TABLE I

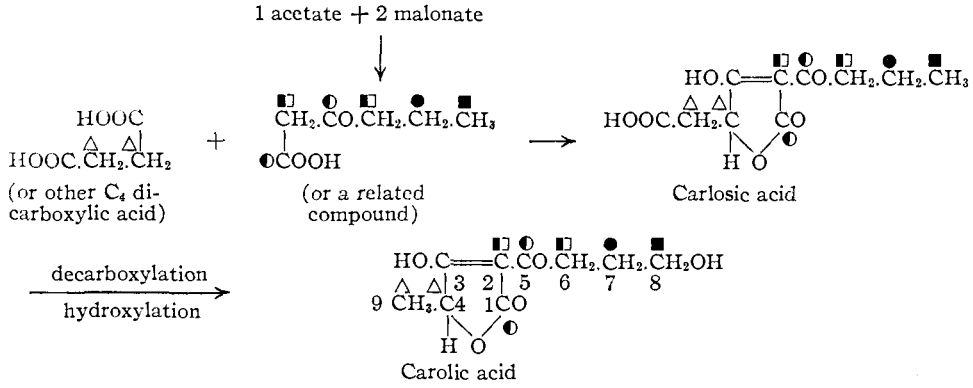
Precursor	% of total activity[a] C1	C2	C3	C4	C5	C6	C7	C8	C9
Acetate-1-C^{14}	35.3	—	—	—	35.9	—	28.6	—	—
Acetate-2-C^{14}	—	41.6	—	—	—	33.0	—	21.4	—
Malonate-1,3-C^{14}	50.6	—	—	—	46.7	—	—	—	—
Malonate-2-C^{14}	—	53.6	—	1.8	—	40.0	—	—	—
Succinate-2,3-C^{14}	3.9	19.8[b]	2.6	38.4	3.5	—	—	—	25.8

[a] Values below 1.0% are indicated by a dash. [b] The exact significance of this value is not clear. The figure is derived indirectly by difference from a mixture of C_2 and C_9 so that any deficit in the C_9 determination will make C_2 appear to be higher than it really is.

glucose after separate addition of acetate-1- and 2-C^{14}, malonate-1,3- and 2-C^{14} and succinate-2,3-C^{14}. The per cent. incorporations of radioactivity were, respectively, for acetates, 25.4 and 32.2; for malonates, 10.8 and 30.6; and for succinate, 5.7. The labeled carolic acids were degraded by hydrolysis to $CO_2(C_1)$, butyrolactone ($C_{5,6,7,8}$) and acetoin ($\overset{2,9}{CH_3}-\overset{3,4}{CHOH}-\overset{3,4}{CO}-\overset{2,9}{CH_3}$). To obtain separate C^{14} values for each carbon atom, butyrolactone was reduced to butyric acid for degradation by successive Schmidt reactions.[4] Acetoin was degraded by the method of Gross and Werkman.[5] Carolic acid also was converted to α-bromo-γ-methyltetronic acid which gave C_9 as the methyl and C_4 as the carboxyl of acetate on Kuhn-Roth oxidation and Schmidt degradation. The observed C^{14} distributions are given in Table I.

Carbons 3, 4, and 9 are not significantly labeled by either acetate or malonate. Lybing and Reio[2] reported a similar finding for carolic acid derived from acetate-1-C^{14} with *P. charlesii* P 146. Although labeling of carbons 3, 4 and 9 by acetate would have been expected from operation of the citric acid cycle, Lybing and Reio suggested that these atoms originate from one of the C_4 dicarboxylic acids of the cycle. The experiment with succinate-2,3-C^{14} shows clearly that this compound is an important precursor of carolic acid, and that C_4 and C_9 can, in fact, be derived from a dicarboxylic acid.

The results with labeled acetate, by themselves, would indicate that carbons 1, 2, 5, 6, 7, and 8 are derived by "head to tail" condensation of three acetate units. However, although malonate is well utilized for carolic acid biosynthesis, the malonate carbons contribute to only four of the six "acetate derived" carbons, namely, 1, 2, 5, and 6. The six carbon chain evidently is derived from one acetate unit and two malonate units, as is the case in fatty acid synthesis. Malonate apparently



■, carbon from acetate methyl only; ●, carbon from acetate carboxyl only; ◧, carbon from acetate methyl or malonate methylene; ◐, carbon from acetate or malonate carboxyl; △, carbon from succinate methylene.

The labeled acetate, by themselves, would indicate that carbons 1, 2, 5, 6, 7, and 8 are derived by "head to tail" condensation of three acetate units. However, although malonate is well utilized for carolic acid biosynthesis, the is not converted to acetate to any significant extent so that the unique role of acetate in initiating the synthesis of the fatty acid chain is clearly demonstrated. A similar participation of both acetate and malonate has been reported for the biosynthesis of penicillic acid[6] and of stipitatonic acid.[7] A possible outline of the pathway for carolic acid synthesis is shown in the accompanying reaction sequence. This hypothesis is strengthened by our finding that neither carbon of acetate significantly labels the COOH group of carlosic acid, also isolated in these experiments.

(4) E. F. Phares, *Arch. Biochem. Biophys.*, **33**, 173 (1951).

(5) N. H. Gross and C. H. Werkman, *Arch. Biochem.*, **15**, 125 (1947).

(6) R. Bentley and J. G. Keil, *Proc. Chem. Soc.*, 111 (1961); J. G. Keil, *Federation Proc.*, **20**, 80 (1961).

(7) R. Bentley, *Federation Proc.*, **20**, 80 (1961).

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RECEIVED MAY 25, 1961

PURIFICATION AND CHARACTERIZATION OF SHEEP PROLACTIN

Sir:

Sheep prolactin preparations previously have been shown to be mixtures of three electrophoretic components,[1,2] each reported to possess equal crop-sac-stimulating activity.[1] Recently, however, other workers[3] found a noticeable difference in the

(1) R. D. Cole and C. H. Li, *Biochim. Biophys. Acta*, **38**, 563 (1959); R. D. Cole, *J. Biol. Chem.*, **236**, 1369 (1961).

(2) J. G. Pierce and M. E. Carstens, *J. Am. Chem. Soc.*, **80**, 3482 (1959).

(3) K. A. Ferguson and A. L. C. Wallace, *Acta Endocrinol.*, Suppl. **51**, 293 (1960).

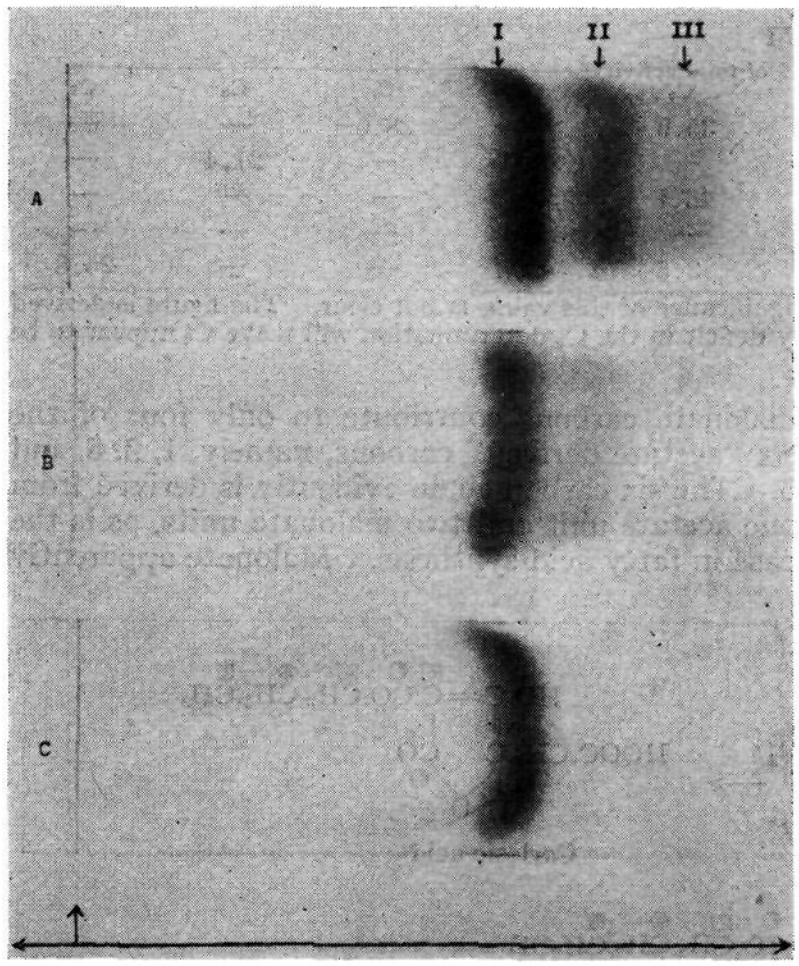

Fig. 1.—Isolation of component I: A, starting material (component I, 60%; component II, 30%; component III, 10%); B, fractions 40-90 obtained from DEAE-cellulose column (component I, 90%; component II, 10%); C, component I, obtained by rechromatography of fractions 40-90.

biological activity of the respective components of the prolactin mixture. We wish to record here fractionation studies on material of this type which have led to the isolation in substantially pure form of a major component of prolactin with markedly greater biological activity than that of the accompanying substances. This component, which appears to be homogeneous by the criteria of free boundary electrophoresis and ultracentrifugal analysis, exhibits a significant "growth hormone-like" activity in causing an increase in the body weights of plateaued female rats. It also causes a rise in the level of nonesterified fatty acids in the blood of fasted dogs which received 2 mg./kg. of body weight.

Prolactin isolated from sheep pituitary glands[4] was fractionated by column chromatography with DEAE (diethylaminoethyl)-cellulose, utilizing a 9-chamber variable gradient device[5] to obtain a compound elution gradient. Each chamber contained 100 ml. of eluent. One gram of starting material was applied to a 21 × 2.5-cm. column. We obtained good chromatographic resolution with a tris-(hydroxymethyl)-aminomethane–EDTA–borate buffer, "TEB" (0.05 *M* Tris, 0.016 *M* EDTA-disodium salt, 0.075 *M* H_3BO_3, 0.05 *M* NaOH), *p*H 9.0, $\Gamma/2 = 0.15$. The volume fraction of this buffer in each chamber of the gradient device is given in the following series: 0.26, 0.33, 0.33, 0.33, 0.33, 0.33, 0.33, 1.0, 1.0. The last two chambers contained in addition to the buffer 0.3 and 1.35 *M* NaCl, respectively. Protein concentration in each 5-ml. fraction of eluate was estimated by measuring absorption at 280 mμ. Fractions 40 to 90, comprising the first major chromatographic peak, were dialyzed, lyophilized and rechromatographed under identical conditions as described above. Fractions 40 to 100 obtained by rechromatography were combined, dialyzed and lyophilized. The progress of fractionation was followed by free boundary and by paper strip electrophoresis, the latter employing TEB-buffer, *p*H 9.0, $\Gamma/2 = 0.05$. Figure 1 depicts the progress of a typical fractionation, the components being named I, II, and III in order of increasing electrophoretic mobility. Component I behaved as a homogeneous protein in free boundary electrophoresis with an electrophoretic mobility of 3.80 × 10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1} in glycine–NaOH buffer, *p*H 10, $\Gamma/2 = 0.1$. The sedimentation constant in this same buffer was found to be 2.18 *S* with the sample sedimenting as a single, sharp symmetrical boundary. The isoelectric point of component I, as determined by free boundary electrophoresis, was *p*H 5.75. The N-terminal amino acid was found to be threonine by the fluorodinitrobenzene[6] and by the phenyl isothiocyanate[7] method. The number of moles of N-terminal threonine per mole of prolactin (mol. wt. 25,000) was found to be unity, after suitable corrections had been made for the partial destruction of this amino acid during hydrolysis. Action of carboxypeptidase on component I did not reveal a C-terminal amino acid. When performic acid-oxidized component I was treated with fluorodinitrobenzene, the only dinitrophenylated α-amino group found was threonine, while treatment with carboxypeptidase revealed cysteic acid at the C-terminal. Performic acid-oxidized component I sedimented as a single, sharp boundary during ultracentrifugation. These observations are in agreement with those reported previously by Cole and Li,[8] who found highly purified prolactin to be a single polypeptide chain with an intrachain disulfide loop at the C-terminal. Whereas these same authors found little or no difference in the biological and chemical properties of the three electrophoretic components of prolactin,[1] we noticed differences in both. Thus, highly purified preparations of components II and III, in contrast to component I, do show aspartic acid at the C-terminus upon direct digestion with carboxypeptidase. Furthermore, components II and III showed decidedly less biological activity than component I as illustrated by determination of lactogenic activity and of "growth hormone-like" activity. The latter was ascertained by determination of the net increase in body weights of plateaued female rats during a period of 10 days.[9] Lactogenic activity was determined by intramuscular injection of prolactin into crop sacs of white Carneaux pigeons.[10] These activities are summarized in Table I.

(4) R. D. Cole and C. H. Li, *J. Biol. Chem.*, **213**, 197 (1955).

(5) E. A. Peterson and H. A. Sober, *Anal. Chem.*, **31**, 857 (1959).

(6) H. Fraenkel-Conrat, J. I. Harris and A. L. Levy, "Methods of Biochemical Analysis," **2**, 370 (1955).

(7) H. Fraenkel-Conrat, J. I. Harris and A. L. Levy, "Methods of Biochemical Analysis," **2**, 393 (1955).

(8) R. D. Cole and C. H. Li, *J. Biol. Chem.*, **224**, 399 (1957).

(9) W. Marx, M. E. Simpson and H. M. Evans, *Endocrinology*, **30**, 1 (1942).

TABLE I

Preparation	Lactogenic activity I.U./mg.	"Growth hormone-like" activity: Total dose (mg.)	"Growth hormone-like" activity: Net change in body weight (g.)
Starting material (3 components)	20	0.75	+ 4
Component I	30–35	0.75	+15
Components II and III	5–10	0.75	− 1

When the temperature was maintained at −20° during the initial preparation of starting material from sheep pituitaries, our product contained a considerably greater proportion of component I than when the temperature was held at 5°. Also, highly purified component I can be converted easily into 3-component material upon standing in 2% aqueous NH_4OH at 5° for two hours.

The data obtained thus far indicate that component I is biologically highly active prolactin, whereas components II and III seem to be altered forms of this component.

Acknowledgment.—The authors wish to thank Dr. A. Segaloff for the crop-sac-stimulating assays, and Dr. D. E. Williams and Miss V. J. Powell for the physical chemical measurements.

(10) O. Riddle, R. W. Bates and S. W. Dykshorn, *Am. J. Physiol.*, **105**, 191 (1933).

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RECEIVED JUNE 7, 1961

FIRST OBSERVATION OF AQUEOUS TETRAVALENT CURIUM[1]

Sir:

We wish to report the first observation of *aqueous tetravalent curium*.

The measured $M^{+3} = M^{+4} + e^-$ potentials of U, Np, Pu and Am become increasingly negative with atomic number.[2,3] The presently unknown value for the $Cm^{+3} = Cm^{+4} + e^-$ potential is expected to be more negative than the $Am^{+3} = Am^{+4} + e^-$ potential of −2.6 to −2.9 volts[4] and to lie considerably beyond the normal limiting value for stability in aqueous solution. Previous attempts to oxidize Cm^{+3} in aqueous systems produced no positive results, confirming the expected stability of the $5f^7$ configuration.[5–9]

It was discovered recently that tetravalent americium (which has only a transient existence in usual aqueous media) exhibits remarkable stability in 15 *M* ammonium or alkali fluoride.[10] Such Am(IV) solutions show self-reduction at a rate of approximately 4%/hour due to the alpha activity of Am^{241}. It was hoped that tetravalent curium could be stabilized sufficiently by high fluoride concentrations to be observed in solution. A few milligrams of Cm^{244} had been brought to a high degree of purity from stocks made available to us by the Argonne National Laboratory. The specific activity of Cm^{244} is about 25 times that of Am^{241} and one would predict a self-reduction rate for Cm(IV) of 1–2%/minute. However, by using reasonably fast techniques, some 30–40 minutes should be available for spectral studies.

In the americium(IV) work, a compound containing americium *already in the tetravalent state* was used as a starting material, *i.e.*, $Am(OH)_4$ was dissolved in 15 *M* ammonium fluoride. Attempts to make $Cm(OH)_4$ by alkaline oxidation of $Cm(OH)_3$ using hypochlorite or ozone were not successful. Therefore dissolution of CmF_4 (prepared under anhydrous conditions) was attempted.

Curium tetrafluoride was prepared by treating CmF_3 with elemental fluorine using methods and apparatus previously described.[11,12] Addition of the resulting CmF_4 to 15 *M* NH_4F, either at 25 or 0°, produced only vigorous bubbling accompanied by the immediate formation of white CmF_3 from the yellow CmF_4. We believe that the Cm(IV) is oxidizing NH_4^+ under these conditions.

However, upon addition of CmF_4 to 15 M CsF at 0°, a solution of tetravalent curium as a fluoride complex is obtained. The light yellow solution shows an absorption spectrum similar to that previously obtained for solid CmF_4[12] (see Fig. 1).

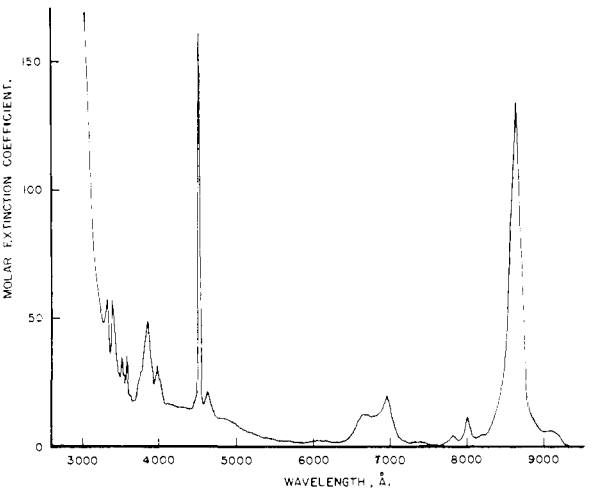

Fig. 1.—Absorption spectrum of Cm(IV) in 15 *M* CsF at 10.5° in 1 cm. quartz cell, 15 *M* CsF reference.

The 15 *M* CsF solution was pretreated with ozone (*ca.* 5% O_3 in O_2) for one hour to oxidize traces of impurities. The ozone stream was continued for about 10 minutes to stir and dissolve

(1) This work was performed under the auspices of the U. S. Atomic Energy Commisssion.

(2) B. B. Cunningham, *Proc. Int'l. Conf. Peaceful Uses of Atomic Energy*, **7**, 225 (1956).

(3) B. B. Cunningham, *XVIIth Int'l. Congress of Pure and Applied Chemistry*, 1, 64 (1960).

(4) R. A. Penneman, J. S. Coleman and T. K. Keenan, *J. Inorg. Nucl. Chem.*, **17**, 138 (1961).

(5) R. A. Penneman and L. B. Asprey, *Proc. Int'l. Conf. Peaceful Uses of Atomic Energy*, **7**, 355 (1956).

(6) J. J. Katz and G. T. Seaborg, "The Chemistry of the Actinide Elements," John Wiley and Sons, Inc., New York, N. Y., 1957, pp. 381–383.

(7) S. W. Rabideau, L. B. Asprey, T. K. Keenan and T. W. Newton, *Proc. Second Int'l. Conf. Peaceful Uses of Atomic Energy*, **28**, 361 (1958).

(8) T. K. Keenan, *J. Chem. Educ.*, **36**, 27 (1959).

(9) R. A. Penneman and T. K. Keenan, "The Radiochemistry of Americium and Curium," NAS-NS-3006 (1960).

(10) L. B. Asprey and R. A. Penneman, *J. Am. Chem. Soc.*, **83**, 2200 (1961).

(11) L. B. Asprey, *et al.*, *ibid.*, **79**, 5825 (1957).

(12) L. B. Asprey and T. K. Keenan, *J. Inorg. Nucl. Chem.*, **7**, 27 (1958).