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THE PATH OF CARBON IN PHOTOSYNTHESIS. XVIII THE IDENTIFICATION OF NUCLEOTIDE COENZYMES

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THE PATH OF CARBON IN PHOTOSYNTHESIS. XVIII
THE IDENTIFICATION OF NUCLEOTIDE COENZYMES*

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The radioactive compounds to be observed when algae or green leaves are allowed to photosynthesize in $C^{14}O_2$ for short periods are almost all phosphorylated derivatives of sugars. Of these, phosphate esters of trioses, sedoheptulose and fructose are the first to incorporate C^{14} followed closely by ribulose diphosphate, glucose-6-phosphate and a phosphate of mannose. It has been noted, in earlier papers of this series,[1] that on radiograms of the products of photosynthesis, a dark area appeared in a position occupied by no known sugar phosphate and which gave glucose on acid hydrolysis or on treatment with a phosphatase preparation. This has hitherto been referred to as an "unknown glucose phosphate." It was found that this substance was more labile to acid than glucose-1-phosphate, itself a readily hydrolysable phosphate, and furthermore that other labile glucose derivatives were formed as intermediates during the acid hydrolysis. Accumulation of labeled glucose in this area precedes that in sucrose and suggests its synthetic relationship to sucrose phosphate synthesis.

A closer study of this radioactive area, which becomes appreciable on radiograms only after about 30 seconds of normal photosynthesis by all the plants studied in this laboratory, has shown that phosphatase treatment causes liberation of galactose and mannose, as well as glucose. Moreover, at least two weakly radioactive spots with high R_f values in phenol were noted, as well as traces of other sugars. When the original spot was removed from a chromatogram of Scenedesmus (60 seconds in $C^{14}O_2$) and rechromatographed, at least eight radioactive areas were found (Figures 1 and 2). Three of these were due to glucose, galactose and mannose. Two of the others each gave glucose and galactose, but no mannose, on hydrolysis by phosphatase. This behaviour led us to suspect the presence of compounds of the uridine diphosphate glucose type, described in a series of papers by Leloir and his co-workers.[2] Should radioactive uridine diphosphate glucose be present, one would expect the hexose moiety to contain the earliest incorporated carbon label. Our attention was therefore directed to the radioactive compounds from longer photosyntheses produced by treatment with phosphatase. Using extracts from sugar beet (5 minutes in $C^{14}O_2$) and Chlorella (10 minutes in $C^{14}O_2$), it has been shown that uridine and some adenosine are products of the enzymatic dephosphorylation of the original unknowns. The nature of the original form in which the adenosine appears on the chromatogram is still uncertain. Although the original unknown area is known to be the region in which ATP would be found, suitable enzymatic treatment of this area after a second purification by chromatography in a third solvent[2] still yielded uridine as well as adenosine.

The uridine has been identified by chromatography with authentic uridine in a 2-dimensional chromatogram as well as by its R_f value in two other solvent systems. The presence of adenosine has been shown by co-chromatography with

authentic adenosine, and by enzymatic deamination to inosine, identified by co-chromatography and by its hydrolysis to give hypoxanthine and ribose. The phosphatase preparation used for enzymatic dephosphorylation was found to contain a deaminase.

Experimental Part

When the plant extract is chromatographed as a band in a unidimensional chromatogram (phenol solvent) and the slow-moving u.v.-absorbing band extracted and hydrolyzed with acid, at least three u.v.-absorbing compounds are produced. These are adenine, adenosine-5'-phosphate and uridine-5'-phosphate. All three compounds have been characterized by their u.v. absorption spectra and R_f values in various solvents. The location of the phosphate group in the uridylic acid has been shown by its reaction with sodium periodate. The adenylic acid has the same R_f value as adenosine-5'-phosphate (muscle adenylic acid) and different from that of yeast adenylic acid.

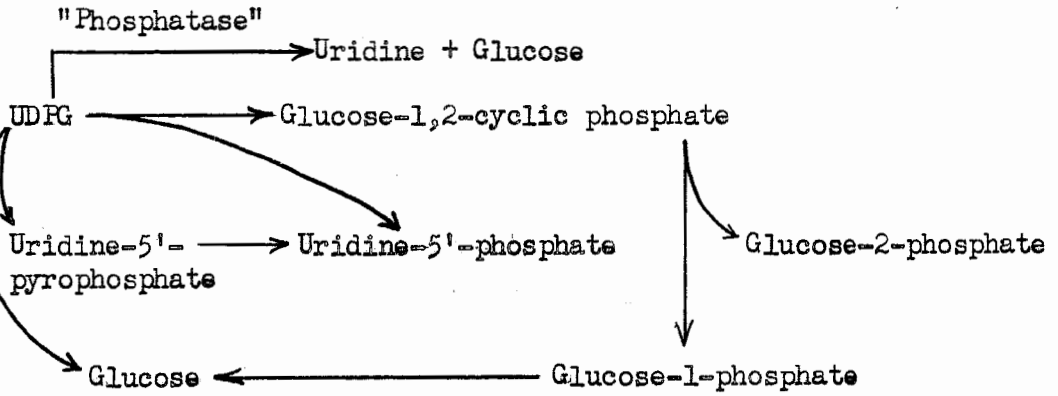
The radioactive compounds produced when the original is subjected to rechromatography have been examined. In particular, the two spots which yielded only glucose and galactose on dephosphorylation were subjected to further study. They had properties very reminiscent of the "fast ester" and "slow ester" of Leloir and his co-workers,[3] which have been shown to be, respectively, glucose-1,2-cyclic phosphate and a mixture of glucose-1- and glucose-2-phosphates. The corresponding phosphates of galactose have very similar properties. Glucose-1,2-cyclic phosphate was prepared by the action of ammonia on uridine diphosphate glucose, and co-chromatographed with the suspected "cyclic phosphate" area, i.e. that with R_f values nearly as great as those of glucose itself.

We have attempted to chromatograph the original unknown in the ethanol/ammonia solvent of Paladini and Leloir.[3] It has not been possible to demonstrate quantitative conversion into cyclic phosphates, probably due to the

insolubility of heavy metal phosphates in alkaline solvents. The heavy metals themselves are extracted from the original 2-dimensional chromatogram.***

The decomposition of the original unknown can be interpreted if it contains at least four compounds, as depicted below

I



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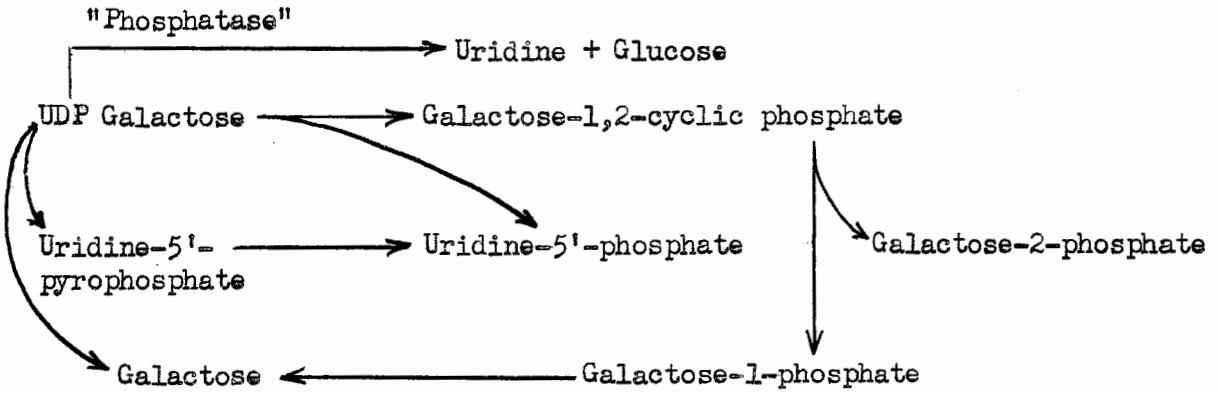

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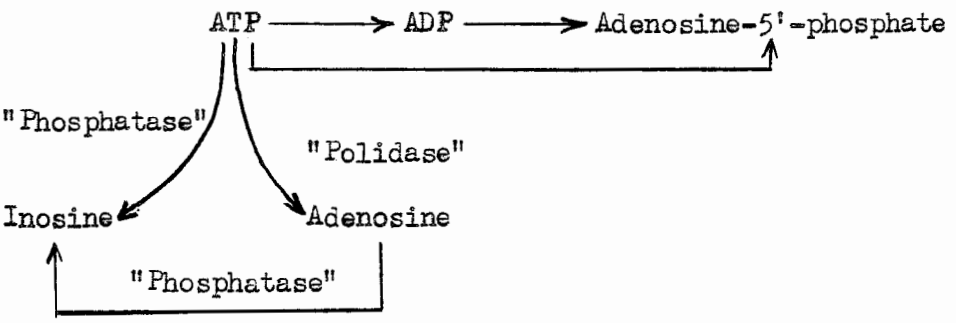


IV A compound containing mannose

The possible nature of the mannose-containing compound is of some interest. From its chromatographic behaviour, it appears not to be a 1,6-diphosphate or a monophosphate of mannose. It may therefore be a mannose-containing nucleotide of the UDPG type. When the "cyclic phosphate" area is hydrolyzed, glucose, galactose, but no mannose are produced. Hydrolysis of the "2-phosphate" area yields little or no mannose. Mannose differs from glucose and galactose in the configuration of the C_2 hydroxyl group, which is concerned in cyclic phosphate formation in the case of glucose and galactose. If the mannose is present in a compound of the UDPG type, with the same glycosidic configuration (viz. α-), then cyclic phosphate formation would be impossible, and the mannose nucleotide could only decompose to give free mannose, and possibly mannose-1-phosphate. Work is in progress to establish whether or not the mannose-containing compound is related in any way to that described by Leloir and Cabib,[4] which gives mannose, guanine and phosphate on hydrolysis.

After photosynthesis for some time (10 minutes or more) in $C^{14}O_2$, many other compounds, probably sugars, are found when the original radioactive nucleotide area is hydrolyzed by phosphatase. Further investigation of these products may lead to the identification of other compounds of the nucleotide-sugar class.

General. - Except where otherwise stated, the chromatographic conditions and radioautograph technique were those used previously in this laboratory.[5] The enzyme preparations used were "Polidase-S" (Schwarz Laboratories, Inc.) and "Phosphatase" (General Biochemicals, Inc.). Both were used in 1% solution, without buffer, and the incubations carried out, under toluene, with 200 μg. of enzyme, for periods of 24-72 hours, at 35° C.

Characteristics of the Unknown Radioactive Area. - The area appears on all radiograms, such as that in Figure 1, from green plants which have undergone photosynthesis in $C^{14}O_2$. Appreciable amounts of radioactivity accumulate

only after thirty seconds with most plants. It is situated below the hexose monophosphate area and is well differentiated from this and the ribulose diphosphate area.

Rechromatography of Unknown. - The original unknown was eluted from a chromatogram of 60 seconds photosynthesis in Scenedesmus (Figure 1). Eight radioactive areas were visible on the radioautograph (Figure 2). The areas (1), (2) and (3) were treated with phosphatase and rechromatographed.

Area (1) gave radioactive compounds in the positions characteristic of glucose, galactose and mannose, and of nucleotides (Figure 3). (2) gave glucose and galactose. (3) gave glucose and galactose, with possibly a trace of mannose.

Identification of the Hexose Sugars Produced by Acid Hydrolysis or Phosphatase Treatment. - In this experiment the unknown spot was obtained from Scenedesmus, and the hydrolysis carried out enzymatically. Many other such identifications have been carried out, using algae and green leaves, all with the same result.

The area (from 60 second Scenedesmus) was extracted and hydrolyzed with "Phosphatase." The hydrolysate was subjected to unidimensional chromatography in butanol-propionic acid, and the dried chromatogram exposed to film. Two main radioactive bands were observed when the film was developed. These were extracted together and divided into three equal samples. To each was added 100 γ of glucose, galactose and mannose respectively. Each sample was subjected to two-dimensional chromatography in phenol (first direction) and n-butanol-pyridine (second direction). The radiogram of each showed three spots, and one of the radioactive spots corresponded in position, size and shape to the brown spot developed by the aniline hydrogen phthalate due to

the reagent hexose carrier. Thus, the radioactive hexoses were identified as glucose, galactose and mannose. The relative amounts of radioactivity in the three sugars can be seen from Figure 3.

Co-chromatography of the "Cyclic Phosphate" Area with Glucose-1,2-cyclic phosphate. - Authentic glucose-1,2-cyclic phosphate was obtained by chromatography of UDPG (Ba salt, 60% pure, 1200 γ) as a band in ethanol-ammonia. The cyclic phosphate, R_f 0.57, whose position was determined by spraying a test strip with phosphate reagent, was eluted and co-chromatographed in two dimensions with the unknown radioactive phosphate and with glucose and galactose (50 γ of each). The solvents used were phenol and t-butanol-picric acid. The radioactive area was found to correspond exactly to the position of the added glucose-1,2-cyclic phosphate.

Identification of Uridine in Phosphatase Hydrolysate. - The nucleotide area from a longer photosynthetic experiment (5 minute sugar beet leaves) was eluted and hydrolyzed with "Phosphatase." When the hydrolysate was chromatographed in the standard solvents in addition to the above sugars, a radioactive spot appeared below the orange dye, tropeolin. (Labeled uridine in Figure 4.) The radioactive area was eluted and the following experiments carried out.

Sample 1. - Sample 1 and 25 γ of each of the nucleosides cytidine, uridine, adenosine and guanosine were chromatographed in one dimension in 5% disodium hydrogen phosphate-iso-amyl alcohol, using the technique of Carter.[6] Table I gives the R_f values found by radioautography and examination of the chromatogram under ultra-violet light.

The radioactive spot was coincident with the leading edge of the cytidine, uridine spot.

Sample 2. - Uridine (50 γ) was added, and the solution chromatographed in t-butanol-HCl. The R_f value of the radioactive spot was 0.58, exactly coincident with the u.v.-absorption. The area was extracted and subjected to two-dimensional chromatography in the standard solvents. After exposure to film, the chromatogram was sprayed with sodium periodate according to the method of Buchanan, Dekker and Long.[7] The color with Schiff's reagent was allowed to develop at room temperature. After three days, a strong blue-purple spot was apparent, exactly coincident with the radioactivity (Figure 5).

Identification of Inosine in "Phosphatase" Hydrolysate. - (1) The radioactive nucleotide area from a 5-minute sugar beet chromatogram was treated with "Phosphatase" and rechromatographed. A radioactive spot beyond uridine (labeled inosine in Figure 4) was extracted and co-chromatographed with inosine (50 γ, prepared by enzymatic deamination of adenosine). The darkened area on the radioautograph and the spot produced by the periodate Schiff's reagent spray were coincident.

(2) An identical nucleoside area (labeled inosine in Figure 4) was eluted from a chromatogram and chromatographed with cytidine, uridine, guanosine and adenosine carriers (25 γ of each) in 5% disodium hydrogen phosphate-iso-amyl alcohol by the technique of Carter.[6] The R_f value was 0.71 which did not correspond to that of any of the carriers (cf. Table I).

(3) Another sample of this nucleoside area together with 100 γ hypoxanthine, 100 γ ribose and 100 γ uridine was hydrolyzed in N HCl at 100° for 10 minutes. The hydrolysate was chromatographed in the standard solvents, and exposed to film. Two radioactive spots were observed. One was coincident with the hypoxanthine spot (seen under u.v. light) and the other was coincident with the ribose, detected by means of the aniline hydrogen phthalate spray.

(4) Adenosine (50 γ) was treated with "Phosphatase" and chromatographed in disodium hydrogen phosphate-iso-amyl alcohol.[6] Only one compound was visible under ultra-violet light. It had an R_f value of 0.70. Adenosine and uridine, on the same chromatogram, had R_f values of 0.53 and 0.79 respectively. The enzyme-treated compound had an absorption spectrum (in 0.1 N HCl) with a maximum at 250 mμ and was undoubtedly inosine. Hypoxanthine has an R_f value close to that of adenosine in this solvent. The specificity of the deaminase present in the phosphatase preparation has not been determined.

(5) Another sample of this nucleoside area was obtained from a chromatogram of a "phosphatase"-treated Scenedesmus (60 seconds in $C^{14}O_2$) nucleotide area and rechromatographed in one dimension in n-butanol-propionic acid. A u.v.-absorbing spot, containing very little radioactivity, was seen, at an R_f value slightly higher than that of the free sugars. It was extracted, and hydrolyzed with N HCl at 100° for 15 minutes. The spot was chromatographed in n-butanol-propionic acid, in which it was found to have the same R_f value (0.31) as hypoxanthine (0.30). It was eluted from the chromatogram and chromatographed in 5% disodium hydrogen phosphate-iso-amyl alcohol in which it had the same R_f value (0.58) as hypoxanthine (0.55). The R_f value of adenine was 0.39. The spot, when extracted from the chromatogram, had a u.v. absorption maximum (in 0.1 N HCl) at 248 mμ. (Figure 6). (Hypoxanthine 249 mμ.)

Identification of Adenosine in Polidase-S Hydrolysate. - The radioactive nucleotide area from a 10-minute Chlorella chromatogram was rechromatographed in ethanol-ammonium acetate at pH 7.5. The slowest-moving radioactive, u.v.-absorbing spot, which had the same R_f value as ATP in this solvent, was extracted and treated with "Polidase-S." On rechromatography in two dimensions in the

standard solvents three spots of approximately equal radioactivity were produced. These were identified as ribose, uridine, and adenosine by co-chromatography with authentic specimens.

Identification of Adenine in Acid Hydrolysate. - The extract from Scenedesmus (1.5 g. wet weight) which had photosynthesized for 2 minutes in $C^{14}O_2$ was chromatographed in phenol as a band. After thorough drying, a slow-moving radioactive band exhibiting u.v.-absorption was observed. No other u.v. absorbing bands were visible. The band was extracted and hydrolyzed with N HCl for 10 minutes at 100°. The hydrolysate was chromatographed in n-butanol-propionic acid. Two u.v.-absorbing bands, A and B, which were also radioactive, were observed.

The faster moving band, A, was extracted and chromatographed in t-butanol/HCl where it gave a spot of R_f 0.29 (adenine 0.29, adenosine 0.27). The spot was extracted and found to have a u.v.-absorption spectrum measured in 0.1 N HCl (max. 262 mμ.) and 0.1 N NaOH (max. 268) (Figure 7), identical with the spectrum of adenine.

Identification of Adenosine-5'-Phosphate in Acid Hydrolysate. - The slower moving band, B, above was extracted and chromatographed in two dimensions in the standard solvents. Two u.v.-absorbing, radioactive spots were produced, in the positions expected for an adenylic acid and a uridylic acid in these solvents (Figure 8).

The faster moving spot was extracted, and the u.v. absorption spectrum, measured in 0.1 N HCl, was found to have a maximum at 259 mμ, corresponding to that of adenosine-5-phosphate.

Identification of Uridine-5-phosphate in Acid Hydrolysate. - The spot suspected to be uridylic acid (see above) was eluted (u.v. max. 261 mμ) and co-chromatographed with authentic uridine-3-phosphate (50 μg.) and uridine-5-

phosphate (50 μg.) using the butanol-propionic acid solvent. The radioactivity coincided with the u.v. absorption by the uridine-5-phosphate (R_f .15) and not with that of uridine-3-phosphate (R_f .33). Addition of 500 μg. sodium metaperiodate to the unknown for thirty minutes before chromatography resulted in a radioactive product with the same R_f value (.47) as obtained from reaction with authentic uridine-5-phosphate. Periodate had no effect upon the R_f of uridine-3-phosphate.

Summary

1. Uridine diphosphate glucose and uridine diphosphate galactose have been identified in green plants.
2. The hexoses of these compounds become labeled rapidly during photosynthesis in $C^{14}O_2$ and constitute a large fraction of the total labeled non-polysaccharide hexose.
3. A mannose-containing compound chromatographically similar to a nucleotide is present.
4. Evidence is presented for the presence of an adenosine-containing nucleotide other than ATP, ADP or AMP.

Table I

R_f Values of Radioactive Hydrolysis Products
in 5% Disodium Hydrogen Phosphate-iso-amyl Alcohol

Compound	Reported R_f values[6]	u.v.-absorbing areas of added carrier	Radioactivity observed upon "Phosphatase" treatment of nucleotide area
Adenosine	.54	.54	
Guanosine	.62	.64	
Cytidine	.76	.80	.80
Uridine	.79		

Footnotes

(*) The work described in this paper was sponsored by the U.S. Atomic Energy Commission.

(**) Present address, Lister Institute, London, England.

(***) Mr. A. T. Wilson, in these laboratories, has found that treatment of the original extract with ammonia results in the almost complete disappearance of the radioactive area below the "hexose monophosphates" with the concommitant appearance of a spot in the "cyclic phosphate" area.

References

(1) Benson, A. A., Kawaguchi, S., Hayes, P.M. and Calvin, M., J. Am. Chem. Soc., 74, 4477 (1952).

(2) Leloir, L. F., "The Metabolism of Hexosephosphates," Phosphorus Metabolism, I, 67, Johns Hopkins Press, 1951.

(3) Paladini, A. C. and Leloir, L. F., Biochem. J., 51, 426 (1952).

(4) Leloir, L. F. and Cabib, E., Phosphorus Metabolism, I, 75, Johns Hopkins Press, 1951.

(5) Benson, A. A., et al., J. Am. Chem. Soc., 72, 1710 (1950).

(6) Carter, C. E., J. Am. Chem. Soc., 72, 1468 (1950).

(7) B uchanan, J. G., Dekker, C. A., and Long, A. G., J. Chem. Soc., 3162 (1950).

Captions to Figures

Fig. 1 - Products of Sixty Seconds Photosynthesis by Scenedesmus.

Fig. 2 - Rechromatography of UDPG Area.

Fig. 3 - Phosphatase Hydrolysate of Area (1).

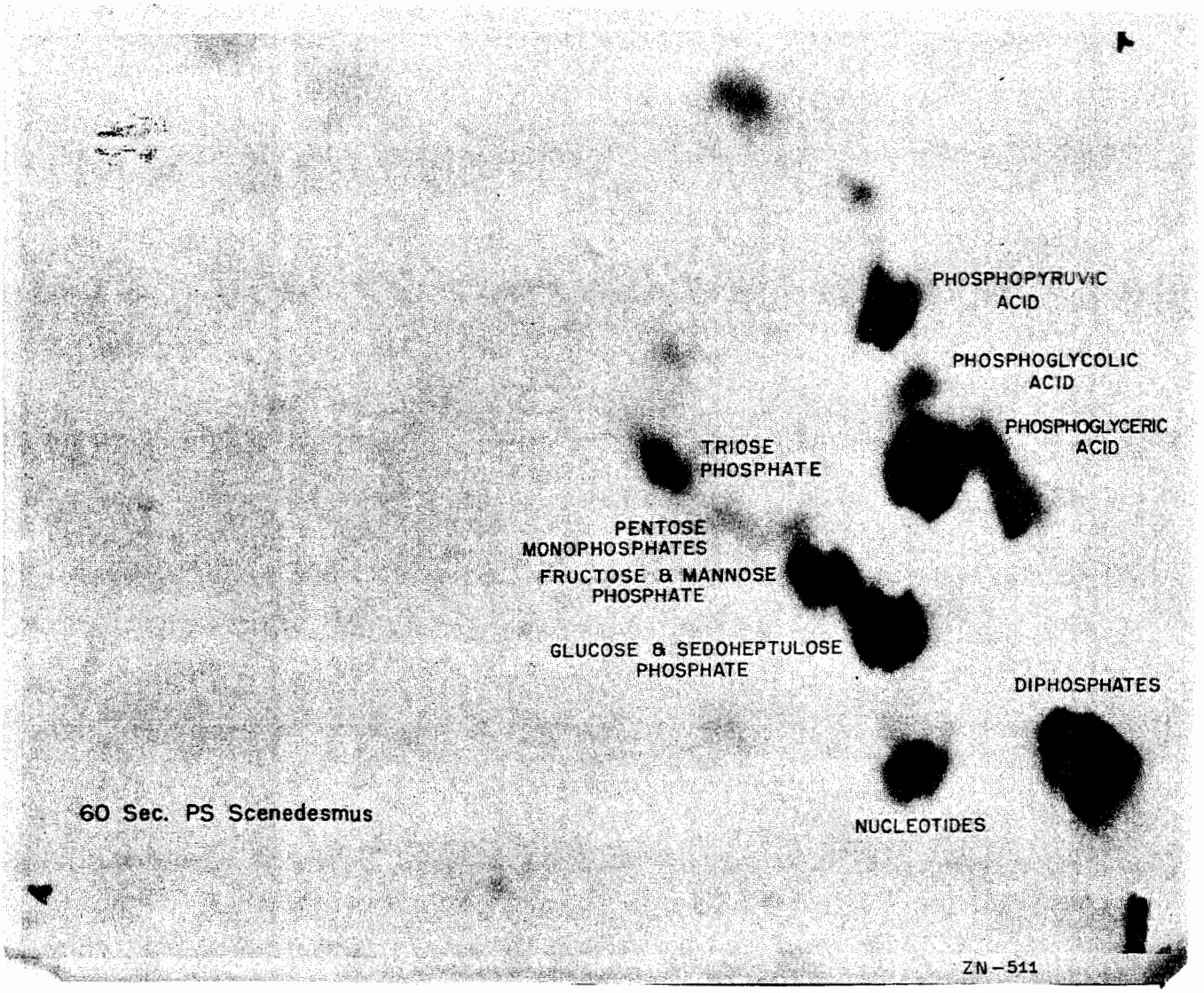
Fig. 4 - Phosphatase Hydrolysate of UDPG Area from 5 minute Sugar Beet Leaf Photosynthesis.

Fig. 5 - Identification of Uridine by Co-chromatography.

Fig. 6 - Absorption spectra of hypoxanthine. White circles represent relative absorption by authentic hypoxanthine in 0.1 M HCl, 0.1 M NaOH. Black circles, relative absorption of unknown in 0.1 M HCl. The values of the ordinates are calculated from absorption measurements on a Cary Recording Spectrophotometer.

Fig. 7 - Absorption spectrum of adenine. Circles represent relative absorption of authentic adenine sulfate. Smooth curves, of unknown. A, in 0.1 M HCl. B, in 0.1 M NaOH. The ordinate values were calculated from absorption measurements with a Beckman spectrophotometer.

Fig. 8 - Separation of Adenylic and Uridylic Acids from Acid-Hydrolysate of Compounds in UDPG Area.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PHOSPHOGLYCOLIC ACID
PHOSPHOGLYCERIC ACID
TRIOSE PHOSPHATE
PENTOSE MONOPHOSPHATES
FRUCTOSE & MANNOSE PHOSPHATE
GLUCOSE & SEDOHEPTULOSE PHOSPHATE
DIPHOSPHATES
NUCLEOTIDES
60 Sec. PS Scenedesmus
ZN-511

Fig. 1

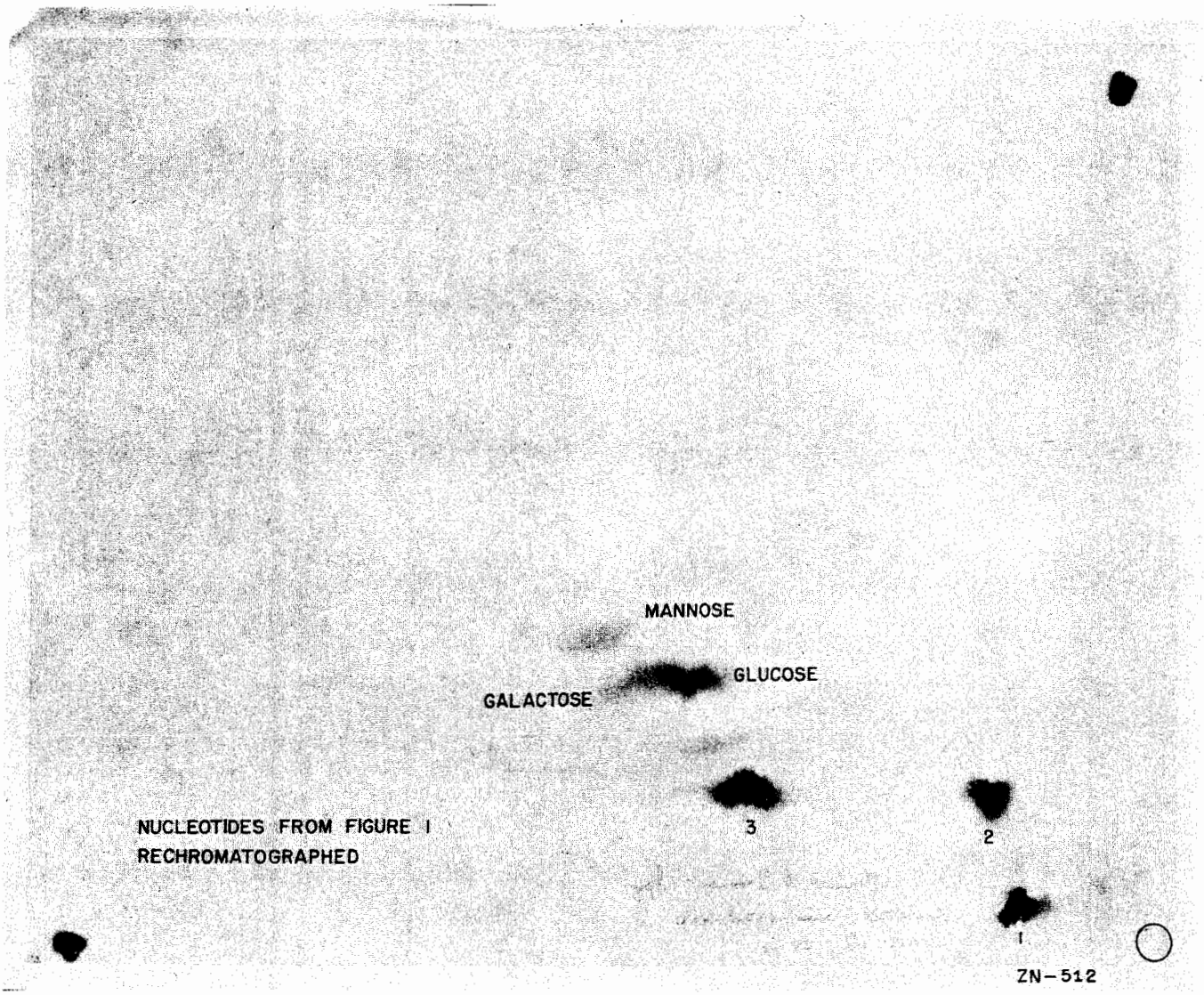
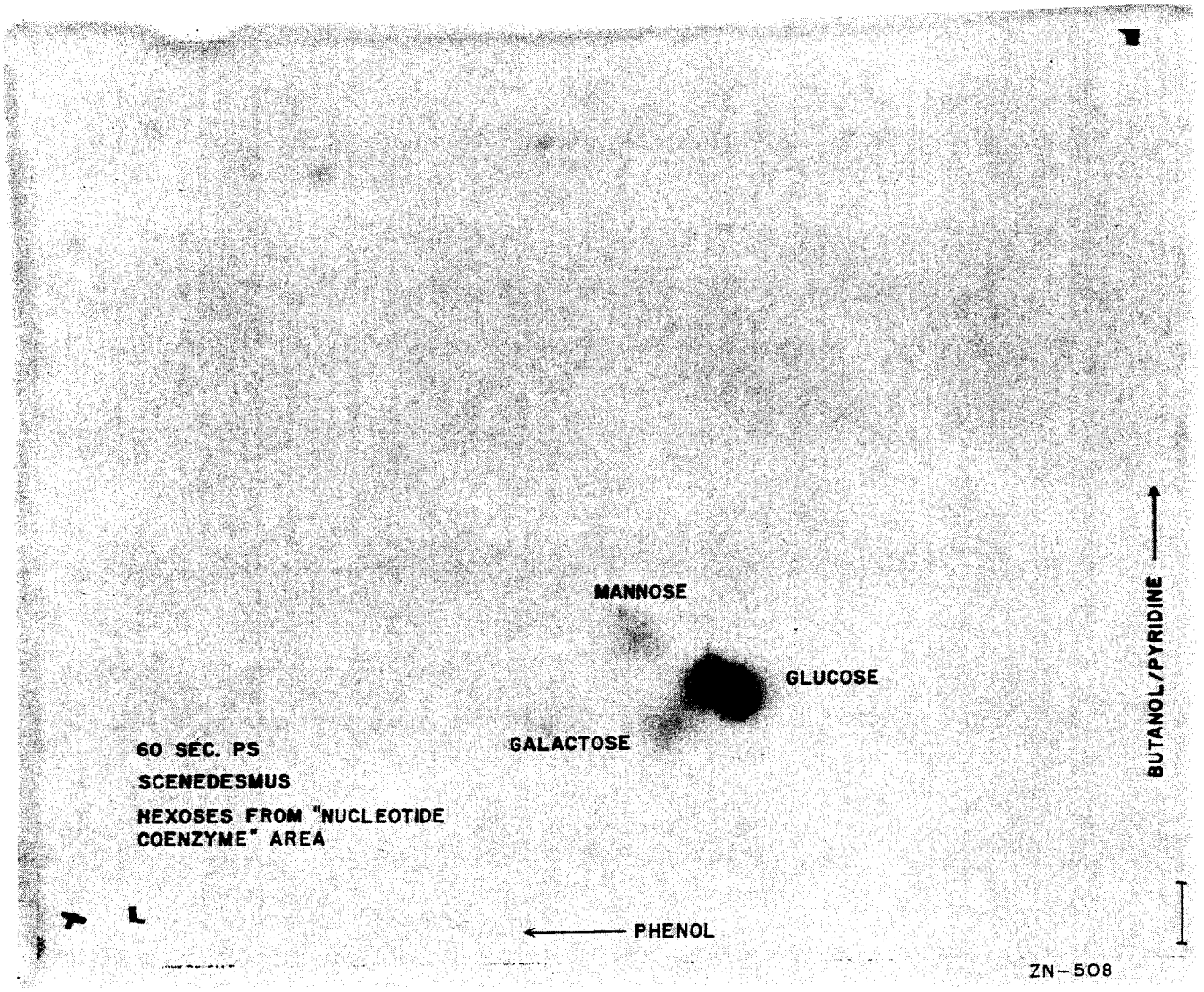

MANNOSE
GLUCOSE
GALACTOSE
NUCLEOTIDES FROM FIGURE 1
RECHROMATOGRAPHED
3
2
1
ZN-512

Fig. 2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PHENOL
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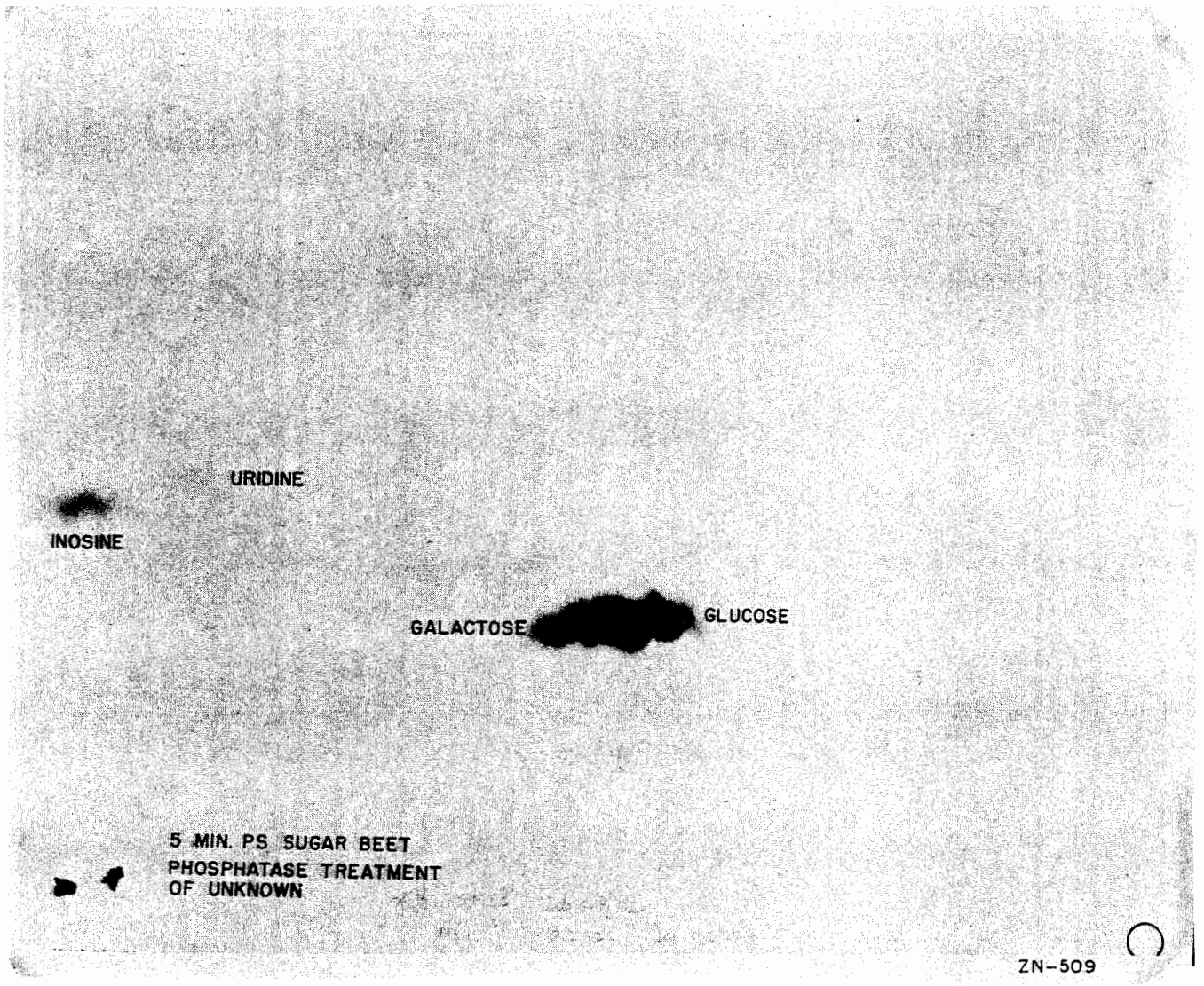
ZN-508

Fig. 3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PHOSPHATASE TREATMENT
OF UNKNOWN
ZN-509

Fig. 4

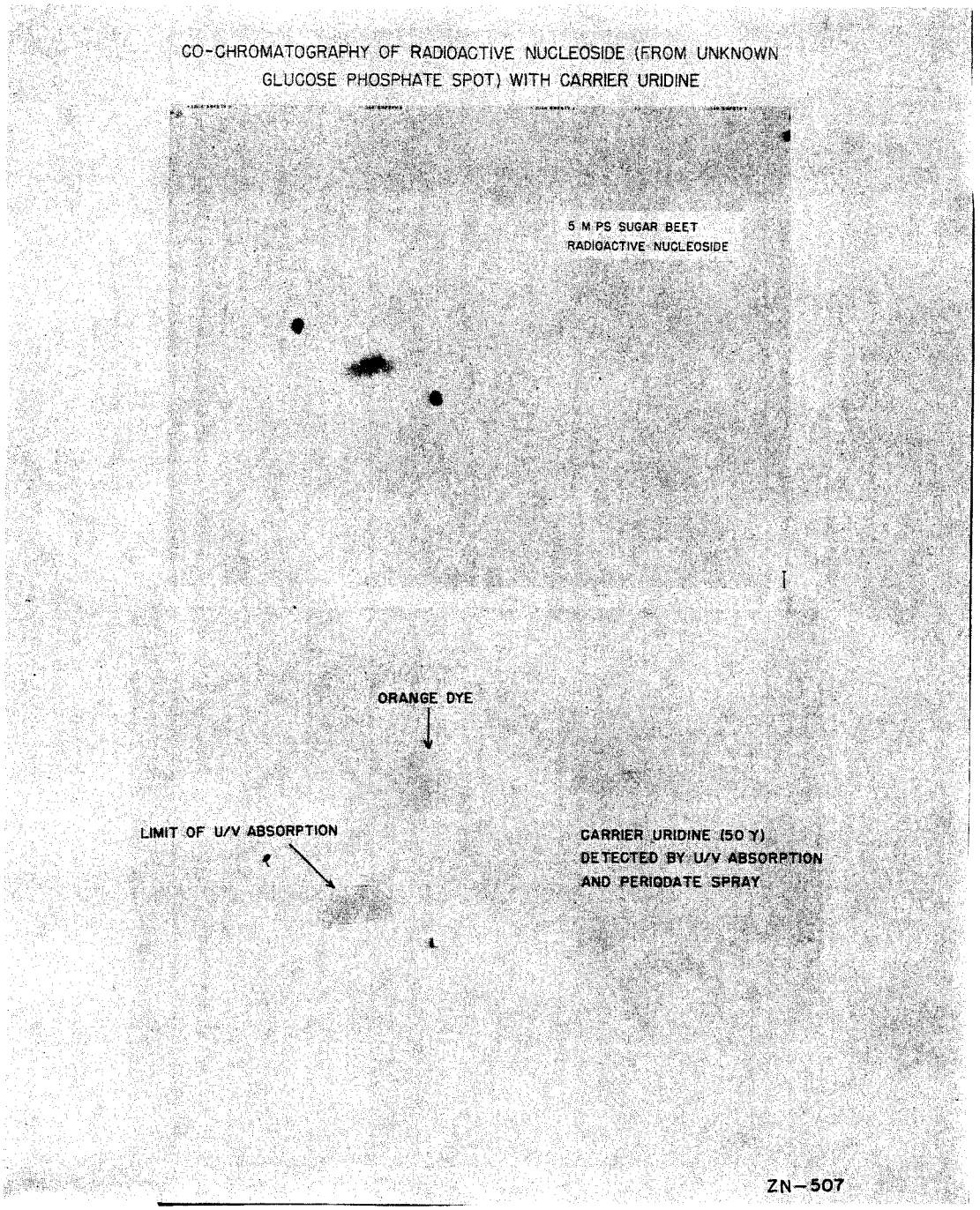
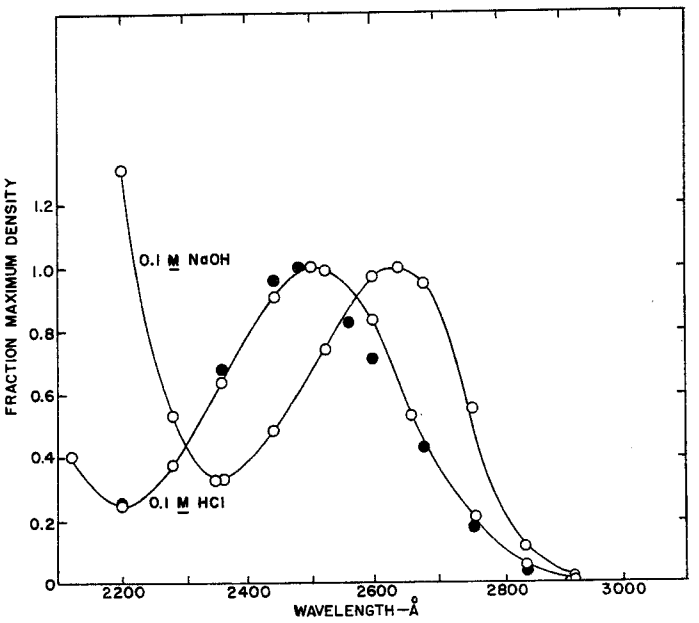

CO-CHROMATOGRAPHY OF RADIOACTIVE NUCLEOSIDE (FROM UNKNOWN GLUCOSE PHOSPHATE SPOT) WITH CARRIER URIDINE
5 M PS SUGAR BEET RADIOACTIVE NUCLEOSIDE
ORANGE DYE
LIMIT OF U/V ABSORPTION
CARRIER URIDINE (50 Y) DETECTED BY U/V ABSORPTION AND PERIODATE SPRAY
ZN-507

Fig. 5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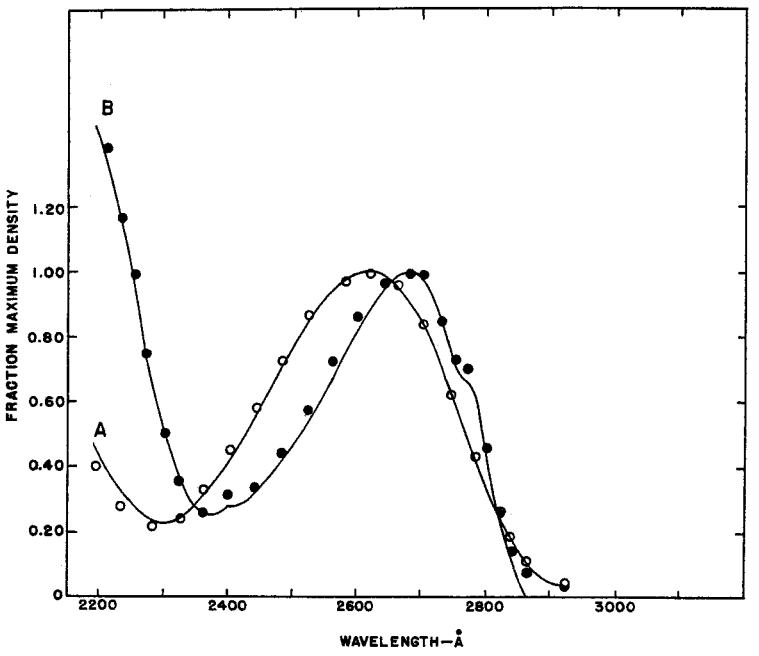
WAVELENGTH—Å

Fig. 6


B
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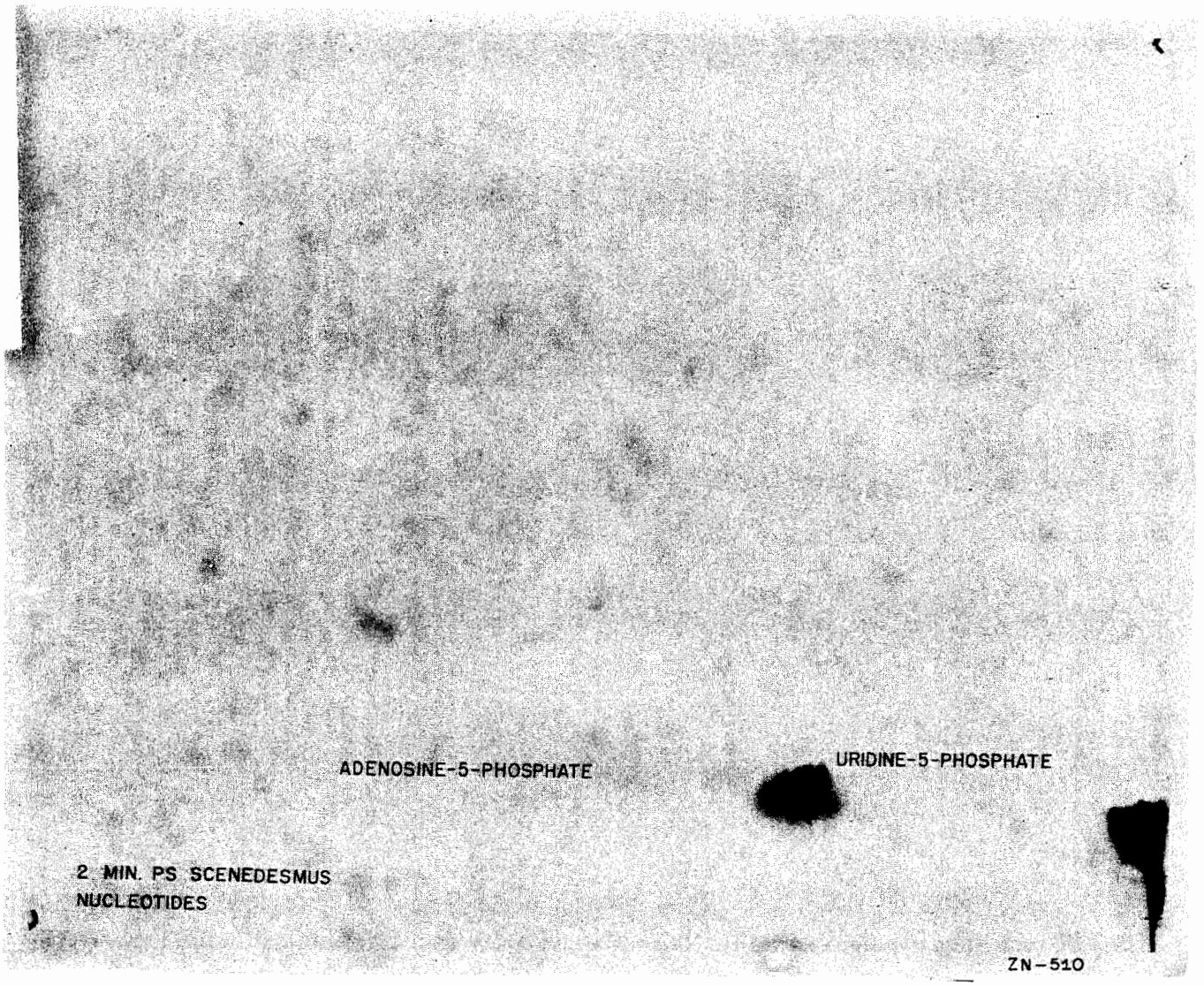
WAVELENGTH—Å

Fig. 7


ADENOSINE-5-PHOSPHATE
URIDINE-5-PHOSPHATE
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NUCLEOTIDES
ZN-510

Fig. 8