was added, the mixture was kept at 58° for 2 hours, evaporated, extracted with methylene chloride, washed with water and dried over $MgSO_4$. Evaporation left 1.62 g. of yellow oil, which was distilled at 8μ pressure to give 1.57 g. (4.46 mmoles, 90%) of colorless liquid, b.p. 110–115°, $n^{25}D$ 1.5698.

Anal. Calcd. for $PS_2O_2C_{17}H_{21}$: C, 57.85; H, 5.97; P, 8.76; S, 18. 14. Found: C, 57.88; H, 5.96; P, 8.61; S, 18.53, 17.83.

O,O,S-Triphenyl Phosphorodithioate.—To 0.575 g. (5.22 meq.) of freshly distilled thiophenol in 50 ml. of *t*-butanol was added 20 ml. (4.80 meq.) of 0.240 *N* potassium *t*-butoxide soln. O,O-Diphenyl phosphorochlorido-thioate (1.21 g., 4.25 meq.) was added, and the mixture shaken and kept at 60° for 6 hours. The solvent was evaporated, the residue extracted with methylene chloride, and the methylene chloride extract washed with water and dried over $MgSO_4$. Evaporation of the solvent left 1.57 g. of yellow oil, which was evaporatively distilled at 5μ pressure to give 1.28 g. (3.58 meq., 84%) of colorless liquid, $n^{25}D$ 1.6340, which solidified on standing to give white prisms, m.p. 66–70°. Recrystallization from methanol raised the m.p. to 71.5–73.0°.

Anal. Calcd. for $PS_2O_2C_{18}H_{15}$: C, 60.40; H, 4.20; P, 8.66; S, 17.89. Found: C, 60.38, 60.51; H, 3.96, 4.11; P, 8.56, 8.79; S, 18.00, 18.20.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY MEDICAL COLLEGE, NEW YORK, NEW YORK]

Tritiation of Oxytocin by the Wilzbach Method and the Synthesis of Oxytocin from Tritium-labelled Leucine[1]

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The preparation of tritium-labelled oxytocin is described. The first approach leading to *randomly* labelled oxytocin involved the tritiation of a sample of synthetic oxytocin by the gas exposure technique of Wilzbach. After purification of the tritiated material by countercurrent distribution, a product was obtained with a specific radioactivity of 12 microCuries per mg. and a potency of 330 units per mg. All of the component amino acids were found to contain radioactivity as would be expected. *Specifically* labelled oxytocin was prepared by a total synthesis in which tritium-labelled L-leucine, obtained by tritiation of N-triphenylmethyldehydro-L-leucine, served as the starting material. The synthesis of the desired nonapeptide was accomplished *via* the tetrapeptide S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide which was coupled by means of dicyclohexylcarbodiimide with the pentapeptide S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparagine. Treatment of the product with sodium in liquid ammonia and subsequent aeration of the reduced nonapeptide yielded biologically active material which was purified by countercurrent distribution. A hormone preparation was obtained with a specific radioactivity of 130 microCuries per mg. and with a biological potency equal to that of our best preparations of nonradioactive oxytocin (approximately 500 units per mg.).

Early in our studies of oxytocin it was realized that an isotopically labelled preparation was desirable for certain chemical and biological investigations. Our first approach almost three years ago to a radioactive preparation was through random labelling of the hormone by the tritium gas exposure method of Wilzbach.[3,4] In this paper we wish to report some results with the application of this method to oxytocin and to present the synthesis of specifically labelled oxytocin.

For the preparation of randomly labelled oxytocin a sample of synthetic oxytocin, possessing approximately 450 U.S.P. units per milligram, was exposed to tritium for seven days. The resulting material, after having been dissolved in dilute acetic acid and lyophilized, was assayed again and found to possess about 330 units per milligram. The preparation was then repeatedly lyophilized from dilute acetic acid in an attempt to remove exchangeable tritium. Through electrophoresis on paper, it was found that only a small fraction of the radioactivity in the sample was bound to the hormone.

The bulk of the lyophilized material was subjected to two consecutive countercurrent distributions in the system butanol–ethanol–0.05% acetic acid (4:1:5). After 425 transfers the fraction present in the tubes representing the central portion of the peak of biological activity was removed and lyophilized. The material was then subjected to a further 100 transfer countercurrent distribution. The distribution curves for the biological activity, radioactivity and the color developed by the Folin–Lowry reaction agreed with the calculated distribution curve. From the active peak of the second countercurrent distribution, after concentration and lyophilization, a product was obtained in about 40% over-all yield having a specific radioactivity of 12.4 microCuries per mg. and a biological potency of 330 units per mg. After hydrolysis and paper chromatography according to the method of Levy and Chung[5] all of the amino acids were found to contain radioactivity. Glycine and tyrosine appeared to be somewhat more highly radioactive than the others.

The availability of *specifically* labelled oxytocin would obviously be of particular advantage in certain types of chemical, enzymic and metabolic studies of the hormone. The preparation of spe-

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(2) (a) Smith-Mundt-Fulbright Fellow under the auspices of the Institute of International Education. (b) Deceased February 3, 1959.

(3) K. E. Wilzbach, *J. Am. Chem. Soc.*, **79**, 1013 (1957).

(4) The application of the Wilzbach method to the random labelling of another posterior pituitary hormone, lysine-vasopressin, has been reported in a preliminary communication by Fong and co-workers (C. T. O. Fong, I. L. Schwartz, E. A. Popenoe, L. Silver, M. A. Schoessler, *J. Am. Chem. Soc.*, **81**, 2592 (1959)). After exposure of lysine-vasopressin to tritium, they obtained after purification an extremely low yield (0.1%) of tritiated lysine-vasopressin with a specific activity of approximately 300 micro Curies per milligram. By use of a modified Wilzbach procedure employing an electric discharge for tritium labelling, a preparation of arginine-vasopressin with high specific radioactivity and full biological activity has been more recently reported (C. T. O. Fong, L. Silver, D. R. Christman and I. L. Schwartz, *Proc. Natl. Acad. Sci. U. S.*, **46**, 1273 (1960)).

(5) A. L. Levy and D. Chung, *Anal. Chem.*, **25**, 396 (1953).

cifically labelled oxytocin of a biological potency equal to that of our best preparations of non-radioactive oxytocin (approximately 500 units per mg.) has been accomplished by a synthesis in which tritium-labelled L-leucine served as the starting material. This synthesis of tritium-labelled oxytocin is the first instance of the total synthesis of a radioactive polypeptide hormone and can be regarded as constituting the first step towards the synthesis of doubly and triply labelled oxytocin containing, in addition to tritium, C^{14} and/or S^{35} for the purposes mentioned.

For the preparation of tritium-containing leucine the intermediate N-acetyldehydro-DL-leucine was prepared. β-Methylallyl chloride (I) was condensed with ethyl acetamidomalonate (II) according to Albertson and Archer[6] to give ethyl 2-acetamido-2-carbethoxy-4-methyl-4-pentenoate (III) as shown in the accompanying equations and Compound III was saponified with aqueous sodium carbonate followed by decarboxylation to give the N-acetyldehydro-DL-leucine.[7]

$$CH_2{=}\overset{\overset{\displaystyle CH_3}{|}}{C}{-}CH_2Cl \ + \ H{-}\overset{\overset{\displaystyle COOC_2H_5}{|}}{\underset{\underset{\displaystyle COOC_2H_5}{|}}{C}}{-}NHCOCH_3 \ \longrightarrow$$

I   II

$$CH_2{=}\overset{\overset{\displaystyle COOC_2H_5}{}}{C}{-}CH_2{-}\overset{}{\underset{}{C}}{-}NHCOCH_3$$

$$\overset{\displaystyle CH_3}{} \qquad \overset{\displaystyle COOC_2H_5}{}$$

III

To avoid the resolution of radioactive acetylleucine the acetyldehydroleucine was resolved by means of hog kidney acylase. The resulting dehydro-L-leucine took up the theoretical amount of hydrogen upon catalytic hydrogenation. The hydrogenated product showed the same optical rotation and the same chromatographic behavior on paper as an authentic sample of L-leucine.

Although it may have been possible to obtain fairly high specific activities by reducing the free dehydroleucine with carrier-free tritium in glacial acetic acid, it was decided to use N-triphenylmethyldehydroleucine (N - trityldehydroleucine), which is soluble in solvents containing no labile hydrogen, and to carry out the reduction in ethyl acetate. N-Trityldehydro-L-leucine was therefore prepared. A trial reduction with hydrogen showed that the cleavage of the trityl group upon hydrogenation was not much slower than the reduction of the double bond in trityldehydroleucine. One hundred milligrams of N-trityldehydro-L-leucine in ethyl acetate solution was then reduced with carrier-free tritium gas in the presence of palladium on charcoal. The resulting crude product contained approximately 30 Curies. We proceeded with two-thirds of this quantity and, after removal of a large amount of exchangeable radioactivity, the radioactive preparation was freed from triphenylmethane and diluted with nonradioactive L-leucine to give a total of one gram of starting material for the synthesis. This amount possessed a total radioactivity of 12.5

Curies. Paper chromatographic and paper electrophoretic studies revealed the presence of radioactivity not attributable to leucine. It was decided, however, to proceed without further purification of the highly radioactive preparation in the expectation that the radioactivity not due to L-leucine would be eliminated during the subsequent synthetic steps as well as during the final purification of the oxytocin by countercurrent distribution.

The synthetic route employed by Cash[8] was used to obtain the C-terminal tripeptide sequence of oxytocin from the radioactive leucine. Coupling of the decarbobenzoxylated tripeptide with p-nitrophenyl S-benzyl-N-carbobenzoxy-L-cysteinate[9] and subsequent decarbobenzoxylation of the product yielded the radioactive tetrapeptide S-benzyl-L-cysteinyl-L-prolyl-L-leucyglycinamide. The desired radioactive protected nonapeptide intermediate S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S- benzyl - L-cysteinyl-L-prolyl-L-leucylglycinamide was obtained by condensation of the pentapeptide S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl- L - isoleucyl - L-glutaminyl-L-asparagine with the radioactive tetrapeptide by means of carbodiimide.[9] The yields in this radioactive synthesis were lower than those obtained in parallel syntheses with nonradioactive compounds. In fact the yield at the tripeptide stage was such as to necessitate a further dilution of the radioactive carbobenzoxy-L-prolyl-L-leucylglycinamide with an equal amount of nonradioactive compound before proceeding to the next step.

Radioactive oxytocin was prepared from the protected nonapeptide by reduction with sodium in liquid ammonia and subsequent oxidation to the disulfide form by aeration in aqueous solution. The crude material was purified by countercurrent distribution. After 1000 transfers in the system butanol–propanol–0.05% acetic acid (2:1:3) a highly purified preparation was obtained with a potency of approximately 500 avian depressor units per mg. and a specific radioactivity of 130 microCuries per mg.

Amino acid analysis of an acid hydrolysate of the material showed the amino acids and ammonia to be present in the expected ratios. Radioactivity was found only in leucine. In addition a portion of the material was subjected to a 400-transfer distribution to test the coincidence of the distribution of radioactivity, biological activity and Folin–Lowry color. The curves representing these values were in close agreement.

It may be noted that no loss of biological activity of the tritium-labelled oxytocin was observed during countercurrent distribution and storage in the countercurrent phases at + 4° for 12 weeks. The radioactive oxytocin was stored at this temperature in aqueous acetic acid solution containing 0.1% benzoic acid for subsequent use in biological studies on the distribution of oxytocin in various tissues and for chemical and metabolic studies of the hormone and its metabolites.

(6) N. F. Albertson and S. Archer, J. Am. Chem. Soc., 67, 308 (1945).

(7) N. F. Albertson, ibid., 72, 1396 (1950).

(8) W. D. Cash, J. Org. Chem., 26, 2136 (1961).

(9) M. Bodanszky and V. du Vigneaud, J. Am. Chem. Soc., 81, 2504 (1959).

Experimental[10]

Labelling of Oxytocin by Exposure to Tritium Gas.—
Synthetic oxytocin, weighing 152 mg. and having a biological
potency of 450 units per mg., was exposed at the new Eng-
land Nuclear Corporation, Boston, to 3 Curies of tritium gas
according to the method of Wilzbach at 0.39 atmosphere
and 4° for seven days. After the tritium gas had been re-
moved, the oxytocin was dissolved in about 5 ml. of 1% ace-
tic acid and lyophilized. At this point the biological potency
was found to be 330 units per mg. and the specific radio-
activity 175 μCuries per mg. The material was then redis-
solved and lyophilized as before. This procedure was re-
peated several times.

A sample of the lyophilized material in 0.2 N acetic acid
was subjected to electrophoresis on Whatman 3MM paper
in 0.2 N acetic acid at 300 volts for 14 hr. The paper was
sprayed with ninhydrin and one spot with some degree of
trailing was observed. A similar electrophoresis was carried
out in which the paper strip was not sprayed with ninhydrin
but was cut crosswise in 1 cm. sections which were then
counted in a windowless flow Geiger counter. The paper
strips were then macerated in 0.9% sodium chloride solution,
and after centrifugation the solution was assayed for biologi-
cal activity. The results showed that most of the radio-
activity was located near the origin, gradually decreasing to
a relatively low level in the region of biological activity.
Only about 10 to 15% of the total radioactivity was found in
the area where the hormonal activity was located.

Another experiment was carried out in which two aliquots
were run parallel to each other on the same paper. The
paper was cut lengthwise and one strip was sprayed with
ninhydrin reagent. A portion of the second strip which
corresponded to the ninhydrin spot on the other half was cut
out of the paper and eluted with 1 ml. of 0.25 N acetic acid.
This solution was evaporated to dryness, redissolved in a
small amount of acetic acid and again subjected to electro-
phoresis under the same conditions. The paper was not
sprayed with ninhydrin but was cut into 1 cm. sections and
each section counted as before. Each section was then
eluted with 2 ml. of 0.25 N acetic acid and the biological
activity and radioactivity were determined. The peak of
radioactivity corresponded with the peak of biological activ-
ity which indicated that some of the tritium was bound to
the hormone itself. An attempt was therefore made to iso-
late the labelled hormone by countercurrent distribution.
One hundred and ten mg. of oxytocin which had been re-
peatedly lyophilized after treatment with tritium gas as al-
ready described was distributed for 425 transfers in a 20 ml.,
200 tube Craig countercurrent machine in the system
butanol–ethanol–0.05% acetic acid (4:1:5). One peak was
obtained on plotting the values for the Folin–Lowry color
reaction, whereas the radioactivity was rather evenly dis-
tributed in all the tubes. The solutions in Tubes 105 to 125
comprising the peak were combined, concentrated to a small
volume and lyophilized. This material, weighing 45.4 mg.,
was then redistributed for another 100 transfers as before.
The distribution curves of the radioactivity, Folin–Lowry
color reaction and biological activity coincided with the
theoretical distribution curve. The solutions in Tubes 20–50
were combined, concentrated and lyophilized, yielding 43.1
mg. of labelled oxytocin, having a biological potency of 330
units per mg. and a specific radioactivity of 12.4 μCuries per
mg.

A two dimensional paper chromatogram of a hydrolyzed
sample of this material was carried out according to the
method of Levy and Chung.[5] The spots developed with
ninhydrin were cut from the paper and counted in a window-
less flow Geiger counter. All of the amino acids appeared to
be labelled. The spots which corresponded with tyrosine
and glycine were more highly radioactive than the others.

N-Acetyldehydro-DL-leucine.—A few small pieces of so-
dium were added to 2.5 liters of absolute ethanol, and 205 g.
(1.28 moles) of ethyl acetamidomalonate was then added.
Additional sodium was added over a period of one-half hour
until 21.6 g. had been used. The reaction mixture was
warmed to a gentle reflux and 111 g. (1.22 mole) of redistilled
methylallyl chloride was added over a 1 hr. period. The
solution was refluxed for 6 hr., cooled and allowed to stand
at room temperature overnight.

The crude ethyl 2-acetamido-2-carbethoxy-4-methyl-4-
pentenoate was subjected to alkaline hydrolysis followed by
decarboxylation. Most of the alcohol was removed *in
vacuo;* 2 liters of an aqueous solution containing 220 g. of
sodium carbonate was added and the mixture was heated at
reflux for 20 hr. Five grams of Norite then was added and
the hot solution was filtered through Solka Floc.[11] The clear
brown solution was acidified to Congo Red with concentrated
hydrochloric acid and kept at 0° for 40 hr. The crystalline
solid which had separated was collected by filtration yielding
70 g. of the product. More material was recovered by ex-
traction of the aqueous filtrate with ethyl acetate and con-
centration of the extract to a small volume. The combined
crude product was recrystallized once from water, yielding
90 g., m.p. 154–157°; lit., 160°,[7] 158–159°.[12]

Dehydro-L-leucine—Acetyldehydro-DL-leucine (70.0 g.)
was suspended in 3 liters of distilled water and the pH was
raised to 7.80 with 3 N LiOH. After the volume was made
up to 4 liters with water, 400 mg. of Acylase powder[13] was
added and the mixture was stored at 38° for 22 hr. The
reaction was followed by measuring the decrease in pH. A
constant pH of 6.90 was reached after 6 hr. The reaction
mixture was treated with charcoal at pH 5 and concentrated
by evaporation. The dehydro-L-leucine was crystallized
out by addition of ethanol;[14] yield, 18.0 g. (68%), m.p. with
decomposition 222–223°, $[\alpha]^{20}D$ −26.8° (c 0.97, 5 N HCl).

When this substance was chromatographed (descending)
on Whatman No. 1 paper in the system butanol–acetic
acid–water (4:1:5), a single sharp spot was obtained after
treatment with ninhydrin. The color of the spot was yellow
but turned violet after several days. An aliquot of the
material (800 mg.) was hydrogenated at 28° in 23 ml. of
glacial acetic acid with 20 mg. of 10% Pd on charcoal as
catalyst. The reaction was completed in 1 hr. at a hydrogen
pressure of 770 mm. The catalyst was filtered off and the
solution was evaporated to dryness *in vacuo* at 50°. The
evaporation was repeated twice, after 5 ml. of water had been
added each time. After a final drying *in vacuo* over P_2O_5 and
NaOH for 5 hr., the rotation of the substance was $[\alpha]^{20}D$
+22.7° (c 2.0, glacial acetic acid), which agreed with that of
an authentic sample of L-leucine. Hydrogenated dehydro-L-
leucine and an authentic sample of L-leucine were chromato-
graphed (descending) on parallel strips of Whatman No. 1
paper in the system butanol–acetic acid–water (4:1:5).
Both substances gave one ninhydrin spot with the same
R_f value (0.63). A small sample of the dehydro-L-leucine
was dissolved in boiling water, treated with charcoal
and crystallized[14]; m.p. with decomposition 222°, $[\alpha]^{20}D$
−27.9° (c 0.97, 5 N HCl).

Anal. Calcd. for $C_6H_{11}O_2N$: C, 55.8; H, 8.52; N, 10.9.
Found: C, 55.8; H, 8.65; N, 10.8. Hydrogen Number.[15]
Calcd.: 129.2. Found: 129.4.

N-Trityldehydro-L-leucine.—The procedure used by Ste-
lakatos, *et al.*,[16] for the preparation of tritylleucine diethyl-
ammonium salt was followed. From 5.2 g. of dehydro-L-
leucine 3.25 g. of N-trityldehydro-L-leucine diethylam-
monium salt (m.p. 135–140°) was obtained and 1.6 g. of
dehydroleucine was recovered from the reaction mixture.
A small sample of the N-trityldehydro-L-leucine diethyl-
ammonium salt was recrystallized twice from dry ether;
m.p. with decomposition 139–141°.

Anal. Calcd. for $C_{29}H_{36}O_2N_2$: C, 78.3; H, 8.14; N, 6.29.
Found: C, 78.4; H, 8.21; N, 6.37.

In order to obtain the free trityldehydro-L-leucine, 3.0 g.
of the diethylammonium salt was dissolved in 150 ml. of 0.05
N NaOH and the solution was left in a desiccator at the
water pump for several hours. Glacial acetic acid was then
added with stirring until the pH reached 5. The precipitate
which formed was filtered off, washed with water and dried

(10) All melting points were determined in capillary tubes and are
corrected.

(11) A cellulose product of Brown Co., New York.

(12) W. D. Celmer and I. A. Solomons, *J. Am. Chem. Soc.*, **77**, 2861
(1955).

(13) Armour Acylase tech. Lot 128-220, with 400 Armour Acylase
Units per gram. A proper amount of Acylase for the digestion was
determined in a preliminary experiment.

(14) For the isolation and purification of the dehydro-L-leucine,
the procedure described in *Biochem. Preparations*, **3**, 88 (1953), for
the resolution of DL-alloisoleucine was followed exactly.

(15) C. L. Ogg and F. J. Cooper, *Anal. Chem.*, **21**, 1400 (1949).

(16) G. C. Stelakatos, D. M. Theodoropoulos and L. Zervas, *J. Am.
Chem. Soc.*, **81**, 2884 (1959).

over $MgSO_4$ for 3 hr. *in vacuo*. It was then dissolved in 30 ml. of tetrahydrofuran which had been redistilled from sodium, and the solution was evaporated to dryness at room temperature *in vacuo*. This procedure was repeated 4 times with tetrahydrofuran and finally with a mixture of benzene–hexane (1:5).[16] The residue weighing 2.5 g. was obtained as a white fluffy foam.

Incorporation of Tritium into N-Trityldehydro-L-leucine. —The tritiation was done by New England Nuclear Corporation, Boston. It was carried out in a sealed 10 ml. flask containing 100 mg. of trityldehydro-L-leucine in 1 ml. of ethyl acetate, 150 mg. of catalyst (10% Pd on C) and 35 Curies of carrier-free tritium. After the mixture was shaken for 2 hr. at room temperature, the excess tritium gas was removed on the vacuum line. The catalyst was filtered off and rinsed with 5 ml. of ethyl acetate. After the catalyst had been washed with 10 ml. of glacial acetic acid, the combined filtrates were evaporated to dryness *in vacuo* and the residue was taken up in 50 ml. of ethanol containing 0.2 ml. of conc. HCl. Two-thirds of this solution, containing 20 Curies of radioactivity, were sent to this Laboratory.

Carbobenzoxy-L-prolyl-L-leucine.*[17]—The solution containing the radioactive material was evaporated *in vacuo* to about 3 ml. in a distillation apparatus at 20° with the capillary dipping into the solution connected to a nitrogen tank. The distillate contained 6 Curies. The distillation was repeated 6 times in the presence of 30 ml. of glacial acetic acid whereupon the radioactivity in the distillates fell to a fairly constant value of 30–50 mCuries.

After the last distillation the remaining solution was transferred to a 125-ml. Erlenmeyer with glacial acetic acid. Nonradioactive L-leucine (200 mg.) was added and the solution (40 ml.) was mixed with 70 ml. of water to remove triphenylmethyl derivatives. A fine white precipitate formed at once, and the mixture was allowed to stand for 2.5 hr. under nitrogen. The precipitate then was filtered off. Aliquots of the filtrate containing 1 μCurie (approximately 0.015 μg.) were chromatographed on Whatman No. 1 paper in the systems (A) butanol–acetic acid–water (4:1:5) (descending), (B) butanol–3% ammonia (1:1) (descending) and (C) acetone–water (4:6) (ascending). On each paper strip a spot of authentic L-leucine (5 μg.), which subsequently was developed with ninhydrin, was run parallel to the radioactive spot. When the paper was scanned for radioactivity, a peak comprising approximately 50% of the total radioactivity detected on the paper was found at the expected place for leucine (at R_f value 0.63 in system A, at R_f value 0.33 in system B and at R_f value 0.9 in system C). The remainder of the radioactivity was found as a peak with R_f 0.86–0.89 (system A), as a radioactive zone extending from the leucine peak to the solvent front with a peak at the front (system B) and as a sharp peak at the solvent front (system C). Another aliquot of the material (1 μCurie) was subjected to electrophoresis on paper at 0° for 16 hr. with the use of a potential gradient of 7 volts/cm. in 0.2% acetic acid. Only one radioactive peak was obtained at the expected place for leucine. There was, however, some high radioactive background on the paper between the origin and the peak.

The filtrate, which was obtained after removal of the triphenylmethyl derivatives, was evaporated to dryness *in vacuo* at 20–30° in a nitrogen atmosphere. The residue was diluted with 780 mg. of L-leucine to give 1.0 g. of radioactive leucine. The total radioactivity in this material was found to be 12.5 Curies. The preparation was kept prior to use in 25 ml. of glacial acetic acid containing a few ml. of benzene.

The glacial acetic acid–benzene mixture was removed *in vacuo* from the radioactive leucine preparation and the resi-

due was dissolved in a mixture of water (7.6 ml.) and triethylamine (1.55 ml.). To this solution the mixed anhydride of carbobenzoxy-L-proline and isobutylcarbonic acid was added. The mixed anhydride was prepared immediately before use by adding isobutylchloroformate (1.045 g.) in tetrahydrofuran (3 ml.) to a cold solution (−15°) of carbobenzoxy-L-proline (1.90 g.) in tetrahydrofuran (9.5 ml.) and triethylamine (1.1 ml.). The mixture was stirred for 20 minutes at −5° and after its temperature had been lowered to −20°, it was added to the radioactive leucine solution. The reaction mixture was stirred for 10 minutes at −10° and then 100 minutes at room temperature. Hydrochloric acid was added until the *p*H reached 3 and the tetrahydrofuran then was removed *in vacuo*. From the residual aqueous solution a heavy oil separated out which solidified overnight. The solid was washed by decantation with a few ml. of 0.05 N HCl. It was recrystallized (without heating) from approximately 200 ml. of acetic acid–water (1:20) and from approximately 100 ml. of chloroform–hexane (1:5). The purified product (1.67 g.) melted at 135–136.5° and showed $[\alpha]^{20}$D −76.0° (*c* 5.3, chloroform). It contained a total of 5.6 Curies of radioactivity. Nonradioactive carbobenzoxy-L-prolyl-L-leucine prepared by the same method showed $[\alpha]^{20}$D − 75.8° (*c* 6.0, chloroform), $[\alpha]^{20}$D −58.0° (*c* 1.0, ethanol) and melted at 137.5–139°.

Carbobenzoxy-L-prolyl-L-leucine* (1 μCurie [approximately 0.3 μg.]) and 50 μg. of nonradioactive carbobenzoxy-L-prolyl-L-leucine were chromatographed parallel to one another descending on the same strip of Whatman No. 1 paper in the solvent system butanol–3% aqueous ammonia (1:1). The radioactive run was scanned and the nonradioactive control run was treated with Bromocresol green according to Hiscox and Berridge.[18] A radioactive peak at the place of nonradioactive carbobenzoxy-L-prolyl-L-leucine representing approximately 70% of the radioactivity detected on the paper and additional activity between the peak and the front were obtained.

Carbobenzoxy-L-prolyl-L-leucyl*-glycinamide.—To the cooled solution (−15°) of carbobenzoxy-L-prolyl-L-leucine* (1.67 g.) in tetrahydrofuran (21 ml.) and triethylamine (0.67 ml.), isobutylchloroformate (0.633 g.) was added. The mixture was stirred for 10 minutes at −10° and its temperature was then lowered to −20°. Glycinamide hydrochloride (0.565 g.) in a mixture of water (5 ml.) and triethylamine (0.76 ml.) was added and the reaction mixture was stirred for 10 minutes at −10° and then for 80 minutes at room temperature. Hydrochloric acid was added until the *p*H reached 4 and the tetrahydrofuran was removed *in vacuo*. The crude, partially oily product which separated from the residual aqueous solution was washed with water and then dissolved in dichloromethane (25 ml.). The dichloromethane solution was extracted with 10 ml. of 0.5 N HCl, 10 ml. of water, 10 ml. of 0.5 M $KHCO_3$ and then 3 times with about 10 ml. of water. It was then stored in a calcium chloride desiccator for several hours. The dry solvent was removed *in vacuo* and the residue was crystallized from 100 ml. of chloroform–hexane (1:6). The dry substance melting at 151–156°, weighed 600 mg. and showed $[\alpha]^{20}$D −70.0° (*c* 2.0, glacial acetic acid). It contained 1.19 Curies. Nonradioactive carbobenzoxy-L-prolyl-L-leucylglycinamide prepared by the same method melted at 161–163° and showed $[\alpha]^{20}$D −73.0° (*c* 2, 95% ethanol), $[\alpha]^{20}$D −81.2° (*c* 2, glacial acetic acid).

S-Benzyl-N-carbobenzoxy-L-cysteinyl-L-prolyl-L-leucyl*-glycinamide.—To 0.60 g. of carbobenzoxy-L-prolyl-L-leucyl*-glycinamide in glacial acetic acid the same amount of nonradioactive carbobenzoxy-L-prolyl-L-leucylglycinamide was added. The solution was concentrated to a sirup by evaporation *in vacuo* at 20°. Hydrogen bromide (3 N) in acetic acid (5 ml.) was added and the solution was stirred for 100 minutes. Dry ether (20 ml.) then was added and the precipitate which formed was washed with ether and dissolved in methanol (30 ml.). Amberlite IRA 400 in the OH form was added to the solution until the reaction for bromide ion became negative.

The resin was filtered off and washed with methanol. The filtrate was evaporated *in vacuo* leaving a partially crystalline residue of the L-prolyl-L-leucyl*-glycinamide. This material was dissolved in dimethylformamide (1.2 ml.) and coupled with *p*-nitrophenyl carbobenzoxy-S-benzyl-L-cysteinate (1.35 g.). Two hours after the latter compound had

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(17) Where an asterisk is employed it is to indicate the presence of the radioactive leucine residue. Throughout the synthetic steps, care was taken not to leave or store peptide intermediates in the solid state for prolonged periods of time. Up to the tripeptide stage no crystallization was extended for more than 16 hr. The intermediates were dried *in vacuo* to constant weight and then dissolved immediately in a suitable solvent. The possible effect of air was reduced by storing all peptide solutions in an atmosphere of tank-nitrogen. Prolonged stirring or other manipulations were carried out while nitrogen was either bubbled through the solutions or flushed through the vessels used. All operations were carried out in an efficient hood. All steps of the synthetic work were tested at least twice with somewhat larger amounts prior to the radioactive synthesis, with the same reagents and materials except for the tritiated leucine. Yields and purities in these "cold runs" were obtained as described in the literature.

(18) E. R. Hiscox and N. J. Berridge, *Nature*, 166, 522 (1950).

been added the yellow solution had become dark brown and by the next day the semisolid mass of crystals appeared nearly black. Ethyl acetate (22 ml.) was added with stirring and after being allowed to stand for 1.5 more days at room temperature the mixture was stored in the refrigerator overnight. The crystalline mass was filtered off and washed with ethyl acetate (20 ml.). This product was grey, with the dark color remaining in the mother liqu r. The crystals were dissolved in warm methanol (15 ml.) and after the solution had been filtered with charcoal, water (9 ml.) was added to the warm filtrate. The following day the colorless crystals thus obtained were separated. They weighed 330 mg. (19%), melted at 170–171.5°, showed $[\alpha]^{20}D$ −84.0° (c 2.0, glacial acetic acid) and contained 0.154 Curie (13%). Previously prepared nonradioactive S-benzyl-N-carbobenzoxy-L-cysteinyl-L-prolyl-L-leucylglycinamide showed $[\alpha]^{20}D$ −59.0° (c 2.0, dimethylformamide), $[\alpha]^{20}D$ −81.8° (c 2.0, glacial acetic acid) and melted at 170–171.5°.

S-Benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucyl*-glycinamide.—The carbobenzoxy group was removed from the protected tetrapeptide S-benzyl-N-carbobenzoxy-L-cysteinyl-L-prolyl-L-leucyl*-glycinamide with HBr in glacial acetic acid as already described for the preparation of L-prolyl-L-leucyl*-glycinamide. The free tetrapeptide (5 μ Curies [approximately 10 μg.]) and parallel to it 10 μg. of nonradioactive free tetrapeptide were chromatographed (descending) on the same strip of Whatman No. 1 paper in the solvent system butanol–acetic acid–water (4:1:5). The radioactive run was scanned and the nonradioactive control run was sprayed with ninhydrin. A radioactive peak at the place of nonradioactive tetrapeptide representing approximately 85% of the radioactivity detected on the paper and a small peak near the solvent front were obtained. The free tetrapeptide was dissolved in dimethylformamide (2.2 ml.). After 0.384 g. of the pentapeptide S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparagine[9] had been added and dissolved, the solution was cooled to −10° and 0.24 g. of dicyclohexylcarbodiimide was added with stirring. The reaction mixture was allowed to stand at room temperature for 4 hr. and then at 0° overnight. The solidified mass was then mixed with glacial acetic acid (0.25 ml.) and ice-cold water (22 ml.). The precipitate was washed with cold water (30 ml.) on the filter and dried *in vacuo* over calcium chloride at room temperature. After extraction of the dry product with several portions of methanol (total 25 ml.) 330 mg. of solid was obtained.

This material was dissolved in dimethylformamide (4.7 ml.) and filtered, and the filter was rinsed with 3 ml. of the same solvent. Cold water (63 ml.) then was added with stirring, followed by glacial acetic acid (0.05 ml.) after 30 minutes. The white precipitate was washed on the filter with water and dried over calcium chloride at room temperature *in vacuo*. After extraction of this material with hot methanol (25 ml.) in several portions, 120 mg. of slightly grey solid remained.

Removal of the Protective Groups from the Labelled Protected Nonapeptide and Preparation of Biologically Active Material.—The protected nonapeptide (120 mg.) was dissolved in 150 ml. of liquid ammonia (redistilled from sodium), and the protective groups were removed by adding sodium from a glass capillary at the boiling point of the solution.[19] Ammonium chloride (80 mg.) then was added. The ammonia was evaporated in a partial vacuum, the last 50 ml. being removed *in vacuo* from the frozen state. The white, fluffy residue was dissolved in 200 ml. of 0.1% acetic acid, the pH was adjusted to 6.5 with 1 N ammonia and the solution was aerated with CO_2-free air for 13 hr. The solution then was acidified with glacial acetic acid to pH 4, filtered and evaporated *in vacuo* at room temperature to a small volume (23 ml.). This concentrated solution contained a total of 22,000 avian depressor units.[20] The amount of radioactivity found was 13 mCuries.

The concentrated solution was placed in the first 8 tubes of a 6 ml., 200 tube Craig countercurrent distribution machine and submitted to 640 transfers in the solvent system butanol–propanol–0.05% acetic acid (2:1:3) at +2°. A separation into 3 peaks as detected by Folin–Lowry color and radioactivity measurement was obtained with K values of 0.21, 0.35 and 0.44, the middle peak being biologically active. The tubes comprising the inactive peaks were emptied and refilled with fresh solvent and the distribution was continued for a total of 1,000 transfers with recycling. The biologically active peak found in Tubes 235–305 was removed from the machine in three portions. The contents of Tubes 235–255 (Fraction I), 256–280 (Fraction II) and 281–305 (Fraction III) were pooled separately and the upper phase of each fraction was extracted repeatedly with fresh lower phase which then was combined with the original lower phase. A total of 20,000 units of biological activity and 5.9 mCuries of radioactivity were recovered in the three fractions.

The side fractions containing a total of one-third of the biologically active material were approximately 30% less active in the fowl than the middle fraction, when dilutions of the same intensity of Folin–Lowry color were compared.

The three fractions showed on paper chromatography in butanol–acetic acid–water (4:1:5) and paper electrophoresis in 0.2% acetic acid and in pyridine acetate only one radioactive peak at the expected place for oxytocin.

An aliquot of the middle fraction was concentrated *in vacuo* and lyophilized. This material showed an avian depressor activity of approximately 500 units per mg.[9],[21] and a specific radioactivity of 130 μ-Curies mg. An amino-acid analysis of the lyophilized oxytocin was performed on a Beckman–Spinco amino acid analyzer according to the procedure of Spackman, Stein and Moore.[22] The following amino acid molar ratios (with the ratio of leucine taken as 1) were obtained: Aspartic acid 1.0, glutamic acid 0.9, proline 1.0, glycine 1.1, cystine 0.9, isoleucine 0.9, leucine 1.0, tyrosine 0.7, ammonia 2.8. The effluent from the column after having passed the spectrophotometer was collected in a fraction collector. The radioactivity of the fractions was measured by liquid scintillation counting. A single peak of radioactivity, corresponding to the position of leucine was found. As a further test of the homogeneity of the material, an aliquot of Fraction II equivalent to 9 mg. of oxytocin was subjected to countercurrent distribution for an additional 400 transfers in the same solvent system. The resulting distribution was analyzed by measurement of Folin–Lowry color, avian depressor activity and radioactivity. The three curves obtained by plotting these values were in close agreement with each other and with the calculated curve.

Measurement of Radioactivity.—Liquid scintillation counting was carried out in a Tracerlab scintillation counter using the Dioxane–Xylene Phosphor of Jacobson, *et al.*[23]; 10 ml. of phosphor solution was added to 5 ml. of dioxane containing the active sample (5–100 μl.). The efficiency was between 20 and 25% and was controlled with tritiated toluene (New England Nuclear Corp.) as an internal standard. Radiochromatograms were scanned with a scanning device using a windowless gas flow detector (Actigraph II, Nuclear Chicago Corp.).

values between 175 and 240 units per mg. of nonapeptide were obtained after aeration.

(21) Avian depressor activity was assayed by the method described in "The Pharmacopeia of the United States of America," 16th revision, Mack Printing Company, Easton, Pa., 1960, p. 546. However, the chicken was prepared according to the procedure of R. A. Munsick, W. H. Sawyer and H. B. van Dyke, *Endocrinology*, **66**, 860 (1960).

(22) D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

(23) H. I. Jacobson, G. N. Gupta, C. Fernandez, S. Hennix and E. V. Jensen, *Arch. Biochem. Biophys.*, **86**, 89 (1960).

(19) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts and P. G. Katsoyannis, *J. Am. Chem. Soc.*, **76**, 3115 (1954).

(20) Therefore 175 units of avian depressor activity had been obtained per mg. of protected nonapeptide. In previous "cold runs"

Studies on Polynucleotides. XII.[1] Experiments on the Polymerization of Mononucleotides. A Comparison of Different Polymerizing Agents and a General Improvement in the Isolation of Synthetic Polynucleotides[2]

BY H. G. KHORANA,[3] J. P. VIZSOLYI AND R. K. RALPH[3]

The polymerization of a standard mixture of 3'-O-acetylthymidine-5' phosphate and thymidine-5' phosphate in anhydrous pyridine has been studied with different activating agents. The reagents used were dicyclohexylcarbodiimide (DCC) as previously described, p-toluenesulfonyl chloride, 2,5-dimethylbenzenesulfonyl chloride, 2,4,6-trimethylbenzenesulfonyl chloride, diphenylphosphorochloridate and diisopropylcarbodiimide. Of these, dicyclohexylcarbodiimide is most satisfactory. A general improvement in the isolation of synthetic polynucleotides is described which involves the treatment of the total mixture of polymeric products with an excess of acetic anhydride in dry pyridine. This step leads to the cleavage of the frequently encountered side products which consist of oligonucleotides joined together by pyrophosphate linkages between their phosphomonoester groups. With the acetic anhydride-pyridine step, pure thymidine and deoxycytidine oligonucleotides were obtained directly by chromatography of the total polymeric mixtures on DEAE-cellulose (bicarbonate) columns.

In several previous papers of this series[1,4] the polymerizations of a number of deoxyribomononucleotides and the isolation and characterization of the resulting homologous series of polynucleotides have been described. The reagent used in most of the work has been dicyclohexylcarbodiimide (DCC) and the reaction mixtures, at least initially, have been heterogeneous. A further more or less common feature of the polymerization experiments described is that side products are obtained in which some of the oligonucleotides are joined together by means of a pyrophosphate bond between the phosphomonoester groups. The process of internucleotide (phosphodiester) bond formation starting with the mononucleotide is believed to pass through a number of steps, the first one of which is the formation of the symmetrical P¹,P²-dinucleoside pyrophosphates (I). Of the mononucleotides studied,

$$
\begin{array}{cc}
O & O \\
\| & \| \\
RO\overset{}{P}\!-\!O\!-\!\overset{}{P}OR \\
| & | \\
O^- & O^-
\end{array}
$$

I, R = nucleoside
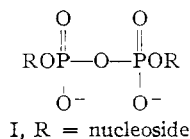
the process of phosphodiester bond formation reached nearest completion in the case of thymidine-5' phosphate.[4c] In the study of the polymerization of thymidine-3' phosphate, for example, significant amounts of oligonucleotides containing pyrophosphate bonds were present in the final products.[4b] In continuing our studies on the methods for the polymerization of mononucleotides, we have sought to compare the efficacy of some alternative reagents and the present communication describes the observations we have made on this subject. Further, a procedure involving treatment of the polymeric mixtures with acetic anhydride-pyridine is described which is effective in cleavage

of the pyrophosphate bonds present in the polymeric products. Consequently, the isolation of pure homologous polynucleotides from the polymeric mixtures is rendered easier.

Comparative Study of Activating Agents.—In the first study of the polymerization of thymidine-5' phosphate, p-toluenesulfonyl chloride and DCC were used as the reagents.[4a] However, thus far detailed investigations have been reported[4] only of the products obtained using DCC. Since, in contrast with the use of DCC, the reaction mixture using p-toluenesulfonyl chloride is homogeneous, a further examination of the latter reagent with a view to increasing the yields of the higher polymers has been undertaken. The polymerization of a mixture of 3'-O-acetylthymidine-5' phosphate and thymidine-5' phosphate was carried out under the conditions recently described for DCC.[4c] The elution pattern obtained after chromatography on a DEAE-cellulose column under the standard conditions[4c] is shown in Fig. 1. A comparison of this elution pattern with that published recently for the analogous experiment with DCC shows that the polymerization did not go as far as it did using DCC. Furthermore, the pattern with the sulfonyl chloride in the range of hexa- and higher polynucleotides (Fig. 1) indicated greater complexity and evidently much lower yields of these higher members were present. In addition there was evidence for the appearance of a new series of compounds with altered ultraviolet absorption characteristics. Thus peaks No. 1, 2, 4 and 7 (Fig. 1) had ultraviolet absorption spectra showing shifts in the λ_{max} from that characteristic of the thymidine chromophore (peak 1, λ_{max} 257 and 263 mμ; peak 2, λ_{max} 252 mμ; peak 4, λ_{max} 252 mμ; peak 7, λ_{max} 260 mμ). It is possible that two of these peaks represent mono- and dinucleotides bearing p-toluenesulfonyl groups at the terminal 3'-hydroxyl groups, and that higher homologs of similar compounds also are present in the latter peaks. It should be mentioned that these side products are different from those that contain the pyridinium chromophore at the $C_{3'}$ or $C_{5'}$ carbon of the terminal nucleoside (cf. ref. 4b, 4c and the following paper).

2,5-Dimethylbenzenesulfonyl chloride and 2,4,6-trimethylbenzenesulfonyl chloride also were tried

(1) Paper XI, R. K. Ralph and H. G. Khorana, J. Am. Chem. Soc., 83, 2926 (1961).

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