Incubation of NAD(P)H_2: glutathione oxidoreductase (*EC* 1.6.4.2) with flavin adenine dinucleotide for maximal stimulation in the measurement of riboflavin status

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1. Some modifications to the erythrocyte glutathione reductase assay for riboflavin status are described.
2. Cusum analysis of results collected on a quality-control (QC) haemolysate, analysed separately at the beginning and end of each batch of samples over a period of 20 weeks, suggested that the activation coefficient (AC) was higher at the end of a batch than at the beginning.
3. The higher AC was due to higher FAD-stimulated enzyme activities of the QC samples measured at the end of the day, by comparison with the beginning, and this suggested that the conditions of assay were not optimal.
4. The conditions required to achieve maximal coupling of FAD to glutathione reductase (NAD(P)H_2: glutathione oxidoreductase; *EC* 1.6.4.2) were therefore examined and found to be 15 min at 35° by comparison with the 5–7 min incubation used by most workers.
5. Alternatively, where samples are prepared in batches, the enzyme and FAD should be pre-incubated in the reaction mixture for 2 h at 4° or 1 h at 25° before the standard incubation of 5 min at 35°.
6. Additionally, the use of cummulative sum (cusum) analysis on the QC results suggested that there was a slight deterioration of QC sample after 4-weeks storage. However, the QC results obtained, remained within 2 standard deviations of initial results over a 20-week period, suggesting that the deterioration was very slight.

The use of erythrocyte glutathione reductase (NAD(P)H_2:glutathione oxidoreductase; *EC* 1.6.4.2; EGR) to assess riboflavin status is now well established. Many workers have used the method described by Glatzle *et al.* (1970) but modifications of this or other methods have also been proposed (Nichoalds & Lawrence, 1974; Bayoumi & Rosalki, 1976). Our own method is based on that of Glatzle *et al.* (1970) and although the method has proved highly satisfactory, participation in collaborative experiments with workers in other laboratories has resulted in consistent differences, the causes of which are elusive.

Some studies were recently undertaken to examine the riboflavin status of Chinese subjects collected in an area of high risk for oesophageal cancer. Problems in the transport of these samples to Europe necessitated our handling the samples with great care and this meant that low temperatures were used in case of instability up to the time of assay. Cumulative sum (cusum) analysis of quality control (QC) samples assayed over the same period and using the same conditions showed that control results obtained at the end of the day were consistently different from those at the beginning. The results suggested that the binding of FAD to the EGR was not always optimal.

This paper describes experiments which examined the influence of time and temperature at the preparative stages of the assay on the subsequent measurement of riboflavin status. The results are discussed in relation to conditions used by other workers.

EXPERIMENTAL

Subjects

Blood used in the study was obtained from apparently healthy volunteers in the Department (control samples) or from Chinese subjects collected in Linxian County, Chinese Peoples

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Republic in a collaborative study involving the International Agency for Research on Cancer, Lyon and the Chinese Academy of Medical Sciences. Studies on the riboflavin status of the Chinese subjects will be described elsewhere.

Assay for riboflavin status

The method used is that described by the Department of Health and Social Security (1979) except for the concentration of FAD. Haemolysate (0·1 ml of 1:20 dilution) was mixed with 0·1 M-potassium phosphate buffer, pH 7·4, 2·3 mM-potassium EDTA, 0·889 mM-oxidized glutathione (GSSG) with or without 3 μM-FAD, in a total volume of 3·6 ml. Haemolysate samples (twenty to thirty) were set up in duplicate in the reagents mentioned previously in batches and maintained at 4–10° until assayed. Samples were then incubated at 35° for 5 min before the addition of 80 μM-NAD(P)H_2 to start the reaction. Where variations in these basic conditions occur, they are indicated in the text. An activation coefficient (AC) was calculated from the ratio, EGR activity plus FAD: activity measured in the absence of FAD. Values of 1·30 or more are regarded as indicative of riboflavin deficiency (Department of Health and Social Security, 1979).

Preparation of haemolysate

Venous blood was centrifuged, the plasma removed and the buffy layer on the surface of the erythrocytes (RBC) discarded. Remaining RBC were then stirred gently with a glass rod and a haemolysate prepared, by mixing 1 ml RBC with 1 ml water, for transport or storage or both at −20° or below. Before use, haemolysates were diluted a further ten times with water to a final dilution of 1:20.

QC haemolysate

RBC were prepared as described previously but mixed with water to give a 1:20 haemolysate immediately. Portions (1 ml) were dispensed into small plastic tubes which were capped and stored at −20° until required.

RESULTS

Intrabatch variation, obtained by measuring twenty samples of the QC haemolysate in duplicate, gave a coefficient of variation (CV) for the AC of 5%. Means ($\pm$SD) of the unstimulated and FAD-stimulated EGR activities and AC of the QC were $7{\cdot}78 \pm 0{\cdot}42$, $8{\cdot}66 \pm 0{\cdot}67$ i.u./g haemoglobin and $1{\cdot}11 \pm 0{\cdot}06$ respectively. Use of QC haemolysate samples at the beginning and end of all batches analysed over a 20-week period, gave results which lay within 2 SD of the mean for all but one sample (Rathakette, 1981). However, cusum analysis of this data showed clear differences between results of the two QC samples analysed each day.

Figs. 1 and 2 show cusum plots of the AC and EGR activities of the QC haemolysates obtained irregularly over a period of 4 months. The reference or zero line represents the mean value obtained when intrabatch variation was measured.

The cusum plot represents the cumulative sum of the differences from the reference point. When the method is under control then the difference from the reference value will be fairly constant and the plot will maintain a steady slope which may rise, fall or be horizontal. When a change occurs in the QC, the average difference from the reference point will change and the slope of the cusum plot will change direction.

Fig. 1 provides evidence of two aspects concerning the QC haemolysate. In the initial phase of the plot, the QC analysed at the end of the batch had a higher AC than the reference thus the cumulative sum of the differences caused the slope of the plot to rise. In contrast, the QC analysed at the beginning of the batch tended to be the same as the reference and

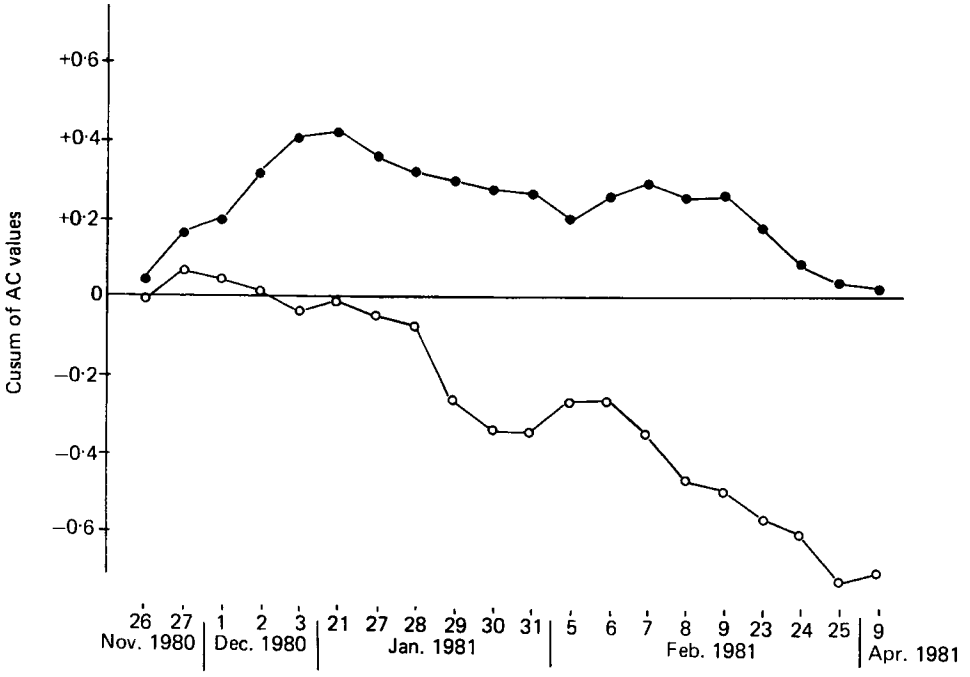

Fig. 1. Cumulative sum (cusum) plots for the activation coefficients (AC) of quality-control (QC) haemolysates measured at the beginning (○—○) and end (●—●) of each batch of samples over a period of 20 weeks. The period of time which the two QC samples stood at 4–10° before analysis on each day was variable and not recorded. The first (○—○) is unlikely to have stood for less than 1 h while the second (●—●) may have stood for 3–4 h. The zero horizontal line represents an AC of 1·11. Test and QC samples were set up as described on p. 460.

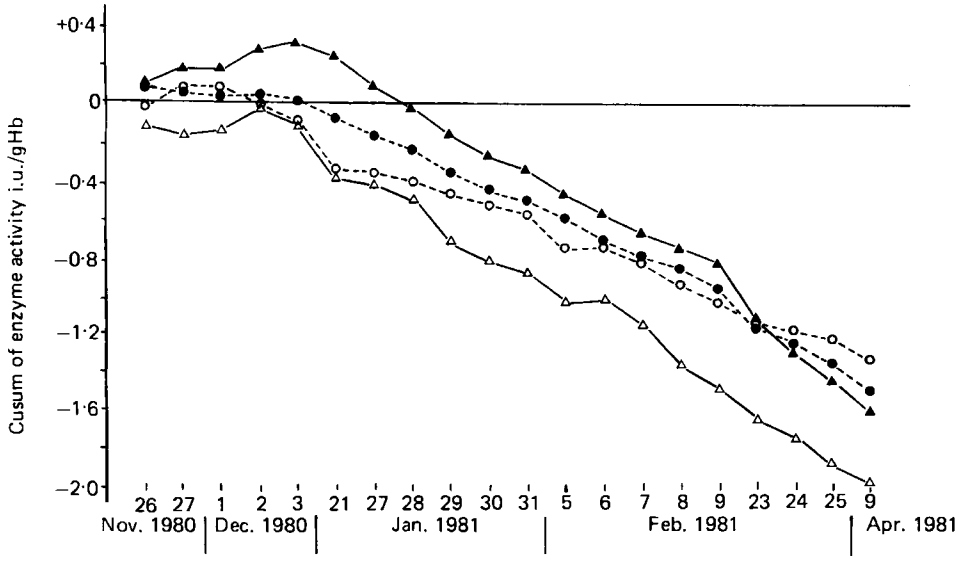

Fig. 2. Cumulative sum (cusum) plots of glutathione reductase (NAD(P)H_2: glutathione oxidoreductase; *EC* 1.6.4.2; EGR) activities of quality-control (QC) haemolysates measured with (△, ▲) and without (○, ●) FAD at the beginning (△, ○) and end (▲, ●) of each batch of samples over a period of 20 weeks. Samples were set up as described on p. 460 and stood approximately 1 h (△, ○) or 3–4 h (▲, ●) at 4–10° before assay. The zero horizontal line for FAD-stimulated enzyme activity was 8·66 and unstimulated activity 7·78 μmol NAD(P)H_2 oxidized/min (i.u.) per g haemoglobin (Hb).

Table 1. *Influence of temperature and period of time standing on the measurement of the activation coefficient*

Sample	Temperature	Period of time standing (h)					
		0	0·5	1	2	3	4
L5	25	1·61	1·82	1·85	1·87	1·94	1·87
		1·53	1·83	1·83	1·89	1·85	1·86
	4	1·63	1·65	1·58	1·88	1·79	1·83
		1·64	1·59	1·58	1·80	1·81	1·82
L32	25	1·39	1·54	1·53	1·64	1·67	1·67
		1·39	1·54	1·64	1·65	1·74	1·71
	4	1·31	1·39	1·43	1·41	1·58	1·52
		1·32	1·38	1·43	1·49	1·49	1·51
L10	25	1·19	1·18	1·30	1·25	1·22	1·26
		1·27	1·30	1·33	1·26	1·30	1·26
	4	1·14	1·15	1·23	1·24	1·27	1·22
		1·20	1·18	1·20	1·25	1·26	1·22
L35	25	1·52	1·76	1·78	1·82	1·74	1·87
		1·63	1·73	1·86	1·84	1·78	1·74
	4	1·58	1·47	1·49	1·63	1·70	1·67
		1·53	1·49	1·53	1·67	1·72	1·66
L24	25	1·59	1·84	1·91	2·0	1·91	2·03
		1·68	1·75	1·87	2·08	1·96	2·06
	4	1·49	1·56	1·51	1·68	1·52	1·63
		1·57	1·56	1·55	1·68	1·60	1·65
Control	25	1·20	1·21	1·23	1·22	1·26	1·26
		1·25	1·19	1·17	1·23	1·25	1·25
	4	1·15	1·16	1·15	1·13	1·17	1·12
		1·16	1·13	1·14	1·19	1·15	1·15

Activation coefficients (AC) were measured in two experiments on six haemolysate samples after samples had been mixed with reagents and stood for intervals shown at 4–10° (Expt 1) or 25° (Expt 2) before pre-incubation at 35° for 5 min. $NAD(P)H_2$ was then added to start the reaction as described on p. 460. AC values obtained following a standing time of 2 h or more for respective samples at 4–10° ($P = 0{\cdot}91$) or 1 or 2 h or more at 25° ($P = 0{\cdot}0505$, $0{\cdot}49$) were not significantly different (F tests). When results from samples stood for 2 h or more at both temperatures were combined, however, there was a significant difference ($P < 0{\cdot}001$, F test).

hence the plot remained horizontal. At the time of the 6th or 7th observation, however, the slope of both plots changed in a more downward direction. That is the AC recorded at both the beginning and end of a batch of samples tended to give lower results than those initially recorded. This change in slope occurred after QC haemolysates had been stored for approximately 4 weeks, suggesting that there may have been some deterioration in the sample at this point. However, throughout the entire study, the QC AC recorded at the end of a batch tended to be greater than that at the beginning. In a similar manner the FAD-stimulated EGR activity of the QC haemolysates at the end of a batch was also frequently greater that that at the beginning, whereas there was no difference between the unstimulated enzyme activities.

These results suggested that conditions used for assay were not optimal at the beginning of the day. The fact that basic activities were similar at the start and end of the day suggested that the temperature of assay was identical throughout the day. Therefore, it seemed as if samples analysed early in the day had received an inadequate exposure to FAD before measurement of EGR activity.

Table 1 shows the results of two experiments. The first was set up to investigate the influence of standing the prepared haemolysates in reagents at 4–10° before the 5 min period

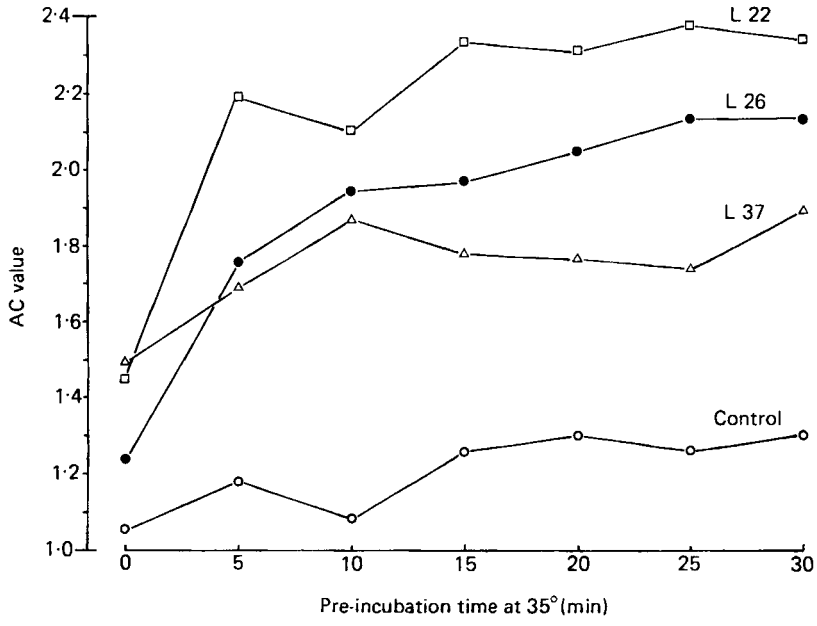

Fig. 3. Mean activation coefficients (AC) of four haemolysate samples which, after addition of reagents (p. 460), were immediately pre-incubated at 35° for the intervals shown. Reaction was initiated using $NAD(P)H_2$ as described on p. 460.

of 35° temperature equilibration. For five of the six samples there was a direct relationship between the period of standing at 4–10° and the AC. The latter tended to plateau in samples stood for more than 2 h and analysis of variance confirmed that there was no significant change in AC of samples stood for 2 h or more at 4–10°.

The second experiment shows the results obtained when the same samples were stood at room temperature (25°) before the 35° temperature equilibration. Similar results were obtained and the F test again suggested that samples stood for 2 h achieved a maximum EGR-AC. However, closer examination of the 25° experiment (Table 1) shows that only one sample, L32, showed a marked increase in AC after 60 min standing at 25°. In fact AC values of samples stood for 60 min or more at 25° were not significantly different ($P > 0{\cdot}05$). (F test). Thus it would appear that samples stood at room temperature achieve optimal binding between FAD and EGR more quickly than those stood at 4–10°.

A further analysis of variance on the combined 2, 3 and 4 h results in Table 1 suggested that there were highly significant differences between results obtained under the different conditions ($P < 0{\cdot}001$). A closer examination of results in Table 1 does indicate that three of the samples (L24, L32 and L10) achieved a higher plateau AC when stood at 25° before the incubation. The experiment shown in Fig. 3 was done, therefore, to re-examine the period of time required for samples to be exposed to 35° to attain a maximum AC in the absence of any waiting period at 4–10° or room temperature post-mixing of haemolysate and reagents. The results suggest that at least 15 min incubation at 35° is required to achieve the maximum AC. Analysis of variance confirmed that there was no significant difference between results obtained from those samples incubated for 15 min or more at 35°.

Unfortunately, there was insufficient sample to use the same one in all the three experiments described previously. A final experiment therefore was prepared in which six haemolysates were selected to cover a wide range of AC values. Each was mixed with

Table 2. *Comparison of activation coefficients obtained using three optimized methods of sample preparation*

Sample no.	Treatment*†					
	1		2		3	
7	1·55	1·55	1·60	1·54	1·56	1·57
8	1·92	1·95	1·72	1·87	1·99	2·01
34	1·43	1·43	1·44	1·46	1·40	1·32
51	1·43	1·40	1·40	1·41	1·49	1·48
55	1·22	1·23	1·28	1·26	1·29	1·34
81	1·50	1·46	1·50	1·41	1·42	1·56

* Haemolysates were diluted with reagents and treated as follows prior to assay:
1. Incubated immediately at 35° for 15 min.
2. Stood at 4–10° for 2 h then incubated at 35° for 5 min.
3. Stood at 25° for 2 h then incubated at 35° for 5 min.

† Results obtained were not significantly different (F test; $P = 0{\cdot}058$).

reagents and exposed to the optimal conditions described for the three different methods of preparation. Table 2 shows that the results obtained were the same for all three methods of sample preparation (F test, not significant $P > 0{\cdot}05$).

DISCUSSION

The method we use in our laboratory is based on the method described by Glatzle *et al.* (1970). These workers used a temperature of 35° for the measurement of enzyme activity and for consistency in our work, we have continued to use this temperature. Many others have used 37° (Beutler, 1969; Sauberlich *et al.* 1972; Nichoalds & Lawrence, 1974; Bayoumi & Rosalki, 1976). The higher temperature produces slightly higher enzyme activities, however, the AC is a ratio of the FAD-stimulated to unstimulated activity and not affected by the slight difference in temperature. It is assumed therefore, that the observations described in the results apply equally well to those measuring EGR at 35 and 37°.

The cusum technique is a useful method of detecting changes in the average level of accuracy (Woodward & Goldsmith, 1964). The change in slope after QC haemolysates had been stored for 4 weeks may indicate that EGR is not as stable as has been reported previously. However, most workers are only concerned with the AC and provided the ratio of FAD-stimulated to unstimulated activity remains unaltered, a small fall in enzyme activity is not important. In this study, of the QC AC values, whether measured at the beginning or end of each batch, only one result was outside the 2 SD from the intrabatch mean throughout the 20-week period of study.

However, separate cusum analysis of the results from the two QC haemolysates included with each batch of samples, suggested that the accuracy of the method was poorer at the start of each day. The fact that the QC sample analysed later in the day gave a higher AC suggested that the method being used gave improved accuracy after haemolysate samples had stood with the reagents for a longer period.

The pre-incubation of the assay sample at 35° (or 37°) is probably the most important time in the assay to ensure maximal binding between the enzyme and FAD. However, the results presented here suggest that the period preceeding this incubation is also important particularly where the pre-incubation at 35° is less than 15 min (for example, Glatzle *et al.* 1970; Sauberlich *et al.* 1972; Tillotson & Baker, 1972; and many others).

The function of the pre-incubation period at 35° is twofold; to raise the temperature of

the sample to that at which enzyme activity will be measured and to facilitate binding between the enzyme and FAD. The former will be influenced by the volume of the sample–reagent mixture which has been reported as low as 1·0 ml (Beutler, 1969), from 1·8 to 2·35 ml (Glatzle *et al.* 1970; Nichoalds & Lawrence, 1974) to our own conditions of 3·55 ml (Department of Health and Social Security, 1979). The period of time allowed for the pre-incubation varies from 5 min (Glatzle *et al.* 1970; Department of Health and Social Security, 1979), 8 min (Tillotson & Baker, 1972; Sauberlich *et al.* 1972; Nichoalds & Lawrence, 1974) to 30 min (Bayoumi & Rosalki, 1976). In this study, 5 min at 35° was sufficient to raise the temperature of a volume of 3·55 ml to 35° before the assay.

To achieve maximal stimulation of EGR by FAD, however, these experiments suggest that freshly prepared samples require a minimum of 15 min at 35°. Only two groups of workers report conditions which meet this requirement. Bayoumi & Rosalki (1976) pre-incubated for 30 min and Beutler (1969) reported a two-stage pre-incubation procedure following the addition of FAD and GSSG. In total, Beutler's (1969) samples were exposed to FAD for 20 min pre-incubation. Furthermore, Beutler reported that maximal FAD-stimulated enzyme activities were achieved with 1 μM-FAD which is in contrast to most other workers who use 8–10 μM-FAD. This latter point is worth mentioning for it may be that higher concentrations of FAD enable equilibrium to be reached more rapidly. However, the assay described by Bayoumi & Rosalki (1976) used a 30 min pre-incubation in spite of 10 μM-FAD and in fact other workers have reported that higher concentrations of FAD can have an adverse effect on EGR activity (Schorah & Messenger, 1975).

Most other workers use those methods which employ the 5 or 8 min pre-incubation. The usual time for measurement of enzyme activity in the manual assay is 5 min and presumably the similar pre-incubation time has arisen from convenience. The results shown in Tables 1 and 2 indicate, however, that a 5 min pre-incubation at 35° is only adequate when haemolysate and reagents have been in contact for a sufficient period before the pre-incubation. Where samples are set up and stored at low temperatures, the period of contact before pre-incubation must be at least 2 h. On the other hand, of samples prepared and stood with reagents at room temperature (25°), most had reached optimal binding conditions after 1 h (Table 1).

There is some evidence that a 2 h exposure at low temperatures of some samples was insufficient for maximal stimulation. An analysis of variance on results from samples stood for 2 h or more (Table 1) suggested that the effect of the two temperatures was significantly different ($P < 0{\cdot}001$). That is, some samples, particularly L24 and L32, gave higher results in the 25° experiment than in that at 4–10°. The effects of the two temperatures were measured on different days, therefore experimental error may have contributed to the differences. Sample deterioration was not a factor, however, since the 25° experiment followed the one at 4–10°. The differences may indicate that samples L24 and L32 are not stimulated optimally by FAD even when exposed for 4 h at temperatures of 4–10° and sample 8 in Table 2 may be another example. However, analysis of variance of results in Table 2 suggested that there were no differences between the results obtained. Thus, generally speaking 2 h at 4–10° is sufficient time for most samples to achieve optimal binding with FAD.

The practical significance of these findings is worth considering. In all experiments, samples were chosen with a wide range of AC values. Most of the AC values were much higher than those usually obtained from patients or survey subjects in the UK or other Western countries. The influence of exposure time of sample to FAD was greatest in those samples with the highest AC values (Table 1 and Fig. 3). In other words, the increased sensitivity to be gained by increasing exposure time of sample to FAD may only make a very small difference to AC values in or around the 'normal' range, i.e. that of a

well-nourished population 1·00–1·30. However, as can be seen from sample L10 (Table 1) and the control sample (Fig. 3), slightly altered conditions of pre-incubation will alter the AC even of 'normal' samples. Where comparing results between laboratories, attention to these details is very important.

Obviously, for the assay of a single, freshly-prepared sample, the use of a 15 min pre-incubation at 35° will produce the maximal AC. When large numbers of samples are being analysed, a lengthy pre-incubation is not so easily organized in the manual assay system, particularly when the length of the assay itself is not usually greater than 5 min. The experiments reported here will enable investigators to set up samples in batches and, after a 2 h standing period, use a pre-incubation period of 5 min at 35° to obtain maximum AC values.

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REFERENCES

Bayoumi, R. A. & Rosalki, S. B. (1976). *Clin. Chem.* **22**, 327.
Beutler, E. (1969). *J. clin. Invest.* **48**, 1957.
Department of Health and Social Security (1979). *Rep. Hlth Soc. Subj.* no. 16, 191.
Glatzle, D., Körner, W. F., Christeller, S. & Wiss, O. (1970). *Int. Z. Vitaminforsch.* **40**, 166.
Nichoalds, G. E. & Lawrence, J. D. (1974). *Clin. Chem.* **20**, 624.
Rathakette, P. (1981). Riboflavin status of subjects with oesophagitis in a high risk area of oesophageal cancer. MSc Project Report, University of London.
Sauberlich, H. E., Judd, J. H. Jr, Nichoalds, G. E., Broquist, M. P. & Darby, W. J. (1972). *Am. J. clin. Nutr.* **25**, 756.
Schorah, C. J. & Messenger, D. (1975). *Int. Z. Vitaminforsch.* **45**, 39.
Tillotson, J. A. & Baker, E. M. (1972). *Am. J. clin. Nutr.* **25**, 425.
Woodward, R. H. & Goldsmith, P. L. (1964). *Cumulative Sum Techniques*, 1st ed., ICI Monogr. no. 3. Edinburgh: Oliver & Boyd.

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