The Reactivation of Phosphorylated Chymotrypsin

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Chymotrypsin, like most other enzymes with esterase activity, is inactivated by combination with organophosphates (Balls & Jansen, 1952). The inactive phosphorylated enzyme is relatively stable in water but hydroxylamine and picolino-hydroxamic acid will restore the activity by nucleophilic displacement of the enzyme from the phosphoryl residue (Cunningham, 1954; Jandorf, Crowell & Levin, 1955). The only reported kinetic study of this process is by Cunningham (1954) on reactivation with hydroxylamine, but his conclusions as to mechanism are open to criticism as, at best, only about 30% of the original enzymic activity could be recovered. The present paper describes the complete reactivation of phosphorylated chymotrypsin by oximes and hydroxamic acids. The results necessitate some modification of views on the mechanisms for chymotryptic hydrolysis which have been proposed recently (Cunningham, 1957; Dixon & Neurath, 1957a; Davies & Green, 1958).

EXPERIMENTAL

Materials

Commercial chymotrypsin (The Armour Laboratories) was dialysed against running tap water for 24 hr. to remove $MgSO_4$, and was then freeze-dried. A stock solution of chymotrypsin (50 mg.) in $0.1M$-KH_2PO_4 (10 ml.) was stored in a refrigerator.

N-Acetyltyrosine ethyl ester was prepared from commercial tyrosine ethyl ester by Fischer's method (1904). Hydroxyiminoacetone, salicylohydroxamic acid and picolinohydroxamic acid were prepared as described by Freon (1939), Jeanrenaud (1889) and Hackley, Plapinger, Stolberg & Wagner-Jauregg (1955). 2-*Hydroxyiminomethyl*-N-*methylpyridinium methanesulphonate*, m.p. 155° (Found: N, 11·8. $C_8H_{12}O_4N_2S$ requires N, 12·1%), was prepared by boiling 2-hydroxyiminomethylpyridine with methyl methanesulphonate in benzene.

Methods

Chymotrypsin was assayed by the continuous-titration method (Schwert, Neurath, Kaufmann & Snoke, 1948), whereby the acid produced by enzymic hydrolysis of an ester substrate is titrated, as produced, with $0.1N$-NaOH. The assay mixture consisted of $0.01M$-N-acetyltyrosine ethyl ester (7 ml.), $0.02M$-phosphate buffer, pH 8 (1 ml.), M-NaCl (1 ml.) and enzyme (1 ml. of the stock solution after 500-fold dilution).
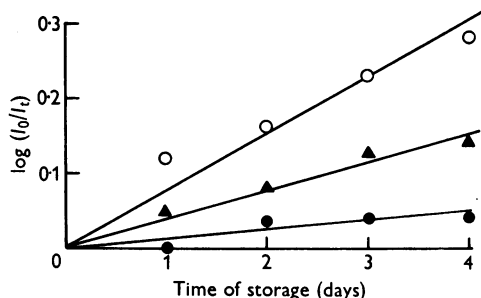

Fig. 1. Spontaneous reactivation at 25° of chymotrypsin inhibited with Sarin. The inhibited enzyme was stored in acetate buffer at pH 5·1 (O), in phthalate buffer at pH 6·3 (▲), or in borate buffer at pH 8·7 (●). Fuller details of the buffer compositions are given in Table 2.
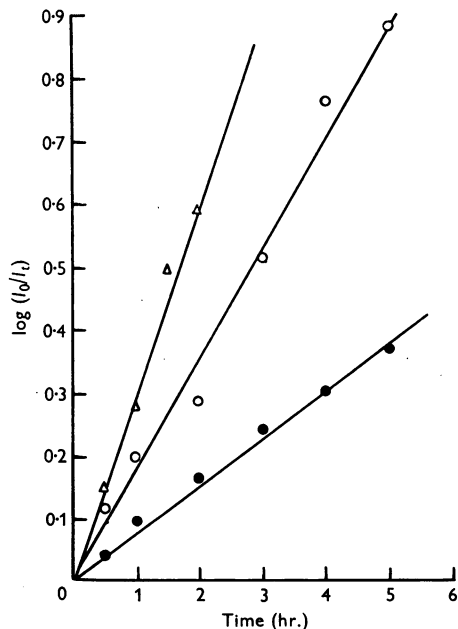

Fig. 2. Reactivation of chymotrypsin inhibited with Sarin by hydroxyiminoacetone (0·5 M) at 25° and at pH 7·5 (△), pH 8·0 (O) or pH 9·0 (●). (For details see Methods.)

The enzyme was inhibited by addition of 0·05 M-*iso*propyl methylphosphonofluoridate (Sarin) in propan-2-ol (0·2 ml.) to the stock solution of the enzyme (2·0 ml.) containing N-NaOH (0·15 ml.) to raise the pH to 7·4. Inhibition was complete within 30 min. at room temperature although there was still some unchanged Sarin present as indicated by Marsh & Neale's (1956) colorimetric method of estimation. After 3 hr. all the excess of Sarin had hydrolysed.

In the spontaneous-reactivation experiments 2 ml. of the inhibited enzyme solution (after contact with Sarin for 3 hr.) was diluted to 10 ml. with an appropriate buffer. At suitable time intervals, samples (1 ml.) were withdrawn and diluted with water to 100 ml.; 1 ml. of the diluted solution was then taken for assay. In the normal-reactivation experiments the buffer was replaced by the oxime or hydroxamic acid adjusted to the required pH. The time of contact between enzyme and inhibitor was reduced to 30 min. as any excess of Sarin would have been hydrolysed almost instantaneously on addition of the reactivator (Green & Saville, 1956). The 100-fold dilution before assay prevented any interference by the reactivator or buffer with the assay. The apparent rate constants were calculated graphically by use of the formula

$$k = \frac{2 \cdot 3}{At} \log (I_0 / I_t),$$

where A is the concentration of the reactivator and I_t / I_0 the fraction of the inhibited enzyme remaining after time t. Typical experiments for spontaneous reactivation and reactivation by hydroxyiminoacetone are shown in Figs. 1 and 2.

RESULTS

Preliminary experiments with some of the oximes and hydroxamic acids which were active in dephosphorylating inhibited cholinesterase (Childs, Davies Green & Rutland, 1955) showed (i) that reactivation was far slower than with cholinesterase, (ii) that *iso*propyl methylphosphonylated chymotrypsin was more readily reactivated than diethylphosphorylated chymotrypsin or di*iso*propylphosphorylated chymotrypsin, and (iii) that among oximes and hydroxamic acids which varied little in their intrinsic reactivity with Sarin itself (Green & Saville, 1956), structural variation produced only minor changes in reactivating power (see Table 1); this is in marked contrast with what was found with cholinesterase (Childs et al. 1955).

Accordingly, for subsequent kinetic work Sarin was chosen as the inhibitor, and hydroxyiminoacetone, which, being highly water-soluble, could be used at high concentration, was chosen as the reactivator.

Although neither diethylphosphorylchymotrypsin nor di*iso*propylphosphorylchymotrypsin appears to hydrolyse on storage in water to regenerate any free enzyme (Balls & Jansen, 1952; Cunningham, 1954), *iso*propyl methylphosphonylchymotrypsin does slowly recover some chymotryptic activity even in the absence of any specific reactivator (see Fig. 1). This recovery by spontaneous hydrolysis was examined further (see Table 2). The results are difficult to interpret kinetically as the rate is markedly affected by the buffer, but the trend is clear, namely that hydrolysis becomes faster as the solution is made more acidic. The experiments were not continued beyond 4 days, as after this time control enzyme solutions in the same buffers had begun to deteriorate, but, as described below, the

Table 1. *Rate constants for the reactivation at 25° of chymotrypsin inhibited with Sarin*

Compound	Concn. (M)	pH	k (l./mole/hr.)
Hydroxyiminoacetone	0·3–0·5	7·5	1·2
	0·2–1·0	8·0	0·96
2-Hydroxyiminomethyl- *N*-methylpyridinium methanesulphonate	0·3–0·5	7·5	0·48
Picolinohydroxamic acid	0·1	8·0	1·1
Salicylohydroxamic acid	0·05	7·4	2·9

Table 2. *Spontaneous recovery at 25° of chymotrypsin inhibited with Sarin*

Inhibited enzyme (2 ml.) in 0·1 M-KH_2PO_4 (adjusted to pH 7·4 with NaOH) was diluted to 10 ml. with buffer. The percentage of reactivation is based on a control non-inhibited enzyme sample in the same buffer.

Buffer	pH of buffer + enzyme	Reactivation after 96 hr. (%)
5 ml. of 0·2 M-sodium acetate + 3 ml. of 0·2 N-acetic acid	5·1	48
4·5 ml. of 0·1 M-Na_2HPO_4 + 3·5 ml. of 0·05 M-citric acid	5·9	47
5 ml. of 0·2 M-potassium hydrogen phthalate + 3 ml. of 0·25 N-NaOH	5·9	40
4 ml. of 0·1 M-potassium hydrogen phthalate + 4 ml. of 0·05 N-NaOH	6·3	26
6·4 ml. of 0·1 M-Na_2HPO_4 + 1·6 ml. of 0·1 M-NaH_2PO_4	7·4	36
2 ml. of 0·2 M-Na_2HPO_4 + 6 ml. of 0·001 N-HCl	7·8	23
5 ml. of 0·2 M-Na_2HPO_4 + 3 ml. of 0·01 N-HCl	8·0	24
5 ml. of 0·2 M-sodium diethylbarbiturate + 3 ml. of 0·12 N-HCl	8·0	<10
5 ml. of 0·2 M-borax + 3 ml. of 0·2 N-HCl	8·7	<10

remaining inhibited enzyme could be reactivated with hydroxyiminoacetone up to the control activity levels.

Table 3 shows the linear dependence on concentration of the rate of reactivation of inhibited chymotrypsin by hydroxyiminoacetone at constant pH. At the pH of these experiments (pH 8) about one-third of the oxime exists in the form of its sodium salt, so that as the oxime concentration is varied the total electrolyte concentration is also

Table 3. *Rate constants for reactivation by hydroxyiminoacetone at* pH 8 *and* 25° *of chymotrypsin inhibited with Sarin*

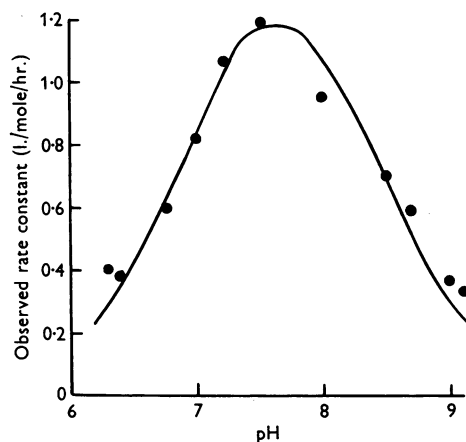| Concn. of hydroxy-iminoacetone (M)	Time (hr.) required for 50 % reactivation	k (l./mole/hr.)
0·2	3·6	0·96
0·3	2·25 2·6 }	0·98
0·5	1·7 1·6 1·45 }	0·89
1·0	0·6 0·8 }	0·99

Fig. 3. Dependence on pH of the rate of reactivation at 25° by hydroxyiminoacetone of chymotrypsin inhibited with Sarin.

varied. In order to check that this did not affect the results, one experiment was carried out with 0·5 M-hydroxyiminoacetone to which had been added 0·5 M-KCl. As no significant change in rate was found in the presence of this additional electrolyte it was not deemed necessary to keep the ionic strength of the solutions constant in subsequent experiments. A linear dependence of rate on concentration was assumed in calculating the rate constants given in Table 1.

The effect of pH on the rate of reactivation by hydroxyiminoacetone is shown in Fig. 3. The bell-shaped curve is similar to that found for reactivation with hydroxylamine (Cunningham, 1954) and for reactivation by hydroxyiminoacetone of inhibited cholinesterase (Davies & Green, 1956), and can be interpreted in the same way. The curve superimposed on the experimental points was drawn on the assumption that reactivation is due solely to a reaction between the anion of the oxime (pK_A 8·3) and a protonated form of the inhibited enzyme with pK_A 7·0.

Cholinesterase, when inhibited with organophosphates, gradually changes from a form which can be reactivated by oximes and hydroxamic acids into one which cannot. This change is accelerated in mildly acidic solution (Davies & Green, 1956; Hobbiger, 1956). In order to see whether inhibited chymotrypsin undergoes a like change solutions of it were inhibited with Sarin at pH 7·4 and were then diluted with buffers to maintain the pH at 7·4 or to alter the pH to 5 or 8. These solutions were then stored at 25° for 4 days, after which time they were treated with hydroxyiminoacetone (0·5 M) at pH 8 for 6 hr. Complete reactivation occurred in each (see Table 4).

DISCUSSION

Organophosphates react with chymotrypsin to give an inactive protein containing a single phosphoryl group per molecule (Balls & Jansen, 1952). Acidic or enzymic hydrolysis of the phosphorylated enzyme gives a number of phosphorylated peptides but the phosphorus atom is invariably found attached to the hydroxylic oxygen of a single

Table 4. *Reactivation of chymotrypsin inhibited by Sarin after storage at* 25°

Chymotrypsin inhibited by Sarin at pH 7·4 was diluted with buffers at different pH values. After storage for 4 days the inhibited enzyme solution was treated with hydroxyiminoacetone (0·5 M) for 6 hr. at pH 8. Enzyme activities are given in arbitrary units. Fuller details of the buffer compositions are given in Table 2.

Buffer	pH	Activity after 0 hr.		Activity after 96 hr.	
		Enzyme control	Inhibited enzyme	Enzyme control	Reactivated enzyme
Acetate (0·1 M)	5·1	200	20	186	180
Phosphate (0·1 M)	7·4	203	0	163	162
Barbitone (0·1 M)	8·0	177	11	177	177

serine residue (Cohen, Oosterbaan, Warringa & Jansz, 1955; Schaffer, Simet, Harshman, Engle & Drisko, 1957). More recently, inactive monoacylated chymotrypsins have been isolated (McDonald & Balls, 1957), which are similar to the phosphorylated enzymes although they are much more readily reactivated. Degradation experiments have shown that in monoacetylchymotrypsin, the acetyl group is attached to the same serine hydroxyl group as is the phosphoryl group in phosphorylated chymotrypsin (Oosterbaan & van Adrichem, 1958). This evidence, together with the similar dependence on pH of phosphorylation, of acylation and of normal substrate hydrolysis (Hartley, 1956; Gutfreund & Sturtevant, 1956; Dixon & Neurath, 1957b), suggests that the initial step in each case is phosphorylation or acylation of a serine hydroxyl group catalysed by the imidazole ring of a histidine residue. In normal substrate hydrolysis the acylated enzyme is assumed to hydrolyse immediately to regenerate the free enzyme. Hydrolysis of the more stable acylated enzymes can be followed kinetically (Gutfreund & Sturtevant, 1956; Dixon & Neurath, 1957b) and it has been shown that hydrolysis occurs more rapidly as the pH is raised and can be explained as a histidine-catalysed hydrolysis of the acylated serine.

If the mechanism of phosphorylation and dephosphorylation is identical with that of acylation and deacylation, then reactivation of the phosphorylated enzyme should occur more readily with increasing pH. In fact, as shown above, quite the reverse is true, the phosphorylated enzyme being hydrolysed less readily as the pH is increased. Furthermore, the rate of reactivation by hydroxyiminoacetone similarly falls in more alkaline solution. These results are consistent with the effect of pH on the hydrolysis of a phosphorylated imidazole group (Atkinson & Green, 1957), but not with an imidazole-catalysed hydrolysis, yet the degradation evidence seems overwhelming that the amino acid which is phosphorylated is serine, not histidine. These discrepancies can be resolved, at least qualitatively, if the acylated (or phosphorylated) active centre in its various possible states of ionization exists as shown in Fig. 4.

In neutral solution or weakly alkaline solution (about pH 8) the acylated enzyme exists primarily as an equilibrium mixture of forms b and e (Fig. 4), which are interconverted via forms a and g or c and g.
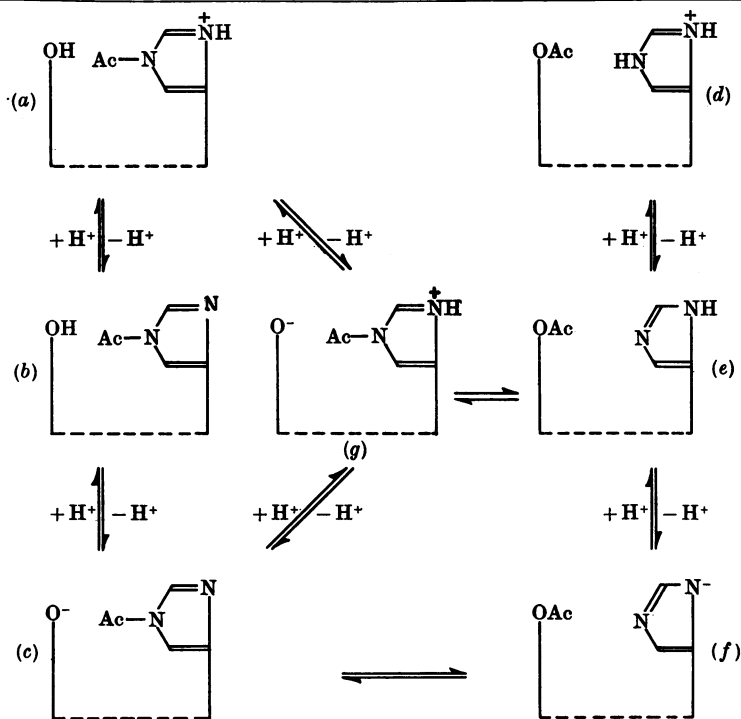
Ac, Acyl or phosphoryl

Fig. 4. The hydroxyl group is that of a serine residue, the imidazole group that of a histidine residue. These are assumed to be held in juxtaposition by the configuration of an unspecified number of amino acids. Equilibrium signs are drawn only where the two structures differ only in the migration of a single atom or group.

The predominant form is assumed to be *e*, so that on degradation the main product would be an acyl- or phosphoryl-serine. Forms *c* and *f* will occur to a significant extent only in fairly strong alkali and can be ignored under normal conditions.

In more acidic solution the equilibrium will lie mainly between *a* and *d*, *d* being assumed to be predominant. Since acylated hydroxyl groups are generally fairly stable whereas acyl imidazoles are relatively unstable it is reasonable to assume that before the acyl group can be hydrolysed away from the enzyme it must be located on the imidazole ring. Thus for hydrolysis to occur the predominant *O*-acyl form (*d* and *e*) must be converted via *g* into the *N*-acyl form (*a* and *b*). If the hydrolysis of the *N*-acyl form were more rapid than the acyl-migration reaction (*e* → *g*) the effect of pH on the rate of hydrolysis would be determined by the fraction of the *O*-acylated enzyme in form *e*. This is consistent with the observed increase in the rate of deacylation with increasing pH (Gutfreund & Sturtevant, 1956; Dixon & Neurath, 1957*b*). If, in dephosphorylation on the other hand, the rate-controlling step were the hydrolysis of the *N*-phosphoryl form and not the O → N migration (*e* → *g*) of the phosphoryl group, then the rate of dephosphorylation would be expected to increase and not decrease with decreasing pH, as found and as observed with di-*iso*propylphosphorylimidazole (Atkinson & Green, 1957).

This modified theory of deacylation and dephosphorylation does not necessitate any amendment to previous theories (Cunningham, 1957; Dixon & Neurath, 1957*a*; Davies & Green, 1958) that acylation and phosphorylation involve an imidazole-catalysed acylation or phosphorylation of serine. All the above reactions may be assisted or hindered by hydrogen-bonding but the evidence is insufficient for any categorical assertions as to which groups are bonded in this way.

It is becoming widely accepted that not only are individual esterases acylated and phosphorylated by what is basically the same mechanism, but also that the mechanism of phosphorylation of esterases generally is fundamentally the same. The similarity in the dependence on pH of phosphorylation of a wide range of esterases (Mounter, Alexander, Tuck & Dien, 1957) and the isolation of very similar phosphorylated peptides on the degradation of inhibited esterases (Cohen *et al.* 1955) strongly support this belief. The resemblance between inhibited chymotrypsin · and inhibited cholinesterase in the dependence on pH of the rate of reactivation offers yet further support. If this idea is correct then the above picture of the phosphorylated active centre of chymotrypsin should also be applicable to cholinesterase, but phosphorylated cholinesterase, unlike phosphorylated chymo-

trypsin, slowly changes on storage from a form which can be reactivated to one which cannot. This change has been tentatively attributed (Jandorf, Michel, Schaffer, Egan & Summerson, 1955; Davies & Green, 1956; Hobbiger, 1956) to migration of the phosphoryl group from histidine, which is assumed to be the initial site of phosphorylation, to serine, but if the above picture is correct this explanation is untenable. An alternative possibility (W. N. Aldridge, unpublished work) is that, on storage of the inhibited enzyme, one of the alkyl groups is split off the alkylphosphoryl residue, giving a phosphate anion resistant to nucleophilic attack by the reactivator. This dealkylation may be accelerated in inhibited cholinesterase by interaction between the alkyl group and the anionic-specificity site on the enzyme which is absent in chymotrypsin. This would explain the chemically unexpected fact that conversion from the 'reactivatable' form into the 'non-reactivatable' form occurs much more rapidly with di*iso*propylphosphoryl-cholinesterase and *iso*propyl methylphosphonyl-cholinesterase than with diethylphosphoryl-cholinesterase. This theory could be proved or disproved unequivocally by comparison of the conversion rate with the rate of loss of ^{14}C from purified cholinesterase inhibited with Sarin containing ^{14}C in the *iso*propyl group.

SUMMARY

1. Oximes and hydroxamic acids will completely reactivate chymotrypsin inhibited with organophosphates, at a rate varying linearly at constant pH with the concentration of the reactivator.

2. Hydroxyiminoacetone, 2-hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate, picolinohydroxamic acid and salicylohydroxamic acid have about equal potency as reactivators of chymotrypsin inhibited with *iso*propyl methylphosphonofluoridate (Sarin). This is in line with their roughly equal reactivities with Sarin itself but is in marked contrast with the great difference in their abilities to reactivate inhibited cholinesterase.

3. A bell-shaped pH–activity curve was found for reactivation by hydroxyiminoacetone of chymotrypsin inhibited by Sarin.

4. On storage at 25° for 4 days at various pH values, inhibited chymotrypsin, unlike inhibited cholinesterase, did not lose its ability to be reactivated.

5. Chymotrypsin inhibited with Sarin slowly recovers its activity even in the absence of any specific reactivator. This spontaneous recovery is accelerated by a decrease in pH.

6. A structure is proposed for the phosphorylated or acylated active centre of chymotrypsin which will explain (*a*) the isolation after degradation of

phosphorylated or acylated chymotrypsin of peptides in which the phosphoryl or acyl group is attached to a serine hydroxyl group, (b) the increase in the rate of hydrolysis of acylchymotrypsin as the pH is raised, and (c) the increase in the rate of hydrolysis of phosphorylchymotrypsin as the pH is lowered.

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Substrate Specificity of Rumen Cellulolytic Enzymes

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Cell-free preparations of rumen micro-organisms prepared by butanol extraction have been previously shown to contain at least two types of enzyme involved in the hydrolysis of carboxymethylcellulose, one of these, akin to cellobiase, being selectively inhibited by D-glucono-1 → 4-lactone (Festenstein, 1958a). It has also been shown (Halliwell, 1957a, b) that the type of extract studied is without appreciable action on filter paper or cotton fibres. In the present work, specificity studies have been extended to cellophan and cellulose dextrin and the inhibitory effect of gluconolactone has been examined with reference to specific glucose production from cellulosic substrates, as well as from cellosaccharides up to cellopentaose.

EXPERIMENTAL

Substrates. Soluble cellulose dextrin was prepared from absorbent cotton wool and also from filter-paper powder (Whatman, standard grade) by solution in 72% H_2SO_4, as

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described by Kooiman, Roelofsen & Sweeris (1953); small amounts of insoluble cellulose dextrin were removed along with the excess of $BaCO_3$ added for neutralization of acid and the filtrates were clarified by centrifuging at 10 000 g. The final products, twice precipitated by 80% ethanol, gave values of 20 (filter paper) and 30 (cotton wool) for the degree of polymerization, as determined by the cuprimetric and hypoiodite methods used for determination of reducing sugar (Festenstein, 1958a); they were soluble in the buffered solutions used in the enzyme-assay procedure, but small amounts of dilute H_2SO_4 gave precipitates which did not dissolve on subsequent neutralization.

Cellotriose, cellotetraose and cellopentaose were gifts from the British Rayon Research Association; they were chromatographically pure and gave reducing values of 1·0, 1·18 and 1·18 respectively, expressed in terms of reduction of the Nelson–Somogyi reagent (Somogyi, 1952) by equimolecular quantities of glucose; the value obtained for cellobiose (L. Light and Co. Ltd.) was 1·12.

Sodium carboxymethylcellulose containing an average of 0·5 carboxymethyl unit per glucose residue (Cellofas B, Imperial Chemical Industries Ltd.), cellobiose (L. Light and Co. Ltd.) and cellophan (PT 300, British Cellophane Ltd.) were commercial preparations. Cellophan strips weighing 25 mg. were cut from the sheets supplied and soaked in water for at least 48 hr. before use, in order to