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High-Efficiency Liquid-Scintillation Counting of ^{14}C-Labelled Material in Aqueous Solution and Determination of Specific Activity of Labelled Proteins

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1. Inexpensive scintillation mixtures are described which enable the detection of as little as 40 $\mu\mu$c of ^{14}C in aqueous solution with an efficiency of counting of over 80%. 2. A rapid method for the counting of alkaline, acidic and neutral aqueous solutions of up to 1ml. volume is described. Ethanol or 2-ethoxyethanol is used as blending agent. 3. The scintillation counting of alkaline solutions is applied to the accurate determination of the specific activity of ^{14}C-labelled proteins from plant tissues. 4. Attention has been paid to the importance of a standardized washing procedure for the removal of all traces of radioactive material from glassware.

The use of radioactive materials in biological experiments has become indispensable for investigating many biochemical reactions. In the past qualitative determinations were sufficient but nowadays accurate quantitative measurements are possible.

Investigation of protein synthesis, especially by cell-free systems, often involves the measurement of very low activities of ^{14}C-labelled material. Determination by scintillation counting is very sensitive, especially for non-aqueous material, when greater than 90% efficiency may be obtained. For counting aqueous solutions, however, the efficiency is often drastically reduced. Several systems for the scintillation counting of aqueous solutions have been described, often involving the use of Hyamine salts to obtain a one-phase system, which give efficiencies of about 60% (e.g. Passmann, Radin & Cooper, 1956; Herberg, 1960; Brown & Badman, 1961). A review of various solvents has been presented by Davidson (1958), and use of some of these in aqueous systems has been reported: Bruno & Christian (1961) employed Cellosolve and Takahashi, Hattori & Maruo (1963) note a dioxan system for counting tritiated biological samples in aqueous solution. Suspension of precipitates on glass filter paper has also been used to advantage (Johnson & Smith, 1963).

The use of the chloride salt of Hyamine has several advantages over the free base. However, its use is still complicated by its high viscosity, its quenching effect and especially in the large volumes required for blending after neutralization.

A search was therefore made for scintillation systems which were free from these disadvantages and yet maintained high counting efficiencies. Following the report of Robinson & Novelli (1962) that radioactive proteins could be dissolved in formic acid and counted in a scintillation mixture of toluene, 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene and containing ethanol as blender, it seemed of particular interest to investigate more fully the use of ethanol and 2-ethoxyethanol (Bruno & Christian, 1961), rather than Hyamine 10X, as blenders for the high-efficiency counting of aqueous samples, especially radioactive proteins. The demonstration by Hayes, Ott, Kerr & Rogers (1955) that biphenylylphenyloxadiazole used as a primary solute in toluene gave a considerably higher pulse height than 2,5-diphenyloxazole suggested that its use in a scintillation mixture might well be advantageous. Scintillation mixtures which will maintain a one-phase system with both acidic and alkaline solutions of ^{14}C-labelled material are described with ethanol and 2-ethoxyethanol as blenders. Routine measurements at efficiencies of more than 80% have been made on radioactive samples in 0·1ml. of aqueous solution, and even for volumes of 1ml. efficiencies greater than 60% have been attained.

The high level of efficiency of counting for material dissolved in alkali has been successfully applied to the accurate determination of the specific activity of small amounts of protein labelled with low activities of ^{14}C.

EXPERIMENTAL

Apparatus. The scintillation-counting equipment used comprised: a Panax scintillation counter, type SC–LP (Panax Equipment Ltd., Redhill, Surrey) capable of

measurement of millimicrocurie quantities of low-energy β-emitters; a high-voltage supply, IDL type 1820A (Isotope Developments Ltd., Beenham Grange, Reading, Berks.); an amplifier-selector (IDL type 1830A) and an automatic scaler (model 8041A)–timer (model 6301A) (Research Electronics Ltd., Cleckheaton, Yorks.). No cathode follower was used as the connexion between the counter and the amplifier was kept short.

The settings of 1100v high voltage (E.H.T.) and 7v discriminator bias (D.B.) were chosen so that the count rate was virtually independent of small fluctuations in E.H.T. (Francis, Mulligan & Wormall, 1959, p. 165) and in D.B. (Panax, 1960). Amplification was at the maximum setting of $\times$ 1000. At this amplification setting, however, the system as set up did not satisfy at the same E.H.T. setting the two conditions, (1) that the sample count shows a minimum variation with variation in the E.H.T., and (2) that the background count rate is minimal compared with the sample count rate [ideally (sample count rate)^2/background at a maximum]. Condition (1) was fulfilled at 1100v (E.H.T.), 7v (D.B.), the setting employed throughout this investigation, and condition (2) at 980v (E.H.T.), 7v (D.B.). Consequently assays were carried out at an E.H.T. where, although small fluctuations in applied voltage did not greatly affect the sample counting rate, the effect on the background was slightly more marked. In this context the use of the IDL type 532 high-voltage supply system which has greater stability than the IDL type 1820A might have been advantageous. However, it was observed at the E.H.T. setting of 1100v that a 2v E.H.T. change, which might have been expected from a 10% mains voltage change when using the type 1820A E.H.T. supply, only altered the background count by 5counts/min. Silicone oil (Hopkin and Williams Ltd., MS200/2 centistokes) was used to provide optical contact between the counting vessel and the window of the photomultiplier tube; 2 centistoke oil was found to be more convenient than 20 centistoke oil as it did not cling so strongly to the base of the glass counting vessel, resulting in less loss of oil and easier cleaning of the vessel; also, as was found by Stitch (1959), bubbles were less easily trapped beneath the counting vessel. The Panax counter, silicone oil, scintillator and the vessels were kept between 1° and 4° in a refrigerator (Rosenthal & Anger, 1954).

Scintillator. Scintillation-grade NE toluene [Nuclear Enterprises (G.B.) Ltd., Sighthill, Edinburgh 11] was used as the solvent. 2,5-Diphenyloxazole (NE505) or biphenylylphenyloxadiazole (NE501) was used as primary solute, at concentrations of 4g./l. and 10g./l. respectively, and 1,4-bis-(5-phenyloxazol-2-yl)benzene (NE502), 0·1g./l., as secondary solute (Hayes, Ott & Kerr, 1956). For standardization of the counting equipment NE213 liquid-scintillator was employed.

Reagents. For aqueous samples either 2-ethoxyethanol [ethylene glycol monoethyl ether (Cellosolve, laboratory reagent); British Drug Houses Ltd., Poole, Dorset] or spectroscopically pure ethanol was added to the scintillator as required to give a one-phase solution. Formic acid (analytical reagent, 98–100%; British Drug Houses Ltd.) was added to the scintillation solution when the radioactivity of alkaline solutions was being determined. For protein estimations Folin–Ciocalteu reagent (British Drug Houses Ltd.) diluted 1:1 (v/v) with water was used. Crystalline bovine albumin (Sigma Chemical Co., St Louis, Mo., U.S.A.) and egg albumin (L. Light and Co. Ltd., Colnbrook, Bucks.) were used as protein standards. Pyroneg [Diversey (U.K.) Ltd., London W.1) and RBS25 concentrate (Medical–Pharmaceutical Developments Ltd., Shoreham-by-Sea, Sussex) were used for cleaning glassware.

Radiochemicals. L-[U-^{14}C]Alanine and U-^{14}C-labelled protein hydrolysate from *Chlorella* (The Radiochemical Centre, Amersham, Bucks.) were dissolved in sterile glass-distilled water. *n*-[1-^{14}C]Hexadecane was used as an internal standard.

Biological samples. Samples of tomato-leaf protein, labelled by the uptake of supplied radioactive amino acids, were prepared and washed by the method of Siekevitz (1952). The ether-dried protein was dissolved in dilute sodium hydroxide or formic acid.

METHODS AND RESULTS

Standardization of the counting equipment. Liquid *n*-hexadecane of accurately known activity was used as supplied as an internal standard (Table 1); an efficiency of 91% was obtained for the NE 213 scintillator compared with 81% for the toluene–ethanol scintillator (Table 1). Since day-by-day variation in counting efficiency is known to occur a check count was taken each day before counting samples. For this purpose the difference between the count on a Panax CR/14/28 sealed reference source and a Panax B/21 sealed reference background was noted. Over a period of 3 months our equipment gave 87·4 ± 2·4% (24) of the quoted count rate.

Standard procedure for counting radioactive samples. For all determinations a background count on the vessel containing the required amount of scintillator was taken. The sample was then added, thoroughly mixed with the scintillator, and the count rate determined almost immediately, unless it had been exposed to light or warmed.

When the sample count rate was low the time of counting was increased, as was also the time for the background count, to give a statistically significant figure.

Background counting rate and sample containers. A low background count rate is desirable when counting samples of low activity as the higher the background count, the lower the total count/background count ratio, hence a higher probable error will occur (Francis *et al.* 1959, p. 217). Therefore, for low background counts special glass counting vessels with ground-glass stoppers (Panax Ltd.) were employed. These were made from glass relatively free from natural radioactivity, and gave a mean background count of 148counts/min. (Table 2). Bottles (13ml.) with plastic caps have also been used with the same efficiency, but have higher background counts, varying between 186 and 245counts/min. The background count rates referred to here are all for the vessel plus 5ml. of scintillator.

Table 1. *Standardization of equipment and counting efficiency of various systems for a non-aqueous radioactive source*

Liquid *n*-[1-^{14}C]hexadecane, specific activity 0·97 μc/g., 0·01 ml. (16 662·5 disintegrations/min.) was added to the scintillation mixtures shown below, on which a background count had previously been recorded. PPO, 2,5-Diphenyloxazole; BPD, biphenylylphenyloxadiazole; POPOP, 1,4-bis-(5-phenyloxazol-2-yl)benzene.

Scintillator							
Solvent (%, v/v)	Blender (%, v/v)	Primary solute (g./l.)	Secondary solute (g./l.)	Vol. (ml.)	Other additions (g./l.)	Net count (counts/min.)	Efficiency (%)
NE 213	—	—	—	4	—	15104	90·6
Toluene (100)	—	PPO (4)	POPOP (0·1)	4		15098	89·8
(70)	Ethanol (30)	PPO (4)	POPOP (0·1)	5		13428	80·7
(70)	2-Ethoxyethanol (30)	PPO (4)	POPOP (0·1)	5		13403	80·4
(100)	—	BPD (10)	POPOP (0·1)	4		15590	93·6
(70)	2-Ethoxyethanol (30)	BPD (10)	POPOP (0·1)	4		13949	83·7
(100)	—	BPD (10)	POPOP (0·1)	4	Naphthalene 80	15344	92·1
(70)	2-Ethoxyethanol (30)	BPD (10)	POPOP (0·1)	4	Naphthalene 80	13760	82·6

Table 2. *Effect of type of container used for sample on the background count rate*

All figures are the means of results taken over a period of 3 months for 24 glass vessels and 12 glass bottles washed by the standard procedure described in the text. Toluene–ethanol scintillator was used.

Sample	Mean count rate (counts/min.)	No. of observations	Maximum deviation from the mean count rate	
No vessel or bottle ('castle' empty)	108	22	−9	+10
Vessel empty	132	56	−8	+14
Vessel + 5 ml. of scintillator	148	128	−12	+22
Bottle empty	175	12	−6	+9
Bottle + 5 ml. of scintillator	213	15	−27	+32

Washing procedure. Reproducible background counts are of importance in accurate counting of low activities, and it was found that some carry-over of radioactive material in the vessels occurred from experiment to experiment. Thus in experiments involving plant proteins labelled with ^{14}C (supplied as $^{14}CO_2$) samples of protein in solution and having specific activities of more than 2000 counts/min./mg. were taken. After washing with detergent residual count rates of over 400 counts/min. were recorded for vessels plus 5 ml. of scintillator (Table 3). When radioactive amino acids had been added to incubation mixtures (e.g. 0·1 ml. of L-[^{14}C]alanine solution containing 1·1 million counts/min.; original specific activity 15·2 mc/m-mole) in 1 ml. final volume) the carry-over occasionally ran to several thousand counts/min. unless the vessels were washed by the standard procedure given below. This was also the case when dilutions of highly labelled radioactive materials were used for reference or for calibration of counting efficiency. It was noted that a thin film of silicone oil often covered the inside of the vessels, and the active material appeared to become protected by the silicone oil. Therefore a standard procedure for washing the counting vessels was evolved which removed the silicone oil.

The bottom of the vessel was wiped with absorbent material to remove as much oil as possible, the contents were emptied and the vessel was rinsed three times with hot water. The vessel was then allowed to stand (usually overnight) in a solution of commercial cleaner (Pyroneg) made strongly alkaline with a few sodium hydroxide pellets. The oil rose to the surface and was decanted. The vessel was rinsed seven times in warm water, then a 4% solution of RBS 25 concentrate heated to 60–70° was poured into the vessel and allowed to stand for 10 min. It was then rinsed seven times with water, twice with glass-distilled water and finally dried in an oven. This procedure has given very reproducible results (Table 3).

Counting aqueous samples. A scintillation mixture of toluene–ethanol (7:3, v/v) containing 4g. of

Table 3. *Residual activity in counting vessels after washing*

Count on vessel plus 5 ml. of scintillator after two experiments involving radioactive protein (specific activity approx. 2000 counts/min./mg. of protein). (i) Vessels washed by detergent alone. (ii) Vessels washed by the standard procedure. The values are not corrected for background, which was 132 counts/min.

Vessel no.	Residual count rate (counts/min.) (i)	(ii)
1	800	169
2	450	161
3	483	167
4	201	167
5	280	177
6	287	175
7	325	166
8	814	169
9	221	177

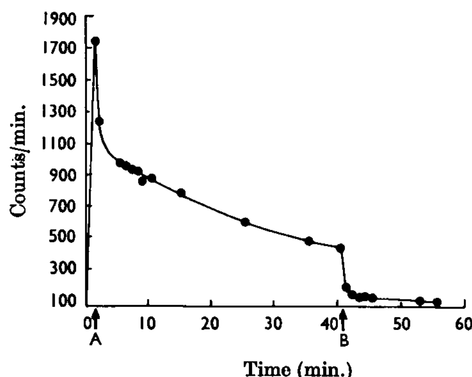

Fig. 1. Effect of alkali on background count rate. A portion (0·1 ml.) of 0·5 N-NaOH was added (arrow A) to 5 ml. of toluene–ethanol (7:3, v/v) scintillator and the scintillations so induced were followed by consecutive counts. After 40 min. 0·1 ml. of formic acid was added (arrow B) to the mixture and the rapid decrease in scintillations recorded.

2,5-diphenyloxazole/l. and 0·1 g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene/l. (Robinson & Novelli, 1962) was used. A similar mixture in which ethanol was replaced by an equal percentage of 2-ethoxyethanol was also assayed.

For 0·1 ml. samples 30% ethanol or 2-ethoxyethanol is the minimum practicable proportion required to maintain a one-phase solution in 4–5 ml. of scintillator. To count alkaline samples formic acid was added to the scintillator. Formic acid (0·1 ml. of 23 N) will acidify most alkaline samples

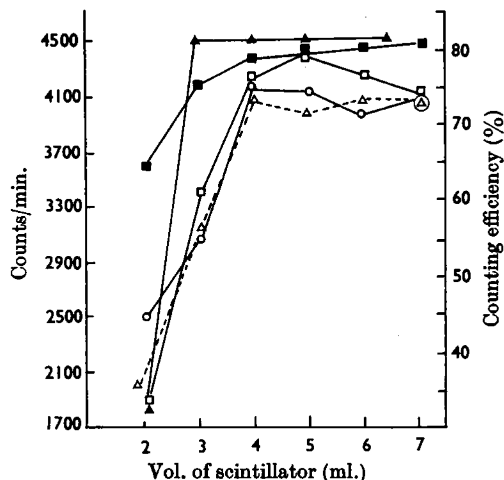

Fig. 2. Effect of scintillator volume on counting efficiency for various scintillation systems. Samples (0·1 ml.) of ^{14}C-labelled protein hydrolysate solution containing 2·5 μmc (5550 disintegrations/min.) were added to the scintillator. ○, 2·5 μmc in water was added to toluene–ethanol (7:3, v/v) scintillator; △, 2·5 μmc in water was added to toluene–ethanol (7:3, v/v) scintillator and 0·1 ml. of formic acid; □, 2·5 μmc in 0·5 N-NaOH was added to toluene–ethanol (7:3, v/v) scintillator and 0·1 ml. of formic acid; ▲, 2·5 μmc in 0·5 N-NaOH was added to toluene–2-ethoxyethanol (7:3, v/v) scintillator and 0·1 ml. of formic acid; ■, 2·5 μmc in 95% formic acid was added to toluene–ethanol (24:1, v/v) scintillator.

likely to be used (≡1 ml. of 2·3 N alkaline solution). On addition of an alkaline sample to the acidified scintillator a whitish blob may form, but this dissolves immediately on swirling. No spurious counts have then been noted, in contrast with the effect when the alkaline solution was not acidified (Fig. 1).

Fig. 2 compares the effect of scintillator volume on counting efficiency for 0·1 ml. samples of acidic, neutral and alkaline solutions and also demonstrates that material in all three systems may be counted with similar efficiencies. For samples dissolved directly in formic acid only 4% ethanol or 2-ethoxyethanol is required for blending, and the efficiency is slightly higher. Little change in efficiency occurs between 4 and 6 ml. of scintillator volume. There is only a slight rise in background count as the scintillator volume is increased from 2 to 7 ml. Radioactive alanine of activity varying between 40 μmc and 40 $\mu\mu$c was counted as 0·1 ml. samples in 5 ml. of toluene–2-ethoxyethanol scintillator at a mean efficiency of 82%.

When the count rate of a sample is very low it is advantageous to take a larger portion, although this is often not possible in biosynthetic reactions.

Table 4. *Effect of sample volume on counting efficiency for a range of scintillation systems*

All figures are for samples containing 0·25 μmc of ^{14}C-labelled protein hydrolysate (555 disintegrations/min.) added to 5 ml. of scintillator. Abbreviations, see Table 1.

Scintillator...	Toluene–ethanol (7:3, v/v) PPO POPOP		Toluene–ethanol (7:3, v/v) PPO POPOP*		Toluene–ethanol (7:3, v/v) PPO POPOP		Toluene–2-ethoxyethanol (7:3, v/v) PPO POPOP		Toluene–2-ethoxyethanol (7:3, v/v) BPD POPOP	
Sample in...	95% Formic acid		0·5 N-Sodium hydroxide		Water		Water		Water	
Vol. of sample (ml.)	Total amount of ethanol added to maintain one phase (ml.)	Efficiency (%)	Total amount of ethanol added to maintain one phase (ml.)	Efficiency (%)	Total amount of ethanol added to maintain one phase (ml.)	Efficiency (%)	Total amount of 2-ethoxyethanol added to maintain one phase (ml.)	Efficiency (%)	Total amount of 2-ethoxyethanol added to maintain one phase (ml.)	Efficiency (%)
0·1	—	86·1	—	80·7	—	78·6	—	81·5	—	84·2
0·2	—	82·5	0·8	76·0	0·5	77·3	0·2	79·6	0·3	80·7
0·3	—	73·0	1·5	72·3	1·0	74·3	0·6	76·6	0·8	78·5
0·4	—	69·2	2·0	69·0	1·5	67·6	0·9	76·8	1·0	74·5
0·5	—	64·0	2·5	64·5	2·0	61·8	1·2	70·9	1·5	71·7
0·6	—	59·7	3·0	57·5	2·5	61·1	1·6	70·0	1·8	71·8
0·7	—	57·7	3·5	55·7	3·0	53·8	1·9	64·6	2·0	71·7
0·8	—	51·2	4·0	48·4	3·5	47·3	2·1	63·4	2·3	70·9
0·9	—	48·8	4·5	46·5	—	—	—	—	2·5	67·9
1·0	—	44·9	5·5	42·4	4·3	40·5	2·5	62·2	2·6	65·4

* Formic acid (0·1 ml.) was added per 5 ml. of scintillator.

Table 5. *Effect of trichloroacetic acid-precipitated protein on counting efficiency*

The count rate on a vessel containing scintillator plus 0·1 ml. of formic acid, or 0·1 ml. of formic acid solution containing 2·1 mg. of protein/ml., was taken. A 0·1 ml. portion of ^{14}C-labelled protein hydrolysate (2·5 μmc, 5550 disintegrations/min.) was added and the new count rate determined.

Scintillator	Vol. (ml.)	Protein (μg.)	Radioactivity before addition of active material (counts/min.)	Radioactivity after addition of active material (counts/min.)	Net (counts/min.)	Efficiency (%)
Toluene–ethanol	5	—	143·4	4688	4545	81·9
Toluene–ethanol	5	210	149·2	4835	4686	84·4
Toluene–2-ethoxyethanol	4	—	173·5	4698	4525	81·5
Toluene–2-ethoxyethanol	4	210	171·6	4748	4576	82·5

For aqueous samples larger than 0·1 ml., extra blender must be used to produce a one-phase system, and in consequence there is some decrease in efficiency (Table 4). Even with samples in formic acid, where no additional blender is required, a drop in efficiency is noted.

As in the systems reported by Brown & Badman (1961), a marked quenching occurred with some common organic solvents. Acetone (0·5 ml.) caused a drop of 64%, chloroform (0·5 ml.) a drop of 40%, and ether (0·5 ml.) caused a drop of only 1·8% in the counting efficiency. No quenching has, however, been found when the sample was counted in the presence of protein prepared by trichloroacetic acid precipitation and washed by the method of Siekevitz (1952), as is indicated in Table 5.

Table 6. *Effect of light on the background count rate*

Scintillations were induced by exposing a counting vessel, a pipette containing scintillator or vessel containing scintillator to light. When the scintillator was exposed in a pipette it was transferred to a vessel which had been kept under 'dark' conditions. In each case the vessel was quickly placed in the scintillator counter after exposure, and the decay of light-induced scintillations was followed by consecutive counts. The toluene–ethanol scintillator (5ml.) was used. All figures include the thermal noise background count rate of 108 counts/min.

Time after exposure	Vessel only exposed to light (counts/min.)	Scintillator only exposed to light (counts/min.)	Vessel + scintillator exposed to light (counts/min.)
(a) Exposed to artificial light (1500ft.candles) for 3min.			
(min.)			
1	3862	654	4197
2	592	395	1608
3	362	281	903
4	254	249	792
5	203	214	644
6	207	213	523
7	189	229	418
8	173	178	381
9	162	202	372
10	144	188	362
(b) Exposed to daylight (200ft.candles) for 3min.			
(sec.)			
20	336	465	705
40	228	435	456
60	234	417	450
80	180	336	330
100	174	318	372
120	165	318	360
180	156	276	291
240	—	246	222

Light-induced scintillations. Light-induced scintillation has been noted by Davidson (1958), and scintillations were found to occur as a result of exposure to light of both the counting vessels and the scintillator. The effects appeared to be approximately additive (Table 6) and the severity of induced scintillation was related to the intensity of the light. The effect of intense light on scintillator containing a radioactive sample was also studied. The induced scintillations fell off very rapidly at first, but after about 10min. the decrease was small and often obscured by variations in the count rate, and usually reached a steady state some 60min. after exposure.

Accurate determination of specific activity of ^{14}C*-labelled protein.* The radioactive protein is dissolved in either formic acid or in sodium hydroxide. If formic acid is used it should be noted that polysaccharides (such as starch) are very readily soluble (Gregory & Cocking, 1965) and, especially in radioactive experiments involving green plant material, small amounts may have a high specific activity.

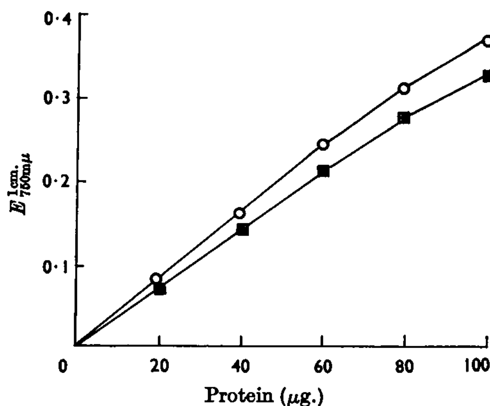

Fig. 3. Comparison of colour production for different proteins in the Lowry method. The maximum amount of protein used was 100μg. ●, Egg albumin in water; ○, bovine albumin in water; □, tomato-leaf protein in 0·1N-NaOH. Experimental details are given in the text.

Many proteins will dissolve in dilute alkaline solutions and in certain instances up to 0·5N-sodium hydroxide may be used. A 0·1 ml. sample is added to the toluene–ethanol or toluene–2-ethoxyethanol scintillator containing 0·1 ml. of formic acid, as previously described, and the mixture counted. A further portion, in the same pipette, is made to 1 ml. with water, or, if necessary, to 1 ml. with 0·5N-sodium hydroxide.

Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951). A stable copper solution may be prepared by the addition of 1 g. of sodium D-tartrate in aqueous solution to an aqueous solution of 0·5g. of copper sulphate pentahydrate, adding 10ml. of N-sodium hydroxide and finally making up to 100ml. Sodium citrate may be used instead of sodium D-tartrate (Eggstein & Kreutz, 1955), in which case no sodium hydroxide is required to maintain a stable copper solution.

Very close agreement has been attained in colour production with solutions containing similar concentrations of bovine albumin, egg albumin and tomato-leaf protein prepared by trichloroacetic acid precipitation (Fig. 3). The final strength of the sodium hydroxide in the protein solution will affect the colour production, but the effect is reproducible, and can be compared with a standard curve. Usually the final concentration is 0·1N-sodium hydroxide or less, and at this concentration there is only a small decrease in the colour produc-

tion compared with an aqueous solution (Fig. 4). The specific activity as counts/min./μg. of protein may then be directly calculated (Table 7). In experiments in which only one labelled amino acid is employed the number of μmoles of this amino acid incorporated into protein can be accurately determined provided that the counting efficiency is known.

For solutions of protein in formic acid the procedure is essentially as before, except that the scintillator need not contain formic acid. The portion of protein solution for spectrophotometric estimation is neutralized with 5vol. of 4·12N-sodium hydroxide (Smit & Stocken, 1964). Occasionally precipitation due to high ion concentration may be encountered on addition of the Folin copper reagent.

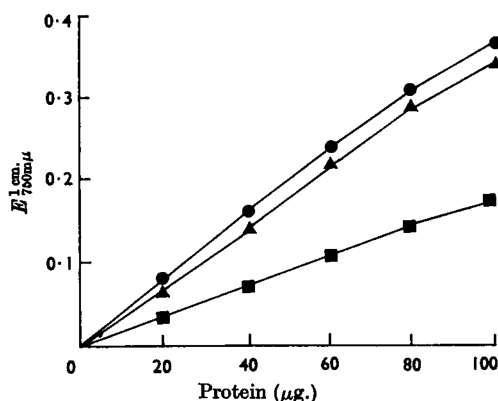

Fig. 4. Effect of alkalinity of protein sample on colour production in the Lowry method. Egg albumin was dissolved in ●, water, ▲, 0·1N-NaOH, and ■, 0·5N-NaOH, and diluted with appropriate solution to give final concentrations between 20 and 100μg./ml. Portions (1ml.) were taken for estimation as described in the text.

DISCUSSION

Several scintillation systems were developed which gave high efficiencies for the counting of aqueous samples and which were much less sensitive to sample volume than the NE 213–Hyamine 10X system. Moreover, the counting efficiency was not so critically dependent on the volume of ethanol or 2-ethoxyethanol added to give a one-phase system. Table 4 summarizes the relative merit of each of these systems, and it may be noted that for small volumes (0·1 ml.) there is only a very slight difference in efficiency between each system used, and for such quantities individual preference would dictate the system to be used. Ethanol is readily available and is more pleasant to use than 2-ethoxyethanol, which has the disadvantage that it may form dangerous peroxides. For sample volumes greater than 0·5ml., however, 2-ethoxyethanol shows distinct advantages in that a smaller volume is required to maintain a one-phase system, and also in giving 10–20% higher efficiency than ethanol for such samples.

The scintillation solution containing biphenylylphenyloxadiazole in place of 2,5-diphenyloxazole as the primary solute gives the highest counting efficiency attained, being some 3–7% better than in otherwise comparable scintillation mixtures. For a non-aqueous sample of ^{14}C the former mixture gave 93% efficiency in our equipment (Table 1), whereas with 2-ethoxyethanol as blender, 0·1 ml. portions of aqueous solutions containing ^{14}C, ^{35}S and ^{3}H have been counted at efficiencies of 84, 72 and 5% respectively, under the same voltage and temperature conditions. However, the high concentration of biphenylylphenyloxadiazole required (10g./l. compared with 4g. of 2,5-diphenyloxazole/l.) resulted in a rather viscous solution which had a strong tendency to creep from the counting vessel, leaving a white film of solutes when the solvents evaporated. Biphenylylphenyloxadiazole is

Table 7. *Determination of protein specific activity*

Tomato seedlings (12-day-old) were grown under aseptic conditions for 5 days on an inorganic medium containing ^{14}C-labelled protein hydrolysate. The leaves were cut off, ground in a pestle and mortar and protein was precipitated with trichloroacetic acid and washed by a standard procedure (Siekevitz, 1952). After the final ether wash the protein was dissolved in 1ml. of 0·5N-NaOH. A portion (0·01ml.) of the alkaline protein solution was used for the determination of radioactivity by the procedure described in the text by adding it to 5ml. of toluene–ethanol scintillator containing 0·1ml. of formic acid. A further portion (0·01ml.) was diluted to 1·0ml. and the protein content estimated colorimetrically. The presence of radioactivity in the constituent amino acids of the protein was confirmed by hydrolysis of the protein followed by chromatography and radioautography.

Sample volume (ml.)	Background (counts/min.)	Sample (counts/min.)	Net count	$E^{1\,cm.}_{750m\mu}$	Protein (μg.)	Specific activity (counts/min./μg. of protein)
0·01	173·5	1030·8	857·3	0·180	46	18·64

also more expensive than 2,5-diphenyloxazole and for these reasons it is only really of advantage when the lowest activities are to be determined.

Naphthalene has been recommended as an intermediate solvent (Kallmann & Furst, 1958), and has in certain cases been observed to reduce quenching (Dobbs, 1963). The addition of 8% naphthalene to the scintillation mixture was not found, however, to give any enhancement of efficiency in this instance (Table 1), nor was there any reduction in the quenching observed when the sample volume was increased. Formic acid was found to be very convenient for acidifying samples without causing precipitation or a significant drop in counting efficiency. Alkaline solutions if not acidified give spurious counts and formic acid was therefore added when alkaline aqueous samples were to be counted.

The use of a routine counting procedure involving the determination of the background count rate before the addition of the radioactive sample to the counting vessel containing the scintillator allows very accurate counting of low activities. The standard washing procedure gives a very reliable removal of radioactivity from the glassware, there being no carry-over from one experiment to the next, and thus the background count rate remains virtually constant for all counting vessels.

The Panax scintillation counter used incorporated a single photomultiplier tube which was specially selected for low thermal noise. The thermal noise was further decreased by maintaining the counting 'castle' in a refrigerator at 1–4°. For the accurate determination of the specific activity of labelled proteins dissolved in alkali the investigation of the counting of alkaline samples at high efficiency was coupled with an investigation of the effect of the concentration of alkali on the sensitivity of the Lowry colorimetric method for the estimation of proteins. The use of portions of this alkaline solution for the determination of radioactivity and for the estimation of protein has provided an accurate method for the determination of low specific activities.

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