Fatty Acid Synthesis in Adipose Tissue Incubated in Tritiated Water*

Robert L. Jungas

ABSTRACT: Measurements have been made of the amount of radioactivity incorporated into fatty acids during their synthesis in adipose tissue incubated in a medium containing tritiated water and [U-^{14}C]glucose. The incorporation of tritium was proportional to the total rate of fatty acid synthesis as estimated by the tissue net gas exchange. Tritium appeared only in those fatty acids which also contained ^{14}C. When insulin was present medium glucose served as the only important precursor for fatty acid synthesis and the ratio of tritium to ^{14}C incorporation was constant and equal to 0.87. Degradation of the fatty acids revealed that the tritium incorporation was not uniform along the carbon chain. Even-numbered positions contained 0.96 tritium atom/^{14}C atom and odd-numbered positions 0.71. When the H_2O of the medium was replaced by D_2O the tritium incorporation was increased to 1.28 atoms/^{14}C atom. The additional tritium appeared mainly at the even-numbered positions which now contained 1.8 tritium atoms/^{14}C atom. From these data it was estimated that in the absence of an isotope effect 23 of the hydrogen atoms of palmitate would be derived from water. Measurement of the incorporation of deuterium from D_2O into fatty acids confirmed this conclusion. The glyceride glycerol of tissue incubated with tritiated water, [U-^{14}C]glucose, and insulin contained 1.10 tritium atoms/atom of ^{14}C. It is proposed that the incorporation of ^{3}H into fatty acids can be used as a reliable measure of the total rate of fatty acid synthesis in both control and insulin-treated adipose tissue.

Despite the fairly widespread use of deuterium- or tritium-labeled water as a means of demonstrating the occurrence of fatty acid synthesis there is little information concerning the quantitative reliability of this means of measuring fat synthesis. In view of the stability of the C–H bonds of fatty acids to proton exchange (Van Heyningen *et al.*, 1938), it is evident that the incorporation of hydrogen isotopes into fatty acids can occur only during their metabolism. Early workers were able to show that when deuterated water was administered to animals substantial amounts of label appeared in the fatty acids of various tissues (Schoenheimer and Rittenberg, 1936; Waelsch *et al.*, 1940; Stetten and Salcedo, 1944), and subsequent *in vitro* studies using deuterated or tritiated water have amply documented this finding (Shapiro and Wertheimer, 1948; Bloom, 1959; Foster and Katz, 1966). Whether this label incorporation is stoichiometric with the process of fatty acid synthesis or reflects in addition the partial reversal of the β-oxidation sequence of fatty acid oxidation or other enzyme-catalyzed exchange reactions has remained unclear. Recent advances in our understanding of the mechanism of fatty acid synthesis (Vagelos, 1964) permit more detailed predictions as to the extent of labeling which should accompany this process. However inadequate knowledge concerning the details of some of the reactions involved and the possible occurrence of kinetic isotope effects make it impossible to define precisely the amount of labeling to be expected. On the contrary careful quantitation of the labeling process may be expected to contribute to our knowledge of the detailed mechanisms of fatty acid synthesis.

In the course of studies on the action of insulin on the metabolism of adipose tissue (Jungas and Ball, 1964) a need became evident for a method to measure quantitatively the *total* rate of fatty acid synthesis. Although the measurement of the extent of fatty acid synthesis from a particular precursor such as medium glucose or acetate may be easily accomplished by the use of ^{14}C-labeled substrates no satisfactory procedure for measuring the total rate of fat synthesis has been described. Measurements of the net gas exchange of isolated tissues have occasionally proven useful for this purpose (Folley and McNaught, 1958; Flatt and Ball, 1964) though such measurements will obviously be affected by many other cellular processes. It appeared that the incorporation of tritium from labeled water into fatty acids might provide a more satisfactory method, and the extent of this incorporation has therefore been investigated in some detail. The results presented here indicate that this is a valid procedure for control or insulin-treated rat epididymal adipose tissue.

Materials and Procedures

Rats were obtained from the Charles River Breeding Laboratories, North Wilmington, Mass. The pro-

* From the Department of Biochemistry, Harvard Medical School, Boston, Massachusetts 02115. *Received April 17, 1968.* Supported by U. S. Public Health Service Grant AM-08076. The author is the recipient of Public Health Service Research Career Development Award AM-10556. A preliminary report was presented at the 150th National Meeting of the American Chemical Society, Atlantic City, N. J., Sept 1965.

cedures used for their maintenance and for obtaining tissue samples have been described previously (Jungas and Ball, 1963). Except where indicated all incubations were performed in Warburg vessels containing 1.30 ml of the "Pardee bicarbonate" medium (Jungas and Ball, 1964) which is 10 mM in bicarbonate. When desired, tritiated water, uniformly labeled D-glucose-^{14}C, and insulin were added from the vessel side arms following a 15-min period of gassing and thermoequilibration. Except where otherwise noted the additions were adjusted to provide the following concentrations: glucose, 1 mg/ml and 0.3 μCi/ml; tritiated water, 1–2 mCi/ml; and insulin, 1 munit/ml. Isotopes were obtained from New England Nuclear Corp. and beef insulin was the gift of Dr. Otto K. Behrens, Eli Lilly & Co. Incubation was continued 120 min following the additions after which the tissues were washed five times in 10-ml portions of ice-cold 0.15 M NaCl. For each washing the tissue was gently agitated for 10 min or more in order to allow thorough removal of intracellular tritiated water (Crofford and Renold, 1965). A suitable aliquot of the final incubation medium of each vessel was assayed to determine the specific radioactivity of medium water. Medium glucose specific activity was determined from an aliquot of a replicate vessel which did not contain tissue or tritiated water.

The total lipids of the washed tissue were extracted with chloroform–methanol and washed as described by Flatt and Ball (1964). An aliquot was removed for counting and the remainder was saponified in 9 ml of 1.1 N NaOH in 89% ethanol for 15 min at 50°. Extraction of the nonsaponifiable lipids from the alkaline digest with petroleum ether (bp 30–60°) was omitted after preliminary studies showed only a negligible amount of radioactivity was recovered in this fraction. The digest was acidified with H_2SO_4 and the fatty acids were extracted into diethyl ether, washed with water, and assayed for radioactivity. The remaining digest was neutralized with NaOH and evaporated to 3–4 ml. Aliquots were taken by weight for the determination of glyceride-glycerol radioactivity.

All isotope measurements were formed with liquid scintillation counters. The solution described by Bray (1960) was used as the counting fluid for aqueous samples whereas lipid samples were counted in toluene containing 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis-[2-(5-phenyloxazolyl)]benzene. Corrections for quench-in the case of aqueous samples were obtained either by adding internal standards or by counting aliquots of different sizes and extrapolating to zero sample concentration as suggested by Peng (1964). In all cases excellent agreement was found between the sum of the radioactivity in glyceride glycerol and fatty acids with that in the total lipid fraction.

The separation of fatty acids according to the number of double bonds present was achieved by silica gel thin-layer chromatography (Mangold and Kammereck, 1961) of the mercuriacetoxy derivatives prepared according to Goldfine and Bloch (1961).

In the experiments in which saturated fatty acids were degraded the amount of tritiated water added per Warburg vessel was increased to 5 mCi and glucose-^{14}C to 0.7 μCi. About 150 mg of tissue was used in each of two vessels and insulin was added to each. After the incubation and tissue washings were completed the tissue fragments were combined and the total fatty acids were recovered as just described. The acids were esterified with diazomethane (Lipsky and Landowne, 1963) and the mercuriacetoxy derivatives were prepared and streaked along the edge of a 20 × 20 cm chromatographic plate of silica gel (1 mm thick). The plate was developed in diethyl ether–ligroin (bp 66–75°) (4:1), and the saturated esters which move with the solvent front were recovered by diethyl ether extraction of the gel. As much as 1.5 g of total fatty acids of adipose tissue could be fractionated in this manner on a single plate.

The saturated esters were saponified as described above and the acids degraded according to the following sequence of reactions.

$$2RCH_2CO_2H + HgO + 2Br_2 \xrightarrow{CCl_4} 2RCH_2Br + HgBr_2 + H_2O + 2CO_2$$

$$RCH_2Br + AgOCOCH_3 \xrightarrow[(CH_3CO)_2O]{CH_3CO_2H} RCH_2OCOCH_3 + AgBr$$

$$RCH_2OCOCH_3 + NaOH \xrightarrow{CH_3CH_2OH} RCH_2OH + NaOCOCH_3$$

$$3RCH_2OH + 4CrO_3 + 12CH_3CO_2H \longrightarrow 3\,RCO_2H + 9H_2O + 4Cr(OCOCH_3)_3$$

The initial reaction is a modification of the Hunsdiecker reaction recently introduced by Cristol and Firth (1961). The dry acids were taken up in 5 ml of dry CCl_4 and added to a twofold excess of red HgO. A 10% excess of bromine dissolved in CCl_4 was added and the mixture was refluxed in the dark for 1 hr. The solution was chilled on ice, filtered, and taken to dryness. The residue was dissolved in 1 ml of ice-cold petroleum ether and the solution was again filtered to remove remaining $HgBr_2$. Yields of 85–95% of the alkyl bromides were obtained.

The bromides were refluxed for 5 hr with a 50% excess of silver acetate in 25 ml of glacial acetic acid containing 1 ml of acetic anhydride (Anker, 1952). The solution was cooled and filtered and two volumes of water added. The precipitated acetates were recovered by extracting vigorously three times each with 15 ml of petroleum ether. The combined extracts were washed with 10 ml of water and contained the acetates in 90–95% yield. The esters were saponified as described above and the fatty alcohols were extracted into petroleum ether from the alkaline digest. The product was washed with water and carefully taken to dryness.

The oxidation of the alcohols was performed by a modification of the procedure of Pattison *et al.* (1956). A 50% excess of CrO_3 was dissolved in 0.15 ml of water and 4.85 ml of glacial acetic acid added. This solution was added to the dry fatty alcohols and allowed to stand at room temperature overnight in a stoppered flask. Water (2 ml) was added and the fatty acids were recovered by three extractions with 8 ml of petroleum

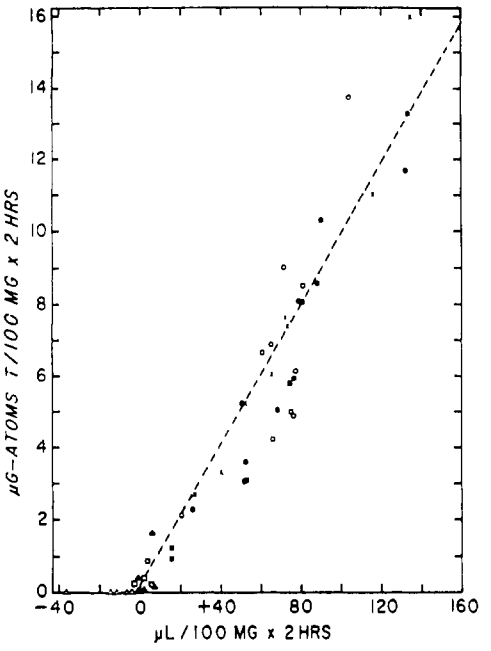

FIGURE 1: The relation between net gas exchange and the incorporation of tritium from tritiated water into fatty acids. The abscissa represents the microliters of net gas exchange corrected for CO_2 release accompanying the production of lactic acid. The line has been drawn by eye. Tissue from refed rats: (○) control, (●) insulin (1 munit/ml), (×) niacin (10 μg/ml); normally fed rats: (□) control, (■) insulin; or fasted rats: (△) control, (▲) insulin.

ether. The combined ether extracts were washed with 1 ml of water which was returned to the oxidation flask. The contents of this flask were chilled and neutralized by the addition of KOH pellets. The mixture was frozen and the water was collected by lyophilization using an apparatus of the type described by Calvin *et al.* (1949). The fatty acid products were purified by extraction into 1 N NaOH in 90% ethanol (Borgstrom, 1952). Following acidification with H_2SO_4 the acids were reextracted into petroleum ether and washed with water. Acids shorter than ten carbons in length are lost in this procedure (Wakil *et al.*, 1957). The over-all yield of fatty acids shorter by one carbon atom was 65–70%.

Results

Flatt and Ball (1964) have demonstrated that an excellent correlation exists between the net gas exchange of adipose tissue corrected for CO_2 release resulting from lactic acid production and the incorporation of uniformly labeled glucose-^{14}C into fatty acids. These results were obtained with tissue from normally fed rats incubated in the presence of insulin so that essentially all of the fatty acids synthesized were made from medium glucose. Under these conditions therefore the tissue net gas exchange serves as a valid measure of the total rate of fatty acid synthesis.

The incorporation of tritium from tritiated water into tissue fatty acids may also reflect the total rate of fatty acid synthesis. Studies of the relationship between these two methods of monitoring fat synthesis are summarized in Figure 1. In order to obtain as wide a range of values of net gas exchange as possible, data from normally fed rats, 3-day-fasted rats, and rats fasted 3 days and refed 2 days on a high carbohydrate diet (Wooley and Sebrell, 1945) have been included. In some cases insulin or niacin has been added to the incubation medium. It is apparent from the figure that over a very wide range of experimental conditions a good correlation exists between these two indices of fat synthesis. Thus it appears that tritium incorporation into fatty acids is as good a measure of the total rate of fatty acid synthesis as is the net gas exchange. Limitations in the interpretation of the net gas exchange data prevent a precise assessment of the relationship between fatty acid synthesis and tritium incorporation from this type of experiment.

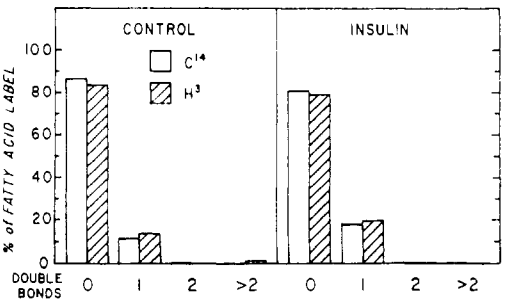

FIGURE 2: Distribution of radioactivity incorporated into fatty acids according to the number of double bonds present. Results from two closely agreeing experiments have been averaged for presentation.

If the tritium incorporation is a valid measure of fatty acid synthesis there should be no label incorporation when synthesis does not occur. Two types of experiments were performed to examine this requirement. The first of these employed tissue removed from rats fasted for 3 days and incubated in a medium containing tritiated water but no substrate and no insulin. Such fasted tissue is nearly devoid of glycogen (Gutman and Shafrir, 1964) and thus fat synthesis should be minimal under these conditions. In three experiments of this type the tritium incorporation into fatty acids was 0.0022, 0.0002, and 0.0014 or an average of 0.0013 μg-atom/100 mg of fresh tissue in 2 hr. These values are four orders of magnitude smaller than some of those presented in Figure 1 and less than 1% of the lowest values seen with normal tissue under the same conditions. Since the fasted tissue does combust fatty acids as shown by respiratory measurements (Ball and Jungas, 1963) it may be concluded that reactions associated with the β-oxidation sequence do not introduce appreciable label into the tissue fatty acids.

The experiment summarized in Figure 2 leads to a similar conclusion. In this case tissue from normally fed rats was incubated with and without insulin in a medium containing both uniformly labeled glucose-^{14}C and tritiated water. The tissue fatty acids were isolated and separated into classes according to the number of double bonds present. The failure of the tissue to synthesize essential fatty acids from glucose is shown by the virtual absence of ^{14}C in the fatty acids containing

TABLE I: Fatty Acid Synthesis in Normally Fed Rats.[a]

	μg-atoms Incorporated into Fatty Acids/100 mg in 120 min					
	Control			Insulin		
Expt	^{14}C	^{3}H	$^{3}H/^{14}C$	^{14}C	^{3}H	$^{3}H/^{14}C$
1	0.295	0.611	2.07	7.44	6.61	0.89
2	0.125	0.237	1.90	3.54	3.09	0.87
3	0.211	0.285	1.35	1.23	1.06	0.85
4	0.070	0.112	1.61	3.84	3.19	0.83
5	0.644	0.833	1.30	6.76	5.83	0.86
6	0.111	0.242	2.19	3.34	3.02	0.90
Av	0.243	0.387	1.74	4.36	3.80	0.87
Std error	±0.087	±0.112	±0.15	±0.95	±0.84	±0.01

[a] Adipose tissue was incubated 140 min with 1 munit/ml of insulin present from the start. Tritiated water and glucose-U-^{14}C were added after 20 min to give 1.80×10^4 dpm per μg-atom of hydrogen or carbon and 1 mg/ml of glucose. Experiments 2 and 6 were performed in Krebs–Henseleit buffer containing half the recommended calcium (Krebs and Henseleit, 1932).

two or more double bonds, as has been observed previously (Gellhorn *et al.*, 1962; Gliemann and Dole, 1964). A similar labeling pattern was seen for tritium indicating that tritium is incorporated into tissue fatty acids only during their synthesis. This result confirms the findings of Bernhard and Schoenheimer (1940a) and Bernhard and Bullet (1942) who observed that when mice or rats were administered D_2O no deuterium appeared in the carcass fatty acids containing two or three double bonds.

Experiments were next carried out to determine how many tritium atoms were incorporated into each fatty acid molecule during its biosynthesis and whether this number is constant. The results of these experiments are summarized in Table I. Tissue from normally fed rats was incubated in the presence of uniformly labeled glucose-^{14}C and tritiated water with and without insulin. As shown in the table the tissue incubated without insulin showed a highly variable rate of incorporation of both isotopes into fatty acids and the ratio of tritium to ^{14}C incorporated also varied somewhat. When insulin was added the incorporation of both isotopes remained highly variable but now the ratio T:^{14}C was constant within experimental error and equal to 0.87. The same ratio was observed when the experiments were repeated using tissue from rats fasted for 3 days.

These results may be interpreted as follows. When insulin is present there is very little dilution of the large quantities of radioactive glucose entering the cell by intermediates arising from endogenous sources. This is demonstrated by the fact that nearly all of the CO_2 released under these conditions is radioactive (Flatt and Ball, 1964). Consequently the incorporation of isotope from medium glucose into tissue fatty acids serves as an accurate measure of the total rate of fat synthesis. Because the ratio T:^{14}C is constant under these conditions it follows that the tritium incorporation is an equally precise index of this process and that for each ^{14}C atom incorporated into fatty acids $^7/_8$ of a tritium atom is also incorporated. Resolution of the labeled fatty acids by gas chromatography revealed that the average chain length of the newly synthesized fatty acids was 16.0, as expected from earlier observations (Martin *et al.*, 1961). Thus an average of 14 tritium atoms are incorporated into each newly synthesized fatty acid, or one for each methylene group present. In the control tissues glucose uptake is small and the breakdown of glycogen is not restrained by insulin (Jungas and Ball, 1964). As a result an appreciable dilution of the carbon label occurs and the T:^{14}C ratio observed in the fatty acids is increased. The amount of dilution to be expected can be estimated from the data of Flatt and Ball (1964). Working under similar conditions these workers estimated that 58% of the total acetylcoenzyme A units produced were derived from ^{14}C-labeled glucose. Thus the T:^{14}C ratio in the fatty acids of the control tissues may be expected to be about twice that of the insulin-treated tissues, as was in fact observed. It would appear therefore that the tritium incorporation into fatty acids may be used as an accurate measure of the total rate of their synthesis in both control and insulin-treated tissues.

These results raised the question of whether in fact each methylene-^{14}C group did contain one tritium atom or whether some other pattern of isotope distribution prevailed. The experiments with insulin were therefore repeated using higher levels of radioactivity. Following the incubation the tissue saturated fatty acids were isolated and degraded carbon by carbon using the procedures already described. Two largely independent methods were used to determine the amount of tritium associated with each carbon atom. The first of these consisted of measuring the tritium released as water during the oxidation of fatty alcohols to the corresponding

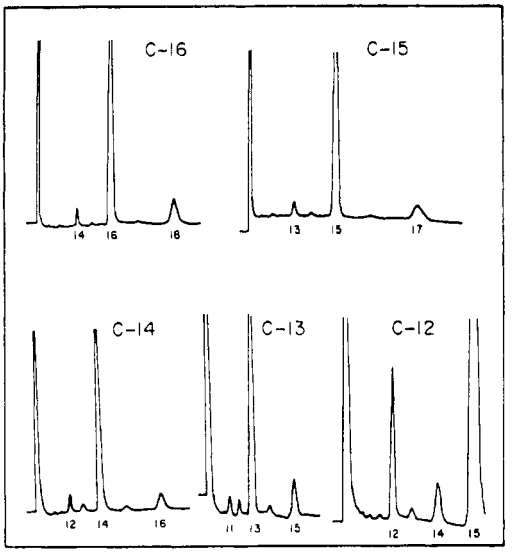

FIGURE 3: Gas chromatography of fatty acid methyl esters produced during the degradation of the saturated fatty acids of adipose tissue. Methyl esters were separated on a column of 15% ethylene glycol succinate on a Gas Chrom P support. The tracing labeled C-16 refers to esters made from the saturated fatty acids prior to the first cycle of degradation; C-15 refers to esters made from fatty acids subjected to one cycle of degradation, etc. Numbers under the peaks indicate the number of carbon atoms in the fatty acid skeleton. Carrier palmitate was added prior to the final degradation cycle and appears as a 15-carbon acid in the lower right-hand frame.

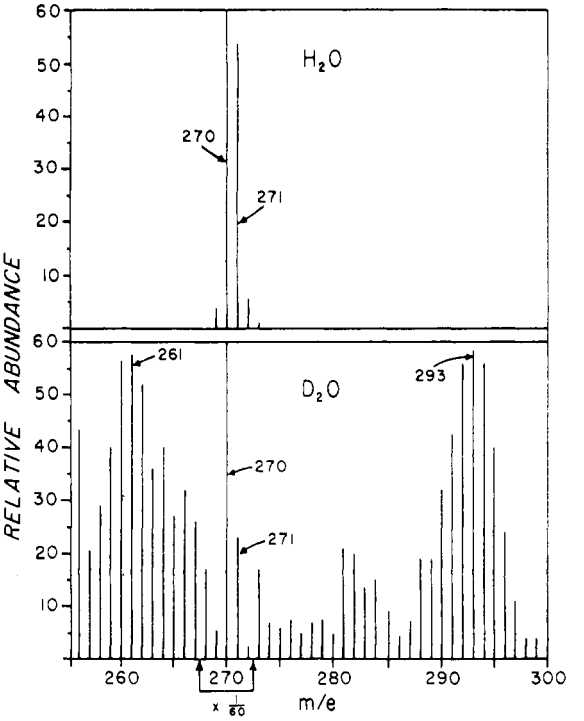

FIGURE 4: Portions of the mass spectra of methyl palmitate isolated from adipose tissue incubated in water or D_2O. The tissue-saturated esters were injected into a gas chromatographic column (OV-1) with the effluent passing in part into an LKB Model 9000 mass spectrometer. The upper frame is from a control experiment and represents a mass scan taken just as the palmitate peak emerged from the column. The lower frame shows the spectrum of palmitate derived from tissue incubated in D_2O. The deuterated forms exited from the column well ahead of the major palmitate peak. In the lower figure the mass peaks from 269 to 272 have been reduced 60-fold to bring them on scale. In both figures the mass 270 peak remains off scale.

fatty acids. This procedure has often been used in fatty acid degradation (Brady *et al.*, 1960; Foster and Bloom, 1963) for it offers a direct measurement of the tritium originally present on the α-carbon of the fatty acid from which the alcohol was derived. It suffers from the disadvantage that it has not been possible to carry out the oxidation with yields greater than about 80%. Consequently unless all the reaction products are identified and measured the interpretation of the tritium yield in the recovered water is somewhat inexact.

The second procedure relies solely on measurements of the T:^{14}C ratio in the various intermediate products as the fatty acids are degraded. While in principle this method is exact, in practice its accuracy is limited by the requirement that small changes in isotope ratios be precisely measured. For example, if the isolated saturated fatty acids yielded a T:^{14}C ratio of 14:16 or 0.875, degradation by one carbon should yield a ratio of 13:15 or 0.867 if 1.0 tritium atom were present on each ^{14}C atom α to the carboxyl group. A measured result of 13.1:15 or 0.874 would correspond to a 10% underestimate of the α-tritium content.

The adequacy of the chemical procedures employed for the degradation of the fatty acids was investigated by gas chromatography of the intermediate products. Representative results are presented in Figure 3. The observations confirm the fact that the degradation cycles proceeded smoothly. A very small amount of overoxidation apparently occurred as indicated by the gradual accumulation in the mixture of the fatty acid shorter by one carbon atom than the major constituent. Isotopic measurements of the products of a degradation experiment are presented in detail in Table II. In this experiment the T:^{14}C ratio in the total tissue fatty acids was 0.897 while the average chain length of the ^{14}C-labeled acids was 16.0 so that 14.35 tritium atoms were present per molecule of labeled 16-carbon acid. Removal of the unsaturated acids increased the T:^{14}C ratio slightly. The constancy of the tritium content of the acids, bromides, and alcohols in each degradation cycle demonstrates that little tritium is lost by exchange during the course of the chemical conversions. During each cycle of degradation the yield of fatty acids shorter by one carbon as calculated on a weight basis was the same as that calculated from the ^{14}C recovery. Hence the ^{14}C must have been evenly distributed along the carbon chain. The two methods of analysis agree well with each other and indicate clearly that the tritium incorporation is nonuniform along the carbon chain. Approximately one tritium atom was found associated with the even-numbered ^{14}C atoms, whereas about two-thirds of that amount was present on the odd-numbered positions. Similar results were obtained when the experiment was repeated. The average tritium distribution in the fatty acids was as follows: α-carbon, 1.00; β-

TABLE II: Position of Tritium in Fatty Acids Synthesized in Tritiated Water.

Degradation Product	Ratio Method T/Mole[a]	Ratio Method T/C	H_2O Method T/C	Average T/C
Total fatty acids	14.35			
16-Carbon acid[b]	14.59			
15-Carbon bromide	14.66, 14.66[c]			
15-Carbon alcohol	14.72			
15-Carbon acid	13.70			
14-Carbon bromide	13.61, 13.67	0.99	0.96	0.98 (α-T)
14-Carbon alcohol	13.71			
14-Carbon acid	13.02			
13-Carbon bromide	13.00, 13.02	0.65	0.78	0.72 (β-T)
13-Carbon alcohol	13.04			
13-Carbon acid	12.10			
12-Carbon bromide	12.08, 12.10	0.92	0.95	0.94 (γ-T)
12-Carbon alcohol	12.13			
12-Carbon acid	11.44	0.66	0.74	0.70 (δ-T)

[a] The units are microgram-atoms of tritium per mole of ^{14}C-labeled product. [b] The average chain length of the ^{14}C-labeled saturated fatty acids was 16.0 with about 80% of the total ^{14}C present in palmitate, 8% in myristate, and 12% in stearate. The tritium distribution was similar to that of the ^{14}C. Hence the saturated acids are labeled "16-carbon acid" although no attempt was made to remove other minor components which were present (see Figure 3). [c] Averages have been taken for the groups of compounds which may be expected to be identical in tritium content.

carbon, 0.74; γ-carbon, 0.96; and δ-carbon, 0.70, expressed as atoms of tritium per atom of ^{14}C.

Attempts to decipher the meaning of these incorporation data as they relate to the intimate mechanism of the condensation, reduction, and dehydration reactions of fatty acid synthesis met with uncertainties arising from the possible existence of large kinetic isotope effects in the tritium incorporation. We have therefore attempted to gain some information about the magnitude of the isotope effect involved. For this purpose the tissue was incubated in a medium in which the water was replaced by heavy water, D_2O. Uniformly labeled glucose-^{14}C and tracer amounts of tritiated water were present as before. If there is a discrimination between T and H atoms for incorporation into fatty acids, the effect should be smaller when H is replaced by D. Thus an increase in the tritium incorporation would be predicted when the experiment is done in D_2O and from the magnitude of the increase it should be possible to estimate the size of the isotope effect. It was not possible to eliminate all the H_2O from the incubation vessels as tritiated water was available only in H_2O solution and some water was no doubt introduced with the tissues despite a prior washing in a D_2O solution of the incubation medium. It is estimated that more than 95% of the H_2O was replaced by D_2O, and no corrections have been applied to allow for the remaining H_2O.

The results indicated that there was in fact a sizeable isotope effect. On the average of four experiments performed in D_2O the T:^{14}C ratio in the total fatty acids was increased to 1.28, indicating that now 20.4 $\pm$ 0.5 (std error) tritiums were incorporated per mole of fatty acids. From this value and the theory of kinetic isotope effects outlined by Swain *et al.* (1958) it may be estimated that in the absence of an isotope effect 23 of the hydrogen atoms of fatty acids would be derived from the tritiated water. The fatty acids recovered in these experiments were also analyzed for their deuterium content by mass spectrometry. The upper end of the mass spectrum obtained from the palmitate peak in a control experiment in which no D_2O was used is shown in the upper half of Figure 4. The molecule ion of *m/e* 270 and the associated 271 peak resulting from the natural occurrence of ^{13}C are the only main peaks visible. A corresponding spectrum from the experiment employing D_2O is shown in the lower half of the figure. Besides the methyl palmitate molecule ion there is a scatter of heavier ions present which must therefore have contained deuterium. The major components of these heavier ions center around mass 293 indicating that an average of 23 deuterium atoms was incorporated per mole of palmitate in agreement with the tritium data. Note that a much higher gain setting was employed during the scan shown in the lower half of the figure. This was necessary because only about 1 in 500 of the palmitate molecules present was newly synthesized during the *in vitro* incubation and hence contained deuterium.

In view of the rather large isotope effect it became of interest to reexamine the distribution of tritium atoms

TABLE III: Distribution of Tritium in Fatty Acids Synthesized in D_2O Medium.

Position	Expt	Ratio Method T/C[a]	H_2O Method T/C	Average
α-Carbon	1	1.88	1.51	1.70
	2	2.00	1.96	1.98
	Av	1.94	1.74	1.84
β-Carbon	1	1.06	0.88	0.97
	2	0.65	0.87	0.76
	Av	0.86	0.88	0.87

[a] The units are microgram-atoms of tritium per microgram-atom of ^{14}C incorporated at each position.

in the fatty acids synthesized in D_2O to see where the additional tritium appeared. The results of two degradations are summarized in Table III. The major change is clearly at the even-numbered carbon atoms. On the average 1.84 tritium atoms were found on the α position which when corrected for the remaining tritium *vs.* deuterium isotope effect means that all of the hydrogen atoms at that position were derived from water. At the β position the situation is less clear. The isotope effect is smaller than at the α position as 0.87 atom of tritium was found as compared with the previous value of 0.74. This difference is probably within the error of the methods used.

In some of the experiments the radioactivity in the glyceride-glycerol moiety of the tissue fat was also determined. On the average of six experiments conducted with tissue from normally fed rats incubated in the presence of insulin, glucose-U-^{14}C, and tritiated water, the T:^{14}C ratio of the glycerol was 1.10 ± 0.11. When the α-carbons were counted separately as their dimedon derivatives (Katz and Rognstad, 1966) they yielded a ratio of 1.20. The formic acid derived from the β-carbon had a ratio of 0.94. Similar ratios were seen when the experiment was conducted in D_2O indicating that the isotope effect was small during glycerol formation.

Discussion

The data presented here clearly demonstrate that the incorporation of tritium from water into fatty acids may be used as an accurate measure of the total rate of fatty acid synthesis in adipose tissue from normal rats incubated in the presence of insulin and glucose. As far as is known fatty acid synthesis proceeds by the same reaction sequence whether or not insulin is present; only the rate of the process is affected by the hormone. Thus it seems highly likely that the tritium incorporation in the control tissue will also accurately reflect the total rate of fatty acid synthesis. A direct confirmation of this prediction is not currently possible because of the lack of any independent means of assessing the total rate of fatty acid synthesis in the untreated tissue. As discussed above, however, the values for the T:^{14}C ratio in the control tissue presented here taken in conjunction with the data of Flatt and Ball (1964) leave little doubt that this prediction must be at least approximately correct. The net gas exchange data presented in Figure 1 support the view that this is also true for adipose tissue from fasted refed rats. In this figure the points lying above 2 μg-atoms/100 mg in 2 hr are drawn almost exclusively from refed tissue and largely determine the slope of the line shown. By combining these data with the similar graph of Flatt and Ball (1964) relating the conversion of glucose-U-^{14}C into fatty acids and the net gas exchange an estimate of 0.90 for the T:^{14}C ratio of the refed tissue may be obtained. This value is in good agreement with that measured directly in normal tissue in the presence of insulin. Because of the rich glycogen content of the refed tissue it is uncertain whether or not there will be significant dilution of medium glucose carbon entering this tissue even in the presence of insulin. The recent report of Foster and Katz (1966) of a T:^{14}C ratio of 0.95 in the fatty acids of refed tissue incubated with tritiated water, glucose-U-^{14}C, and insulin suggests that this dilution may be less than 10%.

The isotope effect observed during the incorporation of tritium into fatty acids appears to be of no great practical consequence as it seems to be a constant factor. The extent of the discrimination appears to be quite independent of the rate of fatty acid synthesis as shown by the constancy of the T:^{14}C ratio in normal and fasted tissue treated with insulin despite a greater than tenfold variation in rate. Therefore a good estimate of the total carbon flow into fatty acids may be obtained simply by multiplying the tritium incorporation by 3/7. It should be pointed out however that no measurements of the fatty acid T:^{14}C ratio have been reported for tissue incubated under conditions in which the contribution of the pentose cycle to glucose metabolism is known to be small, for example, in the presence of large amounts of epinephrine (Katz and Rognstad, 1966). It is possible that under such conditions the T:^{14}C ratio may be somewhat increased (see below).

The use of D_2O to estimate the tritium isotope effect is not strictly valid for a process as complicated as the synthesis of fatty acids. Undoubtedly there are several reactions between pyruvate and palmitate at which hydrogen atoms may exchange with water. At each of these tritium may be introduced, and substituting D_2O for H_2O will favor the tritium incorporation. The difficulty lies in those steps in which tritium previously introduced may be lost by water exchange. In this case competition may occur between the tritium atom and hydrogen atoms which derive from the starting glucose and the replacement of H_2O by D_2O will not then affect the rate at which tritium is lost. Thus substituting D_2O for H_2O may lead to an overestimate of the isotope effect. The agreement between the tritium data and the deuterium measurements suggests that this effect is not serious. Moreover the isotope effect reported here is in good agreement with that found by previous workers. Glascocke and Duncombe (1952) injected both deuter-

ated and tritiated water into nursing rats and found 20% less tritium than deuterium in fatty acids isolated from the mammary gland. In a similar experiment Eidinoff *et al.* (1953) found 15% less tritium in rat liver fatty acids. The present data would predict that 17% less tritium would be found.

In their classic experiments Rittenberg and Schoenheimer (1937) found that when mice were fed a low-fat diet and administered D_2O for a long period of time the amount of deuterium in the carcass-saturated fatty acids gradually attained a plateau in which 43% of the hydrogen atoms had been derived from the body water. Subsequently a value of 45% was observed for the saturated fatty acids of mouse liver (Bernhard and Schoenheimer, 1940b) and 42% for the saturated fatty acids of the carcass of rats (Bernhard and Bullet, 1943). From the T:^{14}C ratio of 0.87 observed in the total fatty acids in the present experiments and the data in Table II it may be calculated that on the average 46% of the hydrogen of the newly synthesized saturated fatty acids was derived from water. The agreement between these values appears to be fortuitous. From the present estimates of the isotope effect involved in fatty acid synthesis it may be estimated that 53% of the hydrogens of newly synthesized fatty acids would be derived from water as assayed with deuterium. Apparently the time interval used in the classic experiments (21–98 days) did not allow for the *de novo* synthesis of the entire carcass pool of saturated fatty acids. Rather about 15% of the saturated acids behaved like the monoenoic fatty acids (Bernhard and Bullet, 1943) and did not turn over completely within the period of observation.

The results of the degradation experiments show clearly that tritium is present at the odd-numbered positions in the fatty acid chain. A similar finding had been reported for liver slices (Foster and Bloom, 1963) and more recently also for adipose tissue (Foster and Katz, 1966). The pathway by which tritium is incorporated into this position is of great interest. It is well established that the hydrogen atoms which enter the fatty acids by way of TPNH[1] will appear exclusively on the odd-numbered carbons (Brady *et al.*, 1960; Foster and Bloom, 1963; Foster and Katz, 1966). Moreover in adipose tissue the hydrogen from the TPNH generated *via* the pentose cycle is quantitatively recovered in fatty acids (Katz and Rognstad, 1966) indicating that no exchange between water and the hydrogens of TPNH occurs in this tissue. Consequently the amount of tritium incorporated into the odd-numbered positions from water reflects the extent to which reduced coenzymes derived from sources other than the pentose cycle contribute to the reductive processes of fatty acid synthesis. On the average of all the degradation experiments it was found that 0.81 of the 2 hydrogen atoms at the β position of the fatty acids was derived from water. Thus the pentose cycle contributed at most 1.19/2 or 60% of the reduced coenzymes used for fatty acid synthesis under these conditions, in good agreement with other estimates obtained from the analysis of metabolic products derived from glucose-^{14}C (Flatt and Ball, 1964; Katz and Rognstad, 1966). From the balance studies of Flatt and Ball (1964) and of Rognstad and Katz (1966) it may be inferred that the major part of the remaining reduced coenzymes used for fat synthesis are obtained from the oxidation of glyceraldehyde phosphate. The DPNH produced when this oxidation occurs in the presence of tritiated water would be expected to contain tritium as a result of the exchange catalyzed by aldolase between the hydrogen of position 1 of glyceraldehyde phosphate and water (Rose and Rieder, 1955). Whether this DPNH contributes to fatty acid synthesis directly or more likely following transfer of the tritium to TPN *via* malic dehydrogenase and the malate enzyme (Kornacker and Ball, 1965) is unknown. Remarkably, the isotope effect associated with these reactions of the pyridine nucleotides appears to be small. This is in agreement with the earlier studies of Hoberman and D'Adamo (1960) who found little difference in the rates of incorporation of label into glycogen from lactate-2-T and lactate-2-D.

Degradation of the fatty acids from tissues incubated in heavy water indicated that nearly all of the hydrogen at the even-numbered positions of fatty acids are derived from water. Rapid exchange of these hydrogens with water may occur at the β-ketoacyl-ACP[2] stage of fatty acid synthesis. Marcus *et al.* (1958) found that, when acetoacetyl pantetheine was reduced by DPNH and pig heart L-(+)-β-hydroxybutryl coenzyme A dehydrogenase in a medium containing D_2O, $^7/_8$ of the hydrogen at the 2 position had exchanged with water after only 2 min. On the other hand previous measurements of the incorporation of tritium from acetate-2-T-2-^{14}C to fatty acids in liver have provided evidence that some substrate hydrogen may remain bound to the even-numbered carbon atoms. If this were not so, when palmitate is synthesized from acetate not more than $^1/_8$ of the tritium should accompany the ^{14}C into the fatty acid and this tritium would all be present on the ω-methyl position. However, Foster and Bloom (1962) found a fatty acid T:^{14}C ratio of 0.20 of that of the starting acetate. In adipose tissue values of 0.14–0.40 have been reported starting from glucose-6-T-6-^{14}C (Landau and Katz, 1964; Katz and Rognstad, 1966). Similar findings have also been reported for lactating mammary gland (Abraham *et al.*, 1963; Bartley *et al.*, 1965). The spread in the values reported is considerable but on the average they lie well above 0.13, indicating some incorporation of tritium into the interior of the fatty acid molecule. It is likely that isotope discrimination effects favor retention, and no quantitative estimates can be based on these ratios. Degradation experiments with these precursors have not been described.

On the average it was found that the T:^{14}C ratio in glyceride glycerol in the presence of insulin was 1.1. This value is in good agreement with what is known about the reactions involved in the conversion of glucose to glycerol. Of the seven carbon-bound hydrogens in glucose four may be expected to exchange with water

[1] See *Biochemistry 5*, 1445 (1966).

[2] ACP = acyl-carrier protein.

during this process. The hydrogen at C-2 and C-5 is of course absent in dihydroxyacetone phosphate. The hydrogen at position three is expected to be exchanged with water in the triose isomerase reaction (Rieder and Rose, 1959) while aldolase will exchange the number four hydrogen (Rose and Rieder, 1955). No reactions are known which exchange the hydrogens at positions 1 and 6. In fact, retention of the two hydrogens at position 6 has been demonstrated. Glucose-6-T-6-^{14}C is converted to glyceride glycerol in adipose tissue with no change in the T:^{14}C ratio (Landau and Katz, 1964; Katz and Rognstad, 1966). The lack of exchange of the hydrogen at position one may be inferred from the data of Bartley *et al.* (1965). They found that when glucose-1-T-1-^{14}C and glucose-6-T-6-^{14}C were converted into fatty acids in mammary gland slices the ω-methyl group of the fatty acids showed identical T:^{14}C ratios. Since the pathways of these labeled precursors converge at the triose phosphate level and the 6-T is not lost prior to that, the 1-T must also not be subject to exchange upon conversion into triose phosphates. Thus when glucose is converted into 2 moles of glycerol three of the eight hydrogens on the glycerol α-carbons must derive from glucose. The results show that the remaining five α-hydrogens and the two β-hydrogens exchange with water so that a T:^{14}C ratio of $^{7}/_{6}$ or 1.1. is observed. It is not clear why Katz and Rognstad (1966) observed extensive incorporation of tritium from glucose-3-T-U-^{14}C into the α-carbons of glycerol. These workers explained their findings by postulating that the triose isomerase of adipose tissue and mammary gland had a different mechanism from the liver enzyme studied by Rose *et al.* (1962). The present data do not support this speculation.

Acknowledgments

I am grateful to Dr. James Orr for assistance in obtaining the mass spectra. Mr. Hans Schären provided skillful technical assistance throughout these studies.

References

Abraham, S., Katz, J., Bartley, J., and Chaikoff, I. L. (1963), *Biochim. Biophys. Acta 70*, 690.

Anker, H. S. (1952), *J. Biol. Chem. 194*, 177.

Ball, E. G., and Jungas, R. L. (1963), *Biochemistry 2*, 586.

Bartley, J., Abraham, S., and Chaikoff, I. L. (1965), *Biochem. Biophys. Res. Commun. 19*, 770.

Bernhard, K., and Bullet, F. (1942), *Helv. Chim. Acta 25*, 1313.

Bernhard, K., and Bullet, F. (1943), *Helv. Chim. Acta 26*, 1185.

Bernhard, K., and Schoenheimer, R. (1940a), *J. Biol. Chem. 133*, 707.

Bernhard, K., and Schoenheimer, R. (1940b), *J. Biol. Chem. 133*, 713.

Bloom, B. (1959), *J. Biol. Chem. 234*, 2158.

Borgstrom, B. (1952), *Acta Physiol. Scand. 25*, 111.

Brady, R. O., Bradley, R. M., and Trams, E. G. (1960), *J. Biol. Chem. 235*, 3093.

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.

Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M., and Yankwich, P. E. (1949), Isotopic Carbon, New York, N. Y., Wiley, p 93.

Cristol, S. J., and Firth, W. C. (1961), *J. Org. Chem. 26*, 280.

Crofford, O. B., and Renold, A. E. (1965), *J. Biol. Chem. 240*, 14.

Eidinoff, M. L., Perri, G. C., Knoll, J. E., Marano, B. J., and Arnheim, J. (1953), *J. Amer. Chem. Soc. 75*, 248.

Flatt, J. P., and Ball, E. G. (1964), *J. Biol. Chem. 239*, 675.

Folley, S. J., and McNaught, M. L. (1958), *Brit. Med. Bull. 14*, 207.

Foster, D. W., and Bloom, B. (1962), *Biochim. Biophys. Acta 60*, 189.

Foster, D. W., and Bloom, B. (1963), *J. Biol. Chem. 238*, 888.

Foster, D. W., and Katz, J. (1966), *Biochim. Biophys. Acta 125*, 422.

Gellhorn, A., Benjamin, W., and Wagner, M. (1962), *J. Lipid Res. 3*, 314.

Glascocke, R. F., and Duncombe, W. G. (1952), *Biochem. J. 51*, XL.

Gliemann, J., and Dole, V. P. (1964), *J. Biol. Chem. 239*, 4062.

Goldfine, H., and Bloch, K. (1961), *J. Biol. Chem. 236*, 2596.

Gutman, A., and Shafrir, E. (1964), *Am. J. Physiol. 207*, 1215.

Hoberman, H. D., and D'Adamo, A. F. (1960), *J. Biol. Chem. 235*, 523.

Jungas, R. L., and Ball, E. G. (1963), *Biochemistry 2*, 383.

Jungas, R. L., and Ball, E. G. (1964), *Biochemistry 3*, 1696.

Katz, J., and Rognstad, R. (1966), *J. Biol. Chem. 241*, 3600.

Kornacker, M. S., and Ball, E. G. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 899.

Krebs, H. A., and Henseleit, K. (1932), *Z. Physiol. Chem. 210*, 33.

Landau, B. R., and Katz, J. (1964), *J. Biol. Chem. 239*, 697.

Lipsky, S. R., and Landowne, R. A. (1963), *Methods Enzymol. 6*, 513.

Mangold, H. K., and Kammereck, R. (1961), *Chem. Ind. (London)*, 1032.

Marcus, A., Vennesland, B., and Stern, J. R. (1958), *J. Biol. Chem. 233*, 722.

Martin, D. B., Horning, M. G., and Vagelos, P. R. (1961), *J. Biol. Chem. 236*, 663.

Pattison, F. L. M., Stothers, J. B., and Woolford, R. G. (1956), *J. Am. Chem. Soc. 78*, 2255.

Peng, C. T. (1964), *Anal. Chem. 36*, 2456.

Rieder, S. V., and Rose, I. A. (1959), *J. Biol. Chem. 234*, 1007.

Rittenberg, D., and Schoenheimer, R. (1937), *J. Biol. Chem. 121*, 235.

Rognstad, R., and Katz, J. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 1148.

Rose, I. A., Kellermeyer, R., Stjernholm, R., and Wood,

H. G. (1962), *J. Biol. Chem. 237*, 3325.
Rose, I. A., and Rieder, S. V. (1955), *J. Am. Chem. Soc. 77*, 5764.
Schoenheimer, R., and Rittenberg, D. (1936), *J. Biol. Chem. 114*, 381.
Shapiro, B., and Wertheimer, E. (1948), *J. Biol. Chem. 173*, 725.
Stetten, D., and Salcedo, J. (1944), *J. Biol. Chem. 156*, 27.
Swain, C. G., Stivers, E. C., Reuwer, J. F., and Schaad, L. J. (1958), *J. Am. Chem. Soc. 80*, 5885.
Vagelos, P. R. (1964), *Ann. Rev. Biochem. 33*, 139.
Van Heyningen, W. E., Rittenberg, D., and Schoenheimer, R. (1938), *J. Biol. Chem. 125*, 495.
Waelsch, H., Sperry, W. M., and Stoyanoff, V. A. (1940), *J. Biol. Chem. 135*, 291.
Wakil, S. J., Porter, J. W., and Gibson, D. M. (1957), *Biochim. Biophys. Acta 24*, 453.
Wooley, J. G., and Sebrell, W. H. (1945), *J. Nutr. 29*, 191.

The Circular Dichroism of Polypeptide Films*

Linda Stevens, Robert Townend, Serge N. Timasheff† Gerald D. Fasman, and Joan Potter

ABSTRACT: Circular dichroism spectra were obtained on films of polypeptides in the β conformation. In agreement with previous optical rotatory dispersion results, these polypeptides fall into two categories: I-β and II-β. Poly-L-tryptophan was also examined in film form, identifying the side-chain bands above 250 mμ.

In recent years, a major effort has been devoted to the characterization of polypeptide and protein structures by the method of optical rotatory dispersion (Urnes and Doty, 1961; Fasman, 1963; Harrington *et al.*, 1966). The recent availability of instrumentation capable of measuring circular dichroism in the far-ultraviolet region has made it possible to identify the circularly dichroic bands which give rise to the Cotton effects in the optical rotatory dispersion spectra of various conformations. As a result, solutions of polypeptides in various conformations have been examined and the proper circular dichroism bands identified. Thus, it is known that, in solution, the α-helical conformation is characterized by a positive band at 190–191 mμ and two negative bands at 207 and 221 mμ, while the random (or disordered) conformation has a strong negative band at 196 mμ, a weak positive band at 217 mμ, and a very weak negative band at 238 mμ (Holzwarth *et al.*, 1962; Brahms and Spach, 1963; Grosjean and Tari, 1964; Beychok and Fasman, 1964; Holzwarth and Doty, 1965; Velluz and Legrand, 1965; Townend *et al.*, 1966; Sarkar and Doty, 1966; Timasheff *et al.*, 1967). Polyproline I and II, as well as the collagen triple helix, have been similarly characterized (Timasheff *et al.*, 1967). The β or pleated-sheet structure received attention after the discovery that poly-L-lysine will assume that conformation in dilute aqueous solution when heated gently at alkaline pH values (Rosenheck and Doty, 1961; Applequist and Doty, 1962; Davidson *et al.*, 1966). It was found that the antiparallel pleated-sheet β conformation (Susi *et al.*, 1967) assumed by that polypeptide in solution has a circular dichroism spectrum consisting of a negative band at 217 mμ (Townend *et al.*, 1966; Sarkar and Doty, 1966) and a positive band at 195 mμ (Townend *et al.*, 1966). The latter may split into two components (Timasheff *et al.*, 1967) as predicted by theory (Pysh, 1966).

Recently, Fasman and Potter (1967) reported that films of polypeptides, known from infrared dichroism to be in the antiparallel β structure, give nonidentical optical rotatory dispersion spectra which may be grouped into two families, designated as I-β and II-β. The first has an optical rotatory dispersion spectrum similar to that of β-structured poly-L-lysine in solution (trough at 230 mμ, peak at 205 mμ) (Davidson *et al.*, 1966). The second family (II-β) has an optical rotatory dispersion spectrum composed of a trough close to 240 mμ and a peak between 210 and 215 mμ. The latter optical rotatory dispersion spectrum is similar to that of β-structured poly-*S*-carboxymethyl-L-cysteine in aqueous solution at pH values below 5 (Ikeda and Fasman, 1967). It seemed of interest, therefore, to examine the circular dichroic spectra of β-structured polypeptides cast as films and to establish the band shifts which occur between forms I and II.

* From the Eastern Regional Research Laboratory, Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Philadelphia, Pennsylvania 19118 (L. S, R. T., and S. N. T.), and Publication No. 596 from the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154 (G. D. F. and J. P.). *Received June 11, 1968.* This work was supported in part by grants from the National Institutes of Health (AM 5852), the National Science Foundation (GB 5576), and U. S. Army Medical Research Grant No. DA49-193 MD-2933.

† Present address: Graduate Department of Biochemistry, Brandeis University, Waltham, Mass. 02154.