into compound II or compound III if the concentration is high enough—i.e., greater than 1.0 mM, as was found in the hydrogen peroxide system alone (*5*).[1(c)]

These results cannot be due to the initial formation of peroxides through the attack of the oxidizing agents on reducible groups present, for peroxides themselves do not react in an identical way. They reveal further the similarity that exists between compound II of peroxidase and the metmyoglobin intermediate, for both can be formed using single electron transfer oxidizing agents.

Compared with metmyoglobin, the appearance of the first intermediate when potassium chloriridate is used presents an additional feature of great importance, because its chemical properties parallel those of compound I in the H_2O_2 or alkyl hydroperoxide systems (*6*). It is reduced to compound II by ferrocyanide in accord with the above reaction scheme *a*, and appears again when compound II is oxidized by potassium chloriridate. It differs from compound I in the peroxide systems by its far greater stability and certain details of its absorption spectrum (*7*). Although it has a Soret band of similar low intensity, the absorption is somewhat greater below 405 mμ. There can be little doubt that it is a compound of the same chemical type, and in conjunction with the spectroscopic indications that an intermediate of low Soret band intensity is also formed with the other oxidizing agents, an enzyme-substrate-complex structure for compound I, Per-H_2O_2 or Per-OOH, appears less satisfactory compared with more general structures in which the iron has the effective oxidation number +5.

Further experiments showed that intermediates are only produced using potassium chloriridate in solutions of pH greater than 7.0, and that at pH 11.6, where the peroxidase is present in its alkaline form, the very rapid production of compound II via the first intermediate still occurs. This behavior is in contrast to the action of peroxides where reaction with the acid form of peroxidase is pH-independent and the alkaline form does not react (*6*). This suggests that the intermediates can be produced by electron transfer from the acid or alkaline form of peroxidase with chloriridate, whereas the peroxide reactions involve hydrogen atom transfer which is unfavored with the alkaline form. Since the redox potential of the chloriridate ion is independent of pH, these observations also confirm the participation of H in the redox reactions of the intermediates for which there are clear indications in the previous experiments.[1(a)]

No evidence could be found for the reduction of the first intermediate by the enzyme itself in the reaction

$$\text{First intermediate} + \text{enzyme} \rightarrow 2 \text{ compound II} \quad \ldots b$$
$$(\text{formally: } Fe^{5+} + Fe^{3+} \rightarrow 2\ Fe^{4+}).$$

An over-all reaction of this type is undoubtedly favored in the peroxide systems,[1(a)] for at low values of the H_2O_2/enzyme ratio the formation of compound II approximates to the stoichiometry

$$1 \text{ mol } H_2O_2 \equiv 2 \text{ mol compound II.}$$

The absence of reaction *b* can be regarded as a reason for the appearance of the first intermediate as a stable state. In terms of free energy, reaction *b* will be favored if the redox potential $E^{\circ}_{5+/4+} > E^{\circ}_{4+/3+}$. Compound formation by the +5 state in the chloriridate system would lower $E^{\circ}_{5+/4+}$, and the stability of the first intermediate would follow on the reversal of this relationship when $E^{\circ}_{5+/4+} < E^{\circ}_{4+/3+}$.

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Centrifugation in Field-aligning Capsules: Preparative Microcentrifugation[1]

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Preparative high-speed centrifugation is commonly employed for fractionating and concentrating many biological entities of small size. Some of these materials, notably viruses, may be obtained as highly active agents even in extremely small quantities. This circumstance makes it appear that microcentrifugation methods can be advantageously applied to those cases where only fractional milliliter samples of source material are available—quantities too small to be handled with the usual preparative centrifugation methods. A microtechnique for improving the specific activity of minute amounts of sedimentable materials serves also as a useful adjunct to microchemical and microphysical assay methods. Severe difficulties arise in standard centrifugation procedures when attempts are made to secure successive depositions of a small amount of material in round-bottom plastic tubes of the size commonly employed in angle rotors, since excessive losses of recoverable material occur owing to "shelving" and back-diffusion from the thinly spread deposits. Capillary centrifugation in swinging-cup rotors has previously been employed (*1–3*), but the largest values of centrifugal acceleration obtainable with such rotors are too small to pellet most viruses and macromolecules.

A method that might be described as "field-aligning capsule centrifugation" has been employed in this laboratory for the subfractionation of very small pellets obtained from 10-ml volumes of clarified supernatant fluid of virus-infected tissue cultures and controls. Pellets obtained initially by use of conventional equipment are resuspended in 0.01–0.1 ml of diluent

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fluid, and centrifugation of this resuspension in small glass capsules compacts the particulate components into tips tapering to microscopic dimensions. The capsules are so prepared for centrifugation as to include an air bubble which serves initially to float the capsules vertically in standard plastic centrifuge tubes filled with a liquid adjusted to a suitable density. During the centrifugation cycle the long axis of the capsule is constantly aligned with the vector resultant of the three forces acting upon it: gravitation, and the radial and tangential forces resulting from the rotational motion. Effectively, the alignment of the capsule is as perfect as though it were a cup swinging on a truly universal bearing.

Two stages of preparation are required prior to centrifugation: (*a*) encapsulation of the material to be centrifuged and (*b*) flotation of the filled capsule. The stepwise procedure for encapsulation is illustrated in Fig. 1. The capsule is first prepared as a micropipette, with capillary stems drawn from each end of the body, which remains as a segment of thin walled glass or quartz tubing. Suitable thin-wall glass stock may be prepared by using glass apparatus-weight tubing of 15-mm diameter, and by drawing out a 2- or 3-cm section of it to a length of about 20 cm. In drawing out the capillary stems one must take care not to produce too thin a capsule wall near its ends. For centrifugation in fields above approximately 40,000 *g* the capsule walls must be of uniform thickness and circular in cross sections. Quartz capsules are notably stronger than glass ones and can be centrifuged at over 100,000 *g* without breakage. They can be readily prepared from thin-walled quartz tubing by use of a fine oxygen-gas flame. It has been found advantageous to make a number of pipettes with varying capacities to facilitate matching the capsule volume with the sample volume.

Material for encapsulation is first clarified of gross particulate material and then siphoned into one of the capsules of adequate capacity to contain the sample and also to enclose a bubble of air, as indicated

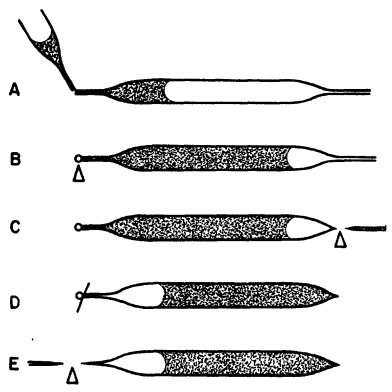

FIG. 1. Steps in the preparation of glass capsules for micro-centrifugation. *A*, filling the open-ended capsule; *B*, sealing the solvent-containing end; *C*, sealing an air bubble into the other end; *D*, breaking elongated end after transposing bubble by momentary centrifugation; *E*, final sealing of air-bubble end.

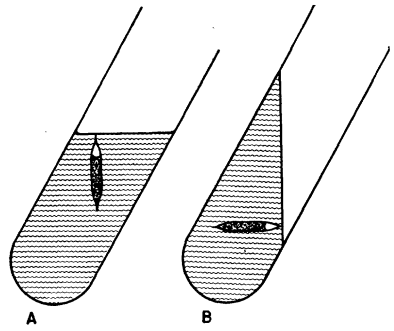

FIG. 2. Capsule floating in partially filled centrifuge tube. *A*, rotor at rest; *B*, rotor at speed. For high-speed centrifugation the tube must be filled by floating mineral oil over the salt solution shown.

in Fig. 1 *A* and *B*. The optimum size of the enclosed bubble clearly depends upon the average density of the capsule compared with the medium in which the capsule is to float, but a considerable latitude in bubble size may be compensated for by variation in the density of the flotation medium. A minute amount of clean solvent sufficient to displace the previously admitted solution from the stem into the body of the capsule is admitted, and the entrance tip of the long stem is sealed quickly in a fine, hot flame. The open capillary stem is sealed off close to the body by first drawing it out at the point of sealing to a thread, followed by fusing (Fig. 1 *C*). The fused end is immediately quenched with a drop of water to prevent warming the capsule contents excessively. If the capsule is of short body length it is ready for use at this stage, since the material sedimenting during centrifugation will collect in the elongated tip through which the sample was admitted. But if capsules are to be made of the greatest possible capacity, further preparation is required to allow them to fit closely across the plastic tubes of the angle rotor employed. The bubble is transposed to the opposite end of the capsule by a momentary centrifugation at low speed, the tip-seal of the remaining capillary stem is severed (Fig. 1 *D*), and the stem removed in the manner described previously. The completed capsule is shown in Fig. 1 *E*. Two fractions may be obtained from one encapsulation in the following manner: the capsule is first used at the stage shown in Fig. 1 *C* in a rotor provided with plastic tubes of diameter sufficient to permit horizontal orientation of the capsule with stem attached; after centrifugation the stem and its contents are removed; the capsule is then resealed for a subsequent sedimentation in a smaller rotor capable of higher values of centrifugal field.

The suspension of the capsule is illustrated in Fig. 2. A plastic centrifuge tube is half filled with a salt solution adjusted to a density such that the capsule is supported in a vertical position with the upper tip slightly above the surface of the salt solution. During centrifugation the capsule must be free to swing through 90° of arc in a plane containing the rotor axis. The greatest possible length of a capsule floating

freely in a tube of an angle rotor is determined by the diameter of the centrifuge tube containing the capsule and the angle at which the tube is held (the "rotor angle"). A 45° rotor angle permits the greatest capsule length for a given tube diameter.

When high centrifugal fields ($> 20{,}000\ g$) are employed the possible collapse of the plastic centrifuge tubes is prevented by filling them completely, with mineral oil layered above the aqueous level. After centrifugation the capsule may conveniently be removed from the centrifuge tube by first lowering over it an open end of a closely fitting section of glass tubing. When the tube is withdrawn, the capsule and some salt solution come with the tube. The capsule is rinsed and blotted dry and then enveloped in a piece of transparent Scotch tape. It is then scored through the tape at a position slightly above the meniscus, and broken apart. Supernatant fluid can be readily removed by capillary siphoning, and the sediment resuspended in fresh solvent with the aid of glass probes.

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Establishing Long-Term Cultures of Mammalian Normal, Solid Tumor, and Ascites Tumor Cells on Glass

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By embedding his tissue fragments in a clot of lymph Harrison (*1*) was the first to succeed in growing or maintaining animal cells *in vitro*. Shortly thereafter, Burrows (*2*) suggested the use of a plasma clot as the solid phase of tissue culture media, and this technique has become very widely adopted by tissue culture workers.

The practice of embedding the tissue to be cultured in a clot, although originally the difference between success and failure, has greatly restricted the dimensions of tissue cultures and has placed serious limitations on experimental applications, especially in the field of metabolism. To cope with the problem, various investigators have tried replacing the plasma clot with simpler materials or else have sought means for separating the cells from the plasma. Outstanding among these have been Evans and Earle and their associates, who introduced perforated cellophane (*3*) as a solid substrate for cell growth, and later found that certain lines of cells, after growing on the cellophane, could be induced to grow directly on the glass surface of the culture vessel. Interest in obtaining animal cell cultures of the type described by Earle (strain L) led to the development of the techniques presented in this paper for establishing and maintaining the growth of several cell strains directly on glass.

A slight modification in the customary roller tube culture technique was employed for the original cultures. It is the general practice to coat the entire inner surface of the roller tube with clotted plasma; this was modified by using only a strip of plasma, about ¼ in. wide, streaked down one side of the tube with a capillary pipette. Three explants approximately $6 \times 2 \times 1$ mm were seated in this strip, and coagulation was induced by the addition of a drop of 50% chick embryo extract. One ml of medium of the following composition was added to each tube: 10% chick embryo extract (final concentration), 50% Earle's balanced salt solution, and 40% horse serum. The tubes were incubated at 37° C on a conventional roller tube apparatus. Fluid changes were made 3 times weekly unless the condition of the cultures indicated that a departure from this routine was desirable. Good growth was noted generally within 48 hr in the plasma around the explants, and later, as the area of growth increased, the cells were observed to migrate out of the plasma onto the glass. After about 2 months' incubation, growth on the glass usually extended completely around the tube, covering all, or nearly all, the surface of the lower half. Covering of the area was not always accomplished by a steadily advancing sheet of cells, but often through random scattering. Apparently cells or cell clumps became detached from areas of dense growth, floated free, and resettled in other open areas. These islets then started new growth centers and thus speeded up total population of the available surface. After this amount of growth had occurred, nearly all evidence of the original explants had disappeared and most of the narrow strip of plasma had been lysed or otherwise lost. Thus a dense culture of cells growing directly on glass had been achieved.

After obtaining growth of the cells on glass, the next and more perplexing problem was that of subculture by the scraping and pipetting technique. Most experiments fail at this point, apparently because the cells do not survive such harsh physical treatment or change of environment. To facilitate this step, two recommendations found in the literature of Sanford and Earle and their associates were followed. The first of these involved the use of "conditioned medium," as described for single cell experiments (*4*); i.e., the cells were scraped into the old medium, and this suspension was then transferred to the new vessel with no further addition of medium. In most instances the pH of this medium was quite low; hence adjustment was made to approximately pH 7.6 by adding a few drops of sodium bicarbonate.

The second step was based on Earle's (*5*) finding that a heavier cell suspension favors transplantability. In order to utilize this principle, the first subcultures were made to vessels with a smaller surface area than that of the parent culture flask, thereby concentrating