REFERENCES

[1] L. RÉVÉSZ, A. FORSSBERG AND G. KLEIN, *J. Natl. Cancer Inst.*, 17 (1956) 37.
[2] J. GREENLEES AND G. A. LEPAGE, *Cancer Research*, 15 (1955) 256.
[3] K. MOLDAVE, *Proc. Soc. Exptl. Biol. Med.*, 92 (1956) 783.
[4] A. D. BABSON AND T. WINNICK, *Cancer Research*, 14 (1954) 606.
[5] D. STEINBERG AND M. VAUGHAN, *Biochim. Biophys. Acta*, 19 (1956) 584.
[6] M. HEIMBERG AND S. F. VELICK, *J. Biol. Chem.*, 208 (1954) 725.
[7] M. V. SIMPSON AND S. F. VELICK, *J. Biol. Chem.*, 208 (1954) 61.
[8] D. S. HOGNESS, M. COHN AND J. MONOD, *Biochim. Biophys. Acta*, 16 (1955) 99.
[9] B. ROTMAN AND S. SPIEGELMAN, *J. Bacteriol.*, 68 (1954) 419.
[10] A. L. KOCH AND H. R. LEVY, *J. Biol. Chem.*, 217 (1955) 947.
[11] A. LEVAN AND T. S. HAUSCHKA, *Hereditas*, 38 (1952) 251.
[12] L. GOLDBERG, E. KLEIN AND G. KLEIN, *Exptl. Cell. Research*, 1 (1950) 543.
[13] G. KLEIN AND L. RÉVÉSZ, *J. Natl. Cancer Inst.*, 14 (1953) 229.
[14] H. J. TJIO AND A. LEVAN, *Lunds Univ. Årsskr.*, 50/15 (1954) 1.
[15] A. FORSSBERG AND G. KLEIN, *Exptl. Cell. Research*, 7 (1954) 480.
[16] D. STEINBERG, M. VAUGHAN AND C. B. ANFINSEN, *Science*, 124 (1956) 389.

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THE GUANIDINATION OF SOME BIOLOGICALLY ACTIVE PROTEINS*

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S-Methyl isothiourea was first employed by SCHÜTTE[1] to convert the free amino groups in proteins to guanidino groups, and has subsequently been used for a similar purpose by CHRISTENSEN[2] and by ROCHE *et al.*[3,4]. The reaction of O-methyl isourea with amino acids and peptides was investigated by GREENSTEIN[5,6], and later this reaction was employed by HUGHES *et al.*[7] for the preparation of guanidinated human serum albumin. Recently CHERVENKA AND WILCOX[8] reported the complete guanidination with O-methyl isourea of the free ε-amino groups in chymotrypsinogen.

Although a number of biologically active substances have been guanidinated, with the exception of the aforementioned study on chymotrypsinogen[8] no reports have appeared on the effect of guanidination on biological activity. It is the purpose of this communication to present information relative to the guanidination of two pituitary hormones, lactogenic hormone (prolactin) and growth hormone (somatotropin), and of the enzyme lysozyme.

MATERIALS AND METHODS

O-Methyl isourea acid sulfate was prepared from O-methyl isourea hydrochloride[9] by the method of HUGHES *et al.*[7]. O-Methyl isourea neutral sulfate was synthesized by the method of BELLO[10]. In the initial experiments, the acid sulfate was employed; it was converted to the free base with $Ba(OH)_2$, and used according to the conditions employed by HUGHES[7]. Other experiments were

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carried out with the neutral sulfate, which was either converted to the free base with $Ba(OH)_2$, or used directly in concentrated NH_4OH[1,3], or at pH 10.5–11.0 (adjusted with KOH). Most of the experiments were performed by this last method. All reactions were carried out at 0° for from 3 to 5 days. The extent of guanidination was determined by the following methods: (a) determination of arginine and homoarginine in acid hydrolysates of proteins by the method of SAKAGUCHI[11]; (b) reaction of the protein with fluorodinitrobenzene (FDNB)[12] and determination of the amount of free ε-DNP lysine present following acid hydrolysis of the dinitrophenylated protein; and (c) determination of the homoarginine that is formed and of whatever free lysine may still be present, by means of dinitrophenylation of the acid hydrolysates of the proteins and two-dimensional paper chromatography of the dinitrophenylated amino acids[13]. In the "toluene" system, which is used for developing the first dimension in this procedure[13], DNP-homoarginine has a greater R_F than does DNP-arginine, the only other water-soluble DNP-amino acid present. A semi-quantitative estimation of the homoarginine formed and of the lysine remaining could also be obtained from the pattern of spots revealed after chromatography of an acid hydrolysate in the two-dimensional paper chromatographic system of LEVY AND CHUNG[14]. Homoarginine has a slightly greater R_F than arginine in the butanol–acetic acid–water system, and a much greater R_F than arginine in the buffered cresol–phenol system, which is used in the second dimension. In all experiments hydrolysis was carried out in a vacuum in constant boiling HCl for 24 hours at 110°.

Quantitative N-terminal group analysis was performed on the hydrolysates of the dinitrophenylated proteins by means of the two-dimensional paper chromatographic procedure of LEVY[13].

Acetylation was carried out with acetic anhydride according to the procedure of HUGHES[15,16]. Deamination[17] was effected in N $NaNO_2$ at pH 4 for 30 minutes at 0°. Oxidation with periodate was carried out at room temperature as previously described[18]. In some instances the excess periodate was removed by adjusting the pH of the chilled solution to the region of maximum protein insolubility (pH $\simeq$ 5.5) and then thoroughly washing the precipitate with water.

Subsequent to all the reactions except the dinitrophenylation, the proteins were thoroughly dialyzed; in the case of lysozyme, special care was exercised in the choice of dialysis membranes.

Growth hormone was prepared from the anterior lobes of beef pituitaries by the method previously described[19], and lactogenic hormone was derived from whole sheep pituitaries by previously published methods[20,21]. Lysozyme was obtained from the Armour Laboratories.

The potency of the growth hormone was determined in hypophysectomized rats by the tibia test[22,23], and the lactogenic activity was estimated in pigeons by the method of minimal stimulation of the crop sac[24]. The activity of the lysozyme was determined with suspensions of phenol-killed *M. lysodeikticus** and with Difco Lysozyme substrate, by two different procedures[25,26].

RESULTS

Growth hormone

Native growth hormone is known to possess 23 lysine residues[27] and 2 free α-amino groups[28,29] per mole of 46,000 molecular weight. After being treated with O-methyl isourea at pH 10.5 in the cold for 5 days, the hormone was found to possess 21 to 22 homoarginine groups and 1 or 2 lysine residues per mole. Both α-amino groups were still completely available for dinitrophenylation after guanidination of the protein, and the subsequent use of a reaction which detects guanidino groups[30] on the chromatograms of the hydrolysates of the guanidinated protein revealed no positively-reacting groups other than arginine and homoarginine, indicating that there had been no reaction with an α-amino group. Some of these guanidinated preparations (*ca.* 21 homoarginine groups) were submitted to zone electrophoresis on starch in 0.1M Na_2CO_3 at pH 11.2, where, because of the difference between the pK values of the ε-amino and guanidino groups, a distribution or spectrum of the guanidinated species in any single preparation would be expected to be revealed. However, the electrophoretic patterns obtained showed only one major peak with a small percentage of the material appearing as two distinct shoulders of lesser anodic mobility. No material with a mobility similar to that of the unreacted protein was seen. Free electrophoresis of a similar guanidinated preparation in the Perkin-Elmer model of the Tiselius ap-

* We are indebted to Dr. R. DELEY for culturing the *M. lysodeikticus*.

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paratus in an acetate buffer of pH 4.0 and 0.03 ionic strength gave two components whose mobilities were identical with those previously reported for the components in the partially denatured hormone[31]. This may be taken to mean that the hormone has been partially denatured (30% or less) in the course of the guanidination but that no alteration of electrophoretic behavior due to the substitution of guanidino groups for ε-NH_2 groups is apparent at this pH.

When the control and the guanidinated preparations were assayed for growth-promoting activity, they were all found to be active (Table I). Whatever small amount of inactivation that may have occurred is undoubtedly due to some denaturation of the protein by alkali[31]. Hence, it appears that the transformation of an ε-amino group to a guanidino group results in no alteration of the biological activity. In contrast to guanidination, acetylation, under conditions which led to the substitution of approximately 13 ε-amino groups, resulted in a complete loss of growth-promoting activity (Table I). When the acetylated growth hormone was submitted to the fluorodinitrobenzene (FDNB) procedure, it was demonstrated that almost all of the α-NH_2 groups had been acetylated. Since there was a possibility that modification of the α-amino groups was responsible for the loss of activity, further experiments were undertaken in which guanidinated growth hormone preparations, which are known to possess free α-NH_2 groups, were submitted to acetylation. It was found that the acetylated-guanidinated protein possessed no free α-amino groups, and that its activity was essentially intact (Table I). The latter preparations also served as controls for the acetylation of the native hormone, showing that when loss of activity occurs it is not due to acetylation of non-amino groups (*e.g.*, tyrosine phenoxyl).

TABLE I

BIOLOGICAL ACTIVITY OF DERIVATIVES OF GROWTH AND LACTOGENIC HORMONES AND OF LYSOZYME

Protein	*Treatment*	*Proportion of amino groups reacted*		*No. of assay animals*	*Proportion of original activity remaining*
		α	ε		
		%	%		%
Growth hormone	Guanidinated	0	94	32 (4)*	80
	Acetylated	>95	57	8 (2)	< 10
	Acetylated-guanidinated**	>95	>99	11 (2)	70
Lactogenic hormone	Guanidinated	0	>99	11 (3)	100
	Acetylated	>95	70	9 (2)	< 10
	Acetylated-guanidinated**	>95	>99	12 (3)	100
	Periodate-oxidized	>95	0	12 (3)	75
Lysozyme	Guanidinated	0	>99	(4)	100
	Acetylated	>95	67	(8)	20***
	Acetylated-guanidinated**	>95	>99	(4)	100

* Number in parentheses is No. of individual assays.
** The actual order of reaction was guanidination followed by acetylation.
*** See DISCUSSION.

Lactogenic hormone

Amino acid analysis and end-group determinations have disclosed the presence of 10 lysine groups[32] and one α-amino group[18] in lactogenic hormone. When the hormone was treated with O-methyl isourea, all the lysine residues were converted to homo-

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arginine. End-group analysis of the treated hormone revealed that the N-terminal threonine [18] was unmodified, and, as in the case of growth hormone, paper chromatography yielded no evidence of any guanidino compounds other than arginine and homoarginine. Even though complete conversion of the ε-amino groups to guanidino groups had occurred, the biological activity was not affected (Table I). On the other hand, acetylation of 8 amino groups in the molecule resulted in a marked loss of activity (Table I). However, no loss of activity resulted from acetylation of the guanidinated hormone. In none of the acetylated preparations was the N-terminal threonine any longer free to react with FDNB. According to these results, the hormone apparently does not require α-amino groups for its biological activity. Additional confirmation of this conclusion could be obtained by allowing the native and guanidinated hormones to react with periodate. Under these conditions, the terminal threonine was quantitatively transformed[18] to acetaldehyde and glyoxylic acid leaving the glyoxylic acid at the N-terminus. The absence of an α-amino group from the periodate-treated hormone was confirmed by dinitrophenylation. Such preparations were uniformly active (Table I).

Lysozyme

The ε-amino groups of lysozyme, like those of lactogenic hormone, were found to be completely guanidinated following treatment with O-methyl isourea. After hydrolysis of the dinitrophenylated guanidinated protein, no ether-soluble DNP amino acids could be extracted from the hydrolysate. Chromatography of the aqueous phase revealed that the N-terminal lysine[33] had been completely transformed into homoarginine, present in the hydrolysate as DNP-homoarginine. Assays of enzymic activity indicated that such preparations were still fully active, in contrast to the extensively acetylated lysozyme preparations, which were shown to possess less than one-third of the original activity (Table I). Acetylation of the guanidinated preparation once again resulted in undiminished activity, even though the α-amino group had been substituted.

DISCUSSION

Extensive acetylation of growth hormone has been found to result in a loss of biological activity, confirming the earlier report of LI AND EVANS[34]. The results summarized in Table I also confirm previous findings that acetylation results in inactivation of lactogenic hormone[35] and of lysozyme[36]. The case of lysozyme deserves special mention. In some preparations in which approximately one-third of the amino groups were acetylated, no loss of biological activity was observed. Even with the extensively acetylated preparations recorded in Table I, about one-quarter of the original activity could still be detected. Since no unmodified lysozyme is present in such preparations, it would appear either that all the lysine groups are not necessary for activity or that the modified lysozyme still possesses some activity, albeit diminished or modified[36].

Similarly it was noted that when the experimental conditions for the acetylation of growth hormone were modified, so that fewer acetyl groups were introduced, no loss of biological activity was observed. The modified procedure was carried out as follows: 50 mg of somatotropin was dissolved in 50 ml of 0.2 *M* sodium acetate solution, and after adding 0.1 ml acetic anhydride, the solution was kept at 0° C for 4 hours. The solution was then dialyzed thoroughly and lyophilized. Dinitrophenylation of the

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product gave evidence that the two N-terminal amino groups and 8 ε-amino groups of lysine were acetylated. From N-acetyl determination*, it was found that the acetylated material contained 1.2% acetyl residues, a value in fair agreement with the value computed from the number of amino groups which were not available for dinitrophenylation. Bioassay of the acetylated hormone indicated that it retained growth-promoting activity. This activity cannot be due to the presence of the native hormone since the acetylated preparation migrated during free electrophoresis essentially as a single component with an electrophoretic mobility of $4.2 \cdot 10^{-5}$ cm^2 per sec per volt in pH 4.0 buffer of 0.03 ionic strength at 1° C, whereas in the same buffer the native hormone has a mobility[31] of $6.5 \cdot 10^{-5}$. From these experiments, it is evident that not all of the ε-amino groups are essential for activity. Apparently, 8 out of 23 ε-amino groups in the hormone molecule can be acetylated by this milder procedure without appreciable loss of growth-promoting activity.

In contrast to the inactivation observed following extensive acetylation of these various proteins, complete retention of biological activity was observed in all instances following guanidination. Furthermore, acetylation after guanidination was now without effect on the activity. These results indicate that (a) whereas loss of the positive charge on the ε-amino groups through acetylation results in loss of activity, conservation of that charge implies conservation of activity despite a conversion of amino to guanidino groups; and (b) loss of the positive charge on the α-amino groups of these proteins through acetylation in no way affects the biological activity, indicating that in these three proteins the α-amino groups are not necessary for activity.

The non-essentiality of the N-terminal amino groups in lactogenic hormone for biological activity has been substantiated by the observation that destruction of the N-terminal threonine by oxidation with periodate does not affect the activity. In order to support the conclusion that α-amino groups are not essential for activity in connection with the other two proteins as well, deamination of the guanidinated proteins with $NaNO_2$ at pH 4 was attempted (see METHODS). In every instance loss of activity was virtually complete. However, since all preparations became markedly red or yellow in color and marked changes in the absorption spectra of the proteins were observed, it was felt that the loss of activity could be attributed to modification of tyrosine and other essential groups and consequently that no conclusions could be reached regarding the specific effects of deamination.

The findings reported here should in no way be subject to generalization; they are derived solely from experience with three specific proteins. In the case of growth hormone, the finding that α-amino groups are not necessary for activity confirms the preliminary findings of REID[37], who reported that in the reaction of growth hormone with only a small excess of acetic anhydride, it was chiefly the α-amino groups that were acetylated, and that under these conditions the activity was unchanged. Loss of activity resulted only when ε-amino groups were extensively acetylated, for which greater excesses of acetic anhydride were required. Thus REID's kinetic method and the method of selective acetylation of the guanidinated protein employed in this study both yield similar results.

The finding that α-amino groups offer an apparently complete resistance to guanidination is in agreement with the results of GREENSTEIN[5], ROCHE *et al.*[4], and CHERVENKA AND WILCOX[8].

* By Elek micro-analytical laboratories, Los Angeles.

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With respect to the extent of guanidination, growth hormone behaved differently from both lactogenic hormone and lysozyme. The ε-amino groups of the latter proteins could be fully guanidinated, but those of growth hormone were only 90–95% guanidinated. ROCHE *et al.*[4] experienced similar difficulties with thyroglobulin, and preliminary experiments that we have performed with a number of other proteins indicated that under none of the conditions employed could either insulin or α-corticotropin be completely guanidinated. Insulin possesses only a single ε-amino group, yet only 0.2–0.5 moles of homoarginine were found. In α-corticotropin only 3 out of the 4 lysine residues were converted to homoarginine. Guanidination of this pituitary hormone, though obviously incomplete, produced a derivative whose biological activity was similar to that of the control preparations. As with the other hormones, acetylation of α-corticotropin resulted in complete loss of activity. However, unlike the results with the other hormones, acetylation of the guanidinated derivative of α-corticotropin also resulted in complete loss of activity. Although with this hormone guanidination was incomplete, these results may be interpreted as confirming the findings of WHITE[38] and of DIXON[39] that the N-terminal serine of the corticotropins is necessary for their activity.

Acetylation of lactogenic hormone with ketene has been previously reported[35]. However, since this reagent reacted extensively with tyrosine phenolic groups as well as with amino groups, only with reservation could any conclusions be presented with regard to the essentiality of amino groups. Acetylation with acetic anhydride under the conditions described by HUGHES[15] has been found to lead to acetylation of amino groups alone. Investigation of the absorption spectra of all the acetylated proteins gave no evidence of any reaction on the part of the tyrosine phenolic groups, and no indication of an increase in absorption at 263 mμ, which is characteristic of O-acetyl tyrosine, was observed.

Although guanidination of lactogenic hormone proceeded equally well under all the various conditions for guanidination that were employed, poor recovery of activity was encountered after treatment in concentrated ammonia. However, non-guanidinated control preparations were also partially inactivated by the latter treatment. Since reaction at pH 10.5 (adjusted with KOH) permitted complete retention of activity, this apparently milder condition is to be preferred.

The procedures for the assay of the biological activity of growth and lactogenic hormones, as is the case for all pituitary hormones, have limitations in terms of sensitivity and in terms of quantitative determination. Consequently, an enzyme was sought for which a simple and sensitive assay procedure was available. Furthermore, in order to establish that conservation of positive charges was attended by retention of activity whereas removal of such charges was not, it was necessary to find an enzyme whose activity was known to be markedly decreased by acylation. This immediately eliminated from consideration almost all the proteases (*cf.* [16]) including chymotrypsinogen[8]. Lysozyme not only fulfills the first two requirements, but also is already known to react completely during the guanidination procedure[4]. In addition to the studies with lysozyme, one single experiment with ribonuclease was performed; this latter enzyme also fulfills the two requirements mentioned above. A control preparation which had been submitted to the conditions of the guanidination procedure, in the absence of O-methyl isourea, was fully active*. The fully guanidinated preparation,

* We are indebted to Dr. FRANK F. DAVIS for the ribonuclease assays.

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however, was shown to have lost a considerable portion of its activity, with only 20% of the original activity remaining. Both acetylated and acetylated-guanidinated preparations were essentially completely inactive. Thus, of all the biologically active proteins that have been guanidinated and whose potencies have been determined, ribonuclease alone manifested any extensive modification of activity.

The results obtained in the course of these studies suggest that guanidinated proteins may be useful for various types of investigations. One potential use would be for the preparation of active labelled hormones for tracer studies. ^{14}C-Labelled cyanamide can be easily converted to O-methyl isourea[10], which could then be used to guanidinate the protein; derivatives obtained in this manner would possess distinct advantages over the commonly used ^{131}I-labelled derivatives. At least in the cases of growth and lactogenic hormones, and of α-corticotropin, maximal incorporation of the radioactive guanidinated derivative would still leave the hormonal activity intact*. This is not true of iodinated preparations, which also suffer from the limitation that the tagged tracer molecules may themselves possibly be inactive, so that the fate of the radioactive molecules might not be the fate of the native hormone. Furthermore, iodination introduces large iodine atoms which may produce steric effects. Iodination also introduces a change in the charge of the proteins in the physiological pH range, since the pK of the tyrosine phenolic groups (*ca.* 10) is decreased to about 8 in monoiodinated and about 6.5 in diiodinated derivatives[40]. Such derivatives may also be subjected to the action of specific dehalogenating enzymes.

Another area of investigation would be in reactions designed to effect preferential modification of α-amino groups. Just as the acetylation technique discussed in this paper becomes selective when applied to the guanidinated protein, addition of amino acids by the N-carboxy anhydride method[41,42] and removal of amino acids by the phenyl*iso*thiocyanate technique[43,44] also become selective when performed on the guanidinated protein. The phenyl*iso*thiocyanate procedure may then be employed for determining the essentiality of the various amino acids in an N-terminal sequence for the activity of a protein. Ordinarily, where ε-amino groups are necessary for activity, the formation of ε-phenylthioureido groups by phenyl*iso*thiocyanate immediately inactivates the proteins and makes it impossible to determine the effects of stepwise degradation upon activity.

Finally, other types of guanidinated derivatives, for example phenylguanidinated, whereby the lysine side chains can be lengthened and large steric factors introduced with no appreciable effect on the positive charge (compared to unsubstituted guanidino groups a decrease in pK of from 1 to 3 units may be expected[45]), should be useful for studying reactions where a close approach of two molecules (*e.g.*, enzyme–substrate interactions) has been postulated.

SUMMARY

1. The guanidination of pituitary growth and lactogenic hormones and of lysozyme has been described. In all three instances guanidination has had no effect on the biological activity of the proteins.

* Since completely substituted derivatives may not be necessary for such studies, it would be advisable to carry out guanidination at as low a pH (*ca.* pH 10) and for as short a period of time as possible. The partially guanidinated derivatives thus obtained should be more soluble than the completely substituted proteins, and the chances of alkaline denaturation of the protein would be markedly decreased. Excess reagent could be recovered following precipitation as the picrate.

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2. On the other hand, in confirmation of earlier results, acetylation of all three proteins was found to lead to inactivation; however, acetylation of the guanidinated derivatives does not result in inactivation.

3. Since it has been shown that only the ε-amino groups are guanidinated, the results have been interpreted as indicating that the α-amino groups of these proteins are not necessary for biological activity. For maintenance of the activity, what is essential is conservation of the positive charge on the lysine side chain, independent of whether this charge results from the presence of an ε-amino or a guanidino group.

4. The results of preliminary data on the guanidination of insulin, α-corticotropin and ribonuclease have been presented.

5. Some possible uses for the guanidinated derivatives have been suggested.

REFERENCES

1 E. SCHÜTTE, *Z. physiol. Chem.*, 279 (1943) 59.
2 H. N. CHRISTENSEN, *J. Biol. Chem.*, 160 (1945) 75.
3 J. ROCHE AND M. MOURGUE, *Compt. rend.*, 222 (1946) 1142.
4 J. ROCHE, M. MOURGUE AND R. BARET, *Bull. soc. chim. biol.*, 36 (1954) 85.
5 J. P. GREENSTEIN, *J. Biol. Chem.*, 109 (1935) 529, 541.
6 J. P. GREENSTEIN, *J. Org. Chem.*, 2 (1938) 480.
7 W. L. HUGHES, JR., H. A. SAROFF AND A. L. CARNEY, *J. Am. Chem. Soc.*, 71 (1949) 2476.
8 C. H. CHERVENKA AND P. E. WILCOX, *J. Biol. Chem.*, 222 (1956) 635.
9 J. STIEGLITZ AND R. H. MCKEE, *Ber.*, 33 (1900) 1517.
10 J. BELLO, *Biochim. Biophys. Acta*, 18 (1955) 448.
11 S. SAKAGUCHI, *J. Biochem., Japan*, 37 (1950) 231.
12 F. SANGER, *Biochem. J.*, 45 (1949) 563.
13 A. L. LEVY, *Nature*, 174 (1954) 126.
14 A. L. LEVY AND D. CHUNG, *Anal. Chem.*, 25 (1953) 396.
15 W. L. HUGHES, JR., quoted in OLCOTT AND FRAENKEL-CONRAT[16].
16 H. S. OLCOTT AND H. FRAENKEL-CONRAT, *Chem. Revs.*, 41 (1947) 151.
17 J. S. L. PHILPOT AND P. A. SMALL, *Biochem. J.*, 32 (1938) 542.
18 R. D. COLE, I. I. GESCHWIND AND C. H. LI, *J. Biol. Chem.*, 224 (1957) 399.
19 C. H. LI, *J. Biol. Chem.*, 211 (1954) 555.
20 C. H. LI, M. E. SIMPSON AND H. M. EVANS, *J. Biol. Chem.*, 146 (1942) 627.
21 R. D. COLE AND C. H. LI, *J. Biol. Chem.*, 213 (1955) 197.
22 F. S. GREENSPAN, C. H. LI, M. E. SIMPSON AND H. M. EVANS, *Endocrinology*, 45 (1949) 455.
23 I. I. GESCHWIND AND C. H. LI, in R. SMITH, O. GAEBLER AND C. N. H. LONG, *Hypophyseal Growth Hormone, Nature and Actions*, Blakiston Co., Inc., New York, 1955, p. 28.
24 W. R. LYONS, *Cold Spring Harbor Symp. Quant. Biol.*, 5 (1937) 198.
25 E. H. BOASSON, *J. Immunol.*, 34 (1938) 281.
26 A. N. SMOLELIS AND S. E. HARTSELL, *J. Bacteriol.*, 58 (1949) 731.
27 C. H. LI AND D. CHUNG, *J. Biol. Chem.*, 218 (1956) 33.
28 C. H. LI AND L. ASH, *J. Biol. Chem.*, 203 (1953) 419.
29 A. L. LEVY AND C. H. LI, *J. Biol. Chem.*, 217 (1955) 355.
30 J. P. JEPSON AND I. SMITH, *Nature*, 172 (1953) 1100.
31 C. H. LI AND H. PAPKOFF, *J. Biol. Chem.*, 204 (1953) 391.
32 R. D. COLE, *Thesis*, Univ. of California, 1954.
33 F. C. GREEN AND W. A. SCHROEDER, *J. Am. Chem. Soc.*, 73 (1951) 1385.
34 C. H. LI AND H. M. EVANS, in G. PINCUS, *Recent Progress in Hormone Research*, Vol. III, Academic Press, Inc., New York, 1948, p. 3.
35 C. H. LI AND A. KALMAN, *J. Am. Chem. Soc.*, 68 (1946) 285.
36 H. FRAENKEL-CONRAT, *Arch. Biochem. Biophys.*, 27 (1950) 109.
37 E. REID, *Nature*, 168 (1951) 955.
38 W. F. WHITE, *J. Am. Chem. Soc.*, 77 (1955) 4691.
39 H. B. F. DIXON, *Biochem. J.*, 62 (1956) 25P.
40 E. J. COHN AND J. T. EDSALL, *Proteins, Amino Acids and Peptides*, Reinhold, New York, 1943.
41 M. A. STAHMANN AND R. R. BECKER, *J. Am. Chem. Soc.*, 74 (1952) 2695.
42 H. FRAENKEL-CONRAT, *Biochim. Biophys. Acta*, 10 (1953) 180.
43 P. EDMAN, *Acta Chem. Scand.*, 4 (1950) 283.
44 H. FRAENKEL-CONRAT, J. I. HARRIS AND A. L. LEVY, in D. GLICK, *Methods of Biochemical Analysis*, Vol. II, Interscience Publ., Inc., New York, 1955, p. 359.
45 T. L. DAVIS AND R. C. ELDERFIELD, *J. Am. Chem. Soc.*, 54 (1932) 1499.

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