ANALYTICAL BIOCHEMISTRY 60, 15–24 (1974)

# Determination of the Arginine Content of Proteins by the Sakaguchi Procedure

G. TOMLINSON[1] AND T. VISWANATHA[2]

*Department of Chemistry, University of Waterloo, Waterloo, Ontario, Canada*

Received September 10, 1973; accepted December 7, 1973

The arginine contents of a variety of protein samples determined by the quantitative Sakaguchi method were in good agreement with the values obtained by quantitative ion-exchange chromatography, after acidic hydrolysis. One protein, α-chymotrypsin, while possessing a linear relationship between the colour obtained in the Sakaguchi test and the protein concentration, gave colour yields which corresponded to the apparent presence of an additional Sakaguchi-positive residue. The appearance of this phenomenon was found to be associated with the process of zymogen activation. Subsequently, it was found that two other serine esterases, trypsin and subtilisin, also exhibited this behaviour. Possible reasons for this have been discussed.

The applicability of the Sakaguchi reaction (1) and its various modifications to the detection and determination of arginine and monosubstituted guanidines in solution has been documented (2–22). Relatively little attention has been paid to the use of the procedure in the determination of the arginine content of intact proteins. The literature on this point is conflicting. Several earlier workers have suggested that the Sakaguchi method may indeed be applicable to the estimation of arginine in proteins without prior acidic hydrolysis (7–9,19,23,24), whereas Izumi (18) has concluded that it is not. During studies in our laboratory (25) on the chemical modification of arginine residues in proteins, we required a rapid procedure for the estimation of the extent of arginine modification. A study of the Sakaguchi method and some of its variations was therefore undertaken and its applicability to the determination of the arginine content of proteins was tested. The final procedure adopted is essentially Weber's modification (2) of the original Sakaguchi reaction (1), as described by Greenstein and Winitz (26).

[1] Present address: Institute of Molecular Biology, University of Oregon, Eugene, Oregon.

[2] To whom all correspondence should be addressed.

## EXPERIMENTAL

### *Materials*

The following reagent-grade materials were employed in this study: α-naphthol (Fischer), sodium nitrite (Allied Chemical), bromine and urea (J. T. Baker Chemical), thiodiglycol (Pierce Chemical Co.), arginine (General Biochemicals), and *N*-acetyl-L-tyrosine ethyl ester (Mann). All other chemicals and reagents not specifically mentioned above were reagent grade and used without further purification.

Proteins used for arginine determinations were lysozyme, soybean trypsin inhibitor, chymotrypsinogen A, and α-chymotrypsin (all obtained from Worthington Biochemical Corp.). Ribonuclease A and bovine serum albumin were obtained from Sigma Chemical Co. and subtilisin from Nutritional Biochemicals Corp. Extensively acetylated chymotrypsinogen (27), chymotrypsinogen modified by treatment with diacetyl trimer (DT) (28), and anhydro-chymotrypsin (29) were laboratory preparations.

### *Methods*

The Sakaguchi determination was carried out essentially as described by Greenstein and Winitz (26). As the mode of addition of reagents is important, the procedure is described here in detail. The reagents used in the test are as follows:

(A) 0.1% α-naphthol in 50% ethanol (prepared fresh daily),
(B) 10% KOH,
(C) 5% urea,
(D) potassium hypobromite (0.64 ml $Br_2$ in 100 ml 5% KOH, prepared fresh daily).

To 1 ml of test solution containing 0.1–1.0 μmole arginine (either free arginine or the equivalent amount of protein) is added 1 ml of (A) and 1 ml of (B). The solution is mixed well; 1 ml of (C) is added; the solution is mixed again followed by the rapid addition of 2 ml (D) with continuous shaking. (This step is extremely critical and it is absolutely essential to ensure that the addition of reagent (D) and the mixing be carried out as rapidly as possible.) A reagent blank is carried out simultaneously, using distilled water instead of arginine solution. After standing for 20 min at room temperature, the absorbance at 520 nm is measured. The arginine content of the sample is determined by comparison with a standard curve prepared using a 1.00 μmole/ml stock solution of arginine.

The arginine content of the various proteins obtained by use of the Sakaguchi procedure was compared to the values obtained by amino

acid analysis following acidic hydrolysis of identical protein samples. Proteins were hydrolysed with 6 N HCl at 110°C for 24 hr in sealed tubes, and the arginine content was determined by the ion-exchange chromatographic procedure of Spackman *et al.* (30) with the aid of a Beckman Model 120 B amino acid analyser. The instrument was calibrated using the same arginine standards as those used in the colorimetric procedure.

The tryptic activation of chymotrypsinogen was achieved according to the published procedure (31). Enzyme activity was assayed by the method of Schwert and Takenaka (32) using *N*-acetyl-L-tyrosine ethyl ester as substrate.

All spectrophotometric measurements were performed using a Beckman DB-G recording spectrophotometer and 1-cm cuvettes. Protein concentrations were determined by measurement of their absorbance at 280 nm, using the extinction coefficients cited in the literature.

## RESULTS

### *Analytical Procedure*

When excess hypobromite is added to an alkaline solution of arginine in the presence of α-naphthol but in the absence of any stabilizing

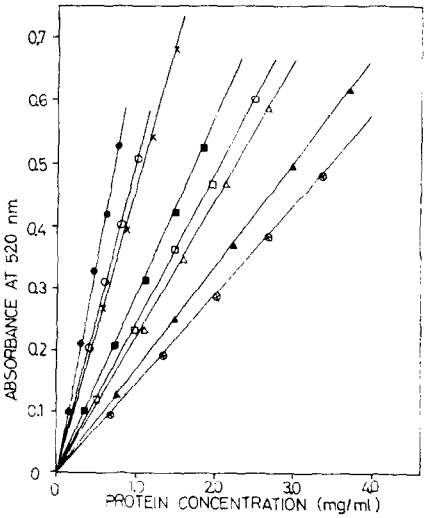


FIG. 1. Relationship between the amount of coloured-complex produced and protein concentration. —●——●—, lysozyme; —○——○—, soybean trypsin inhibitor; —×——×—, bovine serum albumin; —■——■—, ribonuclease; —□——□—, chymotrypsinogen A; —△——△—, α-chymotrypsin; —▲——▲—, trypsin; —⊗——⊗—, subtilisin.

agent, there is an instantaneous production of a red-coloured complex followed by a rapid fading of the colour. In the presence of urea, which reacts with the excess hypobromite and thereby prevents further oxidation of the complex, the decay of the colour is inhibited and after 20 min the rate of decay of colour is slow enough to permit an accurate spectrophotometric measurement of the absorbance at 520 nm.

Using the procedure as described above, excellent standard curves can be obtained using solutions of arginine of concentration up to at least 1.00 μmole/ml. On the basis of twenty such standard curves, a value of the slope of 1.38 (±0.04) has been used as a conversion factor in the estimation of the arginine content of the subsequent protein solutions.

*Estimation of Arginine in Proteins*

All of the protein solutions tested during this study were found to give linear relationships with respect to colour yield obtained by the Sakaguchi procedure for various protein concentrations. Figure 1 shows some typical results.

TABLE 1
Arginine Content of Protein Samples

| Protein | Concentration[a] (mg/ml) | Arginine content (μmole) | |
|---|---|---|---|
| | | Ion-exchange chromatography | Sakaguchi |
| Ribonuclease A | 0.573 | 0.166 | 0.176 |
| Lysozyme | 0.758 | 0.559 | 0.527 |
| Soybean trypsin inhibitor | 1.03 | 0.521 | 0.505 |
| Bovine serum albumin | 1.48 | 0.490 | 0.495 |
| Chymotrypsinogen A | 2.48 | 0.417 | 0.410 |
| DT-modified chymotrypsinogen[b] | 0.803 | 0.085 | 0.088 |
| Acetylated chymotrypsinogen[c] | 2.32 | — | 0.340 |
| Anhydro-chymotrypsin | 2.50 | 0.290 | 0.300 |
| α-Chymotrypsin | 2.65 | 0.312 | 0.423 |

[a] Aliquots (1 ml) of each protein solution were used in the determination of arginine by both the Sakaguchi procedure (see text) and by ion-exchange chromatography after 24 hr 6 N HCl hydrolysis (30). All determinations were carried out in duplicate, the values agreeing to within 3% of each other. Protein concentrations were determined from their absorbancies at 280 nm, using the following $\epsilon_{1\ cm}^{1\%}$ values: lysozyme, 26.5 (33); ribonuclease A, 6.95 (34); soybean trypsin inhibitor, 9.54 (35); bovine serum albumin, 6.60 (36); chymotrypsinogen A and α-chymotrypsin, 20.0 (37,38). DT-modified chymotrypsinogen, acetylated chymotrypsinogen and anhydro-chymotrypsin were assumed to possess the same extinction coefficients as their precursors.

[b] Chymotrypsinogen A treated with diacetyl trimer (28).

[c] 92.5% acetylated, based on reaction with ninhydrin.

In view of this linearity, the amount of arginine in the proteins was calculated by reference to the arginine standard curves and compared to the values obtained by quantitative amino acid analysis (30) after acidic hydrolysis of identical samples. The results contained in Table 1 illustrate that the two procedures compare very favorably with each other, with one exception ($\alpha$-chymotrypsin).

In the case of proteins of known molecular weight, these calculations can be extended to provide the number of arginine residues per mole of protein. The results of several such calculations are shown in Table 2. Again an anomaly is noted in the value obtained for $\alpha$-chymotrypsin by the Sakaguchi procedure. All of the other proteins tested, however, show good agreement with the expected values.

*Arginine Content of Serine Esterases*

The arginine content of two other serine esterases, trypsin and subtilisin, were also determined. The results are compared to those obtained with $\alpha$-chymotrypsin in Table 3.

The reasons for the apparent presence of an additional Sakaguchi-positive component in $\alpha$-chymotrypsin were investigated further by subjecting a solution of chymotrypsinogen to tryptic activation and mon-

TABLE 2
Estimation of the Number of Arginine Residues per Mole of Protein

| Protein | No. of arginine residues/mole of protein[a] | | |
|---|---|---|---|
| | Ion-exchange chromatography[b] | Sakaguchi[c] | Expected[d] |
| Ribonuclease A | 3.97 ± 0.15 | 4.20 ± 0.20 | 4.0 |
| Lysozyme | 10.6 ± 0.30 | 9.64 ± 0.30 | 11.0 |
| Soybean trypsin inhibitor | 10.8 ± 0.40 | 10.2 ± 0.40 | 10.0 |
| Bovine serum albumin | 22.5 ± 0.70 | 22.0 ± 0.80 | 23.0 |
| Chymotrypsinogen A | 4.20 ± 0.13 | 4.12 ± 0.14 | 4.0 |
| DT-modified chymotrypsinogen | 2.65 ± 0.15 | 2.72 ± 0.22 | — |
| Acetylated chymotrypsinogen | — | 3.75 ± 0.26 | 4.0 |
| Anhydro-chymotrypsin | 2.90 ± 0.10 | 3.00 ± 0.14 | 3.0 |
| $\alpha$-Chymotrypsin | 2.94 ± 0.12 | 3.97 ± 0.12 | 3.0 |

[a] Estimated on the basis of the following protein molecular weights: lysozyme, 14,307 (39); ribonuclease A, 13, 683 (40); soybean trypsin inhibitor, 21,500 (41); bovine serum albumin, 66,000 (36); chymotrypsinogen A, DT-modified chymotrypsinogen, acetylated chymotrypsinogen, anhydro-chymotrypsin, and $\alpha$-chymotrypsin, 25,000 (37,38).

[b] Determined at the protein concentrations given in Table 1.

[c] Mean of eight duplicates over a tenfold concentration range, the maximum concentrations being those given in Table 1.

[d] On the basis of the known sequence and/or composition (see above references).

TABLE 3
Arginine Content of Serine Esterases

| Protein | No. of arginine residues/mole of protein[a] | | |
|---|---|---|---|
| | Ion-exchange chromatography[b] | Sakaguchi[c] | Expected[d] |
| α-Chymotrypsin | 2.94 ± 0.12 | 3.97 ± 0.12 | 3.0 |
| Trypsin | 1.86 ± 0.11 | 2.75 ± 0.15 | 2.0 |
| Subtilisin | 1.78 ± 0.10 | 2.69 ± 0.10 | 2.0 |
| Acetylated chymotrypsin[e] | — | 3.95 ± 0.15 | 3.0 |

[a] Estimated on the basis of the following protein molecular weights: α-chymotrypsin and acetylated chymotrypsin, 25,000 (37,38); trypsin, 23,800 (42); subtilisin, 27,537 (43).
[b] See footnote *b*, Table 2.
[c] See footnote *c*, Table 2.
[d] See footnote *d*, Table 2.
[e] Derived from acetylated chymotrypsinogen. Value corrected for one arginine residue released upon activation.

itoring the appearance of esterase activity and increase in Sakaguchi colour simultaneously. The apparent increase in the arginine content was found to parallel the appearance of enzyme activity, as shown in Fig. 2. Similar behaviour was noted when extensively acetylated chymotrypsinogen was used in place of chymotrypsinogen.

## DISCUSSION

The simultaneous reaction of monosubstituted guanidines with α-naphthol and alkaline hypobromite forms the basis of the Sakaguchi reaction. The product of the reaction is a red-coloured complex with a $\lambda_{max}$ at 510 nm, the structure of which has recently been elucidated and a mechanism for its formation proposed (44). Considerable attention has been paid to the improvement of the reproducibility of the method, centering largely on the establishment of optimal conditions and stabilization of the coloured product. Thus 8-hydroxyquinoline, 7-chloro-8-hydroxyquinoline (14,16), and 2,4-dichloro-1-naphthol (19) have been used in place of α-naphthol, while *N*-bromosuccinimide has been suggested as a more reliable source of bromine than hypobromite (15). Urea appears to be the traditional stabilizing agent (2–12,14,15) but thiodiglycol (22), sodium thiosulfate (13) and sodium nitrite (17) have also been used. Efforts have also been made to circumvent the interference caused by the presence of large excess of amino groups (17) as well as that due to side chains of amino acids such as histidine, methionine, tryptophan and tyrosine (19).

In the case of intact proteins, the problem of a large excess of free amino groups does not usually arise since these are blocked in the form

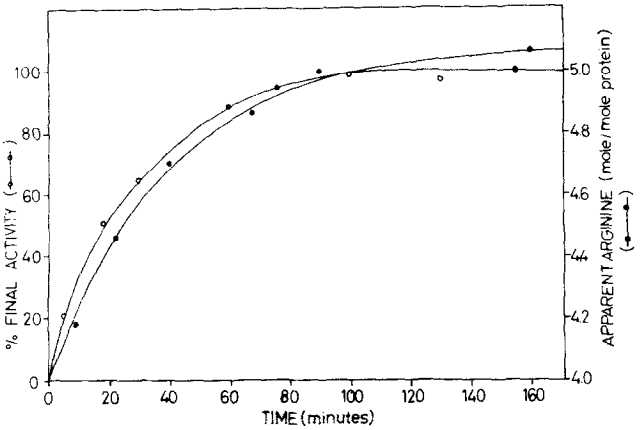


FIG. 2. Variation in chymotryptic activity and arginine content of protein during incubation of chymotrypsinogen A ($9.0 \times 10^{-5}$ M) with trypsin (31). (Temperature, 22°C; 0.02 M phosphate buffer, pH 6.90). Sakaguchi determinations were performed using aliquots of activation mixture.

of peptide linkages. Provided that the arginine side chains are accessible to the reagents, the use of intact proteins should give better results than those obtained using acid hydrolysates. Since the test is carried out in very strong base, it was felt that the proteins should become sufficiently unfolded to permit the reaction of the guanidino groups with the Sakaguchi reagents. Several earlier workers have suggested that this might indeed be the case (7–9,19,23,24), although only one report, to our knowledge, has examined this possibility thoroughly (19).

In the current study, the procedure, essentially the same as Weber's modification (2) of the original method (1), has been found to yield consistently reproducible results. Hence its application to the determination of arginine in the proteins was investigated. In all of the cases studied, a linear relationship between the absorbance at 520 nm and the protein concentration was obtained. With one exception ($\alpha$-chymotrypsin), the amount of protein-bound arginine determined by the quantitative Sakaguchi procedure was in good agreement with that found in the acid hydrolysates by ion-exchange chromatography (30). Furthermore, the numbers of arginine residues per mole of protein obtained by both methods were in mutual agreement and corresponded closely with the known values.

One of the proteins studied, namely, $\alpha$-chymotrypsin, gave anomalous results when subjected to the Sakaguchi determination. Values were consistently obtained which corresponded to the apparent presence of one additional arginine residue per mole of protein compared to the number

obtained by the ion-exchange chromatographic method. Chymotrypsinogen A, the inactive precursor of α-chymotrypsin, behaved normally in that the arginine content determined by the Sakaguchi method was in good agreement with that obtained by the ion-exchange procedure, both values being in close correspondence to the number of arginine residues expected on the basis of the known composition of the protein (38).

In view of these observations, the phenomenon was investigated further. Chymotrypsinogen A was subjected to activation by trypsin. At appropriate time intervals, samples were assayed both for enzymatic activity and for arginine content by the Sakaguchi procedure. The appearance of chymotryptic activity was followed closely by an increase in the colour obtained in the Sakaguchi test. The increase in the Sakaguchi colour became maximal when the enzymatic activity reached a maximum and corresponded to the presence of one additional Sakaguchi-positive component per mole of protein. It is apparent, therefore, that the enhanced colour obtained with α-chymotrypsin is associated in some way with the activation of the zymogen. Extensively acetylated chymotrypsinogen behaves similarly, giving rise to one additional Sakaguchi-positive component after tryptic activation.

The reasons underlying the above observation remain unclear. It is possible that one or more of the arginine residues in α-chymotrypsin are more accessible than others and thereby contribute to the colour obtained in the Sakaguchi test in such a manner that gives rise to a higher value than that usually obtained. However, the excellent agreement between the observed and expected values obtained with the other proteins suggests that, in general, the conditions of the test are such that all of the arginine residues are equally accessible to the reagents. Histidine has been reported to give rise to a positive Sakaguchi test, with a sensitivity of about one-twentieth of that of arginine (23). In view of the widely accepted involvement of histidine in the catalytic mechanism of α-chymotrypsin, it is possible that the enhanced reactivity of this residue may also result in a higher sensitivity towards the Sakaguchi reagents, and thereby give rise to the apparent presence of an additional arginine. Again, however, the conditions of the test are such that it is unlikely that the histidine residue retains this activity, since the native conformation of the protein is almost certainly severely disrupted.

Alternatively, the additional Sakaguchi positive component may be related to the previously reported (45,46) but as yet unconfirmed presence of an unusual basic amino acid at the active site of α-chymotrypsin. In this connection, it is interesting to note that anhydro-chymotrypsin, an inactive protein prepared from α-chymotrypsin by chemical modification of the active site, has an arginine content as determined by the

Sakaguchi procedure which is in agreement with both the value obtained by ion-exchange chromatography and that expected on the basis of the published composition of the protein (38). Evidently modification of the active site of the enzyme brings about a loss of the ability to form an enhanced colour under the conditions of the test.

Since the mechanisms of action of those enzymes known as the serine esterases are considered to possess many common features, it was of interest to determine whether or not any of these other proteins also exhibited anomalous behaviour in the Sakaguchi tests. Both trypsin and subtilisin, in addition to α-chymotrypsin, were found to give rise to a colour yield in the Sakaguchi test which corresponded to approximately one additional Sakaguchi positive residue which was otherwise not detected by amino acid analysis.

## ACKNOWLEDGMENT

These studies were supported by a grant from the National Research Council of Canada.

## REFERENCES

1. SAKAGUCHI, S. (1925) *J. Biochem. (Tokyo)* **5**, 25.
2. WEBER, C. J. (1930) *J. Biol. Chem.* **86**, 217.
3. JORPES, E., AND THOREN, S. (1932) *Biochem. J.* **26**, 1504.
4. DUMAZERT, C., AND POGGI, R. (1939) *Bull. Soc. Chim. Biol.* **21**, 1381.
5. DUBNOFF, J. W., AND BORSOOK, H. (1941) *J. Biol. Chem.* **138**, 381.
6. BBAND, E., AND KASSEL, B. (1942) *J. Biol. Chem.* **145**, 359.
7. ANTHONY, A., ALBANESE, A. A., AND FRANKSTON, J. E. (1945) *J. Biol. Chem.* **159**, 185.
8. MACPHERSON, H. T. (1946) *Biochem. J.* **40**, 470.
9. KEYSER, J. W. (1948) *Biochem. J.* **43**, 488.
10. KRAUT, H., SCHRADER-BEILSTEIN, E. V., AND WEBER, M. (1950) *Z. Physiol. Chem.* **286**, 248.
11. SAKAGUCHI, S. (1950) *J. Biochem. (Tokyo)* **37**, 231.
12. SAKAGUCHI, S. (1951) *J. Biochem. (Tokyo)* **38**, 91.
13. VAN PILSUM, J. F., MARTIN, R. P., KITO, E., AND HESS, J. (1956) *J. Biol. Chem.* **222**, 225.
14. JANUS, J. W. (1956) *Nature (London)* **177**, 529.
15. SZILAGY, I., AND SZABO, I. (1958) *Nature (London)* **181**, 52.
16. SATAKE, K., AND LUCK, J. M. (1958) *Bull. Soc. Chim. Biol.* **40**, 1743.
17. IZUMI, Y. (1965) *Anal. Biochem.* **10**, 218.
18. IZUMI, Y. (1965) *Anal. Biochem.* **12**, 1.
19. MESSINEO, L. (1966) *Arch. Biochem. Biophys.* **117**, 534.
20. SURAN, A. A. (1966) *Arch. Biochem. Biophys.* **113**, 1.
21. DURZAN, D. J. (1969) *Can. J. Biochem.* **47**, 657.
22. ALEXEENKO, L. P., AND OREKHOVICH, V. N. (1970) *Int. J. Prot. Res.* **II**, 241.
23. WATSON, D. (1966) *Experientia* **22**, 76.
24. CHERVENKA, C. H., AND WILCOX, P. E. (1956) *J. Biol. Chem.* **222**, 635.

25. TOZER, N. M. (1973) M. Sc. Thesis, University of Waterloo.
26. GREENSTEIN, J. P., AND WINITZ, M. (1961) Chemistry of the Amino Acids, Vol. 3, p. 1847, Wiley, New York.
27. BALLS, A. K., AND RYAN, C. A. (1963) *Proc. Nat. Acad. Sci. USA* **50**, 448.
28. YANKEELOV, J. A., JR., MITCHELL, C. D., AND CRAWFORD, T. H. (1968) *J. Amer. Chem. Soc.* **90**, 1664.
29. WEINER, H., WHITE, W. N., HOARE, D. G., AND KOSHLAND, D. E., JR. (1966) *J. Amer. Chem. Soc.* **88**, 3851.
30. SPACKMAN, D. H., STEIN, W. H., AND MOORE, S. (1958) *Anal. Chem.* **30**, 1190.
31. DREYER, W. J., AND NEURATH, H. (1955) *J. Amer. Chem. Soc.* **77**, 814.
32. SCHWERT, G. W., AND TAKENAKA, Y. (1955) *Biochim. Biophys. Acta* **16**, 570.
33. STEINER, R. F. (1964) *Biochim. Biophys. Acta* **79**, 51.
34. SHERWOOD, L. M., AND POTTS, J. T., JR. (1965) *J. Biol. Chem.* **240**, 3799.
35. LASKOWSKI, M., AND LASKOWSKI, M., JR. (1954) *Advan. Prot. Chem.* **9**, 203.
36. WETLAUFER, D. B. (1962) *Advan. Prot. Chem.* **17**, 378.
37. WILCOX, P. E., COHEN, E., AND TAN, W. (1957) *J. Biol. Chem.* **228**, 999.
38. DIXON, G. H., AND NEURATH, H. (1957) *J. Biol. Chem.* **225**, 1049.
39. PRAISSMAN, M., AND RUPLEY, J. A. (1968) *Biochemistry* **7**, 2446.
40. HIRS, C. H. W., MOORE, S., AND STEIN, W. H. (1956) *J. Biol. Chem.* **219**, 1623.
41. WU, Y. U., AND SCHERAGA, H. A. (1962) *Biochemistry* **1**, 698.
42. WALSH, K. A., KAUFFMAN, D. L., SAMPATH KUMAR, K. S. V., AND NEURATH, H. (1964) *Proc. Nat. Acad. Sci. USA* **51**, 301.
43. MARKLAND, F. S., AND SMITH, E. L. (1967) *J. Biol. Chem.* **242**, 5198.
44. HEESING, A., AND HOPPE, K. (1967) *Chem. Ber.* **100**, 3649.
45. VISWANATHA, T. (1964) *Proc. Nat. Acad. Sci. USA* **51**, 1117.
46. RICKERT, W. S., AND VISWANATHA, T. (1967) *Biochem. Biophys. Res. Commun.* **28**, 1028.