this contribution (as well as the absorption intensity) may depend on the conformation of the polymer.

In view of the findings with the random poly-L-tyrosine, it seems reasonable that in the helical polymer a side-chain chromophore band at 225 mμ of positive sign is present but obscured by the larger n-π^* peptide transition at 225 mμ. (The other band at about 240 mμ in the random polymer is what we believe to be the 248-mμ band actually observed in the helical polymer.) Since the sign of the side-chain-chromophore band is opposite to the n-π^* band, we may infer that the latter is actually larger than observed. We may not, however, simply subtract the ellipticity found in the random polymer at 225 mμ from the negative band observed in the helical polymer, because in the helix the side chains are helically arrayed (as pointed out by Fasman *et al.*, 1964) with ensuing exciton-band splitting and alteration of magnitude and position of the absorption bands. A quantitative calculation of the magnitude of the n-π^* ellipticity band in helical poly-L-tyrosine thus is not possible.

Discussion

It is hardly surprising that a molecule with an absorption spectrum as complicated as that of poly-L-tyrosine exhibits highly complex ORD and circular-dichroism behavior. Among the puzzling features are the absence in the random polymer of dichroism bands corresponding to the high-wavelength amino acid bands and the absence of a Cotton effect at 294 mμ for the random-ionized polymer, as noted in the preceding paper. Added to these is our inability to decide from the dichroism data whether or not the helical polymer at *p*H 11.2 is *fully* helical. The rotational strength of the n-π^* transition in poly-L-tyrosine is only about one-third of the value for fully helical poly-α,L-glutamic acid but, as noted above, it is entirely likely that an opposite-sign side-chain-chromophore band is effectively reducing a larger rotational strength. Computations on the basis of 100% helix can be made which are compatible with the experimentally observed dichroism band, but these are *ad hoc* and unconvincing.

Finally, we may point out that an apparent paradox in the ORD paper, connected with the decreased value of the rotation at 238 mμ in water compared to 0.2 M salt, may be owing to a diminution of that contribution to the 248-mμ dichroism band which is due to the ionized tyrosine with a comparable increase in the (expected) opposite-sign 225-mμ un-ionized tyrosine, and it may not be necessary to invoke any large change in the n-π^* transition.

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A Deoxyribonuclease Reaction Requiring Nucleoside Di- or Triphosphates*

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An extract of *Micrococcus lysodeikticus* will split DNA in the presence of Mg^{2+} and a single nucleoside di- or triphosphate. The reaction requires native DNA. DNA is degraded from the ends of the molecule, since acid-soluble material is released before either the viscosity or the transforming activity of the DNA is appreciably reduced, and since compounds sensitive to *Escherichia coli* alkaline phosphatase and presumed to be mononucleotides are produced from the start of the reaction. The specific activity of the extract has been increased 25-fold by ammonium sulfate fractionation and DEAE-cellulose chromatography. This partially fractionated extract shows an absolute dependence on nucleoside triphosphate for the degradation of DNA; other chelating agents will not substitute. The amount of DNA degraded to acid-soluble material is strictly determined by the amount of nucleoside triphosphate in the reaction mixture.

We have recently been studying an enzyme prepared from *Micrococcus lysodeikticus* which preferentially inactivates transforming DNA that has been treated with either ultraviolet light or with monofunctional alkylating agents (Strauss, 1962; Strauss and Wahl, 1964). In the course of these experiments we noticed that a mixture of nucleoside triphosphates added to ^{32}P-labeled DNA promoted the liberation of acid-soluble radioactivity from the DNA upon addition of the *M. lysodeikticus* extract. Further investigation disclosed that the release of acid-soluble material from DNA was stimulated by the addition of a single nucleoside di- or triphosphate. We have fractionated the *M.*

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lysodeikticus extract to obtain a preparation with which the release of acid-soluble material from DNA is absolutely dependent on ATP or other nucleoside di- or triphosphate. In this paper we shall document our findings and report some of the characteristics of this novel reaction.

Materials and Methods

Salmon sperm DNA for the viscosity study was obtained from Dr. E. P. Geiduschek; a commercial preparation of salmon DNA from the California Corp. for Biochemical Research was used in other experiments. *Bacillus subtilis* DNA was prepared by a modification of the method of Marmur (1961), inserting an extraction of the preparation with neutralized-water-saturated phenol following treatment with ribonuclease. ^{32}P-Labeled DNA was prepared from *B. subtilis* grown on the medium described by Young and Spizizen (1961) and using the preparation method of Lehman (1960) or of Marmur (1961) followed by overnight dialysis against 2 M NaCl + 0.05 M sodium citrate to remove acid-soluble material. Salmon DNA solutions were made in 0.15 M NaCl; [^{32}P]-DNA was diluted so that the citrate did not interfere with the nuclease reaction. The nucleotides were commercial preparations. Pancreatic DNAase, RNAase lysozyme, and *Escherichia coli* alkaline phosphatase were obtained from the Worthington Biochemical Corp.

DNA was determined by the method of Burton (1956), phosphate by the Fiske-Subbarow reaction (Leloir and Cardini, 1957), and protein with the Folin-Ciocalteu reagent (Lowry *et al.*, 1951). Charcoal for the absorption of nucleotides and nucleosides was prepared by the method of Tsuboi and Price (1959). This charcoal suspension absorbed at least 99% of the nucleosides and nucleotides in the concentrations used in these experiments and retained less than 3% of radioactive inorganic phosphate. Transformation assays followed the protocol of Anagnostopoulos and Spizizen (1961), using *B. subtilis* strain 168 to score *ind*$^+$ transformants.

Deoxyribonuclease activity was measured either by the presence of residual acid-insoluble DNA or, when ^{32}P-labeled DNA was used, by the release of acid-soluble radioactivity. DNA for the analysis of acid-insoluble deoxyribose was precipitated from reaction mixtures by the addition of 0.3 ml of cold 5.0 M $HClO_4$ to each 1.0 ml of chilled reaction mixture to which 0.2 ml of a cold 15 mg/ml solution of bovine serum albumin had been added as a carrier. The precipitate was washed with cold 1.0 M $HClO_4$ and then heated 30 minutes at 75° with 3.0 ml of 1.0 M $HClO_4$. The acid-soluble supernatant was then used for the analysis of DNA by the diphenylamine method. When ^{32}P-labeled substrate was used a portion of the initial supernatant was plated directly after precipitation with cold acid and its radioactivity was determined. A unit of enzyme was defined as the amount releasing 1 μmole of acid-soluble phosphorus in 30 minutes from a reaction mixture containing 10 μmoles glycine buffer, *p*H 9.5, 5 μmoles $MgCl_2$, 2.5 μmoles mercaptoethanol, 100 mμmoles ATP, 89 mμmoles DNA-P (30 μg), and enzyme in a total volume of 0.5 ml. At the end of the reaction 0.2 ml of a 15-mg/ml solution of albumin was added to the mixture followed by 0.3 ml of 1 N $HClO_4$. After 5 minutes at 0° the precipitate was removed by centrifugation at 8000 *g* for 10 minutes and a portion of the supernatant was plated, neutralized with a drop of KOH, and evaporated, and its radioactivity was determined. The amount of acid-soluble radioactivity was proportional to enzyme concentration under these conditions of assay (Fig. 1).

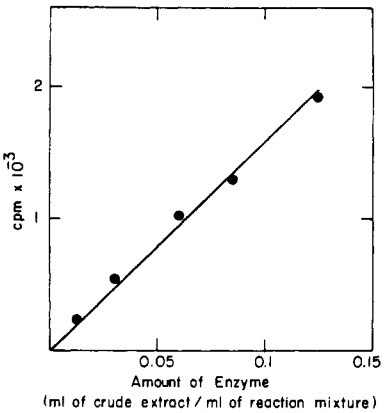

Fig. 1.—Liberation of acid-soluble ^{32}P from [^{32}P]-DNA with varying amounts of extract. The reaction mixture is described in the text.

The extracts used in these experiments were prepared from *M. lysodeikticus* grown in the medium described by Steiner and Beers (1961). Activity has also been observed in extracts of a commercial lyophilized preparation of *M. lysodeikticus* obtained from Miles Chemical Co. Little or no activity was observed in extracts of *B. subtilis*. For many of the preliminary experiments an extract was prepared by lysing a 10% suspension of cells (wet wt) in 0.05 M Tris buffer, *p*H 7.0, with 0.2 mg/ml of lysozyme at 37°. The mixture was treated in a sonic disintegrator (Branson Sonifier) until a free-flowing liquid was obtained. After centrifugation the supernatant was precipitated with protamine sulfate and the precipitate was eluted with 0.07 M potassium phosphate buffer, *p*H 8.0, and precipitated with ammonium sulfate to concentrate the extract. The preparation was dialyzed overnight against 0.05 M Tris buffer and stored frozen. Preparations retained activity for over 2 months. This procedure removed nucleic acids from the preparation but there was very little increase in specific activity (units per mg protein) over the fresh lysate. This preparation is therefore referred to as "crude extract" and typical preparations contained about 11 mg of protein per ml and 4.3 Kornberg units of DNA polymerase per mg of protein.[1]

Table I
Purification of the *M. lysodeikticus* Extract

Enzyme Fraction	Total Volume (ml)	Protein (mg/ml)	Specific Activity[a] (units/mg protein)	Total Units
Crude extract	170	15.4	0.06	174.
Ammonium sulfate, 30–60%	110	11.7	0.12	155.
Ammonium sulfate, 0–45%	65	7.4	0.30	144.
1st DEAE-cellulose	15	7.0	0.76	80.
2nd DEAE-cellulose	8.4	0.6	1.62	8.2

[a] Specific activity = μmoles of DNA-P made acid soluble in the complete reaction mixture described in the text (see Materials and Methods) minus the ATP-independent deoxyribonuclease activity per mg protein.

Results

Purification of Extract.—A 25-fold increase in the specific activity of the nuclease activity has been obtained (Table I). Twenty-nine g of *M. lysodeikticus*

[1] We should like to thank Dr. B. Zimmerman for performing this assay.

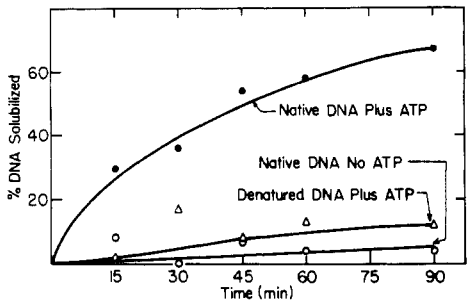

FIG. 2.—Release of acid-soluble material from native and heat-denatured *B. subtilis* DNA by the crude extract. The incubation mixtures (1.0 ml) contained 20 μmoles of glycine buffer, *p*H 9.5, 10 μmoles of $MgCl_2$, 5 μmoles of mercaptoethanol, 0.2 μmole of disodium ATP (where added), 0.05 ml of crude extract (7.1 mg protein/ml), and 129 μg of *B. subtilis* DNA (0.38 μmole). Denatured DNA was prepared by heating the DNA dissolved in 0.15 M NaCl for 15 minutes in a boiling-water bath. Incubation was at 37° in separate tubes. At the times indicated a sample was removed and chilled, and the DNA was precipitated and determined as described under Materials and Methods.

cells in 200 ml of 0.05 M Tris buffer, *p*H 7.0, was lysed with lysozyme at 37°; all subsequent operations were carried out at 0–4°. The viscous suspension was treated in a sonic disintegrator and then centrifuged 30 minutes at 16,000 *g*. Solid ammonium sulfate (39.5 g) was added to 170 ml of supernatant, and after 10 minutes at 0° the precipitate was collected by 60-minute centrifugation at 16,000 *g*. The supernatant was then adjusted to 60% saturation with ammonium sulfate by the addition of solid salt. After standing at 0° for 30 minutes the precipitate was collected by centrifugation (12,000 *g* for 15 minutes) and dissolved in 0.05 M Tris buffer, *p*H 7.5. The resulting solution was dialyzed overnight against the same buffer containing, in addition, 0.002 M mercaptoethanol, and centrifuged at 13,000 *g* for 20 minutes to remove the small amount of precipitate that had formed. The solution so obtained was brought to 45% saturation with ammonium sulfate and the precipitate obtained was dissolved in 0.02 M phosphate buffer, *p*H 7.5, and dialyzed overnight against the same buffer containing 0.002 M mercaptoethanol.

A column of DEAE-cellulose (10 × 2.2 cm) was prepared and equilibrated with 0.002 M potassium phosphate buffer, *p*H 7.5. Sixty-five ml of the dialyzed ammonium sulfate fraction was added to the column and the absorbed material was washed with 10 ml of the same buffer. A linear gradient was applied with 0.1 M and 0.5 M phosphate buffers at *p*H 6.5 as limiting concentrations; 150 ml of each buffer was used with a flow rate of 60 ml/hour. Five-ml fractions were collected.

Seven tubes at the highest specific activity were pooled and concentrated by the addition of ammonium sulfate to 60% saturation. The precipitation was collected by centrifugation, dissolved in 0.05 M Tris buffer, *p*H 7.5, and dialyzed against the same buffer. Seven ml of this first DEAE-cellulose fraction was absorbed on a second DEAE-cellulose column (7 × 1 cm) which had been equilibrated with 0.05 M Tris buffer, *p*H 7.0. A linear gradient was applied with 0.1 M and 0.5 M Tris buffers, *p*H 7.5, as limiting concentrations. Fifty ml of each buffer was used and 2-ml fractions were collected. Two of these fractions had a high specific activity and were pooled. This pooled fraction will be referred to as the "partially fractionated extract." Over a 25-fold increase in specific activity was obtained but there was a poor recovery of total activity in the last step (Table I).

Characteristics of the Reaction.—Nuclease activity was destroyed by heating crude preparations for 10 minutes in a boiling-water bath. The reaction was stimulated by the presence of mercaptoethanol; after 60-minute incubation of crude extract with salmon DNA and ATP we found 46% of added DNA acid-soluble in the presence of 5 μmoles of mercaptoethanol per ml whereas only 32% became acid-soluble in its absence. We routinely add mercaptoethanol to all reaction mixtures.

Optimal activity was obtained with *p*H 9.5 glycine buffer which corresponds to a final *p*H for the reaction mixture (containing glycine buffer, $MgCl_2$, ATP, DNA, mercaptoethanol, and enzyme) of *p*H 8.6. A typical *p*H-activity curve is obtained with 50% of maximal activity obtained with *p*H 8.8 and 10.0 glycine buffers. Mg^{2+} is required for reaction to occur. The Mg^{2+} requirement of crude extract was saturated at a Mg^{2+} concentration of about 5×10^{-3} M and no inhibition of activity was obtained at Mg^{2+} concentrations of 2×10^{-2} M. The reaction mixture with $MgCl_2$ omitted had a *p*H of 8.7 compared to a *p*H of 8.6 for the complete reaction mixture described. About 10% of enzyme activity was obtained when Mn^{2+} was substituted for Mg^{2+}; 0.02 M Ca^{2+} completely inhibited enzyme activity in the presence of Mg^{2+} and did not substitute for Mg^{2+} in its absence. The reaction required native rather than denatured DNA since DNA heated for 15 minutes in a boiling-water bath was not appreciably degraded by the crude extract (Fig. 2).

The Role of Nucleoside Triphosphates.—ATP or some similar "energy-rich" compound must be present along with *M. lysodeikticus* extract to convert DNA to acid-soluble products. Nuclease activity in the absence of ATP is removed early in the fractionation procedure (Table II). deATP is as efficient as ATP and some splitting of DNA occurs when ADP is added, but no reaction is obtained even with crude extract

TABLE II
REMOVAL OF ATP-INDEPENDENT DEOXYRIBONUCLEASE ACTIVITY IN THE COURSE OF THE ENZYME PURIFICATION[a]

Fraction	Enzymatic Activity (cpm × 10^{-5}/ml enzyme) ATP-dependent DNAase	ATP-independent DNAase
Crude extract	11.8	1.6
Ammonium sulfate, 30–60%	11.4	0.6
Ammonium sulfate, 0–45%	17.2	0.8
First DEAE-cellulose	14.2	0
Second DEAE-cellulose	1.7	0

[a] The reaction mixture is described under Materials and Methods. The data are from the fractionation shown in Table I.

when either AMP or pyrophosphate is added (Table III). Potassium phosphate has no effect. The other nucleoside and deoxynucleoside triphosphates will substitute for ATP but the adenine derivatives are the most efficient (Table IV). Since *M. lysodeikticus* has been reported to have an active myokinase (Singer and Guss, 1962), it is possible that the ADP effect is caused by the formation of ATP or the reverse.

The activity of ATP is stable to heating for 5 minutes at 100° at *p*H 10 but is rapidly destroyed at acid *p*H. Different commercial sources of ATP have comparable activity. In order to determine whether the require-

TABLE III
EFFECT OF ATP ON THE FORMATION OF ACID-SOLUBLE PRODUCTS FROM DNA[a]

Addition to Reaction Mixture	DNA Acid-soluble (%)
None	0
ATP (0.2 μmole)	53
ADP (0.2 μmole)	34
AMP (0.2 μmole)	0
deATP (0.3 μmole)	64
Pyrophosphate (0.2 μmole)	4
Pyrophosphate (0.1 μmole)	0
ATP (0.1 μmole) + pyrophosphate (0.1 μmole)	45
deATP (0.15 μmole) + pyrophosphate (0.1 μmole)	55

[a] The system contained 40 μmoles of glycine buffer, pH 9.5, 20 μmoles of $MgCl_2$, 5 μmoles of mercaptoethanol, 0.3 μmole of salmon sperm DNA-P, 0.1 ml of crude extract, plus additions as shown in a total volume of 1.0 ml. Incubation was for 90 minutes at 37°. Residual DNA was determined by the diphenylamine method.

TABLE IV
EFFECT OF VARIOUS NUCLEOTIDES AND NUCLEOSIDES ON NUCLEASE ACTIVITY[a]

Nucleotide or Nucleoside	DNA Acid-soluble (mμmoles) (corrected for control with no nucleoside addition)
ATP	34.2
ADP	29.6
AMP (2′ and 3′)	1.4
Adenosine	1.6
deATP	35.3
deCTP	3.3
deGTP	5.8
TTP	6.0
UTP	13.9
GTP	8.0
CTP	12.1

[a] The system contained 10 μmoles of glycine buffer, pH 9.5, 5 μmoles $MgCl_2$, 2.5 μmoles mercaptoethanol, 89 mμmoles [^{32}P]-DNA, 6.2 μg partially fractionated extract, and 100 mμmoles nucleotide or nucleoside. The total volume of the reaction was 0.5 ml. The mixture was incubated at 37° for 30 minutes. The reaction was stopped by the addition of 0.2 ml of albumin (15 mg/ml) and 0.3 ml of 1 N $HClO_4$. Supernatant (0.5 ml) was used for the determination of liberated ^{32}P from DNA after centrifugation. The control includes acid-soluble ^{32}P present in the DNA preparation.

ment for ATP might be merely incidental to the reaction involved in the chelation of a metal inhibitor, for example, the crude enzyme preparation was dialyzed overnight against 250 volumes of 0.1 M Tris buffer, pH 7.5, containing 10^{-3} M EDTA. The formation of acid-soluble material from DNA still required the addition of ATP and was not stimulated by the addition of EDTA. EDTA did not substitute for ATP even when added at a concentration of 5 μmoles/ml, and in fact at these concentrations EDTA added to complete reaction mixtures inhibited the reaction, presumably by chelating of required Mg^{2+}. An absolute requirement for ATP was also demonstrated after passage of the crude extract through Sephadex G-75 (Table V), a treatment which should remove contaminating metal ions. Five ml of crude extract was poured onto a 1.3 × 7.0–cm column of Sephadex G-75 previously equilibrated with 0.15 M NaCl. The extract was eluted with 0.15 M NaCl and the visibly yellow material appearing up to one bed volume was collected and tested. It should also be noted that in some experiments utilizing [^{32}P]-DNA, the DNA was dissolved in 0.15 M NaCl + 0.015 M sodium citrate. About 1.5 μmoles of citrate, an efficient chelating agent, was therefore present as a constituent of the reaction mixture in which an ATP requirement, saturated at about 0.1 μmole/ml, was demonstrated.

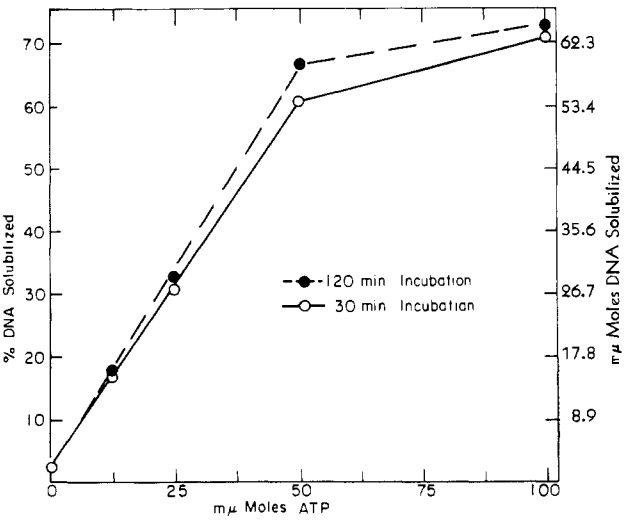

FIG. 3.—Effect of ATP concentration on the formation of acid-soluble products from DNA. The reaction mixture (3 ml) contained 60 μmoles glycine, pH 9.5, 30 μmoles $MgCl_2$, 15 μmoles mercaptoethanol, 534 mμmoles [^{32}P]-DNA, 32 μg enzyme, and ATP as indicated. The mixture was incubated at 37°. At the times indicated, 0.5 ml of sample was removed from the reaction mixture. Albumin (0.2 ml) (15 mg/ml) and 0.3 ml of 1 N $HClO_4$ were added to the sample. After centrifugation, 0.5 ml of supernatant was plated and its radioactivity was determined. Ordinate and abscissa give the results per 0.5 ml of reaction mixture.

TABLE V
EFFECT OF ATP ON THE ACTIVITY OF DIALYZED PREPARATIONS[a]

Extract Treatment	Reaction Mixture	DNA Acid-soluble (%)
Dialyzed against 10^{-3} M EDTA	Complete	29
	−ATP	2
	−ATP + 0.2 μmole EDTA	3
Passed through Sephadex G-75	Complete	45
	−ATP	0

[a] The complete reaction mixture contained 20 μmoles/ml glycine buffer, pH 9.5, 10 μmoles/ml $MgCl_2$, 10.2 μmoles ATP, and 5 μmoles/ml mercaptoethanol. EDTA-dialyzed crude extract (0.46 mg) and 129 μg of salmon DNA were added in a total of 1 ml. Extract passed through Sephadex G-75 was assayed in a total volume of 0.5 ml containing 7.4 μg of ^{32}P-labeled DNA and 0.21 mg of crude extract. Incubation in both cases was for 60 minutes at 37°; salmon sperm DNA was assayed colorimetrically, [^{32}P]-DNA by the determination of acid-soluble radioactivity.

The amount of DNA made acid-soluble by the partially fractionated extract was a linear function of the amount of ATP added up to about 100 mμmoles of ATP per ml. Approximately 1 mole of acid-soluble DNA phosphorus was obtained for each mole of ATP added (Fig. 3). No additional acid-soluble phosphorus was liberated on further incubation of the reaction mixture. Similar results were obtained with the crude

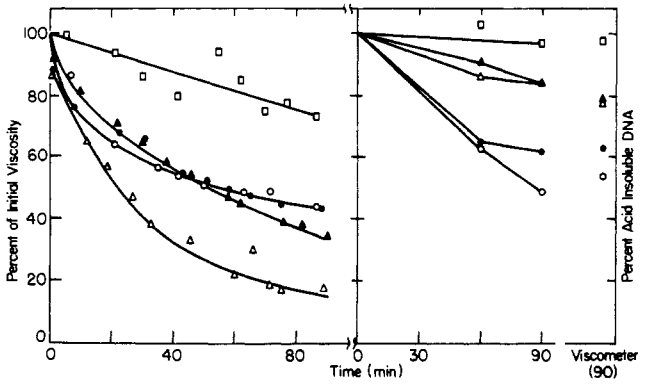

FIG. 4.—Decrease in viscosity and breakdown of DNA to acid-soluble material after treatment with pancreatic deoxyribonuclease and with the crude extract from *M. lysodeikticus* in the presence and absence of ATP. Two reaction mixtures for each set were incubated in the same bath at 37°, one in an Ostwald viscometer, the other in a tube. Each mixture (3.0 ml) contained either 60 μmoles glycine buffer, *p*H 9.5, 30 μmoles $MgCl_2$, 15 μmoles mercaptoethanol, 825 μg salmon sperm DNA, 0.15 ml *M. lysodeikticus* extract (7.1 mg/ml), and the following amounts of ATP: none (boxes), 0.8 μmole (solid circles), 1.6 μmoles (open circles); or for pancreatic deoxyribonuclease 50 μmoles phosphate buffer, *p*H 6.8, 30 μmoles $MgCl_2$, 825 μg salmon sperm DNA, and either 0.025 μg (solid triangles) or 0.05 μg (open triangles) of pancreatic deoxyribonuclease (Worthington Biochemical Corp.). Enzyme and ATP were added to the reaction mixture equilibrated at 37°. Outflow times were measured at the indicated intervals. The total drop in outflow time during the 90-minute period was 20.7 seconds for one of the viscometers containing *M. lysodeikticus* extract plus ATP, and 24.4 seconds for the higher concentration of pancreatic deoxyribonuclease. At the times indicated 0.5-ml samples were withdrawn from the duplicate tubes for analysis of acid-insoluble DNA as described under Materials and Methods. The material in the viscometers after 90 minutes' incubation was also analyzed for DNA.

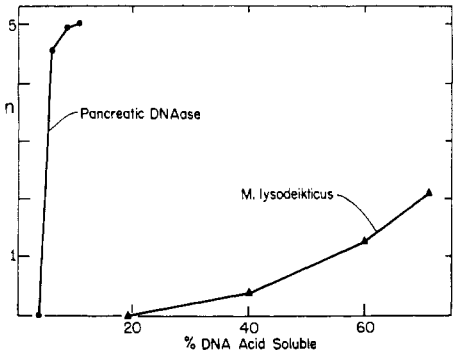

FIG. 5.—Inactivation of transforming DNA by pancreatic deoxyribonuclease and by the crude extract from *M. lysodeikticus*. The incubation mixture (2.0 ml) for pancreatic deoxyribonuclease contained 40 μmoles potassium phosphate buffer, *p*H 6.8, 20 μmoles $MgCl_2$, and 0.05 μg pancreatic deoxyribonuclease (Worthington Biochemical Corp.). The incubation mixture for the "crude extract" contained 40 μmoles glycine buffer, *p*H 9.5, 20 μmoles $MgCl_2$, 10 μmoles mercaptoethanol, 0.4 μmoles ATP, and 0.1 ml extract (7.1 mg protein/ml) in 2.0 ml total volume. All reaction mixtures contained 280 μg *B. subtilis* DNA. Incubation was at 37° and the reaction was stopped by heating for 10 minutes at 60° after the addition of 0.2 ml of 1.5 M NaCl + 0.15 M sodium citrate. The transformation assay was for transformants to indole independence; DNA was determined as usual. The ordinate *n* indicates the number of hits calculated from the relationship $P(O) = N/N_0 = e^{-n}$ where N/N_0 is the fraction of the original transforming activity surviving treatment.

extract. It was also possible to show in other experiments that the initial rate of the nuclease reaction was dependent on the ATP concentration.

More inorganic phosphate could be detected in a reaction mixture containing DNA and ATP than when ATP was present alone. When 0.28 μmole/ml of DNA were incubated with crude extract and 0.2 μmole/ml of ATP, 0.43 μmole/ml of inorganic phosphate was found in the reaction mixture compared to only 0.24 μmole/ml when the DNA was omitted. In this experiment about 0.1 μmole/ml of DNA was transformed to acid-soluble products. The crude extract contained appreciable pyrophosphatase activity. Notwithstanding this result which seems to indicate a DNA-dependent splitting of ATP, we are unable to present reliable stoichiometric data on the splitting of ATP as a function of time and DNA concentration. The amounts of inorganic phosphate which could be liberated from ATP in the range in which DNA splitting is a linear function of ATP concentration are small enough to make quantitative colorimetric work difficult by our procedures.

Identical amounts of acid-soluble material were obtained from DNA regardless of whether ATP and DNA were added simultaneously to the partially fractionated extract or whether the addition of ATP preceded that of DNA by about 30 minutes, indicating that the partially fractionated extract was devoid of ATPase activity under these reaction conditions. The initial rates of splitting of DNA were identical regardless of the time at which the ATP had been added, suggesting that the previous addition of ATP had not changed the conformation of some protein in the system to permit nuclease action.

The Products of Nuclease Action.—The nucleotide-stimulated nuclease reaction differs in its mode of action from pancreatic DNAase. A concentration of pancreatic DNAase adjusted to produce a drop in the viscosity of salmon sperm DNA comparable to that produced by the crude extract did so with far less liberation of acid-soluble material (Fig. 4). Pancreatic DNAase inactivated *B. subtilis*-transforming activity before there was any significant production of acid-soluble material, but about 50% of the DNA was degraded to acid-soluble material before an average of a single lethal hit was observed in transforming DNA treated with crude extract (Fig. 5).

The composition of the products of reaction was investigated using the *E. coli* alkaline phosphatase (Fig. 6), which functions as a monoesterase (Heppel *et al.*, 1962). A complete reaction mixture with ^{32}P-labeled DNA was incubated with crude extract for varying times and the reaction was stopped by immersion of the mixture in a boiling-water bath for 2 minutes. Alkaline phosphatase was then added, and after incubation with phosphatase the DNA was precipitated as usual. The supernatant was divided into two portions; one was plated immediately and its radioactivity was determined. A second portion of supernatant was shaken with charcoal for 30 minutes as described by Tsuboi and Price (1959) and then filtered. Portions of the filtrate and the entire amount of charcoal were plated and the radioactivity of each was determined. Control experiments with this reaction mixture indicated that the incubation with alkaline phosphatase would completely split at least 0.8 μmole/ml of AMP into inorganic phosphate, and that no further splitting occurred in the experimental reaction mixtures incubated for longer periods of time with phosphatase. "Monoesterase-sensitive counts" are defined as radioactivity not absorbed by charcoal after treatment with phosphatase and presumed to be due to inorganic phos-

phate; "monoesterase-insensitive counts" represent the radioactivity retained by charcoal and presumably due to oligonucleotides.

There was a rapid increase in monoesterase-sensitive radioactivity in the acid-soluble fraction from the start of incubation of the crude extract with DNA and ATP (Fig. 6). This monoesterase-sensitive fraction represented about 60% of the total acid-soluble phosphorus at 10, 20, and 30 minutes of reaction time; at 45 and 60 minutes' reaction time about 70% of the total acid-soluble phosphorus was monoesterase sensitive. A doubling in the acid-soluble counts *not* sensitive to phosphomonesterase was obtained during the first 20 minutes of reaction after which the total number of counts absorbed by charcoal gradually declined (Fig. 6).

The monoesterase-sensitive fraction as we have defined it includes inorganic phosphate already present in the reaction products before phosphatase treatment as well as the phosphorus of mononucleotides, nucleoside diphosphates, and the terminal phosphorus of oligonucleotides and of the acid-precipitable DNA. In a separate experiment we found that about 15% of the acid-soluble radioactivity (9.8% of the total radioactivity) was not adsorbed to charcoal after 60 minutes incubation with the crude extract when the reaction mixture was not treated with alkaline phosphatase. This 15% represents inorganic phosphate present among the reaction products; the corrected monoesterase-sensitive radioactivity is therefore about 55% of the total acid-soluble phosphorus. Only 1.6% of the total radioactivity was found as inorganic phosphate (acid-soluble phosphorus not adsorbed by charcoal) after alkaline phosphatase treatment of a reaction mixture incubated with crude extract in the absence of ATP.

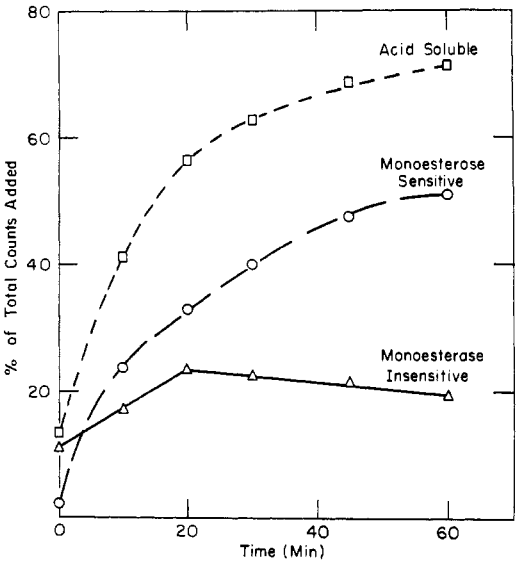

FIG. 6.—Treatment of the products of nuclease action with *E. coli* alkaline phosphatase. 1.2 ml of reaction mixtures containing (per ml) 20 μmoles glycine buffer, *p*H 9.5, 10 μmoles $MgCl_2$, 5 μmoles mercaptoethanol, 0.2 μmoles disodium ATP, 49 μg (0.15 μmoles) *B. subtilis* ^{32}P-labeled DNA (2.36×10^5 cpm/ml of reaction mixture), and 0.067 ml *M. lysodeikticus* "crude extract" (7.1 mg protein/ml) were incubated at 37° for the indicated periods. The reactions were stopped by immersion for 2 minutes in a boiling-water bath. After centrifugation 1 ml of supernatant was incubated for 60 minutes at 37° with 1.3 units of *E. coli* alkaline phosphatase. Albumin and 5 M $HClO_4$ were added as described under Materials and Methods and the mixture was centrifuged; 0.2 ml of supernatant was plated directly while 0.5 ml was added to 0.5 ml of charcoal suspension and shaken 30 minutes at room temperature. The mixture was filtered through a Millipore filter. The filter was washed and assayed for radioactivity directly after attachment to a steel planchet; 0.2 ml of filtrate (undiluted with washings) was plated and radioactivity was counted. The figure is based on the percentage of total counts added; "monoesterase-sensitive counts" are defined as the radioactivity not adsorbed by charcoal; "monoesterase-insensitive counts" represent the ^{32}P adsorbed to charcoal.

DISCUSSION

The experiments described demonstrate the existence of a reaction or reaction system catalyzed by an extract of *M. lysodeikticus* in which the breakdown of DNA is strictly dependent on the amount of ATP or other nucleoside di- or triphosphate present in the reaction. This requirement is not trivial; for example, it is not owing merely to the chelation of some metal-ion inhibitor, since partial purification of the enzyme or enzyme system only increases the requirement for nucleoside triphosphate and since dialysis against EDTA or passage through Sephadex does not lead to diminution of the ATP requirement nor does addition of chelating agents substitute for ATP as might be expected if the ATP acted only to chelate some inhibitory metal. The linear dependence of the amount of DNA made acid-soluble on the amount of ATP added makes it seem reasonable that the nucleoside triphosphate plays a stoichiometric role in the reaction mechanism.

It is unlikely that the reaction as described at its optimum *p*H of 8.6 is caused simply by reversal of the DNA polymerase reaction (Bessman *et al.*, 1958), since DNA polymerase requires about 3×10^{-3} M pyrophosphate to drive the equilibrium in the direction of DNA degradation. The reaction described proceeds in the absence of free pyrophosphate, and added pyrophosphate has no effect on the splitting of DNA by the crude extract. The reaction is limited by the amount of nucleoside triphosphate added and can proceed until over 70% of the added DNA is transformed to acid-soluble products.

The experiments also indicate that the splitting reaction takes place at or near the end(s) of a native DNA molecule. The fact that large amounts of acid-soluble material may be produced before either the viscosity or the transforming activity is appreciably lowered indicates that splitting cannot be random throughout the molecule as in the case of pancreatic DNAase (Thomas, 1956). Over 60% of the total acid-soluble radioactivity liberated from ^{32}P-labeled DNA by the crude extract was not adsorbed to charcoal after treatment with alkaline phosphatase, compared to 15% not adsorbed before treatment. We conclude from this high percentage of monoesterase-sensitive phosphorus that mononucleotides (or nucleoside diphosphates) are produced from DNA from the start of the reaction. It does appear that some ^{32}P is released by the crude extract which is not sensitive to phosphatase action, and this accounts for about 40% of the acid-soluble phosphorus after 20 minutes of reaction. Since mononucleotides are probably present from the start of the reaction it is evident that splitting must occur from the ends of the molecule as required by the transformation results. However, it is not possible to characterize the system as an exonuclease since we have not shown that the products are exclusively mononucleotides nor that they are liberated in sequence.

We cannot suggest a mechanism for this nucleotide-requiring reaction at the present time since the specific

activity of the extract has been increased only 25-fold and we do not yet know whether one or several enzymes in the extract are responsible for the effect. It is clear however that a system can be obtained from *M. lysodeikticus* which is completely dependent on a single nucleoside di- or triphosphate for the breakdown of DNA and that the amount of DNA breakdown is strictly determined by the amount of nucleoside triphosphate in the reaction mixture.

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Metabolism of D-Valine by *Streptomyces antibioticus*: Isolation of *N*-Succinyl-D-valine

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The known inhibitor effect of D-valine on the production of actinomycin by *Streptomyces antibioticus* prompted studies on the metabolism of this amino acid by growing cultures of *S. antibioticus*. A major metabolite of D-valine, isolated from ethyl acetate extracts of the acidified medium, has been identified as *N*-succinyl-D-valine. A simple procedure for the isolation of the dimethyl ester of *N*-succinyl-D-valine is presented, based on separation of this compound using gas-liquid chromatography. The possible significance of *N*-succinylamino acids in peptide synthesis is discussed.

Actinomycin, a chromopeptide antibiotic elaborated by *Streptomyces antibioticus*, consists of a phenoxazinone chromophore attached to two pentapeptide chains (Brockmann, 1960). Among the amino acids present in the peptide portion, D-valine is noteworthy since its precursor has been shown to be L- rather than D-valine (Katz and Weissbach, 1963). In fact, exogenous D-valine inhibits formation of the antibiotic (Katz, 1959, 1960) although the growth of the organism is not impaired. Colored compounds possibly related to actinomycin do accumulate in the medium in the presence of D-valine, however, and as a part of a continuing program concerning the biosynthesis of this antibiotic we have examined therefore the fate of D-valine when added to cultures of *S. antibioticus*. A major metabolite was found to be *N*-succinyl-D-valine.

MATERIALS AND METHODS

S. antibioticus was cultured in a glutamic acid–galactose mineral salts medium as previously described (Katz and Goss, 1959). D-Valine (250 μg/ml of medium) was added at the time actinomycin production began, which generally was after 24–36 hours of cell growth. After 2–3 days of further incubation the organism was separated by filtration and the medium (*p*H 8) was extracted with an equal volume of ethyl acetate in order to remove any actinomycin formed (Katz and Weissbach, 1963). The medium was then acidified with hydrochloric acid to *p*H 2 and again extracted with an equal volume of ethyl acetate. Evaporation of the solvent left a brown oil which was treated as described later.

Hydrolyses were conducted in a nonevacuated tube sealed with a Teflon-lined cap using 6 N hydrochloric acid at 120° (15 psi) for varying periods of time in an autoclave. D-Amino acid oxidase was obtained from Worthington Biochemical Corp. (Burton, 1955) and L-amino acid oxidase from Ross Allen Reptile Institute (*Crotalus adamanteus* snake venom) (Ratner, 1955).

Quantitative determinations of amino acids in solution were performed using the method of Stein and Moore (1948), or with the aid of paper chromatography by the method of Naftalin (1948). Kjeldahl nitrogen was analyzed by the micro-Kjeldahl procedure (Kabat, 1961).[1]

D-[1-^{14}C]Valine was obtained from California Corp. for Biochemical Research with an activity of 9.8 mc/mmole. For incorporation studies 1 μc was added to flasks containing 100 ml of medium.

Ascending chromatography of materials isolated from cultures was performed on Whatman No. 1 paper with the following solvent systems: (A) butanol–acetic acid–water (12:3:5) and (B) butanol-ethanol-water (5:2:10).

[1] We should like to acknowledge the assistance of Mrs. Francis Ondrick of Georgetown University who performed the micro-Kjeldahl determination.