Studies on the Use of Sulphonated Cation-Exchange Resins for the Hydrolysis of Ovomucoid

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Acid hydrolysis of mucoproteins in order to liberate their sugar components tends at the same time to destroy the sugars produced. The carbohydrate residues present (as judged by colorimetric estimations) are not all recovered as monosaccharides after hydrolysis. During such hydrolysis there is usually a formation of black insoluble, or yellow soluble, humins, which probably arise in part from condensation of liberated neutral sugars with amino sugars or amino acids. Tryptophan is particularly liable to combine with neutral sugars in this manner (Lugg, 1938). Underwood & Deatherage (1952) heated the sulphonated cation-exchange resin Dowex-50 with coffee proteins and obtained a good yield of amino acids which were absorbed by the resin. Humins were not formed. Since neutral sugars would not be absorbed, the resin method seemed applicable to mucoproteins, in that the products of hydrolysis would be immediately separated, and their further chemical interaction less likely. Moreover, neutral sugars would be exposed to a high concentration of hydrogen ions only in the immediate vicinity of the insoluble resin: this also might reduce sugar destruction. Preliminary experiments (A. St. J. Dixon & R. W. Jeanloz, unpublished) confirmed that Dowex-50 resin hydrolysis compared favourably with previously used acid-hydrolytic methods in liberating neutral sugars from mucoproteins isolated from human plasma. Accordingly, the following study of the method was undertaken, using ovomucoid as a model.

MATERIALS AND METHODS

Ovomucoid was obtained from Worthington Biochemical Corporation, Freehold, New Jersey, prepared according to Lineweaver & Murray (1947), and had a moisture content of 14-6% as determined by drying to constant weight under 20 mm. Hg at 60°. Its composition, corrected for moisture content, was as follows: acetyl, 4-3; N, 13-4; 13-6; hexosamine 9-0, 9-4 (2N-HCl hydrolysis); hexose (expressed as mannose), 10-0±0-3 (average of five estimations, anthrone and orcinol methods, ± standard deviation).
The preparation inhibited 80% of its own weight of crystalline trypsin (obtained from Armour Laboratories, Chicago, Illinois). At pH 8-6, ionic strength 0-1, in sodium barbiturate buffer, ovomucoid separated under electrophoresis in the Tiselius apparatus into 93% of a component of mobility $-3\times10^{-4}$ cm.$^2$/v$^{-1}$ sec.$^{-1}$, with secondary peaks of 4% at approximately $-3\times10^{-4}$, and 3% at $-1\times10^{-3}$ cm.$^2$/v$^{-1}$ sec.$^{-1}$ towards the anode respectively. At pH 4-0, ionic strength 0-1, sodium acetate buffer, four main components were visible. Thus the analysis of this material corresponds closely to that reported by Lineweaver & Murray, but it cannot be considered homogeneous.

### Resins

The following cation-exchange sulphonated resins were tested: Dowex-50 (X 2 C-L, lot no. 2921-33, 50–100 mesh/in.); Dowex-50 (X 8 C-L, lot no. 2921–16, 50–100 mesh/in.); Dowex-50 (X 16 C-L, lot no. 2993–19, 50–100 mesh/in.); Dowex-50 (cross-linkage unstated, 200–400 mesh/in.). The indications ‘X2, X8, X16, C-L’ refer to the percentage of divinylbenzene added to the resin before polymerization and are a measure of the number of links between adjacent polymer chains. These resins were obtained from the Dow Chemical Company, Midland, Michigan. In addition, tests were made on two samples of the similar resin Amberlite IR 120-H (40–50 mesh/in.) (obtained from Rohm and Haas Company, Washington Square, Philadelphia, Pennsylvania) and one sample of Duolite C-3 (40–50 mesh/in.) (obtained from the Chemical Process Company, 901 Spring Street, Redwood City, California).

All the sulphonated resins tested liberated a yellow-brown colour and an acid if stored damp in the hydrogen phase. Repeated washings with water, or repeated cycling between 4% HCl and 4% NaCl at room temp., did not extract all these soluble components. The resins were incubated with water at 90–100$^\circ$ for several days and the supernatants neutralized with Ba(OH)$_2$. A precipitate formed which was filtered off and characterized as BaSO$_4$ by its insolubility in hot conc. HCl. The yellow filtrate was decolorized with Darco G-60 charcoal (obtained from the Darco Corporation, 60 E. 42nd St., New York, N.Y.). The coloured product removed had a strong ultraviolet light-absorption peak at 275 m$\mu$, and maximum absorption at 400 m$\mu$ in the visible range. All the resins liberated similar breakdown products.

The resins were cycled 4 times between 4% NaCl and 4% HCl, left in acid overnight at 27$^\circ$, washed with distilled water until the washings were free of Cl$^-$, dried with acetone, and stored at 0$^\circ$ during further testing. Portions were sealed in tubes with an equal weight of water and heated at 100$^\circ$ with constant agitation. The tubes were opened each day, the supernatant fluids decanted, and the resin, after washing, resealed with water as before. After 2 days the daily liberation of both colour and acid became constant for each resin. Duolite C-3 showed 3–4 times as much breakdown as the other resins and was not further investigated. The two batches of Amberlite IR 120-H behaved in the same way. Repetition, measuring acid strength and optical density at 400 m$\mu$, gave the results shown in Table 1 after 2 days.

Thus of the remaining resins, Amberlite IR 120-H showed least breakdown and Dowex-50 (X 16 C-L) the most. The Amberlite IR 120-H did not show much advantage over the Dowex-50 main batch (cross-linkage not known). As the particle size of the latter was much finer and more appropriate for use as a hydrolysing agent it was chosen for experimental use. A large standard batch was prepared, dried and stored at 0$^\circ$. This batch did not change appreciably during storage for several months as regards the amount of acid and colour liberated on heating with water to 100$^\circ$ and was used throughout the hydrolysis studies.

### Table 1. Resin breakdown after incubation with water at 100$^\circ$ for 2 days

| Supernatant from | Acid strength | Optical density (
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Amberlite IRC 120-H</td>
<td>0-00070 n</td>
<td>0-055</td>
</tr>
<tr>
<td>Dowex-50 (main batch)</td>
<td>0-00120 n</td>
<td>0-089</td>
</tr>
<tr>
<td>Dowex-50 (X 2 C-L)</td>
<td>0-00083 n</td>
<td>0-125</td>
</tr>
<tr>
<td>Dowex-50 (X 8 C-L)</td>
<td>0-00170 n</td>
<td>0-082</td>
</tr>
<tr>
<td>Dowex-50 (X 16 C-L)</td>
<td>0-00530 n</td>
<td>0-242</td>
</tr>
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### Analytical methods

Hexose carbohydrate was measured colorimetrically by the orcinol method (Sørensen & Haugaard, 1933) as modified by Friedman (1949) or by the modification by Loewus (1952) of the anthrone method. For this latter method to be satisfactory glassware must be acid-washed and capped to exclude dust, solutions must be filtered to remove lint, and a rigid mixing schedule must be followed since colour development (Scott & Melvin, 1953) depends on the heat of mixing of sulphuric acid and water. These methods agreed within 5% for a variety of protein hydrolysate–hexose mixtures. The results are expressed as mannose.

Amino sugars were estimated by a modification (Boas, 1938) of the method of Elson & Morgan (1933). Boas's complicated neutralization schedule was avoided by evaporating HCl (present after the stage of removal of interfering substances) by means of a stream of dry $N_2$ at 60$^\circ$ and making up the residue to a known volume with water.

Hexosamines were identified by the paper-chromatographic method ‘A’ of Stoffyn & Jeanloz (1954) which uses a hot ninhydrin solution containing pyridine to convert glucosamine and galactosamine into arabinoxylose and lyxose respectively. This method is satisfactory even in the presence of an excess of amino acids in the ovomucoid hydrolysate if performed in a sealed tube and if the molar ratio of ninhydrin to total amino groups is about 20:1. After opening the tube, the reaction products were evaporated to dryness, then taken up in a small amount of chloroform and shaken with a few drops of water. The water layer and washings were removed by aspiration, concentrated, and applied directly to the starting line of the chromatogram.

$N$-Acetylglucosamine was measured by the following method, which is a modification of that of Morgan & Elson (1934). Reagents: (1) p-dimethylaminobenzaldehyde (twice crystallized from dilute acetic acid) (1 g.) is freshly dissolved in 47 ml. of acetic acid mixed with 3 ml. of 10% HCl; (2) $N$-acetylglucosamine standard solutions, containing 10–50 μg./ml.; (3) 0·5% Na$_2$CO$_3$. Estimations were done in duplicate in a series of 15 × 150 mm. matched borosilicate tubes. 1 ml. of solution, blank or standard was mixed with 0·1 ml. of (3) in each tube. The tubes (stoppered with glass balls, and still in their rack) were heated in a water bath at 98$^\circ$ for 8 min., then cooled by immersion in water. After 4 ml. of glacial acetic acid followed by 1 ml. of (1) had been
added to each (using a calibrated syringe pipette) the tubes were allowed to stand at 27° for 1 hr. Optical density at 530 m\(\mu\). was measured in a Junior Coleman spectrophotometer.

Sugars, amino sugars, and amino acids were separated by descending paper chromatography at room temperature, using Whatman no. 54 paper, \(\pi\)-butanol–ethanol–water \((4:1:1, \text{ by vol.) as solvent and developing for 24 or 48 hr. Tentative identifications of sugars in this solvent system were confirmed by mixing with known sugars and chromatographing in alternative solvent systems.}

The sugars and amino sugars were detected using aniline hydrogen phthalate spray (Partridge, 1949) or a silver nitrate method (Trevelyan, Procter & Harrison, 1950). If clean fresh reagents were used with the latter, permanent black-on-white chromatograms were obtained suitable for transmission densitometry in which the sugar to be measured was compared with a series of standards included on each side of the same chromatogram, with an error of \(\pm 14\%\) (percentage standard deviation of duplicates). Alternatively, the spots were located by colour development of the standards only, eluted (Wyatt, 1951) and quantitatively estimated by the appropriate method as above.

Total amino groups were measured colorimetrically in terms of DL-alanine by the ninhydrin method of Moore & Stein (1948).

Resin hydrolysis of ovomucoid was carried out in 10 x 100 mm. tubes, each containing 0-9 g. of the prepared selected resin, 4-5 mg. of dry ovomucoid and 3-0 ml. of water. After sealing, the tubes were heated at 100° for 0, 0-5, 1, 2, 4, 16, 64 and 128 hr. with constant mixing. In duplicate runs the tubes were laid horizontally (to expose the greatest resin surface) in a steam bath at 100°. There was little difference in the quantitative results; constant mixing gave a slightly increased rate of hydrolysis. At the end of each period a tube was withdrawn and stored at 0° to prevent further hydrolysis. When the series had been completed, the tubes were centrifuged and opened, and their contents and 60 ml. of washings poured (to form chromatographic columns) into funnel-topped, taper-pointed 200 x 6 mm. tubes prepared by plugging with glass wool and a layer of sand followed by a 15 mg. layer of charcoal (Darco G-60). No appreciable amount of hexose or hexosamine was adsorbed by this amount of charcoal under these conditions.

The effluents and washings (containing neutral sugars, unchanged ovomucoid, and some \(\mathrm{H}_2\mathrm{SO}_4\) from resin breakdown) were neutralized with a small amount of \(\mathrm{BaCO}_3\), filtered through Celite 545 and the filtrate and washings evaporated to dryness at approx. 60° by a stream of dry \(\mathrm{N}_2\). Neutral sugars were separated by dissolving the dried residue in one part of water and adding nine parts by vol. of ethanol, allowing the mixture to stand overnight at 4°. The supernatants and 90% ethanol washings were separated by centrifuging, evaporated to dryness and made up to 10 ml. with water. Samples were used for estimation of total sugars, \(\text{N}-\text{acetyltetraosamine and hexosamine, and for paper chromatography. The 90%}-\text{ethanol-insoluble material was subjected directly to the anthone reaction to estimate unchanged mucoprotein. Hexosamine remaining on the column was eluted by 3-0 ml. of 2-n HCl along with most of the amino acids. The remainder of the amino acids could be eluted with concentrated hydrochloric acid. The eluates were evaporated to dryness, made up to 10 ml. with water and portions used for estimation of hexosamine and total amino groups and for paper chromatography.}

Control tubes subjected to the same procedure contained resin and water only.

Representative acid hydrolysis tubes included for comparison contained 4-5 mg. of ovomucoid in 3 ml. of 0-5\% \(\mathrm{H}_2\mathrm{SO}_4\) or in 3 ml. of 2-0 n-HCl. These were sealed and incubated under the same conditions and for the same periods as the resin hydrolysis series. After opening the tubes, the sulphuric acid hydrolysates were neutralized with excess \(\mathrm{BaCO}_3\) and filtered. Hydrochloric acid hydrolysates were evaporated to dryness over \(\text{NaOH}\) under reduced pressure in the cold (0-4 hr. hydrolysates) or under a stream of dry \(\mathrm{N}_2\) at 60° (16 and 64 hr. hydrolysates). The residues were taken up in a little water. Acid hydrolysates thus neutralized were passed through similar columns containing 0-9 g. of Dowex-50 (acid form) over a 15 mg. layer of charcoal and separated as before.

RESULTS

The procedure was tested for possible losses by means of the anthrone or orcinol methods in the following recovery experiments. It is to be noted that conversion of hexone into another chromogen would not have been detected.

A solution of mannose in water (10 \(\mu g./mL\).) was heated to 100° in sealed tubes with resin for periods up to 64 hr. Losses were: 0-48 hr., nil; 48-64 hr., 15%.

Published reports on the proportions of sugars (Stacey & Woolley, 1940, 1942) and amino acids (Lewis, Snell, Hirschmann & Fraenkel-Conrat, 1950) were used to prepare a simulated ovomucoid hydrolysate, which was heated to 100° with resin in sealed tubes for 16 hr. Loss (average of two), nil.

Sugars were added to ovomucoid solution in amounts sufficient to double the expected concentration of carbohydrate. After hydrolysing the mixture with resin for 18 hr. at 100° the colour value for added carbohydrate was unchanged.

An aqueous solution of galactose (2 ml.; 20 \(\mu g./mL\).) was mixed with varying proportions of charcoal. Provided the ratio of charcoal to galactose did not exceed 250:1 the galactose was not adsorbed. This amount removed at least 80% of the brown-coloured material formed by resin breakdown during a 16 hr. hydrolysis. The remaining 20% was insufficient to affect the colorimetric estimations of neutral or amino sugars.

A solution of mannose, galactose and glucosamine was mixed with resin which had been heated in water at 100° for 16 hr. The sugars were then separated from the resin and its breakdown products, according to the scheme outlined above. No loss of colour value for carbohydrate during separation was demonstrated.

Ovomucoid was hydrolysed with Dowex-50 resin and the sugars isolated according to the above procedure. Figures in brackets below give the percentage orcinol value for carbohydrate (as mannose) at each stage of the procedure, in terms of that given by unhydrolysed ovomucoid:
Ovomucoid solution (100%), resin hydrolysis and decolorization with charcoal (67%), neutralization with barium carbonate and filtration (61%), evaporation to dryness, solution in water, dilution with ethanol to 90%, and separation of supernatant (56%), chromatographic separation with subsequent elution as mannose (36%) and galactose (10%) and a slow-moving reducing substance, possibly a disaccharide (4%).

Mannose, galactose, N-acetylglucosamine, and glucosamine were the only identified sugars recovered from hydrolysis of ovomucoid.

Fig. 1. Comparison of the anthrone colour values for neutral carbohydrate (expressed as mg. mannose/g. of ovomucoid) determined on hydrolysis of ovomucoid with Dowex-50 resin (—) and with 0.5N-H_2SO_4 (—). Note the broken logarithmic time scale.

Fig. 1 compares hydrolysis with 0.5N sulphuric acid and with resin in terms of anthrone colour for carbohydrate. The curves reflect the greater speed of the acid method, but the total 'carbohydrate' liberated is misleading. Only 30-40% of the chromogens from acid hydrolysis were recoverable as hexose on paper chromatography. The disparity was even greater in the case of 2N hydrochloric acid hydrolysis (not illustrated). On the other hand, between 80 and 90% of the anthrone or orcinol colour values for carbohydrate liberated by resin hydrolysis could be accounted for as free mannose and galactose. Moreover, the yield of hexoses, as judged both by colour values of the hydrolysates and by densitometry of hexose spots on chromatograms, was surprisingly constant for the resin method after 4 hr. of hydrolysis, compared with the more erratic yield from 0.5N-H_2SO_4 hydrolysis, which falls off rapidly after 4 hr.

When 0.5N-H_2SO_4 hydrolysis of ovomucoid was allowed to proceed for 16 hr., there was no change in the orcinol colour value for carbohydrate of the reaction mixture as a whole. Between 16 and 64 hr. of hydrolysis the orcinol colour value for carbohydrate increased by about 15%, by which time the characteristic odour of furfural was distinguishable. Compounds such as this give the colour tests for carbohydrates but, being volatile, would be lost in the subsequent separation and chromatographic procedures. Their formation may in part explain the high carbohydrate values given by colorimetric methods.

Both the resin and the sulphuric acid methods of hydrolysis liberated N-acetyllactosamine, the yield of which diminished as the yield of free amino sugar increased (Fig. 2). Values for hexosamine and its acetyl derivative (colorimetric methods) corresponded well with those obtained by quantitative paper chromatography. The resin and sulphuric acid methods gave yields which were 95 and 80% respectively of those obtained by hydrochloric acid hydrolysis. The HCl method also gives a product which produces the cleanest hexosamine chromatograms, and is therefore the most satisfactory of the three methods tested for recovery of amino sugars.

The identity of the hexosamine

Masamune & Yosizawa (1950) and Odin (1951) have stated that ovomucoid contains galactosamine. Our results failed to confirm this. The hexosamine,
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when converted into the corresponding pentose with ninhydrin yielded only arabinose. Results were the same when the hydrolysis schedule of Odin (1951) was followed; paper-chromatographic analysis of three successive crystalline crops showed glucosamine and several amino acids. The mother liquor still contained a trace of amino sugar, all of it glucosamine. The \(N\)-acetylhexosamine, eluted from a paper chromatogram on which it had been separated after resin hydrolysis of ovomucoid, gave a colourless syrup which spontaneously crystallized. After hydrolysis of these crystals to the hexosamine, and conversion into the corresponding pentose, only arabinose was found. When dealing with pure materials, as in this case, the method is sensitive to as little as 2–5% of galactosamine in the presence of 95–98% of glucosamine (Stoffyn & Jeanloz, 1954). This discrepancy between these reports might arise from differences in the method of preparation and purity of the ovomucoid.

**DISCUSSION**

So far, we have used the resin method only for soluble glycoproteins and mucoproteins, but Glegg, Eidinger & Leblond (1953) have applied a similar method to the hydrolysis of reticular fibres. A 48 hr. hydrolysis was used. Elsewhere these workers have published control experiments on hexoses submitted to their procedure (Glegg & Eidinger, 1954). Deuel, Selnis, Angas-Weiss & Huber (1951) were unable to hydrolyse glycogen with Amberlite IR-120, whereas Wadman (1952) demonstrated some hydrolysis of 'soluble starch' by the same resin, and obtained complete methanolysis of methylated starch in the presence of this resin and methanol. These results suggest that the size of the molecule may influence the extent of resin hydrolysis.

The effectiveness of the resin as a hydrolytic catalyst is unlikely to depend on the small concentration (less than 0.0001 N) of sulphuric acid which results from resin decomposition during the hydrolytic procedure in the time (4 hr.) required to achieve maximum sugar liberation from ovomucoid. From 48 hr. onward, however, the amount of free sulphuric acid is such that concentration of the hydrolysate without previous neutralization will destroy a significant proportion of the hexoses. Moreover, if the concentrate be applied directly to a chromatogram it may attack the paper, producing glucose, etc., as artifacts. Preparations submitted to resin hydrolysis must not, of course, be contaminated with salts since by ion exchange equivalent amounts of acids will be set free.

The orcinol and anthrone colour values for total hexose, taken with the ratio of mannose to galactose found by elution after chromatographic separation and with the yield of hexosamine, suggest that the ratio mannose:galactose: \(N\)-acetylglucosamine in ovomucoid is 7:2:9. However, this is unreliable since colorimetric methods may not accurately reflect the total hexose carbohydrate of the mucoprotein. The use of figures for the actual recovery of identified hexose gives the ratios 5:2:14. Stacey & Woolley (1940, 1942) reported 6:2:14 for hydrolysis of a carbohydrate fraction isolated from ovomucoid. These proportions cannot be considered to reflect molecular structure, since ovomucoid is not electrophoretically homogeneous.

An interesting feature of the analysis of ovomucoid is that only half the \(N\)-acetyl groupings present can be accounted for as \(N\)-acetylglucosamine.

If it be assumed that all the glucosamine is present in the molecule as the \(N\)-acetyl derivative, the hydrolysis curves show that all the sugars are liberated at the same rate and achieve maximum values after approx. 4 hr. This contrasts with the rate of liberation of amino acids, which under the same conditions were not maximally released until 16 hr.

**SUMMARY**

1. Sulphonated cation-exchange resins from different manufacturers and having different degrees of cross-linkage were studied and found to be effective for hydrolysing mucoproteins. This action did not depend on traces of sulphuric acid released by resin breakdown under the conditions of hydrolysis. Resin breakdown increased with both degree of cross-linkage and duration of hydrolysis, limiting the usefulness of the method, and making neutralization of the hydrolysate before working up essential.

2. The method was applied to the hydrolysis of ovomucoid. Mannose, galactose and glucosamine (partly acetylated) were obtained in yields which compared favourably with usual acid-hydrolysis methods.

3. Reports of galactosamine in ovomucoid could not be confirmed by a sensitive method.

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**REFERENCES**

An investigation into the behaviour of simple peptide esters on reduction with metal hydrides has been carried out concurrently with the analytical work on proteins to be described later by Chibnall & Rees. Information was required concerning the conditions under which the reagents lithium borohydride (LiBH₄), lithium aluminium hydride (LiAlH₄), and aluminium hydride (AlH₃) reduced ester groups but failed to attack the peptide bond, and concerning methods for isolating pure reduction products for subsequent degradative studies. The following experimental work describes the preparation of β-hydroxyamides from (a) the esters of glycyglycine, L-leucylglycine and L-leucylglycylglycine and (b) the esters of the toluene-p-sulphonyl derivatives of glycyglycine, L-leucyl-L-phenylalanine and L-leucylglycylglycine. Fromaget & Jollès (1952) have reported yields of less than 50% for the diols produced from glutamyl-γ-glycine diethyl ester and glutathione diethyl ester by reduction with LiAlH₄. The conditions for the total reduction of simple peptides to polyamines have been described by Karrer & Nicolaus (1952).

The main products and possible secondary products arising from the action of metal hydrides on a peptide ester may be summarized schematically as follows:

\[ \text{NH}_2\text{CHR}.\text{CO} . \text{NH} . \text{CHR}' . \text{CO}_2\text{Et} \]

\[ \xrightarrow{+ \text{Metal hydride}} \]

\[ \text{NH}_2\text{CHR}.\text{CO} . \text{NH} . \text{CHR}' . \text{CH}_2\text{OH} \]

\[ \xrightarrow{\beta \text{-Hydroxyamide}} \]

\[ \text{NH}_2\text{CHR}.\text{CH}_2\text{NH} . \text{CHR}' . \text{CH}_2\text{OH} \]

\[ \xrightarrow{\text{Hydroxydiamine}} \]

\[ (\text{NH}_2\text{CHR}' . \text{CH}_2\text{OH}, \text{NH}_2\text{CHR}.\text{CH}_2\text{OH}, \text{NH}_2\text{CHR}' . \text{CO}_2\text{H}) \]

\[ \text{NH}_2\text{CHR}.\text{CO} . \text{NH} . \text{CHR}' . \text{CO}_2\text{H} \]

**The Reduction of Esters of Simple Peptides by Metal Hydrides**

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**EXPERIMENTAL AND RESULTS**

**Materials**

Tetrahydrofuran. This solvent (Imperial Chemical Industries Ltd., Billingham) was refluxed over K metal for 24 hr. and distilled, refluxed over LiAlH₄ and distilled, and stored in a dark bottle. The solvent rapidly peroxides and samples that have been kept longer than 2 weeks should be redistilled over LiAlH₄ before use.

Standard solutions of LiBH₄ and LiAlH₄. Solutions between 1 and 2.5 M were prepared by refluxing the reagent (New Metals and Chemicals Ltd., London) (rough weight judged by bulk) with 30–40 ml. tetrahydrofuran for 2 hr., allowing to cool and centrifuging. The clear supernatant was poured off into a bottle from which the air had been displaced by dry N₂. The reducing power of these solutions may be assessed by titration of the Li by standard acid or more accurately by the manometric method of Krynitsky, Johnson & Carhart (1948) depending on the volume of H₂ liberated on decomposition by acid. In the latter estimation a longer reaction time is necessary in the case of LiBH₄. Decomposition of all boranes is assumed complete after about 30 min. The solutions can be kept for a few weeks but long storage brings about some change which is not understood.

Aluminium hydride. A 0.5 M solution of AlCl₃ was prepared by adding the resublimed material to tetrahydrofuran pre-cooled to -20°. On warming to room temperature the solid dissolves giving a practically clear solution. A solution

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