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INCREASED CONVERSION OF URIDINE-5′-PHOSPHATE AND URIDINE-5′-TRIPHOSPHATE TO CYTOSINE NUCLEOTIDES BY A LIVER FRACTION FROM COLCHICINE-TREATED RATS*

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Abstract—Seventeen hours after the injection of colchicine into rats (1·1 mg/kg body weight) the centrifugal high-speed supernatant fraction of the liver had approximately twice the normal capacity for converting uridine-5′-phosphate and uridine-5′-triphosphate to cytosine nucleotides. These results are consistent with those of earlier experiments in which the specific activity of the liver RNA cytosine was approximately twice the control value after orotic acid-^{14}C and uridine-^{14}C were injected into the colchicine-treated rat.

The greater conversion of UMP to cytosine nucleotides could not be explained by a lessened degradation of UMP to uridine and uracil or by a lessened degradation of cytosine nucleotides to cytidine in the preparation from the drug-treated animal. Dialysis experiments ruled out the acquisition by the liver of a freely diffusible activator as a result of injecting colchicine. The greater conversion of UTP could not be explained by a greater stability of this substrate in the preparation from the drug-treated animal. Similarly, greater stability of adenosine-5′-triphosphate, needed for the conversions, was not the basis for the increased activity.

These observations lead to the suggestion that in the liver of the colchicine-treated rat there is an increased concentration of the aminating enzyme which transforms a uracil ribonucleotide to a cytosine ribonucleotide.

IT WAS observed in this laboratory[1] that when rats were treated with colchicine 16–18 hr prior to the injection of a variety of radioactive precursors of nucleic acid purines and pyrimidines, there resulted an increase in the specific activities of the liver RNA bases. When orotic acid-^{14}C was the precursor, the increase in the specific activity of the cytosine above the control value was greater than the increase in the uracil specific activity. When uridine-^{14}C was the precursor, the specific activity of the RNA cytosine was at least twice as high as the control, while that of the uracil was increased only 20 to 30 per cent. Similar results were obtained when orotic acid-^{14}C was incubated with slices of liver from the drug-treated and control animals.

Orotic acid[2] and uridine[3] are converted to UMP in rat liver. Hurlbert and Kammen[4] found that the soluble fraction of a sucrose homogenate of rat liver can convert UMP, UDP, and UTP to cytosine ribonucleotides. Glutamine is the source of the amino group of the cytosine derivatives; and ATP, a guanine ribonucleotide, and Mg^{2+} are

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cofactors for the reaction. The results obtained by analysis of the liver RNA pyrimidines in the experiments with colchicine suggested that the conversion of a uracil ribonucleotide to a cytosine ribonucleotide is accelerated in the liver after treatment with the drug. The examination of this possibility is the subject of the present communication.

MATERIALS AND METHODS

Compounds. Uridine-5′-phosphate uniformly labeled with ^{14}C was obtained from the New England Nuclear Corp. and uridine-5′-triphosphate-2-^{14}C from Schwartz BioResearch, Inc. All other nucleotides and nucleosides were products of P-L Biochemicals, Inc. L-Glutamine was obtained from Schwartz BioResearch, Inc.

Colchicine treatment of animals. Male rats of the Holtzman Sprague–Dawley strain weighing approximately 200 g were treated as described in the previous work.[1] Colchicine dissolved in 0·5 ml isotonic saline was injected s.c. into two rats in amounts to provide a level of 0·11 mg/100 g body weight. Two control animals received injections of saline. Food was withdrawn and tap water was offered *ad libitum*.

Liver supernatant fraction. Seventeen hours after the injections, the animals were anesthetized with ether and the livers perfused *in situ* with ice-cold isotonic saline. After excision they were rinsed with cold saline, blotted, trimmed free of adhering tissue, and weighed. The supernatant fraction was obtained essentially as in the work of Hurlbert and Kammen.[4] The chilled whole organs were minced in iced beakers and homogenized in three volumes (v/w) of ice-cold 0·25 M sucrose–0·01 M tris-Cl^-, pH 8, by means of a motor-driven Potter–Elvehjem-type homogenizer consisting of a Teflon pestle and glass mortar. A single homogenizing arrangement with constant speed and number of strokes was applied in all the experiments. The homogenates were centrifuged in Lusteroid tubes in rotor No. 40 of the refrigerated Spinco model L preparative ultracentrifuge at 105,000 *g* for 1 hr. The supernatant solutions were withdrawn by pipetting to avoid contamination with the pellets and kept at 1°. To 10 ml supernatant was added 0·85 ml of 0·35 M tris-Cl, pH 7·4. Protein was determined by the method of Lowry *et al.*[5] with crystalline ovalbumin as the standard.

Conversion of substrates by liver supernatant fraction. The effect of colchicine on the conversion of UMP to cytosine nucleotides was determined by the use of the standard substrate mixture (SSM) devised by Hurlbert and Kammen[4] and consisted of 0·25 μmole UMP-^{14}C, 10 μmoles ATP, 0·25 μmole GMP, 2·5 μmoles L-glutamine, and 20 μmoles $MgCl_2$ in 0·25 ml neutralized to pH 7·2 with tris-OH. In measuring the conversion as a function of the amount of liver supernatant protein, 0·25 M sucrose–0·035 M tris-Cl^-, pH 7·4, was used to adjust the incubation mixtures to a standard final volume, which was 0·5 ml if 0·13 ml of the SSM was used and 1·0 ml if 0·25 ml was used. The incubation mixtures were prepared at 1° and incubated at 37°. After the desired length of incubation, the mixtures were chilled to 1° and 1 μmole each of carrier CMP and cytidine was added. Removal of protein in 0·4 N $HClO_4$, hydrolysis of cytosine nucleotides to CMP in hot 1 M $HClO_4$, removal of ClO^-_4 ion, and the separation of CMP and cytidine by ion-exchange chromatography on Dowex 50-H^+ were carried out according to the procedures of Hurlbert and Kammen. CMP and cytidine were evaporated to dryness *in vacuo*. When the conversion of UMP to uracil and uridine was examined, these compounds were isolated by ion-exchange chromatography on Dowex 1-formate.[6]

To determine whether colchicine treatment affected the conversion of UTP to cytosine nucleotides, the equivalent amount of UTP-^{14}C replaced UMP-^{14}C in the SSM.

In studying the conversion of UTP-^{14}C to UDP and UMP, glutamine and GMP were eliminated from the SSM and, after incubating at 37°, deprotenization was effected by the addition of 5 ml of ice-cold 6% TCA to 1 ml of the incubation mixture. The protein precipitate was washed with 1 ml of the cold TCA and the washing combined with the bulk of the supernatant solution. TCA was removed by extracting the solution five times with ether; the aqueous solution was then neutralized by dropwise addition of 1 M tris-OH. One μmole each of UMP, UDP, and UTP was added as carrier, and separation of the nucleotides was accomplished on a Dowex-1 formate column as described by Herbert *et al.*[7] The compounds were absorbed from their eluates on Dowex-1 OH^-, stripped from the resin with 1 M HCl, and evaporated to dryness.

The substrate mixture used to study the breakdown of ATP by the liver supernatant fraction contained ATP and $MgCl_2$ in the amounts in which they were present in the SSM, with the elimination of UMP, GMP, and glutamine. After incubating with the liver supernatant fraction at 37°, deproteinizing with TCA, and removal of the TCA as described above, the solutions were neutralized with dilute NH_3 and subjected to ascending chromatography for 16 hr on sheets of Whatman No. 1 filter paper in isobutyric acid : concentrated NH_4OH : H_2O (66 : 1 : 33) with appropriate standards.[8] Areas corresponding to ATP, ADP, and AMP were located by scanning with ultraviolet light. These and appropriate blank areas were cut from the sheets and extracted for 30 min and again for 5 min with 0·01 M phosphate buffer at pH 7·4 in a boiling water bath. After combining the extracts, the nucleotide concentrations were measured by u.v. absorption spectrophotometry with appropriate blanks. Tissue blanks obtained in the absence of ATP were negligible. Inorganic phosphate in the deproteinized incubation mixtures was determined by the method of Fiske and SubbaRow[9] after removing the organically bound phosphate with Norite.[10]

Measurement of radioactivity. The isolated compounds were brought into solution with water. Aliquots giving infinitely thin samples were dried on aluminum planchets, and the radioactivity was assayed in an internal flow counter (Nuclear-Chicago Corp.). Sufficient background and sample counts were recorded until the standard counting error was 5 per cent or less.[11] The data were converted to the total counts of each compound in any one experiment.

RESULTS AND DISCUSSION

The procedure used in the present experiments do not identify the cytosine nucleotide that is formed from the UMP-^{14}C or UTP-^{14}C that is incubated with the liver supernatant fraction. The CMP that is finally obtained is the product resulting from acid hydrolysis of all possible forms of cytosine nucleotides that could arise from these substrates. It is not as yet known whether only one of the uridine 5′-phosphates is the substrate for the reaction in which L-glutamine furnishes the amino group or whether UMP, UDP, or UTP serves equally well. Thus, the conversion of the UMP-^{14}C in the SSM to cytosine nucleotides may include kinase activity which first produces UDP or UTP, one of which or both may then be aminated. Starting with UTP-^{14}C, the

production of cytosine nucleotides could include pyrophosphatase activity which initially provides UDP or UMP as substrate for amination.

The main objective of the present investigation was to determine whether there was any difference between the livers of the normal and colchicine-treated rats in their capacity to convert UMP and UTP to cytosine nucleotides. It was recognized that should a difference be found, questions stemming from the above considerations would immediately arise. For example, if there were a change in the yield of cytosine nucleotides when UMP or UTP was incubated with the liver supernatant fraction after colchicine treatment, could it be due to a change in concentration of the aminating enzyme or could it be the result of altered interconversions of uridine phosphates? Although such questions would form the subject for additional investigation, occasion arose to begin attempts to answer some of them.

Conversion of UMP-^{14}C *and* UTP-^{14}C *to cytosine nucleotides by liver supernatant fraction*

The rates of formation of cytosine nucleotides from UMP-^{14}C by the supernatant fractions of livers from control and colchicine-treated animals appear in Fig. 1. With

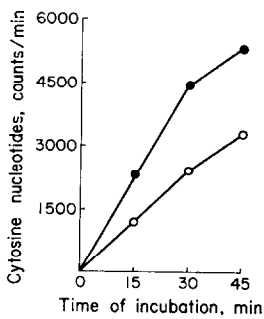

FIG. 1. Rates of conversion of UMP-^{14}C to cytosine nucleotides by liver supernatant fractions from rats treated with saline (open circles) and colchicine (solid circles). There were 10·6 mg and 10·0 mg of protein per tube respectively. The zero-time values for cytosine nucleotides were 95 counts/min and 120 counts/min, respectively, and the data were corrected for them. Incubation occurred at 37° with $1{\cdot}19 \times 10^5$ counts/min of UMP-^{14}C.

equivalent amounts of protein, the rate observed after colchicine treatment was approximately twice that found in the control preparation.

The extent of conversion of UMP-^{14}C to cytosine nucleotides in 30 min of incubation as a function of the amount of supernatant fraction protein is illustrated in Fig. 2. The liver preparation from the drug-treated animals was about twice as active as the control preparation.

In Table 1 are shown the results of additional experiments, some of which include the yields of cytidine and of uracil plus uridine. The increased yield of cytosine nucleotides from UMP-^{14}C after colchicine treatment cannot be explained by their lesser degradation to cytidine. The maximal yield of uracil plus uridine was only 2 per cent of the incubated UMP-^{14}C. Thus, decreased degradation of the substrate UMP cannot provide an explanation for its increased conversion to cytosine nucleotides in the preparation from the drug-treated animals.

With UTP-^{14}C as substrate, the yield of cytosine nucleotides was at least 70 per cent higher after treatment with colchicine (Table 2).

The magnitudes of the increases in the conversions of UMP-^{14}C and UTP-^{14}C to cytosine nucleotides in the liver preparation after colchicine treatment are consistent

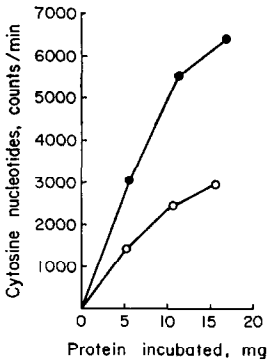

FIG. 2. Conversion of UMP-^{14}C to cytosine nucleotides as a function of the amount of protein of the liver supernatant fractions from saline-treated rats (open circles) and colchicine-treated rats (solid circles). The time of incubation was 30 min at 37° with $1{\cdot}19 \times 10^5$ counts/min of UMP-^{14}C per tube.

TABLE 1. CONVERSION OF UMP-^{14}C TO CYTOSINE NUCLEOTIDES, CYTIDINE, URIDINE, AND URACIL BY LIVER SUPERNATANT FRACTION FROM SALINE-TREATED AND COLCHICINE-TREATED RATS

Pretreatment *in vivo*	Protein incubated (mg)	CMP	Cytidine (counts/min)	Uracil + uridine	Ratio $\frac{\text{CMP after colchicine}}{\text{CMP after saline}}$
Saline	5·0	785	98	2100	2·4
Colchicine	5·1	1920	83	1430	
Saline	12·0	2550	120	1480	1·8
Colchicine	10·0	4480	460	1160	
Saline	12·1	2360	390		2·0
Colchicine	11·7	4710	608		
Saline	10·1	2090			1·9
Colchicine	9·9	3980			
Saline	10·0	2390*	120	1530	1·9
Colchicine	10·6	4450*	190	1480	
Saline	10·4	2470†	130	1270	2·2
Colchicine	11·2	5540†	320	2100	

Incubation occurred at 37° for 30 min in 1·0 ml reaction mixture. The total counts/min of UMP-^{14}C per tube varied from $1{\cdot}19 \times 10^5$ to $1{\cdot}25 \times 10^5$.

* Data from the experiment that provided Fig. 1.

† Data from the experiment that provided Fig. 2.

with the earlier results.[1] When liver slices from drug-treated male animals were incubated with orotic acid-^{14}C, the specific activity of the RNA cytosine was double the control value. It can be concluded that colchicine injected into rats confers on the liver an increased capacity to convert UMP and UTP to cytosine nucleotides.

Stability of UTP *and* ATP *in liver supernatant fractions*

In comparing the rates of conversion of UMP, UDP, and UTP to cytosine nucleotides, Hurlbert and Kammen[4] found only a slight superiority of UTP. A comparison of the data in Table 1 with those in Table 2 again suggests that UTP is not a significantly better precursor than UMP. At an average concentration of 10·9 mg of control

TABLE 2. CONVERSION OF UTP-^{14}C TO CYTOSINE NUCLEOTIDES BY LIVER SUPERNATANT FRACTIONS FROM SALINE-TREATED AND COLCHICINE-TREATED RATS

Pretreatment *in vivo*	Protein incubated (mg)	CMP (counts/min)	Ratio $\frac{\text{CMP after colchicine}}{\text{CMP after saline}}$
Saline	5·8	1610	1·7
Colchicine	5·7	2770	
Saline	5·8	1340	2·2
Colchicine	5·4	2980	
Saline	5·4	1350	1·7
Colchicine	5·2	2320	

Incubation was carried out at 37° for 30 min with 58,100 counts/min of UTP-2-^{14}C in 0·51 ml reaction mixture per vessel.

liver protein/ml there resulted an average of 2370 counts of CMP from $1{\cdot}2 \times 10^5$ counts of UMP in 30-min incubation. At an average concentration of 5·7 mg of control liver protein/0·51 ml, 1440 counts of CMP were formed from $5{\cdot}8 \times 10^4$ counts of UTP in the same period. The only indication that UTP may be the preferred substrate in the amination reaction was the predominance of UTP in the incubation mixtures when UMP-^{14}C was the initial uracil nucleotide.[4] In three experiments, when UMP-^{14}C was incubated with the liver preparations from control and colchicine-treated rats, the ratio of the UDP plus UTP formed to the UMP that remained ranged from 4 to 7, suggesting that the UMP is not a preferred substrate. If in fact UTP is a direct substrate for the reaction, it may be asked whether the greater yield of cytosine nucleotides obtained from UTP when it is incubated with the preparation from the drug-treated animal is a reflection of a greater stability of UTP in that preparation. However, scarcely any difference was found in the extent of breakdown of UTP in the two kinds of preparation (Table 3), and at least 80 per cent of the UTP-^{14}C that was incubated was recovered unchanged.

The difference in the activities of the two preparations toward UMP and UTP with respect to cytosine nucleotide formation cannot be ascribed to a difference in the stability of the ATP that is necessary for the conversion, a conclusion reached from the data in Table 4. In those experiments conversion to AMP could not be detected. The yield of inorganic phosphate higher than that of ADP suggests that any AMP that may have been formed was degraded to adenosine.

Although no difference was found between the two kinds of liver preparations in their small capacity for degrading UTP and ATP, such equivalence may not apply to the GMP which is present in the SSM and is necessary for the conversion of uracil ribonucleotides to cytosine ribonucleotides. Hurlbert and Kammen[4] found no difference between the abilities of GMP and GTP to serve as cofactors for the reaction in

the liver supernatant fraction. In preliminary experiments in this laboratory with a partially purified preparation of the liver enzyme activity, no difference was found between GMP, GDP, and GTP in this regard. Whether the increased yield of cytosine nucleotides in the liver supernatant fraction from the colchicine-treated rat can be

TABLE 3. BREAKDOWN OF UTP-^{14}C TO UMP AND UDP BY SUPERNATANT FRACTIONS OF RAT LIVER HOMOGENATES

Pretreatment *in vivo*	Protein incubated (mg)	UMP + UDP 0-time	UMP + UDP 30 min	UMP + UDP Δ	UMP + UDP formed as % UTP incubated	UTP remaining after 30 min	
		(counts/min × 10^4)				(counts/min × 10^4)	(% of initial)
Saline	6·3	0·348	1·48	1·13	19	5·03	86
Colchicine	5·7	0·460	1·27	0·81	14	5·65	97
Saline	6·7	0·654	2·45	1·80	31	4·83	83
Colchicine	6·0	0·671	1·40	0·73	13	4·85	83
Saline	6·0	0·489	1·60	1·11	19	4·66	80
Colchicine	5·6	0·635	1·45	0·81	14	4·92	84

There were 5·82 × 10^4 counts/min of UTP-2-^{14}C in 0·51 ml reaction mixture per tube. After preparing the mixtures at 1°, the contents of one set of tubes in each experiment were stirred for 30 sec while cold, and then deproteinized (0-time). After stirring, the other set of tubes was incubated at 37° for 30 min and the contents chilled and deproteinized. The values are averages for duplicate samples.

TABLE 4. STABILITY OF ATP WHEN INCUBATED WITH SUPERNATANT FRACTIONS OF RAT LIVER

Pretreatment *in vivo*	Protein incubated (mg)	ATP remaining (μmoles)	ADP formed (μmoles)	Inorganic $PO_4^{\equiv}$ formed (μmoles)
Saline	13·3	9·04	0·68	2·66
Colchicine	12·8	9·15	0·86	2·62
Saline	11·7	8·40	0·77	3·02
Colchicine	11·1	7·79	0·81	3·90

A solution of ATP and $MgCl_2$ was neutralized to pH 7·4 with tris-OH, and 0·25 ml containing 10·2 μmoles ATP and 20 μmoles $MgCl_2$ was supplemented with 0·25 ml of 0·035 M tris-Cl^- buffer pH 7·4–0·25 M sucrose. To the ice-cold mixture was added 0·50 ml of liver supernatant fraction previously adjusted to pH 7·4 with tris-Cl^-. Tissue blanks omitted ATP. After incubation for 30 min at 37° followed by cooling to 1°, protein was removed by the addition of TCA, and the supernatant solutions were assayed for adenosine phosphates and inorganic phosphate as described in Materials and Methods. The values presented are averages of duplicates and have been corrected for the tissue blanks.

attributed to a disposition of guanine ribonucleotides different from that in the control preparation can best be decided by a study of more highly purified enzyme preparations.

Effect of dialysis of liver supernatant fraction on the conversion of UMP *to cytosine nucleotides*

To examine the possibility that an increased activity of the liver preparation might be due to the acquisition of a low-molecular weight activator as a consequence of

injecting colchicine, three experiments were performed in which the supernatant fraction of the livers were dialyzed against 0·02 M acetate buffer, pH 6·5.[4] In all three, the differential between the activities in the preparations from the drug-treated and control animals was maintained. The results of one of the experiments appear in Table 5. The higher activity cannot be explained on the basis that the liver gains a small, freely diffusible activating molecule after injection of colchicine.

TABLE 5. RETENTION AFTER DIALYSIS OF AUGMENTED CAPACITY OF THE LIVER SUPERNATANT FRACTION FROM COLCHICINE-TREATED RATS TO CONVERT UMP-^{14}C TO CYTOSINE NUCLEOTIDES

Pretreatment of animals	Further treatment of liver supernatant	Protein incubated (mg)	CMP (counts/min)	Ratio colchicine saline
Saline	none	10·0	2480	1·8
Colchicine	none	11·1	4340	
Saline	aged	10·2	2460	1·7
Colchicine	aged	10·5	4150	
Saline	dialyzed	5·2	1410	1·8
Colchicine	dialyzed	5·6	2490	

Visking casing was soaked in 0·1 M EDTA for 18 hr and then washed with distilled water before use. The reaction was assayed in the freshly obtained supernatant fractions, in dialyzed aliquots of them, and in aliquots which were permitted to age at 4° during the dialysis period. Dialysis was performed at 4° against a 125-fold volume of 0·02 M acetate buffer, pH 6·5, for 1·5 hr and was repeated with fresh buffer for an additional 1·5 hr. The outer solutions were stirred continuously. After dialysis, the bag contents were adjusted to pH 7·3 by the addition of 0·22 volume of 0·5 M tris-Cl$^-$, pH 8·0. The composition of the reaction mixtures for the non-aged and aged preparations was 0·25 ml of SSM; 0·25 ml of 0·035 M tris-Cl$^-$, pH 7·4, in 0·25 M sucrose; and 0·50 ml of preparation. For the dialyzed material it was 0·25 ml of SSM, 0·35 ml of 0·25 M sucrose, and 0·40 ml of the adjusted bag contents. Incubation occurred for 30 min at 37° with $1·26 \times 10^5$ counts/min of UMP-^{14}C.

The results obtained from the experiments *in vivo* and *in vitro* strongly suggest that there is an elevation in the concentration of a uracil ribonucleotide-aminating enzyme in the liver after treatment of rats with colchicine. In the former experiments[1] two observations created this impression. There was a greater increase in the specific activity of the liver RNA cytosine than of the uracil after the administration of appropriate radioactive precursors to the colchicine-treated rat. In addition, there was no significant change in the concentration of free glutamine in the liver of the drug-treated rat, indicating that the apparent increased synthesis of cytosine ribonucleotides could not have resulted from an increase in the source of the cytosine amino group. In the present experiments it was found that the liver supernatant fraction from the colchicine-treated rat does have an augmented capacity to convert UMP and UTP to cytosine nucleotides. In the case of UMP as substrate, acquisition of a dialyzable activator could not be the explanation for this increased activity. In the case of UTP as substrate, this increase was not the result of lesser substrate breakdown in the preparation from the drug-treated animal. Similarly, no difference in the breakdown of ATP by the two preparations could be seen.

These observations, while highly suggestive, do not constitute firm proof that an increase in the concentration of the aminating enzyme in the liver occurs after treatment of rats with colchicine. More definitive information may be forthcoming from

experiments that combine treatment of colchicine with a block in protein synthesis *in vivo* and that involve direct enzyme isolation.

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REFERENCES

1. H. Weinfeld and A. A. Sandberg, *Biochem. Pharmac.* **13**, 1627 (1964).
2. R. B. Hurlbert and V. R. Potter, *J. biol. Chem.* **209**, 1 (1954).
3. E. S. Canalakis, *J. biol. Chem.* **227**, 329 (1957).
4. R. B. Hurlbert and H. O. Kammen, *J. biol. Chem.* **235**, 443 (1960).
5. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
6. H. O. Kammen and R. B. Hurlbert, *Cancer Res.* **19**, 654 (1959).
7. E. Herbert, V. R. Potter and Y. Takagi, *J. biol. Chem.* **213**, 923 (1955).
8. Pabst Laboratories Circular OR-17 (April 1961).
9. C. H. Fiske and Y. Subbarow, *J. biol. Chem.* **66**, 375 (1925).
10. R. K. Crane and F. Lipmann, *J. biol. Chem.* **201**, 235 (1953).
11. R. Loevinger and M. Berman, *Nucleonics* **9**(1), 26 (1951).