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THE ANALYSIS OF PURINE AND PYRIMIDINE BASES AND THEIR NUCLEOSIDES BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

The analysis of cytosine, uracil, thymine, adenine, guanine, hypoxanthine and xanthine simultaneously with their nucleosides was achieved by high-pressure liquid chromatography using a column packed with a porous-core, strong anion-exchange resin and gradient elution with ammonium acetate solution of pH 9.7. The complete analysis was achieved in 2 h and the reproducibility of the quantitative results was demonstrated. An example of the application of this analysis to an investigation of a genetic disease is reported.

INTRODUCTION

The analysis of nucleotides in cell extracts is now being carried out routinely using high-pressure liquid chromatography (HPLC)[1–8]. Although the purine and pyrimidine bases as well as their ribonucleosides have been separated by this technique[9–11], the rapid analysis of the nucleosides in the presence of their bases is not readily achieved. Cation-exchange systems have been used for analyzing bases and nucleosides[9,12–16] but it was found that uridines and guanosines are not retained with cation exchangers in a low pH range[11]. Murakami *et al.*[16], however, did separate some nucleotides, nucleosides and bases on cation-exchange resin using acetate or citrate buffers. Because the uridines and guanosines are ionized at alkaline pH and move further out in the chromatogram, anion-exchange resins and eluents in the pH range 6–11 were used by Singhal and Cohn[11] in exploring a wide range of experimental conditions for the separation of the four major nucleosides. This system was also used to separate the bases. Compounds such as hypoxanthine and xanthine and their nucleosides, which are of importance in purine metabolism studies, were not included in these investigations.

In studies of normal purine and pyrimidine metabolism and abnormalities in metabolic pathways caused by disease[17–20], the concentrations of purine and pyrimi-

dine bases and/or their nucleosides in cell extracts often must be determined. In addition, a change in the concentration of a specific nucleoside may be accompanied by a concomitant change in its base or in another nucleic acid component. Thus, it is important to be able to monitor the bases simultaneously with the nucleosides in a small sample of tissue or in physiological fluids. Furthermore, in cell extracts both bases and nucleosides may be present. Thus, for the accurate quantitation of a compound, there must be no interference caused by another compound that has the same retention time.

Although the complete separation of the UV-absorbing constituents, including purines and pyrimidines and their nucleosides, in urine has been achieved[21–25], the time required for such analyses prevents their routine use in studies involving many samples. Therefore, the major objective of this research is to develop a rapid, sensitive HPLC analysis of the purine and pyrimidine bases*, adenine, guanine, cytosine, thymine, uracil, hypoxanthine and xanthine, concomitantly with their nucleosides for use in studies of normal and abnormal metabolism of nucleic acid components.

EXPERIMENTAL

Apparatus

A Varian LCS 1000 high-pressure liquid chromatograph with a double-beam UV detector operating at 254 nm was used. The cylindrical flow cell was 1 mm in diameter and had a 10-mm path length. A 10-mV Texas Instruments Servoriter was used. The column, which was purchased from Varian Aerograph (Palo Alto, Calif., U.S.A.), was 25 cm in length and of 2 mm I.D. and was packed with Aminex A-28.

Mode of operation

In order to optimize the operating conditions for the desired separations, both isocratic and gradient elution modes were used. For the isocratic elution mode, potassium and ammonium dihydrogen phosphate and ammonium acetate solutions with concentrations from 0.05 to 0.5 *M* were tried. The pH of the ammonium acetate solutions was varied from 3.0 to 11.0.

For the gradient elution mode, a low-concentration eluent of 0.05 *M* ammonium acetate and a high-concentration eluent of 1.00 *M* ammonium acetate were found most effective in preliminary work. It was also found that the best separations were obtained when the pH of the solution was adjusted to 9.7 with ammonia solution. The mixing chamber in the chromatograph was filled with low-concentration buffer to the 50-ml mark (the starting volume). The column flow-rates (the flow-rate of the gradient from the mixing chamber into the column) and the gradient flow-rates (the flow-rate of the high-concentration buffer into the mixing chamber) were varied from 6 and 3 ml/h to 20 and 10 ml/h, respectively. Full-scale absorbance was 0.08 O.D. unit for the standards and 0.04 O.D. unit for the blood samples. All samples were introduced with a 10-μl Hamilton Syringe using the stop-flow technique.

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* Abbreviations: adenine = Ade; adenosine = Ado; hypoxanthine = Hyp; inosine = Ino; xanthine = Xan; xanthosine = Xao; guanine = Gua; guanosine = Guo; cytosine = Cyt; cytidine = Cyd; uracil = Ura; uridine = Urd; thymine = Thy; thymidine = dThyd.

Chemicals

Reagent-grade ammonium hydroxide (B & A, Morristown, N. J., U.S.A.), ammonium acetate (B & A), potassium dihydrogen phosphate (Mallinckrodt, St. Louis, Mo., U.S.A.) were used. The purine and pyrimidine bases, cytosine, uracil, thymine, adenine, guanine, hypoxanthine and xanthine and their nucleosides, cytidine, uridine, thymidine, adenosine, guanosine, inosine and xanthosine were purchased from Sigma (St. Louis, Mo., U.S.A.). Two stock solutions, one of all the bases and one of all the nucleosides (*ca.* 5 m*M* in each compound) were prepared and stored at −4°.

Blood samples

Fresh blood in acid–citrate–dextrose from which the platelets had been removed was obtained from the Division of Hematologic Research of the Pawtucket Memorial Hospital. Dr. William N. Kelley, Chief, Division of Rheumatic and Genetic Diseases, Duke University Medical Center, kindly supplied us with samples of fresh whole blood (heparinized) from a patient with Lesch Nyhan Syndrome (LNS).

Quantitation of peaks

Each base has its own molar extraction coefficient at 254 nm; thus separate calibration factors were required for quantitating each purine and pyrimidine. As the absorbance is due mainly to the chromophore in the base, the same molar extinction coefficient was used for the respective nucleosides. Areas under the peak were determined by multiplying the height of the peak by the width at half-height.

Incubation studies

Samples of erythrocytes from a normal subject and from a patient with LNS were incubated for 2 h with guanosine according to the procedure described by Brown and Parks[17]. Samples were prepared for HPLC analysis by pipetting 1 ml of the suspension of erythrocytes dropwise into 2 ml of cold 12% trichloroacetic acid and stirring rapidly on a Vortex mixer. The samples were centrifuged and 0.5-ml aliquot portions of the supernatant fluid were extracted with water-saturated diethyl ether. The nucleosides and bases in 20-μl samples of this extract were determined using the experimental conditions described in this paper and the nucleotides were determined with another 20-μl aliquot portion using the experimental conditions previously described by Brown and Parks[17].

RESULTS

Optimal operating conditions

It was found necessary to use a porous-core column packing in order to obtain the desired separations of purine and pyrimidine bases from their nucleosides. With pellicular materials, there was insufficient retention capacity to achieve the desired separations. A column packed with Aminex A-28 gave effective separations. Temperatures from ambient to 75° were tried and it was found that 70° was the optimal temperature for eluting the compounds with longer retention times. Ammonium and potassium dihydrogen phosphate and ammonium acetate were used and the best separations were obtained with ammonium acetate. This is the eluent used by Scott *et al.*[21] for the analysis of UV-absorbing constituents in urine. The pH of the ammo-

nium acetate was varied from 3 to 11, and a pH of 9.7, the optimal pH found for separating adenine, guanine, uracil and cytosine by Singhal and Cohn[11], was the most effective.

Using an isocratic elution mode, the flow-rates and concentrations of ammonium acetate were varied. At a higher concentration than 0.3 *M* ammonium acetate, resolution of the earlier peaks was lost, and at a lower concentration, hypoxanthine and xanthine and their ribonucleosides were retained on the column. Using 0.3 *M* ammonium acetate, xanthine and xanthosine were not separated, nor was hypoxanthine separated from inosine. In addition, the separations of uracil from thymidine (12 and 13 min) and adenosine from guanine (17 and 18 min) were not reproducible. If it is necessary to use an isocratic elution mode (for example, in recycling techniques) the best flow-rate was found to be 9 ml/h; at higher flow-rates, the thymidine peak was not separated from the cytosine–cytidine peak, and at lower flow-rates, retention times were not reproducible.

Therefore, a linear gradient elution mode using 0.05 and 1.0 *M* ammonium acetate (both solutions adjusted to pH 9.7), a gradient delay of 10 min, a column flow-rate of 12 ml/h, a gradient flow-rate of 6 ml/h and a column temperature of 70° were found to be the optimal experimental conditions for achieving the desired separations. Cytidine and cytosine, however, were not resolved under these conditions.

Each base was run separately and in combination with the other bases (Fig. 1a) as well as with the nucleosides (Fig. 1b) in order to check reproducibility of retention times and effectiveness of separations. Using these conditions, each nucleoside was also run separately and together with other nucleosides (Fig. 1c).

Peak areas as well as peak retention times were reproducible, as shown in Fig. 2. There was good linearity of response. For example, in Fig. 3, the concentration (in nanomoles) of dThyd is plotted against the area (in cm^2) of the dThyd peak. Therefore, the procedure can be used for quantitative analysis.

When the flow-rates were increased in an attempt to shorten the time required for an analysis, some resolution was lost (Table I).

Standard solutions containing nucleotides as well as nucleosides and bases were also chromatographed in order to determine the relative retention times of the nucleotides and to make sure that the nucleotides did not interfere in the nucleoside and base analyses. It was found that the nucleotides were retained on the column until after the xanthines were eluted. Therefore, analysis of the nucleosides and bases could be achieved in the presence of nucleotides in cell extracts.

Application

In order to determine the applicability of this procedure to cell extracts, the analysis was used in a preliminary *in vitro* study of purine metabolism in erythrocytes of a patient with LNS in collaboration with Dr. William N. Kelley of Duke University Medical Center. When erythrocytes from normal subjects were incubated with guanosine, large amounts of guanosine 5′-triphosphate (GTP) were formed[17]. When erythrocytes from a patient with LNS were incubated with guanosine, there was no significant change in the GTP concentrations. On monitoring the nucleosides and bases, using the procedure described under Experimental, of the erythrocytes from the normal subjects, the exogenous guanosine concentration decreased and no other base or nucleoside peak could be seen. With the erythrocytes from the LNS patient, it was

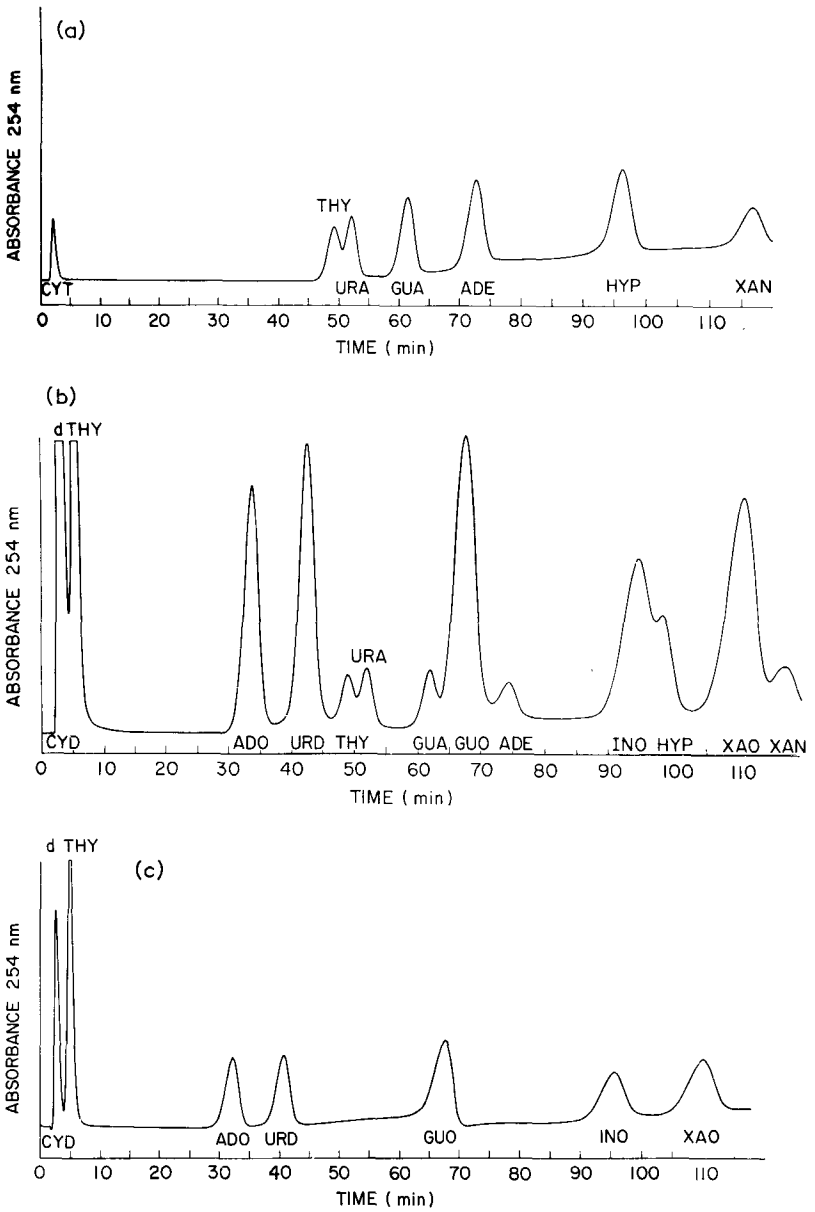

Fig. 1. Separation of purine and pyrimidine bases and nucleosides. (a) Separation of purine and pyrimidine bases: adenine, guanine, hypoxanthine, xanthine, uracil, cytosine, thymine (*ca.* 5 μl of solution of bases, *ca.* 5 m*M* in each). (b) Separation of purine and pyrimidine bases and their ribonucleosides (*ca.* 5 μl of solution of bases, *ca.* 5 m*M* in each; (*ca.* 15 μl of solution of nucleosides, *ca.* 5 m*M* in each). (c) Separation of purine and pyrimidine nucleosides: adenosine, guanosine, inosine, xanthosine, uridine, cytidine and deoxythymidine (*ca.* 15 μl of solution, 5 m*M* in each nucleoside). The operating conditions were as follows. Eluents: low-concentration, 0.05 *M* ammonium acetate, pH adjusted to 9.7; high-concentration, 1.00 *M* ammonium acetate, pH adjusted to 9.7. Flow-rates: column, 12 ml/h; gradient, 6 ml/h. Starting volume: 50 ml. Temperature: 70°. UV output: 0.08 O.D. unit full scale.

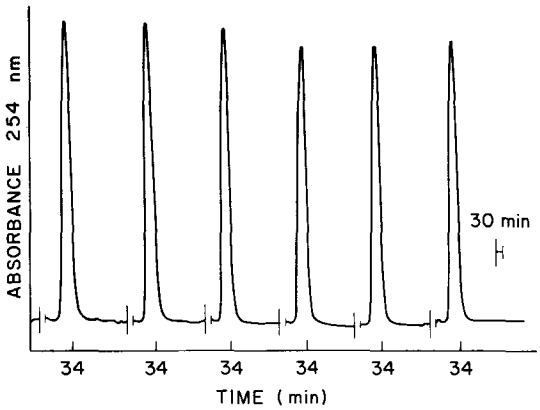

Fig. 2. Reproducibility of peak shape, area and retention time. Chromatograms of adenosine. Operating conditions as in Fig. 1.

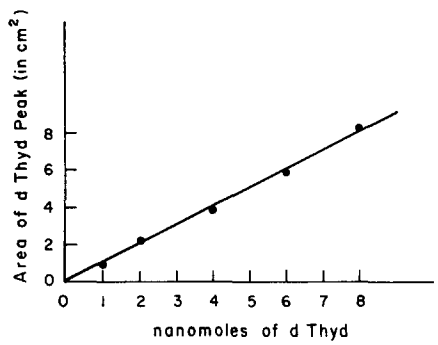

Fig. 3. Plot of concentration (nanomoles) of dThyd *versus* area (cm^2) of dThyd peak.

TABLE I

RETENTION TIMES (min) OF STANDARD PURINE AND PYRIMIDINE BASES AND THEIR NUCLEOSIDES AT DIFFERENT COLUMN AND GRADIENT FLOW-RATES

All other conditions as in Fig. 1.

Compound	*Flow-rates (ml/h)**		
	*12 and 6***	*15 and 7.5****	*20 and 10****
Cyt	3	3	2½
Cyd	3	3	2½
dThyd	6	4	3
Ado	34	11	8
Urd	43	11	12
Thy	49	—	—
Ura	52	19	14
Gua	62	23	18
Guo	68	30	22
Ade	74	35	22
Hyp	97	54	36
Ino	94	54	36
Xao	110	78	48
Xan	117	78	48

* Column flow-rate first, gradient flow-rate second.
** Average of 10 values.
*** Average of 6 values.

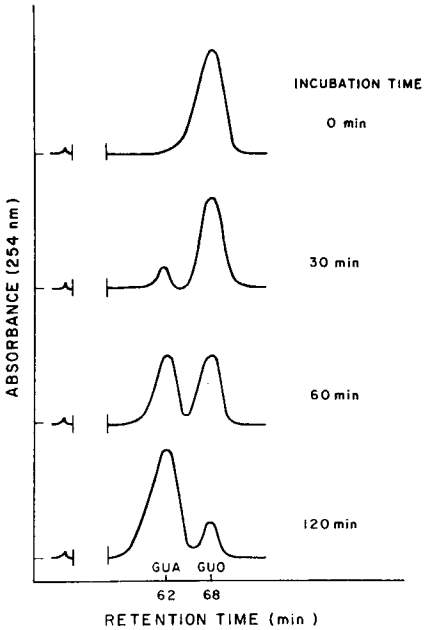

Fig. 4. Chromatogram showing formation of guanine with time after incubation of erythrocytes from LNS patient with guanosine. Operating conditions for HPLC analysis of nucleosides and bases as in Fig. 1.

found that as the guanosine concentration decreased, guanine accumulated (Fig. 4). These results support enzyme research which showed that the enzyme hypoxanthine–guanine phosphoribosyl transferase is either very low or missing in LNS patients[26]. Thus, although guanosine can be converted into guanine by purine nucleoside phosphorylase, the guanine cannot be concerted *in vitro* into its nucleotides in erythrocytes from a patient with this disease.

DISCUSSION

The analytical procedures described can be very useful in investigations of normal purine and pyrimidine metabolism and the abnormalities in the metabolic pathways caused by disease, chemotherapy or other factors such as ageing and pollutants. To date, apart from the studies by the Oak Ridge group on the UV-absorbing constituents in urine[21–25], no comprehensive baseline studies have been carried out on the normal concentrations of nucleosides and bases in other physiological fluids such as blood or spinal fluids and in extracts of tissue. Although the concentrations of nucleosides or bases may be insignificant in blood or tissue from normal subjects, the presence of these compounds in samples of patients with certain diseases may be highly significant and possibly give a clue to the presence of the disease, the mechanism of the disease process or a means of monitoring treatment of the disease. This can be especially important in the study of birth defects involving enzyme deficiencies. For example, in patients with adenosine deaminase deficiency, it has been reported that adenosine, which accumulates in the cells, may be the lethal agent causing immuno-

logical defects[20,27]. Therefore, in this case, a rapid, sensitive and reliable technique for determining adenosine levels could be effectively used in studying this disease. The technique for monitoring the full nucleoside and base profile of the various formed elements of blood may also be important in determining if there are other alterations in nucleoside or base levels occurring as a result of or simultaneously with the increase in adenosine levels. In preliminary work with Dr. Kelley on nucleotide levels in patients with LNS, it was found that blood adenosine nucleotide levels were low. With this technique, it may be possible to monitor a treatment now being used, *i.e.*, the administration of adenine and glucose[19], by following the blood levels of adenine. The nucleotide analysis previously described[4] can be used to determine if the adenine nucleotide concentrations are restored to normal levels with this treatment.

Other possible chemotherapeutic agents may also be investigated, utilizing nucleotide, nucleoside and base analyses in *in vitro* blood studies[18]. In the treatment of cancer and other diseases, purine and pyrimidine analogs are used as chemotherapeutic agents[28]. The HPLC technique described in this paper in combination with the nucleotide analysis described previously[4,5] may prove to be a valuable method for monitoring blood levels of the drug being used, the blood levels of drug metabolites and the concentration of the drug and its metabolites being excreted in the urine.

Although this method, as described for the analysis of nucleosides and purine and pyrimidine bases, can be used in biomedical investigations, we are presently attempting to improve the procedure, *i.e.*, to shorten the time of analysis, to improve the resolution and to increase the sensitivity. Microparticle packings show promise for improved separations and work is under way to establish suitable experimental conditions for the separations of nucleosides and bases using columns packed with particles about 10 μm in diameter. In addition, the separations will be extended to include xanthine analogs such as caffeine, theophylline and theobromine and metabolites of these xanthines.

Baseline studies of the nucleosides and bases in physiological fluids such as whole blood, plasma and spinal fluids as well as extracts of tissues will be carried out. If the concentrations of any nucleosides and/or bases are significant, large numbers of samples from normal subjects will be analyzed so that the results will be statistically valid and can be used as controls in studies of alterations of nucleic acid components caused by disease.

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REFERENCES

1 N. G. Anderson, *Anal. Biochem.*, 4 (1962) 269.
2 N. G. Anderson, J. G. Green, M. L. Barber and L. C. Ladd, *Anal. Biochem.*, 6 (1963) 153.
3 C. G. Horvath, B. Preiss and S. R. Lipsky, *Anal. Chem.*, 39 (1967) 1422.
4 P. R. Brown, *J. Chromatogr.*, 52 (1970) 257.
5 E. M. Scholar, P. R. Brown and R. E. Parks, Jr., *Can. Res.*, 32 (1972) 259.
6 P. R. Brown, R. P. Agarwal, J. Gell and R. E. Parks, Jr., *Compr. Biochem. Physiol.*, 43 (1972) 891.
7 E. M. Scholar, P. R. Brown, R. E. Parks, Jr. and P. Calabresi, *Blood*, 41 (1973) 927.
8 P. R. Brown, J. Herod and R. E. Parks, Jr., *Clin. Chem.*, 19 (1973) 919.
9 C. G. Horvath and S. R. Lipsky, *Anal. Chem.*, 41 (1969) 1227.
10 M. Uziel, C. K. Koh and W. E. Cohn, *Anal. Biochem.*, 25 (1968) 77.
11 R P. Singhal and W. E. Cohn, *Anal. Biochem.*, 45 (1972) 585.
12 W. E. Cohn, *Science*, 109 (1949) 377.
13 P. Reichard and B. Estbarn, *Acta Chem. Scand.*, 4 (1950) 1047.
14 C. F. Crampton, F. R. Fraenkel, A. M. Benson and A. Wade, *Anal. Biochem.*, 1 (1960) 249.
15 M. Uziel and C. Koh, *J. Chromatogr.*, 59 (1971) 188.
16 F. Murakami, S. Rokushika and H. Hatano, *J. Chromatogr.*, 53 (1970) 584.
17 P. R. Brown and R. E. Parks, Jr., *Anal. Chem.*, 45 (1973) 948.
18 R. E. Parks, Jr. and P. R. Brown, *Biochemistry*, 12 (1973) 3294.
19 G. Sperling, in A. D. Vries and J. B. Wyngarden (Editors), *Purine Metabolism in Man, Advances in Experimental Medicine and Biology*, Vols. 41A and 41B, Plenum Press, New York, 1973.
20 *Birth Defects Institute, Symposium IV, Combined Immunological Disease — A Molecular Defect?*, Academic Press, New York, in press.
21 C. D. Scott, J. E. Attrill and N. G. Anderson, *Proc. Soc. Exp. Biol. Med.*, 125 (1967) 181.
22 C. D. Scott, *Clin. Chem.*, 14 (1968) 41.
23 C. D. Scott, R. L. Jolley and W. F. Johnson, *Amer. J. Clin. Pathol.*, 53 (1970) 701.
24 J. E. Mrochek, W. C. Butts, W. C. Rainey and C. A. Burtis, *Clin. Chem.*, 17 (1971) 72.
25 C. D. Scott, D.D. Chilcote and N.E. Lee, *Anal. Chem.*, 44 (1972) 85.
26 L. A. McDonald and W. N. Kelley, *Biochem. Genet.*, 6 (1972) 21.
27 K. Ishii and H. Green, *J. Cell Sci.*, 13 (1973) 1.
28 L. S. Goodman and A. Gilman, *The Pharmacological Basis of Therapeutics*, Macmillan, New York, 1967.