid fats such as tallow it is advisable to add a sufficient quantity of vegetable oil in order to reduce the melting point so that the reaction can be conducted at temperatures between 15° and 30°.

With kola nut lipases Mastbaun claims that the activity increases up to a temperature of 50° when its optimum effect is attained.

V. Dynamics of the Reaction.

Reversibility of the reaction. The hydrolysis of esters by means of strong mineral acids is a balanced one. In many reactions in which enzymes play a part it has been shown that the enzymes can exert not merely an analytical or decomposing action but also a synthetic action. This has been demonstrated in the case of maltase (Croft Hill J. C. S., 1898, *73*, 634; 1903, *83*, 578) which can build up di-from mono-saccharides and of emulsin (Kieble J. C. S., Abstr., 1913, (i), 63; Bourquelot Ann. Chim. Phys., 1913, (viii), *29*, 145; 1915, (ix) *4*, 130) which can produce alkyl glucosides or galactosides and hydroxy-nitriles. Fokin (1906) concludes that the hydrolysis of oils by means of castor seed lipase is not reversible although he states that the reaction proceeds further if the glycerol produced during the hydrolysis is removed. Nicloux has shown that the addition of either product diminishes the rate of hydrolysis (*loc. cit.* p. 32). The investigations of numerous other chemists prove conclusively, however, that the reaction is reversible and that glycerides may be synthesised by means of both animal and vegetable lipases from glycerol and fatty acids. Kastle and Loewenhart (Amer. Chem. J., 1900, *24*, 491) using lipase from pig's pancreas appear to have been the first to demonstrate the synthesising functions of the enzyme in the case of ethyl butyrate, and A. E. Taylor (J. Biol. Chem., 1906, *2*, 87) was the first to draw attention to the synthesis of triacetin by means of castor seed lipase and to point out that the equilbrium points

with lipase are practically the same as those obtained by using normal sulphuric acid at the ordinary temperature in the case of 0·5, 1·0 and 2·0 per cent solutions of triacetin. A. Welter (Zeits., angew. Chem., 1911, *24*, 385 ; J. S. C. I., 1911, 30, 433) using a mixture of 6—7 parts of ferment, 100 parts of the oil and 35—40 parts of water found that 90 per cent of the oil is hydrolysed during the first two days and the reaction then slowly proceeds to equilibrium ; with only 20—25 parts of water the reaction is slower and equilibrium is attained when 80 per cent of the oil is hydrolysed. With 100 parts of fatty acids, 20 parts of pure glycerol and 10 parts of castor ferment the acid value of the mixture diminishes during the first two days and the values obtained indicated that the following percentages of fatty acids had combined with glycerol in the case of the mixtures of acids named :— palm kernel oil acids 30, cocoanut oil acids 21, maize oil acids 22, ground nut oil acids 19, castor oil acids 14, oleic acid 26, cotton seed acids 7. No reaction occurs if the glycerol is omitted, so that the diminution in the acid value is not due to anhydride formation. The experiments with ground nut acids and palm kernel acids were repeated on a larger scale and the resulting glycerides isolated, examined and saponified and the production of glycerol proved. Somewhat similar results were obtained by F. L. Dunlap and L. O. Gilbert (J. Am. Chem. Soc., 1911, *33*, 1787) by Y. W. Jalander (Biochem. Zeits., 1911, *36*, 435) and by M. Krausz (Zeit. angew. Chem., 1911, *24*, 829). S. Iwanow (Ber. deut. Bot. Ges., 1911, *29*, 595 ; J. S. C. I., 1912, *31*, 501.) has shown that similar synthetic effects can be produced by using glycerine extracts of unripe flax, poppy and rape seeds, and the precipitates obtained by adding alcohol to the glycerine extracts were also found to possess both lipolytic and synthetic properties. Careful experiments on the synthetic functions of lipase have also been made by Armstrong and Gosney (Proc. R. S., 1914, B *88*, 176) using the acids derived from olive oil. With the proportions of glycerol and fatty acids required for the formation of a triglyceride, equilibrium is attained when 40 per cent of the acid has entered into combination, and the same equilibrium point is attained by starting with olive oil and the theoretical amount of water required for complete hydrolysis. By the addition of more water the equilibrium is displaced in the direction of greater hydrolysis and the rate of chemical change is greatly retarded. Excess of glycerine also retards the rate and displaces the equilibrium in the opposite direction, for example with equivalent proportions of fatty acids and glycerol 59·2 per cent had combined 50 hours, whilst with 1 molecular excess of glycerol 55·8

per cent of the acid had combined. The retardation of the hydrolysis by excess of water is ascribed to its direct action in preventing contact of enzyme and oil, but its effect in the synthetic experiments is probably due to the withdrawal of glycerine from the system by its solution in the water. Synthesis is not entirely inhibited by using 30 molecular proportions of water to 1 of glycerol, but in the absence of excess of water excess of glycerol beyond two molecular proportions has but little effect in increasing the amount of fat synthesised. The isolation of the synthetic product and the determination of its saponification value point to the formation of mono-and di-glycerides in addition to the fat itself.

Synthetic functions of lipases from other seeds. Other vegetable lipases also possess synthesising properties: this has been shown in the case of the lipase of chelidonium seeds by K. Bournot (Biochem. Zeitsch., 1913, *52*, 172; 1914, *65*, 140) who states that with mixtures of oleic acid and monohydric alcohols 90 per cent. esterification occurs, he also states that secondary alcohols are esterified less readily and tertiary alcohols not at all, similarly di-and tri-hydric alcohols are esterified less readily than monohydric. With oleic acid and glycerine at 35°, 75 per cent. combination takes place within 24 hours. The rate of esterification tends to increase with the number of carbon atoms present. Most acids of the type $R{\cdot}CH_2{\cdot}CO_2H$ are readily esterified and acids of the type $RR'CH{\cdot}CO_2H$ less readily. The synthetic activity of bacterial lipase has been pointed out by Söhngen (J. S. C. I., 1911, *30*, 812).

The reaction velocity. Many attempts have been made to study the rate of the hydrolytic process and to determine the velocity constant. A. Kanitz (Zeitsch. physiol. Chem., 1905, *46*, 486) taking some of the values obtained by Constein, Hoyer and Wartenberg shows that the value $x/t^{\frac{1}{2}}$ is nearly constant whereas x/t is not, where x denotes the amount of oil hydrolysed in the given time (Compare H. Euler *ibid.* 1905, *45*, 421). Nicloux (C. R., 1903, *138*, 1288,) has attempted to show that the equation for a unimolecular reaction, $k=1/t.\log a/(a-x,)$ holds good for the hydrolysis of an oil by his cytoplasm ferment if the reaction is rapid and nearly completed in 7·5 hours, otherwise the values of k decrease appreciably as t increases. Taylor (*loc. cit.*) using solutions of triacetin or ethyl acetate and his dry lipase preparation found that the values for k calculated by means of the ordinary equation for a unimolecular reaction varied considerably, *e. g.* triacetin from 88 x 10^{-4} to 112 x 10^{-4} The temperature coefficient, k_{t+10}/k_t, in the case of triacetin using temperatures

of 18° and 28° is 2·6. Taylor points out that in the case of oils concordant results for k cannot be expected as the fatty acids produced during the reaction affect the solubilities of triolein, tristearin and tripalmitin and thus the substrate alters in composition; experiments made by the same author on triolein at 18° and shaking mechanically the whole time indicate that the value x/t is roughly constant. The theoretical meaning of this is discussed, and the conclusion drawn that the experimental velocity determined is not a reaction velocity in the chemical sense, but a diffusion velocity in the physical sense. An increase in temperature of 10° increases the experimental velocity by only 20 per cent and within limits the relationship between the mass of the ferment and the degree of acceleration is one of direct proportionality.

Jalander (Biochem. Zeitsch., 1911, *36*, 435) claims that for periods of over 60 minutes and within fairly wide limits of concentration of enzymes Schutz's formula, $x/e^{\frac{1}{2}}$ holds approximately when x=amount of oil saponified and e=quantity of enzyme, and that for constant concentration of enzyme x/t^m=constant where m is a constant varying with the conditions. Armstrong and Gosney (Proc. R. S., 1913, *B 86*, 586) discuss the general question of the dynamics of the reaction between oils and water using castor seed lipase as hydrolyst. Their conclusions are that the interaction must be supposed to take place at and between surfaces separated by a thin film of water at most and that both products of the change inhibit the interaction of enzyme and oil. They studied the rate of hydrolysis and conclude that in all probability a given amount of enzyme changes equal amounts of material in successive equal intervals of time *i. e.* x/t is constant and that the observed departures from this rate are due to the inhibiting effects of the products of the change and to gradual destruction of the enzyme. The rate at which action takes place is dependent on the conditions of the colloid, which cannot be expressed in terms of the concentration of the solution. Hence the law of mass action cannot be applied. It is suggested that lipase contains a glycerol nucleus attached to a carboxylic group in proximity to an acidic group.

Tancov (*loc. cit*) has carried out a number of experiments with olive oil and varying amounts of enzymes and of acid and states that the results show that the substrate and the products formed from it alter the behaviour of lipase towards the activating acid and hence form compounds with the enzyme. These compounds are to be regarded as intermediate, since in presence of the products and of comparatively large proportions of substrate the splitting of oil

by the lipase proceeds with concentrations of acid considerably in excess of those which arrest the reaction when the products are absent and the proportions of substrate comparatively small. The action takes place, therefore, not by means of free lipase but by way of intermediate compounds of lipase with the substrate and its products of decomposition. The intermediate compounds of lipase with the products of its action are decomposed more readily by low than by high concentrations of acid, their stability diminishing with the latter to a minimum, which is found to correspond with the optimum activity of enzyme. With high concentration of acid the substrate and its products in the system undergo an irreversible change.

VI. The Products and their Separation.

The products obtained by the hydrolysis of oils by means of water are glycerol and free fatty acids, but as the reaction is reversible, unless a very large excess of water is used, it follows that in practice the yields of products are not theoretical and as a rule the reaction stops when about 90 to 95 per cent of the oil is decomposed. The product is then a stiff emulsion containing unsaponified oil, free fatty acids, glycerol, water, lipase and the various substances introduced with it and any activator which may have been used. E. Hoyer (Seifenfabrikant, 1903, *23*, 1093) describes a method of taking samples for estimating the amount of hydrolysis. A sample of the emulsion is drawn off, warmed in a tube and 4—5 drops of a 25 per cent solution of sulphuric acid added, the mixture is then boiled and the tube placed on a boiling water bath, when the fatty acids settle out and are run into another tube. The oily acids are heated to remove water, filtered and 2 grams taken, dissolved in 95 per cent alcohol and titrated with standard sodium hydroxide. The method recommended for effecting a separation by breaking the emulsion is the addition of a small amount of sulphuric acid and raising the temperature for a short time to 80°; when the mass has stood for some time three distinct layers are obtained (a) an upper layer consisting of the free fatty acids and any unsaponified oil, this layer will solidify on cooling; (b) a lower layer consisting of an aqueous solution of glycerol, but also containing the activator, acetic acid or manganous sulphate, and the sulphuric acid added at the end of the reaction, as well as various soluble protein materials derived from the castor seed. If kept for some time this solution should clarify and can be run off as a clear, pale yellow coloured liquid; (c) a middle layer containing the crushed seeds used as ferment and also containing much glycerine water and a certain amount of fatty acids.

The treatment of this middle layer is one of the difficulties which has to be overcome in attempting to adopt the castor seed ferment process as a commercial undertaking. When the crushed seeds themselves are used the middle layer is relatively large, much glycerine water is retained in this portion and its separation is difficult. In addition the glycerine water which separates contains large quantities of organic matter in solution and its refining becomes difficult. One of the chief reasons for the numerous attempts made to introduce lipase preparations in place of the crushed seed has been the idea of diminishing the amount of the middle layer and improving the quality of the glycerine water.

Details of the methods used in refining and concentrating the dilute glycerine liquor are given in Part II pp. 260, 263)

VII. Animal Lipases.

The various tissues and organs of the animal body contain lipolytic enzymes which are of importance in the physiological activities of the body. Both that obtained from the liver and from the pancreas, more especially pigs' pancreas, are comparatively rapid hydrolysing ferments and many investigations have been made with them. Just as with castor seed lipase a good emulsion is essential for rapid hydrolysis, but the addition of free acid is injurious and it is necessary to keep the liquid neutral or faintly alkaline by means of sodium carbonate. Serum has an accelerating effect (Tsuji). It has been shown that so called aqueous solutions of the enzymes are active; this activity is, however, lost when the turbid solution is repeatedly filtered and can be restored by adding the clear filtrate to the residue. The lypolytic activity is apparently due to a ferment (the residue) and a co-ferment contained in the filtrate. (Magnus and Rosenheim and Shaw Mackenzie). The co-ferment is not destroyed at 100° and consists of bile salts e. g. sodium taurocholate (Loewenhart). The investigations include a study of the dynamics of the reaction (Dietz), the influence on the reaction of the structures of the esters used (Kastle) the selective action of the enzyme (Dakin, Mayer) and the effect of added salts (Pickelharing). The optimum temperature is 40° and the enzyme is destroyed at 65—70° (Kastle and Loewenhart). The enzymes possess synthesising as well as hydrolysing properties, (Kastle & Loewenhart, Pottevin) and it has been found possible to prepare dry lipolytic powders from pigs' pancreas (Baur). The general view is that the process would be much more expensive to run than a similar process using castor seed lipase (Lowkowitsch).

The following is a list of papers dealing with researches on animal lipases. As some of the journals may not be readily obtained for consultation, references are also given to abstracts of the papers in the abstracts of the Journal of the Chemical Society.

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