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Enzyme Markers for the Presence of Circulating Interferon: 2-5A Synthetase in Blood Lymphocytes and Protein Kinase in Platelet-Rich Plasma (41783)

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Abstract. The level of 2-5A synthetase in extracts of peripheral blood lymphocytes and a specific protein kinase activity in platelet-rich plasma were measured in normal individuals and in patients suffering from viral or bacterial infections. The level of these enzymes was tested at different times during the disease. The level of 2-5A synthetase and the protein kinase activity was enhanced by several-fold during viral and bacterial infections and decreased during the course of the disease in parallel with clinical ameliorations and reversal of clinical symptoms. Among the different types of infections studied, higher levels of these enzymes were observed during viral than bacterial infections. Our results emphasize the use of these enzymes as markers to evaluate the state of the disease and recovery. Furthermore, they provide evidence for the production of interferon during different types of infection.

Treatment of cells with interferon results in the induction of two double-stranded (ds) RNA-dependent enzymes, pppA $(2'p5'A)_n$ synthetase (2-5A synthetase) and a specific protein kinase (for references see (1)). The protein kinase activity is manifested by the phosphorylation of an endogenous 67,000-mol wt protein in mouse cells or a 72,000-mol wt protein in human cells. Both the 2-5A synthetase and the protein kinase are present in different organs of mice and their level is enhanced several-fold after treatment of mice with interferon or after injection of inducers of interferon, such as virus and synthetic dsRNAs (2, 3). Accordingly enhanced levels of these enzymes in different organs of mice have been used as convenient markers for the presence and action of interferon in an organism (4, 5).

Recently, we reported the presence of a protein kinase activity in human plasma platelets which is responsible for the phosphorylation of a 70,000- to 72,000-mol wt protein (72K protein) found in blood plasma (6, 7). The protein kinase activity and its substrate bind to poly(G)–Sepharose and can be conveniently assayed in this form (8). The 72K protein has been identified as the α-chain of fibrinogen (9). The interest in this protein kinase system was initiated on the observation that treatment of patients with interferon or inducers of interferon resulted in an enhanced level of such kinase activity (6, 10). Variations in the level of platelet protien kinase activity, therefore, may reflect different levels of circulating interferon. By means of a technique of phosphorylation of an exogenous substrate, calf-thymus histone, here we measured the level of this kinase activity in the platelet-rich plasma (PRP) of normal individuals and of patients with viral and bacterial infections. The level of the protein kinase activity in PRP was assayed in parallel with measurements of 2-5A synthetase in peripheral blood lymphocytes as enhanced levels of this latter enzyme have been shown in patients with different types of infections and following treatment with interferon (11–13). Thus, we employed both of these enzymes to monitor the presence of interferon in patients with different types of infections.

Materials and Methods. [γ-^{32}P]ATP and [^{3}H]ATP were supplied by Amersham International (England). Poly(I)·poly(C)–Sepharose and poly(G)–Sepharose were prepared as described (8).

For the assay of the protein kinase activity, blood was collected in polystyrene tubes containing heparin (100 U/ml) and aprotinin (100 U/ml; Zymofren, Specia) and left 15–30 min at room temperature. Platelet-rich plasma (PRP) was collected after centrifugation (200*g*, 15 min) and was stored at −80°C. The protein kinase activity was assayed in PRP by three different methods: by poly(G)–Sepharose (8) and pH 5 fraction (14) for the phosphorylation

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of proteins in PRP and by histone kinase assay for the phosphorylation of an exogenous substrate.

Histone kinase. Samples (aliquots of 2.5 μl) of PRP were diluted with an equal volume of NP-40 buffer before incubation (30°C, 45 min) with 100 μg of calf-thymus histone (H-III, Sigma) and 10 μ*M* [γ-^{32}P]ATP (25 Ci/mmole) in a total reaction mixture (100 μl) containing: 10 m*M* Hepes, pH 7.6, 50 m*M* KCl, 2.5 m*M* $Mg(OAc)_2$, 5 m*M* $MnCl_2$, 7 m*M* 2-mercaptoethanol, 15% glycerol (v/v), and 5% aprotinin. After the incubation, aliquots (50 μl) of the reaction mix were transferred to a 2.5-cm filter disc (Whatman 3MM paper) and washed (4°C) consecutively with cold 10% trichloroacetic acid containing 0.5 m*M* ATP, 5% trichloroacetic acid containing 0.5 m*M* NaH_2PO_4, and subsequently with ethanol and acetone at room temperature. The filter discs were dried and the radioactivity was measured by liquid scintillation (7). The results are expressed in picomoles of $^{32}PO_4$ incorporated per milligram of histone per assay.

Assay of 2-5A synthetase. Heparinized blood was diluted with an equal volume of Hanks' and layered on a cushion of Lymphopred (Lymphopred TM, Nyegaard and Co. A/S, Oslo, Norway) and centrifuged 30 min at 1500 rpm (18°C). The cells at the interphase were collected and washed twice in 4 ml of Hanks' solution and finally suspended in NP-40 buffer at 10^7 cells/ml. This suspension was then vortexed and kept at −80°C. Aliquots (100 μl) of poly(I)·poly(C)–Sepharose were previously equilibrated in buffer A before addition of lymphocyte extracts (100 μl). Binding of the 2-5A synthetase to poly(I)·poly(C)–Sepharose was carried out in polystyrene tubes at room temperature for 15 min, followed by a further 15-min incubation at 30°C with gentle mixing. Unbound proteins to the poly(I)·poly(C)–Sepharose were eliminated by washing three times in 5 ml buffer A. The supernatant obtained by centrifugation (200*g*, 5 min) after each wash was removed by decanting. The poly(I)·poly(C)–Sepharose bound synthetase fractions were then resuspended in a total reaction mixture (300 μl) containing: 100 μl of poly(I)·poly(C)–Sepharose enzyme; 20 m*M* Hepes, pH 7.6; 50 m*M* KCl; 25 m*M* $Mg(OAc)_2$; 7 m*M* 2-mercaptoethanol; 5 m*M* ATP; 10 m*M* creatine phosphate; 0.16 μg/ml of creatine kinase; 0.1 mg/ml of poly(I)·poly(C); and 1 μl of [^{3}H]ATP (1 mCi/ml). Incubation was for 17–18 hr at 30°C and was terminated by heating at 90°C to form 5 min. ^{3}H-labeled 2-5A was purified by DEAE–cellulose chromatography as described (15) except that the concentration of KCl was 50 m*M* instead of 90 m*M* in the washing buffer. The DEAE–cellulose samples containing 2-5A were then suspended in 3 ml of 0.3 *N* KOH and the radioactivity of the entire sample was then measured after dilution into 10 ml of liquid scintillant cocktail for aqueous samples (INSTA-GEL; United Technologies Packard).

The results are expressed in nanomoles of [^{3}H]AMP incorporated into 2-5A per 10^6 lymphocytes per assay (17–18 hr incubation). In these experiments, we found that it was necessary to purify the commercial [^{3}H]ATP in order to minimize the background counts in the 2-5A assay (16).

Patients. All of the patients included in this study were admitted to "Charles Nicolle" hospital.

(i) Patients suffering from viral infections included symptoms such as (a) rash and fever: measles virus, rubella virus; (b) vesicles and fever: varicella-zoster virus, herpes hominis virus; (c) diarrhea: rotavirus and enterovirus; (d) lymphocytic meningitis: enterovirus, mumps virus, or unidentified virus; (e) encephalitis: herpes hominis virus; (f) fever, adenopathy, lymphocytopenia, purpura: E.B. virus, I.C.M. virus.

(ii) Patients suffering from bacterial infections included (a) meningitis: *Haemophilus influenzae, Streptococcus pneumoniae, Neisseria meningitidis;* (b) Pneumonia: *Haemophilus influenzae, Streptococcus pneumoniae,* gram negative (*Klebsiella*); (c) gram negative septicemia; (d) O.R.L. infections: epiglottitis, otitis, laryngotracheitis.

(iii) Patients suffering from noninfectious diseases included (a) breast and gastrointestinal tract malignancy diseases, Hodgkin, A.L.L.; (b) idiopathic thrombocytopenia, iron deficiency anemia; (c) ischemic heart diseases.

Results. *The protein kinase activity in PRP.* The protein kinase system recovered from PRP by partial purification on poly(G)–Sepharose (8) or by precipitation at pH 5 (pH

TABLE I. HISTONE KINASE ACTIVITY IN THE PRP OF FOUR DIFFERENT PATIENTS (A–D) WITH VIRUS INFECTIONS

Patient	Sample[a]	Histone kinase[b] (pmole PO_4/mg)
A	1	20.57
	2	9.76
B	1	10.71
	2	7.38
C	1	19.1
	2	5.0
	3	2.1
D	1	5.5
	2	4.2

[a] Samples were from four different patients with viral infections. Samples 1 in A to D show the protein kinase activity at the time of diagnosis of viral infections. Samples 2 represent different times during the course of treatment while sample 3 in C represents a point after the recovery.
[b] Histone kinase activity was measured as described under Materials and Methods.

5 fraction; (14)) is manifested by the phosphorylation of the α-chain of fibrinogen (9). The level of such protein kinase activity in normal PRP varies from one individual to the other, but it seems to remain constant under normal conditions (6). Treatment of patients with interferon or inducers of interferon, however, results in an enhanced level of such kinase activity (6, 10). Enhanced levels of protein kinase activity are also detectable in the PRP of patients with different types of viral infections (data not shown).

Proteins phosphorylated in the pH 5 fraction or after purification on poly(G)–Sepharose represent phosphorylation of substrates found in the blood plasma by the platelet protein kinase activity (7, 14). Variations in the level of these endogenous substrates, therefore, may give artifactual results since phosphorylated levels of these proteins would depend on their presence but not on the level of the platelet protein kinase activity. For this reason, we investigated the capacity of the protein kinase activity to phosphorylate an exogenous substrate, calf-thymus histone. We have previously shown that the protein kinase system attached to poly(G)–Sepharose is capable of phosphorylating calf-thymus histone and that variation in the level of phosphorylation of the endogenous substrate could also be reflected in a histone kinase assay (10). A modified version of the histone kinase assay was employed here to measure the level of protein kinase activity in PRP. Small aliquots of PRP were diluted in the kinase buffer and assayed in this crude form (Materials and Methods). Table I shows the level of phosphorylation of histones in the PRP of four patients (A, B, C, and D) with different types of viral infections. The first points (samples 1 in A–D) in each case represent the level of protein kinase activity at the time of diagnosis of viral infection. The other points (samples 2 in A, B, and D; Samples 2 and 3 in C) represent the level of the kinase at different times during the course of infection or after recovery. It is interesting here to note that enhanced levels of protein kinase activity decreased during the course of infection and this decrease coincided with the withdrawal of clinical symptoms. Variations observed in the level of phosphorylation of histones (Table I) by the PRP of these patients

TABLE II. THE LEVEL OF 2-5A SYNTHETASE AND PROTEIN KINASE IN HEALTHY INDIVIDUALS AND IN PATIENTS WITH DIFFERENT DISEASES

		Patients		
Enzyme[a]	Healthy individuals	Viral infections	Bacterial infections	Noninfectious diseases
2-5A Synthetase[b]	2.00 ± 0.98	29.11 ± 22.98 $P < 0.001$	6.77 ± 2.99 $P < 0.001$	4.84 ± 3.85 $P < 0.001$
Protein kinase[c]	2.08 ± 0.53	4.21 ± 1.17 $P < 0.001$	2.89 ± 1.17 $P < 0.001$	3.12 ± 1.51 $P < 0.001$

[a] Statistical analysis was carried out by comparison of the means in a normal distribution.
[b] Values are nmole AMP/10^6 lymphocyte/assay (17–18 hr incubation).
[c] Values are pmole PO_4/mg histone/assay (45 min incubation).

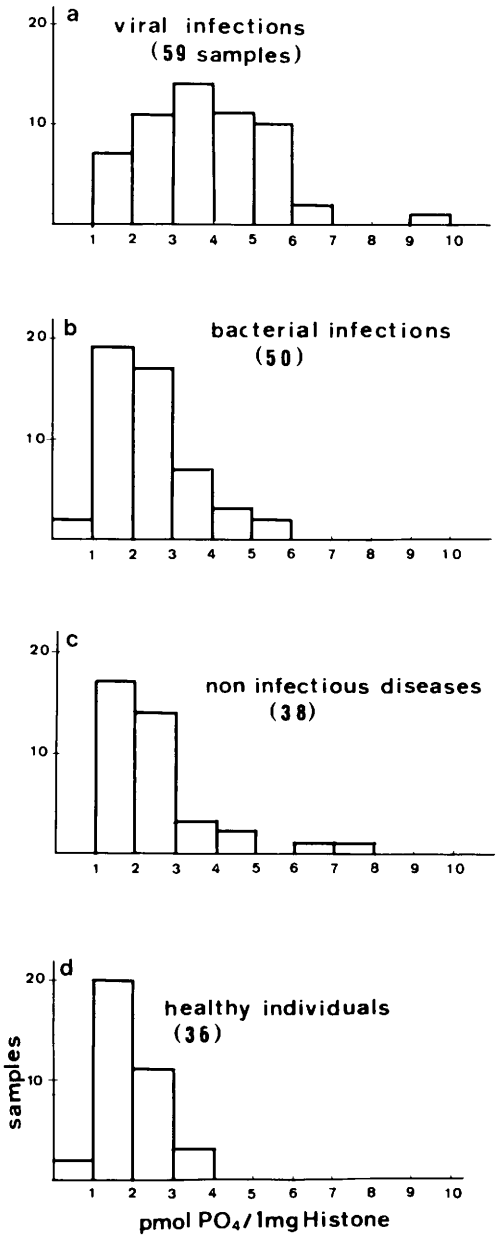

FIG. 1. The level of histone kinase activity in the PRP of healthy individuals as well as of patients with viral infections, bacterial infections and noninfectious diseases. The numbers in parentheses represent the number of cases in each group. The abscissa of each group represents the level of histone kinase activity in pmole PO_4/mg histone (Materials and Methods). The ordinates show the number of samples with the different levels of kinase activity. Viral and bacterial infections were as in Figs. 3 and 4. Non-infectious diseases include various hematological problems (A.L.L., low platelet counts, Hodgkin, anemia), ischemic heart diseases, and malignancy diseases.

were also detectable by the assays of poly(G)–Sepharose and pH 5 fraction (data not shown). The first method (histone kinase), however, is of advantage over the other two techniques since it is rapid and quantitative. Accordingly we used histone kinase assay for estimation of the protein kinase activity in PRP of normal individuals and patients with different types of diseases.

Variations in the level of histone kinase in PRP. The level of histone kinase activity in healthy individuals (control) varies from one person to the other, but it remains constant in one individual, at least for a certain period of time (2–6 days). A study was carried out on 12 healthy individuals in order to find out variations in the level of histone kinase activity

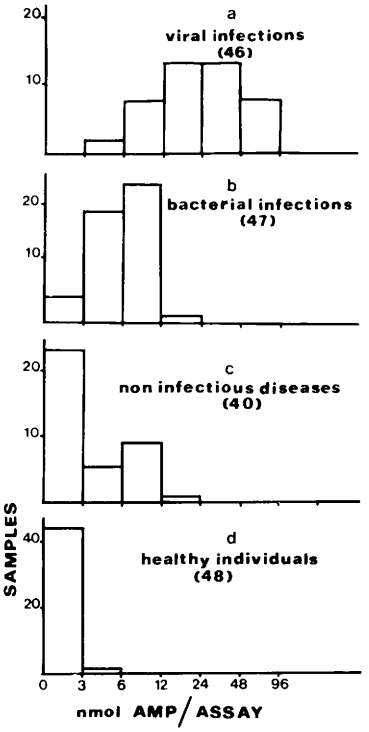

FIG. 2. The level of 2-5A synthetase in lymphocytes of healthy individuals as well as of patients with viral infections, bacterial infections, and noninfectious diseases. The numbers in parentheses represent the number of cases in each group. The abscissa of each group represents the level of 2-5A synthetase in nmole AMP/10^6 lymphocytes/17–18 hr incubation (Materials and Methods). The ordinates show the number of samples with the different levels of 2-5A synthetase. Patients were as in Fig. 1.

between two preparations of PRP from the same individual at 2 to 6 days interval. The mean values obtained were 1.92 ± 0.56 and 1.99 ± 0.64 pmole of PO_4 incorporated per mg of histone for the first and second preparations, respectively. The difference between these two points was found to be statistically insignificant ($P < 0.90$ by Student's t test).

A study carried out on the PRP of 120 healthy subjects without previous history of disease indicated that the level of histone kinase activity in normal PRP is 1.75 ± 0.75 pmole of $^{32}PO_4$/mg of histone. A separate study carried out independently on another group of 59 healthy individuals gave a mean value of 2.08 ± 0.53 pmole of $^{32}PO_4$/mg of histone (Table II). Such a kinase activity is significantly enhanced in patients with viral or bacterial infections or with noninfectious diseases (Table II). In general, it is possible to see that the distribution of histone kinase values tends to move toward higher levels (>2 pmole $^{32}PO_4$/mg histone) in patients compared to healthy individuals (Fig. 1). These enhanced levels return to normal values after recovery (data not shown).

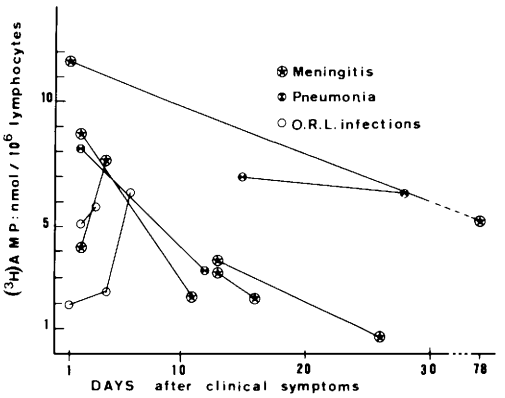

FIG. 4. The level of 2-5A synthetase in the lymphocytes of patients with bacterial infections. The ordinate represents the level of 2-5A synthetase (Materials and Methods) in nanomoles of AMP incorporated into 2-5A per 10^6 lymphocytes per assay (17–18 hr incubation). The abscissa represents the time of each assay after diagnosis and during treatment. O.R.L. infections stand for epiglotitis, otitis, and laryngotracheitis. The meningitis case who showed a high level of 2-5A synthetase (11.7 nmole AMP/10^6 lymphocytes) turned out later on to have a localized herpes infection.

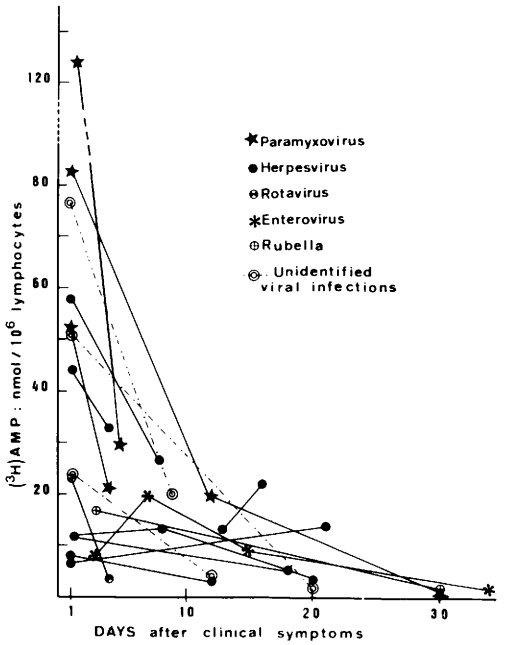

FIG. 3. The level of 2-5A synthetase in the lymphocytes of patients with viral infections. The ordinate represents the level of 2-5A synthetase (Materials and Methods) in nanomoles of AMP incorporated into 2-5A per 10^6 lymphocytes per assay (17–18 hr incubation). The abscissa represents the time of each assay after the appearance of clinical symptoms.

The level of 2-5A synthetase in peripheral blood lymphocytes. Among the 48 healthy individuals studied for the assay of 2-5A synthetase, it was possible to show that the basal level of 2-5A synthetase is 2 ± 0.98 nmole of AMP/10^6 lymphocytes. This level is enhanced significantly in the lymphocytes of patients with viral and bacterial infections (Figs. 2, 3 and Table II). Higher levels of 2-5A synthetase were observed in patients with viral infections (29.11 ± 22.98 nmole of AMP/10^6 lymphocytes) than with bacterial infections (6.77 ± 2.99 nmole of AMP/10^6 lymphocytes). This is probably due to the fact that viruses are better inducers of interferon than bacteria.

When possible, the level of 2-5A synthetase of patients with viral and bacterial infections was assayed immediately after diagnosis, during the treatment period and after recovery. Figures 3 and 4 show that the level of 2-5A synthetase which was enhanced in patients with such infections was decreased during the course of each respective disease. The decline

in the level of 2-5A synthetase in each case corresponded to the state of recovery.

Correlation between the levels of 2-5A synthetase in lymphocytes and protein kinase in PRP. The results discussed above indicated that the level of 2-5A synthetase in lymphocytes and the level of histone kinase in PRP of patients with viral, bacterial, and noninfectious diseases are enhanced significantly compared to mean values observed in normal (healthy) individuals (Table II).

In order to find out a correlation between the level of 2-5A synthetase and the level of protein kinase, we measured the level of these enzymes in parallel in different patients and in normal volunteers. Each test was carried out in duplicate and the results are summarized in Table III. In these experiments, high levels of 2-5A synthetase in lymphocytes and histone kinase activity in PRP were considered to be values more than 10 nmole AMP/10^6 lymphocytes and 3 pmole PO_4/mg histone, respectively. Enzyme levels equal or less than these values were taken as normal levels. Statistical analysis was carried out by chi square (χ^2) test. The high or normal levels of these enzymes were correlated significantly in normal (healthy) individuals ($P > 0.30$) as well as in patients with noninfectious diseases ($P > 0.10$). In contrast the high or normal levels of these enzymes were not correlated in patients with viral ($P < 0.02$) or bacterial ($P < 0.05$) infections.

Discussion. The results described here show that the level of 2-5A synthetase in peripheral blood lymphocytes and the level of protein kinase activity in PRP are enhanced in patients with bacterial and viral infections (Table II). The level of these enzymes is also enhanced in patients treated with interferon and poly(A)·poly(U) (10, 11). In view of these observations, it is possible to suggest that enhanced levels of 2-5A synthetase and protein kinase during viral and bacterial infections reflect the presence of circulating interferon. In addition, they indicate the immediate response of an organism toward interferon (10–13). These enzymes, therefore, can be used as convenient markers to monitor therapy with interferon. This is of great interest since circulating interferon has a very short half-life and furthermore assay of low levels of interferon is not reproducible. In contrast, enhanced levels of these enzymes last several hours and it is possible to assay efficiently any modifications in the enzyme level.

The levels of both 2-5A synthetase and protein kinase vary from one individual to the other under normal and disease conditions. It is preferable, therefore, to measure enzyme levels at different times in the same individual, in order to assess the results efficiently. Enhanced levels of 2-5A synthetase and protein kinase decrease during the course of infection in parallel with recovery. Thus, the level of these enzymes may indicate the state of the disease and its evolution during the period of treatment. Finally, the level of these enzymes may be useful in monitoring the return to a normal physiological condition.

The levels of 2-5A synthetase in lymphocytes and the protein kinase in PRP were found to be correlated in healthy individuals and as well as in patients with noninfectious diseases. In patients with viral or bacterial infections, however, the level (high or normal) of 2-5A synthetase did not correlate systematically with the level of the protein kinase

TABLE III. CORRELATION BETWEEN THE LEVELS OF 2-5A SYNTHETASE IN LYMPHOCYTES AND PROTEIN KINASE IN PRP

2-5A Synthetase[a]	Protein kinase[b]	Viral infections	Bacterial infections	Noninfectious diseases	Normal
>10	>3	32/44	1/43[c]	—	—
>10	≤3	9/44	—	2/36	—
≤10	>3	2/44[c]	6/43	5/36	2/36
≤10	≤3	1/44[c]	36/43	29/36	34/36

[a] Enzyme levels: nmole AMP/10^6 lymphocytes/assay (17–18 hr incubation).
[b] Enzyme levels: pmole PO_4/mg histone/assay (45 min incubation).
[c] Bacterial infection + localized herpes.

activity (Table III). This discrepancy between the level of 2-5A synthetase and the protein kinase may have been due to some altered mechanism during viral and bacterial infections. The level of these enzymes is estimated by their activity in an *in vitro* assay; thus the efficiency of their detection is under the control of several variable agents such as ribonucleases and phosphatases. For example, we have recently shown that the level of 2-5A synthetase and poly(I)·poly(C)–Sepharose-bound protein kinase in the spleen of mice becomes undetectable during rabies virus infection. This decrease in respective enzyme levels was in fact due to the presence of agents interfering with the assay of the enzymes (17).

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