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Kinetics of Adenosine Uptake by Erythrocytes, and the Influence of Dipyridamole

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SUMMARY

The uptake of adenosine into erythrocytes takes place by two different mechanisms. One is saturable by high substrate concentrations, is inhibited by dipyridamole, and can be described in terms of Michaelis-Menten kinetics. The other mechanism is proportional to the adenosine concentration and has the characteristics of diffusion across lipophilic areas of the membrane. The total uptake (Y) fits a model described by the equation

$$Y = \frac{V_{max}}{1 + K_m/S} + P_d S \quad (1)$$

where the apparent K_m and V_{max} values are derived from the Michaelis-Menten equation, P_d is the proportional constant of the simplified law of diffusion, and S is the substrate concentration.

Evidence for the validity of the model was obtained by comparing the uptake of four purine derivatives, inosine, adenosine, N^6-methyladenosine, and N^6-dimethyladenosine. These four derivatives possess different octanol/water partition coefficients as a consequence of the different groups at C-6 of the purine molecule. The uptake by diffusion correlates with the partition coefficients of the four derivatives. Dipyridamole inhibits only the saturable part, which follows Michaelis-Menten kinetics. Which of the two uptake mechanisms predominates depends on substrate concentration, and is important for understanding the mechanism of action of dipyridamole.

INTRODUCTION

Adenosine is taken up rapidly by the heart (1, 2), lungs, liver (3), and erythrocytes (4). The rate of elimination from the extracellular space is important in the role of adenosine as a coronary vasodilator and appears to be related to the mode of therapeutic action of the coronary vasodilators dipyridamole, hexobendine, and lidoflazine (5–10).

The subject of this paper is the study of this elimination mechanism. The experiments were performed on erythrocytes because their adenosine uptake mechanism is similar to that of the heart and they are easier to handle. Erythrocytes of guinea pigs were used because their adenosine metabolism is less rapid than in humans or other species.[1]

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This paper is dedicated to Professor H. J. Bielig in honor of his 60th birthday.

[1] Unpublished observations.

MATERIALS AND METHODS

Experiments with erythrocytes. For preparation of red cells, blood was withdrawn from guinea pigs by cardiac puncture and mixed with heparin, 0.5 g/liter. After centrifugation at 1200 × *g* for 2 min, the supernatant fraction was removed and the red cells were washed five times with an ice-cold solution of the following composition: NaCl, 166 mM, and Tris-HCl, pH 7.4. The red cells were then suspended with the same solution to give a 20–25% hematocrit.

Substrates were prepared as follows. Adenosine was placed in a series of tubes in the amounts listed in Table 3. To each tube were added 0.1 μCi of ^{14}C-labeled adenosine disolved in 0.05 ml of ice-cold 166 mM NaCl in Tris-HCl, pH 7.4, and 0.02 ml of 2 μM dipyridamole. A second series of tubes was prepared without dipyridamole to act as a control. This process was repeated for inosine, N^6-methyladenosine, and N^6-dimethyladenosine. The erythrocyte and substrate preparations were warmed separately to 37°. The red cells were added to each of the substrate preparations and mixed to start the incubation. Under the experimental conditions the uptake of inosine, adenosine, and N^6-methyladenosine was linear up to 4 min. Therefore an incubation time of 3 min was used. N^6-Dimethyladenosine was incubated for only 30 sec because of its very rapid uptake. Incubation was stopped by addition of 7 ml of the ice-cold NaCl-Tris solution, using an automatic pipette (Quickfit, England) to achieve immediate, thorough mixing. The suspension of erythrocytes was then centrifuged at 1600 × *g* and 0° for 45 sec, and the supernatant fluid was carefully removed with a capillary tube and replaced with ice-cold 166 mM NaCl in Tris-HCl, pH 7.4, mixing vigorously. This process took 90 sec and was repeated three times.

The ^{14}C content of the packed erythrocytes was measured after deproteinization with 2 ml of ice-cold 5% trichloracetic acid, using vigorous shaking. After centrifugation, 1 ml of the supernatant solution was added to 9 ml of the scintillation fluid of Bray (11) and counted in a Packard, Tri-Carb model 2000 scintillation spectrometer. Correction for quenching was performed by external standardization. At least 10,000 cpm were registered for each sample. The uptake was calculated in moles per liter of packed erythrocytes from the disintegrations per minute and the specific activity of the added test substance.

Calculations. The experimental values were fitted to our postulated model, as shown in Eq. 1, by the method of least squares. The deviations of the experimental from the calculated values obtained by Eq. 1 were used to test the validity of the model. The method of calculation is given in the APPENDIX.

Materials. [8-^{14}C]Adenosine (specific activity, 49 mCi/mmole) and [8-^{14}C]inosine (specific activity, 264 mCi/mmole) were obtained from the Radiochemical Centre, Amersham, England. [*methyl*-^{14}C]N^6-Methyladenosine and [*methyl*-^{14}C]N^6-dimethyladenosine were synthesized from 6-chloropurine riboside and [*methyl*-^{14}C]methylamine or [*methyl*-^{14}C]dimethylamine, respectively (12). Both substances were purified by thin-layer chromatography. The specific activity was 36 mCi/mmole for methyladenosine and 23 mCi/mmole for dimethyladenosine.

The nonradioactive purine derivatives and dipyridamole (Persantin) were kindly supplied by Fa. PWA/Waldhof, Aschaffenburg, Germany, and Fa. Thomae, Biberach/Riss, Germany.

Octanol/water partition. Octanol (5 ml) was vigorously shaken for 60 sec with 5 ml of ice-cold 166 mM NaCl in Tris-HCl, pH 7.4, containing 0.037 mmole of the unlabeled and 0.1 μCi of the labeled test substance. The radioactivity of 1 ml each of the octanol and water phases was measured as described in the preceding section.

RESULTS

The first question was whether or not adenosine was taken up via diffusion. If so, the addition of increasing amounts of adenosine should result in a linear proportional increase in uptake. However, with low concentrations of adenosine in the incubation medium the uptake was high initially but fell as the adenosine concentration was raised (Fig. 1). This nonlinear dependence of up-

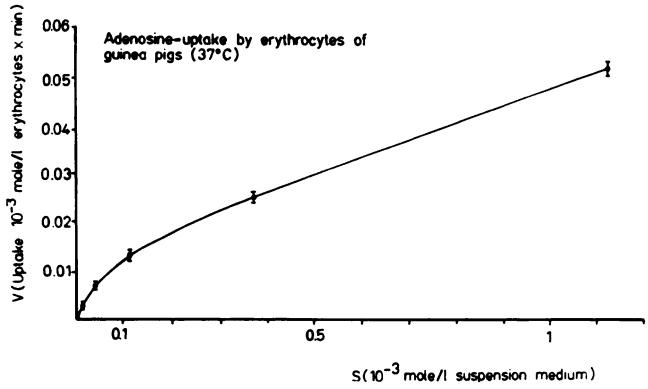

FIG. 1. *Uptake of adenosine by erythrocytes of guinea pigs, depending on concentration of adenosine (S) in suspension medium*

Each point is the mean of five experiments. Vertical brackets represent standard deviations.

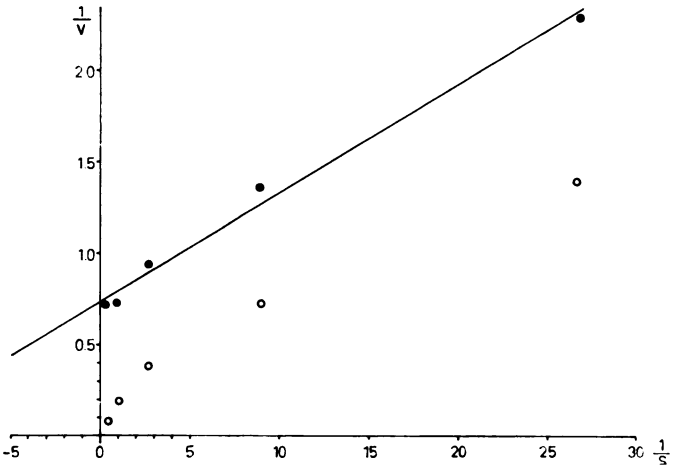

FIG. 2. *Lineweaver-Burk plot of adenosine uptake*

○, data from Fig. 1 (S = data × 10^3; V = data × 10^2); ●, the same data after subtraction of the P_dS component, obtained from Eq. 1.

take on concentration is not in agreement with the law of diffusion, but is similar to a saturable enzymatic reaction. Therefore we tested whether adenosine uptake could be described by a model conforming to Michaelis-Menten kinetics. This treatment was justified because transport mechanisms across cell membranes are similar to enzymatic actions (13). The experimental data for adenosine uptake in a Lineweaver-Burk plot are shown in Fig. 2 (open characters). The correlation betweed $1/v$ and $1/S$ could not be represented by a straight line, especially not at higher concentrations of adenosine. Therefore Michaelis-Menten kinetics does not provide a valid description of adenosine uptake. We have assumed that besides a saturable process a second mechanism—diffusion, directly dependent on concentration—governs the uptake of adenosine. This assumption is based on the observation that the deviations from a straight line increase with higher concentrations. If this hypothesis is true, the uptake of adenosine should follow an equation which is the sum of Michaelis-Menten kinetics plus the law of diffusion:

$$Y = \frac{V_{max}}{1 + K_m/S} + P_dS \qquad (1)$$

TABLE 1

Constants of Eq. 1, describing uptake of four purine derivatives, found by approximation of experimental data

SD = standard deviation of the experimental data from the values calculated by Eq. 1. RD = relative deviation, setting the calculated data at 100%. Values following the plus-or-minus signs represent standard deviations of the constants.

Purine derivative	V_{max}	K_m	P_d	SD	RD
	$\mu M/min$	μM			
Inosine	25.8 ± 1.98	241.3 ± 41.6	0.0019 ± 0.0005	±0.35	±3.18
Adenosine	20.1 ± 1.42	101.8 ± 20.7	0.0293 ± 0.0004	±0.45	±1.87
N^6-Methyladenosine	31.2 ± 3.38	80.8 ± 25.8	0.1654 ± 0.0011	±1.21	±0.98
N^6-Dimethyladenosine	64.3 ± 34.06	21.9 ± 26.7	1.4988 ± 0.0947	±6.16	±1.09

where Y = total uptake of adenosine, composed of Michaelis-Menten kinetics and the law of diffusion

V_{max} corresponds to maximum uptake by erythrocytes per minute (apparent V_{max})

K_m = concentration producing half-saturation of uptake (apparent K_m)

P_d corresponds to proportional constant of a simplified law of diffusion and, for erythrocytes, contains unknown parameters of the diffusion law

S = concentration of adenosine in suspension medium

The constants V_{max}, K_m, and P_d are unknown and can be found by approximating the experimental data for adenosine uptake to Eq. 1 by the method of least squares (Table 1). The mean relative deviation of the experimental data from the theoretical values calculated by the three constants shows a relative deviation of only 1.8% for adenosine uptake. This proves the practicability of the above equation for describing adenosine uptake. Substracting P_dS, using the P_d values given in Table 1, and plotting the remaining hypothetical enzyme-like part by the Lineweaver-Burk method yields the expected linear dependence (Fig. 2, filled characters).

In previous experiments it was shown that the pharmacological effect of adenosine is potentiated by inosine, N^6-methyladenosine, and N^6-dimethyladenosine (12). Therefore it was assumed that these compounds are taken up in the same way. Table 1 shows that the uptakes of inosine, N^6-methyladenosine, and N^6-dimethyladenosine can be fitted to the same model. The standard and relative deviations are small. The constants V_{max} and K_m are in the same order of magnitude. However, there are marked differences among the constants P_d for the four substances.

For a better comparison of the four compounds, the dependence of uptake on concentration is demonstrated graphically in Fig. 3. The solid lines represent the total uptake of the four compounds, and the broken lines show the P_dS component. For inosine P_dS is very small; for adenosine it is 10 times higher, and so forth. Figure 3 reveals that the differences among the four compounds are attributable to diffusion, i.e., P_dS.

The velocity of diffusion of a chemical compound through a membrane depends on molecular weight and physicochemical properties. Hence the constant P_d should correlate with at least one of these properties. The differences in molecular weight of the tested substances are too small to influence the velocity of diffusion appreciably. The OH, NH_2, $NHCH_3$, and $N(CH_3)_2$ groups at C-6 of the purine molecule, however, should affect diffusion because of their charge or solubility in lipophilic areas of the membrane. If so, there should exist a correlation between the oil/water partition coefficients of the four compounds and their uptake as

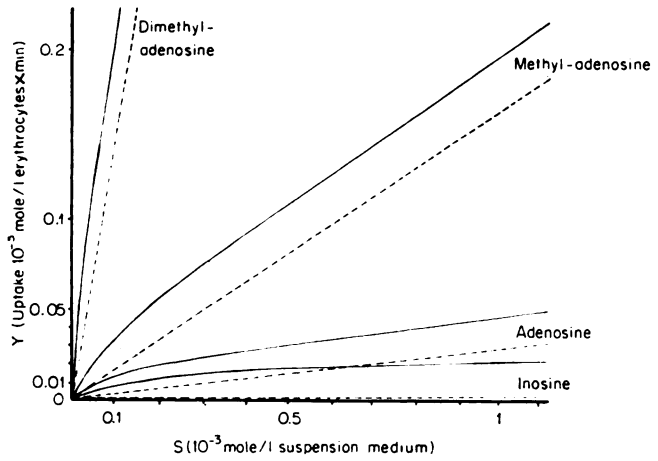

FIG. 3. *Uptake of purine derivates by erythrocytes of guinea pigs*

The uptake of the four purine derivatives, depending on the concentration of the suspension medium, was calculated with Eq. 1 and the constants of Table 1. ——, total uptake = V (millimoles per liter of erythrocytes per minute); - - -, component resulting from P_dS.

represented by P_dS. Octanol was used to study partition, because the solubility of inosine and adenosine in heptane and chloroform, which are commonly used for this purpose, is too low to be comparable with the expected solubility of these nucleosides in cell membranes. There are great differences in the octanol/water partition coefficients, which correlate well with the uptake constant, P_d (Table 2, last column). This result justifies the conclusion that the P_dS component represents diffusion through lipophilic areas of the membrane.

The validity of the assumed uptake mechanism can be tested as follows. The uptake of adenosine is inhibited by concentrations of dipyridamole as low as 1 nM (14). If the P_dS component is diffusion-limited, and the remainder is an enzyme-like reaction, only the latter should be totally inhibited by dipyridamole. Increasing concentrations of dipyridamole up to 1 μM and even higher did not inhibit adenosine uptake completely. With such high concentrations it can be shown that the uptake component following Michaelis-Menten kinetics is exclusively abolished by dipyridamole.

The ratios of control uptake to that in the presence of 2 μM dipyridamole are listed in Table 3. The boldface values are those measured, and the others are those predicted by Eq. 1 with the constants listed in Table 1. The agreement of both values is satisfactory for inosine, adenosine, and N^6-methyladenosine. The uptake of N^6-dimethyladenosine could not be inhibited by dipyridamole. However, the diffusion component of the uptake of this compound is very high (Fig. 3), and its K_m is smaller than the K_m of adenosine. Higher concentrations of dipyridamole may also be necessary to inhibit N^6-dimethyladenosine uptake, but such experiments were not done. The degree of inhibition for adenosine, inosine, and N^6-methyladenosine decreases inversely with higher substrate concentrations, presumably because the dipyridamole inhibition is com-

TABLE 2

Octanol/water partition coefficient at pH 7.4 and room temperature

The suspension medium for erythrocytes was used as the water phase. The concentration of the substrates was 0.112 mM.

Test compound	Octanol/water partition coefficient	P_d (of P_dS component)
Inosine	0.0034	0.0019
Adenosine	0.0381	0.0293
N^6-Methyl-adenosine	0.108	0.1654
N^6-Dimethyl-adenosine	0.363	1.4988

TABLE 3

Substrate concentration dependence of inhibition of uptake of four purine derivatives by 2 μM dipyrimadole

The values in boldface type represent the experimental data. Each is the mean of five experiments. The other values were calculated using P_dS of Eq. 1.

Compound	Ratio of uptake of controls to uptake with dipyridamole				
	0.037 mM	0.112 mM	0.374 mM	1.123 mM	3.745 mM
Inosine	48.7	38.6	22.6	10.8	4.3
	30.0			**12.3**	
Adenosine	5.9	4.2	2.4	1.6	1.2
	6.3	**3.8**	**2.0**	**1.5**	**1.0**
N^6-Methyl-adenosine	2.6	2.0	1.4	1.2	1.0
	1.5			**1.0**	
N^6-Dimethyl-adenosine	1.7	1.3	1.1	1.0	1.0
	0.99			**0.96**	

petitive, as shown in preliminary experiments (12).

DISCUSSION

The experiments described above reveal that the mechanism of adenosine uptake possesses two components. One component is saturable and kinetically resembles an enzymatic reaction. It is described by a Michaelis-Menten type of equation and is inhibited by dipyridamole. A second component corresponds to the law of diffusion, is not saturable, cannot be inhibited, and appears to represent diffusion across lipophilic parts of the membrane. Evidence for the validity of this model was obtained in three different ways: (a) approximation of the experimental data for uptake of the four purine derivatives to the mathematical model (Eq. 1), (b) inhibition of the enzyme-like component of the uptake model by dipyridamole, and (c) correlation of the oil/water partition of the four purine derivatives with the diffusion component (P_dS) of the model.

These results are not in contradiction to the findings of Schrader and Berne (15), who found that 0.1 mM dipyridamole completely blocked the uptake of 0.5–5.0 μM adenosine by red cell ghosts. Their experiments, however, were performed with only 5–50% of the lowest concentration used in our investigations. In this range of very low concentrations, where the enzyme-like component predominates, nearly total inhibition would be expected.

Lassen (16) postulated a two-component system for the transport of hypoxanthine and uric acid across the red cell membrane. This mechanism, however, should be different from that for adenosine, because the uptake of adenine, hypoxanthine, and uric acid cannot be blocked even by high concentrations of dipyridamole (1).

The permeation of uridine, thymidine, cytidine, and cytidine arabinoside across erythrocyte membranes, recently described by Oliver and Paterson (17) and Lieu *et al.* (18), also probably does not follow the same mechanism as adenosine. Compounds using the same transport mechanism should prevent the elimination of adenosine from the receptor site and in this way potentiate the pharmacological effect of adenosine. This has been demonstrated by the potentiation of the effect of adenosine on heart rate (19). Uridine and thymidine did not potentiate adenosine.[1]

The present data do not indicate whether the enzyme-like component is either active transport, passive carrier-mediated transfer, or uptake induced by intracellular phosphorylation. All these possibilities may correspond to Michaelis-Menten kinetics and be inhibited by dipyridamole. Evidence for the first and second mechanisms would be the finding that adenosine is transported against a gradient. However, adenosine and inosine are phosphorylated to mono-, di-, and triphosphates (20), and N^6-methyladenosine and N^6-dimethyladenosine, to the corresponding monophosphates (12). This phosphorylation process occurs on or in the membrane and is very fast (21, 22). No gradient was demonstrable. Inhibitors of ATP metabolism, such as ouabain or 2,4-dinitrophenol, were without effect on adenosine uptake.[1] Thus further experiments, presumably with inhibitors of adenosine phosphorylation or with red cell ghosts, may solve this problem.

The third possibility, intracellular phosphorylation, can be excluded. Adenosine phosphorylation cannot be inhibited even

with very high concentrations of dipyridamole (1 mM). This is shown by experiments that were performed on heart homogenates (12) and confirms the results of Schrader and Berne on red cell ghosts (15).

Studies of temperature dependence did not distinguish between the two remaining mechanisms of uptake. The diffusion component of adenosine uptake showed the same high degree of temperature dependence as the enzyme-like part. Sodium leakage, which is assumed to be a passive process, also is highly temperature-dependent (22). Thus high temperature dependence does not prove the existence of an active process.

What is the meaning of these observations with respect to the action of dipyridamole on the coronary circulation? The adenosine concentration in the smooth muscle of coronary vessels is presumably an important determinant of coronary dilation. Coronary occlusion increases the myocardial adenosine concentration (23), and this increase appears to be due to release of the nucleoside into the extracellular space (21). The amount released exceeds the concentration required to elicit maximal coronary dilation (24). However, as the extracellular adenosine is washed out by the coronary circulation, the adenosine concentration in the blood may fall to levels at which the saturable transport component predominates. Thus the inhibition by dipyridamole of uptake by red cells may lead to an augmentation of adenosine concentration in the smooth muscle of the coronary vessels and coronary dilation.

APPENDIX

Fitting of Measured Values to Postulated Equation

This method is not generally known. It can be applied to other problems, such as resorption from intestine. The aim is to provide evidence for the validity of the hypothetical model in terms of Eq. 1. This can be done by fitting the experimental data to Eq. 1,

$$Y = \frac{V_{max}}{1 + K_m/S} + P_d S \qquad (1)$$

where Y is the total uptake, V_{max} corresponds to the maximum uptake per minute, K_m is the concentration producing half-saturation, P_d corresponds to the proportional constant of the law of diffusion, and S is the concentration of adenosine in the suspension medium.

The relative standard deviation of the calculated from the experimental data indicates the validity of the equation. The experimental data were fitted to Eq. 1 by the method of least squares. The following calculations and special transformations of Eq. 1 were used; approximate values of the true V_{max}, K_m, and P_d can be estimated using the Lineweaver-Burk plot of Fig. 1. These estimations are represented by V, K, and P. If we calculate the uptake from these estimated values we obtain, instead of the true uptake, Y, the estimated uptake, Y'. The difference between Y and Y' is ΔY, which can also be approximated by the total differential:

$$\Delta Y = \left(\frac{\partial Y'}{\partial V_{max}}\right)\Delta V_{max} + \left(\frac{\partial Y'}{\partial K_m}\right)\Delta K_m + \left(\frac{\partial Y'}{\partial P_d}\right)\Delta P_d \qquad (2)$$

From Eq. 1 the derivatives in Eq. 2 are

$$\frac{\partial Y'}{\partial V_{max}} = \frac{1}{1 + K/S} = a$$

$$\frac{\partial Y'}{\partial K_m} = -\frac{V}{(1 + K/S)^2 S} = -b$$

$$\frac{\partial Y'}{\partial P_d} = S = c$$

Notice that a, b, and c depend on the values chosen for V and K, and on the concentration S_i for any particular experimental observation.

Substituting these derivatives for their corresponding values in Eq. 2, we get, for any selected concentration S_i,

$$\Delta Y = Y_i - Y_i' = a\Delta V_{max} - b\Delta K_m + c\Delta P_d \qquad (3)$$

An approximate least-squares solution can be obtained as follows. Let V_i be the difference in the ith measurement between the value Y_i and the observed Y_{0i}. Then V_i is given by

$$V_i = Y_i - Y_{0i} = (Y_i - Y_i') - (Y_{0i} - Y_i') = \Delta Y_i - \Delta Y_i^* \qquad (4)$$

where ΔY_i^* is defined by the above equation as the difference between Y_i', calculated using V, K, and P, and the experimental value Y_{0i}. Values of V, K, and P which give a minimum for the sum over i of V^2 are to be chosen as the best estimates. Hence we wish to find values of ΔV_{max}, ΔK_m, and ΔP_d to add to V, K, and P to estimate V_{max}, K_m, and P_d. This can be achieved by differentiating the expression for the sum of V_i^2.

$$\sum_i V_i^2 = (\sum_i a_i \Delta V_{max} - \sum_i b_i \Delta K_m + \sum_i c_i \Delta P_d - \sum_i \Delta Y_i^*)^2 \quad (5)$$

with respect to the parameters ΔV_{max}, ΔK_m, and ΔP_d, and setting the derivatives equal to zero. This yields the following three normal equations:

$$\Delta V_{max} \sum aa - \Delta K_m \sum ab + \Delta P_d \sum ac = \sum a \Delta Y^* \quad (6)$$

$$\Delta V_{max} \sum ab - \Delta K_m \sum bb + \Delta P_d \sum bc = \sum b \Delta Y^* \quad (7)$$

$$\Delta V_{max} \sum ac - \Delta K_m \sum bc + \Delta P_d \sum cc = \sum c \Delta Y^* \quad (8)$$

(The subscript i is dropped from a, b, c, and Y^* and from the summation sign.)

The calculations in this paper were performed with a Wang 700 desk computer. For the calculation of a, b, and c the estimated values V, K, and P were used. Experience showed that these were not critical. Estimated values that were 5 times too high or too low gave the same results for adenosine uptake. No weighting factors were necessary, all points being considered to be equally precisely measured. The constants can be improved by iteration of the solution of the above normal Eqs. 5–7, using the newly obtained values of V, K, and P. After three iterations no further improvement could be achieved. After the last iteration the standard deviation from Y, calculated with Eq. 1 and the experimental data, Y_0, is obtained by

$$SD = \sqrt{(Y_i - Y_{oi})^2/(n - 1)}$$

and the relative deviation, by setting $Y = 100\%$. The individual deviations SD_i of V_{max}, K_m, and P_d are obtained by (25)

$$SD_i = SD \sqrt{(\cdots)}$$

where $(\cdots)$ represents the diagonal element from the last iteration calculated from the matrix of the normal Eqs. 6–8.

The entire program involves the following steps: (a) storing the data for adenosine uptake and substrate concentrations; (b) storing the estimated values V, K, and P; (c) construction of the matrix for solution of the normal equations from the stored data; (d) correction of the values V, K, and P; (e) five iterations of steps (c) and (d); (f) calculating the standard deviations of the function and of V_{max}, K_m, and P_d; and (g) printout of the stored data and results. As the capacity of the core of the Wang 700 is small, the results of steps (f) and (g) were temporarily stored on magnetic tape.

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