*STUDIES ON THE CONFORMATION OF HUMAN SERUM HIGH-DENSITY LIPOPROTEINS HDL_2 AND HDL_3**

BY A. SCANU†

DEPARTMENT OF MEDICINE, UNIVERSITY OF CHICAGO

Communicated by Leon O. Jacobson, October 15, 1965

Studies from this laboratory[1] and others[2, 3] have indicated that the HDL from human serum can be degraded by Et_2O-ETOH to yield a water-soluble apoprotein containing about 1 per cent lipid and 3.3 per cent carbohydrate by weight.[4] This apoprotein is made of subunits[3, 5, 6] of a minimum average weight of 21,500[6] and can undergo relipidation *in vitro*[7] and *in vivo*.[8]

The structural characteristics of human serum HDL are not known. The possibility of preparing HDL in various states of lipidation offered an opportunity for determining the effect of the lipid on the conformation of the apoprotein. Thus, studies were conducted on the human serum HDL subclasses—HDL_2 and HDL_3—before and after delipidation using the techniques of optical rotatory dispersion and viscometry. The results are the subject of this report.

Materials and Methods.—Source of lipoproteins: Serum was separated by centrifugation from fresh, clotted blood obtained from several fasting, healthy, white male, human donors, 20–30 years of age, A, Rh+. EDTA, neutralized to pH 7, was added to the pooled sera to a final concentration of 0.05 gm per 100 ml.

Preparation of lipoproteins: Within 1 hr after collection, the serum was adjusted to *d* 1.063 gm/ml by addition of solid NaCl and spun in a model L Spinco ultracentrifuge, model 30.2 rotor, 79,488 × *g*, 20 hr at 16°. After separation of the top milliliter (chylomicrons and low-density lipoproteins), the tubes were sliced in the middle clear zone, the undernates adjusted to *d* 1.120 with solid NaBr, ultracentrifuged, and the top milliliters ("impure" HDL_2) removed. The undernates were brought to *d* 1.21 gm/ml with solid NaBr, respun, and the top milliliters ("impure" HDL_3) collected. To obtain "pure" HDL_2 and HDL_3, both fractions were refloated twice at *d* 1.21 gm/ml. Separation and purification of HDL subfractions were conducted in a 40.3 rotor, 114,480 × *g*, 16°, 24 hr. Techniques for assessing purity of the products have been reported elsewhere.[7] Prior to analysis, HDL_2 and HDL_3 were dialyzed against several changes of the buffer selected for study (see *Results*).

Delipidation technique: Lipids from either HDL_2 or HDL_3 were extracted by 3:2 EtOH:Et_2O at −10° by a modification of the technique of Scanu *et al.*[1] The protein-to-lipid distribution of the products before and after delipidation is given in Table 1. Treatment of the lipoproteins with Et_2O alone according to Avigan[2] promoted the release of all triglyceride, 25% cholesterol, and no phospholipids.

Optical rotatory dispersion studies were conducted in a Cary model 60 spectropolarimeter adjusted at 27.5°. In the spectral zone between 600 and 300 mμ a 0.2-cm (thickness of the solution layer) cell was used with protein concentrations between 2 and 4 mg/ml. Within this range, the

TABLE 1

PER CENT LIPID PROTEIN DISTRIBUTION OF HDL_2 AND HDL_3 BEFORE AND AFTER DELIPIDATION

	HDL_2		HDL_3	
	Before	After	Before	After
Total protein*	42.0	98.8	57.0	98.8
Total cholesterol†	27.0	—	21.0	—
Phospholipids‡	26.5	1.2	17.0	1.2
Triglycerides§	4.5	—	5.0	—

* Lowry, O. H., *et al.*[9]
† Abell, L. L., *et al.*[10]
‡ Stewart, C. P., and E. B. Hendry.[11]
§ Van Handel, E., and D. B. Zilversmit.[12]

values of rotation were concentration-independent. For the far ultraviolet, 5-mm and 1-mm cells were used with protein concentrations of 20–40 μg/ml. For other details see *Results*.

Viscometric studies were carried out in an Ostwald capillary viscometer having a flow time for water of 185 sec. Each protein was studied at three different dilutions to permit extrapolation to zero concentration. Densities were determined by pycnometry. For intrinsic viscosity, the relation $[\eta] = \lim_{c \to 0} \eta_{sp}/c$ was used. The values were obtained from the intercept at the ordinate of the plot η_{sp} versus c.

Reagents: Solvents were freshly distilled before use. Reagent-grade urea and guanidine hydrochloride were recrystallized from ethanol and absolute methanol, respectively.

Results.—(A) *Studies on HDL$_2$ and αP$_2$:* (1) *Analysis of optical rotatory dispersion data: Simple Drude equation:* The plot of $[\alpha]_\lambda\lambda^2$ versus $[\alpha]_\lambda$ according to Yang and Doty[13] gave, between 600 and 300 mμ, a complex dispersion curve for both HDL$_2$ and αP$_2$ (Fig. 1). The value of λ_c, estimated from the slope of the straight line between 390 and 310 mμ, was 289 and 281. The figure for HDL$_2$ treated with Et$_2$O was the same as for the native lipoprotein.

Modified two-term Drude equation: Rotatory data above 280 mμ fitted the equation proposed by Shechter and Blout:[14]

$$[R'] = \frac{A_{193}\lambda^2_{193}}{\lambda^2 - \lambda^2_{193}} + \frac{A_{225}\lambda^2_{225}}{\lambda^2 - \lambda^2_{225}}.$$

The parameters A_{193} and A_{225} were obtained by plotting $[R'](\lambda^2 - \lambda^2_{193})/\lambda^2_{193}$ against $\lambda^2_{225}/(\lambda^2 - \lambda^2_{225})$ (see Fig. 2). The line fitted that reported by Schechter and Blout[15] for polypeptides and fibrous proteins in water. αP$_2$ had values of A_{193} and A_{225} slightly lower than those of HDL$_2$ and αP$_2$ (Table 2). The results of HDL$_2$ treated with ether were the same as HDL$_2$ untreated.

Moffitt equation: For rotations at wavelength greater than 300 mμ, the Moffitt-Yang[16] equation

$$[R'] = \frac{a_0\lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2 - \lambda_0^2)^2}$$

was used based on $\lambda_0 = 212$. In the calculation of the reduced mean residue rotation, $[R']$, the value 118 for the mean residue weight was calculated from the amino acid composition.[6] The observed values of $[\alpha]_\lambda$ were corrected for the refractive index of the solvent according to the tables of Fasman[17] or by the Sellmeier formula $n^2 = 1 + [(a\lambda^2)/(\lambda^2 - \lambda_v^2)]$. The values of a_0 and b_0 were calculated from the slope and intercept of the $[R'](\lambda^2 - \lambda_0^2)$ against $1/(\lambda^2 - \lambda_0^2)$ plot (Fig. 3). As shown in Table 1, αP$_2$ had b_0 values slightly lower than those of its parent lipoprotein, either native or after treatment with Et$_2$O.

Dispersion in the far-ultraviolet (Fig. 4): Both HDL$_2$ and αP$_2$ showed a trough at 233 mμ (negative Cotton effect), a crossover point at 225 mμ, a shoulder between 215 and 220 mμ, and a peak at 198 mμ (positive Cotton effect). HDL$_2$ treated with ether had the dispersion curve of untreated HDL$_2$.

Effects of the solvent medium: Urea and guanidine·HCl affected the optical rotatory parameters of αP$_2$ more markedly than HDL$_2$ (Table 3). Solvents shown to produce depolymerization of αP[3, 5, 6] were also investigated. Prior to analysis, either HDL$_2$ or αP$_2$ was extensively dialyzed at 26° against: (*a*) glycine buffer pH 10.5, ionic strength 0.1, (*b*) acetate buffer pH 3, ionic strength 0.1, (*c*) phosphate buffer pH 8.6, ionic strength 0.1 made 0.05 *M* in respect to SDS. Based on the

TABLE 2

OPTICAL ROTATORY PARAMETERS AND INTRINSIC VISCOSITY OF HDL_2 AND HDL_3 BEFORE AND AFTER DELIPIDATION

Material	Solvent	Simple Drude equation	Modified Two-Term Drude Equation		Moffitt Equation		Far Ultraviolet			Intrinsic viscosity
		λ_c	A_{198}	A_{225}	a_0	b_0	$[R']_{233}$	$[R']_{215}$	$[R']_{198}$	$[\eta]$
HDL_2	Phosphate buffer pH 8.6, 1 = 0.1	289	+2196	−1623	−18	−514	−9000 ± 300	+20000 ± 1000	+65000 ± 5000	0.032
HDL_2(E)†	"	286	+2190	−1618	−18	−512	−8900 ± 350	+19800 ± 1000	+60000 ± 5500	0.045
αP_2	"	281	+1928	−1426	−29	−450	−8000 ± 400	+17500 ± 1200	+45000 ± 7500	0.120
HDL_3	"	287	+2192	−1630	−18	−516	−9100 ± 400	+21000 ± 1500	+62000 ± 5600	0.035
HDL_3(E)†	"	282	+2188	−1622	−18	−512	−9000 ± 300	+20000 ± 1000	+60000 ± 5000	0.046
αP_3	"	280	+1948	−1460	−29	−455	−8200 ± 400	+17600 ± 1100	+44000 ± 7000	0.125

* In view of a complex Drude plot, the values were calculated from the straight line in the spectral range between 390 and 310 mμ.
† HDL_2 or HDL_3 treated with diethyl ether.

TABLE 3

OPTICAL ROTATORY PARAMETERS AND INTRINSIC VISCOSITY OF HDL_2 AND αP_2 IN THE PRESENCE AND AFTER REMOVAL OF UREA OR GUANIDINE·HC

N	Solvent medium	HDL_2 λ_c	a_0	b_0	$[R']_{233}$	$[\eta]$	αP_2 λ_c	a_0	b_0	$[R']_{233}$	$[\eta]$
1	Phosphate buffer + urea*	242	−646	−187	−3500	0.16	203	−779	≃0	−2000	0.40
2	(1) After dialysis against phosphate buffer†	291	−46	−500	−8000	0.042	280	−34	−488	−7500	0.19
3	Phosphate buffer + guanidine·HCl‡	240	−720	−200	−2800	0.18	215	−1000	≃0	−2200	0.38
4	(3) After dialysis against phosphate buffer§	289	−45	−507	−8200	0.04	279	−38	−501	−7500	0.19

* Phosphate buffer pH 8.6, ionic strength 0.1, made 8 *M* in respect to urea. The protein solution was allowed to stand for 24 hr at 27° prior to analysis.
† Solution 1 was dialyzed against several changes of phosphate buffer pH 8.6, ionic strength 0.1.
‡ Conditions as in 1, with exception that 8 *M* guanidine HCl, instead of urea, was used.
§ Experimental conditions, same as in 2.

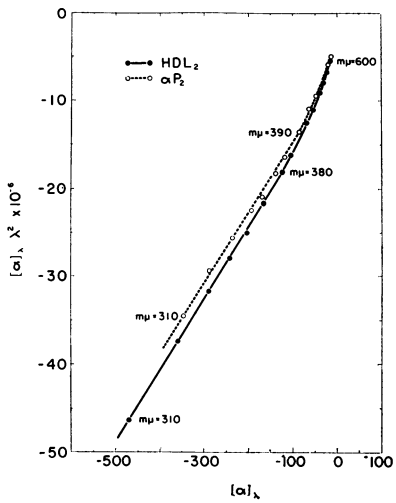

FIG. 1.—Plot of optical rotation according to the simple Drude equation ($\lambda_0 = 212$ mμ).

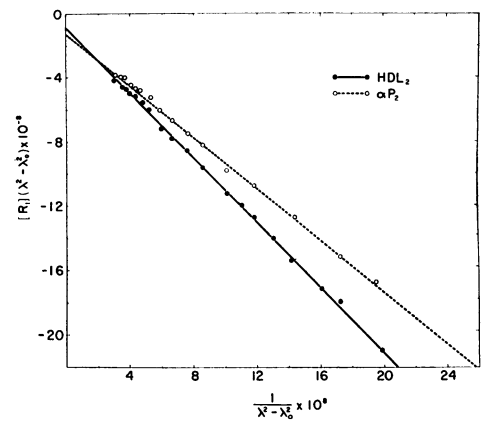

FIG. 3.—Plot of optical rotation according to the Moffitt equation ($\lambda_0 = 212$ mμ).

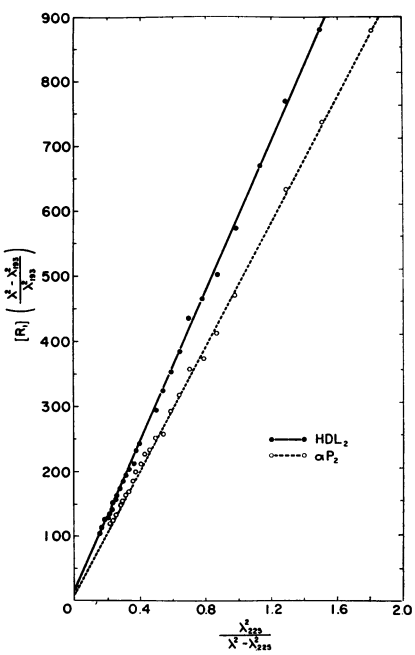

FIG. 2.—Plot of optical rotation according to the "modified" double-Drude equation.

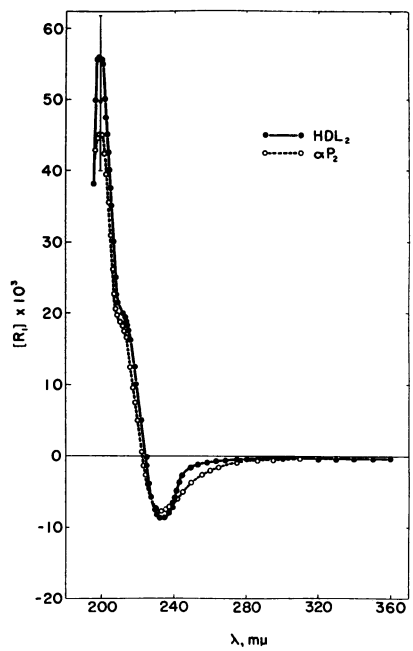

FIG. 4.—Optical rotatory dispersion of HDL_2 and αP_2 plotted as reduced mean residue rotation [R'] against λ(mμ).

values of b_0 and the negative Cotton effect at 233 mμ, the alkaline medium produced little effect on either HDL_2 or αP_2 as compared with the data obtained with phosphate buffer (Table 2). In the acid medium, the values of HDL_2 were $b_0 = -371°$, $[R']_{233} = -4600°$; those of αP_2, $-158°$ and $-3650°$. In the presence of SDS, HDL_2 gave $b_0 = -514$, $[R']_{233} = -7700°$; αP_2, $b_0 = -330°$, $[R']_{233} = -4400°$.

TABLE 4

ESTIMATES OF PER CENT HELIX OF HDL_2, HDL_3, αP_2, AND αP_3

Material	Moffitt Equation, λ_0 = 212 mμ*	Moffitt Equation, λ_0 = 220 mμ†	"Modified" two-term Drude equation‡	Depth of Negative Cotton Effect $[R']_{233}$§	$[R']_{233}$**
HDL_2	64.2	71.0	79.8	71.2	62.0
αP_2	56.0	62.9	71.7	63.5	55.2
HDL_3	64.5	71.2	79.8	71.4	62.7
αP_3	55.5	64.6	71.8	65.0	56.5

* $b_0^{1000} = -700$; $b_0^0 = +100$ (ref. 19).
† $b_0^{100} = -390$; $b_0^0 = +90$ (ref. 20).

‡ $H = \frac{A_{193} - A_{225} + 690}{56.4}$ (ref. 15).

§ −12,600° (ref. 22).
** −14,500 (ref. 18).

Estimation of per cent of helix (Table 4): In view of the uncertainties concerning the calibration of parameters for full helical content in proteins,[18] various estimates were obtained. HDL_2 had a helical content between 60 and 70 per cent and αP_2 between 55 and 65 per cent. Previous data from the amino acid analysis[6] had indicated that the group of amino acid "nonhelix formers" (thr., val., ser., gly., pro., Ile, cys.) represents about 28 per cent of the total 188 residues of αP_2.

(2) *Viscometry:* The low intrinsic viscosity of HDL_2 was modestly affected by treating the lipoprotein with Et_2O, but increased markedly upon lipid extraction by EtOH-Et_2O (Table 2). A further increase of the intrinsic viscosity of αP_2 was observed in the presence of urea or guanidine·HCl (Table 3). The changes were reversed by removing the denaturing agents by dialysis (Table 3).

When urea or guanidine·HCl acted upon HDL_2, the intrinsic viscosity was affected significantly less than with αP_2 (Table 3).

(B) *Studies on HDL_3 and αP_3:* As summarized in Tables 3 and 4, analysis of HDL_3 before and after delipidation by the parameters of optical rotatory dispersion and intrinsic viscosity gave results identical with those of HDL_2. This was also true for the effects of urea, guanidine·HCl, SDS, acid, and alkaline media.

Discussion.—Previous studies on the subclasses of human serum high-density lipoprotein had furnished evidence that HDL_2 has about twice the size and weight of HDL_3[23, 24] and that in the process of $HDL_2 \rightarrow HDL_3$ conversion lipid loss is associated with release of peptide subunits.[24] The present data on optical rotatory dispersion and viscometry now indicate that the apoproteins αP_2 and αP_3 from HDL_2 and HDL_3, respectively, have a high helical content and suggest that in their native state they exist in solution as a mixture of α-helix and random coil. This interpretation is supported by the finding that either HDL_2 or HDL_3 had a complex Drude plot,[17] high λ_c values,[25] high negative values of b_0,[25] high A_{193} and A_{225} parameters,[19] and exhibited positive and negative Cotton effects in the far-ultraviolet with peak maxima in the spectral region reported for α-helical polypeptides and proteins.[22, 26, 27] An exact determination of the helical content of HDL protein was not obtained, because of insufficient theoretical basis for the computation.[17, 18, 25] If one would accept a semiquantitative estimate, the data in Table 4 indicate that 60–70 per cent of the polypeptide chain in either HDL_2 or HDL_3 is folded in a right-handed helix.

The observation that the optical dispersion of HDL_2 and HDL_3 did not markedly differ from that of their delipidated products αP_2 and αP_3 suggests that attachment with lipid does not significantly affect the secondary structure of the HDL apo-

protein. This finding and the knowledge that HDL protein is made of subunits[3, 5, 6] also supports the view[3, 6] that lipids participate in the maintenance of the tertiary structure of the lipoprotein by acting as a bridge between the various protein subunits. The partial shielding effect of lipid on the denaturation of αP by urea, guanidine, SDS, and acid is compatible with this interpretation. HDL subunits have been noted to polymerize readily upon removal of lipids,[6] and this observation may account for the high values of intrinsic viscosity of αP_2 and αP_3 (Table 2).

Urea or guanidine induced profound changes in the intrinsic viscosity and optical rotatory dispersion of αP_2 and αP_3, most probably owing to an increased expansion of these apoproteins consequent to unfolding of the helical structure. The reversibility of this process upon removal of the denaturing agents, an observation analogous with that reported by Harrison and Blout[21] for apomyoglobin, suggests that the secondary conformation of the protein is dependent upon its primary structure.[28] It is interesting to note that in the presence of these denaturing agents, αP retains its capacity to bind lipids.[29]

Summary.—The effect of lipid removal on the conformation of the apoproteins αP_2 and αP_3, of the two high-density lipoprotein subclasses, HDL_2 (*d* 1.063–1.120) and HDL_3 (1.120–1.21), was studied using the techniques of optical rotatory dispersion and viscometry. The data indicated that either in their native state (i.e., in association with lipids) or upon delipidation, αP_2 and αP_3 had a high α-helical content, estimated between 60 to 70 per cent for the native state and 55 to 65 per cent for the delipidated forms. The helical structure, which was disrupted by the action of urea or guanidine·HCl, was to a large extent restored upon removal of the denaturing agents by dialysis. The data were interpreted as consistent with the view that lipids do not participate in the secondary structure HDL apoproteins, but act as bridges between the various apoprotein subunits.

The author wishes to thank Dr. E. Blout, Harvard Medical School, and Dr. G. Smith, Illinois Institute of Technology, for permission to use the Cary model 60 spectropolarimeter in their laboratories. He is also indebted to Dr. E. Blout for helpful advice in the early phase of the experimentation. Mrs. C. Edelstein and Mr. S. Gupta provided excellent technical assistance.

The abbreviations used in the text are HDL, high-density lipoprotein of *d* 1.063–1.21 gm/ml; HDL_2 and HDL_3, the HDL subclasses floating at *d* 1.063–1.120 and 1.120–1.21, respectively; αP, αP_2, and αP_3, apoproteins from HDL, HDL_2, and HDL_3 obtained after treatment with ethanol-diethyl ether; EtOH, ethanol; Et_2O, diethyl ether; SDS, sodium dodecylsulfate.

* Supported by grants HE 08727 from the USPHS, G-64-42 from the Life Insurance Medical Research Fund, and C65-1 from the Chicago and Illinois Heart Association.

† Recipient of Research Career Development Award no. HE-24,867 from the USPHS.

[1] Scanu, A., L. A. Lewis, and M. Bumpus, *Arch. Biochem. Biophys.*, **74**, 390 (1958).

[2] Avigan, J., *J. Biol. Chem.*, **226**, 957 (1957).

[3] Shore, V., and B. Shore, *Biochem. Biophys. Res. Commun.*, **9**, 455 (1962).

[4] Scanu, A., in *Advances in Lipid Research*, ed. R. Paoletti and D. Kritchevsky (New York, N. Y.: Academic Press, 1965), p. 63.

[5] Sanbar, S. S., and P. Alaupovic, *Biochim. Biophys. Acta*, **71**, 235 (1963).

[6] Scanu, A., *J. Lipid Res.*, in press.

[7] Scanu, A., and W. L. Hughes, *J. Biol. Chem.*, **235**, 2876 (1960).

[8] Scanu, A., and W. L. Hughes, *J. Clin. Invest.*, **41**, 1681 (1962).

[9] Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

[10] Abell, L. L., B. B. Levy, B. B. Brodie, and F. E. Kendall, *J. Biol. Chem.*, **195**, 357 (1952).

[11] Stewart, C. P., and E. B. Hendry, *Biochem. J.*, **29**, 1683 (1935).

[12] Van Handel, E., and D. B. Zilversmit, *J. Lab. Clin. Med.*, **50**, 152 (1957).
[13] Yang, J. T., and P. Doty, *J. Am. Chem. Soc.*, **79**, 761 (1957).
[14] Shechter, E., and E. R. Blout, these PROCEEDINGS, **51**, 695 (1964).
[15] *Ibid.*, p. 794.
[16] Moffitt, W., and J. T. Yang, these PROCEEDINGS, **42**, 596 (1956).
[17] Fasman, G. D., in *Methods in Enzymology*, ed. S. P. Colowick and N. O. Kaplan (New York, N. Y.: Academic Press, 1963), vol. 6, p. 952.
[18] Jirgenson, B., *J. Biol. Chem.*, **246**, 1064 (1965).
[19] Schechter, E., J. P. Carver, and E. R. Blout, these PROCEEDINGS, **51**, 1029 (1964).
[20] Urnes, P. J., cited in ref. 21.
[21] Harrison, S. C., and E. R. Blout, *J. Biol. Chem.*, **240**, 299 (1965).
[22] Simmons, N. S., C. Cohen, A. G. Szent-Györgyi, D. B. Wetlaufer, and E. R. Blout, *J. Am. Chem. Soc.*, **83**, 4766 (1961).
[23] Hazelwood, R. N., *J. Am. Chem. Soc.*, **80**, 2152 (1958).
[24] Scanu, A., *Circulation*, **32**, Suppl. 2, 30 (1965).
[25] Blout, E. R., in *Optical Rotatory Dispersion*, ed. C. Djerassi (New York, N. Y.: McGraw-Hill Book Co., 1960), p. 263.
[26] Blout, E. R., I. Schmier, and N. S. Simmons, *J. Am. Chem. Soc.*, **83**, 4766 (1961).
[27] Yang, J. T., and T. Samejima, *J. Biol. Chem.*, **238**, 3262 (1963).
[28] Anfinsen, C. B., in *Brookhaven Symposia on Biology*, No. 15 (1962), p. 184.
[29] Scanu, A., unpublished observations.

---

*BIOCHEMICAL AND AUTORADIOGRAPHIC STUDIES OF DIFFERENT RNA'S: EVIDENCE THAT TRANSFER RNA IS CHROMOSOMAL IN ORIGIN**

BY PHILIP S. WOODS AND GEOFFREY ZUBAY†

DEPARTMENT OF BIOLOGICAL SCIENCES, UNIVERSITY OF DELAWARE, AND DEPARTMENT OF ZOOLOGY, COLUMBIA UNIVERSITY

Communicated by Berwind P. Kaufmann, October 14, 1965

It is well established that three types of RNA—ribosomal, transfer, and messenger —are found in most cells, each performing an essential role in protein synthesis. Annealing studies[1] indicate that the sequential arrangement of nucleotides in each species of RNA is reflected by a complementary sequence of nucleotides located in some portion of the chromosomal DNA. This and other biochemical evidence[1] strongly suggest that some, if not all, of the RNA is made on a DNA template. Autoradiographic studies[2] in higher organisms further indicate that most of the RNA of the cytoplasm is nuclear in origin; some RNA appears to be synthesized in the bulk of the chromatin and some appears to be synthesized in the nucleolus. By adopting a combined biochemical and autoradiographic approach, it should be possible to identify and trace the pathways of each type of RNA separately in the intact cell. This approach has recently been used by Perry[3] in studying ribosomal and messenger RNA. The investigation presented here is a similar attempt to trace the pathways of transfer RNA (tRNA) in cells of *Vicia faba* meristems and is based on an extraction method for removing tRNA without removing other types of nucleic acid from fixed tissue. [Here transfer RNA is considered equivalent to soluble RNA (sRNA).]