1-(*o*-Carboxyphenylamino)-1-deoxyribulose

A COMPOUND FORMED BY MUTANT STRAINS OF *AEROBACTER AEROGENES* AND *ESCHERICHIA COLI* BLOCKED IN THE BIOSYNTHESIS OF TRYPTOPHAN

By C. H. DOY AND F. GIBSON

School of Bacteriology, University of Melbourne, Carlton, N. 3, Victoria, Australia

(Received 29 July 1958)

This paper presents the evidence for assigning the structure 1-(*o*-carboxyphenylamino)-1-deoxyribulose to a compound (Y) formed by mutant strains of *Aerobacter aerogenes* (N3-33, N2-18) and *Escherichia coli* (R19-33). The experiments with *A. aerogenes* have been reported briefly (Gibson, Doy & Segall, 1958). None of these strains would grow in the minimal medium described by Davis & Mingioli (1950) unless this was supplemented with tryptophan or indole, and were therefore presumably unable to convert anthranilic acid into indole. Therefore it is possible that 1-(*o*-carboxyphenylamino)-1-deoxyribulose (A) is an intermediate in the conversion of anthranilic acid into indole, probably as the immediate precursor of indolylglycerol (Yanofsky, 1956), thus:

RESULTS

Formation and concentration of compound Y

The natural compound (Y) was readily formed in an aerated mixture of washed cells of *A. aerogenes* N3-33 with glucose, ammonium chloride, magnesium sulphate and phosphate buffer. After centrifuging the cells compound Y was separated by acidification and extraction of the supernatant with ethyl acetate. Concentrates of the compound could then be prepared by evaporating to small bulk under vacuum, but complete removal of the solvent resulted in decomposition of the compound, as indicated by changes in the spectrum. It has not been found possible to isolate Y, other than as an impure oil, except on paper chromatograms; but its identity has been established by spot reactions on paper chromatograms, isolation of a crystalline 2:4-dinitrophenylhydrazone, ultraviolet-absorption spectroscopy and by a comparison with synthetic compounds.

Preliminary spectroscopic and chromatographic evidence for the structure of compound Y

The intense blue-violet fluorescence and the absorption spectrum suggested that the compound might be a derivative of anthranilic acid. Preliminary experiments in which the ultraviolet-absorption spectra of a number of anthranilic acid derivatives were examined in aqueous solution at pH 7.7 and about pH 2 (unpublished data) indicated that the spectrum of Y was consistent with the structure (I). Substitution of the carboxyl or imino group destroyed the similarity to Y, as did the removal of the carbonyl group from a position β to nitrogen.

The colour reactions of Y on paper chromato-

grams indicated that a sugar fragment was present and that this was probably a pentulose (Table 1). Concentrates of Y instantly decolorized alkaline methylene blue at room temperature, which suggested an Amadori compound (Hodge, 1955).

To detect Y on paper chromatograms it was more convenient to use alkaline 2:3:5-triphenyltetrazolium chloride, which gave an instantaneous red colour without heating. Under these conditions the common sugars, with the exception of fructose, produced only a faint coloration. Fructose, however, was easily differentiated from Y by the bright yellow it gave with anthrone reagent.

Comparison of the properties of glycosylamines and Amadori compounds with compound Y

As intermediates in the syntheses of Amadori compounds and as models for the study of absorption spectra, the glycosylamines, *N*-*o*-carboxyphenyl-D-ribosylamine, -D-arabinosylamine, -L-arabinosylamine, -D-xylosylamine and -D-glucosylamine, were prepared. These gave no colour with 2:3:5-triphenyltetrazolium chloride reagent. The different colour reactions (Table 1), susceptibility to acid hydrolysis and different ultraviolet-absorption spectra (Tables 3, 4; Figs. 1, 2) eliminated the possibility that Y was identical with one of these glycosylamines.

The glycosylamines when refluxed in *NN*-dimethylformamide rapidly yielded a reaction mixture giving the alkaline methylene blue and 2:3:5-triphenyltetrazolium chloride reactions typical of Y. Each reaction product gave a spot on paper chromatograms strongly fluorescent in ultraviolet light and an intense red with 2:3:5-triphenyltetrazolium chloride reagent at room temperature. When alternate spots of the unknown and reference compounds were chromatographed under identical conditions it was found that the substance originating from the ribosylamine, or the arabinosylamine, could not be distinguished from Y either by R_f (Table 2) or colour reactions (Table 1). The analogous compound from the xylosylamine always had a slightly lower R_f , but gave similar colour reactions, whereas that from the glucosylamine was readily differentiated by both R_f and colour reactions. These results were observed in the solvent systems methanol-butan-1-ol-benzene-water and butan-1-ol-acetic acid-water. A reference substance, usually Y, was included on all paper chromatograms and no reliance was placed on absolute R_f values alone since the latter varied slightly from day to day.

The ribosylamine (II) and the arabinosylamine (III) differ only in the configuration of C-2 in the sugar fragment, which difference is lost in the Amadori rearrangement to (V). However, the xylosylamine (IV) differs from both (II) and (III) at C-3 and this difference is retained in the Amadori compound (VI). This supports the relationship found on paper chromatograms. Although (II)-(VI) are drawn with the D configuration, none of the evidence so far presented indicates the configuration of Y. Further support for the Amadori structure was obtained from a detailed study of the ultraviolet-absorption spectra of natural and synthetic products and the preparation of a crystalline 2:4-dinitrophenylhydrazone from both natural and synthetic sources.

The ultraviolet-absorption spectra of the glycosylamines (Table 3, Fig. 1) showed characteristic

Table 1. Colours developed with reagents sprayed on paper chromatograms

Reagent	Compound				References
	Compound Y and the synthetic pentose Amadori compounds	Synthetic hexose Amadori compound	<i>N</i> - <i>o</i> -Carboxyphenyl-D-pentosylamines*	<i>N</i> - <i>o</i> -Carboxyphenyl-D-glucosylamine*	
Ammonium molybdate	Blue	Blue	Blue	Blue	Aronoff & Vernon (1950)
Aniline acetate	Red† changing to red-brown	Red-brown	Pale yellow-brown	Pale yellow-brown	Aronoff (1956) Tracey (1950)
Phloroglucinol	Dark green (keto); purple (aldo)	Dark green	Yellow-brown	Pink, changing to purple	Borenfreund & Dische (1957)
Anthrone	Bright purple (ketoxylase)	Grey-yellow, often changing to purple	Bright yellow	Brown	Johanson (1953)
Alkaline 2:3:5-triphenyltetrazolium chloride	Nil†	Red	Nil	Nil	Trevelyan, Procter & Harrison (1950)

* The glycosylamines were spotted on to Whatman no. 1 paper and not chromatographed.

† Used by the original authors as a quantitative colorimetric method in solution. When applied to paper, as suggested by Aronoff (1956), a red first develops but then changes to red-brown. Glucose gave only pale brown in the same time (6 min. at 100°).

‡ No immediate colour with pentoses unless heat is applied.

§ The references give results of original authors for pentoses. The other results were obtained during the present work.

changes with alteration in pH which were important in differentiating them from the synthetic Amadori compounds (Table 4, Fig. 2). On adjusting the pH of solutions of the glycosylamines in phosphate buffer (pH 7.7) to pH ≤ 2.0 , by the addition of hydrochloric acid, the maximum in the 250 $m\mu$ region is rapidly quenched. Simultaneously the 315–320 $m\mu$ maximum undergoes a bathochromic shift to the 330 $m\mu$ region, but is then also quenched. The final spectrum has no clear maximum, but if the pH is then adjusted to ≥ 12 with

10N-sodium hydroxide maxima appear at 240–242 and 310–312 $m\mu$, which are identical with the maxima of anthranilic acid at this pH. If some of the original solution at pH 7.7 is adjusted directly to pH ≥ 12 there is no change in the wavelength of maximum absorption. This suggests that, at strongly acid pH, hydrolysis to anthranilic acid is very rapid. It is known from other experiments that hydrolysis proceeds even at slightly alkaline pH. The pentose and hexose compounds cannot be readily differentiated by spectral behaviour (Table 3).

Table 2. R_f values of anthranilic acid, compound Y and synthetic Amadori compounds derived from certain N-glycosylamines of anthranilic acid

R_f values below were obtained together on the one sheet of Whatman no. 1 paper developed with methanol–butan-1-ol–benzene–water (2:1:1:1, by vol.); solvent travel approx. 19 cm.

Compound	Parent glycosylamine	R_f
Anthranilic acid	—	0.93
Compound Y (four samples)	—	0.85–0.86
1-(o-Carboxyphenylamino)-1-deoxy-D-ribose	N-o-Carboxyphenyl-D-arabinosylamine	0.86
	N-o-Carboxyphenyl-D-ribosylamine	0.85
1-(o-Carboxyphenylamino)-1-deoxy-L-ribose	N-o-Carboxyphenyl-L-arabinosylamine	0.85
1-(o-Carboxyphenylamino)-1-deoxy-D-xylulose	N-o-Carboxyphenyl-D-xylosylamine	0.84*
1-(o-Carboxyphenylamino)-1-deoxy-D-fructose	N-o-Carboxyphenyl-D-glucosylamine	0.80

* The small difference in R_f between the xylulose and ribulose compounds was confirmed on many chromatograms (see the Experimental section), whereas compound Y and the ribulose derivatives could not be differentiated.

Table 3. *Ultraviolet-absorption spectra of anthranilic acid and some anthranilic acid glycosylamines*

Spectra were measured on 0.1 mm-solutions against a blank of the appropriate solvent. Absorption was recorded at intervals of 5 μ , generally, 1 μ at maxima, over the range 220–400 μ .

Compound	0.1 M-Phosphate buffer, pH 7.7			Ethanol			Ethyl acetate-ethanol (98:2, v/v)		
	$\lambda_{\max}$ (μ)	$\epsilon_{\max}$	$\epsilon_{\max}$ ratio	$\lambda_{\max}$ (μ)	$\epsilon_{\max}$	$\epsilon_{\max}$ ratio	$\lambda_{\max}$ (μ)	$\epsilon_{\max}$	$\epsilon_{\max}$ ratio
<i>N</i> -o-Carboxyphenyl-D-riboseylamine	247 316	9600 3570	2.66/1	250 331	8930 4350	2.05/1	253 335	7750 4740	1.64/1
<i>N</i> -o-Carboxyphenyl-D-arabino-sylamine	247 316	8950 3470	2.58/1	249–250 333	9750 4830	2.02/1	253 336	8300 5080	1.64/1
<i>N</i> -o-Carboxyphenyl-L-arabino-sylamine	246 316	8850 3420	2.59/1	249–250 333	9980 4930	2.02/1	253–254 336	9050 5400	1.68/1
<i>N</i> -o-Carboxyphenyl-D-xylo-sylamine	247 316	9880 3650	2.7/1	249–250 333	9750 4830	2.02/1	253 336	7100 4250	1.67/1
<i>N</i> -o-Carboxyphenyl-D-glucosylamine	247 317	9600 3740	2.57/1	249 333	9500 4570	2.08/1	253 336	7570 4620	1.64/1
Anthranilic acid	240 310	7170 2980	2.41/1	248–249 336	7100 4460	1.59/1	252–253 336	5630 4900	1.15/1

Table 4. *Ultraviolet-absorption spectra of compound Y and synthetic Amadori compounds derived from certain N-glycosylamines of anthranilic acid*

Experimental samples were obtained as eluates from paper chromatograms. Eluates (eluting solvents 0.1 M-phosphate buffer, pH 7.7; ethanol; ethyl acetate) were diluted with pure solvent until an extinction value in the range 0.8–1.0 was recorded at 280 μ . pH adjustments were made by adding conc. HCl or 10N-NaOH to samples in phosphate at pH 7.7. Absorption was recorded at intervals of 5 μ , generally, 1 μ at maxima, over the range 220–400 μ .

Compound	Initial	Parent glycosylamine	pH 7.7		Adjusted to		Ethanol	Ethyl acetate
			Stored for approx. 16 hr., 20–25°	pH ≤ 2*	pH ≥ 12 (1 hr., 20–25°)			
1-(<i>o</i> -Carboxyphenylamino)-1-deoxy-D-ribose	250 324	<i>N</i> - <i>o</i> -Carboxyphenyl-D-riboseylamine	240 309	250 343	240 308	254 347	254–255 346	
	250	<i>N</i> - <i>o</i> -Carboxyphenyl-D-arabinosylamine	240	250	240	253–254	254	
	323		310	343	310	344–345	345	
1-(<i>o</i> -Carboxyphenylamino)-1-deoxy-L-ribose	250 325	<i>N</i> - <i>o</i> -Carboxyphenyl-L-arabinosylamine	240 310	250 345	242 308	254 347	254 346	
1-(<i>o</i> -Carboxyphenylamino)-1-deoxy-D-xylose	249 325	<i>N</i> - <i>o</i> -Carboxyphenyl-D-xylosylamine	240–242 312	249 345	242 310	254 347	254 343	
1-(<i>o</i> -Carboxyphenylamino)-1-deoxy-D-fructose	252 328	<i>N</i> - <i>o</i> -Carboxyphenyl-D-glucosylamine	250 325	253 347–348	246–247 318	255 350	255 350	
Y	250 325	—	240–242 310	250–251 345	240–242 310	254 346–347	254–255 345–347	

* A weak inflexion is found at approx. 280 μ .

In general the spectra of the synthetic Amadori compounds (Table 4, Fig. 2), as determined on eluates from paper chromatograms, bear the expected relationship to the spectra of the glycosylamines. The introduction of the additional chromophore has resulted in a bathochromic shift of both bands of maximum absorption, but more especially the band at longer wavelength, which has shifted 8–10 $m\mu$ (phosphate buffer, pH 7.7). In phosphate buffer (pH 7.7) Y has absorption maxima at 250 and 325 $m\mu$. On adjusting the pH of this solution to ≤ 2 with concentrated hydrochloric acid, the spectrum changes with the maxima now found at 250–251 and 345 $m\mu$. An inflexion found at about 280 $m\mu$ (Fig. 2), but with weak absorption, is probably attributable to the free carbonyl group of the keto form. If some of the original solution at pH 7.7 is adjusted to pH ≥ 12 , with 10N-sodium hydroxide, immediate spectral changes occur with a gradual shift (over about 60 min.) to a maximum at 310–312 $m\mu$ and an inflexion or slight maximum at 240–242 $m\mu$. Readjustment to slightly acid pH results in a spectrum with no prominent features.

Paper chromatography of the Amadori reaction products from each of the pentosylamines separated compounds (2:3:5-triphenyltetrazolium chloride positive) which on elution had identical spectra with natural Y, isolated and eluted in the same manner (Table 4, Fig. 2). The hexose analogue derived from the glucosylamine exhibited similar spectra when examined at slightly alkaline and strongly acid pH. However, the behaviour at strongly alkaline pH was distinctive (Table 4). The spectral changes were similar to, but slower than, those observed with the pentose compounds. This differentiation was more distinct at pH 7.7 as the

rate of change was retarded still further. After elution with buffer, pH 7.7, the spectra measured immediately were similar, but after 16 hr. at room temperature, fresh examination indicated that the samples of Y and the synthetic pentose Amadori compounds now exhibited a spectrum with a maximum at 310–312 $m\mu$ and an inflexion, or slight maximum, at 240–242 $m\mu$. In contrast, the synthetic hexose Amadori compound had shown little change, with maxima found at 250 and 325 $m\mu$ (Table 4, Fig. 2). These changes are interpreted as indicating the liberation of anthranilic acid after enolization of the Amadori compound. From the above observations it would seem that the fructose derivative is more stable to alkali than the pentose compounds. In general, this alkaline lability would be expected of Amadori compounds. The behaviour of Y was in all respects identical with that of the synthetic pentose Amadori compounds.

Preparation and properties of 2:4-dinitrophenylhydrazones from natural and synthetic products

As Y seemed unstable when attempts were made to purify the crude accumulation product, it was necessary to attempt the preparation and isolation of a derivative under mild non-alkaline conditions. Compound Y was quantitatively extracted from ethyl acetate with phosphate buffer (pH 7.7), and the addition of an acid solution of 2:4-dinitrophenylhydrazine to this extract resulted in the formation of an amorphous, orange-red precipitate, which on isolation melted over a wide range (100–200°). Recrystallization from ethanol eventually yielded rather amorphous yellow crystals, m.p. 213–215° (decomp.). Elementary analysis indicated a mono-2:4-dinitrophenylhydrazone,

Fig. 1. Absorption spectra of 0.1 mm-*N*-*o*-carboxyphenyl-D-arabinosylamine measured in (a) 0.1M-phosphate buffer, pH 7.7, max. 247, 316 $m\mu$; (b) ethanol, max. 249.5, 333 $m\mu$; (c) ethyl acetate-ethanol (98:2, v/v) max. 253, 336 $m\mu$. These spectra are typical of the anthranilic acid glycosylamines prepared (see the Experimental section). For details of $\lambda_{\max}$, ϵ values and ratios see Table 3.

$C_{18}H_{19}O_5N_5$. An apparently identical compound was obtained by the addition of 2:4-dinitrophenylhydrazine reagent to the Amadori-rearrangement reaction mixture obtained from *N*-*o*-carboxyphenyl-D-arabinosylamine. The synthetic and naturally derived hydrazones gave no depression of melting point on admixture, could not be

separated by paper chromatography and showed identical ultraviolet-absorption maxima (Fig. 3). At $pH \leq 2$ the spectral changes immediately and on storage were identical.

Although neither the natural nor the synthetic compound has been isolated, the method of preparation of the synthetic compound, the typical

Fig. 2. Comparison of the absorption spectra of compound Y and the synthetic Amadori compounds, showing the identical behaviour of Y and the Amadori compounds derived from *N*-*o*-carboxyphenyl-pentosylamines. The hexose analogue, derived from *N*-*o*-carboxyphenyl-D-glucosylamine, is differentiated by the rate of hydrolysis at pH 7.7, which is slower for the hexose compound. Experimental samples were eluted from paper chromatograms with 0.1M-phosphate buffer, pH 7.7. For details of λ_{max} , see Table 4, and for preparation of samples see the Experimental section.

Fig. 3. Absorption spectra of the 2:4-dinitrophenylhydrazone derivatives from compound Y and a synthetic source of 1-(*o*-carboxyphenylamino)-1-deoxy-D-ribulose. The spectrum at pH ≤ 2 was recorded after adjusting the pH of a solution in 0.1M-phosphate buffer, pH 7.7, with conc. HCl, and initially shows rapid changes. —, Spectrum recorded immediately; ---, spectrum after 2 days' (natural), overnight (synthetic) storage at pH ≤ 2 . $\lambda_{\max}$ (pH 7.7): 245, 280, 373 m μ . Spectra are qualitative (different *E* values).

reaction with redox reagents and the behaviour of products from the pentosylamines, together with the evidence of the 2:4-dinitrophenylhydrazones, support the view that Y is an Amadori compound with the structure 1-(*o*-carboxyphenylamino)-1-deoxyribulose (1-anthranilino-1-deoxyribulose).

Configuration of compound Y

Attempts were made to assign a configuration to the molecule. Paper chromatography did not differentiate the D and L synthetic compounds, although this was attempted with optically active substances either impregnated on the paper or present in the developing solvent. Attempts to obtain the specific rotation of the 2:4-dinitrophenylhydrazones failed because of the intense colour in pyridine. A study of the optical rotations of the glycosylamines in pyridine showed that the D-pentosylamines were laevorotatory and suggested that the D-Amadori compound would

behave similarly. The optical rotation of an ethyl acetate concentrate of Y was *laevo*. This indirect evidence is not considered sufficient to assign the D configuration definitely to 1-(*o*-carboxyphenylamino)-1-deoxyribulose.

Formation of compound Y by *Escherichia coli*

Towards the conclusion of this work a mutant strain (R19-33) of *E. coli* was found to accumulate a compound shown by ultraviolet-absorption spectroscopy, colour reactions and R_f values to be identical with the 1-(*o*-carboxyphenylamino)-1-deoxyribulose accumulated by the *A. aerogenes* strains.

DISCUSSION

Yanofsky (1956) has postulated 1-(*o*-carboxyphenylamino)-1-deoxyribulose as an intermediate in tryptophan biosynthesis, suggesting that the phosphorylated compound is involved in the con-

version of anthranilic acid into indolyl 3-glycerol phosphate. At the same time he suggested *N*-*o*-carboxyphenylribosylamine 5-phosphate as the immediate precursor of 1-(*o*-carboxyphenylamino)-1-deoxyribulose 5-phosphate. We have found no evidence to suggest that the compound as accumulated was phosphorylated. It is possible that it is phosphorylated within the cells, since Lingens, Burkhardt & Hellmann (1957) have shown that indolylglycerol phosphate within the cells is excreted as indolylglycerol. Yanofsky (1956) has shown the formation of indolylglycerol phosphate from anthranilic acid by cell-free extracts of *E. coli*. That the ribosylamine was accumulated by N 3-33 but then underwent spontaneous conversion into the Amadori compound is unlikely. It has been shown that under the conditions of accumulation the ribosylamine readily hydrolyses to a diazotizable amine which has an ultraviolet spectrum identical with that of anthranilic acid.

Intermediates in biosynthetic pathways have usually been isolated after the accumulation in culture fluids. The results above would suggest that the use of cell suspensions may be of particular value in the detection and isolation of more labile compounds.

EXPERIMENTAL

All melting points are corrected. Elementary analyses are by The Australian Microanalytical Service, Commonwealth Scientific and Industrial Research Organization, Division of Industrial Chemistry, Melbourne.

Accumulation of compound Y

A light suspension (approx. 4×10^6 cells/ml.) of *A. aerogenes* N3-33 was made in water, from the growth on a heart-infusion-agar slope (Gibson, Jones & Teltscher, 1956) incubated for 16 hr. at 37°. Thirty heart-infusion-agar plates (4 in. diam.) were inoculated by spreading 0.2 ml. of the suspension over the surface of each plate. The inoculated plates were incubated for 16 hr. at 37° and the cells washed off with a total of 5 ml. of 0.9% NaCl soln./plate. Freshly harvested cells were used for each experiment. The cells were centrifuged and washed twice with 0.9% NaCl soln., resuspended in a mixture of substrates (Na_2HPO_4 , 25.6 g.; KH_2PO_4 , 2.72 g.; D-glucose, 36 g.; 0.1 M- MgSO_4 , 2 ml.; water to a total volume of 2 l.); the turbidity was measured with an EEL portable colorimeter (625 m μ filter, galvanometer-scale reading 17-22, with $\frac{1}{2}$ in. diam. tubes). The mixture was incubated for 24 hr. at 37° with vigorous aeration through a sintered-glass disk. Cells were then removed by centrifuging the accumulation mixture in a Sharples supercentrifuge to give a clear supernatant.

Attempted isolation of compound Y

Extraction. The supernatant (approx. 1.8 l.) was acidified with conc. HCl (18 ml.) and was extracted by shaking with an equal volume of ethyl acetate. The aqueous layer was discarded and the strongly fluorescent organic layer dried over anhydrous Na_2SO_4 (overnight). After filtration the

ethyl acetate extract was concentrated about tenfold by distillation of the solvent at about 60 mm. Hg. Such concentrates were somewhat yellow in colour and were the stock form in which Y was stored in the refrigerator.

Further procedure. Most of the remaining ethyl acetate was removed by continuing the distillation at reduced pressure. Usually a rather waxy material would separate during concentration, especially if the concentrate was chilled. Any such precipitate was filtered off and discarded. As concentration proceeded the colour deepened until finally a dark-red oil was obtained. This residue would not crystallize, even after prolonged storage in the 'deep-freeze', but degenerated to a dark semi-solid mass. The original oil rapidly decolorized methylene blue and gave an intense red with 2:3:5-triphenyltetrazolium chloride at room temperature (20-25°). It produced an oily red precipitate with 2:4-dinitrophenylhydrazine reagent (0.5% in 2N-HCl).

Alternatively the ethyl acetate concentrate was extracted with an equal volume of aqueous 0.1 M-phosphate buffer (Na_2HPO_4 - KH_2PO_4 ; pH 7.7) to remove Y quantitatively. Compound Y could be concentrated by reducing the quantity of buffer used in the extraction. The buffer extract was acidified (0.1 ml. of conc. HCl/10 ml. of buffer) and extracted with an equal volume of ethyl acetate. If the organic solvent was removed at reduced pressure the product was a red oil with similar properties to the oil isolated from the primary ethyl acetate extract. It was found that Y could best be precipitated as its 2:4-dinitrophenylhydrazone by the addition of 2:4-dinitrophenylhydrazine reagent to the buffer extracts (see below).

Paper chromatography

Paper chromatograms (Whatman no. 1 or no. 3 paper) were developed with ascending solvent. Because of slight day-to-day variation in the R_F of a given compound it was thought advisable to rely on comparison of R_F values with a reference compound on each sheet. The reference compound was usually Y, applied as the stock ethyl acetate concentrate; however, anthranilic acid and the common sugars were sometimes used, depending on the reason for running the chromatogram. For the chromatography of the 2:4-dinitrophenylhydrazones buffered paper was used to prevent 'tailing'. The paper was prepared by drawing the sheet through 0.1 M-phosphate buffer (pH 7.7), blotting to absorb the surplus and then allowing to dry at room temperature until slightly moist.

Solvent systems. The principal system used was methanol-butan-1-ol-benzene-water (2:1:1:1, by vol.; Mason & Berg, 1951). If two compounds appeared to have an identical R_F this was confirmed with the organic layer from butan-1-ol-acetic acid-water (5:4:1, by vol.). Butan-1-ol-propan-1-ol-water (1:2:1, by vol.) made 0.005N with respect to ammonia (Partridge, Bonner & Yanofsky, 1952), and also butan-1-ol saturated with water, were used as solvents in some experiments. For development of 2:4-dinitrophenylhydrazones aqueous 83% ethanol was used (Kun & Garcia-Hernandez, 1957).

Detection of compounds. All the compounds of interest fluoresced in ultraviolet light and this property was used for their detection. In seeking a guide to chemical structure, colour reactions were carried out on the paper and the colour reactions of Y were compared with synthetic compounds (Table 1). Reagents were prepared as described by

the original authors but when heating was required to develop the colour it was more convenient to use an oven at 90–100° rather than to vary the temperature from test to test. These deviations in technique appeared only to alter the time required for development. Use was made of the strong reducing power of Amadori compounds to detect them on paper. The alkaline 2:3:5-triphenyltetrazolium chloride reagent of Trevelyan, Procter & Harrison (1950) was used. An intense red was instantaneously produced at room temperature. The common reducing sugars gave a similar colour but the development at room temperature was slow. It was found that the colour reactions were centred on the Y or synthetic Amadori compound spot detected in ultraviolet light and that sensitivity, as measured by area, was less than that observed with ultraviolet light. The fluorescence of the Amadori compounds was so intense that as chromatograms were developed it was often possible to follow migration in daylight.

Comparison of known compounds with compound Y. For this purpose pairs of Y spots were alternated with pairs of known compound along the origin. After development, vertical strips were cut so as to include a Y and known spot. A comparison of R_F was then made under ultraviolet light and a colour reaction carried out. Because spots were compared under identical conditions the results were comparable irrespective of minor variations in technique. All the colour reactions were reproducible, with the exception of the test with anthrone reagent, which sometimes gave a grey-yellow reaction and at other times a purple with Y and the synthetic pentose Amadori compounds. The hexose Amadori compound always gave a bright yellow with this reagent. The most characteristic apart from the 2:3:5-triphenyltetrazolium chloride reaction was that with the phloroglucinol reagent of Borenfreund & Dische (1957). For colour reactions see Table 1.

R_F values. It was impossible to differentiate 1-(*o*-carboxyphenylamino)-1-deoxy-D-ribose from the L-compound by R_F or colour reaction. Although 1-(*o*-carboxyphenylamino)-1-deoxy-D-xylulose has only a slightly lower R_F than the ribulose analogue (Table 2) the two were readily differentiated. The difference was clearly seen when a series of sheets were chromatographed with alternate spot pairs of the two compounds.

Performance. In general, development with the solvents listed above gave well-defined spots; some tailing could be observed under ultraviolet light but was not usually detected with chemical reagents. The glycosylamines were difficult to chromatograph because of the tendency to hydrolyse to anthranilic acid and sugar. It cannot be said with certainty that a spot attributable to a glycosylamine was observed in this work.

Syntheses

Glycosylamines of anthranilic acid. In general, the preparations of the glycosylamines were based on the methods of Berger & Lee (1946). No difficulty was found in obtaining good yields under moderate conditions and without the addition of catalysts. Since maximum yields were not the primary concern, preparations were not exhaustively treated. It was found difficult to recrystallize the products and thorough washing with water and ethanol gave crystals at least the equal of recrystallized material (as indicated by melting point and elementary analysis). At all stages care was taken to exclude foreign matter.

N-o-Carboxyphenyl-D-arabinosylamine. Anthranilic acid (5.9 g.) in absolute ethanol (50 ml.) was added to D-arabinose (5.0 g.) and refluxed on a water bath. The sugar rapidly dissolved and after 5 min. the reaction mixture solidified. After cooling, the crystalline mass was filtered off, washed with ethanol (approx. 20 ml.), water (approx. 20 ml.) and a further few millilitres of ethanol. Drying over silica gel at room temperature and under vacuum gave a mass of fine white needles (9 g., 100% yield on D-arabinose), m.p. 167–168° (decomp.); $[\alpha]_D^{25} - 62^\circ$ in pyridine (c, 1.25, rotation recorded within 15 min. of solution), -48° (48 hr.) (Found: C, 53.7; H, 5.8; O, 36.0; N, 5.1. $C_{12}H_{15}O_6N$ requires C, 53.5; H, 5.6; O, 35.7; N, 5.2%).

N-o-Carboxyphenyl-L-arabinosylamine. This compound was prepared in the same manner as the D-arabinosylamine, but also as follows. L-Arabinose (0.2 g.) in water (1 ml.) was added to anthranilic acid (0.22 g.) in ethanol (2 ml.) and the mixture shaken at room temperature (22°). After 25 min. crystals began to separate, giving a semi-solid mass at 30 min. After removal from the shaker at 45 min., the product was left at room temperature overnight. After washing with ethanol (15 ml.), water (15 ml.) and a further few millilitres of ethanol the product was sucked dry and drying completed at room temperature over silica gel under vacuum. This treatment yielded fine white needles (0.31 g., 86.4% on L-arabinose), m.p. 161° (decomp.); $[\alpha]_D^{25} + 61.2^\circ$ in pyridine (c, 1.25, rotation recorded within 15 min. of solution), $+52.3^\circ$ (24 hr.), $+48.5^\circ$ (69 hr.) (Found: C, 53.4; H, 5.8; O, 35.8; N, 5.13. $C_{12}H_{15}O_6N$ requires C, 53.5; H, 5.6; O, 35.7; N, 5.2%).

N-o-Carboxyphenyl-D-ribosylamine. Anthranilic acid (0.2 g.) was stirred into a solution of D-ribose (0.2 g.) in 95% ethanol (2.7 ml.). The anthranilic acid rapidly dissolved and the mixture was allowed to stand at room temperature (20°) for 3 hr. and then overnight at 4°. During the next day the mixture became semi-solid but was kept at 4° for a further 3 days. The product was filtered off, washed with ethanol and then water, and sucked dry and finally dried at room temperature over silica gel under vacuum to give a mass of white needles (0.3 g., 81% on D-ribose), m.p. 121° (decomp.); $[\alpha]_D^{25} - 58.2^\circ$ in pyridine (c, 1.25; rotation recorded within 15 min. of solution), $+2.6^\circ$ (48 hr.) (Found: C, 52.2; H, 5.9; O, 37.4; N, 5.0. $C_{12}H_{15}O_6N$ requires C, 51.8; H, 5.8; O, 37.4; N, 5.0%).

N-o-Carboxyphenyl-D-xylosylamine. D-Xylose (0.21 g.) in 95% ethanol (1 ml.) was added to anthranilic acid (0.22 g.) in ethanol (2 ml.) and the mixture shaken for 15 min. at room temperature, by which time a mass of white crystals had separated. After standing overnight at room temperature the crystals were filtered off, washed with ethanol (30 ml.), water (30 ml.) and a further few millilitres of ethanol, and sucked dry and then dried at room temperature over silica gel under vacuum to give white needles (0.28 g., 78% on D-xylose), m.p. 167° (decomp.); $[\alpha]_D^{25} - 42.6^\circ$ in pyridine (c, 1.25, rotation recorded within 15 min. of solution), -23.4° (48 hr.) (Found: C, 53.2; H, 5.6; O, 35.5; N, 5.2. $C_{12}H_{15}O_6N$ requires C, 53.5; H, 5.6; O, 35.7; N, 5.2%).

N-o-Carboxyphenyl-D-glucosylamine. D-Glucose (5 g.) was added to anthranilic acid (5.7 g.) in ethanol (50 ml.) and the mixture refluxed on a water bath until slightly red in colour (15 min.). Some of the sugar was undissolved, but the bulk of the product was decanted and stored in the

refrigerator. Over a period of about 3 weeks the mixture became plastic and then partially solidified. The mass was filtered off and triturated with water, ethanol, water (each 100 ml.) and finally washed with a little ethanol. After suction the product was dried at room temperature over silica gel under vacuum to give white crystals (3.4 g., 39% on D-glucose), m.p. 137–138° (decomp.); $[\alpha]_D^{25} + 102.6^\circ$ in pyridine (*c*, 1.25, rotation recorded within 15 min. of solution), +81.9 (48 hr.) (Found: C, 49.2; H, 6.1; O, 40.5; N, 4.6. $C_{13}H_{17}O_7N \cdot H_2O$ requires C, 49.2; H, 6.0; O, 40.4; N, 4.4%).

Rearrangement of the glycosylamines to Amadori compounds. This was accomplished by refluxing the glycosylamines in *NN*-dimethylformamide.

Glycosylamine (0.5 g.) in *NN*-dimethylformamide (2 ml.) was refluxed for about 5 min. During this time the mixture passed from colourless through yellow to deep red, at which stage the reaction mixture was cooled and poured into a test tube. Any remaining glycosylamine was decomposed by adding a drop of conc. HCl to the product and allowing the mixture to stand for several hours at room temperature. These products were used directly for paper-chromatographic separation of the Amadori compound. Chromatography indicated that the reaction products were anthranilic acid, the Amadori compound, the free sugar and at least two coloured compounds, one travelling at the front (methanol–butan-1-ol–benzene–water) and the other between anthranilic acid and the Amadori compound.

Properties of the glycosylamines. Solutions of the glycosylamines in pyridine, or *NN*-dimethylformamide, did not decolorize alkaline methylene blue or produce a colour with alkaline 2:3:5-triphenyltetrazolium chloride at room temperature. All the compounds showed a slow mutarotation in pyridine (not specially dried), but this was not followed to the equilibrium value. The specific rotation of *N*-*o*-carboxyphenyl-D-glucosylamine, H_2O was found to be of similar order but of opposite sign to that reported by Sannié (1948) in pyridine. Our material melted at 137–138° and that of Sannié at 125–127°. Inoue, Onodera & Kitaoka (1951–52) report a compound with m.p. 137–138°, $[\alpha]_D + 68$ in ethanol–water. The ultraviolet-absorption spectra (Table 3, Fig. 1) of the unrecrystallized but reasonably pure glycosylamines (see elementary analyses) showed no significant difference between the pentose and hexose compounds. Hydrolysis to anthranilic acid appeared to proceed rapidly at strongly acid pH. An experiment to determine the stability of the ribosylamine (5 mM in 0.067M-phosphate buffer) at pH 8.04, 6.98, 5.91 and 5.29 at 37° indicated rapid hydrolysis at the lowest pH and about 50% hydrolysis, after 2½ hr., at pH 8.04. The rate of hydrolysis increased with decrease in pH. Hydrolysis was followed by observing the hypsochromic shifts of the bands of maximum absorption (247 → 240 mμ, 316 → 310 mμ; 1 in 40 dilution measured in 0.1M-phosphate buffer, pH 7.7) and the measurement of diazotizable amine by the method of Bratton & Marshall (1939). On the basis of a standard curve for anthranilic acid, the colour values indicated complete hydrolysis within 20 hr. at all pH values. The experiment indicated that if *N*-*o*-carboxyphenyl-D-ribosylamine was accumulated in substrates at approx. pH 7 it would finally be detected as anthranilic acid.

Properties of the Amadori compounds. The Amadori compounds were easily detected on paper chromatograms by the intense blue–violet fluorescence in ultraviolet light and the

immediate formation of intense red when sprayed with 2:3:5-triphenyltetrazolium chloride (0.5% in $CHCl_3$) followed by ethanolic NaOH soln. Heating was unnecessary. Reaction products from the rearrangement of the glycosylamines rapidly decolorized alkaline methylene blue at room temperature (20–25°), and this property was evident in increasing degree from the time the rearrangement mixture refluxed. No extensive attempt was made to isolate the pure Amadori compounds other than on paper chromatograms. A dark-red oil was precipitated from rearrangements in pyridine or pyridine–ethyl malonate by addition of light petroleum (b.p. 40–60°). Such oils were very similar in behaviour to the oils obtained by purifying the natural product. None of these oils could be induced to crystallize. A 2:4-dinitrophenylhydrazone was prepared (see below) but was difficult to purify. Details of the ultraviolet-absorption spectra of the Amadori compounds is discussed elsewhere in this paper. The data obtained in a number of solvents and pH values are presented in Table 4 and Fig. 2. In contrast with the glycosylamines, the pentose and hexose compounds could be differentiated by their spectral behaviour and were less stable at alkaline pH than at acid. It was concluded that in alkali enolization occurred, followed by hydrolytic cleavage of the molecule. There was evidence from the spectral changes that anthranilic acid was one product of cleavage. From paper chromatograms the eluting power of solvents was in the descending order: 0.1M-phosphate buffer (pH 7.7), ethanol, ethyl acetate.

Preparation of 2:4-dinitrophenylhydrazones

From naturally accumulated compound Y. The Y spot on paper chromatograms reacted fairly slowly with 2:4-dinitrophenylhydrazine reagent to give a deep orange and suggested the possibility of isolating a hydrazone from a bulk source. If reagent (0.5% 2:4-dinitrophenylhydrazine in 2N-HCl) was added to a solvent-free Y concentrate a viscous red oil and some amorphous orange crystals separated. Efforts to recrystallize this product failed. As described above, Y was easily obtained as a solution in 0.1M-phosphate buffer (pH 7.7), and this was found to be the best medium from which to precipitate the 2:4-dinitrophenylhydrazone. An equal volume of 0.5% 2:4-dinitrophenylhydrazine in 2N-HCl was added to the buffer extract. The mixture rapidly became turbid and after about 1 hr. an amorphous, orange–red ppt. began to separate. After standing at room temperature overnight, the product was collected and dried (1–3 g.; m.p. 100–200°). Recrystallization of the crude product was difficult, probably because of interaction with the recrystallizing solvent. It was possible to recrystallize from ethanol with large losses of material, but subsequent concentration of the mother liquor yielded amorphous yellow crystals. These were washed with ethanol, sucked dry and finally dried over silica gel under vacuum (m.p. 213–215° decomp.). An elementary analysis was consistent with the product (VIIa) being the mono-2:4-dinitrophenylhydrazone of a 1-(*o*-carboxyphenylamino)-1-deoxypentulose (Found: C, 48.0; H, 4.4; O, 32.0; N, 15.2. $C_{18}H_{19}O_8N_5$ requires C, 48.1; H, 4.2; O, 32.0; N, 15.6%).

*From a synthetic source of 1-(*o*-carboxyphenylamino)-1-deoxy-D-ribulose.* An Amadori-rearrangement reaction (see above) was carried out on *N*-*o*-carboxyphenyl-D-arabino-sylamine and excess of 2:4-dinitrophenylhydrazone reagent

(0.5% 2:4-dinitrophenylhydrazine in 2N-HCl) was added to the products. The amorphous orange-red solid formed was washed with acetone and recrystallized from acetone to give yellow-orange needles 'blushing' to orange-red, m.p. 210–211° (decomp.) (Found: C, 47.9; H, 4.5; O, 32.2; N, 14.6%). Ethanol was added till nearly all the crystals were dissolved at the boiling point and the mixture was filtered whilst hot. The clear filtrate gave a few orange-yellow crystals (m.p. 211°, decomp.) after 2 days in the deep freeze. Addition of a little water to the mother liquor caused long needle-like crystals to form in association with some amorphous material. This material, after drying over silica gel under vacuum, had m.p. 214° (decomp.) (VIIb) (Found: C, 47.6; H, 4.5; O, 32.5; N, 14.9. $C_{18}H_{19}O_9N_5$ requires C, 48.1; H, 4.2; O, 32.0; N, 15.6%). VIIa ('natural') and VIIb ('synthetic') gave no observable depression in mixed melting point although the analysis of the 'synthetic' material did not fit the expected structure as well as that of the 'natural' product. The ultraviolet-absorption spectra of (VIIa) and (VIIb) were very similar in phosphate buffer (pH 7.7). On adjustment to pH < 2 with conc. HCl the immediate and long-term changes were identical (Fig. 3). Paper chromatograms of (VIIa) and (VIIb) developed with aqueous 83% ethanol gave spots of identical R_F (0.58; solvent front travelled 19.4–20.4 cm.).

Ultraviolet-absorption spectroscopy

Spectra were obtained with a Unicam model SP. 500 spectrophotometer and 1 cm. silica cells. Solvents were commercial absolute ethanol, ethyl acetate (analytical reagent) and 0.1 M-phosphate buffer (pH 7.7) prepared from Na_2HPO_4 and KH_2PO_4 of analytical grade. Experimental samples were read against solvent from the same source as used to prepare the sample. Extinction values were recorded at 5 m μ intervals, except at maxima and some minima, when the interval was usually 2 m μ , but sometimes 1 m μ . Because the pH of experimental samples was often varied just before recording the spectrum, it was standard practice to record from short to long wavelengths. When available as crystals the experimental samples were weighed, made up and diluted in solvent to a concentration of 0.1 mm. With ethyl acetate it was necessary to make the primary solution in ethanol and then to add a portion to ethyl acetate to give a final concentration of 0.1 mm in ethyl acetate-ethanol (98:2, v/v). A solvent blank with these proportions was used. With the Amadori compounds crystals were not available and all spectra were determined on eluates from paper chromatograms. Eluates were adjusted to give an extinction of approx. 0.9 at 250 m μ . With ethyl acetate eluates the extinction was often below this value even without dilution, in which case the values could not be adjusted. After reading spectra in buffer at pH 7.7 it was usual to adjust the pH to ≤ 2 by adding a drop of conc. HCl and then to record the spectrum again. Similarly, adjustments were made, to pH ≥ 12 , with 10N-NaOH, a fresh portion of the sample at pH 7.7 being used. The dilution effected by these additions (< 1%) was ignored. pH measurements were made with a Jones model B pH electrometer. It was particularly noticeable with the 2:4-dinitrophenylhydrazones derivatives that after acidification the spectrum was influenced by the time taken and the direction of recording. However, the behaviour was characteristic in itself and seen with derivatives from both natural and synthetic sources.

SUMMARY

1. A new compound was found to be accumulated by washed cell suspensions of mutant strains of *Aerobacter aerogenes* (N3-33, N2-18) and *Escherichia coli* (R19-33) requiring tryptophan or indole for growth. This compound has been identified as 1-(o-carboxyphenylamino)-1-deoxyribulose and may be the immediate precursor of indolyl-glycerol phosphate in tryptophan synthesis.

2. Colour reactions of the natural compound (Y), isolated on paper chromatograms, indicated the presence of a sugar fragment, probably a pentulose. Reducing properties suggested an Amadori compound.

3. A number of glycosylamines of anthranilic acid were synthesized and used as model compounds for ultraviolet-absorption spectrophotometry and as parent compounds in Amadori-rearrangement reactions.

4. The spectral and other characteristics of the Amadori-rearrangement products were determined with material from paper chromatograms.

5. A comparison of the properties of the synthetic Amadori compounds with those of compound Y indicated that the latter was identical with 1-(o-carboxyphenylamino)-1-deoxyribulose. The differentiation from 1-(o-carboxyphenylamino)-1-deoxy-D-fructose was on the basis of colour reaction, R_F and stability at alkaline pH. A xylulose structure was rejected on grounds of R_F .

6. The preparation of an identical 2:4-dinitrophenylhydrazone derivative from compound Y and from synthetic 1-(o-carboxyphenylamino)-1-deoxyribulose confirmed the pentose Amadori structure and the identity of compound Y.

We are grateful to Mr G. Morrison for technical assistance, to Mrs B. Segall for her assistance in some of the early experiments and to Dr T. M. Reynolds for suggestions for the preparation and detection of Amadori compounds. Our thanks are due to Professor S. D. Rubbo for his encouragement of this work and to the Australian National Health & Medical Research Council for a grant.

REFERENCES

- Aronoff, S. (1956). *Techniques of Radiobiochemistry*. Ames, Iowa: Iowa State College Press.
- Aronoff, S. & Vernon, L. (1950). *Arch. Biochem. Biophys.* **28**, 424.
- Berger, L. & Lee, J. (1946). *J. org. Chem.* **11**, 75.
- Borenfreund, E. & Dische, Z. (1957). *Arch. Biochem. Biophys.* **67**, 239.
- Bratton, A. C. & Marshall, E. K. (1939). *J. biol. Chem.* **128**, 537.
- Davis, B. D. & Mingioli, E. S. (1950). *J. Bact.* **60**, 17.
- Gibson, F., Doy, C. H. & Segall, S. B. (1958). *Nature, Lond.*, **181**, 549.
- Gibson, F., Jones, M. J. & Teltscher, H. (1956). *Biochem. J.* **64**, 132.

- Hodge, J. E. (1955). *Advanc. Carbohydr. Chem.* **10**, 169.
- Inoue, Y., Onodera, K. & Kitaoka, S. (1951-52). *J. Agr. chem. Soc. Japan*, **25**, 291. Cited by Ellis, G. P. & Honeyman, J. (1955). *Advanc. Carbohydr. Chem.* **10**, 139.
- Johanson, R. (1953). *Nature, Lond.*, **172**, 956.
- Kun, E. & Garcia-Hernandez, M. (1957). *Biochim. biophys. Acta*, **23**, 181.
- Lingens, F., Burkhardt, H. J. & Hellmann, H. (1957). *Z. Naturf.* **12b**, 493.
- Mason, M. & Berg, C. P. (1951). *J. biol. Chem.* **188**, 783.
- Partridge, C. W. H., Bonner, D. M. & Yanofsky, C. (1952). *J. biol. Chem.* **194**, 269.
- Sannié, C. (1948). *C.R. Acad. Sci., Paris*, **226**, 182. Cited by Ellis, G. P. & Honeyman, J. (1955). *Advanc. Carbohydr. Chem.* **10**, 139.
- Tracey, M. V. (1950). *Biochem. J.* **47**, 433.
- Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
- Yanofsky, C. (1956). *J. biol. Chem.* **223**, 171.

Intermolecular and Intramolecular Reactions of Human-Serum Albumin after its X-Irradiation in Aqueous Solution

By D. ROSEN

Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London, S.W. 3

(Received 15 December 1958)

The work detailed below deals with changes found in the ultraviolet-absorption spectra of aqueous solutions of human-serum albumin after X-irradiation at concentrations where the action of the radiation has been shown to be entirely indirect (Rosen, Alexander & Brohult, 1957). This matter has been variously studied in the past 30 years (Spiegel-Adolf & Krumpel, 1927, 1929; review by Arnow, 1936; Barron & Finkelstein, 1952). The purpose of the present paper is threefold: to demonstrate the important part in the absorption spectra of irradiated solutions played by light scattered from aggregated protein; to show how pH affects the aggregation and consequent light-scattering; and from these results to show how irradiation produces both intermolecular and intramolecular effects on the protein, in a proportion varying with circumstances. Although it is known that serum albumin and other proteins in solution are aggregated by ionizing radiation (Carroll, Mitchell & Callanan, 1952; Barron & Finkelstein, 1952; Rosen *et al.* 1957), the fact that this leads to a greatly enhanced scattering of light has been mentioned only by Passynskii (1955) and by Pavlovskaya & Passynskii (1957). It has been considered, however, in a study of ultraviolet irradiation of proteins (Claesson, 1956).

MATERIALS AND METHODS

Chemical reagents. Freeze-dried human-serum albumin was obtained by courtesy of Dr M. E. Mackay from a pharmaceutical preparation made at the Lister Institute, London; it was pure on examination by electrophoresis and by ultracentrifuge. Water used was redistilled in an all-glass apparatus. Other reagents were of the highest purity available commercially. All glassware before use was

washed in chromic acid, very thoroughly rinsed with tap water and distilled water and then oven-dried. All solutions were prepared on the day of use and were irradiated while open to the air. The concentrations indicated below are with respect to the protein as supplied. The moisture content of the material was 6-8%, but absolute determinations of this figure and of the final concentrations of protein were not attempted.

Radiation source and its calibration. The X-ray machine used was a Newton KX 10 run at 140 kv and 5 ma with no added filtration (half-value layer 2.86 mm. aluminium), run to give a dose rate between 3000 and 3500 rads/min. (The rad is the unit of absorbed dose, 100 ergs/g.) The output of the machine was calibrated chemically, by the oxidation of ferrous sulphate solution, with the solution occupying the same position and volume as the protein solution irradiated later. The *G* value (number of molecules changed per 100 ev of energy deposited by the radiation in the solution) was taken as 15.6 (Lefort, 1958).

Spectrophotometry and light-scattering. Absorption spectra were measured on a Unicam SP. 500 spectrophotometer in 1 cm. quartz cells. The relation between the weight-average molecular weight, M_w , and the light-scattering effect in the transmitted light is given by a modification, due to Debye and Cachin, of the standard Rayleigh formula (see Stacey, 1956). At a concentration c (g./ml.), the turbidity τ (that part of the absorption coefficient due solely to light-scattering) is given by

$$\frac{H_0}{\tau} = \frac{1}{M_w} \cdot \frac{1}{T},$$

where

$$H = \frac{32\pi^2 n_0^2 (dn/dc)^2}{3\lambda^4 N}$$

and

$$\frac{1}{T} = 1 + \frac{16\pi^2 r^2 n^2 n_0^2}{3\lambda^4}.$$

Here n_0 and n are the refractive indices of the solvent and solution, N is Avogadro's number, λ is the wavelength (in vacuo) of the light in which measurement is being made, and r is the radius of gyration of a molecule of the solute.