Liquid Scintillation-Counting for [^{14}C]Steroids

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The measurement of ^{14}C (β = 0·158 Mev) and of tritium (β = 0·018 Mev) is limited by the low energy of the emitted β particles. Standard techniques for the assay of these isotopes may be divided into the following three broad categories: (*a*) end-window and windowless flow gas counters operating in the proportional or Geiger region, which involve plating of the source at either infinite thinness or infinite thickness; (*b*) assay of the isotope in the gas phase after combustion of the biological sample; (*c*) liquid-scintillation techniques, of more recent introduction, in which the radioactive substance is incorporated into a liquid phosphor and the scintillations are 'observed' by means of a photomultiplier.

Method (*a*) suffers from the well-known difficulties in obtaining uniform and reproducible plating of the material to be assayed. Moreover, with plating techniques, it is considered necessary to convert the samples into a common form by combustion and incorporation into barium carbonate to obtain accurate and comparable specific radioactivities. Abrams & Clarke (1951) observed inaccuracies of 30% when attempting to count samples containing ^{14}C not subjected to combustion. However, Karnovsky *et al.* (1955) reported much smaller correction factors when counting samples, not subjected to combustion, by a windowless flow gas counter than have been reported for end-window Geiger counters. Where the latter method is employed the absorption of the low-energy β particles in the thinnest window available renders the method highly inefficient for ^{14}C assay and presents 'tremendous if not insurmountable difficulties for the assay of tritium' (Haigh, 1958). The estimation of a tritiated steroid in a methane flow proportional counter has, however, been described by Eidenoff & Knoll (1950). A windowless flow type of counter operating in the Geiger region for assay of low-energy β rays has been described by Damon (1951) and by Banks, Blow & Francis (1956), and applied to the estimation of tritium by Banks, Crawhall & Smyth (1956). Ayres, Pearlman, Tait & Tait (1958) have assayed tritiated progesterone and corticosteroids in a windowless flow counter.

Method (*b*), the assay of ^{14}C and tritium in the gas phase, overcomes the difficulties of plating the samples and the low sensitivity associated with end-window counters. Several methods have been described with ion chambers (e.g. Jesse, Hanum, Fostat & Hart, 1947); Janney & Moyer, 1947) and Geiger–Müller tubes (e.g. Brown & Miller, 1947; Bernstein & Ballentine, 1950; Glascock, 1952; Bradley, Holloway & McFarlane, 1954; Apelgot, Roumegous, Patureau & Moustacchi, 1955; Broda, 1955; Korshunov, Amenitskaya & Aĭvazov, 1955; Banks *et al.* 1956). These methods, although very sensitive and accurate, are time-consuming and unsuitable for routine use where large numbers of samples have to be assayed accurately, e.g. eluates from chromatographic columns. Method (*c*), the liquid scintillation-counting of ^{14}C and tritium, is gaining increasing popularity because of the high sensitivity and reproducibility due to a fixed 4π geometry and because of the elimination of window absorption, the necessity for plating and the elaborate technique required for gas counting. Many uses of the method have been described (e.g. Reynolds, Harrison & Salvini, 1950; Kallman & Furst, 1950; Bluh & Terentiuk, 1952; Farmer & Bernstein, 1952; Hayes & Gould, 1953; Rosenthal & Anger, 1954; Wagner & Guinn, 1955; Weinberger, Davidson & Ropp, 1956; White & Helf, 1956; Hodgson, Gordon & Ackerman, 1958). A balanced review has been given by Davidson & Feigelson (1957), who have discussed the advantages and disadvantages of liquid scintillation-counting in detail. The latest developments in this field are described by Bell & Hayes (1958).

The method has been applied to the estimation of ^{14}C-labelled steroid hormones (e.g. Werbin, Bergenstal, Gould & LeRoy, 1957; Chen, Schedl, Rosenfeld & Bartter, 1958; Kabara, Okita & LeRoy, 1958) and to some tritiated sterols by Hayes & Gould (1953), but there appears to be no systematic record of the variables affecting the method, especially as applied to this class of compound.

The advantages of liquid scintillation-counting for the detection of steroids are the high solubility of these compounds in the organic phosphor, the high overall efficiency and the convenience of the method. With the Ekco type N612 scintillation counter 65–69% efficiency has been obtained for the detection of ^{14}C (Table 1), based on [^{14}C]-naphth-2-ol and [^{14}C]testosterone sources obtained from The Radiochemical Centre, Amersham, Bucks. All counting was carried out at room temperature, under the optimum experimental conditions

described below, with a background of 1·5–2·0 counts/sec. There are a number of important experimental variables, however, which affect the absolute efficiency and the reproducibility of the method. It is thought that these variables may be of general application and that experiences in this respect may be of value to other workers in this and other fields.

APPARATUS AND MATERIALS

Apparatus. The Ekco type N612 Counter was connected to a stable power source (Harwell type 1007 or type 532B), timer (Harwell type 1003F), scaler (Harwell type 1009D) and decatron unit (Harwell type 1221c). The N612 counter uses a 13-stage photomultiplier, E.M.I. type 95145, with a simple linear amplifier offering alternative gains of 250, 500 and 1000 with an attenuation of 10 to 1 on these values if required. Details of its use and construction have been described by Haigh (1957).

Reagents. These must be purified to avoid possible ambiguities due to trace impurities. Ethyl acetate, light petroleum (b.p. 60–80°), acetone and methylene dichloride of A.R. grade were redistilled in Pyrex apparatus, the first and last 10% of the distillates being rejected. Chloroform and carbon tetrachloride of A.R. grade were fractionally distilled through a 150 cm. fractionating column. Benzene, A.R. grade, was redistilled twice, the first and last 10% of the distillates being rejected. Ethanol was dried and purified from volatile aldehydes and ketones by the method of Cook, Dell & Wareham (1955). Methanol, A.R. grade, was refluxed over CaO for 2 hr. and fractionally distilled through a 150 cm. fractionating column. Toluene of reagent grade, sulphur-free, was distilled three times in Pyrex apparatus, the first and last distillates being rejected in each case.

^{14}C-labelled compounds. [^{14}C]Testosterone and [^{14}C]-oestrone (The Radiochemical Centre, Amersham, Bucks.) were dissolved in benzene. [^{14}C]Naphth-2-ol (The Radiochemical Centre) was dissolved in toluene.

Organic phosphor. Scintipak (Nash and Thompson Ltd., Chessington, Surrey) was dissolved in the recommended quantity (4·2 g. in 1 l.) of purified toluene. The Scintipak consists of a mixture of 4 g. of *p*-terphenyl and 0·2 g. of *p*-bis-[2-(5-phenyloxazolyl)]benzene. Unless otherwise stated 'the scintillator' refers to the Scintipak solution described above.

EXPERIMENTAL

Effect of p-*bis*-[2-(5-*phenyloxazolyl*)]*benzene on counting efficiency.* No comparison of various primary and secondary solutes was made; these have been adequately described in the literature (e.g. Hayes, Ott, Kerr & Rogers, 1955; Hayes, Ott & Kerr, 1956). The efficiency of *p*-bis-[2-(5-phenyloxazolyl)]benzene in increasing the relative pulse height has been described by Hayes *et al.* (1956) with Dumont 6292 photomultipliers. That this compound is similarly effective with the E.M.I. 95145 photomultiplier is indicated from Fig. 1, which shows the increase in counting efficiency of the N612 counter when *p*-bis-[2-(5-phenyloxazolyl)]benzene is added to a primary solute of *p*-terphenyl in toluene. Although *p*-bis-[2-(5-phenyloxazolyl)]benzene and other secondary solutes may function as wavelength shifters, a report by Ziegler, Seliger & Jaffe (1955) indicated that the successful reversal of O_2 quenching may be one of the ways in which these compounds function. No allowance has been made for this quenching of the scintillator by dissolved O_2

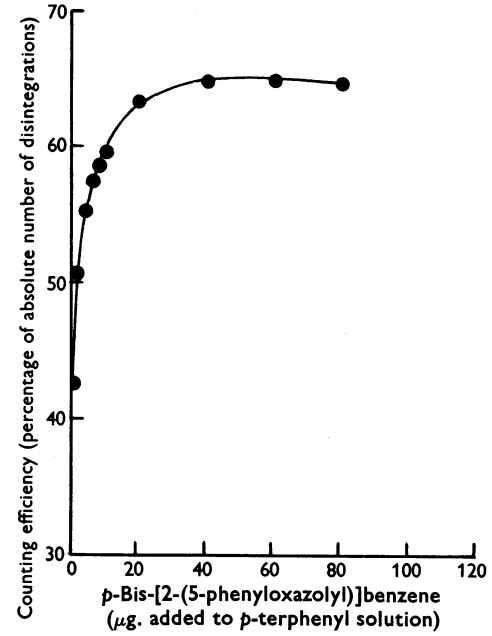

Fig. 1. Effect of *p*-bis-[2-(5-phenyloxazolyl)]benzene on the counting efficiency for [^{14}C]testosterone dissolved in 3 ml. of a solution of *p*-terphenyl in toluene (4 g./l.). Increasing amounts of *p*-bis-[2-(5-phenyloxazolyl)]benzene in toluene were added (2 g./l.).

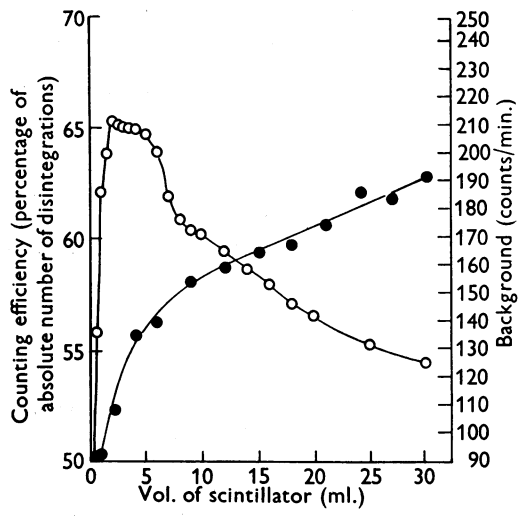

Fig. 2. Effect of increasing the volume of the phosphor, at constant concentration of scintillator, on the counting efficiency for a [^{14}C]testosterone source. ○, [^{14}C]Testosterone; ●, background count rate.

(Pringle, Black, Funt & Sobering, 1953; Arnold, 1954). Experiments were carried out to test the effects of bubbling N_2 (O_2-free) through the scintillator but no significant improvement in counting efficiency was obtained. Bubbling O_2 through the scintillator, however, reduced the counting efficiency by about 10%.

Variation of counting efficiency with volume of phosphor. Increasing volumes of the scintillator were added to a [^{14}C]testosterone source and the count rate was observed at each volume (Fig. 2). The efficiency rose rapidly to an optimum which corresponded to a volume of 2–4 ml. of the scintillator. The efficiency thereafter decreased with increasing volume. The background count rose linearly with increasing volume of the scintillator (Fig. 2). For routine counting a volume of 3 ml. was selected.

Variation of counting efficiency with concentration of Scintipak in liquid phosphor. The efficiency of the count rose rapidly with increasing concentration of the Scintipak in the toluene (Fig. 3). For routine counting the concentration recommended by Nash and Thompson Ltd. (4·2 g./l.) was adopted.

Quenching of the scintillator. The two main problems in scintillation-counting are insolubility of the sample and quenching. The former is not troublesome in connexion with the steroid hormones but the latter was investigated at some length. The importance of solvents in liquid scintillators has been described by Hayes, Rogers & Sanders (1955) and the quenching of liquid scintillator solutions by a variety of organic compounds has been described by Kerr, Hayes & Ott (1957).

Effect of solvent impurities in the scintillator on the counting efficiency. In biochemical experiments with labelled compounds, the final material for assay may be presented in any of a number of various organic solvents following extraction from tissues or complex incubation systems. The effect of such solvents on the counting efficiency, when added to 3 ml. of the scintillator containing [^{14}C]testosterone, can be seen from Fig. 4. The chlorinated hydrocarbons tested have a dramatic quenching effect, whereas solvents such as benzene, diethyl ether and light petroleum have effect only by dilution of the phosphor (Fig. 4). A wide range of solvents have been examined by Hayes, Rogers & Sanders (1955) for their effect on the relative pulse height from a liquid phosphor.

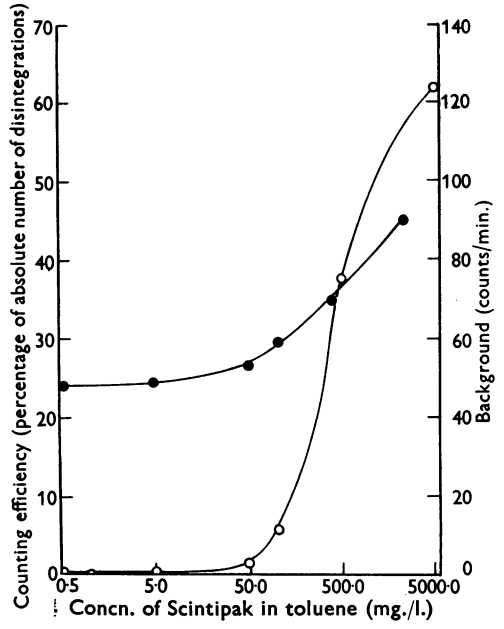

Fig. 3. Effect of the concentration of the scintillator at constant volume (3 ml.) on the counting efficiency. Scintipak of increasing concentration in toluene. ○, [^{14}C]Testosterone sources (10^{-5} μc); ●, background.

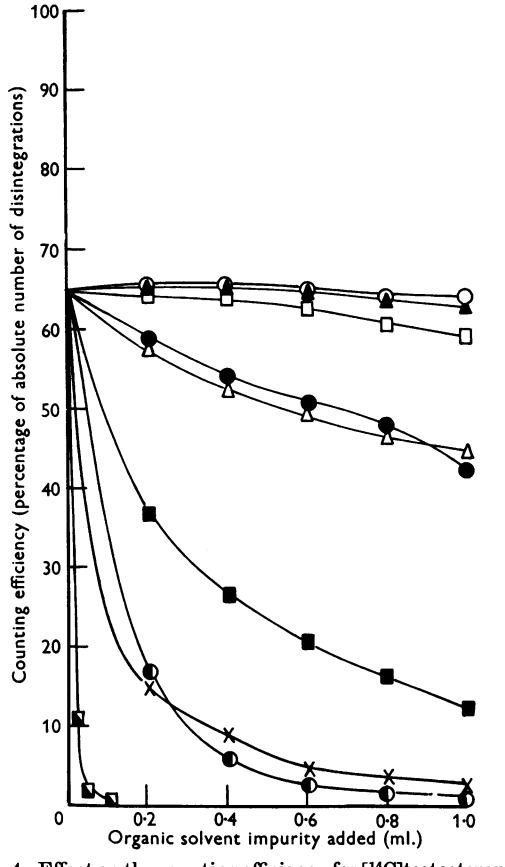

Fig. 4. Effect on the counting efficiency for [^{14}C]testosterone of the addition to the scintillator (3 ml.) of some organic solvent impurities. ○, Benzene; ▲, diethyl ether; □, ethyl acetate; ●, ethanol; △, methanol; ■, methylene dichloride; ◐, chloroform; ×, acetone; ◪, carbon tetrachloride.

Table 1. *Efficiency of counter for* ^{14}C

[^{14}C]Naphth-2-ol and [^{14}C]testosterone sources were added either in solution to phosphor (3 ml.) or first evaporated to dryness in the counting pot before adding the phosphor (3 ml.).

^{14}C Source	Apparent counting efficiency (percentage of absolute number of disintegrations observed)	
	Evaporated in pot and redissolved	Added in solution to phosphor
Naphth-2-ol	58·0	65·0
	60·0	65·0
Testosterone	68·5	68·0
	68·5	68·5

When biological extracts are obtained in solution in a quenching solvent, this may be evaporated to dryness in the counting pot under infrared light. The sample may then be dissolved in the liquid phosphor and counted in the usual manner. A word of caution is necessary in this respect, however, since the ^{14}C source, especially when of high specific activity, may become partially adsorbed on the surface of the glass container. For such adsorbed material, 2π counting geometry may operate only, with a consequent loss of counting efficiency (Table 1).

Self-quenching from steroid hormones. To determine the suitability of the liquid scintillation method for the general assay of labelled steroids it was necessary to determine if self-quenching occurred with any of a large variety of steroids. The compounds listed in Table 2 were dissolved (1 mg./ml.) in benzene or benzene–diethyl ether (1:1, v/v) and 0·5–1·5 ml. of the solution of each steroid was added to the scintillator containing a standard [^{14}C]testosterone source. The decrease, if any, in absolute counting efficiency is shown in Table 2.

DISCUSSION

The liquid scintillation method has been found very convenient for the assay of labelled steroids because of their ready solubility in the organic phosphor. The method is very reproducible when attention is paid to the experimental factors given above and to some additional factors given below. The results given in Table 2 show that the method is of general

Table 2. *Effect of quenching by steroids on the counting efficiency for* ^{14}C

The steroid was dissolved* (1 mg./ml.) in benzene or benzene–diethyl ether (1:1, v/v) and 0·5, 1·0 and 1·5 ml. of the solution was added to the phosphor (3 ml.) containing [^{14}C]testosterone. The addition of 1·5 ml. of pure benzene or ether to the phosphor reduces the absolute counting efficiency by approx. 1·2 and 1·8%, respectively. The counting efficiency without added steroid solution was 65%.

Steroid added	Counting efficiency after addition of steroid (percentage of absolute number of disintegrations)		
Amount added (mg.) ...	0·5	1·0	1·5
Prednisone (17α:21-dihydroxypregna-1:4-diene-3:11:20-trione)*	64·5	64·1	63·1
9α-Fluoro-11β-hydroxy-17-methyltestosterone*	64·0	63·7	62·9
3α:17β-Dihydroxy-5α-androstane*	64·3	63·2	62·6
3α:17β-Diacetoxyandrost-5-ene	64·2	63·6	62·5
9α-Fluorocortisone acetate (9α-fluoro-17α-hydroxy-21-acetoxypregn-4-ene-3:11:20-trione)*	63·9	63·2	62·5
Hydrocortisone (11β:17α:21-trihydroxypregn-4-ene-3:20-dione)	64·1	63·1	62·3
3β:17α:21-Trihydroxypregn-5-en-20-one*	63·8	63·1	62·2
Oestradiol (3:17β-dihydroxyoestra-1:3:5(10)-triene	64·1	63·0	62·0
Androsterone (3α-hydroxy-5α-androstan-17-one)	64·0	62·7	61·9
Cholesterol (3β-hydroxycholest-5-ene)	64·0	62·9	61·9
Cortisone (17α:21-dihydroxypregn-4-ene-3:11:20-trione)*	64·0	63·0	61·8
3α:17α-Dihydroxy-5β-pregnan-20-one)	64·1	62·8	61·8
9α-Fluoro-11β-hydroxytestosterone (9α-fluoro-11β:17β-dihydroxyandrost-4-en-3-one)*	63·9	63·2	61·8
Testosterone (17β-hydroxyandrost-4-en-3-one)	64·2	62·9	61·8
5α-Androstane-3:17-dione	64·8	63·2	61·7
Oestriol (3:16α:17β-trihydroxyoestra-1:3:5(10)-triene)*	64·5	63·7	61·6
Compound S (17α:21-dihydroxypregn-4-ene-3:20-dione)	63·9	63·0	61·4
Progesterone (pregn-4-ene-3:20-dione)	64·3	62·6	61·3
Cholest-4-en-3-one	63·8	62·2	61·2
Pregnenalone (3β-hydroxypregn-5-en-20-one)	64·2	62·8	61·2
Androst-4-ene-3:17-dione	63·9	62·4	60·7
Androsta-1:4-diene-3:17-dione	63·9	62·3	60·4
Oestrone (3-hydroxyoestra-1:3:5(10)-trien-17-one)	62·7	62·0	60·2
5α-Androst-1-ene-3:17-dione	63·8	61·6	59·9
Equilenin (3-hydroxyoestra-1:3:5(10):6:8-pentaen-17-one)	63·0	61·0	58·7

* Steroids were added in slight suspension owing to partial insolubility in the solvents used.

application for the assay of ^{14}C-labelled steroids, since strong quenching of the scintillator was not caused by any of the 25 compounds tested. Definite quenching was, however, shown by certain steroids, notably oestrone and equilenin. There appears to be no systematic relationship between structure and quenching potential with most of the compounds tested. The compounds listed in Table 2 were tested only to a concentration of 1·5 mg. in the scintillator. Where assays are to be made of compounds of low specific activity, however, where the carrier is in excess of 1·5 mg., then further tests would be necessary at the appropriate concentration. Correction for quenching should be made for any compound where its concentration in the scintillator may have a significant effect. Caution should be exercised also when using the method for the assay of crude biological extracts where interfering or quenching compounds or colour (Kinnory *et al.* 1958) may accompany the radioactive product under investigation.

It is a simple matter to test such extracts by addition of a source of accurately known value and to make the appropriate correction for suppression of the expected count.

The Ekco N612 counter uses a small (46 mm. diameter) counting pot. Optical contact of the base of this pot with the top of the photomultiplier is made with silicone oil (Hopkin and Williams Ltd.; MS 200/2 centistokes). The silicone fluid (MS 200/20 centistokes) recommended earlier by the manufacturer (E. K. Cole Ltd.) was less satisfactory since bubbles tended to be trapped beneath the counting chamber in this oil of higher viscosity. Despite the fact that these chambers are manufactured with slightly convex bases it is still possible to trap an occasional bubble beneath the vessel, especially when the silicone fluid is low. It is necessary to pay particular attention to the elimination of all such bubbles, since they have a marked effect on the counting efficiency and thereby upon the reproducibility of the method. In this connexion it should be pointed out that this silicone oil does, in time, become contaminated with atmospheric dust and dirt and extraneous matter conveyed in with the counting vessel. In initial experiments the vessels were carefully wiped with paper tissue to remove dirt and in later experiments with a lens tissue. In both cases reproducibility of the method was unsatisfactory and this was traced to the accumulation, during several weeks' use, of particles of the paper or lens tissue in the silicone fluid. In addition a minute amount of swarf or particles of paint worked into the oil initially and may have added to this trouble. This sort of contamination of the oil gives rise to two effects: (*a*) an intermittent lack of reproducibility due to the disturbance of the contaminating particles every time the shutter mechanism is operated in the silicone oil and (*b*) a long-term increase in interfering material which is reflected in a gradual reduction of the observed count from a standard source.

To overcome these difficulties a chamois leather is now used for wiping the counting vessels before they are introduced into the counter and, most important, a ^{14}C standard test source is maintained. This is of the order of 2×10^4 counts/min., and assay at regular intervals reveals accumulation of contaminating dust necessitating a change of the silicone fluid.

A great deal has been said about possible interference from fluorescence of the glass counting chambers. Under the conditions employed for determination of ^{14}C, however, we have failed to induce deliberately any significant fluorescence effects into these containers either by sunlight or fluorescent lighting.

If attention is paid to the details mentioned above, liquid scintillation-counting is regarded as the most convenient method for the determination of ^{14}C-labelled steroids, despite the rather high background as compared with other methods. Under the optimum experimental conditions described in this paper, greater efficiency (65–69%) has been obtained than can be achieved by end-window or windowless flow gas counters, and much greater convenience than with apparatus for the determination of this isotope in the gas phase. The reproducibility and the efficiency with the scintillation technique at room temperature are sufficiently high for all practical purposes and the added expense and inconvenience of refrigeration would not seem justified for assay of ^{14}C.

SUMMARY

1. The assay of ^{14}C-labelled steroids with the Ekco type N612 liquid scintillation counter is described.

2. The effects of scintillator volumes and concentration on the efficiency of the method are given.

3. Self-quenching by 25 different steroids has been examined. None of the steroids, at the levels tested, exerted strong quenching effects, although slight quenching was apparent with some, in particular with equilenin and oestrone.

4. The effect of impurities in the scintillator on the efficiency of the method has been investigated.

5. Some additional variables which affect the reproducibility are discussed.

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Studies on Carbohydrate-Metabolizing Enzymes

2. TRANS-α-GLUCOSYLATION BY EXTRACTS OF *TETRAHYMENA PYRIFORMIS**

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In continuation of studies on transglucosylation reactions (Duncan & Manners, 1958; Anderson & Manners, 1959; Duncan, Manners & Thompson, 1959) we now describe an investigation of the metabolism of maltose by cell-free extracts of the ciliate *Tetrahymena pyriformis*. The presence of a maltase in such extracts has been noted by Ryley (1952).

* The paper by Anderson & Manners (1959) is regarded as Part 1.

Since maltase activity may be regarded as a trans-α-glucosylation reaction in which water provides the acceptor substrate:

$$\text{Maltose} + \text{enzyme-H} \rightarrow \text{glucose} + \text{glucosyl-enzyme}$$
$$\text{Glucosyl-enzyme} + \text{H·OR} \rightarrow \text{enzyme-H} + \text{glucosyl·OR}$$

where H·OR is the glucosyl acceptor (Gottschalk, 1958), it follows that, if oligosaccharide synthesis takes place in concentrated solutions of maltose,