Journal of Chromatography, 229 (1982) 77—85
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1180

RAPID ASSAY OF CYCLIC AMP PHOSPHODIESTERASE AND 5'-NUCLEOTIDASE BY MEANS OF CHROMATOGRAPHY ON CELLULOSE-NITRATE MEMBRANE STRIPS

ZENON SCHNEIDER*, DANUTA LESZCZYŃSKA and JERZY SOCHA

**Institute of Biochemistry, University of Agriculture, 35 Wołyńska, 60 637 Poznań, and Children's Health Center, 04 736 Warsaw (Poland)*

(First received July 28th, 1981; revised manuscript received November 24th, 1981)

SUMMARY

A simple chromatographic procedure with the use of modified cellulose-nitrate membrane strips, 80 × 40 mm, has been worked out for the rapid isotopic assay of cyclic AMP (cAMP) phosphodiesterase (EC 3.1.4.17) and 5'-AMP nucleotidase (EC 3.1.3.5) in crude extracts of various tissues from animals and plants. The assay is based on enzymatic conversion of the product to adenine, a relatively inert compound which, in contrast to cAMP and 5'-AMP, is strongly adsorbed by the cellulose-nitrate membrane. Due to this property rapid separation of adenine from the unconverted substrate (cAMP or 5'-AMP) is possible. Commercial 5'-nucleotidase and easily obtainable crude extract of adenosine nucleosidase from barley leaves are used as coupling enzymes for the phosphodiesterase assay. The assay of phosphodiesterase in 0.5—2 μl of blood (10^{-5} to $4 \cdot 10^{-5}$ units) has been demonstrated on several examples.

INTRODUCTION

It was recently reported from our laboratory that a number of enzymes can be estimated rapidly by means of chromatography on modified cellulose-nitrate membrane strips [1—3]. This technique has been found to be applicable in the isotopic assay of cyclic AMP (cAMP) phosphodiesterase (EC 3.1.4.17) and 5'-AMP nucleotidase (EC 3.1.3.5) over a broad range of substrate concentrations. The procedure described in this paper is based on our observation that cellulose-nitrate membrane treated with aqueous solutions of aliphatic alcohols strongly adsorb adenine and a few bases but to a much lesser extent the nucleotides derived from them [1, 4]. In this assay the products of these two enzyme actions, 5'-AMP and adenosine, respectively, are converted by coupling enzymes into adenine which in contrast to the substrates is readily adsorbed by the membrane strip. Prepared cellulose-nitrate

0378-4347/82/0000—0000/$02.75

membrane strips, 8 × 40 mm, are used in this method. The application procedure is very simple: 3—5 μl of the incubation mixture are applied onto one end of the strip followed by chromatography with the use of phosphate buffer. The adenine-labeled substrate and the product are separated rapidly and quantitated by counting separately the two halves of the strip. Besides rapidity, the method is advantageous in several aspects in comparison to other phosphodiesterase assays using labeled substrates [5—11]. These advantages are, in particular: (1) fairly good counting efficiency of tritium-labeled compounds on the cellulose-nitrate membrane; (2) high sensitivity; (3) very small amounts of enzyme required (down to $2 \cdot 10^{-5}$ units); (4) insensitivity to a broad range of salt concentrations and pH [2]. This technique seems to be particularly useful for clinical investigations and kinetic studies whenever small amounts of enzyme are available. There is also the potential of adapting it for routine assays by means of automatization.

MATERIALS AND METHODS

Materials

[2,8-^{3}H] Adenosine 3′,5′-cyclic phosphate specific activity 35 Ci/mmol, [adenine U-^{14}C] adenosine 3′,5′-cyclic phosphate specific activity 85 mCi/mmol, [8-^{14}C] adenosine 5′-monophosphate specific activity 53 mCi/mmol, and [8-^{14}C] adenosine specific activity 45 mCi/mmol were from the Radiochemical Centre, Amersham, Great Britain. The [^{3}H] cAMP was purified before use by treatment with 5′-nucleotidase and ascending chromatography on Whatman 3 MM paper with the solvent system *n*-butanol—acetic acid—water (12:5:3). Adenosine 3′,5′-cyclic phosphate was from Calbiochem, San Diego, CA, U.S.A. Cellulose-nitrate membrane sheets (BA 85, 0.45 μm pore size) were from Schleicher and Schüll, Dassel, G.F.R. The sheets were cut into strips 8 × 40 mm in size and modified as described below. 2-Ethoxyethanol was from BDH, Poole, Great Britain. Other chemicals of analytical grade were from Polskie Odczynniki Chemiczne (POCH), Poland.

Source of enzymes

5′-Nucleotidase from *Crotalus adamanteus* venom was from Sigma, St. Louis, MO, U.S.A. The 5′-nucleotidase from barley leaves was a crude preparation obtained in a mixture together with adenosine nucleosidase as described in ref. 2. The partially purified preparations were free of cAMP phosphodiesterase, although, as will be shown in Results, freshly obtained extract of barley leaves showed phosphodiesterase activity. The enzymes dried as described in ref. 12 were kept at room temperature for three years with little loss of activity. Phosphodiesterase from blood was used here as model enzyme for studies of the usefulness of the assay technique. It was obtained from heparinized fresh blood of healthy and ill subjects diluted four times with water to cause osmotic disruption of the cells. It was then diluted several times with 50 m*M* Tris—HCl buffer (pH 7.2) to a concentration of enzyme appropriate to the sensitivity of the method. Phosphodiesterase activity was also examined in freshly prepared crude extracts of barley leaves obtained by rupture of the plant cells as described in ref. 13 and extraction of the enzyme

with the buffer as above followed by centrifugation for 10 min at 10,000 *g*. The supernatant solution was dialyzed against the extraction buffer for 1 h and used with no further purification. As shown in Results, both enzyme preparations appeared to be rather unstable. Commercial 3',5'-cyclic nucleotide phosphodiesterase from bovine heart and adenosine deaminase from calf intestine were from Sigma.

Phosphodiesterase assay

In this assay the product of diesterase action, 5'-AMP, is converted to adenine in coupled enzymatic reactions with the use of two enzymes — 5'-nucleotidase and adenosine nucleosidase — according to the scheme

$$3',5'\text{-cAMP} \xrightarrow[H_2O]{} 5'\text{-AMP} \xrightarrow[P_i]{} \text{adenosine} \xrightarrow[H_2O]{} \text{adenine} + \text{ribose}$$

The unconverted cAMP and one of the final products, adenine, are rapidly separated by chromatography on cellulose-nitrate membrane strips 8 × 40 mm. Since adenine is adsorbed by the membrane it remains at the origin, while cAMP moves with the front of the chromatographic buffer towards the other end. The labeled product and the unconverted substrate are easily assessed by measuring separately the radioactivity of the two halves of the strip. Owing to the very high specific radioactivity of the applied [^{3}H]cAMP the assay could be elaborated for very small volumes of the incubation mixture and tiny amounts of enzyme. The following standard assay was worked out. The incubation mixture contained in a total volume of 15 μl: 0.5 m*M* [2,8-^{3}H]-cAMP (30,000 cpm); 5 m*M* $MgCl_2$; 0.05 *M* Tris—HCl buffer, pH 7.2; 0.001 unit of 5'-nucleotidase of barley leaves or snake venom; 0.003 unit adenosine nucleosidase; and appropriate amounts of the phosphodiesterase. The tubes, protected from evaporation, were incubated for 30 min at 25°C and the reaction was then stopped by inserting the tubes into boiling water for 30 sec. The tubes were next centrifuged and 3—5 μl of the clear supernatant solution were applied on the membrane strip at a distance of about 7 mm from one of its ends. The strip was then kept for 10 min in a moist atmosphere followed by chromatography with 0.02 *M* K_2HPO_4. The chromatography was done on the bottom of a suitable PTFE or polypropylene box containing a strip of chromatographic paper soaked with the above buffer [2]. The membrane strip was attached by one end to the wet paper in order to allow the buffer to flow along it. The unconverted cAMP moved with the front of the buffer towards the other end of the strip, while the adenine remained adsorbed at the start. When the buffer reached the other end, the strip was dried under an infrared lamp and cut into two pieces of equal length. The pieces A and B, containing the product and the unconverted substrate, respectively, were put into standard scintillation vials and solubilized with 0.5 ml of 2-ethoxyethanol followed by addition of 5 ml of toluene scintillator and measurement of the radioactivity. Blank tubes containing no phosphodiesterase were run simultaneously. The activity of the enzyme was assessed on the basis of percentage of the radioactivity in sector A of the strip and the standard curve, as described in the legend to Fig. 1.

5'-AMP nucleotidase assay

The procedure described for phosphodiesterase assay was adopted for the assay of 5'-nucleotidase. The adenosine produced by the enzyme action was converted by adenosine nucleosidase into adenine. Phosphatase-free adenosine nucleosidase from barley leaves was obtained by further purification of the preparation described above on a Sephadex G-100 column [13]. The incubation mixture contained in a total volume of 15 μl was 0.1 *M* Tris—HCl buffer (pH 7.2), 5 m*M* $MgCl_2$, 0.1 m*M* [8-^{14}C] 5'-AMP (30,000 cpm), adenosine nucleosidase 0.003 unit, and 5'-nucleotidase preparation containing 10^{-5} to $5 \cdot 10^{-5}$ activity units. After an appropriate incubation time at 25°C, during which not more than 30% of the substrate was converted, the reaction was stopped by inserting the tubes for 30 sec into boiling water. Further the procedure was followed as described for phosphodiesterase assay except that the strips were not dissolved prior to counting.

Adenosine nucleosidase assay

The assay of this enzyme is described in ref. 2.

Preparation of cellulose-nitrate membrane strips

The membrane sheets described in Materials were cut into strips, 8 × 40 mm, with a scalpel. In order to make the strips suitable for chromatography, they were inserted for 15 min into a 2% aqueous solution of 2-methyl-1-propanol (isobutanol) heated to 60°C, followed by washing four times with cool distilled water and bathing for 10 min in 5% glycerol at room temperature. The strips were removed with philatelic forceps, pressed gently against a filter paper and dried on PTFE foil at room temperature. The strips were kept at 5°C in closed vials for three years with no change in chromatographic performance. Commercial cellulose-nitrate membrane sheets might differ, however, from batch to batch in performance. To ensure uniform performance of the membranes for a longer period of time it is reasonable to keep a reserve of the same batch in stock. On the other hand, the membranes become hydrophobic during storage. This can be overcome by storing the membranes in the presence of isobutanol vapour; in practice, a strip of filter paper soaked with isobutanol was inserted into each membrane package wrapped into a polyethylene foil. A quantity of 1 ml of isobutanol per litre of package space has been found sufficient. The addition of excessive, uncontrolled amounts of isobutanol poured directly onto the membranes damages them.

RESULTS AND DISCUSSION

The interference of purine-catabolizing enzymes in the phosphodiesterase assay

Accurate assay of the phosphodiesterase in crude biological material is difficult to achieve due to the presence of catabolizing enzymes which degrade the primary product, 5'-AMP. Instant conversion of 5'-AMP to adenine, a compound relatively inert under the assay conditions used, seemed to be a solution to this problem. To achieve this, a large excess of the coupling enzymes is required in the assayed sample. According to refs. 14 and 15, a 100-

fold excess of the first coupling enzyme and several thousand-fold excess of the second enzyme relative to the assayed enzyme are required to obtain a farily accurate measurement in a one-step procedure. The two coupling enzymes — 5′-nucleotidase and adenosine nucleosidase — used in our assay of phosphodiesterase in hemolyzed blood, have been found to be very effective even at low concentrations. This is presumably due to their low Michaelis constants ($1.2 \cdot 10^{-5}$ *M* and $2 \cdot 10^{-6}$ *M*, respectively) exceeding by one or two orders of magnitude the K_m values of the potentially interfering enzymes like 5′-AMP deaminase and adenosine deaminase. Hemolyzed blood has been found to contain an appreciable level of adenosine deaminase [2] which, in terms of enzyme activity units, is up five times greater than that of cAMP diesterase. Thus, it seemed to be particularly important to find what level of this interfering enzyme is acceptable in our standard assay. For this purpose the assayed sample was supplemented with commercial adenosine deaminase. The data in Table I allowed us to assess that a supplement of 0.002 unit of commercial adenosine deaminase together with about 0.002 unit of this enzyme introduced with the sample of the hemolyzed blood causes about a 7% underestimation of the phosphodiesterase level measured by the membrane-strip method. This is due to conversion of part of the adenosine into inosine, a compound less effectively adsorbed by the membrane strips. The above deviation is within the range of methodological errors derived from volume and radioactivity measurements and has been considered still acceptable, although near the edge of the given limit. The presence of relatively higher levels of the interfering enzyme would require an increase of the coupling enzyme adenosine nucleosidase in the standard assay in order to fit into the assumed 10% error range. The quantities of the coupling enzymes worked out experimentally and the data in Table I have been found to be in fair agreement with the data calculated on the basis of the known equation combining the Michaelis constant with concentration of the substrate and the velocity of enzymatic conversion:

$$v = \frac{V \cdot s}{K_m + s}$$

Calculation of the results

The mole fraction of cAMP converted by phosphodiesterase was read from a graph (Fig. 1) obtained experimentally from two controls: one with unconverted, and the second with completely converted, substrate in the presence of excess phosphodiesterase. The diagram allows the elimination of errors caused by non-specific binding of cAMP to the membrane and the presence of radioactive contamination in the substrate due to radiolysis of cAMP. The calculation of the enzyme level is described in detail in the legend to Fig. 1. A new graph was routinely plotted every two weeks. Another batch of the modified membrane strip or a fresh portion of the tritium-labeled substrate necessitates a new graph as well.

Examples of diesterase assay

The method has been found to be applicable to the direct assay of cyclic

TABLE I

EFFECT OF ADENOSINE DEAMINASE ON THE DETERMINATION OF cAMP PHOSPHODIESTERASE IN BLOOD, ASSESSED BY PAPER CHROMATOGRAPHY AND THE MEMBRANE STRIP ASSAY

A volume of 300 μl of the standard incubation mixture containing 40 μl of hemolyzed blood from a child with inherited gastrointestinal disorders (patient G in Table II) containing approximately 0.002 unit of cAMP diesterase, 0.012 unit of adenosine deaminase, and 0.5 m*M* [8-^{14}C]cAMP (2,000,000 cpm) instead of the tritium-labeled cAMP used in the standard assay, was divided into six portions. The operation was carried out at 0°C. One portion serving as control for zero time of incubation was immediately denatured by heat treatment. The remaining portions were supplemented with adenosine deaminase or water as specified in the table and incubated at 25°C for 30 min followed by heat denaturation. The sixth portion was exposed to a second incubation for 30 min after addition of 5'-nucleotidase and adenosine nucleosidase as specified. Two 5-μl aliquots of each tube were exposed to chromatography on membrane strips as described in Methods. The quantity of the converted cAMP was calculated as described in the legend to Fig. 1. The residue in the incubation tubes was supplemented with carriers of the compounds indicated in the table and subjected to ascending chromatography on paper Whatman 3 MM using the solvent specified in Methods for purification of commercial [^{3}H]cAMP. The spots visualized by UV light were cut out and the radioactivity was measured. The following R_F values were found: 5'-AMP (0.1), cAMP (0.16), inosine (0.32), adenosine (0.46) and adenine (0.56). The 5'-AMP spot overlapped somewhat that of cAMP, thus the sixth experiment with the additional incubation is the presence of 5'-nucleotidase and adenosine nucleosidase was included. The quantity of substrate converted to the compounds indicated was calculated on the basis of the percentage of radioactivity in the particular spots.

Additives or conditions	Substrate or product in pmoles				
	Paper chromatography				Membrane-strip product
	Substrate cAMP	Products			
		Inosine	Adenosine	Adenine	
Control (no enzyme)	7500	—	—	—	0
None	5115	38	53	2295	2175 ± 98
Adenosine deaminase					
0.0005 unit	5190	60	38	2213	2100 ± 90
0.002 unit	5250	143	30	2078	2025 ± 60
0.005 unit	5265	300	0	1935	1875 ± 38
Second incubation after addition					
0.001 unit 5'-nucleotidase	5063	38	0	2390	1875 ± 83
0.001 unit adenosine nucleosidase					

nucleotide diesterase in crude extracts from animals and plants. The diesterase assay in plants is simpler since plants do not have adenosine deaminase, considered the major cause of error. Most plants contain 5'-nucleotidases and 3'-nucleotidases as well as adenosine nucleosidase so that, nolens volens, the incubation of cyclic nucleotide with crude plant extract usually ends with adenine as the final product [2, 4]. In the case of diesterase assay in blood,

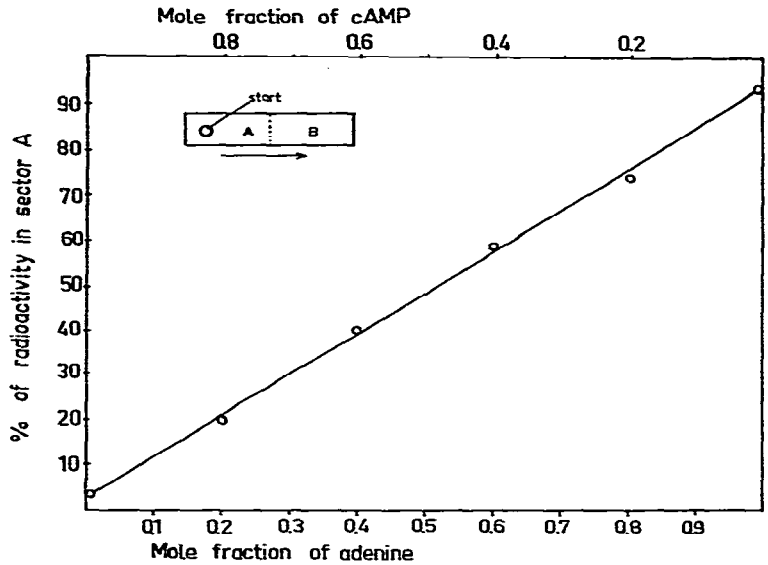

Fig. 1. The standard curve. The points indicated were obtained on the basis of two 30-μl controls, one with zero time of incubation and the second exposed to complete conversion of cAMP to adenine; 30 min of incubation under standard conditions with 0.002 unit of commercial diesterase was satisfactory. The contents of these controls were mixed with each other in ratios to obtain the mole fractions indicated, followed by chromatography on membrane strips and measurement of the radioactivity in both halves (A and B) of the strip (see insert). From the obtained curve the mole fraction of the adenine formed (converted cAMP) was read. The cAMP diesterase (PDE) level (L) in blood expressed in pmoles per min per μl of blood was calculated from the mole fraction of adenine formed (k), time of incubation (t), the volume of undiluted blood (v) and the amount of substrate (m), according to the equation $L = (k \cdot m)/(t \cdot v)$. In routine procedures it was sufficient to draw a graph from two points only, obtained for unconverted and completely converted substrate.

it seemed to be important to know the adenosine deaminase level in order to assess its possible interference. Table II presents examples of routine assay of these two enzymes in blood of children affected with hereditary gastrointestinal disorders. The level of adenosine deaminase is about four to five times greater than that of diesterase. It can be assessed from the data in Table I that the ratio of the levels of these two enzymes as 1:10 is still acceptable within the standard assay.

The stability of diesterase seems to be a critical factor affecting reproducible assay. Since it was observed that the diesterase activity in both blood and barley leaf extract changes noticeably during storage, it seemed important to know the rate of enzyme activity loss in these sources at various temperatures. As shown in Fig. 2 the half-life of this enzyme in the plant extract and in blood at room temperature is about 3.5 and 8 h, respectively, while at 2°C the respective values are 4.5 and 6.5 days. With regard to blood, more often explored as a source of phosphodiesterase in various physiological studies, the curve of enzyme activity decay may be helpful for planning reproducible assay conditions. In contrast to diesterase, the activity of adenosine deaminase changes very little during storage.

TABLE II

LEVELS OF cAMP DIESTERASE AND ADENOSINE DEAMINASE IN BLOOD OF CHILDREN WITH INHERITED GASTROINTESTINAL DISORDERS

The samples of blood were assayed for diesterase and adenosine deaminase as described in Methods and in ref. 2, respectively. Each sample was run in duplicate. The content of each incubation tube was chromatographed on membrane strips in triplicate. The level of the diesterase was calculated from the graph shown in Fig. 1 and the equation given in the legend of Fig. 1.

Subject	pmoles per min per μl of blood (mean ± S.D.)	
	Phosphodiesterase	Adenosine deaminase
10 controls	29—54 (range)	50—120 (range)
A	31± 2	96± 5
B	48± 3	162± 6
C	50± 3	172± 6
D	56± 4	162± 5
E	60± 4	200± 6
F	66± 4	152± 5
G	68± 4	248± 7
H	75± 5	143± 5

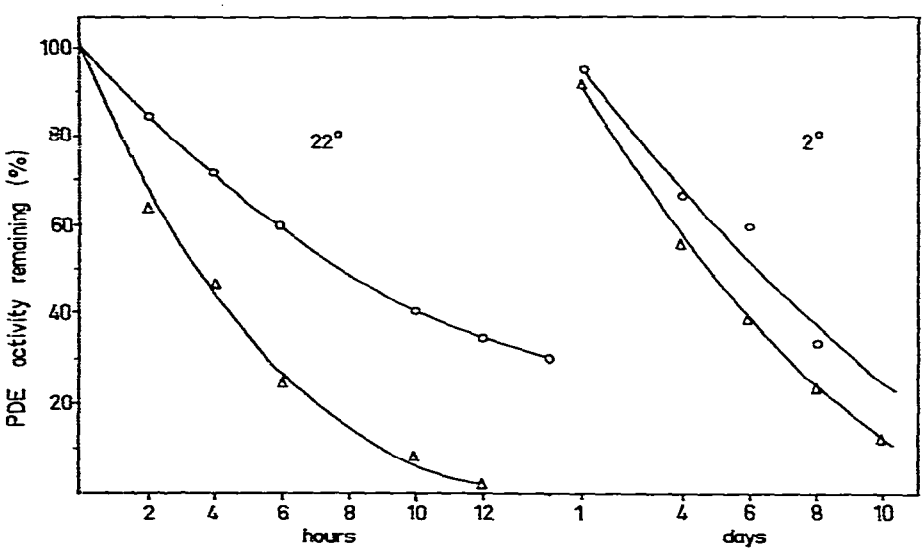

Fig. 2. Time—course of inactivation of cAMP diesterase in blood and barley leaf extract at 2°C and 22°C. Heparinized blood of patient G (see Table II) was kept in small sterile at 2°C and 22°C. The crude extract of barley leaves in 0.1 *M* Tris—HCl buffer (pH 7.2) containing cyclic nucleotide diesterase activity 0.003 unit/ml was handled in a similar manner. At the time indicated, the blood sample was hemolyzed by diluting five times with water. Two 5-μl aliquots of the diluted blood as well as of the barley leaf extract were subjected to standard diesterase assay. The points indicated are mean values of two assays. (○) Blood diesterase; (△) barley leaf diesterase.

ACKNOWLEDGEMENT

The support of the Polish Ministry of Science, Higher Education and Technology, via Grant 40/81/R, is acknowledged.

REFERENCES

1 Z. Schneider, Anal. Biochem., 108 (1980) 96.
2 Z. Schneider, Anal. Biochem., 108 (1980) 104.
3 Z. Schneider, E. Maćkowiak and W. Walerych, Anal. Biochem., 108 (1980) 112.
4 Z. Schneider and A. Guranowski, Anal. Biochem., 68 (1975) 493.
5 D. Monard, J. Janecek and H.V. Rickenberg, Biochem. Biophys. Res. Commun., 35 (1969) 584.
6 G. Brooker, L.J. Thomas and Appleman, Biochemistry, 7 (1972) 4177.
7 C.R. Filburn and J. Karn, Anal. Biochem., 52 (1973) 505.
8 W.Y. Cheung and T.J. Lynch, Anal. Biochem., 67 (1975) 130.
9 R.J. Boudreau and G.J. Drummond, Anal. Biochem., 63 (1975) 388.
10 W.Y. Cheung and A.L. Pichard, J. Biol. Chem., 251 (1976) 5726.
11 J.A. Beavo, J.G. Hardman and E. Sutherland, J. Biol. Chem., 245 (1970) 5649.
12 Z. Schneider and H.C. Friedmann, J. Appl. Biochem., in press.
13 A. Guranowski and Z. Schneider, Biochim. Biophys. Acta, 482 (1977) 145.
14 H.U. Bergmeyer (Editor), Methods of Enzymatic Analysis, Academic Press, New York, 1963, p. 10.
15 K.G. Oldham, Radiochemical Methods of Enzyme Assay, Review 9 of the Radiochemical Centre, Amersham, Whitefriars Press, 1968, p. 6.