role in evaluation of K exchange in this preparation. Furthermore, the data support the thesis that the rapidly exchanging K fraction does not represent significant extracellular localization of this ion.

Summary. Rates of washout of Na^{24+} and I^{131-} were measured in the working perfused guinea pig heart *in vitro* at 37° and 25°C. Washout occurred at 2 or more rates with at least 80% of the ions comprising the fast component and exchanging at a mean rate of 1.03 min^{-1}. There were no significant differences between the washout rates of the Na^{24+} and I^{131-} at the 2 temperatures studied. Washout of tissue slices 400-500 μ thick occurred at a single rate one-tenth that of the fast component in the working perfused heart. The data indicate that ion diffusion delay in passing through the extracellular spaces does not play a significant role in evaluation of K transfer in this preparation, and they also support previous findings that the fast component of K exchange does not represent extracellular localization.

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Received December 16, 1963. P.S.E.B.M., 1964, v116.

Use of Tris(Hydroxymethyl)Aminomethane Buffers in Cultures of Diploid Human Fibroblasts.* (29191)

GEORGE M. MARTIN (Introduced by E. P. Beneditt)
Department of Pathology, University of Washington School of Medicine, Seattle

The control of the concentration of hydrogen ions is one of many chronic frustrations of cell and tissue culture workers. All of the commonly employed media rely upon a bicarbonate system for buffering and ordinarily employ an atmosphere of 5% CO_2 in air. Although some ingenuous feed-back devices have been invented for control of the CO_2 concentration in open systems(1), many experiments are more conveniently carried out in closed systems and it is certainly desirable to maintain stock cultures in sealed containers to minimize contamination and evaporation. We describe here our experience with tris (hydroxymethyl)aminomethane buffer systems in cell cultures with the hope that others will be encouraged to investigate tris-containing media. We have confirmed the results of Swim(2,3) indicating the relatively low toxicity of tris for cultured human skin fibroblasts. When used in conjunction with low concentrations of bicarbonate (which is essential), excellent pH control is obtained with good, but variable growth. Our preliminary experience suggests that the variable results might be attributable to the mode of preparation of stock salt solutions with resulting variations in concentrations of calcium and magnesium.

Materials and methods. All cell cultures were diploid human "fibroblasts" established for 2-10 months from newborn foreskins by either trypsinization or explant techniques and incubated at 36°-37°C. Stock cultures were maintained in 6 ounce prescription bottles and were free of pleuropneumonia-like

* A portion of this work was performed in the Genetics Dept. of Glasgow University during tenure of U. S. Public Health Service Fellowship and was supported by U.S.P.H.S. Research Grants.

organisms. For the growth rate experiments illustrated in Fig. 1 and 2, replicate 22 mm square cover slip monolayers (four back-to-back pairs) were grown in a vertical array in Columbia staining jars (A. Thomas, Philadelphia) containing 10 ml of culture medium. Half the medium was changed and a coverslip sampled daily. Details of these techniques will be described elsewhere; only slightly decreased rates of growth were observed when monolayers were grown vertically as compared to the customary horizontal monolayers.

Protein was determined by a modification of the method of Oyama and Eagle(4).

In the preparation of Waymouth's medium, Hanks' balanced salt solution was substituted for the original Waymouth's salt solution(5). Various tris-buffered modifications of Waymouth's medium were prepared. The one finally employed for routine feeding (medium 10-H) was prepared as follows: to 80-86 ml of Hanks' BSS, 10 ml of a stock solution of Waymouth's constituents (dissolved in Hanks' BSS) were added. To this acid solution (pH of approximately 2.1), 1.0 ml of 6.6% $NaHCO_3$ was added to give a pH of about 7.2. Tris-HCl buffer (1.25 ml of 2 M stock, pH 7.4, 37°C) and 10 ml of heat-inactivated (56°C for 30 min) calf serum were added, giving a final pH of approximately 7.3. A freshly thawed aliquot of glutamine was then added, as well as antibiotics (to give a final concentration of 50 units of penicillin and 50 μg of streptomycin per ml). Compared to complete Waymouth's medium (with 10% calf serum), the final concentration of all constituents (except glutamine and salts) in the tris-buffered medium (10-H) was reduced by approximately 23%; the final concentration of bicarbonate was approximately 7 mM and of tris, approximately 25 mM. In closed systems, the pH is maintained in an atmosphere of air; in open systems, an atmosphere of approximately 2% CO_2 in air is required.

Tris(hydroxymethyl)aminomethane was purchased from either L. Light (Colnbrook, England) or Sigma (St. Louis). Maleic and citric acids were obtained from British Drug Houses (London).

Results. Preliminary investigations. Three types of tris buffer, with or without bicarbonate, and in concentrations ranging from 0.02

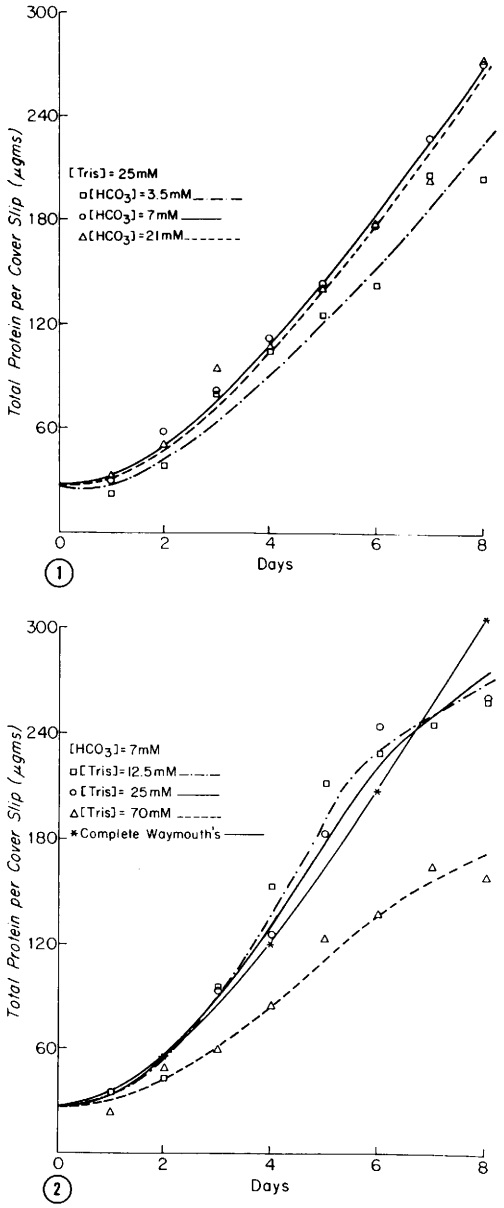

FIG. 1. Growth of diploid human fibroblasts in a tris-buffered modified Waymouth's medium at various concentrations of bicarbonate. Half the medium was replenished daily.

FIG. 2. Growth of diploid human fibroblasts in complete Waymouth's medium as compared to a modified Waymouth's medium containing 7 mM bicarbonate and various concentrations of tris-HCl buffer. Half the medium was replenished daily.

M to 0.08 M (pH 7.3) were tested on 6 different strains of fibroblasts at various population densities. In most cases, closed systems were used (screw-capped prescription bottles) but in a few experiments, petri dish cultures were employed. Qualitative assessments were made of the effects of the buffers on 1) attachment of cells after trypsinization, 2) growth of monolayers and 3) maintenance of pH.

Tris-maleate buffer(6) was by far the most toxic of the 3 varieties of tris buffer. At concentrations of 0.04 M only a rare cell attached to the glass and there was no apparent growth. Tris-citrate buffer (cp. 6) was somewhat less toxic; at concentrations of 0.02 M, there was a markedly diminished growth compared to controls (complete Waymouth's medium) and only a few trypsinized cells managed to stick to the glass. The toxicity was manifested with replicate cultures of all strains.

Very different results were observed with tris-HCl buffer(7), however. Concentrations up to 0.08 M were readily tolerated, although such media, in the absence of bicarbonate, resulted in markedly diminished growth rates, even with closed systems of culture. When bicarbonate was added, growth was greatly improved. Medium containing 0.06 M tris-HCl and 13 mM bicarbonate could not be readily differentiated from control media, either in terms of the attachment of trypsinized cells or growth of monolayers.

Growth in medium 10-H: It was apparent from the preliminary investigations that a satisfactory medium could be designed based primarily upon a tris-HCl buffer but containing also a secondary bicarbonate buffer system at relatively low concentrations. The preparation of such a medium (10-H) is given in the section on materials and methods. Fig. 1 shows that the amount of bicarbonate in this medium (7 mM), although about ⅓ the concentration present in complete Waymouth's medium, permits growth comparable to that obtained with cultures fed with tris-buffered media containing 21 mM bicarbonate. A slight fall in growth rate is observed when the bicarbonate is decreased to 3.5 mM. Fig. 2 shows that the concentration of tris in medium 10-H (25 mM) permits growth comparable to that observed in the presence of only 12.5 mM tris. When the concentration of tris is increased to 70 mM, however, there is a definite fall in growth rate.

A variable decline in growth rate of cultures fed with medium 10-H frequently occurs at about day 4-6 under the conditions of these experiments. Such a decline is shown in the experiment summarized by Fig. 2. Note that the control cultures, fed with complete Waymouth's medium under otherwise identical conditions, did not show this decline in growth rate. In some experiments, this decrease in growth is quite marked; after 8 days of growth, cultures fed with 10-H may have less than half the number of cells and less than half the total protein as cultures fed with complete Waymouth's medium. Doubling the concentration of calcium (from approximately 1.2 mM to 2.4 mM) has prevented this decline in growth with some batches of 10-H; in controlled experiments, growth was further improved when the concentration of magnesium was also doubled (from approximately 0.9 mM to 1.8 mM). However, these results did not obtain with all batches of medium.

Experience with medium 10-H now extends over a period of 20 months. A dozen strains have been continuously cultured in medium 10-H for intervals of up to 6 months. There has been no apparent "delayed" toxicity except when the cells were stored at —75°C in glycerol-containing tris-buffered media, when there was a decreased viability obviously related to the marked alkaline shift which tris buffers undergo at low temperatures(8).

Tris-HCl buffer has also been regularly employed to control the pH of trypsinizing media without apparent effect on cell attachment or growth. Monolayers are first rinsed with 0.54 mM versene in a solution containing 0.8 g % NaCl, 0.04 g % KCl, 0.002 g % phenol red and 20-25 mM tris-HCl buffer (pH 7.4 at 37°C). They are then trypsinized with 0.05% Difco 1:250 trypsin dissolved in an identical solution. With gentle mechanical agitation (pipetting), confluent

monolayers can be converted into single cell suspensions in 1-4 minutes.

In connection with recent electrophoretic studies of the alkaline phosphatase of aged, unfed, confluent monolayers ("self-induced" cultures; 9, 10) it has been necessary to control the pH of such cultures for periods of up to 5 weeks without changing the medium. The periodic addition of tris-HCl buffer (up to total concentrations of 70 mM) has proven quite useful for the purpose. When the pH is thus controlled, monolayers of most strains of diploid human fibroblasts have an extraordinary capacity to remain intact. (If the pH is allowed to fall below 7.0, the monolayers begin to peel.) The only evidence of degenerative changes within such "aged" cultures are accumulations of neutral lipids and lipochrome pigments within the cytoplasm. These are quite evident upon centrifugation of trypsinized monolayers; the concentrated masses of cells have a distinct yellow to orange color. Pathologists interested in these degenerative alterations should find such cultures useful materials for investigation.

Discussion. The present studies and those of Swim(2,3) suggest that it should ultimately be possible to design a cell culture medium buffered by tris (hydroxymethyl) aminomethane in an atmosphere of air which should permit optimal or nearly optimal growth of human skin fibroblasts. The latter have become increasingly important as standard materials for human somatic cell genetic investigations(11,12). Such a medium would be extremely convenient compared to conventional media buffered by bicarbonate in an atmosphere enriched with CO_2.

Medium 10-H, although not optimal for the growth of cultured skin fibroblasts, has already proven quite useful for routine maintenance of stock cultures. Catastrophic fluctuations in pH, so common with bicarbonate buffers, seldom occur. Closed containers do not require gassing. The stock bottles do not have to be fed and trypsinized as often as those fed with complete Waymouth's medium.

Other investigators are urged to carry on this work, especially 1) experiments designed to test various Krebs cycle intermediates and their isomers for their ability to replace bicarbonate buffers as a source of CO_2 and 2) investigations of interactions of tris with various cations. With respect to 1) above, our experience has been limited to citrate and maleate and the results have been uniformly poor. However, in view of the reports(13, 14) that oxalacetate can replace the bicarbonate of media used for growth of certain mammalian cell lines, the effects of this and related compounds upon growth of human diploid skin fibroblasts should be investigated. With respect to 2) above, the improvement in growth of medium 10-H obtained by means of calcium and magnesium supplements warrants further investigation. The inconsistent results of such experiments may be a reflection of variable precipitation of calcium and magnesium salts which we have frequently observed during preparation of concentrated stock solutions of Hanks' balanced salt solutions, even though exercising the precautions recommended by Paul(15). Small but variable quantities of fine white precipitate can usually be observed in Hanks' balanced salt solution after it is autoclaved. Ammonium ferrocyanide spot tests(16) have revealed calcium and/or magnesium in these precipitates. Tests for other constituents have not been performed.

Summary. Tris-maleate and tris-citrate buffers were found to be exceedingly toxic to cultures of diploid human fibroblasts. Tris-HCl buffers had relatively low toxicity, however; cultures continued to grow, although at reduced rates, at concentrations as high as 0.07 M in the presence of 7 mM bicarbonate. Small amounts of bicarbonate appeared to be essential for growth. A useful modification of Waymouth's medium was designed by titrating a somewhat reduced concentration of stock amino acids and vitamins to a pH of approximately 7.1 with $NaHCO_3$ and adjusting the final pH of the medium with tris-HCl buffer to a pH of 7.3; the final concentration of bicarbonate was 7 mM, of tris, 25 mM and of serum, 10%. Excellent control of pH was obtained in closed systems in an atmosphere of air and in open systems in an atmosphere of approximately 2% CO_2; no toxicity has been observed with a dozen

strains in continuous culture for periods of up to 6 months. Compared to Waymouth's medium (with 10% serum), growth in the tris-buffered modification was less predictable. Confluent monolayers were regularly obtained but they were seldom as thick as those fed with Waymouth's medium. With some batches of tris-buffered media, greatly improved growth was obtained by increasing the concentrations of magnesium and calcium. Tris-HCl buffers have also proven useful for controlling the pH of trypsinization media and of aged, unfed confluent monolayers; the latter could be maintained intact for at least 5 weeks when the pH was not permitted to fall below 7.0.

The author is indebted to Dr. John Paul for suggesting the use of tris-citrate buffer, which he has successfully employed in the culture of certain cell lines. The encouragement and advice of Prof. G. Pontecorvo are gratefully acknowledged. Expert technical assistance was provided by Mrs. Mary Ann Derr.

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Received January 8, 1964. P.S.E.B.M., 1964, v116.

Response of Monkeys to Erythropoietin of Rabbit, Sheep, and Human Origin.* (29192)

DONALD VAN DYKE

Donner Laboratory of Medical Physics, University of California, Berkeley

Although it has been shown that erythropoietin prepared from the serum of monkeys will stimulate erythropoiesis in monkeys(1) and that erythropoietin from human urine will stimulate erythropoiesis in monkeys(2) and man(3), there has been no demonstration of the effectiveness of erythropoietin from non-primate sources in primates. Since some species differences for erythropoietin have been demonstrated(4), it was thought worthwhile to determine the effectiveness in monkeys of erythropoietin prepared from the plasma of rabbits and sheep as well as from human urine.

Materials and methods. Three young cynomolgous monkeys (3 kg each) in which the 3 preparations of erythropoietin were tested were first made polycythemic by being kept in a decompression chamber at a simulated altitude of between 15,000 and 18,000 feet for 100 days (15,000 feet for the first 14 days, increased to 18,000 feet for the remainder of exposure). The monkeys were then returned to sea level and the assay begun when erythropoiesis had become almost completely suppressed, as judged by the near absence of

* This work was supported in part by the U. S. Atomic Energy Commission.