Polyvinylpyrrolidone (PVP) as Adjunct in Centrifugal Separation and Enzymic Assay of Subcellular Components.* (21288)

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Sucrose solutions have long been used in the centrifugal separation of subcellular components, the sucrose concentration varying with the tissue and ends sought(1-4). Sucrose tends to maintain morphologic and enzymic integrity of subcellular particulates. However, in certain tissues separation of larger particulates (nuclei, mitochondria) from the microsomal fraction is made difficult by extensive agglutination of the latter. This may occur even in the absence of added electrolytes. In such cases it is often difficult, or impossible, to remove microsomal contamination from the mitochondrial fraction even by repeated resuspensions in fresh medium. This difficulty, which was encountered in mouse brain and tumors (melanoma, hepatoma) was materially overcome by adding a polymer of 1-vinyl-2-pyrrolidone (polyvinylpyrrolidone or PVP) to the sucrose solution.† PVP, presumably in part by increasing colloid osmotic pressure of the medium, counteracts irreversible agglutination of microsomes, facilitates centrifugal resolution of larger cell components (mitochondria, nuclei, etc.) and acts as a metabolic stabilizer. This paper briefly reports a method of using PVP which permits relatively simple separations of subcellular components of mouse brain, liver, and tumor (melanoma, hepatoma). Many modifications in procedure are feasible, but their desirability will depend upon the particular tissue and problems involved. PVP aids in preserving mitochondrial form, and has been used to advantage in studies of tissue slice metabolism(5).

Experimental. Freshly prepared 10% solutions of PVP in 20% sucrose (g/100 ml of aqueous solution) had a pH of about 4. These were adjusted to pH 8-8.5 with KOH. Fragments of mouse brain macerated in this medium, and examined by phase contrast microscopy, showed clean well preserved mitochondria and nuclei with little aggregation and no evidence of microsomal agglutination. On the other hand, brain macerates prepared in 10-30% sucrose solution developed extensive microsomal agglutination (evidenced by formation of a fine gray precipitate visible with phase contrast microscopy). Those prepared with PVP alone, without sucrose, showed swelling of mitochondria (5 to 10% PVP) or poorly dispersed cellular components (20% PVP).

Homogenates. Enzymic activities of brain

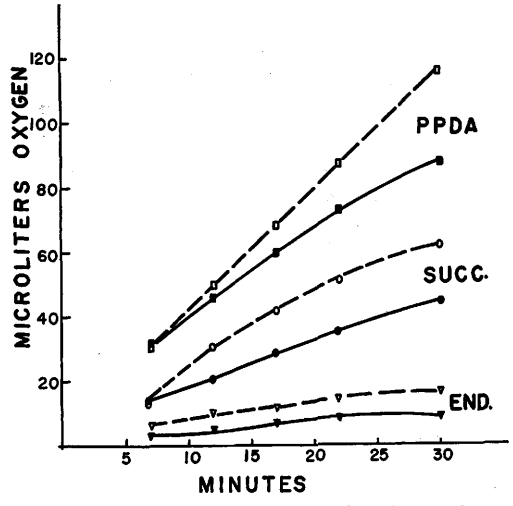

FIG. 1. Cumulative curves for microliters of oxygen consumed/0.6 ml of mouse brain homogenate in 20% sucrose (solid lines), or in 20% sucrose plus 10% PVP (broken lines). PPDA, 10% homogenate with 0.05M paraphenylene diamine plus 2.4×10^{-4} M cytochrome C. SUCC., 10% homogenate with 0.05M succinate plus cytochrome C. END., 20% homogenate with cytochrome C but no added substrate. (100% O_2, plus 2N KOH, in center wells, 38°C)

* This study reports work carried out in both the National Microbiological and National Cancer Institutes of the National Institutes of Health.

† The possible use of polyvinylpyrrolidone for subcellular extractions was suggested to the author in 1951 by Dr. Harold A. Levine. Special thanks are due to Mr. Daniel B. Witwer of the General Aniline and Film Corp., Easton, Pa., for making available at that time an imported sample (1687-202) of PVP and later samples of domestic manufacture (mol. wt. ca. 40,000). All were satisfactory.

homogenates simultaneously prepared in 20% sucrose, or in 20% sucrose plus 10% PVP, are illustrated in Fig. 1. These, and other measurements, showed that PVP had no deleterious effects on enzyme activity, provided, in the case of aerobic systems, an adequate oxygen tension was maintained. In some instances respiration in PVP was actually higher than in plain sucrose (Fig. 1).

Centrifugal separation. The results of an experiment involving a simplified method for mouse brain will be described. Brains of 15 mice were collected in 20 ml of ice-cold 20% sucrose plus 10% PVP (pH 8). The tissue (8 ml in volume) after chilling was removed, quickly drained, and ground at 0°C in a glass Potter-Elvehjem homogenizer with 28 ml of fresh sucrose-PVP solution, and filtered with suction through glass wool. The filtrate (28.5 ml), following removal of an aliquot for enzymatic activity measurements (homog., Table I), was then fractionated at 0°C in an International refrigerated centrifuge (PR-1) with multispeed attachment.

Step (a). Two one-minute cycles were run at maximum speed (R.C.F. ca. 25000 x g). The pellets contained almost all of the red cells and nuclei together with some large mitochondria (P_1, Table I).

Step (b). Two pellets were then obtained from the cleared homogenate by centrifuging 2 aliquots for 45 minutes at maximum speed. One pellet was suspended in 10% sucrose plus 5% PVP and used for enzyme assay (P_2 unwashed, Table I). Microscopically this suspension showed well-preserved, non-agglutinated mitochondria and no nuclei. The supernatant of this run contained a few mitochondria and much microsomal material. The visible particulates together with some microsomal material were removed by a second spin at maximum speed for 40 minutes (cleared supernatant = SN, Table I).

Step (c). One of the original pellets from step b (derived from ⅔ of the cleared homogenate) was resuspended in 7 ml of 20% sucrose plus 10% PVP, rehomogenized briefly and then 7 ml of distilled water (0°C) rapidly stirred into the suspension. Following a rehomogenization the suspension was divided into 2 parts and spun at maximum velocity for 40 minutes. The supernatant contained only a trace of mitochondria, but considerable vague gray, rapidly moving material below the limit of optical resolution (resuspended microsomes). This supernatant was largely cleared of visible mitochondria by a second spin at the same speed for 40 minutes (this cleared supernatant = P_2SN_1, Table I). One of the 2 original pellets was resuspended in 10% sucrose plus 5% PVP (P_2 washed once, Table I). Microscopic analysis showed well-preserved mitochondria with little evidence of precipitated microsomal material.

Step (d). The other original pellet from step c was homogenized in 4 ml of 20% sucrose plus 10% PVP, 4 ml of cold distilled water added with stirring, rehomogenized, and then spun at maximum speed for 40 minutes. The supernatant (P_2SN_2, Table I) contained very few (3 or 4 per oil-immersion field) visible granules and much less microsomal material than the previous wash supernatant. The resuspended pellet (in 10% sucrose and 5% PVP) consisted almost entirely of well-preserved mitochondria (P_2 washed twice, Table I).

TABLE I. Enzymic Activities of Subcellular Fractions of Mouse Brain Isolated in Sucrose-PVP and Expressed as Microliters Gas Exchange/Hour/ml of Homogenate Equivalent (12.5% Tissue by Vol.).

Fraction	Cytochrome oxidase activity* $\mu l\ O_2$	% of homog.	Anaerobic glycolytic activity† $\mu l\ CO_2$‡	% of P_2, unwashed
Homogenate	327	100	—	—
P_1	70	21	—	—
P_2, unwashed	364	111	801	100
P_2, washed once	314	96	533	67
" " twice	322	98	568	71
P_3	17	5	63	8
P_2SN_1	0.5	0.1	62	8
P_2SN_2	0	0	27	3
SN	8	2	35	—

* Vessels contained 0.03M PPDA, 4.8×10^{-4}M cytochrome C., 100% O_2 + KOH, 38°C.

† Vessels contained ATP 0.0019M, DPN 0.0006 M, Mg salt HDP 0.0068M, niacinamide 0.04M, dextrose 0.04M, KH_2PO_4 0.003M, K_2HPO_4 0.008M, $MgCl_2$ 0.01M, glutathione 0.01M, and $KHCO_3$ 0.04 M (95% N_2 + 5% CO_2, 38°C).

‡ (Activity of fraction plus SN)−(activity of SN run alone) = activity of fraction in presence of supernatant (*cf.* ref. 5, p. 348).

Step (e). The small, chiefly microsomal, clearance pellets from the supernatants of steps b and c were combined and suspended in 10% sucrose plus 5% PVP (P_3, Table I). The centrifugal procedure employed here yields a mitochondrial fraction (P_2 washed twice) nearly free of microsomes (microsomal contamination is quickly evidenced by precipitation following cautious acidification of the suspension under phase microscopy), small pellets of nearly pure microsomal material (P_3), but leaves the mitochondrial wash supernatants ($P_2SN_{1 \text{ and } 2}$), and the original supernatant (SN), heavily contaminated with microsomes. The microsomes can be sedimented in sucrose-PVP by centrifugation at a R.C.F. of ca. 100000 x g (Spinco centrifuge) or by addition of $MgCl_2 \cdot 6H_2O$ (1 to 2 mg per ml) and spinning at ca. 25000 x g. This latter treatment promotes agglutination of microsomes without precipitating solubilized enzymes as *e.g.*, those of the glycolytic system.

The distribution of cytochrome oxidase activities in the various fractions as determined by Warburg manometry (Table I) were as expected from the microscopic determinations. The removal of microsomal material during washing of the mitochondrial pellet (P_2 unwashed) was evidenced microscopically by inducing microsomal precipitation in the wash supernatants ($P_2SN_{1 \text{ and } 2}$), and by determining the glycolytic activity of these fractions in the presence of the original supernatant(5). It may be of interest to note that rabies virus in subcellular fractions obtained by sucrose-PVP fractionation of infected mouse brains was not measurably reduced in infectivity by the presence of PVP(6). A modification of the methods reported here has been used to study intracellular distribution of catalase(7).

Summary. Addition of polyvinylpyrrolidone (PVP) to sucrose solutions used in extraction of subcellular components improved centrifugal resolution of particulates, helped to preserve morphologic integrity of subcellular elements, and aided in preservation of enzymic activities. PVP was particularly useful in counteracting irreversible agglutination of microsomes.

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W - V Variation Induced in *S. typhi, S. paratyphi C* and *Paracolobactrum ballerup.* (21289)

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The Vi antigen occurs in a number of Salmonellae and other Enterobacteriaceae. Colonies with this antigen are designated as V colonies, morphologically and serologically different from W forms which lack the Vi antigen and react only with 0 sera. A change of V cultures to W, called V-W variation, is commonly observed in the laboratory. Little is known, however, about the reverse process, *i.e.*, W-V variation. It does not occur spontaneously. Booy and Wolff(1) succeeded in inducing Vi antigen in *S. typhi* W by growing it in media containing live or killed *S. typhi* V (vi) and *Ballerup* Vi organisms. Working with *Escherichia coli,* an organism related to Salmonellae, Boivin *et al.*(2) showed that transformation experiments with this microbe are successful only when unstable but smooth