where $[N_0]$ is the initial concentration of N. The time dependence of [R] is different in the 2 proposed mechanisms. In the case (a) it is:

$$[R]=\frac{k_1[N_0]}{k_2-k_1}(e^{-k_1t}-e^{-k_2t}) \quad (d)$$

In the case (b) it can be obtained by integrating the following differential equation:

$$\frac{d[R]}{dt}=k_1[N_0]e^{-k_1t}-k_2'[R]^2 \quad (e)$$

which was solved by Chien[8], as follows:

$$[R]=[N_0]\sqrt{\frac{\tau}{\kappa}}\frac{iJ_1(2i\sqrt{\kappa\tau})-\beta H_1^{(1)}(2i\sqrt{\kappa\tau})}{J_0(2i\sqrt{\kappa\tau})+\beta iH_0^{(1)}(2i\sqrt{\kappa\tau})}, \quad (f)$$

where, $\tau=e^{-k_1t}$; $\kappa=[N_0]k_2'/k_1$; $\beta=\{iJ_1(2i\sqrt{\kappa})\}/\{H_1^{(1)}(2i\sqrt{\kappa})\}$; $J_0(ix)$, $-iJ_1(ix)$, $-H_1^{(1)}(ix)$ and $iH_0^{(1)}(ix)$ are Bessel functions whose values are tabulated[9].

On the basis of the time-course for the R-form reported in figure 3, the model (b) seems to be the operative one. Actually in the last part of the curve, when the N-form is practically lacking, a linear relationship between 1/[R] and time exists, that is characteristic of 2nd order kinetics. Moreover experimental data fit fairly well with equation (f), derived from the uni-bimolecular model (figure 3, solid line), while the best curve calculated according to the uni-unimolecular model (d) does not fit to the experimental data (figure 3, dashed line). Therefore, the denaturation of yeast glucose-6-phosphate dehydrogenase by saturated fatty acids apparently involves a 2-step mechanism and consequently the existence of an intermediate enzyme form (R), inactive but reactivable in a suitable medium. R molecules aggregates with each other leading to an irreversibly denaturated form of the enzyme. In the present paper the existence of such aggregates is indirectly supported by the 2nd order kinetics of the disappearance of the R-form. Actually the best fit to the experimental data was obtained with a uni-bimolecular model. On the other hand the existence of high molecular weight products was previously reported following the inactivation of the enzyme by fatty acids[2]. These aggregates presumably correspond to the products of interaction of the R-form.

For another multimeric enzyme also, i.e. swine heart fumarase, the role of the chemical composition of the reactivation medium as far as the interconversion of enzymatic forms is concerned has been pointed out[10]. Moreover, preliminary data indicate that for yeast glucose-6-phosphate dehydrogenase, as well as for fumarase, the denatured form(s) are more susceptible to specific proteolytic attack[11].

Work is in progress in our laboratory on this topic, in order to elucidate the mechanistic aspects of selective enzyme denaturation, as well as its possible physiological significance.

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2 P. Tortora, G.M. Hanozet, A. Guerritore, M.T. Vincenzini and P. Vanni, Biochim. biophys. Acta *525*, 297 (1978).
3 R.H. Yue, E.A. Noltmann and S.A. Kuby, Biochemistry *6*, 1174 (1967).
4 R.H. Yue, E.A. Noltmann and S.A. Kuby, J. biol. Chem. *244*, 1353 (1969).
5 Th. Bücher, W. Luh and D. Pette in: Handbuch der Physiologisch- und Pathologisch-Chemischen Analyse, vol. 6/A, p. 292. Eds F. Hoppe-Seyler and H. Thierfelder. Springer, Heidelberg 1964.
6 Y. Hatefi and W.G. Hanstein, Proc. natl. Acad. Sci. USA *62*, 1129 (1969).
7 P.H. von Hippel and T. Schleich, in: Structure and Stability of Biological Macromolecules, p. 417. Eds S.N. Timasheff and G.D. Fasman. Marcel Dekker Inc., New York 1969.
8 J.Y. Chien, J. Am. chem. Soc. *70*, 2256 (1948).
9 E. Jahnke and F. Emde, Table of Functions. Dover Publications, New York 1943.
10 S. Yamato and T. Murachi, Eur. J. Biochem. *93*, 189 (1979).
11 N. Burlini, P. Tortora, G.M. Hanozet and A. Guerritore, Proc. 26th Congr. Soc. ital. Biochim. Bologna 1980, p. 60.

Measurement of polyethylene glycol 4000: Effect of storage and freeze thawing in biological fluids

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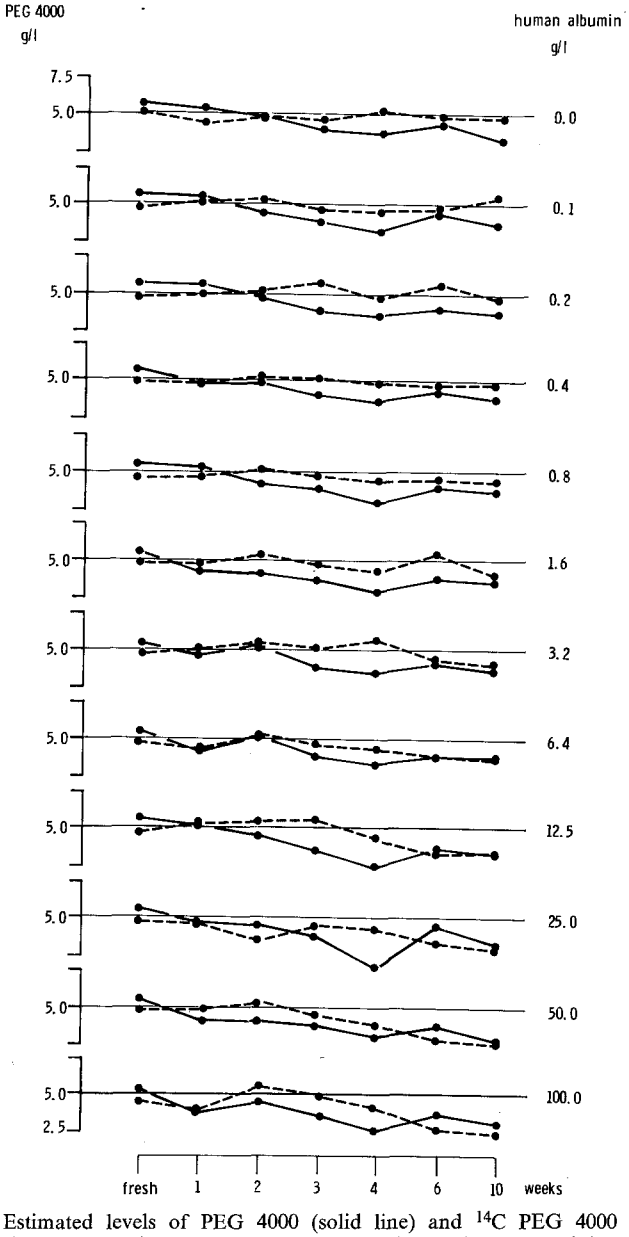
Summary. The effects of storage and freeze-thawing on polyethylene glycol 4000 (PEG 4000) and ^{14}C PEG 4000 in a solution of NaCl (150 mmoles/l) containing varying amounts of human albumin were studied. Results showed that the analysis of both PEG 4000 and ^{14}C PEG 4000 is likely to be inaccurate in these fluids if the specimens have been freeze-stored, thawed and refrozen several times during a period of several weeks. This seems to be due to the freeze-thawing process itself rather than the actual storage. The amount of protein in the samples may increase the fall in estimated levels of polyethylene glycol observed.

During the past 3 decades polyethylene glycol of mol.wt 4000 (PEG 4000) has been used extensively in medical research as a nonabsorbable marker for estimating recovery and calculating fluid volumes in intestinal perfusion studies[1-3]. PEG 4000 remains chemically unchanged in the intestinal lumen and is nontoxic[4]. In intestinal perfusion studies PEG 4000 has been used both in a radiolabeled form (labeled with ^{14}C or ^{3}H)[5,6] and an unlabeled form. Unlabeled PEG 4000 has been measured by the turbidimetric method of Hydén[7]. Doubts have been raised, however, as to whether the chemical analysis of PEG 4000 is reliable when performed on thawed samples which have been stored frozen (−20 °C) for a variable period of time[8,9]. It is known that freeze-thawing may cause precipitation of proteins[10] and that PEG may bind to proteins in biological fluids and cause precipitation of proteins itself[11,12]. Freeze-thawing of biological samples might enhance this process and cause loss of PEG from the fluid.

We have studied the effect of storage and freeze-thawing of solutions of PEG 4000 (5 g/l) doped with ^{14}C PEG 4000 (5 μCi/l) in NaCl (150 mmoles/l) containing varying amounts of human albumin (0.0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.5, 25.0, 50.0 and 100.0 g/l, respectively). All samples were measured in duplicate and data expressed as average

of duplicates (fig.). Data on ^{14}C PEG 4000 were made comparable to PEG 4000 by transformation to g/l. The intra-assay reproducibility (as coefficient of variation, CV) for determination of ^{14}C PEG 4000 in saline (150 mmoles/l) was 2.3% (n=24), and the difference between duplicates 1.0% ± 0.5, SD, n=28. The corresponding figures for the chemical method (Hydén) in determining PEG 4000 were 3.7% (CV, n=24) and 4.2% ± 3.2 (SD, n=28), respectively. The inter-assay reproducibility for determining ^{14}C PEG 4000 and PEG 4000 in freshly prepared standards over 10 weeks were 7.2% (CV, n=10) and 8.1% (CV, n=10), respectively.

Results. The figure illustrates the measured concentration of PEG 4000 and ^{14}C PEG 4000 in samples containing different concentration of albumin (0.0–100.0 g/l) and which were thawed, assayed and then refrozen at weekly intervals for 10 weeks. Thus, by the radiochemical method, the mean initial concentration of 4.77 g/l ± 0.11, SD, n=12 (for all samples) fell to 4.12 g/l ± 0.93, SD, n=12 ($p < 0.05$) by 6 weeks and to 3.86 g/l ± 0.96 ($p < 0.01$) by 10 weeks. By the chemical analysis, a significant fall in concentration was apparent by 2 weeks, 5.43 g/l ± 0.15 initially, falling to 4.64 g/l ± 0.35 by 2 weeks ($p < 0.01$); 3.79 g/l ± 0.11 by 3 weeks ($p < 0.001$) and 3.40 g/l ± 0.24 by 10 weeks ($p < 0.001$). In an attempt to differentiate between the possible effects of the storage itself and the freeze-thawing process on the estimated recovery levels by the assays, samples containing the same fixed amount of PEG 4000 (5 g/l) and ^{14}C PEG 4000 (5 µCi/l) with varying amounts of albumin (range 0.0–100.0 g/l, n=12) were prepared as described before. They were kept frozen at −20 °C for 8 weeks, and during that time only thawed a single time, i.e. just prior to analysis. Treating the samples in this way the radiochemical assay showed an initial mean concentration of 5.29 g/l ± 0.16, SD, n=12 (for all samples), and at 8 weeks a concentration of 5.21 g/l ± 0.29, n=12 ($p < 0.2$); the chemical assay showed an initial concentration of 5.42 g/l ± 0.14, SD, n=12, and at 8 weeks a concentration of 5.13 g/l ± 0.43 ($p > 0.05$). These results indicated that no apparent fall in concentration resulted from freeze-storage over 8 weeks and a single thawing prior to assay.



Estimated levels of PEG 4000 (solid line) and ^{14}C PEG 4000 (broken line) in a solution of NaCl (150 mmoles/l) containing varying amounts of human albumin. Each dot expresses an average of duplicate.

Discussion. Based on these results we conclude that the analysis of both PEG 4000 and ^{14}C PEG 4000 in biological fluids is likely to be inaccurate if the samples are stored and refrozen more than once prior to assay. The results cannot be explained by inter-assay variation (long-term drift or trend) since the standard curves performed for each assay showed no such variation. The underestimate observed in the levels of PEG 4000 and ^{14}C PEG 4000 seem to be due to the thawing and refreezing process rather than the storage itself. The protein content of the samples may increase the underestimate of polyethylene glycol in multiple freeze-thawed samples. The fall in estimated concentrations of polyethylene glycol was greater with the chemical than the radiochemical method, and in fact was observed there after only 2 freeze-thaw cycles. On the basis of our results we recommend that PEG 4000 and ^{14}C PEG 4000 are measured on fresh samples. Alternatively, a standard specimen of the perfusion fluid should be stored and assayed in the same way as the intestinal samples, and used to correct for the underestimation of polyethylene glycol observed here.

1 B. Borgström, A. Dahlqvist, G. Lundh and J. Sjövall, J. clin. Invest. *36,* 1521 (1957).
2 V.L.W. Go, A.F. Hofmann and W.H.J. Summerskill, Gastroenterology *58,* 321 (1970).
3 Ö.G. Björnsson, T. Adrian, J. Dawson, R.F. McCloy, G.R. Greenberg, S.R. Bloom and V.S. Chadwick, Eur. J. clin. Invest. *9,* 293 (1979).
4 C.B. Shaffer, F.H. Critchfield and J.H. Nair, J. Am. pharm. Ass. *39,* 340 (1950).
5 D.L. Miller and H.P. Schedl, Gastroenterology *58,* 40 (1970).
6 E. Krag, B. Krag and K. Lenz, Scand. J. Gastroent. *10,* 105 (1975).
7 S.J. Malawer and D.W. Powell, Gastroenterology *53,* 250 (1967).
8 D.L. Wingate, R.J. Sandberg and S.F. Phillips, Gut *13,* 812 (1972).
9 E. Oddsson, Herlev Hospital, University of Copenhagen, Copenhagen, personal communication.
10 R. Murphy and A.R. Battersby, University Chemical Laboratory, University of Cambridge, Cambridge, unpublished observations.
11 P.H. Iverius and T.C. Laurent, Biochim. biophys. Acta *133,* 371 (1967).
12 B. Chesebro and S.-E. Svehag, Clinica chim. Acta *20,* 527 (1968).