THE APPLICATION OF QUANTITATIVE HISTOCHEMISTRY TO THE PHARMACOLOGY OF THE NERVOUS SYSTEM

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IF it is important anywhere in the body to study drugs at the cellular level, it is surely important to do so in the nervous system. It would be hard to imagine a more complex structure than the brain with its axons, dendrites, and cell bodies, from nerve cells of every size and shape, all mixed together with several different kinds of glial cells. When we consider the possibility that drugs may affect each of these histological elements to a different degree, we are sympathetic with the slow progress in learning about basic mechanisms of drug actions on the central nervous system.

It may be that we need to know more about the nervous system itself, both at the chemical and at the physiological level. We teach that hypnotic and anesthetic agents have a selective action on the central nervous system. Actually it is the other way around. It is the central nervous system which is selective in response to drugs. The more we learn about the individual elements of the nervous system, their chemical nature and their physiological mechanisms, the easier it becomes to understand how drugs can influence their behavior.

The histological complexity of brain presents an interesting challenge to the chemist. Several approaches to the histochemical study of the nervous system have been discussed by others on this symposium. I will limit myself to exploration of possibilities for direct chemical analysis of individual histological elements of the brain or cord, and to a short discussion of applications to the study of drugs.

There are three basic requirements for direct histochemical analysis. (1) The histological structure to be analyzed must be isolated without loss or alteration of the chemical components concerned. (2) The size of the sample to be analyzed must be measured. (3) Analytical methods of sufficient sensitivity must be available. An outline of a general scheme for meeting these requirements is given in Table I. The method of obtaining material for analysis is derived from the proceures originated

TABLE I

Steps in quantitative histochemical analysis

1. Tissue quick frozen in CCl_2F_2 that has been chilled to its freezing point ($-150°$) with liquid N_2.
2. Frozen tissue sectioned in a cryostat at $-15°$ to $-25°$ (5 to 25 μ).
3. Frozen sections dried under vacuum at $-40°$.
4. Sections brought to room temperature under vacuum, and structure to be analyzed dissected free under a wide-field microscope.
5. Samples to be analyzed weighed on a quartz fish-pole balance.
6. Samples subjected to direct chemical analysis.

by Linderstrøm-Lang and Holter. The procedure departs from that of Linderstrøm-Lang and Holter after frozen sections have been prepared. Instead of using whole sections for analysis, the sections are dried under vacuum while still frozen, after which they are brought to room temperature and the desired histological elements are isolated by dissection under a microscope. In this way samples as small as nuclei of single large nerve cells can be obtained unchanged except for loss of moisture. Although the dry sections are unfixed and unstained, many of the histological details are visible, and dissection of structures down to 10 μ in diameter is not difficult. In some parts of the nervous system, such as cerebellum and retina, where histological elements are grouped into discrete layers, sections can be made with orientation in the plane of the layers. From such sections specimens of 0·1 to 2 μg dry weight can be dissected which are pure samples of each of the layers present. Similarly substantial pieces can be obtained from a wide variety of central tracts. Elsewhere, however, it is necessary to isolate and analyze individual histological elements if any worthwhile histochemical information is to be obtained.

Since whole sections are not used for analysis, the size of the isolated fragments must be determined. This is readily accomplished with simple "fish-pole" balances which are merely quartz fibres, of suitable length and thickness, mounted horizontally. The most sensitive balance of this type consists of a 4 mm long fiber of about 0·3 μ diameter, mounted in a 1 ml. tuberculin syringe. It has a sensitivity of 2 picograms, i. e. it is capable of weighing one dry red blood cell to within 10%.

The analytical requirements vary enormously with the size of sample and the substance to be measured. There are few places in the brain where samples larger than 2 or 3 μg can be obtained which are appreciably simpler than whole brain. Two micrograms or so might then be taken as the upper limit of useful sample size. The lower limit *pro tem* might be taken as that of the smallest cell body, or about 0·0001 μg. Most of the enzymes so far studied in brain can produce in an hour between 0·1 and 100 moles of product per kg. dry weight of brain. This

amounts to 2×10^{-10} to 2×10^{-7} moles for a 2 μg sample and 10^{-14} to 10^{-11} moles for a 0·0001 μg sample. Substrate concentrations are much lower than these enzyme activities. Most of those measured thus far range between 10^{-5} and 10^{-1} moles per kg dry weight. Thus the amount of any given substrate present in a 0·0001 μg sample would lie between 10^{-18} and 10^{-14} moles.

These requirements serve as a guide for choice of analytical tools. The Warburg apparatus, for example, with sensitivity limited to measurement of 10^{-6} or 10^{-7} moles, is scarcely suitable for histochemical studies of the type under consideration. Colorimetric methods with microcells are sensitive enough to measure down to 10^{-9} or 10^{-10} moles. The sensitivity would permit measurement of almost any enzyme in a 1 μg sample, but would scarcely suffice for substrate measurements.

The Cartesian diver has been refined by Giacobini to the point where it is capable of measuring 10^{-12} to 10^{-14} moles of material. Its use for histochemical studies is discussed elsewhere in this book[2].

Fluorometry is inherently much more sensitive than colorimetry. The pyridine nucleotides DPN and TPN, for example, can be measured fluorometrically down to levels of 10^{-11} moles in 1 ml. or 10^{-12} moles in micro cuvettes[3]. Consequently fluorometric methods would have enough sensitivity to measure the most active enzymes in the smallest cell body, or substrates present at the highest concentrations in 1 or 2 μg samples.

The use of DPN and TPN as analytical tools has considerable advantage. Almost any biologically important substance can be induced through an enzyme system either to reduce or to oxidize DPN or TPN. If it is reduced coenzyme that is formed, the unchanged excess of the oxidized coenzyme is easily destroyed with weak alkali without loss of the reduced form. If it is oxidized coenzyme that is formed, the excess reduced coenzyme is destroyed with weak acid without loss of the oxidized form. In either case the nucleotide which remains is measured fluorometrically.

The use of DPN and TPN in this manner has another advantage. Whenever the amount of substance to be measured is less than the necessary 10^{-11} or 10^{-12} moles, sensitivity can be increased almost without limit by using the final pyridine nucleotide product to catalyze an enzymatic dismutation of two substrates. After which one of the products can be measured, again with a DPN or TPN system. In this way sensitivity can be increased 10,000 fold, and if necessary the process may be repeated to give 100,000,000 fold increase in sensitivity[4]. This would therefore permit measurement of 10^{-19} moles of almost any substance.

The following example illustrates the entire process. The problem is to measure the amount of glucose (let us say 10^{-14} moles) in a fragment of frozen-dried axoplasm weighing 0·0005 μg.

1. Sample is weighed and treated with weak acid to destroy enzymes present.

2. Hexokinase, ATP, glucose-6-P dehydrogenase and 5×10^{-14} moles of TPN^+ are added. During brief incubation TPNH is formed equivalent to the amount of glucose present, i. e. 10^{-14} mole.

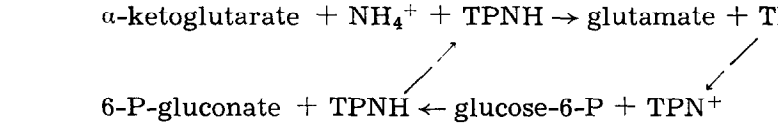
3. The excess TPN^+ is destroyed by heating at 60° in 0·01 N NaOH.

4. The entire sample is now added to a solution containing the following two enzyme systems:

(a) Glucose-6-P, glucose-6-P dehydrogenase.

(b) α-Ketoglutarate, NH_4^+, and glutamic dehydrogenase.

During subsequent incubation the following cyclic process occurs:



After 60 min the cycle has been repeated 10,000 times or more with the formation of at least 10^{-9} moles of 6-P-gluconate from the 10^{-14} moles of TPN present.

5. After heating to 100° to destroy the enzymes, the sample is added to a ml. of reagent containing 6-P-gluconate dehydrogenase and 10^{-8} moles of TPN^+. After incubation, 10^{-9} moles of TPNH are formed, and this is measured by its fluorescence.

It is clear that if more sensitivity is needed that after step number 5 the excess of TPN^+ could be destroyed with alkali and the remaining TPNH cycled again. Such double cycling has been only carried out on a few occasions, but the results show that it is practical and highly reproducible.

It would seem from the above that there are no major obstacles to the performance of direct analyses of structures in the brain as small as the smallest nerve cell body for almost any enzyme or substrate. The problems that such analyses might be expected to resolve include (1) The locus of action of many kinds of drugs; (2) possible chemical differences between areas more susceptible or less susceptible to a given drug; (3) the local chemical effects of a given drug action.

Most of the reported quantitative histochemical results to date have concerned enzyme activities rather than substrate levels.

It may be useful to contrast enzyme and substrate measurements and what pharmacological information each may be expected to give. Of the two, enzymes are easier to measure. The histological distribution of enzymes may indicate local enzyme capability and possibly the local susceptibility to drug action. Substrates are harder to measure not only because the amounts may be very small, but because many of them are subject to very rapid change, which creates difficulties in preparing

tissues for analysis. However, the very fact that substrates may undergo rapid change makes them potentially very useful as indicators of drug action.

Some of these potentialities are suggested by the reults of measurements with whole mouse brain frozen in CCl_2F_2 at — 150° at different time intervals after cutting off the blood supply by decapitation (Figs. 1–5). Decapitation results in an almost immediate shift from oxidative

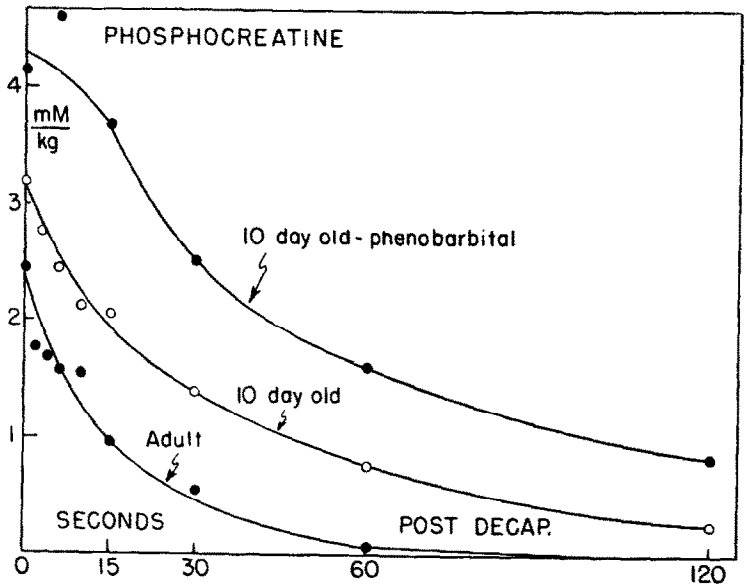


FIG. 1. Phosphocreatine concentrations in whole mouse brain at various times after decapitation. When phenobarbital was used it was administered subcutaneously in sufficient amount to overcome the righting reflex. Animals were kept under anesthesia for at least an hour before decapitation.

to glycolytic metabolism, and also induces a rather extreme degree of stimulation. The brain during the minute or two of survival is forced to derive its total energy from glycolysis and its reserves of P-creatine and ATP. Measurements of lactate, glucose, glycogen, P-creatine, ATP and ADP permit an almost complete appraisal of (1) the relative rates at which each of the possible sources of energy are recruited and (2) the total rate of energy used, i. e. the metabolic rate (Fig. 5). As is well known CrP and glucose fall very rapidly in ischemia (Fig. 1 and 2) and are the chief contributors to the energy supply in the first few seconds[5–8]. Subsequently glycogen and ATP are tapped (Figs. 2 and 4) as the more labile reserves approach exhaustion. The effect of phenobarbital is to diminish the rate of use of all the sources of energy particularly glycogen and ATP, which scarcely fall at all for 2 min under the circumstances of these experiments. This confirms the recent studies of

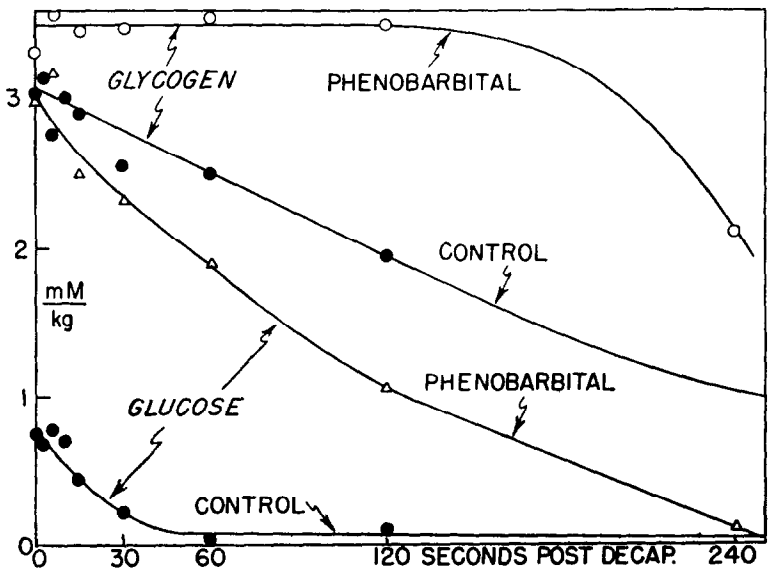


FIG. 2. Glucose and glycogen concentrations in whole mouse brain at intervals after decapitation. Glycogen is recorded as glucose liberated on hydrolysis. The values for glycogen are about 0·4 mM per kg too great since by the method used some of the glucose from ganglioside is also included.

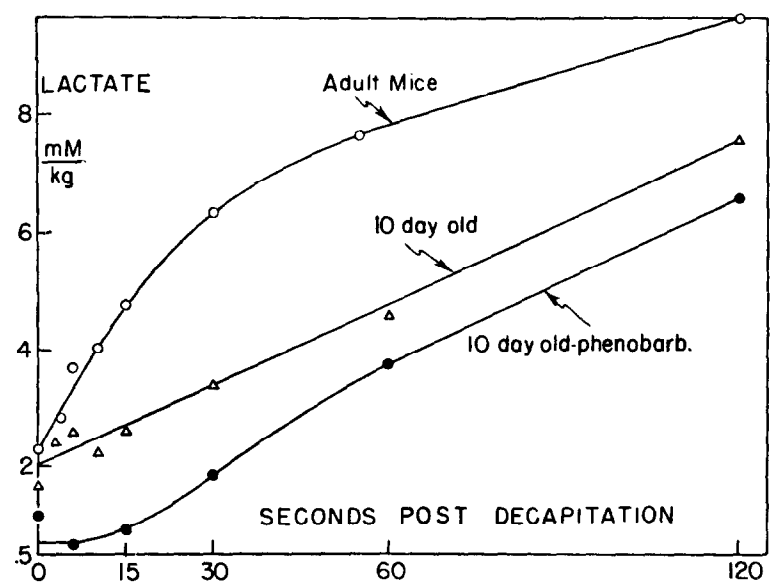


FIG. 3. Lactate concentrations in whole mouse brain at intervals after decapitation.

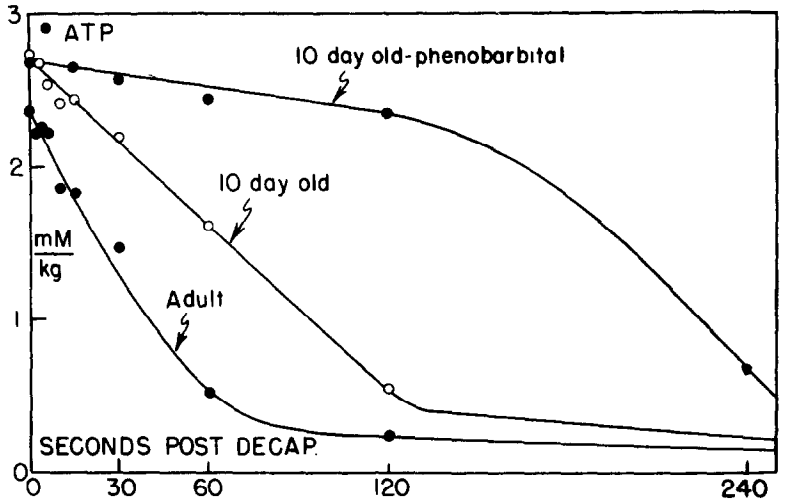


FIG. 4. ATP concentrations in whole mouse brain at intervals after decapitation.

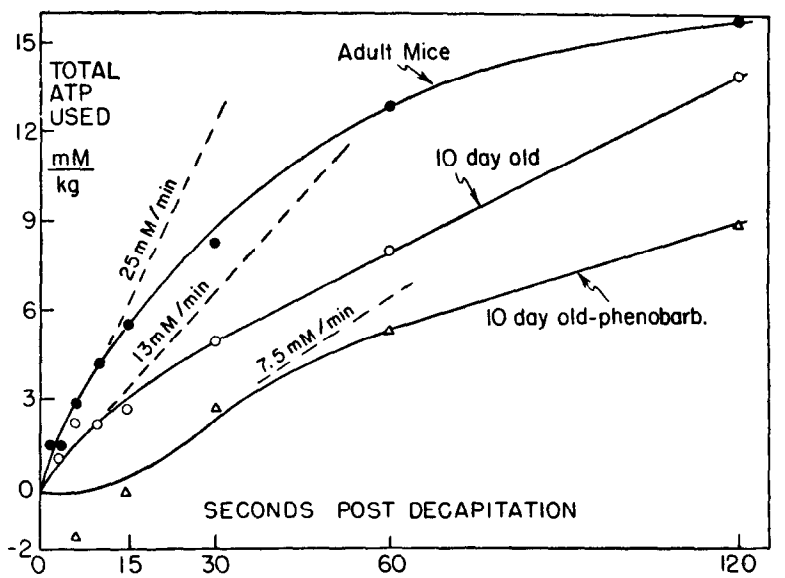


FIG. 5. Calculated use of ATP in whole mouse brain during the first two minutes after decapitation. ATP use is calculated from the net change in ATP, ADP, and PCr plus the ATP that would be formed from conversion of glucose and glycogen to lactate, i.e. 1 mole of ATP per mole of lactate from glucose, and 1·5 mole of ATP per mole of lactate from glycogen. A small correction was also made for changes in concentrations of the intermediates between glucose and lactate.

Minard and Davis[9] with the rat. The effect of age clearly shown in all figures is not directly relevant to the present discussion except it illustrates the extreme sensitivity of substrate measurements as indices of nervous activity. Presumably the immature brain has fewer neurones which are brought into activity by the anoxia and stimulus of decapitation.

It seems obvious that if the metabolites under discussion were measured in histologically defined elements of the brain at appropriate times after decapitation, the local metabolic rate could be determined; furthermore, that if such measurements were made with and without depressant drug, that the degree of local depression could be assessed.

It will be noted that glucose is initially much lower in control mice than in those under phenobarbital. This is not an artefact due to glycolysis during freezing of the normal mouse brain, since the sum of glucose, lactate (and glucose to lactate intermediates, not shown) is much less in the control animals. Therefore, in addition to histochemical measurements on the rate of change wtih and without depresant drug, the simple sum of glucose plus lactate at zero time would indicate the areas of greatest or least drug effect.

Certain drugs might be expected to change not only the rate of local energy use, by depression or stimulation, but some might affect the enzyme systems themselves which are responsible for mobilization of energy. If so, this would result in a local alteration in the pattern of metabolic change during ischemia.

This discussion can only suggest the many possibilities for pharmacological study through various quantitative histochemical measurements in the nervous system. The time seems ripe for vigorous investigation of the biochemical effects of drug action at the cellular level.

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