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An Unlimited Microanalytical System

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This is a description of an analytical system, designed for biological purposes, which we immodestly claim has unlimited sensitivity. It has long been a challenge to a certain kind of chemist to devise ways of analyzing small amounts of material. In my own case, and I suspect in that of others, the urge to smaller and smaller things has existed apart from practical objectives. The microchemical pursuit becomes a kind of game, a competition to see who can analyze the smallest object.

In spite of this questionable motivation, it has been easy to find excuses for inventing micromethods. Pregl justified the development of his analytical system by the scarcity of many organic compounds that needed to be analyzed. In the biological world the development of micromethods can be justified on at least two grounds: (1) the unit living system, the cell, is very small, and (2) critically important substances in living tissues are often present at exceedingly low concentrations. For example, a liver cell has a mass of the order of 10^{-9} g. It contains the important control substance, cyclic adenylic acid, at a concentration in the 10^{-6} M range. The amount of cyclic adenylic acid in one liver cell is thus only about 10^{-18} mol. To measure this would be a real challenge—if a valid excuse could be found to do so. There is, in fact, a reasonable justification for analyzing individual liver cells, or at least groups of a few liver cells. It is well known that cells in different parts of the liver lobule differ in many respects: in appearance, response to stimuli, sensitivity to poisons, and enzyme content.

In the case of other organs, for example the brain, there is even more reason to be curious about individual cells. There are many kinds of brain cells, and these are mixed in such fashion as to make separation of large numbers for analysis a hopeless proposition. Moreover, each nerve cell functions as an integral unit in the system, and its composition may differ at different times according to the functional state of its neighbors. To add one more justification, every living organism originated from a unique single cell.

Thus a biochemist with microchemical aspirations can easily rationalize his schemes, and may even be able to persuade a granting agency to support him.

Many ingenious ways to achieve high analytical sensitivity, and to apply them to practical problems, have been devised. It is not the purpose of this Account to review all these, but some of the more significant must be mentioned. In discussing them, a point should be kept in mind: it is one thing to have potential sensitivity, it is another to find the means to realize that full potential. For example, a mass spectrograph may be capable of recording the presence of a few hundred molecules of material in the beam, but it requires many orders of magnitude more of material in the original sample for a practical quantitative analysis.

Micro is a relative term. Micromethods developed by Pregl achieved sensitivities in the 10^{-5} mol range (the amount of nitrogen in 3 mg of morphine). Colorimetric micromethods introduced by Folin were one or two orders of magnitude more sensitive. (There are 5×10^{-7} mol of glucose in 0.1 ml of blood plasma). These would scarcely be regarded as micromethods today.

The list of sensitive means for analysis is long: colorimetry, fluorometry, polarography, gas chromatography, gasometry, mass spectrometry, radioisotopic techniques, bioassay, etc. Any one of these can be made more sensitive with appropriate devices. As an example, gasometric analysis, as employed by Warburg, was suitable for measuring 10^{-6} mol of material. By carrying out reactions in very small Cartesian divers the sensitivity has been increased a millionfold.[1] Similarly, colorimetry as ordinarily practiced is capable of measurements in the 10^{-8} mol range. Ornstein and Lehrer[2] conceived of carrying out colorimetry in droplets which were caused to shrink to much smaller sizes, with corresponding increase in optical density (measured through a microscope). This increased potential sensitivity 3 or 4 orders of magnitude.

In our own case, after struggling with other possibilities, we have achieved all the sensitivity we can

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(1) E. Giacobini, *Acta Physiol. Scand.*, **45**, 311 (1959).

(2) L. Ornstein and G. M. Lehrer, *J. Histochem. Cytochem.*, **8**, 311 (1960).

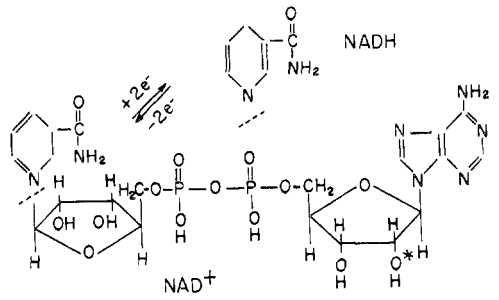

Figure 1. Structures of NAD^+ and NADH. In $NADP^+$ and NADPH the starred hydroxyl is phosphorylated.

use by a combination of fluorometry (in itself inherently sensitive) with a chemical amplifier scheme that has no theoretical limit.

Basic Principles of the System. The system is designed to measure substances present in living material. It happens that almost every substance in a cell is either subject to enzyme attack or can influence an enzyme acton. It also happens that almost every enzyme action involves reduction or oxidation of a pyridine nucleotide (NAD or NADP, nicotine adenine dinucleotide and nicotine adenine dinucleotide phosphate, Figure 1) or can be caused to do so with the aid of other enzymes. For example

$$\text{glucose} + \text{ATP} \xrightarrow{Mg^{2+}} \text{glucose 6-phosphate} + \text{ADP} \quad (1)$$

$$\text{glucose 6-phosphate} + NADP^+ \longrightarrow \text{6-phosphogluconolactone} + \text{NADPH} + H^+ \quad (2)$$

Reaction 1 can be made a measure of either glucose, ATP, Mg^{2+}, or the enzyme which catalyzes this reaction, hexokinase. Reaction 2, the "indicator" reaction, results in a stoichiometric production of NADPH, according to the amount of glucose 6-phosphate produced in reaction 1. Such reaction sequences have become standard among biochemists since they were first introduced by Warburg in the 30's. The NADPH is measured either by its absorption in the near ultraviolet or, less commonly (but much more sensitively), by its fluorescence. This alone can provide sensitivity to measure 10^{-10} to 10^{-11} mol of material.

Higher sensitivity is achieved by first destroying excess $NADP^+$ with alkali (see below), followed by "enzymatic cycling" of the NADPH, *i.e.*, the NADPH is caused to catalyze a two-enzyme system

$$\text{NADPH} + H^+ + \alpha\text{-ketoglutarate} + NH_4^+ \longrightarrow NADP^+ + \text{glutamate} \quad (3)$$

$$NADP^+ + \text{glucose 6-phosphate} \longrightarrow \text{NADPH} + \text{6-phosphogluconolactone} + H^+ \quad (4)$$

With high levels of the two required enzymes (glutamic and glucose-6-phosphate dehydrogenases) reactions 3 and 4 repeat each other in a cyclic fashion at rates as high as 20,000 cycles per hour. Finally, the cycling is stopped and one of the products is measured with the aid of 6-phosphogluconate dehydrogenase and extra $NADP^+$

$$\text{6-phosphogluconate} + NADP^+ \longrightarrow \text{ribulose 5-phosphate} + CO_2 + \text{NADPH} + H^+ \quad (5)$$

The NADPH is measured by its fluorescence. The overall sensitivity permits assay of samples down to 10^{-15} mol. If still more sensitivity is needed, the cycling step can be repeated. In this case, the excess $NADP^+$ in reaction 5 is destroyed with alkali and the NADPH is again cycled with a possible 400,000,000-fold overall amplification (if each cycling step is an hour). This in principle would permit assay of 10^{-19} mol of material (60,000 molecules). It is difficult to see the need for further amplification, although this is possible in principle by a means of a third cycling step.

Amplification systems are also available for NAD (see below). If, as stated above, almost any substance in a cell can cause the oxidation or reduction of a pyridine nucleotide, then almost any substance can in principle be measured with unlimited sensitivity.

There are, however, some practical problems. First, the necessary enzymes must be available. Fortunately, these are being provided in increasing numbers by commercial concerns, according to demands. Second, there are technical problems in bringing the sensitivity to bear on particular problems (for example, the measure of ATP in a single nerve cell).

Current solutions to these practical problems follow.

Stability Properties of Oxidized and Reduced Pyridine Nucleotides. The cycling systems for NAD and NADP obviously do not distinguish between oxidized and reduced forms. It is, therefore, an exceedingly fortunate circumstance that NADH and NADPH are unstable in acid, whereas NAD^+ and $NADP^+$ are unstable in alkali. The differences are enormous. At pH 2, NADH is 100,000 times less stable than NAD^+; at pH 13, there is a difference of the same order of magnitude in the opposite direction. Consequently, whether the product of the indicator reaction is reduced or oxidized nucleotide, the excess substrate nucleotide can be selectively destroyed without detectable loss of the product nucleotide. (For example, NADH is 99.99% destroyed in 2 min at pH 2 and 25°, without detectable loss in NAD^+; conversely, NAD^+ is 99.99% destroyed in 4 min at pH 12 and 60°, now without detectable loss of NADH.)

Fluorometric Measurement of Pyridine Nucleotides. In the microchemical system, whatever the particular method, a pyridine nucleotide must finally be measured, and this is done fluorometrically. If the end product is NADH or NADPH, these can be measured by their native fluorescence. Although their fluorescence is low in comparison to that of many fluorescent compounds, the inherent sensitivity of fluorometry is so great that reduced nucleotides can easily be measured with precision in the 10^{-7} *M* range. This provides roughly 100 times the sensitivity of spectrophotometric measurement.

If the final analytical reaction yields NAD^+ or $NADP^+$, these can also be measured fluorometrically by brief treatment with strong alkali, which converts them to fluorescent cyclic products.[3,4] These prod-

(3) N. O. Kaplan, S. P. Colowick, and C. C. Barnes, *J. Biol. Chem.*, **191**, 461 (1951).

ucts are approximately 10 times as fluorescent as native NADH or NADPH, and can therefore be measured in the 10^{-8} M range. If needed this higher sensitivity can also be obtained with reduced nucleotides by treatment with strong alkali and peroxide. The peroxide oxidizes the reduced nucleotide to NAD^+ or $NADP^+$ which is then converted by the alkali into the fluorescent cyclic product.

Greater useful sensitivity could be achieved by performing these fluorescence measurements in small volumes in micro cells. However, it has proven to be easier to provide whatever additional sensitivity may be required by enzymatic cycling.

Enzymatic Cycling. The principle of enzymatic cycling is not new. Warburg, *et al.*,[5] in 1935 measured NADP by the oxygen consumed when the nucleotide was alternatively reduced by glucose 6-phosphate dehydrogenase and oxidized by "old yellow enzyme." Amplification by enzymatic cycling has turned out to be comparatively trouble free and can give surprisingly reproducible results.[6] It is not difficult to keep the standard deviation to within 1 or 2%. It is unnecessary to know the exact cycling rate, since calculations are based on standards carried through the entire procedure with each batch of samples. This also makes control of incubation time and temperature of minor importance and corrects for possible influence on the cycling rate of components carried over from earlier steps.

The number of potential enzyme pairs for an amplifying cycle is almost unlimited. The pair described above for amplifying NADP is almost ideal because the enzymes are stable, their turnover numbers are high, and one of the steps is essentially irreversible. This means that the system can accommodate a wide range of sample sizes. The cycling rate can be varied a hundredfold or more by simply increasing or decreasing the amounts of the two enzymes of the system; the response is nearly linear from 10^{-7} M to less than 10^{-9} M NADP concentration. Therefore it is unnecessary to estimate sample sizes ahead of time with any exactitude.

An equally good cycle for NAD uses alcohol and malic dehydrogenases to catalyze the reactions[7]

$$NAD^+ + \text{ethanol} \longrightarrow NADH + \text{acetaldehyde} + H^+ \quad (6)$$

$$NADH + \text{oxalacetate} + H^+ \longrightarrow NAD^+ + \text{malate} \quad (7)$$

Malate is finally measured fluorometrically with malic dehydrogenase and NAD^+ or malic enzyme and $NADP^+$.

The NAD and NADP cycles can be used with exceedingly low concentrations of the pyridine nucleotides, 10^{-9} M or less. In fact the danger of contamination with the nucleotide becomes the ultimate limiting factor. The capability to work in the 10^{-9} M range is useful for applications in which the total amount of the material to be measured is ample but the concentration is very low.

Cycles of other types have also proved analytically useful. One of these is used to measure glutamate or α-ketoglutarate.[8]

$$\text{glutamate} + \text{oxalacetate} \longrightarrow \alpha\text{-ketoglutarate} + \text{aspartate} \quad (8)$$

$$\alpha\text{-ketoglutarate} + NH_4^+ + NADH + H^+ \longrightarrow \text{glutamate} + NAD^+ \quad (9)$$

Here the NAD^+ is measured after destroying excess NADH with acid. The cycling rate is low, but it offers a simple way to increase sensitivity 50- to 100-fold.

Another useful cycle described by Breckenridge[9] measures the sum of ATP and ADP (eq 10 and 11).

$$ADP + \text{phosphopyruvate} \longrightarrow ATP + \text{pyruvate} \quad (10)$$

$$ATP + \text{glucose} \longrightarrow \text{glucose 6-phosphate} + ADP \quad (11)$$

The glucose 6-phosphate which accumulates is subsequently measured with glucose 6-phosphate dehydrogenase and $NADP^+$. Breckenridge and others have used this cycle to measure exceedingly small amounts of cyclic adenylic acid in tissues. ATP, ADP, and normal adenylic acid are first removed, after which cyclic adenylic acid is converted enzymatically to ADP. A similar cycle for GTP and GDP has been introduced by Goldberg, *et al.*,[10] for measuring small concentrations of cyclic guanylic acid.

Volume Reduction. To utilize high sensitivity requires that volumes be reduced; otherwise problems arising from reagent blank values become excessive. In most cases, the practical concentration limit for the first specific reaction step is 10^{-6}–10^{-7} M. Therefore, if 10^{-10} mol of substance is to be measured the volume for the first step needs to be reduced to 0.01 to 0.1 μl. Fortunately the Lang-Levy constriction pipet is highly accurate to well below this volume. We have made constriction pipets as small as 0.05 nl (0.00005 μl) with 1% reproducibility. The factor that makes precision possible down to such minute volumes is surface tension. As the pipet diameter decreases, surface tension becomes an increasingly powerful force to sweep the walls nearly free of fluid during delivery.

Oil Well Technique. Volume reduction introduces a new problem, evaporation. It is possible by working in slender open tubes to readily control evaporation down to the 1 μl range. The happiest solution of the evaporation problem with smaller volumes (or, as a matter of fact with volumes as large as 10 μl) is to work under oil. For this we have come to what we call the oil-well technique.[11] The reaction vessels are small holes drilled in rows nearly through a 4 or 5 mm thick block of Teflon or other hydrophobic plastic. (A 2 × 12 cm block may contain 60 to 80 wells.) The wells are partly filled with mineral oil. The sample is introduced with a micropipet into the oil. The fluid delivered into the oil clings to the pipet tip

(4) O. H. Lowry, N. R. Roberts, and J. I. Kapphahn, *J. Biol. Chem.*, **224**, 1047 (1957).
(5) O. Warburg, W. Christian, and A. Griese, *Biochem. Z.*, **282**, 157 (1935).
(6) O. H. Lowry, J. V. Passonneau, D. W. Schulz, and M. K. Rock, *J. Biol. Chem.*, **236**, 2764 (1961).
(7) T. Kato and O. H. Lowry, *Anal. Biochem.*, in press.
(8) O. H. Lowry and J. V. Passonneau, "A Flexible System of Enzymatic Analysis," Academic Press, New York, N. Y., 1972.
(9) B. M. Breckenridge, *Proc. Nat. Acad. Sci. U.S.*, **52**, 1580 (1964).
(10) N. D. Goldberg, J. Larner, H. Sasko, and A. G. O'Toole, *Anal. Biochem.*, **28**, 523 (1969).
(11) F. M. Matschinsky, J. V. Passonneau, and O. H. Lowry, *J. Histochem. Cytochem.*, **16**, 29 (1968).

until it is stripped off by the oil surface and falls to the bottom when the tip is withdrawn. (To facilitate this stripping, the pipet tip is made more slender than usual or is siliconized on the outside to reduce surface tension.) As required, volumes of successive reagents are delivered into the original droplet. (These additions are accomplished without any difficulty; the combined volume clings to the pipet and is stripped off by the surface as before.) When necessary, the entire rack of samples can be heated nearly to 100° with little or no evaporation.

Analyses can be carried out more rapidly in oil wells than in tubes, and it is much easier to clean one rack of 80 wells than to clean 80 test tubes. In practice, pipettings are made under a low-power wide-field microscope with light from below. Reagents are placed in view beside the rack in a small vessel (a short length of glass tubing sealed off except for a small hole at each end opening upwards).

As an example of the oil well technique, an analysis for ATP in the 10^{-14} mol range would proceed somewhat as follows.

Step 1. The sample is introduced in a volume of 0.01 μl into an oil well. An equal volume of reagent is added which contains all the enzymes and cofactors necessary to carry out stoichiometric reactions 1 and 2 above. The result is the formation of 1 mol of NADPH for each mole of ATP.

Step 2. After incubating to allow these reactions to occur, 0.05 μl of NaOH is added and the rack is heated to destroy excess $NADP^+$.

Step 3. A 5-μl volume of complete cycling reagent is added and allowed to incubate for 1 hr, after which time 1 μl of NaOH is added and the rack heated again to terminate the cycling.

Step 4. An aliquot (3 or 4 μl) is transferred to a fluorometer tube containing 1 ml of reagent to measure the 6-phosphogluconate formed in the cycle (reaction 3 above).

Notice that, although the initial reaction must be carried out in a small volume, the amplification achieved by cycling permits the final reading to be made in 1 ml (a 100,000-fold larger volume). It is not difficult in 1 day to make 50 to 100 such analyses with standard deviations of 2 to 5%.

For the analysis of small frozen-dried tissue samples (see below) a droplet of appropriate reagent is first introduced into the oil well. The sample is then pushed through the oil until it touches the droplet to which it immediately adheres. The oil is displaced from the porous tissue by the aqueous reagent, soluble substances are dissolved, and insoluble substances are spread thinly on the surface of the droplet. No enzyme, of many so far tested, has been inactivated in this process.

Application to Tissue Studies. With sufficiently sensitive methods available there still remain technical problems in the application of such methods to specific problems. Most of our experience has been with the analysis of small regions or small groups of cells or single cells within a complex tissue. For this purpose, we have come to the following sequence of steps. (1) The tissue to be studied is quick frozen. (2) Frozen sections are cut at −20° with a microtome at a thickness of 5 to 25 μm according to the size of the structure to be studied. (3) The sections are dried under vacuum at −40°. (4) At room temperature the structures to be analyzed are dissected free. (5) Each sample is weighed (see below) and then subjected to analysis.

This general procedure is applicable to measurements of enzymes, metabolites, and cofactors, even when these are biologically labile (phosphocreatine, glucose, NADPH, etc.). The procedure has the advantage that tissue components are fixed in position in the native state, first by the freezing and then by the drying. (If, instead, small structures are dissected out of fresh tissue there is always the danger of damage to cell membranes with possible diffusion of cell contents and alteration of the native concentration or state.) Once the frozen sections have been dried, they can be stored under vacuum at −20° for years without change in enzyme activities or metabolite or cofactor concentrations. Free hand dissection of the dry tissue is remarkably easy. It has been possible to cleanly dissect samples as small as nuclei of large nerve cells, weighing less than 1 ng (10^{-9} g).

The Fishpole Balance. The size of the tissue sample to be analyzed is most easily determined by weight. For this purpose a fishpole balance[8,12] is well suited. This is nothing more than a fine quartz fiber mounted horizontally with one end free. The sample is placed on the free end and the displacement is measured on a scale in the eye piece of a low power (horizontal) microscope. This is a relatively crude device (accurate only to perhaps 0.5%) but is capable of far greater useful sensitivity than any other balance type. The most sensitive fishpole balance to date is capable of weighing a single dry red blood cell (3×10^{-11} g) to within 3%. One of the reasons for the high useful sensitivity is that the balance can be mounted in a very small chamber and thereby escape serious disturbance from air currents.

The sensitivity is determined by fiber thickness and length. As length increases, the free end of the fiber will droop more and more under its own weight. There is, therefore, a maximum useful length (and sensitivity) for a fiber of any given diameter. A fiber of a balance for the 2- to 10-μg range (sensitivity 0.02 μg) would typically be 35 μm in diameter and 100 mm long. The free tip would droop 30% of the fiber length. A fiber for the 0.1×10^{-9} to 0.5×10^{-9} g range (sensitivity 10^{-12} g) would be only 0.3 μm in diameter and 4 mm long, and the tip would also droop 30% of the fiber length. This latter balance would be mounted in a chamber with a volume of only 0.1 to 0.2 ml; it would be useless, because of disturbing air currents, if counted in a chamber as large as 5 ml in volume. Static electrical changes, which would otherwise also be disturbing, are dispelled by a small amount of radium in the balance case.

Balances for weighing samples of 0.02 μg or less do not need a pan because samples of this size will adhere to the fiber tip as the result of surface forces. Advantage is taken of this phenomenon in transferring small samples on and off the balance. The tool for the transfer is a short piece of sable hair mounted in a holder. This tapers naturally to a diameter of 15 or 20 μm at the tip. To this tip, in turn, is sealed a

(12) O. H. Lowry, *J. Histochem. Cytochem.*, 1, 420 (1953).

short piece of quartz fiber 2 or 3 μm in diameter. A small tissue sample (2×10^{-8} g or less) is easily picked up with this tool and transferred to the balance tip. A similar quartz-tipped "hair point" is also used for transferring weighed samples into the oil wells for analysis.

Specificity. The specificity of the overall analysis rests largely on the first enzymatic step. As in any enzymatic analysis this specificity depends on the properties and purity of the enzymes used and on the conduct of the assay. Increasing sensitivity does not increase the specificity problem and may make it less. For example, at higher dilutions relatively less enzyme may be required with less danger from contaminants. Similarly, with amplification by cycling the "signal" is increased relative to the "noise" (tissue blank).

Performance and Applications. The microanalytical system described is not only unlimited in theory but has been shown in practice to be capable of very high sensitivity and quite satisfactory precision over a ten-million-fold range in sample size (10^{-9} to 10^{-16} mol). The following are typical examples. In the 10^{-10} mol range Karlsson and coworkers[13] have made extensive studies of muscle metabolites in small human biopsy samples, and numerous enzymes have been measured in different segments of single kidney tubules.[14,15] Metabolite levels have been measured in discrete layers of mouse cerebellum[16] (10^{-11}–10^{-12} mol), in different layers of retina (10^{-12}–10^{-13} mol),[11] and in single mouse pancreatic islets (10^{-12}–10^{-14} mol).[17] In one study[18] single nerve cell bodies from mouse spinal cord were analyzed for ATP, phosphocreatine, glucose, and glycogen under a number of different experimental conditions. The sample sizes ranged from 1×10^{-12} to 3×10^{-12} g dry weight and the amounts of the four substances measured were all in the 10^{-12} mol range. Mrs. Elizabeth Barbehenn in this laboratory is currently measuring metabolite levels ranging down to 2×10^{-15} mol in single mouse ova. Measurements have been made of NAD in single nerve cell nuclei in the 10^{-16} mol range.[19] In an unpublished study, Dr. Frank A. Welsh determined the activity of single molecules of glucose 6-phosphate dehydrogenase. This required the measurement of 10^{-17} mol of enzyme product and a 10,000,000-fold amplification by two cycling steps.

This performance exceeds that attainable at present with methods based on other analytical principles. Of these, radioactive tracer methods are perhaps the most sensitive and may be used for comparison with the above. In the case of labeled amino acids having the highest available specific activity, the limit for accurate assay is about 10^{-12} mol with ^{14}C, 10^{-14} mol with ^{3}H, and 10^{-15} mol with ^{35}S (methionine).

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Deoxyribonucleic Acid Renaturation Kinetics and Hybridization. Probes to the Structure of the Eukaryotic Chromosome

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The cell nucleus is defined as that part of a living cell containing the genetic information or, in molecular terms, the DNA. In bacteria and in blue-green algae the nucleus is not separated by a nuclear membrane from the rest of the cell, the cytoplasm. Under the microscope the nucleus appears amorphous and spread throughout the cell. Cells without nuclear membranes are called prokaryotes. Until approximately 5 years ago the major discoveries of molecular biology were made in experiments on bacteria and their viruses.

Eukaryotes are cells with nuclear membranes. Such cells are found in most of the remaining single-cell organisms not mentioned above, such as fungi, protozoa, and most algae. Eukaryotic cells are also found in all higher plants and animals. The molecular biology of the eukaryotes is only now emerging as an area of useful experimentation. There are many similarities with the prokaryote, for the central dogma of molecular biology is common to all cells: DNA codes for DNA, DNA codes for RNA, and RNA codes for protein. The differences between the two

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