EFFECTS OF BUFFER SYSTEMS ON THE INTERACTION OF THE POTENT NEPHROTOGENIC AMINONUCLEOSIDE OF PUROMYCIN WITH RAT KIDNEY CORTEX MICROSOMES*

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SUMMARY

Study of the effects of pH on the interaction of the nephrotogenic aminonucleoside of puromycin with rat kidney cortex microsomes clearly indicates that binding of aminonucleoside to the microsomal fraction is sharply reduced when equilibrium dialysis experiments are conducted in the presence of the Good series of buffers, and in the presence of glycylglycine or phosphate buffers. Marked similarity in binding values, obtained in the presence of the Good series of buffers over the pH range 6.21 to 8.71, suggests a common mechanism of interference of these buffers with the binding of aminonucleoside to the microsomal fraction, and a buffer effect independent of pH. In experiments conducted with any one buffer compound, adjusted to different pH values, however, a pH effect was discernible.

INTRODUCTION

Although the working biochemist is well aware of the necessity of selecting a buffer system that provides sufficient buffering capacity for a given biochemical reaction, he may be unaware of, or fail to consider, the side effects of certain buffers on the biochemical or biological system being studied (1,2). Thus the improper selection of buffer may have been responsible for the failure of many *in vitro* attempts to duplicate *in vivo* effects of a variety of drugs or other chemical agents on enzymic reactions and mechanochemical parameters. Consequently progress in understanding the mechanisms of physiological reactions may have been delayed.

Good *et al* (3) have contributed significantly to the design of new buffers covering the pH range of 6 to 8, and have evaluated the non-buffering

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side effects of a considerable series of buffers in the Hill reaction, in mitochondrial oxidative phosphorylation, and in protein synthesis in cell-free extracts of E. coli. Since the Good buffers proved more efficient than conventional ones in the foregoing reactions (3), we utilized this series of buffers in a study of the effects of pH on the interaction of the amino-nucleoside of puromycin (6-dimethylamino-9-[3'-amino-3'-deoxy-β-D-ribo-furanosyl]-purine) with rat kidney cortex microsomes. Quite unexpectedly binding of the aminonucleoside to the microsomal fraction was sharply reduced in equilibrium dialysis experiments conducted in the presence of the Good series of buffers. The present report includes these findings and, in addition, observations on the effects of phosphate and glycylglycine buffers on the interaction of aminonucleoside with rat kidney microsomes.

METHODS

Preparation of Microsomes. Normal adult Sprague-Dawley, virgin female rats were fasted 24 hours prior to sacrifice by stunning and decapitation. Both kidneys were immediately decapsulated in situ and perfused with 0.44 M sucrose (0-3° C). The kidneys were then removed, and the pelvis and medulla excised. After weighing, the cortex was homogenized in ice-cold 2.25 mM citric acid in 0.44 M sucrose solution (4 vol. citric acid-sucrose solution/g wet wt kidney cortex). The homogenate was filtered through cheese cloth, diluted with an equal volume of ice-cold 0.44 M sucrose, and centrifuged at 380 x g for 10 minutes to remove nuclei. The supernatant was separated and centrifuged at 27,000 x g for 15 minutes to remove the mitochondrial fraction Supernatant from the mitochondrial pellet was then centrifuged at 114,000 x g to sediment the microsomes. Microsomal pellets were resuspended in 0.44 M sucrose solutions of the test buffer.

Equilibrium Dialysis Procedure. Visking-Cellulose dialysis bags were prepared by washing five times with boiled, glass-distilled water, and then equilibrating for 24 hours with ice-cold 0.44 M sucrose. For dialysis, one volume of the microsomal suspension was placed in a prepared dialysis bag

and mixed with one volume of aminonucleoside-8-^{14}C, dissolved in the 0.44 M sucrose-test buffer solution. Dialysis bags were placed in 10 ml of buffer-sucrose solution and dialyzed in the cold-room at 3° C for 24 hours (time required to attain equilibrium as indicated in preliminary studies). The initial concentration of aminonucleoside in the dialysis bag was 5 mM (specific activity: 78,000 cpm/mmole).

After dialysis, the volume of the contents of the bag and of the surrounding buffer-sucrose solution were measured, and aliquots counted for carbon-14 activity in a Tri-Carb Scintillation Spectrometer (Packard Model 3003), equipped with Automatic Standardization Accessory (Model 526).

Protein content of the solution inside the bag was determined by the procedure described by Rowsell (4). Solutions outside the bag were always checked for protein in order to detect leaks which might have occurred during equilibrium dialysis. No difficulty was experienced.

Binding values were calculated from the radioactivity measurements and the protein content of the fraction inside the bag, and expressed in terms of mμmole aminonucleoside bound per gram microsomal protein.

RESULTS AND DISCUSSION

Table I summarizes binding data for equilibrium dialysis experiments with rat kidney cortex microsomes in several different buffer systems over the pH range 6.21 to 8.75. Clearly, maximum binding of aminonucleoside to the microsomal fraction occurred when the latter was suspended in unbuffered 0.44 M sucrose solution at pH 7.05. In the presence of the Good series of buffers, over the pH range 6.21 to 8.71, binding values were reduced to one-fourth to one-fifth of the values with unbuffered sucrose.

In this respect, the markedly similar binding values for the various pH values in the Good series, not only suggest that the interference of these buffers with the binding of aminonucleoside to the microsomal fraction operates via a common mechanism, but also reveal a buffer effect that is largely independent of pH. However, in experiments using the same buffer at

Table I. Effects of Buffer System* and pH on Interaction of Aminonucleoside-8-^{14}C† with Rat Kidney Cortex Microsomes

Microsomes Suspended in:	pH‡	Binding Values mμmole Aminonucleoside/g Microsomal Protein
0.44 M Sucrose	7.05	1680
0.44 M Sucrose	7.05	1134
MES	6.21	397
TES	7.54	349
HEPES	7.05	298
HEPES	7.70	455
TRICINE	8.38	435
BICINE	8.71	246
Glycylglycine	8.20	270
Glycylglycine	8.75	108
Phosphate Buffer	6.41	790
" " "	7.17	859
" " "	7.28	695

*All buffers were prepared in 0.44 M sucrose solution. Final buffer concentrations were 0.03 M. (See Table II for chemical names of MES, TES, HEPES, TRICINE, and BICINE.)

†Concentration of aminonucleoside in dialysis bag at start of experiment, 5 mM.

‡All pH measurements were made at 3° C, the temperature at which equilibrium dialysis was conducted.

different pH values, there was an alteration in the binding value whenever a pH value was altered. The direction of these alterations was not, however, related to the direction of the changes in pH. For example, increasing the pH of the glycylglycine buffer from 8.20 to 8.75 resulted in a 60% reduction in the aminonucleoside binding value; although in the case of the HEPES buffer, a shift of pH from 7.05 to 7.70 resulted in an increase of approximately 50% in the binding value. Moreover, in the case of equilibrium studies conducted in the presence of phosphate buffers, no consistent pH effect was discernible, and interference with aminonucleoside binding to microsomes was much less than that observed in the presence of the Good series of buffers.

In seeking a common mechanism to explain the fact that both the Good series of buffers and the structurally unrelated glycylglycine and phosphate buffers interfere with the binding of aminonucleoside to microsomes, we can only conclude that interaction of aminonucleoside with the microsomes involves a positively charged site on the latter and a negatively charged functional group on aminonucleoside and on the buffering compounds. For purposes of comparison, Table II summarizes the trivial and chemical names of the Good series of buffers, and indicates the functional groups.

While in the cases of BICINE, TRICINE, TES and HEPES, interference with aminonucleoside binding may be partially explained by competition of the negatively charged $-CH_2O^-$ grouping for a positive site on the microsomes, the interference of MES, glycylglycine and phosphate buffers with the binding can not be explained in this way. In these cases, negatively charged carboxyl, sulfonic acid, and phosphate functional groupings must be mechanistically involved.

Table II. Comparison of Structure and Functional Groups of Aminonucleoside and the Good Series of Buffers

Trivial Name of Compound	Chemical Name of Compound	Number & Type of Functional Groups
Aminonucleoside	6-dimethylamino-9-(3'-amino-3'-deoxy-β-D-ribofuranosyl)-purine	1 ($-CH_2OH$) 1 ($-NH_2$)
MES	2-(N-morpholino)-ethane sulfonic acid	1 ($-SO_3H$)
TES	N-Tris-(hydroxymethyl)-methyl-2-aminoethane sulfonic acid	3 ($-CH_2OH$) 1 ($-SO_3H$)
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid	1 ($-CH_2OH$) 1 ($-SO_3H$)
TRICINE	N-Tris-(hydroxymethyl)-methyl glycine	3 ($-CH_2OH$) 1 ($-COOH$)
BICINE	N,N-Bis-(2-hydroxyethyl)-glycine	2 ($-CH_2OH$) 1 ($-COOH$)

The unexpected observation that the Good series of buffers, as well as the glycylglycine and phosphate buffers, interferes with the interaction between the nephrotogenic aminonucleoside of puromycin and rat kidney cortex microsomes emphasizes the necessity of being alert to side effects of buffering compounds on biochemical or biophysical parameters, and focuses attention on the importance of the proper selection of buffer in in vitro attempts to confirm in vivo effects of drugs or other chemical agents on a specific reaction system.

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