Biochem. J. (1964), **92**, 550

The Activation of L-Threonine Dehydrogenase by Potassium Ions

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(*Received* 9 *December* 1963)

In the preceding paper (Green & Elliott, 1964) two effects of potassium chloride on partially purified *Staphylococcus aureus* threonine dehydrogenase were demonstrated: an increase in enzymic activity and a stabilization of the enzyme against thermal inactivation. In the present paper a more detailed study of the activation of threonine dehydrogenase by inorganic salts is described. It has been found that the active form of the enzyme is probably a metal ion–enzyme complex formed by a slow reaction between a univalent metal ion and the enzyme protein. In the present paper the term 'activation' means 'apparent activation'. It may be, in fact, true activation of the enzyme or stabilization of it or both, as the present experiments do not distinguish between these effects. This point is discussed below.

A preliminary account of this work has already appeared (Green, 1963).

MATERIALS AND METHODS

Chemicals. NAD^+ was obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A., and crystalline lactate dehydrogenase from L. Light and Co. Ltd., Colnbrook, Bucks. Potassium pyruvate was prepared by the method of Korkes, del Campillo, Gunsalus & Ochoa (1951). All salts were of analytical-reagent grade.

Tris–HCl buffers were prepared by adjusting tris, of the concentration indicated, to pH 8·5 with HCl of the same concentration.

Enzyme preparations. Staphylococcus aureus Duncan crude extracts and partially purified threonine dehydrogenase were prepared as described by Green & Elliott (1964). Dialysed enzyme was prepared by dialysis of the appropriate enzyme preparation against 100 vol. of water at 4° for 12 hr.; it was used immediately.

Assay of threonine-dehydrogenase activity. Unless otherwise stated, the following method was used. The reaction mixture contained, in a total volume of 3·0 ml., 100 μmoles of tris–HCl buffer, pH 8·5, 300 μmoles of L-threonine, 1 μmole of NAD^+, 2 μg. of crystalline lactate dehydrogenase (EC 1.1.1.27), 30 μmoles of potassium pyruvate, 0·75 ml. of enzyme solution (except for the partially purified enzyme when 0·1 ml. was used) and sufficient KCl to bring the final concentration to 0·3 M. Incubations were carried out in 25 ml. Erlenmeyer flasks shaken aerobically at 37° for 15 min. The reaction was stopped by the addition of 0·6 ml. of 25% (w/v) $HClO_4$, the precipitated protein and $KClO_4$ were removed by centrifugation and aminoacetone was determined in a sample of the neutralized supernatant as described by Mauzerall & Granick (1956).

Determination of potassium. Potassium determinations were kindly carried out by Dr W. V. Macfarlane, using an EEL flame photometer.

RESULTS

Time-dependent activation of the enzyme by K^+ *ions.* For the work described in this section crude *Staph. aureus* extract was used, unless otherwise stated.

When the crude extract was dialysed against 100 vol. of water at 4° for 12 hr. most of the enzymic activity was lost, but part of it could be recovered by incubation of the dialysed enzyme with potassium chloride at 0° for relatively long periods.

Time required for activation of the enzyme by potassium chloride. The activation of water-dialysed enzyme by potassium chloride is very slow and it was necessary to incubate the enzyme with potassium chloride for up to 24 hr. at 0° to obtain maximal activity (Fig. 1). The rate of activation was linear over the first 4–5 hr., at least with potassium chloride concentrations up to 0·3 M.

The conditions for assay of the enzyme were chosen so as to give a true measure of the enzymic activity at all stages of activation (with a possible exception that is discussed below). The final potassium chloride concentration of the reaction mixture was adjusted to a standard level of 0·3 M and was, therefore, independent of the potassium chloride content of the enzyme solution. At the incubation time chosen (15 min.) the reaction was proceeding at the initial rate, so there was no appreciable activation or inactivation of the enzyme during the assay, and the rate of reaction was proportional to the amount of enzyme added.

Activation of the enzyme as a function of the potassium chloride concentration. As shown in Fig. 1, both the rate and extent of activation depended on the concentration of potassium chloride with which the dialysed enzyme was incubated. Fig. 2 shows the activity of the enzyme after incubation

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with different concentrations of potassium chloride for 3·7 hr. (this being within the period during which activation was approximately linear with time) and 28·8 hr. (when the extent of activation was maximal). Maximal activity of the enzyme was obtained after incubation with 0·5 M-potassium chloride for 28·8 hr., when the final activity was 4·4 times that immediately after dialysis against water. Analysis of the water-dialysed enzyme showed that the K^+ ion concentration was 0·3 mM and the residual activity may have been due to this.

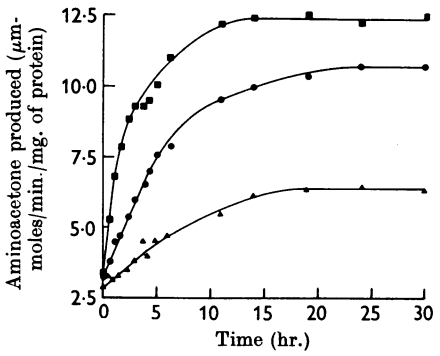

Fig. 1. Time-course of activation of dialysed enzyme by KCl. Equal volumes of the dialysed enzyme were added to the appropriate KCl solutions (at 0°) to give final KCl concentrations of 0·05 M (▲), 0·30 M (●) and 1·00 M (■) respectively. The solutions were incubated at 0°, and samples were withdrawn for assay at the times indicated.

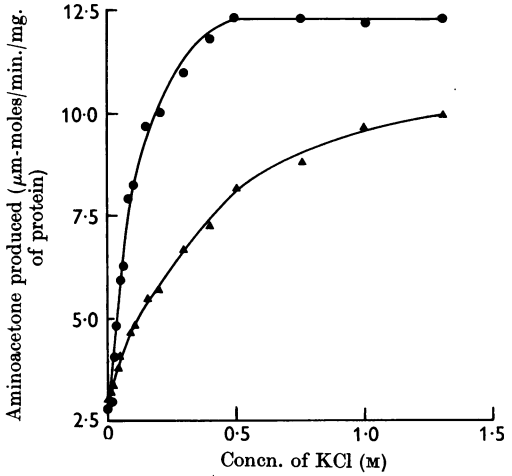

Fig. 2. Extent of activation of the dialysed enzyme as a function of the concentration of KCl with which it was incubated. The enzymic activities were assayed after incubation of the dialysed enzyme, at 0°, with the concentrations of KCl indicated for 3·7 hr. (▲) and 28·8 hr. (●) respectively.

Effect of ethylenediaminetetra-acetic acid on the rate and extent of activation of the enzyme by potassium chloride. To eliminate the possibility that a bivalent or tervalent metal ion impurity in the potassium chloride, rather than potassium chloride itself, might be the active agent, incubation of the enzyme with potassium chloride was carried out in the presence of EDTA, pH 7·0, over the range 1 μM–10 mM. EDTA had no effect on either the rate or extent of activation.

Activation of the partially purified enzyme by potassium chloride. Partially purified *Staph. aureus* threonine dehydrogenase can be activated by potassium chloride, after dialysis against water, in a similar manner to the crude enzyme. In the presence of 0·3 M-potassium chloride, maximal activation of the enzyme required 24–30 hr. and the activity was 2·7 times as great as that of the enzyme immediately after dialysis against water.

Specificity of activation of the enzyme for both cations and anions. Cations were tested as chlorides and anions as potassium salts for capacity to activate threonine dehydrogenase. In each case the salt was incubated, at concentrations of 0·05, 0·15 and 0·50 M, with water-dialysed enzyme at 0° for periods of 3·5 hr. and 26 hr. Since certain salts have either an inhibitory or a stimulatory effect on the assay procedure used to determine the enzymic activity, a correction factor, measured as described in Table 1, was applied to adjust the measured enzymic activities to those expected if the only salt present in the assay mixture was potassium chloride at a concentration of 0·3 M. The correction factors exceeded 20 % only for Li^+ ions (at 0·5 M), NH_4^+ ions (at 0·5 M), Ca^{2+} ions (at all concentrations), Br^- ions (at 0·5 M), I^- ions (at all concentrations) and NO_3^- ions (at 0·5 M); all of these ions were inhibitory.

As shown in Table 1, all of the alkali-metal chlorides activated the enzyme to some extent and, except possibly for Li^+ and Na^+ ions, the activation was very slow, being incomplete in 3·5 hr. Both Li^+ and Na^+ ions caused much weaker activation than K^+ ions, NH_4^+ ions were approximately the same as K^+ ions, whereas Rb^+ and Cs^+ ions were more effective than K^+ ions. With Ca^{2+} ions the values are inaccurate owing to a very large correction factor and probably this ion has no activating effect. As shown in Table 2, potassium chloride could be replaced by various other potassium salts with comparatively little effect on either the rate or extent of activation, except with potassium iodide where inhibition occurred. This indicates that the activation of the enzyme is probably caused by univalent cations.

Apparent 'instantaneous' activation of the enzyme by K^+ ions. For the work described in this section, partially purified *Staph. aureus* enzyme that had been fully activated by K^+ ions was used.

Effect of the addition of potassium chloride to the mixture for assay of the enzyme. When potassium chloride was added to the enzyme assay mixture, the rate of the reaction was increased (Fig. 3). For maximal activity 0·06–0·08 M-potassium chloride was required; in the presence of 0·088 M-potassium chloride the activity was 54 % greater than that in the presence of 0·008 M-potassium chloride. (The latter value was the lowest that it was possible to test, since this amount of potassium chloride was added with the enzyme.) In all cases the reaction rate was constant for at least 5 min. after the start of the reaction.

Specificity for cations. Various metal chlorides, added to the assay mixture at concentrations of 0·02 and 0·08 M, were tested for capacity to increase the activity of the enzyme. As shown in Table 3, all of the alkali-metal ions were effective to some degree; Li^+ and Na^+ ions caused much weaker

Table 1. *Specificity of the time-dependent activation of the enzyme with respect to cations*

Equal volumes of the dialysed enzyme were added to the appropriate metal chloride solutions (at 0°) to give final concentrations of salt of 0·05, 0·15 and 0·50 M respectively. The solutions were incubated at 0° and the activities were assayed after 3·5 hr. (Expt. A) and 26 hr. (Expt. B). The results are corrected for the effect of added salts on the assay procedure. For the determination of the correction factors, the final compositions of the reaction mixtures were the same as those used for the enzymic assays, but the enzyme had been incubated at 0° with 1·2 M-KCl for 26 hr. and the other salts were added directly to the reaction mixtures.

		Activity (% of that of the water-dialysed enzyme)		
Concn. of salt		0·05 M	0·15 M	0·50 M
Expt. A	No added salt	(100)	(100)	(100)
	LiCl	100	155	238
	NaCl	137	146	246
	KCl	146	229	366
	RbCl	192	246	392
	CsCl	192	256	360
	NH_4Cl	174	229	365
	$CaCl_2$	119	82	185
Expt. B	No added salt	(100)	(100)	(100)
	LiCl	100	146	283
	NaCl	119	128	237
	KCl	283	458	614
	RbCl	420	511	668
	CsCl	430	521	640
	NH_4Cl	266	448	640
	$CaCl_2$	110	110	366

Table 2. *Specificity of the time-dependent activation of the enzyme with respect to anions*

The experimental details were as described for Table 1, except that potassium salts were substituted for metal chlorides. The results are corrected as described for Table 1.

		Activity (% of that of the water-dialysed enzyme)		
Concn. of salt		0·05 M	0·15 M	0·50 M
Expt. A	No added salt	(100)	(100)	(100)
	KF	150	183	291
	KCl	150	217	349
	KBr	175	246	390
	KI	183	233	165
	KNO_3	192	266	366
	$KO_2C{\cdot}CH_3$	158	192	299
Expt. B	No added salt	(100)	(100)	(100)
	KF	250	408	515
	KCl	275	458	566
	KBr	291	440	582
	KI	275	250	150
	KNO_3	333	491	566
	$KO_2C{\cdot}CH_3$	291	399	525

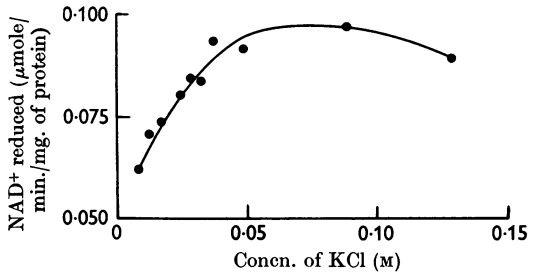

Fig. 3. Effect of the concentration of KCl in the assay mixture on the activity of enzyme which had been fully activated by the time-dependent process. The reaction mixture contained, in a total volume of 3·0 ml., 500 μmoles of tris–HCl buffer, pH 8·5, 100 μmoles of L-threonine, 2 μmoles of NAD^+, KCl to give the final concentration indicated, and 0·05 ml. (0·037 mg. of protein) of purified enzyme that had been dialysed against 0·5 M-KCl for 3 days. The cuvette, which contained all the components of the reaction mixture except enzyme, was incubated for 2 min. at 25°. The reaction was started by the addition of enzyme. The reduction of NAD^+, at 25°, was followed by measurement of the extinction at 340 mμ, recording being commenced within 30 sec. of the addition of the enzyme.

Table 3. *Specificity of the instantaneous activation of the enzyme with respect to cations*

The experimental details were as described for Fig. 3, except that the KCl added to the assay mixture in excess of that added with the enzyme was replaced by 0·02 or 0·08 M metal chloride as indicated.

	Activity (% of that with no added salt)	
Concn. of salt ...	0·02 M	0·08 M
No added salt	(100)	(100)
LiCl	98	102
NaCl	102	109
KCl	133	144
RbCl	146	142
CsCl	140	135
NH_4Cl	126	146
$CaCl_2$	66	38

activation than K^+ ions, NH_4^+ ions were approximately the same as K^+ ions, and Rb^+ and Cs^+ ions were more effective than K^+ ions at the lower concentration; Ca^{2+} ions inhibited the enzyme.

DISCUSSION

There are three possible reasons for the apparent instantaneous activation of threonine dehydrogenase by potassium chloride; the first is an activation that is independent of the time-dependent effect; the second is an activation that is related to the time-dependent effect; and the third is a stabilization of the enzyme against thermal inactivation at 25°. The third possibility is eliminated since no inactivation was observed during the reaction and none could have occurred before the start of the reaction as the enzyme was added last. Of the remaining possibilities, the second seems more probable since the instantaneous and time-dependent activations show a similar specificity for cations. A possible relationship between the two activating effects is described below.

It is not possible to conclude, from the present experiments, whether or not the enzyme completely free from K^+ ions would have any activity. However, if it is assumed that the residual activity of the dialysed enzyme is entirely due to the K^+ ions present, a straight line can be obtained for the Lineweaver & Burk (1934) plot of enzymic activity after equilibration with different concentrations of potassium chloride (Fig. 4). This suggests that each active centre of the enzyme combines with one K^+ ion. If, as has been supposed, the only active form of the enzyme is the K^+ ion–apoenzyme complex and the enzymic activity gives a true measure of the amount of this complex present, then the value obtained for K_m (K^+ ion), 55 mM, is equal to the dissociation constant of this complex. (This dissociation constant refers to the whole process of activation and, if more than one step is involved, not only to that in which K^+ ions combine with the enzyme.)

Several different mechanisms for the time-dependent activation of the enzyme by K^+ ions can explain the observed results. The simplest is a very slow combination of K^+ ions with inactive apoenzyme to give an active K^+ ion–apoenzyme complex. However, it seems unlikely that such a reaction would be slow; a more probable reason for the slow rate would be a change in the structure of the protein molecule. Such a mechanism was suggested by Malmstrom & Westlund (1956) to explain the results obtained by Smith (1951) on the slow activation of leucine aminopeptidase (EC 3.4.1.1) by Mn^{2+} ions. It involves an instantaneous reaction between metal ion and protein to give a complex that is slowly converted into a more active form. A third scheme was put forward by Rabin (1958); it is shown, in a form adapted to the present case, in Scheme 1. One inactive form of the enzyme (A) is slowly converted into a second inactive form (B) by a change in the structure of the protein. This second form can react very rapidly with the metal ion to give active enzyme.

All the results obtained in the present case, including the instantaneous activation, can be explained by Scheme 1. In this it is assumed that the equilibrium of reaction (1) is strongly to the left, only a very small amount of enzyme B being present at any time. This could explain the residual activity of the dialysed enzyme; the equilibrium concentration of enzyme B would immediately be converted into active enzyme on the addition of K^+ ions. Similarly, the higher the concentration of K^+ ions present, up to a saturating level, the more the second equilibrium would be forced to the right. This would result in the first equilibrium also being forced to the right and, therefore, the overall activation would increase in rate with increase in K^+ ion concentration. The final equilibrium of the overall reaction would also depend on the concentration of K^+ ions present, up to a saturating level at which effectively all the enzyme would be present in the active K^+ ion–enzyme form. These conclusions agree with the observed facts. In addition, this scheme would predict that, when fully activated enzyme (i.e. that almost entirely in the form K^+

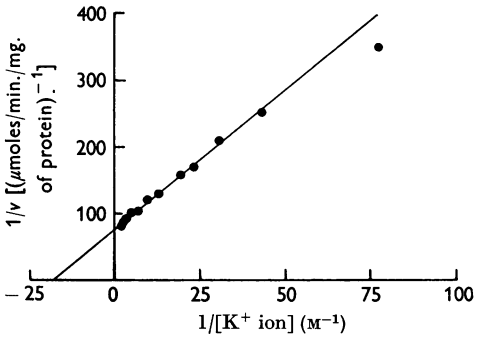

Fig. 4. Lineweaver–Burk plot of the enzymic activity after equilibration of threonine dehydrogenase with different concentrations of KCl. The results are those used for the 28·8 hr. curve of Fig. 2, except that they are corrected for the residual K^+ ion content of the dialysed enzyme. Values up to 0·5 M-KCl are plotted. The best line was calculated by the method of Wilkinson (1961).

$$\text{Enzyme A (inactive)} \overset{\text{slow}}{\rightleftharpoons} \text{Enzyme B (inactive)} \quad (1)$$

$$\text{Enzyme B} + K^+ \overset{\text{fast}}{\rightleftharpoons} K^+ \text{ ion–enzyme complex (active)} \quad (2)$$

Scheme 1

ion–enzyme) is diluted in a K^+ ion-free medium, immediate dissociation of the complex, to an extent depending on the dissociation constant, would occur. The K^+ ions in the medium would prevent, or partially prevent, this dissociation. Thus the observed instantaneous activation could be explained.

Rosenberg (1961) has suggested that metal ions that apparently activate an enzyme may rather be functioning as stabilizers of the enzyme at the temperature of the assay. The stabilization of threonine dehydrogenase by potassium chloride against thermal inactivation has already been observed (Green & Elliott, 1964), and the possibility that the time-dependent activation of the enzyme by K^+ ions may really be due to stabilization must be considered.

The present experiments do not enable this point to be settled as they do not eliminate the possibility that free enzyme (i.e. that not bound to K^+ ions) is instantaneously destroyed at the temperature of the enzymic assay. However, if the idea that the apparent instantaneous activation by potassium chloride is simply maintenance of the equilibrium in favour of K^+ ion–enzyme formation is correct, then the results shown in Fig. 3 demonstrate that K^+ ions do activate the enzyme, since an apparent activation by potassium chloride was observed under conditions where thermal inactivation is known not to occur. Therefore the simplest explanation of the observed results seems to be that the active, and also the most stable, form of the enzyme is the K^+ ion–apoenzyme complex.

Many enzymes, including pyruvate kinase (EC 2.7.1.40) (Kachmar & Boyer, 1953), fructokinase (EC 2.7.1.4) (Parks, Ben-Gershom & Lardy, 1957), tryptophanase (Happold & Struyvenberg, 1954), phosphopyruvate carboxylase (EC 4.1.1.31) (Woronick & Johnson, 1960), β-galactosidase (EC 3.2.1.23) (Cohn & Monod, 1951), formiminotetrahydrofolate cyclodeaminase (EC 4.3.1.4) (Tabor & Wyngarden, 1959), aldehyde dehydrogenase (EC 1.2.1.5) (Black, 1951), homoserine dehydrogenase (EC 1.1.1.3) (Patte, Le Bras, Loviny & Cohen, 1963) and 5,10-methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5) (Ramasastri & Blakley, 1962), have been shown to be activated by K^+ ions and other univalent cations. In those cases where it has been investigated (pyruvate kinase, fructokinase, phosphopyruvate carboxylase, β-galactosidase and formiminotetrahydrofolate cyclodeaminase) it has been shown that one K^+ ion is bound/active centre. However, in all cases except aldehyde dehydrogenase, where there is a 10–30 sec. lag, activation is apparently instantaneous.

The only reported cases of slow activation of enzymes by metal ions are the activation of certain peptidases by Mn^{2+} ions, where there is evidence that one ion is bound/active centre (Smith, 1951), and of arginase (EC 3.5.3.1) by Mn^{2+} or Co^{2+} ions (Mohamed & Greenberg, 1945). With leucine aminopeptidase the time required for activation decreases with the degree of purification of the enzyme (Spackman, Smith & Brown, 1955).

SUMMARY

1. *Staphylococcus aureus* threonine dehydrogenase is partially inactivated by dialysis against water, but the dialysed enzyme can be activated by incubation with potassium chloride at 0°. Maximal activation requires incubation in 0·5 M-potassium chloride for 24 hr.

2. The activation is unaffected by the presence of EDTA.

3. The enzyme is activated by Rb^+, Cs^+, K^+, NH_4^+ and to a smaller extent by Li^+ and Na^+ ions.

4. As well as the time-dependent activation, the enzymic activity is stimulated by the addition of potassium chloride to the assay mixture. The specificities of the two activating effects are closely similar.

5. The results indicate that the active form of the enzyme is a K^+ ion–apoenzyme complex. The K^+ ions present in the assay mixture may act by maintaining the equilibrium in favour of complex-formation.

I thank Dr W. H. Elliott for his interest and advice, Professor A. G. Ogston for helpful discussions and Mr J. Lee for skilled technical assistance. The author held an Australian National University Scholarship.

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Condensed Tannins

19. CONFIGURATION OF DIOL GROUPS IN FLAVAN-3,4-DIOLS BY PAPER CHROMATOGRAPHY AND PAPER IONOPHORESIS*

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(*Received* 17 *October* 1963)

The significance attached to the formation of isopropylidene derivatives in high yield from methylated flavan-3,4-*trans*-diol precursors of wattle tannins results in unreliable conclusions regarding the stereochemistry of the diol group, owing to inversion of the 4-hydroxyl group under acid conditions (Drewes & Roux, 1964). Accurate conclusions are, however, possible from comparative oxidation rates of 3,4-*cis*- and 3,4-*trans*-diols with lead tetra-acetate (Bokadia *et al.* 1961) or periodate (Drewes & Roux, 1964), or from the spin–spin coupling constants of the 2-, 3- and 4-protons obtained from their nuclear-magnetic-resonance spectra (Corey, Philbin & Wheeler, 1961; Clark-Lewis & Jackman, 1961; Lillya, Drewes & Roux, 1963). Moreover, formation of isopropylidene derivatives by ketal exchange offers a possible alternative for deciding between alternative arrangements (Brown & MacBride, 1963).

The present work shows that correlation of both paper-chromatographic and paper-ionophoretic behaviour of methylated flavan-3,4-diols with their known stereochemistry offers simple but apparently reliable criteria for differentiating between *cis*- and *trans*-glycol arrangements.

EXPERIMENTAL AND RESULTS

Nuclear-magnetic-resonance spectra were recorded on a Varian A-60 spectrometer with deuterochloroform as solvent and tetramethylsilane as internal standard. Band positions are expressed as p.p.m. downfield with the standard as origin (Table 1). Coupling constants are measured with an accuracy of ±0·2 cyc./sec. C, H, methoxyl and acetyl estimations are by K. Jones, Microanalytical Laboratory, C.S.I.R., Pretoria. All melting points are uncorrected.

Synthesis of (±)-3′,4′,5′,7-*tetramethoxy- and* (±)-3′,4′,7-*trimethoxy*-2,3-cis-*flavan*-3,4-cis-*diols*

(±)-3′,4′,5′,7-*Tetramethoxy*-2,3-cis-*flavan*-3,4-cis-*diol*. (±)-Dihydrorobinetin from *Robinia pseudacacia* was methylated with diazomethane to give (±)-3′,4′,5′,7-tetramethoxyflavan-3-ol-4-one, m.p. 165°. The methylated dihydroflavonol (500 mg.) in ethanolic N-H_2SO_4 (20 ml.) was refluxed for 6 hr. while O_2 was bubbled through the solution. 3′,4′,5′,7-Tetramethoxyrobinetin (3′,4′,5′,7-tetramethoxyflavonol), m.p. 194–195° (300 mg.), crystallized from the solution (Found: C, 63·7; H, 5·1. Calc. for $C_{19}H_{18}O_7$: C, 63·7; H, 5·0%). Dean & Nierenstein (1925) and Shah & Kulkarni (1958) record m.p. 193° and 194–195° respectively for the same compound.

Raney nickel, freshly prepared from Raney alloy (Hopkin and Williams Ltd.: Ni–Al; 42, 58), was used for reduction of the flavonol. To tetramethoxyrobinetin (192 mg.) dissolved in ethanol–methanol (3:1, v/v) (50 ml.) was added freshly prepared Raney nickel (0·4 g.) and the mixture heated with shaking under H_2 for 4·75 hr. at 90–95° and 38·5 kg./cm.^{2}. After removal of the catalyst the solution was concentrated to small volume (2 ml.). White crystals (92·4 mg.), m.p. 160°, separated at room temperature (48% yield). Recrystallization from ethanol raised the m.p. to 165° (Found: C, 63·5; H, 6·3; OCH_3, 33·5. $C_{19}H_{22}O_7$ requires: C, 63·0; H, 6·1; OCH_3, 34·2%).

(±)-3,4-cis-*Diacetoxy*-3′,4′,5′,7-*tetramethoxy*-2,3-cis-*flavan*. Acetylation of the diol (100 mg.) with acetic anhydride (0·4 ml.) and pyridine (0·4 ml.) gave the diacetate (90 mg.), which crystallizes from ethanol, m.p. 169° (Found: C, 61·9; H, 5·8; OCH_3, 27·6; $CO{\cdot}CH_3$, 19·6. $C_{23}H_{26}O_9$ requires: C, 61·9; H, 5·8; OCH_3, 27·8; $CO{\cdot}CH_3$, 19·3%). Details of the nuclear-magnetic-resonance spectrum of this compound are listed in Table 1.

* Part 18: Drewes & Roux (1964).