Biochemical Pharmacology, 1963, Vol. 12, pp. 97–104. Pergamon Press Ltd., Printed in Great Britain.

A BIOCHEMICAL BASIS FOR RESISTANCE OF L1210 MOUSE LEUKEMIA TO 6-THIOGUANINE*

PATRICIA STUTTS† and R. W. BROCKMAN

Kettering-Meyer Laboratory,‡ Southern Research Institute, Birmingham, Ala., U.S.A.

(*Received* 22 *September* 1962; *accepted* 23 *October* 1962)

Abstract—Enzyme preparations from ascites cell lines of L1210 mouse leukemia were examined for pyrophosphorylase activity. The synthesis of inosinic, guanylic, azaguanylic, and thioguanylic acids from the corresponding purine bases and 5-phosphoribosyl-1-pyrophosphate was significantly less when the reactions were catalyzed by enzyme preparations from 6-thioguanine-resistant and 8-azaguanine-resistant lines than by similar preparations from the sensitive parent line. Experiments *in vivo* showed that there was also a pronounced decrease in the incorporation of radioactivity from thioguanine-^{35}S into the nucleic acids of thioguanine-resistant L1210 cells as compared with those of the sensitive line.

AMONG the proposed mechanisms of resistance of experimental neoplasms to purine antagonists are (1) decrease or loss of pyrophosphorylase activity for the synthesis of fraudulent ribonucleotide and (2) increased degradation of antimetabolite.

Results of studies of the metabolism *in vivo* of 8-azaguanine and 6-mercaptopurine by bacteria and experimental neoplasms resistant to these purine analogs led to the conclusion that resistance to these inhibitors was associated with failure to convert the fraudulent bases to the corresponding ribonucleotides.[1–4] Extension of these studies to cell-free enzyme systems showed further that the activity of certain of the ribonucleotide pyrophosphorylases was significantly decreased in drug-resistant L1210 leukemias or was absent as in the case of certain bacterial mutants.[5–8]

An example of resistance to azaguanine which was accompanied by increased azaguanine-deaminase activity has been reported also.[9] In contrast, however, no significant differences in capacities for azaguanine degradation were seen in some other sensitive and resistant neoplasms.[10]

In earlier studies with 6-thioguanine, Sartorelli, LePage and Moore[11, 12] found that Ehrlich ascites cells which were resistant to this analog synthesized less thioguanylic acid *in vivo* than did the susceptible neoplasm; these authors also observed more catabolic products in resistant than in sensitive cells and concluded that this increased degradation was a factor in resistance in Ehrlich ascites cells.

Since studies in this laboratory have shown that resistance of L1210 mouse leukemias to other purine antagonists was associated with decreased capacity for fraudulent ribonucleotide formation, and in view of the fact that there are reported examples of resistance both with and without concomitant increased degradation of inhibitor,

* This study was supported by Contract SA-43-ph-2433, Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda, Md.

† Present address: Department of Biochemistry, Duke University, Durham, N.C., U.S.A.

‡ Affiliated with Sloan-Kettering Institute for Cancer Research.

the present study was undertaken to attempt to elucidate the mechanism(s) in L1210 cell lines which are resistant to the growth-inhibitory action of 6-thioguanine. A preliminary report of the results of this study has been presented.[13] In recent investigations Ellis and LePage[14] also found that when a relatively high level of thioguanine was employed for selection, the resistant Ehrlich ascites cell population that resulted lacked guanylic acid pyrophosphorylase.

EXPERIMENTAL

Materials

The 6-thioguanine-^{35}S used in this study was obtained from Isotopes Specialties Co., Burbank, Calif. Adenine-8-^{14}C and hypoxanthine-8-^{14}C were purchased from Volk Radiochemical Co., Chicago, Ill.; guanine-8-^{14}C was obtained from Nuclear-Chicago Corp., Des Plaines, Ill.; and xanthine-8-^{14}C and 8-azaguanine-2-^{14}C were synthesized at Southern Research Institute by Dr. John Montgomery and Mr. Harry T. Baker, respectively.

The compounds used as references in the electrophoretic analyses and their sources were as follows: 6-thioguanine and 6-thioxanthine from California Corporation for Biochemical Research, Los Angeles; 6-thiouric acid from Dr. T. Li Loo, National Cancer Institute, Bethesda, Md.; and 6-thioguanosine from the Cancer Chemotherapy National Service Center, Bethesda, Md. The magnesium salt of PRPP* used in the enzyme incubation mixtures was purchased from Pabst Laboratories, Milwaukee, Wis.

All the neoplasms used in this work were ascites tumor lines obtained from Dr. L. W. Law of the National Cancer Institute. L1210S is the parent, drug-sensitive neoplasm, while both L1210/TG and L1210/8-Aza are cross-resistant to 8-azaguanine and 6-thioguanine.

Methods

1. *In vivo-experiments.* The procedures employed in the studies of the metabolism of 6-thioguanine-^{35}S *in vivo* are essentially the same as those previously described[3]; briefly, they are as follows: a group of seven neoplasm-bearing mice received intraperitoneal injections of 6-thioguanine-^{35}S (20 μc/mouse) in two equal doses 1 hr apart; at the end of the second hour the animals were sacrificed by CO_2 asphyxiation. The ascites cells were harvested, washed once with cold physiological saline, and extracted with boiling 80% ethanol, following which the cell residue was separated by centrifugation. The supernatant alcohol extracts, after spin-evaporation and lyophilization to dryness, were redissolved, and aliquots were analyzed by two-dimensional paper chromatography and by paper electrophoresis.

From the cell residue that remained after ethanol extraction, nucleic acids were isolated as previously described,[3, 4] except that the cells were subjected to rapid freezing and thawing three times in an alcohol-dry ice mixture prior to NaCl extraction.

2. *Enzyme experiments.* The enzyme preparations used in this study were made from ascites cells harvested from DBA or CDF_1 mice. After separation from the ascitic fluid, the cells were washed with cold physiological saline. When erythrocytes were present in significant numbers they were ruptured by subjecting the cells to hypotonic shock treatment. This procedure, which is that described by Feigelson

* Abbreviations used in this paper: PRPP, 5-phosphoribosyl-1-pyrophosphate; 8-AzaGMP, 8-azaguanylic acid; and 6-TGMP, 6-thioguanylic acid.

et al.,[15] as modified by Fallon *et al.*,[16] differentially lyses the erythrocytes to permit their removal by centrifugation, leaving the ascites cells intact.

To each volume of packed ascites cells was then added four volumes of 0·05 M sodium phosphate buffer, pH 7·5; the resulting suspensions were rapidly frozen and thawed as described above, following which they were centrifuged for 90 min at approximately 95,000 × *g* in a refrigerated Spinco centrifuge (4°). The clear supernatant solutions thus obtained constituted the crude enzyme preparations. The L1210S and L1210/TG preparations (Tables 3 and 4) contained 6·7 and 18·8 mg protein/ml, respectively, as determined by the Oyama-Eagle modification[17] of the method of Lowry *et al.*[18] using crystalline bovine albumin (Armour) as the reference standard.*

In some cases enzyme preparations were made using 0·1 M Tris buffer of pH 7·6 for the cell suspensions (4×10^8 cells/ml); in these instances cell rupture was accomplished via sonic vibration at 0° for 10 min. In determining the protein content of these enzyme preparations, a Tris buffer blank was used to compensate for the color developed by Tris with the reagents used. Protein content of L1210/S, L1210/TG, and L1210/8-Aza preparations (Table 2) were 4·3, 1·8 and 1·2 mg/ml, respectively.

The enzyme incubation mixtures are described in the footnotes following the tables. After incubation for 1 hr at 37°, enzyme action was stopped by heat denaturation, the precipitated protein was removed by centrifugation, and aliquots of the supernatants were analyzed by paper chromatographic or paper electrophoretic techniques.

3. *Chromatographic, electrophoretic, and radioautographic analyses.* In the analysis of the enzyme incubation mixtures one-dimensional descending chromatography on Whatman 3MM paper using 70% isopropanol in an ammonia atmosphere[19, 20] was used to separate bases from nucleotides. Incubation mixtures and alcohol extracts from the experiments *in vivo* were also analyzed by two-dimensional descending chromatography using phenol:water in the first dimension and *n*-butanol:propionic acid:water in the second dimension as described elsewhere.[21, 22] Since the separation of guanine and xanthine by the solvent systems just described is frequently inadequate, the radioactive guanine-xanthine areas were resolved by overnight ascending rechromatography on Whatman no. 1 paper with a solvent consisting of 90 ml of 0·04 N ammonium hydroxide and 10 ml of *n*-butanol.

Paper electrophoresis on Whatman 3MM paper with 0·05 M sodium tetraborate buffer of pH 9 as described by Markham[19, 20] was used for the separation and identification of metabolites of 6-thioguanine, since these do not behave satisfactorily when chromatographed as described above. Table 1 presents data on the electrophoretic behavior of 6-thioguanine and some of its derivatives.

Radioactive compounds on paper chromatograms and paper electrophoresis strips were located by exposure of the papers to sheets of X-ray film (Eastman Kodak, single emulsion, blue-sensitive). Measurements of radioactivity were made in gas-flow proportional counters and quantitative distribution of radioactivity was determined as previously described.[21]

RESULTS

It was observed that cell-free enzyme preparations from L1210S ascites tumor cells catalyzed the reaction of guanine and of 6-thioguanine with PRPP to produce guanylic

* We are indebted to Dr. Glen J. Dixon and Miss Elizabeth A. Dulmadge for these protein assays.

and thioguanylic acids. Similarly prepared enzyme preparations from the 6-thioguanine- and 8-azaguanine-resistant neoplasms, L1210/TG and L1210/8-Aza, converted relatively less of these substrates to ribonucleotides than to catabolic products (Table 2).

TABLE 1. ELECTROPHORETIC BEHAVIOR OF SOME METABOLIC DERIVATIVES OF 6-THIOGUANINE

Compound	Migration relative to GMP*
6-Thioguanine	0·53
6-Thioxanthine	0·67
6-Thiouric acid	0·82
6-Thioguanosine	0·82
6-Thioguanylic acid†	1·14
Guanylic acid (reference)	1·00

* Results obtained by electrophoresis on Whatman 3MM paper using 0·05 M sodium tetraborate as buffer at an applied potential of 750 V (potential gradient of approximately 15 v/cm) for 1 hr. Better separation could be achieved at 1,000 V (approx. 20 v/cm) for 90 min; relative migration distances at the higher voltage were the same as those reported above.

† Biosynthesized by reaction of 6-thioguanine and 5-phosphoribosyl-1-pyrophosphate in the presence of extracts of L1210 cells.

TABLE 2. METABOLISM OF GUANINE AND 6-THIOGUANINE BY CELL-FREE ENZYME PREPARATIONS FROM DRUG-SENSITIVE AND DRUG-RESISTANT L1210 ASCITES TUMOR CELLS

Radioactive substrate	Chromatographic* or electrophoretic† analysis of distribution of radioactivity (% of total) in enzyme reaction mixtures‡			
		L1210S	L1210/TG	L1210/8-Aza
Guanine-8-^{14}C	Guanine	26	53	66
	Xanthine	9	22	28
	Uric acid	2	1	<1
	Allantoin	trace	0	<1
	Guanosine	19	13	<1
	Guanylic acid	43	7	1
6-Thioguanine-^{35}S	6-Thioguanine	0	59	70
	6-Thioxanthine	4	18	20
	6-Thiouric acid / 6-Thioguanosine§	5	9	7
	6-Thioguanylic acid	90	15	3

* Guanine incubation mixtures were analyzed by two-dimensional chromatography-radioautography.

† Thioguanine incubation mixtures were analyzed by electrophoresis on Whatman 3MM paper, using a 0·05 M sodium tetraborate buffer at an applied potential of 750 V (potential gradient of approximately 15 V/cm) for 1 hr.

‡ Reaction mixtures contained 0·25 μM radioactive substrate, 2 μM PRPP (magnesium salt), and 0·05 ml of enzyme preparation in 0·1 M Tris buffer, pH 7·5; final volume, 0·5 ml; 1-hr incubation at 37°.

§ 6-Thiouric acid and 6-thioguanosine were not separated.

The quantities of catabolic products formed by L1210S enzyme mixtures from guanine and thioguanine were less than 50 per cent as great as the amounts of xanthine and thioxanthine formed by L1210/TG and L1210/8-Aza enzymes. A possible explanation for this apparently increased catabolism in the resistant lines is suggested in the Discussion section.

Since the L1210/TG ascites tumor line used in this study is cross-resistant to 8-azaguanine, it was considered of interest to compare the metabolism of this compound by cell-free enzyme preparations of L1210/TG and of the parent line, L1210S. The results of this experiment are presented in Table 3 and are analogous to those

TABLE 3. METABOLISM OF 8-AZAGUANINE-2-^{14}C BY CELL-FREE ENZYME PREPARATIONS FROM L1210S AND L1210/TG ASCITES TUMOR CELLS

Compound assayed*	mμM compound/mg protein after 1-hr incubation L1210S	L1210/TG
8-Azaguanine	2,823	1,200
8-Azaxanthine	276	96
8-Azaguanosine	2	0
8-Azaguanylic acid	604	0

* Incubation mixtures contained 0·25 μM 8-azaguanine-2-^{14}C, 2 μM PRPP (magnesium salt), and 0·05 ml of enzyme preparation in 0·05 M phosphate buffer, pH 7·5; final volume, 0·5 ml.; 1-hr incubation at 37°.

TABLE 4. CONVERSION OF PURINES AND PURINE ANALOGS TO RIBONUCLEOTIDES BY CELL-FREE ENZYME PREPARATIONS OF L1210S AND L1210/TG ASCITES TUMOR CELLS

Experiment	Enzyme-catalyzed reaction	mμM ribonucleotide formed/mg protein in 1 hr L1210S	L1210/TG	$\frac{\text{L1210/TG}}{\text{L1210S}} \cdot 100$
1*	Adenine → AMP (→ IMP)†	1,022	267	26
	Hypoxanthine → IMP	686	75	11
	Guanine → GMP	1,649	153	9
	8-Azaguanine → 8-AzaGMP	694	32	5
	6-Thioguanine → 6-TGMP	2,104	70	3
2*	Adenine → AMP	525	157	30
	Hypoxanthine → IMP	701	7	1
	Guanine → GMP	673	14	2
	8-Azaguanine → 8-AzaGMP	502	3	<1
	6-Thioguanine → 6-TGMP	707	22	3

* Incubation mixtures for experiment 1 contained 0·25 μM radioactive purine or purine analog, 2 μM PRPP (magnesium salt), and 0·01 ml of the respective enzyme preparations; reaction mixtures for experiment 2 were the same except that each contained 0·05 ml of enzyme extract. All incubation mixtures were 0·5 M with respect to phosphate buffer, pH 7·5; final volume, 0·5 ml.

† IMP accounted for 5 to 8% of the ribonucleotide from adenine.

seen for thioguanine metabolism in that L1210S enzymes formed significant amounts of ribonucleotide, whereas there was no azaguanylic acid synthesized by the L1210/TG extracts. In addition, the amounts of azaxanthine formed by each of the two enzyme systems represented in each case less than 10 per cent of the total radioactivity. These results indicate that for this system, at least, the operative mechanism of resistance is probably not increased degradation of the inhibitor, but more likely a decrease in the capacity for ribonucleotide formation by the L1210/TG cells.

The data in Table 4 compare the conversion of radioactive purines and some purine analogs to their ribonucleotides by enzyme preparations from L1210S and L1210/TG. It was observed that adenine was converted to ribonucleotide in significant amounts by both cell lines, although to an unexplained lesser extent by the resistant L1210/TG line.

In L1210/TG there was a considerable decrease in the capacity for the formation of ribonucleotides of guanine, hypoxanthine, and azaguanine, as well as of thioguanine. This is analogous to the results of other studies[3-6, 23] which have shown that bacterial and mammalian cell lines resistant to azaguanine or mercaptopurine, or both, fail to synthesize significant amounts of ribonucleotide from these purine analogs or from the above purines.

Although a definite decrease in pyrophosphorylase activity in general was seen for L1210/TG, the fact that this decrease was not so great for adenylic acid is consistent with the interpretation that in these L1210 cell lines the pyrophosphorylase that catalyzes the conversion of adenine to adenylic acid is distinct from the enzyme(s) that catalyze the synthesis of ribonucleotides of guanine, hypoxanthine, azaguanine, and thioguanine.

Two experiments were performed *in vivo* to compare the incorporation of radioactivity from thioguanine-^{35}S into the nucleic acids of L1210S and L1210/TG; the results of these experiments are presented in Fig. 1. The striking observation in both

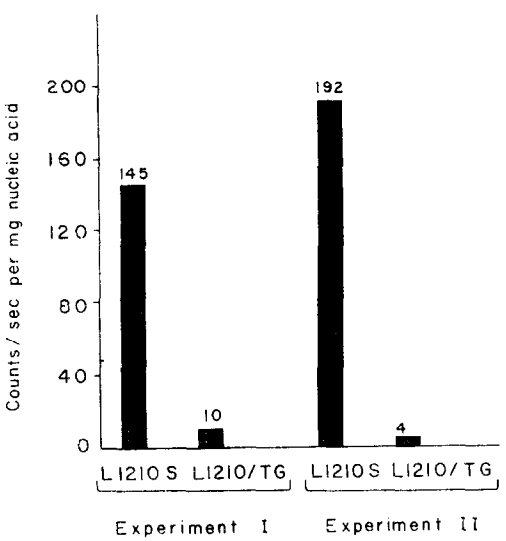

FIG. 1. Radioactivity of the nucleic acid fraction of sensitive and thioguanine-resistant L1210 cells exposed *in vivo* to 6-thioguanine-^{35}S.

experiments is that the specific activity of the nucleic acids from L1210S tumor cells was far greater than that of L1210/TG nucleic acids. An attempt was made to determine whether the radioactivity incorporated into these nucleic acids was thioguanylic acid-^{35}S; the radioactive nucleic acid samples were subjected to KOH hydrolysis and the hydrolysates were subsequently chromatographed. However, although two faint radioactive bands appeared which migrated chromatographically in the purine

ribonucleotide area, the amount of radioactivity in these bands was not sufficient for the execution of further identifying procedures. LePage[12] has shown that thioguanylic acid is indeed incorporated into the nucleic acids of Ehrlich ascites cells.

DISCUSSION

The present study indicates that of the postulated mechanisms of resistance to purine antagonists, in the L1210 lines examined, decreased capacity of resistant cells to synthesize thioguanylic acid is one mechanism by which these cells might become resistant to thioguanine. This finding is in agreement with the previously mentioned one of Ellis and LePage,[14] namely, that a selected thioguanine-resistant Ehrlich ascites cell population had much less guanylic acid pyrophosphorylase activity.

Since catabolism of guanine and thioguanine occurred to a greater extent in the resistant than in the sensitive L1210 cells, the possibility that increased degradation of antimetabolite is a resistance mechanism in these lines perhaps should not be completely eliminated. A possible explanation for this observed effect, however, is that since much less nucleotide was formed by enzymes from the resistant neoplasms, significantly greater amounts of the purine bases remained available as substrate for degradative enzymes for a longer period of time. About 60 to 70% of the thioguanine substrate was recovered unchanged from the L1210/TG and L1210/8-Aza incubation mixtures, whereas none remained as substrate for degradative enzyme action in the L1210/S reaction mixture.

Although there may be evidence that resistance of particular cell lines to given purine analogs is probably not due to increased degradation of the inhibitor, it of course cannot be assumed that this resistance mechanism can be excluded when other purine antagonists are considered. However, with regard to the conclusions of the present study, it is interesting to note evidence that in L1210 ascites cells increased catabolism of inhibitor apparently was not a major factor in resistance to azaguanine[3] or to mercaptopurine.[1] Also, Feigelson *et al.*[15] observed that in L1210 leukemic lymphocytes the levels of xanthine oxidase and uricase were essentially the same in strains that were sensitive to or dependent on azaguanine. It is of additional interest that Ellis and LePage[11, 24] reported examples of resistance of Ehrlich ascites, sarcoma 180, and adenocarcinoma 755 cells to thioguanine which apparently was due to some mechanism other than increased degradation of inhibitor or loss of nucleotide-forming capacity.

No explanation is apparent for the observed decrease in synthesis of adenylic acid by L1210/TG as compared with L1210S; on the basis of the results of previous studies with L1210 and *Streptococcus faecalis*, [3, 4, 6] it was expected that adenine anabolism would be similar in the sensitive and resistant lines. However, it is significant that in the resistant line adenylic acid pyrophosphorylase did not show as great a decrease as did the other pyrophosphorylase(s) and hence is probably distinct from them. Kallé and Gots[25] reported that in resistant mutants of *Salmonella typhimurium* loss of capacity for hypoxanthine and mercaptopurine conversion to ribonucleotides could be distinguished from loss of capacity for guanine and azaguanine conversion. Thus, it remains to be demonstrated whether the resistant L1210 lines examined in the present study show decreased activity of only one or of more than one enzyme.

The diminished pyrophosphorylase activity of the L1210/TG cells was correlated with markedly lower specific activity of the nucleic acids after treatment with

6-thioguanine-^{35}S. In recent investigations LePage and Jones[26] found that in both thioguanine-sensitive and -resistant ascites cell populations, purine sysnthesis was inhibited by thioguanine and certain other purinethiols via a feedback mechanism which presumably first requires conversion of the bases to nucleotides. These authors[27] also demonstrated that thioguanine-sensitive Ehrlich ascites cells which incorporated thioguanine into their nucleic acid retained viability for a considerable time but were unable to replicate their DNA, results suggesting that susceptible tumors were inhibited as a consequence of synthesis of "fraudulent" nucleic acids. From the results of the present study also, such mechanisms of action of thioguanine might be postulated.

While it is clear that other mechanisms of resistance may be encountered, the present study has shown that in the L1210 neoplasms examined, resistance to thioguanine was accompanied by (1) decreased pyrophosphorylase activity for ribonucleotide synthesis, (2) decreased specific activity of nucleic acids after thioguanine-^{35}S treatment, and (3) increased deamination of thioguanine.

Acknowledgements—The authors are grateful to Miss Tommie Lou Barker for performing the animal work and to Miss Linda Simpson for supervising the radioassays. We also acknowledge the helpful suggestions of Dr. L. L. Bennett, Jr., and Dr. G. P. Wheeler concerning the manuscript; and we are indebted to Dr. G. A. LePage, Stanford Research Institute, for valuable discussions of aspects of the work.

REFERENCES

1. R. W. BROCKMAN, M. C. SPARKS and M. S. SIMPSON, *Biochim. biophys. Acta* **26**, 671 (1957).
2. R. W. BROCKMAN, M. C. SPARKS, D. J. HUTCHISON and H. E. SKIPPER, *Cancer Res.* **19**, 177 (1959).
3. R. W. BROCKMAN, L. L. BENNETT, JR., M. S. SIMPSON, A. R. WILSON, J. R. THOMSON and H. E. SKIPPER, *Cancer Res.* **19**, 856 (1959).
4. R. W. BROCKMAN, *Cancer Res.* **20**, 643 (1960).
5. R. W. BROCKMAN, R. A. ROOSA, L. W. LAW and P. STUTTS, *J. cell. comp. Physiol.* **60**, 65 (1962).
6. R. W. BROCKMAN, M. C. SPARKS, M. S. SIMPSON and H. E. SKIPPER, *Biochem. Pharmacol.* **2**, 77 (1959).
7. R. W. BROCKMAN and D. J. HUTCHISON, *Fed. Proc.* **18**, 197 (1959).
8. R. W. BROCKMAN, M. C. SPARKS, P. STUTTS and F. M. SCHABEL, JR., *Bact. Proc.* p. 52 (1959).
9. E. HIRSCHBERG, J. KREAM and A. GELLHORN, *Cancer Res.* **12**, 524 (1952).
10. B. SHACTER and L. W. LAW, *J. nat. Cancer Inst.* **18**, 77 (1957).
11. A. C. SARTORELLI, G. A. LEPAGE and E. C. MOORE, *Cancer Res.* **18**, 1232 (1958).
12. G. A. LEPAGE, *Cancer Res.* **20**, 403 (1960).
13. R. W. BROCKMAN and P. STUTTS, *Fed. Proc.* **19**, 313 (1960).
14. D. B. ELLIS and G. A. LEPAGE, *Cancer Res.* **23**, in press (1963).
15. P. FEIGELSON, J. E. ULTMAN, S. HARRIS and J. DASHMAN, *Cancer Res.* **19**, 1230 (1959).
16. H. J. FALLON, E. FREI, III, J. D. DAVIDSON, T. S. TRIER and D. BURK, *J. Lab. clin. Med.* **59**, 779 (1962).
17. V. I. OYAMA and H. EAGLE, *Proc. Soc. exp. Biol., N.Y.* **91**, 305 (1956).
18. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193**, 265 (1951).
19. R. MARKHAM, in *Modern Methods of Plant Analysis*, K. PAECH and M. V. TRACEY, Eds., vol. IV, p. 246. Springer-Verlag, Berlin (1955).
20. R. MARKHAM and J. D. SMITH, *Biochem. J.* **52**, 552 (1952).
21. R. W. BROCKMAN, C. S. DEBAVADI, P. STUTTS and D. J. HUTCHISON, *J. biol. Chem.* **236**, 1471 (1961).
22. A. A. BENSON, J. A. BASSHAM, M. CALVIN, T. C. GOODALE, V. A. HAAS and W. J. STEPKA, *J. Amer. chem. Soc.* **72**, 1710 (1950).
23. R. W. BROCKMAN, G. G. KELLEY, P. STUTTS and V. COPELAND, *Nature, Lond.* **191**, 469 (1961).
24. D. B. ELLIS and G. A. LEPAGE, *Fed. Proc.* **21**, 164 (1962).
25. G. P. KALLÉ and J. S. GOTS, *Biochim. biophys. Acta* **53**, 166 (1961).
26. G. A. LEPAGE and M. JONES, *Cancer Res.* **21**, 642 (1961).
27. G. A. LEPAGE and M. JONES, *Cancer Res.* **21**, 1590 (1961).