Methods

The trace metal determinations were carried out by a procedure involving immiscible solvent extraction in the presence of chelating agents. An appropriate sample, ranging in volume from 10 to 1000 ml., was wet ashed with nitric and perchloric acids and the residue taken up in water. A non-specific extraction with diphenyl thiocarbazone (dithizone) in carbon tetrachloride at pH 8.5, in the presence of citrate, fractionated the trace metals and left iron, aluminum, and manganese in the aqueous phase. The carbon tetrachloride phase was shaken with dilute HCl to transfer zinc and lead to the aqueous phase. These metals were then determined spectrophotometrically as dithizonates. The residual carbon tetrachloride phase was oxidized with perchloric acid and the residue taken up in dilute nitric acid. On portions of this aqueous phase, copper was determined with diethyl dithiocarbamate, cobalt with nitroso-R salt, and nickel with dimethylglyoxime. The aqueous phase from the non-specific dithi-
zone extraction was oxidized with perchloric acid to destroy citrate, and the residue was dissolved in water. On portions of this aqueous phase, manganese was determined as permanganate, and iron and aluminum were determined as oxinates after adjustment of the pH and extraction with 8-hydroxyquinoline (oxine) in chloroform. The aqueous phase from the extraction of aluminum would contain sodium, potassium, calcium, and magnesium. Assays for these metals were not carried out.

All spectrophotometric determinations were made in a Hilger Uvispek set at the appropriate wave-length for the colored metal derivative. In all cases, the determinations were based on standard curves which obeyed the Beer-Lambert law within the concentration ranges employed. Full details of the assay procedure and the standard curves will be reported separately.

The exact composition of the various synthetic mixtures has already been described (1). The natural media tested included horse serum, chicken plasma, chicken serum, and chick embryo extract (4). The chicken plasma and serum were obtained from year-old cockerels.

**Results**

**Trace Metal Content of Synthetic Mixtures**—Trace metal determinations were made on a balanced salt mixture containing glucose (a modified Tyrode's solution) and on several synthetic mixtures of increasing complexity (1) that were devised by the systematic addition to the balanced salt mixture of amino acids (Mixture 22), vitamins and Tween 80 (Mixture 81), purines, pyrimidines, and cholesterol (Mixture 88), certain intermediary metabolites and accessory growth factors and iron (Mixture 199). The results of these assays are shown in Fig. 1, from which it is evident that there is a progressive increase in the total trace metal content as the synthetic mixtures have become more complex. It is interesting to note that, although iron and aluminum show the greatest increases, the concentration of copper remains practically unchanged from that present in the modified Tyrode's solution. Preparation of the balanced salt solution from a completely different set of chemicals reduced appreciably the level of copper but increased considerably the amounts of iron and aluminum. Cobalt, which is not shown in Fig. 1, was present to the extent of 1.0 \( \gamma \) per 1000 ml. in Mixture 199, but could not be detected in the simpler synthetic mixtures tested. It is probable that this trace of cobalt was introduced as a contaminant of the ferric nitrate incorporated in Mixture 199.

**Trace Metal Content of Natural Media**—Assays were carried out to determine the amount of certain trace metals present in various naturally occurring substances commonly employed in non-synthetic tissue culture media. The results, presented in Table I, indicate slight differences in
Fig. 1. Amount of certain trace metals in synthetic tissue culture media of increasing complexity devised by the systematic addition to a modified Tyrode's solution of amino acids (Mixture 22), vitamins and Tween 80 (Mixture 81), purines, pyrimidines, and cholesterol (Mixture 88), certain intermediary metabolites and accessory growth factors and iron (Mixture 199). Each value represents the average of several determinations.

TABLE I

<table>
<thead>
<tr>
<th>Substance tested</th>
<th>Metal concentration, γ per 100 ml.*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zn</td>
</tr>
<tr>
<td>Chicken plasma</td>
<td>195</td>
</tr>
<tr>
<td>&quot; serum</td>
<td>178</td>
</tr>
<tr>
<td>Horse serum</td>
<td>47</td>
</tr>
<tr>
<td>Chick embryo extract</td>
<td>123</td>
</tr>
</tbody>
</table>

* Each value represents the average of several determinations.
the trace metal content of chicken plasma and chicken serum. Marked differences were found between fowl serum and horse serum in their content of zinc, lead, copper, and cobalt but not in the amounts of manganese, iron, or aluminum that were present. Chick embryo extract was found to contain amounts of lead, cobalt, manganese, and iron that were comparable with those present in chicken plasma and serum, but the copper content was considerably higher and the levels of zinc and aluminum were appreciably lower.

*Trace Metal Content of Water after Various Methods of Purification*—Since the synthetic mixtures were all in aqueous solution, the quality of the distilled water became a matter of the utmost importance. Accord-
ingly, trace metal assays were carried out, on Toronto tap water, on water passed through a metal Barnstead still, on water from the Barnstead still redistilled in an all-Pyrex apparatus, and on water from the Barnstead still subsequently passed through a mixed bed ion exchange column. In each instance, the volume was concentrated from 1000 ml. to approximately 10 ml. before the assay was performed. From the results, shown in Fig. 2, it is apparent that passage through a Barnstead still has greatly reduced the trace metal content of the water, although the aluminum content is still appreciable. It is interesting to note that passage through the metal still has increased the copper concentration by approximately one-fifth. Redistillation in an all-Pyrex apparatus reduced the trace metals to very small amounts. Water from the Barnstead still subsequently passed through an ion exchange column was slightly lower in trace metals than water redistilled in Pyrex.

Effect of Storage on Trace Metal Content of Water—In connection with studies on synthetic media now being carried out in this laboratory, large quantities of water low in trace metals are required for the preparation of media and for the rinsing of glassware. As the demand for such water increased, it became necessary to run the stills continuously and to store any excess water in the refrigerator until needed. It was important, therefore, to determine whether the trace metal content of the water in-

<table>
<thead>
<tr>
<th>Sample</th>
<th>Metal concentration, ( \gamma ) per 1000 ml.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barnstead water redistilled in Pyrex</td>
<td>Zn 1.4  Pb 0.0  Cu 0.5  Fe 0.0  Al 1.0</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot; &quot; then stored in Pyrex bottle</td>
<td>Zn 4.6  Pb 3.0  Cu 1.2  Fe 4.5  Al 10.2</td>
</tr>
<tr>
<td>Barnstead water redistilled in Pyrex, then stored in paraffin-lined bottle</td>
<td>Zn 5.6  Pb 4.1  Cu 1.4  Fe 10.5  Al 15.0</td>
</tr>
<tr>
<td>Barnstead water passed through mixed bed ion exchange column</td>
<td>Zn 0.5  Pb 0.5  Cu 0.5  Fe 0.5  Al 1.0</td>
</tr>
<tr>
<td>Water from ion exchange column stored in polyethylene bag</td>
<td>Zn 1.5  Pb 1.5  Cu 0.6  Fe 1.5  Al 1.3</td>
</tr>
</tbody>
</table>

* Each value represents the average of several determinations.

2 The apparatus used was the Filtr-Ion unit (containing a mixture of Amberlites IR-120 and IRA-400) obtained from the LaMotte Chemical Products Company, Towson, Maryland.
creased during storage. Batches of water freshly redistilled from glass were stored in 10 liter Pyrex bottles, and in 10 liter bottles lined with paraffin wax. Other batches of water that had been passed through the ion exchange column were stored in 10 liter bottles lined with polyethylene plastic bags. After a 2 week period in the refrigerator, 1000 ml. samples were removed from each bottle and concentrated to approximately 10 ml. for the trace metal assays. From the results of these assays, which are presented in Table II, it is evident that water stored in either Pyrex or paraffin-lined bottles becomes unsuitable because of the marked increase in trace metals. Water stored in bottles lined with polyethylene plastic bags shows very little trace metal contamination and this method appears to be satisfactory for routine use.

**DISCUSSION**

The purpose of the present investigation was to determine the level of trace metals in various synthetic and natural mixtures in order to provide a basis for further studies on the mineral requirements of animal cells in tissue culture. For this reason, the assays were restricted to metals of possible nutritional significance (5) and no attempt was made to determine exhaustively all trace metals present. By the method employed, total metal was estimated in each instance and no distinction was made between bound and free metal or between the different oxidative states of cations such as iron and manganese. Within these limitations, the method was found to give consistent and reproducible results and close agreement was obtained between replicate assays.

Trace metal assays on synthetic mixtures of increasing complexity (Fig. 1) showed that there was a progressive increase in individual and total metals as the mixtures became more complex. This increase was a gradual one and could not be attributed to any one substance or group of substances, although the greater part of the total metals present was contributed by the salts of the modified Tyrode's solution. These results suggest that control of the metals present in the complex synthetic mixtures might best be achieved by the use of chelating or sequestering agents rather than by exhaustive repurification of the individual components of the mixtures.

Assays on natural tissue culture media (Table I) indicate that chicken plasma, horse and chicken serum, and chick embryo extract are relatively high in trace metals. Although references to comparable studies on avian serum and plasma have not been found in the literature, the values obtained in the present investigation are in general agreement with the normal ranges for trace metals previously established for mammalian blood (6, 7). It should be pointed out that considerable variation might be
expected among samples of natural media obtained under different conditions and from different animal species. It is believed, however, that the present assay values will serve as a general indication of the trace metal content of other media of natural origin.

Investigation of the trace metal content of water after various methods of purification (Fig. 2) showed that passage of water from the Barnstead still through an ion exchange column reduced the metal content to a lower level than did redistillation in an all-Pyrex apparatus. It was also observed (Table II) that storage of such water in either Pyrex or paraffin-lined bottles resulted in a marked increase in the trace metal content. This accumulation of metals was prevented by lining the storage bottles with inexpensive polyethylene plastic bags. Water purified by the ion exchange method has been used on several thousand tissue cultures without harmful effect. Since animal cells cultivated in synthetic media have been shown to be extremely sensitive test objects (8), it would appear that ion exchange water may be safely employed in other biological systems. In fact, Liebig, Vanselow, and Chapman (9) have recommended such water for use in studies on plant nutrition.

Although water forms the basis for all media used in nutritional and biochemical studies, surprisingly little attention has been paid to factors that may affect its quality. It is generally assumed that redistillation from an all-Pyrex apparatus results in water of a uniformly high quality. The results of the present investigation show that water of high purity may be obtained by redistillation from an all-Pyrex apparatus or by passage of ordinary distilled water through a mixed bed ion exchange column, but that subsequent conditions of storage may render such water unfit for biological use. The storage of purified water in plastic bottles or in glass bottles lined with plastic bags is recommended as a simple and inexpensive means of preventing the accumulation of undesirable amounts of trace metals.

**SUMMARY**

1. Trace metal determinations were made on several synthetic tissue culture media of increasing complexity. Trace metal determinations were also made on chicken plasma, horse and chicken serum, and chick embryo extract, substances commonly used in non-synthetic media.

2. Various methods of water purification have been studied and the lowest trace metal content was found in water from a Barnstead still subsequently passed through a mixed bed ion exchange column.

3. The trace metal content of redistilled and ion exchange water was determined before and after storage in different types of containers. The use of polyethylene bags as bottle liners was found to prevent the accumulation of trace metals.
BIBLIOGRAPHY

TRACE METAL CONTENT OF SOME
NATURAL AND SYNTHETIC MEDIA
George M. Healy, Joseph F. Morgan and
Raymond C. Parker