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EFFECT OF GAMMA IRRADIATION ON INDOLE ACETIC ACID SYNTHESIZING SYSTEM IN POTATOES

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Abstract—The sprout inhibition of potato achieved by 10 Krad gamma irradiation can be completely reversed by treatment of the tubers with 20 ppm IAA. Studies on the level of IAA synthesizing system in unirradiated and irradiated potatoes showed that on irradiation the enzyme activity steadily decreased and fell to zero at the end of 5 weeks storage. When irradiated potatoes were soaked with 20 ppm of IAA, the loss in enzyme activity was not discernible but, with tryptophan, there was no protection of enzyme activity. The enzyme was purified 12 fold, from control and from irradiated potatoes immediately after irradiation. The product of the reaction was identified as IAA by co-chromatography with authentic sample. The reaction required pyridoxal phosphate and a keto acid. Among the keto acids, α-ketoglutaric acid and phenylpyruvic acid were the best as substrates. The optimum pH for the reaction was 8, and optimum temperature was 37°. The two enzyme preparations differed in their response to substrates and co-enzymes; the Michaelis constant for tryptophan was 6×10^{-4} M for the enzyme from control and 4×10^{-4} M for enzyme from irradiated potatoes. The reaction was inhibited by NH_2OH, INH and EDTA. Inhibition by EDTA was reversed by Mn^{++}.

INTRODUCTION*

HORMONE-INDUCED reversal of radiation effects following low dose irradiation of fruits and vegetables is a well known phenomenon. Mathur[1] reported the reversal of radiation-induced dormancy in potatoes by application of exogenous gibberellic acid. Earlier, Skoog,[2] King and Galston[3] and Kelly[4] have shown depletion of the auxin, 3-indoleacetic acid, (IAA) on irradiation, which remains one of the most sensitive of the cellular responses to radiation. Gordon[5] has adduced evidence to support the acute susceptibility of IAA synthesizing system to ionizing radiation. But to date, there are no reports on the effects of IAA on radiation-induced sprout inhibition of potatoes. Also, information is lacking on the effect of gamma irradiation on the synthesis of auxin in potatoes. With this in mind, we have investigated the reversal of sprout inhibition by gamma irradiation and the status of the auxin synthesizing system in irradiated and unirradiated potatoes.

* Abbreviations used: IAA=3-indoleacetic acid; IAM=3-indoleacetamide; IAN=3-indoleacetonitrile; IpyA=3-indolepyruvic acid; IAAld=3-indoleacetaldehyde; TPP=Thiamine pyrophosphate; EDTA=Ethylenediaminetetraacetic acid; INH=Isonicotinic acid hydrazide.

[1] P. B. MATHUR, *Nature* **190**, 547 (1961).
[2] F. J. SKOOG, *J. Cell. Comp. Physiol.* **7**, 227 (1935).
[3] J. W. KING and A. W. GALSTON, *Proc. 3rd Australasian Conf. on Radiobiology*, pp. 238–244, Butterworth, London (1960).
[4] L. S. KELLY, In *Fundamental aspects Radiosensitivity*, pp. 32–46, Brokhaven Natl. Lab. (1961).
[5] S. A. GORDON, *Quart. Rev. Biol.* **32**, 3 (1957).

RESULTS

Effect of Soaking in IAA on Reversal of Sprout Inhibition and IAA Synthesizing Capacity

Mathur[6] has shown that susceptibility to decay by irradiation can be reversed by treatment with 1000 ppm of methyl ester of IAA. At this high concentration of IAA, there is no reversal of sprout inhibition. Our studies on the reversal of sprout inhibition with IAA (Table 1) clearly indicate that soaking of potatoes in a solution of very low concentration of IAA, can reverse the sprout inhibition achieved by gamma irradiation. 20 ppm of IAA is the optimum amount required for complete reversal. These potatoes sprouted within 45 days in the same manner as unirradiated potatoes. However, higher concentration of IAA were not as effective as 20 ppm of IAA in reversing the inhibition.

TABLE 1. REVERSAL OF SPROUT INHIBITION OF POTATOES WITH IAA

Samples	Days of storage				
	10	20	30	40	60
	Percent sprouted				
Control	Nil	10–20	30–40	50–60	90–100
Irradiated	Nil	Nil	Nil	Nil	Nil
Irradiated + IAA					
at concn 1 ppm	Nil	Nil	0–10	10–20	20–30
at concn 10 ppm	Nil	Nil	10–20	40–50	60–70
at concn 20 ppm	Nil	Nil	10–20	40–50	80–90
at concn 50 ppm	Nil	Nil	10–20	30–40	30–40
at concn 100 ppm	Nil	Nil	0–10	20–30	20–30
at concn 150 ppm	Nil	Nil	0–10	0–10	0–20

20 potatoes were selected in each lot. One lot was left as control. Others were irradiated at 10 Krads. The irradiated ones were dipped in different concentration of IAA for 2 hr and stored along with control and irradiated at 25° and 50–60 per cent R.H. The number of potatoes sprouted in each lot were counted after different days of storage and results expressed as percentage sprouted.

The effect of irradiation and treatment with auxin after irradiation on the IAA synthesizing system was then studied. The data in Fig. 1 suggests that during the storage period control tubers exhibit a steady state level of enzyme activity. When irradiated, the initial enzyme activity is increased to about 30 per cent. On storage, there is fast decline in the enzyme activity. At 24 hr, 40 per cent decrease in activity was observed. Finally, at the end of 5 weeks, less than 10 per cent of the initial activity was detectable. As the irradiated tubers were treated with 20 ppm of IAA solution, the pattern of the enzyme activity is very similar to that of control potatoes, although there is slight initial activation. Nevertheless, treatment with tryptophan (100 ppm), the precursor of IAA, does not have any effect in protecting the enzyme activity.

Properties of IAA Synthesizing System

A typical purification of the enzyme from unirradiated tubers is outlined in Table 2. For comparative studies, the enzyme was purified by the same procedure from potatoes

[6] P. B. MATHUR, *Nature* **199**, 1007 (1963).

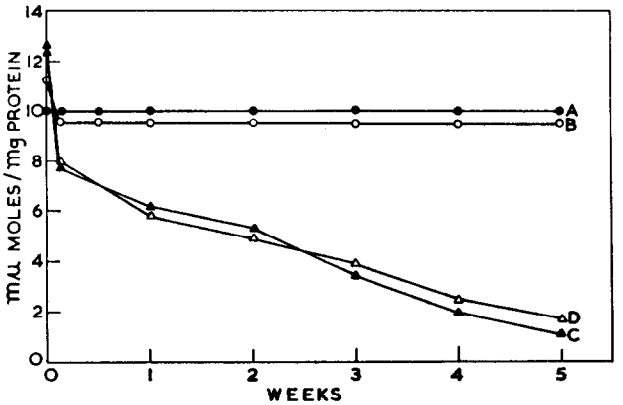

FIG. 1. EFFECT OF GAMMA IRRADIATION ON THE STATUS OF THE ENZYME SYNTHESIZING IAA. For further details, see Experimental.
Curve A, Control, Curve B, Irradiated and treated with IAA, 20 ppm. Curve C, Irradiated; Curve D, Irradiated treated with tryptophan 100 ppm.

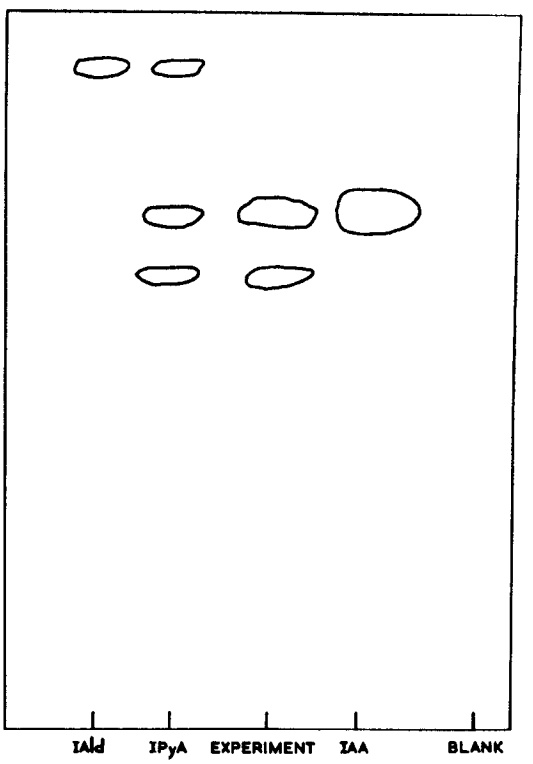

FIG. 2. CHROMATOGRAM OF THE PRODUCT OF IAA SYNTHESIZING SYSTEM FROM POTATOES.
The reaction mixture after acidification to pH 2·8–3 with 3 N HCl, and removal of proteins, was extracted thrice with equal volume of peroxide free ether. For a chromatogram 4 reaction mixtures were extracted, and the ether layer pooled and dried. The residue was dissolved in ether and evaporated to a small volume and spotted on a Whatman No. 1 filter paper. The chromatogram developed with Isopropanol–NH_3–water (10:1:1) solvent in the dark for 14 hr, along with authentic IAA using ascending chromatography. The spots were detected by spraying with *p*-dimethylaminobenzaldehyde reagent.

Table 2. Progress of purification of the enzyme system converting tryptophan to IAA

Fraction	Volume (ml)	Protein (mg/ml)	Specific activity	Total activity (m μmoles)
Homogenate	100	14	5·7	8000
0–50% $(NH_4)_2SO_4$ fraction	65	18	7·8	8650
Calcium phosphate gel Eluate	45	4·5	17·0	5280
30–40% $(NH_4)_2SO_4$	25	1·75	64·8	2825

Conditions were as in Experimental. The activity was determined after 3 hr incubation period.

just after irradiation. This procedure gave us a 12-fold purified enzyme with 35 per cent recovery of original activity.

Purified enzyme gave IAA as the main product of the reaction. The transamination product of tryptophan, IPyA being very unstable, can be converted, either enzymatically or non-enzymatically, by spontaneous dismutation to IAA.[7] The chromatography of the ether extract of the reaction mixture showed IAA as the main constituent. The intensity of the spot corresponding to IPyA was very much less than that of IAA in all chromatograms (Fig. 2).

The pH optima for this reaction was found to be 8. There was a sharp drop in activity in alkaline pHs. The reaction was linear up to 3 hr of incubation. The studies on temperature dependency of the reaction showed, that enzyme had a very high temperature optimum, the maximal activity being at 37°. At 50° there was slight inactivation of the reaction.

The formation of IAA in potatoes is catalysed by transamination of tryptophan as first step. This is evident from the finding that α-ketoglutaric acid and pyridoxal phosphate activate the reaction (Table 3). Recent studies on the synthesis of IAA from tryptophan in pea seedling by Moore and Shamer.[8] have shown that addition of TPP along with other cofactors, enhances activity. This was not observed in the case of the potato enzyme. Of the different keto acids tried, only α-ketoglutaric acid and phenylpyruvic acid showed maximum activity (Table 4). The possible existence of two separate transaminases specific for α-ketoglutaric acid and phenylpyruvic acid, as in the case of *Agrobacterium tumefaciens*[9]

Table 3. Requirements for IAA synthesis

Omission of reagent	m μmoles of IAA formed
*Complete system	36
Pyridoxal phosphate	8·6
α-Ketoglutaric acid	12
Pyridoxal phosphate + α-Ketoglutaric acid	4·6

* Addition of 2 μmoles of TPP to the standard assay mixture did not activate the reaction, any further. Standard assay conditions were used.

[7] J. M. Kaper and H. Veldstra, *Biochem. Biophys. Acta* **30**, 401 (1958).
[8] T. C. Moore and C. A. Shamer, *Arch. Biochem. Biophys.* **127**, 613 (1968).
[9] N. K. Sukanya and C. S. Vaidyanathan, *Biochem. J.* **92**, 594 (1964).

TABLE 4. EFFECT OF DIFFERENT KETO ACIDS ON IAA SYNTHESIS

Keto acid	m μmoles IAA formed
α-Ketoglutaric acid	38
Phenylpyruvic acid	37
Glyoxalic acid	28
Pyruvic acid	24
Oxaloacetic acid	24

In the standard assay mixture α-ketoglutaric acid was replaced with each of the other keto acid at a concentration of 4 μmoles.

cannot be excluded. Further purification of the enzyme is required to resolve this issue. Pyruvic acid, glyoxalic acid and oxaloacetic acid were less active as acceptor of the amino group. They showed only 80 and 70 per cent activity respectively when compared with α-ketoglutaric acid.

Effect of Substrate and Cofactor Concentration on the Activity

Irradiation caused about 30 per cent activation of the enzyme activity. In order to investigate this further, the enzyme was purified from potatoes just after irradiation and its rate of reaction with different substrates and cofactor was compared with the enzyme obtained from control sample (Table 5). In these experiments the enzyme from irradiated potatoes showed more affinity towards its substrate tryptophan, whereas higher concentration of the keto acid and pyridoxal phosphate were required for the activity of this enzyme. The difference in the K_m value of the enzymes from unirradiated and irradiated potatoes obtained here was always reproducible. The K_m value observed in the present case is in excellent agreement with the value (4×10^{-4} M) reported for a cell free enzyme from mung bean seedlings by Wightman and Cohen.[10] The latter enzyme was exclusively localized in the mitochondrial fraction. This observation may explain the slight activation of the enzyme caused by irradiation of whole potatoes. However, there was no activation observed when the isolated enzyme was irradiated at a dose of 10 Krads.

TABLE 5. COMPARISON OF MICHAELIS CONSTANT FOR IAA, SYNTHESIS FROM CONTROL AND IRRADIATED POTATOES

Substrate	K_m	
	Control	Irradiated
Tryptophan	$5{\cdot}74 \times 10^{-4}$ M	4×10^{-4} M
α-Ketoglutaric acid	$8{\cdot}33 \times 10^{-4}$ M	9×10^{-4} M
Pyridoxal phosphate	$4{\cdot}3 \times 10^{-4}$ M	$5{\cdot}5 \times 10^{-4}$ M

The enzyme was isolated from potatoes just after irradiation at 10 Krad.

[10] F. WIGHTMAN and D. COHEN, *Biochemistry and Physiology of Plant Growth Substances* (edited by F. WIGHTMAN and G. SELTERFIELD), pp. 273–288b, Runge Press, Ottawa, Canada (1968).

Inhibitors

Carbonyl reagents like hydroxylamine, INH inhibit the enzyme. The concentration of these reagents for 50 per cent inhibition is 1×10^{-4} M (Table 6). This observation supports the involvement of pyridoxal phosphate in the reaction. Inhibition by EDTA and its partial reversal with Mn^{++} alone, suggest that there is requirement for a metal ion presumably Mn^{++} for this transaminase (Table 7).

TABLE 6. EFFECT OF INHIBITORS ON THE REACTION

Additions	Final concentration (μM)	Percentage inhibition
Hydroxylamine	250	100
	100	50
Isonicotinic acid hydrazide	250	100
	200	80
	100	50
EDTA	500	100
	350	75
	200	50

TABLE 7. EFFECT OF METAL IONS ON THE ENZYME ACTIVITY

Additions	Final concentration (μM)	m μmoles of IAA formed
None	—	36
Mn^{++}	500	36
Zn^{++}	500	32
Mg^{++}	500	28
Fe^{++}	500	28
EDTA	200	18
EDTA + Mn^{++}	200 + 300	30
EDTA + Fe^{++}	200 + 300	14
EDTA + Mg^{++}	300 + 300	15
EDTA + Zn^{++}	200 + 300	17

The enzyme activity was determined after 3 hr incubation period, of the standard assay mixture.

DISCUSSION

The enzymatic steps leading to the biosynthesis of IAA in plants are not well understood due to the very low capacity of the healthy tissue to synthesize this compound. However, there is evidence for the enzyme mediated formation of IAA from several naturally occurring precursors like tryptophan, tryptamine, IAN and IAM. L-Tryptophan is well established

as the precursor for IAA both in higher plants[8,11,12] and in microorganism.[9,13,14] The formation of IAA from tryptophan according to two major pathways follow via IPyA or tryptamine both of these give IAAld as an intermediate. Natural occurrence of IPyA has been established by Winter[15] in *Zea mays* seedlings. In recent years there are a number of studies[7–9] on the intermediary formation of IPyA from tryptophan during its conversion to IAA. Due to its high lability IPyA cannot be detected as the product unless it is complexed and stabilized as a derivative. In this way Moore[8] has established the formation of IPyA and IAAld as intermediates in this reaction.

In IAA synthesizing system from potato, the transamination of tryptophan is the first step and pyridoxal phosphate and a keto acid are essential requirements (Table 3). Addition of TPP did not accelerate the reaction any further. With the pea enzyme Moore and Shamer[8] got an activation of the reaction with TPP suggesting IPyA is enzymatically converted into IAAld. In the present instance the fact that IAA is only the major product suggests that spontaneous breakdown of IPyA occurs under our reaction conditions. Similar observation has been made in *E. coli* system where IAA was the only product, and also there was no evidence for enzymatic conversion of IPyA to IAA.[16] The involvement of pyridoxal phosphate in this reaction is confirmed by the observation that carbonyl reagents like hydroxylamine and INH are potent inhibitors of the enzyme reaction (Table 6). Most interesting finding is the inhibition of the reaction by EDTA and its reversal with Mn^{++} (Table 7). A metal ion requirement is known for the Schiff's base formation in non enzymatic transamination.[17] There are only very few reports on transaminase where a metal ion has been shown to activate the enzyme reaction.[18,19] But in the case of the potato enzyme, addition of Mn^{++} ion to reaction did not have any effect on the reaction. This may be due to the fact that enzyme is saturated with respect to the metal.

The physiological effect of gamma irradiation is to prolong the period of dormancy in potato tubers. The impairment of auxin metabolism is one of the causes attributed to this effect. Free auxin in the tissue is sensitive to ionizing radiation, and it is in a dynamic pool maintained as a steady state system by concomitant biosynthesis and depletion. Gordon[5] has shown that ionizing radiation not only lowers free auxin concentration but affects the auxin synthesizing system as well as inhibiting the aldehyde oxidase which converts IAAld to IAA in mung bean seedlings. Mathur[6] has suggested that gamma irradiation reversibly inactivates the endogenous IAA synthesizing system in potatoes. Our studies on IAA synthesizing system in gamma irradiated potatoes showed that there is slight activation of the enzyme activity just after irradiation (Fig. 1). This is followed by a fast decrease in activity in 24 hr period and then a gradual decline to almost nil in 5 weeks. As expected the control tubers showed a steady state level of enzyme activity. Treatment of irradiated potatoes with 20 ppm IAA enables the auxin synthesizing system to function normally. However, when the tubers were treated with tryptophan instead of IAA there was no reactivation of the IAA synthesizing system. When the enzyme obtained from 5 week-stored

[11] S. A. Gordon, *Plant Physiol.* **33**, 23 (1958).
[12] J. H. M. Henderson and J. Bonner, *Am. J. Botany* **39**, 444 (1952).
[13] B. B. Stowe, *Biochem. J.* **61**, IX (1955).
[14] N. P. Kefford, J. Brockwell and J. A. Zwar, *Australian J. Biol. Sci.* **13**, 456 (1960).
[15] A. Winter, *Arch. Biochem. Biophys.* **106**, 131 (1964).
[16] S. Mahadevan In *Modern Methods of Plant Analysis* (edited by K. Paech and M. V. Tracy), Vol. 7, p. 242, Springer-Verlag, Berlin (1964).
[17] D. E. Metzler, M. Ikava and E. E. Snell, *J. Am. Chem. Soc.* **76**, 468 (1954).
[18] M. V. Patwardhan, *Nature* **181**, 187 (1958).
[19] L. V. S. Sastry and T. Ramakrishnan, *J. Sci. Ind. Res. India* **20c** (10), 277 (1961).

irradiated potato was incubated with tryptophan no ether extractable indole compounds were present. This clearly indicates that irradiation affected the synthesis of the enzyme which involved in the conversion of tryptophan to IAA. When IAA is exogenously supplied, it stimulates the machinery which synthesize the enzyme responsible for its formation. It is known that auxin increased the template activity of DNA, which in turn produced certain RNA, and proteins.[20] Further work is necessary to explore this interesting phenomena.

EXPERIMENTAL

Potatoes used were 15–20 days old after harvest and were stored at 0–4° in the dark for 2–3 weeks. Before gamma irradiation, potatoes were thawed to room temp. and irradiated in a gamma cell 220, at a dose rate of 2290 rads/min. The tubers received 10 Krad dose and after this they were stored at 25° and 50–60% relative humidity.

Assay Method

The IAA formation was estimated with a reaction mixture consisting of L-tryptophan, 2 μmoles; α-ketoglutaric acid, 4 μmoles; pyridoxal phosphate, 1 μmole; Tris-HCl buffer, pH 8·0, 50 μmoles and enzyme proteins, about 500 μg, in a final volume of 2 ml. The mixture was incubated at 37° in the dark for 3 hr. The reaction was stopped with the addition of 0·1 ml 3 N HCl and the final pH was 3·0. The IAA formed was estimated in 1·5 ml aliquot of the reaction mixture using *p*-dimethylaminobenzaldehyde according to the method of Larsen.[21] In this method, there was no change in the concentration of IAA observed when estimated without extraction of the reaction mixture or after extraction with ether, at the concentration of tryptophan employed in the reaction mixture. However, when tryptophan concentration was over 4 μmoles there was interference. In these cases the estimations were done after extracting the indole compounds into ether. In routine assay, where tryptophan concentration is only 2 μmoles, the extraction procedure was excluded. Suitable controls were included in all assays.

Reversal of Sprout Inhibition with Auxin

Potatoes were irradiated at 10 Krads. They were distributed in lots of 20. Each lot was dipped in a particular concentration of IAA for 2 hr and stored at 25° and 50–60% R.H. Concentrations of IAA used were 1, 10, 20, 50, 100, 150 ppm. Number of potatoes sprouted on storage was compared with irradiated control samples. IAA synthesizing capacity was determined in control, irradiated, irradiated treated with 20 ppm IAA, and finally, irradiated treated with 100 ppm tryptophan. The enzyme used in the experiments was purified only up to first ammonium sulfate step described below.

Purification of the Enzyme

All operations were done between 0–4°. The unit of enzyme is defined as that amount which will catalyse the formation of 1 m μmole of IAA under standard assay conditions. Specific activity is expressed as units of enzyme per mg of protein.

Since IAA synthesizing activity was localized mainly in the peel, peelings were used for enzyme extraction. 200 g of potato peelings were macerated in a Waring Blendor with 100 ml of cold sodium phosphate buffer 0·1 M, pH 7·0. The homogenate was filtered through a double layer of cheese cloth and centrifuged at 10,000 *g* for 20 min. To 100 ml of supernatant 31·3 g of $(NH_4)_2SO_4$ was added and dissolved with stirring. The solution was kept for 10 min and the precipitated proteins were centrifuged out at 10,000 *g* for 20 min. The precipitate was dissolved in 60 ml of 0·1 M Na-phosphate buffer pH 7·0 and again centrifuged at 18,000 *g* for 20 min to remove insoluble proteins. The solution thus obtained was dialysed against 4 l. with 0·02 M Na-phosphate buffer pH 7·0 for 4 hr with change of buffer every hour. Total volume after dialysis was 65 ml.

60 ml of the above solution was adjusted to pH 5·8 with 2 N acetic acid and centrifuged at 20,000 *g* for 20 min to remove precipitated proteins. The supernatant was adsorbed on calcium phosphate gel (protein–gel ratio 1 : 5) with stirring for 15 min. The enzyme was eluted from the gel with 45 ml of 0·1 M Na-phosphate buffer pH 7·5. Finally, to 40 ml of gel eluate, 7·04 g $(NH_4)_2SO_4$ was added. The precipitated proteins were discarded after centrifugation. To the supernatant 2·48 *g* $(NH_4)_2SO_4$ was again added. After 10 min, the precipitate was collected by centrifugations at 20,000 *g* for 20 min. The precipitate was dissolved in 20 ml

[20] Y. MASUDA and S. KAMISAKA, *Plant Cell Physiol.* **10,** 79 (1969).

[21] P. LARSEN, *Modern Methods in Plant Analysis* (edited by K. PAECH and M. V. TRACY), Vol. III, p. 614, Springer-Verlag, Berlin.

0·1 M Na-phosphate buffer pH 7·0 and the solution was dialysed against 3 l. of 0·02 M Na-phosphate buffer pH 7·0 with frequent changes of the buffer. The final volume was 25 ml. This preparation can be stored at −20° for a week without any change in activity. However, the enzyme was inactivated on repeated freezing and thawing. Protein was estimated by biuret method.[22]

Acknowledgement—The authors are grateful to Dr. A. Sreenivasan for his helpful suggestions and criticisms.

[22] A. G. GORNALL, C. J. BARDAWELL and M. M. DAVID, *J. Biol. Chem.* **177**, 751 (1949).