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A Simple Competitive Protein-Binding Assay for Cyclic Adenosine 3′:5′-monophosphate

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A cyclic adenosine 3′:5′-monophosphate (Ado-3′:5′-P) assay is proposed basing on the principle of competitive protein-binding.
Free and bound Ado-3′:5′-P is separated by coated (Dextran T 70, bovine serum albumine) charcoal. The Ado-3′:5′-P binding protein is obtained from bull adrenals. The detection limit is 0.25 pmol in the incubation tube. The affinity constant of the protein-Ado-3′:5′-P complex is $2.8 \cdot 10^8$ l/mol. The free enthalpy is approximate 10 kcal/mol. The high specificity is tested with other similar nucleotides. There is a great difference between the N^6-mono- and N^6-2′O-dibutyryl derivatives of Ado-3′:5′-P with respect to their affinities for the binding protein, or protein kinase.
The criteria of reliability are given by precision, detection limit, specificity and accuracy data.
The method is suitable for serial determinations. One person is able to measure 100 samples (including the standard curve) per day.
For linearisation of the dose response curve a new method is described. The proposed plot has the advantages of simplicity and ease of curve calculation. In addition, the plot yields the affinity constant and binding capacity of the binding protein. Moreover, this method allows the estimation of the affinity constant of any competitor of a labeled ligand. This method for linearisation is discussed regarding its limitations and advantages.

Es wird eine Bestimmungsmethode für cyclisches Adenosin-3′:5′-monophosphat (Ado-3′:5′-P) vorgeschlagen, die auf einer kompetitiven Verdrängungsreaktion von ^3H- und nichtmarkiertem Ado-3′:5′-P an einem spezifisch Ado-3′:5′-P bindenden Protein beruht, das aus Nebennieren von Stieren erhalten wird. Die Trennung von freiem und gebundenem Ado-3′:5′-P erfolgt durch mit Dextran T 70 und Rinderserumalbumin vorbehandelter Aktivkohle. Die Nachweisgrenze für Ado-3′:5′-P liegt bei 0,25 pMol ($p \leqq 0{,}05$) im Bestimmungsansatz. Die Affinitätskonstante beträgt $2{,}8 \cdot 10^8$ l/Mol. Die freie Enthalpie für die Reaktion der Anlagerung von Ado-3′:5′-P an das bindende Protein beläuft sich auf etwa 10 kcal/Mol. Die Spezifität der Methode ist hoch; ähnlich gebaute Nucleotide sind weitgehend ohne Einfluß auf die Reaktion. Für die beiden Ado-3′:5′-P-Derivate N^6-Mono- und N^6-2′O-Dibutyryl-Ado-3′:5′-P besteht ein großer Unterschied in der Affinität zum bindenden Protein bzw. der Proteinkinase.
Die Zuverlässigkeit der Methode wird mit Daten für die Präzision, die Nachweisgrenze, die Spezifität und die Richtigkeit belegt.
Die Methode ist für Serienbestimmungen geeignet; eine Assistentin kann etwa 100 Proben (einschließlich Standard-Kurve) am Tag messen.
Zur Linearisierung der Dosis-Wirkungskurve wird eine neue Methode beschrieben, deren Vorteile und Grenzen diskutiert werden.
Die vorgeschlagene graphische Darstellung erlaubt eine einfache Kurvenberechnung sowie die Ermittlung der Affinitätskonstante und der Bindungskapazität des Bindungsproteins. Darüberhinaus kann unter Verwendung nur eines markierten Liganden die Affinitätskonstante für jede kompetitiv wirksame Verbindung bestimmt werden.

The original and most commonly used analytical method for Ado-3′:5′-P detection is based on phosphorylase kinase (EC 2.7.1.38) activation (1, 2). Another method depends on the ability of specific protein kinases (EC 2.7.1.) to phosphorylate histones or casein with ATP-[γ-^{32}P] (3, 4). The following methods have been developed for detecting Ado-3′:5′-P: The nucleotide is enzymatically converted to ADP-[β-^{32}P] (5), or to ATP, and the latter is measured either by a radioactive phosphate exchange reaction with coupled enzymes (6) or luciferase method (7). Alternatively the enzymatic cycling developed by Breckenridge (8), or the resulting ATP, is assayed by $^{14}CO_2$ evolved from glucose-[1-^{14}C] by sequential enzymatic reactions (9). Chromatographic methods are proposed by Bradham and Wooley (10) and Krishna and coworkers (11). A modification of isotopic dilution principle is developed by Brooker and coworkers (12) for Ado-3′:5′-P determination.

Many of the methods mentioned above are less than ideal from the standpoint of standardisation, sensitivity and specificity, and all are laborious.
Recently a method for determination of Ado-3′:5′-P has been proposed, based on a specific Ado-3′:5′-P binding to a protein. Both a radioimmuno-assay (Steiner and coworkers (13)) and a competitive protein-binding assay (Gilman (14), Walton and Garren (15)) were developed. In the latter case Ado-3′:5′-P is specifically bound on a protein kinase from muscle or adrenal gland (16—20).
The assay proposed by us makes use of the principle of competitive protein binding.
The binding protein is extracted from bull adrenal-gland homogenates by centrifugation. It can be used without further purification for a sensitive and specific determination of Ado-3′:5′-P. Dextran T 70 bovine serum albumin-coated charcoal is used to accomplish a convenient and time-saving separation of the free from

the bound Ado-3':5'-P without reducing the sensitivity of the method.
Thus our assay, compared with the methods l. c. (13—15) is easy, rapid, and yet sensitive.

Material and Methods

Chemicals

Adenosine 3':5'-monophosphate, Ado-3':5'-P
Guanosine 3':5-monophosphate, Guo-3':5'-P
Cytidine 3':5'-monophosphate, Cyd-3':5'-P
Uridine 3':5'-monophosphate, Urd-3':5'-P
N^6-monobutyryl-adenosine 3':5'-monophosphate
N^6-2'O-dibutyryl-adenosine 3':5'-monophosphate
Adenosine 5'-triphosphate, ATP
Guanosine 5'-triphosphate, GTP
Adenosine 5'-monophosphate, Ado-5'-P
Adenosine 3'-monophosphate, Ado-3'-P
Phosphodiesterase from bovine heart, Orthophosphoric diester phosphohydrolase (EC 3.1.4.).
All these substances were obtained from Boehringer/Mannheim Bovine serum albumine (Fa. Hoechst).

Binding protein preparation

The Ado-3':5'-P binding protein was obtained from bull adrenal glands. The adrenal glands were removed immediately after slaughtering and placed on ice. The fresh adrenal glands were chopped and homogenized in two volumes of ice-cold buffer I. The homogenate was centrifuged at 3000 *g* for 10 min, followed by a 6000 *g* centrifugation of the supernatant for 15 min. Then the supernatant was removed and aliquots of 2 or 3 ml were placed in polyethylene bottles, shock-frozen in a cold-mixture (e. g. methanol-solid CO_2 bath), and stored at —20°. Before use a bottle is thawed and centrifuged at 30,000 *g* for 15 min. The supernatant is normally used in a dilution of 1:5 with buffer II for assay.
No loss of binding-protein activity has been observed after 9 months.

Protein determination

Protein concentration was determined by the method of Lowry and coworkers (21) with bovine serum albumin as standard.

Buffers

Buffer I

50 mmol/l Tris, 0.25 mol/l Sucrose, 25 mmol/l KCl, 5 mmol/l $MgCl_2$, pH-value was adjusted to 7.4 using HCl.

Buffer II

50 mmol/l Tris, 8 mmol/l theophylline, 6 mmol/l 2-mercaptoethanol, pH-value was adjusted to 7,4 using HCl

Buffer III

50 mmol/l Tris, 8 mmol/l theophylline, 6 mmol 2-mercaptoethanol, 0.25% bovine serum albumine, pH-value was adjusted to 7.4 using HCl.

Tracer

^{3}H-Ado-3':5'-P, specific activity 24.1 Ci/mmol (NEN-Chemicals). The substance was shown to be carrier-free (> 99%) and chemically pure (thin-layer chromatography on PEI-cellulose with the solvent system saturated $(NH_4)_2SO_4$/1 mol/l sodium acetate/isopropanol (80:18:2, v/v)).
The saturating concentration for assay was 40 nCi (88,800 dpm)

Scintillation mixture

PPO 5.0 g, POPOP 200 mg, naphthalin 100 g in 1 l dioxan.

Separation mixture

5.0 g charcoal (Norit A, Serva)
0.5 g Dextran T 70 (Pharmacia, Sweden)
50.0 ml Tris-buffer III.

Tissue preparation

Tissue samples (liver, kidney) from ether anesthetized Wistar rats were frozen in liquid N_2 immediately after removing.
Then pieces were weighed (about 10 mg) and homogenized in 1 ml 6% trichloroacetic acid. After centrifugation the supernatant was extracted 8 times with 5 ml water saturated diethylether. An aliquot of the aqueous phase was dried and redissolved in 50 µl buffer II for determination of Ado-3':5'-P (see Tab. 1).
Recovery of added ^{3}H-Ado-3':5'-P showed that extraction procedure is free from losses (94—108%).

Procedure (Tab. 1).

Results

The competitive binding protein assay of Ado-3':5'-P results in a nonlinear dose response curve, when the bound over free (B/F) ratio or the percent-binding $\left(Y = 100 \cdot \frac{B - N}{B_0 - N}\right)$ of labeled compound are plotted versus dose (Fig. 1).

Tab. 1

Steps in procedure for measurement of Ado-3':5'-P

1—2 total activity; 3—4 control value; 5—20 standard values 21—22 control value of unknown sample; 23ff. unknown samples

Sample No.	1—2	3—4	5—6	7—8	9—10	11—12	13—14	15—16	17—18	19—20	21—22	23—24	25—26	27—28	29—30	31—32
Ado-3':5'-P standard (µl)	0	0	0	50	50	50	50	50	50	50	0	0	0	0	0	etc.
Ado-3':5'-P (pmol)	0	0	0	0.25	0.625	1.25	2.5	5.0	7.5	12.5						
Unknown sample (µl)	0	0	0	0	0	0	0	0	0	0	50	50	50	50	50	etc.
^{3}H-Ado-3':5'-P (µl) (40 nCi)	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	etc.
Tris buffer (µl)	250	250	150	100	100	100	100	100	100	100	200	100	100	100	100	etc.
Binding protein (µl)	100	0	100	100	100	100	100	100	100	100	0	100	100	100	100	etc.
	Mix gently. Incubation time 2 hours at 4° C															
Separation: Charcoal suspension (µl)	0	100	100	100	100	100	100	100	100	100	100	100	100	100	100	etc.
	Mix gently and centrifuge at 2000 *g* for 15 minutes at 4° C															
	200 µl of the supernatant are counted in 10 ml of scintillation liquid. All samples are precisely counted to 1% statistics. Quenching is always the same. The efficiency is 43 ± 1%															

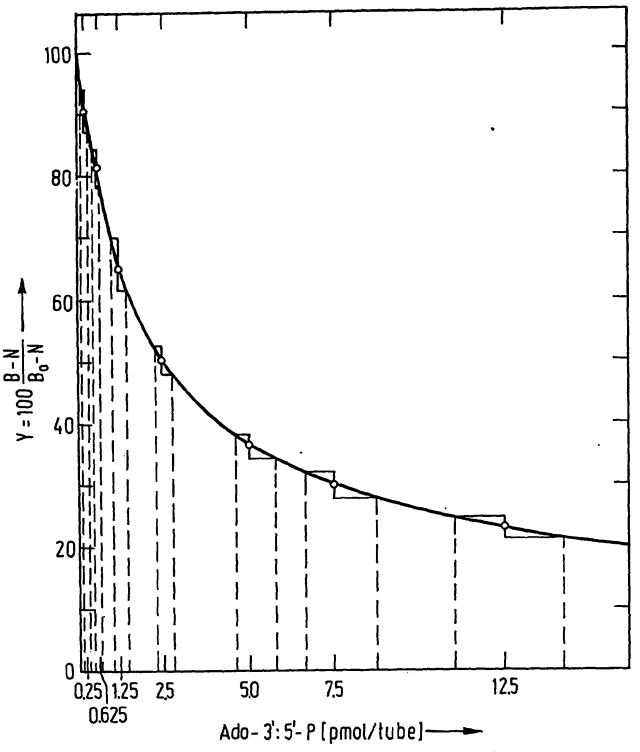

Fig. 1
Standard curve for Ado-3′:5′-P assay. Standard deviation is shown in the curve for $p \leqq 0.05$
Calculation: We use the response variables suggested by ODELL and coworkers (22)
B = number of counts per minute (c. p. m.) bound in the presence of standard or unknown unlabeled Ado-3′:5′-P
B_0 = number of c. p. m. bound in the absence of unlabeled Ado-3′:5-P
T = the total number of c. p. m. (bound and free)
N = blank: treated as value of nonspecific binding. It is subtracted from values of B, B_0, and T

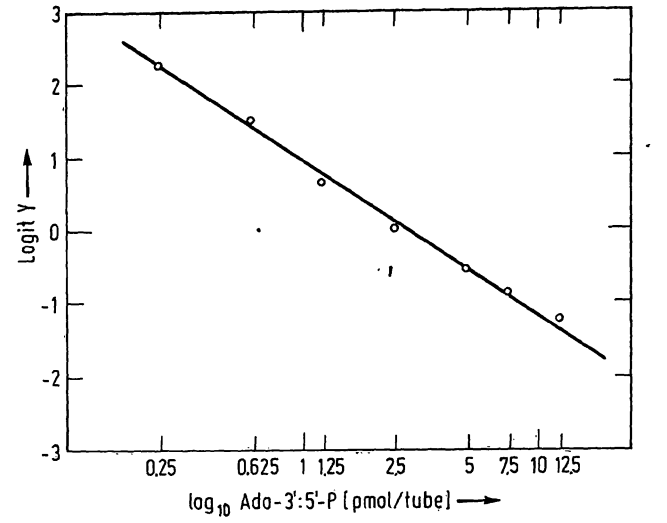

Fig. 2
Standard curve for Ado-3′:5′-P assay, logit plot
Ordinate: $Y = 100 \cdot \frac{B - N}{B_0 - N}$ on logit scale (left). Logit $Y = \log_e \frac{Y}{100 - Y}$
Abscissa: Dose on $\log_{10}$ scale
The means of 10 replicates at each dose are shown

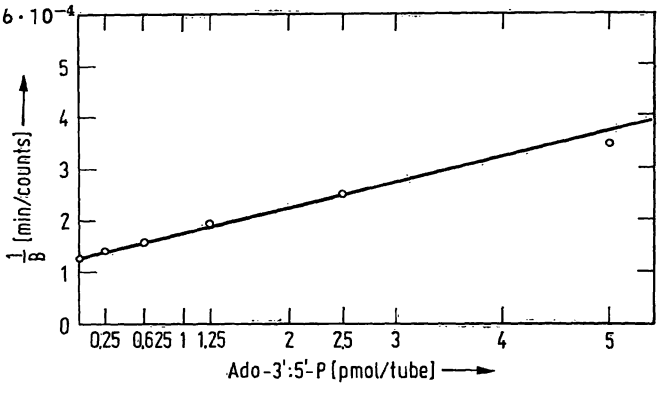

Fig. 3
Linearisation of the standard curve by a "single reciprocal plot". Affinity constant calculated from slope and intercept of the ordinate is $K = 4.3 \cdot 10^8$ l/mol

For more routine dose interpolation the linearisation according to the method "logit plot" suggested by RODBARD and coworkers (23) can be used (Fig. 2).
Another method for linearisation is the "single reciprocal plot" suggested by us. This model has provided a satisfactory fit for a wide range of the dose response curve, when the binding protein is near saturation.
This method is exact, provided that one is dealing with an univalent homogenous reaction between Ado-3′:5′-P and the binding protein.
Our plot has the advantage of easy and simple curve calculation. In addition, the plot yields the affinity constant and the binding capacity of the binding protein from the ordinate-intercept and the slope of the curve, if the constant amount of ^{3}H-Ado-3′:5′-P is known (Fig. 3).

Analysis of data

Precision

In Figure 1 the 95% confidence limits were calculated assuming normal distribution of the 10 values. The range of the dose is shown on the abscissa.
The intraassay coefficient of variation averaged 2.6% ($p \leqq 0.05$). The interassay coefficient of variation obtained from more than 90 duplicate samples of Ado-3′:5′-P averaged 6% ($p \leqq 0.05$) over the range of measurement.

Detection limit

Sensitivity has been usually defined as the smallest amount of substance being measured that can be significantly distinguished from zero. Under present conditions the detection limit is 0.25 pmol per tube. This value is significantly ($p \leqq 0.05$) different from the blank value.
We have included the other aspect of sensitivity, the resolving power, (the ability of the assay to distinguish between slightly different concentrations of Ado-3′:5′-P over the whole range of standard curve), in the concept of precision.

Specificity

In Figure 4 a dose response curve of Ado-3′:5′-P and related compounds are shown. The ability to compete with ^{3}H-Ado-3′:5′-P for binding site(s) of protein kinase was determined.

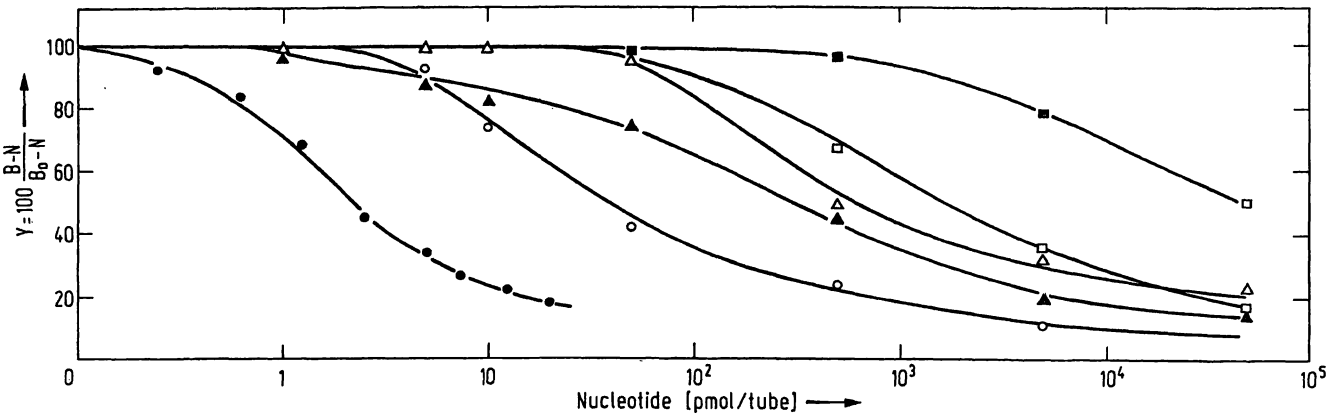

Fig. 4
Dose response curve of Ado-3′:5′-P and related nucleotides
• Ado-3′:5′-P; ○ Ino-3′:5′-P; ▲ Guo-3′:5′-P; △ Cyd-3′:5′-P; □ Urd-3′:5′-P; ■ ATP

It can be shown that the tested compounds do not cross-react in our assay at the concentrations which have been found e. g. for Guo-3′:5′-P (24, 25) in many tissues.

Accuracy

Accuracy is an important criterion of assay reliability. It depends on sensitivity, precision, specificity, and the quality of tissue homogenate. A criterion for accuracy is given by recovery.

In Figures 5 and 6 the recovery is tested by known amounts of Ado-3′:5′-P added at the time of homogenisation. The coefficient of variation was 9.5% (for recovery).

Since competitors or unspecific inhibitors of Ado-3′:5′-P binding may exist in the tissue extract, the assay could yield estimates of Ado-3′:5′-P which were too high. The influence of such compounds can be tested by specific hydrolysis with phosphodiesterase. After treatment with phosphodiesterase, the samples show the blank value. It can be concluded that the tissue extracts do not contain any significant amounts of competitors or unspecific inhibitors of Ado-3′:5′-P that would affect ^{3}H-Ado-3′:5′-P binding to the specific protein. The possibility that phosphodiesterase inactivated competing substances in addition to Ado-3′:5′-P is not completely excluded.

Discussion

Technically more demanding procedures for preparation of binding protein, e. g. the separation of adrenal cortex from medulla, are not so useful as one could believe. There is only a small difference between cortex and medulla with respect to the specific activity of binding protein (15). More highly purified protein (from adrenals (20) or from muscle (14)) and a more rigorous separation method of free and bound Ado-3′:5′-P do not yield essentially higher sensitivity and reliability. The sensitivity of the assay described here

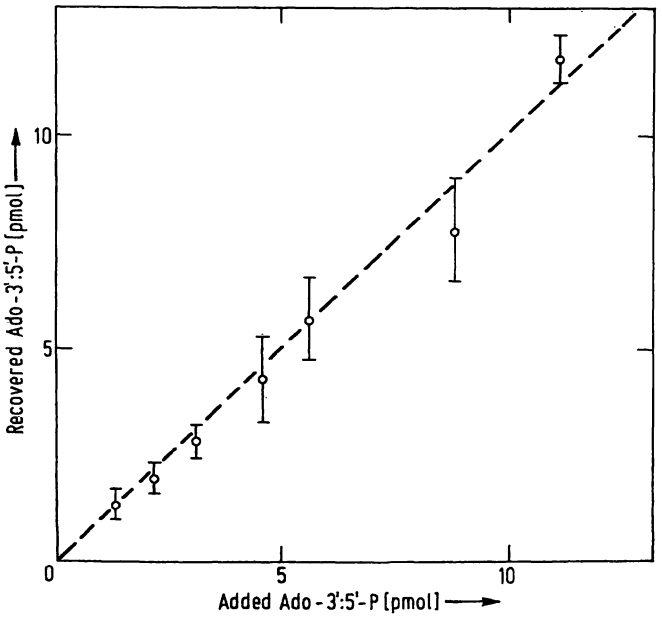

Fig. 5
Recovered amount ($p \leq 0.05$) of Ado-3′:5′-P as a function of Ado-3′:5′-P added to liver homogenate. The mean value of Ado-3′:5-P in the rat liver was 0.8 pmol/mg wet weight. This value is subtracted from the total amount of Ado-3′:5′-P. The dashed line gives 100% recovery

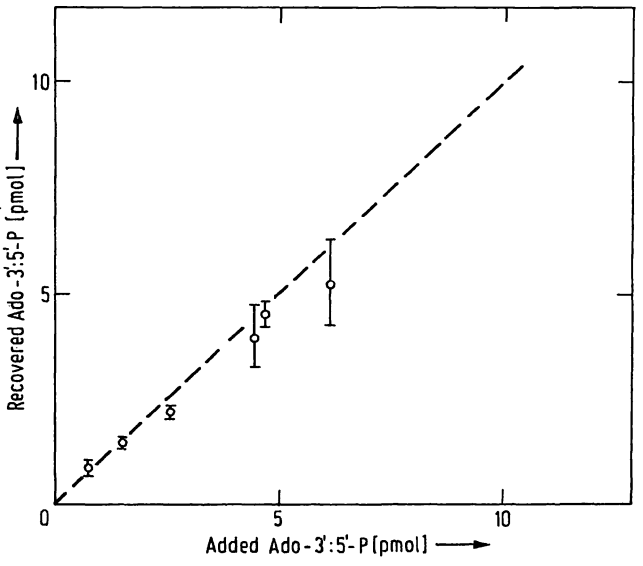

Fig. 6
Recovered amount ($p \leq 0.05$) of Ado-3′:5′-P as a function of Ado-3′:5′-P added to kidney homogenate. The mean value of Ado-3′:5′-P in the rat kidney was 1.5 pmol/mg wet weight. This value is subtracted from the total amount of Ado-3′:5′-P. The dashed line gives 100% recovery

can be enhanced by reducing the tracer concentration and the reaction volume.
We find a 0.4 ml reaction volume the suitable to work with. We generally carry out all assays at 4—6°, because of the higher association constant at low temperature.
Undoubtedly a main problem of Ado-3′:5′-P assay concerns the values of the blanks. This blank (i. e. the apparent amount of Ado-3′:5′-P found in a sample which does not contain Ado-3′:5′-P) consists of

1. competing nucleotide or other material in the tissue preparation. Material present in the tissue preparation may either compete for the binding site or may denaturate a part of the binding protein (tending to cause overestimation of amount of Ado-3′:5′-P present in the tissue extract)
2. substances present in the extract that influence the adsorbing activity of the unspecific adsorbent (resulting in a lower unbound Ado-3′:5′-P fraction and finally in underestimation of the amount of Ado-3′:5′-P).

The first point is treated by the specificity and the accuracy of the assay. The other point can be compensated by the control value of the assay.
The strong affinity and the high specificity of binding protein for Ado-3′:5′-P are the basis for an convenient and sensitive competitive protein binding assay. Considering this we determined the affinity-constant of ^{3}H-Ado-3′:5′-P for the binding protein in a SCATCHARD-plot (26) (Fig. 7). The ratio of bound to free ^{3}H-Ado-3′:5′-P is plotted against the bound ^{3}H-Ado-3′:5′-P. The linearity suggests single site binding kinetics. The intercept on the x-axis equals the molarity of binding sites and the slope equals the negative value of the binding constant. The affinity constant determined from SCATCHARD-plot (Fig. 7) is $1.3 \cdot 10^8$ l/mol (4°). The molarity of binding sites equals $1.13 \cdot 10^{-8}$ mol/l.
The ^{3}H-Ado-3′:5′-P affinity was tested under assay conditions. For separation of bound and free ^{3}H-Ado-3′:5′-P, the adsorbent coated charcoal is added to the system at equilibrium for a very short time only, in order to prevent the dissociation of the protein-^{3}H-Ado-3′:5′-P complex. The equilibrium-state has to be maintained. The charcoal concentration used is relatively high, compared with the literature values (27). Smaller charcoal concentrations produced unsatisfactory preparation results. The maintenance of strictly controlled conditions (stirring, temperature, duration of contact) is necessary for reproducibility. The equilibrium study with binding protein and ^{3}H-Ado-3′:5′-P has shown that the free energy of binding has the relatively high value of 10 kcal/mol. The free energy change can be calculated by the formula $\Delta G^\circ = -RT \ln K$ (R = gas-constant, T = abs. temperature, K = affinity constant). It is composed of a very high negative enthalpy change in association with a negative change of entropy. The thermodynamic data are interpreted as indicative of very tight fit of the interacting components so that the enthalpy is drastically

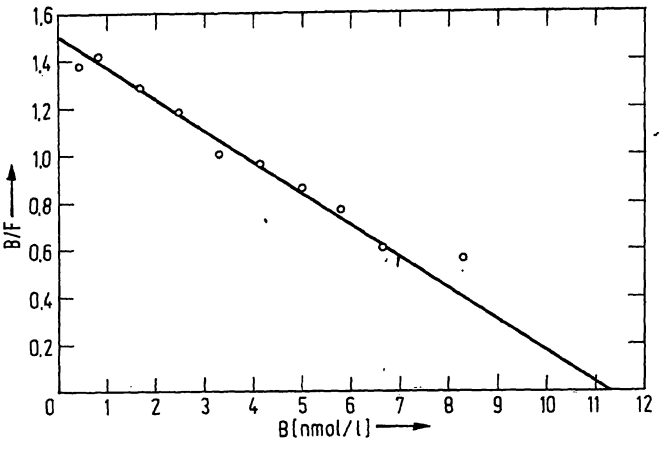

Fig. 7
SCATCHARD-plot.
Ratio B/F is plotted versus B
Affinity constant calculated from slope is $K = 1.3 \cdot 10^8$ l/mol. The molarity of binding sites calculated from the intercept of abscissa is $1.1 \cdot 10^{-8}$ mol/l

reduced and the total order of the system is much increased resulting in a negative entropy change.
The binding protein is relatively specific for Ado-3′:5′-P. Unlabeled Ado-3′:5′-P competes with the labeled form for binding sites. It can be assumed that labeled and unlabeled Ado-3′:5′-P have the same affinity to the binding protein.
Other nucleotides do not compete effectively, as shown in Figure 4 and Table 2, except the 10fold quantities of Ino-3′:5′-P and 100fold quantities of Guo-3′:5′-P which displace labeled Ado-3′:5′-P from the binding protein by 50%. The competition by Ino-3′:5′-P and, to a lesser extent, by Guo-3′:5′-P, and the total lack of competition of ATP, ADP, Ado-5′-P and Ado-3′-P, indicate that the 3′:5′-ring is responsible for specificity of the binding to the protein, but not the adenine part of the adenine nucleotides.
A very interesting fact is the great difference between the binding of N^6-mono- and N^6-2′O-dibutyryl derivatives of Ado-3′:5′-P to the protein kinase. The

Tab. 2
Relative binding of various nucleotides to Ado-3′:5′-P binding protein. The ability of Ado-3′:5′-P to inhibit ^{3}H-Ado-3′:5′-P binding was arbitrarily set at 100%. Relative binding affinity of any given compound is calculated as $\frac{\text{(Ado-3′:5′-P)}}{\text{(compound)}} \cdot 100$ for 50% inhibition of ^{3}H-Ado-3′:5′-P binding. Protein concentration assayed for binding was 400 µg

Compound	Relative binding affinity (%)
Ado-3′:5′-P	100.0
Ino-3′:5′-P	8.0
Guo-3′:5′-P	1.42
Cyd-3′:5′-P	0.50
Urd-3′:5′-P	0.36
ATP	0.005
GTP	< 0.005
Ado-5′-P	< 0.005
Ado-3′-P	< 0.005
ADP	< 0.005
Ado-3′:5′-P derivatives:	
N^6-monobutyryl-	20.0
N^6-2′O-dibutyryl-	0.625

Klinik und Therapie der Vergiftungen

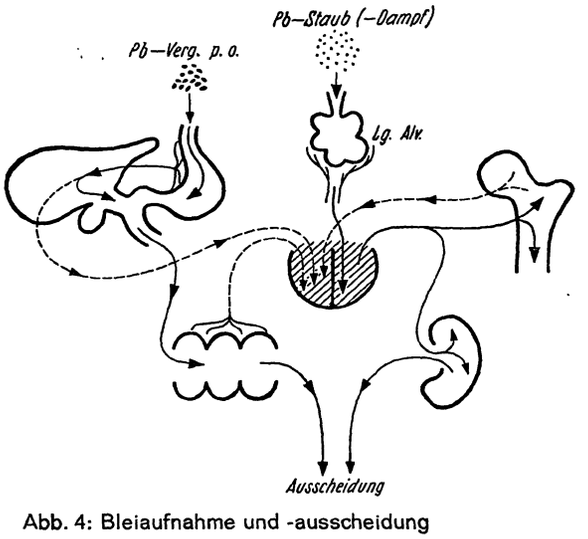

Abb. 4: Bleiaufnahme und -ausscheidung

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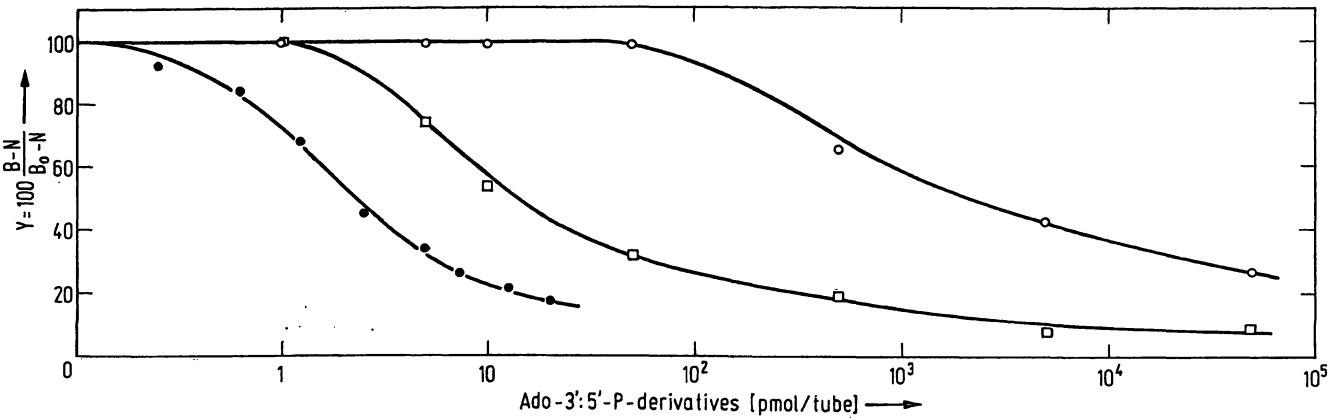

Fig. 8
Dose response curve of Ado-3′:5′-P and its butyryl derivatives
● Ado-3′:5′-P; □ N⁶-monobutyryl Ado-3′:5′-P derivative ○ N⁶-2′O-dibutyryl Ado-3′:5′-P derivative

N^6-monobutyryl derivative competes very effectively with ^{3}H-Ado-3′:5′-P, but the dibutyryl derivative requires a 400fold higher concentration for 50% inhibition (see Fig. 8).

It can be suggested, that the Ado-3′:5′-P derivative monobutyrylated in position 2′O is not so effective for protein kinase activity as the derivative monobutyrylated in position N^6, e. g. the finding by HENION and coworkers (28) that the N^6-derivatives in the liver phosphorylase system retained some activity, but those substituted at the 2′O-position were inactive, could be interpreted in this way.

The "single reciprocal plot" presented in Figure 3 is a new method for linearisation of the dose response curve. This linearisation has the advantage that it is exact provided that one is dealing with the single site binding kinetics. In the SCATCHARD-plot (Fig. 7) and the "single reciprocal plot" (Fig. 3) the data yield a straight line, if dose response is limited to the given range, in which Ado-3′:5′-P is determined. Considering the linearity in both plots, it is reasonable to assume a single site binding kinetic.

A binding system which obeys the law of mass action and in which the binding sites are assumed to be independent can be treated similarly to the equilibrium treatment of enzyme action. Where such equilibrium treatment is appropriate, the classical graphical methods are often used, e. g. the double reciprocal plot (first represented by LINEWEAVER and BURK (29) in enzyme kinetics) and the SCATCHARD-plot. The "single reciprocal plot" proposed by us (also used in enzyme inhibition studies) provides that the labeled and unlabeled compounds possess affinity for the binding protein and are able to displace one another from the binding site of the protein. In other words the protein can form a complex PL* with the labeled compound (L*) and a complex PL with the unlabeled compound (L), but can not form a complex in which both L* and L are attached to the binding protein. This is comparable to the equilibrium treatment of competitive inhibition of an enzyme reaction.

The scheme applicable to a competitive reaction is as follows:

$$\begin{array}{ccc} P + L^* & \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} & PL^* \\ + & & \\ L & & \\ k_{+1} \downarrow\uparrow k_{-1} & & \\ PL & & \end{array}$$

P = binding protein
L = ligand unlabeled
L* = ligand labeled
PL* = binding protein labeled ligand complex (can be measured)
PL = binding protein ligand complex

$K = \frac{k_{-1}}{k_{+1}}$ = dissociation constant of the PL*-complex

$K_i = \frac{k_{-i}}{k_{+i}}$ = dissociation constant of the PL-complex.

In order to derive the rate equation at equilibrium, the following three simultaneous algebraic equations (eq. 1—3) must be solved in terms of the measurable variables ([L*], [L], and total protein ($[P]_0$)) and equilibrium constants

$$\frac{d\,[PL^*]}{dt} = 0 = k_{+1}\,[P]\,[L^*] - k_{-1}\,[PL^*] \qquad \text{(eq. 1)}$$

$$\frac{d\,[PL]}{dt} = 0 = k_{+i}\,[P]\,[L] - k_{-i}\,[PL] \qquad \text{(eq. 2)}$$

$$[P]_0 = [P] + [PL^*] + [PL] \qquad \text{(eq. 3)}$$

The equation for [PL*] at equilibrium is

$$[PL^*] = \frac{[P]_0\,[L^*]}{K\left(1 + \frac{[L]}{K_i}\right) + [L^*]} \qquad \text{(eq. 4)}$$

This equation (eq. 4) can be transformed in

$$\frac{1}{[PL^*]} = \frac{1}{[P]_0}\left(1 + \frac{K}{[L^*]}\right) + \frac{K}{K_i\,[P]_0\,[L^*]}\,[L] \qquad \text{(eq. 5).}$$

If the same compound is given in labeled and unlabeled form, it can be assumed that $K = K_i$. Consequently the eq. 5 is simplified to

$$\frac{1}{[PL^*]} = \frac{1}{[P]_0}\left(1 + \frac{K}{[L^*]}\right) + \frac{1}{[P]_0\,[L^*]}\,[L] \qquad \text{(eq. 6).}$$

Under the conditions given, a straight line results if the data of the saturation analysis are plotted according to equation (eq. 6). The variable PL* is measured after separation of free and bound labeled compound, PL* being equivalent to the bound part (B) of the labeled compound. The other variable L is varied by known concentrations of unlabeled compound. Since the labeled compound concentration is known (from specific activity), the concentration of the binding sites (P_0) and the affinity constant (1/K) are available from slope and the intercept of the straight line in the "single reciprocal plot".

The dose interpolation is readily done with the use of equation 6. The "single reciprocal plot" provides a satisfactory fit for a considerable portion of a dose-response curve. The chief virtues of this method are the ease and the simplicity with which the calculation may be done on a hand calculator and with which the estimation of the parameters K and P_0 is possible. A computer program for fitting the curve with the use of least-squares regression analysis is very easy, too. Moreover, this method allows the estimation of the affinity constant $\left(\frac{1}{K_i}\right)$ of any competitors of a labeled ligand, if its concentration is varied. Upon extrapolation the resulting straight lines intersect at one point with the coordinates $\left(-K_i/\frac{1}{[P]_0}\right)$.

Finally, it should be mentioned that over an unusually wide dose range the linearisation in the "single reciprocal plot" is not satisfactory. The "single reciprocal plot" provides an optimal fit for low and medium dose region of the curve but not for high dose region. Considering that we have derived equation 6 on condition that a univalent homogeneous reaction occurs, but may be there two distinct orders of protein binding sites each with its own characteristic binding constant.

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