Direct biochemical method for enzyme-assays in living cell cultures

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Although these techniques have been developed to measure enzyme — changes in virus infected tissue cultures (TC) 1, 2 the general usefulness of the methods has prompted us to publish first the findings on normal cells. The improvements of our earlier 3, 4, 1 or recently published methods of others 5, 6 may be of interest for those, who intend to study the activity of biocatalysts in cultivated living cells under rigidly controlled experimental conditions. The use of chemically defined "substrate-media" avoids the inconveniences of natural nutrients containing serum, proteins, nucleic acids and especially enzymes 5, 6 without interfering with cell life. An organic compound was incorporated however, serving as substrate for biocatalytic activities of the TC. The number and viability of the cells was controlled during the experiments by microscopic inspection before and after vital staining. Spectrophotometry and various other biophysical tests may be easily carried out on the "substrate media" or on the lysates of the cultures 7. Further reports on other places will deal with problems regarding virus-infected cells 8, 9.

Material and Methods

Five cell-lines were tested during these experiments: 1. subcultures* of HeLa cells 10, 2. and 3. Primary ** and secondary cultures *** of Rhesus kidney epithelium. 4. and 5. Primary and subcultures of human amniotic membranes ****. The cells were cultivated with complete media in 3-8 cm rectangular flasks (Fig. 1 a) of 200 ml capacity, according to the combination of generally used techniques 11, 12. The seed consisted of 1.5-10⁶ cells per ml in 15 ml total nutrient fluid. After 24 hours incubation at 37 °C the growth medium 11, 12 was replaced with 20 ml Seitz-filtered bovine amniotic fluid (BAF) containing antibiotic 12 to wash away the remnants of precedent colored medium, which contained

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2 E. Kovács and D. Wulf, unpublished.  
4 E. Kovács, Biochem. Z. 330, 113 [1958].  
11** human placenta were procured through the courtesy of the Staff of Universitätsfrauenklinik, München.  
12*** human placenta were procured through the courtesy of the Staff of Universitätsfrauenklinik, München.  

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with 1 to 5% bovine embryonic extract (BEE). These cells were also assayed as 72 hours' outplants in BAF.

BEE, was prepared as follows: 1 to 2 months old bovine embryo was grinded and suspended with equal weight of PBS (0.05 M NaH₂PO₄ + 0.145 M NaCl) than shaken mechanically for 24 hours at 4 °C after addition of 1/2 dose of antibiotica. The "brei" was allowed to stand in refrigerator for 3 to 5 days then decanted, centrifuged at 4 °C/300 rpm for 30'. The supernatant provided with full dose of antibiotica was stored frozen until needed.

Before and during biochemical experiments the cultures were inspected through a special microscope, which allowed direct observation of cell morphology at various powers.

"Analytical" nutrient solutions

For enzyme-assays various "substrate media" (SM) were used, suitable for the biocatalyst to be tested. Their composition is shown in Table 1. Tris-buffered isotonic sucrose served (if not otherwise stated) for rincing the TCs. The SM Iso I lacking of salts and glucose but containing sucrose, glutamine bicarbonate and a substrate substance allowed survival of HeLa cells for about 6 to 12 hours; thus its use with above cell-line is recommended for assays ending within a few hours. However human amnion cells survived in good condition over many days (Fig. 2 a). Monkey kidney cells adhered to the glass intactly till about 48 hours.

SM Iso II consisted of 3 vol. SM Iso I and 1 vol. of a watery solution containing glucose, glutamine, amino acids and antibiotica (Table 1). The final amount of bicarbonate was the physiological level (0.025 M); in

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* Courtesy of Dr. N. WOLF, Chief Veterinär, and his Staff, Municipal Slaughter House of München.
1. Tris-buffered-isotonic Sucrose: ("wash"-medium) Sucrose 0.24 M in Tris-HCl 0.01 M; pH 7.3 ± **.

2. Iso I (Substrate-resp. Buffer-medium): Sucrose 0.019 M, Tris-HCl 0.01 M, Na-beta-glycerophosphate 0.0159 M, l-glutamine 0.0007 M, NaHCO\(_3\) 0.025 M, HCl 0.0042 M, pH 7.7 ± ; in buffer-medium, phosphate replaced by glycerol (0.0159 M). (For acid phosphatase NaHCO\(_3\) omitted, sucrose increased to 0.223 M, HCl to 0.0046 M; in buffer-medium glycerophosphate omitted, sucrose increased to 0.2389 M, HCl to 0.0016 M; pH 6.5 ± ***.

3. Iso II (SM — resp. BM): Sucrose 0.1788 M, glucose 0.011 M, Tris-HCl 0.01 M, Na-beta-glycerophosphate 0.0159 M, l-glutamine 0.0007 M, l-glutamic acid 0.0007 M, l-arginine 0.0008 M, l-cysteine 0.0008 M, l-isoleucine 0.001 M, NaHCO\(_3\) 0.025 M (in buffer-medium 0.0159 M of glycerol replacing phosphates); pH 7.7 ± (for acid phosphatase as above) ***.

4. Media 1, 2 or 3 combined with balanced salt solution ± P\(_4\O\): (1:1) with a modified Hanks' solution: NaCl 8.0, KCl 0.4, CaCl\(_2\) 0.14, MgSO\(_4\) 0.155, MgCl\(_2\) 0.125, glucose 1.0, NaHCO\(_3\) 0.39 g/liter—when NaHPO\(_4\) and KH\(_2\)PO\(_4\) was added both in 0.05 g/liter bicarbonate was compensatorily reduced.

5. Synthetic "Maintenance" Medium: (SM resp. BM ± P\(_4\O\)) adapted from RAPPAPORT\(^{17}\) containing salts trace, metals, sugars, glutamine, aminoacids and Tris-buffer.

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**Table I.** Composition of the chemically defined analytical media*. * all Seitz-filtered + antibiotica\(^{11}\); pH measured in this final state. ** for a simple way of preparation see ref.\(^{15}\). *** for SU used in nucleotidase and other assays see methods.

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**Enzyme techniques**

Acid and alkaline phosphatase were tested with glycerophosphate substrate at pH 5.5 to 6.8 resp. 7.3 to 7.8, incorporated in SM Iso I —, Iso II or other nutrients. After decantation of the former medium (generally BAF) and eventual rincing with tris-sucrose "wash-medium" (Table I) 12 to 25 ml preheated SM (24 to 37 °C) was added to the TC; 2 to 4 ml (20 vol.-%) withdrawn immediately for zero-time determination and the rest incubated at 37 °C. Portions centrifuged for 5' at 4 °C were deproteinized by equal volume of 10% icecold tri-

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16 M. KUNITZ, J. gen. Physiol. 24, 15 [1940].
chloroacetic acid, filtered through acid extracted paper; inorganic Phosphorus (IP) was determined on aliquots of the filtrate by a colorimetric method. For total phosphorus determination Kings procedure was used.

Control TC received instead of SM a buffer-medium (BM) (Table I) where for instance in phosphatase assays glycerol and bicarbonate replaced Na-glycerophosphate in equivalent quantities. By this way eventual changes in inorganic Phosphorus content of the TCs were revealed and the difference between the system and the controls (cells with BM + the IP value of the SM alone) calculated. The increase in inorganic phosphorus microgram per ml of the TC-fluid was the measure of the relative activity of phosphomonoesterases. Aliquots removed for IP determination at graded intervals were replaced by the same volume of fresh SM resp. BM. This procedure assured constant volumes and pH, but involved a correction of 20% after the second replacement because of the absence of IP and proteins from the SM. The use of buffer controls could be limited to one or two TCs in the same experiment because IP values were fairly constant during 24 hours, if no massive cell destruction occurred.

The TC controls served also for the assay of the diffusion of intracellular enzymes. Supernatant of centrifuged portions from BM in contact with cells for various length of time were reincubated for 5 to 60' at 37 °C with substrate (Iso I resp. Iso II or for instance for phosphatase tests not-isotonic buffered glycerophosphate according to SHINOWARA et al. 

In other experiments for each time-intervals pools of 2 parallel TCs were assayed, e.g. 12 flasks for 6 determinations; or when controls with buffer were also tested in parallel — 1 cell culture served as control for each assay — period. The TCs were dissolved and total protein resp. specific activities determined also for each point (v. i.). With the help of the latter values average specific activity may be calculated for the whole experiment as a good comparative measure of various TCs.

Protein was estimated by the biuret method and served for the calculation of average specific activities of the enzymes, (mg IP liberated/mg Protein in 1 hour at 37 °C). For this purpose the whole TC was dissolved in Urea-Desoxycholate (UDC) reagent eventually after freezing the TC in inverted position. Rapid freezing and thawing may be repeated to disrupt the cells; UDC was brought directly on the cell-layer before thawing, then mixed with melted culture fluid to dilute the reagent. The desoxycholate content was raised to 10% in a modified solvent applied with success. Dilution till 10% Urea concentration is carried out within 1 hour at 25 °C or 3 hours at 4 °C. The spectrophotometry of the lysate was made as described.

The physiological state of TC was controlled by direct microscopic inspection of the cells on special chemical microscope and by vital staining. Trypan blue, methylene blue, Janus green, were used in 1% concentration in Hank's solution autoclaved 10' with 10 lbs. pressure and stored at 4 °C. Neutral red was dissolved at 25 °C, autoclaved and centrifuged. Before use 10⁻⁹ dilution was made with BAF or Hank's solution. The stains were added to TC medium or the nutrient replaced by diluted dye. The cells were examined immediately and at various time-intervals after reincubation at 37 °C. Trypan blue negativity was the criterion of morphological and physiological integrity in contrast with coloration by neutral red and methylene blue or Janus green staining, followed by destaining. Cell count was made before and after some enzyme-sactivities; the cells of 2 flasks were suspended by the addition of verose 34, 11 or trypsin 12 and counted on conventional cytometers with an without addition of trypsin blue to have the percentage of viable and nonfunctioning or dead (stained) cells.

The general techniques described were valid for acid and alkaline phosphatase, 5-nucleotidase, glucose-6-phosphatase and ATP-ase. Both direct and indirect procedures were used. The TC was controlled by direct microscopic inspection of the cells on special chemical microscope and by vital staining. Trypan blue, methylene blue, Janus green, were used in 1% concentration in Hank's solution autoclaved 10' with 10 lbs. pressure and stored at 4 °C. Neutral red was dissolved at 25 °C, autoclaved and centrifuged. Before use 10⁻⁹ dilution was made with BAF or Hank's solution. The stains were added to TC medium or the nutrient replaced by diluted dye. The cells were examined immediately and at various time-intervals after reincubation at 37 °C. Trypan blue negativity was the criterion of morphological and physiological integrity in contrast with coloration by neutral red and methylene blue or Janus green staining, followed by destaining. Cell count was made before and after some enzyme-sactivities; the cells of 2 flasks were suspended by the addition of verose 34, 11 or trypsin 12 and counted on conventional cytometers with an without addition of trypsin blue to have the percentage of viable and nonfunctioning or dead (stained) cells.

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The general techniques described were valid for acid and alkaline phosphatase, 5-nucleotidase, glucose-6-phosphatase and ATP-ase. Both direct and
direct assays were carried out with various substrate concentrations. The RN-ase assays were made with 0.01% purified RNA suspended in isotonic media and the increase of total acid soluble phosphorus (TASP) was measured following precipitation by uranylacetate reagent. Spectrophotometric techniques are suitable also for demonstration of RN-ase activity. All solutions and reagents used during the experiments described were prepared with ion-exchanged water bidestilled in quartz-glass apparatus.

Results

The assays demonstrate the relative usefulness of the various substrate-media of Table I, supporting biocatalytic function and cell life, thus help to standardize the direct enzyme-techniques for living tissue cultures. Pertinent examples will be given of the various phases of this work.

Alkaline phosphatase assays

1. The effect of salts

The relative value of substrate-media containing or lacking salts is illustrated in Fig. 3. The systems displayed equal phosphatase activities during the first hours of incubation. However the TCs assayed with SM Iso II + modified Hanks’ solution exhibited a linear increase with time in inorganic phosphorus concentration of the supernatant fluid.

During the same experimental period the HeLa cells incubated with the salt-free medium did show a dissimilar behaviour, namely a marked decrease in IP content after 4 hours incubation. The substrate-concentration was equal in both media, thus the difference in activities may be due to the addition of inorganic ions (especially Magnesium, a known activator of alk. phosphatase). Furthermore the decrease of IP may mean an upperhand of phosphorylative reactions; this phenomenon occurs much later in the presence of salts, therefore the use of SM Iso II with inorganic adjuvants is recommended as the assay-medium of predilection, especially for the work with HeLa cells.

The absence of PO₄ from the medium however did not influence adversely the alk. phosphatase activity, as demonstrated with typical examples in Fig. 4. Both, relative and specific activities were of lower order in Iso II + salt mixture containing orthophosphate, thus the omission of IP from the “analytical” nutrient-fluids was experimentally justified. Although the total protein values for the two TCs at 24 hours exhibited 154.5 mcg/ml difference (ab. 22%) in favor of the cells assayed with salts (including orthophosphates) vital staining properties were about the same of both, so one could not decide for the better nutritional value of the former or the letter medium.

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2. Alk. phosphatase activity of various cell-cultures

Fig. 5 illustrates the quantitative differences between the various cell-lines examined. Further the effect of different media on the same kind of TCs are also shown. The extremely high activities of FL-cultures bathed by SM Iso II (no salts) are striking because about 92% of the added substrate were hydrolyzed within 24 hours. The alk. phosphatase activity in presence of the relatively simple (sucrose) SM Iso I as an adjunct to the original bovine amnion-fluid (1:1) was reduced in a similar culture (20 ml system). The substrate hydrolysis reached here only 59% in 24 hours and the flattening of the activity curve is evident after 6 hours. Even if a correction for the different volumes would be allowed, there remains a significant difference between the (relative) activities of the two systems. These observations underline the superiority of the more complex sucrose medium with FL-cells also. The completion of the simpler Iso I by a "natural" nutrient (BAF) did not last more than about 6 hours incubation. The prolonged contact of the amnion-fluid with the cells before Iso I was added may allow the accumulation of inhibitor-substances diffused out of the cells. Thus the complete replacement of the old nutrient-fluid, as a basic principle of our procedures represents a more satisfactory approach to the direct assay of the enzymes of cultivated cells, than the mixing with a natural, but used nutrient fluid.

The low alk. phosphatase activity of the Rhesus subcultures is a surprising feature of these experiments. The results are in agreement with similar values in primary TCs of Rhesus kidney epithelium (not illustrated).

At the comparison of specific activities a similar order is evident in the phosphatase-values of 3 different cell-types (FL-HeLa-Rhesus, Table II). In somewhat different experimental set-up of primary cultures of Rhesus kidney cortex assayed in Iso I or Iso II (without salts) gave similar low results*. The cause of these variations is not yet known.

Acid phosphatase in TC

Unlike alk. phosphatase the addition of salts did not increase the acid phosphatase activity of HeLa cells. Fig. 6 illustrates typical findings. Each point in the curves corresponds to the average value of 6 individual TCs assayed in parallel for phosphomonoesterase with the standard deviation from mean indi-

<table>
<thead>
<tr>
<th>Specific activity as mg of IP/mg of Protein/1 Hour incubation at 37°C</th>
<th>FL cells (subcult. of h. amnion epith.)</th>
<th>HeLa cells (subcult. of h. carcinoma)</th>
<th>Rhesus kidney epith (subcultures)</th>
</tr>
</thead>
<tbody>
<tr>
<td>in SM Iso II (Ø salts)</td>
<td>in SM Iso I + BAF</td>
<td>in SM Iso II + salts (–PO₄)</td>
<td>in SM Iso II + BM (Ø salts)</td>
</tr>
<tr>
<td>67·10⁻² mg</td>
<td>2.2·10⁻² mg</td>
<td>1.08·10⁻⁵ mg</td>
<td>4.7·10⁻⁸ mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.76·10⁻⁴ mg</td>
</tr>
</tbody>
</table>

Table II. Specific activities of alk. phosphatase in TCs. Abbreviations: mg = milligram, IP = inorganic phosphorus, BAF = bovine amnionfluid, BM = buffer medium.

* Unpublished observation.
mice space. The implications of these findings will be further discussed.

In Fig. 7 relative activities are compared with the average specific activities, calculated on the basis of total proteins (determined by the UDC-lysate techniques in pool of two individual TCs for each time interval). This type of experiments illustrate on 12 individual cell-cultures of the same set-up that there is a linearity with time of incubation and relative activity, but the specific activity in presence of salts is rather moderate (average sp. a = $5.38 \cdot 10^{-7}$ mg) and fluctuating.

Similar low values were observed in HeLa cells with Rappaport's maintenance-medium. Both HeLa and primary resp. secondary TCs of Rhesus kidney in SM Iso I exhibited somewhat higher activities (HeLa > Rh.)*, than with SM Iso II.

**Total Proteins in parallel cultures**

Fig. 8 illustrates the individual variations in protein-content of duplicates during incubation at 37 °C. There is a remarkable stability, if only one of the parallel TCs is considered especially during the first phase of the experiment. On the other hand the deviation between two individual assays of the same period of time is ranging from 6 to 50%. The consistently highest values at the end could be interpreted as due to growth, if the fluctuation of the protein concentrations in the various TCs would not cast doubt on such suggestion. Really the compari*

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* Unpublished observation.
son of individual and mean values from 0 to 24 hours, reveals a similar variation, as the one of the parallel assays. This finding explains the differences in specific activities of phosphatase presented in Fig. 7. The cause of the discrepancies is inherent mainly in the cell cultivation techniques (see Fig. 6 also) and only in minor degree in the methods of protein determination. Further attempts for standardization will be shown in Fig. 12 and will be discussed further.

**Enzymes in the TC-fluid**

The findings of simultaneous direct and indirect assays presented in Fig. 9 may explain many points. The acid phosphatase being mostly of nucleolar and lysosomal localization\(^{32,33}\) its activity may depend from the morphological integrity of the cells. In a very simple SM (Iso I without salts) a check on the protein content of the medium and an indirect assay of the enzymes present in the same supernatant TC fluid revealed, that the peaks of protein concentration and enzyme activities coincide after 3 hours incubation (Fig. 9). The direct assay of the whole TC however demonstrated low but progressively rising activities, independent from protein values of the supernatant. Glucose-6-phosphatase (mainly a microsomal enzyme) makes its appearance only after 6 hours in the supernatant. These activities may depend on the damage of the organelles where these biocatalysts are localized or on cell destruction during incubation with deficient media. Direct assay of Glucose-6-phosphatase will be reported\(^9\).

The features illustrated in Fig. 9 are absent when Iso II is used as experimental medium and especially when applied together with inorganic ions; thus the enzyme activities observed in direct assay of the TC with the latter fluid are mainly the function of intracellular biocatalysts, as revealed by the independence from protein concentration and the enzymes diffused into the supernatant. However the findings reemphasize the only relative value of some assay-media, which allow or provoke the diffusion of enzymes into the "milieu". In other instances a tenfold dilution of the contact-media, both by isotonic and hypotonic solutions, revealed high activities, which may mean a masking or inhibition of the diffused biocatalysts (especially with alk. phosphatase...
Fig. 10. 5-NT-ase activity in various cell-lines and effect of salts (relative activities). Legend: •—• 72 Hrs' outplants of h. amnion cell subcultures (FL) with 20 ml SM Iso I + BAF (1:1) p_H 7.8 ±, ○—○ 48 Hrs' outplants of HeLa cells with 10 ml SM Iso II + salts -P_O_4 (1:1) p_H 7.8 ±, •—• similar TC with SM Iso II + BM (no salts), △—△ 6 days old primary cultures of h. amnion cells with 10 ml SM Iso II + salts -P_O_4 ; p_H 7.7 ±, •—• similar TC with SM Iso II + BM (no salts), x—x 72 Hrs' outplants of Rhesus Kidney epith. subcultures with SM Iso II + salts —P_O_4 ; p_H 7.8 ±. Relative activities; mean of duplicates.

mean the reversion of the reaction after almost complete hydrolysis of the substrate. The specific activities illustrated in Table III revealed quantitative differences between the various cell lines, Rhesus exhibiting the highest values.

2. ATP-ase-assays

The Mg^{2+}-activated (mitochondrial?) ATP-ase was measured in indirect assay in the supernatant of HeLa cells with SM Iso II (no salts) and with a substrate medium added directly to the TC. It was a considerable fluctuation in the activity demonstrated by the indirect techniques (Fig. 11). It seems that at 2 hours after incubation with SM Iso I a peak activity can be revealed, followed by a minimum at 4 hours and a rise at the end of the experiment. A short direct assay with the mixture proposed by KIELLEY added to the cells, revealed fluctuating values in HeLa cultures. Peaks were observed at zero-time and after 90' incubation followed by a steady state and some decrease at 2 hours incubation. This medium however was "hypotonic" which fact may be responsible for the fluctuations. Further studies are designed with substrate in Iso II to reveal the pattern of this important enzyme in cultivated cells. The difficulties will be discussed in the light of the newest technical developments.

RN-ase-assays

Fig. 12 illustrates the activity curve of RN-ase at p_H 7.6 assayed in direct assays in SM Iso II, using 0.01% purified RNA as substrate. When TASP concentration as difference between systems and controls was plotted against time of incubation a steady high activity was observed in the first 3 hours. When

<table>
<thead>
<tr>
<th>Specific activity: mg P/mg Protein</th>
<th>Human amnion (primary cultures)</th>
<th>Rhesus kidney cortex (subcultures)</th>
<th>HeLa cell (subcultures)</th>
<th>FL cells (h. amnion subcultures)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Hour at 37°C</td>
<td>1.30 · 10^{-4} mg in SM Iso II + Hanks' solution (−P_O_4)</td>
<td>3.85 · 10^{-4} mg in SM Iso II + modif. solution (−P_O_4)</td>
<td>0.79 · 10^{-6} mg 20 ml system</td>
<td>1.9 · 10^{-6} mg in SM Iso I + BAF*</td>
</tr>
</tbody>
</table>

Table III. Specific activities of 5-NT-ase of various cell-lines. * BAF = bovine amnion fluid.
the cell-count and "alkaline RN-ase" activity were related, the activity per cell calculated was relatively low. There was no attempt made however to see the correlation with the percentage of living cells. In other studies the activity of acid RN-ase was measured at pH 5.8 by similar technique exhibiting analogous behaviour during incubation at 37°C.

Discussion

The usefulness of sucrose-containing isotonic substrate-media for a direct assay of various biocatalysts in cultivated living cells were demonstrated. Great differences in the sensitivity of the outplants against these "skeleton" or "analytical" fluids have been observed. The variations may be due to nutritional and other physicochemical factors. HeLa cells were very sensitive against the lack of glucose and inorganic ions, thus the absence of the latters and not the metabolic inerty of sucrose may be responsible for their low adherence to glass or intact survival in buffered sucrose alone. This cell-line seems not to be able to metabolize sucrose but it is not known if this inability is due to absence of saccharase or low penetration of the disaccharide. These points need clarification in view of the fact that other cell-strains (Rhesus and h. amnion epithelium) in primary- or in sub-cultivation persisted intactly for days or weeks in the absence of glucose. The inpenetrability of sucrose is an old rule of the Cell-Physiology, although for isolated mitochondria (possessing double membrane) this holds only when strongly hypertonic solutions are used. The protection is due probably to the preservation of structurally bound DPN and is ion- and temperature-dependent. Furthermore the beneficial effect of small amount of inorganic salts during cell fractionation was demonstrated by various experimental designs different, more or less complex "analytical" media can be chosen. For instance the effect of inorganic ions, glutamine, aminoacids etc. may be studied replacing them with equivalent amounts of sucrose. The substrate-media supplemented with salts support virus production, thus enzyme-studies in virus-infected cells can be carried out by the direct method.

As regards the lack of trace-metals from SM prepared by ion-exchanged bidistilled water or vitamins there is no danger of carency, because there may be a sufficient "leakage" of such compounds from living and especially desintegrating cells. Similar the omission of inorganic phosphates from the physiological salt-mixture did not show adverse morphological or biochemical effects; the substrate incorporated was almost immediately attacked by cellular hydrolases eliminating the initial deficiency of the nutrient. Otherwise the IP values in TC with buffer-media (lacking of added organic phosphates) remained remarkably constant in agreement with the findings of Cooper although under experimental conditions differing from ours. This relative stability allowed the reduction of the number of buffer-

35 P. D. Cooper, J. gen. Microbiol. 17, 353 [1957].
36 P. D. Cooper, J. gen. Microbiol. 17, 353 [1957].
controls without jeopardizing the results of the experiments.

The role of phosphorylatic processes during the observed hydrolytic reactions needs clarification. It was assumed during these studies that dephosphorylation and phosphorylation work hand-in-hand and phosphomonoesterases set free sufficient amount of IP immediately to continue glycolysis, respiration and oxidative phosphorylation undisturbed, till sufficient endogeneous and exogeneous nutrients, metabolites and cofactors are present. The independence of "internal milieu" assures that the results may not be distorted by excessive phosphorylation which would diminish the amount of IP liberated. Preliminary assays with Dinitrophenol in SM Iso II suggest that there is no excessive accumulation of IP in the first hours, but later when the cytotoxic effect the drug becomes manifest sharp increase in IP concentration of the TC-fluid occurred. This question is under further study, together with the respiration of the cells in "analytical" media.

From indirect evidence we assumed that low molecular substrates pass the cell-wall and the membranes of the organelles although there may exist great difference between various compounds in this respect. The small loss of intracellular proteins and enzymes into the improved salt-containing media and the independence of enzyme activities from the protein concentration of the supernatant emphasize that mainly membrane- and intracellular-enzymes were measured with the direct methods. However diffusion and reabsorption of biocatalysts have to be considered also as "vehicles" in active transport, because permeation of glycero phosphate or ATP through the wall of mammalian cell is, according to data of the literature, problematic and insufficient. The fluctuating activity of enzymes present in the supernatant (Fig. 9, Fig. 11) may support this supposition, although increased proteolysis, diffusion of inhibitors may play a part in these events. Impenetrability may account for the low initial phosphatase values in salt-free media. The linear increase with time however may be due to complex mechanism. The factors being perhaps altered permeability, adaptive stimulation of enzyme synthesis by the substrate, detachment or rounding up of cells modifying the kinetics of the enzymes. The vital-stainings however revealed the morphological and functional integrity of the cells. With these data in hand we might confidently say that with the improved SM the enzyme activities are measured in situ. This suggestion involves that the methods may be comparable with the radiobiological assays in efficiency and refinery and superior of homogenate or slice-techniques. Another great advantage of the direct methods is that the same cells can be assayed consecutively for various enzymes with some variation of the techniques.

Because various enzymes may act on the same compounds the point of the specificity of substrates is of capital importance. Therefore instead of phenol phosphates, which may be attacked by glucose-6-phosphate also, β-glycerophosphate was preferred as substrate for phosphatases, muscle adenyl acid for 5-NT-ase and 3-adenyl acid as an alternate substance for acid phosphatase. In spite of these precautions one- or more similar, but differently located biocatalysts may be responsible for the final results. Recent data cast some doubt on the value of ATPase determinations with older techniques; revision of the latter assays is planned.

Reproducibility of the findings as regards relative activity is not satisfactory. This is inherent in the techniques and mainly in biological variation, which may cause about 12 to 50% deviation in total protein content respectively phosphatase activities of parallel cell-cultures of the same outplantation. Similar inhomogeneity in out-growth was described with other cell-lines also. The variations can be diminished by using as many parallel assays as possible but as a valid comparative measure calculation of specific activities, related to total protein, cell-count or DNA was recommended. Total proteins as reference may give a relative approximation of the TC-mass. The cells however contain a lot of non-enzymic proteins, which fact may be responsible for the

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39 F. Seelisch and K. Letnaska, Naturwissenschaften 44, 450 [1957].
42 E. Kovács et al., to be published.
45 B. B. Marsh, Biochim. biophysica Acta [Amsterdam] 32, 357 [1959].
46 E. Edlinger and S. Dietel, Naturwissenschaften 45, 524 [1958].
relatively low specific activities observed. The reference to DNA-content resp. DNA-phosphorus concentration or preferably with the number resp. morphology of the cell nuclei may be of great value. Both protein and DNA determination however involve the destruction of the TC and may be cumbersome procedures for routine work. The cell-count is a good measure, needs only dispersion of the cultures and can be combined with protein and NA determinations without destruction of the whole cell-population. We hope to report soon on further technical improvements using suspended cultures where cell-number, proteins and DNA, resp. viability can be controlled at any time by direct-count or -tests and vital staining.

The aforesaid does not diminish the value of the techniques described which represent the most useful approach, up-to-date, for enzyme-assay in intact stationary-cultures. Great care was exercised to approach the “normal” state of cells, especially to use of near-physiological temperature and . Latter obviously differed markedly in some instances from the optimium of purified enzymes, but not from the constant internal milieu.

The effect of substrate concentrations was investigated also. Initially 20 ml volumes of assay-media were used but they were reduced later to 10 ml to facilitate the determination of breakdown products. The change in the cell/medium relationship resulted in high enzyme values; e.g. spec, activity of 5-nucleotidase in HeLa cells with 20 ml SM was . Although the amount of substrate is doubled with the larger volume, the contact of the cells with the solutes of the medium becomes more close in smaller assay-volume and the amount of substrate hydrolyzed during the same period of time was almost doubled. These single observations illustrate the complex nature of the problem, the results obtained in living cells not being directly comparable to the in vitro assay of (unnatural?) purified enzymes. Otherwise the substrate concentrations were similar to those generally used for phosphatases, but lower than those preferred for 5-nucleotidase and nuclease. With further reduction of the volume of the SM the cell/substrate ratio could be further modified at will, which fact is important in the case of expensive material or for assays on single cells. Furthermore the usual replacements of the SM by 20 volumen-percent supplies new nourishment, substrates, ions and buffer for the cells, thus almost a steady state is assured during the experiments. This constancy is secured by the interplay of other factors also, namely the uniformity of the outplantage of TCs the moderate growth after the cell-medium was replaced by amnion fluid and the almost complete arrest of mitosis in the sucrose-media (Fig. 2). These circumstances exclude further disproportion in the total number of cells. On the other hand one may ask how far can be cultivated cells with arrested mitosis considered as normal.

We were the first to call attention on the huge loss of specialized cell-material (e.g. intracellular enzymes) during cultivation procedures. The change of the glucose metabolism in cultivated cells toward the “cancerous” type-one was emphasized by and the mutational transformation by the Parker-school. The uniformity of 5-nucleotidase assays (mainly a nuclear enzyme) in various cell-lines is another example that the biochemical set-up of the cultivated cells tend to be modified. Assays with other enzymes however revealed differences in various cell-lines which may depend from species or organs. The decrease of inorganic phosphorus regularly observed at the end of 5-Nucleotidase or some phosphatase tests was explained by eventual upperhand or phosphorylation. Obviously a not mutually exclusive explanation would be the reversion of the enzyme-reaction. This drop occurs only after almost 100% hydrolysis of 5-adenylic acid in 5-nucleotidase-assays, but generally with much smaller rate in case of alk. phosphatase. Both of these phosphomonoesterases did show activation through ions, which may depend on general improvement of the physiological value of the SM supplemented with salts, as revealed by vital staining and

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The inspection of the TC. Furthermore, the activator Mg ions may reach easily the sites of 5-nucleotidase or alk. phosphatase activity in the nucleus, resp. cytoplasm. The decrease in ac. phosphatase values with salt-containing SM is not in contradiction with the aforesaid. In presence of salts may be a tendency of chelating, which may inhibit the acid phosphatase activity in the nucleus, resp.


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