Biosynthesis of Intestinal Mucins

3. FORMATION OF 'ACTIVE SULPHATE' BY CELL-FREE EXTRACTS OF SHEEP COLONIC MUCOSA*

By P. W. KENT AND C. A. PASTERNAK†
Department of Biochemistry, University of Oxford

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Previous results (Pasternak, Kent & Davies, 1958) showed that $^{35}\text{SO}_4^{2-}$ ion is utilized in differing degrees by surviving intestinal tissues and that the ion is incorporated as sulphate esters of intestinal mucins (Pasternak & Kent, 1958). The incorporation is energy-dependent and inhibitors of respiration also prevent the uptake of sulphate. A relatively high level of incorporation of SO_4^{2-} ion was found in colonic mucosa, and on account of its availability and ease of homogenization this tissue was selected for an investigation of the individual stages of the sulphation mechanism.

In liver, formation of phenolic sulphates involves the enzymic activation of SO_4^{2-} ions by combination with adenosine triphosphate (ATP), forming adenosine 3'-phosphate 5'-phosphosulphate (Hilz & Lipmann, 1955). D'Abramo & Lipmann (1957) secured, from chick-embryo condyles, cell-free preparations which promote the incorporation of $^{35}\text{SO}_4^{2-}$ ion into chondroitin sulphate, with indications that a similar sulphate-activation mechanism participates.

MATERIALS AND METHODS

Preparation of tissue homogenates. A portion of the large intestine (approx. 30 cm. from the anus) of adult sheep was obtained immediately after slaughter and placed in ice-cold saline. Within 30 min. the intestine was emptied, rinsed with water and transferred to ice-cold KCl solution (0.9%) brought to pH 7.5 with a little 2-amino-2-hydroxy-methylpropane-1:3-diol (tris). The mucosa (12.5 g.), removed by scraping with a scalpel, was immersed in 25 ml. of 0.25 M-sucrose or KCl (0.9%) at 0°, each solution being brought to pH 8.5 by addition of tris. The tissue was then homogenized (12 strokes) in a chilled Potter-type stainless-steel apparatus.

Fractionation of homogenates. The homogenate (37 ml.) was centrifuged at 0° for 20 min. at 24 000 g. The clear supernatant fraction (22 ml.) was decanted and used in the experiments described below. The precipitate was resuspended in 12 ml. of 0.25 M-sucrose before investigation.

Incubations. Experiments (Table 1) were performed under O_2 in Warburg manometer flasks at 37° for 30–60 min., the centre well containing NaOH on filter paper. Each flask contained 0.1 M-sodium phosphate buffer (pH 7.6, 0.4 ml.), 0.2 M- MgCl_2 (0.2 ml.), 0.154 M- MgSO_4 (0.036 ml.), 0.02 M-ATP (0.2 ml.), $10\mu\text{C}$ of $\text{Na}_2^{35}\text{SO}_4$ (approx. 0.1 ml.) and 0.2 ml. of a solution containing 0.5 M-sodium pyruvate and 0.04 M-sodium fumarate. In each case 3 ml. of a suspension of mucosal scrapings or other enzyme fraction was added. In appropriate cases, 0.5 M-sodium fluoride (0.3 ml.) was added also. Subsequent experiments (Tables 2–4) with an appropriate buffer solution, additions and supernatant enzyme fraction, were performed in stoppered tubes. The protein content of enzyme preparations (1 ml.) was determined separately by precipitation with 1 ml. of trichloroacetic acid (10%, w/v). The filtered trichloroacetic acid insoluble precipitate was weighed after being dried at 100° for 12 hr. The contents of each flask, at the end of incubation, were heated to 100° for 1 min. or were treated with ethanol (1 ml.). After centrifuging (2000 rev./min.) for 2 min. the supernatant solution was examined by paper electrophoresis and paper chromatography. (Imidazole buffers were prepared by the method of Klemperer, 1955.)

Uridine diphosphate glucose. This was obtained from California Biochemicals Inc., Berkeley, Calif., U.S.A.

Uridine diphosphate N-acetyl-D-glucosamine. This was kindly given by Dr Pontis Videla.

Paper electrophoresis. Buffers employed were: sodium diethylbarbiturate (pH 8.8, 0.07 M), ammonium acetate (pH 5.6, 0.1 M) and sodium citrate (pH 5.6, 0.1 M). At low voltage (4.3 v/cm.), experiments were carried out on Whatman no. 1 paper held between glass plates. At the higher voltage (17 v/cm.), Whatman no. 3 paper bathed in chlorobenzene (Cremer & Tiselius, 1950) was employed and the separations were carried out at 3°. Ethanediol (30 ml.) was added to the buffer (500 ml.) to prevent freezing. In analytical experiments 0.025–0.05 ml. of the test solution was used and in preparative experiments 1 ml. was applied across the paper. At the end of each experiment the paper was dried in warm air and the radioactive components were assayed as described previously (Pasternak & Kent, 1958).

Isolation of components separated by paper electrophoresis. The region of dried paper carrying the separated radioactive component was cut out and eluted continuously with glass-distilled water until the radioactivity of the paper had fallen to background count. This was usually achieved within 4 hr. The eluate was concentrated to dryness by lyophilization. Though efficient separations of $^{35}\text{SO}_4^{2-}$ ion and other ^{35}S -labelled products was achieved by the use of

* Part 2. Pasternak & Kent (1958).

† Present address: Department of Pharmacology, School of Medicine, Yale University, New Haven, Conn., U.S.A.

any of the buffers described, ammonium acetate was employed in preparative experiments since it could be removed easily during the freeze-drying procedure, provided that a trap containing solid KOH was attached to the apparatus to maintain neutrality of the specimen.

Paper chromatography. Systems employed were: (a) butan-1-ol-ethanol-water (water-poor phase, 4:1:5, by vol.) in descending elution (Partridge, 1946); (b) ethanol- α -ammonium acetate (5:2, v/v), descending elution (Paladini & Leloir, 1952); (c) propan-2-ol-5.7*N*-HCl (13:7, v/v), descending elution (Wyatt, 1951). Whatman no. 1 paper was used throughout; reducing sugars were detected with aniline hydrogen phthalate (Partridge, 1949), u.v.-absorbing substances by contact photography (Markham & Smith, 1949) and phosphorylated substances as described by Hanes & Isherwood (1949). Sulphated phenols were located as described by Loewi & Kent (1957).

Glucose, glucose 6-sulphate and SO_4^{2-} ions were conveniently separated by ascending chromatography in ethanol-0.1*M*-ammonium acetate (4:1, v/v) (Cohen & Scott, 1950), in which the R_f values were 0.74, 0.65 and 0.27 respectively.

Radioactive Assays. These were as described by Pasternak *et al.* (1958).

RESULTS

A preliminary survey was made of the enzymic activity of whole mucosal scrapings, whole homogenate, and of the soluble and insoluble fractions of tissue homogenates. In each case the ^{35}S -labelled products were examined by paper electrophoresis and the $q_{0.5}$ values were measured during incubation (Table 1) and expressed as $\mu\text{l. of O}_2/\text{hr./mg. of trichloroacetic acid-insoluble material}$. By electrophoresis at pH 8.8 in diethylbarbiturate buffer three radioactive anionic substances were detected,

Table 1. Incorporation of [^{35}S]sulphate by, and $q_{0.5}$ values of, preparations of sheep colonic mucosa

All figures are averages of duplicate experiments.

Incubation medium was that described in the Methods section (containing 85 000 counts/min.).

[^{35}S]Mucin and ^{35}S -labelled component A were determined after incubation for 30 min. by assay of ^{35}S after paper electrophoresis (sodium diethylbarbiturate buffer) and are expressed as counts/100 000 added/mg. of protein. $q_{0.5}$ refers to $\mu\text{l. of O}_2/\text{mg. (dry wt.) of trichloroacetic acid-insoluble material/hr.}$

	With fluoride		
	[^{35}S]Mucin	[^{35}S]Component A	$q_{0.5}$
Scrapings	3	12	-2.5
Homogenate	10	0	-3.2
Supernatant	10	198	0.0
Sediment	1	0	0.0
	Without fluoride		
	[^{35}S]Mucin	[^{35}S]Component A	$q_{0.5}$
Scrapings	62	5	-9.8
Homogenate	10	10	-2.4
Supernatant	9	463	0.0
Sediment	3	0	0.0

(1) A fast-moving component which was shown to be unchanged $^{35}\text{SO}_4^{2-}$ ions, (2) an immobile component, ^{35}S -mucin and (3) an intermediately mobile ^{35}S -labelled substance, designated component A. It appears from Table 1 that the supernatant enzyme fraction is the most active in fixing inorganic sulphate and that the formation of component A is inhibited by F^- ion. The results obtained from homogenates prepared in potassium chloride or sucrose are shown in Fig. 1. It is clear that either medium is suitable and that an incubation period of 20 min. is the optimum time for the production of the labelled component A under the conditions of the experiment. Subsequently all the experiments were performed with potassium chloride homogenates.

Fig. 1. Formation of ^{35}S -labelled component A by sheep colonic mucosa homogenized in 0.9% KCl (O) and 0.25*M*-sucrose (x). The incubation medium contained 10 μmoles of ATP, 4 μmoles of MgCl_2 , 110 μmoles of MgSO_4 , 80 μmoles of phosphate buffer (pH 7.6), 24 μC of $\text{Na}_2^{35}\text{SO}_4$, 7.5–12 mg. in 3 ml. of supernatant enzyme fraction in 7 ml. (total volume). ^{35}S -Labelled component A was determined by assay of ^{35}S after paper electrophoresis (sodium diethylbarbiturate buffer).

Table 2. *Formation of [^{35}S]component A by supernatant fraction of homogenized sheep colonic mucosa*

Incubation medium: 100 μmoles of tris, pH 8; 20 μC of $\text{Na}_2^{35}\text{SO}_4$, 10.7 mg. of enzyme in 1 ml., incubated for 20 min. at 37°. ^{35}S -labelled component A was determined by assays of ^{35}S after paper electrophoresis (ammonium acetate buffer) and is expressed as counts/100 000 added/mg. of trichloroacetic acid-insoluble material.

Additions	^{35}S -labelled component A (counts)
No addition	0
MgCl_2 (6.5 μmoles)	0
ATP (5.0 μmoles)	1200
MgCl_2 (6.5 μmoles) + ATP (5 μmoles)	2400
MgCl_2 (6.5 μmoles) + ATP (5 μmoles) (heated inactivated enzyme)	36

As a result of attempts to simplify the incubation medium, it was found (Table 2) that ATP and Mg^{2+} ions appear to be essential for the formation of the intermediate. In the boiled-enzyme control or ethanol-inactivated control no intermediate was formed.

In the light of these results, incubation of ATP, $^{35}\text{SO}_4^{2-}$ and Mg^{2+} ions with the supernatant enzyme fraction was carried out on a preparative scale and the ^{35}S -labelled component A was isolated. The substance had λ_{max} at 255 $\text{m}\mu$ (0.01N-HCl). It proved to be markedly unstable and was readily hydrolysed by acidic conditions with the liberation of SO_4^{2-} ions. Hydrolysis occurred even during the freeze-drying stage of its isolation unless precautions were taken to maintain neutrality during the process. During attempted preparative electrophoretic separations in sodium citrate at 17 v/cm., a second labelled intermediate (component B) was detected. This was less radioactive and moved more slowly than A but was not investigated further.

Complete release of sulphate resulted when component A was heated with 0.1N-HCl at 37° for 30 min. (Fig. 2) or with 0.1N-HCl at 100° for 15 min. but the compound appeared to be stable to 0.01N-HCl at 100° for several minutes. Acid hydrolysis yielded, in addition to SO_4^{2-} ions, a compound having a u.v.-absorption maximum at 260 $\text{m}\mu$ in 0.01N-HCl.

The product of acid hydrolysis moved more slowly than component A in paper electrophoresis at pH 5.6 and had a mobility similar to that of adenosine diphosphate (ADP). The u.v. spectrum was unaffected by addition of bromine and the product was therefore considered not to be a uridine nucleotide (Caputto, Leloir, Cardini & Paladini, 1950). More vigorous hydrolysis of component A with 2.5N-HCl at 100° for 6 hr., followed by paper-chromatographic examination of the products

(freed from HCl under high vacuum) in propan-2-ol-HCl, revealed the presence of adenine (R_f 0.27, λ_{max} 257 $\text{m}\mu$ in 0.01N-HCl). Under these conditions inorganic phosphate was also released.

Transfer of sulphate from component A. In order to test whether component A was an 'active sulphate', its reactions with phenol and o-nitrophenol were examined with a liver transferase system (DeMeio & Tkacz, 1950). The enzyme preparation was the supernatant fraction prepared from rat liver (4 g.), homogenized in 20 ml. of 0.9 % potassium chloride and centrifuged at 2400 g for 30 min. at 0°; 18 ml. of supernatant was obtained, of which 0.7–1 ml. was used in each test. In the presence of this enzyme preparation ^{35}S -labelled component A, transfers its sulphate group to an added phenol (Table 3) in the absence of

Fig. 2. Rate of hydrolysis of ^{35}S -labelled component A by 0.1N-HCl at 37°. $^{35}\text{SO}_4^{2-}$ ions were estimated after separation by paper electrophoresis in ammonium acetate.

Table 3. *Formation of phenyl [^{35}S]sulphate by supernatant fraction of homogenized rat liver*

Incubation medium: 10 μmoles of MgSO_4 , 2.5 μmoles of MgCl_2 , 10 μmoles of cysteine, 1 μmole of phenol, 200 μmoles of tris, pH 8; 5 mg. of enzyme in 1 ml., incubated for 30 min. Phenyl [^{35}S]sulphate was determined by assay of ^{35}S after paper chromatography (butanol-ethanol-water) and is expressed as counts/100 000 added/mg. of trichloroacetic acid-insoluble material.

Additions	Phenyl [^{35}S]sulphate (counts)
$\text{Na}_2^{35}\text{SO}_4$ (2 μC)	0
$\text{Na}_2^{35}\text{SO}_4$ (2 μC) + ATP (10 μmoles)	600
^{35}S -labelled component A (200 $\mu\text{m-moles}$)	9400

ATP, without the intervention of inorganic sulphate. It was also found (Table 4) that the supernatant enzyme fraction from sheep colonic mucosa catalysed the formation of phenolic sulphates from component A.

Attempted sulphation of carbohydrate substrates. A series of experiments was performed in which isolated ^{35}S -labelled component A (or $^{35}\text{SO}_4^{2-}$ ions, ATP and Mg^{2+} ions) and supernatant mucosal enzyme preparation were incubated with a number of potential carbohydrate acceptors. The latter were derivatives of sugars which are either known, or related to known constituents of sulphated mucins (Werner, 1953), and included D-glucose, D-glucosamine hydrochloride, D-galactosamine hydrochloride, N-acetyl-D-glucosamine, methyl N-acetyl- α -D-galactosaminide, methyl N-acetyl- β -D-glucosaminide, α -D-glucose 1-phosphate, D-glucos-

amine 6-O-phosphate, uridine diphosphate glucose, uridine diphosphate N-acetyl-D-glucosamine and hyalobiuronic acid. The extruded and extracted guinea-pig colonic mucins (5 mg.) (Pasternak *et al.* 1958) were also examined. The following system was used to investigate the sulphation of these substances: the carbohydrates (1 μmole), cysteine (10 μmoles), imidazole (100 μmoles), $\text{Na}_2^{35}\text{SO}_4$ (5–20 μC) and 8–12 mg. of enzyme, in a final volume of 0.6–1.0 ml. (pH 7). After incubation for 60–90 min. at 37°, the products were examined by electrophoresis in ammonium acetate and by chromatography in ethanol-ammonium acetate. No evidence was obtained of the formation of a sulphated carbohydrate, either with the supernatant enzyme or with whole mucosal homogenates. However, added glucose 6-O-sulphate is still present after incubation with these enzyme preparations.

Table 4. *Formation of phenyl [^{35}S]sulphate from component A by supernatant fraction of rat liver and sheep colon*

Supernatant fractions of homogenized liver and colonic mucosa were prepared as described in the Methods section. Incubation medium 10 μmoles of MgSO_4 , 2.5–5 μmoles of MgCl_2 , 10 μmoles of cysteine, 1 μmole of substrate, 100–200 μmoles of imidazole or tris buffer, 0.3–20 μmoles of ^{35}S -labelled intermediate, 3.4–10.7 mg. of trichloroacetic acid-insoluble material in 0.7–1.0 ml. of enzyme preparation, incubated for 30–60 min. [^{35}S]Sulphate substrate was determined by assay of ^{35}S after paper chromatography (butanol-ethanol-water or ethanol-ammonium acetate) and is expressed as counts/100 000 added/mg. of protein.

Supernatant fraction	Substrate	pH	[^{35}S]Sulphated substrate (counts)
Rat liver	Phenol	8.0	9400
Rat liver	<i>o</i> -Nitrophenol	7.5	8600
Sheep colonic mucosa	Phenol	8.0	7600
Sheep colonic mucosa	<i>o</i> -Nitrophenol	7.0	3900

Table 5. *Spectral data of non-radioactive nucleoside components of reaction mixture*

Reaction mixture: 5 μmoles of ATP, 6.5 μmoles of MgCl_2 , 100 μmoles of tris (pH 8), 20 μC of $\text{Na}_2^{35}\text{SO}_4$, 10.7 mg. of trichloroacetic acid-insoluble material (supernatant fraction of homogenized sheep colonic mucosa) in 1 ml., incubated for 20 min. at 37°. Chromatographic system was butanol-ethanol-water.

	R_F 0		R_F 0.12		R_F 0.23	
	0.01 N-HCl	0.01 N-NaOH	0.01 N-HCl	0.01 N-NaOH	0.01 N-HCl	0.01 N-NaOH
$\lambda_{\text{max.}}$ (m μ)	252	252	248	251	248	258
$\lambda_{\text{min.}}$ (m μ)	230	229	227	228	228	233
E_{250}/E_{260}	1.16	1.11	1.59	1.11	1.45	0.92
E_{280}/E_{260}	0.23	0.19	0.26	0.10	0.20	0.08

Table 6. *Spectral data of components of reaction mixture separated by chromatography in ethanol-ammonium acetate (pH 7.5)*

Reaction mixture: 5 μmoles of ATP, 6.5 μmoles of MgCl_2 , 100 μmoles of tris (pH 8), 20 μC of $\text{Na}_2^{35}\text{SO}_4$, 10.7 mg. of trichloroacetic acid-insoluble material (supernatant fraction of homogenized sheep colonic mucosa) in 1 ml., incubated for 20 min. at 37°.

	R_F 0.23		R_F 0.67	
	0.01 N-HCl	0.01 N-NaOH	0.01 N-HCl	0.01 N-NaOH
$\lambda_{\text{max.}}$ (m μ)	249	251	249	252, 257
$\lambda_{\text{min.}}$ (m μ)	228	228	227	231
E_{250}/E_{260}	1.69	1.37	1.52	1.02
E_{280}/E_{260}	0.19	0.20	0.16	0.18

Breakdown of adenosine triphosphate by mucosal enzymes. In addition to the ^{35}S -labelled components A and B, a number of other u.v.-light-absorbing compounds were detected electrophoretically and chromatographically after the supernatant enzyme preparation from colon had acted on $\text{ATP} + \text{SO}_4^{2-}$ ions. These products were not radioactive and their formation was observed in the absence of SO_4^{2-} ions.

Electrophoresis showed a stationary and a mobile u.v.-light-absorbing component; the former was phosphorus-free and appeared to be a mixture of nucleosides or purines. The mobile substance was a phosphate ester having $\lambda_{\text{max.}}$ at $248\text{ m}\mu$, $\lambda_{\text{min.}}$ at $222\text{ m}\mu$ in 0.01N-HCl and $\lambda_{\text{max.}}$ at $252\text{ m}\mu$, $\lambda_{\text{min.}}$ at $225\text{ m}\mu$ in 0.01N-NaOH , and behaved electrophoretically as an inosine monophosphate.

Paper chromatography of the incubation mixture in butanol-ethanol-water revealed a stationary component and two substances with R_f values of 0.12 and 0.23. The R_f values of these substances and their spectral characteristics agreed with those of inosine and hypoxanthine (Table 5). In ethanol-ammonium acetate, the inosine monophosphate (first recognized electrophoretically) had R_f 0.23, and is clearly distinguished from ATP and ADP. The isolated nucleotide had spectral data (Table 6) in agreement with published values.

DISCUSSION

Hitherto, the study of the mechanism of enzymic sulphation in mammalian systems has been confined largely to the formation of phenolic sulphates by liver enzymes. Hilz & Lipmann (1955) showed that 'active sulphate' is first formed from SO_4^{2-} ions and 2 moles of ATP (Wilson & Bandurski, 1956; Bandurski, Wilson & Squires, 1956), and that another enzyme is involved in this subsequent transfer of sulphate from the 'active' derivative to phenolic acceptors. The active sulphate was shown to be adenosine 3'-phosphate 5'-phosphosulphate (Robbins & Lipmann, 1956*a, b*). Similar sulphate activation has been described in yeast (Wilson & Bandurski, 1956) and in *Neurospora crassa* (Hilz & Lipmann, 1955). Liver is known to contain sulphated polysaccharides, notably those of the heparin group. The intestinal mucosa, on the other hand, are sites of high metabolic activity, producing extracellular, soluble, sulphated mucins. It is of interest therefore that here also evidence of sulphate activation has been found. The 'active sulphate', formed enzymically under conditions similar to those described for liver, is an adenine nucleotide carrying a labile sulphate group. The rate of hydrolysis of the latter is substantially greater than that of alkyl *O*- or *N*-sulphates (Meyer & Schwartz, 1950) and is suggestive of an

anhydride linkage. The substance has the same electrophoretic mobility (0.6 that of SO_4^{2-} ions at pH 5.6) as adenosine 3'-phosphate 5'-phosphosulphate. The 'active sulphate' of the mucosa also closely resembles adenosine 3'-phosphate 5'-phosphosulphate in its ability to act as a sulphate donor in the formation of phenolic sulphates either by liver or by mucosal enzyme preparations. It is concluded that the active sulphate of colonic mucosa is either identical with or closely similar to adenosine 3'-phosphate 5'-phosphosulphate. The yield of phenyl sulphate from 'active sulphate' did not exceed 40% with unfractionated soluble mucosal enzymes, and was accompanied by some hydrolysis of the intermediate. Under the conditions necessary for the sulphation of phenols, no evidence of sulphation of simple mono- and disaccharides was obtained. This did not appear to be due to the presence of a glycosulphatase in the enzyme preparation, since D-glucose 6-*O*-sulphate was not hydrolysed. Recent results obtained by D'Abramo & Lipmann (1957) suggest that in the formation of chondroitin sulphate, oligo- or poly-saccharide acceptors engage with 'active sulphate'.

Circumstantial evidence indicates that the same mechanism of sulphate activation may be involved in the biosynthesis of aryl sulphates as in that of sulphated polysaccharides. In cartilaginous tissue (Layton & Frankel, 1951; Loewi & Kent, 1957), and in the small intestine (Werner, 1953; Williams, 1947) formation of both types of sulphated product has been observed. The biosynthesis of D-glucose sulphate by enzymes from the partly autolysed mucous glands of *Charonia lampas* has been demonstrated by Suzuki, Takahashi & Egami (1957). Here, *p*-nitrophenyl [^{35}S]sulphate functioned as a donor (in contrast to adenosine 3'-phosphate 5'-phosphosulphate, cf. Gregory & Nose, 1957), and the results suggest the participation of transulphatases.

The soluble fraction from the colonic mucosa contains a number of enzymes besides the system which activates sulphate. Such enzyme preparations catalyse the breakdown of ATP to inosine, inosine monophosphate and hypoxanthine.

SUMMARY

1. Supernatant enzyme fractions from sheep colonic mucosa catalyse the formation of an 'active sulphate' from adenosine triphosphate and SO_4^{2-} ions.
2. The 'active sulphate' has been isolated and found to be an adenine nucleotide carrying a very labile sulphate ester. The compound is identical with or closely similar to adenosine 3'-phosphate 5'-phosphosulphate.

3. The 'active sulphate' acts as donor in the formation of phenolic sulphates by liver or mucosal enzymes. No evidence for the sulphation of simple mono- and di-saccharides could be obtained.

4. Mucosal enzymes catalyse breakdown of adenosine triphosphate to inosine, inosine monophosphate and hypoxanthine.

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The Structure of the Naturally Occurring Phosphoglycerides

4. ACTION OF CABBAGE-LEAF PHOSPHOLIPASE D ON OVOLECITHIN AND RELATED SUBSTANCES*

By FLORA M. DAVIDSON† AND C. LONG‡

Department of Biological Chemistry, University of Aberdeen

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The enzyme phospholipase D, which liberates choline from the naturally occurring lecithins, has been found in several plants, including carrot root, cabbage, spinach and sugar-beet leaves, the latex of *Hevea brasiliensis* and defatted cottonseed meal (Hanahan & Chaikoff, 1947a, b, 1948; Smith, 1954; Kates, 1954; Tookey & Balls, 1956). In those plants investigated by Kates (1954), the enzyme was reported to reside exclusively in the plastids, and its activity was greatly increased by the addition of ether.

* Part 3: Long & Penny (1957).

† Present address: Dept. of Pathology, Institute of Orthopaedics, Royal National Orthopaedic Hospital, Stanmore, Middlesex.

‡ Present address: Physiology Department, Institute of Basic Medical Sciences, Royal College of Surgeons of England, London, W.C. 2.

In the work to be described, an examination of several plant species for phospholipase D activity revealed that the leaves of Savoy cabbage and Brussels sprouts were much the richest sources. Furthermore, the enzyme was present in the cell sap as well as in the plastids. The enzyme from the cell sap has been partially purified and found to exhibit an absolute requirement for calcium ions. This enzyme preparation, which removes all the choline from ovolecithin and from synthetic L- α - and DL- α -lecithins, also acts upon egg phosphatidyl-ethanolamine.

Evidence has been obtained for the presence of another enzyme in the supernatant from a Savoy-cabbage homogenate; this appears to be a phosphodiesterase, different from phospholipase D, which hydrolyses water-soluble phosphodiesters.